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Chemical synthesis and biochemical properties of cholestane-5 α ,6 β -diol-3-sulfonate, a non-hydrolysable analogue of cholestane-5 α ,6 β -diol-3 β -sulfate.

Philippe de Médina^{1,2,3#§}, Silia Ayadi^{1,2,3#}, Régis Soulès^{1,2,3}, Bruno Payre⁴, Sandrine Rup-Jacques⁵, Sandrine Silvente-Poirot^{1,2,3§}, Mohammad Samadi^{5§} and Marc Poirot^{1,2,3§}.

¹ Cancer Research Center of Toulouse (CRCT), Inserm, CNRS, University of Toulouse, Team INOV: "Cholesterol Metabolism and Therapeutic Innovations", Toulouse, France.

² Equipe labellisée par la Ligue Nationale contre le Cancer

³ French network for Nutrition physical Activity And Cancer Research (NACRe network)

⁴ Centre de Microscopie Electronique Appliquée à la Biologie, Faculté de Médecine Rangueil, Toulouse, France.

⁵ Laboratory of Chemistry and Physics Multi-Scale Approach to Complex Environments, Department of Chemistry, University Lorraine, 57070 Metz, France.

Corresponding authors[§]:

marc.poirot@inserm.fr

mohammad.samadi@univ-lorraine.fr

sandrine.poirot@inserm.fr

philippe.de-medina@inserm.fr

these authors contribute equally to this work

Abstract

Cholestane-3 β ,5 α ,6 β -triol (CT) is a primary metabolite of 5,6-epoxycholesterols (5,6-EC) that is catalyzed by the cholesterol-5,6-epoxide hydrolase (ChEH). CT is a well-known biomarker for Niemann-Pick disease type C (NP-C), a progressive inherited neurodegenerative disease. On the other hand, CT is known to be metabolized by the 11 β -hydroxysteroid-dehydrogenase of type 2 (11 β -HSD2) into a tumor promoter named oncoesterone that stimulates the growth of breast cancer tumors. Sulfation is a major metabolic transformation leading to the production of sulfated oxysterols. The production of cholestane-5 α ,6 β -diol-3 β -*O*-sulfate (CDS) has been reported in breast cancer cells. However, no data related to CDS biological properties have been reported so far. These studies have been hampered because sulfate esters of sterols and steroids are rapidly hydrolyzed by steroid sulfatase to give free steroids and sterols. In order to get insight into the biological properties of CDS, we report herein the synthesis and the characterization of cholestane-5 α ,6 β -diol-3 β -sulfonate (CDSN), a non-hydrolysable analogue of CDS. We show that CDSN is a potent inhibitor of 11 β -HSD2 that blocks oncoesterone production on cell lysate. The inhibition of oncoesterone biosynthesis of a whole cell assay was observed but resulted from the blockage by CDSN of the uptake of CT in MCF-7 cells. While CDSN inhibits MCF-7 cell proliferation, we found that it potentiates the cytotoxic activity of post-lanosterol cholesterol biosynthesis inhibitors such as tamoxifen and PBPE. This effect was associated with an increase of free sterol accumulation and the appearance of giant multilamellar bodies, a structural feature reminiscent of Type C Niemann-Pick disease cells and consistent with a possible inhibition by CDSN of NPC1. Altogether, our data showed that CDSN is biologically active and that it is a valuable tool to study the biological properties of CDS and more specifically its impact on immunity and viral infection.

Key words: sterols, oxysterol, oxysterol sulfate, cholesterol, uptake, metabolism, cell death, MLB, cancer, NPC1, proliferation, cell death, autophagy.

Abbreviations: 5,6 α -EC: 5,6 α -epoxycholesterols; 5,6 β -EC: 5,6 β -epoxycholesterols; 11 β -HSD2: 11 β -hydroxysteroid-dehydrogenase of type 2; CDS: cholestane-5 α ,6 β -diol-3 β -*O*-sulfate; CDSN: cholestane-5 α ,6 β -diol-3 β -sulfonate; ChEH: cholesterol-5,6-epoxide hydrolase; CT: cholestane-3 β ,5 α ,6 β -triol; Ez: Ezetimibe; NPC: Niemann-Pick type C; OCDO: oncoesterone, 6-oxo-cholestan-3 β ,5 α -diol.

1. Introduction

Oxysterols represent an expanding family of bioactive oxygenated cholesterol metabolites that impact on a plethora of diseases including atherosclerosis, cancer, neurodegenerative diseases and aging [1, 2]. Depending on their chemical structures, these metabolites can exert their biological properties through various molecular mechanisms that could involve either nuclear receptors (e.g., LXR, ROR, ER α , GR) [3-6], G-protein coupled receptors (e.g., SMO, CXCR2, EBI2) [7-11], enzymes (e.g., SOAT/ACAT, ChEH and HMGCoA reductase) [12-15] or transporters (e.g., ions transporters, INSIG, oxysterol binding proteins, NPC1 and NPC1L1) [16-21]. Cholestane-3 β ,5 α ,6 β -triol (CT) and its obligatory precursors 5,6-epoxycholesterols (5,6-ECs) [22] occupy an important place among the oxysterol family, because 5,6-EC are among the major OS found in processed food and in circulating blood [4, 15, 23]. In addition CT is a biomarker of several pathologies such as Niemann-Pick type C (i.e., an inherited neurodegenerative disease), allergic asthma and breast cancers [24-26], and a potential marker of other diseases [27-31]. Looking at the biological properties of CT, both beneficial and deleterious effects have been reported. For example, CT displays antiproliferative, cytotoxic and mutagenic activities and promote vascular smooth muscle cells calcification, a process associated with atherosclerosis [32, 33]. On the other hand, this oxysterol acts as a neuroprotectant and an anti-epileptic agent [34, 35]. Although CT can be form through the acidic hydrolysis of 5,6-epoxycholestan-3 β -ol (5,6-ECs) in strong acidic conditions, in mammalian tissues their hydrolysis requires the obligatory intervention of a microsomal enzyme: the cholesterol-5,6-epoxide hydrolase (ChEH) [14], indeed, the epoxide ring of 5,6-EC was surprisingly found extremely stable towards the attack of nucleophilic compounds by contrast with other aliphatic epoxides [23, 36]. Interestingly, in cancer, CT was shown to be oxidized by the 11 β -hydroxysteroid-dehydrogenase of type 2 (11 β -HSD2) to form an oncometabolite named oncoesterone, that promotes breast cancer proliferation and invasiveness both *in vitro* and *in vivo* [3, 22, 37]. CT was also shown to be sulfated in cells by the sulfotransferase SULT2B1b leading to the production of cholestane-5 α ,6 β -diol-3 β -sulfate (CDS) [38-40]. While genetic evidences supported an inactivation of steroids and sterols through sulfation[41, 42], other data evidenced that sulfation generates biologically active signaling molecules. Cholesterol sulfate was shown to be important in keratinocyte physiology [43] and to control tumor infiltration of T cells[44]. The sulfation of side chain oxysterols led to compounds that display trophic and proliferative properties [45]. B-ring oxysterols led to the production of LXR antagonists on a gene reporter assay[46]. Interestingly the sulfated form of 5,6 α -EC was shown to control breast cancer cell differentiation and death [39, 47]. The 25-hydroxycholesterol-3-sulfate, a sulfated side chain oxysterol, was shown to be an epigenetic regulator that activates fatty acid oxidation, and this compound is under clinical evaluation for the treatment of Non-Alcoholic Steatohepatitis and Alcoholic Hepatitis [48-50]. In addition, the expression of SULT2B1b in tumors inhibits tumor growth and restore antitumor immune response highlighting the implication of a SULT2B1/LXR pathway potentially involving yet unidentified sulfated oxysterols [51]. We previously reported that the sulfated metabolite of CT (cholestane-5 α ,6 β -diol-3 β -sulfate; CDS; Fig 1A) is produced in breast cancer cells highlighting that this compound is a metabolite and a modulator of LXR signaling[39]. Despite this observation, studies aiming to evaluate the biological properties of CDS have never been reported to date. Studies on CDS have been hampered because sulfated (oxy)sterols are rapidly hydrolyzed by the steroid sulfatase (STS) which is widely expressed in various tissues and cancer cells (Fig 1A) [39, 52]. To circumvent this problem, we decided to synthesize a non-hydrolysable analogue of CDS. The strategy to produce a non-hydrolysable CTS analogue is by removing the oxygen in 3 β position of

steroid backbone involved in the esterification to eliminate the point of cleavage by STS (Fig 1) as previously reported for estrone sulfate [53], and we report herein for the first time the chemical synthesis of cholestane-5 α ,6 β -diol-3 β -sulfonate (CDSN) (Fig 1B) as a stable and non-hydrolysable chemical analogue of CDS. We evaluate the impact of CDSN on BC cells on the biosynthesis of oncoesterone, on the cellular uptake of several oxysterols and on cell fate.

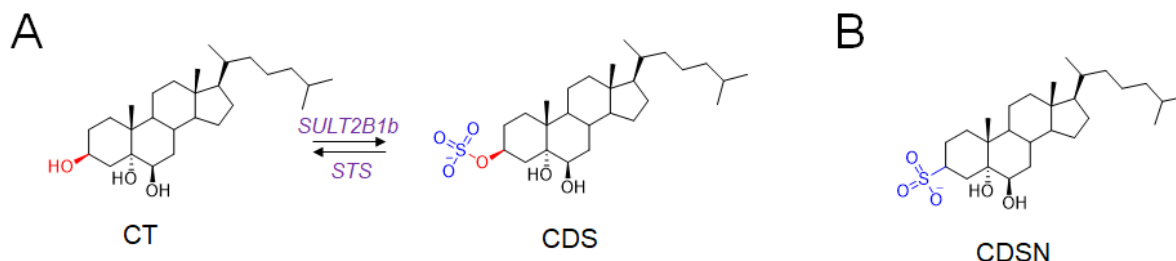


Figure 1: A) The reaction of sulfation of cholestane-3 β ,5 α ,6 β -triol (CT) is catalyzed by the sulfotransferase SULT2B1b to give cholestane-5 α ,6 β -diol-3 β -sulfate (CDS). The desulfation of CDS is catalyzed by the steroid sulfatase (STS). B) Chemical structure of cholestane-5 α ,6 β -diol-3 β -sulfonate (CDSN), a non-hydrolysable analogue of CDS.

2. Experimental section

2.1. General

All reagents were obtained from commercial suppliers and used without further purification. PBPE was produced as reported before [54]. Flash chromatography was carried out using silica gel (Merck Kieselgel 60, 230-400 mesh). Thin layer chromatography (TLC) analyses were performed on thin-layer analytical plates 60 F₂₅₄ (Merck). Melting points were measured on a Stuart melting point apparatus SMP30. Infrared (IR) spectra were recorded on a Nicolet iS5 FT-IR spectrometer. ¹H and ¹³C NMR spectra were recorded with a Bruker AvanceNeo 400 spectrometer and are shown on the Supplementary Material section.

2.2. Chemical synthesis of cholestane-5 α ,6 β -diol-3 β -sulfonate (CDSN)

Thiocholesterol (4): Thiocholesterol was prepared according to the procedure described by King and al[36], from cholesterylisothiuronium *p*-toluenesulfonate [55] **2** (2.156 g, 3.5 mmol). The solid crud product was purified over silica gel using hexane as eluent to yield **4** as a white solid (1.210 g; 86%). mp 95.5 °C (lit [36] 97.5 °C). IR (neat) ν : 2930, 2899, 2865, 2849, 1465, 1375 cm⁻¹. ¹H NMR (400 MHz, CDCl₃) δ 5.31 (m, 1H, H-6), 2.69 (m, 1H, H-3), 2.31(m, 2H, H-5), 1.00 (s, 3H, 19-CH₃), 0.91 (d, *J* = 6.6 Hz, 3H, 21-CH₃), 0.86 (dd, *J* = 6.6, 1.8 Hz, 6H, 26-CH₃ and 27-CH₃), 0.67 (s, 3H, 18-CH₃). ¹³C NMR (101 MHz, CDCl₃) δ 142.06, 121.19, 56.90, 56.30, 50.35, 44.36, 42.45, 40.08, 39.90, 39.67, 39.60, 36.49, 36.34, 35.94, 34.23, 31.95 (CH₂), 31.95 (CH), 28.38, 28.17, 24.42, 23.98, 22.97, 22.71, 21.05, 19.48, 18.87, 12.00. the ¹H and ¹³C NMR data were identical to that reported in lit [38] MS (EI, CH₂Cl₂): *m/z*: 402 ([M]⁺, 43%)

5 α , 6 β - dihydroxy- cholesteryl sulfonate (5):

Method A: A solution of 35% H₂O₂ (1 mL) was dissolved in 98% formic acid (4 ml) at 0 °C and the mixture was stirred at rt for 1 h to afford peroxyformic acid. To this was added a solution of thiocholesterol **4** (1 mmol, 402 mg) in CH₂Cl₂ (5 ml) at 0 °C. The reaction was stirred at rt overnight. The reaction content was transferred to a 250 ml flask and the solvent

was carefully (foam formation) evaporated below 25 °C and dried under vacuum. The residue was dissolved with 20 ml of MeOH and heated at 60 °C in a water-bath for 1h. The solvent was evaporated and purified on silica gel using CH₂Cl₂-MeOH (80-20 then 70-30) to yield **5** as a white solid (446 mg; 92%),

Method B: A solution of 35% H₂O₂ (1 mL) was dissolved in 98% formic acid (1 ml) at 0 °C and the mixture was stirred at rt for 1 h to afford peroxyformic acid. To this was added a solution of thiocholesterol **4** (1 mmol, 402 mg) in THF (8 ml) at 0 °C and stirred at rt for 1h. The solution was warmed to 50-60 °C in a water-bath for 3h and stirred at rt overnight. The solvent was evaporated and treated as described above to yield **5** as a white solid (460 mg; 95%), mp 275.3 °C. IR (neat) v: 3404, 2932, 2866, 1228, 1164, 1048 cm⁻¹. ¹H NMR (400 MHz, MeOD) δ 3.49 (d, *J* = 3.4 Hz, 1H, H-6), 3.20 (tt, *J* = 12.5, 4.5 Hz, 1H, H-3), 2.37 (t, *J* = 13.1 Hz, 1H, 2-Hax), 1.15 (s, 3H, 19-CH₃), 0.94 (d, *J* = 6.4 Hz, 3H, 21-CH₃), 0.88 (d, *J* = 6.5 Hz, 6H, 26-CH₃ and 27-CH₃), 0.71 (s, 3H, 18-CH₃). ¹³C NMR (101 MHz, MeOD) δ 76.42, 75.37, 57.67, 57.51, 56.14, 46.66, 43.89, 41.49, 40.70, 39.35, 37.38, 37.15, 35.45, 34.10, 33.62, 31.63, 29.34, 29.14, 25.19, 24.94, 23.41, 23.20, 22.95, 22.08, 19.24, 17.00, 12.61.

2.3. Cell culture

MCF7 cells were chosen because they express a functional SULT2B1b enzyme [39]. HEK293T cells were chosen for transfection assays and overexpression of HSD11B2 as reported in [56]. MCF-7 and HEK293T cells were from the American Tissue Culture Collection and cultured until passage 30. MCF-7 cells were grown in RPMI 1640 supplemented with 5% fetal bovine serum (FBS). HEK293T cells were grown in DMEM 10% FBS. All media were supplemented with penicillin and streptomycin (50 U/mL). Cells were cultured in a humidified atmosphere with 5% CO₂ at 37°C. Cell lines were tested once a month for mycoplasma contamination using Mycoalert Detection (Lonza).

2.4. Measure of sterol metabolism in MCF-7 Cells.

We have used MCF-7 cells for whole cell oncosterone biosynthesis assays from [¹⁴C]-CT as described before [37]. MCF-7 cells were plated into six-well plates (1.5×10⁵ cells per well) in the appropriate complete medium. Two days after seeding, this medium was replaced with complete medium and cells were treated with 1 μM [¹⁴C]-CT (26 mCi/mmol; 0.052 μCi/well) for 5 h in the presence or absence of CDSN at 20 μM. After incubation, cells were washed and scraped, and neutral lipids were extracted with a chloroform-methanol mixture as described previously and then separated by TLC using either ethyl acetate as the eluent [57]. The radio-labeled sterols were revealed by autoradiography. For quantification, silica zones at the expected R_f values corresponding to authentic [¹⁴C]-labeled standards were scraped and radioactivity was measured using a β-counter.

2.5. Cell Transfection.

HEK-293T cells (5.10⁶) were transfected with 5 μg of the plasmid pCMV6-XL5 HSD2 (SC122552, OriGene Technologies, Inc., Rockville, MD, USA) using the Neon Transfection System (Thermo Fisher Scientific, Waltham, MA, USA) with 2 pulses at 1300 V for 20 ms. 48 h after transfection, the media was replaced by DMEM without phenol red supplemented with 10 % dextran-coated charcoal-stripped FBS. Cells were harvested 72 h after transfection, washed with PBS and stored at -80°C.

2.6. Measure of oncosterone biosynthesis inhibition by CDSN

We have chosen to test the cell lysate from HEK293T cells transfected with a plasmid encoding 11β-HSD2 to measure the impact of inhibitors of oncosterone biosynthesis from [¹⁴C]-CT at the enzyme level [37]. Cell lysates were prepared from HEK293T cells transiently

expressing human 11 β -HSD2 as described before[37]. Briefly, 2.10⁶ cells were resuspended in 150 μ L of activity buffer (Tris-HCl 25 mM PH 7.4, glycerol 20%, sucrose 25 mM, NaCl 200 mM, MgCl₂ 1 mM, CaCl₂ 1 mM) with 1% protease inhibitor mixture (Sigma-Aldrich). Cells were lysed by four cycles of freeze/thaw (nitrogen-rt) and samples were centrifuged at 10000 rpm for 10 minutes at 4°C. Supernatant were collected, aliquoted and stored at -80°C before use. Protein concentration was measured using the Bradford method. Enzymatic activity was measured in 11 β -HSD2 activity buffer (final volume 0.2 mL) containing the substrate ([¹⁴C]-CT (1 μ M), cell lysate protein (20 μ g), NAD⁺ (0.5 mM) and DMSO 1% in the absence or presence of the tested compounds. After 10 minutes of incubation at 37°C, the reaction was stopped by immersing the sample in ice-water and adding 1.5 ml chloroform/methanol (2:1) and 300 μ l of aqueous KCl (8.8%). Organic layer was washed with water (1 mL) and reduced to dryness under a flux of nitrogen. Lipids were then separated by TLC using either ethyl acetate as the eluent. The radioactive sterols were revealed by autoradiography. For quantification, silica zones at the expected R_f values corresponding to authentic [¹⁴C]-labeled standards were scraped and radioactivity was measured using a β -counter (tri-carb; Perkin-Elmer).

2.7. Uptake of Cholesterol, 5,6-ECs, and CT

MCF-7 cells were plated into twelve-well plates (7.5 \times 10⁴ cells per well) in the appropriate complete medium. Four days after seeding, the medium was replaced with 5% FBS medium or medium without FBS and cells were incubated with [¹⁴C]-cholesterol, [¹⁴C]-5,6 α -EC, [¹⁴C]-5,6 β -EC or [¹⁴C]-CT (1 μ M; 26 mCi/mmol; 0.026 μ Ci/well) in the presence or absence of 40 μ M Ezetimibe (Ez) (Sigma-Aldrich) or 20 μ M of CDSN, for 5 hours. Then, cells were washed with PBS and were re-suspended in 1 ml of SDS 0.1 %. Radioactivity was measured using a β -counter (tri-carb; Perkin-Elmer).

2.8. Cell death assay.

MCF-7 cells were seeded in RPMI with 5% FBS into 6-well plates at 1.5 \times 10⁵ cells per well. The cells were then treated with solvent vehicle (0.1% ethanol/0.1 % DMSO), 2.5 μ M Tam or 10 μ M PBPE alone or in the presence of 10 μ M CDSN. Cell death was determined by the Trypan blue exclusion assay. The cells were trypsinized and resuspended in the Trypan blue solution (0.25% (w/v) in PBS) and counted in a Malassez cell under a light microscope.

2.9. Filipin staining procedures

Cell were grown on glass coverslips and treated with drugs for 72 h and then fixed with 3.7% paraformaldehyde for 15 min at room temperature followed by washing twice with PBS (Euromedex), and stained with filipin (50 mg/ml) for 75 min at room temperature followed by washing twice with PBS as described before [58]. Cells were analysed using a Nikon eclipse 90i microscope. The pixel intensities of the images were measured using NIH Image software and reported on a graph as in [59].

2.10. Transmission Electron Microscopy

Cells were fixed with 2% glutaraldehyde in 0.1 mol/L Sorensen phosphate buffer (pH 7.4) for 1 h and washed with the Sorensen phosphate buffer (0.1 mol/L) for 12 h. The cells were then postfixed with 1% OsO₄ in Sorensen phosphate buffer (0.05 mol/L Sorensen phosphate buffer, 0.25 mol/L glucose, 1% OsO₄) for 1 h. The cells were then washed twice with distilled water and prestained with an aqueous solution of 2% uranyl acetate for 12 h. Samples were then treated exactly as described previously[60].

2.11. Statistical analysis.

Values are the mean \pm S.E. of three independent experiments each carried out in duplicate. Statistical analysis was carried out using a Student's t-test for unpaired variables. *, ** and *** in the figures refer to statistical probabilities (P) of <0.05, <0.01 and <0.001, respectively, compared with control cells that received solvent vehicle alone.

3. Results and discussion

3.1. Chemical synthesis of CDSN

Chemical synthesis of CDSN is shown in Fig 2. Initially, we examined the preparation of format cholesteryl sulfonate **3** by oxidation of cholesterylisothiuronium *p*-toluenesulfonate **2**[55] with performic acid according to Yoder procedure [41]. ^1H NMR analysis of **3** reveals the presence of *p*-toluenesulfonic acid as by product. Tentative to remove *p*-toluenesulfonic acid which accompanied compound **3**, either by crystallization or purification over silica gel was unsuccessful. To circumvent this problem, we turned to the oxidation of thiocholesterol **4** which was prepared from **2** by alkali hydrolysis [61]. Thus, oxidation of thiocholesterol with performic acid in CH_2Cl_2 or THF produced a mixture of **3** and the deprotected sulfonate **5**. Heating the resulting mixture at 60 °C in MeOH affect the deformylation of **3** which is catalyzed by sulfonic acid [62] moiety and furnished the desired $5\alpha,6\beta$ -dihydroxy-cholesteryl sulfonate **5** (Scheme 1). In conclusion, the synthesis of $5\alpha,6\beta$ -dihydroxy-cholesteryl sulfonate **5** was achieved in four steps from commercially available cholesterol **1**. Facile deprotection of **3** due to the presence of sulfonic acid moiety, avoiding the use of strong base (e.g. NaOH, KOH, K_2CO_3) is worthy-mentioning. ^1H NMR and ^{13}C NMR spectra are presented in supplementary material.

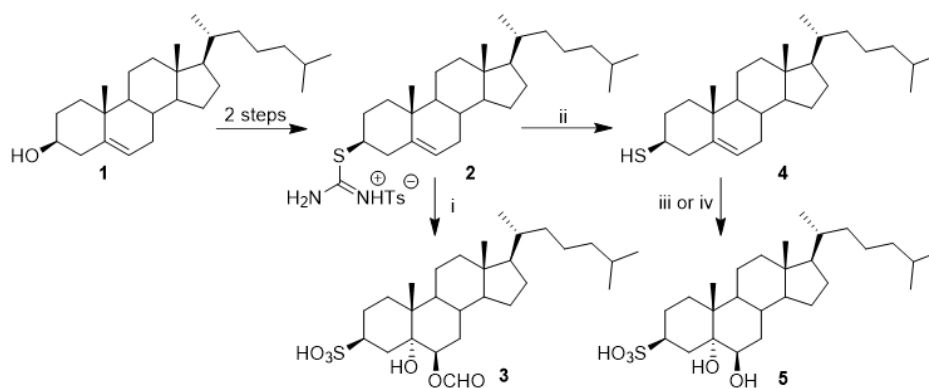


Figure 2: Chemical synthesis of CDSN. i: HCOOH , H_2O_2 , 50 °C, 1h. ii: 3 equiv NaOH, EtOH, reflux, 2h, 86%. iii: a: HCOOH , H_2O_2 , CH_2Cl_2 , 0 °C- rt, 24h. b: MeOH, 60 °C, 1h, 92%. iv: a: HCOOH , H_2O_2 , THF, 0 °C-rt 1h, 50-60 °C, 3h, rt, 24h. b: MeOH, 60 °C, 1h, 95%.

3.2. CDSN is an inhibitor of oncoesterone biosynthesis

We have previously demonstrated that CT is metabolized toward the oncometabolite oncoesterone through 11β -HSD2 [37]. The inhibition of oncoesterone production constitutes an a putative new therapeutic strategy for the treatment of breast cancer [3, 22]. Consequently, we evaluated if CDSN can inhibit oncoesterone production. We evaluated the impact of CDSN on oncoesterone production catalyzed by 11β -HSD2. Oncoesterone synthase activity was carried out on cell lysate from HEK-293T transiently transfected with a plasmid encoding human 11β -HSD2 (HEK293T-11HSD2 lysate). The conversion of [^{14}C]-CT into [^{14}C]-

Oncosterone in HEK293T-11HSD2 lysate was measured alone or with increasing concentrations of CDSN ranging from 1 to 10 μM . CDSN inhibit the production of [^{14}C]-Oncosterone from [^{14}C]-CT in a dose-dependent manner ($\text{IC}_{50} = 2.55 \pm 0.63 \mu\text{M}$) (Fig 3A-B). Consequently, CDSN is a potent inhibitor of oncosterone synthase activity.

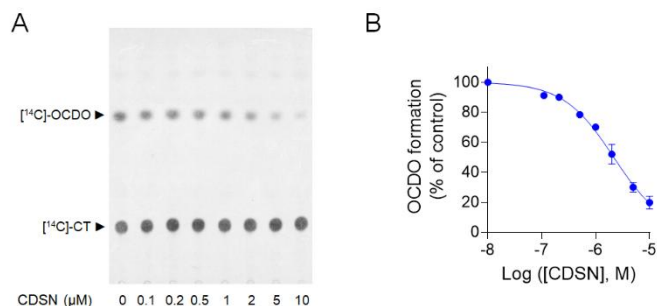


Figure 3: Effect of CDSN on OCDO biosynthesis. HEK293T-11HSD2 cells lysate (20 μg) were incubated with 1 μM of [^{14}C]-CT at 37°C for 10 minutes with increasing concentrations of CDSN ranging from 1 to 10 μM . OCDO biosynthesis was assayed by measuring the conversion of [^{14}C]-CT to [^{14}C]-OCDO by thin-layer chromatography (TLC) and quantified as described in Materials and Methods. **A)** On the left panel, a representative autoradiogram of a dose-dependent inhibition of Oncosterone production by CDSN in HEK293T-11HSD2 cells lysate. **B)** on the right panel, quantification of the dose-dependent inhibition of OCDO biosynthesis activity measured by TLC with increasing concentrations of CDSN. Values are the mean \pm S.E. of three independent experiments each carried out in duplicate.

3.3. Impact of CDSN on CT metabolism in MCF-7 cells

We previously reported that the human breast cancer cell line MCF-7 cells expressed the SULT2B1b protein, produced sulfated oxysterols [30] and respond to the stimulation of sulfated oxysterols. We have thus chosen this cell line to study the effect of CDSN. We measured the impact of CDSN on Oncosterone formation on a whole cell assay by incubating MCF-7 cells with [^{14}C]-CT alone or in the presence of 20 μM CDSN for 5 hours. After 48 hr, and in the absence of CDSN, 19 \pm 6% of the radioactivity was incorporated into cells (Fig 4A). The intracellular radioactivity corresponds at 16 \pm 2% to [^{14}C]-Oncosterone formed by 11 β -HSD2 in MCF-7 cells (Fig 4B). Surprisingly, the treatment of cells with CDSN triggers a strong diminution of the intracellular radioactivity (Fig 4A) whereas the percentage of intracellular [^{14}C]-OCDO was not affected by CDSN (Fig 4B). Indeed, CDSN reduces the intracellular level of both [^{14}C]-CT and [^{14}C]-OCDO by 69 % and 44% respectively in MCF-7 cells (Fig 4C). These results showed that CDSN does not inhibit the biosynthesis of oncosterone on a whole cell assay but inhibits CT uptake in MCF-7 cells.

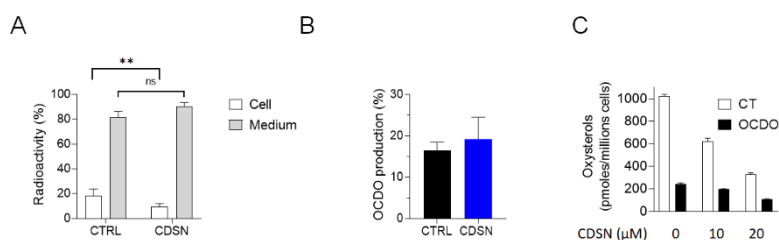


Figure 4: Measure of the impact of CDSN on [^{14}C]-CT uptake and metabolism in MCF-7 cells. MCF-7 cells were incubated with 1 μM of [^{14}C]-CT for 5 hours with or without 20 μM of CDSN. 11 β -HSD2 activity (OCDO synthase) was assayed by measuring the conversion of [^{14}C]-CT to [^{14}C]-OCDO by TLC and quantified as

described in Materials and Methods. (A) Quantification of intracellular radioactivity from lipidic extracts of MCF-7 treated with solvent vehicle and CDSN (20 μ M); (B) Percentage of intracellular conversion of [14 C]-CT into [14 C]-OCDO on MCF-7 treated with solvent vehicle and CDSN (20 μ M); (C) Quantification of the intracellular level of [14 C]-CT and [14 C]-oncosterone on MCF-7 treated with solvent vehicle or 20 μ M CDSN. Values are the mean \pm S.E. of three independent experiments each carried out in duplicate. Statistical analysis was carried out using a Student's t-test for unpaired variables. *, ** and *** in the figures refer to statistical probabilities (P) of <0.05, <0.01 and <0.001, respectively, compared with control cells that received solvent vehicle alone.

3.4. Impact of CDSN on oxysterols and cholesterol uptake in breast cancer cells

To get more insight on the ability of CDSN to inhibit the cellular uptake of oxysterols, we evaluate the impact of CDSN in comparison with ezetimibe (Ez) (i.e., a well-known inhibitor of NPC1L1) that was shown to inhibit the uptake of B-ring oxysterols [63]. MCF-7 cells were treated with [14 C]-cholesterol, [14 C]-5,6 α -EC, [14 C]-5,6 β -EC and [14 C]-CT alone or in the presence of 20 μ M CDSN or with 40 μ M Ez for 5 hours. Cells were then lysed by SDS 0.1% and the intracellular radioactivity was measured. We found that Ez slightly reduces the uptake cholesterol by approximately 20% but did not inhibit the uptake of 5,6 α -E, 5,6 β -EC and CT. By contrast, we found that CDSN induced of 70% reduction of cholesterol uptake and a 17%, 24% and 36% inhibition of 5,6 α -E, 5,6 β -EC and CT uptake respectively (Fig 5A-D). We next found that the absence of FBS, which provides lipids including cholesterol to cells, CDSN totally blocked sterols uptake suggesting that added sterols are uptaken by cells when associated to FBS components such as lipoproteins. In conclusion, we show that CDSN is an inhibitor of CT, 5,6 α -E, 5,6 β -EC and cholesterol uptake in MCF-7 cells while ezetimibe is slightly active to block only cholesterol uptake. These data suggest that the control by CDSN of the uptake of these sterols is independent of NPC1L1 and may required the LDLR endocytic pathway.

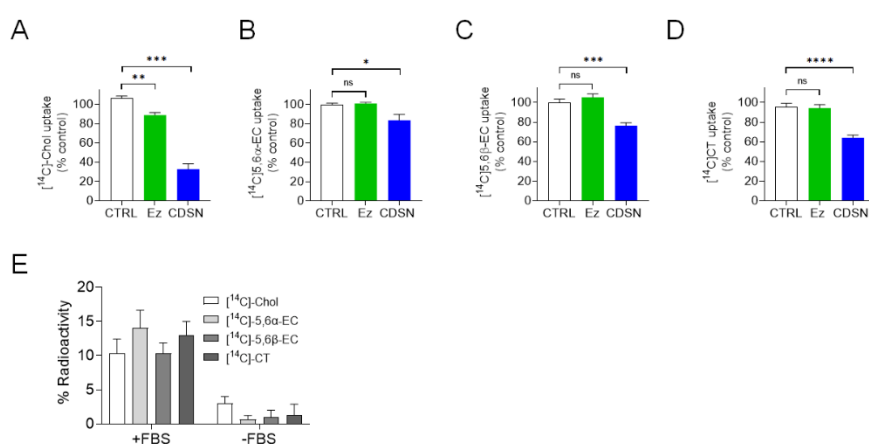


Figure 5: MCF-7 cells were incubated with 1 μ M of [14 C]-cholesterol, 1 μ M of [14 C]-5,6 α -EC, 1 μ M of [14 C]-5,6 β -EC or 1 μ M of [14 C]-CT for 5 hours with or without 40 μ M of ezetimibe, or 20 μ M of CDSN. Cells were lysed with 0.1 % SDS and intracellular radioactivity counted with a β -counter. Quantification of intracellular radioactivity from MCF-7 cells exposed to [14 C]-Cholesterol (A), [14 C]-5,6 α -EC (B), [14 C]-5,6 β -EC (C) and [14 C]-CT (D). The intracellular radioactivity was expressed as the percentage of the intracellular radioactivity measured in the absence of tested compounds (control with solvent vehicle). Values are the mean \pm S.E. of three independent experiments each carried out in duplicate. Statistical analysis was carried out using a Student's t-test for unpaired variables. *, ** and *** in the figures refer to statistical probabilities (P) of <0.05, <0.01 and <0.001, respectively, compared with control cells that received solvent vehicle alone.

3.5. Effect CDSN alone or in combination with post-lanosterol cholesterol biosynthesis inhibitors on MCF-7 cells proliferation and viability

We report here that CDSN inhibits MCF-7 proliferation but was not cytotoxic up to 20 μM (Fig 6A-C). We previously reported that Tam and PBPE triggered the inhibition of cholesterol biosynthesis and a massive accumulation of free sterols [39, 64-68]. This was characterized at the cellular level by an increased vesicular filipin staining associated the presence multilamellar bodies (MLB) in the cytoplasm of MCF-7 cells [39, 64-68]. Since the endocytic LDLR pathway involved NPC1 transporters [69], we were interested to determine if CDSN could enhanced sterol storage in MCF-7 cells. Cells were exposed to Tam, PBPE with or without CDSN for 72 h and then cell proliferation and viability was measured. We observed that single treatment with Tam or PBPE inhibit cell proliferation but not viability at these concentrations. We observed that the combination of CDSN with Tam or PBPE with induces anoikis that was not observed with single treatment (Fig 6A) and affect drastically cell viability (Fig 6C). These data showed that CDSN inhibits MCF-7 cell proliferation and this effect was potentiated by the inhibitors of cholesterol biosynthesis Tam and PBPE which led to anoikis and massive cell death.

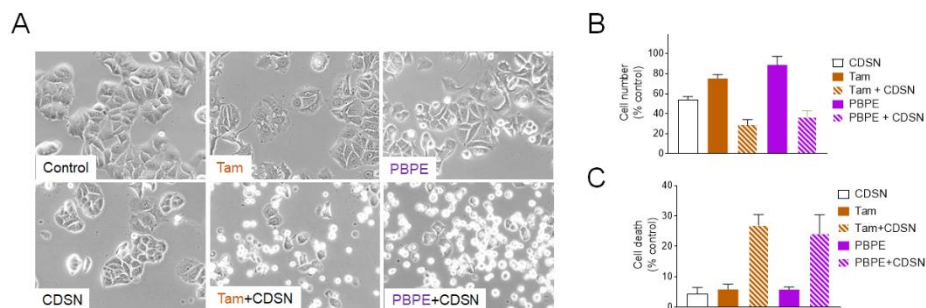


Figure 6: Effect CDSN on MCF-7 cells proliferation and viability. MCF-7 cells were exposed for 3 days to solvent vehicle (Control), 10 μM CDSN alone or a combination with 2.5 μM Tam, or 10 μM PBPE. **A)** Morphological changes observed by light microscopy (x40); **B)** Antiproliferative activity. Cells were harvested by trypsinization and counted on a Malassez chamber. Data were expressed as the percentage of cell number relative to control cells; **C)** Cell death measurement. Cell death was determined by Trypan blue exclusion test. Data were expressed as the percentage of cell death relative to control cells. Values are the mean \pm S.E. of three independent experiments each carried out in duplicate. Statistical analysis was carried out using a Student's t-test for unpaired variables. *, ** and *** in the figures refer to statistical probabilities (P) of <0.05, <0.01 and <0.001, respectively, compared with control cells that received solvent vehicle alone.

3.6. The combination of CDSN with Tam and PBPE induces free sterol accumulation and the appearance of giant multilamellar bodies

We next investigated the impact of CDSN treatment of MCF-7 cells on intracellular sterol accumulation, filipin labeling and MLB formation. CDSN alone does not trigger an increased filipin labeling or the appearance of MLB. Tam or PBPE alone triggers the increased filipin labeling (Fig 7A) confirming previously published observations [64, 65]. As expected, combination treatments of MCF-7 cells led to an increased filipin labeling (Fig 7B) and the accumulation of MLB (Fig 8). These data show that CDSN impact on the dynamic of sterol homeostasis in MCF-7 possibly involving NPC1.

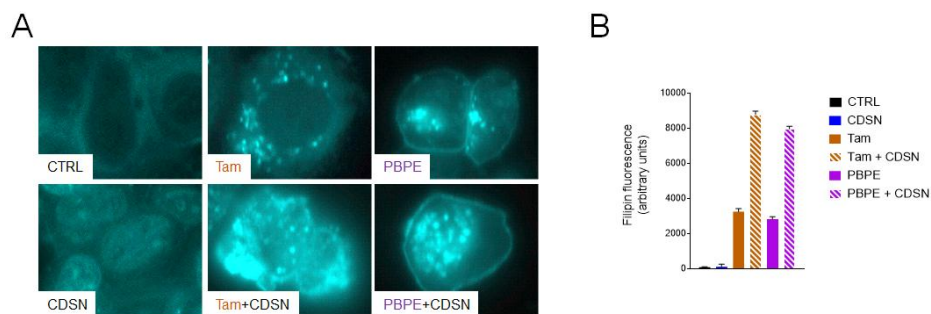


Figure 7: Representative pictures of cells stained with filipin and analyzed by fluorescent microscopy, magnification x 40. MCF-7 cells were exposed for 2 days to solvent vehicle (Control); PBPE (10 μ M), CDSN (10 μ M) or a combination of PBPE (10 μ M) with CDSN (10 μ M).

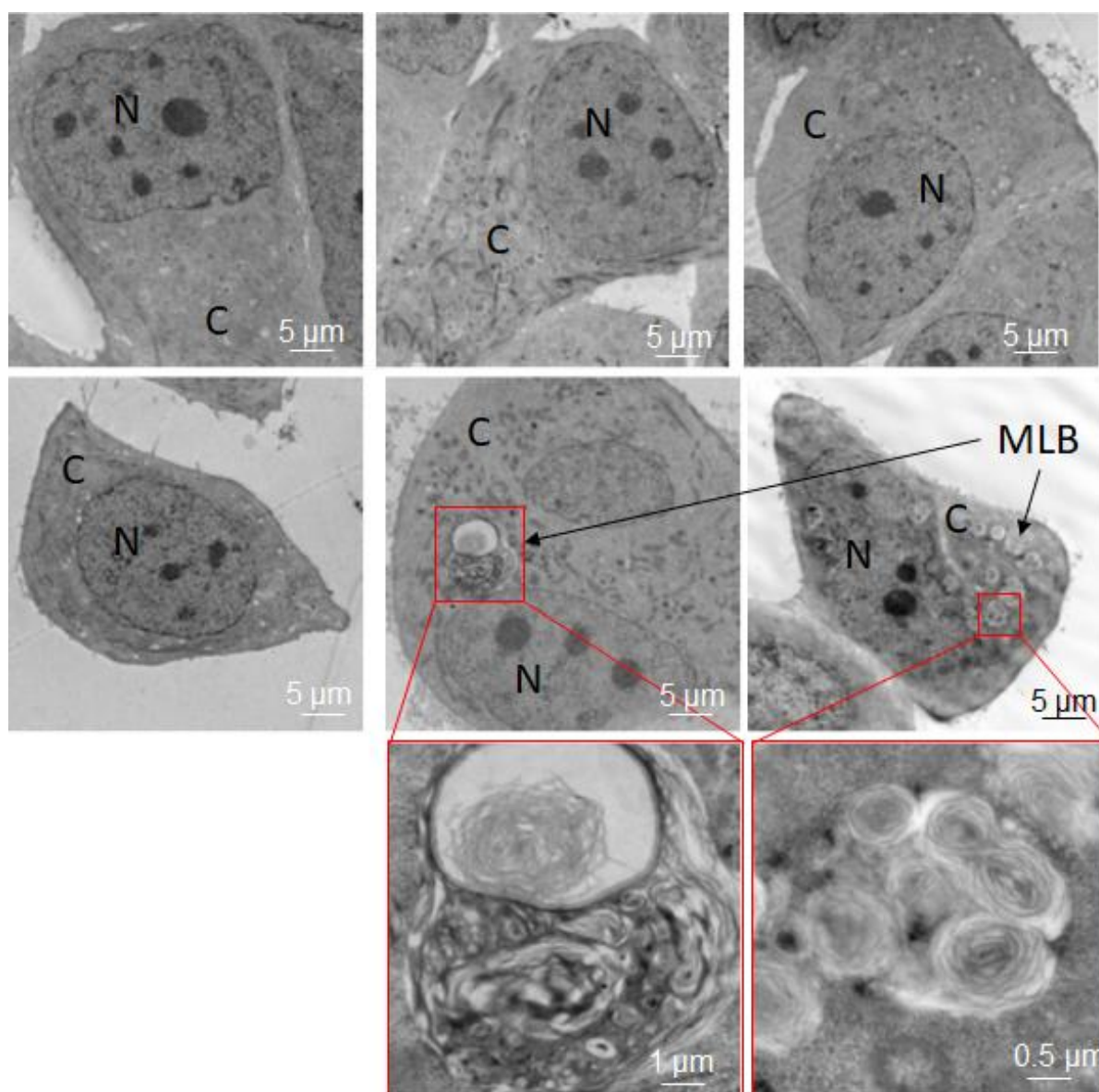


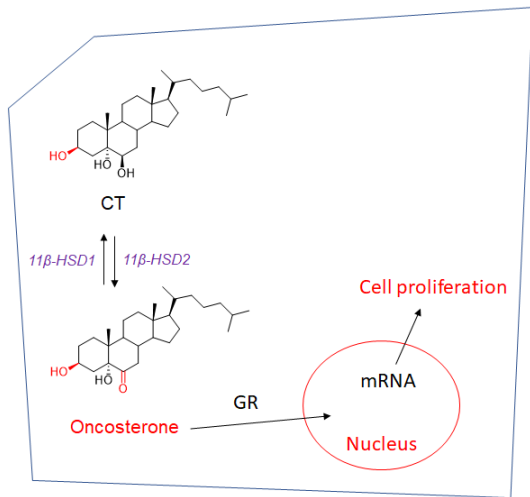
Figure 8: Transmission electron microscopic ultrastructural analysis of MCF7 cells exposed for 2 days to solvent vehicle (Control); PBPE (10 μ M), CDSN (10 μ M) or a combination of PBPE (10 μ M) with CDSN (10 μ M). N, nucleus; C, cytoplasm; LD, lipid droplet; MLB, multilamellar bodies. Experiments were repeated at

least three times in duplicate with comparable results. The images presented are representative of three independent experiments.

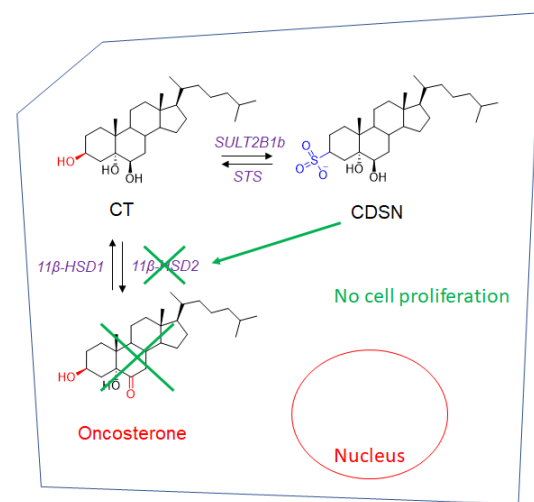
4. Conclusion

We report herein the chemical synthesis of CDSN as a non-hydrolysable analogue CTS. The synthesis is original and gave the product of interest in 1 step from thiocholesterol oxidation with peroxyformic acid with a yield of 92 and 95 % when using CH_2Cl_2 or THF respectively as solvent for the reaction. We next evaluate the biological properties of CDSN which is a non-hydrolysable analogue of CTS. We first look at the impact of CDSN on the transformation of CT into oncoesterone and found is a potent inhibitor of the enzyme in cell lysates suggesting it could be a substrate or an endogenous inhibitor of the oncoesterone synthase (11β -HSD2) (Fig 9A-B). While we observed that the oncoesterone level decreased drastically under CDSN treatment, a careful analyses of this effect ruled out an inhibition of the oncoesterone synthase in that case because we found that CDSN inhibited drastically the uptake of CT by MCF-7 cells. In addition, we found that CDSN inhibited not only the uptake of CT but also the uptake of cholesterol, 5,6 α -EC and 5,6 β -EC. These data suggest that exogenous CTS could control 5,6-EC and CT homeostasis. The uptake of these sterol can be mediated through several mechanism using the endocytic pathway via the LDLR [70], or transporters such as NPC1L1 which is express in MCF-7 cells [71]. We found that CDSN reduces the uptake of cholesterol which can be partly inhibited by Ezetimide suggesting that NPC1L1 control the uptake of 20% of cholesterol in MCF-7 cells but Ezetimide was inefficient to block the uptake of CT and 5,6-EC suggesting that another mechanism was required. On the other hand, we found that FBS was required for the uptake of sterols suggesting that tested sterols were uptaken by cells via lipoproteins such as low density lipoproteins (LDL). LDL are uptaken by cells via LDL receptor which is controlled by NPC1[69]. NPC1 is a transporter involved in the transport of cholesterol from the reticulum endoplasmic to lysosomes. It is also involved in the clathrin-coated pits endocytic pathway controlling lipoproteins an virus uptake which led to the development of NPC1 inhibitors. NPC1 has been identified as pharmacological targets that can block the host cell entrance and the infectivity of several viruses such as ebola virus [72-74], bluetongue virus [75], reovirus [76], african swine fever virus [77], flovovirus [78], HIV [79] and SARS-Cov-2 [80, 81]. Interestingly, CT was also used as a chemotype for the development of inhibitors of NPC1 transporters [82, 83]. These data support that CDSN inhibits NPC1 and the uptake of 5,6-EC and CT complexed with LDL as summarized on Fig 9C-D.

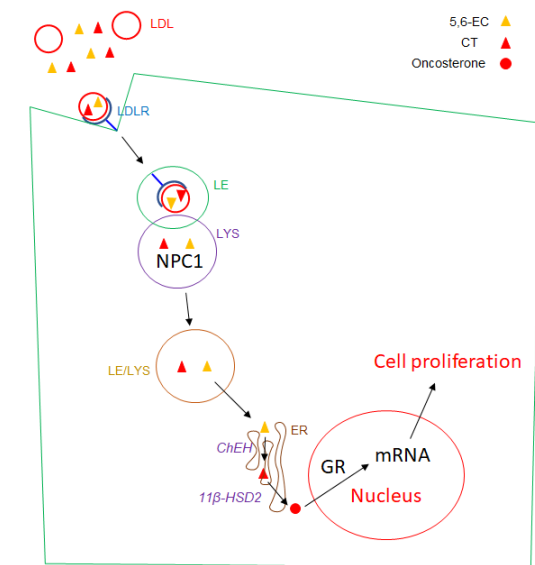
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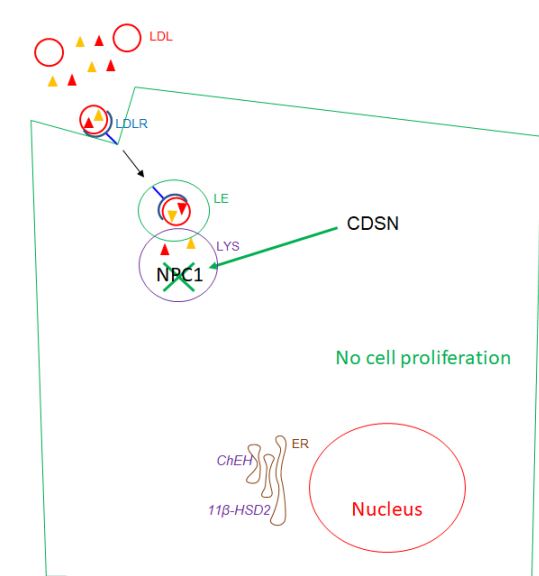
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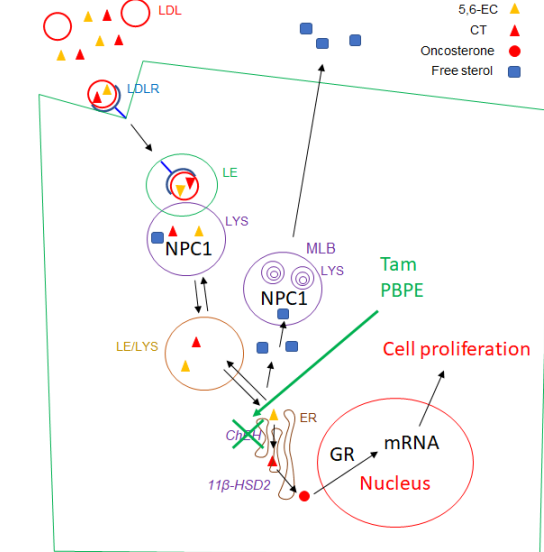
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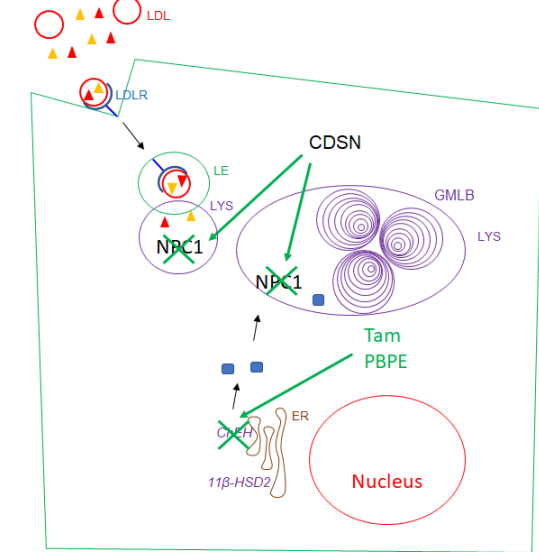


Figure 9: **A)** Biosynthesis of oncoesterone from CT and stimulation of cell proliferation through the activation of GR by oncoesterone. **B)** CDSN inhibits the biosynthesis of oncoesterone at the 11β -HSD2 level and cell proliferation. **C)** LDL transport 5,6-EC and CT into cells that can be further metabolized into oncoesterone that stimulate cell proliferation via the activation of GR. **D)** CDSN inhibits 5,6-EC and CT uptake by blocking NPC1, and thus inhibiting oncoesterone production and cell proliferation. **E)** Tamoxifen and PBE inhibit the ChEH/EBP complex which blocks oncoesterone biosynthesis and induce free sterol accumulation due to EBP inhibition. Free sterols accumulation led to the appearance of MLB. **F)** Co-treatment of Tam or PBPE with CDSN led to the overaccumulation of free sterols into giant MLB. GR: glucocorticoid receptor; LDL: low density lipoproteins; LDLR: LDL receptor; LE: late endosomes; LYS: lysosomes; ER: endoplasmic reticulum; MLB: multilamellar bodies; GMLB: giant MLB.

CDSN inhibits cell proliferation but was found weakly cytotoxic. The fact that NPC1 may control the uptake of sterols of interest led us to test compounds that are inhibitors of cholesterol biosynthesis and that induced the accumulation of free sterols, to determine if CDSN amplified or not sterol accumulation. Tam and PBE have been shown to induce the accumulation of sterols due to their inhibition of cholesterologenic enzymes [64-68, 84]. This accumulation was associated to the induction of autophagy in MCF-7 cells and to the appearance of MLB in the cytoplasm of cells [47]. Tam or PBPE combination treatment with CDSN of MCF-7 cells induce the appearance of giant MLB that may result from the inhibition of sterol trafficking in these cells (Fig 9E-F). The accumulation of sterols and the presence of MLB recapitulates ultrastructural modifications reported for Type C Niemann-Pick disease fibroblasts [85, 86].

Altogether, our data strongly suggest that CDSN and therefore CDS could display antitumor activity on breast cancer through the inhibition of oncoesterone production. This latter could arise through two mechanisms: 1°) the inhibition of oncoesterone biosynthesis by intracellular CDS (Fig 9A-B) and 2°) the inhibition of cellular uptake of oncoesterone precursors (5,6-EC and CT) by extracellular CDS (Fig 9C-D). This strongly suggests that CDS is a natural CT metabolite that can regulate oncoesterone biosynthesis and LDLR-mediated oxysterol cellular uptake.

The impact of CDSN in combination with Tam, PBPE *in vivo* on tumor implanted in mice would be necessary to validate the potential therapeutic interest of CDSN for BC treatment and suggests that the endogenous production of CTS by BC cells could represent a predictive factor of sensitivity to hormone therapy with tamoxifen and eventually other SERMs. The impact of combination treatments on other BC cells with various phenotypes in terms of cholesterol/oxysterols homeostasis, biosynthesis, metabolism, and sterol receptor is worthy of investigation. In particular, BC cell lines that are representative of the different BC subtypes *in vitro* and *in vivo* deserve further investigations. These studies would help to determine if CDSN could be a specific or general tool with a therapeutic potential and would paved the way for exploring the importance of the CDS pathway in the physio-pathology and in anticancer strategies.

The potential role of CDS on different pathologies such as Niemann-Pick Type C disease, viral infection and cancer deserves further investigation.

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