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Mixture effects at very low doses with combinations of anti-androgenic pesticides, antioxidants, industrial pollutant and chemicals used in personal care products

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ABSTRACT

Many xenobiotics have been identified as *in vitro* androgen receptor (AR) antagonists, but information about their ability to produce combined effects at low concentrations is missing. Such data can reveal whether joint effects at the receptor are induced at low levels and may support the prioritisation of *in vivo* evaluations and provide orientations for the grouping of anti-androgens in cumulative risk assessment. Combinations of 30 AR antagonists from a wide range of sources and exposure routes (pesticides, antioxidants, parabens, UV-filters, synthetic musks, bisphenol-A, benzo(a)pyrene, perfluorooctane sulfonate and pentabromodiphenyl ether) were tested using a reporter gene assay (MDA-kb2). Chemicals were combined at three mixture ratios, equivalent to single components' effect concentrations that inhibit the action of dihydrotesterone by 1%, 10% or 20%. Concentration addition (CA) and independent action were used to calculate additivity expectations. We observed complete suppression of dihydrotestosterone effects when chemicals were combined at individual concentrations eliciting 1%, 10% or 20% AR antagonistic effect. Due to the large number of mixture components, the combined AR antagonistic effects occurred at very low concentrations of individual mixture components. CA slightly underestimated the combined effects at all mixture ratios. In conclusion, large numbers of AR antagonists from a wide variety of sources and exposure routes have the ability of acting together at the receptor to produce joint effects at very low concentrations. Significant mixture effects are observed when chemicals are combined at concentrations that individually do not induce observable AR antagonistic effects. Cumulative risk assessment for AR antagonists should apply grouping criteria based on effects where data are available, rather than on criteria of chemical similarity.

Introduction

Cryptorchidisms and hypospadias are the most frequent congenital malformations in boys. Although there are marked differences in regional prevalence, several countries have experienced increases in the incidence of cryptorchidisms (reviewed in: Main et al., 2010) and hypospadias (Boisen et al., 2004; Nassar et al., 2007; Nelson et al., 2005; Pierik et al., 2004). Alcohol consumption, low birth weight, premature birth and diets lacking in protein (Pierik et al., 2004) are well recognised risk factors, but these alone cannot explain the continuing rises in incidence. Skakkebaek et al. (2001) have proposed that cryptorchidism and hypospadias are part of the testicular dysgenesis syndrome, hypothesised to arise from insufficient androgen action in foetal life, and that exposures to anti-androgenic chemicals are an etiological factor. Although evidence for links between exposure to specific chemicals and testicular dysgenesis syndrome in humans is currently limited (reviewed in: WHO, 2012), support for the plausibility of an involvement of androgen receptor (AR) antagonists comes from experimental studies using a developmental toxicity model in the rat. In foetal life, steroidal androgens are key drivers of the differentiation of the Wolffian duct system into the vas deferens, epididymis, seminal vesicles and external genitalia. Exposure of male rats to AR antagonists and other anti-androgens in foetal life leads to incomplete masculinisation and severe malformations of the reproductive organs, similar to some of the disorders seen in humans,

Abbreviations: AR, androgen receptor; CA, concentration addition; DHT, dihydrotestosterone; IA, independent action; IC01, IC10, and IC20, concentrations that inhibit the androgenicity of DHT by 1, 10 or 20%.

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such as cryptorchidisms and hypospadias (*e.g.* Gray et al., 1999; Hass et al., 2007). These observations have provided the stimulus to assess human health risks associated with AR antagonists.

The realisation that humans are typically exposed to numerous anti-androgens simultaneously (Schlumpf et al., 2010; Woodruff et al., 2011) has motivated the consideration of possible combination effects. In animal experiments, anti-androgens are known to produce combination effects (Christiansen et al., 2009, 2012; Hass et al., 2007; Metzdorff et al., 2007; Rider et al., 2008), but there are obvious limitations to studying the joint effects of larger numbers of agents *in vivo*, even though such information is essential for risk assessment. However, to predict the effects of large multi-component mixtures on the basis of the toxicity of its components will stretch the resources for *in vivo* studies. Such resource limitations do not come into play with *in vitro* assays with AR responsive reporter gene constructs and their use has considerably advanced our knowledge about the ways in which AR antagonists can act together (Birkhoj et al., 2004; Ermler et al., 2011; Kjærstad et al., 2010; Orton et al., 2012). The experimental results with anti-androgen mixtures have stimulated interest in cumulative risk assessment for these chemicals (NRC, 2008). Cumulative risk assessment cannot proceed without addressing which chemicals should be considered together, and which criteria should be used to build common assessment groups. In the USA, chemicals with similar structures have been grouped together (USEPA, 2006a, 2006b, 2006c, 2007, 2011), but more recently, alternative approaches which place an emphasis on common adverse outcomes have been suggested (EFSA, 2013; NRC, 2008). Although the data published in the literature show that combination effects can arise from quite diverse AR antagonists (Ermler et al., 2010; Orton et al., 2012), the development of common assessment groups for these agents will also require evidence that chemicals from a range of sources and exposure routes can together antagonise the AR.

A combination of 30 AR antagonists was selected for testing, which comprised 13 pesticides (herbicides, insecticides, fungicides) and 17 non-pesticides (antioxidants, parabens, UV-filters, synthetic musks, bisphenol A (BPA), benzo(a)pyrene (BaP), perfluorooctane sulfonate (PFOS) and pentabromodiphenyl ether (BDE100)). While human exposure to the 13 selected pesticides was inferred from their wide use in the EU (no biomonitoring data available: Orton et al., 2011), the 17 non-pesticides were selected based on their high levels in human tissues (Ermler et al., 2011). These chemicals together covered a wide range of sources and exposures routes, including oral (pesticides, antioxidants: McKinlay et al., 2008; BPA: Geens et al., 2012), dermal (UV filters: Giokas et al., 2007; synthetic musks: Roosens et al., 2007; parabens: Darbre and Harvey, 2008; brominated flame retardants: Buttke et al., 2013) and inhalation (BaP: Ravindra et al., 2008). The large number of chemicals included in our mixtures provided the opportunity to assess the combined effects of AR antagonists at low concentrations, particularly at concentrations where the effects of the single components are below the detection limit of the assay. Such data is important to contribute to future efforts of modelling the effects of untested mixtures composed of xenobiotics with known anti-androgenicity at relevant concentrations.

We used a fixed-mixture ratio experimental design, in which we employed two common concepts for predicting the (additive) effects of mixtures: concentration addition (CA, also called dose addition) and independent action (IA, also called response addition). CA assumes that all compounds have a similar mechanism of action (*e.g.*, binding to the same receptor), whereas IA presumes that all mixture components affect the same endpoint *via* different sites or modes of action (dissimilar action). Both additivity models assume that there is no interaction between the compounds, neither on a physico-chemical level nor in their toxicokinetics and toxicodynamics. If this condition is fulfilled, agreement between observed and expected outcomes can be expected (for review see: Kortenkamp, 2007). Although there are exceptions (Christiansen et al., 2009; Kjærstad et al., 2010), the majority of studies have shown that the effects of mixtures can be approximated fairly well by using the concept of CA when the effects of individual mixture components are known (Christiansen et al., 2008; Ermler et al., 2011; Hass et al., 2007; Howdeshell et al., 2008; Orton et al., 2012). In light of the features of the *in vitro* AR antagonist assay used here, it can be hypothesised that CA would be an appropriate prediction concept, but we also calculated mixture effects by using IA for comparative purposes.

To realise the aims of our low dose mixture experiments, we had to develop criteria for what should constitute a "low dose". One option was to choose concentrations of all single mixture components associated with effect magnitudes around the limit of detection of the MDA-kb2 assay. We previously reported that the statistical power afforded by the MDA-kb2 assay in our laboratory can reliably detect a reduction by 10% of the effects of the reference androgen dihydrotesterone (DHT) (Ermler et al., 2010). Therefore, we tested two effects levels (10% inhibition and 20% inhibition) where associated concentrations of individual components could be detected statistically. In addition, we were interested in examining effects at 1% inhibition, as this results in very low concentrations of individual pollutants (range: 0.012–39.8 μM, Table 1), which may be more environmentally relevant. Since such small effects cannot be measured directly with the MDA-kb2 assay, the respective effect concentrations had to be estimated by regression. This is the first study to investigate mixtures of such a large number of components and how such mixtures behave when combined at very low levels.

Table 1
Chemicals selected for mixture studies. Shown are effect concentrations (mole/L) for individual mixture components required to produce 1% AR antagonistic effects (IC01). The concentrations at which these chemicals are present in the three mixtures where these produce a combined effect of 10% AR antagonism are also shown ("IC01 mix", "IC10 mix", "IC20 mix").

	Individual	Concentration in mixture at 10% inhibition		
Compound	IC01	IC01 mix	IC10 mix	IC20 mix
3-BC	4.50E−06	7.68E−07	8.26E−07	4.97E−07
4-MBC	7.63E−06	1.30E−06	1.10E−06	6.14E−07
AHTN	1.52E−06	2.60E−07	3.20E−07	2.35E−07
BDE100	7.72E−08	1.32E−08	2.37E−08	1.75E−08
Benzo(a)pyrene	2.93E−08	5.01E−09	4.15E−08	6.55E−08
Benzophenone 2	6.70E−08	1.14E−08	3.29E−08	3.24E−08
Benzophenone 3	3.63E−06	6.21E−07	4.67E−07	2.77E−07
BHA	1.31E−06	2.24E−07	2.59E−07	1.87E−07
BHT	1.86E−06	3.17E−07	1.12E−06	9.35E−07
Bisphenol A	4.08E−07	6.97E−08	6.90E−08	4.68E−08
Chlorpropham	2.59E−06	4.43E−07	3.77E−07	2.41E−07
Cyprodinil	3.83E−06	6.55E−07	7.42E−07	4.59E−07
Dimethomorph	6.01E−08	1.03E−08	1.12E−08	8.05E−09
Ethyl paraben	3.98E−05	6.80E−06	4.10E−06	2.18E−06
Fenhexamid	8.22E−08	1.41E−08	6.32E−08	6.07E−08
Fludioxonil	1.54E−07	2.64E−08	3.30E−08	2.50E−08
HHCB	3.83E−07	6.55E−08	1.15E−07	9.74E−08
Imazalil	2.91E−07	4.98E−08	1.24E−07	9.77E−08
Linuron	2.75E−07	4.70E−08	6.32E−08	5.03E−08
Methiocarb	2.28E−06	3.90E−07	3.26E−07	2.03E−07
Methyl paraben	2.69E−05	4.59E−06	4.25E−06	2.76E−06
n-Butyl paraben	1.73E−05	2.95E−06	1.91E−06	9.70E−07
n-Propyl paraben	3.22E−05	5.51E−06	3.03E−06	1.53E−06
PCB138	1.27E−06	2.17E−07	1.80E−07	1.14E−07
PFOS	4.89E−06	8.36E−07	7.64E−07	4.36E−07
Phenylphenol	3.28E−07	5.61E−08	1.31E−07	1.03E−07
Pirimiphos-methyl	1.07E−06	1.84E−07	2.31E−07	1.73E−07
Pyrimethanil	3.79E−06	6.48E−07	1.06E−06	8.83E−07
Tebuconazole	6.56E−07	1.12E−07	1.24E−07	8.79E−08
Vinclozolin	1.26E−08	2.15E−09	5.89E−09	4.93E−09
SUM (=IC10)		2.72E−05	2.19E−05	1.34E−05

Abbreviations: BDE = pentabromodiphenyl ether; BHA = butylated hydroxyanisole; PFOS = perfluorooctane sulfonate; 3-BC = benzylidene camphor; 4-MBC = 4-methylenbenzylidene camphor; BHT = butylated hydroxytoluol.