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## Original article

## Bismuth nitrate-induced novel nitration of estradiol: An entry to new anticancer agents



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## ABSTRACT

Direct nitration of estradiol was carried out using metal nitrates on solid surfaces under mild condition, and a combination of bismuth nitrate pentahydrate impregnated KSF clay was found to be the best reagent to synthesize 2- and 4-nitroestradiol effectively. Furthermore, various basic side chains were introduced, through O-linker at C-3, to these nitroestradiols. The ability of these derivatives to cause cytotoxicity in Estrogen Receptor (ER)-positive and ER-negative breast cancer cell lines, as well as cancer cell lines of other origins, was examined. Qualitative structure activity relationship (SAR) has also been studied. We found that a basic side chain containing either a piperidine or morpholine ring, when conjugated to 2-nitroestradiol, was particularly effective at causing cytotoxicity in each of the cancer cell lines examined. Surprisingly, this effective cytotoxicity was even seen in ER-negative breast cancer cells.

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## 1. Introduction

According to World Health Organization, cancer was the leading cause of death worldwide in 2008, accounting for 7.6 million deaths (around 13% of all deaths) [1]. Furthermore, cancer-related mortalities are projected to continue to rise to over 13.1 million by 2030 [1]. Therefore, there is a continuous need to develop new anticancer agents to reduce both cancer incidence and cancer-related mortalities.

Breast cancer is the most commonly occurring type of cancer globally [2], and estrogens and Estrogen Receptors (ERs), including

ER $\alpha$  and ER $\beta$ , play an important role in numerous cancers, including breast, ovarian, uterine, prostate, and colon cancers [3,4]. In fact, ERs are key targets for the treatment of breast cancer [5], and the selective estrogen receptor modulator (SERM), Tamoxifen (Tam), is an ER $\alpha$  antagonist that has been used to treat breast cancer since the 1970s [6]. However, many breast cancers become resistant to Tam therapy, and Tam therapy leads to an increased risk of other tumor types; therefore, recent studies have focused on identifying novel SERMs that are tissue selective, on targeting other mechanisms of ER function, and on identifying novel compounds that target ER $\beta$  instead of ER $\alpha$  [5].

In addition to modulating SERMs, medicinal chemistry has focused on inhibiting the synthesis of estrogens by inhibiting the activities of Aromatase or 17- $\beta$ -hydroxysteroid dehydrogenase or by modulating sulfotransferase enzymes (e.g., steroid sulfotransferase or steroid sulfatase) that affect the sulfation of estrone and/or estradiol [7]. In this respect, nitroestrogens have potential application in cancer chemotherapy. For example, 2-nitroestradiol was reported to be a more effective inhibitor of a steroid alcohol

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sulfotransferase when compared with 4-nitroestradiol [8], whereas 4-substituted estrogen derivatives have been reported to inhibit the steroid sulfatase enzyme more effectively than 2-substituted estrogen derivatives [9].

We have previously reported that the introduction of small carbon chains with a basic terminal moiety or heterocyclic scaffold to polyaromatic systems can induce significant anticancer activity to the resultant molecules [10–22]. In the present study we have developed (i) a direct procedure for the nitration of estradiol and (ii) introduced carbon linkers on the oxygen at C-3 with basic terminal moieties to the nitroestradiol derivatives. The resultant compounds have been screened against a small panel of cancer cell lines, including ER-positive and ER-negative breast cancer cells, as well as ovarian cancer cells and colon cancer cells. We have also analyzed specified cellular and molecular mechanisms by which certain nitroestradiol derivatives affect these cancer cell lines. Some of these novel nitroestradiol compounds were more potent cytotoxic agents than 4-hydroxytamoxifen (4-HT) when used to treat breast cancer cell lines, and these compounds were also potent cytotoxic agents against ER-negative breast cancer cells. Furthermore, one of these novel nitroestradiol compounds circumvented P-glycoprotein-mediated drug resistance in ovarian cancer cells.

## 2. Results

### 2.1. Synthesis of novel nitroestradiol compounds

Organo-bismuth chemistry is considered an emerging field in synthetic organic chemistry research, and we have previously demonstrated the selective activity of trivalent bismuth nitrate pentahydrate in a number of examples. These experiments resulted in various methods that include nitration of aromatic systems [23–25], Michael reaction [26], protection of carbonyl compounds [27], deprotection of oximes and hydrazones [28], Paal–Knorr synthesis of pyrroles [29], hydrolysis of amide [30], electrophilic substitution of indoles [31,32], synthesis of  $\alpha$ -aminophosphonates [33], Biginelli condensation [34], and selective synthesis of 1,4-dihydropyridines [35]. To determine the best route for the direct nitration of  $\beta$ -estradiol, different metal nitrates were analyzed in combination with various solid supports (Scheme 1). We observed that a mixture of 4- and 2-nitroestradiol was obtained when bismuth nitrate pentahydrate was used as nitrating agent on solid supports, such as florisil, silica gel, molecular sieves 4 Å, montmorillonite KSF clay and alumina in two solvents (benzene and dichloromethane) under refluxing condition using Dean–Stark water separator (Table 1, entries 1,2, 4–8). 2-Nitroestradiol was isolated as the major product (40–50% yield) using the same nitrating agent when molecular sieve was used as solid surface and the reflux was carried out in benzene using Dean–Stark water separator (Table 1, entry 3) or when KSF clay (Table 1, entry 9) or alumina (Table 1, entry 10) was used as solid surface in dichloromethane using Dean–Stark water separator. No reaction was observed when  $\beta$ -estradiol was mixed with solid supports impregnated with bismuth nitrate (Table 1, entries 11–15). Mixture

of the nitro derivatives was obtained under microwave irradiation in solvent free condition (Table 1, entries 16–20). Moderate to excellent yield of both the regioisomers was obtained under reflux in benzene (Table 1, entries 21–25). Taken together, these data indicate that nitration of  $\beta$ -estradiol depends strongly on the nature of the solid support and metal nitrate, with a high to excellent yield of nitroestradiols (70–95%) being obtained when bismuth nitrate is used as nitrating agent impregnated with KSF clay (Table 1).

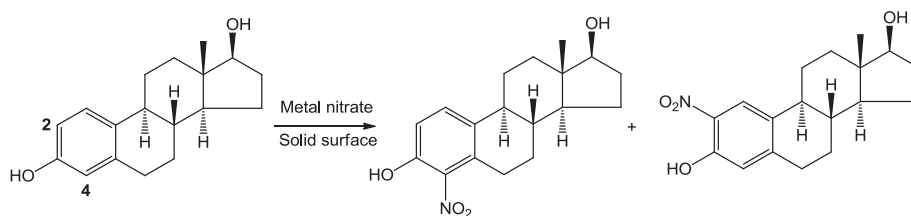
Based on this observation, a comparative study was performed using other metal nitrates, including ammonium cerium (IV) nitrate (CAN), zinc nitrate, calcium nitrate, lanthanum nitrate, sodium nitrate and cupric nitrate, in combination with KSF clay. We found that CAN, lanthanum nitrate and sodium nitrate did not yield nitroestradiols of any kind when used as a metal nitrate in combination with KSF clay (Table 1, entries 26, 29, and 30). Additionally, zinc nitrate and calcium nitrate formed 2-nitroestradiol regioselectively and cupric nitrate formed a mixture of both the isomers in a ratio 1:1 with an overall 60% yield (Table 1, entries 27–28 and 31 respectively).

Upon obtaining 2- or 4-nitroestradiol, we coupled carbon chains with different terminal basic moieties to the phenolic hydroxy group of the nitroestradiol derivatives. This was accomplished by refluxing hydrochloride salt of the corresponding chloride compound in the presence of potassium carbonate in acetone (Scheme 2), and the final products (1–4) were purified by column chromatography. These six novel nitroestradiol derivatives (1–6), including 2- and 4-nitroestradiol, were then analyzed as potential anticancer agents *in vitro*.

### 2.2. Select nitroestradiol compounds decrease viability & block estradiol effects in breast cancer cell lines

Since ERs are a major target for anticancer agents and potential anticancer agents [5], including tamoxifen and estradiol derivatives, we initially analyzed the ability of our novel estradiol derivatives to reduce viability of breast cancer cell lines, including ER-positive (both early and late-stage MCF-7 cells and MD-MBA-453 cells) and ER-negative cells (AU565). Although compounds 1, 5, and 6 did not effectively reduce viability of any of the breast cancer cell lines examined, three compounds (3, 4, and 5) were capable of reducing viability of breast cancer cell lines—compounds 3 and 4 were the most potent cytotoxic agents, reducing viability with an estimated  $IC_{50}$  value of less than 10  $\mu$ M (Table 2). In fact, the estradiol derivatives (3 and 4), as well as a known ER-targeting drug, 4-hydroxytamoxifen (4-HT), reduced the viability of ER-positive and ER-negative cells with similar efficacy, and the toxicity exhibited by 3 was slightly better when compared to that 4-HT, with an  $IC_{50}$  value of approximately 2  $\mu$ M in breast cancer cells (Table 2). These data suggest that selective novel nitroestradiol compounds effectively cause cytotoxicity in breast cancer cells and that this cytotoxicity might occur in an ER-independent manner.

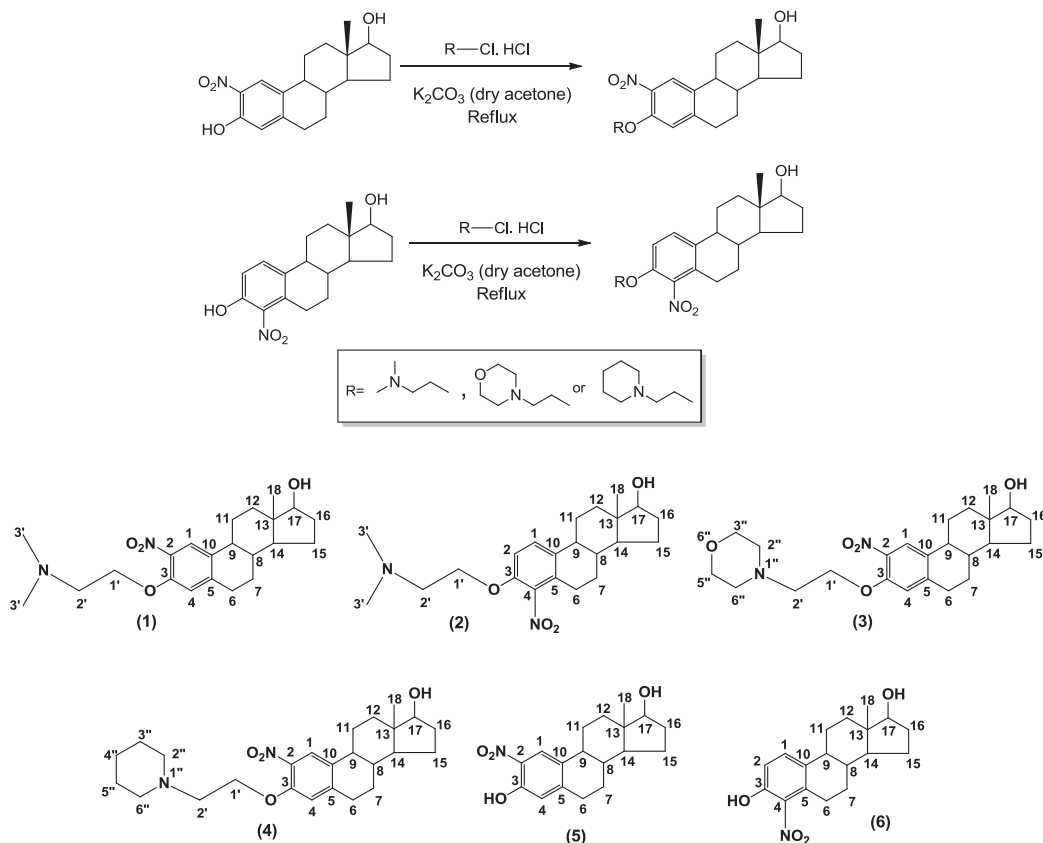
To further examine whether our novel estradiol derivatives could affect breast cancer cell lines in an ER-dependent manner, we compared the ability of compound 3 and 4-HT to inhibit estradiol-



Scheme 1. Reaction whereby  $\beta$ -estradiol is nitrated with metal nitrate on a solid surface.

**Table 1**  
Nitration of  $\beta$ -estradiol with metal nitrate on solid surface via scheme shown in [Scheme 1](#).

Entry	Metal nitrate	Solid surface	Method/Solvent	Product ratio (4-nitro:2-nitro)	Yield (%) <sup>a</sup>
1	Bi(NO <sub>3</sub> ) <sub>3</sub>	Florisil	Dean–Stark/benzene	1:4	50
2	Bi(NO <sub>3</sub> ) <sub>3</sub>	Silica gel	Dean–Stark/benzene	3:1	65
3	Bi(NO <sub>3</sub> ) <sub>3</sub>	Molecular sieves 4 Å	Dean–Stark/benzene	0:1	50
4	Bi(NO <sub>3</sub> ) <sub>3</sub>	KSF clay	Dean–Stark/benzene	1:5	80
5	Bi(NO <sub>3</sub> ) <sub>3</sub>	Alumina	Dean–Stark/benzene	1:3	40
6	Bi(NO <sub>3</sub> ) <sub>3</sub>	Florisil	Dean–Stark/DCM	1:10	20
7	Bi(NO <sub>3</sub> ) <sub>3</sub>	Silica gel	Dean–Stark/DCM	1:4	45
8	Bi(NO <sub>3</sub> ) <sub>3</sub>	Molecular sieves 4 Å	Dean–Stark/DCM	1:6	60
9	Bi(NO <sub>3</sub> ) <sub>3</sub>	KSF clay	Dean–Stark/DCM	0:1	75
10	Bi(NO <sub>3</sub> ) <sub>3</sub>	Alumina	Dean–Stark/DCM	0:1	40
11	Bi(NO <sub>3</sub> ) <sub>3</sub>	Florisil	Dry	No reaction	–
12	Bi(NO <sub>3</sub> ) <sub>3</sub>	Silica gel	Dry	No reaction	–
13	Bi(NO <sub>3</sub> ) <sub>3</sub>	Molecular sieves 4 Å	Dry	No reaction	–
14	Bi(NO <sub>3</sub> ) <sub>3</sub>	KSF clay	Dry	No reaction	–
15	Bi(NO <sub>3</sub> ) <sub>3</sub>	Alumina	Dry	No reaction	–
16	Bi(NO <sub>3</sub> ) <sub>3</sub>	Florisil	Microwave/solvent-free	2:1	35
17	Bi(NO <sub>3</sub> ) <sub>3</sub>	Silica gel	Microwave/solvent-free	2:1	20
18	Bi(NO <sub>3</sub> ) <sub>3</sub>	Molecular sieves 4 Å	Microwave/solvent-free	1; 1	75
19	Bi(NO <sub>3</sub> ) <sub>3</sub>	KSF clay	Microwave/solvent-free	2:1	70
20	Bi(NO <sub>3</sub> ) <sub>3</sub>	Alumina	Microwave/solvent-free	1:1	25
21	Bi(NO <sub>3</sub> ) <sub>3</sub>	Florisil	Reflux/benzene	1:4	60
22	Bi(NO <sub>3</sub> ) <sub>3</sub>	Silica gel	Reflux/benzene	2:1	40
23	Bi(NO <sub>3</sub> ) <sub>3</sub>	Molecular sieves 4 Å	Reflux/benzene	1:6	45
24	Bi(NO <sub>3</sub> ) <sub>3</sub>	KSF clay	Reflux/benzene	1:1	95
25	Bi(NO <sub>3</sub> ) <sub>3</sub>	Alumina	Reflux/benzene	1:1	80
26	CAN	KSF clay	Dean–Stark/benzene	No reaction	–
27	Zn(NO <sub>3</sub> ) <sub>2</sub>	KSF clay	Dean–Stark/benzene	1:5	50
28	Ca(NO <sub>3</sub> ) <sub>2</sub>	KSF clay	Dean–Stark/benzene	1:3	25
29	LaNO <sub>3</sub>	KSF clay	Dean–Stark/benzene	No reaction	–
30	NaNO <sub>3</sub>	KSF clay	Dean–Stark/benzene	No reaction	–
31	Cu(NO <sub>3</sub> ) <sub>2</sub>	KSF clay	Dean–Stark/benzene	1:1	60

<sup>a</sup> Isolated yield.

**Scheme 2.** Reactions and end products in which different side chains with basic terminal moieties were added to nitroestradiols.

**Table 2**

Estimated IC<sub>50</sub> values (μM) for compounds (**1–6**) in breast cancer cell lines. IC<sub>50</sub> values (mean ± standard error) were calculated as described in Materials and Methods.

Compound	Cell lines			
	MDA-MB-453 (ER+)	MCF-7L (ER+)	MCF-7 (ER+)	AU565 (ER-)
<b>4-OH-tamoxifen</b>	5.4 ± 3.8	6.3 ± 5.1	9.2 <sup>a</sup>	6.2 ± 0.6
<b>1</b>	23.9 ± 5.7	28.3 ± 14.4	ND	ND
<b>2</b>	7.1 ± 2.0	11.5 ± 2.5	ND	ND
<b>3</b>	3.5 ± 0.6	2.7 ± 1.2	0.6 ± 0.1	2.3 ± 0.1
<b>4</b>	8.8 ± 3.0	7.7 ± 3.4	4.6 ± 0.3	3.6 ± 0.3
<b>5</b>	14.6 ± 6.4	18.6 ± 14.6	ND	ND
<b>6</b>	>50	>50	ND	ND

<sup>a</sup> IC<sub>50</sub> was generated from one independent experiment.

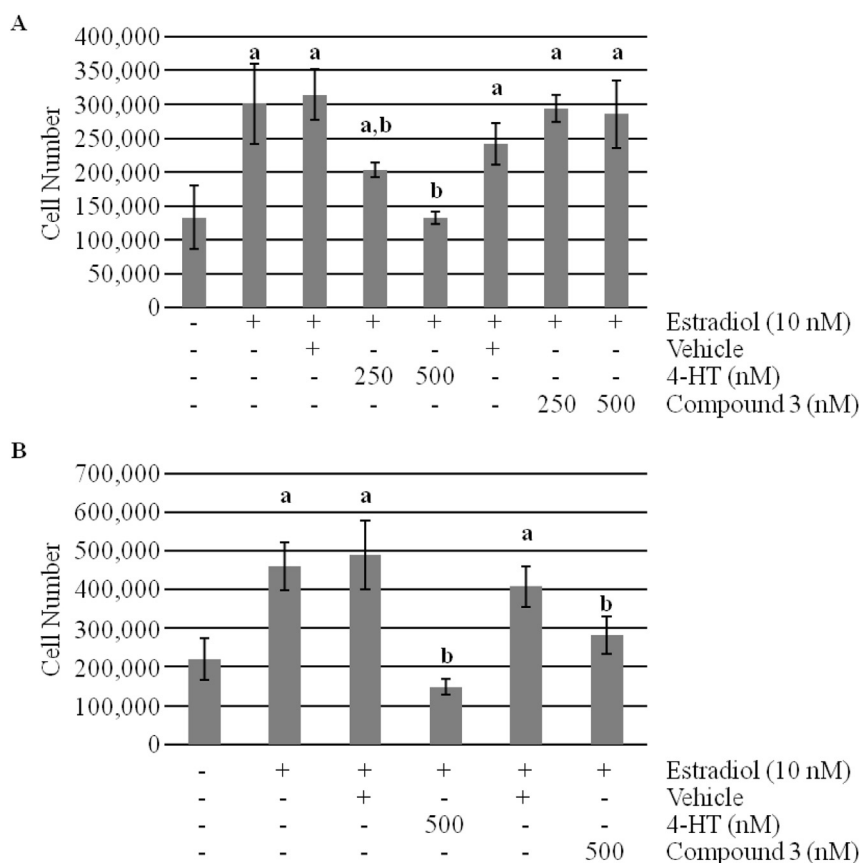
induced cell proliferation of MCF-7 cells when used at sub-lethal doses. After three days or five days of estrogen treatment, the number of Estradiol-treated MCF-7 cells, but not AU565 cells, was increased 2.5- to 3-fold when compared to cells grown in serum free media (Fig. 1A and data not shown). This increase in MCF-7 cell number after 3 days of estradiol exposure was significantly blocked by addition of 4-HT in a dose dependent manner; however, compound **3** had no detectable effect on the increase in cell number in response to estradiol exposure for three days (Fig. 1A). In contrast, both 4-HT and **3** significantly inhibited increased cell numbers in response to estradiol exposure for five days (Fig. 1B), although 4-HT

exhibited a greater inhibition when compared to **3**. Taken together, these data indicate that compound **3** functions, at least in part, as an antagonist of estradiol, albeit less effectively than 4-HT.

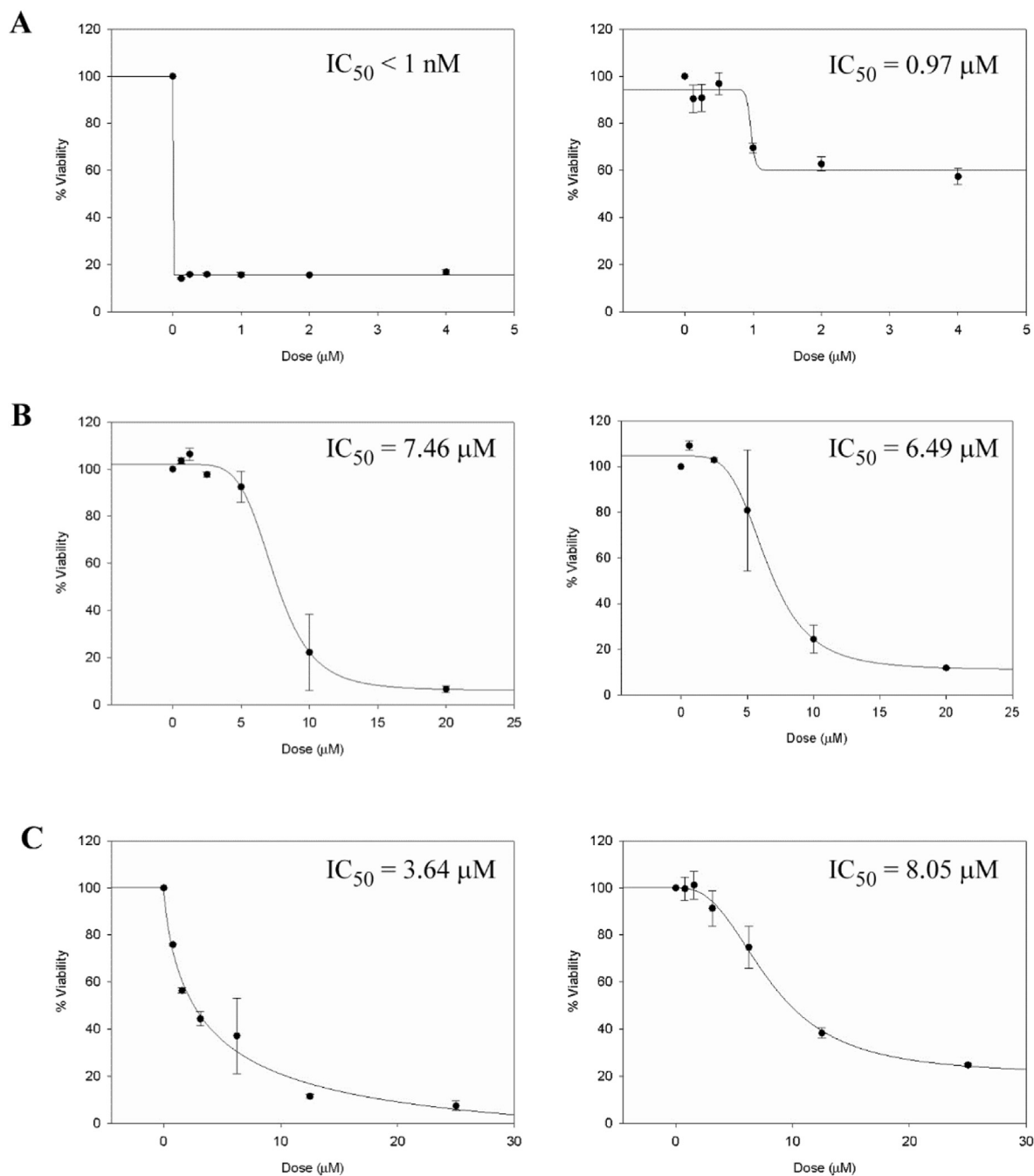
### 2.3. Compound **3** circumvents p-glycoprotein-mediated drug resistance in ovarian carcinoma cells

We also analyzed the ability compound **3** to reduce viability of an ovarian cancer cell line, SKOV3. Similar to the breast cancer cell lines, we found that **3** was a slightly more effective cytotoxic agent against SKOV3 cells when compared with 4-HT, although both **3** and 4-HT were much less potent cytotoxic agents when compared with the microtubule stabilizing agent, Paclitaxel (Fig. 2).

Next, we analyzed the ability compound **3** to reduce viability of a retrovirally transduced SKOV3 cell line overexpressing P-glycoprotein, SKOV3-MDR1-M6/6. These SKOV3-MDR1-M6/6 cells have previously been shown to be resistant to cytotoxicity induced by anticancer drugs, including daunorubicin and paclitaxel, when compared with SKOV3 cells [36,37]. Similarly, we found that SKOV3-MDR1-M6/6 cells were resistant to cytotoxicity in response to paclitaxel treatment when compared with SKOV3 cells, since the IC<sub>50</sub> value for paclitaxel was greater than 1000-fold higher in SKOV3-MDR1-M6/6 cells when compared with SKOV3 cells (Fig. 2A). In addition, paclitaxel was incapable of killing over 45% of SKOV3-MDR1-M6/6 cells, even when used at dosages up to 20 μM (Fig. 2A and data not shown). In contrast, no resistance or only a modest level of resistance was observed for 4-HT or compound **3**, which had IC<sub>50</sub> values equal to or only 2.2-fold higher in SKOV3-



**Fig. 1.** The effect of compound **3** on estradiol-induced cell proliferation in MCF-7 cells. MCF-7 cells were treated with 17-β Estradiol in the absence or presence of sub-lethal doses of 4-HT or compound **3** (dosages are indicated) for 3 days (A) or 5 days (B) as described in Materials and Methods. After treatment, cells were counted and compared statistically using the Mann Whitney U test. A representative experiment is shown, and each data point represents the mean ± standard deviation value of four replicates. <sup>a</sup>Statistically significant ( $p < 0.05$ ) when compared with cells grown in serum free media. <sup>b</sup>Statistically significant ( $p < 0.05$ ) when compared with vehicle-treated cells.



**Fig. 2.** The effect of Paclitaxel, 4-HT, or compound **3** on viability of ovarian cancer cells that overexpress P-glycoprotein. SKOV3 cells (left panels) or SKOV3-MDR1-M6/6 cells (right panels) were treated with increasing concentrations of Paclitaxel (A), 4-HT (B), or compound **3** (C) for 48 h, and then cell viability was analyzed using SRB assay. The dose curve for each compound in each cell line was plotted, and the calculated  $\text{IC}_{50}$  value for each compound is indicated in the top right corner of each graph. A representative experiment is shown ( $n \geq 2$ ).

MDR1-M6/6 when compared to SKOV3 cells, respectively (Fig. 2B and C). These data indicate that compound **3**, as well as 4-HT, bypasses the P-glycoprotein-mediated resistance of SKOV3-MDR1-M6/6 cells.

#### 2.4. Select nitroestradiol compounds decrease viability of multiple mammalian cell lines

Since our novel nitroestradiol derivatives killed both ER-positive and ER-negative breast cancer cell lines with similar efficacy and compound **3** overcame drug resistance in ovarian cancer cells, we

decided to test the effects of these novel compounds on the viability of a small panel of human and mouse cancer cell lines that originated from tissues other than reproductive tissues. These included a colon cancer cell line (HT-29), liver cancer cell lines (HepG2, Hepa1-6, and Hep3B), and a cervical cancer cell line (HeLa). Similar to the cancer cell lines from reproductive tissue, 4-nitroestradiol (**6**) was the least effective in reducing cell viability in this small panel of cell lines as the majority of cell lines had an estimated  $\text{IC}_{50}$  value greater than  $50 \mu\text{M}$  when treated with this compound (Table 3). Also similar to cancer cell lines from reproductive tissues, compounds **2**, **3**, and **4** were the most potent



**Table 3**

Estimated IC<sub>50</sub> values (μM) for compounds (**1–6**) in a small panel of mammalian cell lines. IC<sub>50</sub> values (mean ± standard error) were calculated as described in Materials and Methods.

Compd.	Cell lines				
	HepG2	Hepa1-6	Hep3B	HeLa	HT-29
<b>1</b>	8.2 ± 0.5	7.7 ± 2.0	12.5 <sup>a</sup>	12.2 ± 1.4	11.0 ± 3.5
<b>2</b>	4.9 ± 0.1	3.1 ± 0.1	5.0 ± 0.1	6.3 ± 2.3	4.3 ± 1.7
<b>3</b>	5.9 ± 1.7	0.3 ± 0.1	4.5 ± 4.0	5.4 ± 2.0	0.17 ± 0.05
<b>4</b>	4.9 ± 0.1	3.2 ± 0.3	3.7 ± 1.6	7.6 ± 0.9	3.2 ± 0.2
<b>5</b>	19.0 ± 6.4	10.5 ± 0.1	12.3 ± 3.6	3.6 ± 0.3	6.2 <sup>a</sup>
<b>6</b>	>50	ND <sup>b</sup>	>50	30.2 ± 2.3	28.8 ± 4.8
<b>Cisplatin</b>	7.0 <sup>a</sup>	4.0 <sup>a</sup>	ND	11.7 <sup>a</sup>	16.8 <sup>a</sup>

<sup>a</sup> Single dose curve & IC<sub>50</sub>.

<sup>b</sup> Min > 50% Viability.

cytotoxic compounds when used to treat colon, liver or cervical cancer cells, with an estimated IC<sub>50</sub> value of 8 μM or lower (Table 3).

To determine when compounds **2**, **3**, and **4** were causing cytotoxicity, HepG2 cells were treated with each compound at a concentration of twice the calculated IC<sub>50</sub> value for 24-, 48-, or 72-h time-points. Both **2** and **4** caused cytotoxicity between 24 and 48 h, reducing viability greater than 80% after 48 h (Fig. 3); however, **3** caused a steady reduction in viability, reducing viability greater than 80% only after 72 h (Fig. 3). Treatment of cells with compound **3** only reduced viability approximately 30% after 24 h and 55% after a 48-h treatment (Fig. 3).

### 2.5. Estradiol compounds potentially cause cytotoxicity via induction of apoptosis

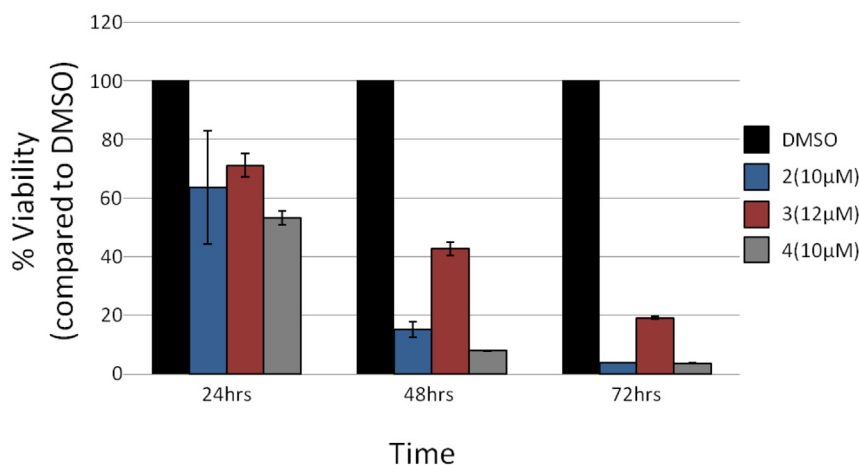
To determine the mechanism by which our novel nitroestradiol derivatives were killing cells, we analyzed the effects of compounds **3** or **4** on plasma membrane blebbing, which is a known indicator of apoptosis, in HeLa cells over the course of 24 h. Both **3** and **4** caused membrane blebbing on the surface of cells, whereas non-treated cells or vehicle-treated cells did not exhibit blebbing on the cell surface (Fig. 4). This blebbing started after 12 h of treatment and continued throughout the 24-h period in which cells were observed, and similar results were observed in HepG2 cells (data not shown). These data suggest that the ability of these novel nitroestradiol derivatives to reduce cell viability occurs through an apoptosis-dependent mechanism.

## 3. Discussion and conclusions

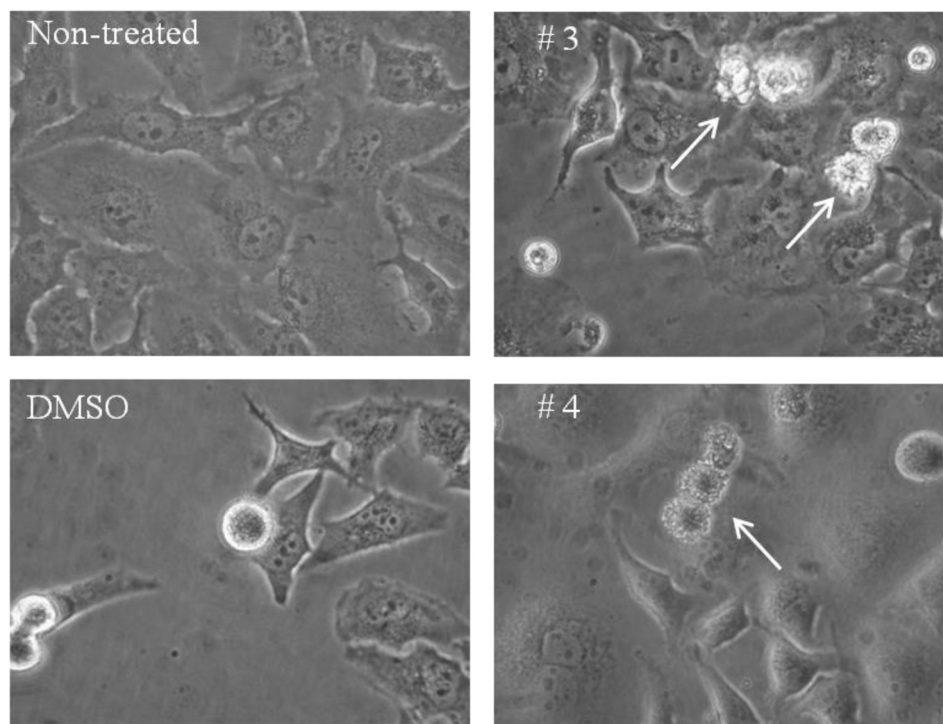
Conventional nitration of aromatic compounds involves nitric acid-sulfuric acid treatment or nitronium tetrafluoroborate, which typically requires a mixture of concentrated or fuming nitric acid with sulfuric acids leading to excessive acid waste streams and added expense. Furthermore, nitration of estrogens has been reported using nitric acid [38,39] or peroxidase/H<sub>2</sub>O<sub>2</sub>/NO<sub>2</sub> [40], and either of these procedures includes the use of hazardous chemicals and utilizes a difficult work-up procedure with low yield. In this study, a total of 31 different reaction conditions were used involving several metal nitrates in combination with various solid supports and techniques to develop a novel, eco-friendly procedure for the nitration of estradiol. Of the 31 reaction conditions tested, we found that bismuth nitrate, when used as a nitrating agent impregnated with KSF clay, could efficiently generate either 2- or 4-nitroestradiol.

We also conjugated three different side chains containing basic moieties to either 2- or 4-nitroestradiol, and we examined the potential anti-cancer effects of these novel agents. 2-nitroestradiol was a more effective cytotoxic agent when compared with 4-nitroestradiol (Tables 2 and 3). These data are similar to a previous study showing that either 2-hydroxyestradiol or 2-methoxyestradiol inhibited cell proliferation and caused cellular apoptosis, whereas 4-hydroxyestradiol or 4-methoxyestradiol had no effect on cell proliferation or apoptosis [41]. Although 2-nitroestradiol wasn't as potent at causing cytotoxicity as previously reported 2-methoxyestradiol [42], the addition a side chain containing either a morpholine or piperidine ring (**3** or **4**, respectively) improved the cytotoxic potency dramatically, making the cytotoxicity of these compounds comparable to 2-methoxyestradiol and, in some cases, better than 4-HT in breast cancer cells as well as cisplatin in colon cancer cells and other cell types [42] (Table 2). Interestingly, the addition of an ethylamine group to 4-nitroestradiol improved its cytotoxicity beyond that of 2-nitroestradiol with the same side chain (Tables 2 and 3). Thus, it would be interesting to conjugate side chains containing either the morpholine or piperidine ring onto 4-nitroestradiol to determine whether the potency could be improved even further.

Our results also indicate that the novel nitroestradiol derivatives **3** and **4**, along with 4-HT, can inhibit estradiol-induced proliferation of ER-positive breast cancer cells, but that these estradiol derivatives can affect toxicity of ER-positive and ER-negative breast



**Fig. 3.** The effect of **2**, **3**, or **4** on HepG2 cell viability over the course of time. HepG2 cells were treated with compounds **2**, **3**, and **4** at 2X IC<sub>50</sub> concentrations for 24, 48, or 72 h, and then cell viability was analyzed using SRB assay. A representative experiment of two independent experiments performed in duplicates is shown. Each data point represents the mean ± standard deviation value.



**Fig. 4.** The effects of **3** or **4** on cell morphology. HeLa cells were treated with the indicated compounds (**3**: 16  $\mu$ M; **4**: 10  $\mu$ M) for 24 h. After treatment, cells were observed using bright-field microscopy and photographed every 3 h. Arrows indicate cells exhibiting membrane blebbing.

cancer cell lines in a similar manner (Table 2). This data supports previous results showing that Tam could inhibit growth of ER-negative AU565 cells [43]. Taken together, these data suggest that, like 4-HT, our novel nitroestradiol derivatives can affect cells by regulation of Estrogen Receptor, but regulation of ER is not likely to be the mechanism by which these agents cause cytotoxicity. This notion is plausible, since Tam has been shown to induce oxidative stress and apoptosis in Jurkat cells, which lack ER expression [44], and another group showed that Tam induced apoptosis via  $\text{Ca}^{2+}$  influx in ER-negative HepG2 cells [45]. In addition, Tam, when used at pharmacological concentrations (above 5  $\mu$ M), was reported to induce cytochrome c release from mitochondria resulting in acute cell death in both ER-positive (MCF-7) and ER-negative (MDA-MB-231) breast cancer cells [46]. Thus, it is possible that our novel nitroestradiol derivatives are causing toxicity through one or several of these mechanisms. In fact, our data indicates that **3** and **4** cause plasma membrane blebbing (Fig. 4), suggesting that these nitroestradiol derivatives, like Tam, are causing apoptosis. One possible ER-independent mechanism by which our novel nitroestradiol derivatives might cause cytotoxicity is by integrating into the plasma membrane of cells, since it is well known that sterols function, at least in part, in this manner [47]. Future studies will focus on elucidating the cellular and molecular mechanisms by which novel estradiol derivatives cause cytotoxicity in cancer cells and in determining the role, if any, that ER plays in cytotoxicity in response to these and other novel estradiol derivatives.

In addition to killing ER-positive and ER-negative breast cancer cell lines, our nitroestradiol compounds, **3** and **4**, also caused cytotoxicity in ovarian and colon cancer cells, as well as cervical and hepatic cancer cell lines. Interestingly, compound **3** was also capable of circumventing the drug resistance in ovarian cancer cells overexpressing P-glycoprotein (Fig. 2), although SKOV3-MDR1-M6/6 cells did display a subtle resistance to **3** when compared with SKOV3 cells. This result is similar to a previous study showing that

SKOV3-MDR1-M6/6 cells exhibited a mild resistance to 2-methoxyestradiol and a robust resistance to paclitaxel [36]. P-glycoprotein (Pgp) is one of nine Multidrug Resistant Proteins (MRPs), and MRPs are expressed in various tissues of the body and capable of transporting numerous physiological substrates or anticancer drugs out of cells [48].

In conclusion, an ecofriendly direct procedure for the nitration of estradiol is reported using KSF clay impregnated bismuth nitrate pentahydrate as nitrating agent. A total of 31 different conditions have been tested to figure out the best condition. In the following step, carbon linkers with diversely substituted basic terminal moieties have been introduced on the oxygen at C-3. The biological evaluation showed that compounds **3** and **4** are cytotoxic for HepG2, Hepa1-6, Hep3B, HeLa and HT-29 cancer cell lines. Interestingly, the compound **3** is more potent than 4-HT (4-hydroxytamoxifen, the well-known anticancer drug) for both ER-positive and ER-negative cells and this compound also circumvented P-gp mediated drug resistance in ovarian cancer cells. Qualitative structure activity relationship (SAR) has also been studied. Thus, it would be interesting to assess whether the nitroestradiol derivatives described here, or other novel nitroestradiol derivatives, circumvented the multidrug resistance mediated by MDR1 and other MDR proteins *in vitro* and *in vivo*. If so, then nitroestradiol derivatives could become chemotherapeutic drugs with clinical usefulness.

## 4. Experimental section

### 4.1. Materials

Dimethylsulfoxide (DMSO; Sigma Aldrich Corp., St. Louis, MO), phosphate-buffered saline (PBS), Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen, Carlsbad, CA), Fetal Bovine Serum (FBS; Invitrogen), McCoy's media (Invitrogen) were purchased. All other



chemicals were purchased from Sigma–Aldrich Corporation (analytical grade). Throughout the project solvents were purchased from Fisher-Scientific. Deionized water was used for the preparation of all aqueous solutions.

Melting points were determined in a Fisher Scientific electrochemical Mel-Temp<sup>®</sup> manual melting point apparatus (Model 1001) equipped with a 300 °C thermometer. FT-IR spectra were registered on a Bruker IFS 55 Equinox FTIR spectrophotometer as KBr discs. <sup>1</sup>H NMR (600 MHz) and <sup>13</sup>C NMR (150 MHz) spectra were obtained at room temperature with Bruker superconducting Ultrashield<sup>™</sup> Plus 600 MHz NMR spectrometer with central field 14.09 T, coil inductance 89.1 H and magnetic energy 1127.2 kJ using CDCl<sub>3</sub> as solvent. Elemental analyses (C, H, N) were conducted using the Perkin–Elmer 2400 series II elemental analyzer, their results were found to be in good agreement (±0.2%) with the calculated values for C, H, N.

## 4.2. Synthesis of the compounds 1–6

### 4.2.1. Nitration of estradiol

A representative procedure (Entry 24, Table 1) for the nitration of estradiol is as follow: Estradiol (1 mmol) and montmorillonite KSF (500 mg) were added to a suspension of bismuth nitrate pentahydrate (1 equiv.) in anhydrous benzene (30 mL). The mixture was refluxed for 2 h and the reaction was monitored by TLC. After completion of the reaction, the mixture was filtered through vacuum and the solid was washed thrice (3 × 10 mL) by dichloromethane and washed with saturated solution of sodium bicarbonate, brine and water (10 mL each) successively. The organic layer was dried over anhydrous sodium sulfate and filtered through cotton. It was then concentrated to afford the crude mixture. The pure products **5** and **6** were isolated by chromatographic column (hexane/ethyl acetate over silica gel).

### 4.2.2. Introduction of carbon linker with basic terminal moiety

A representative procedure leading to the synthesis of compound (**2**) is as follow: 4-Nitroestradiol (1 mmol), N,N-dimethyl-2-chloroethylamine and potassium carbonate (1 equiv.) were mixed in anhydrous acetone (5 mL) and the mixture was refluxed for 4–6 h (monitored by TLC). After completion of the mixture was filtered through vacuum and the solid was washed thrice (3 × 10 mL) by dichloromethane. The solvent was removed by rotavapor and 20 mL of dichloromethane was added to the crude mixture. The organic layer was washed with saturated solution of sodium bicarbonate, brine and water (10 mL each) successively. It was then dried over anhydrous sodium sulfate and filtered through cotton. The organic layer was concentrated through rotavapor again to afford the crude mixture. The pure product (**2**) was isolated by chromatographic column (hexane/ethyl acetate over silica gel (65%)).

## 4.3. Spectroscopic analyses of compounds (1–6)

The physical and spectral data are of the compounds are given below:

### 4.3.1. 3-(2-(Dimethylamino)ethoxy)-13-methyl-2-nitro-7,8,9,11,12,13,14,15,16,17-decahydro-6H-cyclopenta[a]phenanthren-17-ol (**1**)

Brick red solid (71%); mp 142 °C; IR (KBr) 3416, 2916, 1611, 1564, 1517, 1466, 1345, 1263, 1091, 899, 759 cm<sup>-1</sup>; <sup>1</sup>H NMR (600 MHz, d<sub>6</sub>-DMSO) δ 0.66 (s, 3H, H-18), 1.28 (m, 7H, H-7, H-14, 15, 16), 1.59 (m, 1H, H-8), 1.85 (m, 3H, H-9 & H-11), 2.11 (m, 1H, H-17), 2.20 (s, 6H, H-3'), 2.62 (t, J = 5.70 Hz, 2H, H-12), 2.84 (m, 2H, H-2'), 3.52 (t, J = 8.64 Hz, 2H, H-6), 4.17 (m, 2H, H-1'), 4.50 (broad s, 1H, OH), 7.05

(s, 1H, H-4), 7.69 (s, 1H, H-1); <sup>13</sup>C NMR (150 MHz, d<sub>6</sub>-DMSO) δ 11.13 (C-18), 22.66 (C-15), 25.67 (C-11), 26.24 (C-7), 29.22 (C-6), 29.84 (C-16), 36.29 (C-12), 37.88 (C-8), 42.70 (C-9), 42.91 (C-13), 45.55 (C-14), 49.38 (2C-3'), 57.31 (C-2'), 67.82 (C-1'), 79.90 (C-17), 114.92 (C-4), 121.73 (C-1), 132.79 (C-10), 137.24 (C-2), 144.36 (C-5), 149.21 (C-3). Anal. Calcd for C<sub>22</sub>H<sub>32</sub>N<sub>2</sub>O<sub>4</sub>: C, 68.01; H, 8.30; N, 7.21. Found: C, 67.86; H, 8.17; N, 7.16.

### 4.3.2. 3-(2-(Dimethylamino)ethoxy)-13-methyl-4-nitro-7,8,9,11,12,13,14,15,16,17-decahydro-6H-cyclopenta[a]phenanthren-17-ol (**2**)

Reddish yellow solid (65%); mp 133 °C; IR (KBr) 3244, 2947, 1618, 1571, 1529, 1462, 1372, 1290, 1232, 1079, 1017, 814, 790, 669 cm<sup>-1</sup>; <sup>1</sup>H NMR (600 MHz, d<sub>6</sub>-DMSO) δ 0.67 (s, 3H, H-18), 1.26 (m, 6H, H-7, 12, 16), 1.57 (m, 1H, H-14), 1.89 (m, 3H, H-8, H-11), 2.10 (m, 3H, H-9, H-15), 2.18 (s, 6H, H-3'), 2.31 (m, 2H, H-6b, H-17), 2.62 (m, 2H, H-2'), 3.52 (distorted t, J = 8.40 Hz, 1H, H-6a), 4.15 (m, 2H, H-1'), 4.51 (broad s, 1H, OH), 7.13 (d, J = 8.82 Hz, 1H, H-2), 7.44 (d, J = 8.82 Hz, 1H, H-1); <sup>13</sup>C NMR (150 MHz, d<sub>6</sub>-DMSO) δ 11.13 (C-18), 22.63 (C-15), 23.41 (C-11), 25.52 (C-7), 25.88 (C-6), 29.82 (C-16), 36.36 (C-12), 37.55 (C-8), 42.67 (C-9), 43.22 (C-13), 45.38 (C-14), 49.21 (2C-3'), 57.15 (C-2'), 67.55 (C-1'), 79.92 (C-17), 111.51 (C-2), 128.03 (C-5), 131.51 (C-1), 133.68 (C-10), 141.20 (C-4), 146.97 (C-3). Anal. Calcd for C<sub>22</sub>H<sub>32</sub>N<sub>2</sub>O<sub>4</sub>: C, 68.01; H, 8.30; N, 7.21. Found: C, 67.91; H, 8.13; N, 7.27.

### 4.3.3. 13-Methyl-3-(2-morpholinoethoxy)-2-nitro-7,8,9,11,12,13,14,15,16,17-decahydro-6H-cyclopenta[a]phenanthren-17-ol (**3**)

Deep yellow solid (74%); mp 104 °C; IR (KBr) 3240, 2926, 1616, 1541, 1476, 1419, 1245, 1179, 1048, 911, 833, 747 cm<sup>-1</sup>; <sup>1</sup>H NMR (600 MHz, d<sub>6</sub>-DMSO) δ 0.66 (s, 3H, H-18), 1.26 (m, 7H, H-7, 12, 14, 16), 1.47 (distorted t, J = 5.58, 11.04 Hz, 2H, H-15), 1.58 (m, 1H, H-8), 1.84 (m, 3H, H-9, 11), 2.13 (m, 2H, H-6), 2.34 (m, 4H, H-2'', 6''), 2.58 (m, 4H, H-3'', 5''), 2.87 (m, 2H, H-2'), 3.53 (ddd, J = 3.78, 5.82, 9.84 Hz, 1H, H-17), 4.18 (dddd, J = 5.82, 8.94, 13.26, 19.14 Hz, 2H, H-1'), 4.54 (d, J = 4.50 Hz, 1H, OH), 7.07 (s, 1H, H-4), 7.69 (s, 1H, H-1); <sup>13</sup>C NMR (150 MHz, d<sub>6</sub>-DMSO) δ 11.15 (C-18), 22.64 (C-15), 23.91 (C-11), 25.91 (C-7), 26.10 (C-6), 29.85 (C-16), 36.27 (C-12), 38.05 (C-8), 42.66 (C-9), 42.72 (C-13), 46.09 (C-14), 49.30 (C-2'), 49.36 (C-2'', 6''), 53.53 (C-3'', 5''), 66.17 (C-1'), 79.96 (C-17), 117.00 (C-4), 123.05 (C-1), 133.12 (C-10), 135.28 (C-2), 149.59 (C-5), 157.54 (C-3). Anal. Calcd for C<sub>24</sub>H<sub>34</sub>N<sub>2</sub>O<sub>5</sub>: C, 66.95; H, 7.96; N, 6.51. Found: C, 67.06; H, 8.00; N, 6.43.

### 4.3.4. 13-Methyl-2-nitro-3-(2-(piperidin-1-yl)ethoxy)-7,8,9,11,12,13,14,15,16,17-decahydro-6H-cyclopenta[a]phenanthren-17-ol (**4**)

Brownish yellow solid (62%); mp 146 °C; IR (KBr) 3448, 2991, 1617, 1516, 1458, 1350, 1275, 1115, 1068, 944 cm<sup>-1</sup>; <sup>1</sup>H NMR (600 MHz, d<sub>6</sub>-DMSO) δ 0.66 (s, 3H, H-18), 1.23 (m, 10H, H-3'', 5'', 7, 12, 16), 1.46 (m, 3H, H-14, 15), 1.58 (m, 1H, H-8), 2.11 (m, 3H, H-9, 11), 2.25 (distorted d, 2H, H-4''), 2.42 (m, 4H, H-2'', 6''), 2.65 (t, J = 5.82 Hz, 2H, H-6), 2.85 (m, 2H, H-2'), 3.52 (t, J = 8.10 Hz, 1H, H-17), 4.18 (dddd, J = 5.76, 8.64, 12.96, 18.78 Hz, 2H, H-1'), 4.52 (broad s, 1H, OH), 7.07 (s, 1H, H-4), 7.69 (s, 1H, H-1); <sup>13</sup>C NMR (150 MHz, d<sub>6</sub>-DMSO) δ 11.13 (C-18), 22.66 (C-15), 23.81 (C-11), 25.56 (C-3'', 5''), 25.69 (C-7), 26.24 (C-4''), 29.21 (C-6), 29.82 (C-16), 36.28 (C-12), 37.90 (C-8), 42.71 (C-9), 42.92 (C-13), 49.37 (C-14), 54.28 (C-2'), 56.97 (C-2'', 6''), 67.69 (C-1'), 79.91 (C-17), 115.17 (C-4), 121.69 (C-1), 132.84 (C-10), 137.31 (C-2), 144.35 (C-5), 149.26 (C-3). Anal. Calcd for C<sub>25</sub>H<sub>36</sub>N<sub>2</sub>O<sub>4</sub>: C, 70.06; H, 8.47; N, 6.54. Found: C, 69.91; H, 8.39; N, 6.43.

#### 4.3.5. 13-Methyl-2-nitro-7,8,9,11,12,13,14,15,16,17-decahydro-6H-cyclopenta[a]phenanthren-3,17-diol (**5**)

Bright yellow solid (75%); mp 162 °C; IR (KBr) 3363, 2929, 2360, 1631, 1577, 1525, 1480, 1432, 1312, 1266, 1052, 762, 656 cm<sup>-1</sup>; <sup>1</sup>H NMR (600 MHz, d<sub>6</sub>-DMSO) δ 0.66 (s, 3H, H-18), 1.11 (m, 1H, H-14), 1.20 (m, 3H, H-7,15b), 1.26 (m, 2H, H-12), 1.35 (m, 1H, H-16b), 1.57 (m, 1H, H-8), 1.79 (m, 1H, H-15a), 1.87 (m, 2H, H-11), 2.09 (m, 1H, H-16a), 2.24 (m, 1H, H-9), 2.78 (m, 2H, H-6), 3.52 (t, *J* = 8.16 Hz, 1H, H-17), 4.51 (s, 1H, 17-OH), 6.81 (s, 1H, H-4), 7.74 (s, 1H, H-1), 10.52 (s, 1H, 3-OH); <sup>13</sup>C NMR (150 MHz, d<sub>6</sub>-DMSO) δ 11.11 (C-18), 22.67 (C-15), 25.69 (C-11), 26.21 (C-7), 28.88 (C-6), 29.82 (C-16), 36.25 (C-12), 37.87 (C-8), 42.68 (C-9), 42.81 (C-13), 49.42 (C-14), 79.91 (C-17), 118.45 (C-4), 121.41 (C-1), 132.18 (C-2), 133.97 (C-10), 146.06 (C-5), 150.22 (C-3). Anal. Calcd for C<sub>18</sub>H<sub>23</sub>NO<sub>4</sub>: C, 68.12; H, 7.30; N, 4.41. Found: C, 67.99; H, 7.21; N, 4.35.

#### 4.3.6. 13-Methyl-4-nitro-7,8,9,11,12,13,14,15,16,17-decahydro-6H-cyclopenta[a]phenanthren-3,17-diol (**6**)

Brownish yellow solid (48%); mp 224 °C; IR (KBr) 3184, 2925, 1570, 1527, 1497, 1348, 1294, 1046, 794 cm<sup>-1</sup>; <sup>1</sup>H NMR (600 MHz, d<sub>6</sub>-DMSO) δ 0.66 (s, 3H, H-18), 1.15 (m, 1H, H-14), 1.26 (m, 8H, H-7,8,12,15, 16b), 1.86 (m, 2H, H-11), 2.09 (m, 1H, H-16a), 2.25 (m, 1H, H-9), 2.62 (m, 2H, H-6), 3.52 (m, 1H, H-17), 4.50 (broad s, 1H, 17-OH), 6.85 (d, *J* = 8.70 Hz, 1H, H-2), 7.28 (d, *J* = 8.70 Hz, 1H, H-1), 10.49 (s, 1H, 3-OH); <sup>13</sup>C NMR (150 MHz, d<sub>6</sub>-DMSO) δ 11.23 (C-18), 22.76 (C-15), 26.06 (C-11), 26.93 (C-7), 29.13 (C-6), 29.89 (C-16), 36.58 (C-12), 38.68 (C-8), 42.78 (C-9), 43.51 (C-13), 49.52 (C-14), 80.04 (C-17), 114.89 (C-2), 125.96 (C-5), 128.28 (C-1), 130.40 (C-10), 137.08 (C-4), 154.86 (C-3). Anal. Calcd for C<sub>18</sub>H<sub>23</sub>NO<sub>4</sub>: C, 68.12; H, 7.30; N, 4.41. Found: C, 67.96; H, 7.26; N, 4.32.

#### 4.4. Mammalian cell culture

HepG2, Hepa1-6, Hep3B, MCF-7 (less than 100 passages), MCF-7L (over 500 passages), MDA-MB-453 and HeLa cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen, Carlsbad, CA) containing 10% Fetal Bovine Serum (FBS, Invitrogen), and HT-29 cells were cultured in McCoy's media (Invitrogen) containing 10% FBS. SKOV3, and SKOV3-MDR1-M6/6 cells were grown in Basal Medium Eagle (BME) media containing 10% FBS. All cell lines were purchased from American Type Culture Collection (ATCC, Manassas, VA) except MCF-7L, MDA-MB-453, SKOV3, and SKOV3-MDR1-M6/6. MCF-7L and MDA-MB-453 cells were provided by Dr. R.K. Dearth, while SKOV3 and SKOV3-MDR1-M6/6 cells were provided by Dr. Susan Mooberry (UTHSCSA). All cells were incubated at 37 °C with 5% CO<sub>2</sub>.

#### 4.5. Mammalian cell viability assays

Cells were plated (5000 cells/well) onto a 96-well dish and incubated overnight at 37 °C. The following day, cells were treated with increasing dosages (0.1 μM–100 μM) of each estradiol derivative, which had been dissolved in DMSO. The DMSO concentration of treatments was limited to 0.25%, and cells were treated with DMSO alone (0.25%) or 10 μM cisplatin as negative and positive controls for cytotoxicity, respectively. For breast cancer cell lines, estradiol derivatives were compared with 4-hydroxytamoxifen (4-HT). After 48 h, cells were fixed and cell viability was analyzed using the Sulforhodamine B colorimetric assay as described previously [49]. Absorbance of SRB was measured utilizing a SpectraMaxM5 plate reader and absorbance values were normalized to non-treated cells. Normalized cell viabilities, with increasing drug doses, were plotted on a 4-parameter logistical curve, and the IC<sub>50</sub> of each compound in each cell line was calculated using SigmaPlot

software (Systat Software, Inc.). Each compound was used in two independent cell viability assays. The mean IC<sub>50</sub>, with the corresponding standard deviation, of the two independent treatments was then calculated. For time-courses, viability was assessed after treating HepG2 cells (5,000 cells/well) with compounds at twice the IC<sub>50</sub> concentrations for 24, 48, and 72 h. After treatment, cells were fixed and cell viability was analyzed using SRB assay as described above.

#### 4.6. Estradiol-induced cell proliferation

MCF-7 cells were plated onto 12-well dishes at a density of 1.0 × 10<sup>5</sup> cells/well. The following day, cells were placed into phenol red-free, serum-free Improved MEM (IMEM: Mediatech, Manassas, VA) containing 10-mM HEPES, 1-μg/mL apotransferrin, and 1-μg/mL fibronectin for 24 h. Cells were then treated with 10-nM 17β-Estradiol (E<sub>2</sub>) in the absence or presence of 4-HT or **3** (compounds were used at either 250 nM or 500 nM, respectively). Media and treatments were changed and repeated, respectively, every 48 h, and cells were counted on Day 3 and Day 5 of treatments using a cellometer Vision automated cell counter (Nexcelom Bioscience, Lawrence, MA). Both E<sub>2</sub> and 4-HT were dissolved in ethanol (EtOH), and **3** was dissolved using DMSO. Cells were treated with the vehicle for 4-HT (EtOH) or the vehicle for **3** (DMSO) in combination with 10 nM E<sub>2</sub> and neither vehicle affected E<sub>2</sub>-induced cell proliferation. Statistical analyses were performed using SigmaPlot software (Systat Software, Inc.), and *P* values were calculated using a Mann–Whitney *U* test.

#### 4.7. Membrane blebbing

To detect membrane blebbing, HeLa cells were plated onto 6-well dishes at a density of 1.5–2.0 × 10<sup>5</sup> cells/well. Cells were then treated with the compound **3** (16 μM) or **4** (10 μM) and visualized every 3 h using an Olympus CKX31 culture microscope (Olympus Corp., Tokyo, Japan). Images were captured using a Nikon Coolpix E995 eyepiece camera (Nikon, Japan).

#### Conflict of interest

The authors declare no competing financial interest.

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#### Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.ejmech.2014.06.010>.

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