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Synthesis and pharmacological validation of a novel series of non-steroidal FXR agonists

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ABSTRACT

To overcome the known liabilities of GW4064 a series of analogs were synthesized where the stilbene double bond is replaced by an oxymethylene or amino-methylene linker connecting a terminal benzoic acid with a substituted heteroaryl in the middle ring position. As a result we discovered compounds with increased potency in vitro that cause dose-dependent reduction of plasma triglycerides and cholesterol in db/db mice down to 2 × 1 mg/kg/day upon oral administration.

The Farnesoid X Receptor (FXR) is a member of the nuclear hormone receptor superfamily and senses bile acid levels as its endogenous ligands.[1–3] FXR is mainly expressed in tissues which are exposed to bile acids such as the entire gastrointestinal tract, the liver and the gallbladder. FXR mRNA can also be found in adrenals, kidneys, adipose tissue, and possibly the pancreas.[4,5] Activation of FXR by derivatives of natural bile acid ligands such as 6-ethyl-chenodeoxycholic acid (6-ECDCA) or by synthetic non-steroidal agonists like GW4064 results in beneficial metabolic effects such as glucose lowering, insulin sensitisation, triglyceride, and cholesterol lowering[6–8] in various rodent models. Moreover, FXR agonists seem to have specific hepatoprotective functions by preventing lipid accumulation and reducing fibrosis and inflammation in the liver, specifically. Beyond the effects provided by its liver-specific functions, FXR controls the expression of a key cytokine, FGF-15/19 in the intestine, specifically the ileum.[9] FGF-19 has insulin sensitizing, body weight lowering and lipid lowering effects, thereby extending the action of FXR agonists indirectly to all tissues which are generally responsive to FGF-15/19.[10] Recently, the release of positive data from two phase II studies using 6-ECDCA (INT-747), a bile acid derivative and orally available FXR agonist, in patients with Primary Biliary Cirrhosis (PBC), a chronic inflammatory cholestatic condition in the liver, and in Type II diabetics with non-alcoholic fatty liver disease (NAFLD) validated the clinical utility of FXR agonists in these metabolic and hepatic indications. 6-ECDCA administration to Type II diabetic patients for 6 weeks improved insulin sensitivity and lowered body weight in these individuals.[11] However, high doses of 6-ECDCA seems to be associated with the induction of pruritus in both PBC patients and healthy volunteers (phase I study from the same group of investigators[12]). These potential adverse effects of higher doses of 6-ECDCA may limit the clinical utility of this drug, fostering the need for further FXR agonists with potent in vivo activity.

GW4064 is the first synthetic non-steroidal FXR agonist described in the literature.[13] It demonstrates potent FXR binding and activation in biochemical and cellular in vitro assays as well as pharmacological effects in different rodent models of metabolic and other diseases. However, its suitability as a drug for FXR targeted pharmacotherapy is questionable given that this compound harbors a *trans* stilbene moiety as a potential toxicophore and given its photolability.[14] Various groups have tried to overcome the liabilities associated with this prototypical non-steroidal FXR agonist and have generated derivatives of GW4064 without the *trans* stilbene moiety.[15,16] Most of these approaches, however, did not provide compounds with improved potency at FXR.

Using GW4064 as a starting point, we have modified its structure to increase polarity, improve bioavailability, and potentially

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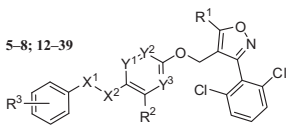
also increase potency at FXR. Here we report the synthesis and pharmacological evaluation of a novel series of FXR agonists wherein the core features of GW4064 are mostly maintained and the *trans* stilbene moiety of GW4064 is replaced by 3- and 4-benzoic acid moieties linked to a substituted pyridine in the middle ring position by oxymethylene or amino-methylene linkers in both possible orientations. For those cases where the –O– or –NH– or –NMe– was the attachment point to the middle ring, which is linked to the isoxazole by another oxymethylene linker, we replaced the 3-Cl-phenyl from the GW4064 by different $-CF_3$ substituted pyridines in order to reduce the potential formation of quinoidal structures. Table 1 shows the compounds which have been synthesized and their activities in a biochemical FXR FRET assay, a cellular Gal4-LBD reporter gene assay (M1H) and a full length FXR direct reporter (DR) assay.

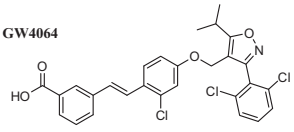
Schemes 1–3 provide an overview on the synthetic strategies chosen for making various derivatives within this structural class of close GW4064 analogs. The pyridine-3-yl-containing analogs are prepared according to Scheme 1. Briefly, commercially available 6-methyl-5-nitropyridin-2-ol (**1a**) or the corresponding trifluoro-methyl analog (**1b**) were alkylated with isoxazole building blocks **2a** or **2b** via a Mitsunobu reaction using DIAD. The nitro group of the intermediates were reduced with zinc in acetic acid to afford the common intermediates **3a–c**. Standard derivatization such as amide bond formation with benzoyl chlorides or reductive alkylation with benzaldehydes, subsequent alkylation of the amide NH or the secondary amine with MeI or EtI and final ester hydrolysis gave analogs **5**–**8**, **12**–**15**, **17**, **19**, **22**, and **24**. The hydroxyethyl analog **18** was prepared in a similar fashion via a reductive alkylation of **3b** with benzaldehyde **4b** followed by a final hydroxyl deprotection. The analog **9** was prepared by a Mitsunobu reaction of 4-methyl-5-nitropyridin-2-ol with **2a** whereas **10** and **11** were prepared by reaction of deprotonated **2a** with 2-chloro-4-methyl-quinoline and 2-chloro-6-methyl-4-(trifluoro-methyl)-pyridine, respectively.

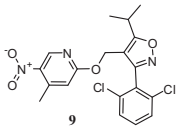
Scheme 2 shows the synthesis of analogs with modified acidic moieties. Acyl sulfonamides **16** and **20** are obtained from **13** and **15**, respectively, by reaction with EDCI and methane sulfonamide, while analog **21** is obtained in a two step sequence, first activation of the carboxylic acid as an ONSu ester and subsequent reaction with *O*-phospho-ethanolamine in the presence of sodium bicarbonate as base. For sulfonic acid derivative **23** the best result was obtained when taurine was coupled to the carboxylic acid using the phosphonium based coupling reagent HATU. The two pyridine-2-yl-containing analogs **27** and **28** were prepared as follows (Scheme 2): 2,3-dichloro-5-nitropyridine was reacted by nucleophilic substitution of the chlorine in 2-position with methyl 3-(aminomethyl)-benzoate or methyl 4-(aminomethyl) benzoate, respectively, followed by diazotation and iodination at the 5-position. A CuI/phenanthroline catalyzed cross coupling reaction of intermediates **26a** or **26b** with **2a**, followed by ester hydrolysis afforded compounds **27** and **28**.

Scheme 3 summarizes the synthesis of analogs containing a phenolether linker element (**35**–**39**) and analogs **33** and **34**, containing inverted amino-methylene linkers. Commercially available tetrahydropyridone derivative **29** was converted to the corresponding 2-hydroxy-pyridine by bromination with NBS and elimination of HBr. This intermediate is alkylated with **2b** via a

Table 1
Overview of compounds made as FXR active GW4064 analogs







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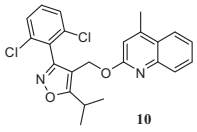

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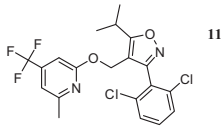

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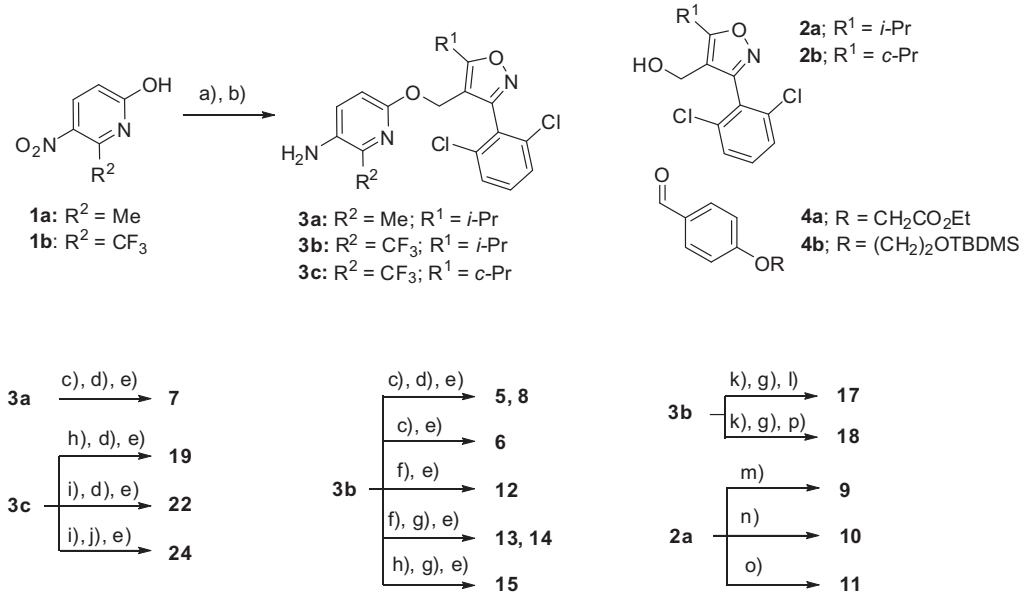
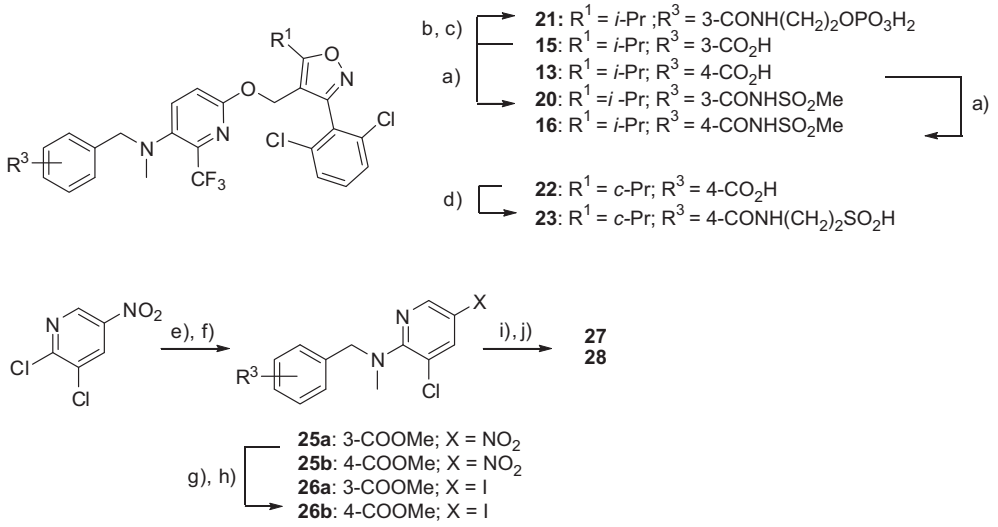
Compd	R^1	R^2	R^3	X^1	X^2	Y^1	Y^2	Y^3	FRET (nM)	Eff (%)	M1H (nM)	Eff (%)	DR (nM)	Eff (%)
GW4064			See extra structure						20	100[a]	35	100[a]	30	100[a]
5	*i*-Pr	CF_3	*p*-COOH	CO	N–CH_3	CH	CH	N	235	101	316	36	208	63
6	*i*-Pr	CF_3	*p*-COOH	CO	NH	CH	CH	N	452	109	474	21	385	48
7	*i*-Pr	CH_3	*p*-COOH	CO	N–CH_3	CH	CH	N	365	95	2030	21	1190	49
8	*i*-Pr	CF_3	*m*-COOH	CO	N–CH_3	CH	CH	N	433	110	571	35	537	55
9			See extra structure						89	101	1000	58	1140	76
10			See extra structure						147	104	536	40	878	68
11			See extra structure						225	98	Inactive	Inactive	Inactive	Inactive
12	*i*-Pr	CF_3	*p*-COOH	CH_2	NH	CH	CH	N	162	97	398	48	101	45
13	*i*-Pr	CF_3	*p*-COOH	CH_2	N–CH_3	CH	CH	N	22	97	90	75	15	57
14	*i*-Pr	CF_3	*p*-$(CH_2)_2$–COOH	CH_2	N–CH_3	CH	CH	N	17	96	35	55	16	66
15	*i*-Pr	CF_3	*m*-COOH	CH_2	N–CH_3	CH	CH	N	12	102	80	89	6	58
16	*i*-Pr	CF_3	*p*-CONH–SO_2–CH_3	CH_2	N-CH_3	CH	CH	N	40	105	221	64	11	67
17	*i*-Pr	CF_3	*p*-OCH_2–COOH	CH_2	N–CH_3	CH	CH	N	35	90	214	69	19	59
18	*i*-Pr	CF_3	*p*-O$(CH_2)_2$–OH	CH_2	N–CH_3	CH	CH	N	164	87	1150	43	155	59
19	*c*-Pr	CF_3	*m*-COOH	CH_2	N-CH_3	CH	CH	N	3	102	9	79	2	43
20	*i*-Pr	CF_3	*m*-CONH–SO_2–CH_3	CH_2	N–CH_3	CH	CH	N	48	110	279	59	20	72
21	*i*-Pr	CF_3	*m* CONH–$(CH_2)_2$–OPO_3H_2	CH_2	N–CH_3	CH	CH	N	36	114	457	48	216	61
22	*c*-Pr	CF_3	*p*-COOH	CH_2	N–CH_3	CH	CH	N	12	109	30	81	6	60
23	*c*-Pr	CF_3	*p*-CONH–$(CH_2)_2$–SO_3H	CH_2	N–CH_3	CH	CH	N	14	105	1420	57	925	49
24	*c*-Pr	CF_3	*p*-COOH	CH_2	N–CH_2CH_3	CH	CH	N	194	94	180	54	113	43
27	*i*-Pr	Cl	*p*-COOH	CH_2	N–CH_3	N	CH	CH	242	103	107	70	120	51
28	*i*-Pr	Cl	*m*-COOH	CH_2	N–CH_3	N	CH	CH	190	105	100	42	61	54
33	*c*-Pr	CF_3	*p*-COOH	NH	CH–CH_3	CH	CH	N	101	99	333	59	202	49
34	*c*-Pr	CF_3	*p*-COOH	N–CH_3	CH–CH_3	CH	CH	N	179	102	208	55	182	57
35	*c*-Pr	CF_3	*o*-COOH	O	CH_2	CH	CH	N	434	91	1710	63	611	33
36	*c*-Pr	CF_3	*m*-COOH	O	CH_2	CH	CH	N	43	98	151	62	33	41
37	*c*-Pr	CF_3	*p*-COOH	O	CH_2	CH	CH	N	76	86	95	59	38	42
38	*c*-Pr	CF_3	*m*-COOH	O	CH–CH_3	CH	CH	N	155	102	107	50	58	49
39	*c*-Pr	CF_3	*p*-COOH	O	CH–CH_3	CH	CH	N	352	89	189	70	611	33

[a] Efficacy is set as 100%. The experimental details can be found in the supplementary data.



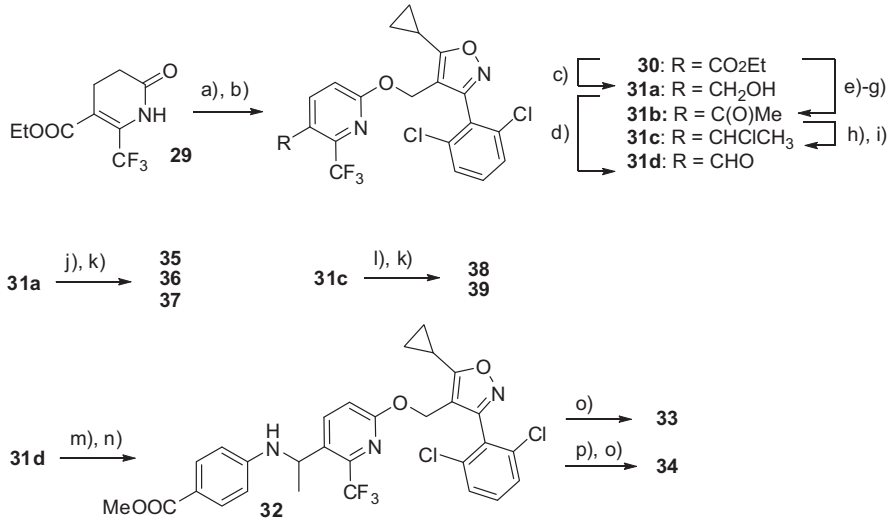
Scheme 1. For advanced starting materials see Ref. [21], reagents and conditions: (a) **2**, PPh_3, C_6H_6 or $PhCH_3$, DIAD, rt, 12 h; 54–88%; (b) Zn, AcOH, MeOH, rt, 2 h, 75–95%; (c) methyl 4-(chlorocarbonyl) benzoate (for **5**) or methyl 3-(chlorocarbonyl)benzoate (for **8**), TEA, CH_2Cl_2, rt, 2 h, 31–65%; (d) NaH, MeI, THF, rt, 17 h; 56–79%; e) NaOH, H_2O, 50 °C, 3 h; 54–100%; (f) methyl 4-formylbenzoate (for **13**) or methyl 3-(4-formyl-phenyl)propanoate (for **14**), $NaBH(OAc)_3$, AcOH, DCE, rt, 24 h, 49%; (g) paraformaldehyde, $NaBH(OAc)_3$, AcOH, DCE, rt, 24 h, 59–70%; (h) methyl 3-formylbenzoate, THF, $BF_3{\cdot}Et_2O$, rt, 12 h, then $Na(CN)BH_3$, rt, 1–2 h, 89–95%; (i) methyl 4-formylbenzoate, THF, $BF_3{\cdot}Et_2O$, rt, 12 h, then $Na(CN)BH_3$, rt, 4 h, 70%; (j) EtI, NaH, DMF, 0 °C, 2 h; (k) **4a** (for **17**) or **4b** (for **18**), THF, $BF_3{\cdot}Et_2O$, rt, 12 h, then $Na(CN)BH_3$, rt, 1 h, 53–70%; (l) LiOH, THF, H_2O, rt, 12 h, 83–92%; (m) 4-methyl-5-nitropyridin-2-ol, PPh_3, DIAD, C_6H_6, rt, 12 h, 56%; (n) 2-chloro-4-methylquinoline, NaH, THF, rt, 12 h, 70%; (o) 2-chloro-6-methyl-4-(trifluoromethyl)pyridine, NaH, THF, rt, 12 h, 55%; (p) TBAF, THF, rt, 5 h, 75%.



Scheme 2. Reagents and conditions: (a) methane sulfonamide, EDCI, DMAP, CH_2Cl_2, rt, 12 h, 50–60%; (b) HOSu, DCC, EtOAc, rt, 12 h, 93%; (c) *O*-phospho-ethanolamine, $NaHCO_3$, THF, H_2O, rt, 48 h, 17%; (d) HATU, DMF, 0 °C, 30 min, then taurine, DIEA, rt, 18 h, 50%; (e) methyl 3-(aminomethyl) benzoate hydrochloride or methyl 4-(aminomethyl)benzoate hydrochloride, DIPEA, *i*-PrOH, 60 °C, 1 h, 92–96%; (f) NaH, MeI, DMF, rt, 2 h, 78–96%; (g) $Na_2S_2O_4$, MeOH, H_2O, 90 °C, 1 h, 36–39%; (h) isoamylnitrite; CH_2I_2, cat. HI, rt, 2 h, 44–47%; (i) 3.5 equiv **3a**, cat. Cu, cat. 1,10-phenanthroline, Cs_2CO_3, $PhCH_3$, 120 °C, 14 h, 65–73%; (j) LiOH, THF, MeOH, H_2O, rt, 6 h, 73–79%.

Mitsunobu reaction to afford common intermediate **30**. After reduction of **30** to the corresponding hydroxymethyl analog **31a** with LAH compounds **35**–**37** were obtained by Mitsunobu alkylation with *o*-, *m*-, and *p*-methyl benzoates and final ester hydrolysis. Analogs **38** and **39** were obtained by alkylation of methyl 3- or 4-hydroxybenzoate with chloride **31c** and final ester hydrolysis. Chloride **31c** in turn was prepared from **30** by transformation of the ethyl ester into the Weinreb amide, reaction with MeMgBr, reduction of the ketone to the alcohol and ensuing chlorination. Analogs **33** and **34**, containing inverted amino-methylene linkers, were prepared by forming first the Schiff-base of methyl 4-amino-benzoate with aldehyde **31d**, followed by addition of a methyl group by reaction with MeLi, then either direct hydrolysis of the ester (**33**) or methylation of the secondary amine and final ester hydrolysis (**34**).

Modification of the original isopropyl at the 5-position of the isoxazole into a cyclopropyl results in an about two to fourfold increase in FXR activation potency (compare **13** to **22** and **15** to **19**). Notably, derivatives that just contain the middle heteroaryl ring either as a bicyclic structure such as in **10** or as substituted pyridyl like in **9** or **11** maintain FXR agonistic capabilities as the FRET biochemical assay data indicate. This suggests that a core



Scheme 3. Reagents and conditions: (a) NBS, CCl_4, reflux 18 h; (b) **2b**, PPh_3, DIAD, THF, rt 1.5 h, 74% (two steps); (c) LAH, THF, rt, 2 h, 86%; (d) MnO_2, $CHCl_3$, reflux, 3 h, 92%; (e) LiOH, MeOH, H_2O, rt, 12 h, 100%; (f) *N,O*-dimethylhydroxylamine hydrochloride, HATU, DIPEA, DMF, rt, 12 h, 82%; g) MeMgBr, THF, −78 °C to rt, 2.5 h, 79%; (h) LAH, THF, 0 °C, 2 h, 94%; (i) $SOCl_2$, $CHCl_3$, 0 °C, 2 h, 92%; (j) methyl hydroxybenzoate (*o*-, *m*- or *p*-), PPh_3, DIAD, THF, rt, 12 h, 60–65%; (k) LiOH, MeOH or THF, H_2O, reflux, 5 h, 60–90%; (l) methyl 3-hydroxybenzoate or methyl 4-hydroxybenzoate, NaH, DMF, 100 °C, 12 h, 22–38%; (m) methyl 4-aminobenzoate, PPTS, $PhCH_3$, Dean–Stark, 16 h; (n) MeLi, THF, rt, 1.5 h, 56%; (o) LiOH, THF, H_2O, rt, 12 h, 36–48%; (p) NaH, THF, MeI, 0 °C to rt, 2 h, 48%.

structure containing the 2,6-dichlorophenyl-isoxazole moiety contributes a major part to the binding as well as to the activation properties of this type of ligands. While omitting the linker and aryl-acid part results in an only minor loss of FXR cell-free activity the loss of potency in the cellular assays is more dramatic.

The substituents R^2 contribute potency with a $-CF_3$ being the best amongst –Cl, $-CF_3$, and $-CH_3$. Halogens have not been tried in the case of the pyridin-3-yl because the proximity to the pyridine nitrogen may cause an undesirable instability of such substituted heteroaryls.

The two pyridin-2-yl derivatives (**27**, **28**) have chlorines in the R^2 position of the middle ring and therefore resemble the original substitution pattern of GW4064 except for the different linker and the pyridine-*N*. Compared to the pyridine-3-yl compounds these two examples are significantly less potent in all three assays and were not further explored.

A strong impact on the FXR activity of these isoxazole derivatives comes from the configuration of the two-atom spacer between the middle (hetero)aryl and the COOH bearing terminal phenyl. The crystal structure of GW4064 suggests to replace the stilbene double bond by a linker of similar length with the additional constraint that the slightly staggered coplanar conformation of the middle aryl and the terminal benzoic acid might be kept in order to maintain FXR agonism and potency. We synthesized (methyl)-amino-methylene and oxymethylene linkers in both orientations between the middle heteroaryl and the terminal benzoic acid except for the oxymethylene with the –*O*– attached to the middle heteroaryl in order to avoid potential benzoquinone-like structure formation in vivo. The *N*-methyl substituted amino-methylene linker with nitrogen attached to the middle aryl yielded by far the best results. Omitting the methyl substituent at the nitrogen results in an appr. 20-fold loss of activity whereas replacement of *N*-methyl by ethyl results in decreased FXR activity, suggesting that there is no larger lipophilic pocket to accommodate the *N*-alkyl substituent (compare **12**, **13**, **22**, and **24**, respectively). Changing the orientation of the heteroatom in the linker decreases potency by about 5- to 10-fold (compare **22** vs **34**).

Considerable attention should be given to the nature and the position of the acid moiety at the terminal phenyl ring. All derivatives that contain a carboxylic acid at the terminal phenyl show a consistent correlation between activities in biochemical and cellular assays. When these benzoic acids are changed into –COOH bioisosteres with similar acidity such as the acylsulfonamides **16** and **20**, this correlation is still maintained. However, compounds which have permanently charged acidic functions with increased polarities such as **21** or **23** display a strong disparity between the FRET results which indicate still good binding to FXR and the M1H and DR data demonstrating a rather poor cellular activity, probably due to their diminished membrane permeability. Just increasing the distance between the phenyl and the $-COO^-$ moiety as exemplified in **14** and **17** as opposed to **13** neither has a strong impact on the potency in the FRET assay nor in the cellular assay.

Molecular modeling studies suggest that the prolonged distance to the acidic function should be incompatible with the formation of the known ionic interaction to Arg331 as described in Refs. 14 and 15 Pellicciari et al.[17] suggest that there is a 'hole' in the FXR ligand binding pocket structure which marks an exit vector for substituents ranging out from the *p*-position of the terminal aryl passing along the Arg331 that forms an important salt bridge with the benzoic acid $-COO^-$ of the ligand towards the aqueous surface of the receptor. If compounds **14**, **21**, and **23** really reach out into the solvent surrounding of the FXR protein, then the only minor differences in the biochemical assay potency might imply that the loss of binding enthalpy (see Fig. 1) by losing the ionic interaction to Arg331 might be compensated by the gain in H_2O solvatisation enthalpy. Docking studies with ligands **13** and **23** suggest, however, that the $-SO_3^-$ group orients towards Arg264 resulting in an ionic interaction in addition to maintaining an H-bond interaction between the amide –C=O and Arg331 as well as forming a new one to Ser342. Since most docking programs cannot precisely account for solvation enthalpy effects it remains unclear whether in reality the solvation effect or the formation of a new charged interaction prevails.

Since the active isoxazole derivatives from this series should mimic the natural ligand bile acids at FXR we speculated that transfection of the bile acid transporter NTCP (= Na-taurocholate cotransport protein) that actively imports taurine conjugated bile acids into hepatocytes[18] might also accept the taurine conjugate **23** as a substrate which could alter **23** potentially into a cellular and possibly in vivo active compound. Indeed, upon transfection of NTCP into the HEK 293 cells which are used for the M1H assay, the EC_{50} value decreased from 1417 nM down to 36 nM, comparable to the 30 nM

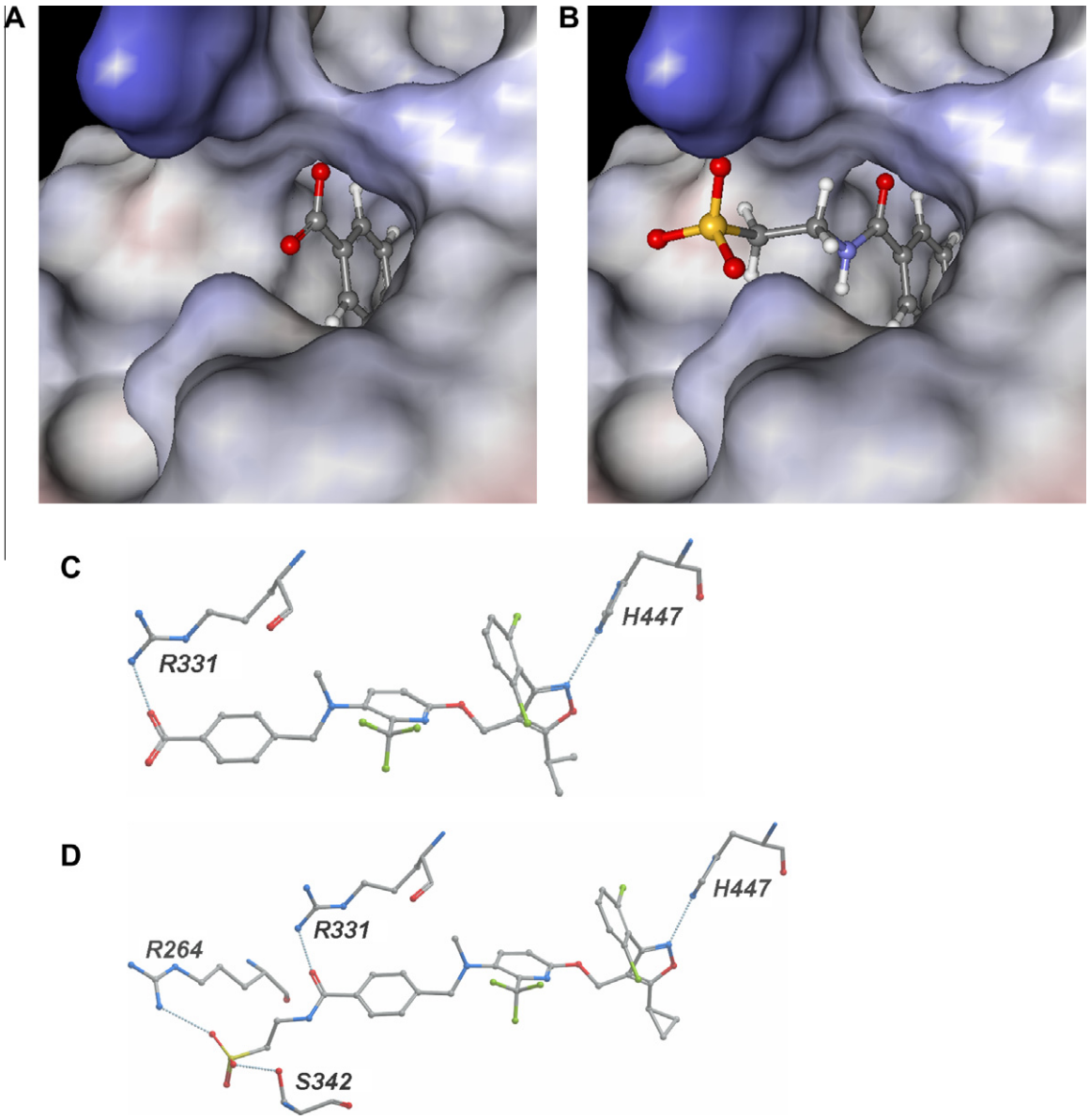


Figure 1. Solvent surface of FXR colored by interpolated charge with the docked compounds **13** (A, C) and **23** (B, D), respectively.

of **22**. This shows that the taurine amide of **22** exerts its good FXR potency in a cellular environment, when the membrane permeability barrier is overcome by an active transport mechanism.

All compounds described in Schemes 1–3, were tested in a broad human nuclear receptor M1H assay panel to check for off-target activity within this target class (see Table 1 in supplementary data). Compounds **13** and **22** were found to have the lowest off-target activity with basically no efficient activation of any other nuclear receptor except for FXR and PXR at appr. 100-fold less potency compared to their FXR activities. Compound **19**, although the most potent in all assays used, shows minor activity at the Estrogen Receptors α and β, PPARα, and PPARγ and was for this reason not considered further for in vivo testing.

Compounds **13**, **23**, and **22** were subject to pharmaco-kinetic and pharmacodynamic studies in mouse. Surprisingly, we could find only minute amounts of the compounds in plasma, independent of the route of administration, po or iv. However, when looking for parent compound and main metabolite levels in the liver and bile fluid, very high concentrations of the parent compound as well as of the direct glucuronides and taurine amides could be detected (see Tables 2A and 2B). Interestingly, **23**, which shows poor cell permeability in cellular assays was found at high levels in the liver and in bile fluid. Compound **23**, the taurine amide of **22**, decomposed to **22** only to a minor extent, whereas **22** was converted into **23** to a large extent upon liver passage. The unusual taurine amidation as one form of Phase II metabolism resembles the taurine conjugation of bile acids. This suggests that these isoxazole derivatives are not only potent FXR agonists but also mimic bile acids as the natural FXR ligands with regard to being recognized by at least the two enzymes involved in bile acid–taurine conjugation and the transporters involved in subsequent transport. Since NTCP, as the major hepatic import pump for conjugated bile acids accepts **23** as a substrate and **22** is found to be converted into **23** in vivo one may assume an enterohepatic circulation of the taurine amidated compounds.[19]

The other relevant key metabolites of **13** and **22**, besides minor amounts of N-demethylation products, were the respective glucuronides. These were found to be acyl-glucuronides since digestion of bile or bile extracts of animals treated with **13** or **22** with glucuronidase yielded the unmodified parent compounds and the COOH

Table 2A
Plasma pharmacokinetics of the compounds **13**, **22**, and **23**

	Compd **13**		Compd **22**		Compd **23**	
	5 (mg/kg) po	1 (mg/kg) iv	5 (mg/kg) po	1 (mg/kg) iv	5 (mg/kg) po	1 (mg/kg) iv
5 (min)[a]	57	1617	427	745	1	72
10 (min)[a]	92	1598	283	437	3	59
30 (min)[a]	420	361	531	325	6	3
90 (min)[a]	267	122	370	398	12	19
240 (min)[a]	14	0	52	94	0	8
V_d (mL/kg)	5260	931	5200	1243	n.a.	31,691
CL (mL/h/kg)	6253	1010	4075	717	196,931	11,385
Bioavailability (%)	16.2		16.1		5.8	

[a] Plasma (parent compd only). All values in ng/mL. n.a. = data not available.

Table 2B
Liver and gall content of the compounds **13**, **22**, and **23** and their key metabolites

Compd	Dose/Route	Liver[a] (carboxylic acid)	Liver[a] (glucuronide)	Liver[a] (taurine amide)	Bile[a] (carboxylic acid)	Bile[a] (glucuronide)	Bile[a] (taurine amide)
13	5 (mg/kg) po	1512	2872	372	1697	18,928	22,443
13	1 (mg/kg) iv	262	0	134	472	4261	11,927
22	5 (mg/kg) po	257	172	0	1446	2807	47,638
22	1 (mg/kg) iv	23	17	0	173	150	10,013
23	5 (mg/kg) po	6	30	262	32	1055	32,442
23	1 (mg/kg) iv	8	7	350	12	169	24,151

[a] Time after dosing 240 min. All values in ng/mL.

moiety presents the only functional group of the parent compounds amenable for direct glucuronidation.

The fact that all three compounds were detected at double digit micromolar concentrations as glucuronides and/or taurine amides in the bile suggests a model wherein these molecules are biologically recognized as bile acids, are taken up by active transport in the intestine and undergo a high first pass liver extraction (most likely also due to NTCP or similar transporter mediated active uptake), phase II conjugation into acyl-glucuronides and taurine amides, respectively, and subsequent export into bile. The taurine conjugates are potent FXR agonists themselves and are likely to be subject to reuptake in the intestine, thereby providing a mechanism for sustained pharmacodynamic action. The inherent danger of multiple rounds of enterohepatic cycling lies in accumulation of compound upon regular daily dosing.[20]

To test for pharmacodynamic effects with regard to lipid lowering and for potential compound accumulation over prolonged exposure, we used the db/db mouse, an accepted model for FXR-mediated effects. Compounds **13** and **22** were administered at doses of 1, 5, and 25 mg/kg b.i.d. and compared with the effects on total plasma triglycerides (TG) and total plasma cholesterol (TC) of 6-ECDCA (see Fig. 2). All three compounds, 6-ECDCA, **13**, and **22**, showed TG and TC lowering effects that were significant for most doses tested (p <0.01 for **13** and 6-ECDCA at 1 mg/kg b.i.d. and p <0.001 for all three compounds at all other doses for TG lowering; p <0.001 for **13** and **22** at 2 × 5 mg/kg b.i.d. and all higher doses and for 6-ECDCA at 25 mg/kg b.i.d. for TC lowering). All three FXR agonists tested showed a clear linear dose response relationship with regards to TG and TC lowering, however, **22** was found to be the most effective in terms of lipid lowering effects

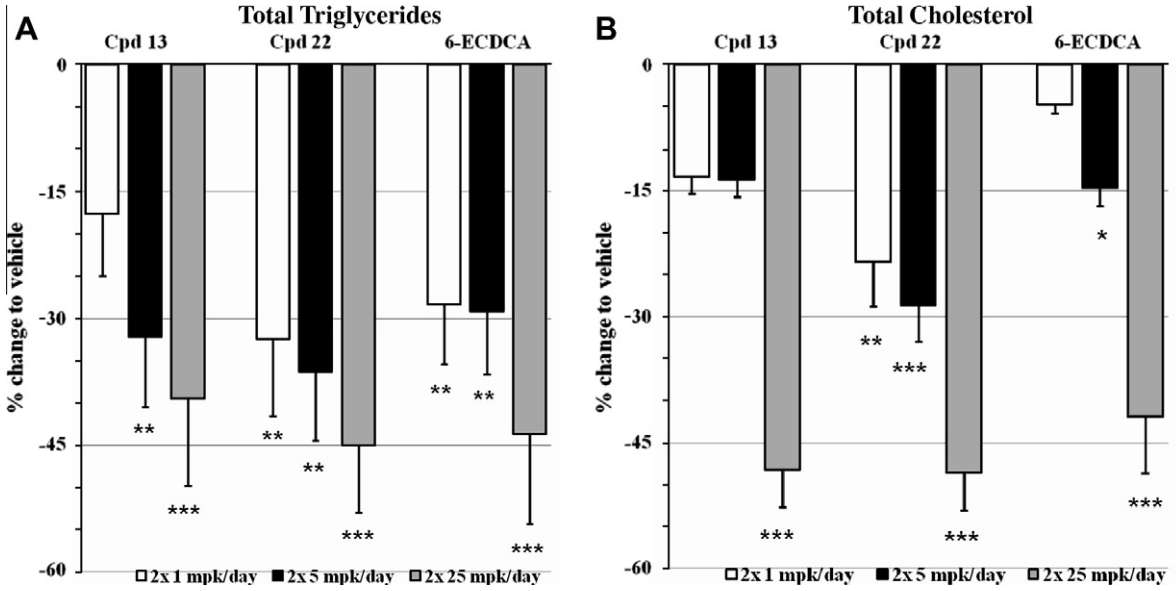


Figure 2. C57BL/KSK-*mLep*^{db}/*mLep*^{db} mice were treated (12 days) with the compounds **13**, **22**, or 6-ECDCA (*p <0.05, **p <0.01, ***p <0.001).

(TC mean for **22** at 1 mg/kg b.i.d. = 97.2 mg/dl vs 121.0 mg/dl for 1 mg/kg b.i.d. 6-ECDCA, p <0.05).

With respect to compound accumulation after multiple dosing the differences are striking (Table 2 in supplementary data): Whereas all three compounds tested show comparable levels of low plasma but very high liver concentrations at 4 h after the last administration, suggesting a high first pass effect for these compounds, organ concentrations at 24 h after the last administration differ dramatically between the isoxazole derivatives and 6-ECDCA. For **13**, **22**, and its metabolite **23** only minute amounts were detectable in either plasma or liver whereas 6-ECDCA showed nearly unchanged levels in plasma and liver, indicative of either highly efficient enterohepatic circulation, unusual stability, or storage in the liver.

The absence of **22** and **23** 24 h after the last administration despite the proposed entereohepatic cycling of the taurine amide may be explained by the fact that the taurine amide is only one of a few key metabolites including the N-demethylated species and the glucuronide. The glucuronide of **22** may not be accepted by the bile acid transporters which would result ultimately in fecal loss given that cleavage of glucuronide by intestinal bacteria is not complete. Despite, the relatively rapid removal of the isoxazoles **13** and **22** from the hepatobilliary system, they show pharmacodynamic effects in the db/db mouse comparable or better than those of 6-ECDCA which has a dramatically longer duration of exposure.

In summary we have made a series of potent FXR agonists that mimic the structure of the parent compound GW4064 but are devoid of the stilbene moiety. A key functional feature of this series is either the free terminal benzoic acid or an amide conjugate thereof that can extend the position of the acidic moiety by two atoms without significantly losing potency in biochemical or cellular FXR assays. When these extended derivatives such as **14**, **17**, **21**, or **23** are docked into the crystal structure of GW4064-bound FXR, the structurally related elements adopt very similar positions to those of GW4064. The terminal acid, however, which is believed to form an ion bridge to Arg331 in the GW4064-FXR co-crystal might change its ion bridge partner to Arg264.

When tested in db/db mice, compounds **13** and **22** showed a linear dose-dependent reduction in total plasma triglycerides and total plasma cholesterol. Surprisingly the non-steroidal FXR agonists **13** and **22** are metabolically transformed (taurine and glucuronic acid conjugation) in a way that resembles that of natural bile acids. The key metabolite of **22**, **23** is a direct ligand of FXR, and a substrate of NTCP as observed by gain-of-function studies. Nevertheless, compared to 6-ECDCA which was present at high levels in liver 24 h after the last administration, neither **22** nor **23** were present at relevant concentrations in plasma or liver at this timepoint. Thus, the risk of sustained non-mechanism related effects with the isoxazoles might be lower compared to 6-ECDA, while the duration of desirable pharmacodynamic effects is clearly adequate to modify lipid homeostasis in these mice.

Overall, these data suggest that the newly identified compounds are useful tools for assessing the effect of small-molecule mediated FXR activation in multiple clinically relevant disease models.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.06.084.

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