Toxicology and Applied Pharmacology 272 (2013) 757–766

Contents lists available at ScienceDirect

Toxicology and Applied Pharmacology

journal homepage: www.elsevier.com/locate/ytaap

In vitro - in vivo correlations for endocrine activity of a mixture of currently used pesticides ☆

Camilla Taxvig [a,*], Niels Hadrup [a], Julie Boberg [a], Marta Axelstad [a], Rossana Bossi [b], Eva Cecilie Bonefeld-Jørgensen [c], Anne Marie Vinggaard [a]

[a] Division of Toxicology and Risk Assessment, National Food Institute, Technical University of Denmark, Mørkhøj Bygade 19, DK-2860 Søborg, Denmark
[b] Department of Environmental Science, Aarhus University, DK-4000 Roskilde, Denmark
[c] Department of Public Health, University of Aarhus, DK-8000 Aarhus C, Denmark

ARTICLE INFO

Article history:
Received 10 May 2013
Revised 12 July 2013
Accepted 24 July 2013
Available online 14 August 2013

Keywords:
Pesticides
Mixtures
Rat
Aromatase
H295R
Females

ABSTRACT

Two pesticide mixtures were investigated for potential endocrine activity. Mix 3 consisted of bitertanol, propiconazole, and cypermethrin, and Mix 5 included malathion and terbuthylazine in addition to the three pesticides in Mix 3.

All five single pesticides and the two mixtures were investigated for their ability to affect steroidogenesis *in vitro* in H295R cells. The pesticides alone and both mixtures affected steroidogenesis with both mixtures causing increase in progesterone and decrease in testosterone. For Mix 5 an increase in estradiol was seen as well, indicating increased aromatase activity.

The two mixtures were also investigated in pregnant rats dosed from gestational day 7 to 21, followed by examination of dams and fetuses. Decreased estradiol and reduced placental testosterone were seen in dams exposed to Mix 5. Also a significant increase in aromatase mRNA-levels in female adrenal glands was found for Mix5. However, either of the two mixtures showed any effects on fetal hormone levels in plasma or testis, or on anogenital distance.

Overall, potential aromatase induction was found for Mix 5 both *in vitro* and *in vivo*, but not for Mix 3, an effect likely owed to terbuthylazine in Mix 5. However, the hormonal responses *in vitro* were only partly reflected *in vivo*, probably due to some toxicokinetic issues, as the pesticide levels in the amniotic fluid also were found to be negatively affected by the number of compounds present in the mixtures. Nonetheless, the H295R assay gives hints on conceivable interference with steroidogenesis, thus generating hypotheses on *in vivo* effects.

Introduction

The high prevalence of disorders related to the endocrine system, *e.g.* fertility problems and congenital malformations of reproductive organs, has been of growing concern for several years. Genetic, environmental and lifestyle factors are likely to be involved in these adverse effects, and one of these factors is developmental exposure to endocrine active compounds (EACs) (IPCS, 2002).

Humans are exposed to a mixture of several EACs (Blount et al., 2000; Swan et al., 2005), and during the last decade, scientific and regulatory focus has gradually shifted towards taking mixture effects into account.

In studies where experimental animals have been exposed simultaneously to several EACs, *e.g.* pesticides, substantial mixture effects on reproductive development have been seen, even though each of the individual compounds was present at low doses, where no effects were seen (Hass et al., 2007, 2012; Jacobsen et al., 2012; Metzdorff et al., 2007; Silva et al., 2002). In addition, there are indications that cumulative exposure to EACs such as pesticides may play a role causing adverse effects on human development. In epidemiological studies possible association between risk of cryptorchidism and maternal pesticide exposure has been reported (Andersen et al., 2008; Carbone et al., 2007; Kristensen et al., 1997; Weidner et al., 1998). Because the adverse effects of some pesticide mixtures occur at exposure levels at which the single pesticides do not cause adverse effects, it raises concerns about their potential combined impact on human health. Thus, there is a need for continued research on the effect of combined exposure in order to gain more knowledge on mixture effects.

The present study forms part of a larger project, in which an initial screening of 13 currently used pesticides was performed, applying a battery of *in vitro* assays, including assays for effects on the estrogen receptor (ER), the androgen receptor (AR), the aryl hydrocarbon receptor (AhR), the thyroid hormone receptor (TR), and steroidogenesis (Kjeldsen et al., 2013). The aim of the screening was to reveal potential mechanisms of action as well as to determine the potency of the

* Corresponding author. Fax: +45 35887001.
E-mail address: camta@food.dtu.dk (C. Taxvig).

0041-008X/$ – see front matter © 2013 The Authors. Published by Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.taap.2013.07.028

pesticides. The selection of test compounds was based on a list of pesticides that were registered and approved for use in Denmark (except for malathion prohibited in 2008) as well as their endocrine disrupting potential, as described both in the open literature and in draft assessment reports (DARs). The overall aim of the project was to evaluate the predictability of the *in vitro* tests for potential endocrine disrupting effects *in vivo*. Based on the initial *in vitro* tests five pesticides were selected for further study. The five pesticides chosen for the current study represent different classes of pesticides, and included the fungicides bitertanol and propiconazole, the insecticides cypermethrin and malathion, and the herbicide terbuthylazine. The selection of pesticides was based on their potency and efficacy in the initial *in vitro* tests, with a special focus on data from the H295R steroidogenesis assay. The five pesticides were mixed in two different mixtures. A mixture named "Mix 3" consisting of: bitertanol, propiconazole, and cypermethrin mixed in the ratio 1:1:1 and a mixture called "Mix 5" consisting of all five pesticides mixed in a 1:1:1:1:1 ratio.

In the present study the aim was to investigate the potential endocrine disrupting effects of the selected pesticide mixtures *in vitro* using the H295R steroidogenesis assay, and *in vivo* in an *in utero* exposure rat study. In the *in vitro* experiment all five single pesticides were tested in addition to the two mixtures. For the *in vitro* study the intention was not to perform any mixture modeling or mixture predictions, but rather to compare the effect of the single pesticides to the effects of the mixtures to see if we by intuition were able to predict the qualitative response of the mixtures.

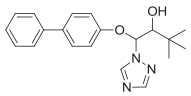
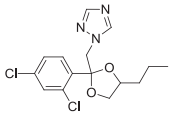
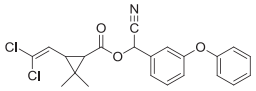
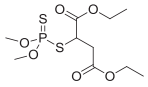
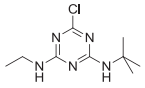
In the *in vivo* study pregnant rats were dosed with the two pesticide mixtures from gestation day (GD) 7 to 21. At GD 21 fetuses were removed by cesarean section, and various endpoints were measured to examine potential endocrine disrupting effects of the mixtures.

Material and methods

Chemicals

Cypermethrin, PESTANAL®, analytical standard (CAS no. 52315-07-8), malathion, PESTANAL®, analytical standard (CAS no. 121-75-5), bitertanol PESTANAL®, analytical standard (CAS no. 55179-31-2), propiconazole PESTANAL®, analytical standard (CAS no. 60207-90-1), and terbuthylazine PESTANAL®, analytical standard (CAS no. 5915-41-3) were all purchased from Sigma-Aldrich (St. Louis, USA). The test compounds were dissolved in corn oil (no. C8267-2.5L) for the *in vivo* study or in dimethyl sulfoxide (DMSO, CAS 67-68-5) for the *in vitro* studies, respectively, both from Sigma-Aldrich (St. Louis, USA). Table 1 lists the names, chemical structures and CAS numbers of the pesticides.

Table 1
Pesticides included in the study.

Pesticide	Chemical structure	CAS number
Bitertanol		55179-31-2
Propiconazole		60207-90-1
Cypermethrin		52315-07-8
Malathion		121-75-5
Terbuthylazine		5915-41-3

H295R assay

Cell culture and chemicals. The effects of the single pesticides and the two pesticide mixtures on the production of progesterone, testosterone, and estradiol were tested in the NCI-H295R human adrenocortical carcinoma cell line (ATCC no. CRL-2128, LGC Standards, Boras, Sweden) as previously described (Hecker et al., 2011). In brief cells were seeded in 24-well culture plates (Costar3524,Corning,NY, USA) with DMEM/F12 medium (Gibco, Paisley, UK) supplemented with 2.0% Nu-serum (BD Sciences Denmark) along with 1% ITS+ premix (containing6.25 μg/ml insulin, 6.25 μg/ml transferrin,6.25 ng/ml selenium,1.25 mg/ml BSA and 5.35 μg/ml linoleic acid; BD Sciences Denmark) and incubated for 24 h at37 °C in a humidified atmosphere of 5% CO2/air. The pesticides and mixtures were added (1 ml) to the cells in triplicates at six concentrations ranging from 1.6 to 100 μM, and left to incubate for 48 h. Control wells (0 μM) contained the same amount of DMSO (0.1%) as exposed cells. After the 48 h incubation period the medium was removed and stored at −80 °C until the hormone analyses. Hormone levels were normalized to the solvent control containing 0.1% DMSO. All hormone measurements for single pesticides and mixtures were repeated in at least three independent experiments. For evaluation of cytotoxicity, cells were added 5 mg/ml MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (cat. no. M2128, Sigma, St. Louis, USA) once the media for the hormone analysis had been removed. After an incubation period for approximately 1.5 h at 37 °C at 5% CO_2 the MTT was removed, and 0.5 ml isopropanol was added and the contents were mixed for 5 min on a plate shaker (Hecker et al., 2011). Fluorescence was next measured (excitation 560 nm, emission 590 nm) on Wallac Victor^2 1420 multilabel counter (PerkinElmer, Massachusetts, USA).

Hormone measurements. Hormones were extracted from cell supernatants using a C18 solid phase extraction (SPE) cartridge (200 mg) (prod. no. 220-0020-B, Mikrolab Aarhus Denmark) as previously described (Vinggaard et al., 2002). The eluate was evaporated for approximately 4 1/2 h in a centrifugal vacuum concentrator (SpeedVac, Thermo Fisher Scientific, Waltham, MA, USA). Samples were resuspended in 200 ml Diluent 1 (PerkinElmer, Waltham, MA, USA) and stored at 4 °C. Samples were next placed in a water bath for 10 min at 45 °C to dissolve the steroid hormones. Estradiol, progesterone and testosterone were measured using commercially available time-resolved fluoroimmunoassay kits (Wallac DELFIA®) purchased from PerkinElmer (Skovlunde, Denmark) according to the description of the manufacturer (Estradiol: prod. no. 1244-056, Progesterone: prod. no. A066-101, Testosterone: prod. no. A050-101).

Animals and exposure

The animal study was performed under conditions approved by the Danish Animal Experiments Inspectorate and by the in-house Animal Welfare Committee. The study included 84 time-mated Wistar rats (HanTac:WH, Taconic Europe, Ejby, Denmark) supplied on gestational day (GD) 3 and upon arrival, randomly distributed in pairs and housed under standard conditions: semitransparent polycarbonate cages (15 × 27 × 43 cm) with Aspen bedding (Tapvei, Denmark) situated in an animal room with controlled environmental conditions (12 h light-dark cycles with light starting at 9 p.m., light intensity 500 lx, temperature 21 ± 2 °C, humidity 50% ± 5%, ventilation 8 air changes per h). A complete rodent diet for growing animals ALTROMIN 1314 (Soy- and alfalfa-free ALTROMIN GmbH, Lage, Germany) and acidified tap water (to prevent microbial growth) were provided *ad libitum*. The animals