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A new non-estrogenic steroidal inhibitor of 17β-hydroxysteroid dehydrogenase type 1 blocks the estrogen-dependent breast cancer tumor growth induced by estrone

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Abstract

17β-Hydroxysteroid dehydrogenase type 1 (17β-HSD1) converts estrone (E1) into estradiol (E2) and is expressed in many steroidogenic tissues and breast cancer cell lines. Since the potent estrogen E2 stimulates the growth and development of hormone-dependent diseases, inhibition of the final step of E2 synthesis is considered a promising strategy for the treatment of breast cancer. Based on our previous study identifying 16β-(m-carbamoylbenzyl)-E2 (CC-156) as a lead compound for the inhibition of 17β-HSD1, we performed a number of structural modifications to reduce its undesired residual estrogenic activity. The steroid derivative PBRM (3-(2-bromoethyl)-16β-(m-carbamoylbenzyl)-17β-hydroxy-1,3,5(10)-estratriene) emerged as a potent inhibitor of 17β-HSD1 with an IC50 value of 68 nM for the transformation of E1 into E2. When tested in the estrogen-sensitive breast cancer cell line T-47D and in mice, PBRM demonstrated no estrogenic activity in the range of concentrations tested. Furthermore, with the purpose of evaluating the bioavailability of PBRM and CC-156 injected subcutaneously (2.3 mg/kg), we measured their plasmatic concentrations as a function of time, calculated the area under the curve (AUC0-12h) and demonstrated a significant improvement for PBRM (772 ng/ml*h) compared to CC-156 (445 ng/ml*h). We next tested the in vivo efficiency of PBRM on the T-47D xenograft tumor model, in female ovariectomized athymic nude mice. After a treatment with PBRM, tumor sizes in mice stimulated with exogenous E1 were completely reduced at the control group level (without E1 treatment). As a conclusion, PBRM is a promising non-estrogenic inhibitor of 17β-HSD1 for the treatment of estrogen-dependent diseases such as breast cancer.

Keywords: Steroid, inhibitor, enzyme, 17β-hydroxysteroid dehydrogenase, hormone, breast cancer.
1. Introduction

Steroid hormones play an important role in the development and differentiation in several tissues (1). They are synthesized by a combined action of enzymes from different families including P450 (CYP) enzymes (2), the short chain dehydrogenases/reductases (SDR) and the aldoketo reductases (AKR) (3-6). 17β-Hydroxysteroid dehydrogenases (17β-HSDs) convert steroids at position 17 in order to modulate their biological potency as estrogens and androgens. In fact, this enzyme family transforms the keto-forms of sexual steroids, usually inactive, into the hydroxyl-forms, active over their receptor (7). To date, there are 15 known isoforms of 17β-HSDs, which are cofactor-dependent (8), and all of these belong to the SDR family except 17β-HSD5 which is an AKR enzyme (1). The enzymatic activities associated with the different isoforms of 17β-HSDs are widespread in human tissues, not only in classic steroidogenic tissues, such as the testis, ovary, and placenta, but also in a large series of peripheral intracrine tissues (9). More importantly, each 17β-HSD isoform has a specific tissue distribution (10-12) and displays a selective substrate affinity, and moreover, in intact cells, its activity is unidirectional (reductive or oxidative) (9, 13). These findings indicate that selectivity of drug action could be achieved by targeting a particular 17β-HSD isoform with selective inhibitors. For cancer therapy, the inhibition of oxidative 17β-HSDs, thus transformation of the most proliferative cell form (hydroxyl) of hormone into a less potent form (ketone), is not suitable. In contrast, the selective inhibition of reductive 17β-HSDs involved in the transformation of ketosteroids into hydroxysteroids must be encouraged (9). The most extensively characterized of 17β-HSDs is type 1 (17β-HSD1), which catalyzes the NAD(P)H-dependent reduction of estrone (E1) into the potent estrogen estradiol (E2) (Fig. 1A) (14). E1 only has a low affinity for the estrogen receptor (ER) and has to undergo pre-receptor activation by 17β-HSD1, a reduction to E2, to bind to the ER with high affinity (15). This enzyme also catalyzes the reduction of dehydroepiandrosterone (DHEA) into 5-androstene-3β,17β-diol (Δ5-diol), a weaker estrogen that becomes more important after menopause (16). Expression of 17β-HSD1 is increased in breast tumors of postmenopausal women and the level of expression has prognostic significance (15, 17, 18). Inhibiting 17β-HSD1 activity could thus constitute a valuable way of reducing E2 level, with the aim of shrinking breast tumors (19-23).

Our group has previously reported the synthesis of a number of E2 derivatives modified at position 16 (19, 24-28) for use as 17β-HSD1 inhibitors. In one of these studies, CC-156 (16β-(m-carbamoylbenzyl)-E2) (Fig. 1B) was identified as a lead compound for the inhibition of 17β-HSD1 (19). Since the carbamoylbenzyl group can be found in the nicotinamide moiety of 17β-HSD1
cofactor (NADPH or NADH), we hypothesized that it could generate key interaction with amino acid neighboring the catalytic site. In fact, the \( m\)-carbamoylbenzyl appears to be an important characteristic of this new class of 17\( \beta\)-HSD1 inhibitors (19, 29). Despite its excellent inhibitory activity, it could only reduce 62\% of the proliferative activity induced by a physiologic concentration of E1 (0.1 nM) in T-47D estrogen-sensitive (ER\(^+\)) breast cancer cells. The cell growth reduction was not 100\% because a weak (38\%) estrogenic activity was induced by CC-156 itself, an E2 derivative having a residual estrogenic activity (19). Based on these results, we performed a number of structural modifications at position 3 of CC-156 in an attempt to modulate interaction with important amino acids belonging to the catalytic site and to reduce the undesired residual estrogenic activity. The steroid derivative PBRM (3-(2-bromoethyl)-16\( \beta\)-(\( m\)-carbamoylbenzyl)-17\( \beta\)-hydroxy-1,3,5(10)-estratriene) (Fig. 1C) emerged as a potent 17\( \beta\)-HSD1 inhibitor without residual estrogenic activity. After publishing the chemical synthesis and the preliminary data of in vitro assays with PBRM (30), these interesting results now require additional in vitro and in vivo studies. Because PBRM with its bromoethyl group at C3 is likely to be more hydrophobic (\( c\log P = 6.26\)) than CC-156 (\( c\log P = 4.82\)), the analog inhibitor with a hydroxyl group at C3, and that this may impinge the physicochemical properties, we assessed both inhibitors. In this paper, we present the in vitro 17\( \beta\)-HSD1 inhibition and estrogenic activity in T-47D cells of this new C3/C16 derivative of E2, as well as the results of in vivo studies evaluating the plasma concentration, the estrogenicity and 17\( \beta\)-HSD1 inhibitory activity in a breast cancer xenograft model.

2. Materials and methods

2.1 In vitro studies

2.1.1 Cell culture

Breast cancer cell line T-47D was obtained from the American Type Culture Collection (ATCC) and maintained in a 175 cm\(^2\) culture flask at 37 °C in a humidified atmosphere at 5\% CO\(_2\). This cell line was not authenticated in the authors’s laboratory. 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 \( \mu \)g/mL) and estradiol (1 nM).
2.1.2 17β-HSD1 inhibition assay

T-47D cells were seeded in a 24-well plate (3000 cells/well) in 990 µL of medium supplemented with insulin (50 ng/mL) and 5% dextran-coated charcoal-treated FBS, which was used rather than untreated 10% FBS, to remove the remaining steroid hormones. Stock solutions of inhibitors CC-156 and PBRM were previously prepared in ethanol and diluted with culture medium to achieve appropriate concentrations prior to use. After 24 h of incubation, 5 µL of the diluted solution were added to the cells to obtain a final concentration ranging from 1 nM to 10 µM to determine the IC50 value. The final concentration of ethanol in the well was adjusted to 0.1%. Additionally, 5 µL of a solution of [14C]-estrone (American Radiolabeled Chemicals, Inc., St. Louis, MO, USA) was added to obtain a final concentration of 60 nM. Cells were incubated for 24 h and each inhibitor was assessed in triplicate. After incubation, the culture medium was removed and labeled steroids (E1 and E2) were extracted with 1 mL of diethyl ether. The organic phases were evaporated to dryness with nitrogen. Residues were dissolved in dichloromethane and dropped on silica gel thin layer chromatography plates (EMD Chemicals Inc., Gibbstown, NJ, USA) and eluted with toluene/acetone (4:1) as solvent system. Substrate [14C]-E1 and metabolite [14C]-E2 were identified by comparison with reference steroids (E1 and E2) and quantified using the Storm 860 system (Molecular Dynamics, Sunnyvale, CA, USA). The percentage of transformation and the percentage of inhibition were calculated as follow: % transformation = 100 x [14C]-E2 / ([14C]-E1 + [14C]-E2) and % of inhibition = 100 x (% transformation without inhibitor - % transformation with inhibitor) / % transformation without inhibitor (31, 32).

2.1.3 Cell proliferation assays (17β-HSD1 inhibitory, estrogenic and antiestrogenic activities)

Quantification of cell growth was determined by using CellTiter 96®Aqueous Solution Cell Proliferation Assay (Promega, Nepean, ON, Canada) following the manufacturer’s instructions. T-47D cells were resuspended with the medium supplemented with insulin (50 ng/mL) and 5% dextran-coated charcoal treated FBS rather than 10% FBS to remove remaining hormones. Aliquots (100 µL) of the cell suspension were seeded in 96-well plates (3000 cells/well). After 48 h, the medium was changed with a new one containing an appropriate concentration of products to be tested and was replaced every 2 days. Cells have grown either in absence or presence of the compounds for 7 days. To determine the proliferative (estrogenic) activity, the estrogen-sensitive T-47D cells were grown in absence (basal cell proliferation was fixed as 100%) or presence of compounds to be tested at 0.5 to 10 µM. The potent estrogen E2 was used as a reference control. To determine the inhibition of E1-
induced cell proliferation, the T-47D cells were grown in the presence of E1 (0.1 nM) without (control) or with the inhibitor at a concentration of 0.5, 1, 2.5, and 5 µM. Stock solutions of PBRM and CC-156 inhibitors were previously prepared in ethanol and diluted with culture medium to achieve appropriate concentrations prior to use. The cell proliferation without E1 ± inhibitor (control) was fixed as 100%. To determine the potential antiestrogenic activity of inhibitor PBRM, the T-47D (ER⁺) cells were grown in the presence of estrogen E2 (0.1 nM) and pure antiestrogen EM-139 (0.5 µM) [33] or inhibitor PBRM (0.5 µM). The cell proliferation without E2 and tested compounds (control) were fixed as 100%.

2.1.4 ERα binding assay

A competitive binding assay using a purified full-length recombinant human ERα (Life Technologies, Grand Island, NY) was done as previously described (34, 35). Briefly, each reaction consisted of 1.2 nM rhERα and 2.5 nM [³H]-estradiol in assay buffer (10 mM Tris, 1.5 mM EDTA, 1 mM dithiothreitol, 10% glycerol, 1 mg/mL BSA, pH 7.5) with different concentrations of the inhibitors or untritiated estradiol (E2) in a total reaction volume of 100 µL. Stock solutions of PBRM and CC-156 inhibitors were previously prepared in ethanol and 10 µL added to the reaction mixture to achieve appropriate concentrations. Non-specific binding was determined by incubation with an excess of E2 (1 µM). After an overnight incubation at 4 °C, 100 µL of cold 50% hydroxyapatite slurry was added to bind the receptor/ligand complex. After 15 minutes, 1 mL of wash buffer (40 mM Tris, 1 mM EDTA, 1 mM EGTA, 100 mM KCl, pH 7.4) was added and the tubes were centrifuged at 4500 rpm for 5 minutes at 4 °C. The washing step was repeated twice. The radioactivity of the pellet was extracted by incubation with 1 mL of ethanol for 1 h at room temperature. The suspension was then put into 10 mL of Biodegradable Counting Scintillant and the radioactivity counted with a Wallac 1411 Liquid Scintillation Counter. IC₅₀ values were obtained using GraphPad Prism 5 and RBA values were obtained by using the following equation: (IC₅₀ of 17β-E2 / IC₅₀ of tested compound) x 100.

2.2 In vivo studies

2.2.1 Animals

All animals were acclimatized to environmental conditions (temperature: 22 ± 3 °C; humidity: 50 ± 20%; 12-h light/12-h dark cycles, lights on at 07:15 h) for at least 3 days before starting the
experiment. The animals were allowed free access to water and a certified commercial rodent food (Rodent Diet #T.2018.15, Harlan Teklad, Madison, WI, USA) and randomized according to their body weight. The experiments with animals were conducted in an animal facility approved by the Canadian Council on Animal Care (CCAC) and the Association for Assessment and Accreditation of Laboratory Animal Care. The study was performed in accordance with the CCAC Guide for Care and Use of Experimental Animals. Institutional approval was obtained.

2.2.2 Plasmatic concentration of inhibitor after a single subcutaneous injection

Six week-old male Sprague-Dawley rats (Crl:CD®(SD)Br VAF/Plus™) weighing approximately 220 g were obtained from Charles-River, Inc. (St-Constant, QC., Canada). The animals were housed 3 per cage. A pharmacokinetic study was carried out following one subcutaneous (s.c.) injection of the inhibitor at one concentration (2.3 mg/kg of body weight in 0.5 mL of vehicle fluid). The inhibitor was first dissolved in ethanol (EtOH) and thereafter we added propylene glycol (PG) to obtain a final concentration of EtOH of 8%. During this experiment, the rats were housed individually and were fasted for 8 h before inhibitor injection but allowed free access to water. Blood samples for determination of inhibitor plasma concentration were collected at the jugular vein (0.4 mL by animal) at target intervals of 3, 7, 12 and 24 h post-dose for PBRM and 3 and 12 h for CC-156, from three rats per time point. After the collection at 7 h, a replacement fluid (0.9% sodium chloride injection USP) was injected in the rat. Blood samples were collected into Microvette potassium-EDTA (ethylenediamine tetraacetic acid)-coated tube (Sarstedt, Aktiegesellschaft & Co, Germany) and centrifuged at 3200 rpm for 10 minutes at 4 °C. The plasma was collected and stored at -80 °C until analyzed by liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS) analysis.

2.2.3 Measurement of plasma concentrations

The concentration of the inhibitors (CC-156 and PBRM) was determined by LC/MS/MS analysis using a procedure developed at CHUQ (CHUL) - Research Center (Québec, QC, Canada). Briefly, for extraction from serum, 100 µL of serum sample is transferred to individual tubes and 600 µL of ammonium acetate (1 mM) is added. A methanolic solution (50 µL) containing a steroidal internal standard (compound 48 in reference 36) is then added to each tube. Samples are transferred on Strata-X SPE columns (Phenomenex, Torrance, CA, USA), which have been conditioned with 2 mL of methanol and 2 mL of water. Each column is washed with 2 mL of methanol:water (10:90, v/v).
The inhibitor is then eluted with 5 mL of methanol containing 5 mM ammonium acetate. Methanol is evaporated at 45 °C under inert atmosphere and the residue dissolved in 100 µL of methanol:water (85:15, v/v). Calibration standard curves for CC-156 and PBRM were prepared in serum by extracting the steroidal inhibitor as reported above for samples. For the steroid analysis, the HPLC system uses a 75 x 4.6-mm, 3-µm reversed-phase Luna Phenyl-Hexyl column (Phenomenex, Torrance, CA, USA) at a flow rate of 0.8 mL/min. The inhibitor is detected using an API 3000 mass spectrometer, equipped with TurboIonSpray (Applied Biosystems, Canada). ESI in positive ion mode was used. The area under the curve (AUC) was calculated using the linear trapezoidal rule.

2.2.4 In vivo estrogenicity assay

Female ovariectomized (OVX) BALB/c mice weighing approximately 20 g were obtained from Charles-River, Inc. (St-Constant, Qc., Canada). The animals were housed 5 per cage. Groups of 5 mice were treated with E1 (0.02 µg/0.1 mL s.c.) or 17β-HSD1 inhibitor at 10, 50 and 250 µg (0.1 mL s.c.) daily for 7 days. The inhibitor or E1 was first dissolved in ethanol (EtOH) and thereafter we added propylene glycol (PG) to obtain the appropriate concentration in vehicle fluid (8% EtOH/92% PG). Animals were killed 24 h after administration of the last dose of compound and uterus and vagina were removed, excised of fat and weighed. Total body weights of mice were also recorded.

2.2.5 Inhibition of E1-stimulated T-47D tumor growth in nude OVX mice (xenograft model)

Female OVX BALB/c athymic nude mice weighing approximately 20 g were obtained from Charles-River, Inc. (St-Constant, Qc., Canada). The animals were housed 5 per cage. For the inhibition of T-47D tumor growth, 24 h after a pre-dose of E1 (0.1 µg/0.1 mL of vehicle (8% EtOH/92% PG)) injected s.c. per mouse, mice were inoculated s.c. with 1x10⁷ T-47D cells in 50 µL Matrigel (BD Biosciences, Bedford, MA) into both flanks of each mouse. T-47D tumor growth was stimulated using E1 (0.1 µg/0.1 mL vehicle s.c.) per mouse per day for 15 days. From day 16, animals with tumors were randomized in function of tumor volume and separated into three groups. Group 1 (control mice) was treated 32 days s.c. with 0.1 mL of vehicle alone (8% EtOH/92% PG) per mouse per day. Group 2 (E1 0.1 µg) was treated 32 days s.c. with E1 (0.1 µg/0.1 mL vehicle s.c.) per mouse per day. Group 3 (E1 0.1 µg + PBRM 250 µg) was treated with E1 (0.1 µg) and PBRM (250 µg) in a combined s.c. injection (0.1 mL of vehicle) per mouse per day for 32 days. The inhibitor of E1 was first dissolved in ethanol (EtOH) and thereafter we added propylene glycol (PG) to obtain the appropriate concentration in vehicle fluid (8% EtOH/92% PG). The mice were weighed at start and
volumes of tumors were determined by external caliper twice a week and the greatest longitudinal
diameter (length) and the greatest transverse diameter (width) were determined. Tumor volume based
on caliper measurements was calculated by the modified ellipsoidal formula: \( \text{Tumor volume} = \frac{1}{2}(\text{length} \times \text{width}^2) \) (37). At the end of the studies the mice were terminally anaesthetized, final body
weights and tumor sizes were determined. Uteri and vagina were removed, excised of fat and weighed
(38–40).

2.3 Statistical analysis

Statistical significance was determined according to the multiple-range test of Duncan–Kramer
(41). \( P \) values which were less than 0.05 were considered as statistically significant.

3. Results and discussion

3.1 17\( \beta \)-HSD1 inhibitory activity

The IC\(_{50}\) values of PBRM and CC-156 were determined using breast cancer T-47D cell line
(Fig. 2), which exerts strong endogenous expression of 17\( \beta \)-HSD1 (23). We can see that PBRM has a
good inhibitory effect on 17\( \beta \)-HSD1 with IC\(_{50}\) value of 68 nM. As a reference, inhibitor compound
CC-156 already synthesized by our research team, inhibited the enzyme with an IC\(_{50}\) of 27 nM. This
IC\(_{50}\) value is in agreement with the previous value of 44 nM obtained using the same cell line but a
different lot of cells and also with a different number of passages (19). PBRM is thus only 2.5-times
less effective in inhibiting the enzyme than CC-156. In fact, the presence of a bromoethyl at position
C3 produces a slight decrease in the potency of PBRM to inhibit the 17\( \beta \)-HSD1 activity. This
suggests that the 3-bromoethyl chain generates another kind of interaction with the catalytic site of the
enzyme rather than the OH of CC-156 or E1, the natural substrate of the enzyme.

3.2 Inhibition of E1-stimulated cell proliferation

We investigated the effectiveness of PBRM and CC-156 to block the proliferative effect
induced by E1 in estrogen-sensitive breast cancer cell line T-47D. We tested the ability of these 17\( \beta \)-
HSD1 inhibitors to inhibit the cell growth induced by the transformation of E1 (0.1 nM) into potent
estrogen E2. This concentration of E1 is close to the intracellular concentration in breast cancer cells
(42). Even though CC-156 inhibitor exerts some estrogenic effects when tested in the absence of E1,
we decided to use it as a reference compound. PBRM was able to inhibit the proliferative effect induced by E1 in a concentration-dependent manner (Fig. 3A). At concentrations of 0.5 and 1 µM, CC-156 showed a stronger effect than PBRM (221% vs 269% and 218% vs 243%, respectively), which is in accordance with its lower IC50 (27 and 68 nM, respectively). At higher concentrations, however, the inhibitory effect of CC-156 is probably counterbalanced by its residual estrogenic-like proliferation effect on ER+ cells and never reached the basal level (100%) of cell proliferation. On the other hand, PBRM reduced the cell growth from 250% to 156 and 125%, at 2.5 and 5 µM respectively.

The reduction of E1-induced cell proliferation obtained when using inhibitor PBRM could also be the result of an antiestrogenic activity of this E2 derivative. Indeed, an antiestrogenic compound will block the proliferative (estrogenic) effect of E2 mediated by its action on the estrogen receptor (ER). We thus verified the antiestrogenic properties of PBRM in order to confirm that the observed inhibition of T-47D cell proliferation was due to the inhibition of 17β-HSD1 and not its action on ER. As illustrated in Figure 3B, the enzyme inhibitor PBRM does not reverse the proliferative effect on ER+ cells of E2 (0.1 nM) like the pure antiestrogen EM-139 (33) does. This result suggests that PBRM does not work as an antiestrogenic compound, but acts instead as an inhibitor of E1 into E2 transformation catalyzed by 17β-HSD1.

### 3.3 Estrogenic activity on T-47D (ER+) cell line and ERα binding affinity

In order to detect any undesirable estrogenic activity of 17β-HSD1 inhibitors, cell proliferative assays were carried out on the T-47D cell line which is known to express the estrogen receptor (ER+) (43). Proliferative activity of compounds PBRM and CC-156 was evaluated at 0.5, 1, 2.5 and 5 µM (Fig. 4A). From the data collected, it is clear that inhibitor CC-156 exerts significant proliferative activities at all concentrations, which is in agreement with our previous studies (19, 30). On the other hand, it is clear that PBRM was not estrogenic at any concentration tested, which underlines the importance of the 3-bromoethyl chain to remove the undesired estrogenicity.

Having assessed the in vitro estrogenic activity of PBRM and CC-156 on ER+ cell proliferation, we next investigated their affinity for ERα (Fig. 4B), the predominant receptor isoform involved in estrogenic effect. The concentration at which the unlabeled natural ligand (E2) displaces half the specific binding of [3H]-17β-E2 on ERα (IC50) was determined by computer fitting of the data using non-linear regression analysis and the relative binding affinity (RBA) then calculated. The RBA
of E2 was established as 100% whereas the RBA for inhibitor CC-156 was 1.5%. Although low, this binding affinity for ERα can explain the proliferative (estrogenic) activity we have measured in the T-47D estrogen-sensitive cell line. Contrary to CC-156, however, no binding affinity was detected for PBRM, the second generation of 17β-HSD1 inhibitor. Thus, these results are clearly in agreement with the findings generated from the in vitro proliferation tests with ER+ cells.

3.4 Estrogenic activity of inhibitors in mice

To verify that the lack of estrogenicity of PBRM observed in vitro in the T-47D cell proliferation assay translates into the in vivo setting, the estrogenicity of PBRM was investigated using the OVX mouse model by measuring the weight of the uterus (Fig. 5A) and vagina (Fig. 5B), two estrogen-sensitive (ER+) tissues. For the OVX mice control group (OVX-CTR) a low weight of 22 mg was observed for the uterus. However, when administrated s.c. to OVX mice, E1 (0.02 μg/mouse/day) is converted into E2 by 17β-HSD1 and we observed a 2.5-time increase in uterine weight compared to OVX-CTR (22 mg vs 55 mg; P < 0.01). We tested CC-156 as reference at a single dose of 50 μg/mouse/day, because we already know that this compound was estrogenic in vitro and we expected a similar action in vivo. In fact, we could see that at a 50 μg/mouse/day dose uterine growth is stimulated from 22 mg for the OVX-CTR group to 29 mg for OVX-CC-156 group (P < 0.05). In counterpart, weights of the uterus from all PBRM dose groups (10, 50 and 250 μg/mouse/day), were not significantly different to those of the OVX-CTR group after seven days of treatment (25, 24 and 23 mg, respectively). Thus, these results confirmed that PBRM is non-estrogenic in vivo. The measurement of vagina weights clearly demonstrated the same tendency for PBRM as previously observed with the uterus.

3.5 Plasma concentration of inhibitors

A single subcutaneous injection (2.3 mg/kg) of inhibitors PBRM and CC-156 was given to two different groups of rats in order to determine the inhibitor bioavailability and whether the structural modification in the substituent at position C3 (bromoethyl vs OH) increases the plasma concentration. The mean plasma concentrations of inhibitors PBRM and CC-156 at different times and the corresponding area under the curve (AUC) are presented in Figure 6A. At first, we found that the maximum plasma concentration (Cmax) was attained at 3 h following injection for both inhibitors. Even if PBRM showed a non-significant different Cmax compared to CC-156 (73.4 ng/mL vs. 65.7 ng/mL), the plasma concentration for the two inhibitors declined differently. CC-156 had almost
disappeared in blood 12 h after the injection (11.3 ng/mL), suggesting that the dose of 2.3 mg/kg is too weak to be administrated only once a day. For PBRM however, values of 73.8 and 50.7 ng/mL were found after 7 and 12 h of injection respectively, the last one was significantly different from the concentration found at the same time for CC-156 \((P < 0.05)\). AUC\(_{0-12h}\) values indicate that PBRM was 1.7 times more available to rats than CC-156 (772 ng\(*h/\text{mL}\) vs 445 ng\(*h/\text{mL}\) \((P < 0.01)\). After 24 h, a plasma concentration of 11.7 ng/mL was measured for PBRM, thus we obtained an AUC\(_{0-24h}\) of 1146 ng\(*h/\text{mL}\) for it.

3.6 Inhibition of E1-stimulated T-47D tumor growth in OVX nude mice

After we established that PBRM inhibitor was found in plasma after a one-day single s.c. injection, we decided to study the efficacy of PBRM \textit{in vivo}. Female OVX Balb/c nude mice were inoculated with \(1 \times 10^7\) T-47D (ER\(^+\)) human breast cancer cells in Matrigel, as in the procedure described by Day \textit{et al} (23), except that inoculation was made into both flanks of mouse. The mice received E1 (0.1 µg/day), which after its transformation to E2 by 17β-HSD1, stimulates tumor growth. Only mice with tumors which were well established after 15 days of treatment with 0.1 µg E1/mouse s.c. were selected to continue the study. We used the dose of 250 µg/mouse of PBRM because this was the highest dose tested in the \textit{in vivo} estrogenicity assay that proved to be non estrogenic. Figure 6B shows the effect of PBRM on the growth of tumors stimulated with 0.1 µg E1/mouse/day. In the first 18 days of treatment, the tumors were not actively growing and maintained their initial size at the beginning of treatment. From day 19, the volume of tumors in the control (CTR) group began to decrease until they reached approximately the 74% of the initial volume after 28 days and continued at the same level until day 32 (77%). In the E1 treated group however, tumors grew reaching 136% of their initial size, whereas in the mice treated E1-PBRM the growth of the tumors was inhibited (74%), decreasing to the level of the CTR group at the end of treatment \((P < 0.01\) at days 28 and 32, E1-PBRM vs E1). Clearly, PBRM blocks the formation of E2 in the tumor through the inhibition of 17β-HSD1 and thus the tumor growth.

At the end of the study, the body weights of the mice were recorded and the estrogen-sensitive tissues (uterus and vagina) were taken for analysis. There was no effect of either E1 or PBRM on mouse weight over the 32-day treatment period (Fig. S1A), indicating that there is no apparent toxicity of PBRM at 250 µg/day/mouse (s.c.). Although uterine and vagina weights were increased significantly in both of the E1-treated groups \((P < 0.01,\) E1 and E1-PBRM vs. CTR), treatment with
PBRM had no effect on the E1-stimulated uterine and vaginal weight increase (Fig. S1B and S1C). Our results are in agreement with those obtained by other research groups (23, 38), which demonstrated that despite the fact that the 17β-HSD1 inhibitor was used at a concentration which produces a decrease in tumor volume (by inhibiting the human 17β-HSD1 in xenograft), the doses of E1 (0.1 μM) used stimulated uterine weight gain which could not be reduced at the end of the study. In fact, the main expression of 17β-HSD1 is in the ovary of female rodents (44) and low levels are detected in the uterus only by RT-PCR (45), but not by in situ hybridization (46). Day et al (23) suggested that the lack of effect of 17β-HSD1 inhibitor on uterine and vaginal weight may therefore be due to either the higher sensitivity of the uterus than the tumor to circulating estrogens. Since the murine 17β-HSD1 is an ortholog of the human 17β-HSD1 presents in xenografted tumor (47), it is also possible that PBRM – always tested on human 17β-HSD1– did not inhibit the murine enzyme present in the uterus. Interestingly, our results obtained with the T-47D xenograft tumor model revealed that 17β-HSD1 inhibitor PBRM completely blocks the tumor growth induced by E1, the precursor of potent estrogen E2.

4. Conclusion

Several groups are working towards the development of 17β-HSD1 inhibitors for clinical use in the treatment of hormone-dependent breast cancer (48). However to date, only the groups of Day et al (23) and Husen et al (38) have demonstrated the efficacy of an inhibitor in the in vivo treatment of E1-stimulated breast tumors in nude mice, but to our knowledge no inhibitor is currently in clinical trial. In our study we describe the in vitro and in vivo evaluation of a new inhibitor of 17β-HSD1. Known as PBRM, this steroid (E2) derivative has two characteristic elements: a carbamoylbenzyl chain at position C16 for 17β-HSD1 inhibition and a 2-bromoethyl side chain at position C3 for removing the residual estrogenic activity associated with its E2 nucleus (30). PBRM has an IC_{50} value of 68 nM in the whole T-47D cell assay, is non-estrogenic both in vitro and in vivo, and gave a higher plasma concentration when compared to the reference inhibitor CC-156 after one-day s.c. injection of 2.3 mg/kg. Most importantly, after 32 days of treatment, tumor sizes of OVX mice treated with E1 and PBRM were completely reduced at the control group level (without E1 treatment). It is noteworthy that for the first time an inhibitor of 17β-HSD1 is able to decrease the final tumor volume by 100%. These results strongly imply that in the experimental setting the tumor growth was mainly mediated via E2, produced by the action of 17β-HSD1 expressed in the T-47D cells, and that the remaining E1 seems not to be able to sustain the tumor growth. Another interesting aspect of
inhibiting 17β-HSD1 came from the fact that this enzyme also transforms DHEA into Δ⁵-diol. As demonstrated by Poulin and Labrie (49), Δ⁵-diol at physiological concentrations, acts as a genuine estrogen in estrogen-sensitive breast cancer cells through its direct interaction with ER. Thus, inhibiting the transformation of DHEA into Δ⁵-diol is another way to deprive breast cancer cells of an estrogenic stimulus that becomes more important after menopause (16). Our in vitro and in vivo results clearly highlighted the potential utility of PBRM, a new 17β-HSD1 inhibitor, for the treatment of breast cancer which could be used alternately or sequentially with other drugs against estrogen-dependent diseases.

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**Figure Legends**

**Figure 1.** A: 17β-Hydroxysteroid dehydrogenase type 1 (17β-HSD1) transforms estrone (E1) to estradiol (E2) in presence of cofactor NAD(P)H. B: 17β-HSD1 steroidal inhibitor CC-156 (16β-(m-carbamoylbenzyl)-E2. C: 17β-HSD1 non-estrogenic steroidal inhibitor PBRM (3-(2-bromoethyl)-16β-(m-carbamoylbenzyl)-17β-hydroxy-1,3,5(10)-estratriene).

**Figure 2.** Inhibitory potency of PBRM and CC-156 in T-47D intact cells. Breast cancer cells expressing 17β-HSD1 were incubated with various concentrations of inhibitors for 24 h in presence of labeled [14C]-E1 (60 nM). IC50 represents the concentration that inhibited 17β-HSD1 activity by 50%. Results are the means (± SEM) of a triplicate.

**Figure 3.** A: Cell growth of T-47D cells induced by a physiologic concentration of E1 (0.1 nM) in the presence or absence of inhibitors PBRM and CC-156 at various concentrations. Control (CTR) is fixed at 100%. Results are expressed as means (± SEM) of triplicate. (a, $P < 0.01$ vs CTR; b, $P < 0.01$ vs E1 (0.1 nM); c, $P < 0.01$ CC-156 vs PBRM). B: Effect of inhibitor PBRM and the pure antiestrogen EM-139 on the inhibition of E2 (0.1 nM)-induced proliferation (antiestrogenic activity) of estrogen-sensitive (ER⁺) human breast cancer T-47D cells. Two days after plating, the cells were incubated for 7 days with the indicated concentration of compounds. Medium was changed every second day. Control (CTR) is fixed at 100%. Results are expressed as means (± SEM) of triplicate. (a, $P < 0.01$ vs CTR; b, $P < 0.01$ vs E2 (0.1 nM).

**Figure 4.** A: Effects of inhibitors PBRM and CC-156 on the growth of estrogen-starved T-47D (ER⁺) human breast cancer cells after 7 days of treatment. Control is fixed as 100%. Results are expressed as means (± SEM) of triplicate (** $P < 0.01$). B: Effects of increasing concentrations of PBRM and CC-156 in displacing [3H]-E2 binding to the human ERα. From the dose-response curves, the relative binding affinity of unlabeled E2 was fixed as 100% whereas the RBA of CC-156 was 1.5%. No binding affinity was detected for PBRM.

**Figure 5.** Effect of inhibitors PBRM and CC156 on uterine (A) and vagina (B) weight of ovariectomized (OVX) mice treated for 7 days (*$P < 0.05$ and **$P < 0.01$, experimental versus OVX control animals (CTR)).
Figure 6. **A:** Plasma concentration of PBRM and CC-156 as a function of time following subcutaneous (s.c.) injection of 2.3 mg/kg in Sprague-Dawley rats. **B:** Effect of PBRM, an inhibitor of 17β-HSD1, on the growth of E1 (s.c.)-stimulated T-47D tumors (xenograft) in ovariectomized (OVX) nude mice. (*$P < 0.05$ and **$P < 0.01$, E1-PBRM and OVX control animals (CTR) versus E1).
Figure 1

A

 Estrone (E1) \[\xrightarrow{17\beta-\text{HSD1}}\] Estradiol (E2)

\[\text{NAD(P)H}\]

\[E1 (X = O)\]
\[E2 (X = \beta-\text{OH}/H)\]

B

C

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Figure 2

% of transformation (14C)-E1 into (14C)-E2

Concentration (log M)

- PBRM (IC50 = 68 ± 6 nM)
- CC-156 (IC50 = 27 ± 4 nM)
Figure 6

A

Plasma concentration of compounds (ng/ml)

Time (h)

B

Tumor volume (%)

17β-HSD1

E1 → E2 → Tumor Growth

1. CTR

2. E1 (0.1 μg)

3. E1 (0.1 μg) + PBRM (250 μg)
Molecular Cancer Therapeutics

A new non-estrogenic steroidal inhibitor of 17β-hydroxysteroid dehydrogenase type 1 blocks the estrogen-dependent breast cancer tumor growth induced by estrone

Diana Ayan, Rene Maltais, Jenny Roy, et al.

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