IDENTIFICATION OF CYTOCHROME P450 AND ARYLAMINE N-ACETYLTRANSFERASE ISOFORMS INVOLVED IN SULFADIAZINE METABOLISM

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ABSTRACT:
Sulfadiazine hydroxylamine has been postulated to be the mediator of the greatly increased rates of adverse reactions to sulfadiazine experienced by people with human immunodeficiency virus infection. Therefore, we investigated the involvement of cytochrome P450 isoforms in sulfadiazine metabolism. Formation of both the hydroxylamine and 4-hydroxy sulfadiazine was NADPH-dependent in human liver microsomes (HLM). The average K_m (±S.D.) and V_max in HLM (n = 3) for hydroxylamine formation was 5.7 ± 2.2 mM and 185 ± 142 pmol/min/mg, respectively. Significant (p < 0.05) inhibition by selective P450 isoform inhibitor sulfaphenazole (2.1 µM; CYP2C9) indicated a role for CYP2C9 in the formation of the hydroxylamine. Hydroxylamine formation correlated strongly with tolbutamide 4-hydroxylation (CYP2C8/9) in HLM (r = 0.76, p < 0.004, n = 12). Fluconazole (CYP2C9/19 and CYP3A4 inhibitor at clinical concentrations) inhibited hydroxylamine formation, with one-enzyme model K_i estimates ranging from 9 to 40 µM. Acetylation of sulfadiazine in human liver cytosol (HLC) correlated strongly with NAT2 activity as measured by sulfamethazine N-acetylation (r = 0.92, p < 0.001, n = 12). The average K_m (±S.D.) and V_max in HLC (n = 3) was 3.1 ± 1.7 mM and 221.8 ± 132.3 pmol/min/mg, respectively. The polymorphic acetylation of sulfadiazine may predispose slow acetylator patients to adverse reactions. Therefore, we predict that fluconazole may prove useful in the clinic as an in vivo inhibitor of sulfadiazine hydroxylamine formation to suppress adverse reactions to this drug.

Sulfadiazine has an important role in acute therapy for Toxoplasmosis gondii encephalitis, the most common opportunistic infection of the brain experienced by AIDS patients. Sulfadiazine is an arylamine antibiotic associated with an extraordinarily high rate of adverse reactions, ~40% in AIDS patients (Haverkos, 1987; Leport et al., 1988). Typical doses for toxoplastic encephalitis in AIDS patients are high and range up to 8 g administered daily in four divided doses. Some of the adverse effects of arylamines are thought to be caused by the formation of the hydroxylamine metabolite that is further oxidized to the highly electrophilic nitroso metabolite, which covalently binds to cellular macromolecules, resulting in adverse reactions (Shear and Spielberg, 1985; Rieder et al., 1988). Although sulfadiazine has been marketed since the 1940s, very little is known about cytochrome P450 isoform involvement in hydroxylamine formation in humans or the arylamine N-acetyltransferase (NAT) detoxification pathway. The only oxidative metabolite previously reported in humans was 4-hydroxy sulfadiazine, which comprised 12% of the dose in a slow acetylator (Vree et al., 1995). The 5-hydroxy sulfadiazine metabolite has been identified in monkeys (Vree et al., 1995), and the 4-hydroxy, 5-hydroxy, and dihydroxy sulfadiazine metabolites have been identified in various animal species (Atef and Nielsen, 1975; Nieuws et al., 1987, 1988; Vree et al., 1991a,b). Leone et al. (1987) reported that 30 to 60% of a dose of sulfadiazine is eliminated as the parent, and 20 to 40% is eliminated as the acetylated metabolite in humans. Sulfadiazine plasma C_max concentrations following a single 2-g oral dose of sulfadiazine are predicted to be 300 µg/ml or 1.2 mM (Vree et al., 1995).

Human NAT is encoded at two different loci. The enzyme encoded at one locus has a wide tissue distribution, is responsible for acetylation of p-aminobenzoic acid (PABA), and is termed NAT1. The second locus encodes an enzyme, which has a more restrictive tissue distribution with higher levels of expression in the liver and red blood cells, is responsible for the acetylation of sulfamethazine (SMZ), and is termed NAT2. NAT2 is associated with the classic form of human NAT polymorphism. Both NAT1 and NAT2 genes have allelic variation (Butcher et al., 2002). Slow acetylator status has been associated with increased rates of adverse reactions to arylamine antibiotics, such as sulfamethoxazole (Wolkenstein et al., 1995). Suppression of hydroxylamine formation through the use of metabolic inhibitors has been explored as a therapeutic strategy to decrease the high rate of adverse reactions to the arylamines dapsone (Mitra et al., 1995, Winter et al., 2004a) and sulfamethoxazole (Mitra et al., 1996, Winter et al., 2004b) in people with AIDS.

The major aim of this study was to identify the cytochrome P450 isoforms involved in sulfadiazine hydroxylation to identify potentially clinically useful in vivo inhibitors of this pathway. A secondary aim

ABBREVIATIONS: NAT, arylamine N-acetyl transferase; PABA, p-aminobenzoic acid; SMZ, sulfamethazine; HPLC, high performance liquid chromatography; EC, electrochemical; HLM, human liver microsomes; HLC, human liver cytosol; SDZ, sulfadiazine; SDZ-HA, N-hydroxy sulfadiazine.
Materials and Methods

Chemicals. 4-Hydroxy sulfadiazine, 5-hydroxy sulfadiazine, N-hydroxy sulfadiazine (the hydroxylamine), and N-acetyl sulfadiazine were obtained from the National Institute of Allergy and Infectious Diseases AIDS Research and Reference Reagent Program (Bethesda, MD). Sulfadiazine was purchased from Sigma-Aldrich (St. Louis, MO). N-Acetyl sulfamethazine was a gift from Dr. Edith Sim, University of Oxford (Oxford, UK), and N-acetyl \( p \)-aminobenzoic acid was obtained from Aldrich Chemical (Milwaukee, WI). All other chemicals were of analytical grade and were obtained commercially.

Human Liver Samples. Livers were procured, processed, and stored as previously described (Rettie et al., 1989). Microsomes (HL123, -126, -135, -141, and -142) were prepared as previously described (Hickman et al., 1998). Reactions were preincubated for 5 min; then 1 mM NADPH was added, and the reaction was terminated after 5 min; the incubation mix did not exceed 1% (v/v). Incubations for mechanism-based inhibitors, such as troleandomycin and diethyldithiocarbamate, were carried out as described by Hickman et al. (1998). Reactions were preincubated for 5 min; then 1 mM NADPH was added, and the reaction was terminated after 5 min to 10 min with 25 \( \mu \)M of 2 N HCl or 1.5% perchloric acid. Samples were vortexed under an atmosphere of argon, left on ice for 10 min, and centrifuged at 20,000 \( \times \) g for 10 min; then the supernatant was injected into preassembled argon-purged, gas-tight amber autosampler vials.

Stock Preparation. Sulfadiazine hydroxyamine was dissolved in argon-purged dimethyl sulfoxide in gas-tight amber autosampler vials into which freshly prepared aqueous 10 mM ascorbic acid (10% v/v) was injected. The head-space of the gas-tight vials was purged with argon, and the vials were kept on ice or stored at \(-70°C\). All dilutions were made in freshly prepared 10 mM ascorbic acid using gas-tight syringes and liquid-to-liquid transfer techniques. All other stocks were prepared as equimolar sodium salts and freshly diluted in buffer, pH 7.4 at 37°C.

Cytochrome P450 Assays. HPLC Assay. The analytical system consisted of a Coulchem II electrochemical detector (EC) (\( G = 200 \) mV, \( E_I = 100 \) mV, \( E_2 = -200 \) mV) coupled to a downstream Shimadzu SPD-6A UV detector (\( \lambda = 266 \) nm; Shimadzu, Kyoto, Japan). Optimal Coulchem performance required a Shimadzu LC600 dual piston pump coupled to an Alltech Free Flow Pulse Dispenser (Alltech Associates, Deerfield, IL) and a Spectra-Physics SP8875 autosampler (Spectra-Physics, Mountain View, CA) using a Rhoedine injector valve. An Ultrasphere C18 reverse phase column (5-\( \mu \), \( 250 \times 4.6 \) mm; Beckman Coulter, Inc., Fullerton, CA) was used with a mobile phase consisting of 5:0.05:95 (v/v/v) acetonitrile/triethylamine/50 mM sodium citrate, 1 mM EDTA, pH 2.21, at a flow rate of 1 ml/min. EC detection was used for the hydroxylamine, and UV detection was used for all other metabolites of interest.

Enzyme Assays. The 250-\( \mu \)l microsomal incubation matrix consisted of 0.1 to 2 mg/ml microsomal protein, 1 mM EDTA, and 1 mM glutathione in 50 mM HEPES buffer, pH 7.4 at 37°C. All inhibitors were prepared as aqueous solutions unless specified. The final concentration of organic solvent in the incubation mix did not exceed 1% (v/v). Incubations for mechanism-based inhibitors, such as troleandomycin and diethylthiocarbamate, were carried out as described by Hickman et al. (1998). Reactions were preincubated for 5 min; then 1 mM NADPH was added, and the reaction was terminated after 5 min to 10 min with 25 \( \mu \)l of 2 N HCl or 1.5% perchloric acid. Samples were vortexed under an atmosphere of argon, left on ice for 10 min, and centrifuged at 20,000 \( \times \)g at 4°C for 10 min; then the supernatant was injected into preassembled argon-purged, gas-tight amber autosampler vials. Samples were injected onto the HPLC within 3 h of the incubation.
Arylamine N-Acetyltransferase Assays. HPLC Assays. The analytical system described above was used for the analysis of N-acetylated sulfadiazine with UV detection at 266 nm. The mobile phase consisted of 10:0.05:90 (v/v) acetonitrile/triethylamine/acetate acid at a flow rate of 1 mL/min.

Enzyme Assays. Assays were conducted by the method reported by Grant et al. (1991). Acetyl coenzyme A concentrations were fixed at 100 μM. Incubations contained 40 μl of liver cytosol diluted to the appropriate concentration with 250 mM sucrose, 20 μM of acetyl-CoA/carnitine/carnitine acetyl transferase cofactor regenerating system dissolved in 225 mM triethanolamine-HCl, 4.5 mM thio-dithiothreitol, pH 7.5, 20 μl of acetyl coenzyme A (450 μM in water), and varying amounts of sulfadiazine, sulfamethazine, or para-aminobenzoic acid diluted as its sodium salt in 20 mM Tris buffer to start reactions. Reactions were terminated with 10 μl of 15% perchloric acid and 50 μl injected onto the HPLC.

Data Analysis. Apparent IC₅₀ (IC₅₀_app) and residual effect model IC₅₀ (IC₅₀_res) estimates were obtained from probit plots and nonlinear regression estimation using the equation below:

\[
\text{% Control activity} = \frac{E_{\text{max}} - (E_{\text{max}} - E_0) \times \left( \frac{I}{I + IC_{50\text{, app}}} \right)}{E_{\text{max}}} \tag{1}
\]

where \(E_{\text{max}}\) was the maximal rate of hydroxylamine production in the absence of inhibitor, \(E_0\) was the uninhibitable hydroxylamine production rate, and \(I\) was the inhibitor concentration. To determine IC₅₀_app, \(E_0\) was assumed to be zero. Initial estimates of \(K_i\) (inhibitor binding affinity for the enzyme) were obtained from Dixon plots, and the mechanism of inhibition was confirmed by Lineweaver-Burk transformation. Nonlinear regression estimation of IC₅₀ and \(K_i\) for one-enzyme models of inhibition was performed using PCnonlin (Scientific Consulting, Apex, NC) and reported as the parameter ± S.E.M.

Nonlinear regression estimation of parameters for two-enzyme models where only one enzyme was competitively (2) or noncompetitively (3) inhibited by fluconazole were also performed using the equations below:

\[
V = \frac{S \times V_{\text{max1}}}{S + K_{\text{m1}} \times (1 + I/K_1)} + \frac{S \times V_{\text{max2}}}{S + K_{\text{m2}}} \tag{2}
\]

\[
V = \frac{S \times V_{\text{max1}}}{S \times (1 + I/K_1) + K_{\text{m1}} \times (1 + I/K_1)} + \frac{S \times V_{\text{max2}}}{S + K_{\text{m2}}} \tag{3}
\]

where \(V\) was the rate of hydroxylamine formation; \(V_{\text{max}(n)}\), the maximal rate of hydroxylamine formation for each enzyme (n) and \(K_{\text{m}(n)}\), a measure of substrate affinity and the substrate concentration (S) that produces \(V_{\text{max}(n)}/2\).

All statistical data are reported as the mean ± S.D. of triplicate determinations. The unpaired t test was used to detect significant differences (\(p \leq 0.05\)).

The Pearson correlation coefficient (\(r\)) and the Spearman rank correlation coefficient (\(r_s\)) were used to determine the significance of substrate and isoform selective probe activity correlations. The F-ratio test was used to discriminate between one- and two-enzyme inhibition models.

Results

Cytochrome P450 Assays. HPLC Sulfadiazine Oxidative Metabolite Assay Development. Oxidation potentials for the hydroxylamine and 5-hydroxy sulfadiazine were 0 and 400 mV, respectively. Oxidation potentials for 4-hydroxy sulfadiazine, N-acetyl sulfadiazine, and sulfadiazine were greater than 600 mV. Because EDTA in the mobile phase becomes significantly oxidized at 400 mV, electrochemical detection at E₁ = 100 mV was used only for the hydroxylamine. When there were significant interfering peaks in the chromatography, the response to reduction of the nitroso species (oxidative product of the hydroxylamine) was monitored with E₂ = −200 mV, which was immediately downstream from E₁. UV detection at 266 nm was used for all of the other metabolites of interest. Sample chromatograms where 4-hydroxy sulfadiazine, N-hydroxy sulfadiazine, 5-hydroxy sulfadiazine, and sulfadiazine eluted at 8.4, 10.2, 11.9, and 12.8 min, respectively, are shown in Fig. 1.

The limit of detection for the hydroxylamine in the microsomal sample matrix was less than 0.2 pmol on column, and calibrations were linear over the range 0.2 to 2 pmol injected on column with \(r^2 = 0.998\). The accuracy and precision for the assay of the hydroxylamine were typically less than 6.1 and 16.8%, respectively. 4-Hydroxy and 5-hydroxy sulfadiazine calibrations were linear over the range of 4 to 75 pmol injected on column with \(r^2 \geq 0.99\).

Determining Linear Conditions and Parameters for Michaelis-Menten Kinetics. The hydroxylamine was stable for only 15 min (10% loss) in the full sample matrix containing denatured protein at

![](image)

FIG. 3. Isoform-specific inhibition of hydroxylamine formation in HL141 at 100 μM sulfadiazine. Isoform specificities were tolbutamide = CYP2C9, sulfaphenazole = CYP2C9, caffeine = CYP1A2, chloroxazone = CYP2E1, p-nitrophenol = CYP2E1, troleandomycin = CYP3A4, quinidine = D6, coumarin = 2A6, S-mephenytoin = CYP2C19, and orphenadrine = CYP2B6. All inhibitors were compared with solvent-matched controls containing not more than 0.3% solvent. Data are presented as mean and standard deviation of triplicate observations.

FIG. 2. Eadie-Hofstee plots for N-hydroxylation of sulfadiazine (SDZ-HA) in human liver microsomes over the range 300 μM to 10 nM. The average \(K_{m}\) (±S.D., n = 3) and \(V_{max}\), estimated by nonlinear regression, were 5.7 ± 2.2 mM and 185 ± 142 pmol/min/mg protein, respectively.
The hydroxylamine spiked into the full sample matrix treated with 0.2 N HCl was stable for greater than 6 h in argon-purged gas-tight autosampler vials.

The hydroxylamine and 4-hydroxy sulfadiazine were produced in human liver microsomes in a reaction that required NADPH, and their formation was eliminated by the presence of 1% Triton, indicating a role for cytochrome P450 metabolism. Formation of 5-hydroxyl sulfadiazine was not detected in human liver microsomes. Formation of the hydroxylamine and 4-hydroxy sulfadiazine was linear up to 15 and greater than 40 min, respectively, with protein concentrations up to 2 mg/ml. Hydroxylamine formation appeared as uniphasic plots on Eadie-Hofstee plots (Fig. 2), with an average $K_m$ (±S.D.) of 5.7 ± 2.2 mM and 7-fold variation in $V_{max}$ of 185 ± 142 pmol/min/mg protein. Formation of 4-hydroxy sulfadiazine in HL123 had an apparent $K_m$ > 10 mM with a formation rate of 69.2 pmol/min/mg protein at 10 mM sulfadiazine. The limited solubility of sulfadiazine in the microsomal incubation matrix (10 mM) did not allow us to determine the $K_m$ for 4-hydroxy sulfadiazine formation with confidence.

**Isoform Selective Inhibition Screening of Hydroxylamine Formation.** Screening with cytochrome P450 isoform selective inhibitors of hydroxylamine formation in human liver microsomes at clinical concentrations of sulfadiazine 100 μM showed significant inhibition by 250 μM tolbutamide (2× $K_m$ of CYP2C9; Veronese et al., 1993) and 2.16 μM sulfaphenazole (CYP2C9), indicating a role for CYP2C9 ($p ≤ 0.05$, see Fig. 3).

Lymphoblast-expressed CYP2C9*1 showed good sulfadiazine hydroxylation activity, with a $K_m$ of 7.2 ± 0.3 mM and $V_{max}$ of 36.9 ± 1.3 pmol/min/pmol P450. Lymphoblast-expressed CYP2C8 and CYP2C19 had activity that was 5 to 7 times greater than the vector control enzyme at 5 mM sulfadiazine. Baculovirus insect cell-expressed CYP2C8 (Supersomes) without coexpressed cytochrome $b_5$ had a $K_m$ of 2.59 ± 0.06 mM and $V_{max}$ of 0.330 ± 0.010 pmol/min/pmol P450. Baculovirus insect cell-expressed CYP2C8 (Supersomes) with coexpressed cytochrome $b_5$ had a $K_m$ of 2.87 ± 0.16 mM and $V_{max}$ of 0.571 ± 0.009 pmol/min/pmol P450. CYP2B6 appeared to have a $K_m$ much greater than 10 mM (rate of hydroxylamine formation at 10 mM was 0.41 pmol/min/pmol P450). All other lymphoblast-expressed enzymes investigated (CYP1A1, CYP1A2, CYP2A6, CYP2D6, CYP3A4, CYP2E1) did not show activity significantly greater than the endogenous activity of the control. A role for CYP2C8/9 in hydroxylamine formation was indicated by a strong correlation between the rate of sulfadiazine hydroxylamine formation (4 mM) and 4-hydroxylation of tolbutamide (500 μM) for 12 human livers (Fig. 4; $r = 0.76, p < 0.004$; $r_s = 0.78, p < 0.002$).

Fluconazole As a Selective Inhibitor of Hydroxylamine Formation. Fluconazole, an inhibitor of CYP2C9/19 and CYP3A4 at clinical concentrations, was investigated as a selective inhibitor of hydroxylation formation. The IC$_{50, app}$ for fluconazole (0–200 μM) at a sulfadiazine concentration of 100 μM in three livers (HL123, HL141, and HL142) was 32.0 ± 13.7 μM (16.2 to 40 μM). The IC$_{50, rem}$ was 11.7 ± 2.0 μM (9.5 to 13.3 μM) with residual activity of 25.7 ± 7.4% (17.2 to 30.0%). The significant residual activity indicated the presence of at least two enzymes in hydroxylation formation; therefore, nonlinear regression analysis was used to determine the fluconazole $K_i$ for one- and two-enzyme models of inhibition. For the one-enzyme models, the fluconazole $K_i$ in HL142 was determined to be 8.6 ± 0.6 μM with a competitive mechanism, and the $K_i$ in HL123 was 39.5 ± 2.8 μM with a noncompetitive mechanism, as shown by Dixon and Lineweaver-Burk plots in Fig. 5. However, the subtle nonlinearities in the data for HL123 are also consistent with a two-enzyme model in which one enzyme is competitively inhibited by fluconazole, but the other enzyme is not inhibited (eqs. 2 and 3). For competitive inhibition of one of the enzymes responsible for hydroxylamine formation in HL123 (enzyme 1), the parameters for $V_{max1}$, $K_{m1}$, $V_{max2}$, $K_{m2}$, and the $K_i$ were estimated to be 253 ± 21 pmol/min/mg, 8.6 ± 1.3 mM, 51.3 ± 5.8 pmol/min/mg, 2.1 ± 0.4 mM, and 10.4 ± 2.5 μM, respectively. When simulations were performed for an enzyme model with these parameters, apparent one-enzyme noncompetitive profiles were obtained for both the Dixon and Lineweaver-Burk plots (data not shown). The average IC$_{50, rem}$ agrees closely with the $K_i$ obtained for the two-enzyme model for HL123, $K_{m2}$ for the fluconazole inhibitable enzyme is close to the estimate of 7.2 mM for lymphoblast-expressed CYP2C9, and $K_{m2}$ is close to the estimate of 2.6 mM and 2.9 mM obtained for baculovirus insect cell-expressed CYP2C8 with and without supplemental cytochrome $b_5$, respectively. The data were also fit to a two-enzyme model in which fluconazole inhibition was noncompetitive for one of the enzymes (eq. 3 with the parameters for $V_{max1}$, $K_{m1}$, $V_{max2}$, $K_{m2}$, and the $K_i$ estimated to be 203 ± 18 pmol/min/mg, 6.0 ± 0.9 mM, 71 ± 7 pmol/min/mg, 4.7 ± 0.7 mM, and 16.2 ± 1.1 μM, respectively. As determined by the F-ratio test, both the two-enzyme models (eqs. 2 and 3) were a significantly ($p < 0.001$) better fit to HL123 data versus the one-enzyme models. Since fluconazole inhibited the one-enzyme model of HL142 with a competitive mechanism, the two-enzyme competitive model appears to be the model most consistent with our HL123 data.

**Arylamine N-Acetyltransferase Assays.** HPLC N-Acetyl Sulfadiazine Metabolic Assay Development. Retention times for sulfadiazine and N-acetyl sulfadiazine were 8.0 and 11.5 min, respectively. Calibrations were linear and reproducible over the range of 8.5 to 1000 pmol injected on column ($r^2 ≥ 0.992$). Cloned NAT enzymes expressed in *Escherichia coli* produced a time-dependent interfering peak caused by an interaction between perchloric acid and the bacterial protein. Cloned enzyme incubations were therefore terminated with 90 μl of acetonitrile and then diluted to a final concentration of 20% acetonitrile, and the supernatant was injected on the HPLC.

**Determining Linear Conditions and Parameters for Michaelis-Menten Kinetics.** Formation of N-acetyl sulfadiazine was linear up to
1 h with cytosolic protein concentrations up to 2 mg/ml. N-Acetyl sulfadiazine formation appeared uniphasic on Eadie-Hofstee plots (Fig. 6), with an average $K_m$ (± S.D.) and $V_{max}$ of 3.1 ± 1.7 mM and 221.8 ± 132.3 pmol/min/mg, respectively, at an acetyl coenzyme A concentration of 100 μM. Enzyme kinetic parameters for bacterially expressed wild-type NAT1*3 and NAT2*4 are listed in Table 1, where both enzymes had similar $K_m$ values of 5.8 ± 0.2 mM and 5.4 ± 0.2 mM, respectively, for sulfadiazine acetylation.

Isoform-Selective Inhibition Screening of N-Acetyl Sulfadiazine Formation. Selective inhibition screening in three livers using 250 μM SMZ as the NAT2 probe and 100 μM PABA as the NAT1 probe showed a dominant NAT2 component in two livers and an apparent dominant NAT1 component in one liver (HL142) (Fig. 7). The acetylation of sulfadiazine in 12 human livers was not correlated with NAT1 activity, as shown by p-aminobenzoic acid acetylation, but was correlated with NAT2 activity as shown by sulfamethazine acetylation (Fig. 8; $r = 0.96$, $p < 0.001$; $r_s = 0.92$, $p < 0.001$).

Discussion

We developed a highly sensitive, selective electrochemical HPLC assay that enabled us to detect and quantify the highly unstable hydroxylamine of sulfadiazine. Our human liver microsomal data are consistent with a significant role for cytochrome P450 in both hydroxylamine and 4-hydroxy sulfadiazine formation. Detection of 4-hydroxy sulfadiazine is consistent with a report by Vree et al. (1995). Despite the $K_m$ for the hydroxylamine being in the millimolar range, the reported recovery of 14% of a sulfadiazine dose as 4-hydroxy sulfadiazine (which appears to have an even higher $K_m$ in human liver microsomes) in a slow acetylator (Vree et al., 1995) suggests that hydroxylamine production would be significant in vivo.

The isoform selective screening profile and the correlation of the rate of hydroxylamine formation with tolbutamide 4-hydroxylation strongly indicates a role for CYP2C8/9 in hydroxylamine formation. Tolbutamide 4-hydroxylation has been demonstrated to be a reliable measure both of in vitro and in vivo CYP2C9 activity even when CYP2C9 allelic variants are present (Gill et al., 1999; Kirchheiner et al., 2002; Shon et al., 2002). In support of CYP2C9 involvement is a clinical study in which phenytoin clearance, which is largely CYP2C9-mediated with a minor contribution by CYP2C19 (Levy, 1995), was inhibited significantly (~50%, $n = 8$) by a 4-g oral dose of sulfadiazine (Hansen et al., 1979). When fluconazole (a CYP2C9/19 and CYP3A4 inhibitor at clinical concentrations) was explored as a potentially useful clinical inhibitor of hydroxylamine formation, significant residual enzyme activity ranging from 17.2 to
droxylation (Kunze et al., 1996). The conazole IC50 profiles and the apparently noncompetitive Dixon and enzyme activity. The significant residual activity shown by the fluconazole because of the inhibition screening profile and the undetectable clone.

In the case of sulfadiazine, a major role for CYP3A4 was discounted due to the variability in the range of Kp values for one-enzyme models (8.6 to 39.5 μM) determined. In a previous study on dapsone hydroxylamine formation (Winter et al., 2000), we found that CYP2C8, unlike other members of the CYP2C family, was not inhibited by fluconazole at concentrations up to 200 μM. Since sulfaphenazole is not an inhibitor of CYP2C8, our observation that toltubamide 250 μM (2 × Km of 2C9; Miners et al., 1988) inhibited sulfadiazine hydroxylamine formation greater than sulfaphenazole (18 × Kp of 2C9; Miners et al., 1988) also supports a potential role for CYP2C8 in sulfadiazine hydroxylamine formation.

The fluconazole IC50 values determined using a residual effect model was 11.7 ± 2 μM (9.5–13.3), which agrees closely with the fluconazole Kp of 8 μM determined for CYP2C9-mediated S-warfarin 7-hydroxylation (Kunze et al., 1996). The Kp for fluconazole inhibition of cloned CYP3A4 has been reported to be 9.21 μM (Gibbs et al., 1999). In the case of sulfadiazine, a major role for CYP3A4 was discounted because of the inhibition screening profile and the undetectable clone enzyme activity. The significant residual activity shown by the fluconazole IC50 profiles and the apparently noncompetitive Dixon and Lineweaver profiles for HL123 are completely consistent with a two-enzyme model in which there is competitive inhibition of CYP2C9 and a lack of fluconazole inhibition of CYP2C8 (and/or other enzymes) in hydroxylamine formation. The two-enzyme model will be discussed in detail below.

Acetylation of sulfadiazine in human liver is mediated by NAT2, as shown by a strong correlation with the NAT2-specific sulfamethazine N-acetylation. Because the bacterially expressed human wild-type NAT enzymes had very similar Km values, in vivo NAT enzyme must have much greater activity toward sulfadiazine acetylation, either in the form of a higher catalytic rate constant (kcat) and/or a much greater level of enzyme expression in human liver. Sulfadiazine acetylation would be expected to be a predominantly NAT2-mediated process as sulfamethazine (our NAT2 probe) is actually 4,6-dimethyl sulfadiazine. Methylation of the para-substituent must enhance affinity for NAT2, since the reported Km for sulfamethazine acetylation is only 120 μM (Grant et al., 1991). NAT2 is expressed predominantly in the liver and red blood cells, whereas NAT1 is expressed ubiquitously. It is possible that NAT1-mediated acetylation may predominate in nonhepatic tissues. The Km for sulfadiazine N-acetylation is comparable with that found for hydroxylamine formation. Slow acetylator status may predispose patients to sulfadiazine adverse reactions by allowing more parent drug to be available for oxidative pathways and hydroxylamine formation.

The cause of the extremely high rate of adverse reactions to sulfadiazine and other arylamine drugs, such as sulfamethoxazole and dapsone, in HIV-infected patients has not been determined. If the rate of adverse reactions is related to the total body burden of cytochrome P450-mediated production of the hydroxylamine, then potent inhibitors of CYP2C9 activity suitable for use in the clinic would be predicted to decrease the rate of adverse reactions and allow patients to complete their antibiotic therapy. Inhibition of a metabolite’s formation clearance by an inhibitor (I) with an in vivo-determined Ki can be predicted for a one-enzyme Michaelis-Menten model based on the following equation derived by Shaw and Houston (1987) for both

![Graph](image.png)

**FIG. 6.** Eadie-Hofstee plots for N-acetylation of sulfadiazine (AcSDZ) in human liver cytosol over the range 156 μM to 5 mM. The average Kp (± S.D.) and Vmax were determined to be 3.1 ± 1.7 mM and 221.8 ± 132.3 pmol/min/mg protein at an acetyl coenzyme A concentration of 100 μM.

30% was found, indicating a role for one or more non-fluconazole-inhibitable enzymes. The variability in fluconazole IC50 app estimates (16.2 to 40 μM) at clinical sulfadiazine concentrations in vitro agreeing with the variability in the range of Kp values for one-enzyme models (8.6 to 39.5 μM) determined. In a previous study on dapsone hydroxylamine formation (Winter et al., 2000), we found that CYP2C8, unlike other members of the CYP2C family, was not inhibited by fluconazole at concentrations up to 200 μM. Since sulfaphenazole is not an inhibitor of CYP2C8, our observation that toltubamide 250 μM (2 × Km of 2C9; Miners et al., 1988) inhibited sulfadiazine hydroxylamine formation greater than sulfaphenazole (18 × Kp of 2C9; Miners et al., 1988) also supports a potential role for CYP2C8 in sulfadiazine hydroxylamine formation.

The fluconazole IC50 values determined using a residual effect model was 11.7 ± 2 μM (9.5–13.3), which agrees closely with the fluconazole Kp of 8 μM determined for CYP2C9-mediated S-warfarin 7-hydroxylation (Kunze et al., 1996). The Kp for fluconazole inhibition of cloned CYP3A4 has been reported to be 9.21 μM (Gibbs et al., 1999). In the case of sulfadiazine, a major role for CYP3A4 was discounted because of the inhibition screening profile and the undetectable clone enzyme activity. The significant residual activity shown by the fluconazole IC50 profiles and the apparently noncompetitive Dixon and Lineweaver profiles for HL123 are completely consistent with a two-enzyme model in which there is competitive inhibition of CYP2C9 and a lack of fluconazole inhibition of CYP2C8 (and/or other enzymes) in hydroxylamine formation. The two-enzyme model will be discussed in detail below.

Acetylation of sulfadiazine in human liver is mediated by NAT2, as shown by a strong correlation with the NAT2-specific sulfamethazine N-acetylation. Because the bacterially expressed human wild-type NAT enzymes had very similar Km values, in vivo NAT enzyme must have much greater activity toward sulfadiazine acetylation, either in the form of a higher catalytic rate constant (kcat) and/or a much greater level of enzyme expression in human liver. Sulfadiazine acetylation would be expected to be a predominantly NAT2-mediated process as sulfamethazine (our NAT2 probe) is actually 4,6-dimethyl sulfadiazine. Methylation of the para-substituent must enhance affinity for NAT2, since the reported Km for sulfamethazine acetylation is only 120 μM (Grant et al., 1991). NAT2 is expressed predominantly in the liver and red blood cells, whereas NAT1 is expressed ubiquitously. It is possible that NAT1-mediated acetylation may predominate in nonhepatic tissues. The Km for sulfadiazine N-acetylation is comparable with that found for hydroxylamine formation. Slow acetylator status may predispose patients to sulfadiazine adverse reactions by allowing more parent drug to be available for oxidative pathways and hydroxylamine formation.

The cause of the extremely high rate of adverse reactions to sulfadiazine and other arylamine drugs, such as sulfamethoxazole and dapsone, in HIV-infected patients has not been determined. If the rate of adverse reactions is related to the total body burden of cytochrome P450-mediated production of the hydroxylamine, then potent inhibitors of CYP2C9 activity suitable for use in the clinic would be predicted to decrease the rate of adverse reactions and allow patients to complete their antibiotic therapy. Inhibition of a metabolite’s formation clearance by an inhibitor (I) with an in vivo-determined Ki can be predicted for a one-enzyme Michaelis-Menten model based on the following equation derived by Shaw and Houston (1987) for both

**TABLE 1**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>Vmax</th>
<th>Km</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAT1<em>3</em></td>
<td>PABA</td>
<td>6.4</td>
<td>687</td>
</tr>
<tr>
<td>NAT1<em>3</em></td>
<td>Sulfadiazine</td>
<td>18.3</td>
<td>5.8</td>
</tr>
<tr>
<td>NAT2<em>4</em></td>
<td>SMZ</td>
<td>0.8</td>
<td>136</td>
</tr>
<tr>
<td>NAT2<em>4</em></td>
<td>Sulfadiazine</td>
<td>2.98</td>
<td>5.4</td>
</tr>
</tbody>
</table>

*We used exactly the same source of cloned NAT enzyme, within a similar time frame as reported by Palamanda et al. (1995).*

**FIG. 7.** Isoform-specific inhibition of N-acetyl sulfadiazine formation in human liver cytosol at 50 and 1000 μM sulfadiazine (SDZ). Probe specificities were p-aminobenzoic acid (PABA) = NAT1 and sulfamethazine (SMZ) = NAT2. N.D., not detectable.
assuming that the substrate concentrations are much less than the 
K_m for the enzyme. At expected fluconazole clinical concentrations of 25 
µM (Winter et al., 2004a,b) and an observed one-enzyme model K_i 
range of 8.6 to 39.5 µM, we would predict approximately 40 to 70% 
half inhibition of sulfadiazine hydroxylamine formation in vivo. Flucon-
azole has similar estimated apparent in vitro apparent IC_50, K_i, estimates for inhibiting the production of hydroxylamine metabolites 
of two arylamine drugs, dapsone and sulfamethoxazole (Winter et al., 
2000, 2004b). We have also shown that fluconazole can inhibit the in 
vivo hydroxylamine production of both dapsone and sulfamethoxa-
azole by ~33 to 60% (Winter et al., 2004a,b). On the basis of the 
above data, we predict that fluconazole, which is only 10% plasma 
protein bound, will substantially inhibit sulfadiazine hydroxylamine 
formation and may therefore be a clinically useful strategy to decrease 
adverse reactions to sulfadiazine in vivo. To test this strategy will 
require both an in vivo pharmacokinetic study to confirm significant 
inhibition of hydroxylamine production by fluconazole, followed by a 
larger clinical trial when fluconazole and sulfadiazine are chronically 
coadministered and the adverse effects of sulfadiazine are measured.

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