RE-EVALUATION OF THE FIRST SYNTHETIC ESTROGEN, 1-KETO-1,2,3,4-TETRAHYDROPHENANTHRENE, AND BISPHENOL A, USING BOTH THE OVARIECTOMISED RAT MODEL USED IN 1933 AND ADDITIONAL ASSAYS

by

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Summary

1-Keto-1,2,3,4-tetrahydrophenanthrene (THP-1) was reported by Cook et al in 1933 as the first synthetic estrogen. Estrogenic activity was assessed by the induction of vaginal cornification in ovariectomised rats. The corresponding 4-isomer (THP-4) was shown to be inactive. Both chemicals have been re-synthesised and assessed for hormonal activity. Each chemical bound weakly and to the same extent to isolated estrogen receptors, but only at high concentrations. However, they each lacked estrogenic or anti-estrogenic activity when evaluated *in vitro* using a yeast hER assay, and both failed to induce vaginal cornification or uterotrophic effects in ovariectomised rats. THP-1, and to a lesser extent THP-4, were shown to possess weak androgenic and anti-androgenic activity *in vitro* when evaluated using an hAR yeast assay. Estrogenic activity for bisphenol A (BPA) was subsequently demonstrated by Dodds and Lawson (1936) using the same ovariectomised rat protocol, and this activity has been confirmed and supplemented by positive uterotrophic effects for BPA in the same bioassays.

The present results illustrate the complexity of deriving conclusions regarding the hormonal activities of chemicals. First, some activities observed in isolated hormonal receptor binding assays may not be expressed in functional hormonal assays. This indicates the need for functional hormonal assays in any screening programme. Second, that activities observed for a chemical in one hormonal assay may not be reflected in related hormonal assays. This indicates the need to define assay protocols with some precision when incorporating them into screening batteries. Finally, that some literature reports of hormonal activity for chemicals may not be capable of independent confirmation under apparently identical conditions of test. This illustrates the need to use lists of hormonally active chemicals with care

Introduction

We noted recently (Ashby 1998) the prescient ideas associated by Cook et al (1933) with their demonstration of the first synthetic estrogen. Cook et al had evaluated the two tetrahydrophenanthrene compounds THP-1 and THP-4 (for structures see Figure 1) as possible active analogues of estrone, the main natural estrogen known at that time. In their studies ovariectomised rats were exposed to the agents as a single subcutaneous (s.c.) injection, and estrus-inducing activity was determined by assessment of vaginal cytology 54h after dosing. THP-1 induced vaginal cornification in 50% of the animals at a total dose of 50 mg/rat, and in 100% of the animals at a total dose of 100 mg/rat. THP-4 was without activity at the only dose level tested (total dose of 50 mg/rat). Three years later, Dodds and Lawson (1936) demonstrated similar effects on vaginal cytology for bisphenol A (BPA), but by then their test protocol had been enlarged to involve twice daily injections of the test agent for three days (total dose of 100 mg/rat BPA).

The weakly estrogenic activity of BPA to rodents has since been confirmed, most recently by demonstration of the uterotrophic activity of this same total dose of BPA in the immature rat (Ashby and Tinwell 1999). However, the activity of THP-1 has not hitherto been re-evaluated. The chemical structure of THP-1 is only loosely related to that of estradiol; in particular, it bears no phenolic substituent, a

characteristic of most synthetic estrogens. It therefore became of interest to re-synthesise THP-1 and THP-4 (Haworth 1933) in order to confirm their relative estrogenicity. The activity of both chemicals in the ovariectomised rat has been re-evaluated using vaginal cornification and uterotrophic activity as the criteria of estrogenicity. The activity of both chemicals in the yeast hER and hAR agonist and antagonist assays, as described by Sohoni and Sumpter (1998), was also evaluated.

The lack of functional estrogenic activity for both of the phenanthrenes in the present studies led to repetition of a similar ovariectomised rat experiment performed on BPA by Dodds and Lawson (1936), with extension of the protocol to include the uterotrophic endpoint.

Methods

Chemicals 1,2,3,4-tetrahydrophenanthren-1-one (THP-1) and 1,2,3,4-tetrahydro-phenanthren-4-one (THP-4) were synthesised by the method described by Haworth (1933). THP-1 had mp 93.6-94.3^{o}C [Haworth (1933) reports mp 95-96°C]. NMR spectroscopy (400MHz, CDCl$_3$) confirmed the structure and indicated the presence of about 3% of the THP-4. THP-4 had mp 66-67°C [Haworth (1933) reported mp 69°C]. NMR spectroscopy (400MHz, CDCl$_3$) confirmed the structure and indicates the presence of about 5% of THP-1. In each case C and H content was found to be within 0.2% of the calculated values. Estradiol (E$_2$), dihydrotestosterone (DHT), 4-hydroxytamoxifen and flutamide were purchased from Sigma Chemical Company Ltd. (Poole, Dorset, UK). Bisphenol-A (BPA, purity > 99%) was supplied by Aldrich Chemical Company, Gillingham, Dorset, UK. [2,4,6,7-^{3}H]oestradiol (88Ci/mmole) was obtained from Amersham Pharmacia Biotech UK Ltd, Bucks, UK.

Competitive Binding Assay Uterine post-microsomal supernatant, isolated from the tissue of immature female Alpk:APfSD rats (21-25 days old) was used as a source of cytosolic ER in a competitive binding assay as previously described (Shelby et al, 1996; Ashby et al, 1999). Cytosol extracts were incubated with [2,4,6,7-^{3}H]oestradiol (5nM) and varying concentrations of unlabelled putative competitors (0.5nM-5μM E$_2$; 5nM-500μM THP 1 and 4) at 4°C for 18h. The precipitated receptor complex was extensively washed in buffer before being suspended in Optiphase-MP scintillant (LKB Scintillation Products) and the radioactivity determined a described earlier (Ashby et al 1999).

Yeast Assays Details of the yeast human estrogen receptor (hER) and human androgen receptor (hAR) agonist and antagonist assays have been described previously (Sohoni and Sumpter, 1998). In outline, the DNA sequence of hER or hAR is integrated stably into the yeast genome and associated with an estrogen or androgen responsive expression system linked to β-galactosidase. When an estrogenic or androgenic ligand is bound to the receptor, β-galactosidase is synthesised which catalyses the conversion of the yellow assay detector substrate to a red product. For the antagonistic assays, E$_2$ or DHT (the main natural ligands for hER and hAR, respectively) are added to the medium at a concentration which gives a sub-maximal stimulatory response (E$_2$ at 2.5x10^{-10}M or DHT at 2.5x10^{-9}). Chemicals which are

able to inhibit the activity of the these natural ligands lead to a dose-dependent decrease in β-galactosidase expression and a reduction in the rate of formation of the red product. Both test chemicals were assayed at least twice in each of the 4 types of yeast-based assay and representative data are shown.

Animal studies Alpk:APfSD (Wistar derived) rats were obtained from the AstraZeneca breeding unit (Alderley Park). 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 and as described previously (Ashby et al. 1997). Rats were ovariectomized at 6-8 weeks of age and used after a 2 week recovery period. All administrations were by subcutaneous (s.c.) injection in either arachis oil (AO, as used in our earlier studies) or sesame oil (as used by Cook et al 1933 and Dodds and Lawson 1936). The ovariectomized rat uterotrophic assays were carried out as described previously (Ashby et al. 1997) with the additional observation of vaginal smears, as described below.

Four studies were conducted. The first represented a small modification of the experiments described by Cook et al. (1933) for THP-1 and THP-4, with E_2 as a positive control agent. In the second and third experiments the precise experimental conditions used by Cook et al. (1933) were used, with the uterotrophic endpoint added. The final experiment represented an exact repeat of the vaginal cornification study reported by Dodds and Lawson (1936) for BPA, also with the uterotrophic endpoint added. Vaginal smears were taken daily from all animals and at termination. All animals were killed 72 h after the first (or only) dose of test agent. Quantification of vaginal cornification was determined on the terminal smears as described previously (Routledge et al, 1998).

Experiment 1
Rats received either THP-1 or THP-4 (50 mg/rat, s.c) on three consecutive days, or 100 mg/rat of either chemical as a single s.c dose. The vehicle control group received AO alone (0.75 ml/rat, s.c for 3 days) and the positive control group received E_2 (0.011 mg in 0.75 ml AO /rat, s.c for 3 days or 0.022 mg in 1.5 ml AO/rat, s.c as a single dose). In this experiment the vehicle and fixed dose volumes were extrapolated from the dose volume of 5ml/kg/day for 3 days used in our previous studies (Routledge et al, 1998). The dose volume for the single dose group was doubled because of solubility problems with THP 1 & 4. The fixed doses of E_2/rat were also based on previous studies where 0.04 mg/kg/day E_2 was used as an s.c. positive control agent (Routledge et al, 1998) and was doubled for the single dose group.

Experiments 2 and 3
Rats received THP-1 (100 mg/rat, s.c) or THP-4 (50 mg/rat, s.c) dissolved in sesame oil (2.0 ml/rat) as a single dose split between 2 sites. The control group received sesame oil alone (2.0 ml/rat, s.c) and the positive control group received 0.022 mg E_2/rat, s.c in 2.0 ml sesame oil/rat as a single dose.

Experiment 4
Rats received BPA at a total dose of 100 mg/rat, s.c (over 3 days) in sesame oil (total volume of 3.0 ml sesame oil) administered twice daily (0.5 ml/dose) for 3 days. The control group received sesame oil twice daily (0.5 ml/rat/dose, s.c) and the positive

control group received E_2 at a total dose of 0.034 mg/rat, s.c (over 3 days), administered twice daily for 3 days in sesame oil.

Statistical analysis of data Analysis of variance was determined using the GLM procedure described in SAS (1989). Differences from control values in all cases were assessed statistically using a 2-sided Student's t-test based on the error mean square from the analysis of variance.

Results

Competitive binding Assays Representative ER binding data are illustrated in the Figure 2. Both THP-1 and THP-4 were relatively weak ER ligands, competing with ^{3}H-E_2 only at high concentrations. There was little difference between the relative affinities of the two compounds.

Yeast Assays THP-1 and THP-4 were inactive in the hER estrogen and anti-estrogen screens (Figure 3A and 3B). The estrogen E_2 and the antiestrogen 4-hydroxytamoxifen acted as positive control agents for these two sets of experiments, respectively. Although the results (Figure 3B) seem to suggest that both test chemicals were weakly anti-oestrogenic at very high concentrations (above $1x10^{-5}$M), in fact the results probably indicate a degree of toxicity of the test chemicals to the yeasts at these high concentrations. Turbidity of the medium was measured at the end of each assay, as an indirect measure of yeast cell number. This showed reduced turbidity in the wells containing high concentrations of both test chemicals, indicating that the yeast cells did not divide as rapidly in these wells. As yeast cell number affects the degree and rate of response of the assay to active chemicals (see Beresford et al, 2000, for a full discussion of this point), the results probably do not suggest that the test chemicals are weakly anti-oestrogenic, but rather that they are toxic (to yeast cells) at concentrations above about $1x10^{-10}$M. In contrast, THP-1 was clearly androgenic and THP-4 showed marginal androgenic activity (Figure 4A). Both THP-1 and THP-4 showed dose-related anti-androgenic activity comparable with that of the positive control, flutamide (Figure 4B). The androgen DHT and the antiandrogen flutamide acted as positive control agents for these two sets of experiments, respectively.

Ovariectomised rat assays. THP-1 and THP-4 were tested in three ovariectomised rat assays and were without either uterotrophic activity or estrus-inducing (vaginal cornification) activity (Table 1). The conditions of test involved the precise conditions used by Cook et al (1933; Expts. 2 and 3) where full vaginal cornification was observed 54 h after dosing and persisted for 150 h. In our experiments vaginal smears were examined 24, 48, 54 and 72 h after the first (or only) dose, but neither THP-1 nor THP-4 produced any increase in vaginal cornification at any timepoint (data only shown for 72 h; Table 1).

In contrast, when tested under the same conditions as those described by Dodds and Lawson (1936), BPA elicited both uterotrophic and estrus-inducing activity (Table 1).

Discussion

Cook et al (1933) did not specify the source of their samples of THP-1 and THP-4 (Figure 1). However, they were probably donated by Haworth who described their synthesis that same year (Haworth 1933). NMR analysis of the present samples revealed that each was contaminated by ~5% of the alternative isomer, however, they are each of the same melting point as described by Haworth (1933) and can be considered to be the same as the samples used by Cook et al (1933). Further, the activity of each isomer was similar when activity was seen, and that eliminates the chance that the contaminant isomer was dominating the activity of the other isomer. Both chemicals bound equally and weakly to isolated estrogen receptors (Figure 2), but both were without functional activity as estrogens or anti-estrogens *in vitro* or *in vivo* (Table 1 and Figure 3). This unexpected finding led to re-evaluation of BPA in the ovariectomised rat, as described by Dodds and Lawson (1936). The activity observed for BPA (Table 1), coupled to its established activity as an estrogen in the present yeast hER assay (Sohoni and Sumpter 1998) and the immature rat uterotrophic assay (Tinwell and Ashby 1999) indicates that our vaginal cornification assay was of similar sensitivity to the assay used by Cook et al to evaluate THP-1 (Cook et al 1933, Dodds and Lawson 1936).

Sohoni and Sumpter (1998) have shown that some chemicals that show activity as estrogens in the yeast hER assay also elicit anti-androgenic activity in the corresponding hAR assay. THP-1 showed AR agonist and antagonist activity, with reduced levels of activity for THP-4. Shortage of the test chemicals prevented further evaluation of these activities in rodents.

In summary, THP-1, reported by Cook et al to be the first synthetic estrogen, has been shown to lack functional estrogenic activity *in vitro* and *in vivo*, but to possess androgenic and anti-androgenic activity *in vitro*. THP-4, reported by Cook et al not to be estrogenic *in vivo*, has been confirmed to be non-estrogenic both *in vitro* and *in vivo* and to possess weak androgenic and anti-androgenic activity *in vitro*. Both chemicals bound equally and weakly to isolated estrogen receptors.

The present results illustrate the complexity of deriving conclusions regarding the hormonal activities of chemicals. First, some activities observed in isolated hormonal receptor binding assays may not be expressed in functional hormonal assays. This indicates the need for functional hormonal assays in any screening programme. Second, that activities observed for a chemical in one hormonal assay may not be reflected in related hormonal assays. This indicates the need to define assay protocols with some precision when incorporating them into screening batteries. Finally, that some literature reports of hormonal activity for chemicals may not be capable of independent confirmation under apparently identical conditions of test. This illustrates the need to use lists of hormonally active chemicals with care

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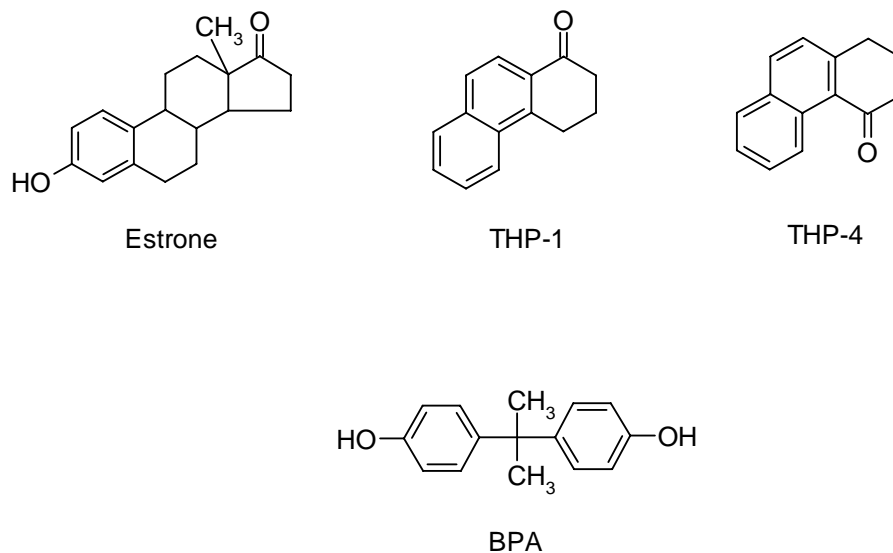
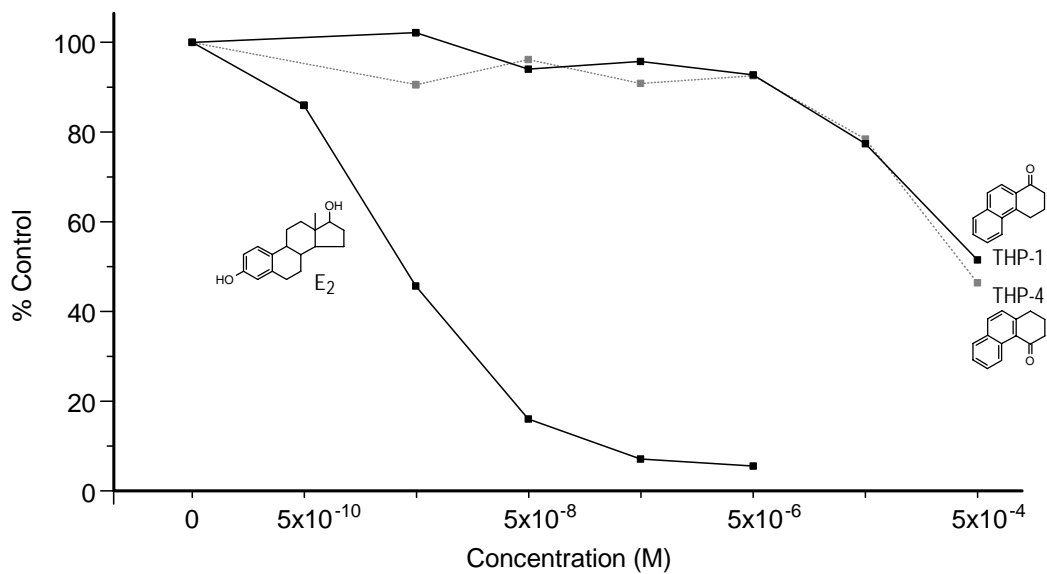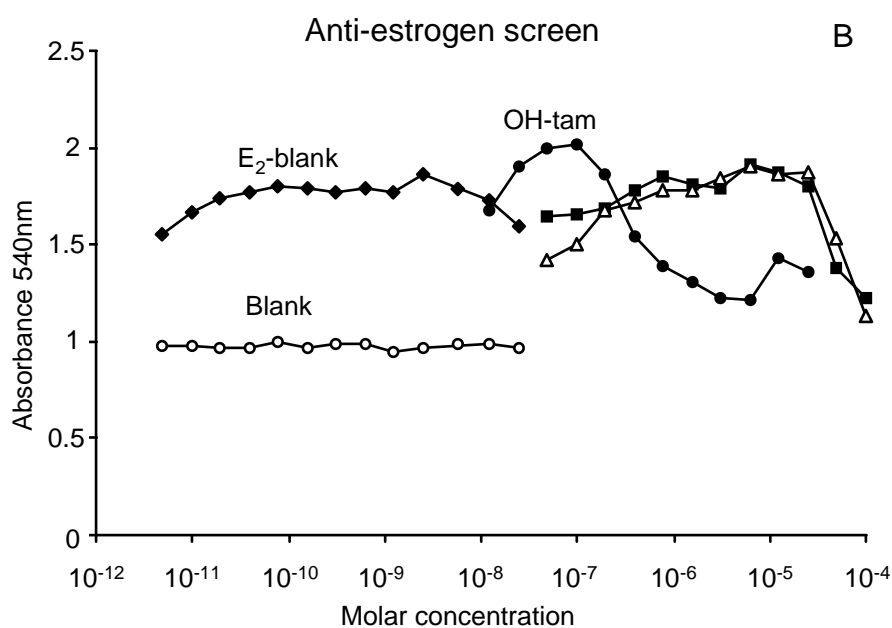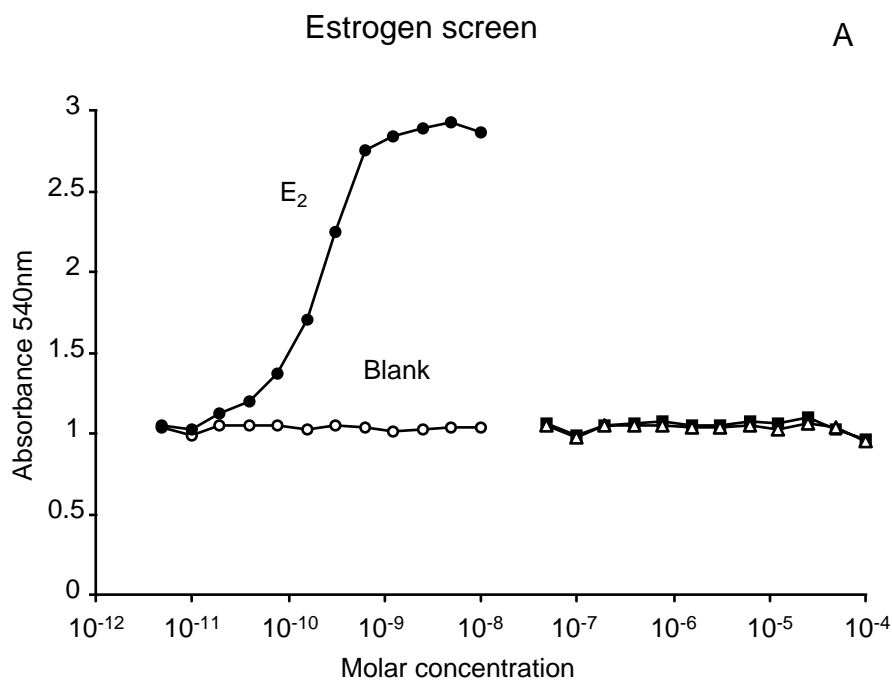FIGURE 1.Structures of the chemicals discussed in the text.

FIGURE 2: Relative binding affinity of THP-1 and THP-4 to isolated estrogen
receptors derived from rat uterine cytosol. Estradiol is shown as reference agent.

Estrogen screen
A

Absorbance 540nm

3

2.5

E_2

2

1.5

Blank

1

0.5

0

10^{-12} 10^{-11} 10^{-10} 10^{-9} 10^{-8} 10^{-7} 10^{-6} 10^{-5} 10^{-4}

Molar concentration

Anti-estrogen screen
B

Absorbance 540nm

2.5

2

OH-tam

E_2-blank

1.5

Blank

1

0.5

0

10^{-12} 10^{-11} 10^{-10} 10^{-9} 10^{-8} 10^{-7} 10^{-6} 10^{-5} 10^{-4}

Molar concentration

FIGURE 3: Estrogenic (A) and anti-estrogenic (B) activity of THP-1 (■) and THP-4 (▲) in the yeast hER screen. Blank wells contained solvent only (O). E_2 and 4-hydroxytamoxifen (● ; OH-tam) were used as positive controls in A and B respectively. In B, E_2 was present in all wells (except blanks) at a single concentration of 2.5 x 10^{-10}M, E_2-blanks (◆) contained E_2 only. Values are the means of duplicate wells taken from a representative experiment. Both THP-1 and THP-4 were concluded to lack either estrogenic or anti-estrogenic activity.
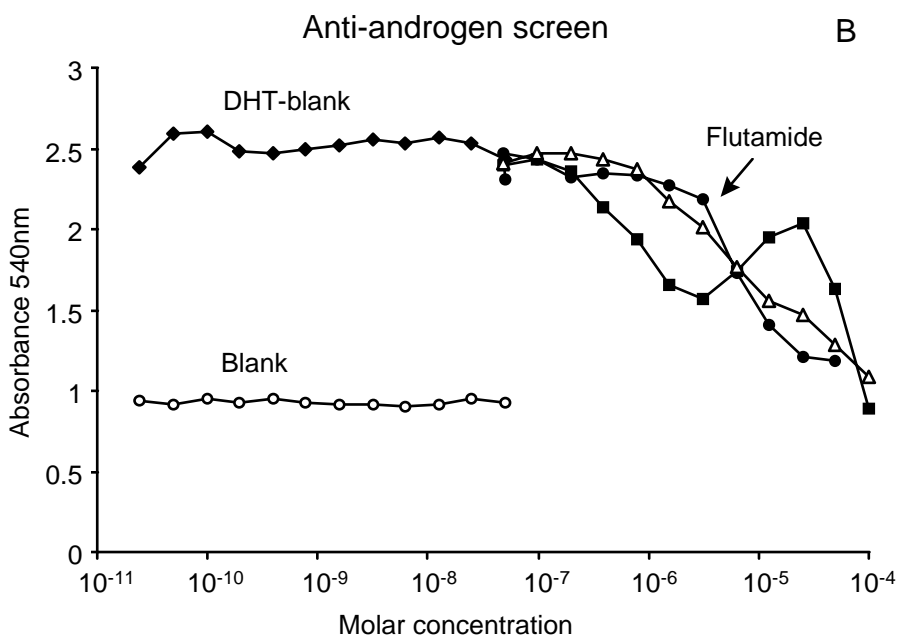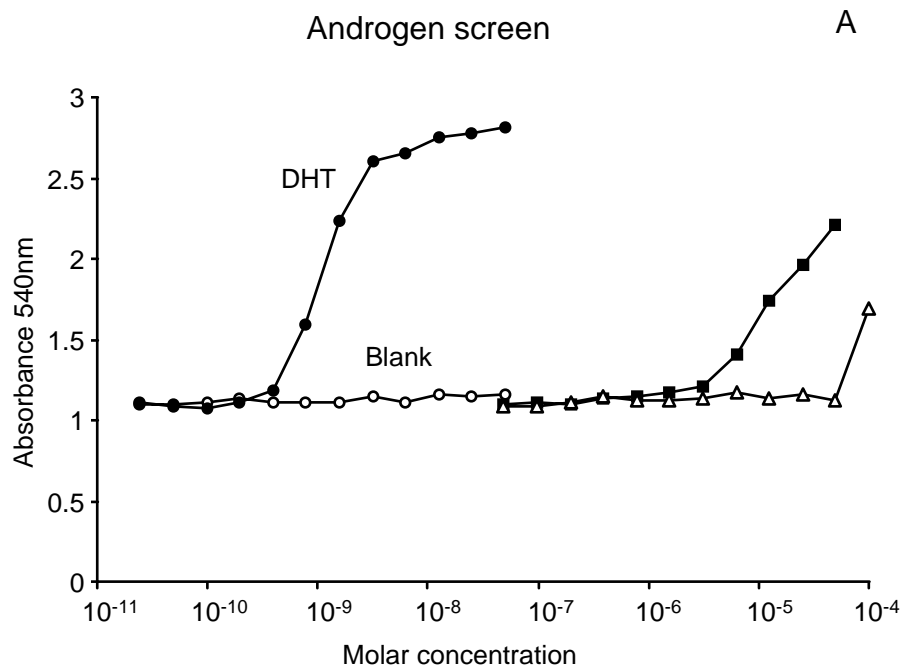
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FIGURE 4: Androgenic (A) and anti-androgenic (B) activity of THP-1 (■) and THP-4 (Δ) in the yeast hAR screen. Blank wells contained solvent only (O). DHT and flutamide (●) were used as positive controls in A and B respectively. In B, DHT was present in all wells (except blanks) at a single concentration of 2.5×10^{-9}M, DHT-blanks (◆) contained DHT only. Values are the means of duplicate wells taken from a representative experiment.

Compound	Dose/rat and duration of dosing	Uterus wet weight (mg)	Uterus dry weight (mg)	Vaginal cornification (% cornified cells)	Initial body weight (g)	Final body weight (g)
EXPERIMENT 1						
Arachis oil	0.75 ml/day for 3 days	68.8±6.4 (6)	16.6±1.5	18.5±5.7	288.2±19.5	300.0±21.7
THP-1	50 mg/day for 3 days	72.0±11.3 (7)	17.2±2.7	17.8±10.1	277.3±14.7	283.0±15.4
THP-1	100 mg/day for 1 day	65.0±6.3 (7)	15.8±1.6	21.9±13.4	277.6±14.2	285.4±16.2
THP-4	50 mg/day for 3 days	69.1±10.5 (7)	16.8±2.7	8.9±8.6	273.0±21.3	275.6±24.5
THP-4	100 mg/day for 1 day	63.7±7.6 (6)	15.1±1.8	16.5±7.2	273.7±16.4	276.3±20.2
E_2	0.011mg/day for 3 days	231.6±32.2** (3)	44.5±6.5**	100±0**	286.0±8.0	292.0±12.0
E_2	0.022 mg/day for 1 day	144.2±5.5** (3)	29.6±0.7**	100±0**	286.0±8.2	289.7±6.8
EXPERIMENT 2						
Sesame oil	2.0 ml/day for 1 day	70.7±8.8 (6)	16.2±2.0	18.9±4.7	269.0±20.3	284.0±22.9
THP-1	100 mg/day for 1 day	68.8±4.7 (4)	16.2±2.2	20.0±5.6	266.8±18.1	278.3±17.1
THP-4	50 mg/day for 1 day	71.3±7.7 (5)	16.2±1.5	15.2±6.9	264.0±8.8	277.6±11.2
E_2	0.022 mg/day for 1 day	127.4±4.9** (3)	25.9±0.6	100.0±0**	265.3±10.5	267.3±9.7
EXPERIMENT 3						
Sesame oil	2.0 ml/day for 1 day	67.7±9.5 (7)	15.5±2.0	11.5±3.9	279.8±18.6	296.1±18.2
THP-1	100 mg/day for 1 day	67.1±5.5 (4)	15.6±1.6	12.6±5.4	289.8±13.7	307.5±16.3
THP-4	50 mg/day for 1 day	70.2±11.9 (5)	16.4±2.4	17.3±7.0	283.0±12.4	296.2±13.3
E_2	0.022 mg/day for 1 day	115.5±4.8** (3)	23.2±0.7**	100±0**	284.3±11.4	293.0±9.8
EXPERIMENT 4						
Sesame oil	0.5 ml/dose, twice daily, for 3 days	70.9±12.9 (7)	15.8±3.1	8.2±3.0	279.5±13.4	295.3±14.0
BPA	16.7 mg/dose, twice daily, for 3 days	135.6±24.4** (7)	26.8±5.9**	100±0**	281.8±24.1	291.4±25.0
E_2	0.0056 mg/dose, twice daily, for 3 days	221.9±3.5** (3)	40.8±1.2**	100±0**	293.7±14.5	296.7±14.7

TABLE 1. The effect of THP-1, THP-4 and BPA on uterus weights and vaginal cornification (% cornified cells present in the vaginal smear). Estradiol (E_2) was used as a positive control. Compounds were administered s.c. in oil (arachis or sesame as specified). Animals were killed 72h after the first (or only) dose. The vaginal cornification data shown were determined at sacrifice. Data are mean ± SD, values in parentheses are numbers of animals, which were the same for all determinations. * $p< 0.05$, ** $p< 0.01$.