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Synthesis of New Alkylaminooxysterols with Potent Cell Differentiating Activities: Identification of Leads for the Treatment of Cancer and Neurodegenerative Diseases

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We describe here the syntheses and the biological properties of new alkylaminooxysterols. Compounds were synthesized through the trans-diaxial aminolysis of 5,6-α-epoxysterols with various natural amines including histamine, putrescine, spermidine, or spermine. The regioselective synthesis of these 16 new 5α-hydroxyl-6β-aminoalkylsterols is presented. Compounds were first screened for dendrite outgrowth and cytotoxicity in vitro, and two leads were selected and further characterized. 5α-Hydroxy-6β-[2-(1*H*-imidazol-4-yl)ethylamino]cholestan-3β-ol, called dendrogenin A, induced growth control, differentiation, and the death of tumor cell lines representative of various cancers including metastatic melanoma and breast cancer. 5α-Hydroxy-6β-[3-(4-aminobutylamino)propylamino]cholest-7-en-3β-ol, called dendrogenin B, induced neurite outgrowth on various cell lines, neuronal differentiation in pluripotent cells, and survival of normal neurones at nanomolar concentrations. In summary, we report that two new alkylaminooxysterols, dendrogenin A and dendrogenin B, are the first members of a class of compounds that induce cell differentiation at nanomolar concentrations and represent promising new leads for the treatment of cancer or neurodegenerative diseases.

The microsomal antiestrogen binding site (AEBS[a]) is a nanomolar target for the antitumor and chemopreventive drug tamoxifen (Tam) that was first described 30 years ago.[1] The AEBS was shown to be different from the estrogen receptor α which is known as the primary target of Tam for the hormonotherapy of breast cancers.[2,3] Pharmacological studies established that the AEBS bound a number of drugs belonging to various pharmacological classes in addition to Tam, including selective estrogen receptor modulators, inhibitors of cholesterol biosynthesis, and selectively binds diphenylmethane derivatives of tamoxifen such as PBPE and tesmilifene.[4−9] We and others established that the AEBS was involved in the cytotoxicity induced by its cognate ligands.[6,7,9−15] One selective AEBS ligand, tesmilifene, was developed for the treatment of breast and prostate cancers despite the lack of a clear understanding of its molecular mechanism of action at that time.[16−21] The molecular characterization and the identification of the AEBS established that it was a multienzyme complex involved in cholesterol metabolism.[4,22−25] We established that in mammalian cells, the AEBS subunits involved in Tam binding are the 3β-hydroxysterol-Δ^8-Δ^7-isomerase (D8D7I) and the 3β-hydroxysterol-Δ^7-reductase (DHCR7)[4] and we recently showed that it contained the cholesterol-5,6-epoxide hydrolase (ChEH) (de Medina et al., unpublished results), which catalyzes the hydration of 5,6-epoxysterols such as 5,6α- and 5,6β-epoxycholestan-3β-ol (CEs). Post-lanosterol cholesterol biosynthesis enzymes such as D8D7I and DHCR7 are involved in genetic syndromes[26] in which organ developments are impaired suggesting a possible link between the AEBS/ChEH complex and cell differentiation. This led us to show that the AEBS controlled the differentiation and death of breast cancer cells through the modulation of cholesterol metabolism and the production of sterol autoxidation products such as CEs.[10,27,28] These results raised the possibility that defects in these enzymes may affect the catalytic properties of the ChEH and the metabolism of CEs, which opens up the possibility of the involvement of CEs or their metabolites in mammalian developmental programs. CEs in the plasma of healthy human subjects occurred at levels of 1−100 nM.[29] At these concentrations, we showed that CEs did not induce tumor cell differentiation (reported in the present paper). Interestingly, the AEBS was earlier described as a binding site for histamine,[30] suggesting that CE and histamine may be concentrated at the level of the AEBS. The spatial proximity between CE bearing a putative electrophilic oxirane group (epoxide) and histamine that contains nucleophile nitrogen groups led us to hypothesize a possible proximity induced catalysis[31] of a ring-opening reaction of CEs with histamine. In that case, the reaction would give 6-histaminylcholestan-3,5-diols (HiCD) that might be active to induce cell differentiation. In the present study, we reported the synthesis of HiCD and the synthesis of the product of aminolysis of 5,6α-epoxysteroids with various natural amines. The synthesized compounds were tested for differentiation of mammalian cells.

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[a] Abbreviations: AEBS, antiestrogen binding site; Tam, tamoxifen; PBPE, *N*-pyrrolidino-2-[4-(benzyl)phenoxy]ethanamine·HCl; D8D7I, 3β-hydroxysterol-Δ^8-Δ^7-isomerase; DHCR7, 3β-hydroxysterol-Δ^7-reductase; ChEH, cholesterol-5,6-epoxide hydrolase; CE, 5,6-epoxycholestan-3β-ol; HiCD, 6-histaminylcholestan-3,5-diols; DDA, dendrogenin A; DDB, dendrogenin B; DHA, docosahexaenoic acid; ATRA, all-trans-retinoic acid; BDNF, brain derived neurotrophic factor.

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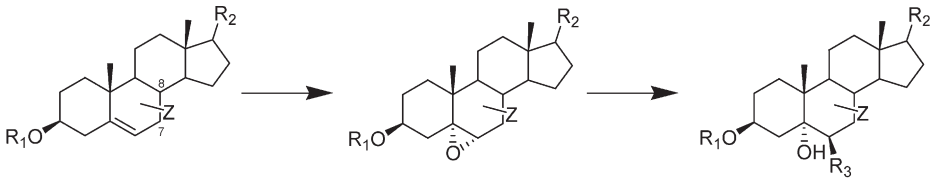
Chart 1. Epoxidation and Aminolysis of Steroids



Z = single bond in C8-C7, double bond in C8-C7 ($\Delta^{8\text{-}7}$); R1 = H, acetyl, butyryl; R2 = OH, 6-methylhept-2-yl; R3 = histamine, putrescine, spermidine, spermine

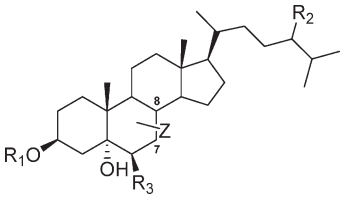
Chemistry

Sterol-5,6-epoxides (**1**−**6**) were synthesized as described previously (Chart 1)[32,33] and gave a 75:25 mixture of α- and β-epoxisterol isomers. α-Epoxysterols **1**−**6** were purified by recrystallization and used for coupling with alkylamines, using 3 equiv of $LiClO_4$ in ethyl alcohol as solvent,[34] and afforded the corresponding 6β-aminoalkyloxysterols through trans diaxial opening of the oxirane ring (Table 1). No reactions were obtained under these conditions with the β-CE. The absence of reactivity of β-CE can be explained by the impossibility of a trans diaxial opening with the amine in the transition state. New alkylaminooxysterols varied in their steroid structures by the presence or the absence of a double bond in the C8−C7 position, the esterification of the 3β hydroxyl by an acetyl or a butyryl group, and the presence of an ethyl group on the C24 position. Histamine, putrescine, spermidine (SPD), and spermine were used for the aminolysis of sterol epoxides. SPD contains three different nucleophilic groups; thus, in order to avoid mixtures of regioisomers, the synthesis of three protected spermidines were prepared as described in Scheme 1 and used as reported above to obtain the expected 6β-aminoalkylsterols. The deprotection of the amines was done with trifluoroacetic to obtain the expected free amines (**23**−**27**). The molecular structures of synthesized compounds are presented in Table 1. The compounds were then tested for biological applications.

Biology

Induction of Dendrite Outgrowth and Cytotoxicity on U-937 and P19 Cells. U937 is a monocytic-like cell line that can undergo differentiation into macrophages upon treatment with 100 nM phorbol myristyl acetate for 1 day,[35,36] and then a treatment with IL-4 and GM-CSF induced a dendrite extension (Table 1). P19 is a pluripotent mouse carcinoembryonic cell line that can be differentiated into neurons with micromolar concentrations of all-trans-retinoic acid (ATRA). The main characteristic of this effect is the appearance and the extension of dendrites.[37,38] As a preliminary screening, compounds were tested for dendrite outgrowth stimulation on U937 and P19 cells, which allows a rapid morphological assessment of cell differentiation. Compound **14** corresponds to the product of aminolysis of α-CE with histamine by its aminoethyl side chain. **14** was a potent inducer of dendrite outgrowth on cells. Modification of cell morphology was observed on 40−50% of adherent cells of U937 and 65−70% of P19 cells. In both cases the length of the dendrites was greater than 200 μm after 48 h of treatment. We have investigated the impact of treatment with the synthesized compounds on U937 and P19 cell morphology and have measured their cytotoxicity. **14** was cytotoxic with IC_{50} of 0.8 and 2.4 μM in U937 and P19 cells, respectively. The presence of a double bond on C7−C8 on ring B of the steroid backbone (**15**) induced a drastic diminution of dendrite outgrowth and 10-fold reduction in cytotoxicity compared with **14**. The addition of an ethyl group on C24 of the isooctyl side chain (**16**) has no incidence on the biological activities compared with **14**, showing that the increase in steric hindrance of the isooctyl side chain of the sterol backbone is not deleterious for activity. Compound **17** is a regioisomer of **14** in which histamine was grafted on by its imidazole ring. **17** was less potent in inducing dendrite outgrowth on U937 and P19 cells and had a 10-fold decrease in cytotoxicity compared with **14** showing that regioisomers **14** and **17** were not equipotent. Acetylation of the hydroxyl in C3 of ring B of the steroid backbone (**18**) did not change the biological properties of **14**, whereas the replacement of the acetyl group by a butyryl group (**19**) led to a strong decrease of both the dendritogenesis and cytotoxicic potencies on the two tested cell lines. This indicates that the presence of a bulky substituent on the C3 position decreases the biological activities. The elimination of the isooctyl side chain and its replacement by a ketone group (**20**) abolished the dendrite outgrowth properties and decreased the cytotoxicity compared with **14**. Replacing histamine by 1,4-diaminobutane (putrescine) (**21**) on **14** decreased dendrite outgrowth potencies and cytotoxicity. Compound **21** has a stronger potency to induce dendrite outgrowth on P19 cells than on U937 cells. This tendency was more pronounced in the presence of a double bond between C7 and C8 on ring B of the steroid backbone (**22**). Compounds obtained by the aminolysis of **1** or **2** with spermidine grafted through the two possible primary amines gave compounds **23**, **24**, **25**, and **26**. These compounds showed weak potencies to stimulate dendrite outgrowth on U937 cells but high potencies to stimulate dendrite outgrowth on p19 cells with no cytotoxicity on both cell lines, the most potent being **24**. Compound **27** is a spermidine conjugate, but in this case spermidine was conjugated by the secondary amine. **27** was highly efficient at inducing dendrite outgrowth on both U937 and P19 cells but, as observed for the histamine and putrescine conjugates, was cytotoxic. This showed that regiosiomers **24**, **26**, and **27** were not equipotent for the induction of dendrite outgrowth on both cell lines. Coupling spermine to epoxide **1** or **2** gave compounds **28** and **29** that were potent inducers of dendrite outgrowth on P19 cells. Compounds **28** and **29** were not cytotoxic and weakly efficient in inducing dendrite outgrowth on U937 cells. From these studies, it can be concluded that the products of conjugation through epoxide ring opening with biogenic amines gave products with potent capacities to induce dendrite outgrowth at nanomolar concentrations. Two families of compounds have been delineated depending on the biogenic amines that were grafted: (1) histamine derivatives (**14**, **16**, **19**) that were potent inducers of dendrite outgrowth on U937 and P19 cells and were cytotoxic; (2) spermidine derivatives (**24** and **25**) that induced selectively dendrite outgrowth on P19 cells without being cytotoxic.

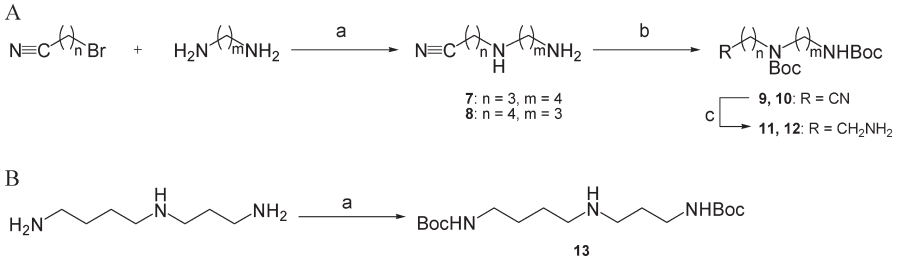
Table 1. Effect of Compounds on Dendrite Outgrowth of Lymphoid U937 Cells and Carcinoembryonic P19 Cells[a]



					U937		P19	
compd	R_1	R_2	R_3	Z	dendrite outgrowth	cytotoxicity, IC_{50} (μM)	dendrite outgrowth	cytotoxicity, IC_{50} (μM)
ATRA					−	−	−	−
IL4 + GMCSF					+	−	−	−
cholesterol	H	H	−	−	−	−	−	−
α-CE	H	H	−	−	−	−	−	−
β-CE	H	H	−	−	−	−	−	−
2	H	H	−	$\Delta^{7,8}$	−	−	−	−
3	H	C_2H_5	−	−	−	−	−	−
4	CH_3CO	H	−	−	−	−	−	−
5	*n*-C_3H_5CO	H	−	−	−	−	−	−
6	H	−	−	−	−	−	−	−
histamine	−	−	−	−	−	−	−	−
spermidine	−	−	−	−	−	−	−	−
spermine	−	−	−	−	−	−	−	−
putrescine	−	−	−	−	−	−	−	−
α-CE + histamine	−	−	−	−	−	−	−	−
α-CE + spermidine	−	−	−	−	−	−	−	−
14	H	H	-$NH(CH_2)_2$-(1H-imidazol-5-yl)	−	++++	0.8 ± 0.2	++++	2.4 ± 0.2
15	H	H	-$NH(CH_2)_2$-(1H-imidazol-5-yl)	$\Delta^{7,8}$	+	7.6 ± 0.4	++	5.3 ± 0.5
16	H	C_2H_5	-$NH(CH_2)_2$-(1H-imidazol-5-yl)	−	++++	1.1 ± 0.1	++++	2.5 ± 0.3
17	H	H	-N-2-aminoethyl-(1H-imidazol-1-yl)	−	+	7.7 ± 0.6	+	8.9 ± 0.4
18	CH_3CO	H	-$NH(CH_2)_2$-(1H-imidazol-5-yl)	−	++++	0.9 ± 0.1	++++	2.5 ± 0.2
19	$_nC_3H_7CO$	H	-$NH(CH_2)_2$−(1H-imidazol-5-yl)	−	−	8.2 ± 0.6	−	9.4 ± 0.6
20	H	−	-$NH(CH_2)_2$-(1H-imidazol-5-yl)	−	−	9.8 ± 0.7	−	7.3 ± 0.5
21	H	H	-$HN(CH_2)_4NH_2$	−	++	2.1 ± 0.2	+++	2.6 ± 0.2
22	H	H	-$HN(CH_2)_4NH_2$	$\Delta^{7,8}$	++	2.6 ± 0.2	++++	2.8 ± 0.2
23	H	H	-$HN(CH_2)_3NH(CH_2)_4NH_2$	−	+	−	++++	−
24	H	H	-$HN(CH_2)_3NH(CH_2)_4NH_2$	$\Delta^{7,8}$	+	−	+++++	−
25	H	H	-$HN(CH_2)_4NH(CH_2)_3NH_2$	−	+	−	++++	−
26	H	H	-$HN(CH_2)_4NH(CH_2)_3NH_2$	$\Delta^{7,8}$	+	−	++++	−
27	H	H	$_2HN(CH_2)_3$-$N((CH_2)_4NH_2$	$\Delta^{7,8}$	+	7.5 ± 0.6	++++	8.9 ± 0.7
28	H	H	-$HN(CH_2)_3HN(CH_2)_4NH(CH_2)_3NH_2$	−	+	−	+++	−
29	H	H	-$HN(CH_2)_3HN(CH_2)_4NH(CH_2)_3NH_2$	$\Delta^{7,8}$	+	−	+++	−

[a] Z is the status of the C8−C7 bond (single or double). $\Delta^{7,8}$: double bond between C8 and C7 of the steroid backbone. IC_{50}: concentration of a compound required to induce 50% of the cytotoxicity. Cells were treated for 48 h with 100 ng/ml IL-4 and GM-CSF. Dendrite outgrowth was measured using a light microscope after 48 h of incubation of cells with 100 nM of each indicated compounds. −: dendrites are not detectable on cells. +: the length of dendrites is $< 5\,\mu$m. ++: the length of dendrites is between 5 and 10 μm. +++: the length of dendrites is between 10 and 50 μm. ++++: the length of dendrites is between 50 and 100 μm, or the length of dendrites is $> 100\,\mu$m. +++++: the length of dendrites is $> 100\,\mu$m, and a dendritic contact network is observable. Cell death was determined by the trypan blue exclusion test.

Scheme 1. Protection of Spermidine[a]



[a] Reagents and conditions. A: (a) MeOH, 0 °C, then room temp, overnight, 26−47%; (b) Boc_2O, DCM, 0 °C, then room temp, 4 h, 82−85%; (c) $LiAlH_4/Et_2O$, 0 °C, then room temp, 1 h, 65−82%. B: (a) BocON, THF, 0 °C, then room temp, overnight, 39%.

The potencies of these new alkylaminooxysterols were peculiar to their structure because treatment of cells with reactant alone (sterol epoxides and amines) or in association was unable to induce dendrite outgrowth on a precursor of a

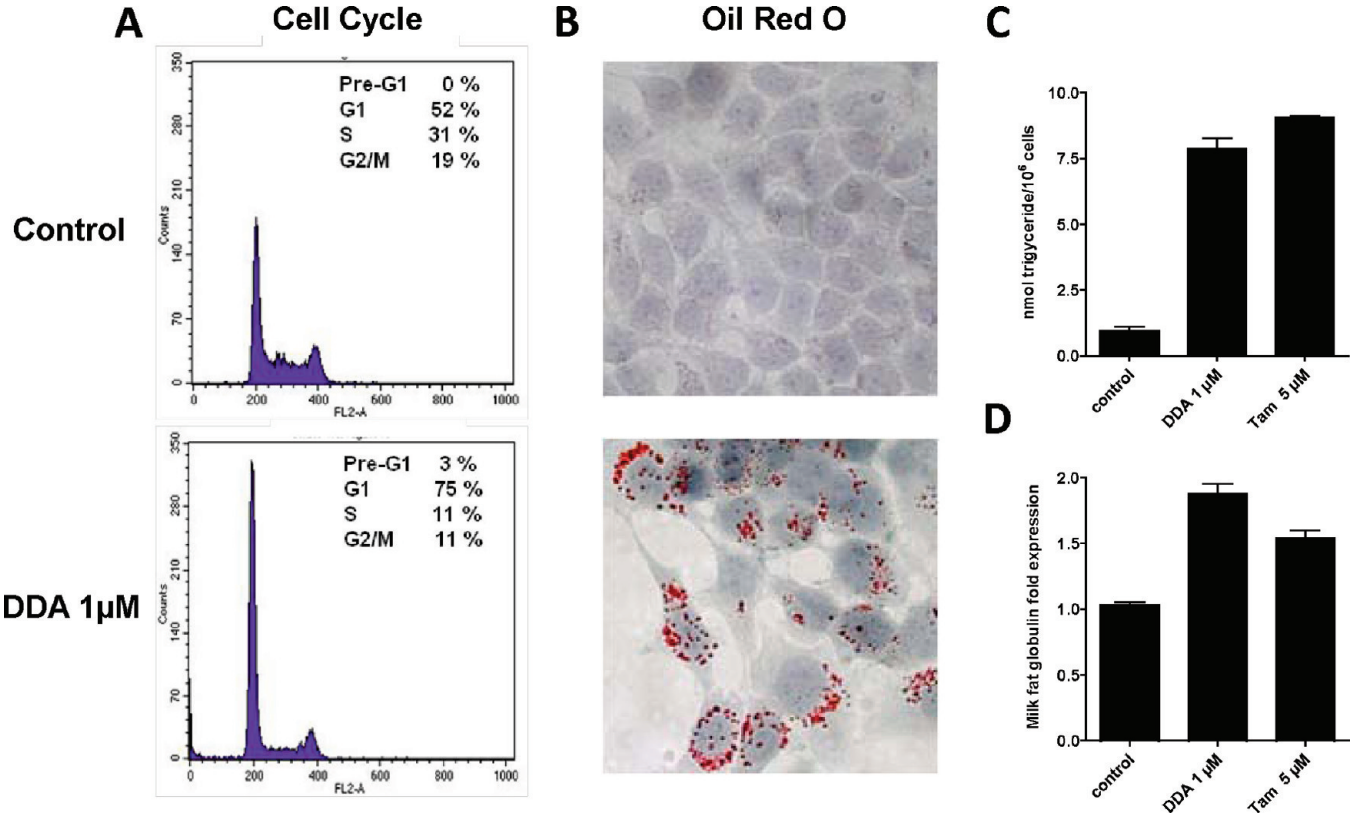


Figure 1. DDA (**14**) induced growth control and characteristics of differentiation in MCF-7 cells, a human breast adenocarcinoma cell line. (A) Cell cycle distribution of MCF-7 cells treated with solvent vehicle (control) or 1 μM DDA for 48 h. Cell cycle distribution was measured as described in the Experimental Section by FACS flow analysis using a Becton Dickinson FACS system. (B) Staining of neutral lipids with oil red O (ORO) in MCF-7 cells treated with solvent vehicle (control) or 1 μM DDA for 48 h. Morphologic and biochemical changes were evaluated by light microscopy ($\times$40) of ORO stained cells counterstained with Mayer's hematoxylin as described in the Experimental Section. (C) Quantification of triglycerides present in MCF-7 cells treated for 48 h with solvent vehicle (control), 1 μM DDA, or 5 μM tamoxifen (Tam) was carried out as described in the Experimental Section. (D) Quantification of milk fat globulin expressed in MCF-7 cells treated for 48 h with solvent vehicle (control), 1 μM DDA, or 5 μM tamoxifen (Tam) was carried out as described in the Experimental Section.

Chart 2. Structure of Dendrogenin A

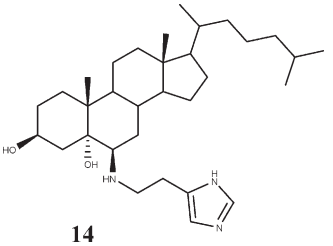


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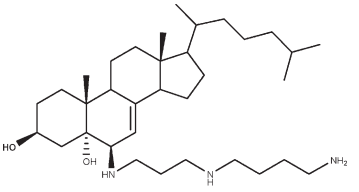
Chart 3. Structure of Dendrogenin B



24

neuron and they were not cytotoxic (up to 20 μM) (Table 1). This indicated that these new alkylaminooxysterols displayed specific effects. Compounds **14** and **24** were further characterized for their pharmacological properties because they were the most potent compounds of their series and were named dendrogenin A (DDA) (Chart 2) and dendrogenin B (DDB) (Chart 3), respectively, because of their ability to stimulate dendrite outgrowth.

DDA Induces Cell Differentiation. Since we showed that DDA induced characteristics of differentiation in U937 and P19, we studied its effects on differentiation of other tumor cell lines. We have investigated the effects of DDA on the differentiation of human breast cancer cells (MCF-7). Figure 1 shows that DDA induced the arrest of cells in the G0−G1 phase of the cell cycle, modified the cell morphology, and stimulated the accumulation of neutral lipid containing intracytoplasmic vesicles stained in red with oil red O. We determined that the nature of the neutral lipids that accumulated in cells were triacyl glycerols (Figure 1), the major lipids found in milk, and that DDA stimulated the expression of milk fat globulin which is one of the major proteins found in milk (Figure 1). Altogether, these data established that DDA induced differentiation in MCF-7 cells. Interestingly, the concentrations of tamoxifen and PBPE that were required to obtain a similar effect as 1 μM DDA were 5 and 20 μM, showing that DDA was much more active. We next evaluated the impact of DDA on the differentiation of human melanoma cells (SK-Mel-28). DDA induced cell cycle arrest in the G0−G1 phase (Figure 2A). DDA dramatically changed in the cell morphology from fibroblastic (Figure 2B, panel 1) to dendritic that resembled normal melanocytes (Figure 2B,

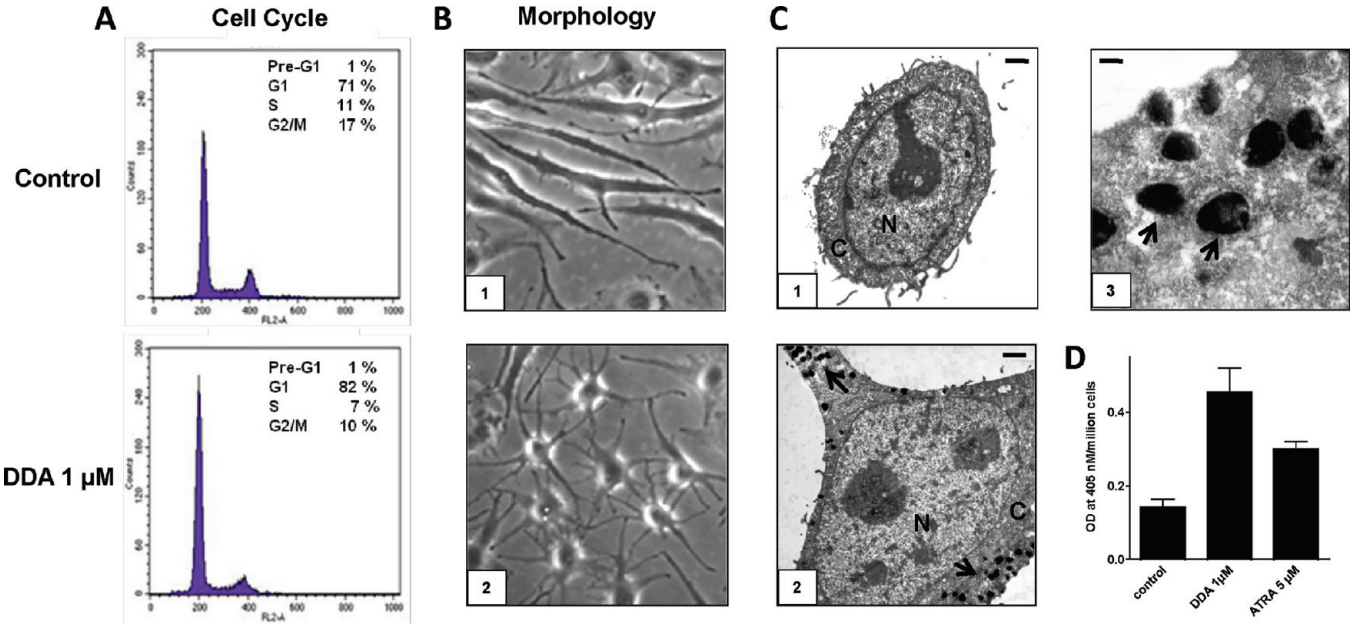


Figure 2. DDA (**14**) induced growth control and characteristics of differentiation in SK-Mel-28 cells, a human melanoma cell line. (A) Cell cycle distribution of SK-Mel-28 cells treated with solvent vehicle (control) or 1 μM DDA for 48 h. Cell cycle distribution was measured as described in the Experimental Section by FACS flow analysis using a Becton Dickinson FACS system. (B) Morphologic changes of SK-Mel-28 treated with solvent vehicle (1) or with 1 μM DDA for 48 h (2) were evaluated by light microscopy (×40) as described in the Experimental Section. (C) Electron micrographs of SK-Mel-28 cells treated with solvent vehicle (1) or 1 μM DDA for 48 h (2, 3). After fixation of the cells and embedding in Epon 812, ultrathin sections of the cells were prepared, stained with uranyl acetate and lead citrate, and examined in a Hitachi electron microscope: N, nucleus; C, cytoplasm; bars, 1.25 μm for panel 1, 0.5 μm for panel 2, and 0.1 μm for panel 3. Arrows indicate the presence of types III and IV melanosomes. (D) Melanogenesis in SK-Mel-28 cells treated with solvent vehicle (control), 1 μM DDA, or 5 μM ATRA for 48 h. Melanogenesis was measured in cell lysates by measuring the absorbance at 405 nm as described in the Experimental Section.

panel 2). Ultrastructure analysis by electron microscopy revealed the presence of type IV melanosomes in the DDA-treated cells (Figure 2C, panels 2 and 3), and biochemical studies confirmed that DDA stimulated melanogenesis at lower concentrations than ATRA (Figure 2D). Altogether these data established that DDA induced differentiation of SK-Mel-28 cells toward melanocytes and also that at higher concentrations, DDA killed MCF-7 and SK-Mel-28 cells. DDA shows promising properties in vitro as a potent inducer of tumor cell differentiation.

DDA Is Cytotoxic against Various Tumor Cell Lines. Having shown that DDA induced cell differentiation and death in tumor cell lines of different origins as described above, we measured the cytotoxicity of DDA (**14**) on a panel of tumor cell lines representative of different cancers. We report in Table 2 that DDA was active against every cell line tested in the micromolar range. It is noted that these tumor cells of human and mouse origins were sensitive to DDA in the hundreds of nanomolar to the micromolar range. This effect was specific to DDA in our series because its regioisomer (**17**) was only weakly cytotoxic and DDB was not toxic to these cells up to 20 μM for 72 h. DDA shows promising properties in vitro as a potent inducer of tumor cell death.

Effect of DDB on Neuritogenesis, Neuron Differentiation, and Neuron Survival. Given that DDB induced dendrite outgrowth on P19 cells (Table 1), the differentiation of P19 cells was further investigated phenotypically. Figure 3 shows that 48 h of exposure of P19 cells to 10 nM DDB produced dendrite expansion and the appearance of a network with contact between cells (Figure 3A). Immunocytochemistry using antibodies directed against MAP2A and β3-tubulin showed that DDB induced the expression of MAP2A (Figure 3B) and β3-tubulin (Figure 3C) in dendrites, indicating that the dendrites may be neurites. The neuronal phenotype of P19 cells treated with DDB was confirmed by showing that DDA stimulated the expression of the *N*-methyl-D-aspartate receptor (NMDAR1) (Figure 3C) which is a characteristic of P19 differentiation into neurons.[39] Thus, DDB induced P19 cells to differentiate into neurons and the dendrite extensions were neurites.

We next investigated whether the stimulation of neurite outgrowth could be observed on a set of tumor cell lines of

Table 2. Effect of DDA on the Induction of Cytotoxicity on a Panel of Tumor Cell Lines[a]

cell	cell type	IC_{50} (μM)
MCF-7	breast carcinoma, human	2.31 ± 0.2
TSA	breast carcinoma, mouse	1.28 ± 0.2
SK-MEL-28	melanoma, human	2.12 ± 0.2
B16F10	melanoma, mouse	0.68 ± 0.1
A549	lung carcinoma, human	2.56 ± 0.6
P19	embryonal carcinoma, mouse	1.80 ± 0.4
SK-N-SH	neuroblastoma, human	2.24 ± 0.5
SH-SY5Y	neuroblastoma, human	1.35 ± 0.5
Neuro2A	neuroblastoma, mouse	2.32 ± 0.2
U87	glioma, human	0.74 ± 0.2
U937	monocytic leukemia, human	0.79 ± 0.3
NB4	promyelocytic leukemia, human	1.32 ± 0.4
KG1	acute myeloid leukemia, human	5.53 ± 0.8
HCT-8	colon carcinoma, human	0.89 ± 0.2
SW620	intestinal carcinoma, human	0.42 ± 0.1

[a] Cells were incubated with increasing concentrations of DDA ranging from 10 nM to 20 μM for 72 h, and cytotoxicity was quantified by trypan blue exclusion tests (see Experimental Section).

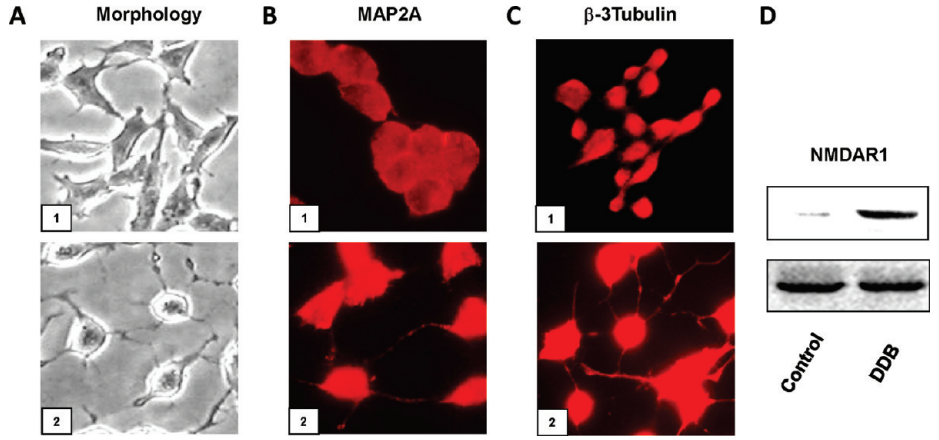


Figure 3. DDB induce the differentiation of the P19 carcino embryonic cells into neurons. Cells were treated with solvent vehicle (1) or 10 nM DDB (2) for 48 h and (A) analyzed by light microscopy for morphology or (B) analyzed by immunocytochemistry for the expression of MAP2A and (C) the expression of β3-tubulin. (D) The expression of NMDAR1 was shown by Western blotting after extraction of the protein from treated cells.

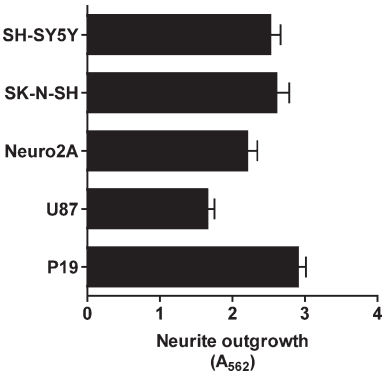


Figure 4. DDB induced neurite outgrowth on cells from various cell lines. Cells were treated for 48 h with 100 nM DDB. Neurite outgrowth was quantified by measuring the absorbance (A_{562}) reading of crystal violet extracted from stained isolated neurites.

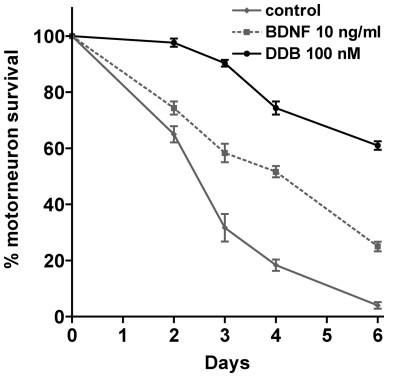


Figure 5. DDB promotes mouse purified motoneuron survival. Solvent vehicle, 100 ng/mL BDNF, or 10 nM DDB was added to the culture immediately after seeding. Cells were counted at days 2, 3, 4, and 6. Results are expressed as a percentage of surviving motoneurons.

neuronal and human origins. Figure 4 shows that DDB induced neurite outgrowth not only on P19 cells but also on human (SH-SY5Y, SK-NS-H) and mouse (Neuro2A) neuroblastoma and human glioma (U87) cells. No toxicity was observed up to 20 μM with DDB. Under the same conditions, 2−5 μM ATRA and 40−60 μM docosahexaenoic acid (DHA) were required to produce an effect comparable to that obtained with DDB. ATRA and DHA are nonpeptidic compounds known to induce neurite outgrowth.[40] This established that DDB acts as a neurotrophin on both mouse and human cells at very low concentrations.

The effect of DDB on the survival of purified mouse motoneurons was tested, and as can be seen in Figure 5, DDB stimulated motoneuron survival. A time course study was performed and showed that DDB was extremely potent and the effect challenged peptidic neurotrophin BDNF. DDB shows promising properties in vitro as a potent inducer of neurite outgrowth, neuron differentiation, and neuron survival that justify further investigations into its potential to treat neurodegenerative diseases and for neuroregenerative therapy.

Conclusion

We describe here the synthesis of new alkylaminooxysterols. We hypothesized that these compounds may be inductors of cell differentiation and confirmed this hypothesis through a series of in vitro experiments. Compounds were first tested for their ability to stimulate dendrite outgrowth and for their cytotoxicity on two different cell lines (U937 and P19). Structure−activity studies were done by synthesizing various 5,6-epoxysterols and -steroids that were conjugated to different biogenic amines, and protected forms of SPD were synthesized to obtain selective regioisomers. Two families of compounds were obtained on the basis of their biological properties. The first class included compounds containing a sterol backbone and a histamine (grafted by the primary amine) or a putrescine on C6. These compounds were potent inducers of dendrite outgrowth and were cytotoxic. The second class included sterols containing or not containing a double bond at C7−C8 and SPD or SPM grafted through a primary amine. These compounds induced neurite outgrowth on P19 cells without cytotoxicity but did not induce dendrite outgrowth on monocytic U937 cells. From these compounds, the most potent compounds of each series were selected and tested more specifically. Dendrogenin A (DDA) (**14**) induced differentiation and cell death in the human breast adenocarcinoma cell line MCF-7 and the human metastatic melanoma SK-Mel-28. Dendrogenin A (DDA) (**14**) was tested for cytotoxicity on tumor cell lines representative of different cancers and showed

a high potency to kill tumor cells (at nanomolar to submicromolar concentrations). Dendrogenin B (DDB) (**24**) induced neurite outgrowth on different cell lines of neuronal origin, more specifically inducing the differentiation of pluripotent embryonic carcinoma stem cells (P19) into neurons at nanomolar concentrations. Finally we showed that DDB helped motoneurons to survive. Altogether, these data showed that dendrogenin A and dendrogenin B are new alkylaminooxysterols with high potency in in vitro models which deserve further investigations on preclinical models of cancer and neurodegenerative diseases. Moreover, since these compounds were synthesized on the basis of a hypothesis of a metabolic transformation of 5,6-epoxysterols with natural amines, the question that remains to be answered is whether the active alkylaminooxysterols synthesized here are natural metabolites. Studies are ongoing in our laboratory to detect these compounds in mammalian tissues.

Experimental Section

Chemical Synthesis, Materials, and Instrumentation. Reagents were purchased from Sigma-Aldrich (St. Louis, MO). The progress of the reactions was monitored by TLC using Merck silica gel 60 F254 (0.040−0.063 mm) with detection with 50% methanolic H_2SO_4 and 0.2% ethanolic ninhydrin. Melting points were determined with a Kofler apparatus and are uncorrected. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker spectrometer AC250, AC300, or AC400. Solutions were prepared in deuteriochloroform ($CDCl_3$), deuterated methanol (CD_3OD), or deuterated dimethyl sulfoxide (DMSO-d_6) with TMS as internal standard. ESI-MS and CI-MS spectra were obtained with a quadrupolar NERMAG R10-10 spectrometer. Infrared (IR) spectra were recorded on a Perkin-Elmer IR-881 spectrometer. Optical rotations were measured with a Perkin-Elmer 241 polarimeter. Microanalyses were carried at the Ecole Nationale Superieure de Chimie at Paul Sabatier University in Toulouse (France), and all the values were within ±0.3% of the calculated compositions. High resolution mass spectrometry (HRMS) was performed with a Waters LCT spectrometer, and the observed mass yields referred to purified products and are not optimized. All moisture-sensitive reactions were carried out under an argon atmosphere using oven-dried glassware and anhydrous solvents. All the organic layers were dried using anhydrous magnesium sulfate. All test compounds showed ≥95% purity.

General Procedure for the 5,6-Epoxidation. *m*-Chloroperbenzoic acid (*m*CPBA) (4.25 mmol) in methylene chloride (10 mL) was added dropwise to a solution of the sterol or the steroid (2.5 mmol) in methylene chloride (25 mL) over a period of 1 min at room temperature. The reaction mixture was stirred over 20 min and then washed with aqueous sodium sulfite (10%) and sodium hydrogenocarbonate (5%) and dried over anhydrous sodium carbonate. The solvents were evaporated under reduced pressure to give the crude product.

5,6α-Epoxycholestan-3*β*-ol (1). Recrystallization of the crude product in acetone/water (9:1) gave the α-epoxide **1** as white needles (0.58 g, 58%): mp 140−141 °C (lit. 142 °C); R_f[EtOAc] = 0.54; NMR δ_H (300 MHz, $CDCl_3$) 3.84 (1H, m), 2.83 (1H, d, J = 8.6 Hz), 0.99 (3H, s), 0.83 (3H, d, J = 6.5 Hz), 0.81 (3H, d, J = 1.5 Hz), 0.78 (3H, d, J = 1.5 Hz), 0.54 (3H, s); NMR δ_C (75 MHz, $CDCl_3$) 68.68, 65.76, 59.33, 56.85, 55.87, 42.57, 42.34, 39.86, 39.49, 39.41, 36.14, 35.76, 34.86, 32.41, 31.06, 29.89, 28.82, 28.07, 27.99, 24.05, 23.84, 22.81, 22.55, 20.64, 18.64, 15.93, 11.86; m/z 403.6 $[M + H]^+$, 420.6 $[M + NH_4]^+$.

5,6α-Epoxycholest-7-en-3*β*-ol (2). Recrystallization of the crude product in acetone produced white needles of the α-epoxide (0.7 g, 70%): mp 146−148 °C (lit. 144−146 °C[33]); $[\alpha]_D$ −113 (lit. −112) (c 0.59, $CHCl_3$);[33]) IR $CHCl_3$ 3620, 3440, 2955, 2875, 1643, 1465, 1382, 1038, 898, 868 cm^{-1}. R_f[EtOAc] = 0.64; NMR δ_H (400 MHz, $CDCl_3$) 5.45 (1H, d, J = 4.0 Hz), 3.84 (1H, m), 3.02 (1H, d, J = 4.1 Hz), 1.03 (3H, s), 0.92 (3H, d, J = 6.4 Hz), 0.88 (3H, d, J = 2.0 Hz), 0.87 (3H, d, J = 2.0 Hz), 0.55 (3H, s); NMR δ_C (100 MHz, $CDCl_3$) 148.29, 114.94, 68.60, 67.17, 56.05, 55.12, 54.77, 42.46, 41.56, 39.99, 39.69, 38.95, 36.33, 36.25, 35.14, 33.68, 31.39, 28.21, 27.95, 24.08, 23.41, 23.03, 22.76, 21.15, 19.01, 16.63, 12.01; m/z 401.6 $[M + H]^+$, 418.6 $[M + NH_4]^+$.

5,6α-Epoxystigmastan-3*β*-ol (3). Recrystallization of the crude product in acetone/water (9:1) gave the α-epoxide as white needles (0.54 g, 54%): mp 109−110 °C (lit. 109−110 °C[41]); $[\alpha]_D$ −40 (lit. −40[41]); IR (CCl_4), 3620, 3438, 2940, 2870, 1466, 1377, 1089, 1037, 966 cm^{-1}; R_f[EtOAc] = 0.65; NMR δ_H (300 MHz, $CDCl_3$) 4.00−3.80 (1H, m), 2.89 (1H, d, J = 4.4 Hz), 2.06 (1H, m), 2.00−1.75 (4H, m), 1.70−0.90 (28H, m), 0.89 (3H, d, J = 6.6 Hz), 0.84 (3H, t, J = 7.2 Hz), 0.82 (3H, d, J = 6.4 Hz), 0.80 (3H, d, J = 6.6 Hz), 0.61 (3H, s); NMR δ_C (75 MHz, $CDCl_3$) 68.74, 65.67, 59.29, 56.85, 55.78, 45.81, 43.45, 42.55, 42.33, 39.87, 39.40, 36.13, 34.85, 33.89, 32.40, 31.10, 30.16, 29.89, 29.13, 28.82, 28.09, 26.89, 26.08, 24.05, 23.04, 20.64, 19.82, 19.02, 18.69, 15.92, 11.96, 11.85; m/z 431.4 $[M + H]^+$, 448.4 $[M + NH_4]^+$.

5,6α-Epoxycholestan-3*β*-acetate (4). Evaporation of the solvent gave a white solid corresponding to a 70:30 mixture of α-**4** and *β*-epoxide (66%). The mixture was used without further purification for aminolysis: mp 112 °C; R_f[EtOAc] = 0.6; NMR δ_H (300 MHz, $CDCl_3$) 4.97 (1H, m), 2.90 (1H, d, J = 4.2 Hz), 2.03 (3H, s), 1.09 (3H, s), 0.92 (3H, d, J = 6.6 Hz), 0.89 (3H, d, J = 1.5 Hz), 0.86 (3H, d, J = 1.5 Hz), 0.63 (3H, s); NMR δ_C (75 MHz, $CDCl_3$) 170.17, 71.38, 63.55, 59.14, 56.77, 56.17, 50.96, 42.23, 42.27, 39.49, 37.99, 36.66, 36.12, 35.72, 34.98, 32.46, 32.13, 29.86, 28.76, 28.06, 27.22, 24.14, 22.81, 22.55, 21.92, 18.67, 17.02, 15.85, 12.20; m/z 445 $[M + H]^+$, 462 $[M + NH_4]^+$.

5,6α-Epoxycholestan-3*β*-butyrate (5). Evaporation of the solvent gave a white solid corresponding to a 70:30 mixture of α-**5** and *β*-epoxide (64%). The mixture was used without further purification for aminolysis: mp 86 °C; R_f[EtOAc] = 0.9; NMR δ_H (300 MHz, $CDCl_3$) 4.96 (1H, m), 2.88 (1H, d, J = 4.2 Hz), 2.23 (3H, m), 2.12 (1H, m), 1.07 (3H, s), 0.60 (3H, s); NMR δ_C (75 MHz, $CDCl_3$) 172.85, 71.12, 65.24, 59.87, 59.19, 56.80, 42.46, 42.35, 39.52, 39.40, 38.10, 36.52, 36.15, 35.79, 35.05, 32.16, 29.89, 28.79, 28.10, 28.03, 27.30, 24.07, 23.87, 22.84, 22.58, 20.61, 18.64, 18.54, 15.89, 13.64, 11.87; m/z 473 $[M + H]^+$, 490 $[M + NH_4]^+$.

5,6α-Epoxyandrostan-3*β*,17*β*-diol (6). The crude product was purified by column chromatography [EtOAc] to give **6** as a white powder (0.58 g, 55%): R_f[EtOAc] = 0.6; NMR δ_H (300 MHz, $CDCl_3$) 3.76 (1H, m), 3.56 (1H, t, J = 5.6 Hz), 2.95 (1H, d, J = 4.5 Hz), 1.13 (3H, s), 0.72 (3H, s); NMR δ_C (75 MHz, $CDCl_3$) 80.79, 67.76, 66.03, 59.24, 51.69, 43.03, 42.55, 39.21, 36.22, 34.73, 32,33, 30.44, 39.81, 29.09, 28.15, 22.72, 20.04, 14.90, 10.13; m/z 307.6 $[M + H]^+$, 329.6 $[M + Na]^+$.

***N*^{4}-(2-Cyanoethyl)-1,4-diaminobutane (7).** Acrylonitrile (1.19 g, 22.5 mmol) was added to a stirred solution of 1,4-diaminobutane (1.32 g, 15 mmol) in methanol (35 mL) at 0 °C. The mixture was stirred at room temperature overnight. The solvent was evaporated and the resulting oil purified by column chromatography [isopropylamine/MeOH/$CHCl_3$, 1:5:15] to give compound **7** as a colorless oil (1.01 g, 47%): R_f[isopropylamine/MeOH/$CHCl_3$, 1:5:15] = 0.5; NMR δ_H (250 MHz, $CDCl_3$) 2.92 (2H, t, J = 6.5 Hz), 2.70 (2H, t, J = 6.5 Hz), 2.65 (2H, t, J = 6.5 Hz), 2.52 (2H, t, J = 6.5 Hz), 1.45 (4H, m); NMR δ_C (75 MHz, $CDCl_3$) 118.94, 49.22, 45.24, 42.20, 31.51, 27.57, 18.93; m/z 142.2 $[M + H]^+$.

***N*^{4},*N*^{8}-Di-*tert*-butyloxycarbonyl-*N*^{4}-(2-cyanoethyl)-1,4-diaminobutane (8).** A solution of di-*tert*-butyl dicarbonate (895 mg, 4.1 mmol) in CH_2Cl_2 (3 mL) was added dropwise to solution of **7** (283 mg, 2 mmol) in CH_2Cl_2 (3 mL) at 0 °C. The mixture was stirred at room temperature for 4 h. Water (20 mL) was added, and the mixture was extracted with

CH_2Cl_2. The solvent was evaporated and the residue was purified by column chromatography [MeOH/$CHCl_3$, 1:3] to give compound **8** as a colorless oil (560 mg, 82%): R_f[MeOH/$CHCl_3$, 1:3] = 0.39; NMR δ_H (250 MHz, $CDCl_3$) 3.44 (2H, t, J = 6.8 Hz), 3.26 (2H, t, J = 6.8 Hz), 3.12 (2H, m), 2.60 (2H, m), 1.54−1.42 (4H, m), 1.44 (9H, s), 1.42 (9H, s); NMR δ_C (75 MHz, $CDCl_3$) 156.01, 155.41, 118.41, 80.47, 79.18, 47.24, 43.44, 40.03, 28.40, 27.37, 25.99, 16.96; m/z 342.4 $[M + H]^+$.

N^4,N^8-Di-*tert*-butyloxycarbonylspermidine (9). To a stirred solution of lithium aluminum hydride (7.12 mmol) in Et_2O (100 mL) at 0 °C, compound **8** (608 mg, 1.78 mmol) in Et_2O (50 mL) was added dropwise over a 30 min period. After 1 h, a 1 M aqueous solution of sodium hydroxide was added and the precipitate filtered. The organic phase was extracted, washed with brine, and dried over anhydrous sodium carbonate. The solvent was evaporated to give compound **9** as a colorless oil (400 mg, 65%): R_f[isopropylamine/MeOH/$CHCl_3$, 1:5:15] = 0.60; NMR δ_H (400 MHz, $CDCl_3$) 3.13 (6H, m), 2.70 (2H, t, J = 6.8 Hz), 1.66−1.26 (6H, m), 1.46 (9H, s), 1.44 (9H, s); NMR δ_C (100 MHz, $CDCl_3$) 156.21, 155.61, 79.65, 79.01, 46.74, 43.20, 40.44, 39.3, 31.80, 28.63, 27.64, 25.80; m/z 346.4 $[M + H]^+$.

N^3-(2-Cyanopropyl)-1,3-diaminopropane (10). Compound **10** was prepared as described above for compound **7**, using 1,3-diaminopropane (2 g, 2.25 mL, 27 mmol), 4-bromobutyronitrile (4.2 g, 29 mmol), and other reagents scaled accordingly. Evaporation of the solvent gave an oil that was purified by chromatography [isopropylamine/MeOH/$CHCl_3$, 1:5:15] to give compound **10** as a colorless oil (991 mg, 26%): R_f[isopropylamine/MeOH/$CHCl_3$, 1:5:15] = 0.32; NMR δ_H (250 MHz, $CDCl_3$) 2.82 (2H, t, J = 6.8 Hz), 2.72 (2H, t, J = 6.8 Hz), 2.63 (2H, t, J = 6.5 Hz), 2.46 (2H, t, J = 6.5 Hz), 1.52 (4H, m); NMR δ_C (75 MHz, $CDCl_3$) 118.91, 49.20, 45.34, 42.22, 31.48, 27.56, 19.00; m/z 142.3 $[M + H]^+$.

N^1,N^3-Di-*tert*-butyloxycarbonyl-N^3-(3-cyanopropyl)-1,3-diaminopropane (11). **11** was prepared as compound **8** above, using compound **10** (353 mg, 2.5 mmol) and other reagents scaled accordingly. Evaporation of the solvent gave an oil that was purified by column chromatography [MeOH/$CHCl_3$, 1:3] to give compound **11** as a colorless oil (725 mg, 85%): R_f[MeOH/$CHCl_3$, 1:3] = 0.42; NMR δ_H (400 MHz, $CDCl_3$) 3.31 (4H, m), 3.12 (2H, m), 2.37 (2H, t, J = 7.2 Hz), 1.90 (2H, m), 1.70 (2H, m), 1.49 (9H, s), 1.45 (9H, s); NMR δ_C (100 MHz, $CDCl_3$) 146.95, 145.41, 120.02, 80.56, 79.46, 45.77, 37.70, 28.62, 27.61, 23.20, 14.95; m/z 342.4 $[M + H]^+$.

N^1,N^4-Di-*tert*-butyloxycarbonylspermidine (12). **12** was prepared as compound **9** above, using **11** (830 mg, 2.43 mmol) and other reagents scaled accordingly. Evaporation of the solvent gave compound **12** as a colorless oil (690 mg, 82%). R_f[isopropylamine/MeOH/$CHCl_3$, 1:5:15] = 0.60; NMR δ_H (400 MHz, $CDCl_3$) 3.26−3.12 (6H, m), 2.72 (2H, t, J = 6.8 Hz), 1.56−1.41 (6H, m), 1.47 (9H, s), 1.44 (9H, s); NMR δ_C (100 MHz, $CDCl_3$) 156.21, 155.61, 79.65, 79.01, 47.05, 43.60, 42.07, 37.72, 31.05, 28.82, 28.66, 26.22; m/z 346.3 $[M + H]^+$.

N^1,N^8-Di-*tert*-butyloxycarbonylspermidine (13). 2-(*tert*-Butoxycarbonyloxyimino)-2-phenylacetonitrile (508 mg, 2.7 mmol) in anhydrous THF (5 mL) was added dropwise to a stirred solution of spermine (150 mg, 1.03 mmol) in anhydrous THF (20 mL) at 0 °C. The mixture was stirred and allowed to rise to room temperature overnight. The solvent was then removed. The crude product was diluted in ethyl acetate (20 mL), extracted with 5% aqueous sodium hydroxide solution (3 times) and then water (3 times), and dried. The solvent was evaporated and the resulting oil was purified by chromatography [EtOAc/MeOH, 1:1] to give compound **13** as a light-yellow oil (213 mg, 60%). Further recrystallization from light petroleum gave the product as white crystals (139 mg, 39%): mp 84−85 °C (lit. 85.5−86.5 °C); R_f[EtOAc/aceton/acetic acid/water, 5:3:1:1] = 0.56; NMR δ_H (400 MHz, $CDCl_3$) 3.16−3.02 (4H, m), 2.60 (2H, t, J = 6.6 Hz), 2.54 (2H, t, J = 6.6 Hz), 1.59 (2H, m), 1.45 (4H, t, J = 6.6 Hz), 1.37 (18H, s); NMR δ_C (100 MHz, $CDCl_3$) 156.32, 79.16, 49.64, 47.95, 40.64, 39.44, 30.08, 28.65, 28.04, 27.58; m/z 346.4 $[M + H]^+$.

General Procedure for the Aminolysis of Epoxides. To a solution of the epoxide (**1**−**6**) (0.25 mmol) in anhydrous ethanol (3 mL) was added lithium perchlorate (0.75 mmol) and the amine (histamine, *Nω*-acetylhistamine, putrescine, **9**, **12**, or **13**) as a free base (1 mmol) dissolved in anhydrous ethanol (1 mL). The reaction mixture was stirred and refluxed for 4 days and periodically monitored by TLC (eluted with the appropriate solvent system). The solvent was evaporated, and the residue was diluted in ethyl acetate, washed three times with water, and dried. The solvent was then removed and the crude product purified by either column chromatography or reverse phase HPLC. Column chromatography was done at atmospheric pressure, using 60 Å silica (Aldrich) deactivated by mixing with 2.5% v/v triethylamine for 1 h and using a gradient of MeOH in EtOAc for elution.

5α-Hydroxy-6β-[2-(1*H*-imidazol-4-yl)ethylamino]cholestan-3β-ol (14). Compound **1** and histamine were used as reactants and treated as described in the general procedure. The crude product of the reaction was purified by column chromatography to give compound **14** as a white powder (69 mg, 54%). Compound **14** was further purified by RP-HPLC using an acetonitrile gradient (40% B for 8 min, then to 100% B in 20 min; A is 95:5 water/acetonitrile, 0.1% TFA; B is 95:5 acetonitrile/water 0.1% TFA) with a retention time of 18 min. The purity was ≥98%. $[\alpha]_D^{20}$ −13.0 (EtOH); R_f[MeOH/NH_3, 28%, 8:2] = 0.82; NMR δ_H (400 MHz, MeOD) 7.64 (1H, s), 6.90 (1H, s), 4.03 (1H, m), 3.10 (1H, m), 2.81 (3H, m), 2.52 (1H, m), 2.52 (1H, dt), 1.09 (3H, s), 0.94 (3H, d, J = 6.5 Hz), 0.90 (3H, d, J = 1.2 Hz), 0.89 (3H, d, J = 1.2 Hz), 0.72 (3H, s); NMR δ_C (100 MHz, MeOD) 135.75, 130.70, 118.06, 75.82, 67.10, 64.90, 56.47, 55.96, 49.35, 45.58, 42.78, 40.63, 40.23, 39.51, 38.30, 36.18, 35.94, 32.74, 30.45, 30.40, 29.47, 28.14, 27.96, 25.85, 23.99, 23.77, 22.03, 21.78, 18.06, 16.56, 11.56; HRMS m/z 514.7373 (calculated for $C_{32}H_{56}N_3O_2$ 514.7376).

5α-Hydroxy-6β-[2-(1*H*-imidazol-4-yl)ethylamino]cholest-7-en-3β-ol (15). Compound **2** and histamine were used as reactants and treated as described in the general procedure. The crude product of the reaction was purified by column chromatography to give compound **15** as a white powder (66 mg, 52%): $[\alpha]_D^{20}$ −45.3 (EtOH); R_f[MeOH/NH_3, 28%, 8:2] = 0.82; NMR δ_H (400 MHz, MeOD) 8.83 (1H, s), 7.52 (1H, s), 5.44 (1H, d, J = 4.0 Hz), 4.1 (1H, m), 3.62−3.50 (2H, m), 3.40 (1H, m), 3.37−3.31 (2H, m), 1.13 (3H, s), 1.01 (3H, d, J = 6.0 Hz), 0.93 (3H, d, J = 0.7 Hz), 0.91 (d, J = 0.7 Hz), 0.69 (3H, s); NMR δ_C (100 MHz, MeOD) 135.58, 130.70, 118.09, 110.10, 75.78, 67.64, 64.70, 56.19, 54.42, 47.92, 44.51, 40.30, 38.09, 33.50, 31.51, 24.10, 22.93, 22.15, 19.30, 18.13, 12.65; HRMS m/z 512.4205 (calculated for $C_{32}H_{54}N_3O_2$ 512.4211).

5α-Hydroxy-6β-[2-(1*H*-imidazol-4-yl)ethylamino]stigmastan-3β-ol (16). **3** and histamine were used as reactants and treated as described in the general procedure. The crude product was purified by column chromatography to give compound **16** as a white powder (61 mg, 45%): R_f[MeOH/NH_3, 28%, 8:2] = 0.82; NMR δ_H (300 MHz, MeOD) 8.64 (1H, s), 7.40 (1H, s), 4.11 (1H, m), 3.55 (1H, dt, J = 6.0, 3.0), 3.40 (1H, m), 3.30−3.19 (2H, m), 3.03 (1H, d, J = 6.6 Hz), 1.16 (3H, s), 0.79 (3H, s); NMR δ_C (75 MHz, MeOD) 118.67, 73.67, 66.17, 64.77, 55.99, 55.57, 45.88, 44.65, 42.67, 39.79, 39.39, 38.77, 37.85, 36.01, 32.26, 31.99, 31.36, 30.11, 29.93, 28.98, 27.21, 23.51, 22.75, 22.31, 20.86, 20.72, 19.16, 18.79, 17.99, 17.85, 15.09, 11.33; HRMS m/z 542.4670 (calculated for $C_{34}H_{60}N_3O_2$ 542.4680).

5α-Hydroxy-6β-[4-(2-Aminoethyl)imidazol-1-yl]cholestan-3β-ol (17). Compound **1** and *Nω*-acetylhistamine were used as reactants and treated as described in the general procedure. The crude product of the reaction was purified by column chromatography to give the N-acetylated-form of compound **17** as a white powder (39 mg, 28%): mp 170−172 °C; $[\alpha]_D^{20}$ −11.7 (EtOH); R_f[AcOEt/MeOH, 1:1] = 0.64; NMR δ_H (300 MHz, MeOD) 7.68 (1H, br), 7.05 (1H, br), 4.05 (1H, m), 3.98 (1H, d, J = 6.0 Hz), 3.42 (2H, t, J = 7.1 Hz), 2.74 (2H, t, J = 7.1 Hz), 1.19 (3H, s), 0.99 (3H, d, J = 6.6 Hz), 0.93 (3H, br), 0.91 (3H, br), 0.83 (3H, s), 0.75 (3H, s);

NMR δ_C (75 MHz, MeOD) 171.78, 137.69, 137.09, 117.25, 75.24, 66.63, 62.16, 56.25, 26.18, 44.79, 42.31, 41.69, 39.96, 39.34, 39.08, 38.02, 36.01, 35.76, 62.86, 32.57, 30.38, 30.12, 28.02, 27.79, 27.38, 23.72, 23.61, 21.87, 21.62, 21.26, 20.93, 17.87, 15.02, 11.48; *m/z* 556.45 $[M + H]^+$. The intermediate was then diluted in a mixture of ethanol (10 mL) and 1 N HCl (2 mL) and refluxed for 10 h. The solvent was then removed in vacuo, and the crude product was diluted in EtOAc, washed with brine, and dried. Removal of the solvent gave a yellow solid which was subsequently purified by column chromatography to give **17** as a white powder (21 mg, 58%): R_f[MeOH/NH_3, 28%, 8:2] = 0.72; NMR δ_H (300 MHz, MeOD) 9.04 (1H, s), 7.60 (1H, s), 4.18 (1H, d, J = 6.0 Hz), 4.08 (1H, m), 3.30 (2H, t, J = 7.1 Hz), 3.15 (2H, t, J = 7.1 Hz), 0.98 (3H, d, J = 6.3 Hz), 0.91 (3H, br), 0.89 (3H, br), 0.85 (3H, s), 0.76 (3H, s); NMR δ_C (75 MHz, MeOD) 129.20, 128.87, 121.32, 74.56, 66.33, 64.82, 56.31, 56.11, 44.54, 42.66, 40.86, 39.94, 39.28, 38.24, 37.71, 35.93, 32.55, 32.08, 30.02, 29.70, 27.93, 27.75, 23.55, 23.40, 22.32, 21.79, 21.54, 20.84, 17.79, 16.96, 15.24, 11.51; HRMS *m/z* 514.7357 (calculated for $C_{32}H_{56}N_3O_2$ 514.7376).

5α-Hydroxy-6β-[2-(1*H*-imidazol-4-yl)ethylamino]cholestan-3β-acetate (18). Compound **4** and histamine were used as reactants and treated as described in the general procedure. The crude product of the reaction was purified by column chromatography to give compound **18** as a white powder (55 mg, 40%): R_f[MeOH/NH_3, 28%, 8:2] = 0.84; NMR δ_H (300 MHz, MeOD) 7.57 (1H, s), 6.85 (1H, s), 5.17 (1H, m), 3.15−3.10 (1H, m), 2.99−2.92 (1H, m), 2.82 (2H, t, J = 6.3 Hz), 2.62 (1H, m), 1.91 (3H, s), 1.04 (3H, s), 0.84 (3H, d, J = 6.5 Hz), 0.79 (3H, d, J = 1.2 Hz), 0.77 (3H, d, J = 1.2 Hz), 0.64 (3H, s); NMR δ_C (75 MHz, MeOD) 169.17, 136.05, 134.81, 114.75, 74.97, 71.25, 64.85, 56.19, 55.62, 49.55, 46.76, 42.63, 40.16, 39.92, 39.29, 38.03 35.95, 35.72, 32.40, 30.15, 28.66, 28.21, 27.90, 27.75, 23.92, 23.64, 23.55, 21.82, 21.57, 20.81, 19.93, 17.84, 15.99, 11.44; HRMS *m/z* 546.4459 (calculated for $C_{34}H_{58}N_3O_3$ 556.4473).

5α-Hydroxy-6β-[2-(1*H*-imidazol-4-yl)ethylamino]cholestan-3β-butyrate (19). Compound **5** and histamine were used as reactants and treated as described in the general procedure. The crude product of the reaction was purified by column chromatography to give compound **19** as a white powder (61 mg, 42%): R_f[EtOAc/MeOH, 8:2] = 0.43; NMR δ_H (300 MHz, MeOD) 7.45 (1H, s), 6.73 (1H, s), 5.12 (1H, m), 2.91 (1H, m), 2.71−2.65 (3H, m), 2.35 (1H, m), 2.18 (2H, t, J = 7.0 Hz), 1.08 (3H, s), 0.89 (3H, d, J = 6.5 Hz), 0.80 (3H, d, J = 1.2 Hz), 0.78 (3H, d, J = 1.2 Hz), 0.61 (3H, s); HRMS *m/z* 584.4771 (calculated for $C_{36}H_{62}N_3O_3$ 584.4786).

5α-Hydroxy-6β-[2-(1*H*-imidazol-4-yl)ethylamino]androstan-3β,17-diol (20). Compound **6** and histamine were used as reactants and treated as described in the general procedure. The crude product of the reaction was purified by column chromatography to give compound **20** as a white powder (44 mg, 42%): R_f[MeOH/NH_3 28% 8:2] = 0.77; NMR δ_H (300 MHz, MeOD) 4.09 (1H, m), 3.62 (1H, m), 3.49 (2H, m), 3.03 (2H, t, J = 6.8 Hz), 2.91 (1H, m), 1.18 (3H, s), 0.82 (3H, s); NMR δ_C (75 MHz, MeOD) 136.58, 129.11, 113.53, 80.83, 74.04, 66.32, 34.91, 50.10, 49.93, 45.10, 43.05, 39.95, 38.12, 37.40, 36.59, 32.37, 30.20, 30.11, 29.20, 26.96, 22.66, 20.42, 15.68, 10.64. HRMS *m/z* 418.3068 (calculated for $C_{24}H_{40}N_3O_3$ 418.3064).

5α-Hydroxy-6β-(4-aminobutylamino)cholestan-3β-ol (21). Compound **1** and 1,4-diaminobutane were used as reactants and treated as described in the general procedure. The crude product was purified by column chromatography to give compound **21** as a white powder (43 mg, 35%): R_f[MeOH/NH_3, 28%, 8:2] = 0.45; NMR δ_H (300 MHz, $CDCl_3$) 3.99 (1H, m), 2.81 (2H, t, J = 7.5 Hz), 2.67 (1H, m), 2.43 (1H, m), 2.34 (1H, m), 1.06 (3H, s), 0.87 (3H, d, J = 6.3 Hz), 0.83 (3H, d, J = 0.9 Hz), 0.81 (3H, d, J = 0.9 Hz), 0.66 (3H, s); NMR δ_C (75 MHz, $CDCl_3$) 67.52, 63.77, 58.34, 56.41, 55.79, 47.87, 45.66, 42.75, 41.29, 40.66, 40.02, 39.53, 38.46, 36.24, 35.92, 33.01, 30.64, 30.41, 29.80, 28.29, 28.10, 27.91, 27.10, 24.37, 22.88, 22.47, 21.18, 18.65, 17.47, 12.27; HRMS *m/z* 491.4577 (calculated for $C_{31}H_{59}N_2O_2$ 491.4571).

5α-Hydroxy-6β-(4-aminobutylamino)cholest-7-en-3β-ol (22). Compound **2** and 1,4-diaminobutane were used as reactants and treated as described in the general procedure. The crude product was purified by column chromatography to give compound **22** as a white powder (62 mg, 51%): $[\alpha]_D^{20}$ −43.6 (EtOH); R_f[MeOH/NH_3, 28%, 8:2] = 0.36; NMR δ_H (300 MHz, MeOD) 5.40 (1H, br), 4.01 (1H, m), 2.93−2.82 (1H, m), 2.85 (2H, t, J = 7.2 Hz), 2.67−2.57 (2H, m), 1.03 (3H, s), 0.99 (3H, d, J = 6.3 Hz), 0.92 (3H, d, J = 0.9 Hz), 0.90 (3H, d, J = 0.9 Hz), 0.64 (3H, s); NMR δ_C (75 MHz, MeOD) 139.78, 117.55, 76.54, 64.04, 54.42, 49.59, 43.31, 43.14, 40.13, 39.99, 39.51, 39.31, 37.07, 31.12, 35.90, 32.80, 30.43, 27.78, 27.63, 27.60, 27.19, 23.59, 22.75, 21.84, 21.63, 24.56, 18.04, 11.16; HRMS *m/z* 489.4401 (calculated for $C_{31}H_{57}N_2O_2$ 489.4415).

5α-Hydroxy-6β-[3-(4-aminobutylamino)propylamino]cholestan-3β-ol (23). Compounds **1** and **9** were used as reactants and treated as described in the general procedure. The crude product was purified by column chromatography to give the intermediate as a yellow powder (36 mg, 20%): R_f[EtOAc] = 0.51; NMR δ_H (300 MHz, MeOD) 4.06 (1H, m), 3.35−3.23 (7H, m), 3.08 (2H, t, J = 6.8 Hz), 1.50 (9H, s), 1.46 (9H, s), 1.16 (3H, s), 0.96 (3H, d, J = 6.6 Hz), 0.92 (3H, d, J = 1.2 Hz), 0.90 (3H, d, J = 1.2 Hz), 0.75 (3H, s); NMR δ_C (75 MHz, MeOD) 157.17, 154.71, 79.74, 78.45, 66.95, 65.75, 56.28, 55.90, 46.89, 46.21, 45.43, 42.59, 40.49, 40.06, 39.63, 39.31, 38.14, 35.97, 35.75, 32.28, 30.22, 29.39, 27.95, 27.77, 27.43, 27.42, 27.01, 25.62, 25.14, 23.90, 23.56, 21.81, 21.56, 20.88, 17.83, 16.22, 11.37; *m/z* 748.80 $[M + H]^+$, 770.85 $[M + Na]^+$. The intermediate was then diluted in a DCM/TFA (1:1 v/v) mixture and stirred at room temperature for 1 h. The reaction was quenched with 5 mL water. The aqueous layer was basified with 5% aqueous sodium hydrogenocarbonate solution and extracted with butanol and the organic layer dried. Evaporation of the solvent gave compound **23** as a yellow powder (83 mg, 71%): R_f[isopropanol/NH_3, 28%/water, 7:3:1] = 0.62; NMR δ_H (300 MHz, $CDCl_3$) 4.10 (1H, m), 3.18−2.98 (8H, m), 1.12 (3H, s), 0.96 (3H, d, J = 6.6 Hz), 0.92 (3H, d, J = 0.9 Hz), 0.89 (3H, d, J = 0.9 Hz), 0.76 (3H, s); NMR δ_C (75 MHz, $CDCl_3$) 73,66, 66.28, 64.36, 56.13, 55.69, 46.91, 46.03, 44.64, 44.47, 42.67, 39.88, 39.29, 38.55, 37.76, 35.94, 35.71, 34.39, 32.05, 30.05, 29.81, 27.89, 27.75, 27.23, 24.11, 23.55, 23.43, 22.73, 21.95, 21.82, 21.57, 20.75, 17.82, 14.92, 11.30; HRMS *m/z* 548.5141 (calculated for $C_{34}H_{66}N_3O_2$ 548.5150).

5α-Hydroxy-6β-[3-(4-aminobutylamino)propylamino]cholest-7-en-3β-ol (24). Compounds **2** and **9** were used as reactants and treated as described in the general procedure. The crude product was purified by column chromatography to get the intermediate as a yellow powder (142 mg, 76%): R_f[EtOAc] = 0.38; NMR δ_H (300 MHz, MeOD) 5.40 (1H, br), 3.35−3.22 (4H, m), 3.07 (2H, t, J = 6.8 Hz), 2.85 (1H, m), 2.54 (2H, t, J = 6.6 Hz), 1.49 (9H, s), 1.46 (9H, s), 1.04 (3H, s), 0.99 (3H, d, J = 6.0 Hz), 0.92 (3H, d, J = 0.9 Hz), 0.90 (3H, d, J = 0.9 Hz), 0.64 (3H, s); NMR δ_C (75 MHz, MeOD) 157.15, 156.06, 139.74, 117.70, 79.44, 78.42, 76.58, 67.19, 64.13, 56.14, 54.44, 46.62, 45.19, 44.75, 43.42, 43.34, 43.17, 40.36, 39.97, 39.65, 39.32, 37.08, 36.12, 35.91, 32.88, 30.41, 27.79, 27.63, 27.46, 27.00, 23.60, 22.79, 21.85, 21.65, 21.61, 18.05, 17.55, 11.24; *m/z* 746.95 $[M + H]^+$. The intermediate was then treated as described for **23** above to give the desired product as a light-yellow powder (91 mg, 64%). Compound **24** was further purified by RP-HPLC using an acetonitrile gradient (25−100% B in 60 min; A is 95:5 water/acetonitrile, 0.1% TFA; B is 95:5 acetonitrile/water) with a retention time of 33 min. The purity was ≥98%. $[\alpha]_D^{20}$ −34.2 (EtOH); R_f[isopropanol/NH_3 28%/water, 7:3:1] = 0.62; NMR δ_H (300 MHz, $CDCl_3$) acidic form 5.44 (1H, d, J = 4.2 Hz), 4.11 (1H, m), 3.69 (1H, d, J = 4.2 Hz), 3.40 (2H, m), 3.26−3.15 (6H, m), 1.14 (3H, s), 1.03 (3H, d, J = 6.0 Hz), 0.96 (3H, d, J = 0.6 Hz), 0.93 (3H, d, J = 0.6 Hz), 0.73 (3H, s); NMR δ_C (75 MHz, $CDCl_3$) 149.13, 109.00, 73.83, 66.69, 62.77, 56.06, 54.84, 46.99, 45.39, 44.61, 43.76, 43.29, 43.15, 39.27, 38.66, 36.68, 36.00, 35.80, 32.20, 31.36, 30.07, 27.76, 27.48, 24.19,

23.53, 22.84, 22.25, 21.80, 21.55, 21.42, 17.95, 16.83, 11.34; HRMS m/z 546.4990 (calculated for $C_{34}H_{64}N_3O_2$ 546.4993).

5α-Hydroxy-6β-[4-(3-aminopropylamino)butylamino]cholestan-3β-ol (25). Compounds **1** and **12** were used as reactants and treated as described in the general procedure. The crude product was purified by column chromatography to give the intermediate as a yellow powder (116 mg, 62%); R_f[EtOAc] = 0.54; NMR δ_H (300 MHz, MeOD) 4.06 (1H, br), 3.34 (3H, m), 3.25 (4H, m), 3.06 (2H, t, J = 6.8 Hz), 1.49 (9H, s), 1.47 (9H, s), 1.15 (3H, s), 0.96 (3H, d, J = 6.6 Hz), 0.92 (3H, d, J = 0.9 Hz), 0.90 (3H, d, J = 0.9 Hz), 0.75 (3H, s); NMR δ_C (75 MHz, MeOD) 157.08, 156.06, 79.60, 78.57, 75.82, 66.97, 64.52, 56.29, 55.90, 49.01, 46.44, 45.43, 44.76, 44.21, 42.58, 40.41, 40.07, 39.31, 38.14, 37.56, 35.98, 35.75, 32.46, 30.29, 30.24, 37.96, 27.77, 27.43, 27.42, 23.90, 23.57, 21.83, 21.58, 20.89, 17.85, 16.29, 11.35; m/z 748.80 $[M + H]^+$, 770.85 $[M + Na]^+$. The intermediate was then treated as described for compound **23** above to give compound **25** as a light-yellow powder (23 mg, 64%): R_f[isopropanol/NH_3 28%/water, 7:3:1] = 0.61; NMR δ_H (300 MHz, $CDCl_3$) 4.04 (1H, m), 3.40−3.26 (7H, m), 3.10 (2H, t, J = 6.8 Hz), 1.16 (3H, s), 0.97 (3H, d, J = 6.8 Hz), 0.92 (3H, d, J = 0.6 Hz), 0.90 (3H, d, J = 0.9 Hz), 0.75 (3H, s); NMR δ_C (75 MHz, $CDCl_3$) 72,99, 67.08, 64.39, 56.30, 55.49, 46.81, 44.74, 44.42, 42.69, 39.99, 39.32, 38.35, 37.69, 35.94, 35.62, 34.67, 32.14, 30.17, 29.97, 27.89, 27.65, 27.22, 24.06, 23.66, 23.39, 22.73, 21.95, 21.82, 21.57, 17.82, 14.92, 11.30; HRMS m/z 548.5139 (calculated for $C_{34}H_{64}N_3O_2$ 548.5150).

5α-Hydroxy-6β-[4-(3-aminopropylamino)butylamino]cholest-7-en-3β-ol (26). Compounds **2** and **12** were used as reactants and treated as described in the general procedure. The crude product was purified by column chromatography to give the intermediate as a yellow powder (90 mg, 48%): R_f[EtOAc] = 0.38; NMR δ_H (300 MHz, MeOD) 5.39 (1H, br), 4.06 (1H, m), 3.25 (4H, m), 3.07 (2H, t, J = 6.8 Hz), 2.62 (1H, m), 2.05 (2H, m), 1.49 (9H, s), 1.47 (9H, s), 1.15 (3H, s), 0.96 (3H, d, J = 6.3 Hz), 0.92 (3H, d, J = 0.9 Hz), 0.90 (3H, d, J = 0.9 Hz), 0.75 (3H, s); NMR δ_C (75 MHz, MeOD) 157.06, 156.06, 79.41, 78.59, 75.83, 66.98, 64.54, 56.30, 55.90, 49.02, 46.45, 45.43, 44.69, 42.59, 40.42, 40.08, 39.32, 38.15, 37.53, 35.99, 35.76, 32.48, 30.30, 31.25, 27.98, 27.77, 27.45, 27.44, 23.91, 23.58, 21.85, 21.61, 20.91, 17.88, 16.32, 11.38; m/z 746.95 $[M + H]^+$. The intermediate was then treated as described for compound **23** above to give compound **26** as a light-yellow powder (85 mg, 94%). It was further purified by RP-HPLC as described for compound **24** and presents a retention time of 32 min: $[\alpha]_D^{20}$(EtOH) −33.5; R_f[isopropanol/NH_3 28%/water, 7:3:1] = 0.63; NMR δ_H (300 MHz, $CDCl_3$) 5.40 (1H, br), 4.07 (1H, m), 3.76−3.46 (2H, m), 3.40 (1H, m), 3.21 (t, J = 6.8 Hz, 2H), 3.15 (t, J = 6.9 Hz, 2H), 3.04 (t, J = 6.8 Hz, 2H), 1.11 (s, 3H), 0.99 (d, J = 6 Hz, 3H), 0.92 (d, J = 0.9 Hz, 3H), 0.90 (d, J = 0.9 Hz, 3H), 0.69 (s, 3H); NMR δ_C (75 MHz, MeOD) 149.12, 109.00, 73.83, 66.39, 62.87, 62.76, 56.05, 54.83, 45.39, 44.61, 43.75, 43.14, 39.26, 39.13, 38.66, 36.67, 35.99, 35.80, 32.20, 30.07, 27.76, 27.47, 24.18, 23.53, 22.84, 22.61, 22.24, 21.79, 21.54, 21.41, 17.95, 16.84, 11.34; HRMS m/z 546.4999 (calculated for $C_{34}H_{64}N_3O_2$ 546.4993).

5α-Hydroxy-6β-[(4-aminobutyl)(3-aminopropyl)amino]cholest-7-en-3β-ol (27). Compounds **2** and **13** were used as reactants and treated as described in the general procedure. The crude product was purified by column chromatography to give the intermediate as a yellow powder (87 mg, 47%): R_f[EtOAc/MeOH, 3:1] = 0.90; NMR δ_H (300 MHz, MeOD) 5.37 (1H, br), 4.04 (1H, m), 3.33−3.17 (6H, m), 2.82 (1H, m), 2.10 (2H, m), 1.49 (9H, s), 1.47 (9H, s), 1.13 (3H, s), 0.94 (3H, d, J = 6.3 Hz), 0.90 (3H, d, J = 0.9 Hz), 0.88 (3H, d, J = 0.9 Hz), 0.73 (3H, s); NMR δ_C (75 MHz, $CDCl_3$) 157.06, 156.06, 79.41, 78.59, 73.83, 66.39, 62.87, 62.76, 56.05, 54.83, 49.02, 46.45, 45.43, 44.69, 42.59, 40.42, 40.08, 39.32, 38.15, 37.53, 35.99, 35.76, 32.48, 30.30, 31.25, 27.98, 27.77, 27.45, 27.44, 23.91, 23.58, 21.85, 21.61, 20.91, 17.88, 16.32, 11.38; m/z 746.85 $[M + H]^+$. The intermediate was then treated as described for compound **23** above to give the product as a light-yellow powder (62 mg, 71%). Compound **27** was further purified by RP-HPLC as described for compound **24** and presents a retention time of 26 min: R_f[isopropanol/NH_3 28%/water, 7:3:1] = 0.63; NMR δ_H (300 MHz, $CDCl_3$) acidic form 5.13 (1H, d, J = 3.9 Hz), 3.96 (1H, m), 3.51 (1H, d, J = 4.0 Hz), 3.40 (4H, m), 3.24 (4H, m), 1.10 (3H, s), 0.94 (3H, d, J = 6.4 Hz), 0.83 (6H, d, J = 0.6 Hz), 0.57 (3H, s); NMR δ_C (75 MHz, MeOD) 149.12, 109.00, 73.83, 66.39, 62.87, 62.76, 56.05, 54.83, 45.39, 44.61, 43.75, 43.14, 39.26, 39.13, 38.66, 36.67, 35.99, 35.80, 32.20, 30.07, 27.76, 27.47, 24.18, 23.53, 22.84, 22.61, 22.24, 21.79, 21.54, 21.41, 17.95, 16.84, 11.34; HRMS m/z 546.5006 (calculated for $C_{34}H_{64}N_3O_2$ 546.4993).

5α-Hydroxy-6β-*N*-{3-[4-(3-Aminopropylamino)butylamino]-propylamino}cholestan-3β-ol (28). Compound **1** and *N,N′*-bis-(3-aminopropyl)butane-1,4-diamine were used as reactants and treated as described in the general procedure. The crude product was purified by column chromatography to give the intermediate as a yellow powder (48 mg, 32%). Compound **28** was further purified by RP-HPLC using a acetonitrile gradient (0% B for 10 min, then to 100% B in 60 min; A is 95:5 water/acetonitrile, 0.1% TFA; B is 95:5 acetonitrile/water) with a retention time of 45 min: R_f[isopropanol/NH_3 28%/water, 7:3:1] = 0.37; NMR δ_H (300 MHz, MeOD) 4.11 (1H, m), 3.39−3.23 (2H, m), 3.19−3.06 (10H, m), 2.91 (1H, d, J = 1.5 Hz), 1.11 (3H, s), 0.97 (3H, d, J = 6.6 Hz), 0.92 (3H, d, J = 0.9 Hz), 0.90 (3H, d, J = 0.9 Hz), 0.77 (3H, s); NMR δ_C (75 MHz, MeOD) 73.67, 66.19, 64.76, 56.06, 55.58, 45.88, 44.65, 44.65, 42.67, 39.79, 39.37, 37.85, 31.99, 31.36, 30.11, 29.92, 28.98, 27.21, 22.31, 20.80, 20.73, 19.16, 17.85, 11.33; HRMS m/z 605.5709 (calculated for $C_{37}H_{73}N_4O_2$ 605.5728).

5α-Hydroxy-6β-*N*-{3-[4-(3-Aminopropylamino)butylamino]-propylamino}cholest-7-en-3β-ol (29). Compound **2** and *N,N′*-bis(3-aminopropyl)butane-1,4-diamine were used as reactants and treated as described in the general procedure. The crude product was purified by column chromatography to give the intermediate as a yellow powder (57 mg, 38%). Compound **29** was further purified by RP-HPLC as described for 1 h and presents retention time of 45 min: R_f[isopropanol/NH_3 28%/water, 7:3:1] = 0.37; NMR δ_H (300 MHz, MeOD) 5.34 (1H, br), 4.05 (1H, m), 3.38−3.21 (2H, m), 3.18−3.03 (11H, m), 1.06 (3H, s), 0.97 (3H, d, J = 6.0 Hz), 0.90 (3H, d, J = 0.6 Hz), 0.87 (3H, d, J = 0.9 Hz), 0.65 (3H, s); NMR δ_C (75 MHz, MeOD) 150.89, 110.13, 75.18, 67.67, 64.61, 58.37, 57.46, 56.21, 48.33, 48.26, 46.88, 45.97, 45.88, 45.18, 44.55, 40.70, 38.10, 37.85, 37.43, 37.23, 33.53, 31.55, 29.20, 28.86, 25.42, 24.96, 24.25, 23.96, 23.72, 23.22, 22.97, 22.85, 19.37, 18.42, 18.14, 12.68; HRMS m/z 603.5552 (calculated for $C_{37}H_{71}N_4O_2$ 603.5572).

Cell Culture. All cell lines used in this study were from the ATCC with the exception of TS/A (murine mammary adenocarcinoma) which was kindly provided by P. Lollini (Bologna, Italy). A549 (human lung pulmonary cancer cells), HT29 (human colon adenocarcinoma), MCF-7 (human breast ductal cells), TS/A (murine mammary adenocarcinoma), SW 620 (mouse colon cancer cells), and HCT-8 (human colon adenocarcinoma) cells were grown in RPMI 1640 medium supplemented with 1.2 mM glutamine, 5% fetal bovine serum, penicillin, and streptomycin (50 units/mL). U937 (human myeloid lymphoma), SH-SY5Y (human neuroblastoma), and SK-N-SH (human neuroblastoma) were grown in RPMI 1640 medium supplemented with 1.2 mM glutamine, 10% fetal bovine serum, penicillin, and streptomycin (50 units/mL). U87 (human glioblastoma), Neuro2A (mouse neuroblastoma), B16-F10 (mouse melanoma), SK-Mel-28 (human melanoma), NB4 (myeloid lymphoma), and KG1 (myeloid lymphoma) cells were grown in DMEM medium supplemented with 3.2 mM glutamine, 10% fetal bovine serum (FBS), penicillin, and streptomycin (50 units/mL). Cells were incubated in a humidified atmosphere with 5% CO_2 at 37 °C.

Cell Death Assays. Cells were seeded in RPMI with 5% FBS into 12-well plates at 30 000 cells/well. The cells were then treated with solvent vehicle (0.1% ethanol), 40 μM PBPE, or 10 μM Tx for 3 days. Treated cells were incubated in the presence

or in the absence of 2.5 μg/mL cycloheximide, 2.5 pg/mL actinomycin D, 100 μM z-VAD-fmk, 100 μM z-DEVD-fmk, 500 μM vitamin E, 1 mM *N*-acetylcysteine, 500 μM butylated hydroxytoluene, 10 mM 3-MA, or 50 nM Baf A1. Cell death was determined by trypan blue exclusion assay. Cells were scraped and resuspended in trypan blue solution (0.25% w/v in PBS) and counted in a Malassez cell under a light microscope.

Dendrite Outgrowth on U937. Cells were suspended at 1.1×10^6 cells/mL in RPMI medium supplemented with 10% of heat inactivated serum and cultured in 12-well plates with 10 ng/mL of PMA (Sigma) for 48 h. Adherent cells were washed with complete medium and then were treated with 100 ng/mL IL-4 and 100 ng/mL GM-CSF or with indicated compounds. Cultured cells were observed by phase-contrast microscopy for evidence of increasing size, formation of clusters, and neurite outgrowth. At the end of culture, cell count was measured on an automated cell counter (Sysmex, Milton Keynes, U.K.). Cytocentrifuge preparations of unmanipulated and cultured blasts were stained with Giemsa. Examination of these cytospin preparations allowed assessment of viability and maturation. Cells that had increased in size, acquired copious gray cytoplasm without cytoplasmic granules or vacuoles, and developed long cytoplasmic processes were defined as mature leukemic DCs morphologically.

Phenotypic Characterization by Immunocytochemistry. P19 cells were seeded into six-wells culture plates (10 000 cells/well) and treated for 24 h with the solvent vehicle or indicated compounds. Cultured cells were fixed for 10 min in 4% paraformaldehyde, washed with PBS, and permeabilized with PBS 1% BSA and 0.05% saponin for 10 min. Cells were saturated with PBS 3% BSA for 30 min at room temperature. Cells were then incubated with mouse polyclonal antibody to mouse MAP2A (Chemicon, MAB3418, 1/100), β3-tubulin (Sigma, T8660, 1/200), in PBS 1% BSA, 0.05% saponin for 1 h at room temperature. Slides were washed with PBS 0.1% BSA and 0.05% saponin and incubated for 1 h with TRITC-conjugated secondary antibody. The glass coverslips were mounted onto slides using fluorsafe (Calbiochem) as mounting medium. Cells were observed by fluorescence microscopy using a Zeiss LSM 510 microscope (Zeiss, Gottingen, Germany). Staining of MCF-7 cells for milk fat globulin was performed exactly as previously described using monoclonal antihuman milk fat globulin (MFG) from (Chemicon, ABA4087).[28]

Cell Cycle Analysis. Cell cycle analysis was performed by flow cytometry. Cells were plated onto 100 mm plates at a density of 250 000 cells and then treated with solvent vehicle or the indicated compounds for 24 and 48 h. Cells were trypsinized and washed once with phosphate-buffered saline (PBS), fixed in 70% ethanol, and stored at 4 °C for subsequent cell cycle analysis. Fixed cells were washed with PBS and incubated with PBS containing 100 μg/mL RNaseA for 30 min at 37 °C, then stained with 0.25% Tween-20 and 50 μg/mL propidium iodide (PI) for 30 min at 37 °C in the dark. The DNA content of 1×10^6 stained cells was analyzed by flow cytometry. The fractions of cells in the sub-G1, G0/G1, S, and G2/M phases were calculated using Cell Quest software (Becton Dickinson, Mansfield, MA).

Triglyceride Quantification. MCF-7 cells (5 million) were treated for 48 h with the solvent vehicle or tested compounds. Cells were then washed three times in PBS, and lipids were extracted by liquid extraction according to the method of Bligh and Dyer.[42] Triglycerides analyses were done according to previously published procedures.[28]

Assay for Neurite Protein. Cells were plated on inserts with a 3 μm pore membrane at their base coated with poly-D-lysine. The inserts were then placed into 24-well plates. During the 24 h incubation in the presence of the test compounds, neurons extended their neurites through the microporous membrane to the lower chamber. Membrane inserts were then removed, neurons were fixed in methanol, and the neurites were stained with a cresyl violet solution. Cell bodies were removed from the top of the membrane, and the dye was extracted from the neurites and quantified at 562 nm on a spectrophotometer.[43]

Melanin Measurement. Melanin measurement was done as previously described.[44] Briefly, cells were treated for 24 h with increasing concentrations of DDA, then were collected, washed with PBS, and centrifuged for 5 min at 1200 rpm. Pellets (corresponding to 2×10^6 cells) were solubilized in 200 μL of 1 M NaOH and incubated for 1 h at 80 °C. The melanin content was measured spectrophotometrically at 405 nm on a Labsystems FluoroSkan Ascent FL microplate fluorimeter.

Oil Red O and Staining Procedures. Cells were grown on glass coverslips and treated with drugs for 72 h and then fixed with 3.7% paraformaldehyde for 1 h at room temperature followed by washing twice with PBS (Euromedex) and stained with oil red O in 60% (v/v) isopropyl alcohol and hematoxylin. Quantification of lipid accumulation was achieved by extracting oil red O from the stained cells with isopropyl alcohol and measuring the extinction of the extract at 510 nm. The value obtained using a control culture was subtracted from the resulting values. The oil red O absorbance was corrected by costaining DNA with SYBR green dye (Molecular Probes) and quantified on a Labsystems FluoroSkan Ascent FL microplate fluorimeter. The cell number was determined from a standard curve.

Western Blotting. Cells were plated onto 100 mm plates at a density of 250 000 and then treated with indicated compounds for 48 h. Cells were lysed with 50 mM Tris, pH 7.4, 150 mM NaCl, 0.1% SDS, 1% deoxycholic acid, 1% Triton X-100, 1 mM PMSF, 1 mM EDTA, and a cocktail of protease and phosphatase inhibitors (Roche). For the detection of NMDAR1, proteins were separated on 7.5% SDS−PAGE gels, electrotransferred onto polyvinylidene difluoride membranes, and incubated overnight at 4 °C with the mouse antimouse NMDAR1 (1:1000) or the mouse antihuman glyceraldehyde 3-phosphate dehydrogenase (1:1000). Visualization was achieved with an Enhanced Chemiluminescence Plus kit (Amersham Biosciences), and fluorescence was measured by either autoradiography or using a PhosphorImager (Storm 840, Amersham Biosciences).

Proliferation Assays. Cells were seeded in the appropriate medium into 12-well plates at 30 000 per well. The cells were then treated for 3 days with increasing test drug concentrations (10 nM to 10 μM). The drugs and medium were changed after 48 h. Cells were harvested by trypsinization and counted on a Coulter counter. Experiments were repeated in triplicate.

Transmission Electron Microscopy. Cells were fixed with 2% glutaraldehyde in 0.1 M Sorensen phosphate buffer (pH 7.4) for 1 h and washed with the Sorensen phosphate buffer (0.1 M) for 12 h. The cells were then postfixed with 1% OsO_4 in Sorensen phosphate buffer (0.05 M Sorensen phosphate, 0.25 M glucose, 1% OsO_4) 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 previously described.[45]

Biostatistical Analysis. Values are the mean ± SE of three independent experiments each carried out in duplicate. Statistical analysis was carried out using a Student's *t* test for unpaired variables. 1 and 11 in the figures refer to statistical probabilities (P) of < 0.001 and < 0.0001, respectively, compared with control cells that received the solvent vehicle alone.

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Supporting Information Available: Results from elemental analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

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