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Synthesis and Biological Activity of 3-N-Substituted Estrogen Derivatives as Breast Cancer Agents

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Abstract

3-*N*-substituted-estrogen derivatives were synthesized and characterized. Their antiproliferative activities against human ER (+) MCF-7 (Breast), ER (–) MDA-MB-231 (breast) and Ishikawa (endometrial) cancer cell lines were determined after 72 hours drug exposure employing CellTiter-Glo assay at concentrations ranging from (0.01-100,000 nM). The antiproliferative activities of these compounds were compared to tamoxifen (TAM), 4-hydroxytamoxifen (4-OHT, active metabolite of tamoxifen) and raloxifene (RAL). *In vitro* results indicated that compound **5** (IC₅₀ = 12μM) displayed comparable antiproliferative activity against MDA-MB 231 cell line; while compounds **6**, **7** and **13** (IC₅₀ = 12μM) displayed higher activity against MCF-7 and Ishikawa cell lines, in comparison to TAM activity (19-33μM).

Keywords

3-*N*-substituted estrogen derivatives; antiproliferative activity; breast cancer

INTRODUCTION

Estrogens are known to affect growth, development and maintenance of many tissues and organs [1-3]. The physiological effects of these steroids are mediated by two estrogen receptor (ER) subtypes; estrogen receptor alpha (ER-α) and estrogen receptor beta (ER-β) with differences in tissue distribution and transcriptional activity [4-7]. Estrogens stimulate the proliferation of normal and malignant cells through the ER *via* the induction of nucleic acid synthesis and activation of growth regulatory genes. Estrogen functions primarily by binding to the ER that dimerizes; then binds to estrogen responsive elements (EREs) in DNA, followed by the regulation of estrogen responsive transcription genes [7].

There is strong evidence that estrogen plays a role in the development and progression of breast cancer, the most frequently diagnosed cancers among women [8]. Currently one of way of blocking estrogen action on tumor cells is to prevent the binding of estrogen to ER by using an antiestrogen compound capable of blocking the effects of 17β-estradiol (E2) without displaying any estrogenic activity on their own [9-11]. Antiestrogenic compounds display an antagonist action at the ER, exhibit antitumor effect and are widely used in the

CONFLICT OF INTEREST

There is no conflict of interest associated with this publication.

treatment of hormone-dependent ER (+) breast cancer [12, 13]. Example of antiestrogenic compounds are Selective estrogen receptor modulators (SERMs) drugs such as tamoxifen (TAM, **1**) and Raloxifen (RAL, **2**) [14, 15]. TAM (Fig. **1**), a triphenylethylene (TPE) nonsteroidal antiestrogen, is the first chemotherapeutic drug used in the treatment of estrogen receptor positive (ER +) breast cancer [16]. It reduces the risk of contralateral breast cancer, behaves as ER antagonist in the breast tissue and as ER agonist in bone and lipids [17, 18]. TPE antiestrogen compounds contain a dialkylaminoethoxy side chain group, which is essential for their physiological activity. Studies have shown that these compounds lose their antiestrogenic activity and become potent estrogenic compounds in the absence of such groups [19, 20]. For example, replacement of dimethylaminoethoxy group of TAM with a methoxy group decreases TAM inhibitory growth activity in MCF-7 cells, while the addition of dimethylaminoethoxy group to gem-diphenyldichloroethylene, a weak estrogenic compound, resulted in an enhanced inhibitory growth activity in MCF-7 cells [21, 22].

E2 (the most biological active estrogen) and derivative are used by millions of women in Hormone Replacement Therapy (HRT) for the treatment of peri- and post-menopausal related symptoms [23-25]. E2 also served as a framework for the attachment of various substituents for ER therapeutic applications in the treatment of hormone-dependent breast cancer [26, 27]. Studies have shown that E2 derivatives ICI-182,780 (Fulvestrant, **4a**) and ICI-164,384 (**4b**) are as effective as tamoxifen in the treatment breast cancer [28, 29]. This finding and others led to Food & Drugs Administration (FDA) approving Fulvestrant for the treatment of post-menopausal ER (+) breast cancers [30].

Recently, our group has demonstrated that 3-*N*-alkylaminoethoxy derivatives of E2 showed sufficient potent activity in inhibiting the E2-stimulated proliferation of MCF-7 cancer cells [31]. Therefore, based on this and other previous investigations involving the attachment of side chain group to biologically active E2, we herein report the synthesis of new series of estrogen derivatives (Scheme **1**) with various alkylaminopropoxy groups at the Carbon-3 position. These compounds were also evaluated for *in vitro* antiproliferative activities against MCF-7, MDA-MB-231 human breast cancer cell lines, and Ishikawa human endometrial cell line. These cell lines are widely accepted models for assessing potent anti-proliferative and antiestrogenic compounds. TAM, 4-OHT and RAL were used as standards for comparison purposes in all these studies.

MATERIALS AND METHODS

Experimental Section

General—Commercial grade solvents and reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) or Alfa Aesar (Ward Hill, MA, USA) and used without further purification. The NMR spectra were recorded on Varian 300 MHz spectrometer (¹H: 300 MHz, ¹³C: 75MHz). The appropriate deuterated solvents are indicated in the procedure, and line positions recorded in *ppm* from the reference signal. ESI-TOF Mass Spectrometer was recorded on Finnigan LCQ - Quadrupole Ion Trap (Thermo Finnigan, San Jose, CA), and the HPLC pump was an Agilent (HP) 1100 series pump or Applied Biosystems model 400 pump. The purification was performed using Flash column chromatography with silica gel 60 (160–200 mesh) obtained from Sigma-Aldrich (St. Louis, MO, USA).

General Procedures for the Synthesis of 3-*N*-alkylaminopropoxyl Derivatives of Estrogen

Method A: To a solution of estradiol/estrone (1 eq) in anhydrous acetone were added 1-(3-chloropropyl)-alkyl amino hydrochloride (1.1 eq), anhydrous K₂CO₃ (2.5 eq) and NaI (catalytic amount). The mixture was heated under reflux overnight. After TLC indicated that

the reaction was completed, the solvent was removed in vacuum and extracted with ethyl acetate (3 times). The combined organic layer was washed with brine water, dried over anhydrous magnesium sulfate and the solvent removed to afford the crude product that was purified using silica gel flash column chromatography [eluting with mixture of CH₂Cl₂ - MeOH (9:1, V/V)] to provide the title product.

3-(3-piperidinylpropoxy)estra-1,3,5(10)-trien-17 β -ol (5) as white solid—¹H-NMR (300MHz, CDCl₃) δ 7.18 (d, J=8.4 Hz, 1H, 1-CH), 6.69 (dd, J=8.4, 2.7 Hz, 1H, 2-CH), 6.62 (d, J=2.7 Hz, 1H, 4-CH), 3.97 (t, J=6.3Hz, 2H, 3-OCH₂-), 3.73 (td, J=8.6, 5.1Hz, 1H, 17 α -CH-), 2.87-2.81 (m, 2H, 6-CH₂), 2.46 (t, J=7.5Hz, 2H, -NCH₂CH₂O-), 2.40 (t, J=5.4 Hz, 4H, -NCH₂CH₂- of piperidinyl), 2.35-2.26 (m, 1H), 2.25-2.06 (m, 2H), 1.99-1.83 (m, 4H), 1.74-1.16 (m, 15H), 0.78 (s, 3H, 13-CH₃); ¹³C-NMR (75MHz, CDCl₃) δ 157.1, 138.1, 132.9, 126.5, 114.8, 112.3, 82.0, 66.7, 56.3, 54.8 (two carbon), 50.3, 44.2, 43.5, 39.1, 37.0, 30.8, 30.0, 27.5, 27.1, 26.6, 26.1 (two carbon), 24.6, 23.4, 11.3; HRMS (ESI-MS) calculated for C₂₆H₄₀NO₂ [(M+H)⁺] 398.3054, found 398.3029.

3-(3-N,N-dimethylaminopropoxy)estra-1,3,5(10)-trien-17 β -ol (6) as white solid—¹H-NMR (300MHz, CDCl₃) δ 7.18 (d, J=8.4 Hz, 1H, 1-CH), 6.70 (dd, J=8.4, 2.7 Hz, 1H, 2-CH), 6.63 (d, J=2.7 Hz, 1H, 4-CH), 3.98 (t, J=6.3 Hz, 2H, 3-OCH₂-), 3.72 (t, J=8.4 Hz, 1H, 17 α -CH-), 2.87-2.81 (m, 2H, 6-CH₂), 2.49 (t, J=7.4 Hz, 2H, -NCH₂CH₂O-), 2.29 (s, 6H, -NCH₃), 2.22-2.06 (m, 3H), 2.00-1.83 (m, 4H), 1.74-1.64 (m, 1H), 1.56-1.13 (m, 8H), 0.78 (s, 3H, 13-CH₃); ¹³C-NMR (75MHz, CDCl₃) δ 157.0, 138.2, 132.9, 126.6, 114.6, 112.2, 82.0, 66.2, 56.7, 50.2, 45.6 (two carbon), 44.2, 43.5, 39.1, 37.0, 30.8, 30.0, 27.6, 27.5, 26.6, 23.4, 11.3; HRMS (ESI-MS) calculated for C₂₃H₃₅NO₂ [(M+H)⁺] 358.2741, found 358.2755.

3-(β -N,N-dimethylaminopropoxy)estra-1,3,5(10)-trien-17 δ -ol (7) as white solid—¹H-NMR (300MHz, CDCl₃) δ 7.19 (d, J=8.4 Hz, 1H, 1-CH), 6.72 (dd, J=8.4, 2.7 Hz, 1H, 2-CH), 6.64 (d, J=2.7 Hz, 1H, 4-CH), 4.02 (ddd, J=9.6, 5.7, 2.1 Hz, 1H, 3-OCH₂-), 3.82 (ddd, J=9.6, 6.0, 2.4 Hz, 1H, 3-OCH₂-), 3.73 (t, J=8.4 Hz, 1H, 17 α -CH-), 3.00 (m, 1H, CH₃CHNCH₃ of isopropyl), 2.87-2.78 (m, 2H, 6-CH₂), 2.37 (s, 6H, -CHNCH₃), 2.23-2.06 (m, 2H), 1.98-1.84 (m, 3H), 1.74-1.65 (m, 2H), 1.57-1.20 (m, 6H), 1.24 (d, J=6.6Hz, 3H, -NCHCH₃), 0.78 (s, 3H, 13-CH₃); ¹³C-NMR (75MHz, CDCl₃) δ 156.9, 138.2, 133.0, 126.6, 114.7, 112.3, 82.1, 69.8, 58.7, 50.2, 46.4, 44.2, 43.5, 41.7 (two carbon), 39.0, 37.0, 30.8, 30.0, 27.5, 26.6, 23.4, 11.4; HRMS (ESI-MS) calculated for C₂₃H₃₅NO₂ [(M+H)⁺] 358.2741, found 358.2754.

3-(3-N,N-dimethylaminopropoxy)estra-1,3,5(10)-trien-17-one (8) as white solid—¹H-NMR (300MHz, CDCl₃) δ 7.18 (d, J=8.4 Hz, 1H, 1-CH), 6.71 (dd, J=8.4, 2.7 Hz, 1H, 2-CH), 6.65 (d, J=2.7 Hz, 1H, 4-CH), 3.98 (t, J=6.3 Hz, 2H, 3-OCH₂-), 2.91-2.86 (m, 2H, 6-CH₂), 2.43 (t, J=7.4 Hz, 2H, -NCH₂CH₂O-), 2.49 (dd, 1H, J=8.7, 18.5 Hz), 2.43 (t, J=7.4 Hz, 2H, -NCH₂CH₂CH₂O-), 2.41-2.36 (m, 1H), 2.24 (s, 6H, -NCH₃), 2.21-2.19 (m, 1H), 2.16-2.08 (dd, 1H, J=8.7, 18.5 Hz), 2.05-1.88 (m, 5H), 1.66-1.39 (m, 6H), 0.91 (s, 3H, 13-CH₃); ¹³C-NMR (75MHz, CDCl₃) δ 221.2, 157.3, 138.0, 132.1, 126.5, 114.7, 112.4, 66.4, 56.7, 50.6, 48.2, 45.8 (two carbon), 44.2, 38.6, 36.1, 31.8, 29.9, 27.9, 27.0, 26.2, 21.8, 14.1; HRMS (ESI-MS) calculated for C₂₃H₃₃NO₂ [(M+H)⁺] 356.2584, found 356.2598.

Method B: To a solution of estradiol (1 eq) in anhydrous acetone was added 1-bromo-3-chloropropane (1.1 eq), anhydrous K₂CO₃ (2.5 eq) and NaI (catalytic amount). The mixture was heated under reflux overnight. After TLC indicated that the reaction was completed, the solvent was removed in vacuum and extracted with ethyl acetate (3 times). The combined organic layer was washed with brine water, dried over anhydrous magnesium sulfate and the

solvent removed to afford the crude product that was purified by recrystallization from hexane to provide 3-chloropropoxyl-estradiol.

To a solution of 3-chloropropoxyl-estradiol (1 eq) in anhydrous acetone was added alkyl amino (1.5 eq), anhydrous K_2CO_3 (2.5 eq) and NaI (catalytic amount). The mixture was heated under reflux overnight. After TLC indicated that the reaction was completed, the solvent was removed in vacuum and extracted with ethyl acetate (3 times). The combined organic layer was washed with brine water, dried over anhydrous magnesium sulfate and the solvent removed to afford the crude product that was purified using flash column chromatography [eluting with mixture of CH_2Cl_2 - MeOH (9:1, V/V)] to provide the title product.

3-(3-(4-methylpiperazinyl)-propoxy)estra-1,3,5(10)-trien-17 β -ol (9) as white solid— ^1H -NMR (300MHz, $CDCl_3$) δ 7.18 (d, $J=8.4$ Hz, 1H, 1-CH), 6.69 (dd, $J=8.4$, 2.7 Hz, 1H, 2-CH), 6.62 (d, $J=2.7$ Hz, 1H, 4-CH), 3.98 (t, $J=6.6$ Hz, 2H, 3-OCH₂-), 3.73 (t, $J=8.7$ Hz, 1H, 17 α -CH-), 2.87-2.81 (m, 2H, 6-CH₂), 2.54-2.47 (m, 10H, -NCH₂-), 2.35-2.30 (m, 1H), 2.28 (s, 3H, CH₃N- of piperazinyl), 2.21-2.04 (m, 3H), 1.98-1.83 (m, 4H), 1.74-0.88 (m, 8H), 0.78 (s, 3H, 13-CH₃); ^{13}C -NMR (75MHz, $CDCl_3$) δ 157.1, 138.1, 132.9, 126.5, 114.8, 112.3, 82.0, 66.4, 55.4, 55.3 (two carbon), 53.4 (two carbon), 50.3, 46.2, 44.2, 43.5, 39.1, 37.0, 30.8, 30.0, 27.5, 27.1, 26.6, 23.4, 11.3; HRMS (ESI-MS) calculated for $C_{26}H_{41}N_2O_2$ [(M+H)⁺] 413.3163, found 413.3191.

3-(3-morpholinylpropoxy)estra-1,3,5(10)-trien-17 β -ol (10) as white solid— ^1H -NMR (300MHz, $CDCl_3$) δ 7.19 (d, $J=8.4$ Hz, 1H, 1-CH), 6.70 (dd, $J=8.4$, 2.7 Hz, 1H, 2-CH), 6.62 (d, $J=2.7$ Hz, 1H, 4-CH), 3.99 (t, $J=6.3$ Hz, 2H, 3-OCH₂-), 3.71 (t, $J=4.8$ Hz, 4H, -OCH₂CH₂N- in morpholinyl; m, 1H, 17 α -CH-), 2.87-2.82 (m, 2H, 6-CH₂), 2.50 (t, $J=6.9$ Hz, 2H, -NCH₂CH₂O-), 2.46 (t, $J=4.5$ Hz, 4H, -NCH₂CH₂ of morpholinyl), 2.35-2.25 (m, 1H), 2.21-2.05 (m, 2H), 1.99-1.84 (m, 4H), 1.64-1.74 (m, 1H), 1.51-1.41 (m, 8H), 0.78 (s, 3H, 13-CH₃); ^{13}C -NMR (75MHz, $CDCl_3$) δ 157.1, 138.1, 132.8, 126.5, 114.7, 112.2, 81.9, 67.1 (two carbon), 66.2, 55.9, 53.9 (two carbon), 50.3, 44.2, 43.5, 39.1, 37.0, 30.7, 30.0, 27.5, 26.7, 26.5, 23.3, 11.3; HRMS (ESI-MS) calculated for $C_{25}H_{38}NO_3$ [(M+H)⁺] 400.2846, found 400.2805.

3-(3-pyrrolidinylpropoxy)estra-1,3,5(10)-trien-17 β -ol (11) as white solid— ^1H -NMR (300MHz, $CDCl_3$) δ 7.18 (d, $J=8.4$ Hz, 1H, 1-CH), 6.70 (dd, $J=8.4$, 2.7 Hz, 1H, 2-CH), 6.63 (d, $J=2.7$ Hz, 1H, 4-CH), 3.99 (t, $J=6.3$ Hz, 2H, 3-OCH₂-), 3.73 (t, $J=8.4$, 1H, 17 α -CH-), 2.87-2.81 (m, 2H, 6-CH₂), 2.61 (t, $J=7.5$ Hz, 2H, -NCH₂CH₂O-), 2.55-2.47 (m, 4H, -NCH₂CH₂ of pyrrolidinyl), 2.33-2.28 (m, 1H), 2.22-2.07 (m, 2H), 2.03-1.83 (m, 4H), 1.80-1.72 (m, 4H, -NCH₂CH₂- in pyrrolidinyl), 1.71-1.16 (m, 8H), 0.78 (s, 3H, 13-CH₃); ^{13}C -NMR (75MHz, $CDCl_3$) δ 157.1, 138.1, 132.8, 126.5, 114.8, 112.3, 82.1, 66.6, 54.5 (two carbon), 53.4, 50.3, 44.2, 43.5, 39.1, 37.0, 30.9, 30.0, 29.1, 27.5, 26.6, 23.7 (two carbon), 23.4, 11.3; HRMS (ESI-MS) calculated for $C_{25}H_{39}NO_2$ [(M+H)⁺] 384.2897, found 384.2867.

3-(3-N,N-diisopropylaminopropoxy)estra-1,3,5(10)-trien-17 β -ol (12) as white solid— ^1H -NMR (300MHz, $CDCl_3$) δ 7.18 (d, $J=8.4$ Hz, 1H, 1-CH), 6.70 (dd, $J=8.4$, 2.7 Hz, 1H, 2-CH), 6.63 (d, $J=2.7$ Hz, 1H, 4-CH), 3.97 (t, $J=7.2$ Hz, 2H, 3-OCH₂-), 3.72 (t, $J=8.4$ Hz, 1H, 17 α -CH-), 3.02 (m, $J=6.9$ Hz, 2H, -NCH(CH₃)₂ of isopropyl), 2.85-2.80 (m, 2H, 6-CH₂), 2.60 (t, $J=6.9$ Hz, 2H, -NCH₂CH₂CH₂O-), 2.35-2.25 (m, 1H), 2.20-2.06 (m, 3H), 1.98-1.79 (m, 3H), 1.74-1.65 (m, 1H), 1.40-1.07 (m, 8H), 1.01 (d, $J=6.9$ Hz, 12H, -NCH(CH₃)₂), 0.78 (s, 3H, 13-CH₃); ^{13}C -NMR (75MHz, $CDCl_3$) δ 157.0, 138.2, 133.0, 126.5, 114.8, 112.4, 82.0, 66.3, 64.8, 50.4, 48.7, 44.2, 43.5, 41.9, 39.1, 37.0, 30.8, 30.0, 27.6, 27.5,

26.6, 23.4, 21.0 (four carbon), 11.4; HRMS (ESI-MS) calculated for $C_{27}H_{44}NO_2 [(M+H)^+]$ 414.3367, found 414.3367.

3-(3-N,N-diethylaminopropoxy)estra-1,3,5(10)-trien-17 β -ol (13) as white solid

^1H -NMR (300MHz, $CDCl_3$) δ 7.18 (d, $J=8.4$ Hz, 1H, 1-CH), 6.69 (dd, $J=8.4, 2.7$ Hz, 1H, 2-CH), 6.63 (d, $J=2.7$ Hz, 1H, 4-CH), 3.97 (t, $J=6.6$ Hz, 2H, 3-OCH₂-), 3.72 (t, $J=8.4$ Hz, 1H, 17 α -CH-), 2.87-2.82 (m, 2H, 6-CH₂), 2.60 (t, $J=7.2$ Hz, 2H, -NCH₂CH₂O-), 2.54 (q, $J=7.2$ Hz, 4H, -NCH₂CH₃), 2.34-2.22 (m, 1H), 2.17-2.05 (m, 2H), 1.98-1.83 (m, 4H), 1.74-1.64 (m, 1H), 1.56-1.10 (m, 8H), 1.02 (t, $J=7.2$ Hz, 6H, -NCH₂CH₃), 0.78 (s, 3H, 13-CH₃); ^{13}C -NMR (75MHz, $CDCl_3$) δ 157.2, 138.1, 132.8, 126.4, 114.8, 112.3, 81.9, 66.6, 52.1, 50.3, 47.2 (two carbon), 44.2, 43.5, 39.1, 37.0, 30.8, 30.0, 27.5, 27.2, 26.6, 23.4, 12.0 (two carbon), 11.3; HRMS (ESI-MS) calculated for $C_{25}H_{40}NO_2 [(M+H)^+]$ 386.3054, found 386.3058.

3-(3-N-methylaminopropoxy)estra-1,3,5(10)-trien-17 β -ol (14) as white solid

^1H -NMR (300MHz, $CDCl_3$) δ 7.18 (d, $J=8.4$ Hz, 1H, 1-CH), 6.70 (dd, $J=8.4, 2.7$ Hz, 1H, 2-CH), 6.63 (d, $J=2.7$ Hz, 1H, 4-CH), 4.01 (t, $J=6.3$ Hz, 2H, 3-OCH₂-), 3.72 (t, $J=8.6$ Hz, 1H, 17 α -CH-), 2.87-2.81 (m, 2H, 6-CH₂), 2.77 (t, $J=6.9$ Hz, 2H, -NCH₂CH₂O-), 2.46 (s, 3H, CH₃NH-), 2.35-2.25 (m, 1H), 2.20-2.05 (m, 2H), 2.00-1.83 (m, 4H), 1.74-1.65 (m, 1H), 1.56 (br, s, 1H, -NHCH₃), 1.52-1.14 (m, 8H), 0.78 (s, 3H, 13-CH₃); ^{13}C -NMR (75MHz, $CDCl_3$) δ 157.0, 138.2, 132.9, 126.5, 114.7, 112.3, 82.1, 66.5, 50.3, 49.3, 44.2, 43.5, 39.1, 37.0, 36.6, 30.8, 30.0, 29.7, 27.5, 26.6, 23.4, 11.3; HRMS (ESI-MS) calculated for $C_{22}H_{33}NO_2 [(M+H)^+]$ 344.2584, found 344.2598.

3-(3-aminopropoxy)estra-1,3,5(10)-trien-17 β -ol (15) as white solid

^1H -NMR (300MHz, $CDCl_3$) δ 7.18 (d, $J=8.4$ Hz, 1H, 1-CH), 6.70 (dd, $J=8.4, 2.7$ Hz, 1H, 2-CH), 6.63 (d, $J=2.7$ Hz, 1H, 4-CH), 4.01 (t, $J=6.0$ Hz, 2H, 3-OCH₂-), 3.72 (t, $J=8.7$ Hz, 1H, 17 α -CH-), 3.38 (t, $J=6.8$ Hz, 1H, H₂NCH₂-), 2.89 (t, $J=6.8$ Hz, 1H, H₂NCH₂-), 2.85-2.81 (m, 2H, 6-CH₂), 2.34-2.26 (m, 1H), 2.18-1.87 (m, 6H), 1.73-1.64 (m, 3H), 1.56-1.13 (m, 8H), 0.78 (s, 3H, 13-CH₃); ^{13}C -NMR (75MHz, $CDCl_3$) δ 157.0, 138.2, 132.9, 126.6, 114.6, 112.2, 82.0, 66.0, 50.2, 44.2, 43.5, 39.6, 39.1, 37.0, 33.2, 30.8, 30.0, 27.5, 26.6, 23.4, 11.3; HRMS (ESI-MS) calculated for $C_{21}H_{31}NO_2 [(M+H)^+]$ 330.2428, found 330.2437.

Antiproliferative Activity Studies

The antiproliferative activity of compounds **5-15** was evaluated at the Southern Research Institute (SRI, Birmingham, Alabama, USA) according to procedures [32]. The compounds were screened against human ER (+) MCF-7 (breast), ER (-) MDA-MB-231 (breast) and Ishikawa (endometrial) cancer cell lines in comparison to TAM, 4-OHT and RAL.

Material—Human MCF-7 and MDA-MB-231 breast cancer cell lines were purchased from the NCI. The human Ishikawa endometrial cancer cell line was purchased from Sigma Aldrich company. All three-cell lines were cultured in phenol red-free RPMI-1640 (Hyclone) (500 mL) supplemented with L-glutamine-dipeptide (Hyclone) (5 mL), and 10% fetal bovine serum (Atlanta Biologicals) (50 mL).

Method—All three cell lines were cultured and treated with compounds **5-15**, TAM, 4-OHT and RAL ranging from 0.01-100,000 nM concentration in the presence of 10 nM estradiol using the previous reported method [33]. The results expressed as IC₅₀ (Inhibitory concentration of 50%) values were the averages of three data points for each concentration and were calculated using GraphPad Prism 4.0.

RESULTS

Chemistry

The 3-*N*-aminopropoxy derivatives of estrogen **5-8** were synthesized according to the general procedure outlined scheme 1. In this investigation, the alkylation of the 3-position was achieved by reacting 17 β -estradiol/estrone with chloropropyl-alkylamino group in the presence of potassium carbonate (K₂CO₃) and NaI (catalytic amount) in acetone (method A) to afford the desired compounds in moderate to good yields. This method has also been widely using in the alkylation of phenolic group to afford the desired product in up to 80% yield and is said to involve a Finkelstein reaction, followed by an S_N2 reaction to afford the desired product [34-36]. Compounds **5-8** were purified using silica gel flash column chromatography eluting with mixture of CH₂Cl₂-MeOH (9: 1 V/V) to afford the pure analogs between 54-77%, which were characterized using proton (¹H) NMR, carbon (¹³C) NMR and high resolution mass spectra (ESI-MS, Electrospray Ionization) analysis as indicated in the experimental section. Mass spectroscopic (ESI, Electrospray Ionization) analysis revealed molecular ion at m/z 398.3029 [M+H]⁺ corresponding to compound **5**, m/z 358.2755 [M+H]⁺ corresponding to compound **6**, m/z 358.2754 [M+H]⁺ corresponding to compound **7** and m/z 356.2598 [M+H]⁺ corresponding to compound **8**.

In order to synthesize the 3-*N*-alkylaminopropoxyl E2 derivatives **9-15**, we first synthesized the key intermediate, 3-chloropropoxy substituted E2, by reacting a mixture of 1-bromo-3-chloro propane and E2 in the presence of potassium carbonate (K₂CO₃) in acetone (Scheme 2). The intermediate, 3-chloropropoxy substituted E2, was then reacted with appropriate tertiary amino groups to afford 3-*N*-aminopropoxy E2 **9-15** in good to moderate yields (65-78%). The advantage of this method is that it does provide access to other derivatives with increase -CH₂ group at the tertiary aminoalkoxy side chain group.

Antiproliferative Activity—*In vitro* antiproliferative activity of compounds **5-15** were evaluated against human ER (+) MCF-7 (breast), ER (–) MDA-MB-231 (breast) and Ishikawa (endometrial) cancer cell lines at concentration ranging from (0.01 – 100,000 nM) in the presence of 10 nM E2 using CellTiter-Glo assay (E2 was used for competitive growth inhibitory studies). As shown in Table 1, compounds **5-9** and **11- 15** (IC₅₀ = 10-25 μ M) demonstrated significant antiproliferative activity against human ER (+) MCF-7 breast cancer cell line in comparison to TAM (IC₅₀ = 26 μ M), but were less potent than 4-OHT and RAL (IC₅₀ = 2 and 3 μ M, respectively) (Note: IC₅₀ is the concentration of test drug where a 50% reduction is observed in cell growth compared to the untreated control after a 72 h period of exposure to test drug). Compounds bearing dimethylaminopropoxy (**6**) and diethylaminopropoxy (**13**) groups, the most active of the series in this cell line, exhibit higher antiproliferative activity against MCF-7 cell line based on the IC₅₀ value (Fig. 2a).

The antiproliferative activity of compounds **5-15** against human ER (–) MDA-MB-231 breast cancer cell lines were also investigated to shed some light on their mechanism of action. It was observed in this cell line that (i) compounds **6-9**, **11**, **12**, **14** and **15** (IC₅₀ = 20-29 μ M) showed comparable cytotoxicity to the standard drugs, TAM (IC₅₀ = 19 μ M), 4-OHT (IC₅₀ = 22 μ M), and RAL (IC₅₀ = 28 μ M), respectively (Table 1); and (ii) compound **5** (IC₅₀ = 12 μ M) possessing piperidinylpropoxyl group, the most active of the series in this cell line, exhibits similar antiproliferative activity based on the IC₅₀ value in comparison to TAM, 4-OHT and RAL (Fig. 2b). This result suggests that compound **5** may also inhibit cell proliferation *via* ER-independent mechanism in comparison to TAM.

Furthermore, the antiproliferative activity of these compounds against human Ishikawa endometrial cell line was also evaluated and results (Table 1) revealed that (i) compounds **7**,

8, 13 and **15** (IC_{50} = 16, 19, and 18 M; respectively) were more active than TAM (IC_{50} = 33 μ M), 4-OHT (IC_{50} = 20 μ M), and RAL (IC_{50} = 23 μ M) in inhibiting the E2 stimulated proliferation of the endometrial cells and (ii) compound **7** possessing β -dimethylaminoisopropoxyl group, the most active of the series in this cell line, exhibits higher antiproliferative activity (Fig. 2c). This result indicates that compound **7** may lower the risk of developing uterine cancer based upon the IC_{50} value in comparison with TAM.

DISCUSSION

One of the chemical features of antiestrogenic compounds known as ER ligands is the basic amino side chain (tertiary aminoalkoxy group) [37-39]. The nature of the side chain and its orientation relative to the ligand backbone play an important role in the determination of their tissue-selective activity [40, 41]. In view of the above facts and in order to further develop an understanding of the structural requirement for E2 derivative containing amino the side chain group, we proposed the incorporation of the tertiary aminopropoxy group at the C-3 position of E2 to afford 3-*N*-alkylaminopropoxyl estradiol derivatives (Scheme 1-2). The antiproliferative activities of these compounds **5-15** against MCF-7, MDA-MB-231 and Ishikawa cell lines as a test models were evaluated using CellTiter-Glo assay [32]. Antiproliferative activities of these compounds in MCF-7 cell line indicates that compounds **6** and **13** were more active than TAM and may act as ER antagonists based on the antiproliferative mechanism (ER-dependent mechanisms) of TAM against ER (+) MCF-7 breast cancer cells; inhibition of E2 binding to the ER [42]. However, further studies are needed to ascertain this claim.

Recent studies have also shown that TAM may inhibit cell proliferation *via* ER-independent mechanisms against ER (–) MDA-MB-231 breast cancer cell line [43-46]. This cell line constitutes an original model for identifying the ER-independent mechanisms of TAM antiproliferative effects [47, 48]. The results obtained against MDA-MB-231 breast cancer cell line indicate that the antiproliferative activity of compound **5** were comparable to the standard drugs, thus suggesting that they may also inhibit cell proliferation *via* ER-independent mechanism. Also, in the Ishikawa cell line, compound **5** showed a higher IC_{50} value that indicates possible risk for uterine cancer development.

In the development of antiestrogenic SERM drugs, it is critical that the candidate drug minimize undesirable uterine effect (agonist activity), which could lead to both increase in uterine bleeding and an increased risk of developing uterine cancer. The Ishikawa cell line is an endometrial adenocarcinoma cell line, which has been used as test model in evaluating this effect [48]. Finally, this present results further confirm the importance of the basic amino side chain groups with respect to the antiproliferative activity of the compounds.

CONCLUSION

3-*N*-substituted-estrogen derivatives were synthesized, and characterized using proton (^1H) NMR, carbon (^{13}C) NMR and high-resolution mass spectra (ESI-MS, Electrospray Ionization). Their antiproliferative activities against human ER (+) MCF-7 (breast), ER (–) MDA-MB-231 (breast) and Ishikawa (endometrial) cell lines were determined and compared to that of the standard antiestrogen drugs TAM, 4-OHT, and RAL. *In vitro* results indicated that the synthesized E2 derivatives containing piperidinylethoxy **5**, displayed slightly higher antiproliferative activity against human MDA-MB-231 (hormone-independent), MCF-7 (hormone-dependent); as evident by the lowest IC_{50} value in comparison with TAM. Nevertheless, compound **5** has a higher liability in causing uterine cancer based upon the higher IC_{50} value against Ishikawa cell line in comparison with TAM. Furthermore, it is worth noting that compound **5** displayed lower antiproliferative

activities against MCF-7 (hormone-dependent) in comparison to RAL and 4-OHT. Compound **15** displayed moderate activities against Ishikawa (endometrial), MCF-7 and MDA-MB-231 (breast) cell lines, indicating that it is the overall best candidate to be considered for future studies. These studies will involve ER binding studies, uterotrophic and antiuterotrophic activities in immature rat in order to further understand how these compounds interact with the ER.

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Fig. (1).
Structures of tamoxifen (TAM, **1**), 4-hydroxytamoxifen (4-OHT, **2**), raloxifene (RAL, **3**), 17β-estradiol (E2), ICI-182,780 (fulvestrant, **4a**), and ICI-164,384 (**4b**).

Fig. (2).

In vitro antiproliferative activity of (a) compound **6** against (ER+) MCF-7 cell line, (b) compound **5** against (ER-) MDA-MB-231 cell line and (c) compound **7** against Ishikawa cell line; in comparison to TAM, 4-OHT and RAL.

Scheme (1).

Reagents and Conditions: (i) Appropriate alkyl halide (R-Cl), K_2CO_3 , NaI, acetone, reflux (method A).

Scheme (2).

Reagents and Conditions: (i) 1-bromo-3-chloro propane, K_2CO_3 , acetone, reflux, (ii) Appropriate tertiary amino groups, K_2CO_3 , NaI, acetone, reflux (method B).

Table 1
IC₅₀ Values (μM) for Compounds 1-15 Tested against Human (ER+) MCF-7 (Breast), (ER-) MDA-MB231 (Breast) and Ishikawa (Endometrial) Cancer Cells

Compounds No.	Ishikawa (μM)	MDA-MB-231 (μM)	MCF-7 (μM)
1 (TAM)	33	19	26
2 (4-OHT)	20	22	2
3 (RAL)	23	28	3
5	39	12	20
6	25	25	16
7	16	28	25
8	19	26	21
9	25	26	22
10	>100	82	>100
11	21	25	25
12	28	29	18
13	19	36	16
14	24	29	28
15	18	20	20

¹The data represent the average of triplicate determinations at various concentrations.

²The IC₅₀ values were determined from the graphs (GraphPad Prism) using mean values of data points at various concentrations.