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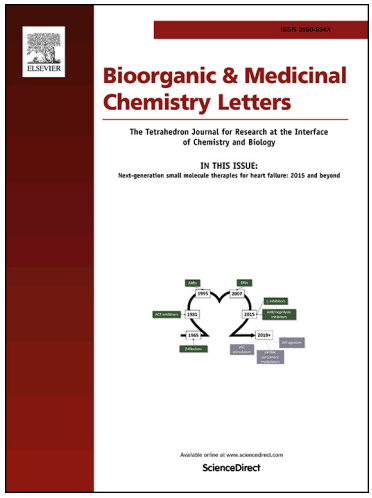

Structural requirements of cholenamide derivatives as the LXR ligands

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Graphical Abstract

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Structural requirements of cholanamide derivatives as the LXR ligands

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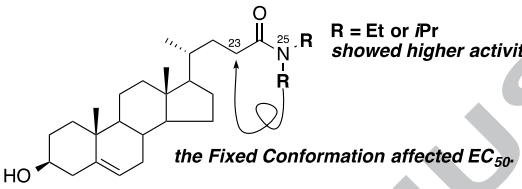

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ABSTRACT

A study of the structural requirements of cholic acid derivatives as liver X receptor (LXR) ligands was performed. A model of cholenamide derivative **1** complexed with LXR showed that the C24 carbonyl oxygen forms a hydrogen bond with His435 located close to Trp457. The *N,N*-dimethyl group is located in a hydrophobic pocket. Based on these data, we designed compounds with high affinity for LXRs. Cholenamide derivatives **1**–**11** were synthesized from 3β-acetyl-Δ^5-cholenic acid **20**, and lactams **12**–**19** were synthesized from alcohol **25**. Tertiary amides **3** and **4** showed higher activity in reporter assays, and compounds with hydrophobic residues exhibited the highest activity of all derivatives. The stereochemistry at C23 was found to be an important determinant of EC_{50} and gene transactivation, as each isomer exhibited different activity.

Liver X receptors (LXR)α (NP1H3) and LXRβ (NP1H2) are members of the nuclear receptor superfamily[1] that regulate the expression of genes involved in lipid, glucose, and cholesterol metabolism and homeostasis. Whereas LXRα is primarily expressed in the liver, intestines, adipose tissue, and macrophages, LXRβ is expressed in all tissues and organs.[2,3] LXRs regulate pathways through interactions with naturally occurring oxysterols. After forming obligate heterodimers with the retinoic X receptor (RXR),[4,5] LXRs bind to LXR response elements within the promoters of target genes (e.g., *ABCA1*, an ATP binding cassette protein,[6–11] and *SREBP1c*, which encodes sterol regulatory element binding protein 1c).[12–14]

Several transcriptional activators of LXRs, such as endogenous oxysterol 24(*S*), 25-epoxycholesterol (eCH),[15] and synthetic nonsterol T0901317,[16] have been shown to increase the expression of several genes, including *ABCA1*, which is involved in lipid metabolism and reverse cholesterol transport (RCT), resulting in reduced atherosclerosis (Figure 1).[17] However, these transcriptional activators also activate triglyceride (TG) synthesis in the liver via upregulated expression of the *SREBP1c* gene and fatty acid synthase (*FAS*).[18] The utility of these LXR agonists is limited as drug targets, however. A study has revealed that selective LXRβ activation improves RCT in LXRα-knockout mice, while LXRα contributes to lipogenesis in liver.[19] Thus, therapeutically useful LXR agonist would be an LXRβ-selective agonist that induces RCT without elevating TG levels in the plasma and liver. In fact, several investigations have been directed towards the development of LXRβ-selective agonists.[20]

A recent investigation revealed that the synthetic sterol *N,N*-dimethylcholenamide (**1**), which contains an electronegative oxygen atom on C24, binds LXRα and LXRβ with *K*i values of 130 and 100 nM, respectively, and is more potent in modulating the activity of LXRs (Figure 1).[21] Compound **1** is also a gene-selective LXR modulator exhibiting minimal effects on *SREBP1c* while mediating potent transcriptional activation of *ABCA1* both in vitro and in vivo in mice.[22a] More importantly, compound **1** has been described to enhances cholesterol efflux in macrophages without stimulating lipogenesis circumventing the negative effects such that the synthetic sterol, T0901317 (**T**) activates triglyceride synthesis.[22b] Therefore, analogues of compound **1** would be interesting candidates for therapeutic modulators of LXRs.[22c]

In this study, we modified the amide moiety of compound **1** to seek novel modulators of LXRβ based on the computer-simulated model of the ligand-receptor complex calculated with the reported crystal structure (PDB ID: 1P8D).[23] We further

tested these compounds for the LXR-dependent transcriptional activity and calculated EC50 for LXRβ.

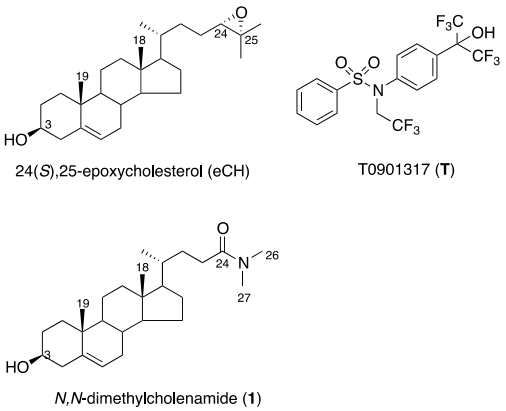


Figure 1. LXRs Ligands

X-ray crystallographic analysis of the structure of the eCH–hLXRβ complex revealed that LXRβ becomes active upon formation of a hydrogen bond between the epoxide oxygen atom and the imidazole Nε2 of His435 (Figure 2a),[23] while the A-ring in eCH is oriented toward helix 1, with the 3-hydroxyl forming a hydrogen bond with Glu281. Subsequent mutagenesis research showed that Trp443 in the LXRα (Trp457 in LXRβ) AF2 helix is essential for activation of LXR.[24] The imidazole moiety of His435 holds the indole moiety of Trp457 of the AF2 helix via a CH–π interaction. With respect to LXR ligands, oxygen atoms on C24 of several steroidal LXR ligands appear to form a hydrogen bond with Nε2 of the imidazole moiety of the His residue, which induces the CH–π interaction between the His and Trp residues in the AF2 helix. As a result, the LXRs assume an active structure. T091317 was shown to fit into a position occupied by the C and D rings of eCH, and the bis-trifluoromethyl carbinol is involved in a hydrogen bond interaction with His435. Interestingly, however, T091317 does not make contact with Glu281. The first crystal structure of a human LXRα-RXRβ heterodimer complexed with T091317 was also described.[25]

We first constructed a complex model involving *N*,*N*-dimethylcholenamide **1** based on the crystal structure of eCH–LXRβ (PDB ID: 1P8D, Figure 2b and 2c).[23] The C24–C27 moieties of eCH in the X-ray crystallographic structure were replaced with an *N*,*N*-dimethylamide moiety. This initial complex was then solvated in a truncated octahedral water box with a thickness of 8 Å around the protein under neutral conditions. The energy of the system was minimized and MD ,molecular dymamics, calculations were performed with AMBER11/SANDER under periodic boundary conditions of 300 K and 1 atm and a non-bonded interaction cut-off distance of 14 Å. The complex structure model showed that the carbonyl oxygen of the amide forms a hydrogen bond with Nε2 of His435, locating the CH proton of the imidazole moiety of His435 above the 5-membered ring moiety of Trp457.

The abovementioned conformation suggests that the amide group is arranged in an appropriate position to form a proper CH–π interaction between His435 and Trp457. By contrast, the *N*,*N*-dimethyl group is located in a hydrophobic pocket formed by Leu442, Phe268, Phe271, and Thr272 (Figure 2c), which suggests that the hydrophobic moieties of the *N*,*N*-substituted groups affect the affinity of ligands for LXRs and/or the conformation of the amide moiety of the ligands. Furthermore, we found that the corresponding hydrophobic pocket is located in the ligand-binding space, also suggesting that the space induces a restricted conformation.

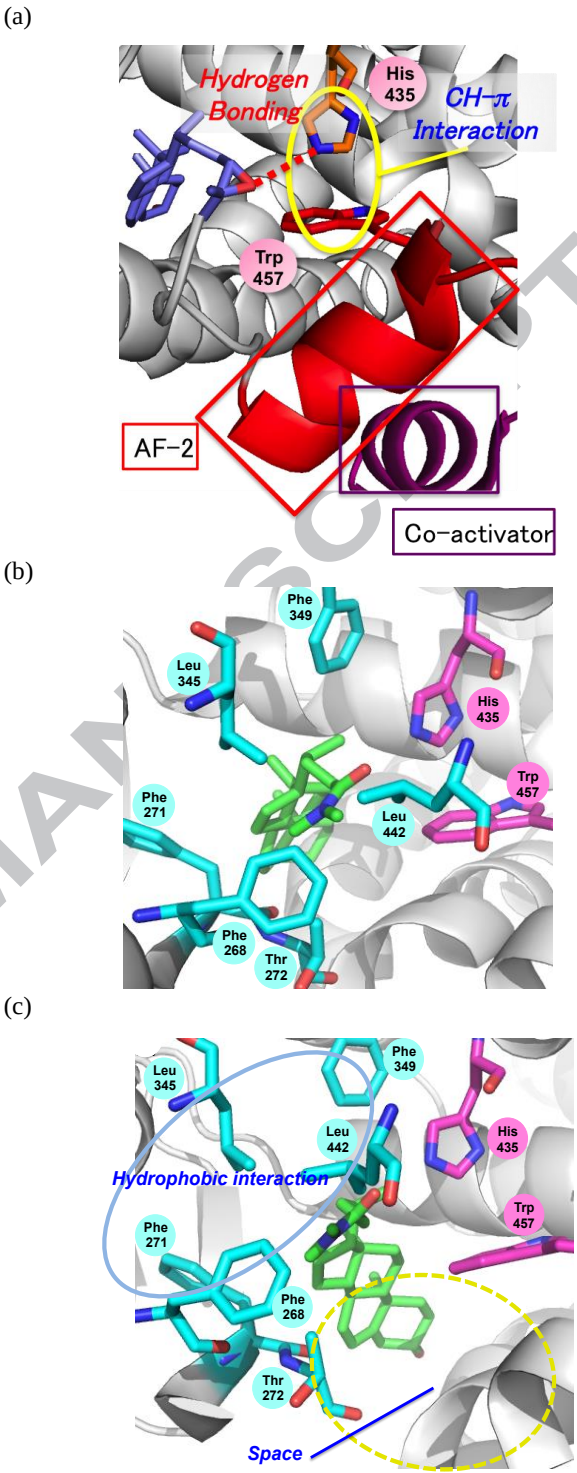


Figure 2. (a) LXR ligand binding pokect of eCH. The epoxide oxygen (red) of eCH (violet) interacts with His435.
The AF-2 helix is highlighted in red. The Co-activator is colored in pink violet. (b) Binding model of **1** in hLXRβ. Compound 1 is colored in yellow green. (c) A space of the opposite side of the hydrophobic pocket.

Thus, we designed *N*,*N*-alkyl groups to investigate the effects of the hydrophobic moieties of the cyclic and noncyclic *N*,*N*-dialkyl groups (Figure 3). We then introduced a methyl group at the C23 position to restrict the conformation of the side chain including the amide carbonyl group and also introduced cyclic structures connecting the C23 and *N*-alkyl group restraining the conformation of the amide (lactam) carbonyl group.

We synthesized compound **2** and *N*,*N*-disubstituted compounds **3**–**9**. Compounds **8** and **9** have cyclized *N*,*N*-dialkyl groups, and compounds **10**–**19** are lactams cyclized between C23

and the *N*-alkyl group (Figure 4). These newly designed cholenamide derivatives were evaluated by measuring stimulation of LXR transcription using a cell-based reporter assay.[26]

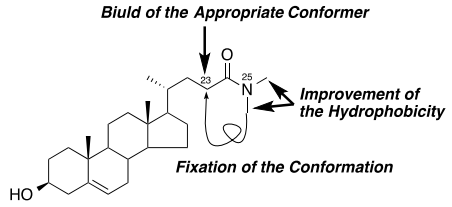


Figure 3. The plan to increase the affinity with LXR.

The cholenamide derivatives were synthesized from 3β-acetyl-Δ^5-cholenic acid **20** in 2 steps (Table 1). Condensation of **20** and various secondary amines using HATU gave the corresponding amides, from which the acetyl group at C3 was removed using K_2CO_3 to afford compounds **1**–**4** and **6**–**9** (Table 1). Compound **5** was obtained by *N*-methylation of compound **2** using NaH and MeI (Scheme 1, eq. a). Amides α-methylated at C23 were derived from **6** via methylation using MeI and LDA and obtained at 85% yield. The diastereomeric ratio at C23 was 1:1.2, with a slight excess of the more-polar product. The TBDPS group was removed using TBAF to afford the products at 87 and 85% yield respectively (Scheme 1, eq. b).

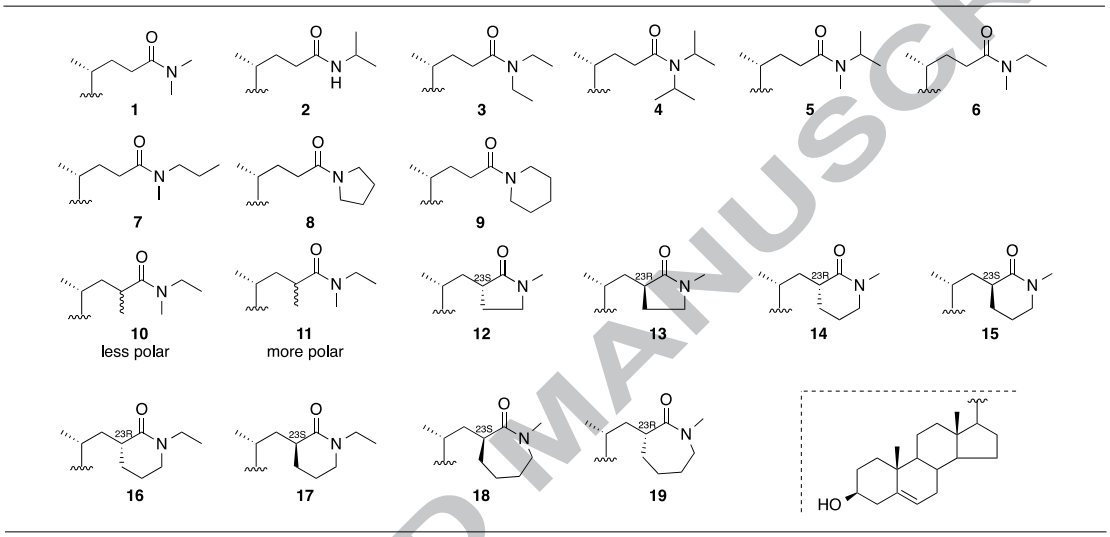
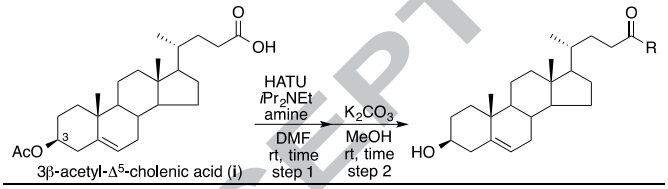


Figure 4. Structure candidates of cholic acid derivatives for LXRβ lingands.

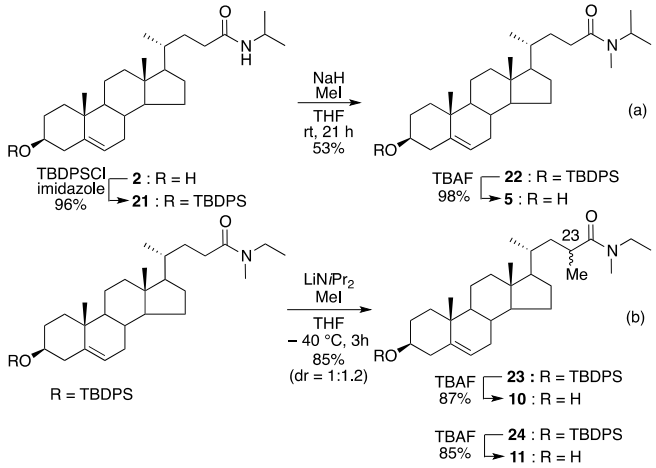
Table 1. Synthesis of cholenamide deriveatives



Amine	Step 1		Step 2		cpd[a]
	Time/h	Yield/%	Time/h	Yield/%	
Me_2NH	2	93	4	74	**1**
i$PrNH_2$	1	84	2	87	**2**
Et_2NH	4	84	2	69	**3**
iPr_2NH	4	50	2	96	**4**
EtNHMe	1	78	4	94	**6**
PrNHMe	1	81	14	98	**7**
Pyrrolidine	1.5	88	5	88	**8**
Piperidine	1	92	2	80	**9**

[a] compound number.

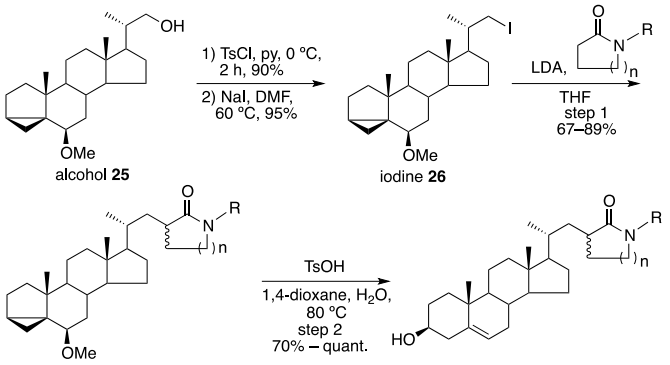
Several lactams were synthesized from alcohol **25**, as described in Scheme 2 and Table 2. After treatment of **25** with TsCl, the resulting tosylate was converted to an iodide using NaI. This iodide was reacted with the enolates derived from several γ-, δ-, and ε-lactams using LDA to give a diastereomeric mixture. In this reaction, a more-polar compound was produced in slight excess in the case of the γ- and δ-lactams with an *N*-methyl group. For the δ-lactam with an *N*-ethylated compound and ε-lactam, a less-polar compound was the major product. The diastereomers were easily separated by silica gel chromatography other than γ-lactam derivative.



Scheme 1. Synthesis of amide derivatives

Although in case of the alkylation of iodine **26** with γ-lactam gave two separable spots (more- and less- polar ones) by normal SiO_2-phase tlc (thin layer chromatogram) analysis, each of spots was realized to be a diastereomer mixture after separation. Subsequent conversion of the AB-ring using TsOH gave the desired alcohols at C3. In case of γ-lactam derivative, conversion of less polar mixture gave compound **12** and **13** in 72% and 11% whereas more polar mixture gave compound **12** and **13** in 31% and 39% respectively. The configuration at C23 for each diastereomer was assigned from the CD spectrum and structural optimization by MD/MM calculation using CHARMm installed in Discovery Studio as a force field. Thus, the more polar γ-lactam showed a negative Cotton effect at 216 nm, indicative of

an *R*-configuration at C23 according to the octant rule of the CD spectrum.[27] By contrast, a positive Cotton effect was observed for the less-polar compound at 217 nm (Figure 5). Configuration of other lactams were also determined by the same methods.[27]



Scheme 2. Synthesis of lactams

Table 2. Summary of Step 1 and 2 in Scheme 2

step 1

n	R	eluent of tlc[a]	less polar product		more polar product	
			rf value	yield/%	rf value	yield/%
1	Me	A	0.20	31	0.14	49
2	Me	B	0.35	36	0.27	46
2	Et	C	0.48	52	0.37	37
3	Me	C	0.48	53	0.36	14

step 2

n	R	eluent of tlc[a]	less polar product			more polar product		
			rf value	yield/%	cpd[d]	rf value	yield/%	cpd[d]
1	Me	D	0.20	72[b] 31[c]	**12**	0.14	11[b] 39[c]	**13**
2	Me		0.25	71[b]	**14**	0.22	quant.[c]	**15**
2	Et		0.42	83[b]	**16**	0.37	79[c]	**17**
3	Me		0.42	70[b]	**18**	0.26	82[c]	**19**

[a] A: hexane/EtOAc = 5/5, B: hexane/EtOAc = 6/4, C: hexane/EtOAc = 7/3, D: hexane/Et_2O/CH_3CN = 4/5/1. [b] less polar compound in step 1 was the starting material. [c] More polar compound in step 1 was the starting material. [d] compound number.

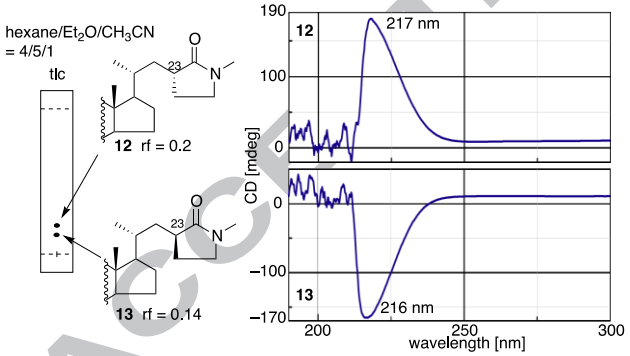


Figure 5. CD spectrum of compound **12** and **13**

LXR-dependent transcription stimulated by compounds **2**–**9** was assessed using 293 FT human embryonic kidney cells transfected with a reporter vector harboring LXR-responsive elements fused to luciferase and a rat LXRα or LXRβ (rLXRα; rLXRβ) expression vector. As a reference, stimulation with *N*,*N*-dimethylcholenamide (**1**) was included in every assay (Figure 6). Compounds **3** and **4**, which possess bulkier alkyl substituents, showed higher transcription stimulation activity compared with compound **1**, whereas compound **2**, with a secondary amide, and compounds **8** and **9**, which possess an amide with cycloalkyl moiety, were less active, suggesting that the hydrophobic pocket prefers bulkier substituents. Notably, the transcription stimulation activity of compound **4** in the LXRα assay was 2-fold higher than in the LXRβ assay. All compounds tested showed higher activity in the LXRα assay compared with the LXRβ assay.

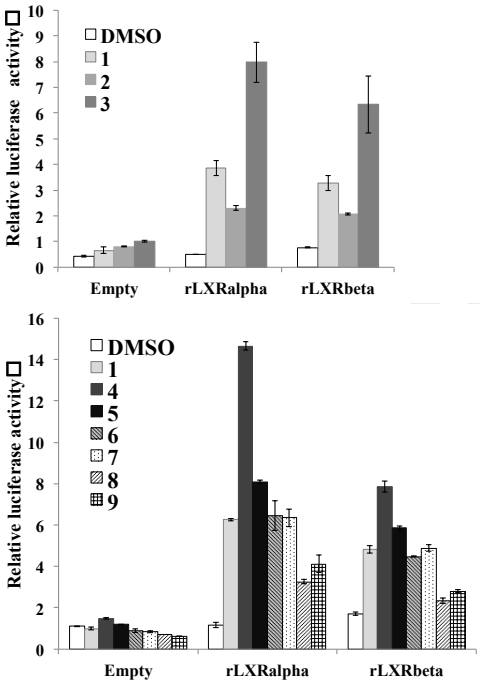


Figure 6. LXRα and LXRβ-dependent transcription activity stimulated by cholic acid derivatives.
293FT cells were cotransfected with p3xLXRE-Luc (50 ng), *Renilla* luciferase vector (10 ng), and empty vector (pIRESNeo3; 150 ng), pCMXrLXRα (100 ng) or pCMXrLXRβ (100 ng) . After transfection, 5 μM compounds indicated Figure 4 (1 to 9) or DMSO (D) were added to the media for 14-18 hours. Cells were harvested for *Firefly* luciferase assay normalizing with *Renilla* luciferase. Data are the mean of 2 independent experiments ± standard error.

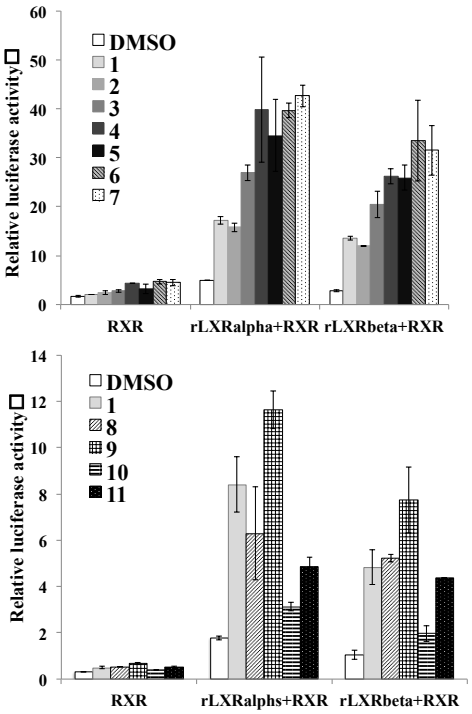


Figure 7. LXRα and LXRβ-dependent transcription activity stimulated by cholic acid derivatives in RXR-expressed conditions.
293FT cells were cotransfected with p3xLXRE-Luc (50 ng), *Renilla* luciferase vector (10 ng), pSG5mRXR (75 ng), and, pCMXrLXRα (75 ng) or pCMXratLXRβ (75 ng). After transfection, cells were treated with 5 μM compounds listed in Figure 4 (1 to 10) and assayed as in Figure 5.

We also expressed RXR along with LXR to confirm the ligand-binding specificity to LXR but not RXR in the LXR-RXR heterodimer (Figure 7). Compounds **3**–**7**, which have a tertiary amide, showed stronger activity compared with compound **1** in both the LXRα and LXRβ assays. Once again, they showed stronger activity in the LXRα assay than the LXRβ assay. The activity of compounds **5**, **6**, and **7** under conditions of RXR co-expression was higher than that of LXRα or LXRβ alone.

Next, we calculated EC_{50} by dose-curve using the similar luciferase assay with cells expressing LXRβ. Presumably due to the differences in the number of expressed receptors, the EC_{50} values deviated in each time of assay. Therefore, we included compound **1** in each assay as a control to normalize the deviation in each assay. T0901317 (**T**) was also included along with compound 1 (Table 3).

Table 3. Kinetic analyses of cholic acid derivatives for LXRβ-dependent transcription stimulating activity

exp.	cpd	LXRβ		exp.	cpd	LXRβ	
		EC_{50} (μM)	Efficacy/%			EC_{50} (μM)	Efficacy/%
a	**1**	0.423	100	b	**1**	0.464	100
	2	0.862	85		**5**	0.623	109
	3	0.344	211		**6**	0.786	108
	4	1.107	148		**7**	1.024	102
c	**1**	0.141	100	d	**1**	0.206	100
	8	0.165	99		**10**	3.896	39
	9	0.127	134		**11**	1.992	65
e	**1**	0.124	100	f	**1**	0.154	100
	12	1.291	66		**14**	0.239	117
	13	–	0.54		**15**	–	0.69
g	**1**	0.382	100	h	**1**	0.334	100
	17	0.643	62		**T**	0.030	98
	18	0.462	80				

EC50 (μM) are calculated based on the luciferase assay with dose-response curve. Efficacy (%) is expressed as percentage of maximal activity relative to **1** . Compound 1 was included in each set of experiment to normalize deviation observed in each assay. Each set of experiment (exp.) was indicated as (a) to (h).

Compound **3**, with a diethyl amide, and compound **4**, with a diisopropyl amide, showed higher efficacy. Compound **2**, with a secondary amide, was less active than the tertiary amides. The *N*-methyl compounds **5** and **6** have an *N*-isopropyl or -ethyl group, respectively, whereas compound **7** has an *N*-propyl group. These compounds showed EC_{50} and efficacy similar to those of compound **1**. Compound **3**, which harbors a flexible side chain, showed 2-fold higher efficacy and lower EC_{50} values as compared with compound **1**, whereas compound **8**, which harbors a cyclized structure, showed efficacy similar to compound **1**. Compound **9**, with a piperidine ring, showed higher efficacy than compound **1**. These results suggest that the size of the *N*,*N*-alkyl substituents is critical for binding to the receptor. The effect of stereochemistry at C23 was examined by introducing a methyl group or cyclic structure (compounds **10**–**19**). The transactivation activity of the more-polar compound **11** was 2-fold higher than that of the less-polar compound **10** (Table 3). Among the lactam compounds examined, only the *S*-isomer compound **12** and *R*-isomer compound **14** exhibited stimulatory activity. Importantly, compounds **13** and **15** showed no transactivation activity (Table 3). Although compound **12** was less potent compared with compound **1** (Table 3), compound **14** was more efficacious than compound **1** (Table 3). By contrast, δ-lactam compound **17** and ε-lactam compound **18** showed lower transactivation activity than compound **1**. These results indicate that among the compounds with restrained conformations, only lactam compound **14** should have a conformation of the amide oxygen suitable for binding the receptor. Comparison of those amide (lactam) derivatives led us to determine the receptor-bound conformation of cholenamide compound **1**. This putative receptor-bound conformation was consistent with that of the chloenamide complexed in the ligand-binding site of the receptor, as shown in Figure 3.

In summary, we designed compounds exhibiting good affinity for LXRs by modeling structures of LXRs in complex with their ligands. In luciferase assays, the tertiary amide compounds **3** and **4** exhibited higher activity. In LXR-RXR assays, compounds with hydrophobic moieties showed higher activity than compound **1**. The stereochemistry of the C23 of the lactams was found to be important for determining the EC_{50} values and transactivation of the genes, thus providing information regarding the receptor-bound conformation of the side chain of the cholenamide derivatives.

Acknowledgments

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Supplementary Material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/xxxx/xxxx/j.bmclxxx.

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