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In vivo estrogen bioactivities and in vitro estrogen receptor binding and transcriptional activities of anticoagulant synthetic 17β-aminoestrogens

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Abstract

Estrogenic activities of the two 17β-aminoestrogen (AE) derivatives, prolame and butolame, were studied upon coagulation, serum luteinizing hormone (LH) and uterine weight, including endometrial morphology in castrated female rats. We have also investigated the ability of these two compounds, as well as another AE pentolame, to activate transcription through the estrogen receptor α (ERα) and the estrogen receptor β (ERβ). Administration of prolame and butolame to castrated animals increased significantly ($P < 0.01$) the mean clotting time when compared with that obtained in the group of control animals. Butolame was a more potent anticoagulant than prolame ($P < 0.01$), as judged by their corresponding IC_{50} (5.4 ± 0.65 and 66.6 ± 2.57 μg/animal, respectively). In contrast, estradiol significantly shortened blood clotting times ($P < 0.005$). Both prolame and butolame caused a significant inhibition of serum LH levels (EC_{50} 8.10 ± 0.79 and 17 ± 64 μg/animal, respectively), and restored castration-induced reduction in uterine weight of ovariectomized rats (EC_{50} 4.14 ± 1.57 and 17.0 ± 1.78 μg/animal, respectively). In terms of the effects of prolame, butolame and pentolame in transient transfection assays, all the three AE activated ER dependent reporter gene expression, however, only at high concentrations. Prolame had the highest activity followed by butolame and pentolame. Induction of transcription by these compounds was preferentially mediated through the ERα, especially in the case of pentolame where little, if any, activation occurred through the ERβ. None of the compounds showed antagonistic activities through either ER subtype. The overall data suggest that modifications in the structure and length of the amino-alcohol side-chain at C-17 might have an impact on the affinity and estrogenic intrinsic properties of AE at the level of diverse target tissues. © 2000 Elsevier Science Ltd. All rights reserved.

1. Introduction

Synthetic 17β-aminoestrogens (AE) differ from natural estrogens in their ability to affect blood-clotting times. In contrast to 17β-estradiol (E_2), AE induce long lasting anticoagulant effects also inhibiting, in vitro, the process of platelet aggregation [1–6]. To our knowledge, only one of these steroids (pentolame) has been proved in terms of its estrogenic potencies upon the neuroendocrine and reproductive organs of female rats. In a previously published study by our group [6], pentolame, an AE derivative containing an amino-alcohol side chain (–NH–CH_2)*n*–OH at C-17 with five methylenes, demonstrated a low degree of estrogenicity on the pituitary and endometrium. At the same time, pentolame showed significant opposite effects as compared to E_2 upon blood coagulation. These obser-

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vations, especially those concerned with pentolame's relative capability of suppressing pituitary gonadotropin release, are potentially important in providing opportunities to improve the design of new agonistic and/or antagonistic analogues of sex steroids with tissue specific actions and disease-prevention benefits. In this regard, it is known that differences in blood clotting times can be obtained by small changes within the chemical structure of the 17β-amino side-chain [3]. This observation suggests that modification in the structure, including the length of the amino-alcohol side-chain at C-17, might also have an impact on the intrinsic estrogenic properties of these compounds. In order to test this hypothesis, we have studied the anticoagulant and estrogenic effects under in vivo conditions, including the relative binding affinity (RBA) to intracellular steroid receptors, as well as estrogen receptor(ER)-mediated transcriptional activities, of three structurally different AE with side chains of 3, 4 and 5 methylenes.

2. Material and methods

2.1. Reagents

Non-radioactive estradiol: 1,3,5(10)-estratrien-3,17β-diol and estrone: 3-hydroxy-1,3,5(10)-estratrien-17-one were purchased from Sigma Chemical (St. Louis, MO) and its chemical purity established by paper chromatography. The 3-amino-1-propanol, 4-amino-1-butanol and sodium borohydride were obtained from Aldrich (Milwaukee,WI). [2,4,6,7-^{3}H]estradiol ([^{3}H]-E_2) s.a. 97 Ci/mmol was purchased from Amersham (Buckinghamshire, UK). Radiochemical purity of labeled steroids was established by thin-layer chromatography and repeated crystallizations to constant s.a. Radioactivity was determined in a Packard Tri-Carb liquid scintillation spectrometer (Model 1900 TR) with a counting efficiency for [^{3}H] of 60%, using Ready Solv HP (Beckman Instruments, Palo Alto, CA). All other solvents and reagents used were of analytical grade.

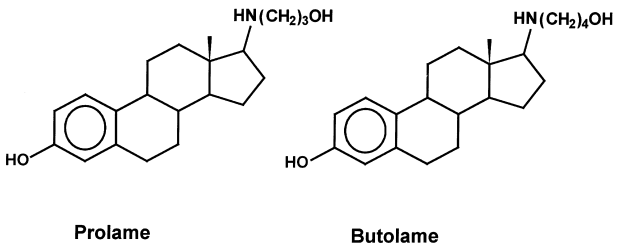
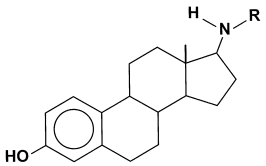


Fig. 1. Structural formulae of R_1: prolame [17β-(3-hydroxy-1-propylamino)-1,3,5(10)-estratrien-3-ol], R_2: butolame [17β-(4-hydroxy-1-butylamino)-1,3,5(10)-estratrien-3-ol] and R_3: pentolame [17β-(5-hydroxy-1-pentylamino)-1,3,5(10)-estratrien-3-ol].

2.2. Chemical synthesis

17β-(3-hydroxy-1-propylamino)-1,3,5(10)-estratrien-3-ol (prolame), 17β-(4-hydroxy-1-butylamino)-1,3,5(10)-estratrien-3-ol (butolame) (Fig. 1) and 17β-(5-hydroxy-1-pentylamino)-1,3,5(10)-estratrien-3-ol (pentolame) were synthesized from estrone according to the methods described previously [1,7]. Chemical purity was established by spectral (IR/NMR/MS) and chromatographic (HPLC, TLC) techniques as previously reported [1,7].



R_1= $(CH_2)_3OH$
R_2= $(CH_2)_4OH$
R_3= $(CH_2)_5OH$

2.3. Constructs

The expression vectors for ERα and ERβ (pCMV5-hER and pCMV5rERβ) containing the full-length coding sequence of the ERα and ERβ, respectively, kindly provided by Dr. B.S. Katzenellenbogen of the University of Illinois (Urbana, IL), were prepared for transfection into HeLa cells as previously described [8]. The estrogen responsive reporter plasmid (ERE-E1bCAT) contains a fragment of the vitellogenin A_2 gene promoter (positions −331 to −87) upstream of the adenovirus E1b TATA box fused to the chloramphenicol acetyltransferase (CAT) gene [9].

2.4. Animals and tissues

Adult female Wistar rats (200–250 g) used in this study were housed under a lighting schedule of 12-h light/12-h darkness with free access to food and water. Gonadectomies were performed under anesthesia with 4% chloral hydrate, 20 days prior to hormonal treatment. Animals were subcutaneously injected for five consecutive days with 0.3 ml of propyleneglycol containing either prolame, butolame or E_2 at different concentrations. Control animals received the vehicle alone. At the desired times, animals were killed by decapitation. Blood collected from the cervical wound (trunk blood) was centrifuged and serum stored individually at −20°C until assayed. The appropriate tissues were immediately removed, blotted, weighed and processed as described below. Uteri were fixed in 10% formaldehyde and submitted for histological examination.

2.5. Hormonal assays

Serum concentrations of pituitary luteinizing hormone (LH) were measured in duplicate by specific radioimmunoassay (RIA) using reagents and protocols provided by the National Hormone and Pituitary Program (Rockville, MD, USA) as previously described [10]. The results were expressed as the mean ± SE in ng/ml according to NIAMDD rat LH R_p−3, used as reference preparation. The intra- and inter-assay coefficients of variation were 4.6 and 8.2%, respectively.

2.6. Receptor binding studies

Immature female intact rats (80–100 g) without estrogen priming were used for these studies. Uterine cytosols were prepared after tissue homogenization in TEDM buffer (20 mM Tris–HCl, pH 7.4 at 4°C, 1.5 mM EDTA, 0.25 mM dithiothreitol, 10 μg/ml leupeptine and 10% glycerol) using 10 s bursts of a polytron homogenizer (Brinkmann Instruments, Westbury, NY) in a tissue buffer ratio of 1:6 (w/v). The homogenates were centrifuged at 105,000 × *g* for 1 h at 2°C in a SW 50.1 rotor (Beckman Instruments). The RBA of prolame and butolame to cytosol ER were evaluated by incubations containing 1 nM [^{3}H]-E_2 in the presence of increasing concentrations (10^{-9}–10^{-3} M) of the non-radioactive corresponding steroids for 18 h at 4°C. Bound and free steroids were separated by the addition of Dextran-coated charcoal suspension (250 mg Norit-A and 25 mg Dextran T-70 in 100 ml of TEDM buffer). After centrifugation at 800 × G for 15 min at 4°C, the supernatants were assayed for radioactivity determination. The RBA of each competitor was calculated from their abilities to displace [^{3}H]-E_2 bound from its receptor as previously described [11].

2.7. Transfections and CAT assay

HeLa cells were plated the day before transfections at a density of 3.0×10^5 cells/well/six-well plate in Dulbecco's modified Eagle's medium without phenol red, which was supplemented with 5% stripped FBS (HyClone Laboratories, Logan, UT) and 10,000 U of Penicillin and Streptomycin (DMEM-HG) and maintained in 5% CO_2 at 37°C. The next day, the HeLa cells were visualized on a microscope to verify that the cell density was between 30 and 50% confluent. Transfections were performed in triplicate using SuperFect™ (Qiagen, Valencia, CA), using reagents and protocols provided by the manufacturer. Briefly, serum-free media (0.1 ml) was aliquoted and DNA samples added, after vortexing; 10 μl of SuperFect™ reagent was added and vortexed for 10 s. After incubation at room temperature for 5–10 min, 0.6 ml of supplemented DMEM-HG was added to each sample. The medium containing the transfection complexes (1 μg of the reporter gene plasmid, 25 ng of ER expression vector) was added to the cell monolayer, which had previously been rinsed with PBS. The plates were incubated for 3 h at 37°C in 5% CO_2. After incubation, the plates containing the transfection complexes were rinsed with PBS and 3 ml of complete growth medium was added to each well. Twenty-four hours later, the medium was replaced with complete medium containing the compounds of interest. Dimethyl sulphoxide (DMSO) or ethanol (EtOH) were used as steroid vehicles. CAT activity using 5 μg of protein, 25 μg of butyryl coenzyme-A (Sigma Chemical, St. Louis, MO), 2×10^5 cpm of xylene-extracted [^{3}H]-chloramphenicol (Dumont-NEN Research Products, Boston, MA) in 0.25 M Tris–HCl, pH 8.0, was assayed as described [12–14].

2.8. Blood clotting time

Blood-clotting time was measured in vehicle and steroid-treated castrated female rats 24 h after the last steroid administration. After warming the tail of the animal in water at 40°C, a small cut was made at the tip using a sharp razor blade as reported earlier [6]. A blood sample (25 μl) was collected from the tip of the tail into a microhematocrit glass capillary tube, and was made to flow with gravity by tilting the capillary

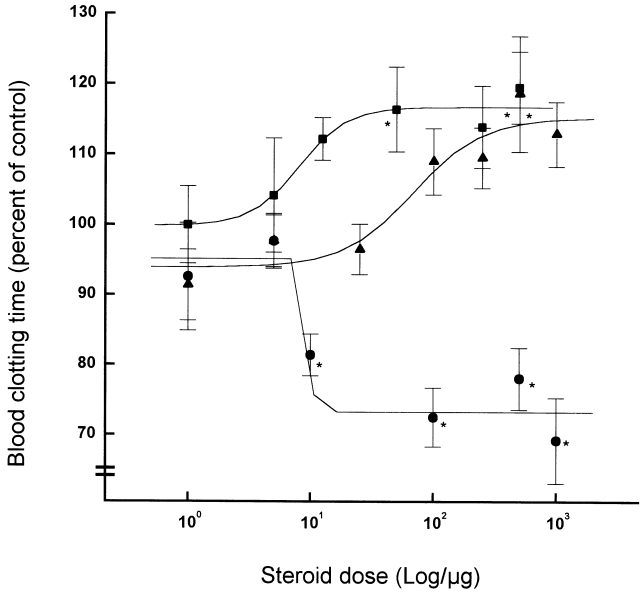


Fig. 2. Effects of prolame (▲), butolame (■) and estradiol (●) upon blood clotting times in castrated female rats. Twenty days after ovariectomy, rats were s.c. injected with 0.3 ml of propyleneglycol containing various concentrations of the corresponding steroid (range 1–1000 μg/animal) for five consecutive days. Blood clotting times were recorded 24 h after the last injection. Each point represents the mean ± SD of seven rats. Control rats (100%) received only the vehicle alone. $*\pm P < 0.01$ vs. vehicle.

tube to angles of $+60^{\circ}$ and -60° with respect to the horizontal plane until the blood ceased to flow (reaction end-point).

2.9. Statistical analysis

Statistical significance among groups were analyzed employing one-way analysis of variance (ANOVA) and differences between the control and the treated groups were estimated by Dunnet's or Student–Newman–Keul's methods as appropriate. Differences were considered significant at a level of P less than or equal to 0.05. Data are representative of at least three independent experiments.

3. Results

3.1. Effects of prolame and butolame on blood clotting times

Fig. 2 shows the dose–response effects of prolame, butolame and E_2 on blood clotting times in long-term ovariectomized rats. Different doses of steroids were administered daily for five consecutive days and evaluations on blood coagulation were done 24 h after the last injection. As shown, both prolame and butolame increased significantly the mean time of bleeding when compared to that observed in the vehicle-treated control group (100%). Doses above 10 μg/animal of both AE resulted in values significantly different to those observed in the control group. In addition, butolame was a more potent anticoagulant than prolame ($P < 0.01$) as judged by their corresponding IC_{50} upon blood coagulation (5.4 ± 0.65 and 66.6 ± 2.57 μg/animal, respectively). In contrast, mean clotting times were significantly shortened ($P < 0.01$ vs. vehicle) in the group of E_2 treated rats, whose values were always significantly lower ($P < 0.005$) when compared to those obtained with similar doses of both AE.

3.2. In vivo estrogenicity

In order to determine the effects of AE on the hypothalamic–pituitary unit, we measured the serum concentrations of radioimmunoassayable LH in long-term castrated female rats treated for five consecutive days with different doses of prolame and butolame. As illustrated in Fig. 3, administration of both AE on ovariectomized rats at doses 25–1000 μg/animal caused a significant decrease ($P < 0.05$) in serum LH levels compared to those in the ovariectomized control group. In terms of LH suppressing activities of these two AE, prolame showed a significantly higher potency ($P < 0.05$) than butolame with IC_{50} of 8.10 ± 0.79 and 17 ± 2.64 μg/animal, respectively. In addition,

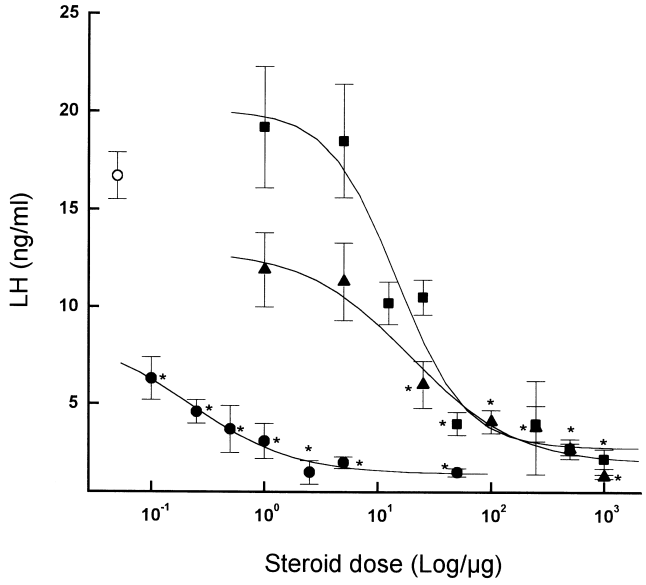


Fig. 3. Effects of different doses of prolame (▲), butolame (■) and estradiol (●) upon circulating LH levels of castrated female rats. Twenty days after gonadectomy, rats were s.c. injected with 0.3 ml of propyleneglycol containing different concentrations of steroids (range 0.1–1000 μg/animal) for five consecutive days. One day after the last injection, rats were killed by decapitation and trunk blood collected and processed in duplicate for LH measurements. Control rats received the vehicle alone (○). Each point represents the mean ± SD of seven to eight rats. $* \pm P < 0.05$ vs. control.

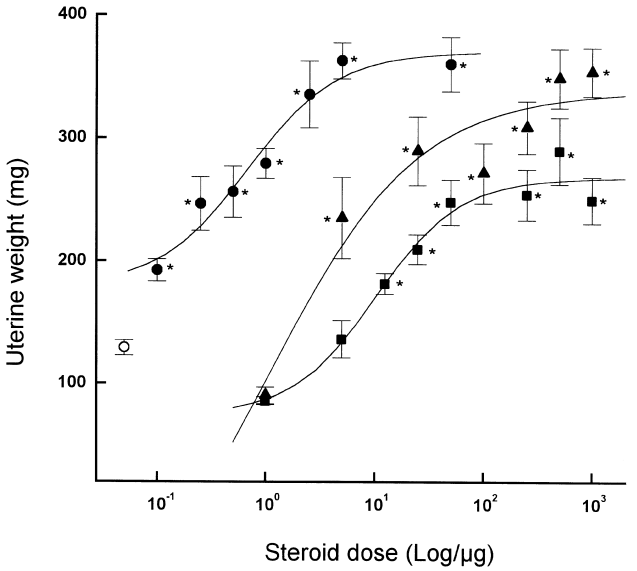


Fig. 4. Dose-dependent effects of prolame (▲), butolame (■) and estradiol (●) on uterine weight in castrated female rats. Twenty days after castration, 5-day treatment was started at the indicated doses. Control ovariectomized rats received the vehicle alone (○). Each point represents the mean ± SD of seven to eight rats. $* \pm P < 0.05$ vs. control rats.

administration of E_2 effectively suppressed the high postovariectomy concentrations of LH (IC_{50} 0.21 ± 0.1 μg/animal). As shown in Fig. 3, E_2, at the dose of 2.5 μg/animal, brought LH up to or below the limits of detection of the assay.

The estrogenic effects of prolame and butolame on the reproductive female tract were studied by their ability to restore the castration-induced reduction in uterine weight. Fig. 4 shows the pattern of uterine weights of ovariectomized rats treated with different doses of both AE derivatives and E_2. As shown in this figure, E_2 treatment caused a major increase in uterine weight (EC_{50} 0.6 ± 0.3 μg/animal), whereas prolame and butolame were less effective in terms of their uterotropic activity (EC_{50} 4.14 ± 1.57 and 17.0 ± 1.78 μg/animal, respectively). However, both AE significantly increased the uterine weight compared to that seen in ovariectomized rats ($P < 0.05$ at the EC_{50}, of both AE, respectively). Again, prolame showed a significantly higher estrogenic potency than that obtained with butolame ($P < 0.05$ at their corresponding EC_{50}). The endometrial hystological changes induced by both prolame and butolame were characterized by an increase in both stromal and epithelial cells. Morphologically, the major findings consisted of enlargement of endometrial gland, the epithelial cells showed a columnar pattern and there was an increase in the number and volume of stromal cells. These morphological findings were similar to those obtained in rats treated with E_2 (data not shown) and to those previously described for pentolame [6].

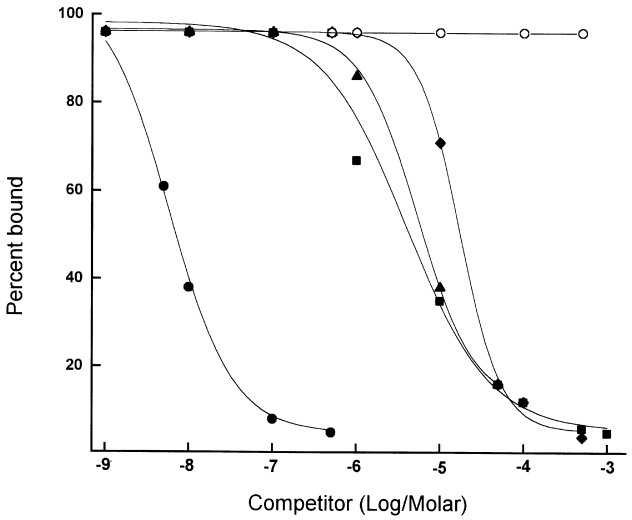


Fig. 5. Estrogen receptor binding activity of prolame (▲), butolame (■), pentolame (◆), estradiol (●) and ORG-2058 (○). Uterine cytosols from immature female rats were labeled with [^{3}H]-E_2 at 4°C in the absence or presence of increasing concentrations of the corresponding unlabeled steroid. Bound and free fractions were separated by the addition of a dextran-coated charcoal suspension. Each point represents the mean of three determinations.

3.3. Receptor binding characteristics

In order to assess whether prolame and butolame interact with specific intracellular ER, we used cytosol fractions prepared from uteri obtained from immature intact non-steroid treated rats. Fig. 5 shows competition curves of non-labeled synthetic AE and E_2 for specific estrogen binding sites in the rat uterus. The results demonstrated that all synthetic AE tested competed with [^{3}H]-E_2 for the ER. The ability of prolame and butolame to displace [^{3}H]-E_2 bound from its cytosolic receptor, expressed as their RBA, was substantially lower than that of unlabeled E_2 (0.11 and 0.14, respectively). Pentolame showed the lowest ability to displace [^{3}H]-E_2 from its receptor. As expected, addition of increasing concentrations of unlabeled ORG-2058, a synthetic specific ligand for progesterone receptors, did not displace [^{3}H]-E_2 bound from uterine cytosolic estrogen-binding sites.

3.4. Estrogen receptor-mediated transcription activation by 17β-aminoestrogens

To determine whether AE would regulate transcription through the ERα or ERβ, a reporter plasmid containing a canonical estrogen response element driving expression of the chloramphenicol acyltransferase reporter was transiently transfected into HeLa cells. In the absence of cotransfected expression vectors for both subtypes of human ER, induction of CAT reporter transcription by the different compounds was not observed. However, cotransfection with an expression vector for the human ERα with the reporter construct resulted in a significant dose-dependent CAT transcriptional activation after incubation with E_2, prolame and butolame (EC_{50} 4.0×10^{-11} m, 4.7×10^{-9} m and 1.2×10^{-8}M, respectively). In this study, we have also tested, for transcriptional activation, another similar AE derivative (pentolame) whose in vivo estrogenic activity has been previously described by this laboratory [6]. As shown in Fig. 6A addition of pentolame to cotransfected HeLa cells was followed by a lower CAT transcriptional potency (EC_{50} 1.1×10^{-7}M) than that observed with similar doses of prolame and butolame, respectively. However, similar responses were obtained when higher concentrations (1 μM) of all AE were added. Estradiol induced the highest six-fold increment in CAT transcriptional activation. In terms of the ERβ (Fig. 6B), E_2, prolame and butolame increased CAT activity in a dose-dependent manner; however, higher concentrations of these ligands were required (EC_{50} 2.1×10^{-10}, 9.0×10^{-8} and 1.16×10^{-7} M, respectively). Pentolame even at high concentrations showed the lowest ability to increase CAT activity through activation of the ERβ (EC_{50} 9.1×10^{-7} M). In order to investigate whether these compounds, especially

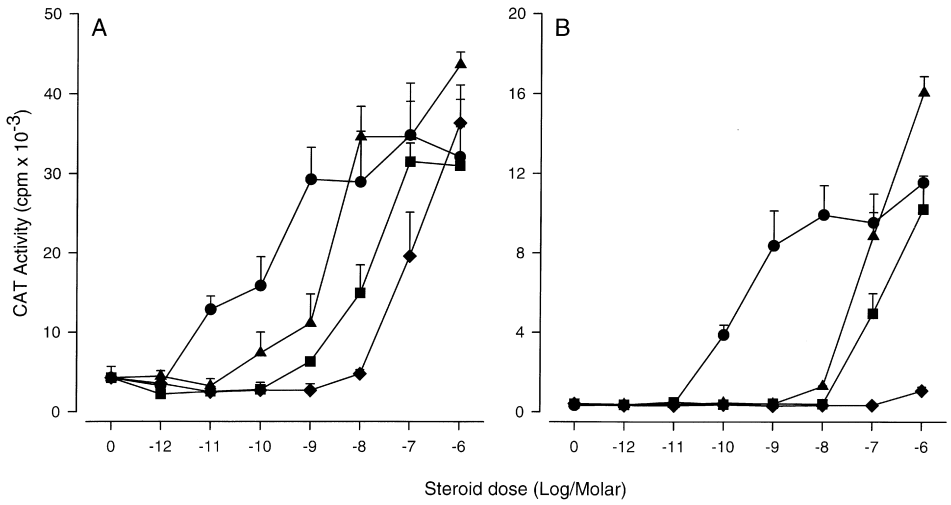


Fig. 6. Effect of 17β-estradiol (●), prolame (▲), butolame (■) and pentolame (◆) on ERα (Panel A) and ERβ (Panel B) mediated reporter activity. HeLa cells were transfected with the expression vector for ERα or ERβ and an (ERE) CAT reporter gene (EREelbCAT). After 24 h, cells were harvested and assayed for CAT activity. Values are the mean ±SD of a representative experiment per triplicate.

pentolame, act through the ERβ as antagonists, HeLa cells transfected with an expression vector for ERβ, together with the estrogen-responsive reporter gene, were treated with E_2 (1×10^{-9} M) in the presence or absence of increasing concentrations of prolame, butolame or pentolame, respectively (10^{-8}–10^{-6}M), or the antiestrogen 4-hydroxytamoxifen (10^{-7}M). As shown in Fig. 7, with the exception of hydroxytamoxifen (Panel A), none of the AE tested significantly inhibited estradiol-induced reporter gene activation (Panel B). Similar results were obtained with the ERα (data not shown).

4. Discussion

In the process of investigating tissue-selective activity of contraceptive synthetic steroids, our group has discovered the anticoagulant properties of certain estrogen derivatives that could be of potential therapeutic use [1–6]. In this regard, several observations indicate that administration of natural and synthetic steroids (estrogens and progestins) are associated with a decreased incidence of endometrial and ovarian cancer due to the progestin component in the formulation and the suppression of gonadotropin secretion.

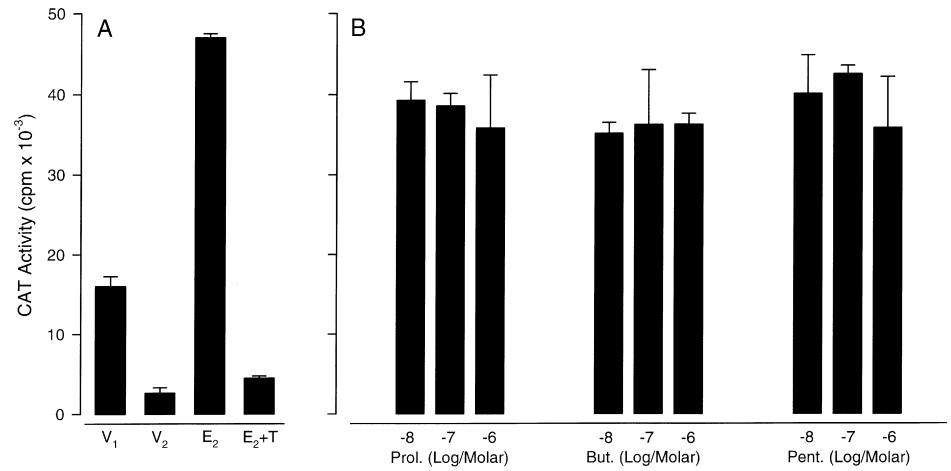


Fig. 7. ERβ-mediated CAT inhibition. HeLa cells transfected with the expression vector for ERβ and an (ERE) CAT reporter gene (EREelbCAT) were incubated with 1nM E_2 in the presence or absence of 4-hydroxytamoxifen (T) (Panel A), and various concentrations (10^{-8}–10^{-6}M) of prolame (Prol), butolame (But) or pentolame (Pent) (Panel B). Control incubations were performed in the presence of only the vehicles (V1: DMSO and ethanol) or the vehicle (ethanol) plus T (V2) (Panel A). Values are the mean ± SD of a representative experiment per triplicate.

Although most of the metabolic studies demonstrate favorable effects of estrogen therapy, its prescription in subjects with pre-existing metabolic abnormalities may be contraindicated. Therefore, the design and development of hormones with tissue-specific actions might be of importance for individuals with high risks of sex-steroid related diseases. In this study, two additional AE derivatives were investigated in terms of their estrogenicity at the neuroendocrine level and reproductive organs of the female rat. The overall objective of this work was to discriminate, among the available non-toxic synthetic AE, compounds with unique agonist and/or antagonist properties at the level of steroid responsive tissues in an effort to develop new drugs for targeted sub-populations. The results of this study allowed us to support and extend previous observations [3] indicating that actions of AE upon blood coagulation might be due to changes in the structure and length of the amino-alcohol side-chain at C-17. Indeed, a recent report from this laboratory [6] indicated that pentolame, an AE with five methylenes at the 17β amino-alcohol side-chain, elicited a significant increase in blood clotting times when injected into adult castrated female rats. However, when compared with E_2, its effects on the pituitary and endometrium showed a low degree of estrogenicity. These observations, together with the results presented in this communication, suggest that a short side-chain is associated with lower anticoagulant but higher estrogenic potencies of these compounds. From the results in previous studies, including those presented herein, it is not possible to convincingly explain the mechanisms by which AE function as agonists in reproductive organs but not in the coagulation system. Interestingly, a number of observations suggest that natural and synthetic steroids act in a tissue-specific manner, as in the case of androgens [15,16] and synthetic compounds, such as tamoxifen [17] or members of the 19-nor testosterone family [18,19]. These observations may correspond to a general phenomenon of more than one hormone action that are determined by the presence of tissue-specific transcription factors, coactivators or corepressors, receptors sub-types or implying also the bioconversion to metabolites with different receptor–ligand interactions [18,20].

It was clear from the data reported herein that prolame and butolame have the abilities to interact with specific E_2 receptors with RBA significantly lower than those for the natural hormone. It has been firmly established that ER complexes are not always recognized and transcribed similarly in all cells [21] leading to the concept that a number of ligands acting through the same receptor can express distinct effects in different cells [21,22]. These observations may, in part, explain the relative antagonistic and agonistic effects of AE upon coagulation and the reproductive system, respectively.

In this study, we have determined whether prolame, butolame and pentolame, that differ from each other in their biological potencies to alter coagulation and pituitary LH release, were able to regulate transcription through the ER. The overall results demonstrated that all AE through interaction with the ERα up-regulated gene CAT expression in a reporter plasmid containing a consensus ERE. Interestingly, pentolame had a lower potency to activate transcription indicating most likely a direct effect of the amino-alcohol side-chain composition on ligand–receptor interactions and activation. The question remains, however, whether difference in responses of these two AE observed either in vivo or in vitro are mediated directly at the transcriptional level or through changes in steroid–receptor interaction.

From a more general perspective, the data presented herein demonstrate that AE modulate transcription via E_2 receptors, whose transcriptional activities are most probably affected by the amino-alcohol side-chain composition at C-17. In addition, it seems plausible that further identification of E_2 receptor modulators, such as AE at the molecular level, could allow the development of therapeutic tissue-selective new drugs for targeted sub-populations.

Acknowledgements

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