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Original article

Improved synthesis and in vitro evaluation of the cytotoxic profile of oxysterols oxidized at C4 (4α- and 4β-hydroxycholesterol) and C7 (7-ketocholesterol, 7α- and 7β-hydroxycholesterol) on cells of the central nervous system

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ABSTRACT

Whereas the biological activities of oxysterols oxidized at C7 (7-ketocholesterol (7KC), 7β-hydroxycholesterol (7β-OHC), 7α-hydroxycholesterol (7α-OHC)) are well documented, those of oxysterols oxidized at C4 (4β-hydroxycholesterol (4β-OHC), 4α-hydroxycholesterol (4α-OHC)) are not well known, especially on the cells of the central nervous system. Therefore, an improved methodology has been validated for 4β-OHC and 4α-OHC synthesis, and the effects on cell viability and cell growth of these molecules were studied on immortalized, tumoral and normal brain cells (158N, C6 and SK-N-BE cells, and mixed primary cultures of astrocytes and oligodendrocytes). Whereas inhibition of cell growth with 7KC, 7β-OHC, and 7α-OHC is associated with a decrease of cell viability (cytotoxic activities), our data establish that 4β-OHC and 4α-OHC have no effect on cell viability, and no or minor effect on cell growth evocating cytostatic properties. Thus, comparatively to oxysterols oxidized at C7, the toxicity of oxysterols oxidized at C4 is in the following range of order: 7KC ≥ 7β-OHC > 7α-OHC > (4β-OHC ≥ 4α-OHC). Interestingly, to date, 4β-OHC and 4α-OHC are the only oxysterols identified with cytostatic properties suggesting that these molecules, whereas not cytotoxic, may have some interests to counteract cell proliferation.

1. Introduction

Cholesterol is an important structural element of cell membranes and it is also an essential substrate for biosynthesis of numerous molecules such as bile acids, steroid hormones and neurosteroids, which are potent and effective neuromodulators that are synthesized from cholesterol in the brain [1–5]. Moreover, the cholesterol molecule is easily oxidized and may be transformed into numerous oxidation products known as oxysterols. Thus, oxysterols are 27-carbon-atom cholesterol oxidation products which can be produced endogenously by enzymatic reactions or by autoxidation [6–8]. They also can be provided by food. The enzymatic pathways can form both A-ring, B-ring and side-chain hydroxylated oxysterols depending on the enzyme and the tissue, while the non-enzymatic pathways form mainly B-ring oxysterols [8,9]. In human circulation, the quantitatively dominating oxysterols are 27-hydroxycholesterol, 24-hydroxycholesterol, 7α-hydroxycholesterol and 4β-hydroxycholesterol (4β-OHC) [10,11].

To date, there are some evidences that oxysterols contribute to the regulation of numerous biological activities including cholesterol homeostasis, inflammation, cell differentiation, and proliferation, and that they are also involved in the development of different pathologies such as osteoporosis, age-related macular degeneration, cardiovascular and neurodegenerative diseases like Alzheimer, Parkinson, and multiple sclerosis [12,13]. The oxysterols

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also participate in the Hedgehog and wnt signaling pathways [14,15]. They are also important components of lipid rafts, and potent activators of associated metabolic pathways [16–18]. The ability of some oxysterols, such as 7β-hydroxycholesterol, to induce cell death on different cancerous cells [12,19,20] leads to design new anti-tumoral drugs, and to precise the roles of these molecules on different types of cancer cells [20–23]. On the basis of the oxidation state on rings A and B of the sterol nucleus, and on the oxygenated groups known to be essential for cytotoxicity in natural occurring oxysterols, synthesized oxysterols were evaluated for cytotoxicity in several cancer and noncancerous cells [24,25]. Among these molecules, 4β-hydroxycholesterol was studied and minor cytotoxic effects were observed on HT-29 cells deriving from human colorectal adenocarcinoma [25]. In vivo, 4β-hydroxycholesterol is formed from cholesterol by cytochrome P4503A4 (CYP3A4) and cytochrome P4503A5 (CYP3A5) [26,27] while its isomer 4α-hydroxycholesterol would be produced by cholesterol autoxidation [27,28]. The cytotoxic activity of 4β-hydroxycholesterol, which is a potent Liver X Receptor α (LXRα) and Liver X Receptor β (LXRβ) agonist [29,30], and which can therefore contribute to the regulation of numerous genes [31], remains however not well known on normal or tumoral cells of the central nervous system (CNS), which can constitute potential targets of these compounds when the blood brain barrier is altered either in pathological conditions or under the action of different physical or chemical treatments.

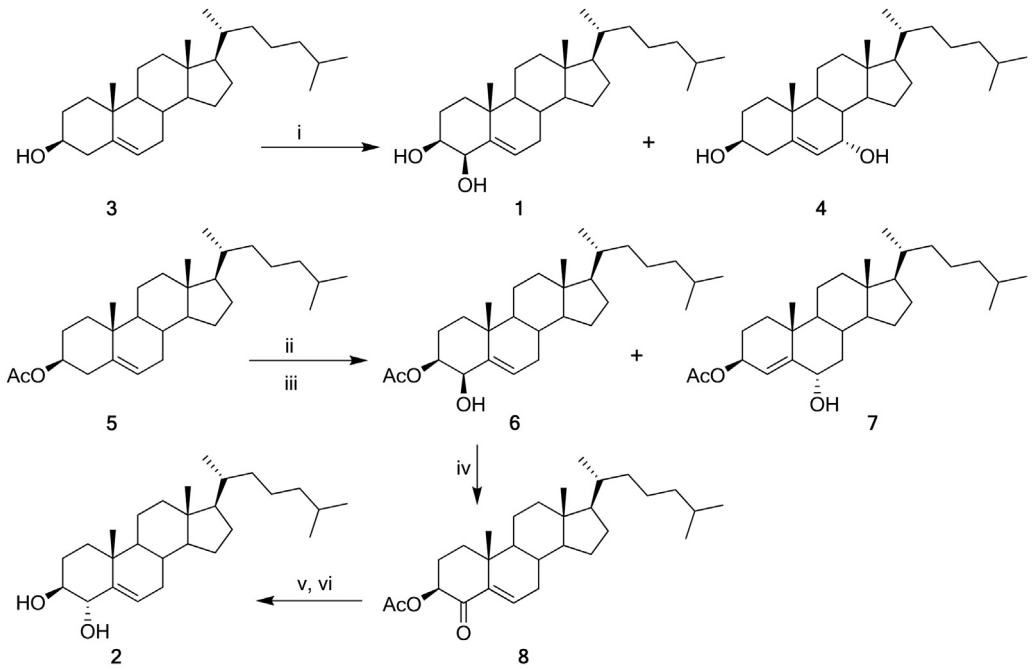
Therefore, in the present investigation an improved methodology is describe to synthetize 4β-hydroxycholesterol (4β-OHC), and its isomer 4α-hydroxycholesterol (4α-OHC), and the ability of these molecules to induce cell death and to modulate cell proliferation was studied on different immortalized, tumoral and normal cells of the CNS (158N murine oligodendrocytes immortalized with the SV40 large T-antigen [32,33], C6 rat glioma cells, SK-N-BE human neuroblastoma cells, and mixed murine primary culture of glial cells (astrocytes and oligodendrocytes)). In those conditions, the effects of 4β-OHC and 4α-OHC were compared with those of oxysterols known to have strong cytotoxic activities: 7-ketocholesterol (7KC) and 7β-hydroxycholesterol (7β-OHC) [12,13].

As the biological activities of these compounds are not well known [30], and as there is a growing interest for oxysterols and oxysterol derivatives for cancer treatment [34], the effects of 4β-OHC and 4α-OHC on cell viability and cell growth were determined on various normal and tumoral cells of the CNS comparatively to oxysterols oxidized at C7. Indeed, 7β-OHC is a potent inducer of cell death on C6 rat glioma cells [20]. Noteworthy, at the opposite of oxysterols oxidized at C7 (mainly 7KC and 7β-OHC), and in agreement with data obtained on other cell types [19,24,25], our study does not show cytotoxic activities (induction of cell death associated with reduced cell growth) of 4β-OHC and 4α-OHC on immortalized, tumoral, and normal cells of the CNS used. However, it reveals cytostatic activities (absence of cell death associated with reduced cell growth) in a range of concentration from 5 to 160 μM, especially on 158N and C6 cells, and no effect on normal glial cells, suggesting that 4β-OHC and 4α-OHC may have some interests to counteract cell proliferation.

2. Results and discussion

2.1. Chemistry

The reaction of SeO_2 with cholesterol was reported to produce 4β-hydroxycholesterol as the only product [35,36]. Recently, Ghosh et al. have reported that the oxidation of cholesterol using excess of SeO_2 produced the 4β-hydroxycholesterol **1** and 3β,4β,7α-



i: 1.4 equiv SeO_2, $CHCl_3$, reflux, 48 h (62% for **1** and 17% for **4**); ii: 1.4 equiv SeO_2, $CHCl_3$, reflux, 24 h, (50% for **6** and 25% for **7**); iii: 1.4 equiv SeO_2, 3 equiv N-methylmorpholine, $CHCl_3$, 70°C, 48 h, (60% for **6** and 5% for **7**; iv: 0.05 equiv TPAP, 1.5 equiv NMO, MS 4 Å, CH_2Cl_2, rt, 10 h, (90%), v: 2 equiv $NaBH_4$, THF-MeOH, 0°- rt, 1h: vi: 2 equiv K_2CO_3, MeOH 24h (80%) two steps from **8**.

(**1**): 4β-hydroxycholesterol ; (**2**): 4α-hydroxycholesterol

Fig. 1. Synthesis of ring B oxygenated sterols at C4 position: 4α-hydroxycholesterol (4α-OHC) and 4β-hydroxycholesterol (4β-OHC).

dihydroxycholesterol [37]. In our hands, treatment of cholesterol with SeO_2 (1.5 equiv) in $CHCl_3$ produced 4β-hydroxycholesterol **1** and the 7α-hydroxycholesterol [38] **4** in 62% and 17%, respectively. It is important to mention that the formation of compound **4** from cholesterol by SeO_2 has not been reported previously. In contrast, treatment of cholesteryl acetate **5** with the SeO_2 furnished 4β-hydroxylated **6** and 6α-hydroxylated **7** in 50% and 25% respectively [39–41].

Surprisingly, treatment of cholesteryl acetate **5** with SeO_2 in the presence of *N*-methylmorpholine ($pK_b = 7.38$) at 70 °C decreases the formation of compound **7** to 5% and increases the amount of compound **6** to 60% yield, whereas no reaction occurred in the presence of triethylamine ($pK_b = 11$). These results show that the formation of **7** is dependent on the reaction medium (SeO_2, $pK_a = 2.62$), and it seems that the formation of 6α-hydroxylated **7** was produced from 4β-hydroxylated **6** rather than by oxidation of cholesteryl acetate **5** with SeO_2 (the mechanism of the formation of 6α-hydroxylated **7** from cholesteryl acetate **5** is currently under study in our laboratory).

Finally, the 4α-hydroxycholesterol **2** was obtained as followed: catalytic oxidation of compound **6** with tetrapropylammonium perruthenate (TPAP) [42] in the presence of 4-methylmorpholine *N*-oxide (NMO) afforded the expected ketone **8** in very good yields, which was reduced selectively by $NaBH_4$ and in situ hydrolysis of the remaining acetate gave the 4α-hydroxyl cholesterol **2** [43] (Fig. 1).

2.2. Structure activity relationship on cell viability and cell growth

On the basis of the oxidation state at C4 and C7 on ring A and B, respectively, of the sterol nucleus and on the oxygenated groups known to be essential for the side effects of different oxysterols, various oxysterols were evaluated for their cytotoxicity (effects on cell viability and cell growth). The purpose of this study was to evaluate the effects of 4α-OHC and 4β-OHC, which are not well known, comparatively to oxysterols with well described biological activities (7KC, 7β-OHC, and 7α-OHC) [13] on cells of the CNS.

Therefore, to evaluate the impact of the hydroxyl at C4, and the incidence of its position (α or β), a panel of immortalized, tumoral and normal cells of the CNS was used: murine oligodendrocytes 158N immortalized with the SV-40 large T-antigen [44]; C6 rat glioma cells; SK-N-BE human neuroblastoma cells; mixed murine primary culture of astrocytes and oligodendrocytes. The neuroblastoma derived cell line (SK-N-BE) was chosen because of the role of cholesterol and of a particular oxysterol, 24S-hydroxycholesterol, in the normal brain cellular functions and also because of the influence of oxysterol imbalance in neurodegenerative processes [45]. Moreover, neuroblastoma as well as glioma (taken in consideration in the present study with the use of C6 rat glioma cells) are common malignancies in childhood [46] with generally low cure rates due to inefficient therapies as a result of the impermeable specific characteristics of the blood brain barrier. Oxysterols, with amphiphilic properties and rapid exchange rates between membranes are expected to cross easily the blood brain and the brain hematotumoral barriers being potentially useful as a chemotherapeutic alternative for neuroblastoma and glioma treatment. Murine oligodendrocytes (158N) cells with some characteristics of well differentiated oligodendrocytes [44], and mixed primary culture of astrocytes and oligodendrocytes were used as model of noncancerous cells to take in consideration the potential side effects on myelin synthesis (oligodendrocytes are myelin synthetizing cells) and on cholesterol homeostasis, which is tightly regulated in the neurons via tight connections with the astrocytes [45].

On transformed and tumoral cells (158N cells, C6 cells, SK-N-BE cells), under treatment with 7β-OHC and 7KC, as previously observed on different cells of the vascular wall [13], the most potent effects, both on cell viability and cell growth, were observed with 7KC and 7β-OHC, and at a lower extend with 7α-OHC (Figs. 2–4A–C; Figs. 2–4F–H). Interestingly, 7α-OHC which is not toxic on human monocytic leukemia cells (U937) [47] was cytotoxic on 158N and C6 cells but not on SK-N-BE cells. Previously, 7β-OHC was also reported to induce cell death on the human neuroblastoma bone marrow derived cell line (SH-SY5Y) [25]. Noteworthy, under treatment with 4α-OHC and 4β-OHC, no effect on cell viability, and more or less pronounced effects on cell growth were found on 158N and C6 cells in a range of concentrations from 5 to 160 μM (Figs. 2–4 DE; Figs. 2–4 IJ), supporting a potential cytostatic activity of these molecules (Table 1). These later data are in agreement with those obtained on HT-29 human colon carcinoma cells and ARPE-19 human retinal pigmentary epithelial cells [24,25]. On mixed primary culture of murine glial cells (astrocytes and oligodendrocytes), no effects of 7α-OHC, 4α-OHC and 4β-OHC (used at 50 μM; in the range of the LD50 and IC50 of 7β-OHC) were revealed whatever the cell type considered, whereas pronounced cytotoxic effects were detected with 7β-OHC (marked decrease of cell viability and inhibition of cell growth), and at a lower extend with 7KC (Fig. 5A and B).

Overall, our data underline that the hydroxyl substrate in C7, on ring B of the sterol nucleus, provides the highest cytotoxicity, even on immortalized and normal glial cells calling thus into question its selective cytotoxicity [25,48] (Table 1). They also show that hydroxyl position at C4, on ring A of the sterol nucleus, has no effect on cell viability, and either more or less pronounced effects on cell growth of immortalized or tumoral cells (158N and C6 cells, respectively), or no effects on cell proliferation of normal glial cells (astrocytes, oligodendrocytes) (Table 1). Noteworthy, to date, 4β-OHC and 4α-OHC are the only oxysterols identified with cytostatic properties suggesting that these molecules, whereas not cytotoxic, may have some interests to counteract cell proliferation. As 4β-OHC is a quantitatively dominating oxysterol in human circulation [10,11], it is suggested that it could be involved in the control of cell proliferation. In addition, as 4β-OHC has no cytotoxic effects, whatever the cell type considered, whereas it is a potent LXRα and LXRβ agonist [29,30], this oxysterol or some of its derivatives may have some pharmacological interests to trigger LXRα and/or LXRβ associated metabolic pathways without interference on cell viability.

3. Conclusions

We described and validated a new methodology for 4α-OHC and 4β-OHC synthesis, and we bring new information establishing the absence of effects of these oxysterols on cell viability as well as more or less pronounced effects on cell growth (cytostasis) of immortalized and tumoral cells, and no effect on normal cells of the CNS. The cytostatic properties of 4α-OHC and 4β-OHC may have some pharmacological interests.

4. Experimental methods

4.1. Materials and general methods

7-Ketocholesterol (7KC) was from Sigma–Aldrich (St. Louis, MO, U.S.A.). The following oxysterols, 7β-hydroxycholesterol (7β-OHC) and 7α-hydroxycholesterol (7α-OHC) were either from Sigma–Aldrich or a generous gift from Prof. M. Samadi (Département de Chimie, LCP – A2MC, Université de Lorraine, Metz, France). The

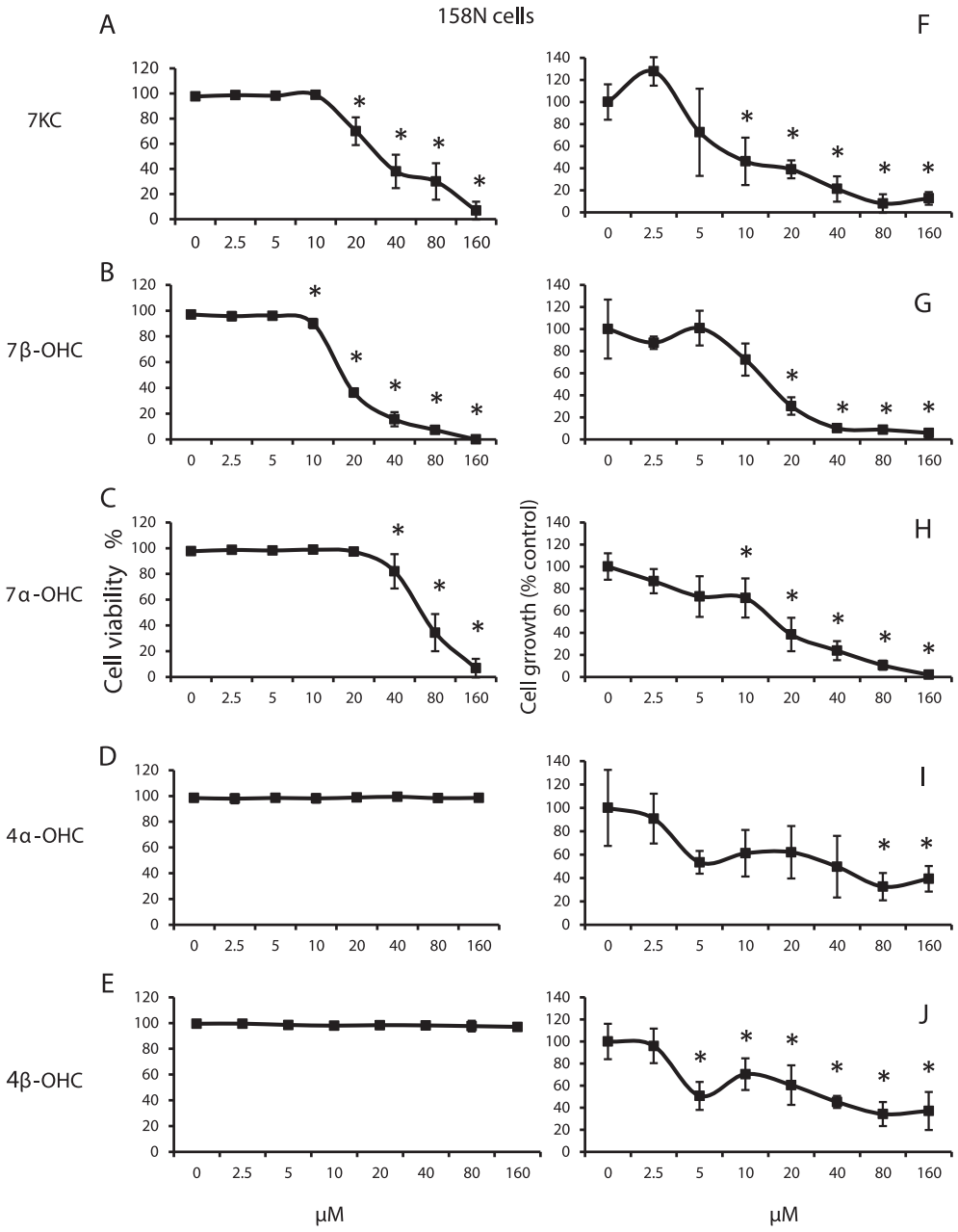


Fig. 2. Effects of 7-ketocholesterol (7KC), 7-β-hydroxycholesterol (7β-OHC), 7α-hydroxycholesterol (7α-OHC), 4α-hydroxycholesterol (4α-OHC) and 4β-hydroxycholesterol (4β-OHC) on cell growth and viability of 158N murine oligodendrocytes cultured for 24 h in the absence or presence 7KC, 7β-OHC, 7α-OHC, 4α-OHC, and 4β-OHC in a range of concentrations from 2.5 to 160 μM. Cell growth and viability were determined by cell counting in the presence of trypan blue. Data shown are mean ± SD from three independent experiments. Significance of the difference between untreated- and oxysterol-treated cells (Mann–Whitney test; $^{*}P < 0.05$ or less). No difference was observed between absolute control and vehicle (ethanol: 0.05–0.32% corresponding to each oxysterol concentration).

purity of these oxysterols was determined to be 100% by gaseous phase chromatography–mass spectrometry.

All of the reactions leading to the synthesis of 4α-hydroxycholesterol (4α-OHC) and 4β-hydroxycholesterol (4β-OHC) were carried out under an argon atmosphere. All reagents were obtained from commercial suppliers and used without further purification. Flash chromatography was carried out using silica gel 60 F_{254} (Merck) with mixtures of ethyl acetate and hexane as eluent unless9 specified otherwise. TLC analyses were performed on thin layer analytical Plates 60 F254 (Merck). Melting points were measured on a Reichert Kofler Thermopan apparatus and are uncorrected. ^{1}H and ^{13}C NMR spectra were recorded with a Bruker Advance 250 spectrometer. High-resolution mass spectra (HRMS) were taken in electron ionization (EI) mode on Jeol GCmate.

*4.1.1. The general procedure for the synthesis of **1**, **4**, **6** and **7** was as follows*

To a solution of cholesterol or cholesterol acetate (3 mmol) in $CHCl_3$ (30 mL) was added SeO_2 (4.2 mmol). The mixture was heated at reflux for 48 h for **3** and 24 h for **5**. The reaction mixture was then cooled and activated carbon was added and then filtered off. The solvent was evaporated under reduced pressure and the residue was purified over silica gel using EtOAc–hexane as eluent.

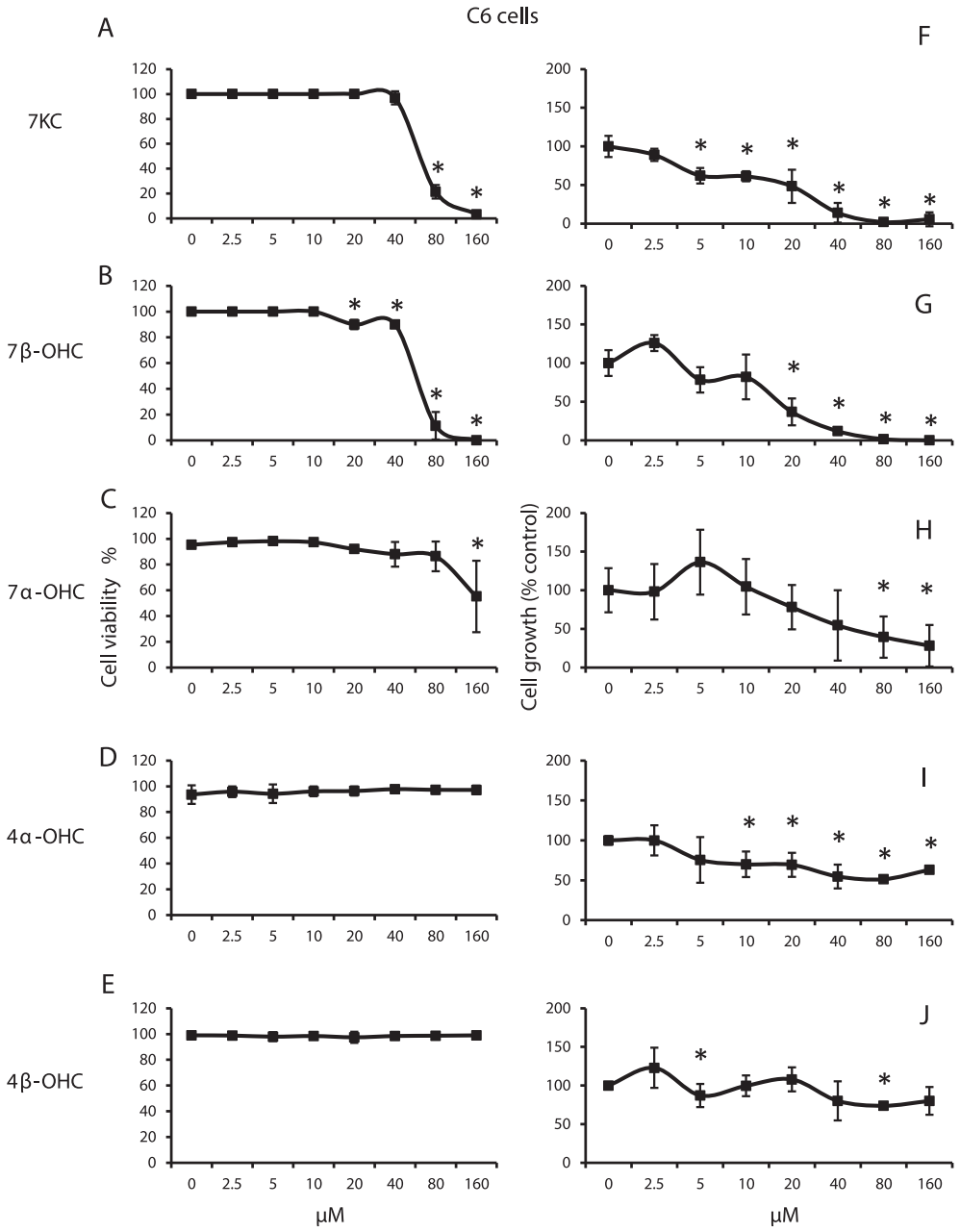


Fig. 3. Effects of 7-ketocholesterol (7KC), 7-β-hydroxycholesterol (7β-OHC), 7α-hydroxycholesterol (7α-OHC), 4α-hydroxycholesterol (4α-OHC) and 4β-hydroxycholesterol (4β-OHC) on cell growth and viability of C6 rat glioma cells cultured for 24 h in the absence or presence 7KC, 7β-OHC, 7α-OHC, 4α-OHC, and 4β-OHC in a range of concentrations from 2.5 to 160 μM. Cell growth and viability were determined by cell counting in the presence of trypan blue. Data shown are mean ± SD from three independent experiments. Significance of the difference between untreated- and oxysterol-treated cells (Mann–Whitney test; $^{*}P < 0.05$ or less). No difference was observed between absolute control and vehicle (ethanol: 0.05–0.32% corresponding to each oxysterol concentration).

*4.1.2. Cholest-5-ene-3β,4β-diol (**1**) (also named 4β-hydroxycholesterol (4β-OHC))*

Purified over silica gel using EtOAchexane (50–50). White solid (62% yield), mp 170–172 °C, IR (KBr): *ν*: 3411, 2940, 1659 cm^{-1}. ^{1}H NMR ($CDCl_3$): *δ* 5.64 (dd, *J* = 5.0, 2.0 Hz, 1H, 6-H), 4.1 (d, *J* = 4.0 Hz, 1H, 4α-H), 3.52 (m, 1H, 4-H), 1.18 (s, 3H, 19-CH_3); 0.91 (d, *J* = 6.5 Hz, 3H, 21-CH_3); 0.87 (d, *J* = 6.5 Hz, 6H, 26-CH_3 and 27-CH_3); 0.68 (s, 3H, 18-CH_3) ppm. ^{13}C NMR ($CDCl_3$) *δ* 142.9; 128.9; 77.4; 72.6; 57.0; 56.2; 50.3; 42.4; 39.8; 39.6; 37.0; 36.3; 36.1; 35.9; 32.2; 31.9; 28.3; 28.1; 25.5; 24.4; 23.9; 22.9; 22.7; 21.1; 20.6; 18.8; 12.0 ppm. HRMS (EI): *m*/*z* calcd for $C_{27}H_{46}O_2$ [M] 402.3498, found 402.3489.

*4.1.3. Cholest-5-ene-3β,7α-diol (**4**)*

Purified over silica gel using EtOAc–hexane (70–30). White solid (17% yield), mp 187–189 °C; IR (KBr): *ν*: 3603, 3396, 2934, 2867, 1663, 1467, 1383, 1056 cm^{-1}. ^{1}H NMR ($CDCl_3$) *δ* 5.5 (d, *J* = 5.16 Hz, 1H, 6-H), 3.85 (broadt, 1H, 7β-H), 3.57 (m, *J* = 11.0, 5.4 Hz, 1H, 3-H), 0.99 (s, 3H, 19-CH_3), 0.92 (d, 3H, *J* = 6.4 Hz, 21-CH_3), 0.86 (d, 6H, *J* = 6.6 Hz, 26-CH_3 and 27-CH_3), 0.68 (s, 3H, 18-CH_3) ppm. ^{13}C NMR ($CDCl_3$) *δ* 146.2, 123.8, 71.2, 65.3, 55.8, 49.4, 42.2, 42.1, 42.0, 39.5, 39.1, 37.5, 37.4, 37.0, 36.1, 35.7, 31.3, 28.2, 28.0, 24.3, 23.7, 22.8, 22.6, 20.7, 18.7, 18.2, 11.60 ppm. HRMS (EI): *m*/*z* calcd for $C_{27}H_{46}O_2$ [M] 402.3498, found 402.3501.

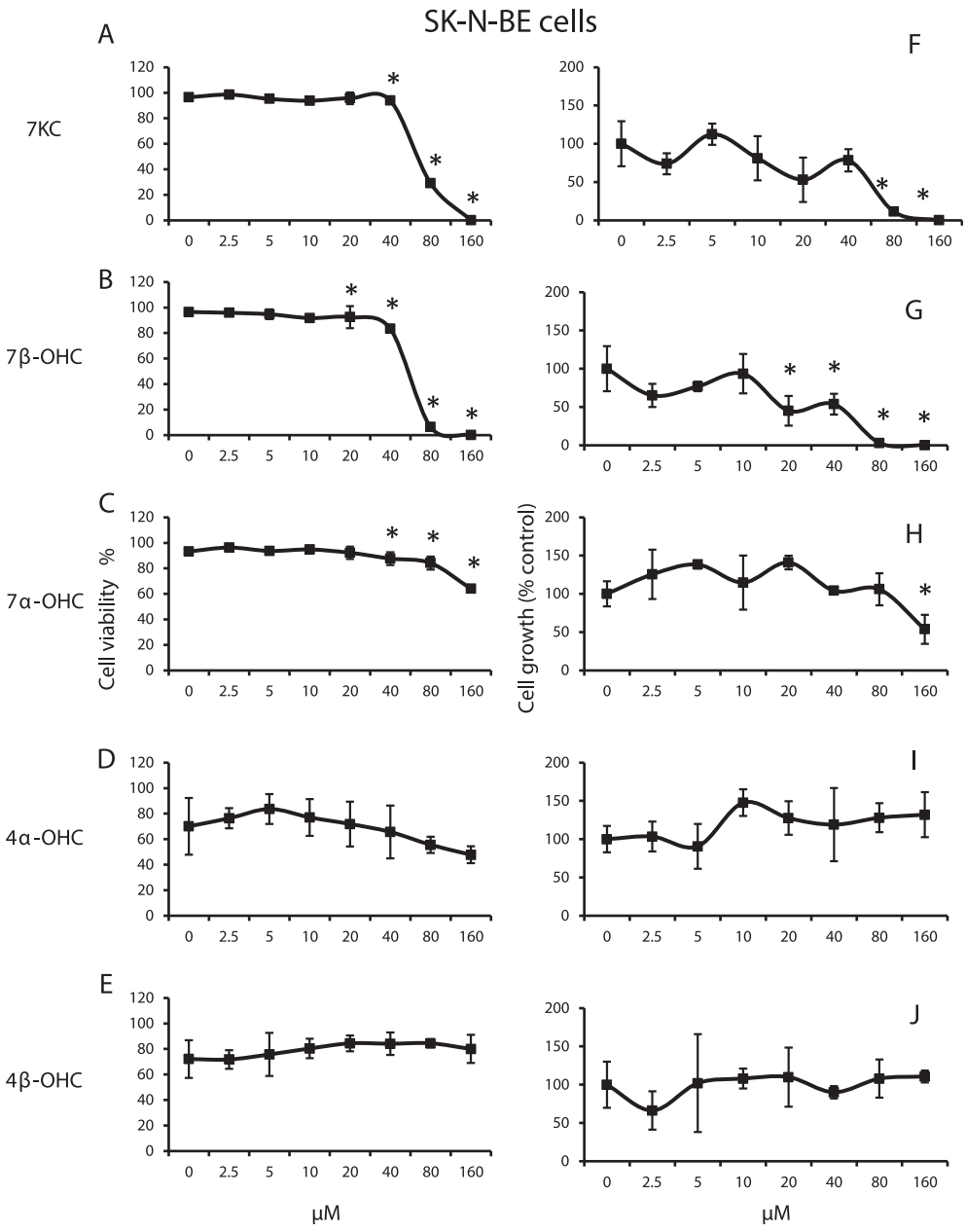


Fig. 4. Effects of 7-ketocholesterol (7KC), 7-β-hydroxycholesterol (7β-OHC), 7α-hydroxycholesterol (7α-OHC), 4α-hydroxycholesterol (4α-OHC) and 4β-hydroxycholesterol (4β-OHC) on cell growth and viability of human neuroblastoma cells (SK–N-BE) cultured for 24 h in the absence or presence 7KC, 7β-OHC, 7α-OHC, 4α-OHC, and 4β-OHC in a range of concentrations from 2.5 to 160 µM. Cell growth and viability were determined by cell counting in the presence of trypan blue. Data shown are mean ± SD from three independent experiments. Significance of the difference between untreated- and oxysterol-treated cells (Mann–Whitney test; $^*P < 0.05$ or less). No difference was observed between absolute control and vehicle (ethanol: 0.05–0.32% corresponding to each oxysterol concentration).

*4.1.4. 3β-Acetoxy-4β-hydroxy-5-cholestene (**6**)*

Purified over silica gel using EtOAc–hexane (20–80). White solid (50% yield), mp 175–176 °C. IR (KBr): *ν*: 3527, 3466, 2954, 1740, 1714 cm^{-1}. ^1H NMR ($CDCl_3$): *δ* 5.62 (d, *J* = 4 Hz, 1H, H-6), 4.65 (td, *J* = 12.6, 4.2. 3.6 Hz, 3-H), 4.17 (d, *J* = 3 Hz, 1H, 4α-H), 2.03 (s, 3H, OCOCH3), 1.15 (s, 3H, 19-CH_3), 0.84 (d, *J* = 6.5 Hz, 3H, 21-CH_3), 0.79 (d, *J* = 6.5 Hz, 6H, 26-CH_3 and 27-CH_3), 0.61 (s, 3H, 18-CH_3) ppm. ^{13}C NMR ($CDCl_3$): *δ* 170.1, 141.5, 129.4, 75.6, 75.4, 56.8, 56.1, 56, 50.2, 42.2, 39.6, 39.5, 36.9, 36.16, 36.14, 32, 31.7, 28.1, 27.9, 24.2, 23.8, 22.8, 22.5, 21.6, 21.3, 21, 20.4, 18.6, 11.8 ppm. HRMS (EI): *m*/*z* calcd for $C_{29}H_{48}O_3$ [M] 444.3604, found 444.3607.

*4.1.5. 3β-Acetoxy-6α-hydroxy-4-cholestene (**7**)*

Purified over silica gel using EtOAc–hexane (30–70). White solid (25% yield), mp 140–142 °C. IR (KBr): *ν*: 3457, 2936, 1742, 1713, 1377, 1263 cm^{-1}. ^1H NMR ($CDCl_3$): *δ* 5.83 (m, *J* = 5.2, 4.8, 3.2 Hz, 1H, 3-H), 5.38 (dd, *J* = 3.2, 0.8 Hz, 1H, 4-H), 3.6 (m, *J* = 12.0, 4.8 Hz, 1H, 6β-H), 2.08 (s, 3H, OCOCH3), 1.18 (s, 3H, 19-CH_3), 0.91 (d, *J* = 6.6 Hz 3H, 21-CH_3), 0.87 (d, *J* = 6.6 Hz 6H, 26-CH_3 and 27-CH_3), 2.08 (s, 1H), 0.68 (s, 3H, 18-CH_3) ppm. ^{13}C NMR ($CDCl_3$): *δ* 171.2, 138.7, 131.5, 79.2, 71.7, 56.7, 56, 501, 42.2, 39.6, 39.4, 36.8, 36.1, 35.9, 35.7, 32, 31.6, 28.1, 28, 25.7, 24.2, 23.8, 22.8, 22.5, 21.6, 20.5, 20.3, 18.6, 11.8 ppm. HRMS (EI): *m*/*z* calcd for $C_{29}H_{46}O_2$ [M − 18] 426.3498, found 426.3497.

Table 1
Effects of oxysterols oxidized at C7 and C4 on cell viability and cell growth of different cell types of the central nervous system.

	Cells			
Oxysterols	Murine oligodendrocytes **158N**	Rat glioma **C6**	Human neuroblastoma **SK-N-BE**	Mixed murine primary culture **(astrocytes / oligodendrocytes)**
7KC	**LD50** (around 30 μM) **IC50** (around 10 μM) **- Marked effect on cell viability** **- Marked cell growth inhibition** **Cytotoxic**	**LD50** (around 60 μM) **IC50** = 20 μM **- Slight effect on cell viability** **- Marked cell growth inhibition** **Cytotoxic**	**LD50** (around 60 μM) **IC50** (around 20 μM) **- Slight effect on cell viability** **- Marked cell growth inhibition** **Cytotoxic**	7KC (50 μM) **70.5±9.1% viable cells and reduction of cell growth** **- Decrease of cell viability and cell growth** **Cytotoxic**
7β-OHC	**LD50** (around 15 μM) **IC50** (around 15 μM) **- Marked effect on cell viability** **- Marked cell growth inhibition** **Cytotoxic**	**LD50** (around 60 μM) **IC50** (around 15 μM) **- Slight effect on cell viability** **- Marked cell growth inhibition** **Cytotoxic**	**LD50** (around 55 μM) **IC50** (around 20 μM) **- Slight effect on cell viability** **- Marked cell growth inhibition** **Cytotoxic**	7β-OHC (50 μM) **19.9±16.2% viable cells and reduction of cell growth** **- Marked decrease of cell viability and cell growth** **Cytotoxic**
7α-OHC	**LD50** (around 60 μM) **IC50** (around 15 μM) **- Slight effect on cell viability** **- Marked cell growth inhibition** **Cytotoxic**	**LD50** ≥ 160 μM **IC50** = 40 μM **- No effect on cell viability** **- Marked cell growth inhibition** **Cytotoxic**	**LD50** > 160 μM **IC50** ≥ 160 μM **- No effect on cell viability** **- Slight cell growth inhibition (160 μM only)**	7α-OHC (50 μM) **- No effect on cell viability and cell growth**
4β-OHC	**LD50** > 160 μM **IC50** (around 30 μM) **Cytostasis**	**LD50** > 160 μM **IC50** ≥ 160 μM **Cytostasis**	**LD50** > 160 μM **IC50** > 160 μM **- No effect on cell viability and cell growth**	4α-OHC (50 μM) **- No effect on cell viability and cell growth**
4α-OHC	**LD50** > 160 μM **IC50** (around 40 μM) **Cytostasis**	**LD50** > 160 μM **IC50** ≥ 160 μM **Cytostasis**	**LD50** > 160 μM **IC50** > 160 μM **- No effect on cell viability and cell growth**	4β-OHC (50 μM): **- No effect on cell viability and cell growth**

In agreement with previous investigations [**24, 25, 50**], an oxysterol was considered as a cell death inducer when its lethal dose 50% (LD50) was lower than 50 μM (marked effect on cell viability); it was considered as an inhibitor of cell proliferation when its inhibitory concentration (IC50) was lower than 50 μM (marked cell growth inhibition). Thus, an oxysterol was considered cytotoxic when he was characterized by a marked effect on cell viability and/or a marked effect on cell growth inhibition. An oxysterol was considered as cytostatic when the inhibition of cell proliferation was not associated with a decrease of cell viability.

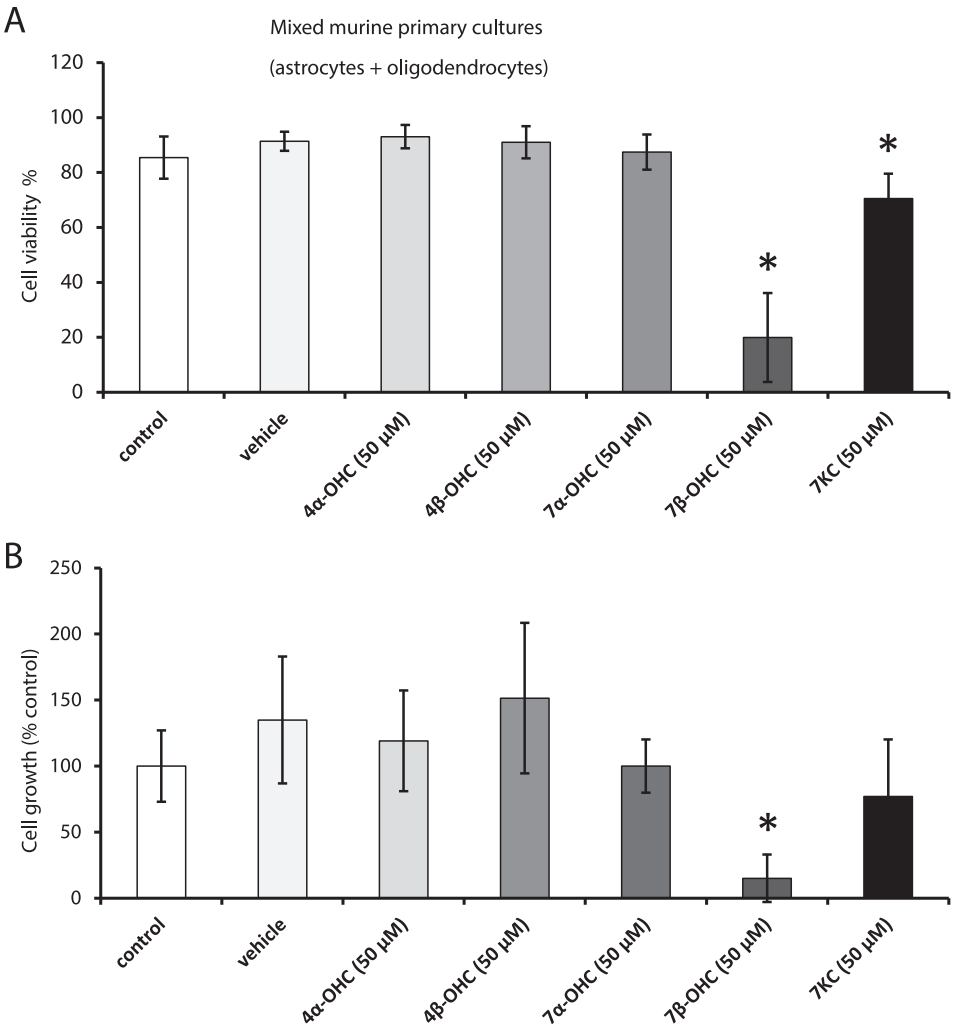


Fig. 5. Effects of 7-ketocholesterol (7KC), 7-β-hydroxycholesterol (7β-OHC), 7α-hydroxycholesterol (7α-OHC), 4α-hydroxycholesterol (4α-OHC) and 4β-hydroxycholesterol (4β-OHC) on cell growth and viability of mixed murine primary culture of astrocytes and oligodendrocytes. After 10 days of culture, murine astrocytes/oligodendrocytes primary cells were cultured for 24 h in the absence or presence 7KC, 7β-OHC, 7α-OHC, 4α-OHC, and 4β-OHC used at 50 μM. Cell growth and viability were determined by cell counting in the presence of trypan blue. Data shown are mean ± SD from three independent experiments. Significance of the difference between untreated- and oxysterol-treated cells (Mann–Whitney test; $^*P < 0.05$ or less). No difference was observed between absolute control and vehicle (ethanol: 0.1%).

*4.1.6. 3β-Acetoxy-cholest-5-en-4-one (**8**)*

TPAP (17.6 mg, 0.05 mmol) was added to a solution of alcohol **5** (1 mmol, 444 mg), NMO (1.5 mmol, 175 mg), and activated Molecular sieves, 4 Å (200 mg) in CH_2Cl_2 (5 mL). The mixture was stirred for 10 h at rt under argon, filtered, concentrated, and purified over silica gel using EtOAc–hexane (10–90) as eluent to yield the keto compound **8** (398 mg, 90%) as a white solid. mp 118–120 °C, IR (KBr): *ν*: 2953, 2867, 1753, 1705, 1692, 1625, 1373, 1225 cm^{-1} ^1H NMR ($CDCl_3$): *δ* 6.35 (dd, *J* = 2.5, 4.7 Hz 1H, 6-H), 5.19 (dd, *J* = 7.2, 12.0 Hz, 1H, 3-H), 2.1 (s, 3H, OCOCH3), 0.97 (s, 3H, 19-CH_3), 0.9 (d, *J* = 6.5 Hz, 3H, 21-CH_3), 0.84 (d, *J* = 6.5 Hz, 6H, 26-CH_3 and 27-CH_3), 0.67 (s, 3H, 18-CH_3) ppm. ^{13}C NMR ($CDCl_3$) *δ* 197.7, 170.1, 144.6, 134.1, 76.1, 56.3, 56, 49.1, 42.3, 39.5, 39,4, 39.1, 36.1, 35.7, 34.5, 31.6, 31, 29.6, 28.1, 27.9, 25.6, 24.1, 23.7, 22.5, 21.3, 21.1, 20.8, 18.6, 11.9 ppm. HRMS (EI): *m/z* calcd for $C_{29}H_{46}O_3$ [M] 442.3447, found 442.3401. Anal. Calc. for $C_{29}H_{46}O_3$: C 78.12, H 10.63. Found. C 78.48, H 10.47.

*4.1.7. Cholest-5-ene-3β,4α-diol (**2**)*

To solution of compound **8** (133 mg, 0.3 mmol) in THF–MeOH (1:1) 5 mL, was added $NaBH_4$ (23 mg, 0.6 mmol) at 0 °C and the mixture was stirred at this temperature for 30 min. After 30 min stirring at room temperature K_2CO_3 (83 mg, 0.6 mmol) was added and the mixture was stirred over night. The solvent was evaporated under reduced pressure and the residue was dissolved in CH_2Cl_2 and the organic layer was washed with water, brine dried over Na_2SO_4 and the solvent was evaporated under reduced pressure. The residue was purified over silica gel using EtOAc–hexane (40–60) to give compound **8** 96 mg (80%) as a white solid. mp 220–222 °C, IR (KBr): *ν*: 3378, 2937, 1670, 1466, 1383 cm^{-1}. ^1H NMR ($CDCl_3$): *δ* 55.53 (dd, *J* = 5.0, 2.0 Hz, 1H, 6-H), 3.77 (dd, *J* = 8.0, 2.0 Hz, 1H, 4β-H), 3.50 (m, 1H, 3-H), 0.95 (s, 3H, 19-CH_3), 0.84 (d, *J* = 6.4 Hz, 3H, 21-CH_3), 0.79 (d, *J* = 6.6 Hz, 6H, 26-CH_3 and 27-CH_3), 0.62 (s, 3H, 18-CH_3) ppm. ^{13}C NMR ($CDCl_3$) *δ* 141.9, 117, 76.5, 75, 56.7, 56.12, 50.4, 42.2, 39.7, 39.5, 38, 36.6, 36.1, 35.7, 31.5, 31.3, 28.2, 28, 27.9, 24.2, 23.8, 22.8, 22.5, 20.8, 20.2, 18.7, 11.8 ppm. HRMS (EI): *m/z* calcd for $C_{27}H_{46}O_2$ [M] 402.3498, found 402.3470.

^1H and ^{13}C NMR spectra of compounds **1**, **2**, **4**, **6**, **7** and **8** are given as Supplementary data.

4.2. Cell cultures and treatments

Murine oligodendrocytes (158N), with some characteristics of differentiated oligodendrocytes [44], were immortalized with the

SV40 large T-antigen derived from Tabby male (Ta/Y) control mice [32,33]. They were seeded at 120,000 cells per well in 12 wells microplates containing 1 mL of culture medium, constituted by Dulbecco's modified Eagle medium (DMEM) (Lonza) supplemented with 5% (v/v) heat-inactivated fetal bovine serum (FBS) (Pan Biotech) and 1% antibiotics (100 U/mL penicillin, 100 mg/mL streptomycin) (Pan Biotech).

Rat glioma cells (C6) were seeded at 250,000 cells per well in 12 wells microplates containing 1 mL of culture medium, constituted by Dulbecco's modified Eagle medium F12 (DMEM F12) (Lonza) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) (Pan Biotech) and 1% antibiotics (100 U/mL penicillin, 100 mg/mL streptomycin) (Pan Biotech).

Human neuroblastoma cells (SK-N-BE) were seeded at 400,000 cells per well in 12 wells microplates containing 1 mL of culture medium, constituted by Dulbecco's modified Eagle medium (DMEM) (Lonza) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) (Pan Biotech) and 1% antibiotics (100 U/mL penicillin, 100 mg/mL streptomycin) (Pan Biotech).

158N, C6, and SK-N-BE cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2, and passaged twice a week. At each passage, cells were trypsinized with a (0.05% trypsin-0.02% EDTA) solution (Pan Biotech).

The conditions of treatment of murine oligodendrocytes 158N cells, C6 rat glioma cells, and SK-N-BE human neuroblastoma cells with the different oxysterols (4β-OHC, 7KC, 7β-OHC, 7α-OHC) were realized as previously defined [49,50]. Initial concentrations of oxysterols (800 μg/mL = 2 mM) were prepared as follows: 1 mg oxysterol + 50 μL absolute ethanol + 1.2 mL culture medium. As 4α-OHC was more difficult to solubilize than the other oxysterols used, initial concentration of 4α-OHC (400 μg/mL = 1 mM) was prepared as follows: 1 mg oxysterol + 100 μL absolute ethanol + 2.4 mL culture medium. Briefly, after plating 158N cells, C6 cells, and SK-N-BE cells for 24 h, cells were further treated for 24 h with various concentrations of these compounds (1–64 μg/mL) corresponding to (2.5–160 μM).

Mixed primary cultures of murine astrocytes and oligodendrocytes, and conditions of treatments were performed as follows [30]. One to two-days-old neonatal BALB/cJRj mice pups (Janvier-Europe) were washed with 70% ethanol and quickly beheaded. Briefly, the brains were removed and placed in a Petri dish containing 10 mL DMEM medium. Meninges and blood vessels were removed by rolling on a sterilized filter paper. The cleaned brains were removed and placed in Petri dish containing 10 mL of fresh medium (DMEM/10% FCS/1% antibiotics). Brain tissue was gently diced into small pieces and crushed on a 100 μm cellular sieve. Cells were harvested in 10 mL of DMEM/10% FCS/1% antibiotics in a 50 mL tube. These 10 mL were used to seed one 12 wells plate (800 μL/well). Primary cultures were incubated in wet atmosphere at 37 °C, 5% CO_2. After 4 days of culture, the medium was renewed, and then changed twice a week.

After 10 days of culture, different treatments were realized. Mixed primary cultures of murine astrocytes and oligodendrocytes were realized in 12 wells plate for 24 h in the absence or in the presence of 4α-OHC, 4β-OHC, 7KC, 7β-OHC, 7α-OHC (50 μM).

4.3. Evaluation of cell viability and cell growth

After trypsinization, cells were centrifuged and resuspended in culture medium. The percentage of viable cells (cell viability % = number of trypan blue negative cells/total number of cells) and cell growth (cell growth (% control) = number of trypan blue negative cells in the assay/number of trypan blue negative cells in the control) were determined with trypan blue under an inverted phase contrast microscope Diaphot (Nikon). These parameters were determined after 24 h of culture in the absence or in the presence of 4α-OHC, 4β-OHC, 7KC, 7β-OHC, and 7α-OHC used in a range of concentrations from 2.5 μM to 160 μM. This range of concentrations was chosen in order to calculate with accuracy the 'lethal dose 50%' (LD50) as well as the half maximal inhibitory concentration (IC50). The lethal dose 50% (LD50) corresponds to the amount of compound required to kill 50% of the test population, and the inhibitory concentration (IC50) corresponds to the concentration required to reduce by 50% the number of viable cells [30]. In agreement with previous investigations [24,25,50], an oxysterol was considered as a cell death inducer when its LD50 was lower than 50 μM; it was considered as an inhibitor of cell proliferation when its IC50 was lower than 50 μM. Thus, an oxysterol was considered cytotoxic when its LD50 was lower than 50 μM and/or when its IC50 was lower than 50 μM; it was considered as cytostatic when the inhibition of cell proliferation was not associated with a decrease of cell viability.

4.4. Statistical analysis

Analyses were carried out with WinSTAT® for Microsoft® Excel (version 2012.1). Since the biological variables were not normally distributed, nonparametric Mann–Whitney test was used. P values <0.05 were considered significant in all tests.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2013.09.028.

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