Steroids 77 (2012) 1403–1418

Contents lists available at SciVerse ScienceDirect

Steroids

journal homepage: www.elsevier.com/locate/steroids

Solid-phase chemical synthesis and *in vitro* biological evaluation of novel 2β-piperazino-(20*R*)-5α-pregnane-3α,20-diol N-derivatives as anti-leukemic agents

Hajer Jegham [1], René Maltais [1], Philippe Dufour, Jenny Roy, Donald Poirier *

Laboratory of Medicinal Chemistry, Oncology and Genomic Unit, CHUQ-CHUL Research Center and Laval University, Québec, Canada G1V 4G2

ARTICLE INFO

Article history:
Received 22 May 2012
Received in revised form 17 July 2012
Accepted 26 July 2012
Available online 14 August 2012

Keywords:
Aminosteroid
Pregnane
Leukemia
Cell viability
Lymphocytes
Apoptosis

ABSTRACT

The steroid nucleus is an interesting scaffold for the development of new therapeutic agents. Within the goal of identifying anticancer agents, new pregnane derivatives were prepared by using a sequence of liquid and solid-phase reactions. After we dehydrated epi-allopregnanolone in one step with diethylaminosulfur trifluoride and generated a 2,3α-epoxide, the regio- and stereo-selective aminolysis of this epoxide enabled us to obtain a 2β-piperazino-pregnane, whose secondary amine was protected as N-Fmoc-derivative. Using the difference in reactivity between OHs 3 and 20, we linked the pregnane nucleus-selectively on the polystyrene diethylbutylsilane resin via the OH in position 20. We next achieved in parallel the coupling of an amino acid (1st level of diversity) and the coupling of a carboxylic acid (2nd level of diversity) to generate two libraries of pregnane derivatives. The compounds inhibited the HL-60 leukemia cell growth and the most potent were three compounds (PD, LPC-37 and LPC-48) with a L-proline as first level of diversity and a cyclohexyl-carbonyl, a naphthalene-2-carbonyl or a 3-acetylbenzoyl as second level of diversity. LPC-48 efficiently inhibited HL-60 cell proliferation with IC_{50} value of 1.9 μM and exhibited a low toxicity on normal peripheral blood lymphocytes (IC_{50} = 31 μM). These results encouraged us to further evaluate the biological activity of these new aminosteroids by investigating their preliminary mechanism of action.

1. Introduction

Leukemia starts in blood-forming tissue such as the bone marrow and causes large numbers of blood cells to be produced and enter the bloodstream [1]. Approximately 9000 deaths from acute myeloid leukemia were predicted for 2009 in the United States, almost all in adults [2]. The therapeutic approach to acute leukemia is usually chemotherapy by using anticancer agents; however, severe side-effects and complications such as serious infection and bleeding are major problems linked to this type of approach in the clinical setting. In particular, the side effects of current chemotherapeutic treatments are sometimes fatal, especially among older and immunocompromised patients. Further studies are still required to explore novel and safer potent anticancer agents for the treatment of leukemia [3].

The steroid nucleus constitutes an interesting scaffold for the development of new therapeutic agents [4–6]. However, until now, few steroidal compounds have been investigated for potential antileukemic activity. In 1992, a novel class of aminosteroids has incited interest owing to its ability to inhibit the proliferation of breast cancer cells *in vitro* [7]; a few years later, a C19-steroid derivative 2β-(4-methylpiperazinyl)-5α-androstane-3α,17β-diol was reported to inhibit the proliferation of HL-60 cells (human acute myeloid leukemia cells) and to promote cell differentiation [8]. Taking advantage of our expertise in steroid chemistry, we synthesized various 2β-derivatives of 5α-androstane-3α,17β-diol and investigated their antiproliferative potential on HL-60 cells [9–12]. From these compounds, the aminosteroid RM (Fig. 1) was one of the most potent compounds as it exhibited an IC_{50} value of 0.6–2.1 μM [11,12]. Interestingly, RM showed cytotoxicity on tumor cell lines but a very low cytotoxicity on normal cells [12]. In order to extend our structure–activity relationship study of this new family of cytotoxic agents, we generated pregnane derivatives that were closely related to the androstane derivative RM and bearing two levels of molecular diversity at position 2β (Fig. 1).

In this report, we have optimized the use of diethylaminosulfur trifluoride as dehydrating reagent, we described the chemical synthesis of two libraries containing 40 new pregnane derivatives and we presented data on their antiproliferative activity on HL-60 cells. The toxicity of three members belonging to the two libraries (PD, LPC-37 and LPC-48) was also evaluated on normal peripheral blood lymphocytes and two compounds were selected for a preliminary investigation of their mechanisms of action on HL-60 cells.

* Corresponding author. Address: Laboratory of Medicinal Chemistry, Oncology and Genomic Unit, CHUQ-CHUL Research Center (Bloc T), 2705 Laurier Boulevard, Québec, Canada G1V 4G2. Tel.: +1 418 654 2296; fax: +1 418 654 2761.
E-mail address: donald.poirier@crchul.ulaval.ca (D. Poirier).
[1] These authors contributed equally to this work.

0039-128X/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.steroids.2012.07.012

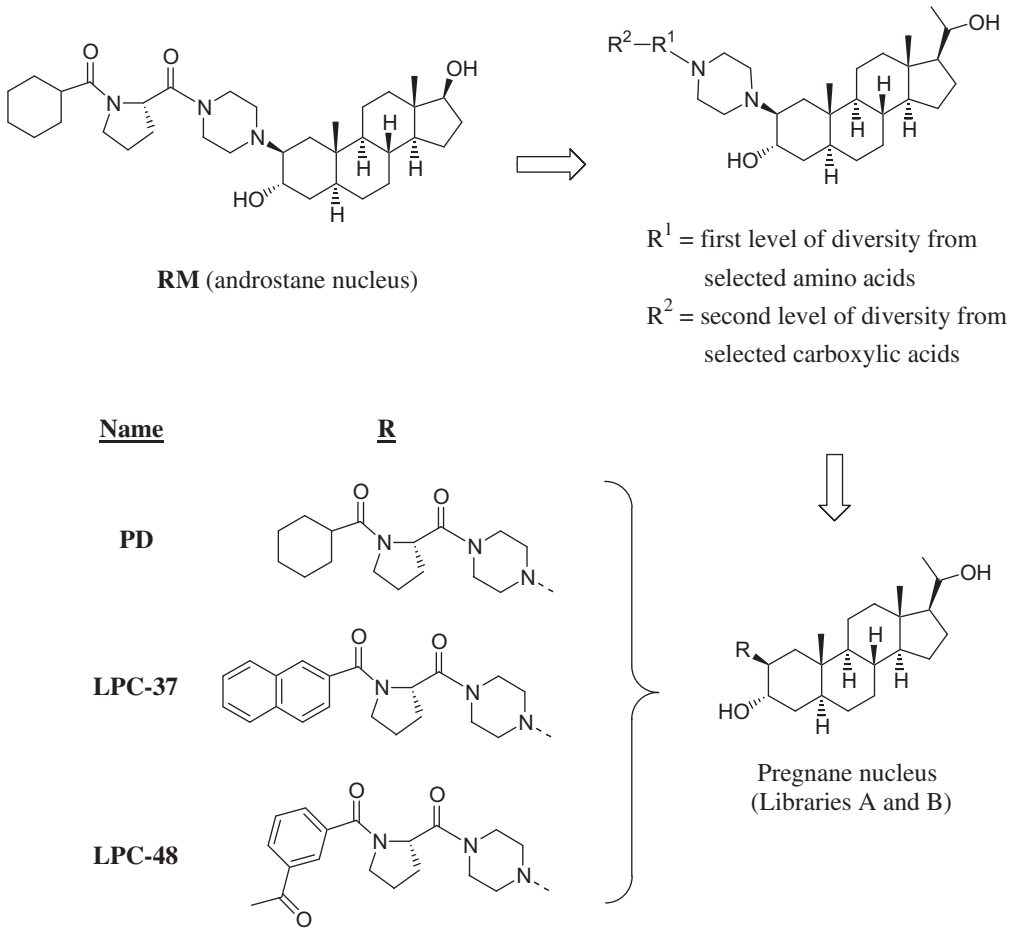


Fig. 1. Chemical structures of the androstane derivative RM (compound B10 or **14** reported in reference 11 or 12, respectively), the general representation of pregnane library members, and structures of three pregnane derivatives (PD, LPC-37 and LPC-48) selected for additional biological *in vitro* studies.

2. Material and methods

2.1. Chemical synthesis

2.1.1. General

Epi-allopregnanolone was purchased from Steraloids (Wilton, NH, USA). The polystyrene diethylbutylsilane (PS-DES) resin with a loading of 1.58 mmol/g was supplied by Argonaut Technologies (San Carlos, CA, USA). Chemical reagents were purchased from Sigma–Aldrich Canada Ltd. (Oakville, ON, Canada) and Calbiochem-Novabiochem Corp. (San Diego, CA, USA). The usual solvents were obtained from Fisher Scientific (Montréal, QC, Canada) and were used as received. Anhydrous dichloromethane (DCM) and dimethylformamide (DMF) were obtained from Sigma–Aldrich. The loading of steroid **5** on resin was performed in peptide synthesis vessels (25 mL) with frit equipped for vacuum filtration (ChemGlass Inc.; Vineland, NJ, USA). The reaction vessel was shaken with a Burrell wrist-action shaker model 75 (Pittsburgh, PA, USA). The libraries were synthesized in parallel fashion using an automated synthesizer (model 'The solution' from aapptec, Louisville, KY, USA) with a reaction block (Ares block) of 96 wells (4 mL per well). The work-up following final cleavage from the solid support was assisted with a separator phase syringe (10 mL) from Biotage (Charlottesville, VA, USA). Thin-layer chromatography (TLC) and flash-column chromatography were performed on 0.20-mm silica gel 60 F254 plates and with 230–400 mesh ASTM silica gel 60, respectively (E. Merck; Darmstadt, Germany). Melting points were recorded on an Electrothermal IA9300 SERIES Digital Melting Point Apparatus and are uncorrected. Infrared spectra (IR) by attenuated total reflectance (ATR) were recorded on ABB model MB3000 FT-IR spectrophotometer (Québec, Qc, Canada) and the significant band reported in cm^{-1}. Nuclear magnetic resonance (NMR) spectra were recorded at 400 MHz for ^{1}H and 100.6 MHz for ^{13}C on a Bruker Avance 400 digital spectrometer (Billerica, MA, USA). The chemical shifts (δ) were expressed in ppm and referenced to chloroform (7.26 and 77.0 ppm), acetone (2.05 and 29.2 ppm) or methanol (3.31 and 49.0 ppm), respectively, for ^{1}H and ^{13}C NMR. Low-resolution mass spectra (LRMS) were recorded on a PE Sciex API-150ex apparatus (Foster City, CA, USA) equipped with a turbo ion-spray source. High-resolution mass spectra (HRMS) were provided by Pierre Audet at the Laval University Chemistry Department (Québec, QC, Canada). The purity of selected aminosteroids LPC-37 and LPC-48 was determined by high-performance liquid chromatography (HPLC) (Waters Associates Milford, MA, USA) using a Nova Pak C18 reversed-phase column (150 mm × 3.9 mm id, 4 μm, 60 Å). The names of steroid derivatives were obtained using ACD/Labs (Chemist's version) software (Toronto, ON, Canada).

2.1.2. Synthesis of solid-phase precursor **5**

*2.1.2.1. (5α)-pregn-2-en-20-one (**1**).* To a solution of 5α-pregnan-3β-ol-20-one (3.75 g, 11.8 mmol) in anhydrous DCM (200 mL) at −78 °C under argon atmosphere was added diethylaminosulfur trifluoride (DAST) (2.30 mL, 17.7 mmol) and the resulting solution was stirred for 1 h at −78 °C. The solution was then directly evaporated in presence of silica gel and purified by flash chromatography with EtOAc/hexanes (2:98) as eluant to give **1** (880 mg, 25%) as a white solid (mp = 113–114 °C) with a ratio of C_2—C_3 vs. C_3—C_4 alkene isomer of 96:4. Data reported are for the major C_2—C_3 isomer.

IR (ATR) *v*: 1704 (C=O). ^{1}H NMR (acetone-d_6) δ: 0.60 (s, 18-CH_3), 0.78 (s, 19-CH_3), 0.75–2.15 (residual CH and CH_2), 2.07 (s, 21-CH_3), 2.60 (t, J = 9.1 Hz, 17α-CH), 5.57 (m, 2-CH and 3-CH). ^{13}C NMR ($CDCl_3$) δ: 11.7, 13.4, 20.9, 22.7, 24.4, 28.6, 30.2, 31.6, 31.7, 34.6, 35.6, 39.1, 39.7, 41.4, 44.2, 53.9, 56.7, 63.9, 125.8, 125.9, 209.8. LRMS for $C_{21}H_{32}O$ $[M+H]^+$: 301.1 *m*/*z*.

*2.1.2.2. (5α,20R)-pregn-2-en-20-ol (**2**).* To a solution of ketone **1** (860 mg, 2.86 mmol) in anhydrous DCM (120 mL) at −78 °C under argon atmosphere was slowly added DIBAL-H (1.0 M in hexane) (5.72 mL, 5.72 mmol). The solution was stirred at −78 °C for 3 h and successively washed with an aqueous HCl solution (5%) and a Rochelle salt solution. The organic phase was then filtered using a phase separator syringe (Biotage) and evaporated. Purification by flash chromatography (EtOAc/hexanes, 5:95) yielded 672 mg (78%) of **2** as a white solid (mp = 132–133 °C). IR (ATR) *v*: 3487 (OH). ^{1}H NMR ($CDCl_3$) δ: 0.76 (s, 18-CH_3 and 19-CH_3), 1.13 (d, J = 6.1 Hz, 21-CH_3), 0.70–2.10 (residual CH and CH_2), 3.73 (m, 20-CH), 5.58 (m, 2-CH and 3-CH). ^{13}C NMR ($CDCl_3$) δ: 11.7, 12.5, 20.8, 23.6, 24.4, 25.6, 28.7, 30.3, 31.9, 34.6, 35.4, 39.8, 40.1, 41.4, 42.4, 54.0, 55.9, 58.6, 70.6, 125.8, 126.0. LRMS for $C_{21}H_{33}$ $[MH-H_2O]^+$: 285.2 *m*/*z*.

*2.1.2.3. (2α,3α,5α,20R)-2,3-epoxypregnan-20-ol (**3**).* To a solution of alkene **2** (660 mg, 2.18 mmol) in anhydrous DCM (20 mL) at 0 °C under an atmosphere of argon was added in six portions *m*-chloroperbenzoic acid (*m*-CPBA) (733 mg, 3.27 mmol). The solution was stirred for 1 h at 0 °C and then allowed to return to room temperature overnight. The resulting solution was diluted with DCM (30 mL) and successively washed with a $Na_2S_2O_3$ aqueous solution (10%) and a Na_2CO_3 aqueous solution (10%), dried with $MgSO_4$, filtered and evaporated to dryness. Purification by flash chromatography (EtOAc/hexanes, 5:95) yielded 511 mg (73%) of **3** as a white solid (mp = 154–156 °C). IR (ATR) *v*: 3475 (OH). ^{1}H NMR (acetone-d_6) δ: 0.62 (CH), 0.74 (s, 18-CH_3), 0.79 (s, 19-CH_3), 1.07 (d, J = 6.1 Hz, 21-CH_3), 0.70–2.30 (residual CH and CH_2), 3.06 (m, 2 × CH of epoxide), 3.62 (m, 20-CH). ^{13}C NMR ($CDCl_3$) δ: 12.4, 12.9, 20.7, 23.6, 24.4, 25.6, 28.4, 29.0, 31.7, 33.6, 35.4, 36.2, 38.2, 39.9, 42.3, 51.1, 52.4, 53.7, 55.7, 58.5, 70.6. LRMS for $C_{21}H_{34}O_2$ $[M+H]^+$: 319.1 *m*/*z*.

*2.1.2.4. (2β,3α,5α,20R)-2-(piperazin-1-yl)-pregnane-3,20-diol (**4**).* To the epoxide **3** (500 mg, 1.57 mmol) were added piperazine (3.5 g, 40.6 mmol) and water (1 mL). The suspension was heated overnight at 150 °C and the resulting solution was cooled to room temperature, diluted with DCM (50 mL) and washed three times with water (100 mL). The organic layer was dried using a phase separator syringe (Biotage). Purification by flash chromatography (DCM/MeOH/triethylamine, 95:4:1) yielded 529 mg (83%) of **4** as a white solid (mp = 114 °C). IR (ATR) *v*: 3410 (OH and NH). ^{1}H NMR (acetone-d_6) δ: 0.75 (s, 18-CH_3), 0.99 (s, 19-CH_3), 1.07 (d, J = 6.1 Hz, 21-CH_3), 0.70–2.25 (residual CH and CH_2), 2.35, 2.50 and 2.77 (3 m, 4 × CH_2N and 2α-CH), 3.62 (m, 20-CH), 4.01 (m, 3β-CH). ^{13}C NMR (CD_3OD) δ: 12.8, 14.7, 22.0, 23.8, 25.5, 26.8, 29.2, 33.3, 34.3, 36.4, 37.2, 40.5, 41.1, 43.8, 46.7 (2×), 51.8 (2×), 57.0, 57.5, 59.4, 66.4, 66.9, 70.9. LRMS for $C_{25}H_{44}N_2O_2$ $[M+H]^+$: 405.4 *m*/*z*.

*2.1.2.5. 9H-fluoren-9-ylmethyl-4-[(2β,3α,5α,20R)-3,20-dihydroxypregnan-2-yl]piperazine-1-carboxylate (**5**).* To a solution of piperazino derivative **4** (520 mg, 1.27 mmol) in THF (12 mL) was added water (3 mL), $NaHCO_3$ (322 mg, 3.83 mmol) and Fmoc-*O*-succinimide (518 mg, 1.53 mmol). The solution was stirred overnight at room temperature. The resulting solution was diluted with EtOAc (50 mL), washed twice with water (70 mL), once with brine (30 mL), dried with $MgSO_4$, filtered and evaporated to dryness. Purification by flash chromatography (DCM/MeOH, 98:2) yielded 530 mg (65%) of **5** as a white solid (mp = 84–87 °C). IR (ATR) *v*: 3445 (OH) and 1709 (OCON). ^{1}H NMR (acetone-d_6) δ: 0.76 (s, 18-CH_3), 1.01 (s, 19-CH_3), 1.07 (d, J = 6.1 Hz, 21-CH_3), 0.70–1.75 (residual CH and CH_2), 2.21 (m, CH), 2.40 and 2.52 (2 m, 2 × CH_2N and 2α-CH), 3.41 (broad s, 2 × CH_2NCO), 3.65 (m, 20-CH), 4.04 (m, 3β-CH), 4.28 (t, J = 6.5 Hz, CH of Fmoc), 4.42 (d, J = 6.6 Hz, CH_2O of Fmoc), 7.34 (t, J = 7.4 Hz, 2 × CH of Fmoc), 7.42 (t, J = 7.4 Hz, 2 × CH of Fmoc), 7.67 (d, J = 7.3 Hz, 2 × CH of Fmoc), 7.87 (d, J = 7.5 Hz, 2 × CH of Fmoc). ^{13}C NMR ($CDCl_3$) δ: 12.6, 17.3, 21.2, 23.6, 24.4, 25.6, 28.3, 31.6, 32.8, 34.7, 35.4, 35.7, 38.4, 40.1, 42.6, 47.4, 48.0, 55.8, 56.0, 58.5, 63.7, 64.9, 67.2, 70.6, 120.0 (2×), 125.0 (2×), 127.0 (2×), 127.7 (2×), 141.3, (2×), 144.0 (2×), 155.1. LRMS for $C_{40}H_{54}N_2O_4$ $[M+H]^+$: 627.4 *m*/*z*.

*2.1.3. Solid-phase synthesis of pregnane derivatives **9** (libraries A and B)*

*2.1.3.1. 9H-fluoren-9-ylmethyl-4-[(2β,3α,5α,20R)-3,20-dihydroxypregnan-2-yl]piperazine-1-carboxylate loaded resin **6**.* 1,3-Dichloro-5,5-dimethylhydantoin (5.85 g, 29.7 mmol) in dry DCM (60 mL) was added to PS-DES resin (5.44 g, 1.58 mmol/g theoretical loading) that had been previously dried under vacuum over a period of two days, placed into a 100 mL peptide flask under argon and allowed to swell in dry DCM (25 mL). After a period of 1 h, the resulting chlorosilyl resin was washed under argon with dry DCM (3 × 75 mL). The disappearance of the Si-H band at 2100 cm^{-1} was confirmed by the IR spectrum. The resin was next used for the loading step. The chlorosilyl resin was swollen in dry DCM (20 mL) under argon atmosphere. A solution of imidazole (1.16 g, 17.0 mmol) and diol **5** (10.7 g, 16.8 mmol) in DCM (20 mL) was subsequently added. The mixture was vortexed overnight at room temperature using a Burrell wrist-action shaker. The loaded resin was washed with DCM (3 × 75 mL) and dried overnight under vacuum to provide 7.3 g of resin **6** (loading of 0.40 mmol/g). IR (KBr): *v* 3442 (OH, alcohol), 1702 (C=O, carbamate) cm^{-1}. The free diol **5** (8.7 g) was easily recovered after flash chromatography using EtOAc/hexanes (1:1) as eluent.

*2.1.3.2. Procedure for the preparation of resin-bound derivatives **7**.* A solution of piperidine in DCM (20% v/v) (70 mL) was added to resin **6** (7.3 g, 0.4 mmol/g) and the suspension was vortexed using a Burrell wrist-action shaker for 1 h at room temperature. The resin was then filtered and washed successively with DCM (5 × 75 mL) and MeOH (5 × 75 mL), and finally dried overnight to provide 6.5 g of Fmoc deprotected resin. The resin was divided into portions (1.80 g, 0.4 mmol/g in a 50 mL peptide flask). To each portion was added a solution of the appropriate amino acid (Fmoc-L-proline-OH (2.5 g, 7.5 mmol), Fmoc-D-proline-OH (2.5 g, 7.5 mmol), Fmoc-L-phenylalanine-OH (2.9 g, 7.5 mmol), Fmoc-D-phenylalanine-OH (2.9 g, 7.5 mmol) or Fmoc-L-tetrahydro-isoquinoline-3-carboxylic acid (3.0 g, 7.5 mmol), benzotriazole-yl-oxy-tris-pyrrolidinophosphonium hexafluorophosphate (PyBOP) (3.9 g, 7.5 mmol) and *N*-hydroxybenzotriazole (HOBt) (1.0 g, 7.5 mmol) in DMF (25 mL) under argon atmosphere. Diisopropylethylamine (DIPEA) (2.6 mL, 15 mmol) was added to the suspensions and the peptide flasks were vortexed with a Burrell wrist-action shaker for 5 h at room temperature. The resins were then filtered and washed successively with DCM (5 × 25 mL) and MeOH (5 × 25 mL), and finally dried overnight to give the resins **7**. The coupling reaction was repeated a second time in each case in order to ensure complete coupling.

*2.1.3.3. Procedure for the preparation of resin-bound derivatives **8**.* To each of the resin-bound derivatives **7** (2.5 g) was added 30 mL of a 20% (v/v) solution of piperidine in DCM (30 mL). The suspensions were vortexed with a Burrell wrist-action shaker for 1 h at room temperature. The resins were then filtered, washed successively with DCM (5 × 30 mL) and MeOH (5 × 30 mL) and finally dried

overnight to provide Fmoc deprotected resins. For library A, portions of 100 mg of the resins **7** were placed in tagged individual syringe reactor (3 × 4 = 12 syringes; tagged L-Pro, L-Phe, L-Tic). For library B, portions of 100 mg of the resin **7** of the appropriate resin-bound amino acid derivatives (L-Pro, D-Pro, L-Phe, D-Phe) were placed in reactor wells (4 mL) of the automated synthesizer reaction block (4 × 7 = 28 wells). To each reactor syringe (library A) or reactor well (library B) was successively added 0.7 mL of a 0.3 M solution of the appropriate carboxylic acid in DMF, 0.7 mL of a 0.3 M solution of PyBOP and HOBt in DMF, and finally 0.7 mL of 0.6 M solution of DIPEA in DMF. The resulting suspensions were vortexed at 600 rpm for 3 h under argon atmosphere. The well and syringe reactors were then filtered to remove the reaction solution from the resin. The resins were subsequently washed successively with DMF (2 × 3 mL), DCM (2 × 3 mL) and MeOH (2 × 3 mL). This procedure was repeated a second time for each of the resin-bound derivatives **8**.

*2.1.3.4. Cleavage of the resin-bound derivatives **8** to obtain library members of general formula **9**.* To each of the resin-bound derivatives **8** was added 2 mL of an acid solution of 2 M methanolic HCl (AcCl + MeOH) in DCM (20:80, v/v) and the resulting suspensions were vortexed at 600 rpm for 1 h. DCM (1 mL) was added and the suspensions were filtered and the recovered filtrate was neutralized with 0.5 mL of 10% aqueous $NaHCO_3$ (pH 8). The biphasic solution was filtered using a phase separator syringe (Biotage) and the resulting organic solution evaporated under reduced pressure. The 12 (3 × 4) crude amide compounds of library A (Table 2) were purified by filtration over a silica gel plug (10 mL) using EtOAc/hexanes (1:1) (15 mL) and then EtOAc (20 mL). In another experiment, the 28 (4 × 7) amide compounds of library B (Table 3) were evaporated to dryness and judged sufficiently pure by TLC and ^{1}H NMR analyses for direct screening on HL-60 cells. All members of libraries A and B were analyzed by TLC, ^{1}H NMR and LRMS.

2.1.3.5. Library A (12 aminosteroids; Table 2).

2.1.3.5.1. 1-[(2S)-2-({4-[(2β,3α,5α,20R)-3,20-dihydroxypregnan-2-yl]piperazin-1-yl}carbonyl)pyrrolidin-1-yl]-3-methylbutan-1-one (PA): 7.0 mg. ^{1}H NMR ($CDCl_3$) δ: 0.74 (s, 18-CH_3), 0.84 (s, 19-CH_3), 0.96 and 0.98 (2 d, J = 6.2 Hz, (CH_3)$_2$CH), 1.13 (d, J = 6.1 Hz, 21-CH_3), 0.69–2.28 (residual CH and CH_2), 2.35–2.75 (broad m, 2 × CH_2N and 2α-CH), 3.48 and 3.70 (2 m, 3 × CH_2NCO and 20-CH), 3.85 (m, 3β-CH), 4.89 (dd, J_1 = 3.3 Hz, J_2 = 8.1 Hz, NCHCO of Pro). LRMS for $C_{35}H_{60}N_3O_4$ [M+H]$^+$: 586.5 *m/z*.

2.1.3.5.2. N-[(2S)-1-{4-[(2β,3α,5α,20R)-3,20-dihydroxypregnan-2-yl]piperazin-1-yl}-1-oxo-3-phenylpropan-2-yl]-3-methylbutanamide (FA): 6.0 mg. ^{1}H NMR ($CDCl_3$) δ: 0.76 (s, 18-CH_3), 0.81 (s, 19-CH_3), 0.93 (d, J = 6.0 Hz, (CH_3)$_2$CH), 1.14 (d, J = 6.0 Hz, 21-CH_3), 0.70–1.85 (residual CH and CH_2), 2.10 and 2.53 (2 m, 2 × CH_2N, 2α-CH and CH_2CO), 2.93 and 3.04 (2 m, CH_2Ph), 3.30 and 3.74 (2 m, 2 × CH_2-NCO, 20-CH and 3β-CH), 5.17 (m, NHC*H*CO of Phe), 6.32 (d, J = 8.2 Hz, NH) 7.20 (m, 3 × CH of CH_2*Ph*), 7.28 (m, 2 × CH of CH_2-*Ph*). LRMS for $C_{39}H_{62}N_3O_4$ [M+H]$^+$: 636.5 *m/z*.

2.1.3.5.3. 1-[(3S)-3-({4-[(2β,3α,5α,20R)-3,20-dihydroxypregnan-2-yl]piperazin-1-yl}carbonyl)-3,4-dihydroisoquinolin-2(1H)-yl]-3-methylbutan-1-one (TA): 1.9 mg. ^{1}H NMR ($CDCl_3$) δ: 0.75 (s, 18-CH_3), 0.84 (s, 19-CH_3), 0.96 and 0.98 (2 d, J = 6.6 Hz, (CH_3)$_2$CH), 1.14 (d, J = 6.0 Hz, 21-CH_3), 0.69–1.70 (residual CH and CH_2), 1.75–2.70 (broad m, 2 × CH_2N, 2α-CH and CH_2CO), 3.05 (d, J = 6.1 Hz, CHCH_2Ar), 3.30–3.78 (broad m, 2 × CH_2NCO and 20-CH), 3.85 (3β-CH), 4.67 (q of AB system, J = 15.8 Hz, NCOCH_2Ar), 5.57 (t, J = 5.6 Hz, NHCHCO of Tic), 7.20 (m, 4 × CH of Ar). LRMS for $C_{40}H_{62}N_3O_4$ [M+H]$^+$: 648.4 *m/z*.

2.1.3.5.4. 3-cyclohexyl-1-[(2S)-2-({4-[(2β,3α,5α,20R)-3,20-dihydroxypregnan-2-yl]piperazin-1-yl}carbonyl)pyrrolidin-1-yl]propan-1-one (PC): 7.9 mg. ^{1}H NMR ($CDCl_3$) δ: 0.74 (s, 18-CH_3), 0.83 (s, 19-CH_3), 1.13 (d, J = 6.0 Hz, 21-CH_3), 0.69–2.25 (residual CH and CH_2), 2.32–2.75 (broad m, 2 × CH_2N, 2α-CH and CH_2CO), 3.50 and 3.70 (2 m, 3 × CH_2NCO and 20-CH), 3.85 (m, 3β-CH), 4.87 (dd, J_1 = 3.3 Hz, J_2 = 8.3 Hz, NCHCO of Pro). LRMS for $C_{38}H_{64}N_3O_4$ [M+H]$^+$: 626.5 *m/z*.

2.1.3.5.5. 3-cyclohexyl-N-[(2S)-1-{4-[(2β,3α,5α,20R)-3,20-dihydroxypregnan-2-yl]piperazin-1-yl}-1-oxo-3-phenylpropan-2-yl]propanamide (FC): 6.4 mg. ^{1}H NMR ($CDCl_3$) δ: 0.76 (s, 18-CH_3), 0.81 (s, 19-CH_3), 1.14 (d, J = 6.0 Hz, 21-CH_3), 0.69–1.70 (residual CH and CH_2), 2.20 (t, J = 7.8 Hz, CH_2CO), 2.15 and 2.54 (2 m, 2 × CH_2N and 2α-CH), 2.93 and 3.03 (2 m, CH_2Ph), 3.35 and 3.75 (2 m, 2 × CH_2NCO, 20-CH and 3β-CH), 5.17 (m, NHC*H*CO of Phe), 6.33 (d, J = 8.2 Hz, NH) 7.20 (m, 3 × CH of CH_2*Ph*), 7.28 (m, 2 × CH of CH_2*Ph*). LRMS for $C_{42}H_{66}N_3O_4$ [M+H]$^+$: 676.5 *m/z*.

2.1.3.5.6. 3-cyclohexyl-1-[(3S)-3-({4-[(2β,3α,5α,20R)-3,20-dihydroxypregnan-2-yl]piperazin-1-yl}carbonyl)-3,4-dihydroisoquinolin-2(1H)-yl]propan-1-one (TC): 1.4 mg. ^{1}H NMR ($CDCl_3$) δ: 0.75 (s, 18-CH_3), 0.84 (s, 19-CH_3), 1.14 (d, J = 6.0 Hz, 21-CH_3), 0.72–1.85 (residual CH and CH_2), 2.00–2.70 (broad m, 2 × CH_2N, 2α-CH and CH_2CO), 3.05 (d, J = 5.3 Hz, CHCH_2Ar), 3.30–3.78 (broad m, 2 × CH_2NCO and 20-CH), 3.86 (m, 3β-CH), 4.68 (q of AB system, J = 15.7 Hz, NCOCH_2Ar), 5.54 (t, J = 5.7 Hz, NHCHCO of Tic), 7.18 (m, 4 × CH of Ar). LRMS for $C_{43}H_{66}N_3O_4$ [M+H]$^+$: 688.3 *m/z*.

2.1.3.5.7. cyclohexyl[(2S)-2-({4-[(2β,3α,5α,20R)-3,20-dihydroxypregnan-2-yl]piperazin-1-yl}carbonyl) pyrrolidin-1-yl]methanone (PD): 8.0 mg. ^{1}H NMR ($CDCl_3$) δ: 0.74 (s, 18-CH_3), 0.84 (s, 19-CH_3), 1.13 (d, J = 6.1 Hz, 21-CH_3), 0.70–2.22 (residual CH and CH_2), 2.34–2.70 (broad m, 2 × CH_2N, 2α-CH and CH_2C*H*CO), 3.30–3.78 (broad m, 3 × CH_2NCO and 20-CH), 3.85 (m, 3β-CH), 4.87 (dd, J_1 = 3.5 Hz, J_2 = 8.2 Hz, NCHCO of Pro). ^{13}C NMR ($CDCl_3$) δ: 12.6, 17.1(17.2), 23.6, 24.4, 24.8, 25.6, 25.8, 28.3, 28.7, 28.8, 29.0, 31.6, 32.9, 34.6, 35.3, 35.8, 38.4, 40.1, 42.5, 42.6, 42.7, 46.0, 46.9, 48.3, 48.4, 52.5, 55.7, 55.8, 56.0, 58.5, 61.1, 63.8, 64.9, 70.6, 170.4, 179.4. LRMS for $C_{37}H_{62}N_3O_4$ [M+H]$^+$: 612.6 *m/z*.

2.1.3.5.8. N-[(2S)-1-{4-[(2β,3α,5α,20R)-3,20-dihydroxypregnan-2-yl]piperazin-1-yl}-1-oxo-3-phenylpropan-2-yl]cyclohexanecarboxamide (FD): 5.1 mg. ^{1}H NMR ($CDCl_3$) δ: 0.76 (s, 18-CH_3), 0.81 (s, 19-CH_3), 1.14 (d, J = 6.0 Hz, 21-CH_3), 0.70–1.87 (residual CH and CH_2), 2.15 and 2.54 (2 m, 2 × CH_2N, 2α-CH and CH_2C*H*CO), 2.93 and 3.02 (2 m, CH_2Ph), 3.30 and 3.74 (2 m, 2 × CH_2NCO, 20-CH and 3β-CH), 5.14 (m, NHC*H*CO of Phe), 6.35 (d, J = 8.0 Hz, NH), 7.20 (m, 3 × CH of CH_2*Ph*), 7.27 (m, 2 × CH of CH_2*Ph*). LRMS for $C_{41}H_{64}N_3O_4$ [M+H]$^+$: 662.5 *m/z*.

2.1.3.5.9. cyclohexyl[(3S)-3-({4-[(2β,3α,5α,20R)-3,20-dihydroxypregnan-2-yl]piperazin-1-yl}carbonyl)-3,4-dihydroisoquinolin-2(1H)-yl]methanone (TD): 1.2 mg. ^{1}H NMR ($CDCl_3$) δ: 0.75 (s, 18-CH_3), 0.84 (s, 19-CH_3), 1.14 (d, J = 5.9 Hz, 21-CH_3), 0.70–1.90 (residual CH and CH_2), 1.95–2.73 (broad m, 2 × CH_2N, 2α-CH and CH_2C*H*CO), 3.05 (d, J = 5.9 Hz, CHCH_2Ar), 3.30–3.78 (broad m, 2 × CH_2NCO and 20-CH), 3.86 (m, 3β-CH), 4.67 (q of AB system, J = 15.6 Hz, NCOCH_2-Ar), 5.54 (m, NHCHCO of Tic), 7.18 (m, 4 × CH of Ar). LRMS for $C_{42}H_{64}N_3O_4$ [M+H]$^+$: 674.3 *m/z*.

2.1.3.5.10. 1-[(2S)-2-({4-[(2β,3α,5α,20R)-3,20-dihydroxypregnan-2-yl]piperazin-1-yl}carbonyl)pyrrolidin-1-yl]nonan-1-one (PN): 8.5 mg. ^{1}H NMR ($CDCl_3$) δ: 0.75 (s, 18-CH_3), 0.84 (s, 19-CH_3), 0.87 (t, J = 6.8 Hz, CH_3CH$_2$), 1.13 (d, J = 6.0 Hz, 21-CH_3), 0.69–2.25 (residual CH and CH_2), 2.30 (m, CH_2CH_2CON), 2.35–2.70 (broad m, 2 × CH_2N and 2α-CH), 3.52 and 3.70 (2 m, 3 × CH_2NCO and 20-CH), 3.86 (m, 3β-CH), 4.87 (dd, J_1 = 3.3 Hz, J_2 = 8.2 Hz, NCHCO of Pro). LRMS for $C_{39}H_{68}N_3O_4$ [M+H]$^+$: 642.5 *m/z*.

2.1.3.5.11. N-[(2S)-1-{4-[(2β,3α,5α,20R)-3,20-dihydroxypregnan-2-yl]piperazin-1-yl}-1-oxo-3-phenylpropan-2-yl]nonanamide (FN): 5.2 mg. ^{1}H NMR ($CDCl_3$) δ: 0.76 (s, 18-CH_3), 0.81 (s, 19-CH_3), 0.88 (t, J = 6.7 Hz, CH_3CH$_2$), 1.14 (d, J = 6.0 Hz, 21-CH_3), 0.70–1.85 (residual CH and CH_2), 2.16 and 2.53 (2 m, 2 × CH_2N, 2α-CH and CH_2CH_2CON), 2.94 and 3.03 (2 m, CH_2Ph), 3.31 and 3.75 (2 m,

2 × CH_2NCO, 3β-CH and 20-CH), 5.16 (m, NHC*H*CO of Phe), 6.33 (d, *J* = 8.1 Hz, NH), 7.20 (m, 3 × CH of CH_2*Ph*), 7.28 (m, 2 × CH of CH_2-*Ph*). LRMS for $C_{43}H_{70}N_3O_4$ $[M+H]^+$: 692.5 *m*/*z*.

2.1.3.5.12. 1-[(3S)-3-({4-[(2β,3α,5α,20R)-3,20-dihydroxypregnan-2-yl]piperazin-1-yl}carbonyl)-3,4-dihydroisoquinolin-2(1H)-yl]nonan-1-one (TD): 2.1 mg. ^{1}H NMR ($CDCl_3$) δ: 0.75 (s, 18-CH_3), 0.85 (s, 19-CH_3), 0.88 (t, *J* = 6.7 Hz, CH_3CH$_2$), 1.14 (d, *J* = 6.0 Hz, 21-CH_3), 0.71–1.85 (residual CH and CH_2), 2.00–2.68 (broad m, 2 × CH_2N, 2α-CH and CH_2CH_2CON), 3.05 (d, *J* = 4.9 Hz, CHCH_2Ar), 3.32–3.74 (broad m, 2 × CH_2NCO and 20-CH), 3.86 (m, 3β-CH), 4.67 (q of AB system, *J* = 15.7 Hz, NCOCH_2Ar), 5.55 (m, NHCHCO of Tic), 7.18 (m, 4 × CH of Ar). LRMS for $C_{44}H_{70}N_3O_4$ $[M+H]^+$: 704.4 *m*/*z*.

2.1.3.6. Library B (28 aminosteroids; Table 3).

2.1.3.6.1. {4-[(2β,3α,5α,20R)-3,17-dihydroxypregnan-2-yl]piperazin-1-yl}[2S)-1(naphtalen-1-ylcarbonyl)pyrrolidin-2-yl]methanone (LPC-37): 10 mg. ^{1}H NMR ($CDCl_3$) δ: 0.73 (s, 18-CH_3), 0.84 (s, 19-CH_3), 1.12 (d, *J* = 6.1 Hz, 21-CH_3), 0.70–2.30 (residual CH and CH_2), 2.40–2.80 (broad m, 2 × CH_2N and 2α-CH), 3.40–3.95 (broad m, 3 × CH_2NCO, 20-CH and 3β-CH), 5.14 (dd, J_1 = 5.9 Hz, J_2 = 8.0 Hz, NCHCO of Pro), 7.53 (m, 2 × CH of Aryl), 7.67 (dd, J_1 = 1.3 Hz, J_2 = 8.3 Hz, CH of Aryl), 7.86 (m, 3 × CH of Aryl), 8.10 (s, CH of Aryl). LRMS for $C_{41}H_{58}N_3O_4$ $[M+H]^+$: 656.4 *m*/*z*. See Section 2.1.4 for additional characterization of this compound.

2.1.3.6.2. N-[(2S)-1-{4-[(2β,3α,5α,20R)-3,20-dihydroxypregnan-2-yl]piperazin-1-yl}-1-oxo-3-phenylpropan-2-yl]naphthalene-2-carboxamide (LFC-37): 7.0 mg. ^{1}H NMR ($CDCl_3$) δ: 0.75 (s, 18-CH_3), 0.82 (s, 19-CH_3), 1.14 (d, *J* = 6.1 Hz, 21-CH_3), 0.70–1.85 (residual CH and CH_2), 2.09, 2.21 and 2.57 (3 m, 2 × CH_2N and 2α-CH), 3.05–3.82 (4 m, 2 × CH_2NCO, CH_2Ph, 20-CH and 3β-CH), 5.43 (m, NCHCO of Phe), 7.28 (m, CH_2*Ph*), 7.56 (m, 2 × CH of Aryl), 7.90 (m, 4 × CH of Aryl), 8.31 (s, CH of Aryl). LRMS for $C_{45}H_{60}N_3O_4$ $[M+H]^+$: 706.6 *m*/*z*.

2.1.3.6.3. {4-[(2β,3α,5α,20R)-3,20-dihydroxypregnan-2-yl]piperazin-1-yl}[(2R)-1-(naphthalen-2-ylcarbonyl)pyrrolidin-2-yl]methanone (DPC-37): 8.5 mg. ^{1}H NMR ($CDCl_3$) δ: 0.73 (s, 18-CH_3), 0.84 (s, 19-CH_3), 1.13 (d, *J* = 6.0 Hz, 21-CH_3), 0.70–2.30 (residual CH and CH_2), 2.45–3.10 (broad m, 2 × CH_2N and 2α-CH), 3.30–3.95 (broad m, 3 × CH_2NCO, 20-CH and 3β-CH), 5.14 (dd, J_1 = 5.9 Hz, J_2 = 8.1 Hz, NCHCO of Pro), 7.52 (m, 2 × CH of Aryl), 7.67 (dd, J_1 = 1.3 Hz, J_2 = 8.5 Hz, CH of Aryl), 7.86 (m, 3 × CH of Aryl), 8.09 (s, CH of Aryl). LRMS for $C_{41}H_{58}N_3O_4$ $[M+H]^+$: 656.5 *m*/*z*.

2.1.3.6.4. N-[(2R)-1-{4-[(2β,3α,5α,20R)-3,20-dihydroxypregnan-2-yl]piperazin-1-yl}-1-oxo-3-phenylpropan-2-yl]naphthalene-2-carboxamide (DFC-37): 6.5 mg. ^{1}H NMR ($CDCl_3$) δ: 0.76 (s, 18-CH_3), 0.82 (s, 19-CH_3), 1.14 (d, *J* = 6.0 Hz, 21-CH_3), 0.70–1.85 (residual CH and CH_2), 2.07, 2.40 and 2.58 (3 m, 2 × CH_2N and 2α-CH), 3.05–3.80 (broad m, 2 × CH_2NCO, CH_2Ph, 20-CH and 3β-CH), 5.42 (m, NCHCO of Phe), 7.28 (m, CH_2*Ph*), 7.56 (m, 2 × CH of Aryl), 7.90 (m, 4 × CH of Aryl), 8.31 (s, CH of Aryl). LRMS for $C_{45}H_{60}N_3O_4$ $[M+H]^+$: 706.5 *m*/*z*.

2.1.3.6.5. 2-{[(2S)-2-({4-[(2β,3α,5α,20R)-3,20-dihydroxypregnan-2-yl]piperazin-1-yl}carbonyl)pyrrolidin-1-yl]carbonyl}-4H-chromen-4-one (LPC-38): 6.4 mg. ^{1}H NMR ($CDCl_3$) δ: 0.74 (s, 18-CH_3), 0.85 (s, 19-CH_3), 1.13 (d, *J* = 6.1 Hz, 21-CH_3), 0.70–2.35 (residual CH and CH_2), 2.30–2.78 (broad m, 2 × CH_2N and 2α-CH), 3.30–4.10 (broad m, 3 × CH_2NCO, 20-CH and 3β-CH), 5.02 (m, NCHCO of Pro), 6.84 (s, COCH═C—O), 7.44 (m, CH of coumarin), 7.51 (d, *J* = 8.5 Hz, CH of coumarin), 7.72 (t, *J* = 8.5 Hz, CH of coumarin), 8.21 (d, *J* = 8.0 Hz, CH of coumarin). LRMS for $C_{40}H_{56}N_3O_6$ $[M+H]^+$: 674.4 *m*/*z*.

2.1.3.6.6. N-[(2S)-1-{4-[(2β,3α,5α,20R)-3,20-dihydroxypregnan-2-yl]piperazin-1-yl}-1-oxo-3-phenylpropan-2-yl]-4-oxo-4H-chromene-2-carboxamide (LFC-38): 6.0 mg. ^{1}H NMR ($CDCl_3$) δ: 0.76 (s, 18-CH_3), 0.82 (s, 19-CH_3), 1.14 (d, *J* = 6.0 Hz, 21-CH_3), 0.70–1.90 (residual CH and CH_2), 2.00–2.60 (m, 2 × CH_2N and 2α-CH) 2.98–3.80 (broad m, 2 × CH_2NCO, 20-CH, 3β-CH and CH_2Ph), 5.33 (m, NHC*H*CO of Phe), 7.16 (s, COCH═C—O), 7.25 (m, 3 × CH of CH_2*Ph*), 7.31 (m, 2 × CH of CH_2*Ph*), 7.47 (t, *J* = 7.1 Hz, CH of coumarin), 7.58 (d, *J* = 8.5 Hz, CH of coumarin), 7.75 (t, *J* = 8.6 Hz, CH of coumarin), 7.84 (d, *J* = 8.0 Hz, NH), 8.22 (dd, J_1 = 1.6 Hz, J_2 = 8.0 Hz, CH of coumarin). LRMS for $C_{44}H_{58}N_3O_6$ $[M+H]^+$: 724.4 *m*/*z*.

2.1.3.6.7. 2-{[(2R)-2-({4-[(2β,3α,5α,20R)-3,20-dihydroxypregnan-2-yl]piperazin-1-yl}carbonyl)pyrrolidin-1-yl]carbonyl}-4H-chromen-4-one (DPC-38): 9.6 mg. ^{1}H NMR ($CDCl_3$) δ: 0.74 (s, 18-CH_3), 0.85 (s, 19-CH_3), 1.13 (d, *J* = 6.1 Hz, 21-CH_3), 0.70–2.40 (residual CH and CH_2), 2.45–2.90 (broad m, 2 × CH_2N and 2α-CH), 3.30–4.10 (broad m, 3 × CH_2NCO, 20-CH and 3β-CH), 5.03 (m, NCHCO of Pro), 6.84 (s, COCH═C—O), 7.45 (m, CH of coumarin), 7.51 (d, *J* = 8.2 Hz, CH of coumarin), 7.72 (t, *J* = 8.6 Hz, CH of coumarin), 8.21 (dd, J_1 = 1.6 Hz, J_2 = 8.0 Hz, CH of coumarin). LRMS for $C_{40}H_{56}N_3O_6$ $[M+H]^+$: 674.4 *m*/*z*.

2.1.3.6.8. N-[(2R)-1-{4-[(2β,3α,5α,20R)-3,20-dihydroxypregnan-2-yl]piperazin-1-yl}-1-oxo-3-phenylpropan-2-yl]-4-oxo-4H-chromene-2-carboxamide (DFC-38): 2.2 mg. ^{1}H NMR ($CDCl_3$) δ: 0.76 (s, 18-CH_3), 0.82 (s, 19-CH_3), 1.14 (d, *J* = 6.0 Hz, 21-CH_3), 0.68–2.20 (residual CH and CH_2), 2.08, 2.40 and 2.57 (3 m, 2 × CH_2N and 2α-CH), 2.95–3.80 (broad m, 2 × CH_2NCO, 20-CH, 3β-CH and CH_2Ph), 5.33 (m, NHC*H*CO of Phe), 7.17 (s, COCH═C—O), 7.30 (m, CH_2*Ph*), 7.47 (t, *J* = 7.6 Hz, CH of coumarin), 7.58 (d, *J* = 8.3 Hz, CH of coumarin), 7.75 (t, *J* = 8.6 Hz, CH of coumarin), 7.83 (d, *J* = 8.1 Hz, NH), 8.22 (dd, J_1 = 1.4 Hz, J_2 = 8.0 Hz, CH of coumarin). LRMS for $C_{44}H_{58}N_3O_6$ $[M+H]^+$: 724.4 *m*/*z*.

2.1.3.6.9. 6-{[(2S)-2-({4-[(2β,3α,5α,20R)-3,20-dihydroxypregnan-2-yl]piperazin-1-yl}carbonyl)pyrrolidin-1-yl]carbonyl}-2,2-dimethyl-2,3-dihydro-4H-pyran-4-one (LPC-39): 9.3 mg. ^{1}H NMR ($CDCl_3$) δ: 0.74 (s, 18-CH_3), 0.84 (s, 19-CH_3), 1.13 (d, *J* = 6.0 Hz, 21-CH_3), 1.50 (s, $(CH_3)_2$C), 0.70–2.25 (residual CH and CH_2), 2.30–2.75 (broad m, 2 × CH_2N, 2α-CH and CCH_2CO), 3.30–3.90 (broad m, 3 × CH_2NCO, 20-CH and 3β-CH), 4.90 (dd, J_1 = 4.5 Hz, J_2 = 7.4 Hz, NCHCO of Pro), 5.91 (s, C═CHCO). LRMS for $C_{38}H_{60}N_3O_6$ $[M+H]^+$: 654.4 *m*/*z*.

2.1.3.6.10. N-[(2S)-1-{4-[(2β,3α,5α,20R)-3,20-dihydroxypregnan-2-yl]piperazin-1-yl}-1-oxo-3-phenylpropan-2-yl]-2,2-dimethyl-4-oxo-3,4-dihydro-2H-pyran-6-carboxamide (LFC-39): 10 mg. ^{1}H NMR ($CDCl_3$) δ: 0.76 (s, 18-CH_3), 0.81 (s, 19-CH_3), 1.14 (d, *J* = 6.0 Hz, 21-CH_3), 1.48 and 1.49 (2 s, $(CH_3)_2$C), 0.70–1.88 (residual CH and CH_2), 2.12 and 2.54 (2 m, 2 × CH_2N, 2α-CH and CCH_2CO), 2.95–3.80 (broad m, 2 × CH_2NCO, 20-CH, 3β-CH and CH_2Ph), 5.20 (m, NHC*H*CO of Phe), 6.29 (s, C═CHCO), 7.22 (m, 3 × CH of CH_2*Ph*), 7.29 (m, 2 × CH of CH_2*Ph*), 7.51 (d, *J* = 8.3 Hz, NH). LRMS for $C_{42}H_{62}N_3O_6$ $[M+H]^+$: 704.4 *m*/*z*.

2.1.3.6.11. 6-{[(2R)-2-({4-[(2β,3α,5α,20R)-3,20-dihydroxypregnan-2-yl]piperazin-1-yl}carbonyl)pyrrolidin-1-yl]carbonyl}-2,2-dimethyl-2,3-dihydro-4H-pyran-4-one (DPC-39): 11 mg. ^{1}H NMR ($CDCl_3$) δ: 0.74 (s, 18-CH_3), 0.84 (s, 19-CH_3), 1.13 (d, *J* = 6.0 Hz, 21-CH_3), 1.49 and 1.50 (2s, $(CH_3)_2$C), 0.70–2.20 (residual CH and CH_2), 2.30–2.84 (broad m, 2 × CH_2N, 2α-CH and CCH_2CO), 3.25–3.90 (broad m, 3 × CH_2NCO, 20-CH and 3β-CH), 4.90 (m, NCHCO of Pro), 5.91 (s, C═CHCO). LRMS for $C_{38}H_{60}N_3O_6$ $[M+H]^+$: 654.5 *m*/*z*.

2.1.3.6.12. N-[(2R)-1-{4-[(2β,3α,5α,20R)-3,20-dihydroxypregnan-2-yl]piperazin-1-yl}-1-oxo-3-phenylpropan-2-yl]-2,2-dimethyl-4-oxo-3,4-dihydro-2H-pyran-6-carboxamide (DFC-39): 5.1 mg. ^{1}H NMR ($CDCl_3$) δ: 0.76 (s, 18-CH_3), 0.81 (s, 19-CH_3), 1.14 (d, *J* = 6.0 Hz, 21-CH_3), 1.48 and 1.50 (2 s, $(CH_3)_2$C), 0.68–1.85 (residual CH and CH_2), 2.10, 2.35 and 2.54 (3 m, 2 × CH_2N, 2α-CH and CCH_2CO), 2.95–3.80 (broad m, 2 × CH_2NCO, 20-CH, 3β-CH and CH_2Ph), 5.20 (m, NHC*H*CO of Phe), 6.29 (s, C═CHCO), 7.22 (m, 3 × CH of CH_2*Ph*), 7.30 (m, 2 × CH of CH_2*Ph*), 7.51 (d, *J* = 8.1 Hz, NH). LRMS for $C_{42}H_{62}N_3O_6$ $[M+H]^+$: 704.4 *m*/*z*.

2.1.3.6.13. 4-{[(2S)-2-({4-[(2β,3α,5α,20R)-3,20-dihydroxypregnan-2-yl]piperazin-1-yl}carbonyl)pyrrolidin-1-yl]carbonyl}benzonitrile (LPC-41): 12 mg. ^{1}H NMR ($CDCl_3$) δ: 0.74 (s, 18-CH_3), 0.84 (s, 19-

CH_3), 1.13 (d, J = 6.0 Hz, 21-CH_3), 0.68–2.30 (residual CH and CH_2), 2.45, 2.60 and 2.68 (3 m, 2 × CH_2N and 2α-CH), 3.35–3.90 (broad m, 3 × CH_2NCO, 20-CH and 3β-CH), 5.04 (dd, J_1 = 5.5 Hz, J_2 = 8.2 Hz, NCHCO of Pro), 7.69 (q of AB system 4 × CH of 4-CN-Ph-CO). LRMS for $C_{38}H_{55}N_4O_4$ $[M+H]^+$: 631.4 *m/z*.

2.1.3.6.14. 4-cyano-N-[(2S)-1-{4-[(2β,3α,5α,20R)-3,20-dihydroxypregnan-2-yl]piperazin-1-yl}-1-oxo-3-phenylpropan-2-yl]benzamide (LFC-41): 6.2 mg. ^{1}H NMR ($CDCl_3$) δ: 0.76 (s, 18-CH_3), 0.82 (s, 19-CH_3), 1.14 (d, J = 6.1 Hz, 21-CH_3), 0.70–1.88 (residual CH and CH_2), 2.10, 2.22 and 2.56 (3 m, 2 × CH_2N and 2α-CH), 3.00–3.84 (broad m, 2 × CH_2NCO, 20-CH, 3β-CH and CH_2Ph), 5.33 (m, NHCHCO of Phe), 7.16 (d, J = 7.9 Hz, NH), 7.23 (m, 3 × CH of CH_2*Ph*), 7.29 (m, 2 × CH of CH_2*Ph*), 7.74 and 7.88 (2 d, J = 8.4 Hz, 4 × CH of 4-CN-Ph-CO). LRMS for $C_{42}H_{57}N_4O_4$ $[M+H]^+$: 682.4 *m/z*.

2.1.3.6.15. 4-{[(2R)-2-({4-[(2β,3α,5α,20R)-3,20-dihydroxypregnan-2-yl]piperazin-1-yl}carbonyl)pyrrolidin-1-yl]carbonyl}benzonitrile (DPC-41): 14 mg. ^{1}H NMR ($CDCl_3$) δ: 0.73 (s, 18-CH_3), 0.84 (s, 19-CH_3), 1.13 (d, J = 6.0 Hz, 21-CH_3), 0.68–2.32 (residual CH and CH_2), 2.40–2.90 (broad m, 2 × CH_2N and 2α-CH), 3.35–3.90 (broad m, 3 × CH_2NCO, 20-CH and 3β-CH), 5.05 (dd, J_1 = 5.5 Hz, J_2 = 8.2 Hz, NCHCO of Pro), 7.69 (q of AB system, 4 × CH of 4-CN-Ph-CO). LRMS for $C_{38}H_{55}N_4O_4$ $[M+H]^+$: 631.4 *m/z*.

2.1.3.6.16. 4-cyano-N-[(2R)-1-{4-[(2β,3α,5α)-3,20-dihydroxypregnan-2-yl]piperazin-1-yl}-1-oxo-3-phenylpropan-2-yl]benzamide (DFC-41): 5.4 mg. ^{1}H NMR ($CDCl_3$) δ: 0.76 (s, 18-CH_3), 0.82 (s, 19-CH_3), 1.14 (d, J = 6.0 Hz, 21-CH_3), 0.72–1.85 (residual CH and CH_2), 2.10, 2.39 and 2.60 (3 m, 2 × CH_2N and 2α-CH), 3.00–3.80 (broad m, 2 × CH_2NCO, 20-CH, 3β-CH and CH_2Ph), 5.30 (m, NHC*H*CO of Phe), 7.26 (m, CH_2*Ph* and NH), 7.74 and 7.88 (2 d, J = 8.4 Hz, 4 × CH of 4-CN-Ph-CO). LRMS for $C_{42}H_{57}N_4O_4$ $[M+H]^+$: 681.5 *m/z*.

2.1.3.6.17. (2E)-1-[(2S)-2-({4-[(2β,3α,5α,20R)-3,20-dihydroxypregnan-2-yl]piperazin-1-yl}carbonyl)pyrrolidin-1-yl]-3-phenylprop-2-en-1-one (LPC-42): 11 mg. ^{1}H NMR ($CDCl_3$) δ: 0.75 (s, 18-CH_3), 0.85 (s, 19-CH_3), 1.13 (d, J = 6.0 Hz, 21-CH_3), 0.70–2.30 (residual CH and CH_2), 2.43 and 2.69 (2 m × CH_2N and 2α-CH), 3.40–3.97 (m, 3 × CH_2NCO, 20-CH and 3β-CH), 4.99 (dd, J_1 = 3.7 Hz, J_2 = 8.2 Hz, NCHCO of Pro), 6.77 (d, J = 15.5 Hz, CH=C*H*CO), 7.36 (m, 3 × CH of Aryl), 7.51 (m, 2 × CH of Aryl), 7.70 (d, J = 15.5 Hz, C*H*=CHCO). LRMS for $C_{39}H_{58}N_3O_4$ $[M+H]^+$: 632.4 *m/z*.

2.1.3.6.18. (2E)-N-[(2S)-1-{4-[(2β,3α,5α,20R)-3,20-dihydroxypregnan-2-yl]piperazin-1-yl}-1-oxo-3-phenylpropan-2-yl]-3-phenylprop-2-enamide (LFC-42): 4.5 mg. ^{1}H NMR ($CDCl_3$) δ: 0.75 (s, 18-CH_3), 0.81 (s, 19-CH_3), 1.14 (d, J = 6.0 Hz, 21-CH_3), 0.70–1.88 (residual CH and CH_2), 2.15 and 2.55 (2 m, 2 × CH_2N and 2α-CH), 2.96–3.80 (broad m, 2 × CH_2NCO, 20-CH, 3β-CH and CH_2Ph), 5.30 (m, NHC*H*CO of Phe), 6.42 (d, J = 15.6 Hz, CH=C*H*CO), 6.59 (m, NH), 7.25 (m, CH_2*Ph*), 7.37 (m, 3 × CH of Aryl), 7.51 (m, 2 × CH of Aryl), 7.63 (d, J = 15.6 Hz, C*H*=CHCO). LRMS for $C_{43}H_{60}N_3O_4$ $[M+H]^+$: 682.4 *m/z*.

2.1.3.6.19. (2E)-1-[(2R)-2-({4-[(2β,3α,5α,20R)-3,20-dihydroxypregnan-2-yl]piperazin-1-yl}carbonyl)pyrrolidin-1-yl]-3-phenylprop-2-en-1-one (DPC-42): 6.4 mg. ^{1}H NMR ($CDCl_3$) δ: 0.74 (s, 18-CH_3), 0.84 (s, 19-CH_3), 1.14 (d, J = 6.0 Hz, 21-CH_3), 0.72–2.30 (residual CH and CH_2), 2.40–2.90 (broad m, 2 × CH_2N and 2α-CH), 3.35–3.95 (broad m, 3 × CH_2NCO, 20-CH and 3β-CH), 5.00 (m, NCHCO of Pro), 6.77 (d, J = 15.5 Hz, CH=C*H*CO), 7.35 (m, 3 × CH of Aryl), 7.52 (m, 2 × CH of Aryl), 7.70 (d, J = 15.5 Hz, C*H*=CHCO). LRMS for $C_{39}H_{58}N_3O_4$ $[M+H]^+$: 632.5 *m/z*.

2.1.3.6.20. (2E)-N-[(2R)-1-{4-[(2β,3α,5α,20R)-3,20-dihydroxypregnan-2-yl]piperazin-1-yl}-1-oxo-3-phenylpropan-2-yl]-3-phenylprop-2-enamide (DFC-42): 4.1 mg. ^{1}H NMR ($CDCl_3$) δ: 0.76 (s, 18-CH_3), 0.81 (s, 19-CH_3), 1.14 (d, J = 6.0 Hz, 21-CH_3), 0.71–1.88 (residual CH and CH_2), 2.08, 2.38 and 2.57 (3 m, 2 × CH_2N and 2α-CH), 2.98–3.80 (m, 2 × CH_2NCO, 20-CH, 3β-CH and CH_2Ph), 5.30 (m, NCHCO of Phe), 6.42 (d, J = 15.6 Hz, CH=C*H*CO), 6.58 (d, J = 8.0 Hz, NH), 7.26 (m, CH_2*Ph*), 7.38 (m, 3 × CH of Aryl), 7.51 (m, 2 × CH of Aryl), 7.63 (d, J = 15.6 Hz, C*H*=CHCO). LRMS for $C_{43}H_{60}N_3O_4$ $[M+H]^+$: 682.4 *m/z*.

2.1.3.6.21. {4-[(2β,3α,5α,20R)-3,20-dihydroxypregnan-2-yl]piperazin-1-yl}[(2S)-1-{[(4S)-4-(prop-1-en-2-yl)cyclohex-1-en-1-yl]carbonyl}pyrrolidin-2-yl]methanone (LPC-46): 12 mg. ^{1}H NMR ($CDCl_3$) δ: 0.74 (s, 18-CH_3), 0.83 (s, 19-CH_3), 1.13 (d, J = 6.0 Hz, 21-CH_3), 1.74 (s, CH_2=CCH_3), 0.70–2.30 (residual CH and CH_2), 2.32–2.75 (m, 2 × CH_2N and 2α-CH), 3.30–3.90 (m, 3 × CH_2NCO, 20-CH and 3β-CH), 4.73 (d, J = 13.4 Hz, CH_2=CCH$_3$), 4.91 (t, J = 6.9 Hz, NCHCO of Pro), 6.14 (s, CH=C—CO). LRMS for $C_{40}H_{64}N_3O_4$ $[M+H]^+$: 650.5 *m/z*.

2.1.3.6.22. (4S)-N-[(2S)-1-{4-[(2β,3α,5α,20R)-3,20-dihydroxypregnan-2-yl]piperazin-1-yl}-1-oxo-3-phenylpropan-2-yl]-4-(prop-1-en-2-yl)cyclohex-1-ene-1-carboxamide (LFC-46): 7.5 mg. ^{1}H NMR ($CDCl_3$) δ: 0.76 (s, 18-CH_3), 0.81 (s, 19-CH_3), 1.14 (d, J = 6.0 Hz, 21-CH_3), 1.74 (s, CH_2=CCH_3), 0.72–2.20 (residual CH and CH_2), 2.22–2.60 (broad m, 2 × CH_2N and 2α-CH), 2.90–3.82 (broad m, 2 × CH_2NCO, 20-CH, 3β-CH and CH_2Ph), 4.74 (d, J = 16.2 Hz, CH_2=CCH$_3$), 5.23 (m, NHC*H*CO of Phe), 6.60 (d, J = 8.1 Hz, NH), 6.70 (s, CH=C—CO), 7.20 (m, 3 × CH of CH_2*Ph*), 7.29 (m, 2 × CH of CH_2*Ph*). LRMS for $C_{44}H_{66}N_3O_4$ $[M+H]^+$: 700.5 *m/z*.

2.1.3.6.23. {4-[(2β,3α,5α,20R)-3,20-dihydroxypregnan-2-yl]piperazin-1-yl}[(2R)-1-{[(4S)-4-(prop-1-en-2-yl)cyclohex-1-en-1-yl]carbonyl}pyrrolidin-2-yl]methanone (DPC-46): 6.2 mg. ^{1}H NMR ($CDCl_3$) δ: 0.74 (s, 18-CH_3), 0.84 (s, 19-CH_3), 1.13 (d, J = 6.0 Hz, 21-CH_3), 1.74 (s, CH_2=CCH_3), 0.70–2.30 (residual CH and CH_2), 2.32–2.87 (broad m, 2 × CH_2N and 2α-CH), 3.35–3.90 (broad m, 3 × CH_2NCO, 20-CH and 3β-CH), 4.73 (m, CH_2=CCH$_3$), 4.93 (d, J = 7.7 Hz, NCHCO of Pro), 6.12 (s, CH=C—CO). LRMS for $C_{40}H_{64}N_3O_4$ $[M+H]^+$: 650.6 *m/z*.

2.1.3.6.24. (4S)-N-[(2R)-1-{4-[(2β,3α,5α,20R)-3,20-dihydroxypregnan-2-yl]piperazin-1-yl}-1-oxo-3-phenylpropan-2-yl]-4-(prop-1-en-2-yl)cyclohex-1-ene-1-carboxamide (DFC-46): 2.6 mg. ^{1}H NMR ($CDCl_3$) δ: 0.76 (s, 18-CH_3), 0.81 (s, 19-CH_3), 1.14 (d, J = 6.0 Hz, 21-CH_3), 1.75 (s, CH_2=CCH_3), 0.70–2.20 (residual CH and CH_2), 2.25–2.62 (broad m, 2 × CH_2N and 2α-CH), 2.90–3.80 (broad m, 2 × CH_2NCO, 20-CH, 3β-CH and CH_2Ph), 4.74 (d, J = 16.2 Hz, CH_2=CCH$_3$), 5.22 (m, NHC*H*CO of Phe), 6.60 (d, J = 8.0 Hz, NH), 6.69 (s, CH=C—CO), 7.22 (m, 3 × CH of CH_2*Ph*), 7.29 (m, 2 × CH of CH_2*Ph*). LRMS for $C_{44}H_{66}N_3O_4$ $[M+H]^+$: 700.6 *m/z*.

2.1.3.6.25. 1-(3-{[(2S)-2-({4-[(2β,3α,5α,20R)-3,20-dihydroxypregnan-2-yl]piperazin-1-yl}carbonyl)pyrrolidin-1-yl]carbonyl}phenyl)ethanone (LPC-48): 11 mg. ^{1}H NMR ($CDCl_3$) δ: 0.74 (s, 18-CH_3), 0.84 (s, 19-CH_3), 1.13 (d, J = 6.0 Hz, 21-CH_3), 0.70–2.35 (residual CH and CH_2), 2.40–2.75 (broad m, 2 × CH_2N and 2α-CH), 2.62 (s, CH_3CO), 3.40–3.90 (broad m, 3 × CH_2NCO, 20-CH and 3β-CH), 5.07 (dd, J_1 = 5.7 Hz, J_2 = 8.1 Hz, NCHCO of Pro), 7.51 (t, J = 7.7 Hz, CH of Aryl), 7.79 (d, J = 7.6 Hz, CH of Aryl), 8.02 (d, J = 7.8 Hz, CH of Aryl), 8.16 (s, CH of Aryl). LRMS for $C_{39}H_{58}N_3O_5$ $[M+H]^+$: 648.5 *m/z*. See Section 2.1.4 for additional characterization of this compound.

2.1.3.6.26. 3-acetyl-N-[(2S)-1-{4-[(2β,3α,5α,20R)-3,20-dihydroxypregnan-2-yl]piperazin-1-yl}-1-oxo-3-phenylpropan-2-yl]benzamide (LFC-48): 4.0 mg. ^{1}H NMR ($CDCl_3$) δ: 0.76 (s, 18-CH_3), 0.82 (s, 19-CH_3), 1.14 (d, J = 6.1 Hz, 21-CH_3), 0.70–1.85 (residual CH and CH_2), 2.10, 2.20 and 2.57 (m, 2 × CH_2N and 2α-CH), 2.65 (s, CH_3CO), 3.02–3.82 (m, 2 × CH_2NCO, 20-CH, 3β-CH and CH_2Ph), 5.37 (m, NHC*H*CO of Phe), 7.16 (d, J = 8.0 Hz, NH), 7.28 (m, CH_2*Ph*), 7.55 (t, J = 7.8 Hz, CH of Aryl), 7.98 (d, J = 7.9 Hz, CH of Aryl), 8.11 (d, J = 7.8 Hz, CH of Aryl), 8.36 (s, CH of Aryl). LRMS for $C_{43}H_{60}N_3O_5$ $[M+H]^+$: 698.4 *m/z*.

2.1.3.6.27. 1-(3-{[(2R)-2-({4-[(2β,3α,5α,20R)-3,20-dihydroxypregnan-2-yl]piperazin-1-yl}carbonyl)pyrrolidin-1-yl]carbonyl}phenyl)ethanone (DPC-48): 11 mg. ^{1}H NMR ($CDCl_3$) δ: 0.73 (s, 18-CH_3), 0.84 (s, 19-CH_3), 1.13 (d, J = 5.9 Hz, 21-CH_3), 0.68–2.30 (residual CH and CH_2), 2.44–2.90 (broad m, 2 × CH_2N and 2α-CH), 2.62 (s, CH_3CO), 3.40–3.92 (broad m, 3 × CH_2NCO, 20-CH and 3β-CH), 5.07 (dd,

J_1 = 5.7 Hz, J_2 = 8.1 Hz, NCHCO of Pro), 7.51 (t, J = 7.7 Hz, CH of Aryl), 7.79 (d, J = 7.6 Hz, CH of Aryl), 8.02 (d, J = 7.8 Hz, CH of Aryl), 8.16 (s, CH of Aryl). LRMS for $C_{39}H_{58}N_3O_5$ $[M+H]^+$: 648.4 *m/z*.

2.1.3.6.28. 3-acetyl-N-[(2R)-1-{4-[(2β,3α,5α,20R)-3,20-dihydroxypregnan-2-yl]piperazin-1-yl}-1-oxo-3-phenylpropan-2-yl]benzamide (DFC-48): 3.0 mg. ^{1}H NMR ($CDCl_3$) δ: 0.76 (s, 18-CH_3), 0.82 (s, 19-CH_3), 1.14 (d, J = 6.1 Hz, 21-CH_3), 0.68–1.85 (residual CH and CH_2), 2.08, 2.40 and 2.60 (3 m, 2 × CH_2N and 2α-CH), 2.65 (s, CH_3CO), 3.02–3.80 (m, 2 × CH_2NCO, 20-CH, 3β-CH and C*H*$_2$Ph), 5.37 (m, NHC*H*CO of Phe), 7.16 (d, J = 7.9 Hz, NH), 7.29 (m, CH_2*Ph*), 7.55 (t, J = 7.7 Hz, CH of Aryl), 7.98 (d, J = 7.8 Hz, CH of Aryl), 8.11 (d, J = 7.8 Hz, CH of Aryl), 8.36 (s, CH of Aryl). LRMS for $C_{43}H_{60}N_3O_5$ $[M+H]^+$: 698.4 *m/z*.

2.1.4. Purification and additional characterization of LPC-37 and LPC-48

The two aminosteroids selected for a preliminary investigation of their mechanism of action on HL-60 cells, LPC-37 and LPC-48, were resynthesized using 2 × 375 mg of resin **7** (0.56 mmol/g) following the general procedure described above for **8** and **9**. The two compounds released from the resin were purified by flash chromatography using DCM/MeOH (95:5) as eluent to give LPC-37 and LPC-48.

2.1.4.1. LPC-37: 49 mg. IR (ATR) ν: 3433 (OH) and 1636 (CON). ^{1}H NMR and LRMS data are reported in previous section. ^{13}C NMR ($CDCl_3$) δ: 12.2, 17.2, 21.2, 23.6, 24.4, 25.6, 28.3, 29.4, 29.7, 31.6, 32.9, 34.6, 35.3, 35.8, 38.4, 40.1, 42.6, 46.3, 48.2, 48.8, 50.3, 55.8, 56.0, 56.2, 58.5, 63.8, 64.9, 70.6, 124.5, 126.5, 127.1, 127.4, 127.7, 128.0, 128.6, 132.5, 133.7, 133.9, 169.5, 170.4. HRMS for $C_{41}H_{58}N_3O_4$ $[M+H]^+$: 656.4422, found 656.4425. HPLC purity: 93.4%.

2.1.4.2. LPC-48: 52 mg. IR (ATR) ν: 3425 (OH), 1690 (CO) and 1632 (CON). ^{1}H NMR and LRMS data are reported in previous section. ^{13}C NMR ($CDCl_3$) δ: 12.6, 17.2, 21.2, 23.6, 24.4, 25.5, 25.6, 26.7, 28.3, 29.4, 31.6, 33.1, 34.7, 35.3, 35.8, 38.4, 40.1, 42.6, 46.1, 48.0, 48.7, 50.2, 55.8, 55.9, 56.0, 56.4, 58.5, 63.9, 65.0, 70.5, 127.4, 128.7, 129.7, 131.8, 136.8, 137.1, 168.4, 170.1, 197.5. HRMS: calculated for $C_{39}H_{58}N_3O_5$ $[M+H]^+$: 648.4371, found 648.4376. HPLC purity: 92.4%.

2.2. Biological evaluations

2.2.1. Cellular material

2.2.1.1. Human promyelocytic leukemia cells. HL-60 (ATCC, Rockville, MD, USA) were routinely grown in suspension in 90% RPMI 1640 (Sigma, Saint Louis, MO, USA) containing L-glutamine (2 nM), and antibiotics (100 IU penicillin/mL, 100 μg streptomycin/mL) and supplemented with 10% (v/v) fetal bovine serum (FBS), in a 5% CO_2 humidified atmosphere at 37 °C. Cultures were maintained two times per week by addition of fresh medium or replacement of medium.

2.2.1.2. Peripheral blood lymphocytes isolation. Normal human peripheral blood lymphocytes (PBL) were isolated from buffy coats kindly provided by Dr. Jean Sévigny (CHUQ-CHUL Research Center). In summary, mononuclear cells were isolated on Histopaque (Sigma–Aldrich, Oakville, ON, Canada) density gradient (d = 1.077) by centrifugation (300*g*, 30 min). They were then washed in 0.02 M phosphate-buffered saline (PBS) at pH 7.2. Further purification of lymphocytes from peripheral blood mononuclear cells was performed by adhesion of monocytes to plastic plates for 4 h at 37 °C. Lymphocytes were grown in RPMI 1640 medium (1 × 10^6 cells/mL) supplemented with 10% FBS, L-glutamine (2 nM) and antibiotics (100 IU penicillin/mL, 100 μg streptomycin/mL) and stimulated by leucoagglutinin (PHA-L) (Sigma–Aldrich, Oakville, ON, Canada) in a 5% CO_2 humidified atmosphere at 37 °C [13,14].

2.2.2. Cell proliferation and viability assay

2.2.2.1. Inhibition of HL-60 cells proliferation. Triplicate cultures of 1 × 10^4 cells in a total of 100 μL medium in 96-well culture plates were incubated at 37 °C, 5% CO_2. Doxorubicin (Novapharm, Toronto, ON, Canada) was used as positive control. Compounds were dissolved in ethanol to prepare the stock solution of 1 × 10^{-2} M. These compounds and doxorubicin were diluted at multiple concentrations in culture medium, added to each well, and incubated for 72 h. Following each treatment, 20 μL of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2-H-tetrazolium (MTS) reagent (Promega, Madison, WI, USA) were added to each well and the reaction was stopped after 4 h. MTS is converted to water-soluble colored formazan by dehydrogenase enzymes present in metabolically active cells. Subsequently, the plates were read at 490 nm using a microplate reader (Molecular Devices, Sunnyvale, CA, USA). Data were reported as the percentage of cell proliferation inhibition compared to the control (basal cell proliferation) for two concentrations of 1 μM and 10 μM. For IC_{50} determination, the percentage of cell proliferation was plotted against the drug concentration ranging from 10^{-8} to 10^{-4} M and the value resulting in 50% cell growth inhibition was determined.

2.2.2.2. Inhibition of peripheral blood lymphocytes proliferation. The antiproliferative activity of tested compounds and doxorubicin in PBL was assessed by culturing cells (1 × 10^5 cells/100 μL culture medium) in 96-well culture plates in presence of leucoagglutinin (PHA-L) (2 μg/mL culture medium) for 48 h, compounds to test were then added and treated cells were incubated for 72 h. After each incubation period, MTS (20 μL) was added to each well. The reaction was stopped after 4 h. Absorbance was plotted against the drug concentration and IC_{50} values were determined [13,15].

2.2.2.3. Assay for cell viability. Cell viability was determined by the trypan blue dye exclusion test. HL-60 cells were plated into a 6-well plate at 2.5 × 10^5 cells/mL in a total volume of 2 mL. Cells were then exposed or not (control) for 24, 48 or 72 h at 37 °C to LPC-37 or LPC-48. After each incubation period, the number of cells that did not take up trypan blue was counted using a haemocytometer [16].

2.2.3. Flow cytometric cell cycle analysis

HL-60 cells were plated at a density of 1 × 10^6 cells per 100 mm × 15 mm culture dish in a total volume of 10 mL and exposed or not (control) for 24, 48 or 72 h at 37 °C to LPC-37 or LPC-48. After treatment, cells were collected by centrifugation for 5 min at 400*g*, resuspended in 300 μL of ice-cold PBS, fixed in ice-cold 95% ethanol and stored at −20 °C for at least 30 min. Fixed cells were collected by centrifugation and pellets were resuspended in PBS containing RNase A (500 UI/mL). Samples were kept at room temperature for 30 min. To determine cellular DNA content, cells were stained with propidium iodide (50 μg/mL), incubated for 20 min on ice then analyzed by flow cytometry using a Coulter EPICS XL (Beckman-Coulter, Miami, FL, USA). The percentages of cells in G0/G1, S and G2/M phases and the percentage of cells in sub-G0/G1 peak were calculated using MultiCycle AV Software (Phoenix Flow Systems, San Diego, CA, USA) that eliminated the debris effect [17].

2.2.4. Morphological analysis with fluorescence microscopy

To evaluate the apoptotic activity of LPC-48, we performed nuclear staining with the DNA-binding dye Hoechst 33342 (Invitrogen, Burlington, ON, Canada). HL-60 cells were plated into 6-well

plates at 10^6 cells/well and exposed or not (control) to increasing concentrations of LPC-48 for 72 h. Cells were collected by centrifugation at 200*g* for 5 min, washed with ice-cold PBS and then fixed with 2% paraformaldehyde in PBS for 10 min at 4 °C. Fixed cells were washed with PBS, incubated with Hoechst 33342 (10 μg/mL) for 15 min in the dark, then placed on slides and observed under a fluorescence microscope (excitation 352 nm, emission 461 nm; *NIKON TE2000-E*). Apoptotic cells were identified by condensation of chromatin and fragmentation of nuclei. Pictures were obtained at 40×, using a video camera Q-imaging (Burnaby, BC, Canada) [18].

3. Results and discussion

3.1. Synthesis of new pregnane derivatives

3.1.1. Synthesis of solid phase precursor **5**

Compound **5**, the pregnane to be linked on a polymeric support for the solid-phase synthesis, was obtained following a 5-step sequence of reactions (Fig. 2). In the first step, the 3β-OH of epi-allopregnanolone was dehydrated regioselectively using diethylaminosulfur trifluoride (DAST) as dehydrating reagent. The procedure was adapted from a previously published result showing that a C-2,3 alkene was obtained as a minor side-product when androsterone (ADT) was fluorinated with DAST [19,20]. In fact, we optimized the reaction conditions using the steroid scaffold ADT and epi-ADT (Fig. 3) in order to maximize the C-2,3 vs. C-3,4 alkene ratio (inseparable mixture) as well as the reaction yield (Table 1). The use of 1.2 equivalent of DAST at −78 °C in dichloromethane (DCM) gave interesting ratio for ADT (95:5) and epi-ADT (96:4) with a higher yield of alkene for ADT (57%) than for epi-ADT (30%) (entries 3 and 12). Changing DCM for THF or toluene did not improve the quantity of C-2,3 alkene (entries 3, 8 and 9). The use of dimethylaminosulfur trifluoride (Me-DAST) instead of DAST gave the same yield and alkene ratio (entries 15–17). Although the best yield of alkene was obtained when dehydrating a 3α-OH group, such as ADT, instead of a 3β-OH group, such as epi-ADT, it was found too much costly to use allopregnanolone (3α-OH) to generate **1**. In fact, considering the cost of this commercially available natural product, we found advantageous to use epi-allopregnanolone as starting material rather than allopregnanolone (1200-fold more costly), even with an expected twice lowest yield of **1**. Thus, the reaction on epi-allopregnanolone with 1.2 eq of DAST at −78 °C in DCM gave the pregnene **1** with a high C-2,3 ratio (98:2) but a moderate yield of 25% (entry 18). The DAST procedure was found more appropriate than the classical methods for alcohol dehydratation previously used [10], which always gave a higher amount (>10%) of undesired steroidal C-3,4 androstene. We also favored the one-step DAST procedure over the five-step classical procedure that we previously used starting from dihydrotestosterone, a 3-keto androstane derivative [11].

The carbonyl of **1** was stereoselectively reduced with DIBAL-H to give the corresponding alcohol **2** in 78% yield (Fig. 2). Following Cram's rule [21], and in accordance with NMR data [22], the 20*R* isomer (or 20β-OH) was isolated. The subsequent epoxidation of alkene **2** with *m*-CPBA stereoselectively afforded the 2,3α-epoxide **3** (83% yield), which was next regio- and stereo-selectively opened with piperazine to give **4**. N-Fmoc protection of the NH of piperazino derivative **4** completed the synthesis of the solid-phase precursor **5**.

3.1.2. Solid-phase synthesis of libraries A and B

N-Fmoc-2β-piperazino-5α-pregnan-3α,20β-diol (**5**) was coupled to chlorosilyl resin, which was previously generated *in situ* from butyldiethylsilane polystyrene (PS-DES) resin. In the coupling reaction, the resin was swelled in dry DCM and treated with imidazole and the steroid **5** (Fig. 4) to give the coupled steroid **6**. Similarly as previously observed for the synthesis of androstane derivatives [11], the low reactivity of the 3α-OH group at proximity of a bulky *N*-Fmoc-piperazine group allowed a regioselective coupling at the more reactive 20-OH. The Fmoc protecting group of **6** was easily removed with a solution of 20% piperidine in DMF and the compound **7** was obtained by coupling a *N*-Fmoc amino acid thus introducing the first level of diversity. In the next step, the *N*-Fmoc group of **7** was hydrolyzed and the steroid-bound resin

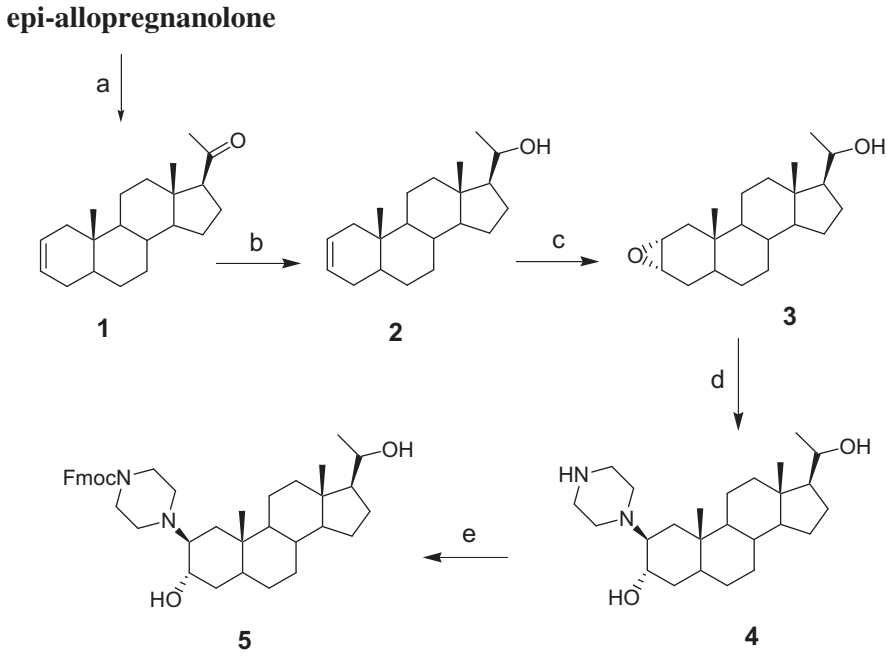


Fig. 2. Synthesis of **5** from epi-allopregnanolone. *Reagents and conditions:* (a) DAST, DCM, −78 °C, 1 h, 25% yield; (b) DIBAL-H, DCM, −78 °C, 78% yield; (c) *m*-CPBA, DCM, 0 °C, 73% yield; (d) piperazine, H_2O, 160 °C, 24 h, 83% yield; (e) Fmoc-OSu, $NaHCO_3$ (1 M), THF/H_2O (5:1, v/v), rt, 65% yield.

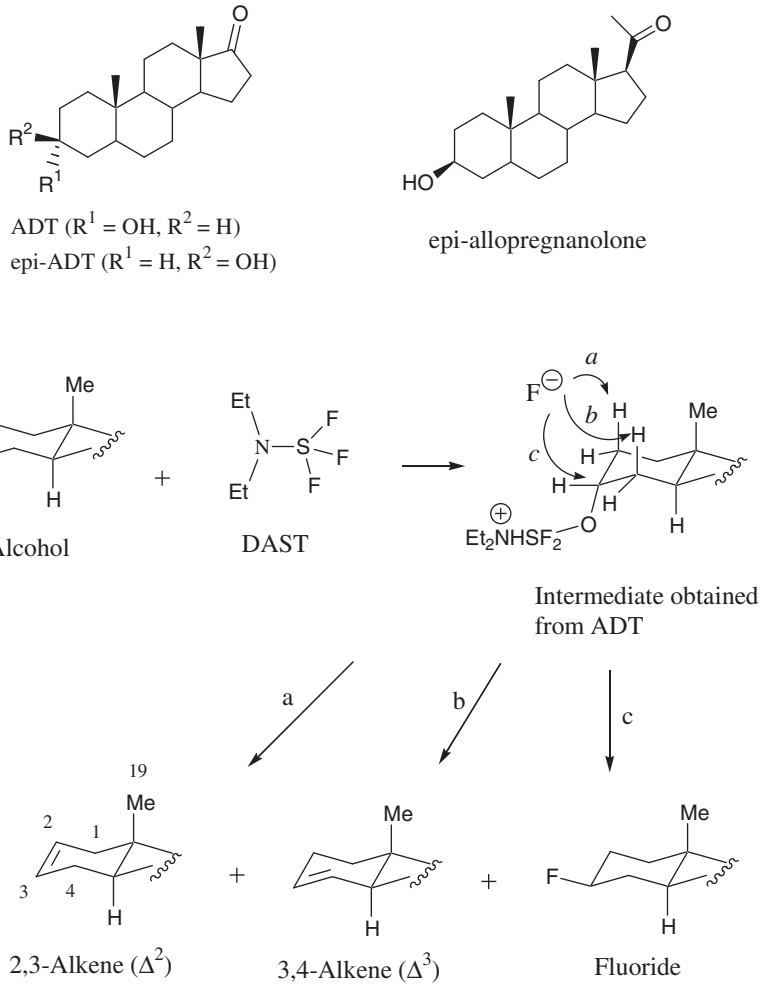


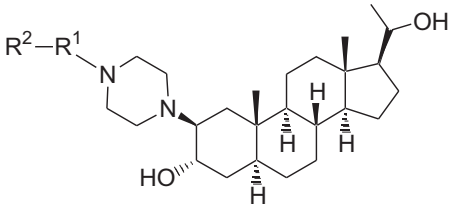
Fig. 3. Synthesis of C-2,3 and C-3,4 alkenes from the corresponding steroidal alcohol using DAST as dehydrating agent.

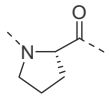
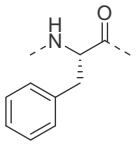
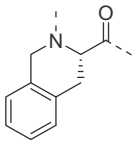
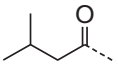
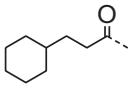
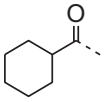
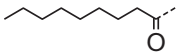
Table 1
Optimisation of dehydration reaction (synthesis of steroidal alkenes from the corresponding C-3 alcohol).[a]

Entry	Steroidal alcohol[b]	Reagent[c]	Eq.[d]	*T* (°C)	Solvent	Yield of alkene (%)[e]	Steroidal alkene Δ^2:Δ^3 ratio[f]
1	ADT (3α-OH)	DAST	1.2	0	DCM	55	91:9
2	ADT (3α-OH)	DAST	1.2	−40	DCM	ND	93:7
3	ADT (3α-OH)	DAST	1.2	−78	DCM	57	95:5
4	ADT (3α-OH)	DAST	1.2	−90	DCM	56	94:6
5	ADT (3α-OH)	DAST[g]	1.2	−78	DCM	60	93:7
6	ADT (3α-OH)	DAST[h]	1.2	−78	DCM	ND	94:6
7	ADT (3α-OH)	DAST[i]	1.2	−78	DCM	21	87:13
8	ADT (3α-OH)	DAST	1.2	−78	THF	ND	88:12
9	ADT (3α-OH)	DAST	1.2	−78	Toluene	ND	92:8
10	Epi-ADT (3β-OH)	DAST	1.2	0	DCM	31	94:6
11	Epi-ADT (3β-OH)	DAST	1.2	−40	DCM	ND	95:5
12	Epi-ADT (3β-OH)	DAST	1.2	−78	DCM	30	96:4
13	Epi-ADT (3β-OH)	DAST	1.2	−90	DCM	26	97:3
14	Epi-ADT (3β-OH)	DAST	2.4	−78	DCM	40	95:5
15	Epi-ADT (3β-OH)	Me-DAST	1.2	−78	DCM	29	97:3
16	ADT (3α-OH)	Me-DAST	1.2	−78	DCM	78	94:6
17	Epi-alloPREG (3β-OH)	Me-DAST	1.2	−78	DCM	28	97:3
18	Epi-alloPREG (3β-OH)	DAST	1.2	−78	DCM	25	98:2

[a] The reaction was performed in anhydrous conditions (argon atm.). After 1 h, the mixture was evaporated in presence of silica gel and the solid added on the top of a silica gel column to perform a flash chromatography.
[b] Androsterone (ADT) is an androstane C-19 steroid; epi-allopregnanolone is a pregnane C-21 steroid.
[c] DAST, diethylaminosulfur trifluoride; Me-DAST, dimethylaminosulfur trifluoride.
[d] Equivalent (Eq.) of reagent vs. the steroidal alcohol.
[e] Yield of 2,3- and 3,4-alkenes isolated after a silica gel flash-column chromatography. ND: yield not determined.
[f] Ratio of 2,3-alkene (Δ^2) and 3,4-alkene (Δ^3) determined by ^{1}H NMR spectroscopy.
[g] Concentrated.
[h] Diluted (5×).
[i] Triethylamine was added to the reaction mixture.

Table 2
Library A compounds with their elements of molecular diversity (R^1 and R^2) and % of HL-60 cell growth inhibition reported at two concentrations (1 μM/10 μM).[a]

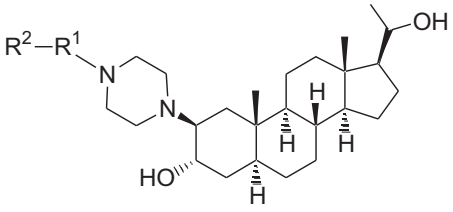


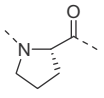
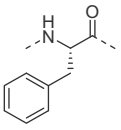
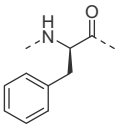
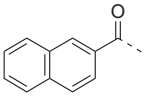
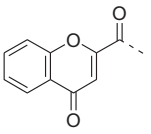
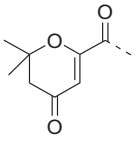
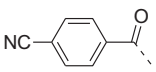
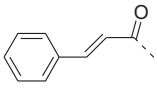
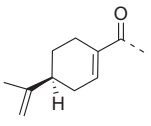
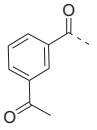
R_2	R_1		
			
	L-Pro (P)	**L-Phe (F)**	**L-Tic (T)**
 **Isovaleric acid (A)**	PA 0/75[a]	FA 0/81	TA 0/62
 **Cyclopentylpropionic acid (C)**	PC 0/93	FC 0/19	TC 0/66
 **Cyclohexylcarboxylic acid (D)**	PD 13/88	FD 5/46	TD 0/42
 **Nonanoic acid (N)**	PN 2/76	FN 0/0	TN 0/45

[a] Human promyelocytic leukemia (HL-60) cells were incubated for three (3) days with tested compounds and the cell growth inhibition reported in % at two concentrations (1 and 10 μM). The number of metabolically active cells was measured using the MTS methodology. The reference compound doxorubicin inhibited 90% and 93% of cell concentration at 1 and 10 μM, whereas the aminosteroid RM inhibited 28% and 85% of cell growth at the same concentrations.

submitted to an acylation with a carboxylic acid (second level of diversity) providing **8**. The final compound **9** was released with an acid treatment and neutralized with a 10% aqueous bicarbonate solution using an automated work-up procedure. The pregnane derivatives were prepared in parallel and are represented by the model libraries A ($3 \times 4 = 12$ compounds, Table 2) and B ($4 \times 7 = 28$ compounds, Table 3). All members of the libraries were analyzed by TLC, and the results confirmed the good reactivity of all building blocks used in the elaboration of the libraries, except for library A where the acylation with the bulky *N*-Fmoc-L-tetrahydro-isoquinoline-3-carboxylic acid did not reach completion. For those incomplete reactions, we thus proceeded to a fast purification on silica gel to remove the unreacted piperazino-steroid. All library members showed the expected M+H signal by LRMS analyses whereas TLC analyses confirmed the library uniformity and allowed us to perform a random sampling. These selected library members were then characterized by ^{1}H NMR. Furthermore, the aminosteroids LPC-37 and LPC-48, showing the best antiproliferative activities on HL-60 cells, were resynthesized, purified by chromatography and fully characterized by IR, ^{1}H NMR, ^{13}C NMR, HRMS, and their purity determined by HPLC. Additional NMR

Table 3
Library B compounds with their elements of molecular diversity (R^1 and R^2) and % of HL-60 cell growth inhibition reported at two concentrations (1 μM/10 μM).[a]



R_2	R_1			
				
	L-Pro	**L-Phe**	**D-Pro**	**D-Phe**
 **C-37**	LPC-37 6/86[a] (IC_{50} = 1.9 μM)	LFC-37 0/0	DPC-37 0/0	DFC-37 0/0
 **C-38**	LPC-38 0/89	LFC-38 0/32	DPC-38 0/24	DFC-38 0/0
 **C-39**	LPC-39 0/0	LFC-39 0/94	DPC-39 0/0	DFC-39 0/75
 **C-41**	LPC-41 0/85	LFC-41 0/10	DPC-41 0/0	DFC-41 0/46
 **C-42**	LPC-42 7/83	LFC-42 0/0	DPC-42 0/72	DFC-42 0/0
 **C-46**	LPC-46 0/87	LFC-46 0/0	DPC-46 0/78	DFC-46 0/0
 **C-48**	LPC-48 7/82 (IC_{50} = 1.9 μM)	LFC-48 0/55	DPC-48 0/0	DFC-48 0/23

[a] Human promyelocytic leukemia (HL-60) cells were incubated for three (3) days with tested compounds and the cell growth inhibition reported in % at two concentrations (1 and 10 μM). The number of metabolically active cells was measured using the MTS methodology. The reference compound doxorubicin inhibited 78% and 84% of cell growth at concentrations of 1 and 10 μM, respectively.

experiments (HSQC, HMBC, COSY and NOESY) of the representative compound PD allowed the assignment of all carbon signals and key proton signals (Fig. 5) as well as to confirm the 2β-piperazino-3α-OH substitution (Fig. 6).

3.2. Biological evaluation

3.2.1. Primary screening

The cytotoxic activity of newly synthesized aminosteroids from libraries A and B was evaluated on the human promyelocytic leukemia HL-60 cell line. We chose this cell line because the first steroid derivative reported to exert an inhibitory activity on leukemic cells was tested on HL-60 cells [11]. In addition, the latter cells are well characterized and required simple maintenance *in vitro*. The cell proliferation assay was performed at concentrations of 1 and 10 μM for each compound of libraries A and B, and the results were expressed as the percentage of cell growth inhibition (Tables 2 and 3). Our results show that the 40 compounds (libraries A and B) were not similarly effective against HL-60 cells. The aminosteroids from both libraries have two levels of diversity. The first level is provided by an amino acid (L-proline, L-phenylalanine or L-tetrahydrozisoquinoline-3-carboxylic acid) whereas the second level originated from a carboxylic acid (isovaleric acid, cyclopentylpropionic acid, cyclohexyl-carboxylic acid or nonanoic acid). From the % of HL-60 cell growth inhibition reported in Table 2, it appears that eleven compounds out of twelve inhibited the cell growth at 10 μM, but only the pregnane derivative PD, with L-proline as building block, inhibited the cell growth at 1 μM (13%). For comparison, the androstane derivative RM, which has the same side-chain at position 2β than PD but a different steroid nucleus, inhibited 28% and 85% of HL-60 cell proliferation at 1 and 10 μM, respectively.

The data from the first library indicated that the antiproliferative effect is modulated by the nature of the amino acid. We then extended our structure–activity relationship study by testing compounds from library B. The molecular diversity was enriched by adding D-proline, D-phenylalanine and new carboxylic acids as building blocks (Table 3). Interestingly, the combination of L-proline with a naphthalene-2 carbonyl, a 3-phenylacryloyl or a 3-acetylbenzoyl resulted in three hits (LPC-37, LPC-42 and LPC-48). When inverting the stereochemistry of the L-proline, the activity of the related compounds drastically dropped. In fact, compounds LPC-37, LPC-42 and LPC-48 inhibited >81% of cell growth at 10 μM whereas their analogues bearing a D-proline as first level of diversity had no antiproliferative effect except DPC-42 which inhibited 72% of cell growth at 10 μM. Only a weak potency was exhibited by compounds bearing a phenylalanine as amino acid. However, compounds LFC-39 and DFC-39 having a 6,6-dimethyl-4-oxo-5,6-dihydro-4,4-pyran-2-carbonyl as second level of molecular diversity inhibited >75% of cell growth at 10 μM, but they had no effect at 1 μM.

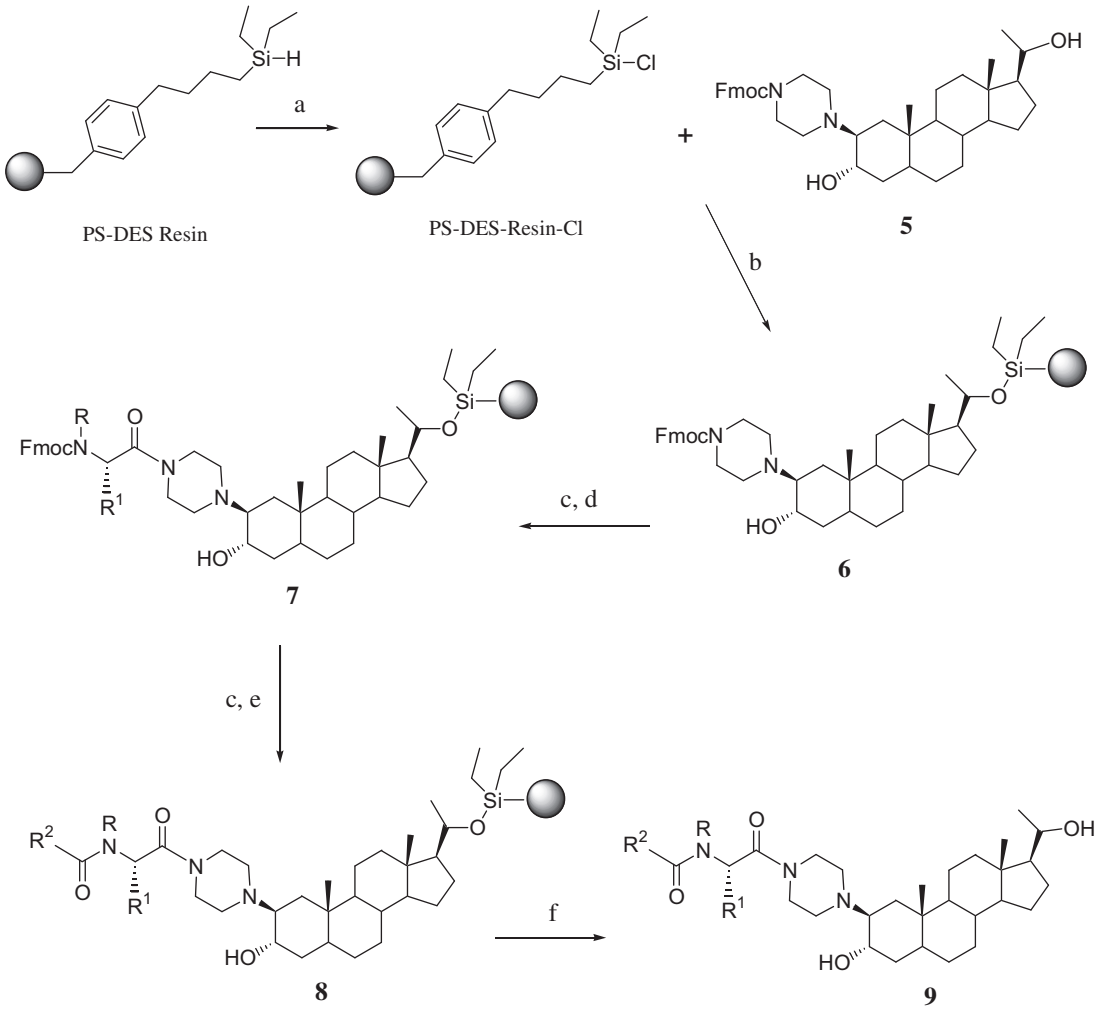


Fig. 4. Synthesis of libraries A and B. *Reagents and conditions:* (a) 1,3-dichloro-5,5-dimethylhydantoin, DCM, rt; (b) imidazole, DCM, rt; (c) piperidine 20% (v/v), DCM, rt; (d) *N*-Fmoc-aminoacid, PyBOP, HOBt, DIPEA, DMF, rt; (e) carboxylic acid, PyBOP, HOBt, DIPEA, DMF, rt; (f) (i) HCl (2 M)/MeOH (AcCl + MeOH) in DCM (20:80, v/v); (ii) 10% $NaHCO_3$.

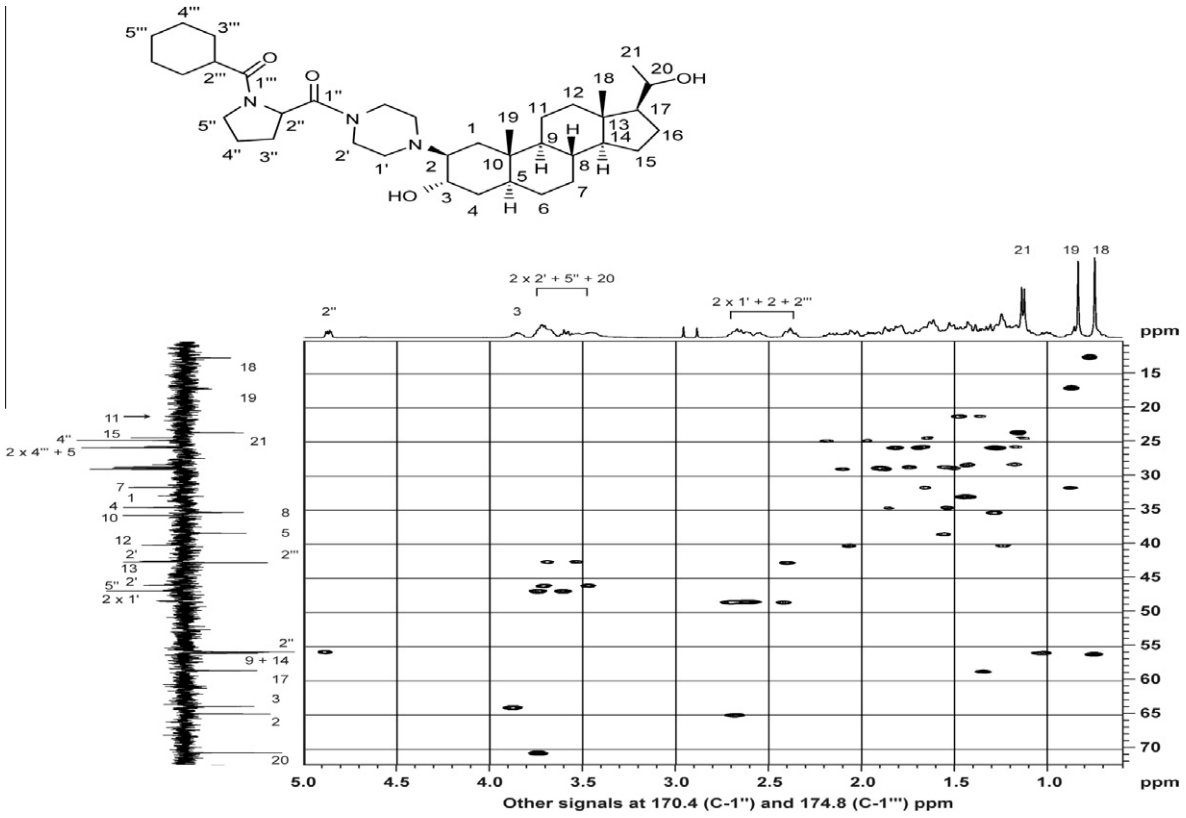


Fig. 5. NMR (HSQC) experiment of the representative aminosteroid PD showing all carbon (^{13}C) signals and key proton (^{1}H) signals.

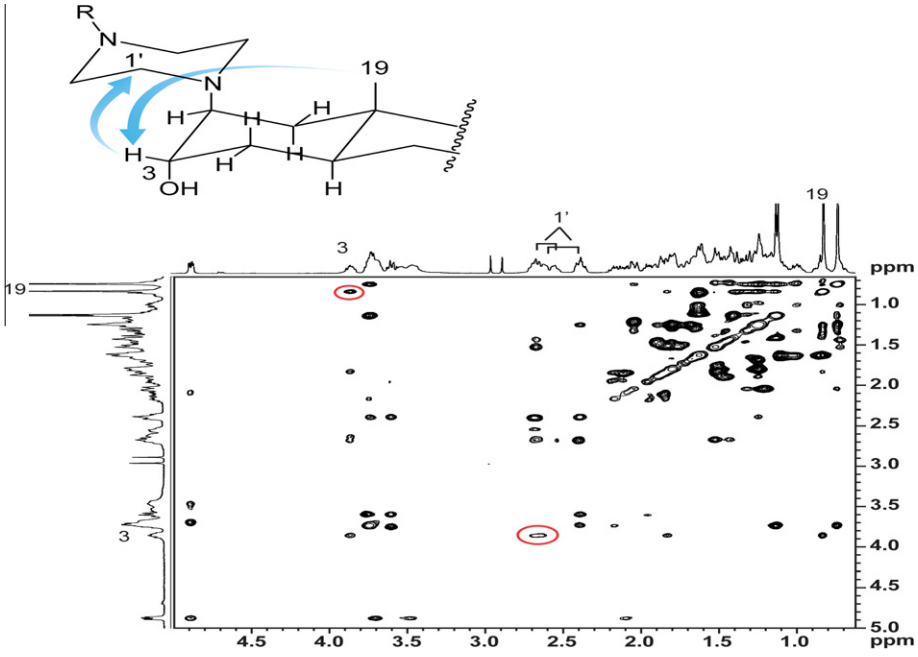


Fig. 6. NMR (NOESY) experiment with PD showing the key interactions between 19-CH_3 and 3β-CH (3α-OH) and between 3β-CH and CH_2-1′ (2β-piperazine).

The whole results obtained from this primary screening assessed the key role of the amino acid L-proline (first level of molecular diversity) in the cytotoxic activity. The role of the carboxylic acid introduced in the second level of molecular diversity is however less important. We then selected the two representative aminosteroids LPC-37 and LPC-48 for a series of preliminary mechanistic investigations. These two aminosteroids were consequently synthesized in larger quantities, purified by chromatography and their purity assessed by HPLC.

3.2.2. IC_{50} values determination and selective-cytotoxicity of LPC-37 and LPC-48

As a complementary part for the primary screening, it was thought worthwhile to evaluate the differential growth inhibitory

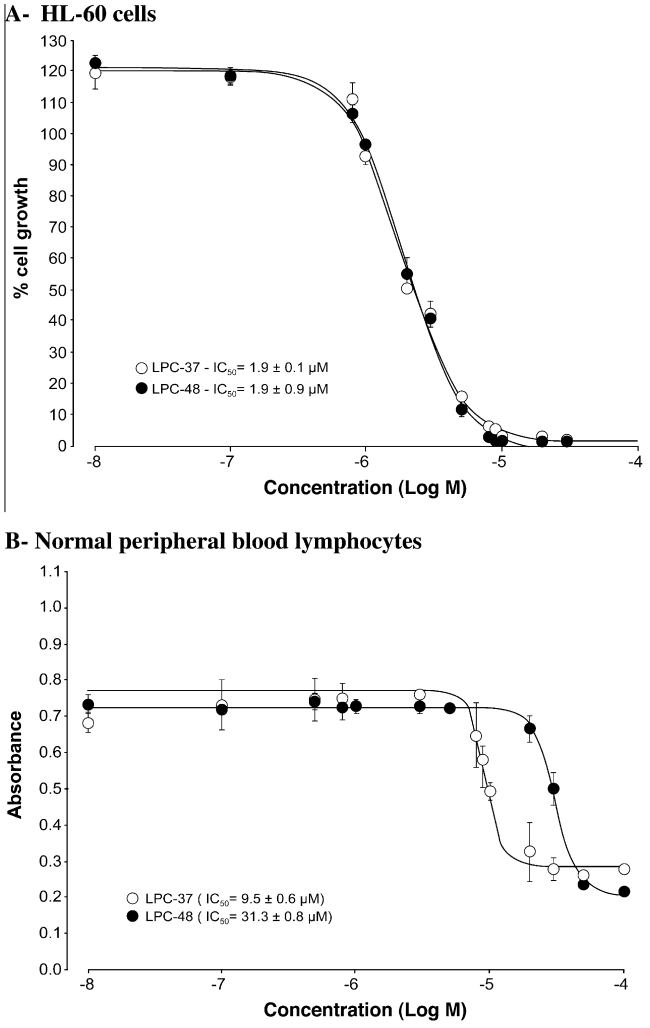


Fig. 7. Selective cytotoxicity of compounds PD, LPC-37 and LPC-48 towards HL-60 cells (A) and normal PBL (B). Cells were exposed to the aminosteroid at concentrations ranging from 10^{-8} M to 10^{-4} M, for 72 h. Single points are average of triplicate.

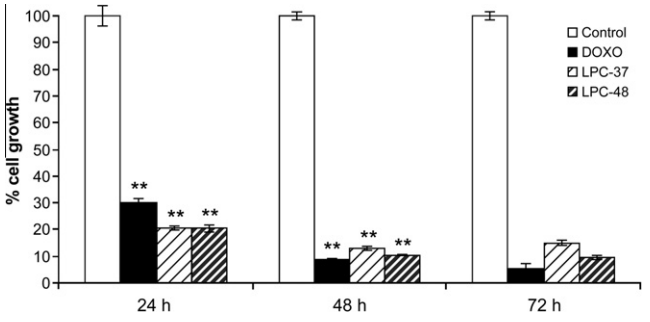


Fig. 8. Inhibition of HL-60 cell growth by compounds LPC-37, LPC-48 and doxorubicin (Doxo). The cells (5×10^4) were seeded in a total volume of 100 µL and exposed to 10 µM of each compound for 24, 48 and 72 h. The cell proliferation was measured by MTS method. Each data point represents the mean of a triplicate (mean ± SD). The results are significantly different from the control. $^{**}p < 0.01$ vs. control.

effect of aminosteroids LPC-37 and LPC-48 on both cancerous and normal cell lines. We first determined their IC_{50} values (the concentration required to inhibit cell growth by 50%) on cancerous (HL-60) cells as well as on normal PBL. Both LPC-37 and LPC-48 gave the same cell growth inhibition (IC_{50} = 1.9 µM) on HL-60 cells

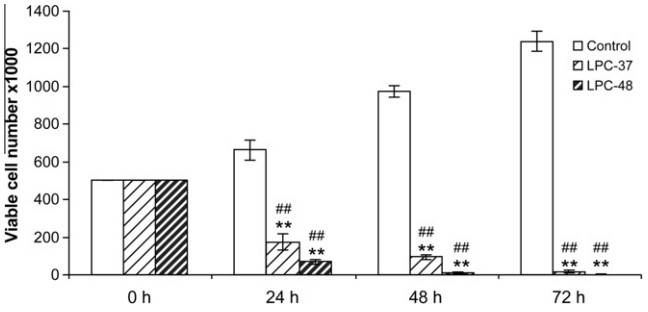


Fig. 9. Number of viable HL-60 cells after a treatment with LPC-37 or LPC-48. The cells (5×10^5) were exposed to 10 µM of each compound for 24, 48 and 72 h. The viable cells were measured by the trypan blue exclusion method using a haemocytometer. Each data point represents the mean of a triplicate (mean ± SD). The results are significantly different from the control. $^{**}p < 0.01$ vs. control, $^{\#\#}p < 0.01$ vs. 0 h.

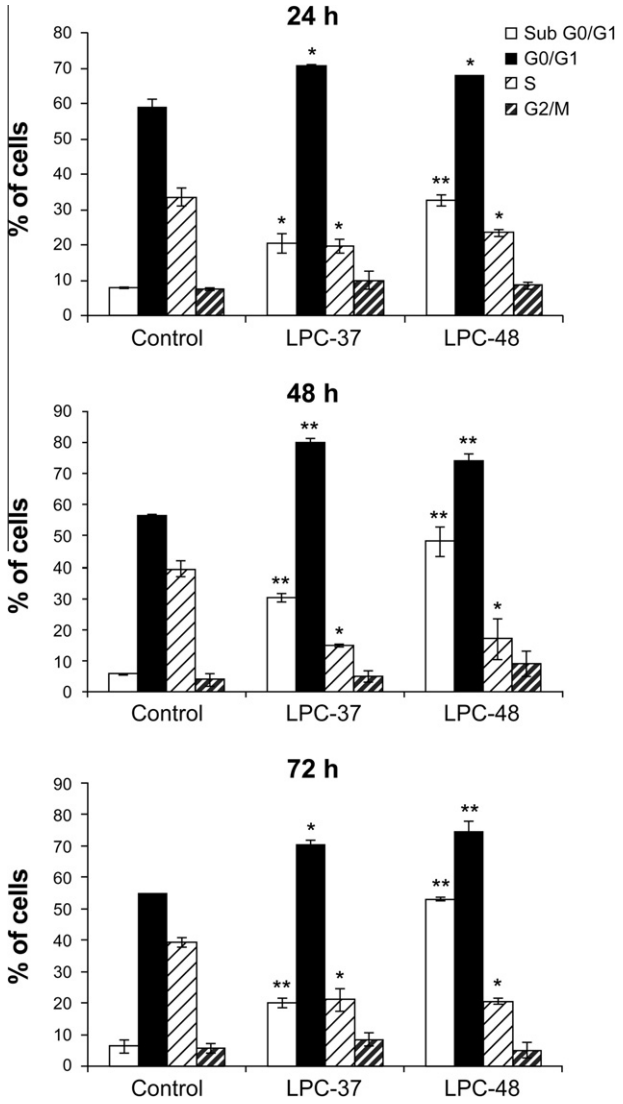


Fig. 10. Cell cycle analysis of HL-60 cells treated with LPC-37 (5 µM) or LPC-48 (5 µM). After 24 h, 48 h and 72 h of treatment, cells were labelled with propidium iodide and analyzed by flow cytometry. $^{**}p < 0.01$ vs. control, $^{*}p < 0.05$ vs. control.

after 72 h of incubation (Fig. 7A). Since chemotherapy very often induces severe side effects, which are in part a consequence of destruction of normal cells, we also tested the aminosteroids on

normal cells. Our findings point to a selective effect between tumor and normal cells of the aforementioned compounds. Indeed, as illustrated in Fig. 7B, the normal PBL were about 16-fold less sensitive to the inhibitory activity of LPC-48, with an IC_{50} of 31.3 μM. LPC-37 was slightly less selective as it displayed IC_{50} value of 9.5 μM.

After assessing that the growth inhibitory effect of LPC-37 and LPC-48 on HL-60 cells was dose-dependent, we examined whether it was also time-dependent. As shown in Fig. 8, a treatment with 10 μM of either LPC-37 or LPC-48 led to almost 80% cell growth inhibition upon the first 24 h of treatment. Longer incubation resulted in a weak increase in growth inhibition. Indeed, after 72 h of treatment, LPC-37 and LPC-48 inhibited 85% and 90% of cell growth, respectively. Thus, the maximum of cell growth inhibition is reached in the first 24 h of treatment. In addition, the two tested compounds proved to be nearly equipotent to doxorubicin at 10 μM concentration.

3.2.3. LPC-37 and LPC-48 induce cell death in HL-60 cells

To determine whether LPC-37 and LPC-48 growth inhibition of HL-60 cells was related to a cytotoxic or a cytostatic effect, we evaluated the cell viability of HL-60 after treatment with 10 μM of either compound for 24, 48 and 72 h. Apart from the MTS method, which evaluates cell growth but cannot distinguish between growth arrest and apoptosis or necrosis; cell viability can be assessed using the trypan blue exclusion test, which is based on the principle that living cells possess intact cell membranes that exclude this dye, whereas, non-viable cells (late apoptotic and necrotic cells) with damaged plasma membrane take it up [23]. The trypan blue staining revealed that the two compounds had a similar cytotoxic effect on HL-60 cells (Fig. 9). It is noteworthy that cell viability underwent a drastic decline in the early hours of treatment, as LPC-37 and LPC-48 reduced the viability to 35% and 14%, respectively, after 24 h. An increasing effect was observed for the two compounds with an increase in exposure time. In fact, the number of viable cells treated with LPC-37 decreased to 95,000 cells, and there were roughly, no viable LPC-48 treated cells after 48 h of treatment. Thus, the cytotoxicity of the two compounds was confirmed.

3.2.4. LPC-37 and LPC-48 induce a G0/G1 cell cycle arrest

Having assessed the cytotoxicity of the two pregnane derivatives LPC-37 and LPC-48, we next investigated their preliminary mechanism of action. Particular interest was focused on their effect on the cell cycle and on apoptosis induction. We performed cell-cycle analysis of HL-60 cells treated with 5 μM of either of the two compounds at different time periods. The percentages of cells in Sub-G0/G1, G0/G1, S and G2/M were calculated and presented in Fig. 10. The two aminosteroids significantly increased the amount of G0/G1-phase cell population (59% in control vs. 70% and 68% in cells treated with LPC-37 and LPC-48, respectively). Concomitant with this increase in the amount of G0/G1 cells, we observed a decrease in the amount of S cells (33% in control vs. 19% and 23% in cells treated with LPC-37 and LPC-48, respectively). Moreover, a significant accumulation of sub-G0/G1 cells was detected (20% and 32% in cells treated with LPC-37 and LPC-48, respectively, vs. 8% in control). Cells in sub-G0/G1 are hypodiploid cells and their accumulation suggests that they have undergone apoptosis. The pattern of cell cycle distribution changes was the same after 48 h and 72 h of treatment. Interestingly, the percentage of Sub-G0/G1 cells at the different time periods was more important in LPC-48 treated cells than in LPC-37 treated cells, which points to an enhanced and faster occurring toxicity for LPC-48. These results suggest that the two pregnane derivatives LPC-37 and LPC-48 induce a strong G0/G1 cell cycle block followed by apoptosis in HL-60 cells.

3.2.5. Effects of LPC-48 on cell nuclear morphology and DNA fragmentation

Apoptotic cells were first identified by a series of typical morphological changes, and morphology still constitutes important experimental proof of the underlying process [24]. To further investigate the mechanism of this novel family of pregnane

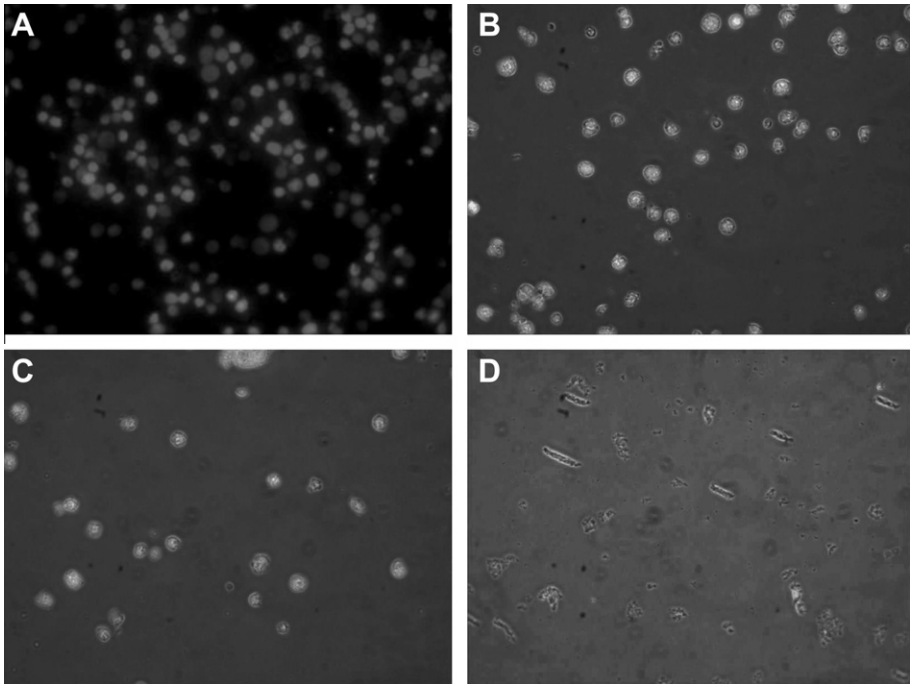


Fig. 11. Nuclear morphology of HL-60 cells treated with 0 (A, control), 30 (B), 50 (C) and 80 (D) μM of LPC-48 for 72 h and stained with Hoechst 33342 dye. Fluorescent pictures were taken at 40× magnification using a NIKON TE2000-E microscope. Nuclear condensation is evident in cells with bright, condensed and rounded nuclei. Membrane blebbing and apoptotic bodies are also evident.

derivatives inducing cytotoxicity, we selected compound LPC-48, and used fluorescence microscopy to determine whether it induced apoptosis or necrosis of HL-60 cells at the single cell level. LPC-48 treated and untreated HL-60 cells were stained with the synthetic fluorescent bisbenzimidazole dye Hoechst 33342. This molecule is a cell membrane permeant, A–T base pair selective, minor groove binding, double stranded DNA-selective binding and live-cell stain [25]. Fig. 11 shows the representative morphology of HL-60 cells when exposed to 0, 30, 50 and 80 μM of LPC-48 for 72 h. Numerous morphological changes typical to apoptosis were detected: plasma membrane blebbing, cellular shrinkage, mitochondria depolarization, chromatin condensation and DNA fragmentation. The control HL-60 cells appeared normal with round and homogeneous nuclei (Fig. 11A). After treatment with 30 μM of LPC-48, alterations in the structure, size and shape of the cell nucleus were detected. Chromatins of condensed appearance, cell shrinkage and nuclear fragmentation as well as a formation of apoptotic bodies were thus observed (Fig. 11B). In addition to these morphological changes, the cells treated with 50 and 80 μM of LPC-48 showed an impairment of the plasma membrane and cell disintegration (Fig. 11C and 11D).

The sub-G0/G1 peak on flow cytometry and the nuclear morphology of LPC-48 treated cells strongly suggest that the new synthesized pregnane derivatives trigger apoptosis in HL-60 cells [26]. The process by which LPC-37 and LPC-48 block cell cycle progression is still to be clarified. A Western-blot analysis would determine whether the two compounds regulate the expression of G1-phase associated proteins. In addition, in order to assess that the two aminosteroids induce apoptosis in treated HL-60 cells, the translocation of phosphatidylserine at the cell surface can be examined by an annexine binding assay.

4. Conclusion

The solid-phase synthesis of two focused libraries of novel amino-pregnane derivatives (Library A; 12 members, and Library B; 28 members) was performed to extend our SAR data on aminosteroid antileukemic activities [11,12]. Improvement of the chemical synthesis of the pregnene **1** was obtained using DAST as regioselective dehydrating reagent to obtain a high ratio (98:2) of the C-2,3 alkene. This reaction allowed us to improve the chemical synthesis of the solid-phase precursor **5** from one step for the generation of the intermediate **1** rather than the four (or six) steps required when using alternative known sequences of reaction. The solid-phase precursor **5** was then coupled to polystyrene diethylbutylsilane (PS-DES) resin and submitted to diversification using different amino acids (first level of diversity) and carboxylic acids (second level of diversity). The choice of the different building blocks composing the libraries was made from our previous SAR studies on piperazino-androstane scaffold by choosing the building blocks showing good activity in previous screenings on HL-60 cells [11,12]. We selected three amino acids (L-Pro, L-Phe, L-Tic) as first level of diversity and four different carboxylic acids as capping groups for library A and four amino acids (L-Pro, D-Pro, L-Phe, D-Phe) and seven carboxylic acids capping groups for library B. These two libraries are small in size, but their preparation opens the door to the synthesis of bigger libraries of pregnane derivatives.

When we compared the pregnane derivative PD (Library A) to RM, a first generation aminosteroid with an androstane nucleus [11,27] in the same protocol, they gave similar antiproliferative activity on HL-60 cells showing the potential of both pregnane and androstane nuclei as molecular scaffold. When tested on normal lymphocytes, the pregnane derivatives LPC-37 and LPC-48 (Library B) were found to preferentially inhibit the proliferation of cancerous HL-60 cells. Our data presented in this study show that this new family of pregnane derivatives induces a G0/G1 cell cycle arrest of HL-60 cells leading to apoptosis. Additional studies will be necessary to elucidate their exact mechanism of action. Identification of this mechanism would help us to rationalize our results and design other active compounds more efficiently.

Acknowledgments

We thank the Canadian Institutes of Health Research (CIHR) and the Cancer Research Society (CRS) for their financial support. We also thank Dr. Charles Doillon (CHUQ-CHUL Research Center) for providing the Hoechst 33342 dye, the use of the inverted microscope and for helpful discussions. We greatly appreciated the gift of buffy coats from Dr. Jean Sévigny (CHUQ-CHUL Research Center). Thanks to Marie-Claude Trottier and Micheline Harvey for NMR analyses and for careful reading of the manuscript, respectively.

References

[1] National Cancer Institute. Available at: www.cancer.gov/cancertopics/types/leukemia.
[2] American cancer society. Available at: www.cancer.org/docroot/CRI/content/CRI_2_2_1x_What_Is_Acute_Myeloid_Leukemia_AML.asp?rnav=cri.
[3] Xian M, Ito K, Nakazato T, Shimizu T, Chen CK, Kizaki M. Zerumbone, a bioactive sesquiterpene, induces G2/M cell cycle arrest and apoptosis in leukemia cells via a Fas- and mitochondria-mediated pathway. Cancer Sci 2007;98:118–26.
[4] Maltais R, Tremblay MR, Ciobanu LC, Poirier D. Steroids and combinatorial chemistry. J Comb Chem 2004;6:443–56.
[5] Poirier D, Maltais R. Solid-phase organic synthesis (SPOS) of modulators of estrogenic and androgenic action. Mini-Rev Med Chem 2006;6:37–52.
[6] Hanson JR. Steroids: partial synthesis in medicinal chemistry. Nat Prod Rep 2007;24:1342–9.
[7] Kim RS, Zaborniak CL, Begleiter A, LaBella FS. Antiproliferative properties of aminosteroid antioxidants on cultured cancer cells. Cancer Lett 1992;64:61–6.
[8] He Q, Jiang D. A novel aminosteroid is active for proliferation inhibition and differentiation induction of human acute myeloid leukemia HL-60 cells. Leukemia Res 1999;23:369–72.
[9] Thibeault D, Poirier D. An efficient method for the regioselective aminolysis of 2,3α-steroid epoxide. Synlett 2003;8:1192–4.
[10] Thibeault D, Roy J, DeRoy P, Poirier D. Chemical synthesis of 2β-amino-5α-androstane-3α,17β-diol N-derivatives and their antiproliferative effect on HL-60 human leukemia cells. Bioorg Med Chem 2008;16:5062–77.
[11] Roy J, DeRoy P, Poirier D. 2β-(N-substituted piperazino)-5α-androstane-3α,17β-diols: parallel solid-phase synthesis and antiproliferative activity on human leukemia HL-60 cells. J Comb Chem 2007;9:347–58.
[12] Roy J, Maltais R, Jegham H, Poirier D. Libraries of 2β-(N-substituted piperazin)-5α-androstane-3α,17β-diol: chemical synthesis and cytotoxic effects on human leukemia HL-60 cells and on normal lymphocytes. Mol Divers 2011;15:317–39.
[13] Kagialis-Girard S, Mialou V, Chebel A, Ffrench M. Inhibition of normal lymphocyte proliferation by indirubin-3′-monoxime: a multifactorial process. Leuk Lymphoma 2007;48:605–15.
[14] Rocha DD, Militao GC, Veras ML. F. Selective cytotoxicity of withaphysalins in myeloid leukemia cell lines versus peripheral blood mononuclear cells. Life Sci 2006;79:1692–701.
[15] Cwynarski K, Laylor R, Macchiarulo E, Goldman J, Lombardi G, Melo JV, Dazzi F. Imatinib inhibits the activation and proliferation of normal T lymphocytes in vitro. Leukemia 2004;18:1332–9.
[16] Bezerra DP, Militao GC, de Castro FO, Pessoa C, de Moraes MO, Costa-Lotufo LV. Piplartine induces inhibition of leukemia cell proliferation triggering both apoptosis and necrosis pathways. Toxicol In Vitro 2007;21:1–8.
[17] Park S, Chapuis N, Bardet V, Tamburini J, Gallay N, Willems L, et al. PI-103, a dual inhibitor of Class IA phosphatidylinositide 3-kinase and mTOR, has antileukemic activity in AML. Leukemia 2008;22:1698–706.
[18] Murata T, Itoigawa M, Ito C, Nakao K, Tsuboi M, Kaneda N, Furukawa H. Induction of apoptosis in human leukaemia HL-60 cells by furanone-coumarins from Murraya siamensis. J Pharm Pharmacol 2008;60:385–9.
[19] Hudlicky M. Fluorination with diethylaminosulfur trifluoride and related aminofluoro-sulfuranes. Org React 1988;35:513–641.
[20] Bird TGC, Fredericks PM, Jones ERH, Meakins GD. Convenient general preparation of oxygenated monofluoro- and gem-difluoro-5α-androstanes using diethylaminosulfur trifluoride. J Chem Soc Chem 1979:65–6.
[21] Fieser LF, Fieser M, editors. Steroids. New York: Reinhold Publishing Corp.; 1959. p. 339.
[22] Ciobanu LC, Boivin RP, Luu-The V, Poirier D. 3β-Sulfamate derivatives of C19 and C21 steroids bearing a *t*-butylbenzyl or a benzyl group: synthesis and evaluation as non-estrogenic and non-androgenic steroid sulfatase inhibitors. J Enzyme Inhib Med Chem 2003;18:15–26.

[23] Heinrich MC, Scheer M, Heckmann M, Kuefner MA, Uder M. Iodixanol induces apoptotic and antiproliferative effects but no necrotic cell death in renal proximal tubular cells in vitro. RoFo 2009;181:349–54.
[24] Vermeulen K, Van Bockstaele DR, Berneman ZN. Apoptosis: mechanisms and relevance in cancer. Ann Hematol 2005;84:627–39.
[25] Garner DL. Hoechst 33342: the dye that enabled differentiation of living X-and Y-chromosome bearing mammalian sperm. Theriogenology 2009;71:11–21.
[26] Park H, Aiyar SE, Fan P, Wang J, Yue W, Santen RJ. Effects of tetramethoxystilbene on hormone-resistant breast cancer cells: biological and biochemical mechanisms of action. Cancer Res 2007;67:5717–26.
[27] Jegham H, Roy J, Maltais R, Desnoyers S, Poirier D. A novel aminosteroid of the 5α-androstane-3α,17β-diol family induces cell cycle arrest and apoptosis in human promyelocytic leukemia HL-60 cells. Invest New Drugs 2012;30:176–85.