

Evidence of Mechanism-Based Inhibition by the Highly Selective Human CYP3A Inhibitor Azamulin

David Stresser, Ph.D.*, Thuy Ho, Catherine Cargill, Andrew Blanchard, Raman Sharma, Andre Dandeneau, Marc Broudy, Joseph Goodwin, Stephanie Turner, Christopher Patten*, Ph.D., Shangara Dehal, Ph.D., Charles Crespi, Ph.D.*
BD Biosciences Discovery Labware, Woburn, MA 01801

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 μ M) were at least 100-fold lower than all other non-CYP3A enzymes except CYP2J2 (~50-fold lower). The IC_{50} value with heterologously expressed CYP3A4 was 15-fold and 13-fold less than CYP3A5 and CYP3A7, respectively. The reference inhibitor ketoconazole was less selective and exhibited potent inhibition (IC_{50} values < 10 μ M) 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 μ M azamulin in the presence of NADPH for 10 minutes inhibited ~95% of testosterone 6 β -hydroxylase activity compared to 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 spectra with a spectral dissociation constant of 3.5 μ M 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 μ M. Azamulin represents an important new chemical tool for use in characterizing the contribution of CYP3A to the metabolism of xenobiotics.

Introduction

Cytochrome P450 isoform-selective chemical inhibitors are used primarily in reaction phenotyping studies to determine P450 isoform contribution to a metabolic reaction.¹ Chemicals are a simpler and more cost-effective alternative to immunoinhibitory antibodies, but kinetic information is usually required to ensure proper use. Azamulin (AZA) (Figure 2), an azole derivative of the pleuromutilin class of anti-infectives, has been suggested as a selective CYP3A inhibitor.² BD Biosciences previously determined that AZA is a highly potent inhibitor of CYP3A and that selectivity is superior to ketoconazole in a competitive inhibition model.³ The objectives of the current work were to 1) extend the panel of enzymes examined to include CYP4F12 and CYP2J2, and 2) characterize selectivity for major CYPs in a time- and NADPH-dependent inhibition model.

Materials and Methods

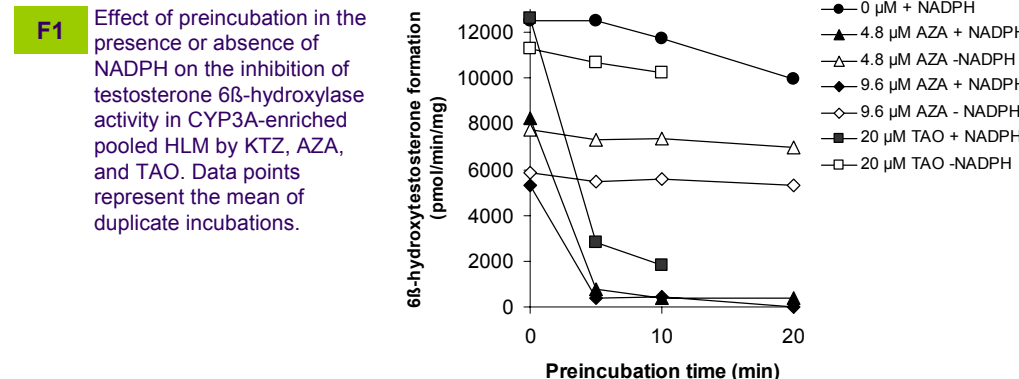
Chemicals and Enzymes

Human liver microsomes (HLM), microsomes from baculovirus-infected insect cells (BD Supersomes™ Enzymes) and AZA were obtained from BD Biosciences Discovery Labware (Woburn, MA). All other chemicals (reagent grade) were obtained from reputable sources.

Enzyme Inhibition Assays

Determination of IC_{50} values in a competitive inhibition model. The percent inhibition of enzyme activity by ketoconazole (KTZ) and AZA for several CYP isoforms was determined using pooled HLM or BD Supersomes Enzymes (CYP4F only) as described.⁴ The IC_{50} values were determined by linear interpolation with 3, 4, or 8 point curves, where concentrations were spaced by log or half log units, respectively. Inhibition assays were conducted at substrate concentrations at or near the K_m value.

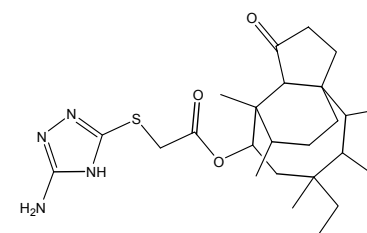
Time- and NADPH-dependent inhibition. Incubations with testosterone (Figure 1) were performed in phosphate buffer with 1 mg/mL HLM protein (1:1 pool of two donors, rich in CYP3A) with and without a NADPH generating system. Solvent only, 4.8 μ M and 9.6 μ M AZA, representing 20X and 40X the IC_{50} values were tested in duplicate. Triacetyloleandomycin (20 μ M) was used as a positive control. After 0, 5, 10, and 20 minutes of incubation (0, 5, and 10 minutes for TAO), 0.05 mL was added to 0.95 mL testosterone assay mixture, containing a NADPH regenerating system and 200 μ M testosterone. The reaction was stopped after 10 minutes by the addition of 500 μ L acetonitrile. A portion of the supernatant was analyzed by HPLC. This same approach was carried out again for CYP3A4 and several other enzymes using AZA concentration of 5 μ M and 10 μ M in HLM (pool of 22 donors) and a fixed preincubation period of 10 minutes. After the preincubation period, the mixture was diluted 1:20 (except CYP2C19, which was 1:2) and the reactions carried out. The probe substrate concentrations were 2 to 10 times higher than the K_m values.



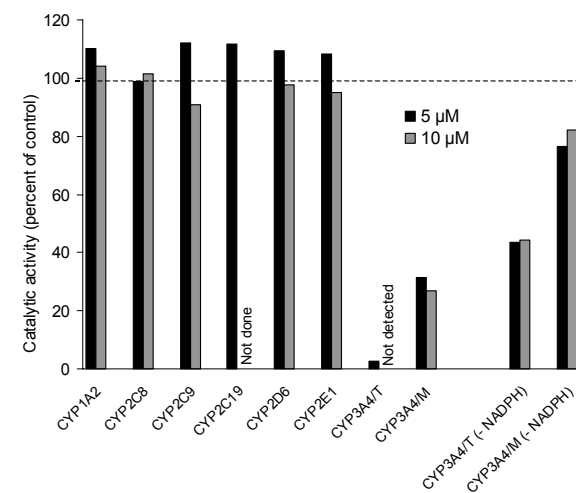
T1 Summary of AZA Specificity in cDNA-expressed Enzymes or HLM in Comparison to KTZ in a Competitive Inhibition Model

Enzyme	Enzyme Source	Substrate	IC_{50} (μM) KTZ	IC_{50} (μM) AZA	IC_{50} Ratio AZA/KTZ
CYP1A1	BD Supersomes™	BzRes	0.017	> 50	>2900
CYP1A2	BD Supersomes	CEC	> 50	> 200	-
CYP1A2	HLM	Phenacetin	55	> 100	> 1.8
CYP1B1	BD Supersomes	BzRes	5.6	> 50	> 8.9
CYP2A6	BD Supersomes	Coumarin	> 50	> 50	-
CYP2B6	BD Supersomes	EFC	6.3	> 50	> 7.9
CYP2B6	HLM	Bupropion	ND	>50	-
CYP2C8	BD Supersomes	DBF	4.4	4.1	9.3
CYP2C8	HLM	Paclitaxel	8.6	73	8.5
CYP2C9	BD Supersomes	MFC	5.1	57	11.2
CYP2C9	HLM	Diclofenac	9	> 100	> 11
CYP2C19	BD Supersomes	CEC	13	47	3.6
CYP2C19	HLM	S-mephenytoin	57	> 100	> 1.8
CYP2D6	BD Supersomes	AMMC	ND	> 200	-
CYP2D6	HLM	Bufuralol	67	> 100	> 1.5
CYP2E1	BD Supersomes	MFC	ND	> 50	-
CYP2E1	HLM	p-Nitrophenol	> 50	> 50	-
CYP2J2	BD Supersomes	Terfenadine	4.6	6.6	1.4
CYP3A4	BD Supersomes	BFC	0.023	0.026	1.1
CYP3A4	BD Supersomes	Terfenadine	< 0.1*	0.12	1.6
CYP3A4/5	HLM	Testosterone	0.06	0.24	4.0
CYP3A4/5	HLM	Midazolam	0.04	0.15	3.7
CYP3A5	BD Supersomes	BFC	0.11	0.38	3.5
CYP3A7	BD Supersomes	BFC	0.16	0.34	2.1
CYP4A11	HLM	Lauric acid	> 100	> 100	-
CYP4F2	BD Supersomes	LTB ₄	< 1 [†]	46	57
CYP4F3b	BD Supersomes	LTB ₄	22	>100	> 4.5
CYP4F12	BD Supersomes	Terfenadine	0.74	29	39

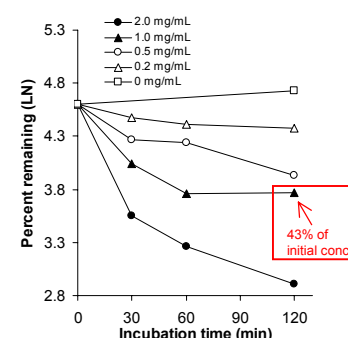
F2 Structure of Azamulin



F3 Effect of preincubation of 5 μ M or 10 μ M AZA in pooled HLM (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; CYP3A, testosterone (T) or midazolam (M). For CYP3A, the effect of preincubation in the absence of NADPH is also shown. Values for 5 μ M represent means of two experiments conducted in duplicate; Values for 10 μ M represent the mean of duplicate samples from a single experiment.

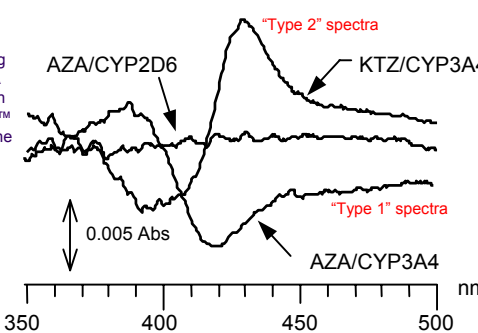


F4 Metabolic stability of 5 μ M AZA in the presence of Helms. The HLM protein concentration in the incubations is as shown in the legend.



F5

Overlays of absorbance spectra recorded from 500 nm to 350 nm showing spectral interaction of AZA (28 μ M) or KTZ (5 μ M) with CYP3A4 BD Supersomes™ Enzymes. Also shown is the overlay of the spectrum produced upon addition of AZA (22 μ M) to CYP2D6.



Results and Discussion

Enzyme selectivity. AZA was found to be a potent and highly selective inhibitor of human CYP3A and superior to KTZ as determined by comparing IC_{50} values in a competitive inhibition model (Table 1). Time/NADPH-dependent inhibition of CYP3A by AZA was demonstrated by the experiment shown in Figure 1. Preincubation of AZA with HLM in the presence of NADPH greatly reduced CYP3A4/5 catalyzed testosterone 6 β -hydroxylase activity compared to preincubation in absence of NADPH. In the same model, inhibition was also time- and NADPH-dependent with an alternative CYP3A substrate midazolam, but inhibition was less potent. No inhibition of CYP1A2, 2C8, 2C9, 2C19, 2D6, or 2E1 was found (Figure 3), demonstrating AZA is CYP3A selective in a time-/NADPH-dependent model in addition to a competitive inhibition model.

Solubility, metabolic, and chemical stability. Aqueous solubility was found to be > 300 μ M. AZA easily dissolved in DMSO and acetonitrile for preparation of 30 mM and 1 mM stock solutions, respectively. Ten mM stocks could be prepared in acetonitrile with mild heat and sonication. AZA exhibited no detectable instability when stored in acetonitrile for up to 12 days at ambient temp or -20°C. Metabolic stability analysis (Figure 4) showed AZA initially underwent relatively rapid metabolism but slowed and was not depleted after even long incubation times.

Spectral interaction analysis. Azamulin bound to CYP3A4 protein (BD Supersomes™ Enzymes) resulting in absorbance MAX and MIN of approx. 388 nm and 418 nm, respectively, with an isobestic point near 405 nm (Figure 5), typical of a 'type I' spectral change. Binding was saturable and yielded a K_s APP of 3.5 μ M. No evidence of interaction was found with CYP2D6 at concentrations up to 22 μ M. By contrast, KTZ exhibited a 'type II' spectral interaction with CYP3A4 (MAX and MIN of approx. 429 nm and 395 nm, respectively; isobestic point ~ 415 nm) as expected, believed to result from coordination of the imidazole moiety with the heme and a shift in equilibrium to a low spin hemoprotein. Our findings demonstrate that ligation of the triazole moiety of AZA to the heme is not required for potent inhibition of CYP3A.

When CYP3A4 was supplemented with NADPH, addition of 50 μ M TAO gave an absorption maxima of 455 nm. This spectral perturbation is characteristic of a metabolite intermediate complex (MIC), a catalytically inactive form of the enzyme. However, when 5 μ M or 50 μ M AZA was added to CYP3A4, no MIC formation was observed up to 30 minutes later. Subsequent addition of 50 μ M TAO to same incubations did not yield MIC formation. Thus MIC formation was not required for time-/NADPH-dependent inhibition.

Summary and Conclusions

1. AZA is a potent and highly selective *in vitro* inhibitor of human CYP3A. Selectivity exceeds that of ketoconazole.
2. The potency and selectivity of enzyme inhibition by AZA is greatly enhanced by preincubation in the presence of NADPH. Relative to triacetyloleandomycin, AZA is much better characterized and exhibits greater potency.
3. AZA exhibits excellent solubility, metabolic, and chemical stability which facilitates use as an *in vitro* tool.
4. Unexpectedly, AZA did not coordinate with the heme iron of CYP3A4, demonstrating this is not required for potent inhibition by an azole.
5. The use of 5 μ M AZA should block CYP3A activity under most assay conditions while having little effect on non-target CYP enzyme activity. One exception to this is CYP2J2, which might be partially inhibited under these conditions.

References

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2. Clarke, S.E. Open discussion, 7th EUPERS Conference on Optimizing Drug Development: Strategies to Assess Drug Metabolism/Transport Interaction Potential - Towards a Consensus, November 13-15, 2000, Basel, Switzerland (2000).
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4. Stresser, D.M., et al., Highly selective inhibition of human CYP3A *in vitro* by azamulin and evidence that inhibition is irreversible. *Drug Metab. Dispos.* In press (2004).