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Communications to the Editor

Identification of a Chemical Tool for the Orphan Nuclear Receptor FXR

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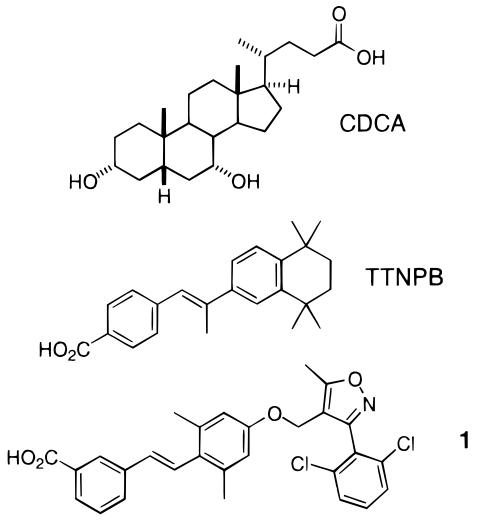
Bile acids are the amphipathic terminal metabolites of hepatic cholesterol metabolism. In humans, cholic acid and chenodeoxycholic acid (CDCA; Chart 1) are the primary bile acids found in the enterohepatic circulation. These bile acids play important roles in the regulation of intestinal lipid absorption, bile flow, and biliary lipid secretion.[1,2] Bile acids are synthesized in the liver and secreted into the intestine, where their physical properties facilitate the absorption of fats and vitamins through micelle formation. Cholesterol disposal from the liver is also dependent on the bile acid composition of the secreted bile. Impairments in the biosynthesis of bile acids, due to hereditary genetic defects, have been identified in children with severe cholestatic diseases and vitamin maladsorption.[3,4]

In addition to their role in the solubilization of fat and cholesterol, bile acids are signaling molecules that regulate the expression of genes involved in their biosynthesis and transport. In the liver, bile acids down-regulate the transcription of the *CYP7A* gene, which encodes the enzyme that catalyzes the rate-limiting step in hepatic bile acid synthesis.[5] In the intestine, bile acids induce the expression of a bile acid-binding protein (I-BABP), which is involved in the active transport of bile acids in the ileum.[6] Recently FXR (NR1H4),[7] an orphan member of the nuclear receptor gene family, was identified as a receptor for CDCA and other bile acids.[8−10] CDCA is found at micromolar concentrations in the liver and intestine, which matches the dose required to bind and activate FXR. A binding site has been identified in the promoter of the *I-BABP* gene, through which FXR regulates its transcription.[9,11] FXR has also been shown to repress transcription of the *CYP7A* gene,[9,10] although the mechanism may be complex since no FXR-binding site has been found in this gene promoter.[12]

Chart 1. Chemical Structures of FXR Agonists



Analysis of the role of FXR in the regulation of bile acid and cholesterol homeostasis has been hampered by the lack of chemical tools to study the pharmacology ofthe receptor. CDCA, the most potent of the bile acids on FXR, is not well suited for this purpose, since it interacts with bile acid binding and transport proteins and is extensively metabolized in the intestine to form lithocholic acid.[13] The only known nonsteroidal FXR ligand is the retinoid TTNPB (Chart 1).[8,14] However, TTNPB is only a weak FXR agonist (EC_{50} > 1 μM) and

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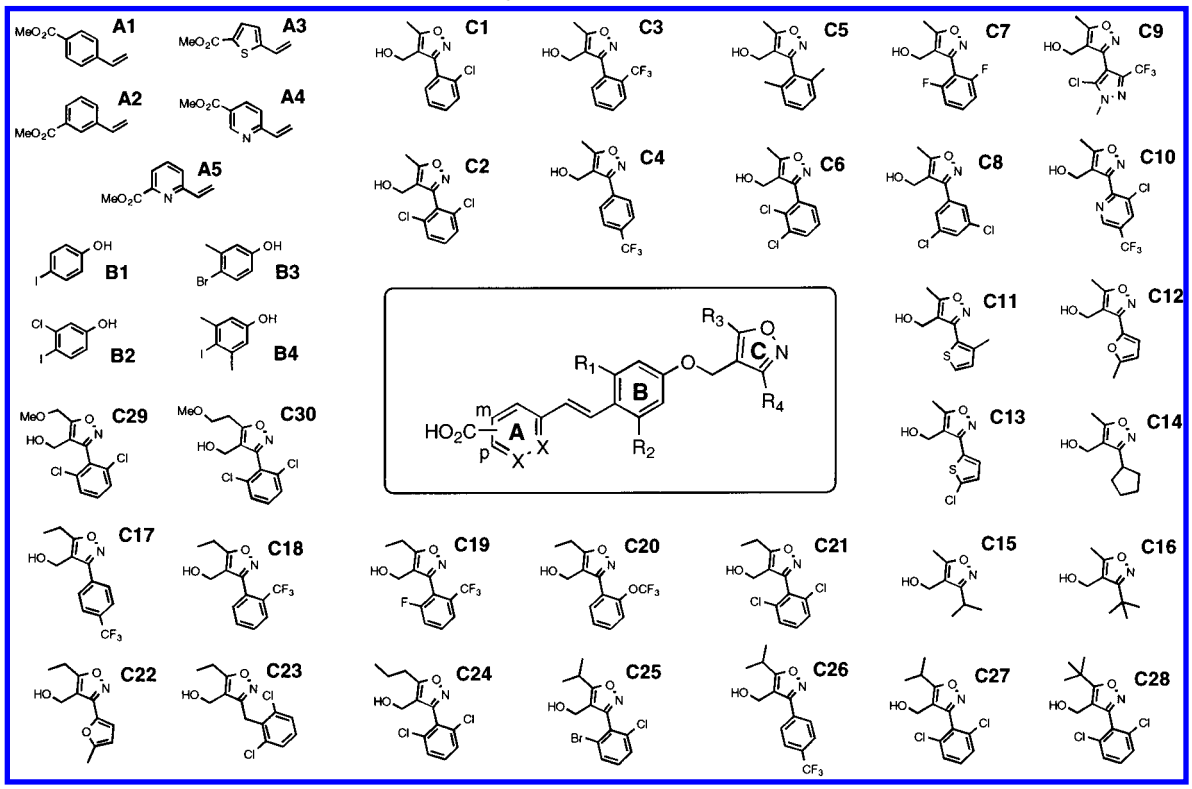
Chart 2. Structures of the Oxazole Focused Library



is 100-fold more potent as an agonist for the retinoic acid receptor.[15] We decided, therefore, to identify a potent and selective nonsteroidal FXR ligand for use as a chemical tool in the functional analysis of this orphan receptor by reverse endocrinology.[16]

The isoxazole **1** (Chart 1) was identified as an FXR partial agonist from a combinatorial library of 9900 stilbene carboxylic acids.[17] Using an established cell-free ligand-sensing assay (LiSA),[8] which measured the ligand-dependent recruitment of the $SRC1^{676-700}$ peptide to FXR by fluorescence resonance energy transfer, **1** showed an $EC_{50} = 70$ nM but only 60% of the efficacy of CDCA. The low efficacy of **1** in the SRC1 recruitment assay may contribute to its weak FXR agonist activity ($EC_{50} = 4.1$ μM) in a cell-based assays.[18] A focused three-component library of isoxazoles was synthesized to explore the structural requirements for FXR activity (Chart 2), with a goal of increasing potency and efficacy. Five vinyl-substituted aryl and heteroaryl carboxylates (**A1**−**A5**) were coupled with four halophenols (**B1**−**B4**) by palladium-catalyzed Heck reaction. Allylation of the phenol followed by saponification of the ester generated 20 stilbene acids, which were loaded onto Sasrin resin.[19] Removal of the allyl protecting group yielded 20 resin-bound phenols. A series of 4-hydroxymethylisoxazoles (**C1**−**C13**, **C17**−**C22**, and **C24**−**C30**) were synthesized by reaction of β-ketoesters with aryl and heteroaryl α-chlorooximes,[20] followed by reduction of the ester. Since this approach was not suitable for alkyl or benzyl α-chlorooximes, the corresponding alkyl or benzyl nitriles were used to synthesize additional 4-hydroxymethylisoxazole derivatives (**C14**−**C16** and **C23**).[21] The individual resin-bound phenols were reacted under Mitsunobu conditions with each of the 4-hydroxymethylisoxazoles. The final resins were cleaved with TFA to yield a library of 2−3 mg of 600 discrete isoxazole derivatives. Each compound was judged to be >80% pure by HPLC/MS analysis.

The isoxazole library was screened at a concentration of 1 μM using the cell-free FXR LiSA,[8] and 37 compounds were identified with activity equal to 50 μM CDCA. The active compounds were derived from alkene monomers **A1**−**A4**, phenol monomers **B1**−**B4**, and isoxazole monomers **C19**−**C21**, **C23**, **C25**, and **C27**−**C29**. Thus, most of the alkene and phenol monomers, but only a subset of the isoxazole monomers, were represented. No 3-methylisoxazoles, from monomers **C1**−**C16**, were active, indicating a clear preference for larger lipophilic substituents at this position. The active isoxazoles contained ethyl, isopropyl, *tert*-butyl, or methoxymethyl as their 3-substitutents, from monomers **C19**, **C20**, **C23**, **C25**, and **C27**−**C29**. These monomers also contained 5-phenyl- or 5-benzylisoxazole substituents, each of which were additionally substituted in the ortho-position. These ortho-substituents are likely to twist the aryl group out of the plane of the isoxazole in their low-energy conformations. Notably, all of the isoxazoles containing para-substituted aryl groups were inactive. Although the original lead **1** was included in the oxazole library, it was judged to be inactive, since it showed only 60% of the activity of the CDCA control.

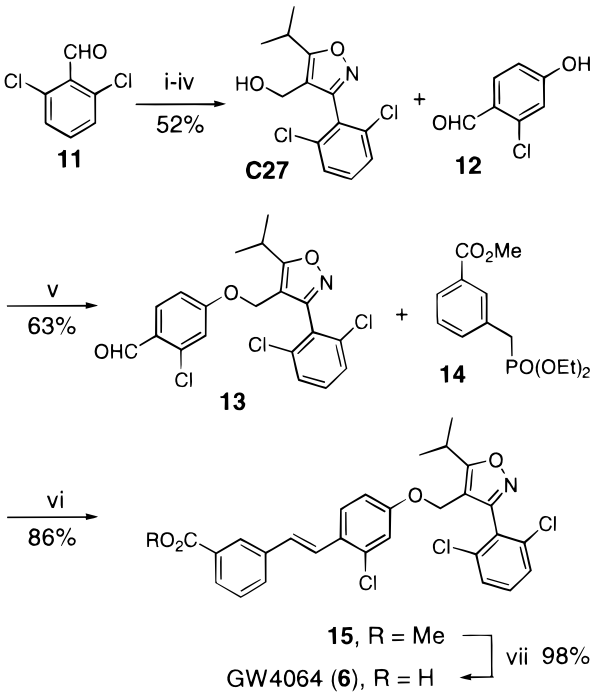
Several of the active isoxazole analogues were resynthesized on a 50-mg scale and purified to homogeneity by chromatography (Table 1). Although this was not an exhaustive resynthesis, the isoxazole analogues **2**−**10** contained examples of monomers **C19**, **C20**, **C23**, **C25**, and **C27**, which were represented in 34/37 (92%) of the active compounds. The resynthesized isoxazoles incorporated only monomers **A1**, **A2**, and **B2**−**B4**, since little preference was seen among the alkene and phenol monomers in the active compounds. Isoxazoles **1**−**10** were assayed in the cell-free FXR LiSA (Table 1). All of

Table 1. FXR Activity of Isoxazole Analogues

	structure[a]					FXR activity[b]	
no.	CO_2H	R_1	R_2	R_3	R_4	EC_{50} (nM)	RE
CDCA						3400 ± 200	1.0
1	*m*	CH_3	CH_3	CH_3	2,6-Cl_2Ph	70 ± 2	0.6
2	*m*	H	Cl	CH_3CH_2	2-F,6-CF_3Ph	54 ± 8	1.3
3	*m*	H	Cl	CH_3CH_2	2-OCF_3Ph	48 ± 3	1.6
4	*m*	H	Cl	CH_3CH_2	CH_2(2,6-Cl_2Ph)	60 ± 7	1.2
5	*m*	H	Cl	CH_3CHCH_3	2-Br,6-ClPh	12 ± 1	1.4
6	*m*	H	Cl	CH_3CHCH_3	2,6-Cl_2Ph	15 ± 1	1.4
7	*m*	H	CH_3	CH_3CHCH_3	2,6-Cl_2Ph	47 ± 1	1.0
8	*m*	CH_3	CH_3	CH_3CHCH_3	2,6-Cl_2Ph	100 ± 8	1.0
9	*p*	CH_3	CH_3	CH_3CHCH_3	2,6-Cl_2Ph	37 ± 5	1.0
10	*p*	CH_3	CH_3	CH_3CH_2	2,6-Cl_2Ph	100 ± 10	0.7

[a] Compound structures from Chart 2, where X = CH. [b] Compounds were assayed by fluorescence resonance energy transfer for recruitment of the $SRC1^{676-700}$ peptide to human FXR using a cell-free LiSA as described.[8] EC_{50}, the concentration of test compound that gave 50% of the maximum fluorescence ± SE, n = 3; RE, maximum efficacy of the test compound relative to 50 μM CDCA.

Scheme 1[a]



[a] (i) $HONH_2$·HCl, NaOH, EtOH, H_2O, reflux; (ii) NCS, DMF; (iii) methyl 4-methyl-3-oxopentanoate, NaOMe, THF; (iv) DIBAL-H, THF; (v) **12**, TPP, DIAD, CH_2Cl_2; (vi) NaH, THF; (vii) LiOH, H_2O, THF.

the isoxazoles were active over a similar dose range; however, isoxazoles **5** and **6** were 5-fold more potent than the original lead **1**. Notably, an increase in efficacy was seen when the 3-methylisoxazole **1** was replaced by the 3-isopropylisoxazoles **5**−**9**. The 2-fold increase in efficacy suggests that the 3-substituent binds to a lipophilic pocket that is important for receptor activation. The isoxazoles **5** and **6**, which had the best combination of potency and efficacy, were >200-fold more potent than CDCA in the FXR LiSA.

Isoxazole **6** was chosen for further characterization due to its potent FXR activity and relative ease of synthesis on a multigram scale (Scheme 1). Isoxazole **C27**, which was readily available in four steps from 2,6-dichlorobenzaldehyde (**11**),[20] was reacted with 2-chloro-4-hydroxybenzaldehyde (**12**). The resulting aldehyde **13** was subjected to a Horner−Emmons reaction with phosphonate **14** to generate the stilbene **15**. Finally, saponification of the methyl ester yielded **6** as a white powder. Isoxazole **6** was a full agonist with EC_{50} values of 80 and 90 nM, respectively, in CV-1 cells transfected with mouse and human FXR expression vectors and an established reporter gene.[8,14] There was no activity of **6** on other nuclear receptors, including the retinoic acid receptor, at concentrations up to 1 μM.[22] Thus, **6** is a potent and selective nonsteroidal FXR agonist.

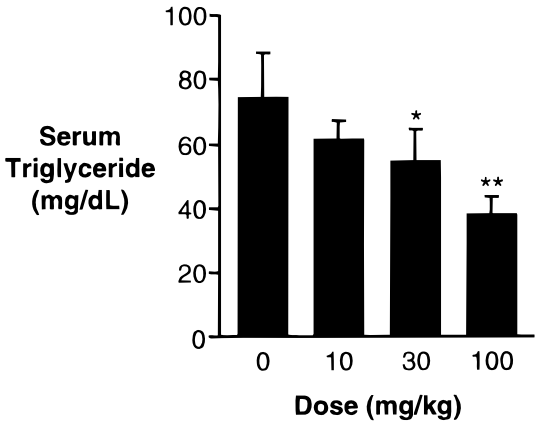


Figure 1. Pharmacological effect of FXR agonist GW4064 (**6**). Fisher rats (groups of 6) were dosed orally, b.i.d., with **6** as a suspension in 0.5% methyl cellulose. After 7 days, the rats were fasted for 18 h before blood collection. Triglycerides were analyzed by standard procedures on an Ilab 600; *P = 0.07; **P = 0.001.

Pharmacokinetic analysis in rats showed that **6** possessed an oral bioavailability of 10% with a $t_{1/2}$ = 3.5 h. To demonstrate that **6** had utility as a chemical tool, we studied its effects on serum triglycerides, since CDCA was reported to show modest lowering of triglycerides in humans undergoing bile acid therapy for gallstones.[23] Fisher rats were dosed with **6** by oral gavage. After 7 days, a dose-dependent lowering of serum triglycerides was observed in the rats receiving **6**, with an ED_{50} = 20 mg/kg (Figure 1). Although the mechanism of triglyceride lowering in humans by CDCA is unknown,[23] these results suggest that it may be due, in part, to FXR regulation of the bile acid metabolism.[22]

In conclusion, we have identified the first high-affinity nonsteroidal FXR agonist through use of high-throughput screening and combinatorial chemistry. GW4064 (**6**) will be a valuable chemical tool for studying the role of FXR in mammalian physiology and disease.[22] Finally, our data establishes triglyceride lowering as a surrogate pharmacological response to the activation of FXR.

Acknowledgment. We thank Ronda Ott for pharmacokinetic data on **6**.

Supporting Information Available: Detailed experimental procedures for the synthesis of the oxazole library and **6**; list of the active compounds identified in the oxazole library; analytical data for compounds **1**−**10**; dose−response data for CDCA, **1**, and **6** in the FXR LiSA; and dose−response data for the activation of human and mouse FXR by **6** in a cell-based reporter assay. This material is available free of charge via the Internet at http://pubs.acs.org.

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