Short Communication

Is 1-Aminobenzotriazole an Appropriate in Vitro Tool as a Nonspecific Cytochrome P450 Inactivator?

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ABSTRACT:

1-Aminobenzotriazole (1-ABT) is generally considered to be a nonselective mechanism-based inactivator of both human and nonhuman cytochrome P450 (P450) enzymes. Thus, 1-ABT is routinely used when conducting in vitro reaction phenotyping studies with new chemical entities in drug discovery to decipher P450 from non-P450-mediated metabolism. Experiments with pooled human liver microsomes (HLMs) demonstrated that carbon monoxide binding, although substantially reduced after a 30-min preincubation 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 after 30-min preincubation with either 1-ABT (1 mM), a positive control time-dependent inhibitor, or a competitive inhibitor. Whereas P450 2A6 and 3A4 activity was essentially eliminated upon 30-min pretreatment with 1-ABT, the other human P450s were less affected, with at least 20% activity remaining after pretreatment. In contrast, most of the known P450 selective time-dependent inhibitors were more effective inactivators than 1-ABT at lower concentrations. A particularly interesting finding was that 1-ABT was quite ineffective at inactivating P450 2C9, with roughly 60% activity remaining after pretreatment, 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 nonselective P450 inhibitor in vitro is risky, and false conclusions regarding remaining metabolic activity being non-P450 mediated after 1-ABT pretreatment may be made.

In vitro reaction phenotyping, where enzymes contributing to the biotransformation of new chemical entities 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 interpatient variability in drug exposure. One critical issue in conducting these in vitro studies is having the appropriate tool substrates and inhibitors of the various human cytochrome P450 (P450) enzymes. A particularly useful tool inhibitor historically has been 1-aminobenzotriazole (1-ABT), which is thought to inactivate P450 enzymes nonselectively 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 preincubate either human liver microsomes (HLMs) or hepatocytes with high concentrations of 1-ABT (~1 mM) before the introduction of test compounds (substrate-depletion approach) to decipher P450 from non-P450 mediated metabolism (Dalmadi et al., 2003; Williams et al., 2003; Kostrubsky et al., 2006). Although 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 nonspecific inactivator of P450 by determining the remaining activity of the major human P450 enzymes (CYP1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, and 3A4) in HLMs after pretreatment with 1 mM 1-ABT. These 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, NADP⁴⁺, isotropic acid, isotropic dehydrogenase, magnesium chloride, 1-aminobenzotriazole, sodium dithionite, tolbutamide, diclofenac, 4’-hydroxydiclofenac, sulfaphenazole, dextromethorphan, dextrophan, paroxetine, quinidine, phencetin, acetaminophen, furafylline, α-naphthoflavone, midazolam, 1’-hydroxymidazolam, erythromycin, ketoconazole, buproplion, ticlopidine, clotrimazole, paclitaxel (Taxotol), amidodarone, (S)-mephénytoin, 4’-hydroxymephénytoin, (−)-N-3-benzyl nirvanol, coumarin, 7-hydroxycoumarin, 8-methoxypsoralen, translycypromine, chlorzoxazone, 6-hydroxychlorzoxazone, and diethylidithiocarbamate were all purchased from Sigma-Aldrich (St. Louis, MO). Montelukast was purchased from Sequoia Research Products (Pangbourne, UK). Pooled HLMs (pool of 50 donors), 6a-hydroxyhaldothiazol, and hydroxybupropion were purchased from BD Biosciences (San Jose, CA), whereas tiencil 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 HLMs 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 (CYP1A2, 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 (Eppendorf AG, Hamburg, Germany) and consisted of HLMs (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 MgCl₂, 1 mM, NADP⁴⁺).

ABBREVIATIONS: P450, cytochrome P450; 1-ABT, 1-aminobenzotriazole; HLM, human liver microsomes; TDI, time-dependent inhibitor; LC/MS/MS, liquid chromatography/tandem mass spectrometry; CO, carbon monoxide.
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 \( K_a \)), 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), 7-hydroxychlorzoxazone (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 3000g for 10 min, supernatant was transferred to a separate analytical plate, and metabolite formation was analyzed by liquid chromatography/tandem mass spectrometry (LC/MS/MS) as described below. After treatment with 1-aminobenzotriazole, positive control TDI, and competitive inhibitor, the percentage of remaining P450 activity was calculated by comparing the metabolite formation after 0- and 30-min preincubation with solvent controls without inhibitor. Data were plotted for visualization using GraphPad Prism (GraphPad Software Inc., San Diego, CA) software.

**Spectrophotometric Analysis.** Reduced carbon monoxide (CO) difference spectra were recorded on a Hitachi (Danbury, CT) U3300 dual-beam spectrophotometer, and data were analyzed using UV Solutions 1.2 software (Hitachi). In brief, a 5-min incubation (containing 1 mg/ml HLMs) and 1 mM 1-ABT in 100 mM potassium phosphate buffer (pH 7.4) was prepared, and 2100-μl aliquots were taken at 0 (−NADPH) and 30 min after initiation of incubation with 1 mM 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 1 min). Next, absorbance was scanned from 400 to 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. Percentage of 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 (Applied Biosystems, Foster City, CA) triple-quadrupole instrument, with the exception of 4′-hydroxymephenytoin, which was analyzed on a PE Sciex API-4000. The mass spectrometer was equipped with an electrospray ionization interface connected in line with a Shimadzu LC20AD (Shimadzu Scientific Instruments, Columbia, MD) pump and a Leap Technologies CTC PAL (Carrboro, NC) autosampler. A 10-μl aliquot of the 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 (Agilent Technologies, Santa Clara, CA) C18 2.1 × 50 mm column, with the exception of 4′-hydroxymephenytoin, which was separated on an Alltima (Grace Davison Discovery Science, Deerfield, IL) 3-μm C18 2.1 × 50 mm column. The source temperature was set to 400°C, and mass spectral analyses were performed using multiple reaction monitoring. 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-ABT is often used as a nonselective 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 after pretreatment 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) after preincubation with 1 mM 1-ABT for 30 min. After the aforementioned preincubation conditions with 1-ABT, roughly 20% of the spectrally detectable 450 is remaining (~80% loss), indicated by the peak absorbance differences at 450 nm (Fig. 1), which suggests that measurable enzymatic activity probably still remains. Regardless of the loss in spectrally detectable P450, it is still unknown which of the P450 enzymes are most affected by 1-ABT. As demonstrated in Table 2, all of the human P450 enzymes tested were inactivated after preincubation with 1-ABT, albeit to differing degrees, as evidenced by a lower percentage of remaining activity after preincubation with inhibitor compared with no preincubation. In addition, incubations with control TDIs resulted in loss of P450 activity after 30 min, whereas incubations with selected competitive inhibitors resulted in minor, if any, differences in enzymatic activity after 30-min preincubation (Table 2). In the cases of P450s 2A6 and 3A4, enzyme activity was essentially completely inhibited (remaining activities of ~2%) after the preincubulation with 1-ABT. However, for the remaining P450 enzymes, residual activity ranged between 19 and 58%. In addition, whereas 1-ABT produced an equal or greater loss of activity in comparison with 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 TDI 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, thienic acid as well as the competitive inhibitor sulfaphenazole were significantly more effective, with 11 and 27% remaining activity, respectively, compared with 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, 2005). Prototypical substrates of P450 2C9 are carboxylic acids such as flurbiprofen, naproxen, ibuprofen, and diclofenac (Tracy et al., 1996, 1997; Klose et al., 1998), which are anionic at pH 7.4, and proposed to be involved in a key binding interaction with Arg108, a
cationic residue in the active site (Ridderström 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 that competitive inhibition, indicated by reduced activity at time 0 (i.e., no preincubation) compared with solvent control incubations without 1-ABT, was observed for numerous P450s. For example, P450s 2A6 and 3A4 demonstrated 30% activity without a preincubation at time 0 (Table 2), which is not surprising considering the fact that even after a 20-fold dilution into the secondary incubation, 1-ABT is present at a 50 μM concentration. Therefore, it must be acknowledged that the percentage of remaining activity that is measured after preincubation is probably a combination of time-dependent and competitive inhibition. It is anticipated that if the residual 1-ABT were to be washed away and the P450 activity assayed, then the activity after 30-min preincubation would most likely be higher. Taking these data together, 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 the remaining enzymatic activity after an extended pretreatment (30 min) with a high concentration of 1-ABT (1 mM) is markedly measurable, despite a significant loss of spectrally detectable P450, thus challenging the common assumption that when using 1-ABT in vitro as a pan-P450 inhibitor, the remaining enzymatic activity is non-P450 mediated.

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**TABLE 2**

Percentages of remaining P450 activity observed in HLMs after a 30-min preincubation with either 1 mM 1-ABT or selected concentration of a specific positive control P450 TDI, or competitive inhibitor

<table>
<thead>
<tr>
<th>Probe Reaction</th>
<th>1-ABT (1 mM) Preincubation</th>
<th>Positive Control TDI Preincubation</th>
<th>Competitive Inhibitor Preincubation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P450</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0 min</td>
<td>30 min</td>
<td>0 min</td>
</tr>
<tr>
<td>Phenacetin O-deethylase</td>
<td>1A2</td>
<td>91 (2.0)</td>
<td>28 (3.0)</td>
</tr>
<tr>
<td>Coumarin 7-hydroxylase</td>
<td>2A6</td>
<td>22 (4.0)</td>
<td>2.3 (0.1)</td>
</tr>
<tr>
<td>Bupropion hydroxylase</td>
<td>2B6</td>
<td>80 (3.4)</td>
<td>30 (1.7)</td>
</tr>
<tr>
<td>Taxol 6α-hydroxylase</td>
<td>2C8</td>
<td>71 (0.3)</td>
<td>37 (2.6)</td>
</tr>
<tr>
<td>Diclofenac 4'-hydroxylase (S)-Mephenytoin 4'-hydroxylase</td>
<td>2C9</td>
<td>90 (12)</td>
<td>58 (4.2)</td>
</tr>
<tr>
<td>Dextromethorphan O-demethylase</td>
<td>2C19</td>
<td>52 (11)</td>
<td>19 (1.4)</td>
</tr>
<tr>
<td>Chlorzoxazone 6-hydroxylase</td>
<td>2D6</td>
<td>75 (11)</td>
<td>29 (2.5)</td>
</tr>
<tr>
<td>Midazolam 1'-hydroxylase</td>
<td>2E1</td>
<td>53 (4.0)</td>
<td>25 (2.9)</td>
</tr>
<tr>
<td>(S)-Mephenytoin 4'-hydroxylase</td>
<td>3A4</td>
<td>26 (0.8)</td>
<td>1.7 (0.3)</td>
</tr>
</tbody>
</table>

**FIG. 1.** The graphic illustrates CO binding before (0 min) and after preincubation (30 min) for control incubation without 1-ABT, where ~30% loss in spectrally detectable P450 was observed (A), and incubation with 1 mM 1-ABT, resulting in ~80% loss in spectrally detectable P450 (B).
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