Synthesis, *in vitro* and *in vivo* Biological Evaluation of new Oxysterols as Modulators of the Liver X Receptors

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Abstract: Liver X Receptor (LXR) modulators have shown potential as drugs since they target genes affecting metabolism and fatty acid synthesis. LXR antagonists are of particular interest since they are able to reduce the synthesis of complex fatty acids and glucose uptake. Based on molecular modeling, five new cholesterol mimics were synthesized, where four contained a hydroxyl group in the 22-*S*-position. The new compounds were screened *in vitro* against several genes affecting lipid metabolism. The compound that performed best *in vitro* was a dimethylamide derivative of 22(*S*)-hydroxycholesterol and it was chosen for *in vivo* testing. However, the blood plasma analysis from the *in vivo* tests revealed a concentration lower than needed to give any response, indicating either rapid metabolism or low bioavailability.

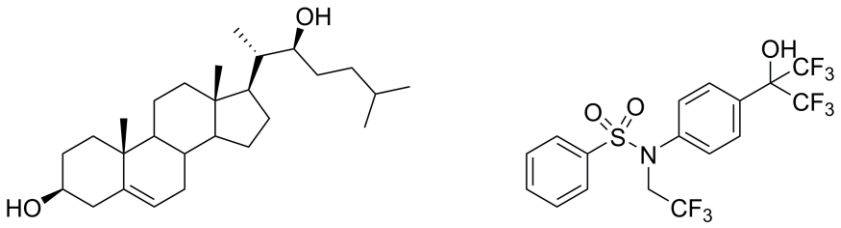
INTRODUCTION

The Liver X Receptors (LXRs) belong to the nuclear receptor superfamily. LXRs have been identified as promising drug targets due to their involvement in regulation of cholesterol, lipid metabolism and glucose metabolism.[1, 2] LXR consists of two isoforms, LXRα and LXRβ.[3] LXRα is the main isoform found in the liver, but the receptor is also found in adipose tissue, skeletal muscle, macrophages, kidney and the small intestine. LXRβ is, however, found throughout the body.[4] The endogenous ligands for LXRs are oxysterols and bile acids.[5-8]

LXR agonists have been developed as potential treatments for e.g. metabolic and cardiovascular disorders, and have shown promising regarding treatment of atherosclerosis and diabetes.[9-11]

Several studies have shown that LXR activation over a prolonged period of time results in elevated uptake of glucose and fatty acids, leading to increased storage of fatty acids.[12] LXRs are also suggested to be involved in the pathogenesis of type 2 diabetes.[12] Specific LXR antagonists could reduce the synthesis of complex fatty acids, an underlying part of the pathogenesis of type 2 diabetes. However, only a few compounds have been described as LXR antagonists[13-16], e.g. 5α,6α-Epoxycholesterol (and derivatives), 22(*S)*-hydrocycholesterol (**22SHC**, **1**) and GSK2033.[13, 17, 18]

A selective LXR antagonist may have beneficial effects on glucose uptake and lipid metabolism, two processes of importance for obesity and type 2 diabetes.[1, 2] LXR target genes stearoyl–CoA desaturase 1 (SCD1) and fatty acid synthase (FAS) code for enzymes that are key regulators in lipid metabolism; compounds repressing these genes could be lead drug candidates for treatment of type 2 diabetes and metabolic syndrome.[18] A small modification in the stereochemistry of an endogenous LXR agonist (22(*R*)-hydroxycholesterol) resulted in a compound (**22SHC**, Figure 1-1) with selective antagonistic properties on lipogenesis, reducing or abolishing the effect of the potent LXR agonist **T0901317** (Figure 1-2).[18] This indicated that synthetic modulators could alter gene expressions and increase the lipid metabolism and glucose uptake in human cells. Thus, the main focus in this work was to continue our search for new LXR modulators[19, 20] and explore whether newly synthesized derivatives of **22SHC** (**1**), based on molecular modelling, showed similar or more potent effects on lipid and glucose metabolism both *in vitro* and *in vivo* than the parent compound. Such compounds would have great potential as new clinical candidates if intellectual patent rights can be secured.



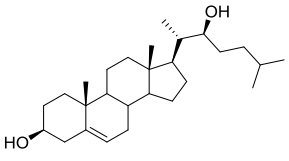
22SHC, **1** T0901317, **2**

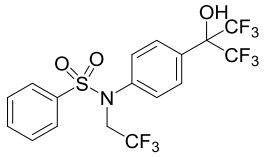
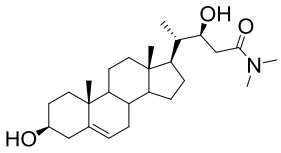
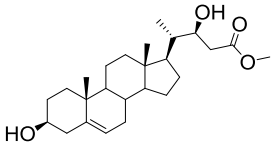
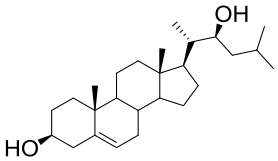
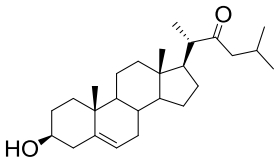
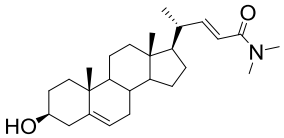
Figure 1. Structure of the known LXR modulators **22SHC** (**1**) and LXR agonist **T0901317** (**2**).

RESULTS AND DISCUSSION

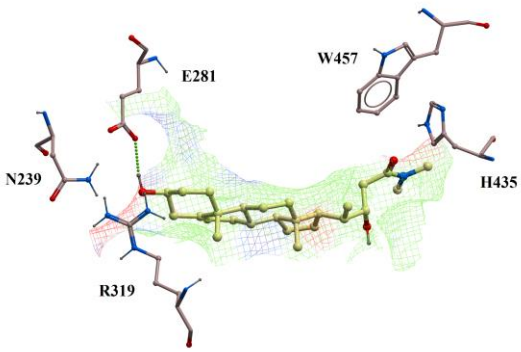
Since LXRβ is found throughout the whole body, we focused our molecular modelling efforts on this isoform. In addition, the structure of 24(*S*),25-epoxycholesterol in complex with LXRβ is known X-ray crystallography (PDB code: 1P8D) and could be used as guide in the evaluation of the docked complexes. A series of steroid based compounds were constructed *in silico* and the compounds were docked into LXRβ structure after removal of 24(*S*),25-epoxycholesterol. Compound **10** (one of the compounds with highest docking score,) can be seen docked into the ligand binding pocket of LXRβ in Figure 2. Some of the highest scoring compounds (docking score below -35) from the docking are listed in table 1.

Table 1: Docking scores of selected modulators to LXRβ (PDB-code 1PQ6). The results are the best score obtained during 3 simulations.

Compound number	**Chemical structure**	**Docking score LXRβ**
22SHC, **1**		-36.73

T0901317, **2**		-22.60
10		-42.00
14		-35,36
16		-36.11
18		-37.73
21		-42.61

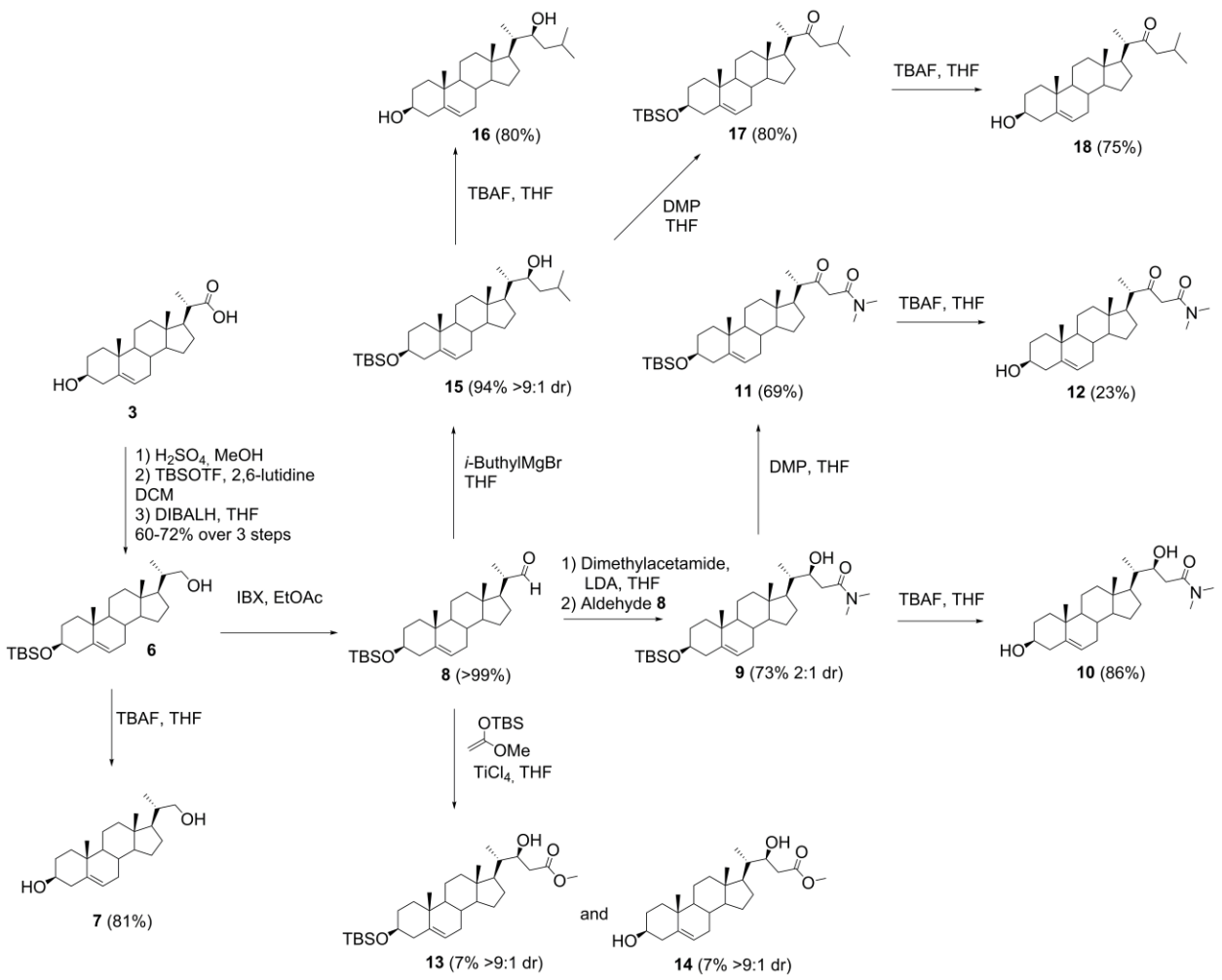
1



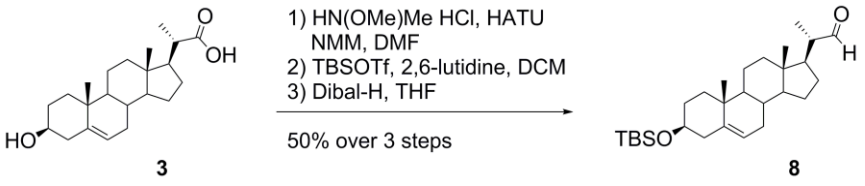
2

Figure 2. Compound **10** docked to the ligand binding pocket of LXRβ including relevant amino acid side chains. The Ligand Surface (mesh) will be displayed colored by binding property - white=neutral surface, green=hydrophobic surface, blue=hydrogen bonding acceptor potential, and red=hydrogen bond donor potential.

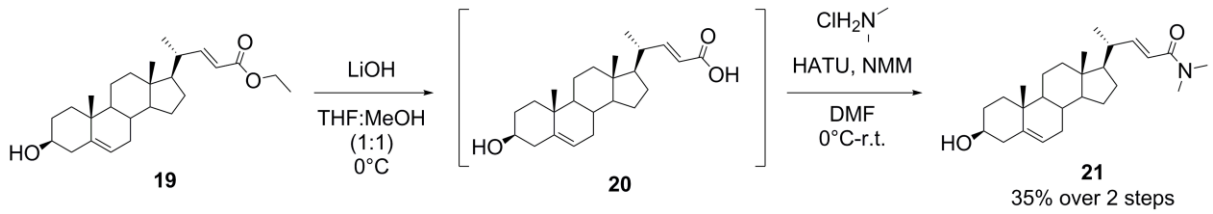
Some of the compounds with the best results from the molecular modeling were chosen for synthesis, as these compounds were not described in the literature. The commercially available Fernholtz acid (**3**) was used as a synthetic starting point, which conveniently could be converted to the key aldehyde intermediate (**8**).[21] Lithium enolate addition to **8** provided the protected amide **9** in good yield and 2:1 d.r. of 22*S* and 22*R*, which after recrystallization could be obtained as a single diastereomer. Treatment of **9** with TBAF afforded compound **10** in good yield. A Mukaiyama aldol reaction gave the β-hydroxy esters **13** and **14** directly, although in poor yield (reaction not optimized). A Grignard-reaction provided compound **15**, which after deprotection afforded nor-22SHC (**16**). Compound **15** could also be oxidized to ketone **17**, which after deprotection gave the β-keto-alcohol **18**. Dimethylamide **21**, an unsaturated analogue of **10**, which lack the 22(*S*)-hydroxy group, was prepared according to scheme 3 in a slightly different way starting from the ester **19**. The synthesis of all new modulators is shown in scheme 1-3 and the detailed experimental procedures can be found in the supporting information.



Scheme 1: Synthesis of some selected new modulators from the key aldehyde **8**.



Scheme 2: Alternative synthesis of the key aldehyde **8** via a Weinreb amide intermediate.



Scheme 3: Synthesis of unsaturated dimethylamide **21**.

We have previously shown that **22SHC** (**1**) behaved as an antagonist in skeletal muscle cells and HepG2 cells giving reduced lipogenesis and increased glucose uptake.[22] In this study, we have synthesized cholesterol mimetics of **22SHC** (**1**) and tested them in the same cell systems. The results confirm that **22SHC** (**1**) reduces lipogenesis in myotubes and that the two dimethyl amides **10** and **21** mimick this effect. Compounds **10** and **21** have the same ability as **22SHC** (**1**) to abolish the effect of the LXR-agonist **T0901317** (Figure 3). This indicates that 22SHC, **10** and **21** are LXR modulators. While the precise mechanisms of action for the new compounds are still unknown, the *in silico* docking results suggests they compete with **T0901317** for the LXR binding site. However, since not all LXR target genes are affected, the binding mode might be different from the other known LXR binders. Similar effects were observed with regards to HepG2 cells, especially compound **10** shows similar effects as **22SHC** (**1**) both on reducing lipogenesis and abolish the effect of **T0901317**, while compound **21** only abolished the effect of **T0901317** (Figure 4A). Further, both compounds **10** and **21** reduce lipogenesis in a dose-response like manner similar to **22SHC** (figure 4B and C). Expression of genes important for lipogenesis (FASN and SCD1) and reverse cholesterol transport (ABCA1) in the cells was also studied (Figure 5). Again, we observed that compound **10** and **21** behaved similar to **22SHC** (**1**), especially in that they neutralized the effect of **T0901317** on both FASN and SCD1 (Figure 5A and B). The ABCA1 codes for a cholesterol transporter important for reversing the cholesterol transport in organisms. It is essential that the expression of this gene is not reduced since it could enhance the development of atherosclerosis especially in humans and rodents. Neither **22SHC** (**1**) nor the new 22SHC cholesterol mimics seem to reduce the levels of ABCA1 alone or in combination with **T0901317** (Figure 5C). The detailed mechanism for the different regulation of

FAS and SCD1 versus ABCA1 by 22SHC (**1**) is still unknown. One explanation offered is that different co-regulators are involved in regulating the activity of LXR, thereby modulating the genes involved in different metabolic pathways. But this requires further investigation.

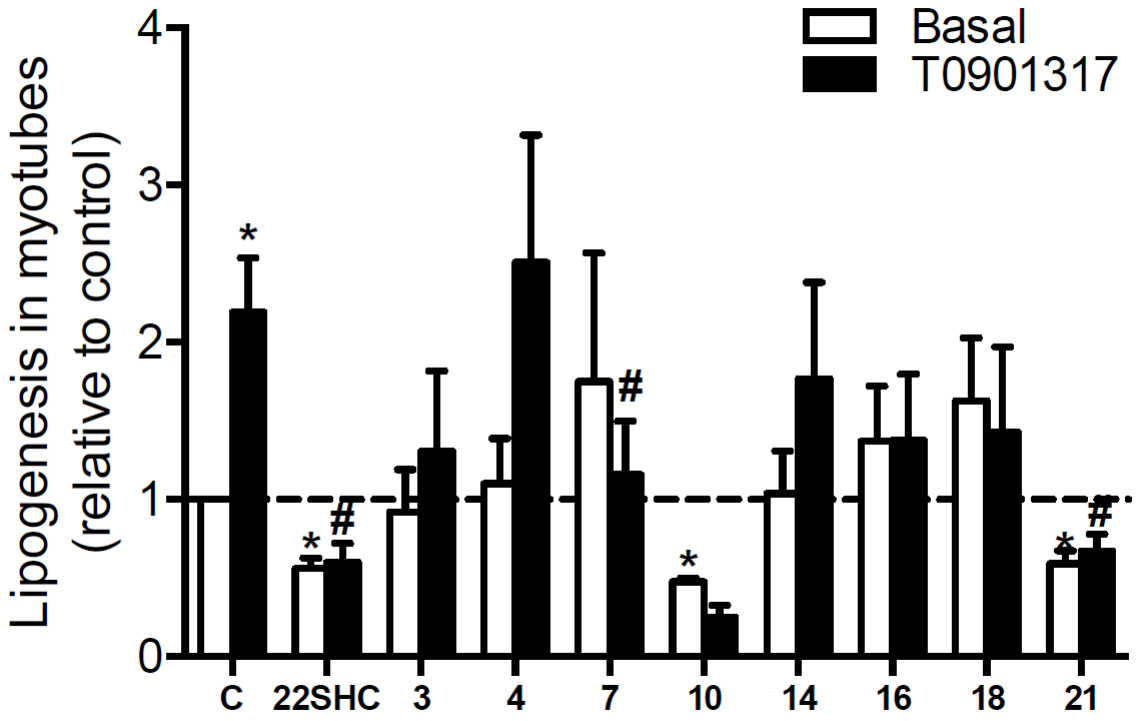


Figure 3. ***De novo*** **lipogenesis in myotubes was reduced by 22SHC, compound 10 and 21.** Myotubes were treated with DMSO (0.1 %) control (C), 10 µM 22SHC and compounds **3**, **4**, **7, 10, 14, 16, 18** and **21** ± 1 µM T0901317 for 4 days. The cells were incubated with [1-^{14}C]acetate (1 µCi/mL, 100 µM) for 24 h before lipids were isolated by filtration through hydrophobic MultiScreen® HTS plates. The levels of lipids were determined by scintillation counting. Values represent fold change relative to control for total lipids synthesized from acetate given as means ± SEM, n = 3-5 separate experiments. *P < 0.05 vs. control (DMSO) and $^{\#}P$ < 0.05 for **T0901317** vs treatment + **T0901317**.

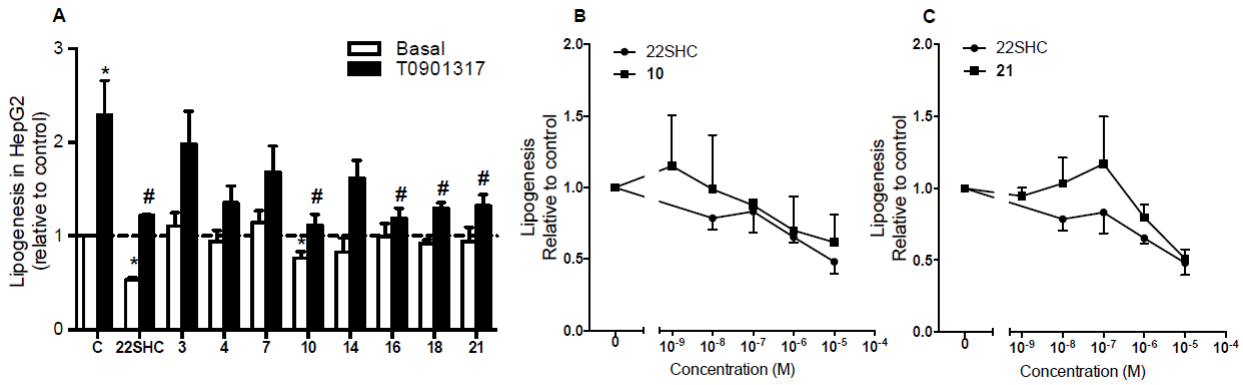


Figure 4. ***De novo*** **lipogenesis in HepG2 cells was reduced by 22SHC, compound 10 and 21.** HepG2 cells were treated with DMSO (0.1 %) control (C), 10 µM 22SHC, compounds **3**, **4**, **7**, **10**, **14**, **16**, **18** and **21** ± 1 µM T0901317 for 24 h. Thereafter, the cells were incubated with [1-^{14}C]acetate (1 µCi/mL, 100 µM) for 4 h before lipids were isolated by filtration through hydrophobic MultiScreen® HTS plate. The levels of lipids were determined by scintillation counting. Values represent fold change relative to control for total lipids synthesized from acetate given as means ± SEM from (A) lipogenesis in HepG2 cells, n=4-8 separate experiments (B) dose-response for 22SHC and compound 10, n=3, (C) dose-response for 22SHC and compound 21, n=3 . *P < 0.05 vs. control (DMSO) and $^{\#}P$ < 0.05 for **T0901317** vs treatment + **T0901317**.

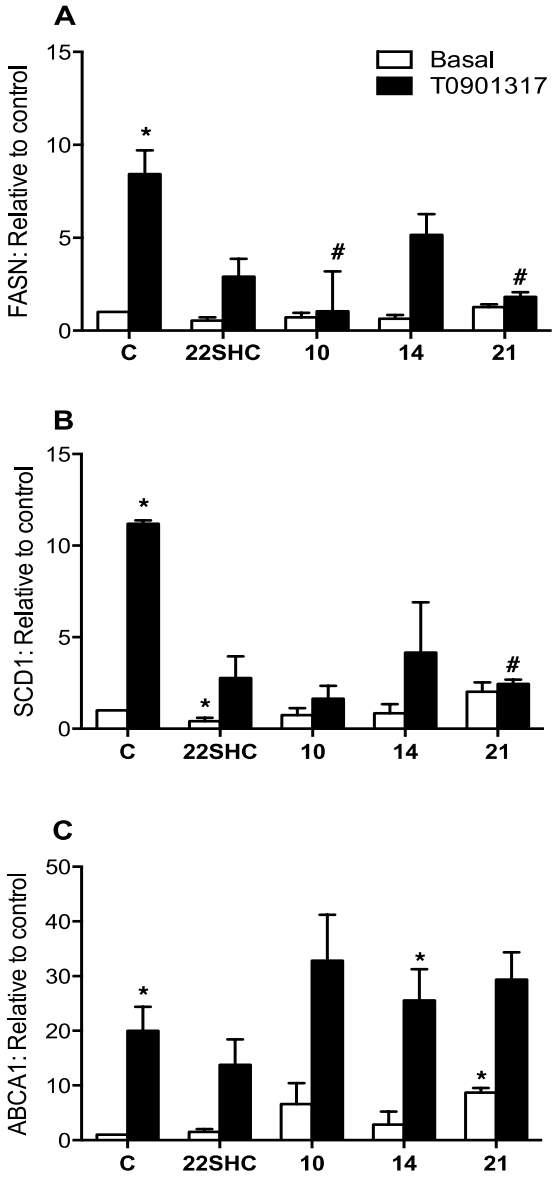


Figure 5. Effects of 22SHC and 22SHC-mimics on basal and T0901317-induced gene expression

Myotubes were treated with DMSO (0.1%) control (C), 1 μM T0901317, 10 μM 22SHC, compounds **10, 14** and **21**, for 4 days. Total RNA was then isolated from the cells and analyzed by qPCR as described in Materials and Methods. Gene expressions were normalized to 36B4. Values represent fold change relative to control given as means ± SEM (n=3-6). Analyzed LXR

target genes were (A) fatty acid synthase (FASN), (B) stearoyl-CoA desaturase 1 (SCD1) and (C) ATP-binding cassette transporter A1 (ABCA1). *$P < 0.05$ vs. control (DMSO) and $^{\#}P < 0.05$ for T0901317 vs. treatment + T0901317.

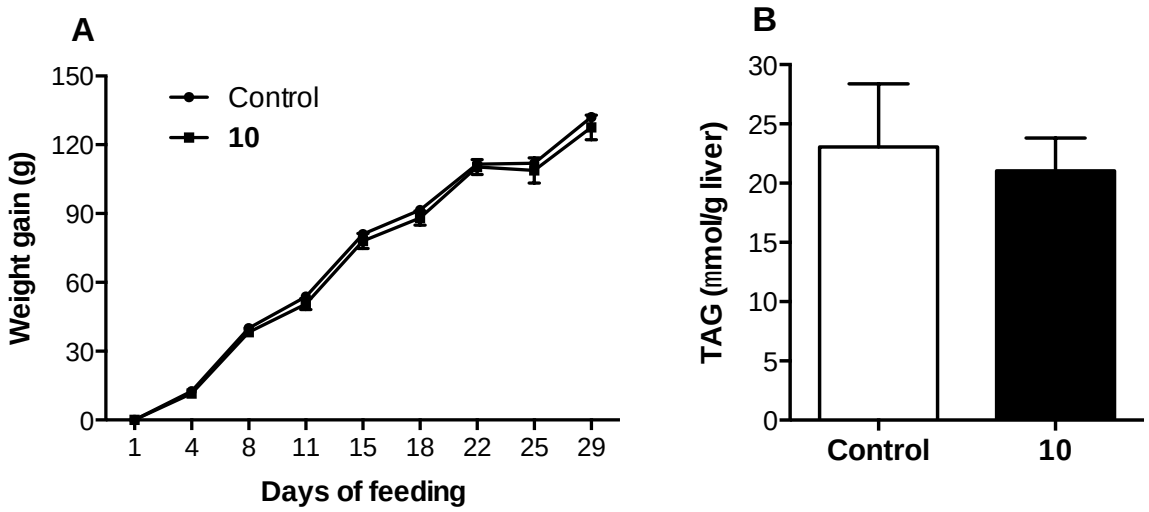


Figure 6. Effects of compound 10 *in vivo* in Wistar rats.

Rats were randomly divided into two groups with 8 animals in each, receiving high-fat diet (HFD) ± **10** for 28 days. The rats were given free access to tap water and were weighed in the morning on day 1, 4, 8, 11, 15, 18, 22, 25 and 29 to measure weight gain (A). Liver samples were prepared as described in methods for triacylglycerol (TAG) analysis (B). Data are presented as mean ± SEM (n=6).

A previous *in vivo* study showed that **22SHC** (**1**) reduced both body weight gain and triacylglycerol (TAG) levels in rats on a high-fat diet.[23] Therefore, based on our *in vitro* results, the effect of compound **10** was examined in Wistar rats fed a high-fat diet identical to how **22SHC** (**1**) was tested previously. Compound **21** was rejected as test compound due to structural similarities with previously tested cholesterol derivatives.[24] During and after the treatment period

with compound **10**, we could not observe the same beneficial effects on body weight or levels of TAG in the liver as observed for **22SHC** (**1**) (figure 6). There were no effects on plasma levels of glucose, TAG, cholesterol or non-esterified fatty acids (NEFA) either (data not shown). The plasma analysis from the rats taken on the last day of the experiment showed that levels of compound **10** were very low and hardly detectable, approximately 1-2% of what was previously found for **22SHC** (data not shown). 22SHC (1) has an oral bioavailability of about 50% and was easily detected in plasma after oral administration.[23] Possible explanations for reduced *in vivo* effect of **10** could be related to low bioavailability, e.g. increased metabolism in liver and/or gut. Previously, Griffett and co-workers have reported that an inverse LXR agonist, with similar effects on lipogenesis as observed for **10**, did not survive the first pass metabolism, but could be detected in liver.[25] The observed differences in cLogP values (calculated using ChemDraw Ultra 13.0, CambridgeSoft) of **1** (cLogP 7.3) and **10** (cLogP 4.6) could also affect metabolism, thereby partly explaining the lack of effects on body weight gain and levels of TAG in the liver. Gastrointestinal (GI) amide hydrolysis of 10, leading to the corresponding carboxylic acid with a cLogP of 1.6, may also explain the reduced plasma concentration of **10**. Rats have an efficient gastro-intestinal metabolism, and GI cleavage of amides by GI enzymes is well described in the literature.[26-28]

CONCLUSION

Molecular modeling identified several new potential LXR modulators. Some of the best hits (docking score < -35) were then synthesized in moderate to good yields and subjected to *in vitro* testing in myotubes and HepG2 cells. Compounds **10** and **21** showed similar results as the known compound **22SHC** (**1**) on both the ability to reduce lipogenesis and regulation of lipogenic genes. Therefore, compound **10** was chosen for an *in vivo* study to compare it with the reported

effect of **22SHC** (**1**) in mice on a high fat diet. The results from the *in vivo* study showed no significant difference in body weight gain between control mice and animals that were subjected to treatment with **10**. When analyzing the plasma concentrations for **10**, the average concentration was found to be 1.1±0.5 ng/mL (mean 0.77 ng/mL). The low plasma concentration observed, is likely due to low uptake by the intestine and/or rapid first pass metabolism by the liver. The bioavailability of **10** needs to be improved and better described before further *in vivo* studies can be performed. In addition, in order to avoid similar problems, bioavailability studies of the lead compounds should be performed before future *in vivo* tests.

EXPERIMENTAL SECTION

Materials. Dulbecco's modified Eagle's medium (DMEM-Glutamax™, 5.5 mM), DMEM, foetal bovine serum, Ultroser G, penicillin-streptomycin-amphotericin B, and trypsin-EDTA were obtained from Gibco, Life Technologies (Paisley, UK). [1-^{14}C]acetic acid (54 mCi/mmol) and D-[^{14}C(U)]deoxy-D-glucose (6.0 Ci/mmol) were purchased from ARC (American Radiolabeled Chemicals, St. Louis, MO, USA). Insulin Actrapid was from Novo Nordisk (Bagsvaerd, Denmark). Bovine serum albumin (BSA) (essentially fatty acid-free) and 22(*S*)-hydroxycholesterol (**22SHC**, **1**) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fernholtz acid (**3**) was purchased from Steraloids Inc, (Newport, RI USA). Ester **19** was purchased from Synthetica AS (Oslo, Norway). RNeasy Mini kit and RNase-free DNase were purchased from Qiagen Sciences (Oslo, Norway). Agilent Total RNA isolation kit was from Agilent Technologies (Santa Clara, CA, USA). The primers were purchased from Invitrogen (Paisley, Scotland, UK), while SYBR® Green and TaqMan® reverse-transcription reagents kit were from Applied Biosystems (Foster City, Canada). **T0901317** was obtained from Cayman Chemical Company (Ann Arbor, MI, USA). Hydrophobic MultiScreen®

HTS plates were from Millipore (Billerica, MA, USA). Corning® CellBIND® tissue culture plates were obtained from Corning Life-Sciences (Schiphol-Rijk, The Netherlands). The protein assay reagent was obtained from BioRad (Copenhagen, Denmark). All other chemicals used were used as received and of high quality.

Docking experiment. The ligands were docked into the crystal structure of the LXRβ ligand binding domain in complex with 24(*S*),25-epoxycholesterol (PDB id 1P8D) using Internal Coordinate Mechanics (ICM) software version 3.[29] The ligands were docked into the crystal structure of the LXRβ ligand binding domain in complex with 24(*S*),25-epoxycholesterol (PDB id 1P8D). To set up the receptor grid maps, amino acids within 5 Å of the co-crystallized ligand were selected. The ligands were charged using ICM auto pKa macro (pH 7) and converted to 3D before docking. Due to the stochastic docking method, three parallel docking runs were performed and the best-scored ligand from the parallels was selected as the best orientation.

Synthesis of new modulators. The synthesis of the new oxysterols **10**, **14**, **16** and **18** are based on nucleophilic additions to the known aldehyde **8**[21], which could be made in two separate ways. The amide **21** was made from ester **19**. The detailed experimental procedures can be found in the supporting information.

Culturing of human myotubes. Satellite cells were isolated as previously described[30] from the *M. obliquus internus* abdominis of 6 healthy donors, age 39.9 (± 2.9) years, body mass index 23.5 (± 1.4) kg/m^2, fasting glucose 5.3 (± 0.2) mM, insulin, plasma lipids and blood pressure within normal range and no family history of diabetes. The muscle biopsies were obtained with informed consent and approval by the National Committee for Research Ethics, Oslo, Norway. The cells were cultured in DMEM-Glutamax™ (5.5 mM glucose), 2 % foetal bovine serum, 2 % Ultroser G, penicillin (100 units/mL), streptomycin (100 µg/mL), and amphotericin B (1.25

μg/mL) for proliferation. At 70-80 % confluence the growth medium was replaced by DMEM-Glutamax™ (5.5 mM glucose) supplemented with 2 % foetal bovine serum, penicillin (100 units/mL), streptomycin (100 μg/mL), amphotericin B (1.25 μg/mL), and insulin (25 pM) to induce differentiation. The cells were cultured in humidified 5 % CO_2 atmosphere at 37°C, and the medium was changed every 2–3 days. Experiments were performed after 7 days of differentiation.

Culturing of HepG2 cells. The human hepatoblastoma cell line HepG2 (HB-8065, ATCC, Manassas, VA, USA) was cultured in DMEM-Glutamax™ (5.5 mM glucose) supplemented with 10 % foetal bovine serum, streptomycin (100 μg/mL) and penicillin (100 units/mL) at 37°C in 5 % CO_2.

RNA isolation and analysis of gene expression by TaqMan® real-time qPCR. Myotubes were treated with DMSO (0.1 %), 1 μM **T0901317**, 10 μM **22SHC** (**1**), compounds **3**, **4**, **7**, **10**, **14**, **16**, **18** and **21**, for 4 days, harvested and total RNA was isolated by Agilent Total RNA isolation kit (Agilent Technologies, Santa Clara, CA, USA) according to the supplier's total RNA isolation protocol. Total RNA (1 μg/μL) was reversely transcribed with hexamere primers using a Perkin-Elmer Thermal Cycler 9600 (25°C for 10 min, 37°C for 1 h, 99°C for 5 min) and a TaqMan reverse-transcription reagents kit (Applied Biosystems). DNA expression was determined by SYBR® Green (Applied Biosystems). Primers (36B4, ABCA1, FASN, GAPDH and SCD1) were designed using Primer Express® (Applied Biosystems). Primer sequences are available upon request. Each target gene were quantified in duplicates and carried out in a 25 μL reaction volume according to the supplier's protocol. All assays were run for 40 cycles (95°C for 12 s followed by 60°C for 60 s). The transcription levels were normalized to the reference control genes 36B4 and GAPDH.

***De novo* lipogenesis.** Cells were treated with DMSO (0.1 %), 1 μM **T0901317**, 10 μM **22SHC** (**1**), compounds **3**, **4**, **7**, **10**, **14**, **16**, **18** and **21**, for 4 days for myotubes and 24 h for HepG2 cells, before exposure to DMEM supplemented with [1-^{14}C]acetic acid (1 μCi/mL, 100 μM) for 24 h for myotubes and 4 h for HepG2 cells. Thereafter, cells were harvested in 0.1 M NaOH, assayed for protein [31] and total lipids were isolated by filtration of the cell lysate through a hydrophobic MultiScreen® HTS plate (Millipore, Billerica, MA, USA). The levels of lipids were determined by scintillation counting, and lipogenesis from acetate was calculated by use of protein levels for standardization.

Glucose uptake. Myotubes were treated with DMSO (0.1 %), 1 μM **T0901317**, 10 μM **22SHC** (**1**), compounds **3**, **4**, **7**, **10**, **14**, **16**, **18** and **21**, for 4 days. Thereafter, cells were exposed to D-[^{14}C(U)]deoxy-D-glucose (1 μCi/mL, 100 μM) for 4 h. After incubation the cells were washed two times with ice-cold phosphate buffered saline (PBS), lysed in 0.1 M NaOH, and radioactivity was counted by scintillation counting. The protein content of each sample was determined,[31] and glucose uptake was calculated using protein levels for standardization.

Animals for the compound 10 effect study. Male Wistar rats were purchased from Scanbur AS, Norway. The rats were fed *ad libitum* a regular maintenance diet (Special Diets Services (SDS), Witham, Essex, UK) for 5 days after arrival to our animal facilities. Then a feeding regimen was adopted using a high-fat diet (HFD, see below) for 28 days. The rats were about 9 weeks old at the start of the experimental feeding. Wistar rats were randomly divided into two groups with 8 animals each receiving HFD ± **10**. There were 4 animals in each cage and they had free access to tap water. The experimental protocol (Id: 5904) was approved by the National Animal Research Authority.

Diets. The animals were given HFD. In total, HFD consisted of 1.9% gelatin, 5.7% wheat bran, 7.7% vitamin and mineral mix, 25.1% cornstarch, 25.7% casein, 26.8% beef tallow and 7.1% sunflower oil. The HFD provided approximately 60% of the energy from fat.

Experimental protocol. The rats (n=16) were fed *ad libitum* on the experimental diets, and the total feed intake for each group (n=8) was recorded at the end of the experiment. Rats were given **10** in 45% water solution of 2-hydroxypropyl-β-cyclodextrin by gavage. They received 30 mg/kg/day of **10** calculated at the beginning of the feeding period. Body weight was registered twice a week. Controls and treated rats were weighed at the same day during the experimental feeding. Blood samples were collected from a vein in the leg in the morning between 08:30 and 9:00 am once a week. Immediately after termination, blood samples (cardiac puncture) and tissues (snap frozen in liquid nitrogen) were collected between 10:00 am and 12:00 pm.

Serum analysis. After anaesthetizing the rats with 20 mg pentobarbital (i.p. 50 mg/mL), blood was collected from all 16 animals by aortic puncture and left in room temperature to coagulate. Serum was prepared and stored at -20ºC prior to analysis. Serum lipids and glucose were measured on the MaxMatPL system (ILS Laboratories Scandinavia AS, Oslo, Norway) using the following kits: glucose, triacylglycerol (TAG), total cholesterol and non-esterified fatty acids (NEFA) (all from ILS Laboratories Scandinavia AS).

Determination of liver triacylglycerol content. Frozen liver samples from all 16 animals were weighed and homogenized in 1 mL ice cold 1 mmol/L EDTA buffer with Precellys®24 bead beater (5800 beats, 30s, www.precellys.com) and spun down for 10min, 1000g at 4°C. The supernatant was transferred to new tubes. Triacylglycerol content was measured with a TG PAP 150-kit (BioMerieux, Marcy l´Etoile, France) according to the supplier´s protocol.

Plasma concentration analysis. The detailed experimental procedure for the plasma concentration analysis for compound **10** can be found in the supporting information.

Presentation of data and statistical analysis. Data in text and figures are given as mean (± SEM) from n = number of separate experiments. At least 3 parallels were included in each experiment. Comparisons of different treatments were evaluated by two-tailed, paired Student's t-test, and $P < 0.05$ was considered significant.

ASSOCIATED CONTENT

Supporting Information. Additional synthetic details and μLC-MS data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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