Interactions of endosulfan and methoxychlor involving CYP3A4 and CYP2B6 in human HepaRG cells

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Running title: Differential CYPs modulation by pesticides

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List of Abbreviations

CAR, constitutive androstane receptor; CYP, cytochrome P450; DMSO, dimethylsulfoxide; END, endosulfan; FCS, fetal calf serum; MXC, methoxychlor; PXR, pregnane X receptor.
Abstract

Humans are usually simultaneously exposed to several pesticides; consequently, combined actions between pesticides themselves or between pesticides and other chemicals need to be addressed in the risk assessment. Many pesticides are efficient activators of PXR and/or CAR, two major nuclear receptors that are also activated by other substrates. In the present work, we searched for interactions between endosulfan and methoxychlor, two organochlorine pesticides whose major routes of metabolism involve CAR- and PXR-regulated CYP3A4 and CYP2B6, and whose mechanisms of action remain poorly understood in humans. For this purpose, HepaRG cells were treated with both pesticides separately or in mixture for 24h or 2 weeks at concentrations relevant to human exposure levels. In combination they exerted synergistic cytotoxic effects. Whatever the duration of treatment both compounds increased CYP3A4 and CYP2B6 mRNA levels while they differently affected their corresponding activities. Endosulfan exerted a direct reversible inhibition of CYP3A4 activity that was confirmed in human liver microsomes. By contrast, methoxychlor induced this activity. The effects of the mixture on CYP3A4 activity were equal to the sum of those of each individual compound, suggesting an additive effect of each pesticide. Despite CYP2B6 activity was unchanged and increased with endosulfan and methoxychlor respectively, no change was observed with their mixture, supporting an antagonistic effect. Altogether, our data suggest that CAR and PXR activators endosulfan and methoxychlor can interact together and with other exogenous substrates in human hepatocytes. Their effects on CYP3A4 and CYP2B6 activities could have important consequences if extrapolated to the in vivo situation.
Introduction

Pesticides are major and ubiquitous contaminants of the human environment. The human population is usually simultaneously exposed to low doses of several pesticides via food and to some extent, via inhalation and cutaneous contact. These compounds can be substrates, inhibitors and inducers of hepatic enzymes and also causative agents of various toxic effects. Interactions between pesticides themselves or between pesticides and other chemicals are known to occur, frequently through the generation of reactive intermediates that may exert their effects in the liver itself or in other tissues. Therefore, combined actions of pesticides need to be addressed in the risk assessment (Reffstrup et al., 2010; Lokke et al., 2013). Indeed, if the effects of mixtures are often equal to the arithmetic sum of the effects of each component in certain cases the observed toxicity deviated significantly from expected additivity, indicating synergistic or antagonistic effects (Kortenkamp et al., 2009). In the present work, we have studied whether the two organochlorine pesticides endosulfan and methoxychlor could interact together. As numerous other chemicals and endogenous substrates they are activators of two major nuclear receptors, pregnane X receptor (PXR) and/or constitutive androstane receptor (CAR) (Casabar et al., 2010; Kublbeck et al., 2011), and their major routes of metabolism involve cytochrome P450 (CYP) 3A4 and CYP2B6 that are known to be regulated by the two nuclear receptors (Blizard et al., 2001; Casabar et al., 2006).

Both pesticides are classified as endocrine disrupters. Endosulfan has been reported to affect a variety of organ systems and physiological functions (Moon and Chun, 2009). Animal studies have shown its toxicity to the liver, kidney, nervous, blood, immune and reproductive systems (Choudhary and Joshi, 2003; Singh et al., 2008; Briz et al., 2011). Methoxychlor induces follicular atresia, reduces ovulation rate and decreases embryo implantation in rats and mice (Tiemann, 2008). It also reduces the weight of testes, prostate and seminal vesicles and causes disorders of spermatogenesis in male rats (Okazaki et al., 2001). However, mechanisms of action of endosulfan and methoxychlor remain poorly understood in humans. Studies using primary human hepatocytes have shown that endosulfan caused an oxidative stress and that both endosulfan and methoxychlor enhanced transcription of CYP3A4 and...
CYP2B6 genes but effects on their corresponding enzyme activities remained unclear (Dehn et al.,
2005; Casabar et al., 2010; Kublbeck et al., 2011; Rouimi et al., 2012).

In this study, we showed that endosulfan and methoxychlor up-regulated CYP3A4 and CYP2B6
transcripts but differently affected their corresponding activities in the metabolically competent human
HepaRG cells after either single or 2-week repeated treatment.
Materials and methods

Chemicals: Endosulfan (68.3 % alpha-endosulfan, 30.9 % beta-endosulfan) was purchased from ChemService (West Chester, PA). Methoxychlor (PESTANAL®, analytical standard), dimethyl sulfoxide (DMSO), testosterone, 6β-hydroxy-testosterone, nifedipine, oxidized nifedipine, midazolam, bupropion, and ketoconazole were from Sigma Aldrich (St. Quentin Fallavier, France). 1-hydroxy-midazolam, 1-hydroxy-midazolam 13C3, hydroxy-bupropion and hydroxy-bupropion d6 were supplied from LGC Standard (Molsheim, France). All other chemicals were of the highest quality available.

Cell cultures and pesticide treatments

HepaRG cells were cultured at a density of 2.6 x 10^4 cells/cm² in 12- or 24-well plates as described previously (Gripon et al., 2002; Aninat et al., 2006). They were first incubated in the Williams’ E medium supplemented with 10% fetal calf serum (FCS), 100 units/ml penicillin, 100 µg/ml streptomycin, 5 µg/ml insulin, 2 mM glutamine, and 5 x 10^5 M hydrocortisone hemisuccinate for 2 weeks. Maximal liver-specific activities were attained after two additional weeks in the same medium added with 2% DMSO. The culture medium was renewed every 2 or 3 days. At that time HepaRG cells were used for pesticide treatments.

HepG2 cells were used for cytotoxicity comparison with HepaRG cells. Briefly, they were seeded at a density of 100,000 cells/cm² in 24-well plates. The growth medium was composed of minimum essential medium, nonessential amino acids, 100 units/ml penicillin, and 100 µg/ml streptomycin, and supplemented with 10% FCS. The cells were used at the time they reached confluence.

Endosulfan and methoxychlor were dissolved in DMSO; both control and treated cultures received the same final concentration of vehicle. The binary mixture was designed as [E+M]. Thus, 20 µM [E+M] was composed of 20 µM of each pesticide. For mRNA and activity measurements HepaRG cells were treated in a serum-free medium containing only 0.1% DMSO for 24 or 48 h.
Preparation of microsomal and cytosolic fractions

Human liver tissue samples and HepaRG cells were homogenized in 50mM Tris-HCl buffer (pH 7.4) containing 0.25M sucrose and 1mM EDTA. Microsomal and cytosolic fractions were the sediment and supernatant, respectively from the last of three successive centrifugations at 4 °C (3000 g, 10 min; 8000 g, 20 min; and 30000 g, 60 min).

Cytotoxicity assay

Cytotoxicity of pesticides was evaluated by the methylthiazol tetrazolium colorimetric assay (Aninat et al., 2006).

Isolation of RNA and RT-qPCR analysis

For the determination of cytochrome P450 mRNA levels, HepaRG cells were treated for 24 h or 14 days with the pesticides. Total RNA was extracted from 10^6 cells with the SV total RNA isolation system (Promega, Madison, WI), which directly included a DNase treatment step. RNAs were reverse-transcribed into cDNA using a High-Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA). RT-q PCR for all genes was performed by the fluorescent dye SYBR Green methodology using the SYBR Green PCR Master Mix (Applied Biosystems) and the ABI Prism 7000 (Applied Biosystems). The primer forward and the primer reverse used for CYP3A4, 2B6 and 18S were the followings: CYP3A4: forward 5’-CTTCATCCAATGGACTAGCATAAAT-3’ and reverse 5’-TCCCAAGTATAACACTCTACACAGACAA-3’; CYP2B6: forward 5’-TTCTACTGCTTCCGTCTATCAA-3’ and reverse 5’-GTGCAATCCCACAGCTCA-3’; and for control 18S: forward 5’- CGCCGCTAGAGGTGAAATTC-3’ and reverse 5’-TTGGCAAATGCTTGCCTC-3’.

The amplification curves were read with the ABI Prism 7000 SDS software using the comparative cycle threshold method. The relative quantification of the steady-state mRNA levels was calculated after normalization against 18S RNA. Furthermore, a dissociation curve was performed after the PCR to verify the specificity of the amplification. Results were expressed as a fold change of mRNA levels measured in controls arbitrarily set at 1.
Determination of CYP activities

For the determination of P450-related activities, HepaRG cells were cultured in 24-well plates and treated for 48 h with pesticides and then incubated with specific substrates for each CYP in phenol red-free medium deprived of FCS and DMSO for 2 h. Several substrates of CYP3A4 were used. Cultures were incubated with either 200 µM testosterone, 200 µM nifedipine, or 50 µM midazolam, three substrates of CYP3A4, or with 100 µM bupropion, a specific substrate of CYP2B6. Oxidized-nifedipine was quantitated by HPLC-UV. 6β-OH-testosterone, OH-midazolam and its internal standard and OH-bupropion and its internal standard were directly measured in the culture medium by HPLC-MS/MS (Galetin et al., 2003). CYP activities were determined as pmol/mg protein/min and are reported as a fold change of activity measured in controls arbitrarily set at 1. Each 24-well contained around 0.4x10^5 cells corresponding to 250 mg protein.

Statistical analysis.

Data are presented as means ± standard error of the mean (SEM). Significant differences were evaluated using the Mann-Whitney U test. *P < 0.05 was considered as statistically significant. To evaluate whether the effects caused by the mixture of endosulfan and methoxychlor were additive, more than additive or less than additive, it was possible to calculate the expected effect of the mixture under the hypothesis of simple additivity response. The expected value was the sum of the effects observed for each individual compound (Dumont et al., 2010). The Mann–Whitney U test was then used to test whether any observed response was significantly different from the expected response. Effects stronger than expected were designated as resulting from synergism whereas effects smaller than expected were designated as resulting from inhibition. For analysis of cytotoxicity values, the CATAM mixture model (http://service004.hpc.ncsu.edu/toxicology/faculty/leblanc/web1/) was also used.
Results and discussion

Cytotoxicity of endosulfan, methoxychlor and the mixture.

Preliminary studies were performed to estimate viability of HepaRG cells after a 24 h exposure to endosulfan and methoxychlor individually and in mixture; varying concentrations from 5 up to 500 µM were tested (Fig. 1A). Endosulfan cytotoxicity sharply increased from 100 µM to reach a 100% loss of cell viability at 200 µM (IC_{50}=123 µM) in HepaRG cells. Methoxychlor was significantly less cytotoxic than endosulfan: indeed, no effect was observed at 100 µM and 45% cells were still viable in the presence of 200 µM (IC_{50}=189 µM). The equimolar mixture was significantly more cytotoxic than individual pesticides at 100 µM and a 100% loss of cell viability was observed in the presence of 150 µM (IC_{50}=77 µM). When HepaRG cells were treated every 2-3 days for 14 days with varying pesticide concentrations from 1 up to 100 µM, cytotoxicity was exacerbated (Fig. 1B). IC_{50} fell to 72 µM and 38µM for endosulfan and the mixture respectively and a 40% cell loss was observed with 100µM methoxychlor. Increased cytotoxicity after repeated treatment with the two pesticides could be explained by continuous generation of toxic metabolites. Indeed, endosulfan and methoxychlor cytotoxicity has been associated, at least in part, to their metabolites, including endosulfan sulfate for the former and demethylated derivatives for the latter (Miller et al., 2006; Key et al., 2010). As previously reported (Josse et al., 2008) major CYP activities were well maintained in differentiated HepaRG cells over a 2-week period. Noticeably, as expected HepG2 cells which did not express major CYPs, were much less sensitive to the two pesticides and their mixture (IC_{50} = 406 µM, >500µM and 250 µM for endosulfan, methoxychlor and the mixture respectively after a 24h treatment (Fig 1C). A synergistic cytotoxic effect was observed in HepaRG cells while it was only additive in HepG2 cells after exposure to 100 µM of each pesticide in mixture (Fig. 1D).

Based on these cytotoxicity data, non-cytotoxic concentrations of the two pesticides ranging from 1 to 20 µM were used for measuring CYP transcripts and activities in HepaRG cells; these concentrations were similar as those used by others in in vitro studies (Casabar et al., 2010; Craig et al., 2013) and relevant to human exposure levels (Botella et al., 2004; Carreno et al., 2007). Noticeably, endosulfan
and its metabolites have been found to be concentrated as much as ten times in liver than in blood (Nath et al., 1978).

**Effects on CYP3A4 and CYP2B6 after single exposure**

CYP3A4 and CYP2B6 are both implicated in metabolism of endosulfan and methoxychlor (Blizard et al., 2001; Casabar et al., 2006). We evaluated whether endosulfan, methoxychlor and their mixture could modulate their expression and/or activity after single and repeated exposure. After a single 24 h exposure, a concentration-dependent increase in CYP3A4 mRNA levels was observed in HepaRG cells treated with endosulfan, methoxychlor and their mixture, reaching respectively 7.7-, 9.9- and 22.4-fold at 20 µM (Fig. 2A). Both pesticides also induced CYP2B6 mRNA expression in a concentration-dependent manner (Fig. 2B). These data are in agreement with previous studies (Coumoul et al., 2002; Lemaire et al., 2005; Casabar et al., 2010; Rouimi et al., 2012).

Activity of both CYPs was measured after a 48 h exposure. Despite an induction at the transcript level, endosulfan showed a strong concentration-dependent inhibition of CYP3A4 activity in HepaRG cells, as shown by quantification of 6β-hydroxy-testosterone, the metabolite of testosterone formed by CYP3A4 (Fig. 2C). Indeed, 5, 10 and 20 µM endosulfan inhibited 45, 60 and 75% of CYP3A4 activity respectively. This decrease of CYP3A4 activity observed by determination of 6β-hydroxy-testosterone formation was confirmed by measurement of hydroxyl-midazolam and oxidized nifedipine, two metabolites specifically formed by CYP3A4 from midazolam and nifedipine respectively (Fig. 2D, E).

On the other hand, methoxychlor induced CYP3A4 activity except when using testosterone as a substrate (Fig. 2C, D, E). The most likely explanation was a competitive inhibition of 6β-testosterone hydroxylation by methoxychlor and its metabolites as previously reported by Li et al. (1993). However, a reduced cooperativity in the binding of testosterone molecules to its binding site has been observed in the presence of some other substrates (Galetin et al., 2003) and consequently cannot be excluded with methoxychlor. Noticeably, the two other substrates nifedipine and midazolam that do not bind to the same active site as testosterone (Galetin et al., 2003) did not interact with methoxychlor and therefore could be considered as more appropriate substrates than testosterone to evaluate the effects of this pesticide on CYP3A4 activity.
To confirm a direct inhibition of CYP3A4 activity by endosulfan, microsomes prepared from human liver samples and HepaRG cells were incubated with this pesticide at 20 µM for 20 min. A 28% and 60% inhibition of 6β-hydroxy-testosterone formation was observed with microsomes from human liver and HepaRG cells, respectively (Fig. 2F). This inhibition was not NADPH-dependent, suggesting a non-mechanism-based inhibition. These results provide the first demonstration of a direct inhibitory effect of endosulfan on CYP3A4 activity in human hepatocytes. Surprisingly, previous studies have only analyzed mRNA and protein levels in endosulfan-treated human hepatocytes (Dehn et al., 2005; Casabar et al., 2010; Rouimi et al., 2012). A lack of direct correlation has been reported between transcripts and enzyme activity levels for other pesticides, especially organophosphate insecticides which are similarly CAR and PXR activators (Abass et al., 2012).

Both endosulfan and methoxychlor caused increase of CYP2B6 activity as shown by quantification of OH-bupropion formation (Fig. 2G). Although expected, to our knowledge an induction of CYP2B6 activity by these two CAR activators had never been reported in human hepatocytes.

Effects on CYP3A4 and CYP2B6 after repeated exposure

CYP transcripts and activities were also measured in HepaRG cells after 2-week repeated treatment with the two pesticides individually and in mixture. As shown in Fig. 3A and B an increase of CYP3A4 and CYP2B6 transcripts was observed after treatment with 5 and 10 µM of endosulfan, methoxychlor and their mixture.

After 14 days repeated treatment, CYP3A4 activity dropped by 60 and 70% of control values in response to 5 and 10 µM endosulfan respectively while it was increased with 10 µM methoxychlor (Fig. 3C). CYP2B6 activity was unchanged with endosulfan and induced by methoxychlor (Fig. 3D). Therefore, it might be concluded that the effects of endosulfan and methoxychlor on transcripts and activity of CYP3A4 and CYP2B6 were comparable after single or 2-week repeat treatment of HepaRG cells when exposed to the same pesticide concentrations (Fig. 2 and 3).
Effects of the mixture on CYP3A4 and CYP2B6 after single and repeated exposure

Changes in CYP3A4 and CYP2B6 activities after exposure to an equimolar mixture of endosulfan and methoxychlor should correspond to the addition of changes measured with each compound separately, if no interaction.

For CYP3A4, additive effects were observed whatever the concentration and the duration of the treatment (Fig. 2E and 3C). Total activity with the mixture represented the sum of a decrease with endosulfan and an increase with methoxychlor. As an example after single exposure, at 20 μM endosulfan decreased 0.65-fold while methoxychlor increased 2.85-fold CYP3A4 activity. No significant difference was found between observed (1.77 ± 0.25-fold) and theoretical additive (2.55 ± 0.2-fold) effects (Supplemental Table 1).

For CYP2B6, our results showed additive effects only for the lowest concentrations (5 μM after single and 1μM after repeat exposure). However, with 10-20 μM and 5-10 μM [E+M] mixture after single and repeat exposure respectively this activity was significantly lower than expected (Fig 2G, 3D). For instance, 20 μM endosulfan and 20 μM methoxychlor increased CYP2B6 activity (1.3- and 1.7-fold respectively). Although a 2-fold augmentation was expected no effect was observed with the mixture (1.1-fold) supporting an interaction (antagonism) between the two pesticides on this CYP activity (Supplemental Table 1).

Although contaminants are recognized as having usually much less affinity to CYPs than pharmaceuticals, our data clearly showed that both endosulfan and methoxychlor affected CYP activities in HepaRG cells at concentrations relevant to human exposure levels.

In summary, our results obtained with the metabolically competent human HepaRG cells exposed to single or repeated doses of endosulfan and methoxychlor individually or in mixture, provide the first demonstration that these two pesticides can exert opposite effects on CYP3A4 and CY2B6 activities. They also bring further support to the occurrence of metabolic interactions between environmental contaminants themselves and between environmental contaminants and other chemicals, including drugs and endogenous compounds, especially those which are agonists of the same nuclear receptors.
Such effects on main xenobiotic metabolizing enzymes activities could have important consequences if extrapolated to the *in vivo* situation.
Acknowledgements

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Authorship Contributions.

Participated in research design: Camille Savary, Rozenn Josse, André Guillouzo
Conducted experiments: Camille Savary,
Contributed new reagents or analytic tools: Fabrice Guillet, Arnaud Bruyère
Performed data analysis: Camille Savary, Fabrice Guillet
Wrote or contributed to the writing of the manuscript: Camille Savary; Rozenn Jossé, Marie-Anne Robin, Andre Guillouzo.
References


Footnotes

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Legends to Figures

Fig. 1: Cytotoxic effects of endosulfan (END), methoxychlor (MXC) and their mixture [E+M] in HepaRG cells after 24 h (A) and 14 days (B) of treatment and for comparison in HepG2 cells (C) after 24 h. (D): Expected versus observed cytotoxic effects based on the CATAM mixture model (http://service004.hpc.ncsu.edu/toxicology/faculty/leblanc/web1/); in parentheses values obtained by using the method of Dumont et al, 2010 as described in material and methods section. 100 µM [E+M] means that the mixture contained 100 µM of each pesticide. Cytotoxicity was assayed using the methylthiazoletetrazolium test. Results are expressed as % of the value found in control cells arbitrarily set at 100 %. Data are means ± S.E.M. of 3 independent experiments. *p<0.05 compared with control cells.

Fig. 2: Effects of endosulfan, methoxychlor and their mixture [E+M] on CYP mRNAs and/or activities in HepaRG cells and microsomes. HepaRG cells were exposed to the vehicle (0.1% DMSO) (CTR), endosulfan, methoxychlor and their mixture [E+M] for 24 or 48 h. CYP3A4 and CYP2B6 mRNAs (A, B) and activity (C, D, E, F, G) were measured 24 and 48 h after the last treatment, respectively. CYP3A4 activity was estimated by determination of 6β-hydroxy-testosterone (C), OH-midazolam (D) and OH-nifedipine (E). CYP2B6 activity was estimated by determination of OH-bupropion (G). Microsomes were prepared from human liver and HepaRG cells, then pre-incubated with 20µM endosulfan (END) or 0.5 µM ketoconazole (KETO), used as a reference inhibitor, for 20 min with or without NADPH before incubation with testosterone (F). All results are expressed as fold changes compared to corresponding controls. Data are means ± S.E.M of at least 3 independent experiments. *P<0.05 compared to control cells.

Fig. 3. Effects of endosulfan, methoxychlor and their mixture [E+M] on CYP3A4 and CYP2B6 mRNAs and activities after repeat treatment. HepaRG cells were exposed to the vehicle (0.1% DMSO) (CTR), endosulfan, methoxychlor and their mixture [E+M] for 14 days. CYP3A4 activity was estimated by determination of OH-nifedipine (C). CYP2B6 activity was estimated by determination of OH-bupropion (D). Data are means ± S.E.M of three independent experiments. *P<0.05 compared to control cells.
Figure 1

A

HepaRG cells
24h

B

HepaRG cells
14 days

C

HepG2 cells
24h

D

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(All concentrations in µM)
Figure 2: Single treatment effects on CYP3A4 and CYP2B6 mRNA and activity.

(A) CYP3A4 mRNA expression with treatments: CTR, endosulfan, methoxychlor, and [E+M] at different concentrations.

(B) CYP2B6 mRNA expression with treatments: CTR, endosulfan, methoxychlor, and [E+M] at different concentrations.

(C) CYP3A4 activity for 6β-OH-testosterone at different treatments and concentrations.

(D) CYP3A4 activity for OH-midazolam at different treatments and concentrations.

(E) CYP3A4 activity for OH-nifedipine at different treatments and concentrations.

(F) CYP3A4 activity for 6β-OH-testosterone with preincubation with NADPH in human liver and HepaRG cells.

(G) CYP2B6 activity for OH-bupropion at different treatments and concentrations.

* indicates statistical significance.
Repeat treatment

**CYP3A4**

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