

# Several environmental oestrogens are also anti-androgens

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## Abstract

There is presently considerable interest in endocrine disruption which is a new area of endocrinology concerned with chemicals that mimic hormones, in particular sex steroids. It has been hypothesised that exposure to such chemicals may be responsible for adverse effects in both humans and wildlife. Until now, chemicals that mimic oestrogens (so-called xenoestrogens) have been the main focus of endocrine disruption research. However, recent evidence suggests that many abnormalities in the male reproductive system may be mediated via the androgen receptor. By blocking androgen action, exposure to an anti-androgen may cause changes similar to those associated with oestrogen exposure.

We have used *in vitro* yeast-based assays to detect oestrogenic, anti-oestrogenic, androgenic and anti-androgenic activities in a variety of chemicals of current interest. We show that many of the so-called 'environ-

mental oestrogens' also possess anti-androgenic activity. The previously reported anti-androgenic activities of vinclozolin and p,p'-1,1-dichloro-2,2-bis(p-chlorophenyl) ethylene (DDE) were confirmed. We also found that o,p'-1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane (DDT), bisphenol A and butyl benzyl phthalate were anti-androgenic. However, not all xenoestrogens are also anti-androgenic, because nonylphenol was found to be a weak androgen agonist. Our results demonstrate that hormone-mimicking chemicals can have multiple hormonal activities, which may make it difficult to interpret their mechanisms of action *in vivo*.

Although not a specific objective of this study, our results also demonstrate that yeast-based assays are powerful tools with which to investigate both agonist and antagonistic hormonal activities of chemicals.

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## Introduction

Much of the current focus in the endocrine disruption field surrounds the oestrogenic action of various man-made chemicals. The ability of a chemical to bind to the oestrogen receptor, either *in vivo* or *in vitro*, has been used as a definition of oestrogenicity. Of concern is whether exposure to chemicals with steroid-like activity can disrupt normal endocrine function, leading to altered reproductive capacity, infertility, endometriosis, and cancers of the breast, uterus, and prostate (Colborn 1995, Jensen *et al.* 1995, Safe 1995).

Although most work has focused on the oestrogen receptor, recent evidence suggests that some abnormalities in male sexual development may be mediated at the level of the androgen receptor (Kelce & Wilson 1997). The presence of a potent anti-androgen can create an 'oestrogenic environment', thus producing symptoms indicative of oestrogen exposure.

Vertebrates synthesise steroids via a pathway that involves the sequential degradation of cholesterol to progesterins, then androgens (e.g. testosterone) and finally oestrogens (e.g. 17 $\beta$ -oestradiol). This pathway is found in both sexes, and circulating plasma concentrations of sex

steroids are representative of the relative conversion of androgens to oestrogens. Females have elevated plasma oestrogens compared with androgens, whereas males have the opposite ratio. It is the ratio of androgens to oestrogens that creates a male versus female hormonal milieu (Guillette *et al.* 1994). The presence of a potent androgen antagonist would create an overall oestrogenic effect (Kelce *et al.* 1995).

Because of the multiple biological effects of oestrogens and androgens and the influence of absorption, metabolism, distribution and excretion on the manifestation of their activity, any single assay can provide only limited information on these activities. For example, *in vitro* assays can provide valuable insights on the mechanisms of action, but are restricted in their capacity to mimic whole animal metabolism and distribution. In contrast, it can be difficult to infer the mechanism of action of a chemical from whole-animal studies, due partly to the complexity of the regulatory processes in an animal, and partly due to the possibility that a chemical has multiple hormonal activities. The growing interest in chemicals that mimic hormones will mean that many of these chemicals will be tested *in vivo* in the foreseeable future, in both short-term and multi-generation studies, with a variety of reproductive

parameters as the endpoints. Validated, reproducible *in vitro* assays can be used to investigate and define the hormonal activities of chemicals, and this information used to aid both design and interpretation of *in vivo* experiments. To this end, we have used two recombinant yeast screens, one for oestrogens, the other for androgens, to investigate the ability of some chemicals with reported hormone-mimicking properties to interact, both as agonists and antagonists, with the human oestrogen and androgen receptors.

## Materials and Methods

### The recombinant yeast screens

Both strains were developed in the Genetics Department at Glaxo Wellcome, plc. (Stevenage, Herts, UK).

**(1) Yeast expressing the human oestrogen receptor (hER)** Details of the oestrogen-inducible expression system have been described previously (Routledge & Sumpter 1996). In outline, the DNA sequence of the human oestrogen receptor was stably integrated into the yeast (*Saccharomyces cerevisiae*) genome. This yeast also contained expression plasmids carrying oestrogen-responsive elements regulating the expression of the reporter gene *lacZ* (encoding the enzyme  $\beta$ -galactosidase). Thus, when an active ligand (in this case oestradiol or an oestrogen-mimicking substance) bound to the receptor,  $\beta$ -galactosidase was synthesised and secreted into the medium, and as a result the chromogenic substrate chlorophenol red  $\beta$ -D-galactopyranoside (CPRG) underwent a colour change from yellow to red. The absorbance of the end product was measured at 540 nm; this changes from a value of about 1 (yellow: no activity) to about 2.8 (red: maximum activity). The use of this oestrogen-sensitive yeast to demonstrate anti-oestrogenic activity has also been reported (Routledge & Sumpter 1997).

**(2) Yeast expressing the human androgen receptor (hAR)** The main principles of the hAR screen were essentially the same as those of the hER screen described, unless stated otherwise.

The yeast strain PGK<sub>h</sub>AR contained a gene for the human androgen receptor (hAR), which was constitutively expressed (Purvis *et al.* 1991). In the presence of a ligand such as dihydrotestosterone (DHT), the androgen receptor bound to an androgen responsive element on a plasmid, thereby initiating transcription of the reporter gene *lacZ* (encoding the enzyme  $\beta$ -galactosidase). This produced a colour change from yellow to red, which was measured by absorbance at 540 nm.

For both recombinant yeast strains, ten times concentrated stock cultures were stored at  $-20^{\circ}\text{C}$  in 0.5 ml aliquots in Nalgene 1.2 ml sterile cryogenic vials (Merck

Ltd, Lutterworth, Leics, UK). Both hAR and hER yeast strains were stored with a shelf-life of four months, before being replaced with new  $-20^{\circ}\text{C}$  stocks.

**Preparation of medium components** All components except CPRG (Boehringer Mannheim, Lewes, East Sussex, UK) were purchased from Sigma Chemical Company Ltd (Poole, Dorset, UK), and were research grade biochemicals suitable for cell culture.

Minimal Medium (pH 7.1) was prepared by adding 13.61 g  $\text{KH}_2\text{PO}_4$ , 1.98 g  $(\text{NH}_4)_2\text{SO}_4$ , 4.2 g KOH pellets, 0.2 g  $\text{MgSO}_4$ , 1 ml  $\text{Fe}_2(\text{SO}_4)_3$  solution (40 mg/50 ml  $\text{H}_2\text{O}$ ), 50 mg L-leucine, L-histidine and adenine, 20 mg L-arginine-HCl and L-methionine, 30 mg L-tyrosine, L-isoleucine and L-lysine-HCl, 25 mg L-phenylalanine, 100 mg L-glutamic acid, 150 mg L-valine, and 375 mg L-serine to 1 l purite double-distilled water. Aliquots of 45 ml were dispensed into 150 ml bottles, sterilised at  $121^{\circ}\text{C}$  for 10 min, and stored at room temperature.

Vitamin solution was prepared by adding 8 mg thiamine, 8 mg pyridoxine, 8 mg pantothenic acid, 40 mg inositol, and 20 ml biotin solution (2 mg/100 ml  $\text{H}_2\text{O}$ ) to 180 ml double-distilled water. The solution was then filter-sterilised through 0.2  $\mu\text{m}$  pore size disposable filters (Whatman, Maidstone, Kent, UK), and 10 ml aliquots were stored at  $4^{\circ}\text{C}$  in sterile glass bottles.

A 20% w/v solution of D-(+) glucose was sterilised in 20 ml aliquots at  $121^{\circ}\text{C}$  for 10 min and stored at room temperature. Stock solutions of L-aspartic acid (4 mg/ml) and L-threonine (24 mg/ml) were sterilised in 20 and 5 ml aliquots respectively, at  $121^{\circ}\text{C}$  for 10 min. L-Aspartic acid stock solutions were stored at room temperature and L-threonine stock solutions were stored at  $4^{\circ}\text{C}$ . A 20 mM copper (II) sulphate solution was prepared and filter-sterilised through 0.2  $\mu\text{m}$  pore size disposable filters (Whatman). The solution was stored in 5 ml aliquots at room temperature in sterile glass bottles. A 10 mg/ml stock solution of CPRG was made in sterile distilled water and stored at  $4^{\circ}\text{C}$  in sterile glass bottles.

Growth medium was prepared by adding 5 ml glucose solution, 1.25 ml L-aspartic acid solution, 0.5 ml vitamin solution, 0.4 ml L-threonine solution, and 125  $\mu\text{l}$  copper (II) sulphate solution to 45 ml single-strength Minimal Medium in a sterile conical flask. The growth medium was then inoculated with 125  $\mu\text{l}$  ten times concentrated yeast stock, and incubated at  $28^{\circ}\text{C}$  for approximately 24 h on an orbital shaker (250 r.p.m. with a 50 mm throw).

The final assay medium was prepared by adding 0.5 ml chromogenic substrate (CPRG) to 50 ml fresh growth medium. This medium contained  $4 \times 10^7$  cells/50 ml.

### Compounds tested

Steroid hormones, used to determine the specificity and sensitivity of both screens, were purchased from Sigma, except the hydrocortisone and progesterone, which were

purchased from BDH (Poole, Dorset, UK) and Halewood Chemicals Ltd (Staines, Middlesex, UK) respectively. All were >99% pure.

Bisphenol A (99% pure), butyl benzyl phthalate (BBP; 98% pure), flutamide (99% pure), hydroxytamoxifen (98% pure) and diethylstilboestrol (DES; 99% pure) were purchased from Sigma. Pesticides *o,p'*-1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane (*o,p'*DDT) (99% pure), *p,p'*-1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene (*p,p'*DDE) (99% pure) and vinclozolin (99.8% pure) were purchased from Greyhound Chem Service (Birkenhead, Merseyside, UK). 4-Nonylphenol (4-NP; 95% pure) was supplied by Dr John Ashby (Zeneca, UK) who obtained it from Fluka (Gillingham, Dorset, UK).

#### Assay procedure

To minimise aerosol formation, yeast assays were carried out within a type II laminar flow cabinet. Stock solutions of chemicals were serially diluted in ethanol, in a 96-well optically flat-bottomed microtitre plate (Linbro/Titertek, ICN FLOW, Basingstoke, Hants, UK). Aliquots of 10  $\mu$ l were then transferred to two identical sterile plates and the ethanol allowed to evaporate to dryness. It is unknown whether all the chemicals redissolved completely when the assay medium was added. To address this potential problem, we also conducted some assays in which the medium was added first to the wells, followed by the chemical in ethanol; thus, the chemical presumably remained in solution throughout. When the two methods were compared, using several chemicals, no significant differences in the potencies of the chemicals were observed, suggesting that allowing the ethanol to evaporate before adding the medium does not produce artifactual results due to the relative insolubility of some chemicals. In each experiment, the same solutions were tested in both the hER and hAR screens so that results could be reliably compared. 17 $\beta$ -Oestradiol and DHT were used as positive controls in the hER and hAR screens respectively, and a negative control (ethanol) was included in all experiments, in order to eliminate the possibility that the results obtained were due to direct conversion of the CPRG by the medium components alone. Aliquots of 200  $\mu$ l of the assay medium (containing recombinant yeast and CPRG) were added to the appropriate plates (hER and hAR). The plates were sealed with autoclave tape and shaken vigorously for 2 min on a titre plate shaker before incubation at 32 °C.

The hAR plates were removed after 24 h and incubated overnight at 28 °C, in order to optimise the reaction without risking a significant increase in background expression of  $\beta$ -galactosidase. The hER plates were removed after 72 h. This was done because the colour change (yellow to red) occurs faster in the hAR screen than in the hER screen.

Colour development of the medium was checked at regular intervals at an absorbance of 540 nm, and also at

620 nm, to allow for subsequent correction for turbidity (a measure of growth rate of the yeast), using a Titertek Multiskan MCC/340 plate reader, (corrected value = chemical absorbance at 540 nm – (chemical absorbance at 620 nm – blank absorbance at 620 nm)). High concentrations of some chemicals can inhibit growth of the yeast, or even lyse the cells (for example, see Routledge & Sumpter 1996). However, in the assays reported in this paper, no concentrations of any of the test chemicals significantly reduced the growth of the yeast; thus, none of the results can be accounted for by alterations in the growth rate of the yeast in the presence of chemicals. Following incubation, a deep red colour denotes positive activity and, due to a slight background production of  $\beta$ -galactosidase, the negative control wells appear light orange in colour. Growth of yeast is accompanied by a turbid appearance. Chemicals which are toxic to the yeast usually lead to clear yellow wells, with no growth and lysis of the cells.

The agonistic abilities of each chemical were assessed first, and the concentration range producing a full dose–response curve (if any was obtained) was then used in the assays conducted to assess antagonistic activities. This use of the same concentration in both the agonist and antagonist assays allowed us to determine, for each chemical, which activity predominated (if a chemical had more than one activity).

#### Anti-androgen and anti-oestrogen assay procedure

To determine whether any of the chemicals possessed anti-oestrogenic and/or anti-androgenic activities, the natural ligand (17 $\beta$ -oestradiol or DHT) was added to the medium of the appropriate assay at a concentration that produced a sub-maximal response (65%); the ability of chemicals to inhibit the colour change induced by the natural ligand was then determined. As the two screens vary slightly in their sensitivities (the oestrogen screen is about ten times more sensitive than the androgen screen), the chosen background concentrations of 17 $\beta$ -oestradiol and DHT also varied; they were  $2.5 \times 10^{-10}$  M 17 $\beta$ -oestradiol and  $1.25 \times 10^{-9}$  M DHT respectively.

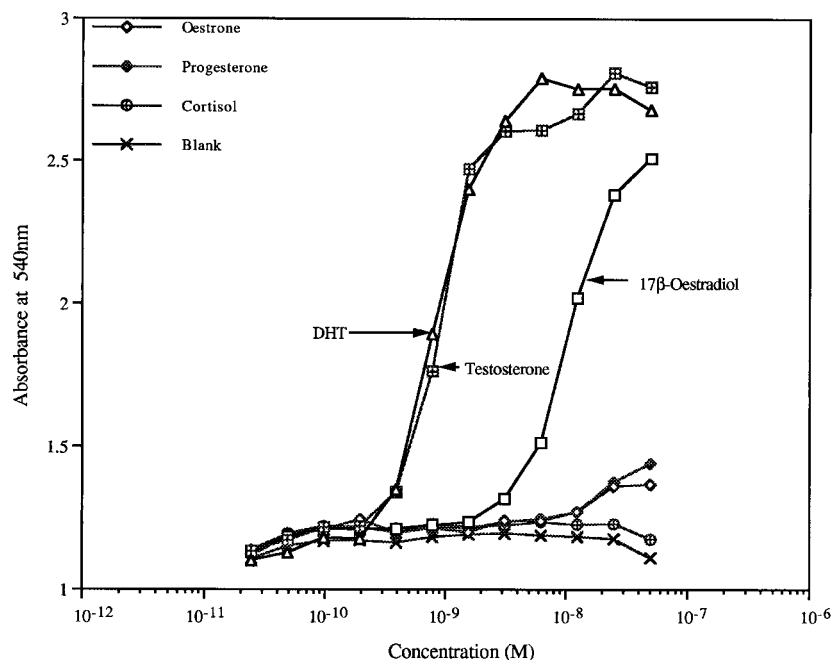
## Results

The potencies expressed here are in molar terms, and are based on ED<sub>50</sub> values.

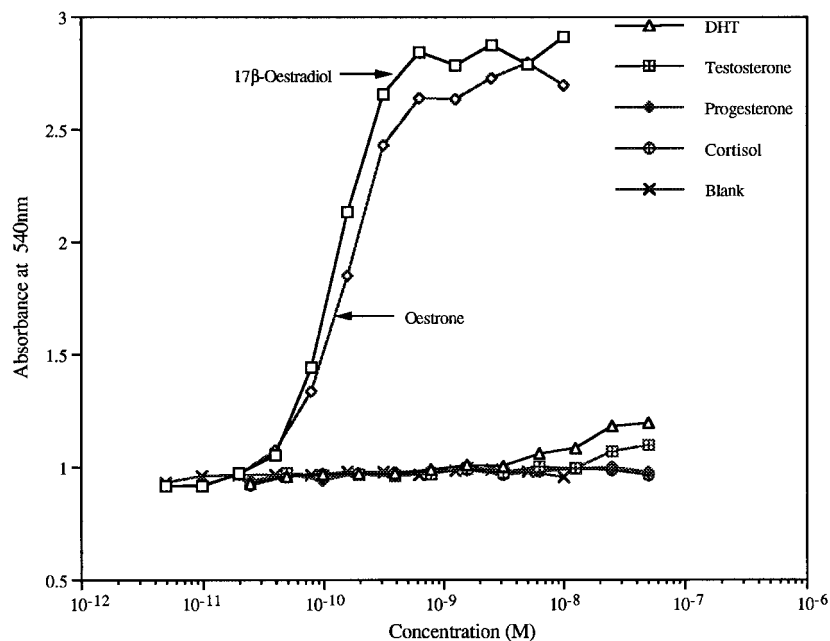
#### Specificity of the hAR and hER recombinant yeast screens

The specificities of both screens were determined by investigating the ability of a range of steroids to stimulate synthesis of  $\beta$ -galactosidase in the yeast.

Figure 1 demonstrates the specificity of the androgen screen. DHT and testosterone had similar activity. 17 $\beta$ -Oestradiol was able to stimulate  $\beta$ -galactosidase synthesis



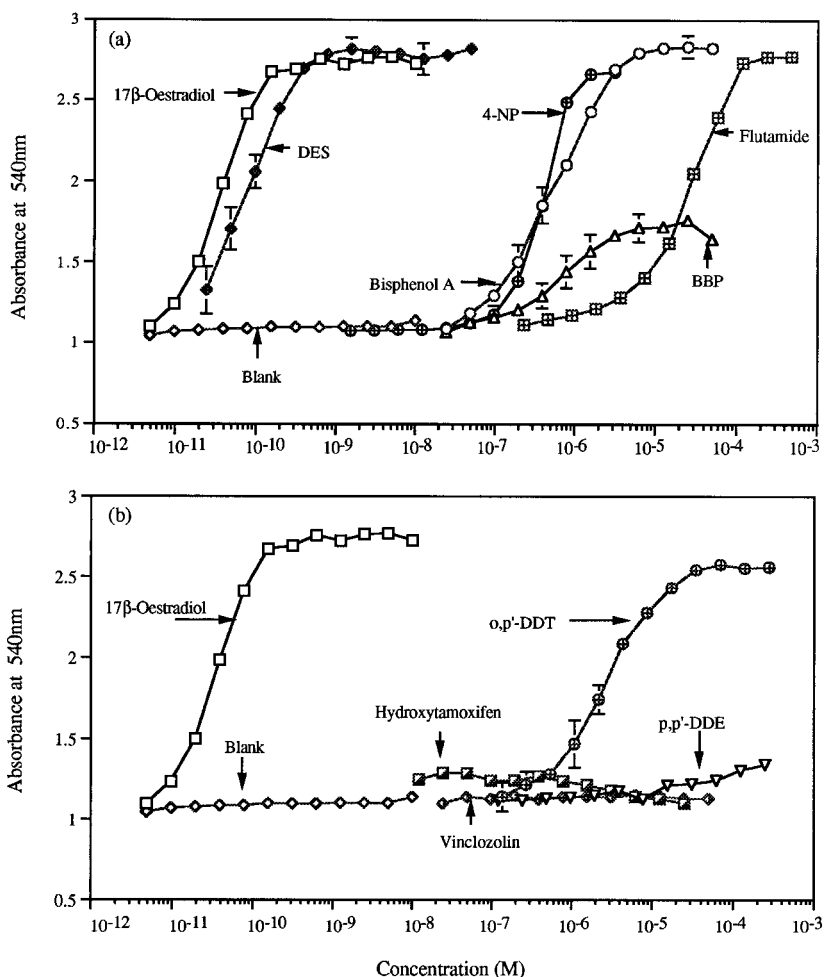
**Figure 1** The specificity of the hAR yeast screen. The steroids tested were serially diluted in two-fold steps from  $5 \times 10^{-8}$  M. The results are representative of four experiments.



**Figure 2** The specificity of the hER yeast screen. The steroids tested were serially diluted in two-fold steps from  $5 \times 10^{-8}$  M. The results are representative of four experiments.

and was found to be approximately 30-fold less potent than DHT and testosterone. Progesterone and oestrone showed very slight activity at concentrations above  $10^{-8}$  M, but not at lower concentrations. Cortisol was inactive in the hAR screen at the concentrations tested.

The data presented in Fig. 2 show that, in the oestrogen screen,  $17\beta$ -oestradiol was approximately 50-fold more potent than oestrone. DHT and testosterone showed some minimal activity, but were many orders of magnitude less potent than the oestrogens tested.



**Figure 3** Activity of xenobiotics in the hER yeast screen, shown in two panels (a and b) for clarity. Preliminary experiments determined the approximate oestrogenic activity of each chemical. Based on the information provided, different concentrations were serially diluted in order to obtain, wherever possible, full dose–response curves. Blank values are the means of wells containing solvent (ethanol) only. Error bars represent S.E.M., to provide an illustration of the reproducibility of the data.

Cortisol and progesterone were inactive in the hER screen.

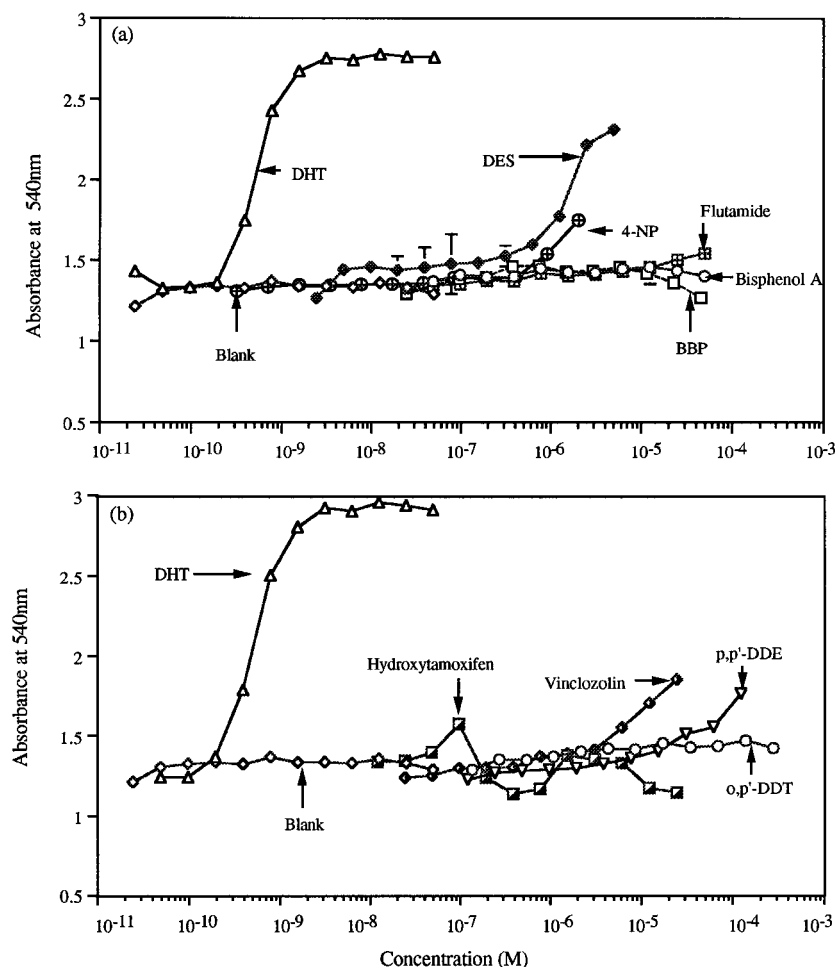
#### Response of the hER screen to xenobiotics

The responses of the hER assay to all the test chemicals are shown in Fig. 3a,b. DES was the most potent synthetic chemical tested, and was found to be five times less potent than 17β-oestradiol. Bisphenol A and 4-nonylphenol were found to be approximately equipotent, and about 10 000 times less potent than 17β-oestradiol. BBP and o,p'-DDT were both approximately 100 000 times less potent than 17β-oestradiol, although BBP produced a shallow dose–response curve, making an accurate estimate of its potency very difficult. Flutamide was found to be a very weak

agonist, although it produced a full dose–response curve, and was approximately 100 000 to 1 000 000 times less potent than 17β-oestradiol. Hydroxytamoxifen showed some minimal activity, but this was not obviously dose-dependent. Vinclozolin showed no activity in the hER screen.

#### Response of the hAR screen to xenobiotics

The responses of the hAR assay to all chemicals tested are shown in Fig. 4a,b. DES was found to be weakly androgenic, being approximately 80 000 times less potent than DHT. Vinclozolin, p,p'-DDE and 4-NP also showed some activity, albeit they were even weaker agonists than DES. Flutamide and o,p'-DDT were essentially inactive at



**Figure 4** Activity of xenobiotics in the hAR yeast screen, shown in two panels (a and b) for clarity. Preliminary experiments determined the approximate androgenic activity of each chemical. Based on the information provided, different concentrations were serially diluted in order to obtain, wherever possible, full dose-response curves. Blank values are the means of wells containing solvent (ethanol) only. Error bars represent S.E.M., to provide an illustration of the reproducibility of the data.

the concentrations tested. Hydroxytamoxifen showed a similar response to that which it displayed in the hER screen, with no obvious dose-response but some inconsistent weak activity. Bisphenol A and BBP showed no activity in the hAR screen.

#### Response of chemicals in the anti-oestrogen screen

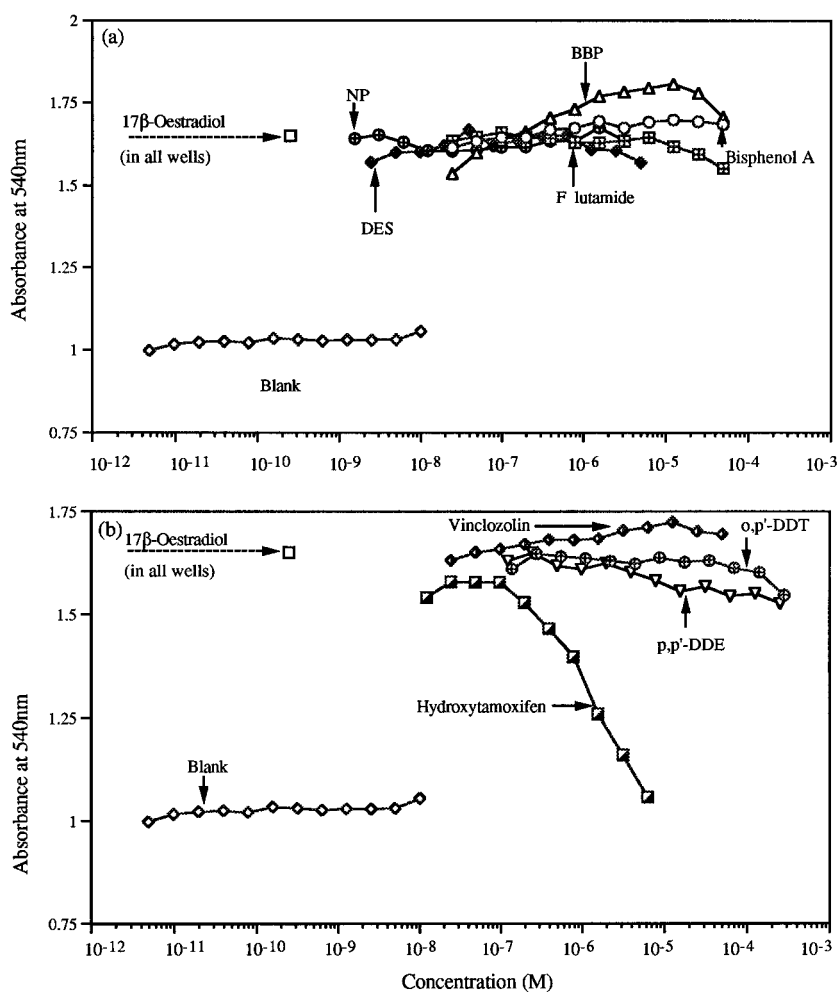
Of all the chemicals tested, the results of which are shown in Fig. 5a,b, the only chemical to inhibit the activity of 17 $\beta$ -oestradiol in this assay was hydroxytamoxifen, which produced a full dose-response curve, with the highest concentrations tested completely inhibiting the activity of the 17 $\beta$ -oestradiol. p,p'-DDE showed evidence of slight

competition, but nothing as pronounced as that displayed by hydroxytamoxifen.

#### Response of chemicals in the anti-androgen screen

Figure 6a,b displays data showing the abilities of the test chemicals to inhibit the activity of DHT. The clinical anti-androgen, flutamide, was successful in inhibiting the activity of DHT in a dose-dependent manner. Bisphenol A, BBP, o,p'-DDT and hydroxytamoxifen also all successfully inhibited the action of DHT; in all cases a full dose-response curve was obtained, and the chemicals were approximately as potent as the anti-androgen, flutamide. Vinclozolin, p,p'-DDE and DES all displayed





**Figure 5** Anti-oestrogenic activity of the test chemicals, shown in two panels (a and b) for clarity. With the exception of the blank (solvent only),  $17\beta$ -oestradiol was present throughout at a single concentration ( $2.5 \times 10^{-10}$  M); this produced a sub-maximal response (oestradiol alone). Serial dilutions of the test chemicals were then assessed for their ability to inhibit their response to  $17\beta$ -oestradiol.

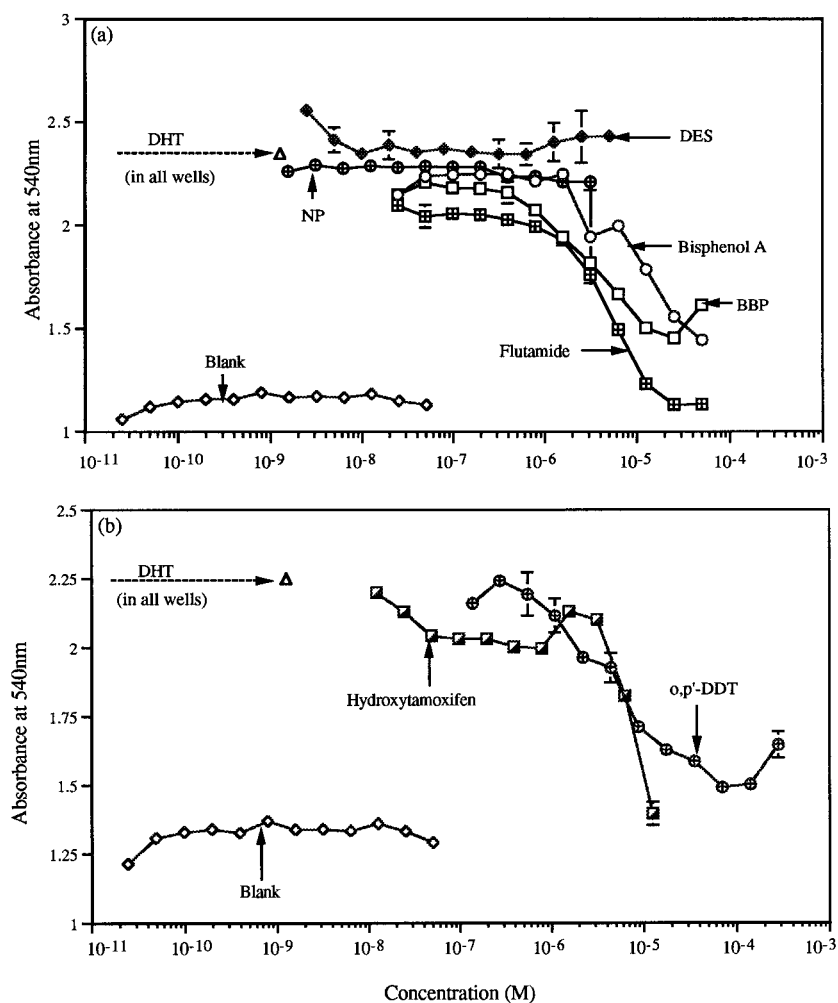
anti-androgenic activity early on in every assay in which they were tested (up to and including day 4), but as the assay continued to develop, the DHT overcame the anti-androgenic activity of these chemicals. This phenomenon, in which incubation time influences the degree of anti-androgenicity observed, is illustrated in Fig. 7. It is best seen in Fig. 7a, where p,p'-DDE appears initially to be a good anti-androgen; concentrations between  $1 \times 10^{-4}$  M and  $1 \times 10^{-6}$  M produced a full dose-related inhibition of the androgenic activity of DHT. However, one day later (when the response to DHT was more developed), the inhibitory activity of p,p'-DDE was much less obvious, and after another day (day 6) it was largely lost. Similar results were obtained with vinclozolin. However, in this case, a U-shaped dose-response

developed with time, in which low concentrations showed anti-androgenic activity but higher concentrations did not (Fig. 7b). This unusually shaped dose-response curve was not observed with any other test chemical, but was not obtained consistently with vinclozolin (in four assays).

The results demonstrate that many of the chemicals investigated had multiple mechanisms of action. The relative potencies of the chemicals in the different assays have been semi-quantitatively summarised in Table 1.

## Discussion

Exposure to substances which can mimic or disrupt the normal functioning of reproductive hormones has been

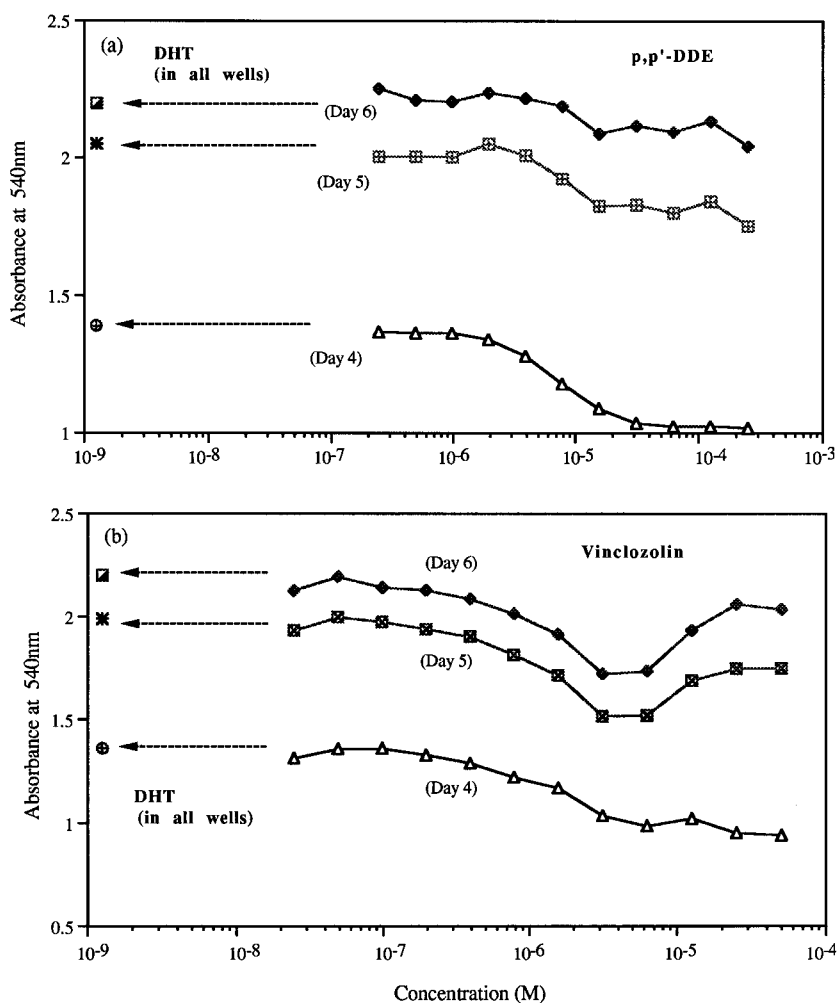


**Figure 6** Anti-androgenic activity of the test chemicals, shown in two panels (a and b) for clarity. With the exception of the blank (solvent only), DHT was present throughout at a single concentration ( $1.25 \times 10^{-9}$  M); this produced a sub-maximal response (DHT alone). Serial dilutions of the test chemicals were then assessed for their ability to inhibit their response to DHT. The data for p,p'-DDE and vinclozolin are not shown, because they are presented in Fig. 7. Error bars represent S.E.M.

linked to effects such as a reduction in fertility, reduced hatch rate in fish and bird eggs, as well as alterations in hormone levels, and disrupted adult mating behaviour, all of which could have serious implications for wildlife and human population dynamics (Fry & Toone 1981, Rattner *et al.* 1984, Carlsen *et al.* 1992, Purdom *et al.* 1994, Guillette 1995). Furthermore, numerous morphological effects have been noted in the offspring of rats exposed to sex hormone disrupting substances, including deformities of the reproductive tract, such as undescended testicles and hypospadias (Kelce *et al.* 1994), alterations in bone mass (Migliaccio *et al.* 1995), as well as the other effects which are not so immediately apparent, such as reduced sperm counts in males (Sharpe & Skakkebaek 1993).

Oestrogen and androgen generally act by binding at receptor sites, and synthetic chemicals may disrupt this action in a number of ways. For example, a chemical may mimic the action of the natural ligand by being able to act on this receptor site, or it may interfere with the receptor in some other way, and be able to block the action of the hormone (Iguchi 1992). Some chemicals can also perturb the normal functioning of the sex hormones by inhibiting the enzymes responsible for steroid hormone biosynthesis and/or inducing enzymes responsible for steroid metabolism (Majdic *et al.* 1996). Furthermore, it has also been suggested that some substances, such as lindane, might influence the metabolic breakdown pathway of the natural or endogenous oestrogen, such that more potent products





**Figure 7** Change in the anti-androgen screen with time. DHT was present throughout at a single concentration ( $1.25 \times 10^{-9}$  M). Serial dilutions of p,p'-DDE (a) and vinclozolin (b) were then assessed for their ability to inhibit the response to DHT. The absorbance of the same microtitre plate was read on days 4, 5 and 6, during which time the response to DHT progressively developed. Wells containing solvent only were included but the data have been omitted for clarity.

are formed (Bradlow *et al.* 1995). In addition, it seems that oestrogen action can be stimulated by other signalling mechanisms, such as via growth factors. This means that there may be numerous possible mechanisms of action for sex hormone disruption (McLachlan & Korach 1995).

One chemical can have a number of different mechanisms of action; for example o,p'-DDT, which was shown originally to be a weak oestrogen (Bitman *et al.* 1968), also possesses anti-androgenic activity (Kelce *et al.* 1995), and a number of alkyphenolic chemicals shown initially to be weakly oestrogenic (White *et al.* 1994), have also recently been shown to be anti-progestagenic (Tran *et al.* 1996b). Until very recently, most of the interest in xenobiotics acting as endocrine disrupters was concen-

trated on their oestrogenic activities (see, for example, Jobling *et al.* 1995), although the anti-androgenic properties of some xenobiotics have also been reported (Gray *et al.* 1994, Kelce *et al.* 1995). We have used two recombinant yeast strains, one containing a gene for the human oestrogen receptor and the other a gene for the human androgen receptor, to investigate whether many of the endocrine disrupting chemicals of current intense interest possess more than one type of endocrine activity.

We found the specificities of the yeast assays to be as expected. As already demonstrated (Routledge & Sumpter 1996, 1997), the hER yeast assay used here shows a high degree of specificity for oestrogens. In contrast, the hAR yeast assay was not as specific; oestrogens (but not

**Table 1** Semi-quantitative assessment of the agonist and antagonist activities of the test chemicals in the oestrogen receptor and androgen receptor assays. The most potent chemical in each screen has been assigned a potency of four plus signs (++++), and the potency of all the chemicals expressed relative to this

Chemical	Oestrogenic activity	Anti-oestrogenic activity	Androgenic activity	Anti-androgenic activity
17 $\beta$ -Oestradiol	++++	—	+++	—
DHT	+	—	++++	—
Hydroxytamoxifen	—	++++	+	+++
Flutamide	+	—	—	++++
Bisphenol A	++	—	—	++++
BBP	+	—	—	++++
Nonylphenol	++	—	+	—
Vinclozolin	—	—	+	+++
o,p'-DDT	++	+	—	+++
p,p'-DDE	+	+	+	++
DES	++++	—	++	—

progesterone or cortisol) showed considerable activity. This relative lack of specificity of the hAR was demonstrated some years ago (Wilson & French 1976) and confirmed recently (Gaido *et al.* 1997). The authors of the latter paper showed 17 $\beta$ -oestradiol to be about 1/30<sup>th</sup> the potency of DHT, just as we have found.

Using the hER yeast assays, we found the agonistic activities of our test xenobiotics to be as expected, and similar to the results of others using yeast assays (Routledge & Sumpter 1996, Gaido *et al.* 1997, Harris *et al.* 1997), and also to results obtained using other oestrogen-responsive assays, such as receptor-binding and cell proliferation assays (Kelce *et al.* 1995, Soto *et al.* 1995, Majdic *et al.* 1996, Shelby *et al.* 1996, Coldham *et al.* 1997).

Much less data is available on the (possible) androgenic activity of xenobiotics. Those which are available (Gaido *et al.* 1997) suggest that fewer chemicals possess androgenic activity than possess oestrogenic activity; our results support this conclusion. Intuitively this seems surprising, in view of the fact that the androgen receptor is less specific than the oestrogen receptor (at least to endogenous steroid hormones). We found that 4-NP, reported to date only as weakly oestrogenic (Soto *et al.* 1991, White *et al.* 1994), also had weak androgenic activity. The finding that p,p'-DDE is a weak androgen agonist has already been reported (Gaido *et al.* 1997) and was not particularly surprising, given that Kelce *et al.* (1995) have reported that it binds to the androgen receptor. Vinclozolin also had weak androgenic activity *in vitro*, although its overall effect *in vivo* is anti-androgenic (Gray *et al.* 1994, see also comments below).

None of the xenobiotics we tested possessed any anti-oestrogenic activity. As far as we are aware, there are no reports concerned with (possible) anti-oestrogenic activity of these chemicals. However, one other group of environmentally-persistent man-made chemicals, the

polyaromatic hydrocarbons, does contain some members which possess anti-oestrogenic activity (Tran *et al.* 1996a).

Many, but not all, of the xenobiotic chemicals possessed anti-androgenic activity. For example, bisphenol A and butyl benzyl phthalate, both so-called 'environmental oestrogens' or 'xenoestrogens' (Jobling *et al.* 1995), also possess anti-androgenic activity. It is thus worth considering whether some, or even all, of the reported *in vivo* endocrine effects of these chemicals (Piersma *et al.* 1995, Sharpe *et al.* 1995, Nagel *et al.* 1997, Steinmetz *et al.* 1997) are due not to their oestrogenicity, but rather to their abilities to block the action of androgen at the androgen receptor. For example, both BBP and bisphenol A demonstrated anti-androgenic activity over the concentration range (approximately  $1 \times 10^{-5}$  M to  $1 \times 10^{-7}$  M) in which they also showed oestrogenic activity. These chemicals were as potent anti-androgens as flutamide, which although a relatively weak anti-androgen (the clinical dose is around 500 mg per day compared with 25  $\mu$ g for ethinyl oestradiol when used as a contraceptive) is, nevertheless, widely used clinically to inhibit androgen. However, not all xenoestrogens also have anti-androgenic activity; 4-NP did not, nor did the synthetic oestrogen DES, or the natural oestrogen 17 $\beta$ -oestradiol. Even when tested at very high concentrations (up to  $1 \times 10^{-4}$  M), comparable to the highest concentrations of the xenobiotics tested, 17 $\beta$ -oestradiol lacked any anti-androgenic activity (data not shown). These results are perhaps best explained by suggesting that many xenoestrogens possess some structural feature (or features) that also gives them anti-androgenic activity.

Our results with p,p'-DDE and o,p'-DDT were similar, but not identical, to the results reported by Kelce *et al.* (1995). We also found both p,p'-DDE and o,p'-DDT to possess anti-androgenic activity, but whereas Kelce *et al.* found p,p'-DDE to be somewhat more potent than

o,p'-DDT, we found them approximately equipotent, although it should be emphasised that obtaining precise potency estimates from our data is unwise, especially when potency varies with time in at least some cases (see Fig. 7). In transient transfection assays (which are probably not too dissimilar to yeast assays), Kelce *et al.* (1995) found p,p'-DDE and hydroxyflutamide to be equally effective inhibitors of transcriptional activity. We also found p,p'-DDE to be essentially equipotent to flutamide (which is almost certainly hydroxylated by the yeast, because it is hydroxyflutamide which is the more potent anti-androgen). We also found bisphenol A and BBP to be as potent anti-androgens as flutamide.

The results obtained with vinclozolin support earlier reports (Gray *et al.* 1994) of the anti-androgenic activity of this chemical. The work of Gray and colleagues has shown that it is the main metabolites of vinclozolin, rather than vinclozolin itself, which are the active anti-androgens (Kelce *et al.* 1994). Therefore, our results probably indicate that the yeast we used can metabolise vinclozolin into its anti-androgenic metabolites. The unusual U-shaped dose-response curve obtained with vinclozolin in the anti-androgen screen (which was repeated a number of times) is difficult to interpret presently, but cautions against using *in vitro* assays to label chemicals as possessing (or not possessing) specific types of activity, when different concentrations can obviously show different activities. This effect might also depend upon the concentration of the agonist (in this case DHT) with which the test chemical is competing.

The length of the incubation time, prior to reading the plates, can also affect the results. Chemicals which demonstrated clear anti-androgenic activity initially (such as p,p'-DDE and vinclozolin) appear not to be anti-androgenic if the incubation time is extended. Anti-androgenic (and anti-oestrogenic) activity is more obvious when the response to the agonist (DHT or 17 $\beta$ -oestradiol) is sub-maximal; under the assay conditions used here, this was an absorbance of between 1.5 and 2.0. If the plate is incubated for longer, the response to the agonist continues to increase until it reaches the maximum (about 2.8), at which time it swamps the anti-androgenic activity of any test chemical which is also present. The yeast assays used here, in which the  $\beta$ -galactosidase is secreted into the medium and causes a colour change, allow continuous monitoring of the experiments, whereas in the other yeast assays developed to date (for example Arnold *et al.* 1996, Gaido *et al.* 1997) the  $\beta$ -galactosidase is not secreted and hence the cells must be lysed to free the enzyme before it is measured; thus, time courses are much more difficult to conduct.

Our results create an enigma in this area of research; namely, why there are so few androgen mimics, yet such an apparent abundance of oestrogen mimics, and why there are so few chemicals that block the oestrogen receptor effectively, yet so many chemicals that appear to act in an antagonistic manner with the androgen receptor.

In summary, the main findings of this work are that some of the so-called 'environmental oestrogens', such as bisphenol A and BBP, which up to now have been reported to possess oestrogenic activity only, also possess anti-androgenic activity. This might suggest that some structural feature of these chemicals (and also of o,p'-DDT and p,p'-DDE) provides them with both activities. All of the results presented here were derived from *in vitro* assays, and should ideally be confirmed (or refuted) in *in vivo* assays. However, distinguishing oestrogenic from anti-androgenic effects *in vivo* may not be easy. Until recently, the uterotrophic assay (Ashby *et al.* 1997) would have been used to identify oestrogenic activity, and alterations in male sex differentiation (reviewed by Kelce & Wilson 1997) used to identify anti-androgens. However, the finding of a second oestrogen receptor (Kuiper *et al.* 1996), which is very prominently expressed in the male reproductive tract (and the ovary of females) cautions against assuming that the uterus is sensitive only to oestrogens (and anti-oestrogens), and the prostate (and other components of the male reproductive system) is sensitive only to androgens and anti-androgens. Nevertheless, multi-generation studies incorporating a range of endocrine endpoints should help in elucidating the major mechanisms of actions of those chemicals which possess two (or more) types of activity.

Although the research reported here was aimed primarily at investigating whether xenobiotics of present interest have narrow or wide profiles of endocrine activity (the latter appears most common), it also demonstrates the usefulness of yeast-based assays. There is presently considerable debate as to the most appropriate way to screen xenobiotic chemicals for endocrine activity. The yeast-based assays used in this study are specific, extremely sensitive, reliable, robust and fairly cheap to do. Our results also show that yeast-based assays can be used to screen for antagonistic as well as agonistic activities.

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