Nongenomic Actions of Bile Acids. Synthesis and Preliminary Characterization of 23- and 6,23-Alkyl-Substituted Bile Acid Derivatives as Selective Modulators for the G-Protein Coupled Receptor TGR5

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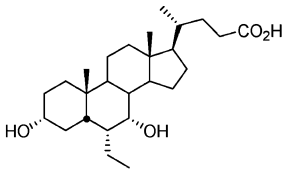
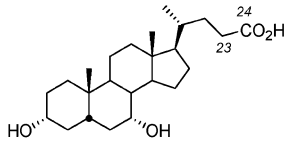
Abstract: 23-Alkyl-substituted and 6,23-alkyl-disubstituted derivatives of chenodeoxycholic acid are identified as potent and selective agonists of TGR5, a G-protein coupled receptor for bile acids (BAs). In particular, we show that methylation at the C-23(*S*) position of natural BAs confers a marked selectivity for TGR5 over FXR, while the 6α-alkyl substitution increases the potency at both receptors. The present results allow for the first time a pharmacological differentiation of genomic versus nongenomic effects mediated by BA derivatives.

Considered for many years as the final products of cholesterol catabolism and involved in dietary lipid and vitamin adsorption, bile acids (BAs[a]) are experiencing a new lease of life, being now recognized as key elements of paracrine and endocrine functions related to the homeostasis of cholesterol levels, control of lipid and carbohydrate metabolism, and regulation of the immune system.[1] BAs exert their role as signaling molecules through interactions with membrane and nuclear receptors, as well as with components of the mitogen-activated protein kinase pathway. In 1999, the orphan farnesoid X receptor (FXR) was adopted as a BA nuclear receptor,[2−4] which is now recognized as a master regulator of the pleietropic actions of endogenous BAs within the systemic endocrine apparatus and the enterohepatic circulation.[5] In 2002, two groups independently reported the identification and the preliminary characterization of a membrane-type, G-protein coupled receptor for BAs, named M-BAR or TGR5.[6,7] Thus, BAs potently induced cyclic adenosine monophosphate (cAMP) formation in CHO cells transiently expressed with TGR5 in a dose-dependent manner; the cAMP formation is independent of nuclear receptor activation and the rank order of potency for BAs activating TGR5 is clearly different from that of BA activating FXR. TGR5 is broadly expressed in human tissues, including those that are not a target for BAs. In particular, TGR5 is highly expressed in the adipose tissue and in macrophages.[7] While this latter observation may allow a reconsideration of earlier reports occasionally indicating an immunosuppressive effect for BAs,[8−13] the former one has been linked to a report stating that cholate inhibits high-fat diet-induced hyperglycemia and obesity in rats.[14] Indeed, administration of BAs to mice increased energy expenditure in the brown adipose tissue (BAT) and prevented diet-induced obesity and insulin resistance. This effect was ascribed to the increased cAMP-dependent induction of the type 2 iodothyronine deiodinase (D2) enzyme, which converts thyroxine (T4) into 3,5,3′-triiodothyronine (T3), thus giving rise to increased thyroid hormone activity. The up-regulation of D2 depends on the activation by BAs of membrane-bound TGR5, which mobilizes cAMP.[15] Consistent with a role for TGR5 in energy homeostasis, female TGR5 deficient mice, although not obese under chow fed conditions, are predisposed to accelerated obesity when fed a high fat diet.[16] Although the translation of this mechanism toward clinically exploitable strategies is still far, TGR5 is emerging as a very promising target for metabolic control and energy homeostasis, thus allowing the potential unlinking of metabolism control from the diet.

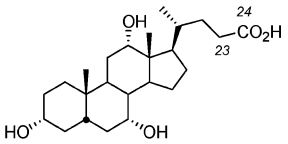
Chart 1. Synthetic and Natural Bile Acids



6α-Ethyl-Chenodeoxycholic Acid
(6ECDCA, **1**)



Chenodeoxycholic Acid
(CDCA, **2**)



Cholic Acid
(CA, **3**)

The further pharmacological and pathophysiological characterizations of TGR5, as well as its validation as clinically useful target requires the availability of potent and selective ligands. While screening of nonsteroidal libraries may provide suitable and chemically diverse templates for further optimization,[17] chemical elaboration around the steroid nucleus of BAs remains an effective route toward potent BA receptors ligands, although the selectivity among different receptors (i.e., FXR vs TGR5) is a relevant issue.

We have previously reported extensive structure activity relationship studies on BAs derivatives as FXR modulators[18−20] and discovered 6ECDCA (INT747, **1**, Chart 1) as the most potent steroid ligand for FXR so far available.[18] We have also accumulated a considerable experienwhichce in the design, synthesis and biological evaluation of unnatural BA derivatives modified in the side chain, reporting for some of them interesting properties, such as reduced hepatic amidation, alternative conjugation pathways for sustained biliary secretion, and altered ability to enter the enterohepatic circulation.[21−27] Furthermore, we noticed regio- and stereoselective biological properties for

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[a] Abbreviations: BA, bile acid; BAT, brown adipose tissue; CA, cholic acid; cAMP, cyclic adenosine monophosphate; CDCA, chenodeoxycholic acid; D2, type 2 iodothyronine deiodinase; FXR, farnesoid X receptor; LBD, ligand binding domain; M-BAR, membrane-bile acid receptor; 6ECDCA, 6α-ethyl-chenodeoxycholic acid.

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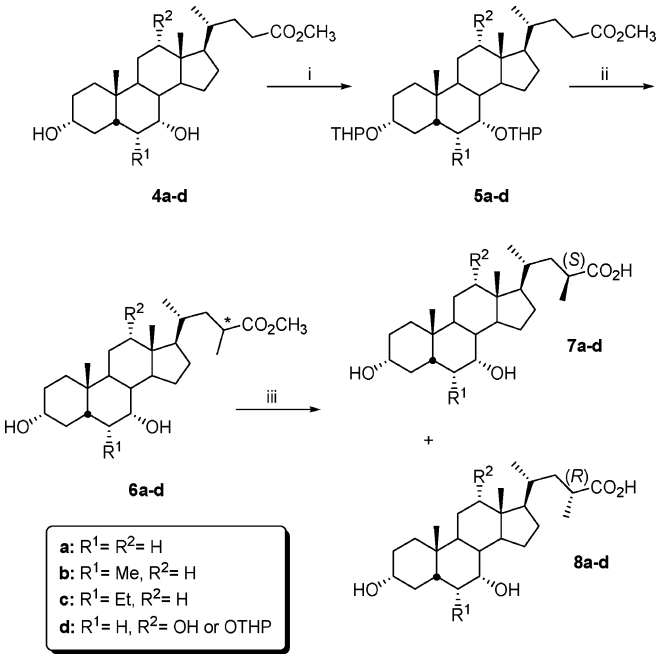
Scheme 1[a]



[a] Reagent and conditions: (i) 3,4-DHP, p-TSA, dioxane, rt; (ii) (a) LDA, CH_3I, THF, −78 °C; (b) HCl, CH_3OH, rt; (iii) NaOH, CH_3OH, reflux.

some of the side-chain modified BA derivatives, thus inferring the potential for a fine-tuning modulation of their biological activities. Based on these considerations, and given the potential differences between TGR5 and FXR in terms of either different tissue expression or topological requirements for ligand interaction, we deemed appropriate to initially evaluate side chain modified analogs of BAs in search for TGR5/FXR selective modulators. Given the order of potency of naturally occurring BAs at TGR5,[6,7] we initially focused our attention on chenodeoxycholic acid (CDCA, **2**, Chart 1) and cholic acid (CA, **3**, Chart 1) derivatives, which are expected to have a high potency at TGR5, while keeping a reasonable hydrophobic/hydrophilic balance. As a result of the screening, we identified a subset of C-23 alkyl-substituted analogs of CDCA and CA.[28] Their synthesis and preliminary evaluation as TGR5 ligands is reported herein.

Chemistry. The synthetic route to the 23(*R*)- and 23(*S*)-methyl-BA derivatives (**7a**−**d** and **8a**−**d**) is outlined in Scheme 1. Thus, treatment of the methyl cholanoates **4a**−**d**[18,19] with 3,4-dihydro-2*H*-pyran in dioxane in the presence of a catalytic amount of *p*-toluenesulfonic acid (*p*-TSA) afforded the corresponding hydroxy-protected BA derivatives **5a**−**d** in quantitative yields.[29] Reaction of **5a**−**d** with methyl iodide at −78 °C using lithium diisopropylamide as a base and tetrahydrofuran (THF) as solvent, followed by treatment with methanolic HCl afforded the corresponding 23-methylated BA esters **6a**−**d**. Hydrolysis of the methyl esters **6a**−**d** with NaOH in methanol at reflux yielded, respectively, a mixtures of two compounds, which were separated by flash chromatography into less polar acids, 23(A)-methyl BA derivatives **7a**−**d**, and more polar acids, 23(B)-methyl BA derivatives **8a**−**d**.

The absolute configuration assignment to the eight diastereoisomeric BAs **7a**−**d** and **8a**−**d** was based upon the single-crystal X-ray analysis performed on a suitable selected compound chosen depending on the quality of its crystal. Thus, X-ray analysis of the 23(A)-methyl-3α,7α,12α-trihydroxy-5β-cholan-24-oic acid (**7d**) confirmed the *S*-chirality of the carbon at the 23-position (Figure 1) and allowed to fix the absolute configuration of the other BA diastereoisomers by ^{13}C NMR comparison.

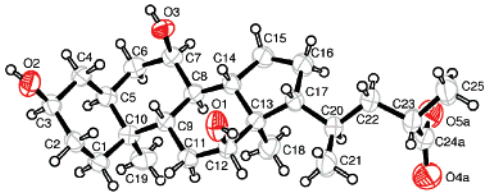


Figure 1. Drawing of the crystal structure of 23(*S*)-methyl-3α,7α,12α-trihydroxy-5β-cholan-24-oic acid (**7d**) × CH_3OH. Ellipsoids enclose 50% probability. C(24a), O(4a), and O(5a) have s.o.f. equal to 0.55(1). The solvate methanol molecule has been omitted for clarity.

Table 1. TGR5 and FXR Activities of Bile Acids and Bile Acid Derivatives[a]

	TGR5[b]		FXR[c]		EC_{50} ratio
index	EC_{50}	efficacy	EC_{50}	efficacy	(TGR5/FXR)
2	6.71	105	13.0	62	0.52
1	0.755	101	0.361	100	2.1
7a	3.58	110	>100	0[d]	<0.036
8a	25.5	100	10.5	49	2.4
7b	0.140	105	11.6	23	0.012
7c	0.095	102	11.8	73	0.0081
3	13.6	101	n.d.[e]	n.d.[e]	n.d.[e]
7d	4.39	105	n.d.[e]	n.d.[e]	n.d.[e]
8d	>51.9	75[d]	n.d.[e]	n.d.[e]	n.d.[e]

[a] Data represents average values of at least three independent experiments. [b] Units are μM for EC_{50} and % of 10 μM LCA value for efficacy. [c] Units are μM for EC_{50} and % of 10 μM 6ECDCA value for efficacy. [d] Plateau activation level not reached; the maximum concentration tested was 125 μM for **8d** and 100 μM for **7a**. [e] n.d. = not determined.

Biology. The newly synthesized compounds were evaluated, along with CDCA (**2**) and CA (**3**) as reference controls, for their ability to increase cAMP-responsive element (CRE)-driven luciferase reporter activity in CHO cells, which transiently transfected with hTGR5 or to activate FXR on COS1 (ATCC) cells in cell-based luciferase assay in the case of CDCA derivatives (Table 1).

Interestingly, a significantly different behavior is observed between the respective C-23-methyl BA epimers. In particular, while **8a** is moderately active at both FXR and TGR5, with an TGR5/FXR EC_{50} ratio of 2.4, the corresponding C-23(*S*)-epimer **7a** is inactive at FXR up to a 100 μM concentration, while activating TGR5, with an EC_{50} = 3.58 μM (TGR5/FXR EC_{50} ratio < 0.036) and 110% of efficacy with respect to 10 μM LCA. Thus, 23(*S*)-methyl-CDCA (**7a**) resulted to be highly selective TGR5 agonist. We previously reported that the insertion of alkyl groups at the 6α-position of CDCA (**2**), greatly enhances the potency at FXR and discovered the most potent steroid ligand for FXR, 6ECDCA (**1**).[18] Analogously, we wondered whether a similar modification would have improved the potency and selectivity of **7a**. Thus, the 6α-methyl (**7b**) and 6α-ethyl (**7c**) derivatives of **7a** were synthesized and evaluated in the above assays. As shown in Table 1, the introduction of the 6α-methyl or 6α-ethyl group had a considerable impact on the TGR5 activity, with an increase in potency of 1 or 2 orders of magnitude, respectively. Thus, 6α-ethyl-23(*S*)-methyl-3α,7α-dihydroxy-5β-cholan-24-oic acid (**7c**) turned out to be the most potent and selective TGR5 agonist so far reported.

Molecular Modeling. With the aim of gaining insights into the structural basis for the observed TGR5/FXR selectivity, we docked both the 23(*S*)- and 23(*R*)-Me-CDCA (**7a** and **8a**, respectively) epimers into the canonical binding pocket of the ligand binding domain (LBD) of FXR (pdb code: 1OSV). Both compounds were superimposed to the experimentally determined

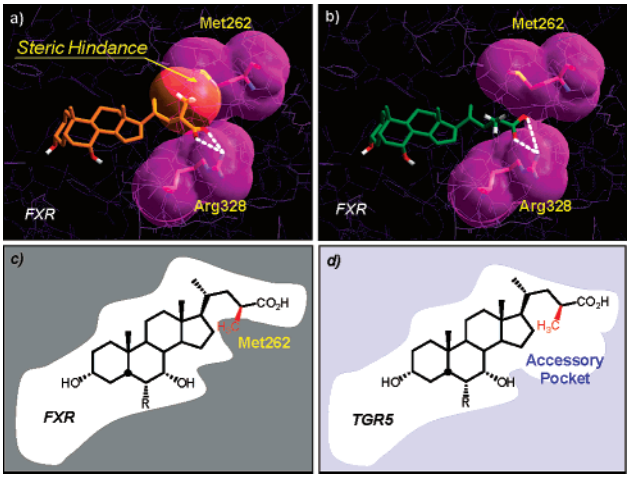


Figure 2. (a) Docking experiments of 23(*S*)-Me-CDCA (**7a**) and (b) 23(*R*)-Me-CDCA (**8a**) into the crystal structure of FXR (1osv, chain A). The main steric (orange and magenta surfaces) and electrostatic hydrogen bonding (dashed lines) interactions with Met262 and Arg328 are highlighted. (c,d) Cartoons representing the bile acid binding site of FXR (c) and TGR5 (d) as resulting from structure−activity relationships of 23(*S*)-methyl CDCA derivatives (**7a**−**c**).

disposition of the potent FXR agonist 6ECDCA (**1**), cocrystallized with the LBD in 1OSV.

While **8a** could be neatly docked into the LBD, **7a** displayed a severe steric clash between the 23(*S*)-methyl group and the side chain of Met262 (Figure 2a,b). This unfavorable interaction could be relieved by changing the conformation of the side chain of **7a**, but this could only be achieved by paying a considerable energy cost (ca. 30 kcal/mol from the global minimum). It is interesting to observe (Table 1) that the 6α-methyl (**7b**) and 6α-ethyl (**7c**) derivatives of **7a** show a significant FXR activity, thus indicating that the energy gain induced by the 6α-alkyl substituent can partially overcome the unfavorable interaction of the 23(*S*)-methyl group with the side chain of Met262.

Taken together, these data indicate that the binding pocket for BAs is not entirely conserved between TGR5 and FXR and that minor modification on the side chain can result in a significant selectivity (Figure 2c,d), thus allowing a pharmacological differentiation between genomic and nongenomic effect of BA derivatives. Studies aimed at further elucidating the structure−activity relationship of BA derivatives in regard to TGR5 activation are under way.

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Supporting Information Available: Description of the synthetic procedures, biological methods, computational methodologies, and analytical analysis of all target compounds, and full crystal data for **7d** × CH_3OH. This material is available free of charge via the Internet at http://pubs.acs.org.

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