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Obstacles to the Prediction of Estrogenicity from Chemical Structure: Assay-Mediated Metabolic Transformation and the Apparent Promiscuous Nature of the Estrogen Receptor

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ABSTRACT. Information on structure–activity relationships (SAR) and pathways of metabolic activation would facilitate the preliminary screening of chemicals for estrogenic potential. Published crystallographic studies of the estrogen receptor (ER) imply an essential role of the two hydroxyl groups on estradiol (17β-E_2) for its binding to ER. The influence of these hydroxyl groups on ER binding and estrogenicity was evaluated by the study of 17β-E_2 with one or both of these hydroxyl groups removed (17β-desoxyestradiol and 3,17β-bisdesoxyestradiol, respectively). 6-Hydroxytetralin (17β-E_2 with its C- and D-rings removed) and other synthetic estrogens were also studied. The estrogenicity assays comprised a yeast ER-mediated transcription assay, mammalian cell transcription assays incorporating either ER α or ER β, and the immature rat uterotrophic assay. With the exception of 6-hydroxytetralin in the uterotrophic assay, all the chemicals were active in all the assays. Hydroxylation of the two desoxy compounds to estradiol was shown to occur in immature female rats, but metabolism was not implicated in the responses observed in the ER-binding and yeast systems. It is concluded that the 3-hydroxyl and 17β-hydroxyl groups of 17β-E_2 are not absolute requirements for estrogenicity. It would therefore be of value to the derivation of SAR for estrogenicity were the crystal structure of the bisdesoxy-E_2/ER complex to be evaluated. BIOCHEM PHARMACOL **60**;10:1519–1530, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. estrogenicity; structure–activity relationship; metabolism; estrogen receptor; 17β-estradiol

A surprising number of synthetic chemicals are able to mimic the biological activity of 17β-E_2,‖ as exemplified by the plasticizer BBP [1, 2] and the detergent precursor NP [3, 4] (Fig. 1). This creates a particular need for knowledge of the SAR of estrogenicity [5–7]. However, the estrogenic potency of most of the recently defined synthetic estrogens is low, and many of them bear little structural similarity to 17β-E_2 [7, 8]. Such structural comparisons are complicated further by the general absence of knowledge regarding the metabolic conversion of test chemicals in the many assays for estrogenicity.

Historically, SAR for xenobiotic estrogens were based on 17β-E_2 as the lead structure, and although they are able to explain the estrogenic activity of agents such as the hydroxylated polychlorinated biphenyls (PCBs) [9–11], they are less useful when studying estrogens such as kepone and BBP. It may be possible to adapt these SAR to accommodate monophenols such as NP and non-phenols such as BBP, but such adaptations concomitantly weaken the specificity of the SAR to the point that probably most phenols (and most aromatic compounds capable of metabolism to a phenol) become potential ER agonists. This potential loss of specificity cannot currently be assessed because no single chemical has yet been internationally agreed to be devoid of estrogenicity.

The crystal structures of the LBD of ER α and ER β in complex with either 17β-E_2 or partial ER agonists were recently described [12, 13]. These data revealed an unexpectedly large and plastic hydrophobic central cavity in bound ER and the formation of specific hydrogen-bonded

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‖ *Abbreviations:* SAR, structure-activity relationships; ER, estrogen receptor; 17β-E_2, 17β-estradiol; hER, human estrogen receptor; BBP, butyl benzyl phthalate; NP, 4-nonylphenol; LBD, ligand-binding domain; 17desoxy-E_2, 17β-desoxyestradiol; bisdesoxy-E_2, 3,17β-bisdesoxyestradiol; tosyl-NP, 4-nonylphenol-*p*-toluene sulfonate; NB, nonylbenzene; 17α-E_2, 17α-estradiol; DES, diethylstilbestrol; SIM, single-ion monitoring; Vit, vitellogenin; LH, luteinizing hormone; and ERE, estrogen response element.

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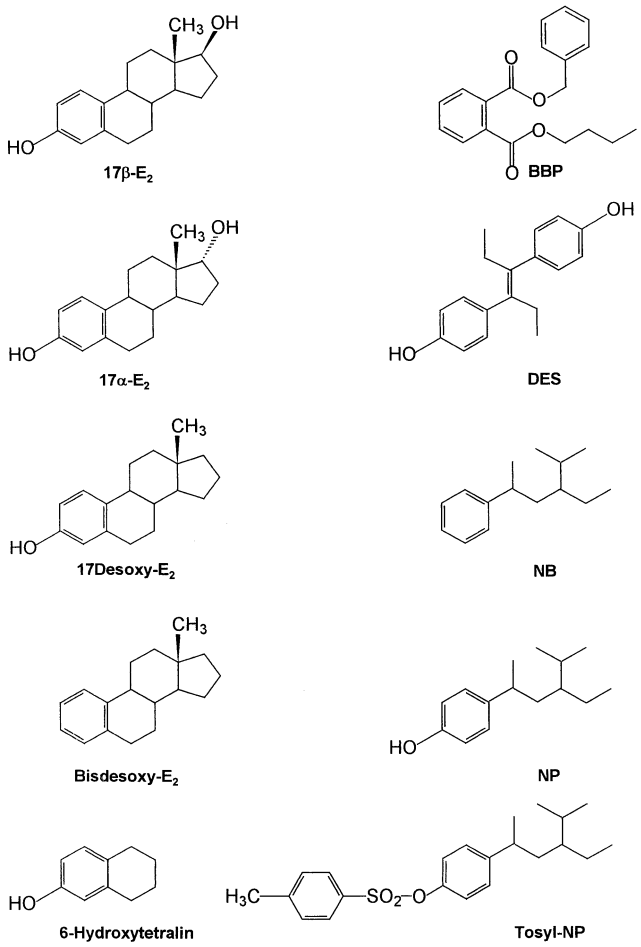

FIG. 1. Chemical structures of compounds 17β-E_2, 17α-E_2, 17desoxy-E_2, bisdesoxy-E_2, 6-hydroxytetralin, BBP, DES, NB, NP, and tosyl-NP.

interactions between the receptor and the 3- and 17β-hydroxyl groups of 17β-E_2. Brzozowski *et al.* [12] noted that the size of this cavity might account for its 'catholic/promiscuous' binding properties, and they suggested that knowledge of the specific sites of hydrogen bonding would aid the design of more potent, or more selective, therapeutic agents. However, these same hydrogen-bonding requirements obscure further the activity of non-hydroxylated estrogens such as BBP, at least in the absence of data indicating metabolic hydroxylation during the course of their assessment for estrogenicity.

Within the above context, it was decided to conduct basic SAR studies in which the importance of the two hydroxyl groups on 17β-E_2 could be assessed. For this purpose, the 17β-desoxy derivative of 17β-E_2 (17desoxy-E_2) and the bisdesoxy derivative of 17β-E_2 (bisdesoxy-E_2) were evaluated in an ER–ligand displacement assay and a range of estrogenicity assays. The possible metabolism of these chemicals was also evaluated in order to distinguish intrinsic estrogenicity from the estrogenicity of metabolites. Limited experiments were also conducted on 6-hydroxytetralin (17β-E_2 without its aliphatic C- and D-rings), the tosyl derivative of NP (tosyl-NP), and NB (derived from NP by removal of its phenolic hydroxyl group).

MATERIALS AND METHODS

Chemicals

17β-E_2, 17α-E_2, estrone, 2-methoxyestrone, estrone 3-β-D-glucuronide, DES, Glucurase (bovine liver β-glucuronidase in acetate buffer; 5000 units/mL), ascorbic acid, and NADPH were obtained from Sigma. 6-Hydroxytetralin (5,6,7,8-tetrahydro-2-naphthol) was supplied by Aldrich Chemical Co. 17Desoxy-E_2 (1,3,5(10)-estratriene-3-ol) was synthesized by the method of Francois and Levisalles [14] and had a melting point (mp) of 138–139° (in [14], mp 138–140°). NMR and mass spectrometry confirmed its structure. Bisdesoxy-E_2 (1,3,5(10)-estratriene) was synthesized by the method of Dannenburg and Köhler [15] and had a mp of 74.5–75.5° (in [15], mp 76.0°). It had the molecular ion and NMR spectrum corresponding to the required structure. NP (85% of mixed branched *p*-isomers) was obtained from Fluka Chemicals. Tosyl-NP (mixed branched isomers) was prepared by standard methods from the same batch of NP. The reaction mixture was poured onto ice and 2 M hydrochloric acid and extracted with ether. After drying, the extract was evaporated to produce a yellow oil whose structure was confirmed by NMR and elemental analysis. Without further purification, tosyl-NP (3 g) was dissolved in ethanol and converted to NB by the action of Raney nickel (30 g, pore size ~50 μ; Aldrich Chemical Co.) with stirring and heating to reflux temperature for three days. After removal of the nickel residues, the filtrate was evaporated to an oil which was purified by chromatography on a Florisil column, eluting with petroleum ether bp-40–60° and collecting the least polar component. The structure was confirmed by C and H analysis (within 0.5% of theory), NMR, and mass spectrometry. [6,7-^{3}H]17β-E_2 was obtained from DuPont NEN and HPLC-grade solvents were obtained from Fisher Scientific. All other chemicals were purchased from BDH.

Animals

Immature female Wistar rats (21–25 days old) were obtained from a breeding colony maintained by the Biomedical Services Unit, University of Liverpool. Immature female Alpk.AP rats (21–22 days old) belonging to a Wistar-derived strain were obtained from the breeding unit at AstraZeneca, Alderley Park, and were acclimatized for 24 hr before being dosed. Animal studies were performed in accordance with the UK "Animals (Scientific Procedures) Act." Animal care and procedures were carried out according to in-house standards. All animals were housed in wire-mesh cages except that solid bottoms were provided for the immature animals. Temperature was controlled at 22 ± 3°. Humidity was controlled at 30–70%. A 12-hr light/dark cycle was maintained. Food and water were available *ad lib*.

Estrogen Receptor Competitive Binding (Ligand Displacement) Assay

The competitive binding assay was carried out as described previously [16], adapted for use with rat uterine cytosol [17], using increasing concentrations of unlabelled competitor (0–5 μM) to displace [6,7-^{3}H]17β-E_2 from its receptor.

Yeast Transcription Assay

The estrogenic activity of test chemicals was determined by the yeast hER-mediated transcription assay of Routledge and Sumpter [18]. Briefly, the DNA sequence of hER α is integrated into the yeast genome. Upon binding an active ligand, the ER interacts with the ERE of a reporter gene encoding β-galactosidase. Thus, β-galactosidase is secreted into the medium where it hydrolyzes the chromogenic substrate chlorophenol red-β-D-galactopyranoside, resulting in a colour change from yellow to red (540 nm) that can be measured spectrophotometrically. The criterion for activity in the assay is a reproducible and dose-related increase in positive wells. In the case of marginal positive responses, the emphasis is on the reproducibility of the response rather than on the statistical analysis of individual datasets.

COS-1 Cell Transcription Assays

The estrogenicity of test chemicals was assessed using transcription assays utilizing the methods employed by Pennie *et al.* [19].

Uterotrophic Assay

Immature female Alpk.AP rats were dosed orally with varying amounts of 17β-E_2, 17α-E_2, 17desoxy-E_2, bisdesoxy-E_2, or 6-hydroxytetralin as described previously [20]. Compounds were administered in arachis oil and dosing volumes were 5 mL/kg body weight. All experiments were accompanied by a vehicle and a 17β-E_2-positive control group

Uterine Cytosolic Incubations

Studies to investigate if metabolism of test compounds (to 17β-E_2) occurred in the ER competitive binding assay utilized incubations in 12-mL glass tubes containing 200 μL rat uterine cytosol and 1 mM 17desoxy-E_2 or bisdesoxy-E_2 in TEGM (10 mM Tris, 1.5 mM EDTA, 10% glycerol, and 3 mM $MgCl_2$) buffer [16] (pH 7.6) to give a final volume of 1.0 mL. Substrate or cytosol was omitted from controls. Following incubation at 4° for 18 hr, the reaction was terminated by the addition of methyl *tert*-butyl ether (4 mL). This final reaction mixture was subject to rotation mixing for 15 min. The combined organic phases of two extractions were evaporated to dryness under a stream of N_2 at 40° and the residue was reconstituted in methanol (200 μL) for immediate analysis by HPLC. Aliquots (20 μL) were eluted from an Ultracarb 5-μm C-8 column with acetonitrile (50–70%, 12 min; 70–80%, 1 min) in 0.1% acetic acid at 1 mL/min and the analytes detected at 280 nm (0.05 absolute units of full scale).

Yeast Incubations

Studies to investigate whether the yeast was able to metabolize test compounds (to 17β-E_2), under the conditions of the assay [18], utilized incubations in 100-mL glass flasks containing 20 mL growth medium and either 100 μM 17desoxy-E_2 or 1 mM bisdesoxy-E_2. Yeast or substrate was omitted from controls. Following incubation at 32° in a naturally ventilated incubator for three days, aliquots (200 μL) from each flask were taken and their absorbance at 540 nm determined. Reaction mixtures were centrifuged at 1700 *g* for 10 min to sediment the yeast. Yeast cells were resuspended in H_2O (10 mL) and sonicated. The lysed yeast or growth medium (2 mL aliquots) was extracted with methyl *tert*-butyl ether (8 mL × 2) and the combined organic phases evaporated to dryness under a stream of N_2 at 40°. The residue was reconstituted in methanol (200 μL) for analysis by HPLC. Aliquots (20 μL) were eluted from an Ultracarb 5-μm C-8 column with acetonitrile (50–70%, 12 min; 70–80%, 1 min; 80%, 10 min; 80–100%, 1 min; 100%, 10 min) in 0.1% acetic acid at 1 mL/min.

Animal Experiments

Immature female Wistar rats (21–25 days old) were anaesthetized with sodium pentobarbitone (60 mg/kg, i.p.). Cannulae were inserted into the femoral vein and common bile duct. [6,7-^{3}H]17β-E_2 (41.6 μmol/kg; 4 μCi), 17desoxy-E_2 (41.6 μmol/kg), or bisdesoxy-E_2 (41.6 μmol/kg) in DMSO was given by i.v. injection over 5 min. Bile was collected for 30 min prior to administration of the drug to obtain an appropriate 'drug blank' sample for mass spectral comparisons. Vehicle control animals were given DMSO at a volume appropriate to their weight. Thereafter, bile was collected as a single 3-hr fraction followed by a 1-hr fraction and stored at −80° if not analyzed immediately by LC–MS.

Liquid Chromatography–Mass Spectrometry

The configuration of the LC–MS system and parallel radioactivity detector (Radiomatic A250, Canberra Packard) has been described previously [21]. A Quattro II tandem quadrupole instrument (Micromass Ltd.) fitted with the standard LC–MS interface and electrospray source was employed in the positive-ion mode for SIM (dwell time of 200 msec; inter-channel delay of 20 msec). Eight channels were monitored, corresponding to ammonium adducts ($[M + NH_4]^+$) of the analytes: m/z 448 (desoxyestrone glucuronide), m/z 450 (desoxyestradiol glucuronide), m/z 464 (estrone glucuronide), m/z 466 (estradiol

glucuronide), m/z 480 (hydroxyestrone glucuronide), m/z 482 (hydroxyestradiol glucuronide), m/z 494 (methoxyestrone glucuronide), and m/z 496 (methoxyestradiol glucuronide). Metabolites were characterized as ion chromatographic peaks that were absent from pre-dose control bile. Biliary metabolites were resolved on an Ultracarb 5-μm C-8 column (25 × 0.46 cm) with a gradient of methanol (20–60%, 25 min) in 0.1 M ammonium acetate, pH 6.9. The flow rate was 0.9 mL/min. Fragmentation spectra were acquired between m/z 200–550 over a scan time of 5 sec. In the case of biliary metabolites of [6,7-^{3}H]17β-E_2, the remainder of the column effluent was mixed (1:1, v/v) with Ultima-flo AP (Canberra Packard) for detection of radioactivity.

Quantification of Estrone Glucuronide by LC–MS

Calibration graphs for estrone glucuronide were constructed on each day of analysis with dilutions of a freshly prepared methanol solution (0–100 μM). Bile aliquots (50 μL) were eluted from an Ultracarb 5-μm C-8 column with methanol (20–60%, 25 min) in 0.1 M ammonium acetate, pH 6.9. The mass spectrometer monitored *inter alia* m/z 464 for estrone glucuronide, identified by co-elution with the authentic compound. Peak areas for estrone glucuronide were computed with Masslynx integration software (Micromass Ltd.). Calibration graphs were linear ($r^2 > 0.98$) over the specified concentration range. Endogenous estrone glucuronide (0–3.83 nmol/mL), when detected in the pre-dose bile, was subtracted from the glucuronide found after administration of the desoxy compounds.

Enzymic Hydrolysis of Biliary Conjugates

Bile (200 μL) from animals given [6,7-^{3}H]17β-E_2, 17desoxy-E_2, or bisdesoxy-E_2 was incubated with Glucurase (500 μL) in glass test tubes at 37° for 16 hr. Incubations were extracted with methyl *tert*-butyl ether (4 mL × 2), and the ether extracts were evaporated to dryness under N_2 and reconstituted in methanol (200 μL) for analysis by HPLC. Control incubations were carried out in the absence of enzyme. Deconjugated metabolites (20–50 μL aliquots) and co-injected standards were analyzed on an Ultracarb 5-μm C-8 column with acetonitrile (20–75%, 40 min) in 0.1% acetic acid at 1 mL/min.

Preparation of Microsomes

Microsomes were prepared from the livers of immature female Wistar rats according to the method of Gill *et al.* [22]. Microsomal protein concentrations were determined by the method of Lowry *et al.* [23].

Microsomal Incubations

Incubations were carried out in 12-mL glass tubes and contained 1 mg microsomal protein, 100 mM $MgCl_2$, 1 mM ascorbic acid, and 500 μM 17desoxy-E_2 or bisdesoxy-E_2 in 67 mM phosphate buffer (pH 7.4) to give a final volume of 1 mL. Substrate or NADPH was omitted from control incubations. Following preincubation at 37° for 2 min in a shaking water bath, the reaction was initiated by the addition of NADPH (1 mM). After 90 min, with additions of NADPH after 30 min and 60 min, the reaction was terminated by adding methyl *tert*-butyl ether (4 mL). This final reaction mixture was subjected to rotation mixing for 15 min. The combined organic phases of two extractions were evaporated to dryness under a stream of N_2 at 40° and the residue was reconstituted in methanol (200 μL) for immediate analysis by HPLC. Aliquots (20 μL) of the methanol solutions were eluted from an Ultracarb 5-μm C-8 column with acetonitrile (50–70%, 12 min; 70–80%, 1 min) in 0.1% acetic acid at 1 mL/min. Analytes were also chromatographed with ethyl acetate (20%, 10 min; 20–30%, 10 min; 30%, 10 min) in hexane at 1 mL/min on a LiChrosorb 5-μm diol column.

HPLC

HPLC was performed with an Ultracarb 5-μm C-8 column (25 × 0.46 cm; Phenomenex) or a LiChrosorb 5-μm diol column (25 × 0.46 cm; Phenomenex) connected to a Spectra-Physics SP8800 ternary solvent delivery system, a Spectra-Physics analytical UV1000 UV detector (Spectra-Physics), and a Radiomatic A250 data system (Canberra Packard). Metabolites were characterized as chromatographic peaks that were absent from control incubations (minus substrate or NADPH).

RESULTS

Estrogen Receptor Competitive Binding Assay

The rank order of competitive binding activity for 17β-E_2 and various xenoestrogens was DES > 17β-E_2 > NP > tosyl-NP > BBP > NB. NB had marginal activity (Fig. 2A). 17β-E_2 > 17desoxy-E_2 > 17α-E_2 > bisdesoxy-E_2 > 6-hydroxytetralin was the rank order of activity for the steroidal analogues (Fig. 2B), which produced ID_{50} values as shown in Table 1.

Yeast Transcription Assay

17β-E_2, DES, NP, tosyl-NP, and BBP were active in the yeast hER-mediated transcription assay (Fig. 3A). 17β-E_2, 17α-E_2, 17desoxy-E_2, bisdesoxy-E_2, and 6-hydroxytetralin were active (Fig. 3B), with ED_{50} values as shown in Table 1. Figure 4 shows the activity of 17β-E_2 and NP after either the normal three- or extended six-day incubations, and the weak activity of NB observed only after six-day incubations.

The calculation from Andrews allows a simple estimate of the contribution of a hydrogen bond to a drug–receptor interaction [24]. The average binding energy for a single bond is between 1.6–9.5 kcal mol^{-1} [25]. The difference in energy between bisdesoxy-E_2 and 17desoxy-E_2 was 4.2 kcal

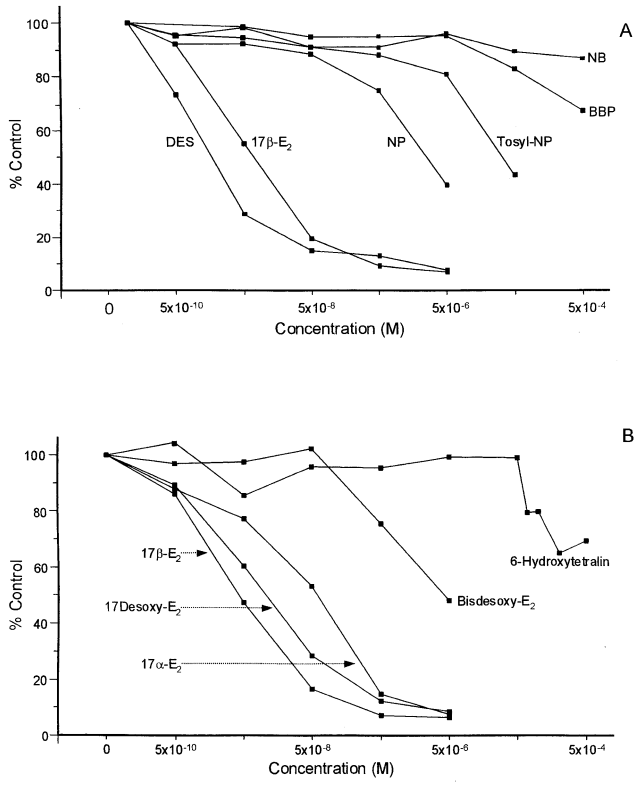

FIG. 2. Competitive binding of (A) 17β-E_2 and non-steroidal xenoestrogens and (B) 17β-E_2, 17α-E_2, and steroidal analogues to rat uterine cytosolic estrogen receptors. Data are derived from duplicate samples taken from a representative experiment.

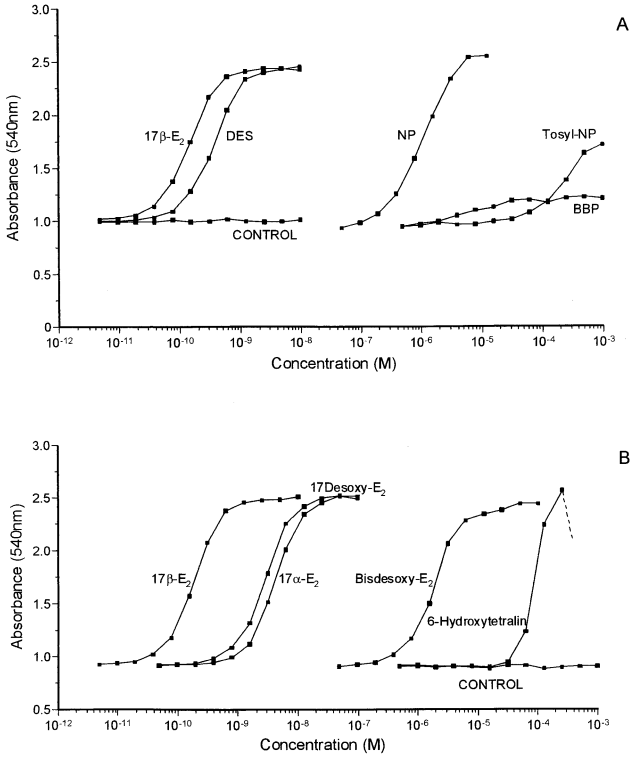

FIG. 3. Response of the yeast hER assay to (A) 17β-E_2, DES, NP, tosyl-NP, BBP and to (B) 17β-E_2, 17α-E_2, 17desoxy-E_2, bisdesoxy-E_2, and 6-hydroxytetralin. Data are derived from duplicate samples taken from a representative experiment. The dotted line represents the onset of toxicity of the compound towards the yeast, as seen by a decrease in turbidity at 620 nm.

mol^{-1}, whereas the difference between 17desoxy-E_2 and 17β-E_2 was 1.4 kcal mol^{-1}.

COS-1 Cell Transcription Assays

The data shown in Fig. 5A are from a representative experiment. Each experiment was repeated at least twice. We have previously reported that ER β preferentially activates the consensus Vit ERE, whereas ER α shows greater activation at the divergent LH ERE [19]. In the present study, data are presented for the receptor/ERE combination which gave the greatest degree of transcriptional activation, i.e. ER β/Vit and ER α/LH. Increasing concentrations of 17desoxy-E_2 caused a significant increase in transcription through ER α, which was of a similar magnitude to that induced by 17β-E_2. 6-Hydroxytetralin and bisdesoxy-E_2 caused somewhat lower inductions compared with 17desoxy-E_2 and 17β-E_2. The degree of transcriptional activation by 17β-E_2 through ER β was less than

TABLE 1. The relative ligand displacement and estrogenicity activities of 17β-E_2 and structural analogues in *in vitro* and *in vivo* assays

	Estimated activity relative to 17β-E_2		
Chemical	ER–ligand displacement assay (M)*	Yeast transcription assay (M)†	Uterotrophic assay (mg/kg)‡
17β-E_2	5×10^{-9} [100]	2×10^{-10} [100]	0.1 [100]
17α-E_2	1×10^{-8} [50]	4×10^{-9} [5]	0.4 [25]
17Desoxy-E_2	9×10^{-9} [56]	2×10^{-9} [10]	0.8 [12.5]
Bisdesoxy-E_2	5×10^{-6} [0.1]	2×10^{-6} [0.01]	1.0 [10]
6-Hydroxytetralin	NA§	9×10^{-5} [0.0002]	NA‖

*Concentration giving 50% inhibition of 17β-E_2 binding.
†Concentration giving 50% of maximal activity.
‡Dose required to give a twofold increase in uterine weight.
§Not applicable as 6-hydroxytetralin did not give 50% inhibition of 17β-E_2 binding.
‖Not applicable as 6-hydroxytetralin was not active in the uterotrophic assay.

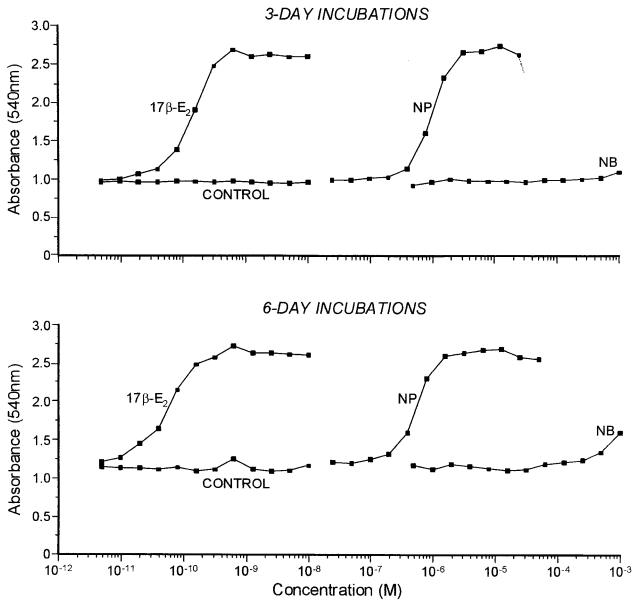

FIG. 4. Response of the yeast hER assay to 17β-E_2, NP, and NB. Data are derived from duplicate samples taken from a representative experiment. Data are shown for the normal three-day incubation period and for the extended six-day incubation.

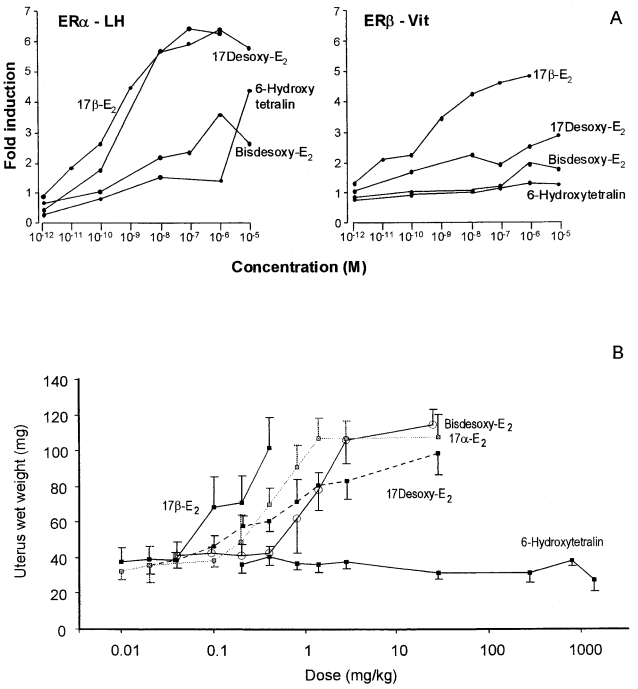

FIG. 5. (A) Response of the COS-1 cell ER transcription assay for 17β-E_2, 17desoxy-E_2, bisdesoxy-E_2, and 6-hydroxytetralin. Assays using ER α and ER β with the LH and Vit ERE, respectively, are shown. (B) The activity of 17β-E_2, 17α-E_2, 17desoxy-E_2, bisdesoxy-E_2, and 6-hydroxytetralin in the immature rat uterotrophic assay. Data are means ± SD (N = 5 to 25 animals). Control uterine weights ranged from 25 to 41 mg (mean 35 mg).

through ER α, a result which agrees with previous findings [19, 26]. In contrast to ER α, ER β-mediated transcription in response to 17desoxy-E_2 was significantly less than for 17β-E_2. 6-Hydroxytetralin and bisdesoxy-E_2 were extremely weak activators of ER β.

Uterotrophic Assays

All chemicals tested, except 6-hydroxytetralin, gave a positive assay response. The results of several experiments are combined in Fig. 5B. The activities (relative to 17β-E_2) of the test agents as estrogens are shown in Table 1.

Uterine Cytosolic Incubations

Based upon ID_{50} values (Table 1), 5 nM 17β-E_2 (representing a conversion of 56% for the incubated steroid) would have to be formed to give the ER–ligand displacement we report for 17desoxy-E_2, and either 5 nM 17β-E_2 (0.1%) or 9 nM 17desoxy-E_2 (0.18%) for the competitive binding of bisdesoxy-E_2. However, incubations of 17desoxy-E_2 (1 mM) or bisdesoxy-E_2 (1 mM) with immature rat uterine cytosol did not result in detectable metabolism to 17β-E_2 (data not shown). The high substrate concentration was utilized so that 17β-E_2 would be formed at approximately 560 μM (56%) or 1 μM (0.1%) if it was responsible for the competitive binding seen with 17desoxy-E_2 or bisdesoxy-E_2, respectively, and thus be detectable above the limit of sensitivity of the HPLC method (0.5 μM 17β-E_2). 17Desoxy-E_2 and bisdesoxy-E_2 eluted with retention times of 20.9 and 35.3 min, respectively.

Yeast Incubations

Incubations of 17desoxy-E_2 (100 μM) or bisdesoxy-E_2 (1 mM) with yeast under the conditions of the yeast assay (three days, 32°) did not result in metabolism of either chemical to 17β-E_2 or of bisdesoxy-E_2 to 17desoxy-E_2 (data not shown). Although high substrate concentrations were employed to allow for the limit of sensitivity of the HPLC method, 17desoxy-E_2 and bisdesoxy-E_2 both produced the characteristic colour change associated with exposure to estrogens, indicating that the yeast cells were still viable. 17Desoxy-E_2 and bisdesoxy-E_2 eluted with retention times of 22.1 and 29.6 min, respectively. Bisdesoxy-E_2 yielded one unidentified metabolite with a retention time of 23.1 min.

Identification of the Biliary Metabolites of [6,7-^{3}H]17β-E_2 in Immature Female Rat

Radiochromatographic analysis of bile taken from immature female Wistar rats treated with [6,7-^{3}H]17β-E_2 (41.6 μmol/kg, i.v.) resolved several metabolites: 52.2 ± 9.1% (mean ± SD, N = 4) of the radiolabel was recovered in bile over 4 hr. The 3-hr recovery of radioactivity was 48.3 ± 9.3%. LC–MS analysis of the bile (0–3 hr collection) and

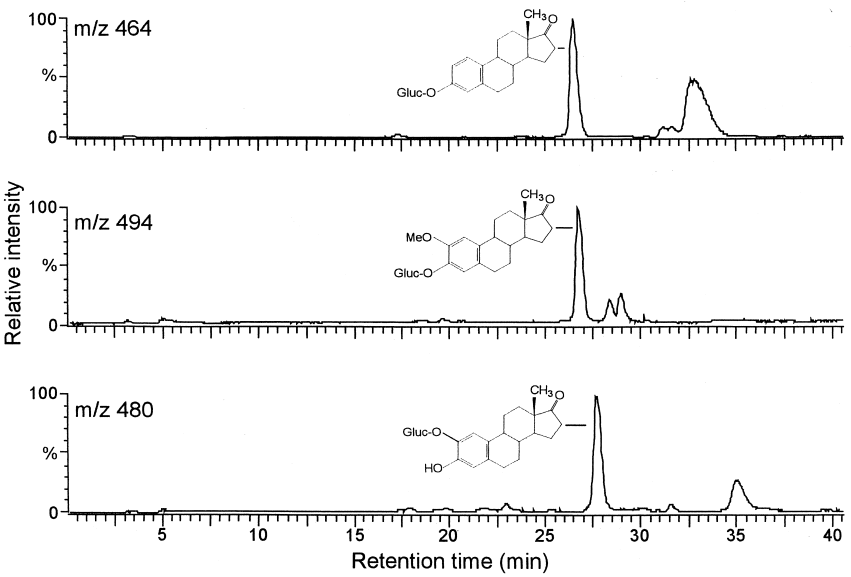

FIG. 6. HPLC separation (methanol–ammonium acetate) with electrospray MS detection of the biliary metabolites of [6,7-^{3}H]17β-E_2 (41.6 μmol/kg, i.v.) in immature female rat (0–3-hr collection). The SIM chromatograms represent $[M + NH_4]^+$ for estrone glucuronide (m/z 464), 2-methoxyestrone glucuronide (m/z 494), and 2-hydroxyestrone glucuronide (m/z 480). Biliary metabolites were defined as ion chromatographic peaks that were absent from pre-dose and vehicle control bile. Ion chromatographic peaks that are unlabelled are endogenous to bile.

interpretation of the data, as described previously [27, 28], revealed the three major metabolites to be glucuronides (Fig. 6) of estrone (m/z 464 $[M + NH_4]^+$, R_t = 26.5 min; 9.5 ± 1.3% of the eluted radioactivity), 2-methoxyestrone (m/z 494, R_t = 26.8 min; 43.0 ± 1.6%), and 2-hydroxyestrone (m/z 480, R_t = 27.7 min; 26.9 ± 2.1%). Estrone glucuronide was quantified by LC–MS, and its combined recovery (mean ± SD) from the 3- and 1-hr bile collections (275.94 ± 50.1 nmol, N = 4) was found to be 10.0 ± 1.5% of the dose (Table 2). The vast majority of the metabolites formed by the liver were excreted in the bile as demonstrated by the recovery of radioactivity, which is in agreement with previous work [27, 28]. The major biliary metabolites of administered [6,7-^{3}H]17β-E_2 were used as 'metabolic markers' to assess whether metabolism to 17β-E_2 had occurred following administration of either 17desoxy-E_2 or bisdesoxy-E_2; any 17β-E_2 formed would be rapidly metabolized [27, 28].

Identification of the Biliary Metabolites of Bisdesoxy-E_2 in Immature Female Rat

LC–MS analysis of bile collected for 4 hr from immature female Wistar rats administered bisdesoxy-E_2 (41.6 μmol/kg, i.v.) showed the production of several metabolites (Fig. 7A). SIM of m/z 464 revealed the formation of two metabolites with retention times of 27.2 and 29.6 min. One metabolite (R_t = 27.2 min) co-eluted with authentic estrone glucuronide. Estrone glucuronide in bile, quantified by LC–MS, (15.67 ± 8.37 nmol, N = 4) represented 0.42 ± 0.17% of the dose (Table 2). SIM of m/z 494 indicated the formation of 2-methoxyestrone glucuronide (R_t = 27.9 min). Two metabolites (R_t = 26.5 min and 28.4 min) were located at m/z 480; the metabolite with a retention time of 28.4 min corresponded to 2-hydroxyestrone glucuronide. No metabolites were observed in the remaining five channels monitored by the mass spectrometer. The fragmentation pattern for each glucuronide was identical to that of the corresponding biliary metabolite of 17β-E_2 (data not shown).

Identification of the Biliary Metabolites of 17Desoxy-E_2 in Immature Female Rat

LC–MS analysis of bile taken from immature female Wistar rats dosed with 17desoxy-E_2 (41.6 μmol/kg, i.v.) revealed the production of metabolites which yielded ions at m/z 464, 494, and 480 (Fig. 7B). SIM for m/z 464 indicated the

TABLE 2. The recovery of estrone glucuronide in bile of immature female Wistar rats administered [6,7-^{3}H]17β-E_2, 17desoxy-E_2, or bisdesoxy-E_2*

Compound	Estrone glucuronide (nmol)†			Estimated production of 17β-E_2 (% of dose)‡
	0–3 hr bile	3–4 hr bile	% of dose	
17β-E_2	251.16 ± 50.04	24.80 ± 5.94	10.0 ± 1.5	NA
17Desoxy-E_2	1.86 ± 0.91	0.33 ± 0.22	0.11 ± 0.05	1.0
Bisdesoxy-E_2	13.33 ± 8.18	2.33 ± 0.31	0.42 ± 0.17	4.0

*Anaesthetized and cannulated immature female Wistar rats were dosed (41.6 μmol/kg) i.v.

†Estrone glucuronide was quantified by LC–MS. Values represent means ± SD (N = 4 rats).

‡Quantification of estrone glucuronide enabled an estimate to be made for the production of 17β-E_2, calculated on the basis that estrone glucuronide in bile constituted approximately 10% of an equivalent dose of 17β-E_2 administered i.v.

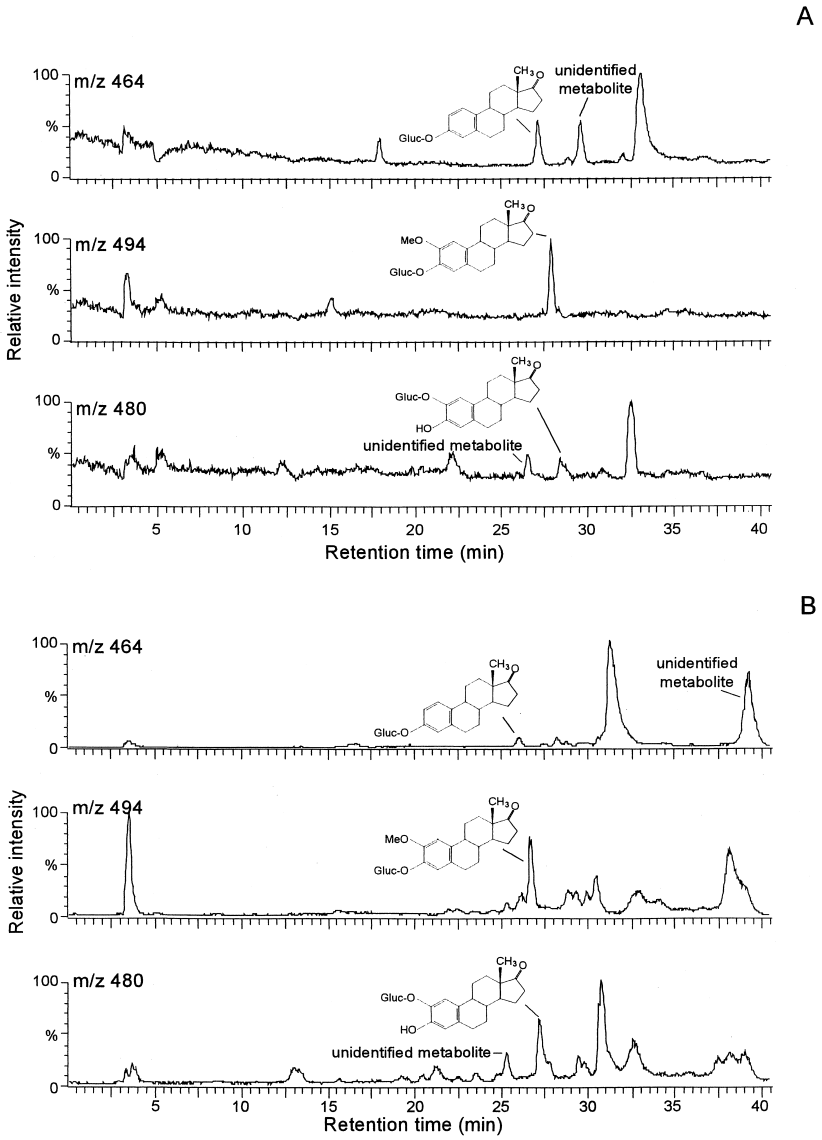

FIG. 7. HPLC separation (methanol–ammonium acetate) with electrospray MS detection of the biliary metabolites of (A) bisdesoxy-E_2 (41.6 μmol/kg, i.v.) and (B) 17desoxy-E_2 (41.6 μmol/kg, i.v.) in immature female rat (0–3 hr-collection). The SIM chromatograms represent $[M + NH_4]^+$ for ions with masses corresponding to estrone glucuronide (m/z 464), 2-methoxyestrone glucuronide (m/z 494), and 2-hydroxyestrone glucuronide (m/z 480). Biliary metabolites were defined as ion chromatographic peaks that were absent from pre-dose and vehicle control bile. Ion chromatographic peaks that are unlabelled are endogenous to bile.

production of two metabolites (R_t = 26.1 min and 39.4 min). Authentic estrone glucuronide co-eluted with one of them (R_t = 26.1 min). The estrone glucuronide in bile (2.03 ± 0.91 nmol, N = 4) equaled 0.11 ± 0.05% of the dose (Table 2). SIM for m/z 494 (2-methoxyestrone glucuronide) revealed the formation of one metabolite (R_t = 26.8 min). Two metabolites (R_t = 25.5 min and 27.4 min) were located at m/z 480 (2-hydroxyestrone glucuronide). LC–MS comparison with the known major metabolites of 17β-E_2 indicated that two of these metabolites (R_t = 26.8 and 27.4 min) were 2-methoxyestrone glucuronide and 2-hydroxyestrone glucuronide, respectively. No metabolites were observed in the remaining five channels.

Identification of Deconjugated Biliary Metabolites of [6,7-^{3}H]17β-E_2 Bisdesoxy-E_2, and 17Desoxy-E_2

Enzymic hydrolysis of conjugates in bile from rats given [6,7-^{3}H]17β-E_2 liberated three major metabolites corresponding chromatographically to 2-hydroxyestrone (R_t = 22.4 min), estrone (R_t = 27.8 min), and 2-methoxyestrone (R_t = 28.6 min). Hydrolysates of bile from rats given bisdesoxy-E_2 or 17desoxy-E_2 contained two metabolite peaks (R_t = 27.8 min and 28.6 min) which co-eluted with estrone and 2-methoxyestrone, respectively (data not shown). This indicated that the glucuronides identified in bile were not isomers of estrone or 2-methoxyestrone.

Hepatic Microsomal Incubations

Incubation of 17desoxy-E_2 with hepatic microsomes from immature female rats in the presence of NADPH yielded four metabolites with retention times of 9.1, 12.1, 16.8, and 18.7 min. 17Desoxy-E_2 had a retention time of 19.7 min. One minor metabolite (R_t = 9.1 min) co-eluted with 17β-E_2. Analysis on a diol column also resolved four metabolites with retention times of 7.8, 10.8, 20.2, and 26.3

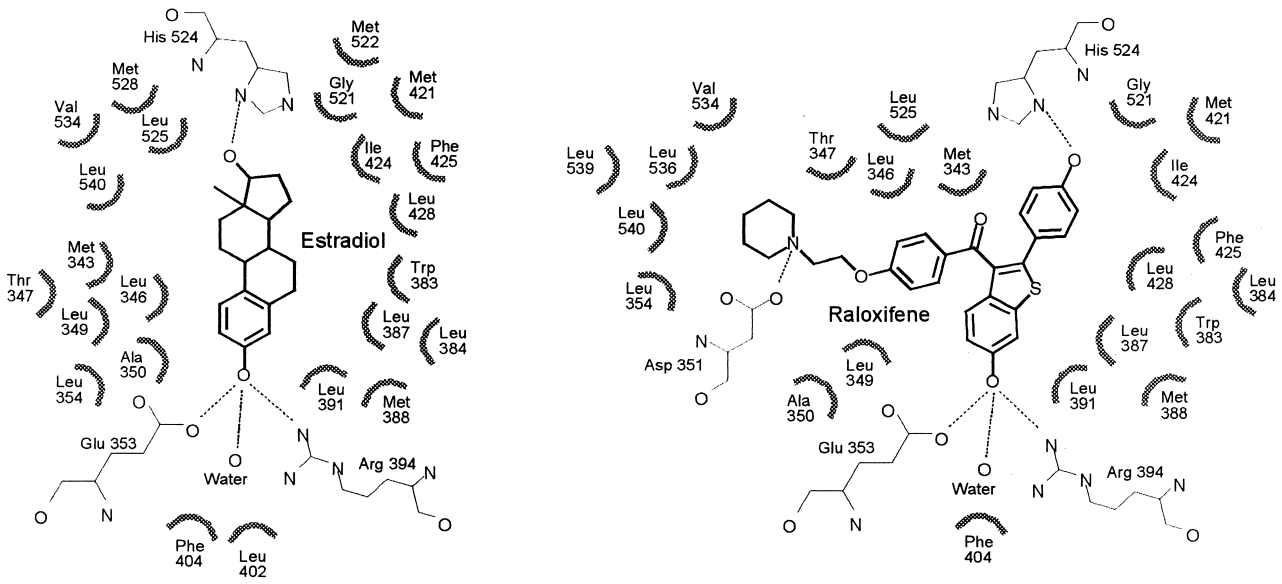

FIG. 8. Re-drawn from Fig. 2, c and d of Brzozowski *et al.* [12], indicating the interactions of estradiol and raloxifene with ER. Hydrogen-bonded interactions are shown as dotted lines.

min. 17Desoxy-E_2 eluted with retention time of 7.3 min. One of the metabolites (R_t = 26.3 min) also co-chromatographed with authentic 17β-E_2 (data not shown).

Incubation of bisdesoxy-E_2 with microsomes, in the presence of NADPH, resulted in the formation of two metabolites (R_t = 18.6 and 19.6 min). Bisdesoxy-E_2 had a retention time of 33.0 min. One of the metabolites (R_t = 19.6 min) co-eluted with authentic 17desoxy-E_2. Analysis on a diol column resolved one metabolite with a retention time of 7.4 min. Bisdesoxy-E_2 eluted with a retention time of 3.5 min. The metabolite co-eluted with authentic 17desoxy-E_2 (data not shown).

DISCUSSION

There are four basic ways to predict the estrogenic activity of a chemical in the absence of animal studies: determination of its potential to displace tritiated 17β-E_2 from ER (assumed ER binding of the agent), measurement of ER-mediated transcription induced by the agent in an appropriate *in vitro* assay, comparison of the two- or three-dimensional structure of the agent with that of 17β-E_2, and modelling of the agent's ability to interact with the LBD of ER. The activities predicted by these methods can be compared with estrogenicity obtained *in vivo* in the rodent uterotrophic assay. The present paper discusses the interrelationship of these several end points with a variety of synthetic chemicals.

The crystal structure of the LBD of ER α in complex with either 17β-E_2 or the partial ER agonist raloxifene was recently described by Brzozowski *et al.* (adapted in Fig. 8) [12]. Similar data have been presented by Pike *et al.* [13] for ER β in complex with 17β-E_2. These data revealed an unexpectedly large and plastic hydrophobic central cavity in ligated ER and the formation of specific hydrogen-bonded interactions with the 3- and 17β-hydroxyl groups of 17β-E_2. In the case of raloxifene, there is an additional H-bonding interaction between the ER and the piperidine substituent of raloxifene, demonstrating the plasticity of the cavity. Brzozowski *et al.* [12] noted that the size and apparent plasticity of the central hydrophobic cavity in ER might account for what they called its 'catholic/promiscuous' binding properties. The wide range of structures that can interact with ER is illustrated by the ER-binding and yeast transcription assay data for the potent estrogens 17β-E_2 and DES, the weak phenolic estrogen NP, and the very weak estrogens tosyl-NP and BBP. BBP and tosyl-NP lack hydroxyl groups and bear little obvious structural relationship to 17β-E_2. The inability of NB to bind to ER confirms the importance of the phenolic hydroxyl group in NP for its estrogenicity. However, although NB is inactive in the standard three-day protocol of the yeast assay, it does show weak activity when the assay is extended to six days. These six-day data emphasize that the definition of estrogenic activity is dependent, in part, upon the conditions of its assessment, an important point when most estrogenicity assays do not have fixed protocols.

The importance of the phenolic hydroxyl group for the estrogenicity of NP agrees with Brzozowski *et al.* [12], who suggested that the 'pincer-like' arrangement of specific hydrogen bonds around the A-ring hydroxyl of 17β-E_2 imposed an absolute requirement on ER ligands to contain a hydroxyl-substituted aromatic ring. However, these same specific hydrogen-bonding requirements isolate even further the estrogenic activity of non-hydroxylated estrogens such as BBP and tosyl-NP. In summary, the present data illustrate that molecules lacking a structural relationship to 17β-E_2 and devoid of phenolic hydroxyl groups can still

possess estrogenic activity, presumably due to an ability to fit into the large, plastic hydrophobic cavity of the receptor. This confirms that ER is relatively indiscriminate in its binding to chemicals, a fact that complicates the derivation of SAR for non-hydroxylated estrogens.

The role of the two hydroxyl groups and C/D-rings in 17β-E_2's estrogenic activities was investigated using 17desoxy-E_2 and bisdesoxy-E_2, and 6-hydroxytetralin, respectively. The two desoxy compounds were known to bind to human uterine ER [29, 30] and 6-hydroxytetralin to rat uterine ER [31]. 17Desoxy-E_2 possesses uterotrophic activity similar to that of 17α-E_2 when injected for seven days into hypophysectomized rats [32], and an early study mentioned unspecified estrogenic activity for both of the desoxy compounds in female rats [33]. 17α-E_2 has also been reported to be positive in the ovariectomized rat uterotrophic assay [34]. However, Clark *et al.* [34] suggested that this activity of 17α-E_2 may be due to its partial metabolism to 17β-E_2, and Hajek *et al.* [35] have shown that 17α-E_2 is only estrogenic in immature animals. Despite the several reports of estrogenic activity, no concomitant evaluation of these 3 partial derivatives of 17β-E_2 using current estrogenicity assays has been described.

The data presented here established that each of the compounds was bound by rat uterine ER. Bisdesoxy-E_2 revealed that the steroidal estrogen skeleton need neither possess a C-3- nor C-17-hydroxyl group for binding, indicating that it is hydrophobic interactions that are responsible for the binding of bisdesoxy-E_2 to ER. The importance of such hydrophobic interactions was demonstrated following removal of the C/D-rings. Although both NP and 6-hydroxytetralin contain a hydroxylated A-ring, NP's partial C/D-ring structure provides a larger surface area for hydrophobic interactions, perhaps accounting for its greater relative binding affinity for ER compared to that of 6-hydroxytetralin. A similar rank order of activities was seen for the chemicals in both the yeast transcription and mammalian cell transcription assays.

Crystallographic studies of the ER–17β-E_2 complex have shown that 17β-E_2 forms three hydrogen bonds with its receptor, two with the C-3-hydroxyl and one with the C-17β-hydroxyl group [12]. Using this model, it might be anticipated that 17desoxy-E_2 forms two hydrogen bonds with the receptor. From this, it might be predicted that the rank order of activity in both the ligand displacement and yeast transcription assays would correspond with the rank order of predicted hydrogen bond formation. The ascending rank order of activities, namely bisdesoxy-E_2 $\ll$ 17desoxy-E_2 $<$ 17β-E_2, conforms with the importance of the hydrogen bonds in binding 17β-E_2 to its receptor. Furthermore, in the yeast assay, the relative potencies of the three compounds are consistent with simple estimates of the contribution of a hydrogen bond to ligand affinity [24]. However, the intrinsic estrogenic activity of bisdesoxy-E_2, in particular, is inconsistent with the notion of an absolute requirement for hydrogen-bonding interactions with the receptor [12, 13].

Both 17desoxy-E_2 and bisdesoxy-E_2 were active in the immature rat uterotrophic assay when administered orally. In contrast, 6-hydroxytetralin was inactive, probably because of a combination of its weak estrogenic activity measured *in vitro* and its rapid clearance. Thus, at the empirical level, each of the three reduced versions of 17β-E_2 is weakly estrogenic *in vitro* and the two desoxy compounds are also relatively potent estrogens *in vivo* (Table 1). Evaluation of estrogenic activity *in vitro* could yield misleading data if there was metabolism of the two desoxy compounds to active estrogenic metabolites (including formation of 17β-E_2) *in vivo*. It therefore became important to establish whether metabolism of these partial derivatives of 17β-E_2 occurred in the ER-binding and yeast assays.

As might be expected, studies failed to detect any metabolism of the desoxy compounds when incubated with rat uterine ER at 4°. Likewise, in the yeast assay, no evidence was found for the formation of 17β-E_2 from either compound or of 17desoxy-E_2 from bisdesoxy-E_2. The amount of 17β-E_2 required to be formed from 17desoxy-E_2 and bisdesoxy-E_2 to account for their activities in the ER-binding and yeast assays is far greater than the detection limits of the analytical procedure. It is therefore concluded that the activities of the two desoxy compounds reflect activities of the parent compounds.

In contrast to the situation *in vitro*, both 17desoxy-E_2 and bisdesoxy-E_2 undergo metabolism *in vivo* to 17β-E_2. The *in vivo* metabolism studies matched the conditions of the uterotrophic assay in terms of dose, strain, sex, and age of rat. The differences were the route of administration and the time–course. However, it was necessary to use bile-cannulated animals and intravenous administration for the metabolic study [27, 28]. Microsomal metabolism of 17desoxy-E_2 and bisdesoxy-E_2 to 17β-E_2 and 17desoxy-E_2, respectively, conformed with transformation to 17β-E_2 *in vivo*. 17β-E_2 is only a minor metabolite of both 17desoxy-E_2 and bisdesoxy-E_2 in immature female rats. Other metabolites that are formed are probably isomers of 17β-E_2 and its metabolites, but it is not known whether these isomers are estrogenic.

It is clear that the uterotrophic activity of the two desoxy compounds could be accounted for, at least in part, by the intermediate formation of 17β-E_2: 17desoxy-E_2 and bisdesoxy-E_2 are intrinsically active in the absence of metabolism. The unexpected potency (increased a thousandfold) of bisdesoxy-E_2 is consistent with its superior rate of conversion to 17β-E_2, a conversion that is not uniquely achieved via 17desoxy-E_2 (Fig. 9). A separate pathway might involve 17β-hydroxylation to give the intermediate 3desoxy-E_2, which could undergo further hydroxylation to 17β-E_2.

The findings of the present study illustrate the complexity of deriving SAR for predicting estrogenicity, and indicate the need to integrate the following concepts into any developing SAR models. (1) The presence of hydroxyl substituents on a molecule is not a universal requirement for ER binding. (2) The possible role of metabolism of test

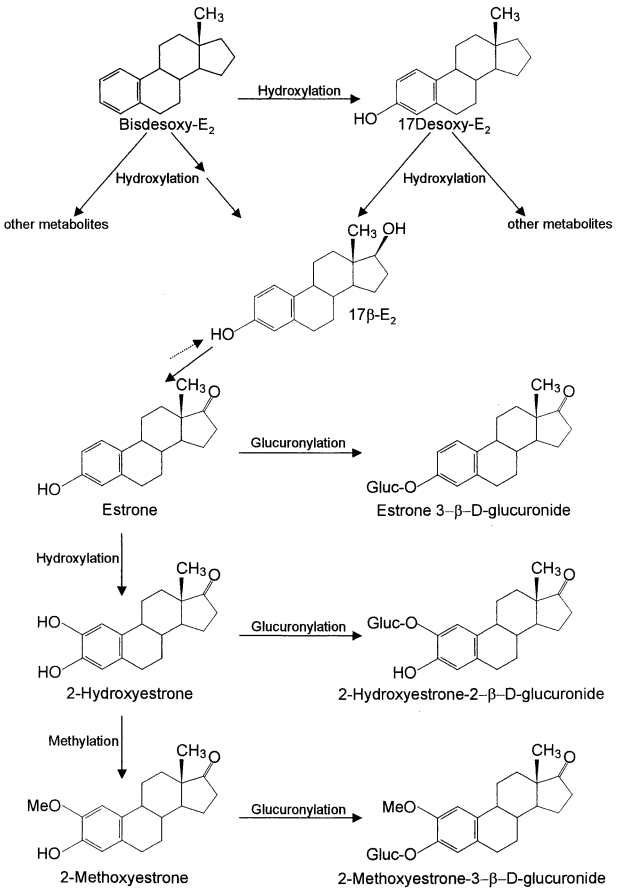

FIG. 9. A representation of the known metabolic pathway for 17β-E_2 [27, 28] in addition to the proposed mechanism of formation of 17β-E_2 from 17desoxy-E_2 and bisdesoxy-E_2.

chemicals during their bioassay for estrogenicity, and the precise assay protocol used, can influence the experimental outcome. (3) Some chemicals may be estrogenic *in vitro* for different reasons than they are estrogenic *in vivo* (e.g. bisdesoxy-E_2), and some may be so weakly estrogenic *in vitro* that the dose levels required for activity *in vivo* cannot be achieved (e.g., perhaps, 6-hydroxytetralin) (4) The estrogenic activity of some chemicals, e.g. BBP, tosyl-NP, and bisdesoxy-E_2, cannot currently be predicted with any confidence from their chemical structure.

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