0026-895X/92/050975-06$03.00/0

MOLECULAR PHARMACOLOGY, 41:975-980

Human Liver Microsomal *N*-Hydroxylation of Dapsone by Cytochrome P-4503A4

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Received November 15, 1991; Accepted February 11, 1992

SUMMARY

One of the major routes of elimination of dapsone (4,4′-diaminodiphenylsulfone) is by *N*-oxidation, to produce a hydroxylamine metabolite. The specific form of cytochrome P-450 (P-450) involved in this oxidation reaction was examined in human liver microsomal preparations previously characterized with respect to their content of several known P-450 enzymes. Among five preparations, the rank order of activity for dapsone hydroxylamine formation was most well correlated with the immunochemically determined level of P-4503A4 ($r = 0.94$, $p < 0.03$). Moreover, inhibition of microsomal oxidation was observed with antibodies specific to P-4503A, with a maximum reduction of >90%, but was not produced by antibodies specific to P-4501A2, P-4502C_{MP}, or P-4502E1. Prior incubation of microsomes with gestodene (100 μM) or troleandomycin (20 μM), known selective mechanism-based inhibitors of P-4503A enzymes (in the presence of NADPH), led to 75% and 40% reductions in catalytic activity, respectively. In contrast, preincubation with increasing concentrations of α-naphthoflavone, a known activator of P-4503A4, increased dapsone *N*-hydroxylation in a concentration-dependent manner, with 5-fold activation being observed at 50 μM α-naphthoflavone. Finally, P-4503A4 isolated from human liver microsomes and cDNA-expressed P-4503A4 (in yeast) were both able to catalyze dapsone *N*-hydroxylation, with the latter preparation exhibiting a 3-fold activation in the presence of 100 μM α-naphthoflavone. Collectively, these findings demonstrate that *N*-oxidation of dapsone in human liver is predominantly mediated by P-4503A4, and they suggest that quantitative measurement of this metabolic pathway *in vivo* might serve as an index of the activity of this enzyme.

Dapsone (4,4′-diaminodiphenylsulfone) is used clinically to treat a variety of diseases including leprosy (1), chloroquine-resistant malaria (2), autoimmune diseases (3, 4), and a number of skin diseases, including dermatitis herpetiformis (5) and local skin necrosis due to brown recluse spider bites (6). More recently, dapsone has become of increasing importance in the treatment of Kaposi's sarcoma (7) and *Pneumocystis carinii* (8) in patients with acquired immunodeficiency syndrome. Such therapeutic use is limited by the frequent occurrence of side effects such as methemoglobinemia and hemolytic anemia, which have been associated with metabolic formation of dapsone hydroxylamine (9, 10).

Recent studies with human liver microsomes have found that the activity of P-4501A2[1] is responsible for the activation, through *N*-oxidation, of many known arylamine procarcinogens (14, 15). This raises the possibility that P-4501A2 may also be responsible for the *N*-hydroxylation of dapsone, and its *in vivo* activity could play a crucial role in determining individual susceptibility to toxicity from this drug.

The objective of this study was to determine which, if any, of the known P-450 enzymes are involved in the *N*-hydroxylation of dapsone. Such knowledge could lead to a better understanding of dapsone-related toxicities and, in addition, it could also indicate whether dapsone *N*-hydroxylation might serve as a useful model for arylamine *N*-hydroxylation in general.

Materials and Methods

Chemicals. Dapsone was purchased from Sigma Chemical Co. (St. Louis, MO). Meta-dapsone (3,3′-diaminodiphenylsulfone) and α-naphthoflavone were obtained from Aldrich Chemical Co. (Milwaukee, WI). The hydroxylamine of dapsone was prepared as previously described, for use as an analytical standard (16). Gestodene was provided by Dr. H. Kuhl, Johannes Wolfgang Goethe University (Frankfurt, Germany), and TAO was a gift from Dr. P. H. Beaune, INSERM-Necker (Paris, France).

Liver microsomes. Human liver samples were obtained, through Tennessee Donor Services (Nashville, TN), from organ donors who met accidental deaths. The processing of these samples and preparation of microsomes have been described previously (17).

This work was supported in part by United States Public Health Service Grants GM31304, CA44353, and ES00267.

[1] Nomenclature of the individual P-450 enzymes is as recommended by Nebert *et al.* (11). For a recent review of some of the human P-450 enzymes and their relative activities, see the work of Guengerich and Shimada (12), and references cited therein. (*S*)-Mephenytoin 4′-hydroxylase is referred to as P-4502C_{MP} because it is generally accepted that this is a member of the P-4502C subfamily, but its primary sequence is currently unknown (13).

ABBREVIATIONS: P-450, cytochrome (heme-thioate protein) P-450; TAO, troleandomycin.

Enzymes. P-4503A4 was expressed in *Saccharomyces cerevisiae* transformed with human cDNA clone NF25 (18) and was partially purified from microsomes isolated from the yeast (19). Human liver P-4503A4 (20), rabbit NADPH-cytochrome P-450 reductase, and human cytochrome b_5 were purified as previously reported (21).

Antibodies. Rabbit antibodies to specific human P-450s were prepared and assessed for specificity as previously described; i.e., anti-P-4501A2 (22), anti-P-4502C_{MP} (21), anti-P-4502E1 (23), and anti-P-4503A4 (20). In all cases, these antibodies should be considered to be specific for each P-450 subfamily (i.e., 1A, 2C, 2E, and 3A); the issue of which of the P-4503A enzymes was of most significance in the *N*-hydroxylation of dapsone was addressed by the use of other experimental approaches.

Assays. A panel of human liver microsomes from five different donors, coded as HL-102, HL-105, HL-110, HL-124, and FH-96 (14), were screened for dapsone *N*-hydroxylase activity. These microsomal preparations had been previously well characterized with respect to the activity and/or immunochemically determined levels of P-450s 1A2, 2C_{MP}, 2D6, 2E1, and 3A4 (14). Each microsomal preparation (2 mg of microsomal protein/ml) was incubated with 0.1 mM dapsone in 0.1 M potassium phosphate buffer (pH 7.4), in the presence of an NADPH-generating system consisting of 0.5 mM $NADP^+$, 1 unit of glucose 6-phosphate dehydrogenase/ml, and 10 mM glucose-6-phosphate, in a final volume of 0.5 ml. Before the addition of the glucose-6-phosphate, all assay samples were preincubated for 3 min at 37°. The reaction was then allowed to continue for 20 min at 37°, in a shaking water bath. Reactions were stopped by the addition of 1 volume of cold, water-saturated, ethyl acetate. Meta-dapsone (1.5 μl of a 0.12 mM solution in methanol) was added to each assay sample as an internal standard, and proteins and other debris were separated out by centrifugation at 1000 × *g* for 5 min. The organic layer was stored at −20° until analysis within 48 hr.

Determination of the formation of dapsone hydroxylamine was performed using a previously described high performance liquid chromatography procedure (24). Briefly, the organic solvent of the extracted sample was evaporated by using a gentle stream of N_2, at 25°. The residue was dissolved in 100 μl of mobile phase (see below), and a 50-μl aliquot of this solution was injected onto a 4.6- × 250-mm octadecylsilyl (C-18), μ-Bondapack, stainless steel column (Waters-Millipore, Milford, MA) (flow rate, 1 ml/min), fitted with a reverse phase guard column (Whatman, Piscataway, NJ). The mobile phase consisted of deionized water, acetonitrile, acetic acid, and triethylamine (81.5:17.5:1:0.05). Under these chromatographic conditions, the retention times of dapsone hydroxylamine, dapsone, and internal standard were 12, 16, and 22 min, respectively. Linear regression analysis was used to examine the relationship between the rate of formation of dapsone hydroxylamine and the marker activity or immunochemically determined amount of each individual P-450 enzyme, with $p < 0.05$ being taken as the criterion of statistical significance.

Immunoinhibition of dapsone *N*-hydroxylation was examined by preincubating sample HL-110 human liver microsomes (0.5 mg of protein/ml) with various concentrations of preimmune serum (2 or 10 mg of IgG/nmol of P-450) or specific antibodies raised to P-4501A2, P-4502C_{MP}, P-4502E1 (2 or 10 mg of IgG/nmol of P-450), or P-4503A4 (1, 2, 5, 10, or 15 mg of IgG/nmol of P-450), in 0.1 M potassium phosphate buffer (pH 7.4), for 20 min at 23° (14). Dapsone (0.1 mM) and the components of the NADPH-generating system were then added, and the reactions were carried out as described above. Meta-dapsone (0.2 nmol) in methanol was added as an internal standard, and the samples were assayed as described above.

The ability of known mechanism-based inhibitors of P-4503A to inhibit dapsone *N*-hydroxylation was determined by preincubating liver sample HL-110 microsomes (0.5 nmol of total P-450 protein) with various concentrations of gestodene (0, 20, or 100 μM) or TAO (0 or 20 μM), in 0.1 M potassium phosphate buffer (pH 7.4), in the presence or absence of the standard NADPH-generating system, for 30 min at 37° (25). Samples were then diluted 20-fold with 0.1 mM potassium phosphate buffer (pH 7.4) containing 0.1 mM dapsone and additional components of the NADPH-generating system (25). The reaction was stopped after 20 min, and meta-dapsone was added to each sample as an internal standard before analysis.

Liver sample HL-110 microsomes (0.25 nmol of total P-450 protein), in 0.1 M potassium phosphate buffer (pH 7.4), were incubated with dapsone (0.1 mM) in the presence of increasing concentrations of α-naphthoflavone (0–0.1 mM), a known activator of P-4503A4 (26), and the NADPH-generating system, in a final volume of 0.5 ml. The assay samples were preincubated at 37° for 3 min before the addition of glucose-6-phosphate and were processed as described above.

P-4503A4 preparations purified from human liver microsomes (100 pmol) and cDNA-expressed P-4503A4 (43 pmol) were reconstituted with 14 pmol of human cytochrome b_5, 25 pmol of rabbit NADPH-cytochrome P-450 reductase, and 90 nmol of dilauroylphosphatidylcholine, in 0.1 M potassium phosphate buffer (pH 7.4). These reconstituted enzymes were assayed for dapsone *N*-hydroxylase activity after 20 min of incubation at 37°, in the presence of 0.1 mM dapsone and the NADPH-generating system. α-Naphthoflavone (100 μM) was added to some of the reactions with both reconstituted enzyme systems.

Results

Dapsone hydroxylamine was formed in measurable amounts by all five of the human liver microsomal preparations. Such activity correlated with both the immunoquantified level of P-4503A4 ($r = 0.94$, $p < 0.03$) (Table 1) and the oxidation of nifedipine ($r = 0.93$, $p < 0.03$) (Table 1; Fig. 1). On the other hand, no significant correlations were seen between dapsone *N*-hydroxylase activity and the activities of P-4501A2, P-4502C_{MP}, P-4502D6, or P-4502E1 or the immunochemically determined levels of P-4501A2, P-4502C_{MP}, or P-4502E1 (Table 1).

Human liver microsomal preparation HL-110 possessed the highest dapsone *N*-hydroxylase activity and was used for all subsequent assays. When HL-110 microsomes were preincubated with a specific antibody raised to P-4503A4, dapsone *N*-hydroxylase activity was reduced to approximately 10% of control levels at a concentration of only 2 mg of IgG/nmol of P-450. In contrast, the preimmune serum and the antibodies to P-4502C_{MP}, P-4501A2, and P-4502E1 failed to inhibit microsomal dapsone *N*-hydroxylation at concentrations up to 10 mg of IgG/nmol of P-450 (Fig. 2).

Based on these findings, further experiments were carried out to assess the effects of known mechanism-based inhibitors (TAO and gestodene) and an activator (α-naphthoflavone) of

TABLE 1

Linear correlation coefficients between microsomal dapsone *N*-hydroxylase activity and the immunochemically determined amount or activity of various P-450s in microsomes from five different livers

P-450	Correlation with protein level	Correlation with enzyme activity[a]
1A2	0.01	0.75
2C_{MP}	0.76	−0.46
2D6	ND[b]	−0.56
2E1	−0.47	−0.01
3A4	0.94[c]	0.93[c]

[a] The ranges of the marker enzyme activities (nmol of product formed/min/nmol of P-450) in the microsomal preparations used (14) were as follows: P-4501A2 (phenacetin *O*-deethylation), 0.01–0.19; P4502C_{MP}, [*S*)-mephenytoin 4′-hydroxylation], 0.09–0.53; P4502D6 [(±)-bufuralol 1′-hydroxylation], 0.06–0.55; P4502E1 (*N*,*N*-dimethylnitrosamine *N*-demethylation), 0.13–2.01; and P-4503A4 (nifedipine oxidation), 1.25–764.

[b] ND, not determined.

[c] $p < 0.03$

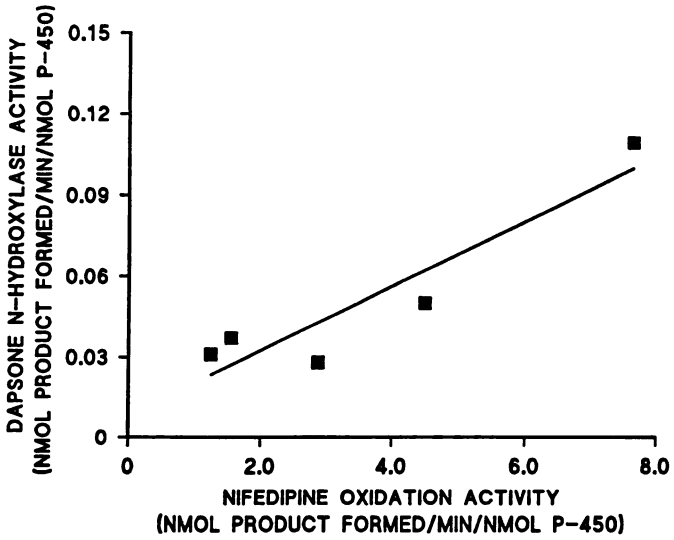

Fig. 1. Relationship between dapsone *N*-hydroxylase and nifedipine oxidase activity in the microsomal enzyme preparations described in Table 1 ($r = 0.93$, $p < 0.03$).

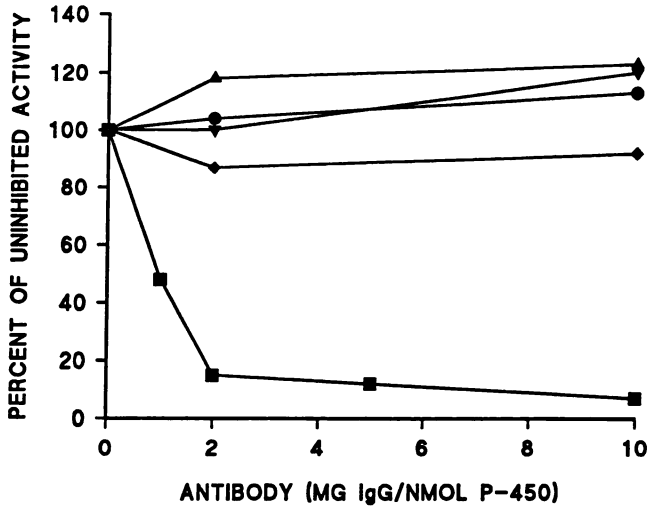

Fig. 2. Dapsone hydroxylase activity, relative to control levels of activity, in the presence of preimmune serum (▲) or specific antibodies to P4501A2 (●), P4502C$_{MP}$ (▼), P4502E1 (◆), or P4503A4 (■).

P-4503A4. A concentration of 20 μM TAO inhibited dapsone *N*-hydroxylation by only 10–15% in the absence of an NADPH-generating system in the preincubation medium; the modest inhibition that was observed might have occurred after the NADPH and dapsone were added. However, in the presence of an NADPH-generating system in the preincubation medium, 20 μM TAO consistently inhibited dapsone *N*-hydroxylation by 40% (Fig. 3). Similar results were obtained with gestodene (Fig. 3). In the absence of an NADPH-generating system in the preincubation with gestodene, little inhibition of dapsone *N*-hydroxylase activity was seen. However, when such a system was present in the preincubation, dapsone *N*-hydroxylation was inhibited in a concentration-dependent manner, exhibiting only 25% of control levels of activity at a concentration of 50 μM gestodene.

Increasing concentrations of α-naphthoflavone, added to microsomal incubations, stimulated dapsone *N*-hydroxylase activity (Fig. 4), with a 5-fold maximal activation occurring at 50 μM α-naphthoflavone.

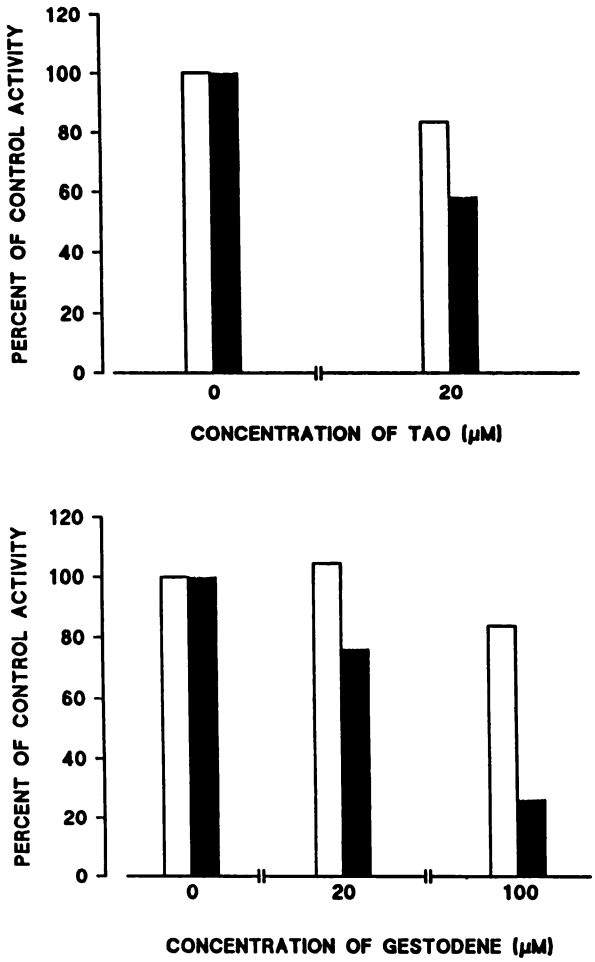

Fig. 3. Dapsone *N*-hydroxylase activity, relative to control values, in the presence (■) or absence (□) of an NADPH-generating system, in a preincubation with TAO (*upper*) or gestodene (*lower*). Control activity was considered to be the dapsone *N*-hydroxylase activity when the inhibitor and an NADPH-generating system were absent during the preincubation. Data represent the results of four assays under each condition.

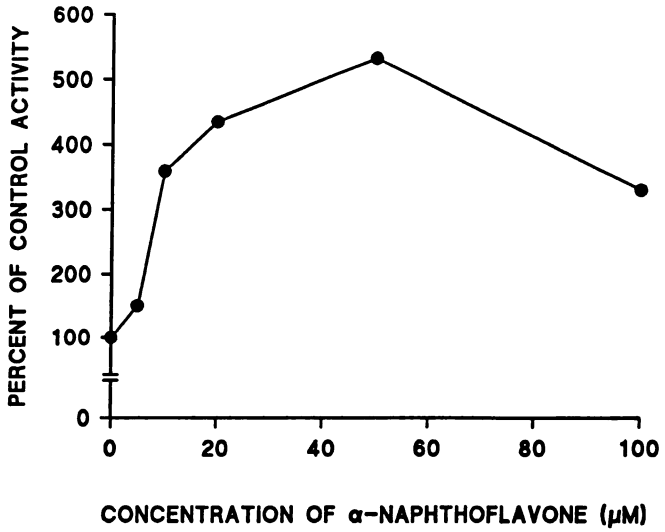

Fig. 4. Dapsone *N*-hydroxylase activity, relative to control, in the presence of increasing concentrations of α-naphthoflavone in the microsomal incubation with dapsone.

P-4503A4 enzyme purified from human liver microsomes and cDNA-expressed P-4503A4 were both able to *N*-hydroxylate dapsone, with the latter exhibiting 5-fold greater activity (0.04 and 0.20 nmol of product/min/nmol of P450, respectively). In the presence of α-naphthoflavone, cDNA-expressed P-4503A4 activity showed a 3-fold increase in dapsone *N*-hydroxylase activity, a finding that contrasted with that with P-4503A4 purified from liver microsomes, which showed no such activation.

Discussion

This study, using several different approaches, suggests that human hepatic dapsone *N*-hydroxylation is mediated by P-4503A4. Thus, a significant correlation between dapsone *N*-hydroxylase activity and both the immunochemically determined level of P-4503A4 and nifedipine oxidase activity was observed in the several microsomal preparations studied. The extensive and almost complete inhibition of microsomal metabolite formation by anti-P-4503A4, the inhibition of dapsone *N*-hydroxylation by TAO (27, 28) and gestodene (25), known inhibitors of P-4503A4, the activation by α-naphthoflavone, a known activator of P-4503A4 (14, 26), and the ability of purified microsomal and cDNA-expressed P-4503A4 to *N*-hydroxylate dapsone *in vitro* provide further supportive evidence.

The oxidation of nifedipine serves as the prototypic activity by which P-4503A4 enzyme is identified and, thus, is the activity by which this protein has been isolated for purified enzyme assays and development of antibodies (20). Recent studies have found that there is another human P-450 in the 3A subfamily, denoted P-4503A5, that exhibits significant nifedipine oxidase activity (29). This enzyme has been found in only approximately 25–30% of human livers (29–31) and is 84% identical in amino acid sequence to P-4503A4 (31). The antibodies used for the current assays, although specific to P-4503A enzymes, do not distinguish P-4503A4 and P-4503A5 (29). Thus, if P-4503A5 can *N*-hydroxylate dapsone and P-4503A5 is present in the HL-110 microsomal preparation used, this activity would also have been inhibited in the assays presented here. However, in other studies done in collaboration with Dr. S. A. Wrighton (Lilly Research Laboratories, Indianapolis, IN), sample HL-105 was found not to contain P-4503A5, as judged by assays with an adsorbed anti-P-4503A5 preparation that does not recognize P-4503A4 (29). Because this particular microsomal sample (HL-105) does not contain P-4503A5 and has also been shown to be devoid of P-4503A3 mRNA (32), it appears that P-4503A4 is the most likely enzyme responsible for dapsone *N*-oxidation in liver sample HL-105 (the catalytic activity of this preparation is the second highest of the five samples measured and falls near the value expected from consideration of nifedipine oxidation) (Fig. 1). This does not exclude a contribution from P-4503A5, because preliminary studies with a purified P-4503A5 preparation provided by Dr. Wrighton indicate that this enzyme can also catalyze dapsone *N*-oxidation.[2] However, the relative contributions of P-4503A4 and P-4503A5 *in vivo* are not clear (further *in vitro* analyses with cDNA-expressed proteins may determine the relative capabilities of these two enzymes to catalyze dapsone *N*-hydroxylation).

The finding that dapsone is *N*-hydroxylated by the P-4503A subfamily of enzymes is somewhat unexpected, in that previous *in vitro* studies, using the same methodology, have demonstrated that P-4501A2 is primarily responsible for the *N*-hydroxylation of a number of other arylamines, including many known procarcinogens (14, 15). No significant role for P-4503A4 in the *N*-hydroxylation of arylamines has previously been found, although the enzyme does appear to oxidize 6-aminochrysene to a genotoxic product (14) and contributes to the *N*-hydroxylation of 4,4′-methylene-bis(2-chloroaniline) (33). These results suggest that the P-450 enzymes do not recognize the arylamine function as a distinct chemical entity.

We have recently obtained evidence that the metabolism of dapsone to its hydroxylamine is a major determinant of intersubject variability in the total clearance of dapsone. In fact, in the subjects studied, almost 90% of the variability in dapsone clearance was accounted for by differences in the fractional metabolic clearance to dapsone hydroxylamine (24). This knowledge, combined with the present results, suggests that it may be feasible to develop a simple, single-point, noninvasive measurement of P4503A4 activity, similar to approaches previously used with other enzymes such as P-4502D6 (34) and P-4502C_{MP} (35). Such a probe may be valuable, in that the activity of P-4503A4 varies at least 10–15-fold among different individuals (14, 36, 37). Thus, an *in vivo* probe of its activity could be helpful in determining individualized doses for drugs with low therapeutic indices, such as cyclosporin (38–40), lidocaine (41, 42), and quinidine (43), that are also metabolized by P-4503A4.

Nifedipine, the first identified substrate for P-4503A4 (20), has been used as a probe for the *in vivo* activity of the enzyme (37). However, this requires repeated blood sampling to determine the areas under the plasma concentration-time curves for nifedipine and its primary metabolite. Because of this and other complicating factors, the routine use of nifedipine as an *in vivo* probe is impractical, especially for large population studies. A "breath test" has been developed that measures the $^{14}CO_2$ formed by P-4503A4-mediated *N*-demethylation of erythromycin (44). Although relatively simple, rapid, and noninvasive, this approach requires a hospital setting to administer an intravenous dose of radioactivity, factors that pose signficant limitations. Similar considerations also apply to measurement of the plasma concentration of monoethylglycinexylidide after intravenous administration of lidocaine (45). The urinary excretion of 6β-hydroxycortisol formed by P-4503A-mediated metabolism of cortisol (19, 36) has also been suggested as an approach for assessing the activity of this enzyme. However, the involvement of extrahepatic 6β-hydroxylase(s) complicates the application of this approach for assessing hepatic enzyme activity (46). Accordingly, a simple, noninvasive measurement of the conversion of dapsone to its hydroxylamine, based, for example, on the excretion of the metabolite relative to that of unchanged drug (24), would have potential advantages over current *in vivo* probes for P-4503A4 activity. Moreover, the use of dapsone as an *in vivo* probe has already been extensively demonstrated with respect to *N*-acetylation (34, 47).

A number of drugs are also known to modulate the *in vivo* activity of P-4503A4, for example, rifampicin (36, 39, 48), erythromycin (19), and TAO (49). Administration of these drugs with dapsone would be expected to alter dapsone clearance. A study in patients with leprosy has shown that coadministration of rifampicin with dapsone leads to an increased clearance of dapsone (48), consonant with the *in vitro* results

[2] Unpublished observations.

presented here and providing support for the development of a dapsone probe of P-4503A4 activity to anticipate potential drug interactions between such drugs and dapsone or other drugs known to be metabolized by P-4503A4.

Such a probe might also be useful in determining the ability of an individual to activate procarcinogens such as the aflatoxins, which are known to be activated by P-4503A4 *in vitro* (12, 14, 26), or the ability to metabolize carcinogens such as 1,6-dinitropyrene, a chemical produced in combustion reactions that is known to be inactivated by P-4503A4 *in vitro* (50). Finally, an *in vivo* probe of P-4503A4 activity would make it possible to assess whether this activity contributes to determining which patients develop dapsone-related toxicities, such as methemoglobinemia and hemolytic anemia, that can occur after the administration of therapeutic doses of dapsone (2, 10).

In conclusion, evidence obtained with human liver microsomes strongly suggests that P-4503A4 is responsible for dapsone *N*-hydroxylation in humans, and measurement of such oxidation may serve as a useful probe of P-4503A4 activity *in vivo*. If such a probe is established, it will be possible to use it under a variety of circumstances. These include dosage optimization for important drugs with low therapeutic indices, prediction of the occurrence of dapsone-related toxicities, and prediction of potential drug interactions. In addition, it may be possible to estimate relative risk for cancer in individuals known to be exposed to carcinogens that P-4503A4 is capable of activating or detoxifying.

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