The Metabolism of Pyrazoloacridine (NSC 366140) by Cytochromes P450 and Flavin Monooxygenase in Human Liver Microsomes

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ABSTRACT
Pyrazoloacridine (PZA) is an experimental antitumor agent presently under investigation for treatment of solid tumors on the basis of its unique mechanism of action and selectivity for human solid tumor xenograft in mice. Using capillary electrophoresis coupled with electrospray ionization mass spectrometry, we have identified three oxidative metabolites of PZA, 9-desmethyl-PZA, N-demethyl-PZA, and PZA N-oxide. The cytochrome P450 (CYP) isoforms involved in PZA metabolism were characterized by studies with CYP chemical inhibitors, correlation of marker activities for selected CYPs with formation of the metabolites using a human liver panel, and PZA metabolism by cDNA-expressed CYPs. 9-Desmethyl-PZA formation was catalyzed by CYP1A2, whereas N-demethyl-PZA formation was catalyzed by CYP3A4. PZA N-oxide formation was catalyzed by flavin monooxygenase (FMO) rather than CYP, as determined by studies with chemical inhibitors of FMO and metabolism by cDNA-expressed human flavin monooxygenase. After administration of [10b-14C]PZA to mice, six urinary metabolites were detected by high-performance liquid chromatography UV and radiochromatograms including 9-desmethyl-PZA, N-demethyl-PZA, and PZA N-oxide. Trace concentrations of 9-desmethyl-PZA and PZA N-oxide were detected in mouse plasma. PZA N-oxide and N-demethyl-PZA were detected in urine from patients after PZA administration. PZA, 9-desmethyl-PZA, and PZA N-oxide inhibited growth of A375 human melanoma cells. IC50 values were 0.17, 0.11, and 7.0 μM, respectively, for the three molecules.

INTRODUCTION
Pyrazoloacridine [PZA (NSC 366140)] is an acridine analog presently under investigation in Phase II clinical trials. In preclinical studies, PZA had greater cytotoxicity against solid tumor cell lines than against leukemia cell lines and selective cytotoxicity against hypoxic cells compared with nonhypoxic cells (1, 2). In vitro activity was schedule independent against early- and advanced-stage colon 38 and pancreatic carcinoma 03 tumor models. PZA was only weakly cross-resistant against multidrug-resistant tumor cells (3). In a recent study, PZA was active in multidrug-resistant neuroblastoma cell lines. That activity was substantially increased after prolonged exposure (4). PZA was equally effective against noncycling and exponentially growing Chinese hamster ovary cells (1). Consistent with this observation, PZA inhibited RNA synthesis more effectively than DNA synthesis (1). PZA inhibited topoisomerases I and II by a mechanism that is distinct from other dual topoisomerase I/II inhibitors (5), possibly due to direct enzyme inhibition (5, 6). PZA also induced apoptosis in p53-deficient Hep3B human hepatoma cells (7).

In preclinical murine studies, in vivo activity was schedule independent against early- and advanced-stage colon 38 and pancreatic carcinoma 03 tumor models (2). Central nervous system toxicity, attributed to high PZA peak plasma concentrations, was dose-limiting after bolus administration but was not observed after 4–6-h infusions. Hematological toxicity was dose-limiting when the drug was administered by 4–6-h infusion (2).

In Phase I studies, neurological toxicity was dose-limiting after a 1-h infusion, whereas hematological toxicity was dose-limiting after a 3-h (8, 9) or 24-h infusion (10). Hematological toxicity was also dose-limiting in pediatric patients given either a 1-h or 24-h infusion of PZA (11). In contrast to adults, mild neurological toxicity was observed in only 1 of 22 pediatric patients given a 1-h infusion (11). Pharmacokinetic data from the adult trials revealed rapid clearance and possibly enterohepatic recirculation of drug after i.v. PZA administration (8, 9). Substantial interpatient variation was observed for PZA pharmacokinetics in adults and children (8–11). PZA metabolism was not investigated in those Phase I studies.

PZA demonstrated modest antitumor activity against cisplatin and Taxol-resistant ovarian cancer in one of the adult Phase I trials (8, 9). In subsequent Phase II trials, one complete response was observed in a study with hormone-refractory prostate cancer patients (12), and modest activity was noted in patients with platinum-resistant (13) or recurrent platinum-sensitive ovarian cancer (14). Several other studies reported insignificant activity (15–18). PZA is presently under investigation in two Phase II trials for glioblastoma and one Phase I trial for neuroblastoma. PZA metabolism was not characterized during preclinical and Phase I development, primarily due to the difficulty in isolation and identification of putative metabolites.
We previously developed a capillary electrophoresis (CE) method that effectively separated PZA and several metabolites (19). Recent technical developments allow coupling of CE with electrospray ionization (ESI)-mass spectrometry (MS) providing a configuration ideal for rapid on-line metabolite characterization (20, 21). Using this methodology, we identified the oxidative PZA metabolites from mouse and human hepatic microsomes and characterized the reaction phenotype for those metabolites with combined data from studies with selective chemical inhibitors, correlation analysis, and metabolism by recombinant P450s and flavin monooxygenase (FMO3). We also characterized the growth inhibition of these metabolites against human tumor cell lines for comparison with parent drug cytotoxicity data.

MATERIALS AND METHODS

Chemicals and Reagents

PZA methanesulfonate and [10b-14C]PZA methanesulfonate hydrate (specific activity, 18 mCi/mmol) were provided by the Pharmaceutical Resources Branch, Division of Cancer Treatment, National Cancer Institute (Bethesda, MD). 9-Desmethyl-PZA was generously provided by Warner-Lambert (Ann Arbor, MI). Metyrapone, α-naphthoflavone, coumarin, tolbutamide, quinidine, disulfiram, erythromycin, quercitin, chlorozoxanze, phenacetin, toluamide, methimazole, paclitaxel, and sulfaphenazole dissolved in methanol; and α-naphthoflavone dissolved in methanol and inosine prepared in 0.25M sucrose (24). The pellet obtained after ultracentrifugation was resuspended in 0.15 M KCl. Protein content was determined by the Lowry method (25).

Preparation and characterization of the liver microsomal fraction. A liver microsomal fraction from 10 individual human livers with defined catalytic activities for several CYP-selective substrates was obtained from Human Biologics, Inc. (Phoenix, AZ). Microsomal suspensions were performed in the reaction buffer to achieve a final protein concentration of 1 mg/ml and incubated with PZA (100 μM) for a 30-min reaction period as described above.

In vitro metabolism by mouse liver microsomes

Mouse Liver Microsomes. Mouse liver homogenates were prepared in 0.15 M KCl or 0.25 M sucrose after excision from mice sacrificed by cervical dislocation. S-9 supernatant fractions were obtained by centrifugation (9000 × g, 10 min) of liver homogenates prepared in 0.15 M KCl. Hepatic microsomes were obtained by differential centrifugation of liver homogenates prepared in 0.25 M sucrose (24). The pellet obtained after ultracentrifugation was resuspended in 0.15 M KCl. Protein content was determined by the Lowry method (25).

In Vitro Metabolism by Mouse Liver Microsomes

Human Liver Microsomes. Human liver microsomes used to characterize PZA oxidative metabolism (HL8) were provided by Jerry M. Collins (Center for Drug Evaluation and Research, United States Food and Drug Administration, Rockville, MD). Human liver samples, medically unsuitable for liver transplantation, were acquired under the auspices of the Washington Regional Transplant Consortium (Washington, D.C.). Preparation and characterization of the liver microsomal fractions have been described previously (23).

Incubation Conditions. Incubations of human microsomal suspensions were performed in amber glass vials maintained at 37°C in a shaker bath. Each 100-μl incubation mixture contained human liver microsomes (0.1 mg of protein), NADP+ (0.4 mM), glucose-6-phosphate (25 mM), glucose-6-phosphate dehydrogenase (0.7 unit/ml), magnesium chloride (5 mM), and potassium phosphate (100 mM) buffer adjusted to pH 7.4. Control incubation mixtures contained boiled microsomes or active microsomes with a nitrogen or CO/O2 atmosphere. The incubation mixtures were preincubated for 2 min before the initiation of the reaction upon the addition of PZA. At the end of the incubation period, reactions were terminated by the addition of a 20-μl aliquot to 40 μl of ice-cold methanol. The aqueous methanol supernatants obtained after centrifugation (10,000 × g for 2 min) were diluted 1:1 with HPLC mobile phase for analysis. In selected experiments, P450-selective chemical inhibitors [caffeine metyrapone and SKF525A dissolved in water; coumarin, quinidine, erythromycin, quercitin, chlorozoxanze, phenacetin, toluamide, methimazole, paclitaxel, and sulfaphenazole dissolved in methanol; and α-naphthoflavone dissolved in methanol] were incubated with PZA (100 μM) in reaction buffer for 30 min as described above. Appropriate controls containing methanol or DMSO only were performed in parallel incubations.

Correlation of PZA Metabolism with Marker Activities of Selected Cytochrome P450 (CYP) Forms. A panel of microsomal fractions from 10 individual human livers with defined catalytic activities for several CYP-selective substrates was obtained from Human Biologics, Inc. (Phoenix, AZ). Microsomal suspensions were prepared in the reaction buffer to achieve a final protein concentration of 1 mg/ml and incubated with PZA (100 μM) for a 30-min reaction period as described above.

Metabolite Formation by cDNA-Expressed Human P450 Enzymes. Microsomal suspensions from the B-lymphoblastoid cell line AHH-1 TK+/− expressing cDNA constructs for CYP1A1, CYP1A2, CYP2B6, CYP2C8, CYP2E1, and CYP3A4 were obtained from Gentest Corporation (Woburn, MA). After dilution with the reaction buffer to achieve a final protein concentration of 1 mg/ml, microsomal suspensions were incubated with PZA (100 μM) for reaction periods of 30 and 120 min. Control reactions included microsomal preparations from cells that contained the expression vector alone.
sium chloride (5 mm) and Tris buffer (50 mm, pH 7.4). Where appropriate, nicotinamide (16.7 mm) and/or flavin-mononucleotide (0.5 mm) was added to incubation mixtures.

In some experiments, the above mixtures were incubated under reductive conditions. Air was replaced by nitrogen in incubation flasks by alternating vacuum (2 min × 3 times) with nitrogen (2 min × 3 times) via a needle through the septum of the sealed flask. After a 2-min preincubation period, drug was added to the sealed flask, and aliquots were removed at appropriate times with needle and syringe and frozen immediately for later HPLC analysis.

Nitro-reductase activity in microsomal or S-9 preparations was monitored by measuring the amount of p-aminobenzoic acid formed from p-nitrobenzoic acid by the Bratton and Marshall method (26) as modified by Fouts and Brodie (27). Microsomal or S-9 incubation aliquots were added to screw-capped glass vials that contained H2O (3 ml) and 15% trichloroacetic acid (0.8 ml) to stop the reaction and precipitate proteins. Vials were allowed to stand on ice for 5 min. Standard curve samples were prepared by serial dilution of stock p-aminobenzoic acid (1 mg/ml) such that the final concentration of p-aminobenzoic acid in the acid extracts was 0.1–1 μg/ml. After the addition of 0.1% sodium nitrate (0.4 ml) to 4 ml of the acid extract, the mixture was allowed to stand at room temperature for 3 min. After a 2-min reaction with 0.5% ammonium sulfamate (0.4 ml), 0.1% N-(1-naphthyl)ethylene diamine dihydrochloride (0.4 ml) was added to the reaction mixtures, and the absorbance of the samples was monitored spectrophotometrically at 540 nm. The unknown sample was compared with an appropriate standard curve that was treated as described above.

Urinary Excretion of PZA

Non-tumor-bearing male CD2F1 mice (20–30 g), supplied by the National Cancer Institute, were housed 5 animals/cage on commercially obtained pure wood shaving bedding in an on-site facility with light provided from 6 a.m. to 8 p.m. Food (Purina Rodent Chow) and tap water were provided ad libitum.

PZA was prepared in sterile water or normal saline, and doses of 37.5 mg/kg were administered slowly (3–5 min) via the tail vein to male CD2F1 mice. In selected experiments, mice were given [10b-14C]PZA (1 μCi/dose). Urine was collected from mice (4 mice/cage) placed in glass metabolism cages after drug administration. At the end of each 24-h collection period, the urine volume was recorded, and the samples were stored frozen (−20°C) until analysis.

Human subjects were three patients with advanced cancer participating in a Phase I trial of PZA at Wayne State University (Detroit, MI). PZA (600 mg/m2) was given by a 1-h i.v. infusion. Urine was collected for a 24-h period beginning at the start of the infusion.

Metabolite Purification

Urine was extracted with ethyl acetate/isopropanol (9:1, v/v), and the organic layer was removed, dried over magnesium sulfate, and concentrated under reduced pressure. Microsomal incubation mixtures were deproteinized by the addition of methanol (2:1). After centrifugation (1000 × g, 5 min), the supernatant was transferred to a conical centrifuge tube and evaporated to dryness under a gentle stream of nitrogen. Urine and microsomal extracts were reconstituted in HPLC mobile phase, and peak fractions with retention times corresponding to putative metabolites (determined from the elution pattern of radioactivity in mice that had received radiolabeled PZA) were pooled and concentrated to dryness. Individual peak fraction concentrates were extracted with ethyl acetate/isopropanol (9:1, v/v) and chromatographed by HPLC until the final organic extract exhibited a single peak by HPLC (usually 3 organic extraction/HPLC cycles).

Analytical Methods

Reverse-phase HPLC separations were achieved on a Lichrosorb C2 or C8 (E.M. Science) analytical column (250 × 4.0-mm inner diameter, 10-μm particles) fitted with a Brownlee RP2 (Chromtech) guard column (15 × 3.2-mm inner diameter, 7-μm particles). The mobile phase consisted of acetonitrile: tetrahydrofuran:100 mM potassium phosphate (20:5:80) adjusted to an apparent pH 4.0 with 10% phosphoric acid after mixing the solvents and was delivered at a flow rate of 1.0 ml/min. UV absorbance of the column effluent was monitored at 450 nm.

Mouse urine (0.2 ml) was incubated with 0.4 ml of 500 units/ml type VII β-glucuronidase (Sigma Chemical Co.) in 75 mM potassium phosphate (pH 6.8) overnight at 37°C to investigate the presence of glucuronide conjugates. Active β-glucuronidase in the incubation mixtures was ascertained in parallel incubations of phenolphthalein glucuronide in urine from untreated mice.

CE was performed on a Beckman P/ACE 2100 instrument through an uncoated fused silica capillary (65-cm × 50-μm inner diameter). The separation buffer consisted of methanol containing 20 mM ammonium acetate with 1% acetic acid, and the separation voltage was 30 kV. The CE capillary was maintained at ambient temperature (25°C). Capillary flow to the electrospray ion source was supplemented with a coaxial delivered liquid sheath of 60:40:1 (v/v/v) isopropanol-water-acetic acid at a flow rate of 2 μl/min through the ESI needle and served as a ground for the CE capillary as described previously (20, 21). The ESI voltage was ~3400 V. The CE-MS scan range was 80–600 Da (exponential magnet scan from low to high mass) at a rate of 2 s/decade, and the instrument resolution was ~1000. MS was performed on a Finnigan MAT 900 mass spectrometer (Bremen, Germany) of EB configuration (where E and B are electric and magnetic sectors, respectively). A position- and time-resolved ion counter PATRIC focal plane detector was used with an 8% mass window. A modified Analytica electrospray ion source (Branford, CT) operated in a positive ion mode with the needle assembly at ground potential was used throughout. The sample needle of the ESI source was replaced by the CE capillary, from which 2–3 mm of the polyamide coating at the MS end had been removed by treatment with hydrofluoric acid.

Growth Inhibition Assay

Exponentially growing A375 (human melanoma) tumor cells in DMEM (100 μl, without phenol red) with 10% FCS and 1% penicillin-streptomycin were added to each well of 96-well
cell culture plates at a cell density of 1000 cells/well and placed in an incubator (37°C, 5% CO2/95% air, 100% relative humidity) for a 24-h incubation period. Fresh medium (100 μl) containing appropriate concentrations of drugs or PZA metabolites was added to each well. After a 72-h incubation period, 50 μl of PBS were added to each well. After 4 h, medium was removed carefully from the wells, leaving 50 μl in each well, which was mixed with 150 μl of DMSO by repeated pipetting. After 5 min, absorbance at 570 nm was determined using a microtiter plate reader.

Each experiment included 6 wells/drug concentration and 6 control wells and was repeated two to three times. IC50 values were calculated as the concentration of drug required to cause a 50% decrease in absorbance by drug-treated cells compared with untreated cells.

RESULTS

Identification of PZA Oxidative Metabolites. Three oxidative metabolites were detected by HPLC analysis after incubation of PZA with NADPH-fortified human liver microsomal incubations (Fig. 1). Efforts to characterize these metabolites by HPLC/MS analysis were unsuccessful due to poor ionization under ESI and atmospheric pressure chemical ionization conditions. The CE-ESI-MS method was developed to overcome this limitation by using on-line isotachophoresis pre-concentration before separation and MS (19). Accordingly, these metabolites (also among those detected in mouse and human urine after an i.v. dose of PZA; see below) were identified by CE-ESI-MS analysis of the corresponding HPLC peak fractions. The “soft” ionization of each product as it was analyzed by on-line CE-ESI-MS yielded prominent pseudo-molecular ion (MH+) responses. In addition, the bond linking the substituted alkyl nitrogen moiety to the ring was rather weak, causing fragmentation during ionization processes. Hence, for each product, there was facile loss of the substituted alkyl nitrogen moiety. These fragment ions were used in conjunction with MH+ responses to identify the metabolic products of PZA (Fig. 2). 9-Desmethyl-PZA was identified by m/z 354 and m/z 309 ions representing MH+ and loss of the dimethylamino [-N(CH3)2] moiety, respectively (Fig. 2C). N-Demethyl-PZA was identified by m/z 354 and m/z 323 ions representing MH+

and the loss of monomethylamino [-NH(CH3)] moiety, respectively (Fig. 2B). PZA N-oxide was identified by m/z 384 and m/z 323 ions representing MH+ and loss of the -N(CH3)2 moiety, respectively (Fig. 2D).

Characterization of the in Vitro Metabolism of PZA. Metabolism of PZA by human and mouse liver microsomes was dependent on the presence of NADPH and oxygen during the incubation. In descending order, the quantity of metabolite formed by human liver microsomes was PZA N-oxide > N-demethyl-PZA > 9-desmethyl-PZA (Fig. 1). The classical P450 inhibitor SKF525A (1000 μM) almost entirely abolished N-demethyl-PZA formation and reduced 9-desmethyl-PZA formation by 40%, but it substantially increased PZA N-oxide formation. Metyrapone selectively reduced N-demethyl-PZA formation by 88%. Incubation of PZA with boiled microsomes abolished formation of all three PZA metabolites. Mild heating, which selectively inactivates FMO but not P450 (28), inhibited formation of PZA N-oxide but not of 9-desmethyl-PZA or N-demethyl-PZA. These data are consistent with P450-catalyzed metabolism of PZA to form 9-desmethyl-PZA and N-demethyl-PZA and FMO-catalyzed metabolism of PZA to form PZA N-oxide.

The kinetics of PZA N-oxide, N-demethyl-PZA, and 9-desmethyl-PZA formation by human liver microsomes are summarized in Table 1. The Km values for formation of each metabolite were similar. The Vmax value for PZA N-oxide formation was 15-fold greater than the value for 9-desmethyl-PZA formation, whereas the Vmax for N-demethyl-PZA could not be determined due to absence of an authentic standard.

Because PZA exhibits antiproliferative activity under hypoxic conditions (1) and contains a nitro (-NO2) moiety, reductive metabolism of PZA was assessed with mouse liver microsomal preparations, using the conversion of p-nitrobenzoic acid to p-aminobenzoic acid as a positive control. Reductive metabolism of PZA was not observed in those incubation mixtures as determined by either parent drug disappearance or metabolite formation detected by HPLC analysis.

Inhibition of PZA Metabolism by Form-Selective Chemical Inhibitors. To characterize the involvement of CYP and FMO in PZA metabolism by human liver microsomes, studies were conducted with selective inhibitors of the two enzyme systems. Results of microsomal PZA N- and O-demethylation and N-oxidation studies are summarized in Table 2. Chemicals with the greatest inhibitory effect on PZA metabolism were known substrates or inhibitors of CYP1A [α-naphthoflavone (29), quercitin (30)], CYP3A [α-naphthoflavone (29), erythromycin (31), quercitin (32), quinidine (33), and paclitaxel (23)], and FMO [methimazole (34)]. Specifically, 9-desmethyl-PZA formation was reduced by α-naphthoflavone and quercitin, but not by erythromycin, quinidine, paclitaxel, or methimazole. N-Demethyl-PZA formation was reduced to the greatest extent by erythromycin, quercitin, and methimazole; reduced to a lesser extent by α-naphthoflavone, quinidine, and paclitaxel, but not reduced by the selective CYP1A inhibitors phenacetin or caffeine. PZA N-oxide formation was reduced substantially by methimazole and α-naphthoflavone.

Correlation of PZA Metabolism with Marker Activities of Selected CYP Forms. Correlation of PZA metabolite formation with subfamily-selective catalytic activities within a
A panel of 10 human livers was examined to further characterize P450 subfamilies responsible for PZA metabolism (Table 3). 9-Desmethyl-PZA formation was best correlated with the CYP1A subfamily marker activities 7-ethoxyresorufin O-dealkylation ($r^2 = 0.779$) and caffeine N3-demethylation ($r^2 = 0.823$). $N$-Demethyl-PZA formation was best correlated with testosterone 6β-hydroxylation ($r^2 = 0.993$) and tolbutamide methyl-hydroxylation ($r^2 = 0.789$), marker activities of CYP3A and CYP2C subfamilies, respectively. PZA $N$-oxide formation was best correlated with coumarin 7-hydroxylation ($r^2 = 0.640$) and $S$-mephenytoin 4′-hydroxylation ($r^2 = 0.722$), marker activities of CYP2A and CYP2C subfamilies, respectively.

**Metabolism of PZA by Recombinant Human CYPs and FMO3.** PZA was incubated with selected recombinant human CYPs, based on chemical inhibition and correlation data. Of the recombinant P450s evaluated, only CYP1A2 catalyzed formation of 9-desmethyl-PZA, and only CYP3A4 catalyzed formation of $N$-demethyl-PZA (Fig. 3). None of the P450s examined (including CYP1A1, CYP1A2, and CYP2C8) catalyzed the formation of PZA $N$-oxide. However, recombinant human FMO3 catalyzed conversion of PZA to PZA $N$-oxide (Fig. 3), consistent with findings that mild heating and methimazole inhibited formation of PZA $N$-oxide, whereas certain P450 inhibitors increased PZA $N$-oxide formation.

**In Vivo Metabolism of PZA by Humans and Mice.** PZA $N$-oxide, $N$-demethyl-PZA, and 9-desmethyl-PZA (peaks C, E, and F, respectively) were among the metabolites detected in the 24 h urine of male CD2F1 mice treated with 37.5 mg/kg radiolabeled PZA (Fig. 4A). The unknown metabolite represented by peak A disappeared after incubation with $β$-glucuronidase, but concomitant appearance of a new metabolite peak or

![Fig. 2](image_url) Figure 2: Ion electropherograms of pyrazoloacridine (PZA) and into oxidative metabolites after purification of urine from humans (PZA, $N$-demethyl-PZA, and PZA $N$-oxide) and mice (9-desmethyl-PZA). A, the PZA pseudo-molecular ion was monitored at $m/z$ 368. B, the $N$-demethyl-PZA pseudo-molecular ion was monitored at $m/z$ 354. C, the 9-desmethyl-PZA pseudo-molecular ion was monitored at $m/z$ 354. D, the PZA $N$-oxide pseudo-molecular ion was monitored at $m/z$ 384. The *inset structures* illustrate the major ions observed by electrospray ionization mass spectrometry.

**Table 1.** Enzyme kinetics of PZA metabolism by human liver microsomes

<table>
<thead>
<tr>
<th>Metabolism</th>
<th>$V_{max}$ (nmol/min/mg protein)</th>
<th>$V_{max}/K_m$ (ml/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PZA $N$-oxidation</td>
<td>147 (5)</td>
<td>1.32 (0.12)</td>
</tr>
<tr>
<td>PZA 0-demethylation</td>
<td>156 (32)</td>
<td>0.089 (0.023)</td>
</tr>
<tr>
<td>PZA $N$-demethylation</td>
<td>201 (40)</td>
<td>5.66 (0.43) \times 10^{-4}</td>
</tr>
</tbody>
</table>

$^a$ PZA, pyrazoloacridine.
increased peak height of other putative metabolites was not observed by HPLC analysis. As noted earlier, HPLC/MS analysis could not be used to characterize PZA metabolism, and without authentic standards it was not possible to develop conditions to detect unknown metabolites in CE electropherograms. All detected PZA metabolites most likely contain the intact aromatic nucleus because the radiolabel was retained, and the UV absorbance spectra of each peak were similar to those of the parent drug (data not shown). The three identified PZA oxidation products were also found in the urine of cancer patients treated with 600 mg/m² PZA (Fig. 4B). Mouse plasma extracts contained each of the metabolites detected in urine as illustrated for the 5-min plasma sample (Fig. 4C).

**Growth-Inhibitory Activity of PZA and Its Metabolites.** The antiproliferative activities of PZA, 9-desmethyl-PZA, and PZA N-oxide were evaluated after incubation with A375 human melanoma tumor cells. Of note, 9-desmethyl-PZA (Table 4) was almost equally as active as parent drug with regard to growth inhibition of the A375 tumor cell line. The antiproliferative activities of PZA and 9-desmethyl-PZA were similar (Table 4). The antiproliferative activity of PZA N-oxide was 40-fold less active than that of the parent drug. We were not able to isolate adequate quantities of N-demethyl-PZA to evaluate antiproliferative activity.

**DISCUSSION**

The primary purpose of this study was to characterize the in vitro oxidative hepatic metabolism of PZA because metabolism plays a major role in plasma clearance of PZA (8, 9). In those studies, total body clearance (1000–2500 ml/min) exceeded liver blood flow, urinary recovery of parent drug was <2%, and PZA plasma profiles were reminiscent of enterohepatic recirculation. Three in vitro oxidative metabolites, 9-desmethyl-PZA, N-demethyl-PZA, and PZA N-oxide, have been identified in human and mouse liver microsomal incubations. The structure of each metabolite was determined by on-line CE-ESI-MS analysis. 9-Desmethyl-PZA and PZA N-oxide were characterized by ESI-MS/MS analysis. 9-Desmethyl-PZA and PZA N-oxide were characterized by ESI-MS/MS analysis.

**Table 2** Chemical inhibition of PZA metabolism

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>CYP</th>
<th>Inhibitor (mM)</th>
<th>Metabolite formation (% of control)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>9-Des methyl-PZA</td>
</tr>
<tr>
<td>α-Naphthoflavone</td>
<td>1A, 3A</td>
<td>20</td>
<td>74</td>
</tr>
<tr>
<td>Caffeine</td>
<td>1A, 3A</td>
<td>200</td>
<td>54</td>
</tr>
<tr>
<td>Coumarin</td>
<td>2A, 2D, 3A</td>
<td>20</td>
<td>86</td>
</tr>
<tr>
<td>Quinidine</td>
<td>2D, 3A</td>
<td>200</td>
<td>91</td>
</tr>
<tr>
<td>Chlorozoxazone</td>
<td>2E, 1A</td>
<td>200</td>
<td>96</td>
</tr>
<tr>
<td>Tolbutamide</td>
<td>2C</td>
<td>200</td>
<td>84</td>
</tr>
<tr>
<td>Sulfaphenazole</td>
<td>2C</td>
<td>200</td>
<td>99</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>3A</td>
<td>200</td>
<td>102</td>
</tr>
<tr>
<td>Quercitin</td>
<td>3A, 1A</td>
<td>200</td>
<td>92</td>
</tr>
<tr>
<td>Methimazole</td>
<td>FMO</td>
<td>200</td>
<td>92</td>
</tr>
</tbody>
</table>

* PZA, pyrazoloacridine; CYP, cytochrome P450; FMO, flavin monooxygenase.

* Values in the table represent metabolite formation in the presence of inhibitors as a percentage of metabolite formation in the absence of inhibitors after incubation of PZA with human liver microsomes.

* CYP isoforms inhibited by the chemicals listed in column 2. Methimazole is an inhibitor of FMO, but not CYP.

* If <15% inhibition at the high concentration, data for low concentration are not shown.

**Table 3** Correlation of PZA metabolism with marker activities of selected CYP subfamilies in a panel of 10 human liver microsomal preparations

<table>
<thead>
<tr>
<th>Compound</th>
<th>Correlation coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>7-Ethoxyresorufin O-dealkylation (CYP1A)</td>
<td>0.779</td>
</tr>
<tr>
<td>Caffeine N-demethylation (CYP1A)</td>
<td>0.823</td>
</tr>
<tr>
<td>Coumarin 7-Hydroxylation (CYP2A)</td>
<td>0.457</td>
</tr>
<tr>
<td>Tolbutamide methyl-hydroxylation (CYP2C)</td>
<td>0.230</td>
</tr>
<tr>
<td>S-Mephenytoin 4-hydroxylation (CYP2C)</td>
<td>0.256</td>
</tr>
<tr>
<td>Dextromethorphan 0-demethylation (CYP2D)</td>
<td>0.256</td>
</tr>
<tr>
<td>Chlorozoxazone 6-hydroxylation (CYP2E)</td>
<td>0.204</td>
</tr>
<tr>
<td>Testosterone 6p-hydroxylation (CYP3A)</td>
<td>0.594</td>
</tr>
<tr>
<td>Lauric acid 12-hydroxylation (CYP4A)</td>
<td>0.169</td>
</tr>
</tbody>
</table>

* PZA, pyrazoloacridine; CYP, cytochrome P450.

* Values in the table represent metabolite formation in the presence of inhibitors as a percentage of metabolite formation in the absence of inhibitors after incubation of PZA with human liver microsomes.
oxide were further identified by similarities of chromatographic retention times, electrophoretic mobilities, and UV absorbance spectra with authentic standards. A synthetic standard was not available for N-demethyl-PZA. NADPH- and oxygen-dependent disappearance of PZA from microsomal incubation mixtures was consistent with a role for CYP in the metabolism of PZA. Heat-sensitive and NADPH- and oxygen-dependent formation of PZA N-oxide was indicative of a role for FMO in the metabolism of PZA. We did not observe reductive metabolism of PZA by mouse liver nitroreductase.

CYP1A2 was the predominant enzyme responsible for 9-demethyl-PZA formation, based on strong inhibition by the CYP1A-selective inhibitor α-naphthoflavone, high correlations between 9-demethyl-PZA formation and two CYP1A marker activities (7-ethoxyresorufin O-dealkylation and caffeine N3-demethylation), and the observation that CYP1A2 was the only recombinant CYP to catalyze formation of 9-demethyl-PZA.

The predominant enzyme responsible for N-demethyl-PZA formation was CYP3A4, based on selective inhibition by the CYP3A-selective inhibitors erythromycin, quinidine, and quercitin; a high correlation with the CYP3A4 marker activity testosterone 6β-hydroxylation; and the observation that CYP3A4 was the only human cDNA-expressed CYP to catalyze N-demethylation of PZA. Enzymes in the CYP2C subfamily may have a modest role in N-demethyl-PZA formation because quinidine, quercitin, and paclitaxel inhibit CYP2C isoforms and there was a correlation with tolbutamide methyl hydroxylation (a marker for CYP2C activity).

Neither inhibitor, correlation, nor cDNA studies clearly defined a P450 catalytic activity responsible for PZA N-oxide formation. PZA N-oxide formation was inhibited by α-naphthoflavone and quercitin, but not other CYP1A, CYP3A, and CYP2C subfamily-selective inhibitors. Moreover, PZA N-oxide formation was increased during incubation with the classical P450 inhibitor SKF525A correlated with coumarin 7-hydroxylation and S-mephenytoin 4′-hydroxylation, markers for CYP2A6 and CYP2C19, respectively, and PZA N-oxide was absent after incubation with recombinant CYP3A4.

Given the conflicting findings, we investigated the catalytic activity of FMO, a NADPH-dependent enzyme that catalyzes N-oxidation. PZA N-oxide formation was inhibited by mild heating and methimazole, both of which inhibit FMO (34). Most importantly, PZA N-oxide, but not 9-demethyl-PZA or N-demethyl-PZA, was detected in incubations with recombinant human FMO3. These observations are consistent with FMO catalytic activity for PZA N-oxidation.

Whereas the in vitro metabolism data are consistent with CYP1A2, CYP3A4, and FMO3 in the oxidative metabolism of PZA, the urinary excretion data clearly implicate additional metabolic pathways for PZA metabolism. Palomino et al. (35) detected a PZA reduction product, 5-aminopyrazoloacridine, in mouse urine. In our studies, PZA was not metabolized under reductive conditions. Because p-aminobenzoic acid formed p-nitrobenzoic acid in parallel reactions, it would appear that PZA is not metabolized by mouse hepatic nitroreductase. These observations suggest that intestinal microflora catalyze the reduction of the PZA nitro moiety because other investigators found that nitroreductase activity present in isolated cecal contents was much greater than that present in the liver (36, 37). We were also unable to identify PZA conjugates in mouse urine based on enzyme hydrolysis studies.

Interindividual variation in drug metabolism and differences in biological activity of the resulting metabolites may contribute to differences among patients in efficacy and/or toxicity of anticancer agents (38). In this regard, the equivalent cytotoxicity of PZA and 9-demethyl-PZA suggests that PZA activity is probably maintained after metabolism by CYP1A2 to the 9-demethyl metabolite. In contrast, the markedly reduced cytotoxicity of PZA N-oxide compared with the parent molecule suggests that oxidation by FMO3 may contribute to deactivation of PZA. Furthermore, FMO3 exhibits high levels of expression in brain, and enzyme variants with low catalytic activity have been identified (39). Because primate studies found that PZA crosses the blood-brain barrier (40), individuals that have low FMO3 activity variants may be more susceptible to PZA-related neurological toxicity.

In conclusion, we characterized the in vitro metabolism of PZA by mouse and human liver microsomes. PZA N-oxide and N-demethyl-PZA were the major products of NADPH-dependent human liver microsomal metabolism of PZA.Formation of

### Table 4 Cytotoxicity of PZA and its metabolites against A375 tumor cells

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>IC₅₀ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doxorubicin</td>
<td>0.128 ± 0.03</td>
</tr>
<tr>
<td>PZA</td>
<td>0.171 ± 0.01</td>
</tr>
<tr>
<td>9-Desmethyl PZA</td>
<td>0.114 ± 0.02</td>
</tr>
<tr>
<td>PZA N-Oxide</td>
<td>7.10 ± 1.2</td>
</tr>
</tbody>
</table>

*PZA, pyrazoloacridine.*
these metabolites was catalyzed by FMO3 and CYP3A4, respectively. 9-Desmethyl-PZA was a minor metabolite, and formation was catalyzed by CYP1A2. Whereas N-oxidation is a deactivation pathway, and PZA antiproliferative activity is maintained after O-demethylation, the role of N-demethylation in the activity of PZA is presently unknown. Based on these data, we have incorporated pharmacokinetic studies in two clinical trials of PZA. A Phase I/II trial of the combination of PZA and carboplatin for patients with recurrent glioma, initiated based on in vitro synergy between the two drugs against T98G human glioblastoma cells (41), incorporated pharmacokinetic studies to investigate the effect of enzyme-inducing anticonvulsants on the disposition of PZA and its metabolites. A Phase I trial of PZA and stem cell or bone marrow transplantation to treat children with
ACKNOWLEDGMENTS

We thank Dr. David E. Zembower for technical assistance and Wanda L. Rhodes for preparation of the manuscript.

REFERENCES


The Metabolism of Pyrazoloacridine (NSC 366140) by Cytochromes P450 and Flavin Monooxygenase in Human Liver Microsomes

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