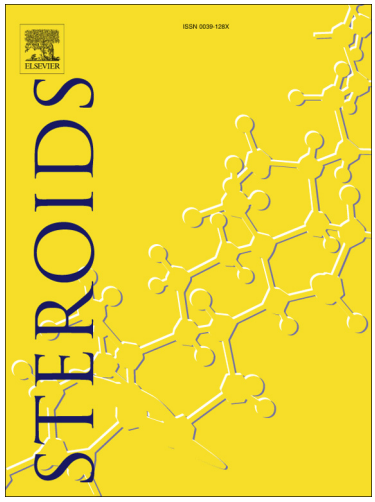

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Regulation of liver X receptor target genes by 22-functionalized oxysterols. Synthesis, *in silico* and *in vitro* evaluations.

Elvar Örn Viktorsson,[a] Mari Gabrielsen,[b] Nugalya Kumarachandran,[c] Ingebrigt Sylte,[b] Pål Rongved,[a] Ove Alexander Høgmoen Åstrand, [*#a] and Eili Tranheim Kase[#c]

[a] *School of Pharmacy, Department of Pharmaceutical Chemistry, University of Oslo, PO Box 1068 Blindern, N0316 Oslo, Norway. E-mail: o.a.h.astrand@farmasi.uio.no*
[b] *Department of Medical Biology, Faculty of Health Sciences, University of Tromsø, Tromsø, Norway*
[c] *Department of Pharmaceutical Biosciences, School of Pharmacy, University of Oslo, Oslo, Norway*
[*] *Corresponding author*
[#] *Contributed equally.*

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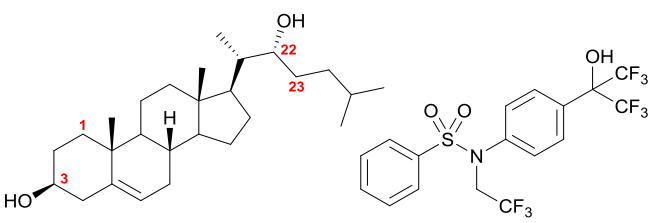
Abstract:

The endogenous oxysterol 22(*R*)-hydroxycholesterol (22RHC, **1**) is an LXR agonist which upregulates genes of critical involvement in human cholesterol- and lipid metabolism. In contrast, its synthetic epimer 22(*S*)-hydroxycholesterol (22SHC, **8**) has shown specific antagonistic effects in recent studies, avoiding unwanted side effects provided by potent LXR agonists. In terms of LXR modulation, the aim of this study was to compare 22SHC (**8**), 22RHC (**1**) and synthesized ligands with keto- and amide functionality in the 22nd position of the cholesterol scaffold. 22SHC (**8**) and 22RHC (**1**) performed as expected while 22-ketocholesterol (22KC, **10)** revealed an attractive *in vitro* profile for further investigation in terms of anti-atherosclerotic properties as selective upregulation of the ATP-binding cassette transporter ABCA1 was observed. A new synthesized amide derivate, Fernholtz cyclohexylamide (**13)** was shown to reduce lipogenesis in a dose-responsive manner and abolish the effect of the potent LXR agonist T0901317 when administered simultaneously.

1. Introduction

The liver X receptor (LXR) is a ligand-activated transcription factor and a member of the nuclear receptor (NR) superfamily.[1] Two isoforms are known, LXRα (NR1H3) and –β (NR1H2) and the pathways they regulate *in vivo* rely on activation by endogenous oxysterols.[2, 3] LXRs are involved in regulating expression of cohorts of genes by interacting with LXR-responsive elements.[4, 5] Evidences collected over the past decades confirm the crucial role of LXRs in the metabolism of cholesterol, lipids, bile acids and carbohydrates.[6-9] LXR-induced gene expression is tissue specific as LXRα is highly expressed in liver, kidneys, small intestine, macrophages and skeletal muscle, while the β-isoform is expressed more widely and has been identified in most tissues throughout the human body.[2, 10, 11] The importance of LXRs as pharmacological targets has been considered in several disease states, such as atherosclerosis,[12] diabetes mellitus type 2 (T2DM) and obesity,[13, 14] cancer,[15, 16] inflammation[17], skin disorders and Alzheimer's disease.[19, 20] Moreover, novel



22-(*R*)-hydroxycholesterol (1) (Natural endogenous LXR-agonist)

T0901317 (2) (Potent synthetic LXR-agonist)

Fig. 1 Two documented LXR-agonists. On the left is the endogenous LXR agonist 22(*R*)-Hydroxycholesterol (**1**) with key positions of the cholesterol scaffold numbered. On the right, T0901317 (**2**), a potent synthetic LXR agonist is shown.

genes expressed upon LXR activation are still discovered.[1, 4] The LXRs have relatively recently been considered as a drug targets, as it still was unknown in 1995 whether these receptors had endogenous ligands.[21] In the active state, LXR bound to its ligand forms an obligatory heterodimer with retinoid X receptor (RXR) that assistantly can be activated by retinoic acid. The activated complex is bound to DNA responsive elements in order to initiate gene expression.[21] Thus, the target is of high complexity. Advances in drug development of LXR-modulators has suffered from the lack of options for targeting either isoform of the receptor selectively as the α and $-\beta$ isoforms share 74% of the same amino acid sequence in the ligand binding domain (LBD) and only differ in 2 residues at the ligand binding site.[22] Further, the ligand binding pocket is of large volume compared with other NRs[23] and high flexibility can be seen observing the great chemical diversity of ligands known to bind and activate LXRs. An array of ligands, natural and synthetic ones have been explored in order to address their influence on LXR-induced gene expression.[22, 24] LXR agonists such as T0901317 (**2**)(fig. 1) and GW3965 (structure not shown) are potent inducers of gene expression of key regulatory genes involved in certain metabolic processes.[25, 26] Central target genes of LXR transcription involve the ATP binding cassette transporters (ABC transporters ABCA1/G1 and ABBCG5/G8), sterol regulatory element-binding protein-1c gene (SREBPC1c) and others of known importance in lipogenesis including fatty acid synthase (FAS) and stearoyl-CoA desaturase (SCD). Additionally, lipidation of Apolipoprotein E (ApoE) which is highly expressed in the human brain and connected to the pathogenesis of Alzheimer´s disease is LXR regulated.[19] To date, LXR activation (LXR-agonism) has particularly been of interest due to desirable up-regulation of ABCA1 expression in the light of anti-atherosclerotic properties.[27] However, their therapeutic values are largely hampered by undesirable side effects, such as hypertriglyceridemia, enhanced lipogenesis and hepatic steatosis, which have shown to be hard to avoid, leaving full LXR agonists only as research tools.[13, 28]

The effect of the synthetic derivative 22(*S)*-hydroxycholesterol (22SHC, **8**) on the LXR receptors and lipid metabolism has been discussed and is reported in prior to be inactive.[3, 5, 29] In contrast, recent studies report that 22SHC (**8**) may possess antagonistic effects on LXRs and have the ability to selectively down-regulate the expression of genes involved in lipid formation, reduce lipid metabolism and increase glucose uptake.[6, 30] The corresponding epimer 22(*R)*-hydroxycholesterol (22RHC, **1**), is a well-known endogenous agonist for lipid production.[1] These biological findings are of interest, since they suggest that a single change in stereochemistry of the steroidal side chain manipulates gene expression of key LXR target genes oppositely. The literature compound 22-ketocholesterol (22KC, **10**) has earlier been shown[31] to be oxidized from 22SHC (**1**) by NADPH dependent oxygenase [32] and has been described as a potent inhibitor of P-450-driven cleavage of cholesterol.[33] To our best knowledge, 22KC (**10**) has not been evaluated in terms of LXR-modulation.

We hypothesized that further investigation of the cholesterol side-chain functionality specifically at the 22-position could lead to new therapeutic candidates beneficial for diabetes and obesity. The first step further was to investigate 22-oxo functionalized cholesterol derivatives. To achieve this, our recently developed synthetic pathway from Fernholtz acid (**3**) to TBS-protected Fernholtz Weinreb amide (**5**) proved resourceful in order to provide 22-carbonyl-cholesterol analogs such as the TBS-protected Fernholtz aldehyde (**6**) and TBS-protected 22KC (**9**). Molecular modelling studies were undertaken to explore ligand docking scores and interaction modes for LXRβ. To evaluate performance of ligands in biological systems, their modulating effects on LXR controlled gene expression was measured by several assays using both human skeletal muscle cells and HepG2 cells.

2. Experimental procedures

2.1. Materials

General information regarding the materals for the synthetic part of this work is found within the supplementary information. 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). [1-14C]acetic acid (54 mCi/mmol) and D-[14C(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 (**8**) (and 22(R)-hydroxycholesterol (**1**) 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 (**2**) 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.

2.2. Docking experiments

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 3.8-4.[35] To set up the receptor grid maps for docking, 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. Three parallel docking simulations were performed and the best-scored ligand from the parallels was selected as the best orientation. The ICM virtual ligand screening (VLS) scoring function was used.

2.3. Synthetic procedures

2.3.1. Fernholtz Weinreb amide (4)

Fernholtz acid **3** (400 mg, 1.15 mmol, 1 eq) was dispersed in anhydrous DMF (20 mL) stirring at r.t. under argon atm. To the suspension, HATU (483 mg, 1.27 mmol, 1.1 eq) and HOBt monohydrate (195 mg, 1.27 mmol, 1.1 eq) were added followed by drop wise addition af DIPEA (0.24 mL, 1.38 mmol, 1.2 eq) upon which a transparent solution was obtained. The resulting solution was left stirring for 2 hours or until no starting material was observed by TLC. Then, *N*,*O*-dimethylhydroxylamine hydrochloride (561 mg, 5.75 mmol, 5 eq) was added, followed by another drop wise addition of DIPEA (0.98 mL, 5.75mmol, 5 eq). The resulting mixture was stirred overnight. The reaction mixture was transferred to a separatory funnel, diluted by H_2O (400 mL) and extracted by EtOAc (4x25 mL). The organic phases were pooled, washed with brine (3x300 mL), dried over $MgSO_4$ and filtered before solvents were removed *in vacuo*. The crude product was further purified by flash column chromatography using 50% EtOAc/Heptane as eluent yielding 348 mg (77%) of the desired white solid. R_f: 0.40 (EtOAc/Heptane 8:2). $[\alpha]^{20}_D$ = -34.2 (1.05 mg/mL, $CHCl_3$). ^{1}H NMR (300 MHz,

$CDCl_3$) δ 5.40 – 5.24 (m, 1H), 3.66 (s, 3H), 3.59 – 3.41 (m, 1H), 3.14 (s, 3H), 3.03 – 2.75 (m, 1H), 2.34 – 0.76 (m, 27H), 0.71 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 178.3, 141.0, 121.6, 71.8, 61.5, 56.3, 52.7, 50.2, 42.4, 42.4, 39.7, 37.4, 37.1, 36.6, 32.3, 32.0, 31.9, 31.7, 27.2, 24.4, 21.2, 19.5, 17.4, 12.3. HRMS: e/z calculated for $C_{24}H_{39}NO_3$ 389.2930, found 389.2924 (1.5 ppm).

2.3.2. TBS-Fernholtz Weinreb amide (5)

2,6-Lutidine (0.21 mL, 1.79 mmol, 1.8 eq) and *tert*-butyldimethylsilyl triflate (0.26 mL, 1,13 mmol, 1.2 eq) were added drop wise to a stirring solution of Fernholtz Weinreb amide **4** (383 mg, 1.06 mmol) in anhydrous DCM at -40°C under Ar-atm. The reaction mixture was stirred for 30 minutes until no starting material was observed by TLC and gradually allowed to reach rt and stirred for 1 additional hour. The reaction mixture was absorbed on silica gel and solvents removed *in vacuo*. The crude product was purified by flash column chromatography on silica using 20% EtOAc/heptane as the eluent yielding 384 mg (78%) of white solid. R_f: 0.52 (EtOAc/heptane 1:4). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.40 – 5.25 (m, 1H), 3.69 (s, 3H), 3.57 – 3.41 (m, 1H), 3.16 (s, 3H), 3.05 – 2.84 (m, 1H), 2.36 – 2.07 (m, 2H), 2.07 – 0.79 (m, 33H), 0.73 (s, 3H), 0.06 (s, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 178.4, 141.6, 121.2, 72.8, 61.5, 56.4, 52.8, 50.3, 43.0, 42.4, 39.8, 37.5, 37.2, 36.7, 32.3, 32.2, 32.1, 32.0, 27.2, 26.1, 24.5, 21.2, 19.6, 18.4, 17.4, 12.4, -4.4. HRMS (TOF MS ES$^+$): Exact mass calculated for $C_{30}H_{53}NO_3SiNa$ $[M+Na]^+$: 526.3692, found 526.3696 (0.68 ppm).

2.3.3. TBS-Fernholtz aldehyde (6)

1M DIBALH solution in THF (4.1 mL, 4.1 mmol) was added drop wise to a stirring solution of TBS-Weinreb amide **5** (0.50 g, 1.02 mmol) in DCM (20 mL, anhydrous) at -78 °C under argon atm. After 2 h of stirring, the reaction mixture was gradually allowed to reach rt and consequently quenched ($NaHCO_3$ and Na-K-tartrate saturated aqueous sol., 20 mL of each) and left stirring for 1 h. The crude mixture was transferred to a separatory funnel and further diluted with H_2O (200 mL). The aqueous phase was extracted by EtOAc (4x25 mL), dried over $MgSO_4$, filtered and concentrated *in vacuo*. The resulting crude solution was absorbed on silica gel and further purified by flash column chromatography on silica (EtOAc/heptane 1:9) affording 375 mg (83%) of white solid. R_f: 0.68 (1:1 EtOAc/heptane v/v). ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.57 (d, J = 3.2 Hz, 1H), 5.31 (m, 1H), 3.48 (m, 1H), 2.43 – 2.10 (m, 3H), 2.08 – 0.77 (m, 34H), 0.72 (s, 3H), 0.05 (s, 6H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 205.2, 141.7, 121.1, 72.7, 56.2, 51.2, 50.3, 49.7, 43.1, 43.0, 39.7, 37.5, 36.7, 32.2, 32.1, 32.0, 27.2, 26.1, 24.8, 21.2, 19.6, 18.4, 13.6, 12.4, -4.4. ^{1}H and ^{13}C NMR data are in accordance with literature.[36]

2.3.4. TBS-22(S)-hydroxycholesterol (7)

2M Isopentyl MgBr solution in THF (0.68mL, 1.35mmol) was added drop wise to a stirring solution of TBS-Fernholtz aldehyde 6 (120 mg, 0.27 mmol) in anhydrous THF (3 mL) at -78° C under N_2 atm. The reaction mixture was left stirring for 3.5 hrs, and then quenched with NH_4Cl (10 mL of 10% aqueous sol.). The aqueous phase was extracted with EtOAc (3x20 mL). Organic phases were pooled, dried over MgSO4 and filtered. The crude solution was absorbed onto silica gel. Flash column chromatography (EtOAc/heptane 1:9) yielded 73 mg (53%) of a white solid. R_f: 0.29 (EtOAc/heptane 1:9) 1H NMR (400 MHz, $CDCl_3$) δ 5.36 – 5.27 (m, 1H), 3.68 – 3.55 (m, 1H), 3.54 – 3.42 (m, 1H), 2.33 – 2.10 (m, 2H), 2.09 – 0.76 (m, 46H),

0.69 (s, 3H), 0.05 (s, 6H). 13C NMR (101 MHz, $CDCl_3$) δ 141.7, 121.2, 74.1, 72.8, 56.9, 52.8, 50.3, 43.00, 42.4, 40.3, 40.00, 37.5, 36.7, 35.8, 33.4, 32.3, 32.1, 32.1, 28.3, 27.9, 26.1, 24.4, 22.9, 22.7, 21.3, 19.6, 18.4, 11.9, 11.7, -4.4.

2.3.5. 22-Hydroxycholesterol (22SHC, 8)

TBAF (215 mg, 0.68 mmol) was added to a stirring solution of TBS-22SHC **7** (70.4 mg, 0.14 mmol) in THF (2 mL). The reaction mixture was stirred at rt for 3 h until no starting material was observed by TLC. The reaction mixture was concentrated *in vacuo* before the solid crude material was dispersed in H_2O and extracted by EtOAc (3x25 mL). The pooled organic phases were dried over $MgSO_4$, filtered and concentrated *in vacuo*. The crude compound was asbsorbed onto silica gel and further purified by flash column chromatography on silica (20-50% EtOAc in Heptane) to afford 37 mg (67%) as a white solid. R_f: 0.55 (50% EtOAc/Heptane). ^{1}H NMR (600 MHz, $CDCl_3$) δ 5.37 – 5.32 (m, 1H), 3.62 (t, *J* = 6.3 Hz, 1H), 3.56 – 3.47 (m, 1H), 2.34 – 2.18 (m, 2H), 2.04 – 1.79 (m, 5H), 1.68 – 0.81 (m, 33H), 0.69 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 140.9, 121.8, 74.8, 71.9, 56.8, 52.7, 50.3, 42.4, 42.4, 40.3, 40.0, 37.4, 36.6, 35.8, 33.4, 32.1, 32.0, 31.8, 28.3, 27.9, 24.4, 22.8, 22.7, 21.3, 19.6, 11.9, 11.7.

2.3.6. TBS-22-keto cholesterol (9)

Dess-Martin periodinane (127 mg, 0.30 mmol, 1.5 eq) was added to a stirring solution of TBS-22SHC **7** (103 mg, 0.30 mmol, 1eq) in DCM (20 mL) at r.t. The reaction mixture was stirred for 20 h, and then diluted by 0.5M $NaHCO_3$ (30 mL) and extracted by DCM (3x20 mL). Organic phases were pooled, dried over $MgSO_4$ and filtered before solvent was removed *in vacuo*. The resulting crude compound was purified by flash column chromatography (EtOAc/heptane 1:9) yielding 87 mg (85%) of the white solid. $[\alpha]^{20}$ = -46.2 (*c* = 1.04 mg/mL, $CHCl_3$). R_f: 0.51 (EtOAc/heptane 1:1). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.36 – 5.27 (m, 1H), 3.54 – 3.42 (m, 1H), 2.62 – 2.06 (m, 5H), 2.06 – 0.76 (m, 42H), 0.70 (s, 3H), 0.05 (s, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 215.1, 141.7, 121.2, 72.8, 56.3, 52.2, 50.3, 49.6, 43.0, 42.7, 39.8, 37.5, 36.7, 32.6, 32.2, 32.1, 32.0, 27.9, 27.7, 26.1, 24.7, 22.6, 22.5, 21.2, 19.6, 18.4, 16.8, 12.2, -4.4. HRMS (TOF MS ES$^+$): Exact mass calculated for $C_{33}H_{58}O_2SiNa$ $[M+Na]^+$: 537.4103, found 537.4109 (0.97 ppm).

2.3.7. 22-ketocholesterol (10)

TBAF (237 mg, 0.75 mmol) was added to a stirring solution of TBS-22-Ketone **9** (77mg, 0.15 mmol) in THF (3 mL). The reaction mixture was stirred at r.t. for 24 hrs. THF was removed in vacuo and the resulting crude dispersed in 10% NH_4Cl (30 mL). The aqueous layer was extracted by DCM (3x30 mL). The collected organic phases were combined and solvents removed under reduced pressure before further purification by flash column chromatography (20-50% EtOAc/heptane) to yield 42.2 mg (70%) of a white solid. R_f: 0.18 (30% EtOAc/heptane). $[\alpha]^{20}_D$ = -26.5 (0.83 mg/mL, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.37 – 5.28 (m, 1H), 3.58 – 3.43 (m, 1H), 2.58 – 2.12 (m, 5H), 2.02 – 1.89 (m, 1H), 1.87 – 1.78 (m, 3H), 1.72 – 1.34 (m, 9H), 1.33 – 1.21 (m, 1H), 1.19 – 0.91 (m, 8H), 0.87 (d, *J* = 6.5 Hz, 8H), 0.69 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 215.1, 140.9, 121.6, 71.8, 56.2, 52.2, 50.2, 49.6, 42.6, 42.4, 39.8, 39.8, 37.4, 36.6, 32.5, 32.0, 32.0, 31.6, 27.8, 27.7, 24.6, 22.5, 22.5, 21.2, 19.5, 16.7, 12.2. HRMS e/z calculated for $C_{27}H_{44}O_2$: 400.3341, found 400.3348 (-1.7 ppm).

2.3.8. General method for the synthesis of amides (11-18)

Fernholtz acid **3** (0.50 mmol, 1.0 eq) was dispersed in DMF (15 mL) stirring at rt under Ar-atm. To this suspension, HBTU (0.55 mmol), HOBt monohydrate (0.55 mmol) and the corresponding amine (0.50-2.5 mmol) were added. The resulting solution was stirred for 5 minutes before DIPEA (0.50 mmol) was added drop wise. The resulting mixture left stirring overnight. The resulting reaction mixture was diluted with H_2O (200 mL) and extracted by EtOAc (4x30 mL). Organic phases were pooled and washed with 0.5M $NaHCO_3$ (200 mL), brine (2x200 mL), dried over $MgSO_4$ and filtered before solvents were removed *in vacuo*. The resulting crude compound was dry loaded on silica gel and further purified by flash column chromatography. Eluent systems are stated individually below for compounds **11-18**.

2.3.9. Fernholtz isobutyl amide (11)

Flash column chromatography (Heptane/EtOAc 1:4 - 1:1) gave 155mg (75%) of the cream-white solid. $[\alpha]^{20}_D$ = -45.3 (*c* = 1.14 mg/mL, $CHCl_3$). R_f: 0.38 (80% EtOAc/heptane). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.45 (t, *J* = 6.1 Hz, 1H), 5.38 – 5.28 (m, 1H), 3.59 – 3.44 (m, 1H), 3.19 – 2.92 (m, 2H), 2.34 – 2.16 (m, 2H), 2.13 – 0.75 (m, 35H), 0.69 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 176.7, 140.9, 121.7, 71.9, 56.5, 52.9, 50.2, 46.8, 45.3, 42.4, 42.4, 39.7, 37.4, 36.6, 32.1, 31.9, 31.8, 28.7, 27.9, 24.5, 21.2, 20.3, 19.5, 17.9, 12.2. HRMS: e/z calculated for $C_{26}H_{43}NO_2$ 401.3294, found 401.3300 (-1.6 ppm)

2.3.10. Fernholtz n-butyl amide (12)

Flash column chromatography on silica (heptane/EtOAc 1:4) gave 157 mg (82%) of the cream-white solid. R_f: 0.53 (EtOAc/heptane 8:2). $[\alpha]^{20}$ = -58.0 (1.90 mg/mL, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.79 – 5.39 (m, 1H), 5.39 – 5.26 (m, 1H), 3.61 – 3.42 (m, 1H), 3.36 – 3.10 (m, 2H), 2.65 – 0.76 (m, 44H), 0.68 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 176.8, 140.9, 121.6, 71.8, 56.5, 52.9, 50.2, 45.0, 42.5, 42.4, 39.7, 39.2, 37.4, 36.6, 32.1, 31.9, 31.9, 31.8, 27.7, 24.5, 21.2, 20.2, 19.5, 17.8, 13.9, 12.2. HRMS: e/z calculated for $C_{26}H_{43}NO_2$ 401.3294, found 401.3291 (0.7 ppm).

2.3.11. Fernholtz cyclohexyl amide (13)

Flash column chromatography (Heptane/EtOAc 1:4) gave 120 mg (64%) of cream-white solid. R_f: 0.48 (EtOAc/heptane 8:2). $[\alpha]^{20}_D$ = -46.9 (1.02 mg/mL, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.56 – 5.13 (m, 1H), 3.83 – 3.67 (m, 1H), 3.60 – 3.43 (m, 1H), 2.35 – 2.14 (m, 3H), 2.14 – 0.88 (m, 43H), 0.69 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 175.8, 140.9, 121.7, 71.9, 56.5, 53.0, 50.2, 48.0, 45.1, 42.5, 42.4, 39.7, 37.4, 36.6, 33.4, 33.3, 32.1, 32.0, 31.8, 27.6, 25.7, 25.0, 25.0, 24.5, 21.2, 19.5, 17.8, 12.3. HRMS: e/z calculated for $C_{28}H_{45}NO_2$ 427.3450, found 427.3438 (2.9 ppm).

2.3.12. Fernholtz pentafluoropropan amide (14)

Flash column chromatography on silica (50% Heptane/EtOAc) gave 123mg of the desired white solid (45%). R_f: 0.38 (EtOAc/heptane 1:1). $[\alpha]^{20}_D$ = -39.4 (1.27 mg/mL, $CHCl_3$). ^{1}H NMR (400 MHz, Methanol-d_4) δ 5.39 – 5.30 (m, 1H), 4.04 – 3.81 (m, 2H), 3.47 – 3.34 (m, 1H), 2.45 – 2.32 (m, 1H), 2.30 – 2.12 (m, 2H), 2.09 – 0.81 (m, 26H), 0.76 (s, 3H). ^{13}C NMR (101 MHz, Methanol-d_4) δ 180.1, 142.2, 122.3, 120.3 (qt, *J* = 285.8, 36.0 Hz), 114.6 (tq, *J* = 253.2, 36.0 Hz), 72.4, 57.8, 53.9, 51.7, 44.9,

43.5, 43.0, 41.0, 39.1 (t, *J* = 24.5 Hz), 38.6, 37.7, 33.3, 33.0, 32.3, 28.2, 25.4, 22.2, 19.9, 17.9, 12.5. HRMS: e/z calculated for $C_{25}H_{36}F_5NO_2$ 477.2666, found 477.2658 (1.6 ppm).

2.3.13. Fernholtz glycine ethyl ester amide (15)

Flash column chromatography on silica using 1:1 EtOAc/heptane as eluent yielded 140 mg of the purified white solid (56%). R_f: 0.42 (EtOAc/heptane 8:2). $[\alpha]^{20}_D$ = -46.2 (1.17 mg/mL, $CHCl_3$). ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.96 (t, *J* = 5.2 Hz, 1H), 5.41 – 5.27 (m, 1H), 4.20 (q, *J* = 7.1 Hz, 2H), 3.99 (d, *J* = 5.2 Hz, 2H), 3.61 – 3.41 (m, 1H), 2.36 – 2.09 (m, 3H), 2.09 – 0.75 (m, 28H), 0.69 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 176.9, 170.3, 140.9, 121.6, 71.8, 61.6, 56.5, 52.9, 50.2, 44.7, 42.5, 42.4, 41.3, 39.7, 37.4, 36.6, 32.0, 31.9, 31.8, 27.7, 24.5, 21.2, 19.5, 17.7, 14.3, 12.2. HRMS: e/z calculated for $C_{26}H_{41}NO_4$ 431.3036, found 431.3032 (0.7 ppm).

2.3.14. Fernholtz dodecyl amide (16)

Flash column chromatography using 1:1 EtOAc/heptane as eluent yielded 119 mg (40%) of the purified cream white solid. R_f: 0.33 (EtOAc/heptane 1:1). $[\alpha]^{20}_D$ = -41.4 (1.11 mg/mL, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.41 (t, *J* = 5.8 Hz, 1H), 5.37 – 5.29 (m, 1H), 3.57 – 3.45 (m, 1H), 3.31 – 3.03 (m, 2H), 2.34 – 2.15 (m, 2H), 2.14 – 1.37 (m, 17H), 1.37 – 1.20 (m, 21H), 1.17 (d, *J* = 6.7 Hz, 3H), 1.13 – 1.02 (m, 3H), 1.00 (s, 3H), 0.97 – 0.91 (m, 1H), 0.91 – 0.82 (m, 3H), 0.68 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 176.6, 140.9, 121.6, 71.8, 56.5, 53.0, 50.2, 45.2, 42.4, 42.4, 39.7, 39.4, 37.4, 36.6, 32.1, 31.9, 31.8, 29.8, 29.8, 29.8, 29.7, 29.7, 29.5, 29.4, 27.7, 27.1, 24.5, 22.8, 21.2, 19.5, 17.8, 14.3, 12.2. HRMS: e/z calculated for $C_{34}H_{59}NO_2$ 513.4546, found 513.4550 (-0.7 ppm).

2.3.15. Fernholtz benzyl amide (17)

The resulting crude product was dry loaded on silica and purified by flash column chromatography on silica using EtOAc/heptane 1:1 as eluent yielding 164 mg of white solid (65%). R_f: 0.57 (50% EtOAc/heptane). $[\alpha]^{20}_D$ = -57.4 (0.94 mg/mL, $CHCl_3$). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.40 – 7.20 (m, 5H), 5.73 (t, *J* = 5.8 Hz, 1H), 5.40 – 5.28 (m, 1H), 4.52 – 4.31 (m, 2H), 3.59 – 3.44 (m, 1H), 2.37 – 0.73 (m, 31H), 0.68 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 176.5, 140.9, 138.7, 128.8, 128.0, 127.6, 121.6, 71.8, 56.4, 53.0, 50.2, 45.1, 43.5, 42.5, 42.4, 39.7, 37.4, 36.6, 32.0, 31.9, 31.8, 27.8, 24.5, 21.2, 19.5, 17.9, 12.2. HRMS: e/z calculated for $C_{29}H_{41}NO_2$ 435.3137, found 435.3137 (0.2 ppm).

2.3.16. Fernholtz picoyl amide (18)

Flash column chromatography using EtOAc/heptane (8:2-10:0) as eluent yielding 127mg of the desired white solid (50%). R_f: 0.09 (EtOAc/heptane 8:2). $[\alpha]^{20}_D$ = -44.0 (1.00 mg/mL, $CHCl_3$). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.60 – 8.48 (m, 1H), 7.65 (td, *J* = 7.8, 1.8 Hz, 1H), 7.26 (d, *J* = 7.8 Hz, 1H), 7.23 – 7.13 (m, 1H), 6.67 (t, *J* = 5.0 Hz, 1H), 5.39 – 5.28 (m, 1H), 4.53 (d, *J* = 5.0 Hz, 2H), 3.60 – 3.44 (m, 1H), 2.36 – 2.12 (m, 3H), 2.11 – 0.76 (m, 25H), 0.71 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 176.8, 156.7, 149.1, 140.9, 136.9, 122.5, 122.4, 121.7, 71.9, 56.5, 53.0, 50.2, 45.0, 44.4, 42.5, 42.5, 39.7, 37.4, 36.6, 32.1, 32.0, 31.8, 27.7, 24.5, 21.2, 19.5, 17.8, 12.2. HRMS: e/z calculated for $C_{28}H_{40}N_2O_2$ 436.3090, found 436.3078 (2.7 ppm).

2.4. Culturing of human myotubes

Satellite cells were isolated as previously described[37] 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.

2.5. 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.

2.6. RNA isolation and analysis of gene expression by TaqMan® real-time qPCR

Myotubes were treated with DMSO (0.1 %), 1 µM T0901317 (**2**), 10 µM 22SHC (**8**), 22RHC (**1**) and compounds **10 - 18** 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.

2.7. De novo lipogenesis

Cells were treated with DMSO (0.1 %), 1 µM T0901317 (**2**), 10 µM 22SHC (**8**), 22RHC (**1**) and compounds **4**, **10 – 18** 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[38] 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.

2.8. Glucose uptake

Myotubes were treated with DMSO (0.1 %), 1 µM T0901317 (**2**), 10 µM 22SHC (**8**), 22RHC (**1**) and compounds **10** and **11** 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[38] and glucose uptake was calculated using protein levels for standardization.

2.9. 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.

3. Results and discussion

3.1. Molecular modelling

Currently, a number of LXRα and –β crystal structures have been resolved and are accessible within protein databanks, all of which are co-crystallized with either a steroidal- or non-steroidal LXR-agonist.[22] In the present work, molecular modeling experiments were performed on LXR*β* (PDB

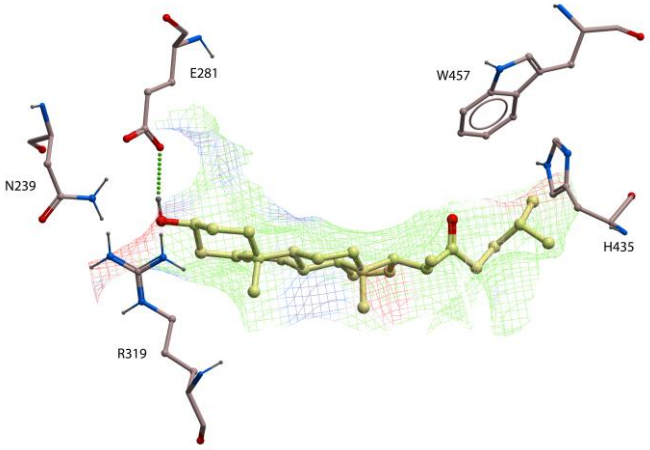


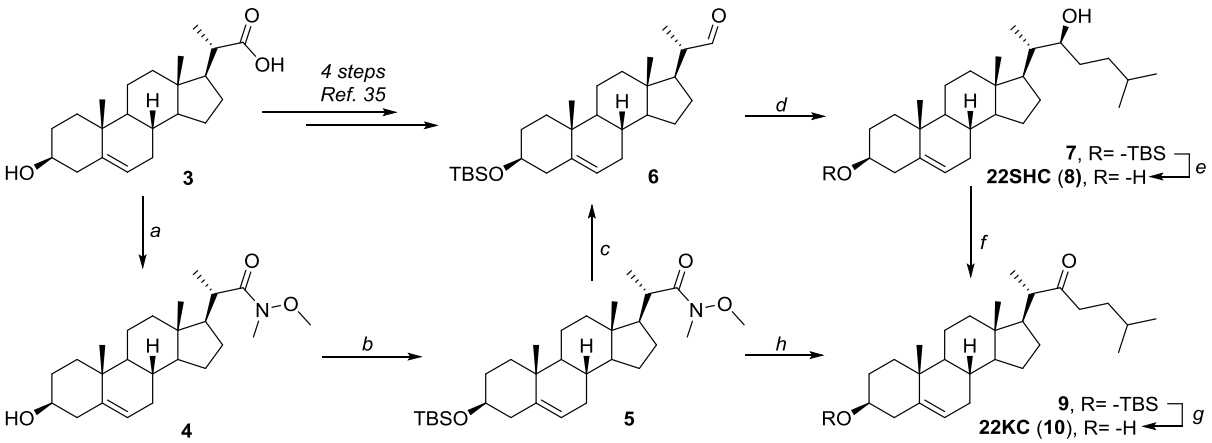
Fig 2. 22-ketocholesterol (**10**) docked into the ligand binding domain of LXRβ (PDB 1P8D) where relevant amino acidic side chains are depicted. The left side shows a polar cavity where the 3rd positioned secondary alcohol of 22KC (**10**) forms a hydrogen bond (shown in green) with Glu281. Oxygen atoms are colored in red and carbon atoms of the ligand in yellow. Carbon atoms for adjacent amino acids in grey and nitrogen atoms blue. The ligand surface (mesh) is displayed in colors by binding property. White – neutral surface, green - hydrophobic, blue – hydrogen bond acceptor potential and red – hydrogen bond donor potential.

code: 1P8D)[23] complexed with the endogenous oxysterol agonist 24(*S*),25-epoxycholesterol (eHC) within the LBD. The rationale for this choice relates to the structural similarity between eHC and the compounds synthesized in the present work. The molecular structures of 22KC (**10**) and 22-Fernholtz-22-isobutylamide (**11**) along with 22RHC (**1**) and 22SHC (**8**) were docked into the LXRβ structure after removal of 24(*S*),25-epoxycholesterol. Further, the amide derivatives **4** and **11-18** were plotted and docked as well. All docking scores are available in **table S-1** in the supporting information. The docking scores included are the best score obtained during three docking simulations. The Fernholtz-

benzyl amide (**17**) (see table 1) resulted in highest score, most likely as result of π-π interactions between the terminal aromatic side chain of the synthetic steroid side chain and aromatic moieties of Trp457/His435 which are currently considered as an essential "switch" activating the receptor upon LXR-agonism.[23] Since the two epimers 22RHC (**1**) and 22SHC (**8**) promote gene expression oppositely for certain genes, a special interest relates to the performance of 22KC (**10**) *in vitro* and *in silico*. **Fig. 2** shows 22KC (**10)** in the LBD of LXR*β*. 22KC (**10**) scored better *in silico* than both 22RHC (**1**) and 22SHC (**8**).

3.2. Synthesis of new modulators

Aldehyde **6** was synthesized from Fernholtz acid **3** (scheme 1) in up to 67% yield over 4 steps in accordance to described methods**[36]** (Synthetic procedures, NMR spectra and experimental data for intermediate compounds of this route (**19-21**) are given in supporting information). Aldehyde **6** (regardless of hydroxyl protection in the 3rd position) has been utilized for synthesis of multiple steroid compounds for various purposes.[39-42] Our recently developed method transforming Fernholtz acid **3** to its corresponding Weinreb amide (**4**) proved favorable.[34, 43] Consequent alcohol protection of position 3 to form **5** and DIBALH reduction gave aldehyde **6** in 50% overall yield over 3 steps. TBS-protected 22SHC (**7**) was formed under standard Grignard conditions using isopentylmagnesium bromide followed by TBAF mediated TBS-deprotection which afforded 22SHC (**8**) in moderate yields. Dess-Martin oxidation of **7** followed by TBAF mediated TBS-deprotection of **9** gave 22KC

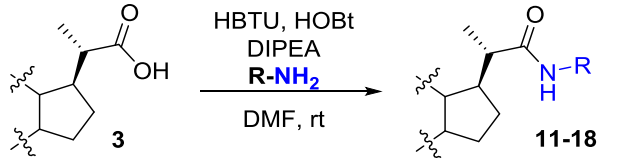


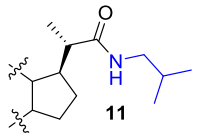
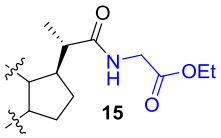
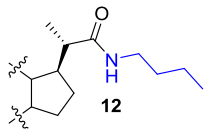
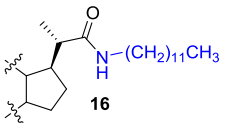
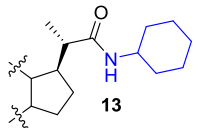
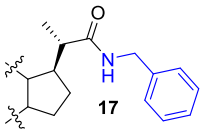
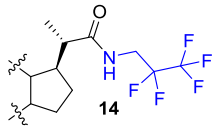
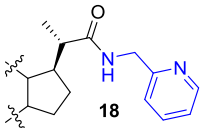
Scheme 1: Synthesis outline and reaction conditions a) MeNHOMe·HCl, HATU, HOBt, DIPEA, DMF, rt, overnight, 77% b) TBS-OTf, 2.6-lutidine, DCM, -40 °C, 78% c) DIBALH, THF, -78 °C - rt, 83 % d) Isopentyl-MgBr, THF, -78 °C,52% e) TBAF, THF, rt , 67% f) DMP, DCM, rt, 78% g) TBAF, THF, rt , 70% h) Isopentyl-MgBr, THF, -0 °C - rt, 29%

(**10**) in moderate yields as well. TBS-protected 22KC (**9**) was also formed directly from the TBS-protected Weinreb amide (**5**). However, this method (provided in supporting information) only afforded 29% yield using 10 molar equivalents of the Grignard reagent at relatively high temperature (0 °C).

Amide derivatives of Fernholtz acid (FA, **3**) were prepared by nucleophilic substitution on the activated ester formed upon reaction between FA (**3**) and HBTU in DMF. The poor solubility of FA (**3**) in common organic solvents including DMF was greatly enhanced upon instant transformation to activated benzotriazole ester. By this general method, nucleophilic substitutions were carried out using different amine nucleophiles to afford amides **11-18**. Structures and yields of isolated products are given in table 1.

Table 1: Synthesis of LXR-amides **11-18.**



Product	Yield†	Product	Yield†
 **11**	75%	 **15**	56%
 **12**	82%	 **16**	40%
 **13**	65%	 **17**	65%
 **14**	45%	 **18**	50%

†Isolated yields from non-optimized reactions

3.3. In vitro evaluations

Previous work in human skeletal muscle cells has shown that 22SHC (**8**) acts as an antagonist on lipid metabolism, while its epimer 22RHC (**1**) acts as a weak agonist on the same metabolic pathways.[44] In this work, we have studied expression of genes in human skeletal muscle cells and hepatocytes (HepG2 cells) important for *de novo* lipogenesis (FASN and SCD1) and reverse cholesterol transport (ABCA1) (**fig. 3**).

The results confirm that 22SHC (**8**) acts as an antagonist by downregulating genes important for lipogenesis (**fig. 3B** and **C**) while it does not affect the expression of another major LXR target gene ABCA1 (**fig. 2A**).[6, 44] From a physiological point of view, it is important that ABCA1 is not reduced since this could affect reverse cholesterol transport and ultimately increase the risk of atherosclerosis *in vivo*.[45] However, the mechanism for the different regulation of FAS and SCD1 versus ABCA1 by 22SHC (**8**) is still unknown. A possible explanation offered is that different co-regulators are involved in regulating the activity of LXR in the regulation of genes involved in different metabolic pathways, but this requires further investigation to unravel. We observe that the effect of the potent LXR agonist T0901317 (**2**) on SCD1 and FAS is abolished by 22SHC (**8**) as expected by an antagonist, but this is not observed for the expression of ABCA1, supporting the theory

that 22SHC (**8**) does not regulate gene expression exclusively through activation of LXR. The reduction of *de novo* lipogenesis by 22SHC (**8**) confirmed the functional relevance of the downregulation of FAS and SCD1 in HepG2-cells (**fig. 3D** and **E**). The epimer 22RHC (**1**) on the other hand, up-regulates all three LXR target genes tested, but only partly reduces the effect of T0901317 (**2**) on the expression of FAS and SCD1 (**fig. 3A-C**). This can be explained by that 22RHC (**1**) is a weaker agonist than T0901317 (**2**), and therefore will the expression of the target gene be reduced when 22RHC (**1**) partly replaces T0901317 (**2**) through competition for the same seat on the LXR receptor. However, 22RHC (**1**) does not seem to up-regulate *de novo* lipogenesis as expected by the up-regulation of FAS and SCD1 (**fig. 3D**). To further explore the chemical functionality of the 22nd position, we also tested the effects of 22KC (**10**) and Fernholtz-isobutyl amide (**11**) on the same

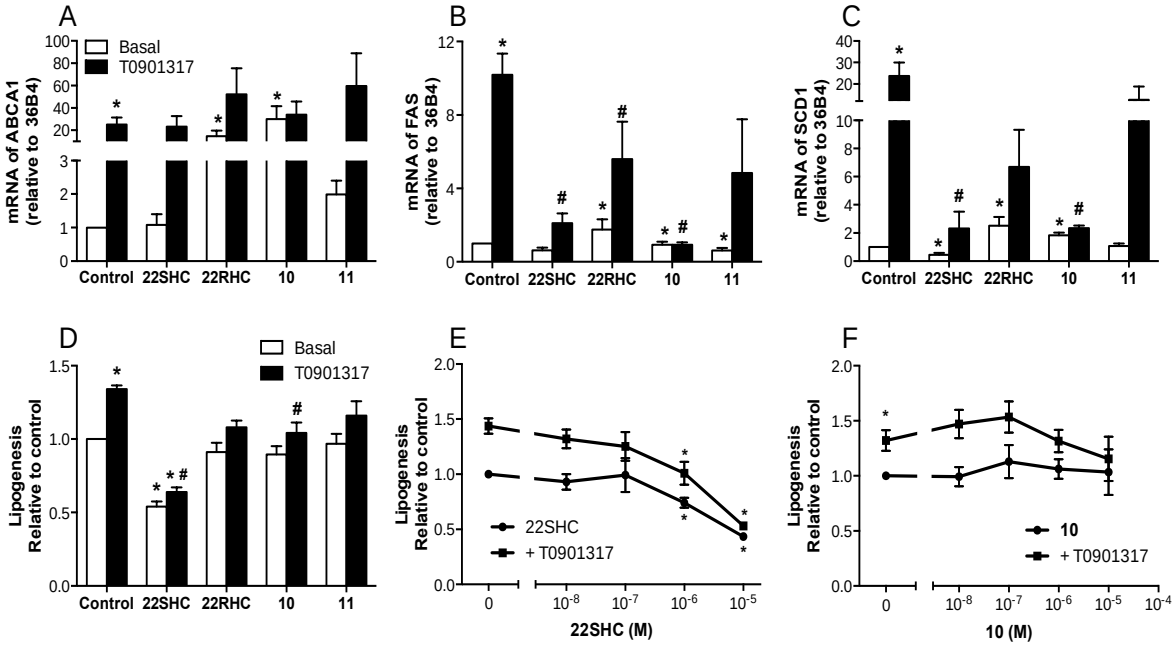


Fig. 3. Effects of 22SHC (**8**) and analogs on basal and T0901317-induced gene expression in skeletal muscle cells and de novo lipogenesis in HepG2 cells. Human skeletal muscle and HepG2 cells were treated for 4 days and 24h, respectively, with DMSO (0.1%) control, 1 µM T0901317 (**2**) and 10 µM 22SHC (**8**), 22RHC (**1**), 22KC (**10**), Fernholtz-isobutyl amide (**11**) ± 1 µM T0901317 (**2**). Total RNA was then isolated from human skeletal muscle cells and analyzed by qPCR as described in experimental procedures. Gene expressions were normalized to 36B4. For lipogenesis, HepG2 cells were incubated with [1-14C]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 given as means ± SEM (n=3-6). Analyzed LXR target genes were (A) ATP-binding cassette transporter A1 (ABCA1), (B) fatty acid synthase (FASN) and (C) stearoyl-CoA desaturase 1 (SCD1), while (D) represent lipogenesis in HepG2 cells, (E) dose-response for 22SHC (**8**) ± T0901317 and (F) dose-response for 22KC (**10**) ± T0901317 (**2**). *P < 0.05 vs. control and #P < 0.05 for T0901317 (**2**) vs. treatment + T0901317 (**2**).

parameters. 22KC (**10**) up-regulated the ABCA1 expression (**fig. 3A**) as 22RHC (**1**) while behaved similar as 22SHC (**8**) on the FAS gene expression. 22KC (**10**) reduced the expression of FAS and abolished the T0901317-effect significantly (**fig. 3B**). The T0901317-effect was also abolished by 22KC (**10**) on the expression of SCD1, but slightly increased the expression of SCD1 from basal. However, it does not seem like 22KC (**10**) or Fernholtz-isobutyl amide (**11**) have any effects on *de novo* lipogenesis as 22SHC (**8**) (**fig. 3D** and **F**).

The upregulation of ABCA1 by 22KC (**10**) shown in **Fig. 3A**, where expression of the other studied LXR-target genes mostly remains uninfluenced (**Fig. 3B** and **C**), point towards an attractive *in vitro* profile. Further investigation of 22KC (**10**) in terms of anti-atherosclerotic properties should be performed as the compound could bias unwanted side effects provided by potent LXR-agonists, such as T0901317 (**2**) which upregulates both FAS, SCD1 and lipogenesis potently.

Previously, we have also seen an increased glucose uptake in skeletal muscle cells after treatment with 22SHC (**8**),[6] but this was not observed for neither 22RHC (**1**), 22KC (**10**) nor Fernholtz-22-isobutyl amide (**11**) (data not shown).

To further explore the potential of preserving the 22-oxo-23-aza group with several different side chains (Table 1), compounds **4** and **12-18** were screened for effects on *de novo* lipogenesis in HepG2 cells with 22SHC (**8**) and T0901317 (**2**) as control compounds (**fig. 4A**). As expected, T0901317 (**2**) increased *de novo* lipogenesis while 22SHC (**8**) decreased the synthesis and abolished the T0901317-effect. Fernholtz-cyclohexyl amide (**13**), seemed to have similar effect as 22SHC (**8**) on both reducing *de novo* lipogenesis and abolish the T0901317-upregulation. None of the other compounds showed any effect. The dose-response curve of Fernholtz-cyclohexyl amide (**13**) on lipogenesis in HepG2 cells confirms that the compound indeed is able to suppress the lipogenesis in a dose-dependent manner (**fig. 4B**).

To confirm that the effect of Fernholtz-cyclohexyl amide (**13**) on lipogenesis most likely is regulated through LXR, changes in expression of ABCA1, FAS and SCD1 were studied (**fig. 4C-E**). The effects of the control substances 22SHC (**8**) and T0901317 (**2**) were as expected, while Fernholtz-*N*-butyl amide (**12**) **12** and Fernholtz-cyclohexyl amide (**13**) counteracted the effect of T0901317 (**2**) significantly on FAS, an indication that the mode of action on reduction of lipogenesis is LXR regulated. None of the tested compounds reduced the basal gene expression of neither FAS nor SCD1

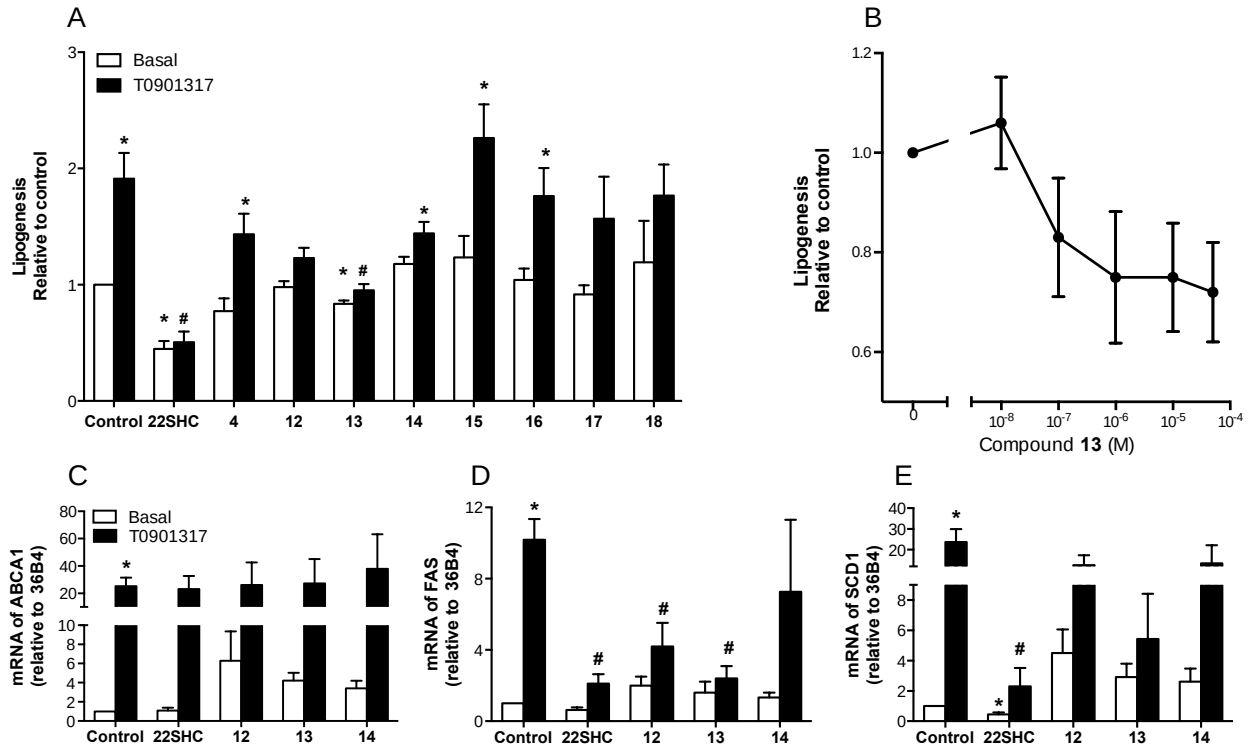


Fig. 4. De novo lipogenesis and effects of 22SHC (**8**) and amide analogs on basal and T0901317-induced gene expression. Human skeletal muscle and HepG2 cells were treated for 4 days and 24h, respectively, with DMSO (0.1%) control, 1 µM T0901317 (**2**) and 10 µM 22SHC (**8**), **4**, **12** - **18** ± 1 µM T0901317 (**2**). Total RNA was then isolated from human skeletal muscle cells and analyzed by qPCR as described in experimental procedures. For lipogenesis, HepG2 cells were incubated with [1-14C]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 lipid synthesis given as means ± SEM n=4 separate experiments from (A) lipogenesis (B) dose-response for Fernholtz-cyclohexyl amide (**13**). Analyzed LXR target genes were (C) ATP-binding cassette transporter A1 (ABCA1), (D) fatty acid synthase (FASN) and (E) stearoyl-CoA desaturase 1 (SCD1). Values represent fold change relative to control for gene expression is given as means ± SEM (n=3-6). Gene expressions were normalized to 36B4. *P < 0.05 vs. control and #P < 0.05 for T0901317 (**2**) vs. treatment + T0901317 (**2**).

(**fig. 4B-C**).

4. Conclusions

In summary, we have shown that LXR-**expression** of key target genes involved in human lipid metabolism is affected differently depending on the stereochemistry and functionality of the 22nd position on the cholesterol scaffold. Moreover, 22/23 oxo-aza amide compounds **4** and **11-18** were evaluated *in silico* and *in vitro* as well. Several of the ligands tested *in silico* performed well in terms of docking scores (**table S-1**, supporting information) using the LXRβ complex (PDB code: 1P8D).[23] New synthetic methods were established to provide new shorter and robust pathway to TBS-protected aldehyde **6**, a common intermediate for a large variety of different steroidal structures. In addition, one step method for the preparation of various Fernholtz amides **4** and **11-18** was established.

In vitro experiments were conducted in human hepatocytes and skeletal muscle cells. The experiments showed 22SHC (**8**) to downregulate gene expression of FAS, SCD1 and *de novo* lipogenesis. The endogenous agonist 22RHC (**1**) upregulated expression of all target genes tested. Interestingly, 22KC (**10**) behaved as 22RHC (**1**) (an agonist) in terms of ABCA1 upregulation. This is important as reduced expression of ABCA1 would affect reverse cholesterol transport and ultimately increase the risk for development of atherosclerosis. Moreover, the 22KC (**10**) had only minor influence on gene expression of FAS, SCD1 and no effect on lipogenesis. The *in vitro* profile of 22KC (**10**) presented in this work therefore suggests that 22KC (**10**) should be investigated further in terms of anti-atherosclerotic features as unwanted side-effects provided by potent agonists like T0901317 (**2**) could be biased. Moreover, agonistic effects of T0901317 (**2**) were mostly abolished by 22KC (**10**).

Of the new amides tested *in vitro* (**4** and **11-18**), Fernholtz-cyclohexyl amide (**13**) downregulated *de novo* lipogenesis. No other Fernholtz amides were shown to possess biological activity in terms of LXR-modulation.

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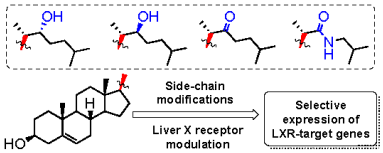
Graphical abstract



Highlights for

Regulation of liver X receptor target genes by 22-functionalized oxysterols. Synthesis, *in silico* and *in vitro* evaluations.

New version:

- **New oxysterol based LXR-modulators have been synthesized**
- **Importance of the 22th position of the oxysterol scaffold has been investigated**
- **Compounds were evaluated *in silico* and *in vitro* for regulation of key LXR-target genes**
- **22-Ketocholesterol downregulates ABCA1 selectively**
- **A new oxysterol analog was found to reduce lipogenesis**

Old version:

From abstract:

The endogenous oxysterol 22(*R*)-hydroxycholesterol (22RHC) is an LXR agonist which upregulates genes of critical involvement in human cholesterol- and lipid metabolism. In contrast, its synthetic epimer 22(*S*)-hydroxycholesterol (22SHC) has shown specific antagonistic effects in recent studies, avoiding unwanted side effects provided by potent LXR agonists.

Highlights

- We have synthesized different oxysterol LXR-modulators which differ in their functional moiety at the 22th position on the steroidal scaffold.
- We have also synthesized a new class of compounds including an amide functionality at the 22-23 position of the cholesterol scaffold
- All compounds have been evaluated *in silico* and moreover, tested for regulation of LXR target genes *in vitro*.
- 22-ketocholesterol which to our knowledge has not been evaluated in such studies before, shows an attractive profile to investigate further in terms of anti-atherosclerotic properties as it upregulates the ATP-binding cassette transporter ABCA1 selectively and might therefore bias unwanted side effects provided by classic LXR-full agonists as discussed within the manuscript. Another compound of the new synthesized class, cyclohexylamide **13** was shown to reduce lipogenesis in a dose responsive manner.