Stereoselective Metabolism of Endosulfan by Human Liver Microsomes and Human Cytochrome P450 Isoforms

Hwa-Kyung Lee, Joon-Kwan Moon, Chul-Hee Chang, Hoon Choi, Hee-Won Park, Byeoung-Soo Park, Hye-Suk Lee, Eul-Chul Hwang, Young-Deuk Lee, Kwang-Hyeon Liu, and Jeong-Han Kim

School of Agricultural Biotechnology, Seoul National University, Seoul (H.-K.L., J.-K.M., C.-H.C., H.C., H.-W.P., B.-S.P., J.-H.K); College of Pharmacy, Wonkwang University, Iksan (H.-S.L); College of Natural Resources and Life Science, Dong-A University, Busan (E.-C.H); Division of Life and Environmental Science, Daegu University, Gyeongsan, Korea (Y.-D.L) and Department of Pharmacology and PharmacoGenomics Research Center, Inje University College of Medicine, Busan (K.-H.L)
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Address co-correspondence to: Dr. Jeong-Han Kim or Kwang-Hyeon Liu, School of Agricultural Biotechnology, Seoul National University, Seoul 151-742, South Korea (J-H Kim), Department of Pharmacology and Pharmacogenomics Research Center, Inje University College of Medicine, Busan 614-735, South Korea (K-H Liu). E-mail: kjh2404@snu.ac.kr; dstlkh@inje.ac.kr

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Abbreviations:
P450: cytochrome P450; HLM: human liver microsomes; Cl	\text{int}: intrinsic clearances; GC: gas chromatography
Abstract

Endosulfan (6,7,8,9,10,10-hexachloro-1,5,5a,6,9,9a-hexahydro-6,9-methano-2,3,4-benzo(e)dioxathiepin-3-oxide) is a broad-spectrum chlorinated cyclodiene insecticide. This study was performed to elucidate the stereoselective metabolism of endosulfan in human liver microsomes and to characterize the cytochrome P450 (P450) enzymes that are involved in the metabolism of endosulfan. Human liver microsomal incubation of endosulfan in the presence of NADPH resulted in the formation of the toxic metabolite, endosulfan sulfate. The intrinsic clearances (Cl_{int}) of endosulfan sulfate from β-endosulfan were 3.5-fold higher than those from α-endosulfan, suggesting that β-endosulfan would be cleared more rapidly than α-endosulfan. Correlation analysis between the known P450 enzyme activities and the rate of the formation of endosulfan sulfate in the 14 human liver microsomes showed that α-endosulfan metabolism is significantly correlated with CYP2B6-mediated bupropion hydroxylation and CYP3A-mediated midazolam hydroxylation, and that β-endosulfan metabolism is correlated with CYP3A activity. The P450 isoform selective inhibition study in human liver microsomes and the incubation study of cDNA-expressed enzymes also demonstrated that the stereoselective sulfonation of α-endosulfan is mediated by CYP2B6, CYP3A4 and CYP3A5, and that that of β-endosulfan is transformed by CYP3A4 and CYP3A5.
The total Cl\text{int} values of endosulfan sulfate formation catalyzed by CYP3A4 and CYP3A5 were consistently higher for β-endosulfan than for the α-form (Cl\text{int} of 0.67 versus 10.46 \mu l/min/pmol P450, respectively). CYP2B6 enantioselectively metabolizes α-endosulfan, but not β-endosulfan. These findings suggest that the CYP2B6 and CYP3A enzymes are major enzymes contributing to the stereoselective disposition of endosulfan.
Introduction

In general, pesticides undergo extensive metabolic transformations in living organisms through various metabolic reactions. Microsomal mixed function oxidase is the primary enzyme for the phase one reactions that convert xenobiotics into more soluble products. Although these products are generally less toxic than the parent compound, more toxic metabolites may also occur, as in the cases of parathion (Chambers et al., 1991) and malathion (Buratti et al., 2005). Such in vitro studies with microsomal preparations provide specific details of the chemical identity of metabolites and intermediates, the pattern of their formation, and the metabolic pathways of xenobiotics (Liu et al., 2002; Liu and Kim, 2003; Kim et al., 2005a).

In recent years, a number of papers have been published on the activity of human P450s involved in the metabolism of some pesticides. It has been demonstrated with human liver microsomes and recombinant human P450s that CYP1A2, CYP2B6, and CYP3A4 are major metabolizing enzymes responsible for pesticide metabolism (Tang et al., 2001; Tang et al., 2002; Tang et al., 2004; Usmani et al., 2004a; Usmani et al., 2004b; Buratti et al., 2005). No information is available on the metabolism of endosulfan in humans, despite the fact that its widespread use makes the potential for human exposure high, both for agriculture workers and for the general population.
Endosulfan (6,7,8,9,10,10-hexachloro-1,5,5a,6,9,9a-hexahydro-6,9-methano-2,3,4-benzo(e)dioxathiepin-3-oxide) is a broad-spectrum chlorinated cyclodiene insecticide developed by Farbwerke Hoechst AG in Germany (Barnes and Ware, 1965). Because of its abundant usage and its potential for environmental transport, endosulfan contamination is found throughout the environment (Kullman and Matsumura, 1996). It is rapidly metabolized and excreted by chemical or biological systems in a living body as oxidation or hydrolysis products like endosulfan-sulfate, endosulfan-alcohol, endosulfan-ether, endosulfan-lactone, and endosulfan-hydroxyether (Martinez Vidal et al., 1998). Endosulfan alcohol is a metabolite nontoxic to fish and other organisms; thus, hydrolysis producing endosulfan alcohol may be an important detoxification pathway of endosulfan. However, endosulfan sulfate is nearly as toxic as the parent chemical, and as it does not undergo any further degradation, its residues tend to increase in the environment (Kennedy et al., 2001).

The technical grade of endosulfan contains two stereoisomers, α-endosulfan and β-endosulfan, in an approximate ratio of 7:3. Although the commercial product is applied as a 7:3 isomeric mixture, the fates of the α and β forms vary, and the observed ratio of the isomers is dependent upon the physical state of environmental components. The differences between the two isomers have been attributed to various factors, such as
their differential volatilization, photodecomposition, and alkaline hydrolysis, as well as their biotic metabolism (El Beit et al., 1981).

Metabolism may be an important determinant of pesticide toxicity. Hepatic metabolism of endosulfan in humans has not been previously investigated in vitro; nor have the contributions of P450 isoforms to metabolic pathways been elucidated. An understanding of the metabolic pathways and the varying contributions of specific P450 isoforms involved will enable a better understanding of the differences in metabolism among individuals; this will provide important information relative to the metabolic interactions of endosulfan with other chemicals. Therefore, the present study was carried out to elucidate the stereoselective metabolism of endosulfan in human liver microsomes and to identify the cytochrome P450 isozymes responsible for the formation of the metabolite.

**Materials and Methods**

**Chemicals and Reagents.** α-Endosulfan, β-endosulfan, endosulfan sulfate, endosulfan diol, endosulfan ether, and endosulfan lactone were purchased from Chem Service, Inc. (West Chester, PA). Coumarin, diethyldithiocarbamate, 6β-
hydroxytestosterone, α-naphthoflavone, ketoconazole, quercetin, quinidine, sulfaphenazole, testosterone, Thio-TEPA, β-nicotinamide adenine dinucleotide phosphate (β-NADP), glucose-6-phosphate, and glucose-6-phosphate dehydrogenase were purchased from Sigma-Aldrich (St. Louis, MO). The solvents were HPLC grade (Fisher Scientific Co., Pittsburgh, PA) and the other chemicals were of the highest quality available. S-Benzyl Nirvanol, pooled and single-donor human liver microsomes (HLM) and ten different human recombinant P450s, 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4, and 3A5 (Supersomes®), were purchased from BD Gentest Co. (Woburn, MA). All human P450 isoforms in Supersome® are co-expressed with human P450 reductase. The manufacturer supplied information regarding protein concentration and P450 content.

Metabolism of Endosulfan Enantiomers in Human Liver Microsomes or cDNA-Expressed P450 Isoforms. The optimal conditions for microsomal incubation were determined in the linear range for the formation of metabolites of endosulfan. In all experiments, endosulfan was dissolved and then serially diluted with acetonitrile to the required concentrations; the solvent was subsequently removed by evaporation to dryness under reduced pressure with an AES2010 SpeedVac (Savant Instruments Inc., Holbrook, NY).

The incubation mixtures, containing either 25 µl of microsomes (2 mg protein/ml of stock, prepared from two different human liver microsomal preparations) or 25 µl of cDNA-expressed P450 (diluted to 200 pmol/ml with phosphate buffer, pH 7.4), were
pre-incubated in 100 μM phosphate buffer (pH 7.4) in the presence of an NADPH-generating system (including 1.3 mM NADP, 3.3 mM glucose-6-phosphate, 3.3 mM MgCl₂, and 1.0 unit/ml glucose-6-phosphate dehydrogenase) for 10 min at 37°C in a shaking water bath. The control incubations were conducted in the absence of the NADPH-generating system. The reactions were initiated by the addition of various concentrations of endosulfan enantiomer (0.5 to 100 μM), and the reaction mixtures (final volume of 250 μl) were incubated for 30 min at 37°C. The reactions were terminated by adding 10 μl of 85% phosphoric acid. The samples were extracted with ethyl acetate (0.5 ml) by vortexing for 1 min, and were centrifuged at 15,000 g for 5 min; this procedure was repeated once to increase the extraction efficiency. The combined organic phase was analyzed by gas chromatography.

**Analytical Procedures.** The concentrations of endosulfan sulfate were measured by the gas chromatography (GC) method (Kaur et al., 1997). The system consisted of an Agilent 4890 GC (Agilent, Wilmington, DE) with an electron capture detector (ECD). The compounds were separated on a DB-1 bonded-phase fused silica capillary column (30 m × 0.25 mm i.d., df = 0.25 μm; J & W Scientific, Folsom, CA). The injector and detector temperatures were 260 and 320°C, respectively. The oven temperature was programmed from 100°C (3 min isothermal) to 280°C (held for 3 min at the final temperature) at 10°C/min. The flow rate of the nitrogen carrier gas was 1 ml/min at a
split ratio of 1:100. Quantitative analysis was performed with external standard calibration method. Working standard solutions of endosulfan sulfate were prepared at the concentration of 0.01, 0.05, 0.2, 0.5, 2.5, 10, and 30 μM by serial dilution with ethyl acetate. The calibration curve was fitted with high linearity ($r^2 > 0.98$).

**Chemical Inhibition Studies with Human Liver Microsomes.** The inhibitory effects of known P450 isoform-selective inhibitors on the formation of endosulfan sulfate were evaluated to determine the P450 isoform(s) involved in the metabolic pathway. The formation ratio of endosulfan sulfate from α- and β-endosulfan was determined from the reaction mixtures incubated in the presence or absence of known P450 isoform-selective inhibitors. The P450 isoform-selective inhibitors used were α-naphthoflavone (10 and 100 μM) for CYP1A2, coumarin (100 and 1000 μM) for CYP2A6, thio-TEPA (5 and 50 μM) for CYP2B6, quercetin (1 and 10 μM) for CYP2C8, sulfaphenazole (10 and 100 μM) for CYP2C9, S-benzynirvanol (1 and 10 μM) for CYP2C19, quinidine (10 and 100 μM) for CYP2D6, diethyldithiocarbamate (10 and 100 μM) for CYP2E1 and ketoconazole (1 and 10 μM) for CYP3A. Except for the addition of the P450 isoform-selective inhibitors, the incubation conditions were similar to those described previously (Shin et al., 1999).

**Correlation Experiments.** Endosulfan enantiomers (10 μM) were incubated with microsomes from 14 different livers to test the correlation of endosulfan metabolism
with the activity of individual P450s. The activities of each P450 isoform were determined using cocktail incubation and tandem mass spectrometry, as described previously (Kim et al., 2005b). Isoform-specific reaction markers were used to determine the activity of each P450: phenacetin O-deethylation (CYP1A2), coumarin 7-hydroxylation (CYP2A6), bupropin hydroxylation (CYP2B6), paclitaxel 6α-hydroxylation (CYP2C8), tolbutamide 4-methylhydroxylation (CYP2C9), S-mephenytoin N-demethylation (CYP2C19), dextromethorphan O-demethylation (CYP2D6), chlorzoxazone 6-hydroxylation (CYP2E1), midazolam 1’-hydroxylation (CYP3A), and testosterone 6β-hydroxylation (CYP3A). The correlation coefficients between the formation rates of endosulfan sulfate and the activity of each P450 isoform in the different HLM were calculated by nonparametric regression analysis (SAS version 8.01; SAS Institute Inc., Cary, NC). A p-value less than 0.05 was considered statistically significant.

Data Analysis. Results are expressed as means ± standard deviations (S.D.) of estimates obtained from two different human liver microsomes in triplicate experiments. In the microsomal incubation studies, the apparent kinetic parameters of endosulfan biotransformation (K_{m} and V_{max}) were determined by fitting a one-enzyme Michaelis-
Menten equation or a Hill equation. The formation rates of endosulfan sulfate from α–
endosulfan and β–endosulfan were best fitted to the Hill equation \( V = \frac{V_{max} \cdot S^n}{(K_m + S^n)} \). The calculated parameters were the maximum rate of formation \( (V_{max}) \), the
Michaelis constant (apparent \( K_m \)), the intrinsic clearance (\( Cl_{int} = \frac{V_{max}}{\text{apparent } K_m} \)), and
Hill coefficient (n). Calculations were performed using WinNonlin software (Pharsight,
Mountain View, CA). The percentages of inhibition were calculated by the ratio of the
amounts of metabolites formed with and without the specific inhibitor. In the incubation
study of the cDNA-expressed P450 isoforms, a Hill equation was fitted to the
unweighted data on the formation rate of endosulfan sulfate to estimate the enzyme
kinetic parameters.

Contributions of each cytochrome to endosulfan sulfonation were normalized for mean
values of the relative abundance of individual cytochromes in the liver (Rodrigues,
1999). Briefly, the reaction rates measured with individual cDNA-expressed P450
isoforms were normalized with respect to the nominal specific content of the
corresponding P450 in native human liver microsomes. In this study, we adapted the
data of immunologically determined P450 isoform liver contents reported by other
researchers (Wrighton et al., 1990; Shimada et al., 1994; Hanna et al., 2000); i.e., 1.7%
for CYP2B6, 23.0% for CYP3A4, and 5.8% for 3A5. In turn, the normalized rates for
each cDNA-expressed P450 were summed, yielding a ‘total normalized rate’ (TNR = \(\sum f_i \cdot V_i\)), and the normalized rate for each P450 isoform (\(= f_i \cdot V_i\)) was expressed as a percentage of the net reaction rate (\(= 100 \cdot f_i \cdot V_i/\sum f_i \cdot V_i\), where \(f_i\) indicates the fraction of each P450 isoform content in the human liver, and \(V_i = V_{\text{maxi}} \cdot [S_i]/K_{mi} + [S_i]\)).

**Results**

**Identification of the P450 Isoforms Involved in the Metabolism of Endosulfan.**

Human liver microsomal incubation of endosulfan in the presence of NADPH resulted in the formation of endosulfan sulfate (Fig. 1). The rates of formation of metabolite were proportional to incubation times up to 45 min and protein concentrations up to 0.3 mg/ml at 30 min. A P450 isoform-selective inhibition study was performed to evaluate which P450 isoforms are involved in the stereoselective metabolism of endosulfan in human liver microsomes (Fig. 2). Among the nine inhibitors tested, ketoconazole, a well-known CYP3A-selective inhibitor, inhibited endosulfan sulfate formation from both enantiomers. The CYP2B6-selective inhibitor, thio-TEPA, inhibited only the metabolism of \(\alpha\)-endosulfan. After treatment with various concentrations of ketoconazole and thio-TEPA, the formation rates of endosulfan sulfate decreased in a concentration-dependent manner (Fig. 3). After treatment with 1 \(\mu\)M ketoconazole, the
level of endosulfan sulfate formation from β–endosulfan (5 µM) markedly decreased to 22.9% of that in the controls, as compared with 59.2% from the α–form, indicating the stereoselective inhibitory effect of ketoconazole on the formation of endosulfan sulfate. Endosulfan sulfate formation from α–endosulfan was also inhibited by thio-TEPA as well as ketoconazole, suggesting that the stereoselective sulfonation of α–endosulfan is mediated by more than one enzyme.

Correlations between the rate of endosulfan metabolism and the activities of P450s in HLM are summarized in Table 1. The formation rates of endosulfan sulfate from α–endosulfan was significantly correlated with the activity of CYP3A and CYP2B6, while that of β-endosulfan was significantly correlated with the activity of CYP3A (Fig. 4).

Endosulfan sulfate formation from endosulfan enantiomers were also studied using the human cDNA-expressed P450 isoforms, P450s 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4, and 3A5 (Fig. 5). The endosulfan sulfate formation rates from the β–endosulfan by rCYP3As were significantly greater than those from the α–form. The cDNA-expressed CYP2B6, however, catalyzed endosulfan sulfate formation with a higher formation rate from the α–enantiomer than from the β-form. Similar to the chemical inhibition study, the other P450 enzymes exhibited no significant metabolic activity.
Enzyme Kinetic Analysis. The formation of endosulfan sulfate from endosulfan was clearly stereoselective in the incubation of human liver microsomal preparations (Fig. 6). The formation rates of endosulfan sulfate from $\beta$–endosulfan were significantly higher than those from $\alpha$–endosulfan. The estimated $V_{\text{max}}$ and Cl$_{\text{int}}$ values of $\beta$–endosulfan for the formation of endosulfan sulfate were higher, and the $K_m$ value was slightly lower, than those of the $\alpha$-endosulfan (Table 2), which resulted in 3.5-fold higher formation Cl$_{\text{int}}$ values from $\beta$-endosulfan, as compared with the $\alpha$-form. The values of the formation Cl$_{\text{int}}$ of the endosulfan sulfate were 0.20 and 0.69 µl/min/pmol P450 for $\alpha$– and $\beta$-endosulfan, respectively, indicating the metabolic stereoselectivity.

Next, we examined the enzyme kinetic parameters for the formation of endosulfan sulfate from $\alpha$- and $\beta$-endosulfan upon incubation with cDNA-expressed human CYP3A4, CYP3A5, and CYP2B6 (Fig. 7). Under the experimental conditions used, the metabolism of endosulfan by these P450 isoforms was best described by a Hill equation. CYP3A4 and CYP3A5 metabolized both endosulfan enantiomers to endosulfan sulfate, whereas endosulfan sulfate formation from $\alpha$–endosulfan was catalyzed only by CYP2B6 (Table 3). The total Cl$_{\text{int}}$ values of all three P450 isoforms were higher for $\beta$-endosulfan than for the $\alpha$–form. The CYP3A4-catalyzed endosulfan sulfate formation
of β-endosulfan showed a higher $V_{\text{max}}$ and $K_m$ than those of the α–enantiomer, resulting in a higher formation of $\text{Cl}_{\text{int}}$ from the β–form than from the α–form (4.80 versus 0.62 µl/min/pmol of CYP3A4). The $\text{Cl}_{\text{int}}$ value for endosulfan sulfate formation by CYP3A5 was 113.2-fold higher for β–endosulfan than for the α-form.

Contributions of P450 isoforms were determined after normalization for the predicted relative abundance of each P450. This simulation shows that CYP3A4 is the major P450 isoform responsible for endosulfan sulfate formation from both α– and β–endosulfan (Fig. 8). For α-endosulfan, the percentage of the net reaction catalyzed by CYP3A4 (around 85%) decreased with increasing substrate concentration, and reached about 70% at 100 µM. However, in the case of β–endosulfan, the percentage of the net reaction catalyzed by CYP3A4 (around 74%) increased slightly to 84% at 100 µM with increasing substrate concentration.

**Discussion**

The metabolism studies of endosulfan have been reported for various organisms, such as mammals, fish, insects, and some microorganisms (Barnes and Ware, 1965; Sutherland et al., 2000; Awasthi et al., 2003). As the result of previous research, different major metabolites were determined according to the organisms, and they were endosulfan ether, endosulfan hydroxyether, endosulfan sulfate, and endosulfan alcohol.
In the present study, we determined that the major metabolite of human liver microsomal incubation was endosulfan sulfate. This metabolite has a toxicity comparable to the parent compound, endosulfan.

The present in vitro incubation studies using human liver microsomes and cDNA-expressed human P450s clearly demonstrate the stereoselective metabolism of α and β-endosulfane in the endosulfan sulfate formation pathway. The mean metabolic intrinsic clearance rates obtained in human liver microsomes, as estimated by $V_{max}/K_m$, indicated that β-endosulfan was metabolized to endosulfan sulfate more efficiently than α-endosulfan. The $Cl_{int}$ values of the β-endosulfan were 3.5-fold higher for endosulfan sulfate formation than those of the α-endosulfan (0.20 and 0.69 µl/min/pmol P450, respectively), indicating the metabolic stereoselectivity.

The differences in stereoselective disposition between α- and β-endosulfan seem to be largely due to their characteristic stereoselective metabolisms. In the present study, the stereoselective metabolism of α-endosulfan was mediated by three P450 isoforms, CYP2B6, CYP3A4, and CYP3A5, and β-endosulfan was transformed by CYP3A4 and CYP3A5. Correlation analysis conducted on 14 single-donor HLM samples revealed significant correlations for CYP3A (midazolam 1'-hydroxylation) and/or CYP2B6 (bupropion hydroxylation). When testosterone is used as other probe substrate of
CYP3A (Maenpaa et al., 1993), the rates of formation of endosulfan sulfate from endosulfan enantiomers also correlated very well with the CYP3A activity, as shown in Table 1. Further verification of the importance of CYP2B6 and CYP3A in the metabolism of endosulfan was obtained by the P450 isoform-selective inhibition study. Ketoconazole (2 $\mu$M) inhibited up to 59.7% and 89.1% of endosulfan sulfate formation from $\alpha$- and $\beta$-endosulfan, respectively. The remaining metabolic activity of $\alpha$-endosulfan following ketoconazole inhibition may be attributed to CYP2B6 activity. After treatment with 25 $\mu$M thio-TEPA, the level of endosulfan sulfate formation from $\alpha$-endosulfan decreased to 52.9% of that in the control, as compared with 89.5% from the $\beta$ form, indicating the stereoselective inhibitory effect of thio-TEPA on the formation of endosulfan sulfate. In the inhibition study with sulfaphenazole, selective CYP2C9 inhibitor, $\alpha$-endosulfan sulfonation was slightly inhibited (~30%) by sulfaphenazole. This inhibition may be due to non-selective inhibition of CYP2B6 activity by sulfaphenazole. A similar inhibition of CYP2B6 activity by sulfaphenazole was observed in metabolism study of efavirenz, where CYP2B6-mediated efavirenz 8-hydroxylation was also weakly inhibited (~20%) by sulfaphenazole (Ward et al., 2003). Therefore, slight inhibition of $\alpha$-endosulfan sulfonation might be minor inhibitory effect of CYP2B6 by sulfaphenazole. The total formation $C_{\text{int}}$ values of all three or two of
these P450 isoforms were consistently higher for the β-endosulfan than for the α-\endosulfan. The Cl_{int} of CYP2B6 for α-endosulfan (1.85 \mu l/min/pmol P450) was extremely high compared with those of CYP3A4 and CYP3A5, and the Cl_{int} of CYP3A4 for β-endosulfan was similar to that of CYP3A5 (4.80 and 5.66 \mu l/min/pmol P450).

Taken together, these results suggest that CYP3A4 is the major enzyme involved in the sulfonation of both endosulfan enantiomers at concentrations in the usual experimental range. These results are similar to those from metabolism studies of fipronil (Tang et al., 2004), omeprazole (Abelo et al., 2000) and lansoprazole (Kim et al., 2003), where CYP3A4 was shown to have a high degree of sulfonation activity. In addition, CYP2B6 is also important P450 isoform responsible for endosulfan sulfate formation from α-endosulfan (Fig. 5 and 7). The percentage of the net reaction by CYP2B6 (around 15%) increased with substrate concentration dependent manner and reached 26% at 100 \mu M. Similarly, it is known that CYP2B6 is involved in the sulfoxidation of tazofelone (Surapaneni et al., 1997) and S-methyl N,N-diethylthiocarbamate (Pike et al., 2001).

Expression of CYP3A4 and CYP2B6 has been shown to be highly variable among human liver samples (Wrighton et al., 1990; Shimada et al., 1994; Faucette et al., 2000;
Lang et al., 2001). This variability is probably due to effects of genetic polymorphisms or exposure to drugs that are inducers or inhibitors of CYP3A4 (Neuvonen et al., 1998; Westlind et al., 1999; Hariparsad et al., 2004) and CYP2B6 (Gervot et al., 1999; Hesse et al., 2001; Lang et al., 2001). Therefore, the genetic polymorphisms may affect the stereoselective metabolism of endosulfan. Some researcher reported that CYP2B6 represents ~6% of the total liver P450 content (Stresser and Kupfer, 1999), in contrast to earlier estimates that it represented less than 1% (Shimada et al., 1994). Hence, subjects with a high liver CYP2B6 level would be expected to exhibit higher \( \alpha \)-endosulfan sulfonation by CYP2B6 than that by CYP3A4.

In conclusion, our study clearly demonstrated stereoselectivity in the formation of endosulfan sulfate from \( \alpha \)- and \( \beta \)-endosulfan; the intrinsic clearances of both metabolic pathways were consistently and significantly higher for \( \beta \)-endosulfan than for the \( \alpha \)-form in human liver microsomal fractions. The P450 isoform-specific chemical inhibition study, correlation analysis, and incubation study of cDNA-expressed P450 enzymes demonstrated that CYP2B6 metabolized \( \alpha \)-endosulfan, but not \( \beta \)-endosulfan, in an enantioselective manner, and that CYP3A4 and CYP3A5 were responsible for the enantioselective formation of endosulfan sulfate from both isomers. In addition, even though the intrinsic clearance rates for CYP2B6 were greater for \( \alpha \)-
endosulfan than those observed for CYP3A4, CYP3A4 could contribute substantially to
the metabolism because of its high relative abundance in liver tissue.
References


Figure captions.

**Fig. 1.** GC/ECD chromatogram of endosulfan and its metabolites from in vitro incubation with human liver microsomes, and the proposed metabolic pathway of endosulfan by human liver microsomes.

Endosulfan (30 µM) was incubated with HLMs (0.2 mg/ml protein) in the absence (—) or presence (▬) of an NADPH-generating system for 30 min at 37°C. The appearances of metabolite peaks were monitored by GC with electron capture detection.

**Fig. 2.** Stereoselective inhibition of known P450 isoform-selective inhibitors on sulfonation from α–endosulfan (A) and β–endosulfan (B) in human liver microsomal incubations.

Pooled human liver microsomes (0.2 mg/ml, Gentest) were incubated with 10 µM endosulfan enantiomers in the absence or presence of various chemical inhibitors at 37°C for 30 min. The chemical inhibitors used were as follows: α-naphthoflavone (10, 100 µM) for CYP1A2; coumarin (100, 1000 µM) for CYP2A6; thio-TEPA (5, 50 µM) for CYP2B6; quercetin (1, 10 µM) for CYP2C8; sulfaphenazole (10, 100 µM) for
CYP2C9; S-benzylirvanol (1, 10 µM) for CYP2C19; quinidine (10, 100 µM) for CYP2D6; diethyldithiocarbamate (10, 100 µM) for CYP2E1; and ketoconazole (1, 10µM) for CYP3A. Data shown are averages of remaining activity relative to the control metabolite formation rate estimated from duplicate experiments.

**Fig. 3.** Inhibition of oxidative metabolism of endosulfan by ketoconazole (A) and thioTEPA (B). Results are expressed relative to uninhibited control rates. Data points are the mean values (n = 3).

**Fig. 4.** Correlation analysis between the known P450 enzyme activities and the rate of formation of endosulfan sulfate from α-endosulfan (A) and β-endosulfan (B) in 14 human liver microsomes.

**Fig. 5.** Representative plot of the formation of endosulfan sulfate from endosulfan enantiomers by cDNA-expressed P450 isoforms.

Human cDNA-expressed P450s 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4, and 3A5 were incubated with 10 µM endosulfan enantiomers at 37°C for 30 min. Data
shown are averages of duplicate experiments.

**Fig. 6.** Kinetics for the formation rate of endosulfan sulfate from endosulfan enantiomers in human liver microsomes.

An increasing concentration of endosulfan (0 – 100 µM) was incubated with HLMs (0.2 mg/ml) and an NADPH-generating system for 30 min at 37°C. Left panel, rate of formation of endosulfan sulfate versus endosulfan concentration curves where the kinetic data were fit to a Hill equation. Right panel, the corresponding Eadie-Hofstee plots [rate versus (rate/endosulfan concentration)]. Each data point is the averages obtained from two different human liver microsomes.

**Fig. 7.** The formation of endosulfan sulfate from endosulfan enantiomers in recombinant human CYP2B6 (A), CYP3A4 (B), and CYP3A5 (C).

An increasing concentration of endosulfan (0 – 100 µM) was incubated with recombinant P450s and an NADPH-generating system for 30 min at 37°C. Upper panel, rate of formation of endosulfan sulfate versus endosulfan concentration curves where
the kinetic data were fit to a Hill equation. Lower panel, the corresponding Eadie-Hofstee plots [rate versus (rate/endosulfan concentration)]. Each data point represents the average of triplicate incubations.

**Fig. 8.** Abundance-adjusted simulations of the relative contributions of three or two P450 isozymes to endosulfan sulfonation in relation to α- and β-endosulfan concentrations.

The contribution of each enzyme was expressed as a percentage of the net reaction rate estimated from the ratio of normalized rates for each P450 isoform and the total normalized rate, as described in the Discussion section (Rodrigues, 1999).
Table 1. Correlation of formation rates of endosulfan sulfate from endosulfan enantiomers (10 µM) with the P450 maker activities in human liver microsomes ($n = 14$).

Data were analyzed using the nonparametric correlation test (Spearman $r$). The activity of each isoform was determined using the respective specific substrate probe reaction, as described previously (Kim et al., 2005b).

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<tr>
<th>Activity</th>
<th>P450 isoforms</th>
<th>Correlation coefficient ($r$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>α-Endosulfan</td>
</tr>
<tr>
<td>Phenacetin O-deethylation</td>
<td>CYP1A2</td>
<td>0.55</td>
</tr>
<tr>
<td>Coumarin 7-hydroxylation</td>
<td>CYP2A6</td>
<td>0.28</td>
</tr>
<tr>
<td>Bupropion hydroxylation</td>
<td>CYP2B6</td>
<td>0.87*</td>
</tr>
<tr>
<td>Paclitaxel 6α-hydroxylation</td>
<td>CYP2C8</td>
<td>0.65</td>
</tr>
<tr>
<td>Tolbutamide 4-methylhydroxylation</td>
<td>CYP2C9</td>
<td>0.58</td>
</tr>
<tr>
<td>S-Mephenytoin N-demethylation</td>
<td>CYP2C19</td>
<td>0.20</td>
</tr>
<tr>
<td>Dextromethorphan O-demethylation</td>
<td>CYP2D6</td>
<td>0.10</td>
</tr>
<tr>
<td>Chlorzoxazone 6-hydroxylation</td>
<td>CYP2E1</td>
<td>0.56</td>
</tr>
<tr>
<td>Midazolam 1’-hydroxylation</td>
<td>CYP3A</td>
<td>0.68*</td>
</tr>
<tr>
<td>Testosterone 6β-hydroxylation</td>
<td>CYP3A</td>
<td>0.73*</td>
</tr>
</tbody>
</table>

*p < 0.05
Table 2. Mean enzyme kinetic parameters of the formation of metabolites from α- and β-endosulfan in human liver microsomes.

<table>
<thead>
<tr>
<th></th>
<th>α-Endosulfan</th>
<th>β-Endosulfan</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_{max}^a$</td>
<td>1.48 ± 0.07</td>
<td>4.40 ± 0.18</td>
</tr>
<tr>
<td>$K_m$</td>
<td>7.34 ± 1.29</td>
<td>6.37 ± 0.88</td>
</tr>
<tr>
<td>$Cl_{int}$</td>
<td>0.20 ± 0.07</td>
<td>0.69 ± 0.28</td>
</tr>
<tr>
<td>$n$</td>
<td>0.80 ± 0.07</td>
<td>0.83 ± 0.06</td>
</tr>
</tbody>
</table>

Values are means ± S.D. of estimates from two different human liver microsomes.

$^aV_{max}$ is expressed as pmol/min/pmol P450, $K_m$ as µM, and $Cl_{int}$ as $V_{max}/K_m$ (µl/min/pmol P450).
Table 3. Mean enzyme kinetic parameters of the formation of endosulfan sulfate from endosulfan isomers from the cDNA-expressed P450s.

<table>
<thead>
<tr>
<th></th>
<th>CYP2B6</th>
<th>CYP3A4</th>
<th>CYP3A5</th>
<th>Total Cl$_{int}$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>α-Endosulfan</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$V_{max}^a$</td>
<td>10.31</td>
<td>2.04</td>
<td>0.50</td>
<td></td>
</tr>
<tr>
<td>$K_m$</td>
<td>5.58</td>
<td>3.30</td>
<td>10.54</td>
<td></td>
</tr>
<tr>
<td>Cl$_{int}$</td>
<td>1.85</td>
<td>0.62</td>
<td>0.05</td>
<td>2.52</td>
</tr>
<tr>
<td>$n$</td>
<td>0.23</td>
<td>0.97</td>
<td>0.98</td>
<td></td>
</tr>
<tr>
<td><strong>β-Endosulfan</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$V_{max}$</td>
<td>-</td>
<td>34.75</td>
<td>25.36</td>
<td></td>
</tr>
<tr>
<td>$K_m$</td>
<td>-</td>
<td>7.25</td>
<td>4.48</td>
<td></td>
</tr>
<tr>
<td>Cl$_{int}$</td>
<td>-</td>
<td>4.80</td>
<td>5.66</td>
<td>10.46</td>
</tr>
<tr>
<td>$n$</td>
<td>1.26</td>
<td>1.29</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^aV_{max}$ is expressed as pmol/min/pmol P450 isoform, $K_m$ as µM, and Cl$_{int}$ as $V_{max}/K_m$ (µl/min/pmol P450).
Fig. 1.
(A) α-Endosulfan

(B) β-Endosulfan

Fig. 2.
Fig. 3.
Fig. 4.
Fig. 5.

Endosulfan sulfate formation rate $V$ (pmol/min/pmol P450)

α-Endosulfan
β-Endosulfan

CYP1A2  CYP2A6  CYP2B6  CYP2C8  CYP2C9  CYP2C19  CYP2D6  CYP2E1  CYP3A4  CYP3A5
Kinetics of endosulfan sulfate formation

Eadie-Hofstee plot

Fig. 6.
Fig. 7.

(A) CYP2B6

(B) CYP3A4

(C) CYP3A5

V (pmol/min/pmol P450)

[S] (μM)

V (pmol/min/pmol P450)

V/[S] ((pmol/min/pmol P450)/μM)
Fig. 8.