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The 1,2,4-triazole as a scaffold for the design of ghrelin receptor ligands: development of JMV 2959, a potent antagonist

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Received: 15 May 2012 / Accepted: 27 June 2012 / Published online: 14 July 2012
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Abstract Ghrelin is a 28-residue peptide acylated with an *n*-octanoyl group on the Ser 3 residue, predominantly produced by the stomach. Ghrelin displays strong growth hormone (GH) releasing activity, which is mediated by the activation of the so-called GH secretagogue receptor type 1a (GHS-R1a). Given the wide spectrum of biological activities of Ghrelin in neuroendocrine and metabolic pathways, many research groups, including our group, developed synthetic peptide, and nonpeptide GHS-R1a ligands, acting as agonists, partial agonists, antagonists, or inverse agonists. In this highlight article, we will focus on the discovery of a GHS-R1a antagonist compound, JMV 2959, which has been extensively studied in different *in vitro* and *in vivo* models. We will first describe the peptidomimetic approach that led us to discover this compound. Then we will review the results obtained with this compound in different studies in the fields of food intake and obesity, addictive behaviors, hyperactivity and retinopathy.

Keywords Addiction · Antagonist · Anti-obesity · Ghrelin receptor · Peptidomimetic · 1,2,4-Triazole

Introduction

Ghrelin is a 28-residue peptide acylated with an *n*-octanoyl group on the Ser 3 residue and is predominantly produced by the stomach (Kojima et al. 1999) (Fig. 1).

Ghrelin displays strong growth hormone (GH)-releasing activity, which is mediated by the activation of the so-called GH secretagogue receptor type 1a (GHS-R1a) (Howard et al. 1996). GHS-R1a is concentrated in the hypothalamus–pituitary unit, but is also distributed in other central and peripheral tissues (Howard et al. 1996; Guan et al. 1997; Yokote et al. 1998; Gnanapavan et al. 2002; Dass et al. 2003). Indeed, apart from stimulating GH secretion, ghrelin exhibits hypothalamic activities that result in the stimulation of prolactin (PRL) and adrenocorticotrophic hormone (ACTH) secretion (Arvat et al. 2001; Broglio et al. 2003). It effects a negative feedback on the pituitary–gonadal axis (Furuta et al. 2001; Fernandez-Fernandez et al. 2004), stimulates appetite and a positive energy balance (Tschop et al. 2000; Wren et al. 2000; Inui 2001; Nakazato et al. 2001; Tschop et al. 2001; Akamizu et al. 2004), influences sleep and behavior (Asakawa et al. 2001a, b; Carlini et al. 2002, 2004; Tolle et al. 2002; Weikel et al. 2003), controls gastric motility and acid secretion (Masuda et al. 2000; Asakawa et al. 2001a, b; Fujino et al. 2003; Peeters 2003; Inui et al. 2004), modulates pancreatic exocrine and endocrine function, and affects glucose levels (Broglio et al. 2001, 2003; Adeghate and Ponery 2002; Date et al. 2002; Murata et al. 2002; Ukkola 2003; Salehi et al. 2004). Ghrelin has an influence on the cardiovascular system (Nagaya et al. 2001; Enomoto et al. 2003; Nagaya and Kangawa 2003) and has been shown to modulate the proliferation of tumor cells (Jeffery et al. 2002; Duxbury et al. 2003; Volante et al. 2003; Cassoni et al. 2004).

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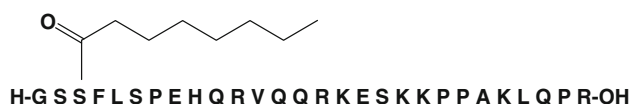


Fig. 1 Human Ghrelin

Given this wide spectrum of biological activities, it is evident that the discovery of ghrelin and its receptor has opened up many avenues for neuroendocrine and metabolic research. Furthermore, many research groups, including our group, developed synthetic peptide, and nonpeptide GHS-R1a ligands, acting as agonists, partial agonists, antagonists, or inverse agonists (Moulin et al. 2007b).

In this highlight article, we will focus on the discovery of a GHS-R1a antagonist compound, JMV 2959, which has been extensively studied in different in vitro and in vivo models.

We will first describe the process that led us to discover this compound, thanks to a peptidomimetic approach. Then we will review the results obtained with this compound in different studies in the fields of food intake and obesity, addictive behaviors, hyperactivity and retinopathy.

Development of JMV 2959: from peptide to peptidomimetics

First lead: EP 51389

The story started in our laboratory in collaboration with the Europeptides Company.

Starting from GHRP-6, the first synthetic growth hormone secretagogue active in vitro and in vivo discovered by Bowers in 1984 (Bowers et al. 1984), scientists from Mediolanum Farmaceutici synthesized an analog compound, named Hexarelin (Deghenghi et al. 1994), which differs with GHRP-6 by the introduction of (*D*)-2-methyltryptophan in place of (*D*)-tryptophan, which confers a better stability and a reduced toxicity to the molecule, compared to GHRP-6.

Starting from this result, Europeptides worked at the reduction of Hexarelin size to obtain compounds with a better oral bioavailability. Their studies led to the discovery of tripeptide EP 51389 (Deghenghi 1999). Collaboration started between Europeptides and our laboratory, with the aim to design an original family of growth hormone secretagogues derived from EP 51389 (Fig. 2).

From peptide to pseudo-peptide: JMV 1843 (ARD-07, AEZS-130)

Compound EP 51389 was modified at its C-terminal part: a gem-diamino tryptophane residue was incorporated, the amine function was then formylated to give compound JMV 1843 (Guerlavais et al. 2003) (Fig. 3).

The idea of introducing this modification was inspired by results obtained in our laboratory concerning studies on Bombesin analogs (Azay et al. unpublished results). Bombesin is a 14-residue peptide hormone from the large family of neuropeptides of the gastro-intestinal tract. Indeed, structure–activity relationship studies showed that the C-terminal nonapeptide of Bombesin was sufficient to

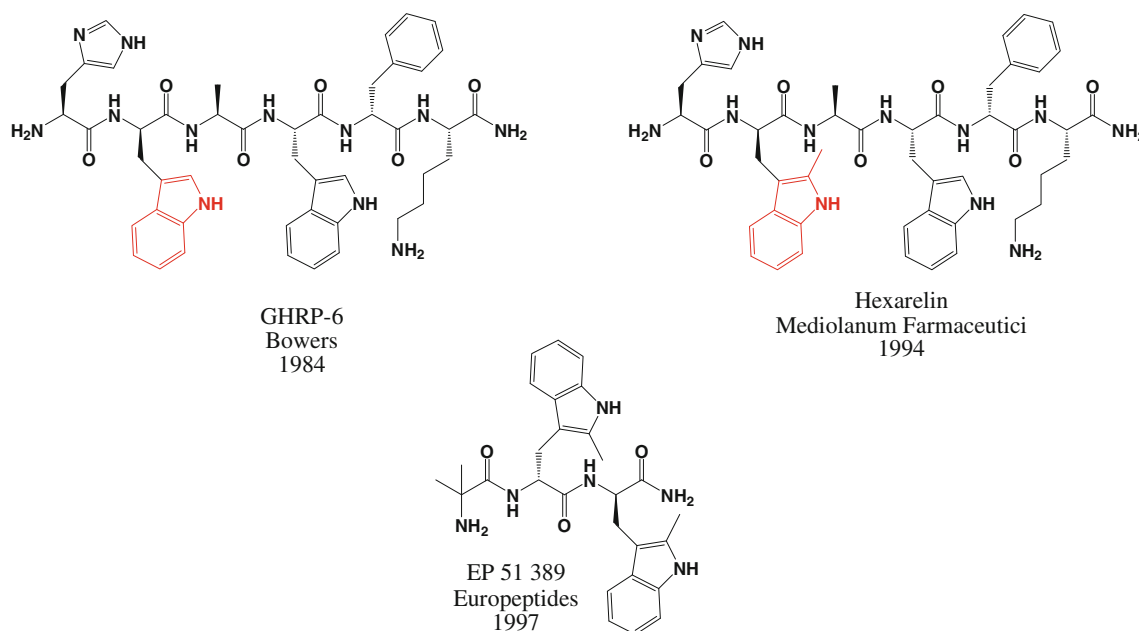


Fig. 2 Structure of GHRP-6, Hexarelin and EP 51389

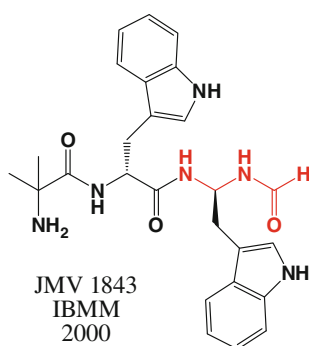


Fig. 3 Compound JMV 1843

have the total biological activities of the hormone. The importance of specific residues as well as the importance of C-terminal dipeptide was also observed according to a general strategy that we developed for gastrin (Rodríguez et al. 1987): the most potent Bombesin antagonists were obtained by modifying this C-terminal dipeptide. In our laboratory, among all the modifications introduced in the C-terminal dipeptide of bombesin, the substitution of the amide function of the C-terminal by a formylated amine function led to a potent bombesin receptor agonist. Interestingly, this compound showed remarkable *in vivo*

activity. This modification was applied to compound EP 51389 to give compound JMV 1843.

From pseudo-peptide to peptidomimetics: JMV 2215

It was then discovered that the C-terminal part moiety was not necessary for binding to the receptor. Suppression of this part of the molecule was suggested from Novo-Nordisk studies (Peschke et al. 2000) concerning NN 703 derivatives, displaying a very strong *in vitro* activity on growth hormone release (Fig. 4).

Indeed, the corresponding compound, named JMV 2215, displays a GHSR-1a binding affinity similar to that of compound JMV 1843. Moreover, this compound does not induce any growth hormone secretion in rat per subcutaneous route.

Compound JMV 2215 was chosen as a lead compound because of the simplicity of its structure. As ghrelin and its implication in food intake stimulation were discovered at that time, the interest for the discovery of receptor antagonists prompted us to adopt a classical medicinal chemistry approach. (Hruby and Balse 2000) As peptidic structures present several disadvantages such as limited stability toward proteolysis, poor transport properties and inherent flexibility, we decided to rigidify the structure by introducing

Fig. 4 Suppression of the C-terminal part moiety in compounds NN 703 and JMV 1843

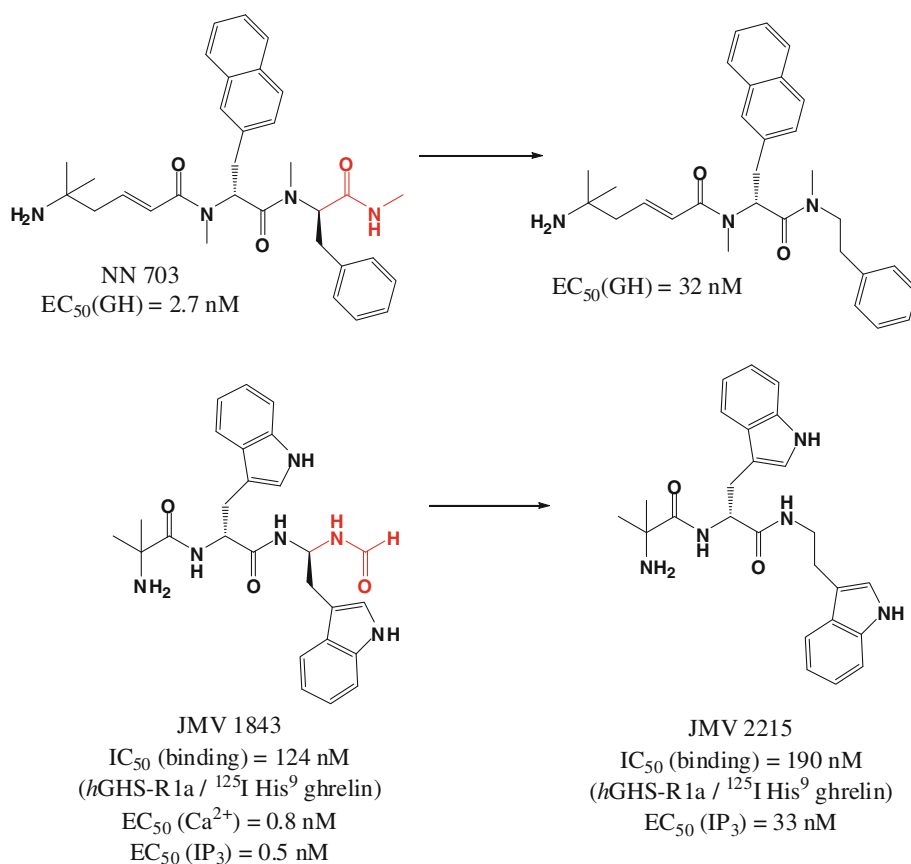
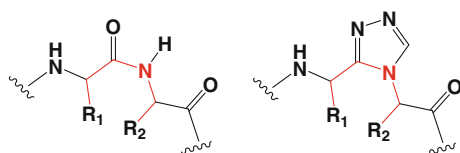
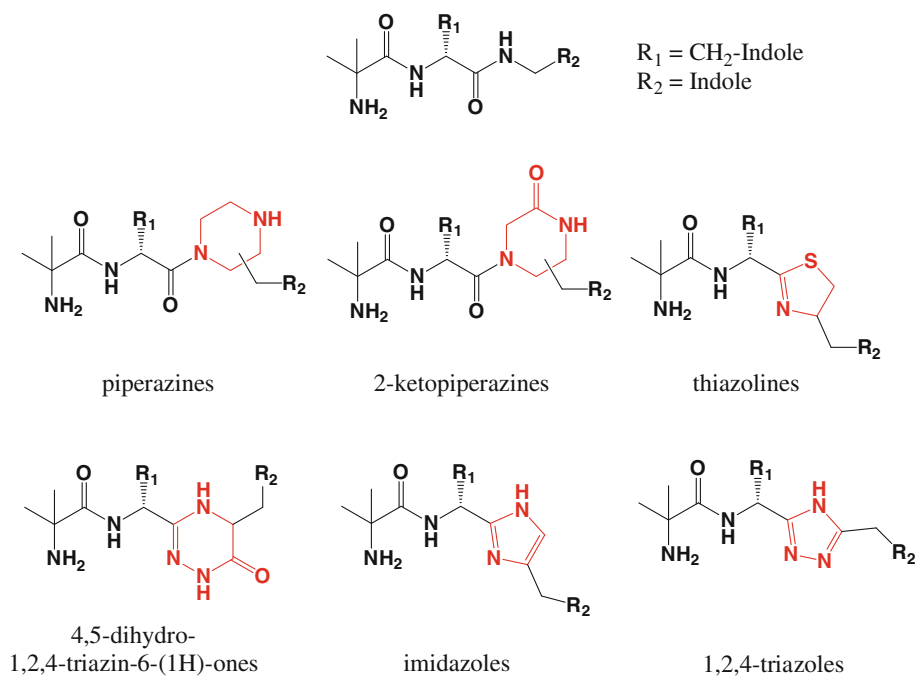
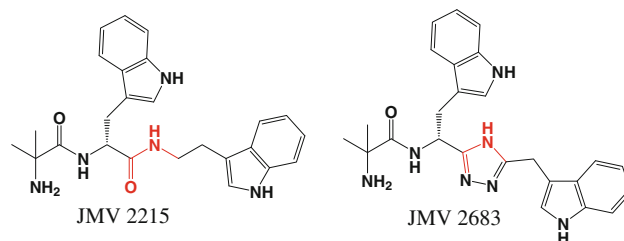
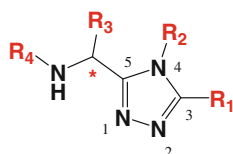


Fig. 5 Introduction of heterocyclic scaffolds**Fig. 6** Mimicking of the cis configuration of amide by the triazole scaffold**Fig. 8** Structure of compounds JMV 2215 and JMV 2683**Fig. 7** General triazole scaffold

various heterocycles bearing the needed side chain groups for receptor binding (Fig. 5, unpublished results).

These original heterocyclic structures were supposed to present a better selectivity and stability compared to their peptidic counterparts. Among all these molecules, several presented interesting binding affinities toward the ghrelin receptor, and particularly those belonging to the triazole family.

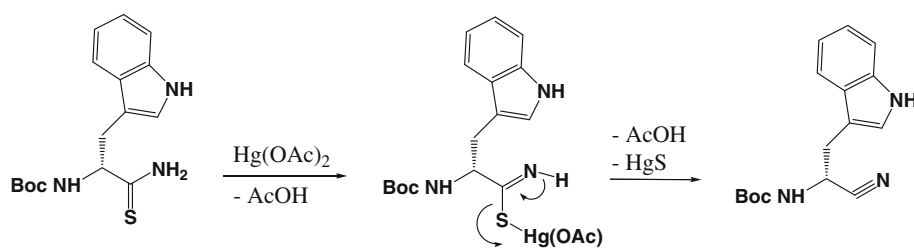
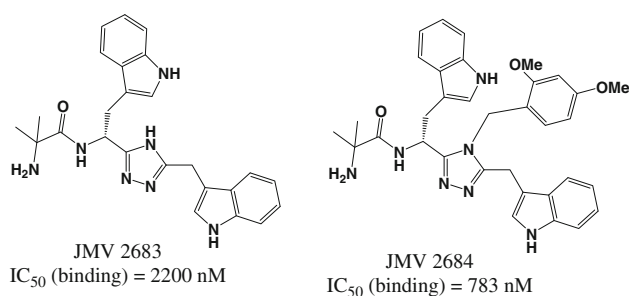
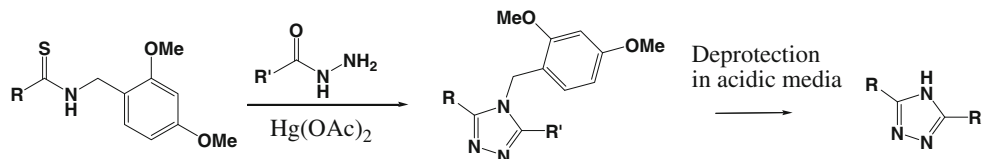
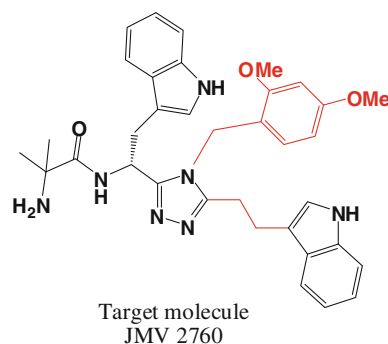
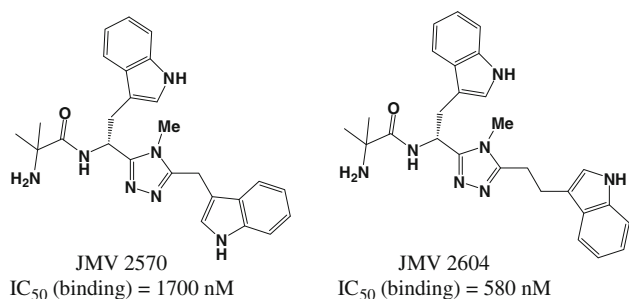
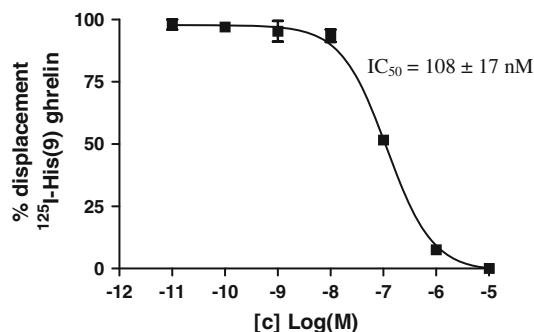
First triazole antagonist: JMV 2760

We started to work on the triazole family by the end of 2004, in collaboration with Æterna-Zentaris GmbH, with the following aims:

- To find new ghrelin receptor ligands displaying a binding affinity lower than 10 nM.
- To look for ghrelin receptor antagonists to develop a compound able to be tested in feeding behavior.
- To synthesize molecules having a molecular weight in the 500–600 g/mol range.
- The synthetic scheme leading to these compounds has to be easy to handle and modular.

We chose to work on the 1,2,4-triazole moiety, for the following reasons:

- Interesting results were preliminary obtained with this family in our laboratory.
- The 1,2,4-triazole moiety presents a strategic interest because no patent claimed this scaffold as ghrelin receptor ligand.
- This scaffold is well known in medicinal chemistry for various biological properties: antifungal (Collin et al. 2003), antimicrobial (Papakonstantinou-Garoufalas

Scheme 1 Degradation of primary thioamide into nitrile**Scheme 2** Protection/deprotection by an *o,p*-dimethoxybenzyl group**Fig. 9** Compounds JMV 2683 and JMV 2684**Fig. 11** Target molecule JMV 2760**Fig. 10** Compounds JMV 2570 and JMV 2604**Fig. 12** Displacement curve of compound JMV 2760

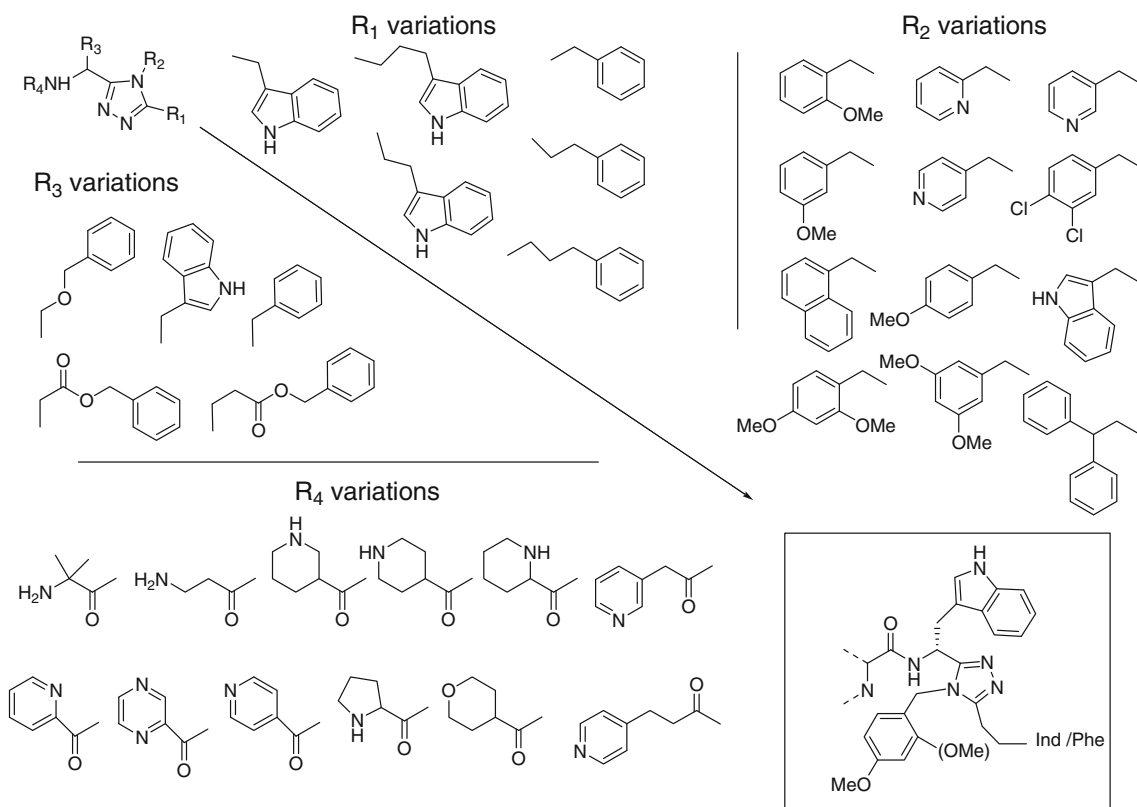
et al. 2002), antiviral (De Clercq 2004), antiasthmatic (Naito et al. 1996) and hypotonic activities (Hester et al. 1971). 1,2,4-triazoles are also used as amide bond isostere for the design of peptide ligands with the aim of enhancing their pharmacological properties (Boyd et al. 1994; Thompson et al. 1994), or to mimic the *cis* configuration of the amide bond, which is present in some peptides (Hitotsuyanagi et al. 2002) (Fig. 6).

The 1,2,4-triazole moiety presents 3 potential points of diversity in positions 3, 4 and 5. An aminoalkyl group in

position 5 will allow a fourth point of diversity. When the amino-alkyl is branched, the molecule displays an asymmetric center. The presence of these sites of diversity is essential when a structure–activity relationship study is considered (Fig. 7).

Once chosen the general scaffold, we then had to choose the first lead compound. For this purpose, we carefully examined the results previously obtained in our laboratory with the triazole family.

Figure 1 consists of two graphs. The left graph is a dose-response curve showing Fluorescence signal (% maximal response) on the y-axis (0 to 100) versus Concentration Ghrelin (log M) on the x-axis (-12 to -6). Four sigmoidal curves are plotted, each with different symbols: open circles (top), filled circles, filled squares, and open squares (bottom). The right graph is a Schild plot showing log(Dose Ratio-1) on the y-axis (-1 to 2) versus Conc. Antagonist (log M) on the x-axis (-8 to -6). Three data points are plotted as filled circles, and a straight line is fitted through them.

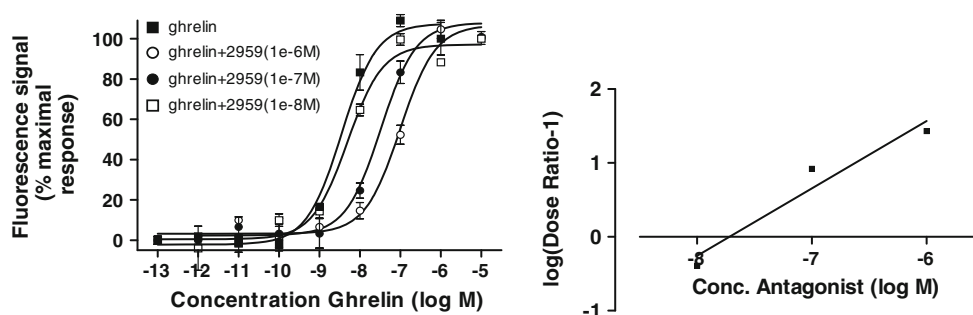
COc1ccc(cc1)CCn2nc3ccccc3nc2[C@@H](c4c[nH]c5ccccc45)C(=O)NCC

JMV 2959
IC₅₀ (binding) = 32 nM
Antagonist

Log(M)	% displacement ^{125}I -His(9) ghrelin
-12	100
-11	98
-10	100
-9	90
-8	80
-7	48
-6	12
-5	2

Fig. 16 Displacement curve of compound JMV 2959

Fig. 17 Calcium-induced ghrelin dose response curves in the absence or presence of different concentrations of JMV 2959 and Schild Plot of compound JMV 2959



Starting from compound JMV 2215, a first target was compound JMV 2683, in which amide bond was replaced by the triazole scaffold (Fig. 8).

The synthetic scheme started from a primary thioamide, which was anticipated to cyclize as described by Hitotsuyanagi et al. (2002). Unfortunately, the primary thioamide spontaneously degraded into nitrile (Avalos et al. 1997) in the presence of mercury (II) acetate (Scheme 1).

It was then considered to protect the primary amide by an *o,p*-dimethoxybenzyl group, cleavable in acidic media (Scheme 2).

Both the amino-protected and unprotected compounds were tested to determine their binding affinity toward GHS-1a receptor. It was found that the N-protected triazole presented a better binding affinity for the GHR-R1a (i.e. compound JMV 2684 vs. JMV 2683, Fig. 9).

Several other triazole compounds were synthesized, and two of them retained our attention, compounds JMV 2570 and JMV 2604 (Fig. 10).

Indeed, by introducing an additional carbon atom between the triazole ring and the indole moiety, the binding affinity of the molecule was significantly improved.

By combining these two observations:

- Trisubstituted triazole with *o,p*-dimethoxybenzyl group in position 4;
- 2 carbon atoms between the triazole scaffold and the indol moiety;

we considered synthesizing the corresponding compound, which was named JMV 2760 (Fig. 11).

Compound 2760 was tested (Demange et al. 2007) to determine its ability to inhibit ^{125}I -[His⁹] ghrelin radioligand binding on hGHS-1a receptor transiently expressed on LLC-PK1 cells. The obtained inhibition curve indicated an inhibitory constant IC_{50} of 108 ± 17 nM. This binding affinity was far better from that obtained with JMV 2684 and JMV 2604 (IC_{50} 783 and 580 nM, respectively) (Fig. 12).

The biological activity of compound JMV 2760 was then determined. For this purpose, the percentage of activation of intracellular calcium caused by the compound in saturating conditions (concentration of 10 μM) was measured.

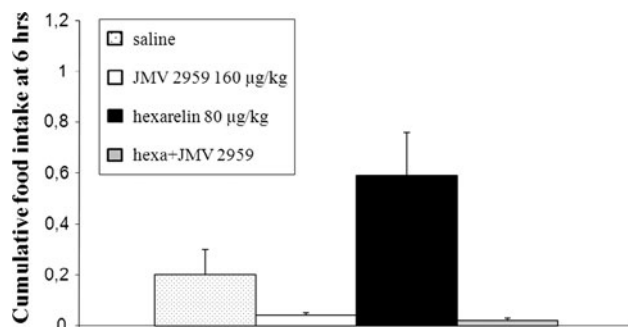


Fig. 18 Inhibition of hexarelin (80 $\mu\text{g}/\text{kg}$) induced cumulative food intake (g of food/100 g body weight) at 6 h by acute administration (sc) of compound JMV 2959 (160 $\mu\text{g}/\text{kg}$)

Compound JMV 2760 did not induce any intracellular calcium mobilization by itself. It was then tested at various concentrations on ghrelin-stimulated $[\text{Ca}^{++}]_i$ mobilization. The dissociation constant of the receptor/JMV 2760 complex was determined by the Schild method (Fig. 13).

This experiment revealed a dissociation constant K_b of 14 ± 5 nM.

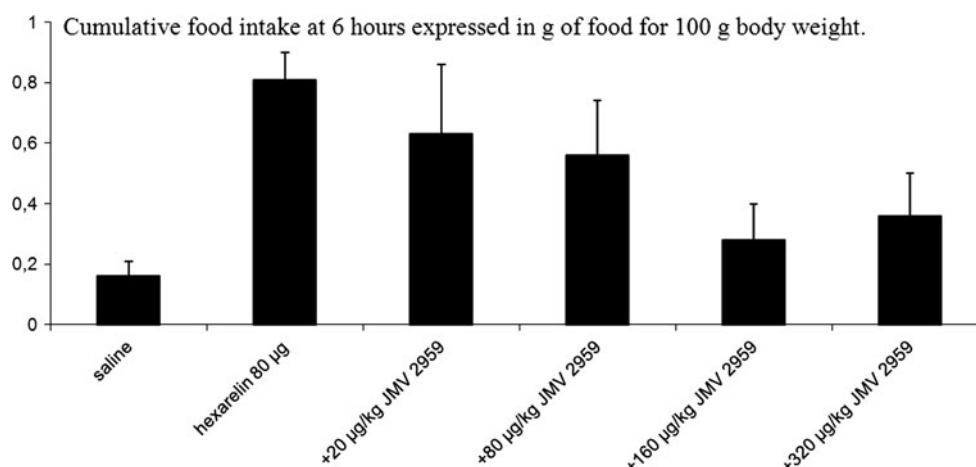
Compound JMV 2760 presented interesting binding affinity toward GHS-R1a, and behave as a ghrelin receptor antagonist on the $[\text{Ca}^{++}]_i$ mobilization. This compound was the starting point of a structure–activity relationship study for searching ghrelin receptor antagonists.

Structure–activity relationship studies on the triazole series: discovery of compound JMV 2959 (AEZS-123)

Starting from this lead compound, extensive structure–activity relationship studies were performed (Demange et al. 2007; Moulin et al. 2007a, 2008a, b) (Fig. 14).

Variation of the R_1 moiety was performed: the nature of the aromatic group and the carbon chain length was tested. For the R_2 position, various groups, with diverse steric hindrance and substitutions, were tested. In R_3 position, the nature of the aromatic group was modified, as well as the configuration of the carbon atom. We also studied the R_4 part of the molecule: on the scheme below are displayed

Fig. 19 Dose-response study for JMV 2959 on food intake stimulated by 80 µg/kg hexarelin



several carboxylic acids that were introduced. By this mean, more than 300 compounds were synthesized. The structure–activity relationship study allowed to rationalize, at least in part, the binding affinity of compounds toward the ghrelin receptor.

This study led to privilege a CH₂–CH₂–indole or a CH₂–CH₂–phenyl group in R₁ position. In R₂ position, a *p*-methoxy or *o,p*-dimethoxybenzyl group was preferred. It also clearly appeared that a (*D*)-tryptophan precursor was preferred at the R₃ position. Concerning the R₄ position, it appeared that to obtain an antagonist compound with high affinity, an aliphatic or aromatic nitrogen atom in position 2 relative to the amide carbonyl function was necessary.

One of these compounds JMV 2959 (Fig. 15) was chosen for further investigation.

In vitro characterization of compound JMV 2959

This compound was tested (Moulin et al. 2007a) to determine its ability to inhibit [¹²⁵I]-[His⁹] ghrelin radioligand binding on hGHS-1a receptor transiently expressed on LLC-PK1 cells (IC₅₀ 32 ± 3 nM).

Compound JMV 2959 did not induce any intracellular calcium mobilization by itself. The dissociation constant of the receptor/JMV 2959 complex was determined by the Schild method. This experiment revealed a dissociation constant K_b of 19 ± 6 nM (Figs. 16, 17).

The aqueous solubility of the hydrochloride salt of JMV 2959 was determined to be 5.0 mg/mL, and the clogP = 2.67.

First in vivo properties of compound JMV 2959

This compound was extensively studied in vivo by several research groups and for several different potential applications.

In vivo effects on food intake and fat accumulation

Ghrelin, first isolated from the stomach (Kojima et al. 1999), has emerged as an important gut-brain signal for the regulation of food intake (Wren et al. 2000; Nakazato et al. 2001) and fat mass (Tschöp et al. 2000). Circulating ghrelin levels increase preprandially in healthy subjects (Cummings et al. 2001), and this finding is consistent with a role for ghrelin in food-seeking behavior and meal initiation.

Indeed, the central ghrelin signaling system receives considerable interest as a potential therapeutic target for the regulation of appetite and fat mass (Vincent and Le Roux 2007). A number of studies have shown that suppressed ghrelin signaling has beneficial effects to prevent/reverse obesity in animal studies (Asakawa et al. 2003; Esler et al. 2007).

Compound JMV 2959, as a ghrelin receptor antagonist, can be of great interest for this type of studies. Compound JMV 2959 was evaluated for in vivo effects on food intake and obesity (Moulin et al. 2007a). JMV 2959 was tested in feeding behavior in rats. It was first evaluated alone for its effect on food intake and compared to hexarelin, and then for its ability to inhibit hexarelin-stimulated food intake. As expected, when administered alone, JMV 2959 was without effect, it did not increase food intake. When inhibition of food intake induced by hexarelin was evaluated, JMV 2959 was found to totally suppress food intake induced by hexarelin administration [hexarelin (80 µg/kg, cumulative food intake (g of food/100 g of body weight) at 6 h by acute administration (sc) of compound JMV 2959 160 µg/kg)] (Fig. 18).

A dose-response study was performed for this compound. It showed a well-correlated progressive inhibition of hexarelin-stimulated cumulative food intake with increasing doses (20, 80, and 160 µg/kg). Almost total inhibition was observed at a dose of 160 µg/kg. Higher doses did not improve the inhibition (Fig. 19).

JMV 2959 was also evaluated for its activity on GH secretion in infant rats after sc administration, in the presence or not of hexarelin. Indeed, it is known that in the baby rats the pulsatility of the GH secretion is not yet activated at the hypothalamic level, and therefore, a constant GH blood level is measured in these young animals. As expected, when administered alone, JMV 2959 did not significantly stimulate GH release. Unexpectedly, it was unable to antagonize hexarelin-stimulated GH secretion.

No straightforward correlation could be drawn between *in vitro* results and *in vivo* experiments on GH secretion and food intake. JMV 2959 defined as an *in vitro* GHS-R1a antagonist had no effect on GH secretion or food intake in rats when administered alone. However, it was able to totally suppress hexarelin-induced food intake in rats, while it was unable to inhibit GH secretion elicited by hexarelin. All these findings strongly suggested the existence of either ghrelin receptor subtypes or a particular signalisation pathway of the ghrelin receptor correlated with GH secretion on one side and food intake on the other side. It is a possibility that different signaling pathways could be activated according to the interaction of the ghrelin receptor with another specific receptor present in the same tissue.

The first description of the central acute effects of JMV 2959 to suppress food intake induced by exogenous ghrelin administration and also by fasting-induced food intake was provided by Salomé et al. (2009).

Central (i.c.v.) injection of JMV 2959 to rats suppressed food intake induced by i.c.v. ghrelin injection (1 µg) in a dose-dependent manner with a total blockade at a 8 µg dose. JMV 2959 also reduced fasting-induced food intake in rats (i.e. the amount of food eaten during the first hour of food exposure after 16 h fast).

At the single cell level, the effects of JMV 2959 to suppress ghrelin (0.5 µM)-induced changes in electrical activity of arcuate nucleus cells recorded extracellularly in a slice preparation was also explored: preincubation with ghrelin followed by perfusion with JMV 2959 suppressed the responsiveness of arcuate cells to ghrelin.

Thus, JMV 2959 suppresses ghrelin-induced and fasting-induced food intake at the level of the central nervous system. This appeared to be mediated, at least in part, by a modulation of the activity of ghrelin-responsive arcuate nucleus cells.

In another study (Salomé et al. 2009), the central nervous system mechanism underlying the chronic central effects of ghrelin, with respect to increasing body weight and body fat, was explored. JMV 2959 was used to investigate the role of GHS-R1a in mediating the effects of ghrelin on energy balance and on hypothalamic gene expression.

As expected, in adult male rats, chronic central treatment with ghrelin for 14 days, when compared to vehicle-treated control rats, resulted in an increased body weight, lean mass and fat mass (assessed by dual X-ray absorptiometry), dissected white fat pad weight, cumulative food intake, food efficiency, respiratory exchange ratio and a decrease of energy expenditure. Co-administration of JMV 2959 suppressed the majority of these effects, with the notable exception of ghrelin-induced food intake and food efficiency.

This effect of chronic JMV 2959 on food intake was difficult to interpret because, even though the antagonist appears to suppress ghrelin-induced food intake back to the same level as the control animals (ghrelin + JMV 2959 group vs. control), Salomé et al. could not detect a suppression of ghrelin-induced food intake by the antagonist (ghrelin + JMV 2959 vs. ghrelin). Similar results were obtained for the food efficiency. Thus, these data suggest that JMV 2959 did not completely block ghrelin's effects of chronic central ghrelin administration on food intake, although the trends in the data would make it difficult to conclude that ghrelin-induced food intake is independent of GHS-R1a. Such effects could be explained by additional ghrelin receptor types (Yokote et al. 1998).

By contrast to the feeding data, the ghrelin-induced weight gain was totally blocked by JMV 2959. Both fat mass and lean mass were significantly increased by i.c.v. ghrelin treatment, but only the effect on fat mass was blocked by the antagonist. These data suggest that ghrelin is able to exert these beneficial effects on lean mass by a direct CNS action that does not only involve GHS-R1a (i.e. co-administration with JMV 2959 did not block these effects).

To further explore the dynamic changes in gene expression of hypothalamic peptide systems in rats chronically administered ghrelin (with/without JMV 2959), that have a positive energy balance, high throughput RT-PCR analysis of dissected hypothalamic tissue was performed, exploring a large number of candidate genes that may mediate the metabolic effects of ghrelin. It was found that central ghrelin infusion increased the gene expression of various hypothalamic peptides and their precursors (NPY, CART, prodynorphin, proenkephalin, prolactin-releasing hormone, orexin A and somatostatin), signaling molecules involved in GABA and glutamate signaling (e.g GAD1 and Vglut2) and UCP2 as well as various receptors (CRH-R1, CRH-R2, GHS-R1a, GLP-1R, insulin receptor, leptin receptor, and MC3R). Because ghrelin induces a positive energy balance and activates the hypothalamic-pituitary growth axis, it was impossible to distinguish between those changes that are a direct consequence of ghrelin action (and may be directly involved in ghrelin-induced changes in fat utilization) as opposed to

those that are secondary to the adiposity or other endocrine changes (e.g. circulating adipokines).

Some of the hypothalamic gene expression which had increased after 2 weeks of ghrelin treatment also showed an increased expression even in rats in which the ghrelin was co-infused with JMV 2959 (and that did not have increased adiposity), namely the genes for CRH-R2, GAD1, GHS-R1a, GLP-1R, insulin receptor, NPY, orexin A, prodynorphin, prolactin-releasing hormone, Vglut2, somatostatin and UCP-2. These genes are regulated by chronic central ghrelin treatment, but this does not appear to involve signaling via GHS-R1a alone.

Co-administration of JMV 2959 was able to suppress some of the genes that were up-regulated by ghrelin. These included the genes for CART, MC3R and proenkephalin.

Although the effects of ghrelin on gene expression of the orexigenic peptide AgRP did not reach statistical significance, it was found a suppression of AgRP gene expression when ghrelin was co-administered with JMV 2959, consistent with the established literature that AgRP may be an important hypothalamic target for ghrelin (Kamegai et al. 2001; Tschöp et al. 2002).

Regarding the reported effects of ghrelin that were not blocked by JMV 2959, including effects on hypothalamic gene expression, the authors suggested that these may be mediated by unknown ghrelin receptors (including receptor subtypes). They also pointed out the possibility that these effects were mediated by desacyl ghrelin, the unacylated form of ghrelin that does not bind to GHS-R1a and has anorexigenic properties (Asakawa et al. 2005). The extent to which desacylation of exogenous ghrelin occurs in the brain remains to be determined, because the available evidence to date indicates that key circulating enzymes involved in desacylation do not gain access to the CNS (Duysen and Lockridge 2008). However, GHS-R1a interaction with other receptors expressed in the same tissues may also result in different intracellular pathways that could explain these behavioral differences.

In vivo effects on addictive behavior

A variety of human studies suggests that common neurobiological mechanisms underly different forms of addictive behaviors, including compulsive overeating, pathological gambling, alcoholism, nicotine dependence, and other forms of chemical addiction (Holderness et al. 1994; Volkow and Wise 2005). Given the hyperghrelinemia associated with certain forms of compulsive overeating (Cummings et al. 2002) and also with alcohol dependence (Kaur 2005; Kim et al. 2005), it was hypothesized that a common mechanism, involving the central ghrelin signaling system, underlies the pathophysiology of these diseases. Indeed, GHS-R1a antagonists such as JMV 2959

may have therapeutic potential in drug dependence disorders.

The first report concerning JMV 2959 and addictive behavior was performed by Jerlhag et al. (2009). They provide evidence that the central ghrelin signaling system is required for alcohol reward. Central ghrelin administration (to brain ventricles or to tegmental areas involved in reward) increased alcohol intake in a 2-bottle (alcohol/water) free choice limited access paradigm in mice. By contrast, central or peripheral administration of JMV 2959 suppressed alcohol intake in this model. Alcohol-induced locomotor stimulation, accumbal dopamine release and conditioned place preference were abolished in models of suppressed central ghrelin signaling: GHS-R1a KO mice and mice treated with JMV 2959.

As the target circuits for ghrelin in the brain include a mesolimbic reward pathway that is intimately associated with reward-seeking behavior, the same authors further sought to determine whether the central ghrelin signaling system is required for reward from drugs of abuse other than alcohol, namely cocaine or amphetamine (Jerlhag et al. 2010).

They found that amphetamine as well as cocaine-induced locomotor stimulation and accumbal dopamine release were reduced in mice treated with JMV 2959. Moreover, the ability of these drugs to condition a place preference was also attenuated by JMV 2959.

In another study (Clifford et al. 2011) it was examined the development of cocaine locomotor sensitization in rats, by pharmacologic antagonism by compound JMV 2959. During 7 consecutive days, adult male rats were injected per i.p. route with either 0.3 or 6.0 mg/kg JMV 2959, 20 min later, they were injected per i.p. route with either vehicle or 10 mg/kg cocaine HCl. Rats pretreated with JMV 2959 showed significantly attenuated cocaine-induced hyperlocomotion.

Collectively, these findings raised important questions regarding the physiological role of ghrelin influencing not only food intake and appetite, as described earlier, but also clearly having a broader role in reward induced by addictive drugs such as alcohol, amphetamine, and cocaine.

These results were enlarged by another study, where the effect of peripheral administration of JMV 2959 on the nicotine-induced locomotor stimulation, accumbal dopamine release and conditioned place preference was investigated (Jerlhag and Engel 2011).

It was found that the ability of nicotine to increase the locomotor activity, accumbal dopamine release and to condition place preference was reduced in mice treated with JMV 2959.

Thus GHS-R1a appeared to be required not only for alcohol, cocaine and amphetamine-induced reward, but also for reward induced by nicotine.

Paul Wellman research team also examined the role of ghrelin receptor in the behavioral sensitizing effects of nicotine (Wellman et al. 2011). For this purpose, adult male rats were injected with either 0.3 or 6.0 mg/kg of JMV 2959 per i.p. route and 20 min later with either vehicle or 0.4 mg/kg nicotine hydrogen tartrate per sc route, on each of 7 consecutive days.

It could be observed that rats treated with nicotine alone showed robust locomotor sensitization, as expected, whereas rats pretreated with JMV 2959 showed significantly attenuated nicotine-induced hyperlocomotion.

Skibicka et al. (2011) sought to determine whether ghrelin and its receptors are important for reinforcing motivation for natural sugar reward by examining the role of ghrelin receptor stimulation and blockade for sucrose progressive ratio operant conditioning, a procedure used to measure motivational drive to obtain a reward.

Peripherally and centrally administered ghrelin significantly increased operant responding and therefore, incentive motivation for sucrose. Utilizing JMV 2959, it was demonstrated that blockade of GHS-R1a signaling significantly decreased operant responding for sucrose. These findings suggested that ghrelin antagonists, such as JMV 2959, have therapeutic potential to suppress the overconsumption of sweet food.

These data were confirmed by Landgren et al. (Landgren et al. 2011). They present a rodent study where a group of alcohol-consuming individuals selected from a population cohort was investigated for genetic variants of the ghrelin signaling system in relation to both their alcohol and sucrose consumption. Moreover, the effect of JMV 2959 on voluntary sucrose intake and operant self administration, as well as saccharin intake was investigated. It was found associations with the ghrelin gene haplotypes and increased sucrose consumption, and a trend for the same association was seen in the high alcohol consumers. The preclinical data show that JMV 2959 reduces the intake and self-administration of sucrose in rats as well as saccharin intake in mice. Collectively, the data provided a clear indication that GHS-R1a such as JMV 2959 reduces the intake of rewarding substances.

Egecioglu et al. (2011) also showed that mice with a disrupted gene encoding the ghrelin receptor (GHS-R1a) and rats treated peripherally with JMV 2959 both show suppressed intake of rewarding food in a free choice (chow/rewarding food) paradigm. The ability of rewarding food to condition a place preference was suppressed by JMV 2959 in rats. These data supported the hypothesis that central ghrelin signaling at the level of the ventral tegmental area is important for the reward system. Indeed, the neurocircuitry underpinning food-motivated behavior involves, in particular, the dopamine cells of the ventral tegmental area (VTA) that project to the nucleus accumbens (Nacc).

Skibicka et al. (2011) further sought to determine if ghrelin acts directly within these mesolimbic reward areas to increase food reward/motivation in studies that combine feeding behavior, pharmacology and neuroanatomy. It was found that motivated behavior for a sucrose reward, assessed in an operant conditioning paradigm in rats, was increased when ghrelin was microinjected directly into the VTA but not into the NAcc. By contrast, ghrelin administration to both areas increased the free feeding of chow. Importantly, in a state of overnight food restriction, where endogenous levels of ghrelin are increased, ghrelin receptor blockade by JMV 2959 in the VTA was sufficient to decrease the motivation to work for a sugar reward. Blockade of GHS-R1a in VTA or NAcc was not sufficient to reduce fasting-induced chow hyperphagia. Taken together these data identified the VTA but not the NAcc as a direct, necessary and sufficient, target site for ghrelin's action on food motivation.

In vivo effects on hyperactivity

In patients suffering from anorexia nervosa, total plasma ghrelin concentrations of underweight subjects are increased and tend to normalize with the recovery of body weight (Otto et al. 2001). It has been reported by Misra et al. (2005) that high plasma levels of total ghrelin induced by fasting in humans are negatively associated with the percentage body fat and with low levels of leptin and insulin, which might play an important role in the pathophysiology of anorexia nervosa. Important characteristics of this disease include, in particular, increased locomotor activity and reduced food consumption (Routtenberg and Kuznesof 1967), together with similar endocrine abnormalities (de Rijke et al. 2005).

Studies in GHS-R1a KO mice have implicated the central ghrelin system in food anticipatory activity (Blum et al. 2009; LeSauter et al. 2009). Therefore, it was envisaged to use GHS-R1a antagonists, such as JMV 2959, to suppress anorexia-induced hyperlocomotor activity.

Verhagen et al. (2011) demonstrated that the central ghrelin signaling system, involving GHS-R1a, is required for food anticipation, measured in the ABA model (activity-based anorexia, an animal model of anorexia nervosa). Thus, food anticipatory activity was attenuated both in chronic models of suppressed ghrelin signaling (GHS-R1a KO mice and mice chronically treated with JMV 2959) and in an acute model (rats given an acute central injection of JMV 2959). Collectively these data suggest that the gut-brain signal provided by ghrelin is important for food anticipation and that GHS-R1a antagonists such as JMV 2959 provide a potential therapy for suppressing food anticipatory hyperlocomotor activity independently of food intake.

Anorectic patients often display abnormally high physical activity levels (Kron et al. 1978), which hinder the process of recovery (Kaye et al. 1988; Holtkamp et al. 2004). Thus, reducing hyperactivity in severely ill patients suffering from anorexia nervosa could be beneficial for therapeutic outcome.

In vivo effects on retinopathy

Vascular degeneration and the ensuing abnormal vascular proliferation are central to proliferative retinopathies, consisting of neovascularization of the retina extending into the vitreous body. Given the metabolic discordance associated with these diseases, Zaniolo et al. (2011) explored the role of ghrelin and GHS-R1a in proliferative retinopathy.

In a rat model of oxygen-induced retinopathy, the contribution of ghrelin and GHS-R1a was investigated using stable ghrelin agonists and antagonists, including JMV 2959.

This study allowed disclosing new role for the ghrelin-GHS-R1a pathway in the preservation of retinal vasculature during the vaso-obliterative phase of OIR and during the angiogenic phase of OIR. These findings suggest that the ghrelin-GHS-R1a pathway can exert opposing effects on retinal vasculature, depending on the phase of retinopathy, and thus holds therapeutic potential for proliferative retinopathies.

Perspectives

We described in this paper the process that led us to design ghrelin receptor antagonist JMV 2959. A first very interesting agonist compound, JMV 1843 (ARD-07) was published in 2003. In 2007, Scottish drugmaker Ardana has initiated a US registrational study of this compound, which is being developed as a treatment for growth hormone deficiency in adults. Earlier the same year, JMV 1843 was granted Orphan Drug status by the US Food and Drug Administration (<http://www.thepharmaletter.com/file/46317/ardana-begins-us-study-of-ard-07-.html>).

Further extensive structure–activity relationship studies led us to the discovery of a second very interesting compound, named JMV 2959 (AEZ-123), a ghrelin receptor antagonist, structure of which was published in 2007. As reviewed in the second part of this paper, this compound turned out to be an interesting pharmacological tool allowing researchers to better understand the way ghrelin and its receptor are involved in food intake and obesity, addictive behaviors, hyperactivity and retinopathy. This compound is also claimed in AeternaZentaris preclinical pipeline for the treatment of obesity and related pathologies

(<http://www.aeternazentaris.com/en/page.php?p=32&prod=28>). We believe this compound will follow the same successful runway as compound JMV 1843, to rapidly reach clinical development.

Conflict of interest The authors declare that they have no conflict of interest.

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