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Is 1-Aminobenzotriazole (1-ABT) an Appropriate *In Vitro* Tool as a Non-Specific Cytochrome P450 Inactivator?

Collette D. Linder, Nicole A. Renaud, and J. Matthew Hutzler*

Pharmacokinetics, Dynamics and Metabolism (PDM),

Pfizer Global Research and Development, St. Louis Labs

Running Title: 1-ABT Inactivation of Human P450

*Address Correspondence to:

Dr. J. Matthew Hutzler

Pharmacokinetics, Dynamics and Metabolism

Pfizer Global Research and Development,

700 Chesterfield Parkway West, T3A, Lab 319E

Chesterfield, MO 63017

Tel: 314-274-0261

Fax: 314-274-4426

Email: J.Matt.Hutzler@Pfizer.com

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Abbreviations: 1-ABT, 1-aminobenzotriazole; P450, cytochrome P450; NCE, new chemical entity; HLM, human liver microsomes; TDI, time-dependent inhibitor; LC/MS/MS, liquid chromatography/tandem mass spectrometry; DDC, diethyldithiocarbamate; ESI, electro-spray ionization

Abstract

1-Aminobenzotriazole (1-ABT) is generally considered to be a non-selective mechanism-based inactivator of both human and non-human cytochrome P450 enzymes. Thus, 1-ABT is routinely used when conducting *in vitro* reaction phenotyping studies with new chemical entities (NCEs) in drug discovery to decipher P450 from non-P450-mediated metabolism. Experiments with pooled human liver microsomes (HLMs) demonstrated that carbon monoxide (CO) binding, while substantially reduced following a 30 min pre-incubation with 1-ABT, was still measurable. Thus, remaining activity of nine major human P450s (1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, and 3A4) in HLMs was determined using established selective probe substrates following 30 min pre-incubation with either 1-ABT (1 mM), a positive control time-dependent inhibitor (TDI), or a competitive inhibitor. While P450 2A6 and 3A4 activity was essentially eliminated upon 30 min pre-treatment with 1-ABT, the other human P450s were less affected, with at least 20% activity remaining after pre-treatment. In contrast, most of the known P450 selective time-dependent inhibitors were more effective inactivators than 1-ABT at lower concentrations. Particularly interesting was that 1-ABT was quite ineffective at inactivating P450 2C9, with roughly 60% activity remaining following pre-treatment, which suggests that 1-ABT is much less selective for certain human P450s. This collection of data clearly demonstrates that assuming 1-ABT is a non-selective P450 inhibitor *in vitro* is risky, and false conclusions regarding remaining metabolic activity being non-P450 mediated following 1-ABT pre-treatment may be made.

In vitro reaction phenotyping, where enzymes contributing to the biotransformation of new chemical entities (NCEs) are identified, is a routine practice in drug discovery (Williams et al., 2003; Zhang et al., 2007). This information is essential for understanding the relative contributions of metabolic pathways to overall clearance, and thus, the risk of pharmacokinetic drug-drug interactions and/or inter-patient variability in drug exposure. Critical to conducting these *in vitro* studies is having the appropriate tool substrates and inhibitors of the various human P450 enzymes. A particularly useful tool inhibitor historically has been 1-aminobenzotriazole (1-ABT), which is thought to inactivate P450 enzymes non-selectively by covalent modification of the heme prosthetic group following bioactivation (Ortiz de Montellano and Mathews, 1981). Thus, it has become a common *in vitro* practice to pre-incubate either human liver microsomes or hepatocytes with high concentrations of 1-ABT (~1 mM) prior to the introduction of test compound (substrate-depletion approach) in order to decipher P450 from non-P450 mediated metabolism (Dalmadi et al., 2003; Williams et al., 2003; Kostrubsky et al., 2006). While it is acknowledged that this approach is useful for determining P450-mediated metabolism, the characterization of the specific effects of 1-ABT on the major human P450 enzymes *in vitro* has been limited (Emoto et al., 2005). The purpose of the current research was to evaluate the use of 1-ABT as a non-selective inactivator of P450 by determining the remaining activity of the major human P450 enzymes (CYPs 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, and 3A4) in human liver microsomes following pre-treatment with 1 mM 1-ABT. This data will hopefully serve as a caution for the assumptions commonly made when using 1-ABT for *in vitro* reaction phenotyping studies.

Materials and Methods

Chemicals. Potassium phosphate buffer, $NADP^+$, isocitric acid, isocitric dehydrogenase, magnesium chloride, 1-aminobenzotriazole, sodium dithionite, tolbutamide, diclofenac, 4′-hydroxydiclofenac, sulfaphenazole, dextromethorphan, dextrorphan, paroxetine, quinidine, phenacetin, acetaminophen, furafylline, α-naphthoflavone, midazolam, 1′-hydroxymidazolam, erythromycin, ketoconazole, bupropion, ticlopidine, clotrimazole, paclitaxel (taxol), amiodarone, (S)-mephenytoin, 4′-hydroxymephenytoin, (+)-N-3-benzyl nirvanol, coumarin, 7-hydroxycoumarin, 8-methoxypsoralen, tranylcypromine, chlorzoxazone, 6-hydroxychlorzoxazone, and diethyldithiocarbamate (DDC) were all purchased from Sigma-Aldrich (St. Louis, MO). Montelukast was purchased from Sequoia Research Products (United Kingdom). Pooled human liver microsomes (pool of 50 donors), 6α-hydroxypaclitaxel, and hydroxybupropion were purchased from BD-Biosciences (San Jose, CA), while tienilic acid was available in the chemical bank at Pfizer Global Research and Development. All other chemicals were obtained from commercial sources and were of the highest purity available.

P450 Time-Dependent Inhibition Assays. Remaining enzyme activity using selective cytochrome P450 probe substrates was determined after a primary incubation in human liver microsomes with 1-ABT (1 mM), a positive control time-dependent inhibitor (TDI), and a competitive inhibitor (0.05% total organic v/v) in triplicate for nine major human P450 enzymes (CYPs 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, and 3A4). Incubations were conducted in a 96-well plate format at 37°C using an Eppendorf MTP ThermoMixer (Hamburg, Germany), and consisted of human liver microsomes (1 mg/ml

primary incubation, 0.05 mg/ml secondary incubation) in 100 mM potassium phosphate buffer, pH 7.4, with a NADPH regenerating system (3 mM $MgCl_2$, 1 mM, $NADP^+$, 5 mM isocitric acid, and 1 unit/ml isocitrate dehydrogenase). At selected times (0 and 30 min) 15 µl aliquots were taken out of the primary incubation mixture and diluted 20-fold into a secondary incubation mixture (285 µl) containing the selective probe substrate (concentration ≥ 5-times the published apparent K_m), to measure residual activity. Substrate concentrations and secondary incubation times for each P450 probe substrate were as follows: phenacetin (150 µM, 30 min), coumarin (10 µM, 6 min), bupropion (350 µM, 20 min), paclitaxel (50 µM, 15 min), diclofenac (25 µM, 5 min), (S)-mephenytoin (250 µM, 45 min), dextromethorphan (20 µM, 20 min), chlorzoxazone (300 µM, 20 min), and midazolam (10 µM, 6.5 min). Secondary incubations were quenched with an equal volume of cold acetonitrile (300 µl) containing internal standard (0.2 µM tolbutamide). Plates were centrifuged at 3000 x *g* for 10 min, supernatant transferred to a separate analytical plate, and metabolite formation analyzed by LC/MS/MS as described below. Following treatment with 1-aminobenzotriazole, positive control time-dependent inhibitor, and competitive inhibitor, percent (%) remaining P450 activity was calculated by comparing metabolite formation after 0 min and 30 min pre-incubation to solvent control incubations without inhibitor. Data were plotted for visualization using GraphPad PRISM (San Diego, CA) software.

Spectrophotometric Analysis. Reduced carbon monoxide difference spectra were recorded on a Hitachi (Danbury, CT) U3300 dual-beam spectrophotometer, and data were analyzed using UV Solutions 1.2 software. Briefly, a 5 ml incubation containing 1 mg/ml human liver microsomes (HLM), and 1 mM 1-ABT in 100 mM

potassium phosphate buffer pH 7.4 was prepared, and 2100 µL aliquots were taken at 0 min (-NADPH) and 30 min after initiation of incubation with 1mM NADPH at 37°C. Aliquots were reduced by addition of sodium dithionite, and then evenly divided between two 1 ml 1-cm path length cuvettes. A baseline was recorded, and then carbon monoxide was gently bubbled into the sample cuvette (approximately 1 bubble per second for one minute). Next, absorbance was scanned from 400-600 nm, and peak absorbance at 450 nm was determined. Control studies included incubations in the presence of NADPH without 1-ABT to determine loss of CO binding over the 30 min incubation period. Percent loss in CO-binding was roughly determined by difference in the peak absorbance observed at 450 nm between incubations (corrected for the drift below 0 absorbance units in baseline).

LC/MS/MS Analysis. Metabolites were analyzed on a PE Sciex API-3000 (Foster City, CA) triple-quadrupole instrument, except for 4′-hydroxymephenytoin, which was analyzed on a PE Sciex API-4000. The mass spectrometer was equipped with an electrospray ionization (ESI) interface connected in line with a Shimadzu LC20AD (Columbia, MD) pump and a Leap Technologies CTC PAL (Carrboro, NC) auto-sampler. A 10 µL aliquot of sample was injected onto the column and eluted using a programmed flow rate from 0.4 to 0.6 ml/min. All chromatographic separations were carried out using a gradient elution profile on a Zorbax 3.5 µm Eclipse Plus C_{18} 2.1 x 50 mm column, except for 4`-hydroxymephenytoin which was separated on an Alltima 3 µm C_{18} 2.1 x 50 mm column. The source temperature was set to 400°C and mass spectral analyses were performed using multiple reaction monitoring (MRM). Gradient profiles, ionization modes and fragment transitions for the various metabolites are listed in Table 1.

Tolbutamide was used as the internal standard for both negative and positive ionization modes; the respective transitions were 269.1/170.0 and 271.1/91.2. All data were analyzed using PE Sciex Analyst 1.4.1 software.

Results and Discussion

1-Aminobenzotriazole (1-ABT) is often used as a non-selective inactivator of the human P450 enzymes when conducting reaction phenotyping studies in drug discovery (Williams et al., 2003), yet a full characterization of the remaining activity of the major human P450s following pre-treatment with 1-ABT has yet to be assessed. In the studies described herein, we adopted an incubation condition commonly found in the literature and measured residual enzyme activity for nine of the major human P450s (1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, and 3A4) following pre-incubation with 1 mM 1-ABT for 30 min. Following the aforementioned pre-incubation conditions with 1-ABT, roughly 20% of the spectrally detectable 450 is remaining (~80% loss), indicated by the peak absorbance differences at 450 nm (Figure 1), which suggests that measurable enzymatic activity likely still remains. Regardless of the loss in spectrally detectable P450, it is still unknown which P450 enzymes are most affected by 1-ABT. As demonstrated in Table 2, all of the human P450 enzymes tested were inactivated following pre-incubation with 1-ABT, albeit to differing degrees, as evidenced by lower percent remaining activity following pre-incubation with inhibitor compared to no pre-incubation. In addition, incubations with control time-dependent inhibitors resulted in loss of P450 activity after 30 min, whereas incubations with selected competitive inhibitors resulted in minor, if any, differences in enzymatic activity following 30 min pre-incubation (Figure 2). In the cases of P450s 2A6 and 3A4, enzyme activity was essentially completely inhibited (remaining activities of ~2%) after the pre-incubation with 1-ABT. For the remaining P450 enzymes however, residual activity ranged between 19 and 58%. In addition, while 1-ABT produced an equal or greater loss of activity in

comparison to the control inhibitors of P450 2C19 and 2E1 at the concentrations tested (19 and 25%, respectively), this was not observed for P450s 1A2, 2B6, 2C8, 2C9 and 2D6, where either the positive control time-dependent inhibitor, or competitive inhibitor (or both) displayed more potent inhibition of enzymatic activity than 1-ABT at lower concentrations (Table 2), perhaps arguing against the use of 1-ABT *in vitro*. In particular, for P450 2C9, tienilic acid, as well as the competitive inhibitor sulfaphenazole were significantly more effective, with 11 and 27% remaining activity, respectively, compared to 58% with 1-ABT (Table 2). This notably weak inactivation of P450 2C9 was particularly interesting, and is consistent with previous reports (Emoto et al., 2003; Emoto et al., 2005). Prototypical substrates of P450 2C9 are carboxylic acids such as flurbiprofen, naproxen, ibuprofen, and diclofenac (Tracy et al., 1996; Tracy et al., 1997; Klose et al., 1998), which are anionic at pH 7.4, and proposed to be involved in a key binding interaction with Arg 108, a cationic residue in the active site (Ridderstrom et al., 2000; Wester et al., 2004). The fact that 1-ABT is devoid of a carboxylate negative charge may explain the pronounced lack of inactivation for this P450 isoform. Studies have been initiated to investigate this hypothesis.

It is noteworthy to mention that competitive inhibition, indicated by reduced activity at time zero (i.e. no pre-incubation) compared to solvent control incubations without 1-ABT, was observed for numerous P450s. For example, P450s 2A6 and 3A4 demonstrated <30% activity without a pre-incubation at time zero (Table 2), not surprising considering that even following a 20-fold dilution into the secondary incubation, 1-ABT is present at a 50 μM concentration. It must be acknowledged then that percent remaining activity measured following pre-incubation is likely a combination

of time-dependent and competitive inhibition. It is anticipated that if the residual 1-ABT were to be washed away and then P450 activity assayed, the activity after 30 min pre-incubation would mostly likely be higher. With this said, it was surprising that 1-ABT, a small molecule (134 amu), displayed such profound competitive inhibition of P450 3A4, considering the known large active site cavity of this enzyme (Yano et al., 2004).

In summary, from a practical sense, 1-ABT is clearly useful for determining P450-mediated metabolism *in vitro*, as well as *in vivo* (Strelevitz et al., 2006). However, findings from these studies suggest that remaining enzymatic activity following an extended pre-treatment (30 min) with a high concentration of 1-ABT (1 mM) is markedly measurable, despite significant loss of spectrally detectable P450, thus challenging the common assumption that when using 1-ABT *in vitro* as a pan-P450 inhibitor, remaining enzymatic activity is non-P450 mediated.

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Figure Legend

Figure 1. Carbon monoxide binding prior to (0 min) and after pre-incubation (30 min) for, A) control incubation without 1-ABT where ~30% loss in spectrally detectable P450 was observed, and B) incubation with 1 mM 1-ABT, resulting in ~80% loss in spectrally detectable P450.

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Table 1. Summary of LC/MS/MS analytical conditions for selective P450 probe substrate metabolites measured from secondary incubations.

Metabolite[1]	ESI ion mode	MS Transition *m/z*	Gradient profile %B(min)[2]
Acetaminophen	+	152/110	10(0):10(1.2):98(3.5)
7-Hydroxycoumarin	-	161/133	5(0):5(0.8):95(1.5)
Hydroxybupropion	+	256/131	5(0):5(0.8):98(2.5)
6α-Hydroxypaclitaxel	+	870/286	5(0):5(0.8):98(2.5)
4′-Hydroxydiclofenac	-	310/266	5(0):5(0.3):95(2.5)
4′-Hydroxymephenytoin	-	233/190	5(0):5(0.3):100(3.5)
Dextrorphan	+	258/157	10(0):10(0.8):95(2.0)
6-Hydroxychlorzoxazone	-	184/120	5(0):5(1.0):100(2.0)
1′-Hydroxymidazolam	+	342/203	10(0):10(0.8):95(2.0)

[1] Tolbutamide was used as the internal standard for both negative and positive ionization modes; the respective transitions were *m/z* 269/170 and 271/91.

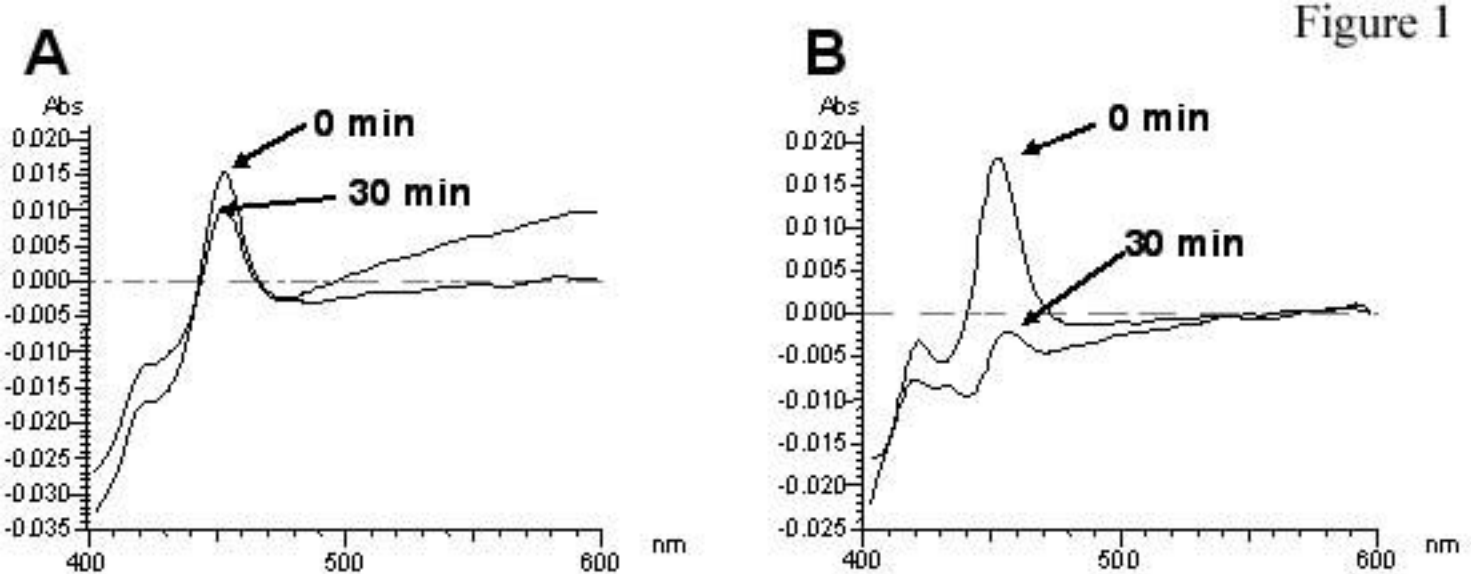
[2] Mobile phases for all analyses were A: 0.1% formic acid in H_2O, and B: 0.1% formic acid in acetonitrile.

Table 2. Percent (%) remaining P450 activity observed in human liver microsomes (HLMs) after a 30 minute pre-incubation with either 1 mM 1-aminobenzotriazole or selected concentration of a specific positive control P450 time-dependent inhibitor (TDI), or competitive inhibitor. Data are presented as mean (S.D.) from triplicate determinations.

		1-ABT (1 mM)		Positive Control TDI			Competitive Inhibitor		
		Pre-Incubation			Pre-Incubation			Pre-Incubation	
Probe Reaction	P450	0 min	30 min		0 min	30 min		0 min	30 min
Phenacetin O-deethylase	1A2	91 (2.0)	28 (3.0)	Furafylline (10 µM)	87 (1.5)	16 (0.8)	α-Naphthoflavone (1 µM)	16 (0.8)	24 (3.3)
Coumarin 7-hydroxylase	2A6	22 (4.0)	2.3 (0.1)	8-Methoxypsoralen (0.5 µM)	60 (2.7)	13 (1.5)	Tranylcypromine (20 µM)	40 (2.0)	53 (6.3)
Bupropion hydroxylase	2B6	80 (3.4)	30 (1.7)	Ticlopidine (20 µM)	50 (3.4)	11 (0.1)	Clotrimazole (10 µM)	52 (8.0)	90 (1.6)
Taxol 6α-hydroxylase	2C8	71 (0.3)	37 (2.6)	Amiodarone (100 µM)	35 (3.6)	16 (3.3)	Montelukast (1 µM)	25 (1.5)	31 (0.8)
Diclofenac 4′-hydroxylase	2C9	90 (12)	58 (4.2)	Tienilic Acid (20 µM)	53 (11)	11 (1.2)	Sulfaphenazole (10 µM)	22 (2.6)	27 (1.5)
(S)-Mephenytoin 4′-hydroxylase	2C19	52 (11)	19 (1.4)	Ticlopidine (25 µM)	65 (1.3)	16 (4.6)	N-Benzyl Nirvanol (10 µM)	45 (7.6)	50 (5.8)
Dextromethorphan O-demethylase	2D6	75 (11)	29 (2.5)	Paroxetine (20 µM)	29 (1.2)	5 (1.2)	Quinidine (1 µM)	53 (5.6)	58 (3.2)
Chlorzoxazone 6-hydroxylase	2E1	53 (4.0)	25 (2.9)	DDC (10 µM)	93 (7.8)	60 (5.6)	Tranylcypromine (10 µM)	60 (6.7)	71 (6.3)
Midazolam 1′-hydroxylase	3A4	26 (0.8)	1.7 (0.3)	Erythromycin (200 µM)	92 (1.9)	52 (2.9)	Ketoconazole (1 µM)	41 (1.3)	32 (1.9)

*Concentrations of 1-ABT and selected TDI and competitive inhibitor controls represent that used in the primary incubation.
DDC – Diethyldithiocarbamate

Figure 1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