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Impact of estradiol structural modifications (18-methyl and/or 17-hydroxy inversion of configuration) on the *in vitro* and *in vivo* estrogenic activity

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ABSTRACT

It is well recognized that the majority of breast cancers are initially hormone-dependent and that 17β-estradiol (17β-E2) plays a crucial role in their development and progression. For this reason, using a compound able to block a specific enzyme involved in the last steps of the biosynthesis of 17β-E2 remains a rational way to treat estrogen-dependent diseases such as breast cancer. The present study describes the biological *in vitro* and *in vivo* evaluation of a structural modification (inversion of C18-methyl group at position 13 from β to α face) of 17β-E2 (**1**) and 17α-estradiol (17α-E2; **2**). The two epimers 18-epi-17β-E2 (**3**) and 18-epi-17α-E2 (**4**) were obtained in two chemical steps by inversion of the C18-methyl of estrone using 1,2-phenylendiamine in refluxing acetic acid and reduction of ketone at position C17 with $LiAlH_4$. The new E2 isomers were tested on estrogen-sensitive cell lines (MCF-7 and T-47D), on estrogen-sensitive tissues (uterus and vagina of mice) and on estrogen receptor (ER) to determine their estrogenic potency relatively to natural estrogen 17β-E2 (**1**). The results show that 18-epi-17β-E2 (**3**) possesses the lower affinity for ER (RBA = 1.2%), the lower estrogenicity on estrogen-sensitive cells (1000 folds less estrogenic than 17β-E2 in MCF-7) and no uterotrophic (estrogenic) activity when tested on mice. In fact, we observed the following order of estrogenicity: 18-epi-17β-E2 (**3**) < 18-epi-17α-E2 (**4**) < 17α-E2 (**2**) ≪ 17β-E2 (**1**). These results suggest that the inversion of C18-methyl of natural 17β-E2 scaffold could be a useful strategy to decrease the estrogenicity of E2 derivatives used as enzyme inhibitors in the context of a treatment of estrogen-dependent diseases.

1. Introduction

Breast cancer is the most frequently diagnosed cancer in women, excluding cancers of the skin. For USA, it was estimated in 2010 that 207,090 women would be diagnosed with breast cancer while 39,840 women were dying from the disease [1]. Therefore, improved strategies not only to treat but also to prevent breast cancer are necessary to achieve control of the disease and to increase survival. Most of breast cancers are initially hormone-dependent diseases, where the hormone 17β-estradiol (17β-E2) (Fig. 1) plays a crucial role in their development and progression [2]. This represents a unique feature of the disease that can be manipulated to effectively control growth and/or prevent tumor development [3]. Accordingly, in this case it is possible to use hormone therapy as an adjuvant to classical treatments such as surgery, radiotherapy and chemotherapy. Consequently, using an antiestrogen to antagonize the estrogen receptor (ER) or using a compound blocking the production of estrogens by inhibiting a specific steroidogenic enzyme, such as aromatase, 17β-hydroxysteroid dehydrogenase type 1 (17β-HSD1) or steroid sulfatase (STS), remains a rational way for the treatment of estrogen-dependent (ER^+) breast cancers [4].

One of the therapeutic approaches for estrogen-dependent diseases is the inhibition of 17β-HSD1, which is involved in the last enzymatic step in the formation of the potent estrogen 17β-E2 (**1**) from estrone (E1) [5–14]. However, there is no inhibitor of 17β-HSD1 currently under clinical investigation although the development of 17β-HSD1 inhibitors started about forty years ago [6]. One of the main problems encountered in their development is that they must be devoid of estrogenic activity. There are two strategies to obtain non-estrogenic inhibitors of 17β-HSD1, one is the synthesis of non-steroidal inhibitors and the other is to modify the E2 nucleus of known steroidal inhibitors to remove their residual estrogenic effect. As examples of modifications, a 2-methoxy or a 2-ethyl group added on the E1 or 17β-E2 (**1**) nucleus reduces the ER-binding affinity and consequently reduces the estrogenicity [14–18]. Another example of modifications is the inversion of 17β-OH of 17β-E2 (**1**) to generate 17α-E2 (**2**), which was considered for quite some time as a hormonally inactive isomer [19,20]. In fact, 17α-E2 (**2**) is the predominant estrogen in some ungulates

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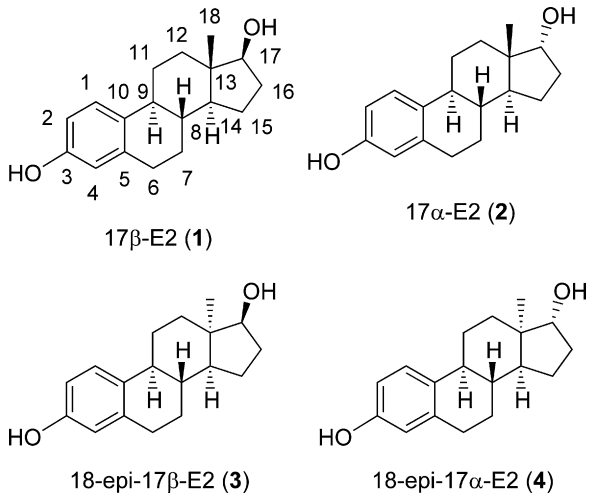

Fig. 1. 2D-Representations of chemical structures of the four estradiol (E2) isomers (compounds **1**–**4**). The carbon numbers are reported only for compound **1** but are the same for compounds **2**–**4**.

and rodents [19,21] and it has only been found under some specific circumstances in the urine and serum of humans [22]. Nevertheless, it has been reported that 17α-E2 (**2**) has non-genomic (smooth muscle relaxation) and genomic (light estrogenic activity) effects, but the latter are lower than those mediated by 17β-E2 (**1**) [20,23]. Administration of 17α-E2 (**2**) to menopausal women and patients on GnRH therapy resulted in a reduction of hot flashes [19,24]. Additionally, 17α-E2 (**2**) is a protecting agent in a wide variety of cell types, including primary neurons, from a diverse array of stressors, including amyloid toxicity, oxidative stress and mitochondrial inhibition, among others [25–29].

It was already known that the 18-methyl group of 17β-E2 (**1**) is not a significant contributor to the receptor-binding affinity, indicating no productive van der Waals contact with the receptor at this site [30], but changing its orientation from the 13-beta face (natural R configuration) to the 13-alpha face (unnatural S configuration) greatly modifies the shape of the C- and D- rings of E2 backbones. Compounds **3** and **4** (Fig. 1), the 13α-methyl (18-epi) isomers of **1** and **2**, were indeed reported to be less neuroprotective and to bind ERα and ERβ less efficiently [27], but their estrogenic properties remain to be further studied. Our interest for using a steroid nucleus with the 18-methyl inversed configuration (18-epi) would be to design new potential non-estrogenic inhibitors of steroidogenic enzymes, especially 17β-HSD1. Here we report the chemical synthesis of two E2 isomers with the 18-methyl group inversed, 18-epi-17β-E2 (**3**) and 18-epi-17α-E2 (**4**), and compare their estrogenic activity to natural 17β-E2 (**1**) and 17α-E2 (**2**) in representative *in vitro* and *in vivo* assays.

2. Experimental

2.1. Steroids

[2,4,6,7 ^{3}H]-17β-estradiol was obtained from American Radiolabeled Chemicals (St. Louis, MO, USA). Natural estradiol (17β-E2; **1**) and the corresponding 17α-isomer (17α-E2; **2**) were purchased from Sigma–Aldrich Canada Ltd (Oakville, ON, Canada) whereas 18-epi-17β-E2 (**3**) and 18-epi-17α-E2 (**4**) were prepared in our laboratory from (13α)-3-hydroxyestra-1(10),2,4-trien-17-one (18-epi-estrone) [31] as reported in Section 2.2 (Fig. 2). The purity of compounds **1**–**4** was determined by high performance liquid chromatography (HPLC) with a Waters' apparatus (Waters Associates Milford, MA, USA) using a Waters 996 Photodiode array detector (207 nm), a phenyl/hexyl-RP column (75 mm × 4.6 mm id, 3 μm) from Phenomenex (Torrance, CA, USA) and a linear solvent gradient from 60:40 to 95:5 of methanol:water.

2.2. Chemical synthesis of 18-epi-17β-E2 (3) and 18-epi-17α-E2 (4)

To a solution of 18-epi-estrone [31] (125 mg, 0.46 mmol) in anhydrous tetrahydrofuran (THF) (5 mL) was added $LiAlH_4$ (2.3 mL of 1.0 M solution in THF) at room temperature. The solution was stirred for 90 min under an argon atmosphere. The resulting solution was then poured into a saturated Rochelle salt solution (50 mL) and then extracted two times with ethyl acetate (EtOAc). The organic phase was washed with brine, dried with sodium sulphate, filtered and evaporated under reduced pressure to give 120 mg of a mixture of alcohols. Purification with a Biotage flash chromatography system (Uppsala, Sweden) using a solvent gradient from EtOAc/Hexanes 1:9 to 3:7 and a silica gel column (KP-Sil, 60A) gave two fractions. The first one contains the 18-epi-17β-E2 (**3**) (35 mg, 28% yield) with HPLC purity of 99.7%. The other fraction (33 mg, 27% yield) contains the compound 18-epi-17α-E2 (**4**) with HPLC purity of 96.5% and 2.8% of undesired 17β-E2 (**1**). To ensure the absence of 17β-E2, this fraction was recrystallized in acetonitrile (1%, w/v) to give 17 mg of compound **4** with an HPLC purity of 99.7%.

(13α,17β)-estra-1(10),2,4-triene-3,17-diol (**3**): HPLC purity of 99.7%, RT = 9.2 min (gradient from 60:40 to 95:5 of methanol:water, phenyl/hexyl-RP column (75 mm × 4.6 mm id, 3 μm; Phenomenex). LRMS for $C_{18}H_{26}O_2Na$ $[M+Na]^+$: 295.4 m/z or for $C_{18}H_{25}O_2$ $(M-H)^-$: 271.4 m/z. ^{1}H NMR-400 MHz (CD_3OD): 0.96 (s, 18-CH_3), 1.10–2.30 (residual CH and CH_2), 2.72 (m, 6-CH_2), 3.76 (dd, J_1 = 4.0 Hz and J_2 = 6.0 Hz, 17α-CH), 6.46 (d, J = 2.3 Hz, 4-CH), 6.55 (dd, J_1 = 2.4 Hz and J_2 = 8.4 Hz, 2-CH), 7.07 (d, J = 8.5 Hz, 1-CH). ^{13}C NMR-100 MHz (CD_3OD): 26.1 (C15), 28.7 (C11), 28.9 (C7), 29.0 (C18), 30.1 (C6), 30.9 (C12), 31.9 (C16), 40.0 (C9), 42.0 (C8), 44.0 (C13), 51.5 (C14), 82.6 (C17), 112.6 (C2), 114.2 (C4), 127.0 (C1), 132.2 (C10), 137.7 (C5), 154.2 (C3). NOESY experiment [32] showed correlations between 14α-CH and 18-CH_3 as well as 18-CH_3 and 17-CH, thus demonstrating the 18-epi-CH_3 and 17α-CH configurations of **3** (18-epi-17β-E2).

(13α,17α)-estra-1(10),2,4-triene-3,17-diol (**4**): HPLC purity of 99.7%, RT = 7.9 min (gradient from 60:40 to 95:5 of methanol:water, phenyl/hexyl-RP column (75 mm × 4.6 mm id, 3 μm; Phenomenex). LRMS for $C_{18}H_{26}O_2Na$ $[M+Na]^+$: 295.4 m/z or for $C_{18}H_{25}O_2$ $[M-H]^-$: 271.3 m/z. ^{1}H NMR-400 MHz (CD_3OD): 0.92 (s, 18-CH_3), 0.95–2.30 (residual CH and CH_2), 2.72 (m, 6-CH_2), 4.19 (t, J = 8.5 Hz, 17β-CH), 6.50 (d, J = 2.6 Hz, 4-CH), 6.57 (dd, J_1 = 2.6 Hz, J_2 = 8.4 Hz, 2-CH), 7.12 (d, J = 8.5 Hz, 1-CH). ^{13}C NMR-100 MHz (CD_3OD): 22.0 (C18), 23.5 (C15), 26.5 (C11), 28.5 (C7), 28.7 (C16), 30.1 (C6), 32.8 (C12), 42.3 (C9), 42.7 (C8), 43.2 (C13), 50.3 (C14), 73.1 (C17), 112.5 (C2), 114.5 (C4), 126.4 (C1), 131.1 (C10), 137.8 (C5), 154.5 (C3). NOESY experiment [32] showed a correlation between 14α-CH and 18-CH_3, but no correlation between 18-CH_3 and 17-CH, thus demonstrating the 18-epi-CH_3 and 17β-CH configurations of **4** (18-epi-17α-E2).

2.3. In vitro estrogenic activity

2.3.1. Cell culture maintenance

Human breast cancer cell lines (T-47D, MCF-7 and BT-20) were obtained from the American Type Culture Collection (ATCC) and maintained in culture flasks (75 cm^3 growth area) at 37 °C under 5% CO_2 humidified atmosphere. The T-47D cells were grown in RPMI medium supplemented with 10% (v/v) fetal bovine serum (FBS), L-glutamine (2 nM), penicillin (100 IU/mL), streptomycin (100 μg/mL) and 17β-E2 (1 nM). The MCF-7 cells were propagated in Dubelcco's Modified Eagle's Medium nutrient mixture F-12

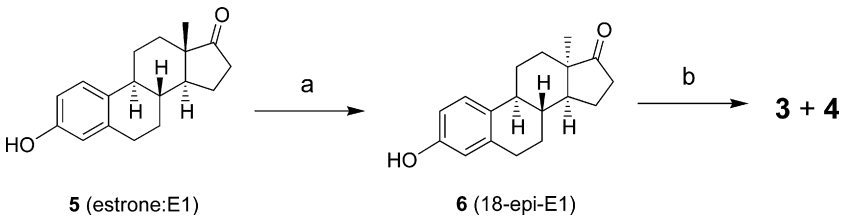

Fig. 2. Chemical synthesis of compounds **3** (18-epi-17β-E2) and **4** (18-epi-17α-E2) from estrone (E1). Reagents and conditions: (a) 1,2-phenylendiamine, acetic acid, reflux, 5 h; (b) $LiAlH_4$, tetrahydrofuran, room temperature.

Ham (DMEM-F12) medium supplemented with 5% FBS, glutamine (2 nM), penicillin (100 IU/mL), streptomycin (100 μg/mL) and 17β-E2 (1 nM). BT-20 cells were grown in minimal essential medium (MEM) supplemented with 10% (v/v) FBS, glutamine (2 nM), penicillin (100 IU/mL) and streptomycin (100 μg/mL).

2.3.2. Cell culture assay

The cells from each breast cancer cell line were seeded into 96-well plates (3000 cells per well). The cells were suspended in the appropriate culture medium reported above, except that FBS was replaced by 5% (v/v) FBS treated with dextran-coated charcoal to remove the endogenous steroids and the medium was supplemented with insulin (50 ng/mL). After 48 h of deprivation, the cells were incubated for 7 days at 37 °C in presence of 17β-E2 (**1**), 17α-E2 (**2**), 18-epi-17β-E2 (**3**) and 18-epi-17α-E2 (**4**) at different concentrations in freshly changed medium. The effects of the drugs on the growth of three different cell lines (MCF-7, T-47D and BT-20) were determined by using 20 μL of 3-(4,5-dimethylthiazol-2-y1)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) reagent (Owen's reagent, Cell Titer 96®, Aqueous One Solution, Promega, USA). MTS was added to each well and the reaction was stopped after 4 h. The reagent is converted to water-soluble colored formazan by dehydrogenase enzymes present in metabolically active cells. The ability of cells to transform MTS is indicative of the degree of mitochondrial/cellular respiration within those cells. Subsequently, the absorbance was recorded at 490 nm with a 96-well plate reader (Molecular Devices, Sunnyvale, CA, USA). The conversion of MTS by untreated cells at the end of the cultured period was set at 100%. Results shown are representative of two separate experiments in triplicate.

2.4. Estrogen receptor (ER) binding

2.4.1. Tissue preparation of ER

Female Sprague-Dawley rats, weighing 200–300 g were obtained from Charles-River (St. Constant, QC, Canada). The rats were gonadectomized under general anesthesia (Isoflurane) and killed by cervical dislocation 24 h later. The uteri were rapidly removed, dissected free from adhering tissue and frozen on dry ice and kept at −80 °C before their use. All subsequent steps needed for the ER preparation were performed at −4 °C. Uteri were homogenized in 10 volume (w/v) of buffer A (25 mM Tris–HCl, 1.5 mM EDTA disodium salt, 10 mM α-monothioglycerol, 10% glycerol, and 10 mM sodium molybdate, pH 7.4), using a Polytron PT-10 homogenizer from Brinkman Instruments (Mississauga, ON, Canada) at a setting of 5 for three periods of 10 s, with intervals of 10 s for cooling. The homogenate was then centrifuged at 105,000 × *g* for 60 min in a Beckman L5-65 ultracentrifuge (Fullerton, CA, USA).

2.4.2. ER binding assay

Estrogen binding was measured using the dextran-coated charcoal adsorption technique as previously described [33,34]. The radioactive 17β-estradiol ([^{3}H]-17β-E2) solubilized in ethanol was diluted into buffer A reported above. Aliquots of uterine cytosol preparation (0.1 mL) were incubated with 5 nM of [^{3}H]-17β-E2 (approximately 200,000 cpm, 0.1 mL) (New England Nuclear-PerkinElmer, Woodbridge, ON, Canada) in the presence or absence of the indicated concentrations of compounds **1**–**4** (0.1 mL, prepared in buffer A containing 10% of ethanol) for 3 h at room temperature. Unbound steroids were then separated by incubation for 15 min at room temperature with 0.3 mL 0.5% Norit-A and 0.005% Dextran T-70 in buffer B (1.5 mM EDTA disodium salt, 10 mM α-monothioglycerol, and 10 mM Tris–HCl, pH 7.4) and centrifuged at 3000 × *g* for 15 min. Aliquots of the supernatant (0.3 mL) were removed for radioactivity measurement. After the addition of 10 mL of Formula-989 scintillation liquid (New England Nuclear-PerkinElmer, Woodbridge, ON, Canada), the radioactivity was measured in a Beckman counter at a counting efficiency of 62%. The concentration of tested compound that inhibited 50% (IC_{50}) of the binding of labeled 17β-E2 was determined from the dose response curves and the relative binding affinity (RBA) was calculated as $IC_{50([^3H]\text{-}17\beta\text{-}E2)}/IC_{50(\text{tested compound})} \times 100$.

2.5. In vivo estrogenic activity (uterotrophic assay)

Female BALB/c mice (42–53 days) weighing 18 g were obtained from Charles River (St. Constant, QC, Canada) and housed four to five per cage in temperature (22 ± 3 °C) and light (12 h/day, light on at 7h15) controlled environment. The mice were fed with rodent chow and tap water *ad libitum*. The animals were ovariectomized (OVX) under isoflurane-anesthesia via bilateral flank incisions and randomly assigned to groups (5 animals by group). Mice in the OVX control group received the vehicle alone (8% ethanol–92% methylcellulose (0.4% aqueous)) during the 7-day period. The possible estrogenic activity of tested compounds was evaluated after their administration by subcutaneous (s.c.) injection (1, 10 and 100 μg/kg, s.c., twice daily (BID)) as a suspension in 8% ethanol–92% methylcellulose (0.4% aqueous) to OVX female mice for 7 days. On day 8, the mice were sacrificed by cervical dislocation. The uteri and vagina were rapidly removed, freed from fat and connective tissue and weighed. Results are the means ± SEM of 5 mice per group.

2.6. Statistical analysis

Data are expressed as the means ± SEM, and statistical significance was determined according to the multiple range test of Duncan–Kramer [35]. *P* values which were less than 0.05 were considered as statistically significant.

3. Results and discussion

3.1. Cell proliferation assay

3.1.1. ER$^+$ cell lines

The proliferative activity of compounds **1**–**4** was evaluated on human breast cancer (ER$^+$) cell lines MCF-7 and T-47D. We chose these cell lines because they express the ER, predominantly ERα, and they proliferate in presence of estrogenic compounds [36].

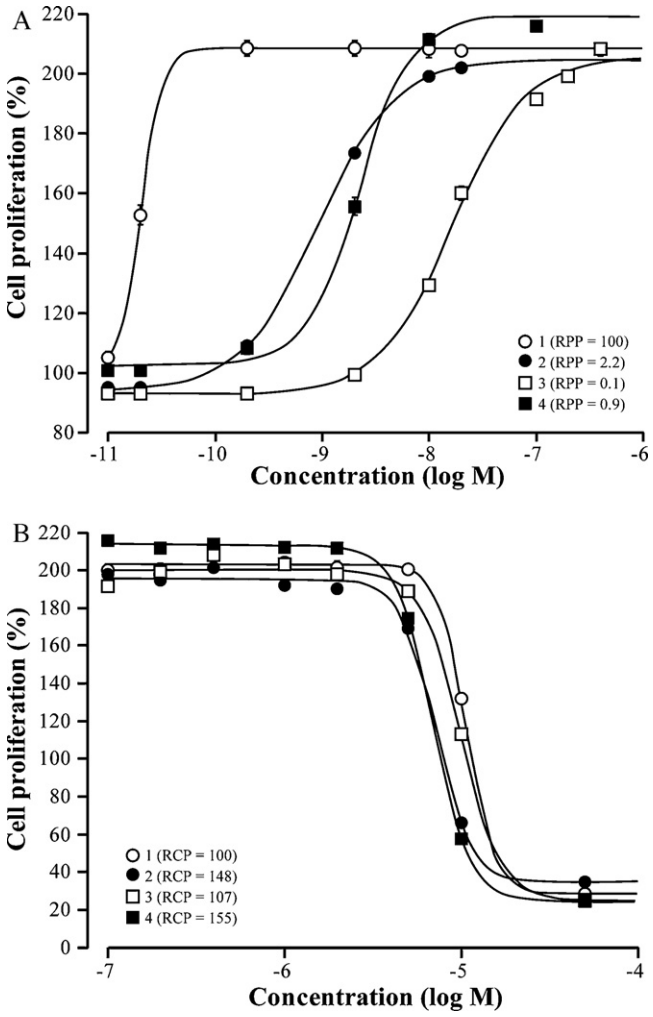

Fig. 3. Effect of compounds **1**–**4** on MCF-7 (ER^+) cells growth at different concentrations. (A) Proliferative (estrogenic) effect at concentrations <1 μM. (B) Cytotoxic effect at concentrations >1 μM. The cell proliferation without product was fixed at 100%. The relative proliferative potency (RPP) or relative cytotoxic potency (RCP) were calculated as $EC_{50\,(17\beta\text{-E2})}/EC_{50\,(\text{compound } \mathbf{1}, \mathbf{2}, \mathbf{3} \text{ or } \mathbf{4})} \times 100$. Estradiol isomers are 17β-E2 (**1**), 17α-E2 (**2**), 18-epi-17β-E2 (**3**) and 18-epi-17α-E2 (**4**).

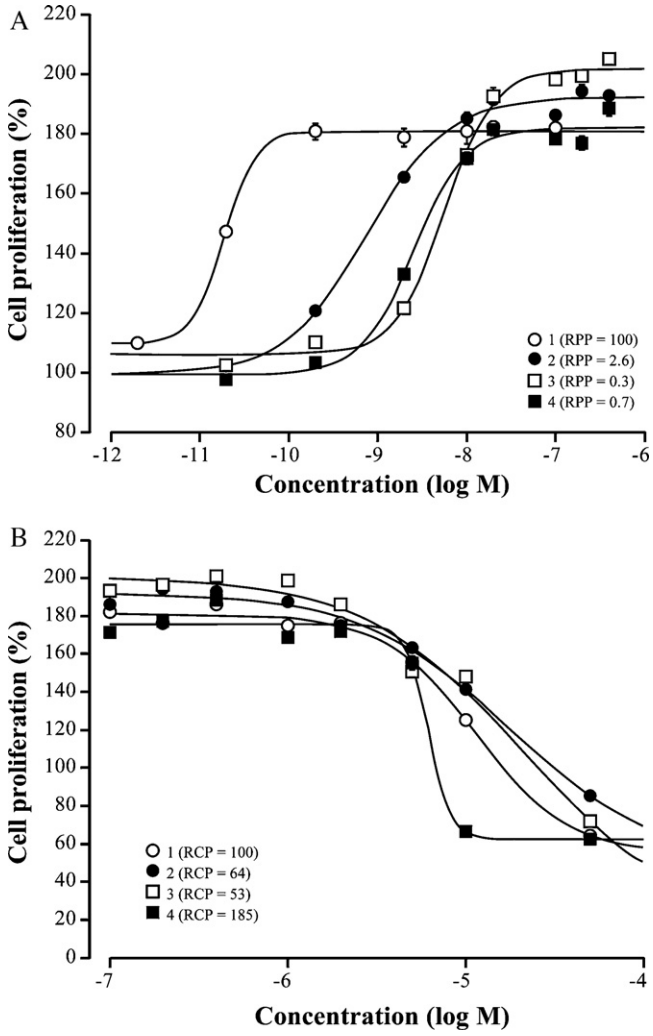

Fig. 4. Effect of compounds **1**–**4** on T-47D (ER^+) cells growth at different concentrations. (A) Proliferative (estrogenic) effect at concentrations <1 μM. (B) Cytotoxic effect at concentrations >1 μM. The cell proliferation without product was fixed at 100%. The relative proliferative potency (RPP) or relative cytotoxic potency (RCP) was calculated as $EC_{50\,(17\beta\text{-E2})}/EC_{50\,(\text{compound } \mathbf{1}, \mathbf{2}, \mathbf{3} \text{ or } \mathbf{4})} \times 100$. Estradiol isomers are 17β-E2 (**1**), 17α-E2 (**2**), 18-epi-17β-E2 (**3**) and 18-epi-17α-E2 (**4**).

Therefore, they are good *in vitro* models to evaluate the effect of the structural modifications of the E2 nucleus on the ER. The assay was performed at concentrations ranging from 0.01 nM to 0.5 μM for each compound and the results were expressed as the percentage of cell proliferation (Figs. 3A and 4A). The cell proliferation without compounds **1**–**4** was fixed as 100% (control). The four E2 isomers clearly modulated the proliferation of MCF-7 and T-47D cells, which are both sensitive to estrogens. However, there are some differences in the pattern of activity according to the range of concentrations and two kinds of proliferation effects, estrogenic and cytotoxic, were observed at low and high concentrations, respectively. At lower concentrations (0.01–5 μM), all tested compounds induced cell growth in different degrees until to reach a plateau (approximately at 200% of cellular proliferation). As expected, 17β-E2 (**1**) was the most estrogenic compound of the four E2-isomers in MCF-7 cells. Compound **2**, with the OH in position 17α, was 45 folds less potent than natural 17β-E2 (**1**). Compound **3**, with an inversion of 18-methyl in position 13 of 17β-E2, was 1000-fold less estrogenic than 17β-E2 (**1**). Finally, with two changes in the structure of 17β-E2 nucleus, an inversion of both 17-OH and 18-methyl groups, compound **4** was surprisingly only 111-fold weaker estrogenic than 17β-E2 (**1**). The same tendency was observed in T-47D cells, compounds **2**, **3** and **4** were 38, 333 and143-folds less estrogenic than compound **1**, respectively. In summary, the four E2 isomers induced estrogen-sensitive (ER^+) cell proliferation in the following order: **1** (17β-E2) ≫ **2** (17α-E2) > **4** (18-epi-17α-E2) > **3** (18-epi-17β-E2). After exposure of ER^+ cells at high concentrations (over 5 μM) of compounds **1**–**4**, an important cytotoxic effect in terms of cell growth inhibition was observed in both cell lines. Thus, compounds **2** and **4** seem to be more cytotoxic than **1** and **3** in MCF-7 cells (Fig. 3B) whereas compound **4** is more cytotoxic in T-47D cells (Fig. 4B). The results obtained from these cell proliferation experiments assessed the role of 18-methyl group orientation (β or α) on the estrogenic activity and cytotoxicity of natural potent estrogens 17β-E2 (**1**) and 17α-E2 (**2**).

3.1.2. ER^- cell line

The BT-20 cells are negative for estrogen receptor (ER^-), but do express an estrogen receptor mRNA that has deletion of exon 5 [37]. We decided to test compounds **1**–**4** in this cell line to demonstrate that the effect observed in ER^+ cell lines was due to the action on the estrogen receptor. When we tested compounds **1**–**4** on BT-20 (ER^-) cells, no proliferative effect was observed at all tested concentrations (Fig. 5). At high concentration (>1 μM), cytotoxic

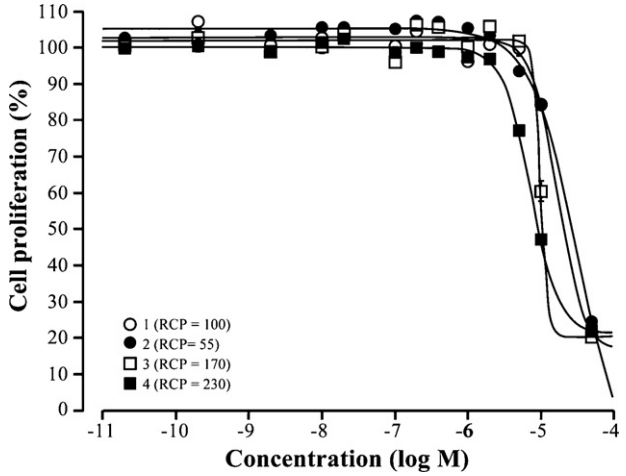

Fig. 5. Effect of compounds **1**–**4** on BT-20 (ER⁻) cell growth at different concentrations. The cell proliferation without product was fixed at 100%. The relative cytotoxic potency (RCP) was calculated as $EC_{50\,(17\beta\text{-E2})}/EC_{50\,(\text{compound } \mathbf{1}, \mathbf{2}, \mathbf{3} \text{ or } \mathbf{4})} \times 100$. Estradiol isomers are 17β-E2 (**1**), 17α-E2 (**2**), 18-epi-17β-E2 (**3**) and 18-epi-17α-E2 (**4**).

effect was observed in BT-20 cell. This effect on ER⁻ cells was observed in the same range of concentrations than for ER⁺ cell lines (Figs. 3B and 4B). The present data demonstrate that the cytotoxic activity observed at high concentrations is acting by a non ER-dependent mechanism.

3.2. ER binding affinity

Having assessed the *in vitro* estrogenic activity for the four E2 isomers, we next investigated their affinity for ER. We performed the binding assay using ER obtained from uteri of gonadectomized rats. The predominant isoform in the mature uterus is ERα, like in the MCF-7 cells, therefore we expressed our results as the affinity for the ERα [38]. The concentration at which the unlabeled ligand displaces half the specific binding of [^{3}H]-17β-E2 on ER (IC_{50}) was determined by computer fitting of the data using non-linear regression analysis. Compounds **1**–**4** bound to the ER in different degree as represented by the dose-response curves (Fig. 6). The relative binding affinity (RBA) of natural 17β-E2 (**1**) for the ER was established at 100%, because it had the highest ER-binding affinity for ER of the four E2 isomers. Compound **2** (17α-E2) had a RBA of 3.6% indicating

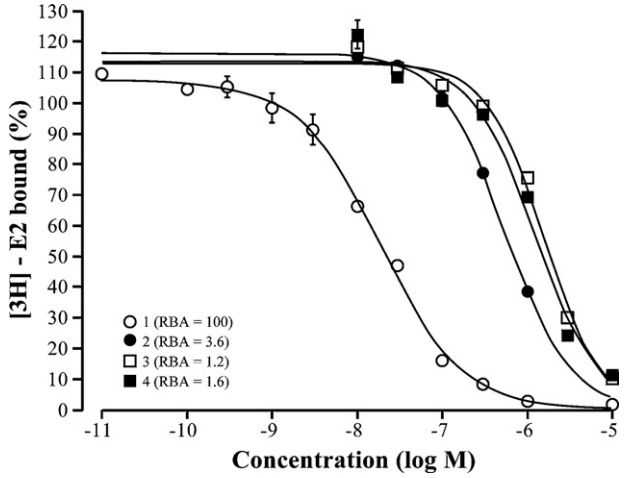

Fig. 6. Effect of increasing concentrations of compounds **1**–**4** in displacing [^{3}H]-17β-E2 binding to the rat uterine estrogen receptor alpha (ERα). Estradiol isomers are 17β-E2 (**1**), 17α-E2 (**2**), 18-epi-17β-E2 (**3**) and 18-epi-17α-E2 (**4**). RBA: relative binding affinity.

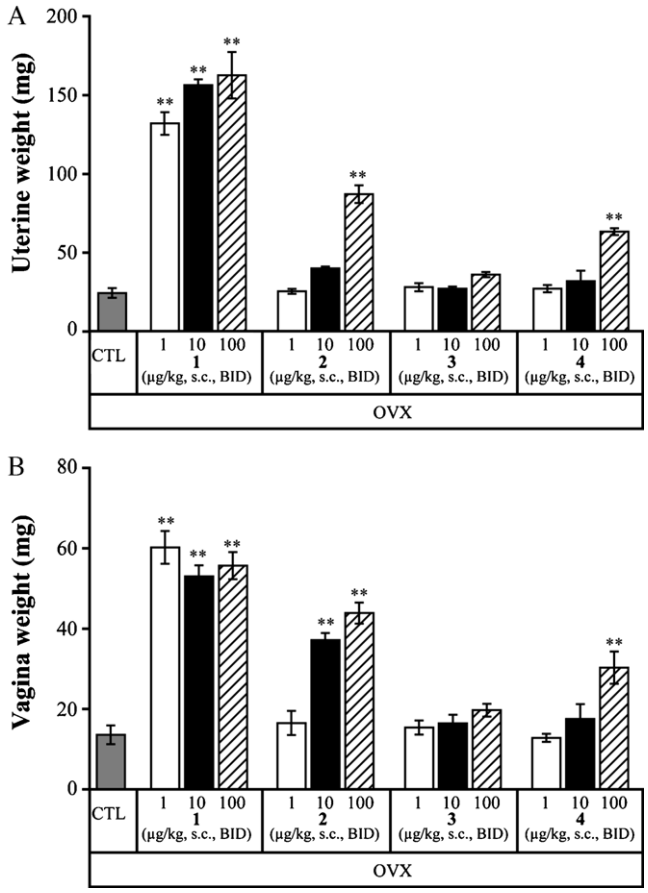

Fig. 7. Effect of compounds **1**–**4** on estrogen-sensitive (ER⁺) tissues. Uterine (A) and vagina (B) weight of mice treated 7 days with 1, 10 and 100 μg/kg (s.c. BID) of compound **1**, **2**, **3** or **4**. $^*P \leq 0.05$ vs control, $^{**}P \leq 0.01$ vs control. Estradiol isomers are 17β-E2 (**1**), 17α-E2 (**2**), 18-epi-17β-E2 (**3**) and 18-epi-17α-E2 (**4**).

that the inversion of 17β-OH to 17α-OH decreases the affinity for ER by 28-fold. This RBA value is in good agreement with that (3.0%) reported recently by Blair et al. [39] using rat uteri as the source of ER for their competitive binding assay. The RBA of compounds **3** and **4** were 1.2% and 1.6%, respectively, thus showing a weak ER binding affinity. Similarly, as reported by Perez et al. [27] for human ERα, the binding affinity of **3** (18-epi-17β-E2) is lower than that of **4** (18-epi-17α-E2). These two compounds share an important modification of the structure of natural estrogen 17β-E2 (**1**), the inversion of 18-methyl in position 13, which is apparently responsible for the major loss, 81- and 66-fold, of binding affinity for the ER. The ranking order is thus 17β-E2 (**1**) ≫ 17α-E2 (**2**) > 18-epi-17α-E2 (**4**) > 18-epi-17β-E2 (**3**) (Table 1). These results are in good agreement with our findings generated from the *in vitro* proliferation tests with ER⁺ cells.

3.3. Estrogenic activity in mice

Another approach to evaluate the estrogenic activity of compounds **1**–**4** was to use the ovariectomized (OVX) mouse model by measuring the weight of the uterus (Fig. 7A) and the vagina (Fig. 7B), two estrogen-sensitive (ER⁺) tissues. When we subcutaneously administrated (s.c.) 17β-E2 (**1**) to OVX mouse, we observed an increase of the uterine weight from 24 mg (control) to 125, 155 and 160 mg depending of the dose (1, 10 and 100 μg/kg, respectively). For 17α-E2 (**2**) and 18-epi-17α-E2 (**4**), the increase in uterine weight was only significantly different ($P \leq 0.01$) to control group (3.6 and 2.6-folds, respectively) at a dose of 100 μg/kg suggesting

Table 1
Structural characteristics of compounds **1**–**4** and radiolabeled ligand assay for ER.

Compound	18-CH_3 orientation	17-OH orientation	IC_{50}[a] (nM)	ERα-RBA[b] (%)
17β-E2 (**1**)	β	β	19	100
17α-E2 (**2**)	β	α	529	3.6
18-epi-17β-E2 (**3**)	α	β	1534	1.2
18-epi-17α-E2 (**4**)	α	α	1188	1.6

[a] The concentration of tested compound inhibiting 50% of the binding of labeled 17β-E2 (IC_{50}) was obtained from dose–response curves.
[b] RBA: relative binding affinity.

an estrogenic effect. This was not the same for the 18-epi-17β-E2 (**3**) which presented a weakly- and non-significant uterotrophic activity (1.5-fold vs control group) at this high dose. Doses of 1 and 10 μg/kg reflect the same pattern of response. Measuring the weight of the vagina demonstrated the same tendency as observed with the uterus. Thus, as for the uterus, the order of estrogenic potencies in the vagina was 17β-E2 (**1**) > 17α-E2 (**2**) > 18-epi-17α-E2 (**4**), whereas no significant uterotrophic activity was obtained with 18-epi-17β-E2 (**3**).

3.4. Structural analysis

It was already known that subtle modifications of the E2 nucleus can modulate the pattern of responses within a cell [40]. From the examination of the structures of compounds having high affinity for ER, it was also suggested that steroid-receptor binding is primarily the result of interaction between the receptor and the steroidal A-ring [41]. Duax et al. also mentioned that the superposition of the phenolic A-ring of six molecules that bind to ER suggests that the variability in D-ring orientation and shape is compatible with receptor binding affinity and some degree of activity. Taking account of all these observations, we attempted to rationalize our results using the 3-dimentional (3-D) structures of compounds **1**–**4**. In fact, the changes in functional group positioning and in C- and D-rings conformation, like those produced by modifying the orientation of 17-OH and 18-methyl group, generate important 3-D structural modifications.

As expected, the ideal position of 17-OH to get an optimal interaction with key amino acids of the ER, which result in the best ER binding and estrogenic potency, is that of the natural ligand of ER (17β-E2; **1**). Changing the 17β-OH orientation of **1** to a 17α-OH orientation (compound **2**) did not modify the shape of the steroid, but moved the OH away from its optimal positioning (Fig. 8A) with the consequence of reducing both the RBA and estrogenic activity (Table 1, Figs. 3 and 4). A greater impact on the steroid shape was however obtained with compounds **3** and **4** having a 18-methyl orientation inversed from the beta face (natural R configuration; 13β) to the alpha face (unnatural S configuration; 13α) (Fig. 8B). These two compounds have a lower binding affinity for ER than 17α-E2 (**2**) and also a lower estrogenicity. Conformational investigations of such 18-epi-E2 nucleus (or 13α-steroid) by X-ray analysis and ^{1}H NMR spectroscopy (NOESY experiments in solution) showed the existence of compounds with the classical steroid conformation (C-ring chair) and others with an atypical ring C twist-boat conformation according to the orientation of the 17-OH [31]. From the results of this study, 18-epi-17α-E2 (**4**) should have a classical C-ring chair conformation and 18-epi-17β-E2 (**3**) a C-ring twist boat conformation. In accordance with the NMR investigation of the Schonecker's group [31], our NOESY experiments showed the characteristic signals (cross-peaks 1-H to 11α-H and 11β-H for **3** and only a cross-peak 1-H and 11α-H for **4**) that support the 3-D structures of **3** and **4** (Fig. 8B).

Having established the 3-D structural conformation of compounds **3** and **4**, they were overlaid with compounds **1** and **2** using Chem3D software and assuming that all A-rings share the same

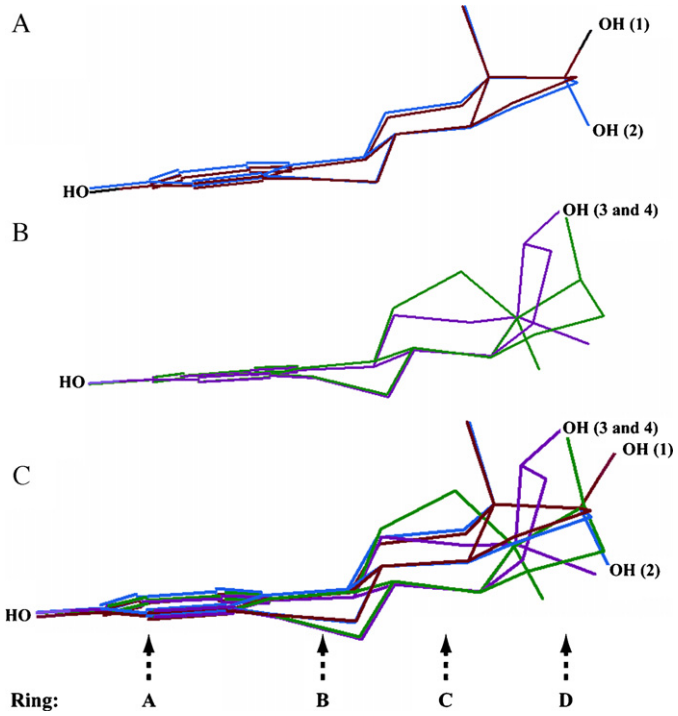

Fig. 8. Different overlapping of 3-D structures of E2 isomers. (A) Compounds **1** and **2**, (B) compounds **3** and **4**, and (C) compounds **1**–**4**. The 17-OH of compound **4** is located 1.157 Å behind the 17-OH of compound **3**. The steroids are reported in red (17β-E2; **1**), blue (17α-E2; **2**), green (18-epi-17β-E2; **3**) and pink (18-epi-17α-E2; **4**). Compounds **1**, **2** and **4** have a classical C-ring chair conformation whereas compound **3** has an atypical C-ring twist boat conformation. The structure energy minimization as well as overlaying was performed using Chem3D (Pro Version 5.0) software. The energy minimization was obtained with the MM2 minimization method (RMS gradient = 0.100). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

place (Fig. 8C). Clearly, the C- and D-rings of both 13α-isomers **3** and **4** have a less planar shape than C- and D-rings of **1** and **2** thus limiting the access to the ER-binding domain by steric hindrance. In other words, the interferences between the ER and the C- and D-rings of compounds with unnatural configuration (18-epi-methyl) are harmful to hydrogen bond formation between key amino acids and 17-OH. It is important to note that 18-epi-17β-E2 (**3**), the E2 isomer having an atypical C-ring twist boat conformation, is the compound having the lower ER-binding affinity, the lower proliferative (estrogenic) potency on estrogen-sensitive cells and, interestingly, the only E2 isomer producing no stimulation of estrogen-sensitive tissues (uterus and vagina) in mouse.

4. Conclusion

In this study, we determined the estrogenic activity of compounds **3** and **4**, two E2 isomers synthesized in our laboratory and having the methyl-18 in opposite (α) orientation than natural 17β-E2. Taken together, the *in vitro* data, *in vivo* data and structural analysis of compounds **1**–**4** demonstrated that inver-

sion of the 18-methyl group, from β to α configuration greatly affected the estrogenicity of the E2 nucleus. This seems to be due to steric hindrance produced by the non-planar shape of C- and D-rings of 18-epi-isomers. We demonstrated that 18-epi-17β-E2 (**3**) is the less estrogenic compound tested according to the following order: 18-epi-17β-E2 (**3**) < 18-epi-17α-E2 (**4**) < 17α-E2 (**2**) ≪ 17β-E2 (**1**). These results encourage us to modify the methyl-18 stereochemistry of estrane nuclei to obtain weakly- or non-estrogenic compounds, which could be used in the design of new inhibitors of key steroidogenic enzymes.

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