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Novel substituted isoxazole FXR agonists with cyclopropyl, hydroxycyclobutyl and hydroxyazetidinyl linkers: Understanding and improving key determinants of pharmacological properties

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ABSTRACT

Several isoxazole-containing series of FXR agonists have been published over the last 15 years, subsequent to the prototypical amphiphilic “hammerhead”-type structure that was originally laid out by GW4064, the first potent synthetic FXR agonist. A set of novel compounds where the hammerhead is connected to the terminal carboxylic acid-bearing aryl or heteroaryl moiety by either a cyclopropyl, a hydroxycyclobutyl or a hydroxyazetidinyl linker was synthesized in order to improve upon the ADME properties of such isoxazoles. The resulting compounds all demonstrated high potencies at the target receptor FXR but with considerable differences in their physicochemical and in vivo profiles. The structure-activity relationships for key chemical features that have a major impact on the in vivo pharmacology of this series are discussed.

The Farnesoid X Receptor (FXR) is a member of the nuclear hormone receptor superfamily and senses bile acids such as chenodeoxycholic acid (CDCA) and their taurine or glycine amide conjugates as its endogenous ligands.[1–3] FXR mRNA can be detected throughout the entire gastrointestinal tract from the esophagus to the rectum, and in other tissues with exposure to bile acids such as liver and kidneys.[4,5] FXR heterodimerizes with RXR (Retinoid X-Receptor) and this dimer complex acts as a ligand-activated transcription factor to control the expression of various target genes which are involved in bile acid, cholesterol, triglyceride and lipoprotein homeostasis in the liver and circulation.[6,7] Furthermore, FXR regulates complex biological processes beyond metabolism such as liver regeneration and intestinal barrier integrity, and has been shown to influence the enterohepatic immune system through anti-inflammatory effects.[8-11]

Thus, FXR has been implicated as a target for novel pharmacotherapies that address metabolic diseases such as dyslipidemias, Type 2 Diabetes or the related Metabolic Syndrome.[12] Recently, 6-Et-CDCA (synonyms INT-747 or Obeticholic Acid, OCA), which is a semi-synthetic derivative of CDCA, that is about 80-fold more potent as a human FXR agonist[13], demonstrated significant improvements in insulin sensitivity and other beneficial metabolic effects in a phase IIa study in patients with Non-Alcoholic Fatty Liver Disease (NAFLD).[14] The same compound also yielded a significant reduction in Alkaline Phosphatase (a biomarker for the extent of liver impairment) in patients with Primary Biliary Cirrhosis (PBC) in phase IIb and phase III studies.[15,16] Further phase IIb data indicate histopathological improvements in patients with Non-alcoholic Steatohepatitis (NASH) upon treatment with OCA over 72 weeks.[17]

Here we describe the rationale and medicinal chemistry program that resulted in a series of FXR agonists with improved physicochemical and pharmacological properties relative to earlier compounds.

The first potent synthetic FXR agonist, GW-4064, established an isoxazole-containing prototype.[18] Many subsequent patents and publications sought to address the liabilities of GW4064, such as limited bioavailability and photolability.[19–25] Taken together, several general features have emerged in isoxazole-type FXR agonists that are necessary for maintaining activity (see Figure 1). The isoxazole core is preserved in all journal publications. In the patent literature, however, isoxazole replacements such as 1,2,3 triazoles or pyrazoles with similar substitution patterns and similar FXR potency have been described.[26] The 2,6-dichlorophenyl substituent of the GW4064 template is generally conserved as the most potent motif at this position, but alkylene spacers between this and the isoxazole ring can be introduced without significant loss in FXR activity (e.g. **2**, Figure 1).[21] Another approach to improve potency and reduce lipophilicity in this region has been to incorporate dichloropyridines and -pyridine *N*-oxides (e.g. **3**, Figure 1) as phenyl replacements, resulting in analogs with comparable potency and improved permeability.[20] However, *in vivo* data were not provided. In contrast to the isopropyl moiety at the 5-position of the isoxazole ring, which can be replaced only by cyclopropyl but no other small alkyl groups without loss in potency,[23] there is wide tolerability for structural variation in linking the oxymethylene substituent at the 4-position of the isoxazole core to the terminal acidic entity that seems to be a mandatory hallmark of potent FXR agonists of this type (Figure 1, **4**-**6** as examples).[21–26] A comprehensive review of isoxazole-type FXR agonists has been published.[31]

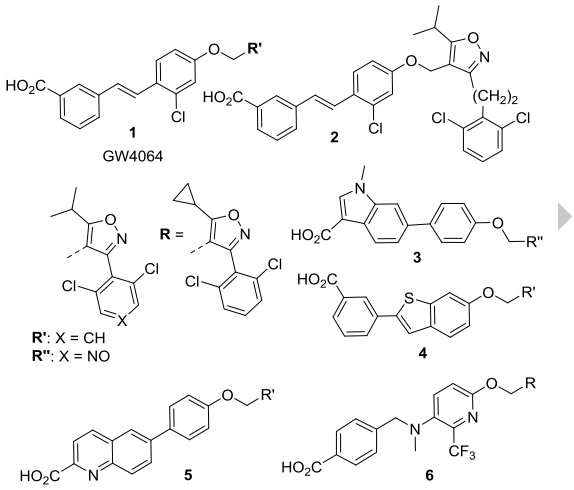


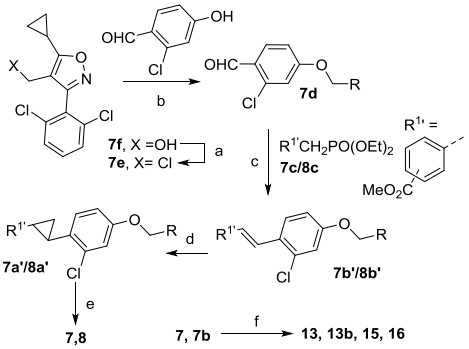
Figure 1
Representative FXR agonists of the isoxazole type.

In general, these compounds are lipophilic carboxylic acids whose amphiphilic properties limit their utility as drug candidates with regard to aqueous solubility and formulation for oral administration. Furthermore, off-target effects with membrane proteins in general and anion membrane transporters in particular are believed to increase with increasing lipo- and amphiphilicity.[27,28] Apart from GW4064, whose limited plasma exposure upon oral dosing has been described,[33] very limited data addressing *in vivo* pharmacological properties of isoxazole-type FXR agonists have been published.

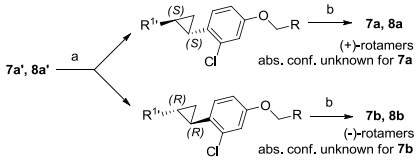
We have previously drawn attention to the similarity between synthetic isoxazole-type FXR agonists and natural bile acids by shape and polarity distribution, and this similarity manifests not only in FXR potency but also in recognition by Bile Acyl: Coenzyme A Synthase (BACS) and Bile Acyl Coenzyme A: Amino Acid N-acyltransferase (BAAT), enzymes that conjugate bile acids: taurine and glycine amides were found as phase II metabolites of **6** *in vivo*.[23] These earlier isoxazoles turned out to be very potent with regard to lipid lowering in a high fat diet mouse model, and the challenge here was to find novel isoxazole-type FXR agonists of similar or improved potency with the additional requirements of increased polarity, reduced amphiphilicity and consequently improved ADME properties.

The structures of the newly identified FXR agonists are shown in Table 1. The syntheses of analogs containing cyclopropyl as a linker element are depicted in Schemes 1–6. Racemic **7** and **8** were prepared in four steps. Alkylation of 2-chloro-4-hydroxy-benzaldehyde with chloro-methyl-isoxazole building block **7e** was followed by a trans-selective Horner-Wadsworth-Emmons (HWE) reaction with phosphonates **7c** or **8c** to furnish stilbenes **7b'** and **8b'**. Cyclopropanation was accomplished by reaction with diazomethane under palladium catalysis, whereby the trans geometry of the two phenyl substituents was maintained. Derivatives **13**, **15** and **16** were prepared from **7** by standard procedures (see Scheme 1). Eutomer **13b** was prepared from **7b** by the same procedure as **13** was prepared from **7**. The enantiomers of **7a'/8a'** were separated by chiral HPLC prior to ester hydrolysis (Scheme 2).[32]

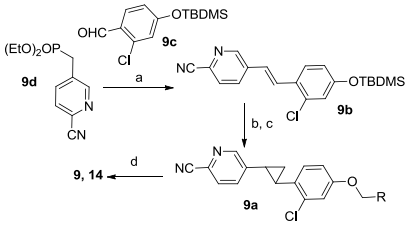
Pyridine analog **9** was introduced via the 2-cyano-pyridyl phosphonate **9d**, following a similar route as for **7/8**, leading to **9a**, which was either hydrolysed to carboxylic acid **9** or transformed into tetrazole **14** (Scheme 3). Pyridine analog **10** was prepared first by a HWE reaction but with inverted functionalities (Scheme 4), followed by phenol deprotection, alkylation with **7e**, then cyclopropanation and final ester hydrolysis.



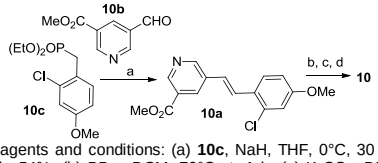
Scheme 1: Reagents and conditions: (a) $SOCl_2$, benzotriazole, DCM, 0–25°C, 1.5 h, 80%; (b) K_2CO_3, DMF, 60°C, O/N, 90%; (c) **7c/8c**, NaH, THF, 0°C, 30 min, then **7d**, 0°C–rt, 3 h, 60–77%; (d) CH_2N_2, $Pd(OAc)_2$, Et_2O, -50-0°C, 4–8 h, 55–60%; (e) LiOH, THF, H_2O (5:1 v/v), 50°C, O/N; (f) for **13**: $(COCl)_2$, DCM, cat. DMF, 0°C, then $MeSO_2NH_2$, DIEA, DMAP, 9%; for **15**: (I) HATU, DMF, 0°C, 30 min, then $NH_2CH_2CO_2Me$, DIEA; (II) LiOH, THF, H_2O (5:1, v/v), rt, O/N, 28%; for **16**: HATU, DMF, 0°C, 30 min, then $NH_2(CH_2)_2SO_3H$, rt, 18 h, 42%.



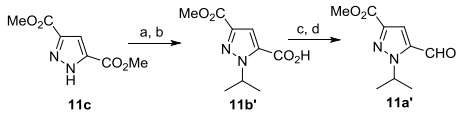
Scheme 2: Reagents and conditions: (a) prep. chiral HPLC, Chiralpak ODH, hexane/IPA; (b) LiOH, THF, H_2O (5:1 v/v), 50°C, O/N.



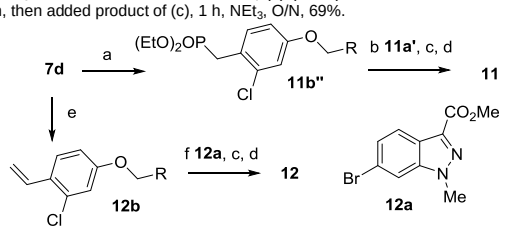
Scheme 3: Reagents and conditions: (a) **9d**, NaH, THF, 0°C, 30 min, then **9c** , 0°C–rt, 3 h, 37%; (b) CH_2N_2, $Pd(OAc)_2$, Et_2O, -50–0°C, 77%; (c) NaH, DMF, 0°C, 1 h, then **7e**, rt, O/N, 44%; (d) for **9**: KOH, EtOH/H_2O 3:1 v/v, reflux, 4 h, 29%; for 14: NaN_3, NH_4Cl, DMF, 100°C, O/N, 12%.



Scheme 4: Reagents and conditions: (a) **10c**, NaH, THF, 0°C, 30 min, then **10b** , 0°–rt, 1 h, 54%; (b) BBr_3, DCM, 70°C–rt, 1 h; (c) K_2CO_3, DMF, 60°C, O/N, 27% over two steps; (d) CH_2N_2, $Pd(OAc)_2$, Et_2O, -50–0°C, 4 h, 37%.



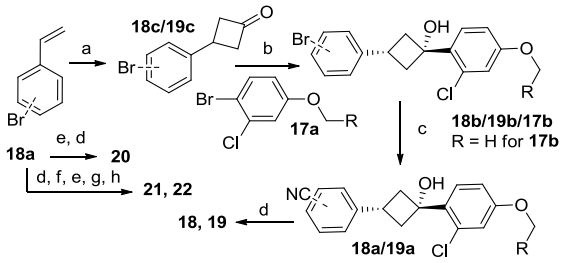
Scheme 5: Reagents and conditions: (a) **11c**, NaH, DMF, 0°C, 30 min, then 2-iodopropane in DMF, 0°C–rt, 2.5 h, 18%; (b) KOH, MeOH, 20°C, 24 h, 62%; (c) BH_3•Me_2S, THF, 0°C, rt, O/N, 70%; (d) $(COCl)_2$, DMSO, DCM, -30°C, 20 min, then added product of (c), 1 h, NEt_3, O/N, 69%.



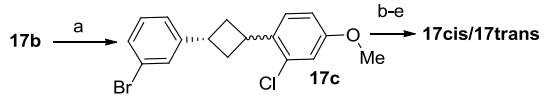
Scheme 6: Reagents and conditions: (a) *(I)*. $NaBH_4$, EtOH, 3 h, rt; *(II)*. PBr_3, Et_2O, 30 min, 0°C; *(III)*. $P(OEt)_3$, 175°C, 3 h; (b) **11b''**, NaH, THF, 0°C, 30 min, then **11a'**, 0°–rt, 3 h, 33%; (c) CH_2N_2, $Pd(OAc)_2$, Et_2O, -50–0°C, 4 h, 65–70%; (d) LiOH, THF, H_2O (5:1 v/v), 50°C, O/N, 17–48%; (e) n-BuLi, THF, $MeP(Ph)_3^+Br^-$, -60°C, 4 h, **7c**, -60°C–rt; (f) **12b**, **12a**, tri(orthotoloyl)phosphine, TEA, DMF, $Pd_2(dba)_3$, 100°C, 16 h, 23% over two steps.

The pyrazole containing analog **11** was prepared following the same approach from aldehyde **11a'**, which was prepared from the pyrazole diester **11c** by alkylation, selective ester hydrolysis and reduction (Schemes 5,6). The eutomer of **11**, **11b** was prepared by chiral HPLC separation of the racemic methyl ester of **11** and final ester hydrolysis, analogous to Scheme 2. The indazole analog **12** was prepared by a Heck reaction between vinyl building block **12b** and bromo-indazole **12a**, with the resultant olefin being transformed into **12** by methods analogous to those for **11**.

The syntheses of the FXR agonists containing cyclobutyl or azetidinyl as linker elements are depicted in Schemes 7–11. Cyclobutanone intermediates **18c/19c** were generated by cycloaddition with ketene in modest yields. Selective lithiation of bromo-chloro building block **17a** and addition to **18c/19c** at low temperature afforded hydroxycyclobutyl intermediates **18b/19b** in which the aryl substituents were trans. Palladium-catalysed cyanation and hydrolysis afforded analogs **18** and **19**. Further standard transformations were applied to obtain derivatives **20** to **22** from **18** or **18a** (Scheme 7). The des-hydroxycyclobutyl analog **17** was prepared by reduction of **17b** with sodium borohydride/TFA, affording **17c** as a mixture in which the cis isomer predominated (ca. 8:1). After phenol deprotection and cyanation the isomers were separated by preparative HPLC and then transformed into analogs **17cis** and **17trans** via alkylation with **7e** and final hydrolysis (Scheme 8).

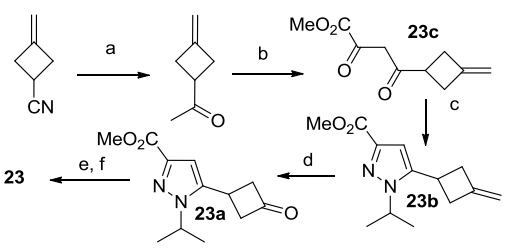


Scheme 7: Reagents and conditions: (a) DMF, $Cl(CH_2)_2Cl$, $(TfO)_2O$, 0°C, 1 h, then bromo-vinylbenzene, 2,4,6-collidine, reflux, O/N, 27%; (b) **17a**, n-BuLi, THF, -78°C, 1 h, then **18c** or **19c**, -78°C, 1 h, 37–39%; (c) DMF, $Zn(CN)_2$, $Pd_2(dba)_3$, Xantphos, 115°C, 10 h, microwave, 42%; (d) NaOH, EtOH, reflux, O/N, 56–90%; (e) NaH, DMF, MeI for **20**, EtI for **21**, $Br(CH_2)_2OTHP$ for **22**, 50°C, O/N, 41%; (f) CH_2N_2 (2M in Et_2O), THF, 0°C–rt, 1 h, 98%; (g) NaOH, MeOH, reflux, O/N, 26–50%, (h) for **22**: AcOH, THF, 40°C, 12 h, 10%.

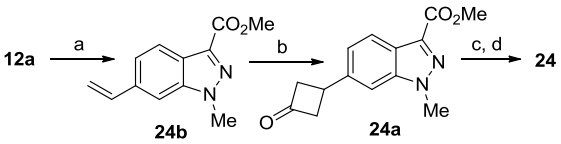


Scheme 8: Reagents and conditions: (a) $NaBH_4$, TFA, rt, O/N, 65%; (b) $Zn(CN)_2$, $Pd(PPh_3)_4$, DMF/H_2O (10:1 v/v), 90°C, O/N, 76%; (c) BBr_3, DCM, -78°C, cis/trans isomer separation by HPLC, 13% trans isomer; (d) **7e**, K_2CO_3, NaI, DMF, 60°C, O/N, 82%; (e) NaOH, EtOH/H_2O, reflux, O/N, 50%.

The synthesis of analog **23** started with 3-methylenecyclobutanecarbonitrile (Scheme 9). After reaction with methyl magnesium bromide the resultant ketone underwent a Claisen condensation with diethyl oxalate to afford diketone **23c**. A cyclisation with hydrazine hydrochloride in ethanol afforded the desired pyrazole regioisomer **23b**, and olefin oxidation then provided the cyclobutanone intermediate **23a** in good yield. Organometallic coupling of **17a** with **23a** was best achieved by preparation of the Grignard reagent from **17a** with *i*PrMgCl•LiCl, to which **23a** was added.



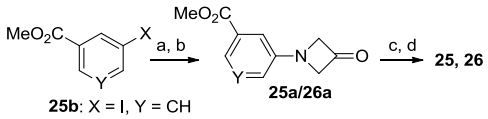
Scheme 9: Reagents and conditions: (a) MeMgBr, Et_2O, 0°C–rt; (b) NaOEt, EtOH, 15 min, then $(CO_2Et)_2$ added, 67°C, 4.5 h, rt, O/N, 56%, over two steps; (c) EtOH, N_2H_4-HCl, rt, 3 h; (d) $NaIO_4$, $RuCl_3$ x H_2O, EtOH/H_2O (77:13, v/v), rt, 45 min, $Na_2S_2O_3$, 78% over two steps; (e) *i*PrMgCl•LiCl, THF, **17a**, 0°C–rt, 4 h, then **23a**, -10°C–rt, 90 min, 61%; (f) THF, MeOH, H_2O, LiOH, rt, 4.5 h, 92%.



Scheme 10: Reagents and conditions: (a) tributyl(vinyl)tin, $Pd(Ph_3)_4$, DMF, 90°C, 4 h, 88%; (b) DMF, $Cl(CH_2)_2Cl$, $(TfO)_2O$, 0°C, 1 h, then **24b**, 2,4,6-collidine, reflux, O/N, 40%; (c) **17a**, n-BuLi, THF, -78°C, 1 h, then **18c** or **19c**, -78°C, 1 h, 40%; (d) THF, MeOH, H_2O, LiOH x H_2O, rt, 4.5 h, 45%.

This procedure resulted in a higher yield compared to the addition of lithiated **17a** and could be run at room temperature (Scheme 9). Analog **24** was prepared following the same route as for **18/19** (Scheme 10).

Hydroxyazetidine analogs **25** and **26** were prepared as depicted in Scheme 11, exploiting Cu- or Pd-catalysed coupling reactions of aromatic halides **25b/26b** with azetidin-3-ol. After oxidation to the azetidinones **25a/26a** the syntheses followed the same route as for **18/19**.

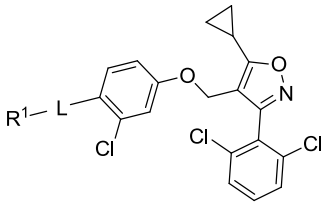


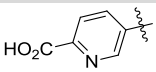
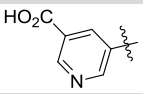
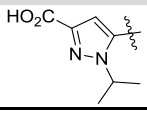
25b: X = I, Y = CH
26b: X = Br, Y = N

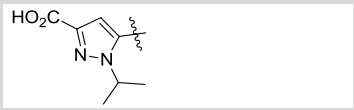
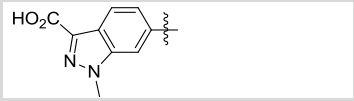
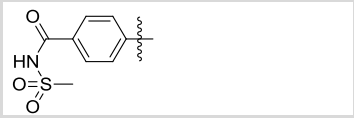
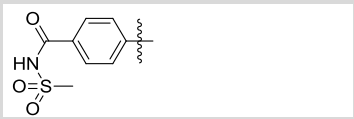
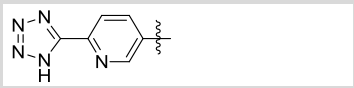
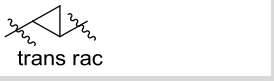
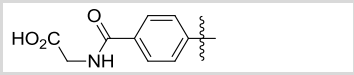
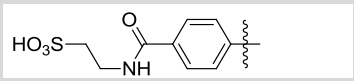
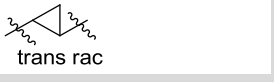
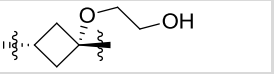
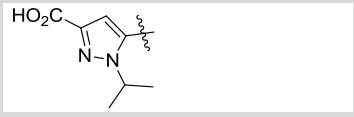
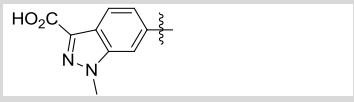
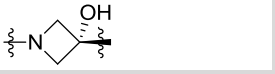
Scheme 11: Reagents and conditions: (a) for **25b**: 3-azetidine-3-ol HCl-salt, Cs_2CO_3, CuI, L-proline, DMSO, 90°C, 18 h, 66%, for **26b**: 3-azetidine-3-ol, Cs_2CO_3, BINAP, $Pd(OAc)_2$, dioxane, 85 °C, 12 h, 13%; (b) Dess-Martin periodinane, DCM, rt, 2 h (57–75%); (c) **17a**, n-BuLi, THF, -78 °C, 1 h, then **25a** or **26a**, -78 °C, 1 h (32–37%); (d) LiOH, THF, H_2O, 50°C, 12 h (23–58%).

The FXR agonistic activities of these analogs are shown in Table 1. The replacement of the stilbene olefin in GW4064 with a cyclopropyl linker element provided a series (**7**-**8b**) whose potency was dependent upon both the absolute stereochemistry of the cyclopropyl substituents, and upon the point of attachment of the carboxylic acid. Despite high lipophilicity (see Table 2), compounds **7** and **8** demonstrated pharmacokinetic parameters in the mouse which were suitable to investigate their in vivo pharmacology (see Table 3).

Table 1
Overview of new isoxazole-type FXR agonists.



Cpd	R_1	L	FRET EC_{50} [nM]	Eff (% Ref.)	M1H EC_{50} [nM]	Eff (% Ref.)
1	Structure see Figure 1		20	100	35	100
7	4-carboxy-phenyl	trans rac	131	100	76	85
7a	4-carboxy-phenyl	(S)*, (S)*, (+)-rotamer	1094	100	186	71
7b	4-carboxy-phenyl	(R)*, (R)*, (-)-rotamer	80	99	47	89
8	3-carboxy-phenyl	trans rac	32	102	34	87
8a	3-carboxy-phenyl	(S), (S), (+)-rotamer	219	101	90	88
8b	3-carboxy-phenyl	(R), (R), (-)-rotamer	18	100	29	98
9		trans rac	66	101	57	68
10		trans rac	10	103	14	73
11		trans rac	7	102	6	81

11b		 , (-)-rotamer	4	101	4	79
12		 trans rac	13	104	14	84
13		 trans rac	90	100	44	88
13b		 , (-)-rotamer	48	94	44	81
14		 trans rac	131	101	111	79
15		 trans rac	150	100	209	58
16		 trans rac	76	98	1372	34
17 trans	3-carboxy-phenyl		24	100	15	89
17 cis	3-carboxy-phenyl		77	99	92	88
18	3-carboxy-phenyl		29	100	25	79
19	4-carboxy-phenyl		24	105	63	71
20	3-carboxy-phenyl		79	93	102	75
21	3-carboxy-phenyl		277	94	220	89
22	3-carboxy-phenyl		373	97	377	68
23			9	100	110	78
24			19	99	50	82
25	3-carboxy-phenyl		54	97	86	94
26			7	102	552	71

FRET: Biochemical ligand dependent Nuclear Receptor-Cofactor peptide interaction assay, using the biotinylated SRC-1 peptide b-CPSSHSSLTERHKILHRLLQEGSPS-COOH (0.4 µM) and purified FXRaa$^{187–472}$-LBD fused to GST (2.5 ng) together with 200 ng streptavidine-allophycocyanine and 6 ng europium labeled-anti-GST as reagents in 25 µL assay buffer (20 mM Tris/HCl at pH 7.5; 5 mM $MgCl_2$; 60 mM KCl; 1 mM DTT; 0.9 g/L BSA). FRET values are given in nM and Eff (%) is the maximum efficacy of the compound relative to GW4064 are means of at least 2 assays.
M1H: Mammalian one hybrid assay; A cellular transient transfection assay were the human FXRaa$^{187–472}$-LBD is fused C-terminally to a Gal4 DNA-binding domain under transcriptional control of the CMV promoter in pCMV-BD (Stratagene). This chimeric plasmid construct is co-transfected into HEK293 cells together with pFRluc (Strategene) encoding a Gal4 promoter driven firefly luciferase. Cells were treated with serial dilutions of the test compound. EC_{50} values were calculated from at least three experiments.
Eff: % Efficacy is the extrapolated maximum signal generated by the test compound in a dose response dilution. Efficacy of GW4064 in the respective assay is set as 100%.
* Absolute configuration not known.

A first SAR exploration of the left hand side aromatic moiety identified several carboxy-bearing heteroaromatic analogs with further improved potencies such as **10**, **11**, and **12**. The greater polarity of these heteroaromatic moieties is reflected in a significant increase in aqueous solubility (measured for **10** and **11**, see Table 2) and an increase in membrane permeability (accompanied by a decrease in PAMPA membrane accumulation). Changing the position of the carboxy group from 3 (rel. to the cyclopropyl, **10**) to 4 (**9**) resulted in a drop in potency, highlighting the importance of the exact position of the acidic moiety on the terminal aromatic ring. A similar trend can be seen when comparing the corresponding phenyl analogs **7** and **8**.

The carboxylic acid moiety can be replaced by the acyl-sulfonamide (**13**) or tetrazole (**14**) isosteres without major loss of *in vitro* activity, opening up possibilities for minimizing phase II metabolism *in vivo* - indeed, **13** is slightly more potent than the corresponding carboxylic acid **7**.

When comparing the racemic mixtures **7**, **8**, **11** and **13** with their corresponding eutomers **7b**, **8b**, **11b** and **13b** a similar increase in potency in the FRET assay is observed (1.64 to 1.88 fold). Although the absolute configuration of the eutomers have not been elucidated for **7b**, **11b** and **13b** they exhibit the same sense of optical rotation, pointing towards a same absolute configuration of the cyclopropyl linker. (**7b** and **13b** have the same absolute configuration since **13b** is prepared from **7b**, see Scheme 1). Together, these findings indicate that the difference in activity between distomers and eutomers is mainly caused by a more favourable binding of the cyclopropyl linker in the eutomer series ((-)-rotamers).

The glycine and taurine conjugates of **7** (**15** and **16**), both of which have been detected *in vivo* in mice (data not shown; see also ref 23), display similar potency compared to the parent in the FRET assay. The activity of **16** in the cellular assay is significantly reduced, likely due to lower cellular permeability as a result of the strongly acidic sulfonic acid. This effect is similar to that observed with natural bile acids and their taurine conjugates.[1,2]

Since the cyclopropyl linker element is chiral in its nature and no reliable enantioselective synthesis to access the single enantiomers in a cost efficient manner was available, we considered achiral alternatives. The introduction of a 1,4-disubstituted cyclobutyl linker led to compounds **17trans** and **17cis**. Both compounds displayed encouraging biochemical and cellular FXR potency, with the trans isomer being 3-fold more potent in the FRET assay and 6-fold more potent in the M1H assay. Compound **18** was synthesized opportunistically from the hydroxylated cyclobutyl intermediates generated in the synthesis of **17** and was also tested for activity. Surprisingly, it showed a very similar potency compared to **17trans**, despite the substantial polarity increase that is reflected in improved aqueous solubility. The presence of a hydroxyl substituent in this area of isoxazole-type FXR ligands is unprecedented. The suitability of the newly identified hydroxybutyl moiety as a potent and polar achiral linker element was further corroborated by the synthesis of direct homologs to the cyclopropyl examples, yielding the additional pairs **7**/**19**, **11/23**, and **12/24**. In all cases, the hydroxybutyl analogs retained FXR activity with increased aqueous solubility, improved membrane permeability and decreased PAMPA membrane accumulation.

The similar FXR activity of these pairs of ligands is suggestive of a specific interaction of the hydroxyl group with the FXR ligand binding domain (LBD), so as to compensate for the energy penalty of desolvation of the hydroxyl group upon binding. This hypothesis is further supported by a small set of ether derivatives (**20–22**): removal of the H-bond donor in the methyl ether **20** reduces FXR activity by approximately 3- to 4-fold, and ethyl ether **21** and hydroxyethyl ether **22** show even further reduced activity.

Accordingly, FXR LBD cocrystal structures with similar ligands (GW4064, GW8062 from Ref. 29) were used as a template for docking studies with **17trans** and **18** (which differ only in the presence of the hydroxyl group) to search for potential novel interactions. These experiments generated structures in which conformational changes revealed a beneficial H-Bond interaction with the side chain of Serine[329] (see Figure 2). An interaction with this serine residue has previously been described for a structurally unrelated class of FXR agonists as well as for bile acid derivatives.[30]

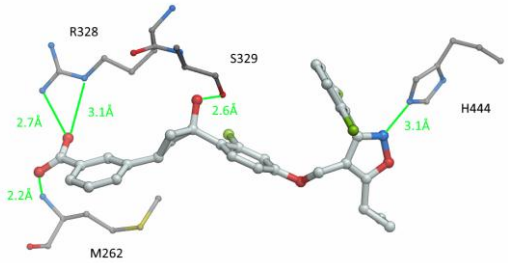


Figure 2
Ball and stick representation of compound **18** docked into the LBD of human FXR. The hydroxyl substituent at the cyclobutyl moiety of **18** points to the hydroxyl functionality of Ser329 with a calculated distance of 2.6 Å, suggesting a H-bond interaction. For docking experiments we used ICM 3.8-4a modeling software (MolSoft LLC, San Diego, CA).[35] The complex of FXR with GW4064 (PDB:3DCT) was used as reference structure. During the docking simulation standard parameters were applied and Ser329 were allowed to be flexible.

Building on these encouraging results, compounds **25** and **26** were synthesized as hydroxyazetidinyl analogs of **8/18** and **10** in which stereoisomers are absent. While **25** has a slightly reduced activity in the FRET and M1H assay compared to **18**, compound **26** is equipotent with **10** in the FRET assay (7 vs. 10 nM EC_{50}), although it shows reduced activity in the cellular assay.

The calculated physicochemical parameter clogD of a subset of these analogs is provided in Table 2. A gradual increase in hydrophilicity from compound **7** to compound **26** can be observed, with introductions of heteroaromatic carboxylic acid moieties, the hydroxy-cyclobutyl linker and the hydroxy-azetidinyl linker representing the major increments. The impact on solubility of the linker modifications is comparable to that of the benzoic acid replacements (compare **8** to **10** or **18**). A further beneficial effect for compounds that bear both linker and benzoic acid replacements simultaneously can be seen in the PAMPA assay: compounds **23** and **26** demonstrate the highest membrane flux and the lowest membrane accumulation in the set. The low membrane accumulation of only 3 fold for compound **26** is remarkable, given the general tendency of these types of amphiphilic compounds to insert into artificial bilayer membranes.

Interestingly, the PAMPA membrane accumulation, together with the clogD seem to correlate with the observed shift from the biochemical to the cellular FXR activity: Compounds **7**, which has a high clogD (5.2) and a high membrane accumulation (30 fold) is more active in the M1H assay than in the FRET assay, while compound **26** with the lowest clogD (3.2) and the lowest membrane accumulation (3 fold) is two orders of magnitude less active in the cellular assay than in the biochemical assay. There is no standard model which would explain why more lipophilic and membrane-accumulating compounds tend to be more potent at FXR compared to more hydrophilic ones. However, a recent publication provides a hypothesis to explain this phenomenon.[34]

The two most polar analogs **23** and **26** were tested on a panel of 20 nuclear receptors, including PXR and showed no activity up to 5 µM.

Table 2

Physicochemical properties and PAMPA assay data for selected FXR agonists.

Cpd	clogD	aq. solubility [µM]	PAMPA [%flux]*	PAMPA [fold memb. enrich.]**
7	5.2	72	14	30
8	5.1	20	17	33
10	3.8	165	n.d.	n.d.
11	4.2	158	29	22
17trans	5.6	n.d.	0	319
18	4.4	192	24	27
19	4.4	192	21	9
23	3.5	171	46	11
25	4.4	n.d.	27	40
26	3.2	197	35	3

* = $[C_{acceptor}/(C_{donor} + C_{acceptor})]$ x 100 x 2, after 16 h at rt; ** = fold enrichment in the membrane compartment is determined by $C_{membrane} * 2 / (C_{acceptor}+C_{donor})$ with $C_{membrane}$, $C_{acceptor}$ and C_{donor} representing the concentrations of this compound in the membrane phase (extracted into a volume equal to acceptor or donor compartment) or the acceptor or donor compartment, respectively.

The pharmacokinetic profiles of a subset of analogs in mice were also assessed (Table 3). Overall there was a clear general trend that more hydrophilic compounds had an increased plasma exposure, a reduced clearance and a reduced volume of distribution (compare **8** with **18**, **26**; **11** with **23**). In addition, the position of the carboxylic acid on the aromatic ring had a dramatic impact on the plasma exposure (compare **18** with **19**). Analogs with the acid in the 3-position relative to the linker element exhibited improved properties compared to their 4-position isomers. Notably, the difference was greater in the more polar hydroxycyclobutyl series compared to the cyclopropyl series (compare **7** with **8** and **18** with **19**).

Table 3
In vivo pharmacokinetic properties of selected FXR agonists in C57BL/6J mice.

Cpd.	AUC [hr*ng/mL]	Vd/F [L/kg]	C_{max} [ng/mL]	Cl/F [mL/min/kg]	$T_{1/2}$ [h]	F%
7	430	3.6	280	85	0.5	44
8	800	4.7	1060	55	1.0	53
11	110	5.5	160	83	0.8	11
18	8600	0.3	9800	4.7	0.6	49
19	180	3.9	96	190	1.1	41
23	960	0,8	1470	16	0.6	18

Dose: 5mg/kg p.o., 1 mg/kg i.v. All parameters except F were calculated from the p.o. arm.

To assess pharmacodynamic properties, compounds **7**, **8**, **11**, **18**, and **23** were administered at a dose of 10 mg/kg for 10 days to C57BL/6J mice pre-fed and maintained on a high fat diet with 1% (w/w) cholesterol. Despite the differences in PK profiles, each of these showed significant improvements in plasma lipid profiles (Figure 3A). Compounds **7** and **11** exhibited the highest cholesterol reduction, followed by compounds **18** and **13**. Compound **8b** turned out to be less active. Liver levels of the compounds 4 hours after the final dose were assessed, but there were no obvious relationships between plasma or liver exposure and the lipid lowering effects observed (Figure 3B and 3C).

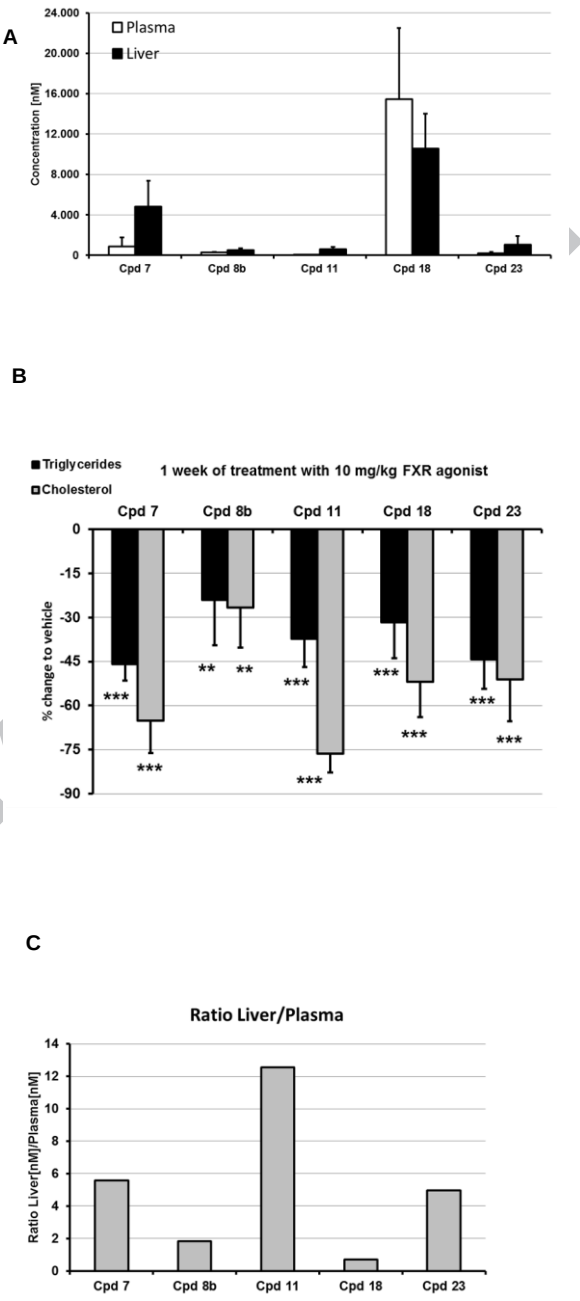


Figure 3
Changes in total plasma cholesterol and total plasma triglycerides upon 10 days of 10 mg/kg p.o. administration of the indicated compounds into C57BL/6J mice prefed and maintained on a high fat diet (60% kcal fat) enriched with 1% (w/w) cholesterol. Shown are the % changes to the vehicle control group (**3A**). Liver levels (**3B**) and liver/plasma ratios (**3C**) of the indicated compounds (parent only) upon final sacrification of the animals, 4 h after the final gavage (* = statistical significance ranges; *p<0.05, **p<0.01, ***p<0.001).

In summary, the challenge of improving polarity, reducing amphiphilicity and thereby improving pharmacokinetic properties while maintaining potency of GW4064-type isoxazole FXR agonists was successfully addressed by introducing a hydroxyl-bearing 4-membered ring (either cyclobutyl or azetidinyl) as a linker between the middle and the terminal aryl rings. The hydroxyl group may engage in an H-bond interaction with Ser^{329} of the FXR LBD. This is an unprecedented interaction within the series of isoxazole FXR agonists and might be further explored to

generate improved analogs within this class of compounds.

The data provided here show that there is no direct correlation between physicochemical and *in vivo* pharmacokinetic and pharmacodynamic properties. However, the more polar isoxazole-type FXR agonists are clearly improved in terms of general drug-likeness (aqueous solubility, membrane permeability), which will likely facilitate pharmaceutical development. These analogs exhibit *in vivo* efficacies that are similar to those of the cyclopropyl linker compounds and other similarly lipophilic FXR agonists.[23]

Clearly, further studies are required that elucidate the mechanistic basis for the observed ADME properties and their relationships to the pharmacodynamic properties of this class of novel FXR agonists.

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