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Metabolism of Endosulfan-α by Human Liver Microsomes and Its Utility as a Simultaneous in Vitro Probe for CYP2B6 and CYP3A4

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ABSTRACT:

Endosulfan-α is metabolized to a single metabolite, endosulfan sulfate, in pooled human liver microsomes (K_m = 9.8 μM, V_{max} = 178.5 pmol/mg/min). With the use of recombinant cytochrome P450 (P450) isoforms, we identified CYP2B6 (K_m = 16.2 μM, V_{max} = 11.4 nmol/nmol P450/min) and CYP3A4 (K_m = 14.4 μM, V_{max} = 1.3 nmol/nmol P450/min) as the primary enzymes catalyzing the metabolism of endosulfan-α, although CYP2B6 had an 8-fold higher intrinsic clearance rate (CL_{int} = 0.70 μl/min/pmol P450) than CYP3A4 (CL_{int} = 0.09 μl/min/pmol P450). Using 16 individual human liver microsomes (HLMs), a strong correlation was observed with endosulfan sulfate formation and *S*-mephenytoin *N*-demethylase activity of CYP2B6 (r^2 = 0.79), whereas a moderate correlation with testosterone 6 β-hydroxylase activity of CYP3A4 (r^2 = 0.54) was observed. Ticlopidine (5 μM), a potent CYP2B6 inhibitor, and ketoconazole (10 μM), a selective CYP3A4 inhibitor, together inhibited approximately 90% of endosulfan-α metabolism in HLMs. Using six HLM samples, the percentage total normalized rate (% TNR) was calculated to estimate the contribution of each P450 in the total metabolism of endosulfan-α. In five of the six HLMs used, the percentage inhibition with ticlopidine and ketoconazole in the same incubation correlated with the combined % TNRs for CYP2B6 and CYP3A4. This study shows that endosulfan-α is metabolized by HLMs to a single metabolite, endosulfan sulfate, and that it has potential use, in combination with inhibitors, as an in vitro probe for CYP2B6 and 3A4 catalytic activities.

Endosulfan is an organochlorine pesticide and a contaminant at toxic superfund sites. It is currently applied as a broad-spectrum insecticide to a variety of vegetables, fruits, cereal grains, and cotton (USEPA, 2002). Endosulfan is sold under the tradename Thiodan and as a mixture of two isomers, namely 70% α- and 30% β-endosulfan (ATSDR, 2000). Endosulfan exposure has been shown to increase rodent liver weights and elevate microsomal enzyme levels (Gupta and Gupta, 1977). In mice, endosulfan exposure resulted in increased testosterone metabolism and clearance (Wilson and LeBlanc, 1998). Studies involving children suggest that long-term environmental exposure to endosulfan causes delayed male sexual maturation and reduced testosterone levels (Saiyed et al., 2003). The mechanism by which endosulfan exerts these effects may involve its ability to activate the human pregnane X receptor and induce the expression levels of cytochrome P450 (P450) enzymes, thereby increasing metabolic rates for steroid hormones.

Before beginning an investigation of endosulfan's possible endocrine-disrupting effects, we wished to examine its metabolic pathway in humans. Until recently, there have been no published data on human metabolism of endosulfan or on the possible contributions of P450 isoforms to its metabolism. Based on animal studies, a proposed metabolic pathway for endosulfan was published by the Agency for Toxic Substances and Disease Registry (ATSDR, 2000) and is shown in Fig. 1. A study using cats reported the immediate presence of endosulfan sulfate in the liver following intravenous administration of endosulfan (Khanna et al., 1979). In rats administered a single oral dose of ^{14}C-endosulfan, the metabolites sulfate, lactone, ether, and diol were detected in their feces 5 days later (Dorough et al., 1978). Analyses of human adipose tissue, placenta, umbilical cord serum, and milk samples demonstrated the presence of parent compound (α- and β-endosulfan) and metabolites endosulfan sulfate, diol, lactone, and ether, although the sulfate was the predominant degradation product (Cerrillo et al., 2005).

The present study determined that endosulfan-α is metabolized to a single metabolite, endosulfan sulfate, in human liver microsomes, and its metabolism is primarily mediated by CYP2B6 (at high efficiency) and CYP3A4 (at low efficiency). CYP2B6 is recognized to be expressed at only 3 to 5% of total P450s in human livers (Gervot et al., 1999; Lang et al., 2001, whereas CYP3A4 is known as the most abundant P450 isoform, expressed at 20 to 60% of total P450s in human liver. The respective levels of CYP2B6 and CYP3A4 in human liver microsomes in combination with their strong affinity to endosulfan-α (K_m = 16.2 and 14.4 μM, respectively) and their corresponding

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[1] This article is dedicated in memory of Dr. Randy Rose, who died in a tragic car accident.

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ABBREVIATIONS: P450, cytochrome P450; rP450, recombinant P450; HLM, human liver microsome; % TNR, percentage total normalized rate; % I, percentage inhibition; ACN, acetonitrile; FMO, flavin-containing monooxygenase; rFMO, recombinant FMO; NR, normalized rate; pHLM, pooled human liver microsome; M-M, Michaelis-Menten; CL_{int}, intrinsic clearance.

clearance rates of endosulfan (CL_{int} = 0.70 and 0.09 μl/min/pmol P450, respectively) presented a unique opportunity of investigating the potential of endosulfan-α to simultaneously probe for the in vitro catalytic activity of both CYP2B6 and 3A4.

Most, if not all, of the information in this communication was presented at the 13th annual ISSX meeting in Maui, HI on October 23 to 27, 2005 (Casabar et al., 2005). Subsequently, after the current communication had been prepared for submission, a manuscript was submitted and published from another laboratory (Lee et al., 2006). Lee et al. (2006) reported on the metabolism of α- and β-endosulfan isomers, whereas the present study only reports on the metabolism of the α-isomer. Although the results from the two laboratories on metabolism of endosulfan-α are in general agreement, the current communication extends the findings in the development of endosulfan-α as a simultaneous probe for CYP2B6 and 3A4 in human liver microsomes.

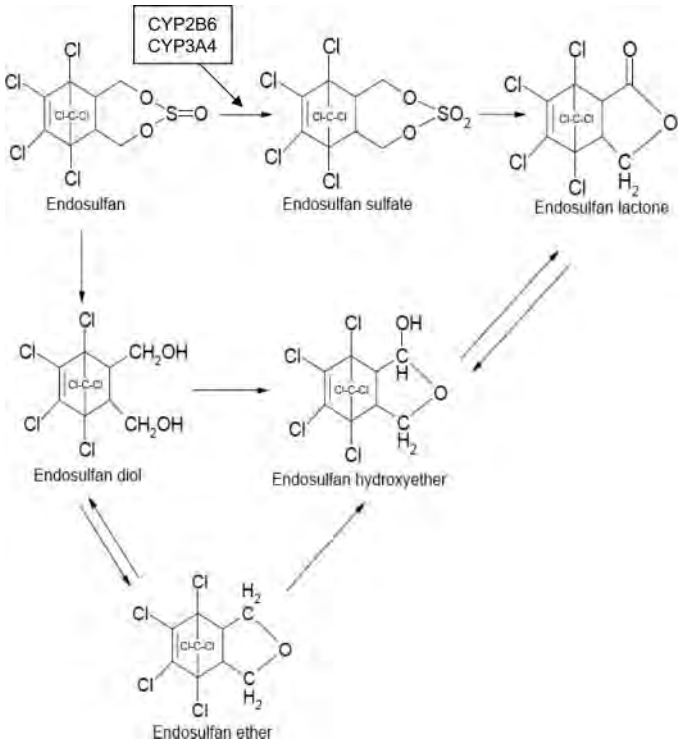

FIG. 1. The proposed metabolic pathway for endosulfan based on animal studies, as published by ATSDR (2000), was modified to show that human CYP2B6 and CYP3A4 primarily catalyze the metabolism of endosulfan-α to endosulfan sulfate, the only metabolite detected in the present study.

Materials and Methods

Chemicals. Endosulfan-α, the predominant isomer (70%) in commercial endosulfan, was used in the study of endosulfan metabolism. Endosulfan-α, endosulfan sulfate, endosulfan diol, endosulfan ether, and endosulfan lactone reference materials were purchased from ChemService (West Chester, PA). Stock solutions of endosulfan-α and metabolites were prepared in acetonitrile (ACN) and stored at −20°C. $NADP^+$, glucose 6-phosphate, and glucose-6-phosphate dehydrogenase were purchased from Sigma-Aldrich (St. Louis, MO). High-performance liquid chromatography (HPLC)-grade water, ACN, EDTA, magnesium chloride, Tris, and all other chemicals not specified were purchased from Fisher Scientific (Pittsburgh, PA).

Ticlopidine, a potent mechanism-based chemical inhibitor to CYP2B6 (Richter et al., 2004), and ketoconazole, a selective chemical inhibitor to CYP3A4 (Baldwin et al., 1995) were purchased from Sigma-Aldrich. Stock solutions of ticlopidine were prepared in distilled water and stored at room temperature. Ketoconazole was dissolved in methanol and stock solutions were stored at 4°C.

Human Liver Microsomes (HLMs) and P450 Isoforms. Pooled HLMs (20 mg/ml) and 16 selected individual HLMs (20 mg/ml each) were purchased from BD Biosciences (San Jose, CA). The individual HLMs chosen for this study were representative of the levels of *S*-mephenytoin *N*-demethylase activity of CYP2B6 as follows: Low, HG32, HG95, HH47, HG74, HK37; Mid, HG43, HG93, HH18, HK25, HH101, HG3; and High, HH13, HG89, HG64, HG112, HG42. Human recombinant P450 (rP450) and recombinant flavin monooxygenase (rFMO) isoforms expressed in baculovirus-infected insect cells (Supersomes) were also purchased from BD Biosciences.

Metabolism Assays. Preliminary studies were performed to determine the times and HLM protein concentrations that produced a linear metabolic rate for 50 μM endosulfan-α. Endosulfan sulfate formation was linear from 0.05 to 0.25 mg/ml protein and from 5 to 60 min of incubation. The solvent effects of dimethyl sulfoxide, acetone, ACN, methanol, ethanol, and isopropanol at 1% solvent concentration were also tested on endosulfan-α metabolism. There were no differences in the rates of endosulfan sulfate formation among the different solvents, with the exception of isopropanol, which slightly inhibited formation of endosulfan sulfate (data not shown).

Based on the results of initial studies, 20 μM endosulfan-α substrate concentration dissolved in ACN, 0.25 mg/ml HLM protein concentration, and 30-min incubation time were used for subsequent metabolism assays, unless

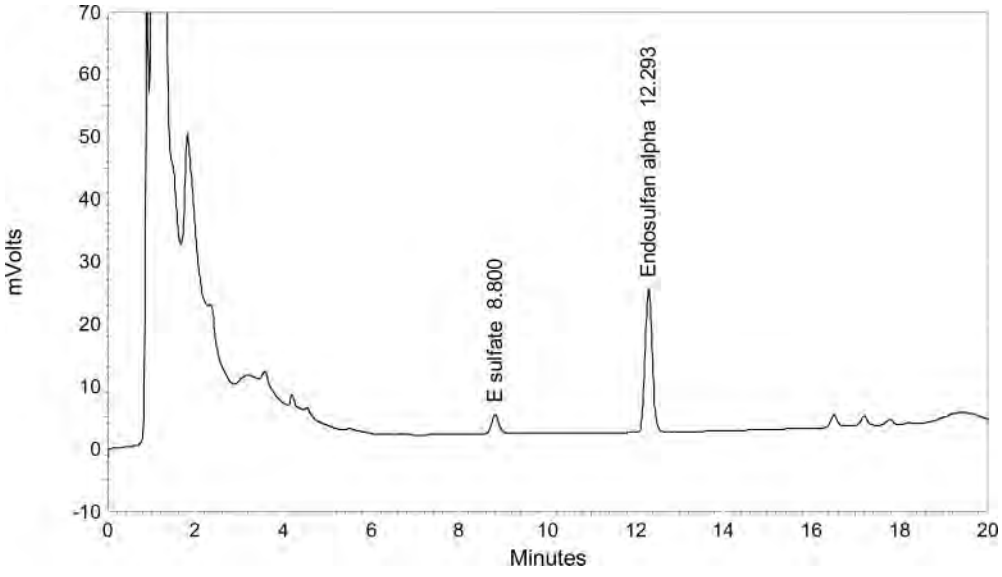

FIG. 2. A representative HPLC chromatogram of endosulfan-α metabolism to endosulfan sulfate, the lone metabolite detected in incubations with HLMs. Endosulfan-α (50 μM) was incubated with 0.25 mg/ml HLMs for 20 min. Endosulfan-α and endosulfan sulfate peaks were detected at retention times of 12.29 and 8.80 min, respectively, in a 20-min HPLC run. The three peaks toward the end of the chromatogram were determined to be contributions from HLMs.

otherwise stated. Metabolism assays with HLMs used 100 mM potassium phosphate buffer (pH 7.4). Metabolism with rP450s and rFMOs used the following buffers as recommended by BD Biosciences: 100 mM potassium phosphate (pH 7.4) for CYP1A1, 1A2, 3A4, 3A7, 2D6*1, 3A5, and SF9 insect control; 50 mM potassium phosphate (pH 7.4) for 2B6, 2C8, 2C19, and 2E1; 100 mM Tris (pH 7.4) for 2C9*1, 2C18, and 4A11; 50 mM Tris (pH 7.4) for 2A6; and 50 mM glycine (pH 9.5) for FMOs 1, 3, and 5. All buffers contained 3.3 mM $MgCl_2$ and 1 mM EDTA.

A preincubation mixture of endosulfan-α (20 μM), HLMs (0.25 mg/ml) or rP450 isoforms (12.5 pmol), and buffer was prepared in 1.5-ml microcentrifuge tubes. This mixture was preincubated for 3 min at 37°C in a water bath with minimal agitation. NADPH-regenerating system (final concentration of 0.25 mM $NADP^+$, 2.5 mM glucose 6-phosphate, and 2 U/ml glucose-6-phosphate dehydrogenase) was added to initiate the reaction. The final assay volume was 250 μl.

Reactions were carried out for 30 min and terminated with 250 μl of ice-cold ACN, followed by pulse-vortexing. Samples were centrifuged at 16,000*g* for 5 min and supernatants were analyzed by HPLC, as described in the HPLC analysis section below.

Inhibition Studies. Protocols for CYP2B6 and CYP3A4 inhibition by ticlopidine and ketoconazole used methods previously established by Richter et al. (2004) and Nomeir et al. (2001), respectively. In the case of ticlopidine, a mechanism-based inhibitor of CYP2B6, a 3-min preincubation at 37°C of ticlopidine (5 μM) with HLMs (100 μg) or rP450s (5 pmol) in 50 mM potassium phosphate buffer (with 3.3 mM $MgCl_2$ and 1 mM EDTA) in combination with an NADPH-regenerating system (final concentration of 0.5 mM $NADP^+$, 5 mM glucose 6-phosphate, and 4 U/ml glucose-6-phosphate dehydrogenase) was carried out before the addition of endosulfan-α (20 μM). In the case of ketoconazole, endosulfan-α (20 μM) and ketoconazole (10 μM) were preincubated along with 100 μg of HLMs or 5 pmol of rP450 in 50 mM potassium phosphate buffer for 3 min at 37°C before the addition of the NADPH-regenerating system (final concentration of 0.25 mM $NADP^+$, 2.5 mM glucose 6-phosphate, and 2 U/ml glucose-6-phosphate dehydrogenase). In both cases, final reaction volumes were 250 μl and reactions were terminated by the addition of 250 μl of ice-cold ACN and processed as described previously.

HPLC Analysis. Metabolite formation was analyzed with a Shimadzu (Kyoto, Japan) HPLC system consisting of an autoinjector (SIL-10AD VP), two pumps (LC-10AT), and a UV detector (SPD-10A VP). Endosulfan-α and metabolites were separated by a Gemini C18 column, 5 μm, 100 × 4.6 mm (Phenomenex, Torrance, CA), and identified with direct injection of reference compounds. The mobile phase for pump A consisted of 99% water and 1% phosphoric acid (pH 2.0) and that for pump B, 100% ACN. The flow rate was 1 ml/min. A gradient methodology was used as follows: 0 to 3 min (60% ACN), 3 to 16 min (60–90% ACN), 16 to 19 min (90–60% ACN), and 19 to 20 min (60% ACN). The injection volume was 50 μl and solutes were detected at 213 nm. Under these conditions, the retention times for endosulfan-α and endosulfan sulfate were 12.4 and 8.9 min, respectively. Endosulfan-α and endosulfan sulfate peaks were quantified with calibration curves constructed from known concentrations of reference materials. The detection limit for endosulfan sulfate following the U.S. Environmental Protection Agency's method detection limit procedure was 0.04 μM (CFR, 2006).

Data Analyses. Michaelis-Menten and Eadie-Hofstee plots were generated using the SigmaPlot Enzyme Kinetics Module (Systat Software, Inc., Point Richmond, CA). Enzyme kinetic parameters K_m and V_{max} were determined using nonlinear regression analysis with the SigmaPlot software.

Correlations of endosulfan sulfate formation with each P450-specific catalytic activity or P450 contents were calculated with simple linear regression using the web-based Statcrunch program (www.statcrunch.com). $p < 0.05$ was considered statistically significant.

To estimate the contributions of different P450 isoforms to the metabolism of endosulfan-α, percentage total normalized rates (% TNR) were calculated using the method described by Rodrigues (1999). In brief, metabolite formation rate (pmol/min/pmol rP450) obtained from rP450 metabolism of the compound of interest is multiplied by the immunoquantified P450 content (pmol P450/mg) in native human liver microsomes, yielding the "normalized rate" (NR) expressed in pmol/min/mg microsomes. The NRs for each P450 involved in the metabolism of the compound of interest is summed up as the "total normalized rate" (TNR) (Rodrigues, 1999). The % TNR for each P450 was then calculated according to the following equation.

$$\%\ \mathrm{TNR} = \frac{\mathrm{NR}}{\mathrm{TNR}} \times 100 = \frac{\text{pmol/min/pmolr P450} \times \text{pmol P450/mg}}{\sum^{(\text{pmol/min/pmol rCYP} \times \text{pmol nP450/mg})}} \times 100$$

Results

Metabolism of Endosulfan-α. Endosulfan-α at 50 μM concentration was metabolized by HLMs to a single metabolite, endosulfan sulfate. Figure 2 shows a representative HPLC chromatogram of this metabolism assay. The retention times for endosulfan-α and endosulfan sulfate were 12.29 and 8.80 min, respectively, in a 20-min HPLC run.

Cytochrome P450 Screening. P450 and FMO contributions to metabolism of endosulfan-α (20 μM) were investigated using 14 rP450 and 3 rFMO commercially available human isoforms. Recombinant CYP2B6 predominantly mediated the formation of endosulfan sulfate by 8-fold (at 6.9 nmol/min/nmol P450) over the next isoform (CYP3A4) with the next highest metabolite formation rate (at 0.8 nmol/min/nmol P450). CYP2C18, 2C19, 2C9*1, and 3A7 also showed metabolic activity, but at negligible levels (Fig. 3). FMOs had no measurable activity toward endosulfan-α.

Kinetics of Endosulfan-α Metabolism. The kinetic parameters K_m and V_{max} were determined by incubating endosulfan-α (0.78–100 μM) with pHLM (0.25 mg/ml), rCYP2B6, or rCYP3A4 (12.5 pmol). Calculated apparent K_m, V_{max}, and CL_{int} values are shown in Table 1.

The respective Michaelis-Menten (M-M) and Eadie-Hofstee plots of endosulfan-α metabolism by pHLM, rCYP2B6, and rCYP3A4 are shown in Fig. 4, A to C. The M-M plot show a hyperbolic curve, indicating saturation of metabolite formation over the substrate concentration range used and suggesting that the data obeyed M-M kinetics. The Eadie-Hofstee plots were linear, indicating either involvement of one enzyme or of more than one enzyme with similar affinity (Ward et al., 2003), and with a slight hook at the bottom end of the curve, suggesting allosteric activation (Faucette et al., 2000).

Correlation of Endosulfan Sulfate Formation with Specific P450 Contents and Selective P450 Activities. Endosulfan-α metabolism was conducted in 16 individual HLMs. Correlations between selective P450 activities from these 16 individual HLMs and specific P450 contents (of a subgroup of 8 HLMs with immunoquantified P450 contents from BD Biosciences) were

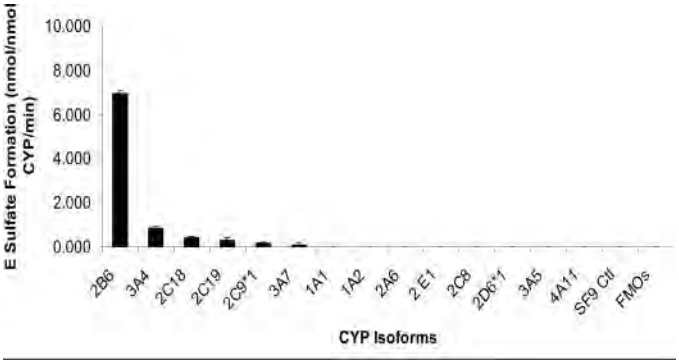

FIG. 3. Rates of endosulfan sulfate formation from endosulfan-α (20 μM) by 14 rP450 and 3 rFMO isoforms. Data shown are the means of two independent determinations.

TABLE 1

Kinetic parameters of endosulfan-α metabolism in pHLMs, recombinant CYP2B6, and 3A4

HLM or P450	K_m	V_{max}	CL_{int}
	μM		
pHLM	9.8	178.5[a]	18.20[b]
CYP2B6	16.2	11.4[c]	0.70[d]
CYP3A4	14.4	1.3	0.09

[a] V_{max} expressed in pmol/min/mg protein for pHLM.
[c] V_{max} expressed in pmol/min/pmol P450 for CYP2B6 and 3A4.
[b] Intrinsic clearance (V_{max}/K_m) expressed in μl/min/mg protein for pHLMs.
[d] Intrinsic clearance (V_{max}/K_m) expressed in μl/min/pmol P450 for CYP2B6 and 3A4.

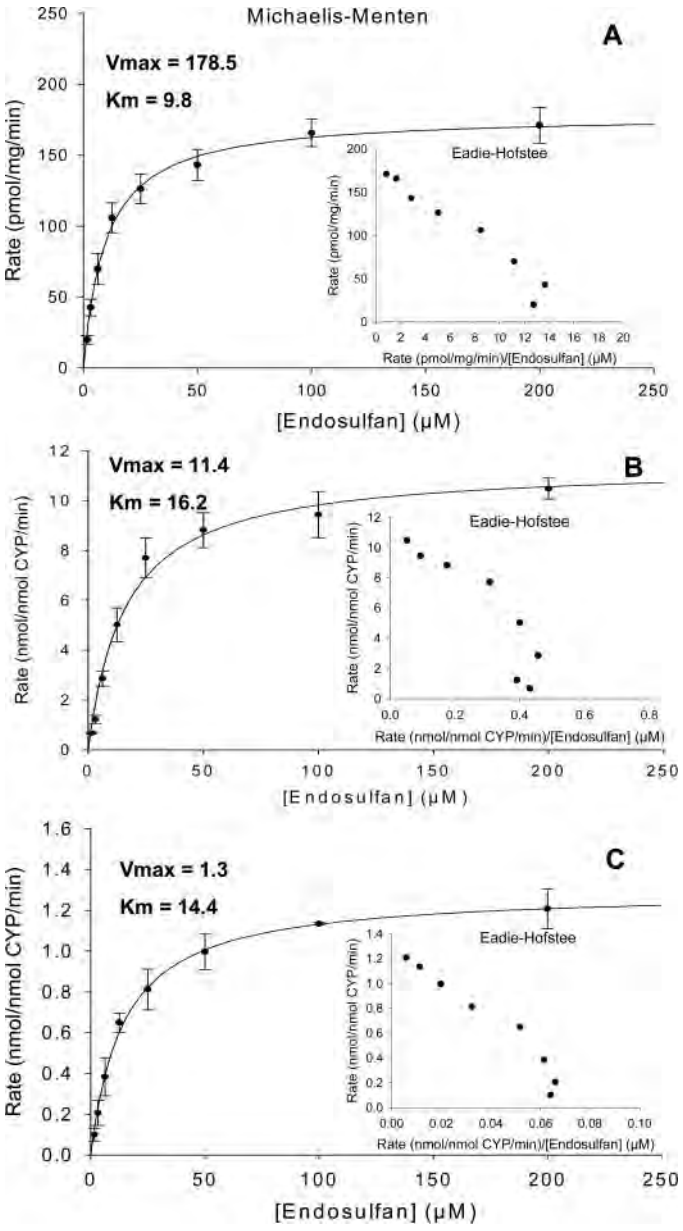

FIG. 4. Velocity of endosulfan sulfate formation versus endosulfan-α concentration in human liver microsomes (A), recombinant CYP2B6 (B), and recombinant CYP3A4 (C). Each point represents the mean of three independent measures.

calculated. A strong correlation was evident between endosulfan sulfate formation and *S*-mephenytoin *N*-demethylase activity of CYP2B6 ($r^2 = 0.79$, $p = 0.0001$). A less significant correlation was found with testosterone 6β-hyroxylase activity of CYP3A4 ($r^2 = 0.54$, $p = 0.001$). Likewise, strong correlations were evident between endosulfan sulfate formation and immunoquantified contents of CYP2B6 ($r^2 = 0.86$, $p = 0.0008$) and 3A4 ($r^2 = 0.81$, $p = 0.002$) (correlation plots shown in Fig. 5, A–D).

No significant correlations were found between endosulfan sulfate formation and diclofenac-4-hydroxylase activity of 2C9 ($r^2 = 0.04$, $p = 0.460$), *S*-mephenytoin 4-hyroxylase activity of 2C19 ($r^2 = 0.01$, $p = 0.743$), and other P450-selective activities (correlation plots not shown). Likewise, no significant correlations were seen with endosulfan sulfate formation and P450 contents of 2C9 ($r^2 = 0.42$, $p = 0.167$), 2C19 ($r^2 = 0.01$, $p = 0.571$), and other P450s. In addition, correlations were calculated for *S*-mephenytoin *N*-demethylase and CYP2B6 content ($r^2 = 0.87$), and testosterone 6β-hydroxylase and CYP3A4 content ($r^2 = 0.97$) in the same subgroup of 8 HLMs (see correlation plots in Fig. 5, E and F).

Inhibition of Endosulfan-α Metabolism by Ticlopidine and Ketoconazole, Selective Chemical Inhibitors for CYP2B6 and 3A4, Respectively. Initially, the optimal concentrations of ticlopidine and ketoconazole needed to obtain maximal inhibition of endosulfan sulfate formation were tested in rCYP2B6 and rCYP3A4. Results of these experiments are shown in Fig. 6, A and B. It was determined that 5 μM ticlopidine and 10 μM ketoconazole were optimal for subsequent inhibition studies. Results of inhibition of endosulfan sulfate formation with ticlopidine (5 μM) and/or ketoconazole (10 μM) are shown in Table 2. Six individual HLMs were chosen for these studies, based on available immunoquantified P450 contents data supplied by the manufacturer. These individual HLMs also represented various ranges of P450 contents (see Table 3). Inhibition of endosulfan sulfate formation by ketoconazole among the six individuals varied from 9 to 38%, implicating varying levels of CYP3A4 among these individuals. Similarly, the range of CYP2B6 involvement varied from 33 to 80%. The results show that inhibition of endosulfan metabolism with ketoconazole and ticlopidine were generally additive in all six HLMs.

% TNR. % TNR was calculated to verify the percentage inhibition (% I) results from this study (Table 3). % TNR obtained from rP450s can be directly related to % I obtained with native HLMs (Rodrigues, 1999). The % I from the combined incubation with ketoconazole and ticlopidine matched the sum of % TNRs of CYP2B6 and 3A4 in the metabolism of endosulfan-α in five of the six HLMs in this study (see Table 4).

Discussion

In the present study, we found endosulfate sulfate as the only metabolite of endosulfan from incubations with HLMs. In mice exposed to a single dose of ^{14}C-endosulfan, endosulfan sulfate concentrations were elevated in the liver, intestine, and visceral fat after 24 h (Deema et al., 1966). A study in rats administered a single oral dose of ^{14}C-endosulfan showed that the endosulfan metabolites diol, sulfate, lactone, and ether were found in the feces 5 days later (Dorough et al., 1978). A recent study, conducted in Spain, in which endosulfan is commonly used identified parent endosulfan and metabolites diol, sulfate, lactone, and ether in adipose tissues, placenta, cord blood, and human milk (Cerrillo et al., 2005). These findings, coupled with results of our study, suggest that the formation of the diol, ether, and lactone metabolites may be the result of metabolic processes beyond those occurring in human liver microsomes.

Our kinetic studies with human liver microsomes as well as with P450 isoforms 2B6 and 3A4 produced monophasic Eadie-Hofstee plots, suggesting that endosulfan-α is metabolized either by one enzyme or by more than one enzyme with similar K_m. A survey of 14 P450 isoforms demonstrated significant metabolism by CYP2B6, followed by 3A4, members of the 2C family, and 3A7. Of these isoforms, CYP2B6 and 3A4 are likely to have the greatest impact based upon activity levels and relative abundance. Although CYP2C18 may be similar to CYP3A4 in its capacity to metabolize endosulfan, it is poorly expressed in human livers (Goldstein, 2001). Our kinetic studies demonstrated that CYP2B6 and CYP3A4 share similar binding affinities (K_m of 16.2 and 14.4 μM, respectively) but vary significantly in maximum velocity. The resulting difference in clearance of endosulfan sulfate demonstrates that CYP2B6 is 8-fold more efficient than CYP3A4 in catalyzing the metabolism of endosulfan-α (see Table 1). The present study determined the kinetic parameters $K_m = 9.8$ μM, $V_{max} = 178.5$ pmol/min/mg HLM, and $CL_{int} = 18.2$ μl/min/mg HLM for endosulfan-α metabolism by human liver microsomes. Lee et al. (2006) reported the following kinetic parameters for endosulfan-α metabolism by HLMs: $K_m = 7.34$ μM, $V_{max} = 1.48$ pmol/min/pmol P450, $CL_{int} = 0.20$ μl/min/pmol P450. Although the K_m values obtained by both laboratories are comparable, the V_{max} and CL_{int} are not comparable because of the different methodologies used by each study in calculation of these kinetic parameters. The

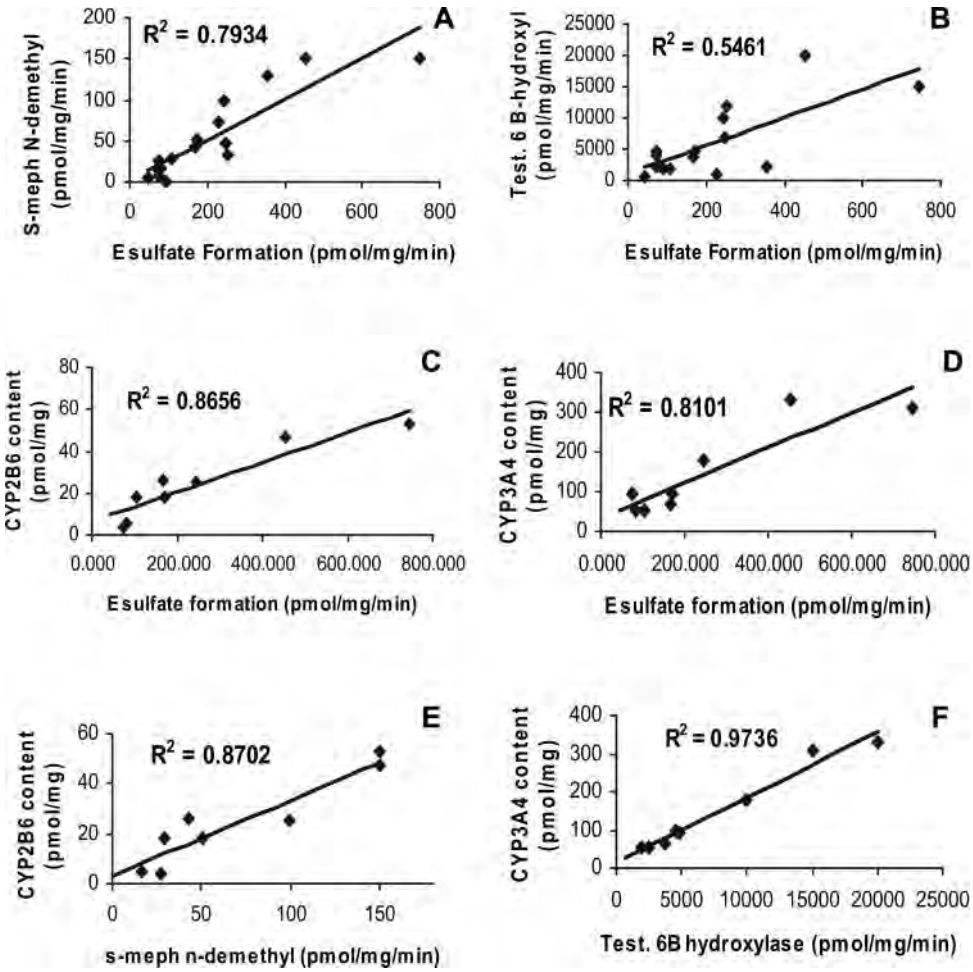

FIG. 5. Correlation plots of endosulfan sulfate formation and selective activities of CYP2B6 (A) and CYP3A4 (B) in 16 individual HLMs or immunoquantified contents of CYP2B6 (C) and CYP3A4 (D) in 8 HLMs. Correlation plots were also generated for *S*-mephenytoin *N*-demethylase and CYP2B6-immunoquantified contents (E) and for testosterone 6 β-hydroxylase and CYP3A4 contents (F) in 8 HLMs. Rates of endosulfan sulfate formation were measured in two independent determinations in each of the HLMs.

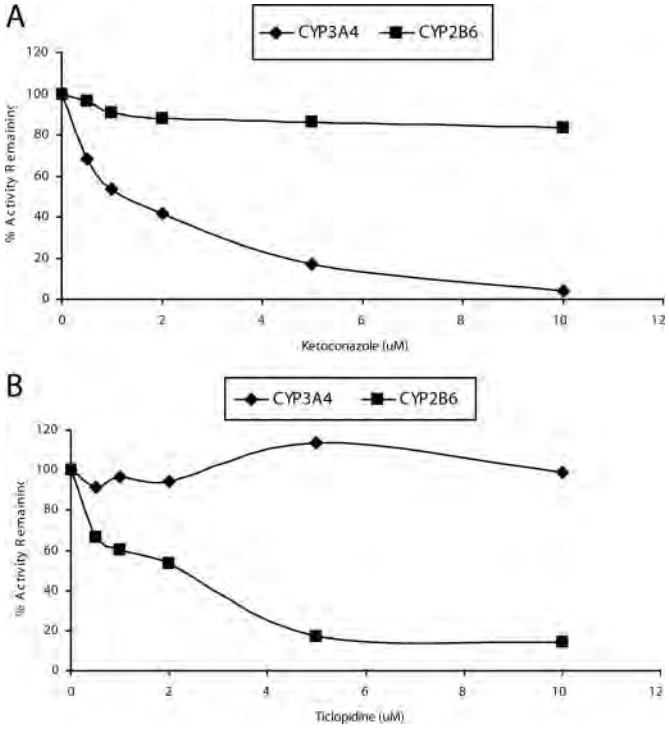

FIG. 6. Inhibition of endosulfan sulfate formation in rCYP2B6 and rCYP3A4 by ketoconazole (0–10 μM) (A) and ticlopidine (0–10 μM) (B). Each point represents the mean of two independent measures.

present study used protein content of HLMs, but Lee et al. (2006) used total P450 content in their calculations.

The correlations for CYP2B6 content and rates of *S*-mephenytoin metabolism ($r^2 = 0.87$) and endosulfan-α metabolism ($r^2 = 0.86$) are comparable, indicating that endosulfan-α is an excellent substrate for CYP2B6. However, the correlations for CYP3A4 content and rates of testosterone metabolism ($r^2 = 0.97$) and endosulfan-α metabolism ($r^2 = 0.81$) differ, suggesting that endosulfan-α is only a moderate substrate, in comparison with testosterone, for CYP3A4. The advantage of endosulfan-α is its utility for simultaneous probing of the activity of both CYP2B6 and CYP3A4.

Initial inhibition studies using monoclonal antibodies to CYP2B6 and 3A4 were abandoned because of their poor ability to inhibit endosulfan sulfate formation in the recombinant P450 isoforms (less than 30%; data not shown). This suggests that these monoclonal antibodies, although specific in inhibiting the metabolism of some substrates, may not be optimal inhibitors for endosulfan or other substrates. Hence, we used ticlopidine and ketoconazole, selective chemical inhibitors for CYP2B6 and 3A4, respectively, to characterize the contributions of these isoforms to endosulfan-α metabolism. Because CYP2B6 has been reported to be partially sensitive to ketoconazole at higher concentrations (Baldwin et al., 1995), we tested the effects of different concentrations of ketoconazole on endosulfan sulfate formation by recombinant CYP3A4 and CYP2B6. The present study determined that at the concentrations used in the inhibition of endosulfan sulfate formation (ketoconazole = 10 μM and ticlopidine = 5 μM), these inhibitors did not significantly inhibit the activity of the other isoform examined (Fig. 5). It is of interest that in the

TABLE 2

Inhibition of endosulfan sulfate formation in HLMs by ketoconazole and ticlopidine

Inhibitors ketoconazole (KTZ; 10 μM) and ticlopidine (TCL; 5 μM) were used alone and combined for metabolism of endosulfan-α in six individual HLMs. Data are means of two independent measurements.

Inhibitor	Percentage Inhibition of Endosulfan Sulfate Formation					
	HG3	HG112	HG42	HG43	HG93	HK23
10 μM KTZ	23.8 ± 0.6	20.5[a]	8.6 ± 1.5	34.9 ± 4.7	37.6 ± 8.8	36.0 ± 0.2
5 μM TCL	67.8 ± 2.6	64.4 ± 0.5	79.2 ± 3.0	57.0 ± 0.4	38.6 ± 3.3	33.0 ± 4.8
5 μM TCL + 10 μM KTZ	92.3 ± 0.4	88.0 ± 1.3	91.5 ± 2.4	85.2 ± 0.6	75.6 ± 6.2	57.0 ± 0.6

[a] No replicate for this measurement due to insufficient HLM HG112 sample.

TABLE 3

Comparison between % TNR and % I in six individual HLMs

% TNR was calculated according to Rodrigues et al. (1999). % I for CYP2B6 was determined with the use of ticlopidine (5 μM) and that for CYP3A4 with ketoconazole (10 μM).

HLMs	rP450	Endosulfan Sulfate Formation Rate[a] in rP450	P450 Content[b] in Native HLMs	Normalized Rate	% TNR	% I
HG42	2B6	9.42	53	499.37	64.8	79.3
	2C9	0.34	80	24.12	3.5	N.D.
	2C19	0.32	6	1.89	0.2	N.D.
	3A4	0.78	310	242.73	31.5	8.6
HG112	2B6	9.42	47	442.83	58.8	64.4
	2C9	0.34	87	29.49	3.9	N.D.
	2C19	0.32	72	22.68	3.0	N.D.
	3A4	0.78	330	258.39	34.3	20.5
HG3	2B6	9.42	18	169.60	65.0	67.9
	2C9	0.34	42	14.24	5.0.4	N.D.
	2C19	0.32	9	2.84	1.1	N.D.
	3A4	0.78	95	74.38	28.5	23.8
HK23	2B6	9.42	7	65.95	42.1	33.0
	2C9	0.34	56	18.98	12.1	N.D.
	2C19	0.32	17	5.36	3.4	N.D.
	3A4	0.78	85	66.56	42.4	36.0
HG43	2B6	9.42	4	37.69	26.3	57.0
	2C9	0.34	51	17.29	12.1	N.D.
	2C19	0.32	47	14.81	10.3	N.D.
	3A4	0.78	94	73.60	51.3	34.9
HG93	2B6	9.42	18	169.60	69.0	38.6
	2C9	0.34	51	17.29	7.3	N.D.
	2C19	0.32	49	15.44	6.7	N.D.
	3A4	0.78	52	40.72	17.0	37.6

N.D., not determined.
[a] Rates in pmol/min/pmol rP450.
[b] Immunoquantified P450 contents in pmol/min/mg protein.

TABLE 4

Sum of CYP2B6 and 3A4 % TNRs vs. % I with ketoconazole and ticlopidine

Comparison between the sum of % TNRs of CYP2B6 and CYP3A4 in the metabolism of endosulfan-α and % I with ketoconazole and ticlopidine in the same incubation. With the exception of HK23, the other five HLMs had matching % TNR and % I.

HLM	% TNR	% I
HG3	94	92
HG112	93	88
HG42	96	92
HK23	84	57
HG43	78	85
HG93	86	76

six HLMs examined, the combined use of ketoconazole and ticlopidine resulted in inhibition of endosulfan sulfate formation, which was generally similar to the results obtained with each inhibitor alone. For four individuals, the combined inhibition of CYP2B6 and 3A4 yielded values from 85 to 92%, yet two individuals retained significant ability to metabolize endosulfan following inhibition (HK23 and HG93 with 57 and 76% inhibition, respectively). To further explore the possibility that other P450s were involved in metabolism for these individuals, the total normalized rates of metabolism for the P450 isoforms identified by screening efforts were investigated.

The % I from the combined incubation with ketoconazole and ticlopidine corresponded well with the combined % TNRs of CYP2B6 and 3A4 (Table 4) in the metabolism of endosulfan-α in five of the six HLMs in this study. With HK23, there was a significantly lower percentage inhibition of endosulfan-α metabolism by CYP2B6 (as demonstrated by % I with ticlopidine) compared with the metabolic contribution of CYP2B6 as predicted by % TNR. This decreased inhibition of CYP2B6 activity in HK23 may be due to a CYP2B6 polymorphism. This is supported by a study in which a 26% decrease was seen in *N*,*N*′,*N*″-triethylene-thiophosphoramide inactivation of *O*-deethylation of 7-ethoxy-4-(trifluoromethyl)coumarin in mutant CYP2B6 compared with wild-type 2B6 (Bumpus et al., 2005). It is now known that CYP2B6 polymorphisms are common in Caucasians and that CYP2B6 is one of the most polymorphic human P450s (Lang et al., 2001).

A number of substrate probes for CYP2B6 have been reported in the literature, including 7-ethoxy-4-trifluoromethylcoumarin (Code et al., 1997), cyclophosphamide, and ifosfamide (Huang et al., 2000), *S*-mephenytoin (Heyn et al., 1996; Ko et al., 1998), bupropion (Faucette et al., 2000; Hesse et al., 2000), and efavirenz (Ward et al., 2003). The known substrate probes for CYP3A4 include testosterone, midazolam, nifedipine, and erythromycin (Yuan et al., 2002). The use of one substrate to simultaneously probe for the in vitro catalytic activity of CYP2B6 and CYP3A4 would be very advantageous. Based

on the results of our inhibition studies, endosulfan-α appears to be a strong candidate for this role.

In conclusion, endosulfan-α is metabolized to a single metabolite, endosulfan sulfate, by HLMs. This metabolism is primarily mediated by CYP2B6 and CYP3A4. The strategies used to demonstrate this were: 1) endosulfan-α metabolism by rP450s, 2) correlation studies of endosulfan sulfate formation and P450-selective activities or P450 immunoquantified contents in individual HLMs, and 3) inhibition studies using CYP2B6- and CYP3A4-selective chemical inhibitors. In addition, endosulfan-α may be used to simultaneously probe for the in vitro catalytic activities of CYP2B6 and CYP3A4. Finally, endosulfan's endocrine-disrupting effects and mechanisms inducing microsomal enzyme activity are currently under investigation.

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