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Characterization of selective estrogen receptor modulator (SERM) activity in two triarylethylene oxybutyric acids

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Abstract

Previously we identified 4-[1-(4-hydroxyphenyl)-2-phenylbuten-1-yl]phenoxy-*n*-butyric acid (4HBA) and its des-hydroxy analog (BA) as potential selective estrogen receptor modulators (SERMs) in the ovariectomized (OVX) rat. The aim of the present study was to characterize comprehensively the effects of 4HBA and BA in both the OVX rat and in estrogen-responsive cells. Thus, 4HBA was found to be an estrogen antagonist with partial agonist efficacy in estrogen-responsive reporter gene and estrogen-dependent proliferation assays (MVLN cells and MCF-7 human breast cancer cells, respectively). In the OVX rat, 4HBA and BA were equally effective and comparable to other known SERMs regarding (a) serum cholesterol reduction and suppression of serum markers of excessive bone metabolism, and (b) partial agonist efficacy in reproductive tissue relative to steroidal estrogens. Like steroidal estrogens, both compounds increased serum triglyceride levels, with BA being more effective in this regard. The maximal effects of 4HBA on all of these parameters except cholesterol lowering were seen at oral doses of 0.4 μmol/kg/day; maximal cholesterol lowering required doses of 10 μmol/kg/day. In OVX rat liver 9S fraction, BA was found to be efficiently converted to a single hydroxylated metabolite, 4HBA. These results suggest that the effects of BA in the OVX rat might, in part, be a consequence of biotransformation to 4HBA, and that those of 4HBA and BA in the OVX rat and in estrogen-responsive cells are qualitatively similar to those of SERMs such as tamoxifen and raloxifene. © 2002 Published by Elsevier Science Inc.

Keywords: SERM; Tamoxifen; Raloxifene; Estrogen receptor; Osteocalcin; Cholesterol

1. Introduction

Tissue-selective ER ligands are exemplified by RAL and TAM (Fig. 1). In estrogen-replacement therapy, RAL appears to have a therapeutic advantage over steroidal estrogens such as E2 and EE2, due to its greatly reduced incidence of reproductive tract side-effects [1]. Thus, RAL is indicated in postmenopausal patients for the prevention of osteoporosis. Previously, TAM was shown to have tissue-selective estrogenicity by virtue of its ability to suppress bone loss and reduce serum cholesterol levels in patients undergoing therapy for breast cancer [2]. These SERMs have clear advantages over conventional nonselective estrogens in long-term therapy. This, together with recognition of certain therapeutic drawbacks associated with their use, has stimulated continuing efforts to characterize novel structural analogs of RAL and TAM [3–5].

In the OVX rat, RAL, TAM, and other SERMs in clinical trials are approximately as efficacious (but generally not as potent) as EE2 in regard to bone loss suppression and serum cholesterol lowering. Also TAM, like E2 and EE2, causes elevation of serum triglycerides and suppression of body weight gain. But the estrogenic efficacy in reproductive tissue of the SERMs is much less than that of E2 or EE2. Because the bone-protective and cholesterol-lowering effects of RAL and TAM in the OVX rat are qualitatively similar to those seen in patients administered these drugs, the OVX rat has emerged as an animal model for experimental characterization of SERMs [3,4,6–8].

In MCF-7 human breast cancer cells, RAL and TAM suppress estrogen-stimulated proliferation [9,10]. Furthermore, studies in other neoplastic cell lines into which ER and reporter genes have been transfected have revealed a

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Abbreviations: ER, estrogen receptor; RAL, raloxifene; TAM, tamoxifen; E2, 17β-estradiol; EE2, ethinyl estradiol; OVX, ovariectomized; SERM, selective estrogen receptor modulator; 4HT, 4-hydroxytamoxifen; LBD, ligand binding domain; ERE, estrogen response elements in DNA; OC, osteocalcin; Dpd, deoxypyridinoline; and RBA, relative binding affinity.

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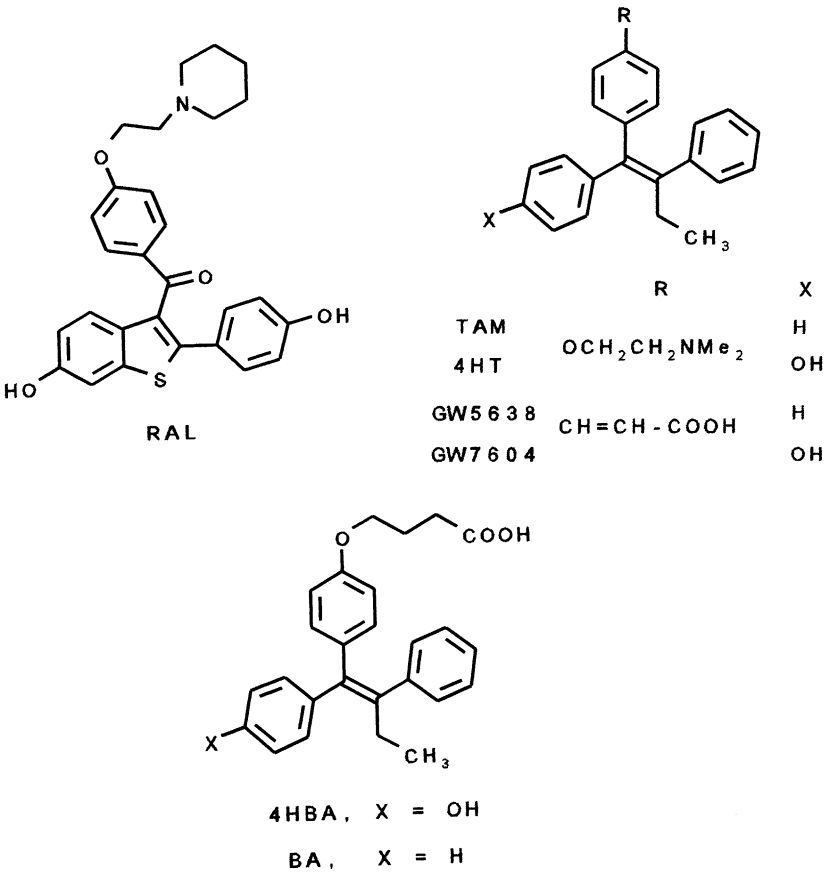

Fig. 1. Chemical structures of raloxifene (RAL), tamoxifen (TAM), 4-hydroxytamoxifen (4HT), 3-[4-(1,2-diphenylbut-1-enyl)phenyl]acrylic acid (GW5638), 3-{4-[1-(4-hydroxyphenyl)-2-phenylbut-1-enyl]phenyl}acrylic acid (GW7604), and triarylethylene oxybutyric acids 4-[1-(4-hydroxyphenyl)-2-phenylbuten-1-yl]phenoxy-*n*-butyric acid (4HBA) and its des-hydroxy analog (BA).

potential mechanistic difference between RAL and TAM and SERMs bearing *acidic* side chains. For example, triarylethylene acrylic acid 3-[4-(1,2-diphenylbut-1-enyl)-phenyl]acrylic acid (GW5638) antagonized the residual partial estrogen agonist effect of either RAL or 4HT [11].

The molecular basis for tissue-selective estrogenic effects expressed by SERMs arises from the distinct conformation they induce in the ER. The ER, after interaction with RAL or TAM, is not as compact in the region of its LBD as when the ER is liganded with E2 [12,13]. Although the resulting SERM–ER complexes were suggested to be as effective as E2–ER complexes in activating ERE in bone-remodelling cells, hepatocytes, and other functionally similar ERE, they were only marginally effective in activating ERE in reproductive tissue, based on studies carried out in the OVX rat [14]. In addition, the SERM–ER complexes could mimic E2–ER complexes at nongenomic sites in bone, liver, and other extra-reproductive tissues.

Using this and related knowledge of structural features conferring SERM activity in ER ligands, we identified the triarylethylene oxybutyric acids 4-[1-(4-hydroxyphenyl)-2-phenylbuten-1-yl]phenoxy-*n*-butyric acid (4HBA) and its des-hydroxy analog (BA) as capable of selective bone-protective effects [15]. Thus, in the OVX rat, 4HBA and BA were each as effective as E2 in lowering serum levels of Dpd and OC. OC is a marker of elevated bone formation that correlates with bone loss [16,17], and Dpd is a collagen catabolite arising solely from the breakdown of bone collagen [18]. However, each compound had only about 30% of the uterotrophic effect of E2.

Thus, we subjected 4HBA and BA to a more comprehensive characterization of their estrogen mimetic effects and potencies in the OVX rat. We also determined the E2 antagonist potency and efficacy of 4HBA in two estrogen responsive *in vitro* cell assays.

2. Materials and methods

2.1. Chemicals

4HBA and BA were prepared as previously described: spectral and elemental analysis data indicated each of these compounds to be >99% pure as its partial hydrate [15]. RAL was obtained from Eli Lilly & Co. E2, EE2, and all biochemicals used in this study were obtained from the Sigma Chemical Co.

2.2. Animals and dosing

OVX Sprague–Dawley rats, 10- to 12-weeks-old, 229–245 g body weight, were obtained from Harlan, Inc. Animal care and handling were carried out solely at the AAALAC accredited animal facility at the University of Georgia College of Pharmacy, in accordance with a protocol approved by the University of Georgia Institutional Animal Care and Use Committee. Rats were housed 2–3/cage at 25° with a light/dark cycle of 13 hr/11 hr. Food (Teklad 22/5 Rodent Diet), with calcium and phosphate contents of 0.95 and 0.67%, respectively, and water were available *ad lib.* to all animals.

The first experiment included seven animals in each of three treatment groups. Each member of the first group was injected s.c., once daily, with vehicle (5% benzyl alcohol in corn oil), 0.1 mL/100 g body weight, 5 days per week for 3 weeks. Members of the remaining two groups were subjected, in turn, to the same treatment regimen with E2 (0.35 μmol/kg) or 4HBA (10 μmol/kg). Treatment regimens were initiated 4 days after the animals were received from the supplier. The body weight of each animal was recorded on the first day of treatment. Dose levels of E2 and 4HBA were chosen based on those of respective structurally related ER ligands found to give maximal estrogenic effects in the OVX rat using protocols similar to the present one [19,20]. All compounds were fully soluble as 20× concentrated stock solutions in benzyl alcohol and after dilution with corn oil.

A second experiment was performed in exactly the same manner described above, except that dosing solutions were administered p.o., and six groups of animals were used. Stock solutions (10×) of 4HBA and BA were prepared by dissolving 0.32 mmol of each compound in 2.5 mL of ethanol and diluting the resulting solution with a solution of 0.32 mmol of tromethamine base in 0.7 mL of water. Daily dosing solutions of BA were obtained by dilution of its stock solution with 9 vol. of 0.11% aqueous methylcellulose. Stock and dosing solutions of EE2 and vehicle were prepared similarly. Dosing solutions of 4HBA were prepared by diluting aliquots (800, 160, and 32 μL) of its stock solution with sufficient 0.11% aqueous methylcellulose to make 8 mL. Members of the six treatment groups received, in turn, vehicle, EE2 (0.35 μmol/kg), 4HBA (0.4, 2, and 10 μmol/kg), and BA (10 μmol/kg) administered by oral gavage.

2.3. Necropsy procedures

At the end of the 21-day study period, animals were killed under carbon dioxide. The body weight of each animal was recorded, as was uterine wet weight. Blood samples were allowed to coagulate at room temperature (2 hr) in a Vacutainer tube. Serum was obtained by centrifugation at 1100 *g* for 10 min at room temperature, and samples were stored at −80° until analyzed, as were uteri.

For drug metabolism studies, livers from four of the aqueous vehicle-treated animals were dissected, combined, minced, and homogenized in 3 vol. of 1.15% ice-cold aqueous KCl using a tissue homogenizer. The homogenate was centrifuged at 9000 *g* for 25 min at 4°. Aliquots (5 mL) of the supernatant (9S fraction) were lyophilized and stored at −80° prior to use.

2.4. ELISAs for serum OC and Dpd

Serum samples were thawed by placing containers on ice for 2 hr. Properly diluted samples were assayed for OC using an enzyme immunoassay kit (Biomedical Technologies, Inc.). The procedure was carried out in a 96-well polystyrene plate in which a monoclonal antibody to the N-terminal region of rat OC was bound to each well surface. After overnight incubation with the diluted serum sample, wells were washed and incubated with a second antibody (goat polyclonal), which interacted with the C-terminal region of the immobilized OC. Subsequent incubation with horseradish peroxidase (HRP) conjugated donkey-anti goat IgG, and then a solution of HRP substrate, 3,3′,5,5′-tetramethylbenzidine, was carried out. Absorbance at 450 nm, which accompanied substrate oxidation, was determined using a plate reader. The amount of OC in the sample was calculated by comparing its absorbance with those of standards that contained known amounts of rat OC. Absorbance intensity was directly proportional to the amount of OC present in the sample.

Alternatively, thawed serum samples were analyzed for total Dpd, using a sequential hydrolysis/competitive enzyme immunoassay procedure (Metra Biosystems, Inc.). Each serum sample was mixed with 6 N HCl plus solubilizing agent. Precipitated protein was separated by centrifugation at 10,000 *g* for 10 min at room temperature. An aliquot of the supernatant was heated at 99° for 22 hr, in a capped 0.5-mL polypropylene centrifuge tube, to hydrolyze that portion of serum Dpd linked to polypeptides. This was neutralized by the addition of 10 N NaOH, and an aliquot was transferred to the 96-well assay plate, each well containing monoclonal anti-Dpd antibody. Then a fixed amount of Dpd-alkaline phosphatase conjugate was added. After a 2-hr incubation, wells were washed, and a solution of *p*-nitrophenyl phosphate was added. After a second 2-hr incubation, alkaline stop solution was added, and the absorbance of the *p*-nitrophenoxide formed was determined at 405 nm using a plate reader. The amount of Dpd in the sample was calculated by comparing absorbance of the sample with that of standards, run in parallel, which contained known amounts of Dpd. Absorbance intensity was inversely proportional to the amount of Dpd originally present in the sample.

2.5. Colorimetric assays for serum cholesterol and triglycerides

Materials and procedures for these determinations were obtained from Sigma. Total serum cholesterol

was determined by initial enzymatic hydrolysis of esterified cholesterol, followed by cholesterol oxidase catalyzed formation of an equimolar amount of hydrogen peroxide that accompanied conversion of cholesterol to cholest-4-en-3-one. The hydrogen peroxide produced was coupled with 4-aminoantipyrine and *p*-hydroxybenzenesulfonate, in the presence of HRP, to give a quinoneimine adduct that was quantitated at 500 nm. Amounts of cholesterol in serum samples were calculated by comparing absorbance of the sample with those of standards, run in parallel, which contained known amounts of cholesterol. All samples and standards were run in duplicate. Serum triglycerides were determined by initial enzymatic formation of glycerol, followed by sequential enzymatic conversion of this to dihydroxyacetone phosphate, with concomitant reduction of NAD to NADH. Diaphorase-catalyzed dehydrogenation of NADH was accompanied by simultaneous oxidation of 2-(*p*-iodophenyl)-3-*p*-nitrophenyl-5-phenyltetrazolium chloride to formazan, which was quantitated at 500 nm. Amounts of triglycerides in serum samples, run in duplicate, were calculated by comparing sample absorbance with those of triglyceride standards, run in parallel.

2.6. Statistical analysis

Pharmacodynamic data are expressed as the means ± SD for each treatment group. Statistical differences between groups were evaluated using a customized spreadsheet prepared using Quattro® Pro 6.0 for Windows. The range of each experimental value was calculated using appropriate t-scores for checking both upper and lower limits at a 95% level of certainty. Differences were considered significant at $P < 0.05$.

2.7. Biotransformation of BA

Triplicate incubations were run in 12 × 75 mm polypropylene tubes. The standard incubation mixture (1.0 mL) contained 20 mM potassium phosphate buffer, pH 7.05, 90 mM potassium chloride, 5 mM magnesium chloride, 0.4 mM NADP, 6.5 mM glucose 6-phosphate, and 9S fraction equivalent to 50 mg of wet liver. Each incubation was started by the addition of BA in 20 μL of *N,N*-dimethylacetamide to give a final concentration of 0.1 mM (38 μg/mL). In control incubations, either the cofactor mixture (NADP and glucose-6-phosphate) or BA was omitted. Incubations were shaken at 70 cycles/min at 37° for 20 min, and then to each was added 0.1 mL of 50 mM EDTA disodium salt. Mixtures were vortexed and heated at 60° for 10 min (tubes were capped). Each mixture was then cooled to room temperature and shaken for 5 min with 3 mL of ether. Mixtures were centrifuged at 450 *g* for 10 min at room temperature. The ether layer (2.0 mL) from each extract was concentrated under a stream of compressed nitrogen gas at 40°. Each residue was reconstituted in 100 μL of mobile phase (below) and subjected to HPLC. Column: 10 μm Whatman® Partisil® ODS-3 (Mitchell modification); mobile phase: methanol/50 mM sodium phosphate buffer, pH 2.45 (75:25, v/v), 1.0 mL/min; UV detection at 277 nm; 20 μL flushed loop injection. Retention times: 4HBA, 10.73 min; BA, 25.37 min. Amounts of 4HBA and BA were estimated based on respective peak areas in chromatograms in comparison with calibration curves in which integrated area as a function of the amount (μg) of each compound analyzed was plotted. The extraction efficiency of BA from cofactor-absent incubations was 78 ± 8%.

2.8. Effects of 4HBA on proliferation of estrogen-responsive cells

Stock cultures of an MCF-7 E3 clone [21] and MVLN cells were maintained in Dulbecco's Modified Eagle's Medium containing 20 mM HEPES, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 0.5 mM gentamycin (Life Technologies) 10% FBS (HyClone) and no phenol red in a 37° incubator with 5% CO_2. To remove contaminating estrogens prior to plating for experiments, both cell lines were passed at low density and then withdrawn from the above estrogen-rich medium for 6 days by washing three times every day with Dulbecco's phosphate-buffered saline (DPBS; Life Technologies) followed by incubation with medium containing dextran-coated charcoal stripped FBS (DCC medium; HyClone).

For proliferation studies, withdrawn MCF-7 E3 cells were utilized in a modified version of an assay previously reported [21]. Cells were plated in DCC medium on day 0 in 96-well plates (Costar) at a density of 4.5×10^3/well and then fed with medium containing the test compounds [0.1% (v/v) ethanol] on days 1 and 4. The relative cell number was determined on day 7 by exposure to Alamar Blue dye [22] for 3 hr followed by detection of fluorescent product on a Dynatek FL-1000 plate reader (Dynex Technologies).

Reporter gene assays utilized the MVLN variant of MCF-7, which has an estrogen-responsive luciferase reporter gene stably integrated [23]. Cells were plated in DCC medium on day 0 in 96-well plates (Costar) at a density of 6×10^4/well and then fed with medium containing test compounds [0.1% (v/v) ethanol] on days 1 and 2. Cells were lysed on day 3 (Cell Lysis Reagent, Promega Corp.), and relative luciferase activity was determined (Luciferase Assay System, Promega Corp.) utilizing a Dynex MLX luminometer (Dynex Technologies).

2.9. Fluorescence polarization determinations of ER binding

The ability of 4HBA and BA to displace specifically bound fluormone ES2 from recombinant human ERα or ERβ was determined, in comparison with E2, using a Beacon 2000 instrument (PanVera Corp.) [24].

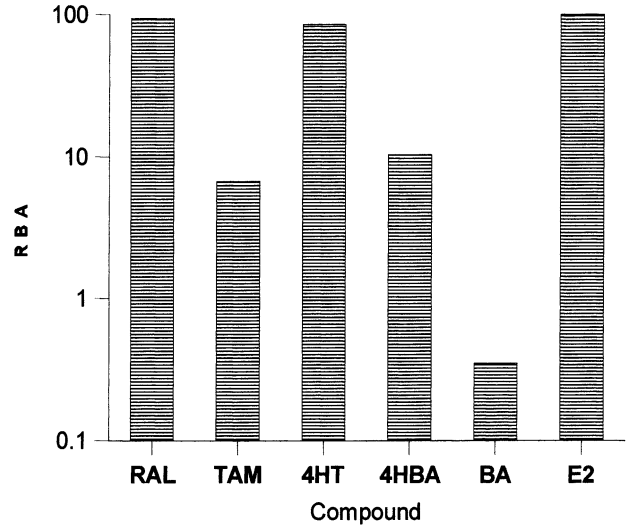

Fig. 2. Comparative ER affinities of 4HBA, BA, and standard ER ligands. The ability of each compound to displace specifically bound [^{3}H]E2 from hERα was determined as described previously [25]. The relative binding affinity (RBA) of each compound was calculated by dividing the concentration of E2 (3.3 nM) required to displace 50% of specifically bound [^{3}H]E2 by the concentration of test compound required to do this, and multiplying the result by 100. Data for the estimation of RBA values were obtained from triplicate determinations of specific [^{3}H]E2 binding as a function of test compound concentration.

3. Results

3.1. Interaction of 4HBA and BA with estrogen receptors

Affinity of 4HBA for human ERα was about one-tenth that of RAL or E2, but was comparable to that of TAM (Fig. 2) [25]. The RBA of BA for the ER was over one order of magnitude lower than that of 4HBA.

To assess the relative degree to which BA and 4HBA interacted with the two ER isoforms, the ratio of RBAs (hERα/hERβ) for each of these compounds was also assessed, using a fluorescence polarization assay [24]. Accordingly, BA and 4HBA had respective RBA ratios (hERα/hERβ) of 1.3 and 3.4.

3.2. Comparative estrogen mimetic/antagonist potency and efficacy of 4HBA in vitro

In the MCF-7 cell proliferation assay, 4HBA exhibited a proliferative efficacy about one-half that of E2, and antagonized the growth-stimulating effect of E2 by 60% (Fig. 3). In the concentration range tested, the standard estrogen antagonist (4HT) had 10–20% of the estrogen mimetic efficacy of E2, and antagonized E2 fully at a concentration of 1 μM. In the MVLN cell reporter assay, 4HBA and 4HT each had comparatively less agonist (and greater antagonist) efficacy than in the MCF-7 proliferation study, with the latter compound showing cytotoxicity at concentrations $\geq$ 1 nM. In both cell lines, 4HT was a more potent and effective E2 antagonist than was 4HBA.

3.3. Partial agonist effects of 4HBA and BA in reproductive tissue

As shown in Fig. 4, the weights of uteri from OVX rats treated with E2 (s.c.) or EE2 (p.o.) were markedly greater than those from respective vehicle-treated controls. Uterine weights in animals administered either 4HBA (s.c. or p.o.) or BA (p.o.) were also greater than those from vehicle-treated animals. But respective uterine weights were 25–29 and 29% of those from animals subjected to estrogen treatment.

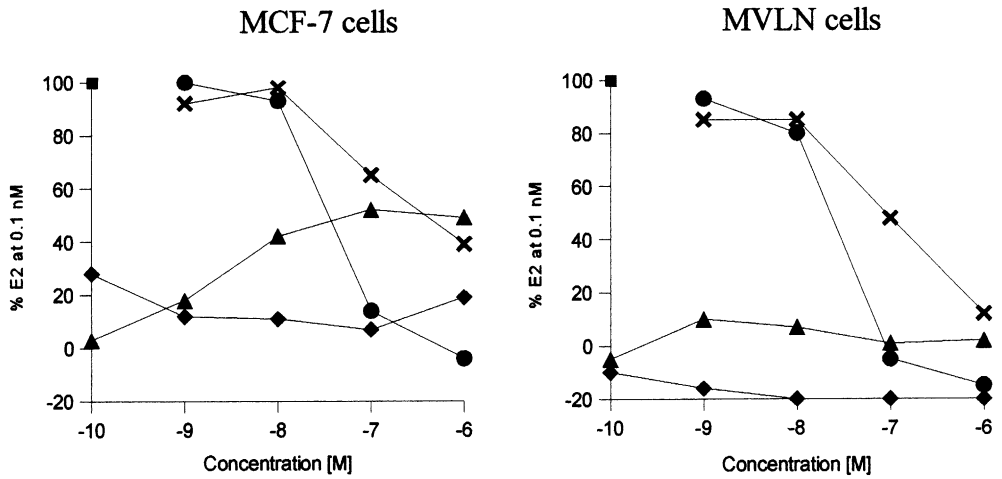

Fig. 3. Agonist and antagonist effects of ER ligands in E2 responsive cells. Extent of cell proliferation was determined at increasing concentrations of 4HT (◆,●), or 4HBA (▲,✖), in the absence (◆,▲) and presence (●,✖) of E2 (■): 1×10^{-10} M in MCF-7 cells, 1×10^{-10} M in MVLN cells. In MCF-7 cells, control E2-induced proliferation in each experiment was 2- to 3-fold over blank at 7 days; in MVLN cells, control E2 induction of the luciferase reporter was 3- to 5-fold over blank (2-day exposure). Negative reporter gene induction by 4HT was the result of cytotoxic effects on MVLN cells. Results shown for each cell line are representative of multiple experiments carried out under uniform conditions.

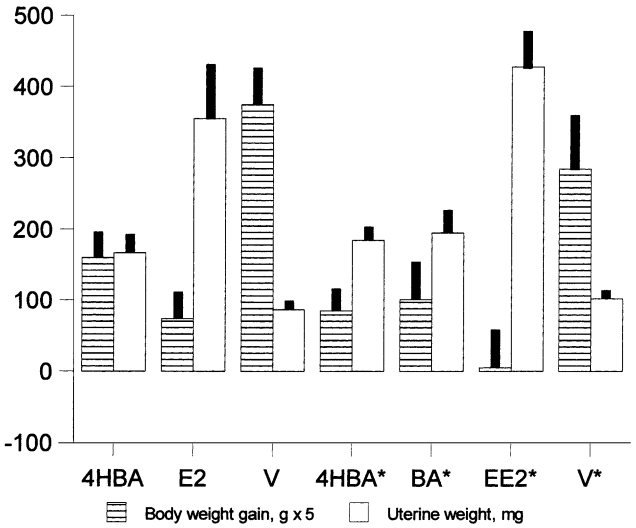

Fig. 4. Effect of treatment regimen on body weight gain and uterine weight. The dose of each test compound was 10 μmol/kg, and that of either E2 or EE2 was 0.35 μmol/kg. Animals (seven/group) received vehicle (V), standard estrogen, or test compound s.c., or p.o. (*), once a day, 5 days/week for 3 weeks. Standard deviations are indicated by vertical lines. For each test compound, $P < 0.05$ for each effect, compared with both the applicable vehicle-treated group and the applicable estrogen-treated one. For each estrogen-treated group, $P < 0.05$ compared with its respective vehicle-treated group.

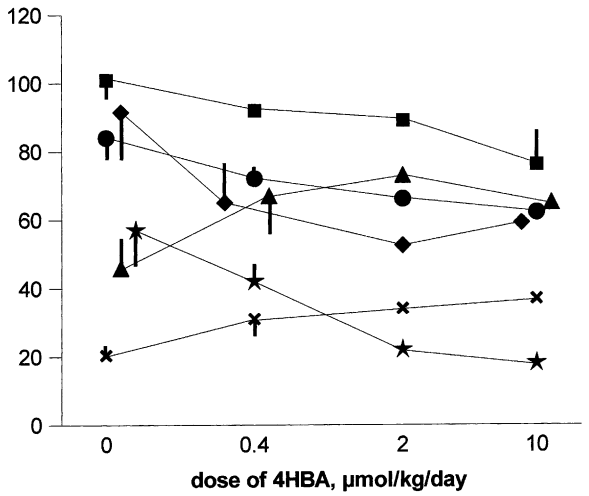

Fig. 6. Influence of the oral dose level on reproductive and extra-reproductive estrogen mimetic effects of 4HBA. [Key: body weight gain, g (★), uterine weight, mg/5 (✖), serum cholesterol, mg/dL (■), serum triglycerides, mg/dL (▲), serum OC, ng/mL (●), serum total Dpd, pmol/mL × 5 (◆).] There were seven animals per treatment group; serum samples from each animal were analyzed in duplicate for each of the indicated serum markers. Standard deviations for effects determined in the vehicle-treated (control) groups, and those associated with the *lowest* dose level at which $P < 0.05$ compared to respective control values, are indicated by vertical lines.

3.4. Equal effectiveness of 4HBA and BA in most other measures of estrogenicity in vivo

4HBA and BA suppressed, to about the same extent, the body weight gain seen in OVX rats administered only vehicle, although they were not as effective as EE2 in this regard (Fig. 4). At 10 μmol/kg oral doses (Figs. 5 and 6), 4HBA and BA each lowered serum cholesterol by about 30% with respect to vehicle-treated controls. Similarly, 4HBA and BA each lowered serum markers of excessive bone metabolism, Dpd and OC, by about 38 and 28%, respectively. On the other hand, treatment of OVX rats with BA resulted in a 100% increase in serum triglycerides (Fig. 5), but with 4HBA the maximal increase was 41% (Fig. 6).

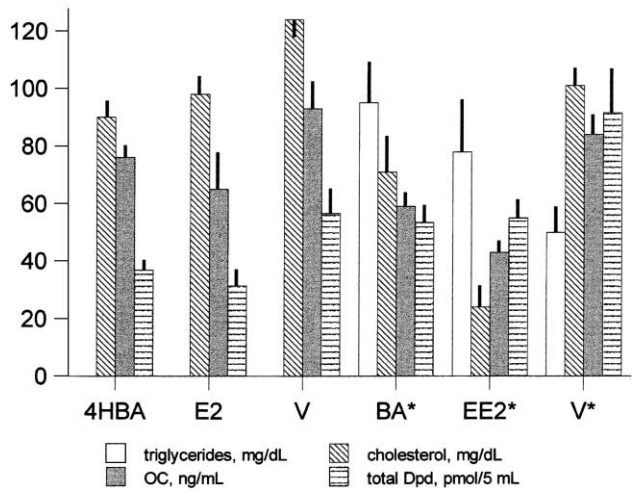

Fig. 5. Effect of treatment regimen on serum levels of markers of estrogen (mimetic) efficacy. Treatment regimens were given s.c., or p.o. (*), as summarized in Fig. 4. There were seven animals per treatment group; serum samples from each animal were analyzed in duplicate for each of the indicated serum markers. Standard deviations are indicated by vertical lines. For all effects of (a) 4HBA and E2 vs. V, (b) BA^* and $EE2^*$ vs. V^*, $P < 0.05$.

BA had *qualitatively* similar effects relative to those of EE2 on serum markers of estrogenic activity (Fig. 5). However, the relative magnitude of each of these was dependent upon the particular marker in question. Thus, although BA was less effective than EE2 in suppressing cholesterol and OC, its effects on Dpd suppression and triglyceride elevation were similar to those of EE2.

3.5. Variable estrogenic efficacy of 4HBA

At a daily dose of 0.4 μmol/kg, 4HBA exhibited its maximal effects (vs. vehicle-treated controls) on uterine weight (52% increase), serum OC and Dpd (14 and 29% decreases, respectively), and serum triglycerides (42% increase) (Fig. 6). Higher dose levels were required to attain maximal effects on serum cholesterol and body weight.

3.6. Biotransformation of BA to 4HBA in vitro

Incubation of BA with NADPH-fortified liver 9S fraction from OVX rats resulted in recovery of a single product

not found in incubations from which BA and/or the NADPH-generating system were omitted. This product had an HPLC retention time identical to that of authentic 4HBA. Its rate of formation was 37.8 ± 6.5 nmol/g of wet liver/20 min, corresponding to a conversion rate of 2.5%/20 min.

4. Discussion

Neither BA nor 4HBA exhibited a qualitative preference for one ER isoform over the other, findings similar to those reported for RAL and TAM [26,27]. Because 4HBA's RBA for hERα approached those of other SERMs and E2 (Fig. 2), further studies of its agonist/antagonist potency and efficacy were carried out in estrogen-responsive cells.

4HBA exhibited greater estrogen antagonist potency and efficacy in MCF-7 cells than did members of a series of triarylethylene oxyacetic acids in which estrogen mimetic activity was dominant [28]. Still, 4HBA was less effective (or potent) than 4HT in antagonizing E2 in the MCF-7 proliferation response or induction of the luciferase reporter gene in MVLN cells (Fig. 3). On the other hand, triarylethylene acrylic acid GW5638 had virtually no partial agonist efficacy and antagonized the residual partial estrogenic effect of 4HT in estrogen responsive cells [11,29]. These last results were obtained using a different line of estrogen-responsive cells than the ones used in the present study. Even with this qualification, it appears that 4HBA retained greater partial estrogen mimetic efficacy than did GW5638. This also seems to be the case in rat uterine tissue, in which 4HBA exhibited a partial estrogenic effect (25–29% that of E2) in the OVX rat (Fig. 4), an efficacy similar to that seen previously with TAM [30,31], but substantially greater than that reported for GW5638 [29]. Together, these results suggest that the ER complex of 4HBA has greater intrinsic activity than that of GW5638 in these cell/tissue types. This might arise from the greater ability of the oxybutyrate side chain of 4HBA, relative to the acrylate side chain of GW5638, to maintain the conformation and/or surface charge in the region of the ER LBD critical to coactivator docking. Indeed, molecular modeling studies of the interaction of the ER LBD with the phenolic analog of GW5638, 3-{4-[1-(4-hydroxyphenyl)-2-phenylbut-1-enyl]phenyl}acrylic acid (GW7604), revealed a strong repulsion of Asp 351 by the carboxyl group of GW7604 [32]. Because Asp 351 has been implicated in coactivator recruitment by the ER, this repulsion was proposed to account for the reduced partial estrogenicity of GW7604, and might also account for that of GW5638, compared to TAM [33].

In OVX rats, SERMs such as RAL and TAM exhibit reduction in serum cholesterol, OC, and urinary Dpd with an efficacy approaching that of nonselective ER modulators such as E2 and EE2, but have less effect on reproductive (uterine) tissues than do these steroidal estrogens. The maximal uterotrophic effect of either 4HBA or BA was slightly greater than that reported for RAL or TAM [30,31,34]. Both 4HBA and BA appeared to reduce serum cholesterol with an efficacy similar to that of the above SERMs, although potency of one of these (4HBA) seemed to be less than that of RAL and TAM [6,30,31]. Similarly, 4HBA and BA caused reduction in serum OC to about the same degree as reported for RAL and TAM, but none of these compounds were as effective as EE2 (cf. Fig. 5) [7]. However, the established SERMs (and 4HBA and BA) were as effective as EE2 in lowering urinary (serum) Dpd. Incidentally, although measurement of serum OC and Dpd levels as a function of treatment regimen has served as a proxy for the assessment of bone density maintenance [7,19,35], measurement of bone density and microanatomic parameters associated with the state of maintenance of cancellous bone might reveal differences between treatment groups having similar OC and Dpd levels. Thus, unequivocal comparison of bone loss suppressive effects of the above ER ligands would require evaluation of cancellous bone histomorphometry as a function of the treatment regimen. Nevertheless, these results indicate that the (hydroxy)triarylethylene oxybutyric acids 4HBA and BA exhibit tissue selective effects in the OVX rat that are comparable to those of RAL and TAM.

In the OVX rat and in humans, EE2 and other steroidal estrogens as well as TAM increase serum triglyceride levels, but RAL has no effect on this type of serum lipid [14,34,36–38]. The clinical significance of elevated triglycerides occurring during hormonal/antihormonal therapy is not clear [39,40]. This might be a consequence of a favorable influence of estrogens or TAM on serum triglyceride *composition* (such as unsaturated/saturated fatty acid ratio) because, in human subjects in general, serum triglyceride elevation has been identified recently as only a minor risk factor in coronary heart disease, which was less important in this regard than elevation of serum cholesterol [41]. Compounds 4HBA and BA, like TAM, were found in the present study to cause an elevation in serum triglycerides. Neither the cellular nor the molecular basis for this effect has been identified, except that TAM-stimulated triglyceride elevation in OVX rat serum was shown to be mediated *via* the ER [14].

Suppression of body weight gain in the OVX rat by estrogens and SERMs is a complex process that also appears to be mediated by the ER [42]. The compounds 4HBA and BA were approximately as effective as RAL but less so than TAM in this regard [31,34]. The clinical significance of this effect of steroidal estrogens and SERMs is unclear.

BA was more effective than 4HBA in its ability to elevate serum triglycerides, and was identical to 4HBA regarding all of its other estrogenic effects in the OVX rat, despite having an ER RBA of only 3.4% that of 4HBA (Fig. 2). These findings can best be accounted for by considering other factors, in addition to or besides ER

affinity, that could contribute to efficacy. First, the ability of BA to activate the ER for binding to ERE might be greater than "predicted" by its RBA. In the series of triarylethylene oxyacetic acid homologs of 4HBA referred to earlier, the estrogenic efficacy in MCF-7 cells did not correlate with the ER RBA in a straightforward way [28]. Thus, it must be kept in mind that structural characteristics *in addition to* those responsible for ER affinity can influence efficacy [43]. Second, the effects of BA could be mediated, in part, by interaction with other receptors besides the ER. Complete reversal of the effects of one or both of these compounds by co-administration of a suitable "pure" antiestrogen would rule out this possibility. However, to our knowledge there are no such substances fully capable of antagonizing the effects of E2, EE2, or SERMs in non-reproductive tissues (e.g. bone, liver) [14]. Third, BA might be a prodrug of 4HBA. Such an oxidative conversion was shown to occur in the liver 9S fraction (above), and a closely related homolog of BA also underwent facile 4-hydroxylation in male or female rat liver fractions [44]. Support for this proposition would, however, require tissue disposition studies, enabled by development of an analytic method of sufficient sensitivity and selectivity to allow quantitation of BA and 4HBA in tissue extracts.

We have characterized two triarylethylene oxybutyric acid derivatives, 4HBA and BA, whose nearly identical reproductive and extra-reproductive estrogenic efficacies were similar to those of established SERMs. Further pharmacologic studies in the OVX rat will focus on the ability of BA to maintain (a) bone histomorphometric parameters in comparison with EE2, and (b) hypothalmic and anterior pituitary levels of β-endorphin. Levels of this endogenous opiate, which is considered to be a marker for neuroendocrine function, are maintained by E2 or EE2 administration in the OVX rat, but are suppressed by (co-)administration of RAL or TAM [45,46].

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