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In vivo and *in vitro* evaluation of the estrogenic properties of the 17β-(butylamino)-1,3,5(10)-estratrien-3-ol (buame) related to 17β-estradiol

Cristina Lemini[1], Martha Medina[1], María E. Avila[1], Pablo Cruz-Lemini[1], Enrique Canchola[2], René Santillan[3], Ana E. Lemus[2]

[1]Departament of Pharmacology, Faculty of Medicine, National Autonomous University of Mexico. Av. Universidad No. 3000, Ciudad Universitaria, Colonia Copilco Universidad, Delegación Coyoacán, Mexico City, Mexico

[2]Department of Reproductive Biology, Metropolitan Autonomous University-Iztapalapa, Mexico City, Mexico

[3]Department of Reproductive Biology, National Institute of Medical Sciences and Nutrition Salvador Zubirán, Mexico City, Mexico

Correspondence: Cristina Lemini, e-mail: clemini@servidor.unam.mx

Abstract:
Background: Buame [17β-(butylamino)-1,3,5(10)-estratrien-3-ol] possesses anticoagulant and antiplatelet activities that are potentially antithrombotic. Since its estrogenicity is unknown, it was evaluated by established methods.
Methods: Buame (10, 100, 500, and 1,000 μg/kg), 17β-estradiol (E_2) (100 μg/kg), or propylene glycol (10 ml/kg) were subcutaneously (*sc*) administered for three days to immature Wistar female rats (21 days old). The relative uterotrophic effect to E_2 was 78 (E_2 = 100) with a relative uterotrophic potency of 1.48 (E_2 = 100). Adult ovariectomized Wistar rats received an *sc* injection at 8:00 h (reversed cycle) of: 7.5 μg of E_2 (≈ 30 μg/kg), buame (≈ 750, 1,500, 3,000 μg/kg), or corn oil (≈ 1.2 ml/kg). After 24 h, progesterone (4–5 mg/kg) was administered. Sexual receptivity was assessed 5 to 7 h later, and the lordosis quotient (LQ; number lordosis/number mounts × 100) was evaluated.
Results: Buame induced lordosis (LQmax 85 ± 9; ED50 952 ± 19 μg/kg) and E_2 LQmax 56 ± 8; ED50 10 ± 2 μg/kg; the relative LQ-potency was 0.51 (E_2 = 100). Buame competed with [^{3}H]E_2 for the estrogen receptor (Buame RBA = 0.15 and Ki = 5.9×10^{-7} M; E_2 RBA = 100; Ki = 6.6×10^{-9} M). Buame increased MCF-7 cells proliferation, from 10^{-11} to10^{-9} M, its proliferative effect was 1.73–1.79 (E_2 = 3.0–3.9); its relative proliferative effect to E_2 was 33–40% (E_2 = 100%) and relative potency 10.4–10.7 (E_2 = 100). Tamoxifen and fulvestrant (ICI 182,780) inhibited buame's proliferation indicating mediation through estrogen receptors in this response.
Conclusion: Buame is therefore an estrogen partial agonist with a weak estrogenic activity.

Key words:
17β-aminoestrogens, uterothopic effect, binding, lordosis, MCF7 cells

Introduction

Women during their reproductive age have a lower incidence of cardiovascular disease and thromboembolic events compared with men of similar age [14]. The protective effects have been attributed to circulating estrogen levels. This protection seems to decrease at menopause, when the ovaries decrease their performance, or when surgical removal of the ovaries is re-

quired, leading to a state of hypoestrogenism inducing metabolic changes in various tissues and organs [12].

Exogenous estrogens are commonly used in hormone replacement therapy (HRT) after menopause and as oral contraceptives (OC). Numerous studies have evaluated the benefits and risks associated with their use [3, 27, 30, 32–34].

One of the major adverse effects associated with estrogen therapy is the risk of producing a thrombotic event. HRT is commonly utilized by postmenopausal women to prevent menopausal symptoms, but its use may increase the likelihood of venous thromboembolism (VTE) 2 to 4 times. Among the most common thromboembolisms presented by women are deep vein thrombosis, pulmonary embolism and stroke [34].

In the search of estrogens with both, the beneficial properties shown by the natural hormone and without the thrombogenic effects shown by currently available estrogens, our group has been working in the assessment of the pharmacological properties of the 17β-aminoestrogens (AEs).

These compounds have the capability of increasing clotting times producing prolonged anticoagulant effects in rats and mice. In contrast, E_2 and other synthetic estrogens shorten clotting times, producing procoagulant effects which are potentially thrombogenic in treated animals, [11, 20, 23, 24]. High doses of AEs administered in laboratory animals have shown not to be thrombogenic, indicating to be promising agents to be used in clinical conditions with thrombosis predisposition.

To explore the potential pharmacological use of these compounds, the evaluation of the estrogenic properties of the 17β-aminoestrogens is necessary. In previous reports, we have characterized the estrogenic effects of the homologous series of 17β-aminoestrogens (AEs) prolame, butolame, and pentolame. All of them possess a N-$(CH_2)_n$-OH chain that differs in the length of the chain substitution (n = 3, or 4, or 5, respectively) of the amino group at C17 of the steroid nucleus [13, 18, 20, 21, 23].

The mentioned AEs decrease serum luteinizing hormone (LH), increase uterine weight, produce endometrial morphological changes in ovariectomized (Ovx) rats, and induce progesterone receptors in the anterior pituitary of the Ovx rat [24]. These compounds are also capable of inducing lordotic behavior in female rats [18]. They activate transcription through the estrogen α and β receptors (ERα, ERβ) with preferential action on ERα. Their estrogenic effects are similar to those of 17β-estradiol (E_2) but with lower potency [13].

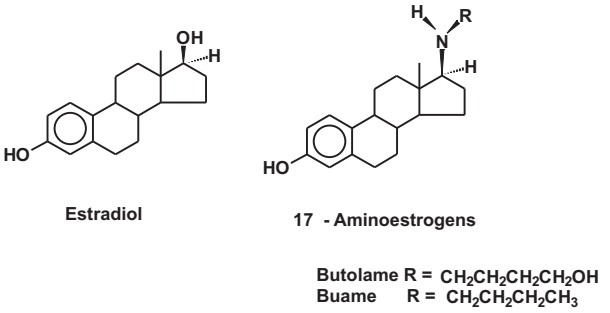

Fig. 1. Structural formulas of 17β-estradiol (E_2), and the 17β-aminoestrogen buame and butolame

Buame is a 17β-aminoestrogen that contains a butyl group as substituent on the amino side chain [–NH–C_4H_{10}] at the C-17 position of the steroid (Fig. 1). This compound is also able to produce anticoagulant effects in mice and has been reported to inhibit platelet aggregation, induced with ADP and in an *in vitro* model [5, 6, 9].

Buame's estrogenic properties have not yet been reported. For this reason, the aim of this work was to characterize the estrogenic properties of buame using the following well known *in vivo* and *in vitro* markers of estrogenicity: 1) uterotrophic assay in the immature Wistar rat, 2) capability to induce adult female rat sexual behavior, 3) binding affinity for the estrogen receptor utilizing pre-pubertal rats uteri, and 4) its capability to induce proliferation of MCF-7 human breast cancer cells whose proliferation rate depends on estrogens. In all the estrogenicity markers, buame's effect was compared with E_2 to estimate its relative effect and relative potency.

Materials and Methods

Reagents

Radioactive material and chemicals: [2,4,6,7-^{3}H]-estradiol ([^{3}H]-E_2), specific activity 72 Ci/mmol was supplied by NEW® Research Products (Boston, MA, USA). 16α-Ethyl-21-hydroxy-19-nor-pregn-4-ene-3,20-dione (ORG-2058) was purchased from Amersham International (England). Non radioactive estradiol (E_2, 1,3,5(10)-estratrien-3,17β diol) was obtained

from Syntex S.A., Mexico City, Mexico. Progesterone (P; pregn-4-ene-3,20-dione), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), sulforhodamine B (SRB), and fulvestrant (ICI 182,780) were purchased from Sigma Aldrich (St. Louis, MO, USA). Buame [17β-(butylamino)-1,3,5(10)-estratrien-3-ol] was prepared from estrone according to reported methods [5, 9, 23]. The estrogen antagonist, tamoxifen, was a gift from Ing. A. Collantes Mirones (ASOFARMA, Mexico).

Animals

Experimental studies were conducted in accordance with the national protection laws of animal welfare: The Animal Protection Law for the Federal District and The General Health Law Related to Health Research (NOM-062-Z00-1999). Adult and immature Wistar rats used in this work were bred in our animal housing facilities. In the experiments, animals were distributed in different groups according to a balanced, Latin square block design, based on body weights, and they were randomly assigned to a different treatment group. The animals were kept at constant temperature (20–22ºC), maintained on standard chow (Nutricubos, Purina) and with water *ad libitum.*

Uterotrophic effect

Immature Wistar female rats (21 days old, 30–40 g, n = 6–8) were kept in a room with 12/12 h light-dark cycle. The groups were treated for three days with subcutaneous (*sc*) administration of 1, 10, 100, or 500 μg/kg body weight (b.w.) of buame, 100 μg/kg of E_2 dissolved in the vehicle substance (V; propylene glycol). The control group was treated with the V only. Twenty four hours after finishing treatment, the uterotrophic activity induced by buame and E_2 was evaluated through the gain in uterine wet weight (Uw). Uterine weights of the treated and the vehicle control groups were expressed in milligrams. The uterotrotrophic effect (UE) was calculated as a percentage difference relative to the control group = [UwBuame × 100/UwV] – 100. The relative uterotrophic effect to E_2 (RUE) was obtained by the relation:

$$\text{RUE} = \text{Relative uterotrophic effect} = [\text{UwBuame- UwV}] \times 100/(\text{UwE}_2 - \text{UwV}).$$

The relative uterotrophic potency (RUP) to E_2 was calculated by the relation:

$$\text{RUP} = \text{DE}_2(\text{UwE}_2 - \text{UwV}) \times 100/\text{Dbuame}\ (\text{UwBuame} - \text{UwV}).$$

The RUE and the RUP values were calculated from the doses (D) that produced the maximal effect; UE, RUE, and RUP were used to define the uterotrophic effects of buame as previously described [21]. The values of RUE and RUP were obtained from the uterine wet weight.

Sexual female behavior

Adult male and female Wistar rats (200 to 250 g body weight) were employed. All the rats were housed four per cage in a reversed 12/12 h light/dark cycle (lights off from 8:00 a.m. to 20:00 p.m.) and maintained on standard chow and water *ad libitum* except during the behavioral test. Female Wistar rats were bilaterally ovariectomized under ether anesthesia 2 weeks prior to hormonal treatment and were randomly assigned to the different groups (n = 6–8).

Steroid treatment and behavioral test design

Steroids were dissolved in corn oil as vehicle and injected subcutaneously in a total volume of 300 μl/animal per day. All drug administrations were performed starting at 8:00 h.

Evaluation of sexual behavior of female rats after single administration of E_2 and buame

Ovx rats received a single *sc* injection at 8:00 a.m. of any of the following: 7.5 μg of E_2/rat/day (approximately 30 μg/kg/day), 750, 1,500, or 3,000 μg/kg of buame or 300 μl/rat/day of corn oil as the vehicle control group (approximately 1.2 ml/kg/day). After 24 h, progesterone (P, 1 mg/rat dissolved in 100 μl of corn oil, approximately 4 to 5 mg/kg) was administered (at 8:00 a.m. starting the dark phase of the reversed cycle). All rats were tested for sexual receptivity 5 to 7 h thereafter.

Evaluation of sexual behavior of female rats subsequent to repeated administration of E_2 and buame

Different groups of Ovx rats under the same conditions described above were *sc* injected for three consecutive days with: 7.5 μg of E_2/rat/day (approximately 30 μg/kg/day), 3,000 μg/kg of buame, or

300 µl/rat/day of corn oil used as control group; 24 h after finishing the treatment, P was administered. Five hours after P administration, all Ovx groups were tested for sexual receptivity, and the LQ mean ± SEM values were calculated.

Behavioral examination

The facilitation of sexual receptivity was estimated in the different Ovx groups, treated with E_2, buame, or the vehicle (control). The Ovx rats were tested with sexually experienced males for sexual behavior at hour 0 (before the first administration), 24 h (before P administration), and 29 h (5 h after P administration). Assessment of times, 24 and 48 h, was performed between 12:00 and 14:00 p.m. All the experiments were done under a dim red light. Each test lasted for either 10 min or until male mounting, 10 times. To measure sexual receptivity, each female was placed in a Plexiglass testing arena (cylindrical plastic cage, diameter = 40 cm, height = 40 cm) with a male (males were acclimated to the arena for 3 min before testing). The male was allowed to mount the female vigorously 10 times. A blind observer recorded lordotic responses of females to male mounting. The number of times the female displayed lordosis (lifting of the head, arching of the back, and movement of the tail to one side) when mounted by a male was counted. For each female, a lordosis quotient (LQ) was calculated (number of lordosis displays/number of mounts × 100) as a measure of sexual receptivity [2].

Data analysis

The LQ maximum response (LQEmax), the LQ effective dose 50 (LQED50), and confidence limits were calculated from the dose-response curves using the sigmoid fitting model (Boltzmann model) using the Origin 6.1 version, Copyright© 1991–2000, Origin Lab Corporation. The percent difference of LQ Emax related to E_2 ($LQEmaxRE_2$) was obtained by the relation:

$$LQEmaxRE_2 = (\text{Buame LQEmax} / E_2\ \text{LQEmax} \times 100) - 100$$

and the LQ relative potency to E_2 = $LQRPE_2$ = [(LQEmax of E_2) (E_2LQED50 (µmol)]/[(LQEmax of buame) (Buame LQED50 (µmol)] × 100 as previously reported for E_2 and other AEs [18].

Cytosol preparations for binding estrogen receptor studies

The binding studies were performed following previously described experiments for other AEs competence assays [24]. Briefly, intact immature Wistar female rats (80–100 g) were used for these studies. Uteri were homogenized at a ratio (w/v) of 1:6 TEDML buffer (20 mM Tris-HCl, pH 7.4, at 4ºC, 1.5 mM EDTA, 0.25 mM dithiothreitol, 10 mM sodium molybdate and 10 µg/ml of leupeptin) with three 10 s bursts of a Polytron homogenizer (Brinkmann Instruments, Westbury, NY, USA). The homogenate was centrifuged at 180,000 × g for 1 h at 2ºC in SW 50.1 rotor (Beckman Instruments, Palo Alto, CA, USA). Cytosol protein content was determined by the Bradford's dye binding method using bovine serum albumin as a standard. Equilibrium parameters of reaction between the radioligand and cytosol limited-capacity binding sites were studied incubating the different uterine cytosol aliquots (2.0–3.4 mg/protein/ml). The preparations were incubated with 1 nM of [^{3}H]-estradiol in the absence or presence of increasing concentrations of the non-radioactive estradiol and buame for 18 h at 4ºC. Bound and free steroids were separated by adding a dextran-coated charcoal suspension (250 mg Norit-A and 25 mg Dextran T-70) in 100 ml of TEDM buffer (20 mM Tris-HCl, pH 7.4, at 4ºC, 1.5 mM EDTA, 0.25 mM dithiothreitol, and 10 mM sodium molybdate), for 5 min at 4°C under continuous shaking, followed by centrifugation at 800 × g at 4ºC for 15 min. Aliquots of the supernatants were assayed for radioactivity determination in a Packard Tri-Carb liquid scintillation spectrometer model 1900 TR, using Instagel Plus (Packard, Downers Grove, IL, USA) as counting solution. Quenching was corrected in all samples by external standardization. Inhibition constant (Ki) and relative binding affinity (RBA) were calculated with the established methods [4] using the Origin_ 6.1 software.

MCF7 cells culture

Estrogen receptor-positive human MCF-7 breast cancer cells from the American Type Culture Collection (ATCC) were kindly provided by Dr. A. Jimenez from our Departament. Stock MCF7 cells were grown as monolayer cultures in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL) supplemented with 10% (v/v) heat inactivated fetal bovine serum (FBS)

from Gibco BRL, 1% (v/v), non essential amino acids, 1% (v/v) sodium pyruvate, 1% (v/v) HEPES at 1 mM (Sigma), and 1% (v/v) penicillin/streptomycin solution 10 mg/l [17]. The cell culture was maintained in a humidified atmosphere of 5% carbon dioxide in air at 37ºC. All cell stocks were sub-cultured at weekly intervals at 70% confluence over a maximum of 10 passages by suspension with 0.05% trypsin-0.02% EDTA (pH 7.3).

The compounds E_2 or buame were prepared as stock solutions 0.1 M in ethanol, and were diluted in culture medium (10^{-13}, 10^{-12}, 10^{-11}, 10^{-10} 10^{-9}, and 10^{-8} M) avoiding ethanol concentration greater than 0.1% (v/v). In order to test the receptor's mediation on the increased proliferation rate of the cells, buame was coincubated with 100 nmol/l of the anti-estrogens tamoxifen or fulvestrant (ICI 182,780) 5 nmol/l.

Cell proliferation experiments

Cells were added to the required volume of phenol red-free DMEM medium containing 10% FBS at a density of 2,500 cells per well and plated in monolayer in 150 µl aliquots into 96 well plastic tissue culture plates (Falcon). After attachment of cells (24 h), the medium was changed and immediately replaced by fresh phenol red-free DMEM medium supplemented with 10% charcoal-treated FBS prepared following the protocol described by Körner et al. [15]. E_2 (1×10^{-13} to 1×10^{-9} M) and buame (1×10^{-12} to 1×10^{-9} M) were added to the cultured cells. The plates were incubated at 37ºC during 6 days. Each concentration was tested in 16 replicates per assay; cells of eight wells without hormones were the control. Two groups of cells also were assigned to the treatment with tamoxifen (100 nmol/l) or fulvestrant (5 nmol/l).

Quantitative evaluation

The proliferation of the MCF-7 cells was estimated after treatment of the cells using the MTT and SRB assays.

The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay, based on the biotransformation of the yellow tetrazolium salt MTT to purple formazan crystals by mitochondrial dehydrogenases only present in viable cells [1]. In this assay, the mitochondrial activity of the cells was estimated by adding 20 µl of MMT solution (5 mg/ml) in phosphate-buffered saline (PBS) for 4 h at 37ºC to the medium. Afterwards, the medium was removed and the formazan crystals were dissolved in dimethyl sulfoxide (DMSO).

The sulforhodamine B dye (SRB assay) described by Skehan et al. [31] is based on the ability of SRB to bind to protein components of cells previously fixed to tissue culture plates by trichloroacetic acid. The SRB binding is stoichiometric, and the dye extracted from stained cells is directly proportional to the cell mass. The SRB assay has been optimized, validated and adapted for MCF-7 cells in 96-well plates by Körner et al. [15] and is a sensitive quantitative method of evaluating total estrogenic activity.

Sulforhodamine (SRB) proliferative assay was performed according to the method of Körner et al. [15]. Cells were fixed with 50 µl of ice-cold 50% trichloroacetic acid solution by gently adding on top of the medium overlaying the cells. The plates were then incubated for 60 min at 4°C. Wells were rinsed five times with tap water and then cells were stained with 0.4% SRB solution (100 µl stain/well) for 30 min at room temperature. After staining, SRB solution was poured off, unbound dye was removed by washing five times with 1% acetic acid solution and left to air dry. The bound SRB dye was then solubilized by adding unbuffered Tris-base solution (200 µl/well), and plates were placed on a plate shaker for 15 min at room temperature.

Curves of optical density (OD) *vs.* number of cells were constructed to test the linearity for both methods according to Rubinstein et al. [29]. OD was read in a UV microtiter plate reader at 492–630 nm (Awareness Technology, Inc.). Experiments were performed at least twice. The number of the cells were expressed as a percentage related to the control cells (0) without treatment by the relation: 100 – treated cells number/number cells of the control × 100.

The proliferative effect (PE), the relative proliferative effect to E_2 (RPE), the relative proliferation potency to E_2 (RPP) were obtained according to Körner et al. [15]. PE was calculated by the relation of the highest cell number achieved with E_2 or buame, and the cell number of the control:

PE = number cells of E_2 or buame/number cells of the control

RPE of buame was estimated comparing the maximal proliferation induced by E_2 with that induced by buame according to the relation:

$$RPE = [PE\text{-}1 \text{ (buame)}/PE\text{-}1 (E_2)] \times 100\%$$

Tab. 1. Uterotrophic activities of E_2 and buame in immature Wistar rats

Group	Dose (μg/kg)	Dose (μM/kg)	N	Uterine wet weight (mg ± SEM)	Uww UE (%)	RUE	RUP	ED50 Uww (μg/kg)
Vehicle	10 ml		10	25 ± 2	–	–	–	
E_2	100	0.36	10	108 ± 20	332	100	100	52[&]
Buame	10	0.031	7	30 ± 3	20	6		
	100	0.31	7	32 ± 5	28	8		
	500	15.5	7	75 ± 4	200*	60*		
	1000	30.1	7	90 ± 7	260*	78*	1.48	365

Uw = uterine weight; UE (%) = uterine effect was obtained as percent difference related to the control group = [UwBuame × 100/UwV] – 100. * $p < 0.05$ *vs.* vehicle group by Dunn's method. RUE = Relative uterotrophic effect = [UwBuame- UwV] × 100/(UwE_2 – UwV). RUP = relative uterotrophic potency = DE_2(UwE_2-UwV) × 100/DBuame(UwBuame – UwV). [&] = Data obtained from Lemini et al. [21]

RPP to E_2 was calculated by the determination of the EC_{50} values of E_2 and buame (concentration of each compound necessary to obtain 50% of the maximum cell proliferation) RPP = EC_{50} [E_2]/EC_{50} [buame].

Statistical analysis

Statistical significance amongst groups was analyzed employing one-way analysis of variance (ANOVA). The significance of the differences among the control and the treated groups was estimated by the appropriate method [38]. Analysis of data between control and treated animals was performed using the Sigma Stat 3.1 program. The ED_{50} values were estimated from dose response curves utilizing the Sigma Plot 11 program. Results were expressed in the means ± standard error (SEM). Values of $p < 0.05$ were considered statistically significant. Data in all cases are representative of at least two independent experiments.

Results

Uterotrophic assay

The effects on uterine weight of immature rats by the treatment with different doses of buame are shown in Table 1. The uterotrophic effect observed by buame administration was compared with those elicited by a 100 μg/kg of E_2 dose that previously has shown to induce maximum uterotrophic effects [21]. The maximum uterotrophic effect induced by buame related to the vehicle control group was 257%. Buame had a relative uterotrophic effect to E_2 (RUE) of 78 and its relative uterotrophic potency (RUP) was 1.48.

Buame induction on female sexual behavior

Single buame administration did not induce sexual behavior of Ovx rats when the traditional scheme of evaluation for facilitation of sexual behavior was

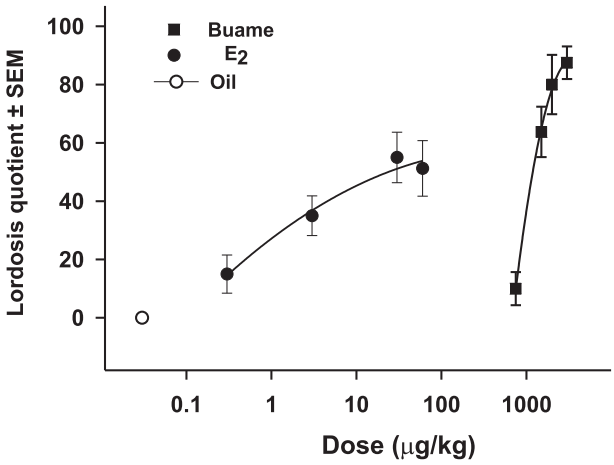

Fig. 2. Five independent groups (n = 7) were treated with a single injection of: the vehicle (V; corn oil; 300 μl/day; ≈ 1.2 ml/kg) as control; 7.5 μg of E_2/rat/day (≈ 30 μg/kg/day), or buame (≈ 750, 1500, 3000 μg/kg). The paradigm used was: administration time (time zero) followed 24 h later by progesterone injection (P, 1 mg/rat dissolved in 100 μl of corn oil, ≈ 4 to 5 mg/kg) and sexual receptivity test was performed 5 h later (29 h) and lordosis quotient (LQ; number of lordosis displays/number of mounts × 100) was determined. Control rats received only the vehicle (V). The V treated rats results were pooled (LQ = 0). Sexual receptivity was tested starting on the first administration day (time 0), 24 h after the administration, and subsequent to progesterone administration (29 h). Each point represents the mean ± SE of eight rats. * $p < 0.01$ *vs.* V

used: 1) estrogen administration; 2) 48 h after P administration; 3) 5 h later, the evaluation of the facilitation of sexual receptiveness. On the other hand, using the previously reported scheme [18]: 1) estrogen administration; 2) 24 h after, P injection and 3) 5 h later, buame was capable of inducing sexual receptiveness in the Ovx rats. Buame induced an LQmax 85 ± 9, while under the same conditions, E_2 induced LQmax 56 ± 8 (Fig. 2). Buame's $LQED_{50}$ was 968 ± 19 µg/kg (compared with E_2 $LQED_{50}E_2$ = 10 ± 2.2 µg/kg; [18] and its $LQRPE_2$ was 0.5 (E_2 = 100).

Three daily administrations of buame followed 24 h later by a sequential administration of P was capable of producing lordosis in the female Ovx rats in a similar way to that observed in the E_2 treated animals. Figure 3 shows the temporal course response. E_2 and buame showed differences in their capabilities of inducing sexual behavior in the Ovx rat. E_2 latency was manifested 48 h after the treatment initiated in a dose and time dependent manner. Meanwhile, buame manifested its effect only after three administrations followed by P administration. Under these conditions, the LQEmax achieved by E_2 and buame were 95 and 80, respectively.

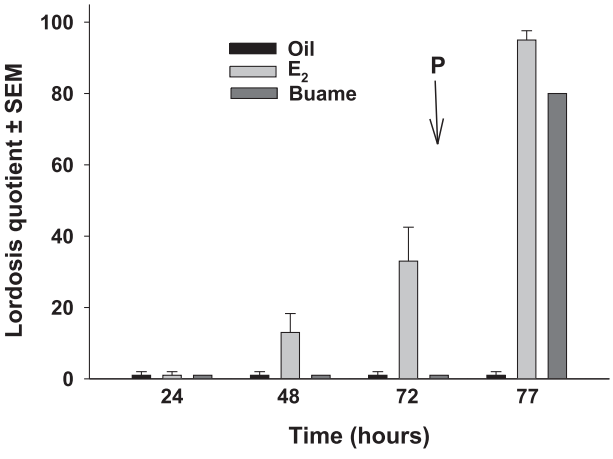

Fig. 3. Repeated administration of E_2, and the 17β-aminoestrogen buame on the sexual behavior of female rats. Twenty days after ovariectomy, rats were *sc* injected for three consecutive days with: E_2 (30 µg/kg/day), buame (1000 mg/kg/day) or the V (corn oil; 300 µl/day; ≈ 1.2 ml/kg) as control; 24 h after finishing the treatment, progesterone (P, 1 mg/rat, approximately 4 to 5 mg/kg) was administered, 5 h after P administration Ovx rats were tested for sexual receptivity and the LQ mean ± SEM values were estimated. Sexual receptivity was tested starting on the first administration day (time 0), through the second (24 h), and third administration (48 h), 24 h after the last administration (72 h), and subsequent to P administration (77 h). Each point represents the mean ± SE of eight rats. Control rats (LQ = 0) received only the vehicle. * $p < 0.01$ *vs.* vehicle. The vehicle (V) results of treated rats were pooled (LQ = 0). * $p < 0.05$ *vs.* V

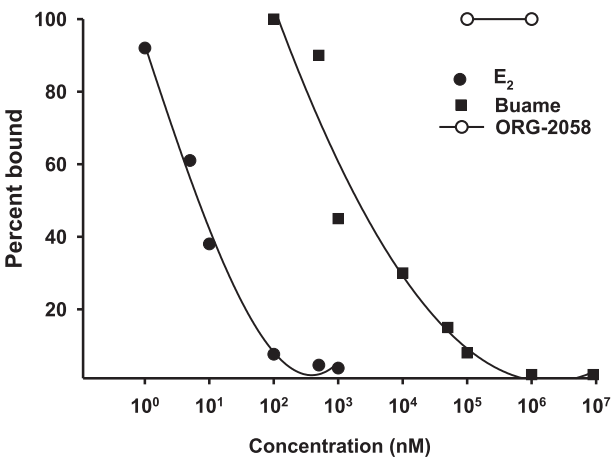

Fig. 4. Competitive binding assay of E_2 (•) the 17β-aminoestrogen, buame (■), and the synthetic progestagen ORG-2058 utilized as a negative control (-o-) since did not displace the $[^3H]E_2$ bound from uterine cytosolic estrogen receptor binding sites

Receptor binding analysis

The competitive binding assay of the 17β-aminoestrogen buame and $[^3H]E_2$ is shown in Figure 4. Buame competed with $[^3H]E_2$ for the estrogen receptor binding sites. The relative binding affinity (RBA) of buame was 0.15 being 666 times less to that shown by E_2 (100). Buame inhibition constant (Ki) was 5.9×10^{-7} M meanwhile in the same conditions E_2 showed a Ki = 6.6×10^{-9} M. As expected, the addition of the synthetic progesterone ORG-2058 did not displace the $[^3H]E_2$ bound from uterine cytosolic estrogen-binding sites.

Proliferation of MCF-7 cells

Buame increased MCF-7 cells proliferation in the range of 10^{-12} to 10^{-9} M, in a concentration dependent manner. Higher concentrations of buame (10^{-8} to 10^{-7} M) did not result in further cell proliferation. Similar results were achieved for E_2 and buame utilizing cells counting evaluation by the MTT or SRB methods (Fig. 5A and 5B). The maximum percent of cell proliferation of E_2 was 130–139% (related to the control = 0) with 10^{-10} M concentration, meanwhile maximum response of buame was 73–79% with 10^{-9} M. The proliferative effect (PE) of buame related to the control MCF7 cells was 1.73–1.78, while E_2 PE was 3.0–3.2. Buame's relative proliferative effect to E_2 (RPE) was

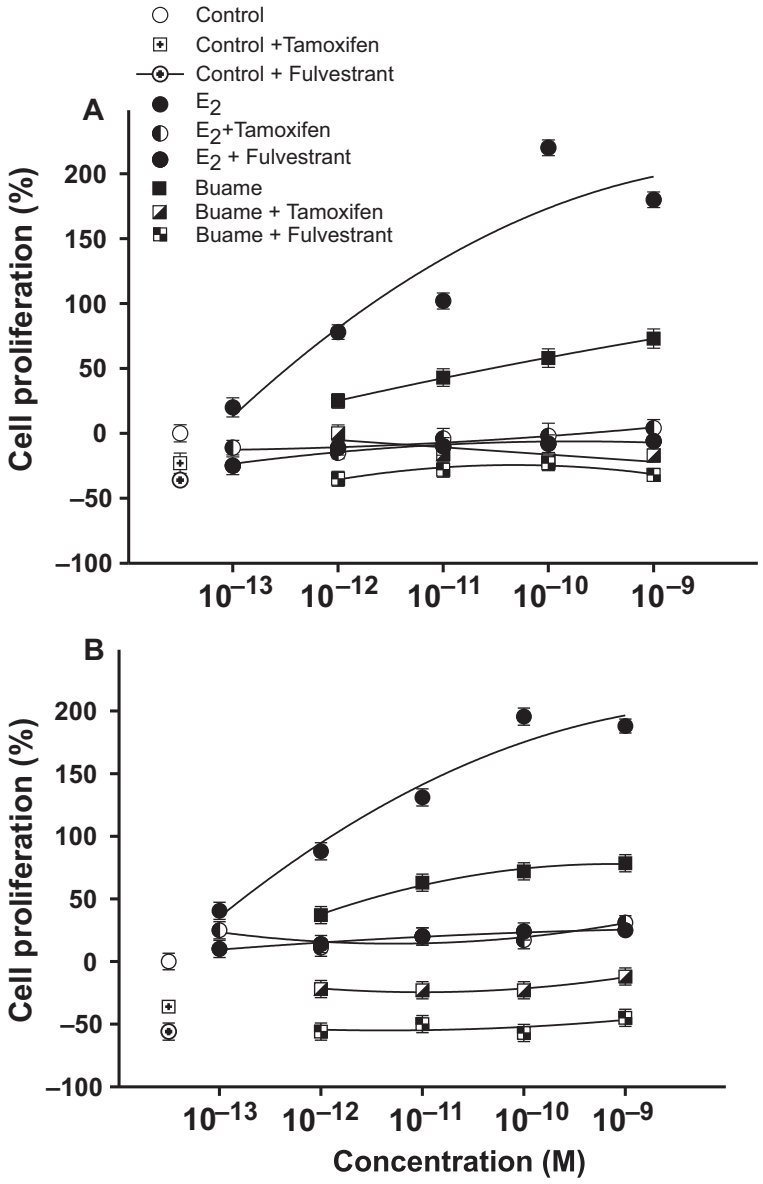

Fig. 5. Dose-response curves of E_2, buame and both compounds coincubated with the antiestrogens tamoxifen or fulvestrant (ICI 182,780) on MCF-7 cells in culture. The results shown in Figure 5A were obtained using the MTT method and 5B results were obtained by the corresponding SRB method

between 33–40%. The relative proliferative potency (RPP) of buame to E_2 obtained from the dose-response curves was 10.4–10.7 (E_2 = 100). The proliferative effects obtained with E_2 and buame were inhibited by the anti-estrogens tamoxifen and fulvestrant (ICI 182,780), these results are shown in Figure 5A and 5B. The depicted results using both MTT (5A) as SRB (5B) for evaluation of the cell proliferation were similar. It could be observed a greater inhibitory response when used the pure antagonist fluvestrant with respect to inhibition than tamoxifen produced. However, in both cases the inhibitory values were similar and not significantly different with respect to their corresponding controls.

Discussion

Buame induced estrogenic responses in all the E_2 targets here explored. *In vivo* testing in reproductive organs of immature rats produced uterotrophic effects and induction of adult female sexual behavior. Both effects are associated with estrogen administration.

Buame also competed with [^{3}H]E_2 for the estrogen receptor and showed it's capability of increasing MCF-7 cell proliferation. This response was inhibited by the antagonists tamoxifen and fulvestrant (ICI 182,780), indicating that buame's capability of inducing proliferative effects on MCF-7 cells is mediated by the activation of estrogen receptors ERα and ERβ.

Buame is structurally related to the 17β-aminoestrogen butolame; both have four carbons on the amino side-chain in C-17 of the steroid nucleus, with an hydroxyl terminal substituent in butolame. Because of this, we expected buame and butolame to have equivalent estrogenic activities, however, buame had a lower degree of estrogenicity in the rat´s uterus compared to butolame [21]. The modification of the substituent on the amino group therefore seems to impact the intrinsic estrogenic properties of this compound.

This difference could be due to the buame alkyl substituent on the amino group instead of the terminal hydroxyl found in butolame. The molecular difference produces changes in the physicochemical properties of the molecules, which consequently changes their biochemical interactions.

It is probable that in buame the butyl group on the amino group in the molecule makes it more lipophilic and less polar than butolame, which would facilitate its passage through biological membranes and consequently show differences in its pharmacokinetic pathway. Therefore, this could suggest possible differences in absorption, metabolism and elimination. If buame´s metabolism and excretion processes were faster than those of E_2 and/or butolame, it would have a shorter elimination half-life, which would not allow us to observe buame´s response after 48 h followed by P (in the classic paradigm) but it would allow us to observe a response after 24 h. To corroborate this hypothesis further investigation of the pharmacokinetic properties of buame and butolame is needed.

The buame induction of sexually receptive behaviors in the Ovx rats showed buame's capability of inducing transcription and translation, leading to an increase of progesterone receptors in the medial preoptic nucleus and the ventromedial hypothalamus, which are responsible for the modulation of female sexual behavior in rats [7, 25, 28].

The lordotic behavior exerted by buame was similar to that elicited by E_2 [2] and included proceptive behaviors such as ear wiggling, hopping and darting, however, the response to buame was more intense, showing more efficacy than E_2. This phenomenon has also been observed with butolame and other AEs and is probably linked to other mechanisms like the oxytocin receptors, pre-proenkephalin peptides, α1B-adrenergic or muscarinic receptors, among others [18, 25, 35, 36]. The response of buame on these targets is still not known and cannot be excluded.

Additionally, the AEs are molecules that can be solvated [8, 18, 22], which confers them the ability to activate membrane receptors in extra hypothalamic structures, which have shown an important influence on the intensity and duration of lordosis [10]. The data here presented cannot explain the action mechanism differences between AEs with respect to E_2 over the facilitation of sexual behavior of the rat, additional work will be necessary to establish them.

On the other hand, although buame displaced E_2 from its estrogen receptors, it had a lower affinity and efficacy than E_2. The binding data obtained from buame are close with those previously obtained from the butolame. Their Ki and RBA for the ER of both compounds were very similar and did not allow us to establish differences between them [24].

Conclusions

Buame possesses an alkyl amino substituent, lacking the OH group at the end of the chain substitution of butolame. The alkyl group probably produces changes in the physicochemical properties of the molecule, changing polarity and liposolubility that have an influence on its pharmacokinetic pathway and probably have an impact on its pharmacodynamic properties, potency and efficacy for the activation of target estrogen receptors.

The phenomena observed here are in agreement with previous observations of estrogenic response in the homologous series of AEs: prolame, butolame and pentolame. Even small changes in the molecular structure related to the length of the amino side-chain at C-17 have an important influence on estrogenic response [13, 18, 24]. Buame estrogenicity is qualitatively similar to that elicited by E_2, however, it has a lower potency than the hormone in the targets here explored, allowing us to infer that buame is a partial agonist with weak estrogenic properties.

The fact that buame showed lower estrogenicity and thrombogenicity than E_2 could be an advantage for its use in HRT, however, it is necessary to evaluate buame on bone and nervous systems where estrogens have shown protective effects to assess its potential clinical use.

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