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) administered i.v. at 20 mg/kg to mice followed by serial and terminal sampling techniques yielded similar results. The ABT concentrations in plasma were sustained at high levels (5–100 μM) for at least 12 h in both species. Pretreatment of animals with ABT 2 h prior to AP administration decreased the plasma AP clearance by about 95% in mice at 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 SJV 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. For serial sampling, 5 jugular vein-cannulated mice were given 20 mg/kg AP, and 30 μl of blood was collected from each mouse at the above time points; the collected blood was deposited directly into Microvette capillary blood tubes (Sarstedt, Inc., Newton, NC) containing lithium heparin. Samples were placed on wet ice and quickly transferred into an Eppendorf Micro Centrifuge (model 5415 D; Eppendorf – 5 Prime, Inc., Boulder, CO) and centrifuged for 2 min at 2000g at the end of each collection interval. Exactly 10 μl of plasma was transferred into polypropylene conical tubes (VWR International, Bridgeport, NJ). Plasma samples were frozen at −20°C immediately following each collection interval. Serial samples of 0.3 ml of blood per time point were drawn from guinea pig (n = 3) for the intravenous AP study, and plasma was separated. This was the same as those for mice, plus 46 and 48 h.

In our last communication (Balani et al., 2002), the dosing regimen of 1-aminobenzotriazole (ABT), a nonspecific inhibitor of cytochromes P450 (P450s) (Huijzer et al., 1989; Constan et al., 1999), was established in rats, dogs, and monkeys to effectively inhibit P450s and hence decrease the plasma clearance and increase the exposure of antipyrine (AP), a nonspecific probe substrate of P450s (Engel et al., 1996; Sharer and Wrighton, 1996; Matzke et al., 2000). All P450s were shown to be affected by ABT treatment (Balani et al., 2002). Due to wide distribution of ABT in rats, P450 inhibition is expected to be general in the body tissues (Town et al., 1993). The literature previously contained varied treatment of animals with ABT (e.g., dosing route, dose level, pretreatment time, and frequency of dosing). The current study extends our previous studies to mice and guinea pigs, which are routinely used for mechanistic PK, toxicity, and pharmacology studies; thus, it is intended to provide guidelines for the pretreatment of animals with ABT to significantly alter the oxidative metabolism of test compounds (e.g., to evaluate metabolite versus parent compound-based toxicities or boost compound concentration available for a target enzyme or receptor). Although the safety assessment of chronic dosing of ABT in mice and guinea pigs has not been reported, its safety in rats has clearly been demonstrated (Mico et al., 1988). This report also highlights the use of serial sampling in mice, thus saving cost and labor and reducing the number of animals in studies. In vitro studies were also conducted in mouse and guinea pig hepatic microsomes to gauge the relative effect of ABT pretreatment on AP intrinsic clearance.

ABBREVIATIONS: ABT, 1-aminobenzotriazole; AP, antipyrine; PK, pharmacokinetic; LC, liquid chromatography; MS/MS, tandem mass spectrometry; AUC, area under the curve.
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 nM, 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 carbamazepine (internal standard). The sample plates were centrifuged at 3000g 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 as mobile phases A and B, respectively. The mass spectrometer was operated in multiple reaction monitoring mode using positive ion electrospray ionization. A major difference in this method compared with the previously reported one was that the sample volume was reduced to 10 μl of plasma for the analysis of AP and ABT concentrations in serially sampled mouse studies. Accordingly, a lower limit of quantitation of 10 nM was achieved. The quantitation limit for guinea pigs for the two analytes was 1 nM. In vitro hepatic microsomal incubation samples were analyzed for 4-hydroxyantipyrine by LC/MS/MS using a SCIEX API-4000 instrument (Applied Biosystems). 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_{obs}) 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 \( k_{obs} = k_{max} \cdot [I] / ([I] + K) \) 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 \( k_{obs} \). Then \( t_{1/2} \) was calculated as \((\text{Ln}2)/k_{obs}\).

**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>CL&lt;sub&gt;ir&lt;/sub&gt; (l/h)</th>
<th>AUC (µM · h)</th>
<th>t&lt;sub&gt;1/2&lt;/sub&gt; (h)</th>
<th>CL&lt;sub&gt;ir&lt;/sub&gt; (l/h)</th>
<th>AUC (µM · h)</th>
<th>t&lt;sub&gt;1/2&lt;/sub&gt; (h)</th>
</tr>
</thead>
<tbody>
<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>1.67 ± 0.56</td>
<td>69.1 ± 21.6</td>
<td>0.9 ± 0.3</td>
</tr>
<tr>
<td>50</td>
<td>Serial</td>
<td>0.08 ± 0.03</td>
<td>1463 ± 515</td>
<td>4.2 ± 2.2*</td>
<td>0.08 ± 0.02</td>
<td>1366 ± 425</td>
<td>ND</td>
</tr>
<tr>
<td>100</td>
<td>Serial</td>
<td>0.10 ± 0.04</td>
<td>1112 ± 456</td>
<td>ND</td>
<td>0.03 ± 0.01</td>
<td>4553 ± 1450</td>
<td>5.0 ± 0.8</td>
</tr>
<tr>
<td>150</td>
<td>Serial</td>
<td>0.03 ± 0.01</td>
<td>ND</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

ND, not determined.

* Based on the last two time points only.
separation, 10/H9262
number of animals required, and increases throughput compared with
macokinetic profiles is practical, saves time and cost, reduces the
studied in mice following serial blood sampling from either jugular
small volume of distribution similar to that of body water
terminal sampling was similar to the one from serial sampling, with
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. 4, 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_i) for ABT as
P450 mechanism-based inactivators were determined to be 45.6 and
193 μM. The maximal inactivation rate constants (k_{max}) were deter-
dined 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. Phar-
macokinetics 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 concentra-
tion 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 compar-
ative 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 phar-
macokinetic 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 avail-
able 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 administra-
tion) 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 concen-
trations 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

![Fig. 3. Plasma concentration-time profiles of antipyrine in mice dosed i.v. at 20 mg/kg along with an oral dose of ABT given 2 h prior. Data from serial sampling.](image1)

![Fig. 4. Plasma concentration-time profiles of ABT dosed orally at 50, 100, and 150 mg/kg in guinea pigs.](image2)
AP plasma concentration-time profiles suggested that a single 50-
mg/kg oral ABT dose 2 h prior to test 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
100, and 150 mg/kg. Thus, because of the long, 2-h pretreatment period
for ABT relative to the short inactivation $t_{1/2}$, there were not big differ-
ences 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 clear-
ance. 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 demonstrat-
ed; therefore, it can be used more routinely to reduce the number
definitions for AP 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 $t_{1/2}$, there were not big differ-
ces in the in vivo plasma clearance values for AP in the two species.

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