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

Frances Orton [a,1], Sibylle Ermler [a], Subramaniam Kugathas [a], Erika Rosivatz [b], Martin Scholze [a], Andreas Kortenkamp [a,*]

[a] Institute for the Environment, Brunel University, Kingston Lane, Uxbridge UB8 3PH, UK
[b] Institute of Chemical Biology, Imperial College London, Exhibition Road, London SW7 2AZ, UK

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 dihydrotestrosterone 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.

© 2013 The Authors. Published by Elsevier Inc. All rights reserved.

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,

---

* Corresponding author.
E-mail address: andreas.kortenkamp@brunel.ac.uk (A. Kortenkamp).
[1] Current address: Biosciences, College of Life and Environmental Sciences, University of Exeter EX4 4QD, UK.

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 infor mation is essential for risk assessment. However, to predict the effects of large multi component mixtures on the basis of the toxicity of its com ponents will stretch the resources for *in vivo* studies. Such resource limi tations 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 pub lished 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 re quire 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 expo sure 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, particu larly at concentrations where the effects of the single components are below the detection limit of the assay. Such data is important to contrib ute 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 compo nents 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 dihydrotestosterone (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 regres sion. 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").

Compound	Individual IC01		Concentration in mixture at 10% inhibition				
			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	06	1.14E	06	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-methylenebenzylidene 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 pollut ants 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 methylenebenzylidene 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. (Leicester shire, 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 me dium (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 lu minescence 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 eth anol (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 inde pendent experimental sessions. To enable the pooling of data from dif ferent 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 distin guish antagonism from interfering cytotoxicity. Cytotoxicity was deter mined 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). Treat ments 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 re porter 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 sup pression of DHT effects by 1%, 10% or 20%, here termed inhibitory concen trations 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 mag nitude where individual components can be reliably identified as AR an tagonists 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 concen tration 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 solu tions 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 indepen dent 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 antag onistic effect of the least efficacious compound present in the combina tion. 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
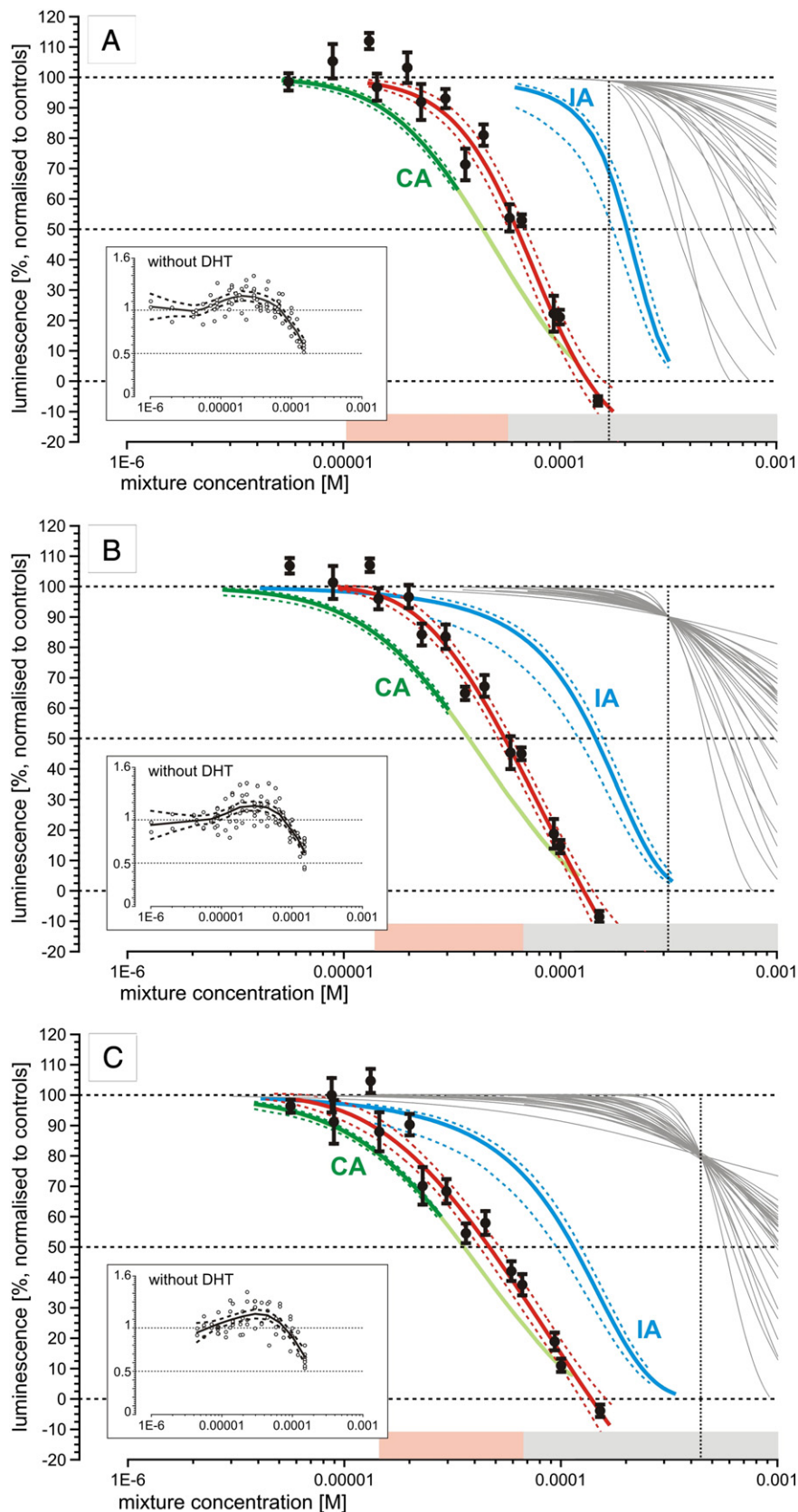
Table 2
Statistical uncertainty of predicted and observed AR antagonistic effect concentrations for 30 compound mixtures.

% 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% agonistic 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 organo phosphates. 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 cumula tive risk assessment.

Conflict of interest

There are no actual or perceived conflicts of interest.

Acknowledgments

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.

References

Birkhoj, M., Nellemann, C., Jarfelt, K., Jacobsen, H., Andersen, H.R., Dalgaard, M., Vinggaard, A.M., 2004. The combined antiandrogenic effects of five commonly used pesticides. Toxicol. Appl. Pharmacol. 201, 10–20.

Boisen, K.A., Kaleva, M., Main, K.M., Virtanen, H.E., Haavisto, A.M., Schmidt, I.M., Chellakooty, M., Damgaard, I.N., Mau, C., Reunanen, M., Skakkebaek, N.E., Toppari, J., 2004. Difference in prevalence of congenital cryptorchidism in infants between two Nordic countries. Lancet 363, 1264–1269.

Buttke, D.E., Wolkin, A., Stapleton, H.M., Miranda, M.L., 2013. Associations between serum levels of polybrominated diphenyl ether (PBDE) flame retardants and environmental and behavioral factors in pregnant women. J. Expo. Sci. Environ. Epidemiol. 23, 176–182.

Casas, L., Fernández, M.F., Llop, S., Guxens, M., Ballester, F., Olea, N., Irurzun, M.B., Rodríguez, L.S.M., Riaño, I., Tardón, A., Vrijheid, M., Calafat, A.M., Sunyer, J., 2011. Urinary concentrations of phthalates and phenols in a population of Spanish pregnant women and children. Environ. Int. 37, 858–866.

Castorina, R., Bradman, A., Fenster, L., Barr, D.B., Bravo, R., Vedar, M.G., Harnly, M.E., McKone, T.E., Eisen, E.A., Eskenazi, B., 2010. Comparison of current-use pesticide and other toxicant urinary metabolite levels among pregnant women in the CHAMACOS cohort and NHANES. Environ. Health Perspect. 118, 856–863.

Christiansen, S., Scholze, M., Axelstad, M., Boberg, J., Kortenkamp, A., Hass, U., 2008. Combined exposure to anti-androgens causes markedly increased frequencies of hypospadias in the rat. Int. J. Androl. 31, 241–247.

Christiansen, S., Scholze, M., Dalgaard, M., Vinggaard, A.M., Axelstad, M., Kortenkamp, A., Hass, U., 2009. Synergistic disruption of external male sex organ development by a mixture of four antiandrogens. Environ. Health Perspect. 117.

Christiansen, S., Kortenkamp, A., Axelstad, M., Boberg, J., Scholze, M., Jacobsen, P.R., Faust, M., Lichtensteiger, W., Schlumpf, M., Burdorf, A., Hass, U., 2012. Mixtures of endocrine disrupting contaminants modelled on human high end exposures: an exploratory study in rats. Int. J. Androl. 35, 303–316.

Cleveland, W.S., Devlin, S.J., Grosse, E., 1988. Regression by local fitting: methods, properties, and computational algorithms. J. Econ. 37, 87–114.

Darbre, P.D., Harvey, P.W., 2008. Paraben esters: review of recent studies of endocrine toxicity, absorption, esterase and human exposure, and discussion of potential human health risks. J. Appl. Toxicol. 28, 561–578.

EFSA, 2013. Scientific opinion on the identification of pesticides to be included in cumulative assessment groups on the basis of their toxicological profile. EFSA J. 11, 3293–3424.

Ermler, S., Scholze, M., Kortenkamp, A., 2011. The suitability of concentration addition for predicting the effects of multi-component mixtures of up to 17 anti-androgens with varied structural features in an in vitro AR antagonist assay. Toxicol. Appl. Pharmacol. 257, 189–197.

Ermler, S., Scholze, M., Kortenkamp, A., 2010. The sensitivity of the MDA-kb2 cell in vitro assay in detecting anti-androgenic chemicals — identification of sources of variability and estimation of statistical power. Toxicol. Vitr. 24, 1845–1853.

Faust, M., Altenburger, R., Backhaus, T., Blanck, H., Boedeker, W., Gramatica, P., Hamer, V., Scholze, M., Vighi, M., Grimme, L.H., 2001. Predicting the joint algal toxicity of multi-component s-triazine mixtures at low-effect concentrations of individual toxicants. Aquat. Toxicol. 56, 13–32.

Geens, T., Aerts, D., Berthot, C., Bourguignon, J.-P., Goeyens, L., Lecomte, P., Maghuin-Rogister, G., Pironnet, A.-M., Pussemier, L., Scippo, M.-L., Van Loco, J., Covaci, A., 2012. A review of dietary and non-dietary exposure to bisphenol-A. Food Chem. Toxicol. 50, 3725–3740.

Giokas, D.L., Salvador, A., Chisvert, A., 2007. UV filters: from sunscreens to human body and the environment. TrAC Trends Anal. Chem. 26, 360–374.

Gray, L.E., Wolf, C., Lambright, C., Mann, P., Price, M., Cooper, R.L., Ostby, J., 1999. Administration of potentially antiandrogenic pesticides (procymidone, linuron, iprodione, chlozolinate, p, p'-DDE, and ketoconazole) and toxic substances (dibutyl- and diethylhexyl phthalate, PCB 169, and ethane dimethane sulphonate) during sexual differentiation produces diverse profiles of reproductive malformations in the male rat. Toxicol. Ind. Heal. 15, 94–118.

Hass, U., Scholze, M., Christiansen, S., Dalgaard, M., Vinggaard, A.M., Axelstad, M., Metzdorff, S.B., Kortenkamp, A., 2007. Combined exposure to anti androgens exacerbates disruption of sexual differentiation in the rat. Environ. Health Perspect. 115 (Suppl. 1), 122–128.

Howdeshell, K.L., Wilson, V.S., Furr, J., Lambright, C.R., Rider, C.V., Blystone, C.R., Hotchkiss, A.K., Gray, L.E., 2008. A mixture of five phthalate esters inhibits fetal testicular testosterone production in the Sprague–Dawley rat in a cumulative, dose-additive manner. Toxicol. Sci. 105, 153–165.

Kjærstad, M.B., Taxvig, C., Andersen, H.R., Nellemann, C., 2010. Mixture effects of endocrine disrupting compounds in vitro. Int. J. Androl. 33, 425–433.

Korner, W., Vinggaard, A.M., Terouanne, B., Ma, R.S., Wieloch, C., Schlumpf, M., Sultan, C., Soto, A.M., 2004. Interlaboratory comparison of four in vitro assays for assessing androgenic and antiandrogenic activity of environmental chemicals. Environ. Health Perspect. 112, 695–702.

Kortenkamp, A., 2007. Ten years of mixing cocktails: a review of combination effects of endocrine-disrupting chemicals. Environ. Health Perspect. 115 (Suppl. 1), 98–105.

Main, K.M., Skakkebæk, N.E., Virtanen, H.E., Toppari, J., 2010. Genital anomalies in boys and the environment. Best Pract. Res. Clin. Endocrinol. Metab. 24, 279–289.

McKinlay, R., Plant, J.A., Bell, J.N.B., Voulvoulis, N., 2008. Calculating human exposure to endocrine disrupting pesticides via agricultural and non-agricultural exposure routes. Sci. Total. Environ. 398, 1–12.

Metzdorff, S.B., Dalgaard, M., Christiansen, S., Axelstad, M., Hass, U., Kiersgaard, M.K., Scholze, M., Kortenkamp, A., Vinggaard, A.M., 2007. Dysgenesis and histological changes of genitals and perturbations of gene expression in male rats after in utero exposure to antiandrogen mixtures. Toxicol. Sci. 98, 87–98.

Nassar, N., Bower, C., Barker, A., 2007. Increasing prevalence of hypospadias in Western Australia, 1980–2000. Arch. Dis. Child. 92, 580–584.

Nelson, C.P., Park, J.M., Wan, J., Bloom, D.A., Dunn, R.L., Wei, J.T., 2005. The increasing incidence of congenital penile anomalies in the United States. J. Urol. 174, 1573–1576.

NRC, 2008. Phthalates Cumulative Risk Assessment — The Tasks Ahead. National Academy of Sciences, National Research Council, Washington DC (ISBN 0-309-12842-0).

Orton, F., Rosivatz, E., Scholze, M., Kortenkamp, A., 2011. Widely used pesticides with previously unknown endocrine activity revealed as in vitro anti-androgens. Environ. Health Perspect. 119, 794–800.

Orton, F., Rosivatz, E., Scholze, M., Kortenkamp, A., 2012. Competitive androgen receptor antagonism as a factor determining the predictability of cumulative antiandrogenic effects of widely used pesticides. Environ. Health Perspect. 120, 1578–1584.

Pierik, F.H., Burdorf, A., Deddens, J.A., Juttmann, R.E., Weber, R.F.A., 2004. Maternal and paternal risk factors for cryptorchidism and hypospadias: a case–control study in newborn boys. Environ. Health Perspect. 112, 1570–1576.

Ravindra, K., Sokhi, R., Van Grieken, R., 2008. Atmospheric polycyclic aromatic hydrocarbons: source attribution, emission factors and regulation. Atmos. Environ. 42, 2895–2921.

Rider, C.V., Furr, J., Wilson, V.S., Gray, L.E., 2008. A mixture of seven antiandrogens induces reproductive malformations in rats. Int. J. Androl. 31, 249–262.

Roosens, L., Covaci, A., Neels, H., 2007. Concentrations of synthetic musk compounds in personal care and sanitation products and human exposure profiles through dermal application. Chemosphere 69, 1540–1547.

Schlumpf, M., Kypke, K., Wittassek, M., Angerer, J., Mascher, H., Mascher, D., Vokt, C., Birchler, M., Lichtensteiger, W., 2010. Exposure patterns of UV filters, fragrances, parabens, phthalates, organochlor pesticides, PBDEs, and PCBs in human milk: correlation of UV filters with use of cosmetics. Chemosphere 81, 1171–1183.

Scholze, M., Boedeker, W., Faust, M., Backhaus, T., Altenburger, R., Grimme, L.H., 2001. A general best-fit method for concentration–response curves and the estimation of low-effect concentrations. Environ. Toxicol. Chem. 20, 448–457.

Skakkebaek, N.E., Rajpert-De Meyts, E., Main, K.M., 2001. Testicular dysgenesis syndrome: an increasingly common developmental disorder with environmental aspects. APMIS 109, S22–S28.

Tamura, H., Ishimoto, Y., Fujikawa, T., Aoyama, H., Yoshikawa, H., Akamatsu, M., 2006. Structural basis for androgen receptor agonists and antagonists: interaction of SPEED 98-listed chemicals and related compounds with the androgen receptor based on an in vitro reporter gene assay and 3D-QSAR. Bioorg. Med. Chem. 14, 7160–7174.

USEPA, 2006a. Chloroacetanilide Cumulative Risk Assessment. Federal Register, pp. 1–74.

USEPA, 2006b. Organophosphate Cumulative Risk Assessment. Federal Register, pp. 43740–43742.

USEPA, 2006c. Triazine Cumulative Risk Assessment. Federal Register, pp. 1–65.

USEPA, 2007. Revised N-methyl Carbamate Cumulative Risk Assessment. Federal Register, pp. 54656–54658.

USEPA, 2011. Pyrethrins/Pyrethroid Cumulative Risk Assessment. Office of Pesticide Programs, Wahington DC, pp. 1–88.

Vonesh, E., Chinchilli, V.M., 1996. Linear and Nonlinear Models for the Analysis of Repeated Measurements. CRC Press.

WHO, 2012. In: Bergman, A., Heindel, J.J., Jobling, S., Kidd, K.A., Zoeller, R.T. (Eds.), State of the Science of Endocrine Disrupting Chemicals (pp.).

Wilson, V.S., Bobseine, K., Lambright, C.R., Gray, L.E., 2002. A novel cell line, MDA-kb2, that stably expresses an androgen- and glucocorticoid-responsive reporter for the detection of hormone receptor agonists and antagonists. Toxicol. Sci. 66, 69–81.

Woodruff, T.J., Zota, A.R., Schwartz, J.M., 2011. Environmental chemicals in pregnant women in the United States: NHANES 2003–2004. Environ. Health Perspect. 119, 878–885.