Short Communication

Interactions of Endosulfan and Methoxychlor Involving CYP3A4 and CYP2B6 in Human HepaRG Cells

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ABSTRACT
Humans are usually exposed to several pesticides simultaneously; 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 pregnane X receptor (PXR) and/or constitutive androstane receptor (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 in humans remain poorly understood. For this purpose, HepaRG cells were treated with both pesticides separately or in mixture for 24 hours 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 differently affecting 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 being 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 exposed to low doses of several pesticides simultaneously 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 may deviate significantly from expected additivity, indicating synergistic or antagonistic effects (Kortenkamp et al., 2009). In the present work, we studied whether the two organochlorine pesticides endosulfan and methoxychlor could interact. In common with numerous other chemicals and endogenous substrates, endosulfan and methoxychlor are activators of two major nuclear receptors, pregnane X receptor (PXR) and/or constitutive androstane receptor (CAR) (Casabar et al., 2010; Kuhlbeck et al., 2011), and their major routes of metabolism involve cytochrome P450s CYP3A4 and CYP2B6, which 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 physiologic functions (Moon and Chun, 2009). Animal studies have shown its toxicity to the liver, kidney, blood, immune, reproductive, and nervous 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 in humans remain poorly understood. 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; Kuhlbeck et al., 2011; Rouimi et al., 2012).

In this study, we showed that endosulfan and methoxychlor upregulated 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% α-endosulfan, 30.9% β-endosulfan) was purchased from ChemService (West Chester, PA). Methoxychlor (PESTANAL, analytical standard), dimethyl sulfoxide (DMSO), testosterone, 6β-hydroxytestosterone, polymerase chain reaction; PXR, pregnane X receptor.

ABBREVIATIONS: CAR, constitutive androstane receptor; DMSO, dimethyl sulfoxide; FCS, fetal calf serum; P450, cytochrome P450; PCR, polymerase chain reaction; PXR, pregnane X receptor.

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nifedipine, oxidized nifedipine, midazolam, propofol, and ketoconazole were from Sigma-Aldrich (St. Quentin Fallavier, France). 1-Hydroxymidazolam, 1-hydroxymidazolam-13C3, hydroxybuproprion, and hydroxybuproprion-D6 were supplied from LGC Standards (Molsheim, France). All other chemicals were of the highest quality available.

**Cell Cultures and Pesticide Treatments.** HepaRG cells were cultured at a density of $2.6 \times 10^6$ cells/cm$^2$ in 12- or 24-well plates as described previously (Grippon et al., 2002; Aninat et al., 2006). They were first incubated in Williams’ E medium supplemented with 10% fetal calf serum (FCS), 100 IU/ml penicillin, 100 µg/ml streptomycin, 5 µg/ml insulin, 2 mM glutamine, and 5 $\times$ 10$^{-5}$ M hydrocortisone hemisuccinate for 2 weeks. Maximal liver-specific activities were attained after 2 additional weeks in the same medium with 2% DMSO added. 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/cm$^2$ in 24-well plates. The growth medium was composed of minimum essential medium, nonessential amino acids, 100 IU/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 hours.

**Preparation of Microsomal and Cytosolic Fractions.** Human liver tissue samples and HepaRG cells were homogenized in 50 mM Tris-HCl buffer (pH 7.4) containing 0.25 M sucrose and 1 mM EDTA. Microsomal and cytosolic fractions were the sediment and supernatant, respectively, from the last of three 80,000 g, 60 minutes. An aliquot of the supernatant was used for CYP3A4, CYP2B6, and 18S were the following: CYP3A4, forward 5'-CCCTCACTGATGGAGGATTT-3' and reverse 5'-TCTCCAGATTAACTCTATCC-3'; CYP2B6, forward 5'-TCTTCTACTTGCTTTCTGCTATCA-3' and reverse 5'-GGCGGACCATGCGCTGTTACCC-3'; and control 18S, forward 5'- CGGCGCTAGGTTGAATTCT-3' and reverse 5'-TGGCAAGATGTTTCTGCTC-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 P450 Activities.** For the determination of P450-related activities, HepaRG cells were cultured in 24-well plates and treated for 48 hours with pesticides and then incubated with specific substrates for each P450 in phenol red-free medium deprived of FCS and DMSO for 2 hours. Several substrates of CYP3A4 were used. Cultures were incubated with either of three substrates of CYP3A4, 200 µM testosterone, 200 µM nifedipine, or 50 µM midazolam, or with 100 µM bupropion, a specific substrate of CYP2B6. Oxidized nifedipine was quantitated by high-performance liquid chromatography (HPLC)-UV. 6β-hydroxytestosterone, hydroxymidazolam and its internal standard and hydroxybuproprion and its internal standard were directly measured in the culture medium by HPLC–tandem mass spectrometry (MS/MS) (Galetin et al., 2003). P450 activities were determined as pmol/mg protein per minute and are reported as a fold change of activity measured in controls arbitrarily set at 1. Each 24-well plate contained around 0.4 $\times$ 10$^5$ cells corresponding to 250 mg of protein.

**Statistical Analysis.** Data are presented as mean ± S.E.M. Significant differences were evaluated using the Mann-Whitney U test. *P < 0.05 was considered 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 antagonism. 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-hour 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 pesticide concentrations from 1 up to 100 µM, cytotoxicity was exacerbated (Fig. 1B). IC$_{50}$ fell to 72 µM and 38 µM for endosulfan and the mixture, respectively, and a 40% cell loss was observed with 100 µM 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, with 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 (Jossie et al., 2008), major P450 activities were well maintained in differentiated HepaRG cells over a 2-week period. Noticeably as expected, HepG2 cells that did not express major P450s were much less sensitive to the two pesticides and their mixture (IC$_{50}$ = 406 µM, less than 500 µM, and 250 µM for endosulfan, methoxychlor, and the mixture, respectively, after a 24 hours treatment (Fig. 1C). A synergistic cytotoxic effect was observed in HepaRG cells, while it was only additive in HepG2 cells after exposure to each pesticide at 100 µM in mixture (Fig. 1D).

Based on these cytotoxicity data, noncytotoxic concentrations of the two pesticides ranging from 1 to 20 µM were used for measuring P450 transcripts and activities in HepaRG cells; these concentrations were similar to those used in other in vitro studies (Casabarr 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; Casabarr 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-hour 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 P450s was measured after a 48-hour 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-hydroxytestosterone, 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-hydroxytestosterone formation was confirmed by measurement of hydroxymidazolam and oxidized nifedipine, two metabolites specifically formed by CYP3A4 from midazolam and nifedipine, respectively (Fig. 2, D and E).

On the other hand, methoxychlor induced CYP3A4 activity except when testosterone was used as a substrate (Fig. 2, C–E). The most probable explanation is competitive inhibition of 6β-hydroxytestosterone 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, which 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 minutes. A 28% and 60% inhibition of 6β-hydroxytestosterone 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.

Effects on CYP3A4 and CYP2B6 after Repeated Exposure. P450 transcripts and activities were also measured 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).

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after 2-week repeated treatments with the two pesticides individually and in mixture. As shown in Fig. 3, A and B, an increase of CYP3A4 and CYP2B6 transcripts was observed after treatment with 5 and 10 \( \mu \text{M} \) endosulfan, methoxychlor, and their mixture.

After 14-day repeated treatments, CYP3A4 activity dropped by 60 and 70% of control values in response to 5 and 10 \( \mu \text{M} \) endosulfan, respectively, while it was increased with 10 \( \mu \text{M} \) 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 treatments of HepaRG cells exposed to the same pesticide concentrations (Figs. 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 occurred.

For CYP3A4, additive effects were observed whatever the concentration and the duration of the treatment (Figs. 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 \( \mu \text{M} \) endosulfan, CYP3A4 activity was decreased by 0.65-fold while methoxychlor increased it by 2.85-fold. No significant difference was found between observed (1.77 \( \pm \) 0.25-fold) and theoretical additive (2.55 \( \pm \) 0.2-fold) effects (Supplemental Table 1).

For CYP2B6, our results showed additive effects only for the lowest concentrations (5 \( \mu \text{M} \) after single and 1 \( \mu \text{M} \) after repeat exposure). However, with 10–20 \( \mu \text{M} \) and 5–10 \( \mu \text{M} \) [E+M] mixture after single and repeat exposure, respectively, this activity was significantly lower than expected (Figs. 2G and 3D). For instance, 20 \( \mu \text{M} \) endosulfan and 20 \( \mu \text{M} \) 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 P450 activity (Supplemental Table 1).

Although contaminants are recognized as usually having much less affinity to P450s than pharmaceuticals, our data clearly showed that both endosulfan and methoxychlor affected P450 activities in HepaRG cells at concentrations relevant to human exposure levels.

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**Fig. 2.** Effects of endosulfan, methoxychlor, and their mixture [E+M] on P450 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 hours. CYP3A4 and CYP2B6 mRNAs (A and B) and activity (C–G) were measured 24 and 48 hours after the last treatment, respectively. CYP3A4 activity was estimated by determination of 6\( \beta \)-hydroxytestosterone (C), hydroxymidazolam (D), and oxidized nifedipine (E). CYP2B6 activity was estimated by determination of hydroxybupropion (G). Microsomes were prepared from human liver and HepaRG cells, then preincubated with 20 \( \mu \text{M} \) endosulfan (END) or 0.5 \( \mu \text{M} \) ketoconazole (KETO), used as a reference inhibitor, for 20 minutes with or without NADPH before incubation with testosterone (F). All results are expressed as fold changes compared with corresponding controls. Data are means \( \pm \) S.E.M of at least three independent experiments. *\( P < 0.05 \) compared with control cells.
In summary, 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 CYP2B6 activities. The results further support the occurrence of metabolic interactions between environmental contaminants themselves and between environmental contaminants and other chemicals, including drugs and endogenous compounds, especially agonists of the same nuclear receptors. Such effects on main xenobiotic metabolizing enzyme activities could have important consequences if extrapolated to the in vivo situation.

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References

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Fig. 3. Effects of endosulfan, methoxychlor, and their mixture [E+M] on CYP3A4 and CYP2B6 mRNAs (A and B) and activities (C and D) 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 oxidized nifedipine (C). CYP2B6 activity was estimated by determination of hydroxybupropion (D). Data are means ± S.E.M of three independent experiments. * P < 0.05 compared with control cells.

Authorship Contributions
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