In Vitro Characterization of Sarizotan Metabolism: Hepatic Clearance, Identification and Characterization of Metabolites, Drug-Metabolizing Enzyme Identification, and Evaluation of Cytochrome P450 Inhibition

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ABSTRACT:

In vitro biotransformation studies of sarizotan using human liver microsomes (HLM) showed aromatic and aliphatic monohydroxylation and dealkylation. Recombinant cytochromes P450 (P450) together with P450-selective inhibitors in HLM/hepatocyte cultures were used to evaluate the relative contribution of different P450s and revealed major involvement of CYP3A4, CYP2C9, CYP2C8, and CYP1A2 in sarizotan metabolism. The apparent \( K_{m,u} \) and \( V_{max} \) of sarizotan clearance, as investigated in HLM, were 9 \( \mu M \) and 3280 pmol/mg/min, predicting in vivo hepatic clearance of 0.94 l/h, which indicates that sarizotan is a low-clearance compound in humans and suggests nonsaturable metabolism at the targeted plasma concentration (\( \leq 1 \mu M \)). This finding is confirmed by the reported human clearance (CL/F of 3.6–4.4 l/h) and by the dose-linear area under the curve increase observed with doses up to 25 mg. The inhibitory effect of sarizotan toward six major P450s was evaluated using P450-specific marker reactions in pooled HLM. \( K_{i,u} \) values of sarizotan against CYP2C8, CYP2C19, and CYP3A4 were >10 \( \mu M \), whereas those against CYP2D6 and CYP1A2 were 0.43 and 8.7 \( \mu M \), respectively. Based on the estimates of sarizotan concentrations at the enzyme active sites, no clinically significant drug-drug interactions (DDIs) due to P450 inhibition are expected. This result has been confirmed in human DDI studies in which no inhibition of five major P450s was observed in terms of marker metabolite formation.

Sarizotan (SZ) hydrochloride is an aminomethyl-chromane that underwent clinical development for levodopa therapy-associated dyskinesia in patients with Parkinson’s disease. The occurrence of dyskinesias, or involuntary movements, is one of the most troublesome and debilitating side effects of prolonged treatment with L-dopa, occurring in up to one-third of all patients. In preclinical studies, SZ was effective in induced Parkinson syndrome in monkeys (Bibbiani et al., 2005). In clinical studies, SZ demonstrated proof of concept in an open pilot study and in a placebo-controlled dose-finding study. Single- and multiple-dose studies in healthy volunteers with orally administered SZ hydrochloride revealed rapid absorption (\( t_{max} 0.5–2.3 \) h). Subsequently, plasma levels declined polyexponentially with a terminal elimination half-life of 5 to 7 h. \( C_{max} \) and \( t_{max} \) varied slightly with formulation and food intake, whereas the area under the curve (AUC) was unaffected by these factors. AUC and \( C_{max} \) increased dose proportionally over the tested dose range of 0.5 to 25 mg (Kroesser et al., 2006a, 2007).

The Food and Drug Administration (FDA) recommends that all new chemical entities (NCEs) in development be characterized with respect to metabolic properties before administration to humans (FDA Guidance for Industry: Drug Interaction Studies—Study Design, Data Analysis and Implications for Dosing and Labeling, http://www.fda.gov/cber/guidelines.htm, 2006). The in vitro characterization involves estimation of the role of metabolism in the clearance, identification of enzymes involved, structural characterization of metabolites, and evaluation of potential drug-drug interaction risks associated with the NCE and its major metabolites with respect to enzyme inhibition and/or induction (Thompson, 2000; Masimirembwa et al., 2001; Björnsson et al., 2003). Metabolic stability studies are used in development to predict in vivo hepatic clearance and drug bioavailability associated with the dosing regimen achieving favorable efficacy and safety properties (Masimirembwa et al., 2003). Accurate approaches

Received August 21, 2009; accepted March 10, 2010

ABBREVIATIONS: SZ, sarizotan; AUC, area under the curve; \( C_{max} \), maximum plasma concentration; \( t_{max} \), time of maximum plasma concentration; NCE, new chemical entity; P450, cytochrome P450; DDI, drug-drug interaction; PK, pharmacokinetic(s); HLM, human liver microsome(s); ABT, aminobenzotriazole; EMD 148107, (\( \pm \))-2-[5-(4-fluorophenyl)-3-pyridylmethylaminomethyl]-6-hydroxychromane, dihydrochloride; EMD 329989, 2R,4R/S)-2-\( \{5-(4-fluorophenyl)-pyridine-3-ylmethyl\}-amino\)-methyl\)-chromane-4-ol; EMD 50929, 3-(4-fluorophenyl)-pyridine-5-carboxylic acid; LC, liquid chromatography; MS/MS, tandem mass spectrometry; HPLC, high-pressure liquid chromatography; RT, retention time; \( K_{i,u} \), inhibitory constant considering \( f_u \) in microsomal incubations; \( K_{m,u} \), unbound Michaelis-Menten constant.
are available for in vitro to in vivo extrapolation of drug clearance (Houston and Galetin, 2003). A battery of reaction phenotyping methods can be applied to identify the enzyme(s) involved in the metabolism of an NCE (Williams et al., 2003). For accurate risk assessments of metabolism-based drug-drug interactions (DDIs), estimation of NCE concentrations and its unbound fractions present at the enzyme site(s) are necessary (Houston and Galetin, 2003). Results from these preclinical studies are used to estimate a safe clinical starting dose for first-in-man studies. Furthermore, the data are invaluable in deciding the need for and guiding the design of in vivo DDI interaction studies.

The aim of the present study was to characterize the in vitro metabolism of SZ in view of predicting human in vivo drug clearance, identification of cytochromes P450 (P450s) involved and metabolites formed, and the risk for P450 inhibition-based DDIs. Based on the results of the present in vitro study, several DDI trials were conducted in the clinical setting. The potential of SZ to act as a P450 inhibitor was investigated in a cocktail study using probe substrates for CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A4. The study confirmed that the likelihood of SZ to interfere with metabolism of substrates of these P450 enzymes is low (Kroesser et al., 2006b). The DDI potential elicited by inhibition of SZ metabolism was investigated in two clinical trials, i.e., one focusing on CYP3A4 inhibition with erythromycin and the other focusing on CYP1A2 inhibition by applying fluvoxamine. The first study showed only small increases in AUC and Cmax at steady state, indicating that coadministration of CYP3A4 inhibitors does not require any adaptation in SZ dosing (Kovar et al., 2006). The second study suggested that besides CYP1A2 the CYP2C family was also inhibited, leading to a moderate increase in SZ exposure (P. Rolan, S. Krösser, A. Kovar, C. Dilger, and M. Fluck, unpublished observations). Furthermore, amplification of SZ metabolism was investigated in a clinical study using St. John’s wort as an inducer of P450, particularly of CYP3A4, and P-glycoprotein. The results showed only a small effect on the PK of SZ that was not considered to be clinically relevant (unpublished observations).

Two placebo-controlled, double-blind phase III studies (PADDY-1 and PADDY-2, 1 mg b.i.d. of SZ for 6 months) did not confirm efficacy shown previously during phase II or preclinical studies. A statistically significant difference in the primary target variables (Unified Parkinson’s Disease Rating Scale measures of severity and duration of patients’ dyskinesia) between SZ and placebo was not demonstrated in these studies. Therefore, SZ hydrochloride has been withdrawn from development at Merck KGaA. However, the comprehensive preclinical in vitro metabolism data set on SZ generated in preparation for this submission is considered interesting per se, because it demonstrate the utility of such studies in predicting in vivo human PK, which was then validated by the comprehensive clinical DDI data set available for SZ.

Materials and Methods

Test Systems. Pooled human liver microsomes (HLM) were obtained from BD Gentest through its supplier in Germany (Nateuc, Frankfurt am Main, Germany), Biopredic International (Rennes, France), or IIAM (Leicestershire, UK). Fresh human hepatocytes were obtained through Biopredic International. Microsomes prepared from baculovirus-infected insect cells (Supersomes) expressing individual human CYP1A2, CYP2A6, CYP2B8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4 were purchased from BD Gentest (German supplier). Control Supersomes expressing no human enzymatic activity were also obtained from BD Gentest.

Chemicals, Reagents, and Buffers. Glucose 6-phosphate, glucose-6-phosphate dehydrogenase (lyophilized powder), and NADP, disodium salt were from Boehringer Mannheim (Mannheim, Germany); ammonium formate, 99.99% was from Fluka (Buchs, Switzerland); NADPH was from Sigma-Aldrich (St. Louis, MO); and 1-aminobenzotriazole (ABT) was from Sigma-Aldrich. All nonradiolabeled reference compounds were from BD Gentest, Sigma-Aldrich, or Ultrafine Chemicals (St. Louis, MO). Nonradiolabeled SZ and metabolites [14C]EMD 148107, [14C]EMD 329989, and [14C]EMD 50929 were prepared in dimethyl sulfoxide or in acetonitrile-methanol-water. Stock solutions of all other P450 substrates, metabolites, or inhibitors were prepared in dimethyl sulfoxide or acetonitrile. Solvent concentration in the final incubations was kept below 0.1%.

The standard medium for Supersome/microsome incubation consisted of potassium phosphate buffer (100 mM, pH 7.4), MgCl2 (3.3 mM), and an NADPH-regenerating system (1.3 mM NADP, 3.3 mM glucose 6-phosphate, and 0.4 U/ml glucose-6-phosphate dehydrogenase) (final concentrations in the incubates). Standard hepatocyte incubation medium consisted of Williams’ medium E + GlutaMAX I medium ( Gibco, Carlsbad, CA) supplemented with 5 × 10−6 M hydrocortisone (Sigma-Aldrich), 100 IU/ml penicillin, 100 μg/ml streptomycin (Gibco), and 4 μg/ml bovine insulin (Sigma-Aldrich).

Incubation Protocols. Plasma protein binding of SZ. The binding of SZ to human plasma proteins was measured using predialized human plasma (70 mM phosphate buffer, pH 7.4, overnight) to stabilize its pH at 7.4. Concentrations of 0.2 to 20 μM SZ were added to this plasma, and the mixture was incubated for 5 h at 37°C. Subsequently, proteins were separated by ultracentrifugation in a Beckman centrifuge at 4°C for 24 h at 245,000g. The clear supernatant was removed and 1 vol% human plasma was spiked to avoid potential adsorption of SZ to the sampling glass vials. SZ concentrations in supernatants and the initial plasma incubate were determined by LC-MS/MS. The fraction unbound was calculated as concentration in supernatant/concentration in plasma.

Effect of ABT on biotransformation of SZ in HLM and formation of metabolites. Incubations were conducted in duplicate in pooled HLM, using the standard buffer and cofactor mix with a microsomal protein concentration of 1 mg/ml and a final incubation volume of 0.5 ml. Incubations were conducted in the presence and absence of the nonselective chemical P450 inhibitor, ABT (Ortiz de Montellano and Mathews, 1981) at 0.25 and 10 mM. Microsomes were preincubated with ABT at 37°C for approximately 5 min before initiation with [14C]SZ (100 μM); negative control incubations in the presence and absence of the inhibitor contained no cofactor mix. Reactions were terminated after 60 min by addition of acetone (0.1 ml) containing nonradiolabeled SZ and metabolites (10 μg/ml) to verify the retention times in each chromatogram. After termination, the samples were centrifuged (8000g; 3 min) to remove the protein precipitate, and the supernatant was analyzed by radio-HPLC. The degree of inhibition of SZ metabolism was determined relative to control samples in the absence of inhibitors.

Determination of kinetic parameters Kmax, and Vmax for SZ clearance and metabolite formation in HLM. To determine the linear range with respect to microsomal protein concentration and incubation time, experiments were conducted under conditions of constant protein concentration (1 mg/ml) with sampling times of 0, 10, 20, 30, 45, and 60 min, followed by a set of incubations with varying protein concentrations (0.1, 0.2, 0.3, and 1.0 mg/ml) and sampling at 0, 5, 10, 15, 20, and 30 min. To investigate Michaelis-Menten parameters, incubations were conducted at a constant concentration of microsomal protein (0.2 mg/ml) and SZ concentrations ranging from 0.0575 to 500 μM. Parent drug and metabolites EM 329989 and EM 148107 were quantified by LC-MS/MS.

Metabolism of SZ by cDNA expressed P450 enzymes (Supersomes). Incubations were conducted in duplicate using Supersomes. Incubations contained [14C]SZ (25 μM) and either microsomes (0.2 mg/ml) or Supersomes [CYP1A2 (100 pmol/ml), CYP2A6 (40 pmol/ml), CYP2C8 (20 pmol/ml), CYP2C9 (120 pmol/ml), CYP2C19 (80 pmol/ml), CYP2D6*1 (20 pmol/ml), CYP2E1 (100 pmol/ml), and CYP3A4 (40 pmol/ml)] in addition to the buffer and cofactor mix described above. Total incubation volumes were 1.0 ml. Before initiation with the cofactor mix, incubations were kept at approximately 37°C for approximately 5 min. Blank incubations were conducted in the absence of cofactor mix. Samples were processed as described above after a total reaction.
time of 60 min. The degree of metabolism of SZ was assessed by appearance of metabolites in test item incubations relative to control incubations using the radio-HPLC method described below.

**Effect of specific P450 inhibitors on SZ metabolism formation in HLM.** Triplicate samples of pooled HLM (0.2 mg/ml) were incubated with [14C]SZ (25 μM) in the presence and absence of the selective (Rendic and Di Carlo, 1997) chemical P450 inhibitors furafylaine (CYP1A2; 30 μM), pirocaine (CYP2A6; 30 μM), quercetin (CYP2C8; 5 μM), sulfaphenazole (CYP2C9; 2 μM), S-mephenytoin (CYP2C19; 300 μM), (+)-N-3-benzylirvinanol (CYP2C19; 2.5 μM), quinidine (CYP2D6; 3 μM), diethyliothiocarbamate (CYP2E1; 10 μM), or ketoconazole (CYP3A4; 3 μM). The concentrations of these inhibitors were determined previously to be sufficiently potent and selective, although it is acknowledged that quercetin and diethyliothiocarbamate may inhibit enzymes other than CYP2C8 and CYP2E1, respectively. For this reason, trimeprprim was used as a more selective inhibitor of CYP2C8 in subsequent hepatocyte experiments (see *Inhibition experiments in fresh human hepatocyte monolayers*). Samples were preincubated at 37°C for approximately 10 min before addition of an NADPH-regenerating system, with the exception of those containing furafylaine and diethyliothiocarbamate. Additional single control incubations were conducted containing either no inhibitor, no inhibitor and no cofactor mix, or no inhibitor and no microsomes. The mechanism-based P450 inhibitors furafylaine and diethyliothiocarbamate were preincubated for 15 min with buffer, microsomes, and cofactor mix before the addition of SZ. A set of duplicate control incubations contained no inhibitor. The total incubation volume was 1.0 ml, and the reaction was terminated after 60 min by the removal of an aliquot (0.4 ml) into a tube containing an equivalent volume of nonradiolabeled SZ metabolite solution (10 μM) dissolved in acetonitrile (V/V). After termination, the samples were centrifuged (8000g; 3 min) to remove the protein precipitate, and an aliquot of supernatant (0.2 ml) was transferred to a clean vial before analysis. The degree of inhibition of SZ metabolism was determined relative to that of control samples in the absence of inhibitors.

**Inhibition experiments in fresh human hepatocyte monolayers.** All incubations were performed at 37°C in a CO₂-air (5:95%) humidified atmosphere. Cells were seeded in 24-well rat collagen I-coated plates (300,000 cells/well, 50 μl/well) in standard hepatocyte medium. SZ was present in incubations at 0.5 ml/well) in standard hepatocyte medium. Cells were seeded in 24-well rat collagen I-coated plates (300,000 cells/well, 50 μl/well) in standard hepatocyte medium. SZ was present in incubations at

**Determination of IC₅₀ and/or Kᵯᵣ values of SZ and its metabolites EMD 50929, EMD 329989 was performed using an Agilent 1100 series HPLC system coupled to a TurboVlanSpray source of the Applied Biosystems API 3000 series (Applied Biosystems, Foster City, CA). The LC-MS/MS system consisted of the following components: Agilent 1100 degasser G1322A, Agilent 1100 binary pump G1312A, Agilent 1100 thermostated well plate autosampler G1368A (sample G1367A and thermostat G1330A; injection volume 50 μl for high calibration batches and sample tray temperature 20°C); and Agilent 1100 thermostated column compartment G1316A (operating temperature 50°C). Selective detection was achieved on an API 3000 triple quadrupole mass spectrometer (PerkinElmerSciex Instruments, Waltham, MA) with Analyst software (version 1.3.2 or 1.4.1; Applied Biosystems). The mass spectrometer was equipped with a TurboVlanSpray interface operating in the positive ion mode, using multiple reaction monitoring for MS/MS detection of the compounds: SZ, precursor ion [M + H]⁺ at m/z 349 and product ion at m/z 186; SZ-d₄, precursor ion [M + H]⁺ at m/z 353 and product ion at m/z 190; EMD 148107 precursor ion [M + H]⁺ at m/z 365 and product ion at m/z 186; EMD 148107-d₄, precursor ion [M + H]⁺ at m/z 369 and product ion at m/z 190; EMD 329989, precursor ion [M + H]⁺ at m/z 365 and product ion at m/z 186; EMD 329989-d₄, precursor ion [M + H]⁺ at m/z 218 and product ion at m/z 172; and EMD 329989-d₄, precursor ion [M + H]⁺ at m/z 222 and product ion at m/z 176.

**Analytical conditions for Kᵯᵣ determination in HLM.** The analytical column was a Luna 3-μm phenylhexyl column (50 × 2 mm; Phenomenex, Torrance, CA). Mobile phases were solvent A (100% acetonitrile) and solvent B (0.1% formic acid, aqueous). The gradient was as follows (time, solvent A, flow): 0.0 min, 14%, 0.60 ml/min; 2.0 min, 14%, 0.60 ml/min; 3.0 min, 40%, 0.60 ml/min; 3.9 min, 90%, 0.60 ml/min; 3.91 min, 90%, 0.60 ml/min; 4.0 min, 90%, 0.60 ml/min; 4.01 min, 90%, 0.60 ml/min; 4.1 min, 90%, 0.60 ml/min; 4.11 min, 90%, 0.90 ml/min; 4.8 min, 90%, 0.90 ml/min; 4.81 min, 14%, 0.90 ml/min; and 6.0 min, 14%, 0.90 ml/min. RTs were determined at approximately 4°C, supernatants were transferred into 5-ml tubes, and acetonitrile (1:1, v/v) containing the appropriate internal standards was added to solubilize the cells. After cell scraping and centrifuging (900g for 5 min at approximately 4°C), supernatants were transferred into 3-ml polypropylene tubes. After termination, the samples were centrifuged (8000g; 3 min) to remove the protein precipitate, and an aliquot of supernatant (0.2 ml) was transferred to a clean vial before analysis. The degree of inhibition of SZ metabolism was determined relative to that of control samples in the absence of inhibitors.

**UV-HPLC methods for determination of P450 activity.** Ethoxyresorufin O-deethylase activity (CYP1A2), ptaatelx 6xo-hydroxy activity (CYP2C8), tobutal-
ml/min; 70 min, 0%, 0.9 ml/min; 80 min, 85%, 0.9 ml/min; and 90 min, 85%, 0.9 ml/min. RTs were 23 min for EMD 50929, 38 min for EMD 148107, 41 min for EMD 329989, and S2 min for SZ. Structures of SZ metabolites were verified by comparison of the fragmentation spectra and chromatographic retention times of HLM metabolites with those of synthetic reference compounds EMD 148107 (aromatic hydroxylation), EMD 250989 (aliphatic hydroxylation), and EMD 50929 (fluorophenylnicotinic acid). If no synthetic reference was available, metabolites were named M (for metabolite) followed by their nominal masses. Multiple metabolites with the identical nominal masses were also assigned the letter a, b, etc. For the identification of the metabolites by ion trap mass spectrometry, the eluate was split approximately 1:2:1 after passing through the radio detector, and the minor part was diverted to an atmospheric pressure chemical ionization interface for mass spectrometric detection. The mass spectrometry system consisted of an ion trap mass spectrometer (LCQ; Thermo Finigan, Waltham, MA) equipped with an atmospheric pressure chemical ionizer (Thermo Finngan) operating in the positive or negative mode.

**Data Analysis.** 
Enzyme kinetic parameters, $K_m$ and $V_{\text{max}}$ for SZ disappearance and formation of metabolites were calculated by nonlinear regression using WinNonlin (Pharsight, Mountain View, CA) and the normal Michaelis-Menten equation (eq. 1):

$$ V = \frac{V_{\text{max}} \times S}{K_m + S} $$

(1)

where $V$ is reaction rate; $S$ is substrate concentration, and $K_m$ is the Michaelis-Menten constant.

**Calculation of microsomal nonspecific binding.** The free fraction of SZ in microsomal incubations ($f_{\text{mic}}$) was calculated according to eq. 2 (Hallifax and Houston, 2006), using logD (pH 7.4) = 4.13 for SZ. The Hallifax equation has been shown to be superior to the Austin equation (Austin et al., 2002) for compounds with $2.5 \leq \text{logD/Ph} \leq 5$ and to deliver accurate predictions for microsomal protein content in the range of $1 \times 10^{-1}$ mg/ml (Gertz et al., 2008):

$$ f_{\text{mic}} = \frac{1}{1 + \frac{P}{10^2} \logD/H + 0.07 \logD/H - 10^2} $$

(2)

where $P$ is microsomal protein content (milligrams per milliliter).

**Calculation of $CL_{\text{int, mic}}$, $CL_{\text{int, mic}, \text{scaled}}$, $CL_{\text{int, hep}}$.** The intrinsic metabolic clearance, $CL_{\text{int, mic}}$ was estimated as the $V_{\text{mic}}/K_m$ and corrected for microsomal binding ($CL_{\text{int, mic}, \text{scaled}}$ in microliters per minute per milligram) according to eq. 3:

$$ CL_{\text{int, mic}, \text{scaled}} = \frac{CL_{\text{int, mic}}}{f_{\text{mic}}} $$

(3)

The apparent intrinsic clearance of the whole liver ($CL_{\text{int, app}}$ in liters per hour) was calculated by eq. 4 using the respective physiological parameters of humans (see below):

$$ CL_{\text{int, app}} = CL_{\text{int, mic}} \times \frac{\text{mg protein}}{\text{g liver}} \times \frac{1}{\text{g liver}} \times \frac{10^3}{60} $$

(4)

The scaled $CL_{\text{int, app}}$ was used for the estimation of hepatic metabolic clearance according to the well stirred model (eq. 5) (Rowland et al., 1973; Wilkinson and Shand, 1975):

$$ CL_{\text{int, app}} = \frac{Q_h \times f_{\text{ub}} \times CL_{\text{int, app}}}{Q_h + f_{\text{ub}} \times CL_{\text{int, app}}} $$

(5)

where $CL_{\text{int, app}}$ is hepatic clearance (liters per hour); $Q_h$ is hepatic blood flow (liters per hour); $CL_{\text{int, app}}$ is apparent intrinsic clearance of the whole liver (liters per hour), and $f_{\text{ub}}$ is the fraction unbound in blood. $f_{\text{ub}}$ was set to 0.001, based on the conservative estimate of plasma $f_{\text{ub}} = 0.002$ (see below), assuming that SZ is almost exclusively distributed to the plasma volume of whole liver because of its extremely high plasma protein binding.

**Evaluation of hepatocyte culture experiments.** Areas under the concentration versus time curves from 0 h to the last time point measured (AUC$_{\text{max}}$) for the human hepatocyte culture experiments in the absence and presence of the three inhibitors were calculated for SZ and its three metabolites EMD 50929, EMD 148107, and EMD 329989 using Kinetics 4.1.1 software (InnaPhase Corporation, Philadelphia, PA). The ascending part of the curves was calculated using the linear trapezoidal rule and the descending segments were calculated using the log-linear trapezoidal rule. AUC ratios for the human hepatocyte culture experiments were calculated from the individual AUC values of each batch during inhibited and control experiments (mean of $n = 2$) according to eq. 6:

$$ \text{AUC ratio} = \frac{\text{AUC}_{\text{inhibited, max}}}{\text{AUC}_{\text{control, max}}} $$

(6)

**Intrinsic metabolic clearance for the human hepatocyte culture experiments was calculated for SZ using Kinetics 4.1.1 according to eq. 7:**

$$ CL_{\text{int, hep}} = \frac{10^6 \cdot \text{Dose}}{AUC \cdot 60} $$

(7)

where $CL_{\text{int, hep}}$ is intrinsic metabolic clearance (microliters per minute per $10^6$ hepatocytes), dose is the initial amount of drug in the incubation medium (nanomoles per $10^6$ hepatocytes), and AUC is area under the curve (nanomolar concentration $\times$ hour).

Free fraction of SZ in hepatocytes ($f_{\text{ub, hep}}$) was calculated according to eqs. 8 and 9 (Kilford et al., 2008), using logD (pH 7.4) = 4.13 for SZ:

$$ f_{\text{ub, hep}} = \frac{1}{1 + 125 \times \text{logD} + 0.067 \times \text{logD} - 112} $$

(8)

$$ V_h = 0.005 \times \frac{n}{V} $$

(9)

where $V_h$ is the ratio of intracellular volume and incubation volume ($\approx 0.005$ at $10^6$ cells per milliliter), $n$ is number of cells per well ($\times 10^6$), and $V$ is incubation volume per well (milliliters).

Unbound intrinsic metabolic clearance, $CL_{\text{int, hep}}$ in human hepatocyte cultures was calculated by eq. 10:

$$ CL_{\text{int, hep}} = \frac{CL_{\text{int, hep}}}{f_{\text{ub, hep}}} $$

(10)

**Determinations of $IC_{50}$ and $K_{m}$**. $IC_{50}$ was determined by plotting the rate of metabolism formation against SZ concentration. The concentration corresponding to 50% inhibition compared with vehicle control incubations (positive controls) can thus be calculated using eq. 11 and represents the $IC_{50}$:

$$ I = I_b + \frac{I_{\text{max}} \times c^n}{c^n + IC_{50}^{-1}} $$

(11)

where $I$ is the inhibitory effect, $c$ is concentration, $I_b$ is the baseline effect, $I_{\text{max}}$ is the maximal inhibitory effect, $n$ is exponent ($c$), and $IC_{50}$ is the inhibitory concentration causing a half-maximal inhibitory effect.

**Model fitting for inhibition type.** $K_{m}$ values for P450 enzymes were determined based on calculated free SZ concentrations in the respective HLM incubations using SigmaPlot 10.0 (Systat Software, Inc., San Jose, CA) and Enzyme Kinetics 1.3 software. For investigation of the most likely mechanism of inhibition, different curve-fitting models assuming either competitive, uncompetitive, or mixed types of inhibition were used for calculating $K_{m}$, $V_{\text{max}}$, and $K_{i, u}$ values. Sum of square values were also calculated as indicators of goodness of fit and were evaluated to identify the most appropriate model(s) to describe the data obtained.

**Calculation of maximal free concentration of drug at enzyme site.** Using the method described by Ito et al., (1998), the potential of SZ to cause clinical interactions with coadministered substrates of the P450 system was evaluated using eqs. 12 and 13 to estimate the maximum free concentration in the liver. The absorption rate constant was set to the maximal achievable value of 0.1 min$^{-1}$ (Anderson et al., 1994), and the fraction absorbed was set to 1, reflecting complete absorption of SZ (Kovar et al., 2006; Kroesser et al., 2006a).
\[
[I]_{\text{in, max}} = \frac{k \times \text{Dose}}{Q_b} \times f_u
\]

\[
[I]_{\text{in, max, u}} = f_u \times [I]_{\text{in, max}}
\]

where \( k \) is the absorption constant, \( Q_b \) is hepatic blood flow, \( f_u \) is the fraction absorbed, \( f_u \) is the fraction unbound, \([I]_{\text{in, max}}\) is the maximal plasma concentration at steady state, \([I]_{\text{in, max, u}}\) is the maximal plasma concentration at steady state, and \([I]_{\text{in, max, ss}}\) is the maximal intracellular (unbound) inhibitor concentration at steady state.

The fraction unbound in plasma \((f_u)\) was set to 0.002, based on conservative evaluation of in vitro plasma protein binding data of SZ. Hepatic blood flow, \( Q_b \), was assumed to be 1.5 l/min, the amount of microsomes per gram of liver was set at 32 mg/g, and the liver weight of a 70-kg person was taken as 1.8 kg in accordance with standard physiological values (Davies and Morris, 1993; Barter et al., 2007). The value of 0.91 \mu M inserted for \( I_{\text{in, max}} \) was obtained from clinical PK data at steady state after a dose of 5 mg of SZ b.i.d. (Kovar et al., 2006).

The clinical drug/drug interaction potential due to P450 inhibition was evaluated considering the FDA approach of \( \frac{I_d}{K_i} \) (FDA Guidance for Industry: Drug Interaction Studies—Design Study, Data Analysis and Implications for Dosing and Labeling, http://www.fda.gov/cebr/guidelines.htm, 2006) using total plasma concentrations. Alternatively, free portal vein concentrations \((I_{\text{in, max, u}})\) and \( K_{u,v} \) were used to evaluate an unbound ratio \( I_{\text{in, max, u}}/K_{u,v} \).

**Results**

Effect of the Nonenzyme-Specific P450 Inhibitor ABT on SZ Metabolism. ABT inhibited the metabolism of SZ at concentrations between 0.25 and 10 mM by 64 and 87%, respectively. The formation of the six quantifiable SZ metabolites was inhibited between 40 and 86% using 0.25 mM ABT and between 70 and 100% using 10 mM ABT.

**Kinetic Parameters for SZ Clearance and Metabolite Formation in HLM.** Biotransformation of SZ to EMD 329989 and EMD 148107 was found to be linear up to 0.3 mg/ml microsomal protein and 10 min of incubation in HLM. The concentration/time-dependent overall clearance of SZ proceeded with a \( K_m \) of 13.7 ± 4.9 \mu M (i.e., \( K_m, u = 9.3 \mu M \), see below) and a maximal rate \( (V_{\text{max}}) \) of 3280 ± 460 pmol/mg/min. Consequently, \( C_{\text{L,initial}} \) was calculated to be 239 ± 92 \mu M/\mu g protein, which translates to \( C_{\text{L,initial, min}} \) of 351 ± 135 \mu M/\mu g protein. Use of the well stirred liver model and \( f_{H} = 0.001 \) results in a hepatic clearance estimate of \( C_{\text{H,1}} = 0.94 ± 0.36 \text{ l/h} \). The concentration/time-dependent formation of the SZ metabolites EMD 148107 and EMD 329989 was fitted to a standard Michaelis-Menten equation. \( K_m \) and \( V_{\text{max}} \) values for EMD 148107 formation were determined to be 31.1 \mu M and 1016 pmol/mg/min. \( K_m \) for EMD 329989 formation was calculated to be 27 \mu M, with a corresponding \( V_{\text{max}} \) of 705 pmol/mg/min (see Supplemental Fig. 1).

Identification of Major Metabolites of SZ Formed in HLM. After incubation of \([14C]SZ\) at 25 \mu M with HLM in the presence of an NADPH-regenerating system, HPLC radiochromatograms showed a number of peaks in addition to that of SZ (Fig. 1), which are depicted in Table 1. The main metabolic pathways of \([14C]SZ\) in HLM are shown in Fig. 2. In all samples investigated, the identity of a major metabolite, M364a, formed by aromatic monohydroxylation in the chromane moiety, was confirmed by comparison of the mass fragmentation and the chromatographic retention time to be identical to the reference compound EMD 148107. Aliphatic monohydroxylation yielded M364c, which was consistent with the mass fragmentation and the chromatographic retention time of the reference compound EMD 329989. Aliphatic monohydroxylation at another position of the chromane moiety produced M364d. Hydroxylation and subsequent dehydrogenation gave rise to the hydroxycromene derivative M362. Cleavage of \([14C]SZ\) by N-dealkylation produced the unlabeled amine methyl-chromane M163, which was detected by mass spectrometry in all samples investigated. As a complementary metabolite to M163, the radiolabeled nicotinic alcohol M203 was observed. The fluorophenyl nicotinic acid metabolite M217 was confirmed to be identical to the reference compound EMD 50929 by comparing the mass fragmentation spectra and the chromatographic retention times.

Because the fluorophenyl nicotinic acid EMD 50929 was observed as a major metabolite, the unlabeled complementary chromane-2-carboxylic acid also was expected to be detected by mass spectrometry. However, this acid was not observed in any of the samples investigated. In addition, no hydroxy derivatives of this acid were observed.

**P450 Enzyme-Specific Metabolism of SZ in Supersomes.** The percentage of parent compound disappearance relative to that in the respective control incubations showed the metabolism of \([14C]SZ\) (25 \mu M) to be catalyzed by CYP1A2, CYP2A6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4 to at least six quantifiable metabolites over a 60-min incubation time (data not shown). The relative contributions of individual P450 enzymes to SZ metabolism in HLM was calculated using relative activity factors for each P450 enzyme as described previously (Venkataraman et al., 2001) and subsequently were expressed as predicted contributions of the individual enzyme in HLM, given in Table 2. Accordingly, direct formation of the metabolite EMD 50929 appeared to be mediated exclusively by CYP1A2, whereas the formation of the other metabolites showed multiple P450 enzyme involvement. Formation of the metabolite EMD 148107 was mediated by CYP2C9 > CYP3A4 > CYP2C8 > CYP2D6, whereas the formation of EMD 329989 was mediated by CYP1A2 > CYP2C8 > CYP3A4 > CYP2A6 > CYP2C9. No other P450 enzyme contributed more than 2% to the relative activity factor-calculated in vitro HLM metabolism.

**Effect of P450 Enzyme-Specific Inhibitors on the Clearance of SZ in HLM.** With experimental error set at an upper limit of 10%, decreases and increases in activity within the ±10% margin were considered insignificant. The overall metabolism of SZ was significantly inhibited by 3 \mu M ketoconazole (CYP3A4; 40.1%). Some inhibition was observed with 5 \mu M quercetin (CYP2C8; 20.7%) and 30 \mu M furafylline (CYP1A2; 15.3%) (Table 3).

The biotransformation of SZ to EMD 50929 was notably inhibited by ketoconazole (CYP3A4; 70.5%) and quercetin (CYP2C8; 52.4%) although moderate inhibition was observed with diethylidithiocarbamate (CYP2E1; 37.3%), furafylline (CYP1A2; 36.9%), sulfaphenazole (CYP2C9; 21.0%), (+)-N-3-benzyl-nirvanol (CYP2C19; 15.2%), and pilocarpine (CYP2A6; 12.4%). The biotransformation of SZ to the major microsomal metabolite M203 was notably inhibited by ketoconazole (CYP3A4; 64.9%). Some inhibition was observed with furafylline (CYP1A2; 13.1%). The biotransformation of SZ to EMD 50929 was evaluated considering the FDA approach of \( \frac{I_d}{K_i} \) (FDA Guidance for Industry: Drug Interaction Studies—Design Study, Data Analysis and Implications for Dosing and Labeling, http://www.fda.gov/cebr/guidelines.htm, 2006) using total plasma concentrations. Alternatively, free portal vein concentrations \((I_{\text{in, max, u}})\) and \( K_{u,v} \) were used to evaluate an unbound ratio \( I_{\text{in, max, u}}/K_{u,v} \).
Sulfaphenazole (CYP2C9; 25.6%). Some inhibition was observed with pilocarpine (CYP2A6; 11.7%). The biotransformation of SZ to EMD 329989 was considerably inhibited by furafylline (CYP1A2; 59.7%). Some inhibition was also observed with quercetin (CYP2C8; 19.6%) and diethyldithiocarbamate (CYP2E1; 16.1%). The biotransformation of SZ to M364d was moderately inhibited by ketoconazole (CYP3A4; 33.8%) and quercetin (CYP2C8; 30.0%). The biotransformation of SZ to M362 was completely inhibited by ketoconazole (CYP3A4; 112%), resulting in metabolite levels as low as those found in the corresponding controls in the absence of cofactor mix. Some inhibition was observed with furafylline (CYP1A2; 23.2%) and pilocarpine (CYP2A6; 11.5%). Quinidine had a significant apparent activation effect on the formation of M362 (41.4%).

Effect of P450 Enzyme-Specific Inhibitors on the Clearance of SZ and Formation of Metabolites in Fresh Human Hepatocyte Cultures. Using P450-selective chemical inhibitors, changes in SZ metabolism were investigated in human hepatocyte cultures. Concentration-time profiles were plotted for the clearance of SZ and the formation of metabolites EMD 148107, EMD 329989, and EMD 50929 in human hepatocyte cultures, as shown in Figs. 3 to 6. AUC ratios were calculated to illustrate and compare the effects of the different enzyme-specific inhibitors on the extent of SZ clearance and metabolite formation (Fig. 7), and unbound intrinsic clearance of SZ was calculated for all experiments using $f_{uhep}/H11005_{0.53}$ (Table 4). The clearance of SZ in human hepatocyte cultures appeared to be relatively unaffected by the presence of enzyme-specific P450 inhibitors (Table 4; Fig. 3). More prominent effects of P450 inhibition were observed with the AUCs of the metabolites. EMD 148107 AUCs increased significantly in the presence of furafylline (CYP1A2 inhibitor) and benzylnirvanol (CYP2C19 inhibitor) (Figs. 4 and 7). Inhibition of CYP3A4 by ketoconazole resulted in a significant increase of EMD 329989 AUC and a decrease of EMD 50929 AUC (Figs. 5–7).

**Determination of IC_{50}/K_{i,u} Values of SZ and Its Metabolites Towards P450 Enzyme.** At 300 μM, SZ inhibited tolbutamide-4-hydroxylase by 35%, suggesting that inhibition of CYP2C9 is not of

<table>
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<th>Metabolite</th>
<th>Putative Structural Formula</th>
<th>RT (min)</th>
<th>Comments</th>
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<td>Hydroxylation position to be confirmed by synthesis</td>
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<td>Hydroxylation position to be confirmed by synthesis</td>
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<tr>
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<td>41</td>
<td>Structure proven</td>
</tr>
<tr>
<td>M364a (EMD 148107)</td>
<td><img src="image" alt="M364a Structure" /></td>
<td>39</td>
<td>Structure proven</td>
</tr>
<tr>
<td>M203</td>
<td><img src="image" alt="M203 Structure" /></td>
<td>32</td>
<td>Nicotinic alcohol: to be confirmed by NMR</td>
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<tr>
<td>M217 (EMD 50929)</td>
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<td>Nicotinic acid structure confirmed by synthesis</td>
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<tr>
<td>M163</td>
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<td>14</td>
<td>Aminomethyl chromane be confirmed by NMR</td>
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</table>
clinical relevance; thus, IC₅₀/Kᵢ,ᵤ for this enzyme was not investigated further. Kᵢ,ᵤ analyses were performed for SZ-mediated inhibition of CYP1A2, CYP2C8, CYP2C9, CYP2D6, and CYP3A4, showing a relatively moderate in vitro inhibition potential for most enzymes investigated with the exception of CYP2D6 (Kᵢ,ᵤ of 0.43 μM) (Table 5; see Supplemental Fig. 2). The metabolites of SZ, EMD 50929, EMD 148107, and EMD 329989 were evaluated for inhibitory effects on the most important drug-metabolizing P450s, namely CYP1A2, CYP2C9, CYP2C19, and CYP3A4 in HLM (see Supplemental Fig. 3). The IC₅₀ values (Table 6) indicate that the metabolites exhibit only minor or negligible inhibitory effects at clinically relevant concentrations.

Estimation of the Maximal Intrahepatic Concentration of SZ after Oral Dosing. According to the method of Ito et al. (1998) (eq. 12), 0.91 μM is the maximally achievable portal vein concentration of SZ after a dose of 5 mg b.i.d., which is considered robust and unlikely to under-represent clinically achievable hepatic concentrations of SZ. With consideration of SZ plasma protein binding (fᵤ = 0.002), [I]ₘₚ,ᵤ was calculated to be 0.0018 μM. Because permeability of SZ is known to be high (Pₐₚ = 110 nm/s), the intracellular hepatic concentration is considered to equal the free plasma concentration calculated above. These data suggest that only extremely low concentrations of free SZ are able to enter interactions at the enzyme site despite its rapid and virtually complete systemic absorption (Kovar et al., 2006; Kroesser et al., 2006a).

The in vivo P450 inhibition potential was estimated based on the simplified ratio of [I]/Kᵢ as well as use of the sophisticated unbound ratio [I]ₘₚ,ᵤ/Kᵢ,ᵤ (Table 7). Accordingly, only CYP2D6 showed an [I]/Kᵢ,ᵤ ratio >0.10, indicating a possible risk of DDI by P450 inhibition. However, evaluation of the risk by the unbound ratio [I]ₘₚ,ᵤ/Kᵢ,ᵤ revealed values <0.01 for all P450s investigated. In vitro Kᵢ estimates for the inhibition of CYP2D6 by the major SZ metabolites were conservatively derived as IC₅₀/2 to be 6.0, 13.7, and >50 μM for EMD 148107, EMD 329989, and EMD 50929, respectively. After dosing SZ at 5 mg b.i.d., the plasma levels of the metabolites were 0.014, 0.05, and 0.2 μM, respectively. Calculated [I]/Kᵢ ratios of 0.002, 0.004, and <0.004, respectively, indicated low risk for DDI by these metabolites.

Discussion

Failure to generate metabolites when SZ was incubated with HLM without NADPH and when incubations were performed in the presence of a general unspecific inhibitor of cytochromes P450, ABT, indicated that the metabolism is mainly catalyzed by P450s. The liver metabolic clearance predicted from in vitro HLM experiments (CL₁₄ =

<table>
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<th>P450 Enzymes</th>
<th>Predicted Contribution</th>
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<tbody>
<tr>
<td></td>
<td>%</td>
</tr>
<tr>
<td>CYP1A2</td>
<td>13 100 5 1 34 6 0</td>
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<td>CYP2A6</td>
<td>1 0 27 0 11 14 0</td>
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</tr>
<tr>
<td>CYP2C19</td>
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</tr>
<tr>
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</tr>
<tr>
<td>CYP2E1</td>
<td>0 0 2 0 1 0 0</td>
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<tr>
<td>CYP3A4</td>
<td>41 0 40 18 15 40 0</td>
</tr>
<tr>
<td>Total</td>
<td>100 100 100 100 100 100 0</td>
</tr>
</tbody>
</table>

RAF, relative activity factor.

Table 2: Predicted contribution of individual P450 enzyme to formation of each metabolite based on Supersomes data and RAF values.
(at 5 mg b.i.d.), the phase I metabolism of SZ is expected to follow
Thus, SZ is considered a low-clearance drug. With a being consistent with that in other articles (Ito and Houston, 2005).
CYP2C8 (•), CYP2C8/9 (○) inhibitors.
CYP2C9 (×) and with (a) CYP1A2 (•), CYP3A4 (○), and CYP3A4 (+) inhibitors.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>SZ</th>
<th>EMD 148107</th>
<th>EMD 32989</th>
<th>EMD 50929</th>
<th>M203</th>
<th>M364d</th>
<th>M362</th>
</tr>
</thead>
<tbody>
<tr>
<td>Furfuryline(^a)</td>
<td>15.3 ± 0.6</td>
<td>7.1 ± 4.4</td>
<td>59.7 ± 6.5</td>
<td>36.9 ± 9.0</td>
<td>13.1 ± 1.9</td>
<td>-0.4 ± 4.8</td>
<td>23.2 ± 16.7</td>
</tr>
<tr>
<td>Pilocarpine</td>
<td>8.5 ± 2.9</td>
<td>11.7 ± 6.1</td>
<td>-13.2 ± 9.1</td>
<td>12.4 ± 10.0</td>
<td>4.3 ± 8.7</td>
<td>-6.2 ± 6.6</td>
<td>11.5 ± 27.9</td>
</tr>
<tr>
<td>Quercetin</td>
<td>20.7 ± 3.3</td>
<td>8.8 ± 3.3</td>
<td>19.6 ± 2.9</td>
<td>52.4 ± 12.9</td>
<td>5.3 ± 4.6</td>
<td>30.0 ± 4.5</td>
<td>-10.3 ± 35.0</td>
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<td>S-Mephenytoin</td>
<td>4.4 ± 7.9</td>
<td>25.6 ± 5.5</td>
<td>-4.6 ± 15.8</td>
<td>21 ± 15.7</td>
<td>-8.2 ± 4</td>
<td>-19.2 ± 6.9</td>
<td>-17.2 ± 12.4</td>
</tr>
<tr>
<td>(+)-3-Benzylnirvanol</td>
<td>-1.0 ± 3.8</td>
<td>-5.5 ± 4.4</td>
<td>-5.0 ± 4.4</td>
<td>-5.7 ± 2.9</td>
<td>-5.7 ± 6.9</td>
<td>-15.4 ± 1.9</td>
<td>-20.7 ± 12.4</td>
</tr>
<tr>
<td>Quinidine</td>
<td>0.6 ± 8.7</td>
<td>-9.2 ± 6.1</td>
<td>-6.8 ± 9.5</td>
<td>7.6 ± 6.6</td>
<td>-7.4 ± 18.5</td>
<td>-7.5 ± 2.5</td>
<td>-41.4 ± 15.0</td>
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<tr>
<td>Diethyldithiocarbamate(^a)</td>
<td>3.5 ± 1.9</td>
<td>-4.7 ± 2.9</td>
<td>16.1 ± 10.5</td>
<td>37.3 ± 9.5</td>
<td>-10.0 ± 7.0</td>
<td>-11.9 ± 8.2</td>
<td>10.1 ± 1.7</td>
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<tr>
<td>Ketoconazole</td>
<td>40.1 ± 4.3</td>
<td>4.0 ± 3.2</td>
<td>-28.8 ± 3.6</td>
<td>70.5 ± 7.2</td>
<td>64.9 ± 2.8</td>
<td>33.8 ± 13.1</td>
<td>112 ± 11</td>
</tr>
</tbody>
</table>

\(^a\) A 15 min preincubation before addition of substrate.

0.94 ± 0.36 l/h) is similar to the reported in vivo clearance (CL/F) of 3.6 to 4.4 l/h (Kroesser et al., 2006a), the range of underprediction being consistent with that in other articles (Ito and Houston, 2005). Thus, SZ is considered a low-clearance drug. With a \(K_{m, S}\) of 9 \(\mu\)M and an estimated maximum free hepatic concentration of 0.0018 \(\mu\)M (at 5 mg b.i.d.), the phase I metabolism of SZ is expected to follow first-order kinetics in vivo. Dose proportionality of \(C_{\text{max}}\) and AUC has, in fact, been observed during a clinical study after single administration of 0.5 to 5 mg of SZ (Kroesser et al., 2006a; Tillner et al., 2006).

SZ is metabolized in HLM via aromatic and aliphatic monohydroxylation at the chrome moiety to form EMD 148107, EMD 32989, and M364d. Subsequent dehydrogenation of EMD 32989 gives rise to M362, whereas M203, EMD 50929, and M163 are produced via \(N\)-dealkylation (Table 1; Fig. 2). The hydroxylated metabolites EMD 148107 and EMD 32989 are not end products of SZ metabolism, because they eventually disappear from hepatocyte cultures (Figs. 4 and 5). Therefore, the highly abundant \(N\)-dealkylated metabolites M203 and EMD 50929 (Figs. 2 and 6) are probably formed not only directly from SZ but also from the chromane-hydroxylated metabolites (Scheme 1).

The extent of SZ clearance through the different possible routes of metabolism may be roughly estimated from the ratio of the individual hydroxylated metabolites to SZ after an incubation period of 1 h (Table 8). This estimate suggests that all three biotransformation routes, i.e., through EMD 148107, M364d, and EMD 32989-M362, are of approximately similar importance in HLM and in fresh human hepatocyte cultures in addition to a possible direct route from SZ to M203 (Scheme 1). By scaling of activities of Supersomes, the metabolism of SZ is considered to be catalyzed primarily by CYP3A4 (41%), CYP2C9 (23%), CYP2C8 (19%), and CYP1A2 (13%) (Table 2). Use of enzyme-specific inhibitors in HLM and hepatocyte incubations also indicated the involvement of these major P450s (Figs. 3–6; Tables 4 and 8).

**TABLE 3**

Effect of P450 enzyme-specific inhibitors on SZ metabolism in HLM

Data are means ± S.D.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>EMD 148107</th>
<th>EMD 32989</th>
<th>EMD 50929</th>
<th>M203</th>
<th>M364d</th>
<th>M362</th>
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<tbody>
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<td>-0.4 ± 4.8</td>
</tr>
<tr>
<td>Pilocarpine</td>
<td>8.5 ± 2.9</td>
<td>11.7 ± 6.1</td>
<td>-13.2 ± 9.1</td>
<td>12.4 ± 10.0</td>
<td>4.3 ± 8.7</td>
<td>-6.2 ± 6.6</td>
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<tr>
<td>Quercetin</td>
<td>20.7 ± 3.3</td>
<td>8.8 ± 3.3</td>
<td>19.6 ± 2.9</td>
<td>52.4 ± 12.9</td>
<td>5.3 ± 4.6</td>
<td>30.0 ± 4.5</td>
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<tr>
<td>S-Mephenytoin</td>
<td>4.4 ± 7.9</td>
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<td>21 ± 15.7</td>
<td>-8.2 ± 4</td>
<td>-19.2 ± 6.9</td>
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<tr>
<td>(+)-3-Benzylnirvanol</td>
<td>-1.0 ± 3.8</td>
<td>-5.5 ± 4.4</td>
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<td>-5.7 ± 2.9</td>
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<td>-15.4 ± 1.9</td>
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<tr>
<td>Quinidine</td>
<td>0.6 ± 8.7</td>
<td>-9.2 ± 6.1</td>
<td>-6.8 ± 9.5</td>
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<td>Diethyldithiocarbamate(^a)</td>
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<td>-4.7 ± 2.9</td>
<td>16.1 ± 10.5</td>
<td>37.3 ± 9.5</td>
<td>-10.0 ± 7.0</td>
<td>-11.9 ± 8.2</td>
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<tr>
<td>Ketoconazole</td>
<td>40.1 ± 4.3</td>
<td>4.0 ± 3.2</td>
<td>-28.8 ± 3.6</td>
<td>70.5 ± 7.2</td>
<td>64.9 ± 2.8</td>
<td>33.8 ± 13.1</td>
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\(^a\) A 15 min preincubation before addition of substrate.

**FIG. 3.** Concentration profile of SZ in human hepatocytes during incubation without inhibitors (○) and with (a) CYP1A2 (•) and CYP3A4 (△) inhibitors and (b) CYP2C8 (○), CYP2C9 (×), CYP2C8/9 (◊), and CYP2C19 (+) inhibitors.

**FIG. 4.** Concentration profile of EMD 148107 during incubation of SZ with human hepatocytes without inhibitors (○) and with (a) CYP1A2 (○) and CYP3A4 (△) inhibitors and (b) CYP2C8 (○), CYP2C9 (×), CYP2C8/9 (◊), and CYP2C19 (+) inhibitors.
the hepatocyte culture is considered closer to the in vivo situation, with hepatocytes. The reason for this finding is unknown, but because
hepatocytes without inhibitors (\(\bullet\)) and with (a) CYP1A2 (\(\triangle\)) and CYP3A4 (\(\Delta\)), CYP2C8 (\(\times\)), and CYP2C19 (\(+\)) inhibitors.

**FIG. 5.** Concentration profile of EMD 329989 during incubation of SZ with human hepatocytes without inhibitors (\(\bullet\)) and with (a) CYP1A2 (\(\triangle\)) and CYP3A4 (\(\Delta\)), CYP2C8 (\(\times\)), and CYP2C19 (\(+\)) inhibitors.

Inhibition of SZ clearance was more pronounced with HLM than in vitro studies, with EMD 50929 as the ultimate and most abundant metabolite. In vivo, the metabolites identified in vitro were also observed in vivo and strongly supported the biotransformation routes proposed from in vitro studies, where ketoconazole resulted in reduced AUC of parent compound, respectively (Kroesser et al., 2006a). Similar to those results are regarded as more predictive for the in vivo situation. As a consequence of the presence of multiple and high-capacity phase I avenues of SZ metabolism (Scheme 1), no single P450 enzyme-specific inhibitor appears to be able to completely ablate overall clearance, as metabolism is redirected along alternative, uninhibited routes of biotransformation. This finding was supported by the in vivo observation that erythromycin, a CYP3A inhibitor, had no effect on formation of EMD 50929 (Fig. 6a), erythromycin caused a 20% decrease in the formation of this metabolite in vivo (Kovar et al., 2006).

TABLE 4

<table>
<thead>
<tr>
<th>Chemical Inhibitor (P450 Enzyme Inhibited)</th>
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<th>Inhibition</th>
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</thead>
<tbody>
<tr>
<td>None (control)</td>
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<tr>
<td>Furafylline (CYP1A2)</td>
<td>11.7 ± 2.4</td>
<td>3</td>
</tr>
<tr>
<td>Ketoconazole (CYP3A4)</td>
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</tr>
<tr>
<td>Trimethoprim (CYP2C8)</td>
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<tr>
<td>Benzylnirvanol (CYP2C19)</td>
<td>12.7 ± 4.5</td>
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<tr>
<td>Sulfaphenazole (CYP2C9)</td>
<td>16.2 ± 6.3</td>
<td>5</td>
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<tr>
<td>Trimethoprim/sulfaphenazole (CYP2C8/9)</td>
<td>12.7 ± 4.5</td>
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<tr>
<td>Quinidine (CYP2D6)</td>
<td>14.8 ± 3.0</td>
<td>5</td>
</tr>
</tbody>
</table>

*This combination was chosen because trimethoprim has been suspected to simultaneously activate CYP2C9.

Metabolites identified in vitro were also observed in vivo and strongly supported the biotransformation routes proposed from in vitro studies, with EMD 50929 as the ultimate and most abundant metabolite. In vivo, the N-dealkylation metabolite EMD 50929 amounted to 63% of the parent drug AUC, whereas the hydroxylated metabolites EMD 148107 and EMD 329989 amounted to 3 and 13% of parent compound, respectively (Kroesser et al., 2006a). Similar to that in vivo experiments, where ketoconazole resulted in reduced formation of EMD 50929 (Fig. 6a), erythromycin caused a 20% decrease in the formation of this metabolite in vivo (Kovar et al., 2006).

Supersomes and HLM inhibition experiments revealed that two SZ metabolites are generated by a single individual P450 enzyme each: EMD 329989 was mostly formed by CYP1A2, and M203 was generated predominantly by CYP3A4 (Scheme 1). Accordingly, the inhibition of CYP1A2 in human hepatocyte cultures caused the AUC of EMD 148107 to substantially increase at the expense of the AUC of EMD 329989 (Fig. 7), indicating redirection of SZ metabolism from the CYP1A2-catalyzed EMD 329989 route to the EMD 148107 pathway (Scheme 1). Likewise, inhibition of CYP3A4 in human hepatocyte cultures resulted in a significant increase of EMD 329989
and some increase of EMD 148107 concentrations, most probably due to redirection from the blocked M364d-M203-EMD 50929 pathway (Figs. 4, 5, and 7). Consequently, the biotransformation end product EMD 50929 common to both blocked pathways was produced to a significantly lesser extent in this experiment (Fig. 6).

The clear effect of CYP2C19 inhibition on EMD 148107 concentration in human hepatocyte culture deserves particular comment (Fig. 4b). In HLM experiments, the two CYP2C19 inhibitors, N-benzyl nirvanol and S-mephentoin had apparently no effect on the formation of any of the metabolites investigated (Table 3). Moreover, in Supersome experiments, hardly any contribution of CYP2C19 was predicted for HLM (data not shown). Therefore, the significant increase in the EMD 148107 AUC in the human hepatocyte experiment is probably not due to its increased formation but rather to its inhibited biotransformation, suggesting that CYP2C19 is involved in the metabolism of this compound. EMD 50929 kinetics in hepatocytes appeared to be virtually unaffected (Fig. 7), whereas EMD 329989 showed a slightly reduced AUC (Fig. 7), which was probably due to the 25% inhibition of CYP1A2 evoked by the presence of N-benzylnirvanol.

In Supersome experiments, the formation of EMD 50929 appeared to be exclusively mediated by CYP1A2 (data not shown). In HLM and human hepatocyte cultures, however, noticeable inhibition of its formation was observed not only in the presence of furafylline (CYP1A2) but also when ketoconazole (CYP3A4) and quercetin (CYP2C8; in HLM) were present. This result clearly indicates that EMD 50929 is formed via several metabolic pathways requiring the involvement of different P450 enzymes. Formation may proceed either directly from the parent drug or via the hydroxylation pathways, which are partly catalyzed by CYP450s other than CYP1A2. Which of these pathways (hatched arrows in Scheme 1) are actually followed in the systems is still unknown.

Phase II reactions of SZ and its metabolites in human hepatocytes were not investigated during this study. However, the similarity of metabolic profiles in HLM and human hepatocyte cultures suggests that significant differences in the initial metabolic steps are not to be expected in the presence of phase II enzymes. However, considerable formation of phase II metabolites at the end of the metabolic cascade cannot be discounted, because formation of the phase I end product EMD 50929 accounts for only approximately one-third of the SZ breakdown occurring during a 6-h incubation in human hepatocyte cultures (Figs. 3 and 6).

Inhibition of six major P450s by SZ and its metabolites has been investigated during the course of this study. Table 7 summarizes [I]/Ki,u values for SZ based on the measured maximal plasma concentration at steady state [I] as well as on calculations of the maximal free inhibitor concentrations at the enzyme site ([I]max,u). Although simple [I]/Ki,u calculations suggest at least one enzyme to be at a moderate risk of drug-drug interactions (i.e., CYP2D6), a more precise definition of [I]i places all P450 enzymes in the moderate- to low-risk category. This differential evaluation is largely brought about by the high degree of plasma protein binding and hence low availability of free drug to partake in drug interactions. Such calculations are not sufficient for the determination of interaction risk when circulating metabolites are concerned. Therefore, simple [I]/Ki estimates for EMD 148107, EMD 329989, and EMD 50929 were performed and suggest that none of the metabolites pose a risk of causing a DDI, even at an SZ dose 5 times higher than the dose used in the phase III studies.
The results demonstrate that the risk of DDIs by SZ or metabolites is low but cannot be completely excluded, especially in the case of CYP2D6. Therefore, a clinical study was conducted, which confirmed the absence of PK interactions with CYP1A2, CYP2C9, CYP2C19, CYP3A4, and CYP2D6, and CYP3A4 substrates in vivo (Kroessler et al., 2006b). Combining SZ in vitro metabolism data from recombinant enzymes, liver microsomes, and hepatocytes has led to the successful prediction of human in vivo drug clearance and identification of major enzymes involved in the metabolism of the drug, and the risk for P450 inhibition-based DDIs. In conclusion, this work demonstrates the high value of using a panel of in vitro assays during drug development to predict potential DDIs in support of phase I clinical studies.

Acknowledgments. We express our gratitude to C. Dyde, K. Janus, T. Scheller, and T. Schirmer for excellent technical assistance, data processing, and the preparation of the laboratory reports. In addition, we gratefully acknowledged the excellent work of M. Millet and F. Brée (Biopredic, Parc d’affaires de la Bretêche, F-35760 Saint Grégoire) for hepatocyte incubations. Finally, we thank Dr. C. Masimirembwa for reviewing this article.

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