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Interaction of endocrine disrupting chemicals, singly and in combination, with estrogen-, androgen-, and corticosteroid-binding sites in rainbow trout (<u>Oncorhynchus mykiss</u>)

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Abstract

The ability of chemicals with known endocrine disrupting activity to interact with three major teleost steroid-binding sites was evaluated. Representative alkylphenols and phthalates, the pesticides dieldrin and toxaphene, the mycoestrogen zearalenone and the phytoestrogen genistein were tested for their ability to displace native ligand from putative estradiol receptor (ER), testosterone receptor (TR) and cortisol receptor (CR) from rainbow trout liver and brain. The ER displayed a higher affinity for alkylphenols than for phthalates, but both groups of compounds were 10^4-2.10^5 times less potent than estradiol in displacing specifically bound [^{3}H]estradiol. The displacement of bound [^{3}H]estradiol did not increase when these compounds were tested in combination. Toxaphene and dieldrin did not bind to the trout ER, either alone or in combination. Zearalenone and genistein were about 10^3-fold less potent than estradiol in displacing specifically bound [^{3}H]estradiol from the trout ER and showed no increase in potency when tested in combination. None of the compounds tested showed evidence of binding to the TR or the CR, failing to displace specifically bound [^{3}H]testosterone and [^{3}H]cortisol respectively. It is concluded that the compounds tested are exclusively estrogenic in rainbow trout, albeit weakly so, and do not display any synergistic effects.

1. Introduction

Many xenobiotics have been demonstrated to have a weakly estrogenic effect in fish (Thomas & Smith, 1993; Jobling et al., 1995; Sumpter and Jobling, 1995; Donohoe and Curtis; 1996; Jobling et al., 1996). These chemicals bind to the fish ER (Thomas and Smith, 1993; White et al., 1994; Jobling et al., 1995), stimulate expression of the vitellogenin gene and synthesis of vitellogenin in fish hepatocytes in vitro (Jobling and Sumpter, 1993; Pelissero et al., 1993), and induce hepatic production of vitellogenin and zona radiata proteins in vivo (Jobling et al., 1996; Arukwe et al., 1997; Harries et al., 1997). Among the most widely distributed of these chemicals in the aquatic environment are alkylphenols, phthalates, metabolites of dichlorodiphenyltrichloroethane (DDT), polychlorinated biphenyls (PCBs) and various pesticides. In particular, relatively high concentrations of alkylphenols and phthalates have been measured in treated sewage effluent (Giger et al., 1984; Giger et al., 1987; Lahl et al., 1988; Blackburn and Waldock, 1995; Petrosky and Vidic, 1996; Vitali et al., 1997) and it is well documented that exposure to treated sewage and waste water from oil industry processes induces the production of vitellogenin and zona radiata proteins in fish (Purdom et al., 1994; Arukwe et al., 1997; Harries et al., 1997; Knudsen et al., 1997). Although recent data suggest that at least some of the estrogenicity of sewage effluent may be ascribed to steroids of human origin (Anon, 1997) there is no doubt that an assemblage of potentially estrogenic chemicals occurs in such effluents. Therefore, evaluating the effect of chemicals in combination, rather than in isolation, is the most realistic approach to assessing environmental impact. Sumpter and Jobling (1995) observed a more than additive effect of a mixture of estrogenic chemicals on the production of vitellogenin by fish hepatocytes in vitro, suggestive of a synergistic effect. The possibility of synergistic relationships between estrogenic chemicals is a topic that has raised much concern and debate recently. Following the publication and subsequent withdrawal of a paper purporting to document very marked

synergistic effects (Arnold et al., 1996; McLachlan, 1997) there is confusion as to whether synergism between estrogenic chemicals exists or not (Arnold et al., 1997; Ashby et al., 1997; Ramamoorthy et al., 1997). However, only a few chemicals have been tested for synergistic effects, and many of the environmentally abundant xenoestrogens have not been screened for such actions.

Interest to date, in both mammals and lower vertebrates, has focused on the interaction of endocrine disrupting chemicals with the estrogen receptor system. Only limited effort has been directed towards assessing the potential disruption of other endocrine axes (e.g. Kelce et al., 1995; Kelce and Wilson, 1997) despite the fact that these are potentially vulnerable to interference by xenobiotics.

The aims of the present study were twofold: to compare the relative binding affinity of a range of estrogenic chemicals for the trout hepatic estrogen receptor, and to investigate whether the ER binding affinity increases when these chemicals are present in combination; secondly, to determine whether chemicals already established to possess estrogenic activity can bind to the trout androgen and corticosteroid receptor.

2. Materials and methods

2.1 Chemicals tested

The chemicals used in this study were obtained from the following sources: phenol (Fluka); 4-n-alkylphenols and butylphenol (Kebo); pentylphenol (Arcos); hexylphenol (TCL); heptylphenol (Eastman); octylphenol (Aldrich); nonylphenol (Arcos); dodecylphenol (Arcos);

butylbenzylphthalate (TCL); all other phthalates (Sigma); dieldrin (Sigma); toxaphene (Aldrich); p,p'-DDT (Aldrich); genistein and zearalenone (Sigma); 17β-estradiol, testosterone, cortisol (Sigma). All radiolabelled compounds were obtained from Amersham Life Science.

2.2 Fish

Mixed-sex rainbow trout (400-600g, Stirling strain) were used throughout these studies. Stock fish were maintained in 1500 l outdoor fiberglass tanks, each supplied with a constant flow of lake water (25 l/min.) at 6 °C and were fed thrice-weekly with commercial trout feed (Trouw Aquaculture; 1% body weight/day).

2.3 Tissue preparation

The interaction of the selected xenobiotics with testosterone-binding sites was assessed using cytosol prepared from whole brain homogenates, while the assessment of interactions with estrogen and corticosteroid binding were evaluated using liver cytosol. The procedures employed for tissue preparation and the characterisation of specific estrogen and corticosteroid binding in rainbow trout liver cytosols has been fully reported previously (Pottinger, 1990; Pottinger and Pickering, 1990). Testosterone binding in whole brain cytosol was measured using the techniques previously described for the quantification of androgen binding in trout olfactory tissue (Pottinger and Moore, 1997) and was fully characterised in terms of affinity, specificity and capacity (Johnsen, Pottinger and Knudsen, unpublished).

2.4 Competition study

Aliquots (200 µl) of cytosol were incubated in triplicate together with 1 pmol of labelled steroid ([2, 4, 6, 7-^{3}H] estradiol, [2, 4, 6, 7-^{3}H]cortisol, or [2, 4, 6, 7-^{3}H] testosterone; Amersham) together with an increasing concentration of competitor, in both the presence (non-specific binding) and absence (total binding) of a 1000-fold excess of inert steroid, for 4 h at 4°C. When chemicals were tested in combination the concentration of the chemicals was halved to obtain the same total molarity in all experiments. After incubation, unbound steroid was removed by the addition to each tube of 200 µl of ice-cold dextran-coated charcoal suspension, followed by incubation on ice for 10 min. and centrifugation at 1000g for 10 min. at 4°C. Aliquots of supernatant (300 µl) were added to 5 ml of scintillant (Ecoscint A, National Diagnostics) in a vial, and counted under standard tritium conditions. Specific binding was calculated from the difference between total and non-specific binding and corrected for protein concentration.

2.5 Protein determination

Protein concentration in the various preparations was determined according to the method of Ohnishi and Barr (1978).

3. Results

Results are presented as means of triplicate incubations. Standard errors are too small to be depicted on the figures. The relative potency of the chemicals tested is referred to as the ratio between the amount of steroid and amount of competitor required to produce the same percentage displacement of the [^{3}H]steroid.

All the alkylphenols tested were found to bind to the ER receptor with a relatively low affinity (Fig. 1.). Concentrations of alkylphenols approximately 10^4-fold greater than those of estradiol were required to produce similar amounts of displacement of specifically bound [^{3}H]estradiol. The maximum displacement achieved was between 40-80% when the concentration of alkylphenols were approximately 50 nmols/tube (165 μM). Higher concentrations of alkylphenols were not tested.

Phthalates also displaced [^{3}H]estradiol from the ER, but higher concentrations were required than for the alkylphenols (Fig. 2.). A concentration of phthalates approximately 2.10^5-fold greater (50 nmols/tube; 165 μM) than that of estradiol was required to produce an equivalent 10-25% displacement of specifically bound [^{3}H]estradiol. The relative potency of both alkylphenols and phthalates in displacing [^{3}H]estradiol showed considerable variation with concentration of the competitor. For this reason, no attempt was made to rank the chemicals in terms of their relative estrogenicity.

When several combinations of alkylphenols and phthalates were employed in the assay system (OP+BBP, PP+BBP, OP+DEHP, nonylphenol+DEP, hexylphenol+DAP), no increase in the ability of the chemicals to displace specifically bound [^{3}H]estradiol was observed, implying an absence of synergistic effects. The results from experiments in which a combination of OP and BBP, and PP and BBP were tested are presented (Figs. 3a and 3b, respectively).

Two pesticides, toxaphene and dieldrin, were tested for binding to the ER individually and in combination. Neither chemical, alone or in combination, displaced specifically bound [³H]estradiol (Fig 4.).

In contrast, the phytoestrogen genistein and the mycoestrogen zearalenone did displace specifically bound [³H]estradiol, displaying a higher affinity for the binding site than the alkylphenols and phthalates (Fig. 5). A 40-60% displacement of [³H]estradiol was achieved at a concentration about 10^3-fold the estradiol concentration required to achieve the same level of displacement. No evidence of synergism was observed when these chemicals were tested in combination.

All the chemicals were tested for their ability to displace specifically bound [³H]testosterone in brain cytosol. No displacement of testosterone was observed at concentrations of competitor up to 10^4 times the concentration of [³H]ligand. Representative results from the testing of DBP, BBP, PP, OP, dieldrin and p,p'- DDT are shown in Fig. 6.

In addition, all chemicals were assessed for their ability to displace specifically bound [³H]cortisol in trout liver cytosol. No displacement of cortisol was found even at a concentration of competitor 5.10^4-fold that of [³H]cortisol. At high concentrations of the chemicals ($>5.10^3$-fold that of the ligand, equivalent to 5 nmols/tube (16 μM) an apparent increase in binding to more than 100% of the initial level observed in the absence of competitor was noted (see discussion). Representative results obtained from testing of PP, OP, DBP, BBP and DEHP are shown in Fig. 7.

4. Discussion

The results of these studies demonstrate that alkylphenols and phthalates bind to the ER of rainbow trout liver with a relatively low affinity. The alkylphenols were between 5.10^3-5.10^4 times less potent than estradiol. This is in agreement with the results of previous studies which utilised trout ER (Sumpter and Jobling, 1995). In the present study the phthalates were found to be as much as 2.10^5 times less potent than estradiol in displacing specifically bound [^{3}H]estradiol, an observation consistent with previous reports (Jobling et al., 1995). However, in the present study BBP showed a similar level of competitiveness to the other phthalates tested whereas others have observed BBP to be a more potent competitor (Jobling et al., 1995; Harris et al., 1997). We cannot explain this disagreement but the source and/or formulation of the BBP used in the studies may be a factor.

Using a recombinant yeast estrogen receptor screen Routledge and Sumpter (1997) were able to rank alkylphenols with respect to their estrogenicity and to demonstrate the importance of certain structural features in determining estrogenicity. Phthalates were not tested. These authors noted differences in estrogenicity among the alkylphenols of a greater magnitude than was observed in the present study and found these differences to be consistent independent of concentration This contrasts with the present study in which the relative estrogenicity of the alkylphenols and phthalates was observed to change with concentration and suggests that the recombinant yeast screen is a system of greater resolution than the in vitro hepatic cytosol receptor assay. The contrasting results obtained using the two assay systems may reflect the possibility that although ER binding affinity among the alkylphenols is similar, the ability of specific chemicals to activate the receptor or associate the ER-ligand-complex to the estrogen response element on the DNA, may be different. However, both these test systems are artificial and neither necessarily reflects the situation in vivo. We have recently screened a

range of alkylphenols for estrogenicity in a fish hepatocyte culture (unpublished results) and found that the range of potency is less than that reported by Routlege and Sumpter (1997) and more akin to that observed in the trout hepatic ER assay. Compared to both the hepatic cytosol receptor assay and the yeast screen, a hepatocyte culture more closely resembles the in vivo situation because it does facilitate at least some of the metabolic transformations undertaken by the liver (Pelissero et al., 1993).

The data presented by Arnold et al. (1996) evoked much speculation regarding the possibility that synergism occurs among estrogenic chemicals, and doubts have been raised that such synergism does occur (Ashby et al., 1997; McLachlan, 1997; Ramamoorthy et al., 1997). The potential for synergistic actions between alkylphenols and phthalates has not previously been tested in assays based on the hepatic ER. In the present study, no evidence of synergistic effects on binding to the ER was observed when alkylphenols and phthalates were combined. Nor was binding of dieldrin and toxaphene to the trout hepatic ER observed, either alone or in combination, despite a report that these environmentally persistent (Paasivirta and Rantio, 1991; Stern et al., 1996) insecticides interact with the human ER synergistically (Arnold et al., 1996). However, it must be noted that this result was not found to be reproducible (McLachlan, 1997). It is therefore reasonable to assume that these compounds will not cause estrogenic effects in fish. This is consistent with the findings of others who have failed to observe synergistic interactions between these chemicals (Ramamoorthy et al., 1997; Ashby et al., 1997). However, Arnold et al. (1997) have reported that a combination of toxaphene and dieldrin bind to the alligator ER in a synergistic manner. These contradictory results require clarification and underline the need for a unifying and reliable method to determine the estrogenicity of chemicals in vertebrate systems.

The binding of genistein and zearalenone to the trout hepatic ER was also assessed. Both these compounds are abundant in many food sources (Livingston, 1978; Verdeal and Ryan, 1979; Kuiper-Goodman et al., 1987), bind to the human ER with a relatively high affinity (Miksicek, 1994), and stimulate growth in estrogen sensitive cell lines (Mayr et al., 1992). Phytoestrogens are also found in fish diet, both of commercial and natural origin, and could thus exert estrogenic effects on fish (Pelissero and Sumpter, 1992). We found that zearalenone and genistein bound to the ER with a higher affinity than the alkyphenols and the phthalates, displaying an apparent affinity of approximately 10^3-fold less than that of estradiol. This is similar to the binding affinity previously reported for these compounds in trout and human ER assays (Miksicek, 1994; Sumpter and Jobling, 1995) and suggests that these compounds could exert more profound estrogenic effects than alkylphenols and phthalates. However, as for the other chemicals tested in this study, no evidence for a synergistic increase in binding affinity for the ER was found when testing these compounds in combination.

None of the compounds tested was successful in displacing [^{3}H]testosterone from a specific binding site in trout brain cytosol suggesting that these chemicals are not likely to manifest androgenic or antiandrogenic effects in fish. Previously it has been reported that p,p'-DDT binds to the TR from rat prostate with a relatively high affinity (Kelce et al., 1995). In the present study p,p'-DDT did not bind to the putative TR from fish brain suggesting that p,p'-DDT is not androgenic in fish. These contrasting observations may reflect species differences in the ligand specificity of the TR, similar to those reported for the binding of other xenoestrogens to steroid receptors. DDT and PCB metabolites displayed no binding to the ER from spotted seatrout (Cynoscion nebulosus; Thomas and Smith, 1993) although the same chemicals have been demonstrated to bind to a mammalian ER (McLachlan et al.,

1984). Steroid receptor structure varies between animals. For example, the fish ER (Pakdel et al., 1989, 1990) contains fewer amino acids than the human ER (Green et al., 1986) although the hormone binding site is conserved (Pakdel et al., 1990; Le Roux et al., 1993). It has been suggested that endocrine disruptors can affect receptor activation through binding to sites on the steroid receptor other than the hormone binding site (McLachlan and Arnold, 1996) and if this is the case, species differences in binding of chemicals to steroid receptors is likely. Furthermore, the rainbow trout ER has a lower affinity for estradiol than the human ER (Le Drean et al. 1995; Petit et al., 1995) which may also give rise to differences in ligand activity in different test systems.

To our knowledge, there have been no studies which have examined the binding of xenobiotics to the CR. However, this receptor system is potentially of some significance with regard to the potential impact of endocrine disrupting chemicals. It has been suggested that exposure of animals to endocrine disruptors can lead to immunosuppression (see Kavlock et al., 1996), but the mechanism underlying this effect is unclear. Cortisol is a well-established immunosuppressant in vertebrates (Parillo and Fauci, 1979; Bateman et al., 1989) including fish (Kaatari and Tripp, 1987; Tripp et al., 1987; Pickering and Pottinger, 1989). The effect of cortisol on the immune system in fish is most likely mediated via a CR (Maule and Schreck, 1990) and therefore susceptible to interference. However, none of the chemicals tested in the present study manifested any affinity for the trout hepatic CR. Nonetheless, the possibility that there are classes of xenobiotic compounds which do interact with the CR cannot be excluded and is worthy of further investigation. Such considerations have to a large extent been overshadowed by the emphasis place on estrogenic effects in current efforts in this field.

A phenomenon occasionally observed in the present study was an apparent <u>increase</u> in binding of labelled cortisol at high concentration of competitor (see Fig. 7). This may be an artefact arising due to the hydrophobic nature of many of the chemicals tested. If at high concentrations the critical micelle concentration (CMC) of these chemicals is reached, micelle formation will occur, with a resulting reduction in the amount of chemical available for exchange with the receptor. Micelle formation could theoretically "upset" the assay in different ways giving rise to spurious results, for example, by "protecting" [^{3}H]steroid from adsorption to the dextran-coated charcoal used to separate bound ligand from free. From our results, it is not possible to provide an explanation for the mechanism behind this artefact.

Conclusion

In conclusion, alkylphenols and phthalates have a low affinity for the trout hepatic ER and should therefore be classified as weak estrogens in trout. No increase in their estrogenicity is apparent when they are in combination, suggesting that synergistic effects are absent. Dieldrin and toxaphene appear not to be estrogenic to fish either alone or in combination. Zearalenone and genistein are more estrogenic than the alkylphenols and phthalates, and are more likely than these compounds to exert estrogenic effects *in vivo*. None of the chemicals tested were found to bind to putative receptors for testosterone and cortisol and it is therefore unlikely that they will exert disruptive effects on processes normally mediated by these hormones. However, this does not exclude the possibility that other classes of compounds have androgenic or corticosteroidogenic effects. In addition, it should be borne in mind that a receptor assay does not take into account metabolism of the chemicals tested, so before final conclusions are made, <u>in vivo</u> screening should be performed.

Acknowledgements

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Figure legends

Fig. 1. Displacement of [^{3}H]estradiol from the trout hepatic ER by a range of

4-n-alkylphenols. The data are presented as the percentage of bound [^{3}H]estradiol for the

given concentration of competitor. Hepatic cytosol was incubated with 1 pmol [^{3}H]estradiol

together with either one of a range of alkylphenols (500, 5000 and 50000-fold the

concentration of [^{3}H]estradiol) or inert estradiol (0.5, 5, 50 and 500-fold the concentration of

[^{3}H]estradiol). The competitors used were phenol, butylphenol (butyl), pentylphenol

(pentyl), hexylphenol (hexyl), heptylphenol (heptyl), octylphenol (octyl), nonylphenol (nonyl)

and dodecylphenol (dodecyl).

Fig. 2. Displacement of [^{3}H]estradiol from the trout hepatic ER by a range of phthalates.

Experimental conditions were as described for Fig. 1. The phthalates tested were Bis

(2-etylhexyl) phthalate (DEHP), diallylphthalate (DAP), dinonylphthalate (DNP),

butylbenzylphthalate (BBP), dibutylphthalate (DBP) and dietylphthalate (DEP).

Fig. 3. Displacement of [^{3}H]estradiol from the trout hepatic ER by (a) pentylphenol (PP),

butylbenzylphthalate (BBP) and PP+BBP in combination, and (b) octylphenol (OP) and bis

(2-etylhexyl) phthalate (DEHP) and OP+DEHP in combination. Note that the concentration

of each of the chemicals when tested in combination was halved. Experimental conditions

were as described for Fig. 1.

Fig. 4. Displacement of [^{3}H]estradiol from the trout hepatic ER by toxaphene, dieldrin and

toxaphene and dieldrin in combination (diel + tox). Note that the concentration of each of the

chemicals when tested in combination was halved. Experimental conditions were as described for Fig. 1.

Fig 5. Displacement of [^{3}H]estradiol from the trout hepatic ER by genistein, zearalenone and genistein and zearalenone in combination (Gen + Zer). Note that the concentration of each of the chemicals when tested in combination was halved. Experimental conditions were as described for Fig. 1.

Fig. 6. Displacement of [^{3}H]testosterone from the high affinity testosterone binding site in rainbow trout brain cytosol by dibutylphthalate (DBP), butylbenzylphthalate (BBP), 4-n-pentylphenol (PP), 4-n-octylphenol (OP), dieldrin and p,p' DDT. Experimental conditions were as described for Fig. 1. except that inert testosterone was employed.

Fig. 7. Displacement of [^{3}H]cortisol from the high affinity cortisol binding site in rainbow trout liver cytosol by pentylphenol (PP), octylphenol (OP), dibutylphthalate (DBP), butylbenzylphthalate (BBP) and bis (2-etylhexyl) phthalate (DEHP). Experimental conditions were as described for Fig. 1. except that inert cortisol was employed.
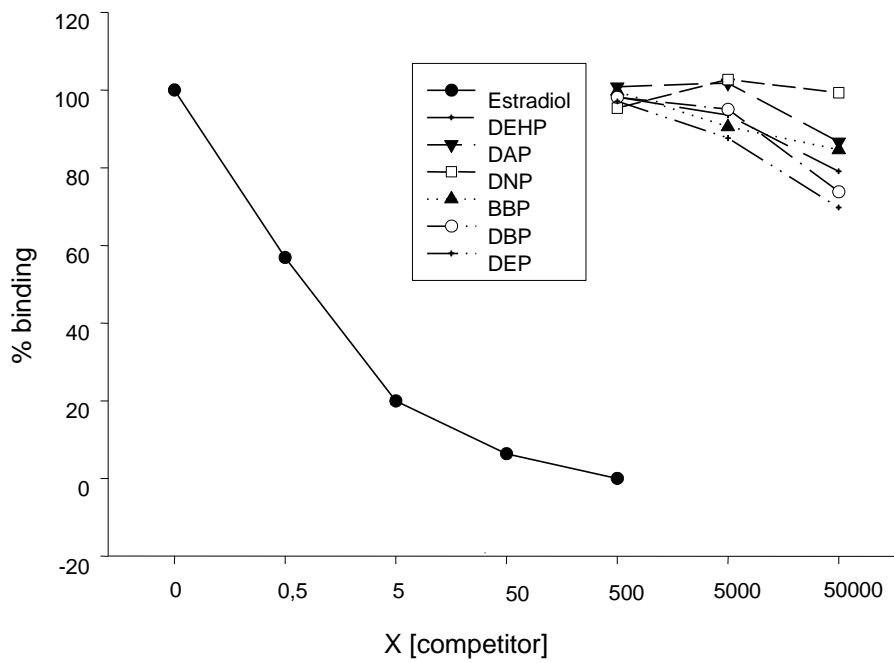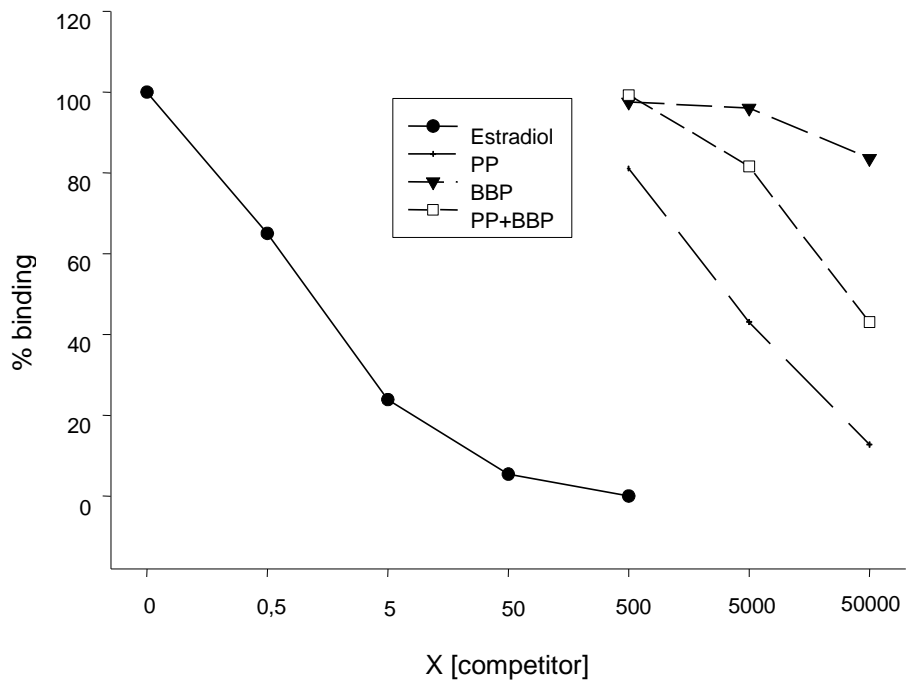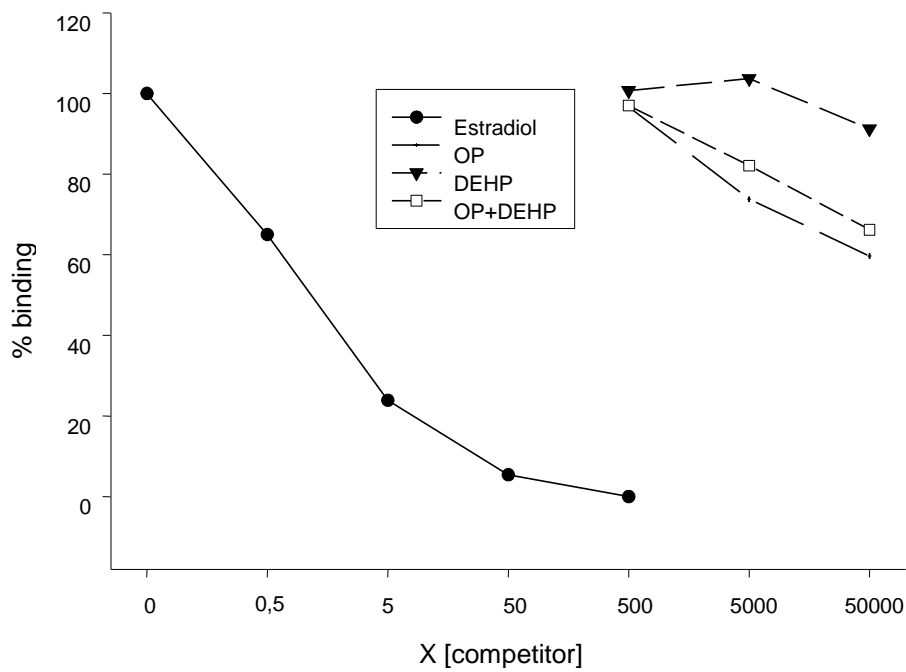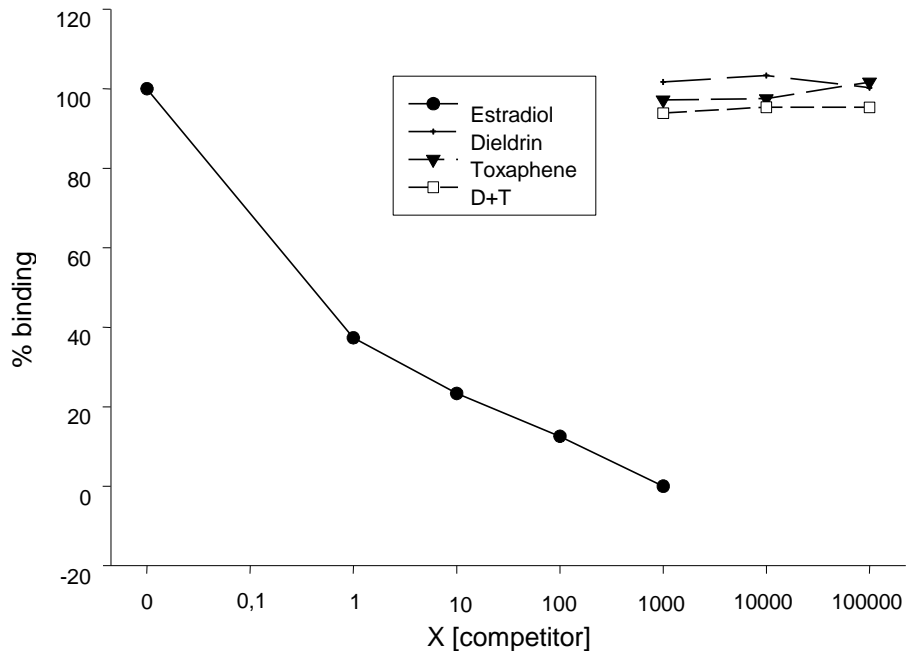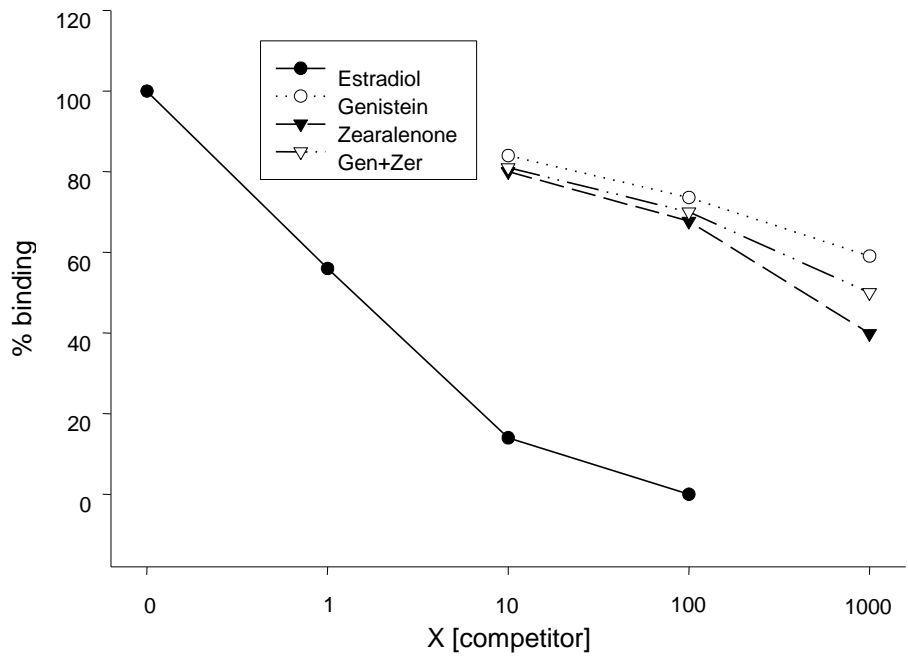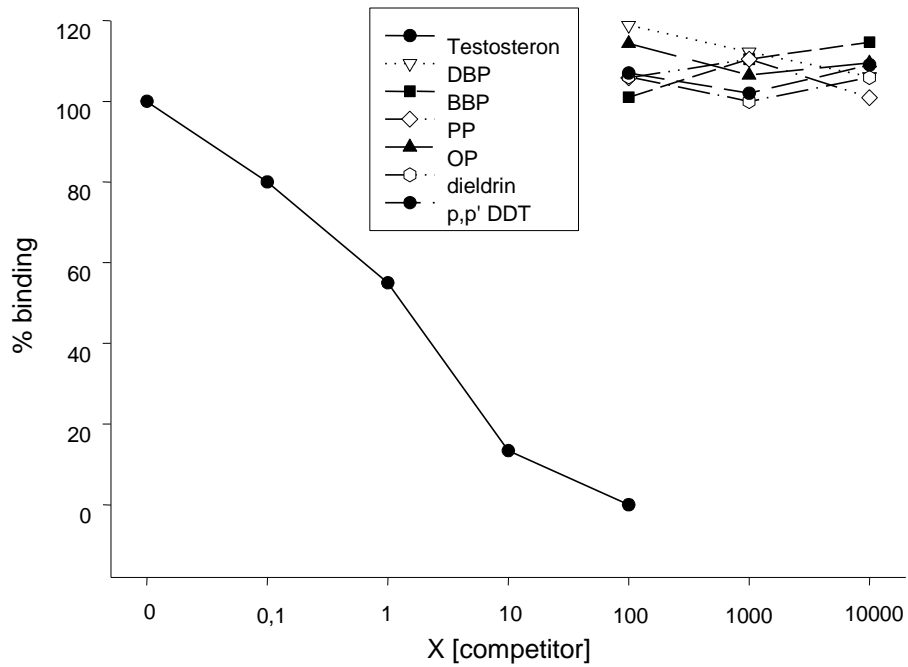
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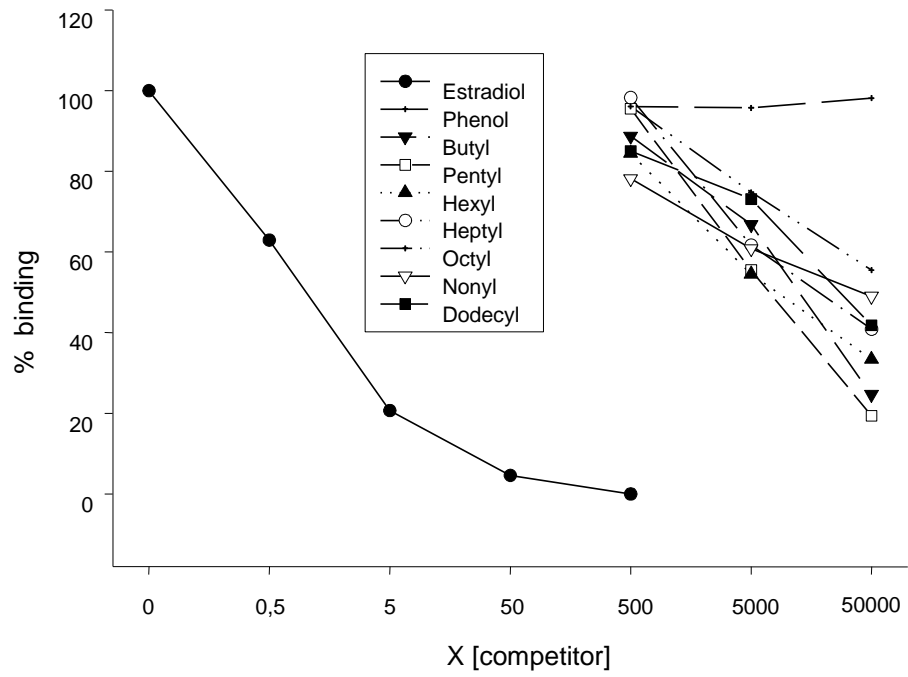
Figure 2.

Figure 3(a)

Figure 3(b)

Figure 4.

Figure 5.

Figure 6.

Figure 7.