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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.

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 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.

Methods

Test compound selection. We have previously shown that 24 current use and environmentally relevant pesticides are AR antagonists (Orton et al., 2011), and that mixtures of these pesticides act in combination (Orton et al., 2012). We have also shown that 17 non-pesticidal pollutants are anti-androgenic and produce combination effects (Ermler et al., 2011). In the present study, we have combined the 13 pesticides assessed earlier (Orton et al., 2011) with a mixture of 17 non-pesticide AR antagonists (Ermler et al., 2011). The selected chemicals are listed in Table 1.

Chemicals. Dihydrotestosterone (DHT; >97% purity) was purchased from Steraloids Ltd. (Croydon, Surrey, UK). Dimethomorph and methiocarb were purchased from Greyhound Chromatography and Allied Chemicals (>98.7% purity; Birkenhead, Merseyside, UK). Ethyl paraben and methyl paraben were purchased from Acros Organics (>99% purity; Geel, Belgium) and hexahydrohexamethylcyclopentabenzopyran (galaxolide/HHCB), 6-acetyl-1,1,2,4,4,7-hexamethyltetraline (tonalide/AHTN) and 2,2′,4,4′,6-pentabromodiphenyl ether (BDE100) from LGC Promochem (>98% purity; Teddington, Middlesex, UK). 3-Benzylidene camphor (3-BC) was purchased from Induchem AG (>97% purity; Volketswil, Switzerland) and 4-methylenbenzylidene camphor (4-MBC) from Merck & Co (>98% purity; Hertfordshire, UK). 2,2′,3,4,4′,5′-Hexachlorobiphenyl (PCB138) was purchased from Riedel-de-Haen (>99% purity; Hanover, Germany). All other chemicals (>97% purity) were purchased from Sigma Aldrich (Poole, Dorset, UK). Ethanol (>99.7% purity) was obtained from VWR International Ltd. (Leicestershire, UK). All test compounds were dissolved in ethanol to make stock solutions to be used in the assays.

MDA-kb2 assay. MDA-kb2 cells are a human breast cancer cell line stably transfected with an androgen responsive firefly luciferase reporter gene (American Tissue Culture Collection, ATCC, LGC standards, Teddington, UK: Wilson et al., 2002). We have adapted and optimized the assay to meet the demands of experimental mixture studies, in terms of high reproducibility and minimal experiment-to-experiment variation. Details of the procedure, together with power analyses were published previously (Ermler et al., 2010). Briefly, cells were seeded at a concentration of 1×10^5 cells/mL in phenol red-free Leibowitz-15 medium (Invitrogen Ltd., Paisley, UK) containing 10% (charcoal-stripped) foetal calf serum (Invitrogen Ltd.) in white luminometer plates. After 28 h of treatment, luciferase activity was determined with SteadyGlo assay reagent (Promega UK Ltd., Southampton, Hampshire, UK) and measured in a plate reader (FLUOstar Optima, BMG Labtech GmbH, Offenburg, Germany). To obtain concentration–response relationships, the effects of test compounds on the luminescence produced by fixed concentrations (0.25 nM DHT) were investigated. A reduction of DHT luminescence in concentration ranges not associated with cytotoxicity (see below) was interpreted as evidence for AR antagonism. Cells were exposed to eight serial dilutions of the selected chemicals either in the presence or the absence of DHT (0.25 nM). Concentration ranges in which the test chemicals exhibited AR antagonistic effects were established initially through screening in the range of 1.17 nM–150 μM, followed by testing of finely spaced concentrations according to the potency and toxicity of each individual mixture component. The following controls were run on each plate: media, media with ethanol (0.25%), DHT (0.25 nM) and DHT serial dilutions (0.009–20 nM) in media with ethanol (0.25%). As positive control for an AR antagonist we used procymidone (0.005–3.2 μM) with DHT (0.25 nM) in media and ethanol (0.25%). All concentrations were tested in duplicate over two plates within each experiment, and were repeated in at least four independent experimental sessions. To enable the pooling of data from different experimental sessions, luminescence readings were normalised to the readings with DHT alone (0.25 nM) and this was taken as the maximum response (100%). Solvent only (ethanol) controls were used to define the minimum response (0%), as described (Ermler et al., 2011; Orton et al., 2012).

Cytotoxicity as a confounding factor. The MDA-kb2 assay measures the decrease in luminescence induction by the DHT agonist that occurs as a result of competitive receptor antagonism. Since the luminescence signal can also be driven down by cytotoxicity, it is important to distinguish antagonism from interfering cytotoxicity. Cytotoxicity was determined in treatments without DHT and reductions in luminescence relative to the solvent (ethanol) controls were measured. The cytotoxic range became apparent from a down-turn of the luminescence readings below the values of solvent controls (shown as insets in Fig. 1). Treatments in the absence of DHT also revealed the AR agonist properties of some of the tested chemicals at higher concentrations. In these cases, cytotoxicity was evaluated in relation to the maximal AR agonistic response. This is an indirect method for determining cytotoxicity in reporter gene assays, and has been reported in the MDA-kb2 assay several times previously (Ermler et al., 2011, 2010; Korner et al., 2004; Orton et al., 2011, 2012).

Test mixtures and prediction of combination effects. A total of 30 chemicals were combined (13 pesticides: fludioxonil, fenhexamid, *ortho*-phenylphenol, tebuconazole, dimethomorph, imazalil, methiocarb, pirimiphos-methyl, cyprodinil, pyrimethanil, vinclozolin, chlorpropham, linuron; 17 non-pesticides: bisphenol-A, *n*-butyl paraben, *n*-propyl paraben, perfluorooctane sulfonate (tetrabutylammonium salt: PFOS), 2,2′,4,4′-tetrahydroxybenzophenone (benzophenone 2), 2-hydroxy-4-methoxybenzophenone (benzophenone 3), butylated hydroxyanisole (BHA), butylated hydroxytoluol (BHT), benzo(α)pyrene (BaP), ethyl paraben, methyl paraben, galaxolide, tonalide, BDE100, 3-BC, 4-MBC and PCB138). Three mixture stock solutions with fixed mixture ratios were prepared. The mixture ratios were chosen in proportion to the concentrations of the individual mixture components that led to a suppression of DHT effects by 1%, 10% or 20%, here termed inhibitory concentrations IC01, IC10 or IC20. Accordingly, the resulting mixtures are referred to as the IC01 mix, the IC10 mix or the IC20 mix. The composition of the mixtures is shown in Supplemental material, Table S1. We utilised the concentration–response relationships of 30 AR antagonistic chemicals (Supplemental material, Table S2) to predict the combined effects of three 30 component mixtures with different mixture ratios (IC01, IC10, IC20). The IC01 mixtures allowed us to assess directly whether mixture effects would arise when all components were combined at their individual IC01 concentrations. The other mixture ratios were chosen to assess whether the predictability of combination effects was affected by mixture ratio. We selected the IC20 mixture because this represents an effect magnitude where individual components can be reliably identified as AR antagonists because their effects are significantly different from the effects seen with cells primed with DHT in the absence of test chemicals. We have previously shown that such effect sizes are demonstrable with the experimental power afforded by the MDA-kb2 assay (Ermler et al., 2010). The IC10 mixture ratio was chosen as a value intermediate between the above two ratios. A list of regression models for the concentration–response relationships of the individual chemicals, together with estimates of their IC10 can be found in Table S2 Supplemental material. Each mixture stock solution was serially diluted and tested at least 3 times in separate experimental sessions and using new stock solutions for each session. Two experimenters conducted the experiments separately.

The mathematical and statistical procedures used for calculating mixture effects according to concentration addition (CA) and independent action (IA) follow those described in Faust et al. (2001). Due to the mathematical features of CA, the concept cannot be used to calculate effect concentrations for the mixture that exceed the maximal AR antagonistic effect of the least efficacious compound present in the combination. For the mixtures investigated here, this limitation was introduced by BaP which showed AR antagonistic effects of a magnitude not

exceeding 60% of the effect seen with DHT, corresponding to an inhibition level of 40% ($\theta_{min} = 0.59$, see Table 2 in Supplementary material). To construct CA prediction curves that covered the entire range of antagonistic effects, we developed a pragmatic solution that extrapolates the toxic units (as defined in Faust et al., 2001) of sub-maximal mixture components to effect levels beyond their maximal efficacy. In short, the contribution of BaP to the overall mixture effect was extrapolated either (i) by setting its toxic unit to zero (assuming no contribution) or (ii) by

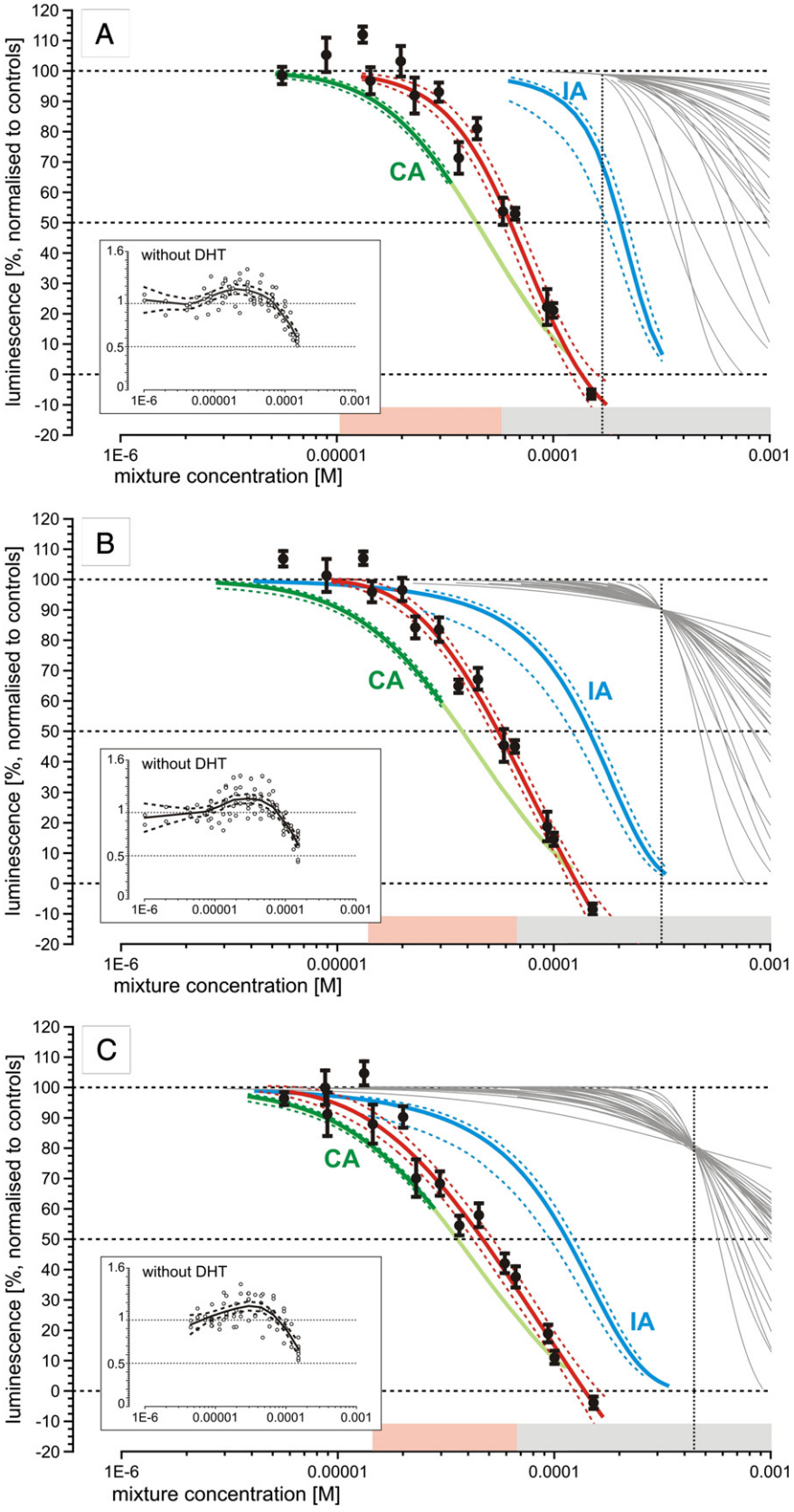

Table 2
Statistical uncertainty of predicted and observed AR antagonistic effect concentrations for 30 compound mixtures.

% suppression of DHT effect	AR antagonistic effect concentrations of mixtures [mole/L]					
	Observed		Predicted by CA		Predicted by IA	
	Mean	95% CI	Mean	95% CI	Mean	95% CI
IC01 mix, as defined in Table S1						
10%	2.72E−5	[2.22E−5–3.22E−5]	1.50E−5	[1.36E−5–1.56E−5]	1.09E−4	[6.50E−5–1.09E−4]
20%	3.76E−5	[3.23E−5–4.29E−5]	2.21E−5	[2.08E−5–2.28E−5]	1.44E−4	[1.06E−4–1.47E−4]
50%	6.34E−5	[5.77E−5–6.87E−5]	4.41E−5–4.42E−5*		2.04E−4	[1.78E−4–2.12E−4]
IC10 mix, as defined in Table S1						
10%	2.19E−5	[1.91E−5–2.48E−5]	1.04E−5	[9.31E−6–1.09E−5]	4.49E−5	[2.38E−5–4.87E−5]
20%	3.00E−5	[2.69E−5–3.31E−5]	1.67E−5	[1.57E−5–1.72E−5]	7.54E−5	[5.02E−5–7.91E−5]
50%	5.52E−5	[5.13E−5–5.92E−5]	3.73E−5–3.74E−5*		1.45E−4	[1.20E−4–1.53E−4]
IC20 mix, as defined in Table S1						
10%	1.34E−5	[9.11E−6–1.76E−5]	8.67E−6	[7.55E−6–9.13E−6]	2.93E−5	[1.48E−5–3.36E−5]
20%	2.06E−5	[1.58E−5–2.48E−5]	1.46E−5	[1.37E−5–1.51E−5]	5.29E−5	[3.53E−5–5.78E−5]
50%	4.66E−5	[4.23E−5–5.07E−5]	3.62E−5–3.63E−5*		1.14E−4	[9.37E−5–1.22E−4]

CA — concentration addition, IA — independent action, CI — confidence interval; *the lowest effect level estimated for benzo[a]pyrene is 59%, preventing an exact calculation of the expected AR antagonistic mixture effect concentration for 50%. Instead the compounds' contribution to the overall mixture effect was extrapolated either (i) by setting its toxic unit to zero (assuming no contribution), or (ii) by setting its toxic unit to a maximum worst-case value (derived from IC mixture estimations for 40% effect levels). The higher values correspond to (i) and the lower to (ii), defining the range of possible CA predictions.

setting its toxic unit to a maximum worst-case value (derived from IC mixture estimations for 40% effect levels). The higher values correspond to (i) and the lower to (ii), defining the range of possible CA predictions. This approach uses the entire information of the dose–response functions of the individual compounds, and makes no assumptions about a common slope model parameter. The regression models for the experimentally observed effects of the three mixtures are listed in Table S3 of supplemental information, together with model parameters, estimated AR antagonistic effect concentrations and effect concentrations for androgenicity and cytotoxicity.

Statistics. To analyse AR antagonist action, raw luminescence readings were normalised on a plate by plate basis to the means of the positive DHT controls (n = 8 wells) and the solvent controls (n = 8 wells) which were placed on the same plate. Luminescence readings from compounds tested in the absence of DHT were divided by the mean of the solvent controls from the same plate and analysed for negative and positive trends (suggestive of cytotoxic or androgenic action, respectively). All data from the same test compound were pooled and statistical concentration response regression analyses were conducted by using the best-fit approach described by Scholze et al. (2001) to derive inhibitory concentrations (IC) for androgenicity. To control for variations between experiments, concentration response data were analysed by using a generalised non-linear mixed modelling approach (Vonesh and Chinchilli, 1996) with plate as a random effect modifier for individual effect data. If readings in the absence of DHT showed indications for cytotoxic or androgenic action, the non-monotonic concentration–response relationship was modelled by non-parametric local regression methods (Cleveland et al., 1988). From this robust fitting method we derived effect concentrations (ECs) for androgenicity, with a 10% increase over the mean solvent mean as the minimum effect criterion, and ECs for cytotoxicity (if present) as 10% reduction of the maximal observed androgenic action. Data points associated with cytotoxicity were not included in regression analysis for anti-androgenicity. Differences between predicted and observed effect concentrations were deemed statistically significant when the 95% confidence belts of the prediction did not overlap with those of the experimentally observed mixture effects. All statistical analyses were performed using the SAS statistical software (SAS Institute Inc., Cary, NC, USA).

Results

All three 30 component mixtures showed AR antagonistic activity in a dose-dependent manner (Figs. 1A–C). As the mixture ratios changed from the IC01 to the IC20 mixtures, the concentration–response relationships shifted towards lower concentrations, and exhibited shallower gradients (Figs. 1A–C, Table 2). Each of the three mixtures also displayed androgenic activity in the concentration ranges associated with approximately half-maximal suppressions of DHT androgenicity (insets and red bars in Fig. 1). As judged by the decrease in luminescence in exposures without DHT, cytotoxicity became apparent at total mixture concentrations exceeding 60 to 70 μM (Supplemental material, Table S3), at concentrations around those required for 50% inhibition of AR antagonistic effects (Figs. 1A–C, Table 2).

Due to the large number of mixture components, we observed striking low dose combination effects. For example, the individual IC01 levels of all 30 compounds summed up to a total mixture concentration of 159 μM, which produced a 100% inhibition of the agonistic effects of DHT (Fig. 1A). Conversely, the concentrations of all components at the point where the IC01 mixture produced a 10% inhibition level were lower by a factor of 5.8 than their corresponding individual IC01 concentrations (Table 1). At such low concentrations, it would be impossible to demonstrate any effects of the individual compounds in the MDA-kb2 assay, yet directly measurable combination effects were produced. Similar

Fig. 1. Predicted and observed anti-androgenic activity of mixtures composed of 30 AR antagonistic compounds with mixture ratios proportional to the effect concentrations of all mixture components individually associated with a 1% antagonistic effect, IC01 (A), a 10% antagonistic effect, IC10 (B) and a 20% antagonistic effect, IC20 (C). Observed mixture effects are from at least four independent mixture experiments and are shown as black dots with error bars (mean ± standard deviation). Red lines are regression fits of the observed mixture effects. Mixture effects were predicted by using concentration addition (CA) (solid green lines). The light green lines show the continuation of the CA predictions assuming that benzo(a)pyrene does not contribute to the mixture effect at antagonistic levels beyond 70% of the luminescence seen with DHT. Predicted effects according to IA are shown as solid blue lines. Dotted lines alongside the CA and IA prediction curves indicate the 95% confidence belts of the predictions. Grey lines show the effects of the single compounds scaled to the concentrations of the total mixtures. Concentration ranges where the mixtures produced androgenic effects are highlighted as red bars above the concentration axis, concentration ranges associated with cytotoxicity are shown as grey bars. Vertical dotted lines depict the mixture concentrations at which all individual concentrations of the compounds are present at concentrations associated with antagonistic effects of 1% (A), 10% (B) and 20% (C). The inset graphs show androgenic effects of the tested mixtures in the absence of DHT, together with the estimated mean effect (solid grey line) and 95% confidence belt (dotted grey line), the concentration axis shows molar concentrations and the y axis shows the luminescence value normalised to the mean solvent control effect. A down-turn of the curves below the horizontal line defining control values (1 on the y axis) indicates cytotoxicity.

relationships became apparent with the IC10 mixture and the IC20 mixture (Table 2).

For the three tested mixtures, we found that the concept of IA produced concentration–response curves with median effect concentrations 4.5- to 3-fold higher than those derived from CA. In addition, while the curves predicted by CA did not differ much for the three mixtures, the IA curves changed slightly towards lower median effect concentrations as the mixture ratio changed from IC01 to IC20 (Fig. 1). The experimentally observed anti-androgenic effects of all three mixtures fell between the window defined by the CA and IA predictions, but came closer to the responses anticipated by CA. The closest agreement between experiment and CA prediction was observed with the IC20 mixture where the measured effect concentrations were approximately 1.3–1.5 times larger than those predicted. In all cases, IA underestimated the experimentally observed effects by a larger margin, with predicted effect concentrations 2–4 times higher than those observed (Table 2).

Discussion

Our study is the first to demonstrate *in vitro* combination effects of mixtures containing a large number of current use AR antagonistic pesticides and a variety of other chemicals with AR antagonist properties. All the chemicals in the mixtures are relevant to human exposures typically experienced in industrialised countries, including the European Union and the USA (see: Ermler et al., 2011; Orton et al., 2011). Our experiments show that large numbers of AR antagonistic chemicals can act jointly at the receptor level when present at concentrations not associated with observable AR antagonism alone. Their joint AR antagonistic effects occurred at concentrations of the individual components that, if tested alone, would have produced effect magnitudes well below 1%. Such effects are too small to be measurable with the power afforded by the MDA-kb2 assay (Ermler et al., 2010). These observations are of relevance in light of realistic environmental exposure scenarios where multiple chemicals are present at low concentrations. With the exception of vinclozolin (*e.g.* Hass et al., 2007), none of the chemicals in our mixtures have been tested *in vivo* for their ability to produce effects typically observed with AR antagonists, it is therefore difficult to say whether any of our selected chemicals, or their mixtures, will produce anti-androgenicity *in vivo*. Even so, our work shows that they have the capability to elicit anti-androgenic effects if they reach the AR in sufficient quantities. Whether this is likely to happen may depend largely on toxicokinetic factors, which are currently unexplored.

Due to a lack of biomonitoring data for pollutants that occur together in human tissues, the number and composition of actual human exposures to xenobiotics with AR antagonistic properties remains largely unknown. The risks from AR antagonists stem mostly from exposures in foetal life, and for this reason, the exposures experienced by pregnant women are of particular relevance. Two recent publications have given an impression of the combined xenobiotic exposures of pregnant women (Castorina et al., 2010; Woodruff et al., 2011). Castorina and co-authors focused on pesticide metabolites in urine, and showed that of the 34 metabolites measured, 7 were detected in over 50% of participants. In this study, positive correlations between some measured analytes were seen, indicating that individuals were exposed to several compounds simultaneously. Woodruff and co-authors reported that the serum of individuals tested for 52 xenobiotics contained a median of 37 compounds (range: 28–45) and those tested for 71 contained a median of 50 compounds (range: 35–60). It appears therefore that co-occurrence of 30 chemicals may be a quite realistic representation of the number of xenobiotics likely to be present in pregnant women in the North American population. Somewhat similar results have been reported from Europe, where 8 out of 11 phthalates/phenols in urine from Spanish pregnant women were detected in all samples (Casas et al., 2011) and UV-filters, synthetic musks, parabens and phthalates were each detected in over 50% of milk samples from nursing mothers in Switzerland (Schlumpf et al., 2010).

Our demonstrations of combination effects of diverse AR antagonists may provide a starting point for defining common assessment groups of AR antagonists in cumulative risk assessment. Ideally, such groupings should be defined on the basis of *in vivo* demonstrations of antiandrogenicity, but in the absence of such data, *in vitro* findings can give some orientation for setting priorities. Common assessment groups have traditionally been defined on the basis of mechanistic criteria and have resulted in groupings with very similar chemical structures. For example, US EPA currently conducts cumulative risk assessment for five groups of pesticides: organophosphorus compounds, N-methyl carbamates, s-triazines, chloroacetanilides and pyrethrins/pyrethroids (USEPA, 2006a, 2006b, 2006c, 2007, 2011). Although they all inhibit acetylcholinesterase, organophosphates and N-methyl carbamates are not evaluated in one group, presumable because carbamylation of the enzyme by carbamates results in rapid recovery not seen with organophosphates. A report on cumulative risk assessment for phthalates and other anti-androgenic chemicals by the US National Research Council discussed the problems associated with using too narrowly focused mechanistic criteria as the basis for groupings and noted that combination effects arise from several phthalates together with other, structurally diverse anti-androgens, including AR antagonists (NRC, 2008). The US National Research Council proposed a physiological grouping concept based on common adverse outcomes and recognised that such grouping criteria go far beyond the criteria derived from similarities in chemical structures. Very recently, similar approaches have been elaborated by the European Food Safety Authority (EFSA, 2013). Our findings support the grouping philosophy proposed by US NRC and EFSA. Due to the features of the AR binding domain, which can accommodate a multitude of chemical structures, assessment groups for this class of agents will have to be based on common outcomes. We propose that the propensity of antagonising the AR *in vitro* provides a useful starting point for creating such groupings which can be refined on the basis of *in vivo* evidence for anti-androgenicity, as and when such evidence is forthcoming.

With predictions based on the AR antagonistic effects of the individual mixture components, the concept of CA provided reasonable approximations of the experimentally observed combined effects, although the agreement between prediction and observation was not perfect. The observed mixture effects fell somewhat short of those anticipated by CA, nevertheless, the joint effects of all three mixture ratios were considerably stronger than those anticipated by IA. We suggest that the slight shortfalls between the observed cumulative effects and the CA predictions may be related to the fact that AR antagonists frequently also exhibit AR agonist properties at higher concentrations (Tamura et al., 2006). Several of the chemicals included in the mixtures fall in that category (Ermler et al., 2011; Orton et al., 2011) and the AR agonistic effects of these mixtures overlapped with the concentration ranges where AR antagonistic effects occurred (Fig. 1, insets). We have previously shown that when chemicals with dual AR agonist and antagonist properties are excluded from the mixtures, and only “pure” AR antagonists are combined, perfect agreement with CA can be obtained (Orton et al., 2012). Since the combination effect predictions are based on the AR antagonist concentration response curves of the single mixture components, the prediction concepts themselves have no facilities to directly account for AR agonism (exposure in the absence of DHT). However, agonistic effects would be indirectly accounted for under the assumption that competing agonist and antagonist effects both contribute to the antagonist concentration response curve, provided that competition is occurring at the same target. We previously showed that when dual action antagonists are included in the mixture, observed deviation is not the result of competition at the ligand binding domain of the AR, but rather unidentified factors cause this phenomenon (Orton et al., 2012). Since the same pesticides as reported in Orton et al. are included in the mixtures tested here, as well as other dual action AR antagonists (Ermler et al., 2011), we hypothesise that similar explanations account for the observed deviation from CA.

Conclusions

We have shown that the anti-androgenic effects of a mixture of AR antagonists from a wide range of sources and exposure routes are additive in this assay at very low concentrations. Since our compound selection was based on either actual or estimated human exposure in Europe and the USA, at least some of the mixture components are likely to be present simultaneously in people. Whether combined effects from exposure to AR antagonists might be produced in foetal life, thus providing a possible explanation for the rising trends in cryptorchidisms and hypospadias, will be determined by the number of AR antagonists present in tissues and their potency. However, our study has shown that the often low levels measured for individual AR antagonists are not a reliable indicator for dismissing risks from this class of chemicals. Renewed efforts of searching for AR antagonists in human tissue will be required for cumulative risk assessment.

Conflict of interest

There are no actual or perceived conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.taap.2013.09.008.

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