7632 J. Med. Chem. 2010, 53, 7632–7638
DOI: 10.1021/jm1007769

Journal of Medicinal Chemistry Article

Sterols as Anticancer Agents: Synthesis of Ring-B Oxygenated Steroids, Cytotoxic Profile, and Comprehensive SAR Analysis

João F. S. Carvalho,[†,§] M. Manuel Cruz Silva,[†,§] João N. Moreira,[‡,§] Sérgio Simões,[‡,§] and M. Luisa Sá e Melo*[,†,§]

[†]*Center for Pharmaceutical Studies, University of Coimbra, Portugal,* [‡]*Center for Neuroscience and Cell Biology, University of Coimbra, Portugal, and* [§]*Faculdade de Farmácia, Universidade de Coimbra, 3000-548 Coimbra, Portugal*

Received April 30, 2010

The cytotoxicity of oxysterols was systematically studied in tumor and normal cells. Synthetic strategies to prepare this library included oxidations at ring B and a new method to yield 6β-hemiphthalates directly from Δ^5-steroids. Most oxysterols were cytotoxic and showed selectivity toward cancer cells, LAMA-84 cells (leukemia) being particularly sensitive to **4**, **8**, **22**, and **27** ($IC_{50} < 5.6\,\mu M$). The structural requirements to induce selective toxicity are discussed to shed light on the development of new anticancer drugs.

Introduction

Oxysterols attract much attention in cell biology and pathophysiology because of the wide range of biological phenomena in which they are involved. Oxysterols can be endogenously produced from cholesterol (Chol[a]) by enzymatic and nonenzymatic oxidative processes or absorbed from diet sources.[1]

Enzymatically formed oxysterols include 27-hydroxycholesterol (27-OHChol), 25-OHChol, 24*S*-OHChol, and the oxidation products 4β-OHChol (**1**) and 7α-OHChol (**7**) (Figure 1). They participate in the biosynthesis of bile acids and steroid hormones, acting also as signaling lipids that regulate Chol biosynthesis, cellular Chol efflux, lipoprotein uptake, and intracellular Chol trafficking,[2] and have been related to neurodegenerative diseases, such as Alzheimer, Parkinson, and multiple sclerosis.[1]

Oxysterols formed by nonspecific oxidative mechanisms have been implicated in atherosclerosis, macular degeneration, and osteoporosis.[1] Nonenzymatic oxidations of Chol occur mainly at ring B, producing the widely studied 5α,6α- and 5β,6β-epoxides of Chol (**2** and **3**, respectively), cholestane-3β,5α,6β-triol (**4**), 7-ketoChol (**6**), and 7β-OHChol (**8**) (Figure 1).

Great effort has been devoted to identify cellular receptors for oxysterols and to correlate their role with as yet undisclosed in vivo functions.[3] Much attention has been given to the cytotoxic potential of oxysterols alone,[4] in combination[5,6] or as a component in oxidized low density lipoprotein,[7] against normal cells, like vascular wall and macrophage cells, because of their believed role in the initial stages of atherosclerosis. However, less attention has been devoted to their ability to inhibit tumor cell growth.

Most common oxysterols are known to interfere with the cell membrane structure and cellular receptors,[3] to inhibit Chol[2,8] and DNA biosynthesis,[9] and to induce cell death in different cell lines by apoptosis or necrosis.[1] The exact cellular mechanisms involved are still largely unknown. It has been suggested that inhibition of Chol synthesis[8] may explain the toxicity induced by oxysterols, since Chol is essential for mitosis progression,[10] although other studies show no correlation between these effects.[11]

Literature on the cytotoxic potential of oxysterols provides only scattered information, mainly related to their activity on vascular or macrophage cells. The present work was undertaken as a systematic study to explore the cytotoxic potential of endogenous and non-natural oxysterols. We synthesized a library of oxygenated steroids to study the structural requirements for a steroid molecule to induce cellular toxicity.

Results and Discussion

Chemistry. Diverse polyhydroxysteroids bearing the 3β, 5α,6β-trihydroxy pattern are found in nature, especially in marine organisms, and some of them are cytotoxic.[12] The endogenous triol **4** is also reported as cytotoxic.[13] Therefore, we synthesized a library of 3β,5α,6β-trihydroxysteroids with different side chains and/or group functionalizations in ring D by applying a straightforward methodology reported by us (**4**, **9**−**16**, **18**, and **19**).[14] Stereoselective reduction of **14** with $NaBH_4/CeCl_3$ afforded **17** (Table 1).

Taking advantage of the formation of phthalic acid in situ when Δ^5-steroids react with MMPP,[14,15] we hypothesized that the phthalic acid could act as nucleophile and attack the epoxide affording hemiphthalate derivatives. The reaction of Chol with an equimolar amount of MMPP in acetonitrile at reflux generated, after 1 day, the 3β,5α-dihydroxycholestan-6β-yl hemiphthalate **24** (Table 1), which was isolated in 39% yield by flash chromatography. The 5α- and 6β-acetamido derivatives and the triol **4** were identified as byproducts, and the 5α-yl hemiphthalate was not detected. The 6β-yl hemiphthalates **25** and **26** were synthesized similarly. In summary, for the first time, a one-pot procedure for the preparation of 5α-hydroxy-6β-yl hemiphthalate cholesteryl derivatives from the corresponding Δ^5-olefin was achieved.

*To whom correspondence should be addressed. Phone: +351 239 488 475. Fax: +351 239 488 471. E-mail: samelo@ff.uc.pt.

[a] Abbreviations: Chol, cholesterol; Doxo, doxorubicin; MMPP, magnesium bis(monoperoxyperphthalic) hexahydrate; OHChol, hydroxycholesterol; SI, selectivity index.

 Published on Web 10/08/2010

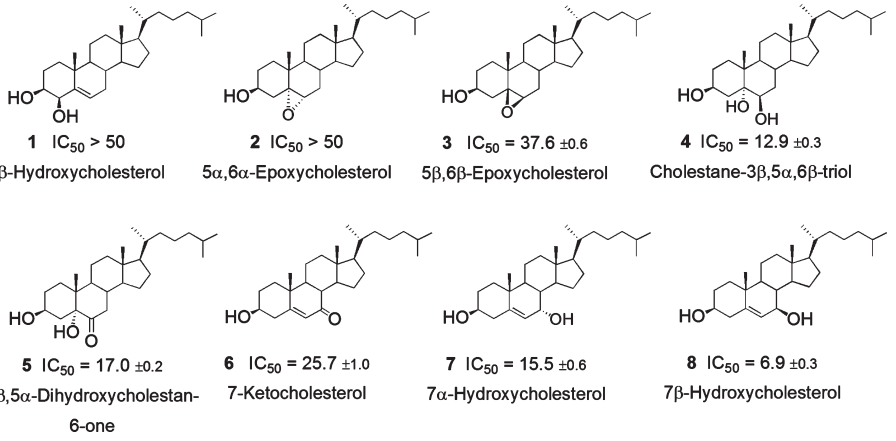
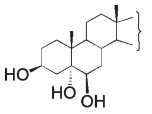
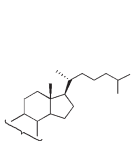
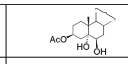
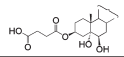
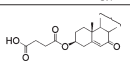
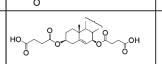
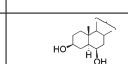
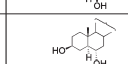
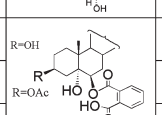
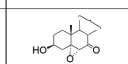
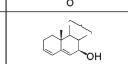
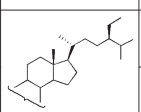
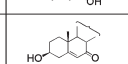
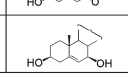


Figure 1. Endogenous ring-B oxysterols. The IC_{50} (μM) was determined on colon cancer HT-29 cells after 48 h.

Table 1. Cytotoxicity of Steroids on the Colon Cancer Cell Line HT-29 (IC_{50} in μM, after 48 h)

3β,5α,6β-Trihydroxysteroids [a]			**IC_{50}**	**Further Synthetic Oxysterols**			**IC_{50}**
	**4**		12.9 ±0.3		**18**		19.4 ±1.2
	9		>50		**19**		18.3 ±0.6
	10		>50		**20**		28.0 ±1.4
	11		>50		**21**		17.6 ±0.9
	12		>50		**22**		12.0 ±0.7
	13		>50		**23**		24.4 ±2.1
	14		>50		**24**		13.5 ±0.5
	15		>50		**25**		17.0 ±0.4
	16		>50		**26**		16.5 ±0.5
	17		>50		**27**		14.2 ±0.3
					28		>50
Cisplatin			13.8 ±0.6		**29**		48.0 ±1.6
Doxorubicin			1.23 ±0.14		**30**		21.4 ±1.1

[a] Synthesis of 3β,5α,6β-trihydroxysteroids through a straightforward methodology described in the literature,[14] with the exception of **17** (see Supporting Information).

The 6-oxo derivative **5** (Figure 1) was accessed by quantitative oxidation of **4** with *N*-bromosuccinimide. The synthesis of the 6β- and 6α-OH derivatives **22** and **23** (Table 1), was achieved in a single reaction by hydroboration of the Chol double bond with the borane−tetrahydrofuran complex reagent in THF at 0 °C, followed by the oxidation with 30% H_2O_2 in 10% NaOH. Although described as affording preferentially the α-hydroxyl isomer, in our hands this hydroboration−oxidation methodology always gave a mixture (~34%) of both isomers.

The Δ^5-7-oxygenated derivatives were prepared by allylic oxidation of cholesteryl or β-sitosteryl acetates by *tert*-butyl hydroperoxide and copper iodide,[16] affording the 7-oxo derivatives in ~50% isolated yields. Alkaline hydrolysis rendered the 3β-OH derivatives **6** (Figure 1) and **29** (Table 1). Reduction of the C7 carbonyl group of the respective 3β-acetoxy-5-ene derivatives with $NaBH_4/CeCl_3$ gave the corresponding 7β-OH derivatives in a stereoselective manner, and alkaline hydrolysis of the 3β-acetate groups afforded the diols **8** (Figure 1) and **30** (Table 1) quantitatively.

Cholesta-3,5-dien-7β-ol (**28**, Table 1) was accessed in a two-step procedure, starting with quantitative 3β-acetate elimination in 7-oxocholest-5-en-3β-yl acetate catalyzed by *p*-toluenesulfonic acid in acetonitrile under reflux followed by stereoselective reduction of the C7 carbonyl, as described above.

The 5α,6α-epoxy-3β-hydroxycholestan-7-one (**27**, Table 1) was synthesized by modifying a known oxidative procedure in aqueous alkaline conditions to overcome substrate solubility problems and to reduce the amount of base used. To a

Table 2. Cytotoxicity (μM) on HT-29 Cells

drug	at 48 h	at 96 h
2	>65	37.8 ± 1.1
3	37.6 ± 0.6	27.5 ± 0.9
4	12.9 ± 0.3	10.3 ± 0.2
8	6.9 ± 0.3	7.2 ± 0.7
18	19.4 ± 1.2	12.4 ± 0.2
24	13.5 ± 0.5	13.2 ± 0.5
25	17.0 ± 0.4	16.7 ± 0.5
26	16.5 ± 0.5	16.1 ± 0.9
27	14.2 ± 0.3	6.9 ± 0.4
pH	7.90[a] → 7.74[b]	7.84[a] → 7.50[b]

[a] pH of the culture medium after noncellular incubation at 37 °C. [b] pH of the culture medium after cellular incubation at 37 °C.

solution of 7-oxocholest-5-en-3β-yl acetate in methanol, an equimolar amount of sodium methoxide and an excess of H_2O_2 were added. After 2 days of stirring at room temperature, **27** was isolated in 20% yield by flash chromatography.

Hemisuccinate derivatives were obtained by direct esterification of Chol and sterols **4**, **6**, and **8** with an excess of succinic anhydride catalyzed by the hindered base *N*,*N*-diisopropylethylamine in dichloromethane under reflux. The reactions afforded quantitatively the cholesteryl hemisuccinate, the oxysterol 3β-yl hemisuccinates **19** and **20**, and the 3β,7β-diyl dihemisuccinate **21** (Table 1). When triol **4** was used as substrate, the 3β,6β-diyl dihemisuccinate derivative was not formed, probably due to steric hindrance of the 6β-OH group.

Biological Evaluation. Cell Proliferation Studies and SAR Correlations. The compounds were evaluated in cultured cells from tumor and nontumor origins, using the Alamar blue assay, as recently described.[16] A period of 48 h of drug exposure was chosen to test cytotoxicity. In some particular cases, a period of 96 h was also tested to disclose significant changes in cytotoxicity over time (Table 2). Cisplatin and doxorubicin (Doxo) were tested in parallel as reference cytotoxic agents.

The human colon cancer cell line (HT-29) was used for screening oxysterols cytotoxicity (Figure 1, Table 1). Several other human tumor cell lines were used, namely, LAMA-84, HepG2, A549, PC3, and MCF-7. Nontumoral cell lines of different histologic origins, ARPE-19, BJ, and HMEC-1 cells, were used as negative controls to give an indication of selectivity for tumor cells (Table 3).

The rationale of this study was to evaluate the influence of the side chain, the oxygenation pattern, and the alcohol conversion into half-esters on the cytotoxicity of the overall steroid molecule. In addition, the combination of an oxysterol with anticancer drugs to potentiate their therapeutic effect was pursued.

Cytotoxicity of Endogenous Oxysterols (Figure 1). The cytotoxicity of eight endogenous oxysterols, **1**−**8**, was evaluated on HT-29 cells. As reported by us,[16] 3β,4β-diol **1** and α-epoxide **2** are not cytotoxic, while β-epoxide **3** is moderately toxic. Triol **4** afforded an IC_{50} of 12.9 μM, and further oxidation to **5** led to a decrease in cytotoxicity. Concerning C7 oxygenated sterols, 7β-OHChol (**8**) was the most potent, followed by its 7α-counterpart **7**, while the 7-oxo analogue **6** was less potent. Although the cytotoxicity of oxysterols has been reported, herein we have evaluated eight endogenous oxysterols under the same experimental conditions, allowing for a direct comparison of their anticancer potential.

Influence of Structural Variations at Side Chain and at Ring D (Table 1). Several 3β,5α,6β-trioxygenated steroids with different side chains (**4** and **9**−**19**) were screened against HT-29 cells. Triol **4**, its 3β-acetyl (**18**), and 3β-hemisuccinyl (**19**) derivatives are cytotoxic. The presence of oxygenated substituents at C17 (**14** and **17**), at C20 (**11**−**13**), or at C16 (**15** and **16**) does not afford significant cytotoxicity (IC_{50} > 50 μM). Accordingly, we conclude that the saturated side chain of Chol is required for cytotoxicity. The C24 ramification of the Chol side chain (**9**), a structural characteristic of abundant phytosterols, and its C22 unsaturation (**10**) led to a significant decrease in activity.

Influence of the Oxygenation Pattern on Ring B: Studies on Synthetic Derivatives (Table 1). Oxysterols **4** and **8** display noteworthy activity toward HT-29 cells, with IC_{50} similar to that of cisplatin. Hence, these molecules were used as templates for further modifications, affording related compounds that were evaluated in HT-29 cells.

The importance of the 5α-OH in **4** was assessed by preparing **22**, and the importance of the stereochemistry of the C6-OH was evaluated by preparing **23**. The results obtained show that the 5α-OH is not crucial for activity while the 6β-alcohol is 2-fold more potent than its 6α-isomer.

To assess the importance of the 3β-OH, we have prepared **28** devoid of this substituent but still bearing the Δ^5-7β-hydroxylation pattern. This compound was not cytotoxic and actually induced cell proliferation by 30% compared to control cells at 50 μM (data not shown).

Moderately cytotoxic 7-ketoChol **6** was converted by epoxidation into **27**, which was almost 2-fold more toxic. Therefore, the α-epoxide when coupled with a C7 carbonyl group increased cytotoxicity.

Hemisuccinate or hemiphthalate derivatives were prepared to confer acidity to the oxysterol and ultimately to improve their cellular uptake in the acidic microenvironment of the tumor.[17] The synthesis of hemisuccinate and hemiphthalate derivatives will also help to compensate for the lipophilicity of oxysterols, potentially improving their druggability. No cytotoxicity was observed for cholesteryl hemisuccinate at 50 μM on HT-29 or A549 cells (data not shown). However, the hemisuccinate derivatives **19** and **20** were cytotoxic to almost the same extent as the sterol counterparts **4** and **6**. Thus, at physiological pH, the 3β-hemisuccinates afford more hydrophilic compounds, as observed by TLC, without significant loss of cytotoxicity.

Concerning the 3β,7β-bis-hemisuccinate **21**, a 2-fold decrease in toxicity against HT-29 and LAMA-84 cells (data not shown, IC_{50} = 7.5 μM) was noticed, compared to the nonesterified diol **8**. This lower activity may be explained by the blockage of the 7β-OH group.

Ester synthesis can also be useful to introduce large polar groups in the sterol nucleus. The presence of a polar substituent at ring B, like a OH group, is essential for the destabilization of the cellular membrane[18] and for cytotoxicity. To further explore the presence of membrane destabilizing groups, 6β-hemiphthalates were synthesized. The cytotoxic effects of **24**−**26** on HT-29 cells reveal that the introduction of a 6β-hemiphthaloyl moiety does not change substantially the cytotoxicity of the parent compounds, bearing a free 6β-OH (**4**, **18**, and **19**).

Besides the sitostane triol **9**, we have also synthesized the sitostanes **29** and **30**. The presence of a 24β-ethyl group led to a significant decrease in cytotoxicity when compared with the cholestane counterparts **6** and **8**. Additionally, in contrast to cholestanes, the 7-keto functionalization allows better cytotoxicity than the 3β,5α,6β-trihydroxy substitution on sitostane derivatives.

Comparison of the Cytotoxic Activities at 48 and 96 h (Table 2). To gain new insights into the cytotoxic potential

of oxysterols, we evaluated the IC_{50} of the most cytotoxic oxysterols found, **4** and **8**, and of a selection of oxysterols susceptible to hydrolysis, specifically, epoxides **2**, **3**, and **27** and esters **18**, **24**, **25**, and **26**, at two incubation periods.

The cytotoxicities of triol **4**, diol **8**, and the 6β-hemiphthaloyl esters **24**−**26** were almost the same at 48 and 96 h, whereas the toxicities of epoxides **2**, **3**, **27** and of the acetoxy derivative **18** improved with incubation time. The increased cytotoxicity of the 3β-acetoxy **18** after 96 h of incubation, resembling the activity of the triol **4**, suggests hydrolysis of the acetate over time. The toxic effects of the 6β-hemiphthaloyl esters **24**, **25**, and **26** at 48 and 96 h indicate that the activities are due to the overall molecules and not to the formation of the hydrolytic products **4**, **18**, and **19**, respectively, revealing new chemical entities. In addition, 3β-acetyl and 3β-hemisuccinyl phthalates **25** and **26** present constant toxicities over time, which does not resemble the activity of the 3β-OH analogue **24**. Therefore, at least in this case, no hydrolysis of the 3β-esters occurs, potentially because of the steric hindrance imposed by the bulky hemiphthalate substituent at the active site of a hydrolytic enzyme.

To reveal the type of hydrolysis, chemical or enzymatic, and to study the stability of oxysterols in the biological fluids and tissues, we monitored the pH drift over time of the culture medium incubated with and without cells (Table 2, pH) and incubated the compounds in aqueous solutions at different pH (7.4, 6.7, 5.7, 4.6, and 3.0) for 4 days at 37 °C, as outlined in Table S1 (Supporting Information). pH 6.7 and pH 4.6 were selected to resemble the pH of in vivo media, specifically, the extracellular media of solid tumors and the acidic lysosomes of cancer cells, respectively, where oxysterols are hypothetically hydrolyzed and deactivated.

Regarding acetoxy **18**, at physiological pH, only traces of the triol **4** were found (Table S1, Supporting Information). Hence, if hydrolysis occurs during incubation, it should be metabolic. Moreover, the fact that other cells tested, A549, PC3, and ARPE-19 cells, presented significantly less sensitivity for **18** ($IC_{50} \geq 50\ \mu M$; data not shown) reinforces the hypothesis of cell specific metabolic process instead of chemical hydrolysis.

We have previously studied epoxy steroids and found that their cytotoxicity is dependent on the position and stereochemistry of the epoxide and on the presence of additional hydroxyl substituents.[16] Epoxides are reactive species that under aqueous acidic conditions can afford hydrolytic products, which can be more cytotoxic. Therefore, epoxysterols can act as prodrugs with increased cytotoxicity against cancer cells in vivo because of the decreased extracellular pH in solid tumors.[17] The cytotoxicity of epoxides **2** and **3** increases over time. This may result from epoxide hydrolysis affording the more active triol **4**. Accordingly, we studied the compounds' stability in aqueous solutions at 37 °C and verified that at physiological pH, the β-epoxide **3** was slightly hydrolyzed whereas the α-epoxide **2** proved to be resistant to hydrolysis, even at lower pH values (Table S1, Supporting Information).

However, the in vitro results after 96 h of incubation for the α-epoxide **2** showed a greater increase in cytotoxicity (> 1.7-fold) than for the β-epoxide **3** (∼1.4-fold), suggesting that hydrolysis was enzymatic rather than chemical. In fact, the ubiquitous Chol epoxide hydrolase has been reported to display a 5-fold preference for the Chol α-epoxide relative to the β-epimer[19] and could thus represent a biological target for prodrug delivery.

Interestingly, a 2-fold increase in cytotoxicity was found for the epoxide **27** at 96 h. Because of the hydrolytic stability of this epoxide at pH 7.4, 6.7, and 4.6 (Table S1), we attribute such increment to metabolic transformation with the possible involvement of Chol epoxide hydrolase.

Cytotoxicity of Selected Oxysterols and Derivatives toward a Panel of Cell Lines (Table 3). The results on HT-29 cells (Figure 1, Tables 1 and 2) prompted the selection of some of the best cytotoxic molecules for further studies in a panel of cancer and noncancer cell lines.

Endogenous oxysterols **4** and **8**, with promising cytotoxicity in HT-29 cells, were studied in five additional cancer cell lines and three normal cell lines. The IC_{50} values found in the different cells are similar for both compounds, with cancer cells being more sensitive than the normal ones except for PC3 cells, which are less sensitive to both oxysterols, and for A549, which are less sensitive to triol **4**.

Hemisuccinate **19**, which was also moderately toxic toward HT-29, was in general much more cytotoxic than the 3β-acetate **18** ($IC_{50} \geq 50$, in A549, PC3, and ARPE19 cells), although less cytotoxic than triol **4**.

The introduction of the 3β-hemisuccinate, as in **19**, led to a different cytotoxic profile when compared to the parent triol **4**. It displayed a much more uniform cytotoxicity among the different cells evaluated except for the LAMA-84 cells, decreasing therefore the selectivity toward the cancer cells studied herein (Figure S1 and S2, Supporting Information). It is worth noting that the cytotoxic evaluation was performed at a pH resembling physiological conditions, and at this pH the hemisuccinate is in the charged form and thus less able to attach to membranes, which could explain the slight decrease in cytotoxicity relative to the 3β-OH analogues. In vivo, because of the increased acidification of the tumor extracellular environment, these esters can potentially act as

Table 3. Cytotoxicity of Selected Oxysterols in 8 Human Cell Lines after 48 h of Exposure[a]

	cancer cells					normal cells			
drug	LAMA-84	HepG2	MCF-7	A549	PC3	HMEC-1	BJ	ARPE-19	SI[b]
4	5.0 ± 0.2	10.9 ± 0.3	13.6 ± 0.2	17.9 ± 0.1	17.4 ± 0.5	15.2 ± 0.2	18.6 ± 0.2	19.1 ± 0.3	3.9
8	4.3 ± 0.2	14.6 ± 0.5	8.7 ± 0.3	13.6 ± 0.2	21.9 ± 0.4	19.1 ± 0.2	20.0 ± 0.4	21.6 ± 0.8	5.0
19	7.1 ± 0.4	18.7 ± 0.3	21.3 ± 0.4	20.3 ± 0.6	22.4 ± 0.3	20.7 ± 0.5	26.2 ± 0.2	24.9 ± 0.4	3.5
22	5.6 ± 0.3	15.6 ± 0.4			21.4 ± 0.4			36.0 ± 0.4	6.4
27	4.7 ± 0.2	15.9 ± 0.6	12.0 ± 05	13.2 ± 0.5	13.7 ± 0.8	13.1 ± 0.2	15.8 ± 0.4	15.1 ± 0.2	3.2
30	17.7 ± 0.9	28.0 ± 1.3	44.8 ± 1.3		30.4 ± 1.3	26.7 ± 0.5	21.7 ± 0.8	25.6 ± 1.0	1.4
Cispl	8.1 ± 0.6	9.8 ± 0.7	27.0 ± 1.3	12.0 ± 0.4	15.9 ± 1.3	7.3 ± 0.3	15.7 ± 1.7	26.7 ± 0.9	3.3
Doxo	0.74 ± 0.08	1.54 ± 0.10	0.85 ± 0.04	0.95 ± 0.06	1.63 ± 0.29	0.36 ± 0.04	2.33 ± 0.28	0.76 ± 0.04	1.0

[a] IC_{50} in μM. Cancer cells: HepG2 from liver, LAMA-84 from myeloid leukemia, A549 from lung, PC3 from prostate metastasis, and MCF-7 from breast metastasis. Normal cells: HMEC-1 from microvasculature, ARPE-19 from eye, and BJ from skin. Reference drugs: cisplatin (Cispl) and doxorubicin (Doxo). [b] Selectivity index = $IC_{50,ARPE\text{-}19}/IC_{50,LAMA\text{-}84}$.

interesting hydrophilic weak acid transporters to target cancer cells.[20]

On the other hand, the 6β-hemiphthalates **25** and **26**, which afforded IC_{50} of 17 μM in HT-29 cells, were found to be more cytotoxic toward the normal ARPE-19 cells, specifically with IC_{50} of 16.0 and 10.2 μM (data not shown). Therefore, the 6β-hemiphthaloyl derivatization, although maintaining the cytotoxicity in HT-29 cells and improving solubility, hampers the selectivity for cancer cells.

Noteworthy, 3β,6β-diol **22** was as cytotoxic as triol **4** to tumor cells but almost 2-fold less toxic to normal ARPE-19 cells. Thus, the removal of the 5α-OH renders the oxysterol more selective for cancer cells. Diols **22** and **8** were found to be interesting molecules against colon adenocarcinoma and leukemia cells, since they were about 3- and 5-fold more active on HT-29 and LAMA-84 cells, respectively, compared to ARPE-19 cells.

Epoxide **27**, with an interesting cytotoxicity for HT-29 cells that improved over time (Tables 1 and 2), was similarly cytotoxic toward the other cancer cell lines tested, with LAMA-84 cells being the most sensitive. However, this modification also led to an increased cytotoxicity toward normal cells.

Many studies highlight the beneficial properties of dietary phytosterols, namely, β-sitosterol.[21] In analogy to Chol,[1] the autoxidation or photo-oxidation of phytosterols in foods also results in the formation of 5-, 6- and 7-oxygenated derivatives.[22] Oxyphytosterols have been shown to be cytotoxic in tumor cells,[22] and different apoptotic mechanisms were reported for 7β-hydroxysitosterol **30** and 7β-OHChol **8**.[23] Thus, we investigated the cytotoxicity of the most potent sitostane derivative, diol **30** (Table 1), on the different cancer and normal cells. We found that, in comparison to the cholestane **8**, the presence of a 24β-ethyl group led to a significant decrease in cytotoxicity on all cancer cell lines tested while retaining cytotoxicity toward normal cells, and so oxyphytosterols may be more harmful than oxysterols. Although some reports compare the cytotoxicity of oxygenated derivatives of the cholestane and sitostane series,[23] herein, for the first time the cytotoxic profile toward normal and cancer cells was achieved for both series.

For the cancer cells HT-29, LAMA-84, A549, and MCF-7, the oxysterol with a Δ^5-7β-OH substitution afforded a more cytotoxic profile than the 3β,5α,6β-trihydroxy substitution, while the opposite effect was observed for the normal cells and PC3 cells.

LAMA-84 cells proved to be quite sensitive to oxysterols **4**, **8**, **22**, and **27**, which were cytotoxic at 4.3−5.6 μM . It is worth pointing out that oxysterols are not only potent but also selective for this cancer cell line. Specifically, a 3- to 6-fold higher cytotoxicity was found in LAMA-84 cells when compared to ARPE-19 cells (SI, Table 3). In this context the diol **22** is a promising lead for LAMA-84 cells.

Combination of an Oxysterol with Chemotherapeutics. A Preliminary Study (Table 4). Having reached a general perspective about the cytotoxic profile of oxysterols against a panel of cancer and noncancer cells, we questioned whether these compounds can interfere with the cytotoxicity of two of the most used anticancer agents, cisplatin and Doxo.

To our knowledge, only one report addresses this issue, showing that 7β-OHChol **8** increases the sensitivity of tumor cells to other anticancer agents and ionizing radiation.[24] Herein, we have chosen triol **4** as oxysterol model to test on HT-29 and ARPE-19 cells as cancer and normal cell models because of their intrinsic low expression of MDR proteins (Table S2, Supporting Information). HT-29 cells were incubated with nontoxic concentrations (1, 3, and 6 μM) of the oxysterol combined with Doxo or cisplatin for 48 h. The combination of **4** with cisplatin results in slightly decreased cytotoxicity, in a dose-dependent manner. Such decrease could be related to the proliferative effects of the oxysterol on HT-29 cells at nontoxic concentrations (Figure S1, Supporting Information). The combination of Doxo with 1 μM **4** resulted in decreased cytotoxicity, as observed for cisplatin. However, the presence of 3 μM **4**, which is a quarter of its IC_{50}, rendered a 30% increase in Doxo's cytotoxicity.

Table 4. Cytotoxicity Study of Drug Combinations with Oxysterol **4** after 48 h of Exposure (IC_{50} in μM)

drug	HT-29	ARPE-19
cisplatin (C)	13.8 ± 0.6	26.7 ± 0.9
C + **4** (1 μM)	15.6 ± 0.7	
C + **4** (3 μM)	17.3 ± 1.2	
C + **4** (6 μM)	20.0 ± 1.2	
Doxo (D)	1.23 ± 0.14	0.76 ± 0.04
D + **4** (1 μM)	1.51 ± 0.18	
D + **4** (3 μM)	0.90 ± 0.10	
D + **4** (6 μM)	0.77 ± 0.07	0.75 ± 0.06

Furthermore, when incubating simultaneously 6 μM **4** and Doxo, a remarkable 60% increase in toxic effect is observed in HT-29 cells, while in ARPE-19 cells such combination does not change the cytotoxicity of Doxo alone. The finding that triol **4** potentiates the toxicity of Doxo toward cancer colon cells without affecting the toxicity toward normal cells is novel and of potential clinical relevance. Indeed, this combination may reduce the overall clinical dose of Doxo to treat colon cancers, improving the ratio between therapeutic benefits and adverse effects.

Conclusions

Research on the cytotoxic potential of oxysterols has been the subject of only disperse reports to date. Herein, a systematic study is disclosed to explore the potential of endogenously found oxysterols and non-natural derivatives to display cytotoxicity.

A library of steroids was synthesized to study the influence of chemical modifications at C17, the oxygenation pattern on ring B, and the esterification of key OH groups with acidic chains on the ability of the oxysterols to induce cell death. Oxygenated steroids at C6 or C7 (**22** and **8**) with a free 3β-OH and the C17 side chain of Chol display more potent cytotoxicity. The synthetic oxysterol **22** has the best selectivity index for LAMA-84 cells among the oxysterols studied herein. A 7β-OH coupled with a Δ^5-double bond, as in **8**, gave an interesting cytotoxic profile with potential selectivity toward most of cancer cells studied. Compound **27** afforded high and selective activity on LAMA-84 cells, and the increasing of cytotoxicity with the incubation time suggests that this compound may act as a prodrug.

The general structure−activity relationships related to ring-A and -B oxygenated sterol derivatives found on HT-29 cells and the comparative in vitro cell sensitivity toward oxysterols action are presented schematically in Figure 2.

Our results show that oxysterols are chemically stable at different pH values, including at the pH of acidic lysosomes which, beyond mitochondria, have been indicated as a subcellular target for oxysterol induction of cell death.[11,25] The cytotoxicity improvement over time observed for epoxides **2**,

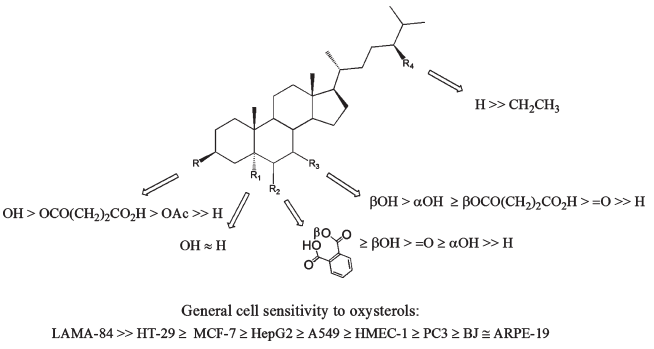


Figure 2. General SAR of sterols for cytotoxicity based on the IC_{50} results on HT-29 cancer cells.

3, and **27** and ester **18** can be due to metabolic transformations.

The co-incubation of a low concentration of oxysterol **4** with Doxo highly favors its cytotoxic potency toward the colon cancer cells HT-29, without affecting the normal ones.

Herein, we have explored and cemented the cytotoxic profile of 30 ring-B oxygenated steroids in a broad array of human cells. New insights on structural modifications favoring the selective cytotoxicity of oxysterols and the disclosure of their ability to increase Doxo's potency toward cancer cells are the main achievements of this work.

Experimental Section

For details, refer to the Supporting Information. The final compounds **1**, **2**, **3**, **7**, **8**[16] and **4**, **9**−**16**, **18**[14] were obtained as described in the literature. The other compounds were obtained as described in the Supporting Information. Purity was confirmed by HPLC to be ≥95% for all final compounds except for 3β,5α,6β,16α-tetrahydroxypregnan-20-one, **16** (84%).

General Procedure for Synthesis of Sterol 6β-Hemiphthalates. To a solution of Chol (250 mg, 0.646 mmol) in acetonitrile (19 mL) under reflux, MMPP (351.5 mg, 0.711 mmol) was added. The mixture was stirred at reflux temperature for 24 h and then stopped by evaporation under vacuum. The resulting white residue was washed with water, filtered, and evaporated to dryness. Careful flash chromatography of the crude material starting with chloroform and then in slow gradient with chloroform/ethanol (50:1 to 1:1) afforded the pure white solid 3β, 5α-dihydroxycholestan-6β-yl hemiphthalate (**24**, 143 mg, 39%). Mp 190−192.5 °C (AcOEt). IR (film) 3403, 3064, 2939, 2867, 1712, 1565, 1404, 1281, 1131, 1078, 1037, 958, 759 cm^{-1}. ^1H NMR (400 MHz, DMSO-d_6) δ ppm 0.59 (3H, s, 18-CH_3), 0.82 and 0.83 (each 3H, 2d, J = 6.6 Hz, 26-CH_3 and 27-CH_3), 0.86 (3H, d, J = 6.5 Hz, 21-CH_3), 1.02 (3H, s, 19-CH_3), 3.84 (1H, m, 3α-H), 4.36 (1H, s, OH), 4.83 (1H, brs, 6α-H), 7.39 (3H, m, Ph), 7.74 (1H, d, J = 7.2 Hz, Ph). ^{13}C NMR (101 MHz, DMSO-d_6) δ ppm 11.9, 16.1, 18.4, 20.6 (CH_2), 22.3, 22.6, 23.2 (CH_2), 23.7 (CH_2), 27.3, 27.8 (CH_2), 30.4, 30.7 (CH_2), 30.8 (CH_2), 31.7 (CH_2), 35.2, 35.6 (CH_2), 37.8 (C), 38.9 (CH_2), 39.6 (CH_2), 40.1 (CH_2), 42.2 (C), 44.1, 55.6, 55.6, 65.4, 73.4 (C-5), 75.9, 126.2, 128.1, 128.9, 129.4, 133.0 (C), 139.2 (C), 167.8 (C=O), 171.0 (C=O). MS m/z (%): 567.3 (100) $[M - H]^+$, 361.5 (15). HRMS (ESI), positive mode, m/z $[M + Na]^+$ calcd for $C_{35}H_{53}O_6Na$, 591.3656; found, 591.3664.

Acknowledgment. We thank Fundação para a Ciência e Tecnologia (FCT), Portugal, through POCI for financial support. We acknowledge "Lab. Espectrometria de Massa do Centro de Estudos Farmacêuticos, Universidade Coimbra" for providing LC−MS data (QITMS) and "Lab. FTICR e Proteómica Avançada, Faculdade Ciências, Universidade Lisboa" for providing FTICR-MS data, both in the National Mass Spectrometry Network. J.F.S.C. thanks FCT for Grant SFRH/BD/18263/2004.

Supporting Information Available: Experimental methods and procedures for chemistry and biology and structural characterization of compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Vejux, A.; Lizard, G. Cytotoxic effects of oxysterols associated with human diseases: induction of cell death (apoptosis and/or oncosis), oxidative and inflammatory activities, and phospholipidosis. *Mol. Aspects Med.* **2009**, *30*, 153–170.
(2) Schroepfer, G. J. J. Oxysterols: modulators of cholesterol metabolism and other processes. *Physiol. Rev.* **2000**, *80*, 361–554.
(3) Olkkonen, V. M.; Hynynen, R. Interactions of oxysterols with membranes and proteins. *Mol. Aspects Med.* **2009**, *30*, 123–133.
(4) Pirillo, A.; Zhu, W.; Roma, P.; Galli, G.; Caruso, D.; Pellegatta, F.; Catapano, A. L. Oxysterols from oxidized LDL are cytotoxic but fail to induce hsp70 expression in endothelial cells. *FEBS Lett.* **1999**, *462*, 113–116.
(5) Larsson, D. A.; Baird, S.; Nyhalah, J. D.; Yuan, X.-M.; Li, W. Oxysterol mixtures, in atheroma-relevant proportions, display synergistic and proapoptotic effects. *Free Radical Biol. Med.* **2006**, *41*, 902–910.
(6) Biasi, F.; Leonarduzzi, G.; Vizio, B.; Zanetti, D.; Sevanian, A.; Sottero, B.; Verde, V.; Zingaro, B.; Chiarpotto, E.; Poli, G. Oxysterol mixtures prevent proapoptotic effects of 7-ketocholesterol in macrophages: implications for proatherogenic gene modulation. *FASEB J.* **2004**, *18*, 693–695.
(7) Steffen, Y.; Wiswedel, I.; Peter, D.; Schewe, T.; Sies, H. Cytotoxicity of myeloperoxidase/nitrite-oxidized low-density lipoprotein toward endothelial cells is due to a high 7β-hydroxycholesterol to 7-ketocholesterol ratio. *Free Radical Biol. Med.* **2006**, *41*, 1139–1150.
(8) Radhakrishnan, A.; Ikeda, Y.; Kwon, H.; Brown, M.; Goldstein, J. Sterol-regulated transport of SREBPs from endoplasmic reticulum to Golgi: oxysterols block transport by binding to Insig. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 6511–6518.
(9) Ishimaru, C.; Yonezawa, Y.; Kuriyama, I.; Nishida, M.; Yoshida, H.; Mizushina, Y. Inhibitory effects of cholesterol derivatives on DNA polymerase and topoisomerase activities, and human cancer cell growth. *Lipids* **2008**, *43*, 373–382.
(10) Fernández, C.; Lobo, M. d. V. T.; Gómez-Coronado, D.; Lasunción, M. A. Cholesterol is essential for mitosis progression and its deficiency induces polyploid cell formation. *Exp. Cell Res.* **2004**, *300*, 109–120.
(11) Lemaire-Ewing, S.; Prunet, C.; Montange, T.; Vejux, A.; Berthier, A.; Bessède, G.; Corcos, L.; Gambert, P.; Néel, D.; Lizard, G. Comparison of the cytotoxic, pro-oxidant and pro-inflammatory characteristics of different oxysterols. *Cell Biol. Toxicol.* **2005**, *21*, 97–114.
(12) Aiello, A.; Fattorusso, E.; Menna, M. Steroids from sponges: recent reports. *Steroids* **1999**, *64*, 687–714.
(13) Wu, Q.; Huang, K. Protective effect of ebselen on cytotoxicity induced by cholestane-3β,5α,6β-triol in ECV-304 cells. *Biochim. Biophys. Acta* **2006**, *1761*, 350–359.
(14) Carvalho, J. F. S.; Silva, M. M. C.; Sá e Melo, M. L. Efficient transdiaxial hydroxylation of Δ^5-steroids. *Tetrahedron* **2010**, *66*, 2455–2462.
(15) Carvalho, J. F. S.; Silva, M. M. C.; Sá e Melo, M. L. Highly efficient epoxidation of unsaturated steroids using magnesium bis(monoperoxyphthalate) hexahydrate. *Tetrahedron* **2009**, *65*, 2773–2781.
(16) Carvalho, J. F. S.; Silva, M. M. C.; Moreira, J. N.; Simões, S.; Sá e Melo, M. L. Efficient chemoenzymatic synthesis, cytotoxic evaluation and SAR of epoxysterols. *J. Med. Chem.* **2009**, *52*, 4007–4019.
(17) Gerweck, L. E. The pH difference between tumor and normal tissue offers a tumor specific target for the treatment of cancer. *Drug Resist. Updates* **2000**, *3*, 49–50.
(18) Massey, J. B.; Pownall, H. J. Structures of biologically active oxysterols determine their differential effects on phospholipid membranes. *Biochemistry* **2006**, *45*, 10747–10758.
(19) Fretland, Adrian J.; Omiecinski, Curtis J. Epoxide hydrolases: biochemistry and molecular biology. *Chem.-Biol. Interact.* **2000**, *129*, 41–59.
(20) Gerweck, L. E.; Vijayappa, S.; Kozin, S. Tumor pH controls the in vivo efficacy of weak acid and base chemotherapeutics. *Mol. Cancer Ther.* **2006**, *5*, 1275–1279.

(21) Quílez, J.; García-Lorda, P.; Salas-Salvadó, J. Potential uses and benefits of phytosterols in diet: present situation and future directions. *Clin. Nutr.* **2003**, *22*, 343–351.
(22) Ryan, E.; McCarthy, F. O.; Maguire, A. R.; O'Brien, N. M. Phytosterol oxidation products: their formation, occurrence, and biological effects. *Food Rev. Int.* **2009**, *25*, 157–174.
(23) Roussi, S.; Winter, A.; Gosse, F.; Werner, D.; Zhang, X.; Marchioni, E.; Geoffroy, P.; Miesch, M.; Raul, F. Different apoptotic mechanisms are involved in the antiproliferative effects of 7β-hydroxysitosterol and 7β-hydroxycholesterol in human colon cancer cells. *Cell Death Differ.* **2005**, *12*, 128–135.
(24) Hyun, J. W.; Holl, V.; Weltin, D.; Dufour, P.; Luu, B.; Bischoff, P. Effects of combinations of 7β-hydroxycholesterol and anticancer drugs or ionizing radiation on the proliferation of cultured tumor cells. *Anticancer Res.* **2002**, *22*, 943–948.
(25) Li, W.; Johnson, H.; Yuan, X.-M.; Jonasson, L. 7β-Hydroxycholesterol induces natural killer cell death via oxidative lysosomal destabilization. *Free Radical Res.* **2009**, *43*, 1072–1079.