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Original article

Development of new LXR modulators that regulate LXR target genes and reduce lipogenesis in human cell models

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ABSTRACT

Four new mimics of 22-*S*-hydroxycholesterol (22SHC) were synthesized and evaluated using molecular modeling and tested in human muscle cells (primary myotubes) and hepatocytes (HepG2 cells). The new compounds (**9**, **12**, **15a** and **15b**) showed good interrelationship between docking scores, to both LXRα and LXRβ, and *in vitro* results. The LXR agonist T0901317 increased the expressions of genes involved in lipogenesis (SCD1, FAS) and cholesterol efflux (ABCA1), but only 22SHC counteracted the up-regulation of SCD1 and FAS by T0901317. Compound **9** and **12** decreased the expression of SCD1, while **9** also decreased the expression of FAS. Compounds **15a** showed a significant antagonistic effect on ABCA1 expression, but neither **15a** nor **15b** were able to counteract the effect of T0901317 on all genes examined. Lipogenesis was increased after T0901317 treatment and only 22SHC significantly counteracted this effect. Treatment with 22SHC and compound **12** reduced lipogenesis compared to control. An increased glucose uptake was observed for all compounds, except for **15b**. In summary, the new synthetic 22SHC mimics showed antagonistic effects similar to that of 22SHC, but the new substances were less potent. The sulfonamide **12** showed similar effects to 22SHC and the best effect on gene expression of the new mimics, however, it was not able to reduce the effect of T0901317 as observed for 22SHC.

1. Introduction

Liver X receptors (LXRα and LXRβ) are key players for increased cellular lipid accumulation and insulin resistance in skeletal muscle, which is a salient feature of type 2 diabetes and metabolic syndrome. The LXRs belong to the nuclear receptor superfamily, which are ligand-activated transcription factors. Known endogenous ligands are oxysterols (cholesterol metabolites) and glucose/glucose-6-phosphate [1,2]. LXR is important for lipid, glucose and cholesterol metabolism [3]. Favorable effects of activating LXR are increased glucose uptake, increased utilization of glucose and lipids and reduced *de novo* synthesis of lipids, while unfavorable effects of LXR activation are increased accumulation of lipids in cells, decreased insulin response and reduced glucose transport in e.g. skeletal muscle [4].

Chronic activation of LXR in human skeletal muscle cells results in increased glucose uptake and oxidation, and increased uptake and storage of fatty acids [5]. Previous observations have shown that the synthetic compound 22SHC have biological activity in human skeletal muscle cells, where 22SHC behaved like an LXR antagonist in these cells. 22SHC repressed genes involved in lipogenesis (i.e. ChREBP, FAS, ASCL1) and lipid handling that resulted in reduced synthesis of complex lipids even when compared to untreated myotubes [6]. We showed that exposure to a synthetic LXR agonist (T0901317) increased lipogenesis more in myotubes established from type 2 diabetics than in myotubes from lean subjects, and that these effects could be efficiently counteracted by 22SHC. Further, 22SHC reduced *de novo* diacylglycerol production below basal, reduced fatty acid uptake and oxidation at the same time as glucose uptake and oxidation were increased, whereas cholesterol transport was unchanged [7]. A drug with similar metabolic properties as observed for 22SHC would represent a new kind of drug treatment and may be an important step in the search toward new treatments against obesity and type 2 diabetes.

Given the desirable properties of 22SHC and the importance of having the correct stereochemistry at the 22 position [8], a series of simpler analogs were made. The new compounds were designed to

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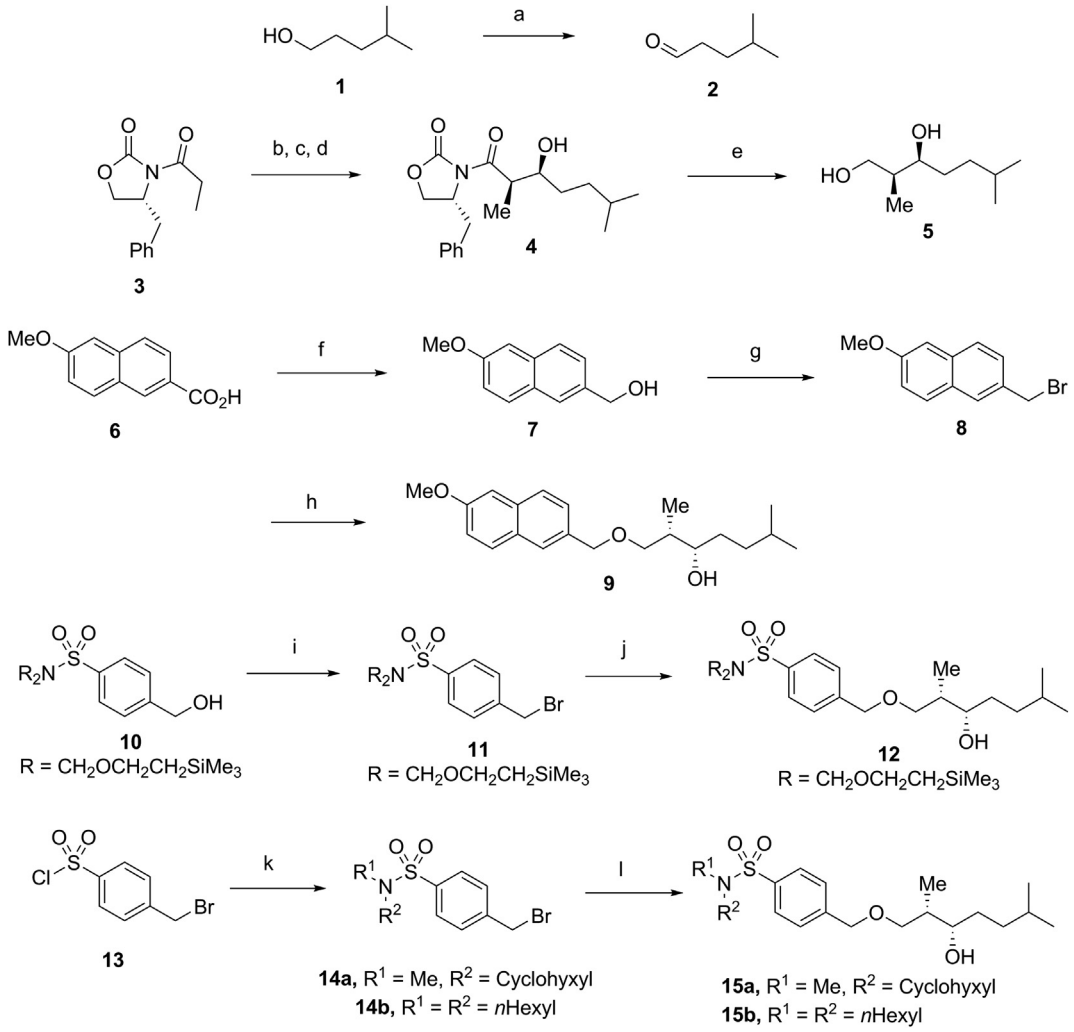


Fig. 1. Synthesis scheme for the new modulators. Experimental conditions: a) PCC, DCM, r.t. 60%. b) nBu$_2$BOTf, DIPEA, DCM, 0 °C. c) **2**, DCM, −78 °C, d) MeOH, H_2O_2, r.t. 62%. e) $LiBH_4$, Et_2O, 0 °C, 67%. f) $LiAlH_4$, THF, 0 °C, 75%. g) PBr_3, CBr_4, 0 °C, 53%. h) **5**, NaH, THF, DMF, 0 °C-r.t. 48%. i) PBr_3, CBr_4, 0 °C, 48%. j) **3**, NaH, THF, DMF, 0 °C-r.t. 18%. k) HNR^{1}R^2, TEA, DCM, 0 °C, 20–50%. l) **5**, NaH, THF, DMF, 0 °C-r.t. 10–50%.

mimic 22SHC and needed to consist of the following structural features; a cholesterol-like side chain incorporating the important hydroxyl group with correct stereochemistry, a lipophilic linker resembling the steroid skeleton and a polar group to mimic the alcohol in the 3-position of 22SHC.

Few compounds have been described as selective LXR antagonists. One potent endogenous LXR antagonist is 5α,6α-epoxycholesterol [9], and one synthetic drug candidate in this class in industrial pipeline is GSK2033 [10]. To our knowledge, none of the LXR modulators currently in the pipeline address the observed ability of 22SHC to stimulate glucose uptake and decrease lipogenesis and it is therefore of great interest to investigate such new compounds.

The aim of this study was to synthesize 22SHC mimics and to test them *in vitro* in human cell model systems whether our new compounds could perform equal or better than the mother substance 22SHC on lipogenesis and on selected genes important for lipid metabolism as well as on glucose uptake.

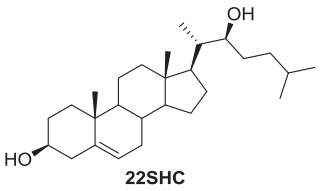
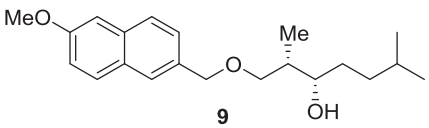
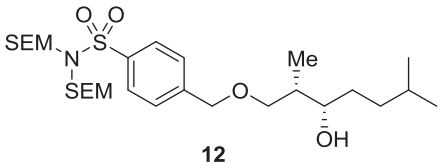
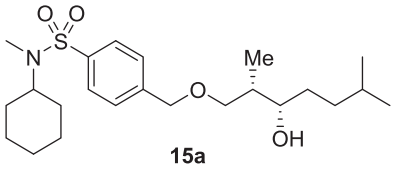
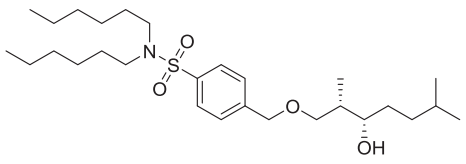
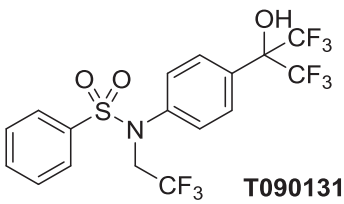
2. Results and discussion

The new LXR regulators were synthesized according to Fig. 1 and the details are listed in the Supporting information. The purity of each compound was assessed by ^{1}H and ^{13}C NMR. All compounds were also analyzed using HRMS.

The 22SHC isomer has been shown to act as an LXR antagonist on certain genes [6,11]. The binding affinities to the LXR receptors were evaluated *in silico* using the Internal Coordinate Mechanics (ICM) program. The results are found in Table 1 and the best docking pose of compound **9** docked into LXRβ is seen in Fig. 2. All compounds except **15a** docks similar to the parent compound 22SHC as previously described [7,11]. The sulfonamide **15a** orients opposite to that of 22SHC (not shown). Compound **9** performed equally to 22SHC and the known agonist T0901317, while compound **12**, **15a** and **15b** only showed moderate affinity for the LXR receptors. Compound **12** gave the lowest binding affinity most likely caused by its partial docking outside the defined binding pocket for both LXR isoforms (not shown).

Previous studies showed that 22SHC could repress genes involved in lipogenesis (SCD1 and FAS) in both myotubes and hepatocytes (HepG2 cells) [7,12]. We wanted to compare the effects of the new compounds to the effect of 22SHC on gene expression of SCD1 and FAS in myotubes. We also explored whether the new compounds influenced the reverse cholesterol transporter ABCA1. The results showed that the known LXR agonist T0901317 increased

Table 1
Structures and corresponding docking scores for LXRα and LXRβ.

Structure	Docking score	
	LXRα	LXRβ
 **22SHC**	−17.5	−29.2
 **9**	−25.4	−25.6
 **12**	−10.0	−7.7
 **15a**	−3.2	−13.0
 **15b**	−12.3	−15.9
 **T0901317**	−24.3	−22.3

the expressions of SCD1, FAS and ABCA1 as expected (Fig. 3A–C). In concordance with previous studies [7,12], 22SHC also increased ABCA1 expression, but had no impact on the effect on T0901317 (Fig. 3C). The compounds **9** and **12** decreased the expression of SCD1 (Fig. 3A), while only **9** decreased the expression of FAS (Fig. 3B). Compound **15a** and **15b** had no effects on the genes tested with the exception that **15a** down-regulated the expression of ABCA1 (Fig. 3C).

For a compound to induce a biological activity, the compound must stabilize the receptor structure in a proper conformation, and it is not necessarily a direct relationship between the receptor binding affinity and the biological effect of the tested compounds. The docking predicted the receptor binding affinity for LXRα and LXRβ, while the *in vitro* testing measured the biological effects. Compound **9** was the only compound with a docking scoring comparable to that of 22SHC. The other compounds were also predicted to bind LXRα and LXRβ, but with a quite low binding affinity. The receptor binding was confirmed by the *in vitro* studies.

These results therefore indicate that simulation of the binding affinity of potential ligands to the LXRs can be used as a tool to predict biological activity when the compounds fit the ligand binding pocket. Molecular modeling has also been used by others with success to evaluate new LXR modulators [13].

Recent data showed that 22SHC reduced *de novo* synthesis of lipids also in liver cells (HepG2 cells), had no effect on intestinal cells (CaCo2) and increased *de novo* synthesis of lipids in adipocytes (SGBS cells) [7]. Therefore, we wanted to explore whether any of the compounds tested on the expression of SCD1 and FAS could influenced lipogenesis in myotubes and HepG2 cells. Compound **12** reduced lipogenesis in myotubes in a dose-dependent manner (Fig. 4A). Lipogenesis in myotubes was increased after T0901317 treatment and tended to increase also in combination with test compounds, except for 22SHC that partly counteracted this effect (Fig. 4B). The effects of test compounds on lipogenesis were more pronounced in HegG2 cells than observed in myotubes; T0901317 increased lipogenesis in general, except when in combination with 22SHC (Fig. 4C). 22SHC and compound **12** reduced lipogenesis compared with control, while compound **9** only tended to do so (Fig. 4C, $p = 0.1$).

The mother substance 22SHC has previously been shown to increase glucose uptake in myotubes, which is thought as a desirable effect [7]. Compounds **9** (Fig. 5A), **12** (Fig. 5B) and **15a** (Fig. 5C) increased glucose uptake in a dose-dependent manner, but not compound **15b** (Fig. 5D). Two of the sulfonamides, **12** and **15a**, gave a significant increase at 1 μM while the 2-methoxynaphtalene analog **9** required 50 μM to give a significant increase in glucose uptake.

3. Conclusion

The new synthetic 22SHC mimics showed antagonistic effects similar to that of 22SHC, but the new substances were less potent. Compound **12** showed the most similar effect and the most desirable profile on gene expression, however, it was not able to reduce the effect of T0901317 to such a degree as that of 22SHC. Interestingly, an increased glucose uptake was observed for all compounds, except for **15b**. The initial studies with novel 22SHC mimetics presented in this study are promising, however, we intend to synthesize and test new cholesterol derivatives before we do *in vivo* testing of selected compounds.

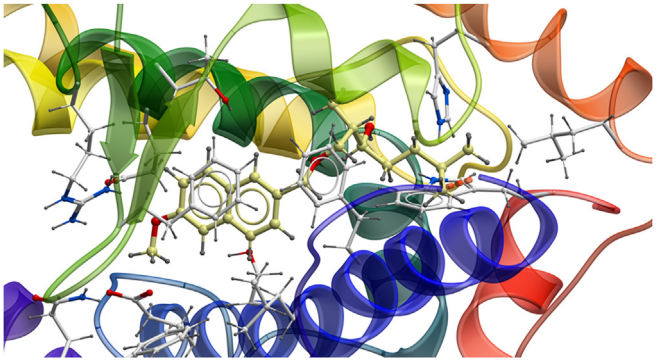


Fig. 2. Compound **9** docked into the LXRβ binding site. The best pose of compound **9** docked into the ligand-binding domain of LXRβ (PDB: 1PQ6). **9** shown in pale yellow while the amino acids near the binding pocket are shown in gray (with red and blue representing oxygen atoms and nitrogen atoms respectively). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

4. Experimental

4.1. 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 were purchased from Sigma–Aldrich (St. Louis, MO, USA). 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 standard commercial high purity quality.

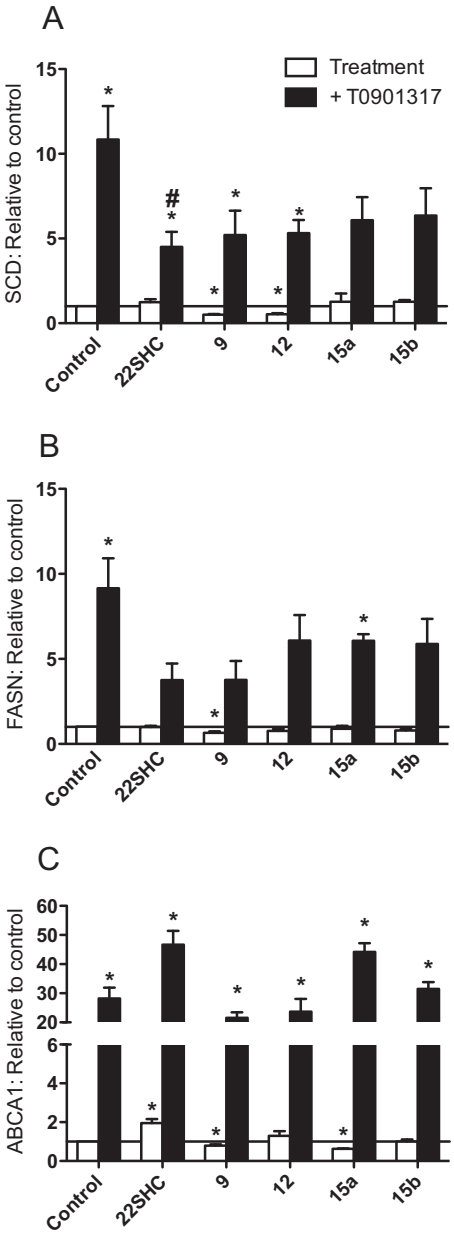


Fig. 3. Effects of 22SHC mimics on gene expression in myotubes and HepG2 cells. Myotubes were treated with DMSO (0.1%), 1 μM T0901317, 10 μM 22SHC, 10 μM of compounds **9**, **12**, **15a** and **15b**, for 4 days. Total RNA was isolated from the cells and analyzed by qPCR as described in Materials and Methods. Gene expressions were normalized to 36B4. Values represent fold change relative to control given as means ± SEM (n = 3–11). Selected LXR target genes: ABCA1, ATP-binding cassette transporter A1; FASN, fatty acid synthase; SCD1, stearoyl-CoA desaturase 1. $^{*}P < 0.05$ vs. control (DMSO) and $^{\#}P < 0.05$ for T0901317 vs. treatment + T0901317.

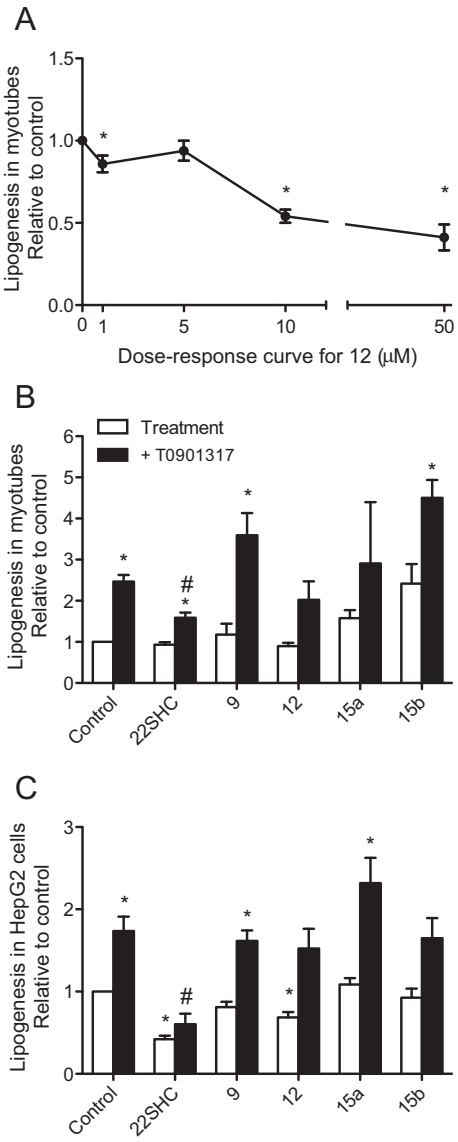


Fig. 4. Effects of 22SHC mimics on lipogenesis in myotubes and HepG2 cells. Cells were treated with DMSO (0.1%), 1 μM T0901317, 10 μM 22SHC, compounds **9**, **12**, **15a** and **15b**, for 4 days for myotubes and 24 h for HepG2 cells. The cells were incubated with [1-^{14}C]acetate (1 μCi/ml, 100 μM) for 24 h for myotubes and 4 h for HepG2 cells 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 from (A) dose–response for compound **12** in myotubes, n = 6, (B) lipogenesis in myotubes, n = 3–6, and (C) lipogenesis in HepG2 cells, n = 3 separate experiments. $^{*}P < 0.05$ vs. control (DMSO) and $^{\#}P < 0.05$ for T0901317 vs treatment + T0901317.

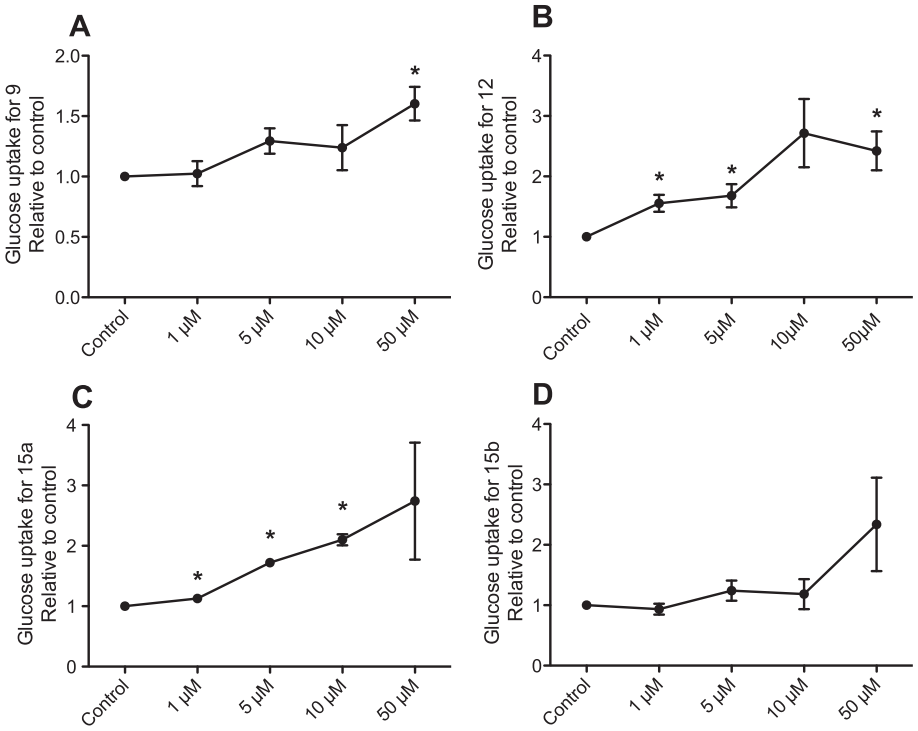


Fig. 5. Effects of 22SHC mimics on glucose uptake in myotubes. Myotubes were treated with DMSO (0.1%), 1 μM T0901317, 10 μM 22SHC, compounds **9**, **12**, **15a** and **15b**, for 4 days. Thereafter, the cells were exposed to D-[^{14}C(U)]deoxy-D-glucose (1 μCi/ml, 100 μM) for 4 h as described in Materials and Methods. The figures show D-[^{14}C(U)]deoxy-D-glucose uptake given as means ± SEM from separate experiments. (A) **9** ($n = 3$), (B) **12** ($n = 6$), (C) **15a** ($n = 3$) and (D) **15b** ($n = 3$). *$P < 0.05$ vs. control (DMSO).

4.2. Docking experiment

The Internal Coordinate Mechanics program [14] was used for docking of 22SHC and the newly synthesized compounds into the ligand binding domain of the human LXR receptors. The X-ray crystal structures of the LXRα ligand binding domain (PDB entry: 1UHL) and LXRβ ligand binding domain (PDB entry: 1PQ6) were used as targets for docking.

A virtual library of 22SHC and the newly synthesized compounds was created using the ICM Chemist package. The crystal structure was loaded, converted to an ICM object, all water molecules removed and all hydrogen atoms optimized using the default conditions set by the program. One of the dimers of the loaded crystal structure was removed prior to the ligand binding pocket was identified. The virtual binding pocket was created by selecting the co-crystallized ligand and creating a ligand pocket that extends 5 Å around the ligand. The ligand was then removed and a virtual docking of the 6 compounds was performed using default conditions. The docking scores were then calculated using the ICM Virtual Ligand Screening add-on and the docking results are the best of three docking runs. The processes were identical for both LXRα and LXRβ.

4.3. Synthesis of LXR modulators

The synthesis of the new LXR modulators and intermediates are described in the Supporting information. All reagents were purchased from Sigma–Aldrich and used without further purification. For column chromatography and filtration trough a pad of silica Merck silica 60 mesh (35–70 μm) is used unless noted otherwise. ^{1}H and ^{13}C NMR were recorded on Bruker DPX 300 and Bruker AVII 400 instrument equipped with a BACS-60 and a BACS-120 automatic sample changer, respectively. All experiments were performed at 25 °C in DMSO-d_6 and $CDCl_3$. High resolution mass spectrometry was obtained using an Autospec Ultima GC–MS (Micromass Ltd. Manchester, England). The MS was equipped with an electron ionization (EI) ion source producing 70 eV electrons. The voltage scan time was 1 s and the inter scan delay time was 0.20 s. The mass spectrometer was tuned to a resolution of 12,000. The ion source temperature was set to 200 °C and the samples were introduced into the instrument via a direct insertion probe, which was cooled with water. The software used was MassLynx version 4.0. (Waters, Milford, MA, USA).

4.4. Cell culturing

4.4.1. Culturing of human myotubes

Satellite cells were isolated as previously described [15] 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) and 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) and 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.

4.4.2. 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.

4.4.3. Treatment of cells

Cells were treated with DMSO (0.1%), 1 μM T0901317, 10 μM 22SHC, compounds **9**, **12**, **15a** and **15b**, for 4 days for myotubes and 24 h for HepG2 cells, before further analyses were performed as described in the following sections. Protein levels were measured as previously described [16] for all wells analyzed for radiolabeled fuel handling and no cell-detachment as sign on toxicity from any of the treatments were detected.

4.5. RNA Isolation and analysis of gene expression by TaqMan® qPCR

Myotubes were treated with DMSO (0.1%), 1 μM T0901317, 10 μM 22SHC, compounds **9**, **12**, **15a** and **15b**, 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 PerkinElmer 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.

4.6. Radiolabeled tracer studies

4.6.1. De novo lipogenesis

Cells were treated with DMSO (0.1%), 1 μM T0901317, 10 μM 22SHC, compounds **9**, **12**, **15a** and **15b**, 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 [16] and total lipids were isolated by filtration of the cell lysate through hydrophobic MultiScreen® HTS plates (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.

4.6.2. Glucose uptake

Myotubes were treated with DMSO (0.1%), 1 μM T0901317, 10 μM 22SHC, compounds **9**, **12**, **15a** and **15b**, 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 twice with ice-cold phosphate buffered saline (PBS), lysed in 0.1 M NaOH, and radioactivity measured by liquid scintillation counting. The protein content of each sample was determined [16], and glucose uptake was calculated using protein levels for standardization.

4.7. 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.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2014.01.003.

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