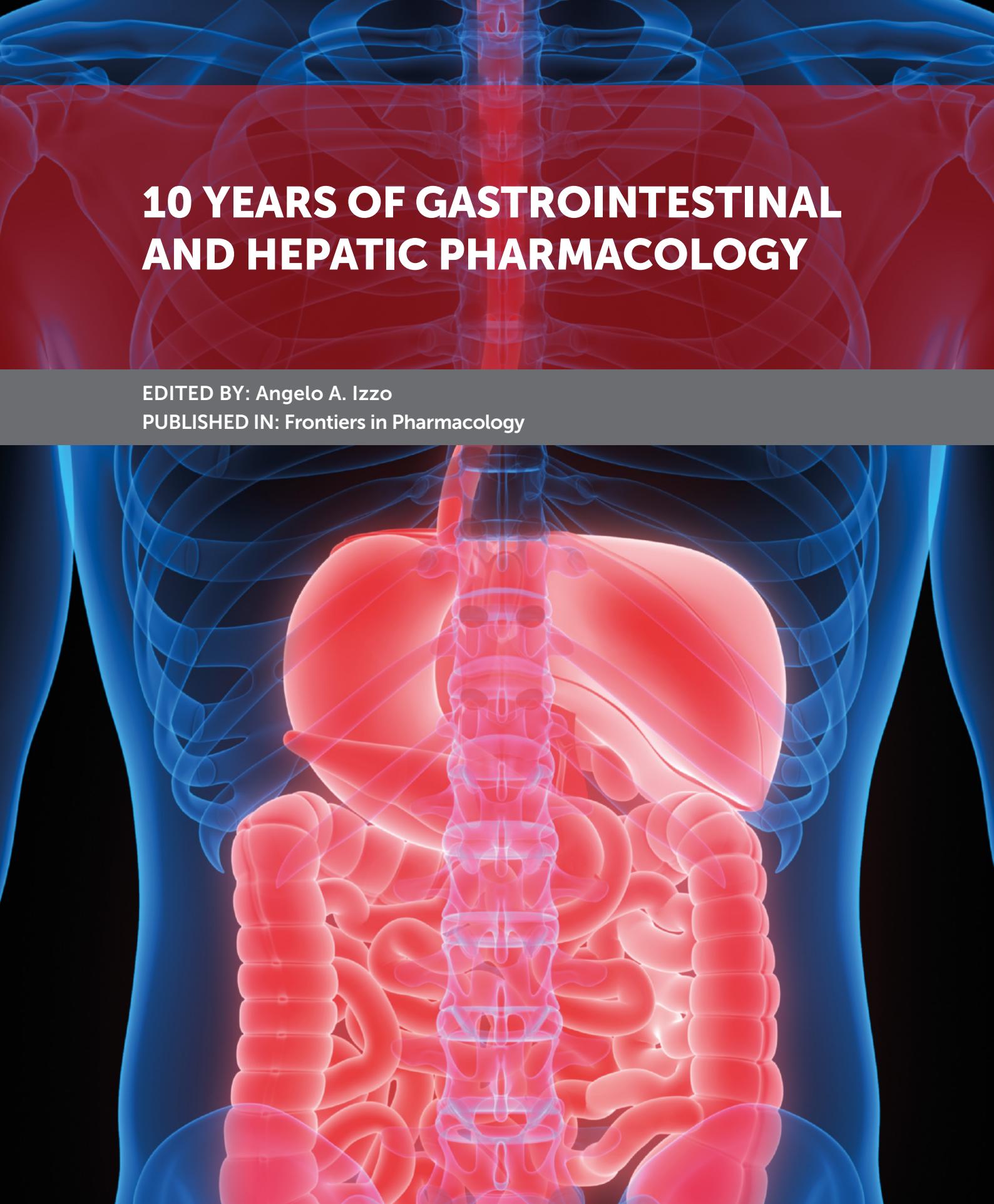


10 YEARS OF GASTROINTESTINAL AND HEPATIC PHARMACOLOGY

EDITED BY: Angelo A. Izzo

PUBLISHED IN: *Frontiers in Pharmacology*





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ISSN 1664-8714

ISBN 978-2-88963-829-1

DOI 10.3389/978-2-88963-829-1

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10 YEARS OF GASTROINTESTINAL AND HEPATIC PHARMACOLOGY

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Cover image: Magic mine/Shutterstock.com

Citation: Izzo, A. A., ed. (2020). 10 Years of Gastrointestinal and Hepatic Pharmacology. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88963-829-1

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A gut feeling about GABA: focus on GABA_B receptors

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γ -Aminobutyric acid (GABA) is the main inhibitory neurotransmitter in the body and hence GABA-mediated neurotransmission regulates many physiological functions, including those in the gastrointestinal (GI) tract. GABA is located throughout the GI tract and is found in enteric nerves as well as in endocrine-like cells, implicating GABA as both a neurotransmitter and an endocrine mediator influencing GI function. GABA mediates its effects via GABA receptors which are either ionotropic GABA_A or metabotropic GABA_B. The latter which respond to the agonist baclofen have been least characterized, however accumulating data suggest that they play a key role in GI function in health and disease. Like GABA, GABA_B receptors have been detected throughout the gut of several species in the enteric nervous system, muscle, epithelial layers as well as on endocrine-like cells. Such widespread distribution of this metabotropic GABA receptor is consistent with its significant modulatory role over intestinal motility, gastric emptying, gastric acid secretion, transient lower esophageal sphincter relaxation and visceral sensation of painful colonic stimuli. More intriguing findings, the mechanisms underlying which have yet to be determined, suggest GABA_B receptors inhibit GI carcinogenesis and tumor growth. Therefore, the diversity of GI functions regulated by GABA_B receptors makes it a potentially useful target in the treatment of several GI disorders. In light of the development of novel compounds such as peripherally acting GABA_B receptor agonists, positive allosteric modulators of the GABA_B receptor and GABA producing enteric bacteria, we review and summarize current knowledge on the function of GABA_B receptors within the GI tract.

Keywords: GABA_B, motility, visceral hypersensitivity, secretion, baclofen, allosteric modulator, agonist

INTRODUCTION

γ -Aminobutyric acid (GABA) is the main inhibitory neurotransmitter in the body and hence GABA-mediated neurotransmission regulates many physiological functions, including those in the gastrointestinal (GI) tract. There are two major classes of GABA receptors and these are classified as either ionotropic GABA_A (including GABA_C) receptors or metabotropic GABA_B receptors (Barnard et al., 1998; Bormann, 2000; Bowery et al., 2002; Cryan and Kaupmann, 2005). It is now over 30 years since these latter receptors were first pharmacologically characterized, and baclofen was identified as a selective GABA_B receptor agonist. GABA_B receptors modulate neurotransmitter release presynaptically by depressing Ca²⁺ influx via voltage-activated Ca²⁺ channels (Bowery et al., 2002; **Figure 1**) while postsynaptic GABA_B receptors couple mainly to inwardly rectifying K⁺ channels (Luscher et al., 1997) and mediate slow inhibitory postsynaptic potentials (Bowery et al., 2002; **Figure 1**). As well as expression in the brain, GABA_B receptors are also abundantly expressed in the GI tract, therefore in this review we will summarize current knowledge on the function of GABA_B receptors in the GI tract.

GABA_B RECEPTOR PROTEINS

The first GABA_B receptor cDNAs were isolated only in 1997 (Kaupmann et al., 1997). The identification of a second GABA_B receptor protein soon after led to the discovery that native GABA_B receptors are heterodimers composed of two subunits, GABA_{B1} and GABA_{B2} (reviewed in Calver et al., 2002; Bettler et al., 2004). In the brain two predominant, differentially expressed splice variants are

transcribed from the *Gabbr1* gene, GABA_{B1a} and GABA_{B1b}, which are conserved in different species including humans (Kaupmann et al., 1997; Bischoff et al., 1999; Fritschy et al., 1999). The human GABA_{B1} gene encodes a third isoform, GABA_{B1c}, a functional role for which has yet to be determined, although it may play a role in the developing human brain (Calver et al., 2002). In the human GI tract there appears to be a similar expression pattern for both GABA_{B1a} and GABA_{B1b} splice variants, with little or no expression of GABA_{B1c} (Calver et al., 2000). The GABA_{B1a} and GABA_{B1b} isoforms differ by the insertion of a pair of tandem "Sushi" domains, which are potentially involved in protein–protein interactions, in the N-terminus of GABA_{B1a}, and differentiate this isoform from GABA_{B1b} (Calver et al., 2002). In the GABA_{B1b} subtype, the N-terminal extracellular domain is the ligand binding domain and differs from the GABA_{B1a} splice variant at the N-terminus by the presence of a tandem pair of CP modules, while the GABA_{B1c} splice variant differs in the fifth transmembrane region and the second extracellular loop by an additional 31 amino acids (Blein et al., 2000). Human GABA_{B1c} is similar to GABA_{B1a} yet lacks one "Sushi" repeat because the splice machinery skips exon 4 and its expression pattern parallels that of GABA_{B1a} (Bettler et al., 2003). It appears at least in some brain regions that GABA_{B1a} and GABA_{B1b} can participate, through heterodimerization with GABA_{B2}, in the formation of both pre- and post-synaptic receptors. Similar heterodimerization has also been postulated to occur in the GI tract between GABA_{B1} and GABA_{B2} (Kawakami et al., 2004) and is further supported by recent immunohistochemical data obtained for both subunits in the upper GI tract (Torashima et al., 2009).

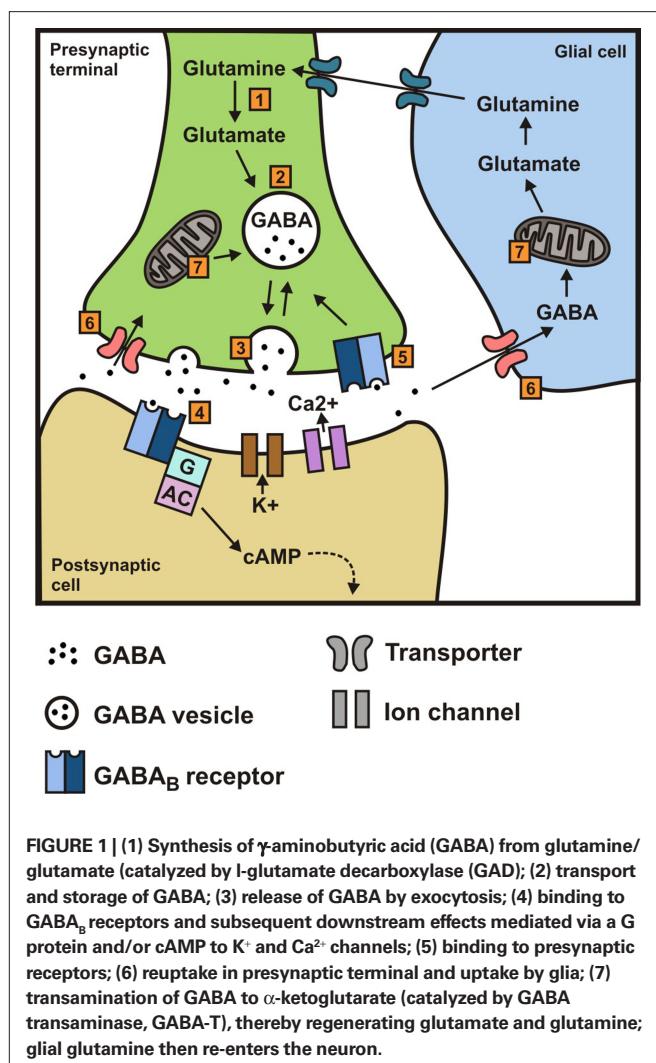


FIGURE 1 | (1) Synthesis of γ -aminobutyric acid (GABA) from glutamine/glutamate (catalyzed by L-glutamate decarboxylase (GAD); (2) transport and storage of GABA; (3) release of GABA by exocytosis; (4) binding to GABA_B receptors and subsequent downstream effects mediated via a G protein and/or cAMP to K⁺ and Ca²⁺ channels; (5) binding to presynaptic receptors; (6) reuptake in presynaptic terminal and uptake by glia; (7) transamination of GABA to α -ketoglutarate (catalyzed by GABA transaminase, GABA-T), thereby regenerating glutamate and glutamine; glial glutamine then re-enters the neuron.

Partial cDNAs corresponding to putative GABA_{B2} splice variants have also been isolated (Clark et al., 2000). However, investigation of the *Gpr51* (*Gabbr2*) gene structure did not provide evidence that these cDNAs correspond to additional GABA_{B2} splice variants (Martin et al., 2001). Furthermore, the absence of an expression profile for GABA_{B2a}, GABA_{B2b}, and GABA_{B2c} in the human GI tract would suggest such splice variants do not play a significant role in GI function (Calver et al., 2000). Therefore, it seems likely that in the brain two major populations of heteromeric GABA_B receptors exist, GABA_{B1a,2} and GABA_{B1b,2}. The behavioral phenotypes of mice with targeted deletions of either the GABA_{B1} (Prosser et al., 2001; Schuler et al., 2001; Mombereau et al., 2004) or the GABA_{B2} subunits (Gassmann et al., 2004; Mombereau et al., 2005) are similar and corroborate the *in vitro* experiments demonstrating that functional GABA_B receptor responses are dependent on the heterodimerization of GABA_{B1} and GABA_{B2} subunits. Additionally, GABA-mediated inhibition of GI motility appears to be dependent on the GABA_{B1} receptor subunit (Sanger et al., 2002). The more recent development of mice lacking both the GABA_{B1a} and GABA_{B1b} receptor splice variants have been generated (Vigot et al., 2006) and are proving to be very useful in understanding the role of

these receptor isoforms in physiological processes (Jacobson et al., 2006, 2007; Vigot et al., 2006), however, such studies have yet to be extended into the GI tract.

LOCALIZATION OF GABA AND GABA_B RECEPTORS IN THE GASTROINTESTINAL TRACT

γ -Aminobutyric acid is located throughout the GI tract and has been localized in enteric nerves as well as in endocrine-like cells implicating GABA as both a neurotransmitter and an endocrine mediator in the GI tract. The primary synthesis pathway for enteric GABA is catalyzed by L-glutamate decarboxylase (GAD; Figure 1) using the substrate glutamate, and has been localized in both Dogiel type I and Dogiel type II enteric neurons (for a review see Krantis, 2000). High affinity plasma membrane GABA transporters (GAT) are also present in the rat GI tract and have been localized to both enteric glia (GAT2) and myenteric neurons (GAT3) of the duodenum, ileum, and colon (Fletcher et al., 2002). In the enteric nervous system (ENS) approximately 5–8% of myenteric neurons, which largely regulate GI motility, contain GABA, and in the colon it predominantly co-localizes with the inhibitory neurotransmitter somatostatin, but also to a lesser extent with enkephalins and nitric oxide (Krantis, 2000). GABA has also been implicated in the regulation of intestinal fluid and electrolyte transport by virtue of its presence in submucosal nerve cell bodies and mucosal nerve fibers (Krantis, 2000). Therefore, it is not surprising that GABA plays a multifunctional role in the regulation of GI activity. In addition to the ENS and endocrine-like sources of GABA, newer endeavors have adapted *Bifidobacteria*, found in the intestines of breast-fed children and healthy adults, to increase GABA production by genetically increasing GAD activity (Park et al., 2005), and GABA-producing bacteria have been exploited in the production of GABA-containing functional foods such as fermented goats milk (Minervini et al., 2009). Genetically exploiting commensal bacteria to elevate intestinal GABA production allows for local delivery of GABA to the GI tract and may therefore be of some therapeutic use in regulating epithelial proliferation (see GABA_B Receptors and Gastrointestinal Carcinogenesis) or may directly alter intestinal secretory activity. Although the current literature would suggest that GABA would need to access the enteric plexi to exert an effect on the latter (see GABA_B Receptor Modulation of Intestinal Electrolyte Transport).

Nakajima et al. (1996) demonstrated using an antibody generated against amino-terminal blocked baclofen, GABA_B receptor immunoreactivity in the rat ENS, muscle and epithelial layers. The 80-kDa antigen against which the antibody was raised was subsequently demonstrated to bind GABA and baclofen, but not the GABA_A antagonist, bicuculline (Nakayasu et al., 1993). Our own studies in mouse intestine, using a different GABA_{B1} receptor antibody (Ab25; Engle et al., 2006) corroborated the findings of Nakajima et al. (1996) with respect to localization of GABA_B receptors on both submucosal and myenteric neurons in the ENS, however we did not detect any mucosal staining in this species (Casanova et al., 2009). In the rat mucosal epithelium, GABA_B receptor positive cells were observed along the length of the GI tract from the gastric body to the colon, decreasing in number in the oral to anal direction, on cells that were morphologically similar to enteroendocrine cells. Both gastric and intestinal regions displayed mucosal GABA_B immunoreactivity, however gastric GABA_B-positive cells tended to contain

somatostatin, in contrast to duodenal GABA_B positive cells which stained positively for serotonin (Nakajima et al., 1996). Therefore, the functional effects of GABA_B receptors are likely to differ along the GI tract, and are likely to be dependant on its colocalization with prominent enteroendocrine cell mediators such as somatostatin and serotonin. Neural GABA_B-positive fibers were observed in the muscle layers of the rat GI tract, and both plexi of the ENS (Nakajima et al., 1996). In the myenteric plexus at least 50% of GABA_B positive neurons display NADPH-diaphorase activity (Nakajima et al., 1996) suggesting that GABA_B receptors may directly modulate inhibitory, nitric oxide-driven neurotransmission. By taking advantage of newly developed transgenic mice expressing GABA_{B1a} and GABA_{B1b} subunits fused to the enhanced green fluorescence protein (eGFP) we also immunohistochemically localized the GABA_{B1} receptor subunit to both myenteric and submucosal neurons in mouse colon and ileum (**Figure 2**). Similar to our studies with an anti-GABA_{B1} antibody, we did not detect any enteroendocrine-like staining for the GABA_B receptor subtype in this species (Casanova et al., 2009).

Analysis of GABA_B receptor subunit expression has been examined in human small intestine and stomach (Calver et al., 2000), rat small and large intestine (Castelli et al., 1999) as well as dog intestine (Kawakami et al., 2004). In the human GI tract GABA_{B1} and GABA_{B2} subunits are differentially expressed (Calver et al., 2000) with the GABA_{B1} receptor subunit, and its splice variants GABA_{B1a} and GABA_{B1b} predominating. GABA_{B2} on the other hand, irrespective of the splice variant examined, was undetectable in

either region of the human GI tract (Calver et al., 2000). Despite the initial findings of Calver et al. (2000) subsequent studies have identified GABA_{B2} message in the human lower esophageal sphincter (LES), cardia and corpus (Torashima et al., 2009) as well as in dog intestine (Kawakami et al., 2004). Furthermore, immunohistochemical analysis identified GABA_{B2} protein on myenteric neurons in human LES and gastric corpus (Torashima et al., 2009).

GABA_B RECEPTORS AND GASTROINTESTINAL FUNCTION

GABA_B-INDUCED SYNTHESIS AND RELEASE OF ENTERIC NEUROTRANSMITTERS AND ENTROCROMAFFIN CELL-DERIVED SEROTONIN

Microdialysis sampling of myenteric plexus neurotransmitter release demonstrated a significant inhibitory effect of the GABA_B receptor agonist, baclofen on canine intestinal acetylcholine (ACh) release and this was sensitive to GABA_B receptor antagonism (Kawakami et al., 2004). Of particular note, in this species at least, was the sensitivity of ACh release (and motility) to the GABA_B receptor antagonist alone (Kawakami et al., 2004). Therefore, in the canine ileum it would appear that GABA_B receptor activation is inhibitory and that GABA via GABA_B receptors tonically inhibits excitatory ACh release. In contrast, release of the inhibitory neurotransmitter, vasoactive intestinal polypeptide from rat colon was insensitive to inhibition by the GABA_B receptor antagonist, phaclofen (Grider and Makhoul, 1992). Similarly, in guinea-pig ileum the production of electrically induced citrulline, as a marker for nitric oxide synthase activity, was insensitive to GABA_B receptor modulation with baclofen, but was reduced by the GABA_A agonist, muscimol (Hebeiss and Kilbinger, 1999). Therefore, with the caveat of species differences, it would appear that GABA_B receptors exert an inhibitory effect on release of ACh, without any significant effect on inhibitory neurotransmitter release or synthesis.

Both GABA_A and GABA_B receptors have also been shown to regulate the release of enterochromaffin cell-derived serotonin from guinea-pig small intestine, although they appear to have opposing effects (Schworer et al., 1989). Baclofen-induced, GABA_B-driven, inhibition of serotonin release occurs via a tetrodotoxin (TTX) insensitive, non-neuronal pathway while GABA_A receptor activation causes a predominant TTX-sensitive, muscarinic receptor-driven release of serotonin (Schworer et al., 1989). Therefore, the potential exists for GABA_B receptors to indirectly regulate ENS activity via release of enteroendocrine-cell derived mediators such as serotonin.

GABA_B RECEPTOR MODULATION OF INTESTINAL MOTILITY

γ -Aminobutyric acid, and as such GABA receptor-mediated effects on GI motility are dependant on an intact ENS as isolated rat smooth muscle cells are unresponsive to addition of GABA (Grider and Makhoul, 1992). Both electrically induced ileal twitch responses and spontaneous colonic smooth muscle contraction (cholinergic in nature) are sensitive to inhibition by baclofen in the guinea-pig (Ong and Kerr, 1982; Allan and Dickenson, 1986; Minocha and Galligan, 1993; **Table 1**). *In vitro* data suggest that this GABA_B-mediated inhibitory effect is countered by GABA_A receptors, as GABA_A receptor activation caused a right-ward shift in the ED₅₀ for baclofen on the ileal twitch response, and this was recovered to some extent in the presence of the GABA_A receptor antagonist, bicuculline (Allan and Dickenson, 1986). In addition to which complex GABA_B

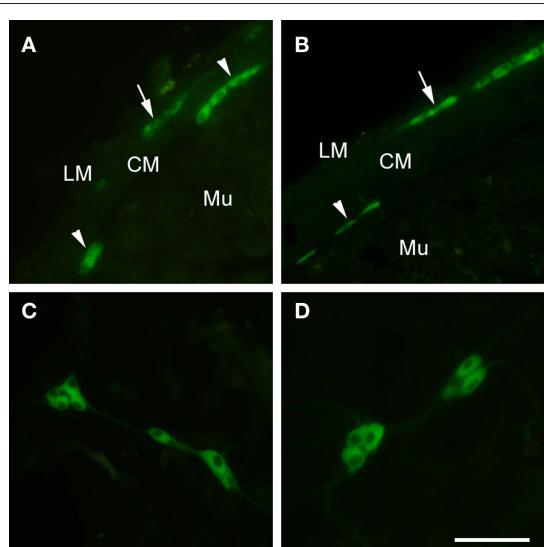


FIGURE 2 | Fluorescence immunohistochemistry using anti-eGFP antibodies revealed GABA_{B1}-eGFP localization in the submucosal (arrowheads) and myenteric plexus (arrows) of GB1^{-/-} mice modified to express GABA_{B1a} and GABA_{B1b} subunits fused to the enhanced green fluorescence protein (eGFP) using a modified bacterial artificial chromosome containing the GABA_{B1} gene (BAC^{+/+}; Casanova et al., 2009) in mouse ileum (A) and colon (B). GABA_{B1}-eGFP was not detected in either the epithelial layer or enteroendocrine cells of GB1^{-/-}, BAC^{+/+} ileum and colon (A,B). Whole mount preparations of ileum (C) and colon (D) revealed a cytoplasmic, non-nuclear, distribution of GABA_{B1}-eGFP in enteric neurons of GB1^{-/-}, BAC^{+/+} mice. Scale bars = 100 μ m. LM, longitudinal muscle; CM, circular muscle; Mu, mucosa. Adapted from Casanova et al. (2009).

Table 1 | Summary of GABA_B receptor-induced effects on gastrointestinal motility.

Region	Species	Baclofen induced-effect	Reference
Duodenum/jejunum	Human	TTX sensitive inhibition of spontaneous and DMPP-induced contraction	Gentilini et al. (1992)
	Rat	Reduction in electrically evoked cholinergic contraction	Krantis and Harding (1987)
		Disruption of migrating motor complex activity (<i>i.v. administration</i>)	Fargeas et al. (1988)
		Atropine-sensitive increase in migrating motor complex activity (<i>i.c.v. administration</i>)	
Ileum	Guinea-pig	Decrease in electrically evoked (cholinergic) twitch response	Ong and Kerr (1982) and Marcoli et al. (2000)
		Relaxation (all levels of the intestine)	Ong and Kerr (1987)
		Inhibition of somatostatin inhibitory activity on cholecystokinin-induced contraction (cholinergic)	Roberts et al. (1993)
		TTX- and hyoscine-sensitive relaxation (basal) and hyoscine-sensitive relaxation following histamine and prostaglandin F _{2α} stimulation	Giotti et al. (1983)
	Mouse	Inhibition of electrically stimulated NO-mediated relaxation	Kilbinger et al. (1999)
		Inhibition of electrically evoked contraction ($GABA_B^{+/+}$)	Sanger et al. (2002)
	Cat	Contraction of longitudinal muscle (distal and terminal ileum; modest if any sensitivity to atropine and TTX) and no effect on circular muscle activity	Pencheva et al. (1999)
Intestine	Dog	No effect (proximal ileum) on longitudinal or circular muscle activity	
		Reduction of circular muscle motor activity coupled with a decrease in ACh release (<i>intra arterial administration</i>)	Kawakami et al. (2004)
Colon	Human	No effect	Gentilini et al. (1992)
	Guinea-pig	Decrease in fecal pellet expulsion and TTX-sensitive relaxation	Ong and Kerr (1982)
		Decrease in basal and physostigmine-induced tone (<i>i.v. administration</i>)	Giotti et al. (1985)
		TTX and scopolamine-sensitive relaxation	Giotti et al. (1985) and Minocha and Galligan (1993)
	Rat	Increase in electrically evoked cholinergic and non-cholinergic circular muscle contraction that is sensitive to nicotinic receptor blockade	Bayer et al. (2003)
	Rabbit	Modest decrease in resting tone and inhibition of electrically-induced (cholinergic) contraction. Inhibition of NANC neurotransmission and decreased transit	Tonini et al. (1989)

ACh, acetylcholine; DMPP, dimethylphenylpiperazinium; *i.v.*, intravenous; *i.c.v.*, intracerebroventricular; NANC, non-adrenergic non-cholinergic; NO, nitric oxide; TTX, tetrodotoxin. Unless otherwise noted in italicize, all drug additions were to *in vitro* preparations.

receptor-dependant signaling pathways, in the guinea-pig ileum at least, have been identified and involve GABA_B receptor-mediated inhibition of somatostatin-sensitive cholecystokinin-induced contraction (Roberts et al., 1993; **Table 1**).

In the human GI tract spontaneous activity of jejunal longitudinal muscle is sensitive to inhibition by both GABA and baclofen. However, spontaneous colonic activity was insensitive to GABAergic modulation (Gentilini et al., 1992) suggesting that GABA_B receptor-mediated inhibition predominates in the small intestine of humans. However, in other species GABA_B receptors have been demonstrated to alter colonic motor activity. For example, desensitization of GABA_B receptors with baclofen, thereby relieving GABA_B-induced effects on motility, resulted in decreased colonic fecal pellet output in the guinea pig (Ong and Kerr, 1982; **Table 1**), potentially due to dysregulation of cholinergic activity and peristalsis as suggested by the authors, or a disinhibition of inhibitory activity. In contrast to the guinea-pig colon, the propulsive velocity

of a distended balloon along rabbit colonic preparations was significantly reduced by GABA_B receptor activation with baclofen, consistent with an inhibitory effect of this receptor on excitatory neurotransmission (Tonini et al., 1989; **Table 1**). In the same species, baclofen had a minor inhibitory effect on colonic longitudinal muscle tone but a more significant inhibitory effect on TTX- and hyoscine-sensitive electrically stimulated responses, suggesting that the inhibitory effects of the GABA_B agonist on colonic activity in the rabbit is dependant on cholinergic neurotransmission (Tonini et al., 1989; **Table 1**). Consistent with modulation of cholinergic enteric nerves, baclofen decreases both GABA_B receptor-induced relaxation of guinea-pig ileal (Giotti et al., 1983) and colonic (Giotti et al., 1985) longitudinal muscle via a TTX-sensitive, cholinergic pathway and *in vivo* inhibited physostigmine-induced colonic tone (Giotti et al., 1985; **Table 1**). GABA receptor-induced relaxation appears to be mediated predominantly via GABA_B receptors in guinea-pig colon, as less than 10% of the GABA-induced relaxant effect is sensitive

to GABA_A receptor blockade (Giotti et al., 1985). However, there is also evidence for GABA_A receptor-mediated activation of inhibitory pathways in guinea-pig colon (Minocha and Galligan, 1993) which one would expect to potentiate GABA_B-mediated relaxation. Non-adrenergic non-cholinergic inhibitory responses also display sensitivity to GABA_B receptor activation in the rabbit (Tonini et al., 1989; **Table 1**), indicative of a co-ordinated regulatory role for GABA_B receptors in the modulation of peristalsis in this species.

The availability of GABA_B subunit receptor deficient mice has led to further characterization of GABA_B receptor-mediated effects in the GI tract (Sanger et al., 2002). Baclofen-induced inhibitory responses were observed in wildtype mouse intestine following electrical stimulation, but were absent in GABA_{B1} subunit deficient animals (Sanger et al., 2002). This unresponsiveness to baclofen does not appear to be due to an overt dysregulation of ileal function in GABA_{B1} mutant mice as these animals respond in a similar manner as wildtype animals to both electrical and cholinergic stimulation (Sanger et al., 2002; **Table 1**). Therefore, the functional dependence of GABA_B receptors in the mouse is dependant on the GABA_{B1} subunit, and this finding is consistent with the preferential expression of this subunit in the GI tract of several species, including humans (Castelli et al., 1999; Calver et al., 2000; Kawakami et al., 2004).

As well as having a peripheral site of action, GABA can exert effects on GI motility via central mechanisms (Fargeas et al., 1988). In unanesthetized rats intracerebroventricular administration of baclofen had a stimulatory effect on GABA_B receptor- and atropine-sensitive migrating myoelectric complexes (MMC) (Fargeas et al., 1988; **Table 1**). While seemingly in disagreement with *in vitro* data, or data from anesthetized animals, which point toward a peripheral inhibitory effect for GABA_B receptors in the GI tract, the authors suggest that this enhancement of MMC activity may represent baclofen-induced adaptation of vagal efferent activity.

GABA_B RECEPTOR MODULATION OF INTESTINAL ELECTROLYTE TRANSPORT

Despite localization of GABA_B receptors in the submucosal plexus of rat (Nakajima et al., 1996) and mouse (GABA_{B1}; Casanova et al., 2009) intestine, they do not appear to be involved in the regulation of electrolyte transport. In guinea-pig intestine, only GABA_A receptor activation, but not baclofen, mimicked GABA-induced elevations in short-circuit current (MacNaughton et al., 1996). A similar bias toward GABA_A receptor-mediated modulation of chloride ion-dependant secretion was also observed in rat small intestine (Hardcastle et al., 1991). However, in this species the GABA-induced effect was dependent on the presence of intact myenteric neurons, suggesting a myenteric reflex is involved in initiating the GABA-induced secretory response (Hardcastle et al., 1991). However, given the paucity of data in this area it is difficult to draw a firm conclusion on the role of GABA_B receptor modulation of intestinal ion transport which may vary among intestinal regions and across species.

GABA_B RECEPTORS AND GASTROINTESTINAL AFFERENT SIGNALING AND NOCICEPTION

Vagal afferent fibers display sensitivity to baclofen and this response is, as expected, sensitive to GABA_B receptor antagonism (Page and Blackshaw, 1999). Further investigation of this vagal afferent pathway elucidated GABA_B receptor-mediated opening of K⁺, and closing of Ca²⁺ channels as contributing to

the inhibitory effect of GABA on afferent activity, although this inhibitory effect varied dependent on the sensitivity of the fibers to mucosal, tension or tension-mucosal stimulation, in addition to which Ca²⁺- and K⁺-independent pathways were also identified (Page et al., 2006). In addition to vagal afferents, GABA_B receptors also regulate spinal afferent signaling (Hara et al., 1990, 1999; Sengupta et al., 2002). Intrathecal injection of baclofen significantly reduced the threshold response to colorectal distension (CRD) in a dose-dependant manner (Hara et al., 1999). Furthermore, when co-administered with morphine, the anti-nociceptive effect of the later was potentiated, indicative of a GABA_B/μ-opioid receptor interaction, which the authors suggest may involve synergistic activation of cAMP and potentiation of the anti-nociceptive effects of both GABA and morphine (Hara et al., 1999). A similar potentiation of the baclofen-induced effect on visceral pain was also observed with the Ca²⁺ channel blocker, diltiazem (Hara et al., 2004). In addition to acting synergistically with morphine and diltiazem, both studies also demonstrated that intrathecal administration of baclofen alone was sufficient to reduce the visceral pain response to CRD (Hara et al., 1999, 2004). These functional data are consistent with subsequent findings describing baclofen-sensitive electrical activity of S₁ dorsal roots following pelvic nerve stimulation during CRD (Sengupta et al., 2002).

Moreover, systemic intravenous (i.v.) administration of baclofen to rats also significantly reduced the visceral pain response, suggesting the GABA_B agonist can potentially exert its anti-nociceptive effects at sites outside the central nervous system, including in the GI tract (Brusberg et al., 2009). The same authors also demonstrated that the positive allosteric modulator of the GABA_B receptor, CGP7930 also displayed efficacy in reducing CRD-induced effects on the visceromotor response, blood pressure, and heart rate following i.v. administration (Brusberg et al., 2009). However, the efficacy of CGP7930 was less than that of baclofen (Brusberg et al., 2009), potentially as its mechanism of action as an allosteric modulator is dependant on the levels of endogenous GABA or GABA tone. In a similar manner to baclofen, CGP7930 does not appear to alter colonic compliance (Brusberg et al., 2009), suggesting the anti-nociceptive effect of CGP7930 is not due to increased accommodation, as a result of muscle relaxation, of the distension stimulus.

In addition to decreasing CRD-induced pain responses, baclofen also alters gut to brain signaling following peripheral colonic inflammation (Lu and Westlund, 2001). Mustard oil-induced colonic inflammation significantly enhanced spinal cord expression of the early gene product Fos, and this response was sensitive to inhibition by baclofen (Lu and Westlund, 2001), suggesting a dampening of afferent signaling from the periphery to the central nervous system. Additionally, baclofen pretreatment *per se*, as well as in the presence of mustard oil, concomitantly increased activity in the rostral nucleus tractus solitarius suggesting that activation of descending anti-nociceptive autonomic pathways or an inhibition of inhibitory activity may also occur, resulting in an enhancement of Fos activity (Lu and Westlund, 2001). Therefore, GABA_B receptor agonists have the potential to exert a dual effect in the GI tract in response to noxious physical or chemical stimuli by decreasing afferent signaling and enhancing anti-nociceptive outflow.

GABA_B RECEPTOR-MEDIATED REGULATION OF GASTRIC MOTILITY, EMPTYING, AND ACID SECRETION

Baclofen exerts a vagus nerve-dependant dual effect on gastric motility that involves an increase in gastric pressure as a result of an inhibition of non-adrenergic non-cholinergic inhibitory neurons in the gastric corpus, as well as an atropine-sensitive stimulation of rhythmic contractions in both the corpus and antrum (Andrews et al., 1987). Moreover, independent of innervation by the central nervous system, peripheral GABA_B receptor activation induces TTX- and atropine-sensitive gastric contractility *in vitro* (Rotondo et al., 2010), suggesting that baclofen locally increases gastric tone through activation of intrinsic cholinergic neurons. Not unexpectedly then, GABA_B receptors have been shown to regulate gastric emptying (in mouse; Symonds et al., 2003). However, this was dependant on the consistency of the diet consumed and on the dose of baclofen administered (Symonds et al., 2003). Lower doses significantly increased gastric emptying of a solid meal, but decreased emptying of a liquid meal at a higher dose (Symonds et al., 2003). This divergent effect of baclofen reflects the different mechanisms that underlie gastric emptying of solid and liquid meals. In a model of delayed gastric emptying, induced by central and peripheral administration of dipyrone, intracerebroventricular baclofen dose-dependently reversed dipyrone-induced gastric retention (Collares and Vinagre, 2005).

Given the evidence for central and peripheral regulation of gastric cholinergic neurons by GABA_B receptors, it is perhaps not surprising that GABA and GABA_B receptors might also influence cholinergic-induced gastric acid secretion. In keeping with such a hypothesis baclofen, or the GABA mimetic PCP-GABA, induce an increase in gastric acid secretion beyond that induced by histamine and cholinergic agonism alone (Goto and Debas, 1983). This effect occurs independently of GABA_A receptors (Hara et al., 1990; Yamasaki et al., 1991) and is accompanied by an increase in vagal cholinergic outflow (Yamasaki et al., 1991). Consistent with such a vagal-cholinergic pathway, systemic baclofen-induced acid secretion (and gastric motility) was inhibited by both atropine and vagotomy (Andrews and Wood, 1986). Similar effects have also been observed in mice, and are mimicked by the GABA_B receptor agonist, SKF-97541 (Piqueras and Martinez, 2004). As predicted by earlier studies Piqueras and Martinez (2004), demonstrated a vagally mediated atropine-sensitive regulation of acid secretion in mouse stomach, however they also demonstrated that GABA_B receptor-induced acid secretion was sensitive to neutralization of gastrin and enhanced in the presence of a somatostatin neutralizing antibody; the former suggesting that GABAergic induced gastric acid secretion occurs via a neurohumoral route which is sensitive to feedback inhibition by the latter. Other studies have identified baclofen-induced acid secretion as also been partially dependant on histamine H₂ receptors, and identified extravagal effects of baclofen on gastric acid secretion in vagotomized rats (Blandizzi et al., 1992).

GABA_B RECEPTORS AS A THERAPEUTIC TARGET IN THE GASTROINTESTINAL TRACT

GABA_B RECEPTORS AND TRANSIENT LOWER ESOPHAGEAL RELAXATION

Modulation of transient lower esophageal sphincter relaxation (TLESR) and the application of GABA_B agonists in the treatment of gastroesophageal reflux disease (GERD) is of particular

translational relevance, being the only case of the use of GABA_B receptors as a clinical target (as recently reviewed by Lehmann, 2009; Lehmann et al., 2010). In pre-clinical studies, intravenous and intragastric administration of baclofen displayed almost equal potencies with respect to inhibition of TLESRs in the dog, despite a concomitant increase in gastric pressure, and these effects were sensitive (to some extent) to GABA_B receptor antagonism and absent when the S enantiomer of baclofen was used (Lehmann et al., 1999). Similar inhibition of TLESRs by baclofen was observed in ferrets (Blackshaw et al., 1999), and the site of action for the GABA_B-mediated effect on TLESR in this species was later demonstrated to involve inhibition of vagal motor output, via GABA_B (GBAB_{B1}) receptors (McDermott et al., 2001), and thought to involve subsequent inhibition of non-adrenergic non-cholinergic activity. However, inhibition of mechano-sensitive gastric vagal afferents and their central synaptic connections with brain stem neurons must also be considered as a site of action for GABA_B receptor agonists in the treatment of GERD. In parallel clinical trials, conducted in and around the same period as pre-clinical studies, data demonstrated that baclofen increased lower esophageal pressure and decreased TLESRs and the number of reflux episodes in healthy human subjects (Lidums et al., 2000). A later study conducted in patients suffering from GERD, similarly demonstrated a significant effect of orally administered baclofen on esophageal pH and on the incidence of reflux episodes and TLESRs, however in this particular study patients did not report any improvement in reflux symptoms (van Herwaarden et al., 2002). Nonetheless, a subsequent study indicated that 4 week treatment with baclofen significantly decreased the intensity of a number of symptoms associated with reflux, including fasting and post prandial epigastric pain, day- and night-time heartburn and regurgitation (Ciccaglione and Marzio, 2003). Despite its efficacy in relieving GERD symptoms, one of the common features associated with baclofen administration in GERD patients is the development of centrally mediated side-effects, with over 80% of baclofen-treated patients reporting neurological events such as dizziness (van Herwaarden et al., 2002). In order to overcome such central side-effects a number of GABA_B receptor agonists have been developed and tested for efficacy in reducing TLESRs, these include the GABA_B agonists AZD9343 (Beaumont et al., 2009), AZD3355 (lesogaberan; Boeckxstaens et al., 2010a,b) and a prodrug of the R enantiomer of baclofen, XP19986 (arbaclofen placarbil; Gerson et al., 2010). The pre-clinical data for AZD9343 favored a decreased side-effect profile as its pharmacology suggested the GABA_B agonist did not readily cross the blood brain barrier and was sequestered intracellularly via a GABA-carrier independent mechanism (Lehmann et al., 2008). Although AZD9343 reduced the number of TLESRs in healthy volunteers, significant side-effects unfortunately remained and included drowsiness and paresthesia (Beaumont et al., 2009). However, other side effects such as the incidence of dizziness in AZD9343-treated subjects were less than those reported in the baclofen-treated group (Beaumont et al., 2009). Of most promise currently in terms of efficacy in treating the symptoms of GERD and having a reduced side-effect profile is lesogaberan. Its pharmacology differs from that of AZD9343 in that lesogaberan displays affinity for GABA carriers, thereby reducing GABA_B-mediated central side effects (Lehmann et al., 2009). Initial trials with lesogaberan in healthy male subjects were positive, with

lesogaberan and baclofen decreasing the number of TLESRs and reflux episodes to a similar extent (Boeckxstaens et al., 2010a). As predicted by pre-clinical studies, subjects treated with lesogaberan had a similar side-effect profile to that observed in those treated with placebo (Boeckxstaens et al., 2010a). Lesogaberan similarly reduced TLESRs in patients with GERD and no significant differences in the side-effect profile between placebo and lesogaberan were observed (Boeckxstaens et al., 2010b). Therefore therapeutically exploiting affinity for GABA-carriers may prove to be beneficial in reducing the central side effects associated with baclofen.

GABA_B RECEPTORS AND GASTROINTESTINAL CARCINOGENESIS

The GABA_B-induced effects on gastric pH may potentially inhibit chemically-induced gastric carcinogenesis observed as a decrease in the incidence and number of gastric tumors (Tatsuta et al., 1990). However, this remains unproven, and the exact mechanism underlying the baclofen-induced decrease in proliferation of antral mucosa has yet to be determined (Tatsuta et al., 1992). In the rat lower GI tract, the same group also observed a GABA_B-induced inhibitory effect on colon tumor growth, but not incidence (Tatsuta et al., 1992).

SUMMARY AND CONCLUSIONS

The diversity of GI functions regulated by GABA_B receptors make it a potentially useful target in the treatment of several GI disorders, but may also limit its therapeutic application due to off target side effects, both in the GI tract and centrally. For example GERD patients and healthy volunteers treated with baclofen reported adverse effects of a neurological nature that included drowsiness and dizziness (Lidums et al., 2000; van Herwaarden et al., 2002; Ciccaglione and Marzio, 2003). However, the development of peripherally acting compounds such as lesogaberan, which by virtue of its affinity for GABA carriers (Lehmann et al., 2009) limits its

effects at central GABA_B receptors, may well overcome the disadvantages associated with traditional GABA_B agonists. Lesogaberan, like baclofen, displays efficacy in the treatment of GERD (Boeckxstaens et al., 2010a,b), but has yet to be tested in other GI disorders where targeting peripheral GABA_B receptors could also be therapeutically useful, i.e., in motility disorders. Furthermore, over the last several years a number of positive allosteric modulators of the GABA_B receptor have been developed (Urwylter et al., 2001, 2003; Malherbe et al., 2008). One of which, CGP7930, reduces the visceral pain response induced by CRD (Brusberg et al., 2009) and may therefore be therapeutically useful in the treatment of functional bowel disorders such as irritable bowel syndrome where visceral pain is a predominant and debilitating symptom. These modulators offer advantages over traditional GABA_B agonists, such as baclofen, as their actions occur following enhancement of endogenous GABA release or transmission, thereby limiting the side-effects that are normally associated with traditional agonist treatment. More novel strategies for delivering GABA to the GI tract in the form of engineered bacteria, such as GAD transfected *Bifidobacterium longum* (Park et al., 2005), or the development of GABA containing functional foods (Minervini et al., 2009) are in their infancy, but may offer potential in treating GI conditions that are GABA or GABA_B receptor-sensitive.

ACKNOWLEDGMENTS

The Alimentary Pharmabiotic Centre is a research centre funded by Science Foundation Ireland (SFI), through the Irish Government's National Development Plan. The authors and their work were supported by SFI (grant nos. 02/CE/B124 and 07/CE/B1368). John F. Cryan is also funded by European Community's Seventh Framework Programme; Grant Number: FP7/2007-2013, Grant Agreement 201714. The authors would like to thank Dr Marcela Julio-Pieper for contributing to the artwork in this manuscript.

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Conflict of Interest Statement: The Alimentary Pharmabiotic Centre and the authors (Niall P. Hyland and John F. Cryan) receive research support from GlaxoSmithKline.

Received: 11 July 2010; paper pending published: 20 August 2010; accepted: 07 September 2010; published online: 04 October 2010.

This article was submitted to Frontiers in Gastrointestinal Pharmacology, a specialty of Frontiers in Pharmacology.

*Citation: Hyland NP and Cryan JF (2010) A gut feeling about GABA: focus on GABA_B receptors. *Front. Pharmacol.* 1:124. doi: 10.3389/fphar.2010.00124*

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Alpha 2 delta ($\alpha_2\delta$) ligands, gabapentin and pregabalin: what is the evidence for potential use of these ligands in irritable bowel syndrome

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Irritable bowel syndrome (IBS) is a complex disorder that is characterized by abdominal pain and altered bowel habit, and often associates with other gastrointestinal symptoms such as feelings of incomplete bowel movement and abdominal bloating, and extra-intestinal symptoms such as headache, dyspareunia, heartburn, muscle pain, and back pain. It also frequently coexists with conditions that may also involve central sensitization processes, such as fibromyalgia, irritable bladder disorder, and chronic cough. This review examines the evidence to date on gabapentin and pregabalin which may support further and continued research and development of the $\alpha_2\delta$ ligands in disorders characterized by visceral hypersensitivity, such as IBS. The distribution of the $\alpha_2\delta$ subunit of the voltage-gated calcium channel, possible mechanisms of action, pre-clinical data which supports an effect on motor-sensory mechanisms and clinical evidence that points to potential benefits in patients with IBS will be discussed.

Keywords: $\alpha_2\delta$ ligands, gabapentin, pregabalin, irritable bowel syndrome, visceral sensitivity, central sensitization, peripheral sensitization, motility

INTRODUCTION

ABDOMINAL PAIN AND VISCERAL HYPERSENSITIVITY

Abdominal pain and discomfort along with altered bowel habit are integral to the diagnosis of irritable bowel syndrome (IBS). Although disordered bowel habit can often be improved in these patients, efficacious treatment of pain, abdominal discomfort, and associated symptoms, such as bloating, remains challenging. The identification and development of new drugs to treat these symptoms has been largely unsuccessful and remains problematic, probably linked to the complexity of the functional gastrointestinal disorders (FGIDs) in which multiple factors appear to contribute to their equally multifarious pathophysiology. For example, genetic predisposition, infection, and traumatic events in early life may all predispose individuals to developing IBS, whilst chronic stress, psychological symptoms, and maladaptive coping mechanisms can increase the frequency and severity of symptoms (Levy et al., 2006; Chitkara et al., 2008; Saito and Talley, 2008; Spiller and Garsed, 2009). Pathophysiologies identified to date include gastrointestinal dysmotility, abnormalities in the inflammatory/immune system, increased intestinal permeability, unstable or altered enteric flora, psychopathology, visceral and somatic hypersensitivity, and abnormal CNS processing (Longstreth et al., 2006; Spiller et al., 2007). Although not all abnormalities are present in all patients, visceral hypersensitivity which often associates with the symptoms of pain (Posserud et al., 2007) and bloating (Posserud et al., 2007; Agrawal et al., 2008) and which is often exacerbated by stress, is thought by many to be a determinant or biological measure of IBS (Mertz et al., 1995; Bouin et al., 2002).

For example, Mertz et al. (1995) showed altered rectal perception in almost all IBS patients in the form of either lowered sensory thresholds, increased sensation intensity or altered viscerosomatic referral, whilst Bouin et al. (2002) suggested that a pain threshold of less than 40 mmHg in the rectum correctly identified IBS from non-IBS subjects. In addition, whilst only approximately half of IBS patients appear to exhibit lowered rectal sensory thresholds to balloon distension (Whitehead and Palsson, 1998; Posserud et al., 2007), almost all patients (70%) show hypersensitivity elsewhere in the gastrointestinal tract, especially in the jejunum (Francis et al., 1995; Hammonds et al., 1998). Moreover, in the latter studies (Francis et al., 1995; Hammonds et al., 1998) within the group as a whole, and especially in those subjects with diarrhea, lower pain thresholds were observed throughout the entire GI tract compared with healthy controls.

CENTRAL SENSITIZATION

These observations of pan-gastrointestinal visceral hypersensitivity and increased viscerosomatic referral, along with reported increases in expression of extra-intestinal symptoms such as headache, dyspareunia, heartburn, muscle pain and back pain (Whorwell et al., 1986; Mayer and Gebhart, 1994), and presence of fibromyalgia in some patients (Whitehead et al., 2002; Almansa et al., 2009) are consistent with a widespread aberrant central processing of pain (central sensitization) in these patients. Further support is provided by the observations that whilst healthy volunteers exhibit an inhibition of the somatic nociceptor flexion reflex (R-III) to slow ramp distension of the rectum, IBS

patients exhibit a facilitation of this reflex, suggesting enhanced spinal processing in IBS (Coffin et al., 2004). In addition, there are an increasing number of studies suggesting that IBS patients may also be hypersensitive to somatic stimuli. One such study, showed hypersensitivity to rectal balloon distension and cutaneous thermal stimulation of the hand and foot in IBS compared with control subjects (Verne et al., 2001). Interestingly foot hypersensitivity was greater than hand hypersensitivity, suggesting greater convergence and overlap of rectal and foot afferents at common lumbosacral levels (greater central hyperalgesia) than rectal and hand afferents at the levels of the cervical spinal (Verne et al., 2001).

POSSIBLE SYNERGISTIC MECHANISMS

Along with the spinal (central) sensitization, other possible synergistic mechanisms of visceral hypersensitivity include disturbances in the cognitive and emotional aspects of pain (e.g., hypervigilance, somatization, catastrophizing, depression), alterations in descending excitatory and inhibitory pathways (e.g., diffuse noxious inhibitory control, DNIC), and sensitization of afferent nerves (e.g., peripheral sensitization due to for example mucosal insult). A quantitative meta-analysis of functional neuroimaging studies in IBS patients during rectal distension showed greater recruitment of attentional (lateral prefrontal cortex), affective (ventral anterior cingulated cortex [ACC], amygdala, dorsal pons), and homeostatic afferent circuits (insula, dorsal ACC, thalamus) compared with controls, with increased regional activity in the insula (INS) and anterior midcingulate cortex (aMCC) being most commonly reported (Labus et al., 2009). More recent studies investigating anatomical differences in the brain between IBS and control subjects have shown morphometric changes in gray matter density predominantly in areas involved in cognition and evaluation, with changes in other areas of the brain being generally explained by anxiety and depression levels in the IBS patients (Seminowicz et al., 2010). In another study by Heymen et al. (2010), in which DNIC was assessed in IBS compared with healthy subjects by measuring the reduction in left hand thermal pain intensity during counter irritation by submersion of the right hand in 12°C water (conditioning stimulus, CS), and controlling for the non-specific effects on pain perception, such as distraction from the CS, psychological symptoms, and cardiovascular reactivity, it was shown that IBS patients demonstrate deficient DNIC probably attributed to disordered central analgesic mechanisms. This deficit has subsequently been shown to directly correlate with visceral hypersensitivity (Piche et al., 2010). Indeed in the morphological study described above significant reductions in gray matter density were observed in the periaqueductal gray, an area known to play a major role in descending pain modulation, which was independent of anxiety and depression (Seminowicz et al., 2010). Other studies have shown that peripheral mucosal insults, such as the presence of inflammation, injury or excess acid do not only increase pain sensitivity at the site of injury (primary hyperalgesia/peripheral sensitization) but also at more remote sites in the gastrointestinal tract (secondary hyperalgesia), via the process of central sensitization (Anand et al., 2007; Knowles and Aziz, 2009). One example in FGIDs, is the observation that pain thresholds to electrical stimulation were not just reduced at the distal end of the esophagus where acid was infused but also in the unexposed

proximal esophagus of patients with non-cardiac chest pain, with this sensitization process being significantly magnified and prolonged compared with healthy volunteers (Sarkar et al., 2000). Another possible example is the onset of IBS following GI infection (post-infectious IBS) where persistent sensitization of the primary afferents due to for example increased mast cells numbers, T lymphocytes, and expression interleukin (IL)-1 β (peripheral sensitization), especially in the presence of risk factors such as depression, hypochondriasis, and adverse life events (Spiller and Garsed, 2009), could lead to central sensitization and the persistence of symptoms, allodynia (pain to a stimulus that does not normally provoke pain), hyperalgesia (increase in intensity of pain to a stimulus that normally provokes pain), and dysmotility long after the resolution of illness.

Thus there appears to be a dyssynergy between the interaction of peripheral and central pain mechanisms, along with influences from cognitive and emotional factors, and abnormalities in descending inhibitory pathways that may all lead to the sensation of abdominal pain and hypersensitivity in IBS. Central mechanisms perhaps play a pivotal role integrating between these processes and thus may represent a promising target for the development of drugs for the treatment of IBS. For a more detailed discussion of central, peripheral, and psychological processes in IBS see the reviews Van Oudenhove and Aziz (2009), Knowles and Aziz (2009), and Anand et al. (2007).

$\alpha_2\delta$ BINDING SITES

Gabapentin (Neurontin) was first introduced as an antiepileptic drug but has more recently been used in the treatment of postherpetic neuralgia, diabetic neuropathy, migraine prophylaxis, and chronic pain conditions (Taylor, 2009; Tzellos et al., 2010). Pregabalin (Lyrica) is a second-generation compound structurally related to gabapentin and approved in the US for the management of neuropathic pain associated with diabetic peripheral neuropathy, postherpetic neuralgia, fibromyalgia, and as adjunctive therapy for adults with partial onset seizures and for generalized anxiety disorder (GAD). In Europe pregabalin is approved for the treatment of neuropathic pain, epilepsy, and generalized anxiety disorder (Taylor, 2009; Tzellos et al., 2010). It has been, or is being, assessed in many clinical trials for disorders such as IBS and neuropathic pain in acute spinal cord injury (see US National Institute of Health, www.ClinicalTrials.gov). Pregabalin has been shown to be 2–10 times more potent than gabapentin and to possess more linear pharmacokinetics (Ben-Menachem, 2004; Huckle, 2004; Taylor, 2009; Tzellos et al., 2010).

Although structurally related to γ -aminobutyric acid (GABA), a major inhibitory neurotransmitter in the CNS, gabapentin, and pregabalin are functionally inactive at GABA_A, GABA_B, or benzodiazepine receptors, and are not converted metabolically into GABA or a GABA receptor agonist (Ben-Menachem, 2004; Huckle, 2004; Taylor, 2009; Tzellos et al., 2010). In addition, clinically effective concentrations of gabapentin and pregabalin have been shown to have no effect on GABA synthesis, uptake or degradation (Ben-Menachem, 2004; Huckle, 2004; Field et al., 2006; Taylor, 2009; Tzellos et al., 2010).

Both gabapentin and pregabalin bind with high affinity to $\alpha_2\delta$ subunits of voltage-gated calcium channels and this has been

proposed as a likely site of their action. Further, in mice with mutations of the $\alpha_2\delta$ subunits that prevent drug binding, pregabalin and gabapentin are devoid of analgesic and anticonvulsant activity (Field et al., 2006). Voltage-gated calcium channels are ubiquitous in the body and are made up of an α_1 subunit, which makes up the ion-conducting pore, coupled together with other subunits including β , γ , and $\alpha_2\delta$. There is great heterogeneity within the family of α_1 subunits, of which 10 members have been described in mammals (for review; Catterall et al., 2005). The $\alpha_2\delta$ subunit appears to play a role not only in the operational characteristics of individual channels, but also to enhance trafficking of the α_1 subunits to the cell membrane, so influencing the number of functional calcium channels (Hendrich et al., 2008; Mich and Horne, 2008). The $\alpha_2\delta$ subunit exists as four distinct subtypes and these are encoded by four distinct genes (Klugbauer et al., 1999; Qin et al., 2002). Only subtypes 1 and 2 have been shown to exhibit binding for gabapentin and pregabalin and therefore might be expected to underwrite the analgesic, anticonvulsant, and anxiolytic activity of these drugs (Gong et al., 2001; Qin et al., 2002).

Despite the widespread localization of voltage-gated calcium channels, the focus of studies to map the distribution of the $\alpha_2\delta$ subunit has largely been restricted to tissues of the central nervous system, with few studies exploring the potential for wider distribution. At both the mRNA and protein level, $\alpha_2\delta$ -subtype 1 is widely distributed throughout human brain (Gong et al., 2001). This widespread distribution of mRNA for the $\alpha_2\delta$ -subtype 1 has been confirmed in the central nervous system of the rat and was reported in regions of the CNS involved in cortical processing, learning and memory, defensive behavior, neuroendocrine secretion, autonomic activation, primary sensory transmission, and general arousal (Cole et al., 2005). These observations have been confirmed at the protein level using immunostaining with an antibody specific for $\alpha_2\delta$ -subtype 1 (Taylor and Garrido, 2008). In this study, the most prominently stained regions of the CNS included those areas involved in pain signaling, including the amygdala, entorhinal cortex, hippocampus, ACC, and insula (Taylor and Garrido, 2008). In addition, a population of small diameter peripheral sensory neurones in the dorsal root ganglia, together with their projections to the spinal cord, stained prominently (Taylor and Garrido, 2008). Immunostaining in the GI tract was also investigated, although only data from the small intestine was reported showing moderate staining in the smooth muscle (Taylor and Garrido, 2008). To date, there has been only one report of neuronal $\alpha_2\delta$ subunit expression in the intestine. In the guinea-pig, *in situ* hybridization has revealed the $\alpha_2\delta$ -subtype 1 localized on the intrinsic primary afferent neurones of the intestine, where they appear to be associated with N-type calcium channels (Needham et al., 2010). In these neurones, pregabalin had inhibitory effects on both the action potential and the after hyperpolarization, raising the possibility that pregabalin may be able to reduce the excitability of these sensory neurones and so potentially inhibit GI hypersensitivity by an effect at these sites. The distribution of $\alpha_2\delta$ -subtype 2 has been less extensively characterized. However, in the rat, mRNA encoding $\alpha_2\delta$ -subtype 2 has been shown to be widely distributed within the CNS, with particularly dense staining in the brainstem, the periaqueductal gray matter, the spinal cord, and dorsal root ganglia (Cole et al., 2005). These are regions

known to play an important role in autonomic function and pain processing. In tissues from human, mRNA for $\alpha_2\delta$ -subtype 2 was detected in several brain regions but not in colon or small intestine (Gong et al., 2001). The absence of $\alpha_2\delta$ -subtype 2 in human jejunum was also confirmed at the protein level.

The functional consequences of the binding of gabapentin/pregabalin with the $\alpha_2\delta$ protein remain controversial. Data from recombinant systems suggests that the function of the $\alpha_2\delta$ subunit is heavily dependent on the subtype of α_1 protein with which the $\alpha_2\delta$ subunit is co-expressed and the cell system into which the proteins are engineered. However, it is widely accepted that the mechanism of action of these agents involves a modulation of calcium conductance, but the precise mechanism for this remains to be elucidated. The modulation of calcium currents by gabapentin has been demonstrated in several studies of isolated neurones (Stefani et al., 1998; Sutton et al., 2002; van Hooft et al., 2002), although other studies have struggled to demonstrate such an effect (Schlicker et al., 1985). More recently, it has been proposed that gabapentin may exert an action through binding to the $\alpha_2\delta$ subunit within the cytosol, rather than at the cell surface, and that this interaction can over time reduce the trafficking of $\alpha_1/\alpha_2\delta$ complexes to functional sites within the cell membrane (Hendrich et al., 2008; Mich and Horne, 2008). Thus gabapentin and pregabalin may exert a range of effects, either acute or chronic, mediated through diverse mechanisms, to modulate calcium flux in nerve terminals. The consequences of this disruption of calcium-mediated membrane depolarization have been investigated extensively. Both gabapentin and pregabalin have been shown to inhibit the release of a wide range of neurotransmitters including noradrenaline, dopamine, 5-HT, acetylcholine, glutamate, substance P, and CGRP from isolated slices of brain and spinal cord from several species following stimulation with either potassium or capsaicin (Dooley et al., 2000; Patel et al., 2000; Fehrenbacher et al., 2003; Brawek et al., 2008). However, the inhibitory effect of gabapentin and pregabalin may be stimulus-dependent, as illustrated in the neocortex, where the magnitude of inhibition of the release of noradrenaline was reduced when neurotransmitter release was evoked by electrical stimulation rather than potassium (Dooley et al., 2000). It has been suggested that $\alpha_2\delta$ ligands may only exert their inhibitory effects on neurotransmitter release in "sensitized" situations and may exert only limited effects in situations of normal physiology. For example, in the spinal cord, gabapentin was only able to exert its presynaptic inhibitory influence on postsynaptic currents in animals in which experimental diabetic neuropathy had been established with streptozotocin and not in unsensitized animals (Patel et al., 2000). Similarly, the pregabalin-mediated reductions in substance P and CGRP release in the spinal cord of the rat are manifest only in animals in which inflammation had been induced following pre-treatment with intraplantar Freund's adjuvant and are absent in untreated animals (Fehrenbacher et al., 2003).

More recently, an additional mechanism of action has been suggested for gabapentin and pregabalin. In cell or neuronal cultures gabapentin and pregabalin were shown to inhibit the activation of the transcription factor NF- κ B evoked by substance P (Park et al., 2008). If confirmed in additional studies, these observations might help explain the increased efficacy of gabapentin and pregabalin

in circumstances of prior inflammation or sensitization, which might be expected to lead to up-regulation of the NF- κ B signaling pathway.

PRE-CLINICAL MODELS OF NON-GI NEUROPATHIC PAIN

The anti-allodynic and anti-hyperalgesic properties of gabapentin and pregabalin have been established in a wide range of animal models and the literature is too extensive to review here. In summary, both of these $\alpha_2\delta$ ligands have been shown to manifest these properties in animal models of inflammatory, surgical, and neuropathic pain, including the inhibition of both the static and dynamic components of allodynia (for example; Field et al., 1997a,b, 1999). Interestingly, in a study of both sympathetically-maintained and sympathetically-independent neuropathic pain, pregabalin was particularly potent at inhibiting both tactile and cold allodynia when given by the intrathecal route, suggesting a predominantly spinal site of action, although the involvement of supraspinal centers cannot be ruled out (Han et al., 2007). Pregabalin was unable to inhibit cold allodynia in the model of sympathetically-independent neuropathic pain when given via the intraperitoneal route (Han et al., 2007). Experiments involving direct recording from spinal neurons have demonstrated the ability of pregabalin to inhibit the C-fiber mediated response of spinal nociceptive-specific neurons, without any effect on the responsiveness of A- δ fibers (You et al., 2009). Further, pregabalin was also able to inhibit central sensitization of the spinal neurons induced by application of bee venom. Spinal transection confirmed that the effect of pregabalin in this study was likely to involve supraspinal centers, mediated through descending inhibitory controls (You et al., 2009).

PRE-CLINICAL MODELS OF GI SENSATION AND MOTILITY

Following the demonstration of the efficacy of gabapentin and pregabalin in animal models of neuropathic pain, investigations of the profile of these agents in small animal models of visceral pain followed. Initial focus was on understanding whether gabapentin and pregabalin might have utility in the treatment of IBS and so studies focused on models of rectal or colonic hypersensitivity. Caution needs to be applied when interpreting these data given the recent history of notable failure of these models to predict the effects of other new drugs on human pain and discomfort (e.g., those acting at neurokinin-1, NK₁, or corticotrophin releasing factor-1, CRF₁, receptors). Despite this in the rat, the intraperitoneal administration of lipopolysaccharide (LPS) results, some 9–12 h later, in hypersensitivity of the rectum. The hypersensitivity can be demonstrated following rectal distension with a small balloon and is manifest as both allodynia as well as hyperalgesia. In this model, pregabalin, following either oral or intraperitoneal administration, suppressed the rectal hypersensitivity response to LPS (Eutamene et al., 2000). Pregabalin (1–30 mg/kg p.o.) dose-dependently inhibited the allodynia observed following distension with the lowest volume of 0.4 mL. However, only the 10 mg/kg dose was able to reduce the nociceptive effect of the larger volumes of distension. These observations were then extended in a more chronic model of colonic allodynia (Diop et al., 2002). Seven days after the administration of trinitrobenzene sulfonic acid (TNBS) into the proximal colon of the rat, an allodynia to distension

was demonstrated in the distal colon of the animal. Histological analysis revealed an inflammatory response in the proximal colon, characterized by the presence of inflammatory cells, necrosis, and hyperemia, 3 days after dosing with TNBS. This response had substantially diminished by day 7 and in the distal colon, no increase in inflammatory cells was observed at any time point. Hyperemia appeared to persist to day 7, the study day for these experiments. Pregabalin (30–200 mg/kg s.c.), given 30 min prior to the balloon distension of the distal colon, dose-dependently reversed the allodynia observed 7 days after TNBS administration. Similar effects were seen following oral administration, 1 h prior to balloon distension. In control animals not pre-treated with TNBS, the highest dose of pregabalin (200 mg/kg s.c.), which fully reversed TNBS-induced allodynia, had no effect on colonic pain thresholds, in contrast to morphine (0.3 and 1.0 mg/kg) which significantly raised thresholds (Diop et al., 2002). In this model, the inflammatory stimulus in the proximal colon establishes a secondary hyperalgesia and allodynia in the distal colon, presumably through central sensitization at the level of the lumbar spinal cord, although possibly also involving higher centers. These central nervous system structures are the likely site of action of pregabalin in this model and in the absence of sensitization, pregabalin had no effect on sensation.

A series of studies with the prototype $\alpha_2\delta$ ligand gabapentin, confirmed that the effects on visceral pain were shared across the class and not restricted to pregabalin. In both mice and rats, gabapentin was shown to reduce the response evoked by intraperitoneal administration of acetic acid, a model of acute visceral pain. Gabapentin (50–200 mg/kg i.p.), dose-dependently inhibited the number of abdominal contractions evoked by intraperitoneal acetic acid when given 40 min ahead of the stimulus (Feng et al., 2003). The maximum effect was seen at 200 mg/kg and inhibited the abdominal response by approximately 75%. This dose of gabapentin impaired the performance of rats on the beam-balance test, suggestive of sedation, which can interfere with the interpretation of tests of analgesia, raising the possibility that the antinociceptive effect of gabapentin was secondary to sedative effects. In this study, the investigators also attempted to develop some mechanistic understanding of these observations and measured acute changes (over a 90-min observation) in the intrathecal levels of several amino acids. Intraperitoneal administration of acetic acid evoked large rises in both aspartate and glutamate. These increases were inhibited completely by prior administration of gabapentin (100 mg/kg i.p.). In addition, acetic acid also increased the intrathecal levels of the inhibitory amino acid serine, an increase that was also inhibited by gabapentin pre-treatment. Intrathecal levels of the inhibitory amino acid, glycine was reduced to below baseline levels by gabapentin. In a broadly similar study, this time conducted in mice, Stepanovic-Petrovic et al. (2008) confirmed the activity of gabapentin to inhibit the nociceptive effects of peritoneal irritation evoked by acetic acid. In this study, intraperitoneal administration evoked a writhing response, characterized by abdominal contractions coupled with elongation of the body and extension of the hindlimbs. Intraperitoneal administration of gabapentin (10–70 mg/kg), 1 h before the acetic acid, dose-dependently inhibited the writhing response. Once again, this group compared the potency of gabapentin to inhibit

acetic-acid-induced writhing with its ability to impair motor function, assessed using the rotarod test (a test of performance in which the rodent is placed on a suspended horizontal rotating rod [not high enough to injure the animal but high enough that the animal avoids falling off] to measure balance, coordination and motor planning). In this study, gabapentin was devoid of activity in the rotarod test, even at doses of 2000 mg/kg, thus making it unlikely that the observed antinociceptive effects were occurring secondary to sedation. In an interesting study from Meymandi and Sepehri (2008), the antinociceptive effect of gabapentin (1–100 mg/kg i.p.) was confirmed in the acetic acid-induced writhing model. In the same study, the dose-response curve to morphine was also constructed and then a low effective dose of gabapentin was given as a combination with a sub-therapeutic dose (0.25 mg/kg i.p.) of morphine. This combination produced a synergistic effect, as writhing was inhibited by over 90% compared to control levels. Similarly, when the sub-therapeutic dose of morphine was combined with a sub-therapeutic dose of gabapentin (10 mg/kg i.p.), a synergistic response was observed, with writhing inhibited by approximately 70%. Interestingly, these synergistic effects were not inhibited by treatment with naloxone. Similar observations, of a synergistic interaction between gabapentin and morphine in models of visceral pain have been made previously in a rat model of experimental pancreatitis induced by bradykinin infusion into the pancreas (Smiley et al., 2004). In this model, gabapentin (100–300 μ g intrathecally) only modestly inhibited the behavioral response to bradykinin. However, when the 300 μ g intrathecal dose of gabapentin was combined with low intrathecal doses of morphine, shown previously to have modest if any inhibitory effects in this model, significant inhibition of all aspects of the behavioral response to bradykinin was observed. These observations taken together illustrate the inhibitory effect of gabapentin on visceral pain, strongly support the concept that this effect is not underwritten or confounded by inhibiting arousal and point to potential synergy with other antinociceptive mechanisms. These data also provide *in vivo* evidence to support the hypothesis that gabapentin reduces the release of excitatory and inhibitory neurotransmitters in the spinal cord.

Recently, a study has been published comparing the effect of pregabalin in the TNBS model of acute hypersensitivity with a model of acute hypersensitivity induced by restraint stress (Ohashi-Doi et al., 2010). As one might predict from previous data, pregabalin (10–100 mg/kg p.o.) reduced colonic nociceptive thresholds dose-dependently in animals sensitized previously with TNBS. In the stress restraint model, increased fecal output in terms of number of pellets and fecal weight was observed during the period of restraint stress. Pregabalin, over the same dose range as examined in the TNBS model, also dose-dependently inhibited the stress-induced increases in fecal output, but had no effect on naive, unstressed rats. This is the only demonstration to date that pregabalin can modulate stress-induced defecation in rats. A comparison of the effects in the two models suggests that pregabalin may be more potent at inhibiting the stress-induced increases in defecation than at inhibiting TNBS-induced colonic hypersensitivity.

The majority of studies of the effects of $\alpha_2\delta$ ligands on visceral pain have been restricted to acute models and largely to

those evoked by chemical irritants or pro-inflammatory stimuli. More recently investigators have studied the effects of gabapentin and pregabalin in models where less invasive and possibly more physiologically relevant stimuli have been used to evoke an acute or chronic phenotype. In a rat model, where repeated tonic colorectal distension induces hypersensitivity, oral pregabalin (10 and 30 mg/kg) inhibited the development of hyperalgesia (Million et al., 2007). Moreover, using Fos staining to indicate neuronal activation, a single oral dose of pregabalin (30 mg/kg) blunted the activation of lumbosacral spinal neurones. These data raise the possibility that in this model, pregabalin inhibits spinal sensitization and so inhibits the development of hyperalgesia. It has been demonstrated that maternal separation of rats in the early neonatal period, a presumably highly stressful stimulus, can give rise to hyperalgesia, revealed by colorectal distension, that is sustained for many weeks after the original stress (Coutinho et al., 2002). In an elegant study, to date only published as an abstract, Coelho et al. (2008) confirmed that a dose of gabapentin (30 mg/kg s.c.) that inhibited acute visceral pain evoked by intraperitoneal acetic acid, was also able to inhibit the pain behaviors evoked by colorectal distension in rats that had undergone maternal separation 9–11 weeks earlier.

New $\alpha_2\delta$ ligands are starting to appear in literature, but to date, only one of these, PD-217014, which has similar binding affinity at the $\alpha_2\delta$ binding site as pregabalin, has been investigated in an animal model of visceral hypersensitivity (Ohashi et al., 2008). In the TNBS model described previously, oral dosing of PD-217014 (3–100 mg/kg) dose-dependently inhibited the reduction in colonic nociception threshold observed 7 days after TNBS administration. Maximum inhibition was reached at 60 mg/kg and the inhibition at this dose was long lasting, reaching a peak at 2 h post-dose and lasting for between 6 and 8 h. Pharmacokinetic/pharmacodynamic (PK/PD) analysis clearly demonstrated that maximum anti-hyperalgesia coincided with peak plasma exposures. Interestingly, whilst the anti-hyperalgesic effect persisted at 6 h post-dose, plasma levels had at this time diminished to low levels. These observations suggest that the persistence of the pharmacological effect of PD-217014 is not simply related to plasma exposure and this phenomenon requires further investigation.

In a recent study, Ravnefjord et al. (2008) demonstrated in normal, unsensitized rats that pregabalin (10–200 μ mol/kg p.o.) inhibited the viscerosomatic response to phasic, noxious colorectal distension at 80 mmHg as well as the viscerosomatic response to ascending (10–80 mmHg), phasic distension. In this study, the highest dose of 200 μ mol/kg p.o. also inhibited the increases in cardiovascular parameters (blood pressure and heart rate) seen in response to noxious distension at 80 mmHg. However, one of the most interesting observations in this study, and one that reveals another potential mechanism of action of pregabalin to reduce pain thresholds in these distension models, was an apparent leftward shift in the colonic pressure-volume relationship. These observations suggest that pregabalin may increase the compliance of the colon in response to distension and by this mechanism could effectively reduce the intensity of the nociceptive stimulus. Similarly, this could be a mechanistic explanation for the observations of antinociceptive activity in models employing colorectal distension as a nociceptive stimulus.

CLINICAL EVIDENCE AND POTENTIAL UTILITY IN IBS

To date only two clinical studies have been published (Lee et al., 2005; Houghton et al., 2007) assessing the effect of these compounds on visceral sensitivity in IBS and one abstract in healthy volunteers (Chua et al., 2009). No results from clinical trials examining the efficacy of $\alpha_2\delta$ ligands on symptoms in IBS patients have yet appeared in literature. However, there is one investigator-sponsored small placebo-controlled trial of pregabalin in IBS (NCT00977197), another investigator sponsored study looking at the effect of pregabalin on colonic sensorimotor function in healthy volunteers (NCT01094808) and a company-sponsored clinical trial assessing the effect of the new generation $\alpha_2\delta$ ligand, PD-217014 in IBS (NCT00139672) currently listed on the US National Institute of Health ClinicalTrial.gov website.

The first study published assessed the effect of gabapentin (300 mg/day for the first 3 days and the 600 mg/day for the subsequent 2 days) on rectal sensitivity to balloon distension in IBS patients with diarrhea diagnosed using Rome II (IBS-D; Lee et al., 2005). The authors reported that the threshold pressures for bloating, discomfort and pain, and rectal compliance all significantly increased after gabapentin but not following placebo. The increase in rectal tone seen after meal ingestion was unaffected. Unfortunately however, no direct comparison was made with placebo in this study, so the significance of their gabapentin findings needs to be viewed with caution. The second study published by the authors assessed the effect of pregabalin (titrated from 50 mg tid to 200 mg tid over 3 weeks) in IBS patients who exhibited rectal hypersensitivity to mechanical distension (Houghton et al., 2007). In comparison to placebo, pregabalin was shown to significantly increase or normalize the sensory thresholds for pain (anti-allodynic effect), along with first sensation and desire to defecate (anti-hyperalgesic effect), without desensitizing (i.e., make hyposensitive) the perception of distension. If confirmed by larger studies and the results from the study currently in progress assessing the effect of pregabalin on colonic sensorimotor function in healthy volunteers (NCT01094808) proves to be negative, then this would suggest that as shown in the animal models, desensitization only occurs in the presence of an hypersensitive state. Such a compound would be most desirable for treatment of IBS and confirms studies in healthy volunteers showing that gabapentin reduces signs of central sensitization induced by intradermal capsaicin (i.e., the area of brush and pinprick hyperalgesia) but not spontaneous or evoked pain induced by capsaicin (Gottrup et al., 2004). Similarly, a more recent study only published in abstract form to date, showed that pregabalin prevents the development of secondary hyperalgesia in the proximal esophagus after distal esophageal acidification but had no effect on the primary hyperalgesia induced in the distal esophagus (Chua et al., 2009), supporting a central mode of action for pregabalin in reduction of pain.

In addition to pregabalin's effect on visceral sensation, and as with the gabapentin rectal motor-sensory study (Lee et al., 2005) and in the animal models (Ravnefjord et al., 2008) described previously, rectal compliance increased following pregabalin, although there appeared to be no association with the observed reduction in visceral pain (Houghton et al., 2007). This suggests additional mechanisms of action, as yet to be explored. Similar observations

have been seen before with both clonidine and nitroglycerine both increasing gastric compliance but only clonidine reducing pain perception (Thumshirn et al., 1999).

Generalized anxiety disorder, as with IBS, is a common disorder. Furthermore studies have shown that 32–58% of patients with IBS meet the diagnostic criteria for GAD (Lydiard, 2001), a condition which has recently been shown to improve following treatment with pregabalin (Stein et al., 2009). As well as improving overall anxiety levels in GAD patients, the study showed that treatment with pregabalin also led to an improvement in GI symptoms that very often coexist in these patients and similar to those seen in functional GI disorders, such as IBS (Stein et al., 2009). This raises the possibility that GI symptoms might improve as a consequence of the treatment of anxiety by pregabalin. Neither the presence of GAD nor levels of co-existing anxiety or depression were measured in the study of rectal hypersensitivity (Houghton et al., 2007), but the observation that pregabalin increased the sensory thresholds for first sensation and the desire to defecate, sensations not normally expected to be under significant psychological influence, might suggest that the anxiolytic properties of pregabalin were not playing a major role in modulating visceral sensation. In support of this hypothesis, other studies using anxiolytic agents, such as buspirone or antidepressants such as amitriptyline have shown no effect on colonic sensitivity to balloon distension (Mertz et al., 1998; Chial et al., 2003; Morgan et al., 2005). However, these observations do not exclude the possibility an anxiolytic effect for pregabalin in amelioration of IBS symptoms, especially in patients with anxiety-induced increased defecation (as implicated by the acute restraint stress animal model; Ohashi-Doi et al., 2010), but clinical trials are required in which psychological symptoms along with the cardinal IBS symptoms are measured to address the true efficacy of these agents in the treatment of IBS.

Other factors that might influence rectal sensation are the adverse effects associated with pregabalin, namely dizziness and/or somnolence. However, in the study of Houghton et al. (2007) these side effects had resolved in the majority of patients by the time sensitivity was assessed, and the change in sensory threshold in these patients was no different from that seen in those still retaining mild/moderate side effects, supporting data from animal studies (Ohashi-Doi et al., 2010).

Furthermore the improvement in sensory threshold tended to associate with a reduction in abdominal pain (Houghton et al., 2007), supporting the observations that pregabalin improves GI symptoms associated with GAD (Stein et al., 2009), and pain in patients with fibromyalgia (Hauser et al., 2009; Smith and Barkin, 2010; Straube et al., 2010). The results from the ongoing clinical trials are eagerly awaited, and whether the patients have been appropriately phenotyped to identify any sub-group improvement based on their hypersensitivity or anxiety status also remains to be revealed.

CONCLUSION

Gabapentin and pregabalin are valuable medicines being used for the treatment of a number of conditions, including neuropathic pain, epilepsy, anxiety, and fibromyalgia. A body of evidence implicates binding to the $\alpha_2\delta$ subunits of voltage-gated calcium channels on presynaptic neuronal membranes as their most likely

mechanism of action. However, recent data also points at potential additional mechanisms within the cell which may underwrite some of their chronic effects and also indicates potential modulation of pro-inflammatory pathways through inhibition of NF- κ B signaling. The modulation of calcium fluxes evoked by gabapentin and pregabalin has been shown to reduce the release of a broad range of both excitatory and inhibitory neurotransmitters, primarily in the central nervous system and hence this mechanism has great potential to influence signaling pathways, including those involved in pain transmission. Emerging data supports a role for the $\alpha_2\delta$ subunit in neurotransmission in the enteric nervous system, but the functional importance of these observations has yet to be fully elucidated.

Data from animal models provides evidence for the inhibition of both visceral nociception and GI function by gabapentin and pregabalin in animals in which hypersensitivity has been induced by either an inflammatory stimulus or stress, but largely illustrates an absence of such activity on basal sensitivity or function.

When particularly strong noxious stimuli are used (acetic acid or distension to high pressures) effects on sensation in unsensitized animals can be observed. These observations are in concordance with earlier experiments performed using isolated *in vitro* preparations from animals in which hyperalgesia had been established. The precise mechanism through which the $\alpha_2\delta$ ligands inhibit

intestinal allodynia and hyperalgesia has only been hinted at and much remains as supposition. The extensive literature that indicates the reduction in the release of neurotransmitters at the spinal and supraspinal level by $\alpha_2\delta$ ligands remains a valid hypothesis to explain the observations, with some supporting data obtained from animal models of visceral pain.

The limited number of clinical studies of visceral pain performed and reported to date support the observations in animals. In patients with IBS, both gabapentin and pregabalin have been shown to reduce rectal sensitivity to balloon distension and in the study with pregabalin, anti-allodynia, and anti-hyperalgesia was demonstrated in subjects with pre-characterized rectal hypersensitivity. Data is expected soon from a similar study in healthy volunteers which will illustrate whether these agents have, like in many animal models, little effect on sensory thresholds in subjects without hypersensitivity. Both animal and clinical data also suggest that $\alpha_2\delta$ ligands may alter intestinal compliance and the significance of this needs further investigation. These data, supported by observations from animal studies, support further investigation of $\alpha_2\delta$ ligands in disorders characterized by visceral hypersensitivity such as IBS. Carefully controlled, randomized clinical trials will be needed to fully understand the potential of these agents to treat these bothersome conditions.

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- Conflict of Interest Statement:** Dr. Jeremy Gale is an employee and shareholder of Pfizer. Professor Lesley A. Houghton has served as a speaker, a consultant, and/or an advisory board member for Novartis, Pfizer, Solvay Pharmaceuticals, GlaxoSmithKline, Clasado, Ono Pharma UK Ltd., Kelloggs UK, Norgine Ltd., and Boehringer Ingelheim; the Neurogastroenterology Unit, University of Manchester/University Hospital of South Manchester NHS Foundation Trust, UK has received research funding from Novartis, Pfizer, Solvay Pharmaceuticals, GlaxoSmithKline, and Danone Research; and the Division of Gastroenterology and Hepatology, Mayo Clinic, Jacksonville, USA has received research funding from Edusa Pharmaceuticals, Inc., USA.
- Received: 08 February 2011; accepted: 29 May 2011; published online: 09 June 2011.*
- Citation: Gale JD and Houghton LA (2011) Alpha 2 delta ($\alpha_2\delta$) ligands, gabapentin and pregabalin: what is the evidence for potential use of these ligands in irritable bowel syndrome. *Front. Pharmacol.* 2:28. doi: 10.3389/fphar.2011.00028*
- This article was submitted to Frontiers in Gastrointestinal Pharmacology, a specialty of Frontiers in Pharmacology.*
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A distinct profile of tryptophan metabolism along the kynurenine pathway downstream of toll-like receptor activation in irritable bowel syndrome

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Irritable bowel syndrome (IBS), a disorder of the brain-gut axis, is characterised by the absence of reliable biological markers. Tryptophan is an essential amino acid that serves as a precursor to serotonin but which can alternatively be metabolised along the kynurenine pathway leading to the production of other neuroactive agents. We previously reported an increased degradation of tryptophan along this immunoresponsive pathway in IBS. Recently, altered cytokine production following activation of specific members of the toll-like receptor (TLR) family (TLR1-9) has also been demonstrated in IBS. However, the relationship between TLR activation and kynurenine pathway activity in IBS is unknown. In this study, we investigated whether activation of specific TLRs elicits exaggerated kynurenine production in IBS patients compared to controls. Whole blood from IBS patients and healthy controls was cultured with a panel of nine different TLR agonists for 24 h. Cell culture supernatants were then analyzed for both tryptophan and kynurenine concentrations, as were plasma samples from both cohorts. IBS subjects had an elevated plasma kynurenine:tryptophan ratio compared to healthy controls. Furthermore, we demonstrated a differential downstream profile of kynurenine production subsequent to TLR activation in IBS patients compared to healthy controls. This profile included alterations at TLR1/2, TLR2, TLR3, TLR5, TLR7, and TLR8. Our data expands on our previous understanding of altered tryptophan metabolism in IBS and suggests that measurement of tryptophan metabolites downstream of TLR activation may ultimately find utility as components of a biomarker panel to aid gastroenterologists in the diagnosis of IBS. Furthermore, these studies implicate the modulation of TLRs as means through which aberrant tryptophan metabolism along the kynurenine pathway can be controlled, a novel potential therapeutic strategy in this and other disorders.

Keywords: irritable bowel syndrome, kynurenine pathway, toll-like receptors, tryptophan, IDO, cytokine

INTRODUCTION

The diagnosis of irritable bowel syndrome (IBS), a highly prevalent functional gastrointestinal disorder (FGID), is currently made based on the presence of a characteristic symptom profile (abdominal pain/discomfort, bloating/distension, alterations in defecatory function) in the absence of a demonstrable organic disease of the gastrointestinal tract (GIT; Drossman and Dumitrescu, 2006). This diagnostic scheme reflects the lack of reproducible biological markers of this heterogeneous disorder, a serious impediment to advancing our understanding of its pathophysiology (Clarke et al., 2009b). The concept of IBS as a disorder of the brain-gut axis is now generally accepted and this has facilitated some progress in the area (Ohman and Simren, 2007, 2010). Recently, indices of a low

grade immune activation have been reported in IBS, including elevations in circulating cytokines (Dinan et al., 2006; Liebregts et al., 2007; Dinan et al., 2008; Scully et al., 2010) and pro-inflammatory polyunsaturated fatty acids (Clarke et al., 2010) as well as evidence of enhanced immune cell activation, both systemically (Ohman et al., 2009a,b) and locally within the GIT (Cremon et al., 2009).

A growing appreciation of the potential impact of this increased inflammatory state on the brain-gut axis (Quigley, 2006; Dantzer et al., 2008; O’Malley et al., 2011) as well as an improved understanding of the influence of the GIT on mood and cognition (Forsythe et al., 2010; Grenham et al., 2011; Mayer, 2011; Bercik et al., 2012; Kennedy et al., 2012), has led to an exploration of a potential role for tryptophan and its associated metabolic

pathways in IBS. Tryptophan is an essential amino acid that serves as a precursor to serotonin (5-HT), a key neurotransmitter within both the enteric nervous system (ENS) and central nervous system (CNS; Ruddick et al., 2006; Forsythe et al., 2010). An alternative and physiologically dominant fate for tryptophan is degradation along the kynurenine pathway leading to the production of neuroprotective compounds like kynurenic acid and neurotoxic compounds like quinolinic acid (Schwarz and Pellicciari, 2002). Dysregulation of tryptophan metabolism is thus poised to impact on mood and cognition within the CNS as well as secretion, motility, and perception in the ENS (Crowell, 2004; Forsythe et al., 2010). Crucially, indoleamine-2,3-dioxygenase (IDO), one of two key primary kynurenine pathway enzymes, is immunoresponsive and studies, to date, have indicated an increase in IDO activity in both male and female subjects with IBS (Fitzgerald et al., 2008; Clarke et al., 2009a).

Mechanistic insights into the inflammation observed in IBS and the associated downstream consequences are currently lacking. Toll-like receptors (TLRs) are pattern recognition receptors integral to the functioning of the innate immune system and respond to a variety of bacterial and viral cell components, resulting in increased production of inflammatory cytokines (Takeuchi and Akira, 2010). Recent evidence from both relevant animal models and biopsies from IBS sufferers have demonstrated altered expression of certain TLRs in the colonic mucosa (McKernan et al., 2009; Brint et al., 2011). Increased TLR2 expression on blood monocytes in IBS patients has also been reported (Ohman et al., 2012). Moreover it has recently been demonstrated that IBS patients have a distinct pattern of peripheral TLR activity as indicated by measurements of cytokine production following whole blood stimulation (McKernan et al., 2011). Although it is known that TLR activation can lead to alterations in IDO expression (Mahanonda et al., 2007), the potential consequences for tryptophan metabolism in IBS remain unknown.

In this study we investigated the potential functional consequences of TLR activation on brain-gut axis signaling in terms of perturbations in tryptophan metabolism. This was based on the hypothesis that IBS patients would exhibit a downstream pattern of tryptophan degradation along the kynurenine pathway subsequent to activation of TLRs with their specific ligands that was distinct from healthy control subjects.

MATERIALS AND METHODS

STUDY POPULATION

The study protocol was approved by the University College Cork (UCC) Clinical Research Ethics Committee. IBS patients were recruited from a university IBS database comprised of individuals who had either attended gastroenterology clinics at Cork University Hospital (CUH) or had responded to direct advertisement on the university campus or a local newspaper regarding participation in IBS research. Thirty seven healthy controls were recruited from staff at both UCC and CUH. Twenty five individuals aged between 18 and 65 years who satisfied Rome II criteria for IBS and in whom organic gastrointestinal diseases and clinically significant systemic diseases had been excluded, were considered for inclusion in the study. Subjects who had undergone any abdominal surgery, with the exception of hernia repair and appendectomy, were also

excluded. No postinfectious IBS (PI-IBS) subjects were included in the recruitment and IBS patients were not selected on the basis of predominant bowel habit, although this was recorded according to the Rome II classifications. All subjects completed a questionnaire to assess both IBS severity (Francis et al., 1997) and current mood (Spitzer et al., 1999).

BIOLOGICAL ASSAYS

Fifteen milliliters of whole blood was collected between 11:00 and 13:00 hours from each healthy control and IBS patient. Collected whole blood (15 mL) was added to an equal volume of Histopaque 1077 (Sigma, St Louis, MO, USA) in a sterile 50 mL tube and centrifuged at $400 \times g$ for 30 min at room temperature. Plasma on the upper layer was transferred to a separate tube and stored at -80°C for future analysis. Collected whole blood (2 mL) was diluted 1:10 in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Dublin, Ireland). Blood was aliquoted into 24 well plates and cultured in a 37°C incubator with 5% CO_2 . Each blood sample was cultured in duplicate in DMEM cell culture medium supplemented with 10% Fetal Calf Serum (Sigma, Dublin, Ireland) with or without the following TLR ligands from a Human TLR agonist kit (Invivogen, San Diego, CA, USA) for 24 h:

TLR1/2-Palmitoyl-3-cysteine-serine-lysine 4 (Pam3Cys); TLR2-heat-killed *Listeria monocytogenes* (HKLM); TLR3-Polyribonucleic polyribocytidylic acid (Poly I:C); TLR4-Lipopolysaccharide (LPS); TLR5-*Salmonella typhimurium* Flagellin; TLR6/2-FSL-1; TLR7-Imiquimod; TLR8-ssRNA40; TLR9-ODN2006. Agonists were reconstituted in endotoxin free water (supplied in kit) to a final concentration of $1 \mu\text{g mL}^{-1}$ except for HKLM (10^8 cells) and Poly I:C ($10 \mu\text{g mL}^{-1}$). Subsequently, supernatants from both untreated and stimulated cells were aspirated and stored at -80°C for future analysis.

HPLC ASSAY FOR TRYPTOPHAN AND KYNURENINE

Tryptophan and kynurenine were determined by high performance liquid chromatography (HPLC): this involved using a system comprising a Waters 510 pump (Waters Ireland, Dublin, Ireland), 717plus cooled autosampler, a 996 PDA detector, a Hewlett Packard 1046A Fluorescent Detector (Waters Ireland, Dublin, Ireland), a waters bus SAT/IN module and a croco-cil column oven. System components were used in conjunction with Waters Empower software (Waters Ireland, Dublin, Ireland). All samples were injected onto a reversed phase Luna $3\mu\text{ C18(2)}$ $150 \times 2 \text{ mm}$ column (Phenomenex, Macclesfield, UK), which was protected by Krudkatcher disposable precolumn filters and security guard cartridges (Phenomenex). HPLC grade acetonitrile, acetic acid, and perchloric acid were obtained from Fisher Scientific Ireland (Dublin, Ireland). The analysis method was based on that by Herve et al. (1996). The mobile phase consisted of 50 mmol L^{-1} acetic acid, 100 mmol L^{-1} Zinc Acetate with 3% (v/v) acetonitrile and was filtered through a $0.45 \mu\text{m}$ Millipore filter (AGB, Dublin, Ireland) and vacuum degassed prior to use. Separations were achieved by isocratic elution at 0.3 mL min^{-1} . The fluorescent detector was set to an excitation wavelength of 254 nm and an emission wavelength of 404 nm. The PDA detector start wavelength was 210 nm and the end wavelength was 400 nm with chromatogram extraction at 330 nm. Working standard dilutions

were prepared from millimolar stock solutions of each standard and stored at -80°C until required for analysis. Samples were deproteinized by the addition of 20 μL of 4 mol L^{-1} perchloric acid to 200 μL of plasma spiked with 3-nitro-L-tyrosine as internal standard. Twenty microliters of either sample or standard was injected onto the HPLC system and chromatograms generated were processed using Waters Empower software. Analytes were identified based on their characteristic retention time and their concentrations determined using Analyte:Internal standard peak height ratios; these were measured and compared with standard injections which were run at regular intervals during the sample analysis. Results were expressed at ng analyte per mL of supernatant/plasma.

STATISTICS

The sample size was determined by a power calculation based on our previous data and aimed at detecting differences between IBS patients and controls at the 0.05 level. Data was expressed as mean \pm SEM. Statistical analysis was carried out using SPSS 18 for Windows (SPSS, Inc., Chicago, IL, US). Plasma tryptophan, kynurene, and the kynurene:tryptophan ratio were compared using an unpaired two-tailed Student's *t*-test and differences considered significant at the $p < 0.05$ level. Bonferroni corrections for multiple comparisons were applied as required. TLR agonist induced alterations in, kynurene and the kynurene:tryptophan ratio were determined using a two-way ANOVA and by Bonferroni *post hoc* tests.

RESULTS

BASELINE CHARACTERISTICS

There were no significant differences between IBS patients (5M, 20F) and controls (13M, 25F) in terms of age (41.32 ± 2.234 vs. 36.63 ± 1.821 , $p = 0.11$) or body mass index (BMI; 24.88 ± 0.7089 vs. 23.96 ± 0.8668 , $p = 0.45$). According to Rome II sub classification of predominant bowel habit, nine had constipation-predominant IBS (IBS-C), eight had diarrhea-predominant IBS (IBS-D), and eight had alternating IBS (IBS-A). There were three current smokers in each group. Eleven IBS subjects (44%) met criteria for a current psychiatric co-morbidity. According to IBS symptom severity scores, 5 patients rated their symptoms as mild, 12 as moderate and 8 as severe.

PLASMA TRYPTOPHAN AND KYNURENE CONCENTRATIONS

There was no significant difference in plasma tryptophan levels between controls and IBS groups (10.0 ± 0.4 vs. $10.5 \pm 0.7 \mu\text{g mL}^{-1}$, $p = 0.51$). Plasma kynurene levels were significantly elevated in comparison to controls levels in IBS patients (0.39 ± 0.02 vs. $0.50 \pm 0.05 \mu\text{g mL}^{-1}$, $p < 0.05$) as was the kynurene:tryptophan ratio (0.03991 ± 0.00222 vs. 0.04795 ± 0.00315 , $p < 0.05$), an index of IDO activity (Figures 1A–C).

TLR AGONIST INDUCED ALTERNATIONS IN TRYPTOPHAN DEGRADATION.

Concentrations of tryptophan and kynurene following TLR stimulation are given in Tables 1 and 2 respectively.

Two-way ANOVA analysis revealed a significant interaction between disease state and treatment ($F_{3,126} = 5.867$, $p < 0.05$) for

the kynurene:tryptophan ratio following stimulation of whole blood with the TLR1/2 agonist Pam3Csk. *Post hoc* analysis indicated a significant reduction in this ratio, in the IBS group, in stimulated preparations compared to the unstimulated samples (0.01774 ± 0.00089 vs. 0.01493 ± 0.00098 , $p < 0.05$) with no alteration in this ratio in the samples taken from the healthy controls (Figure 2A). Two-way ANOVA analysis revealed a significant interaction between disease state and treatment ($F_{3,126} = 8.475$, $p < 0.01$) following stimulation of whole blood with the TLR2 agonist HKLM. *Post hoc* analysis revealed a significant increase in this ratio in the healthy control group in stimulated vs. unstimulated samples (0.01681 ± 0.0006 vs. 0.02159 ± 0.00098 , $p < 0.001$) with no alteration in this ratio in the samples taken from IBS patients (Figure 2B). There was a trend toward a significant interaction between disease state and treatment ($F_{3,126} = 2.887$, $p = 0.092$) following stimulation with the TLR3 agonist Poly I:C. *Post hoc* analysis indicated a significant increase in the ratio in the healthy control group in stimulated vs. unstimulated samples (0.01681 ± 0.0006 vs. 0.01907 ± 0.00092 , $p < 0.05$) with no alteration in the ratio being demonstrated in the samples taken from IBS patients (Figure 2C).

There was no significant interaction between disease state and treatment following stimulation with the TLR4 agonist LPS ($F_{3,125} = 0.157$, $p = 0.962$). *Post hoc* analysis indicated a significant increase in the ratio in the stimulated compared to unstimulated samples from both controls (0.01681 ± 0.0006 vs. 0.02354 ± 0.00112 , $p < 0.001$) and IBS patients (0.01774 ± 0.00089 vs. 0.02367 ± 0.00137 , $p < 0.001$; Figure 3A). There was a trend toward a significant interaction between disease state and treatment following stimulation with the TLR5 agonist flagellin ($F_{3,124} = 3.604$, $p = 0.06$). The kynurene:tryptophan ratio was significantly elevated from unstimulated levels only in the samples taken from IBS patients (0.01774 ± 0.00089 vs. 0.02259 ± 0.0015 , $p < 0.01$; Figure 3B).

There was no significant interaction between disease state and treatment following stimulation with the TL6/2 agonist FSL1 ($F_{1,124} = 1.026$, $p = 0.313$) nor was there any alteration in the kynurene:tryptophan ratio in either healthy controls or IBS subjects (Figure 3C).

There was no significant interaction between disease state and treatment following stimulation with the TLR7 agonist imiquimod ($F_{3,125} = 0.146$, $p = 0.703$) although this agonist did induce an increase in the kynurene:tryptophan ratio in stimulated samples from control subjects (0.01681 ± 0.0006 vs. 0.01954 ± 0.00087 , $p < 0.05$) that was not evident in their IBS counterparts (Figure 4A). There was a trend toward a significant interaction between disease state and treatment following stimulation of samples with the TLR8 agonist ssRNA40 ($F_{3,124} = 3.491$, $p = 0.064$). This TLR ligand induced an increase in the kynurene:tryptophan ratio only in the stimulated samples from IBS patients (0.01774 ± 0.00089 vs. 0.02166 ± 0.00161 , $p < 0.05$; Figure 4B). There was no significant interaction between disease state and treatment following stimulation with the TLR9 agonist ODN2006 ($F_{3,125} = 2.324$, $p = 0.131$) nor was there any alteration in the kynurene:tryptophan ratio in the stimulated samples from either healthy control or IBS subjects (Figure 4C).

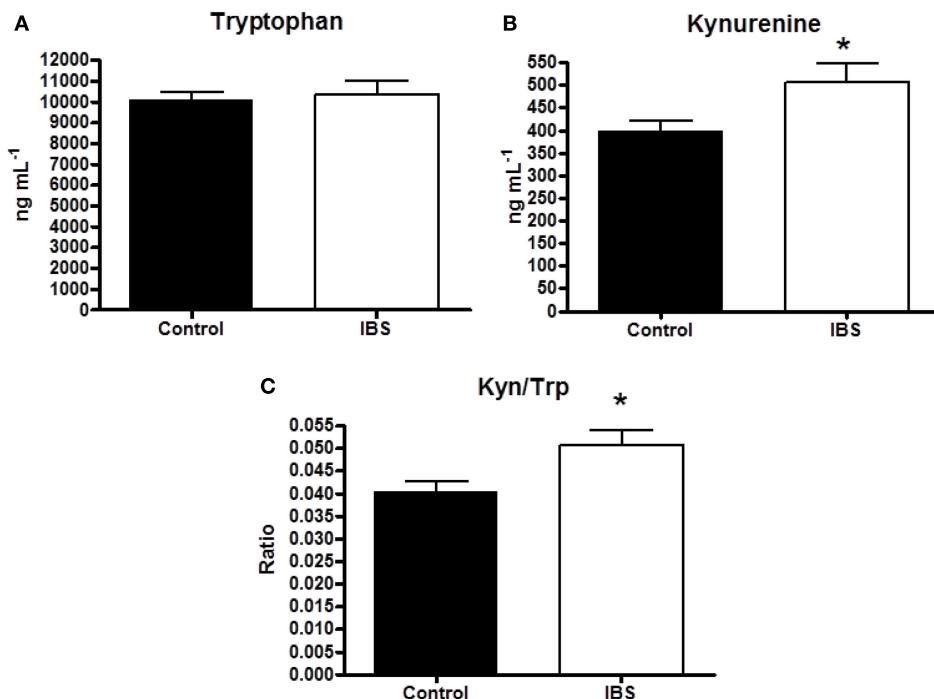


FIGURE 1 | (A) Plasma tryptophan concentrations ($\mu\text{g mL}^{-1}$) in healthy controls and IBS patients. **(B)** Plasma kynurene concentrations ($\mu\text{g mL}^{-1}$) in healthy controls and IBS patients. **(C)** Plasma kynurene:tryptophan

(Kyn:Trp) ratio in healthy controls and IBS patients. Data are expressed as means \pm SEM. Statistical differences between healthy controls and IBS patients were determined using Student's *t*-test. **p* < 0.05.

Table 1 | Tryptophan concentrations ($\mu\text{g mL}^{-1}$) in unstimulated and TLR agonist stimulated whole blood supernatants from healthy controls and IBS patients.

TLR stimulation	Control		IBS	
	Tryptophan	<i>p</i>	Tryptophan	<i>p</i>
Unstimulated	13.5 \pm 0.2	N/A	13.7 \pm 0.3	N/A
1/2 (Pam3Cys)	11.7 \pm 0.2	***	12.0 \pm 0.3	\$\$
2 (HKLM)	13.1 \pm 0.3	—	12.1 \pm 0.3	\$\$
3 (Poly I:C)	11.8 \pm 0.3	***	12.3 \pm 0.2	\$\$
4 (LPS)	13.7 \pm 0.4	—	13.7 \pm 0.4	—
5 (Flagellin)	13.3 \pm 0.3	—	13.3 \pm 0.3	—
6/2 (FSL1)	12.6 \pm 0.2	*	13.2 \pm 0.3	—
7 (Imiquimod)	13.0 \pm 0.2	—	13.0 \pm 0.2	—
8 (ssRNA40)	12.6 \pm 0.3	*	12.9 \pm 0.2	—
9 (ODN2006)	13.4 \pm 0.5	—	13.0 \pm 0.5	—

Data are expressed as means \pm SEM. Statistical differences between stimulated and unstimulated groups were determined using Bonferroni post hoc test. **p* < 0.05, Healthy control unstimulated vs. TLR agonist stimulated; ****p* < 0.001, healthy control unstimulated vs. TLR agonist Stimulated; \$\$*p* < 0.01, IBS unstimulated vs. stimulated; — *p* > 0.05.

DISCUSSION

Previous reports from our laboratory have indicated increased degradation of tryptophan along the kynurene pathway and highlighted the potential utility of these indices as biological

Table 2 | Kynurene concentrations ($\mu\text{g mL}^{-1}$) in unstimulated and TLR agonist stimulated whole blood supernatants from healthy controls and IBS patients.

TLR stimulation	Control		IBS	
	Kynurene	<i>p</i>	Kynurene	<i>p</i>
Unstimulated	0.22 \pm 0.01	N/A	0.24 \pm 0.01	
1/2 (Pam3Cys)	0.21 \pm 0.01	—	0.18 \pm 0.01	\$\$\$
2 (HKLM)	0.27 \pm 0.01	***	0.20 \pm 0.01	\$
3 (Poly I:C)	0.22 \pm 0.01	—	0.19 \pm 0.01	\$\$
4 (LPS)	0.33 \pm 0.01	***	0.32 \pm 0.02	\$\$\$
5 (Flagellin)	0.23 \pm 0.01	—	0.30 \pm 0.02	\$\$
6/2 (FSL1)	0.21 \pm 0.01	—	0.21 \pm 0.01	—
7 (Imiquimod)	0.25 \pm 0.01	—	0.26 \pm 0.02	—
8 (ssRNA40)	0.23 \pm 0.01	—	0.28 \pm 0.02	—
9 (ODN2006)	0.25 \pm 0.01	—	0.21 \pm 0.01	—

Data are expressed as means \pm SEM. Statistical differences between stimulated and unstimulated groups were determined using Bonferroni post hoc test. ****p* < 0.001, Healthy control unstimulated vs. TLR agonist stimulated; \$*p* < 0.05, IBS unstimulated vs. TLR agonist stimulated; \$\$*p* < 0.01, IBS unstimulated vs. stimulated; \$\$\$*p* < 0.001 IBS unstimulated vs. TLR agonist stimulated; — *p* > 0.05.

markers of IBS (Fitzgerald et al., 2008; Clarke et al., 2009a). Here we have confirmed these findings by demonstrating increased plasma kynurene concentrations as well as an elevation in the

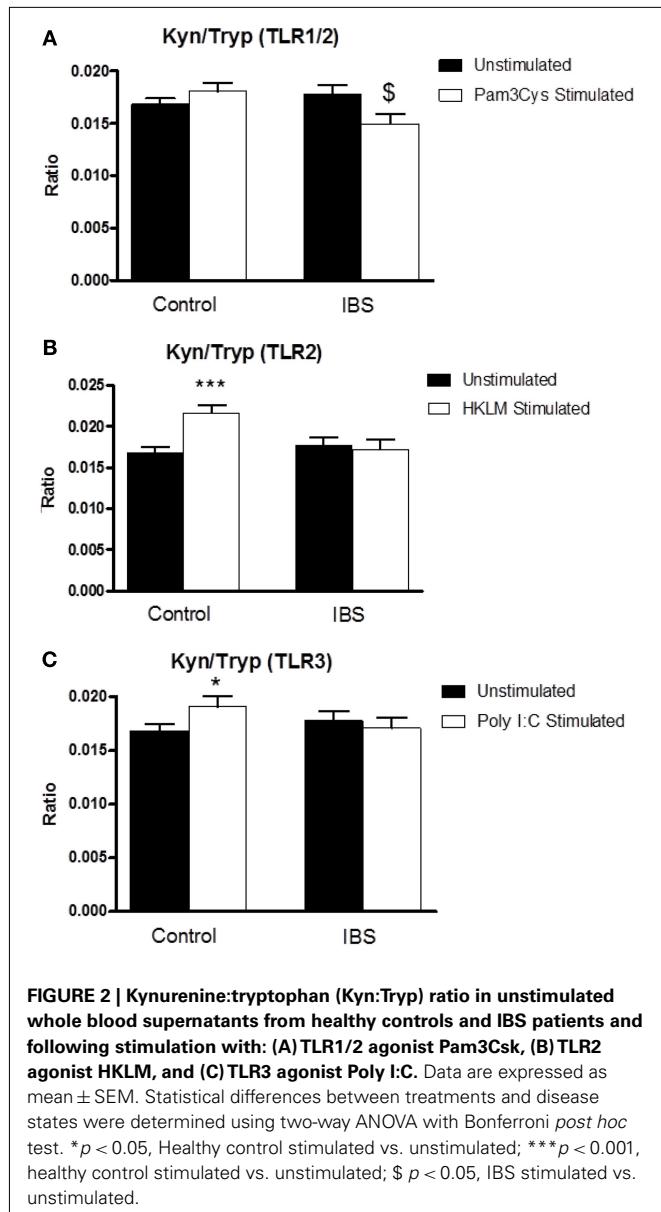


FIGURE 2 | Kynurenine:tryptophan (Kyn:Tryp) ratio in unstimulated whole blood supernatants from healthy controls and IBS patients and following stimulation with: (A) TLR1/2 agonist Pam3Csk, (B) TLR2 agonist HKLM, and (C) TLR3 agonist Poly I:C. Data are expressed as mean \pm SEM. Statistical differences between treatments and disease states were determined using two-way ANOVA with Bonferroni *post hoc* test. * $p < 0.05$, Healthy control stimulated vs. unstimulated; *** $p < 0.001$, healthy control stimulated vs. unstimulated; \$ $p < 0.05$, IBS stimulated vs. unstimulated.

kynurenine:tryptophan ratio in our IBS cohort compared to controls. Moreover, we have demonstrated, for the first time, that IBS patients exhibit a distinct tryptophan degradation profile downstream of TLR activation that is different from that of healthy controls, as indicated by the kynurenine:tryptophan ratio in the supernatants of whole blood preparations.

The pathophysiological relevance of increased activity along the kynurenine pathway in the plasma of IBS patients remains to be defined as does the source of such alterations. Of the TLRs that have previously been demonstrated to be upregulated in the colon in IBS (Brint et al., 2011) and to have an elevated cytokine release profile (McKernan et al., 2011), both TLR5 and TLR8 induced, on activation, an increase in the kynurenine:tryptophan ratio in our experimental system. It has recently been shown that alterations in gastrointestinal IDO activity in Crohn's disease correlate with systemic indices of kynurenine pathway activation (Gupta et al.,

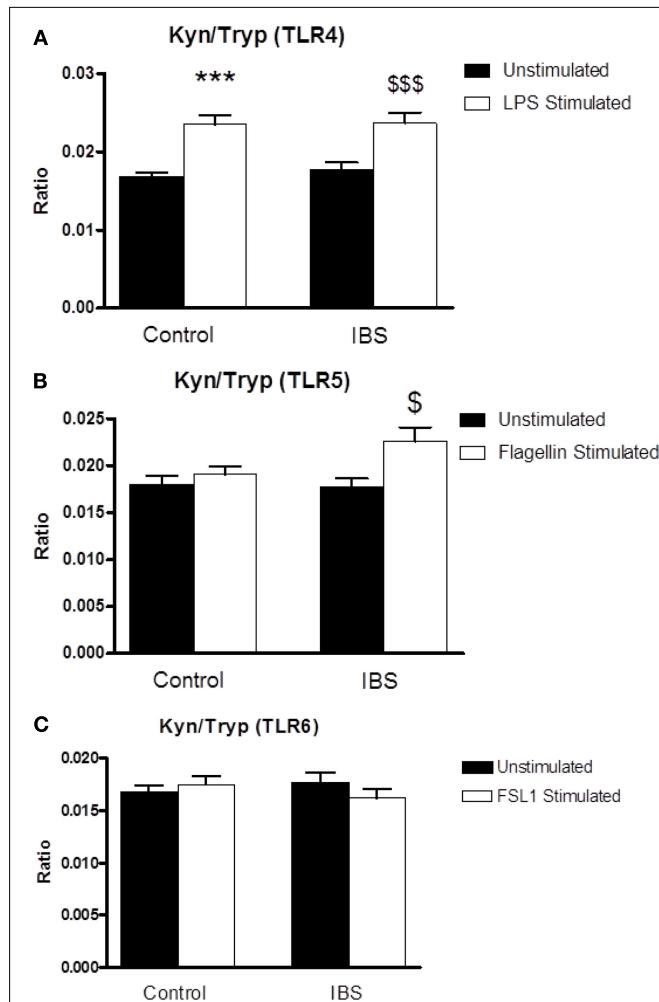


FIGURE 3 | Kynurenine:tryptophan (Kyn:Tryp) ratio in unstimulated whole blood supernatants from healthy controls and IBS patients and following stimulation with: (A) TLR4 agonist LPS, (B) TLR5 agonist flagellin, and (C) TLR6/1 agonist FSL1. Data are expressed as mean \pm SEM. Statistical differences between treatments and disease states were determined using two-way ANOVA with Bonferroni *post hoc* test. *** $p < 0.001$, Healthy control stimulated vs. unstimulated; \$ $p < 0.05$, IBS stimulated vs. unstimulated. There was no significant interaction between disease state and treatment following stimulation with the TL6/2 agonist FSL1 ($F_{1,124} = 1.026$, $p = 0.313$) nor was there any alteration in the kynurenine:tryptophan ratio in either healthy controls or IBS subjects (Figure 3C).

2011). Moreover, it is becoming increasingly apparent that peripheral blood alterations in kynurenine pathway metabolites can manifest at the CNS level (Raison et al., 2009) and that modulation of systemic pathway activity might be a useful therapeutic strategy (Reinhart and Kelly, 2011; Zwilling et al., 2011). Nevertheless, it will be important to determine whether the findings presented here are indeed reflected at the level of the intestinal compartment, an important site of tryptophan metabolism and serotonergic signaling in the periphery and further studies using supernatants from mucosal biopsies will be of value in this regard.

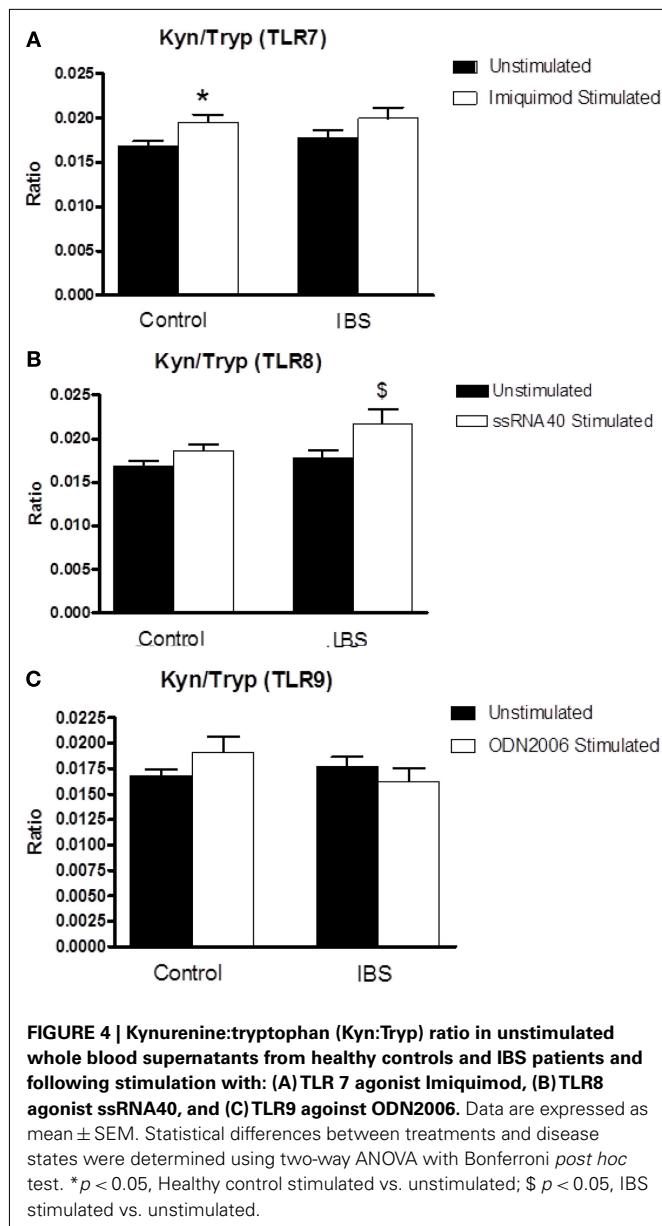


FIGURE 4 | Kynurene:tryptophan (Kyn:Trypt) ratio in unstimulated whole blood supernatants from healthy controls and IBS patients and following stimulation with: (A) TLR 7 agonist Imiquimod, (B) TLR8 agonist ssRNA40, and (C) TLR9 agonist ODN2006. Data are expressed as mean \pm SEM. Statistical differences between treatments and disease states were determined using two-way ANOVA with Bonferroni post hoc test. * $p < 0.05$, Healthy control stimulated vs. unstimulated; \$ $p < 0.05$, IBS stimulated vs. unstimulated.

The differential tryptophan degradation profile at TLR1/2, TLR2, and TLR3 is an important finding. These receptors are expressed at both intracellular and extracellular domains (Akira and Takeda, 2004; Sioud, 2006) and our data suggests the presence, in IBS, of TLR dysfunction at the level of both the cell membrane and the endosome. It is also noteworthy that TLRs can interact at a functional level to limit or inhibit the normal response to a particular ligand (Hajjar et al., 2001), a feature which may be of relevance to the reduced kynurene:tryptophan ratio observed in IBS patients following stimulation with the TLR1/2 ligand. An alternative explanation, given that our ratio alterations following stimulation of TLR1/2 and TLR3 are derived from reductions in tryptophan concentrations in conjunction with *decreased* kynurene, may involve the sequestering of tryptophan for the diverse cellular processes in which it is involved. Certain cytokines, for

example, can upregulate tryptophan hydroxylase expression, the rate-limiting enzyme in the conversion of tryptophan to serotonin (Lisak et al., 2011). Future studies will also need to address whether a differential leukocyte distribution might account for our findings although recent studies suggest that cellular activation might be a more important factor (Ohman et al., 2009a,b).

In any case, the blunted kynurene production at TLR2 and TLR3 in our IBS cohort is perplexing given the enhanced production of TNF- α and IL-8 demonstrated at those receptor subtypes, respectively, in our earlier study (McKernan et al., 2011) and the recently reported increased TLR2 expression on blood monocytes in IBS patients (Ohman et al., 2012). It is, however, worth noting that combinations of elevated cytokines may favour kynurene production over the singular increases described at these receptor subtypes (Taylor and Feng, 1991) and that the availability of certain co-factors is also required (Muller and Schwarz, 2007). Additionally, the spontaneous release of IL-8 from unstimulated samples, a previously reported phenomenon (Molina et al., 2006; Horton and Remick, 2010), from both groups and the baseline differences in the concentration of this cytokine may have obscured the impact that post-stimulation alterations in IL-8 production may have produced. Also of relevance is that repeated stimulation of specific TLRs with their ligands can induce unresponsiveness or immunotolerance, at least in cell line studies (Ehlers and Ravetch, 2007; Gomez-Llorente et al., 2010). This raises the possibility that the defective response at TLR2 and TLR3 in IBS subjects might alternatively be due to prolonged stimulation by endogenous ligands. This is in line with suggestions that activation of TLRs by their endogenous ligands might have a role in the promotion of systemic inflammation (Marshak-Rothstein, 2006). Interestingly host mRNA can activate TLR3 (Kariko et al., 2004) and high mobility group box 1 (HMGB1) is a ligand for TLR2 (Yu et al., 2006). Total plasma mRNA levels remain to be profiled in IBS and although fecal HMGB1 has been proposed as a novel marker of intestinal inflammation, it is also uncharacterised in the disorder.

Interestingly, we found that the downstream consequences of TLR4 activation for tryptophan metabolism are equivalent in both healthy controls and IBS patients. Previously we reported an increased release of the same two cytokines (IL-1B, TNF- α) in IBS patients following stimulation of this receptor with LPS as occurred following TLR5 activation with flagellin (McKernan et al., 2011). At first glance, it appears unusual that an exaggerated release of the same two cytokines would elicit differential responses in terms of kynurene pathway indices at different TLRs if the immunoresponsive enzyme IDO mediates the effects we have observed. However, it should be noted that the magnitude of the cytokine response following TLR4 stimulation is greater than that induced following TLR5 activation suggesting that the more modest cytokine release pattern at the latter receptor may be required to tease apart the differential downstream effects of TLR stimulation on kynurene production. The cellular distribution of the TLRs may also be of importance: it is worth noting that TLR4 is much more highly expressed than TLR5 in monocytes (Hornung et al., 2002) and LPS stimulation of whole blood primarily results in cytokine release from this cell type (Pace and Heim, 2011). In contrast, TLR5 is more highly expressed in NK cells and T cells

than TLR4, although the magnitude of dominance TLR5 enjoys in these cell populations does not match the superiority of TLR4 in terms of monocyte expression patterns. Although we did not analyze the cellular composition of our whole blood preparations, this theory is consistent with the observation that IBS subjects display an increased level of T-cell activation (Ohman et al., 2009a). This explanation also reflects the view in the literature that IBS is a disorder characterised by a low grade inflammation as opposed to an immune disorder *per se* (Clarke et al., 2009b). However it should also be noted that TLR4 stimulation increased kynurenone concentrations without altering tryptophan concentrations which may suggest the involvement of enzymatic components of the kynurenone pathway other than IDO. Indeed differential activation of kynurenone pathway enzymes can be a feature of a systemic immune challenge with LPS (Connor et al., 2008).

A limitation of the current study is that whole blood stimulations of TLRs may only partially model the complexity of the *in vivo* response. This is especially pertinent when one considers that the entire family of receptors may be simultaneously susceptible to activation *in vivo* depending on the combination of ligands present at physiologically relevant concentrations. Indeed, it is known that combinations of TLR ligands can either synergistically induce cytokine gene expression (Makela et al., 2011) or result in a blunted response (Marshall et al., 2007), depending on the particular ligand combination employed. Consequently, our results do not clarify whether the cumulative, sometimes opposing and sometimes synergistic, downstream effects of multiple simultaneous TLR activations are responsible for the enhanced resting state degradation of tryptophan along the kynurenone pathway in IBS. Moreover, it is unclear at present which receptor has the greatest biological influence on kynurenone metabolism *in vivo*. Nevertheless whole blood stimulation of single receptor subtypes is considered a valid strategy to interrogate their responsiveness in general and to determine whether particular patient populations are primed for aberrant responses (Pace and Heim, 2011). Moreover, an assessment of the kynurenone:tryptophan ratio is a previously validated method for the assessment of IDO activity in

cell culture supernatants (Schroecksnadel et al., 2005; Mahanonda et al., 2007; Schroecksnadel et al., 2011). The fact that IBS populations show both basal alterations in this pathway and a distinct profile subsequent to stimulation of individual TLRs with their various ligands only adds to their potential utility as biomarkers of the disorder, albeit as components of a biomarker panel of other promising indices rather than as unique identifiers themselves. Further studies are urgently required, both to define such a biomarker panel and to determine the sensitivity and specificity of such an approach.

In conclusion, we have provided novel evidence demonstrating that TLR activation induces a pattern of downstream tryptophan degradation along the kynurenone pathway that differentiates IBS patients from healthy controls. This bolsters current and previous findings that highlighted baseline disturbances in this pathway and illuminates a mechanism through which TLR responses can functionally impact on brain-gut axis signaling in this disorder. Moreover, this implicates the modulation of TLRs as a novel therapeutic strategy in this debilitating condition.

ACKNOWLEDGMENTS

Timothy G. Dinan, John F. Cryan, and Eamonn M. Quigley designed the study. Eamonn M. Quigley, Declan P. McKernan, and Gabor Gaszner coordinated subject recruitment. Declan P. McKernan, Gabor Gaszner, and Gerard Clarke carried out the research. Gerard Clarke analyzed the data, interpreted the results and wrote the paper. The Alimentary Pharmabiotic Centre is a research centre funded by Science Foundation Ireland (SFI), through the Irish Government's National Development Plan. The authors and their work were supported by SFI (grant nos. 02/CE/B124 and 07/CE/B1368) and by GlaxoSmithKline. Gerard Clarke is in receipt of a research grant from the American Neurogastroenterology and Motility Society (ANMS). Timothy G. Dinan, John F. Cryan and Gerard Clarke are also supported by the Health Research Board (HRB) through Health Research Awards (grant no HRA_POR/2011/23).

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- Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.
- Received:* 27 March 2012; *paper pending published:* 11 April 2012; *accepted:* 26 April 2012; *published online:* 21 May 2012.
- Citation:* Clarke G, McKernan DP, Gaszner G, Quigley EM, Cryan JF and Dinan TG (2012) A distinct profile of tryptophan metabolism along the kynure-nine pathway downstream of toll-like receptor activation in irritable bowel syndrome. *Front. Pharmacol.* 3:90. doi: 10.3389/fphar.2012.00090
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A comprehensive review of the pharmacodynamics, pharmacokinetics, and clinical effects of the neutral endopeptidase inhibitor racecadotril

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Racecadotril, via its active metabolite thiorphane, is an inhibitor of the enzyme neutral endopeptidase (NEP, EC 3.4.24.11), thereby increasing exposure to NEP substrates including enkephalins and atrial natriuretic peptide (ANP). Upon oral administration racecadotril is rapidly and effectively converted into the active metabolite thiorphane, which does not cross the blood-brain-barrier. Racecadotril has mainly been tested in animal models and patients of three therapeutic areas. As an analgesic the effects of racecadotril across animal models were inconsistent. In cardiovascular diseases such as hypertension or congestive heart failure results from animal studies were promising, probably related to increased exposure to ANP, but clinical results have not shown substantial therapeutic benefit over existing treatment options in cardiovascular disease. In contrast, racecadotril was consistently effective in animal models and patients with various forms of acute diarrhea by inhibiting pathologic (but not basal) secretion from the gut without changing gastro-intestinal transit time or motility. This included studies in both adults and children. In direct comparative studies with loperamide in adults and children, racecadotril was at least as effective but exhibited fewer adverse events in most studies, particularly less rebound constipation. Several guidelines recommend the use of racecadotril as addition to oral rehydration treatment in children with acute diarrhea.

Keywords: racecadotril, neutral endopeptidase, analgesia, hypertension, congestive heart failure, diarrhea, loperamide

BACKGROUND

Acute diarrhea is an alteration of normal bowel movements characterized by an increase in the water content, volume, or frequency of stools. The most common causes are bacterial and viral infections, particularly rotavirus infections, but the specific spectrum of infectious agents depends on the clinical setting (Farthing, 2000). Such infections cause intestinal hypersecretion leading to fluid loss and dehydration. Accordingly, oral rehydration is the cornerstone of treatment, and a standardized glucose-electrolyte solution has been developed under the auspices of the World Health Organization and is being used with great success. While this has significantly improved the prognosis of acute diarrhea, it remains a clinical problem in both the developing world and in industrialized countries and, particularly in developing countries, acute diarrhea is still responsible for the death of two to three million individuals per year worldwide (Farthing, 2006).

While the infection underlying acute diarrhea typically is self-limiting, the associated dehydration can be life-threatening, particularly in children or the elderly. Moreover, a shortening of the duration of acute diarrhea can also be an important medical

aim. Therefore, drug treatment can also be a relevant part of the therapeutic approach, in most cases given on top of rehydration treatment. Among anti-diarrhea drugs antibiotics are typically limited to severe cases and other special situations. More frequently, μ -opioid receptor agonists such as codeine, loperamide, and morphine are being employed, among which loperamide has become most frequently used (Baldi et al., 2009). Their main mechanism of action is a reduction of gut motility and accordingly they can cause secondary constipation, abdominal pain, and abdominal distension.

Against this background, racecadotril has been developed as a possible alternative to the use of μ -opioid receptor agonists. Following its original registration as a prescription drug in France in 1992 it meanwhile is available in many countries around the globe, and since 2005 in some of them as a non-prescription drug. The present manuscript reviews the pharmacodynamic, pharmacokinetic, and clinical data for racecadotril and its active metabolite thiorphane. While the clinical focus of the manuscript is on the role of racecadotril in the treatment of diarrhea, we will also discuss other potential uses as they will aid the understanding of the overall clinical profile of the drug. Racecadotril has been reviewed in the past (Lecomte, 2000; Matheson and Noble, 2000; Schwartz, 2000) but those articles had a more limited scope and more than 40 new studies have been published since.

Abbreviations: ANP, atrial natriuretic peptide; i.c.v., intra-cerebroventricular; i.p., intra-peritoneal; i.v., intravenous; NEP, neutral endopeptidase.

MOLECULAR EFFECTS OF RACECADOTRIL

Racecadotril, formerly known as acetorphan, is a prodrug, which is converted to the active metabolite thiorphane (see below; **Figure 1**). Acetyl-thiorphane is another active metabolite of racecadotril but yields only low potency NEP inhibition (Lambert et al., 1993). Racecadotril has stereoisomers, and the *S*- and *R*-isomers of racecadotril are named ecadotril (also known as BP102 or as sinorphane) and retorphan, respectively (Lecomte et al., 1990). Thus, in the subsequent text racecadotril and thiorphane refer to the racemate, whereas ecadotril refers to the *S*-isomer of racecadotril.

At the molecular level racecadotril and thiorphane act by inhibiting the enzyme neutral endopeptidase (NEP, EC 3.4.24.11; see below), which is a membrane-metalloendopeptidase also known as enkephalinase. NEP has various substrates including enkephalins (hence the name enkephalinase) but also atrial natriuretic peptide (ANP), brain natriuretic peptide, substance P, neuropeptides, and neurokinin Y (van Kemmel et al., 1996; Turville and Farthing, 1997). Therefore, NEP inhibition can potentially affect any of these mediators and observed *in vivo* effects in different organ systems may not always relate to the same enzyme substrate (see below).

The first report on thiorphane described an IC_{50} of 4.7 nM for NEP inhibition in striatal membranes (Roques et al., 1980). Inhibition of purified NEP activity from mouse brain yielded affinity estimates (K_i values) of 6.1 and 4500 nM for thiorphane and racecadotril, respectively; however, when racecadotril was pre-incubated with rat brain membranes for 15 min, an apparent K_i value of 8.6 nM was observed, probably reflecting rapid *in vitro* conversion to thiorphane (Lecomte et al., 1986). A similar study reported an IC_{50} of 1.8 nM for thiorphane with racecadotril being 1000 times less potent and acetyl-thiorphane having a value of 316 nM (Lambert et al., 1993, 1995). For *in vitro* inhibition of rat kidney NEP an IC_{50} of 5.4 nM was reported (Fink et al., 1995), apparently reflecting *in vitro* conversion to thiorphane as shown before in rat brain (Lecomte et al., 1986).

A second approach to assess thiorphane affinity for NEP has been radioligand binding studies. In saturation binding studies in various mouse tissues [3 H]-thiorphane exhibited an affinity (K_d value) of 0.46–0.77 nM, and the density of [3 H]-thiorphane binding sites was well correlated with measured NEP activity in a panel of 11 different mouse tissues (de la Baume et al., 1988). Similar saturation binding experiments using [3 H]-racecadotril as the ligand and reported an affinity of 4–5 nM in rats (Fournet-Bourguignon et al., 1992), apparently reflecting conversion of racecadotril to

thiorphane in the assay (Lecomte et al., 1986). A third approach has been to measure occurrence of enkephalin break-down products such as the tripeptide Tyr-Gly-Gly, and in isolated rat brain slices this has yielded an IC_{50} of 9 nM for thiorphane (Giros et al., 1986).

Correspondingly, it has repeatedly been observed that oral, intra-peritoneal (i.p.), or intravenous (i.v.) administration of racecadotril or ecadotril leads to a rapid reduction in NEP activity in plasma (Spillantini et al., 1986; Lecomte et al., 1990; Dussaule et al., 1991, 1993; Stasch et al., 1996; Duncan et al., 1999; Lecomte, 2000), kidney (Gros et al., 1989), and brain (Lecomte et al., 1986; Spillantini et al., 1986). Such studies were performed with consistent results in rats (Lecomte et al., 1986; Stasch et al., 1996; Wegner et al., 1996; Duncan et al., 1999), mice (Lecomte et al., 1986), and humans (Spillantini et al., 1986; Gros et al., 1989; Lecomte et al., 1990; Dussaule et al., 1991, 1993; Lecomte, 2000). *In vivo* inhibition of enkephaline metabolite formation was also observed in rat spinal cord after i.v. racecadotril (Llorens-Cortes et al., 1989) or in mouse striatum after intra-cerebro-ventricular (i.c.v.) thiorphane (Llorens-Cortes et al., 1986). Of note, assessment of NEP inhibition by measuring endogenous enkephalins can yield false negative results as enkephalines can also be metabolized by other aminopeptidases such as EC 3.4.11.2, and this can compensate for NEP inhibition (Bourgoin et al., 1986; Llorens-Cortes et al., 1986). As ANP also is a NEP substrate, NEP inhibition can also be assessed by changes of ANP concentrations in plasma and urine, which are described in detail in Section “Cardiovascular Studies.”

The *R*- and *S*-stereoisomers of thiorphane inhibited purified NEP activity with similar potency 1.7 vs. 2.2 nM, respectively, and occurrence of the enkephalin metabolite Tyr-Gly-Gly with an IC_{50} of 10 nM (Giros et al., 1987). In the same study occurrence of Tyr-Gly-Gly in mouse striatum was also inhibited with similar potency by i.v. administration of ecadotril and retorphan, the stereoisomers of racecadotril (ED₅₀ 0.4 and 0.8 mg/kg, respectively). On the other hand, with the same oral dose of ecadotril and retorphan inhibition of the *in vivo* binding of [3 H]-racecadotril in mouse kidney was somewhat stronger for the *S*-isomer (Lecomte et al., 1990). Similarly, a 30-mg oral dose of ecadotril produced somewhat greater inhibition of NEP activity and ANP levels in human plasma than the same dose of retorphan (Lecomte et al., 1990).

In conclusion, racecadotril and its metabolite acetyl-thiorphane are only low potency NEP inhibitors. However, racecadotril is rapidly converted to the active metabolite thiorphane *in vitro* (Lecomte et al., 1986) and *in vivo* (see Pharmacokinetic and

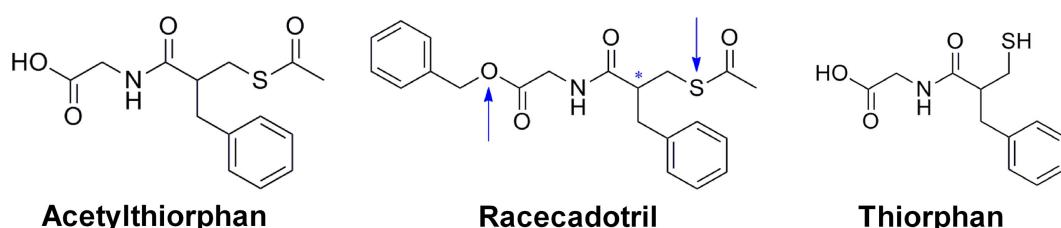


FIGURE 1 | Chemical structures of racecadotril and its two metabolites thiorphane and acetyl-thiorphane. The blue arrows indicate the sites of metabolism; the asterisk indicates the chiral center of the molecule.

Drug–Drug Interaction Studies), and thiorphan is an about 1000 times more potent NEP inhibitor than racecadotril with reported potencies of 0.4–9 nM. The S-isomers of racecadotril and thiorphan may be slightly more potent than the R-isomers.

PHARMACOKINETIC AND DRUG–DRUG INTERACTION STUDIES

ANIMAL PHARMACOKINETIC STUDIES

In mice, following i.v. administration, racecadotril was rapidly metabolized to thiorphan; thus, 30 min after the injection only thiorphan was recovered from the kidney whereas the parent compound racecadotril was not detected (de la Baume et al., 1988). In rats 92% of a single-dose (10 mg/kg) of radioactively labeled racecadotril was eliminated within 24 h (Matheson and Noble, 2000).

HUMAN PHARMACOKINETIC STUDIES

Racecadotril is rapidly absorbed following oral administration. For example, in a single-dose, placebo-controlled, double-blind cross-over study racecadotril doses of 30, 100, and 300 mg reached C_{\max} within 60 min after oral administration; the latter two doses were associated with significant inhibition of plasma NEP activity seen as early as 30 min after ingestion, and that inhibition exhibited a $t_{1/2}$ of 3 h (Lecomte, 2000; Matheson and Noble, 2000). A single-dose study in healthy elderly people reported similar findings (Matheson and Noble, 2000). After ingestion of a single oral dose of 300 mg racecadotril plasma thiorphan levels peaked after 60 min reaching 805–1055 nM; after 240 min plasma levels were still at 92–204 nM (Hinterleitner et al., 1997). In a more recent study, a well validated liquid chromatography/tandem mass spectrometry method has been used to detect thiorphan in human plasma; 20 volunteers received a single oral dose of 200 mg racecadotril, which resulted in a thiorphan C_{\max} of 520 ng/mL, a t_{\max} of 1.35 h and a $t_{1/2}$ of 6.14 h (Xu et al., 2007). The pharmacokinetic properties of racecadotril are similar with chronic dosing as observed in a placebo-controlled, double-blind study with 30, 100, and 300 mg racecadotril being given orally thrice daily for 7 days, where pharmacokinetic parameters were similar on day 1 and day 7 and to the values observed in the single-dose studies (Matheson and Noble, 2000), indicating lack of accumulation upon chronic dosing. Concomitant food intake does not modify the bioavailability of racecadotril but peak NEP inhibition is delayed by about 90 min (data on file).

After oral administration racecadotril is rapidly and effectively metabolized to the active metabolite thiorphan which is the predominant species detected in plasma; the occurrence of thiorphan coincides in time with the inhibition of plasma NEP (Hinterleitner et al., 1997; Xu et al., 2007). Thiorphan has a plasma protein binding of approximately 90% (data on file).

An important question for any drug interfering with the endogenous opioid system is whether it crosses the blood–brain-barrier, i.e., whether upon oral racecadotril administration parent compound or active metabolite reaches relevant levels in the brain to cause NEP inhibition. Animal studies suggest that central nervous effects can occur after parenteral administration of racecadotril but not after oral racecadotril or parenteral thiorphan administration (see Studies on Central Nervous System

Function); this is the apparent result of the combination of the rapid conversion of absorbed racecadotril to thiorphan and the lack of thiorphan passage through the blood–brain-barrier due to its less lipophilic chemical structure (Figure 1). In line with these animal data it has been reported that i.v. administration of racecadotril causes quantitatively similar NEP inhibition in plasma and cerebrospinal fluid in five healthy volunteers as compared to saline infusion (Spillantini et al., 1986), whereas a single high oral racecadotril dose (20 mg/kg) to two volunteers caused a marked reduction of plasma NEP activity within 30 min but did not affect liquor NEP activity (Lecomte, 2000). In accordance with the proposed lack of central nervous effects of orally administered racecadotril, a placebo-controlled cross-over study in 12 subjects being treated with 300 mg/kg racecadotril for 3 days did not detect any impairment of vigilance (Lecomte, 2000).

The active racecadotril metabolite thiorphan is converted to inactive metabolites, but the pathways mediating this conversion have not been characterized in great detail. While two studies using different methodological approaches have reported that racecadotril inhibits CYP 3A4-mediated formation of metabolites of the cancer chemotherapeutic drug irinotecan with an IC_{50} of 46 μ M in human liver microsomes (Haaz et al., 1998a,b), it should be noted that this concentration is equivalent to about 10,000 times the potency of thiorphan for NEP inhibition. Thus, in concentrations which are achieved by therapeutic doses racecadotril is neither an inhibitor nor an inducer of cytochrome P450 enzymes and also not a substrate of the P-glycoprotein transporter (data on file). Accordingly, to date no interactions with other medicinal products have been identified and specifically concomitant treatment with loperamide or nifuroxazide does not affect the pharmacokinetics of racecadotril (data on file). Moreover, racecadotril does not modify protein binding of active substances strongly bound to proteins such as tolbutamide, warfarin, niflumic acid, digoxin, or phenytoin (data on file). The elimination of the inactive thiorphan metabolites occurs mainly via the renal route (data on file).

STUDIES ON CENTRAL NERVOUS SYSTEM FUNCTION

As discussed in Section “Pharmacokinetic and Drug–Drug Interaction Studies,” parenteral administration of racecadotril can have central nervous effects but these are not apparent with oral racecadotril or parenteral thiorphan treatment. Most effects of racecadotril and its metabolites on brain function apparently are mediated by inhibition of enkephalin degradation, as opiate receptor antagonism in many cases abolishes them (see below).

ANALGESIA

Based on the role of morphine and other opiates in pain control, it was an obvious choice to test racecadotril in various pain models. The results have been rather inconsistent across models but much more consistent within models indicating that NEP inhibition selectively targets certain pain pathways. Thus, racecadotril was analgesic in the hot-plate jump test in unspecified mice with i.c.v. administration (Roques et al., 1980) and with i.v. administration in Swiss albino (Lecomte et al., 1986; Costentin et al., 1998), NMRI (Lambert et al., 1993, 1995) and DBA/2J mice but not C57BL/6J mice (Michael-Titus et al., 1989). Interestingly, in the latter study racecadotril increased locomotion in both strains, indicating that

the difference in analgesic effect does not reflect a pharmacokinetic strain difference. In NMRI mice i.v. acetyl-thiorphane and thiorphane were also effective in this model, although thiorphane less than the more lipophilic acetyl-thiorphane and racecadotril (Lambert et al., 1993, 1995). I.v. racecadotril was also analgesic in Swiss albino mice in the tail-withdrawal and the phenylbenzoquinone-induced writhing test (Lecomte et al., 1986). In an arthritis-based pain model in rats (vocalization induced by applying pressure to the left hind paw) racecadotril also exhibited analgesic activity (Kayser and Guilbaud, 1983), but this was weaker than that of the mixed peptidase inhibitor kelatorphan in the same model (Kayser et al., 1989). In the same test racecadotril was not analgesic in non-arthritis rats (Kayser and Guilbaud, 1983). In unspecified mice i.c.v. thiorphane was ineffective in the tail removal test but enhanced the analgesic effects of several enkephalines which are NEP substrates but not of others which are not NEP substrates (Roques et al., 1980). Moreover, racecadotril did not exhibit analgesic properties in the hot-plate licking test in Swiss albino (Lecomte et al., 1986) or NMRI mice (Lambert et al., 1993) or in albino mice in the tail immersion test, but enhanced the anti-nociceptive effect of an exogenously administered enkephalin in the latter model (Livingston et al., 1988). In GB1 mice in the acetic acid-induced abdominal constriction assay racecadotril was effective only at subcutaneous doses of 10 mg/kg and higher (Gray et al., 1998).

Upon chronic stimulation the opioid system can exhibit both desensitization and sensitization. Thus, the analgesic response to i.v. racecadotril was blunted after 14 days of i.c.v. treatment with thiorphane in rats (Bousselma et al., 1991a). However, no cross-desensitization between racecadotril and morphine was observed in mice (Bousselma et al., 1991b) or rats, and in the latter also no cross-sensitization for locomotive effects was observed (Khalouk-Bousselma and Costentin, 1994).

It has also been tested whether racecadotril can enhance analgesic effects of other treatments. Thus, racecadotril or thiorphane enhanced naloxone-sensitive analgesia induced by transcranial electrostimulation in rats (Malin et al., 1989) or by nefopam in mice (Gray et al., 1999), and also enhanced analgesic effects of electroacupuncture in rats (Zhou et al., 1990). In the acetic acid-induced abdominal constriction pain model in GB1 mice racecadotril enhanced the analgesic effect of morphine and of anti-depressants such as dothiepine and amitriptyline (Gray et al., 1998). On the other hand, racecadotril did not enhance naloxone-sensitive analgesic effects in four different pain models in mice (Michael-Titus and Costentin, 1987), and neither i.v. racecadotril nor i.c.v. thiorphane enhanced analgesic effects of dopamine receptor agonists in mice (Michael-Titus et al., 1990a). Another type of interaction between pain-related pathways was suggested by findings in mice in which nociceptin attenuated the analgesic response to racecadotril (Costentin et al., 1998).

In conclusion, racecadotril has direct analgesic effects and can enhance analgesic effects of some other types of drugs in some but not all pain models, but such studies were largely restricted to parenteral administration. Similar to direct opioid receptor agonists, racecadotril-induced analgesia can undergo desensitization upon long-term exposure but despite both morphine and racecadotril effects involving opioid receptors, they did not exhibit cross-desensitization. As the anti-nociceptive effects of racecadotril are

restricted to some model systems, it can be expected that, if at all, racecadotril would be effective only in some forms of pain in patients and only upon parenteral administration; however, a clinical testing of potential analgesic effects of racecadotril in patients has not been reported to our knowledge.

OTHER NERVOUS SYSTEM STUDIES

The administration of natural or synthetic opioid receptor agonists elicits a locomotor response in rodents, which is considered to be an index of the activity of mesolimbic dopaminergic neurons. In both mice and rats i.v. racecadotril was reported to enhance locomotion in mice and rats in a naloxone-sensitive manner, and that response was blocked by a dopamine receptor antagonist and enhanced by a dopamine uptake inhibitor (Michael-Titus et al., 1987, 1990b). Similar to the analgesic racecadotril response (see Analgesia), the locomotor racecadotril response also was desensitized following a 14-day i.c.v. treatment with thiorphane (Bousselma et al., 1991a). In line with the idea that racecadotril can affect dopaminergic transmission in the brain, it was found that i.v. racecadotril modulates dopaminergic transmission in rat olfactory tubercle but not striatum (Dourmap et al., 1990).

Both the opioid and the dopamine system in the brain are prone to addiction. Therefore, it was important to find that racecadotril did not exhibit abuse potential in rats or monkeys in doses up to 50 mg/kg (Knisely et al., 1989). Nevertheless, i.p. racecadotril prevented some but not all withdrawal symptoms in opioid-habituated mice and rats (Livingston et al., 1988; Dzolic et al., 1992). However, racecadotril alone was ineffective in inhibiting naloxone-induced morphine withdrawal symptoms in mice in another study, but a combination of subthreshold doses of racecadotril, CCK-4, and caerulein was effective (Bourin et al., 1999). In a double-blind, double-dummy, clinical proof-of-concept study in 19 heroin-addicted patients comparing 50 mg i.v. racecadotril and 75 µg oral clonidine; racecadotril appeared more effective than clonidine against objective withdrawal symptoms as quantified by the Himmelsbach scale, whereas both treatments were similarly effective against subjective withdrawal symptoms (Hartmann et al., 1991).

Some studies have explored potential metabolic effects of racecadotril. In sheep oral and i.v. administration of racecadotril increased food intake, whereas i.c.v. thiorphane reduced it; as oral racecadotril treatment does not lead to NEP inhibition in the brain and as i.c.v. thiorphane did not mimick the racecadotril effects, this appears to be a peripheral effect (Riviere and Bueno, 1987). Moreover, naltrexone blocked the former but not the latter effect, indicating an involvement of peripheral opioid receptors. A study with i.v. racecadotril in cats reported increases in sham food intake (Bado et al., 1989). In rats i.v. racecadotril caused naloxone-insensitive dose-dependent lowering of blood glucose which was accompanied by increased plasma insulin and C-peptide levels (Wu et al., 2010). While racecadotril did not affect insulin release from isolated pancreatic islets, the muscarinic receptor antagonist atropine blocked and the cholinesterase inhibitor physostigmine enhanced the racecadotril-induced insulin elevation, indicating that they may occur via modulation of parasympathetic nerve activity. In a follow-up study the same investigator group provided evidence that i.v. racecadotril and i.c.v. thiorphane directly inhibit

an insulin-degrading enzyme in the brain, and that brain insulin acts via the vagal nerve on plasma glucose (Lee et al., 2011). The clinical findings of these intriguing observations have not been explored in patients to our knowledge.

In a mouse behavioral dispair test, a model system for anti-depressant effects, immobility time was reduced by 10 mg/kg i.v. or 50 mg/kg i.p. in mice (Lecomte et al., 1986). Moreover, it was reported that thiorphan can provide neuroprotection in newborn mice (Medja et al., 2006).

CARDIOVASCULAR STUDIES

Most effects of racecadotril and its metabolites in the cardiovascular system apparently are largely mediated by inhibition of degradation of the natriuretic peptides. Thus, racecadotril-, ecadotril-, or thiorphan-induced inhibition of ANP break-down and/or elevation of ANP levels have been demonstrated in rats (Fink et al., 1996; Stasch et al., 1996), mice (Gros et al., 1989, 1990a,b; Lecomte et al., 1990; Stasch et al., 1996), and humans (Gros et al., 1989; Dussaule et al., 1991, 1993; Piquard et al., 2002) as assessed in plasma (Gros et al., 1989, 1990b; Lecomte et al., 1990; Dussaule et al., 1991, 1993; Schmitt et al., 1994; Fink et al., 1996; Stasch et al., 1996; Piquard et al., 2002) or in tissues such as kidney (Gros et al., 1989, 1990a). The increase in circulating ANP concentrations is typically associated with increased concentrations of the ANP-generated second messenger cyclic GMP in plasma (Dussaule et al., 1993; Stasch et al., 1995, 1996; Cleland and Swedberg, 1998; Piquard et al., 2002) or urine of animals and patients (Lecomte et al., 1990; Dussaule et al., 1991; Schmitt et al., 1994; Stasch et al., 1995, 1996; Cleland and Swedberg, 1998; Kimura et al., 1998; Duncan et al., 1999). The racecadotril and ecadotril effects on plasma ANP may be even more pronounced in patients (see below). Based on these findings, racecadotril has been studied in various animal models, in healthy volunteers, and in patient groups in which an increased exposure to ANP has been deemed beneficial.

An important physiological effect of ANP is promoting diuresis and natriuresis. Based on the consistent racecadotril effects on ANP, effects of racecadotril have been studied in various animal models and in humans. Thus, oral racecadotril treatment increased natriuresis in normotensive rats, which was accompanied by enhanced diuresis in some (Bralet et al., 1990) but not other studies (Stasch et al., 1995, 1996). Racecadotril-induced diuresis and natriuresis have also been reported in healthy volunteers (Gros et al., 1989; Lecomte et al., 1990). This was accompanied by an elevated glomerular filtration rate and lowered renal blood flow whereas plasma aldosterone concentration, renin activity, and mean arterial blood pressure were not altered (Schmitt et al., 1994).

TREATMENT OF ARTERIAL HYPERTENSION

Racecadotril has been tested in various animal models of hypertension, specifically for its ability to lower blood pressure, improve renal function, and to prevent or reverse organ hypertrophy. These models include spontaneously hypertensive rats (Bralet et al., 1990) and its stroke-prone substrain (Stasch et al., 1995), transgenic rats harboring a mouse renin gene (Stasch et al., 1996), hypertension induced by treatment with the immunosuppressant cyclosporine A in rats (Takeda et al.,

2000), and the rat deoxycorticosterone acetate-salt model of mineralocorticoid-induced hypertension (Ito et al., 1999). Blood pressure lowering by oral racecadotril or ecadotril was consistently shown (Stasch et al., 1995, 1996; Ito et al., 1999; Takeda et al., 2000). In a first pilot study in 12 hypertensive patients receiving increasing sinorphan doses (25–200 mg bid) for a total of 6 weeks a dose-dependent blood pressure reduction was also observed (Lefrancois et al., 1990). In a subsequent randomized, double-blind clinical pilot study with a cross-over design in 16 hypertensive patients racecadotril was less effective than captopril in lowering blood pressure, but the combination of both drugs was more effective than either monotherapy (Favrat et al., 1995).

In line with the effects in normotensive animals and healthy human volunteers, racecadotril also increased diuresis and/or natriuresis in spontaneously hypertensive rats (Bralet et al., 1990), in transgenic rats (Stasch et al., 1996) and in deoxycorticosterone acetate-salt-treated rats (Ito et al., 1999) whereas numerical increases of diuresis and natriuresis did not yield statistical significance in stroke-prone spontaneously hypertensive rats (Stasch et al., 1995).

A prognostically relevant complication of hypertension is the development of hypertrophy of the heart and other cardiovascular organs. In this regard, chronic treatment with racecadotril reduced heart hypertrophy in stroke-prone spontaneously hypertensive rats (Stasch et al., 1995), in transgenic rats (Stasch et al., 1996) and in deoxycorticosterone acetate-salt-treated rats (Ito et al., 1999). A reduction of renal enlargements was observed less consistently in these studies.

Taken together these studies demonstrate beneficial effects of treatment with racecadotril on blood pressure, renal function, and cardiac hypertrophy in various animal models of hypertension, which is in line with the elevated ANP levels in such animals. While clinical pilot studies have confirmed blood pressure lowering effects in hypertensive patients, these were too modest in comparison to established anti-hypertensive treatments to warrant further clinical investigation of racecadotril in this indication; potential enhancement of blood pressure lowering by other drugs was also deemed insufficient to be of clinical relevance.

TREATMENT OF CONGESTIVE HEART FAILURE

Congestive heart failure leads to atrial dilatation which is the most important physiological stimulus for ANP secretion. This enhanced ANP secretion is generally seen as a counter-measure to increase diuresis and natriuresis and thereby lower cardiac after-load. Accordingly, racecadotril has been evaluated in animal models and in patients with heart failure. Animal models of heart failure in which racecadotril or ecadotril have been tested include rats with volume overload due to aortic valve insufficiency (Kimura et al., 1998) or to an atrio-ventricular fistula (Wegner et al., 1996), rats after a myocardial infarction (Duncan et al., 1999), dogs with coronary microembolization (Olivier et al., 2000; Mishima et al., 2002), and dogs with heart failure due to sino-atrial pacing (Solter et al., 2000).

In line with the fluid retention, an activation of the renin-angiotensin system is a hallmark of congestive heart failure. Such activation was mitigated by treatment with racecadotril in animal models (Wegner et al., 1996; Kimura et al., 1998; Duncan et al.,

1999). A similarly reduced activity of the renin–angiotensin system was observed in an early and short-term pilot study in heart failure patients (Kahn et al., 1990) but not in a larger chronic study in such patients (Cleland and Swedberg, 1998). Whether indirectly by reducing activity of the renin–angiotensin system or more directly by increasing ANP exposure, diuretic and/or natriuretic effects of racecadotril were observed in the volume overload rats (Wegner et al., 1996), coronary microembolization dogs (Olivier et al., 2000), and in dogs with pacing-induced heart failure (Solter et al., 2000) but not in post-myocardial infarction rats (Duncan et al., 1999). Accordingly, improvements of the cardiac pump function were observed in most of these models (Kimura et al., 1998; Olivier et al., 2000; Mishima et al., 2002), whereas blood pressure did not change (Mishima et al., 2002).

Some of these heart failure models, particularly those involving volume or pressure overload typically lead to cardiac hypertrophy. In line with the other findings it has been found that chronic racecadotril treatment ameliorates such cardiac hypertrophy in the volume overload rat models (Wegner et al., 1996; Kimura et al., 1998). Similarly, racecadotril treatment also reduced cardiac hypertrophy in the dog microembolization model (Mishima et al., 2002). However, in the post-myocardial infarction rat model neither racecadotril nor perindopril alone reduced development of cardiac hypertrophy whereas their combination did (Duncan et al., 1999).

In accordance with the observed increase in plasma ANP and/or urinary cGMP, three studies have explored whether racecadotril or ecadotril may have therapeutic benefit in heart failure patients. In a series of small pilot studies in patients with severe heart failure (left ventricular ejection fraction 20%), 2 days of ecadotril treatment doubled plasma ANP levels despite starting from a markedly elevated baseline (Kahn et al., 1990). This was accompanied by a reduced renin activity and pulmonary capillary wedge pressure. Based on those encouraging findings a clinical, placebo-controlled dose-ranging study (50–400 mg ecadotril twice daily for 6 months) was performed in 259 patients with moderate heart failure (left ventricular ejection fraction of <35%; Cleland and Swedberg, 1998). This confirmed a dose-dependent increase in plasma and urinary cGMP but patients did not show reduced activation of the renin–angiotensin system or, more importantly, clinical improvement. Another study in a similar population of 50 moderate heart failure patients with 10 weeks of treatment with increasing racecadotril doses (up-titration from 50 to 400 mg twice daily) also failed to demonstrate clinical improvement (O'Connor et al., 1999).

Taken together racecadotril has shown promising findings in animal models of congestive heart failure but patient studies have not confirmed a sufficient clinical potential to warrant further development in this indication.

TREATMENT OF OTHER CARDIOVASCULAR CONDITIONS

Racecadotril has also been tested in animal models and/or clinical pilot studies for various other indications related to cardiovascular function. Studies in rats have reported that i.v. racecadotril may protect the heart against adrenaline-induced arrhythmia (Lishmanov et al., 2001) or against arrhythmia induced by short ischemia–reperfusion episodes (Naryzhnaia et al., 2001), the latter

effect being blocked by a δ -opioid receptor antagonist. In a mouse model of pulmonary hypertension ecadotril was found to have synergistic beneficial effects with sildenafil (Baliga et al., 2008).

In a single-dose pilot study in liver cirrhosis patients racecadotril 30 and 100 mg increased plasma ANP and cGMP and caused a transient diuresis and natriuresis response relative to placebo; the activity of the renin–angiotensin system apparently was not affected in these patients (Dussaule et al., 1991). In a single-dose cross-over pilot study in chronic renal failure patients 100 mg ecadotril inhibited enkephalinase, increased plasma cGMP and natriuresis; aldosterone, glomerular filtration rate, or blood pressure were not affected (Dussaule et al., 1993). Finally, a single dose of 200 mg ecadotril increased plasma endothelin-1, ANP, and cGMP and diuresis and natriuresis in a randomized placebo-controlled study in heart transplant recipients (Piquarel et al., 2002).

STUDIES IN THE GASTRO-INTESTINAL TRACT

Most effects of racecadotril and its metabolites on gastro-intestinal function apparently are mediated by inhibition of enkephalin degradation, as opiate receptor antagonism in many cases abolishes them (see below). However, an inhibition of the degradation of neuropeptide Y and the closely related peptide YY by NEP may also be involved as both of these peptides have anti-secretory effects in the gut (Playford and Cox, 1996).

EXPERIMENTAL STUDIES RELATED TO DIARRHEA

It is well established that enkephalines have potent anti-secretory properties in the gut but do not affect gut motility (Turville and Farthing, 1997). In an initial study in rats, i.v. racecadotril inhibited castor oil-induced diarrhea, an effect which was abolished by the opioid receptor antagonist naloxone (Lecomte et al., 1986). Such findings in the castor oil model of diarrhea were confirmed in rats with oral racecadotril and with i.v. thiorphan; they were blocked by subcutaneous but not by i.c.v. naloxone, indicating that the opioid receptors mediating this effects are located peripherally (Marcais-Collado et al., 1987). Racecadotril also reduced castor oil-induced diarrhea in human volunteers in a placebo-controlled study (Baumer et al., 1992).

To explore the underlying mechanism of anti-diarrhea effects of racecadotril, several studies have been performed. Thus, excessive fluid secretion from the gut is a pathophysiological hallmark of diarrhea. Racecadotril inhibited cholera toxin-induced but not basal secretion in canine jejunum (Primi et al., 1999). This was confirmed in a parallel group study in human volunteers, in which cholera toxin was administered by segmental perfusion directly into the proximal jejunum (Hinterleitner et al., 1997). In another study, racecadotril inhibited secretion induced by rotavirus infection in an *in vitro* model of intestinal secretion, Caco-2 cells (Guarino et al., 2009), a model which may be of value because rotavirus infection is a very frequent cause of childhood diarrhea.

A potential complication of diarrhea treatment is inhibition of intestinal motility as it can lead to secondary constipation and, perhaps even more important, intestinal retention of harmful infectious organisms. In rats oral 40 mg/kg racecadotril was reported not to affect gastro-intestinal transit time, whereas 2 mg/kg loperamide did (Marcais-Collado et al., 1987). Using the

same approach in mice, 20 mg/kg i.v. of racecadotril or thiorphan or 0.5 mg/kg oral loperamide also did not significantly affect transit time, whereas 10 mg/kg oral or 0.5 mg/kg i.v. loperamide significantly prolonged it (Marcais-Collado et al., 1987). A potential consequence of effects on gastro-intestinal transit time was explored in newborn piglets, in which a 4-day oral treatment with 20 mg/kg racecadotril twice daily did not significantly affect *E. coli* content of the proximal jejunum, whereas 1 mg/kg oral loperamide twice daily markedly increased it; accordingly, the *E. coli* content of the stool was significantly reduced by loperamide but not by racecadotril (Duval-Illfah et al., 1999). In placebo-controlled studies in human volunteers racecadotril treatment for up to 1 week also did not modify oro-coecal, colonic or overall gastro-intestinal transit times (Baumer et al., 1989; Bergmann et al., 1992).

In conclusion, both racecadotril and direct μ -opioid receptor agonists have effects on the gut which lead to limitation of pathological fluid loss. While the receptor agonists do so primarily by prolonging transit time and hence providing more opportunity for fluid reabsorption, racecadotril does so by inhibiting fluid secretion; the latter may be preferable as it directly targets the primary pathophysiological mechanism underlying acute diarrhea and also reduces the chance of retention of infectious agents in the gut.

NON-DIARRHEA GASTRO-INTESTINAL STUDIES

i.v. administration of racecadotril was shown to inhibit gastric secretion in cats induced by pentagastrin, histamine or 2-deoxy-D-glucose in a naloxone-sensitive manner, whereas the meal-induced secretion was not affected (Bado et al., 1987). In rats i.v. racecadotril and i.c.v. thiorphan, but not i.v. thiorphan, inhibited gastric acid secretion; this was no longer observed following vagotomy, indicating a central nervous system-mediated effect (Chicau-Chovet et al., 1988). In mice i.p. racecadotril and thiorphan given prior to a fatty meal enhanced gastric emptying in a naloxone-sensitive manner; gastric emptying in response to a non-fat meal was enhanced by low and inhibited by a high thiorphan dose in a naloxone-resistant manner, and racecadotril was without significant effect under these conditions (Liberge et al., 1988).

Effects of racecadotril have also been investigated in the feline gall bladder. In a feline cholecystitis model racecadotril inhibited fluid secretion in a naloxone-sensitive manner, but did not block fluid transport in the normal gall bladder (Jivegard et al., 1989). In that study racecadotril also caused transient gall bladder contraction and increased bile outflow from the liver. In another study in the same species i.v. racecadotril but not i.v. thiorphan caused naloxone-sensitive contraction of the sphincter Oddi, indicating a central nervous system-mediated effect (Thune et al., 1992).

In a controlled cross-over study in 10 healthy volunteers, 2.5 mg/kg i.v. racecadotril attenuated relaxation of the lower esophagus sphincter but did not affect contraction (Chaussade et al., 1988). Racecadotril enhanced the propagation of electrical signals in the distal colon in rats in the fasted and fed state in a naloxone-sensitive manner (Benouali et al., 1993). In a study in healthy volunteers racecadotril produced an atropine-resistant promotion of electrical activity in the rectum, which was absent in patients with Hirschsprung's disease (Grimaud et al., 1989).

PLACEBO-CONTROLLED STUDIES IN ADULTS WITH ACUTE DIARRHEA

The data of all controlled studies with racecadotril in the treatment of acute diarrhea in adults are summarized for efficacy in **Table 1** and for adverse events in **Figure 2**. The original registration of racecadotril as a treatment for acute diarrhea in adults in France in 1992 was based on three placebo-controlled studies. In a double-blind, placebo-controlled, randomized dose-ranging study 49–55 patients per group with acute diarrhea presumed to be due to food poisoning or infection-related received 30, 100, or 300 mg racecadotril or placebo three times per day until recovery for up to 10 days (data on file). The primary outcome parameter was time to cure, which was 68.4, 69.6, 65.0, and 72.0 h with 30, 100, and 300 mg racecadotril and placebo, respectively (not significant). Secondary outcome parameters included number of diarrhoic stools in the first 10 h (2.0–2.2 with the three racecadotril doses as compared to 2.7 days with placebo; $p = 0.06$) and in first 3 days (6.8–7.7 with the three racecadotril doses as compared to 8.6 days with placebo; $p = 0.03$). The incidence of the adverse events anal burning, painful anal contractions, spontaneous abdominal pain, nausea, vomiting, loss of appetite, asthenia, and insomnia did not differ significantly between groups.

In a double-blind, randomized, placebo-controlled study adult out-patients with acute diarrhea of presumed infectious origin having started less than 5 days before (95 on racecadotril, 98 on placebo) were treated with an initial dose of 200 mg racecadotril followed by an additional 100 mg dose after each unformed bowel movement until recovery or for a maximum of 10 days (Baumer et al., 1992). Resolution of diarrhea occurred significantly faster in a Kaplan-Meier type analysis with racecadotril than with placebo, e.g., on day 4 the cumulative probability of recovery was 75% with racecadotril vs. 37% with placebo. Accordingly, mean duration of treatment was 3.0 ± 0.2 days with active treatment vs. 4.4 ± 0.3 days with placebo. Several secondary endpoints including anal burning, spontaneous abdominal pain, nausea, anorexia, pain on abdominal palpation, and abdominal distension were also significantly improved by racecadotril as compared to placebo. The percentage of patients reporting adverse effects with racecadotril and placebo was 16.8 vs. 18.4%, respectively.

In another double-blind, parallel group, placebo-controlled study 70 adult patients with acute diarrhea of presumed infectious origin were included and randomized to receive 100 mg racecadotril or placebo three times daily until recovery for a maximum of 6 days (Hamza et al., 1999). The primary outcome parameter was mean stool weight, which was significantly smaller in the racecadotril as compared to the placebo group (355 ± 35 vs. 499 ± 46 g, respectively). Secondary outcome parameters for which racecadotril was significantly superior to placebo included number of diarrhoic stools after 1 day of treatment (4.3 ± 0.4 vs. 5.4 ± 0.4 , respectively) and percentage of patients passing at least one formed stool on the second day of treatment (15.6 vs. 5.3%, respectively). The incidence of reported adverse events was 3.1% with racecadotril vs. 5.3% with placebo; abdominal distension, not classified as an adverse event in this study, was 5.6% with racecadotril vs. 18.2% with placebo.

Following registration in 1992, one additional double-blind, randomized, placebo-controlled trial was performed in 110 men with cholera, in which 100 mg racecadotril was administered every

Table 1 | Efficacy of racecadotril in the treatment of acute diarrhea in adults.

Outcome parameter	Number of patients	Racecadotril	Comparator	Reference
DOUBLE-BLIND, PLACEBO-CONTROLLED STUDIES IN ADULTS WITH ACUTE DIARRHEA				
Time to recovery, h	54–55 per group vs. 49 [§]	65.0–69.9	72.0	data on file
% Probability for recovery on day 4	95 vs. 98	75*	37	Baumer et al. (1992)
Stool weight, g	32 vs. 38	355 ± 35*	499 ± 46	Hamza et al. (1999)
DOUBLE-BLIND, PLACEBO-CONTROLLED STUDIES IN ADULTS WITH CHOLERA				
Total stool output, g	54 vs. 56	315 ± 31	280 ± 21	Alam et al. (2003)
STUDIES IN ADULTS WITH ACUTE DIARRHEA ASSOCIATED WITH CANCER CHEMOTHERAPY (5-FUOROURACIL)				
Number of stools per day	15 (sequential racecadotril vs. no treatment)	4.9*	6.3	Dorval et al. (1995)
STUDIES IN ADULTS WITH DELAYED DIARRHEA DUE TO CANCER CHEMOTHERAPY (IRINOTECAN)				
Treatment responder	11	36%	—	Saliba et al. (1998)
Prophylaxis of diarrhea	68 vs. 68 no treatment	55%	59%	Ychou et al. (2000)
OCTREOTIDE-CONTROLLED STUDIES IN ADULTS WITH TREATMENT-RESISTANT DIARRHEA IN AIDS PATIENTS				
Stools/day	13 (cross-over)	–2.4*	–1.4	Beaugerie et al. (1996)
DOUBLE-BLIND, LOPERAMIDE-CONTROLLED STUDIES IN ADULTS WITH ACUTE DIARRHEA				
Time to diarrhea resolution, days	37 vs. 32	2.2 ± 0.2	2.3 ± 0.2	Roge et al. (1993)
Number of stools	82 vs. 75	3.5 ± 0.5	2.9 ± 0.4	Vetel et al. (1999)
Duration of diarrhea, h	473 vs. 472	55.0	55.0	Prado (2002)
Duration of diarrhea, h	31 vs. 31	19.5	13.0	Wang et al. (2005)
Time recovery, h	30 vs. 31	36 ± 4*	63 ± 6	Gallelli et al. (2010)

* $p < 0.05$ vs. comparator; [§]dose-ranging study using 30, 100, and 300 mg racecadotril thrice daily. For details on individual studies see main text Section "Studies in the Gastro-Intestinal Tract."

4 h until recovery for a maximum of 72 h as an adjunct to standard treatment (Alam et al., 2003). Both treatments did not differ significantly with regard to total stool output, duration of diarrhea or patients with resolution of diarrhea within 72 h. Adverse events noted as per-protocol such as vomiting, reappearance of dehydration, abdominal pain, headache, or anorexia were not different between the treatment groups. Taken together these studies consistently demonstrate efficacy of racecadotril as compared to placebo with similar adverse event incidences with both treatments. However, it should be noted that treatment regimens and outcome parameter varied considerably between studies.

STUDIES IN OTHER FORMS OF ADULT DIARRHEA

Some studies have been performed with racecadotril in the context of diarrhea associated with cancer chemotherapy. In a pilot study in 15 cancer patients treated with 5-fluorouracil were given a daily dose of 300 mg/d racecadotril for 7 days for the treatment of acute diarrhea (Dorval et al., 1995). As compared to the control period, i.e., earlier cycle of chemotherapy, the number of stools per day was reduced in each patient with a statistically significant reduction of mean number from 6.3 to 4.9 and the number of days with liquid stools significantly dropped from 4.7 to 2.4.

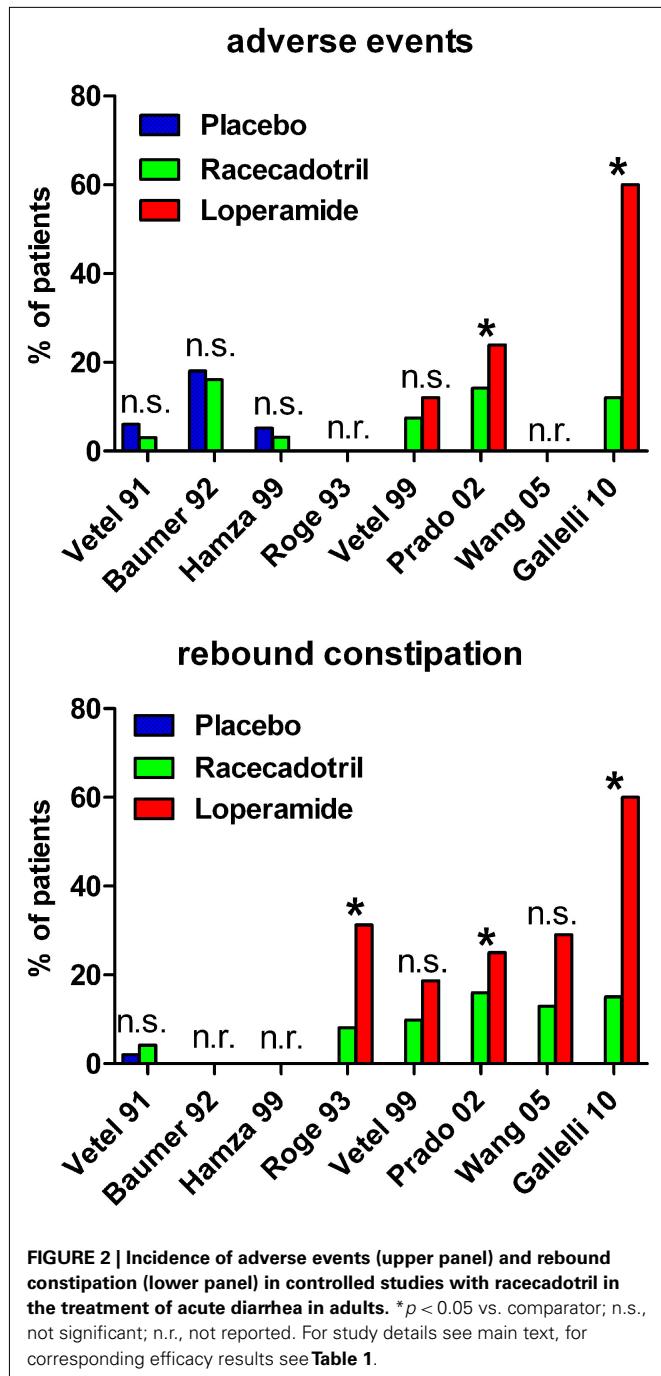
Delayed onset diarrhea is a dose-limiting side-effect of the second-line anti-cancer drug irinotecan, which is often used in the treatment of colon cancer. Among patients exhibiting delayed diarrhea upon irinotecan treatment, 4 out of 11 patients responded to 100 mg racecadotril thrice daily in one cohort, whereas 9 of 10 patients responded to a combination of racecadotril and loperamide ($p < 0.02$ vs. racecadotril alone) (Saliba et al., 1998). In a randomized open-label study 136 patients receiving a total of 714 irinotecan chemotherapy cycles received 300 mg/d racecadotril for

15 days as a prophylactic treatment or no prophylactic treatment but the two groups did not differ significantly in the incidence or severity of delayed diarrhea (Ychou et al., 2000).

In an open, randomized cross-over study the efficacy of 100–300 mg racecadotril thrice daily and 50–150 µg octreotide thrice daily was compared in 13 treatment-resistant diarrhea in AIDS patients (Beaugerie et al., 1996). From a baseline of 7.0 ± 1.2 stools/day racecadotril caused a significant reduction to 4.6 ± 1.1 stools/day, whereas octreotide caused a non-significant reduction to only 5.6 ± 1.2 stools/day. Daily lipid output was increased non-significantly by racecadotril, but was nearly doubled with octreotide.

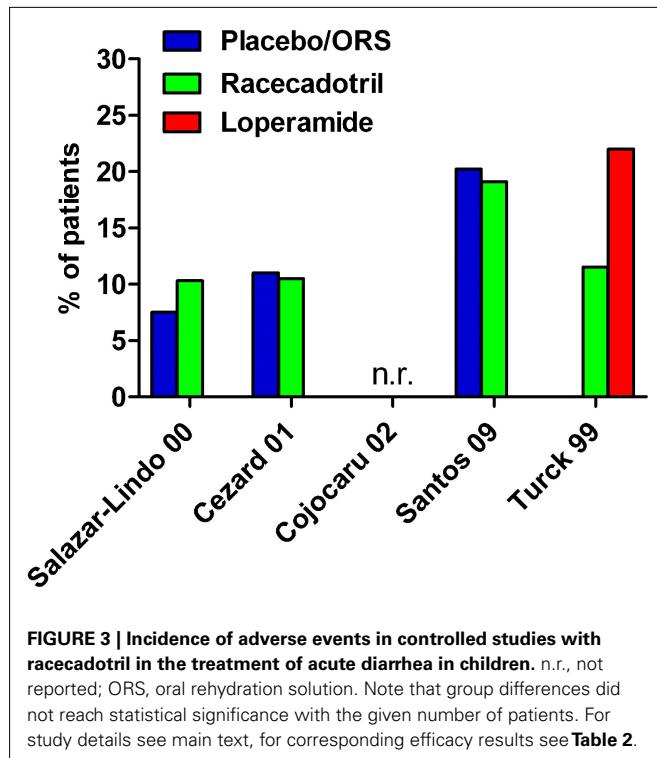
RACECADOTRIL CLINICAL STUDIES VS. PLACEBO AND OPEN STUDIES IN CHILDREN

Based upon the results of racecadotril in the treatment of acute diarrhea in adults, several studies have explored its use in the treatment of acute diarrhea in children. The data of all controlled studies with racecadotril in the treatment of acute diarrhea in children are summarized for efficacy in Table 2 and for adverse events in Figure 3. In the first of such studies 135 boys aged 3–35 months including 73 with a verified rotavirus infection with 1.5 mg/kg racecadotril every 8 h or placebo in a randomized, double-blind study with both treatments being administered on top of oral rehydration solution (Salazar-Lindo et al., 2000). Total stool output in the first 48 h of treatment, the primary study endpoint, was 157 ± 27 g/kg with racecadotril as compared to 331 ± 39 g/kg with placebo ($p < 0.001$). The duration of diarrhea was significantly shorter with racecadotril (28 h regardless of rotavirus status) than with placebo treatment (72 and 52 h in rotavirus-positive and –negative boys, respectively). Moreover, the amount of required oral



rehydration solution was also significantly less with racecadotril treatment. Adverse events were reported in 7 of 68 and 5 of 67 children receiving racecadotril and placebo, respectively; 51 and 52% reported vomiting at least some point during treatment, and study withdrawal occurred in 9 and 14 patients, respectively.

A second double-blind, placebo-controlled randomized study of very similar design with racecadotril on top of oral rehydration solution was performed in 173 infants aged 3 months to 4 years, except that this study included children of both genders (Cezard et al., 2001). Total stool output was significantly lower



by 60% (95% confidence interval 43–88%) with racecadotril as compared to placebo, and this was again independent of rotavirus status. Stool output in the first 24 h of treatment, a secondary endpoint, was also significantly less with racecadotril. The time to recovery was also significantly shorter with racecadotril in a Kaplan–Meier analysis. Moreover, the need for oral rehydration solution on the second day of treatment was also significantly lower with racecadotril treatment. Adverse events were reported by nine patients of each group, but abdominal distension was not noted in either treatment group.

In a third study the effect of racecadotril given as adjunct to oral rehydration solution was compared to rehydration alone in 166 children aged 3–34 months in a randomized open-label study (Cojocaru et al., 2002). The primary endpoint was the number of medical exams within a week after start of treatment which was significantly lower with racecadotril than without (14 vs. 27). Secondary endpoints the number of stools within the first 48 h (6.8 ± 3.8 vs. 9.5 ± 4.5) and the duration of diarrhea (97.2 ± 35.6 vs. 137.7 ± 42.4 h) were also significantly less in the racecadotril group.

In an open-label parallel group study 189 children aged 3–36 months were treated with oral rehydration solution or racecadotril (10 or 30 mg thrice daily for children with 9–13 or >13 kg body weight, respectively) on top of such solution until two normal stools were observed or no bowel movement occurred within 12 h for up to 7 days (Santos et al., 2009). The primary endpoint, number of bowel movements in the first 48 h after initiation of treatment, did not differ significantly between the two treatments (4.1 ± 2.7 vs. 3.8 ± 2.4 for control and racecadotril, respectively), and the duration of gastroenteritis, a secondary

Table 2 | Efficacy of racecadotril in the treatment of acute diarrhea in children.

Outcome parameter	Number of patients	Racecadotril	Comparator	Reference
DOUBLE-BLIND, PLACEBO-CONTROLLED STUDIES IN CHILDREN WITH ACUTE DIARRHEA				
Stool output, g/kg	68 vs. 65	157 ± 27*	331 ± 39	Salazar-Lindo et al. (2000)
Stool output, g/h	84 vs. 82	9*	15	Cezard et al. (2001)
OPEN-LABEL CONTROLLED STUDIES (VS. REHYDRATION ALONE) IN CHILDREN WITH ACUTE DIARRHEA				
Medical exams within 1 week of treatment	81 vs. 83	14*	27	Cojocaru et al. (2002)
Number of stools in first 48 h	88 vs. 91	3.8 ± 2.4	4.1 ± 2.7	Santos et al. (2009)
OPEN-LABEL OBSERVATIONAL STUDIES IN CHILDREN WITH ACUTE DIARRHEA				
Time to relief, h	3873	18.5 ± 12.5	n.a.	Chacon (2010)
DOUBLE-BLIND, LOPERAMIDE-CONTROLLED STUDIES IN CHILDREN WITH ACUTE DIARRHEA				
Number of diarrhoic stools until recovery	52 vs. 50	2.7 ± 0.4	2.1 ± 0.4	Turck et al. (1999)

n.a., Not applicable; * $p < 0.05$ vs. comparator.

endpoint, also differed numerically but not significantly (4.7 ± 2.2 vs. 4.0 ± 2.1 days, respectively). The incidence of adverse events was similar in both treatment groups (20.2 vs. 19.1%, respectively).

In an open-label study 3873 children aged 3 months to 12 years were treated with 1.5 mg/kg thrice daily (Chacon, 2010). The primary endpoint of that study was time to relief, time from start of treatment to last watery bowel movement, which was reached after 18.5 ± 12.5 h (95% confidence interval 17.9–19.0 h). Using the very large number of children in this study, the authors have performed a multiple regression analysis to explore factors affecting drug performance. Among a range of potential explanatory variables diarrhea severity was the only with a significant and independent weight on racecadotril effectiveness, explaining 23% of time to relief variance, but even in severe cases mean time to relief was less than 24 h. Specific adverse event incidence was not reported, but overall tolerability was rated as excellent or good in 95.9% of cases.

While some of the above mentioned randomized pediatric studies have been systematically reviewed in the past (Szajewska et al., 2007; Tormo et al., 2008), those analyses covered only part of the existing trial databases. More importantly, an individual patient meta-analysis of nine pediatric studies with raw data available for analysis from 1384 children has been reported more recently which also included some studies that had not been reported before as full papers (Lehert et al., 2011). The proportion of children with recovery was higher with racecadotril treatment with a hazard ratio of 2.04 (95% confidence interval 1.85–2.32). For in-patient studies, the ratio of mean stool output racecadotril/placebo was 0.59 (0.51–0.74, $p < 0.001$), for out-patient studies, the ratio of the mean number of diarrhoic stools racecadotril/placebo was 0.63 (0.51–0.74, $p < 0.001$).

Accordingly, a survey of treatment patterns among all office-based pediatricians in France found racecadotril to be prescribed by 62% of pediatricians as compared to only 28% prescribing loperamide (Uhlen et al., 2004). In line with the above studies, as an addition to oral rehydration treatment, racecadotril is being recommended for the treatment of acute diarrhea in children by recent guidelines, e.g., from the World Gastroenterology Organisation (World Gastroenterology Association, 2008), the European Society of Pediatric Gastroenterology, Hepatology

and Nutrition/European Society for Pediatric Infectious Diseases (Guarino et al., 2008), a guideline panel from Spain and Latin America (Gutierrez Castrelion et al., 2010), and the German Society for Pediatric Gastroenterology and Nutrition (Koletzko and Lentze, 2008). Similarly, a very recent international panel of experts from France, Ireland, Italy, Malaysia, Peru, Spain, USA, and Vietnam emphasized that oral rehydration solution is the basis of the treatment of acute diarrhea in children; the use of loperamide was discouraged, whereas racecadotril was recognized as an option for additional active treatment on top of rehydration solution (Guarino et al., 2012).

RACECADOTRIL CLINICAL STUDIES VS. LOPERAMIDE (ADULTS AND CHILDREN)

As loperamide has been the primary medical treatment of acute diarrhea for a long time, particularly in adults, six studies have reported direct comparisons of the efficacy and tolerability of racecadotril and loperamide including one study in children.

Roge et al. (1993) reported a double-blind controlled study in which 100 mg racecadotril was compared to 1.33 mg loperamide (two doses at start of treatment, followed by one dose every 8 h) in 37 vs. 32 patients, respectively. The study did not report a primary endpoint but rather several outcome parameters in parallel including physician evaluation of efficacy, time to diarrhea resolution, abdominal pain for more than 1 day, abdominal distension for more than 1 day, duration of abdominal distension and secondary constipation. Racecadotril was numerically superior to loperamide for all of these endpoints, and the difference reached statistical significance for the latter three. The authors did not specifically report incidence of adverse events but secondary constipation can be considered as such and was found significantly less frequent in racecadotril as compared to loperamide-treated patients (8.1 vs. 31.3%).

A second randomized, double-blind, double-placebo-controlled study compared the efficacy and safety of 100 mg racecadotril thrice daily with that of 2 mg loperamide after each diarrhoic stool in 82 and 75 patients, respectively, administered until recovery for a maximum of 7 days (Vetel et al., 1999). Both groups passed a similar number of stools (3.5 ± 0.5 vs. 2.9 ± 0.4) and had a similar duration of diarrhea (14.9 ± 2.0 vs. 13.7 ± 2.2 h). Adverse events

were reported in 7.4% of racecadotril and 12% of loperamide patients, and rebound constipation was experienced by 9.8% of racecadotril vs. 18.7% of loperamide patients.

The third and largest direct head-to-head study compared 473 patients receiving 100 mg racecadotril thrice daily with 472 patient receiving 2 mg loperamide thrice daily in a single-blind design (Prado, 2002). The primary efficacy criterion was duration of diarrhea, defined as time between start of treatment and appearance of first formed stool; this was 55.0 h in both groups (95% confidence interval 50.0–65.0 and 48.0–66.0 h in the racecadotril and loperamide group, respectively). The median duration of abdominal pain was similar in both groups, but difference in pain intensity between start and end of study was significantly in favor of racecadotril; moreover, a significantly greater percentage of patients reported residual pain at study end with loperamide as compared to racecadotril treatment (7 vs. 3%). Rebound constipation (objectively defined as 36 h without passing stool) was significantly more frequent with loperamide than with racecadotril treatment (25 vs. 16%). The incidence of reported adverse events was also significantly greater with loperamide than with racecadotril (23.9 vs. 14.2%).

A fourth randomized study compared 31 patients receiving 100 mg racecadotril thrice daily to 31 patients receiving 2 mg loperamide twice daily in a single-blind manner (Wang et al., 2005). The primary endpoint was duration of diarrhea, which did not differ significantly between treatments (median 19.5 vs. 13.0 h for racecadotril and loperamide, respectively). Duration of abdominal pain, abdominal distension, anal burning, and nausea also did not differ significantly between treatments. Adverse events were reported in 25.0% of racecadotril and 22.0% of loperamide patients. Among these rebound constipation was reported in four and nine racecadotril and loperamide patients, respectively, whereas itching was found in two racecadotril but no loperamide patients.

A fifth double-blind, randomized study compared 100 mg racecadotril thrice daily to loperamide (4 mg starting dose, followed by 2 mg after each unformed stool for a maximum of 8 mg/d) in 30 and 31 patients, respectively, being administered until recovery which was defined by two consecutive normal stools or no stools in a 12-h period (Gallelli et al., 2010). In contrast to the other studies, this one recruited only elderly nursing home residents with acute diarrhea (mean age 82 years). The primary endpoint was time to recovery which was met significantly earlier with racecadotril than with loperamide treatment (36 ± 4 vs. 63 ± 6 h). Secondary endpoints included duration of abdominal pain, number of diarrhea episodes, and total stool output in the intention-to-treat and in the per-protocol populations; racecadotril was numerically superior to loperamide all of these, and this reached statistical significance in several cases. In 50% of patients loperamide was ineffective within 4 days; these were switched to racecadotril resulting in rapid normalization of all symptoms. Adverse events were reported in 12% of racecadotril and 60% of loperamide patients; the latter percentage is much higher than in other loperamide studies, probably due to the elderly population being studied. Specifically, nausea and rebound constipation were noted more frequently with loperamide than with racecadotril, whereas abdominal pain,

headache, and anorexia were seen similarly with both treatments. Based on genotyping for cytochrome P450 3A4 and 2C8 it was excluded that the group differences were not attributable to the presence of ultra-rapid or poor metabolizers. In a pharmacoeconomic analysis the average cost in the loperamide group was twice as high as in the racecadotril group (€ 91.99 vs. € 44.85).

A sixth randomized study directly comparing racecadotril and loperamide in the treatment of acute diarrhea was performed in a pediatric population with a mean age of 4.7 years (range 2–10 years) in a double-blind, double-placebo design (Turck et al., 1999). Fifty-two children received 1.5 mg/kg racecadotril thrice daily and 50 received 0.03 mg/kg loperamide thrice daily. The primary endpoint was number of passed stools until recovery which did not differ significantly between the two groups (2.7 ± 0.4 with racecadotril and 2.1 ± 0.4 with loperamide). The mean duration of diarrhea also did not differ significantly between groups (10.7 ± 1.7 h with racecadotril and 8.8 ± 2.3 h with loperamide). Adverse events were noted in 11.5% of racecadotril and 22% of loperamide patients. There were only statistically significant differences between the two treatments: rebound constipation (36.5% of racecadotril and 58% of loperamide patients) and need for concomitant medication (anti-emetics 5 vs. 8, analgesics 0 vs. 3 patients, oral rehydration 0 vs. 2, and laxatives 0 vs. 1 patient on racecadotril and loperamide, respectively).

Taken together these six studies demonstrate that the efficacy of racecadotril and loperamide in the treatment of acute diarrhea did not differ significantly for some endpoints in some studies, but that racecadotril was significantly more effective for at least some endpoints in some studies. The incidence of adverse events also was similar in some studies but significantly less frequent with racecadotril in some other studies. A much lower frequency of rebound constipation was seen with racecadotril in almost all studies. Thus, in comparison to loperamide racecadotril appeared to be superior in efficacy and tolerability when all studies are taken into consideration.

While the mechanisms underlying differential effects of racecadotril and loperamide in acute diarrhea have not been fully established, two candidates have emerged: Firstly, loperamide preferentially acts on μ -opioid receptors (Dehaven-Hudkins et al., 1999), whereas endogenous enkephalines active both μ - and δ -receptors (Huighebaert et al., 2003). Secondly, NEP inhibition will not only increase exposure to endogenous enkephalines but also to endogenous neuropeptide Y and, possibly, peptide YY, both of which have strong anti-secretory effects in the gut (Playford and Cox, 1996).

SAFETY AND TOLERABILITY

As summarized in Figure 2 for adult and Figure 3 for pediatric patients with acute diarrhea, the incidence of adverse events reported during treatment with racecadotril was consistently similar to that with placebo and similar to or significantly less frequent than that with loperamide. Of note secondary constipation was consistently less frequent with racecadotril than with loperamide; for details see specific study descriptions in section 6. Therefore, the following will summarize published findings on non-clinical toxicity studies and will highlight clinical findings with potential relevance for safety and tolerability.

With regard to general toxicity, no relevant findings were reported for single doses up to 2000 mg/kg and for chronic doses up to 100 mg/kg in dogs (Maertins et al., 2000). In mice no overt toxicity was observed upon i.p. treatment with 50 mg/kg racecadotril for 10 days (Lecomte et al., 1986). In a study with newborn gnotobiotic piglets an oral dose of 130 mg/kg racecadotril produced no signs of neurotoxicity and no deaths, whereas an equivalent high dose of 5 mg/kg loperamide resulted in death in three out of four piglets (Duval-Ilfah et al., 1999). In monkeys 12 months of treatment with up to 100 times the therapeutic human dose did not produce any toxic effects (Lecomte, 2000). Single doses of up to 2000 mg have been administered in healthy volunteers without ill effects (Lecomte, 2000).

Several studies have specifically explored potential adverse events related to airway function, breathing and allergy. As substance P also is a NEP substrate, the effect of racecadotril on responses to exogenous substance P has been explored. In guinea pigs racecadotril enhanced pulmonary substance P response (Lötvall et al., 1990). In humans 200 mg racecadotril enhanced the flare response to substance P in asthmatic subjects (Nichol et al., 1992), and a dose of 300 mg racecadotril enhanced the substance P-induced decrease in nasal conductance in healthy subjects and those with allergic rhinitis (Lurie et al., 1994). There is one case report of a 3-year-old boy weighing 20 kg and reporting generalized edema with itching and aphonia after 2 days of treatment with 30 mg racecadotril; upon additional testing this was classified as a non-allergic hypersensitivity response (Nucera et al., 2006). While respiratory depression is a typical effects of direct opioid receptor agonists with penetration to the central nervous system such as morphine, no respiratory depression was noted with acute i.v. or i.p. racecadotril doses of up to 100 mg/kg in mice (Lecomte et al., 1986).

Finally, there were a few isolated findings from animal studies. In rats racecadotril and thiophan were reported to enhance the duration but not the frequency of uterine contractions in periparturient animals (Adjroud, 1995). Also in rats the angiotensin converting enzyme inhibitor captopril produced plasma extravasation; while racecadotril alone did not mimick this, it enhanced the captopril response (Sulpizio et al., 2004). However, both of these studies are difficult to place into context as no corresponding adverse events have been reported in patients. Thus, the overall non-clinical and clinical studies demonstrate that

racecadotril is a safe drug with an overall tolerability profile similar to placebo.

CONCLUSION

Racecadotril is a low potency inhibitor of NEP, but upon oral administration it is rapidly and effectively metabolized to the potent NEP inhibitor thiophan, with the latter not exhibiting penetration into the central nervous system. NEP inhibition affects the abundance of several endogenous peptides with enkephalins and ANP apparently being most important. Elevated exposure to ANP appears to underly most cardiovascular effects of racecadotril; while these tend to be beneficial they appear quantitatively insufficient to warrant therapeutic use in comparison to other available drug classes. Elevation of enkephalin exposure appears to underly most central nervous effects, most notably analgesia, but the pain relieving effects are inconsistent across animal models. Increased exposure to peripheral endogenous enkephalins appears to underly the gastro-intestinal racecadotril effects. Most prominent among them is an antsecretory effect in the gut which, in contrast to direct μ -opioid receptor agonists, occurs in the absence of effects on gastro-intestinal transit time. The clinical correlate of these findings is therapeutic efficacy against acute diarrhea in adults and children with a tolerability profile similar to that of placebo. In multiple direct comparative studies in different patients populations (children, adults, elderly), countries (Western Europea, Latin Amercia, Asia), and settings (out-patients, inpatients, nursing home residents) racecadotril was at least as effective as loperamide, and in several of those studies exhibited significantly better tolerability than loperamide. Most notably, rebound constipation was consistently less frequent with racecadotril than with loperamide; while this is primarily a tolerability benefit, it may also be relevant with regard to the efficacy of clearance of infectious organisms as demonstrated in one study. Of note, study designs and particularly treatment endpoints differed considerably between studies. This can be seen as a weakness because it makes inter-study comparisons more difficult; however, it can also be seen as a benefit because consistent therapeutic effects across so many different settings witness rather robust efficacy and tolerability. While additional studies appear warranted several guidelines, specifically in pediatric indications, now recommend including racecadotril in the management of acute diarrhea. Whether other forms of diarrhea, e.g., in the context of cancer chemotherapy, also benefit from racecadotril treatment is not fully clear.

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- Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.
- Received: 11 April 2012; accepted: 27 April 2012; published online: 30 May 2012.
- Citation: Eberlin M, Mück T and Michel MC (2012) A comprehensive review of the pharmacodynamics, pharmacokinetics, and clinical effects of the neutral endopeptidase inhibitor racecadotril. *Front. Pharmacol.* 3:93. doi: 10.3389/fphar.2012.00093
- This article was submitted to Frontiers in Gastrointestinal Pharmacology, a specialty of Frontiers in Pharmacology.
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Brain-gut interactions in IBS

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Irritable bowel syndrome (IBS) is a common gastrointestinal disorder with an estimated prevalence of 10–20%. Current understanding of the pathophysiology of IBS is incomplete due to the lack of a clearly identified pathological abnormality and due to the lack of reliable biomarkers. Possible mechanisms believed to contribute to IBS development and IBS like symptoms include physical stressors, such as infection or inflammation, psychological, and environmental factors, like anxiety, depression, and significant negative life events. Some of these mechanisms may involve the brain-gut axis (BGA). In this article we review the current knowledge on the possible involvement of the BGA in IBS and discuss new directions for potential future therapies of IBS.

Keywords: irritable bowel syndrome, brain-gut axis, pathophysiology, autonomic nervous system, hypothalamo-pituitary-adrenal axis

INTRODUCTION

Irritable bowel syndrome (IBS) is a common gastrointestinal (GI) disorder with an estimated prevalence of 10–20% (Philpott et al., 2011). According to Thompson et al. (2000) it accounts for about 3% of all general practice and up to 40% of all GI referrals. IBS causes considerable morbidity amongst its sufferers, who manifest with abdominal pain and altered stool consistency and frequency (Drossman and Dumitrescu, 2006; Lee et al., 2007; Adeyemo et al., 2010). Although not life-threatening, it is a heavy economic burden due to increased work absenteeism and impaired quality of life of its sufferers, as well as increased use of health care services (Sandler et al., 2002).

Current understanding of the pathogenesis of IBS is unsatisfactory due to the lack of demonstrable pathological abnormalities and reliable biomarkers. Traditionally, IBS has been considered a purely functional disorder. A hypothesis based on specimens obtained at endoscopy and in serological cytokine studies views IBS as a localized low grade inflammatory disorder with mast cells (MC) playing a particularly important role (Mayer and Collins, 2002; Philpott et al., 2011). An alternative hypothesis states that food allergy may be responsible (Atkinson et al., 2004). Most recently, the relationship between the neural and immunological networks within the gut and the bi-directional communication

between the gut and the central nervous system (CNS), often related to as the brain-gut axis (BGA) attract most attention (Collins and Bercik, 2009).

In this review we focus on the disturbances in the BGA as a plausible cause of IBS. We overview the pathophysiological mechanisms contributing to symptom perception and generation and the endogenous systems involved. Particular attention is given to stress, emotion and psychological factors in the IBS pathogenesis. We also discuss new directions for potential future therapies of IBS based on discussed mechanisms.

THE BRAIN-GUT AXIS

The BGA constitutes the enteric nervous system (ENS) and the gut wall in the periphery, the CNS, and the hypothalamo-pituitary-adrenal (HPA) axis (Collins and Bercik, 2009). The bi-directional communication between the gut and the CNS is based on the neural, endocrine and neuroimmune pathways. Neuronal pathways include afferent fibers originating in the dorsal root of the ganglia of the thoracic spinal cord (T1–T10) projecting to integrative cortical areas, such as the cerebral, anterior and posterior cingulate, insular, and amygdala cortices and efferent fibers to smooth muscle and glands, originating in nuclei within the brain-stem, as well as S2–S4 spinal levels (parasympathetic) and in the lateral horn of the thoraco-lumbar spinal cord (T1–L3; sympathetic; Mulak and Bonaz, 2004; Gaman and Kuo, 2008; O'Mahony et al., 2011). The main pain signaling pathways in the BGA are the spinothalamic tracts and dorsal columns with descending supraspinal afferents originating from the rostral ventral medulla (Gaman and Kuo, 2008).

In physiological conditions, signals from the GI tract influence the brain, which in turn can exert changes in motility, secretion, and immune function (Mayer et al., 2006). The axis is therefore an important communication system for healthy regulation of food

Abbreviations: ACC, anterior cingulate cortex; ACTH, adrenocorticotrophic hormone; ANS, autonomic nervous system; BDNF, brain-derived neurotrophic factor; BZD, benzodiazepine; CNS, central nervous system; CRD, colorectal distension; CRF, corticotrophin-releasing factor; DLPFC, dorsolateral prefrontal cortex; DRG, dorsal root ganglia; DRN, dorsal raphe nucleus; ENS, enteric nervous system; GI, gastrointestinal; HPA axis, hypothalamo-pituitary-adrenal axis; IBS, irritable bowel syndrome; MC, mast cells; MRI, magnetic resonance imaging; NE, norepinephrine; PAG, periaqueductal gray; PVN, paraventricular nucleus; SERT, serotonin transporter protein; SNRI, serotonin-norepinephrine reuptake inhibitor; SSRI, selective serotonin re-uptake inhibitor; TCA, tricyclic antidepressant; UCN, urocortin.

intake, digestion, gut sensations, and control of the bowel movements. Structural and functional disruptions in the BGA cause changes in perceptual and reflexive responses of the nervous system and may lead to GI disorders, including IBS, which often comorbid with chronic psychiatric diseases (Clarke et al., 2009; Gros et al., 2009).

STRUCTURAL AND FUNCTIONAL ABNORMALITIES IN THE CENTRAL NERVOUS SYSTEM

Visceral hypersensitivity is a key mechanism underlying abdominal pain, one of the main symptoms of IBS (Azpiroz et al., 2007; Barbara et al., 2011). Visceral hypersensitivity is thought to be determined by central and peripheral mechanisms, as it may result from altered transmission within the gut wall, the spinal cord, or the brain. However, the specific contribution of the BGA components to hypersensitive responses in IBS remains unclear.

Direct imaging techniques were recently employed to detect the abnormalities in the structure and functioning of the brain and their possible implications in the pathology of IBS. There is only one structural magnetic resonance imaging (MRI) study (Davis et al., 2008), in which the thinning in the anterior mid-cingulate and insular cortex, structures important for perception of internal body states were observed in the IBS patients. These results were later confirmed by functional MRI (Blankstein et al., 2010). Although the underlying cause of cortical thinning was not elucidated, factors like decreased cell size, apoptosis of neural cells, death of glia and astrocytes, fewer dendritic spines, reduced synaptic density, and excitotoxicity related to enhanced glutamate signaling were suggested as possible contributors. Seminowicz et al. (2010) reported morphometric brain differences between female IBS patients and controls in terms of regional increases and decreases in gray matter density. These alterations occurred primarily in brain areas involved in attention and emotion modulation, as well as cortico-limbic pontine pain modulatory systems and in networks processing interoceptive information. Further studies of Blankstein et al. (2010) evidenced increased gray matter density in the hypothalamus of the IBS patients. Currently it is not possible to discern whether these changes are a predisposing factor for IBS or a secondary change after sustained visceral signals (Fukudo and Kanazawa, 2011).

In their excellent paper on imaging techniques used in the studies of brain-gut interactions, Rapps et al. (2008) reviewed the possible central mechanisms implicated in IBS and found published reports somewhat contradictory. The region that attracted most attention was the anterior cingulate cortex (ACC), one of the six most commonly reported cortical areas that display pain-evoked activity during acute stimulation in humans (Chen et al., 2011). ACC showed altered activity during rectal stimulation in IBS patients in comparison to healthy controls (Rapps et al., 2008 and citations therein). Interestingly, although greater pain by rectal balloon distension was reported by the IBS patients with a history of sexual or physical abuse, changes in their ACC activity were less pronounced than in other IBS patients and the controls (Ringel, 2002). In line with these observation was the study of Mertz et al. (2000), who demonstrated differential activation of the brain between IBS patients and controls. The ACC, the insula,

the prefrontal cortex, and the thalamus were more activated in the IBS patients as compared with healthy controls and the pattern was related to the experience of individuals.

Hall et al. (2010) revealed differences in the central responses in health and in IBS to a single ramp-tonic distension of the colon across a distributed network of regions, involving sensory, striatal, limbic, and frontal areas. The IBS participants showed heightened activation of the ACC, suggesting increased affective responses to painful visceral stimuli. However, it was also observed that the activation of the thalamic, striatal, and dorsolateral prefrontal cortex (DLPFC) regions was relatively greater in control subjects, as compared to IBS patients, which may reflect increased ascending input to the brain, in particular to the cortex and a heightened arousal reaction to distension. Greater recruitment of the DLPFC by controls than IBS patients is consistent with the notion of abnormal descending modulation in IBS.

To further explore the central mechanisms of visceral hypersensitivity in IBS, Lawal et al. (2006) examined total cortical recruitment in response to subliminal (sub-conscious) stepped changes in distension pressure and observed that visceral hypersensitivity in IBS patients is due to increased afferent signaling to the brain, rather than altered processing at the level of the brain. However, the results of the study were later questioned, among others by Lackner et al. (2006), who showed that cognitive behavioral therapy in IBS patients is associated with a reduction of baseline activity in the ACC and accompanied by improvements of GI symptoms. Dorn et al. (2007) showed a contributory part of neurosensitivity in the form of enhanced activity with central neural networks independent of cognitive function.

The most novel findings of Chen et al. (2011) showing that the patients with IBS have white matter abnormalities in the insula, ACC, and other brain areas associated with pain, interoception, and homeostasis indicate that functional gray matter abnormalities in IBS patients are accompanied by white matter aberrations. The white matter deficiencies of the descending modulation of pain and dysfunction of the medial pain system may be responsible for the emotional aspect of pain in IBS.

In conclusion, as evidenced by the results of the meta-analysis performed by Tillisch et al. (2011), a greater engagement of regions associated with emotional arousal and endogenous pain modulation, but similar activation of regions involved in processing of visceral afferent information was observed in patients with IBS compared to controls. These results support a role for structural and functional abnormalities in the CNS in IBS.

COGNITIVE-BEHAVIORAL MODEL OF IBS

IBS is often considered a bio-psychosocial disorder (Engel, 1977; Camilleri and Choi, 1997; Drossman, 1998), which suggests that psychological (e.g., emotions, cognitions, and behavior), social (e.g., modeling, support), and physiological (e.g., cramps, bloating) factors may induce and exacerbate its symptoms (Toner et al., 1998; Mach, 2004). Individual cognitive and emotional responses to recurrent GI symptoms and associated life events may also affect the therapeutical efficacy of anti-IBS treatments (Kennedy et al., 2012).

CENTRAL MECHANISMS

Abnormal activity within higher-order brain systems may alter cognitive and affective processes and contribute to both abnormal pain regulation and higher levels of anxiety and depression, typically reported in chronic pain conditions (Ribeiro et al., 2005) and IBS (Piche et al., 2011, and citations therein). The cognitive-behavioral model of IBS is focused particularly on emotional arousal and organism response to stress and the integrated network of structures, which include the hypothalamus, amygdala, and periaqueductal gray (PAG), as well as a number of neuromodulators and hormones.

Greenwood-Van et al. (2001) showed in animal models that there is a link between the central pathways mediating stress and anxiety and the mechanisms regulating the GI sensitivity. A key component of this link is the amygdala, which is known for its role in the regulation of emotional behavior and the expression of fear and anxiety. Further studies in rodents demonstrated that colonic sensitivity and motility are increased following fear conditioning (Gue et al., 1991; Tyler et al., 2007). In accordance, studies on IBS patients showed substantial activation of the hypothalamus and amygdala, as well as decreased activity of the antinociceptive PAG (Naliboff et al., 2001). More recent investigations employing rectosigmoid balloon distension in IBS patients have shown increased activity in the amygdala, insula, cingulate, and prefrontal cortex, which form a network of brain structures involved in regulating affective and sensory processes (Naliboff et al., 2003; Wilder-Smith et al., 2004; Myers and Greenwood-Van, 2009).

ROLE OF ANS AND HPA AXIS

The autonomic nervous system (ANS) and the hypothalamus-pituitary-adrenal (HPA) axis are commonly regarded as the major components of the stress response system in the vertebrates. Alterations of this complex system have been linked to a variety of anxiety-related psychiatric disorders and stress-sensitive pain syndromes. Stress and stress-related psychosocial factors have also been proposed to act in IBS, particularly its post-infectious variety (PI-IBS), by overarching inflammation and the BGA (Arborelius et al., 1999; Gwee et al., 1999; Fukudo, 2007; Spiller and Garsed, 2009).

The correct function of the ANS and its cross-talk with CNS are important factors preventing from IBS. Disturbances at the ANS level, indicated by decreased parasympathetic and increased sympathetic activity and altered autonomic reflexes often occur in the IBS patients and account for the level of perception to GI stimuli and extra-intestinal symptoms (Azpiroz, 2002; Jarrett et al., 2003; Spaziani et al., 2008).

The key activator of the HPA axis is corticotrophin-releasing factor (CRF), an endogenous 41-amino acid neuropeptide secreted from endocrine cells in the paraventricular nucleus (PVN) of the hypothalamus (Aguilera et al., 2008). The action of CRF is mediated by the CRF1 and CRF2 receptors, which belong to the G protein-coupled receptor family (Kostich et al., 1998). CRF receptor activity can also be modulated by other peptides, like urocortins (UCN; Bale and Vale, 2004; Tache and Brunnhuber, 2008). In the mammalian brain three urocortins have been identified: UCN I, which binds to both receptors, and UCN II and UCN III, selectively binding to CRF2 receptor (Morin et al., 1999; Hsu and Hsueh,

2001; Lewis et al., 2001; Reyes et al., 2001; Bale and Vale, 2004; Dautzenberg et al., 2004). However, the neuroendocrine, autonomic, and behavioral responses to fear and stress are mediated exclusively by CRF and UCN I, which are selective CRF1 receptor ligands (Vale et al., 1981; Bale and Vale, 2004; Tache et al., 2009; Chen et al., 2011).

Corticotrophin-releasing factor and UCN I initiate the signaling cascade in the HPA axis by stimulating the anterior pituitary to secrete adrenocorticotropic hormone (ACTH), which in turn induces synthesis and secretion of glucocorticoids from the adrenal cortex. Growing evidence suggests that also the extra-hypothalamic CRF system is poised to play a critical role in both psychiatric and the BGA disorders (Lowry and Moore, 2006; Bravo et al., 2011).

In rodents, stress-induced release or exogenous administration of CRF and UCN I increased anxiety-like behaviors and stimulated colonic secretion, intestinal motility, and visceral sensitivity (Moreau et al., 1997; Slawecki et al., 1999; Saunders et al., 2002; Vetter et al., 2002; Million et al., 2003; Martinez et al., 2004; Tache et al., 2004, 2009). Johnson et al. (2010) provided evidence that elevated corticosterone levels affected the amygdala and significantly increased brain activation in response to colorectal distension (CRD) compared to that seen in cholesterol-treated controls. Elevated CRF expression was found in the thalamus of the rats exposed to neonatal maternal separation (Tjong et al., 2010). Deletion of the CRF1 gene using transgenic models or intraventricularly administered CRF1 antagonists had anxiolytic effects and attenuated stress- and CRF-induced alterations in gastric and colonic motor function (Smith et al., 1998; Million et al., 2003; Martinez and Tache, 2006; Trimble et al., 2007).

Only a limited number of studies in IBS patients measured basal and stimulated HPA axis hormone levels in response to meal, hormone challenge, or mental stress (Chang et al., 2009, and citations therein) and some of them demonstrated increased HPA axis responses in IBS compared to controls. Fukudo et al. (1998) observed that the intravenous injection of CRF in IBS patients induced exaggerated motility of the colon and increased visceral pain sensitivity compared with healthy controls, whereas administration of a non-selective CRF receptor antagonist ameliorated these responses (Lembo et al., 1996; Sagami et al., 2004). The recent study by Chang et al. (2009) showed that basal levels of plasma ACTH were significantly decreased, while both 24 h basal plasma cortisol levels and stress-induced cortisol levels were mildly elevated upon visceral stimulation in female IBS patients compared to controls, suggesting a dysregulation of the HPA axis in IBS. However, the role of the observed dysregulation of HPA axis in modulating IBS severity or abdominal pain remained unclear.

A meta-analysis performed by Tillisch et al. (2011) revealed that the central nucleus of amygdala indirectly activates the HPA axis and increases ACTH and glucocorticoid secretion via subcortical regions, which relay on PVN (Redgate and Fahringer, 1973; Feldman and Weidenfeld, 1998; Herman et al., 2003; Shepard et al., 2003). The CRF-dependent involvement of the amygdala in the induction of anxiety-like behavior, visceral hypersensitivity, altered bowel habits and other common feature of IBS has been later confirmed in animal studies (Tache et al., 2002; Myers and Greenwood-Van, 2007, 2010; Venkova et al., 2010).

The hippocampus may also be involved in several aspects relevant to the IBS symptomatology, e.g., pain, anxiety, and stress (Prado and Roberts, 1985; Bannerman et al., 2004; Kwan et al., 2005; McEwen, 2007; Niddam et al., 2011). Saito et al. (2002) demonstrated that the induction of visceral pain by CRD increased the release of hippocampal noradrenaline in animal models. Niddam et al. (2011) observed abnormal hippocampal glutamatergic neurotransmission in IBS patients and inverse correlation between glutamate-glutamine concentrations and emotional stress indicators, which was not observed in healthy individuals. It remains possible that the observed hippocampal glutamatergic hypofunction could result from a generally impaired HPA axis tone or it could represent compensatory mechanisms of adaption to enhanced glucocorticoid feedback.

PSYCHOSOCIAL FACTORS AND IBS

According to the cognitive-behavioral model, a history of abuse and other psychosocial factors may induce and aggravate symptoms of IBS, influence illness experience, and affect treatment outcome.

Ringel et al. (2008) showed that patients with IBS and a history of abuse had a significantly lower pain and urge thresholds and a greater tendency to report pain in response to aversive rectal distensions compared with patients with IBS or abuse history alone. However, neuro-sensory sensitivity remained unchanged. These observations suggest that the abuse history in IBS patients may affect central mechanisms of pain amplification or regional brain activation at sites linked to affect and attention, resulting in heightened awareness to visceral and somatic symptoms, greater pain reports, and greater clinical behavioral responses to painful visceral stimuli. Nevertheless, changes in peripheral signaling by nociceptive DRG neurons, including those innervating the colon cannot be excluded, as suggested by several animal studies (Khasar et al., 2008; Winston et al., 2010).

It was also observed that there is a higher prevalence of psychological and psychiatric disorders observed in IBS patients: depression, somatization disorder, generalized anxiety disorder, panic, and phobic disorders and coping difficulties (for review see, Arebi et al., 2008). Drossman et al. (1999) estimated that up to 70% of the patients referred to tertiary centers with IBS meet diagnostic criteria for anxiety or depression. However, Elsenbruch et al. (2006) revealed that women with IBS were characterized by an exaggerated anticipatory anxiety response at baseline, but essentially unaltered anxiety and neuroendocrine responses to a public speaking stressor. These results would suggest that IBS patients show essentially normal emotional responses when faced with challenging psychosocial situations.

Although well-evidenced, the impact of psychosocial factors on the neurochemical responsiveness of visceral nociceptive pathways and the physiological function of the GI remains unclear. It is possible that the psychosocial stressors and/or stressful early life events modulate the immune response of the gut to infectious agents and cause low level inflammation and mast cell infiltration and degranulation in the bowel (Barbara et al., 2004; Ohman and Simren, 2010; Chen et al., 2011; Philpott et al., 2011). This is supported by questionnaire-based studies indicating an increased prevalence of atopic diseases among IBS patients (Philpott et al., 2011, and

citations therein) and a report published by Barbara et al. (2004), demonstrating that there is an increased number of degranulating MC in patients with IBS compared to that in the healthy controls. Increased mucosal immune activation and elevated blood concentrations in pro-inflammatory cytokines are also believed to impact the CNS functioning (for review see; Kennedy et al., 2012). Although these large molecules do not freely pass the blood-brain barrier, a number of studies have provided substantial evidence for their central mechanisms of action, sympathetic arousal and the HPA axis activation (Dinan et al., 2006).

In rodents, early life stress in the form of separation of neonates from the mother results in permanent changes in the CNS, which include unrestrained secretion of CRF and increased expression of its receptors (Owens and Nemeroff, 1993), increased regional norepinephrine release (Southwick et al., 1999), downregulation of β -receptors, decreased benzodiazepine receptor, and γ -aminobutyric acid type A receptor (Caldji et al., 2000). A significant increase in 5-HT-positive cell number and 5-HT content after CRD stimulation was also observed in the colon of animals, which experienced maternal separation (Ren et al., 2007). Videlock et al. (2009) demonstrated that IBS patients and controls with a history of early adverse life events (EAL) have a greater cortisol response to a visceral stressor compared to individuals without EAL, suggesting the involvement of the HPA axis.

CURRENT AND FUTURE MOLECULAR TARGETS FOR IBS TREATMENT

Various classes of drugs, like 5-HT₃ antagonists, tricyclic antidepressants (TCAs), selective serotonin reuptake inhibitors (SSRIs), gabapentinoids, CRF-1 antagonists, β 3 adrenoceptor agonists, somatostatin, N-methyl D-aspartate receptor antagonists, or melatonin are currently in use for the treatment of visceral analgesia and other symptoms of IBS. However, new molecular targets for the future IBS therapeutics are also being investigated.

SEROTONIN RECEPTORS

Serotonin (5-HT) is a key neurotransmitter and a signaling molecule that plays an important role in sensation, secretion, and absorption (for review see, Gershon and Tack, 2007; Garvin and Wiley, 2008). A number of studies reported altered serotonergic signaling activity in the brain and gut in IBS, including increase in plasma 5-HT in IBS-D (diarrhea-predominant) and PI-IBS, reduced levels in IBS-C (constipation-predominant) and changes in plasma and tissue levels of serotonin transporter protein (SERT; Dunlop et al., 2005; Atkinson et al., 2006; Zou et al., 2007; Camilleri, 2011). Drugs aimed at selective modulation of the 5-HT activity (SSRIs, 5-HT₃, and 5-HT₄ receptor antagonists) or both 5-HT and norepinephrine (NE) systems (serotonin-norepinephrine reuptake inhibitors, SNRIs, and tricyclic antidepressants, TCAs) have been used in the treatment of functional GI disorders, as well as in other chronic pain conditions, and psychiatric syndromes. New generation drugs with similar pharmacological profile may soon become novel efficient therapeutics in the treatment of IBS.

Several large clinical trials have demonstrated that serotonin receptor 5-HT₃R antagonists, like alosetron, cilansetron, and ramosetron are among the most effective therapeutic options

to date for both male and female IBS-D patients (Jarcho et al., 2008, and citations therein). The 5-HT3R antagonists alleviate specific IBS symptoms, such as frequent bowel movements, feelings of urgency, and chronic abdominal pain and discomfort, acting through central and peripheral mechanisms. Although the precise mechanisms underlying their effectiveness remain incompletely understood, symptom improvement associated with an interaction with dopamine, cholecystokinin, glutamate, acetylcholine, and GABA (for review see, Barnes et al., 2009) and a reduction in amygdala and emotional arousal circuit activity (Berman et al., 2002) have been suggested. Inhibition of the spinal cord c-fos expression by 5-HT3R antagonists in response to noxious CRD (Kozlowski et al., 2000) suggests that 5-HT3R plays a role in the transmission of noxious information within the spinal cord. Excess 5-HT released from enterochromaffin cells (EC) in the colonic mucosa of both unselected and PI-IBS patients (Spiller, 2007) and decreased expression of SERT (Coates et al., 2004) may also account to this phenomenon.

5-HT3 antagonist-based therapies require the implementation of a risk management plan, as ischemic colitis and complications of constipation may occur (Chang et al., 2010). Therefore, a novel class of compounds (of which the prototype is LX-1031) is being developed that directly inhibits 5-HT synthesis in EC cells, potentially reversing the underlying pathogenetic factor in conditions like IBS-D. Such compounds could become an alternative to the application of classical 5-HT3 receptor antagonists in the treatment of IBS.

Recently, partial 5-HT1 receptor agonists, like buspirone, and antagonists, like robalzotan tartrate monohydrate (AZD7371), attracted much attention as they displayed a potent analgesic effect in the CRD-induced visceral pain model in rats (Sivarao et al., 2004; Lindstrom et al., 2009). However, the clinical development of AZD7371 has been discontinued due to severe adverse events, including hallucinations and the inability to demonstrate significant efficacy in IBS patients compared with placebo (Drossman et al., 2008).

The 5-HT4 receptors in the GI tract are found on enteric neurons and smooth muscle cells. Stimulation of 5-HT4 receptors leads to acetylcholine release and prokinetic effects (Gershon and Tack, 2007). The early generation 5-HT4 receptor agonists, such as cisapride and tegaserod, reversed slow motility and relieved constipation, but they have been withdrawn because of cardiac or vascular adverse effects (Gershon and Tack, 2007). A number of novel 5-HT4 agonists have recently been obtained as potential treatments for patients with IBS-C and appear to be safer than earlier generation agents in these classes (Camilleri et al., 2008; Manini et al., 2010).

The 5-HT7 and 5-HT2B receptors are yet another potential serotonergic target for future IBS treatment. The 5-HT7 receptors are present in humans and other animals and are linked with depression, circadian rhythm, neuroendocrine function, affective behavior and body temperature regulation (for review see, Vanhoenacker et al., 2000). They play an important role in regulating smooth muscle relaxation in the GI and nociceptive pathways (Carter et al., 1995; Meuser et al., 2002) and may thus be involved in the pathological mechanisms of GI dyskinesia, abdominal pain, and visceral paresthesia in IBS. It was demonstrated that 5-HT7

receptors also mediate stress and glucocorticoid-induced effects on hippocampal neurogenesis, which have been implicated in mood. Meanwhile, 5-HT2B receptor blockade was shown to reduce significantly pain behaviors in response to CRD (O'Mahony et al., 2010a).

Recent studies demonstrated that serotonergic neurotransmission can be markedly affected by CRF acting in a CRF receptor-dependent manner (Cryan et al., 2005; Valentino and Commons, 2005). The injection of low doses of CRF in the dorsal raphe nucleus (DRN) reduced the discharge rate of serotonergic neurons in the striatum (Kirby et al., 2000) and the nucleus accumbens (Lukkes et al., 2008) and at a higher dose increased striatal 5-HT release (Price et al., 1998). Additionally, 5-HT levels in the hippocampus were increased by i.c.v. administration of low and high doses of CRF (Penalva et al., 2002). These data suggest a close correlation between the serotonergic system and CRF, which may be taken into consideration when novel anti-IBS therapies are designed.

BENZODIAZEPINE RECEPTORS

One of the newly targeted classes of drugs for the treatment of visceral pain are benzodiazepine (BZD) receptor modulators. BZD receptors are located in subcortical and hypothalamic regions and appear important in controlling autonomic function, such as motor and sensory activity of the gut (for review see, Salari and Abdollahi, 2011). In addition, activation of the central BZD receptors affects GABA interaction with central GABA-A receptors and may influence the ANS, dorsal vagal nuclei, and the ENS. Peripheral BZD receptors were identified on immune cells and other peripheral tissues and may be involved in cell proliferation and immunomodulation (for review see, Zisterer and Williams, 1997).

The BZD receptors and their ligands, which belong to an important regulatory network between the CNS, behavior, and immune response, may thus become an attractive target for future IBS treatments. Recently, a novel BZD receptor ligand dextofisopam was developed for the management of IBS-D (Grundmann et al., 2010) and is currently under investigation.

NEUROKININ RECEPTORS

Substance P (SP) and the neurokinin-1 receptors (NK1R) are located throughout the BGA, including peripheral, spinal, supraspinal, and cortical sites of visceral afferent pathways, as well as brain regions involved in emotional arousal and autonomic function (Tillisch et al., 2012, and citations therein). It was observed that SP and NK1R signaling play an important role in nociceptive responses (hyperalgesia) and the autonomic and behavioral responses to stress in animals and humans.

Recent study by Tillisch et al. (2012) revealed that a 3-week treatment with a novel NK1R antagonist reduced activation of key regions of both the interoceptive afferent and emotional arousal network in response to noxious and non-noxious visceral stimulus in female IBS patients, causing a large decrease in pain-induced negative affect and decreased anxiety and pain ratings. This positive correlation suggests a potential for use of NK1R antagonists in IBS patients to decrease pain related distress.

BRAIN-DERIVED NEUROTROPHIC FACTOR

Neurotrophins promote neuronal survival along with the growth and differentiation of new neurons and synapses. Brain-derived neurotrophic factor (BDNF) may be involved in the integration of excitatory and inhibitory neurotransmission and emerging evidence suggests that amygdaloid BDNF can regulate anxiety-like behaviors (Slack et al., 2004; Pandey et al., 2006).

Yu et al. (2012) recently observed a significant upregulation of BDNF in the colonic mucosa and structural alterations of mucosal innervation in biopsies from patients with IBS, as compared with controls. The enhanced expression of BDNF was closely correlated with the degree of abdominal pain in IBS. These results suggest that endogenous BDNF released in response to inflammation contributes to the development of central sensitization and thus plays a pathophysiological role in the altered gut sensation in IBS. Furthermore, the upregulation of BDNF may also play a role in the structural alterations of mucosal nerve fibers in patients with IBS. Inhibition of the BDNF system could therefore be beneficial for the alleviation of symptoms in the IBS patients.

SEX STEROID RECEPTORS

Because of the sex differences in perceptual responses and a female predominance of the disorder, attention has been drawn to the role of sex steroids, in particular ovarian hormones, in the development of IBS. Previous reports revealed that women with IBS often report exacerbation of symptoms, including visceral and somatic sensitivity during menses (Kane et al., 1998; Mayer et al., 1999; Houghton et al., 2002; Chang et al., 2006; Gustafsson and Greenwood-Van, 2011) and show greater, compared to men, activation of brain areas associated with affective responses including the amygdala and cingulate cortex (Berman et al., 2000; Naliboff et al., 2003). In contrast, male IBS patients show less visceral hypersensitivity than female patients, but have greater sympathetic nervous system responses measured by skin conductance, and decreased cardiovagal activity measured by heart rate variability compared to female IBS patients (Tillisch et al., 2005) and male controls.

Although ovarian steroid receptor levels are higher in some regions of the female brain (Greco et al., 2001; Milner et al., 2008), progesterone and estradiol-induced visceral hypersensitivity does not appear to be sex specific, as males also showed increased visceral sensitivity following hormone implantation on the amygdala (Myers et al., 2011). However, the amygdala may still represent the key supraspinal site mediating the actions of ovarian hormones on visceral pain in both males and females and account for differences in symptom generation in male and female IBS patients (Naliboff et al., 2003; Labus et al., 2008; Kilpatrick et al., 2010). The amygdala may thus become an interesting target for the IBS treatment and alleviation of pain.

TOLL-LIKE RECEPTORS

Toll-like receptors (TLRs) have been localized on mucosal surfaces, including the colonic epithelial cells, and their expression is increased in the colonic mucosa of rat models of visceral hypersensitivity and mucosal biopsies from IBS patients (McKernan et al., 2009; Brint et al., 2011). TLRs are activated by various bacterial and viral cell components (Takeuchi and Akira, 2010), which

stimulate transcription of inflammatory cytokines, like IL-1 β , IL-6, and TNF α and affect transmission in the spinal cord, resulting in central sensitization and hyperalgesia (for review see, Akira and Takeda, 2004; Arebi et al., 2008). Cytokines are also known to cross the blood-brain barrier, to affect the HPA axis and stress response and to stimulate secretion of CRH in rat, as well as in humans (for review see, John and Buckingham, 2003; Dantzer et al., 2008).

Recently, McKernan et al. (2011) demonstrated that TLR agonist-induced cytokine and cortisol release was markedly enhanced in stimulated whole blood from IBS patients compared with healthy controls. These results point out at the TLR as possible targets in the treatment of IBS.

RECEPTORS FOR ACETYLCHOLINE AND CATECHOLAMINES

There is an increasing evidence for the beneficiary role of cholinergic, dopaminergic, and noradrenergic pathways in regulating immunity and cytokine production in IBS, suggesting a positive influence of acetylcholine and catecholamines on the IBS symptoms (Dinan et al., 2008; Rosas-Ballina and Tracey, 2009). However, adrenaline was shown to act directly through adrenergic receptors on DRG neurons or indirectly by increasing levels of pronociceptive mediators following immune activation in the colon or repeated stress, thus increasing the excitability of the neurons and exacerbating pain sensation (Khasar et al., 2008; Winston et al., 2010; Ibeakanma et al., 2011). In contrast, no significant differences in NE responses to sigmoidoscopy were observed in women with IBS-D compared to healthy women (Chang et al., 2009).

These conflicting results point at the necessity of further studies on the involvement of cholinergic, dopaminergic, and adrenergic receptors and their ligands in development of IBS and their possible therapeutical application.

PAST, PRESENT, AND FUTURE OF ANTI-IBS DRUGS TARGETING THE BRAIN-GUT AXIS

For most IBS patients with mild symptoms, lifestyle, and dietary changes may be sufficient; for more moderate symptoms, medications that act on the gut (e.g., anticholinergics, peripheral 5-HT agents) can be considered. However, patients who suffer from severe IBS, characterized by increased levels of pain, poorer quality of life, psychosocial difficulties, or co-morbidity with mood disturbances are usually refractory to first- and second-line therapies (Drossman et al., 2000; Grover and Drossman, 2011). The bidirectional communication between the brain and the gut opens up new treatment possibilities for these patients and directs us to novel pharmacological targets for the anti-IBS drugs.

Almost all IBS patients could benefit from centrally acting treatments, like therapies focused on teaching better stress coping strategies, both at a cognitive and behavioral level (for review see, Larauche et al., 2012), or psychotropic agents. Some of the TCAs, SSRIs, SNRIs, or BZDs have already been employed in the treatment of IBS and proved effective in symptom relief via mood stabilization, modulation of pain perception and amelioration of GI motility and secretion (Ford et al., 2009; Grover and Drossman (2011) estimate that at least every one in eight patients with IBS is offered an antidepressant). However, the effects of psychotropic agents on bowel symptoms and visceral hypersensitivity in IBS

patients have been less robust and less consistent than the benefits reported for global symptoms and abdominal pain/discomfort (Chey et al., 2011). Furthermore, psychotropic agents are not free from undesired side effects. TCAs display anticholinergic properties, including constipation, tachycardia, urinary retention, and xerostomia; patients may also encounter central side effects including sedation, insomnia, agitation, and nightmares (Chey et al., 2011). Compared to TCAs, SSRIs have fewer side effects, but do not improve bloating or visceral pain (Tack et al., 2006). BZDs are used routinely in anxiety disorders, but their efficacy in symptom relief of IBS is under debate (Drossman et al., 2002). New generation of psychotropic agents is therefore anticipated.

Efficacious and safe serotonergic agents may also become future drugs in the treatment of IBS. Recently, novel mixed 5-HT1A agonists/5-HT3 antagonists, 5-HT1B/D agonists, and 5-HT2B antagonists have been proposed as new therapeutics for IBS (Tack et al., 2000; Mulak and Paradowski, 2006; Vera-Portocarrero et al., 2008; Asagarsu et al., 2009; O'Mahony et al., 2010b).

Other endogenous systems, which may become possible new targets in the IBS therapy, include GABA-B, CRF, NK, cannabinoid, and opioid receptors and their ligands. Preliminary data suggest that anxiolytic activity of GABAergic agent, gabapentin may be efficient in reducing central sensitization in hyperalgesia (for review see, Camilleri and Andresen, 2009). CRF receptor

antagonists have also been proposed as a potential treatment of IBS (Martinez and Tache, 2006; Tache et al., 2009). However, due to the failure of treatment with a CRFR1 antagonists to alter colonic transit and the global improvement scale in IBS patients (Sweetser et al., 2009), further studies are required.

The potential use of cannabinoid and opioid receptor ligands as anti-IBS agents has also been considered and has been reviewed in detail elsewhere (Fichna et al., 2009; Izzo and Sharkey, 2010).

CONCLUSION

In summary, there is striking evidence of a crucial involvement of the BGA in the development of IBS and IBS like symptoms. Though the role of the BGA is not fully understood, some concepts are at an advanced stage and allow speculation on possible future treatment options. Future research needs to identify the exact involvement of the discussed neurotransmitter systems and to identify at which level pharmacological treatment may be beneficial to patients with IBS.

ACKNOWLEDGMENTS

This work was supported by the grant from the Deutsche Forschungsgemeinschaft (STO 645/6-1 to Martin A. Storr), and the Iuventus Plus program of the Polish Ministry of Science and Higher Education (0119/IP1/2011/71 to Jakub Fichna).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any

commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 16 March 2012; paper pending published: 28 April 2012; accepted: 15 June 2012; published online: 05 July 2012.

Citation: Fichna J and Storr MA (2012) Brain-gut interactions in IBS. *Front. Pharmacol.* 3:127. doi: 10.3389/fphar.2012.00127

This article was submitted to Frontiers in Gastrointestinal Pharmacology, a specialty of Frontiers in Pharmacology.

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Drug development for the irritable bowel syndrome: current challenges and future perspectives

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Medications are frequently used for the treatment of patients with the irritable bowel syndrome (IBS), although their actual benefit is often debated. In fact, the recent progress in our understanding of the pathophysiology of IBS, accompanied by a large number of preclinical and clinical studies of new drugs, has not been matched by a significant improvement of the armamentarium of medications available to treat IBS. The aim of this review is to outline the current challenges in drug development for IBS, taking advantage of what we have learnt through the Rome process (Rome I, Rome II, and Rome III). The key questions that will be addressed are: (a) do we still believe in the “magic bullet,” i.e., a very selective drug displaying a single receptor mechanism capable of controlling IBS symptoms? (b) IBS is a “functional disorder” where complex neuroimmune and brain-gut interactions occur and minimal inflammation is often documented: do we need to target gut motility, visceral sensitivity, or minimal inflammation? (c) are there validated biomarkers (accepted by regulatory agencies) for studies of sensation and motility with experimental medications in humans? (d) do animal models have predictive and translational value? (e) in the era of personalized medicine, does pharmacogenomics applied to these medications already play a role? Finally, this review will briefly outline medications currently used or in development for IBS. It is anticipated that a more focused interaction between basic science investigators, pharmacologists, and clinicians will lead to better treatment of IBS.

Keywords: drug targets, translational medical research, brain-gut interactions, drug selectivity, biomarkers, 5-hydroxytryptamine, transient receptor potential channels, neuroimmune intestinal interactions

INTRODUCTION

Irritable bowel syndrome (IBS) is a common functional gastrointestinal disorder, characterized by recurrent abdominal pain or discomfort in combination with disturbed bowel habits in the absence of identifiable organic cause. Many medications are used for the treatment of patients with IBS, although their actual benefit is often a matter of debate. In particular, only a few are specifically labeled for IBS. In fact, notwithstanding great progress in our understanding of the pathophysiology of IBS thanks to a large number of preclinical and clinical studies of new drugs, the specific armamentarium of medications available is scant. The aim of this review is to outline the current challenges in drug development for IBS, taking advantage of what we have learned through the Rome process (from Rome I in the 1980s to Rome III published in 2006; Drossman, 2006).

The key questions that will be addressed are: (a) do we still believe in the “magic bullet,” i.e., a very selective drug displaying a single receptor mechanism capable of controlling IBS symptoms? (b) IBS is a “functional disorder” where complex neuroimmune and brain-gut interactions occur and minimal inflammation is often documented: do we need to target gut motility, visceral

sensitivity, or minimal inflammation? (c) are there validated biomarkers (accepted by regulatory agencies) for studies of sensation and motility with experimental medications in humans? (d) do animal models have predictive and translational value? (e) in the era of personalized medicine, does pharmacogenomics applied to these medications already play a role? Finally, this review will briefly outline medications currently used or in development for IBS. It is anticipated that a more focused interaction between basic science investigators, pharmacologists, and clinicians will lead to better treatment of IBS.

THE “MAGIC BULLET”: A CONCEPT THAT NEEDS RETHINKING

The key pharmacodynamics, pharmacokinetic, and safety features for drugs to be used in the treatment of IBS are outlined in **Table 1**.

A selective drug is defined as a compound interacting only with one receptor subtype and leaving other receptors unaffected at concentrations achieved at therapeutic doses. The literature on the treatment of IBS has often resorted to the concept of the “magic bullet,” i.e., a very selective drug displaying a single receptor mechanism capable of controlling IBS symptoms (Camilleri et al., 2006a). This was often considered the key to efficacy avoiding side-effects. This approach is no longer ideal because of several important pitfalls.

First, drug selectivity is always a relative concept, which ignores the basic fact that most molecules, even at therapeutic doses,

Abbreviations: EMA, European Medicines Agency; ENS, enteric nervous system; FDA, food and drug administration; 5-HT, 5-hydroxytryptamine; IBS, irritable bowel syndrome.

Table 1 | Key features for drugs to be used in the treatment of IBS.

Key features	
Pharmacodynamics	The drug should target a whole pathophysiological mechanism rather than a single receptor <i>Possible targets: motility, secretion, visceral sensitivity, neuroimmune interactions/minimal inflammation, brain-gut axis</i> The effect should be maintained over time during treatment
Pharmacokinetics	Good oral bioavailability (unless local action in the gut is specifically wanted) Half-life allows once daily dosing No metabolites with different or unwanted pharmacological actions Avoid CYP substrates with high likelihood of drug interactions Consider interactions with food or herbal products
Safety	Specificity cannot always avoid <i>off-target</i> effects because the same receptor/system also mediates other effects A drug can also hit <i>antitargets</i> (i.e., unwanted targets), another source of side-effects

may have several, sometimes disparate biological effects (i.e., hit a large number of targets in the pharmacological space; Garcia-Serna et al., 2010; Kawasaki and Freire, 2011). These effects may depend on the fact that a single receptor/effect pathway plays a role in different systems, so that even selective compounds have *off-target* effects (Table 1). In addition, there are many instances when the compound is endowed with additional pharmacological properties that hit the so-called *antitargets* (i.e., unwanted targets), responsible for side-effects, which are clarified only after the compound has undergone clinical trials. The classical example is provided by the cardiac side-effects due to hERG K⁺ channel blockade by the early 5-HT₄ receptor agonists (Tonini et al., 1999).

The second issue is that the multifactorial pathophysiology of IBS (with multiple brain-gut and neuroimmune interactions) makes it unrealistic to expect that drugs acting on a single receptor may achieve substantial therapeutic gain over placebo in an area where the placebo response rate is substantial (approaching 40% across all randomized controlled trials; Ford and Moayyedi, 2010). As in other fields (Morphy et al., 2004), evidence suggests that a balanced modulation of multiple targets can provide a superior therapeutic effect and side effect profile compared to the action of a selective ligand. *Designed* multiple ligands that hit a large variety of targets have been produced through rational approaches in which structural features from selective ligands are combined (Morphy et al., 2004). A key challenge in the design of multiple ligands is attaining a balanced activity at each target of interest with a suitable pharmacokinetic profile.

The third issue is that mechanisms underlying symptoms in IBS may differ among patients, hence the need to consider using multiple therapies. With selective drugs, primary clinical endpoints were achieved in less than 70% of patients with the approved agents such as tegaserod or alosetron (Camilleri et al., 2000; Muller-Lissner et al., 2001; Cremonini et al., 2003). On the other hand, it seems reasonable to propose treatment with combination therapy, which is the rule when treating medical conditions such as hypertension or asthma, when monotherapy is no longer adequate. Because of the redundancy of mechanisms controlling neurosensory, neuromuscular, and neuroimmune functions in the gut, it is conceivable that effective treatment of functional gut disorders may require combination therapy.

One example is provided by tachykinin receptor antagonists, which have so far given disappointing results because of inherent differences among animal models and humans: it has been suggested that the analgesic efficacy of multi- or pan-tachykinin receptor antagonists is superior to that of mono-receptor antagonists (Holzer, 2004a).

When drugs address a specific target (e.g., a symptom such as visceral hypersensitivity or motility), heterogeneity in the pathophysiology impacts negatively on the therapeutic gain, if patients are not carefully selected in a clinical trial. Indeed, some of the disappointing results of the past can be ascribed to the lack of understanding of pathophysiology: the same symptom (e.g., diarrhea) does not necessarily depend on the same pathways in all patients.

Thus, new drugs should target a pathophysiological mechanism (provided that it is known!), rather than a specific receptor; on the other hand, recruiting carefully selected patient subgroups may significantly reduce the generalizability of the results of the trial.

Pharmacokinetics may help to achieve gut selectivity and reduce side-effects. This approach is particularly relevant when there are potential actions outside the gut, as it is indeed the case with peripherally restricted opioid receptor antagonists (such as methylnaltrexone and alvimopan), which do not cross the blood brain barrier and, in addition, have very low oral bioavailability (De Ponti, 2002; Holzer, 2004b). An example of minimally absorbed compound is also the guanylate cyclase-C agonist linaclotide (Wensel and Luthin, 2011; Busby et al., 2013), which is now FDA- and European Medicines Agency (EMA)-approved for IBS with constipation.

Another important pharmacokinetic property is the lack of interactions with food or other drugs. Significant interactions with CYP2D6 and CYP3A4 should be predicted in early drug discovery because of their involvement in drug metabolism with important pharmacogenetic aspects.

Finally, as regards safety aspects, apart from the standard safety evaluations, two issues deserve special attention following the experience with cisapride (torsades de pointes associated with QT prolongation; De Ponti et al., 2001) and alosetron (ischemic colitis; Moynihan, 2002). It is clear that even very rare events may negatively impact the risk/benefit balance of drugs that are used to provide symptom improvement of non-serious (though troublesome) diseases such as IBS (De Ponti et al., 2002; Tack et al., 2012). It is remarkable that in IBS with diarrhea, Shah et al. (2012) found that one adverse event resulting in study

discontinuation occurred for every 2.3 and 2.6 patients who benefited, respectively, from tricyclic antidepressants and alosetron, i.e., the number needed to harm was approximately 3. This is quite low, considering the numbers needed to treat reported in the literature for drugs in IBS (Brandt et al., 2009; Camilleri and Mayer, 2009; Menees et al., 2012). Shah et al. (2012) conclude that, rather than simply focusing on the number needed to treat, clinicians should be aware of harm when using pharmacotherapy for IBS.

IBS AS A "FUNCTIONAL DISORDER": NEW PERSPECTIVES AND GLOBAL REGULATORY FRAMEWORK

The classical concept of IBS as a functional disorder derives from the fact that no organic cause can be identified and the diagnosis of IBS is one of exclusion after other disorders have been ruled out. In addition, the precise mechanisms underlying symptom generation are unknown.

However, research of the past 20 years has provided significant advances in the understanding of the pathophysiology of IBS, with an emerging consensus that the various clinical manifestations (including non-gastrointestinal comorbid symptoms) of chronic abdominal pain can best be viewed as a dysregulation in the complex interplay between events occurring in the gut lumen (including microbiota), the mucosa, the enteric nervous system (ENS), and the central nervous system (Mayer and Tillisch, 2011; Matricon et al., 2012). This dysregulation leads to alterations in sensation, motility, brain-gut interactions, and neuroimmune interactions. Considerable evidence documents that sensitizing proinflammatory mediators, mast cells and their products, tryptases, are increased in tissues of patients with colorectal hypersensitivity (Cenac et al., 2007; Balestra et al., 2012; Buhner et al., 2012).

It has been shown that colonic mast cell infiltration and mediator release in proximity to mucosal innervation may contribute to abdominal pain perception in IBS patients (Barbara et al., 2004). Indeed, mucosal mast cell mediators from IBS patients excite rat nociceptive visceral sensory nerves (Barbara et al., 2007). In a recent study (Balestra et al., 2012), mucosal biopsies were obtained from the descending colon of patients with IBS and controls. Mucosal mast cells were identified immunohistochemically. The impact of spontaneously released mucosal mediators on guinea pig electrically stimulated longitudinal muscle myenteric plexus (LMMP) preparations was assessed *in vitro* by means of selective receptor antagonists and inhibitors. Patients with IBS showed an increased mast cell count compared with controls. Application of mucosal mediators of IBS to LMMPs potentiated cholinergic twitch contractions, an effect directly correlated with mast cell counts and mediated by activation of prostanoïd receptors, TRPV1, and P2X receptors. These results support the role of mucosal inflammatory mediators and mast cell activation in altered motor function of IBS.

It is also intriguing that, in patients with IBS, 5-HT spontaneous release was significantly increased irrespective of bowel habit and correlated with mast cell counts and the severity of abdominal pain. This suggests that increased 5-HT release contributes to development of abdominal pain in IBS, probably through mucosal immune activation (Cremon et al., 2011).

Several studies have reported the onset of IBS-like symptoms following established bacterial or viral infections of the GI tract (Barbara et al., 2009). This so-called "postinfectious" IBS occurs in approximately 10% of patients undergoing a documented infectious gastroenteritis, and risk factors to develop symptom persistence are longer duration of the gastroenteritis, female sex, psychosocial stressors at the time of the infection, and psychological factors such as anxiety or depression. Although a causal relationship between abdominal pain and acute or chronic infections cannot be established most of the times, it is tempting to speculate that host-microbial interactions in vulnerable individuals during the early phase of the disorder may lead to permanently altered immune response, which then continues to play a role when symptoms persist in the absence of the infectious organism.

The participation of the gut microenvironment in the pathophysiology of IBS is suggested by studies indicating an interplay between luminal factors including the microbiome, the epithelial barrier, and the mucosal immune system (Stanghellini et al., 2010; Camilleri et al., 2012). In an animal model (McVey Neufeld et al., 2013), microbiota were shown to be necessary for normal excitability of gut sensory neurons and this provides a potential mechanism for the transfer of information between the microbiota and the nervous system.

In postinfectious IBS and in IBS with diarrhea, decreased expression and structural rearrangement of tight junction proteins in the small bowel and colon may lead to increased intestinal permeability. These abnormalities might contribute to the outflow of antigens through the epithelium, causing overstimulation of the mucosal immune system. Accordingly, subgroups of patients with IBS show higher numbers and activation of mucosal immune cells, especially mast cells. Immune factors, released by these cells, including proteases, histamine, and prostanoïds, might also participate in maintaining the permeability dysfunction and contribute to the activation of abnormal neural responses, which, in turn, are involved in abdominal pain perception and changes in bowel habits.

All these mechanisms represent new therapeutic targets in IBS. Here, it is important to remember that probiotics are also currently viewed as an attractive therapeutic option in IBS because of their recognized safety and of their documented biological effects on the host. Preclinical studies have shown that some probiotic strains exhibit potentially useful properties including anti-inflammatory effects, improvement of mucosal barrier homeostasis, beneficial effects on intestinal microbiota, and a reduction of visceral hypersensitivity. However, it remains to be determined to what extent a beneficial effect on these parameters translates to a significant effect on clinical outcomes: although the effect of probiotics on IBS is positive in some randomized, controlled studies, the gain over placebo is small and identification of a tailored probiotic approach for subgroups of patients represents a future challenge.

The complex neuroimmune and brain-gut interactions sometimes associated with minimal mucosal inflammation (Ford and Talley, 2011) and neuroplastic changes in the ENS (Giaroni et al., 1999) pose several questions as regards potential targets for pharmacological intervention: should the therapeutic focus be primarily gut motility, visceral sensitivity, or minimal inflammation? Assessment of these parameters in humans can be undertaken by

using a variety of invasive and non-invasive techniques, some well established and others requiring further validation. By using these techniques, alterations in both sensory and motor function have been reported in IBS and our understanding of sensorimotor dysfunction has indeed increased. Thus, inflammatory, immunologic, and other processes, as well as psychosocial factors such as stress, can alter the normal patterns of sensitivity and motility through alterations in local reflex activity or via altered neural processing along the brain-gut axis. A firm relationship between sensorimotor dysfunction and the production of symptoms, however, has been difficult to show. Thus, the clinical relevance of the former requires further research.

In this context, it is important to remember that in 2003 the EMA adopted a document produced by the Efficacy Working Party on the “*Points to consider on the evaluation of medicinal products for the treatment of the irritable bowel syndrome*” (EMA, 2003). Although the document now needs to be updated (it still refers to Rome II criteria), a key statement is that “*The patient’s global assessment of symptoms and abdominal discomfort/pain should be used as the two primary endpoints. Statistically significant changes must be found in both parameters.*” Thus, clinical efficacy must rely on clinical endpoints in the patient’s perspective, for instance through the global assessment of multiple symptoms. Mechanistic (pathophysiological) studies provide a rationale for drug development, but do not generally predict symptomatic success and do not necessarily identify the most appropriate dose for clinical trials. An important goal is to develop non-invasive tests that identify important pathophysiological mechanisms and assess symptom pattern in short-term (4 weeks) therapeutic trials that pave the way to longer trials. Notably, the EMA document carefully considers the duration of efficacy trials, stating that they must be long enough to determine whether the response is sustained and to determine the effects of treatment withdrawal. A duration of 6 months of active treatment is considered necessary considering the cyclic and non-life threatening nature of the disease.

For inclusion and exclusion criteria for IBS, the current EMA “*Points to consider*” document refers to Rome II criteria (current Rome III criteria differ from Rome II and, notably, the Rome IV process is expected to start in 2013). A revised EMA guideline is awaited soon and should come into force by the end of 2013. A key issue that needs to be addressed, apart from the update to Rome III, is the discrepancy with the FDA guidance issued in 2012 (FDA, 2012). Indeed, the EMA document recommends the two co-primary endpoints indicated above, whereas the FDA guidance recommends a primary endpoint that measures the effect of treatment on two major IBS signs and symptoms: abdominal pain and abnormal defecation (stool frequency or stool consistency, depending on subtype of IBS). The FDA guidance also acknowledges that patient-reported outcome (PRO) measures of the signs and symptoms of the condition are the only currently available measures that can adequately define a treatment effect in a clinical trial. In addition, because of the limitations of using a single-item patient-reported rating of overall change as a primary endpoint, the FDA document recommends the development of a multi-item PRO instrument. The PRO measure(s) should capture all the clinically important signs and symptoms of the target population. The

ongoing regulatory discussion will certainly help all those involved in clinical trials to plan future research.

Linaclotide may serve as an example of the current regulatory situation, with differences in endpoints recommended by the FDA and the EMA: the efficacy and safety of this agent in patients with IBS-C was evaluated in two randomized, placebo-controlled Phase 3 trials. These trials were designed according to both FDA and EMA guidelines and findings based on FDA-recommended endpoints were reported in two recent studies (Chey et al., 2012; Rao et al., 2012), whereas the findings of a planned, separate analysis of both trials based on the distinct efficacy parameters prespecified for EMA submission were published separately (Quigley et al., 2013).

In closing this section, it should be remembered that, over the past 25 years, the Rome process has insisted on clinical features to diagnose IBS on the assumption that grouping of patients with similar features facilitates identification of patients most likely to respond to a given pharmacological agent, but this is not necessarily so because the same symptom (pain) may have several underlying pathophysiological mechanisms. This explains the criticism raised by some investigators against the Rome criteria (Dang et al., 2012). On the other hand, it must be acknowledged that there are several reasons to establish an accurate diagnosis of IBS: to relieve patient uncertainty and initiate the most appropriate treatment, avoiding the burden of unnecessary medications or diagnostic procedures and surgeries (Mearin and Lacy, 2012). In other words, the Rome criteria try to transform the diagnosis of IBS from one of exclusion into a positive diagnosis based on history, physical examination, use of precise diagnostic criteria in the absence of specific alarm features.

Biomarkers for IBS

Biomarkers are objectively measurable indicators of normal or pathological processes or pharmacological responses to a therapeutic intervention (Anonymous, 2001). In order to improve development and usage of biomarkers, a score system of different types of biomarkers has been proposed, depending on their impact on drug development (Wehling, 2009).

Positive modifications of biomarkers which imply improvement of the disease can be taken as endpoints during drug development. To provide a more complete picture, a therapeutic target can coincide with the biomarker (e.g., TNF α) or, as a component of the disease mechanism, modulate it (e.g., NF- κ B).

Unfortunately, as stated in the 2012 FDA document, no validated and accepted biomarker exists in IBS. In addition, the limited repertoire of clinical manifestations of sensorimotor disorders of the gut such as IBS can actually derive from multiple mechanisms, leading in turn to similar symptoms. In many clinical programs of new drugs for IBS, the emphasis was primarily on symptom assessment of broad groups of patients identified by the Rome criteria. As already discussed above, this approach was not ideal and it is not surprising that drugs of potential value have been abandoned.

Certain biomarkers can, in a limited fashion, be used to predict the success of a drug in IBS or to understand its mode of action. These studies may be incorporated in the recommended steps for drug development, but should be viewed only as preliminary/complementary steps of the development program, which

must comply with the regulatory guidance quoted in the previous section.

Currently established tests that can be used as potential biomarkers for clinically relevant endpoints in IBS include the following.

INTRALUMINAL MEASUREMENTS OF COLONIC OR RECTAL MOTILITY AND SENSATION

Intraluminal measurements may serve as biomarkers for motor or sensory modulation in IBS. Manometry has long been used for pathophysiological investigations (Camilleri et al., 2008) and can be used as a useful technique to study the effects of drugs on colonic motility (De Schryver et al., 2002; Dinning and Scott, 2011). Another possible test is intracolonic measurement of postprandial tone using a barostat, which, in healthy subjects (von der Ohe et al., 1994), showed the potential of 5-HT₃ receptor antagonists to prevent diarrhea and other postprandial symptoms in diseases including IBS and carcinoid diarrhea (von der Ohe et al., 1993). This indicates that measuring tone intraluminally may be a useful biomarker for preliminary tests before subsequent trials for efficacy. A recent study in healthy subjects (Sweetser et al., 2009) investigated the effects of lubiprostone on colonic sensation and motility with the following endpoints: colonic compliance, fasting and postprandial tone and motility indexes, pain thresholds, and sensory ratings to distensions. This investigation well exemplifies the potential of pharmacodynamic studies in drug development.

Although testing visceral sensitivity may provide useful mechanistic insights when developing new medications, results always require careful interpretation and are sometimes disappointing. For instance, alosetron was shown to alter colonic compliance, but not colonic sensitivity to isobaric distension (Delvaux et al., 1998). Previously, the κ opioid receptor agonist fedotozine was shown to decrease sensitivity to colonic distension, but the therapeutic gain in placebo-controlled studies in IBS was found to be of insufficient magnitude for further development (Dapoigny et al., 1995; Delvaux et al., 1999; Ness, 1999). Asimadoline is another example of drug tested for its effect on visceral sensitivity in humans (Delgado-Aros et al., 2003).

One disadvantage of sensation biomarkers is that the sample size required to avoid a type 2 error while assessing clinically meaningful effect sizes is higher than with transit endpoints in healthy volunteers and probably even higher in patients. Nevertheless, these sample sizes (12–20 per treatment arm) are still more practical than testing symptom endpoints, which require much larger samples. Thus, a 25–30% effect size can be demonstrated with ~ 20 subjects per treatment arm in sensation-based studies and ~ 12 per treatment arm in studies of transit, on the basis of the variability reported in published studies (Camilleri et al., 2006b).

RADIOPAQUE MARKERS FOR COLONIC TRANSIT

The radiopaque marker test for colonic transit is a widely available test, as shown by early studies with loperamide for diarrhea and fiber for constipation, where radiopaque markers were used to assess whole gut transit time (Cann et al., 1984a,b). The overall effects of drugs for IBS can be predicted by the marker transit test, although transit times are not characteristic of IBS (Horikawa et al., 1999) and other studies addressing more specific endpoints

suggest that the colonic marker transit time (<15 or >60 h) accurately predicts the extremes of stool consistency, with significant overlap for transit times between those extremes (Degen et al., 2001).

RADIOSCINTIGRAPHIC MARKERS FOR COLONIC TRANSIT

Scintigraphic transit measurements are sufficiently well characterized to allow meaningful pharmacodynamic conclusions on the effect of therapeutic agents (Cremonini et al., 2002; Camilleri, 2010a; Vazquez-Roque et al., 2012). Namely, several examples support the use of detailed colonic transit measurement in the development of medications for IBS-associated changes in bowel function. First, alosetron (a 5-HT₃ receptor antagonist) slows overall colonic transit and, on average, increases the time for emptying the ascending colon by 50% (Viramontes et al., 2001a). Second, tegaserod (a 5-HT₄ receptor agonist) accelerates overall colonic transit and, on average, halves the time for emptying the ascending colon (Prather et al., 2000), and several studies showed that this medication was effective in the treatment of IBS with constipation (Muller-Lissner et al., 2001; Novick et al., 2002; Kellow et al., 2003). However, tegaserod was withdrawn by the manufacturer for safety issues in 2007 and was made available only under a restricted access program (Al-Judaibi et al., 2010). Finally, a more recent example of use of scintigraphy to assess transit includes linaclotide (Andresen et al., 2007).

URINE SUGARS FOR IN VIVO GUT PERMEABILITY

Because of the possible role of disruption of intestinal mucosal barrier function in the pathophysiology of IBS, recently a urine sugar (lactulose and mannitol) excretion test was validated in patients with IBS and diarrhea (Rao et al., 2011). Urine sugars at 0–2 and 8–24 h reflect small bowel and colonic permeability, respectively and are increased in patients with IBS and diarrhea vs. controls. This method can be applied to study the effects of agents directed at mucosal pathophysiology, such as mast cell stabilizers or modulators of microbial flora.

ANIMAL MODELS: PREDICTIVITY ISSUES AND CURRENT ROLE

Translational medicine defines conditions and prerequisites to transfer more reliable results obtained from preclinical biomedical research to clinical applications, thus improving patient care through timely and efficient promotion of clinical innovation.

A disease model should mimic the clinical disease condition as much as possible and allow the investigation of unclear pathophysiological mechanisms toward the development of new potential therapeutic options. In spite of undeniable differences among species, animal models still represent the major source of information about biological system and provide an invaluable means to study complex physiological and biochemical interactions.

The predictivity of the disease model itself will substantially depend on the efforts toward optimization and scientific validation of the model, both in terms of resemblance and transferability to humans. Pharmacological targets and biomarkers must be reproduced and be susceptible to pharmacological modulation in the animal model, to ensure that the model is predictive of a therapeutic effect in humans. The real translational value of a biomarker is

represented by its capacity of being thoroughly informative after its validation in clinical studies which provide the bedside to bench approach.

An animal model used as a screening tool in drug development should ideally reproduce different biomarkers providing multiple endpoints to fully assess the spectrum of activity of a test compound. Accurate selection of endpoints plays a pivotal role to assure the translational value of disease models and should take into account the sensitivity and specificity of each endpoint toward prediction of activity.

Although development of new drugs for treatment of IBS can be facilitated by preclinical animal models (Bulmer and Grundy, 2011; Holschneider et al., 2011), it must be acknowledged that a single sufficiently predictive model of efficacy still does not exist and investigators have the option to use multiple models to assess different aspects of the pathophysiology of IBS. This section is only intended as a brief outline of the most commonly used animal models of visceral pain and disturbed gastrointestinal motility, which are reviewed elsewhere (Camilleri et al., 2006a).

MOTILITY

The techniques used to record motility or measure transit in animals may differ from techniques used in humans but the endpoints are identical to those mentioned in the previous section.

VISCERAL PAIN

There are several forms of stimulation and endpoints to measure visceral pain: (a) *mechanical stimuli*: experiments can be performed in awake or anesthetized rats, and the most frequently used stimulus of pain in animals is distension of a gut segment with a balloon connected to a barostat to measure simultaneously compliance and the response to the painful stimulus (Rouzaire et al., 1998). Poor standardization of methods across laboratories is a drawback of these investigations. (b) *Chemical stimuli*: infusing glycerol into the rat colon through a chronically implanted catheter induces abdominal cramps. This model is considered relevant because intracolonic glycerol induces abdominal pain in humans and mimics pain reported by patients with IBS (Louvel et al., 1996). (c) *Other stimuli*: the significance of other models of visceral pain, such as the “writhing test” (consisting of an intraperitoneal injection of an irritant compound such as acetic acid), is questionable.

In the aforementioned models, it is important to distinguish between evaluation of *allodynia* (decrease in the threshold of sensitivity to distension) and *hyperalgesia* (enhanced response to painful stimulus). A commonly used endpoint is the contraction of abdominal muscles induced by rectal or colorectal distension in the rat; the contractions are recorded by electromyography, where the number of spike bursts correlates with the intensity of the stimulus applied (Morteau et al., 1994).

Visceral distension also induces *viscero-visceral reflexes*, such as relaxation of anal sphincters during rectal distension or change in *blood pressure*, which is a pseudoaffective response used to assess visceral pain.

In summary, selection of one or more definitive animal models of visceral hyperalgesia is not possible and using results from more than one animal model may enhance the probability of selecting

effective drugs for further development. Since pain, though a major problem, is not the only symptom affecting quality of life, animal models detailing the effects of drugs on motility and visceral sensitivity may add a further dimension to the assessment of new compounds.

THE ROLE OF PHARMACOGENOMICS

Pharmacogenomics refers to the variability of the expression of individual genes relevant to disease susceptibility as well as drug response at cellular, tissue, individual, or population level. Pharmacogenetics refers more specifically to the study of individual variations in DNA sequence related to drug response. The growing interest in this field is due to the fact that risk/benefit evaluations of drugs are not fully appreciated if one does not fully consider individual variations that may significantly affect pharmacokinetics and pharmacodynamics.

POLYMORPHISMS AS MARKERS OF DISEASE

As an example, patients with IBS had significantly reduced frequencies of the high producer genotype for interleukin 10 than controls (21 vs. 32%; $p = 0.003$): this suggests a genetic predisposition in at least some patients with IBS to produce lower amounts of the anti-inflammatory cytokine interleukin 10 (Gonsalkorale et al., 2003) and lends support to the hypothesis that there may be an inflammatory or genetic component in some cases of IBS (Bashashati et al., 2012).

POLYMORPHISMS AFFECTING DRUG RESPONSE

Genetic variations can affect drug response in at least three different ways (Camilleri and Katzka, 2012): (a) changes in drug metabolism, e.g., through functional CYP450 2D6 genes, which determine the pharmacokinetics and plasma levels of many commonly used agents such as antidepressants; (b) changes in drug transporters, which may affect the response to medications: for instance, polymorphisms in the promoter for synthesis of serotonin transporter (SERT-P) influence response to serotonergic medications in depressed individuals. SERT polymorphisms were associated with a greater colonic transit response in those with long homozygous than those with heterozygous polymorphisms in D-IBS (Camilleri et al., 2002); (c) genetic polymorphism in drug targets. Several examples are provided by recent studies (Camilleri and Katzka, 2012; Vazquez-Roque et al., 2012).

In summary, pharmacogenetics is a rapidly growing field which may provide important pieces of information to fully understand the variability of drug action in patients and cannot be ignored in drug development programs, although its exploitation probably still needs some time.

CLASSES OF DRUGS USED OR UNDER DEVELOPMENT IN IBS

A detailed discussion of all the classes of drugs proposed as potential therapeutic agents in IBS is beyond the scope of this review. The main pharmacological approaches to IBS are summarized in Table 2, which does not include agents traditionally used in IBS, such as laxatives and antidiarrheal agents, respectively for constipation and diarrhea, and probiotics. The gut microbiome as a therapeutic target is covered elsewhere (Floch et al., 2011; Simren

Table 2 | Main pharmacological approaches in IBS.

Drug class	Examples	Pharmacodynamics
5-HT₄ receptor agonists	Prucalopride Naronapride Velusetrag TD-8954	Enteric neurons, smooth muscle cells Increased motility/bowel frequency
5-HT₃-receptor antagonists	Ramosetron	Inhibition of secretion, motility, nociception
TPH₁ blocker	LX-1031	Inhibits 5-HT synthesis by blocking tryptophane hydroxylase 1
Cl-C2 channel activator	Lubiprostone	Increased intestinal water and electrolyte secretion Accelerates transit
Guanylate cyclase-C agonist	Linaclootide	Increased intestinal water and electrolyte secretion Accelerates transit
PAR2 blockers	GB88	Inflammation
TRPV1 blockers	Capsazepine	Inflammation
TRPV4 blockers	RN1734	
Mast cell stabilizers	Ketotifen	Inflammation
μ-Opioid receptor agonists	Loperamide	Enterocyte, enteric neurons, afferent neurons, and inflammation
μ-Opioid receptor antagonists	Naloxone Methylnaltrexone alvimopan	Enteric neurons, afferent neurons, and inflammation
κ-Opioid receptor agonists	Asimadoline	Enteric neurons and afferent neurons Increase in sensory threshold
β₃-Adrenoceptor agonists	Solabegron	Smooth muscle
α₂-Adrenoceptor agonists	Clonidine	Enteric neurons and enterocytes
NK₁ receptor antagonists	Ezlopitant	Enteric neurons, ICC, smooth muscle, immune cells
NK₂ receptor antagonists	Nepadutant	Reduced smooth muscle contractility
NK₃ receptor antagonists	Talnetant	Role in nociception not confirmed in clinical trials in patients with IBS
CCK₁ receptor antagonists	Loxiglumide	Afferent vagal nerves and enteric neurons
Antibiotics	Rifaximin	Poorly absorbed with virtually no systemic effects

et al., 2013). The reader is also referred to a recent detailed review of current and potential pharmacological approaches in IBS (Camilleri, 2012). Herbal preparations used in IBS are covered by another review (Rahimi and Abdollahi, 2012). Brain-gut interactions and possible sites/mechanisms of pharmacological intervention along the brain-gut axis are discussed in a recent review in this Journal (Fichna and Storr, 2012).

Several meta-analyses of pharmacological treatments for IBS have been published in recent years and there is ongoing debate on their interpretation (Lesbros-Pantoflickova et al., 2004; Brandt et al., 2009; Camilleri and Mayer, 2009).

SEROTONERGIC AGENTS

5-Hydroxytryptamine (5-HT) plays a key role in the control of gastrointestinal motility, sensitivity, and secretion (De Ponti, 2004). Several 5-HT receptor types are present on both nerves and

smooth muscle and mediate a number of different actions (De Ponti, 2004). Actions of 5-HT are terminated by a reuptake system, which is inhibited by antidepressants (Gershon and Jonakait, 1979). *Selective serotonin reuptake inhibitors* (SSRIs) alter motility in the stomach, small bowel, and colon (Gorard et al., 1994), but no convincing beneficial therapeutic effects have been reported in IBS. Interestingly, the tryptophane hydroxylase inhibitor LX-1031 was recently reported to have therapeutic potential in IBS (Tack et al., 2011).

5-HT₃ receptor antagonists include alosetron and ramosetron (Camilleri et al., 2000; Hirata et al., 2007; Lee et al., 2011); alosetron delays orocecal and colonic transit times, and reduce colonic compliance but not sensitivity to isobaric distension (Gore et al., 1990; Talley et al., 1990; Scolapio et al., 1995). Shortly after its introduction, alosetron was withdrawn due to suspected side-effects of colonic ischemia (Moynihan, 2002).

5-HT₄ receptor agonists, after withdrawal of tegaserod, now include prucalopride, naronapride, velusetrag (Camilleri, 2010b), and TD-8954 (Beattie et al., 2011). These agents are thought to act on intrinsic neurons to stimulate gastric, small bowel, and colonic transit in health, in constipation and in IBS with constipation (Bouras et al., 1999; Poen et al., 1999; Degen et al., 2001). In the stomach, *5-HT₄* receptor agonists enhance (postprandial) proximal gastric volumes in health, but have no effects on sensation (Tack et al., 2003). Prucalopride was also shown to be effective in the treatment of constipation (Emmanuel et al., 2002).

LINACLOTIDE

This is an example of guanylate cyclase-C agonist (Busby et al., 2013), which has been shown to reduce visceral hypersensitivity in preclinical studies and to improve abdominal pain and constipation symptoms in phase 2 and 3 clinical trials of patients with IBS and constipation (Johnston et al., 2013).

LUBIPROSTONE

This is an oral bicyclic fatty acid selectively activating type 2 chloride channels in the apical membrane of the intestinal epithelial cells, hence stimulating chloride secretion, along with passive secretion of sodium and water, inducing peristalsis and laxation, without stimulating gastrointestinal smooth muscle (Schey and Rao, 2011). It is indicated in IBS with constipation. Considering the importance of epithelial barrier function and cell integrity and the known impact of stressors, the observation that lubiprostone exhibits the additional distinct property of effective protection or repair of the epithelial barrier and cell function after stress suggests potential clinical importance for patients with impaired barrier function, which might occur in IBS (Cuppoletti et al., 2012).

TACHYKININ RECEPTOR ANTAGONISTS

Three distinct receptors, NK₁, NK₂, and NK₃, mediate the biological effects of endogenous tachykinins SP, NKA, and NKB, in the gastrointestinal tract. Through the locations of NK receptors on intrinsic nerves, extrinsic nerves, inflammatory cells, and smooth muscle, inhibition of tachykinin receptors has the potential to inhibit motility, sensitivity, secretion, and inflammation in the gastrointestinal tract (Holzer, 2004a; Lecci et al., 2004). NK₁ receptor antagonists also have antiemetic properties (Holzer, 2004a). Several tachykinin receptor antagonists have been developed so far, but the results, in general, have been disappointing. Recently, chronic treatment with AV608 (NK₁ receptor antagonist) in IBS has been reported to be associated with improved mood and pain ratings and activity of emotional arousal related brain regions (Tillisch et al., 2012).

ADRENOCEPTOR AGONIST

The α_2 -adrenoceptor agonist clonidine was shown to reduce colonic tone and pain sensation in response to distension (Bharucha et al., 1997; Malcolm et al., 2000; Viramontes et al., 2001b). A preliminary study of clonidine in IBS with diarrhea suggested therapeutic potential for clonidine, but clinical application is hampered by dose-limiting side-effects like somnolence or hypotension. Among β_3 -adrenoceptor agonists, solabegron is an example (Grudell et al., 2008).

OPIOID RECEPTOR LIGANDS

Three types of opioid receptors, μ -, δ -, and κ -receptors, located in the ENS as well as on nociceptive pathways, have effects on human gastrointestinal function. Opioid receptor activation reduces visceral pain through peripheral (spinal afferents) and central mechanisms, and inhibits motility through decreased acetylcholine release. κ -Opioid receptor agonists have been proposed as a pharmacological approach to the treatment of altered visceral sensitivity. Acute studies with fedotozine and asimadoline showed decreased sensitivity to gastric or colonic distension (Coffin et al., 1996; Delvaux et al., 1999, 2002; Delgado-Aros et al., 2003). However, therapeutic studies in IBS and FD with fedotozine have been disappointing (Dapoigny et al., 1995; Read et al., 1997). The μ -opioid receptor agonist loperamide, used in the treatment of diarrhea, inhibits secretion, reduces colonic transit, and increases resting anal sphincter tone (Corazziari, 1999). Peripherally acting μ -opioid receptor antagonists (e.g., N-methylnaltrexone and alvimopan) normalize bowel function in opiate-treated patients without compromising central opioid analgesia (Holzer, 2004b). Racecadotril, a neutral endopeptidase (NEP) inhibitor, increases the exposure to NEP substrates including enkephalins: it was found consistently effective in animal models and patients with various forms of acute diarrhea by inhibiting secretion from the gut without changing gastrointestinal transit time or motility (Eberlin et al., 2012). In direct comparative studies with loperamide, racecadotril was at least as effective, but exhibited fewer adverse events in most studies, particularly less rebound constipation (Eberlin et al., 2012). However, its potential in IBS remains to be established.

MISCELLANEOUS AGENTS

CCK has a large number of effects on gastrointestinal contractility and secretion (Walsh, 1994). CCK1 receptor antagonists like loxiglumide and dexloxiglumide enhance gastric emptying in health and in IBS with constipation, though effects on colonic motility are unclear (De Ponti and Malagelada, 1998; Scarpignato and Pelosi, 1999) and clinical usefulness has not been established.

The transient receptor potential ion channel of the *vanilloid type 1* (TRPV1), expressed by primary afferent neurons, is viewed as a trigger for chemonociception and may be upregulated in some functional gut disorders (Chan et al., 2003). TRPV1 and TRPV4 blockade are areas of current investigation (Holzer, 2011; Fichna et al., 2012).

Muscarinic receptor antagonists and *smooth muscle relaxants* are used in some countries for the treatment of IBS. Meta-analysis suggests they are superior to placebo in IBS-related pain (Poynard et al., 2001), though the quality of trials is often questionable.

Cannabinoid CB₁ receptors are expressed on nociceptive afferents and ENS neurons while *CB₂* receptors are expressed on immune cells (Schicho and Storr, 2011). Activation of *CB₁* receptors slows gastrointestinal transit in animals through inhibition of acetylcholine release. The non-specific agonist delta-9-tetrahydrocannabinol has strong antiemetic properties and delays gastric emptying in humans (Frytak et al., 1979; McCallum et al., 1999). The observation that, in an animal model of intestinal inflammation, *CB₁* and *CB₂* receptor subtypes are upregulated opens a new perspective on the possible use of *CB₁* or *CB₂* receptor

agonists in postinfectious IBS with diarrhea (Kimball et al., 2010). Indeed, dronabinol, a non-selective CB agonist, reduces fasting colonic motility in patients with IBS with diarrhea or alternating (Wong et al., 2011).

Finally, $\alpha\delta$ ligands such as gabapentin and pregabalin (Gale and Houghton, 2011) have undergone a number of preclinical and clinical tests for their potential in disorders with visceral hypersensitivity. In fact, voltage-sensitive Ca^{2+} channels are involved in neural function and have an $\alpha\delta$ binding site to which the aforementioned ligands bind potently, reducing Ca^{2+} influx at the nerve terminals. Pregabalin was effective in several animal models of visceral pain (Gale and Houghton, 2011).

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CONCLUSION

From the above overview, it is clear that research in the treatment of IBS is still very active. Although in the past decade some innovative pharmacological agents have not fulfilled their promise because of unexpected side-effects, it is likely that new pathophysiological concepts as well as the publication of new regulatory documents by the FDA and the EMA will be of great help for drug developers.

ACKNOWLEDGMENTS

The original research of the author is supported by a grant from the University of Bologna (Ricerca Fondamentale Orientata 2011).

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Conflict of Interest Statement: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 13 December 2012; **paper pending published:** 20 December 2012; **accepted:** 10 January 2013; **published online:** 01 February 2013.

Citation: De Ponti F (2013) Drug development for the irritable bowel syndrome: current challenges and future perspectives. *Front. Pharmacol.* 4:7. doi: 10.3389/fphar.2013.00007

This article was submitted to Frontiers in Gastrointestinal Pharmacology, a specialty of Frontiers in Pharmacology.

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Alternative functional *in vitro* models of human intestinal epithelia

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Physiologically relevant sources of absorptive intestinal epithelial cells are crucial for human drug transport studies. Human adenocarcinoma-derived intestinal cell lines, such as Caco-2, offer conveniences of easy culture maintenance and scalability, but do not fully recapitulate *in vivo* intestinal phenotypes. Additional sources of renewable physiologically relevant human intestinal cells would provide a much needed tool for drug discovery and intestinal physiology. We compared two alternative sources of human intestinal cells, commercially available primary human intestinal epithelial cells (hInEpCs) and induced pluripotent stem cell (iPSC)-derived intestinal cells to Caco-2, for use in *in vitro* transwell monolayer intestinal transport assays. To achieve this for iPSC-derived cells, intestinal organogenesis was adapted to transwell differentiation. Intestinal cells were assessed by marker expression through immunocytochemical and mRNA expression analyses, monolayer integrity through Transepithelial Electrical Resistance (TEER) measurements and molecule permeability, and functionality by taking advantage the well-characterized intestinal transport mechanisms. In most cases, marker expression for primary hInEpCs and iPSC-derived cells appeared to be as good as or better than Caco-2. Furthermore, transwell monolayers exhibited high TEER with low permeability. Primary hInEpCs showed molecule efflux indicative of P-glycoprotein (Pgp) transport. Primary hInEpCs and iPSC-derived cells also showed neonatal Fc receptor-dependent binding of immunoglobulin G variants. Primary hInEpCs and iPSC-derived intestinal cells exhibit expected marker expression and demonstrate basic functional monolayer formation, similar to or better than Caco-2. These cells could offer an alternative source of human intestinal cells for understanding normal intestinal epithelial physiology and drug transport.

Keywords: human intestinal epithelial cell (hInEpC), induced pluripotent stem cell (iPSC), permeability, Transepithelial Electrical Resistance (TEER), neonatal Fc receptor (FcRn)

INTRODUCTION

The use of *in vitro* cell models for human drug transport studies has focused on intestinal epithelial cells, as these cultures contain primarily absorptive cells. While isolated human intestinal epithelial cells (hInEpCs) retain important *in vivo* anatomical and biochemical features, they are difficult to culture and have limited viability. As a result, immortalized human adenocarcinoma cell lines have been extensively used to study absorption mechanisms. While immortalized cells offer many advantages, extrapolation of data generated with these cell lines to *in vivo* conditions is often difficult, as these cells originated from tumors and are therefore not representative of the true physiological environment (Le Ferrec et al., 2001). In addition, these cells form monolayers that are widely used for small molecule intestinal permeation *in vitro* studies (below). But, with increasing numbers of biotechnology protein therapeutics and novel scaffolds available, which open the possibility for oral delivery, there is a need for alternatives that more closely recapitulate the physiology of the intestinal epithelial cell.

The human colorectal adenocarcinoma cell line Caco-2 is frequently used for drug absorption studies, particularly in the context of small molecules (Le Ferrec et al., 2001; Balimane and Chong, 2005). Caco-2 cells are easy to culture and have the capacity to spontaneously differentiate into cells possessing the morphology and function of enterocytes, the absorptive cells of the intestine (Balinane and Chong, 2005). Caco-2 cells are commonly cultured on semi-permeable inserts in a transwell format, where the cells form a polarized monolayer (Leonard et al., 2000; Le Ferrec et al., 2001), and the transport of molecules between the apical and basolateral chambers can be easily evaluated. While Caco-2 cells are a good model for observation of passive transcellular and paracellular permeability (Balinane and Chong, 2005), there are differences in cytokine production and cytokine receptor expression between Caco-2 cells and normal epithelial cells (Aldhous et al., 2001). In addition, Caco-2 cells under-express transporters and metabolizing enzymes relative to *in vivo* tissue, potentially excluding mechanisms crucial for drug absorption studies (Balinane and Chong, 2005).

Due to the limitations of immortalized intestinal cell lines, many studies have focused on the use of primary hInEpCs as a more physiologically relevant cell-based model (Perreault and Beaulieu, 1998; Aldhous et al., 2001; Ootani et al., 2009; Lahar et al., 2011). However, stocks of these cells are difficult to maintain due to limited donors and low viability in culture. Recently, commercial sources of primary hInEpCs were made available (Lonza; Walkersville, MD), which greatly increase the convenience of obtaining primary cell stocks. Commercial quality control data suggest that these primary hInEpCs have the capacity to form monolayers with tight junctions and express general epithelial markers, such as cytokeratins 8 and 18 (Bosch et al., 1988); however, little characterization has been done on their expression of intestinal cell type-specific markers or transport function. Other efforts to enable long-term culture of primary cells and enhance physiological conditions have led to the development of 3-dimensional (3D) models of the intestinal epithelium, which have focused on the use of primary intestinal stem cells and directed differentiation of pluripotent stem cells.

Stem cells have the capacity to self-renew and differentiate into the various cell lineages that make up specific tissue types. For example, intestinal stem cells are responsible for the self-renewal of the gut epithelium, and have been used in developing 3D intestinal models. Leucine-rich repeat-containing G protein-coupled receptor 5 (LGR-5)-positive stem cells can be isolated from primary intestinal tissue and grown as 3D intestinal organoids with crypt-villi physiology and culturing capacity up to 8 months (Sato et al., 2009). While 3D organoids derived from primary intestinal stem cells appear to possess physiologically relevant phenotypes, they cannot be used to assess classical functionality typically determined within 2-dimensional transwell cultures, such as the formation of monolayers with tight junctions and intestinal permeability and transport.

An additional source of human intestinal cells is possible through directed differentiation of pluripotent stem cells to intestinal cell lineages. The recent advent of human induced pluripotent stem cells (iPSCs) has provided a huge therapeutic potential as a tool for drug discovery, as patient-specific somatic cells can be reprogrammed into an embryonic stem cell-like state that can be directly differentiated to a specific cell type of interest for more physiologically relevant disease modeling. Induced human intestinal organoids (iHIOs) have recently been derived from iPSCs (Spence et al., 2010), which are capable of expressing epithelial and intestinal markers such as caudal type homeobox 2 (CDX2) (hindgut marker), E-Cadherin (cell-to-cell junction marker), and Villin (epithelial brush border marker). However, to our knowledge, the methods for differentiating these into a polarized epithelial monolayer similar to Caco-2 have not been reported.

In this study, we assessed expression of markers and functional activity of the newly commercially available primary hInEpCs and iPSC-derived intestinal cells compared to Caco-2 (**Figure 1**) in cell-based *in vitro* assays. We adapted our previously described 3D intestinal organogenesis to differentiation within transwells.

Intestinal marker expression, formation of monolayers with tight junction formation and functional molecule transport and binding were evaluated.

MATERIALS AND METHODS

CELL CULTURE

Intestinal cells

Human primary small intestinal epithelial cells (Lonza; Walkersville, MD) from 3 donors (Donor A: Lot # 0000258132; Donor B: Lot # 0000256741; Donor C: Lot # 0000256744) were thawed and cultured in transwell inserts for 10–11 days in SmGM-2 media (Lonza), according to manufacturer's instructions. Caco-2 colorectal adenocarcinoma-derived cells (ATCC; Manassas, VA) were cultured in transwell inserts for 14–21 days in Caco-2 media (10% fetal bovine serum, 1X non-essential amino acids, 1X sodium pyruvate, and 6 mM L-Glutamine in Dulbecco's Modified Eagle's Medium (DMEM) High Glucose, reagents from Life Technologies; Carlsbad, CA).

iPSCs

A1145A and B2198A (Johnson & Johnson; Spring House, PA) were produced from human kidney-derived cells using retroviral (Takahashi et al., 2007) and modified mRNA (non-viral) reprogramming methods (Yakubov et al., 2010), respectively. C2198A and C2200B (Johnson & Johnson; Spring House, PA) were produced from human umbilical tissue-derived cells by modified RNA reprogramming methods. D2043A (System Biosciences; Mountain View, CA) was derived from human foreskin fibroblasts by retroviral methods. iPSC lines were maintained in mTeSR1 culture media (STEMCELL Technologies; Vancouver, BC) on culture dishes/flasks coated with Geltrex (Life Technologies) and passaged by Dispase (STEMCELL Technologies) dissociation every 3–5 days as previously described (McCracken et al., 2011).

iPSC DIRECTED DIFFERENTIATION

Transwell differentiation

Semi-permeable transwell inserts were coated apically with Geltrex (Life Technologies) prior to plating iPSCs within 12-well transwell tissue culture plates. iPSCs were plated apically as cell clumps (5–10 cells per clump) at a density of 3000–6000 clumps per transwell insert. Two to three days after plating, iPSCs were differentiated within transwell inserts modified previously reported methods (Spence et al., 2010) into definitive endoderm using GDF8 with GSK3b inhibitor and B27 supplement then differentiated to hindgut using Keratinocyte Growth Factor and Retinoic Acid then EGF, Noggin, and R-Spondin1 for >26 days (Kauffman et al., Submitted) on both sides of the transwell insert; however, the many spheroid structures observed at Stage 2 were maintained as part of the adherent layer throughout Stage 3 differentiation (**Table 1**) for up to 31 days.

MONOLAYER ASSESSMENT

Transepithelial electrical resistance (TEER)

TEER of transwell cultures was recorded in measurements of Ohms using an Epithelial Volt Ohm Meter (EVOM)² and electrode set (World Precision Instruments; Sarasota, FL). Raw

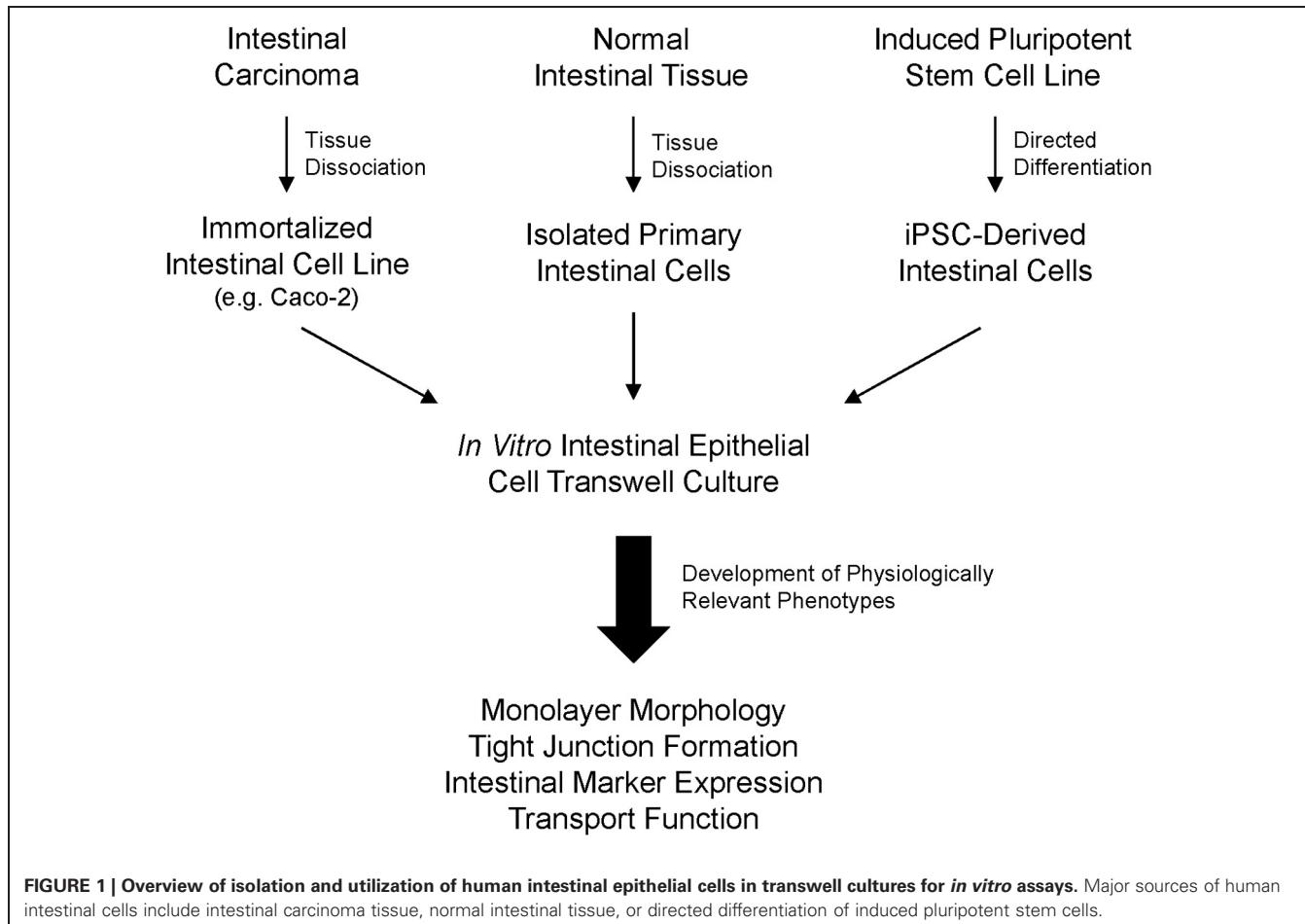


Table 1 | Summary of growth factors and differentiation cell culture conditions used for transwell differentiation, relative to previously established intestinal organogenesis to 3D organoids.

Stage	Growth factors	Days	Cell type	Stage markers	Organoid culture	Transwell culture
1	GDF-8, GSKi, B27	3	Definitive endoderm	SOX17, CXCR4	2D Standard tissue culture plate	2D Transwell
2	KGF, RA	7	Hindgut	CDX2	2D Excise spheroids from standard plate	2D Transwell (No Spheroid Excision)
3	EGF, Noggin, R-Spondin1	26+	Intestinal	E-Cad, CDX2, Villin	3D Intestinal matrigel	2D Transwell

data was converted to $\Omega \times \text{cm}^2$ based on area of transwell plate inserts (1.12 cm^2).

FITC-dextran permeability

Cells in 24-well transwell plates were washed twice with DPBS (Life Technologies). To the apical side, $200 \mu\text{L}$ of 12.5 mg/mL Fluorescein isothiocyanate-dextran, molecular weight 150 kDa (FD150) (Sigma-Aldrich) diluted in Caco-2 media ($pH = 6.0$) was added. To the basolateral side, $500 \mu\text{L}$ of Caco-2 media ($pH = 7.4$) was added. After a 90-min incubation at 37°C , $100 \mu\text{L}$ of media was collected from the basolateral chamber and analyzed for the presence of FITC-Dextran using a SpectraMax M5 microplate reader (Molecular Devices; Sunnyvale, CA).

P-Glycoprotein Transport Assay

Primary hInEpCs were dosed on the apical or basolateral side with a 2 mM mix of Digoxin and Atenolol in HBSSg [2 mM glucose and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid in Hank's Balanced Salt Solution (HBSS)] with calcium and magnesium, $pH = 7.4$ and incubated for 90 min at 37°C in the presence or absence of $10 \mu\text{M}$ Cyclosporin A (CSA). Samples were collected from both the apical/donor and basolateral/receiver chambers and analyzed by LCMS. Digoxin and Atenolol levels in each condition were used to calculate apparent permeability ($P_{app} = \delta Cr / \delta t \times V_r / (A \times C_0)$) in the apical to basolateral (A-B) or basolateral to apical (B-A) direction. δC_r = final receiver concentration; δt = assay time; V_r = receiver volume;

A = transwell growth area; C_0 = initial apical concentration. To ensure monolayer integrity during the assay, all wells were dosed apically with 100 μ g/mL Lucifer Yellow (LY) at the start of Digoxin and Atenolol incubation, and samples were collected from the basolateral chamber for analysis by SpectraMax M5 microplate reader at the end of the 90 min incubation. Only transwells with a LY Papp(A-B) of $< 1 \times 10^{-6}$ cm/s were used in calculating final Papp Ratios for Digoxin and Atenolol. Based on this cutoff, one of 8 wells each for hInEpC Donors A and B, and two of 8 wells for Donor C was excluded from data analysis.

mRNA EXPRESSION

RNA was harvested from Caco-2, primary hInEpCs, or iPSCs before or after differentiation to Stage 3 by RNeasy Mini kit (Qiagen; Germantown, MD), and reverse transcribed to cDNA using the RT² First Strand kit (Qiagen). Using 30 ng/ μ L starting cDNA, samples were used in reactions within Custom RT² Profiler PCR array containing probes supplied by the manufacturer (SABiosciences; Valencia, CA) for intestinal and control markers (Table 2), following reaction cycling conditions outlined in the manufacturer's protocol. Data analysis was performed using the Δ CT method, where raw Ct values were normalized to housekeeping gene 60S acidic ribosomal protein P0 (RPLP0) before comparing expression relative to Caco-2. Expression levels for primary hInEpCs represents the average across all three donors used in this study.

Table 2 | List of probes used in Custom RT² Profiler PCR array mRNA expression analyses of intestinal and control markers in intestinal epithelial cells.

Gene	NCBI reference no	SAB catalog no
E-Cadherin	NM_004360	PPH00135
CDX2	NM_001265	PPH13618
KLF5	NM_001730	PPH00434
Villin	NM_007127	PPH23365
SOX9	NM_000346	PPH02125
LGR5	NM_003667	PPH13346
ASCL2	NM_005170	PPH12852
MUC2	NM_002457	PPH06990
Chromogranin A	NM_001275	PPH01181
LYZ	NM_000239	PPH14748
VIM	NM_003380	PPH00417
FcRn	NM_004107	PPH11194
CXCR4	NM_003467	PPH00621
PDX1	NM_000209	PPH05536
OCT4	NM_002701	PPH02394
TNNT2	NM_000364	PPH02619
PAX6	NM_000280	PPH02598
TUBB3	NM_006086	PPH02607
RPLP0	NM_001002	PPH21138

Probes for the NCBI Reference sequence numbers listed were obtained from SABiosciences (SAB) using the catalog numbers provided.

PROTEIN EXPRESSION

Flow cytometry

Undifferentiated or Stage 1 iPSCs were detached by treatment with Accutase (Sigma-Aldrich; St. Louis, MO) and stained for viability by Near Infrared Live/Dead kit (Invitrogen; Carlsbad, CA) before fixation in Cytofix Buffer (BD Biosciences; San Jose, CA). For pluripotent marker analyses, cells were stained using Human Pluripotent Stem Cell Transcription Factor Analysis or Human Pluripotent Stem Cell Sorting and Analysis kits (BD Biosciences). For definitive endoderm marker analysis, cells were stained with a 1:5 dilution of PE-conjugated mouse anti-human CD184/ C-X-C chemokine receptor type 4 (CXCR4) (306506, Biolegend; San Diego, CA) before permeabilized with Phosflow Perm Buffer (BD Biosciences) and stained with a 1:5 dilution of APC-conjugated goat polyclonal anti-human Sry-related HMG box 17 (SOX17) (IC1924A, R&D Systems; Minneapolis, MN). For analysis of surface expression of neonatal Fc receptor (FcRn), Caco-2 and primary hInEpCs were dissociated from transwell culture by Accutase, incubated with 50 μ g/mL affinity purified rat anti-human FcRn polyclonal antibody (generated in house), followed by incubation with 7.5 μ g/mL FITC Donkey anti-rat IgG (F(ab)2) secondary antibody (109-006-006, Jackson Immunoresearch; West Grove, PA). Fluorescence of stained cells was measured in conjunction with appropriate compensation controls (BD Biosciences) by flow cytometry using an LSR Fortessa FACS Sorter (BD Biosciences). Raw data was analyzed by FlowJo analysis software (Tree Star; Ashland, OR), with gating parameters set based on isotype controls (Figure A1A).

Immunofluorescence

iPSC-derived cells Stage 2 iPSC-derived cells were fixed in 4% paraformaldehyde (Electron Microscopy Sciences; Hatfield, PA) before permeabilization with 0.5% Triton X-100 (Electron Microscopy Sciences). After addition of Image-iT® FX signal enhancer (Invitrogen), cells were treated with 1X Blocking Buffer (Sigma) before exposure to a 1:50 dilution of E-Cadherin, CDX2, or Villin antibodies (Dako), using manufacturer recommended concentrations. Immunoreactivity to primary antibodies was detected with a 1:500 dilution of AF568-conjugated goat anti-mouse antibody (Invitrogen). Prolong GOLD antifade with DAPI (Invitrogen) was added to wells prior to visualizing on a Nikon SMZ-1500 fluorescence dissecting microscope.

FcRn-DEPENDENT IMMUNOGLOBULIN G (IgG) BINDING

Caco-2, hInEpCs (Donor A), or iPSC-derived cells were detached from transwells by Accutase (Sigma) treatment and washed twice with Dulbecco's Phosphate-Buffered Saline (DPBS) (Life Technologies). Cells were transferred in DPBS to MesoScale Discovery (MSD) High Bind plates (MSD; Rockville, MD) at a density of 1×10^4 or 2.5×10^4 cells per well, and incubated at room temperature for 2 h to allow attachment to the plate surface. Plates were then blocked with 20% Fetal Bovine Serum (Life Technologies) and 0.18% Sodium Azide (VWR International; Radnor, PA) for 15 min at room temperature. Wells were washed once with DPBS at pH 6.0 before incubation with FcRn-binding

variants (anti-RSV N434A or anti-RSV H435A in DPBS at pH 6.0) for 90 min at 37°C. Plates were washed 3 times with DPBS at pH 6.0, and cells were incubated with ruthenium-labeled goat anti-human IgG F(ab')2 (1 µg/ml in DPBS at pH 6.0) for 1 h at room temperature. Cells were washed 3 times with DPBS at pH 6.0. Tris-based Read Buffer T without surfactant (MSD) was added to wells immediately before measuring Relative Luminescent Units (RLU) using a Sector Imager 6000 reader and Discovery Workbench software (MSD). For analysis, background signal RLU were subtracted from RLU of samples run in triplicate.

RESULTS

MULTIPLE SOURCES OF HUMAN INTESTINAL EPITHELIAL CELLS

EXHIBIT INTESTINAL MARKER EXPRESSION

Along with limited viability in culture, one of the major drawbacks of routinely using primary hInEpCs for studying intestinal physiology is that these cells must be collected from human donors. A newly available commercial source of primary hInEpCs greatly increases the convenience of obtaining primary cell stocks, but need to be further characterized for intestinal cell-type specific marker expression and functional monolayer formation. Thus, we sought to characterize these commercially available stocks to assess their physiological relevance for use in cell-based *in vitro* assays of intestinal uptake and transport.

As previously reported, a measure of intestinal cell phenotypic quality is the expression of a panel of general intestinal epithelial markers (Spence et al., 2010). We used immunocytochemistry to verify expression and expected localization of several representative intestinal epithelial markers within primary hInEpCs from three different donors (**Figure 2**, top row; **Figure A1B**), relative to the immortalized human intestinal epithelial cell line Caco-2

(**Figure 2**, bottom row). This was visualized for E-cadherin, an epithelial cell adhesion marker found within tight junctions between cells (Zbar et al., 2004), CDX2, a transcription factor that is upstream of signaling promoting intestinal cell fate (Gao et al., 2009) and Villin, an actin-binding protein associated with the intestinal brush border of absorptive enterocytes (Friederich et al., 1999). The localization of these markers within cells was appropriate in primary hInEpCs and Caco-2 (**Figure 2**). Marker expression intensity was variable between the three hInEpC donors within this study, with strongest expression evident in Donor A (**Figure 2**, top row; **Figure A1B**). Expression results for hInEpCs and Caco-2 were used to compare to iPSC-derived iHIOs grown in transwells (below).

In order to evaluate iPSC-derived intestinal cell phenotypes within classical *in vitro* monolayer conditions, we adapted intestinal organogenesis to transwell culture by performing iPSC differentiation through Stage 3 within transwell inserts (**Table 2**, **Figure 3A**). Differentiation of a panel of iPSC lines (A1145A, B2198A, C2128A, C2200B, and D2043A) on matrigel-coated transwell inserts using Myostatin, Glycogen Synthase Kinase 3β inhibitor and B27 supplement resulted in cells expressing definitive endoderm markers SOX17 and CXCR4 (**Figure 3B**), similar to iPSCs differentiated to Stage 1 in standard tissue culture plates (Kauffman et al., Submitted). Further differentiation of iPSC-derived definitive endoderm cells within transwell plates, by addition of Keratinocyte Growth Factor and Retinoic Acid, produced layers of cells with developing spheroid structures at Stage 2 in many of the iPSC lines (**Figure 3C**, top row). CDX2 expression at Stage 2 was relatively uniform within layers of cells differentiating in transwells (**Figure 3C**, bottom row). However, D2043A showed more intense expression around spheroid structures.

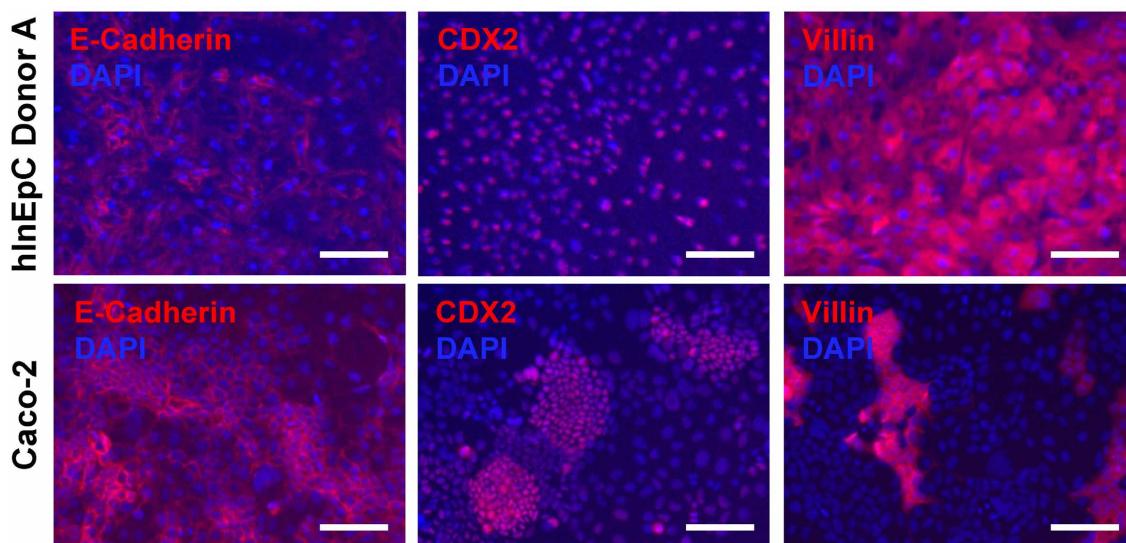
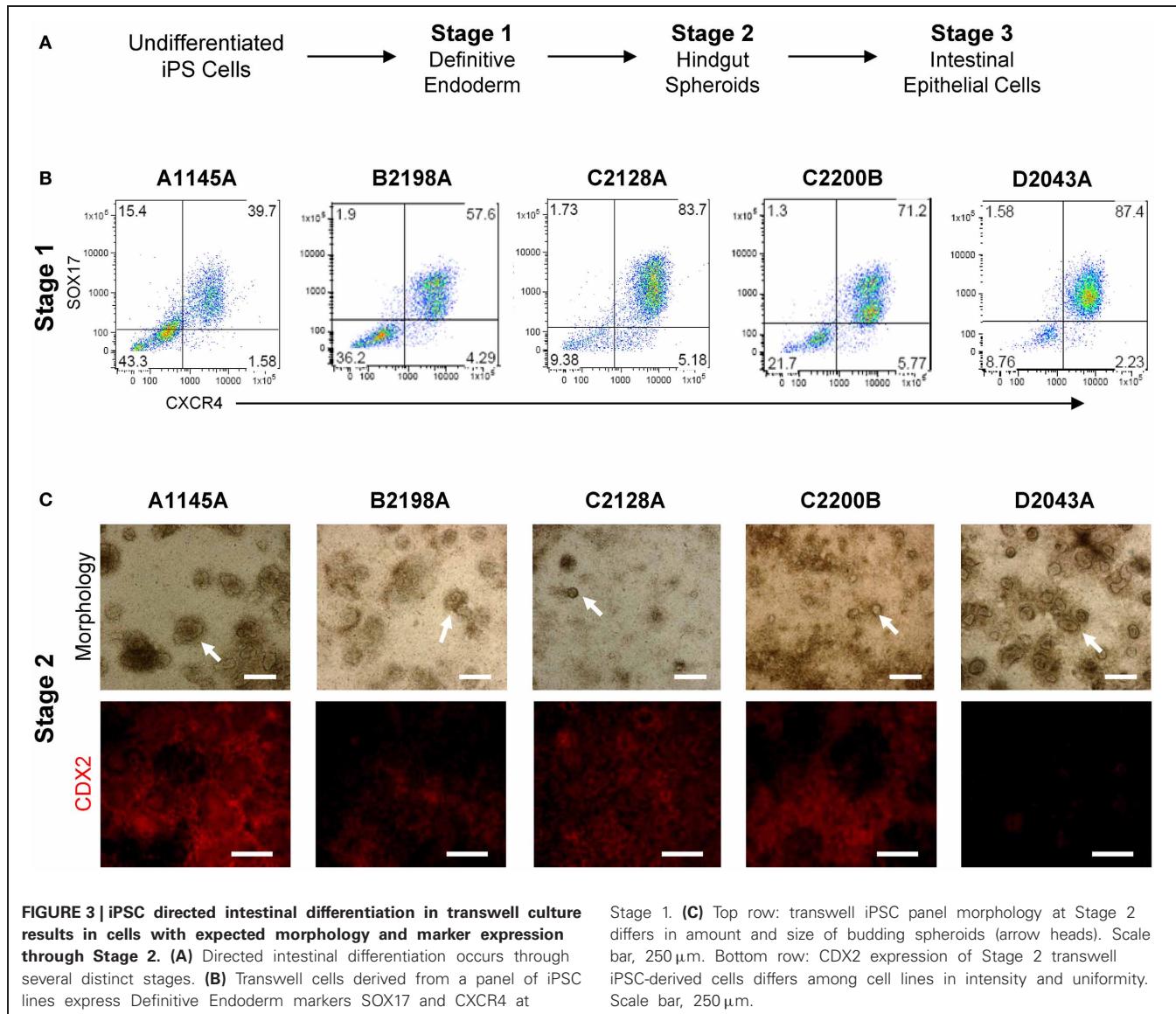


FIGURE 2 | Characterization of intestinal marker expression in commercially available (Lonza) primary human intestinal epithelial cells (hInEpCs) relative to immortalized human intestinal cell line Caco-2. Top row: primary hInEpCs uniformly express intestinal epithelial markers

E-Cadherin (tight junctions), CDX2 (hindgut), and Villin (enterocytes). Primary hInEpC Donor A shown (see **Figure A1B** for Donors B and C). **Bottom row:** Caco-2 show less uniform expression of enterocyte marker Villin. Scale bar, 100 µm.



iPSC-derived Stage 3 3D intestinal organoids exhibit E-Cadherin, CDX2, Villin, and Chromogranin A immunoreactivity with the expected localization (Kauffman et al., Submitted). In this study, a supply of transwell A1145A iPSC-derived Stage 3 Day 31 cells was limited, and did not allow sufficient material for extensive immunocytochemistry analyses of intestinal markers. Thus, mRNA expression analysis was used to directly compare larger panel of known intestinal marker and differentiation control genes Stage 3 A1145A iPSC-derived intestinal marker expression to Caco-2 and primary hInEpCs by RT-PCR (Figure 4). Differentiated Stage 3 iPSCs showed increased marker expression relative to undifferentiated cells, usually reaching a level more similar to primary hInEpCs in the case of 3D iHIOs (Figure 4), or more similar to Caco-2 in the case of transwell-differentiated iPSCs (Figure 4). Intestinal markers that followed this expression pattern included epithelial tight junction marker E-Cadherin (Zbar et al., 2004),

hindgut epithelial marker CDX2 (Gao et al., 2009), enterocyte marker Villin (Friederich et al., 1999), enteroendocrine marker Chromogranin A (O'Connor et al., 1983), and Mucin-2 (MUC2), a marker for intestinal goblet cells (Gum et al., 1999) (Figures 4A–E).

For some intestinal makers, expression in iPSC-derived intestinal cell types was highest in iPSC-derived cells. For example, expression levels of intestinal crypt cell marker Sex determining region Y-box 9 (SOX9), paneth cell marker Lyzosome (LYZ) (Peeters and Vantrappen, 1975), and intestinal stem cell marker LGR5 (Barker et al., 2007) were greatest in iHIOs and transwell differentiated cells compared to Caco-2 or primary hInEpCs (Figures 4F–H). On the other hand, an additional intestinal stem cell marker, Achaete Scute-Like 2 (ASCL2) (van der Flier et al., 2009), was most highly expressed in Caco-2 (Figure 4I). In the case of intestinal epithelial cell differentiation transcription factor Kruppel-like factor 5 (KLF5) (Bell et al., 2013), mRNA expression

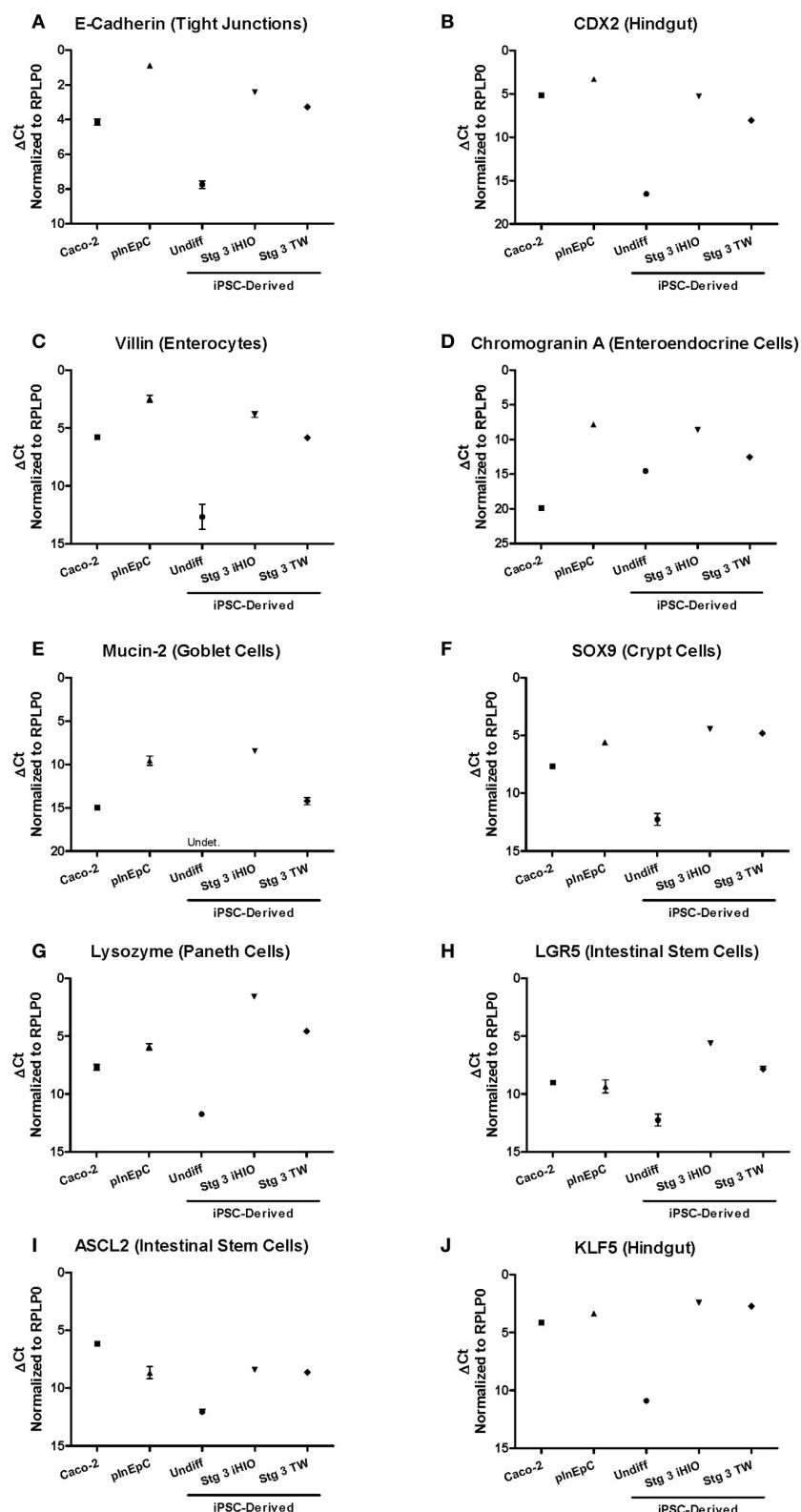


FIGURE 4 | Comparison of intestinal marker mRNA expression in multiple sources of human intestinal epithelial cells. Expression of tight junction marker (A) and hindgut epithelia (B), as well as specific epithelial lineage markers (C–J) intestinal markers of epithelial intestinal lineages was evaluated by RT-PCR in Caco-2, primary hlnEpcCs (average

of Donors A–C), and iPSC-derived undifferentiated (Undiff), Stage 3 induced human intestinal organoids (Stg 3 iHIOs), or Stage 3 transwell intestinal cells (Stg 3 TW). Using the ΔCt method, raw Ct values were normalized to housekeeping gene RPLP0. $N = 3$; Error bars represent SEM.

levels were similar for all intestinal cell types (**Figure 4J**). The expression of several control genes [e.g., pluripotent marker Octamer-binding Transcription factor 4 (OCT4) (Nichols et al., 1998)] confirmed the cell sample quality (**Figure A3**).

PRIMARY hInEpCs AND iPSC-DERIVED INTESTINAL CELLS FORM TRANSWELL MONOLAYERS WITH TIGHT JUNCTIONS

While all three primary hInEpCs donors appeared to be capable of forming confluent cell layers in transwell culture that expression the tight-junction marker E-Cadherin (**Figures 2, 4A**), we assessed tight junction formation functionally through measurements of Transepithelial Electrical Resistance (TEER) and monolayer permeability. As confluent epithelial monolayers form, TEER measurements generally increase, reaching $260\text{--}420 \Omega \times \text{cm}^2$ on average in Caco-2 cultures (Le Ferrec et al., 2001). We found that after 11 days of transwell culture, primary hInEpCs from all three donors exhibited TEER measurements of $> 1500 \Omega \times \text{cm}^2$ (**Figure 5A**), providing a strong indication of tight junction formation. As changes in epithelial TEER can also be explained by changes in transcellular ion permeability (Yu and Sinko, 1997), we confirmed tight junction formation of pInEpC transwell cultures by determining the monolayer permeability to FITC-labeled Dextran at a molecular weight of 150,000 (FD150). Apical side incubation in transwell chambers, resulted in $<3\%$ FITC-labeled Dextran detected on the basolateral side, relative to control transwells with no cells for primary hInEpC Donors A and B (**Figure 5B**). Donor C, which corresponds to the cells with highly variable TEER measurements (**Figure 5B**) showed slightly more FITC-Dextran permeability ($\sim 3\%$ relative to control transwells).

While Stage 3 Day 31 transwell A1145A iPSC-derived cells exhibited intestinal marker expression consistent with other intestinal epithelial cell sources, it was difficult to gauge at which point during Stage 3 differentiation iPSC-derived cells may begin to take on functional phenotypes. Thus, we performed a time course experiment in which A1145A iPSCs were differentiated within transwell culture and assessed for monolayer morphology and evidence of tight junction formation throughout Stage 3 (Days 0, 7, 14, 21, 31). Morphologically, A1145A iPSC-derived cells showed 3D structures and dense patches of cells within transwells at the beginning of Stage 3 that appeared to disappear as differentiation progressed, leaving a flat monolayer-like layer of cells by Day 31 of Stage 3 (**Figure 5C**). To assess tight junction formation in confluent A1145A iPSC-derived transwell monolayers, we measured TEER and FITC-Dextran permeability during iPSC transwell differentiation. At Day 0 of Stage 3 differentiation, A1145A iPSC-derived cells within transwells exhibited low TEER measurements of less than $200 \Omega \times \text{cm}^2$ which steadily increased to measurements reaching $937 \Omega \times \text{cm}^2$ by Day 31 (**Figure 5D**). At Stage 3 Day 0, iPSC-derived cells showed FD150 permeability of $\sim 2\%$ relative to the no cell control (**Figure 5E**), a range similar to that seen for primary hInEpCs (**Figure 5B**). Coinciding with changes in TEER measurements during differentiation (**Figure 5D**), FD150 permeability decreased for iPSC-derived cells to as low as 0.06% of the no cell control (**Figure 5E**).

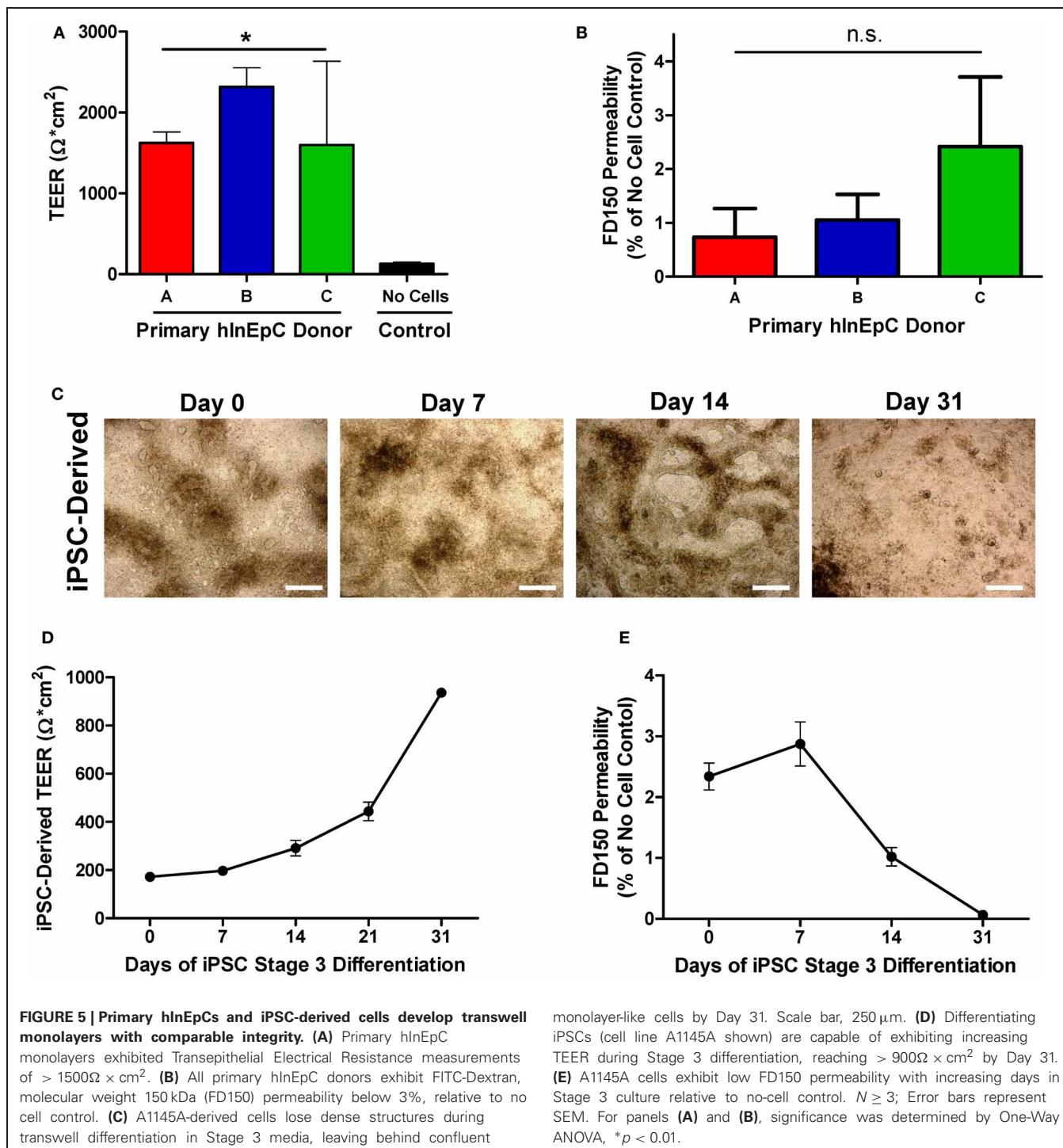
INITIAL ASSESSMENT OF INTESTINAL EPITHELIAL TRANSPORT FUNCTION

To further validate these cell sources functionally, we assessed monolayers for molecule transport or binding. A previously established mechanism influencing small molecule transport by intestinal epithelial cells is efflux by membrane associated ATP-binding cassette P-glycoprotein (Pgp) transporters, which facilitate cellular efflux to prevent accumulation of their substrates (Murakami and Takano, 2008). To assess Pgp transport activity for a given substrate, the basolateral to apical (B-A) permeability is compared to apical to basolateral (A-B), where compounds with efflux ratios (B-A/A-B) greater than 2 or 3 are generally considered to be Pgp substrates (Balimane et al., 2006). Using transwell monolayers of primary hInEpCs, we found that efflux ratios for Digoxin, a compound known to be highly effluxed by Pgp (Balimane et al., 2006), were > 8 for pInEpC Donors A and B (**Figure 6A**). Importantly, efflux of Digoxin was reduced when these cells were also treated with CSA, a known Pgp transporter inhibitor (Watanabe et al., 1997). Atenolol, a poorly Pgp-effluxed compound (Balimane et al., 2006) showed very low efflux ratios of < 1 in all three primary hInEpC donors, which was not further reduced by CSA (**Figure 6B**). During the Pgp transport assay, transwells were also dosed apically with lucifer yellow to confirm primary hInEpC monolayer integrity based on permeability of this fluorescent molecule (**Figure A3**). While the average apparent permeability for all three primary hInEpC donors was below $1.5 \times 10^{-6} \text{ cm/s}$, only transwells below this standard cutoff were used for analysis of Digoxin and Atenolol flux.

Unfortunately, as A1145A iPSC-derived transwell intestinal cells were very limited, there were insufficient cells within this study to perform a properly-controlled Pgp transport assay. Thus, to further validate iPSC-derived cells, we assessed FcRn immunoreactivity and performed a cell surface binding assay for neonatal Fc receptor (FcRn)-mediated transport, which was amenable to the limited cell supply.

Intense FcRn expression in iPSC-derived intestinal organoids was noted as intense single-cell expression within the population of cells (**Figure A4A**) similar that previously reported for human intestinal tissue (Dickinson et al., 1999). We confirmed FcRn expression immunocytochemically in transwell monolayer cultures of Caco-2 and primary hInEpCs both intracellularly (**Figure 6C**) and on the surface of cells (**Figure A4B**). Real-Time RT-PCR analysis showed that FcRn mRNA expression increases to the level close to that of Caco-2 by Stage 3 of transwell differentiation (**Figure 6D**), whereas FcRn expression was highest in primary hInEpCs and iHIOs.

We examined binding of high or low FcRn-binding IgG mAb variants to Caco-2, primary hInEpCs, or iPSC-derived transwell intestinal cells by Meso Scale Discovery assay. Use of this highly sensitive ELISA-like assay allowed us to perform FcRn-dependent binding experiments with proper controls, even with limited-supply iPSC-derived cells. Similar to Caco-2, primary hInEpCs showed significantly higher binding of the IgG variant with a high affinity to FcRn (N434A) than the low FcRn-binding variant (H435A) (**Figure 6E**). iPSC-derived intestinal cells also demonstrated significantly higher binding of a high FcRn-binding IgG variant (M428L) relative to the low FcRn-binding variant



(H435A), however, maximal cell binding was not as high as Caco-2 (**Figure 6F**).

DISCUSSION

The major findings of this study are that marker expression of primary hInEpCs and iPSC-derived intestinal cells were on the same order of magnitude, or better than Caco-2, as determined by immunocytochemistry and mRNA expression analyses. The

iPSC-derived intestinal cells were successfully adapted to differentiate within 2D transwell monolayer culture, and similar to primary hInEpCs, demonstrated functional tight junctions with TEER and low permeability similar to, or better than, Caco-2. Initial assessment suggested functional activity for intestinal transporters such as Pgp transport (primary hInEpCs) or FcRn-dependent binding of molecules (primary hInEpC and iPSC-derived cells). The main conclusions from our study are

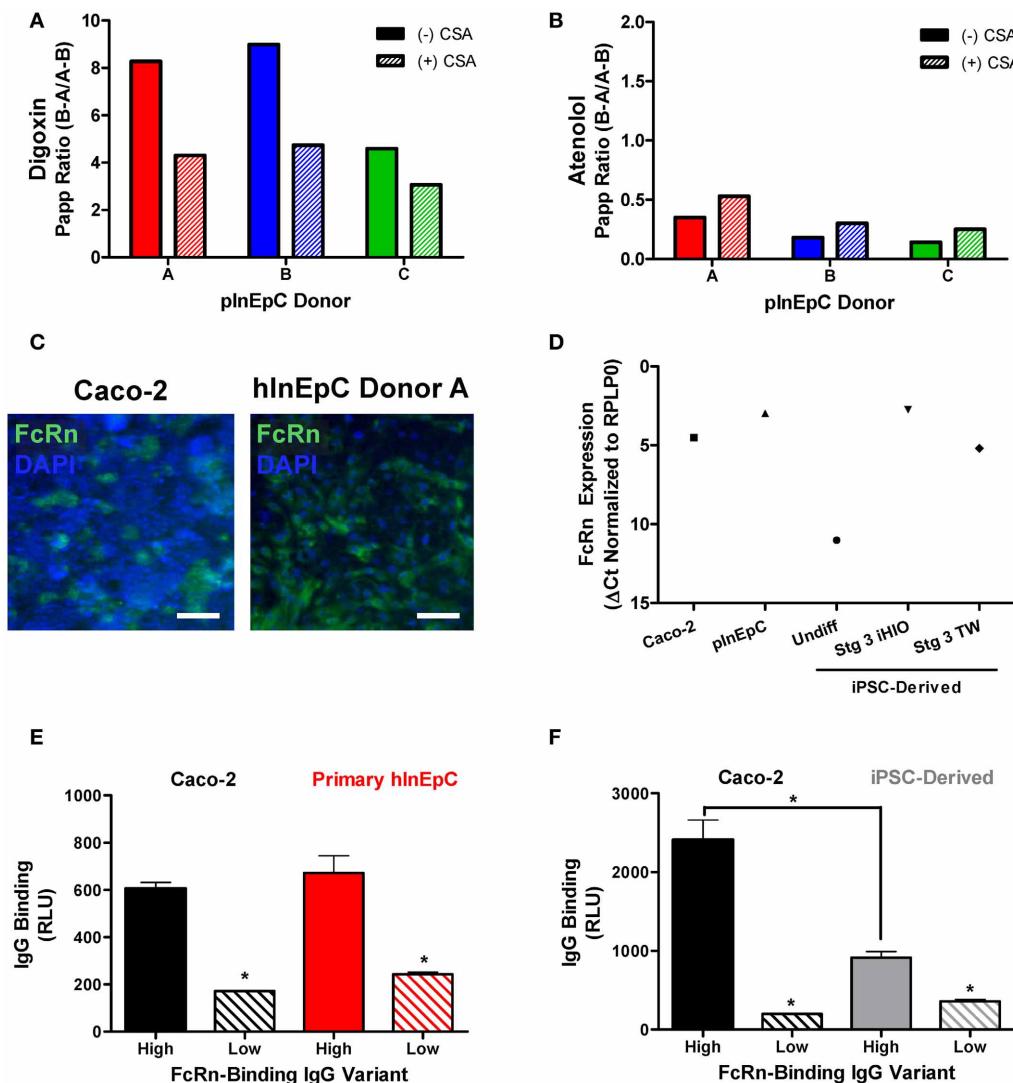


FIGURE 6 | Primary hInEpCs and iPSC-derived intestinal monolayers show functional activity in transwell culture. (A) pInEpC donors A and B show Digoxin Papp Ratios >8, which are decreased in the presence of cyclosporin A (CSA), suggesting that these cell lines have P-glycoprotein (Pgp) transport activity. **(B)** Atenolol Papp ratios for all samples fell below the expected <2 cutoff for Pgp activity. **(C)** Primary hInEpCs (right) show more uniform intracellular FcRn expression than Caco-2 (left), as shown by immunofluorescence. Scale bar, 100 μ m. **(D)** iPSC-derived transwell cells

express FcRn mRNA levels similar to Caco-2, but lower than primary hInEpCs and iPSC-derived 3D induced human intestinal organoids (iHIOs). Caco-2 (E,F), primary hInEpCs (E) and iPSC-derived transwell cells (F) show FcRn-dependent mAb binding by MSD assay, as demonstrated by statistically greater cell binding of high FcRn-binding IgG variants [High, N434A (E) or M428L (F)] than low FcRn-binding variants (Low, H435A). $N \geq 4$; Error bars represent SEM. (E,F), statistical significance determined by t-test, * $p < 0.001$.

further discussed below in relation to previously published studies.

Before using a recently released commercially available source of primary hInEpCs for comparing iPSC-derived intestinal cells, it was necessary to more fully characterize this intestinal epithelial cell source for marker expression and barrier function. The extent to which primary hInEpCs expressed markers by immunofluorescence varied, with expression of E-Cadherin, Villin, and CDX2 strongest in Donor A; however, intestinal marker expression appeared to be more consistent between donors by RT-PCR analysis. While all three

donors exhibited high TEER ($> 1500\Omega \times \text{cm}^2$), TEER and FD150 permeability showed high variability in Donor C. Moreover, Donor C showed poor efflux of Pgp-transported Digoxin, suggesting that this primary hInEpC donor is less useful for intestinal barrier function and transport than Donors A and B.

A 3D directed intestinal organogenesis protocol was successfully adapted to 2D transwell differentiation, based on intestinal marker similarity. Stage 1/Definitive endoderm markers SOX17 and CXCR4 were expressed in 40–87% of the cell population. Stage 2-marker CDX2 was also robustly expressed within

transwell cultures. Stage 3 differentiation within transwells of iPSC-line A1145A (Kauffman et al., Submitted) was similar to the development of iHIOs, except that spheroid structures that formed by Stage 2 of differentiation were not excised for 3D culture, but allowed to continue differentiation within the adherent layer of cells. Interestingly, spheroid structures found at Stage 2 were gradually lost, so that by Stage 3, Day 31, a monolayer-like morphology was found within transwell cultures, which showed TEER measurements more similar to primary hInEpCs and greater than the average TEER for Caco-2 transwell cultures (Le Ferrec et al., 2001). Consistent with this result, low permeability of FD150 for primary hInEpCs and iPSC-derived transwell cells suggests that a barrier function was present in these cell cultures. It is important to note that the TEER obtained in this study for human intestinal epithelial cell sources is several-fold higher than typically found in intestinal tissue *ex vivo*; however, high TEER values may not discount the physiological relevance of these cells *in vitro*, as the presence of several epithelial layers and cell types within intestinal tissue can add multiple impedances to intestinal transmural TEER measurements (Gitter et al., 1998, 2000).

In addition to A1145A, which shows consistent robust intestinal differentiation in 3D and 2D monolayer culture, we also performed a transwell differentiation time course on C2128A because this cell line showed uniform expression of CDX2 at Stage 2 within transwell culture, but demonstrated a distinct morphology. At Stage 2, C2128A cells appeared to already exhibit a monolayer-like morphology with little to no dense structures; therefore, we reasoned that C2128A might be more amenable to the development of iPSC-derived intestinal cells for use in functional transwell monolayer assays. However, as Stage 3 differentiation of C2128A-derived progressed, holes appeared within the layer of cells, which corresponded to low TEER measurements and high FD150 permeability at Stage 3 (**Figure A5**). Thus, differences in iPSC line intestinal differentiation capacity appear to be evident during 2D transwell culture differentiation.

mRNA expression analysis of an extensive panel of intestinal marker and control genes enabled a direct comparison of primary hInEpCs and iPSC-derived cell marker expression to Caco-2 cells. As expected, undifferentiated iPSCs showed levels of marker expression several fold lower than all intestinal samples with the exception of the pluripotent cell control marker OCT4 (Nichols et al., 1998). Differentiated iPSC-derived iHIOs and transwell cells exhibited upregulated intestinal marker expression that was more similar to primary hInEpCs than Caco-2, consistent with a better *in vitro* representation of human intestinal epithelia than Caco-2.

As might be expected, expression of definitive endoderm marker CXCR4 was highest in Stage 3 iHIOs and transwell cells, which went through a definitive endoderm intermediate at Stage 1. Similarly, expression of the mesenchymal marker Vimentin was also most highly upregulated in iPSC-derived intestinal cells, relative to Caco-2. This result is consistent with previously reported increases in expression of Vimentin and another mesenchymal marker, Forkhead Box F1 (Mahlapuu et al., 1998), in

intestinal cells derived from human embryonic stem cells, any may indicate the development of intestinal subepithelial myofibroblasts (Spence et al., 2010).

The intestinal stem cell marker LGR-5 (Barker et al., 2007) was highly upregulated in iPSC-derived intestinal cells relative to Caco-2, which may reflect more active intestinal cell proliferation within these cells, particularly in iHIOs. Expression of Paired Box Gene 6 (PAX6), with known roles in brain development (Mastick et al., 1997), was also highly expressed in iHIOs cells, with less expression in Stage 3 iPSC-derived transwell cells or the other intestinal sources tested. This is consistent with the high Chromogranin A expression in the development of a more mature enteroendocrine system within these cells, as PAX6 also has a known role in the development of Glucagon-like peptide (GLP)-1 and GLP-2 secreting cells (Fujita et al., 2008; Ye and Kaestner, 2009).

While intestinal marker expression in primary of iPSC-derived cells was very encouraging, it was unclear how functional these cells are compared to other established *in vitro* intestinal models, such as Caco-2 or primary tissue. For example, in pluripotent stem-cell derived models of liver or pancreas, progenitor cells must be engrafted into whole animals to produce mature, fully-functional cell types (Liu et al., 2011; Rezania et al., 2011). We realized that adaptation of directed intestinal organogenesis to 2D transwell culture would allow a more direct comparison of iPSC-derived intestinal cell barrier and transport functions to previously characterized intestinal epithelial cell models *in vitro*. Well-studied intestinal transport mechanisms within Caco-2 and primary human intestinal tissue include efflux of small molecules by membrane associated Pgp transporters, (Murakami and Takano, 2008), and intestinal receptor-mediated antibody transcytosis by FcRn (Dickinson et al., 1999; Claypool et al., 2004). These mechanisms were used as an initial assessment of functional quality of primary hInEpCs and iPSC-derived intestinal cells.

Primary hInEpCs A and B showed cyclosporine A-dependent efflux of Digoxin, but not Atenolol, indicative of the presence of Pgp transport activity in these cells. In addition to primary hInEpCs (Donor A), Stage 3 iPSC-derived transwell cells showed FcRn expression and FcRn-dependent mAb binding using high or low FcRn-binding IgG variants; however, unlike primary hInEpCs, iPSC-derived IgG binding did not occur to as large an extent as Caco-2. It should also be noted that in all FcRn-binding experiments, the cell binding signal, while well above background noise, was relatively low for this ELISA-based assay. Furthermore, since FcRn is expressed in neonatal and adult intestinal epithelial cells in primates (Israel et al., 1997; Dickinson et al., 1999) and marker expression during differentiation of iHIOs closely resembles that of embryonic intestinal development (Spence et al., 2010), it is still unclear whether the phenotype of these cells more closely resembles embryonic or adult intestinal tissue.

In summary, our studies indicate that iPSC-derived intestinal cells and newly commercially available primary hInEpCs may provide an alternative source of physiologically relevant hInEpCs. Future studies will be needed to further evaluate the function of primary hInEpCs and iPSC-derived

intestinal cells. Additionally, as these cells are still limited by their viability (hInEpCs) and extensive differentiation time (iPSC-derived cells), large-scale studies will require additional development of methods for scale up or long-term maintenance of these transwell cultures, such as non-oncogenic immortalization strategies.

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- Conflict of Interest Statement:** All investigators are employees of Johnson & Johnson and have no other declarations of interest to disclose.
- Received: 26 April 2013; paper pending published: 17 May 2013; accepted: 03 June 2013; published online: 08 July 2013.
- Citation: Kauffman AL, Gyurdieva AV, Mabus JR, Ferguson C, Yan Z and Hornby PJ (2013) Alternative functional *in vitro* models of human intestinal epithelia. *Front. Pharmacol.* 4:79. doi: 10.3389/fphar.2013.00079
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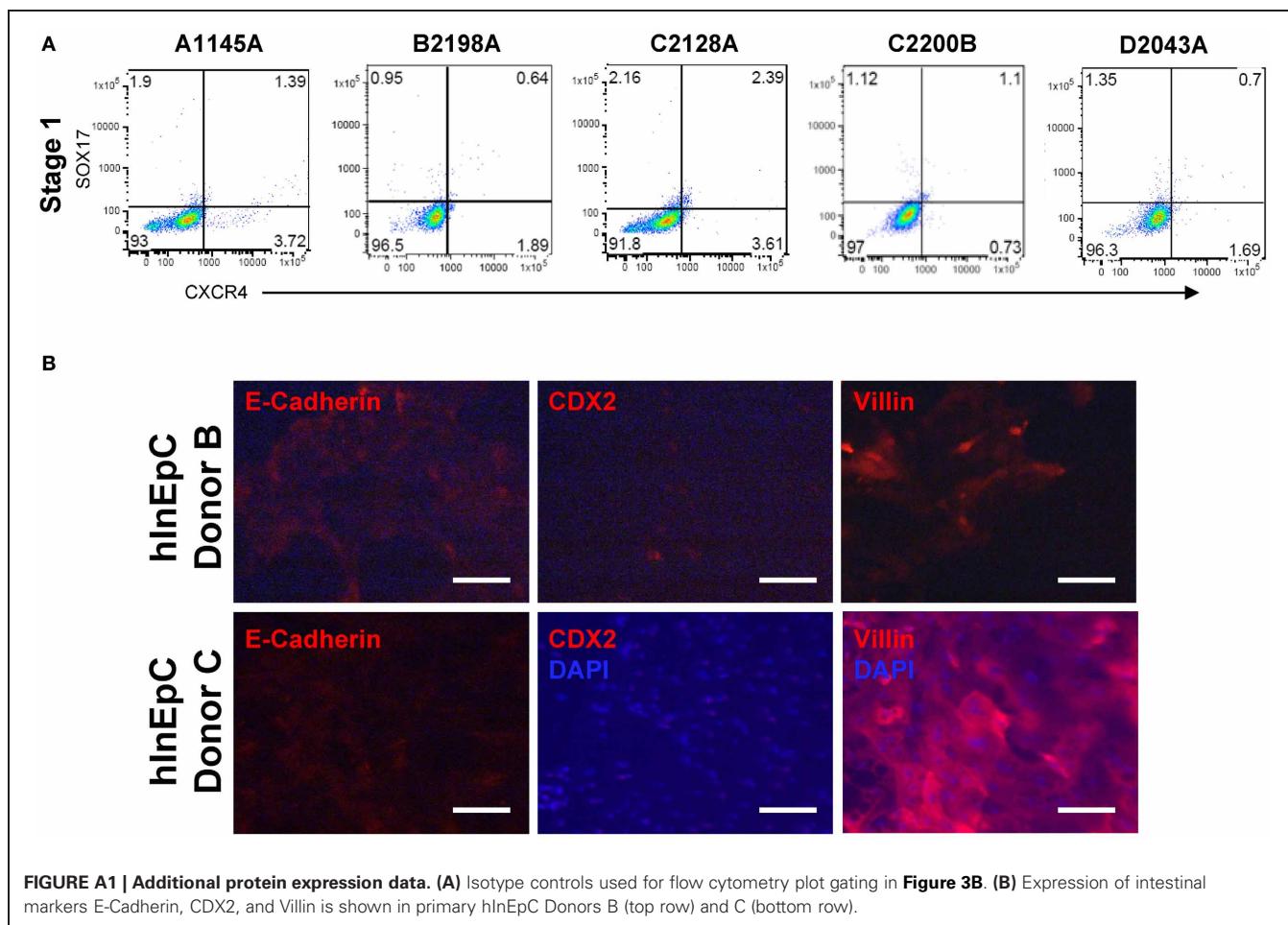
APPENDIX

RESULTS

In addition to intestinal cell-specific markers (**Figure 4**), other control markers were assessed by RT-PCR to evaluate sample quality of iPSC-derived cells, Caco-2, and primary intestinal cells (**Figure A2**). iPSC-derived cells and primary intestinal cells showed very low expression of pluripotent cell transcription factor POU domain, class 5 (OCT4) (Nichols et al., 1998), and about 2000-fold lower than undifferentiated cells (**Figure A2A**). To determine how successfully iPSC-derived intestinal cells differentiated away from a definitive endoderm cell type after Stage 1, expression of CXCR4 was analyzed. As somewhat expected, expression of this definitive endoderm marker was highest in iPSC-derived cells, which went through a definitive endoderm intermediate at Stage 1, with expression levels ranging about

150-fold higher relative to Caco-2 (**Figure A2B**). Expression of the pancreatic foregut marker PDX1 (Miller et al., 1994) was found to be 10–15-fold higher in iPSC-derived cells and primary hInEpCs relative to Caco-2 (**Figure A2C**). Similarly, expression of the mesenchymal marker Vimentin was also highly upregulated in primary hInEpCs and iPSC-derived intestinal cells, relative to Caco-2 (**Figure A2D**).

Neuronal marker Tubulin, beta 3 (TUBB3) (Poirier et al., 2010), expression was higher in iPSC-derived cells and primary hInEpCs relative to Caco-2 (**Figure A2E**). Expression of cardiac cell marker Troponin T, type 2 (TNNT2) (Townsend et al., 1994) was the comparable across intestinal cell types (**Figure A2F**). Expression of Paired Box Gene 6 (PAX6), with known roles in brain development (Mastick et al., 1997), was highly expressed only Stage 3 iHIOs (**Figure A2G**).



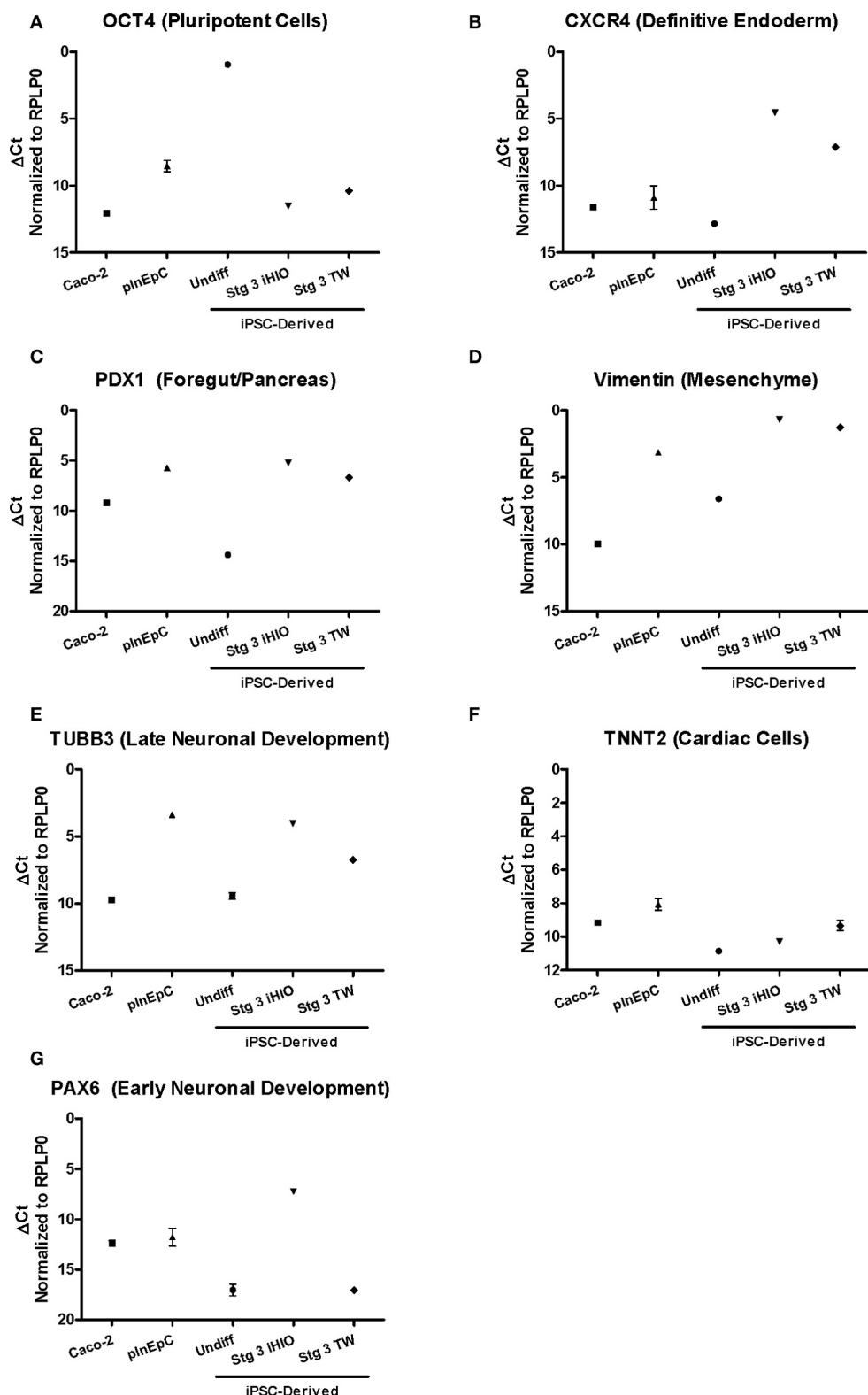


FIGURE A2 | Validation of intestinal cell RT-PCR analysis through a panel of control/non-intestinal markers. Expression of intestinal markers was evaluated by RT-PCR in Caco-2, primary hlnEpc (average of Donors A–C), and iPSC-derived undifferentiated (Undiff),

Stage 3 induced human intestinal organoids (Stg 3 iHIOs), or Stage 3 transwell intestinal cells (Stg 3 TW). Using the DCt method, raw Ct values were normalized to housekeeping gene RPLP0. $N = 3$; Error bars represent SEM.

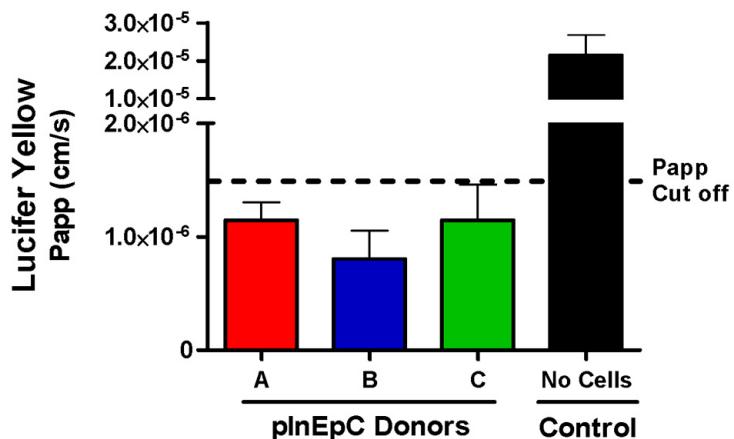


FIGURE A3 | All primary hInEpC donors used within the P-glycoprotein transport assay showed an average lucifer yellow apparent permeability (Papp) below accepted cutoff for intact monolayers

1.5×10^{-6} cm/s. Only transwells that met this cutoff were used to calculate Papp ratios for Digoxin and Atenolol (Figure 6). $N = 3$; Error bars represent SEM.

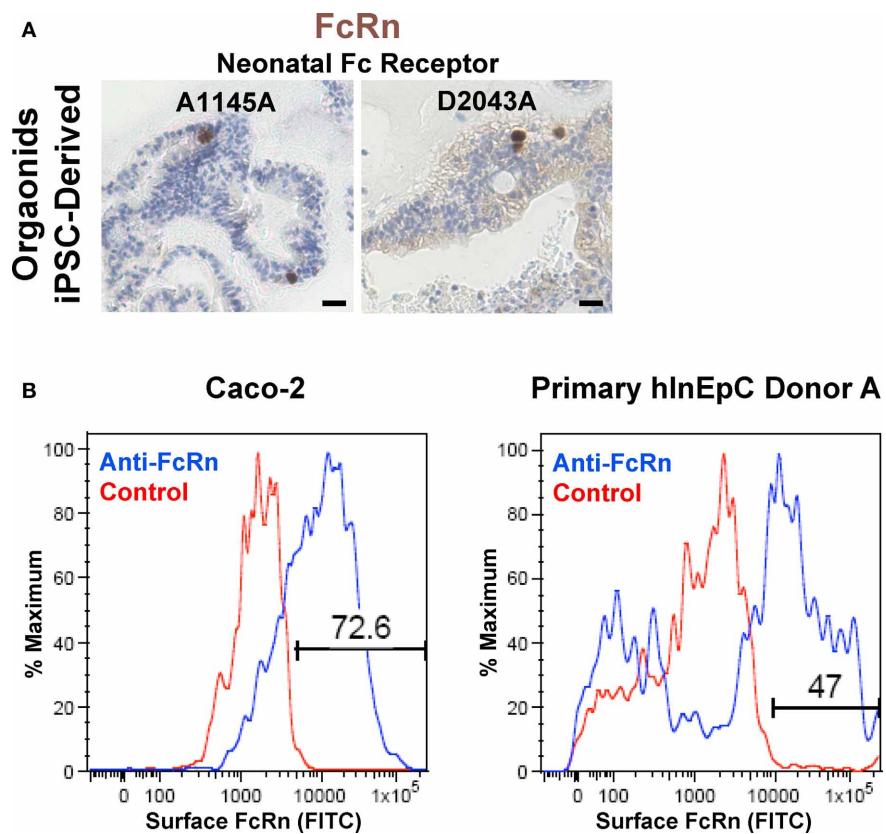
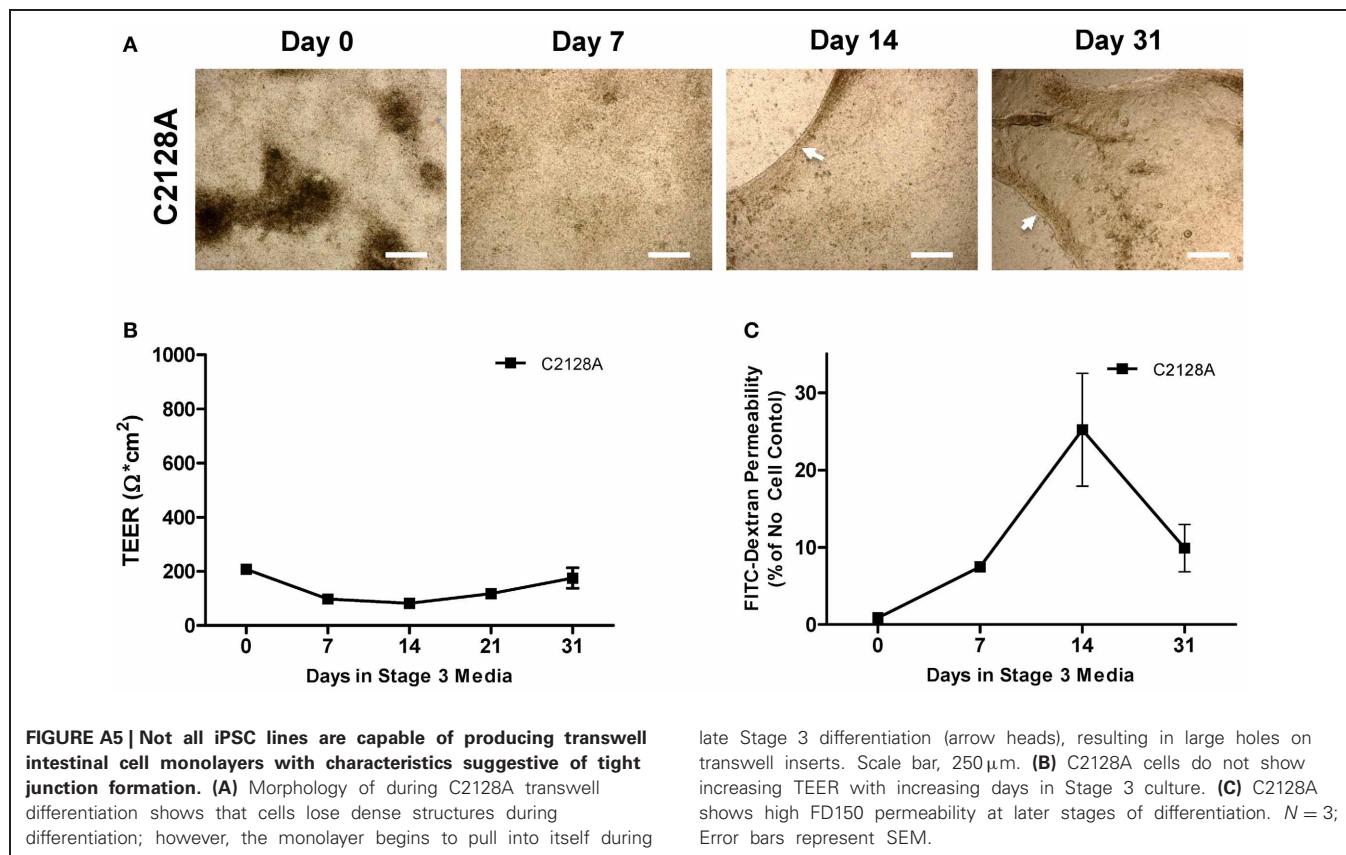


FIGURE A4 | Intestinal epithelial cell expression of FcRn. (A) Immunohistochemical analysis demonstrates iPSC-derived Stage 3 intestinal organoid expression and punctate localization of neonatal Fc Receptor

expression in iPSC-derived organoids. Scale bar, 100 μ m. (B) Flow cytometry analysis shows surface FcRn expression on Caco-2 (left) and primary hInEpCs (right, Donor A shown).



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Celastrol Ameliorates Ulcerative Colitis-Related Colorectal Cancer in Mice via Suppressing Inflammatory Responses and Epithelial-Mesenchymal Transition

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OPEN ACCESS

Edited by:

Angelo A. Izzo,

University of Naples Federico II, Italy

Reviewed by:

Jakub Fichna,

Medical University of Łódź, Poland

Ester Pagano,

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Specialty section:

This article was submitted to

Gastrointestinal Pharmacology,

a section of the journal

Frontiers in Pharmacology

Received: 17 September 2015

Accepted: 23 December 2015

Published: 13 January 2016

Citation:

Lin L, Sun Y, Wang D, Zheng S, Zhang J and Zheng C (2016) Celastrol Ameliorates Ulcerative Colitis-Related Colorectal Cancer in Mice via Suppressing Inflammatory Responses and Epithelial-Mesenchymal

Transition. *Front. Pharmacol.* 6:320.

doi: 10.3389/fphar.2015.00320

Celastrol, also named as tripteterine, is a pharmacologically active ingredient extracted from the root of traditional Chinese herb *Tripterygium wilfordii* Hook F with potent anti-inflammatory and anti-tumor activities. In the present study, we investigated the effects of celastrol on ulcerative colitis-related colorectal cancer (UC-CRC) as well as CRC *in vivo* and *in vitro* and explored its underlying mechanisms. UC-CRC model was induced in C57BL/6 mice by administration of azoxymethane (AOM) and dextran sodium sulfate (DSS). Colonic tumor xenograft models were developed in BALB/c-*nu* mice by subcutaneous injection with HCT116 and HT-29 cells. Intragastric administration of celastrol (2 mg/kg/d) for 14 weeks significantly increased the survival ratio and reduced the multiplicity of colonic neoplasms compared with AOM/DSS model mice. Mechanically, celastrol treatment significantly prevented AOM/DSS-induced up-regulation of expression levels of oncologic markers including mutated p53 and phospho-p53, β-catenin and proliferating cell nuclear antigen (PCNA). In addition, treatment with celastrol inhibited inflammatory responses, as indicated by the decrease of serum tumor necrosis factor-α (TNF-α), interleukin (IL)-1β and IL-6, down-regulation of cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS), and inactivation of nuclear factor κB (NF-κB). Moreover, celastrol obviously suppressed epithelial-mesenchymal transition (EMT) through up-regulating E-cadherin and down-regulating N-cadherin, Vimentin and Snail. Additionally, we also demonstrated that celastrol inhibited human CRC cell proliferation and attenuated colonic xenograft tumor growth via reversing EMT. Taken together, celastrol could effectively ameliorate UC-CRC by suppressing inflammatory responses and EMT, suggesting a potential drug candidate for UC-CRC therapy.

Keywords: celastrol, ulcerative colitis, colorectal cancer, inflammation, epithelial-mesenchymal transition, oncologic proteins

INTRODUCTION

Ulcerative colitis (UC) is a chronic and non-specific inflammatory bowel disease characterized by ulcer and erosion of the rectum and colon (Khor et al., 2011). Epidemiological studies have shown that UC is one of the three highest risk factors for developing colorectal cancer (CRC) due to delayed healing and chronic inflammation (Eaden et al., 2001). CRC is the second most common cancer in women and the third in men worldwide (Ferlay et al., 2010). Although UC-associated CRC (UC-CRC) accounts for only 1–2% of all CRC cases in the general population, it is considered as one of the most serious complications of UC and accounts for approximately 10–15% of all deaths in UC patients (Lakatos and Lakatos, 2008). However, so far, there is no specific and effective treatment for UC and UC-CRC. Therefore, to explore novel drugs with high efficacy and low toxicity against UC and UC-CRC is very imperative and significant.

Celastrol, a triterpene, is a pharmacologically active ingredient extracted from the traditional Chinese medicinal plant *Tripterygium wilfordii* Hook F (also named as Thunder of God Vine) and exhibits significant activities in the treatment of chronic inflammatory, autoimmune diseases, cancer, and neurodegenerative diseases (Allison et al., 2001; Dai et al., 2010; Ge et al., 2010; Venkatesha et al., 2011; Wong et al., 2012). Recently, Shaker et al. (2014) have reported that celastrol ameliorates dextran sulfate sodium (DSS)-induced colitis in mice via modulating intestinal epithelial homeostasis, colonic oxidative stress, and inflammatory cytokines. Meanwhile, several studies have demonstrated that celastrol induces apoptosis in human CRC cells through up-regulation of death receptors and β -catenin pathway and suppresses invasion through down-regulation of CXCR4 chemokine receptor (Sung et al., 2010; Yadav et al., 2010; Lu et al., 2012). The above evidence led us to investigate whether or not celastrol could prevent UC-CRC and if so, through what mechanism.

Chronic inflammation plays a crucial role in the procession of UC tumorigenesis through the induction of cellular DNA damage, telomere shortening, and senescence (Risques et al., 2011). Various initiating factors have been found to be involved in cancer-related inflammation such as nuclear factor κ B (NF- κ B), tumor necrosis factor- α (TNF- α), interleukin (IL)-1 β , and IL-6 (Zhu et al., 2013). The epithelial-mesenchymal transition (EMT) is a process characterized by the loss of epithelial cell markers including E-cadherin, and the acquisition of a mesenchymal phenotype with expression of mesenchymal proteins such as Vimentin, which serves important functions in tumor initiation, progression, invasion, and metastasis (Guarino et al., 2007; Thiery et al., 2009; Chen et al., 2013). Recent studies have demonstrated that EMT also contributes to the pathogenesis of UC and colorectal carcinogenesis, and those factors involved in the development of inflammation are also crucial for the signaling pathways of EMT (Zhu et al., 2013; Tahara et al., 2014). Thus, we hypothesized that the natural agent celastrol might be a promising candidate for the treatment of UC-CRC via suppressing inflammatory responses and epithelial-mesenchymal transition.

In the present work, to test this hypothesis, we developed an azoxymethane (AOM)/DSS-induced UC-CRC mouse model, and demonstrated that celastrol effectively alleviated UC-CRC via suppressing inflammatory response and EMT. In parallel, the *in vivo* and *in vitro* anti-tumor activities and molecular mechanisms of celastrol in human CRC cell lines and xenograft mouse models were further determined. Here, our findings suggest that celastrol has potentials in the treatment of UC-CRC and provide new useful clues regarding its possible mechanisms.

MATERIALS AND METHODS

Cells, Animals, and Materials

Human colorectal adenocarcinoma cell lines HCT116 and HT-29 were obtained from Shanghai Institute of Cell Resource Center of Life Science (Shanghai, China). All cells were cultured in McCoy's 5A medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone, USA), 100 mg/ml streptomycin and 100 U/ml penicillin at 37°C in humidified atmosphere with 5% CO₂.

Male C57BL/6 mice ($n = 65$, 6–8 weeks old) used for UC-CRC models and male BALB/c-*nu* mice ($n = 36$, 5 weeks old) used for colorectal tumor xenograft models were purchased from Vital River Laboratory Animal Technology Co. Ltd (Beijing, China). All animals were housed under controlled conditions (temperature 22 ± 1°C, humidity 40–60% and 12 h dark/light cycle) and free access to a standard laboratory diet and water for 2 weeks. All animal care and experimental procedures were carried out in accordance with the recommendation of the Animal Care Ethics and Use Committee of China Medical University and approved by this Committee.

Celastrol ($\geq 98\%$) was purchased from Dalian Melone pharmaceutical Co., Ltd (Dalian, China). For *in vitro* studies, celastrol was dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich) at a stock concentration of 44 mM. For animal experiments, celastrol was dissolved in DMSO at 20 mg/ml and then diluted with 0.9% saline to the final concentrations (1% DMSO) before administration.

Development of UC-CRC Model and Treatment Procedure

The procedures of induction of UC-CRC model by AOM and DSS were presented in **Figure 1A**. In the study, 65 mice were randomly divided into three groups: 15 mice in the control group, 30 mice in model group (AOM/DSS), and 20 mice in celastrol group (AOM/DSS + celastrol treatment). To develop the UC-CRC model, the mice were given a single intraperitoneal injection of AOM (10 mg/kg body weight in 0.9% saline, Sigma-Aldrich) at first week following adaptation. One week later, the animals were given 3% DSS (Mpbio, Solon, OH, USA) added to the drinking water for 7 days followed by 14 days of drinking water for recovery, and this cycle was repeated twice. Celastrol (2 mg/kg/d) or the vehicle (1%, v/v, DMSO in normal saline) was administrated by gavage daily from first week until the end of 14th week. During the total experimental periods, body weights and survival ratio were measured every week. At the

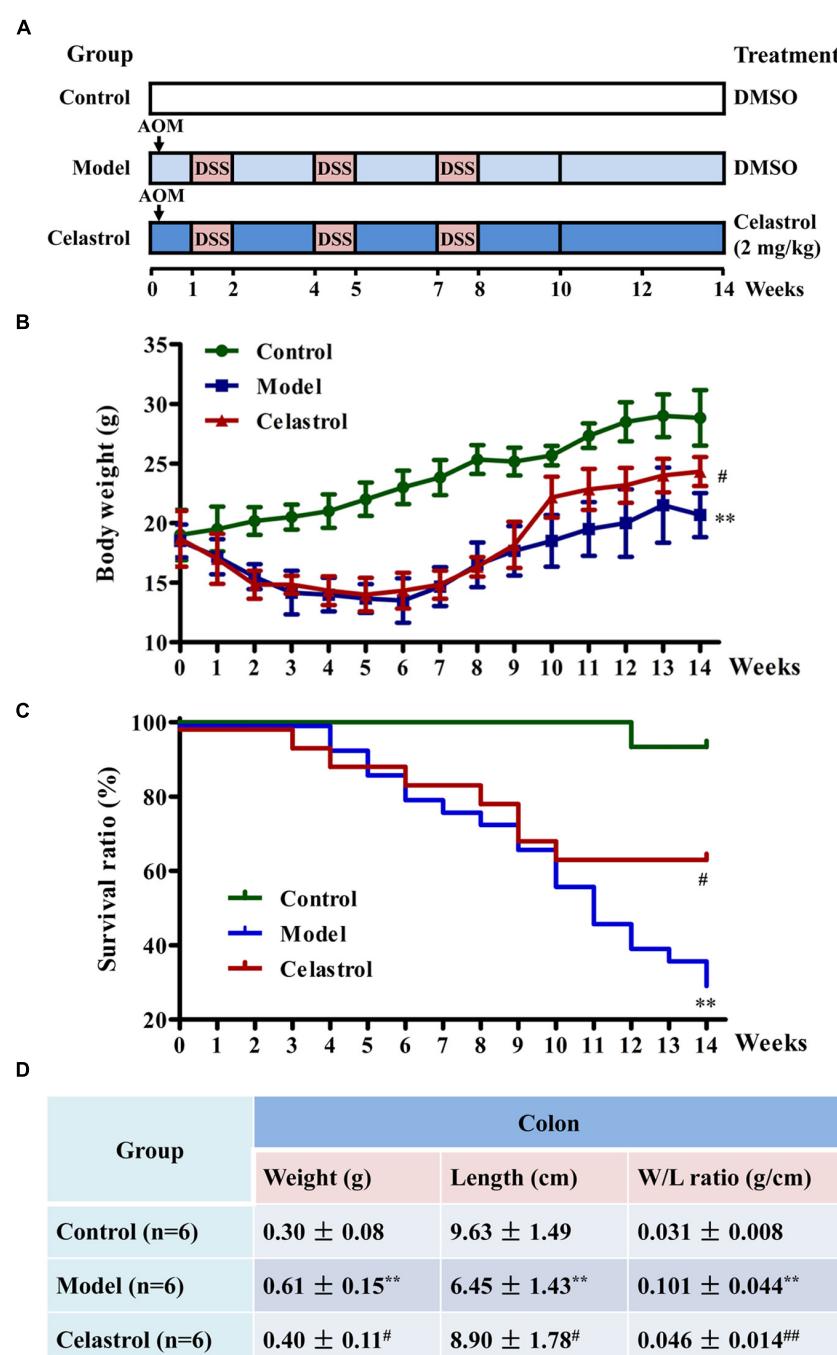


FIGURE 1 | Effects of celastrin on the general health and survival of mice treated with azoxymethane (AOM) and dextran sodium sulfate (DSS).

(A) Experimental protocol for ulcerative colitis-related colorectal cancer (UC-CRC) model and treatment. The details were described in the section “Materials and Methods.” (B) Effect of celastrin (2 mg/kg) on body weight of mice. Body weight of each mouse was measured once per week. (C) Effect of celastrin (2 mg/kg) on survival ratio of mice. Survival status of each mouse was recorded every week. Fifteen mice in the control group, 30 mice in model group, and 20 mice in celastrin group. (D) Effect of celastrin (2 mg/kg) on colon weight and colon length. At the end of experiment, colon tissues were removed and the weight and length were measured. Data are presented as mean ± SD. ** $p < 0.01$ vs. the control group; # $p < 0.05$ and ## $p < 0.01$ vs. the AOM/DSS model group.

end of the experiment, blood was collected for ELISA, then mice were sacrificed and colon tissues were removed. After measuring the weight and length, the colons were slit open longitudinally along the main axis and washed with phosphate

buffer saline (PBS, pH 7.4). The number of tumors in the colons was recorded, and the diameter of each tumor was measured using a sliding caliper, then total tumor area of each colon was calculated. Subsequently, some colon tissues were fixed

in 4% paraformaldehyde buffer for further histopathological examination and immunohistochemical analysis, while others were flash-frozen in liquid nitrogen and kept at -80°C for western blotting analysis.

Histopathological Examination

For histopathology analysis, paraformaldehyde fixed colonic tissues were dehydrated in gradient alcohol, embedded in paraffin and cut into serial sections at 5 μm . Then, these sections were stained with haematoxylin and eosin (H&E) solution and observed under an optical microscope (DP73, OLUMPUS, Japan). Pathological assessment was performed independently and blindly by two pathologists.

Immunohistochemical Staining

For immunohistochemical examination, paraffin-embedded colonic sections were deparaffinized in xylene and hydrated in gradient alcohol. Then, antigen retrieval was performed by heating in pre-boiling buffer in a microwave for 10 min. Next, slides were incubated in 3% hydrogen peroxide solution for 15 min to quench endogenous peroxidase activity and then blocked by 10% goat serum in PBS (pH 7.4) for 15 min at room temperature. Subsequently, slides were incubated with primary antibodies in a humidified chamber at 4°C overnight: cyclooxygenase-2 (COX-2; 1:300), inducible nitric oxide synthase (iNOS; 1:300), β -catenin (1:200), E-cadherin (1:200), N-cadherin (1:200; BOSTER, Wuhan, China), proliferating cell nuclear antigen (PCNA; 1:100), Vimentin (1:300), Snail (1:100; Bioss, Beijing, China), or p53 (1:50), p-p53 (1:50; Santa Cruz, Dallas, TX, USA). After incubation with biotinylated goat anti-rabbit secondary antibody (1:200; Beyotime, Jiangsu, China) and avidin-biotin-horseradish peroxidase (HRP; Beyotime, Jiangsu, China), slides were visualized using diaminobenzidine (DAB), counterstained with haematoxylin and observed under an optical microscope.

Enzyme-Linked Immunosorbent Assays (ELISA) for TNF- α , IL-1 β , and IL-6

The levels of TNF- α , IL-1 β , and IL-6 in the serum were measured using commercial Mouse TNF- α , IL-1 β , and IL-6 ELISA Kits (BOSTER, Wuhan, China), respectively, according to the manufacturer's protocols. Briefly, 100 μl diluent standard or sample serum was added into the antibody-coated wells and incubated for 90 min at 37°C . After washing, samples were incubated with the biotinylated polyclonal antibody for 60 min at 37°C . Then, 100 μl avidin-peroxidase complex solution was added and incubated for 30 min at 37°C . After washing, 90 μl 3,3',5,5'-Tetramethylbenzidine (TMB) color liquid was added, and the mixture was protected from light for 30 min at 37°C . Finally, 100 μl stop solution was pipetted to stop the reaction, and the optical density was determined at 450 nm using a plate reader (ELX-800, BIOTEK, USA).

Cell Viability Assays

MTT assay was used to measure the anti-proliferative effect of celastrol on two kinds of CRC cell lines HCT116 and HT-29. Cells

were seeded in 96-well plates at a density of 3000 cells/well and were allowed to attach for overnight. Then cells were treated with 0–40 μM celastrol for 24 and 48 h. After the treatment, 20 μl of MTT (5 mg/ml, Sigma-Aldrich) dissolved in PBS was added to each well and incubated at 37°C for 4 h. Subsequently, the media with MTT were removed and the formazan granules generated by live cells were dissolved in 200 μl DMSO. The absorbance at 490 nm was measured using a plate reader.

Human Colorectal Tumor Xenograft Model and Treatment

HT-29 or HCT-116 cells (1×10^7) suspended in 0.2 ml of serum-free McCOY's 5A medium were inoculated subcutaneously into the right flank of male 5-week-old BALB/c nude mice. The tumor diameters were measured with digital caliper every 3 days and their volumes were calculated following a standard formula: length \times width $^2/2$. On 10th day after inoculation, for two colorectal tumor models, mice were, respectively, randomized into three groups ($n = 6$) and treated with either vehicle (model group) or celastrol (1 mg/kg or 2 mg/kg) by gavage daily for the duration of the experiment (18 days). By the end of the experiment, mice were sacrificed, and then all tumor xenografts were removed and measured followed by being flash-frozen in liquid nitrogen and kept at -80°C for western blotting analysis.

Cytoplasmic and Nuclear Protein Extraction

Nuclear proteins and cytoplasmic proteins were extracted from the colon tissues using the Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime, Jiangsu, China) according to the manufacturer's instructions. Briefly, colon tissues were cut into small pieces and homogenized with cytoplasmic protein extraction agent A and B. After centrifuging at 1500 g for 5 min at 4°C , the supernatant was collected as partial cytoplasmic protein and the pellet was dissolved with cytoplasmic protein extraction agent A supplemented with PMSF. After incubation on ice for 15 min, cytoplasmic protein extraction agent B was added and incubated for 1 min on ice. Then, the samples were centrifuged at 12,000 g for 5 min at 4°C , and the supernatant was combined with the above cytoplasmic protein. The pellet was re-suspended in nuclear extraction buffer supplemented with PMSF on ice for 30 min and the supernatant containing the nuclear protein were obtained following centrifuging at 12,000 g for 10 min at 4°C . All protein extracts were stored at -80°C .

Western Blot Analysis

Colon tissues and tumor tissues were lysed in RIPA buffer (Beyotime, Jiangsu, China) with protease and phosphatase inhibitors on ice for 1 h. The lysates were centrifuged at 12,000 g for 10 min at 4°C and the supernatant was collected as the total lysate protein. HCT116 and HT-29 cells were treated with celastrol (0–40 μM) for 48 h, then were harvested and lysed in RIPA buffer supplemented with PMSF and phosphatase inhibitors on ice for 1 h. After centrifuging the cell suspension at 12,000 g for 10 min at 4°C , the suspension was collected as the whole cell protein. The protein concentration was determined

with BCA Protein Assay Kit (Beyotime, Jiangsu, China) and a plate reader according to the manufacturer's instructions.

For Western blot analysis, 40 µg of protein from each sample was separated by electrophoresis on 8–13% PAGE-1% SDS gels, and transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). After blocking with 5% non-fat milk in TBST (0.1%) for 1 h at room temperature, the membranes were incubated with appropriate primary antibody overnight at 4°C: COX-2 (1:400), iNOS (1:400), NF-κB p65 (1:500), β-catenin (1:400), E-cadherin (1:400), N-cadherin (1:400; BOSTER, Wuhan, China), PCNA (1:500), Vimentin (1:500), Snail (1:500; Bioss, Beijing, China), or p53 (1:200), p-p53 (1:200; Santa Cruz, Dallas, TX, USA). Then, the blots were washed four times for 5 min each in TBST and incubated with secondary HRP-conjugated goat anti-rabbit or anti-mouse IgGs (1:5000; Beyotime, Jiangsu, China) for 45 min at 37°C. The interest proteins were visualized using enhanced chemiluminescence (ECL; 7Sea, Shanghai, China) and the densitometry of band was analyzed through Gel-Pro-Analyzer system (Liuyi, Beijing, China). Equal loading of protein was confirmed by stripping the blots and re-probing with Histone H3 (1:500; Bioss, Beijing, China) or β-actin antibody (1:1000; Santa Cruz, Dallas, TX, USA). Nuclear NF-κB p65 band densities were normalized to Histone H3, while other band densities were normalized to β-actin.

Statistical Analysis

Data were presented as mean ± SD (standard deviation) of three independent experiments unless otherwise specified. All statistical analyses were performed using GraphPad Prism Software Version 5.0 (GraphPad Software Inc., La Jolla, CA, USA). Data between two groups were compared with two-tailed independent *t*-test and data from more than three groups were analyzed by One-Way ANOVA followed by Bonferroni test. Counting data were analyzed with non-parametric test (Mann–Whitney test). Kaplan–Meier survival analysis was used to evaluate the survival ratio. *p* < 0.05 was considered as statistical significance.

RESULTS

Celastrol Improves the General Health and Survival of Mice Treated with AOM/DSS

As shown in **Figure 1B**, body weight loss was significant in mice treated with AOM in combination with three cycles of DSS during the experimental period compared with the control mice. However, this symptom was alleviated in mice treated with celastrol (2 mg/kg) during the recovery periods when they received tap water without DSS. According to the Kaplan–Meier survival curves (**Figure 1C**), celastrol treatment also significantly increased the survival ratio of AOM/DSS-treated mice from 11th week to the end of experiment. In agreement with previous studies (Li et al., 2010), exposure to AOM and DSS caused a significant increase in colon weight and decrease in colon length,

which was considered as a result of apparent mucosal thickening. Notably, such remarkable increase in colon weight to colon length ratio in mice receiving AOM and DSS was significantly reduced by celastrol treatment (**Figure 1D**).

Celastrol Reduces the Multiplicity of Colonic Neoplasms and the Expression of Oncogenic Proteins

Treatment with AOM and DSS led to 100% incidence of colonic neoplasms with multiplicity of 9.67 ± 2.07 per mouse in model group. Although celastrol administration (2 mg/kg) failed to reduce the incidence of colonic neoplasms, not only did celastrol treatment significantly decrease the number of small neoplasms (diameter < 3 mm) but also the number of large neoplasms (diameter > 3 mm; **Figures 2A,B**). Additionally, celastrol led to an over 40% reduction in the number of total tumors and a more than 50% decrease in tumor area (**Figures 2C,D**). Histologically, crypt destruction, inflammatory cell infiltration, and colon epithelial hyperplasia were observed in the tumor-adjacent colon tissues of AOM/DSS-treated mice. Nevertheless, these symptoms were remarkably mitigated in mice receiving celastrol (**Figure 2E**). There was no colonic tumor observed in the control group.

We also determined the expression levels of neoplastic markers by immunohistochemistry and western blotting. Existing evidence indicate that p53 mutation is an early event of UC-CRC progression, which has been shown to be present in approximately 50% of patients with UC-CRC (Lashner et al., 2003). In general cases, the immunohistochemical staining and western blot of p53 mainly represent the accumulated mutated proteins due to the much longer half-life of mutated p53 than the active wild-type protein (Lashner et al., 2003). As shown in **Figures 3A,B**, the expression levels of p53 and p-p53 proteins in colonic neoplasms of AOM/DSS model mice were significantly increased compared with the control group, implicating the involvement of p53 mutation in our UC-CRC model. Additionally, the expression of oncologic proteins β-catenin and PCNA were dramatically up-regulated in model group. These changes suggested that AOM/DSS-induced UC-CRC was phenotypically similar to human UC-CRC. More noteworthy was that such increase in the expression levels of these neoplastic markers induced by AOM/DSS was significantly suppressed by celastrol treatment (2 mg/kg).

Celastrol Inhibits Inflammatory Responses in AOM/DSS-Induced Mice

Overproduction of pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 by activated macrophages plays an important role in the pathogenesis of UC (Murano et al., 2000). As illustrated in **Figure 4A**, the levels of serum TNF-α, IL-1β, and IL-6 in the AOM/DSS model group were significantly higher than in control group, as assessed by ELISA. Such increase in the levels of these inflammatory makers induced by AOM/DSS was attenuated by treatment with celastrol.

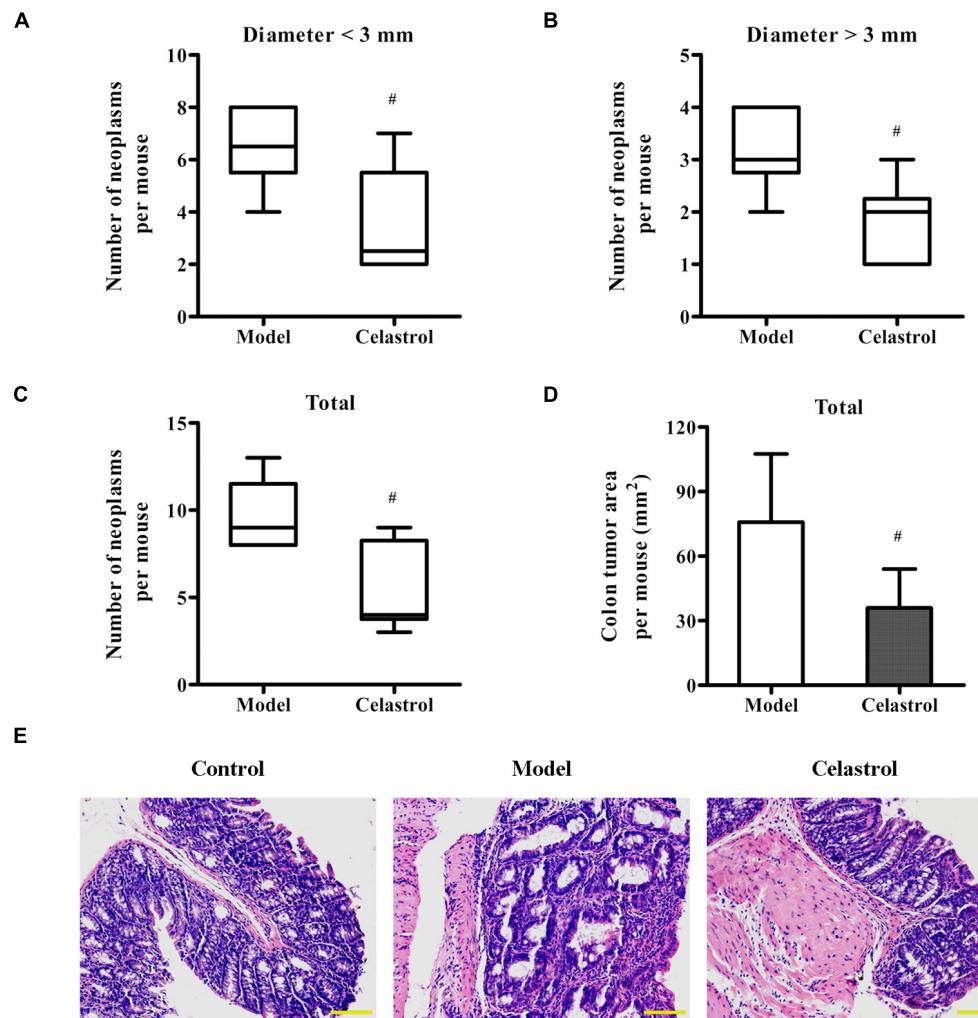


FIGURE 2 | Effects of celastrol on the burden of colonic neoplasms in AOM/DSS-treated mice. Colon tissues were removed, the number and size of tumors in each colon was measured, and the tumor area was calculated. **(A,B)** Effect of celastrol (2 mg/kg) on multiplicity of colonic neoplasms in different sizes (diameter > 3 mm and diameter < 3 mm). **(C)** Effect of celastrol (2 mg/kg) on the total number of tumors per mouse. **(D)** Effect of celastrol (2 mg/kg) on the total tumor area per mouse. **(E)** Representative colonic sections from the control mice, AOM/DSS model mice, and AOM/DSS in combination with celastrol (2 mg/kg) treated mice were stained with haematoxylin and eosin (H&E) for histological assessment. Original magnification was 200 \times . Data are presented as mean \pm SD ($n = 6$). ${}^{\#}p < 0.05$ vs. the AOM/DSS model group.

COX-2 and iNOS are two pro-inflammatory enzymes which are considered to be vital in the pathological process of UC (Dudhgaonkar et al., 2007). Additionally, iNOS acts in synergy with COX-2 to promote the inflammatory response (Sklyarov et al., 2011). Therefore, we evaluated the effects of celastrol on COX-2 and iNOS protein expression in the colonic tissue of AOM/DSS-induced mice. The results of immunochemical and western blot analyses showed that exposure of mice to AOM/DSS led to a significant increase in the expression of COX-2 and iNOS compared with the untreated mice. Oral administration of celastrol was able to obviously reduce the up-regulation of both pro-inflammatory proteins (Figures 4B,C).

Nuclear factor κ B, a key transcription factor that mediates inflammatory signaling pathways, also plays a critical role in

the pathophysiology of UC and CRC (Atreya et al., 2008; Paradisi et al., 2009). Normally, NF- κ B is localized to the cytoplasm in an inactive form. During inflammatory stimulus, NF- κ B is activated and translocates into the nucleus where it regulates the transcription of multiple genes involved in inflammatory response (Karrasch and Jobin, 2008). In order to evaluate whether celastrol also has an effect on NF- κ B activation in our animal model of UC-CRC, the cytoplasmic levels and the nuclear levels of NF- κ B p65 protein in colon tissues were determined, respectively, using western blot analysis. As shown in Figure 4C, compared with control group, the expression of NF- κ B p65 protein in the cytoplasm was significantly decreased, whereas the nuclear NF- κ B p65 levels were obviously increased in the AOM/DSS-treated

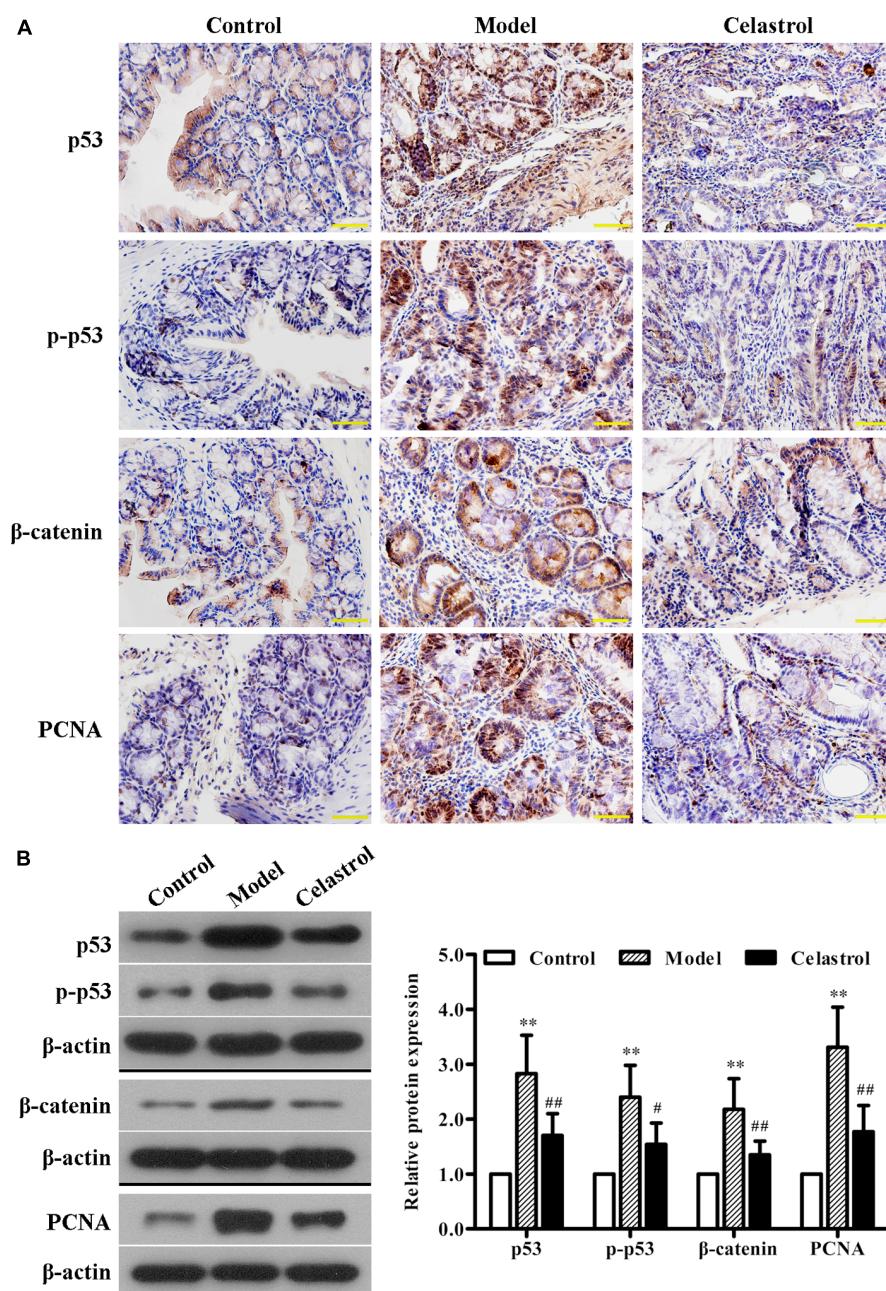


FIGURE 3 | Effects of celastrol (2 mg/kg) on the oncogenic protein expression in AOM/DSS-treated mice. (A) The expression of p53, p-p53, β -catenin, and proliferating cell nuclear antigen (PCNA) in normal colonic tissues or colonic tumor tissues was evaluated using immunohistochemical staining. Shown are representative section of colon tissues from the control group, UC-CRC model group and celastrol-treated group. Original magnification was 400 \times . **(B)** The expression levels of p53, p-p53, β -catenin, and PCNA in colonic tissues from each group were determined by western blot analysis. Representative bands are shown (left), and the relative band intensity ratio was analyzed (right). Data are presented as mean \pm SD ($n = 6$). ** $p < 0.01$ vs. the control group; # $p < 0.05$ and ## $p < 0.01$ vs. the AOM/DSS model group.

model group, suggesting that NF- κ B pathway may undergo activation. Nevertheless, celastrol reversed the decrease of cytoplasmic p65 protein and the increase of nuclear p65 protein induced by AOM/DSS. These results indicate that celastrol may suppress AOM/DSS-mediated activation of NF- κ B signaling.

Celastrol Inhibits AOM/DSS-Induced EMT

A hallmark of EMT is down-regulation of epithelial marker E-cadherin and up-regulation of mesenchymal markers N-cadherin and Vimentin, which is characterized by the loss

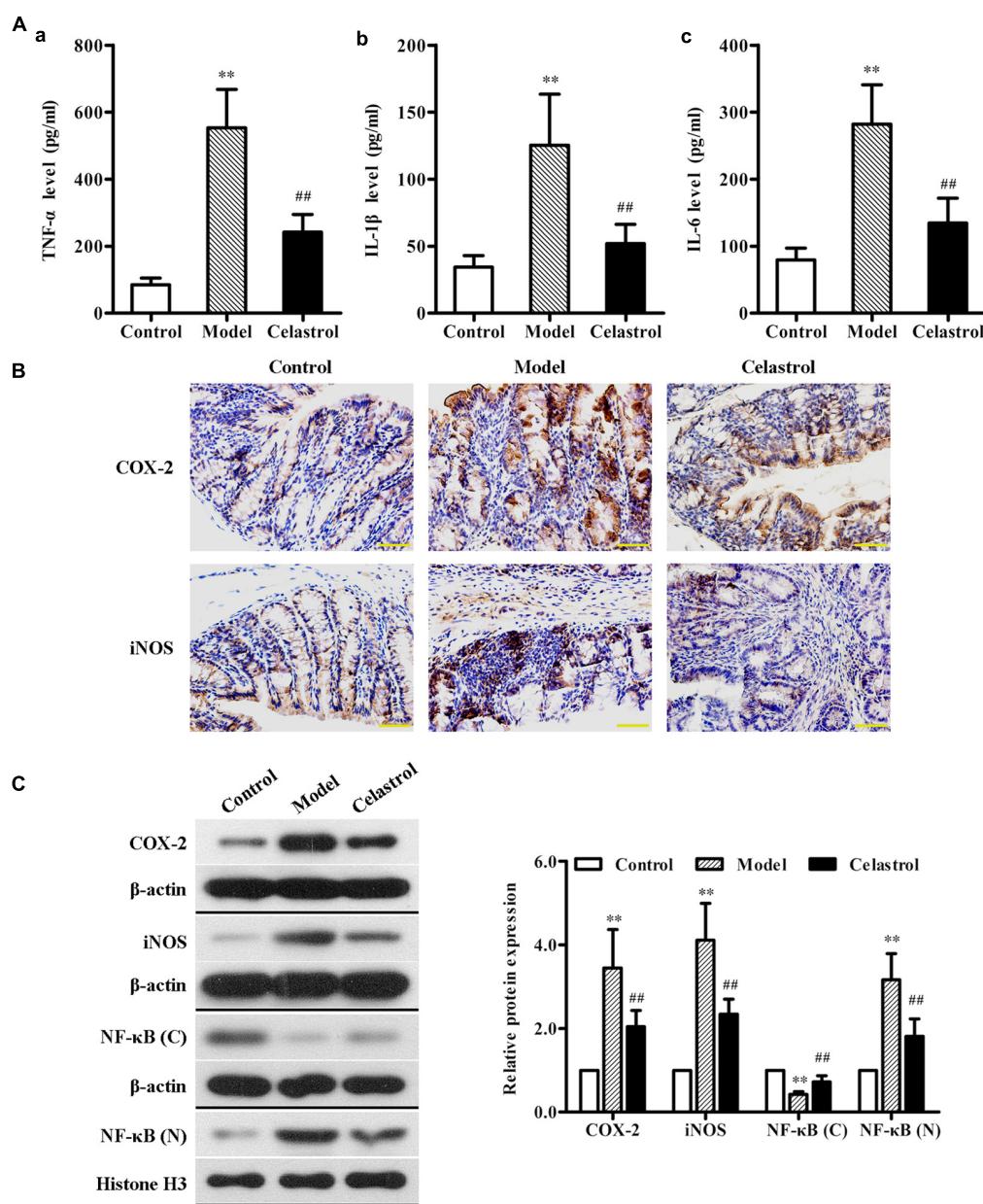


FIGURE 4 | Effects of celastrol (2 mg/kg) on the levels of inflammation cytokines and proteins in AOM/DSS-treated mice. (A) The levels of tumor necrosis factor- α (TNF- α) (a), interleukin (IL)-1 β (b), and IL-6 (c) in serum from the control mice, AOM/DSS model mice and AOM/DSS in combination with celastrol treated mice were determined using ELISA assay. **(B)** The expression levels of cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) in normal colonic tissues or colonic tumor tissues was evaluated using immunohistochemical staining. Representative stained colonic sections from each group are shown. Original magnification was 400 \times . **(C)** The expression levels of COX-2 and iNOS as well as cytoplasmic and nuclear NF- κ B p65 in colonic tissues from each group were determined by western blot analysis. Representative bands are shown (left), and the relative band intensity ratio was analyzed (right). C, cytoplasm; N, nuclear. Data are presented as mean \pm SD ($n = 6$). ** $p < 0.01$ vs. the control group; # $p < 0.01$ vs. the AOM/DSS model group.

of cell-cell adhesion and the gain of migratory and invasive phenotype (Yang et al., 2014). Snail, a zinc finger transcription factor, has been proved as a key regulator for EMT induction in CRCs (Nieto, 2002; Vandewalle et al., 2009). It is demonstrated that Snail suppresses E-cadherin transcription by binding to the E-box site within its promoter, resulting in EMT (Peinado et al., 2007). In this study, the expression of EMT regulatory proteins

in the colon tissue was detected with immunohistochemical staining and western blot analysis. As shown in Figure 5A, significant down-regulation of E-cadherin and up-regulation of N-cadherin, Vimentin, and Snail were observed in AOM/DSS-induced UC-CRC mice compared with control group, suggesting the occurrence of EMT in the model group. Celastrol treatment was shown to dramatically increase the expression of E-cadherin

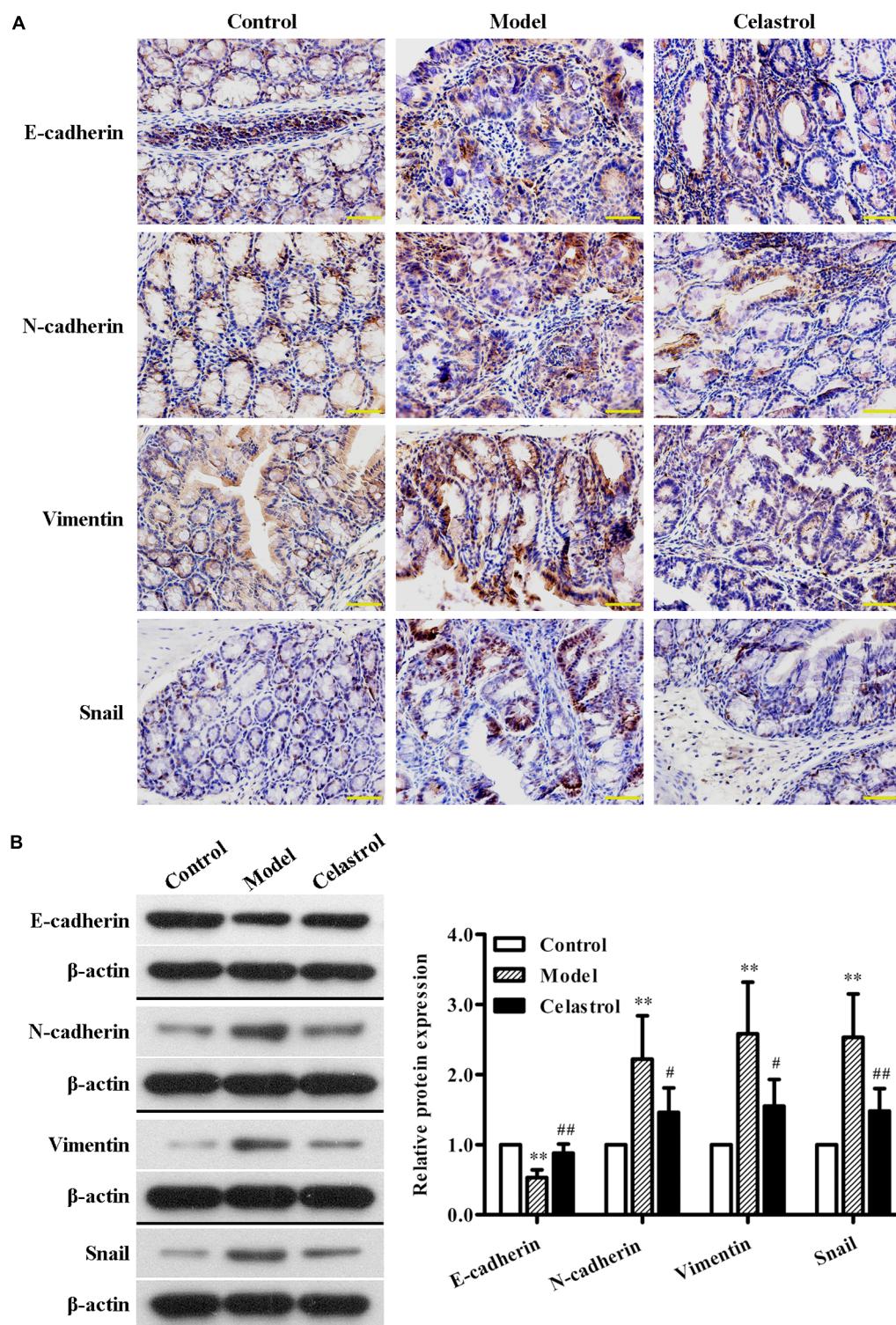


FIGURE 5 | Effects of celastrol (2 mg/kg) on the expression levels of EMT-related proteins in AOM/DSS-treated mice. (A) The expression levels of E-cadherin, N-cadherin, Vimentin, and Snail in normal colonic tissues or colonic tumor tissues was assessed using immunohistochemical staining. Representative colonic sections from the control group, UC-CRC model group and celastrol-treated group are shown. Original magnification was 400 \times . **(B)** The expression levels of E-cadherin, N-cadherin, Vimentin, and Snail in colonic tissues from each group were determined by western blot analysis. Representative bands are shown (left), and the relative band intensity ratio was analyzed (right). Data are presented as mean \pm SD ($n = 6$). ** $p < 0.01$ vs. the control group; # $p < 0.05$ and ## $p < 0.01$ vs. the AOM/DSS model group.

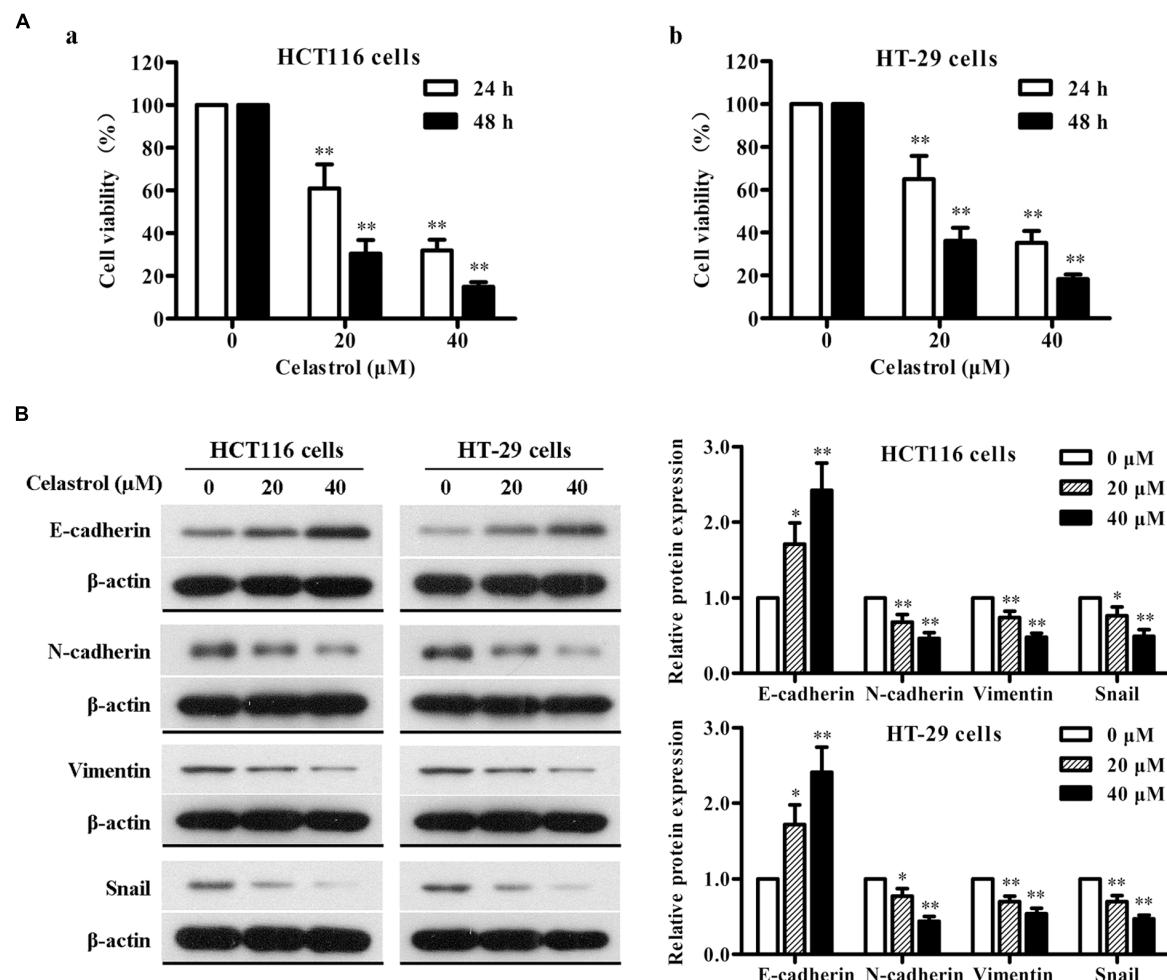


FIGURE 6 | Effects of celastral on cell proliferation and EMT-related protein expression in CRC cells *in vitro*. (A) HCT116 (a) and HT-29 (b) cells were exposed to celastral (0, 20, and 40 μM) for 24 and 48 h, and cell viability was determined by MTT assay. (B) The expression levels of E-cadherin, N-cadherin, Vimentin, and Snail in HCT116 and HT-29 cells treated with celastral (0–40 μM) for 48 h were determined by western blot analysis. Representative bands are shown (left), and the relative band intensity ratio was analyzed (right). Data are presented as mean \pm SD from three independent experiments. * p < 0.05 and ** p < 0.01 vs. control (0 μM).

and decrease the expression of N-cadherin, Vimentin, and Snail. Similarly, western blot analysis further confirmed the effects of celastral on the expression levels of E-cadherin, N-cadherin, Vimentin, and Snail (Figure 5B). Taken together, these observations suggest that celastral can repress EMT in UC-CRC model.

Celastral Inhibits Proliferation and EMT of HCT116 and HT-29 Cells

The effect of celastral on the viability of CRC cells HCT116 and HT-29 was determined using MTT assay. As shown in Figure 6A, celastral treatment led to a significant reduction of cell viability in concentration- and time-dependent manner. It is demonstrated that the inhibitory rates were more than 80% after the treatment with 40 μM celastral for 48 h in both cell lines. These findings indicate that celastral is a potent inhibitor of CRC cell proliferation.

To gain further insight into the effect of celastral on EMT in CRC, we determined the changes in expression of epithelial and mesenchymal markers in CRC cells with celastral treatment for 48 h by western blot analysis. As shown in Figure 6B, celastral significantly increased the expression of epithelial characteristic E-cadherin and decreased the expression of mesenchymal characteristics N-cadherin and Vimentin in both HCT116 and HT-29 cell lines. The transcription factor Snail was also down-regulated in a concentration-dependent manner. Therefore, these results suggest that celastral can ameliorate EMT of CRC cells.

Celastral Inhibits Tumor Growth and EMT in Murine Models of Xenograft Tumor

To test the *in vivo* anti-tumor efficacy of celastral, we established nude mice models bearing inoculated HCT116 and HT-29 tumors. Remarkably, in both CRC models, mice

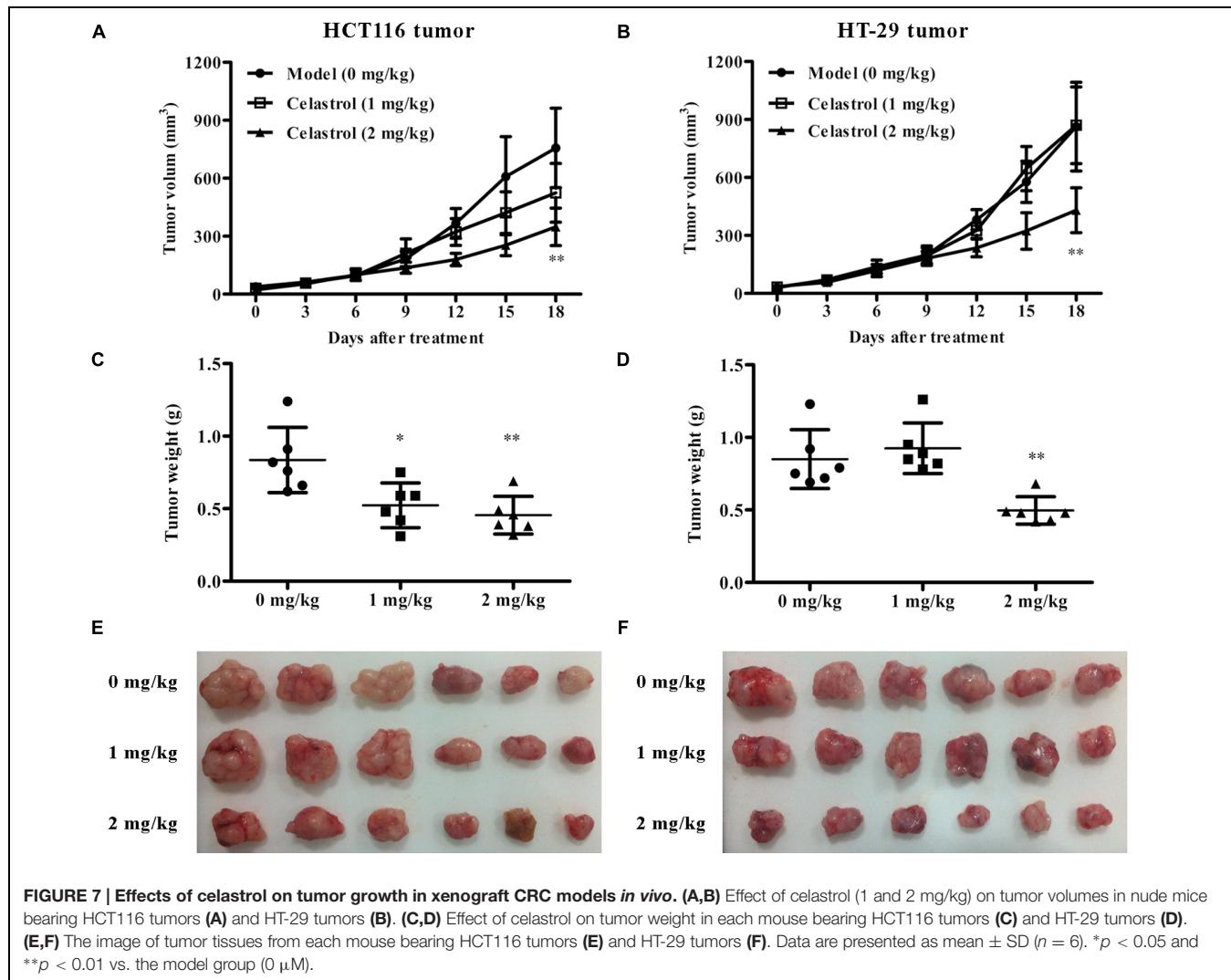


FIGURE 7 | Effects of celastrin on tumor growth in xenograft CRC models *in vivo*. **(A,B)** Effect of celastrin (1 and 2 mg/kg) on tumor volumes in nude mice bearing HCT116 tumors **(A)** and HT-29 tumors **(B)**. **(C,D)** Effect of celastrin on tumor weight in each mouse bearing HCT116 tumors **(C)** and HT-29 tumors **(D)**. **(E,F)** The image of tumor tissues from each mouse bearing HCT116 tumors **(E)** and HT-29 tumors **(F)**. Data are presented as mean \pm SD ($n = 6$). * $p < 0.05$ and ** $p < 0.01$ vs. the model group (0 μ M).

treated with celastrin (2 mg/kg) displayed attenuated tumor growth compared with untreated mice (Figures 7A,B). The overall size and weight of the tumors in the celastrin-treated groups (2 mg/kg) was obviously lower than that of model group (Figures 7C–F). Analysis of tumor weights revealed that the inhibitory rates for HCT116 and HT-29 xenograft mice treated with 2 mg/kg celastrin were 45.5 and 41.6%, respectively. Throughout the treatment schedule, there was no significant difference in mean body weight between celastrin-treated mice and untreated mice (data not shown).

We also investigated the effect of celastrin on EMT in nude mice with CRC and the results were consistent with that of CRC cells. As shown in Figure 8, the expression of E-cadherin was obviously up-regulated and the expression of N-cadherin, Vimentin, and Snail was significantly down-regulated in mice administered with 2 mg/kg celastrin compared to others. Collectively, these data further confirm that celastrin exhibits potent anti-tumor efficacy on CRC by down-regulating EMT.

DISCUSSION

Ulcerative colitis-related CRC is an irreversible malignant colonic disease with high mortality for which there is no effective therapies capable of curing or at least preventing the progressive course. Currently, increasing interest is being focused on exploring underlying mechanisms involved in UC-CRC and novel potential agents in animal models. During the preclinical study, AOM/DSS-induced model is the most commonly used non-hereditary UC-CRC mouse model, which can mimic the development of CRC in human patients. Based on the previous reports (Okayasu et al., 1996; Greten et al., 2004), we established a UC-CRC mouse model by three cycles of DSS administration in combination with AOM pretreatment, which led to 100% incidence of colonic neoplasms as well as marked symptoms including body weight loss, colon weight increase, and colon length shortening. In this study, we found that celastrin treatment significantly reduced the number of colonic neoplasms and tumor area, improved the above symptoms, and increased the survival rate of AOM/DSS-treated

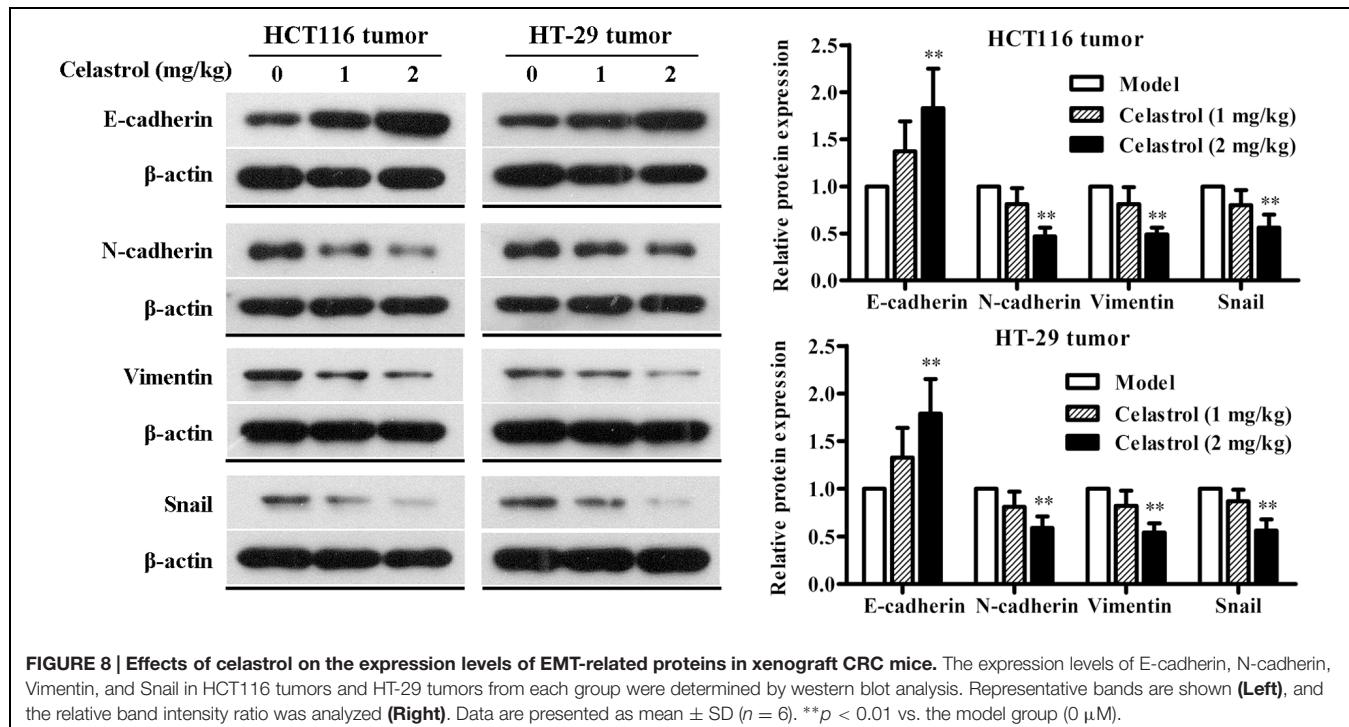


FIGURE 8 | Effects of celastrol on the expression levels of EMT-related proteins in xenograft CRC mice. The expression levels of E-cadherin, N-cadherin, Vimentin, and Snail in HCT116 tumors and HT-29 tumors from each group were determined by western blot analysis. Representative bands are shown (**Left**), and the relative band intensity ratio was analyzed (**Right**). Data are presented as mean \pm SD ($n = 6$). ** $p < 0.01$ vs. the model group ($0 \mu\text{M}$).

mice. In addition, celastrol was also shown to inhibit CRC cell proliferation and attenuate tumor growth in xenograft CRC models. These results suggest that celastrol can be considered as a potential therapeutic drug in ameliorating UC-CRC.

UC-CRC is a well-known multistep process during which the epithelial cells in colon undergo inflammation-dysplasia-carcinoma. An inflammatory environment is considered to play a key role in the initial stage of pathogenesis of UC-CRC. A large body of evidence suggests that activation of NF- κ B is strongly induced in the inflamed colon from UC-CRC patients as well as in experimental UC-CRC models (Wullaert et al., 2011; Lin et al., 2014). The nuclear translocation of NF- κ B can lead to increased levels of certain pro-inflammatory cytokines, such as TNF- α , IL-1 β , and IL-6 in patients with UC (Ogata and Hibi, 2003). NF- κ B activation can also promote expression of pro-inflammatory mediators including COX-2 and iNOS, which further deteriorates inflammatory responses and subsequently results in damage to the colonic tissues (Sakthivel and Guruvayoorappan, 2013). As to the effects of celastrol on inflammatory mediators in serum and colon tissues, we found that celastrol obviously decreased the overproduction of serum TNF- α , IL-1 β , and IL-6, down-regulated the overexpression of COX-2 and iNOS proteins, and inhibited the activation and nuclear translocation of NF- κ B in UC-CRC mice. These findings were in agreement with the previous studies supporting the notion that celastrol functioned as a potent NF- κ B inhibitor in different *in vivo* and *in vitro* models for inflammation and cancer diseases (Pinna et al., 2004; Kannaiyan et al., 2011; Shaker et al., 2014).

There is increasing evidence supporting the promotional role of EMT in UC-CRC progression, which is associated with the

loss of adhesive constraints, enhanced motility, the acquisition of stem cell-like properties, and immune escape (Bates, 2005; Zhu et al., 2013). Among a group of regulators involved in EMT, Snail has been identified as a central mediator of EMT by directly down-regulating E-cadherin in the progression of CRC (Fan et al., 2012). These findings imply that inhibiting EMT may be an ideal strategy for the treatment of UC-CRC. In a recent study, Kang et al. showed that celastrol could markedly inhibit TGF- β 1-mediated EMT through regulating the expression of Snail and E-cadherin in Madin-Darby Canine Kidney (MDCK) and A549 cell lines (Kang et al., 2013). However, the possible effect of celastrol on EMT in UC-CRC model is unclear. As expected, our data demonstrated that the down-regulated E-cadherin as well as the up-regulated N-cadherin, Vimentin, and Snail by AOM/DSS could be inhibited by celastrol treatment. Moreover, the suppression of EMT was also involved in the inhibitory roles of celastrol in human CRC cell proliferation *in vitro* (HCT116 and HT-29 cells) and xenograft colonic tumor growth *in vivo*. Therefore, the results presented here suggest that the alleviative effect of celastrol on AOM/DSS-induced UC-CRC may be partially mediated by suppressing EMT, although the detailed mechanisms need to be explored.

P53 is a tumor suppressor protein which plays an important role in cell cycle, DNA repair, apoptosis, senescence, and angiogenesis (Sionov and Haupt, 1999). Increasing evidence indicates that p53 mutations and loss of heterozygosity are the early events during the progression of UC-CRC (Garrett et al., 2009; Scarpa et al., 2014). It is interesting that wild-type p53 protein has a short half-life and cannot be determined using immunohistochemical

staining, therefore, the positive p53 immunochemical results denote mutated p53 protein (Seyedmajidi et al., 2013). Here, we found that celastral treatment remarkably down-regulated the expression of the dysfunctional p53 and p-p53. Moreover, β -catenin and PCNA, as two important oncogenic transcription factors, have also been reported to play crucial roles during UC-associated colon carcinogenesis (Cappello et al., 2003; Nelson and Nusse, 2004; Clevers, 2006; Georgescu et al., 2007). Our results clearly demonstrated that the expression levels of β -catenin and PCNA in colonic tissues were significantly up-regulated by AOM/DSS, which was in line with previous studies (Lin et al., 2014, 2015). Nevertheless, administration of celastral significantly prevented up-regulation of these neoplastic markers. These data reveals that celastral could execute its protective effect against UC-CRC by preventing the process of carcinogenesis.

During the progression of UC-CRC, there is abundant evidence for the complex relationship among inflammation, EMT, and carcinogenesis. Several inflammatory mediators, such as TNF- α , IL-6, TGF- β , and NF- κ B have been reported to be involved in the whole progression including triggering inflammatory cascade, promoting EMT and facilitating cell transformation and malignancy (Landskron et al., 2014). β -catenin, an important downstream regulator of the Wnt signaling pathway, also participates in EMT procession by binding to membranous E-cadherin (Munding et al., 2012). Our present findings suggest that celastral can prevent the development of UC-CRC in mice via targeting multiple mechanisms across the pathological progression, including alleviating inflammation, intervening EMT, and suppressing carcinogenesis.

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CONCLUSION

Our studies demonstrated for the first time that celastral could effectively prevent UC-related colonic carcinogenesis in AOM/DSS mice model. The mechanisms involved in this effect of celastral on UC-CRC were associated with suppression of inflammatory responses, intervention of EMT as well as down-regulation of mutated p53 and p-p53 proteins, oncogenic proteins β -catenin, and PCNA. Furthermore, the effect of celastral on EMT reversal was also confirmed in CRC cells *in vitro* and the colon cancer xenograft *in vivo*. Based on the data presented here, we believe that celastral may be a potential therapeutic agent for UC-CRC treatment and the research on its more precise mechanisms is ongoing in our group.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: LL, YS, and SZ. Performed the experiments: LL, YS, DW, SZ, JZ, and CZ. Analyzed and interpreted the data: LL, YS, DW, SZ, and JZ. Drafted the paper and revised it critically for important intellectual content: LL, YS, and DW.

ACKNOWLEDGMENT

This work was supported by grants from the Science and Technology Program of Liaoning Province (No.: 2014021083 and 2013225303) and the Science and Technology Program of Shenyang (No.: F14-158-9-49 and F15-199-1-39).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer (EP) and handling Editor (AI) declared their shared affiliation, and the handling Editor states that the process nevertheless met the standards of a fair and objective review.

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Propionate Ameliorates Dextran Sodium Sulfate-Induced Colitis by Improving Intestinal Barrier Function and Reducing Inflammation and Oxidative Stress

OPEN ACCESS

Edited by:

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Specialty section:

This article was submitted to
Gastrointestinal and Hepatic
Pharmacology,
a section of the journal
Frontiers in Pharmacology

Received: 29 March 2016

Accepted: 02 August 2016

Published: 15 August 2016

Citation:

Tong L-c, Wang Y, Wang Z-b, Liu W-y, Sun S, Li L, Su D-f and Zhang L-c (2016) Propionate Ameliorates Dextran Sodium Sulfate-Induced Colitis by Improving Intestinal Barrier Function and Reducing Inflammation and Oxidative Stress. *Front. Pharmacol.* 7:253.
doi: 10.3389/fphar.2016.00253

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Propionate is a short chain fatty acid that is abundant as butyrate in the gut and blood. However, propionate has not been studied as extensively as butyrate in the treatment of colitis. The present study was to investigate the effects of sodium propionate on intestinal barrier function, inflammation and oxidative stress in dextran sulfate sodium (DSS)-induced colitis mice. Animals in DSS group received drinking water from 1 to 6 days and DSS [3% (w/v) dissolved in double distilled water] instead of drinking water from 7 to 14 days. Animals in DSS+propionate (DSS+Prop) group were given 1% sodium propionate for 14 consecutive days and supplemented with 3% DSS solution on day 7–14. Intestinal barrier function, proinflammatory factors, oxidative stress, and signal transducer and activator of transcription 3 (STAT3) signaling pathway in the colon were determined. It was found that sodium propionate ameliorated body weight loss, colon-length shortening and colonic damage in colitis mice. Sodium propionate significantly inhibited the increase of FITC-dextran in serum and the decrease of zonula occludens-1 (ZO-1), occludin, and E-cadherin expression in the colonic tissue. It also inhibited the expression of interleukin (IL)-1 β , IL-6, and tumor necrosis factor- α (TNF- α) mRNA and phosphorylation of STAT3 in colitis mice markedly, reduced the myeloperoxidase (MPO) level, and increased the superoxide dismutase and catalase level in colon and serum compared with DSS group. Sodium propionate inhibited macrophages with CD68 marker infiltration into the colonic mucosa of colitis mice. These results suggest that oral administration of sodium propionate could ameliorate DSS-induced colitis mainly by improving intestinal barrier function and reducing inflammation and oxidative stress via the STAT3 signaling pathway.

Keywords: ulcerative colitis, short-chain fatty acid, propionate, intestinal barrier function, tight junction protein, inflammation, dextran sulfate sodium

INTRODUCTION

Inflammatory bowel disease (IBD), including Crohn's disease and ulcerative colitis, is a group of chronic inflammatory disorders of the gastrointestinal tract characterized by intestinal inflammation and mucosal damage (Quetglas et al., 2015). It is commonly believed that intestinal barrier function destruction, intestinal flora disturbance, and immune dysfunction play important roles in the pathogenesis of IBD (Quetglas et al., 2015; Loddo and Romano, 2015). Glucocorticoids, sulfasalazine, and immunosuppressive drugs have been traditionally used for the treatment and maintenance of ulcerative colitis. However, clinical application of these drugs is limited by their adverse effects (Mao and Hu, 2016), and therefore there is an urgent need to seek alternative remedies.

Increasing the intake of fermentable dietary fibers or short-chain fatty acids (SCFAs) seems to be clinically beneficial to the treatment of colitis (Cabre and Domenech, 2012). SCFAs, predominantly acetate, propionate, and butyrate, are produced in the colonic lumen by anaerobic fermentation of undigested carbohydrates, crude fibers, and polysaccharides (Bolognini et al., 2016). Depending on diet and gut microbiota composition, the intestinal SCFA concentration can range from 60 to 150 mmol/L (Hill, 1995), with butyrate, propionate, and acetate in a nearly constant molar ratio of 15:25:60, respectively (D'Argenio and Mazzacca, 1999). The physiological effects of SCFAs have been well documented, which include reducing the production of proinflammatory factors (Huang and Wu, 1997; Meijer et al., 2010), enhancing intestinal barrier function (Mariadason et al., 1997, 1999; Peng et al., 2007; Suzuki et al., 2008; Van Deun et al., 2008; Elamin et al., 2013), inhibiting oxidative stress (Hamer et al., 2009, 2010), and preventing colon carcinogenesis (Clausen et al., 1991; Hijova and Chmelarova, 2007) *in vitro*, *in vivo*, and in animals. However, most previous studies mainly focused on butyrate, and few studies have devoted their efforts to other SCFAs such as propionate, although it is abundant as butyrate in the gut and blood.

The aim of the present study was to investigate the effects of sodium propionate on intestinal barrier function and the expression of tight junction protein in mice with colitis induced by dextran sulfate sodium (DSS). In addition, the effects of sodium propionate on inflammation and oxidative stress and signal transducer and activator of transcription 3 (STAT3) signaling pathway were also investigated.

MATERIALS AND METHODS

Materials

The main materials used in this study were DSS (molecular weight 36–50 kDa, MP Biomedicals, Inc., Aurora, OH, USA); sodium propionate (Sinopharm Chemical Reagent CO., Ltd., Shanghai, China); Trizol reagent (Invitrogen, Carlsbad, CA, USA); PrimeScript RT Master Mix Perfect Real Time kit (Takara Biotechnology, Dalian, China); FastStart Universal SYBR Green Master (Rox; Roche, Mannheim, Germany); superoxide dismutase (SOD), myeloperoxidase (MPO), and catalase (CAT) kit (Jiancheng, Nanjing, China).

The main reagents used in this study were antibodies for E-cadherin and occluding (Cell Signaling Technology, Danvers, MA, USA; Life Technologies Inc., Gaithersburg, MD, USA); zonula occludens-1 (ZO-1) antibody and β-actin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA; Sigma-Aldrich, St. Louis, MO, USA); mouse anti-CD68 monoclonal antibody (Abcam, Cambridge, England); mouse anti-STAT-3 monoclonal (124H6) and anti-p-STAT-3 (Tyr705) antibodies (Cell Signaling Technology, Danvers, MA, USA).

Animals

C57BL/6J male mice weighing 18–22 g were obtained from SLRC Laboratory Animal Lid (Shanghai, China). All mice were kept under an automated 12 h/12 h dark-light cycle at a controlled temperature of 22°C ± 2°C, relative humidity of 50–60% and allowed free access to standard dry diet and tap water *ad libitum*. All animals received humane care, and experimental procedures were performed in accordance with the guidelines of the Second Military Medical University for health and care of experimental animals.

Dextran Sodium Sulfate-Induced Colitis

Forty mice were equally randomized to four groups: a control group, a propionate group, a DSS group, and a DSS+propionate (DSS+Prop) group. Mice in control and propionate groups received drinking water and sodium propionate [1% (w/v) dissolved in double distilled water], respectively, for 14 consecutive days. Mice in DSS group received drinking water from 1 to 6 days and DSS [3% (w/v) dissolved in double distilled water] instead of drinking water from 7 to 14 days. Mice in DSS+prop group received sodium propionate [1% (w/v)] from 1 to 6 days plus DSS [1% (w/v)] from 7 to 14 days. The disease activity index including weight loss, stool consistency, and fecal blood was evaluated every day. At day 15, all animals were sacrificed by dislocation of the cervical vertebra for blood and organ collection.

Histopathological Assessment

All mice were sacrificed for histological assessment. The colon length was first measured for each group, and then the colon was cut into segments for further detection. Colons in the same position were fixed in 4% neutral formalin, paraffin embedded, and HE stained routinely. Histopathological changes were evaluated by using the histological injury scale as described previously (Wirtz et al., 2007). The criteria for evaluation were as follows: 0: no obvious inflammatory reaction; 1: the presence of low-level inflammatory reaction with a few scattered inflammatory cells; 2: the presence of moderate inflammatory infiltration; 3: the presence of severe inflammatory reaction in the colon tissue as represented by increased vascular density and thickness; 4: the presence of large amounts of inflammation cell infiltration and rupture of goblet cell mass.

In vivo Intestinal Permeability

The *in vivo* intestinal epithelial permeability was determined as described previously (Moussaoui et al., 2014). Briefly, mice were

fasted overnight and FITC-dextran solution (4 kDa, 600 mg/kg) was delivered via gavage. Mice were sacrificed at 4 h after intragastric administration, and blood was harvested via cardiac puncture and then separated by centrifugation. Serum levels of FITC were read at 480 and 520 nm on a microplate fluorometer.

Protein Extraction and Immunoblotting Analysis

Immunoblotting was performed as described previously (Zhang et al., 2011). Briefly, the colon tissue was sliced into sections and washed with PBS. Tissue proteins were extracted by lysing in RIPA buffer containing protease and phosphatase inhibitor cocktail. The mixture was centrifuged at 14,000 × *g* and 4°C for 15 min, and the protein content in the supernatant was determined by Bradford method. An equal amount of protein was separated by 10% (w/v) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. The membranes were blocked for 3 h at room temperature with blocking reagent, and the primary antibodies were incubated overnight at 4°C. The antibodies included anti-ZO-1 (1:1000), anti-E-cadherin (1:1000), anti-occludin antibody (1:2000), anti-STAT3 (1:1000), anti-p-STAT3 (1:1000), and anti-β-actin antibody (1:5000). After washing with PBST, the membranes were incubated with corresponding secondary antibodies (1:10000 dilution) for 50 min at room temperature. Specific bands were scanned and analyzed by Odyssey Infrared Imaging System (LI-COR, Lincoln, NE, USA). β-actin was used as the protein loading control. All immunoblotting experiments were repeated at least three times.

RNA Isolation and Quantitative RT-PCR

Total RNA was extracted from the colon tissue in each group with Trizol reagent according to the manufacturer's instructions. After reverse transcription, complementary DNA was used as templates for PCR. Primers for the inflammatory factors and internal reference were as follows: tumor necrosis factor-α (TNF-α): forward, 5'-CATTCCACG ATTTCCAGA-3'; reverse, 5'-GGAAAGCCCATTGAGTCCT-3'; interleukin (IL)-1β: forward, 5'-CTCACAAAGCAGAGCACAAGC-3', reverse, 5'-CA GTCCAGCCCATA CTTTAGG-3'; IL-6: forward, 5'-CGGAG AGGAGACTTCACAGAG-3', reverse, 5'-CATTTCCACGATT CCCAGA-3'; GAPDH: forward, 5'-GTATGACTCCACTC ACG GCAAA-3', reverse, 5'-GGTCTCGCTCCTGGAAGATG-3'. The housekeeping gene GAPDH was used as internal control, and the amount of RNA was calculated by the comparative threshold cycle method as recommended by the manufacturer. Quantitative real-time PCR was carried out by ABI 7500 real-time PCR system (Applied Biosystems, Foster, CA, USA).

Measurement of Myeloperoxidase (MPO) Level in Colon and Serum

The ability of MPO to modulate the hydrogen peroxide level was used to measure MPO activity by using a modified method

according to the manufacturer's instructions. The freshly excised colon was rinsed, homogenized in tissue lysis buffer, and then centrifuged. Pellets were re-suspended in PBS containing 0.5% hexadecyl-trimethylammonium bromide and then freeze-thawed three times. Absorbance was recorded at 460 nm. The protein concentration was determined using the BCA-100 Protein Determination Kit (Bocai Biotechnology, Shanghai, China). Finally, MPO activity was defined as the quantity of enzyme degrading 1 μmol/ml of peroxide at 37°C, expressed in unit/mg protein.

Detection of CAT and SOD Level in Colon and Serum

The freshly excised colon was rinsed, homogenized in tissue lysis buffer, and then centrifuged. CAT and SOD activities in tissue lysate and serum were measured by using the CAT or SOD kit according to the manufacturer's instruction (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Using a microplate fluorometer, CAT and SOD activities were measured at 405 and 450 nm, respectively. The protein concentration was measured by Bradford method. The concentration of CAT and SOD in colon was presented as picograms per milligram colon protein.

Assessment of Macrophages in Colonic Mucosa by Immunofluorescence

Immunofluorescence was performed as described previously (Liu et al., 2015). The 5 μm paraffin-embedded colonic tissue sections were de-paraffinized in xylene and then rehydrated in ethanol solution. The slides were blocked with 5% BSA in TBS for 90 min. The sections were incubated with anti-CD68 antibody at a dilution of 1:100 overnight at 4°C. After the sections were washed three times with TBS, the slides were incubated with Alexa Fluor 488 secondary antibody diluted 1:200 with TBS and incubated in the dark for 120 min at room temperature. The sections were mounted with mounting medium containing 4,6-diamidino-2-phenylindole (DAPI; Beyotime Institute of Biotechnology, Shanghai, China) for nuclear counterstaining and visualized under a fluorescent microscope (Olympus IX71, Tokyo, Japan). Macrophages were counted per square millimeter (mm²) at a magnification of 400× using a grid ocular. Only cells containing a nucleus stained by DAPI were considered. Counting was performed by two observers independently who were blind to the diagnosis of the specimen. The difference of their results was never greater than 10%, and the mean value was used.

Statistical Analysis

All quantified data were expressed as means ± SD. Data involving more than two groups were assessed by analysis of variance (ANOVA). Values of *P* < 0.05 were considered statistically significant. Statistical analysis was performed using SPSS (Version 18.0 for Windows, SPSS Inc., Chicago, IL, USA).

RESULTS

Effects of Sodium Propionate on Body Weight, Colon Length, and Histopathology in DSS-Induced Colitis Mice

There was no significant difference in body weight, colon length, histological evaluation, and histological score between control and propionate groups.

Compared with control group, mice treated with DSS showed body weight loss on day 13 and day 14 (**Figure 1A**). However, the administration of sodium propionate significantly improved

the body weight loss on day 13 and day 14 as compared with DSS group (19.56 ± 1.43 vs. 16.40 ± 0.44 , 18.06 ± 1.40 vs. 15.14 ± 0.50 , respectively; **Figure 1A**).

Compared with control group, the colon length was significantly shortened in DSS-induced mice.

Compared with DSS group, the administration of sodium propionate significantly increased the colonic length (6.70 ± 0.62 vs. 5.64 ± 0.48 , respectively; **Figure 1C**).

Dextran sulfate sodium-induced mice presented more serious intestinal bleeding than mice in control group.

Compared with DSS group, sodium propionate ameliorated the intestinal bleeding (1.20 ± 0.84 vs. 2.80 ± 0.45 , respectively; **Figure 1B**).

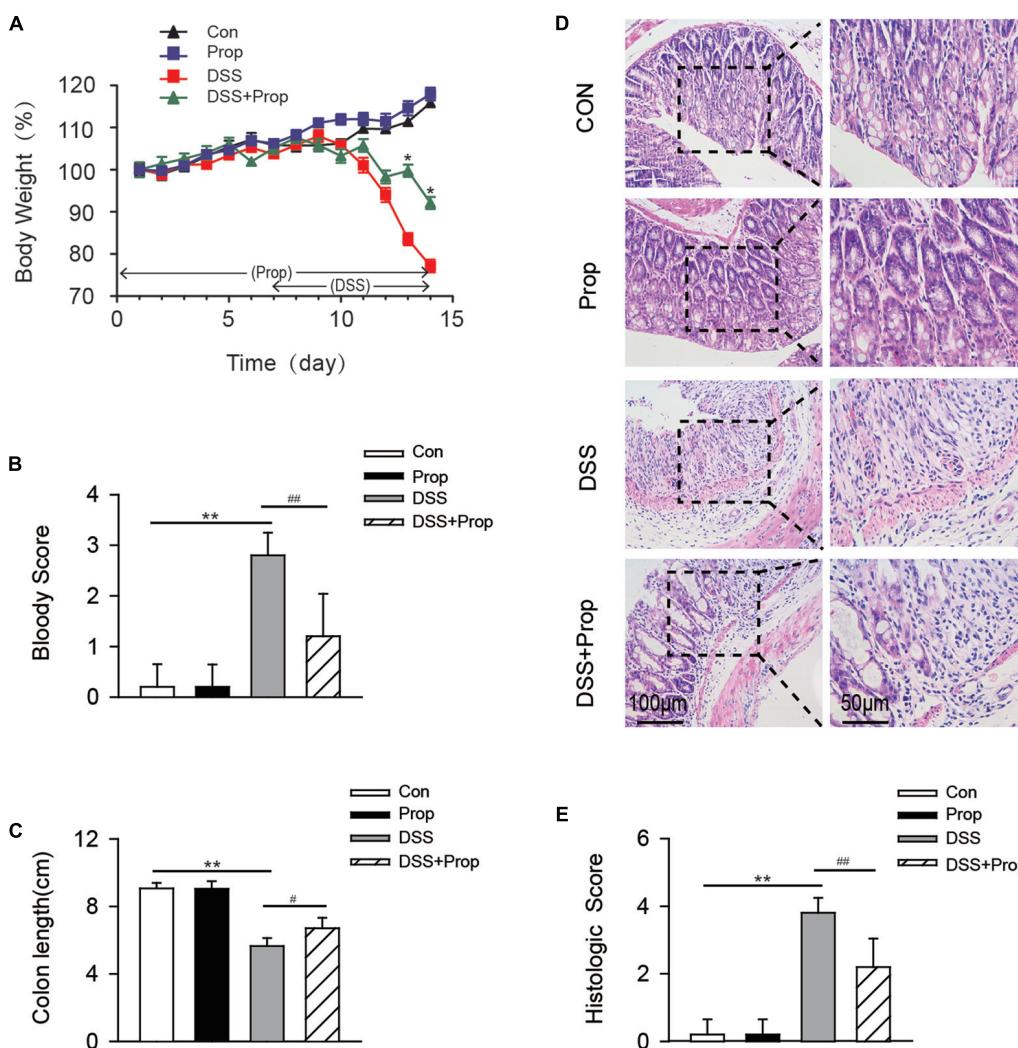


FIGURE 1 | Effects of sodium propionate on body weight, bloody score, colon length, and histopathology in DSS-induced colitis mice. Mice were divided into four groups. Animals in control and propionate groups received water and sodium propionate [1% (w/v) dissolved in water] alone, respectively, during the 14-day treatment period. Animals in DSS group received drinking water from 1 to 6 days and DSS [3% (w/v) dissolved in double distilled water] instead of drinking water from 7 to 14 days. Animals in DSS+propionate (DSS+Prop) group were given 1% sodium propionate for 14 consecutive days and supplemented with 3% DSS solution on day 7–14. Body weight (**A**), bloody score (**B**), and colon length (**C**) were examined. HE stained colon tissue sections were analyzed for histopathology (**D**). Images were representative of 6–8 mice. Colon injury scores were also determined (**E**). Scar bars were 100 and 50 μm , respectively. Data are presented as mean \pm SD, $n = 10$ per group. * $P < 0.05$, ** $P < 0.01$ vs. control group; # $P < 0.05$, ## $P < 0.01$ vs. DSS group.

The histological and morphological characteristics of the colon were assessed by HE staining. The colon from control group showed intact morphology and substantial goblet cells. However, the colon from DSS group presented serious ulcers in the colon membrane, and the number of goblet cells was decreased obviously, causing a high histological score. Compared with DSS group, sodium propionate ameliorated the intestinal ulcer, and blocked neutrophil cell infiltration with minimal loss of goblet cells, resulting in a low histological damage score (2.20 ± 0.84 vs. 3.80 ± 0.45 , respectively; **Figures 1D,E**). These results suggest that sodium propionate could ameliorate tissue injury induced by DSS.

Sodium Propionate Improves Intestinal Barrier Function in DSS-Induced Colitis Mice

Intestinal barrier function plays an important role in maintaining normal bowel function by preventing harmful substances such as intestinal bacteria and toxins from going into other tissues or blood circulation via the intestinal mucosa. The destruction of the intestinal barrier function would lead to colitis and even systemic inflammatory response syndrome (Colgan et al., 2015). There was no significant difference in serum FITC-dextran and the expression of tight junction associated proteins between control and propionate groups. Serum FITC-dextran in DSS-induced colitis mice was higher than that in control mice. However, sodium propionate significantly inhibited the increase of FITC-dextran in serum (0.93 ± 0.02 vs. 1.25 ± 0.07 , respectively; **Figure 2A**). Compared with control group, the expression of tight junction associated proteins including ZO-1, E-cadherin, and occludin, was decreased in DSS-induced colitis mice, indicating that intestinal barrier function was damaged seriously. Compared with DSS group, sodium propionate significantly increased the level of TJ associated proteins include ZO-1 (0.97 ± 0.11 vs. 0.49 ± 0.07 , respectively), E-cadherin (1.03 ± 0.10 vs. 0.57 ± 0.05 , respectively) and occludin (1.07 ± 0.18 vs. 0.61 ± 0.08 , respectively; **Figures 2B–E**), indicating that sodium propionate might contribute to the protection of intestinal barrier function.

Sodium Propionate Attenuates the Trend of Increased Expression of Proinflammatory Factor mRNA and Inhibits the Activation of STAT3 Signaling Pathway

There was no significant difference in mRNA expression level of proinflammatory factors and STAT3 between control and propionate groups.

Compared with control mice, the mRNA expression levels of proinflammatory factors such as TNF- α , IL-1 β , and IL-6 were increased markedly. Compared with DSS group, sodium propionate significantly inhibited the expression of TNF- α (2.15 ± 0.25 vs. 3.43 ± 0.96 , respectively), IL-1 β (3.15 ± 0.69 vs. 5.50 ± 0.60 , respectively), and IL-6 (1.77 ± 1.09 vs. 3.62 ± 0.84 , respectively; **Figures 3A–C**).

Compared with control group, the phosphorylation level of STAT3 was increased in DSS-induced colitis mice. However, sodium propionate significantly inhibited the phosphorylation of STAT3 and decreased inflammatory reaction accordingly (1.00 ± 0.05 vs. 4.96 ± 0.59 , respectively; **Figure 3D**).

Sodium Propionate Inhibits Oxidative Stress in DSS-Induced Colitis Mice

Oxidative stress is considered an important factor in colitis in that it can induce the expression of oxygen free radical and lead to fat, protein, and DNA damage (Balmus et al., 2016). MPO, known as a promoting agent for oxidative stress, was significantly increased in serum and colonic tissue in DSS-induced mice compared with control mice. Sodium propionate decreased the level of MPO in colon (8.69 ± 4.30 vs. 15.61 ± 3.45 , respectively) and serum (0.25 ± 0.05 vs. 0.40 ± 0.07 , respectively) compared with DSS group (**Figures 4A,B**).

Accordingly, the levels of anti-oxidative stress factors including SOD and CAT in serum and colon were significantly decreased in DSS-induced mice compared with the control. Compared with DSS group, sodium propionate increased the level of SOD in colon (8.13 ± 1.56 vs. 6.05 ± 0.46 , respectively) and in serum (0.35 ± 0.03 vs. 0.26 ± 0.04 , respectively); meantime, sodium propionate increased the level of CAT in colon (7.33 ± 1.16 vs. 5.27 ± 1.16 , respectively) and in serum (0.41 ± 0.06 vs. 0.31 ± 0.10 , respectively) (**Figures 4C–F**). There was no significant difference in serum and colon levels of MPO, SOD, and CAT between control and propionate groups.

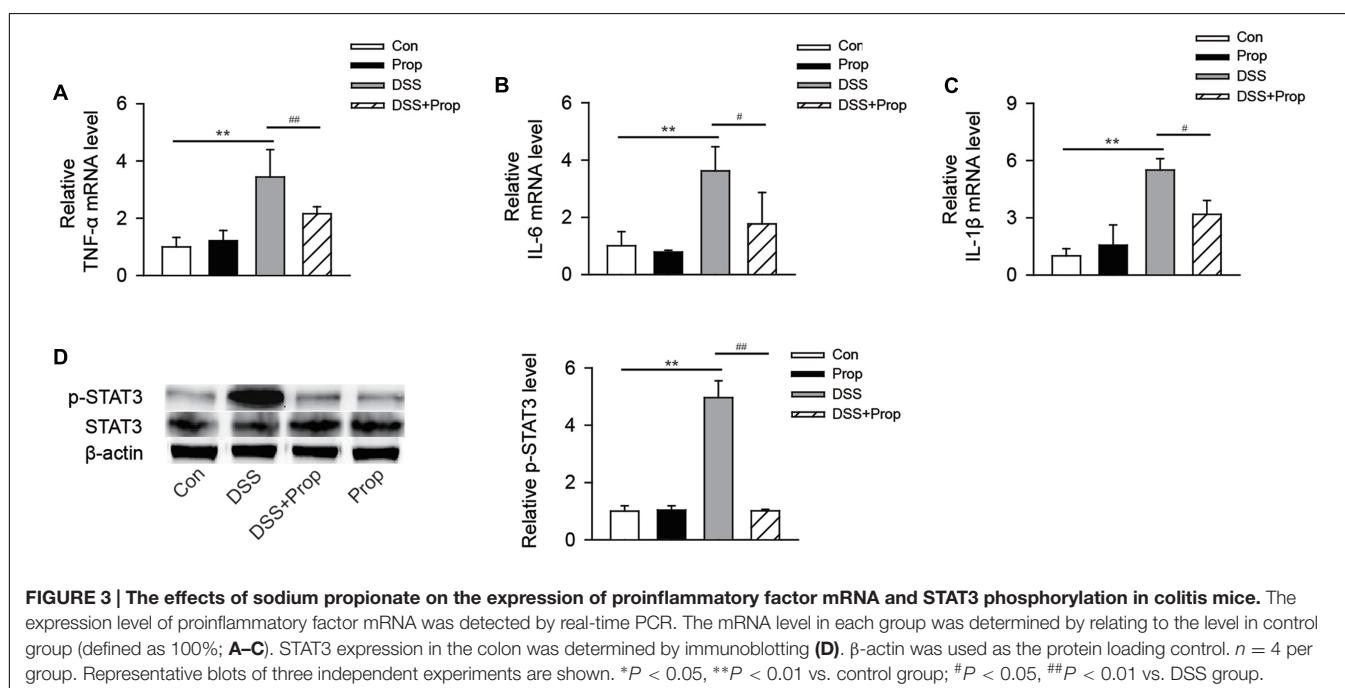
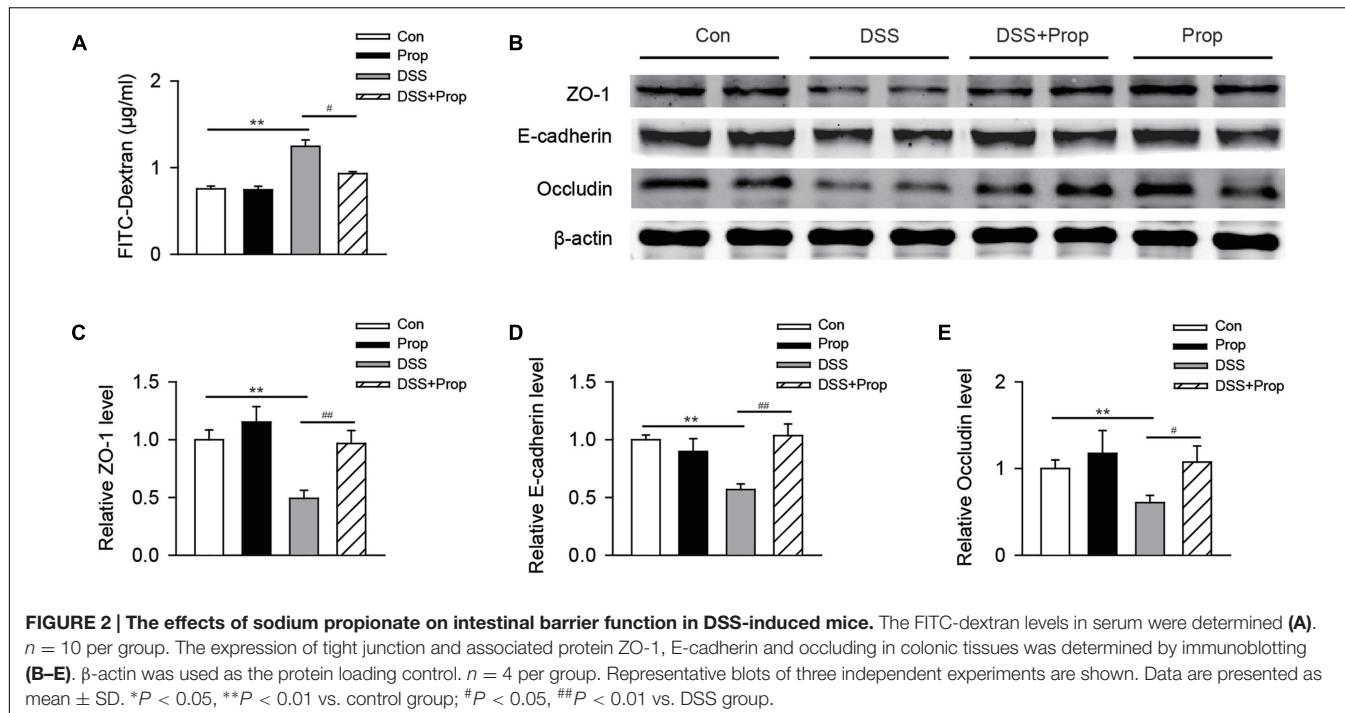
Sodium Propionate Inhibits Expression of CD68 in DSS-Induced Colitis Mice

Destruction of the intestinal barrier can lead to macrophage infiltration into the colon, which can exacerbate the inflammatory process. Expression of CD68 serves as a marker of macrophages/monocytes in the colon. There was no significant difference in CD68 expression between control and propionate groups. The expression of CD68 was significantly increased in DSS-induced mice compared with the control. However, sodium propionate reduced the expression of CD68 in the colon compared with DSS-induced colitis mice. Quantification of CD68 positive macrophages also showed same result (84.33 ± 13.05 vs. 180.67 ± 16.65 , respectively; **Figure 5**).

DISCUSSION

In the present study, we found that sodium propionate inhibited the down-regulation of tight junction proteins such as ZO-1, occludin, and E-cadherin, and improved the impaired intestinal barrier function induced by DSS. Sodium propionate also reduced the expression of pro-inflammatory factors TNF- α , IL-1 β , and IL-6 mRNA in colon tissues. Moreover, sodium propionate inhibited oxidative stress in the colon by reducing MPO activity and enhancing SOD and CAT activities in serum and colon.

Intestinal epithelial barrier defects have been recognized as an important pathogenic factor in a number of inflammatory



conditions of the gut, including CD and UC (Sánchez de Medina et al., 2014). Intestinal epithelial barrier defects are characterized by increased intestinal permeability. Tight junctions and adherence junctions mainly restrict and modulate intestinal permeability (Lee, 2015). In this study, we chose occludin and E-cadherin as the representative proteins of tight junctions and adherence junctions, and used ZO-1 as the representative protein of connecting transmembrane proteins to cytoskeleton

proteins. It was found that sodium propionate increased the serum FITC-dextran level and inhibited the down-regulation of tight junctions and its associated protein occludin, E-cadherin, and ZO-1 in the colon of colitis mice. A recent study showed that multi-fiber mix feeding increased the concentration of total SCFA, acetate, propionate and butyrate in the caecum and epithelial expression and correct localization of tight junction proteins (occludin and ZO-1) in IL-10 $^{-/-}$ mice (Wang et al.,

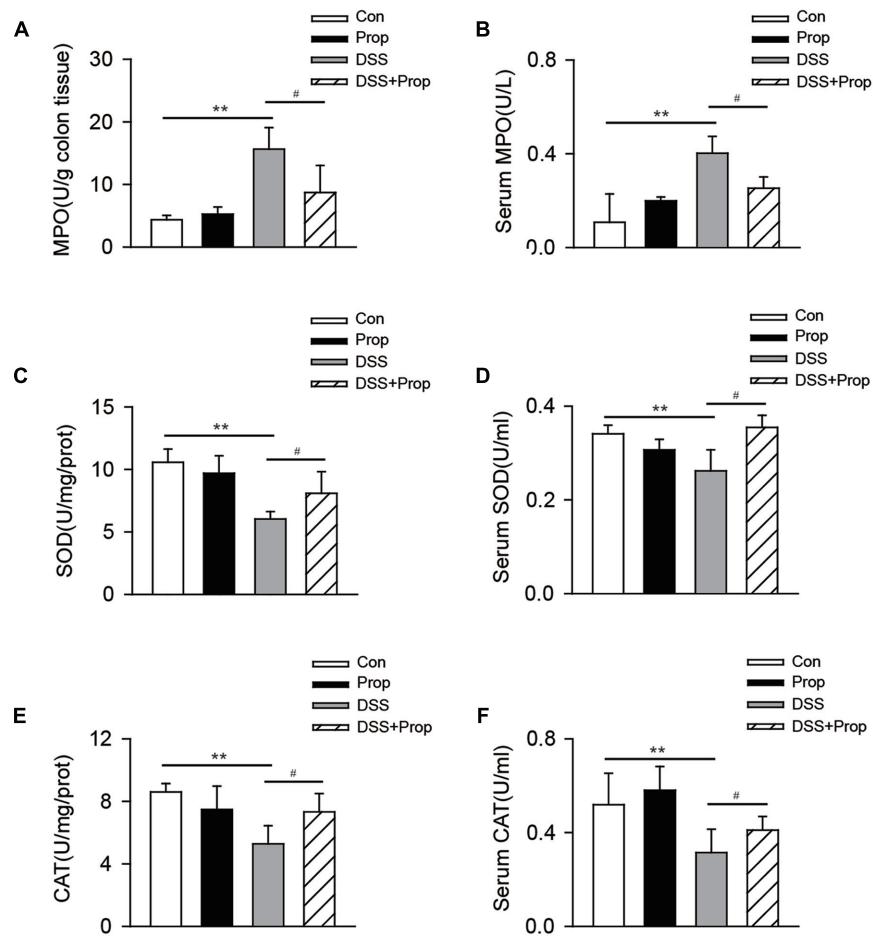


FIGURE 4 | The effects of sodium propionate on oxidative stress response in colitis mice. MPO, SOD, and CAT levels in colonic tissues were determined (**A,C,E**). MPO, SOD, and CAT levels in serum were shown (**B,D,F**). Data are presented as mean \pm SD. $n = 10$ per group, * $P < 0.05$, ** $P < 0.01$ vs. control group; # $P < 0.05$, ## $P < 0.01$ vs. DSS group.

2016). A more previous study also showed that the amount of dietary fiber significantly altered the barrier function by reducing paracellular permeability in the distal colon of normal rats (Mariadason et al., 1999). It was found that applying the SCFA mixture to the intestinal mucosa of anesthetized rats suppressed [3 H] mannitol transport from the caecal lumen to the mesenteric blood in a dose-dependent manner, while propionate alone dose-dependently increased transepithelial electrical resistance in T84 and Caco-2 cells (Mariadason et al., 1997; Suzuki et al., 2008). Elamin et al. (2013) also reported that pretreatment of Caco-2 cells with 4 mmol/L propionate significantly alleviated the ethanol-induced barrier dysfunction, tight junction and F-actin disruption, and metabolic stress. Consistently, our results demonstrated that oral administration of sodium propionate could restore intestinal barrier function, at least in part, through inhibiting the down-regulation of tight junction and its associated protein in colitis mice. It is believed that sodium propionate cannot reach the colon due to its rapid gastric and duodenal absorption. Therefore, to verify whether oral sodium propionate could reach the colon, the concentration

of sodium propionate in the caecum content needs further research.

Chronic inflammation as a hallmark of IBD often results from the recruitment and activation of immune cells from the circulation. It is postulated that intestinal tight junction barrier defects allow for paracellular permeation of noxious luminal antigens that induce inflammatory response. It was found that propionate decreased the generation of proinflammatory cytokines in a co-culture system combining Caco-2 cells with human whole blood (Hamer et al., 2008). Propionate could also diminish TNF- α production and release in neutrophils upon stimulation by lipopolysaccharide (Tedesco et al., 2007; Vinolo et al., 2011). These findings *in vitro* show that propionate has a favorable effect on IBD by attenuating activation of macrophages and neutrophils. The present study *in vivo* found that sodium propionate inhibited the up-regulation of proinflammatory factors IL-6, IL-1 β , and TNF- α mRNA level in the colon of colitis mice.

Additionally, proinflammatory factors activate macrophages and neutrophils to infiltrate into the colonic mucosa, which in

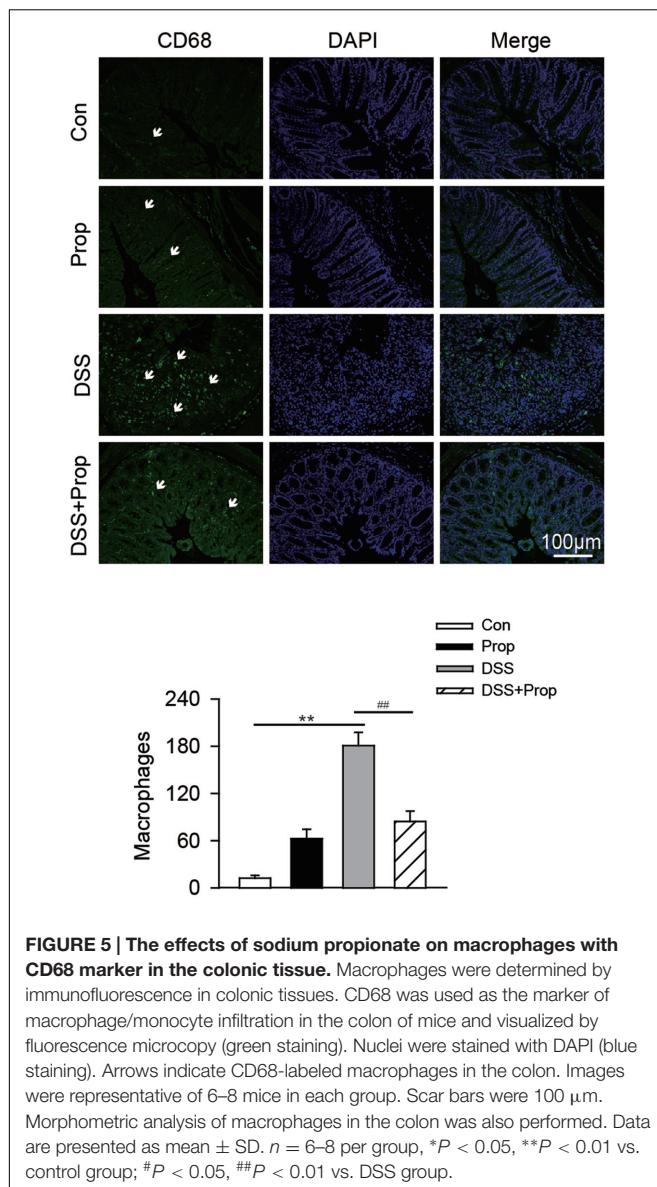


FIGURE 5 | The effects of sodium propionate on macrophages with CD68 marker in the colonic tissue. Macrophages were determined by immunofluorescence in colonic tissues. CD68 was used as the marker of macrophage/monocyte infiltration in the colon of mice and visualized by fluorescence microscopy (green staining). Nuclei were stained with DAPI (blue staining). Arrows indicate CD68-labeled macrophages in the colon. Images were representative of 6–8 mice in each group. Scar bars were 100 μm . Morphometric analysis of macrophages in the colon was also performed. Data are presented as mean \pm SD. $n = 6$ –8 per group, * $P < 0.05$, ** $P < 0.01$ vs. control group; # $P < 0.05$, ## $P < 0.01$ vs. DSS group.

turn stimulates the production of reactive oxygen species (ROS), particularly superoxide, leading to oxidative stress (Roessner et al., 2008). ROS and reactive nitrogen species (RNS) produced by macrophages and neutrophils may further aggravate the inflammatory response and cause intestinal mucosal damage in IBD (Piechota-Polanczyk and Fichna, 2014). CD68 protein is known as a cell surface glycoprotein expressed in mature macrophages in the intestinal lamina propria and serves as a marker of macrophage and monocyte infiltration into the colon (Caprioli et al., 2013). We found that macrophages with CD68 marker were increased in colonic mucosa, and sodium propionate inhibited macrophage infiltration into the colonic mucosa in DSS-induced mice. We also found that sodium propionate decreased the MPO activity and increased the CAT and SOD activities in colon and serum. Taken together, our results indicate that sodium propionate alleviated inflammation

and oxidative stress by inhibiting macrophage infiltration into the intestinal mucosa in DSS-induced colitis mice. However, how sodium propionate decreases macrophage infiltration into the intestinal mucosa and regulates macrophage function needs further investigation.

It was demonstrated that the level of activated STAT3 was higher in intestinal epithelial cells from patients with active ulcerative colitis compared with that in patients with inactive disease or healthy controls, and this level was positively correlated with the severity of colitis (Nguyen et al., 2015). We found that sodium propionate inhibited phosphorylation of STAT3 induced by colitis, which is consistent with the previous report that multi-fiber mix feeding decreased p-STAT3 expression in colonic mucosa of IL-10^{-/-} mice (Wang et al., 2016). STAT3 is a transcriptional activator and activated by a variety of cytokines, growth factors, and oxidative stress as well (Han and Theiss, 2014). But how sodium propionate inhibits STAT3 phosphorylation in colitis needs further study.

Moreover, no *in vivo* toxicity was observed after oral administration of 1% sodium propionate in this study, confirming the safety of sodium propionate. Although the colitis mice treated with propionate could not recover to normal, it is worth noting that low-dose (1%) oral sodium propionate attenuated acute DSS-induced colitis. It is important to avoid overdosing sodium propionate in clinical practice, as it may exacerbate rather than ameliorate colitis. Future studies are needed to compare the efficacy and safety of low-dose vs. high-dose sodium propionate oral therapy.

In summary, the present study demonstrated that oral administration of sodium propionate exerted beneficial effects on the intestinal epithelium by improving intestinal barrier function, inhibiting inflammation, and modulating oxidative stress through STAT3 signal pathway in DSS-induced colitis mice. Our study not only provides *in vivo* evidence for but gains preliminary mechanistic insights into the potential therapeutic benefits of sodium propionate for the management of colitis.

AUTHOR CONTRIBUTIONS

YW, L-cT, and Z-bW contributed to the conception and design of the study, and performed the research. W-yL and SS contributed to the acquisition, analysis and interpretation of data. L-cT and Z-bW drafted the manuscript. LL, L-cZ and D-fS supervised the project and revised the manuscript critically for important intellectual content. All authors have approved the final vision of this manuscript.

ACKNOWLEDGMENTS

This work was supported by grants from the National Natural Science Foundation of China (No. 81273504, 81473258, 81402941); grants from Shanghai Municipal Commission of Health and Family Planning (No. 20144Y0204, 201540294); and grants from the Science and Technology Commission of Shanghai Municipality (No. 15140904600, 16ZR1434400).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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An Orally Active *Cannabis* Extract with High Content in Cannabidiol attenuates Chemically-induced Intestinal Inflammation and Hypermotility in the Mouse

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OPEN ACCESS

Edited by:

Giuseppe Esposito,
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Reviewed by:

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Specialty section:

This article was submitted to
Gastrointestinal and Hepatic
Pharmacology,
a section of the journal
Frontiers in Pharmacology

Received: 27 July 2016

Accepted: 12 September 2016

Published: 04 October 2016

Citation:

Pagano E, Capasso R, Piscitelli F, Romano B, Parisi OA, Finizio S, Lauritano A, Di Marzo V, Izzo AA and Borrelli F (2016) An Orally Active Cannabis Extract with High Content in Cannabidiol attenuates Chemically-induced Intestinal Inflammation and Hypermotility in the Mouse. *Front. Pharmacol.* 7:341. doi: 10.3389/fphar.2016.00341

Anecdotal and scientific evidence suggests that *Cannabis* use may be beneficial in inflammatory bowel disease (IBD) patients. Here, we have investigated the effect of a standardized *Cannabis sativa* extract with high content of cannabidiol (CBD), here named CBD BDS for “CBD botanical drug substance,” on mucosal inflammation and hypermotility in mouse models of intestinal inflammation. Colitis was induced in mice by intracolonic administration of dinitrobenzenesulfonic acid (DNBS). Motility was evaluated in the experimental model of intestinal hypermotility induced by irritant croton oil. CBD BDS or pure CBD were given - either intraperitoneally or by oral gavage – after the inflammatory insult (curative protocol). The amounts of CBD in the colon, brain, and liver after the oral treatments were measured by high-performance liquid chromatography coupled to ion trap-time of flight mass spectrometry. CBD BDS, both when given intraperitoneally and by oral gavage, decreased the extent of the damage (as revealed by the decrease in the colon weight/length ratio and myeloperoxidase activity) in the DNBS model of colitis. It also reduced intestinal hypermotility (at doses lower than those required to affect transit in healthy mice) in the croton oil model of intestinal hypermotility. Under the same experimental conditions, pure CBD did not ameliorate colitis while it normalized croton oil-induced hypermotility when given intraperitoneally (in a dose-related fashion) or orally (only at one dose). In conclusion, CBD BDS, given after the inflammatory insult, attenuates injury and motility in intestinal models of inflammation. These findings sustain the rationale of combining CBD with other minor *Cannabis* constituents and support the clinical development of CBD BDS for IBD treatment.

Keywords: *Cannabis sativa*, cannabidiol, cannabinoids, inflammatory bowel disease, colitis, intestinal motility

INTRODUCTION

Inflammatory bowel disease (IBD) is a chronic immunologically mediated disease with growing incidence and prevalence rates in industrialized countries (Ananthakrishnan, 2015; Taleban et al., 2015). Effective pharmacotherapies for IBD are not always available and existing drugs may cause substantial side-effects leading to poor patient adherence. The anecdotal use of marijuana and/or preparations from *Cannabis sativa* L. (hemp) in IBD patients has been recently confirmed by

investigations in humans (Ravikoff Allegretti et al., 2013; Naftali et al., 2014; Storr et al., 2014; Izzo et al., 2015). For example, a large-scale population based survey has recently suggested that IBD patients experience symptom relief with marijuana use (Weiss and Friedenberg, 2015). The *C. sativa* plant produces over 100 terpenophenolic molecules, i.e., the phytocannabinoids, which accumulates predominantly in the plant glandular trichomes (Russo, 2011). Clearly, the most known among the phytocannabinoids is Δ^9 -tetrahydrocannabinol (THC), whose possible clinical use is hindered by its psychoactivity. This obstacle has addressed further research toward non-psychotropic phytocannabinoids such as cannabidiol (CBD), the versatile pharmacology of which is well established (Esposito et al., 2013; Welty et al., 2014; Brodie et al., 2015; Burstein, 2015; McPartland et al., 2015).

Among numerous pharmacological actions of potential therapeutic interest, pure CBD has been shown to ameliorate experimental colitis (Borrelli et al., 2009; Jamontt et al., 2010; Schicho and Storr, 2012) and to normalize motility in the inflamed mouse gut (Capasso et al., 2008; Lin et al., 2011). It is worthy of note that the beneficial effects of pure CBD in the inflamed gut have been not observed/evaluated after oral gavage (intragastric) administration, a route of drug administration which is easy to be translated to humans for therapeutic use. In the past few years, the contribution of minor phytocannabinoids to the complex *Cannabis* pharmacology has been shown (Pertwee, 2008; Hill et al., 2012; McPartland and Russo, 2014) and a number of examples of additive/synergistic effects among the phytocannabinoids have been reported (Williamson and Evans, 2000; Wilkinson et al., 2003; DeLong et al., 2010; Jamontt et al., 2010; Russo, 2011; McPartland and Russo, 2014). This observation prompted the cultivation of specific *C. sativa* chemotypes (chemical genotypes) with high yields of a specific cannabinoid (Potter, 2014). To produce a *C. sativa* extract, the flowers of female plants are immersed in liquid carbon dioxide at extremely high pressure and the chemical compounds dissolving in this solvent are then separated and purified (Potter, 2014). One of the best studied among such extracts is the standardized *C. sativa* extract with high content of CBD, generally referred to as CBD BDS (an acronym for Botanical Drug Substance). Notably, CBD BDS is a major ingredient of the medicine known with the generic name nabiximols (Sativex®; GW Pharmaceuticals, Cambridge, UK), approved in many countries for the treatment of refractory spasticity in multiple sclerosis (Syed et al., 2014). In the present study, we have examined the effect of CBD BDS in the murine model of colitis induced by dinitrobenzenesulfonic acid. In addition, because motility alterations represent an hallmark in IBD patients, we also investigated the effect of the *C. sativa* extract in the model of intestinal dysmotility induced by the pro-inflammatory agent croton oil.

MATERIALS AND METHODS

Drugs and Reagents

A standardized *C. sativa* extract with high content in CBD (CBD BDS, 63.9% w/w of CBD content) and pure CBD, [purity by

high-performance liquid chromatography (HPLC), 99%] were supplied by GW Pharmaceuticals (Cambridge, UK). The dose of CBD BDS used in the experiments refers to the amount of CBD contained in the extract (e.g., 10 mg/kg of CBD BDS indicates a dose of the BDS that contains 10 mg/kg of CBD). 2,4,6-dinitrobenzenesulfonic acid (DNBS), croton oil and myeloperoxidase (MPO) from human leucocytes, were purchased from Sigma Aldrich S.r.l. (Milan, Italy). Pure CBD and CBD BDS were dissolved in ethanol/Tween20/saline (1:1:8) for i.p. injection (60 μ l/mouse) or in sesame oil (90 μ l/mouse) for oral gavage administration. DNBS was solubilized in 50% ethanol (0.15 ml/mouse). The pure CBD and CBD BDS vehicles had no significant effects on the responses under study.

Plant Material Extraction and Composition of the Cannabis Extract

A *C. sativa* chemotype with a controlled high amount of CBD (de Meijer et al., 2003) was used for the preparation of CBD BDS. Details about the extraction, purification as well as typical HPLC chromatogram are reported elsewhere (Romano et al., 2014). The composition (% w/w) of the main phytocannabinoids in CBD BDS was CBD 63.9 \pm 5.9, Δ^9 -THC 3.0, cannabigerol 2.8, cannabichromene 3.1, cannabidivarin 1.4. The extract was prepared in GW Pharmaceuticals laboratories (Cambridge, UK).

Animals

Male ICR mice, weighing 20–25 g for upper gastrointestinal transit experiments and 25–30 g for colitis experiments, were obtained from Charles River Laboratories (Calco, Lecco, Italy) and housed in polycarbonate cages under a 12 h light/dark cycle with light on at 07:00 a.m., controlled temperature ($23 \pm 2^\circ\text{C}$) and constant humidity (60%). Mice were fed *ad libitum* with standard food, except for the 24 h period immediately preceding the administration of DNBS, for the 12 h period preceding the measurement of intestinal transit and for the 2 h period preceding the oral gavage of drugs. All experiments complied with the Italian D.L. no. 116 of 27 January 1992 and associated guidelines in the European Communities Council Directive of 24 November 1986 (86/609/ECC). According to recent preclinical guidelines in pharmacology, group data subjected to statistical analysis had a minimum of $n = 5$ independent animals per group (Curtis et al., 2015).

Induction of Experimental Colitis

Colitis was induced by the intracolonic administration of DNBS as described before (Borrelli et al., 2015). Briefly, mice were anesthetized with inhaled 5% isoflurane (Centro Agrovete Campania, Scafati, SA, Italy) and DNBS (150 mg/kg) was injected in the distal colon using a polyethylene catheter (1 mm in diameter) via the rectum (4.5 cm from the anus). All animals were sacrificed 3 days after DNBS administration by asphyxiation with CO₂, the mice abdomen was opened by a midline incision and the colon removed, isolated from surrounding tissues, opened along the antimesenteric border, rinsed, weighed, and length measured [in order to determined the colon weight/colon length ratio (mg/cm), used as an indirect marker of inflammation]. Mice

body weight was measured every day throughout the treatment period. All measurements were performed by operators who were unaware of the particular treatment (blinded evaluation). For biochemistry analysis, tissues were kept at 80°C until use. The dose of DNBS (150 mg/kg) and the time point of damage evaluation (i.e., 3 days after DNBS administration) were selected on the basis of preliminary experiments showing a remarkable colonic damage associated with high reproducibility and low mortality for this dosage, and because maximal DNBS-induced inflammation has been reported in mice after 3 days (Massa et al., 2004).

Intestinal Hypermotility Induced by Croton Oil

Increased intestinal motility was induced by the inflammatory agent croton oil as described before (Pol and Puig, 1997; Capasso et al., 2008). Briefly, two doses of croton oil (20 µl/mouse) for two consecutive days were orally administered to mice and 4 days after the first administration of croton oil, upper gastrointestinal transit of mice was measured. This time was selected on the basis of previous work (Pol and Puig, 1997), which reported that the maximal inflammatory response associated to intestinal hypermotility occurs 4 days after the first treatment.

Upper Gastrointestinal Transit

Upper gastrointestinal transit, measured in control, and in mice with intestinal inflammation-induced accelerated intestinal motility (mice treated with croton oil), was evaluated by identifying the leading front of an intragastrically administered charcoal meal marker (10% charcoal suspension in 5% gum Arabic, 10 ml/kg) in the small intestine as previously described (Capasso et al., 2014). Twenty minutes after charcoal administration, mice were killed by asphyxiation with CO₂, and the small intestine was isolated by cutting at the pyloric and ileocaecal junctions. The distance traveled by the marker was measured and expressed as a percentage of the total length of the small intestine from pylorus to caecum.

Pharmacological Treatment

In the experimental model of colitis pure CBD, CBD BDS or vehicle were given intraperitoneally (5–30 mg/kg) or by oral gavage (10–60 mg/kg) for three consecutive days starting 24 h after DNBS administration [Day 0: colitis induction; day 1: CBD BDS (or CBD); day 2: CBD BDS (or CBD); day 3: CBD BDS (or CBD) and mice sacrifice]. The last administration of CBD BDS (or CBD) was given (at day 3) 1 h (for intraperitoneal administration) or 2 h (for oral gavage) before the sacrifice.

In the experimental model of upper gastrointestinal transit pure CBD, CBD BDS, or vehicle were given intraperitoneally (1–10 mg/kg) or by oral gavage (5–60 mg/kg) 30 min (intraperitoneally) or 1 h (oral gavage) before the administration of the marker, to both control mice, and mice with increased intestinal motility induced by the inflammatory agent croton oil. The CBD BDS and pure CBD doses were selected on the basis of previously published work (Borrelli et al., 2009; Romano et al., 2014).

Myeloperoxidase (MPO) Activity

Myeloperoxidase activity, a peroxidase enzyme used to quantify the neutrophil infiltration in whole-tissue colons, was determined as previously described (Borrelli et al., 2015). Full-thickness colons were homogenized in a lysis buffer composed of 0.5% hexadecyltrimethylammonium bromide in 3-(N morpholino) propanesulfonic acid (MOPS) 10 mM in the ratio of 50 mg tissue per mL MOPS. The homogenates were then centrifuged for 20 min at 15,000 × g at 4°C. An aliquot of the supernatant was incubated with sodium phosphate buffer (NaPP pH 5.5) and tetra-methylbenzidine 16 mM. After 5 min, hydrogen peroxide (H₂O₂; 9.8 M in NaPP) was added and the reaction stopped with acetic acid. The rate of change in absorbance was measured by a spectrophotometer at 650 nm. Different dilutions of human MPO enzyme of known concentration were used to obtain a standard curve. MPO activity was expressed as U/mg of tissue.

Tissue CBD Assay: Extraction, Purification, and LC IT-TOF Mass Spectrometry

Colon, liver, and brain samples were dounce-homogenized and extracted with acetone containing internal deuterated standards for CBD quantification by isotope dilution ([²H]₄ CBD). The lipid-containing organic phase was dried down, weighed, and pre-purified by open bed chromatography on silica gel. Fractions were obtained by eluting the column with 99:1, 90:10, and 50:50 (v/v) chloroform/methanol. The 99:1 fraction was used for CBD quantification by LC-MS-IT-TOF analysis using an LC20AB coupled to a hybrid IT-TOF detector (Shimadzu Corporation, Kyoto, Japan) equipped with an ESI interface. We acquired full-scan MSⁿ spectra of selected precursor ions by multiple reaction monitoring (MRM), extracted the chromatograms of the high-resolution [M-H]⁻ values and used the latter chromatograms for calibration and quantification.

HPLC Parameters

Liquid chromatography analysis was performed in the isocratic mode using a KinetexC18 column (10 cm × 2.1 mm, I.D. 5 µm, 100 A; Phenomenex) and methanol:water (75:25) with 0.1% NH₄C₂H₃O₂ as mobile phase with a flow rate of 150 µl/min. The samples were injected with a SIL-20 AC autosampler (Shimadzu Corporation, Kyoto, Japan). The amounts of CBD in tissues, quantified by isotope dilution with the abovementioned deuterated standard, are expressed as ng per mg of tissue weight.

Mass Spectrometry Parameters

Electrosprayed ions were generated using a capillary voltage of 4.66 kV. A curved desolvation line (CDL) was set at a temperature of 250°C to aid desolvation and a heat block temperature of 220°C was also used. To help nebulisation of the electrospray solution nitrogen was pumped into the ion source at a rate of 1.5 L/min. The ToF mass analyzer was used to acquire data in both MS and MS/MS modes. In the MS mode, a 10 ms ion accumulation time was used before ion trapping. In the MS-MS mode, instead, the ion accumulation time was 20 ms and the window used for precursor ion isolation corresponds to

a width of 3 atomic mass unit (amu) and 20 ms. To induce fragmentation of the precursor ion a supplementary alternative current (AC) potential was applied to the end-cap electrodes to induce resonant excitation and argon is used as a collision gas during collision-induced dissociation (CID). The collision was carried out over 30 ms using a *q*-value of 0.251 (45 kHz). Three scans were accumulated in each MS-MS spectrum. In both MS and MS-MS mode data were acquired over a mass range of 200–500 *m/z*. In both regimes of operation ions are pulsed into the time of flight (ToF) with an accelerating potential of 9 kV and the detector voltage is set at 1.7 kV (Piscitelli et al., 2011).

Full details of the quantification of CBD using LC-MS-IT-ToF-MS will be published elsewhere (Piscitelli et al., 2011, manuscript in preparation].

Statistical Analysis

Data are expressed as the mean \pm SEM of *n* experiments. To determine statistical significance, Student's *t*-test was used for comparing a single treatment mean with a control mean,

and a one-way ANOVA followed by a Tukey-Kramer multiple comparisons test was used for analysis of multiple treatment means. *P*-values < 0.05 were considered significant.

RESULTS

Effect of CBD BDS and Pure CBD on Body Weight and Colon Weight/Colon Length Ratio

The administration of DNBS caused both a significant decrease in body weight and a significant increase in colon weight/length ratio when compared to control mice (Figures 1 and 2). Treatment of DNBS mice with CBD BDS, given intraperitoneally at the range dose of 5–30 mg/kg, did not modify the loss of body weight induced by the inflammatory agent (Figure 1A) but reduced DNBS-increased colon weight/length ratio (Figure 1B). The effect was significant at the 30 mg/kg dose (Figure 1B). Likewise, oral gavage administration of CBD BDS (10–60 mg/kg)

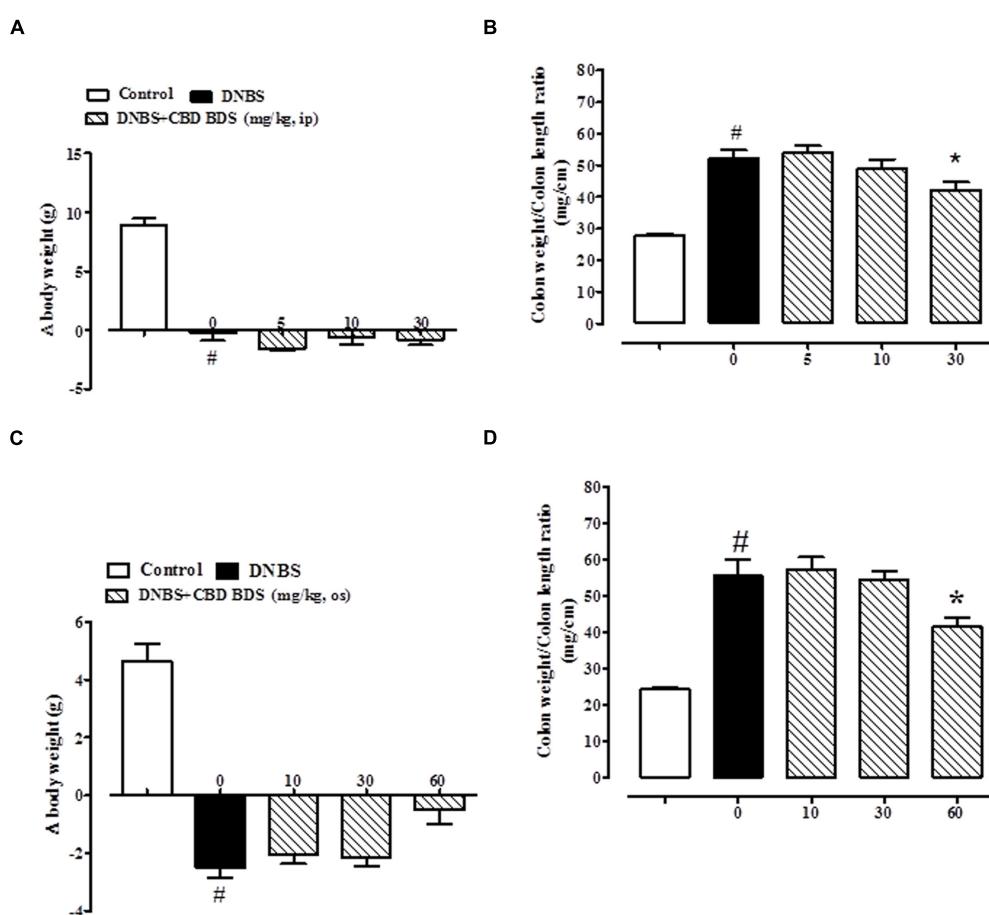


FIGURE 1 | Effect of CBD BDS (5–30 mg/kg, intraperitoneally) on body weight (A) and colon weight/colon length ratio (B) and effect of CBD BDS (10–60 mg/kg, oral gavage) on body weight (C) and colon weight/colon length ratio (D) in DNBS induced colitis in mice (DNBS, 150 mg/kg, intracolonically). CBD BDS was given once a day for three consecutive days starting from 1 day after DNBS administration. Mice were euthanized 3 days after DNBS. Mice were weighted before DNBS (or vehicle) administration and immediately before the sacrifice. Bars are mean \pm SEM of 8–12 mice for each experimental group. $\#p < 0.001$ vs. control and $*p < 0.05$ vs. DNBS alone.

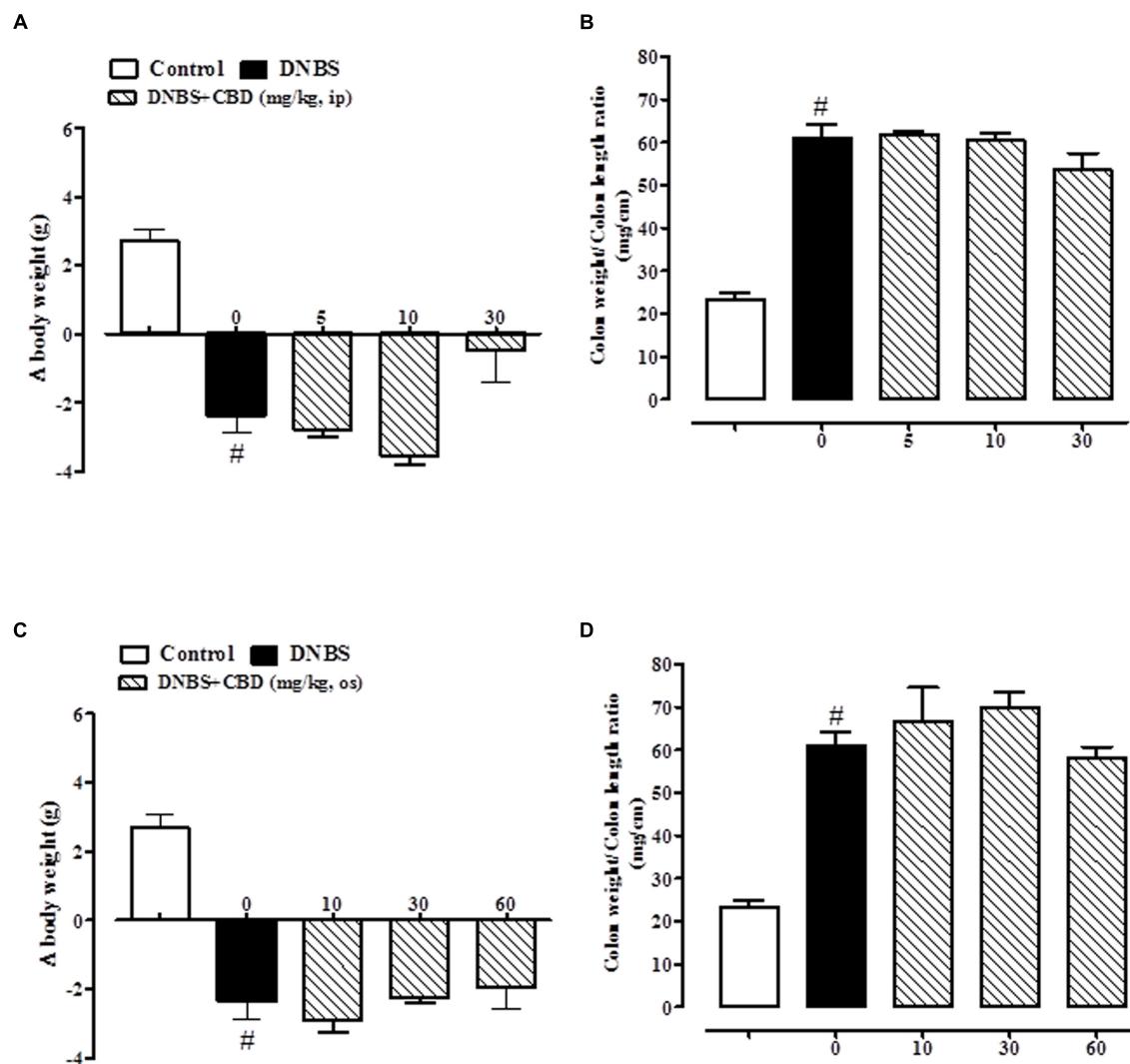


FIGURE 2 | Effect of pure CBD (5–30 mg/kg, intraperitoneally) on body weight (A) and colon weight/colon length ratio (B) and effect of pure CBD (10–60 mg/kg, by oral gavage) on body weight (C) and colon weight/colon length ratio (D) in DNBS induced colitis in mice (DNBS, 150 mg/kg, intracolonically). Pure CBD was given once a day for three consecutive days starting from 1 day after DNBS administration. Mice were euthanized 3 days after DNBS. Mice were weighted before DNBS (or vehicle) administration and immediately before the sacrifice. Bars are mean \pm SEM of 8–12 mice for each experimental group. * $p < 0.001$ vs. control.

had no effect on the DNBS-induced decrease in body weight, although a trend in reducing weight loss was observed at the 60 mg/kg dose (Figure 1C). CBD BDS, given via oral gavage at the 60 mg/kg dose, significantly reduced the colon weight/length ratio increased by DNBS (Figure 1D).

Pure CBD, when both given intraperitoneally (5–30 mg/kg) or by oral gavage (10–60 mg/kg), did not ameliorate DNBS-induced colitis (no variation on body weight and colon weight/colon length ratio; Figure 2).

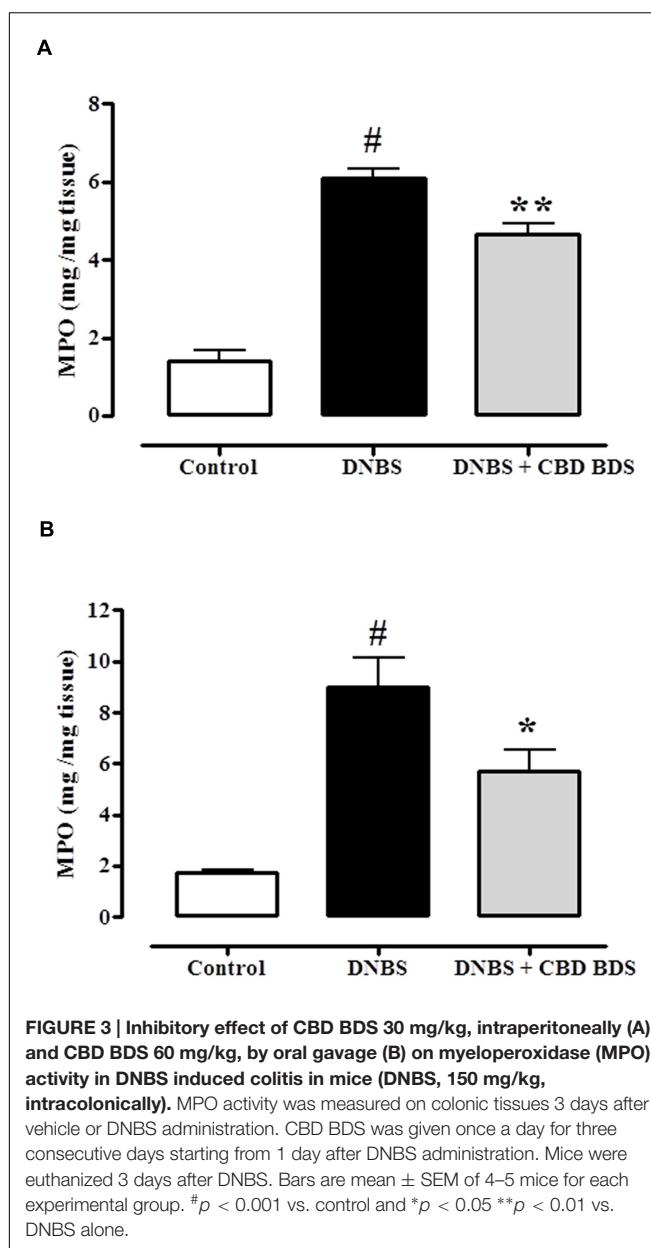
Effect of CBD BDS on MPO Activity

The beneficial effect of CBD BDS on DNBS-induced colitis was further confirmed by the MPO results. DNBS administration produced a threefold increase in MPO activity which was

significantly reduced by either intraperitoneal or oral gavage administration of CBD BDS at the 30 mg/kg and 60 mg/kg dose, respectively (Figure 3).

Effect of Pure CBD and CBD BDS on Upper Gastrointestinal Transit

CBD BDS, given intraperitoneally at the dosage range of 1–10 mg/kg, reduced the intestinal transit in healthy mice, the effect being significant only at the higher dose tested (10 mg/kg; Figure 4A). CBD BDS (1–10 mg/kg), in a dose dependent manner, counteracted the increase in intestinal motility induced by croton oil (Figure 4B). The effect was significant starting from the 1 mg/kg dose. Analysis of the curves representing the inhibitory effect of CBD BDS on transit



in healthy mice and in mice with hypermotility induced by croton oil shows that CBD BDS preferentially inhibited intestinal transit in pathophysiological rather than physiological conditions (**Figure 4**). Likewise, CBD BDS when given by oral gavage at the dosage range of 5–60 mg/kg reduced the intestinal transit in healthy mice, the effect being significant starting from the 10 mg/kg dose (**Figure 5A**). Similar to the intraperitoneal administration, oral gavage administration of CBD BDS (5–60 mg/kg), in a dose dependent manner, reduced the intestinal hypermotility induced by croton oil (**Figure 5B**). The effect was significant starting from the 5 mg/kg dose. Analysis of the curves representing the inhibitory effect of CBD BDS on transit in healthy mice and in mice with hypermotility induced by croton oil shows that CBD BDS, also when given by oral gavage,

preferentially inhibited intestinal transit in pathophysiological rather than physiological conditions (**Figure 5**).

As previously reported (Capasso et al., 2008), pure CBD, given intraperitoneally at the dose range of 1–10 mg/kg, did not affect the intestinal transit in healthy mice (**Figure 6A**) but, significantly and in a dose dependent manner, restored the intestinal motility in mice with hypermotility induced by croton oil (**Figure 6B**). The effect was significant starting from the 5 mg/kg dose (**Figure 6B**). Oral gavage administration of pure CBD did not reduce intestinal transit in healthy mice (**Figure 6C**), and reduced the croton oil-induced accelerated intestinal motility at the 5 mg/kg dose only (**Figure 6D**).

CBD Levels in Tissues of Mice with DNBS-Induced Inflammation after Oral Treatment with Pure CBD or CBD BDS

As shown in **Figure 7**, CBD was detected in the colon, liver, and brain in mice treated with either CBD BDS or pure CBD (oral administration). In the colon (**Figure 7A**) and in the brain (**Figure 7B**), the content increased with the dose in the case of pure CBD, while in the CBD BDS group there were no differences between the two doses.

In the liver, CBD tissue penetration was higher with the oral administration of CBD-BDS, in a dose-dependent manner (**Figure 7C**), while there were no significant differences between pure CBD at 30 and 60 mg. Interestingly, higher CBD levels were achieved with pure CBD in the colon and brain, and much higher CBD levels were achieved with CBD BDS in the liver.

DISCUSSION

The notion that not all of the therapeutic effects of *C. sativa* are due to its many active psychotropic ingredient THC is well established (Wilkinson et al., 2003; Russo, 2011; Brodie et al., 2015). The contribution of non-THC phytocannabinoids to *Cannabis* pharmacology has been scientifically demonstrated in a number of experimental diseases, including ulcerative colitis (Duncan and Izzo, 2015). In previous studies, it has been shown that isolated *Cannabis* constituents, including cannabichromene (CBC), CBD and cannabigerol (CBG), exert favorable effects in experimental models of IBD (Borrelli et al., 2009, 2013; Jamont et al., 2010; Romano et al., 2013). In the present study, we have expanded our knowledge on the intestinal anti-inflammatory effect of phytocannabinoids by showing for the first time that a *Cannabis* extract with high content in CBD, namely CBD BDS, when both given intraperitoneally and by oral gavage, is able to reduce the extent of the damage and to counteract intestinal hypermotility in experimental models of intestinal inflammation. By contrast, we demonstrated that pure CBD, either given intraperitoneally or by oral gavage at matched CBD doses with CBD BDS, after the inflammatory insult does not offer anti-inflammatory effects.

The route of administration for cannabinoids is a major issue since cannabinoids are significantly metabolized by hepatic cytochrome enzymes (Huestis, 2005). There is no evidence

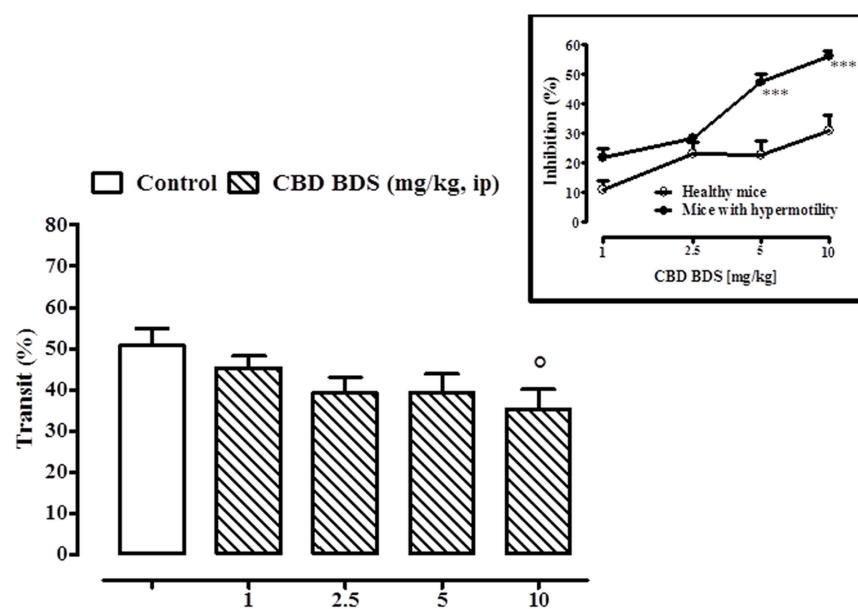
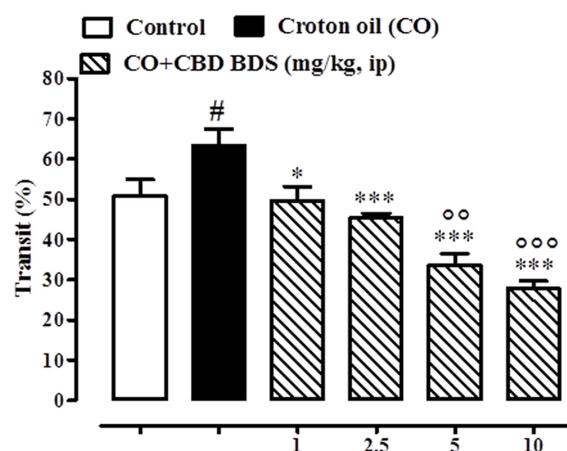
A**B**

FIGURE 4 | Effect of CBD BDS (1–10 mg/kg, intraperitoneally) on intestinal transit in healthy mice (A) and croton oil-treated mice (B). Bars represent the mean \pm SEM of 8–10 animals for each experimental group. $^{\circ}$ and $^{\#}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ vs. control and $*p < 0.05$ and $^{***}p < 0.001$ vs. croton oil alone. Insert: Difference between the curves representing the inhibitory effect of CBD BDS on intestinal transit in healthy mice and mice with hypermotility (mice treated with croton oil). Results are expressed as mean \pm SEM of 8–10 mice for each experimental group. $^{***}p < 0.001$ vs. healthy mice.

in the literature that cannabinoids may exert intestinal anti-inflammatory effects when given orally. While the intraperitoneal route of administration is extensively used in rodent experiments, it appears clear that such a way of delivery is hard to be translated to humans. In the present study, we have shown that CBD BDS, given intraperitoneally or by oral gavage, reduces inflammation associated to DNBS administration. Although we did not provide a microscopic score, as done for CBD in our previous work (Borrelli et al., 2009), the anti-inflammatory effect of CBD BDS was supported by colon length/weight ratio and by the MPO measurements, two well-established marker

of intestinal inflammation (Krawisz et al., 1984; Kristjánsson et al., 2004). In addition, CBD BDS (intraperitoneally or orally) was pharmacologically active when administered after the inflammatory insult, which is clinically relevant in the light of the observation that the main goal of IBD pharmacotherapy is to cure rather than to prevent. In previous studies aimed at investigating the intestinal anti-inflammatory effects of *Cannabis* and its active ingredients, phytocannabinoids, including CBD, were given before the inflammatory insult (i.e., preventive protocol) (Borrelli et al., 2009; Jamontt et al., 2010; Schicho and Storr, 2012). In contrast to CBD BDS, pure CBD does not exert

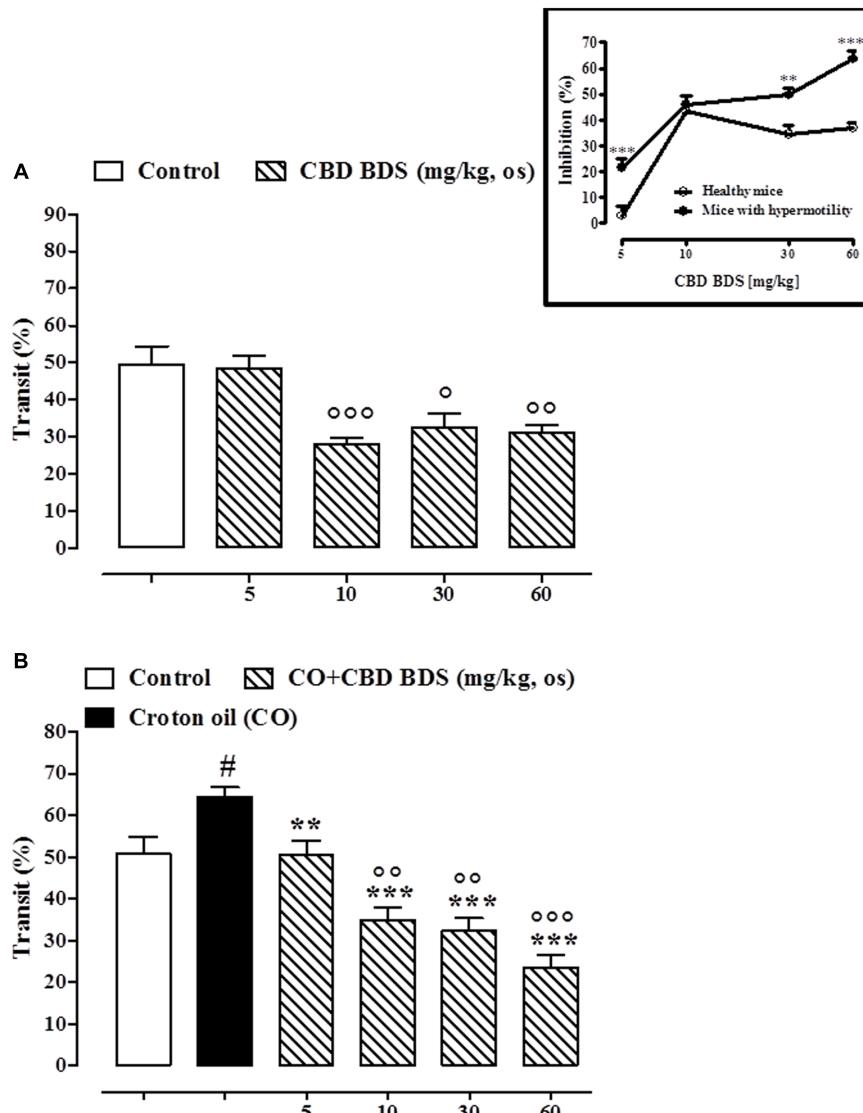


FIGURE 5 | Effect of CBD BDS (10–60 mg/kg, by oral gavage) on intestinal transit in healthy mice (A) and croton oil-treated mice (B). Bars represent the mean \pm SEM of 8–10 animals for each experimental group. $^{\circ}$ and $^{\#}$ $p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ vs. control and $^{**}p < 0.01$ and $^{***}p < 0.001$ vs. croton oil alone. Insert: Difference between the curves representing the inhibitory effect of CBD BDS on intestinal transit in healthy mice and mice with hypermotility (mice treated with croton oil). Results are expressed as mean \pm SEM of 8–10 mice for each experimental group. $^{***}p < 0.001$ vs. healthy mice.

anti-inflammatory effects either when given intraperitoneally or by oral gavage even with the high doses. The lack of effect of pure CBD (up to 20 mg/kg) after oral gavage, but not after intracolonic administration has been previously documented (Schicho and Storr, 2012) and may be not surprising if we consider that this type of administration is subjected to a significant first-pass effect (Huestis, 2005).

The difference in efficacy between pure CBD and CBD BDS is likely due to the presence of pharmacologically active ingredients. Indeed, in addition to CBD, CBD BDS contains other phytocannabinoids, such as THC, CBC, and CBG, which have been previously shown to exert anti-inflammatory effects in experimental models of colitis (Jamontt et al., 2010; Borrelli et al.,

2013; Romano et al., 2013) and whose colonic levels were not determined in the present study. Furthermore non-cannabinoid *C. sativa* constituents, such as flavonoids, phytosterols, and terpenoids, have been shown to ameliorate murine colitis (Reddy, 1976; Somani et al., 2015; Vezza et al., 2016). On the other hand, the higher efficacy of oral CBD BDS does not seem to be ascribable to higher penetration of CBD in the colon when using this drug. In fact, we show here that the exposure to CBD of both the colon and brain after oral administration of either pure CBD or CBD BDS is higher with the highest dose of pure CBD than with the highest dose of CBD BDS, and similar with the lowest doses of the two drugs. Conversely, in the liver, the concentration of CBD, at both doses tested, was higher with CBD BDS than with

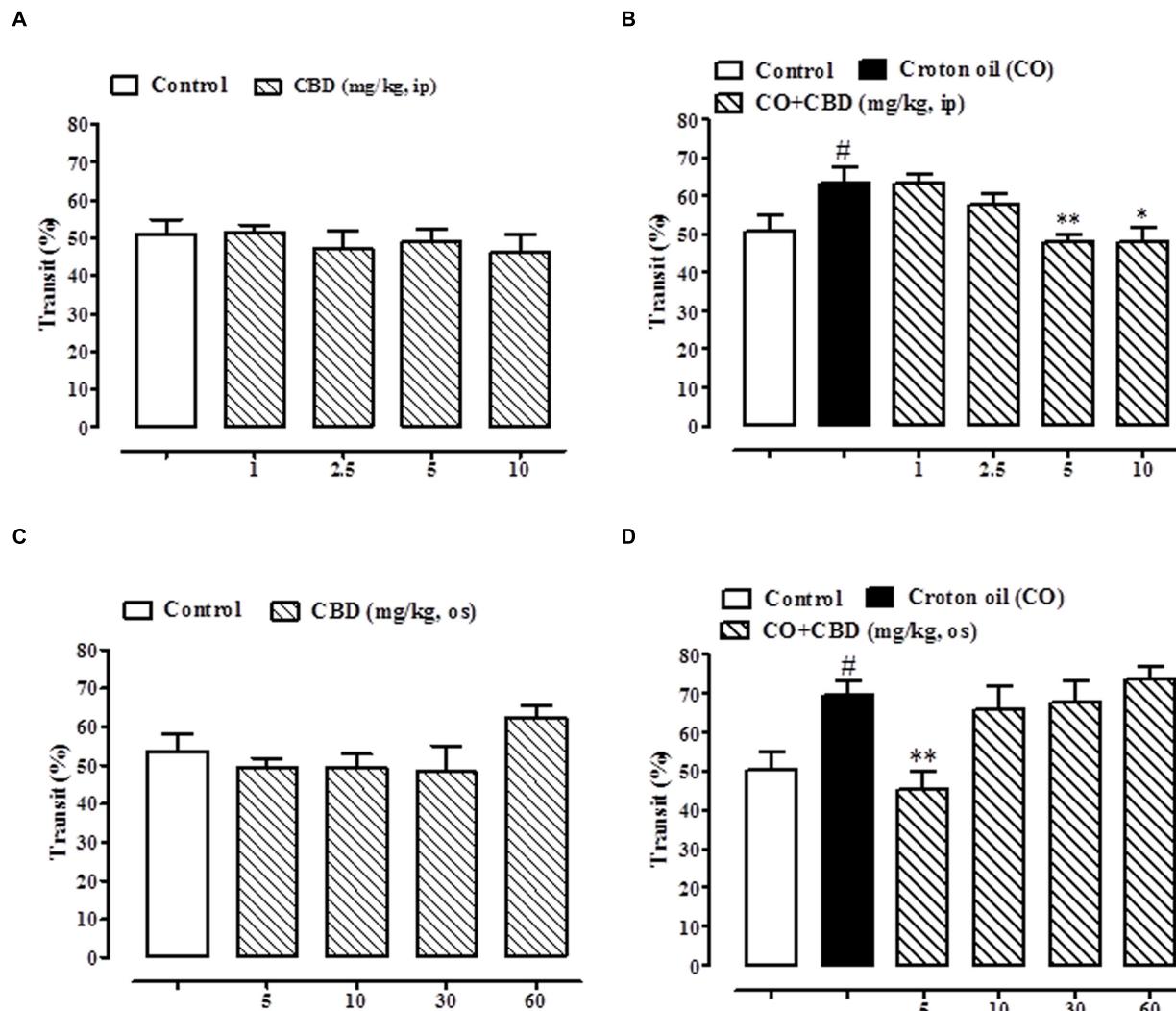


FIGURE 6 | Effect of intraperitoneal administration of pure CBD (1–10 mg/kg) on intestinal transit in healthy mice (A) and croton oil-treated mice (B), and oral gavage administration of pure CBD (5–60 mg/kg) on intestinal transit in healthy mice (C) and croton oil-treated mice (D). Bars represent the mean \pm SEM of 8–10 animals for each experimental group. ${}^{\#}p < 0.05$ – 0.01 vs. control; ${}^{*}p < 0.05$ and ${}^{**}p < 0.01$ vs. croton oil alone.

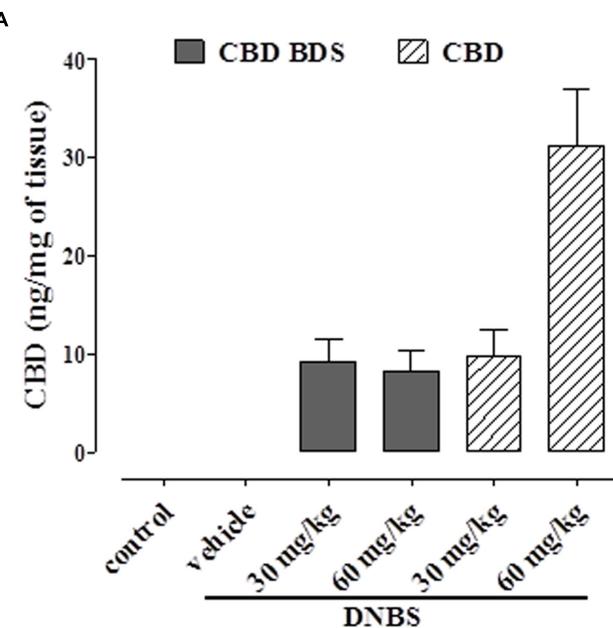
pure CBD. Interestingly, the maximal colonic concentrations of CBD at the end of treatment can be calculated to be \sim 30 nM with CBD BDS and \sim 100 nM with pure CBD, which are not too distant from the potencies of this compound at many of its proposed molecular targets (McPartland and Russo, 2014; Brodie et al., 2015). Others have shown that a non-chemically characterized *Cannabis* extract reduced the severity of rat colitis when administered intracolonically (Wallace et al., 2013).

Inflammatory states in the gut may cause motility disturbances, and alterations in intestinal motility are common debilitating symptoms (Brierley and Linden, 2014). To investigate the effect of CBD BDS on intestinal motility, we adopted the croton oil model of intestinal inflammation-induced hypermotility. This model has been extensively used in the past to evaluate the potential of drugs able to reduce intestinal motility such as opioids (Pol and Puig, 2004) and cannabinoids (Aviello

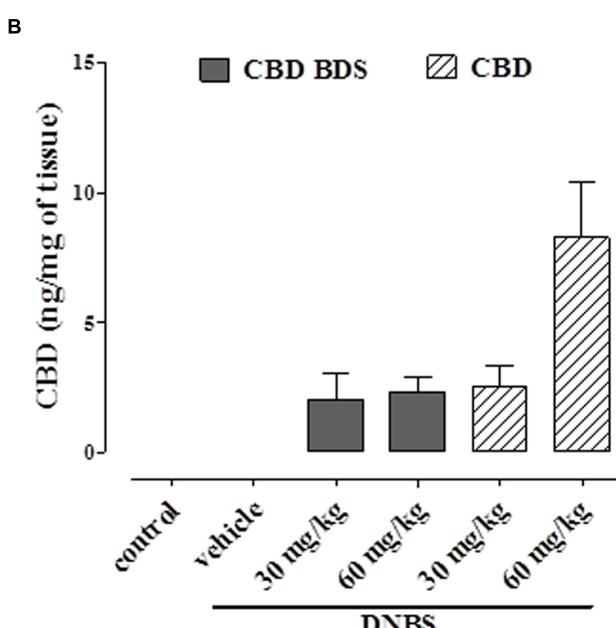
et al., 2008). By using this experimental model, it has been shown that a number of phytocannabinoids, including CBD, CBC, and CBN (Izzo et al., 2000, 2012; Capasso et al., 2008), normalize motility during the inflammatory process, with weak or no effect in control mice. In the present study, we have shown that both CBD BDS and pure CBD, given intraperitoneally or by oral gavage, reduce motility in mice with intestinal inflammation, with weak (CBD BDS) or no (pure CBD) effects in control mice. From a translational viewpoint, the low doses of CBD BDS (and pure CBD) required to normalize motility in the inflamed gut – as well as their pharmacological activity following oral gavage administration – are relevant if we consider that the drugs available to reduce motility may be often associated with constipation (Corsetti and Tack, 2015; Rao et al., 2016).

The anti-inflammatory mode of action of CBD BDS in the gut is still elusive and deserves further *ad hoc* studies. CBD

(COLON)



(BRAIN)



(LIVER)

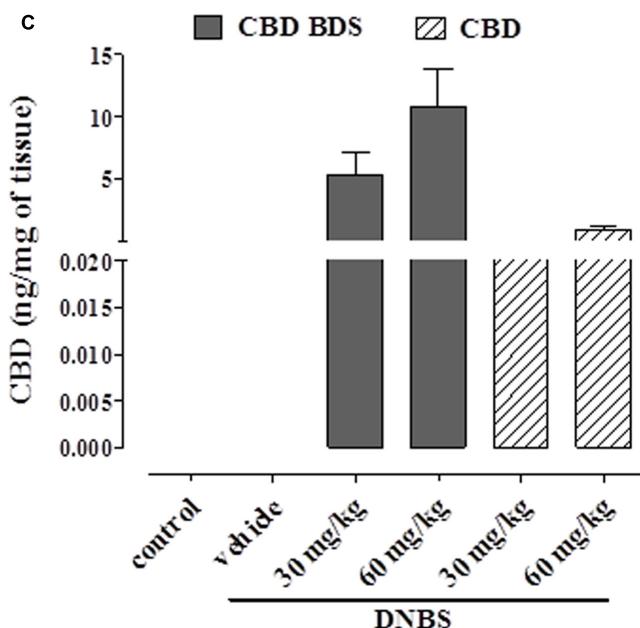


FIGURE 7 | CBD levels in colon (A), brain (B), and liver (C) of DNBS-treated mice after oral administration of either CBD BDS or CBD. Data are means \pm SEM of $N = 5$ –6 mice.

and other minor phytocannabinoids contained in CBD BDS may interact with targets (e.g., components of the so-called endogenous cannabinoid system, TRP channels; De Petrocellis

et al., 2011, 2012) which have been linked to IBD (Izzo et al., 2015; Zielińska et al., 2015). Recently, pharmacological blockade of GPR55 has been shown to protect against experimental gut

inflammation (Stančić et al., 2015), a relevant finding in the light of the observation that CBD is a GPR55 antagonist (Ryberg et al., 2007; Campos et al., 2012). Conversely, the antioxidant effect is believed to not entirely explain *per se* the gut anti-inflammatory actions exerted by CBD (Esposito et al., 2013).

CONCLUSION

The present study reveals for the first time the ability of the CBD BDS to attenuate the severity of inflammation in the DNBS model of colitis, as well as to reduce transit in a model of inflammation-induced dysmotility. The strengths of CBD BDS for a possible clinical use in IBD patients include: (a) its intestinal anti-inflammatory activity following oral gavage administration (in contrast to pure CBD, which was ineffective); (b) its ability to reduce the degree of inflammation in a curative protocol, (c) its ability to reduce motility in the inflamed gut at doses lower than those required to affect motility in control animals. Our results further support the therapeutic rationale for combining

CBD with other minor constituents present in *Cannabis sativa*, also in the light of recent positive effects exerted by CBD BDS (also named GWP42003) in IBD patients (Irving et al., 2015).

AUTHOR CONTRIBUTIONS

EP performed experiments and was responsible for acquisition, analysis and interpretation of data. BR, SF, and OP performed experiments. FP and AL evaluated endocannabinoid levels. RC, AI, VD, and FB were responsible for conception and design, analysis and interpretation of data and redaction of the manuscript.

FUNDING

This research was in part supported by GW Pharmaceuticals, Cambridge.

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- Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.
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Polyunsaturated Fatty Acids and Their Derivatives: Therapeutic Value for Inflammatory, Functional Gastrointestinal Disorders, and Colorectal Cancer

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OPEN ACCESS

Edited by:

Gabriella Aviello,
University of Aberdeen, UK

Reviewed by:

Elisabetta Barocelli,
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Simona Pace,
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Specialty section:

This article was submitted to
Gastrointestinal and Hepatic
Pharmacology,
a section of the journal
Frontiers in Pharmacology

Received: 16 August 2016

Accepted: 14 November 2016

Published: 01 December 2016

Citation:

Michałak A, Mosińska P and Fichna J (2016) Polyunsaturated Fatty Acids and Their Derivatives: Therapeutic Value for Inflammatory, Functional Gastrointestinal Disorders, and Colorectal Cancer. *Front. Pharmacol.* 7:459.
doi: 10.3389/fphar.2016.00459

Polyunsaturated fatty acids (PUFAs) are bioactive lipids which modulate inflammation and immunity. They gained recognition in nutritional therapy and are recommended dietary supplements. There is a growing body of evidence suggesting the usefulness of PUFAs in active therapy of various gastrointestinal (GI) diseases. In this review we briefly cover the systematics of PUFAs and their metabolites, and elaborate on their possible use in inflammatory bowel disease (IBD), functional gastrointestinal disorders (FGIDs) with focus on irritable bowel syndrome (IBS), and colorectal cancer (CRC). Each section describes the latest findings from *in vitro* and *in vivo* studies, with reports of clinical interventions when available.

Keywords: n-3 PUFA, n-6 PUFA, eicosapentaenoic acid, docosahexaenoic acid, arachidonic acid derivatives, inflammatory bowel disease, irritable bowel syndrome, colorectal cancer

INTRODUCTION

Gastroenterology is a rapidly-evolving basic and clinical science that concerns organic and functional gastrointestinal (GI) disorders. The former include inflammatory, infectious and neoplastic diseases and the latter embrace conditions characterized by chronic symptoms and the absence of recognized biochemical or structural explanations.

Inflammatory bowel disease (IBD) has been constricted so far mainly to Europe and US, but currently emerge also in newly industrialized countries in Asia, Middle East, and South America (Kaplan, 2015). North America and Europe also remain the leading regions of the occurrence of functional gastrointestinal disorders (FGIDs), especially irritable bowel syndrome (IBS) (Fox and Muniraj, 2016). In turn, the incidence and mortality of colorectal cancer (CRC) are high in developed countries and are rapidly rising in low-income and middle-income populations (Arnold et al., 2016). Despite an ongoing progress in implementing structured screening in reducing the prevalence rate and developing new treatments, currently available options are not always effective and often prompt adverse effects (Favoriti et al., 2016). Hence, we still need new tools of prevention and therapy.

The aim of this review is to explore the studies on polyunsaturated fatty acids (PUFAs), their metabolites and derivatives, and relate the findings to the pressing problems of gastroenterology. The scientific bibliography included in this review was collected from the PubMed and Web of Science databases. We searched for English language review articles or original research published up to May 2016, using the following keywords alone or in combination: PUFA, arachidonic

acid, linoleic acid, eicosapentaenoic acid, docosahexaenoic acid, fish oil, diet in irritable bowel syndrome, inflammatory bowel disease, Crohn's disease, ulcerative colitis, colorectal cancer, functional gastrointestinal diseases. Clinical trials were searched using ClinicalTrials.gov database.

SYSTEMATIC OVERVIEW OF PUFAs

A fatty acid (FA) comprises aliphatic hydrocarbon chain with methyl and carboxyl groups at opposite ends; PUFAs contain more than one double bond in their structure. There are two main groups of biologically important long chain PUFAs: n-6 PUFA with their first double bond at C6, counting from the methyl C, and n-3 PUFAs with first unsaturated bond at C3. The main representatives of these groups are:

- n-6 PUFAs: linoleic acid (LA, 18:2), arachidonic acid (AA, 20:4),
- n-3 PUFAs: alpha-linolenic acid (ALA, 18:3), eicosapentaenoic acid (EPA, 18:5) and docosahexaenoic acid (DHA, 22:6).

LA and ALA are referred to as essential FAs, because human body lacks the enzymes for their synthesis. Both LA and ALA are the precursors for AA, EPA and DHA, and are of primal biological importance—both FAs must be thus additionally supplemented in case of enzymatic defects or lack of dietary ALA or LA. In humans, diet remains a primary source of DHA and EPA because the efficacy of transforming ALA to longer n-3 PUFAs is low and personally variable (Glaser et al., 2010). EPA and DHA are mostly found in fish, especially salmon, whereas the most important source of ALA are seed oils derived from walnuts, chia, perilla, rapeseeds or soybeans. Currently, due to the suboptimal total PUFAs consumption in most populations,

Abbreviations: 2-AG, 2-arachidonoylglycerol; 12-LO, 12-lipoxygenase; 5,6-EET, 5,6-epoxyeicosatrienoic acid; 5-LO, 5-lipoxygenase; AA, arachidonic acid; AEA, anandamide; ALA, alpha-linoleic acid; CB, cannabinoid; CD, Crohn's disease; CIC, chronic idiopathic constipation; CLA, conjugated linoleic acid; CNS, central nervous system; COX, cyclooxygenase; CRC, colorectal cancer; CSLC, cancer stem like cells; CVD, cardiovascular disease; Cyp450, cytochrome 450; DHA, docosahexaenoic acid; DMPC, dimyristoylphosphatidylcholine; DP, prostaglandin D2 receptor; DPA, docosapentaenoic acid; DSS, dextran sulfate sodium; EETs, epoxyeicosatrienoic acids; ENS, enteric nervous system; EPA, eicosapentaenoic acid; EPA-FFA, free fatty acid eicosapentaenoic acid; EPIC, European Prospective Investigation of Cancer; FA, fatty acid; FAAH, fatty acid amide hydrolase; FAD1, fatty acid desaturase 1; FAP, familial adenomatous polyposis; FDA, Food and Drug Administration; FGIDs, functional gastrointestinal disorders; GI, gastrointestinal; GRAS, generally recognized as safe; HETEs, hydroxyeicosatetraenoic acids; HT-29-EP4, HT-29 human CRC cells with EP4 overexpression; IBD, inflammatory bowel disease; IBS, irritable bowel syndrome; IBS-A, alternating IBS; IBS-C, constipation-predominant IBS; IBS-D, diarrhea-predominant IBS; ICAM, intracellular adhesion molecule 1; iNOS, inducible nitric oxide synthase; LA, linoleic acid; LOX, lipoxygenase; LTB₄, leukotriene B₄; LXs, lipoxins; MPO, myeloperoxidase; NAFLD, non-alcoholic fatty liver disease; NO, nitric oxide; PBMC, peripheral blood mononuclear cells; PGA1, prostaglandin A1; PGD2, prostaglandin D2; PGE2, prostaglandin E2; PGH2, prostaglandin H2; PGJ2, prostaglandin J2; PGs, prostaglandins; PLA2, phospholipase A2; PUFAs, polyunsaturated fatty acids; RvD, D-type resolving; RvD1, resolin D1; Rvs, resolvin; TNBS, 2,4,6-trinitrobenzene sulfonic acid; TRP, transient receptor potential; TRPA, TRP subfamily A receptor; TRPV4, transient receptor potential vanilloid type 4; TXs, thromboxanes; UC, ulcerative colitis; VCAM-1, vascular cell adhesion protein 1; VEGFR-2, vascular endothelial growth factor receptor 2.

there is a growing awareness and support for regular PUFA supplementation (González-Rodríguez et al., 2013; Sioen et al., 2013; Kodentsova et al., 2014).

Physiologically, PUFAs are built into lipid membranes. Once they are released by phospholipase A2 (PLA2), they undergo processing to many biologically active signaling molecules (Gomolka et al., 2011; Maskrey et al., 2013; Capra et al., 2015; Powell and Rokach, 2015). The most important pathways of PUFAs metabolism include:

- cyclooxygenases (COXs)—act on both n-3 and n-6 PUFAs, yielding prostaglandins (PGs), prostacyclins and thromboxanes (TXs); altogether branded prostanoids,
- lipoxygenases (LOXs)—convert AA to lipoxins (LXs) and leukotrienes (LTs). Together with COXs, LOXs also produce protectins, maresins, and resolvins (Rvs) from n-3 PUFAs,
- cytochrome 450 (Cyp 450)—catalyzes the conversion of both n-3 and n-6 PUFAs to epoxyeicosatrienoic acids (EETs). Along with other enzymes, Cyp450 takes part in the synthesis of PUFA derivatives, producing biologically active hydroxyeicosatetraenoic acids (HETEs).

Polyunsaturated fatty acids (PUFAs) can also be processed by non-enzymatic oxidation, creating isoprostanes, and isofurans which also display biological activity (Galano et al., 2015; Roy et al., 2016).

Overall, there is a great variety of PUFAs metabolites which possess pro- and anti-apoptotic properties, play important roles in inflammation, modulate the immune responses and probably affect many yet unknown processes (Figure 1; Masoodi et al., 2015).

PUFAs in Inflammation

So far, the role of PUFAs in inflammation has been mainly explained by the action of AA. One of its most important metabolites are PGs, a heterogenous group of molecules produced by COX-1 and COX-2. Their common precursor is prostaglandin H2 (PGH2) produced from AA and later metabolized into downstream metabolites (Félezou et al., 2011). The main pro-inflammatory one is prostaglandin E2 (PGE2), produced in large quantities by macrophages and neutrophils in response to inflammatory stimuli. PGE2 induces fever, increases vascular permeability and vasodilation, and intensifies pain and oedema mediated by other inflammatory factors, such as histamine and bradykinin. It also enhances its own synthesis and induces production of IL-6 in macrophages. Moreover, PGE2 also plays a role in inducing immune tolerance in the intestine, not mediated by IL-10 or T regulatory cells (Stenson, 2014). Interestingly, prostaglandin D2 (PGD2), displays a strong, purely anti-inflammatory effect (Stenson, 2014) acting through two types of prostaglandin D2 receptors (DP), DP1 and DP2, the latter being identified as a member of the chemokine receptor family (Félezou et al., 2011). Can also be dehydrated to produce prostaglandin J2 (PGJ2), involved in the differentiation of adipocytes by activation of PPAR γ nuclear receptor (Félezou et al., 2011).

Besides PGs, the main AA metabolites are thromboxane A2 (Tx_A2), leukotriene B4 (LTB₄) and LXs. Tx_A2 mainly promotes

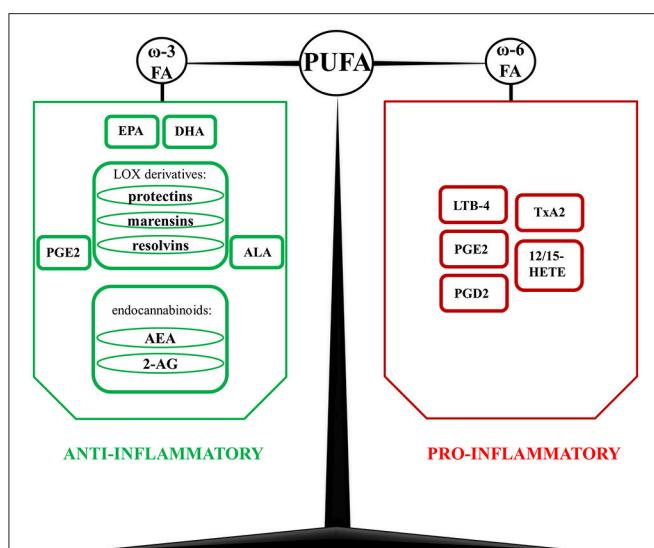


FIGURE 1 | Derivatives of polyunsaturated fatty acids (PUFA) with anti- and pro-inflammatory properties. In contrast to n-3 PUFA-derived mediators, which are mostly considered as anti-inflammatory, n-6 PUFA derivatives promote inflammatory response. However, one of the unique metabolites of n-6 PUFA are lipoxins (Lx)- LxB4 and LxB5, which display a wide spectrum of anti-inflammatory and pro-resolution actions. AA, arachidonic acid; AEA, N-arachidonylethanolamide, anandamide; ALA, alpha-linoleic acid; 2-AG, 2-arachidonoylglycerol; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FA, fatty acid; LOX, lipoxygenases; LTB-4, leukotriene B-4; LxA4, lipoxin A4; LxB4, lipoxin B4; PGD2, prostaglandin D2; PGE2, prostaglandin E2; Tx2, thromboxane A2; 12/15 HETE, 12/15 hydroxyeicosatetraenoic acid.

platelet aggregation and vasoconstriction but is also reportedly involved in allergies, modulation of acquired immunity, atherogenesis, neovascularization, and metastasis of cancer cells (Féltou et al., 2011). LTB4, in turn, recruits neutrophils, promotes vascular leakage, and regulates epithelial barrier function (Cornejo-García et al., 2016), whereas LXs diminish the inflammatory process by limiting leukocyte infiltration (Maderna and Godson, 2009). This shows that n-6 derivatives can both exacerbate and resolve inflammation. In contrast, n-3 PUFAs are considered purely anti-inflammatory FAs, because they serve as substrates for PLA2 in lipid membranes. This causes reduction in AA-derived pro-inflammatory cytokines and suppresses the inflammatory process (Okada-Iwabu et al., 2013).

Nevertheless, PUFAs metabolites gather increasing recognition as prime drivers of inflammation and its resolution. For example, 12-lipoxygenase (12-LO) plays a role in atherosclerosis and adipose tissue inflammation in obesity (Masoodi et al., 2015). In a rat model of obesity, Chakrabarti et al. (2011) reported increased expression of 5- and 12-LO in visceral adipocytes, along with increased amounts of their products: 12- and 5- HETE, and LTB-4.

In addition to pro- and anti-inflammatory properties of PUFAs derivatives, they can also exhibit pro-resolution effects. Contrary to well-established beliefs, resolution of inflammation is also an active process rather than simple cessation of inflammatory stimuli (Shinohara and Serhan, 2016). It is mediated by specialized pro-resolving molecules

(LXs, Rvs, protectins, and maresins) derived from n-3 PUFAs, mainly EPA and DHA (Serhan, 2014). All these molecules mitigate neutrophil influx, stimulate phagocytosis and enhance efferocytosis of cellular debris. Additionally, they also exert class-specific influence on inflammation processes; they counteract eicosanoids, chemokines, and cytokines, regulate specific micro RNAs and act in a receptor-specific manner on human neutrophils and macrophages. In line, administration of RvD1 and 17-hydroxy-DHA to obese diabetic mice not only improved insulin sensitivity but also reduced levels of inflammatory cytokines in WAT and decreased its infiltration by macrophages. Importantly, pro-resolving molecules stimulate heme oxygenase system to produce low concentrations of carbon oxide which acts protectively for tissues (Shinohara and Serhan, 2016).

Another example of signaling molecules involved in inflammatory pathways are endocannabinoids, a heterogeneous group of lipid mediators acting on classical and non-classical cannabinoid (CB) receptors. Two very important representatives of these ligands are N-arachidonylethanolamine named anandamide (AEA), and 2-arachidonoylglycerol (2-AG), both derivatives of AA (Alhouayek and Muccioli, 2012). Both suppress mast cells activation, decrease the production of macrophages and pro-inflammatory cytokines, and modulate T-cell helper activity. Moreover, endocannabinoids play an important role in GI-related inflammation. In line, the activation of classical CB1 and CB2 receptors reduces inflammation-induced hypermotility and attenuates visceral hypersensitivity (Sanso et al., 2006). In colonic cell lines, endocannabinoids promote wound closure, thus becoming an attractive target for treating IBD-related lesions (Alhouayek and Muccioli, 2012).

Overall, PUFAs offer many ways to modulate inflammation. They have also been linked with IBD on an epidemiological level. Analysis of the European Prospective Investigation of Cancer (EPIC)-Norfolk cohort ($N = 25639$) found a positive association of dietary AA intake with ulcerative colitis (UC) incidence and a protective effect of oleic acid intake (de Silva et al., 2014).

PUFAS IN INFLAMMATORY BOWEL DISEASES

Inflammation is a biological response that occurs in many GI disorders and simultaneously underlies both “classical” diseases, such as non-alcoholic fatty liver disease (NAFLD) and non-specific IBD including UC and Crohn’s disease (CD). NAFLD is characterized by a cryptic low-grade inflammation process affecting the liver, with concomitant features of metabolic syndrome: obesity, dyslipidemia, insulin resistance and hypertension (Xu et al., 2015; Yki-järvinen, 2015). NAFLD affects patient’s lifespan by elevating the risk of cardiovascular disease (CVD) (Xu et al., 2015; Yki-järvinen, 2015). The symptoms of NAFLD are usually scarce and the treatment is mainly symptomatic—it includes losing weight, increase in physical activity and diet modifications (Yki-järvinen, 2015). n-3 PUFAs are considered a relevant supplementation in patients with hypertriglyceridemia and many studies suggest the important role of PUFAs in NAFLD pathophysiology (Gambino et al., 2016).

However, there is no consensus from human trials for their systematic use as a drug treatment (Boyratz, 2015; Nakamoto et al., 2016). Nevertheless, this aspect is not the subject of this review; for detailed information please see (Boyratz, 2015; Yki-järvinen, 2015; Musso et al., 2016).

In contrast to NAFLD, the symptoms of UC and CD are very pronounced and significantly affect patient's quality of life (Sobczak et al., 2014). The most common symptoms are diarrhea, fever and fatigue, abdominal pain and cramping, intestinal bleeding, reduced appetite and unintended weight loss. Their core is a constant, low-grade inflammation with temporary exacerbations. Both UC and CD affect 37 adults and 5–11 children per 100 000 annually, leading to increased mortality (Ananthakrishnan, 2015; Ye et al., 2015). The pathogenesis of IBD is not completely defined, but recently the spotlight is on microbiota-stimulated immunoreactivity and adipose tissue hormonal dysregulation (Kostic et al., 2014; Schabeck and Haller, 2015). What is more important, the currently available treatment includes 5-aminosalicylates, steroids, biological therapy and surgical procedures—all aggressive and with significant side effects (George et al., 2015; Khanna and Feagan, 2015; Meyer et al., 2015). Thus, it is crucial to seek new agents that could influence the clinical course of IBD and alleviate or prevent exacerbations. In this field, PUFAs are recognized as promising agents.

Animal Studies

The impact of PUFAs on colitis-associated inflammation has been extensively studied in animal models (Huang et al., 2016). To date, oral administration of dextran sulfate sodium (DSS) and intracolonic administration of 2,4,6-trinitrobenzenesulfonic acid (TNBS) dissolved in ethanol are two most widely used models to induce UC and CD, respectively (Scheiffele and Fuss, 2002; Chassaing et al., 2014; Salaga et al., 2014a,c; Salaga et al., 2016). The main difference between these two models of colitis is that DSS directly damages gut epithelial cells of the basal crypts and disrupts the integrity of the mucosal barrier, whereas in TNBS model, ethanol breaks mucosal barrier and therefore allows TNBS to haptenize colonic proteins in order to stimulate the immune response.

One of the first targets in fighting inflammation may be conjugated LA (CLA) (Viladomiu et al., 2013). The mixture of LA isomers has been categorized by Food and Drug Administration (FDA) as “generally recognized as safe” (GRAS) and can be used as a supplement. In pig models of colitis, CLA was proved to downregulate inflammation by lowering serum levels of tumor necrosis factor alpha (TNF- α) and nuclear factor κ B (NF- κ B) while increasing levels of transforming growth factor β (TGF- β) and upregulating the expression of peroxisome proliferator-activated receptor γ (PPAR- γ) (Hontecillas et al., 2002). These findings were confirmed in DSS- and CD4-induced colitis in mice (Bassaganya-Riera et al., 2004).

Another idea of curbing colonic inflammation is to utilize n-3 PUFAs. This is based on the observation that transgenic mice with inborn ability to convert n-6 PUFA to n-3 PUFA were protected from experimental colitis. Furthermore, in a rat model of TNBS-induced colitis, diet supplemented with ALA

decreased the number of lesions, normalized colon induced NO synthase (iNOS) and COX-2 expression and lowered levels of TNF- α and leukotriene B-4 (LTB-4) to baseline (Hassan et al., 2010). However, it had no effect on PGE2 level and, more importantly, it did not lower the disease inflammation score (Hassan et al., 2010). In TNBS model, ALA supplementation was also proved to decrease the levels of intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion protein 1 (VCAM-1) and vascular endothelial growth factor receptor 2 (VEGFR-2), thus suppressing the angiogenic component of inflammation (Ibrahim et al., 2012).

Other studies concentrate on n-6 to n-3 PUFAs ratio rather than one particular FA. An increase in western high-fat diet consumption worldwide results in dramatic changes in this ratio of FAs from 2:1 in the past, to 10:1 nowadays (Tyagi et al., 2012). In DSS-induced colitis replacement of dietary LA with ALA, and therefore decreasing LA/ALA ratio to 2:1, reduced the severity of colitis and significantly mitigated inflammation, when measured indirectly as shortening of the colon (Tyagi et al., 2012). This change was probably caused by decreased neutrophil infiltration into the colonic tissue, probed by the decrease in myeloperoxidase (MPO) and colonic alkaline phosphatase activity. Supplementation with ALA also prevented the rise in TNF- α and IL-1 β levels, when compared to the control group. Moreover, this diet also partially decreased plasma NO and completely prevented the rise in radical stress in colonic tissue. A reduction in histological score of inflammation was also observed (Tyagi et al., 2012).

Another way to modulate inflammation is to use specific PUFAs metabolites rather than primary FAs. Given the fact that adipose tissue inflammation plays an important role in CD (Michalak et al., 2016), these metabolites may be actively used to suppress existing inflammation or prolong remission in those patients. EPA- and DHA-derived specialized pro-resolving mediators are prime candidates for animal and clinical trials.

A double role of PGE2 in inflammation has also been demonstrated in animals. In DSS-induced colitis PGE2 enhanced proliferation and epithelial healing, whereas in TNBS-induced colitis it promoted Th17 differentiation pathway in lymphocytes and thus exacerbated inflammation (Stenson, 2014). These seemingly inconsistent actions of PGE2 may result from profound biological differences in DSS- and TNBS-induced colitis.

Another group of PUFAs derivatives tested in animal models of IBD are agents targeting the endocannabinoid system. Firstly, it has been demonstrated that CB1- and CB2-knockout mice show increased susceptibility to DSS-induced colitis (Alhouayek and Muccioli, 2012). In line, administration of cannabinoids or inhibitors of cannabinoid degradation decreased intestinal motility, peristalsis and colonic propulsion in rodents in a CB1-dependant manner (Alhouayek and Muccioli, 2012). Salaga et al. (2014c) thoroughly tested fatty acid amide hydrolase (FAAH) inhibitor in TNBS- and DSS-induced colitis. In the TNBS model, orally administered FAAH inhibitor exerted potent anti-inflammatory effect which improved total histological score and decreased tissue MPO activity. Such changes were not observed

in the DSS model, probably due to different mechanisms involved in the inflammatory process (Salaga et al., 2014b,c).

When assessing possible use of PUFAs in therapy, it may be worth to also consider alternative ways of delivery. One of the newest methods includes the encapsulation of n-3 PUFA in liposomes. Notably, by adding radioactive, fluorescent or superparamagnetic agents it is possible to track the drug distribution in the organism and better study its local actions (Calle et al., 2015). Liposomes were already tested in DSS-induced colitis medium, which serves to deliver glucocorticosteroids; unfortunately, this therapy led to worsening of fecal blood loss (Crielaard et al., 2011). However, when imbued with n-3 PUFA, liposomes demonstrated a clear anti-inflammatory effect in DSS-induced colitis in mice and exerted important anti-tumoral effects against glioma. Imaging techniques confirmed the presence of magnetoliposomes in inflamed regions, which opens up many possibilities for targeted, image-guided delivery of medical agents, which may find application in IBD therapy (Calle et al., 2015).

The last study that needs to be mentioned in this chapter may be considered as a bridge toward clinical trials. Meister and Ghosh (2005) incubated biopsies from IBD patients with fish oil and found reduced inflammation manifested by a rise in IL-1 α /IL-1 β ratio in tissues obtained from UC, but not from CD patients. These outcomes indicate differences in a diet that should be taken into consideration while composing nutritional therapy for UC or CD patients.

Clinical Application

Despite promising results in animal studies, translation of PUFA-based interventions into humans is difficult. One of the attempts was to utilize PUFAs against chronic low-grade inflammation in obesity. It has been demonstrated that obesogenic diet concomitant with EPA/DHA supplementation resulted in a more favorable metabolic profile and normalized the levels of endogenous Rvs and protectins, attenuated adipose tissue inflammation and improved insulin sensitivity (Minihane et al., 2015). Another study proved that high dose of long-chain n-3 PUFA, delivered to severely obese non-diabetic patients, increased secretion of anti-inflammatory eicosanoids and decreased expression of inflammatory genes in subcutaneous adipose tissue. Finally, a systematic review by Rangel-Huerta et al. (2012) reported that n-3 PUFAs supplementation affects inflammatory biomarkers in cardiovascular disease and chronic and acute conditions. However, its effects varied in disease-specific manner.

Polyunsaturated fatty acids (PUFAs) have also been evaluated as a factor involved in the course of IBD. Total dietary intake of PUFAs has been shown to positively correlate with the risk of UC, although with marginal significance and without distinguishing between n-3 and n-6 PUFA (Hart et al., 2008). A more detailed big cohort study demonstrated that total dietary n-3 PUFAs, particularly EPA and DHA, protected from UC development in patients older than 45 years (John et al., 2010). Finally, a large prospective cohort study in women also proved the association between higher intake of dietary long chain n-3 PUFAs and a reduced risk of UC (Ananthakrishnan et al., 2014).

Noteworthy, the latest case-control trial reported that dietary intake of fats, especially PUFAs, is associated with increased risk of UC; however, no positive association was seen specifically for n-3 PUFAs intake (Rashvand et al., 2015). Altogether, the above-mentioned studies suggest that n-6 PUFAs facilitate the development of UC while n-3 PUFAs possibly prevent it. This leads to assumption that n-3 PUFAs could be utilized either in inducing or prolonging remission in IBD. Important clinical trials together with summary meta-analysis have been listed in **Table 1**.

In patients with an active form of IBD, administration of duodenal seal oil significantly reduced disease activity index, normalized n-3/n-6 PUFAs ratio and alleviated pain, especially joint-related pain (Arslan et al., 2002; Bjørkkjær et al., 2006). Worth mentioning, in patients with mild to moderate CD, the supplementation with CLA suppressed the ability of T-cells to produce TNF- α , IFN- γ , and IL-17, which decreased disease activity and improved the quality of life of patients (Viladomiu et al., 2013).

When joined with antioxidants, n-3 PUFAs supplementation altered the composition and function of peripheral blood mononuclear cells (PBMCs). Consequently, when stimulated with mitogens, PBMCs produced less IFN- γ , but when stimulated by lipopolysaccharide generated less PGE2 (Treble et al., 2004).

Possible clinical application of PUFAs has been assessed by a few systematic reviews and meta-analyses. The study by Turner et al. (2011) evaluated the effectiveness of n-3 PUFAs in maintaining remission in patients with IBD. They found moderate but significant beneficial effect of omega FA supplementation in patients with CD (relative risk 0.77; 95% confidence interval 0.61–0.98) (Turner et al., 2011). However, conclusions were drawn from only 6 trials comprising 1039 patients altogether, with probably high heterogeneity and publication bias. Analysis of 3 trials on supplementation in 138 patients with UC did not report any significant effect. The same or similar analysis was probably republished in 2014 concluding that n-3 PUFAs may be ineffective for maintenance of remission in CD (Lev-Tzion et al., 2014).

Another systematic review published in 2012 confirmed ineffectiveness of n-3 PUFA supplementation in remission phase of UC or CD. Nonetheless, the supplementation showed many beneficial effects in patients with active phase of UC. Unfortunately, due to high variability of studies and high number of parameters assessed, these results could not be unified (Cabré et al., 2012).

The reason for the shortcomings of the intervention trials may be related to different doses applied in each study and complex interfering metabolic reactions that PUFAs undergo before exerting their biological effects. The key to unlock its power is to utilize alternative, effective ways of drug delivery, such as liposomes. This would allow to evaluate the effects of EPA or DHA action in specific inflammatory environment and thus open up endless possibilities of using particular n-3 PUFA metabolites to achieve more specific effects.

n-3 PUFAs supplementation may also be an attractive option in pediatric patients. In children with CD receiving mesalazine,

TABLE 1 | Clinical trials investigating the use of n-3 PUFAs in IBD.

Condition	Study design	Participants	Intervention	Outcomes	References
IBD-related joint pain	RCT (10 d. treatment period)	17 (9 CD, 10 UC)	Seal oil ($n = 10$) vs. soy oil ($n = 9$) 10 ml 3x daily, administered duodenally	Reduced bodily pain	Bjørkkjær et al., 2006
Active CD	Open label (12 weeks of treatment)	13	CLA 6 g/d	Drop in CD activity index, increase in quality of life	Bassaganya-Riera et al., 2004
CD (patients with currently or recently raised inflammatory markers)	RCT (24 weeks of treatment)	61	EPA and DHA (+ antioxidants) 2.7 g/d for ($n = 31$), Placebo ($n = 30$)	reduction in IFN γ production by mitogen-stimulated PBMC	Treble et al., 2004
Pediatric patients with CD in remission	Double-blind RCT (12 months of treatment)	38	5 ASA (50 mg/kg/d) + EPA + DHA (3 g/d) vs. 5-ASA (50 mg/kg/d) + olive oil (3 g/d)	Lower relapse rate after 1 year	Romano et al., 2005
Active CD	Double-blind RTC (9 weeks of treatment)	31	Nutritional treatment with an isocaloric diet Impact Powder (3 g omega-3 FA, 11.4 g L-Arginine, and 1.2 g RNA per day) Control formula: Nutritional supplement with 7.8 g linoleic acid per day (all patients received systemic steroid therapy)	Significant decrease in CDAI and C-reactive protein in both groups (no difference)	Nielsen et al., 2005
Active UC	Double-blind RTC (6 months of treatment)	121 (86 completed the protocol)	Nutritional supplement with <2.5 g EPA and <1.0 g DHA per day vs. supplement based on sucrose alone (Steroids and 5-ASA allowed, their doses adjusted to clinical response)	Similar clinical improvement, but faster reduction in steroid dose in active group	Seinder et al., 2005
Newly-diagnosed pediatric patients with CD	Double-blind RTC (6 weeks of treatment)	41	Polymeric diet with 1.5% of energy as ALA and 3% as LA vs. elemental diet with 0.4% of energy ALA and 5.4% as LA (No other active therapy for CD allowed)	Similar remission rate between the groups	Grogan et al., 2012
CD patients immediately after reaching remission	Double-blind RTC (58 weeks of treatment)	375	Enteric coated capsules (2–2.4 g EPA/day and 0.6–1 g DHA/day) vs. placebo capsules	47.8% relapse rate vs. 48.8% in placebo	Feagan et al., 2008
CD patients in remission	Double-blind RTC (52 weeks of treatment)	363	Enteric coated capsules (2–2.4 g EPA/day and 0.6–1 g DHA/day) vs. placebo capsules	31.6% relapse rate vs. 35.7% in placebo group	Feagan et al., 2008
CD patients in remission	Metaanalysis of RCT (at least 6 months of treatment)	1039	Fish oil or n-3 PUFA Predefined doses	Risk of relapse - RR 0.77, 95% CI 0.61 to 0.98	Turner et al., 2011 Lev-Tzion et al., 2014
					Cabré et al., 2012

ASA, amino salicylic acid; CD, Crohn disease; CLA, conjugated linoleic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; IBD, inflammatory bowel disease; IFN γ , interferon-gamma; PUFA, polyunsaturated fatty acid; RCT, randomized controlled trial.

supplementation with triglyceride form of n-3 PUFAs in gastro-resistant capsules, containing 400 mg/g of EPA and 200 mg/g of DHA, significantly lowered the relapse rate within 1-year observation with respect to patients supplemented with olive oil placebo capsules (Romano et al., 2005).

Currently, there is no strong evidence that oral n-3 PUFAs supplementation significantly changes the course of either CD or UC, enabling clinicians to drop steroid or 5-ASA therapy. However, n-3 PUFAs may be valuable additions to standard treatment, but more uniformly-devised studies are warranted.

PUFAs IN IRRITABLE BOWEL SYNDROME

Dietary fat also plays an important role in FGIDs (Feinle-Bisset and Azpiroz, 2013). This heterogenous group is generally

defined as conditions with a variable combination of chronic or recurrent GI symptoms. Irritable bowel syndrome (IBS) is the most prevalent FGID noted in the general population, which constitutes 25–50% of gastroenterology outpatients' workload (Wilson et al., 2004). According to the Rome IV criteria, a symptom-based classification system, the clinical symptoms of IBS include abdominal pain, bloating, stool irregularities with concomitant psychiatric and somatic comorbidities (Drossman et al., 2010; Palsson et al., 2016). Depending on the pattern of symptoms, IBS may be classified as diarrhea-predominant IBS (IBS-D), constipation-predominant IBS (IBS-C) or alternating IBS (IBS-A). Among others, one of the putative theories that underlie IBS development is the imbalance within the brain-gut axis, made up of the enteric nervous system (ENS), central nervous system (CNS), and the hypothalamo-pituitary-adrenal

axis (Fichna and Storr, 2012). Such imbalance may be triggered by early life stress, which predisposes to develop stress related disorders, including IBS later in life. A number of studies reported low-grade inflammation as another mechanism implicated in IBS, in which higher infiltration of cytokines e.g., IL-4, IL-6, or TNF- α , pro-inflammatory mediators and mast cells in the colonic mucosa impairs the tight junctions complexity and thus exacerbates IBS symptoms (Camilleri et al., 2012).

The management of IBS requires multimodal approach, including pharmacological, psychological, as well as complementary and alternative medicines. Approved and investigated therapeutics are summarized in **Table 2**.

Currently, dietary interventions are gaining much attention (Böhn et al., 2013; Cuomo et al., 2014). In line, a diet excluding foods high in short-chain carbohydrates termed FODMAPs (Fermentable Oligo-, Di- and Monosaccharides and Polyols) has proven its effectiveness in alleviating IBS symptoms. More recent evidence also indicates the usefulness of dietary lipids (Caldarella et al., 2005; Solakivi et al., 2011; Halmos et al., 2014).

Animal Studies

Visceral hypersensitivity can be mimicked in animal model of IBS. Rats, when separated as neonates from their mother and exposed in later life to acute stress (e.g., in the water avoidance test) display hypersensitivity to mechanical colorectal distension that can be measured electromyographically, and manifest increase in visceral pain. In these animals, elevated levels of pro-inflammatory PUFAs were reported (Clarke et al., 2009). However, in maternally separated rats, dietary supplementation with fish oil reduces neither the response to colon distension nor the level of pro-inflammatory cytokines in colonic tissue (van Diest et al., 2015). Another study combined the supplementation with CLA and probiotics (*B. breve* DPC6330) (Barrett et al., 2012). Groups of 15 rats were either separated from their mothers or not, and fed with probiotic, probiotic together with LA and ALA, or placebo. The supplementation provided significant changes in lipid composition in the rats' serum, colonic tissue and prefrontal cortex, but failed to clearly change the colonic hypersensitivity measured by colon distension (Barrett et al., 2012). Of note, the use of human intestine bacteria (*B. breve*, strain DPC6330) affected the FA metabolism in maternally separated rats to a greater extent than in non-separated animals. The study thus supports the hypothesis that changes in the gut microbiota alter host lipid (e.g., palmitoleic acid, DHA or propionate) composition, and are responsible for the occurrence of IBS symptoms.

Clinical Application

Polyunsaturated fatty acids (PUFAs) can modulate mast cells activity and further mitigate visceral hypersensitivity, a prime feature of IBS. This leads to the question whether supplementing PUFAs could help change the clinical course of IBS and improve patients quality of life. The study by Clarke et al. (2010) reported significant increase in the level of AA and its metabolites in serum samples from IBS patients, compared with healthy controls. An elevated concentration of LTB4 was found across all IBS subtypes, whereas PGE2 showed type-specific elevation

associated solely with IBS-D. It should be noted that PGE2 is able to cross the blood brain barrier, which links FA metabolism and inflammation to brain-gut axis, and therefore may be implicated in the course of IBS (Clarke et al., 2010). There was no correlation between the plasma level of AA and symptom severity. Surprisingly, the same study also found increased concentrations of n-3 PUFAs in serum of IBS patients, however the underlying cause of this augmentation remains unexplained (Clarke et al., 2010).

Polyunsaturated fatty acids (PUFAs) metabolites can bind TRP ion channels, such as transient receptor potential vanilloid 1 (TRPV1), TRPV4, and TRP subfamily A receptor 1 (TRPA1), and signal to sensory neurons to evoke hypersensitivity symptoms seen in IBS. The levels of TRP agonists differ between colon biopsies from IBS patients and healthy individuals. Cenac et al. (2015) demonstrated an increased concentration of a TRPV4 agonist, 5,6-epoxyeicosatrienoic acid (5,6-EET), but not TRPA1 and TRPV4 agonists, in colonic biopsies from IBS-D patients, when compared with control group. This augmentation in 5,6-EET was positively correlated to pain severity and bloating. Biopsies from both IBS-C and IBS-D patients showed elevated levels of PGE-2 and decreased concentration of a TRPV1 agonist. Modulating TRP activity by PUFAs or PUFAs-based ligands may potentiate the production of TRPV4 and its agonist and thus modulate visceral sensation in IBS.

The same study proved that intracolonic administration of supernatants from IBS-D patients' colonic biopsies to mice led to development of hypersensitivity (manifesting as allodynia and hyperalgesia) and increased 5,6-EET, PGE-2 and 15-HETE levels in mouse colonic tissues (Cenac et al., 2015). These changes were prevented by using TRPV4 knockouts or pretreating mice with TRPV4 inhibitors. Finally, the study proved that 5,6-EET stimulates colonic neurons by acting on their TRPV4; the same effect was achieved later with bioptic supernatants from IBS-D patients and hypersensitive mice. The study highlights the potential of PUFA-based therapies in targeting TRPV4 channels.

Currently, there are no clinical trials assessing effects of PUFAs supplementation on clinical course of IBS. Such studies are warranted given the recently-revealed inflammatory factor in IBS.

Apart from supplementation, PUFAs may also serve as base for devising new biologically active agents. Of note, lubiprostone, a PGE1 derivative, has been registered in the treatment of IBS-C and chronic idiopathic constipation (CIC). It activates type 2 chloride channels in apical membrane of intestinal epithelium and thus increases fluid secretion in the GI tract (Li et al., 2016). Lubiprostone also promotes repair of epithelial barrier (Cuppoletti et al., 2012). Most recent metaanalysis by Li et al. (2016) confirmed the effectiveness and safety of lubiprostone in diminishing the severity of constipation and improving stool consistency, degree of abdominal pain and discomfort. Compared to alternative therapies, patients on lubiprostone have improved health-related quality of life, symptom control, reduced symptoms severity and are more satisfied with their therapy (Solem et al., 2016).

TABLE 2 | Currently available and emerging pharmacological treatment options for IBS-C and IBS-D (Sweetser et al., 2009; Chey et al., 2011; Mozaffari et al., 2013; Mosinska et al., 2015, 2016; Shailubhai et al., 2015).

Drug class	Generic name	Mechanism of action	Indication	ClinicalTrials.gov Identifier
BA modulators	Elobixibat	IBAT inhibitor	IBS-C	NCT01833065
	NaCDC	BA analog	IBS-C	NCT00912301
Prokinetics	Itopride	ACh esterase inhibitor; D2 receptor antagonist	IBS-C	NCT01027260
	Relelopripride	5-HT4 partial agonist	IBS-C	NCT02082457
	Mosapride	5-HT4 agonist	IBS-C	NCT01505777
	Tegaserod			approved
	Velusetrag			NCT00391820
	Renzapride	5HT4 agonist and 5HT2b and 5HT3 antagonist	IBS-C	NCT00268879
	Pumosetrag	5-HT3 agonist	IBS-C	not available
	Alosetron	5-HT3 antagonist	IBS-D	NCT00067457
	Ondansetron			not available
	Ramosetron			approved in Japan, Korea and Thailand
PAMORA	Loperamide	μ -opioid receptor agonist	IBS-C	approved
	Eluxadoline	μ -opioid receptor agonist, δ -opioid receptor antagonist	IBS-D	approved
Secretagogue	Dolcanotide	GC-C agonist	IBS-C	not available
	Linaclootide			approved in USA, EU
	Plecanatide			NCT01722318
	Lubiprostone	CIC2 activator	IBS-C	approved in USA
AGENTS WITH OTHER MECHANISMS OF ACTION				
	TC6499	Alpha ₃ beta ₄ NNRs modulator	IBS-C	NCT01149200
	Taranabant	CB1 receptor agonist	IBS-C	not available
	Crofelemer	Chloride channel inhibitor	IBS-D	NCT00461526
	Pexacerfont	CRF receptor antagonist	IBS-D	NCT00399438
	ROSE-010	GLP-1 Analog	IBS-C	NCT01056107
	TU-100	Kampo medicine	IBS-C	NCT01890837
	Asimadoline	κ - opioid agonist	IBS-D	NCT01100684
	Ibudant	NK2 antagonist	IBS-D	NCT01303224
	NHE3 inhibitor	Tenapanor	IBS-C	NCT02727751

PUFAs IN COLORECTAL CANCER

Colorectal cancer (CRC) is the third most common cancer both in men and women, and second in cancer-related deaths (Yan et al., 2016). The risk of CRC is strongly affected by environmental modifiable factors, such as exercise or dietary habits (Teixeira et al., 2014). Fatty acid intake with focus on n-3 PUFAs is one of the main candidate factors affecting CRC incidence and clinical course. It is generally accepted that n-3 PUFAs are associated with protection from CRC, and n-6 PUFAs with its promotion. However, there is a limited number of studies undermining this paradigm and suggesting a more complicated role of PUFAs (Zhang et al., 2015b).

Primary Prevention

The primary prevention studies yield mixed results. A large 2 \times 2 factorial study showed no association between the therapy with insulin glargine or supplementation with n-3 PUFAs and CRC incidence rate (Bordeleau et al., 2014). Similarly, metaanalysis by Shen et al. (2012) did not reveal any significant impact of n-3

PUFAs intake on CRC risk in general. Importantly, the subgroup analysis revealed a significantly reduced risk of CRC among men but this aspect needs further investigation (Shen et al., 2012). In contrast, metaanalyses of prospective cohort studies that investigated the impact of fish consumption or n-3 FAs on CRC prevalence and mortality, provided evidence that fish intake (at least once per week) inhibits CRC carcinogenesis and can lower risk of CRC for about 4% (Park et al., 2013).

On the other hand, PUFAs can still be used as adjuvant agents in therapy or as prevention in high-risk groups, for example in patients with familial adenomatous polyposis, or as a secondary prevention.

Colon Cancer Cell Lines

In human CRC cell lines, n-3 PUFAs modulate both p53-dependent and independent pathways, inhibit COX2 pathway, suppress NF- κ B and downregulate Bcl-2 expression suggesting its anti-proliferative and pro-apoptotic properties (Collett et al., 2001; Sala-Vila et al., 2010; Eltweri et al., 2016). The action of PUFAs on CRC cells is mediated by mitochondrial-dependent

pathways and varies with a degree of cell differentiation. Zhang et al. (2015a) demonstrated that both n-3 and n-6 PUFAs induced apoptosis in LoVo and RKO CRC cell lines when applied at concentrations above 120 μ M (tested at 0–200 μ M). The pro-apoptotic effect of PUFAs is greater in semi-differentiated RKO line compared with undifferentiated LoVo cells. Furthermore, DHA and to lesser extent EPA, enhanced the effects of radiotherapy in two human CRC cell lines (Cai et al., 2014).

Another study by De Carlo et al. (2013) investigated cell-specific action of n-3 PUFAs on differentiated tumor cells and cancer stem like cells (CSLC). The treatment decreased the expression of CD133 in CD133+ colon CSLCs, what suggests a differentiation-stimulating effect (De Carlo et al., 2013). Moreover, EPA also sensitized CRC cells to chemotherapeutics, 5-fluorouracil, and oxaliplatin. An increased sensitivity to 5-fluorouracil was found in CSLCs (De Carlo et al., 2013). Another study demonstrated that EPA coupled with these two cytostatics inhibits cell growth, colonosphere formation, sphere-forming frequency and increases the sphere disintegration in otherwise resistant cells (Vasudevan et al., 2014). Similar synergistic interaction of n-3 PUFAs with chemotherapeutic agents was also supported by other authors; for more information, please see: (Jordan and Stein, 2003; Cai et al., 2014; Eltweri et al., 2016). Currently, 5-fluorouracil and oxaliplatin are first line of treatment in CRC and EPA might be considered a valuable addition. However, clinical trials are needed to determine if the sensitizing effect of EPA is present and clinically relevant *in vivo*.

Additionally, PUFAs also modulate the inflammatory environment in CRC patients. The serum level of n-6 C20:4 PUFAs show positive association with IL-6 and inverse with levels of metalloproteinase-7. In line, there is a positive correlation between C22:5 n-3 PUFAs and IFN- γ , inverse association between C20:5 n-3 PUFA and metalloproteinase-2 and C22:6 n-3 PUFA and levels of IL-8 and metalloproteinase-9 (Jia et al., 2016). This suggests that nutritional intervention may influence the inflammatory signaling and clinical course of CRC.

Animal Models

The *in vivo* results are consistent with *in vitro* studies. In mice and rats, EPA and DHA supplementation reduces cell proliferation and promotes apoptosis (Gutt et al., 2007). This inhibits liver metastasis and leads to decrease in the tumor volume. Importantly, hybrid liposomes (HLs) including PUFAs have demonstrated an inhibitory effect on the growth of tumor cells both *in vitro* and *in vivo* (Ichihara et al., 2014). Tanaka et al. (2008) demonstrated that HLs enriched with n-3, n-6, and n-9 PUFAs inhibit the growth of tumor cells, including colon tumor (WiDr) cells *in vitro*. The highest inhibitory effect was seen in HLs enriched with n-3 PUFA DHA; this action could be attained mostly by increased peroxidation and further necrosis of the cell. However, the effect of DHA-HLs was not totally prevented by addition of antioxidant so their mechanism of action is likely to be more complicated, especially that DHA also demonstrated a pro-apoptotic effect in WiDr colon tumor cells (Tanaka et al., 2008).

More recently, Ichihara et al. reported therapeutic effects of cationic HLs on the hepatic metastases of CRC along

with apoptosis in mice. Female mice with severe combined immunodeficiency were injected intrasplenically with HCT116 cells (5.0×10^6 cells). They were then randomized for intravenous administration of liposomes, containing either L- α -dimyristoylphosphatidylcholine (DMPC) (136 mg/kg/d) or 50% molar DHA (65.7 mg/kg/d) and 50% molar DMPC. After 14 days of therapy, a significant improvement in median survival time and induction of apoptosis in liver metastases were observed (Ichihara et al., 2014).

It is possible that liposome-delivered PUFAs may exert more potent effects on cancer cells than its oral administration; however, this needs further testing and clinical trials. Currently, only orally-supplemented PUFAs are available, but their various forms differ in the efficacy of their absorption from the intestine and incorporation into cells. The EPA in the free fatty acid (EPA-FFA) form is absorbed better from small intestine than the ethyl ester or triglyceride form (Lawson and Hughes, 1988). *In vitro*, EPA-FFA induces apoptosis of HCA-7 human CRC cells by decreasing PGE2 and increasing PGE3 synthesis, what affects their subsequent action on EP4 receptor (Hawcroft et al., 2010). Alone, PGE3 acts as a partial receptor agonist but in the presence of its natural ligand, PGE2, it competes for the receptor and acts as an antagonist. Importantly, HT-29 human CRC cells with EP4 overexpression (HT-29-EP4) do not produce detectable amounts of PGE2 or PGE3 and exhibit no reaction to treatment with EPA-FFA (Hawcroft et al., 2010).

Possibly, an even better form of delivering PUFAs are monoacylglycerols. Morin et al. (2013) showed that this form can be more easily absorbed than FFA. *In vitro*, monoacylglycerol used to deliver docosapentaenoic acid (DPA), an intermediate product between EPA and DHA, to CRC cell lines showed more potent anti-proliferative and pro-apoptotic effect than either EPA or DHA. The action of DPA was also confirmed in a mouse xenograft model of CRC (Morin et al., 2013).

Gounaris et al. (2015) proposed another way to target PUFA metabolism in CRC. In a mouse model of polyposis, the administration of Zileuton (5-LO inhibitor registered for treating asthma) resulted in markedly lowered systemic and local lesion inflammation and led to a decrease in polyp burden (Gounaris et al., 2015). This warrants clinical studies of agents influencing PUFA metabolism in patients with high risk of CRC.

Clinical Application

In humans, n-3 PUFAs have been tested as adjuvants to standard therapy, components of nutritional treatment and supplements for prevention in high-risk groups. Important clinical studies from each field has been summed up in **Table 3**.

When used as an addition to the treatment protocol, oral n-3 PUFAs supplementation (600 mg/day EPA + DHA) reduced inflammation in patients receiving chemotherapy (Mocellin et al., 2013). The results from a metaanalysis by Mocellin et al. (2015) supported n-3 PUFA supplementation as a way to diminish the inflammatory state in CRC patients, either by decreasing levels of IL-6 or CRP/albumin ratio. Moreover, Silva et al. (2012) showed that fish oil supplementation (2 g of fish

TABLE 3 | Recent clinical reports on n-3 PUFA as primary prevention supplements, prospective therapeutics, adjuvants to chemotherapy, nutritional treatment, or prevention in high-risk groups.

n-3 PUFA in CRC					
Condition	Study design	Participants	Intervention	Outcomes	References
PRIMARY PREVENTION					
Patients with diabetes, impaired glucose tolerance or impaired fasting glycemia	2 × 2 RCT (median follow-up 6.2 years)	12,536	Insulin: individual regimen, n-3 PUFA: 900 mg ethyl esters daily	No effect of n-3 PUFA intervention on CRC occurrence	Bordeleau et al., 2014
ADJUVANT TO CHEMOTHERAPY					
Patients with CRC in chemotherapy	RCT (9 weeks of treatment)	11	2 g of fish oil/daily (600 mg/day EPA + DHA) vs. no supplementation	Reduced CRP/albumin ratio	Mocellin et al., 2013
NUTRITIONAL TREATMENT					
Patients with solid tumors	RCT (7 days of treatment)	38	400 ml of medical food, high in protein and leucine, enriched with fish oil and oligosaccharides vs. iso-caloric/iso-nitrogenous product	Reduced PGE2 levels	Faber et al., 2013
Patients referred for CRC elective surgery	Double-blind RCT (7 days of treatment)	138	Oral nutritional supplement (2 g EPA/day, 1 g DHA/day) vs. standard supplement	Increase in LTB5 and 5-HEPE production, decrease in LTB4 production by stimulated neutrophils, no effect on post-operative complications	Sorensen et al., 2014
CLINICAL TRIAL IN METASTATIC CRC					
Patients undergoing liver resection for CRC liver metastases	Double-blind RCT (median 30 days of treatment)	88	EPA-FFA 2 g daily vs. placebo	No difference in Ki-67 proliferative index, better overall survival	Cockbain et al., 2014
PREVENTION IN HIGH-RISK GROUPS					
Patients with FAP after colectomy	RCT (6 months of treatment)	55	EPA-FFA 500 mg twice daily vs. placebo	Reduction in polyp sizes, numbers and global polyp burden	West et al., 2010

5-HEPE, 5-hydroxy-eicosapentaenoic acid; ASA, amino salicylic acid; CD, Crohn disease; CLA, conjugated linoleic acid; CRP, C-reactive protein; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FAP, familial adenomatous polyposis; IBD, inflammatory bowel disease; IFN γ , interferon-gamma; LTB5, leukotriene B-5; PGE2, prostaglandin E2; PUFA, polyunsaturated fatty acid; RCT, randomized controlled trial.

oil, 600 mg/day EPA + DHA) prevented weight loss in patients undergoing chemotherapy, which may influence their quality of life and overall wellbeing. Additionally, another study by Faber et al. (2013) proved that supplementation of EPA (600 mg/day) with DHA (300 mg/day) in medical food to patients receiving radiotherapy effectively lowers serum levels of pro-inflammatory mediator PGE2.

In perioperative conditions, EPA (2 g/day) and DHA (1 g/day) in oral nutritional supplementation also showed anti-inflammatory effects by inducing the conversion of LTB5 to LTB4 in stimulated neutrophils (Sorensen et al., 2014). Nevertheless, the rate of surgical complications was similar between study and control group.

In phase II clinical trials, Cockbain et al. (2014) reported a direct effect of EPA-FFA intake on CRC cells. Patients undergoing liver resection for CRC liver metastases were supplemented with EPA-FFA (2 g/day) or placebo but this supplementation did not change the Ki-67, a proliferation marker index, in CRC. Nonetheless, the therapy was safe, well-tolerated, and reduced tumor vascularization and improved overall survival rate.

PUFAs as Possible Prevention in High-Risk Groups

Age and sedentary lifestyle are established risk factors for developing CRC (Bonnington and Rutter, 2016). There are also hereditary conditions, such as familial adenomatous polyposis (FAP) which are associated with high number of possibly-malignant polyps (Brosens et al., 2015). In general population, detection of pre-cancerous polyps in colonoscopy is a risk factor for the development of CRC later in life (Bonnington and Rutter, 2016). All these patients are possible target groups for intervention with PUFAs. In patients with FAP, a 6-month-long supplementation with 2 g of EPA-FFA daily reduced the number of polyps by 22.4% and their size (diameter) by 29.8%. It also prevented the rise in global polyp burden, when compared with the non-supplemented group (+0.09 vs. -0.34, difference 0.42, statistically significant) (West et al., 2010). Recently, a multicenter double-blinded, placebo-controlled trial was conducted on patients with colonoscopy-detected polyps or aberrant crypt foci (ACF), supplemented with EPA 900 mg or placebo thrice daily for 1 month (Higurashi et al., 2012). The endpoints included formation of ACF, cell-proliferative

TABLE 4 | Summary of ongoing and recently completed clinical trials.

Status; ClinicalTrials.gov Identifier	Title	Study design	Indication	Diet and intervention	Primary endpoint
Recruiting; NCT02534389	Fish oil supplement combined with neoadjuvant chemoradiation for locally advanced rectal cancer	I, R, OP	Rectal neoplasm	Omega-3 fish oil, 2.4 g of EPA + DHA vs. no intervention	Effects of daily consumption of 2.4 g of EPA + DHA for adults with rectal adenocarcinoma in neoadjuvant chemoradiation treatment on Glasgow Prognostic Score
Active, not recruiting; NCT02699047	Gastrointestinal cancer: effects of the fish oil intake on nutritional status, quality of life and immune and metabolic outcomes	I, R, DB	GI cancer, CRC, stomach cancer	Encapsulated fish oil vs. encapsulated olive oil	Change in quality of life, inflammatory response, body weight, body mass index, serum C-reactive protein, tumor markers (CEA, CA-19), serum albumin, survival and others
Active, not recruiting; NCT01661764	Fatty acid desaturase activity, fish oil and colorectal cancer prevention	I, R, DB	Adenomatous colorectal polyps	EPA and DHA vs. oleic acid	Rectal epithelial cell, proliferation, rectal epithelial cell apoptosis
Recruiting; NCT02179372	Modulation of fecal calprotectin by eicosapentaenoic free fatty acid in inflammatory bowel diseases	I, R, DB	UC, CD	EPA vs. MCFA (placebo)	Changes in FC levels
Completed; NCT02069561	Effects of eicosapentaenoic acid on molecular, metabolomics and intestinal microbiota changes, in subjects with long-standing inflammatory bowel disease	I, non-R, OP	UC	EPA vs. no intervention	Changes in RNA profiles, DNA methylation profiles, in cell proliferation and apoptosis
Completed; NCT02349594	Modulation of immune function by parenteral fish oil in patients with Crohn's disease and high inherent tumor necrosis factor-alpha production: a randomized, single blinded, cross-over study	I, R, SB	CD	Omegaven 10% vs. Intralipid 20%	Change in TNF- α production (pg/ml)

CA-19, cancer antigen 19; CD, Crohn's disease; CEA, carcinoembryonic antigen; CRC, colorectal cancer; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FC, fecal calprotectin; GI, gastrointestinal; MCFA, medium chain fatty acid; UC, ulcerative colitis.

Study design: DB, double blinded; I, interventional; non-R, non randomized; OP, open label; R, randomized; SB, single blinded.

and cell-apoptotic activity in colorectal polyps and normal mucosa. No results have been published so far; however, the trial has been registered in UMIN-CTR Search Clinical Trials as UMIN000008172.

Currently, there are also two randomized clinical trials evaluating a possible use of PUFA in patients at increased risk of CRC. The former investigates the effect of EPA and aspirin in a 2×2 factorial randomized controlled trial on colorectal adenoma (Hull et al., 2013). The target group includes patients at increased risk (55–74 y.o., >5 adenomas or >3 adenomas with at least one >10 mm diam. found at the first complete screening colonoscopy). The endpoints of the 12-month supplementation with EPA include, among others, the number of patients with one or more adenomas at surveillance colonoscopy, the number of advanced adenomas and the number of patients reclassified into intermediate risk (Current Control Trials ISRCTN05926847). The second clinical trial is conducted by Harvey Murff from Vanderbilt University (ClinicalTrials.gov Identifier: NCT01661764). It aims to recruit 150 participants with recently identified adenomatous polyps and conduct a

6-month double blind 3×2 factorial randomized controlled trial. The study will assess effects of fish oil supplementation in patients with three different polymorphisms in fatty acid desaturase 1 (FAD1) gene. FAD1 converts LA to AA and its activity determines the levels of AA in tissues. In individuals with inherently lower activity of FAD1, AA levels are substantially lower. The trial will evaluate the efficacy of fish oil supplements on rectal epithelial cell proliferation indexes (measured by Ki-67 labeling) and markers of rectal crypt apoptosis. Moreover, it will provide an insight whether the supplementation is more beneficial in patients with specified genotype. The completion date of this study is due December 2016. Our search also revealed other trials investigating the effect of PUFAs supplementation on clinical course of CRC and IBD; they are summarized in Table 4.

CONCLUSIONS

Polyunsaturated fatty acids (PUFAs) are an important group of bioactive lipids with pleiotropic effects in the organism.

They affect immunity, inflammation and motility of the GI tract. Their usefulness was proven in FGIDs, in which PGE1-derived lubiprostone effectively alleviates the hallmark symptoms of IBS-C and CIC. In IBD and CRC, the potential of PUFAs is recognized but not fully utilized. When it comes to inflammation, the spotlight is currently on n-3 PUFAs, although AA-derived endocannabinoids also provide a promising target for pharmacological interventions. In CRC, the main focus is solely on n-3 PUFAs because of their inflammation- and immune-modulating properties. EPA and DHA are potentially useful as adjuvants to chemotherapy and possibly for CRC prevention in high-risk groups.

Metabolites of PUFAs also exert potent biological effects, more specific than FA themselves and thus should be of interest to pharmaceutical industry. EPA- and DHA-derived substances like Rvs or less known families of PGs could be useful as anti-inflammatory drugs. Finally, liposome-encapsulated n-3 PUFAs not only target specifically inflammatory sites e.g., IBD-related

lesions or tumor mass, but can also be tracked in the human body. These modern drug delivery methods may be the key to unlocking true medical potential of PUFAs.

AUTHOR CONTRIBUTIONS

PM and JF provided the overall concept and framework of the manuscript. AM and PM researched and identified appropriate articles, and participated in writing the manuscript. AM, PM, and JF revised the manuscript. All authors approved the final version of the manuscript.

ACKNOWLEDGMENTS

Supported by the Medical University of Lodz (503/1-156-04/503-01 to JF) and National Science Center (UMO-2013/11/B/NZ7/01301 and UMO-2014/13/B/NZ4/01179 to JF, and 2016/21/N/NZ5/01932 to PM).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Chinese Herbal Medicines Attenuate Acute Pancreatitis: Pharmacological Activities and Mechanisms

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OPEN ACCESS

Edited by:

Ralf Weiskirchen,
RWTH Aachen University, Germany

Reviewed by:

Aftab Ahmad,
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USA

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University of Padua, Italy

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Specialty section:

This article was submitted to
Gastrointestinal and Hepatic
Pharmacology,
a section of the journal
Frontiers in Pharmacology

Received: 18 January 2017

Accepted: 06 April 2017

Published: 25 April 2017

Citation:

Xiang H, Zhang Q, Qi B, Tao X, Xia S, Song H, Qu J and Shang D (2017) Chinese Herbal Medicines Attenuate Acute Pancreatitis: Pharmacological Activities and Mechanisms. *Front. Pharmacol.* 8:216. doi: 10.3389/fphar.2017.00216

Acute pancreatitis (AP) is a commonly occurring gastrointestinal disorder. An increase in the annual incidence of AP has been observed, and it causes acute hospitalization and high mortality. The diagnosis and treatment guidelines for AP recommend conservative medical treatments focused on reducing pancreatic secretion and secondary injury, as a primary therapeutic approach. Unfortunately, the existing treatment options have limited impact on the incidence and severity of AP due to the complex and multifaceted pathological process of this disease. In recent decades, Chinese herbal medicines (CHMs) have been used as efficient therapeutic agents to attenuate AP in Asian countries. Despite early cell culture, animal models, and clinical trials, CHMs are capable of interacting with numerous molecular targets participating in the pathogenesis of AP; however, comprehensive, up-to-date communication in this field is not yet available. This review focuses on the pharmacological activities of CHMs against AP *in vitro* and *in vivo* and the underlying mechanisms. A computational prediction of few selected and promising plant-derived molecules (emodin, baicalin, resveratrol, curcumin, ligustrazine, and honokiol) to target numerous proteins or networks involved in AP was initially established based on a network pharmacology simulation. Moreover, we also summarized some potential toxic natural products for pancreas in order to more safe and reasonable medication. These breakthrough findings may have important implications for innovative drug research and the future development of treatments for AP.

Keywords: acute pancreatitis, Chinese herbal formulas, natural products, pharmacological activities, toxic natural products

INTRODUCTION

Acute pancreatitis (AP) is characterized by a severe inflammatory response with the premature activation of pancreatic digestive enzymes, edema formation, cytoplasmic vacuolization, and infiltration of inflammatory cells into the pancreas (Yadav and Lowenfels, 2006; Banks et al., 2013). The most common risk for AP in adults is gallstones, which increases with age (Lowenfels et al., 2000). Excessive alcohol consumption, as the second-most common cause of AP after gallstones, has been shown to increase the risk of pancreatitis in a dose-dependent manner (Lankisch et al., 2002, 2015). The incidence of AP among subjects with long-term alcoholism is fourfold higher than

those without (Yadav et al., 2007). Other causes include duct obstruction (e.g., related to a tumor or anatomic abnormalities), metabolic aberrations (e.g., hypertriglyceridemia), drug exposure (e.g., thiazides, azathioprine, and estrogens), smoking, and trauma (Yadav and Lowenfels, 2013; Lankisch et al., 2015) (**Figure 1**). Systemic diseases and trauma are particularly common in pediatric patients with AP and differ from those in adults (Snyder, 2000).

Acute pancreatitis is one of the most frequent gastrointestinal diseases leading to total hospital stays, with a reported global annual incidence of 13–45 per 100 000 people (Lankisch et al., 2015). The number of discharges with AP as the principal diagnosis in the USA in 2009 increased 30% compared with that in the year 2000 (Lankisch et al., 2015). Although nearly 80% of patients with AP exhibit mild symptoms and are easy to treat, 20% of those suffer a severe attack with progression to systemic inflammatory response syndrome (SIRS) and multiple organ dysfunction syndromes (MODS), and approximately 10–30% of patients with severe acute pancreatitis (SAP) may die (Whitcomb, 2006). In addition, as shown in **Figure 1**, the associations between AP and certain chronic diseases have garnered attention in AP research in the past 10 years (Yadav et al., 2012; Gillies et al., 2016; Kennedy et al., 2016). Investigations from the US showed a transition to chronic and pancreatic cancer from AP in 32.3% after 3.4 years (Yadav et al., 2012). Findings from a cross-sectional follow-up study and a recent comprehensive systematic review indicate that approximately 40% of patients may develop newly diagnosed prediabetes (e.g., chronic hyperglycemia, insulin resistance) or diabetes after AP, with the risk of diabetes doubling within 5 years (Gillies et al., 2016; Kennedy et al., 2016). Therefore, approaches with high efficacy and minimal side effects are imperative for the treatment of AP.

Since the first international classification of pancreatitis formulated during the 1963 Marseille meeting, the management guidelines of AP have been enacted one after another; so far, a preliminary consensus regarding the treatment for AP has been established (Tenner et al., 2013; Working Group IAP/APA Acute Pancreatitis Guidelines, 2013; Yokoe et al., 2015). Except for a few acute hemorrhagic necrotizing pancreatitis (AHNP) cases that require surgery, the primary therapeutic approach is to recommend conservative treatment focused on reducing pancreatic secretion and secondary injury, including fasting, fluid resuscitation, protease inhibitors, and antibiotics (Whitcomb, 2006; Banks et al., 2013; Lankisch et al., 2015). Although these strategies have been verified in randomized controlled trials, they have a limited impact on the incidence and severity of AP due to their unpredictable side effects and poor patient compliance. So far, AP is still a significant and unresolved challenge to clinicians. Hence, there is a huge demand to explore novel candidates for AP treatment.

Chinese herbal medicines (CHMs), which are abundant sources of biologically active substances, have been commonly used in clinical practices in many countries (Normile, 2003). Presently, more and more CHMs (including Chinese herbal formulas and pleiotropic natural products) have been discovered to have potent effects against AP through targeting numerous protein or biological networks involved in this disease (Tian et al.,

2009; Chen et al., 2011; Xiang et al., 2016). Thus, CHMs are promising candidate drugs for the treatment of AP compared with western medicine, which usually focuses on a single target. For the future development of innovative medicines and to extend the influence of CHMs worldwide, we systematically summarize the available Chinese herbal formulas and pleiotropic natural products used in the treatment of AP and discuss their underlying mechanisms in this review. Within this frame, the underlying mechanisms of AP will also be covered.

UNDERLYING MECHANISMS OF AP

For centuries, many theories have been proposed to explain the underlying mechanism of AP. As presented in **Figure 2**, the pathogenesis of AP seems to be related to a series of complex and multifaceted pathological processes, involving pancreatic self-digestion (Lerch and Gorelick, 2000), inflammatory response, oxidative stress (Xiang et al., 2016), intracellular calcium overload, endoplasmic reticulum (ER) stress (Wu J.S. et al., 2016), pancreatic acinar cell apoptosis and necrosis (Xiang et al., 2016), and microcirculation disorder (Tomkotter et al., 2016). Current therapies, which target the above pathological mechanisms, may improve the prognosis of AP.

Pancreatic Self-digestion Theory

The “pancreatic self-digestion theory” was proposed by Chiara et al. (1886) for the first time (Jin et al., 2013), and this theory suggested that pancreatic duct obstruction and the blockage of pancreatic juice outflow under pathological conditions are associated with exocrine pancreatic hyperstimulation and active trypsin reflux and result in the autodigestion of the pancreas and pancreatitis (Lerch and Gorelick, 2000). This theory was supported by research executed in cerulean-induced AP mice with or without the trypsinogen isoform 7 (T7) gene ($T^{-/-}$). Unlike zymogen activation and AP in wild-type mice, necrosis and cell death were significantly reduced in $T^{-/-}$ mice (Dawra et al., 2011). Most researchers accept that the direct trigger for the onset and exacerbation of AP relates to the inappropriate activation of trypsinogen to trypsin, the key enzyme in the activation of additional zymogens, and a lack of prompt elimination of active zymogens (Lerch and Gorelick, 2000; Whitcomb, 2006). Pancreatic acinar cells are specialized for the production, storage, and release of pancreatic zymogens (Hofbauer et al., 1998). During AP, lysosomal enzymes are mistargeted to the organelles containing zymogens within the acinar cell. The lysosomal hydrolase cathepsin-B prematurely activates trypsinogen, whereas the inhibition or knockout of cathepsin-B has been shown to relieve trypsinogen activation and acinar cell damage (Saluja et al., 1997; Van Acker et al., 2002).

In addition, there are a variety of protective mechanisms against premature zymogen activation *in vivo* through the inhibition or degradation of activated trypsin (Hirota et al., 2006; Binker et al., 2015). These mechanisms include inhibition by pancreatic secretory trypsin inhibitor (PSTI), also known as a serine protease inhibitor Kazal-type 1 (SPINK1) or

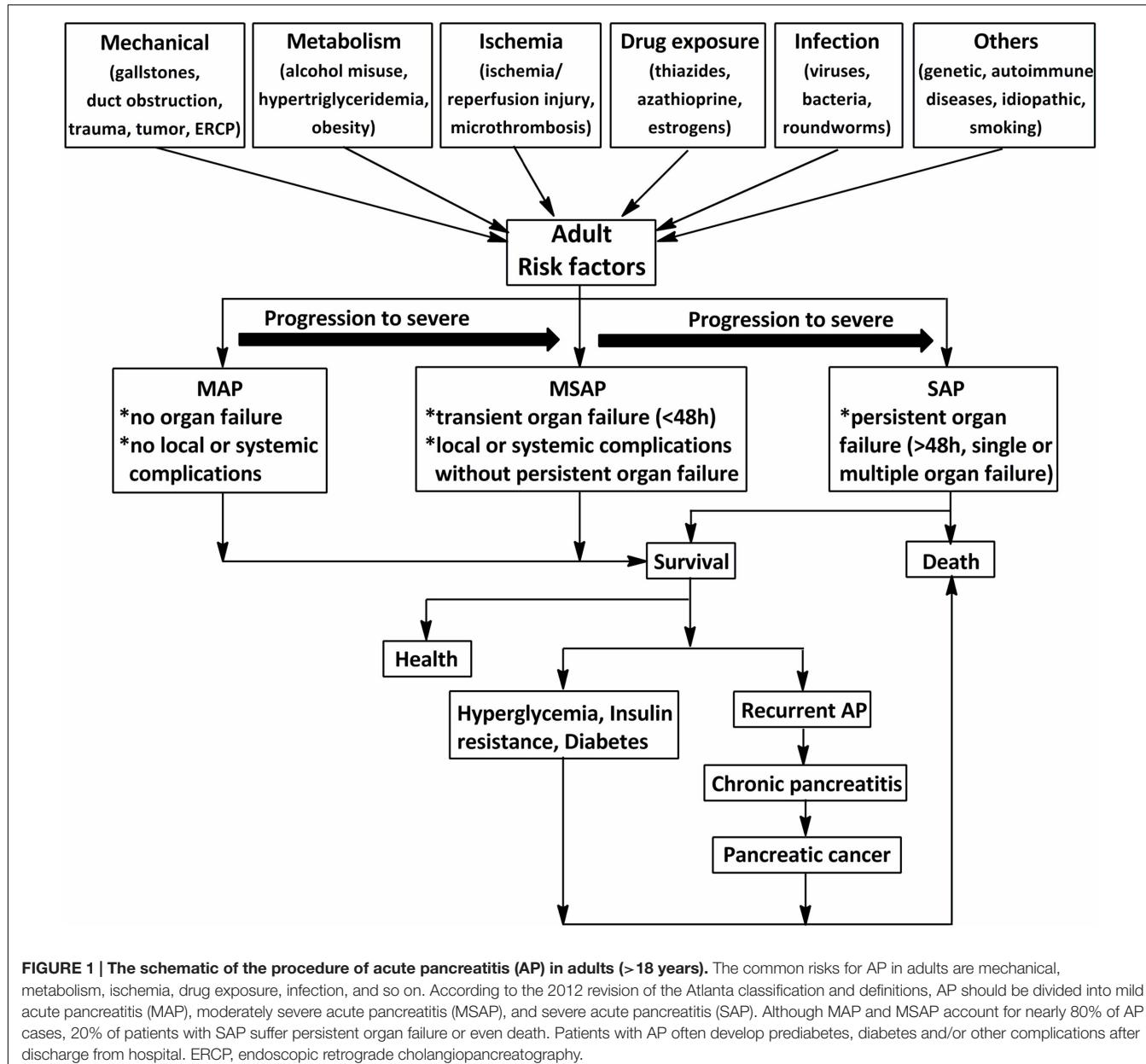


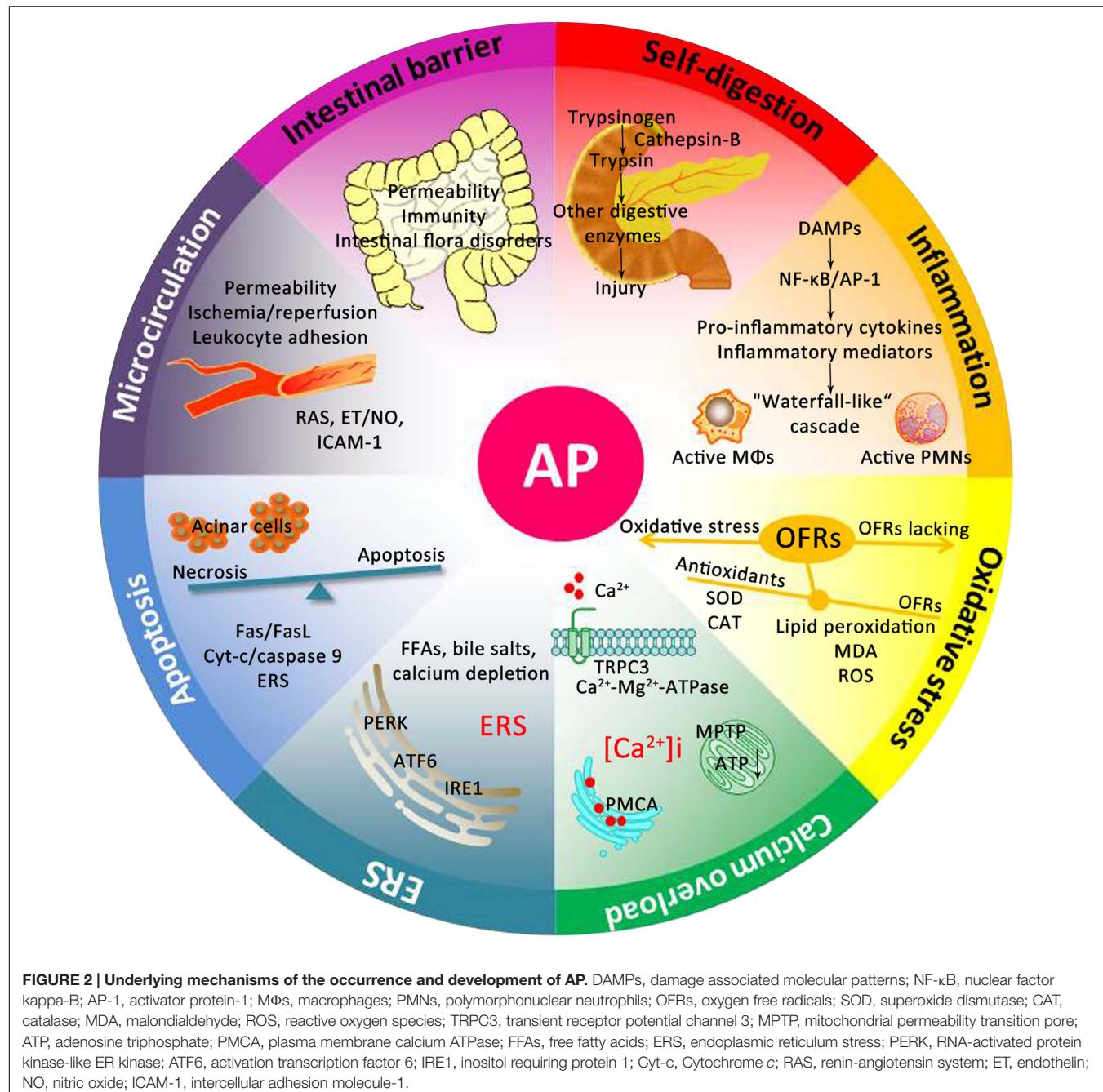
FIGURE 1 | The schematic of the procedure of acute pancreatitis (AP) in adults (>18 years). The common risks for AP in adults are mechanical, metabolism, ischemia, drug exposure, infection, and so on. According to the 2012 revision of the Atlanta classification and definitions, AP should be divided into mild acute pancreatitis (MAP), moderately severe acute pancreatitis (MSAP), and severe acute pancreatitis (SAP). Although MAP and MSAP account for nearly 80% of AP cases, 20% of patients with SAP suffer persistent organ failure or even death. Patients with AP often develop prediabetes, diabetes and/or other complications after discharge from hospital. ERCP, endoscopic retrograde cholangiopancreatography.

tumor-associated trypsin inhibitor (TATI) (Hirota et al., 2006), and degradation by chymotrypsin-C (CTRC) (Binker et al., 2015) and the lysosomal hydrolase cathepsin-L (Wartmann et al., 2010). Once these protective mechanisms are expended, there is an increased risk of developing AP.

Inflammatory Response

An acute inflammatory cascade is shown to be the main reason that mild acute pancreatitis progresses to SIRS and MODS in response to pancreatic cell injury (Whitcomb, 2006; Lankisch et al., 2015). In recent years, studies suggested that damage associated molecular patterns (DAMPs), mainly high mobility group box 1 (HMGB1) protein, are released by injured and necrotic acinar cells (Scalfida et al., 2002). HMGB1 is

an intracellular DNA-binding protein involved in neutrophil activation and pro-inflammatory factor secretion via Toll-like receptors (TLRs) in the pathogenesis of AP (Shen and Li, 2015). TLR4 is the first target response to extracellular HMGB1 and can activate the myeloid differentiation primary response gene 88 (Myd88)-dependent pathway, TNF-associated factor 6 and the MAPK signal transduction pathway, which then lead to the activation of nuclear factor kappa-B (NF- κ B) and activator protein-1 (AP-1) (Li et al., 2016b). NF- κ B and AP-1 are known transcription factors with multiple functions, required for the early regulation of inflammatory signaling (Yang et al., 2016). NF- κ B in the cell cytoplasm is bound to the inhibitor protein kappa B (IkB) in an inactive form. During cellular stress, IkB is phosphorylated by specific IkB kinase (IKK) and rapidly



degraded via the proteasome-dependent pathways (Hayden and Ghosh, 2008). NF- κ B activation during AP is capable of up-regulating the expression of cytokines, chemokines and adhesion molecules, the activation of macrophages, and the infiltration of neutrophils and lymphocytes into the pancreas and peritoneum, thus amplifying the inflammatory response (Jakkampudi et al., 2016). AP-1 is a heterodimer complex formed from proteins belonging to the c-Fos, c-Jun, and activating transcription factor (ATF) families (Schraml et al., 2009). AP-1 can be activated by multiple factors such as growth factors, cytokines, and chemokines, which often act in concert with NF- κ B to control

the cascade chain reaction of inflammatory mediators (Koh et al., 2010). The mechanism of AP-1 activation involves the phosphorylation of c-Jun (Schraml et al., 2009).

During AP, neutrophils migrate from the bloodstream to the damaged tissues, significantly increasing the numbers of local neutrophils in the pancreas (Yang Z.W. et al., 2015). Activated neutrophils have the capability to exacerbate the release of many types of pro-inflammatory cytokines and inflammatory mediators, which contain tumor necrosis factor- α (TNF- α), interleukin (IL)-1, IL-6, and IL-8, oxygen free radicals (OFRs), platelet-activating factor (PAF), leukotrienes, and thromboxane

A2 (TXA2), and many enzymes, in particular, elastase and phospholipase A2 (PLA2) (Kolaczkowska and Kubes, 2013; Nauseef and Borregaard, 2014; Yang Z.W. et al., 2015). These released signaling molecules, in turn, activate NF- κ B via highly specific receptor binding patterns, leading to pancreatic inflammation, necrosis and microcirculation disorder, and the release of excess endotoxin (Rotstein, 2014). The inflammatory response is constantly activated, not only in the local pancreas but also in the extrapancreas, and evolves into multiple organ injury and SIRS.

Oxidative Stress

Kishimoto et al. (1995) found that pancreatic OFRs increased after the induction of AP in rats, demonstrating that significant peroxidation occurs in AP. In patients with AP, the involvement of OFRs is independent of the underlying etiology (Park et al., 2003). Upon physiological conditions, the production and elimination of OFRs remain balanced. OFRs accumulate in the pancreas during AP development, which can attack macromolecules, such as proteins, lipids, and polysaccharides inside the biomembrane, initiating lipid peroxidation and resulting in the breakage of membrane stability and the release of zymogen granules in the acinar cell (Chvanov et al., 2005; Perez et al., 2015). OFRs activate PLA that can break down lecithinum inside the cellular membrane and further initiate pancreatic edema, hemorrhage, degeneration, and necrosis (Tsukahara et al., 1999). Moreover, many OFRs released in AP also cause NF- κ B and AP-1 activation, antioxidant consumption, and superoxide dismutase (SOD) activity decrease, which in turn causes lipid peroxidation and pancreas damage (Yu and Kim, 2014; Perez et al., 2015).

Calcium Overload

In general, calcium release, uptake and extrusion mechanisms remain in fine coordination, whereas some factors can influence calcium regulation and increase the intracellular calcium concentration under certain conditions (Cridle, 2016). Cytosolic calcium is a secondary messenger of intracellular signal transduction and applies to many cellular events, including the regulation of trypsin activity (Kruger et al., 2000). In AP, calcium homeostasis is disrupted, resulting in calcium overload within a cell, which can aggravate the disease state (Kruger et al., 2000; Maleth and Hegyi, 2014). Studies have shown that hypercalcemia causes histological damage during AP in a time- and dose-dependent manner (Gerasimenko et al., 2014). Calcium overload within the pancreatic acinar cells can induce mitochondrial impairment through the formation of the mitochondrial permeability transition pore (MPTP) and the depletion of ATP and necrosis, thus leading to serious injury of the acinar cells and pancreatic inflammation (Mukherjee et al., 2016).

The maintenance of intracellular calcium homeostasis depends on a stable mechanism that coordinates calcium release, entry and exit (Cridle, 2016). It is known that calcium release from internal stores occurs via inositol 1,4,5-trisphosphate receptor (IP3R)-dependent calcium oscillations that promote exocrine secretion (Camello-Almaraz et al., 2006). There are

several ion channels (TRPC3 channels, STIM1-Orai complex) in pancreatic acinar cells implicated in important calcium entry mechanisms, which mediate sustained pathophysiological elevations in intracellular calcium causing mitochondrial injury and cell death (Kim et al., 2009; Lur et al., 2009). Considering the benefit of the production and supply of intracellular adenosine triphosphate (ATP) for intracellular calcium homeostasis in the pancreatic acinar cell, recent studies reported that calcium clearance requires ATP-dependent pumps modulated by sustained oxidative stress (Bruce and Elliott, 2007). In AP, the structural integrity of the plasma membrane is destroyed, and the quantity and activity of endocytoplasmic/sarcoplasmic reticulum (SR) membrane Ca^{2+} -ATPase (PMCA) and cytomembrane Ca^{2+} - Mg^{2+} -ATPase are decreased, and the intracellular calcium is not pumped out of the cell or back into the calcium stores in time (Baggaley et al., 2008; Wang et al., 2008).

ER Stress

The acinar cells of the exocrine pancreas have an abundant ER that contributes to the synthesis and secretion of proteins. ER enzymes require optimal ion concentrations (e.g., calcium) and redox conditions for their regulation and function (Kubisch and Logsdon, 2008). Excess free fatty acids (FFAs), bile salts, calcium depletion of the ER stores, and oxidative stress activate the ER stress response and the injury of acinar cells, which initiates a complicated cascade of events during the early stage of AP (Zeng et al., 2012; Wu et al., 2013). RNA-activated protein kinase-like ER kinase (PERK), activation transcription factor 6 (ATF6), and inositol requiring protein 1 (IRE1) are believed to be ER stress sensors, which play a key role in transducing the stress signals from the ER to the cytoplasm (Kubisch and Logsdon, 2008). In AP, these signaling molecules and their downstream molecules TNF receptor-associated factor 2 (TRAF2) and apoptosis signal-regulating kinase 1 (ASK1) are highly activated (Sendler et al., 2013). This activation parallels trypsinogen activation and leads to apoptosis and inflammatory and immune responses (Kubisch and Logsdon, 2007).

Cell Apoptosis

Both necrosis and apoptosis are the two types of crucial cell death in AP, and may interchange under appropriate conditions. Recent studies prove that the regulation of the necrosis/apoptosis switch is beneficial for alleviating the severity of AP because apoptotic cells maintain membrane integrity without triggering an inflammatory cascade, unlike necrotic cells, which release intracellular contents containing pro-inflammatory and immunogenic cytokines (Takeyama, 2005; Fu et al., 2016). Apoptosis is controlled via three known pathways: the death receptor, the mitochondrial pathway, and the ER stress-induced apoptotic pathway. In early SAP, the stimulation of death receptors such as Fas and tumor necrosis factor receptor 1 (TNFR1) leads to the formation of a death-inducing signaling complex and initiator caspase-8, which initiates downstream caspase-3 (Zhang et al., 2007a). Death receptors transmit the death signals to mitochondria resulting in the release of cytochrome c (a precursor of apoptosis) into the cytoplasm through a permeability transition pore in the mitochondrial

membrane, which subsequently binds to the apoptotic protease activating factor-1 (Apaf-1) and procaspase-9 to form the apoptosome and activate caspase-3 (Xia et al., 2002). Bcl-2, an anti-apoptotic member of the Bcl-2 family, can break the opening of the permeability transition pore induced by Bax (Ma et al., 2011). Recent studies suggest several molecules such as IRE1, C/EBP homologous protein (CHOP), Bcl-2 family and caspase-12 play a role in ER stress-induced apoptosis (Morishima et al., 2002). Evidence indicates that the sustained activation of IRE1 enhances cell survival, suggesting a link between ER stress and death cell fate. CHOP transcription is induced by the activation of PERK and ATF6. Apoptosis via the CHOP signaling pathways is mitochondria-dependent because CHOP can break down the membrane potential and promote the release of cytochrome c from mitochondria (Kubisch and Logsdon, 2008). The members of the ER-resident proapoptotic Bcl-2 family, Bak and Bax, can maintain the homeostasis of ER calcium. When ER stress and increased cytosolic calcium correlate with an increase in calpain activation, it contributes to the activation of ER-resident caspase-12, which directly excites caspase-3 and leads to cellular apoptosis (Nakagawa and Yuan, 2000). Bcl-2 can clear OFRs and prevent the release of calcium from ER and leads to necrosis by inhibiting apoptosis (Wu and Tang, 2016).

Microcirculation Disorder

Microcirculation obstruction throughout the development of AP is a systemic response to pancreatic injury and is closely associated with MODS (Zhou and Chen, 2002; Ma et al., 2011; Tomkotter et al., 2016). The pancreas is the main site of the abnormal metabolism of eicosanoids (TXA2 and PGI2), which are decomposed into several types of inflammatory mediators, such as PAF and leukotrienes, thereby aggravating the inflammatory reaction and microcirculatory disturbance (Zhang et al., 2009b). Microcirculatory hypoperfusion causes the inflow of calcium into cells, leading to calcium overload in the pancreatic cells (Zhou et al., 2004). Calcium influx activates the phospholipid cell system, leading to the destruction of the lysosomal membrane and the release of the enzyme and large amounts of toxic media (Maleth and Hegyi, 2014). In addition, significant hemorrhagic changes in a rat model of SAP have been reported, including high hematocrit (HCT) and blood viscosity, as well as microthrombus formation. The possible mechanism relates to the activation of pancreatin that increases the permeability of capillary vessels, resulting in the exudation of lots of plasma-like liquids from the blood circulation to tissue space. Fragmented erythrocytes release tissue factors, such as adenosine diphosphate (ADP), which can activate the coagulation system and consume abundant fibrinogen (Ma et al., 2011). These factors may be the primary causes of progressive pancreatic tissue necrosis and MODS.

Altered Gut Barrier Permeability

The gut mucosal barrier serves as a “natural guard” in the human body, preventing the entrance of potentially harmful intestinal bacteria and endotoxin into the systemic circulation and extra-intestinal tissues. The gut acts as an important target organ in response to SAP, and as the main source of pancreatic

bacterial super infection and related septic complications (Wang et al., 2016). Gram-negative enteric-type organisms are shown to be the culprit in most pancreatic and peripancreatic infections (Hanna et al., 2014). In SAP early, hypovolemia, splanchnic vasoconstriction, and ischemia-reperfusion injury initiates a derangement in gut barrier function. Increased intestinal permeability, physical and chemical factor changes, and cell immune dysfunction induced by impaired gut mucosa barrier destroy normal flora structure and promote intestinal lumen-derived bacteria and endotoxins translocation to other organs through blood circulation or the lymphatic system, or through a pancreatic duct or bile duct directly connected with the intestinal tract (Guo et al., 2014). Intestinal microflora can also directly penetrate the damaged intestinal mucosal barrier into the abdominal cavity. In this scenario, an augmented immune response is triggered, which causes the gut to become a pro-inflammatory organ that releases cytokines, chemokines, and other pro-inflammatory intermediates. These mediators cause a “waterfall-like” inflammation cascade via the excessive activation of macrophages and neutrophils and release of oxidant and proteolytic enzymes, which aggravates secondary infection in the pancreas, eventually resulting in SIRS and MODS (Leveau et al., 1996; Wu et al., 1998).

CHINESE HERBAL FORMULAS

Chinese herbal formulas have upheld the holistic therapeutic philosophy for thousands of years, which consists of two or more appropriate medicinal plants or animals according to the prescription compatibility principle of traditional Chinese medicine (TCM) formulations and determining the dosage and usage of each medicine (Normile, 2003; Yao et al., 2016b). The components of Chinese herbal formulas are complex and diverse, and the main treatment mechanism seems reasonable. These herbal formulas are an organic combination of many effective components having a multi-target effect on the disease in the body by multiple pathways (Cheng et al., 2015; Wu T.Y. et al., 2016; Xiang et al., 2016). An increasing number of Chinese herbal formulas have been reported to have significant anti-AP effects, and have become a treatment option in many hospitals for AP (Table 1).

Dachengqi decoction (DCQD), first documented in “*Shang Han Lun*” (Treatise on Febrile Diseases), is a representative purgative for constipation treatment and for clearing internal heat in the gastrointestinal tract. DCQD consists of *Radix et Rhizoma Rhei*, *Cortex Magnoliae Officinalis*, *Fructus Aurantii Immaturus*, and *Natrii Sulphas*, which have been used as a classical prescription in China to treat AP for more than three decades (Chen et al., 2010). Rhein, naringin, and honokiol may be the major effect components of DCQD in treatment of AP (Zhang et al., 2016). Clinical studies indicated that DCQD could decrease the ratio of lactulose (L)/mannitol (M) and the surrogate of intestinal permeability assays, which suggested that DCQD protect the intestinal mucosal immune barrier and decrease the incidence of pancreatic infection and MODS (Chen et al., 2010; Jiang, 2010).

TABLE 1 | List of traditional Chinese medicine formulas for the treatment of AP.

Formula	Common composition	Mechanisms	Reference
DCQD	<i>Radix Rhei</i> Et Rhizome (Dahuang), <i>Magnolia Officinalis Rehd</i> Et Wils (Houpu), Aurantii Fructus Immaturus (Zhishi), Natrii Sulphas (Mangxiao)	↓CRP, IL-6, TNF- α , L/M ratio, LPS ↓ROS, ↑NO, iNOS ↓HMGB1, TLRs, NF- κ B, p38 MAPK, IL-6, TNF- α	Chen et al., 2010 Wang et al., 2012 Chen et al., 2015
DCQD (Modified)	<i>Radix Rhei</i> Et Rhizome (Dahuang), Thenardite (Xuanmingfen), <i>Magnolia Officinalis Rehd</i> Et Wils (Houpu), Aurantii Fructus (Zhiquiao), Persicae Semen (Taoren), Raphani Semen (Laifuzi)	↓HMGB1, TNF- α	Qin et al., 2015
CQCQD	<i>Radix Bupleuri</i> (Chaihu), <i>Scutellariae Radix</i> (Huangqin), <i>Magnolia Officinalis Rehd</i> Et Wils (Houpu), Aurantii Fructus Immaturus (Zhishi), Artemisiae Scopariae Herba (Yinchen), Fructus Gardeniae (Zhizi), <i>Radix Rhei</i> Et Rhizome (Dahuang), Natrii Sulfas (Mangxiao)	↑SERCA2 ↓NF- κ B, TNF- α , IL6 ↓[Ca ²⁺]i ↓CCKR1, PLC, IP3 ↑Cytochrome c, Caspase-3 ↑nAChRalpha7, Ach, ↓IL6	Xue et al., 2008 Li et al., 2008 Deng et al., 2008a Guo et al., 2015 Lin et al., 2014 Xue et al., 2014
CQCQD (Modified)	<i>Radix Bupleuri</i> (Chaihu), <i>Scutellariae Radix</i> (Huangqin), <i>Magnolia Officinalis Rehd</i> Et Wils (Houpu), Aurantii Fructus Immaturus (Zhishi), <i>Radix Rhei</i> Et Rhizome (Dahuang), Cortex Moutan (Danpi), Corydalis Rhizoma (Yuanhu), Toosendan Fructus (Chuanlian), Kansui Radix (Gansui), Natrii Sulphas (Mangxiao)	↓SAA ↓MMP-9	Wu et al., 2012; Guo et al., 2013
QYD	<i>Gardenia jasminoides</i> Ellis (Zhizi), Cortex Moutan (Danpi), <i>Radix Paeoniae Rubra</i> (Chishao), <i>Aucklandiae Radix</i> (Muxiang), <i>Magnolia Officinalis Rehd</i> Et Wils (Houpu), <i>Corydalis Rhizoma</i> (Yuanhu), <i>Radix Rhei</i> Et Rhizome (Dahuang), Natrii Sulphas (Mangxiao)	↓Genes: Rgs2, Pnlp, Cpa2, Ela2, LOC503278, Sv2b, LOC500909, Cln3, Reg1, Fbxl20 ↑Genes: Glrx1, LOC499457, Txnl2, Eef1g, LOC499793, Rpl10, LOC499906, Dap, Eef1b2, LOC362290	Zhu et al., 2014
QYD (Modified)	<i>Radix Rhei</i> Et Rhizome (Dahuang), Natrii Sulphas (Mangxiao), Kansui Radix (Gansui), <i>Radix Bupleuri</i> (Chaihu), <i>Aucklandiae Radix</i> (Muxiang), <i>Scutellariae Radix</i> (Huangqin), <i>Coptidis Rhizoma</i> (Huanglian), <i>Magnolia Officinalis Rehd</i> Et Wils (Houpu), <i>Gardenia jasminoides</i> Ellis (Zhizi), <i>Paeoniae Radix Alba</i> (Baishao), <i>Persicae Semen</i> (Taoren)	↓NF- κ B, TNF- α , IL-6, IL-8	Yang et al., 2009
QYG	<i>Radix Rhei</i> Et Rhizome (Dahuang), <i>Radix Bupleuri</i> (Chaihu), <i>Hedysarum Multijugum Maxim</i> (Huangqi), Natrii Sulfas (Mangxiao), <i>Paeoniae Radix Alba</i> (Baishao), <i>Aucklandiae Radix</i> (Muxiang)	↓Proteins: Serpinbla 43 KDa, ClpS, Actg1, Eprs, Hadhsc ↑Proteins: Serpinbla 39 KDa, Prx-IV	Yang et al., 2013
YCHD	<i>Artemisia capillaris</i> Thunb. (Yinchen), <i>Gardenia jasminoides</i> Ellis (Zhizi), <i>Rheum officinale</i> Baill. (Dahuang)	↑PPAR γ , ↓NF- κ B	Xiang et al., 2016
LHD	<i>Radix Rhei</i> Et Rhizome (Dahuang), Cortex Phellodendri Chinensis (Huangbai), <i>Rhizoma Bletillae</i> (Baiji), <i>Radix Angelicae Dahuricae</i> (Baizhi), <i>Fructus Mume</i> (Wumei), <i>Herba Menthae</i> (Bohe)	↓TNF- α , IL-6, IL-10, ↑SOD	Peng et al., 2013
TXHYD	<i>Radix Rhei</i> Et Rhizome (Dahuang), Natrii Sulfas (Mangxiao), Aurantii Fructus Immaturus (Zhishi), <i>Curcumae Radix</i> (Yujin), <i>Polygoni Cuspidati Rhizoma</i> Et Radix (Huzhang), Cortex Moutan (Mudanpi), <i>Radix Paeoniae Rubra</i> (Chishao), <i>Corydalis Rhizoma</i> (Yanhusu)	↓ET	Fang et al., 2007
YHQYD	<i>Radix Rhei</i> Et Rhizome (Dahuang), Natrii Sulfas (Mangxiao), <i>Scutellariae Radix</i> (Huangqin), <i>Paeoniae Radix Alba</i> (Baishao), <i>Radix Ophiopogonis</i> (Maidong), <i>Aucklandiae Radix</i> (Muxiang), <i>Radix Bupleuri</i> (Chaihu), <i>Semen Arecae</i> (Binglang), Cortex Meliae (Kuliapi), <i>Fructus Quisqualis</i> (Shijunzi), <i>Rhizoma Chuanxiong</i> (Chuanxiong), <i>Salviae Miltorrhizae</i> (Danshen), <i>Radix Astragali</i> (Huangqi), <i>Radix Ginseng</i> (Renshen)	↑Pancreatic blood flow	Chen et al., 2011

(Continued)

TABLE 1 | Continued

Formula	Common composition	Mechanisms	Reference
CHSHD	Radix Bupleuri (Chaihu), Radix Rhei Et Rhizome (Dahuang), Scutellariae Radix (Huangqin), Coptidis Rhizoma (Huanglian), Phellodendri Chinensis Cortex (Huangbai), Radix Paeoniae Rubra (Chishao), Radix Salviae (Danshen)	↓TNF- α , IL-6, ↑IL10	Wang H. et al., 2009

DCQD, *Dachengqi decoction*; CQCQD, *Chaiqinchengqi decoction*; QYD, *Qingyi decoction*; QYG, *Qingyi granule*; YCHD, *Yinchenhao decoction*; LHD, *Liuhe dan*; TXHYD, *Tongxiahayu decoction*; YHQYD, *Yihuoqingyi decoction*; CHSHD, *Chaihusihuang decoction*; CRP, C-reactive protein; IL, interleukin; TNF- α , tumor necrosis factor- α ; L/M, lactulose/mannitol; LPS, lipopolysaccharide; ROS, reactive oxygen species; NO, nitric oxide; iNOS, inducible nitric oxide synthase; HMGB1, high mobility group box 1; TLRs, Toll-like receptors; NF- κ B, nuclear factor kappa-B; p38 MAPK, p38 mitogen-activated protein kinases; SAA, serum amyloid A; MMP-9, matrix metalloproteinase 9; SERCA2, sarco/endoplasmic reticulum Ca^{2+} -ATPase; CCKR1, cholecystokinin receptor 1; PLC, phospholipase C; IP3, inositol-1,4,5-triphosphate; nAChR α 7, neuronal acetylcholine receptor alpha 7; Ach, acetylcholine; PPAR γ , peroxisome proliferator-activated receptor gamma; SOD, superoxide dismutase; ET, endothelin.

On the molecular mechanism, DCQD decreases pro-inflammatory cytokines and alleviates the severity of AP through inhibiting TLR/HMGB-1 signal pathways (Chen et al., 2010, 2015; Qin et al., 2015). DCQD could also render pancreas more resilient to stress and microcirculation disorder through eliminating excessive reactive oxygen species (ROS), inducing apoptosis and relieving the necrosis in acinar cells (Ren et al., 2009; Wang et al., 2012). Moreover, recent studies have shown that DCQD is an effective digestive kinetic agent that could promote the gastrointestinal motility and the recovery of intestinal mucosal permeability, and affect the bacterial translocation in the animal models of AP (Chen et al., 2010; Qin et al., 2015).

Chaiqinchengqi decoction (CQCQD), modified from DCQD, is a traditional Chinese prescription used as a purgative. In recent decades, CQCQD has shown significant efficacy in the treatment of AP both *in vivo* and *in vitro* (Liu et al., 2004; Deng et al., 2008a; Xue et al., 2008; Wang et al., 2011). Clinical trials have also proven that CQCQD significantly relieves the severity of clinical symptoms, reduces the duration of organ damage, and shortens the hospitalization time of AP patients (Wang et al., 2011). Research showed that CQCQD may have efficient actions on the activation of choline acetyl transferase (ChAT) and neuronal acetylcholine receptor alpha 7 (nAChR α 7) in peritoneal macrophages, which could inhibit the release of active macrophage pro-inflammatory cytokines (Li et al., 2008; Wang et al., 2011; Xue et al., 2014; Wu W.U. et al., 2016). CQCQD also inhibit the exocrine function of the pancreatic acinar cells and relieve pancreatic tissue lesions via reducing the overload of intracellular calcium (Deng et al., 2008a,b; Xue et al., 2008; Guo et al., 2015). In addition, CQCQD also regulates necrosis to apoptosis in pancreatic cells by promoting the release of mitochondrial cytochrome c and increasing pancreatic caspase-3 activity in SAP rats (Lin et al., 2014). Moreover, CQCQD has a protective effect in SAP complicated with acute lung injury (ALI) and acute respiratory distress syndrome by inhibiting ER stress in AMs, attenuating pro-inflammatory cytokine release and paracellular leakage, which involved in the down-regulation of p-Src, p-p85 α , and c-Fos (Wang Z.C. et al., 2009; Jia et al., 2015; Wu W.U. et al., 2016). In addition, the modified CQCQD can relieve the severity of clinical symptoms in patients with SAP via lowering serum amyloid A (SAA) and matrix metalloproteinase 9 (MMP-9) (Wu et al., 2012; Guo et al., 2013).

Another common Chinese herbal decoction, qingyi decoction (QYD) or qingyi granule (QYG) is generally well tolerated by patients and exhibits purgative function, eliminates blood stasis, promotes blood circulation, and reduces inflammation in the pathogenesis of AP (Yang et al., 2009, 2013; Zhu et al., 2014). Moreover, clinical research proved that QYD could ameliorate AP-induced intestinal barrier injury by inhibiting the expression of intestinal secreted phospholipase A2 (sPLA2) (Zhang et al., 2015). Furthermore, an Illumina whole genome expression profile analysis for pancreatic RNA expression of SAP rats screens 575 differential genes between the SAP and QYD group, including 92 up-regulated genes and 483 down-regulated genes; and the Gene Ontology (GO) categories indicated that these genes are involved the MAPK and NLR signaling pathways, metabolic pathways, cell cycle, and oxide reductase activities (Yang et al., 2013; Zhu et al., 2014). In addition, QYD can reduce the extent of extrapancreatic organ injuries in SAP. QYD administration not only reduces SAP-induced ALI by reducing alveolar type II epithelial cell (AEC II) apoptosis, inhibiting the overexpression of secretory type II phospholipase A2 (sPLA2) (Liu et al., 2014), but also improves SAP complicated liver and renal injuries through decreasing HMGB1 expression (Yang Y.S. et al., 2015). In addition, the actions of modified QYD involve in the biological processes include directly neutralizing endotoxins, reducing intestinal endotoxin generation and absorption, inhibiting excessive neutrophil activation and NF- κ B expression, and minimizing the release of inflammatory cytokines (Yang et al., 2009).

Moreover, our previous study found that Yinchenhao decoction (YCHD), known as an anti-inflammatory and choleric agent, may also be a potential therapy for AP through pro-apoptosis, anti-inflammation, anti-oxidation, and regulation of lipid metabolism partially via regulating the NF- κ B/PPAR γ signaling pathway (Xiang et al., 2016). Apart from these, other Chinese herbal formulas including Liuhe dan (Peng et al., 2013), Huoxueyingyidecoction (Ji et al., 2016), Tongxiahayu decoction (Fang et al., 2007), Yihuoqingyi decoction (Chen et al., 2011), Chaihusihuang decoction (Wang H. et al., 2009), Dachaihu decoction (Cheng et al., 2008), and Qingyichengqi decoction (Zhang et al., 2014) are also effective treatments for AP.

Despite the promising effects of Chinese herbal formulas, huge differences between TCM and modern medicine still push TCM away from mainstream medicine (Liu et al., 2016). There

are several issues and challenges in current studies: First, the drawbacks of traditional decoctions and its administration are obvious, such as discommodiousness, unstable efficacy, and uncontrollable quality. Second, the data that harvested from rigorously stochastic, double-blind, placebo-controlled trials with multiple centers and large samples is insufficient (Qiong et al., 2005). Third, the interaction between the complex chemical and biological systems of AP remains ambiguous. Finally, the pharmacokinetics of these formulas and their interactions with other medications should be further evaluated (Yao et al., 2016b).

PLEIOTROPIC NATURAL PRODUCTS

Pleiotropic natural products derived from medicinal plants through extraction, separation, and purification have clear chemical structures that are different from Chinese herbal formulas and crude extracts (de Oliveira et al., 2015; Bulaj et al., 2016; Yao et al., 2016b). Pure natural products, as carriers of modern herbal medicine advanced technology, are considered as the only way for TCM to realize modernization and internationalization (Normile, 2003; Yao et al., 2016b). Natural products have been used in Japan since the 1970s and have been widely developed in Singapore and other places (Normile, 2003). In Europe and the United States, 25% of prescriptions contain at least one extract from higher plants or compounds. In Germany, pure natural products are considered as drugs rather than food supplements and are covered by medical insurance (Yao et al., 2016a). In short, pure natural products in foreign countries have a good application base and a wide range of markets.

Pleiotropic natural products feature the following advantages: (1) well-known molecular weight, physical and chemical characteristics and easy qualitative and quantitative analyses; (2) various technique indexes can be controlled in the preparation process, and strict quality control can be respected; (3) the concentration of TCM effective ingredients can be greatly increased, enhancing medicine absorption in the body, thereby overcoming the fatal weakness of the slow effect of TCM; and (4) the pollution of harmful substances such as heavy metal ions and residual pesticide, can be maximally eliminated (Zhang et al., 2008b, 2009). Many pure natural products such as emodin, baicalin, resveratrol, and curcumin have been found to have significant therapeutic benefits against AP (**Table 2**). However, there is lack of clinical researches about the doses and side effects of these natural products at present.

Emodin

Emodin (1,3,8-trihydroxy-6-methylanthraquinone) (**Figure 3A**) is a natural anthraquinone derivative isolated from the roots and rhizomes of numerous plants, such as *Rhamnaceae*, *Polygonum*, *Liliaceae*, and *Leguminosae*, and especially, from the Chinese herb *Rheum palmatum L.*, as an active compound, which has been utilized to treat critical illness (e.g., AP) in China for many years (Dong et al., 2016). Previous pharmacological studies have shown that emodin possesses biological activities such as purgative, anti-fibrotic, vasorelaxant, and immunosuppressive (Dong et al., 2016). Emodin also possesses an anti-inflammatory property

particular (Li et al., 2015). Early cell culture and animal research have revealed that emodin at the doses of 10–60 mg/kg could significantly reduce the mortality and have anti-AP effects (Wu et al., 2000, 2014; Ni et al., 2014a; Yao et al., 2015). Currently, studies suggest that emodin ameliorates AP through multiple targets; however, the exact mechanism underlying the effect of emodin in AP has not been completely addressed.

The paramount pathological manifestations of AP are pancreatic edema, hemorrhage, necrosis, and inflammatory infiltration, and increased pancreatic enzymes (Whitcomb, 2006). Emodin, as a direct NF- κ B inhibitor, could result in an anti-oxidation response and subsequently suppress the expression of pro-inflammatory cytokines (Yao et al., 2015). Moreover, our previous research discovered 32 differentially expressed proteins from SAP rats with or without emodin treatment by using iTRAQ-based quantitative proteomic analysis, and a new biomarker, serine protease high-temperature requirement A1 (HTRA1), was found to associate with SAP. Further work found that one of the mechanisms underlying emodin against SAP involved inhibiting the HTRA1/TGF- β 1/NF- κ B signaling cascade and subsequent inflammatory responses (Li et al., 2016a).

Emodin can also promote cell apoptosis and reduce necrosis occurring in the pancreatic acinar cell or inflammatory cells, for example, emodin inhibits SIRS in SAP rats via inducing neutrophil apoptosis via the calcium-mediated caspase-12 signaling pathway (Ni et al., 2014b; Yin et al., 2016). In addition, the protective effects of emodin on ER stress responses are mediated by significantly attenuating calcium overload and decreasing the expression of ER chaperone immunoglobulin-binding protein (Bip), PERK, ATF6, IRE1 α , TRAF2 and ASK1, as well as inhibiting the phosphorylation of MAPKs (Wu et al., 2013, 2014). Schmitt et al. (2004) reported that claudin and occludin expression in the pancreas are obviously reduced in AP, suggesting a possible role of tight junction disruption in interstitial edema formation. Emodin administration could increase claudin and occludin expression and reduce paracellular permeability in the pancreas (Xia et al., 2012). Furthermore, previous research indicated that emodin could reduce pancreatic ischemia and tissue injury through decreasing the expression of thromboxane-2 (TXB2) protein, which is a stable metabolite of TXA2 that has abilities to induce the deformation, release, and secretion of platelets (Kusterer et al., 1991; Wu et al., 2000). Interestingly, many studies have shown that the regeneration and repair of pancreatic injury occurs after AP (Konturek et al., 1997). Transforming growth factor- β 1 (TGF- β 1) is found to be involved in the pathogenesis of AP, especially pancreatic regeneration (Riesle et al., 1997). Emodin combined with somatostatin analogs may be beneficial for the up-regulation of TGF- β 1 and EGF expression, which contributes to pancreatic repair and regeneration (Gong et al., 2002). Moreover, results from Konturek et al. (1998) have demonstrated that significantly up-regulated expression of epidermal growth factor (EGF) may restrict the extent of initial tissue damage and then accelerate pancreatic regeneration and repair in AP.

Emodin has a protective effect in pancreatitis-associated secondary organ damage (Wang et al., 2007; Ning et al., 2009; Xia et al., 2010; Xu et al., 2016), which is both a common

TABLE 2 | List of pure natural products derived from medicinal plants for the treatment of AP.

Natural products	Main source	Models	Mechanisms	Reference
Emodin	<i>Rheum palmatum L., Polygonum cuspidatum, Polygonum multiflorum, Aloe vera, Cassia obtusifolia, Radix et Rhizoma Rhei</i>	SAP rats (NaTc)	↓MDA, NF-κB, TNF-α, IL6, IL-1β,	Yao et al., 2015
		AP rats (NaTc)	↑SOD	Xia et al., 2012
		SAP rats (NaTc)	↑Claudin-5, Occludin	Wu et al., 2013
		AR42J cells (caerulein + LPS)	↓Bip, IRE1α, TRAF2, ASK1, phosphorylation of JNK and p38 MAPK	Wu et al., 2014
		ANP rats (NaTc)	↓Bip, PERK, ATF6, IRE1	Wu et al., 2000
		SAP rats (NaTc)	↓TXB2, ↑6-keto-PGF1α	Li et al., 2016a
		AP rats (caerulein)	↓HTRA1/TGF-β1 signaling pathway	Gong et al., 2002
		SAP rats neutrophils (NaTc)	↑TGF-β1, EGF	Yin et al., 2016
		SAP/SIRS rats pMΦ (NaTc)	Ca ²⁺ -calpain 1-caspase 12-caspase 3 signaling pathway	Ni et al., 2014a,b
			↑ICAM-3, mCD14	
Baicalin	<i>Scutellaria baicalensis Georgi</i>	AP rats (caerulein)	↓NF-κB, TNF-α	Xue et al., 2006
		SAP rats (NaTc)	↓TNF-α, IL-6, MDA, PLA2	Zhang et al., 2007c
		SAP rats (NaTc)	↓NO, MDA, TNF-α	Zhang et al., 2008b
		SAP rats (NaTc)	↓P-selectin, TNF-α, ↑Caspase-3	Xiping et al., 2009b
		SAP rats (NaTc)	↓IL-1β, PAF, TXB2, PLA2, ↑PGE2	Zhang et al., 2009b
		SAP rats (NaTc)	↓Bcl2, ↑Bax	Xiping et al., 2009a
Resveratrol	Grapes, berries, peanuts, soya beans, red wine, rhubarb, giant knotweed rhizome, <i>Eranthis hyemalis</i>	AP rats (CCK8)	↓NF-κB, TNF-α, ↑CAT, glutathione	Szabolcs et al., 2006
		AP rats (NaTc)	↓NF-κB, AP-1, TNF-α, IL-6, iNOS	Gulcubuk et al., 2014
		AP rats (caerulein)	↓IL-1β, MDA, ↑IL10, GSH-Px, SOD	Carrasco et al., 2014
		SAP rats (NaTc)	↓MDA, ICAM-1, VCAM-1, TNF-α,	Jha et al., 2012
		SAP rats (NaTc)	↑SOD	Wang et al., 2008
		SAP rats (NaTc) pMΦ	↓PLA2, [Ca ²⁺] _i , ↑Ca ²⁺ -Mg ²⁺ -ATPase, Ca ²⁺ -ATPase	Ma et al., 2005
			↓NF-κB, iNOS, TNF-α, IL-1, NO	
Dihydro-resveratrol	Orchidaceae, <i>Cannabis sativa L.</i> , A metabolite of trans-resveratrol in the human body	AP rats (caerulein)	↓MDA, NADPH oxidase, MPO, TNF-α, NF-κB, IκB degradation, AKT phosphorylation, ↑glutathione, PI3K	Tsang et al., 2016
Curcumin	<i>Turmeric (Curcuma longa)</i>	AP rats (NaTc)	↓NF-κB, AP-1	Gulcubuk et al., 2013
		SAP rats (NaTc)	↓TLR4, NF-κB	Zhong, 2015
		AP rats (NaTc)	↓TNF-α, IL6	Gulcubuk et al., 2006
		AP rats (NaTc)	↓MDA, NO, bacterial translocation	Gulcubuk et al., 2005
		AP mice (caerulein)	↑PPAR γ , ↓NF-κB, TNF-α	Yu et al., 2011
Ligustrazine	<i>Ligusticum chuanxiong Hort., Curcuma aromatica Salisb., Jatropha podagrica Hook</i>	AP rats and acinar cells (caerulein)	↓p38 Erk MAPK pathways	Chen et al., 2016
Honokiol	<i>Magnolia officinalis Rehd. Et Wils</i>	SAP mice (caerulein)	↓TNF-α, IL1, NO, HMGB1, MPO ↑eIF2 α phosphorylated, CHOP, caspase-3	Weng et al., 2012

AP, acute pancreatitis; SAP, severe acute pancreatitis; ANP, acute necrotizing pancreatitis; SIRS, systemic inflammatory response syndrome; NaTc, sodium taurocholate hydrate; LPS, lipopolysaccharide; CCK8, cholecystokinin 8; pMΦ, peritoneal macrophages; MDA, malondialdehyde; NF-κB, nuclear factor kappa-B; TNF-α, tumor necrosis factor-α; IL, interleukin; SOD, superoxide dismutase; Bip, ER chaperone immunoglobulin-binding protein; IRE1, inositol requiring protein 1; TRAF2, TNF receptor-associated factor 2; ASK1, apoptosis signal-regulating kinase 1; JNK, c-Jun N-terminal kinase; p38 MAPK, p38 mitogen-activated protein kinases; TXB2, thromboxane-2; HTRA1, serine protease high-temperature requirement A1; TGF-β1, transforming growth factor-β1; EGF, epidermal growth factor; ICAM, intercellular adhesion molecule; mCD14, membrane-bound CD14; PLA2, phospholipase A2; NO, nitric oxide; PAF, platelet-activating factor; PGE2, prostaglandin E2; CAT, catalase; AP-1, activator protein-1; iNOS, inducible nitric oxide synthase; GSH-Px, glutathione peroxidase; MPO, myeloperoxidase; VCAM-1, vascular cell adhesion molecule 1; IκB, inhibitor protein kappa B; PI3K, phosphatidylinositol 3'-kinase; AKT, threonine kinase; TLR4, Toll-like receptor 4; HMGB1, high mobility group box 1; PPAR γ , peroxisome proliferator-activated receptor gamma; CHOP, C/EBP homologous protein.

serious SAP complication and a main cause of death. Pancreatitis-associated lung injury occurs as a consequence of significant pulmonary hyperemia, edema, and inflammatory infiltration in the lung tissues. Although the strategies in prevention and treatments have been improved, ALI still causes more than 50%

of deaths in SAP (Bhatia, 2002). Emodin attenuates pulmonary edema and enhances alveolar epithelial barrier function by up-regulating the expression of claudin-4, claudin-5, occludin, AQP1, and AQP5 in lung tissue samples from rats with SAP-induced ALI (Xia et al., 2010; Xu et al., 2016). Several

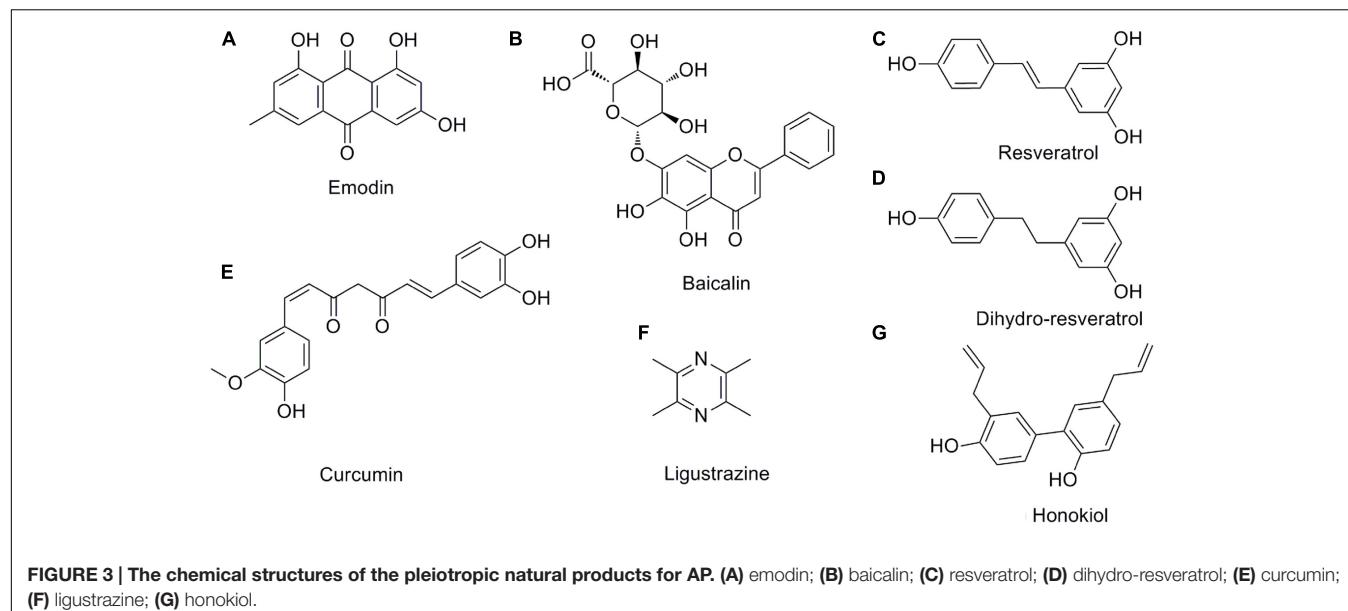


FIGURE 3 | The chemical structures of the pleiotropic natural products for AP. (A) emodin; (B) baicalin; (C) resveratrol; (D) dihydro-resveratrol; (E) curcumin; (F) ligustrazine; (G) honokiol.

in vivo investigations have proposed that emodin can prevent the translocation of bacteria and endotoxins and promote the recovery of intestinal barrier function via inhibiting the intestinal mucosa cell apoptosis and up-regulating the serum leptin content (Ning et al., 2009). In addition, emodin-assisted early enteral nutrition (EAEN) obviously abates the severity of secondary hepatic injury to function in the treatment of AP (Wang et al., 2007). However, emodin could also result in kidney toxicity, hepatotoxicity, and reproductive toxicity, particularly in long-term use with high-doses. Pharmacokinetic studies also indicated that emodin processed poor oral bioavailability because of its wide glucuronidation (Dong et al., 2016).

Baicalin

Baicalin (5,6,7-trihydroxyflavone-7-O-D-glucuronic acid) (Figure 3B) is one of the effective flavonoid compounds extracted from the dried root of *Scutellaria baicalensis* Georgi (Zhang et al., 2009a). *In vitro* experiments of baicalin demonstrated that it features multiple activities, such as resisting bacteria, anti-inflammation, anti-oxidation, and inhibiting platelet aggregation, reducing endotoxin production and inducing apoptosis (Ueda et al., 2002; Shen et al., 2003). The pharmacological actions of baicalin are quite similar to those of octreotide, which is a somatostatin analog that can significantly inhibit pancreatic secretion (Shen et al., 2003; Tian et al., 2009). Fortunately, animal researches indicated that baicalin (50–100 mg/kg) has diverse pharmacological actions associated with antagonizing many stages of SAP onset and is much cheaper than octreotide, thus implying that baicalin is a promising medication for AP treatment. Early studies reported that baicalin could inhibit digestive enzyme activity, reduce pancreas necrosis, restrict lipid peroxidation, and increase the SAP rat survival rate (Zhang et al., 2007c, 2009a).

The beneficial effects of baicalin appear to be mediated by inhibiting NF- κ B and TNF- α activity and increasing caspase-3

expression in multiple organs (Xue et al., 2006). Furthermore, baicalin may be applied for decreasing the expression levels of the P-selectin protein, which serves as a marker for leukocyte-endothelial cell adhesion and active leukocyte-mediated organ injury and plays a key role in the progression of AP (Xiping et al., 2009b). Complementarily, early treatment with baicalin exerts significant protective effects on SAP-induced multiple organ injuries, such as liver (Zhang et al., 2008a), kidney (Zhang et al., 2007b), intestinal mucosa (Zhang et al., 2009a), heart (Xiping et al., 2007), and thymus (Xiping et al., 2010), with their possible mechanisms associated with inhibiting inflammatory mediators and inducing apoptosis.

Resveratrol

Resveratrol (trans-3,5,4'-trihydroxystilbene) (Figure 3C), as a naturally polyphenolic phytoalexin, exists in almost 70 vegetables and fruits or food products, especially in grapes and red wine. It also has been identified as a major active ingredient in giant knotweed rhizome and rhubarb in TCM (Ma et al., 2011). Resveratrol is considered to be a good candidate for the treatment of oxidative and/or inflammatory diseases due to its antioxidant and anti-inflammatory activities and ease of extraction (Ma et al., 2011). Moreover, it has received an increasing amount of attention in treating AP (Meng et al., 2005b; Szabolcs et al., 2006). Previous animal studies have identified and documented the role of resveratrol (10–30 mg/kg) in decreasing the levels of amylase and lipase enzymes, mitigating histological damage in the pancreas and extra-pancreatic organs and reducing the mortality in the case of AP, indicating the therapeutic effect of resveratrol against AP (Li et al., 2006; Wang et al., 2008).

Resveratrol can decrease the production and release of pro-inflammatory mediators, such as IL-1, IL-6, IL-8, TNF- α , and NO, via blocking the activation of NF- κ B and AP-1 and the associated kinases and increasing anti-inflammatory

cytokine IL-10 levels (Ma et al., 2005; Szabolcs et al., 2006; Carrasco et al., 2014; Gulcubuk et al., 2014). At the same time, resveratrol suppressed the expression of intercellular adhesion molecule-1 (ICAM-1), which could mediate leukocyte adhesion to endothelium and reduce the infiltration of leukocytes into inflammatory sites (Meng et al., 2005b; Ma et al., 2011; Jha et al., 2012).

Leonard et al. (2003) demonstrated that resveratrol can clear hydroxyl superoxides and metal inductive radicals in AP as a high-efficiency scavenger. The results from recent studies also demonstrated that resveratrol exerts a protective effect against lipid peroxidation in the membrane and prevents DNA damage via the inhibition of the NF- κ B pathway and subsequent suppression of ROS products (Meng et al., 2005a; Li et al., 2006; Ma et al., 2011). In addition, it has been observed that SOD activity decreases, and the MDA activity significantly increases in organs in the early phases of AP; resveratrol reverses these phenomena to reduce the AP induced oxidative damage in the pancreas (Li et al., 2006).

Another advantage of resveratrol is that it also appears to reduce the intracellular calcium overload through restoring the intracellular calcium regulatory mechanisms, which limit not only pancreatic tissue injury but also secondary organ injury (Wang et al., 2008). Studies of SAP have shown that resveratrol may stabilize erythrocytes, improve the decrease of blood flow, decrease blood viscosity and leukocyte-endothelial interaction, thus decreasing thrombus formation and ameliorating the hypercoagulable state existing in every stage of SAP (Sha et al., 2013). Moreover, resveratrol could suppress microcirculatory disturbance via inhibition of the renin-angiotensin system (RAS) system and regulation of an unbalanced ET/NO status (Ma et al., 2011).

Resveratrol also has the ability to relieve injury in extra-pancreatic organs in SAP such as intestines, liver, brain and lungs through the up-regulation of Bcl-2 and the down-regulation of Bax, cytochrome c, and caspase-3 levels (Meng et al., 2005b; Jha et al., 2008, 2009; Sha et al., 2008). In short, resveratrol has shown potential therapeutic effects in cases of AP by inhibiting the release of inflammatory mediators, promoting antioxidant effects, regulating cytoplasm calcium homeostasis, reversing microcirculatory disturbance, and inducing apoptosis.

When orally consumed, *trans*-resveratrol is rapidly metabolized in the human colon by gut bacteria and converted to dihydro-resveratrol (3,5,4'-trihydroxy bibenzyl), **Figure 3D** (Tsang et al., 2016). Importantly, the solubility of dihydro-resveratrol was at least five times higher than *trans*-resveratrol while exhibiting a much lower cytotoxicity; thus, dihydro-resveratrol is particularly suitable for patients unresponsive to *trans*-resveratrol due to the lack of proper microbial strains (Tsang et al., 2016). Lin et al. (2016) and Tsang et al. (2016) demonstrated that dihydro-resveratrol could significantly ameliorate pancreatic oxidative damage and AP-associated lung injury. The underlying molecular mechanisms involve in the decreased production of intracellular reactive oxidative products and pro-inflammatory cytokines, and the inhibition of the NF- κ B and phosphatidylinositol 3'-kinase (PI3K)-serine/threonine kinase (AKT) signaling pathways.

Curcumin

Curcumin (diferuloylmethane, **Figure 3E**) is a turmeric polyphenol derived from *Curcuma longa* (turmeric), which has been prized in Ayurvedic medicine since ancient times for the treatment of inflammatory conditions due to its various pharmacological benefits including antioxidant and anti-inflammatory properties (Jurenka, 2009; Shehzad et al., 2013). Based on the results of cell cultures, animal models and clinical trials, ample evidence validates that curcumin at the doses of 100 mg/kg may have potential as a therapeutic agent in AP (Gulcubuk et al., 2013; Zhong, 2015). Curcumin's improvement of AP is achieved through repressing the infiltration of inflammatory cells, inhibiting lipid peroxidation, and regulating TLR-4/NF- κ B and PPAR γ /NF- κ B signaling pathway, preventing free radical injury and the prevalence of bacterial translocation, thereby reducing trypsin activation, oxidative enzymes, and tissue injury (Gulcubuk et al., 2005, 2006, 2013; Yu et al., 2011; Zhong, 2015). Moreover, curcumin suggests that the activation of caspase-3 may lead to an increase in apoptosis in the early and late phases of experimental AP.

Others

Ligustrazine (tetramethylpyrazine, **Figure 3F**) (Chen et al., 2016) and honokiol (3,5'-diallyl-4,2'-dihydroxybiphenyl, **Figure 3G**) (Weng et al., 2012) possess special pharmacological activities associated with the acceleration of acinar cell apoptosis at an early phase of AP. Thus, they may soon join the list of candidate drugs in the treatment of AP besides the above-mentioned natural products. However, in-depth research is necessary to confirm this hypothesis.

NETWORK TARGET PREDICTION

Traditional Chinese medicine is one of the promising strategies in treating AP due to its multi-targeting and lower side effects (Yao et al., 2016a). Despite massive investments in TCM research and development, there has been no significant increase in the number of new drugs approved or translated for clinical use. Single targeted drug discovery has proved to be ineffective in combating complex diseases that harbor robust biological networks such as AP. Network pharmacology prediction provides a highly useful method for drug targets, which differs from conventional single target intervention by integrating network biology and polypharmacology, thereby exploring drugs to target numerous proteins or networks involved in a disease (Cheng et al., 2015; Gao et al., 2016). In this review, we also discuss the application of network pharmacology for AP innovative drugs discovery.

We searched the TCMID, DrugBank, GeneCards, and STITCH databases to validate 68 AP targets based on six pure natural products (emodin, baicalin, resveratrol, curcumin, ligustrazine, and honokiol) mentioned above. Subsequently, a compound-target network was constructed using Cytoscape 3.3.0 software (Cheng et al., 2015; Xiang et al., 2016). As shown in **Figures 4A–F**, in this network, red rectangles and different colors-ellipses, respectively, correspond to natural products and

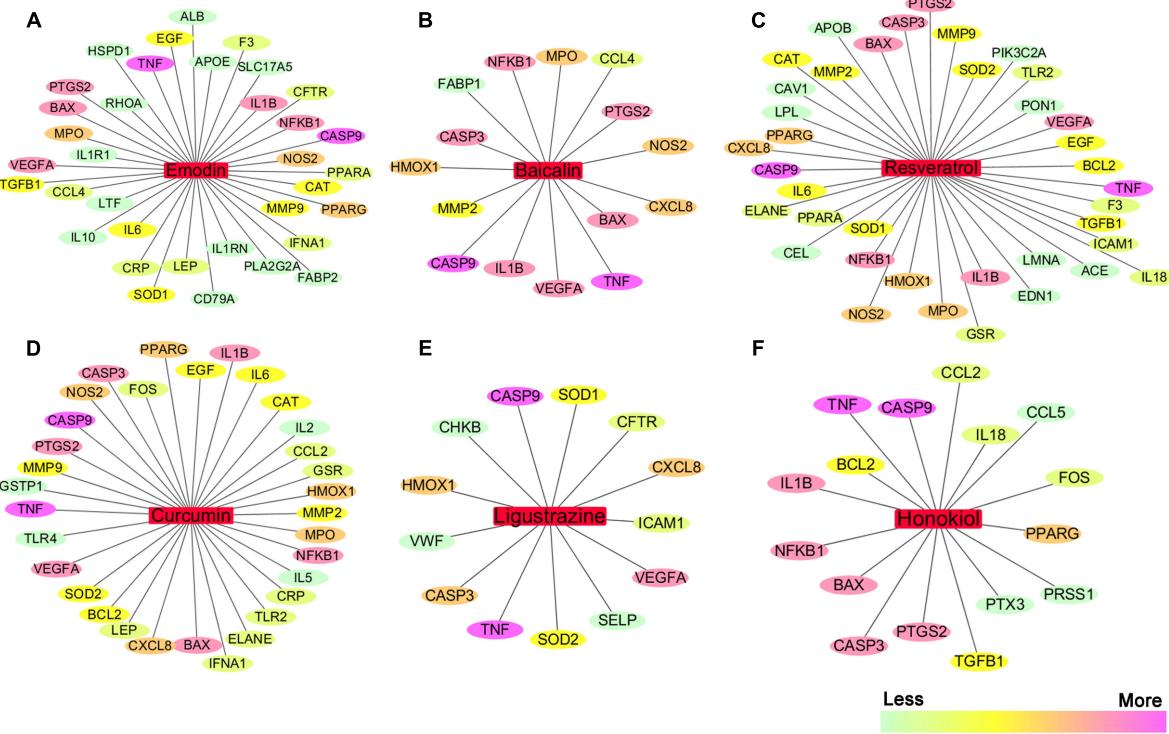


FIGURE 4 | The natural product-target network predicted by network pharmacology. **(A–F)** In this network, red rectangles correspond to natural products (emodin, baicalin, resveratrol, curcumin, ligustrazine, and honokiol), and different colors-ellipses correspond to targets. The colors of each target transit from green to purple, indicating its increased importance in the process of these natural products for AP treatment. In the relationships between natural products and targets, resveratrol corresponds to the highest number of candidate targets (degree = 38), followed by emodin (degree = 35), curcumin (degree = 32), honokiol (degree = 16), baicalin (degree = 15), and ligustrazine (degree = 13). The candidate targets in the progression of AP mainly related to inflammation (TNF, NFKB1, PTGS2, IL1B, IL6, IL10, etc.), apoptosis (CASP9, CASP3, BAX, BCL2, etc.), oxidation-reduction (NOS2, SOD1, MPO, CAT, etc.), and lipid metabolic process (PPARA, PPARG, APOE, APOB, etc.), as well as the maintenance of Ca^{2+} homeostasis (ELANE, CAV1, CCL4, etc.) and microcirculation (IFNA1, SELP, etc.).

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These emphasize the integral function of natural products in the treatment of AP through providing multiple therapeutic effects on multiple targets as compared with Western medicine, which usually focuses on a single target. The candidate targets in the progression of AP mainly related to inflammation (TNF, NFKB1, PTGS2, IL1B, IL6, IL10, etc.), apoptosis (CASP9, CASP3, BAX, BCL2, etc.), oxidation-reduction (NOS2, SOD1, MPO, CAT, etc.), and lipid metabolic process (PPARA, PPARG, APOE, APOB, etc.), as well as the maintenance of Ca^{2+} homeostasis (ELANE, CAV1, CCL4, etc.) and microcirculation (IFNA1, SELP, etc.). Especially, inflammation and apoptosis-related targets (e.g., TNF, CASP9, NFKB1, BAX, IL1B, CASP3) show the higher correlation (degree = 6 or 5), demonstrating the potential therapeutic effect of these natural products on AP through modulating these relevant proteins. Except for the common

targets, each natural product has specific regulation on some targets. For instance, resveratrol has a potential regulatory effect on CAV1 that promotes the maintenance of intracellular calcium homeostasis; emodin maybe benefit to regulate blood lipid levels in AP by regulating the expression and function of APOE that contributes to the lipoprotein biosynthetic, catabolic, and metabolic process; ligustrazine may improve the state of hypercoagulability and microcirculation during AP via regulating VWF and SELP expression. Each natural product acts on multiple targets, and the different natural products share the synergistic targets, which is the basis of action of pure natural products. However, since the compound-target network is only obtained by data mining combined with computer simulation predictions, whether the above natural products against AP through these potential targets still require further experimental validation.

POTENTIAL TOXIC NATURAL PRODUCTS FOR PANCREAS

Recent research pointed out an increasing interest concerning the health benefits of natural products, and they have been

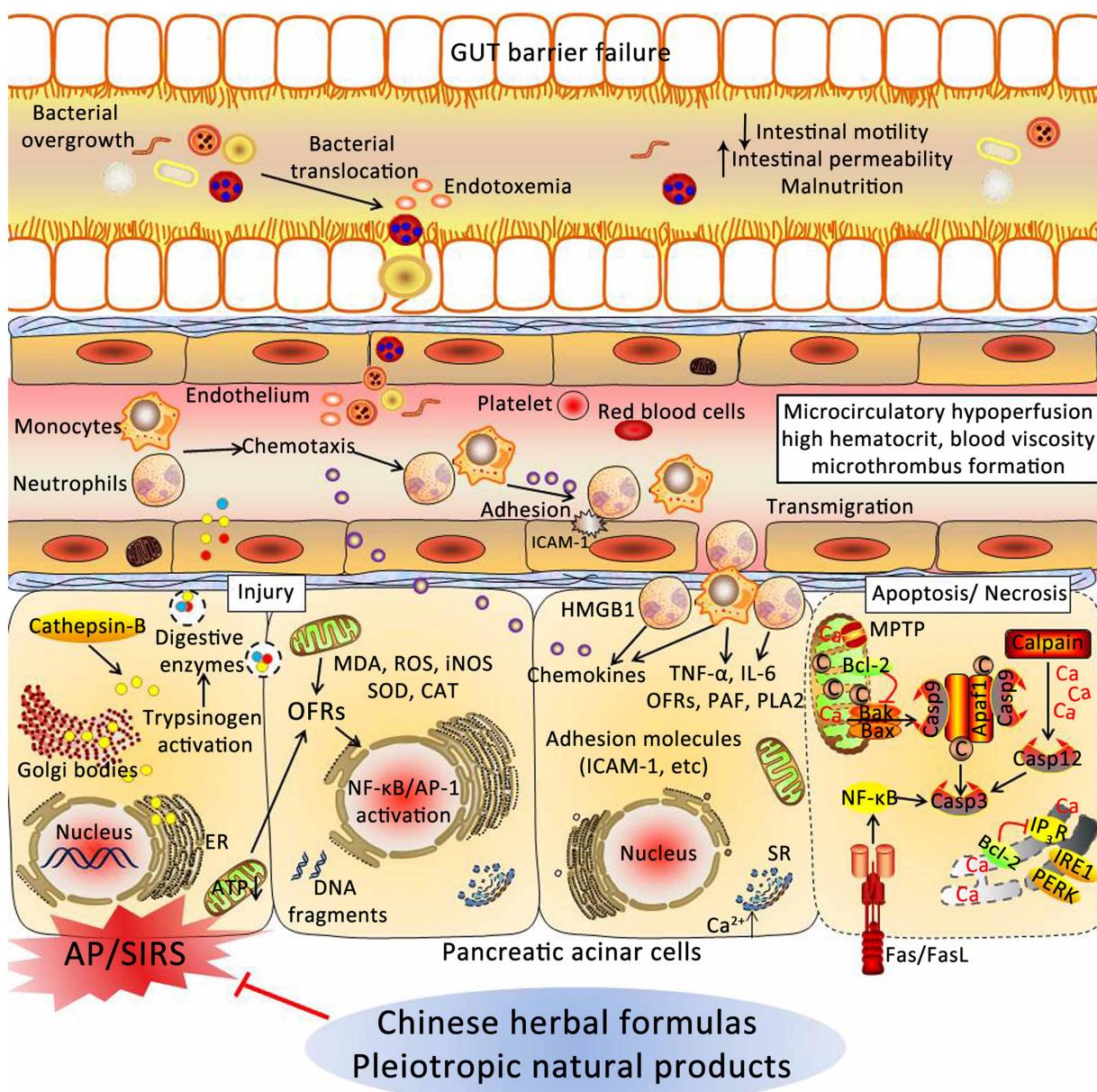


FIGURE 5 | Underlying mechanisms of Chinese herbal medicines (CHMs) for the treatment of AP. CHMs may prevent cellular damage in pancreas associated with AP through a variety of mechanisms, including: (1) depressing the synthesis and secretion of digestive enzymes; (2) depressing oxidative stress through increased antioxidant levels and decreased the excessive OFRs; (3) inhibiting activation of inflammatory pathways; (4) relieving ER stress via PERK and IRE1; (5) restoring the intracellular calcium regulatory mechanisms; (6) inducing the switch from apoptosis to necrosis in pancreatic cells; (7) increasing pancreatic blood flow, and reducing blood viscosity; and (8) restoring intestinal barrier function and blocking bacterial translocation. AP, acute pancreatitis; SIRS, systemic inflammatory response syndrome; ER, endoplasmic reticulum; ATP, adenosine triphosphate; OFRs, oxygen free radicals; MDA, malondialdehyde; ROS, reactive oxygen species; iNOS, inducible nitric oxide synthase; SOD, superoxide dismutase; CAT, catalase; NF-κB, nuclear factor kappa-B; AP-1, activator protein-1; HMGB1, high mobility group box 1; TNF-α, tumor necrosis factor-α; IL-6, interleukin-6; PAF, platelet-activating factor; PLA2, phospholipase A2; ICAM-1, intercellular adhesion molecule-1; SR, sarcoplasmic reticulum; MPTP, mitochondrial permeability transition pore; IP3R, inositol 1,4,5-trisphosphate receptor; PERK, RNA-activated protein kinase-like ER kinase; IRE1, inositol requiring protein 1; Apaf-1, apoptotic protease activating factor-1.

considered a good complementary and alternative medicine in the treatment of AP. As a coin has two sides, exaggerating the safety and non-toxic advantages of natural products leads to the negligence of potential pancreas toxicity for a long

time. There are very limited data of about the potential toxicity of herbs or natural products on the pancreas. Currently, three case reports showed a probable AP induced by saw-palmetto, a phytotherapeutic agent for symptoms related to benign prostatic

hyperplasia (BPH). It has been postulated that saw-palmetto stimulates estrogenic receptors and then increases triglyceride levels or induces a hypercoagulable state that leads to pancreatic necrosis (Jibrin et al., 2006; Wargo et al., 2010; Bruminhent et al., 2011). The evidence derived mainly from random case reports described possible *Ceramium kondoi* or horsetail infusions induced AP (Kim et al., 2013; Garcia Gavilan et al., 2017). Experimental results indicate that l-cyano-2-hydroxy-3-butene (CHB), a nitrile derived from many cruciferous plants, is a selective pancreatotoxin. CHB is also a possible inducer of tissue glutathione in the pancreas, even at toxic doses (200 mg CHB/kg body weight) (Wallig et al., 1988). L-canavanine extracting from *Hedysarum alpinum* seeds processes potential pancreatotoxicity when it's severs as an antitumor. Histological researches of tissues from rats treated with canavanine (3.0 g/kg) for 6 days revealed pancreatic acinar cell atrophy and fibrosis; and serum amylase and lipase levels were increased after one sc injection of 2.0 g/kg canavanine (Thomas and Rosenthal, 1987). Overall, natural products have the potential to be not only beneficial but also harmful under a number of medical conditions. This information should prompt clinicians to consider natural product a potential cause of AP.

CONCLUSION

Acute pancreatitis is the leading cause of hospital admission for gastrointestinal diseases, and the effective strategies are

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Correlation of Proton Pump Inhibitors with Pulmonary Tuberculosis: A Case-Control Study in Taiwan

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OPEN ACCESS

Edited by:

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Reviewed by:

Mario M. D'Elios,
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Specialty section:

This article was submitted to
Gastrointestinal and Hepatic
Pharmacology,
a section of the journal
Frontiers in Pharmacology

Received: 29 May 2017

Accepted: 04 July 2017

Published: 19 July 2017

Citation:

Cheng K-C, Liao K-F, Lin C-L and
Lai S-W (2017) Correlation of Proton
Pump Inhibitors with Pulmonary
Tuberculosis: A Case-Control Study in
Taiwan. *Front. Pharmacol.* 8:481.
doi: 10.3389/fphar.2017.00481

Background and Objectives: Although the relationship between the use of proton pump inhibitors (PPIs) and pulmonary tuberculosis (TB) in Taiwan published in 2014. Due to just only one article and not enough comprehensively, we explore this issue.

Methods: We conducted a population-based case-control study to identify 9,422 subjects aged 20 years or older with newly diagnosed pulmonary TB in 2000–2013 as test cases. We then randomly selected 9,422 subjects aged 20 years or older without pulmonary TB as controls. Both cases and controls were matched in terms of sex, age, and comorbidities. Use of PPIs were defined as subjects who had had at least one prescription for these medications before the index date. No use was defined as subjects who had never had a prescription for PPIs before the index date. The odds ratio (OR) and 95% confidence interval (CI) for pulmonary TB associated with PPI use was estimated using the logistic regression model.

Results: The OR of pulmonary TB was 1.31 for subjects who had used PPIs (95% CI 1.22, 1.41) compared with those with no use of the medications. Sub-analysis revealed the OR of pulmonary TB in subjects using PPI per increasing microgram was 1.25 (95% CI 1.19, 1.30).

Conclusions: PPI use is associated with a 1.3-fold increase in odds of developing pulmonary TB in Taiwan. There is a dose-related response between PPI use and pulmonary TB.

Keywords: pulmonary tuberculosis, proton pump inhibitors, Taiwan National Health Insurance Program

INTRODUCTION

Gastric acid plays a major role in decontaminating the upper gastrointestinal tract. A review article published about 30 years ago sought to determine the relationship between gastric secretion and ingested organisms resulting in possible infection (Howden and Hunt, 1987). Due to progress and recent advances in clinical pharmacology, acid-suppressive agents, including proton pump inhibitors (PPIs), have seen widespread use for treating peptic ulcers and acid reflux esophageal disease (Noguerado et al., 2002; Strid et al., 2003). Over the last 2–3 decades, PPIs were common used for acid suppressive agents in primary and specialty care with excellent safety (Sheen and Triadafilopoulos, 2011). A previous article revealed that PPIs appeared to be more effective than

histamine 2 receptor antagonists (H2RAs) in preventing clinically important and overt upper gastrointestinal bleeding (Alhazzani et al., 2013).

Previous studies have described the relationship between PPI use and several enteric infections, such as *Salmonella enteritis* and *Clostridium difficile* colitis (Dial et al., 2005; Rodríguez et al., 2007), including spontaneous bacterial peritonitis in severe cirrhotic patients (Bajaj et al., 2009). Several research works have indicated that, besides the gastrointestinal system, PPIs are positively associated with infections of the respiratory system, such as community- or hospital-acquired pneumonia (Gulmez et al., 2007; Sarkar et al., 2008; Jager et al., 2012). However, few studies have indicated whether this association was related to low-dose or short-term PPI use (Giuliano et al., 2012; Filion et al., 2013). In addition to hospital- or community-acquired pneumonia, *Mycobacterium tuberculosis* (TB)-associated infection exerts significant burdens on the health-care systems of developing countries, including Taiwan (Hsueh et al., 2006).

Previous articles discussing the association between pulmonary TB and any degree of gastrectomy are scarce, and most of them do not include up-to-date technologies and true mechanism (Boman, 1956; Thorn et al., 1956). To date, the real role of gastric acid in pulmonary TB patients remains unknown. Although the relationship between the use of PPIs and pulmonary tuberculosis (TB) in Taiwan, similar to our study, published in 2014 (Hsu et al., 2014). Due to just only one article and not enough comprehensively (just focused on prescription period of PPIs only), we utilized the Taiwan National Health Insurance Program database to plan and conduct this study for exploring the associations completely and definitely.

METHODS

Data Source

Taiwan is an independent country with a population of over 23 million (Chao et al., 2015; Chen et al., 2015; Ho and Chang, 2015; Hsiao et al., 2015; Hung and Ku, 2015; Lin and Lin, 2016; Lin et al., 2016a; Maa and Leu, 2016; Ooi, 2016; Yu et al., 2016). We conducted a population-based case-control study using data from the Taiwan National Health Insurance Program. This insurance program was established in March 1995 and covers 99% of Taiwan's population (National Health Insurance Research Database, 2017). Details of this program can be found in previous studies (Lai et al., 2010, 2012; Hung et al., 2011; Cheng et al., 2012; Tsai et al., 2016). The present study was approved by the Research Ethics Committee of China Medical University (CMUH-104-REC2-115).

Participants

We identified subjects aged 20 years or older with newly diagnosed pulmonary TB (International Classification of Diseases, Ninth Revision, Clinical Modification, ICD-9 codes 010, 011, 012, and 018) from 2000 to 2013 as test cases. The date of pulmonary TB diagnosis was defined as the index date. Subjects who were not diagnosed with pulmonary TB were randomly selected from the same database as controls. Both

cases and controls were matched in terms of sex, age (5-year intervals), and comorbidities.

Comorbidities Potentially Related to Pulmonary PT

Comorbidities that could potentially be related to pulmonary TB, including alcohol-related diseases, asbestos, chronic kidney disease, chronic obstructive pulmonary disease, diabetes mellitus, human immunodeficiency virus infection, gastrectomy, pneumoconiosis, splenectomy, and chronic liver diseases, such as cirrhosis, hepatitis B infection, hepatitis C infection, and other forms of chronic hepatitis, were assessed. All comorbidities were diagnosed with ICD-9 codes. The accuracy of these codes has been examined in previous studies (Lai et al., 2013a,b, 2014a,b, 2017; Hung et al., 2016; Lai, 2016; Lin et al., 2016a,b; Shen et al., 2016; Hsu et al., 2017; Liao et al., 2017a,b).

Measurements of PPI and H2RA Use

The PPIs available in Taiwan between 2000 and 2013 and considered in this study included esomeprazole, lansoprazole, omeprazole, pantoprazole, and rabeprazole. Patients' prescription histories of PPIs and H2RAs were included in this study. Use of medications was defined as prescription any of the medications studied in this work before the index date. No use of medication was defined as no history of prescription of any of the medications studied in this work before the index date.

Statistical Analysis

We compared the distributions of demographic status, PPI use, H2RA use, and comorbidities between cases and controls using the chi-squared test for categorized variables. The *t*-test was used to test differences in mean age and mean duration of exposure to PPIs between cases and controls. The univariable unconditional logistic regression model was used to measure odds ratios (ORs) and 95% confidence intervals (CIs) and determine the association between pulmonary TB and PPI use. We also analyzed dose-related responses to PPI use. All analyzes were performed using SAS software (version 9.2; SAS Institute, Inc., Cary, NC, USA), and results were considered statistically significant when two-tailed *P*-values were < 0.05.

RESULTS

Characteristics of the Study Population

As shown in Table 1, we identified 9,422 cases with newly diagnosed pulmonary TB between 2000 and 2013 and 9,422 controls without the disease. Both cases and controls showed similar distributions of sex and age. The mean ages (standard deviation) of patients with and without primary TB were 59.6 (17.1) years and 59.5 (17.1) years, respectively, and these values did not show statistical significance (*t*-test, *P* = 0.8). The mean durations of PPI use (standard deviation) of cases and controls were 3.19 (5.96) and 3.49 (6.06) months, respectively; these values did not show statistical significance (*t*-test, *P* = 0.13). While cases with pulmonary TB were more likely to report PPI

TABLE 1 | Information and comorbidities between pulmonary tuberculosis cases and controls.

Variable	Non-tuberculosis <i>N</i> = 9422		Tuberculosis <i>N</i> = 9422		<i>P</i> -value*
	<i>n</i>	%	<i>n</i>	%	
Sex					0.88
Female	3,000	31.8	3,010	31.9	
Male	6,422	68.2	6,412	68.1	
Age group (years)					0.99
20–39	1,551	16.4	1,549	16.4	
40–64	3,474	36.9	3,472	36.9	
65–84	4,397	46.7	4,401	46.7	
Age (years), mean (standard deviation) [†]	59.5	(17.1)	59.6	(17.1)	0.8
Duration of exposure to proton pump inhibitors (months), mean (standard deviation) [†]	3.49	(6.06)	3.19	(5.96)	0.13
Ever use of proton pump inhibitors	1,694	18	2,102	22.3	<0.001
Ever use of histamine-2 receptor antagonists	371	3.94	346	3.67	0.34
COMORBIDITIES					
Alcohol-related disease	717	7.61	745	7.91	0.45
Asbestosis	1	0.01	1	0.01	0.99
Chronic liver disease	1,637	17.4	1,674	17.8	0.48
Chronic obstructive pulmonary disease	4,111	43.6	4,136	43.9	0.71
Chronic kidney disease	459	4.87	468	4.97	0.76
Diabetes mellitus	1,433	15.2	1,477	15.7	0.38
Human immunodeficiency virus infection	21	0.22	21	0.22	0.99
Gastrectomy	9	0.1	9	0.1	0.99
Pneumoconiosis	87	0.92	75	0.80	0.34
Splenectomy	2	0.02	2	0.02	0.99

Data are presented as the number of subjects in each group with percentages given in parentheses, or mean with standard deviation given in parentheses.

*Chi-square test, and [†] t-test comparing subjects with and without pulmonary tuberculosis.

use than controls (22.3 vs. 18%, chi-square test, *P* < 0.001), no significant difference in H2RA use or other comorbidities was observed between cases and controls (Chi-square test, *P* > 0.05).

Pulmonary Tuberculosis Associated with Proton Pump Inhibitor Use

In Table 2, analyzes using the univariable unconditional logistic regression model showed that the OR of pulmonary TB was 1.31 for subjects who had used PPIs (95% CI 1.22, 1.41) compared with subjects who had never used PPIs. Because no other variable was significantly related to pulmonary TB during univariable analysis, we did not perform multivariable unconditional logistic regression analysis.

Sub-analysis of the Association between Pulmonary Tuberculosis and Five Proton Pump Inhibitors

Table 3 shows an analysis of the dose-related responses to PPI use; here, the group with no PPI use was considered the reference group. The OR of pulmonary TB in subjects using PPI per increasing microgram was 1.25 (95% CI 1.19, 1.30). These results indicate a dose-related response between PPI use and pulmonary TB.

DISCUSSION

PPIs have been commonly prescribed to patients with upper gastrointestinal tract bleeding or acid reflux-associated diseases in the last two decades (Hsu et al., 2014). These medications feature an excellent safety profile, and their benefits outweigh their risks in most patients worldwide (Sheen and Triadafilopoulos, 2011). Patients began using PPIs for extended periods of time without considering appropriate indications for primary and specialty care. This problem has intensified in Taiwan, especially in the last two decades (Chen et al., 2003). Achlorhydric stomach is usually accompanied by a weak protective mechanism for ingested organisms, which could bring about gastrointestinal (Howden and Hunt, 1987; Williams and McColl, 2006) and respiratory infections, such as hospital- or community-acquired pneumonia.

Although previous published article in 2014 associated with Taiwan general PPIs user populations and pulmonary TB patients had similar study design and article structure compared with ours (Hsu et al., 2014). Their result revealed that the relationship between PPIs and pulmonary TB patients became gradually faded when the drug prescription period extended. But in our study, we found that cases with pulmonary TB were more likely to have used PPIs only than controls without the disease. General speaking, the relationship between

TABLE 2 | Odds ratio and 95% confidence interval of pulmonary tuberculosis associated with proton pump inhibitors use and comorbidities.

Variable	Crude	
	OR	(95% CI)
Sex (male vs. female)	1.00	(0.94, 1.06)
Age (per 1 year)	1.00	(0.99, 1.00)
PROTON PUMP INHIBITORS (NEVER USE AS A REFERENCE)		
Ever use	1.31	(1.22, 1.41)
HISTAMINE-2 RECEPTOR ANTAGONISTS (NEVER USE AS A REFERENCE)		
Ever use	0.93	(0.80, 1.08)
COMORBIDITIES (YES VS. NO)		
Alcohol-related disease	1.04	(0.94, 1.16)
Asbestosis	1.00	(0.06, 16.0)
Chronic liver disease	1.03	(0.95, 1.11)
Chronic obstructive pulmonary disease	1.01	(0.95, 1.07)
Chronic kidney disease	1.02	(0.89, 1.17)
Diabetes mellitus	1.04	(0.96, 1.12)
Human immunodeficiency virus infection	1.00	(0.55, 1.83)
Gastrectomy	1.00	(0.40, 2.52)
Pneumoconiosis	0.86	(0.63, 1.17)
Splenectomy	1.00	(0.14, 7.10)

CI: confidence interval.

TABLE 3 | Odds ratio and 95% confidence interval of pulmonary tuberculosis in relation to cumulative dosage of proton pump inhibitors use by logistical regression model.

Variable	Case number/ control number	Crude OR	(95% CI)
Never use of proton pump inhibitors as a reference	7320/7728	1.00	(reference)
Proton pump inhibitors use (increase in dosage per mg)	2102/1694	1.25	(1.19, 1.30)

CI: confidence interval.

PPIs (accumulative dose) and pulmonary TB is positive in our study, but negative in theirs (accumulative time). Because of the different results in prescription period or accumulative dosage between both of them, we planned and conducted our study for further investigation. This finding in our study may be explained by several hypotheses as follows.

First, long-term PPI use may weaken the protective mechanism of the stomach because PPIs inhibit gastric secretion. While the acidic environment of the stomach is generally free from bacteria (Vakil, 2009), bacterial colonies gradually develop and increase in achlorhydric or hypochlorhydric stomachs. Frequent and long-term use of PPIs is believed to promote infections in patients prescribed these medications.

Second, the possible explanation might be associated with lower immune status. *M. tuberculosis* shares risk factors similar to those of community-acquired pneumonia, such as diabetes mellitus, alcohol drinking, aging, and HIV infection (Hsu et al.,

2014), although our study showed no significant difference in these comorbidities between cases and controls. A recent large-scale study in Japan and Taiwan revealed that gastrectomy could be associated with increased risk of pulmonary TB (Yokoyama et al., 2004; Huang et al., 2011), leading to poor immunity and nutritional states. We can confirm that the mechanism of gastrectomy is similar to that of PPI use and results in an achlorhydric or hypochlorhydric stomach. Therefore, the risk of developing pulmonary TB may increase because of limited stomach acid secretion and the poor immune status of a patient.

Finally, physicians in hospitals or local clinics may have difficulty in differentiating the symptoms of the initial stages of pulmonary TB and those of acid reflux-associated diseases, such as dry or chronic cough. The patients with chronic cough, disregarding whether pulmonary TB infections or not might be misdiagnosed or ignored, then PPI medication prescribed for treating acid reflux-associated chronic cough by physicians belong reasonable inference. Thus, some patients may still be infected with pulmonary TB even after PPI therapy. On the other words, PPI therapy should be withheld from patients with highly suspect pulmonary TB, even not prescribed PPI before confirm diagnosis of pulmonary TB.

In this study, we analyzed dose-related responses to PPI use and considered controls with no PPI use as the reference group. The dose-related response is understandable (Chou and Talalay, 1984). Patients prescribed high doses of the medications showed more extensive adverse effects than those prescribed relatively lower doses (Sheen and Triadafilopoulos, 2011). As PPI use is gradually increasing in Taiwan (Chen et al., 2003), physicians and specialists should pay more attention to the dose-related responses to and adverse effects of PPIs.

LIMITATION

One of the limitations of this work is underestimation of the numbers of patients with PPI use. Herein, we focused only on Taiwan's National Health Insurance Program database and ignored PPI or H2RA medications prescribed over the counter. Another limitation is diagnosis of pulmonary TB using ICD-9 codes. Miscoding by physicians, an ambiguous definition of pulmonary TB, and lack of sputum culture or chest X-ray could contribute to misdiagnosis of the disease. Finally, history, period, and dosage of PPI therapy were defined arbitrarily and without following any standard guideline. Rigorous evaluation of the definitions of pulmonary TB and manner of PPI use is recommended for future investigations.

STRENGTH

One of the strengths of the present study is that the set of ICD-9 codes used has been validated in previous published studies (Lai et al., 2013a,b, 2014a,b, 2017; Hung et al., 2016; Lai, 2016; Lin et al., 2016a,b; Shen et al., 2016; Hsu et al., 2017; Liao et al., 2017a,b). The long observation period employed in our

study (i.e., from 2000 to 2013) also endows our study with more credibility compared with other similar studies.

CONCLUSION

We conclude that PPI use is associated with a 1.3-fold increase in odds of developing pulmonary TB in Taiwan. There is a dose-related response between PPI use and pulmonary TB. Considering our results, evaluation of the risk of developing pulmonary TB may be necessary prior to prescribing the use of anti-suppressive agents.

AUTHOR CONTRIBUTIONS

KC and KL planned and conducted this study. They participated in the data interpretation, and also critically revised the article. CL conducted the data analysis and critically revised the article.

SL planned and conducted this study. He contributed to the conception of the article, initiated the draft of the article, and critically revised the article.

ACKNOWLEDGMENTS

This study was supported in part by Taiwan Ministry of Health and Welfare Clinical Trial Center (MOHW106-TDU-B-212-133004), China Medical University Hospital, Academia Sinica Taiwan Biobank Stroke Biosignature Project (BM10501010037), National Research Program for Biopharmaceuticals (NRPB) Stroke Clinical Trial Consortium (MOST 105-2325-B-039-003), Tseng-Lien Lin Foundation in Taichung in Taiwan, Taiwan Brain Disease Foundation in Taipei in Taiwan, and Katsuzo and Kyo Aoshima Memorial Funds in Japan. These funding agencies did not influence the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Drugs and Targets in Fibrosis

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Fibrosis contributes to the development of many diseases and many target molecules are involved in fibrosis. Currently, the majority of fibrosis treatment strategies are limited to specific diseases or organs. However, accumulating evidence demonstrates great similarities among fibroproliferative diseases, and more and more drugs are proved to be effective anti-fibrotic therapies across different diseases and organs. Here we comprehensively review the current knowledge on the pathological mechanisms of fibrosis, and divide factors mediating fibrosis progression into extracellular and intracellular groups. Furthermore, we systematically summarize both single and multiple component drugs that target fibrosis. Future directions of fibrosis drug discovery are also proposed.

OPEN ACCESS

Edited by:

Jinyong Peng,
Dalian Medical University, China

Reviewed by:

Xufeng Tao',
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Specialty section:

This article was submitted to
Gastrointestinal and Hepatic
Pharmacology,
a section of the journal
Frontiers in Pharmacology

Received: 28 August 2017

Accepted: 08 November 2017
Published: 23 November 2017

Citation:

Li X, Zhu L, Wang B, Yuan M and
Zhu R (2017) Drugs and Targets in
Fibrosis. *Front. Pharmacol.* 8:855.
doi: 10.3389/fphar.2017.00855

Keywords: fibrosis, drug, target, pathological mechanism, pharmacology

INTRODUCTION

Fibrosis, characterized by excess accumulation of extracellular matrix (ECM), is a common pathological process in many chronic diseases or injuries. Many irritations trigger the pro-fibrotic responses, including persistent infections, radiation, chemical agents, genetic disorders, and autoimmune diseases. The development of fibrosis is accompanied by the loss of a fraction of resident cells and their replacement by ECM, which would finally lead to tissue remodeling and organ failure. Fibrosis contributes to high morbidity and mortality in many diseases such as dilated cardiomyopathy and idiopathic pulmonary fibrosis (IPF) (Gulati et al., 2013; Hutchinson et al., 2015), and inevitably causes a prominent global clinical burden (Raimundo et al., 2016). For example, a study of medicare population aged 65 years and older showed that the incidence of IPF was around 93.7 cases per 100,000 person-years while the cumulative prevalence increased steadily to 494.5 cases per 100,000 person-years across 2001 to 2011 in US (Raghu et al., 2014). Besides, the mortality of non-alcoholic fatty liver disease (NAFLD) patients with a high probability of fibrosis was 69% higher than those without fibrosis (Kim et al., 2013).

As a long-lasting pathological phenomenon, fibrosis occurs in various tissues and organs (**Figure 1**), more often in heart, lung, kidney, liver, skin (Rockey et al., 2015), and less frequently in other tissues and organs such as pancreas, intestine, eye (Wynn, 2008), nerve system (Kawano et al., 2012), mediastinum (Parish and Rosenow, 2002), retroperitoneum (Caiafa et al., 2013), joint and tendon (arthrofibrosis).

Fibrosis contributes to the development of many diseases. First, many studies have demonstrated that the core mechanisms in fibrosis across various tissues and organs are similar. Wang and colleagues found that the interaction between transforming growth factor- β (TGF- β) and connective tissue growth factor signaling is required in kidney, liver, and lung fibrosis (Wang Q. et al., 2011). Makarev and colleagues identified a number of common pathways between lung

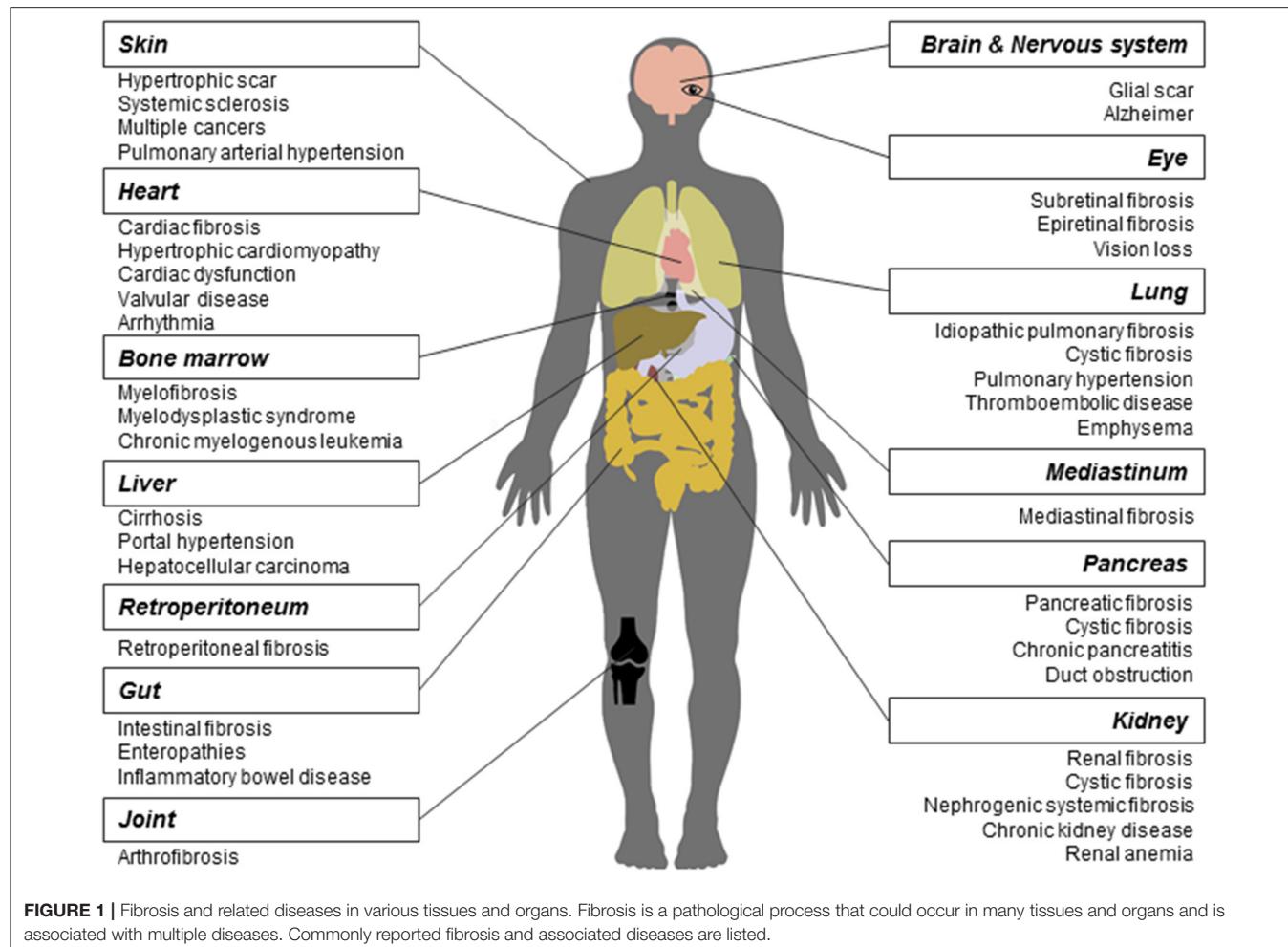


FIGURE 1 | Fibrosis and related diseases in various tissues and organs. Fibrosis is a pathological process that could occur in many tissues and organs and is associated with multiple diseases. Commonly reported fibrosis and associated diseases are listed.

and liver fibrogenesis, such as TGF- β , interleukin-6(IL-6), and integrin-linked kinase signaling (Makarev et al., 2016). Moreover, Wenzke and colleagues detected 90 genes, as well as several networks associated with connective tissue disorders, that play important roles in multi-organ fibrosis including lung, heart, liver, and kidney (Wenzke et al., 2012). On the basis of common pathogenesis across fibroproliferative diseases, some new drugs were proved effective in the treatment of fibrosis across different tissues and organs. For example, Pirfenidone has entered into

Abbreviations: ECM, extracellular matrix; IPF, idiopathic pulmonary fibrosis; NAFLD, non-alcoholic fatty liver disease; TGF- β , transforming growth factor- β ; IL-6, interleukin-6; SSc, systemic sclerosis; PDGF, platelet derived growth factor; EMT, epithelial-mesenchymal transition; HSC, hepatic stellate cell; MMPs, matrix metalloproteinases; α -SMA, α -smooth muscle actin; HGF, hepatocyte growth factor; TNF, tumor necrosis factor; STAT3, signal transducer and activator of transcription 3; TIMPs, tissue inhibitor of metalloproteinases; Hh, signaling, hedgehog signaling; mTOR, mechanistic target of rapamycin; MAPK, mitogen-activated protein kinase; PPAR- γ , peroxisome proliferator activated receptor γ ; FXR, farnesoid-X receptor; LOXL2, lysyl oxidase homolog 2; ROS, reactive oxygen species; CKD, chronic kidney disease; ET, endothelin; BMPER, BMP endothelial cell precursor-derived regulator; TCM, traditional Chinese medicines; FZHY, Fuzhenghuayu capsule; NF κ B, nuclear factor kappa-light-chain-enhancer of activated B cells; QSYQ, Qishenyiqi; QHD, Qushi Huayu Decoction. cryo-EM, cryo-electron microscopy.

the phase II clinical trial for treating the systemic sclerosis(SSc) (Khanna et al., 2016) and the phase III for IPF (King et al., 2014), respectively. Interferon drug Actimmune has been evaluated in patients with IPF (Skaria et al., 2015), liver (Muir et al., 2006), and cystic fibrosis (Moss et al., 2005).

Second, in different tissues and organs, multiple fibrotic diseases are related to each other. They are usually triggered by the same irritation and occur simultaneously. For example, heart and kidney together develop fibrosis (cardiorenal fibrosis) owing to the imbalance of natriuretic peptide system pathway and renin angiotensin aldosterone system/TGF- β 1 pathway in aging (Sangaralingham et al., 2016). Chronic or acute renal failure may induce nephrogenic systemic fibrosis developed from thickening skin (Reiter et al., 2012) to impaired internal organs. In addition, cystic fibrosis, caused by gene mutation, could widely affect multiple organs, such as lung, kidney, and pancreas. Moreover, fibrosis is frequently a common pathological process in NAFLD and inflammatory bowel disease. Replacement of heart tissues by fibrotic protein could alter the ventricle size and shape, leading to hypertrophic cardiomyopathy (Khan and Sheppard, 2006). Cancers such as hepatocellular carcinoma share a series of risk factors with liver fibrosis (De Minicis et al., 2012).

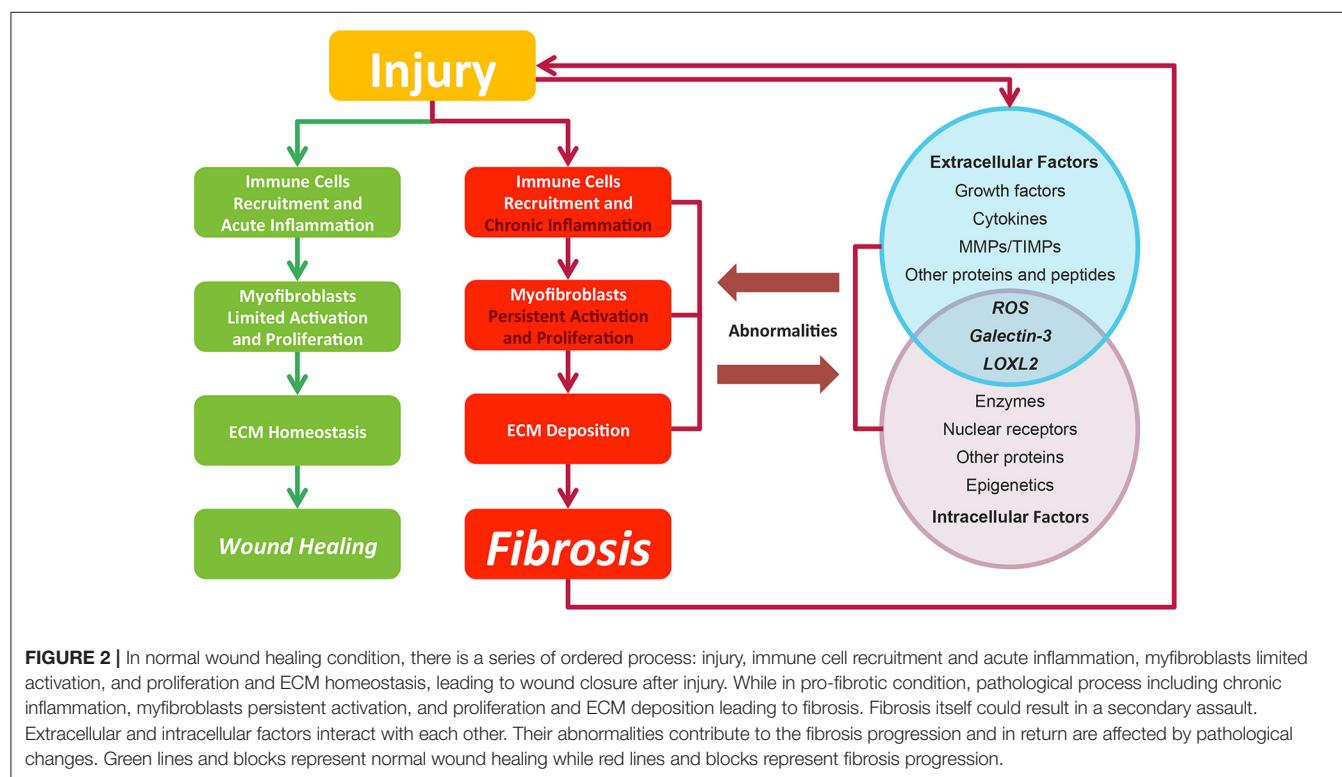
Here we review the current knowledge on the pathological mechanisms of fibrosis and systematically summarize drugs targeting fibrosis in different fibroproliferative diseases. Future directions for fibrosis drug discovery are also proposed.

PATHOGENESIS OF FIBROSIS

Fibrosis is considered as pathological outcomes of normal wound healing (Figure 2). When injuries occur and epithelial and/or endothelial cells are damaged, pro-inflammatory cytokines are released by the coagulation cascade for immune cell recruitment, mainly neutrophils and macrophages. These recruited immune cells function as the scavenger to remove tissue debris and dead cells, resulting in acute inflammation. Meanwhile, immune cells themselves release factors like chemokines and cytokines to amplify inflammatory reactions. Next, the released factors, such as TGF- β (Thannickal et al., 2003), platelet derived growth factor (PDGF) (Tang et al., 1996), interleukin-13 and interleukin-4 (Hashimoto et al., 2001), induce the limited activation and proliferation of myofibroblasts. Besides resident fibroblasts, myofibroblasts are derived from multiple cells (Hinz et al., 2007), including fibrocytes, epithelial cells via epithelial-mesenchymal transition (EMT), endothelial cells via endothelial-mesenchymal transition, pericytes, and smooth muscle cells related to blood vessels. In liver and pancreas, precursor cells like hepatic stellate cells (HSC) (Moreira, 2007) and pancreatic stellate cells (Apte et al., 2012) could also acquire myofibroblastic phenotype. Activated myofibroblasts migrate to injury sites, and their abilities to generate cell traction force enable them to stimulate wound closure (Li and Wang, 2011). Then, the balance of ECM synthesis and degradation could be achieved by myofibroblasts, resulting in ECM homeostasis. Finally, immune cells undergo apoptosis and epithelial/endothelial cells proliferate to regenerate injury sites, leading to wound healing.

In contrast to normal wound healing process, the abnormalities of multiple factors could cause fibrosis (Biernacka et al., 2011) (Figure 2). Under some persistent stimuli, the overexpression of factors like pro-inflammatory cytokines or growth factors would overactivate (Kim et al., 2008) and interact with multiple kinases or nuclear receptors. The deficiencies of some factors would also contribute to fibrosis progression (Allen and Spiteri, 2002). Then, the abnormal signaling sustains to switch normal wound healing process to pro-fibrotic process, acting on the recruitment of excess immune cells, the induction of the myofibroblasts activation and proliferation and the promotion of ECM production. Pro-fibrotic process also promotes the activation of these factors conversely, thereby amplifying inflammatory responses and causing chronic inflammation. Finally, the sustained myofibroblasts activation would generate masses of ECM and tilt the balance in favor of synthesizing ECM to produce fibrosis. Furthermore, the pro-fibrotic process itself could contribute to secondary injury to the wound and cause a chronic vicious circle of pathological responses.

Below we divide factors modulating fibrosis progression into extracellular and intracellular groups, and discuss how they influence fibrosis progression.



Extracellular Factors Mediating the Progression of Fibrosis

The majority of the fibrosis-related extracellular factors are receptor-binding ligands, such as growth factors and cytokines. These factors target adjacent and distant cells in autocrine, paracrine, or endocrine signaling pathways. Then they bind to specific receptors on cell membrane and trigger the intracellular signaling, leading to pro-fibrotic cellular responses. Other extracellular factors, mainly enzymes such as matrix metalloproteinases (MMPs) could degrade ECM to prevent its excessive accumulation.

Growth factors contain a huge family of proteins that stimulate cell growth and proliferation. They are secreted by fibroblasts, immune cells, and epithelial/endothelial cells, and are able to orchestrate cellular responses. While epithelium/endothelium are damaged, cells in these tissues massively upregulate the production of growth factors to promote the proliferation of immune cells and fibroblasts. Among growth factors, TGF- β is the "master" modulator in fibrogenesis (Meng et al., 2016), as it could provoke fibrosis through SMAD-dependent pathway (Lan, 2011) and SMAD-independent pathway related to a number of other pro-fibrotic reactions (Zhang, 2009). TGF- β signaling cascade results in differentiation of effector cells via inducing the expression of myofibroblasts hallmark α -hallmar muscle actin(α -SMA) (Sebe et al., 2008). In addition, TGF- β signaling leads to the transcription of collagen I and III genes (Fine and Goldstein, 1987; Chen et al., 1999) contributing to ECM accumulation. Interacting with TGF- β , many other growth factors have distinct roles in pro-fibrotic process. For example, PDGF induces HSC proliferation and type I collagen expression via downstream focal adhesion kinase/phosphoinositide 3-kinase/protein kinase B signaling (Reif et al., 2003). On the contrary, some growth factors have anti-fibrotic property, such as hepatocyte growth factor (HGF), the overexpression of which alleviates fibrosis in cardiomyopathic hamster through activation of MMP-1 and urokinase-type plasminogen activator (Taniyama et al., 2002). Excessive cytokines are usually secreted by immune cells, such as macrophages, neutrophils, and T cells in inflammation phase of wound healing. Compared with growth factors, cytokines tend to act as cell signaling transmitters to augment immunological responses and then lead to inflammation. One major type of cytokines is chemokines, which guide the recruitment of immune cells and fibroblasts to injury sites. Different immune cells are recruited by different chemokines. Neutrophils could be recruited by chemokine (C-X-C motif) ligand 1 and chemokine (C-X-C motif) ligand 8 by binding glycosaminoglycans with receptors in slightly different ways (Sawant et al., 2016). Chemokine (C-C motif) ligand 5 has been a major factor to induce the migration of HSC in liver fibrosis (Seki et al., 2009). Another type of cytokines is T cell cytokines, which are mainly secreted by activated T lymphocytes. They include interleukins, interferons and tumor necrosis factors (TNF), mediating adaptive immune responses and inflammation that might promote fibrosis. For example, IL-6 has been reported to shift the tissue repair to a chronic inflammatory state by signal transducer and activator of transcription 3(STAT3) signaling

pathway in peritoneal fibrosis (Fielding et al., 2014). In contrast, some cytokines have anti-fibrotic effects. It has been reported that interferon- γ down-regulated the Adenosine A2A receptor signaling to prevent the production of type I collagen in HSC (Block and Cronstein, 2010). Moreover, some T cell cytokines, such as TNF- α , exhibit two-sided effects, pro-fibrotic or anti-fibrotic effect depending on the alternative status of macrophages and micro-environment (Redente et al., 2014).

MMPs are the extracellular endopeptidases degrading ECM including collagens, proteoglycans, laminins, and fibronectin. Tissue inhibitor of metalloproteinases (TIMPs) work as the inhibitors of MMPs. The balance of MMPs and TIMPs modulates the process throughout fibrosis development, including the formation of multiple cell injuries, the activation of latent cytokines and myofibroblasts and mainly, the maintenance of the homeostasis of ECM (Giannandrea and Parks, 2014). Some MMPs have pro-fibrotic functions whereas some have anti-fibrotic according to cell types and phases. The dysregulation of MMP-19 has been proved to cause the degradation of normal liver ECM and initiate liver injury (Jirouskova et al., 2012). Conversely, MMP-2 has been reported to cleave type I collagen and attenuate collagen deposition by HSC, inhibiting liver fibrosis (Radbill et al., 2011). As to TIMPs, they could inhibit or activate fibrosis via MMPs. For example, TIMP-3 inhibits MMPs to induce inflammation (Gill et al., 2010) in lung injury, and TIMP-1 has been found to play a dual role in liver fibrosis (Wang H. et al., 2011).

Other extracellular factors include a wide range of proteins and peptides. They mainly guide the differentiation of myofibroblasts, and are closely related to each other and growth factors. For example, the hedgehog (Hh) signaling pathway mediates EMT during the fetal development, and responds to injury through the repression of epithelial marker epithelial-cadherin by *Snail* and *Twist*. Overactivation of Hh signaling pathway contributes to biliary fibrosis and related liver fibrosis (Omenetti et al., 2008). In fibrotic kidney, the upregulated Wnt signaling has been reported to result in abundant of β -catenin. The signaling regulates genes such as *Twist*, *LEF1* to induce EMT, thus to aggravate disease (He et al., 2009).

Intracellular Factors Mediating the Progression of Fibrosis

Intracellular factors, mainly multiple kinases, propagate the signaling received by cells through phosphorylation and other pathways. A common consequence of the signaling is that, transcription activators or inhibitors translocate into nucleus to regulate fibrosis-related gene expression and cell responses. In certain inflammatory pathways, intracellular factors also modulate the expression of extracellular factors, such as growth factors and cytokines, and secrete them out of cells to amplify inflammatory responses. Besides, epigenetic factors are emerging as a new way to affect fibrosis-related gene expression.

Intracellular factors include a number of kinases. In inflammation phase, many upstream factors, such as TGF- β , TNF- α , and epidermal growth factors initiate mitogen-activated

protein kinase (MAPK) pathway. An element of MAPK pathway, mitogen-activated protein kinase-activated protein kinase-2 mediates myofibroblasts differentiation and regulates the gene expression of several matrix proteins such as *col1a2*, *col3a1*, and *lox* (Vittal et al., 2013). Another important intracellular signaling, mechanistic target of rapamycin (mTOR) pathway is activated by Wnt and TNF- α . Then, mTOR pathway activates ribosomal protein S6 kinase β -1 and modulates protein p21 or p27, which regulates the cell cycle of many cells including fibroblasts. In addition, the inhibition of mTOR pathway reduces collagen deposition and cardiac fibrosis (Chen et al., 2012). Besides serving as amplifiers of signal transduction, some intracellular enzymes such as cathepsin K, which belongs to lysosomal cysteine proteases, have been proved to be able to degrade ECM in lysosome after phagocytosis (Fukumori et al., 2003; Buhling et al., 2004).

Nuclear receptors are receptors located in cytoplasm and nucleus that could receive signals from intracellular ligands and bind to DNA to regulate gene expression. For example, peroxisome proliferator activated receptor γ (PPAR- γ), may directly regulate type I collagen gene (Yang et al., 2006) and block TGF- β signaling (Ghosh et al., 2009). Another nuclear receptor, farnesoid-X receptor (FXR), exhibits anti-fibrotic effect via the reduction of proliferating cholangiocytes and subsequent reduction of TGF- β (Liu et al., 2003). The activation of FXR also decreases a series of pro-fibrotic factors including TIMP-1, collagens, α -SMA, and MMP-2 (Zhang et al., 2009).

Nowadays, epigenetics including microRNAs, DNA methylation and lncRNAs, are found involved in machinery of pro-fibrotic process mainly through regulating fibrosis-related gene expression. Some microRNAs are found to negatively regulate translation of ECM components. Among them, miR-21 induces extracellular-signal regulated kinase/MAPK activity via the inhibition of *Spry1* to protect cardiac fibroblasts survival (Thum et al., 2008). It has been reported that the levels of DNA methylation at specific CpG sites of pro-fibrotic genes (*PPAR α* , *PPAR δ* , *TGF β 1*, *Collagen1A1*, and *PDGF α*) differ among different fibrosis stages in NAFLD (Zeybel et al., 2015).

There are factors that affect fibrosis process both extra- and intracellularly, including reactive oxygen species (ROS), galectin-3 and lysyl oxidase homolog 2 (LOXL2). ROS can be generated through tissue injuries, cell damages and NADPH oxidase activities. Extracellular ROS targets latency-associated peptides and then activates TGF- β signaling while intracellular oxidative stress induces p53-dependent apoptosis in lung fibrosis via the caspases-9/3 activation in mitochondria (Cheresh et al., 2013). As to galectin-3, extracellular galectin-3 induces T cell apoptosis and plays a dual function inside and outside cells (Li et al., 2014). Another factor LOXL2 is generally considered as extracellular enzyme that promotes collagen production and crosslink with collagen fibers in response to mechanical stress (Yang et al., 2016). While on the other hand, intracellular LOXL2 has been reported to induce EMT in carcinoma progression (Peinado et al., 2005).

Many studies proved that different fibroproliferative diseases share common underlying mechanisms (Wenzke et al., 2012). The existence of common mechanisms facilitates the complete

interpretation of fibrosis pathogenesis and enhances our understanding of fibrosis-related diseases. On this basis, it is necessary to reconsider targets involved in these mechanisms and evaluate their potential roles in fibrosis treatment across tissues and organs.

DRUGS AND TARGETS IN FIBROSIS

Motivated by huge clinical burdens, continuous intense researches on drug targeting fibrosis have been conducted, many of which have led to clinical trials. Due to the strong associations between inflammation and fibrosis, more efforts have been devoted to anti-inflammation drugs in the past few years (Dinwiddie, 2005). Nowadays, new targets and drugs for fibrosis are constantly emerging with the progress in understanding fibrosis pathology. Here we summarize them in Tables 1–4, including single (Tables 1–3) and multi component (Table 4) drugs with their verified and potential targets in fibrosis.

Single-Component Drugs Targeting Extracellular Factors Mediating Fibrosis

Nowadays, most approved and investigational drugs are single-component drugs, which only contain one organic component and have distinct targets. As we described previously, fibrosis progression results from a combination of the abnormalities of extracellular and intracellular factors. Drugs targeting extracellular factors are prevalent, about 60% of known targets are receptors located on cell membrane mainly because the extracellular targets are accessible and serve as upstream signals (Overington et al., 2006), and so are the targets of fibrosis-related drugs. The binding of receptors and ligands triggers the downstream signaling, thus the blockade of receptors or ligands is considered to be an effective choice to alleviate fibrosis (Table 1).

The majority of anti-fibrosis drugs targeting extracellular factors are inhibitors of ligands such as growth factors, cytokines and MMPs. Most inhibitors could directly bind to the active sites of targets. The majority of approved inhibitor drugs in Table 1 target TNF, which are widely used in fibrosis-related diseases and could suppress the action of TNF through multiple mechanisms. These drugs include small molecules Thalidomide and Pomalidomide (Weingartner et al., 2012), recombinant protein Etanercept and monoclonal antibodies Belimumab. Thalidomide and Etanercept have completed the phase II trial in IPF (Raghu et al., 2008; Horton et al., 2012) while Belimumab has completed the phase II trial in SSc. Besides, some inhibitor drugs targeting growth factors are still under investigation. For example, Disitertide, a synthetic peptide derived from TGF- β type III receptor, inhibits the binding of TGF- β and its receptor and exhibit anti-fibrotic function (Ezquerro et al., 2003). Drugs that inhibit interleukins are always monoclonal antibodies. Tralokinumab, a human IgG4 monoclonal antibody, shows pro-apoptotic effects via IL-induced apoptotic factors in IPF (Murray et al., 2014). Many natural products act as inhibitor drugs like Dioscin, which is a monomer extracted from *Dioscoreae Rhizoma* and could ameliorate liver fibrosis (Liu et al., 2015; Zhang et al., 2015a,b; Gu et al., 2016; Xu et al., 2017; Yin et al.,

TABLE 1 | Single-component drugs targeting extracellular factors.

Group	Target		Organ ^a	Drug Name	Mechanism	Class	Disease	Clinical trial ^b		Reference/Trial identifier ^c
	Target or mechanism type	mechanism						Phase	2(unknown)	
Growth factors	Extracellular TGF- β signaling	TGF- β ^d	Liver, Kidney, Lung, Heart, Pancreas, Skin, Gut	SHP-627 (FT011)	Inhibitor	Small molecule	Cardiac fibrosis	Preclinical	Zhang et al., 2012	
				Hydronidone (F351)	Inhibitor	Small molecule	Liver fibrosis	2(unknown)	NCT02499562	
		PXS-25			Inhibitor	Small molecule	IPF ^d	Preclinical	Maldonado et al., 2009; Wong et al., 2011	
		Disitertide (P-144)			Inhibitor	Small molecule	Skin fibrosis	2(completed)	NCT00574613	
		Fresolimumab (GC-1008)			Inhibitor	Monoclonal antibody	IPF; SSc ^d	1(completed); 1(completed)	NCT00125385; NCT01284322	
		LY2382770			Inhibitor	Monoclonal antibody	Diabetic kidney disease	2(terminated)	NCT01113801	
		STX-100			Inhibitor	Monoclonal antibody	IPF	2(completed)	NCT01371305	
		CWHM-12			Inhibitor	Small molecule	Liver fibrosis; Lung fibrosis	Preclinical	Henderson et al., 2013	
		ALK5 ^d		SB-431542	Antagonist	Small molecule	Pulmonary fibrosis	Preclinical	Koh et al., 2015	
		BMP-7 ^d		THB-184	Agonist	Small molecule	Renal fibrosis	2(completed)	NCT01830920	
		CTGF		PF-06473871	Inhibitor	Small molecule	Hypertrrophic scar	2(completed)	NCT01730339	
				RXI-109	Inhibitor	Small molecule	Hypertrrophic scar	2(completed)	NCT02030275	
				FG-3019	Inhibitor	Monoclonal antibody	IPF	2(active, not recruiting)	NCT01890265	
				Imatinib	Antagonist	Small molecule	Nephrogenic systemic fibrosis; SSc; IPF	Approved; 2(completed); 3(completed)	NCT00677092; NCT00613171; NCT00131274	
				BOT-191	Antagonist	Small molecule	Liver fibrosis	Preclinical	van Dijk et al., 2015	
				Nilotinib (AMN-107)	Antagonist	Small molecule	SSc	Approved	NCT01166139	
				Dasatinib	Antagonist	Small molecule	Scleroderma pulmonary fibrosis	2(completed)	NCT00764309	
				Nintedanib (BIBF-1120)	Antagonist	Small molecule	Scleroderma; IPF	Approved	NCT02597933; NCT01335464	
		VEGFR ^d / PDGFR		Sorafenib (BAY 43-9006)	Antagonist	Small molecule	Extensive keloids	Approved	NCT01425216	
		TNF ^d		Thalidomide	Inhibitor	Small molecule	IPF	Approved	NCT00162760	
				Pomalidomide	Inhibitor	Small molecule	IPF	Approved	NCT01135199	

(Continued)

TABLE 1 | Continued

Group	Target or mechanism type	Target or mechanism	Organs ^a	Drug Name	Mechanism	Class	Disease	Clinical trial ^b		Reference identifier ^c
								Phase	Reference	
Cytokines	Interleukin	IL-13 ^d	Liver, Kidney, Heart, Pancreas, Skin, Gut	Etanercept	Inhibitor	Recombinant protein	IPF	Approved 2(terminated)	NCT0063869	
	HGF ^d	HGF	Liver, Kidney, Lung, Heart, Skin	Belimumab	Inhibitor	Monoclonal antibody	SSc	Approved 2(terminated)	NCT01670565	
				Refanalin (BB-3)	Stimulant	Small molecule	Liver fibrosis; IPF	Preclinical	Fallowfield, 2011	
	IL-1R1	IL-1R1	Liver, Kidney, Lung, Heart, Skin, Gut	Dectrekuumab (QAX-576)	Inhibitor	Monoclonal antibody	IPF; IPF secondary to SSc	2(terminated); 2(terminated)	NCT01266135; NCT00581997	
	IL-1 β R	IL-1 β R	Anakinra	Takolkinumab	Inhibitor	Recombinant protein	Cystic fibrosis	Approved Preclinical	Iannitti et al., 2016	
			Rilonacept	Anakinra	Antagonist	Recombinant protein	SSc	Approved 2(active, not recruiting)	NCT01538719	
				SAR156597	Inhibitor	Monoclonal antibody	SSc; IPF	2(recruiting); 2(completed)	NCT02921971; NCT01529853	
	CC chemokine	CCL2 ^d	Liver, Kidney, Lung, Heart, Pancreas, Skin, Gut	Carlumab (CINTO-888)	Inhibitor	Monoclonal antibody	IPF	2(completed)	NCT00786201	
				Bindarit	Inhibitor	Small molecule	Myocardial fibrosis; Renal fibrosis	Preclinical	Lin et al., 2009; Zhu et al., 2009	
	CCR5 ^d	CCR5 ^d	Liver, Kidney, Lung	Maraviroc	Antagonist	Small molecule	Liver fibrosis	Approved Preclinical	Gonzalez et al., 2014	
				RS-504393	Antagonist	Small molecule	Renal fibrosis	Preclinical	Kitagawa et al., 2004	
	Interferon	IFN- γ R ^d	Liver, Kidney, Lung, Heart, Pancreas, Skin, Gut	Actimmune	Stimulant	Interferon	IPF; Liver fibrosis; Cystic fibrosis	Approved 3(completed); 2(completed); 2(completed)	NCT00047658; NCT00043303; NCT00043316	
				IFN- α	Interferon alpha oral lozenge	Stimulant	Interferon	2(completed)	NCT01442779	
	MMP ^d /TIMP ^d	MMP/TIMP	Liver, Kidney, Lung, Heart, Pancreas, Skin, Gut	Batimastat (BB-49)	Inhibitor	Small molecule	IPF	Preclinical	Corbel et al., 2001	
				Marimastat	Inhibitor	Small molecule	Liver fibrosis	Approved Preclinical	de Meijer et al., 2010	

(Continued)

TABLE 1 | Continued

Group	Target or mechanism type	Target or mechanism	Organ ^a	Drug Name	Mechanism	Class	Drug		Clinical trial ^b	Phase	Reference/Trial identifier ^c
							Disease	Reference			
Other proteins and peptides	Endothelin	ET-1 receptor ^d	Liver; Kidney, Lung, Heart, Skin, Gut	Macitentan	Antagonist	Small molecule	IPF	Approved, 2(completed)	NCT00903331		
	Bosentan	Antagonist	Small molecule	IPF; SSc;				Approved, 3(completed); 3(completed); 4(completed)	NCT0070590; NCT00319896; NCT01395732		
	Ambrisentan	Antagonist	Small molecule	IPF; SSc				Approved, 3(terminated); 4(unknown)	NCT00879229; NCT01051960		
	Sparsentan (RE-021)	Antagonist	Small molecule	Focal segmental glomerulosclerosis				2(active, not recruiting)	NCT01613118		
	Atrasentan	Antagonist	Small molecule	Renal fibrosis				Preclinical	Samad et al., 2015		
	Losartan	Antagonist	Small molecule	Liver fibrosis; Cystic fibrosis				Approved, 4(completed); 2(not yet recruiting)	NCT00298714; NCT03206788		
Angiotensin II	AT1 receptor ^d	Liver; Kidney, Lung, Heart, Pancreas, Skin, Gut	BMS-986020	Antagonist	Small molecule	SSc; IPF		2(withdrawn); 2(completed)	NCT02588625; NCT01766817		
	LPARD	Liver, Kidney, Lung, Skin	SAR-100842	Antagonist	Small molecule	SSc		2(completed)	NCT01651143		
	PAR1 ^d	Liver; Kidney, Lung, Heart, Pancreas, Skin	PAR1 antagonist	Antagonist	Small molecule	Liver fibrosis		Preclinical	Fiorucci et al., 2004		
	CB1 receptor ^d	Liver	Curcumin*	Antagonist	Small molecule	Liver fibrosis; Renal fibrosis; IPF		Preclinical	Smith et al., 2010; Zhang et al., 2013; Sun et al., 2017		
	Silymarin*	Antagonist	Small molecule	Liver fibrosis				Preclinical	Tsai et al., 2008; Zhang et al., 2013		
	CB2 receptor ^d	β -caryophyllene*	Agonist	Small molecule	Liver fibrosis			Preclinical	Calleja et al., 2013; Mahmoud et al., 2014		
	Prostacyclin receptor	Liver, Kidney, Lung, Heart, Pancreas	Beraprost	Agonist	Small molecule	Renal fibrosis; Cardiac fibrosis		Preclinical	Chen et al., 2014		
		Iloprost	Agonist	Small molecule	IPF; SSc			Approved, 2(completed)	NCT00109681		
		Treprostinal	Agonist	Peptide hormone	Cystic fibrosis			Approved, 2(terminated); 2(completed)	NCT00703339; NCT00775463		
VIP receptor	Lung	Aviptadil	Agonist	Small molecule	IPF			Preclinical	Mathioudakis et al., 2013		
Leukocyte elastase TAFI ^d	Leukocyte elastase	Sivelestat	Inhibitor						Takemasa et al., 2012		
	TAFI	Liver, Kidney, Lung	UK-396082	Inhibitor	Small molecule	Renal fibrosis		Preclinical	Atkinson et al., 2015		

(Continued)

TABLE 1 | Continued

Group	Target or mechanism type	Target or mechanism	Organs ^a	Drug Name	Mechanism	Class	Disease	Clinical trial ^b	Phase	Reference/Trial identifier ^c
Relaxin	Relaxin receptor	Liver, Kidney, Lung, Heart, Skin	Serelaxin	Stimulant	Peptide hormone	Cardiac fibrosis; Renal fibrosis	Preliminary	Samuel et al., 2014; Huskies et al., 2015		
SAP ^d	SAP (mimic)	PRM-151		Stimulant	Recombinant protein	IPF	2/active, not recruiting)	NCT02550873		
Integrin α	Integrin α5	Liver	Diosoin*	Inhibitor	Small molecule	Liver fibrosis	Preliminary	Liu et al., 2015; Zhang et al., 2015a,b; Gu et al., 2016; Xu et al., 2017; Yin et al., 2017		
TGM ^d	TGM2	Kidney, Lung	NTU281	Inhibitor	Small molecule	Renal fibrosis	Preliminary	Johnson et al., 2007		

*Drug belongs to monomer extracted from natural products.

^aOrgans that had study report of corresponding targets in fibrosis treatment.

^bClinical trial resource are from <http://ClinicalTrials.gov>.

^cTrial Identifier is the clinical trial identifier of corresponding drug
^dTGF-β, transforming growth factor-β; IPF, idiopathic pulmonary fibrosis; SSc, systemic sclerosis; ALK5, TGF-β receptor 1; BMP-7, bone morphogenetic protein 7; CTGF, connective tissue growth factor; PDGF, platelet-derived growth factor; PDGFR, platelet-derived growth factor receptor; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor; IFN-γR, interferon-γ receptor; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase; ET-1 receptor, endothelin-1 receptor; AT1 receptor, angiotensin II receptor type 1; LPAR, lysophosphatidic acid receptor; PAR1, protease-activated receptor 1; CB1 receptor, cannabinoid receptor type 1; TAFI, thrombin activatable fibrinolysis inhibitor; SAP, serum amyloid P; VIP, vasoactive intestinal peptide; TGM2, transglutaminase.

2017). In addition, some preclinical drugs inhibit MMPs, like Marimastat, which simultaneously down-regulates MMPs gene expression and MMPs activities. However, Marimastat reduces inflammation and liver injuries while increases fibrosis in mice model. This may result from the indiscriminative inhibition of MMPs, some of which function to degrade ECM (de Meijer et al., 2010).

Compared with inhibitor drugs, antagonists achieve the same inhibition effect by targeting cell membrane receptors to dampen downstream signaling. These small molecules bind to receptors without effectively activating them. The receptors of PDGF, vascular endothelial growth factor, endothelin (ET), and angiotensin all have approved antagonist drugs. An antagonist for tyrosine kinase receptors of PDGF, Imatinib, has showed protective effect by reducing differentiation of resting fibroblasts in SSc mice model (Akhmetshina et al., 2009). However, it did not show efficacy in phase II clinical trial in IPF (Daniels et al., 2010) and the high-dose of Imatinib may lead to severe adverse events (Khanna et al., 2011). Macitentan, a dual antagonist of ET_A and ET_B receptor, is beneficial for lung fibrosis. Similarly, another drug Losartan targeting angiotensin II receptor has been evaluated in IPF patients (Couluris et al., 2012). Some preclinical antagonist drugs including Maraviroc (Gonzalez et al., 2014), Atrasentan (Ritter et al., 2014), and PAR1 antagonists (Fiorucci et al., 2004) were under investigation for fibrosis treatment.

On the contrary, many drugs exert their therapeutic effects by activating their targets. Many anti-fibrotic receptors can be targets of these exogenous agonists that augment the downstream biological responses to suppress fibrosis. An approved agonist drug in this category is Iloprost, which can reverse right ventricle fibrosis by re-establishing collagen balance (Gomez-Arroyo et al., 2015). Another agonist of vasoactive intestinal peptide, Treprostinil, reduces inflammation and collagen deposition (Manitsopoulos et al., 2015). Other anti-fibrotic agonists for cell membrane receptors include Aviptadil, INT-767 (Baghdasaryan et al., 2011) and Beraprost (Kaneshige et al., 2007).

Moreover, a few drugs are synthetic proteins that bind receptors to serve as stimulants and perform the same functions as native proteins. An approved drug, synthetic interferon-γ, Actimmune, has completed phase II or phase III study in multiple fibrosis including IPF (Skaria et al., 2015), liver fibrosis (Muir et al., 2006), and cystic fibrosis (Moss et al., 2005). Another stimulant Refanalin, a HGF mimetic, is a potential drug for liver fibrosis (Fallowfield, 2011; Pellicoro et al., 2014).

Single-Component Drugs Targeting Intracellular Factors Mediating Fibrosis

Compared with extracellular factors, intracellular targets are less popular owing to their inaccessibility. Drugs targeting intracellular factors are less varied because most of them are small molecules. Small molecules could readily translocate into cytoplasm while large molecules such as monoclonal antibodies face more challenges to cross the plasma membrane (Imai and Takaoka, 2006). Nevertheless, more and more studies concerned intracellular factors as targets in recent years, and numerous candidate targets are identified in cytoplasm, nucleus,

TABLE 2 | Single-component drugs targeting intracellular factors.

Group	Target or mechanism type	Target		Organ ^a	Drug Name	Mechanism	Class	Disease	Phase	Clinical trial ^b	Reference/Trial identifier ^c
		Target or mechanism	Organ ^a								
Enzymes	mTOR ^d	mTORC1/2 ^d	Liver; Kidney, Lung, Heart, Skin, Gut	Rapamycin (Sirolimus)*	Inhibitor	Small molecule	Renal interstitial fibrosis	Renal interstitial fibrosis	Approved 3(completed)	NCT01079143	
JAK-STAT ^d	JAK1/JAK2 ^d			Palomid-529 (RES-529)	Inhibitor	Small molecule	Macular degeneration	Macular degeneration	1(completed)	NCT01033721	
				Ruxolitinib	Inhibitor	Small molecule	Myelofibrosis	Myelofibrosis	Approved 3(completed)	NCT00952289	
PI3K-Akt ^d	Akt	Liver; Kidney, Lung, Heart, Skin	Ompalisib (GSK2126458)	Baricitinib	Inhibitor	Small molecule	Renal interstitial fibrosis	Renal interstitial fibrosis	Precclinical	Breyer and Susztak, 2016	
FAK1 ^d	FAK1 ^d	Liver; Kidney, Lung, Heart, Pancreas, Skin	PF-562271	Inhibitor	Inhibitor	Small molecule	IPFd	IPFd	1(completed)	NCT01725139	
JNK ^d	JNK ^d			Tanzisertib (CC-930)	Inhibitor	Small molecule	Pulmonary fibrosis; Cardiac fibrosis; Liver fibrosis	Pulmonary fibrosis; Cardiac fibrosis; Liver fibrosis	Precclinical	Lagares et al., 2012; Fan et al., 2015; Zhao et al., 2017	
MAPK ^d	MAPK	Liver; Kidney, Lung, Heart, Pancreas, Skin, Gut	MMI-0100	Inhibitor	Inhibitor	Small molecule	IPF	IPF	2(terminated)	NCT01203943	
NF-κB ^d	IκB ^d			IMD-1041	Inhibitor	Small molecule	Cardiac fibrosis	Cardiac fibrosis	Precclinical	Xu et al., 2014	
				Bardoxolone methyl (CDDO-Me)	Inhibitor	Small molecule	Pulmonary hypertension	Pulmonary hypertension	2(recruting)	NCT02036970	
NF-κB	Antisense NF-κB			Antisense NF-κB	Inhibitor	Antisense oligonucleotide	Intestinal fibrosis	Intestinal fibrosis	Precclinical	Tanaka et al., 2012	
				Baicalin*	Inhibitor	Small molecule	Renal fibrosis; IPF	Renal fibrosis; IPF	2(recruting)	NCT02036970	
				Sulfasalazine	Inhibitor	Small molecule	Pancreatic fibrosis	Pancreatic fibrosis	Precclinical	Lawrence et al., 2003	
cAMP-PKA ^d	ROCK ^d			Y-27632	Inhibitor	Small molecule	Renal fibrosis; Liver fibrosis	Renal fibrosis; Liver fibrosis	Approved 2(recruting)	Gao et al., 2013; Wang et al., 2015	
Non-kinase enzyme	26S protease Caspase	Liver; Kidney, Lung, Heart, Skin	Bortezomib Emricasan	Inhibitor Inhibitor	Inhibitor Inhibitor	Small molecule Small molecule	SSc pulmonary fibrosis	SSc pulmonary fibrosis	Precclinical	Chavez et al., 2012; Wang et al., 2016 Tada et al., 2001	
				VX-166 Z-VAD-fmk CTP-499						NCT02370693	
PDE ^d	PDE ^d	Kidney, Heart									Barreyro et al., 2015
											Witek et al., 2009
											Kuwano et al., 2001
											NCT01328281

(Continued)

TABLE 2 | Continued

Group	Target or mechanism type	Target		Drug Name	Mechanism	Class	Clinical trial ^b		Reference identifier ^c
		Target or mechanism	Organs ^a				Phase	Reference	
Cathepsin B	Liver, Lung, Heart, Pancreas	VBY-376	Inhibitor	Small molecule	Liver fibrosis	Precclinical	Approved	NCT00492700	Alkhouri et al., 2011
CA-074Me		CA-074Me	Inhibitor	Small molecule	Pancreatic fibrosis; Cardiac fibrosis; Pulmonary fibrosis	Precclinical	2(completed)	NCT01487551	Lerch and Halangk, 2006; Liu et al., 2013; Zhang et al., 2015
S100A9	Liver, Lung, Heart, Skin	Paquinimod	Inhibitor	Small molecule	SSc ^d	2(completed)	Approved	NCT0198301	Matsuura et al., 1997
Procollagen-proline dioxygenase	Liver, Lung	HOE-077	Inhibitor	Small molecule	Liver fibrosis	Precclinical	3(recruiting)	NCT02704403	
Nuclear receptors	PPAR-γ	Liver, Kidney, Lung, Heart, Pancreas, Skin, Gut	Rosiglitazone	Agonist	Small molecule	Liver fibrosis	3(recruiting)	NCT03061721	
		Elatibranor (GFT-505)	Agonist	Small molecule	Liver fibrosis	2(recruiting)	Approved	NCT00719381;	
		Saroglitazar	Agonist	Small molecule	Ostic fibrosis;	1(completed);	1(completed)	NCT01454336	
		Pioglitazone	Agonist	Small molecule	Liver fibrosis	Precclinical	Approved	Zhang et al., 2009	
						Preclinical	Approved	NCT02548351	
						Preclinical	Approved	NCT01978301	
FXR ^d	FXR	Liver, Kidney, Lung, Gut	INT-767	Agonist	Small molecule	Pulmonary fibrosis; Liver fibrosis	3(recruiting)	Zhao et al., 2014	
		PX-102	Agonist	Small molecule	Liver fibrosis	Precclinical	Approved	Baghdasaryan et al., 2011	
		Obeticholic acid* (INT-747)	Agonist	Small molecule	Liver fibrosis	Precclinical	Approved	Ali et al., 2015	
		Turofexorate isopropyl (WAY-362450)	Agonist	Small molecule	Keloid scarring	Precclinical	Approved		
		GW4064	Agonist	Small molecule	Liver fibrosis	Precclinical	Approved		
		Triamcinolone	Agonist	Small molecule	Pulmonary fibrosis; Liver fibrosis	Precclinical	Approved		
GR ^d	GR	Liver, Lung, Heart, Skin	Genistein*	Agonist	Small molecule	IPF; SSc	1(terminated)	Salas et al., 2008;	
ER ^d	ERβ	Liver					Approved	NCT00287729;	
Other proteins	Intracellular TGF-β ^d signaling	SMAD2/3					3(completed);	NCT0193334-	
							2(completed)		

(Continued)

TABLE 2 | Continued

Group	Target or mechanism type	Target	Target or mechanism ^a	Organs ^a	Drug			Clinical trial ^b	Reference
					Drug Name	Mechanism	Class	Disease	Phase
Epigenetics	miRNA methylation	SMAD3	Pentoxyfylline SIS-3 Glycyrrhizin*	Inhibitor Inhibitor	Small molecule Small molecule	Skin fibrosis Renal fibrosis Liver fibrosis	Approved 2(completed) Precclinical 3(terminated)	NCT00001437 NCT00886881	Liu et al., 2010; Chau et al., 2012 NCT02231333
			Anti-miR-21	Inhibitor	Oligonucleotide	IPF; Renal fibrosis Liver fibrosis	Precclinical		
			Transmethylation	Ademetionine (SAM)	Small molecule		Approved Unknown		
			BMPER (gene)	DNA methylation	Methylation	IPF	Precclinical		Huan et al., 2015

^aDrug belongs to monomer extracted from natural products.^bOrgans that had study report of corresponding targets in fibrosis treatment.^cClinical trial resource are from <http://ClinicalTrials.gov>.^dTrial identifier is the clinical trial identifier of corresponding drug.

^aOrganic target of rapamycin complex 1; JAK-STAT, janus kinase/signal transducers and activators of transcription; PI3K-Akt, phosphoinositide 3-kinase/protein kinase B; IPF, idiopathic pulmonary fibrosis; FAK1, focal adhesion kinase 1; MAPK, mitogen-activated protein kinase; JNK, c-Jun N-terminal kinases; NF- κ B, nuclear factor kappa-light-chain-enhancer of activated B cells; IKK, I- κ B kinase; cAMP-PKA, cyclic AMP-protein kinase; ROCK, rho-associated protein kinase; PDE, phosphodiesterase; SSc, systemic sclerosis; PPAR, peroxisome proliferator-activated receptor; FXR, farnesoid X receptor; GR, glucocorticoid receptor; ER, estrogen receptor; TGF- β , transforming growth factor- β .

and mitochondrion. Many approaches, including increasing membrane permeation, combination with supercharged proteins and activating transport through receptors, were implemented to deliver drugs across cell membrane (Mitrugoti et al., 2014).

Fibrosis drugs targeting intracellular factors are summarized and classified into four categories: enzymes, nuclear receptors, other proteins, and epigenetics (Table 2). Many drugs acting through intracellular factors are also inhibitors. These drugs inhibit a wide range of kinases located in cytoplasm, and consequently suppress the translocation of transcription factors that drive the expression of pro-fibrotic genes. Rapamycin and Sirolimus are approved drugs that inhibit mTOR. Rapamycin prevents the activation of macrophages and myofibroblasts and the subsequent release of TGF- β in chronic kidney disease (CKD) (Chen et al., 2012). Sirolimus shows anti-inflammatory and anti-fibrotic effects in IPF (Tulek et al., 2011). Besides kinases, there are many other intracellular proteins that serve as potential targets for fibrosis management. Pirfenidone, one of the IPF drugs, has completed phase III trial in IPF patients with alleviated disease progression and acceptable side effects (King et al., 2014). The potential mechanism of Pirfenidone is inhibiting the nuclear accumulation of intracellular proteins SMAD2/3 to regulate TGF- β signaling (Choi et al., 2012). Other approved inhibitor drugs include Ruxolitinib for bone marrow fibrosis (Wilkins et al., 2013), Paquinimod for SSc (Stenstrom et al., 2016), and Pentoxifylline (Okunieff et al., 2004) combined with vitamin E (Jacobson et al., 2013).

In addition, there are also some nuclear receptors located in cytoplasm and nucleus, which could be activated by small molecule agonists, such as PPAR. Rosiglitazone, a PPAR- γ agonist, has anti-fibrotic effect as a consequence of activating MMP-1 and elevating HGF expression in patients with systemic sclerosis-related interstitial lung disease (Bogatkevich et al., 2012). Other approved PPAR targeting drugs like Elafibranor and Pioglitazone, have entered phase II and phase I studies, respectively. Obeticholic acid is an approved drug entering phase III study that decreases inflammation and fibrosis in NASH patients via activating FXR signaling (Verbeke et al., 2016).

Epigenetics are very different types of fibrosis therapies. The most studied epigenetics-based therapy for fibrosis is microRNA. MicroRNA could be neutralized by specific anti-miRNA oligonucleotides delivered into cells. Among them, anti-miR-21 has been reported to inhibit miR-21 activity and ameliorate fibrosis progression through PPAR signaling in CKD (Chau et al., 2012). Another strategy is the intervention of DNA methylation for proteins such as BMP endothelial cell precursor-derived regulator (BMPER), which acts as the regulator of fibroblasts activation. Altering methylation on BMPER gene has been reported to decrease BMPER level and thus to inhibit fibroblasts activity (Huan et al., 2015). Besides, some drugs targeting both extracellular and intracellular factors are also incorporated (Table 3). A majority of them are antioxidants, including an approved drug N-acetylcysteine (Zhang et al., 2014).

Multi-Component Drugs Used for Fibrosis

Differed from single-component drugs that target a single protein or other simple targets, multi-component drugs contain

TABLE 3 | Single-component drugs targeting both extra- and intracellular factors.

Target or mechanism type	Target or mechanism	Target	Organ ^a	Drug Name	Mechanism	Class	Disease	Clinical trial ^b		Reference identifier ^c
								Phase	Reference	
LOX ^d	LOXL2 ^d	Liver, Kidney, Lung, Heart, Skin, Gut	β-aminopropionitrile (BAPN)	Inhibitor	Small molecule	Cardiac fibrosis	Preclinical	2 completed;	Martinez-Martinez et al., 2016	
		Simtuzumab (GS-6624)	Inhibitor	Monoclonal antibody	Liver fibrosis; IPF ^d	Liver fibrosis; IPF ^d	2(terminated)	NCT01452308; NCT01769196		
ROS ^d	NOX1 ^d / NOX4	Liver, Kidney, Lung, Heart, Pancreas, Skin	GM-CT-01	Inhibitor	Polymer	Liver fibrosis	Preclinical		Traber and Zommer, 2013	
			GR-MD-02	Inhibitor	Polymer	Renal fibrosis	2(completed)	NCT02421094		
		GCS-100	Inhibitor	Polymer	Small molecule	Liver fibrosis	2(completed)	NCT01843790		
		GKT137831	Inhibitor	Small molecule	IPF	Preclinical				
		N-acetylcysteine*	Inhibitor	Small molecule	IPF	Approved		Aoyama et al., 2012		
	Mitochondria	Inhibitor	Small molecule	Liver fibrosis	Preclinical			Dermets et al., 2005; Zhang et al., 2014		
	Salvianolic acid B*	Inhibitor	Small molecule	Liver fibrosis; Renal fibrosis; IPF	Preclinical			Vilaseca et al., 2017		
			Resveratrol*	Inhibitor	Small molecule	Liver fibrosis	3(completed)	Liu et al., 2002, 2016; Pan et al., 2011		
	Vitamin (mimic)	Pyridoxamine Liver, Kidney, Lung, Heart, Pancreas, Skin, Gut	α-tocopherol	Inhibitor	Small molecule	Renal fibrosis	2(completed)	NCT02930977; NCT00320060		
	Collagen (mimic)	Liver, Kidney, Lung, Skin	W001	Inhibitor	Collagen	IPF	Approved Preclinical	Deger et al., 2007		
							1(completed)	NCT01199887		

^aDrug belongs to monomer extracted from natural products.^aOrgans that had study report of corresponding targets in fibrosis treatment.^bClinical trial resource are from <http://ClinicalTrials.gov>.^cTrial identifier is the clinical trial identifier of corresponding drug.^dLOX, lysyl oxidase; LOXL2, lysyl oxidase homolog 2; IPF, idiopathic pulmonary fibrosis; ROS, reactive oxygen species; NOX1, NADPH oxidase 1.

TABLE 4 | Multi-component drugs.

Drug Name	Drug	Clinical trial ^a		Reference
		Disease	Phase	
Fuzhenghuayu capsule (FZHY)	TGF- β ^c /MMP-2 ^c	Liver fibrosis	2(completed); 4(recruiting)	NCT00854087; NCT02241616
Qishenyiqi (QSYQ)	TNF ^c /TGF- β / β -Catenin	Ischemic heart failure	2(recruiting)	NCT02875639
Qushi Huayu Decoction (QHD)	ROS ^c	Liver fibrosis	Preclinical	Feng et al., 2017
Herbal compound 861 (Cpd 861)	TGF- β /MMP-1/TIMP-1 ^c	Liver fibrosis	Preclinical	Hou et al., 2016
Xiao-Chai-Hu Tang (XCHT)	IL-6 ^c /TNF- α /Bax protein	Liver cancer	2(completed)	NCT00040898; Zhou et al., 2012
Dahuangzhechong pill (DHZCP)	α -SMA ^c /TNF- α /IL-13/p38 MAPK ^c /ERK ^c	Liver fibrosis	Preclinical	Cai et al., 2010
Han-dan-gan-le	ROS/collagen	Liver fibrosis	Preclinical	Li et al., 1998
Qianggan-Rongxian Decoction	—	Liver fibrosis	Preclinical	Li et al., 2008
Yi-gan-kang granule	type I collagen/TIMP-1	Liver fibrosis	Preclinical	Yao et al., 2005
Ginkgo biloba extract	TGF- β	Liver fibrosis	Preclinical	Ding et al., 2005
Rosa laevigata Michx (RLTS)	ROS/CYP2E1 ^c /TGF- β /SMAD/FAK ^c /PI3K ^c -Akt ^c -p70S6K ^c /MAPK	Liver fibrosis	Preclinical	Dong et al., 2015
Liuweiwuling (LWWL) tablets	TGF- β /SMAD/ NF- κ B ^c	Liver fibrosis	Preclinical	Liu et al., 2017
Xuefuzhuyu (XFZY) decoction	HIF-Y ^c /DDAH ^c /ADMA ^c /VEGF ^c	Liver fibrosis	Preclinical	Zhou et al., 2014
Diwu Yanggan (DWYG)	TGF- β /BMP-7 ^c	Liver fibrosis	Preclinical	Shen et al., 2014
Ocimum gratissimum extracts (OGEs)	ROS/ α -SMA	Liver fibrosis	Preclinical	Chiu et al., 2014
Yin-Chiao-San (YCS)	ROS/TNF- α	IPF ^c	Preclinical	Yen et al., 2007
Renshen pingfei decoction	TGF- β /SMAD3	IPF	Preclinical	Chen et al., 2016
Hu-qj-yin	TGF- β	IPF	Preclinical	Zhou et al., 2007
Decoction for Strengthening Qi and Replenishing Lung (DSQRL)	—	IPF	Preclinical	Zhang et al., 2008
Modified Kushen Gancao Formula (mKG)	TGF- β /IL-6/IL-17A	IPF	Preclinical	Gao et al., 2016
Sho-seiryu-to (TJ-19)	ROS	IPF	Preclinical	Yang et al., 2010
Hochu-ekki-to (TJ-41)	IL-5/IL-4/IFN- γ	IPF	Preclinical	Tajima et al., 2007
Shenlong Decoction	MMsP/TIMPs	IPF	Preclinical	Lu et al., 2010
Yupingfeng	HMGB1 ^c /TGF- β	IPF	Preclinical	Cui et al., 2015
Danggui-Buxue-Tang (DBTG)	TNF- α /TGF- β	IPF	Preclinical	Lv et al., 2012

^aClinical trial resource are from <http://Clinicaltrials.gov>.^bTrial Identifier is the clinical trial identifier of corresponding drug.

^cTGF- β , transforming growth factor- β ; MMP-2, matrix metalloproteinase 2; TNF, tumor necrosis factor; ROS, reactive oxygen species; TIMP, tissue inhibitor of metalloproteinase; IL-6, interleukin-6; α -SMA, α -smooth muscle actin; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; PDGF-BB, platelet-derived growth factor-BB; IPF, idiopathic pulmonary fibrosis; HMGB1, high mobility group box 1; CYP2E1, cytochrome P450 2E1; FAK, focal adhesion kinase; PI3K, phosphatidylinositol-3-kinase; Akt, amino acid kinase terminal; p70S6K, 70-kDa ribosomal S6 Kinase; NF- κ B, nuclear factor kappa-light-chain-enhancer of activated B cells; HIF- α , hypoxia inducible factors; DDAH, dimethylarginine dimethylaminohydrolase; ADMA, asymmetric dimethylarginine; VEGF, vascular endothelial grow factor; BMP-7, bone morphogenetic protein 7.

more than one active ingredient. Traditional Chinese medicines (TCM) therapies, usually appeared as herbal formula, have been studied for thousands of years as multi-component drugs (Wang et al., 2012). Nowadays, single compound acting on multiple targets and multiple compounds acting on multiple targets are popular strategies in drug development (Hopkins, 2008). Fibrosis includes numerous complicated pathological pathways. Multi-component drugs, aiming at different targets, have the advantage in modulating these pathways simultaneously and producing synergistic effects. Moreover, multi-component drugs are expected to provide great resources for discovering new effective drug molecules. Many studies have revealed the pharmacology of multi-component drugs in the fibrosis treatment (Feng et al., 2009; Yang et al., 2009; Li and Kan, 2017) (**Table 4**). For example, Fuzhenghuayu capsule (FZHY),

a well-known multi-component drug for treating liver fibrosis, inhibits liver fibrosis and improves liver function in patients via inhibition of nuclear factor kappa-B kinase subunit β /nuclear factor κ F and TGF- β signaling (Liu et al., 2005). Another emerging multi-component drug, Qishenyiqi (QSYQ), is under phase II clinical trial for ischemic heart failure. QSYQ attenuates cardiac fibrosis via IL-6/STAT3 and TNF- α /nuclear factor kappa-light-chain-enhancer of activated B cells (NFkB) signalings and anti-apoptosis activities (Wang et al., 2017). Multi-component drugs act on different physiological reactions associated with fibrosis, such as inflammation and angiogenesis, leading to a systematic improvement of disease. Qushi Huayu Decoction (QHD) is a multitargeting drug that alleviates fibrosis by reducing ROS via the induction of glutathione and modulating lipid metabolism and gut barrier function (Feng et al., 2017).

With multiple targets being discovered, it becomes apparent that more common targets will be shared across many fibrotic diseases with common mechanisms, though some proteins will not express in special conditions. Meanwhile, there will be more chance for drug repositioning, which indicate common drugs will be shared across different targets and fibroproliferative diseases.

PERSPECTIVE

Fibrosis is a common pathological process in many diseases, causing a great clinical burden in recent years. The development of state-of-the-art technologies facilitate discovery of fibrosis therapies. Multi-omics analysis provides a more convenient and systematic way for researching on disease mechanisms (Fernandes and Husi, 2017). The seamless combination of traditional transcriptomics approaches with emerging technologies, including proteomics (Ordureau et al., 2014), metabolomics (Shah et al., 2012), and metagenomics (Jiao et al., 2017), will offer unprecedented opportunities to precisely elucidating and dissecting fibrosis mechanisms. Nowadays, the application of cryo-electron microscopy (cryo-EM) in macromolecular structure determination make it easier to identify drug targets (Zheng et al., 2015). Compared with traditional X-ray crystallography, cryo-EM has advantage in determining the structure of more complex and flexible receptors (Huang et al., 2016; Zhang et al., 2017). Finally, with

the advent of the era of big data, artificial intelligence technology, especially deep learning, provides more accurate algorithms for drug repositioning (LeCun et al., 2015; Alaimo et al., 2016). The continuous development and application of the above technologies and methods will make it possible to identify and discover more common mechanisms, targets and drugs in fibrosis.

AUTHOR CONTRIBUTIONS

RZ and LZ conceived and designed the project. Each author has contributed significantly to the submitted work. XL drafted the manuscript. LZ, BW, MY, and RZ revised the manuscript. All authors read and approved the final manuscript.

ACKNOWLEDGMENTS

This work was supported by National Natural Science Foundation of China 31200986 (to RZ), 41530105 (to RZ), Natural Science Foundation, the Shanghai Committee of Science and Technology 16ZR1449800 (to RZ), a departmental start-up fund (to LZ), the Peter and Tommy Fund, Inc., Buffalo, NY (to LZ), and Funds from the University at Buffalo Community of Excellence in Genome, Environment and Microbiome (GEM) (to LZ). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer XT and handling Editor declared their shared affiliation.

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The Flavonoid Quercetin Ameliorates Liver Inflammation and Fibrosis by Regulating Hepatic Macrophages Activation and Polarization in Mice

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OPEN ACCESS

Edited by:

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Dalian Medical University, China

Reviewed by:

Ester Pagano,
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Università degli Studi di Salerno, Italy

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Specialty section:

This article was submitted to
Gastrointestinal and Hepatic
Pharmacology,
a section of the journal
Frontiers in Pharmacology

Received: 11 September 2017

Accepted: 22 January 2018

Published: 09 February 2018

Citation:

Li X, Jin Q, Yao Q, Xu B, Li L, Zhang S and Tu C (2018) The Flavonoid Quercetin Ameliorates Liver Inflammation and Fibrosis by Regulating Hepatic Macrophages Activation and Polarization in Mice. *Front. Pharmacol.* 9:72.

doi: 10.3389/fphar.2018.00072

At present, there are no effective antifibrotic drugs for patients with chronic liver disease; hence, the development of antifibrotic therapies is urgently needed. Here, we performed an experimental and translational study to investigate the potential and underlying mechanism of quercetin treatment in liver fibrosis, mainly focusing on the impact of quercetin on macrophages activation and polarization. BALB/c mice were induced liver fibrosis by carbon tetrachloride (CCl₄) for 8 weeks and concomitantly treated with quercetin (50 mg/kg) or vehicle by daily gavage. Liver inflammation, fibrosis, and hepatic stellate cells (HSCs) activation were examined. Moreover, massive macrophages accumulation, M1 macrophages and their related markers, such as tumor necrosis factor (TNF)- α , interleukin (IL)-1 β , IL-6, and monocyte chemotactic protein-1 (MCP-1) in livers were analyzed. *In vitro*, we used Raw 264.7 cells to examine the effect of quercetin on M1-polarized macrophages activation. Our results showed that quercetin dramatically ameliorated liver inflammation, fibrosis, and inhibited HSCs activation. These results were attributed to the reductive recruitment of macrophages (F4/80 $^{+}$ and CD68 $^{+}$) into the liver in quercetin-treated fibrotic mice confirmed by immunostaining and expression levels of marker molecules. Importantly, quercetin strongly inhibited M1 polarization and M1-related inflammatory cytokines in fibrotic livers when compared with vehicle-treated mice. *In vitro*, studies further revealed that quercetin efficiently inhibited macrophages activation and M1 polarization, as well as decreased the mRNA expression of M1 macrophage markers such as TNF- α , IL-1 β , IL-6, and nitric oxide synthase 2. Mechanistically, the inhibition of M1 macrophages by quercetin was associated with the decreased levels of Notch1 expression on macrophages both *in vivo* and *in vitro*. Taken together, our data indicated that quercetin attenuated CCl₄-induced liver inflammation and fibrosis in mice through inhibiting macrophages infiltration and modulating M1 macrophages polarization via targeting Notch1 pathway. Hence, quercetin holds promise as potential therapeutic agent for human fibrotic liver disease.

Keywords: hepatic fibrosis, quercetin, macrophages, polarization, Notch1, hepatic stellate cells (HSCs)

INTRODUCTION

Liver fibrosis is a typical wound-healing process triggered by liver injury and inflammation resulting from a wide variety of etiologies, such as chronic virus infection (mainly hepatitis B and C viruses), alcoholic and nonalcoholic steatohepatitis (NASH), drugs, cholestasis, and autoimmune hepatitis (Friedman, 2008; Pellicoro et al., 2014; Tsuchida and Friedman, 2017). Nowadays, hepatic fibrosis is viewed as a dynamic process characterized by the massive excess deposition of extracellular matrix (ECM) in the liver (Friedman, 2008; Tsuchida and Friedman, 2017). It has been generally accepted that resident hepatic stellate cells (HSCs), which become activated and transdifferentiate into myofibroblast-like cells in response to chronic liver injury, are the major source of ECM during the process of liver fibrogenesis (Pellicoro et al., 2014; Seki and Schwabe, 2015; Tsuchida and Friedman, 2017). It has become evident that HSCs activation results from the inflammatory activity of liver immune cells, predominantly macrophages (Pellicoro et al., 2014; Seki and Schwabe, 2015; Li et al., 2017b). Of note, hepatic macrophages can directly mediate the behavior of HSCs and other myofibroblasts by producing a range of cytokines, chemokines, and other soluble mediates (Pellicoro et al., 2014). Additionally, activated myofibroblasts can amplify inflammatory responses by inducing the infiltration of macrophages and further secreting cytokines (Duffield et al., 2005; Pellicoro et al., 2014). Given the critical regulatory role of macrophages in HSCs activation and liver fibrosis, we believe that it provides therapeutic targets promising application in the future.

The prevailing concept indicates that hepatic macrophages can arise either from proliferating resident macrophages, or from circulating bone marrow (BM)-derived monocytes, which are recruited to the injured liver (Duffield et al., 2005; Pellicoro et al., 2014; Wynn and Vannella, 2016). Macrophages are highly plastic cells that can be altered depending on the tissue microenvironment (Tacke and Zimmermann, 2014; Wynn and Vannella, 2016). Its polarization status to M1 or M2 is often used to characterize macrophages; in which M1 macrophages exhibit an inflammatory phenotype while M2 macrophages are alternatively activated, including an anti-inflammatory phenotype (Beljaars et al., 2014; Sica et al., 2014; Tacke and Zimmermann, 2014; Tosello-Trampont et al., 2016). Moreover, increasing evidence suggests that M1 macrophages activation plays a critical role in liver inflammation and fibrosis (Beljaars et al., 2014; Sica et al., 2014; Tacke and Zimmermann, 2014). Additionally, inflammatory cytokines, including transforming growth factor- β 1 (TGF- β 1), tumor necrosis factor (TNF)- α , interleukin (IL)-1 β , and IL-6, released from these cells trigger local inflammatory responses and perpetuate inflammation as well as HSCs activation (Sica et al., 2014; Tacke and Zimmermann, 2014; Tosello-Trampont et al., 2016). By contrast, emerging evidence suggested that alternative M2 macrophages attenuated hepatic steatosis and inflammation, and have a pivotal role in the resolution of fibrosis (Beljaars et al., 2014; Sica et al., 2014; Tosello-Trampont et al., 2016; Labonte et al., 2017).

Furthermore, macrophage polarization is regulated by several key molecular mechanisms, including epigenetic regulators,

transcription factors, posttranscriptional regulators, and some signaling pathways (Sica and Mantovani, 2012; Sica et al., 2014; Wijesundera et al., 2014; Xu et al., 2015a). The switch in phenotypes determines their role in liver inflammation and fibrosis, thus controlling M1/M2 macrophage polarization provides potential targets for antifibrotic therapies (Sica et al., 2014; Xu et al., 2015a; Tacke, 2017). Moreover, it has been reported that the M1 macrophage phenotype was controlled by several molecular signaling or transcription factors, including Notch1 signaling, transducer and activator of transcription 1 (STAT1), and interferon-regulatory factor (IRF) 5 (Lawrence and Natoli, 2011; Sica et al., 2014; Xu et al., 2015a; Tacke, 2017), while IRF4 and STAT6 were shown to specifically regulate M2 macrophage polarization (Lawrence and Natoli, 2011). Thus, potential therapeutic approaches might aim to balance M1/M2 macrophages or to regulate macrophage polarization by targeting key macrophage transcription factors (Lawrence and Natoli, 2011; Sica and Mantovani, 2012; Wan et al., 2014; Labonte et al., 2017).

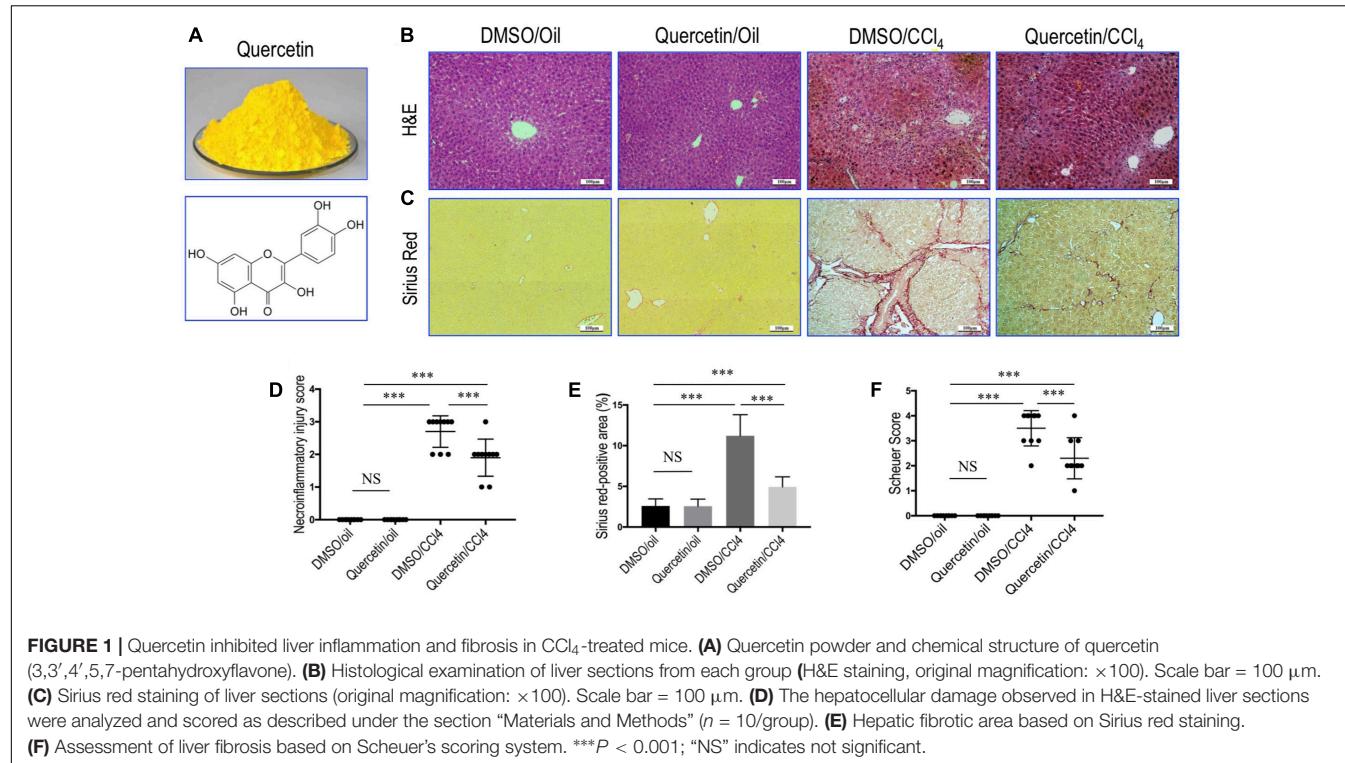
Quercetin (3,3',4',5,7-pentahydroxyflavone; **Figure 1A**) is a well-known flavonoid widely found in many plants and fruits including apples, red grapes, citrus fruit, tomato, onions, and other leafy green vegetables, and a number of berries (Russo et al., 2012; Kim and Park, 2016). Quercetin is known to possess various biological and pharmacological activities including antioxidant, antiviral, anti-inflammatory, anti-proliferative, and antifibrotic effects (Marcolin et al., 2012; Russo et al., 2012; Li et al., 2016a). Indeed, the beneficial effects of quercetin on liver injury and fibrosis have been confirmed by several animal models (Hernandez-Ortega et al., 2012; Marcolin et al., 2012; Li et al., 2016b). Recently, we reported that quercetin inhibited liver inflammation and fibrosis in mice by modulating high mobile group box 1 (HMGB1) and toll-like receptor (TLR) 2/TLR4 signaling pathways (Li et al., 2016b).

However, the precise mechanisms of quercetin on liver fibrosis are incompletely understood. Thus, further studies are needed to define the mechanisms underlying anti-inflammatory and antifibrotic activity of quercetin that hold promise for translation into human treatments. Notably, it has been reported that quercetin attenuated inflammation in human and mouse macrophages *in vitro* upon injury (Overman et al., 2011; Kim and Park, 2016) and reduced mice adipose tissue macrophage infiltration and inflammation in diet-induced obesity (Dong et al., 2014). In the light of these findings, we thus hypothesized that the antifibrotic effects of quercetin should be involved in regulating activation and polarization of hepatic macrophages.

MATERIALS AND METHODS

Reagents and Antibodies

Carbon tetrachloride (CCl₄), quercetin, dimethyl sulfoxide (DMSO), olive oil, 1,4-diazabicyclo[2.2.2]octane (DABCO), and lipopolysaccharide (LPS; from *Escherichia coli* 0727: B8) were purchased from Sigma Chemical, Co., Ltd. (St. Louis, MO, United States). For *in vivo* and *in vitro* experiments, quercetin was diluted immediately in DMSO solution before administration.



Antibodies used in this study comprised: mouse anti-desmin monoclonal antibody (DakoCytomation, Glostrup, Denmark); rabbit anti-collagen III polyclonal antibody, rabbit anti-collagen IV polyclonal antibody, rabbit anti-CD68 monoclonal antibody, rat anti-F4/80 monoclonal antibody, mouse anti-CD11c polyclonal antibody, mouse anti-IRF5 monoclonal antibody, rabbit anti-Ym-1 monoclonal antibody, rabbit anti-CD163 monoclonal antibody, and rabbit anti-GAPDH monoclonal antibody (Abcam, Cambridge, MA, United States); rabbit anti-IL12a monoclonal antibody, rabbit anti-Notch1 monoclonal antibody, and rabbit anti- β -actin monoclonal antibody (Cell Signaling Technology, Boston, MA, United States).

Animal Experimental Protocols

Male BALB/c mice (weight 20–22 g) were purchased from the Shanghai Laboratory Animal Research Center (Shanghai, China). All animals were housed in standard cages ($23 \pm 2^\circ\text{C}$ at a humidity of $55 \pm 10\%$) with a 12 h light/12 h dark cycle. Mice had unrestricted access to food and water. Fibrosis in mice was injected intraperitoneally (i.p.) biweekly for 8 weeks with 0.5 $\mu\text{L/g}$ body weight of CCl_4 , which was dissolved in olive oil at a concentration of 25% v/v (Li et al., 2016b). Fifty-five mice were randomly divided into four groups as described previously [20]. Briefly, Group I ($n = 10$) and Group II ($n = 15$) were given twice weekly injections of olive oil, and received equal volume of DMSO and quercetin (50 mg/kg) by orally, respectively; Group III ($n = 15$) and Group IV ($n = 15$) were injected with CCl_4 and received quercetin and DMSO, respectively. After 8 weeks of treatment with CCl_4 , mice were sacrificed with pentobarbital, mouse livers were removed to examine for fibrosis. The dose of

quercetin for this experiment was based on the previous studies in mice (Hernandez-Ortega et al., 2012; Li et al., 2016a). All animal experiments were performed according to institutional guidelines and regulations and approved by the Animal Care Committee of Fudan University (Shanghai, China).

Cells Culture and Treatment

Raw 264.7 cells were purchased from Sigma Chemical, Co., Ltd. (St. Louis, MO, United States) and cultured in undifferentiated Raw macrophages conditioned medium. Briefly, Raw 264.7 cells were cultured in T25 flasks in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), L-glutamine (2 mM), penicillin (50 U/mL), and streptomycin (50 $\mu\text{g/mL}$) at 37°C and 5% CO_2 . All incubations were performed in cells under the three or four passages (Li et al., 2017b).

In experiments assessing the effects of quercetin on macrophages activation and polarization macrophages, Raw 264.7 cells were polarized by culturing 300,000 cells/well overnight in 24-well plates before replacing the medium with complete culture medium supplemented with M1-differentiated macrophages conditioned medium as described previously (Wan et al., 2014; Tosello-Trampont et al., 2016; Labonte et al., 2017). Briefly, using LPS (100 ng/mL) to induce M1 differentiation. For selective experiments, cells were co-cultured with quercetin (50 μM); and parallel cultures were treated with an equivalent volume of DMSO (0.05%) served as negative controls. Quercetin concentration (50 μM) for macrophage treatment was used in our cell experiments based on previous *in vitro* bioactivity work (Kobuchi et al., 1999; Kim and Park, 2016; Li et al., 2016b). After 24 h of co-culture at 37°C , cells were then washed and

harvested by centrifugation for immunofluorescence analysis, RNA harvesting, and protein isolation (Li et al., 2017b). All measurements were performed in triplicate using different batches of wells. Staining and quantitative RT-PCR analysis were performed on three independent experiments.

Cell Viability Assay

Cell Counting Kit-8 (CCK-8) Assay Kit was used to assess cell viability according to the manufacturer's instructions as described previously (Li et al., 2017a). Briefly, Raw 264.7 cells were seeded into a 24-well culture plate at a density of 1×10^5 cells/well and incubated with quercetin (50 μM) or vehicle (0.05% DMSO) at 37°C for 0, 12, and 24 h; then, the cells were incubated in 10% CCK-8 that was diluted in normal culture medium at 37°C until the visual color conversion occurred. The absorbance at 450 nm was measured with Flexstation 3 Multimode Microplate Reader (Molecular Device). Experiments were conducted in triplicate independently, and data are presented as means $\pm SD$.

Histopathologic Analysis

The left lobe of liver was excised immediately after mice were euthanized, fixed in 10% neutral buffered formalin, and embedded in paraffin. Four-micron sections were stained with hematoxylin and eosin (H&E) and Sirius red according to standard protocols. The H&E-stained liver tissues were evaluated by an experienced pathologist completely blinded to the identity of the samples, according to criteria in four categories for necroinflammation as previously described (Horrillo et al., 2007), and scored for grade 0 (absent), grade 1 (spotty necrosis), grade 2 (confluent necrosis), and grade 3 (bridging necrosis). Liver fibrosis was assessed by measurement of the Sirius-red positive area, which was measured in six low power ($\times 100$) fields per slide using ImageJ 1.49 software (NIH, Bethesda, MD, United States). Fibrosis staging was also classified by a pathologist according to the Scheuer histological scoring system on a scale from 0 to 4 (0 = normal, 4 = cirrhosis) (Scheuer, 1991). The assessment of the preceding scores was uniformly performed under 100 \times magnification in 10 fields per sample.

Immunohistochemistry and Quantitative Analysis of Histological Markers

For immunohistochemical analysis, sections of formalin-fixed, paraffin-embedded liver tissue were cut 4 μm , dewaxed, hydrated, and subjected to heat-induced antigen retrieval according to standard protocols as previously reported (Li et al., 2016b, 2017a). Subsequently, sections were treated with 3% hydrogen peroxide for 10 min, then blocked and incubated overnight at 4°C with primary antibodies as follows: rabbit anti-Desmin (1:100), rabbit anti-collagen III (1:100), rabbit anti-collagen IV (1:100), rat anti-F4/80 (1:50), rabbit anti-CD68 (1:100), mouse anti-CD11c (1:100), rabbit anti-IL-12 (1:100), rabbit anti-IRF5 (1:100), rabbit anti-Ym-1 (1:200), and rabbit anti-CD163 (1:100). The sections were subsequently washed and incubated with HRP-conjugated goat anti-rabbit and anti-mouse IgG secondary antibodies, followed by incubation for 5–10 min

with 3,3'-diaminobenzidine tetrachloride and visualization of specific staining by light microscopy.

The intensity of collagen III and IV immunostaining in tissue sections was quantified using five representative sections of each slide and determined for five animals in each group, and the area of staining was analyzed as a percentage of the total area. Desmin-positive area was quantified in five random non-overlapping $\times 100$ fields and determined for six animals in each group. The immunostaining signaling was quantified at a fixed threshold using free software NIH ImageJ 1.49 (NIH, Bethesda, MD, United States). For quantification of the numbers of hepatic macrophages in sections, six non-overlapping randomly selected fields of view per slide at $\times 400$ magnifications (F4/80 $^{+}$ cells) or $\times 200$ magnifications (CD68 $^{+}$, IL-12 $^{+}$, CD11c $^{+}$, IRF5 $^{+}$, CD163 $^{+}$, and Ym-1 $^{+}$ cells) were examined and expressed as cells per field of view; and five mice of each group were examined (Miura et al., 2012; Li et al., 2016b, 2017b).

Immunofluorescence

Details on the immunofluorescence methodology can be found in our previous reports (Li et al., 2017b). Briefly, freshly dissected liver tissues were OCT-embedded and the sections (10 μm in thickness) were cut with a cryotome Cryostat (Leica, 1900, Germany). After blocking in PBS with 3% Bovine serum albumin (BSA), the liver sections were labeled with primary antibody F4/80 (1:100 dilution) overnight at 4°C, and subsequently incubated with antibody Notch1 (1:200 dilution) for 1 h at room temperature (RT) in case of double staining. Alexa Fluor 594 Donkey anti-mouse and Alexa Fluor 488 Donkey anti-rabbit secondary antibodies (Yeasen Biotechnology, Shanghai, China) were incubated at 1:200 in PBS for 1 h at RT. After washing, sections were counterstained with DAPI (4',6-diamidino-2-phenylindole)-Fluoromount-GTM (SouthernBiotech, United States). Finally, the stained tissues were analyzed by fluorescence microscopy (BX51, Olympus, Japan).

Raw 264.7 cells were fixed and permeabilized in 4% paraformaldehyde, 0.2% TritonX-100 in PBS for 10 min. Nonspecific binding was unmasked with 3% BSA for 1 h at RT, and then the cells were incubated with primary antibodies for IL-12 (dilution 1:200), IRF5 (dilution 1:100), and Notch1 (dilution 1:150) overnight at 4°C. Sections were washed twice with PBS and incubated with fluorescein-labeled secondary antibody at a dilution of 1:500 for 1 h at RT in the dark. Slides were mounted in mounting media with DAPI for 40 min at RT. After washing twice with PBS, the slides were covered with DABCO and images were captured by fluorescence microscopy (IX51, Olympus, Japan).

Western Blot Analysis

Frozen liver tissue was homogenized in radioimmunoprecipitation assay buffer (RIPA buffer) by adding protease inhibitor Cocktail (Roche) and phosphatase inhibitors Cocktail (Sigma, St. Louis, MO, United States), and then centrifuged at 10,000 $\times g$ at 4°C for 20 min (Li et al., 2016b, 2017a). Protein extraction from Raw 264.7 cells was as previously described (Li et al., 2016b, 2017b). Protein concentration was quantified with the Bicinchoninic Acid Protein Colorimetric Assay kits (BBI, Shanghai, China) with BSA as the standard.

Equal amounts of proteins were separated by electrophoresis on 7.5–12% SDS-PAGE gels and transferred onto polyvinylidene difluoride (PVDF) membranes. The membranes were then incubated in blocking buffer [5% nonfat milk powder in tris-buffered saline Tween-20 (TBST)] for 2 h at RT followed by incubation with primary antibody in TBST at 4°C overnight with the specific primary antibodies against Desmin, IRF5, IL-12, and Notch1 (all 1:1000 dilution). The membranes were washed with TBST and then incubated with goat anti-rabbit, anti-mouse, or anti-rat secondary antibodies (1:1500) for 2 h at RT. GAPDH or β -actin (1:5000 dilution) was used as internal control, respectively. After washing off the unbound antibody with TBST, the expression of the antibody-linked protein was determined by an ECL™ Western Blotting Detection Reagents (Amersham Pharmacia Biotech Inc., NJ, United States). The intensity of the western blot bands was performed using NIH ImageJ software. Expression levels were evaluated by quantification of the relative density of each band normalized to that of the corresponding GAPDH or β -actin band density (Li et al., 2016b).

RNA Isolation and Quantitative RT-PCR

Total RNA was extracted from frozen liver tissue or cultured cells using Trizol reagent (Life Technologies, Grand Island, NY, United States) following manufacturer's protocol. RNA was reverse-transcribed with random hexamers and avian myeloblastosis virus reverse transcriptase using a commercial kit (Perfect Real Time, SYBR® PrimeScript™ TaKaRa, Japan). Quantitative RT-PCR was performed for assessment of mRNA expression on the ABI Prism 7500 Sequence Detection system (Applied Biosystems, Tokyo, Japan) as previously reported (Li et al., 2017a). Sequences of primers for target genes were purchased from Sangon Biotech Co., Ltd. (Shanghai, China) and listed in **Table 1**. The reactions were run in triplicates using SYBR green gene expression assays. The relative change was normalized to endogenous GAPDH mRNA using the formula $2^{-\Delta\Delta C_t}$ (Li et al., 2017a).

Statistical Analysis

All data are presented as the mean \pm SD. Comparisons among multiple groups (three or more) were performed by one-way ANOVA with *post hoc* test (Bonferroni or Dunnett's correction for multiple tests). For comparison between two groups, the two-tailed unpaired Student's *t*-test was used for normally distributed data; and the Wilcoxon–Mann–Whitney *U*-tests or Kruskal–Wallis tests were used for non-normally distributed data. Statistical analysis was performed with GraphPad Prism 7.0 (La Jolla, CA, United States). In all comparisons, a *P*-value less than 0.05 was considered as statistically significant.

RESULTS

Quercetin Inhibited Liver Inflammation and Fibrosis in CCl₄-Treated Mice

Remarkably, histological examination revealed that repeated administration of CCl₄ induced the formation of necrotic areas and inflammation in the liver, with obvious alteration of the

sinusoidal and lobular architecture of the liver (**Figure 1B**). However, these morphological changes and inflammation were markedly ameliorated in CCl₄-induced mice also given oral quercetin (50 mg/kg daily). Control oil-injected mice treated with quercetin did not show any liver injury and inflammation; similar to oil-treated animal administrated with vehicle (**Figure 1B**). Consistent with these results, the necroinflammatory injury score was lower in fibrotic mice treated with quercetin than that in fibrotic mice treated with DMSO (2.7 \pm 0.48 vs. 1.9 \pm 0.57, *P* < 0.001; **Figure 1D**).

Fibrillar collagen deposition in livers could reflect the severity of fibrosis, which was assessed by examining Sirius red-stained liver sections. Our results revealed that mice-repeated injections of CCl₄ for 8 weeks induced obviously ECM proteins accumulation, with the formation of bridging fibrosis (**Figure 1C**). While there are only thin layers of collagen surrounded the portal tracts and central veins in the liver from normal control animals. However, fibrotic mice given oral quercetin treatment displayed thinner septa and more preserved hepatic parenchyma than fibrotic animals given vehicle (DMSO) treatment (**Figure 1C**). Furthermore, collagen deposition in the liver of CCl₄-treated mice was confirmed by computerized image analysis of the fibrotic area, whereas fibrotic mice treated with quercetin markedly attenuated the progression of CCl₄-induced fibrosis when compared with vehicle-treated fibrotic mice (4.93 vs. 11.22%, *P* < 0.001; **Figure 1E**). Similarly, we observed that the mean fibrosis score was significantly lower in fibrotic mice also given quercetin treatment than that in fibrotic mice given vehicle treatment (2.3 \pm 0.8 vs. 3.5 \pm 0.7, *P* < 0.001; **Figure 1F**).

Additionally, immunohistochemical evaluation revealed that the deposition of intrahepatic collagen III and IV was increased in fibrotic mice induced by CCl₄ for 8 weeks, whereas co-treatment

TABLE 1 | Mouse primer sequences used for quantitative RT-PCR.

Target gene	Forward primers (5'-3')	Reverse primers (5'-3')
CTGF	GCGCCTGTTCTAACAGCCTGT	TTCATGATCTGCCATCGGG
Collagen 4 α 1	AACAAACGCTCTGCACACTTCGC	CTTCACAAACCGCACACCTG
Collagen 3 α 1	CCTTCTACACCTGCTCCT	CTTCCTGACTCTCCATCCT
TIMP-1	CCAGAACCGCAGTGAAGAGT	TCTGGTAGTCCTCAGAGCCC
Desmin	CCTACACCTGCGAGATTG	ATCATCACCGTCTCTTGG
Vimentin	TTCTCTGGCACGCTCTGACC	CTCCCTGGAGGTTCTGGCAG
F4/80	TCTGGGGAGCTTACGATGGA	GAATCCCGCAATGATGGCAC
CD68	GGGGCTCTGGGAACTACAC	GTACCGTCAACACCTCCCTG
TNF α	GACGTGAAACTGGCAGAAGA	ACTGATGAGAGGGAGGCCAT
IL-1 β	GTGCAAGTGTCTGAAGCAGC	CAAAGGTTGGAAAGCAGCCC
IL-6	GGAGTCACAGAAGGGAGTGGC	CGCACTAGGTTCCGGAGTA
MCP-1	AGCCAACCTCTCACTGAAGCC	GGACCCATTCTCTTGGGG
Notch1	CCTTCGTGCTCTGTTCTTGTG	GGGCTCTCCGCTCTCTTGTG
NOS2	GAGCAACTACTGCTGGTGGT	CGATGTCATGAGCAAAGGCG
GAPDH	TCTCTGCGACTTCAACA	TGTAGCCGTATTCTTGTC
Arg-1	CGTTGTATGATGCACAGCCG	CCCCACCCAGTGTCTTGAC
Ym-1	CTCACTCCACAGGAGCAGG	AGCTGCTCCATGGCCTTC

with quercetin attenuated these collagen accumulations in the liver when compared with DMSO-treated control (**Figure 2A**). These results were further confirmed by quantification of collagen III or collagen IV immunopositive areas; indicating that fibrotic mice treated with quercetin significantly reduced the deposition of collagen in the liver when compared with vehicle-treated control animal (**Figure 2B**). Moreover, we also assessed the expression levels of the markers of profibrogenic genes, such as *Col3α1*, *Col4α1*, connective tissue growth factor (*Ctgf*), and tissue inhibitor of metalloproteinase-1 (*Timp-1*). We found that the levels of those profibrogenic genes were observably enhanced in CCl₄-induced mice when compared with oil-treated normal control; however, quercetin treatment obviously inhibited the profibrogenic effects of CCl₄ injection and decreased the abundance of these genes expression as compared to vehicle-treated animals (**Figure 2C**).

Taken together, these results indicated that quercetin strikingly attenuated liver inflammation and fibrogenesis in CCl₄-induced liver fibrosis mouse model.

Quercetin Inhibited Activation of HSCs in CCl₄-Treated Mice

In order to investigate whether quercetin affects the activation of HSCs in the liver, we examined the expression of HSC-specific marker with immunohistochemical (IHC) staining. In our previous study, we demonstrated that quercetin inhibited α-SMA expression at gene and protein level both *in vivo* and *in vitro* (Li et al., 2016b). To provide additional support evidence, we here examined other HSCs activated markers such as desmin and vimentin (Bansal et al., 2015; Wilhelm et al., 2016). Indeed, as revealed by immunostaining, there were markedly strong desmin signals in the fibrotic septa in the CCl₄-induced livers, while only faint staining for desmin in livers from normal mice; however, there was relatively weak intensity of desmin staining in livers from fibrotic mice receiving quercetin treatment when compared with those fibrotic mice receiving DMSO treatment (**Figure 3A**). Furthermore, computer-assisted semi-quantitative analysis showed that the number of desmin-positive cells was markedly decreased in livers from quercetin-treated fibrotic mice than those from vehicle-treated control mice (3.52 ± 0.16 vs. 7.83 ± 0.23 , $P < 0.001$; **Figure 3B**). These results were also confirmed by western blot analysis and quantitative RT-PCR experiments, indicating that there was lower expression in the levels of desmin gene and protein after chronic CCl₄ mice receiving quercetin compared with those mice receiving vehicle (**Figures 3C,D**). In addition, there was a corresponding reduction in mRNA expression levels of vimentin (**Figure 3D**).

Collectively, these findings indicated that quercetin treatment efficiently reduced HSC-derived myofibroblasts activation in mice induced by CCl₄.

Quercetin Inhibited Massive Macrophages Recruitment into the Fibrotic Livers of CCl₄-Induced Mice

To assess whether the effects of quercetin on liver fibrogenesis were related to the infiltration of macrophages in livers,

liver sections were stained with antibodies against macrophage markers, F4/80 and CD68. IHC staining results revealed a higher number of F4/80⁺ or CD68⁺ cells in livers after chronic CCl₄ damage compared to the normal control livers (**Figure 4A**). Remarkably, these positive macrophages were predominantly observed in the scars of fibrotic livers. However, the number of macrophages infiltration in livers was markedly reduced in fibrotic mice receiving quercetin treatment when compared with those mice given DMSO treatment (**Figures 4A,B**). This observation was further verified by quantification of the F4/80⁺ or CD68⁺ staining cells, indicating that repeated CCl₄ injection for 8 weeks significantly promoted macrophages recruitment into the livers, and that the increased number of macrophages was significantly lower in CCl₄-induced mice with quercetin treatment when compared with vehicle-treated fibrotic control (**Figures 4A,B**). Consistent with these IHC findings, mRNA levels of F4/80 and CD68 in total liver also demonstrated that quercetin treatment to fibrotic mice blocked the up-regulated F4/80 and CD68 expression (**Figure 4C**). Taken together, these findings suggested that quercetin treatment significantly reduced massive hepatic macrophage recruitment to the injured liver.

Quercetin Inhibited M1 Macrophage Polarization and Expression of Inflammatory Properties in Fibrotic Livers

To determine whether quercetin restricts hepatic injury, inflammation, and fibrosis through switching macrophages phenotype and influencing its function, we assessed the status of M1 macrophages and expression of proinflammatory cytokines associated with M1 markers in the fibrotic liver [such as TNF-α, IL-1β, IL-6, and monocyte chemotactic protein-1 (MCP-1)]. Of note, IL-12, an important cytokine produced by classically activated macrophages, could be served as an IHC marker to detect the M1-dominant subset in livers (Beljaars et al., 2014); and other M1 macrophage markers were strongly confirmed by previous studies including CD11c and IRF5 (Beljaars et al., 2014; Alzaid et al., 2016). We found that chronic CCl₄ injection increased M1 macrophages, as determined by IHC staining with antibodies against CD11c, IL-12, and IRF5 (**Figure 5A**) and by quantification of CD11c⁺ cells, IL-12⁺ cells, and IRF-5⁺ cells in liver sections (**Figure 5B**), while these positive cells were restricted to liver sinusoids and fibrotic septa in CCl₄-induced fibrotic mice, and barely detectable in healthy livers; however, quercetin-treated CCl₄ mice suppressed M1 macrophages polarization as confirmed by M1-associated markers (**Figures 5A,B**). Notably, these macrophages were found solely in the fibrotic collagen bands. We also confirmed these results via quantitative RT-PCR and found that repeated CCl₄ injection has been associated with enhanced proinflammatory cytokine markers in the liver, including TNF-α, MCP-1, IL-6, and IL-1β mRNA, as compared with the normal control. However, compared with fibrotic mice receiving DMSO treatment, fibrotic mice receiving quercetin decreased the levels of TNF-α, IL-1β, IL-6, and MCP-1 mRNA expression by 4.85-, 2.78-, 1.24-, and 1.43-fold, respectively (**Figure 5C**).

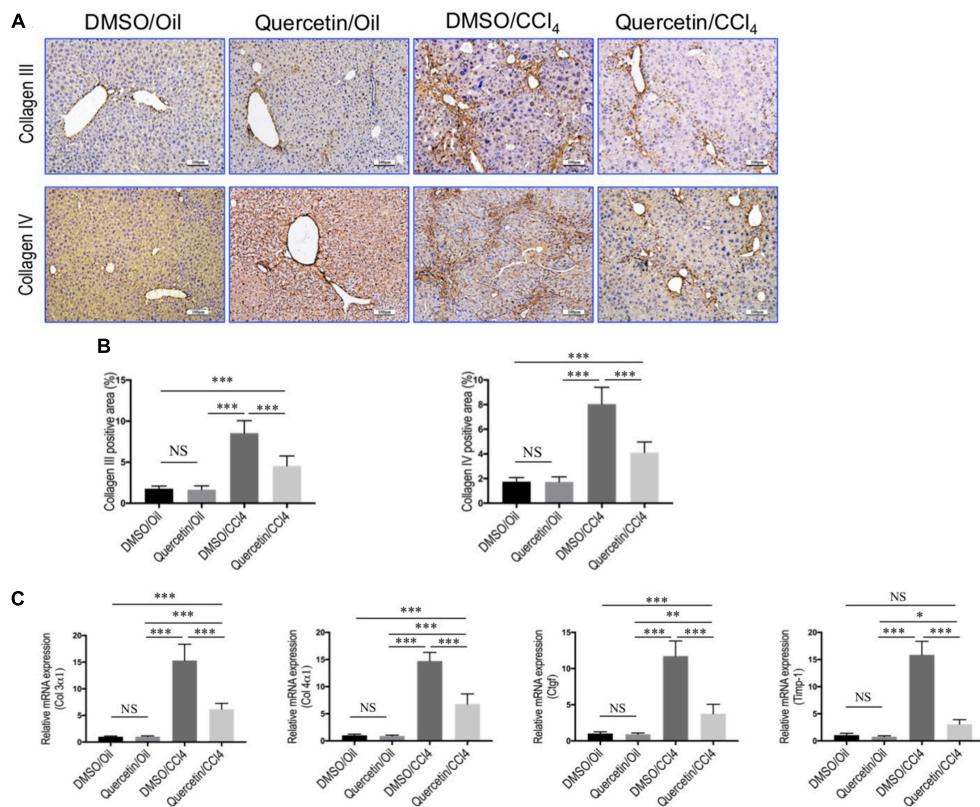


FIGURE 2 | Quercetin inhibited liver fibrotic markers expression in CCl₄-induced mouse fibrotic liver model. **(A)** Representative microscopy images of Collagen III and Collagen IV immunohistochemistry in the liver (original magnification, $\times 100$). Scale bar = 100 μ m. **(B)** Quantitative analysis of Collagen III- and Collagen IV-positive area by ImageJ software (NIH). $n = 5$ /group. **(C)** Expression of fibrotic markers (*Col3α1*, *Col4α1*, *Ctgf*, and *Timp-1*) was examined by quantitative RT-PCR in whole liver samples from each group ($n = 6$ /group). Results were normalized to GAPDH mRNA and expressed as fold change compared to DMSO/oil mice. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; "NS" indicates not significant.

Taken together, these results show that quercetin inhibited M1 polarization of macrophage and reduced the expression of inflammatory properties in fibrotic livers following repeated injection CCl₄.

Quercetin Attenuated M2 Macrophages Polarization and Expression of Immunosuppressive Genes in Fibrotic Livers

In addition, we also evaluated the effect of quercetin on M2-polarized macrophages and activation in the development of liver fibrosis *in vivo*. We used chitinase-3-like 3 (Chi3l3; also known as Ym-1; mouse only) and CD163 (Beljaars et al., 2014; Alzaid et al., 2016) as molecular M2 macrophage markers; and our results demonstrated that the expression of M2 markers in livers was clearly higher after chronic CCl₄ damage as demonstrated by immunostaining with antibodies against Ym-1 and CD163 (Figure 6A); however, mice given quercetin decreased those M2 macrophages staining signaling when compared with the vehicle-treated mice (Figure 6A). These results were further confirmed by quantification of the Ym-1⁺ and CD163⁺ staining cells (Figure 6B), indicating quercetin-treatment obviously reduced

the M2 macrophages recruitment into the liver following 8-week CCl₄ administration. Moreover, the M2 skewing was further confirmed with quantitative RT-PCR for selective M2 typical markers such as Arginase I (Arg I) and Ym-1; and we observed the levels of M2 marker genes (Arg I and Ym-1) in fibrotic livers decreased by quercetin-treated fibrotic mice when compared with DMSO-treated animals (Figure 6C). Together, these results show that quercetin inhibited M2-dominant macrophage polarization in fibrotic livers and limited immunosuppressive genes following CCl₄ administration in mice.

Quercetin Treatment Suppressed M1-Polarized Macrophages *in Vitro*

To further interrogate whether quercetin treatment may prevent M1-polarized macrophages in fibrotic liver, we used the RAW 264.7 cell line as the model of M1 macrophages *in vitro*, as the cells can be reliably polarized to M1 macrophages *in vitro* by stimulation with LPS (Wan et al., 2014; Tosello-Trampont et al., 2016; Labonte et al., 2017). Indeed, our experiment demonstrated that incubation with quercetin to the cells markedly suppressed M1-macrophages polarization as shown in immunostaining with anti-IL12 and anti-IRF5 (Figure 7A).

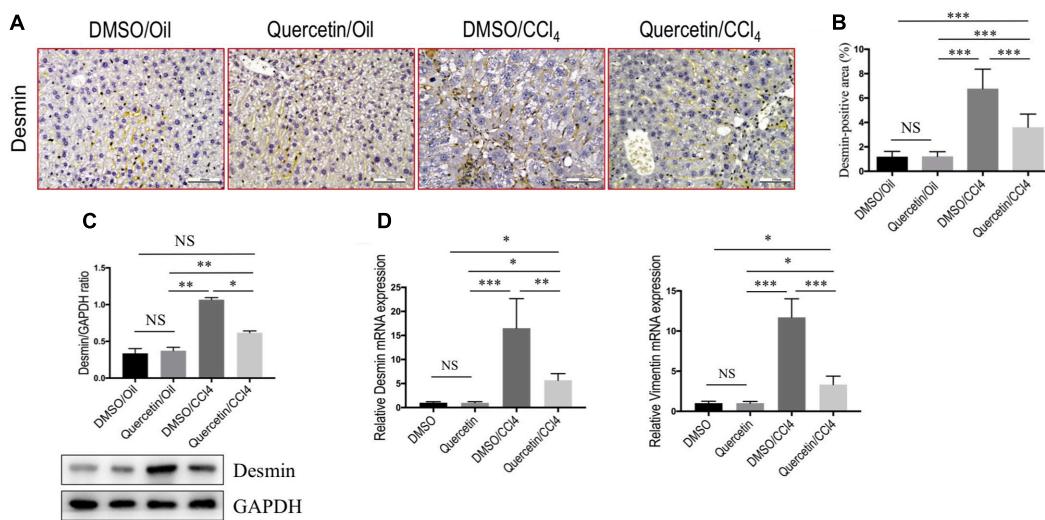


FIGURE 3 | Quercetin inhibited hepatic stellate cells (HSCs) activation in CCl₄-treated mice. **(A)** Representative microscopy images of desmin staining (magnification: $\times 200$) in the liver. Scale bar = 100 μ m. **(B)** Quantification of desmin-positive area by ImageJ software (NIH). Results mean of six fields and $n = 5$ /group. **(C)** Western blotting analysis of desmin expression in lysed liver tissues, with results normalized relative to the expression of GAPDH ($n = 3$). **(D)** Expression of desmin and vimentin mRNA was determined in the liver by quantitative RT-PCR ($n = 6$). Results were normalized relative to GAPDH expression and expressed as mean \pm SD fold change over normal control mice. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; "NS" indicates not significant.

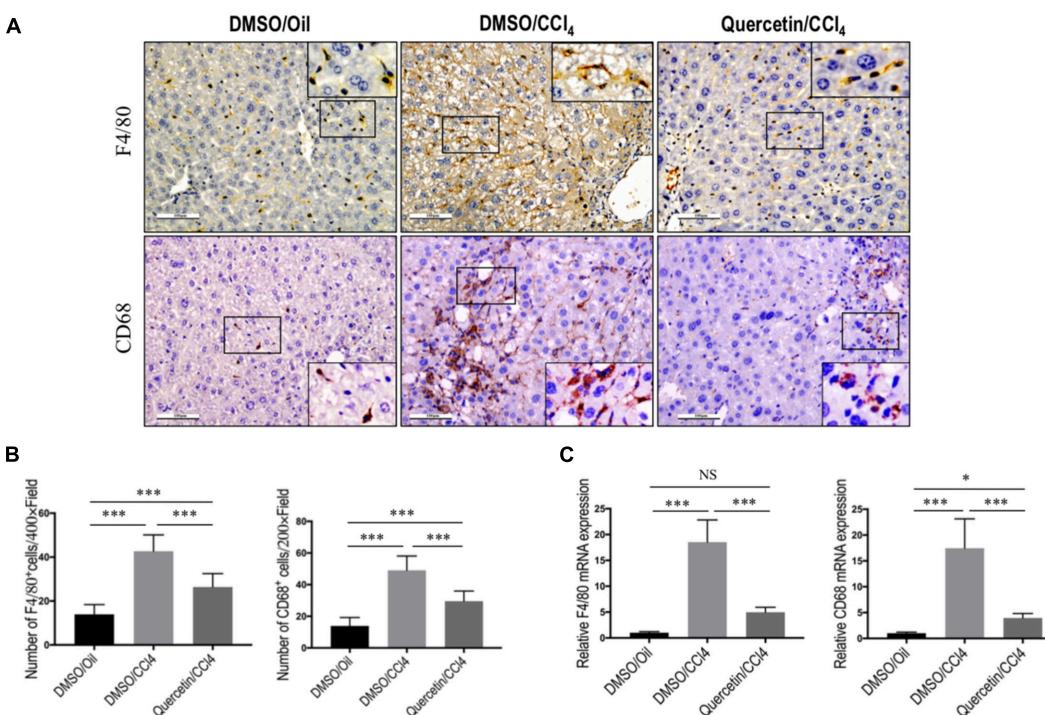
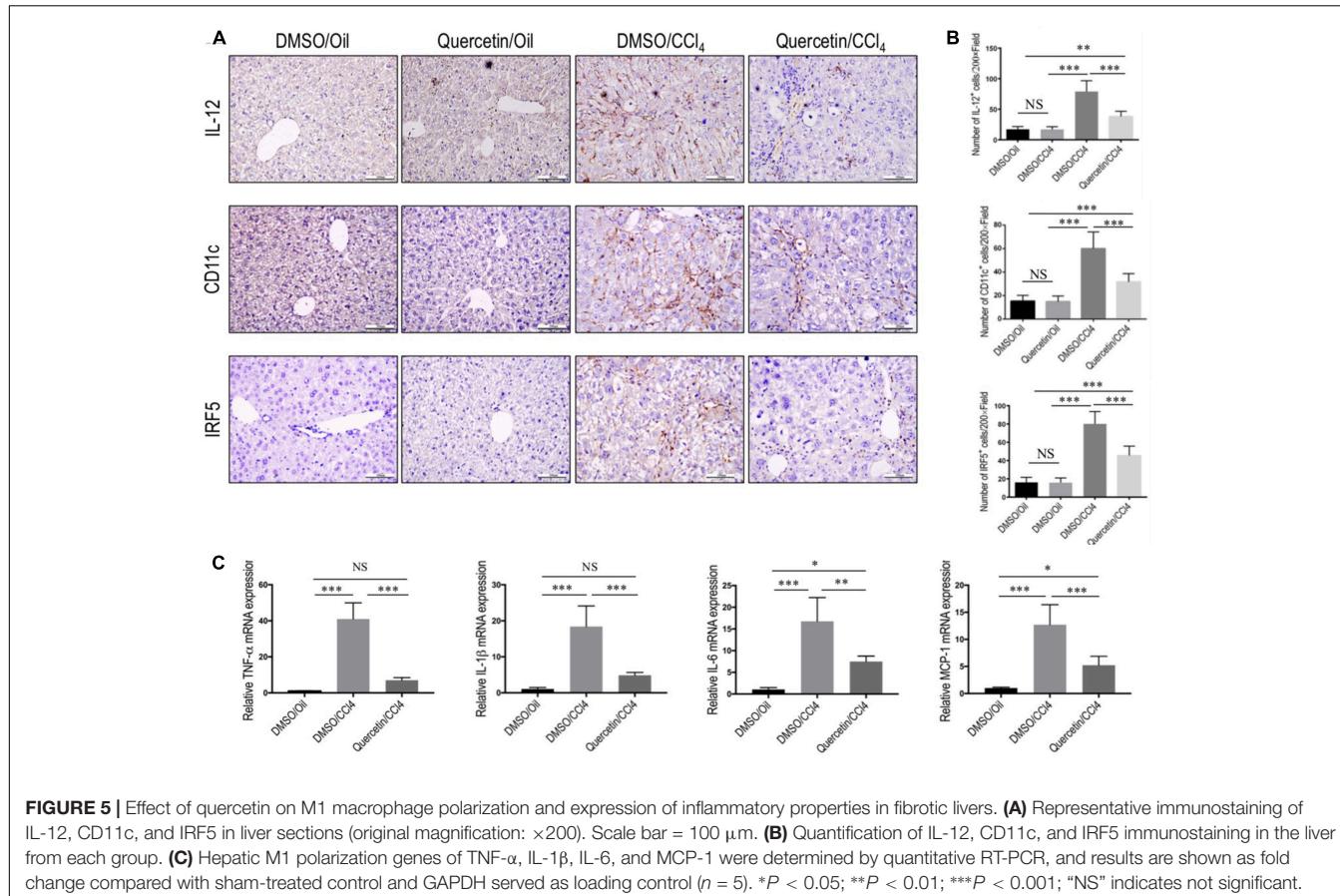


FIGURE 4 | Quercetin inhibited massive macrophage recruitment into the fibrotic livers of CCl₄-induced mice. **(A)** Immunohistochemical detection of F4/80- and CD68-positive cells in liver sections from each group (original magnification: $\times 200$). Insert (magnification: $\times 400$) shows typical morphology of positive macrophages. Scale bar = 100 μ m. **(B)** Quantification of F4/80- and CD68-positive cells in liver sections. Results mean of six fields and $n = 5$ /group. **(C)** Gene expression of macrophage marker F4/80 and CD68 was determined in livers by quantitative RT-PCR, and the results are shown as fold change compared with sham-treated control and GAPDH served as loading control ($n = 6$). * $P < 0.05$; *** $P < 0.001$; "NS" indicates not significant.



Notably, we observed that quercetin, at this dosage, did not affect the cell viability of macrophages *in vitro* (Figure 7B). We then examined the expression of M1 macrophages markers such as IL12 and IRF5 by western blotting; our results demonstrated that quercetin treatment significantly decreased the levels of those markers expression on macrophages when compared with vehicle-treated cells (Figure 7C). Furthermore, we also revealed that quercetin led to the substantially reduced M1-polarized macrophages as depicted in M1-related markers such as *TNF- α* , *IL-1 β* , *IL-6*, and nitric oxide synthase 2 (*NOS2*) (Figure 7D). Taken together, these data suggested that quercetin treatment may regulate the M1-polarized macrophages upon injury *in vitro*.

Quercetin Inhibited Hepatic Macrophages Activation and Suppressed M1-Polarization through Regulating the Expression Notch1 on Macrophages

Recent data have suggested that Notch1 signaling was widely known as a key transcription factor related to M1 macrophage activation (Lawrence and Natoli, 2011; Bansal et al., 2015; Xu et al., 2015a). To verify the involvement of quercetin in regulating M1 macrophages in liver fibrogenesis through Notch1 signaling pathway, we first examined the Notch1 expression and subcellular location in the liver. Double-staining of liver

sections from fibrotic mice showed that Notch1 was localized predominantly in resident macrophages (F4/80), the staining signal of Notch1 in fibrotic liver is stronger than oil-treated normal liver (Figure 8A). Then, we assessed the expression of Notch1 in livers from each group by quantitative RT-PCR and western blots. Our data revealed that the levels of Notch1 expression in fibrotic livers were marked increase when compared with those in the normal control livers; however, quercetin-treated fibrotic mice decreased the levels of Notch1 gene and protein expression when compared with vehicle-treated fibrotic animals (Figures 8B,C).

Finally, we determined whether quercetin inhibits M1 polarization macrophages through regulating Notch1 expression on macrophages *in vitro*. RAW 264.7 macrophages were incubated with quercetin prior to induction of M1-polarized macrophages. The results showed that the expression of Notch1 was increased in RAW 264.7 cells by treatment with LPS, whereas quercetin (50 μ M) in combination to treatment cells significantly abrogated the increase of Notch1 gene and protein expression, as measured by immunofluorescence (Figure 9A), western blots (Figure 9B), and quantitative RT-PCR (Figure 9C). Moreover, these alterations in expression of Notch1 are paralleled by the reduced genetic expression of the M1-specific markers in macrophages, such as IL-1 β , IL-6, and NOS2 (Figure 7D). Collectively, these results indicated that quercetin inhibited M1-polarized macrophages via targeting Notch1.

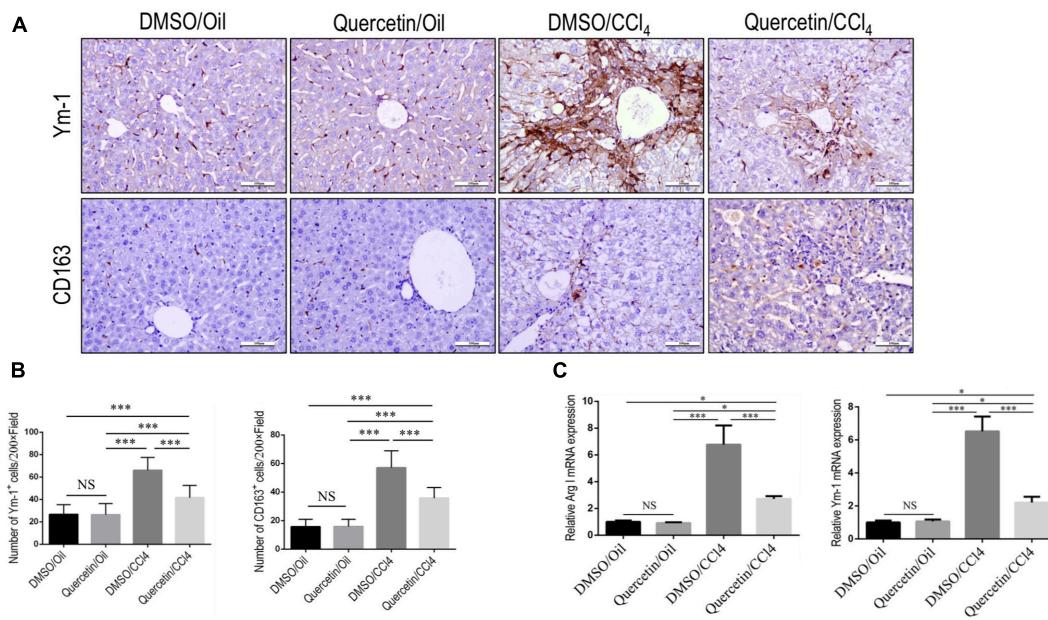


FIGURE 6 | Effect of quercetin on M2 macrophages polarization and expression of immunosuppressive genes in fibrotic livers. **(A)** Representative immunostaining of Ym-1 and CD163 in liver sections (original magnification: $\times 200$). Scale bar = 100 μ m. **(B)** Quantification of Ym-1 and CD163 immunostaining in the liver from each group. **(C)** M2-polarized gene expression of Arginase I (Arg I) and Ym-1 was determined by quantitative RT-PCR, and the results are shown as fold change compared with sham-treated control and GAPDH served as loading control ($n = 5$). * $P < 0.05$; *** $P < 0.001$; “NS” indicates not significant.

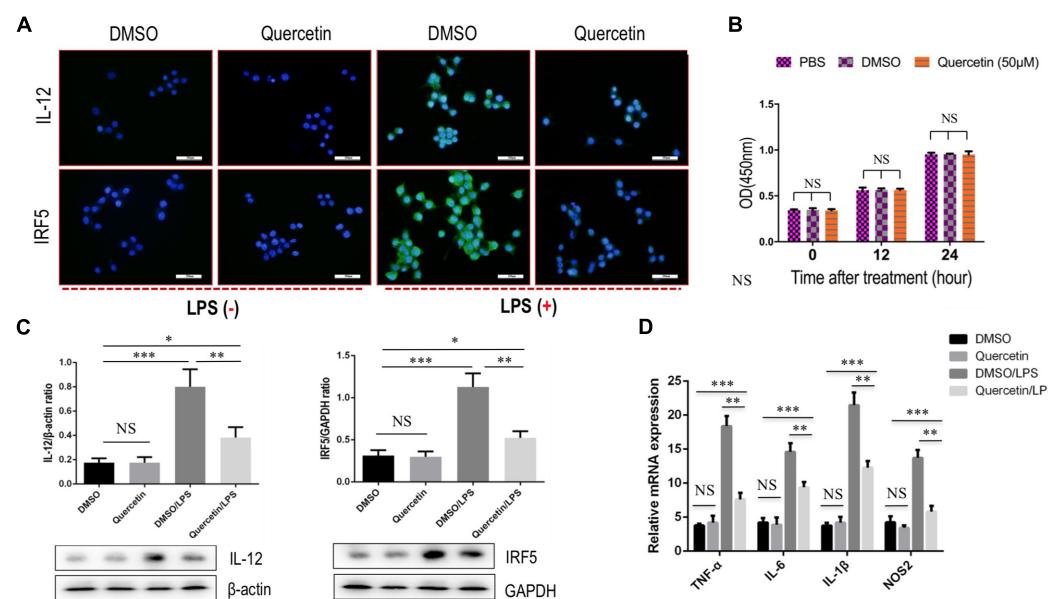
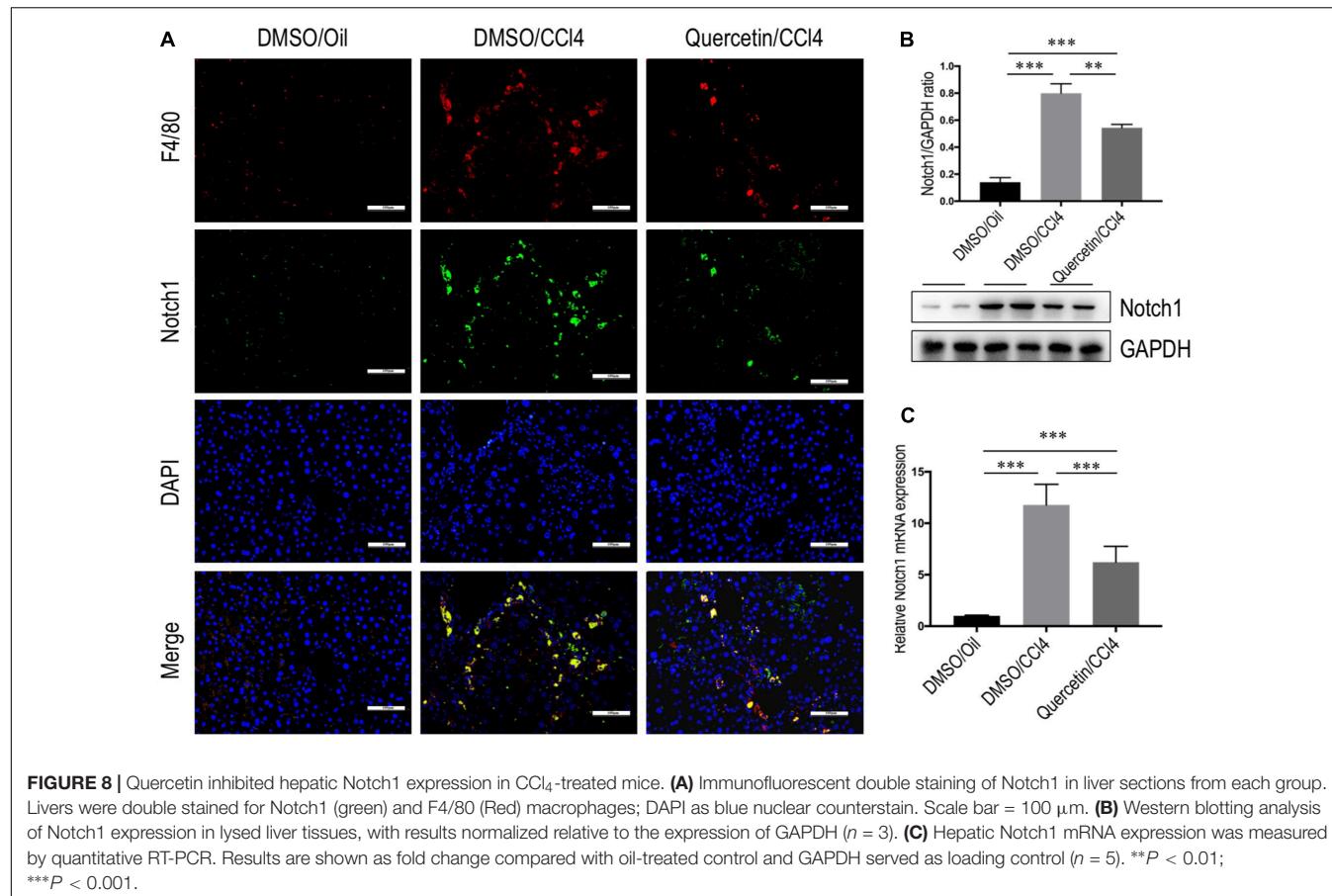


FIGURE 7 | Quercetin treatment suppressed M1 polarization of macrophages *in vitro*. **(A)** Representative fluorescence microscopic images of RAW macrophages with anti-IL12 and anti-IRF5 whole-mount staining. Undifferentiated RAW macrophages conditioned medium, and using LPS (100 ng/mL) to induce M1 differentiation. Quercetin- (50 μ M) or DMSO-treated M1-differentiated macrophages conditioned medium. Bars represent mean \pm SD of at least three independent experiments. Scale bar = 200 μ m. **(B)** Effect of quercetin (50 μ M) on the cell viability of macrophages. Cell viability was then determined by the CCK-8 assay as described in the “Materials and Methods” section. **(C)** Western blotting analysis of M1-markers IL12 and IRF5 protein expression in macrophages RAW 264.7 cells, with results normalized relative to the expression of β -actin or GAPDH ($n = 3$). **(D)** Quantification gene expression analysis of M1-specific markers TNF- α , IL-1 β , IL-6, and NOS2. The mRNA levels were normalized to GAPDH mRNA levels and presented as fold stimulation (mean \pm SD) versus vehicle-treated control. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; “NS” indicates not significant.



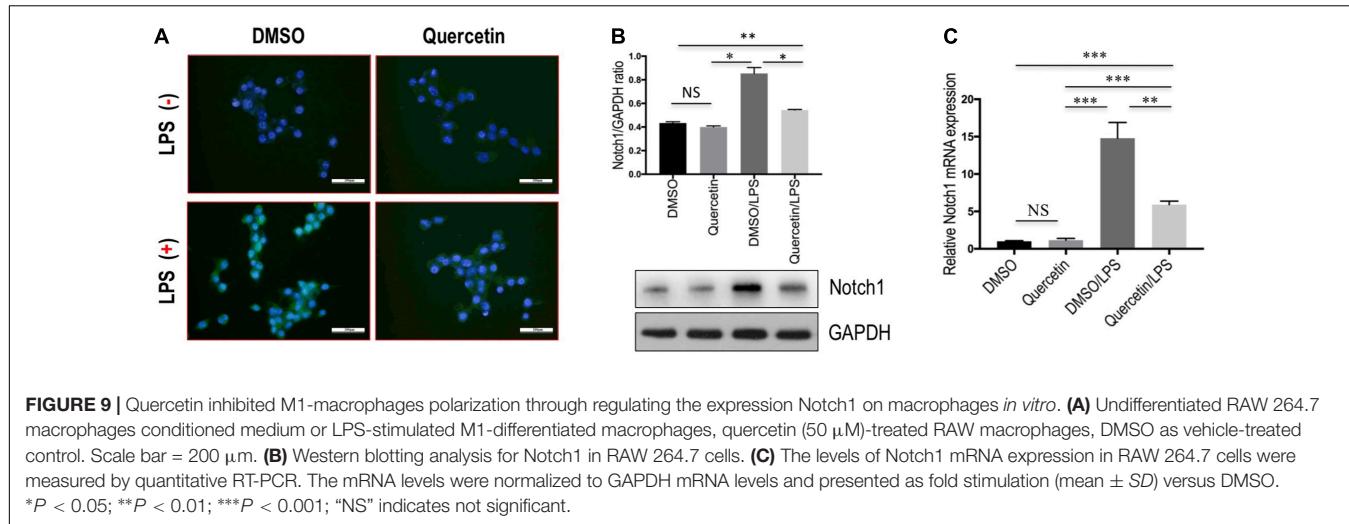
DISCUSSION

In this study, we have provided both *in vivo* validation and mechanistic insights regarding the protective effects of the flavonoid quercetin in CCl_4 -induced liver injury and fibrosis in mice. Importantly, our current finding provides a novel insight for understanding the antifibrotic activity of quercetin, which owing to its inhibition of hepatic macrophages activation and infiltration, and modulation of M1-polarized macrophages via the Notch1 pathway. In addition, we also have strongly reinforced the notion that hepatic macrophages play a critical role in the development of liver fibrosis, and strategies restraining M1 macrophage polarization phenotype may protect against exacerbated inflammation and thus restrict liver fibrosis (Sica et al., 2014; Tacke and Zimmermann, 2014; Tacke, 2017).

Quercetin, a polyphenol (diferuloylmethane), has manifested a diverse range of pharmacological activities including anti-inflammatory, antioxidant, antibacterial, and antitumor properties (Russo et al., 2012; Wu et al., 2017). In the present experiment, using the well-established liver fibrosis model by injection CCl_4 in mice, we provided more evidence that quercetin ameliorated liver inflammation and fibrosis (Figure 1). In liver fibrogenesis, excess ECMs, including collagen, are mainly produced by activated HSCs (Friedman, 2008; Tsuchida and Friedman, 2017). Our results also demonstrated that quercetin

inhibited the activation of HSCs by CCl_4 intoxication (Figure 3). Moreover, when we examined collagen synthesis by measuring the levels of expression of mRNA encoding Collagen $\alpha 3(\text{I})$ and Collagen $\alpha 4(\text{I})$, and the activated HSC markers, we found that the expression levels of these fibrogenic markers were markedly lower in quercetin- than in vehicle-treated mice after 8 weeks of CCl_4 injection (Figure 2C). These data are consistent with the observation that quercetin treatment inhibited HSC activation *in vitro* as shown for the expression of genetic markers such as Collagen $\alpha 1(\text{I})$, TGF- $\beta 1$, and α -SMA (Li et al., 2016b). Furthermore, the data demonstrated that the mechanism of inhibition of liver fibrosis by quercetin was surmised to be direct downregulation of the HMGB1-TLR2/4-NF κ B signaling pathway (Li et al., 2016b). Recently, an interesting study demonstrated that quercetin restricted liver fibrosis by inhibiting HSC activation and reducing autophagy through regulating crosstalk between the TGF- $\beta 1$ /Smads and PI3K/Akt pathways (Wu et al., 2017). Here, we provided evidence that quercetin inhibited liver fibrosis through regulating macrophage polarization and function via Notch1 pathway.

Emerging data have recently demonstrated that macrophages play a complex role in liver fibrogenesis, involved in progression and resolution of hepatic fibrosis (Sica et al., 2014; Tacke and Zimmermann, 2014; Tacke, 2017). Inflammatory cytokines released from those cells perpetuate inflammation as well as



activating HSCs (Pradere et al., 2013; Pellicoro et al., 2014; Tacke, 2017). In this study, we demonstrated that quercetin reduced hepatic macrophage number and ameliorated liver fibrosis following CCl₄ treatment (Figure 4). Notably, our data further suggested that quercetin suppressed M1-polarized macrophages that have inflammatory properties and mediate excessive liver inflammation and fibrosis (Figure 5). Consistent with the inhibition in M1 macrophages activation and shift, the inflammatory cytokines were decreased in quercetin-treated fibrotic livers and in quercetin-treated macrophages when compared with the respective controls (Figure 5C). In order to investigate the effect of quercetin on M1-polarized macrophages, we used Raw 264.7 cell line as *in vitro* model to study. We found that quercetin indeed blocked LPS-mediated M1 macrophages activation as measured by immunofluorescence, western blots, and quantitative RT-PCR (Figure 7). Therefore, our data indicated that quercetin could serve as a regulator of macrophage recruitment and polarization in injury liver.

We also assessed the effect of quercetin on M2-polarized macrophages in fibrotic livers in mice induced by CCl₄ for 8 weeks. Our results showed that the number of Ym-1⁺ and CD163⁺ macrophages in fibrotic livers was obviously increased with displayed higher hepatic expression of M2-macrophage genes (Arg I and Ym-1), as compared to the normal control mice. However, treatment of fibrotic mice with quercetin inhibited M2 macrophages polarization and decreased expression of classic M2 genes in fibrotic livers (Figure 6). On the contrary, previous *in vitro* study has demonstrated that quercetin could induce M2 polarized macrophages (Dong et al., 2014). It is worth to note that inactivation of the M2 macrophages contributed to diet-induced NASH *in vivo* studies and data have recently demonstrated that M2-polarized macrophages promote resolution of inflammation and tissue repair (Beljaars et al., 2014; Tacke, 2017). Consist with our results, previous studies have also demonstrated that Ym-1⁺, CD206⁺, or CD163⁺ macrophages were increased and had the potential effect on liver inflammatory changes in different liver inflammation and fibrosis animal models (Beljaars et al., 2014; Ohtsuki et al., 2015;

Svendsen et al., 2017). Additionally, a recent report suggests that dietary quercetin ameliorated high-fat diet-induced obesity and insulin resistance in mice by regulating the balance of M1/M2 polarization in liver macrophages and reducing the levels of proinflammatory cytokines (Dong et al., 2014). Those different results indicated there is remarkable heterogeneity of liver macrophages with diverse functions, and that function varies according to the phage of injury and depending on the hepatic microenvironment, and is also influenced by the nature of the underlying liver injury (Tacke and Zimmermann, 2014; Tacke, 2017). In addition, there certainly existed functionally distinct macrophage subtypes, which are not simply a subpopulation of macrophages (CD163⁺ or Ym-1⁺) in this study that could be explained the role of M2 macrophages in the development of pathological fibrosis. Thus, understanding of macrophage polarization and function is a keystone of deciphering liver fibrogenesis. Future studies are needed to further confirm whether quercetin mediated the M2 macrophages polarization both *in vivo* and *in vitro*.

Recent investigations have focused on elucidating the molecular mechanisms that suppress inflammation and prevent the development of fibrosis (Wynn and Vannella, 2016). It has revealed that macrophage differentiation and activation is subjected to tight control by several mechanisms, including signaling molecules, transcription factors, epigenetic mechanisms, and posttranscriptional regulators (Lawrence and Natoli, 2011; Wynn et al., 2013; Xu et al., 2015b). Of note, emerging evidence has suggested that Notch pathway plays an important role in macrophage-mediated inflammation (Palaga et al., 2008; Xu et al., 2015b; Kimball et al., 2017). A recent study demonstrated that Notch1-mediated signaling regulation of M1 macrophage activation contributed to the inflammatory pathologies in alcoholic liver disease and obesity-induced liver disease (Xu et al., 2015a,b). It has also been reported that suppressor of cytokine signaling 3 (SOCS3) may play an important role in Notch signaling-mediated M1 macrophage polarization (Eun and Jeong, 2016). In this study, we demonstrated that Notch1 expression on macrophages

was increased during liver injury in mice; however, quercetin treatment abrogated the increased level of Notch1 expression (**Figure 8**). Additionally, in the presence of LPS-induced macrophage activation *in vitro*, in line with M1 polarization, the expression of Notch1 on macrophages was increased. However, quercetin treatment inhibited M1 polarization and the Notch1 expression on macrophages (**Figure 9**). Of note, we have recently demonstrated that quercetin could inhibit HMGB1-TLR2/TLR4-NFkB signaling pathway in the fibrotic liver (Li et al., 2016b). Given that TLR2/TLR4 signaling was also involved in proinflammatory macrophages activation (Palaga et al., 2008; Kimball et al., 2017), we deduced that this inhibitory effect of quercetin might partially mediate in suppressing M1 polarization of macrophages. Collectively, our data highlight a key role for the Notch1 pathway in regulating M1 macrophage polarization in liver injury and fibrosis, indicating that blockade of Notch1 signaling may represent a promising therapeutic target for chronic liver inflammation and fibrosis.

CONCLUSION

Our data lead the evidence for supporting the concept that the hepatic macrophages play a key role in the development of liver fibrosis; and quercetin treatment could be a potential agent for chronic liver inflammation and fibrosis, at least in part, by manipulating macrophage phenotype and activation.

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- This quercetin's antifibrotic activity should be studied in treating human chronic liver diseases in future.

ETHICS STATEMENT

The present study was approved by the Animal Care Committee of Fudan University (Shanghai, China).

AUTHOR CONTRIBUTIONS

XL and CT conceived the study and wrote the manuscript. XL, SZ, and CT contributed to the work designing, performing, analyzing, and interpreting data from all the experiments. QY, QJ, SZ, and LL participated in the design, acquisition, analysis, and interpretation of the data. CT and XL carried out the animal model and all the *in vivo* animal experiments. CT, SZ, and XL interpreted the data and finalized the article. All authors have critically revised and approved the final manuscript and agreed to be accountable for all aspects of the work.

FUNDING

This work was supported by the National Natural Science Foundation of China (grant no. 81170398).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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MicroRNA-129-5p Regulates Glycolysis and Cell Proliferation by Targeting the Glucose Transporter SLC2A3 in Gastric Cancer Cells

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Edited by:

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Specialty section:

This article was submitted to
Gastrointestinal and Hepatic
Pharmacology,
a section of the journal
Frontiers in Pharmacology

Received: 21 February 2018

Accepted: 26 April 2018

Published: 15 May 2018

Citation:

Chen D, Wang H, Chen J, Li Z, Li S, Hu Z, Huang S, Zhao Y and He X (2018) MicroRNA-129-5p Regulates Glycolysis and Cell Proliferation by Targeting the Glucose Transporter SLC2A3 in Gastric Cancer Cells.

Front. Pharmacol. 9:502.
doi: 10.3389/fphar.2018.00502

Tumor cells increase their glucose consumption through aerobic glycolysis to manufacture the necessary biomass required for proliferation, commonly known as the Warburg effect. Accumulating evidences suggest that microRNAs (miRNAs) interact with their target genes and contribute to metabolic reprogramming in cancer cells. By integrating high-throughput screening data and the existing miRNA expression datasets, we explored the roles of candidate glycometabolism-regulating miRNAs in gastric cancer (GC). Subsequent investigation of the characterized miRNAs indicated that miR-129-5p inhibits glucose metabolism in GC cells. miRNA-129-5p directly targets the 3'-UTR of SLC2A3, thereby suppressing glucose consumption, lactate production, cellular ATP levels, and glucose uptake of GC cells. In addition, the PI3K-Akt and MAPK signaling pathways are involved in the effects of the miR-129-5p/SLC2A3 axis, regulating GC glucose metabolism and growth. These results reveal a novel role of the miR-129-5p/SLC2A3 axis in reprogramming the glycometabolism process in GC cells and indicate a potential therapeutic target for the treatment of this disease.

Keywords: miR-129-5p, gastric cancer, cancer metabolism, proliferation, SLC2A3

INTRODUCTION

Gastric cancer (GC) is the fifth most common malignancy in the world (Ferlay et al., 2015). In China, GC is the top three leading cause of cancer death among both men and women, causing approximately 679,100 new cancer cases and 498,000 deaths per year (Chen et al., 2016). Although the incidences and mortality trends for GC have declined in recent years (Siegel et al., 2014), the outcomes of this disease are still among the poorest of all solid-organ tumors, predominantly due to the frequent presence of advanced stage disease with lymphatic or distant metastasis. Current treatment strategies define curable GC as disease (stage 0–III) without distant metastasis (Shen et al., 2013), whereas stage IV disease remains incurable and carries a very poor prognosis despite

Abbreviations: 3'-UTR, 3'-untranslated region; GC, gastric cancer; GSEA, Gene Set Enrichment Analysis; SLC2A3, solute carrier family 2 member 3; TCGA, The Cancer Genome Atlas.

the advent of molecularly targeted biological therapy and novel chemotherapy (Sonnenblick et al., 2015). As there are limited therapeutic approaches for treating advanced GC, it is urgent to elucidate new molecular mechanisms and develop more therapeutic targets for this lethal disease.

Cancer cells exhibit unrestrained growth, which are supported by metabolic adaptations that promote their survival. Aerobic glycolysis, or the Warburg effect (Warburg et al., 1927; Warburg, 1956a), a shift from oxidative phosphorylation to glycolysis and the concomitant accumulation of lactate by-products in the surrounding microenvironment, represents the best-characterized alteration of tumor cell metabolism (Gillies et al., 2008; Cairns et al., 2011b). Altered glucose metabolism is characterized by increased glucose uptake, enhanced glycolysis, and dysregulated mitochondrial oxidative phosphorylation, as well as accumulated lactate production, events that are widespread in various cancer types (Warburg, 1956a,b; Shaw, 2006; Adekola et al., 2012). Recent studies have indicated that several key rate-limiting enzymes of glycometabolic pathways are dysregulated in GC cells, contributing to increased cell proliferation and metastasis (Cui et al., 2015; Jiang et al., 2015; Shiroki et al., 2017). MicroRNAs (miRNAs) are approximately 21–25 nucleotide-long, non-coding RNA molecules that negatively regulate gene translation by binding to the 3'-untranslated region (3'-UTR) of target messenger RNAs (mRNAs), causing either enhanced mRNA degradation or inhibited protein translation (Tay et al., 2008). miRNAs have critical effects that limit the glucose metabolism of cancer cells (Fong et al., 2015; Qiu et al., 2015), and emerging evidences suggest that aberrant miRNAs participate in the altered glycometabolism and pathogenesis of GC (Liu et al., 2016; Li C.Y. et al., 2016).

In this study, we explored potential glycometabolism-regulating miRNAs in GC by integrating our previous high-throughput screening results and the existing GC miRNA expression datasets. miR-129-5p was identified as a candidate suppressor miRNA for glucose metabolism and cell proliferation, and SLC2A3 was a direct functional target gene of this miRNA in GC cells. The miR-129-5p/SLC2A3 axis may serve as a candidate therapeutic target for GC treatment.

MATERIALS AND METHODS

Cell Culture

The HEK 293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, New York City, NY, United States), and SGC-7901 and MGC-803 cells were cultured in RPMI-1640 medium (HyClone, Beijing, China). The media were supplemented with 10% fetal bovine serum (Gibco), 100 IU/mL penicillin G, and 100 µg/mL streptomycin sulfate (Sigma-Aldrich, St. Louis, MO, United States). PDGF-BB (Peprotech, Rocky Hill, CT, United States) was applied at the concentration of 50 ng/mL in RPMI-1640. Cells were cultured in a humidified 37°C incubator with 5% CO₂.

Transfection of Oligonucleotides

The miR-129-5p mimic (5'-CUUUUUGCGGUCUGGG CUUGC-3') and inhibitor (5'-GCAAGCCCAGACCGCA AAAAG-3') were designed and synthesized by Ambion (Austin, TX, United States). Three independent small interfering RNAs (siRNAs) targeting SLC2A3 were synthesized by Ribobio (Guangzhou, China), and the targeting sequences are as follows: 5'-GCTCTTCCAATTGGC TA-3', 5'-CCGACAGCCCATCATCATT-3' and 5'-GCTTCC TCATTACCTTCTT-3'. Cells were transfected with the indicated oligonucleotides or a siRNA pool (3 siRNAs were mixed in an equimolar ratio) with final concentration of 50 nM, using Lipofectamine 2000 (Life Technologies, Darmstadt, Germany). At 48 h post-transfection, cells were harvested for further detection.

Lactate Production and Glucose Consumption

Cells were cultured in DMEM without phenol red (Gibco, New York City, NY, United States) for 15 h, and then the culture medium was collected for lactate or glucose measurement. Quantification of lactate levels was performed utilizing Lactate Assay Kit (BioVision, Mountain View, CA, United States), and glucose levels were determined by Glucose Assay Kit (Sigma-Aldrich, Cat. No. GAHK20-1KT). All values were normalized by the corresponding total protein level (BCA Protein Assay Kit; Thermo Scientific, Waltham, MA, United States).

Cellular ATP Levels

The cellular levels of ATP were determined using CellTiter-Glo® Luminescent Cell Viability Assay (Promega, Madison, WI, United States), following the manufacturer's instructions. All values were normalized by the corresponding total protein level.

Glucose Uptake

Cells were treated as indicated for 24 h, then seeded in a 96-well plate (13,000 cells/well) and cultured for overnight. The culture medium was removed and cells were washed with PBS. Then, cells were incubated with fresh-prepared 500 µM 2-deoxyglucose (2-DG, 50 µL/well) for 20 min at room temperature. The uptake process was stopped and neutralized, and luciferase activities were measured by Glucose Uptake-Glo Assay (Promega). Rate of glucose uptake was analyzed according to the manufacturer's instructions.

Colony Formation Assays, Cell Proliferation, and EdU Incorporation Assay

For colony formation assay, cells were plated in 6-well plate (1,000 cells/well) and incubated 1 week, then fixed with 4% paraformaldehyde and stained with 1% crystal violet (Sigma-Aldrich). Megascopic cell colonies were counted. Cell proliferation was measured by Cell Counting Kit-8 (CCK-8, Dojindo, Kumamoto, Japan), following the manufacturer's instructions. For EdU incorporation assay, cells were incubated with EdU (final concentration of

10 μ mol/L) for 2 h and then analyzed by Click-iT EdU Alexa Fluor[®] Imaging Kit (Molecular Probes, Eugene, OR, United States). Images were acquired using an Olympus DP71X microscope (Olympus, Tokyo, Japan), and EdU-positive cells were counted.

Western Blot

Proteins were separated on 7.5 or 10% sodium dodecyl sulfate-polyacrylamide gel and transferred to nitrocellulose membrane (Bio-Rad, Hercules, CA, United States). Then, the membrane was blocked with 5% non-fat milk and incubated with indicated primary antibodies. The proteins were detected using enhanced chemiluminescence reagents (Thermo Scientific). The primary antibodies used were anti-phospho-Akt (Ser473, #9271; Cell Signaling Technology, Beverly, MA, United States), anti-Akt (#9272, Cell Signaling Technology), anti-Phospho-Erk1/2 (Thr202/Tyr204, #4370, Cell Signaling Technology), anti-p44/42 MAPK (Erk1/2, #9102, Cell Signaling Technology), and anti- β -actin (60008-1-lg, Proteintech).

RNA Extraction and Quantitative Real-Time Polymerase Chain Reaction (qPCR) Analysis

Total RNA was extracted from cells with TRIzol[®] reagent (Invitrogen, Carlsbad, CA, United States). Reverse-transcribed complementary DNA was synthesized by PrimeScriptTM RT Reagent Kit (TaKaRa, Tokyo, Japan). qPCR analyses were performed with SYBR Premix Ex Taq II Kit (TaKaRa, Tokyo, Japan). The detailed primer sequences are listed in Supplementary Table S1. Mature miR129-5p were quantified with specific primers and probes using TaqMan MicroRNA Assays (Applied Biosystems, Foster City, CA, United States), with U6 small nuclear RNA as internal control.

Vector Construction

The pri-miR-129 sequence was amplified from normal human genomic DNA and cloned into the pWPXL lentiviral vector (a generous gift from Dr. Didier Trono) to generate Lenti-miR-129-5p. pWPXL-SLC2A3 was constructed by inserting the open reading frame (ORF) of SLC2A3. The 3'-UTR sequence of SLC2A3 was amplified and inserted into the psiCHECK-2 vector (Promega, Madison, WI, United States). Detailed sequences of the primers and oligonucleotides are listed in Supplementary Table S1.

Lentivirus Production and Transduction

Lentiviral particles were harvested 48 h after Lenti-miR-129-5p or pWPXL-SLC2A3 co-transfection with the packaging plasmid psPAX2 and the VSV-G envelope plasmid pMD2.G (psPAX2 and pMD2.G were gifts from Dr. Didier Trono) into HEK 293T cells using Lipofectamine[®] 2000. SGC-7901 and MGC-803 cells were infected with the resultant recombinant lentivirus in the presence of 6 μ g/ml polybrene (Sigma-Aldrich).

Genome-Wide Transcriptional Profiling via the Complementary DNA (cDNA) Microarray

The SGC-7901 and MGC-803 cells were transfected with miR-129-5p mimics or control mimics. At 48 h post-transfection, cells were harvested for total RNA extraction. For microarray analysis, cells in TRIzol were shipped on dry ice to KangChen Bio-Tech (Shanghai, China) for analysis via the Agilent Whole Human Genome Oligo Microarray (one-color) platform. The RNA preparation and microarray hybridization were performed according to the manufacturer's instructions. Differentially downregulated genes were identified through fold-change filtering ($\log_2\text{Foldchange} < -1$). Pathway analysis and GO analysis were used to explore the roles of these differentially expressed genes.

Luciferase Assays

The HEK 293T cells were cultured in 96-well plates and co-transfected with 10 ng of the psiCHECK-2-SLC2A3-3'-UTR vector and either 5 pmol miR-129-5p mimics or control mimics. After 48 h of incubation, firefly and Renilla luciferase activities were measured using Dual-Luciferase Reporter Assay System (Promega).

Statistical Analysis

Results are presented as the means \pm standard error of the mean (SEM) from at least three independent experiments. Unless otherwise stated, differences between 2 groups or more than 2 groups were determined using Student's *t*-test or one-way analysis of variance (ANOVA), respectively, followed by Dunnett's multiple-comparisons test. *P*-values < 0.05 were considered statistically significant. Statistical analyses were performed using GraphPad Prism for Windows, version 6.00 (GraphPad Software, San Diego, CA, United States).

RESULTS

miR-129-5p Is a Repressor of Glucose Metabolism in GC Cells

Lactate, the final product of aerobic glycolysis, is often overproduced in various types of tumors, which can be used to estimate the specific metabolomic characteristics and acidic microenvironment of tumor cells (Draoui and Feron, 2011). We previously identified 100 glycometabolism-regulating miRNAs using a high-throughput lactate-production screening platform in HeLa cells (Guo W. et al., 2015). By summarizing previously reported miRNA expression datasets, 98 miRNAs were selected based on their frequent dysregulation in GC tissues (Supplementary Table S2). Candidate GC-glycometabolism-related miRNAs were defined as the intersection between the 98 dysregulated miRNAs in GC and the 100 candidate glycometabolism-regulating miRNAs (Figure 1A). In total, seven candidate anti-metabolic miRNAs (miR-29c-3p, miR-34b-5p, miR-124-3p, miR-126-3p, miR-129-5p, miR-148a-3p, and miR-375) and

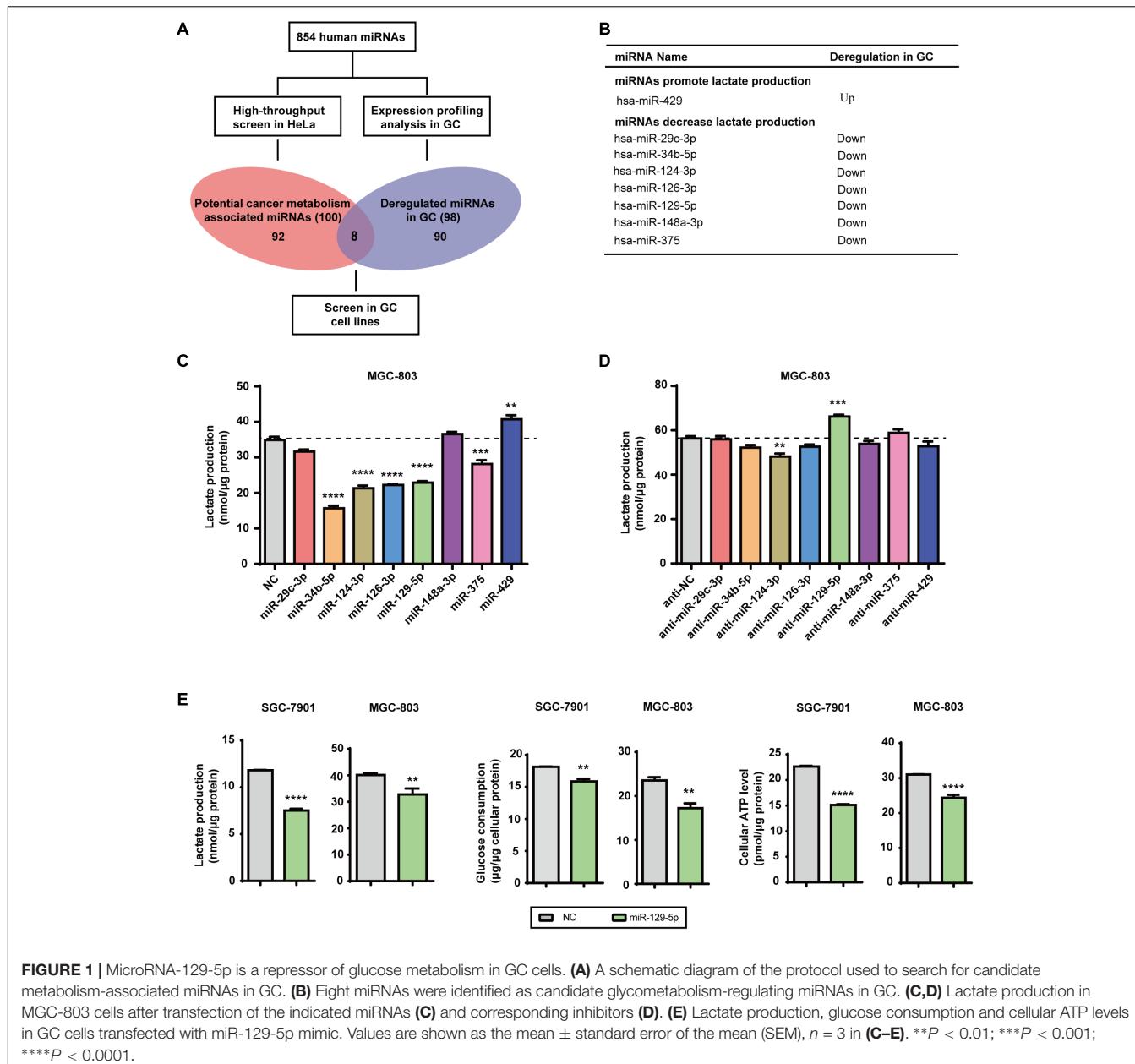


FIGURE 1 | MicroRNA-129-5p is a repressor of glucose metabolism in GC cells. **(A)** A schematic diagram of the protocol used to search for candidate metabolism-associated miRNAs in GC. **(B)** Eight miRNAs were identified as candidate glycometabolism-regulating miRNAs in GC. **(C,D)** Lactate production in MGC-803 cells after transfection of the indicated miRNAs **(C)** and corresponding inhibitors **(D)**. **(E)** Lactate production, glucose consumption and cellular ATP levels in GC cells transfected with miR-129-5p mimic. Values are shown as the mean \pm standard error of the mean (SEM), $n = 3$ in **(C-E)**. ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

one candidate pro-metabolic miRNA (miR-429) emerged (**Figure 1B**).

To further confirm the specific effects of these eight miRNAs on GC glycometabolism, we evaluated lactate production of the MGC-803 cells that were transfected with indicated miRNA mimics or miRNA inhibitors. The results showed that miR-129-5p dramatically repressed lactate production of MGC-803 cells (**Figure 1C**), whereas its inhibitor significantly elevated lactate production of MGC-803 cells (**Figure 1D**). Moreover, miR-129-5p mimics could reduce lactate production, glucose consumption and cellular ATP levels of SGC-7901 and MGC-803 cells (**Figure 1E**), indicating the potential role of miR-129-5p in GC glycometabolism. Taken together, these findings suggest that miR-129-5p inhibits glucose metabolism in GC cells.

SLC2A3 Is the Direct Target of miR-129-5p in GC Cells

To elucidate the mechanisms underlying the inhibitory effects of miR-129-5p on the glycometabolism of GC cells, we next identified its functional target genes. The glycometabolism-related genes were clustered with the annotation of Gene Ontology Biological Function¹. There were 29 glycometabolism-related genes that were upregulated in the GSE13911 dataset ($\text{Log}_2 \text{ FoldChange} > 1$, Supplementary Table S3), and 8 glycometabolism-related genes that were downregulated in miR-129-5p-treated MGC-803 cells ($\text{Log}_2 \text{ FoldChange} < -1$,

¹<http://geneontology.org/>

Supplementary Table S4). Then, these genes were assessed by TargetScan² prediction and miRanda³ prediction, and SLC2A3 was identified as a potential target of miR-129-5p responsible for GC glycometabolism (Figure 2A). To further validate whether SLC2A3 could be directly regulated by miR-129-5p, the wild-type (WT) or mutant (MT) 3'-UTR of SLC2A3 was introduced into luciferase reporter plasmids (Figure 2B). There are two predicted miR-129-5p binding sites in the 3'-UTR of SLC2A3. miR-129-5p dramatically suppressed the luciferase activity of WT SLC2A3 3'-UTR and MT2 SLC2A3 3'-UTR and had a minor effect on MT1 SLC2A3 3'-UTR, but did not affect MT (1+2) SLC2A3 3'-UTR, suggesting that miR-129-5p predominantly binds to the first predicted site (1804–1825 nt) of SLC2A3 3'-UTR (Figure 2C). Moreover, miR-129-5p mimic significantly reduced the mRNA and protein levels of SLC2A3 in GC cells (Figure 2D).

The miR-129-5p/SLC2A3 Axis Regulates Glucose Metabolism in GC Cells

Given that miR-129-5p represses glucose metabolism in GC cells, we next investigated the possible roles of its target gene SLC2A3 in GC glucose metabolism. Silencing of the endogenous SLC2A3 with siRNAs resulted in the dramatic suppression

of the glucose consumption, lactate production, cellular ATP levels, and glucose uptake of GC cells (Figure 3A and Supplementary Figure S1A), which phenocopied the inhibitory effect of miR-129-5p on GC glycometabolism. Furthermore, we established SGC-7901 and MGC-803 cells with stable miR-129-5p overexpression via a lentivirus system (designated as Lenti-miR-129-5p, Supplementary Figure S1B), and constructed a lentivirus plasmid containing an SLC2A3 cDNA sequence without the 3'-UTR to reintroduce SLC2A3 into GC cells that overexpressed miR-129-5p (Supplementary Figure S1C). As expected, miR-129-5p overexpression decreased the lactate secretion, glucose consumption, cellular ATP levels, and glucose uptake of SGC-7901 and MGC-803 cells (Figure 3B), similar to the inhibitory effect of the specific mimics. Moreover, restoration of SLC2A3 in GC cells significantly abolished the miR-129-5p-induced suppression of lactate excretion, glucose consumption, cellular ATP levels, and glucose uptake (Figure 3B). These results suggest that miR-129-5p may regulate glycometabolism through SLC2A3 expression in GC cells.

The miR-129-5p/SLC2A3 Axis Regulates the Proliferation of GC Cells

Over the recent decade, advanced studies of cancer metabolism have broadened our understanding of how disturbed glucose metabolism can be involved in carcinogenesis

²<http://www.targetscan.org>

³<http://www.microrna.org>

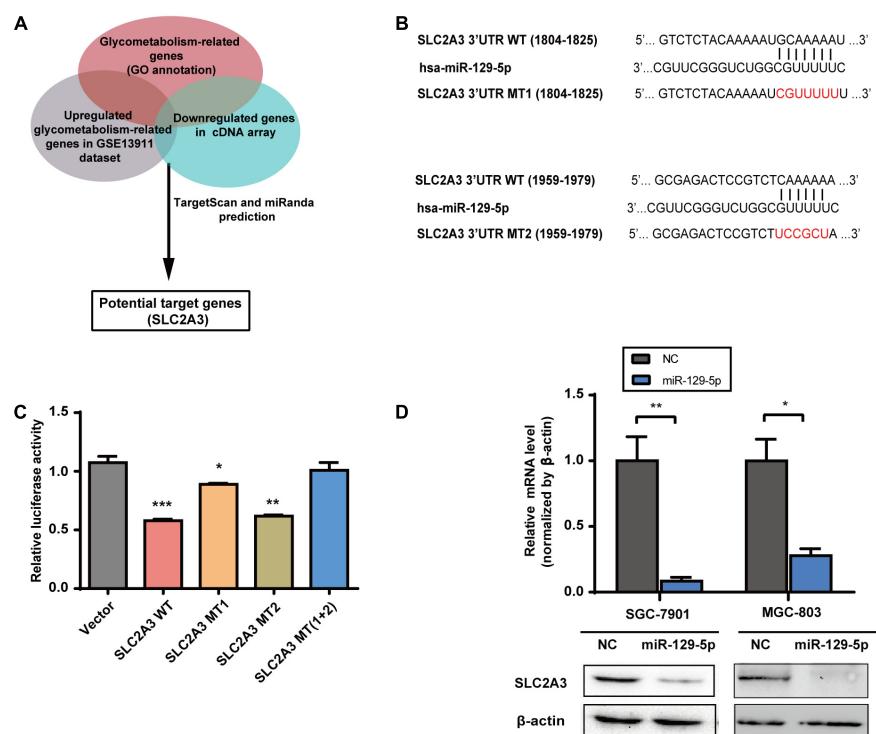


FIGURE 2 | SLC2A3 is the direct target of miR-129-5p in GC cells. **(A)** Schematic representation of the strategy used to identify candidate target genes of miR-129-5p. **(B)** Diagram of putative miR-129-5p binding sites in the 3'-UTR of SLC2A3. The mutant sequences of SLC2A3 3'-UTR used in the luciferase reporter constructs are indicated in red. **(C)** Relative activities of luciferase reporters containing SLC2A3 3'-UTR variants co-transfected with miR-129-5p or negative control mimics in HEK 293T cells. **(D)** SLC2A3 mRNA and protein levels in GC cells transfected with miR-129-5p mimics. Values are shown as the mean \pm SEM, $n = 3$ in **(C,D)**. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

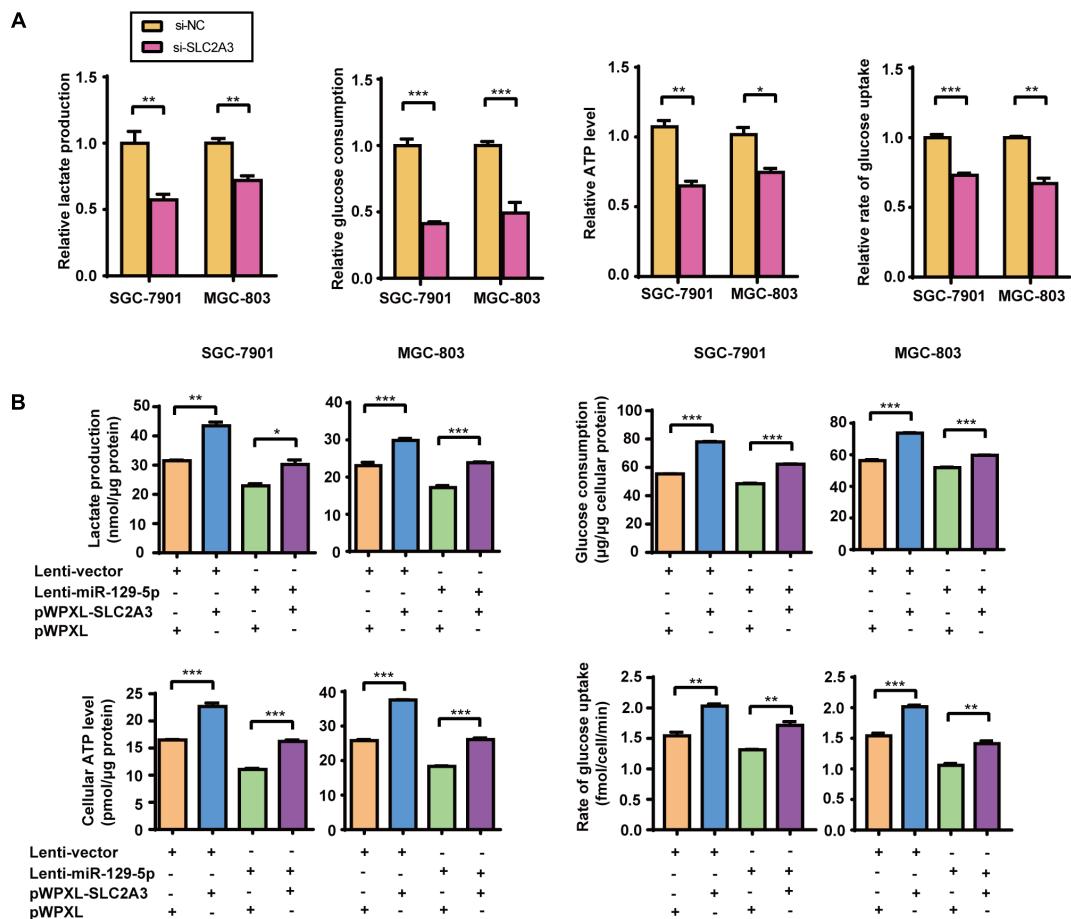


FIGURE 3 | The miR-129-5p/SLC2A3 axis regulates glucose metabolism in GC cells. **(A)** SLC2A3 knockdown suppressed lactate production, glucose consumption, cellular ATP levels and glucose uptake in GC cells. **(B)** The restoration of SLC2A3 protein expression in GC cells significantly abolished the suppressive effects of miR-129-5p on lactate excretion, glucose consumption, cellular ATP levels and glucose uptake in GC cells. Values are shown as the mean \pm SEM, $n = 3$. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

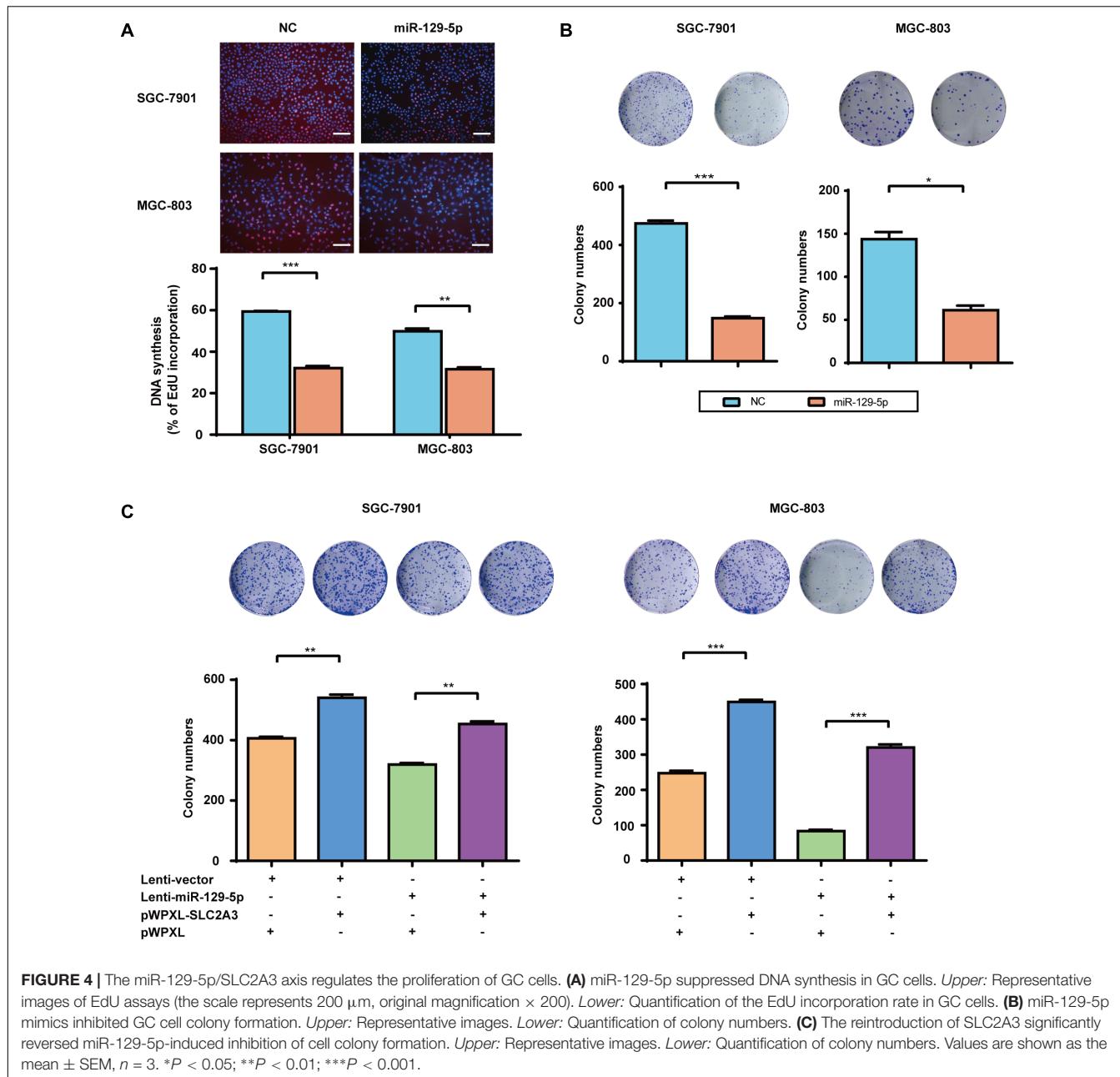
(Ward and Thompson, 2012). miR-129-5p was previously identified as a glycometabolism-related miRNA in GC, then we further characterized its effects on the proliferation of GC cells. EdU incorporation assays showed that miR-129-5p mimic treatment significantly inhibited the proliferation of SGC-7901 and MGC-803 cells (Figure 4A). Moreover, miR-129-5p mimic treatment suppressed the colony formation ability of GC cells (Figure 4B). These results demonstrated that miR-129-5p might act as a suppressor miRNA in GC carcinogenesis. Indeed, miR-129-5p was significantly downregulated in GC tissues in the TCGA cohort (Supplementary Figure S2), compared with that in non-tumor tissues.

Regarding the function of miR-129-5p/SLC2A3 axis in regulating GC glycometabolism, we then examined whether this axis might contribute to the proliferation of GC cells. As shown in Figure 4C, Lenti-129-5p treatment significantly inhibited the colony formation abilities of SGC-7901 and MGC-803 cells, whereas SLC2A3 overexpression dramatically enhanced the colony formation abilities of these cells. Importantly, ectopic expression of SLC2A3

overcame the anti-proliferation effects of miR-129-5p in GC cells (Figure 4C), indicating that targeting SLC2A3 is an important mechanism for the anti-proliferation function of miR-129-5p.

miR-129-5p Reprograms Gene Expression Profiling in GC Cells

To identify the molecular processes and signaling pathways underlying the suppressor activity of miR-129-5p in gastric glycometabolism and carcinogenesis, the gene expression profiling of miR-129-5p-treated MGC-803 cells was analyzed by cDNA microarrays. Functional annotation revealed that signaling pathway gene sets that primarily affected by miR-129-5p include PI3K-Akt signaling pathway, MAPK signaling pathway and Hippo signaling pathways, resulting in the remarkable changes in protein processing in the endoplasmic reticulum and proteoglycans process in cancer (Figure 5A). The cDNA microarray also confirmed that genes in the top-scoring processes, such as those in PI3K-Akt signaling pathway and MAPK signaling pathway (Figure 5B), could



be regulated by miR-129-5p in MGC-803 cells (Figure 5C). Furthermore, miR-129-5p could inhibit the phosphorylation levels of Akt and Erk1/2 in GC cells without obvious changes in the total levels of these proteins, and SLC2A3 knockdown by specific siRNAs suppressed Akt and Erk1/2 phosphorylation (Figure 5D), similar to the effects of miR-129-5p overexpression in GC cells. Moreover, when SGC-7901 and MGC-803 cells were treated with only PDGF-BB, introduction of miR-129-5p in these cells remarkably abolished the promoting effects of PDGF-BB on GC cell proliferation (Figure 5E). These results suggest that the miR-129-5p/SLC2A3 axis exerts its biological activities on glycometabolism and carcinogenesis primarily through

modulating the PI3K-Akt and MAPK signaling pathways in GC cells.

DISCUSSION

Emerging evidences suggest that aberrant miRNAs participate in the pathogenesis of GC (Guo L.L. et al., 2015). Recently, several miRNAs, such as miR-let-7a, miR-148b and miR-181b, were identified as regulators of glycolysis and the citric acid cycle in GC (Tang et al., 2016; Li L.Q. et al., 2016; Ding et al., 2017). We previously identified 100 glycometabolism-regulating miRNAs from an 854-miRNA library using a

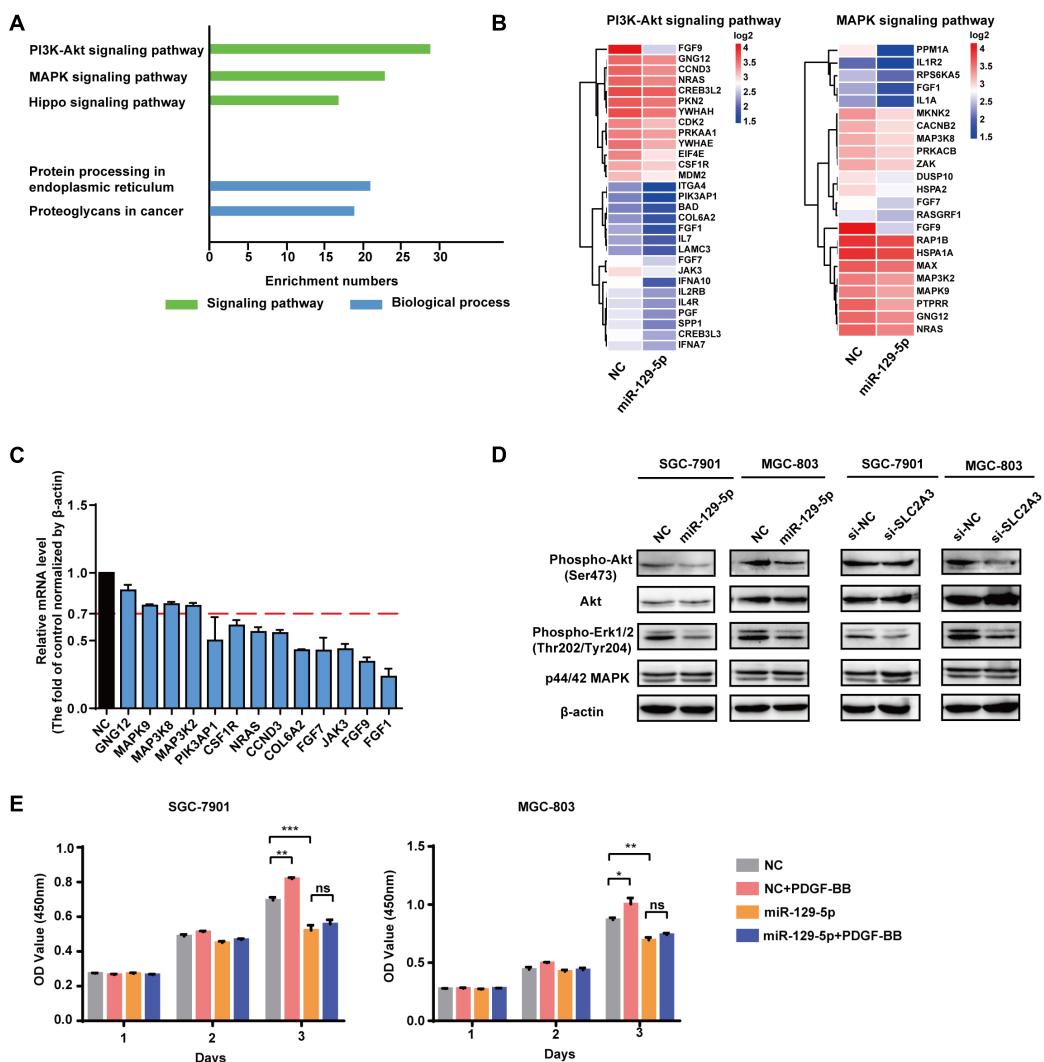


FIGURE 5 | miR-129-5p reprograms gene expression profiling in GC cells. **(A)** Functional gene annotation clustering of genes regulated by miR-129-5p in MGC-803 cells. Significantly enriched groups are ranked based on the group enrichment scores, suggested by the gene ontology terms. Green, signaling pathway. Blue, biological process. **(B)** Expression levels of the subsetting genes involved in PI3K-Akt signaling pathway and MAPK signaling pathway. The genes are shaded with blue or red in the heatmap to indicate low or high expression, respectively. **(C)** qPCR analysis for the selected genes from ranked pathways of MGC-803 cells transfected with miR-129-5p mimics or negative controls. **(D)** Western blotting assays for PI3K-Akt and MAPK signaling pathways in SGC-7901 and MGC-803 cells transfected with miR-129-5p mimics or SLC2A3 siRNAs. **(E)** CCK8 assays for SGC-7901 and MGC-803 cells transfected with miR-129-5p mimics, with or without PDGF-BB treatment. Values are shown as the mean \pm SEM, $n = 3$ in **(C,E)**. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; ns, not significant. β -actin served as internal control.

high-content screening procedure (Guo W. et al., 2015). In the present study, we demonstrated that miR-129-5p could dramatically repress lactate production, glucose consumption, cellular ATP levels, and glucose uptake in GC cells. miR-129 was previously implicated in gastrointestinal cancer (Fesler et al., 2014), due to targeting important genes that are associated with tumorigenesis, disease progression, cell cycle, cell motility, and chemoresistance. The level miR-129-5p was found to be significantly decreased in GC and positively associated with overall survival of GC patient (Li C.Y. et al., 2016). In addition to targeting coding genes, miR-129-5p contributed to lncRNA-AC130710 upregulation in GC tissues (Xu et al.,

2014). Hypermethylation of a miR-129-5p CpG island might play important roles in the development of GC chemoresistance (Wu et al., 2014). As expected, ectopic expression of miR-129-5p significantly inhibited the colony formation abilities and the growth of GC cells. Our findings highlight an additional mechanism for miR-129-5p-inhibited cell growth, which is mediated by the regulation of glucose metabolism in GC cells.

Tumor cells increase their glucose consumption to generate the necessary biomass for their proliferation (Cairns et al., 2011a), and they upregulate facilitative glucose transporter (GLUT) proteins to achieve sufficient glucose uptake (Barron et al., 2016).

GLUT proteins, encoded by the *SLC2* genes, are members of the major facilitator superfamily of membrane transporters (Mueckler and Thorens, 2013), and responsible for the first step of cellular glucose utilization by facilitative diffusion of glucose (Masin et al., 2014). SLC2A3 (GLUT3), a transporter with a high affinity for glucose (K_m approximately 1.5 mM) and the highest calculated glucose turnover rate, transports glucose across the cell membrane in an energy-independent manner (Rodriguez-Enriquez et al., 2009). Including GC, positive staining results for SLC2A3 have been detected in several malignant tumor tissues (Younes et al., 1997), suggesting that SLC2A3 may participate in facilitating glucose uptake in the tumors with intense glucose requirements. Previous studies have shown that SLC2A3 could be directly regulated by miR-106a in glioblastoma (Dai et al., 2013), as well as by miR-195-5p in bladder cancer (Fei et al., 2012). In this study, we demonstrate that SLC2A3 is a direct functional target of miR-129-5p in GC cells and the miR-129-5p/SLC2A3 axis plays important roles in the reprogramming of glycometabolism for tumor bioenergetics and biosynthesis, thus influencing GC cell growth.

Our study further shows that PI3K-Akt signaling pathway and MAPK signaling pathway are involved in mediating the effects of miR-129-5p in GC cells. Gene Set Enrichment Analysis (GSEA) revealed that these two signaling pathways were the top-scoring processes affected by miR-129-5p in GC cells. As Akt and MAPK are pivotal growth-promoting signaling modulators (Wilhelm et al., 2004; Garcia et al., 2006), it is not surprising that they contribute to the proliferation of GC cells as downstream effectors of many stimuli (Wang et al., 2012; Ye et al., 2016). However, the extensive interplay between these pathways and miRNAs, that facilitates the Warburg effect and cell growth in GC cells, is still poorly understood. Our results revealed that the glycometabolism-related miR-129-5p/SLC2A3 axis could significantly affect the phosphorylation of Akt and ERK1/2, and miR-129-5p could erase the promoting effects of PDGF-BB on GC cell proliferation, indicating that PI3K-Akt and MAPK signaling pathways may be implicated in the regulated GC glycometabolism and cell growth by the miR-129-5p/SLC2A3 axis.

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CONCLUSION

We demonstrate that miR-129-5p is a vital regulator of glucose metabolism and cell proliferation in GC cells, and SLC2A3 is the direct functional target of this miRNA. The newly identified miR-129-5p/SLC2A3 axis plays an essential role in GC glucose metabolism and cell growth through the involvement of the PI3K-Akt and MAPK pathways. Our observations provide new insights into the pathogenesis of GC and suggest a novel potential therapeutic target for the treatment of this disease.

ETHICS STATEMENT

Before the experiments, all procedures were approved by the ethical committee of the Fudan University.

AUTHOR CONTRIBUTIONS

XH, YZ, DC, and HW conceived of the presented idea. DC and HW carried out the experiment. DC wrote the manuscript with support from XH, YZ, and HW. DC, YZ, HW, SL and SH analyzed the data. All authors discussed the results and contributed to the final manuscript.

FUNDING

This work was supported by grants from the National Natural Science Foundation of China (81472565, 81672727).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fphar.2018.00502/full#supplementary-material>

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer XT and handling Editor declared their shared affiliation.

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Microbial-Based Therapies in the Treatment of Inflammatory Bowel Disease – An Overview of Human Studies

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OPEN ACCESS

Edited by:

Luca Antonioli,
University of Pisa, Italy

Reviewed by:

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Dapeng Chen,
Dalian Medical University, China

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Specialty section:

This article was submitted to
Gastrointestinal and Hepatic
Pharmacology,
a section of the journal
Frontiers in Pharmacology

Received: 10 October 2018

Accepted: 24 December 2018

Published: 10 January 2019

Citation:

Basso PJ, Câmara NOS and
Sales-Campos H (2019)
Microbial-Based Therapies
in the Treatment of Inflammatory
Bowel Disease – An Overview
of Human Studies.
Front. Pharmacol. 9:1571.
doi: 10.3389/fphar.2018.01571

Inflammatory bowel disease (IBD) is a group of multifactorial and inflammatory infirmities comprised of two main entities: Ulcerative colitis (UC) and Crohn's disease (CD). Classic strategies to treat IBD are focused on decreasing inflammation besides inducing and extending disease remission. However, these approaches have several limitations such as low responsiveness, excessive immunosuppression, and refractoriness. Despite the multifactorial causality of IBD, immune disturbances and intestinal dysbiosis have been suggested as the central players in disease pathogenesis. Hence, therapies aiming at modulating intestinal microbial composition may represent a promising strategy in IBD control. Fecal microbiota transplantation (FMT) and probiotics have been explored as promising candidates to reestablish microbial balance in several immune-mediated diseases such as IBD. These microbial-based therapies have demonstrated the ability to reduce both the dysbiotic environment and production of inflammatory mediators, thus inducing remission, especially in UC. Despite these promising results, there is still no consensus on the relevance of such treatments in IBD as a potential clinical strategy. Thus, this review aims to critically review and describe the use of FMT and probiotics to treat patients with IBD.

Keywords: fecal microbiota transplantation, probiotics, Crohn's disease, Ulcerative colitis, dysbiosis

INTRODUCTION

Inflammatory Bowel Disease (IBD) is a group of immune-mediated diseases mainly represented by Ulcerative colitis (UC) and Crohn's disease (CD) (Mao et al., 2018). IBD presents a multifactorial etiology driven by immunological disturbances, genetic alterations and the influence of environmental factors such as diet, lifestyle, socioeconomic development, intestinal dysbiotic microbiota, among other aspects (Basso et al., 2014). Current therapies are based on pharmacological approaches using traditional medicines such as aminosalicylates, corticosteroids, thiopurines, folic acid antagonists, or biological therapies, aiming at controlling inflammation besides reducing disease relapse (Sales-Campos et al., 2015). However, these approaches are not curative, and patients may become refractory or intolerant to them. In this context, therapies aiming at modulating the microbes inhabiting the human body, especially the intestine, have been suggested as one of the most promising strategies to treat immune-mediated diseases such as IBD

(Ott et al., 2004; Alipour et al., 2016). This is of particular interest because recent investigations demonstrate that conventional treatments fail to completely restore the normal microbiota of patients with IBD, even if associated with special diets (Lewis et al., 2015). Though we understand the importance of other microbial interventions using symbiotics and prebiotics, for example, this review will focus on the human studies using fecal microbiota transplantation (FMT) and probiotics as strategies to restore the normal microbiota in IBD patients.

INTESTINAL MICROBIOTA

Before addressing the role of FMT and probiotics in IBD, it is important to introduce how the intestinal microbiota is able to interact with the vertebrate host, thus influencing health and disease status. Despite the great distribution of microorganisms in different sites of the human body, the most diverse microbial species is found in the gastrointestinal tract (GIT) (Hooper and Gordon, 2001; Hooper et al., 2001). More than 1000 microbial species, including bacteria, virus, and fungal, were identified in the human GIT (Turnbaugh et al., 2007). These commensal and symbiotic communities of microorganisms, also known as microbiota, are able to directly or indirectly influence local and systemic physiology of the human body, including but not limited to the immunologic, endocrine, and nervous systems (Lei et al., 2015). The composition of gut microbiota, in turn, can be influenced by different aspects such as diet, xenobiotics, lifestyle, and genetics (Goodrich et al., 2014; Wen and Duffy, 2017). Thus, it is reasonable to assume the great impact that perturbations in the complex bidirectional relationship between vertebrate hosts and gut microbes may have on host physiology. Further, this complex interaction can also lead to the onset and maintenance of several diseases, including IBD (Eck et al., 2017). Though gut microbiota is colonized by different microorganisms (bacteria, fungi, archaea, and viruses), the term “microbiota” is often used to refer to bacterial species within the GIT, which represents more than 96% of the total microbial population (Turnbaugh et al., 2007). However, fungal and viral dysbiosis have also been implicated in IBD development (Lewis et al., 2015; Duerkop et al., 2018).

To limit inappropriate activation in surfaces with great contact with microbes, like GIT, the human body has developed chemical and physical barriers to anatomically separate the microbiota from immune cells (Hooper et al., 2012). However, this interface is not insurmountable and some commensal microorganisms are able to interact with the immune, endocrine and nervous systems (Cani and Knauf, 2016). So far, two hypotheses were proposed to clarify the mechanisms concerning this interplay: the presence of pattern recognition receptors (PRR) in host cells sensing microbial associated molecular patterns (MAMPs)/danger associated molecular patterns (DAMPS), and the activity of microbial metabolites over different mammalian biological systems (Castro et al., 2015; Rangan et al., 2016). In this context, it is possible to highlight the beneficial role of the polysaccharide A of *Bacteroides fragilis*, which is able to stimulate the differentiation and activity of regulatory

T cells (Treg) in the gut (Donaldson et al., 2016). The presence of Tregs in intestine is of great contribution to the maintenance of a tolerant environment, thus avoiding unnecessary inflammation (Hoepli et al., 2018). Further, the production of immunoglobulin A (IgA) by intestinal plasma cells, which is crucial for the protection against pathogens in intestine, is positively influenced by epithelium-associated bacteria such as *Mucispirillum* and segmented filamentous bacteria (SFB) (Bunker et al., 2015). One of the most studied groups of microbiota-derived metabolites with protective effects toward the mammalian host is the short chain fatty acids (SCFAs) that are mainly derived from fermentation of dietary fibers (Rios-Covian et al., 2016). SCFAs are primarily represented by three compounds acetate, propionate and butyrate, which contribute to the integrity of intestinal epithelium besides directly influencing host metabolic and immune functions (van de Wouw et al., 2018).

INTESTINAL DYSBIOSIS IN THE PATHOGENESIS OF IBD

Dysbiosis has been explored as a causative agent of several systemic and local diseases affecting GIT, including UC and CD (Kostic et al., 2014). The gut microbial changes in IBD are summarized in **Table 1**. In comparison to healthy subjects, IBD patients have reduced microbial composition (up to 25%), diversity, and richness with increased numbers of pathogenic/pathobionts microorganisms (e.g., Proteobacteria, Fusobacteria species, and *Ruminococcus gnavus* – Firmicutes) (Frank et al., 2007), and decreased numbers of beneficial microorganisms such as *Lachnospiraceae*

TABLE 1 | Changes in gut microbiota composition in inflammatory bowel disease patients.

Microorganism (s)	Commensal (C) or pathogenic (P) microorganisms*	UC	DC
Verrucomicrobia	C	↓	↓
Bifidobacterium	C	↓	↓
Roseburia species	C	↓	?
Bacteroides	C	↓↑	↑
Firmicutes	C	↓	↓
Clostridium species (clusters IV and XIVa)	C	↓	↓↑
Saccharomyces cerevisiae	C	↓	↓
Pseudomonas	P	↓	↓
Proteobacteria	P	↑	↑
Fusobacterium	P	↑	↑
<i>Ruminococcus gnavus</i>	P	↑	↑
<i>Candida albicans</i>	P	↑	↑

CD, Crohn's disease; UC, Ulcerative colitis. *Most of the species. References (Gophna et al., 2006; Frank et al., 2007; Kaakoush et al., 2012; Machiels et al., 2014; Lewis et al., 2015; Tahara et al., 2015; Shah et al., 2016; Sokol et al., 2017; Vrakas et al., 2017).

(Firmicutes), *Bifidobacterium* species (Actinobacteria), *Roseburia* (Firmicutes), *Sutterella* (Proteobacteria) (Gilbert et al., 2016), and *Faecalibacterium prausnitzii* (Firmicutes), which are at least 10-fold reduced in IBD (Xiao et al., 2015). To note, *F. prausnitzii* has been suggested as one of the major microbial components of human healthy intestinal microbiota representing almost 5% of the total bacterial population (Louis and Flint, 2009). This bacterium contributes to the maintenance of a regulatory environment in intestine through the production of butyrate, besides providing energy to colonocytes (Sokol et al., 2008). The observation that intestinal microbes cooperate to the maintenance of epithelial integrity in intestine is of great importance since these mechanisms are frequently disrupted in IBD. One of the theories to explain the occurrence of dysbiosis in IBD relies on the inflammation. Results from both experimental and clinical investigations associate inflammatory responses and perturbations in microbial composition in ileum and other intestinal areas to the development of dysbiosis (Gevers et al., 2014; Forbes et al., 2016). On the other hand, a less dysbiotic environment is observed in non-affected areas of diseased subjects (Forbes et al., 2016).

There is evidence suggesting dysbiosis as a cause of IBD. Environmental factors, which directly affects intestinal microbiota composition, have been pointed out as one of the key players in the pathogenesis of IBD. In this regard, early life exposure to breastfeeding and maternal smoking during pregnancy, have been inversely and positively correlated to disease outcome in CD, respectively (Lindoso et al., 2018). Accordingly, patients with UC (Elinav et al., 2011) tend to have a better outcome when treated with microbial-based therapies (i.e., antibiotics, FMT, and probiotics). The mechanisms concerning the influence of dysbiosis in IBD outcome are still a matter of debate and investigation. Some studies suggest the association between the development of inflammation and the presence of some specific bacteria species. The reduction in strict anaerobes (e.g., *Clostridium* groups IV and XIVa), along with the expansion of facultative aerobic or aerobic bacteria, may increase the local concentration of oxygen, thus leading to augmented vascular and mucosal permeability, and promoting intestinal inflammation (Albenberg et al., 2014). Different strains of *Clostridium* species (e.g., IV, XIVa, and XVIII), which lack toxins and virulence factors, have their immunosuppressive activity demonstrated by inducing Treg cells in intestine in a TGF- β -, IL-10- or butyrate-dependent manner (Atarashi et al., 2013; Furusawa et al., 2013). These data suggest that microbial imbalance in IBD favors the development of inflammation by reducing crucial anti-inflammatory players, besides favoring the onset of pro-inflammatory mechanisms. On the other hand, inflammation *per se* also contributes to the onset of a dysbiotic environment. Regardless if inflammation leads to dysbiosis or vice-versa, they have a strong synergistic interaction that must be targeted to develop improved therapeutic strategies. For this reason, therapies aiming at reestablishing the microbial balance may represent the next frontier to treat inflammatory disorders, such as IBD, in which the dysbiosis plays a central role in disease pathogenesis.

FECAL MICROBIOTA TRANSPLANTATION (FMT)

Fecal microbiota transplantation has long been used to treat recurrent *Clostridium difficile* infection (CDI) presenting great effectiveness and significant safeness, with cure rates reaching 90% (Khan et al., 2018). One of the main mechanisms proposed to explain the ability of FMT to treat CDI is attributed to its capacity to restore intestinal microbial balance (Gagliardi et al., 2018). This characteristic has expanded the use of FMT to treat both local and systemic illnesses associated with gut dysbiosis, such as irritable bowel syndrome (IBS) (Mizuno et al., 2017), IBD (Angelberger et al., 2013; Kunde et al., 2013) and metabolic syndrome (Vrieze et al., 2012).

Because of the importance of elucidating how microbiota donors are selected and how FMT is delivered to recipients, these aspects will be clarified first. Then, we are going to present and discuss the most important scientific studies regarding the therapeutic use of FMT in IBD (**Table 2**).

FMT Donor Screening and Routes of Administration

Several aspects must be considered in the search for microbiota donors. Prior to the gut microbial sequencing *per se*, a putative donor must be screened for the presence of infectious agents in feces, including *C. difficile*, intestinal parasites and virus (e.g., Norovirus) (Paramsothy et al., 2015). In blood, aside from the complete blood count, electrolytes, liver, and kidney function tests, the presence of inflammatory markers, and transmissible infectious agents such as HIV, Hepatitis, HTLV, among others, must be performed (Paramsothy et al., 2015). Further, as inclusion criteria, the donor must have no history of suggestive GIT disease, no other major active comorbidities, and preferably, no use of medications, especially, antimicrobials (Paramsothy et al., 2015; Holloran et al., 2018). To ensure that only healthy donors will be selected, additional criteria of exclusion must be used as follows: any family history of colorectal cancer affecting first-degree relatives; use of probiotics 3 months prior the donation period; household members with active GIT infections; any personal or familial history of malignancies, malnutrition, obesity, neurological, or developmental disorders (Paramsothy et al., 2015; Holloran et al., 2018). The difficulties to select FMT donors that fulfills all the stringency criteria along with the costs involved in the screening process have created some important barriers for the broader utilization of this microbial therapeutic approach. Unfortunately, this scenario has stimulated patients to perform FMT in a “homemade” fashion, using inappropriate screened donors, without medical supervision, which often result in serious complications (Hohmann et al., 2014).

For a long time, retention enema was the most used technique for FMT. However, alternative approaches have been used in this regard, including nasogastric tube, capsules, colonoscopy, and self-administered enemas, as previously reviewed (Allegretti et al., 2017). Colonoscopy and retention enema are by far, the most frequently used routes of FMT administration (Gough et al., 2011).

TABLE 2 | Clinical trials of fecal microbiota transplantation for inflammatory bowel disease.

Authors	Diagnosis	Number of patients (P) or studies (S)*#	FMT route	Therapeutic regimen&	Outcome
Paramsothy et al. (2017b)	UC	n = 41 (S)	N.A	N.A	33% of clinical remission
	CD	n = 11 (S)	N.A	N.A	52% of clinical remission
Moayyedi et al. (2015)	UC	n = 70 (P)	Enema	50 g offeces/300 mL of water; once weekly for 6 weeks	24% of clinical remission
Paramsothy et al. (2017a)	UC	n = 85 (P)	Enema	150 mL\$; once a day, 5 days per week for 8 weeks	27% of clinical and endoscopic remission or response
Rosser et al. (2015)	UC	n = 50 (P)	Naso-duodenal tube	60 g of feces/500 mL of saline; two doses (days 0 and 21)	No statistical difference between control and treated patients
Vaughn et al. (2016)	CD	n = 19 (P)	Colonoscopy	50 g of feces/250 mL of saline; one dose	58% of clinical response (control group not included)
Cui et al. (2015)	CD	n = 30 (P)	Endoscopy	150–200 mL\$; one dose	86.7 and 76.7% of clinical improvement and remission, respectively at week 4
Suskind et al. (2015)	CD	n = 9 (P)	Nasogastric tube	30 g of feces/100 or 200 mL of saline; one dose	77.77% of clinical remission at week 2 55.55% of clinical remission at weeks 6 and 12

CD, Crohn's disease; N.A, Not applicable; UC, Ulcerative colitis. *Both total number of patients for clinical trials and number of studies for systematic analysis or meta-analysis were included. #Includes the number of control patients. \$Feces may have undergone additional steps for FMT samples preparation. \$Initial solution concentration is not available.

FMT in IBD

Ulcerative colitis and Crohn's disease are the major entities represented by IBD. The role of FMT has been more explored in the former. From the 307 adult patients pooled in a meta-analysis from 24 UC cohort studies, FMT induced remission in 33%. In 6 pediatric cohort studies, totaling 34 UC patients, clinical remission was slightly reduced to 23% (Paramsothy et al., 2017b). Three randomized controlled trials also presented promising results regarding the use of FMT to treat UC. From a total of 70 UC patients with active disease without infectious diarrhea enrolled in the study, 36 were treated with FMT, and 34 with placebo, once a week for a total of 6 weeks, and remission was induced in 24% of those treated with FMT compared to 5% in the placebo group (Moayyedi et al., 2015). It is important to mention that both placebo and FMT groups were under concomitant anti-inflammatory/immunosuppressive therapy (e.g., corticosteroids, mesalamine, and anti-TNF therapy) while enrolled in the study (Moayyedi et al., 2015). Similar results were observed using enemas 5 days per week for 8 weeks, in a study in Australia that observed a remission rate of 27% in UC patients with active UC treated with FMT when compared to 8% in patients treated with placebo only (Paramsothy et al., 2017a). Regardless if patients had received FMT or not, they were also treated with immunosuppressive drugs such as 5-aminosalicylates, thiopurines, methotrexate, and/or oral prednisone, in a stable dose (Paramsothy et al., 2017a). On the other hand, the remission rates observed in UC patients treated with FMT from healthy donors were similar to those observed in UC patients receiving their own fecal microbiota (Rosser et al., 2015).

Unfortunately, data supporting the role of FMT in CD are scarcer than in UC, and so far, no results from randomized

clinical trials are available. The evidence of the beneficial effects of FMT in CD are all derived from small and uncontrolled studies. A single dose of FMT performed by colonoscopy showed an improvement in clinical outcome of 58% of patients treated with FMT (Vaughn et al., 2016). This observation was followed by increased levels of Tregs in recipients' lamina propria followed by higher microbial diversity (Vaughn et al., 2016), which suggests a reestablishment of microbial balance and a less prominent inflammation. Similarly, a single treatment with FMT induced clinical improvement and remission based on clinical activity in CD patients (Cui et al., 2015). This amelioration was followed by increased patient's body weight after FMT (Cui et al., 2015). For all CD-patients enrolled in the study a 12-week washout period was required for those exposed to immunosuppressive therapies such as cyclosporine, tacrolimus, or infliximab. Antibiotics and probiotics were withdrawn 60 and 30 days before FMT, respectively (Vaughn et al., 2016). The beneficial role of FMT was also addressed in young patients with CD. Nine individuals, aged 12–19 years, presenting mild-to-moderate symptoms received FMT by nasogastric tube once and were followed by 12 weeks (Suskind et al., 2015). Based on the clinical score, 2 weeks after FMT, 7 of 9 patients were in remission, and 5 of 9 patients were in remission at 6 and 12 weeks after FMT. All patients enrolled in the study were allowed to receive immunomodulators during the FMT or placebo treatment (Suskind et al., 2015).

Limitations in FMT Studies

The studies presented here showed promising results regarding the use of FMT to induce remission in UC and to a less extent in CD patients. The differences in the route and

interval of administration, besides of the composition and bacterial load in FMT, may explain the dissimilarities observed among studies. Another important drawback is the lack of comprehensive guidelines to be used globally in the screening and standardization of putative microbiota donors (age, gender, and health status) along with strategies of production, dosage regimen and to evaluate the transplant engraftment. Further, probably because of economic reasons, clinical trials do not deeply investigate the microbial composition of fecal donors using 16S rRNA sequencing and their similarities to the recipients' microbiota. Thus, the observation of similarities between the intestinal microbiota composition of donors and recipients may dictate the successfulness of FMT engraftment. Without the proper identification of the microbial community and the total bacterial load transplanted from a healthy donor to a diseased subject, it is difficult to predict the impact of FMT in IBD or other disorders. Further, as the majority of clinical trials were conducted with concomitant use of immunomodulatory drugs, it is reasonable to assume that FMT may work better as an adjuvant therapy rather than an isolated strategy. To confirm the role of FMT in IBD, more controlled clinical trials with a great number of patients and more standardized fecal samples must be conducted. Additionally, strategies aiming at providing an intestinal microbiota rebalance using well-defined microbial species may represent an improved alternative to total FMT.

FMT Adverse Effects

In general, up to 10% of FMT recipients present minor to mild self-limited adverse effects. The majority of them are related to disturbances in GIT such as diarrhea and abdominal discomfort/pain (Hohmann et al., 2014; Baxter and Colville, 2016). Though less frequently observed, severe side effects can include IBD flares, CDI and other infections, colectomy, small bowel obstruction, pancreatitis, and even death, as recently reviewed (Qazi et al., 2017; Jeon et al., 2018). However, some evidences have shown no differences between FMT and control groups concerning the occurrence of undesirable effects (Narula et al., 2017). Despite the possibility of occurrence of adverse effects, FMT is considered to be safe in IBD. An in-depth screening of donors along with a broader comprehension of the physiopathology in IBD may facilitate the development of strategies to avoid the occurrence of such undesirable effects.

PROBIOTICS

Probiotics are used as safe food additives, pharmaceutical formulations or nutritional supplements defined as "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host" by the World Health Organization (WHO) (Hill et al., 2014). Nevertheless, studies have pointed out that dead microorganisms or their biologically active compounds *per se* can also play protective functions, inferring that the "probiotic" definition should be revisited

or other classifications implemented (Rachmilewitz et al., 2004).

The underlying mechanisms of probiotics are dependent on microbial strain. Moreover, the effects of probiotic mixtures may be complementary (also referred to additive) or synergistic (Ruiz et al., 2009). In general, probiotic strains produce growth factors that strengthen the gut epithelium and antimicrobial substances (e.g., SCFAs, bacteriocins, hydroperoxides, bile acids, and lactic acids) that kill harmful microorganisms (Konieczna et al., 2012a). As a consequence, cellular components (e.g., cellular wall, DNA) are released in the gut environment, which activate immune responses by enhancing the pro-inflammatory cytokines production and immunoglobulin synthesis, besides of improving macrophage and lymphocytes activity (Markowiak and Slizewska, 2017). In this regard, the use of *Bifidobacterium infantis* 35624 in human volunteers increased the amount of IL-10 and FoxP3⁺ cells (Treg) in the circulation (Haskard et al., 2001; Konieczna et al., 2012b). Although immune tolerance is a putative consequence of these enhancements, there is still no consensus on this matter (Castellazzi et al., 2013).

Non-immunological benefits associated to probiotics include the digestion and absorption processes, competition with potential pathogens for nutrients and intestinal adhesion sites, pH alterations, agglutination of pathogenic microorganisms, and sequestration of metabolic toxins (Gagliardi et al., 2018). Animal models and *in vitro* assays describe that probiotics also decrease the apoptosis, increase the mucus synthesis, tissue repair, redistribution and production of tight junctions in gut epithelial cells, thus reducing the intestinal permeability and enhancing the barrier protection and function (Caballero-Franco et al., 2007; Zyrek et al., 2007).

Lactobacillus (e.g., *reuteri*, *rhamnosus*, *casei*, *acidophilus*, *plantarum*, *gasseri*, *paracasei*, *johsonii*, *ghillenii*, and *crispatus*) and *Bifidobacterium* (e.g., *bifidum*, *infantis*, *longum*, *animalis*, *breve*, *lactis*, and *adolescentis*) are the most used strains in probiotics formulations, but multispecies approach has been increasingly applied (Holzapfel et al., 2001). Others strains commonly used include *Streptococcus* spp., *Lactococcus* spp., *Enterococcus* spp., non-pathogenic *Escherichia coli* (strain Nissle), and *Clostridium* spp (Kechagia et al., 2013).

New bacteria genera and species have also been investigated showing good perspectives in preclinical trials. These bacteria are described as new-generation probiotics bringing more complexity to common probiotics in attempt to simulate FMT treatments. The new-generation probiotics comprise *Clostridium* clusters IV, XIVa, and XVIII, *F. prausnitzii*, *Akkermansia muciniphila*, *Bacteroides uniformis*, *B. fragilis*, and *Eubacterium hallii* (El Hage et al., 2017). Technological limitations are current challenges for using these bacteria as probiotics. Importantly, *Clostridium* clusters XIVa and IV are described as promoters of Treg differentiation, critical for immune tolerance as described earlier (Atarashi et al., 2011). Indeed, these bacteria are decreased in the gut of IBD patients (Sokol et al., 2006; Kang et al., 2010; Machiels et al., 2014). Although the number of Tregs is increased in the gut of IBD patients, the expansion is not sufficient to restrain the inflammatory development.

Since gut microbiota is not composed only by bacteria, some formulations and studies use yeasts as probiotics in association with bacteria strains, or even in single-drug formulations. In this context, *Saccharomyces boulardii* is the most commonly used yeast strain and has several anti-inflammatory properties (Pothoulakis, 2009).

Criteria for New Probiotic Development

As pharmaceutical or nutraceutical products, probiotics must meet some criteria to be commercially available. Beyond efficacy, the safety properties of a given drug are the main concern of scientists and regulatory agencies (Doron and Snydman, 2015). Bacterial and yeast strains or their derived-products have distinct levels of regulations according to their purposes and must meet the requirements outlined in published and frequently updated guidelines designed by regulatory agencies (Doron and Snydman, 2015). They can be considered as food (food ingredient, medical food, and dietary supplement), drug (new drugs) or biological product (Degnan, 2008).

As in FMT, safety is a priority, since some inflammatory conditions or patients under immunosuppressive therapy increase the susceptibility to infectious complications, including sepsis (Farina et al., 2001; Riquelme et al., 2003). Probiotics must have human origin, scientifically proven positive effects, be safe even in high-risk populations, cannot cause allergies and must present good technology properties (e.g., feasible culture and large-scale production) (Markowiak and Slizewska, 2017). Several *in vitro* assays may be employed at the first glance to evaluate probiotic potential, epithelium adherence, microbicide activity, ability in reducing the number of pathogenic bacteria and resistance to antibiotic use, bile salts, stomach acids, digestive enzymes and pH (Saarela et al., 2000).

Although not mandatory, studies should also evaluate the adverse effects and drug interactions of probiotics since they have been used as adjuvant therapy in various diseases (Thomsen et al., 2018). For instance, the probiotic *E. coli* strain Nissle 1917 influences the pharmacokinetics of concomitantly taken antiarrhythmic drug amiodarone by increasing the drug bioavailability (Matuskova et al., 2014). Therefore, their presumed safety should be avoided, and the potential risks not neglected.

Probiotics in IBD

In general, probiotics have been effectively used in treating IBD to prevent dysbiosis in patients undergoing prolonged antibiotic or immunosuppressive therapies (Zuo and Ng, 2018). Further, these microorganisms have been used as adjuvant therapy on the attempt to reverse the dysbiotic environment associated with IBD onset and worsening (Yoshimatsu et al., 2015; Tamaki et al., 2016). Although the number of clinical and experimental studies using probiotics in IBD is substantially high, lack of standard practices in therapeutic regimens, low number of samples and poor disease characterization, have limited the relevant conclusions about the efficacy of probiotics in this scenario.

Probiotics have been described as an alternative to induce and maintain the remission in UC, while low or no effects

are observed in CD. The adjuvant use of multispecies probiotic VSL#3, which contains four strains of *Lactobacillus* (*L. casei*, *L. plantarum*, *L. acidophilus* and *L. delbrueckii* subsp. *Bulgaricus*), three of *Bifidobacterium* (*B. longum*, *B. breve*, and *B. infantis*), and one of *Streptococcus* (*S. salivarius* subsp. *Thermophilus*), improved the clinical symptoms in patients with mild to moderately active UC after receiving the daily dose of 3.6×10^{12} CFU (Tursi et al., 2010; Mardini and Grigorian, 2014). Corroborating results were observed after treating mild-to-moderate UC patients with VSL#3 alone, twice a day at the same dose described earlier (Sood et al., 2009). The maintenance of remission rates in UC was also similar in patients under single drug treatment of either non-pathogenic *E. coli* Nissle 1917 ($5-50 \times 10^9$ /day) or mesalazine (1500 mg/day) (Kruis et al., 2004).

However, the systematic review using rigorous statistical methods showed that the beneficial effects of both VSL#3 or *E. coli* Nissle on UC are weak or inconclusive, while there is no positive association in CD (Jonkers et al., 2012), confirming the need for further new randomized controlled trials to increase the significance level of these findings.

The use of *Bifidobacterium*-fermented milk (containing *B. breve*, *B. bifidum*, and *L. acidophilus*) as adjuvant therapy to treat 20 patients (including placebo control) with mild to moderately active UC, showed significant improvement in both clinical and endoscopic activity indexes after 12 weeks (10 billion bacteria/day) (Kato et al., 2004). Interestingly, the SCFAs concentration in feces was higher in the probiotic-treated group compared to the placebo group. However, a recent study using a similar therapeutic strategy (*B. breve*- and *L. acidophilus*-containing fermented milk) showed no efficacy to treat or maintain the remission of UC in 195 patients (Matsuoka et al., 2018). In fact, the use of *B. bifidum* as single strain-containing probiotic was sufficient to increase the levels of fecal SCFAs in healthy volunteers (Gargari et al., 2016), however, the protective role in UC or CD remains unknown. Despite some discrepancies regarding the number of patients used in the studies mentioned above, the first was the only one to confirm the increased number of *Bifidobacteria* in the feces of probiotics-treated patients and to perform endoscopic analysis.

The treatment with *Lactobacillus* GG (18×10^9 viable bacteria/day) alone or associated with mesalazine, prolonged the relapse-free period in UC patients compared to the group treated with immunosuppressant drug alone in a 12-month treatment regimen (Zocco et al., 2006). Similarly, a systematic review of randomized clinical trials showed the use of different lactic acid bacteria and *Bifidobacteria* as adjuvant therapy improved the course of disease and maintenance of clinical remission in UC (Saez-Lara et al., 2015).

As stated above, probiotics have poor or no effects on CD. However, studies have yielded positive results to induce remission by associating probiotics and prebiotics (defined as symbiotics) (Fujimori et al., 2007; Saez-Lara et al., 2015). Additionally, one open-label pilot study containing four children with mildly to moderately active CD had a significant improvement on clinical

TABLE 3 | Effective clinical trials using probiotics for treating inflammatory bowel disease.

Authors	Diagnosis	Number of patients (P) or studies (S)*#	Probiotic	Therapeutic regimen	Outcome
Mardini and Grigorian, 2014	UC	n = 5 (S) n = 441 (P)	VSL#3 ^{&}	Oral; 3.6×10^{12} CFU/day ^{\$}	53.4% of clinical responsiveness and 43.8% of clinical remission
Tursi et al., 2010	UC	n = 144 (P)	VSL#3 ^{&}	Oral; 3.6×10^{12} CFU/day; once a day for 8 weeks	53.4% of clinical improvement and 47.3% of clinical remission
Sood et al., 2009	UC	n = 147 (P)	VSL#3 ^{&}	Oral; 3.6×10^{12} CFU/dose; twice a day for 12 weeks	51.9% of clinical improvement and 42.9% of clinical remission at 12 weeks
Kruis et al., 2004	UC	n = 327 (P)	<i>Escherichia coli</i> Nissle 1917	Oral; $5-50 \times 10^9$ viable bacteria; once a day for 12 months	No differences between probiotic- and mesalazine-treated groups
Kato et al., 2004	UC	n = 20 (P)	Fermented milk (<i>B. breve</i> , <i>B. bifidum</i> and <i>L. acidophilus</i>)	Oral; 10^9 bacteria/day; once a day for 12 weeks	70% of clinical responsiveness and 40% of clinical remission
Zocco et al., 2006	UC	n = 187 (P)	<i>Lactobacillus</i> GG	Oral; 9×10^9 viable bacteria/dose; twice a day for 12 months	No differences between probiotic- and mesalazine-treated groups
Fujimori et al., 2007	CD	n = 10 (P)	<i>B. breve</i> , <i>L. asei</i> and <i>B. longum</i>	Oral; 75×10^9 bacteria/day; once a day for 13 (± 4.5) months	70% of clinical responsiveness and 60% of clinical remission
Gupta et al., 2000	CD	n = 4 (P)	<i>Lactobacillus</i> GG	Oral; 10^{10} CFU/dose; twice a day for 6 months	75% of clinical improvement at weeks 4 and 12

CD, Crohn's disease; UC: Ulcerative colitis. *Both total number of patients for clinical trials and number of studies for systematic analysis or meta-analysis were included.

#Includes the number of control patients. [&]VSL#3 is composed by *L. casei*, *L. plantarum*, *L. acidophilus*, *L. delbrueckii* subsp. *bulgaricus*, *B. longum*, *B. breve*, *B. infantis* and *Streptococcus salivarius* subsp. *thermophilic*. ^{\$}Length of treatments not available.

aspects after treatment with *Lactobacillus* GG (10^{10} CFU/tablet, twice a day for 6 months) (Gupta et al., 2000). However, the low number of samples and the absence of appropriate control (placebo-treated patients or under regular therapy) undermine the rigor of study. Probiotics have no effects in maintaining the remission of CD (Bousvaros et al., 2005; Bourreille et al., 2013).

In conclusion, probiotics are potential options in inducing and maintaining remission of mild to moderately UC, however, seem to be ineffective in DC (Table 3). The results must be considered as preliminary evidence and further randomized double-blind placebo-controlled multicenter trials must be performed to increase the reliability of results.

Limitations on Probiotics Studies

Different therapeutic regimen (including dose and frequency of administration) is an important problem to design treatment protocols. Although doses vary according to bacterial strains, studies have shown that 10^8 – 10^{10} CFU/day are ingested after consuming 100 mL or 100 g of probiotic-containing product (Atarashi et al., 2011). As a consequence, meta-analysis studies have several biases to compare related clinical trials and to draw relevant conclusions.

Unlike FMT therapy, the route of administration is not a potential problem, since the majority of studies use the oral route as the main one, although enemas are also a potential method of probiotic delivery (Oliva et al., 2012).

Another important issue regarding probiotics formulations is the quality control. Several inconsistent data have been described

between label information and product content, contamination, poor quality of strains, among others, as previously reviewed (Kolacek et al., 2017). Moreover, the same strain may show different efficacy in distinct batches as a result of a lack of standardization in bacterial culture procedures used throughout the studies and manufactures. Thus, both guidelines and improvements on supervision are highly encouraged to provide sufficient information on the design of new studies and to prevent unwanted and conflicting outcomes.

The immunosuppressive therapy is also a current challenge for clinicians and researchers. Since long-term use of immunosuppressants causes dysbiosis, it is important to determine whether this factor is a premise for the patient's responsiveness to probiotic treatment (Bhat et al., 2017).

Altogether, these factors represent important limitations in studies setup and the conflicting clinical results found in the literature may derive from poorly designed and standardized studies.

CONCLUSION AND FURTHER DIRECTIONS

Both FMT and probiotics are therapies with good prospects in the medical field, especially in IBD. However, like other newly developed therapies, the challenges encountered for increasing the reliability, safety, and standardization of FMT and probiotics are considerable. Thus, more multicenter studies must be performed to increase the number of samples and variables (features of IBD, phenotypic and genotypic characteristics of

the patients, standardizations in the therapeutic regimen, etc.), generating more significant conclusions.

AUTHOR CONTRIBUTIONS

PB wrote the manuscript, edited, generated the tables, and performed the literature review. NC contributed to review the manuscript and approval of the final version. HS-C designed the aim of the review, wrote the manuscript, performed the literature

review, edited and contributed to final approval for the version to be published.

FUNDING

The authors would like to thank FAPESP for financial support under projects 2015/26682-6 and 2017/05264-7. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Role of Vonoprazan in *Helicobacter pylori* Eradication Therapy in Japan

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OPEN ACCESS

Edited by:

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University of Naples Federico II, Italy

Reviewed by:

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Specialty section:

This article was submitted to
Gastrointestinal and Hepatic
Pharmacology,
a section of the journal
Frontiers in Pharmacology

Received: 24 October 2018

Accepted: 21 December 2018

Published: 15 January 2019

Citation:

Sugimoto M and Yamaoka Y
(2019) Role of Vonoprazan
in *Helicobacter pylori* Eradication
Therapy in Japan.
Front. Pharmacol. 9:1560.
doi: 10.3389/fphar.2018.01560

Complete eradication of *Helicobacter pylori* is important for preventing the development of gastric cancer. The outcome of *H. pylori* eradication therapy is mainly dependent on bacterial susceptibility to antimicrobial agents and potent neutralization of intragastric pH across 24 h, especially when using acid-sensitive antimicrobial agents such as clarithromycin (CLR), amoxicillin and sitafloxacin. However, conventional regimens comprising twice-daily doses (bid) of proton pump inhibitors (PPIs) are generally insufficient for maintaining the required gastric acid secretion for 24 h for successful eradication in all *H. pylori*-positive patients. Further, the increasing prevalence of CLR-resistant strains with each year has led to a decrease in eradication rates of first-line PPI- and CLR-containing therapies in developed countries, including Japan. In 2015, the potassium-competitive acid blocker vonoprazan (VPZ) became clinically available in Japan. VPZ competitively inhibits H⁺/K⁺-ATPase activity more potently than PPIs (e.g., omeprazole, lansoprazole, rabeprazole, pantoprazole, and esomeprazole). Therefore, a VPZ-containing *H. pylori* eradication regimen is expected to increase the eradication rate compared with conventional regimens containing a standard dose of PPI. In fact, a recent meta-analysis that investigated the efficacy of first-line eradication therapy showed that a VPZ-containing regimen achieved a higher eradication rate than a PPI-containing regimen. While the Maastricht V/Florence Consensus Report recommends selecting a bismuth or non-bismuth quadruple therapy and concomitant therapy for patients living in areas with high prevalence of CLR resistance, a VPZ-containing regimen demonstrates effectiveness for patients infected with CLR-resistant strains and patients living in areas where the prevalence of CLR-resistant strains is >15%. As a next step, studies are needed to determine the factors affecting the clinical outcome of VPZ-containing therapy and optimal VPZ-containing alternative regimens for tailored treatments. In this review, we summarize the advantages and disadvantages of VPZ in *H. pylori* eradication therapy.

Keywords: *Helicobacter pylori*, eradication therapy, vonoprazan, intragastric pH, clarithromycin

INTRODUCTION

Rapid and potent acid inhibition after treatment with acid-inhibitory drugs (i.e., proton pump inhibitor [PPI]) is necessary for curing acid-peptic disorders. Treatments that neutralize intragastric pH levels are associated with improved cure rates for peptic ulcers (Barer et al., 1983), gastroesophageal reflux disease (Bell et al., 1992), non-erosive reflux diseases, aspirin-induced and non-steroidal anti-inflammatory drug-induced gastroduodenal mucosal injury (Sugimoto et al., 2012a), and *Helicobacter pylori* infection (Labenz et al., 1995; Sugimoto et al., 2007; Yang et al., 2011). Therefore, an understanding of methods to suppress gastric acid secretion is important in the treatment of acid-peptic disorders.

The new, potent acid-inhibitory drug vonoprazan (VPZ) recently became clinically available in Japan. VPZ competitively inhibits the binding of potassium ions to H⁺/K⁺-ATPase in gastric parietal cells more potently than PPIs (Parsons and Keeling, 2005). VPZ also has two pharmacological advantages over PPIs: it does not require pharmacological activation by gastric acid to inhibit acid secretion, and has a longer half-life (t_{1/2}) due to its slow dissociation kinetics from H⁺/K⁺-ATPase (Sugimoto et al., 2004; Scott et al., 2015). While PPIs typically require more than 75–100 h to exert a maximal gastric acid inhibitory effect (Saitoh et al., 2002; Sugimoto et al., 2006), VPZ produces rapid, strong and long-lasting gastric acid inhibition after administration of the first tablet in a dose-dependent manner (Jenkins et al., 2015; Sakurai et al., 2015). At steady state on Day 7, a once daily dose (oid) of VPZ 40 mg displayed sustained and potent acid inhibition throughout a 24-h period (Jenkins et al., 2015). Moreover, a twice daily dose (bid) of VPZ 20 mg, the standard dosage for *H. pylori* eradication therapy, maintained gastric acid inhibition throughout the 24 h: the pH > 4 and >5 holding time ratio (HTR) was 100 and 99%, respectively, even in *H. pylori*-negative subjects (Kagami et al., 2016). Therefore, VPZ may be an effective first-line acid-inhibitory drug for patients with acid-peptic disorders.

In Japan, *H. pylori* eradication therapies are currently limited to regimens comprising an acid-inhibitory drug such as a PPI or VPZ at a standard dose bid, amoxicillin (AMX) 750 mg bid, and clarithromycin (CLR) 200 mg or 400 mg bid for 7 days as a first-line eradication regimen; and PPI or VPZ bid, AMX 750 mg bid, and metronidazole (MNZ) 250 mg bid for 7 days as a second-line eradication regimen. Unfortunately, the frequent use of CLR in general clinical situations has led to an increase in the prevalence of CLR-resistant *H. pylori* strains in Japan (more than 30%) (Asaka et al., 2001; Murakami et al., 2002), prompting the need for alternative regimens. Because potent neutralization of pH is associated with better outcomes of *H. pylori* eradication therapy, as it is for other acid-peptic disorders (Labenz et al., 1995; Sugimoto et al., 2007; Yang et al., 2011), VPZ may dramatically improve the decreasing *H. pylori* eradication rate in Japan.

Here, we discuss the impact of VPZ in *H. pylori* eradication therapy, as well as some of its disadvantages. We first discuss the general factors influencing the cure rate for *H. pylori* infection due to *H. pylori* eradication therapy and

association with the outcome of eradication and importance of inhibiting acid secretion. We subsequently discuss the efficacy of VPZ-containing eradication therapy of first-, second- and third-line treatments, and in patients with penicillin allergies.

POSSIBLE FACTORS CONTRIBUTING TO THE OUTCOME OF ERADICATION THERAPY FOR *H. pylori* INFECTION

The cure rate for *H. pylori* infection is affected by several possible factors, as below: antibiotic susceptibility (e.g., CLR, AMX, MNZ and levofloxacin) (Asaka et al., 2001; Furuta et al., 2001; Murakami et al., 2002), insufficient acid inhibition during eradication therapy (e.g., CYP2C19 and CYP3A4/5 genotype, dose of drug, treatment schedule and type of acid-inhibitory drug) (Furuta et al., 2001; Sugimoto et al., 2007; Sugimoto and Yamaoka, 2009), the environment (e.g., smoking), and poor adherence to medication and *H. pylori* strain with low virulence activity (e.g., *cagA*-negative strains, *vacA* s2 genotype and *dupA*-negative strains) (Sugimoto and Yamaoka, 2009; Shiota et al., 2012; Table 1). Although much attention is focused on the relationship between resistant strains of *H. pylori* and the success or failure of eradication therapy, potent acid inhibition throughout the 24 h during eradication therapy has become re-recognized as an important outcome of eradication therapy. In fact, the Maastricht V/Florence Consensus Report states that “the use of high dose PPI bid increases the efficacy of triple therapy. Level of evidence: low, and Grade of recommendation: weak” (Malfertheiner et al., 2017).

Control of pH using acid-inhibitory drugs depends on the type of acid-inhibitory drug, dosage (dose and dosing time), combination of drugs (e.g., any of PPI, VPZ and histamine 2 receptor antagonist) and polymorphisms in drug-metabolizing enzyme genes (e.g., cytochrome P450 2C19 (CYP2C19), CYP3A4, and CYP3A5) and polymorphisms in drug transporter genes (e.g., multidrug resistance protein-1 [ABCB1]) that effect the pH during treatment (Table 1; Furuta et al., 1999; Shirai et al., 2001, 2002; Sugimoto et al., 2004, 2005, 2012b; Kodaira et al., 2009; Kagami et al., 2016).

IMPORTANCE OF GASTRIC ACID INHIBITION IN *H. pylori* ERADICATION THERAPY

Helicobacter pylori can survive a periplasmic pH of 4.0–8.0 in the gastric mucosa (Scott et al., 1998). When the bacterial urease activity of *H. pylori* raises the intragastric pH to 4.0–6.0, *H. pylori* survives into the gastric mucosa but does not divide (Scott et al., 1998). Therefore, the consistent and potent action of acid-inhibitory drugs also enables *H. pylori* to grow and become more sensitive to antimicrobial agents against *H. pylori* (Scott et al., 1998). In addition, potent acid inhibition during 24-h increases the stability and bioavailability of acid-sensitive antimicrobial agents by preventing their degradation. Further, PPI and vonoprazan increase gastric mucosal antimicrobial

TABLE 1 | Major potential factors influencing the outcome of *H. pylori* eradication therapy.

Category	Factor		
Antibiotics	Resistance to antibiotics	Clarithromycin Metronidazole Levofloxacin Amoxicillin	A2142G, A2142C, and A2143G mutations in the 23S rRNA gene frxA (hp0642), rdxA (hp0954), and rpsU (hp0562) mutations C261A/G, C271A/T, and A272G mutations in gyrA Multiple point mutations in <i>pbp1</i> gene
Acid inhibition	Insufficient acid inhibition	CYP2C19 type (PPI) CYP2C19*17 (PPI) ABCB1 3435 (PPI) CYP3A5 (VPZ) <i>IL-1B-511</i> <i>IL-1B-31</i> Time of dosing Drug dose	Extensive metabolizer (*1/*1 type) *17 carrier C/C genotype (Caucasian) *1 carrier C/C genotype T/T genotype Low frequency (i.e., oid) Insufficient dose
<i>H. pylori</i> phenotype	<i>H. pylori</i> virulence factors	<i>cagA</i> status <i>vacA</i> genotype <i>dupA</i> status	Negative s2 type Negative
Environment	Volume Smoking Adherence	Volume Smoking Adherence	Much Many Insufficient

ABCB1, multidrug resistance protein-1; CYP2C19, cytochrome P450 2C19; CYP3A5, cytochrome P450 3A5; IL, interleukin.

agents concentration (Grayson et al., 1989; Goddard et al., 1996; Scott et al., 1998). Raising the intragastric pH from 3.5 to 5.5 is shown to increase the *in vitro* antimicrobial efficacy of AMX more than 10-fold (Grayson et al., 1989). The activity of CLR against *H. pylori* is higher at intragastric pH 7.4 than at intragastric pH 5.0, and activity is intermediate at pH 6.8 (Heifets et al., 1992). Recently, the Maastricht V/Florence Consensus Report recommended first-line eradication therapy using a CLR-containing regimen with PPI/AMX or PPIMNZ and an alternative eradication treatment using bismuth-containing quadruple treatment (PPI/bismuth/MNZ/tetracycline) in areas where prevalence of CLR-resistant strains is low (Level of evidence: high, Grade of recommendation: strong), and bismuth or non-bismuth quadruple treatment and concomitant (PPI/AMX/CLR/nitroimidazole) therapies in areas of high (>15%) CLR resistance (Level of evidence: low, Grade of recommendation: strong) (Malfertheiner et al., 2017). These recommendations indicate that there are numerous opportunities to use acid-sensitive antimicrobial agents around the world, and underlines the need to monitor inhibition of gastric acid secretion in eradication treatment.

However, the question of how strongly gastric acid secretion should be inhibited remains. Previously, we showed, using PPI/AMX/CLR triple therapy, a standard eradication regimen around the world, that the median 24-h pH for successful eradication was higher (6.4) and the median pH < 4 HTR (0.5%) was shorter than that for failed eradication (pH 5.2 and pH < 4 HTR 26.7%) (Sugimoto et al., 2007). Therefore, the degree and duration of acid inhibition during eradication therapy are related to the cure rate of *H. pylori*, and we concluded that intragastric pH > 4 should be maintained for 24 h and that the 24-h intragastric pH should be higher than 6.0 (Sugimoto et al., 2007).

Unfortunately, treatment with PPI at standard dose bid does not maintain pH values at higher than 4.0 for long enough to accept the above criteria in all patients receiving eradication therapy (Sugimoto et al., 2004). Therefore, it is necessary to identify the factors that affect acid secretion and to determine the optimal drug therapy to enable the inhibition of acid secretion across 24 h. Because VPZ 20 mg bid inhibits acid secretion across 24 h (pH \geq 4 HTR is 100%), (Kagami et al., 2016) VPZ is an effective acid-inhibitory drug in eradication therapies for *H. pylori* infection. There is no report to investigate direct association with advantage of vonoprazan use and acid inhibition during vonoprazan-containing eradication therapy for better outcome of *H. pylori* eradication therapy.

FIRST-LINE VONOPRAZAN-CONTAINING ERADICATION THERAPY

Study Selection

In this review, we searched for all of relevant studies published up until September 2018 that examined the efficacy of the vonoprazan-containing triple *H. pylori* eradication therapies, using PubMed, EMBASE, and Web of Science. Key words were [“potassium-competitive acid blocker,” “vonoprazan,” or “VPZ”] AND [“*H. pylori* eradication” or “*H. pylori* eradication”]. In addition, we examined the references of the screened articles to identify additional studies. All studies published in English were selected, whereas studies are randomized trial and retrospective observational studies. As the first step of study selection, we excluded irrelevant articles by examining the titles and abstracts of the papers. Next, we screened the full-text of all selected studies. The inclusion criteria were

(1) patients: *H. pylori*-positive patients, (2) eradication therapy: vonoprazan-containing triple therapies (first-, second-, and third-line treatments, and in patients with penicillin allergies), and (3) outcome: eradication rate. The exclusion criteria were (1) non-English language and (2) no detail information, such as sample number.

Efficacy of First-Line Vonoprazan-Containing Eradication Therapy

In recent years, several studies have compared eradication rates between VPZ-containing and PPI-containing triple therapies across centers in Japan. As shown in **Table 2**, up until September 2018, 23 reports had investigated the efficacy of first-line VPZ-containing therapy (21 reports for VPZ/AMX/CLR and 2 reports for VPZ/MNZ/CLR) (Matsumoto et al., 2016; Murakami et al., 2016; Noda et al., 2016; Shichijo et al., 2016; Shinozaki et al., 2016, 2018; Suzuki et al., 2016; Tsujimae et al., 2016; Yamada et al., 2016; Kajihara et al., 2017; Katayama et al., 2017; Maruyama et al., 2017; Nishizawa et al., 2017; Ono et al., 2017; Sakurai et al., 2017; Sue et al., 2017a,b, 2018a; Sugimoto et al., 2017; Tanabe et al., 2017, 2018; Mori et al., 2018; Ozaki et al., 2018), and 19 reports had compared the efficacy between VPZ-containing therapy and PPI-containing therapy, including 3 randomized control trials (Murakami et al., 2016; Maruyama et al., 2017; Sue et al., 2018a) and 16 non-randomized retrospective cohort trials (Matsumoto et al., 2016; Noda et al., 2016; Shichijo et al., 2016; Shinozaki et al., 2016; Suzuki et al., 2016; Tsujimae et al., 2016; Yamada et al., 2016; Kajihara et al., 2017; Nishizawa et al., 2017; Ono et al., 2017; Sakurai et al., 2017; Sue et al., 2017a,b; Mori et al., 2018; Ozaki et al., 2018; Tanabe et al., 2018).

In 2016, a phase III trial of first-line triple therapy in 650 *H. pylori*-positive subjects showed that the first-line eradication rate was 92.6% (95% confidence interval [CI]: 89.2–95.2%) for the VPZ 20 mg/AMX 750 mg/CLR 200 mg or 400 mg regimen compared to 75.9% (95% CI: 70.9–80.5%) for the lansoprazole/AMX/CLR regimen. There was a difference of 16.7% (95% CI: 11.2–22.1%) in favor of VPZ, confirming the non-inferiority of VPZ ($P < 0.0001$) (Murakami et al., 2016). In another randomizes control trial including 141 *H. pylori*-positive patients with VPZ group (VPZ 20 mg, AMX 750 mg, and CLR 200 or 400 mg) or PPI group (rabeprazole 20 mg or lansoprazole 30 mg, AMX 750 mg, and CLR 200 or 400 mg), the eradication rate was significantly higher in VPZ group (95.8 and 95% CI: 88.3–99.1%) than PPI group (69.6 and 95% CI: 57.3–80.1%, $P < 0.001$) in ITT analysis (Maruyama et al., 2017). In a summary of 21 studies investigating the efficacy of the first-line VPZ/CLR/AMX eradication regimen in 7,469 patients who received VPZ-containing therapy and 12,010 patients who received PPI-containing triple therapy, the studies with eradication rate of more than 85% was 61.2% (13/21 studies) in for VPZ-containing therapy and 0% for PPI-containing therapy (**Table 2**). Jung et al. (2017) reported that the pooled *H. pylori* eradication

rate determined by intention-to-treat (ITT) analysis is 88.1% (95% CI: 86.1–89.9%) in the vonoprazan-containing triple therapy and 72.8% (95% CI: 71.0–75.4%) in PPI-containing triple therapy, respectively, as meta-analysis using 10 reports. In addition, the incidence of any adverse events was similar between both regimens (pooled relative risk [95% CI] = 1.02 [0.78–1.34]) (Jung et al., 2017). However, because most of included studies were retrospective observational studies, this review may have a problem with the quality of the studies (Jung et al., 2017). Meta-analysis should avoid to mix randomized case-control studies with cohorts and observational studies.

As shown in **Table 2**, first-line triple VPZ-containing therapies (VPZ/AMX/CLR) therefore show superior efficacy in Japanese individuals in terms of *H. pylori* eradication compared to PPI-containing therapies (Matsumoto et al., 2016; Murakami et al., 2016; Noda et al., 2016; Shichijo et al., 2016; Shinozaki et al., 2016, 2018; Suzuki et al., 2016; Tsujimae et al., 2016; Yamada et al., 2016; Kajihara et al., 2017; Katayama et al., 2017; Maruyama et al., 2017; Nishizawa et al., 2017; Ono et al., 2017; Sakurai et al., 2017; Sue et al., 2017a,b, 2018a; Sugimoto et al., 2017; Tanabe et al., 2017, 2018; Mori et al., 2018; Ozaki et al., 2018). According to a grading system established by Graham et al. (2007) the 69.1–75.0% eradication rate of PPI-containing therapies constitutes an unacceptable grade (grade F). In contrast, the 86.6–91.7% eradication rate of the VPZ-containing therapies reflects an acceptable grade (grade B or C). Although this rate is by no means excellent, it is a positive step for establishing improved treatment methods in the future. These findings suggest that potent acid inhibition using VPZ is a key requirement for a successful therapy, and is effective despite the high rate of CLR-resistance strains in Japanese individuals.

Increasing the duration from 7 to 10–14 days at eradication is known to increase eradication rates by approximately 5% (Calvet et al., 2000; Fuccio et al., 2007). However, given that there is currently no data on the efficacy of eradication regimens on prolonged eradication periods, further studies are needed to clarify the effectiveness of such therapies.

FIRST-LINE VONOPRAZAN-CONTAINING ERADICATION THERAPY FOR PATIENTS INFECTED WITH CLARITHROMYCIN-RESISTANT STRAINS

Implementation of PPI-containing therapy without culture testing is accepted in many countries, whereas *H. pylori* is one of infectious diseases, because eradication treatment based on the culture test requires more time and higher costs compared to non-culture empirical treatment. However, because CLR resistance is becoming a global clinical problem for *H. pylori* eradication in many countries, especially in Japan where the CLR has been used for many patients with bacterial infection, eradication therapy that has been

TABLE 2 | Summary of previous studies for the investigation of the efficacy of first-line eradication therapy for *H. pylori* infection.

First author [ref. no.]	Year	Method	VPZ-containing eradication regimen			PPI-containing eradication regimen			
			Number	Regimen	Eradication rate	Number	Regimen	Eradication rate	
Murakami (Murakami et al., 2016)	2016	RCT	ITT	329	VPZ: 20 mg bid AMX: 750 mg bid CLR: 200 or 400 mg bid	90.9%	321	LPZ: 30 mg bid AMX: 750 mg bid CLR: 200 or 400 mg bid	75.1%
Suzuki (Suzuki et al., 2016)	2016	RST	ITT	181	VPZ: 20 mg bid AMX: 750 mg bid CLR: 200 or 400 mg bid	89.0%	480	LPZ: 30 mg bid or RPZ: 20 mg bid AMX: 750 mg bid CLR: 200 mg bid	74.2%
Shinozaki (Shinozaki et al., 2016)	2016	RST	ITT	117	VPZ: 20 mg bid AMX: 750 mg bid CLR: 200 or 400 mg bid	82.9%	436	LPZ: 30 mg bid, RPZ: 10 mg bid or EPZ: 20 mg bid AMX: 750 mg bid CLR: 200 mg bid	73.9%
Shichijo (Shichijo et al., 2016)	2016	RST	ITT	422	VPZ: 20 mg bid AMX: 750 mg bid CLR: 200 or 400 mg bid	87.2%	2293	LPZ: 30 mg bid, RPZ: 10 mg bid or EPZ: 20 mg bid AMX: 750 mg bid CLR: 200 or 400 mg bid	72.4%
Noda (Noda et al., 2016)	2016	RST		146	VPZ: 20 mg bid AMX: 750 mg bid CLR: 400 mg bid	89.7%	1305	OPZ: 20 mg bid, LPZ: 30 mg bid, RPZ: 10 mg bid or EPZ: 20 mg bid AMX: 750 mg bid CLR: 200 or 400 mg bid	73.9%
Matsumoto (Matsumoto et al., 2016)	2016	RST	ITT	125	VPZ: 20 mg bid AMX: 750 mg bid CLR: 200 mg bid	89.6%	295	LPZ: 30 mg bid, RPZ: 10 mg bid or EPZ: 20 mg bid AMX: 750 mg bid CLR: 200 or 400 mg bid	71.9%
Yamada (Yamada et al., 2016)	2016	RST	ITT	335	VPZ: 20 mg bid AMX: 750 mg bid CLR: 200 mg bid	85.7%	1720	LPZ: 30 mg bid, RPZ: 10 mg bid or EPZ: 20 mg bid AMX: 750 mg bid CLR: 200 mg bid	73.2%
Tsujimae (Tsujimae et al., 2016)	2016	RST	ITT	443	VPZ: 20 mg bid AMX: 750 mg bid CLR: 200 mg bid	84.6%	431	EPZ: 20 mg bid AMX: 750 mg bid CLR: 200 mg bid	79.1%
Katayama (Katayama et al., 2017)	2017	RST	ITT	258	VPZ: 20 mg bid AMX: 750 mg bid CLR: 200 mg bid	90.6%			
Kajihara (Kajihara et al., 2017)	2017	RST	ITT	111	VPZ: 20 mg bid AMX: 750 mg bid CLR: 400 mg bid	94.6%	98	RPZ: 10 mg bid AMX: 750 mg bid CLR: 200 or 400 mg bid	86.7%
Ono (Ono et al., 2017)	2017	RST	ITT	13	VPZ: 20 mg bid MNZ: 250 mg bid CLR: 200 mg bid	92.3%	10	LPZ: 30 mg bid or RPZ: 10 mg bid MNZ: 250 mg bid CLR: 200 mg bid	50.0%
				14	VPZ: 20 mg bid MNZ: 250 mg bid STFX: 100 mg bid	92.9%	20	LPZ: 30 mg bid or RPZ: 10 mg bid MNZ: 250 mg bid STFX: 100 mg bid	100%
Sakurai (Sakurai et al., 2017)	2017	RST	ITT	546	VPZ: 20 mg bid AMX: 750 mg bid CLR: 200 mg bid	87.9%	807	LPZ: 30 mg bid, RPZ: 10 mg bid or EPZ: 20 mg bid	66.9%

(Continued)

TABLE 2 | Continued

First author [ref. no.]	Year	Method	VPZ-containing eradication regimen				PPI-containing eradication regimen			
			Number	Regimen	Eradication rate	Number	Regimen	Eradication rate		
Sugimoto (Sugimoto et al., 2017)	2017	OS	ITT	76	VPZ: 20 mg bid AMX: 750 mg bid CLR: 200 mg bid	82.9%			AMX: 750 mg bid CLR: 200 mg bid	
Maruyama (Maruyama et al., 2017)	2017	RCT	ITT	72	VPZ: 20 mg bid AMX: 750 mg bid CLR: 200 or 400 mg bid	95.8%	69	LPZ: 30 mg bid or RPZ: 20 mg bid AMX: 750 mg bid CLR: 200 or 400 mg bid	69.6%	
Sue (Sue et al., 2017a)	2017	RST	ITT	623	VPZ: 20 mg bid AMX: 750 mg bid CLR: 200 or 400 mg bid	84.9%	608	OPZ: 2- mg bid, LPZ: 30 mg bid, RPZ: 10 mg bid or EPZ: 20 mg bid AMX: 750 mg bid CLR: 200 or 400 mg bid	78.8%	
Nishizawa (Nishizawa et al., 2017)	2017	RST	ITT	353	VPZ: 20 mg bid AMX: 750 mg bid CLR: 200 or 400 mg bid	62.3%	2173	LPZ: 30 mg bid or RPZ: 10 mg bid AMX: 750 mg bid CLR: 200 or 400 mg bid	47.1%	
Tanabe (Tanabe et al., 2017)	2017	OS	ITT	694	VPZ: 20 mg bid AMX: 750 mg bid CLR: 200 or 400 mg bid	82.7%				
Sue (Sue et al., 2017b)	2017	RST	ITT	20	VPZ: 20 mg bid MNZ: 250 mg bid CLR: 200 or 400 mg bid	100%	30	LPZ: 30 mg bid, RPZ: 10 mg bid or EPZ: 20 mg bid MNZ: 250 mg bid CLR: 200 or 400 mg bid	83.3%	
Sue (Sue et al., 2018a)	2018	RCT	ITT	55*	VPZ: 20 mg bid AMX: 750 mg bid CLR: 200 or 400 mg bid	87.3%	51*	LPZ: 30 mg bid, RPZ: 10 mg bid or EPZ: 20 mg bid AMX: 750 mg bid CLR: 200 or 400 mg bid	76.5%	
				41**	VPZ: 20 mg bid AMX: 750 mg bid CLR: 200 or 400 mg bid	82.9%				
Ozaki (Ozaki et al., 2018)	2018	RST	ITT	1688	VPZ: 20 mg bid AMX: 750 mg bid CLR: 200 or 400 mg bid	90.8%	147	EPZ: 20 mg bid or RPZ: 10 mg bid AMX: 750 mg bid CLR: 200 or 400 mg bid	72.8%	
Tanabe (Tanabe et al., 2018)	2018	RST	ITT	363	VPZ: 20 mg bid AMX: 750 mg bid CLR: 200 or 400 mg bid	91.5%	780	LPZ: 30 mg bid, RPZ: 10 mg bid or EPZ: 20 mg bid AMX: 750 mg bid CLR: 200 mg bid	79.4%	
Mori (Mori et al., 2018)	2018	RST	ITT	308	VPZ: 20 mg bid AMX: 750 mg bid CLR: 200 or 400 mg bid	81.2%	272	LPZ: 30 mg bid AMX: 750 mg bid CLR: 200 or 400 mg bid	77.6%	

(Continued)

TABLE 2 | Continued

First author [ref. no.]	Year	Method	VPZ-containing eradication regimen				PPI-containing eradication regimen			
			Number	Regimen	Eradication rate	Number	Regimen	Eradication rate		
Shinozaki (Shinozaki et al., 2018)	2018	OS	ITT	174	VPZ: 20 mg bid AMX: 750 mg bid CLR: 200 or 400 mg bid	83.3%				

All paper to investigate efficacy of first-line vonoprazan-containing eradication therapy up until September 2018 were listed. *Clarithromycin-sensitive strain, **Clarithromycin-resistant strain. AMX, amoxicillin; bid, twice daily dosing; CLR, clarithromycin; EPZ, esomeprazole; ITT, intention to treat analysis; LPZ, lansoprazole; MNZ, metronidazole; OPZ, omeprazole; PP, per protocol analysis; PPI, proton pump inhibitor; OS, observational study; RCT, randomized control trial; RST, retrospective cohort trial; RPZ, rabeprazole; VPZ, vonoprazan.

susceptibility tested may be an effective option. CLR is a key antimicrobial agent of current first-line triple *H. pylori* eradication therapies, exerting its antimicrobial effects by binding to the bacterial ribosome 50S subunit to inhibit protein synthesis. The minimum inhibitory concentration used to define resistance to CLR is generally higher than 1.0 mg/mL (Adamek et al., 1998). The susceptibility to CLR in most *H. pylori* strains is conferred by a single nucleotide polymorphism at either position 2142 or 2143 (i.e., A2142G and A2143G) in the *H. pylori* 23S rRNA gene and associated with MIC > 64 μg/ml. CLR resistance is therefore a potential confounding factor because CLR resistance significantly affects the efficacy of eradication therapy.

Up until September 2018 (PubMed, EMBASE, Web of Science), six reports had compared the efficacy of first-line VPZ-containing therapy between patients infected with CLR-sensitive and -resistant strains, while 4 reports had compared the efficacy of VPZ/AMX/CLR and PPI (omeprazole 20 mg, lansoprazole 30 mg, rabeprazole 10 mg, or esomeprazole 20 mg)/AMX/CLR therapy (Table 3). In a multicenter prospective randomized clinical trial, the ITT analysis of VPZ/AMX/CLR in the patients infected with CLR-sensitive strain were 87.3% (95% CI: 75.5–94.7%) and that of PPI/AMX/CLR were 76.5% (62.5–87.2%), respectively. No significant difference was observed between the VPZ-containing and PPI-containing regimes in terms of the ITT analysis ($P = 0.21$) (Sue et al., 2018a). A meta-analysis of five original articles (Matsumoto et al., 2016; Murakami et al., 2016; Noda et al., 2016; Sue et al., 2017a, 2018a) showed that in patients infected with CLR-sensitive *H. pylori* strains, eradication rates of VPZ- and conventional PPI-containing therapies were similar in two randomized controlled trials (eradication rate: 95.4% [VPZ] and 92.8% [PPI], odds ratio [OR]: 1.63, 95% CI: 0.74–3.61, $P = 0.225$) (Li et al., 2018). However, VPZ-containing therapy was significantly superior to PPI-containing therapy for patients with CLR-resistant strains in both the randomized controlled trials (eradication rate: 82.0% [VPZ] and 40.0% [PPI], OR: 6.83, 95% CI: 3.63–12.86, $P < 0.0001$) (Li et al., 2018). Compared to the efficacy of conventional PPI-based therapy, the risk of VPZ-containing therapy determined using non-randomized controlled trials was greater for *H. pylori*-positive patients infected with CLR-resistant strains (OR: 5.92) than for *H. pylori*-positive patients infected with CLR-sensitive strains (OR:

2.02) (Dong et al., 2017). Based on this evidence, CLR may be overused given that the combination of VPZ and AMX eradicates approximately 80% of *H. pylori* without CLR (Li et al., 2018). However, as an eradication rate of 80% is not satisfactory, additional measures are needed to obtain a higher eradication rate in patients infected with *H. pylori* CLR-resistant strains.

SECOND-LINE VONOPRAZAN-CONTAINING ERADICATION THERAPY

Characteristics of patients who require second-line therapy in Japan are infection with a CLR-resistant strain, CYP2C19 extensive metabolizer (EM) phenotype, and poor adherence when first-line treatment was performed. The second-line regimen PPI/AMX/MNZ is currently covered by the Japanese National Health Insurance system, and while the prevalence of patients with *H. pylori* infected with MNZ resistance strain in Japan is 5–12%, the success rate of this second-line regimen has remained constant at approximately 90% (Tsujimae et al., 2016; Yamada et al., 2016; Nishizawa et al., 2017; Ono et al., 2017; Sakurai et al., 2017; Sue et al., 2017a). Japan differs from many other countries in its prevalence rate of the *H. pylori* MNZ-resistant strain, and the high eradication rate following second-line treatment containing MNZ is thought to be country-specific.

Up until September 2018 (PubMed, EMBASE, Web of Science), twelve reports have investigated the efficacy of second-line VPZ-containing therapy and 5 reports have retrospectively compared the efficacy between VPZ/AMX/MNZ and PPI/AMX/MNZ therapy (Table 4). However, a randomized controlled trial on the efficacy of VPZ/AMX/MNZ as a second-line regimen has not been conducted. Murakami et al. (2016) reported that the eradication rate of second-line VPZ-containing eradication therapy (VPZ 20 mg/AMX 750 mg/MNZ 250 mg, bid, 7 days) was high (98.0 and 95% CI: 89.4–99.9%, $n = 50$) in a phase III trial, ranging from 71.8 to 98.0% in 12 reports (Table 4; Murakami et al., 2016; Tsujimae et al., 2016; Yamada et al., 2016; Katayama et al., 2017; Nishizawa et al., 2017; Sakurai et al., 2017; Sue et al., 2017a; Sugimoto et al., 2017;

TABLE 3 | Summary of previous studies for the investigation of the efficacy of first-line eradication therapy between clarithromycin-sensitive and -resistant strains.

First author [ref. no.]	Year	Method	Clarithromycin- resistance	VPZ-containing eradication regimen			PPI-containing eradication regimen		
				Number	Regimen	Eradication rate	Number	Regimen	Eradication rate
Murakami (Murakami et al., 2016)	2016	RCT	Sensitive	205	VPZ: 20 mg bid AMX: 750 mg bid CLR: 200 or 400 mg bid	97.6%	185	LPZ: 30 mg bid AMX: 750 mg bid CLR: 200 or 400 mg bid	97.3%
			Resistant	100	VPZ: 20 mg bid AMX: 750 mg bid CLR: 200 or 400 mg bid	82.0%	115	LPZ: 30 mg bid AMX: 750 mg bid CLR: 200 or 400 mg bid	40.0%
Noda (Noda et al., 2016)	2016	RST	Sensitive	44	VPZ: 20 mg bid AMX: 750 mg bid CLR: 400 mg bid	100%	25	OPZ: 20 mg bid, LPZ: 30 mg bid, RPZ: 10 mg bid or EPZ: 20 mg bid AMX: 750 mg bid CLR: 200 or 400 mg bid	88.0%
			Resistant	32	VPZ: 20 mg bid AMX: 750 mg bid CLR: 400 mg bid	87.5%	13	OPZ: 20 mg bid, LPZ: 30 mg bid, RPZ: 10 mg bid or EPZ: 20 mg bid AMX: 750 mg bid CLR: 200 or 400 mg bid	53.8%
Matsumoto (Matsumoto et al., 2016)	2016	RST	Sensitive	57	VPZ: 20 mg bid AMX: 750 mg bid CLR: 200 mg bid	100%	212	LPZ: 30 mg bid, RPZ: 10 mg bid or EPZ: 20 mg bid AMX: 750 mg bid CLR: 200 or 400 mg bid	87.8%
			Resistant	46	VPZ: 20 mg bid AMX: 750 mg bid CLR: 200 mg bid	76.1%	97	LPZ: 30 mg bid, RPZ: 10 mg bid or EPZ: 20 mg bid AMX: 750 mg bid CLR: 200 or 400 mg bid	40.2%
Sugimoto (Sugimoto et al., 2017)	2017	OS	Sensitive	19	VPZ: 20 mg bid AMX: 750 mg bid CLR: 200 mg bid	89.5%			
			Resistant	14	VPZ: 20 mg bid AMX: 750 mg bid CLR: 200 mg bid	78.6%			
Sue (Sue et al., 2017a)	2017	RST	Sensitive	180	VPZ: 20 mg bid AMX: 750 mg bid CLR: 200 or 400 mg bid	88.9%			
			Resistant	56	VPZ: 20 mg bid AMX: 750 mg bid CLR: 200 or 400 mg bid	73.2%			
Sue (Sue et al., 2018a)	2018	RCT	Sensitive	55	VPZ: 20 mg bid AMX: 750 mg bid CLR: 200 or 400 mg bid	87.3%	51*	LPZ: 30 mg bid, RPZ: 10 mg bid or EPZ: 20 mg bid AMX: 750 mg bid CLR: 200 or 400 mg bid	76.5%
			Resistant	41	VPZ: 20 mg bid AMX: 750 mg bid CLR: 200 or 400 mg bid	82.9%			

All paper to investigate efficacy of first-line vonoprazan-containing eradication therapy investigated susceptibility to antimicrobial agents up until September 2018 were listed. AMX, amoxicillin; bid, twice daily dosing; CLR, clarithromycin; EPZ, esomeprazole; ITT, intention to treat analysis; LPZ, lansoprazole; MNZ, metronidazole; OPZ, omeprazole; PP, per protocol analysis; PPI, proton pump inhibitor; OS, observational study; RCT, randomized control trial; RST, retrospective cohort trial; RPZ, rabeprazole; VPZ, vonoprazan.

TABLE 4 | Summary of previous studies for the investigation of the efficacy of second-line eradication therapy for *H. pylori* infection.

First author [ref. no.]	Year	Method	VPZ-containing eradication regimen			PPI-containing eradication therapy		
			Number	Regimen	Eradication rate	Number	Regimen	Eradication rate
Murakami (Murakami et al., 2016)	2016	RCT	PP	50	VPZ: 20 mg bid AMX: 750 mg bid MNZ: 250 mg bid	98.0		
Inaba (Inaba et al., 2016)	2016	RST	ITT	37	VPZ: 20 mg bid AMX: 750 mg bid CLR: 200 mg bid	70.2%*		
Yamada (Yamada et al., 2016)	2016	RST	ITT	66	VPZ: 20 mg bid AMX: 750 mg bid MNZ: 250 mg bid	89.6%	386	LPZ: 30 mg bid, RPZ: 10 mg bid, EPZ: 20 mg bid AMX: 750 mg bid MNZ: 250 mg bid
Tsujimae (Tsujimae et al., 2016)	2016	RST	ITT	46	VPZ: 20 mg bid AMX: 750 mg bid MNZ: 250 mg bid	89.1%	54	EPZ: 20 mg bid AMX: 750 mg bid MNZ: 250 mg bid
Katayama (Katayama et al., 2017)	2017	RST	ITT	24	VPZ: 20 mg bid AMX: 750 mg bid MNZ: 250 mg bid	87.0%		
Ono (Ono et al., 2017)	2017	RST	ITT	1	VPZ: 20 mg bid MNZ: 250 mg bid CLR: 200 mg bid	100%	3	LPZ: 30 mg bid or RPZ: 10 mg bid MNZ: 250 mg bid CLR: 200 mg bid
				3	VPZ: 20 mg bid MNZ: 250 mg bid STFX: 100 mg bid	66.7%	24	LPZ: 30 mg bid or RPZ: 10 mg bid MNZ: 250 mg bid STFX: 100 mg bid
Sakurai (Sakurai et al., 2017)	2017	RST	ITT	76	VPZ: 20 mg bid AMX: 750 mg bid MNZ: 250 mg bid	96.1%	185	LPZ: 30 mg bid, RPZ: 10 mg bid or EPZ: 20 mg bid AMX: 750 mg bid MNZ: 250 mg bid
Sugimoto (Sugimoto et al., 2017)	2017	OS	ITT	29	VPZ: 20 mg bid AMX: 750 mg bid MNZ: 250 mg bid	93.1%		
Sue (Sue et al., 2017a)	2017	RST	ITT	216	VPZ: 20 mg bid AMX: 750 mg bid MNZ: 250 mg bid	80.5%	146	LPZ: 30 mg bid, RPZ: 10 mg bid or EPZ: 20 mg bid AMX: 750 mg bid MNZ: 250 mg bid
Nishizawa (Nishizawa et al., 2017)	2017	RST	ITT	85	VPZ: 20 mg bid AMX: 750 mg bid MNZ: 250 mg bid	71.8%	650	LPZ: 30 mg bid or RPZ: 10 mg bid AMX: 750 mg bid MNZ: 250 mg bid
Tanabe (Tanabe et al., 2017)	2017	OS	ITT	73	VPZ: 20 mg bid AMX: 750 mg bid MNZ: 250 mg bid	90.4%		
Ozaki (Ozaki et al., 2018)	2018	RST	ITT	94	VPZ: 20 mg bid AMX: 750 mg bid MNZ: 250 mg bid	86.3%		

All paper to investigate efficacy of second-line vonoprazan-containing eradication therapy up until September 2018 were listed. AMX, amoxicillin; bid, twice daily dosing; CLR, clarithromycin; EPZ, esomeprazole; ITT, intention to treat analysis; LPZ, lansoprazole; MNZ, metronidazole; OPZ, omeprazole; PP, per protocol analysis; PPI, proton pump inhibitor; OS, observational study; RCT, randomized control trial; RST, retrospective cohort trial; RPZ, rabeprazole; VPZ, vonoprazan.

Tanabe et al., 2017; Ozaki et al., 2018). However, there is no significant difference in eradication rates between PPI and VPZ among comparable trials in real-world clinical settings (Tsujimae et al., 2016; Yamada et al., 2016; Nishizawa et al., 2017; Ono et al., 2017; Sakurai et al., 2017; Sue et al., 2017a).

A meta-analysis reported in 2017 showed that the eradication rate of VPZ-containing regimens using ITT analysis was similar to that for PPI-containing regimens (83.4% [VPZ] and 81.2% [PPI], OR: 1.04, 95% CI: 0.77–1.42, $P = 0.79$) (Dong et al., 2017). Per protocol (PP) analysis showed comparable results to

the ITT analysis (89.3% vs. 90.1%, $P = 0.06$). In addition, the eradication rate is similar among various gastrointestinal diseases (Yamada et al., 2016). The above findings suggest that there is no advantage of using VPZ in second-line treatment. This may be because although AMX is an acid-sensitive antimicrobial agent, MNZ is not an acid-sensitive antimicrobial agent, and potent acid inhibition is not required to increase the stability and bioavailability of MNZ. The mechanism of its antimicrobial effect of MNZ is independent of the bacteria's stationary or growth phase distribution. In addition, failure to eradicate *H. pylori* using the VPZ/AMX/CLR regimen as first-line therapy likely limits the efficacy of VPZ in second-line therapy containing MNZ.

In general, second-line therapy with VPZ/AMX/MNZ is well tolerated and patients show good compliance. During the second-line eradication phase, the incidence of treatment-emergent adverse events is 4.0–16.0% (Murakami et al., 2016; Nishizawa et al., 2017). Therefore, in Japan, instead of VPZ/AMX/MNZ, the PPI/AMX/MNZ regimen may be recommended as a second-line treatment due to cost-effectiveness and similar efficacy and safety.

THIRD-LINE VONOPRAZAN-CONTAINING ERADICATION THERAPY

The bismuth-containing quadruple regimen and/or sequential or concomitant regimens recommended by the Maastricht V/Florence Consensus Report cannot be used as eradication treatment in Japan because bismuth is not an approved medical drug. Further, third-line eradication therapy is not accepted by the Japanese National Health Insurance system. Because the *H. pylori* strain that infects most patients who receive third-line therapy is resistant to CLR and MNZ, the eradication therapy using PPI/AMX/sitaflloxacin (STFX) or PPI/MNZ/STFX, where the PPI is selected, is the main third-line regimen in Japan (Murakami et al., 2013; Furuta et al., 2014a; Sugimoto et al., 2015; Mori et al., 2016). STFX is one of a new quinolone

antibacterial agent with anticipated efficacy due to its low MIC for *H. pylori*, including for levofloxacin (LVFX)-resistant strains. Interestingly, STFX has antimicrobial effects to inhibit DNA gyrase and topoisomerase IV, enzymes that are involved in bacteria DNA replication, transcription, DNA repair and recombination (Zhanel et al., 2002). In particular, topoisomerase IV, which consists of ParC and ParE subunits, has an essential role in partitioning replicated chromosomes and is more sensitive than DNA gyrase to some quinolones, such as levofloxacin and ciprofloxacin (Appelbaum and Hunter, 2000). STFX can overcome the resistance of *H. pylori* strains with *gyrA* mutations *in vitro*, (Suzuki et al., 2009) and the low rate of STFX-resistant strains of less than 10% is a strong motivator for its use in eradication therapy (Sugimoto et al., 2015; Mori et al., 2016). Indeed, the effectiveness of STFX-containing eradication therapy has been reported in patients receiving third-line treatment (Murakami et al., 2009, 2013; Suzuki et al., 2009; Hirata et al., 2012; Matsuzaki et al., 2012; Furuta et al., 2014a,b).

Sitaflloxacin is also an acid-sensitive antimicrobial agent, whose stability and bioavailability is increased by potent acid inhibition. Therefore, a VPZ/STFX-containing *H. pylori* eradication regimen is expected to increase the eradication rate compared with a PPI/STFX-containing eradication regimen. As shown in Table 5, two reports have investigated the efficacy of third-line VPZ-containing therapy (Sugimoto et al., 2017; Sue et al., 2018b). In a randomized case-controlled trial, patients were divided into a VPZ group (VPZ/AMX/STFX bid for 7 days) or a PPI group (PPI standard dose/AMX/STFX bid for 7 days) (Sue et al., 2018b). Although sample number was limited ($n = 63$), ITT analysis showed that the eradication rates were 75.8% (95% CI: 57.7–88.9%) in the VPZ group and 53.3% (95% CI: 34.3–71.7%) in the PPI group, respectively (Sue et al., 2018b). No significant difference in the frequency of adverse events was evident between the VPZ-containing and PPI-containing regimens. Therefore, although an eradication rate of around 80% remains unsatisfactory, the VPZ/AMX/STFX regimen is more effective than PPI/AMX/STFX as a third-line regimen in patients in whom CLR-containing first-line and MNZ-containing second-line therapies failed to eradicate *H. pylori*. However, because,

TABLE 5 | Summary of previous studies for the investigation of the efficacy of third-line eradication therapy for *H. pylori* infection.

First author [ref. no.] [ref. no.]	Year	Method	VPZ-containing eradication regimen			PPI-containing eradication regimen		
			Number	Regimen	Eradication rate	Number	Regimen	Eradication rate
Sugimoto (Sugimoto et al., 2017)	2017	OS	ITT	15	VPZ: 20 mg bid AMX: 500 mg qid STFX: 100 mg bid	80.0%		
Sue (Sue et al., 2018b)	2018	RCT	ITT	33	VPZ: 20 mg bid AMX: 750 mg bid STFX: 100 mg bid	75.8%	30 LPZ: 30 mg bid, RPZ: 10 mg bid or EPZ: 20 mg bid AMX: 750 mg bid STFX: 100 mg bid	53.3%

All paper to investigate efficacy of third-line vonoprazan-containing eradication therapy up until September 2018 were listed. AMX, amoxicillin; bid, twice daily dosing; EPZ, esomeprazole; ITT, intention to treat analysis; LPZ, lansoprazole; MNZ, metronidazole; PPI, proton pump inhibitor; OS, observational study; qid, four times daily dosing; RCT, randomized control trial; RST, retrospective cohort trial; RPZ, rabeprazole; STFX, sitafloxacin; VPZ, vonoprazan.

as mentioned above, *H. pylori* was eradicated in 99% or more patients who received both VPZ-containing first- and second-line treatments, the number of *H. pylori*-positive patients requiring third-line therapy is expected to be considerably reduced, to less than 1%.

VONOPRAZAN-CONTAINING ERADICATION THERAPY IN PATIENTS WITH PENICILLIN ALLERGIES

Amoxicillin is the most effective and popular drug used for *H. pylori* infection. Therefore, most recommended regimens for *H. pylori* infection include AMX as a key antimicrobial agent. However, because AMX-containing regimens cannot be used by patients with penicillin allergies, eradication regimens that do not contain penicillin derivatives or agents with beta-lactam rings are selected for *H. pylori* eradication in these patients in Japan (Harris et al., 1996; Furuta et al., 2014b). The incidence of penicillin allergies is 3–7% in Japan (Muranaka et al., 1973). For patients who are allergic to penicillin, the Maastricht V/Florence Consensus Report recommends selecting a PPI/CLR/MNZ regimen in areas with low rates of CLR resistance and bismuth-based quadruple therapy in areas of high CLR resistance (Malfertheiner et al., 2017). However, the PPI/CLR/MNZ regimen is associated with an unacceptably low eradication rate, from 55 to 64% according to PP analysis, in patients with penicillin allergies in areas with high rates of CLR resistance (Gisbert et al., 2005, 2010). In contrast, STFX-based triple therapy is reportedly effective in patients with penicillin allergies. Therefore, a PPI/STFX/MNZ regimen may be potentially useful in patients with penicillin allergies, especially in areas with high CLR resistance rates when PPI is used (Murakami et al., 2009, 2013; Suzuki et al., 2009;

Hirata et al., 2012; Matsuzaki et al., 2012; Furuta et al., 2014a,b).

Two reports have investigated the efficacy of VPZ-containing therapy in patients with penicillin allergies (Table 6). Ono et al. (2017) reported that the eradication rate of VPZ/MNZ/STFX and VPZ/MNZ/CLR was 92.3% ($n = 17$) and 92.9% ($n = 14$), respectively. Sue et al. (2017b) reported that the efficacy of 7-day first-line treatment with VPZ/MNZ/CLR for *H. pylori* eradication was 100% (95% CI: 86.1–100%; $n = 20$). Because the first-line eradication rate of a VPZ-containing regimen in a CLR-resistant population is around 80% (Murakami et al., 2016), VPZ/STFX/MNZ may potentially be effective in patients with penicillin allergies in areas with a high CLR resistance rate, while VPZ/CLR/MNZ may be effective in areas with a low CLR resistance rate.

VONOPRAZAN-CONTAINING ERADICATION THERAPY AND CYP3A4/5 GENOTYPE

Vonoprazan is primarily metabolized to its inactive form by CYP3A4/5, and partially by CYP2B6, CYP2C19, and CYP2D6 (Yamasaki et al., 2016). Although the association between plasma VPZ levels and CYP3A5 genotype is obscure, the elimination rate of VPZ and the formation rate of its major metabolites are significantly correlated with enzyme activity of CYP3A4/5 (Yamasaki et al., 2016), suggesting that CYP3A4/5 activity affects the pharmacokinetics of VPZ, and therefore different clinical outcomes for *H. pylori* eradication among patients with different CYP3A4/5 genotypes. We previously reported that in first-line eradication treatment, the eradication rate in CYP3A5 *1 carriers was 72.7%

TABLE 6 | Summary of previous studies for the investigation of the efficacy of first-line eradication therapy for patients with penicillin allergies.

First author [ref. no.]	Year	Method	VPZ-containing eradication regimen			PPI-containing eradication regimen			
			Number	Regimen	Eradication rate	Number	Regimen	Eradication rate	
Ono (Ono et al., 2017)	2017	RST	ITT	13	VPZ: 20 mg bid MNZ: 250 mg bid CLR: 200 mg bid	92.3%	10	LPZ: 30 mg bid or RPZ: 10 mg bid MNZ: 250 mg bid CLR: 200 mg bid	50.0%
				14	VPZ: 20 mg bid MNZ: 250 mg bid STFX: 100 mg bid	92.9%	20	LPZ: 30 mg bid or RPZ: 10 mg bid MNZ: 250 mg bid STFX: 100 mg bid	100%
Sue (Sue et al., 2017b)	2017	RST	ITT	20	VPZ: 20 mg bid MNZ: 250 mg bid CLR: 200 or 400 mg bid	100%	30	LPZ: 30 mg bid, RPZ: 10 mg bid or EPZ: 20 mg bid MNZ: 250 mg bid CLR: 200 or 400 mg bid	83.3%

All paper to investigate efficacy of vonoprazan-containing eradication therapy for patients with penicillin allergies up until September 2018 were listed. bid, twice daily dosing; CLR, clarithromycin; EPZ, esomeprazole; ITT, intention to treat analysis; LPZ, lansoprazole; MNZ, metronidazole; PPI, proton pump inhibitor; RST, retrospective cohort trial; RPZ, rabeprazole; STFX, sitafloxacin; VPZ, vonoprazan.

(95% CI 54.5–86.7%), which was significantly lower than that in the *CYP3A5*3/*3* type (90.7 and 95% CI 69.4–94.1%, $P = 0.039$) (Sugimoto et al., 2017). In univariate analysis of this study, carriage of *CYP3A5 *3/*3* type was a positive predictive factor for outcome of eradication (OR: 3.656, 95% CI 1.014–13.190, $P = 0.048$) (Sugimoto et al., 2017). *CYP3A5 *3/*3* type may therefore be a significant positive prognostic predictor of successful eradication when VPZ is used in first-line therapy containing CLR and AMX.

TAILORED ERADICATION THERAPY FOR *H. pylori* INFECTION BASED ON CULTURE TEST

Determining the antibiotic susceptibility using either culture or genetic testing or both is useful to increase the eradication rate, particularly in populations with a high rate of infection with drug-resistant strains. Tailored eradication therapies are promising for significantly increasing successful outcomes compared to standard therapies, particularly in areas with a high prevalence of CLR-resistant strains (Furuta et al., 2007; Kawai et al., 2008; Sugimoto et al., 2014; Ferenc et al., 2017; Cho et al., 2018). A tailored treatment regimen based on susceptibility to CLR (PPI/AMX/CLR for patients infected with CLR-sensitive strains and PPI/AMX/MNZ for those with CLR-resistant strains) achieved a 94.3% eradication rate, which is significantly higher than that achieved with standard treatment (71.4%) (Kawai et al., 2008). Currently, no study has examined the efficacy of VPZ-containing tailored eradication therapy based on susceptibility to CLR. Because the eradication rate of VPZ-containing triple regimen in patients infected with CLR-resistant strains is around 80%, tailored eradication is expected to improve the effectiveness of these therapies.

POTENTIAL BENEFITS OR LIMITATIONS OF USING VPZ IN POPULATIONS OUTSIDE JAPAN

Japan is the first country to approve the use of VPZ for clinically acid-peptic disorders, all of studies investigated efficacy of VPZ-containing eradication therapy were reported from Japan. The data on the inhibitory effect on acid secretion of VPZ in Westerners is limited. A recent study showed that at steady state on Day 7, VPZ 40 mg

once daily displayed sustained acid inhibition during a 24-h and intragastric pH > 4 HTR in 100% of Japanese and 93.2% of British individuals (Jenkins et al., 2015). Therefore, the effect of suppressing acid secretion of VPZ may differ between Japanese and Westerners. However, acid inhibitory effects of VPZ administration have advantage in patients with CYP2C19 EM, refractory genotype to PPI therapy, suggesting that VPZ will be expected to show usability to Western population with high prevalence of CYP2C19 EMs.

CONCLUSION

This review focused on the efficacy of VPZ-containing *H. pylori* eradication therapy in relation to intragastric pH. We discussed the factors effecting the therapeutic outcomes of VPZ-containing *H. pylori* eradication therapy and summarized previous findings using first-, second- and third-line therapies. VPZ-containing triple therapy shows high efficacy in terms of *H. pylori* eradication compared to PPI-containing therapy, especially in patients infected with CLR-resistant strains who received VPZ/AMX/CLR as first-line therapy, patients who received VPZ/AMX/STFX as third-line therapy, and patients with penicillin allergies who received VPZ/AMX/STFX. Importantly, however, efficacy is similar between VPZ-containing and PPI-containing eradication therapy in patients infected with CLR-sensitive strains who received VPZ/AMX/CLR as first-line therapy and patients who received VPZ/AMX/MNZ as second-line therapy. We described the potential of a culture test-based and patients' pharmacogenomics-based tailored treatment for achieving an eradication rate exceeding 95%. No report to investigate direct association with advantage of vonoprazan use and acid inhibition during vonoprazan-containing eradication therapy using a pH monitoring study consists. Although one of factors affecting *H. pylori* eradication is potent acid inhibition during PPI-containing eradication therapy, it is required to clarify whether theoretical advantages of vonoprazan is caused by potent acid inhibition during eradication therapy as same as PPI-containing eradication therapy.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Targeting Cytokine Signaling and Lymphocyte Traffic via Small Molecules in Inflammatory Bowel Disease: JAK Inhibitors and S1PR Agonists

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OPEN ACCESS

Edited by:

Luca Antonioli,
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Specialty section:

This article was submitted to
Gastrointestinal and Hepatic
Pharmacology,
a section of the journal
Frontiers in Pharmacology

Received: 20 November 2018

Accepted: 19 February 2019

Published: 13 March 2019

Citation:

Pérez-Jeldres T, Tyler CJ, Boyer JD, Karuppuchamy T, Yarur A, Giles DA, Yeasmin S, Lundborg L, Sandborn WJ, Patel DR and Rivera-Nieves J (2019) Targeting Cytokine Signaling and Lymphocyte Traffic via Small Molecules in Inflammatory Bowel Disease: JAK Inhibitors and S1PR Agonists. *Front. Pharmacol.* 10:212. doi: 10.3389/fphar.2019.00212

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The inflammatory bowel diseases (IBDs) are a chronic, relapsing inflammatory diseases of the gastrointestinal tract with heterogeneous behavior and prognosis. The introduction of biological therapies including anti-TNF, anti-IL-12/23, and anti-integrins, has revolutionized the treatment of IBD, but these drugs are not universally effective. Due to the complex molecular structures of biologics, they are uniformly immunogenic. New discoveries concerning the underlying mechanisms involved in the pathogenesis of IBD have allowed for progress in the development of new treatment options. The advantage of small molecules (SMs) over biological therapies includes their lack of immunogenicity, short half-life, oral administration, and low manufacturing cost. Among these, the Janus Kinases (JAKs) inhibition has emerged as a novel strategy to modulate downstream cytokine signaling during immune-mediated diseases. These drugs target various cytokine signaling pathways that participate in the pathogenesis of IBD. Tofacitinib, a JAK inhibitor targeting predominantly JAK1 and JAK3, has been approved for the treatment of ulcerative colitis (UC), and there are other specific JAK inhibitors under development that may be effective in Crohn's. Similarly, the traffic of lymphocytes can now be targeted by another SM. Sphingosine-1-phosphate receptor (S1PR) agonism is a novel strategy that acts, in part, by interfering with lymphocyte recirculation, through blockade of lymphocyte egress from lymph nodes. S1PR agonists are being studied in IBD and other immune-mediated disorders. This review will focus on SM drugs approved and under development, including JAK inhibitors (tofacitinib, filgotinib, upadacitinib, peficitinib) and S1PR agonists (KRP-203, fingolimod, ozanimod, etrasimod, amiselimod), and their mechanism of action.

Keywords: IBD, small molecules, JAK inhibitors, S1P agonists, MOA

INTRODUCTION

Inflammatory Bowel diseases (IBDs) is a chronic immune-mediated condition of the gastrointestinal tract (Abraham and Cho, 2009). It is potentially caused by a dysregulated mucosal immune response to intestinal microflora in genetically predisposed hosts (Abraham and Cho, 2009). There are currently no curative therapies, and in most cases, lifelong treatment is required (Abraham and Cho, 2009). Non-specific immunomodulatory drugs such as glucocorticoids, sulfasalazine/5-amino salicylates, methotrexate, and thiopurines were among the first drugs used to treat IBD (Soendergaard et al., 2018). The introduction of biologics during the last 20 years has revolutionized the treatment of IBD, and several anti-TNF monoclonal antibodies (mAbs) (including infliximab, adalimumab, certolizumab pegol, and golimumab) are commonly used. More recently, antibodies with a different mechanism of action (MOA), such as anti-integrin $\alpha 4\beta 7$ (vedolizumab) and anti-IL12/IL23 (ustekinumab), became available for clinical use (Olivera et al., 2016). However, mAbs have limitations in terms of safety, cost, and sustained efficacy (Hemperly et al., 2018). In fact, around 10–30% of patients treated with anti-TNF are primary non-responders to therapy, and 23–46% are secondary non-responders (Hemperly et al., 2018). For these reasons, novel orally available drugs are still in great need and are being developed to treat IBD. The present review will focus on new families of chemically synthesized SM drugs already available or under development: Janus Kinases (JAK) inhibitors and sphingosine-1-phosphate receptor (S1PR) agonists, with emphasis on their MOA.

Differences Between Small Molecules and Monoclonal Antibodies

Monoclonal antibodies are large molecules with high molecular weights (~150 kDa) (Samanen, 2013). The mAb structure consists of four polypeptide chains, two identical heavy chains, and two identical light chains. Each mAb molecule has an antigen-binding region (Fab) or variable region, and a constant region or Fc (Ordás et al., 2012). The size and structure of the mAb determines the drug pharmacokinetic, target location, the drug-drug interaction, the antigenicity, and the route of administration. The mAbs are eliminated from the circulation by catabolism, which depends on the rates of proteolysis (extracellular degradation), recycling rates [by interaction with Brambell or the neonatal Fc receptor (FcRn)], and receptor-mediated antibody endocytosis rates (Ordás et al., 2012). Due to the large size of mAb the renal clearance is insignificant (Hemperly et al., 2018). Because of the protein composition of mAbs, the immune system can recognize them as immunogenic foreign antigens, which may lead to the development of specific anti-drug antibodies that nullify their therapeutic effect (Ordás et al., 2012; Yarur and Rubin, 2015; Hemperly et al., 2018). This results in increased drug clearance and ultimately may contribute to treatment failure and/or hypersensitivity reactions (Ordás et al., 2012; Yarur and Rubin, 2015; Hemperly et al., 2018). The addition of immunomodulators can decrease anti-drug

antibody formation but increases the risks associated with immunosuppression (Ordás et al., 2012; Yarur and Rubin, 2015; Hemperly et al., 2018).

The term SM typically refers to organic compounds with low molecular weights, usually <1 kDa, which enables them to diffuse easily through cell membranes to reach intracellular targets (Samanen, 2013; Murphy and Zheng, 2015). Many SM inhibitors can function as immunomodulators due to their ability to specifically block intracellular signaling pathways thought to be pivotal to the pathogenesis of IBD (Samanen, 2013; Murphy and Zheng, 2015; Olivera et al., 2016). SMs have several advantages over conventional immunotherapeutic agents, including ease of administration (oral, without infusion costs), stable structures, non-immunogenic, potentially short half-lives, and usually lower manufacturing costs (Samanen, 2013; Murphy and Zheng, 2015; Olivera et al., 2016). **Table 1** compares the main differences between SM and mAb (Samanen, 2013).

JAK-STAT Pathway and JAK Inhibitors

Cytokines are released by the immune system in response to a stimulus (Abbas et al., 2014b). They bind to specific receptors, triggering activation and initiation of intracellular signaling pathways (Abbas et al., 2014b). Cytokines encompass many structurally unrelated proteins that are grouped based on their binding to distinct receptor super families, which

TABLE 1 | Comparison of properties of SM drugs and mAbs (Samanen, 2013).

	Small molecules	Monoclonal antibodies
Molecular weight	Low (<1000 Da)	High (>1000 Da)
Preparation	Chemical synthesis	Biologically produced
Structure	Small organic compounds	Proteins
Route of administration	Oral	Parenteral
Location of target	Intracellular	Extracellular
Distribution	Variable in organs/tissues/cells	Limited to plasma and/or extracellular fluids
Metabolism	Metabolized typically by liver and gut CYPs into no active and active metabolites	Catabolism by proteolytic degradation to peptides and amino acids
Clearance	The clearance can be by renal excretion, biliary excretion, hepatic metabolism, and intestinal transporters	Mainly involves the reticuloendothelial system (RES) through proteolytic catabolism
Toxicity	Can produce specific toxicity due to parent or metabolites (often “off the target”)	Receptor-mediated toxicity
Antigenicity-hypersensitivity	No antigenic, but can show unpredictable hypersensitivity	Potential
Drug-drug interaction	Pharmacokinetic interactions by competitive clearance mechanism as: –Decreasing clearance by enzyme inhibition –Increasing clearance by enzyme induction	Infrequent
Mechanism of action	Receptor or enzyme inhibition	Depletion

TABLE 2 | Cytokines, receptors, and transduction pathway.

Ligands	Cytokine receptor	Transduction pathway	Function
	Type I		
Epo, Tpo, G-CSF, GH, and PRL	Homodimer receptor	JAK-STAT (JAK2)	Erythropoiesis Myelopoiesis Megakaryocyte/platelet production Growth Mammary development
IL-3, IL-5, and GM-CSF	Common β chain	JAK-STAT (JAK2)	
IL-6, IL-11, IL-23, and OSM	gp-130	JAK-STAT (mainly JAK1 but also JAK2, Naive T cells differentiation T-cell homeostasis TYK2)	Inflammation Granulopoiesis
IL-2, IL-4, IL-7, IL-9, IL-13, IL-15, and IL-21	Common γ chain	JAK-STAT (JAK1, JAK3)	Growth/maturation lymphoid cells Differentiation/homeostasis T cells, NK cells B cells class switching Inflammation
IFNα, IFNβ, IFNγ, IL-10, and IL-22	Type II	JAK-STAT (JAK1, JAK2, TYK2)	Antiviral Inflammation Antitumor
TNFα, TNFβ, LT, CD40, FasL, BAFF, April, Ox40, GITR, nerve growth factor	TNF receptor family	TRAF	Inflammation
IL-1, IL-18, IL-33	IL-1 receptor family	IRAK	Inflammation
Chemokines	Seven transmembrane G-protein-coupled receptors	G proteins	Chemotaxis and lymphocyte migration

Epo, erythropoietin; Tpo, thrombopoietin; G-CSF, granulocyte-colony stimulating factor; GH, growth hormone; PRL, prolactin; IL, interleukin; GM-CSF, granulocyte-macrophage colony-stimulating factor; OSM, oncostatin M; TNF, tumor necrosis factor; LT, lymphotoxin; FasL, Fas ligand, B-cell activating factor; GITR, glucocorticoid-induced TNF receptor; JAK, Janus kinase; STAT, signal transducers and activators of transcription; TRAF, TNF receptor-associated factors; IRAK, interleukin-1 receptor-associated kinases (Abbas et al., 2014d).

include Type I cytokine receptors, Type II cytokine receptors, the TNF receptor family, the IL-1 receptor family, and G-protein-coupled receptors. Each family of receptors utilizes different mechanisms of signal transduction (Table 2; Abbas et al., 2014b). The cytokines bind to the extracellular domain of the receptor, and trigger intracellular changes, resulting in signal transduction that drives changes in gene expression (Clark et al., 2013; Abbas et al., 2014b). Protein kinases have an essential role in the signal transduction pathway of these receptors, and are an attractive target to regulate the inflammatory response (Clark et al., 2013; Abbas et al., 2014b). However, due to the complexity and redundancy inherent to signal transduction networks, some of these kinases may be better therapeutic targets than others (Clark et al., 2013).

The JAK family is a small family of receptor-associated tyrosine kinases that are essential for the cytokine signaling cascade, downstream of Type I and Type II cytokine receptors (Schwartz et al., 2017). The JAK-signal transducers and activators of transcription (STAT) pathway plays an important role in innate immunity, adaptive immunity, and hematopoiesis, participating in cellular processes such as cell growth, survival, differentiation, and migration (Table 2; Banerjee et al., 2017; Olivera et al., 2017). There are four members of the JAK family (JAK1, JAK2, JAK3, and TYK2) and seven signal transducers and transcription activators called signal transducer and activator of transcription, or STAT (STAT 1–4, 5a, 5b, and 6) (Clark et al., 2013; Banerjee et al., 2017; Olivera et al., 2017; Schwartz et al., 2017; Table 3).

The unique structure of each JAK clearly distinguishes them from other members of the protein tyrosine kinase family (Banerjee et al., 2017). The JAKs contain four functional domains: the SH2 domain (a scaffold for STAT), the FERM domain (regulates catalytic activity and mediates association with receptors and other proteins), the pseudo-tyrosine kinase domain, and a catalytically active tyrosine kinase domain

TABLE 3 | STAT and cellular function.

STAT	Cellular function
1	Cell growth and apoptosis TH1 cell-specific cytokine production Antimicrobial defense
2	Mediation of IFNα/IFNβ signaling
3	Cell proliferation and survival Inflammation Immune response Embryonic development Cell motility
4	TH1 cell differentiation Inflammatory responses Cell proliferation
5A	Cell proliferation and survival IL-2Ra expression in T lymphocytes Mammary gland development Lactogenic signaling
5B	Cell proliferation and survival IL-2Ra expression in T lymphocytes Sexual dimorphism of body growth rate NK cell cytolytic activity
6	Inflammatory and allergic immune response B-cell and T-cell proliferation TH2 cell differentiation

TH, T helper; IFN, interferon; IL, interleukin. Table adapted from Miklossy et al. (2013). Therapeutic modulators of STAT signaling for human diseases (Miklossy et al. 2014).

(Banerjee et al., 2017). These last two domains are the basis for the name of the protein family named Janus (the two-faced Roman god of beginnings, endings, and duality), thus JAK exhibits a domain with kinase activity, while the other negatively regulates the activity of the first (Banerjee et al., 2017).

Canonical JAK-STAT signaling starts with the binding between cytokines and their corresponding transmembrane receptors, allowing receptor dimerization and triggering the transactivation of JAK, followed by phosphorylation of the cytoplasmic tails of the receptors that produce coupling sites for STAT, resulting in the tyrosine-phosphorylation (p-Tyr) of the STAT by JAK (Jatiani et al., 2010; Villarino et al., 2017). After these events, STAT (like homo/heterodimers) translocate

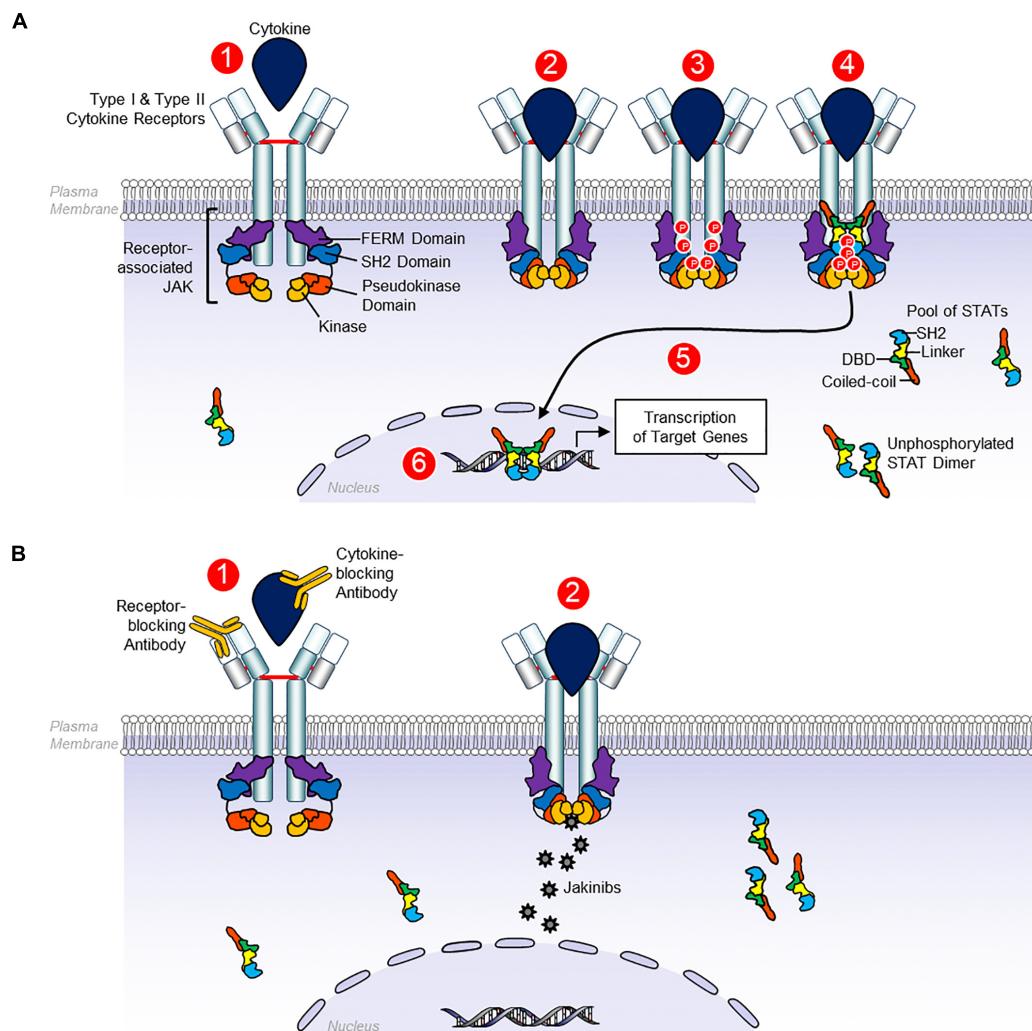


FIGURE 1 | Signaling by receptors Type I and Type II cytokines. **(A)** Type I and type II cytokine receptors comprise subunits that physically associate with Janus kinases (JAKs). Type I and type II cytokines depend on JAKs to transduce intracellular signals. JAK proteins share four components: the kinase domain, the pseudokinase domain, the FERM domain, and the SH2 domain. 1, the canonical JAK-STAT signaling begins with the extracellular association between cytokines and their corresponding transmembrane receptors. 2, the receptor dimerization triggers the transactivation of JAK. 3. Phosphorylation of the cytoplasmic tails of the receptors that create docking sites for STATs. 4–6, STAT binds to JAK, allowing the tyrosine phosphorylation of STAT which results in STAT dimerization, nuclear translocation, DNA binding, and ultimately, modulation of gene transcription. Unphosphorylated STAT dimers also have regulatory functions, although these functions are less well defined. **(B)** 1, monoclonal antibodies can block Type I and Type II cytokines and their receptors. 2, by contrast, JAK inhibitors block cytokine signaling, binding to the kinase domain of JAK in the ATP-binding site, avoiding their phosphorylation and JAK activation, preventing STAT phosphorylation and other substrates, so intracellular signals cannot be transduced (Schwartz et al., 2017).

to the nucleus, bind to DNA, and modulate gene transcription (Jatiani et al., 2010; Villarino et al., 2017). In addition to STAT phosphorylation, other kinases such as Src, phosphoinositide 3-kinases (PI3K), and RAF can be phosphorylated, activating additional signaling pathways involving proteins including Akt, and extracellular signal-regulated kinases (ERK) (Figure 1; Jatiani et al., 2010).

Signal transducers and activators of transcription is under the control of physiological negative regulators such as (i) suppressors of cytokine signaling (SOCS), that inhibit the kinase activity, binding phospho-tyrosine residues and competing with STAT at cytoplasmic level, (ii) protein tyrosine phosphatases

(PTPs) that inactivate JAK and STAT in both the nucleus and the cytoplasm, (iii) protein inhibitor of activated STAT family (PIAS) that interferes at the nuclear level with STAT-mediated transcription and triggers proteasome degradation, and (iv) the modulators SH2B adaptor protein that increase or decrease JAK activation (Villarino et al., 2017).

Many cytokines implicated in the pathogenesis of immune-mediated diseases use the JAK-STAT pathway, representing a potential therapeutic target for these disorders (Jatiani et al., 2010; Clark et al., 2013; Banerjee et al., 2017; Olivera et al., 2017; Schwartz et al., 2017; Villarino et al., 2017). The mAbs can block Type I and Type II cytokines and their receptors. By contrast, JAK

inhibitors block cytokine signaling, binding to kinase domain of JAK at the ATP-binding site, avoiding their phosphorylation and JAK activation, preventing STAT phosphorylation and other substrates, so intracellular signals cannot be transduced (Jatiani et al., 2010; Clark et al., 2013; Banerjee et al., 2017; Olivera et al., 2017; Schwartz et al., 2017; Villarino et al., 2017). Other potential therapeutic candidates include STAT-binding inhibitory peptides, STAT inhibitors, STAT-targeting small interfering RNA (siRNA), and STAT-binding decoy oligonucleotides (Schwartz et al., 2017; Villarino et al., 2017).

The JAK inhibitors have been used in the treatment of hematologic disorders (Jatiani et al., 2010). In recent years, these inhibitors have received attention for the treatment of autoimmune/immune-mediated disorders such as rheumatoid arthritis (RA) (Vanhoutte et al., 2017), systemic lupus erythematosus (SLE) (ClinicalTrials.gov, ClinicalTrials, 2018e), dermatomyositis (Hornung et al., 2014), Sjogren syndrome (ClinicalTrials.gov, ClinicalTrials, 2018n), vasculitis (Zhang et al., 2018), psoriasis (Hsu and Armstrong, 2014), alopecia areata (Divito and Kupper, 2014), atopic dermatitis (Levy et al., 2015), vitiligo (Liu et al., 2017), and IBD (Panés et al., 2017; Sandborn et al., 2017a; Vermeire et al., 2017a).

JAK Inhibitors for the Treatment of IBD

Typically, IBD is associated with chronic inflammation, defined by a dysregulated response of the innate and adaptive immune systems (Abraham and Cho, 2009; Boland and Vermeire, 2017). Chronic inflammation in Crohn's disease (CD) is characterized by a response of helper T cells type 1 (Th1) and helper T cells type 17 (Th17), with inadequate activity of regulatory T cells (Treg), whereas UC has generally been considered a type 2 T helper cell cytokine profile (Th2) (Boland and Vermeire, 2017). In both diseases, many of the cytokines produced by these T cells signal through JAK receptors; therefore, JAK proteins have an important place in the signaling of inflammation in IBD (Boland and Vermeire, 2017).

Key cytokines in the pathogenesis of IBD belong to Type I and Type II cytokines receptors [i.e., IL-6, IL-5, IL-9, IL-10, IL-13, IL-12/23, IL-22, granulocyte-macrophage colony-stimulating factor (GM-CSF), IFN- γ] (Zhou et al., 2007; Dienz and Rincon, 2009; Abbas et al., 2014a,c; Flamant et al., 2017). These cytokines all signal through the JAK/STAT pathway. In contrast, the cytokines TNF, IL-1, and IL-17, which are the major drivers of IBD, do not use the JAK-STAT pathway in their signaling pathways (Zhou et al., 2007; Dienz and Rincon, 2009; Abbas et al., 2014a,c; Flamant et al., 2017). However, these cytokines induce the expression of a wide range of downstream pro-inflammatory cytokines, that in turn depend on JAK/STAT signaling (Zhou et al., 2007; Dienz and Rincon, 2009; Abbas et al., 2014a,c; Flamant et al., 2017).

Interleukin-6 (IL-6) along with oncostatin M (OSM) and IL-11 signal through the gp130-associated receptor family. IL-6 activates JAK1, JAK2, and TYK2 leading to STAT3 transduction, which promotes T cell proliferation, favoring the polarization of Th17 cells (Zhou et al., 2007; Banerjee et al., 2017). Notably, IL-6 can also promote Th2 differentiation (Dienz and Rincon, 2009). In addition, IL-6 has other functions relevant to IBD, such as

regulating intestinal permeability, by its effects on tight junctions, regulating the proliferation of epithelium, and healing of wounds (Flamant et al., 2017).

Interleukin-12 and IL-23 also play an important role in IBD, and JAK2 and tyrosine kinases type 2 (TYK2) are involved in the signaling of these cytokines by activating STAT3 and STAT4, promoting inflammatory reactions through their ability to induce Th1 and Th17 polarization, respectively, and production of IFN- γ , IL-21, and IL-22 (Flamant et al., 2017).

Interleukin-10 is an anti-inflammatory cytokine produced by many immune cell populations, including activated macrophages, dendritic cells, regulatory T cells, Th1 and Th2 cells. IL-10 activates JAK1 and TYK2 proteins, leading to STAT3 phosphorylation (Abbas et al., 2014a). The anti-inflammatory effects of IL-10 results, in part, from its ability to inhibit the production of IL-12 by activated macrophages and dendritic cells as well as inhibiting the expression of costimulatory and class II MHC molecules in these cells (Abbas et al., 2014a).

Interleukin-22 is produced in epithelial tissues, especially in the skin and gastrointestinal tract. IL-22 activates JAK1 and TYK2, transducing signals via STAT3, STAT1, and STAT5. IL-22 has a role in maintaining epithelial integrity, mainly by promoting the barrier function of epithelial cells and by inducing production of anti-microbial peptides (Abbas et al., 2014c). However, IL-22 contributes to inflammation, in part by stimulating epithelial production of chemokines, and may therefore be involved in tissue injury in inflammatory diseases (Abbas et al., 2014c).

Interleukin-9 binding to its receptor leads to activation of JAK1 and JAK3, which in turn phosphorylates STAT1/STAT3 and STAT1/STAT5, respectively (Flamant et al., 2017). IL-9 has been associated with deleterious impact on intestinal epithelial wound healing (Flamant et al., 2017).

Interferon- γ activates JAK1 and JAK2, inducing STAT1 activation, resulting in macrophage activation, Th1 polarization, and increased expression of several proinflammatory cytokines. However, IFN- γ also has a protective function in epithelial healing (Flamant et al., 2017). Moreover, IFN- γ protects from tissue destruction by inhibiting the expression of genes that code for tissue destructive factors such as matrix metalloproteinases (MMPs), serine proteases, coagulation factors, complement components, and enzymes involved in the metabolism of prostaglandin. In addition, IFN- γ decreases neutrophil and monocytes infiltration (Hu and Ivashkiv, 2009). GM-CSF activates JAK2 which phosphorylates STAT5, and STAT3 promoting monocyte/macrophage/granulocyte survival and activation (Kimura et al., 2009; Flamant et al., 2017).

Drugs that block JAK/STAT signaling have the potential to alter multiple inflammatory pathways, being less specific in their action than drugs that target specific cytokines or their receptors (Soendergaard et al., 2018). This complexity is clear for IL-6 (pro-inflammatory) and IL-10 (anti-inflammatory) signaling, where both ligands, despite activating JAK1 and STAT3, have opposing functions (Soendergaard et al., 2018). Consequently, blocking JAK1 affects both IL-6 and IL-10, and may alter the inflammatory balance in both directions (Soendergaard et al., 2018). Additionally, JAK inhibitors can

result in undesirable adverse effects like cytopenia and infectious complications, through its blockade of GM-CSF and IFN- γ signaling, respectively (Clark et al., 2013). On the other hand, a major strength is their effectiveness. Through adequate plasma levels, these drugs induce partial and reversible inhibition of cytokine signaling, resulting in a better balance between the inflammatory and immunomodulatory response (Clark et al., 2013). More selective inhibition of the JAK-STAT pathway is being developed and may overcome the challenges of less selective inhibitors.

The US Food and Drug Administration (FDA), in May 2018, approved tofacitinib as the first JAK inhibitor to treat moderate to severely active UC (Soendergaard et al., 2018). Similar to the FDA, the Committee for Medicinal Products for Human Use (CHMP) at the European Medicines Agency (EMA) had a favorable opinion, and recommended their use in adult patients with moderately to severely active UC with inadequate or loss of response or intolerance to either conventional therapy or biological agents. Currently, no JAK inhibitors are approved for CD; however, other selective JAK inhibitors are in the pipeline for CD (Soendergaard et al., 2018).

Tofacitinib

Tofacitinib (Xeljanz, Pfizer) is a pan-JAK inhibitor, that preferentially inhibits JAK1 and JAK3, in a dose-dependent fashion (Sandborn et al., 2017a). Tofacitinib has a predicted gut availability of 93%, and the clearance is 70% hepatic, whereas the remaining 30% is cleared by renal metabolism (Hemperly et al., 2018). Tofacitinib's half-life is 3 h and neither age, gender, body weight, or disease severity at baseline have an effect on its clearance or plasma levels (Dowty et al., 2014).

A double-blind, placebo-controlled phase 2 study evaluated the efficacy of tofacitinib in patients with UC ($n = 194$) with moderate to severe activity (Sandborn et al., 2012). The patients were randomly assigned during 8 weeks to different tofacitinib doses (0.5, 3, 10, and 15 mg each 12 h) or placebo. The primary outcome at 8 weeks (clinical response established as the decrease of at least three points and at least 30% from the baseline total Mayo score, and decrease of at least one point or an absolute rectal bleeding sub-score of 0 or 1) reported a statistically significant response between the higher doses versus placebo (78% versus 42%, respectively) (Sandborn et al., 2012). These data were supported by phase 3, double-blind placebo-controlled studies; OCTAVE induction 1, 2, and OCTAVE sustain. In the induction trials; OCTAVE 1 ($n = 598$) and 2 ($n = 591$) trials, the patients were randomly assigned to receive 10 mg of tofacitinib twice daily or placebo during 8 weeks (Sandborn et al., 2017a). The primary endpoint was remission at week 8 (a total Mayo score of ≤ 2 , with no subscore > 1 and a rectal bleeding sub-score of 0). This endpoint was achieved in 18.5% in the tofacitinib-treated group versus 8.2% in the placebo group ($P = 0.007$); in the OCTAVE Induction 2 trial, remission was achieved in 16.6% versus 3.6% ($P < 0.001$). A total of 593 patients achieved clinical response after the induction therapy and were recruited in the OCTAVE Sustain trial to randomly receive tofacitinib as maintenance therapy (5 or 10 mg twice daily) or placebo during 52 weeks. The aim endpoint (remission at 52 week) was achieved

in 34.3 and 40.6% (5 and 10 mg twice daily, respectively) versus 11.1% placebo ($P < 0.001$) (Sandborn et al., 2017a). Furthermore, mucosal healing was more frequent in the tofacitinib group, and tofacitinib was effective in both treated and naïve to anti-TNF patients. The safety and efficacy data were evaluated in a phase 3, multicenter, open-label, long-term extension study in patients with severe to moderate UC ($n = 946$). Preliminary data showed that no new safety concerns emerged, compared with those observed in RA. Efficacy results from OLE study (NCT01470612) support sustained efficacy with tofacitinib at both 5 and 10 mg doses twice daily (Lichtenstein et al., 2017).

Similar studies were conducted in patients with moderate to severe CD; In a phase II ($n = 139$) study, patients were randomly assigned to receive tofacitinib (1, 5, or 15 mg twice daily) or placebo during 4 weeks. This study did not show a significant clinical response or remission response (Sandborn et al., 2014). Subsequently, another phase IIb study was performed. In this study, patients were randomized, during 8 induction weeks, to tofacitinib 5 mg twice per day ($n = 86$) or placebo ($n = 91$). The responders were included in the maintenance phase, during 26 weeks, to receive tofacitinib 5 or 10 mg daily or placebo. The majority of enrolled patients were previously treated with anti-TNF (76–79%). In this study, the results were also disappointing, despite the long duration of treatment, the remission rates did not reach significant differences (Panés et al., 2017).

These discouraging results in CD may be due to high placebo response rates or differences in the fundamental immunopathogenesis of CD and UC. Several factors may have contributed to the high placebo response observed, including lack of centralized reading endoscopy and absence of baseline objective markers of disease activity (Panés et al., 2017).

Filgotinib (GLPG0634, Galapagos/Gilead Sciences) is an oral JAK1 inhibitor, with enhanced selectivity for JAK1 over JAK2 and JAK3 (30 and 50 times, respectively) in blood (Vermeire et al., 2017a,b; Hemperly et al., 2018). Filgotinib dosing leads to the formation of active metabolite which exhibits a similar JAK1 selectivity profile as the parent compound, but has less potency (Vermeire et al., 2017a,b; Hemperly et al., 2018). Still, both contribute to the clinical activity of filgotinib. The half-life of filgotinib is 6 h, while the metabolite has a terminal elimination half-life of 21–27 h. Filgotinib and its metabolites are predominantly cleared renally (>80%) (Vermeire et al., 2017a,b; Hemperly et al., 2018).

FITZROY, a double-blind, placebo-controlled study, examined the efficacy and safety of filgotinib for the treatment of active moderate to severe CD (Vermeire et al., 2017a). A total of 174 patients with active CD were enrolled. Disease activity was confirmed by centrally read endoscopy. A proportion of patients achieved clinical remission with filgotinib 200 mg once a day, compared with placebo (47 versus 23%; $p = 0.077$) at week 10. Data also suggested that filgotinib is effective in anti-TNF exposed and naïve patients, being twofold higher in TNF-naïve group (Vermeire et al., 2017a). In addition, a recent *post hoc* analysis showed that clinical remission is still seen in CD, regardless of the disease location or duration (Vermeire et al., 2017b). Currently, there are phase III trials underway in both a CD and UC (ClinicalTrials.gov, ClinicalTrials, 2018h,i,j,m).

Peficitinib (GLPG1205, Janssen) is JAK1 and JAK3 inhibitor (Sands et al., 2018; Soendergaard et al., 2018). The efficacy and safety of the drug has been evaluated for the treatment of moderate to severe UC ($n = 219$) in a multicenter, randomized, double blind, placebo-controlled, phase IIb trial. Nevertheless, the development of this drug was discontinued in 2017 due to disappointing efficacy results (Sands et al., 2018; Soendergaard et al., 2018).

Upadacitinib (UPA) (ABT-494, Abbvie) is a JAK1-selective inhibitor. It is a non-sensitive substrate for cytochrome P450, approximately 20% is eliminated, unchanged, in urine (Hemperly et al., 2018). Its efficacy and safety were assessed in patients with moderate-to-severe CD who had inadequate response, or intolerance, to anti-TNF (Sandborn et al., 2017b). In this study, patients receiving 6 mg twice daily (27%) achieved clinical remission at a higher rate than placebo (11%). A significant dose-response relationship for endoscopic remission was observed in the UPA arm (Sandborn et al., 2017b). In addition, patients with moderate-to-severely active UC ($n = 250$), and history of inadequate response, loss of response or intolerance to corticosteroids, immunosuppressant, or biologic therapies, were included in a phase IIb double-blind placebo-controlled dose-ranging induction study, to assess the safety and efficacy of UPA. At week 8, both the primary objective: clinical remission per Adapted Mayo Score (stool frequency subscore ≤ 1 , rectal bleeding score = 0, endoscopic score ≤ 1) and the secondary objectives: clinical remission per full Mayo score, clinical response per adapted Mayo score and endoscopic improvement were evaluated (Sandborn et al., 2018b). All of these objectives were achieved with different doses ranging from 15 to 45 mg QD. The tolerance to UPA was good and safety was similar to that of other UPA studies (Sandborn et al., 2018b). Phase III studies in CD and UC are ongoing (ClinicalTrials.gov, ClinicalTrials, 2018g,l,o,p).

TD-1473 (Theravance, Biopharma) is a new oral pan-JAK inhibitor being investigated (ClinicalTrials.gov, ClinicalTrials, 2018f; Soendergaard et al., 2018). Unlike other JAK inhibitors, its distribution is limited to the gastrointestinal tract, minimizing systemic toxicity and side effects (ClinicalTrials.gov, ClinicalTrials, 2018f; Soendergaard et al., 2018). Data from phase I study in healthy volunteers have shown that treatment with TD-1473 is safe and well-tolerated (Beattie et al., 2018). The safety, tolerability, and pharmacokinetics of TD-1473 were assessing in a double-blind placebo-controlled multicenter phase Ib study in subjects with moderately to severely active UC ($n = 40$) (ClinicalTrials.gov, ClinicalTrials, 2018f; Sandborn et al., 2018a). In this study, TD-1473 was generally well tolerated over 4 weeks with evidence of intestinal restriction, low systemic exposure, and signals for clinical and biomarker activity in subjects with moderately to severely active UC (ClinicalTrials.gov, ClinicalTrials, 2018f; Sandborn et al., 2018a).

Pf-06651600/Pfizer (JAK3 inhibitor) and **Pf-06700841/Pfizer** (TYK2/JAK1 inhibitor) are being tested in clinical trials to be completed by early 2020 (ClinicalTrials.gov, ClinicalTrials, 2018k,q).

Adverse Effects: Experience From IBD and Rheumatoid Arthritis

Most of the safety information currently for JAK inhibitors belongs to RA and psoriasis literature. For S1PR agonists most of the safety data originated from Multiple Sclerosis and IBD trials, as tofacitinib and fingolimod were approved for those applications years earlier. Otherwise, post-marketing real-world data from clinical practice after the approval of tofacitinib in immune-mediated disease as RA and IBD are available (Hsu and Armstrong, 2014; Charles-Schoeman et al., 2015; Liu et al., 2017; Schwartz et al., 2017; Winthrop, 2017; Cohen et al., 2018; Kang et al., 2018; Verden et al., 2018).

Tofacitinib is the JAK inhibitor whose side effects are best known compared to other more specific JAK inhibitors. Still is unknown if the higher selectivity of the new JAK will result in fewer adverse effects (Winthrop, 2017).

Infections

The risk of serious infections during JAK inhibitor treatments is similar to that of biologics and most infections do not require treatment discontinuation. Nasopharyngitis and influenza are the most frequently reported infection-related adverse events. Tuberculosis and osteomyelitis are infrequent infections that also have been identified, and in this circumstance, the therapy must be interrupted (Winthrop, 2017). In addition, JAK inhibitors increase the risk of herpes zoster infection. However, Shingrix (recombinant zoster vaccine, GlaxoSmithKline) can reduce risk of infection and associated complications in patients treated with JAK inhibitors (Winthrop, 2017).

Other serious viral infections like nephropathy by BK virus (a polyoma virus) have been identified with the use of tofacitinib during renal transplantation (Schwartz et al., 2017). A few cases of cytomegalovirus (CMV) infections, including CMV retinitis, have occurred in patients under treatment with tofacitinib in the long-term extension studies (Sandborn et al., 2014; Schwartz et al., 2017; Winthrop, 2017). Also cases of abscesses, cellulitis, *Clostridium difficile* infection, pneumonia by *Pneumocystis jiroveci*, candida infections, urinary tract infections, and histoplasmosis have been reported (Sandborn et al., 2014).

Malignancy

All immunosuppressants have the potential to increase cancer risk. Accordingly, JAK inhibitors could interfere with T and NK immune vigilance against cancer and the antineoplastic role of IFN- γ (Schwartz et al., 2017).

Recently, the post marketing surveillance (PMS) data of worldwide tofacitinib use in RA, obtained from Pfizer safety database during a 3-year reporting period, was published. The estimated exposure to tofacitinib was 34,223 patient years. The overall relative risk was 0.45 per 100 patients-year, being highest during the first year and stabilizing later. The most notified neoplasms were lymphoma, skin, lung, breast, brain, prostate, uterine and colon cancer, malignant melanoma, squamous, and basal cell carcinoma. During the PMS, the most reported cancer in RA patients receiving tofacitinib therapy was the non-melanoma skin cancer (NMSC) (Cohen et al., 2018).

Dyslipidemia and Cardiovascular Events

A dose-dependent increase in HDL, LDL, and total cholesterol has been observed. Levels normalized after cessation of treatment. This change in lipid profiles has not been found to be associated with an increase of adverse cardiovascular events (Charles-Schoeman et al., 2015).

Thromboembolic events were reported during a placebo-controlled trial of baricitinib, a JAK inhibitor tested in RA. A post-marketing adverse event report from the FDA's Adverse Event Reporting System did not show increased risk of thromboembolic events for tofacitinib, tofacitinib extended-release, or ruxolitinib. However, the data indicated that pulmonary thrombosis and portal vein thrombosis may be a class-wide risk for JAK inhibitors (Kang et al., 2018; Verden et al., 2018).

Anemia and Leukopenia

Because hematopoietic growth factors signal through JAK2, cytopenia is frequent with the use of the first-generation pan-JAK inhibitors. These alterations are usually well tolerated and do not require treatment discontinuation (Schwartz et al., 2017).

Pregnancy

There is a lack of information about the effect of JAK inhibitors during pregnancy since most studies exclude pregnant women, and there is little data available from patients who became pregnant while receiving the medication (Winthrop, 2017). The pregnancy results from patients with UC under tofacitinib exposure were reported. Mahadevan et al. (2018) notified that from 1157 UC patients recruited in interventional trials, 25 cases were reported (11 maternal, 14 paternal) with exposure to tofacitinib. These results include 15 healthy neonates, 2 spontaneous abortions, and 2 medical interruptions. Cases of fetal death, neonate death, and congenital malformations were not described.

The data available to date does not allow to definitive position regarding the tofacitinib effect on pregnancy, and its use is not recommended (Mahadevan et al., 2018).

Others Adverse Events

Intestinal perforation and elevated serum liver enzymes have been reported with the use of tofacitinib (Olivera et al., 2016).

Future Perspectives

The pathogenesis of UC and CD involve different signaling pathways, which may explain the differential response to diverse drugs. The use of a drug with different MOA could be an effective alternative (i.e., tofacitinib for anti-TNF non-responders in UC). Further understanding the main pathways involved in the pathogenesis of IBD may predict the efficacy of specific drugs based on their MOA in the near future (Jabeen et al., 2015).

Janus kinases inhibitors target a broad spectrum of cytokines, and are a safe and effective treatment for immune-mediated disorders, such as IBD. As stated previously the JAK-STAT pathway plays an important role in innate and adaptive immunity, cell growth, survival, differentiation, and migration;

hence, there are concerns of potential off-target effects. However, the safety profile to date is similar to other biological agents (Winthrop, 2017). Selectivity of the new JAK inhibitors may improve safety, while maintaining efficacy. The development of drugs such as TD-1473, with action limited to the gastrointestinal tract and less systemic exposure, may also improve safety.

In cases of refractory illness, an emergent idea is the combined use of drugs that target distinct pathways, such as inhibitors of kinase PI3K or receptor tyrosine kinases (RTKs) (Montor et al., 2018).

Signal transducers and activators of transcriptions do not have intrinsic catalytic activity unlike JAK and RTKs, whose kinase domains are an obvious therapeutic target. A potential and attractive approach is the inhibition of STAT using oligonucleotides, which would sequester STAT away from the nucleus. Small molecules (SMs), inhibitory peptides, and siRNAs that target STATs are also undergoing clinical trials for other diseases (Villarino et al., 2015; ClinicalTrials.gov, ClinicalTrials, 2018b).

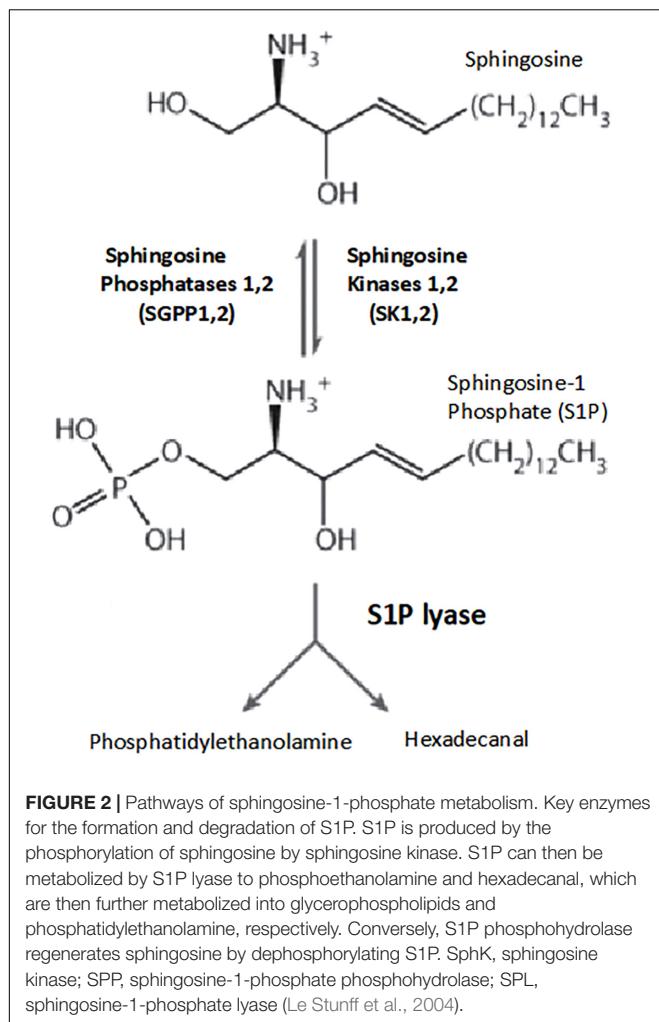
The relative risk and benefits of these drugs as monotherapy, combination, or sequential therapy with other drugs remain incompletely characterized (Barroso et al., 2017).

S1P/S1PR Targeting

Sphingosine-1-phosphate (S1P) is a sphingosine-derived phospholipid that binds to 5 G-protein-coupled receptors (S1PR1-5) (Park and Im, 2017). The S1P receptors are involved in several physiological events and cellular processes, such as adhesion, migration, lymphocyte/hematopoietic cell trafficking, endocytosis, vascular tone and permeability, embryogenesis, angiogenesis, and cardiac function (Sanchez and Hla, 2004; Gonzalez-Cabrera et al., 2014).

Sphingolipids are important elements in the structure of cell membrane, and S1P is a sphingolipid metabolite derived from sphingosine. S1P is phosphorylated by sphingosine kinases 1 and 2, and reversibly dephosphorylated by sphingosine phosphatases 1,2 and by the action of S1P lyase, S1P is irreversibly degraded (Le Stunff et al., 2004; Figure 2).

Sphingosine-1-phosphate/S1PR1 interactions are relevant for lymphocyte trafficking through the thymus, secondary lymphoid organs, circulation, and tissues. S1P mediates the traffic of dendritic cells, B cells, and T cells (naive and central memory-CCR7-positive), but does not have a significant role in the chemotaxis of effector memory CCR7-negative T cells, which maintain tissue immune-surveillance (Abbas et al., 2014a; Perez-Jeldres et al., 2018). The S1P lyase distribution, higher in tissues but absent in the vasculature, favors an S1P concentration gradient between the blood (higher levels), lymph, secondary lymphoid organs, and tissues (lower levels), determining the movement from the areas with low concentration to high S1P concentration (Abbas et al., 2014a; Perez-Jeldres et al., 2018). Elevated S1P levels in blood induce S1PR1 internalization, whereas in the lymph node and tissues S1PR1 is re-expressed after some hours, and during this time the T cell is able to interact with antigen-presenting cells (Abbas et al., 2014a; Perez-Jeldres et al., 2018). Once S1PR1 reappears on the surface of lymphocyte, these cells can leave the lymph node or tissue by



sensing the higher S1P concentration in the blood, determining immune cell egress into the circulation (Horga and Montalban, 2008; Olivera et al., 2016).

Mechanisms of Action of S1PR Modulators

The native ligand S1P induces internalization of S1PR, which are recycled back to the cell surface within several hours, achieving a transitory lymphopenia (Park and Im, 2017). By contrast, S1PR1 agonists lead to the internalization of the receptor and subsequent ubiquitination and proteasome degradation of the receptor, producing sustained lymphopenia that renders lymphocytes incapable of following the S1P gradient and exiting the lymph node. This sequestration potentially prevents their access to sites of inflammation (Abbas et al., 2014a; Park and Im, 2017; Pérez-Jeldres et al., 2018). In addition, S1PR1 is strongly expressed by lymphatic endothelium, where it tightens the lymphatic endothelial barrier. S1PR1 agonists can therefore interfere with lymphocyte trafficking by inhibiting transendothelial migration and blocking lymphocyte egress from the lymph node. These

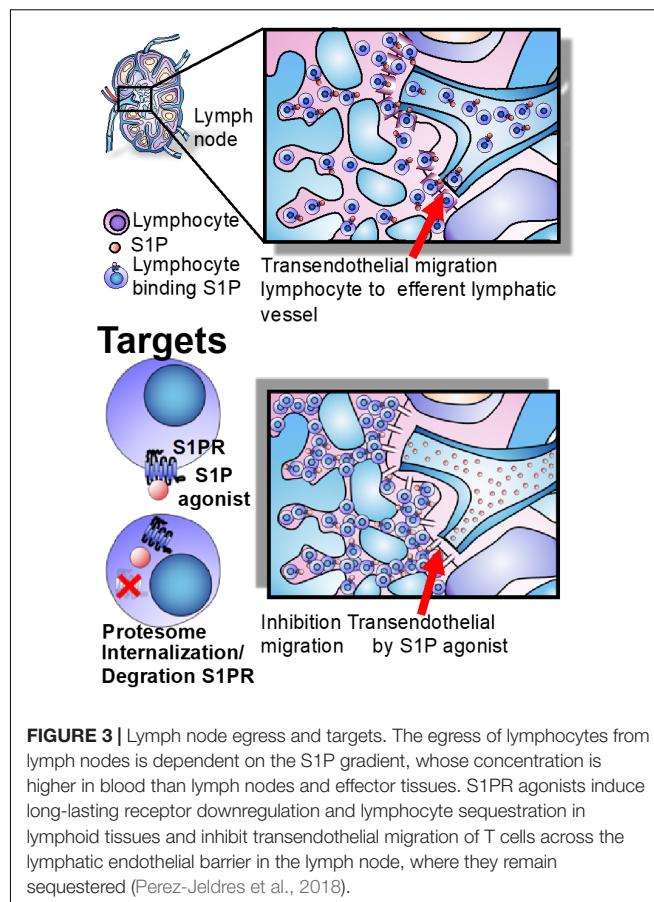


FIGURE 3 | Lymph node egress and targets. The egress of lymphocytes from lymph nodes is dependent on the S1P gradient, whose concentration is higher in blood than lymph nodes and effector tissues. S1PR agonists induce long-lasting receptor downregulation and lymphocyte sequestration in lymphoid tissues and inhibit transendothelial migration of T cells across the lymphatic endothelial barrier in the lymph node, where they remain sequestered (Pérez-Jeldres et al., 2018).

effects are reversed upon withdrawal of the agent (Horga and Montalban, 2008; Pérez-Jeldres et al., 2018; **Figure 3**).

Sphingosine-1-phosphate signaling is involved in multiple immune functions. Therapies targeting the S1P axis may be applicable to treat autoimmune/immune-mediated diseases and have been tested in MS, RA, SLE, psoriasis, and IBD (Pérez-Jeldres et al., 2018).

S1PR Modulators in IBD

Fingolimod/FTY720 (GilenyaTM) is an S1P-analog, acting as non-selective potent agonist of S1PR1,3,4,5. The first S1PR modulator approved for the treatment of relapsing MS was fingolimod (Currò et al., 2017; Peyrin-Biroulet et al., 2017a).

Various preclinical studies have demonstrated its efficacy at ameliorating colitis in animal models of IBD. Treatment of IL-10 knockout mice for 4 weeks efficiently reduced the number of CD4+ T cells in the colonic lamina propria and decreased the production of IFN-γ in the colon (Mizushima et al., 2004; Huwiler and Zangemeister-Wittke, 2018). Similar data were reported with other colitis models, such as dextran sulfate sodium (DSS), trinitrobenzene sulfonic acid, and T cell transfer into immunocompromised mice (Deguchi et al., 2006; Daniel et al., 2007; Radi et al., 2011; Montrose et al., 2013; Huwiler and Zangemeister-Wittke, 2018). The clinical use of fingolimod in IBD has not been tested, and other, more selective,

S1PR modulators are being developed for clinical use in IBD (Carrò et al., 2017).

KRP-203 (NovartisTM) is a S1PR1,4,5 agonist and partial agonist of S1PR3. The safety, tolerance, and efficacy of KRP203 were tested in 27 patients with active moderate UC, in a multicenter, double-blind, placebo-controlled study (Radeke et al., 2016; Perez-Jeldres et al., 2018). KRP203 demonstrated adequate tolerance and safety. While KRP203 was shown to be minimally effective with regards to the clinically relevant threshold (novel Bayesian trial design), a 14% in the KRP203 group achieved clinical remission in comparison a 0% in the placebo group (Radeke et al., 2016; Perez-Jeldres et al., 2018). Based on the results of this small study, further development of KRP-203 for ulcerative colitis (UC) was terminated (ClinicalTrials.gov, ClinicalTrials, 2018a).

Ozanimod/RPC1063 (CelgeneTM) is a S1PR agonist, with enhanced selectivity for S1PR1 and S1PR5 (Perez-Jeldres et al., 2018). Ozanimod is metabolized in humans to form one major active metabolite (CC-112273) and other minor active metabolites (RP101988 and RP101075). CC-112273 is responsible for much of the total activity of ozanimod in human with similar potency and selectivity to ozanimod to S1P1 and S1P5 (Scott et al., 2016). Ozanimod is eliminated primarily via biotransformation, followed by biliary excretion. Renal excretion is limited (Hemperly et al., 2018). Its half-life is 19 h, thus upon drug discontinuation, lymphocyte counts return to normal within 72 h (Scott et al., 2016; Hemperly et al., 2018; seekingalpha.com, 2018). However, new data show that the effect could be prolonged by the metabolite CC112273, which has a long 10–13 day half-life, reducing the competitive advantage of ozanimod on the key safety feature of the lymphocyte recovery profile (Scott et al., 2016; Hemperly et al., 2018; seekingalpha.com, 2018). Currently, there is an ongoing phase 1, randomized, parallel-group, open-label study to evaluate the effect of the modulators of the cytochrome P450 (CYP) 2C8 and/or 3A on the single-dose pharmacokinetics of ozanimod and CC112273 in healthy adult subjects (Scott et al., 2016; ClinicalTrials.gov, ClinicalTrials, 2018r; seekingalpha.com, 2018). The TOUCHSTONE phase 2 trial randomized 197 adults with moderate-to-severe UC to either ozanimod 0.5 or 1 mg or placebo daily for up to 32 weeks (Sandborn et al., 2016; White et al., 2018). After 8 weeks induction, 13.8 and 16.4% of patients (0.5 and 1 mg, respectively) reported clinical remission, versus 6.2% in the placebo group. At the same time, clinical response rates were achieved in 57, 54, and 37% for 1, 0.5, and placebo groups, respectively. Mucosal improvement/healing was observed in approximately 30% of the patients treated in each dose group in comparison with 12% in the placebo group. Moreover, at 32 weeks there was an observed improvement in the rates of clinical remission (21, 26, and 6% for 1, 0.5, and placebo, respectively), and 51% of the patients treated with 1 mg had a clinical response, versus 35 and 20% in the groups treated with 0.5 mg and placebo, respectively. Mucosal improvement/healing did not show major differences in comparison with 8 weeks. Ozanimod treatment (1 mg/kg dose) was associated with a greater proportion of histological remission (defined as a Geboes score ≤ 2) at both 8 and 32 weeks

(Sandborn et al., 2016). The long-term follow-up of patients involved in TOUCHSTONE study demonstrated that ozanimod was safe, effective, and well tolerated (Sandborn et al., 2016; White et al., 2018).

Initial results of a phase 2, open-label study in 69 patients treated with ozanimod for moderate-to-severe CD demonstrated a meaningful clinical improvement at week 4 and endoscopic improvement at week 12 (Feagan et al., 2017). Phase 3 studies investigating the role of ozanimod in IBD are in progress (White et al., 2018).

Etrasimod/APD334 (ArenaTM) is a S1PR1,4,5 selective agonist. Preliminary data from phase 2 OASIS trial in moderate-to-severe UC were reported (prnewswire.com, 2018). The primary objective, defined as an improvement in 3-component Mayo Clinic Score (stool frequency, rectal bleeding, and endoscopy), was met at 12 weeks. In addition, the 2 mg group achieved significant endoscopic improvement compared with placebo (41.8 versus 17.8%), and also the clinical remission was significant in the 2 mg group compared with placebo (24.5 versus 6.0%). Etrasimod was well tolerated and few patients had serious adverse events (SAEs). Arena plans to start a Phase 3 trial for UC (prnewswire.com, 2018).

Amiselimod/MT-1303 (Mitsubishi Tanabe Pharma CorporationTM) is an oral selective S1PR1,5 receptor developed for the therapy of autoimmune/immune-mediated disorders. The efficacy and safety of MT-1303 were studied in a phase 2 trial in CD, but the results have not yet been published (ClinicalTrials.gov, ClinicalTrials, 2018c,d). Amiselimod was also investigated for UC, MS, and other immune-mediated diseases; however, its development was discontinued (Peyrin-Biroulet et al., 2017a).

Safety and Adverse Events

Infections

In general, S1PR modulators maintain immune surveillance against pathogens because their effects on effector memory T cell traffic are limited (Perez-Jeldres et al., 2018). However, serious infections, such as disseminated varicella zoster and herpes are rare, but have been reported with fingolimod (Pelletier and Hafler, 2012). In post-marketing surveillance studies, there have been cases of progressive multifocal leukoencephalopathy (PML) and cryptococcal meningitis with the treatment of fingolimod, and without previous use of natalizumab (Olivera et al., 2016; fda.gov, 2018). However, it is necessary to emphasize that the risk to develop PML is low with fingolimod in the absence of prior natalizumab treatment. It is estimated that the risk with fingolimod use is less 1:10,000 patients. The Novartis safety database has identified 15 cases of PML with the use of fingolimod in monotherapy, as of August 2017 (Berger et al., 2018).

Cardiovascular Events

Reported cardiovascular events include transient bradycardia, atrioventricular block, and hypertension with the fingolimod use (Olivera et al., 2016; Sandborn et al., 2016). These side effects are attributed to S1PR2 and S1PR3 modulation. The development of selective S1PR1 modulators could theoretically bypass these side effects. However, S1PR1

is found in atrial cardiomyocytes, leading to a dose-dependent reduction in heart rate. In the TOUCHSTONE trial, a patient with preexisting bradycardia developed an asymptomatic, transient bradycardia, and first-degree AV block. The episode was self-limited without the need for treatment. These side effects could be minimized with a gradual dose titration regimen (Olivera et al., 2016; Sandborn et al., 2016).

Malignancy

Isolated cases of breast and skin cancer have been identified (Pelletier and Hafler, 2012). Squamous-cell carcinoma of the skin was reported in the TOUCHSTONE trial in one patient on 1 mg of ozanimod. This patient had also received mercaptopurine for more than 2 years (Sandborn et al., 2016).

Leukopenia

A dose-dependent and sustained decrease in lymphocyte count has been reported, consistent with the drug's MOA. However, it is reversible with drug discontinuation (Peyrin-Biroulet et al., 2017b).

Pregnancy

The teratogenicity risk of this group of drugs is unknown, so it is not recommended for use during pregnancy (Scott et al., 2016).

Others Adverse Events

Pulmonary disorders, elevated liver enzymes, and macular edema have been reported (Olivera et al., 2016).

Future Directions for the S1P Pathway

Ponesimod, Ceralifimod, Siponimod AUY954, SEW2871, AUY954, W061, CS-0777, and GSK2018682 are currently being investigated for use in other autoimmune/immune-mediated disorders (Park and Im, 2017). The pathways involved in the synthesis, degradation, and the mechanism of transport of these molecules represent an attractive new area of research (Perez-Jeldres et al., 2018).

Sphingosine Kinases

There are two isoforms of sphingosine kinase (SphK), SphK1 and SphK2. TNF induces SphK1 activation, leading to cyclooxygenase-2 (COX-2) expression and production of prostaglandin E2 (PGE2) that may contribute to mucosal inflammation (Pettus et al., 2003). Moreover, SphK1 expression was found to be elevated in both colonic epithelial cells and inflammatory cells in patients with UC patients correlating with COX2 overexpression (Snider et al., 2009). Data from mice indicate that the SphK1/S1P pathway participates in the development and maintenance of intestinal inflammation (Snider et al., 2009; Wollny et al., 2017). Thus, inhibition of this enzyme could represent a potential new target.

Sphingosine Phosphatase

This enzyme, expressed in the gastrointestinal tract, catalyzes dephosphorylation of S1P to sphingosine, resulting in regulation

of S1P levels. Elevated sphingosine phosphatase expression has been demonstrated in colitis and contributes to its pathogenesis by disrupting barrier integrity, indicating that its inhibition may have beneficial effects in IBD (Huang et al., 2016).

S1P Lyase

Sphingosine-1-phosphate lyase degrades S1P irreversibly. This enzyme is abundant in tissues (Wollny et al., 2017), maintaining low levels S1P in the colonic mucosa in relation with the blood. This favors lymphocyte recirculation from the intestine back into circulation. Its inhibition may impair intestinal lymphocyte egress, but its effect still remains unclear with evidence that shows amelioration of DSS colitis, while other studies show worsening disease (Degagné et al., 2014; Shirakabe et al., 2018).

Spinster Homolog 2

The expression of this intra- and extracellular S1P transporter is upregulated in patients with IBD. Thus, it may represent another way to regulate S1P levels for therapeutic purposes (Miklossy et al., 2013).

Positioning of Small Molecules in the Therapeutic Algorithm of IBD

The choice of IBD treatment must be personalized according to the activity, severity, phenotype, preferences of the patients, comorbidities, history of the therapies used previously, and surgery (Kornbluth and Sachar, 2010; Panes and Alfaro, 2017; Lichtenstein et al., 2018).

The current treatment for IBD is based on aminosalicylates, steroids, immunosuppressants, and biologic therapies (Kornbluth and Sachar, 2010; Panes and Alfaro, 2017; Lichtenstein et al., 2018). The 5-ASA compounds are used as first line in mild-to-moderate UC, and in some cases of IBD-associated arthritis (sulfasalazine). These drugs have an excellent safety profile. Immunosuppressants can be added during maintenance therapy in cases of moderate severity, or in combination with biologic therapy in moderate-to-severe cases due to their synergism or to decrease the immunogenicity of the biologic (Kornbluth and Sachar, 2010; Panes and Alfaro, 2017; Lichtenstein et al., 2018). In recent years, measuring drug and antibody levels has allowed optimization of biological therapies and assisted in avoiding misuse of biologics by under dosing or drug failure (absence of response despite adequate drug level) (Kornbluth and Sachar, 2010; Panes and Alfaro, 2017; Lichtenstein et al., 2018). The calcineurin inhibitors have a limited role in the treatment due their narrow therapeutic window and side effects. Thus, they are mostly being used as a bridge to another maintenance drug in cases of acute severe colitis refractory to corticosteroid. However, in this last case infliximab seems to be a better option, due to less toxicity in comparison with cyclosporine (Kornbluth and Sachar, 2010; Panes and Alfaro, 2017; Lichtenstein et al., 2018).

New SM offers an alternative to the current therapeutic arsenal, especially in cases of steroid-resistance and cases of nonresponse and/or are intolerance to conventional therapies.

Precise positioning the new small drugs in the therapeutic armament for IBD is difficult in the absence of head-to-head randomized controlled trials. The SM have a role in the treatment of moderate-to-severe IBD due to the lack of immunogenicity, and potential intermittent “on-off” dosing without resultant antibody formation and loss of response.

Most information available is for tofacitinib, approved for UC moderate-to-severe active, being a good option in cases refractory to anti-TNFα. Its effectiveness in comparison with anti-TNF as first-line therapy in moderate-to-severe UC, their use as combination therapy for example with other drugs as vedolizumab, its sequential use with other drugs (for example, induction with tofacitinib, followed by vedolizumab), or even it uses in acute severe colitis refractory to steroids, must also be evaluated in clinical trials, before authoritative consensus recommendations. In the absence of head-to-head comparisons, the evidence favors the use of infliximab in hospitalized patients with acute severe colitis in perianal disease.

Furthermore, it is important to consider tofacitinib's safety profile and may be premature recommend its use in combination with immunomodulators, anti-TNFα, and/or cyclosporine, until additional safety information is available.

Improved knowledge of the mechanisms regulating disease, by genome sequencing analysis, improved comprehension of the immunological pathways, and further understanding of the role of the microbiome, may lead to new targets. In fact, it is possible that future therapies will be chosen not only by considering traditional patient characteristics, but also based on the patient's microbiome and immune

genotype, as well as predictive modeling of drug responses validated prospectively.

CONCLUSION

Novel, orally available drugs represent a new and exciting option in the IBD therapeutic arsenal, showing efficacy and reasonable safety. However, more studies are required to define their safety related to infection, malignancy, and pregnancy. One of the clear advantages of SMs is their lack of immunogenicity and their short half-life which represents an advantage when adverse events may mandate interruption of therapy. Other advantages include the administration route, maintenance of T cell effector memory response, potentially lower manufacturing cost, and finally, the new agents are more receptor-specific (Perez-Jeldres et al., 2018). The positioning of these new drugs with relation to existing treatment paradigm remains uncertain.

AUTHOR CONTRIBUTIONS

JR-N, TP-J, WS conception of work. JR-N, TP-J design of work. JR-N, TP-J drafting of manuscript. JR-N, WS, DP, AY, DG, JB, TK, CT, SY, and LL critical revision of manuscript. JR-N, TP-J, WS, DP, AY, JB, TK, CT, DG, SY, and LL final approval of work. JR-N, TP-J, WS, DP, AY, JB, TK, CT, DG, LL, and SY agreement to be accountable for all aspects of presented work.

FUNDING

This work was funded by grants from the National Institutes of Health (DK108670) and VA Merit grant (BLRD-I01 BX003436).

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- Conflict of Interest Statement:** WS reports research grants from Atlantic Healthcare Limited, Amgen, Genentech, Gilead Sciences, Abbvie, Janssen, Takeda, Lilly, Celgene/Receptos; consulting fees from Abbvie, Allergan, Amgen, Boehringer Ingelheim, Celgene, Conatus, Cosmo, Escalier Biosciences, Ferring, Genentech, Gilead, Gossamer Bio, Janssen, Lilly, Miraca Life Sciences, Nivalis Therapeutics, Novartis Nutrition Science Partners, Oppilan Pharma, Otsuka, Paul Hastings, Pfizer, Precision IBD, Progenity, Prometheus Laboratories, Ritter Pharmaceuticals, Roberts Clinical Trials (owned by Health Academic Research Trust or HART), Salix, Shire, Seres Therapeutics, Sigmoid Biotechnologies, Takeda, Tigenix, Tillotts Pharma, UCB Pharma, Vivelix; and stock options from Ritter Pharmaceuticals, Oppilan Pharma, Escalier Biosciences, Gossamer Bio, Precision IBD, Progenity. AJ-R is consultant and part of the speaker bureau for Prometheus Laboratories and employee of Prometheus Laboratories. DP has been a speaker bureau ABBVIE. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.
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Roussel Uclaf Causality Assessment Method for Drug-Induced Liver Injury: Present and Future

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OPEN ACCESS

Edited by:

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University of Naples Federico II,
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Reviewed by:

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Specialty section:

This article was submitted to
Gastrointestinal and Hepatic
Pharmacology,
a section of the journal
Frontiers in Pharmacology

Received: 14 May 2019

Accepted: 04 July 2019

Published: 29 July 2019

Citation:

Danan G and Teschke R (2019)
Roussel Uclaf Causality Assessment
Method for Drug-Induced Liver Injury:
Present and Future.
Front. Pharmacol. 10:853.
doi: 10.3389/fphar.2019.00853

Among the causality assessment methods used for the diagnosis of drug-induced liver injury (DILI), Roussel Uclaf Causality Assessment Method (RUCAM) remains the most widely used not only for individual cases but also for prospective and retrospective studies worldwide. This first place is justified by the characteristics of the method such as precise definition and classification of the liver injury, which determines the right scale in the scoring system, precise definition of the seven criteria, and the validation approach based on cases with positive rechallenge. RUCAM is used not only for any types of drugs but also for herbal medicines causing herb-induced liver injury, (HILI) and dietary supplements. In 2016, the updated RUCAM provided further specifications of criteria and instructions to improve interobserver variability. Although this method was criticized for criteria such as the age and alcohol consumption, recent consensus meeting of experts has recognized their value and recommended their incorporation into any method. While early studies searching for DILI in large databases especially in electronic medical records were based on codes of diseases or natural language without causality assessment, the recommendation is now to include RUCAM in the search for DILI/HILI. There are still studies on DILI detection or the identification of biomarkers that take into consideration the cases assessed as "possible," although it is well known that these cases reduce the strength of the association between the cases and the offending compound or the new biomarker to be validated. Attempts to build electronic RUCAM or automatized application of this method were successful despite some weaknesses to be corrected. In the future, more reflections are needed on an expert system to standardize the exclusion of alternative causes according to the clinical context. Education and training on RUCAM should be encouraged to improve the results of the studies and the day-to-day work in pharmacovigilance departments in companies or in regulatory agencies. It is also expected to improve RUCAM with biomarkers or other criteria provided that the validation process replaces expert opinion by robust standards such as those used for the original method.

Keywords: pharmacovigilance, DILI, drug-induced liver injury, RUCAM, Roussel Uclaf Causality Assessment Method, prospective studies

INTRODUCTION

The diagnosis of drug-induced liver injury (DILI) as any other disease needs to be supported by strong criteria. Until 1993, the main approach was the global introspection, also called expert opinion (EO), with unstructured arguments, absence of score, and no validation or general methods used in pharmacovigilance for any adverse drug reaction. A more objective method was needed and accepted by Council for International Organizations of Medical Sciences (CIOMS) as a topic for consensus meetings organized in 1990 and 1991 (references in Danan and Teschke, 2016). Later on, the Roussel Uclaf Causality Assessment Method (RUCAM) was created and validated with positive rechallenge cases (reference in Danan and Teschke, 2016), but some confusion remains on its name. This came from insufficient reading or misunderstanding of the original article. RUCAM was partly built on the results of consensus meetings, but many criteria were added and the scoring system was established and validated by the Roussel Uclaf team only. CIOMS did not want to endorse RUCAM because the method was not fully established by the members of the consensus meetings. RUCAM should therefore be named as such and not after CIOMS. However, even in a guideline on DILI (Andrade et al., 2019), the name of the method was different according to the chapters. Despite a slow start between 1993 and early 2000s, RUCAM became the most widely used method to support DILI diagnosis in different settings that prompted an updated version to further improve the results (Danan and Teschke, 2016). Several reasons concurred to this situation (Danan and Teschke, 2018): definition and classification of a liver injury, precise criteria, a scoring system, and the validation approach of the original method. RUCAM is now used not only for the diagnosis of DILI in individual cases, case series, registries, or epidemiological studies involving any types of drugs, herbal medicines, or dietary supplements but also as automated RUCAM and other settings such as the modern approach of searching for DILI in electronic medical records (EMRs).

The objectives of this article are twofold: first, to describe, comment on, and highlight how the current utilization of RUCAM can be improved and, second, to consider the future applications of RUCAM beyond individual cases to detect hepatotoxicity of any types of compounds administered to humans.

LITERATURE SEARCH AND DATA REVIEW

Published reports were systematically searched in electronic databases of Medline (source PubMed) from 2014 using the search terms: RUCAM, Roussel Causality Assessment Method, DILI, drug-induced liver injury, pharmacovigilance, and CIOMS. From each searched segment, the publications of the first 30 hits were analyzed and selected for reports in English language. Before the final analysis, the publications were assessed regarding the clinical quality and data completeness. The search was completed on 4 April 2019.

CURRENT RUCAM USE

Why and Where RUCAM Is Used

RUCAM is fairly unique and should be seen in the context of other CAMs to be used in suspected DILI cases and described with their weaknesses (Teschke and Danan, 2018a). In short, to assess DILI, the least is to consider CAMs that are liver specific. Indeed, the general CAMs as proposed by WHO UMC or Naranjo (NAR) are designed to assess any adverse drug reactions, but specific timing, precise definition of dechallenge or rechallenge, list of alternative causes to exclude, and a scoring system are lacking. Furthermore, these methods were not validated or validated against an unstructured opinion. None of them was found better than liver-specific methods. Except the global introspection used by the Drug-Induced Liver Injury Network (DILIN) in the USA (Hayashi et al., 2015), the liver-specific CAMs directly derive from RUCAM, either with some changes to “simplify” RUCAM such as Maria and Victorino (MV) method or to add a controversial diagnostic laboratory test (Lymphoblastic transformation test) as a criterion in the DDW-J only used in Japan by few experts (Das et al., 2018). The strength of RUCAM comes from the precise definition of the criteria and the validation method based on cases with positive rechallenge. RUCAM has been conceived as a step-by-step method similar to a diagnostic approach. It was therefore important to define a liver injury to start off the process and to classify the liver injury because the time to onset and the time course of the biochemical markers are not identical for hepatocellular injury and a cholestatic/mixed liver injury. The causality assessment criteria and the time course of the usual biomarkers for hepatocellular and cholestatic/mixed liver injury are different as indicated in RUCAM. The details are given in the updated RUCAM (Danan and Teschke, 2016) as well as in the work instructions (electronic supplementary material in Danan and Teschke, 2018). Efforts in Europe brought the fascinating topic of DILI back to the roots by consolidating DILI-related science (Björnsson, 2014; Björnsson, 2016; Danan and Teschke, 2016; Andrade et al., 2016; Danan and Teschke, 2018), where DILI was early considered as a challenging disease requiring robust causality assessment method such as RUCAM. This method was used in many prospective DILI studies in Europe and contributed to evidence-based characterization of DILI features. Much support for deep analysis of DILI cases and causality assessment by the updated RUCAM was provided by recognized experts in DILI (Sarges et al., 2016; Shahbaz et al., 2018; Real et al., 2019). Despite strong recommendations, reviews and reports on DILI characteristics are still published each year based on a simple opinion of the author(s) and accepted by reviewers leading to the impression that DILI is not seriously taken. In 2016, a list of DILI cases with validated DILI diagnoses was published, providing a large number of cases published by authors worldwide (Danan and Teschke, 2016). Additional reports were published in many countries, including the USA (Cheetham et al., 2014) and China where RUCAM is included in the national guidelines on DILI (Yu et al., 2017; Shen et al., 2019). Until early 2019, the total number of RUCAM-based DILI cases reported since 1993 is close to 50,000

cases ranking RUCAM as the most commonly used CAM in DILI (Teschke, 2019, *in press*). Beyond chemical drugs RUCAM has been used for other compounds such as herbal medicines or dietary supplements (Teschke and Eickhoff, 2015; Teschke et al., 2016; Zhu et al., 2016).

RUCAM in Clinical Settings

In clinics, for general practitioners or specialists confronted with ALT elevation with or without jaundice but without obvious cause, one of the most frequent issue is to diagnose a DILI. As shown in the UK between 1998 and 2014, the most frequent cause of jaundice and ALT above 400 IU/l on 1,000 consecutive patients in clinics in elderly patients is DILI (Vine and Dalton, 2015). Here, RUCAM is helpful to follow a step-by-step approach, collect prospectively the relevant data, score each suspected drug/herbal medicine, and finally come up with a diagnosis based on transparent criteria and score that allow for a reassessment by colleagues. This approach is supported by independent teams in many countries (Kullak-Ublick et al., 2017). For each patient where DILI is suspected, a RUCAM work sheet should be systematically completed and be part of the patient file.

Clinical trials should not be excluded from the RUCAM use as it was wrongly suggested (Regev et al., 2014). There is no argument to reject RUCAM in clinical trials but just the opposite. Indeed, in this context, it is usual to collect specific and relevant data, and normally, the suspected DILI cases are reported on ongoing basis provided that the flow chart for the management of ALT elevation is available for the investigators and the monitors. It is argued that RUCAM is not adapted to clinical trials although the criteria for causality assessment do not differ qualitatively and quantitatively from the postmarketing setting. The data collected for each case are best used with RUCAM to achieve a correct diagnosis of DILI as shown in a study using a RUCAM-based automated method applied to a dataset from clinical trials (Scalfaro et al., 2017). Moreover, the RUCAM criteria can serve to reach consensus in case of difficult and critical situation in drug development (Teschke and Danan, 2016).

RUCAM has been criticized for the tendency to lower the final score in case of death or liver transplantation due to acute liver failure since the dechallenge is not assessable. This is also true for any CAMs, including EO, but with RUCAM, the exclusion of the alternative causes, if done properly, will increase the final score of the suspect drug. In the case of multiple drugs, RUCAM allows for ranking the suspect drugs. If the drug is indispensable and after a consensus of experts, there is sometimes no other solution than to perform a drug rechallenge test and assess the results according to the strict criteria as provided in RUCAM (Danan and Teschke, 2016; Danan and Teschke, 2018).

Automated RUCAM

As an algorithm with a scoring system, it was tempting to automatize RUCAM. The incorporation of the updated RUCAM in an electronic program would accelerate the evaluation process of large case numbers and likely reduces interrater variability. At the same time, transparency of the evaluation with RUCAM is further enhanced. This was performed by an expert team in DILI to make

the causality assessment independent from individuals (Cheetham et al., 2014). This attempt was a success, with a high agreement between the automatized RUCAM and manual RUCAM scoring. However, the exclusion of alternative causes is recognized as a difficult criterion that should take into consideration the clinical context (Teschke and Danan, 2018b). In the future, a sort of expert system should be built to support this criterion. In addition, the authors rightly suggested to include automated RUCAM into EMRs for the detection of DILI in hospital-based population.

To facilitate case evaluation of suspected DILI in routine pharmacovigilance, an algorithm was proposed based on RUCAM with many supportive tables and named Pharmacovigilance-Roussel Uclaf Causality Assessment Method (PV-RUCAM) (Scalfaro et al., 2017). This project was prompted because, in spontaneous reports, data are usually incomplete and PV professionals are not necessarily experts in DILI. The performance of this method was compared in different settings with regard to its applicability and differentiation capacity. The score was applied in two datasets of individual case safety reports (ICSRs) extracted randomly from clinical trial reports and a third dataset of electronic health records from a global PV database. The results of PV-RUCAM were compared to the original RUCAM and EO and showed 100% sensitivity, 91% specificity, 25% positive predictive value, and 100% negative predictive value. Similar to the original reproducibility test of RUCAM, there was high inter-rater agreement ($Kw = 0.79$) between the two PV-RUCAM assessors. The authors concluded that, compared to other methods, PV-RUCAM is of great help in incomplete ICSRs assessed for DILI by nonexpert PV professionals. This attempt to build a RUCAM-based automated algorithm to be used in any pharmacovigilance department is encouraging and should trigger further research on RUCAM: first, prospective validation of this algorithm as proposed by the authors and, second, fully automatized RUCAM with a sophisticated expert system behind the algorithm to consider (almost) all the clinical situations.

Computerization of causality assessment method was also recently addressed (Tillmann et al., 2018) to decrease variability between raters and improve the results of a CAM. Additional criteria beyond those already included in RUCAM were proposed such as genetics, race, gender, or drug signatures, but the current knowledge on these factors is not strong enough in terms of sensitivity and specificity to be included and scored in any CAM. More prospective studies are needed to add new and validated criteria.

The use of automated RUCAM should be encouraged in prospective and retrospective studies especially in large databases with thousands of potential DILI cases, as they exist in the form of EMR in hospitals (Hunt, 2018) or national health insurance registries. However, it should be used concurrently with strict DILI definitions and verification of the diagnosis in a sample of positive and negative cases.

RUCAM in Databases, DILI Registries, and Epidemiological Studies

A study in the USA at Michigan University developed a novel text searching tool to identify DILI cases in EMR (Heidemann

et al., 2017). RUCAM and EO were applied to suspected DILI cases with good agreement between these methods despite the fact that, in very few cases, the authors were unable to use RUCAM because of missing data on the time to onset of the liver injury. This so-called limitation of RUCAM is not substantiated because it is always possible to assign the lowest score to this criterion in absence of information (Danan and Teschke, 2016). Furthermore, missing data are also an issue for the global introspection method, where it constitutes a limiting step unless assumptions are made that could also be used in RUCAM. A study in Japan using RUCAM and a Japanese method DDW-J to build an algorithm for DILI detection in a medical information database showed an expected agreement between the methods since DDW-J derives from RUCAM and called for further studies on DILI in large EMR database (Hanatani et al., 2014). A meta-analysis of algorithms to identify DILI in EMR (Tan et al., 2018) in 29 studies between 1993 and 2016 included causality assessment methods: EO in 16, RUCAM in 8 (starting in 2000), WHO in 1, RUCAM and WHO in 1, DDW-J in 1 and none in 2 studies. The positive predictive value of DILI detection algorithms calculated on 25 studies was low, ranging from 1.0 to 40.2%. These results were due to considerable variability in case definition of DILI, causality assessment methods, diagnostic codes, and study drugs. Interestingly, the authors concluded that DILI detection algorithms could be improved by the adoption of the internationally agreed DILI definition, the use of the RUCAM, the screening of high-risk drugs, and use of natural language processing and machine learning algorithms. This conclusion was supported by a thoughtful editorial accompanying this article suggesting clues to improve DILI detection algorithms such as drug–drug interactions, drug combinations, patient factors, herbals and dietary supplements that would account for 15% of DILI cases, and the environment (Hunt, 2018).

The first DILI registries were based on EO or general methods used in pharmacovigilance, but very early DILI definition and validated liver-specific CAM were needed to include cases. The adoption of internationally agreed definition of biochemical thresholds in ALT, AST, ALP, and bilirubin and liver-specific CAM improved considerably the reliability of the registries and any type of epidemiological studies. CIOMS then RUCAM introduced liver test thresholds and liver injury classification before causality assessment criteria. RUCAM with its algorithm and scoring system provide easy tools for epidemiologists to ensure homogeneity and reliability of the results. Despite this advance in searching for tools in DILI, the LiverTox database has not systematically used RUCAM but preferably EO to analyze the published cases. In a study based on the original and the updated RUCAM, the quality of a sample of the LiverTox DILI cases was assessed. As a result, some cases included in this database are likely not DILI due to insufficient data quality (Björnsson, 2016; Björnsson and Hoofnagle, 2016). Indeed, this issue was also discussed by others (Real et al., 2019; Teschke and Danan, 2018b) asking for improvements to continue to rely on LiverTox as a worldwide known and popular database on DILI characteristics.

RUCAM is widely used in epidemiological studies for the calculation of DILI incidence rates and to rank drugs according

to their hepatotoxicity. Interestingly, a study to estimate DILI incidence in EMR database jumped over CAMs to use only DILI definition and temporally related criteria (Shin et al., 2013). As the selected codes excluded alternative causes, it was thought that DILI cases were correctly assessed for causality. The diagnosis was based on the assumption that the codes were valid. This was not verified on a sample of cases and constitutes one of the weaknesses of this study. It would have been preferable to use RUCAM as strongly suggested to ascertain the diagnosis of DILI and would make the incidence rates, DILI characteristics, and other calculations more reliable (Hunt, 2018). Another example in a large hospital database is a case–control study designed to detect hepatotoxicity of new drugs and quantify the risk of DILI, RUCAM was used to search for cases and assess causality (Douros et al., 2014). Other RUCAM-based incidence studies performed before 2014 in various countries are summarized in a recent review (García-Cortés et al., 2018). Many epidemiological studies were based on RUCAM to identify DILI cases among which it is worth quoting: registries in Spain, Iceland, Latin America (Bessone et al., 2019), or in hospital data bases in Korea in 2012, Mainland China (Shen et al., 2019), Japan (Aiso et al., 2019), Thailand (Sobhonslidsuk et al., 2016), in a DILI cohort study for liver transplantation (Baekdal et al., 2017), and in a prospective cohort study in India (Rathi et al., 2017). The latter study is a good example that, when RUCAM is used prospectively, the identification of DILI cases as well as causality assessment is easier and more rigorous, and the results case by case are transparent (Teschke and Danan, 2017). Likewise, a prospective cohort study in a tertiary center for liver disease based on RUCAM and compared to studies in other countries (Licata et al., 2017) showed that drug classes involved in DILI are similar in many countries. Wider dissemination of the RUCAM is supported by authors after a study in Brazil, realizing that some epidemiological studies are still based on DILI cases on simple opinion without definition of criteria (Becker et al., 2019).

RUCAM for the Validation of Biomarkers and Risk Factors

To improve our knowledge on DILI, to detect hepatotoxicity of the new therapies such as immunotherapy, and to validate biomarkers and risk factors, high confidence in DILI cases is needed. It is therefore of the utmost importance to define DILI and use validated CAM such as RUCAM in studies testing new markers. Since RUCAM provides for each case the detail of the criteria and how the score has been reached, another expert will be able to reassess the cases. Although it has been repeatedly said that only “probable” (score 6–8) and “highly probable” (score >8) cases should be taken into consideration (Teschke et al., 2017), there are still studies incorporating “possible” cases into DILI cases (Russmann et al., 2014). It is frequently unknown whether these cases are “possible” due to insufficient data on alternative causes, as in retrospective studies, or to concomitant medications because they were given exactly at the same time as for the studied drug. Sometimes to increase the sensitivity of the test and sometimes by misunderstanding of the CAM, these “possible” cases are added to the positive cases. As a result, the

reliability of the association is reduced between the DILI cases and the suspected offending compounds whether it is a drug, an herbal medicine, or a dietary supplement, and the conclusion will not be as convincing as it should be. Genetic and serum biomarkers studies are examples where DILI cases should not suffer approximation in the diagnosis (Tao et al., 2018). The link is commonly weak with studied markers, and it is tempting to add “possible” cases to reach the significant threshold (Liu et al., 2018). This approach should always be refused by reviewers and editors who are confronted with borderline results. Same applies to published DILI cases on a new drug or case series on established drugs where the authors based the assessment on their own opinion and not on RUCAM. The reviewers, as experts, should pay attention to these cases that could enter in DILI databases only because they are published and not more rigorously on the basis of objective criteria and transparent method. Moreover, if the publication includes few cases, the detailed score of RUCAM has to be included in the article or in a supplementary material to ensure the transparency of the assessment. Despite these recommendations (Danan and Teschke, 2018), a number of articles are still published only on the basis of the author’s opinion preventing a reassessment by the reviewer and the editor.

Similarly, regulators should base their evaluation and decision on validated DILI cases assessed with RUCAM. Few national agencies require such assessment when drug hepatotoxicity is discussed after the first case(s) of acute liver injury in postmarketing or clinical trials in drug development. Usually, this risk is considered high enough, depending on the severity and the incidence of the cases, to induce significant changes in the risk benefit balance that could result in a better use of the drug to reduce the risk of acute liver injury or a drug withdrawal from the market. Hepatologists and, more precisely, DILI experts should include an assessment with RUCAM in the drug evaluation and if not done to justify their position. This would ensure transparency of the drug evaluation and the regulatory decision.

FUTURE USE OF RUCAM

Education

The most important step for using RUCAM is to be trained with the tool. The method is simple and user-friendly. The user should follow a stepwise approach, and it is assumed that each criterion is understood according to straightforward instructions given in the supplementary material of the following article (Danan and Teschke, 2018). One of the properties of RUCAM is flexibility to accommodate almost all clinical situations even though the minimum information is missing. Some case studies need to be assessed with a senior user to understand the concept. Nothing new for a hepatologist, but for the staff working in a PV department, the basic education in hepatology is needed. Indeed, understanding the liver tests, the definition and the types of liver injury, the main causes of an acute liver injury, and the need to search for alternative causes including herbal and dietary supplements are necessary to apply intelligently RUCAM. It is worth training one or two staff members in a team

to use RUCAM as the researchers do when a case series is to be identified in databases or to monitor a registry. The recent efforts to automatize RUCAM will be of considerable help.

RUCAM-Based DILI Cohorts for International Harmonization

Although RUCAM is occasionally used retrospectively on DILI cases, it is recommended to use it prospectively, as it constitutes a guide to collect relevant data to assess causality. The example of a prospective study in India shows high quality of the data and subsequent reliability of the results (Rathi et al., 2017). On the opposite, in retrospective studies, the authors struggle to obtain data for causality assessment. This is true not only for RUCAM but also for other CAMs. The particularity of RUCAM is to be available in a worksheet with all the criteria needed to calculate the final score of the suspected DILI case. It is therefore easier to ask on ongoing basis the investigator/reporter for specific data and complete the score. With RUCAM, retrospective or prospective studies involving DILI will benefit for a harmonized approach. The language for international DILI studies could be provided by RUCAM. It would be of great scientific interest to gather the cases from databases in different countries. This cooperation would increase the number of cases and controls so that the power of the detection would increase in parallel as well as the identification of risk factors such as obesity, nonalcoholic fatty liver, diabetes mellitus, HLA variants, and genetic and serum biomarkers. Together with the preceding rule on the incorporation of “probable” and “highly probable” cases only would make significant progress in our knowledge on DILI.

RUCAM Improvements

Responses to comments on the original version of RUCAM of 1993 (Hassan and Fontana, 2019) were provided and already implemented in the updated version (Danan and Teschke, 2016). Other comments were addressed in a summary table (Danan and Teschke, 2018) and in detailed work instructions (Electronic supplementary material in Danan and Teschke, 2018). The introduction of new biomarkers and inclusion into RUCAM were specifically discussed (Teschke et al., 2017) to focus on the method for the validation of new criteria. Another approach proposed by a DILI expert would be to combine RUCAM results with the DILI signature, i.e., the clinical and biochemical characteristics, of a specific drug as provided in articles or in specialized database such as LiverTox (Watkins, 2015). The weakness of this approach is to make sure that the DILI signature is correct and validated. Ongoing international initiatives are working on the modification or addition of criteria to RUCAM. The issue here is, first, the validation of the criteria and, second, the validation of the whole method after the changes. Instead of a qualitative approach of potential changes, it would be necessary to validate the modifications using a gold standard such as the one used to validate the original method with positive rechallenge cases. Although this validation method could be discussed, it has the value to be objective and reproducible and not to be based on EO, which is subjective by definition.

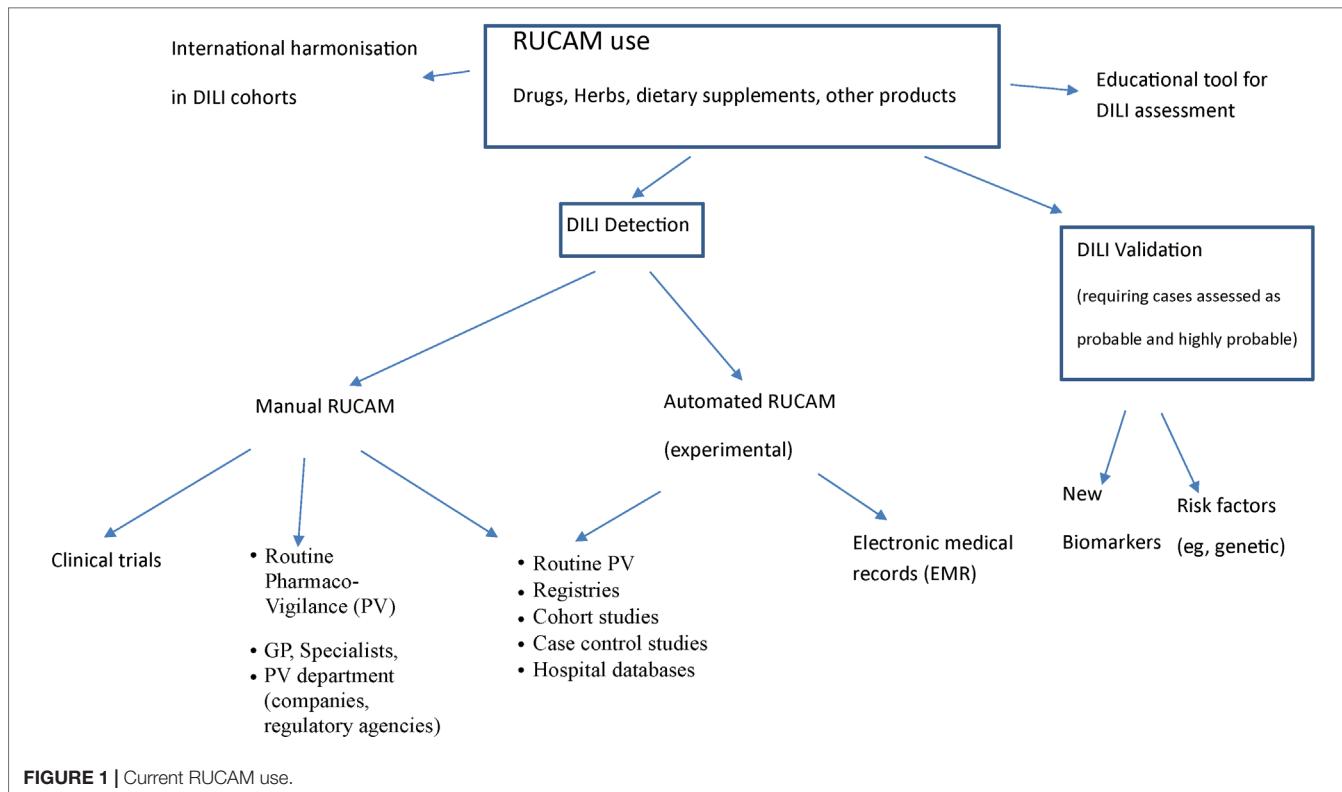


FIGURE 1 | Current RUCAM use.

CONCLUSION

The analysis of the current RUCAM use shows that the method is adapted to the clinical situations as well as to prospective and retrospective studies involving DILI or searching for DILI (**Figure 1**). The method is also used for herb-induced liver injury (HILI) and intoxications to follow a rigorous guide and exclude the alternative causes. RUCAM is a reliable tool for the harmonization of international studies and regulators to help make a decision based on objective criteria in case of drug hepatotoxicity. It was also shown that RUCAM is adapted to liver injury induced by herbs or dietary supplements, which account for an increasing proportion of liver injuries especially in Asian countries. In the future, more educational efforts should be made to introduce the method in training programs for PV experts in companies or national agencies. More studies on automated RUCAM are needed to facilitate the RUCAM use by nonexpert PV professionals and to build expert system to search for alternative causes according to the

clinical circumstances. Studies on DILI cases involving new therapies or biomarkers should take into consideration only the “probable” and “highly probable” cases assessed by RUCAM to be published or strengthen the association between DILI cases and the suspect drug/herb or the biomarker. More studies are warranted to search for RUCAM improvements by adding or modifying criteria, provided that validation process be based on robust standard, such as cases with positive rechallenge, as used for the original method. It is hoped that the use of RUCAM will further increase confidence on the studies performed for DILI detection with new drugs, herbs, and dietary supplements and the identification of risk factors and new biomarkers to be able to take the appropriate measures to reduce the risk of hepatotoxicity.

AUTHOR CONTRIBUTIONS

The authors equally contributed to the manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer JBS declared a shared affiliation, though no other collaboration, with one of the authors, RT, to the handling Editor.

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