

Effects of environmental antiandrogens on reproductive development in experimental animals

L.E.Gray, Jr^{1,5}, J.Ostby¹, J.Furr¹, C.J.Wolf¹, C.Lambright¹, L.Parks¹, D.N.Veeramachaneni², V.Wilson³, M.Price³, A.Hotchkiss³, E.Orlando⁴ and L.Guillette⁴

¹Endocrinology Branch, RTD, ORD, NHEERL, USEPA, RTP, NC, USA, ²Department of Animal Reproduction and Biotechnology, Colorado State University, Fort Collins, CO, USA, ³NCSU/USEPA Cooperative Research Program and ⁴Department of Zoology, University of Florida, Gainesville, FL, USA, ⁵To whom correspondence should be addressed at: MD-72, Endocrinology Branch, RTD, NHEERL, ORD, USEPA, RTP, NC, 27711, USA. E-mail: gray.earl@epa.gov

Chemicals that act as androgen receptor (AR) agonists and antagonists or inhibit fetal steroidogenesis can induce reproductive malformations in humans and laboratory animals. Several environmental chemicals disrupt development in rats and/or rabbits at fetal concentrations at, or near, exposure levels seen in some segments of the human population. In rats, fetal tissues concentrations of 10–20 p.p.m. of the DDT metabolite, p,p'-DDE, are correlated with reproductive abnormalities in male offspring. These concentrations are similar to those measured in first-trimester human fetal tissues in the late 1960s. The pesticides vinclozolin, procymidone, linuron and DDT are AR antagonists. They reduce male rat anogenital distance, and induce areolas at relatively low dosages. Hypospadias, agenesis of the sex accessory tissues and retained nipples are seen in the middle dosages, while undescended testes and epididymal agenesis are seen in the highest doses. Phthalate esters (PE) inhibit testosterone synthesis during fetal life, but do not appear to be AR antagonists. Prenatal administration of a single low dose of dioxin (50–1000 ng TCDD/kg) alters the differentiation of androgen-dependent tissues at p.p.t. concentrations, but the mechanism of action likely involves interaction with a hormone-like nuclear transcription factor, the hormone-like receptor AhR, rather than AR. p,p'-DDT and p,p'-DDE, vinclozolin and di-n-butyl phthalate affect reproductive function in rabbits when administered during prenatal and/or neonatal life. Cryptorchidism and carcinoma in situ-like (CIS) testicular lesions were seen in male rabbits treated during development with p,p'-DDT or p,p'-DDE. Extrapolation of effects from rodents to humans would be enhanced if future studies incorporate determination of tissue concentrations of the active metabolites. Knowledge of the tissue concentrations of the active toxicants also would provide an important link to in-vitro studies, which provide more useful mechanistic information when they are executed at relevant concentrations.

Key words: antiandrogens/pesticides/phthalates/pulp mill effluent/sexual differentiation

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Introduction

Background on the EDC issue

The potential effects of 'endocrine-disrupting chemicals' (EDCs) on human health and the proven effects of EDCs on wildlife are a major focus among the scientific community. Due to data gaps in the current testing of chemicals, in 1996 the United States Environmental Protection Agency (USEPA) was given a mandate under the Food Quality Protection Act and Safe Drinking Water Act to develop protocols to screen for endocrine effects. The

initial impetus for these actions arose from a Work Session in 1991 on 'Chemically Induced Alterations in Sexual Development: The Wildlife /Human Connection' (Colborn and Clement, 1992) which stated that 'Many compounds introduced into the environment by human activity are capable of disrupting the endocrine system of animals, including fish, wildlife, and humans. Endocrine disruption can be profound because of the crucial role hormones play in controlling development' (Colborn *et al.*, 1998). Among these chemicals are pesticides and industrial chemicals, pharmaceuticals, phytochemicals and other anthropogenic products. These scientists 'estimated with confidence' that impairments in humans have resulted from exposure to endocrine disrupters. Laboratory studies corroborate the abnormalities of reproductive development observed in the field and, in some cases, provide mechanisms to explain the effects. In-utero exposure to environmental oestrogens, antiandrogens, phthalates or chemicals such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) could be contributing to the reported decline in sperm counts in some areas (Murature *et al.*, 1987; Carlsen *et al.*, 1992; Giwercman *et al.*, 1993; Toppari *et al.*, 1996; Swan *et al.*, 2000) and the apparent increase in cryptorchid testes, testicular cancer and hypospadias (Paulozzi, 1999; Jegou *et al.*, 2000).

The new approach mandated in 1996 for screening and testing is warranted because some chemicals with endocrine activity are not adequately tested, such that neither claims of 'safety' nor 'eminent hazard to children' can be adequately supported by the available toxicological data. Furthermore, as adequate dose-response data are developed, adverse effects are being noted in laboratory animal models at dosages below or at former no-observed-adverse-effect-levels (NOAELs) which, in some cases, overlap with human exposure levels.

In addition to requiring the implementation of an endocrine screening and testing program, the Food Quality Protection Act of 1996 mandated that the risk assessment process consider combinations of chemicals rather than evaluate the potential risk on a chemical by chemical basis.

Human male reproductive health trends associated with EDCs

Although several reviews (Gray, 1991, 1992, 1998a,b; Colborn and Clements, 1992; Guillette *et al.*, 1994, 1999a,b; Giesy *et al.*, 1995; Guillette, 1995, 2000; Gray and Kelce, 1996; Guillette and Guillette, 1996; Toppari *et al.*, 1996; Monosson, 1997; Ankley and Geisy, 1998; Geisy and Snyder, 1998; Gray and Ostby, 1998; Gray *et al.*, 1998, 1999a; Van der Kraak *et al.*, 1998) have been published within the last decade, new studies are refining our understanding of some of these issues on almost a daily basis. The increases in testicular cancer in many areas of the world have been well documented (Jegou *et al.*, 2000). While it appears that hypospadias and cryptorchidism also are increasing in the industrialized world, one cannot rule out some confounding variables (Paulozzi, 1999). It has been noted that the differences in sperm counts between regions are so large that they cannot be explained by methodological biases, and thus environmental effects are entirely plausible (Jegou *et al.*, 2000).

New classes of EDCs and sources of EDC exposures

We are now not only concerned about pesticides such as DDT [1,1,1-trichloro-2,2-bis-(*p*-chlorophenyl) ethane] and toxic substances like the dioxins and polychlorinated biphenyls (PCBs)

because the issue has broadened as potent human and animal pharmaceuticals have been detected in environmental samples and humans are consuming products that contain EDCs, including phytosterols, food supplements and 'natural products'. Among drugs found in water samples are oestrogens, anabolic steroids, antibiotics, beta-blockers, antiepileptics and lipid-regulating agents (Christian and Ternes, 1999; Metcalfe *et al.*, 1999; Meyer *et al.*, 1999; Ternes, 1999). One study reported that the pharmaceuticals found as contaminants of aquatic systems included 36 of 55 pharmaceuticals and five of nine metabolites measured, including antiepileptic drugs. Another reported that several lipid-regulating agents were present in the effluents at near mg per litre concentrations, while a third reported concentrations of antibiotics in hog lagoons in North Carolina, USA as high as 700 µg/l. Others (Blount *et al.*, 2000) found much higher concentrations of phthalate metabolites in the urine of women of childbearing age than expected. Apparently, phthalates are used extensively in some nail polishes, skin creams and other personal care products. 'Surprising findings following a Belgian food contamination with PCBs and dioxins' were also reported (Schepens *et al.*, 2001). These authors reported 'that 12.1% of Belgian export meat samples from chicken or pork, unrelated to the PCB/dioxin crisis from 1999, contained more than 50 ng polychlorinated biphenyls (PCBs)/g fat and that 6.5% of samples contain more than 20 ng/g fat for the sum of 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane (DDT) and its metabolites'.

Therefore, it is not too surprising that the human fetus is often exposed to some of these banned chemicals (Foster *et al.*, 2000). These authors quantified man-made chemicals in human amniotic fluid during the second trimester. Analytes included common PCB congeners, the DDT metabolites *p,p'*-DDE, *o,p'*-DDE as well as the pesticides hexachlorobenzene (HCB) and the three isomers of hexachlorocyclohexane (alpha-, beta- and gamma-HCH). Overall, one-third of the 53 amniotic fluid samples tested positive for at least one environmental contaminant. The group concluded 'that approximately one in three fetuses in the Los Angeles area is exposed to endocrine modulatory environmental contaminants *in utero* the consequences of which remain unknown at this time'. Other groups (Wasserman *et al.*, 1967; Curley *et al.*, 1969) reported *p,p,m*. concentrations of DDT and its metabolites in first-trimester human fetal tissues.

Concerns about such exposures and the potential latent effects of these chemicals led the National Academy of Sciences to conclude in *Pesticides in the Diets of Infants and Children* that the young are a special concern with respect to pesticide exposures (National Research Council, 1993). We are all now well aware that the basic tenet of toxicology (Paracelcus, 1564; cited in Hayes and Laws, 1991) that 'dose alone determines the poison' is far too limited because the timing and duration of exposure during development, as well as dose, dictates the kinds of effects that can be induced and the severity of the lesions. Chemicals with EDC-activity that have beneficial effects on adults can induce malformations in the fetus.

Review outline

This review initially will provide background information on known effects of EDCs, including androgenic and antiandrogenic drugs on human reproductive development. Following this, the antiandrogenic effects of: (i) pesticides that act as androgen

receptor (AR) antagonists (in rats and rabbits); (ii) toxicants that appear to inhibit fetal testosterone synthesis (in rats and rabbits); (iii) dioxin and dioxin-PCBs (in rats); and (iv) phytochemicals (in rats) are selectively reviewed. In a few cases, an attempt is made to relate human exposures to the tissue levels of toxicants that resulted in adverse effects in the animal studies. While most of the review focuses on environmental chemicals that interfere with the development of androgen-dependent tissues, new evidence is also discussed confirming the existence of environmental androgens, and new information provided on the 'cumulative risk' of a mixture of two antiandrogens that act via different mechanisms of action but affect similar fetal reproductive tissues. In the final section of the review, some of the endocrine screening assays for androgens and antiandrogens are discussed.

Known effects of EDCs

The EDCs of concern

The list of chemicals that are known to affect reproduction and/or development in humans, domestic animals or wildlife via endocrine mechanisms includes TCDD, PCBs and polychlorinated dibenzofurans (PCDFs), tributyl tin, ethinyl oestradiol (EE), anabolic steroids, alkylphenols, plant sterols, fungal oestrogens, chlordecone, dibromochloropropane (DBCP), o,p'-DDD (Mitotane), o,p'-DDT and p,p'-DDE.

Epidemiology studies associating EDCs with human health effects

Several toxicants, including PCBs and PCDFs (Guo *et al.*, 2000) and phthalates (in semen) (Murature *et al.*, 1987) have been associated with sperm abnormalities in men. In females, exposure to oestrogenic chemicals (Hannon *et al.*, 1987) and phthalates (Colon *et al.*, 2000) have been associated with accelerated puberty. TCDD exposure has been associated with skewed sex ratio and an increased incidence of endometriosis (Koninckx, 1999). Several classes of EDCs have been associated with breast cancer (Davis *et al.*, 1993).

A case-control study was conducted (Weir *et al.*, 2000) to investigate the association between exposure to maternal hormones and risk of testicular germ-cell cancer. These authors found that exogenous hormone exposure was associated with elevated risk [Odds ratio (OR)=4.9]. In another case-control study, a six-fold increase was found in the risk for seminoma (one type of testicular cancer) among plastic workers exposed to polyvinyl chloride (PVC) (Ohlson and Hardell, 2000). It was suggested that phthalates, used in PVC as plasticizers, had properties that could promote the growth of endocrine-sensitive tumour cells. Another group (Knight *et al.*, 1996) reported that the incidence of non-seminoma is increasing, and that a significantly increased risk of this form of testicular cancer was associated with several occupations, including miners (OR=12.39), food and beverage processors (OR=3.20), utilities employees (OR=3.15) and leather-industry employees (OR=4.60). Testicular cancer has also been associated with parental use of fertilizers in agriculture (Kristensen *et al.*, 1996). Specific fertilizer regimens on the farm were associated with testicular cancer (rate ratio=2.44), in particular non-seminoma (rate ratio=4.21), with the highest rate ratio estimates displayed for 15- to 19-year-old boys, a subset which was considered more likely to have grown up on a farm.

Recent clinical studies on EDCs

Clinical studies on the effects of background levels of PCBs on the neurobehavioural development of children have documented a number of adverse effects (Brouwer *et al.*, 1995). In one of the few studies of children from a high-exposure population, abnormalities were observed in a population exposed *in utero* and early postnatally to PCBs and PCDFs (Guo *et al.*, 1995). In 1979, Yu-Cheng ('oil-disease') mothers were exposed to PCBs and their heat-degradation products when they ingested contaminated rice oil. Children of these mothers were born growth-retarded, with dysmorphic physical findings, and delayed cognitive development compared with unexposed children. As the male children matured they displayed abnormal sperm morphology, reduced sperm motility, and reduced in-vitro sperm fertilizing capacity (Guo *et al.*, 2000).

Clinical investigations reveal that occupational exposure to chemicals that alter reproductive and endocrine function is not just of historical interest. Although the specific mechanism of action is unknown, 2-bromopropane appears to present a reproductive, endocrine and haematopoietic hazard to both male and female workers (Kim *et al.*, 1996). Korean electronic workers (eight men, 25 women) exposed to solvents containing 2-bromopropane were examined. Most women displayed secondary amenorrhoea with high FSH concentrations and hot flashes, while male workers showed azoospermia or some degree of oligospermia or reduced sperm motility.

Another series of clinical studies from 1996 documents sexual impotence in chemical factory workers exposed to a diethylstilboestrol (DES)-like stilbene derivative. The National Institute of Occupation Safety and Health conducted two studies in response to complaints of impotence and decreased libido among male workers involved in the manufacture of 4,4'-diaminostilbene-2,2'-disulphonic acid (DAS), a key ingredient in the synthesis of dyes and fluorescent whitening agents. Both current and former workers had lower serum testosterone concentrations and reduced libido (Grajewski *et al.*, 1996; Whelan *et al.*, 1996) as compared with control workers.

It was recognized decades ago that one DDT metabolite altered human adrenal function with sufficient potency to be used clinically to reduce adrenal androgen production. o,p'-DDD (Mitotane) is used to treat adrenal steroid hypersecretion associated with adrenal tumours. In addition to this use, lower doses of Mitotane restored menstruation in 13 of 15 women with spanomenorrhoea associated with hypertrichosis. Pregnancies occurred in five of 15 women during the treatment period (Klotz *et al.*, 1971; Hayes and Laws, 1991).

Effects of drugs with EDC activity on human reproductive development

The effects of exposure to endocrine disrupters during sex differentiation is of special concern. This process can be very sensitive to the effects of low doses of EDCs. Reports of U-shaped (non-monotonic), ultra-low dose effects and non-threshold effects for EDCs are challenging assumptions of the risk assessment process for non-cancer endpoints. For example, administration of testosterone produces a well-characterized and reproducible U-shaped dose-response for spermatogenesis (Ewing *et al.*, 1977; Robaire *et al.*, 1979, 1987) in the intact

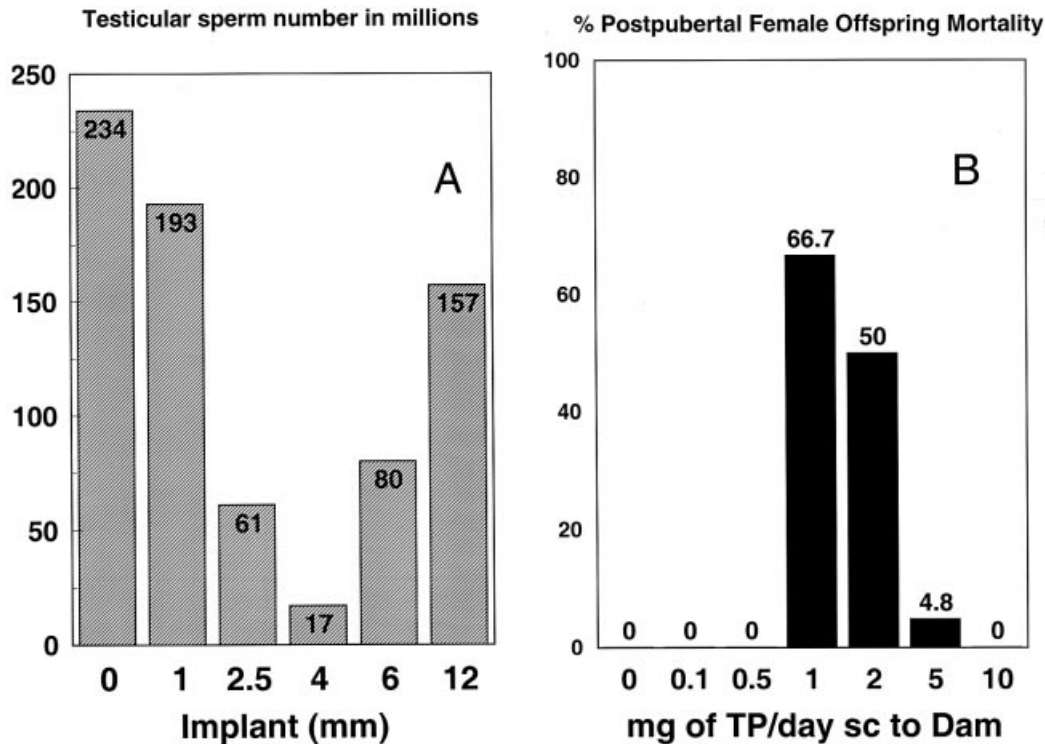


Figure 1. (A) U-shaped dose–response for the relationship between the amount of testosterone (as indicated by the length of the silastic implant on the x-axis) versus the number of testicular spermatids on the y-axis. Data are from Robaire *et al.* (1979). (B) U-shaped dose–response curve for the relationship between postpubertal mortality in female offspring due to reproductive tract (uterine) malformations caused by maternal treatment with testosterone propionate (TP; administered s.c. at the indicated mg/rat per day from gestational day 14–19; Wolf *et al.*, 2000a). Mortality results from hydrometrocolpos (Greene *et al.*, 1939) which develops after puberty. This effect is associated with a blind upper vaginal pouch and lower vaginal agenesis and develops as uterine fluid continuously accumulates as the females cycle, having no excurrent duct. In the higher-dose groups, the females are more severely masculinized and the upper vagina is connected to the urethra via a fistula through which the uterine contents empty.

adult male rat (Figure 1). Administration of testosterone propionate to the dam during gestation induces an inverted U-shaped response in postweaning mortality of female offspring due to malformations of Müllerian duct differentiation (Greene *et al.*, 1939; Wolf *et al.*, 2000a). In-vitro studies show that U-shaped responses do not always involve multiple mechanisms of action. Several AR ligands which are antagonists at low to moderate concentrations became AR agonists at high concentrations (Wong *et al.*, 1995).

The effects of exposure to EDCs are irreversible (the systems are ‘imprinted’ by the fetal hormonal environment); functional alterations of the sex differentiation are often not apparent until after puberty, or even later in life, and the abnormalities—which include malformations and infertility—cannot be predicted from changes in hormone levels produced by similar exposures in adult animals. Over 30 different drugs taken during pregnancy have been found to alter human development as a consequence of endocrine disruption (Schardein, 1993).

One group (Kaufman *et al.*, 2000) found that women who were exposed to DES in the womb ‘are less likely to have had full-term live births and more likely to have had premature births, spontaneous pregnancy losses, or ectopic pregnancies’ than unexposed women. If it is assumed that DES was no longer used extensively in pregnancy after 1971, it has taken three decades to recognize some of these effects. It has been reported that the synthetic oestrogen DES can alter sex differentiation of the human brain, and several behavioural changes have been

observed in DES-daughters (Meyer-Bahlburg *et al.*, 1985; reviewed by Hines and Green, 1991). In addition, it was reported (Meyer-Bahlburg *et al.*, 1985) that women exposed to DES *in utero* were found to have less well established sex-partner relationships, and to be lower in sexual desire and enjoyment, sexual excitability, and coital functioning. In addition, it was found (Hines and Shipely, 1984) that DES-exposed women showed a more masculine pattern of cerebral lateralization on a verbal task than did their sisters.

Drugs known adversely to affect the human fetus are not limited to DES. EDCs are known to alter human development via several mechanisms besides the oestrogen receptor (ER): this includes binding to the androgen (AR), or retinoic acid (RAR, RXR) receptors, and by inhibition of steroidogenic enzymes or the synthesis of thyroid hormones (Schardein, 1993). Exposure to hormonally active chemicals during sex differentiation can produce pseudohermaphroditism (Gray, 1992; Schardein, 1993). Androgenic substances, such as Danazol or methyltestosterone, masculinize human females (i.e. ‘female pseudohermaphroditism’), while progestins act both as androgen antagonists, demasculinizing males such that they display ambiguous genitalia with hypospadias, and as androgen agonists, masculinizing females (Schardein, 1993). The drug aminoglutethimide, which alters steroid hormone synthesis in a manner identical to many fungicides, also masculinizes human females following in-utero exposure (Schardein, 1993).

Mammalian sexual differentiation and the role of androgens

Androgens in the reproductive tract

The role of androgens in sex differentiation in mammals is well understood (Wilson, 1978). Before sex differentiation, the embryo has the potential to develop a male or female phenotype. Following gonadal sex differentiation, testicular secretions induce differentiation of the male duct system and external genitalia. The development of phenotypic sex includes persistence of either the Wolffian (male) or Müllerian (female) duct system, and differentiation of the external genitalia. Other organ systems, like the nervous system, liver and muscles are also 'imprinted'. The male phenotype arises due to the action of two testicular hormones, testosterone and Müllerian inhibiting substance. Testosterone induces the differentiation of the Wolffian duct system into the epididymis, vas deferens and seminal vesicles, while its metabolite, 5- α -dihydrotestosterone (DHT), induces the development of the prostate and male external genitalia. In the absence of these secretions, the female phenotype is expressed. This hypothesis is supported by the observation that when ER α and ER β genes are both knocked out ($\alpha\beta$ ERKO mouse), the female mouse still develops a complete reproductive tract (Couse *et al.*, 1999).

Androgens in the nervous system

In some areas of the central nervous system (CNS), testosterone is aromatized via the steroidogenic enzyme aromatase to oestradiol or reduced via 5 α -reductase to dihydrotestosterone (DH). The relative role of each hormone varies from behaviour to behaviour in a species-specific manner. In some species, all three hormones (testosterone, DHT and oestradiol) play a role in masculinization of the nervous system (Cooke *et al.*, 1998). There are significant species differences in the organizational and activational control of the development of sexually dimorphic behaviours (Cooke *et al.*, 1999). For example, in the rat, the activation of male-like mounting behaviour in the adult female rat does not require an organizational effect of hormones during perinatal life. In at least some strains of rats, this behaviour can be activated by testosterone in adult females (Larson, 1979; Brand *et al.*, 1990). In contrast to the rat, androgens play an important role in the organization of mounting behaviour in the non-human primate (Goy, 1978; Pomerantz *et al.*, 1985), and the fact that perinatal treatments with antiandrogens such as vinclozolin (Gray and Ostby, 1998; Gray *et al.*, 1999b) or diethylhexyl phthalate (Gray *et al.*, 1999a, 2000) do not abolish mounting behaviour in the male rat does not preclude the possibility that such behaviours would be altered in other mammals, including humans. Relevant to this argument, in-utero and lactational exposure to the antiandrogenic pesticide vinclozolin impaired mating behaviour in the male rabbit (Veeramachaneni *et al.*, 1996; Palmer *et al.*, 2000; Veeramachaneni, 2000). Rough-and-tumble play behaviour is one of the few social behaviours which appears to be regulated by androgens in both the rat (Meany *et al.*, 1983) and rhesus monkey (Goy, 1978). Neonatal administration of the AR antagonist vinclozolin during the critical organizational period for play behaviour results in female-like rough-and-tumble play in male rats (Hotchkiss *et al.*, 2001), while maternal administration during late gestation and early lactation did not demasculinize

play behaviour in male rat offspring (Gray and Ostby, 1998). Taken together, these results highlight that the timing for the developmental organization of hormone-mediated behaviours such as play behaviour can differ greatly from species to species, even if the hormonal mechanisms are similar.

A rodent model of the role of androgens on the nervous system

Development of the levator ani (LA)-bulbocavernosus (BC) muscles and their neural regulation has been employed as a model of the organizational and activational roles of testosterone on the ontogeny of sexual dimorphisms in the rat (Breedlove *et al.*, 1999). These tissues are also sexually dimorphic in humans—an effect which develops in the first trimester of pregnancy. The LA and spinal nuclei of the bulbocavernosus (SNB) are considerably larger in male than in female rats, which requires testosterone exposure during prenatal (organizational) and postpubertal (activational) stages of life. As the LA lacks 5 α -reductase, testosterone, and not DHT, is the hormone that initiates the male-like development of the LA and the specific spinal cord nuclei (SNB). While it has been shown that perinatal treatment with a variety of antiandrogens (vinclozolin, procymidone, linuron, DEHP, BBP and p,p'-DDE) and TCDD permanently reduces LABC size, the spinal cord of treated animals (which almost certainly is affected) has not been examined.

Androgen receptor antagonists

Mechanism of action: vinclozolin, procymidone, p,p'-DDE and linuron

The pesticides vinclozolin, procymidone, p,p'-DDT and p,p'-DDE and linuron are AR antagonists. Vinclozolin metabolites, M1 and M2 (Kelce *et al.*, 1994), procymidone (Ostby *et al.*, 1999), p,p'-DDT (and metabolites) (Kelce *et al.*, 1995) and linuron (Lambright *et al.*, 2000; McIntyre *et al.*, 2000) all competitively inhibit the binding of androgens to human AR (hAR) and inhibit androgen-induced gene expression. Of these, it also has been demonstrated that vinclozolin, p,p'-DDE (Kelce *et al.*, 1997) and linuron (Lambright *et al.*, 2000) alter androgen-dependent ventral prostatic gene expression *in vivo*. None of these pesticides or their metabolites (examined to date) appears to display significant affinity for the oestrogen receptor, or to inhibit 5 α -reductase *in vitro* (Kelce *et al.*, 1995; Waller *et al.*, 1996a), although M1 binds the rat progesterone receptor, albeit with relatively low affinity (Laws *et al.*, 1996).

Dose-response effects of vinclozolin on rat reproductive development

Vinclozolin-treated male offspring display female-like anogenital distance (AGD) at birth, retained nipples, cleft phallus with hypospadias, undescended testes, vaginal pouch, epididymal granulomas, and small to absent sex accessory glands. It was found (Wolf *et al.*, 2000b) that the most sensitive period of fetal development to these antiandrogenic effects of vinclozolin was on gestation days (GD) 16–17, but some malformations and other effects also were seen in male rat offspring dosed with vinclozolin on GD 14–15 and GD 18–19. An examination of mating behaviour in these males indicated that vinclozolin treatment

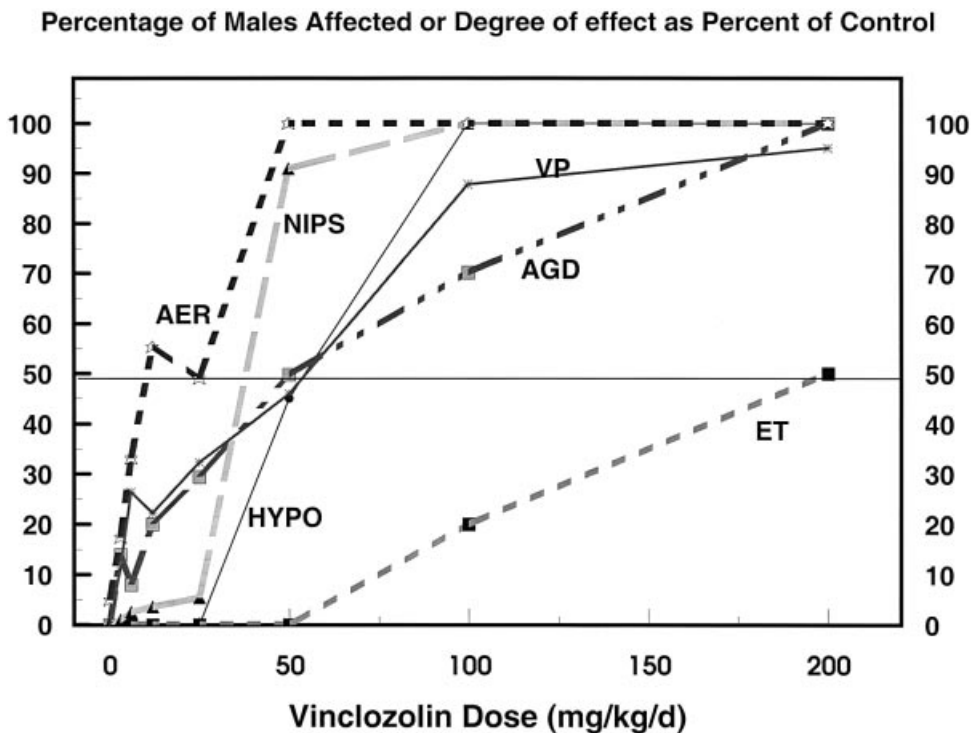


Figure 2. When vinclozolin is administered at varying dosages (from 3 to 100 mg/kg per day) during sexual differentiation (GD 14 to day 3 of lactation), the male rat offspring display alterations of numerous androgen-dependent tissues. Shown here are the dose-response curves for anogenital distance (AGD) at 2 days of age, the percentage of infant males with areolas (AER), ventral prostate weight (VP), percentage of males with permanent nipples (NIPS), and incidence of hypospadias (HYPO) and ectopic testis (ET). For AGD, the data are percent AGD reduced in each dose group ($\times 2$, as only half of AGD is androgen-mediated).

did not alter mounting behaviour, based upon the percentage of male mounting or latencies to mount, but malformed treated males were incapable of attaining intromission (Gray *et al.*, 1994; Gray and Ostby, 1998). In addition, rough-and-tumble play behaviour was not reduced when measured in peripubertal life in vinclozolin-treated male offspring. Apparently, lactational transfer of vinclozolin to the neonatal rat does not provide sufficient concentrations of the active metabolites to affect the organization of this behaviour because pups directly exposed to vinclozolin show female-like rough-and-tumble play levels (Hotchkiss *et al.*, 2001). Dose-response curves for different effects of vinclozolin vary in shape, and ED₅₀ values for different androgen-dependent tissues (Figure 2). Some of these dose-response curves failed to display an obvious threshold (i.e. AGD, induction of areolas and ventral prostate weight (Gray *et al.*, 1999b), and appear linear in the low dose range.

Effect of vinclozolin treatment on pubertal development of the male rat

Androgens play a key role in pubertal maturation in young males (Korenbrodt *et al.*, 1977), and antiandrogens delay this process. Peripubertal treatment with vinclozolin (Monosson *et al.*, 1999), p,p'-DDE (Kelce *et al.*, 1997), methoxychlor (Gray *et al.*, 1989), linuron or di-n-butyl phthalate (Gray *et al.*, 1999a) delay the onset of androgen-dependent preputial separation (PPS). This animal model appears to have potential as an assay to screen for EDCs, being more sensitive than is the Adult Male Assay (O'Connor *et al.*, 1999), but slightly less sensitive than the Hershberger Assay to AR antagonists.

One study was conducted to examine the effects of peripubertal oral administration of vinclozolin on morphological landmarks of puberty, hormone levels and sex accessory gland development in male rats (Monosson *et al.*, 1999). These authors also examined the effects of vinclozolin on AR distribution in the target cells, and measured serum concentrations of vinclozolin, M1 and M2. Vinclozolin treatment delayed pubertal maturation, and retarded sex accessory gland and epididymal growth. Serum LH, testosterone and 5 α -androstane,3 α ,17 β -diol concentrations were increased. These effects were concurrent with subtle, but statistically significant, alterations in the subcellular distribution of AR. In control animals, most AR was in the high-salt cell fraction, apparently bound to the natural ligand and DNA, while in treated males the AR distribution was altered.

M1 and M2 concentrations in the serum of affected animals were below their K_i values for AR. These results suggest that when the vinclozolin metabolites occupy a modest percentage of AR, this prevents maximal AR-DNA binding, alters in-vivo androgen-dependent gene expression and protein synthesis, which in turn, alters morphological development and serum hormone concentrations. Although vinclozolin treatment has been shown to alter both adrenal and liver functions, the mechanism of action for these effects has not been elucidated, and the role of AR (if any) is unknown.

Developmental effects of vinclozolin in the rabbit

Antiandrogenic toxicants alter sexual differentiation and reproductive development in the rabbit. This species offers several useful traits, including a longer gestation, with sexual differentiation *in utero*, a longer period of infantile life (useful for the study

of effects of toxicants during this stage of life), and the ease with which reproductive behaviours and sequential ejaculates can be evaluated. In addition, the rabbit may provide the first animal model of human testicular cancer. Furthermore, the sexual behaviour data suggest that the CNS of this species, more so than the rat, may be altered *in utero* by antiandrogen treatment. Male rabbits exposed *in utero* and/or during infancy to vinclozolin failed to show sexual interest in the females, or did not ejaculate (Palmer *et al.*, 2000).

Procymidone, chlozolate and iprodione

When administered by gavage on GD 14 to day 3 after birth at doses ranging from 25 to 200 mg/kg per day, effects were noted in all dosage groups (Ostby *et al.*, 1999). Procymidone reduced AGD in male pups and induced retained nipples, hypospadias, cleft phallus, vaginal pouch and reduced sex accessory gland size in rat offspring. Procymidone also had marked effects on the histology of the dorsolateral and ventral prostatic and seminal vesicular tissues (at 50 mg/kg per day and above). The effects consisted of fibrosis, cellular infiltration and epithelial hyperplasia (Ostby *et al.*, 1999).

Chlozolate and iprodione are dicarboximide fungicides, similar in structure to the antiandrogens vinclozolin and procymidone. However, when chlozolate and iprodione were administered at 100 mg/kg per day from GD 14 to postnatal day (PND) 3, male rat offspring were not demasculinized or feminized at this dosage level (Gray *et al.*, 1999a).

DDT

Background

Although use of DDT has been banned in some countries, some wildlife populations still display incredibly high total DDT residue levels (Elliott *et al.*, 1994; Guillette *et al.*, 1999b; Williams, 1999) as a result of decades of former use of this persistent, bioaccumulating pesticide. In the sampled orchards and fields (Elliott *et al.*, 1994), birds had high tissue concentrations of p,p'-DDE (up to 103 p.p.m. in fat), but these were even higher in fat from birds at Lake Apopka (Florida, USA). The induction of eggshell thinning in avian and reptilian oviparous vertebrates is the most widely known endocrine effect of p,p'-DDE. Eggshell thinning by p,p'-DDE is caused by the induced inhibition of prostaglandin synthesis in the shell gland, causing calcium deposition around the eggshell membranes to be retarded (Lundholm, 1987, 1994; Lundholm and Bartonek, 1992a,b; Guillette, 2000).

In-utero effects of p,p'-DDE in the rat

When p,p'-DDE is administered at 100 mg/kg per day (on GD 14–18) it reduces AGD and induces hypospadias, retained nipples, and reduces weights of androgen-dependent tissues in treated Long-Evans Hooded (LE) and Sprague-Dawley (SD) male rat offspring (Gray *et al.*, 1999a). While these alterations were evident in both rat strains, only the SD strain displayed hypospadias, and other effects of DDE were of a greater magnitude in this strain. Others (You *et al.*, 1998) also found that p,p'-DDE induced antiandrogenic effects on AGD and areola

development in LE and SD rats, and also noted an increased incidence of chronic suppurative prostatitis in treated male progeny (You *et al.*, 1999a)—not an uncommon observation for males exposed *in utero* to an EDC (i.e. PCBs and procymidone). These adverse developmental effects were correlated with fetal rat tissue concentrations of p,p'-DDE ranging from 1 to 2 µg/g on GD 21 and 10–20 µg/g on GD 20 (You *et al.*, 1999b).

Effects of p,p'-DDE administered to pubertal and adult male rats

When p,p'-DDE was administered at 0, 30 or 100 mg/kg per day from weaning until about 50 days of age, PPS was delayed about 5 days in male rats treated with 100 mg/kg per day, but sex accessory weights and serum hormone levels were not significantly altered (Kelce *et al.*, 1995). p,p'-DDE produces even more marked reductions in androgen-dependent tissue weights in the Hershberger assay (100 mg/kg per day for 7 days). In contrast to the positive responses seen with p,p'-DDE in the 'Pubertal Male' and Hershberger assays, the Intact Adult Male rat assay is unable to detect the antiandrogenic activity of this reproductive teratogen (O'Connor *et al.*, 1999). It was also found (Brien *et al.*, 2000) that p,p'-DDE markedly interferes with erectile function, an androgen-dependent process, in an established rat model of apomorphine-induced erections. A single dose of p,p'-DDE (500 mg/kg, i.p.) decreased apomorphine-induced erections for at least 2 weeks. Testosterone supplementation restored function in castrated rats to pre-castrated levels, but the p,p'-DDE-treated rats required four times as much testosterone to recover erections as compared with control males.

Developmental effects of p,p'-DDT and DDE in the rabbit

In the Dutch Belted rabbit, administration of the antiandrogen p,p'-DDT or p,p'-DDE during gestation and/or lactation produces reproductive abnormalities in male offspring (Veeramachaneni *et al.*, 1996; Palmer *et al.*, 2000; Veeramachaneni, 2000). When does were treated daily from GD 15 through 4 weeks post-kindling with p,p'-DDT at 25 or 250 mg/kg, DDT induced cryptorchidism. Atypical germ cells, some resembling carcinoma-in-situ (CIS) cells were noted in the undescended testes from treated male rabbit progeny, CIS cells were characterized by large nuclei with irregular contours and cytoplasmic inclusions and occasional mitotic figures. CIS cells were first identified and described as atypical germ cells in the human testis that later resulted in testicular germ cell tumours (Skakkebaek, 1972). Similar effects were seen when rabbit does were exposed weekly to p,p'-DDT at 25 mg/kg (from GD1 through 6 weeks post-partum) followed by offspring at 10 mg/kg (from post-natal weeks 6 to 12) (Veeramachaneni *et al.*, 1996). In this study, serum concentrations of p,p'-DDT and its metabolite p,p'-DDE in the offspring were 231 and 38 p.p.b. at 8 weeks, and 187 and 37 p.p.b. at 12 weeks, respectively.

Taken together, these data indicate that adverse developmental reproductive effects are seen in rats and rabbits at levels (based on tissue residues) that are within the range reported for the human fetus in the late 1960s, exposed to DDT at that time through legal applications (Wassermann *et al.*, 1967; Curley *et al.*, 1969).

Potential antiandrogenic effects of p,p'-DDE in wildlife

In the alligator, developmental reproductive abnormalities (small penis, abnormal hormone levels, skewed sex ratio with an

increase in the percentage of intersex and decrease in the percentage of male offspring) in Lake Apopka (Guillette *et al.*, 1994, 1995, 1996, 1999a; Crain *et al.*, 1997, 1998) have been associated with p,p'-DDE egg concentrations of 5.8 p.p.m. (Guillette *et al.*, 1999b). It has been postulated that some of these effects, such as the small phallus size in juvenile male alligators, could result from an antiandrogenic effect of p,p'-DDE. It was also suggested (Guillette *et al.*, 1995) that increased abnormalities could result from an 'oestrogenic environment' created by the mixture of pesticides present in the Lake Apopka ecosystem.

High concentrations of p,p'-DDE (Facemire *et al.*, 1995), coupled with an increased susceptibility of the developing reproductive system to this chemical insult associated with a loss of genetic diversity (O'Brien and Yuhki, 1996) may be contributing to the high incidence of undescended testes in the Florida panther.

Linuron

Mechanistic studies

The urea-based herbicide linuron binds rat prostatic and human AR (hAR) and inhibits DHT-hAR-induced gene expression *in vitro* (Cook *et al.*, 1993; Waller *et al.*, 1996b; Lambright *et al.*, 2000; McIntyre *et al.*, 2000). The antiandrogenicity of linuron is quite apparent when administered during gestation (Gray *et al.*, 1999a; Lambright *et al.*, 2000; McIntyre *et al.*, 2000) or in a Hershberger assay (Lambright *et al.*, 2000). Linuron-treatment (100 mg/kg per day) produces robust reductions in testosterone- and DHT-dependent tissue weights in the Hershberger assay (using castrate-immature testosterone propionate-treated male rats; Hershberger *et al.*, 1953) (Lambright *et al.*, 2000). In contrast, the antiandrogenic effects of linuron and p,p'-DDE on androgen-dependent tissue weights are difficult (if not impossible) to detect in the intact adult male rat except at overtly toxic dosage levels (i.e. 200 mg/kg per day; Cook *et al.*, 1993; O'Connor *et al.*, 1999). The fact that the effects of these AR antagonists on androgen-dependent organ weights in the intact adult male rat are only seen at overtly toxic dosages negates the use of this assay as an animal model for screening antiandrogens and androgens, as proposed by some (O'Connor *et al.*, 1999).

Developmental effects of linuron in the rat

In a multigenerational study, the linuron-treated (40 mg/kg per day) offspring (F₁) (Gray *et al.*, 1999a) sired 40% fewer pups, and treated F₁ males had reduced testicular and epididymal weights, and lower testicular spermatid numbers. These effects were surprising because it has been reported in an earlier multigenerational study that linuron did not produce reproductive malformations (Hodge *et al.*, 1968). Others (Khera *et al.*, 1978) also reported that linuron was not teratogenic at dosages up to 100 mg/kg per day. To resolve this discrepancy, linuron was administered at 100 mg/kg per day through days 14–18 of gestation (Gray *et al.*, 1999a). AGD was reduced in male offspring, and the incidence of areolas (with and without nipples) in infant males was increased in linuron-treated males. Linuron-treatment induced epispadias and reduced the size of the androgen-dependent tissues, including the seminal vesicles,

ventral prostate, levator ani/bulbocavernosus muscles, and epididymides, caused agenesis of the caput and/or corpus epididymides, and some testes were atrophic, fluid-filled and flaccid (Gray *et al.*, 1999a; Lambright *et al.*, 2000; McIntyre *et al.*, 2000). Although linuron is an AR antagonist, it produces a profile of effects that curiously resembles those seen with DBP or DEHP treatment (relatively high incidence of testis and epididymal malformations; see below). It is possible that linuron alters sexual differentiation by dual mechanisms of action.

Methoxychlor

In vitro: an antiandrogenic and oestrogenic pesticide

Methoxychlor is a DDT derivative that provides an interesting example of the multiplicity of EDC action. Methoxychlor is metabolically activated to several monohydroxy- and dihydroxy metabolites that display oestrogenic activity (Bulger *et al.*, 1978). Methoxychlor itself is weakly active or inactive *in vitro* in ER binding and transcriptional activation assays (Maness *et al.*, 1998). HPTE, a methoxychlor metabolite, has high affinity for ER α (Waller *et al.*, 1996b). It is an ER α agonist, an ER β antagonist (Maness *et al.*, 1998) and an AR antagonist. In fact, many natural (i.e. oestradiol) and anthropogenic oestrogens (o,p'-DDT) display affinity for AR (Kelce *et al.*, 1995; Waller *et al.*, 1996b), acting as AR antagonists and agonists in *in-vitro* assays (Danzo, 1997; Sohoni and Sumpster, 1998).

Effects of methoxychlor in male and female rats

In the female rat, methoxychlor displays oestrogenic ER α -mediated activity in many tissues, including the uterus, vagina, brain (behaviour) and bone, but not in the hypothalamic–pituitary axis. When administered to male rats from weaning on, methoxychlor reduces food consumption and growth, delays puberty, reduces sex accessory gland, testicular testosterone production *ex vivo*, epididymal size, and epididymal sperm numbers, and stimulates mating behaviour (Gray *et al.*, 1989, 1999c). In the male rat, methoxychlor appears to be acting both as an oestrogen and as an antiandrogen. However, methoxychlor did not produce any malformations in multigenerational studies (Gray *et al.*, 1989). Unlike oestradiol, methoxychlor fails to induce hyperprolactinaemia, inhibit LH or induce pituitary tumours in the male or female rat after long-term, high-dose treatment (Gray *et al.*, 1988, 1989, 1999c).

Antiandrogenic effects of phthalate esters

Effects of phthalate esters in in-vitro and in-vivo screening assays of antiandrogenic and oestrogenic activity

Although some phthalate esters (PEs) alter reproductive development in an antiandrogenic fashion, the mechanism of action does not appear to involve either AR or ER binding, as neither diethylhexyl phthalate (DEHP) nor monoethylhexyl phthalate (MEHP) bind AR (Parks *et al.*, 1999, 2000). While some have suggested that di-n-butyl phthalate (DBP) was oestrogenic (Jobling *et al.*, 1995), it does not stimulate uterine weight (Meek *et al.*, 1997; Gray *et al.*, 1999a), induce

oestrogen-dependent sex behaviour (lordosis), accelerate vaginal opening, or induce constant oestrus in female rats (Gray *et al.*, 1999a).

Effects of PEs on fetal hormone synthesis and associated malformations

When administered during sexual differentiation, DEHP inhibits fetal Leydig cell testosterone synthesis and reduces fetal testosterone concentrations from GD 18 to 2 days after birth. The reduction in testosterone results in a wide range of malformations of the androgen-dependent tissues in male rats, including reduced AGD, retained nipples, hypospadias, cleft phallus, vaginal pouch, agenesis of the gubernacular cords and sex accessory tissues, underdevelopment of levator ani muscles, undescended testis, epididymal agenesis and testicular atrophy. It has been reported (Arcardi *et al.*, 1998) that DEHP at 3 mg/kg per day produced testicular histopathological alterations in male rat offspring, while others (Poon *et al.*, 1997) reported effects at ~37 mg DEHP/kg per day. Pubertal treatment with DBP or DEHP delays puberty in the male rat (Gray *et al.*, 1999); however, the testis is much less sensitive to the effects of PEs during adult life. It has also been reported (Mylchreest *et al.*, 1998, 1999; Gray *et al.*, 1999a) that in-utero DBP treatment produced malformations in rat offspring, with treatment on GD 16–19 being most effective (Gray *et al.*, 1999a). When administered during sexual differentiation, benzyl butyl phthalate (BBP) and diisononyl phthalate (DINP), but not diethyl phthalate (DEP), dimethyl phthalate (DMP) or dioctyl-terephthalate (DOTP), also cause male reproductive tract malformations (Gray *et al.*, 2000). Male pups from the DEHP and BBP groups displayed shortened AGDs and reduced testis weights. Infant male rats in the DEHP, BBP and DINP groups displayed female-like areolas/nipples. The percentages of males with malformations was 91% for DEHP, 84% for BBP and 7.7% in the DINP group. DEHP and BBP produced a wide range of malformations of the external genitalia, sex accessory glands, epididymides and testes. In the DINP group, two of 52 males displayed nipples, another male displayed bilateral testicular atrophy, and a fourth male displayed unilateral epididymal agenesis with hypospermatogenesis and scrotal fluid-filled testis, devoid of spermatids. In contrast to the above studies, developmental toxicity/teratology studies which only examine fetal animals, failed to detect these malformations (Ema *et al.*, 1993, 1994).

PE in serum and tissues in highly exposed humans and treated rats

Although the current focus is on PEs in toys and cosmetics, these chemicals are ubiquitous in the environment. Some groups have particularly high DEHP exposures, with serum concentrations of MEHP in the p.p.m. range (i.e. patients undergoing dialysis or receiving transfusions, as well as occupational exposures). It was reported in a recent survey that human urine contained surprisingly high (p.p.m. levels) of phthalate monoesters (MBP) (Blount *et al.*, 2000). A study of the exposure of newborn infants to DEHP and MEHP resulting from transfusions found plasma concentrations of DEHP between 3.4 to 11.1 µg/ml, while MEHP ranged from 2.4 to 15.1 µg/ml (Sjoberg *et al.*, 1985a). Similar concentrations of DEHP were observed in the serum of newborn infants after transfusion (6.1 to 21.6 µg DEHP per ml serum) (Plonait *et al.*,

1993). For comparison, the concentrations of DEHP and MEHP in the serum 3 h after a single oral dose of 2.8 g/kg (which induces testicular lesions) are only about four-fold higher than those found in infants on dialysis, being 8.8 ± 1.7 and 63.2 ± 8.7 µg per ml respectively (Teirlynck and Belpaire, 1985). In another study, it was reported that the testicular damage caused by treatment with 1 g DEHP/kg/day for 14 days to 25-day-old rats (40- and 60-day-old rats were unaffected) was associated with concentrations of MEHP in the plasma ranging from 48 to 152 µg/ml (Sjoberg *et al.*, 1985b). As the dosing regimen used by these authors was similar to that in the present study (0.75 g/kg per day for 12 days), it could be predicted that maternal serum concentrations of MEHP in our study would range from 35 to 115 µg/ml—about 10-fold higher than the concentrations seen in dialysis patients.

Developmental effects of DBP in the rabbit

In a study using Dutch-belted rabbits (Higuchi *et al.*, 1999), it was found that in-utero exposure (GD 15–30) to DBP adversely affected reproductive development of the male fetus. At 12 weeks of age, accessory gland weight and AGD were reduced in treated groups, and one animal had undescended testes, ambiguous genitalia, hypospadias and agenesis of the prostate (Veeramachaneni, 2000).

Widespread species sensitivity to PE-induced testicular toxicity

The PEs cause testicular alterations in young adult males from several mammalian species. Affected mammals include several strains of rat (LE, SD and Wistar), mice [including peroxisome proliferator activated receptor α (PPAR α)-knockouts; Ward *et al.*, 1998], hamsters (Gray *et al.*, 1982), ferrets (Lake *et al.*, 1976), guinea pigs (Gray *et al.*, 1982) and rabbits (Higuchi *et al.*, 1999). The testicular toxicity of the PEs appears unrelated to the species-specific expression of PPAR α (Ward *et al.*, 1998).

Inhibitors of steroid hormone synthesis

Background

Some classes of fungicides inhibit sterol synthesis and fungal growth by inhibiting cytochrome (CY) P450 enzymes, especially 14 α demethylation of lanosterol in the sterol pathways. These fungicides are fairly non-specific inhibitors of CYP450 enzymes, so the endocrine effects induced are not always limited to the reproductive system and often include effects on adrenal and liver steroid metabolism in vertebrates and ecdysteroid synthesis in invertebrates. Male pseudohermaphroditism was produced in rats with inhibitors of steroid 17 α -hydroxylase and C17–20 lyase (Goldman *et al.*, 1976). In-utero treatment with drugs that inhibit 5 α -reductase (which is not a P450 enzyme) blocking the conversion of testosterone to dihydrotestosterone produce reproductive malformations in the male rat offspring (Weir *et al.*, 1990; Imperato-McGinley *et al.*, 1992).

Ketoconazole

The antifungal drug ketoconazole inhibits various enzymes which belong to the CYP450-dependent mono-oxygenases, such as side chain cleavage of cholesterol, 11 β -hydroxylase in the adrenal, and 17 α -hydroxylase and C17–20 lyase in the testes. Human testicular mono-oxygenase activities *in vitro* are reduced by 50% from

3.1 $\mu\text{mol/l}$ ketoconazole. Others (Schurmeyer and Nieschlag, 1984) demonstrated that ketoconazole and other imidazole fungicides inhibited testosterone production in men; it was also reported that ketoconazole was useful in the treatment of ovarian hyperandrogenism in women (Pepper *et al.*, 1990). Ketoconazole also has been shown to alter hepatic steroid catabolism in mice (Wilson and Leblanc, 2000).

Administration of ketoconazole from GD 14–18 at 100 mg/kg per day causes whole litter loss (Gray *et al.*, 1999a), an effect that is likely caused by an inhibition of progesterone synthesis. When the dosage of ketoconazole was lowered, the onset of parturition was delayed and the numbers of live pups were reduced at 25 and 50 mg/kg per day; however, treated male pups did not display any reproductive alterations at any dose level (Gray *et al.*, 1999a).

Fenarimol

Some fungicides like fenarimol inhibit the CYP450 enzyme aromatase, preventing the conversion of androgens to oestrogens (Hirsch *et al.*, 1987). When fenarimol was administered continuously from weaning, treated male rats displayed altered mating behaviour (failure to mount a sexually receptive female). As such behaviour is dependent upon the conversion of testosterone to oestradiol in the brain of the male rat, this effect likely resulted from an inhibition in the production of oestrogens in the brain (Gray and Ostby, 1998). Serum hormone concentrations were unaffected by fenarimol treatment, but male rats from the highest dosage group displayed reduced sex accessory gland weights and increased liver size—effects that could indicate that fenarimol was inhibiting CYP450 enzymes in the testis and liver. This chemical also reduced ovarian weight and caused delayed parturition in female rats. In contrast, the F₁ generation, exposed indirectly only during pregnancy and lactation, was unaffected.

TCDD and PCBs: putative antiandrogens

Aryl hydrocarbon (Ah) receptor agonists such as TCDD, PCBs and PCDFs induce developmental toxicity in humans, non-human primates, rodents, fish, mink and other wildlife species (Golub *et al.*, 1991); effects are expressed at p.p.t. concentrations. 2,3,7,8-TCDD and other toxicants bind to a cellular steroid hormone-like receptor, termed the Ah receptor, and alters many hormone levels, growth factors and/or their receptors, as well as hormone synthesis (Birnbaum, 1994) but does not bind AR.

In-utero exposure to very low doses of 2,3,7,8-TCDD produces infertility in rodent progeny (Khera and Ruddick, 1973; Murray *et al.*, 1979; Mably *et al.*, 1992a–c; Bjerke and Peterson, 1994; Bjerke *et al.*, 1994b,c; Gray *et al.*, 1995). A single dose of TCDD ranging from 50 ng to 2 $\mu\text{g/kg}$ during sex differentiation of the rat or hamster results in a number of reproductive alterations in male and female progeny (Gray and Ostby, 1995; Gray *et al.*, 1997a,b; Wolf *et al.*, 1999). The alterations include delayed puberty, reduced ejaculated and epididymal sperm numbers, and reductions in the size of the ventral prostate, seminal vesicle and testis. TCDD-treated males in the above studies did not display hypospadias, reduced AGD or areolas, although epididymal agenesis is induced when the dams are treated with a higher dosage of TCDD (3 $\mu\text{g/kg}$ on GD 15; Wilker *et al.*, 1996). In female offspring, in-utero treatment with TCDD induced clefting of the clitoris with a mild degree of hypospadias in females and a

permanent ‘thread’ of tissue across the opening of the vagina of the progeny (Gray *et al.*, 1997b). Female progeny, treated earlier in gestation (day 8) with TCDD displayed reduced fecundity, a high incidence of constant oestrus, and cystic endometrial hyperplasia at middle-age. Female hamster offspring, from mothers treated with TCDD on day 11 of gestation, also display clitoral clefting and reduced fertility as a result of several functional reproductive problems. These studies indicate that development of the reproductive system is severely altered when fetal TCDD concentrations reach 50 p.p.t. (Hurst *et al.*, 1996, 1997). The two lowest TCDD dosages (0.2 and 0.05 $\mu\text{g/kg}$ on GD 15) produced fetal concentrations of 5 and 13 p.p.t., which lowered sperm counts in male offspring and induced reproductive tract anomalies in female offspring (Gray *et al.*, 1997a,b). The PCB congener 169 is an Ah receptor agonist which has about 0.001 times the potency of TCDD. PCB 169 treatment during pregnancy alters reproductive development of LE hooded male and female rats in a manner nearly identical to TCDD (Gray *et al.*, 1999a).

Although the overall profile of effects in the rats exposed *in utero* to these Ah receptor agonists bears some resemblance to that seen in animals exposed to antiandrogens such as vinclozolin or the phthalates, the fact that Ah agonists fail to reduce AGD or induce areolas, retained nipples, and male hypospadias suggests that TCDD and the dioxin-like PCBs may be affecting the developing reproductive tract via alternative pathways from the AR.

Phytochemicals: plant antiandrogens

Plants contain phytochemicals that alter reproductive performance by displaying antiandrogenic activity. Permixon is an antiandrogenic extract of the saw palmetto used to treat androgen-dependent prostatic diseases (Wilt *et al.*, 1998). It has been reported to act as an AR antagonist (Carilla *et al.*, 1984), to inhibit 5 α -reductase activity (Bayne *et al.*, 1999), and to inhibit oestradiol plus testosterone-induced prostatic growth in castrate male rats (Paubert-Braquet *et al.*, 1996). In contrast, it was reported (Rhodes *et al.*, 1993) that Permixon did not inhibit testosterone- or dihydrotestosterone-stimulated prostate growth in castrated rats, suggesting that it was not antiandrogenic. The developmental effects of this extract have not been determined.

Environmental androgens confirmed

Although numerous pesticides and toxic substances have been identified as ‘environmental antiandrogens’, it is now known that there also are androgens in the environment. Kraft mill effluent (KME) from the Fenholloway river in Florida contains a chemical mixture that binds AR and induces androgen-dependent (but not glucocorticoid-dependent) gene expression *in vitro* (Parks *et al.*, 2001). While masculinized female mosquitofish (*Gambusia holbrooki*) were detected 30 years ago in rivers in Florida downstream from Kraft pulp and paper mills (Davis and Bortone, 1992), the endocrine basis for this effect was only recently confirmed (Howell, 1999; Parks *et al.*, 2000b, 2001). Some 90% of the female mosquitofish from one of the KME sites on this river display some degree of masculinization of the anal fin (Orlando *et al.*, 1999; Parks *et al.*, 2001). Masculinization also can

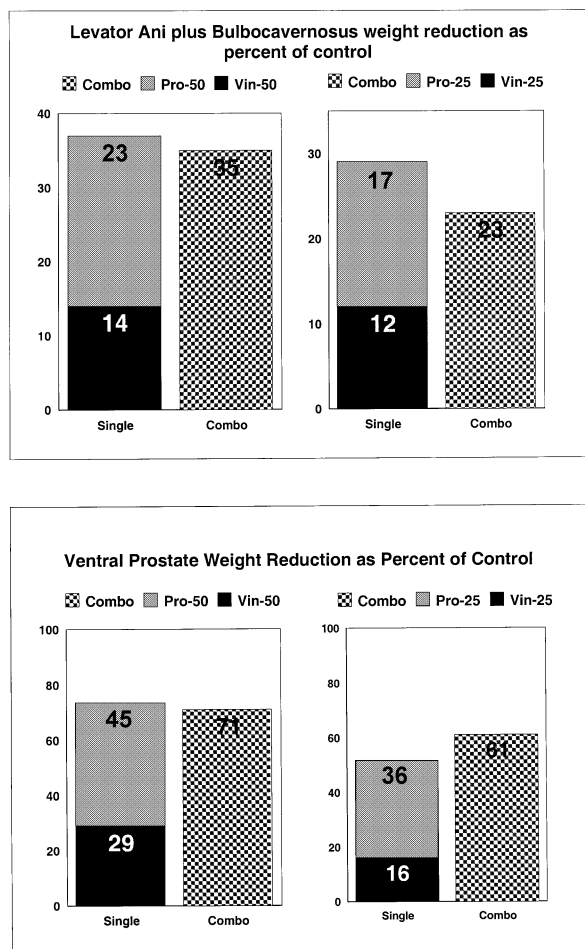


Figure 3. The effects of procymidone (Pro) and vinclozolin (Vin) alone (25 and 50 mg/kg per day) versus mixtures on ventral prostate and levator ani plus bulbocavernosus weight. In all cases shown here, the effects were additive. Organ weights were reduced by the individual compounds or the mixtures in the Hershberger assay using castrate-testosterone propionate (TP)-treated immature male rats (Price *et al.*, 2000).

be achieved in female mosquitofish and killifish in the laboratory with exposures to KME and from microbial (*Mycobacterium smegmata*) metabolites of phytosterols present in the KME (Davis and Bortone, 1992). This novel endocrine activity appears to be associated with some pulp and paper mills on a world-wide basis. Masculinized female fish have been seen in the field in several sites contaminated with KME, including other rivers in Florida (Davis and Bortone, 1992) and bodies of water in Canada, North America and Scandinavia (Wells *et al.*, 1999; Larsson *et al.*, 2000).

Effects of mixtures of antiandrogens: cumulative risk

The Food Quality Protection Act of 1996 mandated that the risk assessment process consider combinations of chemicals that act via the same mechanism, rather than evaluate the potential risk on a individual basis. As we all are aware, no one person is exposed to one chemical at a time, but rather we exposed to mixtures of pesticides and toxic substances at once from many different sources. Hence, we have initiated several studies to examine if mixtures of antiandrogens act in an additive or synergistic

manner. In one of the studies, two 'antiandrogenic' chemicals that altered fetal development via different mechanisms of action were combined to see if this combination also produced cumulative responses.

Cumulative effects of vinclozolin plus procymidone

In the first study, the effects of graded doses of the AR antagonists procymidone and vinclozolin, ranging from 25 to 100 mg/kg per day individually or together, were evaluated in the Hershberger assay (castrate-immature testosterone-treated male rats for 7 days) (Price *et al.*, 2000). At low dosages the mixtures of vinclozolin plus procymidone reduced ventral prostate and levator ani weights in an additive fashion (Figure 3). When the higher doses were combined, the effects were less than additive because each chemical completely inhibited the effects of testosterone by itself. These results provide scientific support for the concept that risk assessments for pesticides that act via a common mechanism of action should consider the 'cumulative' risk of the mixture as opposed to examining risk on an individual chemical-by-chemical basis.

Cumulative effects of an AR antagonist and an inhibitor on fetal testosterone synthesis in utero

One major question in this area is; how all-encompassing should be the definition of 'common mechanism of action'? Should chemicals such as vinclozolin and phthalates such as DBP (which are not AR ligands) be evaluated together in a cumulative risk assessment? To address this question, another mixture study was conducted using an AR antagonist and an inhibitor of fetal testosterone synthesis. When procymidone (50 mg/kg per day, GD 14–18) and DBP (500 mg/kg per day) were administered individually or in combination, hypospadias was present in 1.5% and 0% of the male offspring in the procymidone and DBP groups respectively, whereas 49% of the male offspring in the combination group (procymidone-50 plus DBP 500) displayed this malformation. The incidence of males with a vaginal pouch displayed similar additivity. While these effects may appear to be synergistic, it is believed that these are additive responses to the two toxicants (Figure 4) (Furr *et al.*, 2000). It is clear that toxicants that alter the development of similar reproductive tissues during the same critical period, but act via different mechanisms of action, will produce cumulative effects.

Endocrine screening assays for androgens and antiandrogens

In 1996, legislation was passed that requires that the USEPA establish validated screening and testing procedures for oestrogens and other EDCs. For androgens and antiandrogens, the Endocrine Disruptor Screening and Testing Advisory Committee recommended in 1998 that high priority chemicals be screened (Tier 1) using: (i) AR binding and/or AR-dependent transcriptional activation; (ii) in-vitro assays of steroidogenesis; and (iii) the in-vivo Hershberger assay (Hershberger *et al.*, 1953). Several alternative in-vivo assays were proposed, but these assays are less sensitive to AR ligands and, in addition, none of the alternative assays has been used as extensively or standardized and validated as the Hershberger assay. The Hershberger assay was officially sanctioned for screening in the early 1960s (Dorfman, 1962). This

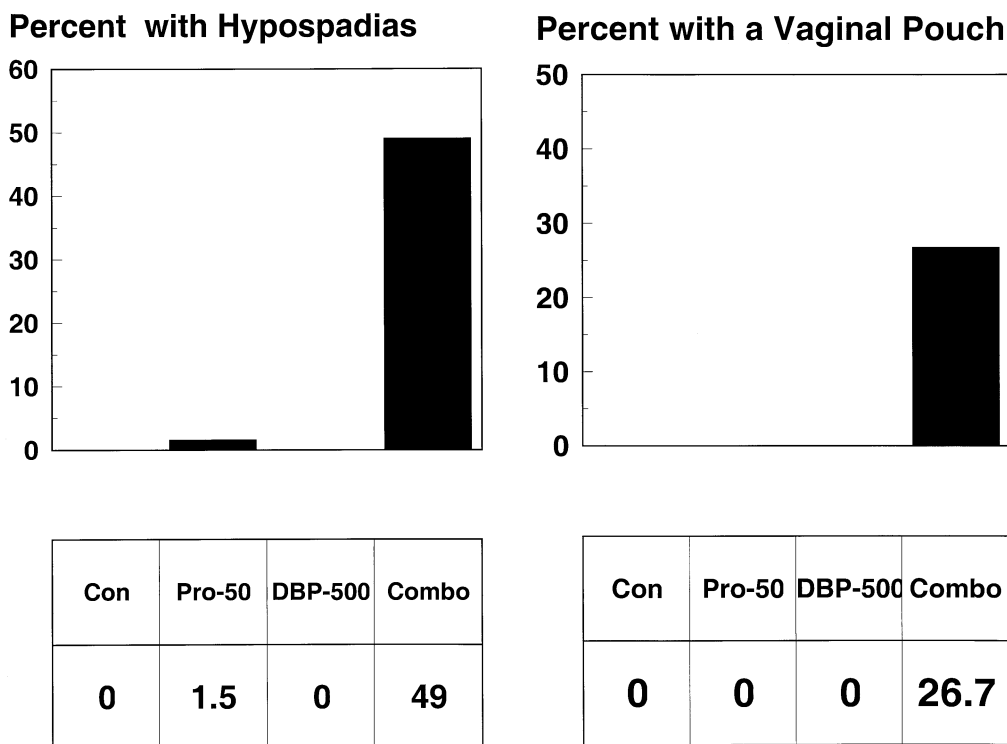


Figure 4. A mixture of two antiandrogens that act via different mechanisms on the differentiation of the reproductive tract of the fetal male rat acts in an additive manner, or greater. Procymidone (Pro) is an AR antagonist. Administered here at 50 mg/kg per day from GD 14–18, only one animal displayed hypospadias (A) and none had a vaginal pouch (B). DBP treatment at 500 mg/kg per day during this interval did not induce hypospadias or a vaginal pouch in any males. However, the combination of Pro plus DBP (Combo) was quite effective, and many males were malformed (Furr *et al.*, 2000). Con=controls.

assay detects androgens by weighing androgen-dependent tissues in immature-castrate male rats that have been dosed for 7–10 days with the test compound. Antiandrogens are detected by examining the ability of the compound to inhibit testosterone-induced growth of the sex accessory tissues in the castrate-immature male rat. Currently, the Organization for Economic Cooperation and Development (OECD) is in the first phase of standardization and validation of the Hershberger assay. The effects of several dosages of testosterone propionate are being compared in a standardized protocol by 17 laboratories, world-wide.

Only chemicals positive in the Tier 1 Screening Battery would be further evaluated in Tier 2 Testing. In Tier 2, chemicals would be evaluated in an ‘enhanced’ multigenerational test. As indicated earlier, the developmental toxicity/teratology test, which only examines fetal animals, are too insensitive to be used to detect antiandrogens. Most, if not all, of the reproductive tract malformations go undetected in this test protocol. In addition, older multigenerational studies also suffer to some degree because they often lacked an assessment of many androgen-sensitive endpoints. Newer multigenerational testing protocols have overcome some, but not all, of the deficiencies of the older guidelines. One of our most significant concerns with the current multigenerational test is that too few F₁ (offspring with developmental exposure) animals are examined after maturity. A standard multigenerational protocol uses 20 litters per dosage group but only examines one animal per sex, per litter. In addition, reproductive tract histopathology is only evaluated in 10 animals per sex, per dose—far too few to detect anything but the most

profound reproductive teratogens. In our ‘transgenerational’ protocol, fewer litters (7–10 per dose group) are used and all the animals in the litter are examined. Our protocol actually uses fewer animals, but provides more statistical power to detect effects in the F₁ generation. In addition, AGD is measured at 2–3 days of age and areolas/nipples at 12–13 days of age in the F₁ generation because these endpoints are extremely useful ‘bio-markers’ of effect. Not only are the effects on AGD and nipples permanent, but they also are highly correlated with malformations and other alterations in androgen-dependent tissues (A.K.Hotchkiss *et al.*, unpublished results).

Summary and research needs for EDCs

Anthropogenic substances in the environment (pesticides, other toxic substances, phytochemicals, pharmaceuticals) with EDC activity have been shown to alter sexual differentiation and/or reproductive function in fish, wildlife and domestic animals. In many cases, such effects can be reproduced by laboratory investigations using surrogate animal models, and several distinct mechanisms of action have been elucidated. For some EDCs, the dosage levels which produce effects in laboratory animals overlap with levels seen in the human population (Hurst *et al.*, 1996, 1997; Wasserman *et al.*, 1967; Curley *et al.*, 1969). If humans were affected by EDCs, how might these effects be expressed? We can make predictions based on: (i) the malformations produced in humans by drugs with EDC activity; and (ii) the effects on animals in the field and the laboratory. In many cases,

we would not expect global changes in human reproductive health. Except for high-dose accidental or occupational exposures, we should anticipate subtle, functional, latent reproductive alterations in the most highly exposed individuals or their progeny, with malformations occurring only in the most susceptible individual(s). Research is needed to address some of the major uncertainties in the risk assessment for EDCs, which include: (i) what are the characteristics of the dose-response curves in the low dose range (threshold or not, monotonic or U-shaped); (ii) how do tissue levels in affected laboratory animals compare with human levels?; (iii) how will real-world mixtures of EDCs and non-EDCs interact?; and (iv) what additional mechanisms of action will be displayed by EDCs?

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