EFFECTIVE DOSING REGIMEN OF 1-AMINOBENZOTRIAZOLE FOR INHIBITION OF ANTIPYRINE CLEARANCE IN GUINEA PIGS AND MICE USING SERIAL SAMPLING

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ABSTRACT:

Single-dose pharmacokinetics of 1-aminobenzotriazole (ABT), a potent nonspecific inhibitor of cytochromes P450 (P450s), were characterized after oral administration to mice and guinea pigs at doses of 50, 100, and 150 mg/kg using serial sampling in both species. Only 30-μl blood samples were drawn from jugular vein-cannulated mice using Microvette capillary tubes containing lithium heparin. A comparison of the pharmacokinetics of antipyrine (AP), a nonspecific probe substrate of P450s, were characterized after oral administration to mice and guinea pigs at doses of 50, 100, and 150 mg/kg using serial sampling in both species. Pretreatment of animals with ABT 2 h prior to AP administration decreased the plasma AP clearance by about 95% in all ABT doses studied and 84, 95, and 95% in guinea pigs at a dose of 50, 100, and 150 mg/kg ABT, respectively. In vitro, the dissociation constants (Kd) for ABT as the P450 mechanism-based inactivator were determined to be 45.6 and 193 μM, and the maximal inactivation rate constants (kmax) were determined to be 0.089 and 0.075 min⁻¹ for the mouse and guinea pig liver microsomes, respectively. The projected P450 inactivations at the plasma Cmax of ABT agreed with the inhibitions of P450-mediated AP clearance observed in vivo. For mechanistic studies in vivo overall, a 2-h prior oral pretreatment with ABT at 50 mg/kg in mice and 100 mg/kg in guinea pigs would provide significant systemic concentrations of the inhibitor over 24 h and inhibition of P450-dependent clearance of test compounds.

Materials and Methods

Materials. Antipyrine, 4-Hydroxyantipyrine, ABT, and NADPH were purchased from Sigma-Aldrich (St. Louis, MO). Mouse and guinea pig liver microsomes were purchased from XenoTech (Lenexa, KS). All other chemicals were of analytical grade.

Animal Pharmacokinetic Studies. Male, jugular vein-cannulated and non-cannulated C57Bl/6 mice and jugular vein-cannulated Hartley SJIC guinea pigs were obtained from Hilltop Laboratory Animals, Inc. (Scottdale, PA). For terminal sampling, mice were dosed intravenously with AP at 20 mg/kg and dissolved in normal saline (1 ml/kg). Three animals per time point of 0, 0.083, 0.25, 0.5, 1, 2, 4, 6, 8, and 24 h were sacrificed, and maximum blood was collected by cardiac puncture into tubes containing lithium heparin as an anticoagulant. Plasma was harvested after centrifugation of the blood. In vitro, the dissociation constants (Kd) for ABT as the P450 mechanism-based inactivator were determined to be 45.6 and 193 μM, and the maximal inactivation rate constants (kmax) were determined to be 0.089 and 0.075 min⁻¹ for the mouse and guinea pig liver microsomes, respectively. The projected P450 inactivations at the plasma Cmax of ABT agreed with the inhibitions of P450-mediated AP clearance observed in vivo. For mechanistic studies in vivo overall, a 2-h prior oral pretreatment with ABT at 50 mg/kg in mice and 100 mg/kg in guinea pigs would provide significant systemic concentrations of the inhibitor over 24 h and inhibition of P450-dependent clearance of test compounds.
Single oral dose studies of ABT were conducted in jugular vein-cannulated mice (n = 4) and guinea pigs (n = 3) at 50, 100, and 150 mg/kg using 0.5% methyl cellulose as a vehicle (5 ml/kg). Serial plasma samples were collected as described above from mice at 0, 0.5, 1, 3, 6, 9, 24, 32, and 48 h and guinea pigs at 0, 0.5, 1, 3, 6, 9, 24, 32, 56, and 72 h.

For the interaction studies, animals were dosed intravenously with AP at 20 mg/kg 2 h after an oral administration of ABT at 50, 100, or 150 mg/kg (n = 3 or 4). Serial plasma sampling ensued as described above for 48 h from both species. Plasma samples from all studies were frozen until analysis.

**Inactivation of Antipyrine Metabolism by ABT.** Inactivation rates were obtained using the conventional two-step incubation methods. All incubations were conducted in 96-well plates. Mouse and guinea pig liver microsomes (2.5 mg/ml) were preincubated with ABT at 0, 10, 50, 100, and 200 μM with or without 2 mM NADPH in a total volume of 100 μl with 0.1 M potassium phosphate buffer, pH 7.4, and 3 mM magnesium chloride. At selected time intervals (0, 5, and 15 min) (at and above 30 min, the kinetics were not in the linear range), 15-μl aliquots of the incubation mixture were transferred into plates containing 135 μl of 0.1 M potassium phosphate buffer, pH 7.4, containing antipyrine (final concentration, 200 μM) and NADPH (2 mM). The samples were incubated for 20 min at 37°C, and the reaction was terminated by the addition of 100 μl of acetonitrile containing 1 μM carbamutide (internal standard). The sample plates were centrifuged at 3000 g for 10 min, and the supernatant was analyzed by LC/MS/MS for 4-hydroxyantipyrine concentration.

**Bioanalysis.** In vivo plasma samples were protein precipitated and analyzed by LC/MS/MS using a method similar to the one reported previously (Balani et al., 2002). In general, the LC/MS/MS system consisted of a binary high-performance liquid chromatography pump (1100; Agilent Technologies, Palo Alto, CA), an HTS PAL autosampler (LEAP Technologies, Carrboro, NC), and a triple-quadrupole mass spectrometer (API-4000; Applied Biosystems, Foster City, CA). Separation was performed on a YMC ODS AQ column (2 × 50 mm; Waters, Milford, MA) using formic acid (0.1%) in water and acetonitrile. The LC conditions involved a Supelco Discovery C18 column (10 × 3 mm; Supelco, Bellefonte, PA), an Agilent pump, and a LEAP autosampler. The mobile phase consisted of a gradient mixture of 0.1% formic acid and acetonitrile.

**Data Analysis.** The pharmacokinetic parameters were calculated with WinNonlin software (Pharsight, Mountain View, CA) using noncompartmental analysis of the data. The observed inactivation rates (k_{inact}) for 4-hydroxyantipyrine formation were calculated from the initial slopes by linear regression analysis of natural log percentage of remaining activity versus preincubation time plot. The inactivation kinetics parameters were obtained by fitting to the hyperbolic equation 

\[ \text{inact} = k_{\text{max}} \times \frac{|I|}{(|I| + K_I)} \]

using Prism Software (GraphPad Software, Inc., San Diego, CA). The projected t_{1/2} for P450 inactivation was then calculated using C_{max} for ABT for the |I| value in the above equation to calculate k_{inact}. Then t_{1/2} was calculated as \((\text{Ln} \, 2/k_{\text{inact}})\).

**Results**

**Mouse PKs.** The plasma concentration-time profiles of antipyrine (20 mg/kg i.v.) by the serial and terminal plasma sampling methods are shown in Fig. 1. For the common time points of the curve, the two plots were practically superimposable. The PK parameters by the two methods also were comparable, as shown in Table 1. The ABT concentration-time profiles following single oral doses of 50, 100, and 150 mg/kg and serial plasma sampling are shown in Fig. 2. The AUC increased in a greater than proportional to dose manner, as shown in

**TABLE 1**

<table>
<thead>
<tr>
<th>ABT Dose (mg/kg)</th>
<th>Sampling</th>
<th>Mouse</th>
<th></th>
<th>Guinea Pig</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>CL&lt;sub&gt;h&lt;/sub&gt;</td>
<td>AUC</td>
<td>t&lt;sub&gt;1/2&lt;/sub&gt;</td>
<td>CL&lt;sub&gt;h&lt;/sub&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>l/h/kg</td>
<td>μM · h</td>
<td>h</td>
<td>l/h/kg</td>
</tr>
<tr>
<td>0</td>
<td>Terminal</td>
<td>1.35 ± 0.18</td>
<td>79.8 ± 10.5</td>
<td>0.5 ± 0.1</td>
<td>0.57 ± 0.16</td>
</tr>
<tr>
<td>50</td>
<td>Serial</td>
<td>1.67 ± 0.56</td>
<td>69.1 ± 21.6</td>
<td>0.9 ± 0.3</td>
<td>0.09 ± 0.02</td>
</tr>
<tr>
<td>100</td>
<td></td>
<td>0.08 ± 0.03</td>
<td>1463 ± 515</td>
<td>4.2 ± 2.2*</td>
<td>0.03 ± 0</td>
</tr>
<tr>
<td>150</td>
<td></td>
<td>0.10 ± 0.04</td>
<td>1112 ± 456</td>
<td>ND</td>
<td>0.03 ± 0.01</td>
</tr>
</tbody>
</table>

ND, not determined.

* Based on the last two time points only.
Table 2. Pretreatment of animals with 50, 100, and 150 mg/kg ABT p.o. 2 h prior to the i.v. administration of 20 mg/kg AP led to a significant drop in the plasma clearance and consequently to an increase in the AUC of AP, as depicted in Fig. 3 and Table 1.

Guinea Pig PKs. The ABT concentration-time profiles following single oral doses of 50, 100, and 150 mg/kg are shown in Fig. 4. The AUC increase was proportional to dose (Table 2). The PK profile after a 20 mg/kg intravenous dose of AP is shown in Fig. 5, and the parameters are given in Table 1. Pretreatment of guinea pigs with 50, 100, and 150 mg/kg of ABT given orally 2 h prior to the i.v. administration of 20 mg/kg AP led to a significant drop in the plasma clearance of AP, as depicted in Fig. 5 and Table 1.

In Vitro Inhibition. Inhibition of AP metabolism to 4-hydroxy AP by ABT (up to 200 μM) was found to be time- and concentration-dependent in both species. The dissociation constants \( K_d \) for ABT as P450 mechanism-based inactivators were determined to be 45.6 and 193 μM. The maximal inactivation rate constants \( k_{inact} \) were determined to be 0.089 and 0.075 min\(^{-1}\) for the mouse and guinea pig liver microsomes, respectively (Table 3).

Discussion

ABT has generally been used as a nonspecific P450 inhibitor. Most laboratories use varied ABT dosing schedules for such studies. Pharmacokinetics of ABT and its interaction with AP were reported for rats, dogs, and monkeys (Balani et al., 2002); based on those data, an effective dosing regimen of ABT was established. The current report extends those studies to other species (mice and guinea pigs), because ABT continues to be used successfully for mechanistic studies in mice (Adler et al., 2000; Hoffler et al., 2003; van Ravenzwaay et al., 2003) and guinea pigs (Knickle and Bend, 1992; Xu et al., 1995; Woodcroft et al., 1997). Thus, the pharmacokinetic parameters were generated for AP in mice using the usual terminal sampling approach and in guinea pigs using serial sampling. The AP pharmacokinetics were also assessed in mice following serial blood sampling from either jugular vein-cannulated or noncannulated animals using tail vein sampling. Only 30 μl of blood was collected at each time point, and after separation, 10 μl of plasma was used for the AP and ABT concentration measurements. The tail vein bleeding frequently led to a degree of hemolysis after the initial couple of time points; as anticipated, this led to the overestimation of plasma concentrations (data not shown) because of the possible partitioning of AP into red blood cells. Sampling from cannulated mice did not cause hemolysis. As shown in Fig. 1 and Table 1, the AP plasma concentration-time profile after the terminal sampling was similar to the one from serial sampling, with low clearance, a volume of distribution similar to that of body water (data not shown), and a short half-life. The results from this comparative study increased the confidence in using the serial micro sampling and analysis for mice studies. All further mice studies were conducted using the jugular vein-cannulated animals. These studies demonstrated that serial sampling in mice to obtain complete pharmacokinetic profiles is practical, saves time and cost, reduces the number of animals required, and increases throughput compared with studies where three animals are sacrificed at each time point in building the PK profile. This serial blood sampling has become possible with the availability of highly sensitive LC/MS/MS assays for microsamples and the commercial availability of jugular vein-cannulated mice. Furthermore, automation in sampling is also available using precise, intelligent automated blood samplers.

As shown in Fig. 2 and Table 2, single 50, 100, and 150 mg/kg oral dose ABT pharmacokinetics in mice were nonlinear, with a greater than dose proportional increase in the exposure. The effect of ABT dosed orally at 50, 100, and 150 mg/kg (2 h prior to AP administration) in mice on 20 mg/kg AP i.v. pharmacokinetics is depicted in Fig. 3, and the parameters are given in Table 1. The plasma AP concentrations stayed at high levels for at least 8 h (and likely longer) compared with that after AP administration alone. The clearance of AP declined by about 95% at all of the ABT doses tested. The magnitude of clearance at the above ABT doses and corresponding
AP plasma concentration-time profiles suggested that a single 50-mg/kg oral ABT dose 2 h prior to the administration of the compound would provide a significant decline in the P450-based clearance in mice. Depending on a need for continuous inhibition of P450, b.i.d. dosing of ABT at 50 mg/kg is suggested.

Pharmacokinetics of ABT given orally at 50, 100, and 150 mg/kg in guinea pigs is described by the parameters shown in Table 2 and the plasma concentration-time profile shown in Fig. 4. The usual serial sampling procedure was followed in this larger animal. The AUC of ABT seemed to increase in a dose-proportional manner in guinea pigs. The plasma clearance of AP in guinea pigs was lower than in mice.

Following the 2-h prior treatment of animals with oral doses of 50, 100, and 150 mg/kg ABT, the AP clearance dropped by 84, 95, and 95%, respectively (Table 1; Fig. 5). Thus, in guinea pigs, a higher AP clearance was observed at plasma concentrations of ABT by both mouse and guinea pig liver microsomes. The respective projected P450 inactivation half-lives in mice (at plasma Cmax) were 9.1, 8.3, and 8.1 min for 50, 100, and 150 mg/kg ABT and 13.3, 12.4, and 11.2 min for guinea pigs. Qualitatively, these t1/2 values indicated that P450s were inactivated very rapidly during the ABT pretreatment period in mice and that this inhibition was faster than in guinea pigs. Thus, because of the long, 2-h pretreatment period for ABT relative to the short inactivation t1/2, there were not big differences in the in vivo plasma clearance values for AP in the two species.

In conclusion, in a single-dose setting, 2-h prior treatment with ABT given orally at 50 mg/kg in mice and 100 mg/kg in guinea pigs is suggested to provide a significant drop in P450-dependent clearance. For chronic, optimal P450 inhibitory effects, 50 mg/kg b.i.d. dosing of ABT in mice and 100 mg/kg q.i.d. in guinea pigs is suggested, considering accumulation of the inhibitor on repeat dosing. The serial plasma sampling in mice was also successfully demonstrated; therefore, it can be used more routinely to reduce the number of mice per study, reduce cost and labor, and increase the throughput.

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References

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