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Abstract

Pesticides have been suspected to act as endocrine disruptive compounds (EDCs) through several mechanisms of action, however data are still needed for a number of currently used pesticides. In the present study, 30 environmental pesticides selected from different chemical classes (azole, carbamate, dicarboximide, oganochlorine, organophosphorus, oxadiazole, phenylureas, pyrazole, pyrimidine, pyrethroid and sulfonylureas) were tested for their ability to alter *in vitro* the transcriptional activity of the androgen receptor in the MDA-kb2 reporter cell line. The responsiveness of the system was checked by using a panel of reference ligands of androgen and glucocorticoid receptors. When tested alone at concentrations up to 10 µM, none of the studied pesticides were able to induce the reporter gene after a 18 hour exposure. Conversely, co-exposure experiments with 0.1 nM dihydrotestosterone (DHT) allowed identifying 15 active pesticides with IC_{50} ranging from 0.2 µM for vinclozolin to 12 µM for fenarimol. Fipronil and bupirimate were here newly described for their AR antagonistic activity.

Keywords: reporter gene assay, pesticides, androgen and glucocorticoid receptors, anti-androgenic potency.

1. Introduction

A number of environmental chemicals, the so-called endocrine disruptive chemicals (EDCs), can alter the normal functioning, synthesis and metabolism of endogenous hormones, and thereby affect growth, development and reproduction in wildlife and humans (Colborn 1995). EDCs are supposed to be active through several molecular mechanisms of action, among which the binding to and subsequent (in)activation of nuclear receptors, including the estrogen (ER) and androgen receptors (AR), has been identified as an important mechanism that mediates endocrine disrupting effects. These receptors are transcriptional factors that play crucial role in the regulation of target gene expression in physiological processes like development, sex differentiation, reproduction, as well as neuro-endocrine system functioning.

A large diversity of environmentally occurring chemicals, including natural and synthetic steroids, pharmaceuticals, industrial and phytopharmaceutical chemicals, have been described as ligands of nuclear receptors (Vos et al., 2000). Among them, the risks for wildlife and human health posed by pesticides is currently a major cause for concern (Colborn and Carroll 2007; McKinlay et al., 2008). Pesticides encompass a large panel of chemical classes that are widely used in both agricultural and non-agricultural applications, and wildlife and human may be exposed through multiple routes including atmosphere, water, occupational, domestic and food consumption. While certain environmental pesticides (e.g. vinclozolin and methoxychlor) or pesticide metabolite (e.g. *p,'p*-DDE) have been early identified as potent anti-androgen and/or estrogen mimicking compounds (Kelce et al., 1995; LeBlanc et al., 1997), the evaluation of currently used pesticides is of more recent concern besides (Andersen et al., 2002; Kojima et al., 2004; Lemaire et al., 2004). McKinlay et al. (2008) recently listed 127 pesticides as having endocrine disrupting properties through various modes of molecular actions in *in vitro* or *in vivo* systems. Although some of the listed pesticides were banned or restricted for use, they are still found in the aquatic environment (Baugros et al., 2008; Kinani et al., 2010). In addition, many pesticides that are authorised for use are now detected in water bodies and may pose a risk to exposed population (Comoretto et al., 2007; IFEN 2007). However, there is still a lack of information on the ED potency of a number of currently used pesticides. In this context, the use of *in vitro* screening assays that inform on specific ED-related mechanisms of action can provide rapid and relevant toxicological information in order to characterise ED property of not yet evaluated pesticides.

In a previous study, we have reported that several currently used pesticides were able to interfere with steroid hormone biosynthesis by altering aromatase enzymatic activity and gene expression in a human placental cell line (Laville et al., 2006). In order to gain further information on their ED potency, these compounds (listed in Table 1) were tested in the present study for their ability to alter the transcriptional activity of the androgen receptor (AR) in the MDA-kb2 reporter cell line (Wilson et al., 2002). This cell line is derived from MDA-MB-453 cells that were stably transfected by the luciferase reporter gene driven by the MMTV promoter. In these cells, this promoter is up-regulated by two endogenous nuclear receptors, AR and glucocorticoid receptors (GR). After calibration of the reporter system in our test conditions using reference AR and GR ligands, the agonistic and AR antagonist actions of pesticides were screened.

2. Materials and methods

2.1. Chemicals

Flutamide, testosterone, 5α-dihydrotestosterone (DHT), androstenedione, dexamethasone, RU846 and pesticides (listed in Table 1) were purchased from Sigma-Aldrich (Saint Quentin Fallavier, France), except azimsulfuron (DuPont de Nemours, France) and sodium arsenite (Alpha Aesar, France). Stock solutions (10 mM) of chemicals were made up in dimethylsulfoxide (DMSO) and stored at –20°C.

2.2. Cell culture and reporter gene assay

The MDA-kb2 cell line (ATCC #CRL-2713) was obtained from LGC Promochem (Molsheim, France). The cells were routinely grown at 37°C under humidified air atmosphere in phenol red containing L15 Medium (Sigma) supplemented with 10 % foetal calf serum (FCS), 1 % (v/v) non essential amino acids, penicillin/streptomycin (50 U/ml each) and 0.1 mg/ml geneticin (Invitrogen, France). The assay procedure for chemical testing is given as follows, accordingly to preliminary assays done in our laboratory in order to optimise assay conditions. Briefly, the cells were seeded at a density of 10^4 cells per well of 96-well white opaque tissue culture plates, and left to incubate for 24 hours at 37°C. Then, for each plate, the cells were dosed in triplicates with solvent alone (negative control), 1 nM and 0.1 nM DHT (as positive controls for AR agonism), 0.1 nM DHT plus 1 µM flutamide (as positive control for AR antagonism), and a range of concentrations of test chemical administrated

either alone (for AR/GR agonistic activity) or in the presence of 0.1 nM DHT in culture medium (for AR antagonistic activity). A screening approach was first used by exposing cells to 1 and 10 µM of test chemicals in order to identify active chemicals. After confirmation of biological effect in second experiments, active compounds were further tested using larger range of concentration (0.01 to 10 µM, up to 30 µM for fenarimol) to establish complete dose-response curves allowing determination of median effective concentration (see section 2.4.). Overall, each chemical has been tested in at least three independent experiments. The final solvent concentration in the medium never exceeded 0.1 % (v/v). At this concentration, DMSO did not alter either luciferase activity or cell viability. After 24 h incubation at 37°C, the medium was removed and replaced by 50 µl of phenol red free medium containing 3.10^4 M D-luciferin (Sigma) and the luminescence signal in living cells was read after 5 minutes with a microtiter plate luminometer (µBeta, Wallac). Results were expressed as percent of maximal luciferase induction by 10 nM DHT. Anti-androgenic activity was examined after co-exposure of the cells to both 0.1 nM DHT and the test chemical. At this concentration, DHT induced about half-maximal luciferase activity in our experiments.

2.3. Cytotoxicity assay

The cellular viability was assessed in independent experiments by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, as described by (Mosmann 1983), with slight modifications. Briefly, cells were seeded at density of 10^4 cells/well in 96-wells culture plates, left to grown for 24 hours, and exposed in triplicates to 1 and 10 µM of test chemicals for 18 hours. After exposure, culture medium was removed and 0.5 mg/ml MTT dissolved in culture medium was added to the wells. After two hours of incubation at 37°C, the medium was removed and the purple formazan product was dissolved by isopropanol addition. Plates were read at 570 nm against a 660 nm reference wavelength with a microplate spectrophotometer reader (BioTek Instruments, France). Cell viability was expressed as a percentage of the value measured in untreated control cells (solvent control).

2.4. Data analysis

Statistical significance of the effects of chemicals was assessed by using the non parametric Mann-Whitney test. A value of $p < 0.05$ was considered significant. The SPSS™ software version 10.1 for Windows was used for the statistical analysis. For reporter gene response, dose-response curves were modelled by using the Regtox 6.3 Microsoft Excel macro (Vindimian 2010), which allows calculation of EC_{50} (concentration that induces 50 %

of maximal response) and IC_{50} (concentrations that inhibits 50 % of maximal response). The relative anti-androgenic potency (RAAP) was determined as the ratio of IC_{50} value of flutamide to that of test compound.

3. Results

3.1. Activity of reference AR and GR ligands

In order to calibrate the use of the MDA-kb2 cell line in our laboratory conditions, reference AR and GR ligands were first tested. Typical dose-response curves that were obtained for AR agonists and antagonists are shown in Figures 1 and 2, respectively. In these experiments, DHT was the most potent AR agonist, followed by testosterone (T), methyltestosterone (MT) and androstenedione (AD), in a decreasing potency order. Maximum fold-induction of luciferase by AR full agonists varied from 5 to 10 across experiments, while the GR agonist dexamethasone induced luciferase 50 to 80 times as compared to untreated control cells (not shown). The EC_{50} and the ranges of fold-induction of luciferase determined in our study for both well-known AR and GR ligands were in line with those previously published using the same cellular model (summarised in Table 2, references therein). These results validated the use of this cell model in our experimental conditions, which involves slight differences in the assay procedure (*i.e.* detection of luciferase activity in living cells) comparing to that previously described for the use of this reporter cell line for the testing of chemicals (Wilson et al., 2002).

3.2. (Anti-)androgenic activity of pesticides

At 10 µM, no significant cytotoxicity was observed after exposure to individual pesticides, as determined by the MTT assay (data not shown). When tested alone for their agonist activity, none of the pesticides significantly induced the luciferase activity in MDA-kb2 cells (data not shown). Conversely, 14 out of 30 pesticides behaved as AR antagonists as they were able to significantly inhibit DHT-induced luciferase in a concentration dependent manner. Active compounds were arbitrarily grouped in two groups of chemicals according to their inhibitory potencies, i.e. as moderate (chemicals that inhibited less than 50 % of DHT-induced luciferase at 10µM) (Figure 3), and potent anti-androgenic pesticides (chemicals that inhibited more than 50% of DHT-induced luciferase at 10µM) (Figure 4). Their IC_{50} ranged from 0.19 µM for vinclozolin, the most potent pesticide, up to 12 µM for fenarimol (Table 1). By regards to their relative anti-androgen potency (RAAP), most of the pesticides were found 3 (*o,p'*-DDE)

to 100 (fenarimol) times less potent than the reference anti-androgen flutamide (Table 1), except for vinclozolin and the methoxychlor metabolite HPTE, that were more or equally potent than flutamide, respectively. Interestingly, bupirimate and fipronil were newly identified as potential antiandrogens.

It is noteworthy that flutamide is a moderate anti-androgen but is metabolised *in vivo* in hydroxyflutamide (HF), a stronger anti-androgen. Using this compound as a reference to determine RAAP of a given compound could be misleading as it is not known whether MDA-kb2 cells can metabolise it. However, Ma et al. (2002) reported that HF was about ten times more potent than flutamide suggesting that metabolism of flutamide in this cell system has minor influence on its anti-androgenic activity. Nonetheless, in the present study, RAAP based on flutamide as a reference should first be viewed as an index allowing relative ranking of active chemicals rather than as an absolute value of their intrinsic anti-androgenic activity.

4. Discussion

By using the MDA-kb2 cell line established by (Wilson et al., 2002), we were able to assess the ability of various selected pesticides to interact with the human AR transcriptional pathway. This study shows the usefulness of the MDA-kb2 cell line to detect AR antagonistic property of several pesticides. The main outcomes of this study are:

- the suitability of the MDA-kb2 cell line to detect anti-androgenicity of pesticides in a screening approach,
- the anti-androgenicity of 15 out of the 30 tested pesticides from various chemical classes, among which several were previously poorly or not described as such,
- the newly identified anti-androgenic property of two currently used pesticides, fipronil and bupirimate.

In first experiments using reference AR and GR ligands, we could establish a very good adequacy between our results and those previously published by using the same cellular model (Table 2). Moreover, the use of a slightly divergent procedure assay (*i.e.* detection of luciferase activity in living cells) than that one originally described by Wilson et al (2002) did not yield divergent results when comparing the IC_{50} values for the reference compounds. In a practical way, the detection of luciferase in living cells has been previously reported for the assessment of luciferase activity in other reporter cell lines (Balaguer et al., 2001; Cosnefroy et al., 2009; Creusot et al., 2010; Pillon et al., 2005) and can thus be recommended to enhance the screening capacity of stable luciferase reporter assays using MDA-kb2 cells. This

comforted the validity of the MDA-kb2 assay in our laboratory conditions and allowed us to further explore the effects of pesticides of environmental interest.

When tested alone, none of the studied pesticides significantly induced the luciferase activity in MDA-kb2 cells (data not shown), which is not an unexpected finding. Indeed, the lack of AR agonistic activity by the tested pesticides is in line with the frequently reported absence of *in vitro* androgenic potency for a large range of organic environmental contaminants, such as pesticides from various chemical classes (Kojima et al., 2004; Lemaire et al., 2004; Xu et al., 2008), benzophenone derivatives (Molina-Molina et al., 2008), alkyphenols and parabens (Satoh et al., 2005). Additionally, by using the *in vitro* AR-EcoScreen reporter gene assay, (Araki et al., 2005) reported that only two out of 253 tested industrial chemicals exerted slight AR agonist activity.

Conversely to the absence of agonistic effects, fifteen out of 30 tested pesticides were able to inhibit in a concentration dependent manner the DHT-induced luciferase in MDA-kb2 cells (Figure 2, Table 1). Vinclozolin and HPTE, which are well-described antagonists of the AR in various cellular models (Andersen et al., 2002; Kelce et al., 1994), were the most active compounds with experimental IC_{50s} equal to (HPTE) or lower (vinclozolin) than the reference AR antagonist flutamide. The 12 other active pesticides presented lower affinities for the AR than flutamide, with relative potencies ranging from 31.5 % for *o,p'*-DDE to 4.3 % for fenarimol. Some of them, such as *o,p'*-DDT, *o,p'*-DDE, endosulfan, methoxychlor, fenarimol or prochloraz, are well known EDCs and their *in vitro* anti-androgenic potency has been previously reported, albeit in different cellular models, which confirms our results (see Table 1 for references).

HPTE was more potent than its parent compound methoxychlor to act as an AR antagonist in MDA-kb2 cells. HPTE is a di-hydroxylated metabolite that is generated *in vivo* by demethylation of methoxychlor by cytochrome P450 monooxygenases (van den Berg et al., 2003). Thus, the comparison of their effects in MDA-kb2 cells suggest that this cell line does not bioactivate such xenobiotic compounds, likely due to low phase I metabolic capacities. To our knowledge however, no information is available regarding xenobiotic metabolic of this cell line.

In MDA-kb2 cells, few recent studies have reported the AR antagonist activity of pesticides. Tamura et al (2006) reported the anti-androgenic activities for fenarimol and methoxychlor in this cell line, although with higher IC_{50} than in our study. Chlordane was not active in our study, confirming its lack of anti-AR activity recently reported in MDA-kb2 cells (Tamura et al, 2006), while it has been shown to slightly inhibit R1881-induced luciferase activity in

other cell models, like the stable reporter PALM cells (Lemaire et al., 2004) and CHO using a transient transfection assay (Kojima et al., 2004). These discrepancies may be due to the use of different cellular models, which have been largely unexplored for *in vitro* AR-luciferase assays.

Permethrin and cypermethrin were ineffective to exert either estrogenic or (anti)androgenic in rat *in vivo* (Kunimatsu et al., 2002). Other authors recently reported the ability of the two pesticides to partially inhibit DHT-induced luciferase at 10 µM in an androgen-responsive reporter cell line (Xu et al., 2008). In our study, no effect was observed at 10 µM for both pesticides.

Fipronil and bupirimate were newly identified as possible anti-androgen chemicals. Fipronil is a phenylpyrazole insecticide and is currently authorised for use in many applications in Europe and other areas. Apart from its potency to interfere with the AR signalling pathway, fipronil is also able to activate pregnane X receptor (PXR) activity *in vitro* (Lemaire et al., 2006). Since it has been well described as both environmental (surface waters, soil) and food chemical contaminant, the present data reinforce the need to consider environmental hazard due to fipronil with regards to its endocrine disrupting potential. Bupirimate is a pyrymidine fungicide that acts by inhibiting fungal growth through interference with auxines. To our knowledge, almost no data are available regarding its endocrine disrupting potency. It has only been described as a potent PXR activator in vitro (Lemaire et al., 2006). We show here that it may also potentially interfere with the AR signalling pathway.

Overall, it is noted that several of identified active chemicals only partially inhibited DHT-induced luciferase at high concentrations, hence posing the question of possible false positives. Although no cytotoxic effect was noted at the highest tested concentration and no alteration of basal luciferase expression was observed in cell exposed to pesticides alone, it cannot be excluded that some of the observed effects could be related to unspecific inhibition of reporter enzyme independently from the AR signalling pathway. Further experiments, such as AR competitive binding assay, would thus be useful to confirm the data reported in the present study and elucidate underlying mechanisms.

In summary, this study shows the suitability of the MDA-kb2 cell line for a screening purpose to assess anti-androgenic property of chemicals, including pesticides. Among the pesticides that were found active in this study, several are still in use despite data on their endocrine disrupting potencies are very recent or scarce. It is however acknowledged that the present assay describes potential (anti)androgens and does not necessarily inform on global endocrine disruptive potential of test chemical. In addition, weakly active pesticides *in vitro* are likely to

exert limited anti-androgenic effect *in vivo*. However, together with other previous studies that addressed the effects of this set of molecule on other molecular targets of EDCs (Laville et al, 2006, Lemaire et al, 2006), our present results strengthen the need to further explore their endocrine disruptive effects *in vivo*, especially for chemical that are able to alter multiple targets for EDCs.

Acknowledgements

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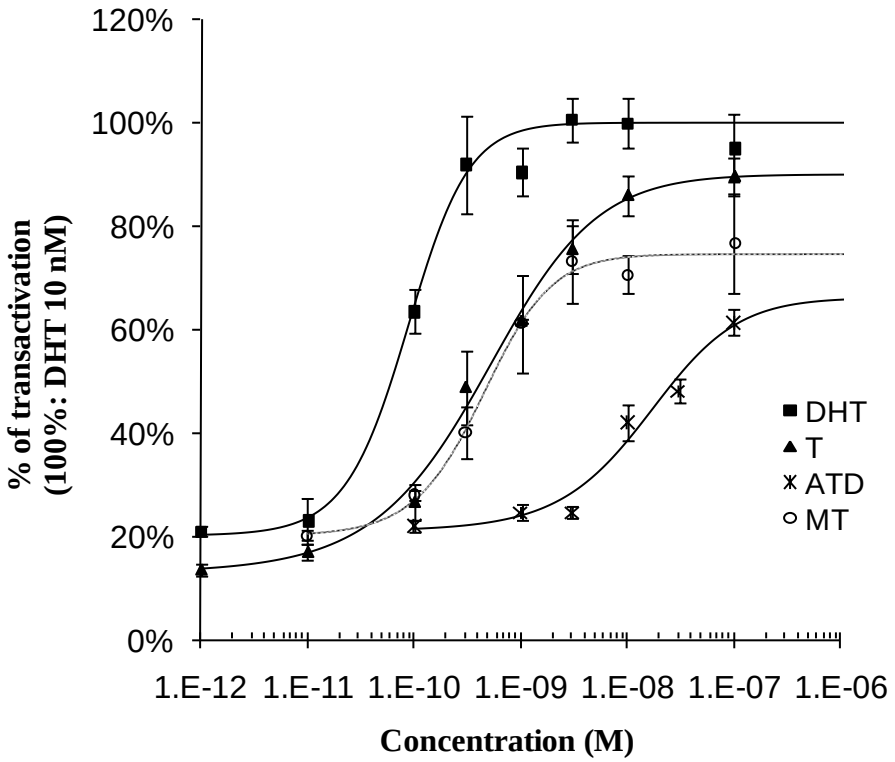

Figure 1: Luciferase induction by the reference AR ligands. MDA-kb2 cells were exposed for 18 h to testosterone (T), 5α-dihydrotestosterone (DHT), 17α-methyltestosterone (MT) and androstenedione (ATD). Data are representative of at least three independent experiments.

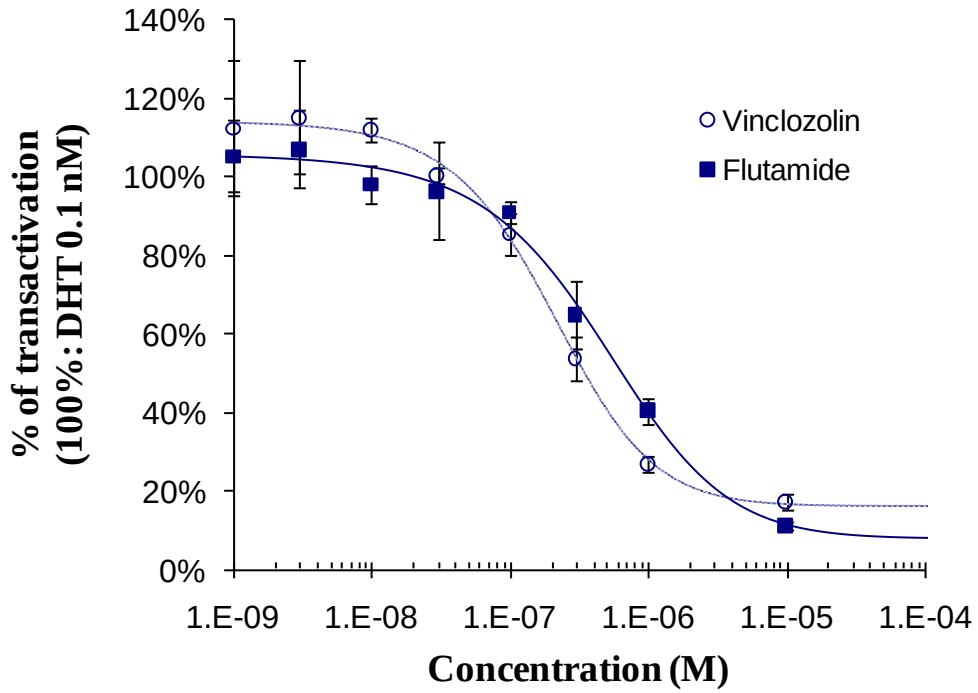

Figure 2: Luciferase inhibition by reference AR antagonists in MDA-kb2 cells. The cells were co-exposed DHT 0.1nM and various concentration of antagonist (flutamide or vinclozolin) for 18 hours. The results are expressed percentage of luciferase induced by DHT alone Data are representative of at least three independent experiments.

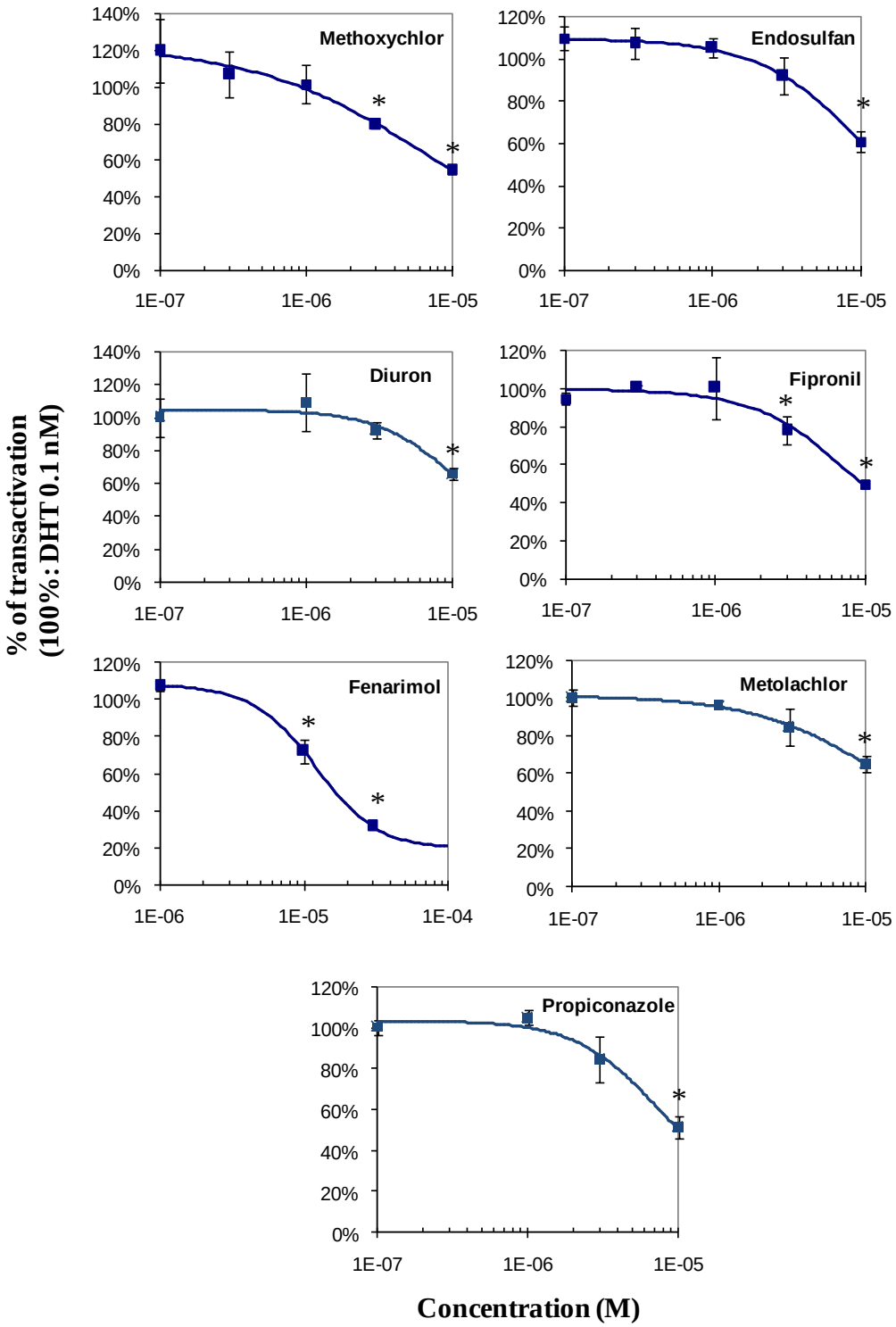

Figure 3: Dose-response curves of luciferase inhibition by moderately active pesticides in MDA-kb2 cells. The cells were co-exposed for 18 hours to both 0.1 nM DHT and various concentrations of pesticides. Calculated IC_{50}s are reported in Table 1. *: different from solvent control ($p<0.05$). Data are representative of at least three independent experiments.

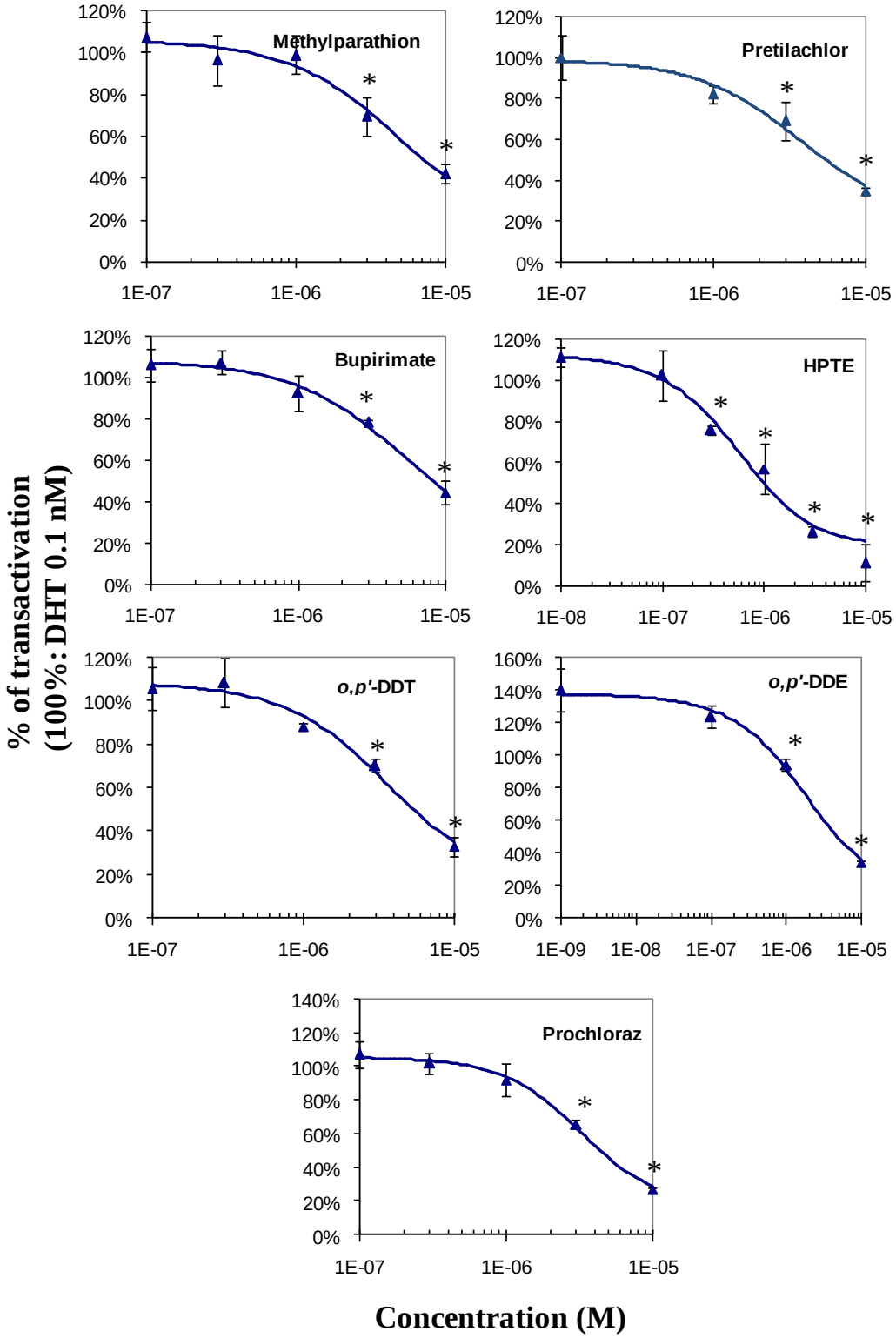

Figure 4: Dose-response curves of luciferase inhibition by the most active pesticides in MDA-kb2 cells. The cells were co-exposed for 18 hours to both 0.1 nM DHT and various concentrations of pesticides. Calculated IC_{50}s are reported in Table 1. *: different from solvent control ($p<0.05$). Data are representative of at least three independent experiments.

Table 1: Summary of anti-androgenic potency of pesticides tested in MDA-kb2 cells and comparison with previously published activities using other *in vitro* biological models[a].

Pesticides	CAS #	IC_{50} (µM) [b]	RAAP (relative to flutamide)	Reported anti-androgenicity (in vitro cell model)	Ref. [c]
Flutamide	13311-84-7	0.51	1		
Vinclozolin	50471-44-8	0.19	2.68	A-	1
HPTE	2971-36-0	0.54	0.94	A- (CHO)	3
o,p'-DDE	3424-82-6	1.62	0.32	A-	4
Prochloraz	67747-09-5	3.09	0.17	A- (CHO)	3, 5
o,p'-DDT	50-29-3	3.32	0.15	A- (CHO)	5
Methyl Parathion	298-00-0	4.26	0.12	A- (CHO, HepG2)	5, 6
Methoxychlor	72-43-5	4.43	0.12	A- (CHO, PALM)	5, 7
Bupirimate	41483-43-6	4.80	0.11	no data	
Pretilachlor	51218-49-6	5.46	0.093	n.e. (CHO)	5
Fipronil	120068-37-3	6.82	0.075	no data	
Diuron	330-54	6.83	0.075	A- (CHO)	5
Propiconazole	262-104-4	7.71	0.066	A- (CHO)	5
Endosulfan	115-29-7	8.74	0.058	A- (CHO)	3, 5
Metolachlor	51218-45-2	9.92	0.051	n.e. (CHO)	5
Fenarimol	60168-88-9	11.80	0.043	A- (CHO)	3, 5
Aldrin	309-00-2	n.e.	n.e.	A ± (CHO, PALM)	5, 7
Atrazine	1912-24-9	n.e.	n.e.	n.e. (PALM)	5, 8
Benomyl	17804-35-2	n.e.	n.e.	n.e. (HeLa)	5, 9
Chlordane	57-74-9	n.e.	n.e.	A- (CHO, PALM)	5, 7
Cypermethrin	67375-30-8	n.e.	n.e.	weak A- (CV-1)	10
Heptachlor	76-44-8	n.e.	n.e.	n.e. (CHO)	5
Permethrin	52645-53-1	n.e.	n.e.	weak A- (CV-1)	10
Toxaphene	8001-35-2	n.e.	n.e.	n.e. (binding assay)	11
Aminotriazole	61-82-5	n.e.	n.e.	no data	
Arsenite Na	7784-46-5	n.e.	n.e.	no data	
Azimsulfuron	120162-55-2	n.e.	n.e.	no data	
Fenbuconazole	114369-43-6	n.e.	n.e.	no data	
Isoproturon	34123-59-6	n.e.	n.e.	no data	
Mecoprop	16484-77-8	n.e.	n.e.	no data	
Oxadiazon	19666-30-9	n.e.	n.e.	no data	

a: RAAP: relative anti-androgenic potency; A±: described as either agonist or antagonist for the AR; A-: AR antagonist; n.e. : non effective pesticide; no data : no data could be found in literature.

b: Reported IC50 were derived from single experiments (Figures 3 and 4); anti-androgenic activity of pesticides have been confirmed in at least three independent experiments.
c: 1: (Kelce et al., 1994); 2: (Sohoni and Sumpter 1998); 3: (Andersen et al., 2002); 4: (Kelce et al., 1995); 5: (Kojima et al., 2004); 6: (Tamura et al., 2003); 7: (Lemaire et al., 2004), 8: (Sultan et al., 2001); 9: (Yamada et al., 2005); 10: (Xu et al., 2008); 11: (Scippo et al., 2004).

Table 2: EC_{50} or IC_{50} of reference AR and GR ligands in MDA-kb2 cells. Comparison with data from previous studies using the same cellular model.

Reference ligands	EC_{50} (nM) ±S.D.	IC_{50} (nM) ±S.D.	*n* [a]	Bibliographic data (EC_{50} or IC_{50} in nM)
AR ligands				
5α-dihydrotestosterone	0.15 ± 0.07	-	9	0.21 [b], 0.136 [c], 0.6 [d]
Testosterone	0.40 ±0.07	-	5	1.3 [d]
17α-methyltestosterone	0.57; 0.44	-	2	0.53 [b], 1.25 [c]
Androstenedione	37.9 ± 28.2	-	3	140 [b], 73.5 [c]
Flutamide	-	512 ± 102	3	788 [c]
Vinclozolin	-	187 ± 55	3	110 [b], 109 [c]
GR ligands				
Dexamethasone	4.40 ± 0.62	-	6	12.6 [c]
RU486	-	8.8± 4.1	3	-

a : n : number of independent experiments

b (Korner et al., 2004)

c (Ma et al., 2003)

d (Satoh et al., 2005)