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Synthesis and antiproliferative activity of novel steroidal dendrimer conjugates

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ABSTRACT

We describe the synthesis of steroidal dendrimer conjugates of first and second generation with tetramethylene core and 5-hydroxy-isophtalic acid dimethyl ester as branching unit modified to incorporate ethynylestradiol or 17α-estradiol as terminal units. The steroidal dendrimer conjugates, the free drug (steroids) and dendrimer were tested against a panel of cancer cell lines (CEM, MCF7, HeLa) and normal human fibroblast (BJ). The steroidal dendrimer conjugates of first generation exhibited cytotoxic activity and induced apoptosis in chronic leukemia (CEM) as resultant activation of caspase cascade which is mainly provoked in G2/M arrested cells.

1. Introduction

More than 11 million people are diagnosed with cancer each year and the incidence of this disease is projected to rise continuously to 16 million by 2020 [1]. For this reason, the discovery of new active drugs and the development of delivery devices capable of improving the therapeutic index of biologically active molecules and decreasing unwanted side effects [2] are of crucial importance.

Dendrimers are synthetic macromolecules possessing well defined branching architectures in nanometric size that can be easily tailored to endow specific properties [3–7]. They have attracted great attention due to their potential in the delivery of anticancer drugs because their high multivalency enhances cellular interactions and promotes a faster endocytosis [8–11]. As drug delivery systems, three strategies [12–14] have been employed: (i) formation of dendrimer networks around the drug; (ii) drug encapsulation inside the dendrimer mediated by electrostatic and van der Walls interactions; (iii) drug "conjugation" by covalent attachment or electrostatic binding at the periphery. However, several reports suggest [13,14] that, when a drug is encapsulated or electrostatically attached to a dendrimer, it may be released prematurely in the body as a result of a small pH change. Consequently, the observed therapeutic effect is lower than one would expect. On the contrary, the covalent conjugation, with or without the assistance of target moieties, improves the selective drug accumulation, increases the circulation time in the body, favoring a sustained liberation [2,12,14] and inducing apoptosis [15–17].

Synthetic estrogens, such as 17α-ethynylestradiol and 17α-estradiol have been used to prevent or reduce menopause symptoms, as oral contraceptives, in the treatment of alopecia, and in neurodegenerative disorders such as Alzheimer and Parkinson diseases [18]. Interestingly steroidal compounds have also shown antiproliferative activity and the ability to induce apoptosis [19]. In particular, the cytotoxicity of 17α-estradiol toward human leukemia Jurkat T cells has been attributed to apoptosis, mainly induced in G2/M-arrested cells [20], while 17α-ethynylestradiol has been evaluated with promising results as inhibitor of human prostate [21] and colon cancer [22]. The combination of dendritic compounds with steroidal derivatives has given rise to new architectures with a wide-range of biological effects when used as drug delivery systems [23–26], including the treatment of malaria [27] and in vitro in the lung inflammatory process [28].

With the aim to exploit the high drug payload of dendrimers without loss of monodispersity and well defined structure, to explore the possibility to enhance the cytotoxic activity of 17α-ethynylestradiol and 17α-estradiol, and bearing in mind the advantages of covalent conjugation in drug delivery, four new steroidal dendrimer conjugates were synthetized in this work. The dendrimers were prepared using a flexible tetramethylene core, and 5-hydroxy-isophtalic acid dimethyl ester as branching unit modified to incorporate 4 units of 17α-ethynylestradiol or

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17α-estradiol for first generation (**8** and **9**) and 8 units for the second (**10** and **11**). Their preliminary biological assays show that all new dendrimers and dendrimeric conjugates of G1 are noncytotoxic against normal fibroblastic cells (BJ), but the conjugates of first generation are able to induce apoptosis in leukemia cancer cells (CEM) displaying a higher cytotoxic activity than the free drug.

2. Experimental

2.1. General

The ^{1}H and ^{13}C NMR spectra were recorded on JEOL 400 and 500, and Bruker 300 using $CDCl_3$, DMSO-d_6 as solvent. Chemical shifts are reported in parts per million (ppm) relative to internal TMS. Mass spectra were recorded with an Agilent Technologies MS TOF using the ESI(+) technique. IR spectra were recorded using a Perkin–Elmer Spectrum GX FTIR spectrometer.

All reagents were commercially available. THF was refluxed over sodium/benzophenone and distilled under reduced pressure in a nitrogen atmosphere prior to use. Column chromatography was carried out with silica gel (70–230 mesh). 5-Hydroxy-isophthalic acid dimethyl ester (**1**) was synthesized following the literature [29]. Compounds **2** and **4** have been reported in the literature, however, using the method described herein the yields were optimized [30,31]. Unambiguous NMR spectral assignment was attained using one and two dimensional spectra.

2.2. Synthesis of compounds

2.2.1. 1,4-Bis(3,5-bis(carboxymethyl)phenoxy)butane (**2**)

5-Hydroxy-isophthalic acid dimethyl ester (**1**) (4.29 g, 20.41 mmol) and potassium carbonate (8.29 g, 59.98 mmol) were stirred in acetonitrile, followed by slow addition of 1,4-dibromobutane (1.1 mL, 9.26 mmol) and 18-crown-6 (1 mg). The mixture was heated at 60 °C during 48 h, filtered and rinsed with CH_2Cl_2. The solvent was removed under reduced pressure to obtain 4.30 g (9.06 mmol, 98%) of compound **2** as beige solid. M.p. 152–153 °C, in agreement with the literature [24].

^{1}H NMR ($CDCl_3$, 400 MHz) δ(ppm): 8.24 (2H, t, J = 1.5 Hz, H-6), 7.71 (4H, d, J = 1.5 Hz, H-4), 4.11 (4H, m, H-2), 3.91 (12H, s, H-8), 2.01 (4H, m, H-1). ^{13}C NMR ($CDCl_3$, 100 MHz) δ(ppm): 166.2 (C-7), 159.0 (C-3), 131.8 (C-5), 123.0 (C-6), 119.8 (C-4), 68.0 (C-2), 52.4 (C-8), 25.8 (C-1). IR (ν_{max}/cm^{-1}): 2951, 2852, 2360, 1725 (O–C=O), 1596, 1453, 1433, 1337, 1312, 1238 (C–O), 1111 (C-O), 1050, 1018, 1000, 902, 875, 753, 671, 628. HR-ESI-TOF-MS (m/z): calcd for $C_{24}H_{26}O_{10}$[M+H]$^+$: 475.1604; found: 475.1583.

2.2.2. 1,4-Bis(3,5-bis(hydroxymethyl)phenoxy)butane (**3**)

A solution of tetraester **2** (1.52 g, 40.00 mmol) in dry THF was added dropwise to a magnetically stirred suspension of $LiAlH_4$ (3.00 g, 6.32 mmol) in dry THF under N_2. The reaction was stirred 24 h at room temperature, and quenched by the slow addition of a solution of ammonium chloride (10 mL) and ethyl acetate (40 mL) with cooling. The aluminum salts were filtered and the solvents were then removed under reduced pressure to give 1.52 g (4.19 mmol, 56%) of compound **3** as a white solid. M.p. 147–149 °C.

^{1}H NMR (DMSO-d_6, 300 MHz) δ(ppm): 6.82 (2H, s, H-6), 6.73 (4H, s, H-4), 4.42 (8H, s, H-7), 3.99 (4H, brs, H-2), 3.35 (OH), 1.84 (4H, brs, H-1).^{13}C NMR (DMSO-d_6, 75 MHz) δ(ppm): 159.0 (C-3), 144.3 (C-5), 116.9 (C-6), 111.0 (C-4), 67.4 (C-2), 63.2 (C-7), 25.9 (C-1). IR (ν_{max}/cm^{-1}): 3291, 3170, 2943, 2873, 2853, 1595, 1449, 1297, 1265, 1168, 1064, 1027, 979, 918, 841, 701, 677, 607. HR-ESI-MS (m/z) calcd for $C_{20}H_{26}O_6$[M+Na]$^+$: 385.1627; found: 385.1609.

2.2.3. 1,4-Bis(3,5-bis(bromomethyl)phenoxy)butane (**4**)

Compound **3** (1.28 g, 3.53 mmol) was dissolved in a 2:1 solution of HBr: H_2SO_4 (100 mL), The mixture was heated at 100–110 °C for 1 h, allowed to cool and diluted with H_2O (100 mL). The aqueous solution was extracted with CH_2Cl_2 (3 × 150 mL) and the combined organic layers were washed with saturated aqueous $NaHCO_3$ solution (100 mL), dried ($MgSO_4$), filtered and concentrated to yield a brown solid. The crude product was purified by column chromatography using hexane to obtain 1.84 g (3.01 mmol, 84%) of **4** as a white crystalline solid. M.p. 126–128 °C.

^{1}H NMR ($CDCl_3$, 300 MHz) δ(ppm): 6.99 (2H, brs, H-6), 6.85 (4H, brs,H-4), 4.42 (8H, s, H-7), 4.04 (4H, m, H-2), 1.98 (4H, m, H-1).^{13}C NMR ($CDCl_3$, 75 MHz) δ(ppm): 159.3 (C-3), 139.6 (C-5), 121.8 (C-6), 115.2 (C-4), 67.5 (C-2), 32.9 (C-7), 25.8 (C-1). IR (ν_{max}/cm^{-1}): 2928, 2871, 1593, 1452, 1388, 1328, 1296 (C-O), 1166 (C–O–C), 1055, 1019, 940, 870, 848, 732, 697, 671, 648 y 633 (C–Br).

2.2.4. 1,4-Bis(3,5-bis(3,5-bis(carboxymethyl)phenoxymethyl) phenoxy) butane (**5**)

5-Hydroxy-isophthalic acid dimethyl ester (**1**) (0.13 g, 0.62 mmol) and potassium carbonate (0.30 g, 2.17 mmol) were stirred in acetonitrile, followed by slow addition of 1,4-bis(3,5-bis(bromomethyl)phenoxy)butane (0.10 g, 0.16 mmol) and tetrabutylammonium fluoride (1 mg) of. The mixture was refluxed 72 h, filtered and rinsed with CH_2Cl_2. The solvent was removed under reduced pressure to obtain 0.15 g (0.13 mmol, 82%) of compound **5** as a beige solid. M.p. 176–177 °C.

^{1}H NMR ($CDCl_3$, 400 MHz) δ(ppm): 8.27 (4H, s, H-11), 7.81 (8H, s, H-9), 7.08 (8H, s, H-7), 6.96 (4H, s, H-4), 5.10 (2H, s, H-6), 4.08 (4H, brs, H-2), 3.92 (24H, s, COOMe), 2.00 (4H, brs, H-1). ^{13}C NMR ($CDCl_3$, 100 MHz) δ(ppm): 166.1 (O=COMe), 159.7 (C-3), 158.7 (C-8), 138.1 (C-5), 131.9 (C-10), 123.4 (C-11), 120.1 (C-9), 118.5 (C-6), 113.3 (C-4), 70.2 (C-7), 67.6 (C-2), 52.5 (COOMe), 26.0 (C-1). IR (ν_{max}/cm^{-1}): 2954, 1721 (O–C=O), 1597, 1431, 1339, 1311, 1239 (C-O), 1170, 1113, 1064, 1043, 1000, 907, 875, 836, 792, 752, 721, 682, 564. HR-ESI-TOF-MS (m/z): calcd for $C_{60}H_{58}O_{22}$[M+Na]$^+$: 1153.3317; found: 1153.3315.

2.2.5. 1,4-Bis(3,5-bis(3,5-bis(hydroxymethyl)phenoxymethyl) phenoxybutane (**6**)

A solution of **5** (0.10 g, 0.09 mmol) in dry THF was added dropwise to a magnetically stirred suspension of $LiAlH_4$ (0.04 g, 1.05 mmol) in dry THF under N_2. The reaction was stirred 24 h at room temperature, and quenched by the slow addition of a solution of ammonium chloride (10 mL) and ethyl acetate (40 mL) with cooling. The aluminum salts were filtered and the solvents were then removed under reduced pressure to give 0.04 g (0.05 mmol, 53%) of compound **6** as a white solid. M.p. 146–147 °C.

^{1}H NMR (DMSO-d_6, 300 MHz) δ(ppm): 7.18 (2H, brs, H-6), 7.08 (4H, brs, H-11), 6.85 (4H, brs, H-4), 6.82 (8H, brs, H-9), 5.04 (8H, brs, H-7), 4.44 (16H, brs, H-12), 4.05 (4H, brs, H-2), 2.48 (8H, brs, OH), 1.87 (4H, brs, H-1). ^{13}C NMR (DMSO-d_6, 75 MHz) δ(ppm): 159.2 (C-3), 158.7 (C-8), 144.4 (C-10), 139.4 (C-5), 118.9 (C-6), 117.3 (C-11), 113.2 (C-4), 111.3 (C-9), 69.3 (C-7), 67.6 (C-2), 63.3 (C-12), 25.8 (C-1). IR (ν_{max}/cm^{-1}): 3317 (OH), 2918, 2850, 2359, 1720, 1595, 1451, 1375, 1294, 1256, 1154, 1110, 1021 (C-O), 843, 801, 752, 684. HR-APCI-TOF-MS (m/z): calcd for $C_{52}H_{58}O_{14}$[M+Na]$^+$: 929.3724; found: 929.3720.

2.2.6. 1,4-Bis(3,5-bis(3,5-bis(chloromethyl)phenoxymethyl) phenoxybutane (**7**)

To a magnetically stirred solution of **6** (0.75 g, 0.83 mmol) in dry CH_2Cl_2 was added dry pyridine (0.59 mL, 7.28 mmol), followed by dropwise addition of $SOCl_2$ (0.52 mL, 7.28 mmol) under N_2. The reaction was stirred 48 h at room temperature, and quenched by the slow addition of water. The organic phase was extracted with

CH_2Cl_2, and dried over Na_2SO_4. The product was purified by column chromatography using hexane:ethyl acetate (85:15) to give 0.58 g (0.55 mmol, 67%) of **7**, as a white solid. M.p. 107–109 °C.

^{1}H NMR ($CDCl_3$, 500 MHz) δ(ppm): 7.06 (2H, brs, H-6), 7.01 (4H, brs, H-11), 6.95 (8H, brs, H-9), 6.94 (4H, brs, H-4), 5.04 (8H, brs, H-7), 4.53 (16H, brs, H-12), 4.07 (4H, brs, H-2), 1.99 (4H, brs, H-1). ^{13}C NMR ($CDCl_3$, 125 MHz) δ(ppm): 159.6 (C-3), 159.2 (C-8), 139.4 (C-10),138.5 (C-5), 121.3 (C-11), 118.5 (C-6), 115.0 (C-9), 113.2 (C-4), 69.9 (C-7), 67.6 (C-2), 45.8 (C-12), 26.0 (C-1). IR (ν_{max}/cm^{-1}): 2957, 2876 (C–H), 1729, 1594(C=C), 1259 (C–O eter), 1454, 1326, 1300, 1259, 1158, 1043, 958, 842, 708 (C-Cl), 674, 583. MALDI TOF MS (m/z) calcd for $C_{52}H_{50}Cl_8O_6$[M+H]$^+$: 1051.1194; found: 1050.1887.

2.2.7. 17α-Ethynylestradiol-dendrimer conjugate of first generation (**8**)

17α-Ethynylestradiol (0.19 g, 0.64 mmol) and potassium carbonate (0.30, 2.17 mmol) were stirred in acetonitrile. To this solution were added **4** (0.10 g, 0.16 mmol) and tetrabutylammonium fluoride (1 mg). The reaction was refluxed 3 days, and rinsed with CH_2Cl_2. The solvent was removed under reduced pressure and the crude product was purified by column chromatography using EtOAc in hexane to give 0.18 g (0.12 mmol, 74%) of **8** as a pale yellow solid. M.p. 153–155 °C.

^{1}H NMR ($CDCl_3$, 500 MHz) δ(ppm): 7.20 (4H, d, J = 8.6 Hz, H-1'), 7.05 (2H, s, H-6), 6.92 (4H, s, H-4), 6.76 (4H, dd, J_o = 8.6 Hz, J_m = 2.7 Hz, H-2'), 6.70 (4H, d, J = 2.7 Hz, H-4'), 4.98 (8H, s, H-7), 4.05 (4H, brs, H-2), 2.60 (4H, brs, H-20'), 0.87 (12H, s, Me-18'). ^{13}C NMR ($CDCl_3$, 125 MHz) δ(ppm): 159.5 (C-3), 156.7 (C-3'), 139.2 (C-5), 138.1 (C-5'), 132.9 (C-10'), 126.5 (C-1'), 118.5 (C-4), 114.9 (C-4'), 112.9 (C-6), 112.4 (C-2'), 87.6 (C-19'), 79.9 (C-17'), 74.2 (C-20'), 69.9 (C-7), 67.6 (C-2), 49.5 (C-14'), 47.2 (C-13'), 43.6 (C-9'), 39.4 (C-8'), 39.0 (C-16'), 32.8 (C-12') 29.9 (C-6'), 27.3 (C-7'), 26.4 (C-11'), 26.0 (C-1), 22.9 (C-15'), 12.8 (Me-18'). IR (ν_{max}/cm^{-1}): 3282 (≡C–H), 2923, 2855, 2161 (C≡C), 1716, 1662, 1601, 1496, 1456, 1377, 1289, 1231, 1159, 1058, 1021, 844, 734, 624. HR-APCI-TOF-MS (m/z): calc for $C_{100}H_{114}O_{10}$[M+Na]$^+$: 1497.8309; found: 1497.8284.

2.2.8. 17α-Estradiol-dendrimer conjugate of first generation (**9**)

To a mixture of 17α-estradiol (0.18 g, 0.66 mmol), and potassium carbonate (0.30 g, 2.17 mmol) in acetonitrile was added **4** (0.10 g, 0.16 mmol) and tetrabutylammonium fluoride (1 mg). The reaction was refluxed 3 days, the solvent was removed under reduced pressure and the crude product was purified by column chromatography using methanol/CH_2Cl_2 to give 0.18 g (0.13 mmol, 80%) of 9 as a pale yellow solid. M.p. 154–156 °C.

^{1}H NMR ($CDCl_3$, 300 MHz) δ(ppm): 7.20 (4H, d, J = 8.6 Hz, H-1'), 7.04 (2H, s, H-6), 6.92 (4H, s, H-4), 6.75 (4H, dd, J_o = 8.6 Hz, J_m = 2.6 Hz, H-2'), 6.70 (4H, d, J = 2.6 Hz, H-4'), 4.99 (8H, s, H-7), 4.05 (4H, brs, H-2), 0.70 (12H, s, Me-18'). ^{13}C NMR ($CDCl_3$, 75 MHz) δ(ppm): 159.5 (C-3), 156.7 (C-3'), 139.2 (C-5), 138.1 (C-5'), 133.1 (C-10'), 126.4 (C-1'), 118.4 (C-4), 114.9 (C-4'), 112.9 (C-6), 112.3 (C-2'), 80.1 (C-17'), 69.8 (C-7), 67.5(C-2), 47.8 (C-14'), 45.6 (C-13'), 43.7 (C-9'), 39.1 (C-8'), 32.5 (C-12'), 31.5 (C-16'), 30.0 (C-6'), 28.1 (C-7'), 26.3 (C-11'), 26.0 (C-1), 24.3 (C-15'), 17.1 (C18'). IR (ν_{max}/cm^{-1}): 3399 (OH), 2929, 2865, 1602,1496, 1231, 1156,1033, 609. HR-APCI-TOF MS (m/z): calcd for $C_{92}H_{114}O_{10}$[-M+Na]$^+$: 1401.8304; found: 1401.8315.

2.2.9. 17α-Ethynylestradiol-dendrimer conjugate of second generation (**10**)

17α-Ethynylestradiol (0.56 g, 1.90 mmol) and potassium carbonate (0.86 g, 6.3 mmol) were stirred in acetonitrile. To this solution was added **7** (0.25 g, 0.24 mmol) and tetrabutylammonium fluoride (1 mg). The reaction was refluxed 3 days, the solvent was removed under reduced pressure and the crude product was purified by column chromatography using EtOAc/hexane to give 0.56 g (0.18 mmol, 75%) of **10** as a pale yellow solid. M.p. 175–177 °C.

^{1}H NMR ($CDCl_3$, 500 MHz) δ(ppm): 7.18 (8H, d, J = 8.6 Hz, H-1'), 7.06 (4H, s, H-11), 7.05 (2H, s, H-6), 6.99 (8H, s, H-9), 6.93 (4H, s, H-4), 6.75 (8H, dd, J_o = 8.6, J_m = 2.5 Hz,H-2'), 6.68 (8H, d, J = 2.5 Hz, H-4'), 5.01 (8H, s H-7), 4.97 (16H, s, H-12), 4.05 (4H, brs, H-2), 2.85 (16H, m, H-6'), 2.59 (8H, s, H-20'), 0.86 (24H, s, Me-18'). ^{13}C NMR ($CDCl_3$, 125 MHz) δ(ppm): 159.6 (C-3), 159.2 (C-8), 156.7 (C-3'), 139.3 (C-10), 138.8 (C-5), 138.1 (C-5'), 132.9 (C-10'), 126.5 (C-1'), 118.8 (C-11), 114.9 (C-4'), 113.23 (C-9), 113.16 (C-4), 112.4 (C-2'), 87.6 (C-19'), 79.9 (C-17'), 74.2 (C-20'), 69.9 (C-7), 69.7 (C-12), 67.6 (C-2), 49.5 (C-14'), 47.2 (C-13'), 43.6 (C-9'), 39.4 (C-8'), 39.0 (C-16'), 32.8 (C-12') 29.9 (C-6'), 27.3 (C-7'), 26.4 (C-11'), 26.0 (C-1), 22.9 (C-15'), 12.8 (Me-18'). IR (ν_{max}/cm^{-1}): 3285, 2930, 2868, 1598, 1496, 1454, 1291, 1231, 1145, 1050, 1020, 843, 786, 571. MALDI TOF MS (m/z): calcd for $C_{212}H_{234}O_{22}$[M+Na+NH$_3$]$^+$: 3171.7355; found: 3171.7646.

2.2.10. 17α-Estradiol-dendrimer conjugate of second generation (**11**)

17α-estradiol (0.045 g, 0.17 mmol) and potassium carbonate (0.07 g, 0.50 mmol) were stirred in acetonitrile. To this solution were added the compound **7** (0.02 g, 0.019 mmol) and tetrabutylammonium fluoride (1 mg). The reaction was refluxed 48 h, filtered and rinsed with water and hexane to obtain 0.05 g (0.017 mmol, 89%) of **11** as a pale yellow solid. M.p. 169–171 °C.

^{1}H NMR ($CDCl_3$, 300 MHz) δ(ppm): 7.19 (8H, d, J = 6.9 Hz, H-1'), 7.07 (4H, s, H-11), 7.03 (2H, s, H-6), 6.99 (8H, s, H-9), 6.94 (4H, s, H-4), 6.75 (8H, d, J = 6.9 Hz, H-2'), 6.69 (8H, s, H-4'), 5.02 (8H, s, H-7), 4.05 (4H, brs, H-2), 2.82 (16H, m, H-6'), 0.68 (24H, s, Me-18'). ^{13}C NMR ($CDCl_3$, 75 MHz) δ(ppm): 159.5 (C-3), 156.7 (C-3'), 139.2 (C-5), 138.1 (C-5'), 133.1 (C-10'), 126.4 (C-1'), 118.4 (C-4), 114.9 (C-4'), 112.9 (C-6), 112.3 (C-2'), 80.1 (C-17'), 69.8 (C-7), 67.5(C-2), 47.8 (C-14'), 45.6 (C-13'), 43.7 (C-9'), 39.1 (C-8'), 32.5 (C-12'), 31.5 (C-16'), 30.0 (C-6'), 28.1 (C-7'), 26.3 (C-11'), 26.0 (C-1), 24.3 (C-15'), 17.1 (C18'). IR (ν_{max}/cm^{-1}): 3451(O-H), 2928, 2864 (C-H), 1598 (C=C), 1496, 1453, 1376, 1295, 1233, 1155, 1036, 846, 788, 710, 573. MALDI TOF MS (m/z): calcd for $C_{196}H_{234}O_{22}$[M+Na]$^+$: 2962.7089; found: 2962.8896.

2.3. Biological tests

2.3.1. Cell culture

Stock solutions (10 mmol/L) of dendrimer conjugate derivatives were prepared by dissolving an appropriate quantity of each substance in DMF. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), trypsin, L-glutamine, penicillin and streptomycin were purchased from Sigma (MO, USA). Calcein AM was obtained from Molecular Probes (Invitrogen Corporation, CA, USA).

The cell lines for screening (T-lymphoblastic leukemia cell line CEM, breast carcinoma cell line MCF-7, cervical carcinoma cell line HeLa, and human fibroblasts BJ) were obtained from the American Type Culture Collection (Manassas, VA, USA). All cell lines were cultured in DMEM medium (Sigma, MO, USA), supplemented with 10% heat-inactivated fetal bovine serum, 2 mmol/L L-glutamine, 10,000 U penicillin and 10 mg/mL streptomycin. The cell lines were maintained under standard cell culture conditions at 37 °C and 5% CO_2 in a humid environment. Cells were subcultured twice or three times a week using the standard trypsinization procedure.

2.3.2. Calcein AM assay

Suspensions of the tested cell lines (ca. 1.0×10^5 cells/mL) were placed in 96-well microtiter plates and after 3 h of stabilization (time zero) the compounds to be tested were added (in four 20 μL aliquots) in serially diluted concentrations in dimethylformamide (DMF). Control cultures were treated with DMF alone, and

the final concentration of DMF in the incubation mixtures never exceeded 0.6%. The test compounds were typically evaluated at six 3-fold dilutions and the highest final concentration was generally 50 μM. After 72 h incubation, 100 μL Calcein AM solution (Molecular Probes, Invitrogen, CA, USA) was added, and incubation was continued for a further hour. The fluorescence of viable cells was then quantified using a Fluoroskan Ascent instrument (Labsystems, Finland). The percentage of surviving cells in each well was calculated by dividing the intensity of the fluorescence signals from the exposed wells by the intensity of signals from control wells and multiplying by 100. These ratios were then used to construct dose–response curves from which IC_{50} values, the concentrations of the respective compounds that were lethal to 50% of the tumor cells, were calculated. The results obtained for selected compounds are shown in Table 1.

2.3.3. Flow cytometric analysis of the cell cycle and apoptosis

CEM leukemia cancer cells were seeded in 100-mm culture dishes, and immediately incubated with the test compounds. After 24 h, the cells were washed, fixed and stained in 0.1% [m/v] sodium citrate, 0.1% [v/v] Triton X-100, 0.2 mg/mL RNase A and 10 μg/mL propidium iodide in PBS. Their DNA contents were then assessed with a flow cytometer (Cell Lab Quanta SC – MPL, Beckman Coulter, CA USA), and the distribution of cells in $subG_1$ ("apoptotic cells"), G_0/G_1, S and G_2/M peaks were quantified, by histogram analysis, using MultiCycle AV software (Phoenix Flow Systems, CA, USA).

2.3.4. Activities of caspases 3 and 7 (3/7)

Treated CEM cells were harvested by centrifugation and homogenized in an extraction buffer (10 mM KCl, 5 mM HEPES, 1 mM EDTA, 1 mM EGTA, 0.2% CHAPS, plus protease inhibitors: aprotinin, leupeptin, PMSF; pH 7.4) on ice for 20 min. The resulting homogenates were clarified by centrifugation at 10000 × g for 20 min at 4 °C. The protein contents of the samples were quantified by the Bradford method and they were diluted to the equivalent protein concentrations. Lysates were then incubated for 1 h with 100 mM Ac-DEVD-AMC as a substrate (Sigma–Aldrich) in an assay buffer (25 mM PIPES, 2 mM EGTA, 2 mM $MgCl_2$, 5 mM DTT, pH 7.3). For negative controls, the lysates were supplemented with 100 mM Ac-DEVD-CHO (Sigma–Aldrich) as a caspase 3/7 inhibitor. The fluorescence of the product was measured using a Fluoroskan Ascent microplate reader (Labsystems, Finland) at 346/442 nm (ex/em).

Table 1
IC_{50} (μM) values obtained from the calcein AM assays with the tested cancer and normal cell lines.

Compound	Cell line[a]			
	CEM	MCF7	HeLa	BJ
17α-ethynylestradiol	19.1 ± 1.7	>50	1.4 ± 0.1	>50
17α-estradiol	25.2 ± 6.4	>50	>50	>50
G1				
3	>50	>50	>50	>50
8	3.5 ± 1.3	>50	>50	>50
9	2.1 ± 0.2	>50	>50	>50
G2				
6	>50	>50	>50	3.3 ± 1.2
10	26.8 ± 4.6	34.6 ± 4.9	5.5 ± 0.1	5.1 ± 1.3
11	>50	>50	>50	>50

[a] The cells were treated for 72 h with serial concentrations of the tested compounds. The values quoted represent means ± SD, these were obtained from three independent experiments performed in triplicate using 17α-ethynylestradiol, 17α-estradiol, 3 and 6 as a control in chronic leukemia (CEM), breast adenocarcinoma (MCF7) and, cervical carcinoma (HeLa) tumor cell lines and against normal human fibroblasted cells (BJ).

2.3.5. Western blotting

The cells were seeded into culture medium in 100-mm culture dishes at a density of 1.5×10^6 cells/mL and treated immediately with dendrimer conjugate derivatives. DMF was used as a vehicle for controls. After 24 h of treatment, the cells were washed three times with cold PBS (10 mM, pH 7.4) and lysed in ice-cold RIPA protein extraction buffer (20 mM Tris–HCl, pH 7.4, 5 mM EDTA, 2 mM EGTA, 100 mM NaCl, 2 mM NaF, 0.2% Nonidet P-40, 30 mM PMSF, 1 mM DTT, 10 mg/mL of aprotinin and leupeptin). The lysate was collected into a microfuge tube and incubated on ice for 1 h. It was then cleared by centrifugation at 10,000*g* for 30 min at 4 °C and the supernatant was collected. Proteins in lysates were quantified by the Bradford method and diluted with Laemmli electrophoresis buffer. The proteins were then separated on 10% or 12% SDS–polyacrylamide gels, transferred to nitrocellulose membranes (Bio-Rad Laboratories, CA, USA) and stained with Ponceau S to check equal protein loading. The membranes were blocked with 5% (w/v) non-fat dry milk and 0.1% Tween-20 in PBS for 2 h and probed overnight with specific primary antibodies (Santa Cruz Biotechnology, CA, USA). After washing in PBS and PBS with 0.1% Tween-20, the membranes were probed with horseradish peroxidase-conjugated secondary antibodies and visualized with West Pico Supersignal chemiluminescent detection reagent (Thermo Fisher Scientific, Rockford, USA). To confirm equal protein loading, immunodetection was performed with anti-β-actin monoclonal antibody (Santa Cruz Biotechnology, CA, USA). The experiments were repeated three times. The protein expression in treated cells was compared to untreated controls.

3. Results and discussion

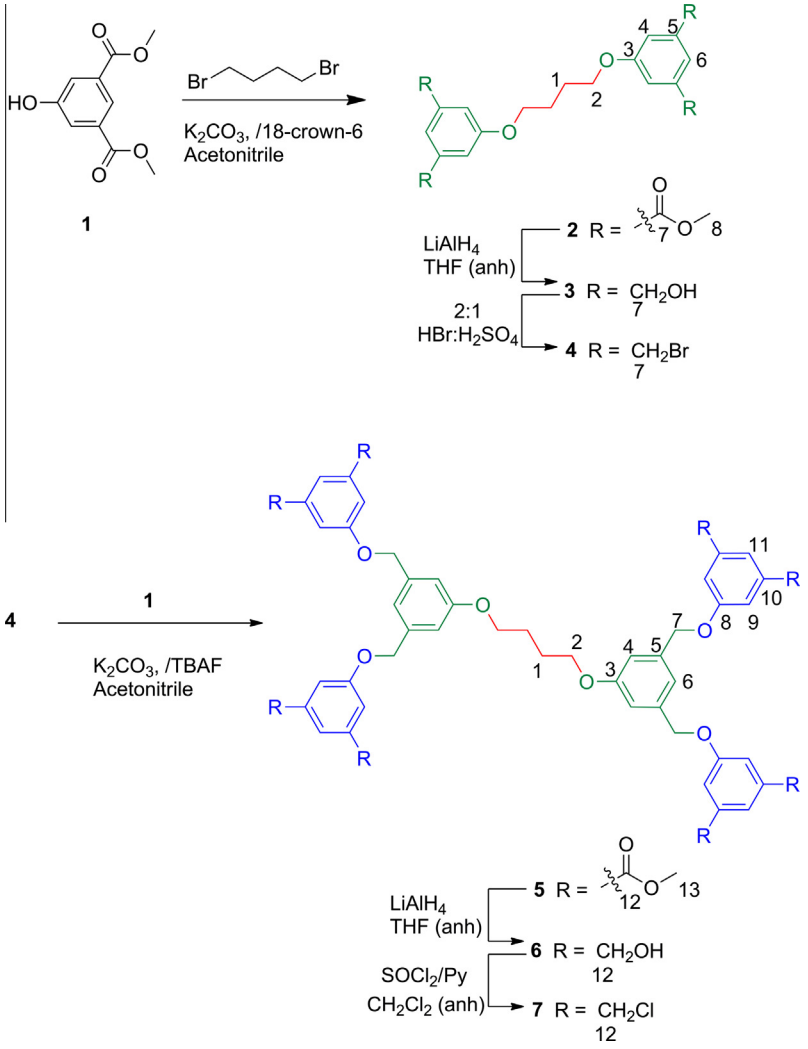
3.1. Chemical synthesis and characterization

The dendrimeric structure consists of a core that determines the shape and steric hindrance in the structure, promoting a spherically or ellipsoidal shape as function of generation. Noteworthy, the PAMAM dendrimer at G1 with 8 NH_2 groups (1.430 KDa and 22 Å) presents an irregular geometry and adopts a spherical shape that increases the steric hindrance and the molecular rigidity in the higher generations, complicating the drug payload at the periphery [33]. Hence, we focused in the synthesis of dendrimeric structures with tetramethylene core in order to promote an ellipsoidal shape and minimize steric hindrance. We used dimethyl 5-hydroxyisophthalate as branching unit which undergoes modification of the functional groups at the periphery via nucleophilic reactions with the steroids.

Following the synthetic route shown in Scheme 1, the dendrimer of first generation (tetramethyl ester **2**) was obtained in high yield (98%) by reaction of dimethyl 5-hydroxyisophthalate (**1**) with 1,4-dibromobutane in the presence of potassium carbonate, and 18-crown-6 as the phase transfer catalyst. Subsequent reduction of **2** with $LiAlH_4$ afforded tetraol **3** (56% yield) which was treated with H_2SO_4/HBr to give tetrabromo derivative **4** (84% yield) as a solid ready for coupling with the steroids or to the second iteration of analogous transformations for the preparation of the second dendrimer generation.

Dendrimers of second generation (**5**, **6** and **7**) were prepared by repeating the synthetic procedures described for the first generation, to give the methyl ester **5** in high yield (82%). The ester was reduced to give hydroxyl derivative **6**, which was converted into benzylic chlorides to give **7**.

The structures of all compounds obtained in this work were unambiguously established using 2D NMR experiments (^1H and ^{13}C, COSY, HETCOR, DEPT 90° and 135°), in conjunction with IR and high resolution mass spectrometry.



Scheme 1. Synthesis of the dendrimeric structures of first and second generation.

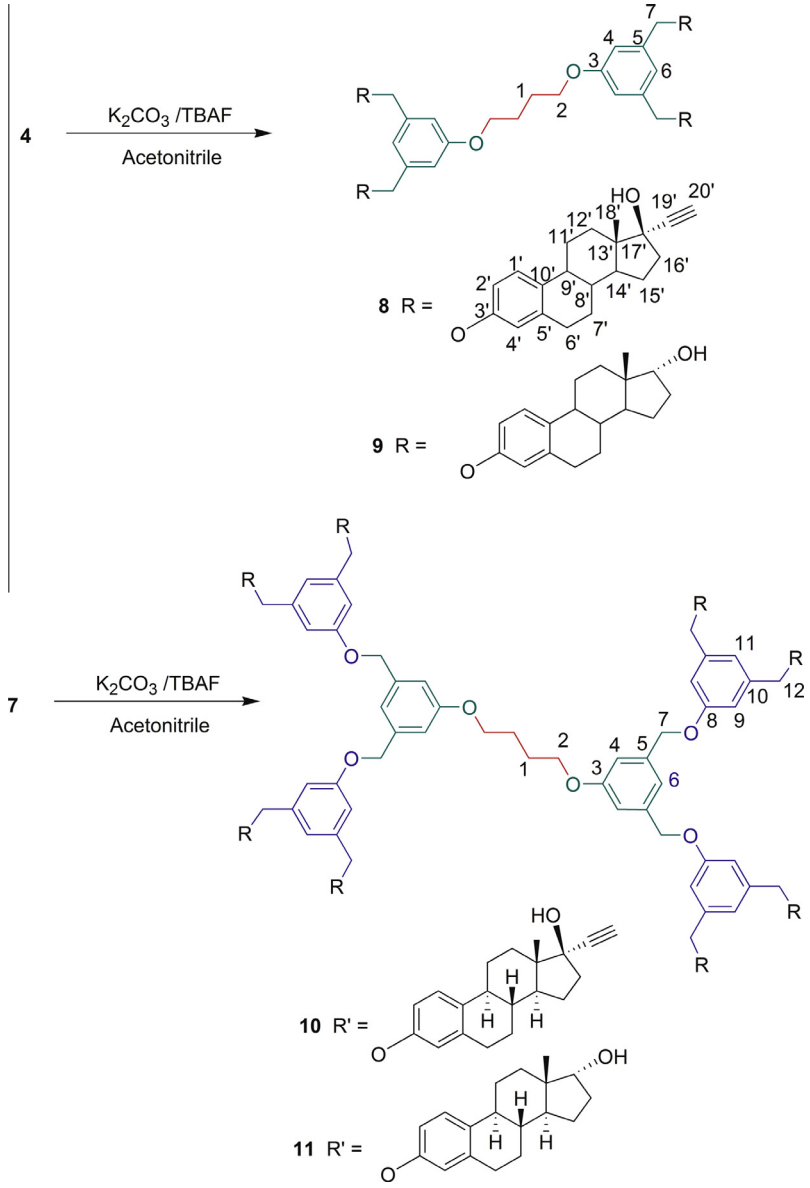
The symmetry present in the molecules simplifies their NMR characterization. The structures were assigned based on the multiplicity, chemical shift and integration of the signals. Because the dendrimers of G1 and G2 consist of a tetramethylene core and dimethyl 5-hydroxyisophthalate as branching units, they have similar ^{1}H and ^{13}C NMR spectra. Nonetheless, there are characteristic signals like the new methylene at 5.04 ppm in G2 dendrimers that allow distinguishing one from the other.

The main difference between the two generations is the coupling of one additional branching unit that results in a spectrum with twice the intensity of the signals compared with the precursor of G1.

Reduction of the ester groups to give G2 dendrimer with 8 OH terminal groups (**6**) was corroborated by the signal at 4.44 (H-12) ppm attributed to new methylene groups and the absence of the carbonyl group in the ^{13}C NMR spectrum. The presence of the hydroxyl group in dendrimers **3** and **6** was demonstrated by IR spectra which showed broad absorption bands at 3291 and 3317, respectively. Substitution of the hydroxyl group in **6** by chlorine (**7**) induced a $\Delta\delta$ of approximately 0.09 for the directly bonded methylene.

Once dendrimers of first and second generation with halogenated surface functionality were obtained, and considering the advantages of steroidal payload in covalent conjugation, they were coupled with the 17α-ethynylestradiol or 17α-estradiol using the hydroxyl group at positions 3 of both steroids for conjugation with the dendrimer, without compromising binding affinity of the estrogen [34]. The covalently bound conjugates of first (**8** and **9**) and second generation (**10** and **11**) were synthesized from estrogens 17-α-ethynylestradiol and 17-α-estradiol and the corresponding halogenated compound **4** or **7** which were linked via ether functionalities, giving first and second generation conjugates in yields of 74% and 86% (Scheme 2). It should be mentioned that a previous publication has reported the synthesis of a dendrimeric conjugate in which 12 molecules of the corticosteroid, methylprednisolone (MP), were linked via ester to the PAMAM G4-OH dendrimer. In order to obtain this conjugate, it was necessary to couple previously glutaric acid to the steroid as spacer since the alternative route incorporating the spacer to the dendrimer afforded lower conjugation ratio of MP, presumably due to steric hindrance at the periphery [28]. In contrast, the steroidal conjugates **8**, **9**, **10** and **11**, synthesized in this work, are relatively non-hindered molecules with high payload of steroid in the dendrimer.

The formation of the steroidal dendrimer conjugates containing 4 and 8 units of 17α-ethynylestradiol (**8**, **10**), or 17α-estradiol (**9**, **11**) at the periphery was confirmed by the appearance of the steroidal signals in the ^{1}H NMR and ^{13}C NMR spectra which were unambiguously assigned using 2D experiments and are in agreement with the shifts reported for the parent steroid [35,36]. The aromatic protons of the dendrimer were assigned according to



Scheme 2. Synthesis of two steroidal dendrimer conjugates derivatives of 17α-ethynylestradiol and 17α-estradiol of first (**8** and **9**) and second generation (**10** and **11**).

the integral and the coupling constants, and the signal shifted to high frequencies at 4.98 (H-7) ppm in **8** and, at 5.02 (H-12) ppm in **10** provided evidence for the formation of the conjugates.

The ^{13}C NMR spectrum of **8** showed 27 different carbons which were assigned using COSY, HETCOR and DEPT experiments. The signal corresponding to the benzyl ether C-7 (–OCH_2–) at 69.8 ppm confirms the formation of G1 steroidal dendrimer conjugates. The ^{13}C NMR spectrum of G2 conjugate derivative of 17α-ethynylestradiol exhibits 32 different carbons and were assigned as previously described.

For compounds **9** and **11** the expected 25 and 30 different carbons are observed in the ^{13}C NMR spectrum and the signals for both steroidal fragments were in agreement with the data reported in the literature [31,32] and showed very slight variations. The signals corresponding to the aromatic ring of the dendrimer and the newly formed ether linkage were also in agreement with those of conjugates **8** and **10** previously discussed.

Additionally to NMR, the structure of the new dendrimeric conjugates was corroborated by using HRMS for **8** and **9** and MALDI TOFF for **10** and **11** since their molecular weights fall in the upper detection limit of the equipment used in HRMS.

3.2. Biological evaluation

In order to evaluate the potential enhancement in cytotoxic activity of 17α-ethynylestradiol and 17α-estradiol when they are anchored to dendrimers of first and second generation, the steroidal dendrimer conjugates of first (**8** and **9**) and second generation (**10** and **11**), steroidal precursors, and hydroxyl terminated dendrimers (**3** and **6**) were screened against chronic leukemia (CEM), breast adenocarcinoma (MCF7) and cervical carcinoma (HeLa) tumor cell lines and against normal human fibroblast (BJ) as control cells. The results are summarized in Table 1.

These results show the innocuity of all dendrimers, except for dendrimer **6**, against normal BJ cells. Also, the highest sensitivity of CEM cell line to the tested compounds, allow us determine that the cytotoxic activity in dendrimer conjugates is due to the steroidal moiety, since dendrimers are non-cytotoxic. Compounds **8** and **9** were more effective against CEM at micromolar concentrations as compared with their steroidal precursors. However, in contrast to expectations, when the generation is increased, the higher payload of steroidal residues does not have a favorable impact in cytotoxic activity, as can be seen in Table 1.

With the aim to have an illustrative 3D image of conjugates than can help to explain the decrease of activity as a consequence of generation increase, possible conformations of steroidal dendrimer conjugates were explored by conformational search calculations in aqueous medium. The Monte-Carlo [37,38] algorithm in Spartan 08 was used, where random changes were made in torsion angles, respecting the steroidal stereochemistry during the search. The conformers of minimum energy of **8**, **9**, **10** and **11** were optimized at MMFFaq level [39]. Fig. 1 shows that the steroids are more exposed in the first generation (**8**) than in the second (**11**), whereby the steroids interact by π–π stacking with the dendrimer forming a very compact structure that precludes interaction with cellular receptors.

These results suggest that our steroidal dendrimer conjugate of first generation could potentially have therapeutic anticancer applications against chronic leukemia and the second generation does not show advantage due to the fact that the increase of dendrimer size promotes hydrophobic and π–π interactions with the steroidal moieties, inhibiting the interaction with cells.

In the context of the results reported herein (Table 1), we decided to test G1conjugates **8** and **9** to determine how these conjugates disturbed the cell cycle and the possible pathway to induce apoptosis in CEM cells by activities of caspase 3/7 and the *Western blot* analysis.

To determine the phase in which the dendrimeric conjugates induce apoptosis, CEM cells were incubated with steroidal dendrimer conjugates **8** and **9**, flow cytometric analysis was used to quantify the distribution of CEM cells in the different phases of the cell cycle, and to determine the sub-G_1 fraction as a marker of the proportion of apoptotic cells. The results show that treatment with **8** and **9** increased the proportions of S-phase cells in all concentrations, along with reductions in proportions of G_2/M cells. In addition, the proportion of cells with sub-G_1 amounts of DNA (apoptotic cells) increased following treatment with the tested conjugates **8** and **9**, showing a threefold increase when treated with 9 50 μM relative to the untreated controls after 24 h (Fig. 2). Thus, the tested steroidal dendrimer conjugate derivatives of first generation effectively disturbed cell cycle and induced apoptosis in CEM cells, via mitochondrial cytochrome c release and resultant activation of caspase cascade, as evidenced by the activities of caspases 3 and 7, which is mainly provoked in G2/M-arrested cells [20].

The action of caspase 3 that cleaves and inactivates the poly ADP-ribose polymerase-1 (PARP) that is a nuclear enzyme involved in DNA repair, led to increased DNA damage, followed by the DNA fragmentation through uncontrolled endonuclease activity resulting in apoptosis [40,41]. We determined the activities of effector caspases 3 and 7 (3/7) in CEM cells exposed to **8** or **9** using a fluorogenic substrate, Ac-DEVD-AMC, and/or the caspase 3/7 inhibitor Ac-DEVD-CHO. Cells were treated with a series of concentrations of the compounds exceeding their IC_{50} values: 5, 25 and 50 μM for 24 h. Conjugate 9 induced a ca. 3.7- and 4.3-fold increase in the activity of caspase-3/7 after 24 h at 25 and 50 μM compared with untreated controls. Compound **8** induced a ca. 2.5-fold increase after 24 h at the highest tested concentration (Fig. 3). The observed increase in the expression of caspase 3/7 (Fig. 3) in conjunction with the DNA fragmentation data (Fig. 4) confirmed that both conjugates of first generation are able to induce apoptosis in leukemia cancer cells (CEM) increasing the cytotoxic activity compared to the free drug [20] suggesting that both conjugates have a common mechanism of action in tumor cells.

Finally Western blot analyses were used to detect changes in expression of apoptosis-related proteins in the CEM leukemia cancer cell line 24 h after treatment with steroidal dendrimer conjugate derivatives **8** and **9** (5, 25 and 50 μM). As shown in Fig. 4, 25 and 50 μM treatments with **8** and **9** induced cleavage of PARP after 24 h correlated with decreased levels of procaspase 3. In addition increased activity of caspase 3 was observed over the same treatment time and concentrations for both compounds (Fig. 3). Expression of a tumor suppressor protein p53 was observed in controls of leukemia cancer cell line CEM and 8 caused its enhanced expression after 24 h. The protein expression of p53 and Mdm2 increased strongly following treatment with 25 μM of 8, but at 50 μM protein expression decreased. For both conjugates (**8** and **9**) at 50 μM, after 24 h, a decreased expression of antiapoptotic proteins Mcl-1 and Bcl-2 was observed, indicating the commencement of apoptosis. As reported previously, Mcl-1 is necessary for cell viability and its decrease could be the cause of cell death in CEM cells [32]. This antiapoptotic protein Mcl-1 has a crucial role in regulating the apoptosis of T-cells [42]. It is known that apoptosis is mediated by caspase cascade activation and that caspase-3 is an executioner protease that cleaves PARP, resulting in DNA degradation and apoptotic death [32]. Our Western blot analysis demonstrated the dose-dependent decrease of procaspase-3 and cleavage

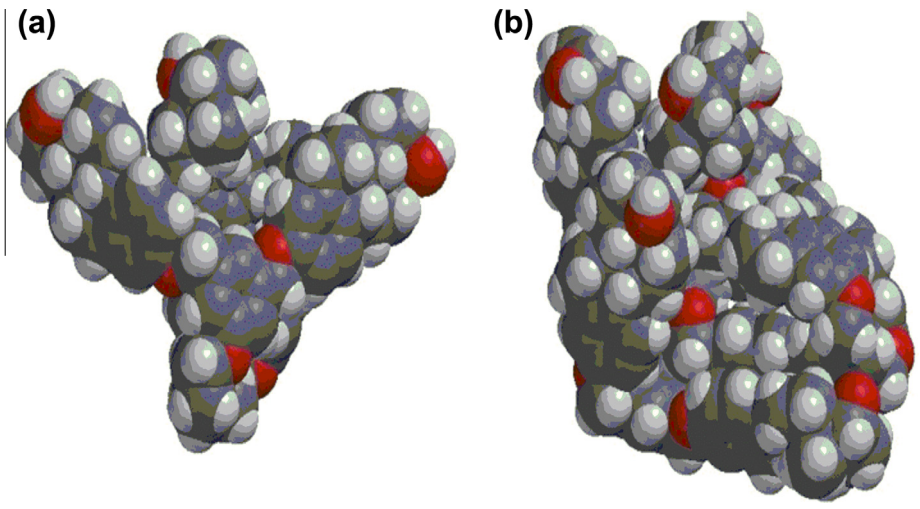


Fig. 1. Minimum energy conformer optimized at the MMFFaq level in Spartan 08 (wavefunction Inc., CA) of 17-α estradiol dendrimer conjugate; a) first generation (9) and b) second generation (11).

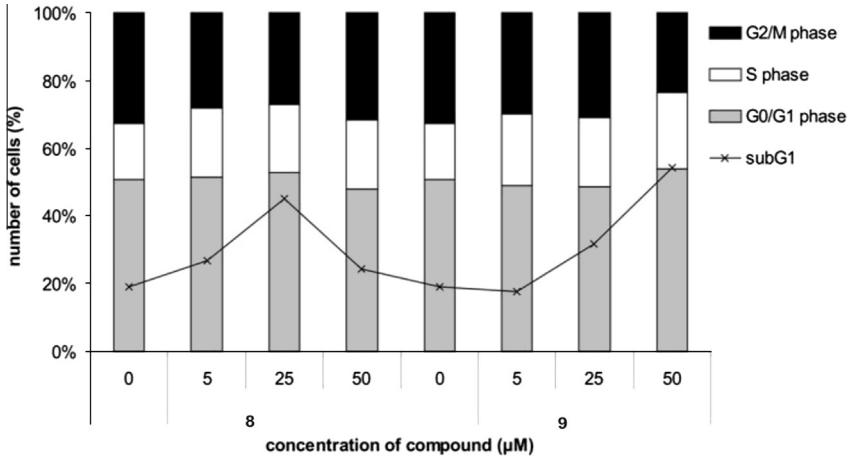


Fig. 2. Histograms, obtained by flow cytometric analysis, showing the distributions of CEM cells across the G0/G1, S, and G2/M phases of the cell cycle after 24 h treatment with **8** and **9** and the sub-G1 fraction of cells (relative to untreated controls). The bars show the percentage of cells (%) in the corresponding phase.

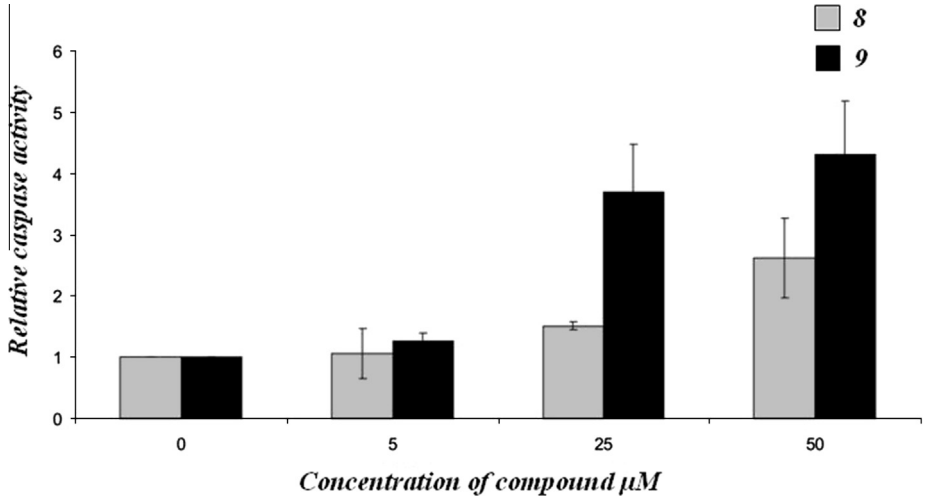


Fig. 3. Increases in the activities of caspase 3 and 7 in CEM cells treated with **8** and **9** for 24 h, relative to untreated controls. The data shown represent averages from experiments performed in triplicate.

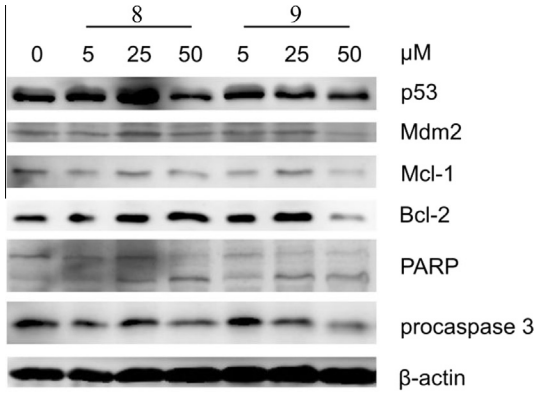


Fig. 4. Western blot analysis of apoptosis-related proteins (PARP and procasapase-3) in leukemia CEM cells treated with steroidal derivatives **8** and **9** compared to their expression in untreated control cells. The expression of β-actin was monitored as a protein loading control.

of PARP after 24 h treatment with steroidal dendrimer conjugates **8** and **9** in the leukemia cancer cell line CEM (Fig. 4).

Thus, the results confirm that these conjugates can induce caspase-3 activated apoptosis (Fig. 3).

In summary, we have prepared two generations of novel dendrimers with flexible tetramethylene core and rigid dimethyl 5-hydroxyisophthalate branching units. In order to test the utility of these compounds, as platforms for improving the therapeutic index of biologically active molecules, we modified the surface of the dendrimer with 17α-ethynylestradiol and 17α-estradiol to obtain four novel steroidal dendrimer conjugates **8**, **9**, **10** and **11**. The steroidal dendrimer conjugates, the free drug and dendrimer were tested against CEM, MCF7, HeLa cancer cell lines and BJ as normal cells. Conjugates **8** and **9** confirmed the enhancement of the steroids activity against CEM when they are conjugated to first generation of dendrimer. However in the case of second generation a decrease in cytotoxic activity was observed. This could be attributed to unfavorable conformations in the steroidal moieties which in the case of G2 lead to a folded structure while the four steroidal fragments are available for cell interaction in G1, as determined by conformational search.

The observation that conjugates of first generation (**8**, **9**) are able to induce apoptosis in leukemia cancer cells (CEM) and show increased cytotoxic activity compared to the free drug, suggests that both conjugates have a common mechanism of action in CEM cells. The interesting anti-cancer activity of the prepared conjugates, suggest that the steroidal dendrimer conjugates **8** and **9** could be of potential use as new anticancer drugs.

Acknowledgments

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Appendix A. Supplementary data

The spectroscopic characterization of all compounds ^{1}H, ^{13}C NMR, MS (ESI) data are given. Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.steroids.2013.09.001.

References

[1] World Health Organization: Cancer. http://www.who.int/cancer/en. (16-July-2013).
[2] Park JH, Lee S, Kim J-H, Park K, Kim K, Kwon IC. Polymeric nanomedicine for cancer therapy. Prog Polym Sci 2008;33:113–37.
[3] Bosman AW, Janssen HM, Meijer EW. About dendrimers: structure, physical properties, and applications. Chem Rev 1999;99:1665–88.
[4] Tomalia DA, Baker H, Dewald J, Hall M, Kallos G, Martin S, Roeck J, Ryder J, Smith P. Dendritic macromolecules: synthesis of starburst dendrimers. Macromolecules 1986;19:2466–8.
[5] Hawker CJ, Fréchet JMJ. Preparation of polymers with controlled molecular architecture. A new convergent approach to dendritic macromolecules. J Am Chem Soc 1990;112:7638–47.
[6] Buhleier E, Wehner W, Vögtle V. Cascade and Nonskid-chain-like syntheses of molecular cavity topologies. Synthesis 1978;2:155–8.
[7] Tomalia DA, Fréchet JMJ. Discovery of dendrimers and dendritic polymers: a brief historical perspective. J Polym Sci Part A Polym Chem 2002;40:2719–28.
[8] Tekade RK, Kumar PV, Jain NK. Dendrimers in oncology: an expanding horizon. Chem Rev 2009;109:49–87.
[9] Bharali DJ, Khalil M, Gurbuz M, Simone TM, Mousa SA. Nanoparticles and cancer therapy: a concise review with emphasis on dendrimers. Int J Nanomed 2009;4:1–7.
[10] Lee CC, MacKay JA, Fréchet JMJ, Szoka FC. Designing dendrimers for biological applications. Nat Biotechnol 2005;23:1517–26.
[11] Nanjwade BK, Bechra HM, Derkar GK, Manvi FV, Nanjwade VK. Dendrimers: emerging polymers for drug-delivery systems. Eur J Pharm Sci 2009;38:185–96.
[12] D'Emanuele A, Attwood D. Dendrimer–drug interactions. Adv Drug Delivery Rev 2005;57:2147–62.
[13] Boas U, Heegaard PMH. Dendrimers in drug research. Chem Soc Rev 2004;33:43–63.
[14] Medina SH, El-Sayed MEH. Dendrimers as carriers for delivery of chemotherapeutic agents. Chem Rev 2009;109:3141–57.
[15] Choi MR, Maxey KJS, Stanley JK, Levin CS, Bardhan R, Akin D, Badve S, Sturgis J, Robinson JP, Bashir R, Halas NJ, Clare SE. A cellular Trojan horse for delivery of therapeutic nanoparticles into tumors. Nano Lett 2007;7:3759–65.
[16] Majoros IJ, Williams CR, Baker JR. Current dendrimer applications in cancer diagnosis and therapy. Curr Top Med Chem 2008;8:1165–79.
[17] Wolinsky JB, Grinstaff MK. Therapeutic and diagnostic applications of dendrimers for cancer treatment. Drug Delivery Rev 2008;60:1037–55.
[18] Moos WH, Dykens JA, Nohynek D, Rubinchik E, Howell N. Review of the effects of 17α-estradiol in humans: a less feminizing estrogen with neuroprotective potential. Drug Dev Res 2009;70:1–21.
[19] Malíková J, Swaczynová J, Kolář Z, Strnad M. Anticancer and antiproliferative activity of natural brassinosteroids. Phytochemistry 2008;69:418–26.
[20] Jun DY, Park HS, Kim JS, Kim JS, Park W, Song BH, Kim HS, Taub D, Kim YH. 17α-Estradiol arrests cell cycle progression at G2/M and induces apoptotic cell death in human acute leukemia Jurkat T cells. Toxicol Appl Pharmacol 2008;231:401–12.
[21] Izumi K, Kadono Y, Shima T, Konaka H, Mizokami A, Koh E, Namiki M. Ethinylestradiol improves prostate-specific antigen levels in preteated castration-resistant prostate cancer patients. Anticancer Res 2010;30:5201–6.
[22] Lointier P, Wildrick DM, Boman BM. The effects of steroid hormones on a human colon cancer cell line in vitro. Anticancer Res 1992;12:1327–30.
[23] Zhang K, Wang Y, Yu A, Zhang Y, Tang H, Zhu XX. Cholic acid-modified dendritic multimolecular micelles and enhancement of anticancer drug therapeutic efficacy. Bioconjugate Chem 2010;21:1596–601.
[24] Ropponen J, Tamminen J, Lahtinen M, Linnanto J, Rissanen K, Kolehmainen E. Synthesis, characterization, and thermal behavior of steroidal dendrons. Eur J Org Chem 2005;1:73–84.
[25] Harrington WR, Kim SH, Funk CC, Erdogan ZM, Schiff R, Katzenellenbogen JA, Katzenellenbogen BS. Estrogen dendrimer conjugates that preferentially activate extranuclear, nongenomic versus genomic pathways of estrogen action. Mol Endocrinol 2006;20:491–502.
[26] Kim SH, Katzenellenbogen JA. Hormone–PAMAM dendrimer conjugates: polymer dynamics and tether structure affect ligand access to receptors. Angew Chem Int Ed 2006;45:7243–8.
[27] Kop T, Pocsfalvi G, Šolaja BA. Synthesis of a steroidal dendrimer core. J Serb Chem Soc 2004;10:769–75.
[28] Khandare J, Kolhe P, Pillai O, Kannan S, Lai ML, Kannan RM. Synthesis, cellular transport, and activity of polyamidoamine dendrimer–methylprednisolone conjugates. Bioconjugate Chem 2005;16:330–7.
[29] Bodwell GJ, Bridson JN, Houghton TJ, Kennedy JWJ, Mannion MR. 1,7-Dioxa [7](2,7)pyrenophane: the pyrene moiety is more bent than that of C70. Chem Eur J 1999;6:1823–7.
[30] Bodwell GJ, Bridson JN, Cyraski MK, Kennedy JWJ, Krygowski TM, Mannion MR, Miller DO. Nonplanar aromatic compounds. 8.1 Synthesis, crystal structures, and aromaticity investigations of the 1, *n*-Dioxa[*n*](2,7) pyrenophanes. How does bending affect the cyclic π-electron delocalization of the pyrene system? J Org Chem 2003;68:2089–98.
[31] Hawxwell SM, Espallargas GM, Bradshaw D, Rosseinsky MJ, Prior TJ, Florence AJ, Van de Streek J, Brammer L. Ligand flexibility and framework rearrangement in a new family of porous metal–organic frameworks. Chem Commun 2007;15:1532.
[32] Opferman JT, Letai A, Beard C, Sorcinelli MD, Ong CC, Korsmeyer SJ. Development and maintenance of B and T lymphocytes requires antiapoptotic MCL-1. Nature 2003;426:671–6.
[33] Tomalia DA. Birth of a new macromolecular architecture: dendrimers as quantized building blocks for nanoscale synthetic polymer chemistry. Prog Polym Sci 2005;30:294–324.
[34] Anstead GM, Carlson KE, Katzenellenbogen JA. The estradiol pharmacophore: ligand structure-estrogen receptor binding affinity relationships and a model for the receptor binding site. Steroids 1997;62:268–303.
[35] Ciuffreda P, Casati S, Manzocchi A. Complete ^{1}H and ^{13}C NMR spectral assignment of 17-hydroxy epimeric sterols with planar A or A and B rings. Magn Reson Chem 2004;42:360–3.
[36] Dionne P, Poirier D. ^{13}C nuclear magnetic resonance study of 17 alpha-substituted estradiols. Steroids 1995;60:830–6.
[37] Chang G, Guida WC, Still WC. An internal-coordinate Monte Carlo method for searching conformational space. J Am Chem Soc 1989;111:4379–86.
[38] Saunders M, Houk KN, Wu YD, Still WC, Lipton M, Chang G, Guida WC. Conformations of cycloheptadecane. A comparison of methods for conformational searching. J Am Chem Soc 1990;12:1419–27.
[39] Kim HY, Sohn J, Wijewickrama GT, Edirisinghe P, Gherezghiher T, Hemachandra M, Lu PY, Chandrasena RE, Molloy ME, Tonetti DA, Thatcher GRJ. Click synthesis of estradiol–cyclodextrin conjugates as cell compartment selective estrogens. Bioorg Med Chem 2010;8:809–21.
[40] Allen RT, Cluck MW, Agrawal DK. Mechanisms controlling cellular suicide: role of Bcl-2 and caspases. Cell Mol Life Sci 1998;54:427–45.
[41] Budihardjo I, Oliver H, Lutter M, Luo X, Wang X. Biochemical pathways of caspase activation during apoptosis. Annu Rev Cell Dev Biol 1999;15:269–90.
[42] Inuzuka H, Shaik S, Onoyama I, Gao D, Tseng A, Maser RS, Zhai B, Wan L, Gutierrez A, Lau AW, Xiao Y, Christie AL, Aster J, Settleman J, Gygi SP, Kung AL, Look T, Nakayama KI, DePinho RA, Wei W. SCF(FBW7) regulates cellular apoptosis by targeting MCL1 for ubiquitylation and destruction. Nature 2011;471:104–9.