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Widely Used Pesticides with Previously Unknown Endocrine Activity Revealed as *in Vitro* Anti-Androgens

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Keywords: fungicide, AR-Lux, anti-androgen, biomonitoring, endocrine disruption

Abbreviations:

AR – Androgen Receptor

DDE – 1,1-*bis*-(4-chlorophenyl)-2,2-dichloroethene

DHT – dihydrotestosterone

EC20 – Concentration that produces a 20% effect

ERR – Environmental Relevance Ratio

IC20 – Concentration that inhibits the androgenicity of DHT by 20%

QSAR – Quantitative Structure Activity Relationship

YAS – Yeast Androgen Screen

Abstract

Background: Evidence suggests that there is widespread decline in male reproductive health and anti-androgenic pollutants may play a significant role. There is also a clear disparity between pesticide exposure and endocrine disrupting data, with the majority of the published literature focused on pesticides that are no longer registered for use in developed countries.

Objective: The aim of this study was to utilise estimated human exposure data to select pesticides to test for anti-androgenic activity, focusing on highest use pesticides.

Methods: We used European databases to select 134 candidate pesticides based on highest exposure, followed by a filtering step according to known or predicted receptor mediated anti-androgenic potency, based on a previously published quantitative structure-activity relationship (QSAR) model. In total, 37 pesticides were tested for *in vitro* androgen receptor (AR) antagonism. Of these, 14 were previously reported to be AR antagonists ("active"), 4 were predicted AR antagonists using the QSAR, 6 were predicted to not be AR antagonists ("inactive"), and 13 with unknown activity, which were "out of domain" and therefore could not be classified with the QSAR ("unknown").

Results: All 14 pesticides with previous evidence of AR antagonism were confirmed as anti-androgenic in our assay and 9 previously untested pesticides were identified as anti-androgenic (dimethomorph, fenhexamid, quinoxyfen, cyprodinil, λ-cyhalothrin, pyrimethanil, fludioxonil, azinphos-methyl, pirimiphos-methyl). In addition, 7 compounds were classified as androgenic.

Conclusions: Due to estimated anti-androgenic potency, current use, estimated exposure, and lack of previous data, we strongly recommend that dimethomorph, fludioxonil, fenhexamid, imazalil, *ortho*-phenylphenol and pirimiphos-methyl be tested for anti-androgenic effects *in vivo*. The lack of human biomonitoring data for environmentally relevant pesticides presents a barrier to current risk assessment of pesticides on humans.

Introduction

Evidence suggests that prenatal and early life exposure to pesticides may be causative factors in a variety of human disorders. For example, a meta-analysis reported that maternally exposed offspring have increased risk of childhood leukaemia [OR (CI) = 2.64 (1.4-5), Wigle et al. 2009].

There are also indications that reproductive abnormalities, expressed as cryptorchidisms, hypospadias and decreased penile length, may be linked to pesticide exposure, most strikingly in maternally exposed boys (Andersen et al. 2008; Damgaard et al. 2006; Rocheleau et al. 2009;). This is significant because male fertility is thought to be declining in many countries (Andersson et al. 2008) and perinatal hypospadias/cryptorchidisms are risk factors for reduced sperm quality and testicular cancer in adulthood (Skakkebaek et al. 2001). Banned persistent organochlorines (*p,p*-DDT, *p,p*-DDE, B-HCH, HCB, alpha-endosulfan, cis-HE, oxychlordane, dieldrin) were detected in all samples of breast milk in a case control study of mothers in Denmark/Finland. Also levels were significantly higher in samples from mothers of sons with cryptorchidism, than in samples from matched controls (1997-2001, Damgaard et al. 2006). Female Danish greenhouse workers exposed to current-use pesticides were more likely to give birth to a son with cryptorchidism than a random sample of mothers from the Copenhagen area (6.2% and 1.9%). Furthermore, sons of mothers who directly handled treated plants or were engaged in spraying pesticides had significantly smaller penises than sons of mothers who had non-contact roles in the greenhouse industry (Andersen et al. 2008). Lastly, a recent meta-analysis of studies from USA and Europe reported that maternal occupational exposure to pesticides was associated with a 36% increased risk of hypospadias relative to the risk in mothers without exposure (risk ratio: 1.36; confidence interval: 1.04-1.77; Rocheleau et al. 2009). The risk of developing cryptorchidisms (Pierik et

al. 2004) and hypospadias (Brouwers et al. 2007) was also associated with paternal exposures to pesticides, mainly in greenhouses for the production of vegetables and flowers.

The term "testicular dysgenesis syndrome" (TDS) has been proposed to explain the interrelated nature of these abnormalities (Skakkebaek et al. 2001). It is conceivable that estrogenic and/or anti-androgenic contaminants play a role in TDS. Experimental studies with rats have shown that maternal exposure to flutamide (a pharmaceutical anti-androgen) affects androgen-dependent developmental outcomes such as ano-genital distance and nipple retention (McIntyre et al. 2001). However, ethinylestradiol has not been shown to affect these endpoints (Howdeshell et al. 2008). Furthermore, hormone receptor screening *in vitro* suggests a preponderance of anti-androgenic activity compared to estrogenic activity in non-organochlorine (current-use) pesticides. For example, Kojima et al. (2004) screened 161 pesticides and reported that 52 were anti-androgenic whereas only 29 were estrogenic, and Orton et al. (2009) reported that 6 of 12 pesticides screened were anti-androgenic and none were estrogenic. There is a good correlation between androgen receptor (AR) antagonist properties and *in vivo* anti-androgenic effects and there is also good evidence that androgen sensitive endpoints are demasculinised in male rats when exposed *in utero* to a wide range of pesticides. Anti-androgenic effects both *in vitro* and via maternal exposure *in vivo* have been reported in response to the herbicide linuron (Lambright et al. 2000; Gray et al. 1999), the fungicides prochloraz (Vinggaard et al. 2005), procymidone (Ostby et al. 1999), tebuconazole (Taxvig et al. 2007) and vinclozolin (Uzumcu et al. 2004; Anway et al. 2006), the organochlorine insecticides DDE (Gray et al. 1999) and endosulfan (Sinha et al. 2001), the organophosphate dimethoate (Verma and Mohanty 2009), and the pyrethroid insecticide deltamethrin (Andrade et al. 2002). However, with the exception of linuron, dimethoate, deltamethrin and tebuconazole, the pesticides listed above have not been authorised for use in Europe during the past 5 years (except for DDE), which should result in lower occupational,

residential and dietary exposures. Endocrine relevant data on current use pesticides is minimal, and in some cases completely absent, with the majority of the published literature focused on pesticides that are no longer registered for use.

Therefore, the aim of this study was to test the anti-androgenic activity of currently used pesticides, with a view to informing future studies to determine their likely role in causing testicular dysgenesis syndrome. We selected compounds for testing based on evidence of human exposure (dietary intake data for Europe) and predicted AR antagonism according to the quantitative structure-activity relationship (QSAR) model developed by Vinggaard et al. (2008). Compounds predicted to be AR antagonists and compounds with high exposure scores were analysed for AR antagonist properties using the MDA-kb2 assay (Ermler et al. 2010, Wilson et al. 2002). In addition, the yeast anti-androgen screen (YAS) was used to further test a subset of pesticides that were newly identified as AR antagonists or had MDA-kb2 assay results that were discordant with QSAR predictions.

Methods

Test compound selection

Pesticides were selected using a combination of exposure scores and data about receptor-mediated anti-androgenic activity (Supplemental Material, Figure 1). First, we identified 134 pesticides with data suggesting relevant human exposures, including 58 pesticides identified at the highest concentrations and most frequently in European foods [European Commission (EC) 2008], 30 additional pesticides with relatively high daily dietary intakes (> 0.0004 µg/kg/day) identified in the FAO/WHO joint meeting on pesticide residues (JMPR) (WHO 2009), 44 additional pesticides identified in >0.4% of fruits and vegetables during routine testing [European Food Safety Authority (EFSA) 2009], and *o,p'*- and *p,p'*-DDE, which we

included due to known adipose tissue levels (Fernandez et al. 2004). Each pesticide was assigned four scores, with each ranging from 1-10: (a) maximum food residue level (EC 2009), (b) estimated daily dietary intake (WHO 2009), (c) frequency of detection in fruits and vegetables (EFSA 2009), with a score of 5 assigned when data were not available; and (d) a score according to the number of times pesticides were listed as one of the top 10 pesticides identified in fruits and cereals in Europe (a frequency score), with a score of 0 assigned if they were never listed (EC 2009). The four scores were summed to generate a "total exposure score", with a maximum possible score of 40 (Supplemental Material, Table 1).

The second stage of compound selection for testing was an assessment of *in vitro* evidence of AR interaction in the available literature (Andersen et al. 2002; Bauer et al. 2002; Kojima et al. 2004; Okubo et al. 2004; Orton et al. 2009; Vinggaard et al. 2008). Compounds previously shown not to be AR antagonists *in vitro* (n = 43) were removed from the list, which reduced the number of candidate pesticides from 134 to 91. Compounds previously reported to be AR antagonists (n = 27) were removed if the ratio of their total exposure score to their published IC20 [total exposure score/published IC20 = "environmental relevance ratio (ERR)"] was less than 3 (ERR was re-calculated using our experimental data after the selection process). This left 14 previously reported AR antagonists for testing by the MDA-kb2 assay. For pesticides without published data (n = 64), AR antagonist activity was predicted using the QSAR developed by Vinggaard et al. (2008). These pesticides were tested using the MDA-kb2 assays if they were predicted to have AR antagonist activity (n = 4) or if they had high exposure scores (>8) regardless of their QSAR status, including 6 pesticides that were predicted not to have AR antagonist activity and 13 pesticides that could not be predicted because they were out of the domain of the QSAR model. In total, 37 compounds were selected for testing in the MDA-kb2 assay. Finally, 8 pesticides that were newly described as highly active anti-androgens in the MDA-kb2 assay and 4 pesticides for which the QSAR

prediction differed from the experimental result (including 1 out of the model domain) were subjected to further testing using the YAS (n = 14). For a summary of the selection process see Supplemental Material, Figure 1.

Chemicals

Dihydrotestosterone (DHT: > 97% purity) was purchased from Steraloids Ltd., novaluron, dimethomorph, pp-DDE, methiocarb and indoxacarb were purchased from Greyhound Chromatography and Allied Chemicals (> 98.7% purity) and all other pesticides were purchased from Sigma Aldrich (>97%). Ethanol was obtained from VWR International Ltd. (> 99.7% purity). All test compounds were dissolved in ethanol to make stock solutions to be used in the assays.

MDA-kb2 Assay

MDA-kb2 cells are human breast cancer cells, stably transfected with a firefly luciferase reporter gene that is driven by an androgen-response element containing promoter (Wilson et al. 2002). Details of the modified assay were published previously (Ermler et al. 2010). Briefly, cells were seeded at a concentration of 1 x 10^5 cells/ml in phenol red-free Leibowitz-15 medium (Invitrogen Ltd.) containing 10% (charcoal stripped) fetal calf serum (Invitrogen Ltd.) in white luminometer plates and allowed to attach for 24 hours. Cells were then exposed to 8 serial dilutions of selected pesticides with or without DHT (0.25 nM). After 24 hours luciferase activity was determined with SteadyGlo assay reagent (Promega UK Ltd.) and measured in a plate reader (FLUOstar Optima, BMG Labtech GmbH). The following controls were run on each plate: media, ethanol, DHT co-exposure concentration (0.25 nM), DHT serial dilutions (0.002 – 10 nM) and flutamide (0.013 – 8 µM) or procymidone (0.005 - 3.2 µM) serial dilutions. All concentrations were tested in duplicate over two plates, and each

pesticide was measured at least twice in separate experiments. For comparative purposes, luminescence was normalised to DHT alone at co-exposure concentration (maximum response - 100%) and solvent only (Ethanol) controls (minimum response - 0%). Initially flutamide was used as the internal quality control for anti-androgenicity, however, due to overlap of toxic effects on the cells with anti-androgenic activity, it was replaced by procymidone, which is more potent (IC50: flutamide = 1.56 µM, procymidone = 0.53 µM) but non-toxic to MDA-kb2 cells in the concentration range associated with receptor antagonism. Pesticides were initially tested over a concentration range of 0.64 nM - 50 µM (5 x dilutions) as a range finding exercise. Subsequently, the concentration ranges were modified to reflect the potency and toxicity of each individual compound. As cytotoxic effects could not be distinguished from anti-androgenic effects in the co-exposed treatments, any readings of the pesticide statistically significantly below the mean ethanol control level (0%) were considered toxic to MDA-kb2 cells and the corresponding co-exposure data were not classified as anti-androgenic. 60% of the pesticides were repeat tested using the same product but with new stock solutions and by a different experimenter.

Yeast androgen screen

The methods for the YAS were described previously (Sohoni and Sumpter 1998). Briefly, stimulation of the transfected AR causes a colour change in the media, which is measured by absorbance at 540 nm (Labsystems Multiskan© Multisoft). Plates were also measured at 620 nm to measure cell growth (turbidity) to check for any cytotoxic effects that may have occurred. Pesticides were co-incubated with DHT (6.4 nM). Controls run on each experiment were: ethanol, DHT serial dilutions (0.0026 - 100 nM) and flutamide serial dilutions (0.19 - 100 µM). Pesticide concentration range varied according to potency observed in MDA-kb2 assay, but was between 0.016 – 750 µM for all test compounds. Incubation time was 53 hours

at 28^0C. Where turbidity readings were significantly depressed, toxicity was indicated and the effect could not be considered anti-androgenic; therefore, these dilutions were removed from analysis. Pesticide serial dilutions were tested in duplicate over two plates, and were tested in two separate experiments.

Statistics

To analyse anti-androgenic action, raw luminescence readings were normalised on a plate-by-plate basis to the means of the positive DHT controls (n=8) and the solvent controls (n=8) (Ermler et al. 2010). All data from the same test compound were pooled and statistical concentration response regression analyses were conducted by using the best-fit approach (Scholze et al. 2001). Specifically, a variety of non-linear regression models were fitted independently to the same data set and the best fitting model was selected using a statistical goodness-of-fit criterion. Concentration-response data from different experimenters were first analysed one-by-one using regression models and differences in regression analyses due to data from different experimenters were judged as statistically significant when the 95% confidence intervals of the regression curves did not overlap. Such statistical differences between experimenters were not observed, and thus data were pooled for final analysis. Luminescence readings from pesticides 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) by using non-parametric contrast tests (Neuhaeuser et al. 2000). If considered to be statistically significant at $p < 0.05$, data were analysed using the best-fit approach as described. All statistical analysis was performed using the SAS statistical software (SAS Institute Inc., Cary, NC, USA). From the

best fitting model we derived inhibitory concentrations (IC) for anti-androgenicity and effect concentrations (EC) for cytotoxicity.

Results

The within-plate variation from readings of the positive DHT controls was derived as a coefficient of variation (CV), with 95% of all CVs falling between 2.1% and 12.9% (mean: 6.5%). Out of the 37 tested compounds, 24 pesticides were anti-androgenic in the MDA-kb2 assay, 9 of which are newly described (Table 1, Figure 1). The most potent *in vitro* AR antagonist was fenitrothion (IC20 = 0.098µM), and the least potent was pyrimethanil (IC20 = 27.2µM). All 14 compounds previously reported in the literature as anti-androgenic were confirmed in our test system. Two of 4 previously untested pesticides that were predicted to be AR antagonists in the QSAR were positive in the MDA-kb2 assay, and 3 of 13 pesticides that could not be predicted using the QSAR (i.e., that were out of the model domain) were also anti-androgenic. Five of 6 pesticides predicted to be inactive based on the QSAR were AR antagonists in the MDA-kb2 assay, but 3 were out of the QSAR prediction range because they were anti-androgenic at a concentration higher than the exclusion criterion of the QSAR (LOD: IC25 = < 10 µM; IC20: cyprodinil = 15.1 µM; pyrimethanil = 27.2 µM; cyhalothrin = 23.1 µM).

All 14 pesticides tested using the YAS were anti-androgenic, including two that lacked activity in the MDA-kb2 assay [tolylfluanid (out of domain of QSAR) and bifenthrin (predicted active in QSAR)] (Table 1).

Twenty-two of the 37 pesticides analysed in the MDA-kb2 assay were cytotoxic. The concentrations required to elicit cytotoxicity were between 2.1- (quinoxyfen) and 50-times (bromopropylate) higher than the concentrations associated with anti-androgenicity (based on

the ratio of EC20 for cytotoxicity and IC20 for anti-androgenicity). Seven of the chemicals analysed in the MDA-kb2 assay showed AR agonist activity when tested in the absence of DHT co-exposure, including two (cyprodinil and chlorpropham) with androgenic activity occurring at lower concentrations than anti-androgenic activity (Table 1, Figure 1). Four of 14 pesticides were cytotoxic in the YAS (cyprodinil, pyrimethanil, tolylfluanid, difenoconazole), while AR agonism was never observed in this assay (Table 1).

Discussion

Our results indicate that systematic testing for anti-androgenic activity of currently used pesticides is urgently required. For example, 20 of the 50 pesticides with the highest exposure scores were anti-androgenic in at least one assay, including 8 that have not been identified as anti-androgens previously (Supplemental Material, Figure 2). In previous *in vitro* screenings of current-use pesticides, proportions of anti-androgenic pesticides were broadly similar (32% (out of 161), Kojima et al. 2004; 50% (out of 12), Orton et al. 2009; 62% (out of 61), Vinggaard et al. 2008), further supporting the possibility that a large fraction of untested pesticides may be anti-androgenic. In contrast, estrogenic activity appears to be less common in current-use pesticides (18%, Kojima et al. 2004; 0% (out of 100), Nishihara et al. 2000; 0%, Orton et al. 2009). Some discrepancy between our data and published data exists, for example, pirimiphos-methyl was previously reported to have no anti-androgenic activity (Kojima et al. 2004) and chlorpropham has been reported to have no activity (Kojima et al. 2004) and to be anti-androgenic (Orton et al. 2009). These differences are most likely due to differences among the assay systems used. We also observed differences between findings based on the MDA-kb2 assay and the YAS. However, IC20 values based on the two assays never deviated by more than one order of magnitude with the exception of 2 pesticides

(tolylfluanid, bifenthrin) that were cytotoxic in the MDA-kb2 assay, and dimethomorph, for which a large divergence was observed in AR antagonist activity (IC20: MDA-kb2 = 0.263 µM; YAS = 38.5 µM).

Although our study was not designed to evaluate the QSAR by Vinggaard et al. (2008), and the number of chemicals falling within the applicability domain of the model was low, we note that several pesticides with anti-androgenic activity *in vitro* were not predicted by the QSAR, in part because some of the compounds were less potent than the prediction domain of the QSAR, which classifies chemicals with an IC25 > 10 µM as devoid of anti-androgenicity. The large proportion of pesticides for which the QSAR was not able to provide predictions (45 out of 64) suggests that extending the applicability domain would increase the usefulness of the model.

The ranking according to our exposure scoring system was similar to the listed "adjusted theoretical maximum dietary intake" of pesticides (58% concordance among the top 40 compounds) previously reported by Menard et al. (2008), which is based on actual French consumption data and maximum residue levels. Consequently, the ERR was similar using either our exposure scores, or the adjusted theoretical dietary intake published by Menard et al. (2008) (Supplemental Material, Table 2) Both our exposure data and that used by Menard et al. (2008) are sourced from before 2008 (except JMPR reports from 2008/2009), and therefore may not be fully representative of current exposures. Indeed, from 2005-2010 the authorisations for use granted by EU authorities expired for 12 of the tested pesticides, including several *in vitro* AR antagonists (procymidone, prochloraz, vinclozolin, ethoxyquin, endosulfan, azinphos-methyl, bromopropylate, dicofol, fenitrothion) and 3 without evidence of anti-androgenic activity (bifenthrin, propargite, profenofos). Thus, exposure to some of the tested compounds should decrease, whereas exposure to replacement products may increase. For example, a pesticide formulation called "Switch", which contains cyprodinil and

fludioxonil (both of which were anti-androgenic in our test system), was recommended as a replacement for the vinclozolin formulation "Ronilan" (Shah 2002).

To the authors' knowledge, except for two reports to date (Heudorf and Angerer 2001; Saieva et al. 2004), there is a complete absence of published human biomonitoring data for pesticides in Europe and, therefore, it is impossible to predict how the levels eliciting an effect *in vitro* may correspond to human internal concentrations. Similarly, although the National Health and Nutrition Examination Survey (NHANES) in the US incorporates human biomonitoring of pesticides, exposure concentrations at human target tissues are very poorly understood, due to the almost complete lack of toxicokinetic data, short half lives of current use pesticides, unspecific urinary metabolites, and unknown metabolic pathways (see Barr 2008). Pesticides with relatively large ERR, including dimethomorph (expiration of EU authorisation: Sept 2017), fludioxonil (Oct 2018), fenhexamid (May 2011), imazalil (Dec 2011), linuron (Dec 2013), *ortho*-phenylphenol (Dec 2019), tebuconazole (Aug 2019) and pirimiphos-methyl (Sept 2017) may be important anti-androgenic pollutants at present and in the future (Table 1). Linuron and tebuconazole are known *in vivo* anti-androgens (Lambright et al. 2000; Taxvig et al. 2007), however, data on the other pesticides are much more limited. This is especially true of dimethomorph, fludioxonil and fenhexamid, for which no previous publications regarding endocrine disruption could be identified. These compounds are newly formulated fungicides (2007, 2008, 2001), which are stable on food commodities (> 70% of the parent compound) and remain unchanged on the commodity when reaching the consumer (EFSA 2010a, 2007, 2010b). Dimethomorph and fenhexamid belong to the fungicide group of sterol biosynthesis inhibitors (Leroux 2004), as do the *in vivo* anti-androgenic conazoles (e.g. Taxvig et al. 2007) and imidazoles (Vinggaard et al. 2005). A study of the sterol biosynthesis inhibitors imazalil, propiconazole, triadimefon, triadimenol, and prochloraz indicated that all inhibited aromatase in human placental microsomes (Vinggaard et al. 2000),

but to our knowledge effects of dimethomorph and fenhexamid on steroidogenesis in mammalian cells have not been assessed. Imazalil and the *in vivo* anti-androgen prochloraz (Vinggaard et al. 2005) are both classified as imidazole fungicides and *in vitro* potency estimates for the two compounds were similar (imazalil IC20 = 3.23 µM, prochloraz = 2.39 µM), but the possible effects of imazalil *in vivo* have not been evaluated. Therefore, it is our view that dimethomorph, fludioxonil, fenhexamid, imazalil should be tested *in vivo* as a matter of urgency. Another relevant pesticide may also be *ortho*-phenylphenol, which is used as a fungicide in agriculture and as a wood preservative, and also has a wide variety of industrial applications (e.g. preservation of glues, plastic additives in flame retardants, disinfectant in hospitals) (LANXESS 2010). In our exposure ranking system it was ranked 12th out of 37 test compounds (Table 1). Considering it was highly ranked by exposure, and that non-agricultural sources were absent from our exposure scores, it is not surprising that it was detected in all human urine samples tested in two studies (mean concentration of 2.9 nM from a total of 30 samples (Ye et al. 2005); 35.2 nM from a total of 22 samples (Bartels et al. 1997), 85% of breast milk samples (mean concentration of 10.6 nMfrom a total of 20 samples, Ye et al. 2006) and 30% of amniotic fluid samples (mean of 0.76 nM from a total of 20 samples, Bradman et al. 2003) in the USA. It was previously identified as a receptor-mediated anti-androgen (Kojima et al. 2004), but there are no data available on its possible effects *in vivo*. Pirimiphos-methyl is an organothiophosphate insecticide that is stable on stored grain (<24 weeks 70% unchanged parent compound: EFSA 2005). There are also indications that it may be anti-androgenic *in vivo* as maternal and post-natal exposure of rats to 12 mg/kg b.w./day caused testicular tubular atrophy (EFSA 2005). In addition, treatment of adult male rats for 90 days resulted in decreased sperm density and mobility (125 mg/kg b.w./day), testicular atrophy (LOAEL 41.67 mg/kg b.w./day) and decreased fertility (125 mg/kg b.w./day) (Ngoula et al. 2007). There is insufficient evidence to assess the risk of

tested pesticides to human health due to a lack of data. However, to the authors' knowledge, all of the pesticides (with the possible exception of fenitrothion; Okahashi et al. 2005; Turner et al. 2002) that were identified as *in vitro* AR antagonists in our study, have also been reported to have anti-androgenic effects *in vivo* in animal models (Anway et al. 2006; Gray et al. 1999; Lambright et al. 2000; McIntyre et al. 2002; Ostby et al. 1999; Sinha et al. 2001; Taxvig et al. 2007; Uzumcu et al. 2004; Vinggaard et al. 2005). We also identified 7 compounds that appeared to be androgenic as they stimulated activity in the absence of DHT. The mechanism of action for this response is not well characterised, however, it has been previously detected in this assay (Tamura et al. 2006; Wilson et al. 2002), and was proposed to be due to conformational change of the ligand binding pocket in such a way that simultaneous androgenic and anti-androgenic activity were possible (Tamura et al. 2006). We are unable to confirm or reject these data however, preliminary data from our laboratory suggests that the stimulatory response is neither via stimulation of the receptor, as we have not observed evidence of androgenic effects in the YAS for any compounds, nor due to cell proliferation, as evidenced by transient transfection of cells with a non-androgenic responsive element. Cyprodinil and chlorpropham were more potent AR agonists (EC20 = 1.91 and 2.67, respectively) than antagonists (IC20 =15.1 and 7.66, respectively) in the MDA-kb2 assay.

Conclusions

In addition to identifying new candidate anti-androgens, our findings highlight important data gaps that prevent accurate assessment of male reproductive health risks from pesticides. The most important of these are the absence of *in vivo* studies and human biomonitoring data for environmentally relevant pesticides. In addition, fungicides typically had high exposure scores and were thus well represented in the testing set, presumably because they are often

applied just before or after harvest to food commodities. They are typically applied as mixtures in order to increase effectiveness and prevent development of resistant strains (FRAC 2010) and therefore, human exposure to mixtures of these *in vitro* anti-androgens may be considerable. In conclusion, the contribution of pesticides to declining male reproductive health requires further investigation, particularly in order to clarify the relationship between effective concentrations *in vivo* and exposure.

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Table 1. Receptor-mediated anti-androgenic activity and cytotoxicity in the MDA-kb2 and YAS

Compound	**Expiration**[a]	**Score**[b]	**QSAR Prediction**	**A-androgen IC20**[c]		**Cytotox. EC20**[c]		**Androgen EC20**[c]	**ERR**
Fungicides				**MDA-kb2**	**YAS**	**MDA-kb2**	**YAS**	**MDA-kb2**	
*Cyprodinil	04.2017	33	Inactive	15.1	1.34	> 50	27.8	1.91	2.2
Procymidone	06.2008	33	AA	0.163	0.956	> 50	> 160	neg	202.5
Imazalil	12.2011	32	AA	3.23	------	19.0	------	neg	9.9
*Pyrimethanil	05.2017	28	Inactive	27.2	9.15	> 125	167	27.8	1.0
*Fludioxonil	10.2018	25	OD	0.801	0.730	28.5	> 160	neg	31.2
Azoxystrobin	12.2011	24	Inactive	neg	------	2.9	------	neg	na
*Fenhexamid	05.2011	24	Active	2.02	------	21.6	------	neg	11.9
Tolylfluanid	09.2016	24	OD	neg	0.234	8.09	1.14	neg	na
O-Phenylphenol	12.2019	21	AA	3.43	------	> 50	------	neg	6.1
Prochloraz	12.2010	18	AA	2.39	------	12.5	------	neg	7.5
Pyraclostrobin	05.2014	17	OD	neg	------	0.089	------	neg	na
Mandipropamid	07.2011	18	OD	neg	------	8.92	------	neg	na
Tebuconazole	08.2019	16	AA	2.89	------	38.9	------	neg	5.5
Difenoconazole	12.2018	13	Active	neg	neg	2.91	0.109	neg	na
Vinclozolin	01.2007	13	AA	0.163	------	> 50	------	2.9	79.8
*Dimethomorph	09.2017	12	Active	0.263	38.5	> 25	> 50	neg	45.6
*Quinoxyfen	08.2014	12	Inactive	4.79	1.21	10.1	> 75	neg	2.5
Spiroxamine	12.2011	9	OD	neg	------	9.29	------	neg	na
Ethoxyquin	03.2008	8	AA	10.7	11.1	> 50	> 200	neg	0.75
Insecticides				**MDA-kb2**	**YAS**	**MDA-kb2**	**YAS**	**MDA-kb2**	
*Pirim.-methyl	09.2017	30	OD	5.49	3.08	> 50	> 200	neg	5.5
Endosulfan	06.2006	19	AA	6.05	------	33.8	------	neg	3.1
Methiocarb	09.2017	17	AA	6.82	------	> 46	------	neg	2.5
Spirotetramat	Pending	17	OD	neg	------	> 50	------	neg	na
**Azin.-methyl*	01.2007	16	OD	5.38	2.25	33.9	> 150	neg	2.9
Bifenthrin	05.2010	16	Active	neg	99.8	22.2	> 200	neg	na
Indoxacarb	03.2016	16	OD	neg	------	11.3	------	neg	na
Spinosad	01.2017	16	OD	neg	------	13.1	------	neg	na
*λ-Cyhalothrin	12.2011	15	Inactive	23.1	95.4	51.4	> 200	neg	0.65
Dicofol	03.2009	15	AA	1.43	------	29.0	------	neg	10.5
Bromopropylate	07.2007	13	AA	0.540	------	27.2	------	neg	24.1
Propargite	12.2010	13	OD	neg	------	0.487	------	neg	na
Fenitrothion	11.2007	11	AA	0.098	------	> 50	------	4.9	112.2
Novaluron	07.2011	9	OD	neg	------	> 50	------	neg	na
Profenofos	07.2003	8	OD	neg	------	8.61	------	neg	na
p p'-DDE	1986	**	AA	0.948	------	> 50	------	3.6	na
Herbicides				**MDA-kb2**	**YAS**	**MDA-kb2**	**YAS**	**MDA-kb2**	
Chlorpropham	01.2015	22	Inactive	7.66	10.2	> 50	> 40	2.67	2.9
Linuron	12.2013	12	AA	1.74	------	> 50	------	3.48	6.9

[a]Expiration date is taken from Annex 1 of Council Directive 91/414/EEC concerning the "placing of plant protection products on the market", if that date is in the past, the compound can no longer be used in Europe (indicated by pesticides that are in italics). [b]See text and Tables S1/S2 for details of exposure score. [c]IC20/EC20, values in µM. A-androgen = anti-androgenic, androgen = androgenic in the absence of DHT, cytotox = cytotoxicity, ERR = environmnental relevance ratio (described in text). YAS = Yeast Androgen Screen, AA = anti-androgenic and refers to known anti-androgens (not assessed by QSAR), OD = Out of Domain (QSAR was not able to predict activity for this compound), neg = no response was observed, * = a newly described anti-androgenic compound, ** DDE not included in ranked exposure. Azin.-methyl = azinphos-methyl, pirim.-methyl = pirimiphos methyl. na = not applicable.

Figure Legend

Figure 1. Regression curves showing anti-androgenic pesticides, and accompanied by stimulatory activity for chlorpropham & cyprodinil. Graphs I-IV are grouped by exposure scores (see Table 1), from highest (group I) to lowest (group IV), with procymidone added to graphs II-IV as a point of reference. Compounds in bold and italic in the legend indicate newly described anti-androgens. Regression lines end at the toxic threshold. Dashed lines indicate pesticides with lapsed registration, solid lines indicate pesticides with current registration. Graphs for chloropham and cyprodinil are shown to demonstrate overlap of androgen receptor antagonism (solid data points and curves) with receptor agonism (light curves). Anti-androgenicity data in graphs I-IV are shown as mean ±standard error

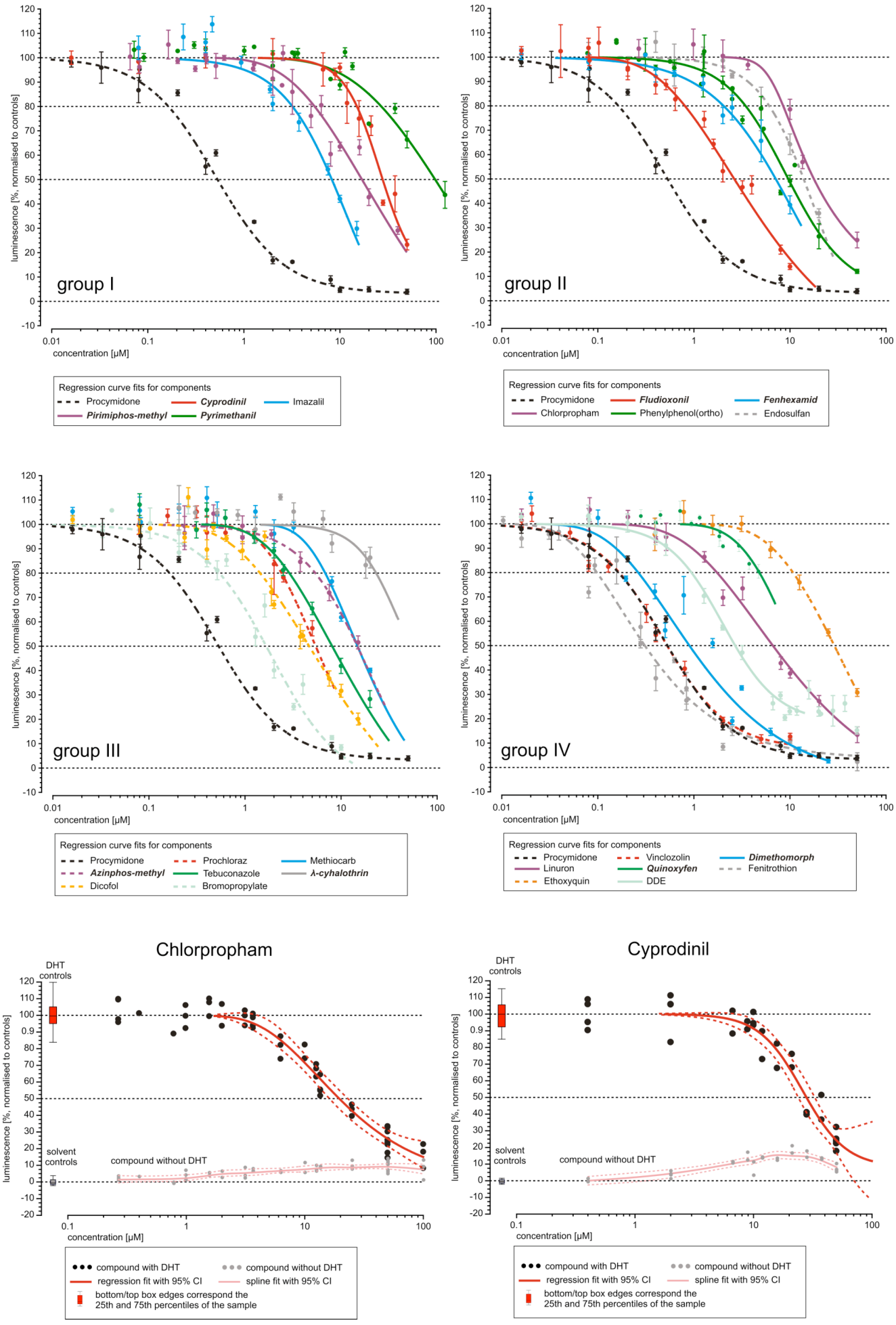
group I
luminescence [%, normalised to controls]
concentration [µM]
Regression curve fits for components
Procymidone
Cyprodinil
Imazalil
Pirimiphos-methyl
Pyrimethanil
group II
Fludioxonil
Fenhexamid
Chlorpropham
Phenylphenol(ortho)
Endosulfan
group III
Prochloraz
Methiocarb
Azinphos-methyl
Tebuconazole
λ-cyhalothrin
Dicofol
Bromopropylate
group IV
Vinclozolin
Dimethomorph
Linuron
Quinoxyfen
Fenitrothion
Ethoxyquin
DDE
Chlorpropham
Cyprodinil
DHT controls
solvent controls
compound without DHT
compound with DHT
regression fit with 95% CI
spline fit with 95% CI
bottom/top box edges correspond the 25th and 75th percentiles of the sample

Figure 1