Novel Compounds Inhibit Estrogen Formation and Action

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ABSTRACT

Estrogens are well known to play a predominant role in human breast cancer. The current endocrine therapy of breast cancer consists in administering an antiestrogen which blocks the action of estrogens at the receptor level. However, the currently available antiestrogens possess mixed estrogenic and antiestrogenic activity, thus limiting their potential therapeutic efficacy. The present data show that a series of new estrogen derivatives demonstrate not only pure antiestrogenic activity in the sensitive in vivo mouse uterus assay, but simultaneously exert potent inhibitory effects on 17β-hydroxysteroid dehydrogenase activity, the enzyme responsible for the formation of 17β-estradiol from estrone, the last step in estrogen formation. Such compounds having a dual site of inhibitory action, namely on estrogen formation and on the estrogen receptor, could well lead to an improved endocrine therapy of breast and other estrogen-sensitive cancers as well as other nonmalignant estrogen-sensitive diseases.

INTRODUCTION

Breast cancer is the most frequent cancer in women. In fact, it is predicted that 155,000 new cases of breast cancer will be diagnosed in the United States in 1991, while 44,500 women are expected to die from the disease during the same period (1). The most recent statistics indicate that one out of nine women will suffer from breast cancer during their lifetime. Unfortunately, the present therapies of breast cancer show positive results in only 30 to 40% of cases and, when present, the positive response is of short duration (2–5).

Among all factors, estrogens are well recognized to play a predominant role in breast cancer development and growth (2–7). Considerable attention has thus focused on the mechanisms of action of estrogens and on the development of blockers of estrogen biosynthesis or estrogen action at the target cell level, since the existing ablative procedures, either surgical or medical, do not permit complete elimination of estrogens (8, 9). Since the first step in the action of estrogens in target tissues is binding to the estrogen receptor (9, 10), a logical approach for the treatment of estrogen-sensitive breast cancer is the use of antiestrogens, or compounds which block the interaction of estrogens with their specific receptor. Unfortunately, until very recently, no agent with pure antiestrogenic activity under in vivo conditions has been available. In fact, tamoxifen, the compound currently used for the treatment of breast cancer in women, behaves as a mixed agonist/antagonist of estrogen action, thus limiting its therapeutic potential and possibly explaining the limited success obtained with this compound in the treatment of breast cancer in women (2–5, 11–13).

In women, mammary gland estrogens originate from both the adrenal and the ovary. The adrenals secrete large amounts of the relatively inactive precursor steroids DHEA4 and especially DHEA-S, which are converted into Δ4-dione and then into active androgens and estrogens in peripheral tissues (14–16). As shown in Fig. 1, the two active estrogens in mammary gland epithelial cells are 17β-estradiol and Δ4-diol, another estrogen recently recognized as being a potent stimulator of breast cancer growth (7, 17–20). Since 17β-HSD (21, 22) catalyzes the last enzymatic step in the biosynthesis of the active estrogen 17β-estradiol from estrone and is also responsible for the formation of Δ4-diol from DHEA, a second logical site of blockade of estrogens is likely to be 17β-HSD. The interest of this approach is strengthened by the observation that the two in tandem human 17β-HSD genes are located on the q11-q12 region of chromosome 17 (21–23), thus making the 17β-HSD gene a candidate for inherited susceptibility to breast cancer in families with early-onset disease (24).

Based on the observation that estradiol derivatives possessing a 7α-alkyl substituent retain their affinity for the estrogen receptor during affinity chromatography (25), 7α-alkyl derivatives of estradiol (26) or estradiol derivatives possessing additional changes designed to increase their affinity for the estrogen receptor and/or decrease their degradation (27–29) have been synthesized and shown to possess pure and potent antiestrogenic activity in the most rigorous in vitro and in vivo systems, including human breast cancer cells (26–29).

The present data show that a series of novel steroidal derivatives which possess both a 7α-undecanamido group as well as a halogen atom at C-16 or a double bond at C-14–C-15 or C-15–C-16 exert, in addition to pure antiestrogenic activity in the sensitive in vivo mouse uterus assay, potent inhibitory effects on 17β-HSD activity in the same tissue. With the ability to block estrogens at two independent sites, such compounds offer new hope for a more efficient therapy of estrogen-sensitive diseases, especially breast cancer.

MATERIALS AND METHODS

Animals. Female BALB/c mice (BALB/cAnNCrI BR) approximately 50 days old and weighing 19–20 g were obtained from Charles River, Inc. (St-Constant, Quebec, Canada) and housed 4–5/cage in a temperature (23 ± 1°C)- and light (14 h light/day, lights on at 6 a.m.)-controlled environment. The mice were fed rat chow and tap water ad libitum. The animals were ovariectomized under general anesthesia (Avertin) via bilateral flank incisions and randomly assigned to groups of 9–10 animals.

For the experiment described in Figs. 3 and 4, treatment with the inhibitors (INH) (5 or 20 μg, twice daily) was initiated on the day of ovariectomy, while treatment with estrone was started 5 days later, after which time both compounds were administered (estrone, 0.06 μg, twice daily) in combination for a 10-day period. Compounds were dissolved in ethanol and administered in 1% (w/v) gelatin-0.9% NaCl solution. The experiment was performed in a blind manner, i.e., the identity of the compounds administered was revealed after completion of the experiments. Mice were killed by decapitation on the 15th morning following ovariectomy, 2–4 h after the last injection. For the

4 The abbreviations used are: DHEA, dehydroepiandrosterone; Δ4-dione, androstenedione; Δ4-diol, androst-5-ene-3,17β-diol; 17β-HSD, 17β-hydroxysteroid dehydrogenase; E1, estrone; E2, 17β-estradiol; DHEA-S, dehydroepiandrosterone sulfate.

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Fig. 1. Dual site of inhibitory action of the novel compounds and illustration of the biosynthetic steps involved in the formation of the active estrogens 17ß-estradiol and 17α-diol from the inactive precursors DHEA and A4-dione by 17ß-HSD (Arrow 2). 3β-HSD, 3β-hydroxysteroid dehydrogenase/17β-steroid dehydrogenase; TESTO, testosterone. DHEA is of adrenal origin while A4-dione is secreted by the adrenals and the ovaries in pre- as well as in postmenopausal women.


Antiuterotrophic and 17β-HSD-inhibitory Activity of D-Ring Unsatuated Compounds. A series of novel 1α-alkyl estradiol derivatives (Fig. 2) were tested for their antiestrogenic activity as well as their potential 17β-HSD-inhibitory activity in ovariec-tomized mice treated with E1, the immediate precursor of E2. The compounds examined were the D-ring unsaturated compound EM-140 (17β-hydroxy-14-ene) as well as compound EM-123 (17β-hydroxy-15-ene). In an attempt to prolong the metabolic half-life of these compounds, a dibenzoate derivative of EM-140, namely EM-226 (3,17β-dibenzoxy-14-ene), and a monobenzoate derivative of EM-123, EM-227 (3-benzoxyloxy-17β-hydroxy-15-ene), were synthesized. The halogenated compound EM-139 (16α-chloro,17β-hydroxy) was used as reference. All of these compounds, with the exception of EM-226, were quite potent in reversing the stimulatory effect of E1 on uterine weight (Fig. 3).

Statistical Analysis. Statistical significance was measured according to the multiple-range test of Duncan-Kramer (31). Data are expressed as means ± SEM.

RESULTS

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Assay of 17β-Hydroxysteroid Dehydrogenase Activity. Individual or pooled (2–3) uteri were homogenized with a Polytron in 1.0 ml phosphate buffer (20 mM KH2PO4, 0.25 mM sucrose, 1 mM EDTA, pH 7.5) containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride as well as antipain, pepstatin A, and leupeptin, 0.5 mg/ml each) and centrifuged for 30 min at 1000 × g. Aliquots of the supernatant were incubated for 60 min at 37°C in 0.5 ml phosphate buffer (12.5 mM KH2PO4, 20% glycerol, 1 mM EDTA, pH 7.5), containing 25 nM [3H]E1, [3H]E2, [3H]α-dione, or [3H]testosterone obtained from Amersham.

Radioactivity was measured in a scintillation spectrometer and the rates of product formation were calculated and expressed as percentage of the uterine 17β-HSD activity measured in E1-treated mice. Protein content was determined by the method of Bradford, using bovine serum albumin as standard (30). No correction was made for endogenous E1 or E2 concentrations which, following a 150-fold dilution of the original tissue, were less than 0.005 nM and 0.025 nM, respectively, while the final concentration of added substrate was 25 nM. The appropriate cofactors used were 1 mM NADH plus NADPH for the reduction of E1 and A4-dione or 1 mM NAD plus NADP+ for the oxidation of E1 and testosterone. The enzymatic reaction was stopped by chilling the incubation mixture in an ice-water slurry and adding 2 ml CH2Cl2. Twenty μg of unlabeled E2 and E1 or testosterone and A4-dione were added as carrier steroids. After first extraction with CH2Cl2, the organic phase was kept while the aqueous phase was extracted again with CH2Cl2. The two organic phases were then pooled and evaporated to dryness under nitrogen. The residue was suspended in 0.1 ml of chloroform:methanol (2:1, v:v) and an aliquot (20 μl) was chromatographed on thin-layer plates in a benzene:acetone (4:1, v:v) system. E1 and E2 were revealed by iodine staining while A4-dione and testosterone were visualized by UV light. The thin layer chromatography areas corresponding to E1 and E2 or A4-dione and testosterone were scraped and transferred to scintillation vials containing 0.5 ml ethanol to which 10 ml scintillation fluid were added. The radioactivity was measured in a scintillation spectrometer and the rates of product formation were calculated and expressed as percentage of the uterine 17β-HSD activity measured in E1-treated mice. Protein content was determined by the method of Bradford, using bovine serum albumin as standard (30). Statistical Analysis. Statistical significance was measured according to the multiple-range test of Duncan-Kramer (31). Data are expressed as means ± SEM.

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Fig. 3. Effect of treatment with EM-139, EM-140, EM-226, EM-123, or EM-227 at the twice daily dose of 5 or 20 μg on estrone-induced uterine weight in mice. **P < 0.01 versus E1-treated control. The weight of the control uteri in E1-treated ovariectomized mice was 63.4 ± 3.10 mg. INH, inhibitor.
(0.06 μg, twice daily) for 10 days, was 60 to 70% reduced by the 5-μg dose of all compounds (except EM-226), while the 20-μg dose reduced uterine weight by 95–100%.

We next examined, in the same experiment, the ability of these compounds to inhibit uterine 17β-HSD activity. As mentioned earlier, 17β-HSD catalyzes the interconversion of DHEA and Δ⁴-diol as well as E₁ and E₂. In addition, 17β-HSD catalyzes the conversion of Δ⁴-dione into the androgen testosterone which in turn can be transformed into the highly active estrogen E₂ by the enzyme aromatase (Fig. 1). For this reason, uterine 17β-HSD activity was assayed by using both estrogenic and androgenic substrates.

Uterine 17β-HSD activity in ovariectomized animals favors the formation of E₂ from E₁ (Fig. 4A) over the reverse reaction (Fig. 4B). On the other hand, treatment of ovariectomized mice for 10 days with E₁ (0.06 ng, twice daily) caused a marked 3- to 5-fold increase in the conversion of E₁ into E₂ (Fig. 4A) and Δ⁴-dione into testosterone (Fig. 4C) by 17β-HSD. Conversion by 17β-HSD of E₂ into E₁ (Fig. 4B) and testosterone into Δ⁴-dione (Fig. 4D) was increased 30-fold by treatment with E₁. With the exception of EM-226, the 3,17β-dibenzoate derivative of EM-140 which had no significant effect on uterine weight (Fig. 3), the D-ring unsaturated compounds proved to be very potent inhibitors of uterine 17β-HSD activity. In fact, EM-139, EM-140, EM-123, and EM-227 potently inhibited the formation of E₂ from E₁ by 55 to 60% at the low 5-μg dose and 90 to 95% at the 20-μg dose, while EM-226 had less potent but still significant inhibitory effects (Fig. 4A). The inhibitory potency of the same compounds on E₁ formation from E₂ (Fig. 4B) ranged from 50 to 55% at the 5-μg dose and 90 to 95% at the highest dose used (20 μg). An even higher degree of inhibition was observed on androgenic (Δ⁴-dione-testosterone) 17β-HSD activity, the percentage of inhibition of testosterone formation ranging from 85 to 90% at the 5-μg dose and 95 to 100% at the 20-μg dose (Fig. 4C). Similarly, Δ⁴-dione formation from testosterone (Fig. 4D) was 80–85% reduced by the low dose and 95–100% by the high dose.

Antiuterotrophic and 17β-HSD-inhibitory Activity of Halo- genated Compounds. The second experiment was carried out over a period of 11 days, during which a series of 16α-halogenated steroidal antiestrogens, namely EM-220 (16α-fluoro,17β-hydroxy), EM-221 (16α-fluoro,17α-hydroxy), EM-139 (16α-chloro,17β-hydroxy), EM-170 (16α-chloro,17α-hydroxy), EM-105 (16α-bromo,17β-hydroxy), and EM-156 (16α-iodo,17β-hydroxy), were tested for their antiuterotrophic and 17β-HSD-inhibitory properties. Since, as observed in the previous experiment (Fig. 3), the 20-μg dose of the antiestrogenic compounds nearly completely reversed the stimulatory effect of E₁ on uterine weight, lower doses (3 and 10 μg) of the compounds were used in order to measure more accurately relative differences in their potency. While 3 μg of EM-220, EM-221, EM-139, and EM-170 caused a 35 to 65% inhibition (P < 0.01) of E₁-stimulated uterine weight (Fig. 5), EM-105 inhibited only by 30% (P < 0.01), while EM-156 had no significant effect at this low dose. Treatment with the 10-μg dose, on the other hand, led to significantly greater inhibitions by EM-220, EM-221, EM-139, and EM-170, while no additional inhibitory effect was seen with EM-221 and EM-105. It should be mentioned that no significant stimulatory effect was observed on uterine weight with any of these novel compounds in the absence of E₁, thus demonstrating their pure antiestrogenic effect, at least at doses which can completely reverse the stimulatory effect of "physiological" doses of estrone.

Treatment of ovariectomized mice with E₁ (0.06 μg, twice daily) for 6 days led to a 6-fold increase in uterine 17β-HSD activity as measured by conversion of E₁ into E₂ (Fig. 6A), while a 3-fold increased conversion of Δ⁴-dione into testosterone was observed (Fig. 6B). Treatment with the twice daily
Fig. 5. Effect of treatment with EM-220, EM-221, EM-139, EM-170, EM-105, or EM-156 at the twice daily dose of 3 or 10 μg for 10 days on E<sub>1</sub>-induced uterine weight in mice. **, P < 0.01 versus E<sub>1</sub>-treated control (CONT). The weight of the control uteri in E<sub>1</sub>-treated ovariectomized mice was 56.2 ± 4.16 mg.

Fig. 6. Effect of treatment with EM-220, EM-221, EM-139, EM-170, EM-105, or EM-156 administered as described in legend to Fig. 5 on 17β-hydroxysteroid dehydrogenase activity in mouse uterus. A, conversion of E<sub>1</sub> into E<sub>2</sub>; B, conversion of Δ<sup>4</sup>-dione into testosterone (TESTO). The measurement of 17β-hydroxysteroid activity was performed as described in Fig. 4. INH-inhibitor, **, P < 0.01 versus E<sub>1</sub>-treated control (CONT).

dose of 3 μg of EM-220, EM-221, EM-139, and EM-105 led to respective 50, 48, 52, and 40% inhibitions (P < 0.01) of estrogenic (E<sub>1</sub>-E<sub>2</sub> conversion) 17β-HSD activity, while EM-170 and EM-156 had no significant effect. When the 10-μg dose was used, respective inhibitions of 83, 75, 85, 70, 45, and 40% (P < 0.01) were measured on the same parameter for EM-220, EM-221, EM-139, EM-170, EM-105, and EM-156. When Δ<sup>4</sup>-dione was used as substrate (Fig. 6B), the inhibition observed with the 3-μg dose was similar to that achieved on estrogenic 17β-HSD activity with the 10-μg dose. In fact, at the 10-μg dose, all compounds, except the weaker EM-156, led to a 95–100% inhibition of E<sub>1</sub>-induced 17β-HSD activity (P < 0.01).

Comparison between Tamoxifen and EM-139. Since tamoxifen is the antiestrogen currently used for the therapy of breast cancer in women (2–5), we have compared its activity to that of the pure antiestrogen EM-139 on E<sub>1</sub>-induced uterine weight (Fig. 7A) and 17β-HSD activity (Fig. 7, B and C). It can be seen that while EM-139 caused 85 and 90% reversals of the stimulatory effect of E<sub>1</sub> on uterine weight (P < 0.01), at the 3- and 10-μg doses, respectively, tamoxifen, at the same doses, had no significant inhibitory effect. It can also be seen in Fig. 7 that while EM-139 inhibited uterine estrogenic (Fig. 7A) and androgenic (Fig. 7B), 17β-HSD activities by 55 to 100% at the two doses used, tamoxifen was without significant inhibitory effect on the same parameters.

DISCUSSION

The present data show that a series of new estrogen derivatives demonstrate not only pure antiestrogenic activity in the sensitive in vivo mouse uterus assay, but simultaneously exert potent inhibitory effects on 17β-hydroxysteroid dehydrogenase. As mentioned above and illustrated in Fig. 1, the enzyme 17β-HSD is responsible for the last step in the biosynthesis of the two active estrogens in women, namely E<sub>2</sub> from E<sub>1</sub> and Δ<sup>4</sup>-dilol
from DHEA. Despite its moderate estrogenic potency, Δ4-diol, due to its relatively high plasma concentration, has substantial estrogenic activity in women. Although 17β-HSD was first purified from human placenta more than 20 years ago (32–34), it is only recently that the structure of the enzyme (21, 22), as well as of its genes and its chromosomal localization (21, 23), could be determined.

Most importantly, 17β-HSD is not only expressed in classical steroidogenic tissues such as the ovary, testis, and placenta, but also in a large series of peripheral target tissues (23), including normal and cancerous breast tissue (35–38). Since estrogens are thought to play a major role in breast cancer (2–7) and 17β-HSD is the enzyme catalyzing the formation of estrogens, it is likely that 17β-HSD is a major factor controlling intracellular estrogen concentration and breast cancer growth. While the formation of E1 from E2 is predominant in most tissues, including the endometrium, there is a higher level of reductive activity of 17β-HSD in breast cancer cells (39, 40). In addition, 17β-HSD activity has been reported to be higher in cancerous as compared to normal breast tissue (41, 42), thus further indicating the importance of blocking this enzymatic step.

The situation of a high secretion rate of precursor adrenal androgens in women is completely different from most animal models used in the laboratory, namely rats, mice, guinea pigs, or others, where sex steroids are secreted exclusively by the gonads. These findings opened a new field of endocrinology, namely intracrinology or "intracrine" secretion (15, 16), a terminology complementary to the well known autocrine, paracrine, and endocrine activities, where a hormone acts at the surface of the producing cells (autocrine), a hormone acts on neighboring cells (paracrine), or a hormone is released in the circulation before reaching distant target tissues (endocrine). Through intracrine activity, locally produced androgens and/or estrogens exert their action inside the same cells where their synthesis takes place.

The daily secretion rate of Δ4-dione in normal cycling women is 1.5 mg, equal amounts being of ovarian and adrenal origins in premenopausal women (43, 44). In postmenopausal women, about 70% of circulating Δ4-dione is of adrenal origin, the rest originating from the ovary (45). Δ4-Dione can either be transformed into the androgens testosterone and dihydrotestosterone or into the estrogen E2, depending upon the ratio of 17β-HSD, 5α-reductase and aromatase activities present in peripheral tissues (Fig. 1). In addition to E2, another important estrogen derived from DHEA is Δ5-diol. This steroid has in fact been shown to exert direct estrogenic effects in both normal and malignant estrogen-sensitive tissues at concentrations found in the circulation of normal adult women (7, 14, 17–20, 46). From 80 to 100% of Δ5-diol in the circulation has been found to derive from circulating DHEA-S and DHEA in postmenopausal as well as young women (14, 46). Such transformation of DHEA-S and DHEA into Δ5-diol requires the activity of steroid sulfatase and 17β-HSD.

In addition to the classical steroidogenic tissues, namely the ovariess, testes, adrenals, and placenta, a large series of human peripheral tissues possess all the enzymatic systems required for the formation of active androgens and estrogens from a relatively constant supply of precursor steroids provided by the adrenals. While, so far, most therapeutical approaches have been aimed and limited at controlling steroid formation by the classical steroidogenic tissues, it is clear that most efforts should now be turned toward steroid formation in peripheral target tissues, or intracrinology, in order to better understand the physiological mechanisms controlling local steroid formation and thus be in a position to develop novel therapeutical approaches which take into account the high proportion of steroids made locally and responsible for the growth and function of normal as well as cancerous tissues. The field of intracrinology should generate major interest in the pharmaceutical field in order to develop specific inhibitors of enzymatic activity as well as more potent and specific antiestrogens and antiandrogens. After all, the classical sources of sex steroids, namely the testes and ovaries, can be easily controlled by administration of the exceptionally well tolerated and reversible luteinizing hormone-releasing hormone agonists (47).

The present data show that a series of new steroidal compounds possessing pure and potent antiestrogenic activity in vivo are also potent inhibitors of the activity of 17β-HSD, the enzyme responsible for estrogen biosynthesis. These compounds act as competitive inhibitors of 17β-HSD.3 The availability of such compounds should be useful, not only for a better understanding of estrogen formation and action, but they could well lead to an improved endocrine therapy of breast cancer and other malignant and nonmalignant estrogen-sensitive diseases.

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