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RESEARCH PAPER

Characterization of the oestrogenic activity of non-aromatic steroids: are there male-specific endogenous oestrogen receptor modulators?

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Background and purpose: The endogenous oestrogens have important biological functions in men as well as in women. Because 17β-oestradiol and oestrone are also formed in the male body, these aromatic oestrogens are generally thought to be responsible for exerting the required oestrogenic functions in the male. In the present study, we tested the hypothesis that some of the non-aromatic steroids that are androgen precursors or metabolites with hydroxyl groups at C-3 and/or C-17 positions may also be able to serve as ligands for the oestrogen receptors (ER) in the male.
Experimental approach: A total of sixty non-aromatic steroids (selected from families of androstens, androstans, androstadiens, oestrens and oestrans) were analysed for their ability to bind and activate the human ERα and ERβ *in vitro* and in cultured cells.
Key results: Six of the non-aromatic steroids, that is, 5-androsten-3β,17β-diol, 5α-androstan-3β,17β-diol, 5(10)-oestren-3α,17β-diol, 5(10)-oestren-3β,17β-diol, 4-oestren-3β,17β-diol and 5α-oestran-3β,17β-diol, were found to have physiologically relevant high binding affinity (~50% of that of oestrone) for human ERα and ERβ. These non-aromatic steroids also activated the transcriptional activity of human ERs and elicited biological responses (such as growth stimulation) in two representative ER-positive human cancer cell lines (MCF-7 and LNCaP) with physiologically relevant potency and efficacy. Molecular docking analysis of these six active compounds showed that they could bind to ERα and ERβ in a manner similar to that of 17β-oestradiol.
Conclusions and implications: These results provide evidence for the possibility that some of the endogenous androgen precursors or metabolites could serve as male-specific ER ligands.
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Keywords: non-aromatic steroids; oestrogen receptor; oestrogenic activity; molecular modelling

Abbreviations: AR, androgen receptor; ARE, androgen response element; CoMFA, comparative molecular field analysis; E_1, oestrone; E_2, 17β-oestradiol; ER, oestrogen receptor; ERE, oestrogen response element; LBD, ligand binding domain; QSAR, quantitative structure–activity relationship; RBA, relative binding affinity

Introduction

The endogenous aromatic oestrogens, such as oestrone and 17β-oestradiol (E_1 and E_2, respectively), exert a whole host of physiological and pathophysiological functions in various target tissues in women (Gruber *et al.*, 2002). Most of their hormonal actions are mediated by two specific oestrogen receptor (ER) subtypes, namely, the ERα and ERβ (Nilsson and Gustafsson, 2002; nomenclature follows Alexander *et al.*, 2008). The endogenous oestrogens in women are biosynthesized from androgens through enzymatic aromatization of their *A*-rings, which occurs mostly in the ovaries and, to a smaller extent, also in extragonadal tissues (Simpson and Davis, 2001). In comparison, aromatization of androgens to oestrogens is quantitatively less important in men, and the blood levels of aromatic oestrogens are usually very low (de Ronde *et al.*, 2003). Nevertheless, studies in animals and humans have shown that the ERα and ERβ are present in many tissues or cell types in the male, such as prostate, bladder, certain regions of the brain, lymphatic tissues and heart (Prins *et al.*, 2001; Adams *et al.*, 2002). In addition, ERs present in various male tissues (e.g. pituitary, breast and prostate) are functionally active and can be readily activated by administration of an oestrogen to elicit strong biological effects.

Among all classes of endogenous steroids, only oestrogens (such as E_1 and E_2) contain an aromatic *A*-ring in their

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structures. For decades, it was generally thought that the aromatic *A*-ring in a steroid is a critical structural feature for its specific ER binding activity. However, analysis of the crystallographic structures of human ERα and ERβ in complex with E_2 showed that the ligand binding pocket where the flat aromatic *A*-ring sits is rather large and it can easily accommodate other non-aromatic ring structures (Brzozowski *et al*., 1997; Manas *et al*., 2004). This finding suggests that some of the non-aromatic steroids may also be able to bind and activate the ERs. This was supported by an earlier study showing that 5α-androstan-3β,17β-diol, a non-aromatic androgen derivative, could bind and also activate ERs in the prostate (Weihua *et al*., 2001).

To search for potential ER ligands in men, we set out to test a total of sixty non-aromatic steroids for their ability to bind and activate the human ERα and ERβ in several *in vitro* bioassay systems. These non-aromatic steroids were selected from the steroid families of androstens, androstans, androstadiens, oestrens and oestrans (some of the representative structures are listed in Figure 1), and they all have a hydroxyl group at their C-3 and/or C-17 position because of their known importance in forming hydrogen bonds with the ERs. Notably, all these non-aromatic steroids are potential precursors, intermediates or metabolites that may be produced during the process of steroid biosynthesis and metabolism. A number of enzymes are known to catalyse the reactions responsible for the

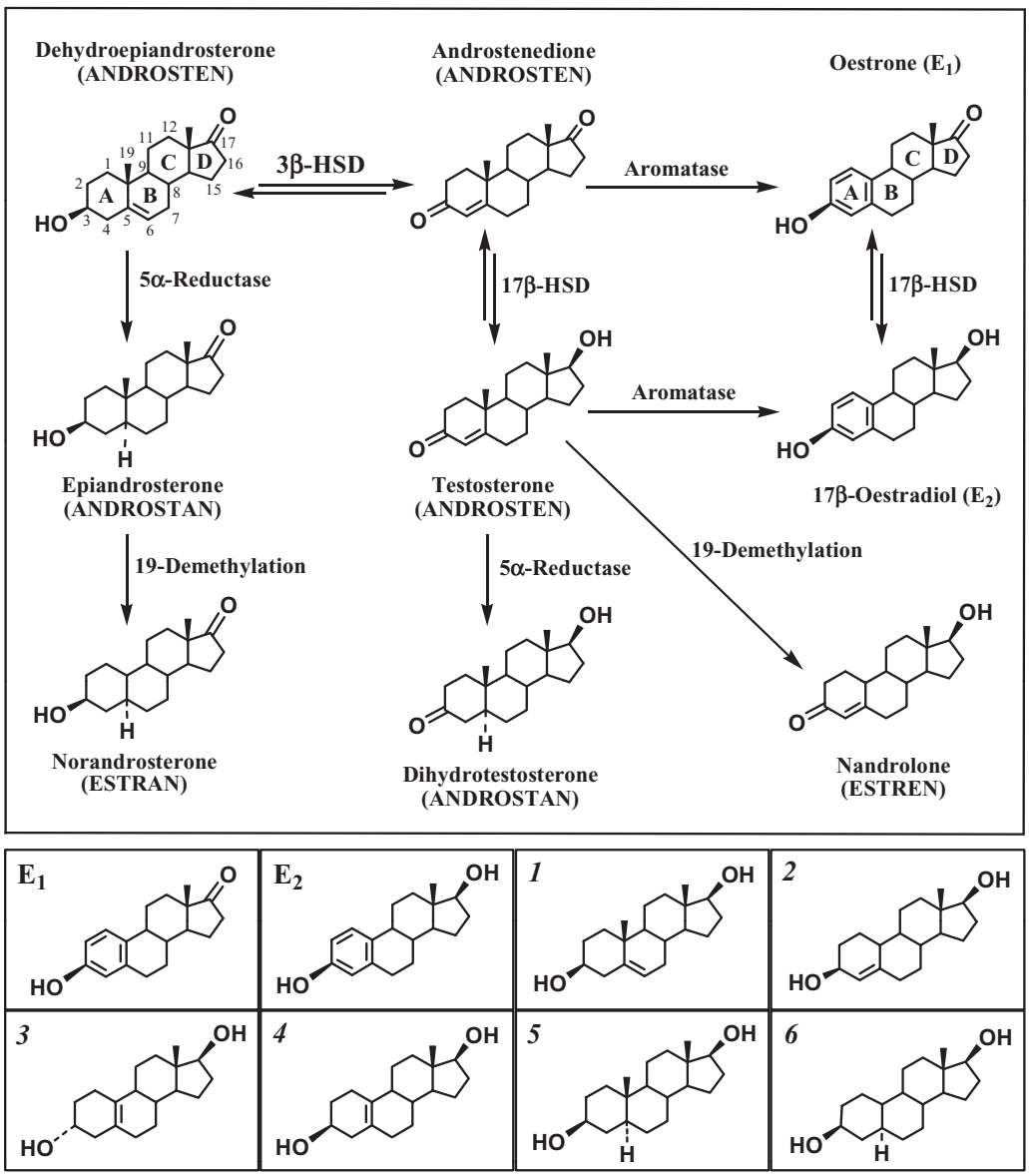

Figure 1 (Upper panel) Potential precursors and metabolic intermediates produced during the biosynthesis and metabolism of endogenous androgens with dehydroepiandrosterone (DHEA) as the starting compound. Note that the name of the corresponding subclass for each non-aromatic steroid was provided under each name (upper case). For simplicity, the representative structures for the androstadien class were not shown in the figure. This subclass of non-aromatic steroids can be produced from the androstens by forming an additional double bond at various positions. (Lower panels) Chemical structures of 17β-oestradiol (E_2), oestrone (E_1) and compounds *1*–*6*. The names of compounds *1*–*6* are as follows. *1*: 5-androsten-3β,17β-diol; *2*: 4-oestren-3β,17β-diol; *3*: 5(10)-oestren-3α,17β-diol; *4*: 5(10)-oestren-3β,17β-diol; *5*: 5α-androstan-3β,17β-diol; *6*: 5α-oestran-3β,17β-diol.

formation of some of these steroids (see Figure 1). For instance, the 3- and 17β-hydroxylsteroid dehydrogenases (3-HSD and 17β-HSD) can catalyse the interconversion of steroids between their ketone and hydroxyl groups at the C-3 and C-17 positions respectively (Peltoketo *et al.*, 1999; Simard *et al.*, 2005). Whereas it is well documented that an androsten can be readily converted to its 5α-androstan product in some of the male target tissues by 5α-reductase (Makridakis *et al.*, 2000), testosterone (an androsten) and epiandrosterone (an androstan) can also be converted to 19-norandrosterone (an oestren) and norepiandrosterone (an oestran), respectively, by the 19-demethylation metabolic pathway (Grosse *et al.*, 2005). In addition, many cytochrome P450 (CYP) enzymes can readily catalyse the addition of a hydroxyl or keto group to various carbon positions in the steroid core structure (Lee *et al.*, 2003).

In the present study, we demonstrated that some of the metabolic derivatives of endogenous androgens could bind to and activate human ERα and ERβ to elicit hormonal responses. These observations suggest that some of the endogenous androgen precursors or metabolites may serve as ER modulators in men.

Methods

ERα and ERβ binding assays

The ERα and ERβ competitive binding assays were performed according to the method described in our recent study (Zhu *et al.*, 2006). The ER binding buffer used for dilution of the receptor preparations consisted of 10% glycerol, 2 mM dithiothreitol, 1 mg·mL^{-1} bovine serum albumin and 10 mM Tris–HCl (pH 7.5). The ERα washing buffer contained 40 mM Tris–HCl and 100 mM KCl (pH 7.4), but the ERβ washing buffer contained only 40 mM Tris–HCl (pH 7.5). The 50% (v/v) hydroxylapatite (HAP) slurry was prepared by using the 50 mM Tris–HCl solution (pH 7.4). The reaction mixture contained 50 μL of varying concentrations of the test compound in the ER binding buffer, and 45 μL of [^{3}H]E_2 solution (at 22.22 nM) with a final [^{3}H]E_2 concentration at 10 nM. Then 5 μL of ERα or ERβ protein (at final concentration of 2.5 nM) was added and mixed gently. Non-specific binding by [^{3}H]E_2 was determined by addition of a 400-fold concentration of the non-radioactive E_2 (at 8 μM with final concentration 4 μM). The binding mixture was incubated at room temperature for 2 h. At the end of the incubation, 100 μL of the HAP slurry was added and the tubes were incubated on ice for 15 min with three periods of brief vortexing. An aliquot (1 mL) of the washing buffer was added. The tubes were vortexed, centrifuged at 10 000× *g* for 1 min, and the supernatants were discarded. This wash step was repeated twice. The HAP pellets were then resuspended in 200 μL ethanol, and the content was transferred to scintillation vials for measurement of the ^{3}H-radioactivity in a liquid scintillation counter (Packard Tri-CARB 2900 TR; Downers Grove, IL, USA).

The IC_{50} value for each competing ligand was calculated according to the sigmoidal inhibition curve, and the relative binding affinity (RBA) was calculated against E_1 or E_2 using the following equation:

$$\text{RBA} = \frac{IC_{50} \text{ for } E_1 \text{ (or } E_2\text{)}}{IC_{50} \text{ for the test compound}}$$

Androgen receptor (AR) binding assay

The same procedures as described above for the ER binding assay were used for assaying the relative AR binding affinity of various non-aromatic steroids. The non-specific binding by [^{3}H]methyltrienolone was determined by addition of a 400-fold concentration (8 μM) of the non-radioactive methyltrienolone.

Culture of human breast cancer cell lines and prostate cancer cell lines

MCF-7 and LNCaP cell lines were obtained from ATCC (Manassas, VA, USA) and cultured according to the instructions of the suppliers. For cell proliferation assay, cells were cultured with medium containing 10% of dextran-coated charcoal-stripped fetal bovine serum (FBS) for 3 days and then were seeded in 96-well plates with 10^4 cells per well. The cells were treated on the second day and also on the fifth day. On the eighth day after seeding the cells, the cell density was determined by using the crystal violet staining method (Liu and Zhu, 2004). The absorbance values of each well were measured at 560 and 405 nm with a UVmax microplate reader (Molecular Device, Palo Alto, CA, USA), and the differences in the absorbance values at these two wavelengths were used to represent the cell density.

Reporter assays

Oestrogen response element (ERE)-based reporter assay. MCF-7 cells were cultured in medium containing dextran-coated charcoal-stripped FBS for 3 days. Then the cells were seeded in 24-well plates with 2×10^5 cells per well. After 24 h, the cells were transfected with a plasmid containing pGL-basic + ERE + E1b + luciferase by using lipofectamine 2000. After another 24 h, different concentrations of compounds were added to each well. Cells were harvested 20 h later and used for luciferase assay. Protein concentration of each well was measured with the protein assay reagent (Bio-Rad, Hercules, CA, USA). The luciferase activity in each well was normalized by the protein concentration. Notably, in this ERE-based luciferase reporter assay, little or no baseline luciferase expression level was detected when human ERα was not expressed in ER negative cells regardless of whether E_2 was present or not (Peterson *et al.*, 2007).

Androgen response element (ARE)-based reporter assay. LNCaP cells were seeded in 24-well plates at a density of 6×10^4 cells per well. After 24 h, the cell culture medium was replaced with a medium containing 10% FBS that was pretreated with dextran-coated charcoal to remove endogenous hormones. After an additional 24 h, the ARE-Luciferase and *Renilla* plasmids were transfected into LNCaP cells with lipofectamine 2000 according to manufacturer's instructions. Twenty-four hours later, cells were treated with each of the testing compounds. The firefly luciferase and *Renilla* luciferase activities

were determined 24 h later using a dual-luciferase system obtained from Promega (Madison, WI, USA).

Quantitative structure–activity relationship (QSAR) study
All calculations described in the present study were carried out using the *SYBYL* molecular modelling programme (V7.1, Tripos Inc., St. Louis, MO, USA) installed in Red Hat Enterprise Linux WS4.0 (Red Hat Inc., Raleigh, NC, USA) operating system on a Dell Precision 690 workstation.

Molecular models and structural alignment. The chemicals used in this study comprised of a total of 58 non-aromatic endogenous steroids. Note that 5α-androstan-3β-ol-16,17-dione-16-oxime was excluded for alignment because of its unique side chain, whereas 4-androsten-3β,17β-diol and 4,9(11)-androstadien-17β-ol-3-one were considered to be outlier compounds for ERα and ERβ respectively. All molecules were constructed using the building tools of the *SYBYL7.1* molecular modelling software. The geometry of each molecule was optimized using the standard Tripos force field with the conjugate-gradient minimization to an energy change convergence criterion of 0.001 kcal·(mol·Å)$^{-1}$. All atomic partial charges were computed using the Gasteiger-Marsili method. All molecules were aligned by using 5-androsten-3β,17β-diol (compound *1*) as template with the rigid-body least-squares fitting method. After alignment, the molecules were placed in a 3-D cubic lattice with 2 Å spacing. Any calculated steric and electrostatic energies that were >30 kcal·mol^{-1} were truncated to this value.

3-D QSAR/comparative molecular field analysis (CoMFA) analysis. For 3-D QSAR/CoMFA analysis, the method of partial least squares regression was used to analyse the compounds by correlating the percentage inhibition of [^{3}H]E_2 binding for each compound (experimental data listed in Table 1) and the CoMFA fields. 4-Androsten-3β,17β-diol and 4,9(11)-androstadien-17β-ol-3-one were the outlier compounds for ERα and ERβ, respectively, and they were excluded in the analysis. The optimum number of principal components (PCs) was determined by the leave-one-out cross-validation procedure. In this method, each compound was systematically excluded once from the data set, after which its activity was predicted by a model derived from the remaining compounds. Then the q^2 value (i.e. the cross-validated r^2 value) was calculated based on these predictions. This q^2 value measures a model's predictive ability. By setting the number of PCs to this optimum number, the final partial least squares analysis was carried out without cross-validation with correlation coefficient r^2. This r^2 tells the model's goodness of fit to the activity data of the compounds. A model for which $r^2 > 0.9$ and $q^2 > 0.4$ is normally considered to be predictive.

Molecular docking and simulation study
Energy minimization and molecular docking were performed with *InsightII* modelling programme (Version 2005, Accelrys Inc., San Diego, CA, USA) installed in Red Hat Enterprise Linux WS4.0 (Red Hat Inc., Raleigh, NC, USA) operating system on a Dell Precision 690 workstation. Consistent valence force field (*CVFF*) was used for energy minimization.

ERα. The structures of six non-aromatic male-specific steroids (structures shown in Figure 1) were built with the *Builder* module in *InsightII* and minimized with the *Discover* module. The ligands were superimposed onto E_2, which was taken from X-ray crystal structure of ERα in association with E_2 (PDB code: 1ERE) (Brzozowski *et al*., 1997). The water molecule in the binding pocket forming a hydrogen bond with 3-OH group of E_2 was also included. All other water molecules were deleted from the crystal structure. Energy minimization was carried out with Polak and Ribiere conjuate gradients method in the *Discover* module in *InsightII* until the final convergence criterion reached the 0.001 kcal·(mol·Å)$^{-1}$. The heavy atoms of the receptor were fixed during the minimization.

ERβ. The X-ray crystal structure of ERβ in complex with ERB-041 (PDB code: 1X7B) (Manas *et al*., 2004) was used as template for docking its binding interaction with E_2. The flexible docking procedures were carried out using the *Simulated Annealing Docking* method in the *Affinity* module of *InsightII*. The binding pocket was defined to include all residues within the 7 Å reach of the binding ligand ERB-041. The water molecule in the binding pocket was also included, which forms a hydrogen bond with one of the hydroxyl groups of ERB-041. All other water molecules were deleted from the crystal structure. A combination of *Monte Carlo* and *simulated annealing* methods was used to explore all the possible binding conformations of E_2. One hundred conformations were obtained and the one with the lowest potential energy was chosen for further minimization by *Discover*. The other ligands were superimposed onto E_2 in the minimized structure, and energy minimization was carried out with Polak and Ribiere conjuate gradients method in the *Discover* module of *InsightII* until the final convergence criterion reached the 0.001 kcal·(mol·Å)$^{-1}$. The heavy atoms of the receptor were fixed during the minimization.

Materials
Oestrone (E_1), E_2 and all non-aromatic steroids listed in Figure 1 and Table 1 were obtained from Steraloids (Newport, RI, USA). The non-aromatic steroids selected for testing in the present study all contained hydroxyl groups at their C-3 and/or C-17 position, which are critical for them to potentially form C-3 and C-17 hydrogen bonds with ERs. [^{3}H]E_2 (specific activity of 115 Ci·mmol^{-1}), [^{3}H]methyltrienolone (specific activity of 85 Ci·mmol^{-1}) and non-radiolabelled methyltrienolone were obtained from PerkinElmer (Waltham, MA, USA). The recombinant human ERα, ERβ and AR proteins were obtained from Invitrogen (Carlsbad, CA, USA). According to the supplier, the purity of the human ERα, ERβ and AR proteins were higher than 75%. HAP was obtained from Calbiochem (through EMD Biosciences, Inc., San Diego, CA, USA). Eagle's modified minimum essential medium (EMEM, phenol red-free) and RPMI-1640 medium were obtained from Sigma Chemical Co. (St. Louis, MO, USA). FBS used in cell culture was purchased from Hyclone Corporation (Logan, UT, USA). The charcoal-stripping method was employed to remove the endogenous oestrogens present in the serum by using the dextran-coated charcoal (Sigma Chemical Co., St. Louis, MO, USA) as described earlier (Liu *et al*., 2005). The

Table 1 Inhibition of [^{3}H]E_2 binding to human ERα and ERβ *in vitro* by a total of sixty non-aromatic steroids from several steroid classes

	ERα	*ERβ*	*AR*
Oestrone (E_1)	88.79	70.84	10.62
Oestradiol (E_2)	98.71	99.91	–
Testosterone	–	–	77.19
Androsten			
4-Androsten-3β,17β-diol	11.76	50.49	–
4-Androsten-16β,17α-diol-3-one	11.96	42.95	–
4-Androsten-16α-ol-3,17-dione	8.55	10.28	–
4-Androsten-16α,17β-diol-3-one	8.72	11.70	–
5-Androsten-3β,17β-diol (*1*)	60.61	63.85	9.49
5-Androsten-3β,17α-diol	14.94	2.92	–
5-Androsten-3β,4α,17β-triol	12.18	54.42	–
1,5α-Androsten-3,17-dione	NI	NI	–
1,5α-Androsten-1β-methyl-17β-ol-3-one	12.58	2.14	–
1,5α-Androsten-17β-ol-3-one	NI	10.11	–
9(11), (5β)-Androsten-3α-ol-17-one	10.64	–0.34	–
9(11), (5α)-Androstan-3β-ol-17-one	6.53	13.55	–
Androstan			
5α-Androstan-3β,17β-diol (*5*)	61.09	70.54	54.75
5α-Androstan-3β,16α-diol	12.31	13.18	–
5α-Androstan-3β,16β-diol	11.67	15.09	–
5α-Androstan-3α,17β-diol	13.15	20.98	–
5α-Androstan-3β,17α-diol	12.57	11.17	–
5α-Androstan-3β,17β-diol-16-one	15.55	8.10	–
5α-Androstan-3β-ol-16-one	12.53	13.30	–
5α-Androstan-3α,16α-diol-17-one	5.21	5.47	–
5α-Androstan-3β,16α-diol-17-one	16.28	13.94	–
5α-Androstan-17α-methyl-3α,17β-diol	13.84	36.36	–
5α-Androstan-17α-methyl-3β,17β-diol	33.09	41.35	–
5α-Androstan-3β-ol-16,17-dione-16-oxime	13.84	15.71	–
5β-Androstan-3β,17β-diol	NI	14.65	–
5β-Androstan-3β,16α-diol-17-one	10.76	7.74	–
5β-Androstan-3β,17α-diol	12.85	7.09	–
Androstadien			
1,4-Androstadien-3,17-dione	NI	NI	–
1,4-Androstadien-17β-ol-3-one	NI	NI	–
4,6-Androstadien-3,17-dione	NI	NI	–
4,6-Androstadien-17β-ol-3-one	NI	1.50	–
4,9(11)-Androstadien-3,17-dione	1.43	NI	–
4,9(11)-Androstadien-17β-ol-3-one	1.40	35.47	–
5,9, (11)-Androstadien-3β-ol-17-one	NI	0.11	–
Oestren			
4-Oestren-3β,17β-diol (*2*)	78.16	81.68	41.28
4-Oestren-3α,17α-diol	NI	13.61	–
4-Oestren-3β,17α-diol	16.85	30.35	–
4-Oestren-3α,17β-diol	27.03	28.25	–
4-Oestren-17α-ol-3-one	NI	9.58	–
4-Oestren-17β-ol-3-one	NI	12.91	–
4-Oestren-6β-ol-3,17-dione	NI	NI	–
5(10)-Oestren-3α,17β-diol (*3*)	66.61	64.04	11.83
5(10)-Oestren-3β,17β-diol (*4*)	89.24	87.95	1.41
5(10)-Oestren-3,17-dione	2.08	NI	–
Oestran			
5α-Oestran-3β,17β-diol (*6*)	76.14	69.14	31.89
5α-Oestran-3α,17α-diol	11.16	5.63	–
5α-Oestran-3β,17α-diol	35.33	42.36	–
5α-Oestran-3α,17β-diol	34.55	23.90	–
5α-Oestran-3,17-dione	27.30	18.91	–
5α-Oestran-17α-ol-3-one	1.29	3.07	–
5α-Oestran-17β-ol-3-one	20.59	18.95	–
5α-Oestran-3β-ol-17-one	21.90	18.29	–
5α-Oestran-3α-ol-17-one	10.52	7.02	–
5β-Oestran-3α,17β-diol	NI	3.06	–
5β-Oestran-3α,17α-diol	8.05	10.54	–
5β-Oestran-3β,17β-diol	NI	7.47	–
5β-Oestran-3,17-dione	NI	20.18	–
Other			
1,4,6-Androstatrien-17β-ol-3-one	20.64	9.61	–
4,6-Oestradien-3,17-dione	NI	8.28	–
4,6-Oestradien-17β-ol-3-one	2.18	5.73	–

For those steroids with a strong activity for ER binding (highlighted and labelled *1–6*), they were also tested for their relative binding activity for human AR by measuring the inhibition of [^{3}H]methyltrienelone binding to the human AR protein *in vitro*. The binding by a radioligand to the respective receptor protein in the absence of any inhibitor was considered to be 100%, and each value was the mean of duplicate measurements of each test compound (at 1 μM) as the percentage inhibition of the radiolabelled ligand binding. A higher percentage inhibition value means a higher binding activity of the test compound for the respective receptor. For details of the assays, refer to the *Methods* section. The highlighted and numbered compounds are the focus of further analyses described in additional experiments. NI stands for no inhibition detected.
AR, androgen receptor; ER, oestrogen receptor.

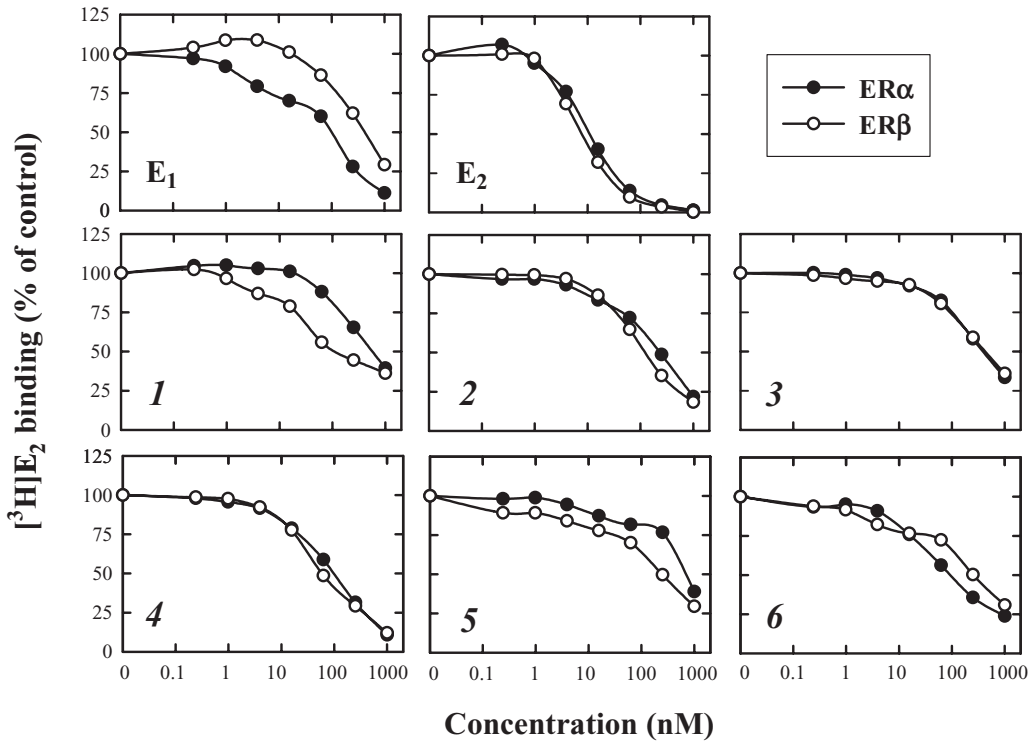

Figure 2 Competition of [^{3}H]E_2 binding to human oestrogen receptor (ER)α and ERβ by various non-aromatic steroids (compounds *1–6*) and also by aromatic steroids 17β-oestradiol (E_2) and oestrone (E_1). Each point was the mean of duplicate measurements. For the chemical names of compounds *1–6*, please refer to the legend of Figure 1.

plasmid containing pGL-basic + ERE + E1b + luciferase was a gift from Dr Carolyn L Smith at the Baylor College of Medicine (Houston, TX, USA). The plasmid containing ARE + luciferase was kindly provided by Dr Benyi Li at the University of Kansas Medical Center (Kansas City, KS, USA). The *Renilla* plasmid used in ARE-based reporter assay was purchased from Promega (Madison, WI, USA). Lipofectamine 2000 was purchased from Invitrogen (Carlsbad, CA, USA), and the luciferase assay system was obtained from Promega (Madison, WI, USA).

Results

Non-aromatic steroids have physiologically relevant high binding affinity for human ERα and ERβ

We tested a total of sixty non-aromatic steroids for their binding affinity for human ERα and ERβ. For each of these compounds, we first assessed its binding affinity for human ERα and ERβ at a high concentration of 1 μM (data are summarized in Table 1). We found that six of the non-aromatic steroids (highlighted in Table 1, with their structures shown in Figure 1 lower panels, referred to as compounds *1–6*) had strong binding activity for both human ERα and ERβ when present at 1 μM. Notably, some of the steroids, such as 5-androstan-3β,4α,17β-triol and 4-androsten-3β,17β-diol, had a weak but selective binding activity for ERβ, with no appreciable binding activity detected for ERα.

Next we determined the whole competition curves (at concentrations of 0.24, 0.98, 3.9, 15.6, 62.5, 250 and 1000 nM) for each of these six active compounds (Figure 2). The data obtained from duplicate measurements were expressed in Figure 2 as the % specific binding of [^{3}H]E_2 compared with the control (in the absence of competing compound). Calculated according to the IC_{50} values determined in this study, the RBAs to E_1 for compounds *1–6* are 16.7%, 39.5%, 24.6%, 98.2%, 12.3% and 115.8%, respectively, for human ERα, and 253.6%, 251.1%, 100.0%, 567.8%, 128.8% and 164.3%, respectively, for human ERβ. Similarly, the RBAs to E_2 for these compounds ranged 1.4–13.2%, for ERα, and 2.8–15.9%, for ERβ. Notably, their binding affinities are physiologically relevant, as they are highly comparable to those of E_1, the quantitatively most important endogenous oestrogen formed in a non-pregnant woman (Zhu and Conney, 1998).

In addition, we also assessed the RBA of these six compounds for the human AR (data summarized in Table 1). Compounds *1*, *3* and *4* had a selective binding affinity for the ERs with little binding activity for the AR. In comparison, compounds *2*, *5* and *6* showed substantial binding activity for human AR (based on their ability to compete with [^{3}H]methyltrienolone for binding to the AR) when they were present at 1 μM, although their RBA was significantly weaker than that of testosterone.

Active non-aromatic steroids can activate ERα in cultured cells

Next we determined whether the non-aromatic steroids that were found to have binding affinity for human ERα and ERβ were receptor agonists or antagonists. It is known that the oestrogen-activated ERs are translocated into the nucleus

and then bind to the specific DNA sequences called ERE to activate the expression of downstream target genes. First, we used a reporter plasmid consisting of an ERE and a luciferase reporter gene to determine the oestrogenic activity of the non-aromatic steroids (Figure 3A). In this assay system, we found that E_1 at 1.6 nM could strongly induce luciferase gene expression. Compounds *1–6* could also induce luciferase gene expression dose-dependently, with EC_{50} values around 5 nM, which were comparable to the EC_{50} value of E_1 (approximately 3 nM).

Similarly, to assess the androgenic activity of the three compounds (i.e. *2*, *5* and *6*) that also retained significant AR binding affinity, an ARE-based reporter assay was employed. The AR-positive LNCaP cells were transfected with an ARE-luciferase reporter plasmid and a *Renilla* plasmid (as the transfection control). In this assay system, the relative activity of compounds *2*, *5* and *6* at 1 nM to induce the transcription of the luciferase reporter gene ranged from over 50% to less than 10% of the activity of 1 nM methyltrienolone, as shown in Figure 3B.

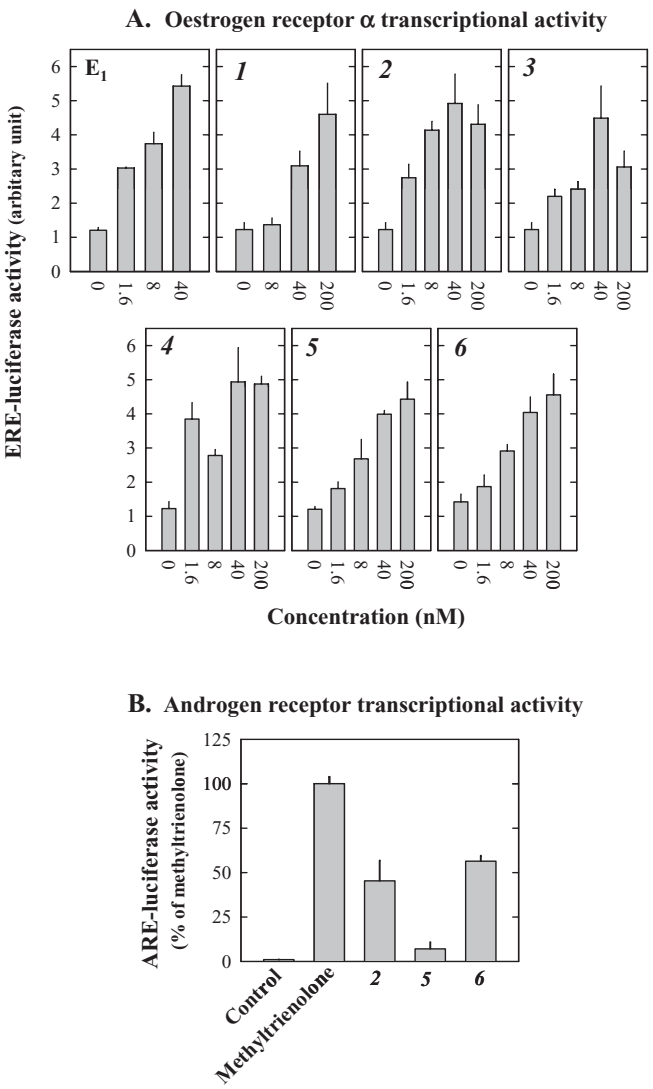

Figure 3 Oestrogen receptor α and androgen receptor transcriptional activity of various non-aromatic steroids. (A) Reporter assay in MCF-7 cells transfected with an oestrogen response element (ERE)-luciferase reporter plasmid and treated with various concentrations of compounds *1–6* and oestrone. The *y*-axis shows the luciferase activity normalized to the total protein concentration. (B) Reporter assay in LNCaP cells transfected with an androgen response element (ARE)-luciferase reporter plasmid and a *Renilla* plasmid and then treated with 1 nM methyltrienolone or compound *2*, *5* or *6*. The *y*-axis shows the firefly luciferase activity normalized to the *Renilla* luciferase activity and expressed as % of the activity of methyltrienolone-treated group. Each value is the mean ± SD of triplicate measurements.

We also used two representative ER-positive human cancer cell lines (i.e. MCF-7 breast cancer cells and LNCaP prostate cancer cells) in culture to determine whether the presence of these non-aromatic steroids could stimulate the growth of these cells (Figure 4). Whereas the rate of cell growth in the absence of exogenous oestrogen was relatively low, treatment of the oestrogen-starved MCF-7 cells (which express predominantly ERα) with E_1 at the lowest concentration tested (0.1 nM) strongly stimulated cell growth (Figure 4A). When these cells were treated with varying concentrations of compounds *1–6*, the rate of their cell growth was also increased in a concentration-dependent manner, with EC_{50} values of approximately 2 nM.

Similarly, in LNCaP cells, a human prostate cancer cell line that expresses both AR and ER and is sensitive to oestrogen-induced growth stimulation, compounds *1–6* exerted a maximal stimulation of their growth, comparable to that induced by E_1 (Figure 4B). Consistent with the proliferative effect seen in MCF-7 cells, the EC_{50} values of compounds *1–6* ranged from 0.5 to 5 nM in LNCaP cells, indicating that they were less potent than E_1. Because all six compounds (some of them have little androgenic activity) exerted a similar maximal proliferative effect in LNCaP cells, this suggested that this effect was largely mediated by the ER signalling pathways. The EC_{50} values of the non-aromatic steroids were well within the physiologically achievable concentrations for androgens and/or their derivatives in men (note that the circulating concentrations of androgens in men are at least 10 times higher than the circulating oestrogens in women).

Molecular modelling study of the binding interactions of non-aromatic steroids with human ERα and ERβ

To probe the structural determinants of various non-aromatic steroids for binding to human ERα and ERβ, we performed the 3-D QSAR/CoMFA analysis of the non-aromatic steroids used in the present study (Figure 5). The 3-D QSAR/CoMFA models for human ERα and ERβ were developed using 58 compounds (excluding two of the compounds as outliers). Of several variations in the structural alignment schemes that were considered and tested in this study, the best results were obtained by superimposing the backbone carbons in the middle *C*-ring. It is likely that this ring of different non-aromatic steroids experiences less change upon binding to the receptors. The statistical results of CoMFA models for both human ERα and ERβ are summarized below. For ERα: $r^2 = 0.907$, $q^2 = 0.402$, PCs = 8, SEE = 9.050, $F = 38.870$; for ERβ: $r^2 = 0.932$, $q^2 = 0.400$, PCs = 10, SEE = 7.280, $F = 49.095$. The correlations of the predicted specific binding values for ERα and ERβ are shown in Figure 5A with the filled squares representing compounds *1–6*. The values of r^2 were higher than 0.9, which was

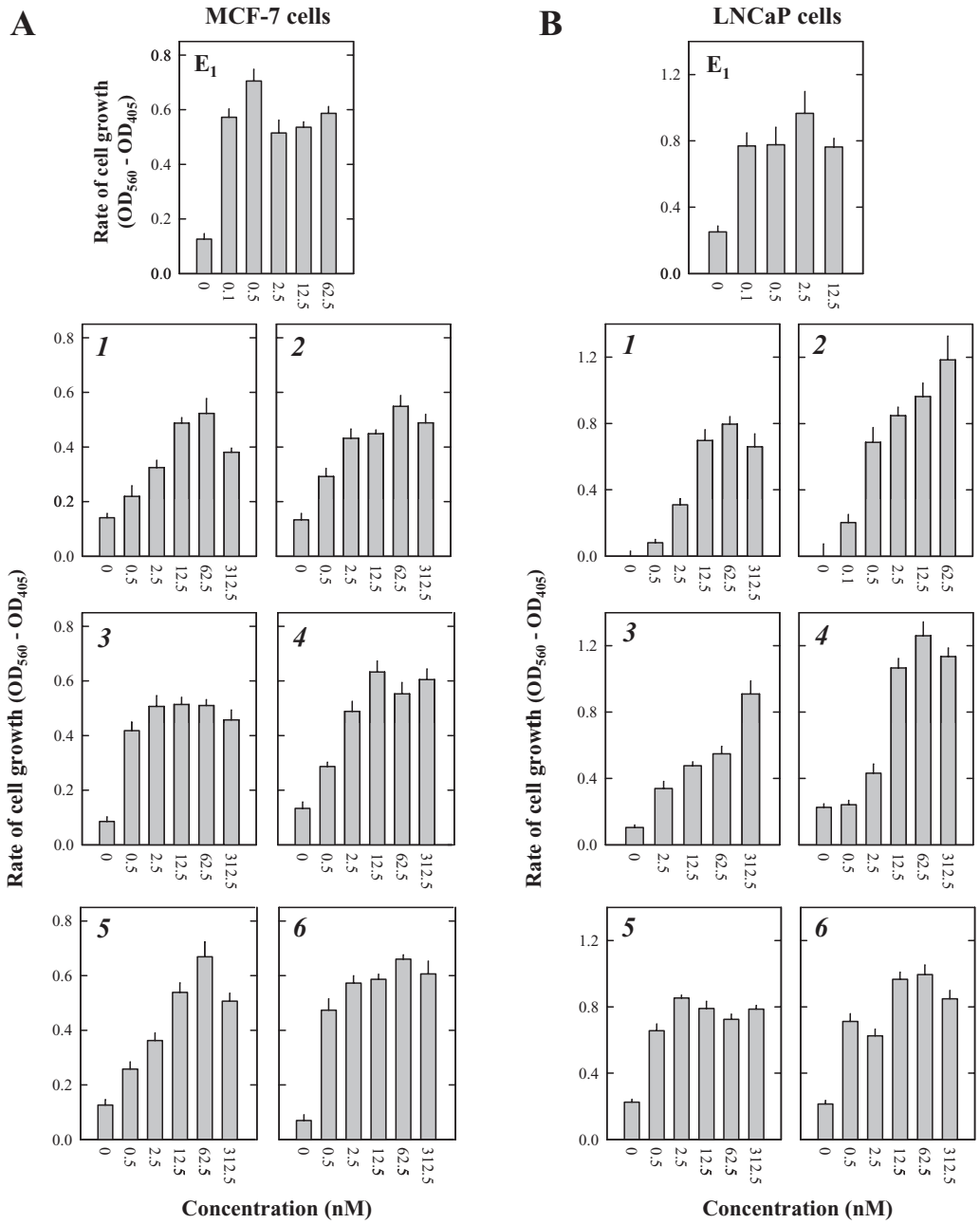

Figure 4 Comparison of the oestrogenic activity of compounds *1–6* with oestrone (E_1). (A) The mitogenic activity in the oestrogen receptor (ER)-positive MCF-7 human breast cancer cells in culture. (B) The mitogenic activity in the ER-positive LNCaP human prostate cancer cells in culture. The *y*-axis shows the cell density measured by crystal violet staining (Liu and Zhu, 2004). Each value is the mean ± SD ($n = 6$).

considered to be a good overall correlation for both ER subtypes between the predicted binding affinity values and the experimentally determined values. The values of q^2 were higher than 0.4, which reflected the good overall predictive ability of the 3-D QSAR/CoMFA models developed in this study. The individual contributions from the steric and electrostatic fields were 52% and 48%, respectively, for the ERα CoMFA model, and 47% and 53%, respectively, for the ERβ CoMFA model.

The colour contour maps derived from the ERα and ERβ CoMFA models are shown in Figure 5B. Note that 5(10)-oestren-3β,17β-diol (compound *4*) is shown inside the field only for demonstration purposes. The contours of the steric map are shown in yellow and green, and those of the electrostatic map are shown in red and blue. Green contours indicate regions where a steric bulk substituent would decrease the inhibition of $[^3H]E_2$ binding with the receptor (decrease the binding affinity of test compound), whereas the yellow contours would indicate areas where a steric bulk substituent would increase the inhibition of $[^3H]E_2$ binding (increase binding affinity of the test compound). The red contours mark regions where a substituent with a strong negative charge would decrease the inhibition of $[^3H]E_2$ binding (decrease the binding affinity of test compound), whereas the

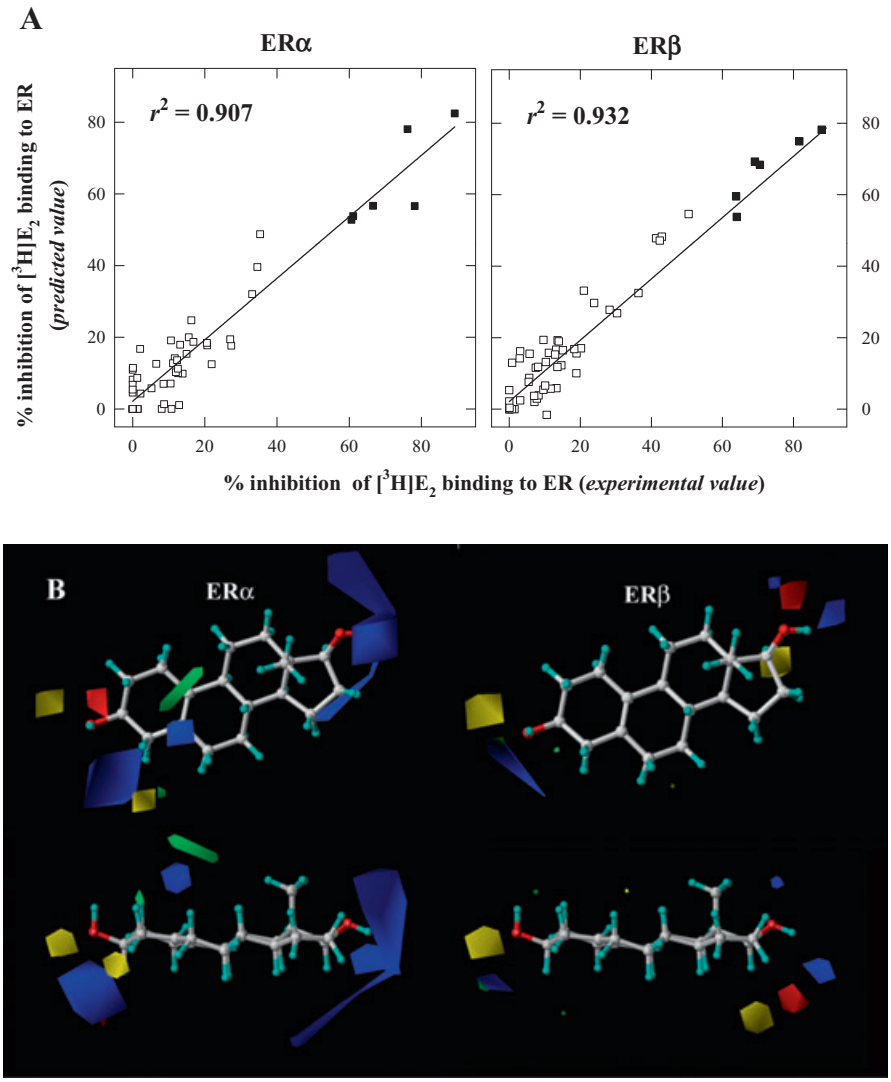

Figure 5 3-D quantitative structure–activity relationship/comparative molecular field analysis (CoMFA) of 58 non-aromatic steroids for their binding with oestrogen receptor (ER)α and ERβ. 5α-Androstan-3β-ol-16,17-dione-16-oxime was excluded because of its unique side chain. 4-Androsten-3β,17β-diol and 4,9(11)-androstadien-17β-ol-3-one were considered to be outlier compounds for ERα and ERβ respectively. (A) The correlations of the predicted % inhibition of [^{3}H]E_2 binding for ERα and ERβ with the corresponding experimental values that were determined in the present study (data listed in Table 1). The filled squares represent data points for compounds *1–6*. (B) The colour contour maps of the CoMFA models for human ERα and ERβ. Note that 5(10)-oestren-3β,17β-diol was shown inside the field only for demonstration purposes. The contours of the steric map were shown in yellow and green, and those of the electrostatic map were shown in red and blue. Green contours indicated regions where a relatively bulky substitution would decrease the inhibition of [^{3}H]E_2 binding of some ligands with the receptor, whereas the yellow contours indicated areas where a bulkier substituent would increase the inhibition of [^{3}H]E_2 binding. The red contours mark regions where a negatively charged substitution likely would decrease the inhibition of [^{3}H]E_2 binding, whereas the blue contours showed areas where a negatively charged substitution would increase the inhibition of [^{3}H]E_2 binding. Ligands with greater values of the inhibition of [^{3}H]E_2 binding (higher binding affinity) were correlated with: (i) less bulk near green; (ii) more bulk near yellow; (iii) less positive charge near blue; and/or (iv) less negative charge near red.

blue contours show areas where a substituent with a strong negative charge would increase the inhibition of [^{3}H]E_2 binding (increase binding affinity of the test compound).

In addition, we have also conducted molecular simulation and docking studies to further characterize the binding interactions of these six non-aromatic steroids with the ligand binding domains (LBDs) of human ERα and ERβ. The known X-ray crystallographic structure of human ERα's LBD in complex with E_2 (PDB code: 1ERE) was directly used for energy minimization with various ligands, including non-aromatic steroids. Because no X-ray crystallographic structure of ERβ's LBD in complex with E_2 was available in the protein data bank, we used the ERβ LBD in complex with the agonist analogue ERB-041 (PDB code: 1X7B) as a template for docking the binding of E_2 and also other non-aromatic steroids (Figure 6A).

Our molecular models showed that the binding of non-aromatic steroids with human ERα and ERβ closely resembled the binding of E_2 in these receptors (Figure 6B and D), especially the interaction of their C-3 and C-17 hydroxyl groups with the receptors by forming four hydrogen bonds between each of the ligands and the receptors (i.e. Glu353, Arg394,

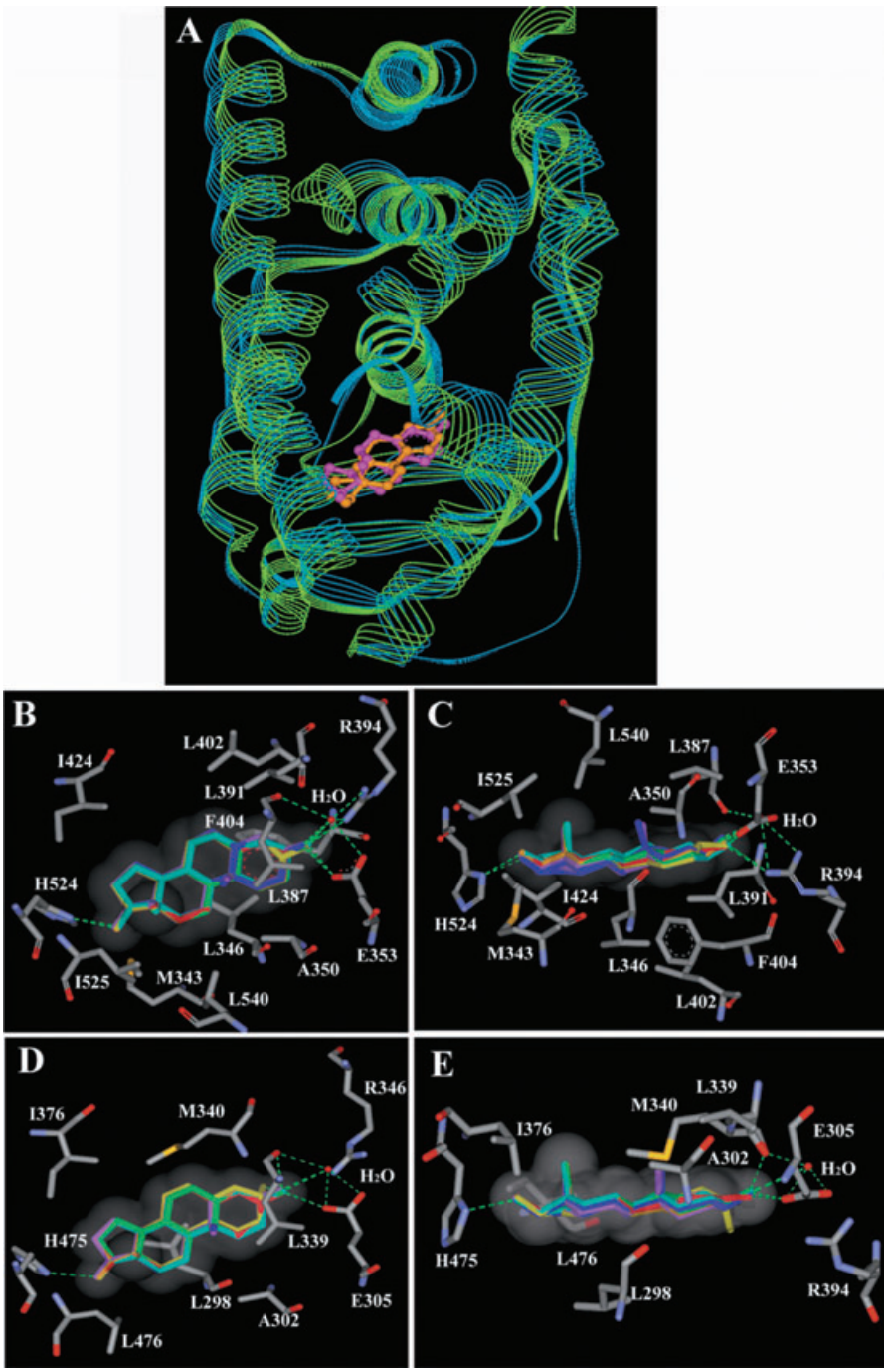

Figure 6 Comparison of oestrogen receptor (ER)α and ERβ binding sites in complex with 17β-oestradiol (E_2) or compounds *1–6* in the docking models developed in this study. The figure was drawn using the *Discovery Studio* software (Version 1.7, Accelrys, Inc. San Diego, CA, USA). (A) Overlay of the ligand binding domains of human ERα and ERβ in complex with E_2. The blue wires represent ERα and the green wires represent ERβ. E_2 in complex with ERα is coloured red and that in complex with ERβ is coloured orange. (B) E_2 and compounds *1–6* in the ERα binding pocket interacting with key amino acid residues Met343, Leu346, Ala350, Glu353, Leu387, Leu391, Arg394, Leu402, Phe404, Ile424, His524, Ile525 and Leu540. (C) Horizontal view of ligands interacting with the ERα binding pocket. (D) E_2 and compounds *1–6* in the ERβ binding pocket interacting with key amino acid residues Leu298, Ala302, Glu305, Leu339, M340, Arg346, Ile376, His475 and Leu476. (E) Horizontal view of ligands interacting with the ERβ binding pocket. E_2 and compounds *1–6* were coloured in red, orange, yellow, green, magneta, dark blue and light blue respectively. Hydrogens were omitted from all molecules. Green dashed lines represent hydrogen bonds between ligands and receptors. The grey shadows represent the van der Waals surfaces of the ligands. For simplicity, the amino acids are labelled with a single letter in the figure.

His524 and a water molecule for ERα; Glu305, Leu339, His475 and a water molecule for ERβ). It was visualized in Figure 6C and E that the non-aromatic *A*-rings, which are not flat as the aromatic *A*-ring of E_2, affected the formation of a strong hydrogen bond between the receptor and the C-3 hydroxyl group of the non-aromatic steroids. Also, the van der Waals interactions between the non-aromatic *A*-rings and the key hydrophobic amino acids of the binding pockets (such as

Phe404 and Leu387 of ERα and Leu339 of ERβ) were much lower compared with their interactions with the aromatic *A*-rings because of the non-planar structure of the non-aromatic *A*-rings. These are the main reasons why the non-aromatic steroids generally have very weak or little binding affinity for the ERα and ERβ, although they can fit inside the binding pocket in a similar manner.

Discussion

In the present study, we identified six non-aromatic steroids, from a total of sixty compounds tested, which have physiologically relevant high binding affinity for human ERα and ERβ, comparable to that of E_1. *In vitro* cell culture-based assays showed that these non-aromatic steroids could effectively activate the ER-mediated genomic actions with low nM EC_{50} values, and they could also elicit hormonal responses (such as strong growth stimulation) in the ER-positive human breast and prostate cancer cell lines (MCF-7 and LNCaP) in culture, with efficacy comparable to that of E_1.

It is known that oestrogens exert a wide range of important biological functions in many organ systems in male animals or men. In ERα-knockout male mice that lacked oestrogen actions, their serum luteinizing hormone (LH) levels were markedly elevated although high levels of circulating testosterone were present in these animals (Lindzey *et al.*, 1998). These phenotypic changes in male ERα-knockout mice are consistent with the well-known physiological functions of oestrogens in the female as crucial feedback regulators of the pituitary secretion of LH and follicle-stimulating hormone. Similarly, studies in men with an aromatase gene mutation also showed higher serum levels of LH and follicle-stimulating hormone, in addition to infertility in these subjects (Rochira *et al.*, 2001). These findings suggest that the endogenous oestrogens present in man have important physiological functions in regulating the release of gonadotropins. In recent years, oestrogens have also been proposed to play an important role in modulating the development of prostate diseases (including cancer) in elderly men (Bosland, 2006; Ellem and Risbridger, 2007). A recent study showed that 5α-androstan-3β,17β-diol (compound *5*) could activate ERβ and inhibit prostate development in animals (Weihua *et al.*, 2002). Notably, whereas some of the non-aromatic steroids (compounds *1*, *3* and *4*) have a selective binding affinity for ERs, some of the others (compounds *2*, *5* and *6*) have binding activity for both human ERs and AR. As AR and ER have both been recognized to play important roles in the normal development as well as pathogenesis of the prostate, it will be of interest to further explore their potential unique biological functions in various other target organs in man.

Our 3-D QSAR/CoMFA analysis of 58 selected steroidal oestrogens (Figure 5) showed high r^2 and q^2 values for human ERα and ERβ, suggesting high degrees of overall correlation and predictability for both ER subtypes between predicted values and experimental values determined in this study for these steroids (Figure 5A). It is of note that there are a number of similarities between the contour maps for human ERα and ERβ (Figure 5B). Firstly, contour maps for both receptors indicate the importance of negatively charged substituents around the regions of the C-3 and C-17 for enhanced ER binding (Figure 5B; blue area). This suggestion is in agreement with the higher ER binding affinities of some of the compounds with C-3 and C-17 hydroxyl groups compared with the compounds with a ketone group. In addition, the contour maps for both receptors also suggest that the slightly bulkier substitutes in the vicinity of the *A*-ring may favour ER binding (Figure 5B, yellow area).

The molecular models developed in this study were based on the known X-ray structures of the LBDs of human ERα and ERβ, which provided valuable information concerning the interactions of these non-aromatic steroids with the ERs. The hydroxyl groups in C-3 and C-17β are key functional groups that form hydrogen bonds with the amino acid residues inside the binding pocket (note that three hydrogen bonds are formed with the C-3 hydroxyl group and one formed with the C-17 hydroxyl group). Based on our recent study (Zhu *et al.*, 2006), the C-3 hydroxyl group appears to be a relatively more important determinant of binding strength compared with the C-17β hydroxyl group because 17-deoxyoestrone (an analogue of E_2 with the 17-hydroxyl group removed) still retained considerable binding affinity for ERα and ERβ, whereas the lack of a free C-3 hydroxyl group completely removed a steroid's ER binding activity. One major difference between the aromatic steroids (such as E_2 and E_1) and the non-aromatic steroids is the aromaticity of their *A*-rings. This difference can be clearly visualized in Figure 6C and E that the non-aromatic *A*-rings are not flat as the aromatic *A*-rings of E_2 and E_1. This difference reduces the strength and even jeopardizes the formation of the hydrogen bonds between the C-3 hydroxyl group of the non-aromatic steroids and the binding pocket residues, resulting in a drastically reduced binding affinity. This is believed to be one of the major determinants for the low binding affinity of the non-aromatic steroids with a C-3α hydroxyl group. Another important determinant of the binding affinity of the non-aromatic steroids is the lower van der Waals interactions between the non-aromatic *A*-ring and the hydrophobic amino acid residues in the binding pockets of human ERα and ERβ. In addition, the *A*- and *B*-rings of 5β-androstans and 5β-oestrans are not on the same plane due to the configuration of their C-5 hydrogen atoms, which also interferes with the formation and strength of the C-3 hydrogen bond, and ultimately contributes to the reduced binding affinity of the 5β-compounds for the ERs compared with the 5α-compounds (see Table 1).

In conclusion, we have identified a group of non-aromatic steroids (potential precursors and/or metabolites of endogenous androgens) that can bind to human ERα and ERβ with physiologically relevant high binding affinity and also activate the ERs and elicit hormonal responses in ERα-positive cell lines in culture. The results of this study suggest an intriguing possibility that some of endogenous androgen precursors or metabolites may serve as ER modulators in men. These findings also call for further studies to determine which of these non-aromatic ER modulators can be produced in men at physiologically relevant quantities and also what are their physiological/pathophysiological functions.

Conflicts of interest

None to declare.

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