HIGHLY SELECTIVE INHIBITION OF HUMAN CYP3A IN VITRO BY AZAMULIN AND EVIDENCE THAT INHIBITION IS IRREVERSIBLE

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ABSTRACT:

Azamulin [14-*O*-(5-(2-amino-1,3,4-triazolyl)thioacetyl)-dihydromutilin] is an azole derivative of the pleuromutilin class of anti-infectives. We tested the inhibition potency of azamulin toward 18 cytochromes P450 using human liver microsomes or microsomes from insect cells expressing single isoforms. In a competitive inhibition model, IC_{50} values for CYP3A (0.03–0.24 μM) were at least 100-fold lower than all other non-CYP3A enzymes except CYP2J2 ($\sim$50-fold lower). The IC_{50} value with heterologously expressed CYP3A4 was 15-fold and 13-fold less than those of CYP3A5 and CYP3A7, respectively. The reference inhibitor ketoconazole was less selective and exhibited potent inhibition (IC_{50} values <10 μM) for CYP1A1, CYP1B1, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP4F2, and CYP4F12. Inhibition of CYP3A by azamulin appeared sigmoidal and well behaved with the substrates 7-benzyloxy-4-trifluoromethylcoumarin, testosterone, and midazolam. Preincubation of 4.8 μM azamulin in the presence of NADPH for 10 min inhibited $\sim$95% of testosterone 6β-hydroxylase activity compared with preincubation in the absence of NADPH. Catalytic activities of CYP1A2, CYP2C8, CYP2C9, CYP2C19, CYP2D6, and CYP2E1 were unaffected by similar experiments. Incubation of azamulin with heterologously expressed CYP3A4 yielded a type I binding spectrum with a spectral dissociation constant of 3.5 μM, whereas no interaction was found with CYP2D6. Azamulin exhibited good chemical stability when stored in acetonitrile for up to 12 days. Aqueous solubility was found to be >300 μM. Azamulin represents an important new chemical tool for use in characterizing the contribution of CYP3A to the metabolism of xenobiotics.

Along with other experimental approaches, enzyme-selective chemical inhibitors are commonly used in reaction phenotyping studies to determine cytochrome P450 isoform contribution to a metabolic reaction (Clarke, 1998). Chemical inhibitors provide a simpler and more cost-effective alternative to immunoinhibitory antibodies and can be used in cells. However, proper use of chemicals may require foreknowledge of the reaction kinetics under investigation. For example, competitive inhibitors should be used with a substrate concentration near or below the apparent K_M. In addition, specificity is often lost when the inhibitor concentration is too high. The effect of microsomal protein concentration and incubation time may also need to be considered, if the inhibitor is also a substrate.

Chemicals used as selective inhibitors of CYP3A include tri-acetyloleandomycin, gestodene, and ketoconazole. Ketoconazole is most widely used, probably because of advantages in potency, selectivity, commercial availability, and ease of use (e.g., preincubation steps are not required) (Maurice et al., 1992; Baldwin et al., 1995; Newton et al., 1995; Bourrie et al., 1996; Sai et al., 2000; Zhang et al., 2002). However, selectivity of ketoconazole for CYP3A is often less than ideal. For example, CYP1B1, CYP2B6, and CYP2C8/9/19 en-zymes are significantly inhibited ($\sim$20% to $\sim$60%) at concentrations needed to inhibit CYP3A[1] by $\sim$ 95% (Newton et al., 1995; Moody et al., 1999; present results). Additionally, ketoconazole inhibits CYP1A1 with potency similar to that of CYP3A (Paine et al., 1999; Sai et al., 2000), a finding that complicates interpretation when addressing CYP3A involvement in tissues expressing CYP1A1, such as intestine and lung. Triacetyloleandomycin, a mechanism-based inhibitor, has been demonstrated to be highly selective for CYP3A in HLMs[2] among a panel of five P450 enzyme activities examined (Newton et al., 1995), and Chang et al. (1994) found that triacetylole-

[1] Unless otherwise specified, the term "CYP3A" refers to CYP3A4 and CYP3A5; other members of this subfamily include CYP3A7 and CYP3A43. It is generally accepted that only CYP3A4 and CYP3A5 can be found in liver at detectable levels and that, of the two, CYP3A4 is predominant in terms of abundance and importance in drug metabolism (Wrighton and Thummel, 2000). The relative contribution of CYP3A5 to the metabolism of drugs is considered to be much less but is an area of active research.

[2] Abbreviations used are: HLM, human liver microsome; P450, cytochrome P450; DBF, dibenzylfluorescein; AMMC, 3-[2-(*N,N*-diethyl-*N*-methylamino)ethyl]-7-methoxy-4-methylcoumarin; BFC, 7-benzyloxy-4-trifluoromethylcoumarin; CEC, 3-cyano-7-ethoxycoumarin; MFC, 7-methoxy-4-trifluoromethylcoumarin; AAA, 94% acetonitrile/6% glacial acetic acid; HPLC, high performance liquid chromatography; TAO, triacetyloleandomycin; DMSO, dimethyl sulfoxide; LTB_4, leukotriene B_4; LTB_4 20-OH, 20-hydroxyleukotriene B_4; $K_{s\ APP}$, apparent spectral dissociation constant; MIC, metabolite intermediate complex; KTZ, ketoconazole; AZA, azamulin.
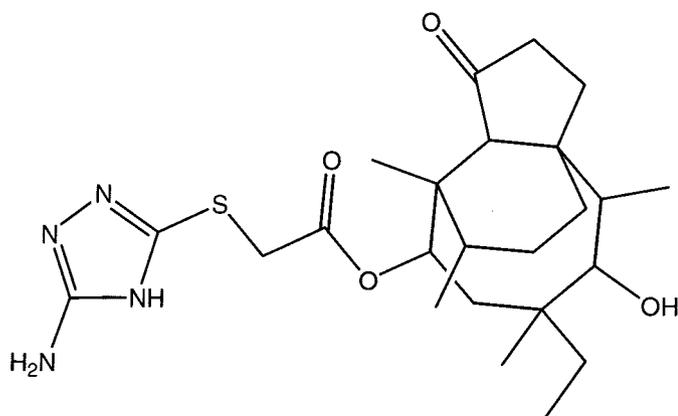
FIG. 1. *Structure of azamulin.*

andomycin selectively inhibited CYP3A4 and CYP3A5 in a panel of cDNA-expressed enzymes using ethoxycoumarin as a substrate. However, relatively high concentrations and long preincubation periods are required to achieve selectivity, and the extent of CYP3A inhibition usually plateaus at 75 to 80% even at concentrations up to 400 μM (Newton et al., 1995; Clarke, 1998, and references therein). Gestodene is another mechanism-based inhibitor shown to be selective for CYP3A (Guengerich, 1990; Newton et al., 1995); however, it has not been as well characterized and, to our knowledge, is not commercially available.

Azamulin [14-*O*-(5-(2-amino-1,3,4-triazolyl)thioacetyl)-dihydromutilin] (Fig. 1) was recently suggested as a highly selective and potent human CYP3A inhibitor (Clarke, 2000). It is a synthetic azole derivative of pleuromutilin, a diterpene antibiotic first isolated from the fungus *Pleurotus mutilis* (now termed *Clitopilus scyphoides*) (Kavanagh et al., 1951). Other members of the pleuromutilin class include the veterinary drugs tiamulin and valnemulin. The objectives of the current work were to examine the suitability of azamulin as a selective inhibitor of human CYP3A in vitro.

Materials and Methods

Chemicals and Enzymes. Pooled human liver microsomes or microsomes from baculovirus-infected insect cells (BD Supersomes enzymes) were obtained from BD Biosciences (Woburn, MA). Supersomes supplemented with cytochrome b_5 were used when available in this format. Azamulin, dibenzylfluorescein (DBF), 3-[2-(*N*,*N*-diethyl-*N*-methylamino)ethyl]-7-methoxy-4-methylcoumarin (AMMC), 7-benzyloxy-4-trifluoromethylcoumarin (BFC), 7-hydroxy-4-trifluoromethylcoumarin, 6α-hydroxypaclitaxel, 4′-hydroxydiclofenac, bupropion, hydroxybupropion, and ($\pm$)-bufuralol were obtained from BD Biosciences. The 7-benzyloxyresorufin, 3-cyano-7-ethoxycoumarin (CEC), 7-methoxy-4-trifluoromethylcoumarin (MFC), ketoconazole, paclitaxel, ($\pm$)-1′-hydroxybufuralol, midazolam, and 1′-hydroxymidazolam were obtained from Ultrafine Chemicals (Manchester, UK). [^{14}C]-(S)-Mephenytoin and [^{14}C]lauric acid were obtained from Amersham Biosciences (Piscataway, NJ). Leukotriene B$_4$ and 20-hydroxyleukotriene B$_4$ were obtained from Cayman Chemical (Ann Arbor, MI). All other chemicals (reagent grade) were obtained from Sigma-Aldrich (St. Louis, MO).

Enzyme Inhibition Assays. *Homogeneous assays with fluorometric substrates.* Assays were conducted in 96-well microplates in duplicate based on the method of Crespi et al. (1997), and as later modified (Crespi et al., 2002). Potassium phosphate buffer, pH 7.4, or Tris-Cl, pH 7.5 containing an NADPH-regenerating system [final concentrations were 1.3 mM NADP+ (or 0.008 mM for AMMC or 0.065 mM for coumarin), 3.3 mM glucose 6-phosphate (or 0.41 mM for AMMC), 0.4 U/ml glucose-6-phosphate dehydrogenase, 3.3 mM magnesium chloride (or 0.41 mM for AMMC)], and control protein from mock-transfected insect cells was added to each well in a volume of 0.1 ml. The inhibitor was then added in acetonitrile (final concentration ≤2%; for CYP2E1 0.5%) and diluted serially 1:3 to provide eight concentrations. The

plate was then warmed to 37°C and the reaction initiated by the addition of 0.1 ml of prewarmed enzyme/substrate mix. The enzyme/substrate mix contained buffer, P450 enzyme, control protein (to standardize final protein concentration to 0.25 mg/ml), and substrate. The final buffer concentration was 100 mM except that 25, 50, 50, and 200 mM potassium phosphate were used for CYP2C8, CYP2C9, CYP2C19, and CYP3A4/5/7, respectively. Reactions were terminated by addition of 75 μl of 80:20 acetonitrile/0.5 M Tris base or, for DBF only, 2 N NaOH. Other assay parameters are provided for each enzyme/substrate pair as shown in Table 1. The substrate concentrations chosen were at or usually within 3-fold of the apparent K_M. In some experiments, the temporal changes in IC$_{50}$ values were monitored using BFC as a substrate probe and either CYP3A4 Supersomes or a mixture of liver microsomes from two donors expressing relatively high levels of CYP3A and low levels of CYP1A2 protein (BD Gentest catalog numbers 452112 and 452003). The final concentration of HLM protein was 25 μg/ml and of Supersomes, 5 nM. Under these conditions, BFC turnover rates for HLMs and Supersomes were similar. In those experiments, no stop solution was added and the plates were read repeatedly within the fluorometer instrument while maintained at 37°C.

Conventional probe substrate assays. The percentage of inhibition by ketoconazole and azamulin with several P450 isoforms was determined in duplicate using pooled human liver microsomes ($n = 22$ donors) or Supersomes in a final volume of 0.5 ml for CYP2E1, 0.1 ml for CYP4A11, 0.05 ml for CYP4F2 and CYP4F3b, 0.2 ml for CYP2J2, CYP4F12, and CYP3A4/terfenadine, and 0.25 ml for all other enzymes. Reactions contained 1.3 mM NADP+, 3.3 mM glucose 6-phosphate, 0.4 U/ml glucose-6-phosphate dehydrogenase, 3.3 mM MgCl$_2$, and 50 mM (for CYP2B6, CYP2C19, CYP4F2, and CYP4F3b) or 100 mM (for CYP1A2, CYP2C8 and CYP2D6, CYP2E1, CYP2J2, CYP3A4/terfenadine, CYP3A4/5, and CYP4F12) potassium phosphate buffer, pH 7.4. For non-CYP3A enzymes, the three concentrations of inhibitor tested were 1 μM, 10 μM, and 100 μM in duplicate (except CYP2B6 and CYP2E1, where the highest concentration was 50 μM). For CYP3A4/5, the concentrations tested were 5000, 1667, 556, 185, 62, 21, 6.9, 2.3, and 0.8 nM. Assays for CYP2C9 were carried out in 100 mM Tris buffer, pH 7.5. To determine the inhibition of CYP1A2, phenacetin (50 μM) was incubated with 0.4 mg/ml HLMs for 15 min and the reaction was stopped by the addition of 0.05 ml of acetonitrile; for CYP2B6 inhibition, bupropion (80 μM) was incubated with 0.2 mg/ml HLMs for 20 min and the reaction was stopped by the addition of 0.025 ml of 70% perchloric acid; for CYP2C8 inhibition, paclitaxel (10 μM) was incubated with 0.4 mg/ml HLMs for 20 min and the reaction was stopped by the addition of 0.5 ml of acetonitrile; for CYP2C9 inhibition, diclofenac (6 μM) was incubated with 0.026 mg/ml HLMs for 10 min and the reaction was stopped by the addition of 0.05 ml of AAA; for CYP2C19 inhibition, *S*-mephenytoin (50 μM) was incubated with 0.8 mg/ml HLMs for 30 min and the reaction was stopped by the addition of 0.05 ml of acetonitrile; for CYP2D6 inhibition, bufuralol (10 μM) was incubated with 0.4 mg/ml HLMs for 20 min and the reaction was stopped by the addition of 0.025 ml of 70% perchloric acid; for CYP2E1 inhibition, *p*-nitrophenol (100 μM) was incubated with 0.4 mg/ml HLMs for 20 min and the reaction was stopped by the addition of 0.1 ml of 20% trichloroacetic acid. The supernatant (0.5 ml) was added to 0.25 ml of 2 N NaOH and the absorbance was measured at 535 nm; CYP3A4/5 with testosterone substrate (120 μM) was incubated with 0.2 mg/ml HLMs for 10 min and the reaction was stopped by the addition of 0.125 ml of acetonitrile; CYP3A4 with midazolam substrate (6 μM) was incubated with 0.05 mg/ml HLMs for 10 min and the reaction was stopped by the addition of 0.05 ml of AAA containing 0.2 μM diazepam; CYP4A11 with lauric acid (10 μM) was incubated with 0.8 mg/ml HLMs for 10 min and the reaction was stopped by the addition of 0.05 ml of AAA; CYP4F2 and CYP4F3b, with leukotriene substrate (30 μM), was incubated with 100 pmol/ml CYP4F2 or 50 pmol/ml CYP4F3b, both for 10 min. Reactions were terminated with the addition of 0.015 ml of AAA. Incubations for CYP2J2, CYP3A4, and CYP4F12 terfenadine hydroxylation consisted of Supersomes (1.5 pmol/ml CYP2J2, 7.5 pmol/ml CYP4F12, and 15 pmol/ml CYP3A4) and terfenadine (1.5 μM). Incubations were carried out for 10 min at 37°C and were terminated by the addition of 50 μl of acetonitrile. After stopping the reactions, incubations were subjected to centrifugation at 14,000 rpm for 3 min to pellet the protein, and the supernatants were retained for HPLC analysis.

Time- and NADPH-dependent inhibition. Incubations were performed in 0.1

TABLE 1

Summary of enzyme inhibition selectivity exhibited by azamulin or ketoconazole in a competitive inhibition model

Enzyme	Enzyme Source	Protein Concentration[a]	Incubation Time	Substrate	[S]	K_M[b]	No. of Inhibitor Concentrations[c]	Upper Concentration[d]	IC$_{50}$ KTZ	IC$_{50}$ AZA	IC$_{50}$Ratio, AZA/KTZ
		min				*μM*			*μM*		
CYP1A1	Supersomes	12.5	30	7-Benzyloxy-resorufin	12.5	12.4	8	50	0.017	>50	>2900
CYP1A2	Supersomes	1	15	CEC	2.5	1.8	8	50/200	>50	>200	–
CYP1A2	HLMs	0.4	15	Phenacetin	50	20	3	100	55	>100	>1.8
CYP1B1	Supersomes	12.5	15	7-Benzyloxyresorufin	12.5	13	8	50	5.6	>50	>8.9
CYP2A6	Supersomes	5	10	Coumarin	3.0	0.7	8	50	>50	>50	–
CYP2B6	Supersomes	5	30	EFC	2.5	2	8	50	6.3	>50	>7.9
CYP2B6	HLMs	0.2	20	Bupropion	80	79	3	50	ND	>50	–
CYP2C8	Supersomes	20	30	DBF	1.0	1.4	8	200	4.4	41	9.3
CYP2C8	HLMs	0.4	20	Paclitaxel	10	4	3	100	8.6	73	8.5
CYP2C9	Supersomes	10	45	MFC	50	55	8	200	5.1	57	11.2
CYP2C9	HLMs	0.026	10	Diclofenac	6	2.9	3	100	9	>100	>11
CYP2C19	Supersomes	5	30	CEC	6.0	5.3	8	200	13	47	3.6
CYP2C19	HLMs	0.8	30	S-mephenytoin	50	43	3	100	57	>100	>1.8
CYP2D6	Supersomes	5	30	AMMC	0.5	0.25	8	200	ND	>200	–
CYP2D6	HLMs	0.4	20	Bufuralol	10	15	3	100	67	>100	>1.5
CYP2E1	Supersomes	10	45	MFC	100	<200	8	50	ND	>50	–
CYP2E1	HLMs	0.4	20	p-Nitrophenol	100	45	3	50	>50	>50	–
CYP2J2	Supersomes	1.5	10	Terfenadine	1.5	0.4	4	100	4.6	6.6	1.4
CYP3A4	Supersomes	5	30	BFC	50	40	8	5	0.023	0.026	1.1
CYP3A4	Supersomes	15	10	Terfenadine	1.5	1.9	4	100	<0.1[e]	0.12	1.6
CYP3A4/5	HLMs	0.2	10	Testosterone	120	53	8	5	0.06	0.24	4.0
CYP3A4/5	HLMs	0.05	10	Midazolam	6	3	8	5	0.04	0.15	3.7
CYP3A5	Supersomes	40	30	BFC	50	33	8	5	0.11	0.38	3.5
CYP3A7	Supersomes	25	45	BFC	50	73	8	5	0.16	0.34	2.1
CYP4A11	HLMs	0.8	10	Lauric acid	10	4.6	3	100	>100	>100	–
CYP4F2	Supersomes	100	10	LTB$_4$	30	90	3	100	<1[f]	46	57
CYP4F3b	Supersomes	50	10	LTB$_4$	30	30	3	100	22	>100	>4.5
CYP4F12	Supersomes	7.5	10	Terfenadine	1.5	1.0	4	100	0.74	29	39

ND, not done; dashes, not applicable.

[a] Units are in mg protein/ml or pmol/ml when HLMs or Supersomes, respectively, are the enzyme source.

[b] K_M values were determined in this laboratory; some of them have been reported previously (Miller et al., 1997).

[c] Spacing between inhibitor concentrations was 1:10, 1:10, or 1:3 when 3, 4, or 8 concentrations, respectively, were tested.

[d] If two values are shown, the first value represents the upper concentration of KTZ; otherwise, concentrations were the same for both KTZ and AZA.

[e] The IC$_{50}$ was not bracketed by two non-zero concentrations and therefore was not calculated; 0.1 μM KTZ inhibited this activity by 68%.

[f] The IC$_{50}$ was not bracketed by two non-zero concentrations and therefore was not calculated; 1 μM KTZ inhibited this activity by 62%.

M potassium phosphate (pH 7.4) with 1 mg/ml HLM protein (50:50 pool of two donors; BD Gentest catalog numbers 452112 and 452089, rich in CYP3A) with and without an NADPH-generating system (as described above). Solvent only, and two concentrations of azamulin, 4.8 μM and 9.6 μM, representing 20 and 40 times the IC$_{50}$ values, were tested in duplicate. Triacetyloleandomycin (TAO; 20 μM) was used as a positive control. After 0, 5, 10, and 20 min of incubation (0, 5, and 10 min only for the TAO), 0.05 ml was removed and added to 0.95 ml of testosterone assay mixture (0.1 M potassium phosphate with 1.3 mM NADP+, 3.3 mM glucose 6-phosphate, 0.4 U/ml glucose-6-phosphate dehydrogenase, 3.3 mM magnesium chloride, and 200 μM testosterone). After 10 min of incubation, the reaction was stopped by the addition of 500 μl of acetonitrile, and the mixture was centrifuged for 3 min. A portion of the supernatant was analyzed as described below. This same approach was carried out for CYP3A4 and several other enzymes using azamulin concentrations of 5 μM and 10 μM in HLMs (pool of 22 donors) and a fixed preincubation period of 10 min. After the preincubation period, the mixture was diluted 1:20 (except CYP2C19, which was diluted 1:2), and the reactions were carried out as described above. The probe substrate concentrations were as described above except that the midazolam and S-mephenytoin concentrations were increased to 50 μM and 200 μM, respectively.

Solubility. Aqueous solubility was evaluated by light scatter analysis of precipitation using a BD Gentest solubility scanner (BD Biosciences). Azamulin was initially dissolved in DMSO at 30 mM. Subsequently, a series of nine secondary DMSO stock solutions were made from the original stock by diluting 1:2 in DMSO. The resulting stock solutions, ranging from 30 mM to 0.06 mM, were then transferred to 100 mM potassium phosphate, pH 7.4 (1:100 dilution), with mixing and evaluated for compound precipitation after 30 min. The DMSO dilution and the aqueous transfer steps were performed using an automated liquid handler (Tecan Genesis; Tecan, Durham, NC). The solubility scanner was set to individually mix each sample before injection (3× mix, medium intensity). The minimum criterion for precipitation was set to 3-fold above the background signal (aqueous buffer with 1% DMSO).

Metabolic stability analysis. Assays were performed in a final volume of 1 ml containing 50 mM potassium phosphate with an NADPH-generating system (as described above). Liver microsomal protein concentrations were 0.2, 0.5, 1.0, or 2.0 mg/ml. Incubations were performed in duplicate with 1 μM ketoconazole or 5 μM azamulin. The acetonitrile solvent concentration was kept constant at 1%. At 0, 30, 60, or 120 min, aliquots of the incubation mixture were added to a 1.5× volume of acetonitrile containing the internal standard carbamazepine and subjected to centrifugation at 14,000g to pellet the protein.

Chemical stability. Azamulin was dissolved in acetonitrile at a concentration of 1 mM and stored at room temperature or at −20°C for 12 days before analysis. The relative stability of azamulin in the stored samples was compared with freshly prepared stocks by liquid chromatography/mass spectrometry analysis of six replicate samples per condition as described below.

Analytical. Metabolite quantification for CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A4/testosterone assays was performed as described previously (Busby et al., 1999). Analysis for the CYP3A4-catalyzed midazolam 1′-hydroxylase assay (Stresser et al., 2000) and CYP4A11-catalyzed lauric acid 12-hydroxylase assay (Crespi et al., 1998) was carried out as previously described. For the CYP4F2 and CYP4F3b assays, a portion of the supernatant was injected directly into a Zorbax (SB) 4.6 mm × 250 mm 5-μm C18 HPLC column. The column was kept at 45°C. The HPLC mobile phases were 30% acetonitrile containing 2 mM perchloric acid (mobile phase A) and 100% methanol (mobile phase B). LTB$_4$ metabolites were separated with initial conditions of 100% mobile phase A, followed by a linear increase in mobile phase B to 70% over a 20-min period. The HPLC flow rate was maintained at

1 ml/min. The product was detected by absorbance at 270 nm and quantitated by comparison to the absorbance of a standard curve for LTB$_4$ 20-OH. The terfenadine alcohol metabolite was separated using HPLC with mobile phases consisting of 0.1% trifluoroacetic acid in water (solvent A) and 0.1% trifluoroacetic acid in acetonitrile (solvent B). Initial HPLC solvent conditions were 90% solvent A and 10% solvent B. The alcohol metabolite was eluted with a linear increase in solvent B to 100% over a 16-min period. The product was detected by UV absorbance monitored at 212 nm. The response was quantitated by comparison to a standard curve of terfenadine alcohol metabolite. For the fluorometric assays in 96-well plates, the fluorescence signal of metabolites was measured using a FLUOstar model 403 fluorescence plate reader (BMG Labtechnologies, Inc., Durham, NC) with appropriate excitation and emission wavelength settings (Stresser et al., 2002). The IC$_{50}$ values were determined by linear interpolation with three, four, or eight concentration point curves, where concentrations were spaced by log, log, or half-log units, respectively. Using those points bracketing 50% inhibition, the IC$_{50}$ values were calculated as follows: IC$_{50}$ = [(50 − A)/(B − A)] × (D − C + C, where A = the first point on the curve, expressed in percentage inhibition, that is less than 50%; B = the first point on the curve, expressed in percent inhibition, that is greater than or equal to 50%; C = the concentration of inhibitor that gives A% inhibition; and D = the concentration of inhibitor that gives B% inhibition. The suitability of using an abbreviated curve for calculating IC$_{50}$ values has been discussed elsewhere (Moody et al., 1999). For metabolic and chemical stability analysis, the HPLC system consisted of a Waters 2790 separations module (Waters, Milford, MA) and was operated in sequential mode. A C18 reverse-phase column (Waters, Symmetry; 4.6 × 50 mm, 3.5 μm) was used to chromatograph the inhibitors incorporating the solvents and gradient as follows: solvent A, 10% acetonitrile, 0.1% formic acid; solvent B, 100% acetonitrile, 0.1% formic acid; 10% B to 100% B in 2.0 min followed by 100% B until 2.25 min before returning to starting conditions at 2.35 min. The column was equilibrated until 3.35 min before injecting the next sample. Ketoconazole and azamulin were analyzed using a Micromass Inc. (Beverly MA) ZMD single quadrupole mass spectrometer using atmospheric pressure chemical ionization. The corona was set to 3.00 μA and cone voltage was set to 25 V. Source temperature was 120°C. Selected ion monitoring was performed at m/z 532.3 (ketoconazole) and m/z 479.4 (azamulin) with dwell times of 0.10 and 0.15 s, respectively. Carbamazepine monitored at m/z 237.5 was used as an internal standard. All analytes were detected as [M + H]$^+$ using atmospheric pressure chemical ionization.

Spectral Interaction Analysis. The spectral interaction of ketoconazole and azamulin with CYP3A4 + OR + b$_5$ Supersomes was determined in Tris-Cl buffer, pH 7.5, at ambient temperature using a Hitachi model U-3010 UV-visible spectrophotometer. Buffer (1.6 ml) and enzyme protein (0.4 ml, 400 pmol) were gently mixed, and 0.98 ml of the mixture was added to both the sample and reference cuvette and a baseline reading was obtained. Increasing amounts of the test substance were added to the sample cuvette from a 0.25 mM (ketoconazole) or 1 mM (azamulin) stock solution prepared in acetonitrile. An equal volume of acetonitrile was added to the reference cuvette. After each addition of test substance, the spectrum was recorded from 350 to 500 nm. In addition, CYP3A4 was replaced with CYP2D6, and the binding of azamulin was assessed. The apparent spectral dissociation constant ($K_{s\ APP}$) was determined by measuring the increase in net absorbance ($\Delta_{418\ nm-388\ nm}$) using SigmaPlot (version 8 for Windows; SPSS Inc., Chicago, IL). To determine whether azamulin was capable of forming a metabolite intermediate complex, experiments were carried out with CYP3A4 + OR + b$_5$ Supersomes in Tris-Cl buffer, pH 7.5, at approximately 30°C. Buffer (1.48 ml), enzyme protein (0.4 ml, 400 pmol), and an NADPH-regenerating system (0.12 ml, as described above) were added to a polypropylene tube and mixed gently. Then, 0.98 ml of the mixture was added to both the sample and the reference cuvette, and a baseline reading was recorded from 500 nm to 380 nm. Azamulin (5 μM or 50 μM) or TAO (50 μM) was added from a 1 mM or 10 mM stock solution prepared in acetonitrile and rescanned at various times up to 60 min.

Results

Characterization of Azamulin P450 Enzyme Inhibition Selectivity and Comparison to Ketoconazole. We examined azamulin or ketoconazole inhibition of P450 isoform-selective metabolism using
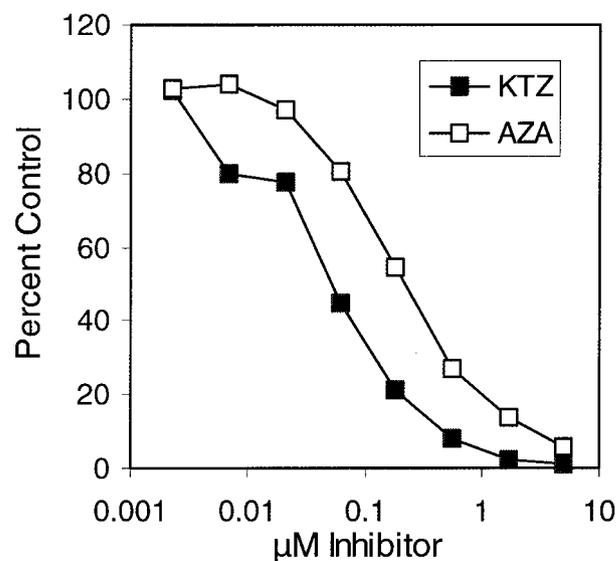

FIG. 2. *Inhibition of HLM-catalyzed testosterone 6β-hydroxylase by ketoconazole or azamulin.*

Data points represent the mean of duplicate incubations.

both cDNA-expressed enzymes and human liver microsomes as the enzyme source. P450 isoform-selective substrate probes were used with HLMs and also with cDNA-expressed enzymes in some cases. To evaluate enzyme selectivity, IC$_{50}$ values were generated from the percentage inhibition determined using three-, four-, or eight-point curves. Eight-point curves were obtained for all CYP3A assays (except CYP3A4/terfenadine) and for all assays in which fluorometric substrates were used with Supersomes as the enzymes source (Table 1). Initially, we compared inhibition potency of azamulin and ketoconazole using a competitive inhibition model. Thus, the probe substrate concentration was used at or near the K_M determined previously in this laboratory. The reaction was initiated by adding enzyme to a mixture of cofactors, inhibitor, and probe substrate (or enzyme and substrate was added to a mixture of cofactors and inhibitor). With this protocol, both ketoconazole and azamulin were found to exhibit selective and potent inhibition of CYP3A enzymes (CYP3A4, CYP3A5, and CYP3A7) with IC$_{50}$ values between 0.02 and 0.38 μM. The selectivity of azamulin for CYP3A4 versus CYP3A5 and CYP3A7 was ~2-fold greater compared to ketoconazole with BFC as the probe substrate. Azamulin displayed potent inhibition of CYP3A4-catalyzed midazolam 1′-hydroxylase and testosterone 6β-hydroxylase in HLMs, and BFC-*O*-dealkylase and terfenadine hydroxylase activity catalyzed by heterologously expressed CYP3A4. Inhibition appeared to be sigmoidal and well behaved, represented by testosterone in Fig. 2. Ketoconazole inhibited CYP1A1 with potency similar to that observed with CYP3A4, whereas azamulin did not inhibit this enzyme. Ketoconazole exhibited relatively potent inhibition of CYP2C and CYP4F enzymes, whereas azamulin was much less inhibitory. Azamulin and ketoconazole were relatively potent inhibitors of CYP2J2 with IC$_{50}$ values of 6.6 and 4.4 μM, respectively. The ratio of azamulin and ketoconazole IC$_{50}$ values was calculated as a means of comparing the selectivity of the two inhibitors. In general, the value of the ratio for nontarget enzymes greatly exceeded those for CYP3A, demonstrating a significant improvement in selectivity over ketoconazole with azamulin in this model.

Evidence of Mechanism-Based Inhibition by Azamulin. We investigated the effect of incubation time on IC$_{50}$ values by using the fluorometric probe substrate BFC, using both CYP3A4 Supersomes
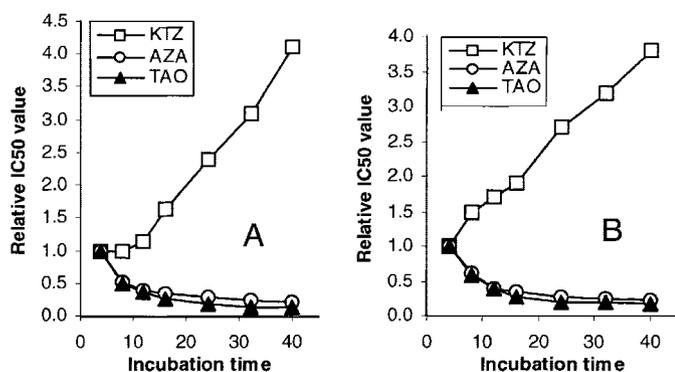
FIG. 3. *Effect of incubation time on IC_{50} values of KTZ, AZA, and TAO in the BFC dealkylation assay.*

A, the enzyme source was CYP3A4 BD Gentest Supersomes enzymes. Initial IC_{50} values after a 4-min incubation were 0.006, 0.130, and 1.36 μM for KTZ, AZA, and TAO, respectively. B, the enzyme source was HLMs (pool of two donors expressing high CYP3A4). Initial IC_{50} values after a 4-min incubation were 0.005, 0.144, and 1.23 μM for KTZ, AZA, and TAO, respectively. All data points represent the mean of duplicate incubations.

FIG. 4. *Effect of preincubation in the presence or absence of NADPH on the inhibition of testosterone 6β-hydroxylase activity in CYP3A-enriched pooled HLMs by KTZ, AZA, and TAO.*

Data points represent the mean of duplicate incubations.

FIG. 5. *Effect of preincubation of 5 μM or 10 μM AZA in pooled HLMs (pool of 22 donors) in the presence of NADPH on the inhibition of several enzyme activities.*

The probe substrates for each enzyme are as follows: CYP1A2, phenacetin; CYP2C8, paclitaxel; CYP2C9, diclofenac; CYP2C19, S-mephenytoin; CYP2D6, bufuralol; CYP2E1, p-nitrophenol; CYP3A4, testosterone (T) or midazolam (M). For CYP3A4, the effect of preincubation in the absence of NADPH is also shown. Values for 5 μM represent means of two experiments conducted in duplicate; values for 10 μM represent the mean of duplicate samples from a single experiment.

and human liver microsomes enriched in CYP3A as the enzyme source (Fig. 3). BFC can be used as a CYP3A-selective substrate probe when used with liver microsomes that are both enriched in CYP3A and relatively low in CYP1A2 (Stresser et al., 2002). Whereas ketoconazole IC_{50} values increased with incubation time approximately 4-fold from 4 to 40 min, azamulin IC_{50} values decreased approximately 5-fold during the same interval and closely tracked the rate of decrease exhibited by triacetyloleandomycin, a mechanism-based inhibitor of CYP3A. Essentially identical results were obtained when using either CYP3A4 Supersomes or the CYP3A-enriched HLM pool, implicating CYP3A as the predominant enzymes involved in inhibitor metabolism.

Preincubation of Azamulin with Human Liver Microsomes. To examine further the inhibitory mechanism of azamulin, we carried out preincubation experiments with HLMs with and without NADPH. Preincubation of 4.8 μM and 9.6 μM azamulin (concentrations 20 and 40 times the IC_{50}, respectively) for 5 min with a CYP3A-enriched HLM pool in the presence of NADPH prior to a 1:20 dilution into a testosterone reaction mix eliminated more than 90% of the testosterone 6β-hydroxylase activity compared with preincubation in the absence of NADPH (Fig. 4). After a 20-min preincubation, more than 95% of activity had been eliminated. Preincubation of azamulin in the absence of NADPH caused no further inhibition of activity than would be expected if the preincubation step was omitted altogether. Preincubation in the presence of NADPH for 20 min in the absence of azamulin caused a 20% decrease in enzyme activity compared with no preincubation. One explanation for these findings is that a metabolite(s) of azamulin binds irreversibly or very tightly to CYP3A and that its formation is dependent on catalytic activity. To determine whether preincubation affected enzyme inhibition selectivity, azamulin was preincubated with HLMs (pool of 22 donors) and NADPH for 10 min followed by a 20-fold dilution into probe substrate reaction mixes for CYP1A2, 2C8, 2C9, 2C19, 2D6, 2E1, and CYP3A (Fig. 5). For non-CYP3A enzymes, inhibition by either 5 μM or 10 μM azamulin was less than 10% compared with preincubation without azamulin. However, testosterone 6β-hydroxylase activity was inhibited by 97% and 100% at 5 μM and 10 μM, respectively. Midazolam 1'-hydroxylase activity was inhibited 69% and 73% by 5 μM and 10 μM, respectively. Inhibition of CYP3A activity was much less when preincubation was conducted in the absence of NADPH as expected.

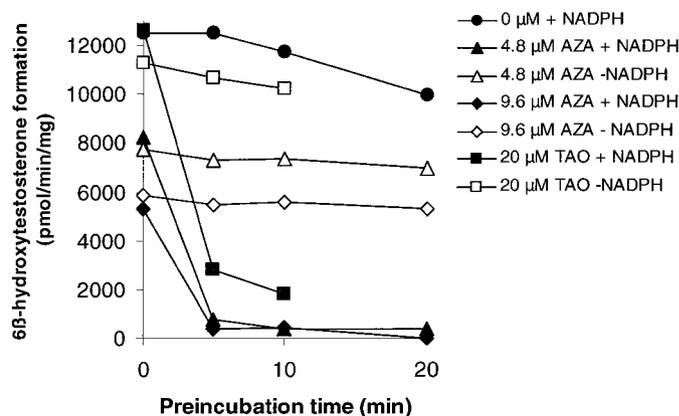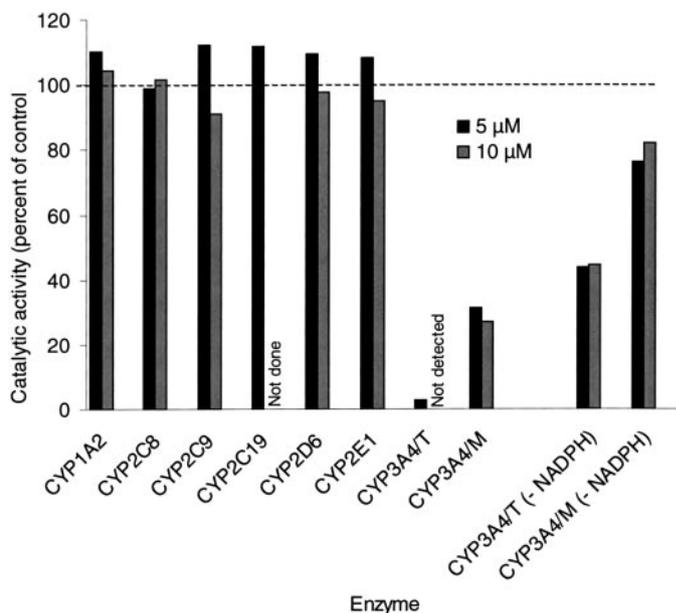**Solubility and Chemical Stability.** Solubility and solution chem-

ical stability are rarely reported for chemical reagents used in vitro. Therefore, we felt it important to evaluate the solubility and solution chemical stability of azamulin. By visual inspection, no evidence of azamulin precipitation was found in aqueous mixes (200 μM) prepared during the course of these experiments, and this was confirmed experimentally. The solubility of azamulin in 100 mM potassium phosphate buffer containing 1% DMSO was found to be >300 μM by light scatter analysis. To evaluate solution chemical stability, azamulin was prepared as 1 mM stocks in acetonitrile and stored at room temperature or −20°C for 12 days. Relative concentrations of azamulin were found to be 89% and 94% for −20°C and room temperature samples, respectively, compared with freshly prepared azamulin, but these differences were not statistically significant (analysis of vari-
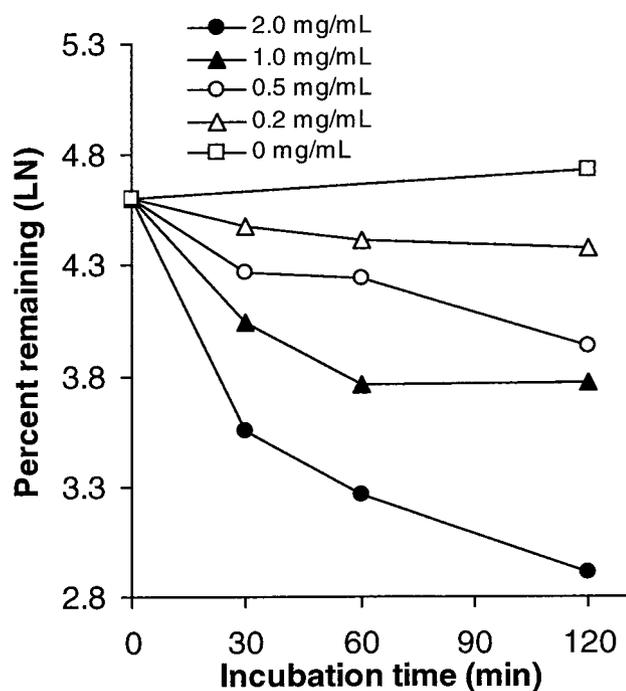
FIG. 6. *Metabolic stability of 5 µM azamulin in the presence of human liver microsomes.*

The HLM protein concentrations in the incubations are shown in the figure. Data points represent the mean of duplicate incubations.

FIG. 7. *Overlays of absorbance spectra recorded from 500 nm to 350 nm showing spectral interaction of azamulin (28 µM) or ketoconazole (5 µM) with CYP3A4.*

Also shown is the overlay of the spectrum produced upon addition of azamulin (22 µM) to CYP2D6.

ance, $p = 0.32$). Azamulin was easily dissolved in acetonitrile for preparation of 1 mM stock solutions, whereas 10 mM stocks could be prepared with mild heat and sonication.

Metabolic Stability of Azamulin. The metabolic stability of 5 µM azamulin was assessed over a 2-h incubation period (Fig. 6) by monitoring loss of parent in 0, 0.2, 0.5, 1.0, and 2.0 mg/ml human liver microsomes. The concentration of 5 µM was chosen as that which might be used to block CYP3A activity and thus determine the metabolic role of this enzyme for the reaction of interest. Azamulin initially underwent relatively rapid metabolism but slowed and was not depleted after even long incubation times (e.g., 43% of the initial concentration remained after a 120-min incubation with 1 mg/ml microsomal protein).

Spectral Interaction Analysis. Spectral interaction analysis demonstrated that azamulin bound to CYP3A4 protein, resulting in an absorbance maximum and minimum of approximately 388 nm and 418 nm, respectively, with an isosbestic point near 405 nm (Fig. 7). This interaction is typical of a "type I" spectral change. Binding was saturable and yielded a $K_{s\ APP}$ of 3.5 µM. No evidence of interaction was found with CYP2D6 at concentrations up to 22 µM. By contrast, ketoconazole exhibited a "type II" spectral interaction with CYP3A4, exhibiting an absorbance maximum and minimum of approximately 429 nm and 395 nm, respectively, with an isosbestic point near 415 nm.

When CYP3A4 was supplemented with NADPH, addition of 50 µM TAO resulted in an absorption maximum of 455 nm. This spectral perturbation is characteristic of a metabolite intermediate complex (MIC), which is a catalytically inactive form of the enzyme. The MIC formation increased with time, exhibiting an absorbance difference of 0.009 (455–490 nm) after 15 min. In contrast, when 5 µM or 50 µM azamulin was added to CYP3A4, no MIC formation was observed after a 30-min incubation. Addition of 50 µM TAO to the same incubations after 30 min did not yield MIC formation.
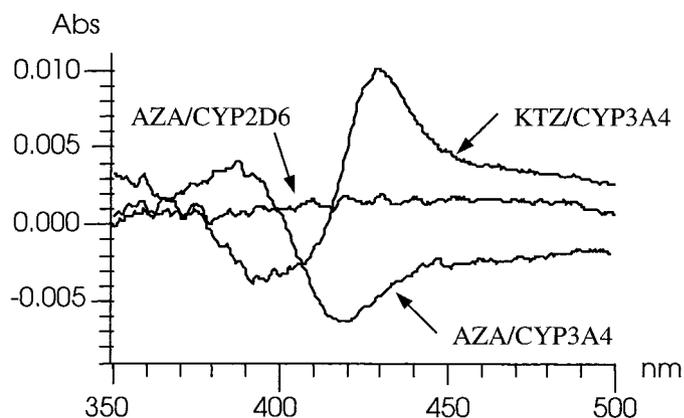
Discussion

Reaction phenotyping studies are designed to determine the contribution of individual P450 isoforms to the metabolism of a chemical (Clarke, 1998). The first of three commonly used experimental approaches is to determine whether heterologously expressed P450 enzymes catalyze the reaction of interest. A second approach involves correlation analysis of the reaction specific activity in a panel of individual donor liver microsomes with the P450 isoform activities ascribed to each donor. Third, the effect of selective chemical or antibody inhibitors on the reaction of interest can be determined. Chemicals provide a powerful and low-cost alternative to monoclonal antibodies but must be used within the "window of selectivity" (concentration range where selectivity is maintained), which may shift depending on the liver microsomal protein concentration (Tran et al., 2002). A recommended list of chemicals for use in reaction phenotyping studies has been compiled (Bjornsson et al., 2003).

In this investigation, we characterized the P450 enzyme inhibition potency and selectivity of azamulin, a derivative of the antibiotic pleuromutilin. To determine selectivity, we compared IC_{50} values among 18 P450 enzymes (28 P450 enzyme/substrate pairs), initially in a competitive inhibition model. Multiple substrates were used to evaluate the inhibition potency of CYP3A4, because of the substrate-dependent inhibition sometimes observed with this enzyme (Kenworthy et al., 1999; Stresser et al., 2000). The IC_{50} values for cDNA-expressed CYP3A4 or HLM-derived CYP3A (0.03–0.24 µM) were at least 100-fold lower than those for all other enzymes except CYP2J2. For that enzyme, IC_{50} values were ~50-fold lower. Although the IC_{50} value with heterologously expressed CYP3A4 was 15-fold and 13-fold less than those of CYP3A5 and CYP3A7, respectively, this is probably not sufficient to differentiate CYP3A4 from these closely related enzymes. The IC_{50} values for nontarget enzymes were generated using abbreviated curves (usually three concentrations), and therefore, the precision of the value may be less than that obtained using a curve with concentrations of closer spacing. However, the difference is expected to be insignificant (Moody et al., 1999; Gao et al., 2002) compared with the interenzyme difference described here. Thus, azamulin exhibits selectivity and potency apparently far greater than CYP3A inhibitors characterized previously.

In this study, ketoconazole was compared alongside azamulin with most of the enzyme/substrate pairs in the competitive inhibition model. Many of the enzymes inhibited by ketoconazole appear to be important primarily in the metabolism of drugs and other xenobiotics

in extrahepatic tissues. These include CYP1A1, CYP1B1, CYP2J2 (which is inhibited approximately equally by azamulin and ketoconazole), and CYP4F12 (Miners and McKinnon, 2000 and references therein; Hashizume et al., 2002; Matsumoto et al., 2002; Ding and Kaminsky, 2003). Ketoconazole also potently inhibited CYP4F2, which is abundant in liver and kidney. This enzyme is associated with the metabolism of endogenous compounds such as arachidonic acid and LTB$_4$ (Christmas et al., 2001), and also drug candidates that structurally resemble endogenous substrates. Therefore, ketoconazole seems particularly ill-suited for elucidating the role of CYP3A in extrahepatic tissues, and azamulin may be a better alternative.

We conducted experiments to examine the effect of incubation time on azamulin potency. Reversible inhibitors that are substrates are subject to metabolic depletion over the course of the incubation, and if metabolites are less inhibitory than the parent, IC$_{50}$ values increase with incubation time. By contrast, when a metabolite is more inhibitory than the parent, a decrease in IC$_{50}$ value with incubation time occurs, and this is characteristic of mechanism-based inhibitors. Azamulin inhibition potency increased with inhibition time, and the rate closely matched that of the mechanism-based inhibitor triacetyloleandomycin. This effect was observed both in HLMs and in CYP3A4 Supersomes, suggesting that CYP3A4 may be mediating the effect within HLMs. By contrast, the inhibition potency of ketoconazole in the same experiments increased with time, consistent with metabolic inhibitor depletion. Therefore, the ratios of the IC$_{50}$ values for ketoconazole and azamulin shown in Table 1 would be expected to converge with longer incubation time (more so with HLM and CYP3A assays). Consistent with these findings, testosterone 6β-hydroxylase and midazolam 1'-hydroxylase activity was essentially eliminated in CYP3A-enriched preincubation with azamulin in the presence of NADPH. Incomplete inhibition in the midazolam 1'-hydroxylase assay may be due to the contribution of other enzymes in generating the 1'-hydroxy metabolite at the concentration of substrate (50 μM) used in this experiment, which is well above the K_M. The susceptibility of other enzymes to inhibition under these experimental conditions was tested. This is because it is conceivable that a putative reactive and inhibitory metabolite generated in the preincubation phase might dissociate from CYP3A and inhibit neighboring enzymes, therefore compromising the selectivity of azamulin. However, preincubation experiments containing 5 μM and 10 μM azamulin caused no significant inhibition of CYP1A2, CYP2C8, CYP2C9, CYP2C19, CYP2D6, and CYP2E1, some of which were weakly inhibited in the competitive inhibition protocol. Whether CYP3A5 was differentially inhibited compared with CYP3A4 within HLMs in the preincubation experiments could not be determined. This is because testosterone and midazolam are metabolized by both enzymes. Detailed experiments with cDNA-expressed CYP3A4 and CYP3A5 enzymes will likely be necessary to fully elucidate whether preincubation (or other assay) conditions can be developed to sufficiently differentiate contribution to metabolism by these two enzymes.

Ketoconazole and azamulin were shown to produce spectral binding complexes when added to CYP3A4 Supersomes enzyme. As expected, ketoconazole yielded a type II difference spectrum, believed to result from coordination of the imidazole moiety with the heme and a shift in equilibrium to a low-spin hemoprotein. By contrast, azamulin exhibited a type I spectrum consistent with a spin equilibrium shift to the high-spin state (Schenkman et al., 1981). Binding of azamulin to CYP3A4 was saturable and did not occur with CYP2D6, which served as a negative control. The low spectral dissociation constant of 3.5 μM was consistent with the low IC$_{50}$ values found with CYP3A. The observation of a type I spectrum was somewhat surprising, since the triazole moiety might be expected to ligate with the heme and

yield a characteristic type II spectrum consistent with other triazoles (Ballard et al., 1988). However, type I interactions with azoles are not unprecedented (Chiba et al., 2001b). Our findings demonstrate that ligation of the triazole to the heme is not required for potent inhibition of CYP3A. Type I inhibitors tend to be metabolically unstable (Chiba et al., 2001a), and azamulin conforms to this model at early time points in an incubation with HLMs. The rapid metabolism may be consistent with a bioactivation step prerequisite for the time-dependent inhibition found with azamulin.

Because azamulin exhibited characteristics of a mechanism-based inhibitor, we attempted to observe formation of a metabolic intermediate complex with CYP3A4 Supersomes (Franklin, 1991). Although troleandomycin caused the characteristic absorption spectrum with a peak around 455 nm, we were unable to detect this peak with azamulin. Interestingly, tiamulin, which is similar in structure to azamulin but lacks the triazolyl group, does form a metabolic intermediate complex in rats both in vivo and in vitro (Witkamp et al., 1996). In addition, it appears to be a highly selective inhibitor of rat CYP3A and inhibits human CYP3A4, but not CYP2C9, in an NIH 3T3 cell line (De Groene et al., 1995). Thus, the high degree of CYP3A selectivity by azamulin may be primarily a function of the pleuromutilin portion of the molecule.

It is important to consider that assay conditions such as buffer type and strength, protein concentration, and incubation time may also affect inhibitor potency (Maenpaa et al., 1998) and may influence interpretation of quantitative data. Because drugs nominated for development are often more stable to metabolism than their predecessors of a few years ago, reaction phenotyping studies increasingly necessitate long incubation times with higher protein concentrations to adequately detect and quantify metabolites. In such cases, the degree of inhibitor probe depletion (via metabolism or microsomal protein binding) should also be considered (Erve et al., 2000; Tran et al., 2002). We assessed the metabolic stability of 5 μM azamulin under conditions that may be employed for reaction phenotyping studies. The 5 μM concentration of azamulin was chosen, based on the data in Table 1 and Fig. 5, to selectively inhibit CYP3A. Our data show that azamulin was metabolized by no more than 57% with a microsomal protein concentration of 1 mg/ml in a 120-min incubation. By contrast, 1 μM ketoconazole was found to be essentially depleted under these conditions (Erve et al., 2000). High concentrations of microsomal protein might be expected to influence inhibition potency as a result of probe binding to protein. A preliminary assessment of microsomal protein binding using equilibrium dialysis indicates that <20% of 5 μM azamulin bound to 0.5 mg/ml microsomal protein after a 2-h incubation in the absence of NADPH (D. M. Stresser and A. A. Dandeneau, unpublished data). These data suggest that azamulin would be resistant to differences in inhibition potency using relatively extreme conditions of incubation time and microsomal protein concentration.

In summary, we have shown that azamulin is a highly potent and selective inhibitor of CYP3A. In addition, we demonstrate that it possesses selectivity that is superior to ketoconazole in a competitive inhibition model. Preincubation of azamulin with liver microsomes in the presence of NADPH greatly enhances CYP3A inhibition potency. Therefore, foreknowledge of the kinetics of the reaction under investigation is unnecessary. The evidence suggests that azamulin is a mechanism-based and irreversible inhibitor, although further studies will be required to meet the criteria as outlined by Silverman (1996) for this to be fully established. The chemical stability and solubility (both in acetonitrile and in aqueous buffer) of this compound are compatible with routine laboratory use. We suggest that the use of 5 μM azamulin should block CYP3A activity under most assay condi-

tions while having little effect on nontarget P450 enzyme activity. One exception to this may be CYP2J2, which might be partially inhibited under these conditions.

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