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Synthesis and antileukemic activity of 16*E*-[4-(2-carboxy)ethoxybenzylidene]-androstene amides

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ABSTRACT

In order to determine the structural requirements for cytotoxicity against various tumor cell lines, a new series of 16*E*-arylidene androstene amides with varying degrees of unsaturation in ring A has been synthesized. Characterization and *in vitro* cytotoxic studies of the newly synthesized compounds are discussed. The compounds on evaluation against various tumor cell lines exhibited significant growth inhibition on leukemia cell lines. 3-Chloro-16*E*-{[4-(4-methylpiperazin-1-yl)-2-oxoethoxy]benzylidene}androst-5-en-17-one **(10)** emerged as the most potent compound of the series with GI_{50} values of 3.94, 2.61, 6.90 and 1.79 μM against CCRF-CEM, K-562, RPMI-8226 and SR leukemia cell lines, respectively.

1. Introduction

Cancer is the leading cause of death in economically developed countries and the second leading cause of death in developing countries [1]. In spite of the large number of available chemotherapeutic antineoplastic agents, the medical need is still largely unmet. The main reasons are lack of selectivity of conventional drugs leading to toxicity, the metastatic spreading implying early tumor implantation in organs other than primary site, the heterogeneity of the disease comprising about 100 types of cancer and the intrinsic or acquired resistance to chemotherapy developed after few therapeutic cycles i.e. multi-drug resistance [2]. Use of steroids is a common practice for the treatment of cancer. Many steroid hormones as well as their agonists and antagonists, such as glucocorticoids (e.g., prednisone), androgens (e.g., fluoxymesterone), antiandrogen (e.g., cyproterone acetate), estrogens (e.g., stilbestrol), aromatase inhibitors (e.g., formestane) and 5α-reductase inhibitors (e.g., finasteride) are already in clinical use and are known to produce beneficial results in the prevention and treatment of cancer [3–9].

Several androstene derivatives with substitution at position C-16 have been described in the literature as potent antineoplastic agents [10,11]. Recently some interesting 16*E*-arylidene androstene derivatives (**1**) have been reported from our laboratory as strong *in vitro* inhibitors of the growth of many types of human tumor cells [12–14]. One of the compounds possessing diethylamino group (**2**, Fig. 1) reached up to *in vivo* xenograft testing stage of Developmental Therapeutic Program of NCI, Bethesda, USA after successfully passing the first two stages i.e. 60 cell line assay and *in vivo* hollow fiber assay [14]. This encouraged us to further explore the structural motif responsible for the anticancer properties of 16-arylidene androstenes. As reported earlier [14], presence of tertiary amino groups enhances the cytotoxic potential of the 16-arylidene compounds; we decided to synthesize amides of 16-arylidene steroidal acid with the anticipation that it may result into enhanced activity. Replacement or substitution of C_3–OH function has a prominent impact on cytotoxic effects of 16-(*para*-substituted)benzylidene series of anticancer compounds [13,14]. Hence it was also envisaged to study the effect of such changes on cytotoxicity profile.

2. Experimental

2.1. Chemistry

Melting points were determined on a Veego melting point apparatus and are uncorrected. Infrared (wavenumbers in cm^{-1}) spectra were recorded on Perkin–Elmer RX1 FTIR spectrophotometer model using potassium bromide pellets (ν_{max} in cm^{-1}). ^1H NMR spectra were obtained on a Bruker Avance II 400 MHz spectrometer using deuterated-chloroform ($CDCl_3$) or deuterated dimethylsulfoxide (DMSO-d_6) as solvents containing tetramethylsilane as internal standard (chemical shifts in δ, ppm). Mass spectra were obtained on an Applied Biosystems API 2000™ Mass spectrophotometer. Elemental analyses were carried out on a Perkin Elmer-2400 model CHN analyzer. Plates for thin layer chromatography (TLC) were prepared with silica gel G according to method of Stahl (E. Merck)

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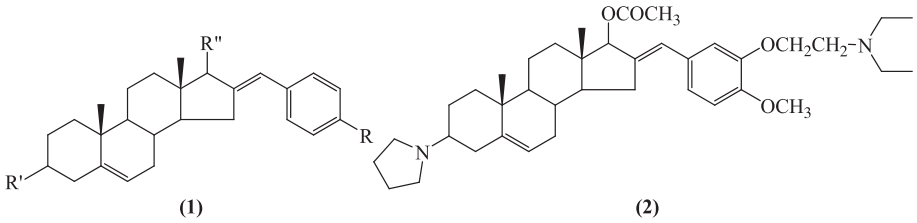


Fig. 1. Strucutres of 16-benzylidene steroids.

using ethyl acetate as solvent and activated at 110 °C for 30 min. Iodine was used to develop the TLC plates. Anhydrous sodium sulfate was utilized as drying agent. All solvents were distilled prior to use according to standard procedures.

2.1.1. Synthesis of 16E-[4-(carboxymethoxy)benzylidene]-3β-hydroxyandrost-5-en-17-one (5) (RB-490)

Methyl chloroacetate (1 ml, in excess) was added to a stirred and refluxing suspension of 4-hydroxybenzaldehyde (0.5 g, 4.09 mmol) and anhydrous potassium carbonate (2 g) in ethyl methyl ketone (100 ml). The reaction mixture was further refluxed for 6 h with continuous stirring. The completion of the reaction was monitored by TLC. The reaction mixture was cooled, filtered and the excess of solvent was removed under reduced pressure to obtain 4-formylphenoxyacetic acid methyl ester (**3**) as an oily residue, which was used as such for further reaction.

A solution of dehydroepiandrosterone (**4**; DHA) (0.5 g, 1.73 mmol), above obtained oily residue and sodium hydroxide (1 g) in methanol (20 ml) was stirred at room temperature for 12 h and the completion of reaction was monitored by TLC. Cold water was added to the reaction mixture and the precipitate obtained was filtered, washed with water, dried and recrystallized from methanol to yield compound **5** (64.9%), m.p. 308–310 °C. FT-IR: 3401, 2937, 1712, 1601, 1510, 1422, 1232, 1055, 832. ^{1}H NMR (DMSO-d_6): δ 0.87 (s, 3H, 18-CH_3), 1.00 (s, 3H, 19-CH_3), 3.33 (m, 1H, 3α-*H*), 4.13 (s, 2H, –OCH_2–), 5.32 (d, 1H, 6-C*H*, *J* = 4.80 Hz), 6.87 (brs, 2H, –C*H*, aromatic), 7.22 (s, 1H, vinylic-*H*, 16-arylidene), 7.5 (brs, 2H, –C*H*, aromatic). ESI-MS *m/z*: 451.1 [M$^+$]. Anal. calcd for $C_{28}H_{34}O_5$: C, 74.64; H, 7.61; found: C, 74.80; H, 7.72.

*2.1.2. General method for the preparation of compounds **7–11***

A mixture of aldol product **5** (0.2 g, 0.44 mmol) and thionyl chloride (3 ml) was stirred at room temperature for 3 h. The excess of thionyl chloride was vacuum evaporated. To the chlorinated residue, 3-chloro-16*E*-[4-(chlorocarbonyl)methoxybenzylidene]androst-5-en-17-one (**6**), dichloromethane (20 ml) was added followed by requisite secondary amine (0.5 ml) and the mixture was stirred overnight at room temperature. Solvent was evaporated and water was added to the obtained residue. The solid so obtained was filtered, washed with water, dried and recrystallized from a mixture of chloroform/methanol (30:70) to yield the corresponding steroidal amide **7–11**.

*2.1.2.1. 3-Chloro-16E-[4-(2-oxo-2-pyrrolidin-1-yl-ethoxy)benzylidene]androst-5-en-17-one (**7**) (RB-430).* White solid. Yield 65.2%. m.p. 190–192 °C. FT-IR: 2941, 1712, 1661, 1510, 1447, 1256, 1086, 829. ^{1}H NMR (CDCl$_3$): δ 0.89 (s, 3H, 18-CH_3), 1.02 (s, 3H, 19-CH_3), 3.46 (m, 4H, –N(CH_2)$_2$, pyrrolidine), 3.70 (m, 1H, 3α-*H*), 4.59 (s, 2H, –OCH_2–), 5.35 (d, 1H, 6-C*H*, *J* = 4.8 Hz), 6.92 (d, 2H, –C*H*, J_o = 8.64 Hz, aromatic), 7.32 (s, 1H, vinylic-*H*, 16-arylidene), 7.43 (d, 2H, –C*H*, J_o = 8.64 Hz, aromatic). ESI-MS *m/z*: 522.2 [M$^+$], 524 [M+2]$^+$. Anal. calcd for $C_{32}H_{40}NO_3Cl$: C, 73.61; H, 7.72; N, 2.68; found: C, 73.50; H, 7.71; N, 2.63.

*2.1.2.2. 3-Chloro-16E-[4-(2-morpholin-4-yl-2-oxoethoxy)benzylidene]androst-5-en-17-one (**8**) (RB-431).* White solid. Yield 44%. m.p. 224–226 °C. FT-IR: 2935, 2851, 1710, 1667, 1510, 1442, 1236, 1110, 1026. ^{1}H NMR (CDCl$_3$): δ 0.96 (s, 3H, 18-CH_3), 1.09 (s, 3H, 19-CH_3), 3.66 (m, 8H, –N–(CH_2)$_2$– and –O–(CH_2)$_2$–, morpholine), 3.77 (m, 1H, 3α-*H*), 4.74 (s, 2H, –OCH_2–), 5.42 (d, 1H, 6-C*H*, *J* = 5.04 Hz), 6.99 (d, 2H, –C*H*, J_o = 8.68 Hz, aromatic), 7.39 (s, 1H, vinylic-*H*, 16-arylidene), 7.50 (d, 2H, –C*H*, J_o = 8.72 Hz, aromatic). ESI-MS *m/z*: 538.2 [M$^+$], 540.1 [M+2]$^+$. Anal. calcd for $C_{32}H_{40}NO_3Cl$: C, 71.42; H, 7.49; N, 2.60; found: C, 71.67; H, 7.32; N, 2.52.

*2.1.2.3. 3-Chloro-16E-[4-(2-oxo-2-piperidin-1-yl-ethoxy)benzylidene]androst-5-en-17-one (**9**) (RB-432).* White solid. Yield 43.5%. m.p. 230–233 °C. FT-IR: 2934, 2857, 1707, 1647, 1600, 1512, 1443, 1250, 748. ^{1}H NMR (CDCl$_3$): δ 0.96 (s, 3H, 18-CH_3), 1.09 (s, 3H, 19-CH_3), 3.53 (m, 4H, –N–(CH_2)$_2$–, piperidine), 3.73 (m, 1H, 3α-*H*), 4.72 (s, 2H, –OCH_2–), 5.42 (d, 1H, 6-C*H*, *J* = 5.44 Hz), 6.99 (d, 2H, –C*H*, J_o = 8.32 Hz, aromatic), 7.39 (s,1H, vinylic-*H*, 16-arylidene), 7.50 (d, 2H, –C*H*, J_o = 8.16 Hz, aromatic). ESI-MS *m/z*: 536.2 [M$^+$], 538 [M+2]$^+$. Anal. calcd for $C_{33}H_{42}NO_3Cl$: C, 73.93; H, 7.90; N, 2.61; found: C, 74.05; H, 7.71; N, 2.59.

*2.1.2.4. 3-Chloro-16E-{[4-(4-methylpiperazin-1-yl)-2-oxoethoxy]benzylidene}androst-5-en-17-one (**10**) (RB-433).* White solid. Yield 52%. m.p. 186–188 °C. FT-IR: 2942, 2857, 1711, 1666, 1510, 1440, 1251, 1175, 828. ^{1}H NMR (CDCl$_3$): δ 0.89 (s, 3H, 18-CH_3), 1.02 (s, 3H, 19-CH_3), 2.10 (s, 3H, –N–CH_3 piperazine), 2.46 (m, 4H, CH$_3$–N–(CH_2)$_2$–, piperazine), 3.59 (m, 4H, –N–(CH_2)$_2$–, piperazine), 3.71 (m, 1H, 3α-*H*), 4.66 (s, 2H, –OCH_2–), 5.35 (d, 1H, 6-C*H*, *J* = 5.16 Hz), 6.92 (d, 2H, –C*H*, J_o = 8.80 Hz, aromatic), 7.32 (s, 1H, vinylic-*H*, 16-arylidene), 7.42 (d, 2H, –C*H*, J_o = 8.80 Hz, aromatic). ESI-MS *m/z*: 551.2 [M$^+$], 533 [M+2]$^+$. Anal. calcd for $C_{33}H_{43}N_2O_3Cl$: C, 71.91; H, 7.86; N, 5.08; found: C, 71.75; H, 7.91; N, 5.12.

*2.1.2.5. 3-Chloro-16E-{4-[2-(N,N-diethylamino)-2-oxoethoxy]benzylidene}androst-5-en-17-one (**11**) (RB-434).* White solid. Yield 65%. m.p. 195–197 °C. FT-IR: 2942, 2852, 1711, 1662, 1509, 1445, 1251, 1075. ^{1}H NMR (CDCl$_3$): δ 0.90 (s, 3H, 18-CH_3), 1.03 (s, 3H, 19-CH_3), 1.07 (t, 3H, –N–CH$_2$CH_3) 1.14 (t, 3H, –N–CH$_2$CH_3), 3.33 (m, 4H, –N(CH_2CH$_3$)$_2$), 3.70 (m, 1H, 3α-*H*), 4.65 (s, 2H, –OCH_2–), 5.36 (d, 1H, 6-C*H*, *J* = 5.44 Hz), 6.92 (d, 2H, –C*H*, J_o = 8.40 Hz, aromatic), 7.32 (s, 1H, vinylic-*H*, 16-arylidene), 7.43 (d, 2H, –C*H*, J_o = 8.28 Hz, aromatic). ESI-MS *m/z*: 524 [M$^+$], 526.1 [M+2]$^+$. Anal. calcd for $C_{32}H_{42}NO_3Cl$: C, 73.33; H, 8.27; N, 2.67; found: C, 73.41; H, 8.40; N, 2.72.

*2.1.3. Synthesis of 3β-hydroxy-16E-[4-(2-methoxy-2-oxoethoxy)benzylidene]androst-5-en-17-one (**12**)*

A mixture of compound **5** (0.4 g, 0.88 mmol), methanol (50 ml) and concentrated H_2SO_4 (5 ml) was heated under reflux for 4 h on a water bath. Methanol was vacuum evaporated, cold water added and resultant solution was neutralized with solid sodium bicarbonate. The solid product so obtained was filtered off, washed with water, dried and recrystallized from methanol to yield the

compound **12** (85%), m.p. 195–197 °C. FT-IR: 3452, 2933, 1769, 1710, 1604, 1511, 1437, 1226, 1178, 831. ^{1}H NMR ($CDCl_3$): δ 0.95 (s, 3H, 18-C*H*$_3$), 1.07 (s, 3H, 19-C*H*$_3$), 3.54 (m, 1H, 3α-*H*), 3.82 (s, 3H, –COOC*H*$_3$), 4.68 (s, 2H, –OC*H*$_2$–), 5.40 (d, 1H, 6-C*H*, *J* = 4 Hz), 6.94 (d, 2H, –C*H*, J_o = 8.80 Hz, aromatic), 7.39 (s, 1H, vinylic-*H*, 16-arylidene), 7.50 (d, 2H, –C*H*, J_o = 8.80 Hz aromatic). Anal. calcd for $C_{32}H_{42}NO_3Cl$: C, 74.97; H, 7.81; N, 17.22; found: C, 74.71; H, 7.91; N, 2.79.

*2.1.4. General method for the preparation of compounds **13–15***

A mixture of compound **12** (0.2 g, 0.43 mmol) and appropriate secondary amine (2 ml) was thermally fused at 100 °C for 5 h. The reaction was monitored by TLC for completion. Cold water was added and solid product so obtained was filtered, washed, dried and recrystallized from a mixture of chloroform/methanol (30:70) to yield the corresponding amide derivative **13–15**.

*2.1.4.1. 3β-Hydroxy-16E-[4-(2-oxo-2-pyrrolidin-1-yl-ethoxy)benzylidene]androst-5-en-17-one (**13**) (RB-435).* White solid. Yield 69%. m.p. 270–273 °C. FT-IR: 2929, 2862, 1708, 1653, 1511, 1445, 1255, 1090, 831. ^{1}H NMR ($CDCl_3$): δ 0.97 (s, 3H, 18-C*H*$_3$), 1.07 (s, 3H, 19-C*H*$_3$), 3.53 (m, 5H, –N(C*H*$_2$)$_2$, pyrrolidine and 3α-*H*), 4.67 (s, 2H, –OC*H*$_2$–), 5.40 (d, 1H, 6-C*H*, *J* = 5.24 Hz), 6.70 (d, 2H, –C*H*, J_o = 8.76 Hz, aromatic), 7.39 (s, 1H, vinylic-*H*, 16-arylidene), 7.50 (d, 2H, –C*H*, J_o = 8.00 Hz aromatic). ESI-MS *m/z*: 504.3 [M$^+$]. Anal. calcd for $C_{32}H_{41}NO_4$: C, 76.31; H, 8.20; N, 2.78; found: C, 76.45; H, 8.26; N, 2.71.

*2.1.4.2. 3β-Hydroxy-16E-[4-(2-morpholin-4-yl-2-oxoethoxy)benzylidene]androst-5-en-17-one (**14**) (RB-437).* White solid. Yield 62.5%. m.p. 220–222 °C. FT-IR: 3466, 2928, 1711, 1654, 1600, 1512, 1442, 1244, 1180, 1033. ^{1}H NMR ($CDCl_3$): δ 0.95 (s, 3H, 18-C*H*$_3$), 1.07 (s, 3H, 19-C*H*$_3$), 3.59 (m, 1H, 3α-*H*), 3.68 (m, 8H, –N–(C*H*$_2$)$_2$– and –O–(C*H*$_2$)$_2$–, morpholine), 4.74 (s, 2H, –OC*H*$_2$–), 5.40 (d, 1H, 6-C*H*, *J* = 5.16 Hz), 6.99 (d, 2H, –C*H*, J_o = 8.80 Hz, aromatic), 7.39 (s, 1H, vinylic-*H*, 16-arylidene), 7.51 (d, 2H, –C*H*, J_o = 8.84 Hz, aromatic). ESI-MS *m/z*: 520.3 [M$^+$]. Anal. calcd for $C_{32}H_{41}NO_5$: C, 73.96; H, 7.95; N, 2.70; found: C, 74.01; H, 7.90; N, 2.74.

*2.1.4.3. 3β-Hydroxy-16E-[4-(2-oxo-2-piperidin-1-yl-ethoxy)benzylidene]androst-5-en-17-one (**15**) (RB-439).* White solid. Yield 75%. m.p. 242–244 °C. FT-IR: 3414, 2935, 2856, 1707, 1644, 1511, 1444, 1249, 1079, 746. ^{1}H NMR ($CDCl_3$): δ 0.97 (s, 3H, 18-C*H*$_3$), 1.07 (s, 3H, 19-C*H*$_3$), 3.50 (m, 5H, –N–(C*H*$_2$)$_2$–, piperidine and 3α-*H*), 4.72 (s, 2H, –OC*H*$_2$–), 5.40 (d, 1H, 6-C*H*, *J* = 5.2 Hz), 6.99 (d, 2H, –C*H*, J_o = 8.80 Hz, aromatic), 7.39 (s, 1H, vinylic-*H*, 16-arylidene), 7.49 (d, 2H, –C*H*, J_o = 8.80 Hz, aromatic). ESI-MS *m/z*: 518.3 [M$^+$]. Anal. calcd for $C_{33}H_{43}NO_4$: C, 76.56; H, 8.37; N, 2.71; found: C, 76.67; H, 8.40; N, 2.69.

*2.1.5. General method for the preparation of compounds **16–18***

The compounds **13–15** (1.92 mmol) were dissolved in a mixture of cyclohexanone (5 ml) and dry toluene (150 ml). Azeotropic distillation was continued at a slow rate while adding a solution of aluminium isopropoxide (1 g) in dry toluene (15 ml) drop wise. The reaction mixture was then refluxed for 5 h and allowed to stand at room temperature overnight. The slurry was filtered and the residue was washed thoroughly with dry toluene. The combined filtrate and the washings were subjected to steam distillation until the complete removal of toluene and cyclohexanone. The solid product obtained was filtered, washed with water, dried and recrystallised from chloroform/methanol (3: 7) to yield compounds **16–18**, respectively.

*2.1.5.1. 16E-[4-(2-oxo-2-pyrrolidin-1-yl-ethoxy)benzylidene]androst-4-ene-3,17-dione (**16**) (RB-436).* White solid. Yield 35%. m.p. 140–142 °C. FT-IR: 2932, 1712, 1648, 1598, 1507, 1428, 1239, 1176, 1090. ^{1}H NMR ($CDCl_3$): δ 1.00 (s, 3H, 18-C*H*$_3$), 1.25 (s, 3H, 19-C*H*$_3$), 3.53 (m, 4H, –N(C*H*$_2$)$_2$, pyrrolidine), 4.67 (s, 2H, –OC*H*$_2$–), 5.76 (s, 1H, 4-C*H*), 6.99 (d, 2H, –C*H*, J_o = 8.80 Hz, aromatic), 7.40 (s, 1H, vinylic-*H*, 16-arylidene), 7.49 (d, 2H, –C*H*, J_o = 8.80 Hz aromatic). ESI-MS *m/z*: 502.3 [M$^+$]. Anal. calcd for $C_{32}H_{39}NO_4$: C, 76.61; H, 7.84; N, 2.79; found: C, 76.42; H, 7.87; N, 2.82.

*2.1.5.2. 16E-[4-(2-morpholin-4-yl-2-oxoethoxy)benzylidene]androst-4-ene-3,17-dione (**17**) (RB-438).* White solid. Yield: 54%, m.p. 196–198 °C. FT-IR: 2932, 2852, 1713, 1661, 1596, 1507, 1435, 1228, 1097, 1026. ^{1}H NMR ($CDCl_3$): δ 1.00 (s, 3H, 18-C*H*$_3$), 1.25 (s, 3H, 19-C*H*$_3$), 3.65 (m, 8H, –N–(C*H*$_2$)$_2$– and –O–(C*H*$_2$)$_2$–, morpholine), 4.74 (s, 2H, –OC*H*$_2$–), 5.76 (s, 1H, 4-C*H*), 6.99 (d, 2H, –C*H*, J_o = 8.80 Hz, aromatic), 7.40 (s, 1H, vinylic-*H*, 16-arylidene), 7.50 (d, 2H, –C*H*, J_o = 8.80 Hz, aromatic). ESI-MS *m/z*: 518.3 [M$^+$]. Anal. calcd for $C_{32}H_{39}NO_5$: C, 74.25; H, 7.59; N, 2.71; found: C, 74.43; H, 7.61; N, 2.75.

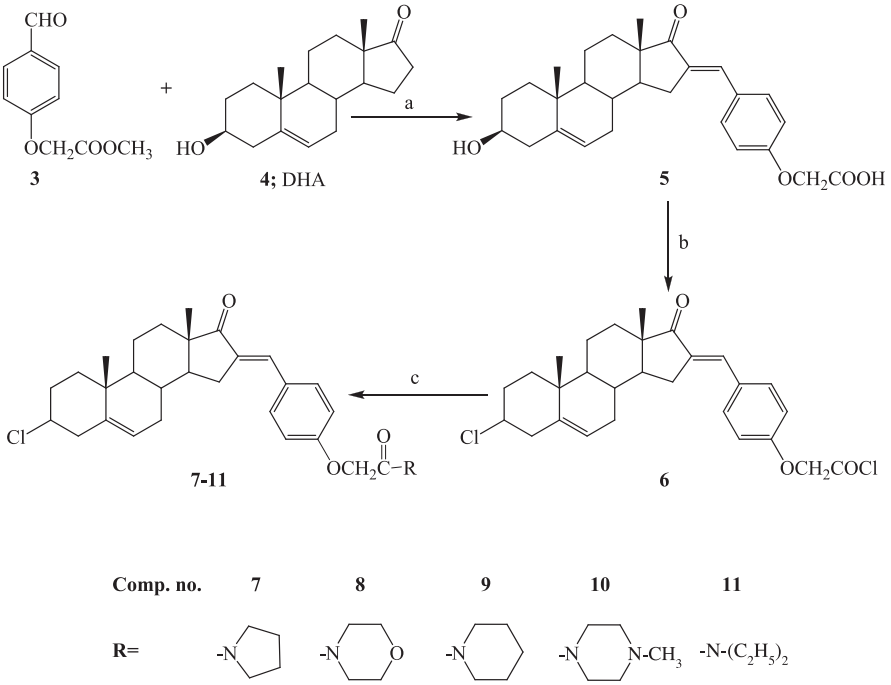
*2.1.5.3. 16E-[4-(2-oxo-2-piperidin-1-yl-ethoxy)benzylidene]androst-4-ene-3,17-dione (**18**) (RB-440).* White solid. Yield: 31%, m.p. 188–190 °C. FT-IR: 2933, 2854, 1714, 1661, 1598, 1508, 1441, 1246, 826. ^{1}H NMR ($CDCl_3$): δ 1.00 (s, 3H, 18-C*H*$_3$), 1.25 (s, 3H, 19-C*H*$_3$), 3.49 (t, 2H, –N–C*H*$_2$–, piperidine), 3.56 (t, 2H, –N–C*H*$_2$–, piperidine), 4.73 (s, 2H, –OC*H*$_2$–), 5.76 (s, 1H, 4-C*H*), 6.99 (d, 2H, –C*H*, J_o = 8.80 Hz, aromatic), 7.40 (s, 1H, vinylic-*H*, 16-arylidene), 7.49 (d, 2H, –C*H*, J_o = 8.80 Hz, aromatic). ESI-MS *m/z*: 516.2 [M$^+$]. Anal. calcd for $C_{33}H_{41}NO_4$: C, 76.86; H, 8.01; N, 2.72; found: C, 76.52; H, 8.06; N, 2.75.

3. Results and discussion

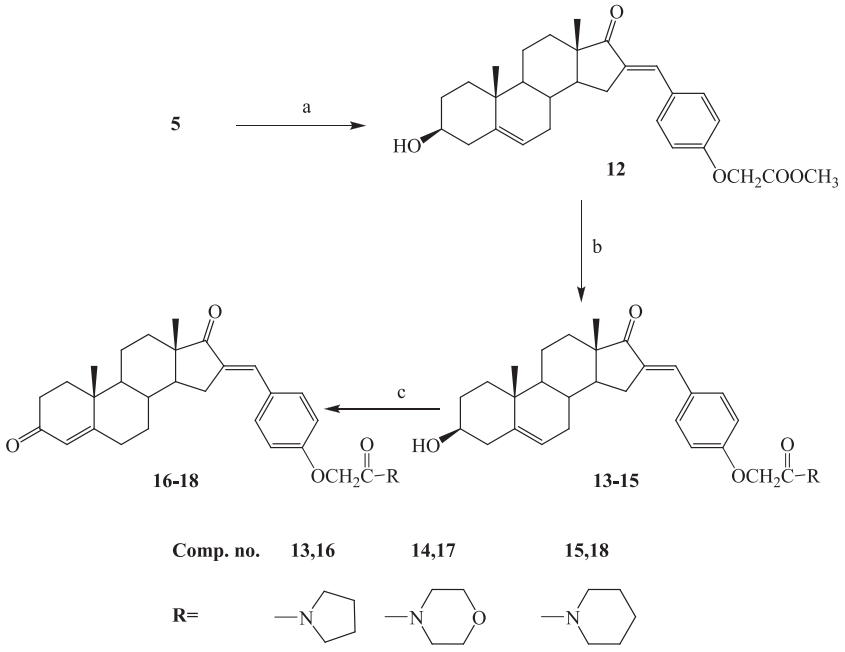
3.1. Chemistry

The synthesis of various 16*E*-arylidenoandrostene derivatives has been carried out as depicted in Schemes 1 and 2. Aldol condensation (Claisen–Schmidt reaction) [15] of DHA (**4**) with 4-formylphenoxyacetic acid methyl ester (**3**) at room temperature in alkaline medium afforded 16*E*-benzylidene steroidal acid **5**. It was observed that during the course of reaction, alkaline hydrolysis of methyl ester took place due to the presence of sodium hydroxide and resulted in the formation of **5** with free carboxylic acid group. The methine-bridged proton at C_{16} appeared at δ 7.22 ppm in the ^{1}H NMR spectrum of **5**. The configuration at C_{16} with respect to the carbonyl at C_{17} has been assigned *E* on the basis of earlier reports from our laboratory [14,16,17].

The carboxylic acid **5** was chlorinated using thionyl chloride, which subsequently yielded 3-chloro-16*E*-[4-(chlorocarbonyl) methoxybenzylidene]androst-5-en-17-one **(6)**. Further treatment with requisite secondary amines such as pyrolidine, morpholine, piperidine, N-methyl piperazine and diethylamine yielded corresponding amides **7–11**. IR absorption band of carbonyl function of parent carboxylic acid shifted from 1721 cm^{-1} to lower wavenumber ~1660 cm^{-1} in compounds **7–11** suggesting formation of amide. The methine-bridged proton of amides appeared at a downfield value at ~δ 7.35 in the ^{1}H NMR spectra in comparison to parent acid, where it resonated at δ 7.22 ppm. Similarly, the –OC*H*$_2$ proton appeared downfield (δ 4.59–4.74 ppm) in compounds **7–11** as compared to the parent aldol compound (δ 4.10 ppm) indicating amide formation. The 3α-*H* was also observed at an upfield δ 3.61–3.77 ppm position in the ^{1}H NMR spectra, which confirmed the conversion of 3-hydroxy substitution to chloro moiety. A [M+2]$^+$ peak due to presence of chlorine was also observed in the mass spectra of compounds **7–11**.



Scheme 1. Reagents and reaction conditions: (a) CH_3OH, NaOH, (b) $SOCl_2$, (c) requisite amine, dichloromethane, RT.



Scheme 2. Reagents and reaction conditions: (a) H_2SO_4, CH_3OH, reflux 4 h, (b) requisite amine, fusion, 100 °C, 6 h, (c) Al(*t*-BuO)$_3$, cyclohexane, toluene, reflux, 4 h.

An alternative synthetic pathway was followed to prepare compounds with enhanced oxidation in ring A as shown in Scheme 2. The 16*E*-benzylidene steroid **5** was esterified to its methyl ester **12** in methanol using H_2SO_4 as dehydrating agent. IR absorption bands of carbonyl of ester function appeared at a higher wavenumber (1769 cm^{-1}) than parent carboxylic acid (1721 cm^{-1}) and a singlet of –O*CH*$_3$ appeared at δ 3.82 ppm in the ^{1}H NMR spectrum of compound **12**. Steroidal ester **12** was further thermally fused with various cyclic secondary amines like pyrolidine, morpholine and piperidine at 100 °C for about 5 h to yield 3-hydroxy substituted target amides **13–15**. Shifting of IR vibrational frequency of carbonyl function to lower wavenumber ~1660 cm^{-1} was observed for these amide derivatives.

Fusion of methyl ester **12** with N-methyl piperazine gave a sticky mass, which could not be crystallized. Also attempts to fuse diethyl amine with the methyl ester **12** remained unsuccessful.

In view of the literature reports [14,18,19] that 3,17-diketo steroids with higher degree of unsaturation in A-ring exhibit potent cytotoxicity, Oppenauer oxidation of **13–15** was carried out to afford α,β-unsaturated-3-keto steroids **16–18**, respectively. A singlet for the 4-C*H* proton at about δ 5.76 ppm and disappearance of 3α-*H* was observed in the ^{1}H NMR spectra of all the Oppenauer products.

3.2. Biology

The compounds **8**, **10**, **11** and **16** were selected by National Cancer Institute, Bethesda, Maryland, USA for evaluation of *in vitro* antineoplastic activity. The screening is a two-stage process, beginning with the evaluation of selected compounds against the 60 cell lines, known as NCI-60 cell lines and include cells from eight melanomas, six leukemia, eight breast cancers, two prostate, nine lung, seven colon, six ovary, eight kidney, and six central nervous system cancers, at a single dose of 10 μM. A 48 h continuous drug exposure protocol was used, and a sulforhodamine B (SRB) protein assay was used to estimate the cell viability or growth. Compounds, which exhibit significant growth inhibition in the single dose were evaluated against the 60 cell panel at five concentration levels in the next phase [20].

The output from the single dose screen is reported as a mean graph and is summarized in table 1. The compounds **8**, **10**, **11** and **16** exhibited significant growth reduction against five leukemia cell lines at a dose of 10 μM. The compounds displayed a mean growth percentage of 58.14, 36.49, 98.18 and 39.00 against these cell lines, respectively. The mean growth percentages against all the 60 cell lines were found to be 90.78%, 66.33%, 101.70% and 68.63%, respectively, for steroidal derivatives **8**, **10**, **11** and **16** suggesting that compound **10** and **16** have considerable inhibitory effect on all the cell lines at 10 μM.

Of all, compound **10** was selected by NCI for further evaluation against the 60 cell panel at five concentration levels at ten fold dilutions, the highest being 10^{-4} M. Mean dose response parameters against all 60 cell lines such as GI_{50} (drug concentration resulting in a 50% reduction in the net protein increase), TGI (drug concentration of total growth inhibition) and LC_{50} (concentration of drug resulting in a 50% reduction in the measured protein at the end of the drug treatment as compared to that at the beginning) are summarized in Table 2. It was observed that the selected compound inhibited growth of leukemia cancer cell lines at very low concentration. The GI_{50} value for compound **10** against four leukemia cell lines CCRF-CEM, K-562, RPMI-8226 and SR were found to be 3.94, 2.61, 6.90 and 1.79 μM, respectively.

As proposed earlier, replacement or substitution of C_3–OH function has a prominent impact on cytotoxic effect, it was found that retaining the hydroxyl group in 3β position of androstene skeleton leads to reduction in growth inhibition as observed for compound **13** which is also in accordance with our previous reports [14]. Whereas, 3,17-diketo compound **16** with higher degree of oxidation in ring A displayed enhanced antiproliferative activity. Incorporation of a chloro group at C_3 as in compound **10** also resulted in increased activity. Significant growth inhibition was observed for compounds **10** and **16** containing N-methyl piperazine and pyrolidine as tertiary amino function with mean %growth of 66.33% and 68.63%, respectively. Compound **10** with N-methyl piperazine group was found to be the most potent compound in comparison to compounds having other tertiary amino function.

In conclusion, it is evident from the results depicted in Tables 1 and 2 that the synthesized amide derivatives of 16*E*-[4-(2-carboxy)ethoxybenzylidene]androstene exhibit potent antileukemic activity, rather than having a generalized cytotoxic effect on the full panel of 60 cell lines as is observed for compound **2** [14]. This may be attributed to the presence of amino function as a labile amide linkage in these compounds. Among the synthesized steroidal amides, compound **10** with C_3 –Cl group was found to have significant antileukemic activity. This study provides valuable information regarding structural requirements to design and develop more potent steroidal anticancer agents in the androstene series.

Table 1
Percentage growth of cancer cells at 10 μM concentration of compounds.

Cell lines	Percentage growth			
	8	10	13	16
Leukemia				
CCRF-CEM	72.57	49.29	93.60	49.01
HL-60 (TB)	66.59	38.25	104.50	14.42
K-562	69.33	18.33	95.30	43.99
MOLT-4	71.39	59.29	98.38	34.52
RPMI-8226	64.55	53.53	99.37	41.14
SR	73.74	0.22	97.94	50.97
Non-small cell lung cancer				
A549/ATCC	78.99	64.97	87.66	59.85
EKVX	77.12	68.22	95.92	69.38
HOP-62	109.05	96.75	98.83	99.16
NCI-H226	84.82	77.19	99.22	65.76
NCI-H23	84.72	71.22	92.69	76.42
NCI-H322M	84.47	85.71	93.48	75.25
NCI-H460	91.89	17.19	103.82	75.95
NCI-H522	76.09	61.51	91.19	66.88
Colon cancer				
COLO 205	100.80	60.50	100.39	63.00
HCC-2998	102.03	90.60	101.67	89.75
HCT-116	81.69	34.58	104.36	54.04
HCT-15	93.58	54.37	104.08	61.41
HT29	76.41	−9.75	114.56	38.24
KM12	89.79	72.87	106.90	68.08
SW-620	96.08	40.38	108.04	87.09
CNS cancer				
SF-268	88.51	73.17	93.21	75.13
SF-295	92.47	82.78	101.91	82.18
SF-539	113.31	93.61	108.59	101.68
SNB-19	117.28	98.04	113.18	85.04
SNB-75	101.44	86.78	93.08	82.14
U251	95.51	34.13	104.82	71.47
Melanoma				
LOX IMVI	84.59	23.46	101.05	57.41
MALME-3M	96.32	73.83	114.20	84.45
M14	103.97	−13.00	107.55	92.20
MDA-MB-435	104.32	56.59	96.70	87.36
SK-MEL-2	76.46	89.82	112.25	72.27
SK-MEL-28	109.12	103.13	103.66	98.32
SK-MEL-5	76.48	80.37	99.50	32.40
UACC-257	81.25	38.82	81.83	50.55
UACC-62	78.91	89.99	110.15	63.97
Ovarian cancer				
IGROV1	112.86	86.64	97.51	80.04
OVCAR-3	97.19	76.09	110.60	70.04
OVCAR-4	117.69	70.73	108.19	51.30
OVCAR-5	106.98	99.69	109.11	96.78
OVCAR-8	93.90	78.91	105.10	72.61
NCI/ADR-RES	82.28	69.21	93.41	71.34
SK-OV-3	107.99	85.83	105.24	93.56
Renal cancer				
786-0	91.60	70.25	106.69	83.47
A498	87.19	75.25	88.24	–
ACHN	92.34	80.08	113.23	87.14
CAKI-1	78.40	70.14	95.46	74.72
RXF 393	104.99	78.98	117.42	77.46
SN12C	90.52	79.75	–	66.52
TK-10	87.09	88.21	100.39	76.24
UO-31	107.09	99.92	101.52	87.03
Prostate cancer				
PC-3	72.00	71.35	90.35	45.87
DU-145	94.62	74.94	97.52	78.60
Breast cancer				
MCF7	85.02	83.87	99.65	76.08
MDA-MB-31/ATCC	122.00	75.25	119.62	77.09
BT-549	87.92	87.33	99.05	36.31
T-47D	87.25	71.23	102.18	40.23
MDA-MB-468	91.75	66.55	102.65	49.49
Mean % growth	**90.78**	**66.33**	**101.70**	**68.63**

Table 2
Dose response parameters such as GI_{50}, TGI and LC_{50} of the 60-cell line assay for compound **10**.

Cell lines	GI_{50} (μM)	TGI (μM)	LC_{50} (μM)
Leukemia			
CCRF-CEM	3.94	>100	>100
K-562	2.61	>100	>100
MOLT-4	–	>100	>100
RPMI-8226	6.90	>100	>100
SR	1.79	–	>100
Non-small cell lung cancer			
A549/ATCC	>100	>100	>100
EKVX	>100	>100	>100
HOP-62	36.4	>100	>100
NCI-H226	10.4	>100	>100
NCI-H23	>100	>100	>100
NCI-H322M	>100	>100	>100
NCI-H460	>100	>100	>100
NCI-H522	>100	>100	>100
Colon cancer			
COLO 205	>100	>100	>100
HCC-2998	>100	>100	>100
HCT-116	3.08	>100	>100
HCT-15	–	>100	>100
HT29	2.04	–	>100
KM12	–	>100	>100
SW-620	–	>100	>100
CNS cancer			
SF-268	>100	>100	>100
SF-295	>100	>100	>100
SF-539	>100	>100	>100
SNB-19	>100	>100	>100
SNB-75	19.0	56.7	>100
U251	3.33	>100	>100
Melanoma			
LOX IMVI	1.61	–	–
MALME-3 M	>100	>100	>100
M14	>100	>100	>100
MDA-MB-435	–	>100	>100
SK-MEL-2	>100	>100	>100
SK-MEL-28	>100	>100	>100
SK-MEL-5	–	>100	>100
UACC-257	–	>100	>100
UACC-62	>100	>100	>100
Ovarian cancer			
IGROV1	>100	>100	>100
OVCAR-3	>100	>100	>100
OVCAR-4	>100	>100	>100
OVCAR-5	>100	>100	>100
OVCAR-8	>100	>100	>100
NCI/ADR-RES	>100	>100	>100
SK-OV-3	>100	>100	>100
Renal cancer			
786-0	88.1	>100	>100
A498	>100	>100	>100
ACHN	>100	>100	>100
CAKI-1	>100	>100	>100
RXF 393	14.3	>100	>100
SN12C	>100	>100	>100
TK-10	27.4	>100	>100
UO-31	>100	>100	>100
Prostate cancer			
PC-3	>100	>100	>100
DU-145	>100	>100	>100
Breast cancer			
MCF7	>100	>100	>100
MDA-MB-231/ATCC	>100	>100	>100
BT-549	96.1	>100	>100
T-47D	>100	>100	>100
MDA-MB-468	6.03	>100	>100

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