ABSTRACT:

Using selective cytochrome P450 (CYP) inhibitors and clinical concentrations (4 μM) of dapsone (DDS), we found a major contribution of CYP2C9 and little or no contribution (<10%) of CYP3A4 and CYP2E1 to dapsone N-hydroxylation (DDS-NHY) in human liver microsomes. Sulphaphenazole (2.16 μM) and tolbutamide (500 μM), selective inhibitors of CYP2C9 (or 2C8/9), inhibited DDS-NHY by 48 ± 14 and 41 ± 15%, respectively. The apparent Michaelis-Menten K_m values for DDS-NHY by cloned CYP2C8, CYP2C9, CYP2C18, and CYP2C19 were 75 μM, 31 μM, 25 μM, and greater than 1 mM, respectively. CYP3A4 and CYP2E1 were incapable of DDS-NHY at 4 μM DDS. S-mephenytoin (360 μM) activated DDS-NHY by human liver microsomes and by CYP2C8 by 43 ± 36 and 193 ± 16%, respectively. This activation was cytochrome b_6-dependent. In contrast, S-mephenytoin inhibited DDS-NHY by CYP2C9, CYP2C18, and CYP2C19 by 27 ± 2, 49 ± 1, and 32 ± 4%, respectively. Because CYP2C18 and CYP19 are expressed at low concentrations in the human liver, these observations indicate that at clinical DDS concentrations, CYP2C9 is a major and CYP2C8 is a likely minor contributor to DDS-NHY in human liver microsomes.

Dapsone (DDS) is used to treat Pneumocystis carinii pneumonia in people with AIDS who are intolerant to co-trimoxazole (sulfamethoxazole/trimethoprim). However, the high rate of adverse reactions to DDS in people with AIDS such as leukopenia, thrombocytopenia, methemoglobinemia, and changes in liver transaminase activity often requires discontinuation of therapy with DDS (Safrin et al., 1996). Dapsone N-hydroxylamine (DDS-HA) has been implicated as the causative agent of the adverse reactions to DDS. Dapsone hydroxylation (DDS-NHY) has been shown to be directly related to methemoglobin formation in vivo (Coleman et al., 1992; Mitra et al., 1995; Rhodes et al., 1995). There is evidence to suggest that a combination of oxidative stress and hydroxylamine formation is responsible for the high rate of adverse reactions to arylamine antibiotics in AIDS patients (Carr et al., 1993; Pace and Leaf, 1995; Rieder et al., 1995).

DDS-NHY has been reported to be catalyzed by cytochrome P450 (CYP)2C9, CYP3A4, and CYP2E1 (Fleming et al., 1992; Gill et al., 1995; Mitra et al., 1995). Unfortunately, this assignment of CYP isoforms to DDS-NHY is confounded by the use of either DDS concentrations much greater than those encountered in the clinic (Fleming et al., 1992; Gill et al., 1995) or use of an inhibitor concentration [diethylidithiocarbamate (DDC); Mitra et al., 1995], which could lead to inhibition of isoforms other than CYP2E1 (Chang et al., 1994; Ono et al., 1996). The use of disulfiram (CYP2E1 inhibitor) as a metabolic inhibitor in vivo, has been explored as a therapeutic strategy to suppress DDS-NHY; it produced a 65% decrease in DDS-NHY in non-HIV-infected subjects (Mitra et al., 1995). However, in a recent clinical study in HIV-infected patients, fluconazole (a CYP2C and CYP3A4 inhibitor that does not inhibit CYP2E1) unexpectedly inhibited DDS-NHY by 50% (Winter et al., 1998), a magnitude greater than expected based on previously reported in vitro and in vivo data (Mitra et al., 1995).

As part of an ongoing series of studies on the prospective in vitro prediction of in vivo drug interactions for drugs used to treat opportunistic infections in AIDS, we have investigated the role of the CYP2C family in mediating DDS-NHY at clinical concentrations (4-8 μM) of DDS.

Materials and Methods

Chemicals. DDS-HA was synthesized and provided by the National Institutes of Allergy and Infectious Diseases (Bethesda, MD). The purity of the hydroxylamine was determined to be >99% by quantitative thin-layer chromatography. DDS was purchased from Sigma Chemical Co. (St. Louis, MO). S-mephenytoin was a gift from Dr. René H. Levy, Department of Pharmacy, University of Washington, Seattle, WA. Human anti-cytochrome b_6 and preimmune antibodies were a gift from Dr. Peter Manyike, University of Washington. All other chemicals were of analytical grade and obtained commercially.

Human Liver Samples. Livers were procured, processed, and stored as previously described (Rettie et al., 1989). Microsomes from the University of Washington's human liver bank from human livers numbered 123, 139, 140, 141, and 142, were prepared as previously described (Hickman et al., 1998).

Cloned Human Enzymes. All cloned enzymes were obtained from Gentest (Woburn, MA). Unless specified, the clones were human lymphoblast-expressed, containing co-expressed CYP reductase and endogenous cytochrome b_6. The CYP2C18 cloned enzyme was baculovirus insect cell-expressed Gentest Supersome preparation lacking coexpressed cytochrome b_6. Therefore, CYP2C18 was always mixed with the Gentest Supersome vector insert control.
Results and Discussion

CYP-mediated DDS-NHY was found to be biphasic in HLM141 microsomes with $K_m$ values of 5.1 ± 3.3 and 189.9 ± 52.5 μM (data not shown), which agrees closely with Mitra and colleagues (Mitra et al., 1995). Because clinical concentrations of DDS therapy are 4 to 8 μM, we conducted all our microsomal incubations at a concentration (4 μM) comparable to the lower $K_m$ value. Of the selective P450 inhibitors (Hickman et al., 1998) used, only sulfaphenazole (2.16 μM; CYP2C9) and tolbutamide (500 μM; CYP2C8/9) significantly inhibited (by 48 ± 14 and 41 ± 15%, respectively) DDS-NHY in human liver microsome (HLM); HL123, 139, 141; Fig. 1A). The inhibition observed reflects CYP2C9 inhibition. It should be noted that tolbutamide at the concentration used was not expected to significantly inhibit CYP2C8, and that sulfaphenazole does not inhibit CYP2C8 (Fig. 1B). In addition, a clinical concentration of fluconazole (20 μM), an inhibitor of CYP2C9, CYP2C19, and CYP3A4 (Fig. 1B) inhibited DDS-NHY in HLM by 41 ± 15% whereas 360 μM S-mephénytoin activated this reaction by 43 ± 37% (Fig. 1A).

Lymphoblast-expressed CYP2C8, CYP2C9, CYP2C18, and CYP2C19 were all capable of forming DDS-NHY from 6.25 μM DDS at a rate of 0.0357, 0.918, 0.684, and 0.0349 pmol/min/pmol of P450, respectively. In contrast, lymphoblast-expressed CYP3A4 and CYP2E1, and Supersome CYP2E1 with coexpressed cytochrome $b_5$ did not show detectable DDS-NHY at a DDS concentration of 4 μM. Velocity profiles for DDS-NHY with increasing substrate concentration (1.6–300 μM) were typical hyperbolic Michaelis-Menten profiles for CYP2C8, CYP2C9, and CYP2C18 (Fig. 2A). DDS-NHY by CYP2C19 displayed autoactivation within the clinical range (4–12.5 μM) as demonstrated by the nonlinear Eadie-Hofstee curve (Fig. 2B). The apparent Michaelis-Menten $K_m$ values for DDS-NHY by CYP2C8, CYP2C9, CYP2C18, and CYP2C19 were 75 μM, 31 μM, 25 μM, and >1 mM, respectively.

To gain insight into the activation of DDS-NHY by S-mephénytoin, we measured DDS-NHY in expressed 2C enzymes. S-mephénytoin (360 μM) inhibited DDS-NHY by CYP2C9, CYP2C18, and CYP2C19 by 27 ± 2, 49 ± 1, and 32 ± 4%, respectively, and activated this reaction by CYP2C8 by 194 ± 16% (all $P$ < .05; Fig. 3A). Cytochrome $b_5$ appears to have an important role in the mechanism of the S-mephénytoin-mediated activation. CYP2C8 Super-
leagues (Mitra et al., 1995; this study) and allosteric autoactivation in the clinical range reported by Korzekwa and colleagues (Korzekwa et al., 1998), makes determination and interpretation of $K_i$ values difficult. Fluconazole (200 $\mu$M) was found to be incapable of completely inhibiting DDS-NHY, suggesting that enzymes other than CYP2C9/19 and CYP3A4 mediate the residual catalytic activity (Fig. 4). The IC$_{50}$ values for fluconazole in HLMs (HL123, 139, 141) was 16.8 ± 3.3 $\mu$M with residual activity of 23.6 ± 8.7%. The residual activity in HLMs is consistent with a probable minor contribution by CYP2C8 as it was activated 33 ± 6% ($P$ < .05) by 360 $\mu$M S-mephenytoin (HL123, 139, 141), but not inhibited by DDC (CYP2E1; data not shown). Moreover, a clinical fluconazole concentration of 20 $\mu$M inhibited DDS-NHY by CYP2C9, CYP2C18, and CYP2C19 by 55 ± 1, 59 ± 1, and 100% ($P$ ≤ .05), respectively, but did not inhibit CYP2C8 (Fig. 1B). A 10-fold greater concentration of fluconazole was also unable to significantly inhibit DDS-NHY by CYP2C8 (data not shown), which is consistent with a role for CYP2C8 as one of the enzymes responsible for the residual activity shown in Fig. 4.

The CYP2C family appears to have a major role in metabolizing DDS to its hydroxylamine at clinical concentrations. Cytochrome $b_5$ has a major role in S-mephenytoin-mediated activation of DDS-NHY. S-mephenytoin activation was CYP2C8-specific. Neither CYP2C18 nor CYP2C19 are likely to contribute significantly to in vivo DDS-NHY because they are expressed at much lower hepatic concentrations than the other CYP2C isoforms (Goldstein and de Morais, 1994). DDS activation of CYP2C9-mediated metabolism of flurbiprofen and naproxen has been reported by Korzekwa and colleagues (Korzekwa et al., 1998), but we were unable to demonstrate sigmoid velocity profiles for any of the lymphoblast-expressed CYP2C clones. The pattern of fluconazole inhibition observed clinically (Winter et al., 1998) is consistent with a major contribution by CYP2C9 and possible minor contribution by CYP2C8 to DDS-NHY at clinical DDS concentrations. These results appear inconsistent with a previous in vivo study where disulfiram (a CYP2E1 inhibitor) was found to

**Fig. 2.** Michaelis-Menten (A) and Eadie-Hofstee (B) velocity profiles for DDS-NHY by Gentest human lymphoblast-expressed 2C8 ( ), 2C9*1 ( ), 2C18 ( ), and baculovirus-insect-cell-expressed (Supersome) 2C18 ( ) human CYP enzymes.

**Fig. 3.** Activation of DDS-NHY by S-mephenytoin.

A. DDS-NHY at 4 $\mu$M DDS in the presence of 360 $\mu$M S-mephenytoin by Gentest 2C8, 2C9*1, 2C18, and 2C19 human CYP enzymes and HLMs. Values represent the mean percentage of control activity ± S.D. of triplicate determinations. All results were statistically significant except for S-mephenytoin activation of CYP2C8 supersomes in the absence of cytochrome $b_5$ ($P$ ≤ .05). Lym, lymphoblast-expressed; s, Supersome (baculovirus insect-cell-expressed); $h_5$, cytochrome $b_5$; anti-$h_5$, IgG, human cytochrome $b_5$ antibody. Antibody experiments were conducted at a ratio of HLM (HL123)/IgG protein of 1:30 w/w. B. Michaelis-Menten velocity profiles for CYP2C8-mediated DDS-NHY for control ( ) and incubations containing 360 $\mu$M S-mephenytoin ( ). Final concentration of methanol in all incubations was 1% v/v.
inhibit DDS-NHY by 65% (Mitra et al., 1995). However, the study by Mitra and colleagues was conducted in healthy volunteers and not HIV-infected patients. There may be disease-related changes in metabolic enzymes involved in DDS-NHY. Alternatively, disulfiram may be an inhibitor of other enzymes (either P450 or non-P450 or both) that may be responsible for in vivo DDS-NHY in healthy volunteers.

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References


Fig. 4. Fluconazole inhibition profile for DDS-NHY in HL142.

Each inhibitor concentration was performed in duplicate. The nonlinear fitted estimates of IC50 (residual effect model), residual activity, and IC50 (apparent) were 18.1 ± 0.9 μM, 19.0 ± 1.1%, and 28.7 ± 2.9 μM, respectively.