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Discovery of 6α-Ethyl-23(*S*)-methylcholic Acid (*S*-EMCA, INT-777) as a Potent and Selective Agonist for the TGR5 Receptor, a Novel Target for Diabesity

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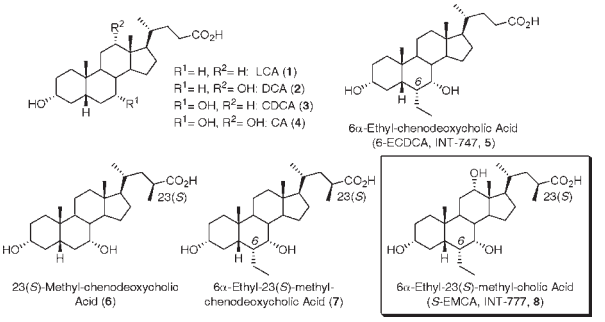
Abstract: In the framework of the design and development of TGR5 agonists, we reported that the introduction of a C_{23}(*S*)-methyl group in the side chain of bile acids such as chenodeoxycholic acid (CDCA) and 6-ethylchenodeoxycholic acid (6-ECDCA, INT-747) affords selectivity for TGR5. Herein we report further lead optimization efforts that have led to the discovery of 6α-ethyl-23(*S*)-methylcholic acid (*S*-EMCA, INT-777) as a novel potent and selective TGR5 agonist with remarkable in vivo activity.

In the quest for improved therapies targeting the variety of pathways involved in obesity, diabetes, and prediabetic insulin resistance in the metabolic syndrome, two bile acid (BA[a]) activated receptors, namely, the nuclear farnesoid-X receptor (FXR) and, more recently, the G protein coupled receptor (GPCR) TGR5, have emerged as especially attractive targets for drug discovery efforts.[1] Indeed, it is well established that FXR and TGR5 have key roles in the regulation of the intricate network governing lipid, cholesterol, and energy homeostasis, the transport and metabolism of fatty acids and triglycerides, and the regulation of glucose homeostasis.[2]

In this paper, we focus on TGR5, a receptor that was discovered in 2002 and further characterized in 2003 by two independent Japanese groups as coupled to $G\alpha_s$-proteins and shown to be mainly activated by lithocholic acid (LCA, **1**) and deoxycholic acid (DCA, **2**) (Chart 1).[3−5] TGR5 is widely expressed but most importantly in metabolic tissues such as liver, muscle, intestine, and brown adipose tissue.[4] In the murine enteroendrocrine cell line (STC-1), in vitro BA-mediated activation of TGR5 leads to a rise in intracellular levels of cAMP, triggering an increase in glucagon-like peptide 1 (GLP-1) release.[6] Additionally, in brown adipocytes TGR5-dependent enhancement of cAMP levels increases type 2 iodothyronine deiodinase-mediated energy expenditure.[6,7]

Chart 1. Natural and Semisynthetic BAs



TGR5 signaling in the muscle, the liver (especially in the sinusoidal endothelial cells (SECs) and in the Kuppfer cells),[8,9] and the gallbladder[10] contributes to the control of energy expenditure,[2c] the regulation of gallbladder and liver functions,[10] the protection of liver against lipid peroxidation, and the reduction of proinflammatory cytokine production.[8,9] Accordingly, TGR5 may have broad therapeutic application, ranging from the treatment of metabolic disorders to liver and inflammatory diseases.[1]

Starting from the discovery of FXR as a BA nuclear receptor in 1999,[11−13] a finding that heralded the paradigm shift of BAs from detergent-like molecules to signaling hormones, and following the disclosure of TGR5 as a BA membrane receptor,[3,4] we have been engaged in the search for potent and selective modulators of these receptors through a strategic approach based on the screening of a large number of BA derivatives prepared by extensive modifications of their core structure.[5,14−19] In the case of FXR, an important breakthrough came with the discovery that the agonist activity at this receptor was dramatically increased when introducing an α ethyl moiety in the C_6 position of chenodeoxycholic acid (CDCA, **3**), a primary BA and FXR endogenous agonist (FXR-EC_{50} = 13 μM). This finding resulted in the disclosure of 6-ECDCA (INT-747, **5**, FXR-EC_{50} = 0.099 μM), a compound endowed with remarkable properties of potency, selectivity, and metabolic stability.[15] Currently, 6-ECDCA (**5**) is in phase II clinical studies for primary biliary cirrhosis and is being advanced in nonalcoholic steatohepatitis (NASH). When **5** was docked into the ligand binding domain of FXR, a nice fit was observed in a small hydrophobic cavity of the receptor.[16]

In our search for novel potent and selective TGR5 agonists, we have followed an analogous evaluative strategy which in this case involved initial screening of natural and semisynthetic BA libraries for TGR5 activity. As a result, we discovered that the incorporation of a methyl moiety with the preference of the *S* over the *R* chiral configuration at the C_{23} position of the CDCA side chain (**3**) afforded selective, albeit not very potent, TGR5 agonist properties

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[a] Abbreviations: BA, bile acid; FXR, farnesoid-X receptor; GPCR, G-protein-coupled receptor; GLP-1, glucagon-like peptide 1; cAMP, cyclic adenosine monophosphate; CA, cholic acid; LCA, lithocholic acid; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; 6-ECDCA, 6α-ethylchenodeoxycholic acid; EMCA, 6α-ethyl-23-methylcholic acid; bw, body weight.

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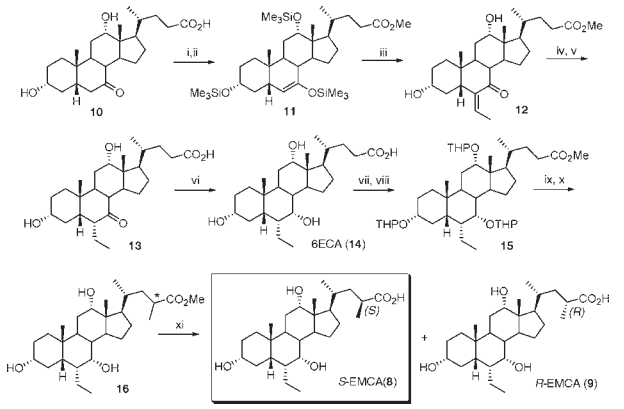
Scheme 1[a]



[a] Reagents and conditions: (i) *p*-TSA, MeOH; (ii) LDA, TMSCl, Et_3N, THF, −78 °C; (iii) MeCHO, $BF_3 \cdot OEt_2$, CH_2Cl_2, −60 °C; (iv) H_2, PtO_2, AcOH/HCl; (v) NaOH, MeOH; (vi) $NaBH_4$, THF/H_2O; (vii) *p*-TSA, MeOH; (viii) 3,4-DHP, *p*-TSA, dioxane, room temp; (ix) LDA, MeI, THF, −78 °C; (x) HCl, MeOH, 45 °C; (xi) NaOH, MeOH, reflux.

(**6**, TGR5-EC_{50} = 3.58 μM, FXR-EC_{50} > 100 μM).[19] When this additional chemical feature was next introduced in 6-ECDCA (**5**), we were pleasantly surprised to discover a remarkable reversal of the activity profile. In the resulting derivative **7**, the FXR activity was remarkably decreased (FXR-EC_{50} = 11.80 μM) and the TGR5 efficacy boosted to nanomolar concentration (TGR5-EC_{50} = 0.095 μM).[19] Further conclusions on the structure−activity relationships (SAR) profile of BA-derived TGR5 agonists were also drawn by the results of our screening.[6,19]

As a continuation to this work and to find a suitable candidate for TGR5 clinical studies, we directed our attention to the optimization of **7** in search for a compound endowed not only with analogous properties of efficacy and selectivity but also with improved pharmacokinetic properties and a more favorable metabolic profile. To this end, we were attracted by the peculiar biological and physicochemical properties of CA (**4**), a primary bile acid in human and many animal species, also reported as one of the main components together with bilirubin of *Calculus Bovis,* a highly valued traditional Chinese medicine (TCM) remedy.[20] CA (**4**) differs from CDCA (**3**) by the presence at C_{12} of an additional α-hydroxyl group oriented on the polar side of the molecule. This "minor" structural difference accounts for the remarkably different physicochemical and biological features of these two BAs. With respect to CDCA (**3**) protonated CA (**4**) is about 10-fold more soluble and relatively less detergent as a result of its hydrophobic/hydrophilic balance and polarity (Table 2).[21−25] Moreover, CA (**4**) is devoid of activity toward FXR receptor (EC_{50} > 100 μM) while showing a moderate agonistic activity on TGR5 (EC_{50} = 13.6 μM).[5] As an even more important consideration, it was previously reported that the pharmacological administration of CA (**4**) at 0.5% w/w in diet-induced obese mice efficiently prevents and treats metabolic syndrome.[6] While this study provided interesting clues about the endocrine functions of bile acids, the high dosage required (0.5% w/w) still limited the proof of concept concerning the therapeutic relevance of TGR5 in the context of metabolic diseases, since the modulation of other and unknown targets could not be ruled out at that dose. An additional issue was also the risk associated with testing a high dose of CA (**4**) in clinical trials due to the production of the toxic secondary BA DCA (**2**) via extensive and efficient intestinal bacteria 7α-dehydroxylation.[24]

Table 1. TGR5 and FXR Activities of Cholic Acid and Its Derivatives[a]

	TGR5[b]		FXR[c]	
compd	EC_{50}[d]	efficacy	EC_{50}	efficacy
CA (**4**)	13.6 (6.66 − 22.87)	101	> 100	0[e]
6-ECA (**14**)	3.44 (2.37 − 5)	95	> 100	49
S-EMCA (**8**)	0.82 (0.54 − 1.24)	166	> 100	18[e]
R-EMCA (**9**)	4.79 (3.44 − 6.66)	86	> 100	114

[a] Data represent average values of at least three independent experiments. [b] Units are μM for EC_{50} and % of 10 μM LCA (**1**) value for efficacy. [c] Units are μM for EC_{50} and % of 10 μM **5** value for efficacy. [d] 95% confidence intervals in parentheses. [e] Curve fit, < 0.5.

Table 2. Physicochemical Properties of BAs[a]

compd	Ws[b] (μM)	cmc[c] (mM)	ST_{cmc}[d] (dyne/cm)	albumin binding (%)
CDCA (**3**)	30	3	45.5	93
CA (**4**)	270	10	48.2	54
S-EMCA (**8**)	99	2	50.1	62

[a] The methods used for the measurements of the physicochemical properties are those reported in ref 26. [b] Ws: water solubility as protonated species in 0.1 M HCl water solution. [c] cmc: critical micellar concentration determined in 0.15 M NaCl water solution. [d] ST_{cmc}: surface tension at cmc in 0.15 M NaCl water solution.

On the basis of the above considerations, we focused our attention on the incorporation of the crucial 6α-ethyl- and 23-methyl moieties in the chemical scaffold of CA (**4**) as key features to improve potency, selectivity, and metabolic stability of the resulting compound. The synthesis, physicochemical properties, and selected biological properties of 6α-ethyl-23(*S*)-methylcholic acid (*S*-EMCA, INT-777, **8**) are herein reported.

The synthetic route to *S*-EMCA (**8**) and *R*-EMCA (**9**) is illustrated in Scheme 1. Treatment of methyl 7-ketodeoxycholate with LDA in THF at −78 °C followed by reaction of the enolate with trimethylchlorosilane afforded the corresponding silyl enol ether **11** in nearly quantitative yield. The intermediate **11** was then reacted with acetaldehyde in the presence of $BF_3 \cdot OEt_2$ at −60 °C in CH_2Cl_2 to obtain the methyl 3α,12α-dihydroxy-6-ethylidene-7-keto-5β-cholan-24-oate (**12**) in 85% yield. Hydrogenation of **12** with PtO_2 in glacial acetic acid/hydrochloric acid, followed by alkali hydrolysis (10% NaOH in methanol) gave selectively the 6α-ethyl derivative **13** in good yield. Selective reduction of the C_7-ketone with $NaBH_4$ in a mixture of THF/H_2O at room temperature afforded the 6-ECA (**14**) in 46% overall yield (from **10**). C_{23}-Methylation of the corresponding 3α,7α,12α-trihydroxy-protected ester **15** and the following acidic and basic hydrolysis reactions[18] gave the desired *S*- and *R*-EMCA (**8**, **9**) in 40% and 22% yield (from 6-ECA), respectively. The absolute configuration assignment to epimers **8** and **9** was based upon the previously reported single-crystal X-ray analysis of 23(*S*)-Me-CA[18] and ^{13}C NMR comparison.

Compounds **8**, **9**, and **14** were evaluated for TGR5 and FXR activities, assessing their abilities to increase cAMP-responsive element (CRE) driven luciferase reporter activity in CHO cells transiently transfected with hTGR5 or to activate FXR on COS1 (ATCC) cells in cell-based bioluminescence assays, respectively (Table 1). In accordance with the reported SAR schemes of TGR5 agonists and FXR modulators,[18,19]

Table 3. Biliary Lipid Secretion Parameters after iv and id Infusion at a Dose of 1 (μmol/min)/kg bw over 1 h of BAs[a]

compd	SV_0 id (iv)	S_{BA} id (iv)	% free id (iv)	% conjug id (iv)
CDCA (**3**)	57 ± 7	0.7 ± 0.2	3 ± 1	96 ± 8
	(51 ± 9)	(0.8 ± 0.1)	(4 ± 1)	(98 ± 5)
CA (**4**)	64 ± 6	1.0 ± 0.4	12 ± 2	90 ± 4
	(78 ± 8)	(1.3 ± 0.2)	(8 ± 3)	(92 ± 6)
S-EMCA (**8**)	112 ± 12	0.5 ± 0.2	94 ± 6	10 ± 5
	(131 ± 11)	(0.7 ± 0.3)	(93 ± 5)	(7 ± 3)
R-EMCA (**9**)	81 ± 8	0.4 ± 0.2	68 ± 8	32 ± 7
	(90 ± 5)	(0.5 ± 0.1)	(65 ± 4)	(26 ± 6)
saline	46 ± 4	0.4 ± 0.1		
	(48 ± 4)	(0.4 ± 0.1)		

[a] Data represent average values and standard deviations of six independent experiments. The vehicle used for the id administration was saline solution. The vehicle used for the iv administration was 3% BSA saline solution, pH 7.2. SV_0: maximum bile secretion rate ((μL/min)/kg bw). S_{BA}: maximum BA secretion rate ((μmol/min)/kg bw). % free: percentage of the administered dose recovered in bile of the molecules as such. % conjugate: percentage of the administered dose recovered as conjugated BA.

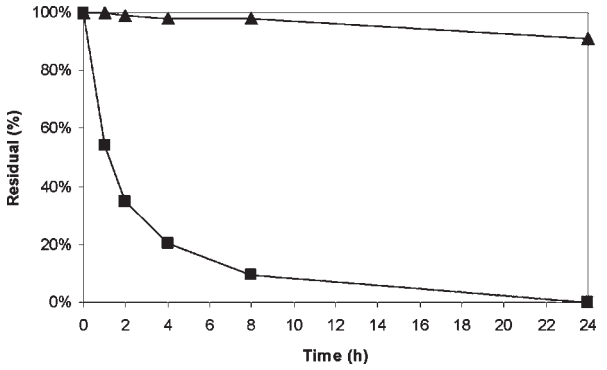


Figure 1. Stability of *S*-EMCA (**9**) (▲) and CA (**4**) (■) in human stool culture.

S-EMCA (**8**) was qualified as a novel potent and selective TGR5 agonist and was next submitted to a preliminary pharmacokinetic characterization and an assessment of the compound's physicochemical properties and metabolic stability (Tables 2 and 3). On the basis of the higher surface tension at the CMC (ST_{CMC}), despite a lower CMC value, *S*-EMCA (**8**) shows a detergency power similar to CA (**4**), albeit with slightly lower water solubility (Table 2).

It is known that intestinal bacteria hydrolyze the C_{24} amide bond of taurine and glycine conjugated BAs and remove the 7α-hydroxyl group of CA, leading to the formation of toxic lipophilic secondary BAs such as DCA (**2**).[25] To determine the sensitivity of *S*-EMCA (**8**) to intestinal flora-mediated 7-dehydroxylation, its metabolic stability was assessed in human stool broth culture as previously described.[26] *S*-EMCA (**8**) appears not to be sensitive to this process and was shown to be highly stable with more than 95% of the compound unmodified after 12 h of incubation. By comparison, more than 50% of CA (**4**) was metabolized after 1 h and up to 90% within 8 h (Figure 1). According to previous studies,[26] it is likely that the extended stability of *S*-EMCA (**8**) is related to the alkylation of the C_6 position which provides steric hindrance to the bacterial 7α-dehydroxylation process.

We next evaluated preliminary pharmacokinetic profiles of *S*-EMCA (**8**) with the aim of gaining insights into the efficiency of its intestinal absorption, hepatic uptake, transport, and biliary secretion upon intravenous administration (infusion in the femoral vein) or oral gavage administration in the duodenum at a dose of 1 (μmol/min)/kg bw for 1 h (bile fistula rat model).[27,28] The results in Table 3 reveal that *S*-EMCA (**8**) has a potent choleretic effect, with the maximum bile secretion rate (SV_0) being significantly higher than those of CDCA (**3**) and CA (**4**). Accordingly, our results show that *S*-EMCA (**8**) is resistant to conjugation, with more than 90% of the compound being secreted into the bile in its unconjugated form after intravenous or intraduodenal infusion (Table 3). In contrast CDCA and CA cannot be secreted into bile as such, requiring the conjugation step. In accordance with previous studies,[29,30] we can envisage that the C_{23}(*S*) methyl group of *S*-EMCA (**8**) prevents carboxyl CoA activation and subsequent conjugation, thereby favoring its cholehepatic shunt pathway with a ductular absorption and a potent choleretic effect.

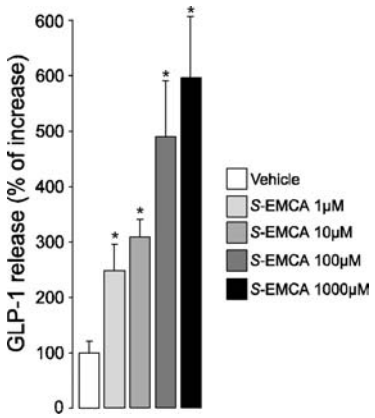


Figure 2. Impact of 1 h exposure to indicated concentration of *S*-EMCA (**8**) on GLP-1 release ex vivo in ileal explants isolated from 18-weeks HF-fed TGR5-Tg male mice (n = 4). The data are represented as mean ± SE: Student's unpaired t-test, (∗) $P < 0.05$, **8** treated ileal explants vs vehicle treated.

To further study the influence of the configuration of the C_{23} methyl group on the side chain amidation and choleretic effect of the compound, similar analyses were also carried out on the other epimer, namely, 6α-ethyl-23(*R*)-methylcholic acid (*R*-EMCA, **9**, Table 3). The inspection of the maximum bile secretion rate (SV_0) shows that the choleretic effect of **9** is still higher than CA (**4**), though lower than **8**. As a result, these data suggest that the orientation of the 23 methyl group is important for the conjugation of the carboxyl group, with the methyl moiety fitting poorly in the catalytic pocket of the conjugating enzyme in the case of the C_{23}(*S*) epimer. Altogether, these results show that *S*-EMCA (**8**) is efficiently absorbed and undergoes enterohepatic cycling albeit with relatively little liver conjugation. The low rate of conjugation may also allow *S*-EMCA (**8**) to escape hepatic first pass clearance and reach the systemic blood circulation.

Given the relatively good albumin binding of *S*-EMCA (**8**) (Table 2), circulation of *S*-EMCA (**8**) in the blood may be facilitated, thereby favoring the systemic targeting of TGR5 in peripheral tissues such as muscle and brown adipose tissue. Supporting this hypothesis, we show in Figure 2 that *S*-EMCA (**8**) dramatically and dose-dependently induces the release of GLP-1 ex vivo. Along with these effects, we have recently demonstrated in a joint paper that the pharmacological targeting of TGR5 by *S*-EMCA (**8**) efficiently increases GLP-1 secretion in vivo.[31] Also consistent with systemic TGR5 activation was an increase in energy expenditure in diet-induced obese mice that resulted in a significant reduction in weight gain and adiposity. Very interestingly, these effects are

associated with an improvement of liver function in high fat fed mice with a concomitant reduction of steatosis and fibrosis.[31]

In conclusion, while our recent studies have univocally demonstrated the promising therapeutic potential of pharmacological activation of TGR5 for the treatment of diabetes, obesity, and related disorders such as NASH, the present results call for additional pharmacokinetic evaluation in support of the selection of *S*-EMCA (**8**) as a novel lead candidate to advance into clinical studies.

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Supporting Information Available: Description of the synthetic procedures, biological methods, and analytical analysis of all target compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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