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Investigation on bile acid receptor regulators. Discovery of cholanoic acid derivatives with dual G-protein coupled bile acid receptor 1 (GPBAR1) antagonistic and farnesoid X receptor (FXR) modulatory activity

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ABSTRACT

Bile acids, the end products of cholesterol metabolism, activate multiple mechanisms through the interaction with membrane G-protein coupled receptors including the bile acid receptor GPBAR1 and nuclear receptors such as the bile acid sensor, farnesoid X receptor (FXR). Even if dual FXR/GPBAR1 agonists are largely considered a novel opportunity in the treatment of several liver and metabolic diseases, selective targeting of one of these receptors represents an attractive therapeutic approach for a wide range of disorders in which dual modulation is associated to severe side effects. In the present study we have investigated around the structure of LCA generating a small library of cholane derivatives, endowed with dual FXR agonism/GPBAR1 antagonism. To the best of our knowledge, this is the first report of bile acid derivatives able to antagonize GPBAR1.

1. Introduction

Bile acids, the end product of cholesterol metabolism, are amphipathic molecules essential for lipid and fat-soluble vitamins solubilization, absorption and metabolism. Bile acids produced in hepatocytes, as primary bile acids, cholic acid (CA) and chenodeoxycholic acid (CDCA), are secreted in the bile ducts, stored in the gallbladder, and then released into the duodenum upon ingestion of a meal to facilitate absorption of triglycerides, cholesterol, and lipid-soluble vitamins [1–5]. In the intestine, primary bile acids undergo a complex metabolism by the intestinal microbiota resulting in generation of secondary bile acids, deoxycholic acid (DCA) and lithocholic acid (LCA). Bile acids are efficiently reabsorbed (>95%) from the intestine, mainly by active transport mediated by the ileal bile acid transporters but also through passive diffusion in the upper small intestine and colon. Synthesis and transport of bile salts are undergo extensive feedback and feed-forward regulation by transcriptional and post-transcriptional mechanisms.

Basic research in the past 2 decades showed that bile acids are signaling molecules activating several cellular networks through the recognition of nuclear and membrane receptors, such as the

Abbreviations: BSEP, bile salt export pump; CDCA, chenodeoxycholic acid; CRE, cAMP response element; CREB, cAMP-response element binding protein; DMT-MM, 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride; 6-ECDCA/OCA, 6-ethylchenodeoxycholic acid/obeticholic acid; ESI-MS, electrospray ionization mass spectrometry; FXR, farnesoid X receptor; GPBAR1, G protein-coupled bile acid receptor 1; HEK293T, human embryonic kidney 293 cells; HepG2, hepatocellular carcinoma human cell line; LCA, lithocholic acid; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; OSTα, organic solute transporter alpha; PBC, primary biliary cirrhosis; PSC, primary sclerosing cholangitis; PXR, pregnane X receptor; RT-PCR, real-time polymerase chain reaction; SHP, small heterodimer partner; TLCA, taurolithocholic acid; VDR, vitamin D receptor.

Reagent lists: CH_3COOK; potassium acetate; DMF, *N*,*N*-dimethylformamide; DMT-MM, 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride; Et_3N, triethylamine; $Et_3N{\cdot}SO_3$, sulfur trioxide triethylamine complex; HCOOH, formic acid; $HClO_4$, perchloric acid; KOH, potassium hydroxide; $LiBH_4$, lithium borohydride; LiBr, lithium bromide; Li_2CO_3, lithium carbonate; MeOH, methanol; $NaNO_2$, sodium nitrite; NaOH, sodium hydroxide; Pd/C, palladium on carbon; $Pd(OH)_2/C$, palladium hydroxide on carbon; TFA, trifluoroacetic acid; THF, tetrahydrofuran; *p*-TsOH, *p*-toluenesulfonic acid monohydrate; *p*-TsCl, *p*-toluenesulfonyl chloride.

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farnesoid X receptor (FXR), pregnane X receptor (PXR), vitamin D receptor (VDR), and the cell surface G protein-coupled receptor (GPCR), GPBAR1 [1–6].

A key regulator of hepatocellular bile salt homeostasis is the bile acid receptor farnesoid X receptor (FXR) [1–3]. FXR, is the main sensor of bile acid levels in hepatocytes and in enterocytes and regulates the expression/activity of a number of downstream targets by binding to specific DNA response elements as an heterodimeric complex with the retinoid X receptor (RXR). One FXR target gene is SHP, which encodes an atypical nuclear receptor that lacks a ligand-binding domain and dimerizes with and inactivates both LRH1 and LXRα, resulting in a decrease in CYP7A1 expression and inhibition of bile acid synthesis through the neutral pathway [1–3]. In addition, FXR ligands negatively regulate basolateral bile acid uptake by hepatocytes via repression of NTCP and OATP-1 and -4, while stimulate the overall gene expression of both canalicular MRP3 (multidrug resistance-associated protein 3) and BSEP and alternative basolateral efflux transporters, MRP4 and OSTα and β (SLC51 solute carrier family 51, α and β subunit). In hepatocytes, FXR activation increases the expression of genes encoding for proteins involved in bile acid detoxification: CYP3A4 (cytochrome P450, family 3, subfamily A, polypeptide 4), UGT1A3, (UDP-glucuronosyltransferase 1A3) and Sult2A1 (sulfotransferase family, cytosolic, 2A member 1) [1–3]. Together, these changes lead to a reduced uptake, reduced *de novo* synthesis and increased excretion of bile acids by hepatocytes. In the intestine, FXR activation modulates the expression of specific transporters by repressing the human apical sodium bile acids transporter (ASBT) and inducing the basolateral organic solute transporters (OSTα and OSTβ). Importantly, activation of intestinal FXR increases the expression and causes the release of FGF-15, (FGF-19 in humans) [1–5] which, after binding to the type-4 of FGF receptor (FGF-R4) in hepatocytes, represses Cyp7A1 [1–3].

FXR is validated target in the treatment of liver and metabolic disorders. Currently, FXR ligands have shown beneficial effects in treating cholestasis, in patients affected by primary biliary cirrhosis (PBC) and primary sclerosing cholangitis (PSC), two immune-mediated disorders characterized by progressive bile duct destruction [7,8]. FXR also has an important role in regulating glucose metabolism through regulation of gluconeogenesis and glycogenolysis in the liver, as well as regulation of peripheral insulin sensitivity in striated muscle and adipose tissue, suggesting potential beneficial effects of FXR agonists in patients with type II diabetes, nonalcoholic fatty liver disease (NAFLD) and nonalcoholic steatohepatitis (NASH) [9–11].

In addition to their role of signal molecules activating the nuclear receptor FXR, bile acids also activates the G-protein coupled receptor GPBAR1 (or TGR5) [6]. GPBAR1 is highly expressed along the intestinal tract, with the highest expression found in the ileum and colon [12]. Despite the liver being a major bile acid target organ, GPBAR1 expression in the liver is very low. GPBAR1 is expressed in liver sinusoidal endothelial cells, gallbladder epithelial cells, and Kupffer cells. GPBAR1 is also expressed in nontraditional bile acid target organs including white and brown adipose, spleen, kidney, pancreas, lung, macrophages, and the central nervous system [12,13]. It is generally recognized that GPBAR1 signaling plays important roles in energy and glucose metabolism as well as anti-inflammation in the digestive system.

Both conjugated bile acids and free bile acids are known to bind and activate FXR and GPBAR1. Indeed the activity toward the two bile acid receptors is structure dependent, with CDCA (EC_{50} approximately 10 μM) and its conjugated forms the most potent endogenous FXR activators and LCA and taurolithocholic acid (TLCA, EC_{50} value of 0.53 μM), the strongest natural agonists of GPBAR1.

In the last years bile acid scaffold has been subjected to intense medicinal chemistry strategies, affording to the identification of potent steroidal derivatives with different pharmacological profiles, from dual to selective agonists toward FXR and GPBAR1 [14]. Sure dual agonists represent a novel opportunity in the treatment of dyslipidemia and related disorders [9–11]. On the other hands, cholestasis is the main therapeutic area for potent FXR agonists. 6-ECDCA/OCA (obeticholic acid) (a dual FXR/GPBAR1 agonist) [15,16] and Px-102 (non steroidal agonist) have been investigated in clinical trials and the results of the phase II trial with OCA support the notion that FXR activation could be beneficial in this setting [17,18]. However, the use of OCA associates with several side effects the most disturbing of which was itching that was not observed in Phase II pilot study in patients with NAFLD with Px102, a non-steroidal ligand [19]. The explanation for this side effect remains unclear but the recent demonstration that GPBAR1 is the physiological mediator of pruritus [20], suggests that the development of highly selective FXR agonists, devoid of GPBAR1 agonism, could be a rational strategy to circumvent the side effect and might have utility in the treatment of PBC.

Intrinsically steroidal ligands are promiscuous covering the same chemical space of the endogenous activators of FXR and GPBAR1. Indeed the recent observation that the elimination of the hydroxyl groups on the tetracyclic core of 5β-cholane scaffold is detrimental in term of GPBAR1 activation [21,22], could be instrumental in generating selective FXR ligands devoid of any activity toward GPBAR1. In this context and in the frame of our interest in the discovery of nuclear receptor modulators, we have decided to manipulate LCA chemical scaffold modifying the functionalities of tetracyclic core, the stereochemistry of A/B rings junction, the length and the functionalization of the side chain. Pharmacological investigations on the so generated small library of cholane derivatives (Fig. 1) resulted in the identification for the first time of potent FXR agonists/GP-BAR1 antagonists.

2. Material and methods

2.1. General experimental procedures

Specific rotations were measured on a Jasco P-2000 polarimeter. High-resolution ESI-MS spectra were performed with a Micromass Q-TOF mass spectrometer. NMR spectra were obtained on Varian Inova 400, 500 and 700 NMR spectrometers (^1H at 400, 500 and 700 MHz, ^{13}C at 100, 125 and 175 MHz, respectively) equipped with a SUN microsystem ultra5 hardware and recorded in CD_3OD ($\delta_H = 3.30$ and $\delta_C = 49.0$ ppm) and $CDCl_3$ ($\delta_H = 7.26$ and $\delta_C = 77.0$ ppm). All of the detected signals were in accordance with the proposed structures. Coupling constants (*J* values) are given in Hertz (Hz), and chemical shifts (δ) are reported in ppm and referred to CHD_2OD and $CHCl_3$ as internal standards. Spin multiplicities are given as s (singlet), br s (broad singlet), d (doublet), or m (multiplet). Through-space ^1H connectivities were evidenced using a ROESY experiment with mixing times of 200 and 250 ms, respectively.

HPLC was performed with a Waters Model 510 pump equipped with Waters Rheodine injector and a differential refractometer, model 401. Reaction progress was monitored via thin-layer chromatography (TLC) on Alugram silica gel G/UV254 plates. Silica gel MN Kieselgel 60 (70–230 mesh) from Macherey–Nagel Company was used for column chromatography. All chemicals were obtained from Sigma–Aldrich, Inc.

All chemicals were obtained from Sigma–Aldrich, Inc. Solvents and reagents were used as supplied from commercial sources with the following exceptions. Tetrahydrofuran and triethylamine were distilled from calcium hydride immediately prior to use. Methanol

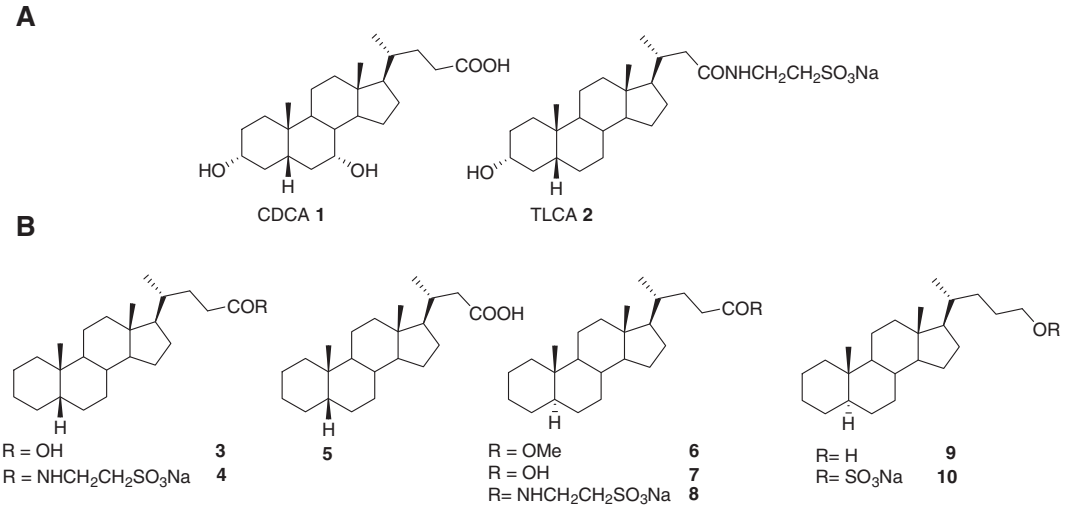


Fig. 1. A: CDCA and TLCA, the endogenous activators of FXR and GPBAR1, respectively. B: 5β- and 5α-cholane derivatives generated in this study.

was dried from magnesium methoxide as follow. Magnesium turnings (5 g) and iodine (0.5 g) are refluxed in a small (50–100 mL) quantity of methanol until all of the magnesium has reacted. The mixture is diluted (up to 1 L) with reagent grade methanol, refluxed for 2–3 h then distilled under nitrogen. All reactions were carried out under argon atmosphere using flame-dried glassware.

The purities of compounds were determined to be greater than 95% by HPLC. Compound **20** was prepared as previously reported [23,24].

2.2. Synthetic methods

2.2.1. Methyl 3α-tosiloxy-5β-cholan-24-oate (12)

To a solution of lithocholic acid (2 g, 5.31 mmol), dissolved in 50 mL of dry methanol was added *p*-toluenesulfonic acid (4.5 g, 26.5 mmol). The solution was left to stand at room temperature for 1 h. The mixture was quenched by addition until the neutrality of $NaHCO_3$ saturated solution. After the evaporation of the methanol, the residue was extracted with ethyl acetate. The combined extract was washed with brine, dried with Na_2SO_4, and evaporated to give the methyl ester as amorphous solid (2.1 g, quantitative yield).

At the solution of the methyl ester (2 g, 5.13 mmol) in dry pyridine (30 mL), tosyl chloride (4.9 g, 25.6 mmol) was added, and the mixture was stirred at room temperature for 8 h. It was poured into cold water (50 mL) and extracted with CH_2Cl_2 (3 × 50 mL). The combined organic phases were washed with saturated $NaHCO_3$ solution (50 mL) and water (50 mL), dried (Na_2SO_4) and concentrated to give 2.57 g of **12** as a white solid (92%). $[\alpha]_D^{25}$ = +59.5 (*c* 0.02, CH_3OH); selected ^1H NMR (500 MHz, $CDCl_3$): δ_H 7.77 (2H, d, *J* = 8.2 Hz), 7.31 (2H, d, *J* = 8.2 Hz), 4.43 (1H, m), 3.64 (3H, s), 2.32 (1H, m), 2.19 (1H, m), 0.88 (3H, d, *J* = 6.6 Hz), 0.86 (3H, s), 0.60 (3H, s); HR ESIMS *m*/*z* 545.3309 $[M+H]^+$, $C_{32}H_{49}O_5S$ requires 545.3301.

2.2.2. Methyl 5β-cholan-24-oate (13)

Lithium bromide (800 mg, 9.2 mmol) and lithium carbonate (680 mg, 9.2 mmol) were added to a solution of compound **12** (2.5 g, 4.6 mmol) in dry DMF (30 mL), and the mixture was refluxed for 2 h. After cooling to room temperature, the mixture was slowly poured into saturated $NaHCO_3$ solution (50 mL) and extracted with ethyl acetate (3 × 50 mL). The combined organic layer was washed successively with water, and then dried over anhydrous $MgSO_4$ and evaporated to dryness to give 1.7 g of oily residue (quantitative yield), that was subjected to next step without any purification.

A solution of compound previously obtained (1.7 g, 4.6 mmol) in THF dry/MeOH dry (10 mL/10 mL, v/v) was hydrogenated in presence of $Pd(OH)_2$ 20% wt on activated carbon Degussa type (30 mg) in PARR apparatus. The flask was evacuated and flushed first with argon and then with hydrogen (about 5 atm). After 12 h, the reaction was complete. The mixture was filtered through celite, and the recovered filtrate was concentrated to give 1.7 g of crude product. Purification by silica gel (hexane/ethyl acetate 95:5) gave compound **13** as a colorless oil (1.27 g, 74%). $[\alpha]_{25}^{D}$ = −3.0 (*c* 0.03, CH_3OH); selected ^1H NMR (400 MHz, $CDCl_3$): δ 3.68 (3H, s), 2.37 (1H, m), 2.24 (1H, m), 0.94 (3H, d, ovl), 0.93 (3H, s), 0.66 (3H, s) ^{13}C NMR (100 MHz, $CDCl_3$): δ 175.2, 56.9, 56.3, 51.8, 44.0 (2C), 40.8, 40.6, 37.9, 36.2, 35.7, 31.4 (2C), 28.5, 27.8, 27.6, 27.4, 26.9 (2C), 24.6, 21.6, 21.2, 18.6, 12.4. HR ESIMS *m*/*z* 375.3267 $[M+H]^+$, $C_{25}H_{43}O_2$ requires 375.3263.

2.2.3. 5β-cholan-24-oic acid (3)

Compound **13** (500 mg, 1.33 mmol) was hydrolyzed with a methanol solution of sodium hydroxide (5%, 5 mL) in H_2O (5 mL) overnight under reflux. The resulting solution was then concentrated under vacuum, diluted with water, acidified with HCl 6 N and extracted with ethyl acetate (3 × 50 mL). The collected organic phases were washed with brine, dried over Na_2SO_4 anhydrous and evaporated under reduced pressure to give 480 mg of compound **3** (quantitative yield). An analytic sample was obtained by HPLC on a Nucleodur 100–5 C18 (5 μm; 10 mm i.d. × 250 mm) with MeOH/H_2O (999.5:0.5) as eluent (flow rate 3 mL/min, t_R = 21 min); $[\alpha]_{25}^{D}$ = +13.3 (*c* 0.32, $CHCl_3$); selected ^1H NMR (400 MHz $CDCl_3$): δ 2.39 (1H, m), 2.26 (1H, m), 0.93 (3H, d, *J* = 6.6 Hz), 0.91 (3H, s), 0.66 (3H, s). ^{13}C NMR (100 MHz $CDCl_3$): δ 179.1, 56.8, 56.2, 50.6, 43.9, 43.0, 40.7, 40.5, 37.8, 36.1 (2C), 35.6 (2C), 31.1, 28.4, 27.7, 27.5, 27.3, 26.8, 24.5, 21.6, 21.0, 18.5, 12.3. HR ESIMS *m*/*z* 359.2956 $[M-H]^-$, $C_{24}H_{39}O_2$ requires 359.2950.

2.2.4. 5β-cholan-24-oyl taurine sodium salt (4)

Compound **3** (50 mg, 0.14 mmol) in DMF dry (3 mL) was treated with DMT-MM (77.5 mg, 0.28 mmol) and triethylamine (70 μL, 0.49 mmol) and the mixture was stirred at room temperature for 10 min. Then to the mixture was added taurine (15 mg, 0.16 mmol). After 24 h, the reaction mixture was concentrated under vacuo and dissolved in water (5 mL). The mixture was purified by HPLC on a Nucleodur 100–5 C18 (5 μm; 4.6 mm i.d. × 250 mm) with

MeOH/H_2O (83:17) as eluent (flow rate 1 mL/min), to give 3.5 mg of compound **4** (t_R = 11 min); $[\alpha]_{25}^{D}$ = +22.5 (*c* 0.05, CH_3OH); selected ^{1}H NMR (400 MHz, CD_3OD): δ 3.58 (2H, t, *J* = 7.0 Hz), 2.95 (2H, t, *J* = 7.0 Hz), 2.24 (1H, m), 2.08 (1H, m), 0.95 (3H, d, ovl), 0.94 (3H, s), 0.68 (3H, s). HR ESIMS *m*/*z* 480.3150 $[M-Na]^-$, $C_{27}H_{46}NO_4S$ requires 480.3153.

2.2.5. 3α-formyloxy-5β-cholan-24-oic acid (14)

A solution of lithocholic acid **11** (500 mg, 1.3 mmol) in 10 mL of 90% formic acid containing 25 μL of 70% perchloric acid was stirred at 47–50 °C for 12 h. The temperature of the heating bath was lowered to 40 °C, then 5 mL of acetic anhydride was added and the mixture was stirred for 15 min. The solution was cooled to room temperature, poured into 50 mL of water and extracted with diethyl ether. The organic layers were washed with saturated $NaHCO_3$ solution (50 mL) and water to neutrality, dried over Na_2SO_4, and evaporated to give 540 mg of **14** (quantitative yield). An analytic sample was obtained by silica gel chromatography eluting with CH_2Cl_2:MeOH 9:1. Selected ^{1}H NMR (400 MHz CD_3OD): δ 8.04 (1H, s), 4.85 (1H, m), 2.39 (1H, m), 2.25 (1H, m), 0.93 (3H, s), 0.92 (3H, d, *J* = 6.7 Hz), 0.65 (3H, s); ^{13}C NMR (100 MHz CD_3OD): δ 178.1, 160.8, 74.4, 56.5, 55.9, 41.9, 40.5, 40.2, 40.1, 35.8 (3C), 35.4 (2C), 32.3, 30.8, 30.7, 28.2, 27.0, 26.3, 24.2, 23.4, 20.8, 18.2, 12.1; HRMS-ESI *m*/*z* 405.2997 $[M+H]^+$, $C_{25}H_{41}O_4$ requires 405.2999.

2.2.6. 3α-formyloxy-24-nor-5β-cholan-23-nitrile (15)

Crude **14** (500 mg, 1.2 mmol), 3.8 mL of cold trifluoroacetic acid, and 1 mL (7.2 mmol) of trifluoroacetic anhydride were stirred at 0 °C until dissolution. Sodium nitrite (248 mg, 3.6 mmol) was added at the solution. The reaction mixture was stirred first at 0–5 °C for 1 h, then at 45–50 °C for 3 h. When the reaction was completed, it was neutralized with NaOH 2 N, then the product was extracted with 50 mL of diethyl ether (3 × 50 mL), followed by washing with brine and dried over anhydrous Na_2SO_4. The ether was removed under reduced pressure to afford 380 mg of **15** (85%), that was subjected to next step without any purification.

2.2.7. 24-nor-lithodeoxycholic acid (16)

Compound **15** (350 mg, 0.94 mmol) was refluxed in *ca*. 50 mL of methanol–water 1:1 with 30% KOH. After 2 h, the basic aqueous solution was neutralized with HCl 6 N. Then methanol was evaporated and the residue was extracted with ethyl acetate (3 × 50 mL) and then with CH_2Cl_2 (3 × 50 mL). The organic layers were washed with brine, dried and evaporated to dryness to give white solid residue, that was purified by silica gel chromatography, eluting with CH_2Cl_2:MeOH 9:1 (340 mg, quantitative yield). An analytic sample was purified by HPLC on a Nucleodur 100–5 C18 (5 μm; 4.6 mm i.d. × 250 mm) with MeOH/H_2O (95:5) as eluent (flow rate 1 mL/min), to give compound **16** (t_R = 10.5 min). $[\alpha]_{25}^{D}$ = +21.9 (*c* 0.58, CH_3OH); selected ^{1}H NMR (400 MHz CD_3OD): δ 3.54 (1H, m), 2.41 (2H, m), 1.00 (3H, d, *J* = 7.0 Hz), 0.94 (3H, s), 0.71 (3H, s). ^{13}C NMR (100 MHz CD_3OD): δ 178.0, 72.5, 57.9, 57.5, 43.6, 42.5, 41.9, 41.4, 37.2, 37.1, 36.5, 35.7, 34.9, 31.1, 29.3, 28.3, 27.6, 25.2, 23.9, 21.9, 19.9, 12.5; HRMS-ESI *m*/*z* 363.2895 $[M+H]^+$, $C_{23}H_{39}O_3$ requires 363.2899.

2.2.8. 24-nor-5β-cholanoic acid (5)

Compound **5** (210 mg, 75% over five steps) was synthesized, starting from compound **16** (300 mg, 0.82 mmol) as described in Scheme 2, by an analogous procedure to that detailed above for compound **3**. An analytic sample was obtained by HPLC on a Nucleodur 100–5 C18 (5 μm; 10 mm i.d. × 250 mm) with MeOH/H_2O (999.5:0.5) as eluent (flow rate 3 mL/min, t_R = 20 min); $[\alpha]_{D}^{25}$ = +24.6 (*c* 0.03, CH_3OH); selected ^{1}H NMR (400 MHz CD_3OD): δ 2.41 (1H, m), 1.00 (3H, d, *J* = 6.0 Hz), 0.95 (3H, s), 0.72 (3H, s); ^{13}C NMR (100 MHz CD_3OD): δ 178.0, 58.0, 57.6, 45.2, 44.0, 42.8, 41.9, 41.5, 38.7, 37.3, 36.5, 35.0, 29.3, 28.6, 28.4, 28.2, 27.7, 25.3, 24.8, 22.4, 21.9, 20.0, 12.5; HR ESIMS *m*/*z* 347.2947 $[M+H]^+$, $C_{23}H_{39}O_2$ requires 347.2950.

2.2.9. Methyl cholan-3,5-dien-24-oate (21)

At a solution of the ditosylate **20** (500 mg, 0.7 mmol) in water (3 ml) and *N*,*N*′-dimethylformamide (DMF; 27 ml) was added CH_3COOK (206 mg, 2.1 mmol) and the mixture was refluxed for 36 h. The solution was cooled at room temperature, then water and ethyl acetate were added and the separated aqueous phase was extracted with ethyl acetate (3 × 20 mL). The combined organic phases were washed with water, dried (Na_2SO_4) and concentrated to give a mixture. Purification by silica gel eluting with hexane–ethyl acetate (99:1) gave the diene **21** (200 mg, 78%). selected ^{1}H NMR (400 MHz CD_3OD): δ 5.92 (1H, d, *J* = 9.7 Hz), 5.58 (1H, d, *J* = 11.3 Hz), 5.38 (1H, m), 3.66 (3H, s), 2.34 (1H, m), 2.22 (1H, m), 0.94 (3H, d, *J* = 5.8 Hz), 0.92 (3H, s), 0.70 (3H, s); ^{13}C NMR (100 MHz CD_3OD): δ 174.8, 141.5, 129.1, 125.2, 123.2, 57.0, 55.8, 51.5, 48.4, 43.4, 39.8, 35.4, 33.8, 31.9, 31.8, 31.7, 31.0, 30.9, 28.1, 24.2, 23.9, 23.0, 20.9, 18.8, 12.0. HR ESIMS *m*/*z* 371.2947 $[M+H]^+$, $C_{25}H_{39}O_2$ requires 371.2950.

2.2.10. Methyl 5α-cholan-24-oate (6)

A solution of methyl chol-3,5-dien-24-oate **21** (150 mg, 0.40 mmol) in absolute methanol (5 mL) and dry THF (5 mL) was added in an oven-dried 50 mL flask, that was charged with 10% palladium on carbon (10 mg). The flask was evacuated and flushed first with argon and then with hydrogen. The reaction was stirred at room temperature under H_2 (1 atm) for 1 h. The mixture was filtered through celite, and the recovered filtrate was concentrated to give 147 mg of compound **6** (quantitative yield). $[\alpha]_{25}^{D}$ = −2.9 (*c* 2.54, CH_3OH); selected ^{1}H NMR (400 MHz $CDCl_3$): δ 3.66 (3H, s), 2.34 (1H, m), 2.21 (1H, m), 0.91 (3H, d, *J* = 6.4 Hz), 0.77 (3H, s), 0.64 (3H, s); ^{13}C NMR (100 MHz $CDCl_3$): δ 176.0, 56.9, 56.2, 55.0, 51.6, 47.3, 40.4, 39.0 (2C), 35.7 (3C), 32.5, 31.4, 31.3, 29.4, 29.3, 28.4, 27.2, 24.5, 22.6, 21.2, 18.6, 12.5, 12.4; HR ESIMS *m*/*z* 375.3269 $[M+H]^+$, $C_{25}H_{43}O_2$ requires 375.3263.

2.2.11. 5α-cholan-24-oic acid (7)

Compound **6** (50 mg, 0.13 mmol) was hydrolyzed with a methanol solution of sodium hydroxide (5%, 5 mL) in H_2O (1 mL) overnight under reflux. The resulting solution was then concentrated under vacuum, diluted with water, acidified with HCl 6 N and extracted with ethyl acetate (3 × 30 mL). The collected organic phases were washed with brine, dried over Na_2SO_4 anhydrous and evaporated under reduced pressure to give compound **7** (40 mg, 87%). An analytic sample was obtained by HPLC on a Nucleodur 100–5 C18 (5 μm; 4.6 mm i.d. × 250 mm) with MeOH/H_2O (92:8) as eluent (flow rate 1 mL/min, t_R = 12 min); $[\alpha]_{25}^{D}$ = +17.7 (*c* 0.14, CH_3OH); selected ^{1}H NMR (400 MHz, CD_3OD): δ 2.31 (1H, m), 2.20 (1H, m), 0.94 (3H, d, *J* = 6.2 Hz), 0.81 (3H, s), 0.69 (3H, s). HR ESIMS *m*/*z* 359.2953 $[M-H]^-$, $C_{24}H_{39}O_2$ requires 359.2950.

2.2.12. 5α-cholan-24-oyl-taurine sodium salt (8)

Compound **7** (10 mg, 27.8 × 10^{-3} mmol) in DMF dry (3 mL) was treated with DMT-MM (16 mg, 58.2 × 10^{-3} mmol) and triethylamine (70 μL, 0.49 mmol) and the mixture was stirred at room temperature for 10 min. Then to the mixture was added taurine (15 mg, 0.16 mmol). After 24 h, the reaction mixture was concentrated under vacuo and dissolved in water (5 mL). The mixture was purified by HPLC on a Nucleodur 100–5 C18 (5 μm; 4.6 mm i.d. × 250 mm) with MeOH/H_2O (83:17) as eluent (flow rate 1 mL/min), to give 3.5 mg (27%) of compound **8** (t_R = 10.4 min); $[\alpha]_{25}^{D}$ = +37. (*c* 0.03, CH_3OH); selected ^{1}H NMR (400 MHz, CD_3OD): δ 3.58 (2H, t, *J* = 7.0 Hz), 2.96 (2H, t, *J* = 7.0 Hz), 2.24 (1H, m), 2.09

(1H,m), 0.95 (3H,d,J = 6.2 Hz), 0.82 (3H,s), 0.69 (3H,s). HR ESIMS m/z 452.2840 $[M-Na]^-$, $C_{25}H_{42}NO_4S$ requires 452.2835.

2.2.13. 5α-cholan-24-ol (9)

Dry methanol (40 μL, 0.94 mmol) and $LiBH_4$ (470 μL, 2 M in THF, 0.94 mmol) were added to a solution of the compound **8** (50 mg, 0.13 mmol) in dry THF (10 mL) at 0 °C under argon and the resulting mixture was stirred for 2 h at 0 °C. The mixture was quenched by addition of NaOH (1 M, 260 μL) and then allowed to warm to room temperature. Ethyl acetate was added and the separated aqueous phase was extracted with ethyl acetate (3 × 15 mL). The combined organic phases were washed with water, dried (Na_2SO_4) and concentrated. Purification by silica gel eluting with CH_2Cl_2–MeOH (85:15) gave the alcohol **9** as a white solid (45 mg, quantitative yield). An analytic sample was obtained by HPLC on a Nucleodur 100–5 C18 (5 μm; 4.6 mm i.d. × 250 mm) with MeOH/H_2O (92:8) as eluent (flow rate 1 mL/min, t_R = 17.6 min); $[\alpha]_{25}^{D}$ = +5.5 (*c* 1.14,CH_3OH); selected ^{1}H NMR (400 MHz $CDCl_3$): δ 3.51 (2H, m), 0.87 (3H, d, J = 6.3 Hz), 0.72 (3H,s), 0.60 (3H,s). ^{13}C NMR (100 MHz $CDCl_3$): δ 63.6, 56.6, 56.1, 54.7, 47.0, 42.6, 40.1, 38.6, 35.6, 35.5 (2C), 32.2, 31.8, 29.3, 29.0 (2C), 28.2, 26.8, 24.2, 22.2, 20.8, 18.6, 12.2, 12.1; HR ESIMS m/z 347.3318 $[M+H]^+$, $C_{24}H_{43}O$ requires 347.3314.

2.2.14. 5α-cholan-24-yl-24-triethylammonium sulfate (10)

At a solution of compound **9** (10 mg, 0.028 mmol) in DMF dry (3 mL) was added triethylamine-sulfur trioxide complex (26 mg, 0.144 mmol) under an argon atmosphere, and the mixture was stirred at 95 °C for 12 h. Most of the solvent was evaporated and the residue was poured over a RP18 column to remove excess SO_3·NEt_3. Fraction eluted with MeOH:H_2O 1:1 contained **10** as ammonium salt. HPLC on a Nucleodur 100–5 C18 (5 μm; 4.6 mm i.d. × 250 mm) with MeOH/H_2O (90:10) as eluent (flow rate 1 mL/min), gave 1.4 mg of compound **10** (18%, t_R = 5 min) as sodium salt. $[\alpha]_{25}^{D}$ = −26.7 (*c* 0.02,CH_3OH); selected ^{1}H NMR (400 MHz,CD_3OD): δ 3.95 (2H,t,J = 6.7 Hz), 0.94 (3H,d,J = 6.5 Hz), 0.81 (3H,s), 0.69 (3H,s). HR ESIMS m/z 425.2730 $[M-Na]^-$, $C_{24}H_{41}O_4S$ requires 425.2726.

2.3. Pharmacological assays

2.3.1. Cell culture

HepG2, an immortalized epatocarcinoma cell line, was cultured and maintained at 37 °C and 5% CO_2 in E-MEM additioned with 10% FBS, 1% glutamine and 1% penicillin/streptomycin.

HEK-293T and Glutag cells were cultured and maintained at 37 °C and 5% CO_2 in D-MEM additioned with 10% FBS, 1% glutamine and 1% penicillin/streptomycin.

2.3.2. Luciferase reporter gene assay

To evaluate FXR mediated transactivation, HepG2 cells were transfected with 100 ng of human pSG5-FXR, 100 ng of human pSG5-RXR, 200 ng of the reporter vector p(hsp27)-TK-LUC containing the FXR response element IR1 cloned from the promoter of heat shock protein 27 (hsp27) and with 100 ng of pGL4.70 (Promega), a vector encoding the human Renilla gene. To evaluate GPBAR1 mediated transactivation, HEK-293T cells were transfected with 200 ng of human pGL4.29 (Promega), a reporter vector containing a cAMP response element (CRE) that drives the transcription of the luciferase reporter gene luc2P, with 100 ng of pCMVSPORT6-human GPBAR1, and with 100 ng of pGL4.70. At 24 h post-transfection, cells were stimulated 18 h with 10 μM CDCA (**1**), TLCA (**2**) and compounds **3**–**10**. In another experimental setting, at 24 h post-transfection, cells were stimulated with 50 μM of compounds **3**–**10** in combination with 10 μM CDCA (**1**) or TLCA (**2**). Luciferase activities were assayed and normalized with Renilla activities.

To calculate the IC_{50} of **3** and **7** *versus* GPBAR1, a dose response curve was performed in HEK-293T transfected as described above and stimulated 18 h with 1, 5, 25 and 50 μM of compounds **3** and **7**. After treatments, 10 μL of cellular lysates were read using Dual Luciferase Reporter Assay System (Promega Italia srl, Milan, Italy) according manufacturer specifications using the Glomax20/20 luminometer (Promega Italia srl, Milan, Italy). Luciferase activities were assayed and normalized with Renilla activities.

2.3.3. Real-Time PCR

Total RNA was isolated from HepG2 or Glutag cells using the TRIzol reagent according to the manufacturer's specifications (Invitrogen). One microgram of purified RNA was treated with DNase-I and reverse transcribed with Superscript II (Invitrogen). For Real Time PCR, 10 ng template was dissolved in 25 μL containing 200 nmol/L of each primer and 12.5 μL of 2× SYBR FAST Universal ready mix (Invitrogen). All reactions were performed in triplicate, and the thermal cycling conditions were as follows: 2 min at 95 °C, followed by 40 cycles of 95 °C for 20 s and 60 °C for 30 s in iCycler iQ instrument (Biorad). The relative mRNA expression was calculated and expressed as 2-(ΔΔCt). Forward and reverse primer sequences were the following: human GAPDH, gaaggtgaaggtcggagt and catgggtggaatcatattggaa; human OSTα, tgttgggccctttccaatac and ggctcccatgttctgctcac; human BSEP, gggccattgtacgagatcctaa and tgcaccgtcttttcactttctg; human SHP, gctgtctggagtccttctgg and ccaatgatagggcgaaagaagag; mouse GAPDH, ctgagtatgtcgtggagtctac and gttggtggtgcaggatgcattg; mouse Pro-glucagon, tgaagacaaacgccactcac and caatgttgttccggttcctc.

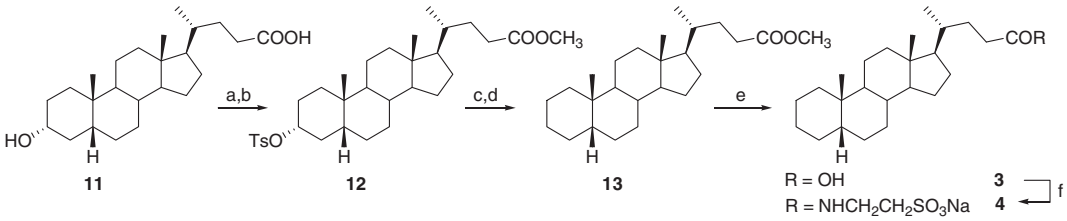
3. Results

3.1. Chemistry

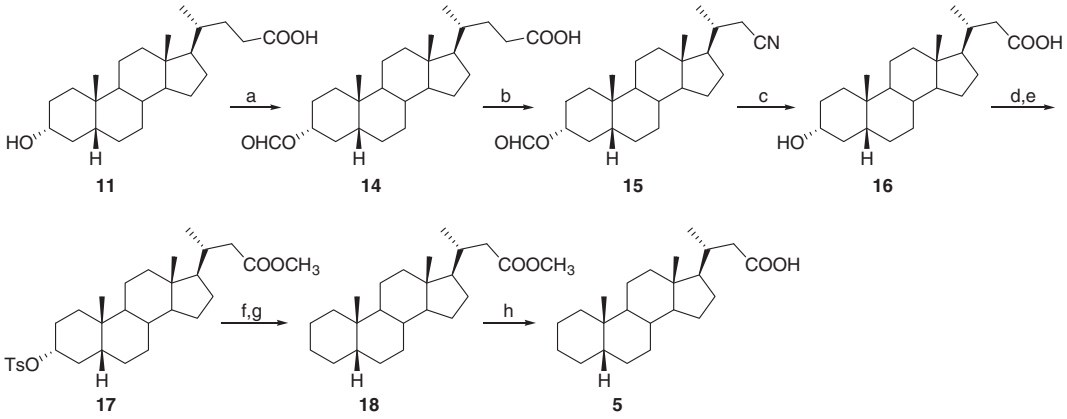
3.1.1. 5β-cholane derivatives (Schemes 1 and 2)

5β-cholanic acid **3** was prepared from LCA in a five steps reaction protocol (Scheme 1). LCA methyl ester intermediate was tosylated at C-3 hydroxyl group furnishing **12** in a 92% yield over two steps. Elimination ($LiBr/Li_2CO_3$) and subsequent hydrogenation (H_2, $Pd(OH)_2$, THF/MeOH) on the crude reaction product furnished methyl 5β-cholanoate **13** that in turn was transformed in the corresponding C24 acid **3** by alkaline hydrolysis (NaOH, MeOH/H_2O) in quantitative chemical yield. A small aliquot of the so obtained 5β-cholanic acid **3** was then subjected to the reaction of amidation with taurine in the presence of the versatile coupling agent DMTMM, 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride [25], giving the amide derivative as ammonium sulfate salt. Purification on RP-18 column followed by HPLC furnished **4** in pure form and as sodium salt.

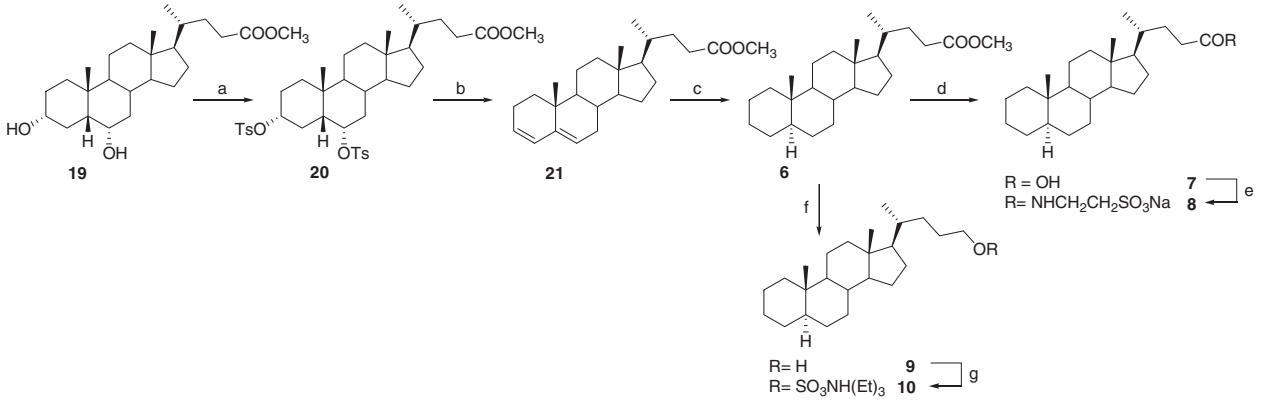
The synthetic protocol towards *nor*-cholanic acid **5** (Scheme 2) encompassed Beckmann one carbon degradation [26,27]. LCA was protected as performate derivative **14** by treatment of LCA with formic acid in presence of catalytic amounts of perchloric acid. Treatment with sodium nitrite in a mixture of trifluoroacetic anhydride and trifluoroacetic acid afforded 23-nitrile intermediate **15** in 85% yield over two steps (Scheme 2) that was in turn transformed in the corresponding *nor*LCA **16** through prolonged alkaline hydrolysis. Methanol/*p*-toluenesulfonic acid treatment gave C23 methyl ester that was subjected to the same synthetic protocol depicted in the Scheme 1. Briefly, tosylation/elimination at C3 and then hydrogenation of the double bond on ring A afforded



Scheme 1. 5β-Cholane derivatives: *Reagents and Conditions*. (a) *p*-TsOH, MeOH dry; (b) *p*-TsCl, pyridine, 92% over two steps; (c) LiBr, Li_2CO_3, DMF, reflux; (d) H_2 3 atm, Pd $(OH)_2$/C degussa type, THF/MeOH 1:1, 74% over two steps; (e) NaOH, MeOH:H_2O 1:1 v/v, quantitative; (f) DMT-MM, Et_3N, taurine, DMF dry.



Scheme 2. Preparation of *nor*-cholanic acid **5** *Reagents and Conditions*. (a) HCOOH, $HClO_4$, quantitative; (b) TFA, trifluoroacetic anhydride, $NaNO_2$, 85%; (c) KOH 30% in MeOH/H2O 1:1 v/v, quantitative; (d) *p*-TsOH, MeOH dry, 97%; (e) *p*-TsCl, pyridine, quantitative; (f) LiBr, Li_2CO_3, DMF, reflux; (g) H_2 3 atm, $Pd(OH)_2$/C degussa type, THF/MeOH 1:1, 78% over two steps; (h) NaOH, MeOH:H_2O 1:1 v/v, quantitative.



Scheme 3. 5α-Cholane derivatives *Reagents and Conditions*. (a) *p*-TsCl, pyridine; (b) CH_3COOK, DMF/H_2O 9:1, reflux, 78% over two steps; (c) H_2, Pd/C, THF/MeOH 1:1, room temperature, quantitative; (d) NaOH 5% in MeOH/H_2O 5:1 v/v, 87%; (e) DMT-MM, Et_3N, taurine, DMF dry; (f) $LiBH_4$, MeOH dry, THF, 0 °C, quantitative yield; (g) $Et_3N{\cdot}SO_3$, DMF, 95 °C.

methyl ester **18**. Finally basic hydrolysis of methyl ester function gave the desiderated nor 5β-cholanic acid **5** in 64% overall chemical yield.

3.1.2. 5α-Cholane derivatives (Scheme 3)

Treatment of methyl hyodeoxycholanoate **19** with tosyl chloride in pyridine afforded the 3α,6α-ditosyloxy-5β-cholanoate (**20**) in satisfactory yield (quantitative yield) (see Scheme 3).

Refluxing in DMF and CH_3COOK for 1 h produced elimination at C-3 and C-6 to give the dyene **21**, which in turn was hydrogenated to afford the required A/B *trans* ring junction in methyl ester **6**.

Hydrolysis at C-23 methyl ester gave nor 5α-cholanic acid **7**, that in a small aliquot was subjected to the reaction of amidation with taurine and purification (RP-18/HPLC) giving the tauro-conjugate **8** sodium salt. An aliquot of methyl ester **6** was transformed in the corresponding C-23 alcohol **9** that in turn was subjected to sulfation affording the side chain sulfate derivative **10**.

3.2. Pharmacological evaluation

Derivatives **3**–**10** were tested for their activity on FXR and GPBAR1 in a luciferase reporter assay on HepG2 and HEK-293T cells transfected with human FXR and GPBAR1, respectively (Figs. 2 and 3).

As previously demonstrated [21], 5β-cholanic acid **3** transactivated FXR with a potency comparable with the endogenus activator CDCA (**1**) and, interestingly this activity was also shared by the corresponding 5α-cholanoic acid **7**, thus demonstrating that, in absence of the hydroxyl groups at C-3 and at C-7, also a flat cholane scaffold could activate FXR. Nor-carboxyl acid derivative **5** was

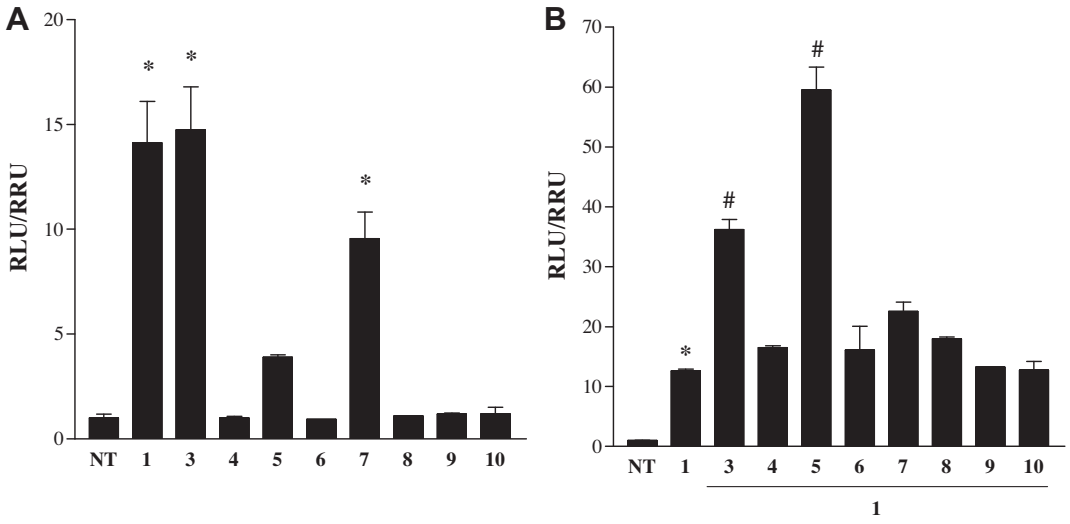


Fig. 2. Transactivation assays on FXR. A, Luciferase reporter assay performed in HepG2 transiently transfected with human pSG5-FXR, pSG5-RXR, pCMV-βgal, and p(hsp27) TKLUC vectors and measured as RLU/RRU (Relative Luciferase Unit/Relative Renilla Unit). Twenty-four hours post transfection, cells were stimulated with compounds **3**–**10** (10 μM). CDCA (**1**, 10 μM) was used as a positive control. Results are expressed as mean ± standard error; $^{*}p < 0.05$ *versus* not treated cells (NT). B, HepG2 cells were transfected as described in A. Twenty-four hours post transfection cells were stimulated with 10 μM CDCA (**1**) alone or in combination with compounds **3**–**10** (50 μM). $^{*}p < 0.05$ *versus* not treated cells (NT). $\#p < 0.05$ *versus* CDCA (1) stimulated cells.

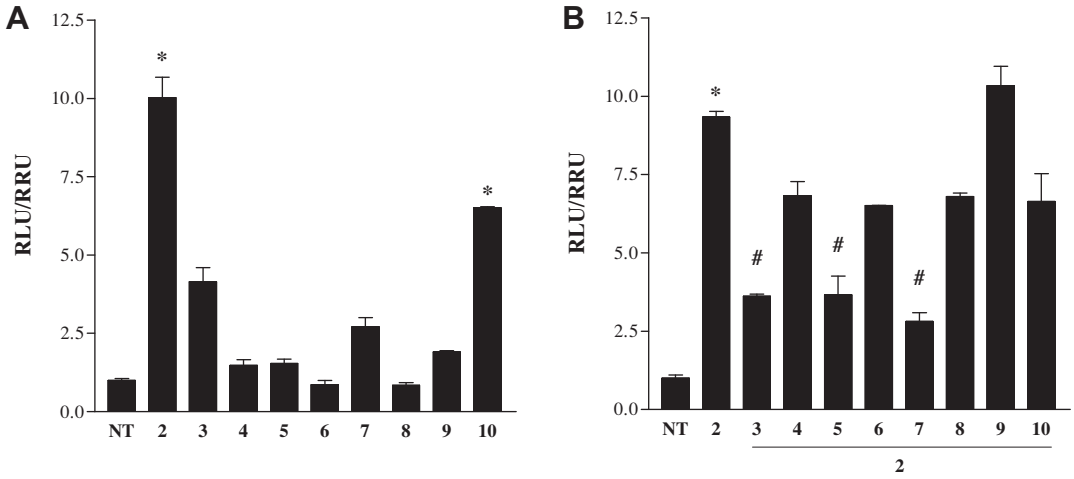


Fig. 3. Transactivation assays on GPBAR1. A, Luciferase reporter assay performed in HEK-293T cells transiently transfected with human GPBAR1 and a reporter gene containing a cAMP responsive element in front of the luciferase gene and measured as RLU/RRU (Relative Luciferase Unit/Relative Renilla Unit). Twenty-four hours post transfection, cells were stimulated with compounds **3**–**10** (10 μM). TLCA (**2**, 10 μM) was used as a positive control. Results are expressed as mean ± standard error. $*p < 0.05$ *versus* not treated cells (NT). B, HEK-293T cells were transfected as described in A. Twenty-four hours post transfection cells were stimulated with 10 μM TLCA (**2**) alone or in combination with compounds **3**–**10** (50 μM). $*p < 0.05$ *versus* not treated cells (NT). $\#p < 0.05$ *versus* TLCA stimulated cells.

almost inactive when tested at 10 μM (Fig. 2A). Moreover, when **5** was tested at 50 μM in the presence of CDCA (Fig. 2B), the ratio RLU/RRU was higher than that of CDCA, indicating that **5** might be a weak FXR agonist.

Data shown in Fig. 3 revealed that the elimination of the hydroxyl group at C-3 on the tetracyclic core of LCA is detrimental in term of activation of the membrane bile acid receptor (compounds **3**–**9** in Fig. 3, Panel A). Indeed data in Panel B are quite interesting. Cell stimulation in presence of TLCA **2** reveals that, among this series, derivatives **3**, **5**, **7** were relatively effective in inhibiting GPBAR1 activation caused by TLCA, thus behaving as antagonists and this result represents the first report of cholanic acid derivatives as dual modulators of FXR/GPBAR1 with an agonistic profile toward the nuclear bile acid sensor and an antagonistic behavior toward the membrane receptor.

Antagonistic activity of compounds **3** and **7** was further investigated by a detailed measurement of concentration–response curve on GPBAR1. As illustrated in Fig. 4, compounds **3** and **7** inhibited the transactivation of GPBAR1 with an IC_{50} of 28 and 22 μM, respectively.

Compound **3** was further investigated in vitro and its effects on FXR and GPBAR1 target genes assessed by RT-PCR. As shown in Fig. 5, panels A-D, 5β-cholanic acid **3** was able to induce the expression of BSEP, SHP and OSTα genes in HepG2 cells whereas antagonized the expression of pro-glucagon mRNA in GLUTAg cells, thus demonstrating that this molecule is an effective FXR agonist endowed with antagonistic activity toward GPBAR1.

4. Discussion and conclusion

FXR senses the intracellular presence of bile acids by activating multiple mechanisms, such as promotion of bile acid export from liver, down-regulation of bile acid import and also attenuation of

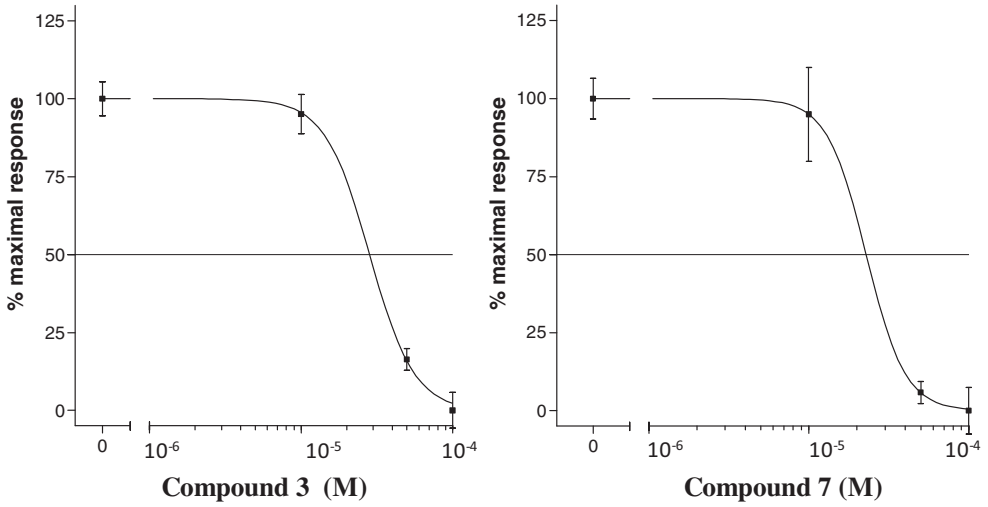


Fig. 4. Concentration–response curve of compounds 3 and 7 on GPBAR1. GPBAR1 activity was measured in HEK-293T cells cotransfected with human GPBAR1 and a reporter gene containing a cAMP responsive element in front of the luciferase gene (CRE). Twenty-four hours post transfection, cells were co-stimulated with 10 μM TLCA (**2**) and increasing concentrations of each compound: range from 1 to 50 μM. Results are expressed as mean ± standard error.

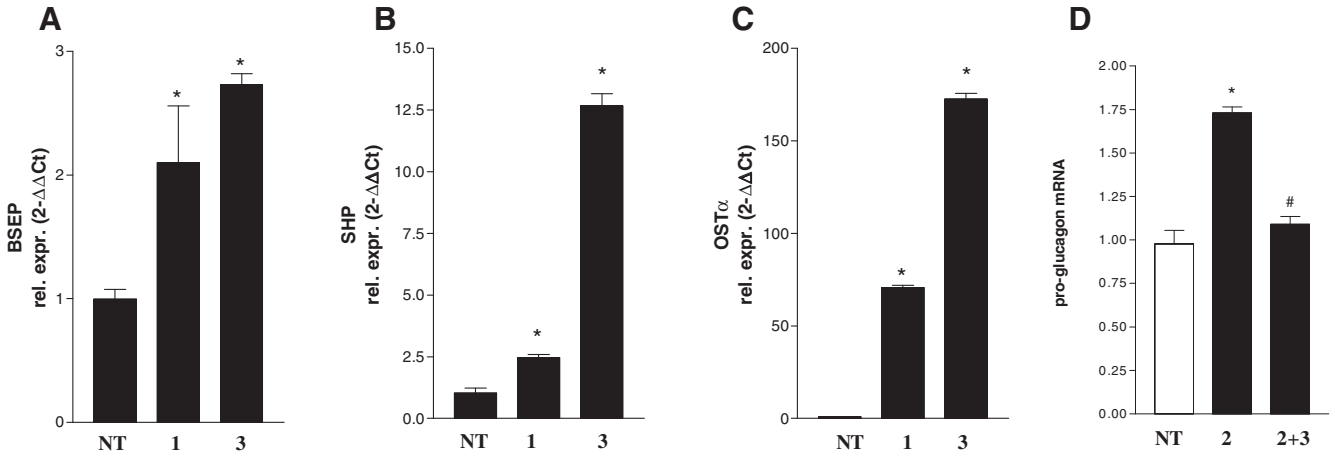


Fig. 5. Effect of compound 3 on FXR and GPBAR1 target genes. (A–C) Real-time PCR analysis of mRNA expression of FXR target genes BSEP (A), SHP (B), and OSTα (C) in HepG2 cells primed with 10 μM of compound **3**. CDCA (**1**) was used as positive control (10 μM). (D) Real-time PCR analysis of mRNA expression of GPBAR1 target gene pro-glucagon in Glutag cells stimulated with 10 μM TLCA (**2**) alone or in combination with 50 μM of compound **3** (**2** + **3**). Values are normalized relative to GAPDH mRNA and are expressed relative to those of not treated cells (NT), which are arbitrarily set to 1. The relative mRNA expression is expressed as 2-(ΔΔCt). $*p < 0.05$ *versus* NT (non treated cells). $\#p < 0.05$ *versus* TLCA (**2**) stimulated cells.

de novo bile acid synthesis. As a consequence, FXR has been identified as an appealing target in the treatment of cholestasis and liver steatosis, where bile acid levels are impaired. In addition, FXR plays a crucial beneficial role in hepatic triglyceride homeostasis, and in glucose metabolism. Therefore, FXR agonists are promising for the treatment of NAFLD, dyslipidemia and type 2 diabetes [7–10]. After FXR de-orphanization by endogenus bile acids [1–3], bile acid scaffold has been subjected to intense medicinal chemistry modifications, producing several steroidal derivatives with different pharmacological profiles, and, of interest, endowed with good physicochemical properties and higher drug-like profiles when compared to non-steroidal ligands.

Since steroidal ligands cover the same chemical space of the endogenous activators, they are intrinsically promiscuous toward FXR and GPBAR1 and, with few exception, this kind of speculation mainly afforded dual modulators [14,22,28].

Besides this promiscuity supports the use of dual FXR/GPBAR1 agonists in the treatment of nonalcoholic steatohepatitis (NASH) and type 2 diabetes [9], the recent identification of GPBAR1 as the physiological mediator of pruritus [20], a common symptom observed in cholestasis, highly limits the pharmacological utility of dual agonists in the treatment of primary biliary cirrhosis (PBC) and related cholestatic disorders. In this context, the discovery of highly selective FXR agonists, devoid of GPBAR1 agonism, represents a good promise in the identification of new pharmacological protocols for PBC, an orphan disease for which therapeutic options are limited and poorly effective. In the present study, we have harnessed on the LCA scaffold modifying the functionalities of tetracyclic core, the stereochemistry of A/B ring junction, the length and the functionalization of the side chain. The results of this investigation has led to the discovery of compounds **3**, **5** and **7**, 5β-cholanoic acid, 5β-norcholanoic acid, and 5α-cholanoic acid, respectively, as the first examples of bile acid derivatives endowed with FXR agonism and GPBAR1 antagonism. Analysis of transactivation data clearly affirms the carboxylic group on the side chain as a key structural feature in GPBAR1 antagonism. Independently by the length of the side chain (C24 in **3** and C23 in **5**) and by the shape of the tetracyclic core, all carboxylic acids generated in

this study were relatively effective in inhibiting GPBAR1 activation caused by TLCA. In fact, both 5β-cholanoic acid **3** and 5α-cholanoic acid **7**, with a bent and a flat shape (A/B *cis* junction in **3** and the A/B *trans* junction in **5**), inhibited TLCA-induced transactivation of GPBAR1 with comparable IC_{50} values (28 μM and 22 μM, respectively, Fig. 4). Of interest is the observation that the presence of a larger negative charged end group such as the sulfate group in **10** and the sulfonate group in the tauro-conjugated derivatives **4** and **8**, is detrimental in term of GPBAR1 antagonism for both 5α- and 5β-cholane derivatives.

In conclusion, on the best of our knowledge, this result represents the first report of cholanoic acid derivatives able to antagonize GPBAR1. These compounds represent novel chemical probes, useful component of today's research arsenal of bile acid derivatives, in dissecting and in shedding light on the complex biological pathways under GPBAR1 control.

The analysis of their in vivo effects could result in the identification of new therapeutical approach to FXR mediated liver disorders in which the concomitant activation of GPBAR1 is associated to severe side-effects.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.steroids.2015.11.003.

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