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Mechanism of action of bolandiol (19-nortestosterone-3β,17β-diol), a unique anabolic steroid with androgenic, estrogenic, and progestational activities☆

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ABSTRACT

Bolandiol is a synthetic anabolic steroid that increases lean body mass and bone mineral density without significant stimulation of sex accessory glands in castrate adult male rats. Since bolandiol suppresses gonadotropins and endogenous testosterone (T) production, we investigated its mechanism of action. We compared the potency of bolandiol *in vitro* and *in vivo* with T, 5α-dihydrotestosterone (DHT), 19-nortestosterone (19-NT) and estradiol (E_2). Bolandiol bound with lower affinity to the recombinant rat androgen receptor (AR) than the other androgens and had low, but measurable, affinity for recombinant human progestin receptors (PR-A, PR-B), and estrogen receptors (ERα and β-1). Functional agonist activity was assessed in transcription assays mediated by AR, PR, or ER. Bolandiol was stimulatory in all these assays, but only 4–9% as potent as T, DHT, and 19-NT via AR, 1% as potent as progesterone via PR, and 3% and 1% as potent as E_2 acting through ERα or ERβ, respectively. In immature castrate rats, bolandiol was equipotent to T in stimulating growth of the levator ani muscle but less potent than T in stimulating growth of the sex accessory glands. Bolandiol also stimulated uterine weight increases in immature female rats, which were partly blocked by ICI 182,780, but it was not aromatized *in vitro* by recombinant human aromatase. In contrast to T, stimulation of sex accessory gland weights by bolandiol was not inhibited by concomitant treatment with the dual 5α-reductase inhibitor dutasteride. As bolandiol exhibits tissue selectivity *in vivo*, it may act via AR, PR, and/or ER, utilize alternative signaling pathway(s) or transcriptional coregulators, and/or be metabolized to a more potent selective steroid.
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1. Introduction

Androgens exhibit anabolic activity in a number of tissues including muscle, bone, prostate and hair follicles. In addition, exogenous androgens interrupt naturally occurring steroid hormone negative feedback leading to suppression of gonadotropin production by the pituitary and profound inhibition of endogenous testosterone (T) production. Steroid analogues are under development to specifically, and independently, target these effects. For example, small molecules with only anabolic properties that spare the prostate, termed selective androgen receptor modulators (SARMs), are currently in development for the treatment of wasting diseases and, potentially, androgen deficiency syndromes [1]. In contrast, an ideal male hormonal contraceptive would achieve complete gonadotropin suppression, thus blocking spermatogenesis, but also maintains the anabolic effects of androgens in muscle, bone, and hair, and inhibits or has no effect on prostate growth.

Bolandiol (19-nortestosterone-3β,17β-diol; 4-estren-3β,17β-diol; 3β-dihydronandrolone) is a synthetic anabolic steroid that until recently was available as a dietary supplement used by athletes to enhance performance. Previously, we compared the activity of bolandiol in a castrated adult male Sprague Dawley rat model to that of 19-nortestosterone (19-NT: nandrolone), a proposed metabolite of bolandiol [2] and dihydrotestosterone (DHT), all administered in Silastic implants [3]. Long-term administration of bolandiol (20–24 wk) prevented the loss of lean body mass (LBM), bone mineral density (BMD), and levator ani (LA) muscle weight that occurs in castrate rats while inhibiting gonadotropin and endogenous T production. The effects of bolandiol were dose-dependent and similar to those observed after administration of DHT or 19-NT to castrate animals. On the contrary, bolandiol resulted in little or no stimulation of ventral prostate (VP) or sem-

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inal vesicles (SV) weights at these doses. Thus, bolandiol exhibited tissue selectivity *in vivo* with a pharmacodynamic profile suitable for potential development as a male contraceptive or a "prostate-sparing" androgen in which suppression of endogenous T might be desirable.

Since bolandiol demonstrated some degree of tissue selectivity with long-term administration, we wanted to further characterize its mechanism of action. In the present study, the potency of bolandiol in various *in vitro* assays was compared with the potencies of T, DHT, and 19-NT. We determined the relative binding affinity (RBA) of these androgens for various classes of steroid hormone receptors as well as their functional activity in transactivation assays mediated by these receptors. The comparative androgenic/anabolic potency *in vivo* was evaluated using stimulation of sex accessory glands and LA muscle weights and suppression of gonadotropin secretion as endpoints in the immature castrate male rat. The possibility that bolandiol manifests estrogenic activity *in vivo* was investigated in the immature female rat by assessing dose-dependent increases in uterine weight following bolandiol or 17β-estradiol (E_2) treatment.

Finally, metabolism of bolandiol could play a role in the selectivity of its *in vivo* activities. It has been suggested that the anabolic activity of bolandiol is attributable to its conversion by 3β-hydroxysteroid dehydrogenase (3β-HSD) to 19-NT [2,4–6], a potent anabolic steroid [7–9], because increased urinary metabolites of 19-NT can be measured after oral dosing of men with bolandiol [10–12]. Interestingly, the prohormone of 19-NT, 19-norandrostenedione, has recently been shown to have tissue selectivity in castrate male rats [13] supporting the notion that precursors and metabolites of these steroids may have unique profiles of tissue activity. Furthermore, by analogy to the major pathways by which T is metabolized, bolandiol could also be converted to tetrahydronandrolone (5β-estran-3α-ol-17-one) by 5α-reductase or to E_2 by aromatase which might contribute to bolandiol's ability to maintain BMD in castrate adult rats [3]. Therefore, we examined the potential metabolism of bolandiol by 5α-reductase *in vivo* and the possible conversion of bolandiol to E_2 by purified recombinant human aromatase *in vitro*.

2. Materials and methods

2.1. Chemicals

Bolandiol (19-nortestosterone-3β,17β-diol; 4-estren-3β,17β-diol; 3β-dihydronandrolone), T, E_2, DHT, 19-NT, dexamethasone, and progesterone were purchased from Steraloids (Newport, RI) and methyltrienolone (R1881, a nonaromatizable androgen), from PerkinElmer Life Sciences (Boston, MA). The antiprogestins, CDB-2914, CDB-4124, and mifepristone and the antiandrogens, hydroxyflutamide and bicalutamide, were received from Southwest Foundation for Biomedical Research (San Antonio, TX) and were >99% pure by HPLC. Nilutamide and ICI 182,780 were from Tocris-Cookson, Ltd. (Bristol, UK). The dual 5α-reductase inhibitor, dutasteride (17β-N-2,5-bis-[trifluoromethyl]-phenylcarbamoyl-4-aza-5a-androstan-1-en-3-one), was from AK Scientific, Inc. (Mountain View, CA) and was >98% pure by HPLC.

2.2. Steroid hormone receptor assays

Competitive binding assays for recombinant human (rh) PR-A and rhPR-B were performed using cytosolic preparations from Sf9 insect cells infected with recombinant baculovirus expressing either hPR-A or hPR-B obtained from Dr. Dean Edwards (Baylor, Houston TX), as described previously [14]. The assay for AR binding employed purified rat AR ligand binding domain (ARLBD), that for ER employed purified recombinant human ERα and ERβ-1 long form, and that for glucocorticoid receptors (GR) employed purified recombinant human GR, all purchased from Invitrogen (Carlsbad, CA) [15,16]. Receptor-bound cpm were determined in a scintillation counter and entered into Packard's RIASmart™ (PerkinElmer, Boston, MA) for calculation of IC_{50}'s using a 4-parameter logistic curve fit. R1881, progesterone, E_2, and dexamethasone were the standards for the AR, PR, ER, and GR assays, respectively, and relative binding affinities (RBA's) were normalized to binding of these standards at 100%.

2.3. Cloning

Full length human ERα and ERβ were cloned into the mammalian expression vector pCR3.1 (Invitrogen). The orientation and sequence of each clone were verified by sequencing. The ERE-LUC construct was generated by cloning the estrogen responsive promoter element (ERE) from the vitellogenin A2 promoter (−331 to −87) into the pGL3-basic reporter construct (Promega, Madison, WI) [17].

2.4. Cell culture and transfection of plasmid DNAs

All cells were cultured in phenol-red free media supplemented with 5–10% charcoal-stripped FBS (Hyclone, Logan, UT). T47Dco human breast cancer cells (a gift of Dr. Kathryn Horwitz, UCHSC, Denver, CO), which express approximately equimolar concentrations of constitutively produced hPR-A and hPR-B [18], were used to assess progestational agonist or antagonist activities. The cells were transiently transfected with PRE_2-tk-LUC, a reporter plasmid containing two copies of a progestin/glucocorticoid/androgen response element (PRE) upstream of the thymidine kinase (tk) promoter and the firefly luciferase (LUC) gene (a gift of Dr. Dean Edwards) using FuGENE™ 6 transfection reagent (Roche, Indianapolis, IN) as described previously [14]. Functional estrogenic activity was also assessed in the T47Dco cell line, which possesses endogenous ER (predominantly ERα, B. Attardi, unpublished obser-

Table 1
Binding of various steroids to AR, PR, and ER.

Steroid	ARLBD		PR-B		PR-A		ERα		ERβ-1	
	IC_{50} (nM)	RBA[a] (%)	IC_{50} (nM)	RBA[a] (%)	IC_{50} (nM)	RBA[a] (%)	IC_{50} (nM)	RBA[a] (%)	IC_{50} (nM)	RBA[a] (%)
R1881 AR standard	6.2 ± 0.6	100[b]	8.7 ± 1.5	139	9.0 ± 0.8	140	ND		ND	
Progesterone PR standard	>500	<1	8.6 ± 0.5	100[b]	9.2 ± 0.6	100[b]	ND		ND	
E_2 ER standard	>500	<1	ND		ND		5.1 ± 0.3	100[b]	5.1 ± 1.0	100[b]
Bolandiol	163.0 ± 10.7	2	115.4 ± 11.0	6	97.2 ± 10.6	5	134.4 ± 12.9	4	254.7 ± 21.6	2
T	27.2 ± 0.3	22	>500	<2	>500	<2	>1000	<1	>1000	<1
5α-DHT	21.4 ± 3.4	33	>500	<2	>500	<2	>1000	<1	>1000	<1
19-NT	11.4 ± 2.6	65	67.8 ± 11.8	12	74.9 ± 19.0	11	>1000	<1	>1000	<1

ND, not done.
[a] Relative binding affinity.
[b] Defined.

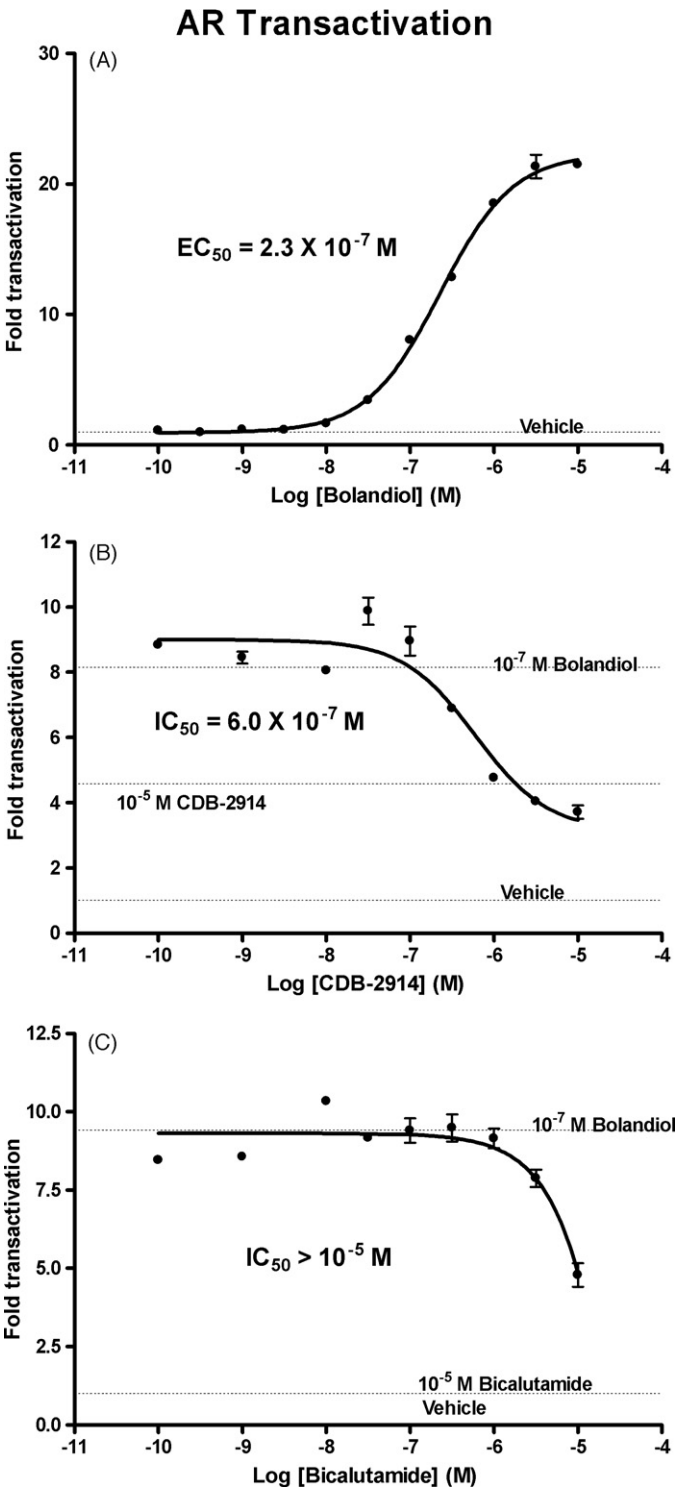

Fig. 1. Androgenic activity of bolandiol. CV-1 cells were transiently transfected with 3XHRE-LUC and a human AR expression vector and treated with various concentrations of bolandiol (A). Inhibition of bolandiol-stimulated transactivation by the antiprogestin CDB-2914 (B) or by the antiandrogen bicalutamide (C).

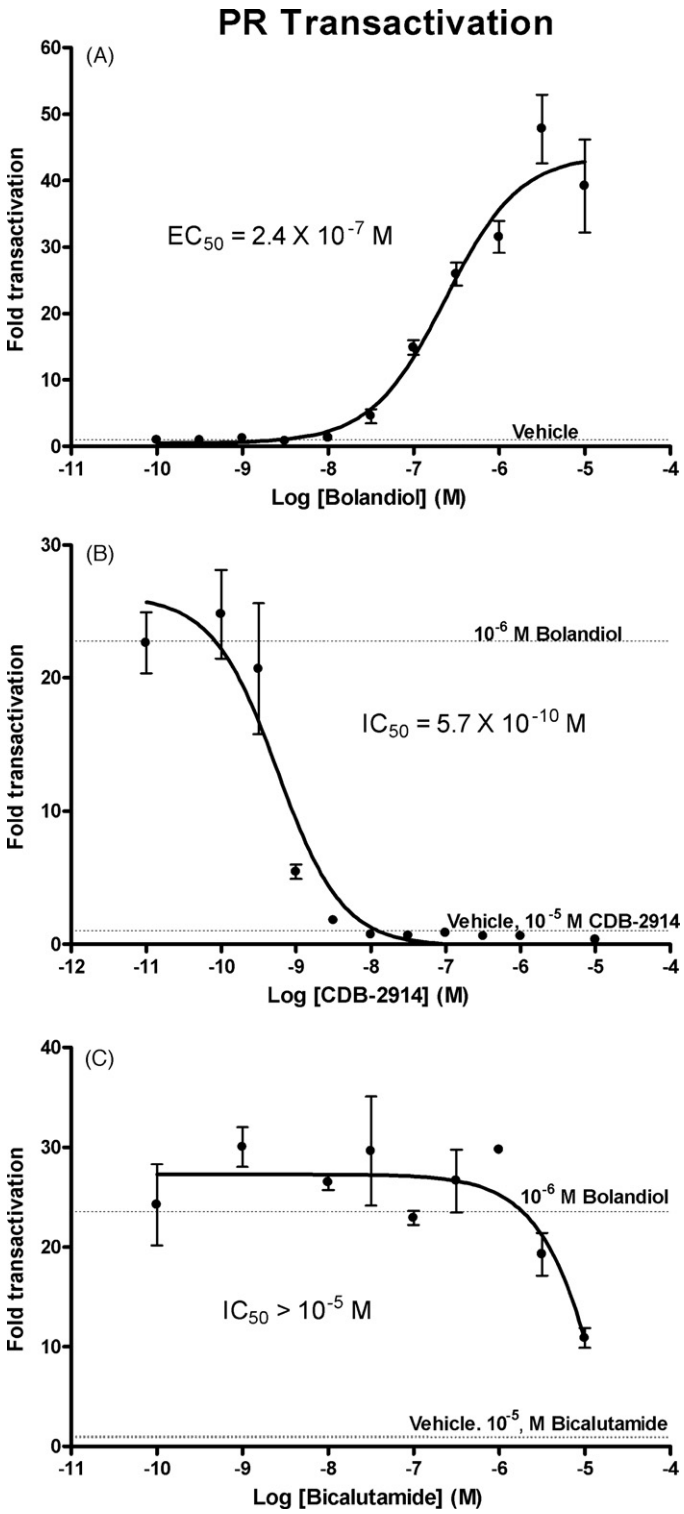

Fig. 2. Progestational activity of bolandiol. T47Dco cells were transiently transfected with PRE_2-tk-LUC and treated with various concentrations of bolandiol (A). Inhibition of bolandiol-stimulated transactivation by CDB-2914 (B) or by bicalutamide (C).

vation) as well as PR. Cells were transfected with 3XERE-LUC, a reporter plasmid containing three copies of an estrogen response element (ERE) upstream of LUC (a gift of Dr. Donald McDonnell, Duke University, Durham, NC) [16]. To evaluate androgenic activity, CV-1 cells were cotransfected with 3XHRE-LUC, containing three copies of a PRE, and a human AR expression vector (pCMV5hAR3.1), both gifts from Dr. Diane Robins (University of Michigan Medical Center, Ann Arbor, MI) using the same conditions. After 6 h, the medium containing FUGENE™ 6 was removed and replaced with medium containing various concentrations of test compounds. Cells were lysed 20 h later, and supernatants were analyzed for protein content and LUC activity [14,16]. Relative light units (RLU) were normalized for differences in protein content per well and plotted

vs concentration of added test compound to determine EC_{50}'s or IC_{50}'s using GraphPad PRISM™, version 4.0 (San Diego, CA).

HEK-293 cells were incubated in 24-well plates with Opti-MEM (Invitrogen) for 30 min before transient transfection using FUGENE HD (Roche) in accordance with the manufacturer's instructions. Aliquots of pGL3-ERE-LUC (0.25 μg) and CMV-Renilla LUC (5 ng) plasmids were cotransfected with either 12.5 ng of empty pCR3.1 (Invitrogen), pCR3.1-hERα or pCR3.1-hERβ plasmids. After 12 h, cells were treated for 24 h with vehicle (0.1% ethanol) or increasing concentrations of E_2, bolandiol or 19-NT. Following treatment, cells were assayed for LUC activity using the Dual-Glo® Luciferase Assay System (Promega) according to the manufacturer's guidelines and a Victor 3 V Multi-label Counter (PerkinElmer). RLU for firefly LUC were normalized to RLU for Renilla LUC, and data were plotted and calculated using SigmaPlot (SPSS Inc., Chicago, IL).

2.5. In vivo androgenic and estrogenic assays

All study protocols were approved by BIOQUAL's IACUC. For assessment of androgenic activity, immature male Sprague Dawley CD rats were purchased from Charles River Laboratories (Kingston, NY) and castrated at 22 days of age. Rats (8/group) were injected sc with either vehicle (10% ethanol in sesame oil), bolandiol, or 19-NT for 8 consecutive days and euthanized by exsanguination 4 h after the last dose. Body weights were obtained, and the VP, SV, and LA muscle were excised, trimmed, blotted, and weighed [19,20]. There was no significant effect of any treatment on final body weights. To determine whether the stimulatory effect of androgens on growth of the VP and SV was reduced by dutasteride, rats (10/group) were injected sc with either vehicle or maximal stimulatory doses of bolandiol, 19-NT, or T for 8 consecutive days. Concurrent with the injections, vehicle or dutasteride was administered orally at l.0 mg/rat/day, a dose which completely suppressed conversion of T to DHT without overt signs of toxicity [21]. Two hours after the final dose, the rats were euthanized, and body, VP, and SV weights were obtained.

Estrogenic activity was assessed in the immature female rat uterotropic assay [22–24]. Briefly, 18-day old Sprague Dawley female rat pups, purchased from Harlan Laboratories (Frederick, MD), were injected sc with vehicle (10% ethanol in sesame oil), or various doses of E_2, or bolandiol for 3 consecutive days (8 rats/group). Additional groups of rats were injected with vehicle, or maximal stimulatory doses of bolandiol or E_2 concurrent with oral administration of the ER antagonist ICI 182,780 at 0.4 mg/rat/day for 3 days. One day following the last treatment, all animals were euthanized. Uteri were excised above the cervix and weighed to the nearest 0.1 mg after expressing fluid.

2.6. LH RIA

LH was measured in rat serum using NIDDK reagents supplied by Dr. A. Parlow following the procedures received with the reagents [20]. The standard was NIDDK-rLH-RP-3. The limit of detection was 0.31 ng/ml based on 200 μl serum.

2.7. Incubations with recombinant human aromatase

T or bolandiol (50 μM) was incubated with GENTEST Human CYP19 + P450 Reductase SUPERSOMES™ (0.1 μM) for various times using conditions specified by the manufacturer (BD Biosciences, Woburn, MA) and described previously [16]. Briefly, reaction mixtures contained an NADPH regenerating system and 3.3 mM $MgCl_2$ in 100 mM potassium phosphate buffer, pH 7.4. After incubation for various times at 37 °C, reactions were stopped by the addition of one-half volume ACN and centrifugation at 14,000 × *g* for 3 min. The supernatant was removed, and 50 μl was injected into a HPLC column. Substrate and potential aromatic A-ring product were separated using a Waters HPLC system (Milford, MA). Peaks of steroids were monitored by UV absorbance. AUC's (areas under the curve integrated for the UV peaks at a specified retention time) were determined using Waters Empower™ software. For quantification of the amount of substrate converted or the amount of product formed, standard curves were constructed according to the manufacturer's instructions. The limit of detection was 20 ng.

2.8. Analysis of data

Data are expressed as mean ± SE ($n \geq 3$) or mean ± SD ($n = 2$). GraphPad PRISM™ was used for graphics and determination of IC_{50}'s and EC_{50}'s for inhibition or stimulation, respectively, of transactivation. In all steroid receptor binding assays, cpm were entered into RiaSmart™ for calculation of IC_{50}'s using a 4-parameter logistic curve fit. Relative binding affinities (RBA, %) for each compound were calculated as follows: IC_{50} of standard/IC_{50} of competitor X 100. Differences in VP, SV, or LA weights between bolandiol- and T-treated rats (Fig. 4) were compared by one-way ANOVA followed by Holm–Sidak multiple comparison tests; SV and LA weights were $\log_{10}$ transformed prior to analysis. Serum LH levels in rats treated with vehicle, bolandiol, or T, or vehicle, 19-NT, or T (Fig. 5) were compared by Kruskal–Wallis one-way ANOVA on ranks followed by the Student–Newman–Keuls test. Uterine weights in rats treated with E_2 or bolandiol with or without ICI 182,780 (Fig. 6) and VP and SV weights in rats treated with androgens and dutasteride or vehicle (Fig. 7) were compared by Student's *t*-test. All tests used SigmaStat 3.5 software (SPSS, Inc.). $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Binding of bolandiol and other androgens to recombinant AR, PR, ER, or GR

Table 1 summarizes the IC_{50} values and RBA's, of bolandiol, T, DHT, and 19-NT for ARLBD, PR-A, PR-B, ERα and ERβ-1. Bolandiol showed weak, but measurable, affinity for all these receptors (2–6% of the standards). T and DHT did not bind to PR or ER, whereas 19-NT bound to PR with about 10% of the affinity of progesterone, the standard in the PR assay. Similar binding of 19-norandrogens to PR has been demonstrated previously [20]. Unlike bolandiol, 19-NT did not bind ER. The RBA of bolandiol for GR was <1% that of the standard, dexamethasone (data not shown).

3.2. Transactivation by bolandiol and other androgens mediated by AR, PR, ER, or GR

The androgenic potencies of these steroids were compared by cotransfecting CV-1 cells with a human AR expression vector and the 3XHRE-LUC reporter vector. In agreement with the results above demonstrating weak binding of bolandiol to AR, bolandiol was considerably less potent than T, DHT, or 19-NT in the transactivation assay. A representative experiment showing concentration-dependent transactivation of 3XHRE-LUC by bolandiol is illustrated in Fig. 1A, and the EC_{50}'s for transactivation of 3XHRE-LUC by bolandiol, T, DHT, and 19-NT from several experiments are summarized in Table 2. Antiprogestins, CDB-2914 (Fig. 1B) and mifepristone, partially reduced bolandiol's transcriptional activity (IC_{50}'s $\sim 10^{-7}$ M), but only to the level of their agonist activity in this cell system. The effects of these antiprogestins are presumably mediated by their weak, but measurable, binding to AR as CV-1 cells contain no

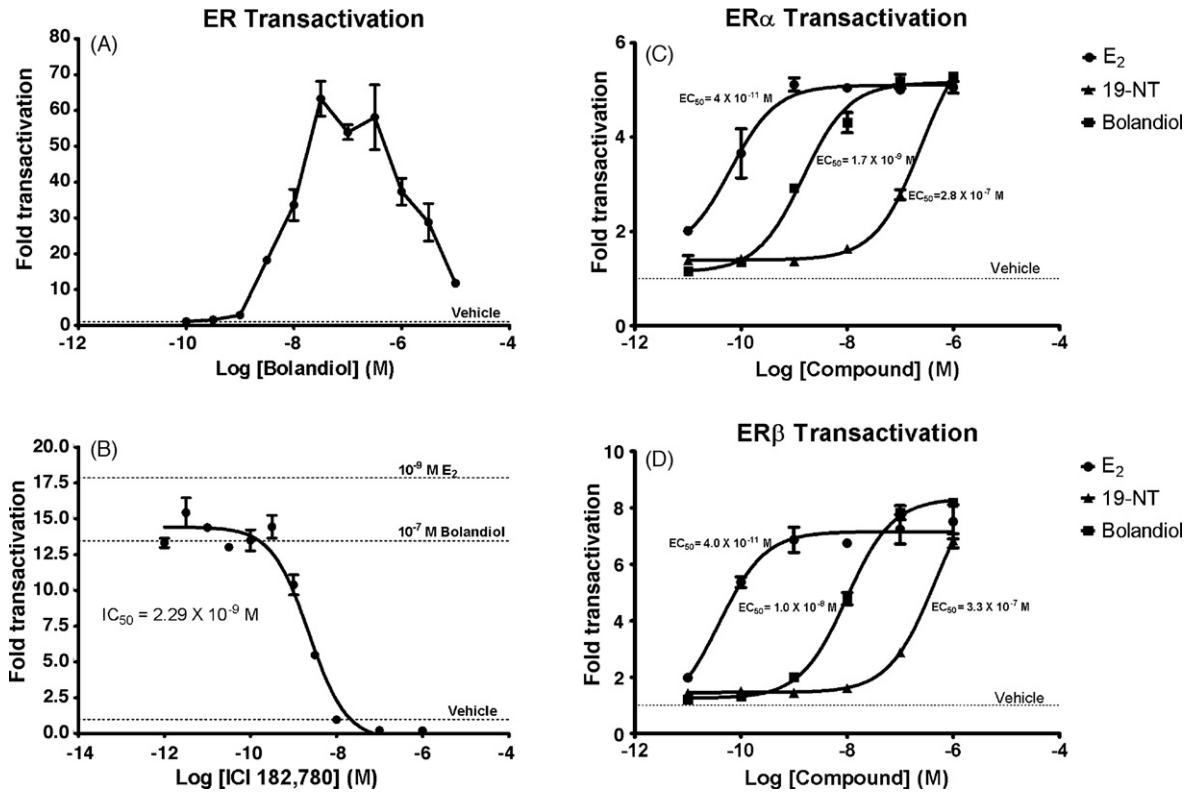

Fig. 3. Estrogenic activity of bolandiol. T47Dco cells were transiently transfected with 3XERE-LUC and treated with various concentrations of bolandiol (A). Inhibition of bolandiol-stimulated transactivation by ICI 182,780 in T47Dco cells (B). HEK-293 cells were cotransfected with ERE-LUC and hERα (C) or hERβ (D) and treated with various concentrations of E_2, bolandiol, or 19-NT.

endogenous steroid hormone receptors [20]. Antiandrogens, bicalutamide (Fig. 1C), hydroxyflutamide, and nilutamide inhibited bolandiol-stimulated transactivation only at very high concentrations.

Like other 19-norandrogens [20], bolandiol and 19-NT also exhibited progestational activity as judged by their ability to stimulate transcription of PRE_2-tk-LUC in T47Dco cells mediated by endogenous PR (Fig. 2A and Table 2). In fact, the EC_{50}'s for transactivation mediated by AR in CV-1 cells and by PR in T47Dco cells were very similar for both compounds. Antiprogestins were potent inhibitors of bolandiol-induced transactivation in T47Dco cells (CDB-2914 in Fig. 2B), whereas antiandrogens again blocked transcription only at the highest concentrations tested ($IC_{50} > 10^{-5}$ M) (bicalutamide in Fig. 2C). The C-19 androgens, T and DHT, showed negligible transcriptional activity in T47Dco cells as expected [20]. Bolandiol was not completely promiscuous as it did not stimulate transcription of PRE_2-tk-LUC in HepG2 cells cotransfected with a rat GR expression vector (data not shown).

Bolandiol bound to the ER subtypes, albeit weakly (Table 1), and also demonstrated ER-mediated transactivation in T47Dco cells. Bolandiol had a biphasic effect on transactivation of 3XERE-LUC (Fig. 3A) which was maximal at about 10^{-7} M and was completely obliterated by concomitant incubation of transfected T47Dco cells with bolandiol and ICI 182,780 ($IC_{50} = 2.3 \times 10^{-9}$ M; Fig. 3B). In contrast, the EC_{50} for transactivation of 3XERE-LUC by E_2 in T47Dco cells was $1.55 \pm 0.57 \times 10^{-11}$ M (B. Attardi, unpublished observation). HEK-293 cells cotransfected with ERα or ERβ expression vectors and pGL3-ERE-LUC were used to determine whether bolandiol-stimulated transcription was mediated by ERα or ERβ or both. As shown in Fig. 3C and D and Table 2, bolandiol was 6–7 times more potent in cells containing ERα than in those containing ERβ and about 33- and 167-fold less potent than E_2 in the presence of ERα or ERβ, respectively. In our previous experience, several other 19-norsteroids (i.e. 5α-dihydronorethindrone, 5α-dihydrodimethandrolone, 5α-dihydro-11β-methyl-19-NT, B. Attardi, unpublished observations) showed similar biphasic trans-

Table 2
Transactivation of 3XHRE-LUC (CV-1 cells), PRE_2-tk-LUC (T47Dco cells), or ERE-LUC (HEK-293 cells) mediated by AR, PR, or ER, respectively, by various steroids.

Steroid	CV-1 cells (AR), EC_{50} (M)	T47Dco cells (PR), EC_{50} (M)	HEK-293 cells (ER)	
			ERα, EC_{50} (M)	ERβ, EC_{50} (M)
R1881	$3.88 \pm 1.53 \times 10^{-10}$	$1.44 \pm 0.08 \times 10^{-10}$	ND	ND
Progesterone	$2.40 \pm 1.13 \times 10^{-6}$	$3.09 \pm 1.42 \times 10^{-9}$	ND	ND
E_2	ND	ND	$6.0 \pm 2.8 \times 10^{-11}$	$8.0 \pm 5.7 \times 10^{-11}$
Bolandiol	$1.73 \pm 0.36 \times 10^{-7}$	$2.75 \pm 0.20 \times 10^{-7}$	$2.0 \pm 0.42 \times 10^{-9}$	$1.34 \pm 0.48 \times 10^{-8}$
T	$1.36 \pm 0.59 \times 10^{-8}$	Negligible	ND	ND
5α-DHT	$6.51 \pm 2.52 \times 10^{-9}$	Negligible	ND	ND
19-NT	$1.62 \pm 0.61 \times 10^{-8}$	$1.30 \pm 0.22 \times 10^{-8}$	ND	ND

Values represent the mean ± SE of 3–7 determinations or the mean ± SD of 2 determinations (R1881 in T47Dco cells, ERα and ERβ). ND, not done.

activation of 3XERE-LUC in T47Dco cells, possibly due to conversion of these synthetic steroids, at high concentrations, to products with inhibitory activity.

3.3. Androgenic activity of bolandiol in the castrate immature male rat

Two androgenic bioassays were carried out to assess the relative potency of bolandiol, T, and 19-NT following daily sc administration to castrate immature male rats. In the first assay, dose-dependent effects of bolandiol and T were determined, and in the second, dose-dependent effects of 19-NT were compared to those of T. The data from the two experiments are combined in Fig. 4. There was no significant effect of these treatments on final body weight. Though both bolandiol and T were effective in stimulating VP and SV weights, the effects of T occurred at lower doses. To obtain similar growth with bolandiol, higher doses were required; for example, there were no significant differences in VP weights in animals treated with bolandiol at 1 or 4 mg total dose compared with T at 0.1 and 0.4 mg total dose ($P > 0.05$). SV weights at 1 mg total dose of bolandiol were not different from those at 0.4 mg total dose of T, and SV weights at 4 mg total dose of bolandiol were not different from those at 1.6 mg total dose of T ($P > 0.05$). In terms of LA muscle weights, the potency lines for bolandiol and T overlapped indicating that the response was equivalent at the same dose. 19-NT was the weakest androgen, whereas bolandiol was about as effective as T in increasing LA muscle and had less of an effect on the prostate than T. In both assays, serum samples obtained 4 h after the final dose on day 8 were assayed to determine treatment effects on circulating LH (Fig. 5A and B). Serum LH levels were significantly suppressed in all rats treated with bolandiol (Fig. 5A) or 19-NT (Fig. 5B), whereas T-induced suppression of serum LH was significant only at a daily dose of 0.2 mg/rat or higher (Fig. 5A and B). Serum T levels in those rats treated with T increased with increasing daily doses and were similar in both assays (data not shown). There was no significant effect of ICI 182,780 on bolandiol-stimulated growth of the VP, SV, and LA ($P > 0.05$), suggesting that ER are not involved in this model (data not shown).

3.4. Estrogenic activity of bolandiol in immature female rats

We investigated the potential estrogenic activity of bolandiol *in vivo* in immature female rats in comparison to E_2 in the presence or absence of the ER antagonist ICI 182,780. Treatment with either E_2 or bolandiol resulted in dose-dependent increases in uterine weight (Fig. 6). The ER antagonist ICI 182,780 alone did not affect uterine growth as expected (data not shown), but it completely inhibited E_2-stimulated increases in uterine weight ($P < 0.05$) confirming our previous results (S. Hild, unpublished observation). ICI 182,780 partially, but significantly ($P < 0.05$), inhibited the bolandiol-stimulated uterine weight increase implicating ER in this effect. However, the failure of ICI 182,780 to completely inhibit bolandiol's action on the uterus is presumably due to androgenic activity of bolandiol on the myometrium as androgens stimulate myometrial muscle growth [24].

3.5. Possible metabolism of bolandiol

By analogy to the metabolism of T, bolandiol could be converted by 5α-reductase to tetrahydronandrolone or by aromatase to E_2. A third possibility is conversion to 19-NT by 3β-HSD [2]. The first possibility was investigated *in vivo* using the castrate 22-day old rat model in which the dual 5α-reductase inhibitor dutasteride was administered concurrently with maximal stimulatory doses of androgens. The effect of these treatments on androgenic endpoints was evaluated (Fig. 7). Dutasteride significantly suppressed

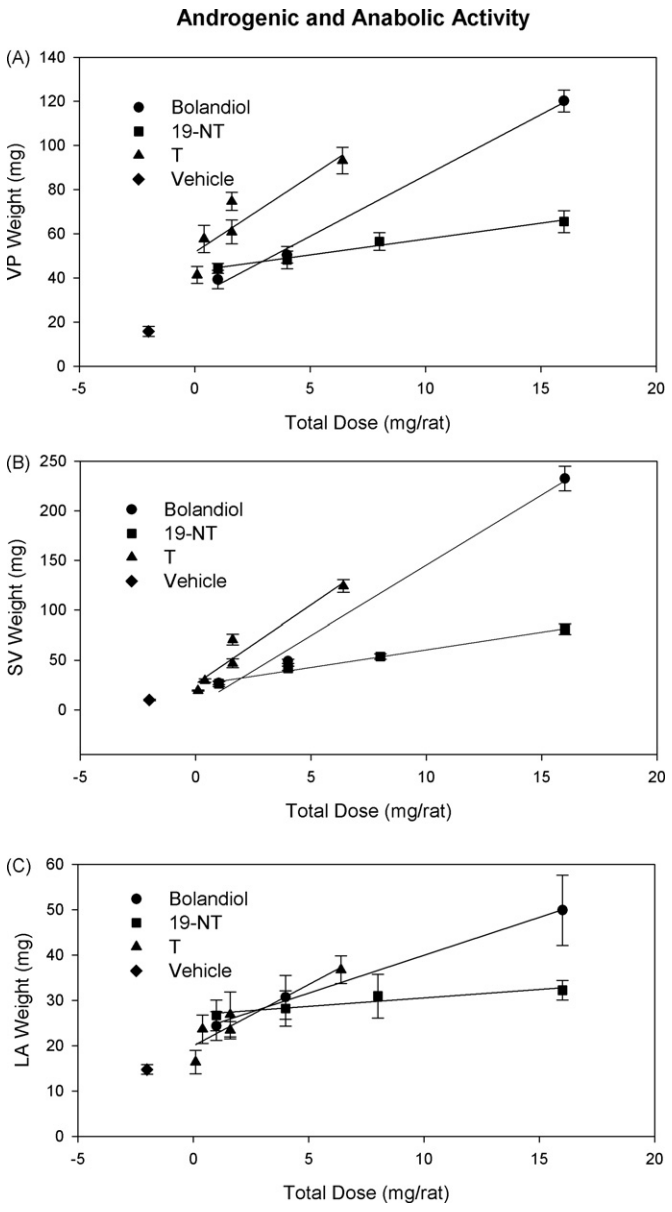

Fig. 4. Dose–response for stimulation of (A) ventral prostate (VP), (B) seminal vesicles (SV), or (C) levator ani muscle (LA) weights by various androgens or vehicle. Twenty-two-day old castrate rats were treated sc for 8 days with bolandiol (0.13, 0.5, or 2.0 mg/rat/day), 19-NT (0.13, 0.5, 1.0, or 2.0 mg/rat/day), T (0.013, 0.05, 0.2, or 0.8 mg/rat/day), or vehicle (10% ethanol in sesame oil), necropsied, and VP, SV, and LA weights determined. Organ weights in vehicle-treated animals have been displaced to the left of 0 dose on the *x*-axis for clarity. There were no significant differences in VP weights in animals treated with bolandiol at 1 or 4 mg total dose compared with T at 0.1 and 0.4 mg total dose ($P > 0.05$). SV weights at 1 mg total dose of bolandiol were not different from those at 0.4 mg total dose of T, and SV weights at 4 mg total dose of bolandiol were not different from those at 1.6 mg total dose of T ($P > 0.05$). There was no significant effect of these treatments on final body weight.

T-stimulated VP ($^*P < 0.05$) and SV ($^*P < 0.001$) weights (Student's *t*-test) as before [21]. However, there was no significant effect of dutasteride on sex accessory gland weights in rats treated with vehicle or bolandiol suggesting that 5α-reduction either does not occur or is not important in contributing to bolandiol's potency. In contrast, dutasteride, in combination with 19-NT, resulted in a small, but significant, increase in VP and SV weights compared to rats dosed with 19-NT alone. Thus, bolandiol and 19-NT do not need to be converted to their 5α-reduced metabolites to exert maximal biological effects.

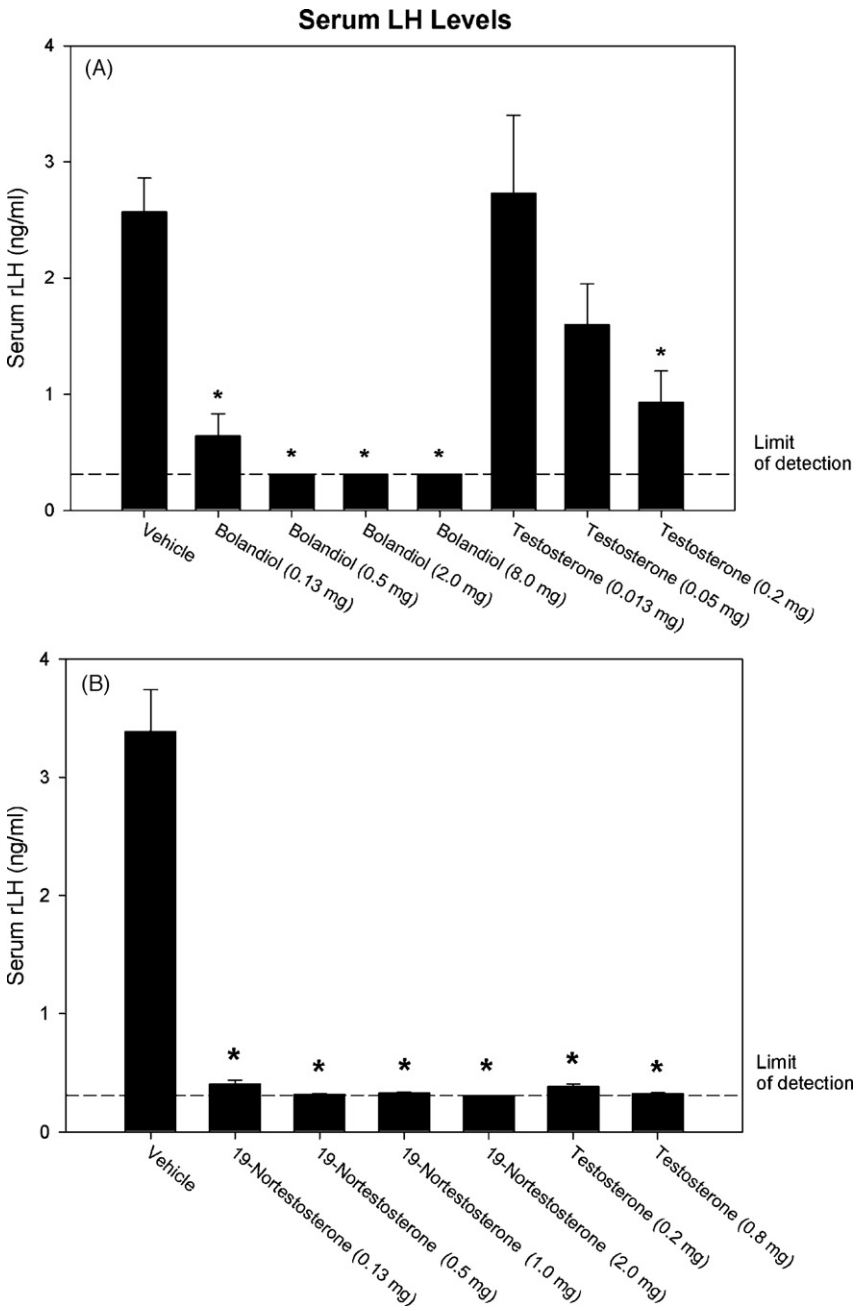

Fig. 5. Serum LH levels in castrate immature male rats treated with various doses of androgens or vehicle. Twenty-two-day old castrate rats were treated sc for 8 days with bolandiol, T, or vehicle (A) or 19-NT, T, or vehicle (B) as indicated in the legend to Fig. 4. *$P < 0.05$ vs vehicle-treated.

To investigate the potential for bolandiol to be converted to E_2 by the aromatase enzyme, we incubated bolandiol or T (as the positive control) for various times with purified human aromatase using conditions established previously [16]. As shown in Fig. 8, conversion of T to E_2 was linear for 60 min and complete by 120 min. Under the same conditions, there was no detectable formation of E_2 from bolandiol for up to 180 min. The limit of detection was 20 ng.

4. Discussion

We showed recently that bolandiol administered via Silastic implants is able to maintain muscle mass and BMD while inhibiting LH production in castrate adult male rats [3]. Moreover, in the same study, VP weights were markedly reduced compared to those of intact controls and castrated rats given T. Thus, bolandiol is an attractive potential treatment for hypogonadism, muscle wasting conditions, and osteoporosis in men, where gains in adiposity and loss of BMD are manifestations of low T levels. As shown in our previous publication [3], there was a dose-dependent decrease in % fat mass and increase in lean mass and BMD in adult rats treated with bolandiol implants. At the highest dose (16-cm implants), these parameters were comparable ($P > 0.05$) to those in intact rats. However, the mechanisms by which bolandiol exerts its tissue selectivity are unknown. In the present study, we investigated the potential pathways by which bolandiol may be acting by examining the specificity of its steroid hormone receptor binding and transcriptional activity *in vitro* and its potential for conversion to active metabolites. The potency and selectivity of bolandiol were compared to those of two physiological androgens, T and DHT, and to two potential metabolites, 19-NT and E_2. Bolandiol

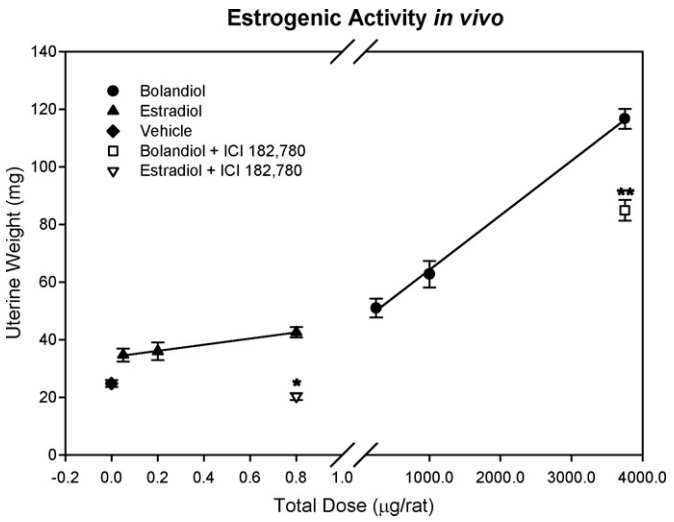

Fig. 6. Dose–response for stimulation of uterine weights by estradiol (E_2), bolandiol, or vehicle. Immature female rats were treated sc for 3 days with E_2 (0.017, 0.067, 0.267 μg/rat/day), bolandiol (0.08, 0.33, 1.25 mg/rat/day), or vehicle (10% ethanol/sesame oil). ICI 182,780 (0.4 mg/rat/day) was administered concurrently with a maximal stimulatory dose of E_2 or bolandiol, and uterine weights were determined. $^*P < 0.05$ vs E_2 alone and $^{**}P < 0.05$ vs bolandiol alone by Student's *t*-test.

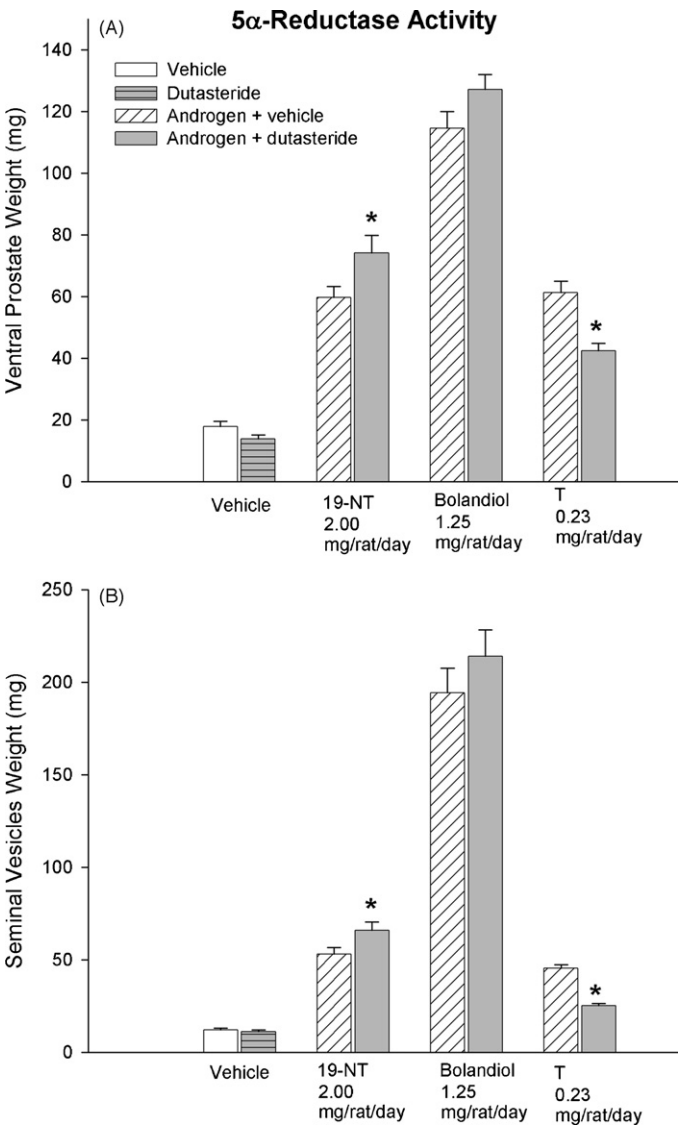

Fig. 7. Effect of dutasteride on (A) ventral prostate (VP) or (B) seminal vesicles (SV) weights in castrate immature rats. Twenty-two-day old castrate rats were treated sc for 8 days with doses of bolandiol, 19-NT, or T that had previously been shown to be stimulatory or with vehicle (10% ethanol/sesame oil), necropsied, and VP and SV weights determined. Dutasteride significantly suppressed T-stimulated VP ($^*P < 0.05$) and SV ($^*P < 0.001$) weights. There was no significant effect of dutasteride on sex accessory gland weights in rats treated with vehicle or bolandiol, whereas there was a significant increase ($^*P < 0.05$) in both VP and SV weights in rats treated with 19-NT. There was no significant effect of these treatments on final body weight.

bound weakly to AR compared to T, DHT, and 19-NT. Like other 19-norandrogens, bolandiol and 19-NT showed low, but measurable, affinity for PR, whereas T and DHT did not bind to PR. These results correlated with the ability of both bolandiol and 19-NT to induce transcription of reporter genes in cell lines containing either AR or PR. Transactivation by bolandiol was approximately equivalent whether mediated by hAR (in CV-1 cells) or hPR (in T47Dco cells). Furthermore, antiprogestins were more potent inhibitors of bolandiol-induced transactivation than antiandrogens, whether mediated by AR or PR. Bolandiol also bound weakly to both ERα and ERβ and demonstrated biphasic transactivation of 3XERE-LUC in T47Dco cells which was completely obliterated by the antiestrogen ICI 182,780. Using ER subtype-specific expression vectors in HEK-293 cells, we determined that transactivation by bolandiol was predominantly mediated by ERα. Thus, bolandiol showed the lowest affinity and the least specificity *in vitro* of the androgens tested for binding to various classes of steroid hormone receptors (AR, PR, and ER) and for transactivation. Bolandiol was not promiscuous for all steroid receptors, however, as it did not bind or activate GR. It is not clear from these results whether bolandiol may act through all these classes of receptor *in vivo*, be metabolized to a derivative with greater specificity, and/or utilize alternative signaling pathways.

In support of our previous results in adult castrate rats, bolandiol also showed tissue selectivity, i.e. preferential anabolic activity, in the immature rat model: Bolandiol was less potent in promoting growth of the VP and SV than T (T had comparable effects at lower concentrations), whereas bolandiol and T showed similar potency on LA growth. Over a range of concentrations, bolandiol effectively suppressed serum LH levels in the immature castrate rat. The potency of bolandiol in the regulation of LH secretion combined with its relatively weaker activity in the prostate makes this compound an attractive target for the development of a male hormonal contraceptive. Male hormonal contraceptives utilizing androgens, progestins, or combinations of these steroids exert negative feedback suppression of the hypothalamic/pituitary/testicular axis, shutting down gonadotropin production while providing sufficient peripheral androgenic activity to maintain normal male health [25]. However, concern exists regarding the effect of long-term T administration on the prostate in men, particularly at the supraphysiologic doses which have been most effective in contraceptive trials [25]. Androgenic compounds which minimize prostatic epithelial cell turnover (which might decrease the risk of prostate cancer development) and prostate gland growth (decreasing the risk of benign prostatic hypertrophy) are attractive candidates for male hormonal contraceptives and are currently in clinical development [1,26]. Subcutaneously administered bolandiol exhibits the critical properties of a SARM-like steroid, including complete suppression of LH and relatively greater potency in muscle compared to prostate. However, additional studies examining the effect of bolandiol on spermatogenesis and fertility and long-term effects on androgen-sensitive tissues are needed to determine the possible clinical utility of bolandiol for these purposes.

Theoretically, bolandiol could be metabolized by three major pathways: by 5α-reductase to tetrahydronandrolone, by aromatase to E_2, or by 3β-HSD to 19-NT. However, from the results presented here, none of these conversions appears to account for bolandiol's

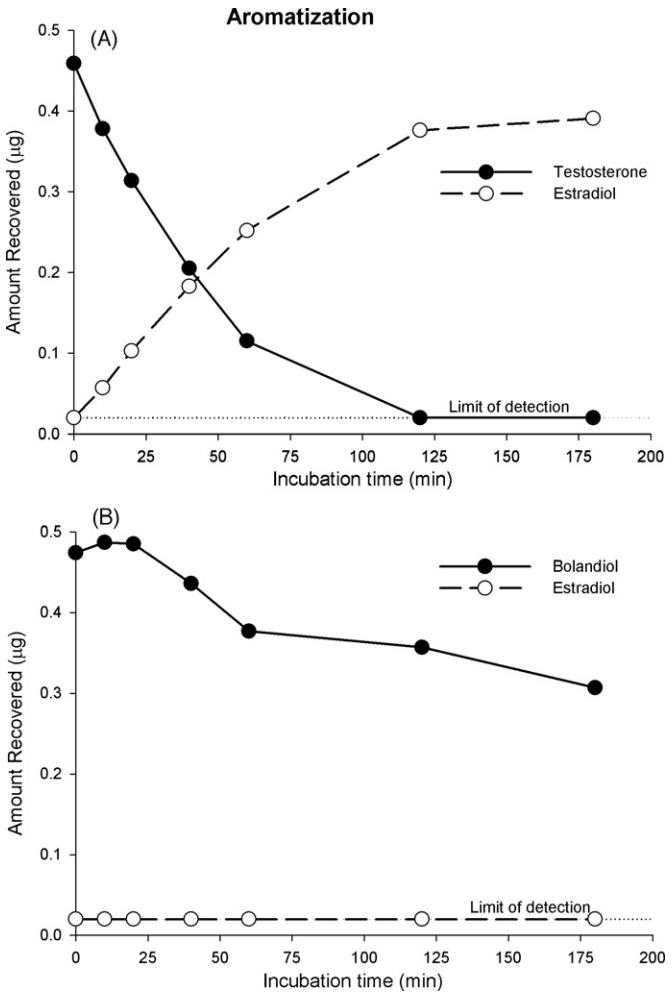

Fig. 8. Time course of incubation of (A) testosterone (T) or (B) bolandiol with recombinant human aromatase. Aromatization of T was complete by 120 min, whereas no aromatization of bolandiol was observed during 180 min incubation. Recovery of all steroids in this system decreased with incubation time (i.e. at 120 min, the amount of T + E_2 was 80% of that at 0 min; the amount of bolandiol was 75% of that at 0 min).

activity *in vivo*. In the first case, bolandiol did not require 5α-reduction to manifest maximal effects on VP and SV weights. The reduced ability of exogenous T in the presence of a 5α-reductase inhibitor to stimulate VP growth demonstrates the importance of 5α-DHT in prostatic androgen signaling. The replacement of endogenous androgens by exogenous bolandiol, a weaker androgen than both 5α-DHT and T, may mimic the prostatic effects of T when 5α-reduction of T is prevented [27]. Therefore bolandiol's lack of 5α-reduction, or prostatic amplification, could in part explain its tissue selectivity, as suggested by others [28]. In the second case, there was no detectable formation of E_2 when bolandiol was incubated for up to 3 h with recombinant human aromatase in contrast to T which was completely converted to E_2 by 2 h under the same conditions. This observation is in agreement with our previous results showing that there is little, if any, conversion of 19-norandrogens, including 19-NT, to their corresponding aromatic A-ring derivatives by aromatase *in vitro* [16]. Finally, as concerns the possible metabolism of bolandiol by 3β-HSD, in the 22-day old castrate rat, a model very sensitive to androgenic stimulation, bolandiol was more potent than 19-NT in promoting growth of the VP, SV, and LA. Thus, it seems unlikely that 19-NT is the major active metabolite of bolandiol in the rat. However, we showed previously [3] that 19-NT produced greater increases than bolandiol in VP, SV, and LA weights in castrate rats when administered as 16-cm Silastic implants over a 24-wk period. In the absence of pharmacokinetic data, any differences in drug exposure between these studies is unknown and therefore cannot be excluded as potentially contributing to bolandiol's disparate efficacy relative to 19-NT. Although currently unavailable, development of an assay to determine bolandiol concentrations in serum using LC–MS/MS is in progress.

As our data do not provide a clear answer as to what intracellular steroid hormone receptor mediates the actions of bolandiol, alternative mechanisms have to be considered. The observation that bolandiol, unique among the androgens tested, binds to ER as well as PR (as did 19-NT), suggests the possibility that these interactions are more important in some tissues than others. This may be the case for the prostate, where both ER and PR are expressed [29]. On the other hand, although the classical action of androgens and other steroid hormones involves activation of transcription (genomic events) through interaction with AR in the cytoplasm or nucleus, recent evidence has pointed to nongenomic events which are independent of transcription. The nature of the receptor(s) mediating the nongenomic effects of androgens is still unclear: both membrane-associated G-protein coupled receptors (GPCR's) and classical AR localized within the cell or within the plasma membrane have been implicated [30–32].

Androgens have been demonstrated to activate various kinases in signaling cascades in diverse cell types. The precise nature of an androgen-induced signal (genomic vs nongenomic) may depend on the type of target cell, the intracellular localization of the AR, and the ligand itself. Whereas skeletal muscle is a target for androgen action via intracellular receptors which mediate a variety of genomic responses, androgens also produce nonclassical effects in this tissue. Rapid effects of T involving second messengers have been reported in skeletal muscle [32], cardiac myocytes [33], and other cell types implicating nongenomic mechanisms of signal transduction. These rapid effects of T in rat myotubes and cardiac myocytes, resulting in increased intracellular Ca^{2+}, are through activation of plasma membrane receptors associated with a Pertussis toxin-sensitive G-protein-phospholipase C/inositol 1,4,5-trisphosphate signaling pathway [32,33]. In rat myotubes, a T derivative which does not cross the cell membrane or bind to intracellular receptors (testosterone–BSA) produced intracellular calcium increases. 19-NT, but not other classes of steroid hormones, mimicked the effect of T on myotubes. The possibility that bolandiol may also exert rapid nongenomic effects on selected tissues needs to be considered.

Another potential explanation for the tissue-specific pharmacology of bolandiol, or SARM's in general, is differential recruitment of coactivators or corepressors [31]. Pools of available transcriptional coregulators, known to differ in expression among cell and tissue types, drive transcriptional outcomes of nuclear hormone receptor ligand binding [34]. Also recent evidence demonstrates that AR receptor conformation, dependent upon ligand binding, can drive differential coregulator recruitment [35]. In this model, upon receptor binding, bolandiol and canonical androgens (T, 5α-DHT) assume receptor conformations facilitating coactivator recruitment resulting in "agonistic" responses in anabolic tissues such as LA. However, in sex accessory tissues like VP or SV, bolandiol AR binding, but not T or 5α-DHT, confers corepressor recruitment and an "antagonistic" transcriptional response, resulting in tissue-specific pharmacology. Other work suggests that in addition to coregulator expression, phosphorylation of coactivators that amplify androgen action or phosphorylation of AR itself could also explain in part bolandiol's molecular mechanism [36,37]. Further studies will be needed to investigate these alternatives.

In our previous study [3], we found that bolandiol appeared to be more effective in maintaining BMD than T or DHT. Although bolandiol is not aromatized to E_2 as demonstrated here, it binds

directly to, albeit weakly, and activates ER in T47Dco and HEK-293 cells. Transactivation in the former cells was mediated by ER as it was completed abrogated by the antiestrogen ICI 182,780. This is in agreement with our *in vivo* results showing induction by bolandiol of uterine weight increases in the immature female rat, an endpoint which was also sensitive to inhibition by ICI 182,780. Furthermore, bolandiol stimulated transcription via ER in U2OS human bone osteosarcoma epithelial cells (C. Coss, unpublished observation). Hence, bolandiol's intrinsic estrogenic activity may contribute to maintenance of BMD *in vivo* providing an advantage over some SARM's in the long-term treatment of hypogonadism and osteoporosis in men.

In conclusion, we have shown in this study that bolandiol injected sc is effective in suppressing LH in castrated young rats and in maintaining LA development while having less of a stimulatory effect on the prostate and SV than T. Bolandiol does not require aromatization or 5α-reduction to support these physiologic endpoints. The mechanism of action of bolandiol is likely through a combination of AR, PR, and ER as it binds weakly to, but exhibits transactivation of all these receptors, in contrast to T and E_2. Future experiments examining the effect of bolandiol in steroid receptor knock-out mice may help to further clarify its mechanism of action. Collectively, bolandiol has properties consistent with its characterization as a tissue selective anabolic steroid or a SARM-like steroid.

Acknowledgements

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