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IN VIVO INHIBITION OF OXIDATIVE DRUG METABOLISM BY, AND ACUTE TOXICITY OF, 1-AMINOBENZOTRIAZOLE (ABT)

A TOOL FOR BIOCHEMICAL TOXICOLOGY

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Abstract—One hour following intravenous pretreatment of rats with 50 mg/kg of the cytochrome P-450 suicide substrate 1-aminobenzotriazole (ABT), the metabolism of phenacetin to acetaminophen is inhibited completely [B. A. Mico *et al.*, *Drug Metab. Dispos.* **15**, 274 (1987)]. Here we report an examination of the time–course of inhibition of phenacetin elimination by ABT, a demonstration of dose-dependent inhibition of phenacetin and antipyrine clearances by ABT, and an examination of the acute toxicity of ABT in rats, as well as the effect of ABT on phenacetin metabolism in beagles. After a 1-, 12-, 24- or 36-hr pretreatment of rats with ABT (50 mg/kg, i.v.), the clearance of phenacetin was decreased 85, 88, 81 and 48%, respectively, from control values. Twelve hours after intraperitoneal pretreatment of rats with 0.3, 1.0, 5.0, 20, and 50 mg/kg of ABT, the total systemic clearance of phenacetin was suppressed 39, 47, 60, 75, and 79%, respectively, from control values. The clearance of intravenously administered antipyrine was decreased 38 and 66% after a 12-hr intraperitoneal pretreatment of rats with 10 or 50 mg/kg of ABT. In rats, no hematological, clinical chemistry, macroscopic, or microscopic abnormalities were detected 1, 2, 3, and 9 days after a single i.v. dose of ABT (50 mg/kg). A 1-hr pretreatment of beagles with ABT (20 mg/kg) decreased the clearance of intravenous phenacetin 50% and completely prevented the formation of acetaminophen. These results demonstrate that ABT pretreatment causes long-lasting inhibition of oxidative drug metabolism without disruption of normal physiological processes. Profound inhibition of oxidation in two species suggests that ABT may have general utility as an inhibitor of oxidative drug metabolism in biochemical pharmacology and toxicology studies.

The heterocyle 1-aminobenzotriazole (ABT) yields the reactive intermediate benzyne (C_6H_4) upon mild chemical oxidation [1], and inactivates cytochromes P-450 by prosthetic heme alkylation in a process which requires enzyme catalysis [2–6]. The chemical reactivity of benzyne and the proficiency of cytochrome P-450 metabolism of amines combine in ABT to yield a suicide substrate of cytochromes P-450 of unusual potency and efficiency. In studies with intact rats and rat liver microsomes [4, 7], ABT inactivates approximately 80% of cytochromes P-450 in rat liver microsomes from control and from phenobarbital- and 3-methylcholanthrane-pretreated rats. In addition, the forms of cytochrome P-450 responsible for lauric acid hydroxylation [4] are inactivated by ABT. With highly purified enzymes [7], ABT inactivates both phenobarbital and 3-methylcholanthrane cytochrome P-450 isozymes. This profound loss of cytochrome P-450, however, is *not* accompanied by measurable changes in hepatic cholesterol 7-alpha-hydroxylase activity [8], adrenal cholesterol side-chain cleavage activity [8], cytochrome b_5 [2], rabbit lung flavin monooxygenase activity [5], or serum transaminase activity [3]. The specific action of ABT, as well as its water solubility, lack of apparent toxicity [3, 9], and potential for long lasting inhibition through enzyme loss, make ABT a potential tool in biochemical toxicology studies in which inhibition of cytochromes P-450 is desired.

Despite the rapidly growing number of chemical functionalities capable of suicidal inactivation of cytochrome P-450 [10, 11], only a few studies exploiting suicide substrates to probe the pathways of xenobiotic metabolism or mechanism of drug action have been published [12, 13]. We have shown recently that a 1-hr pretreatment of rats with ABT causes an 85% inhibition of phenacetin clearance, completely blocks the formation of acetaminophen from phenacetin, but does not affect the rate of elimination of acetaminophen [14]. In the present report we have characterized the duration of inhibition of phenacetin metabolism, determined the dose–response relationship for ABT inhibition of oxidative metabolism of phenacetin and antipyrine, conducted a detailed evaluation of the potential acute toxicity of ABT in rats, and examined the effect of ABT on the pharmacokinetics and metabolism of phenacetin in male beagles.

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METHODS

Animals. Male Sprague–Dawley rats (250–350 g, cesarean section derived) were supplied by the Charles River Breeding Laboratory, Inc., Kingston, NY. After each rat was anesthetized with methoxyflurane (Pitman-Moore, Washington Crossing, NJ), a polyethylene cannula (PE50) was placed in the jugular vein. The rats were allowed to recover overnight before they received the first intravenous dose through this cannula. Three adult male beagles (10 kg, obtained from Marshall Research Animals, NorthRose, NY) were also used for all studies. The dogs were fasted for 18 hr before intravenous administration of the drugs; water was available *ad lib.* The usual diet was resumed 8 hr after dose administration. Crossover experiments in dogs were separated by 1 week.

Analytical methods. The concentrations of phenacetin, acetaminophen and antipyrine in plasma were measured by high pressure liquid chromatography with ultraviolet detection as described previously [14, 15]. The coefficient of variation for these analytical procedures was less than 10%.

Pharmacokinetics. Rats were pretreated with intravenous or intraperitoneal ABT (in saline, 5 ml/kg) at selected time intervals before intravenous (40 mg/kg in propylene glycol, 2 ml/kg) or oral (100 mg/kg) phenacetin. Antipyrine (15 mg/kg) was given intravenously in saline. Intravenous phenacetin or antipyrine was given between 8:00 and 9:00 a.m. Blood samples were taken at ten time intervals after phenacetin and antipyrine administration. Blood was collected with a heparinized syringe by cardiac puncture under methoxyflurane anesthesia. An individual rat was used for each data point. The pharmacokinetic parameters of these drugs in rats were determined from the average data points at each time interval. After the concentration data were normalized to a 1 mg/kg dose, the half-life and concentration at time zero were estimated by unweighted linear regression of the log-transformed data. The area under the concentration time curve from time zero to infinity (AUC) was calculated with a linear trapezoidal rule. The apparent total body clearance was obtained by dividing the dose by the AUC.

Dogs received approximately 20 mg/kg of phenacetin (in propylene glycol) in a 5-min continuous infusion (10 ml total volume). Blood samples were taken at the times indicated in Fig. 2 after phenacetin administration. The same dogs were used to determine the effect of ABT (20 mg/kg, i.v., 1-hr pretreatment) on phenacetin pharmacokinetics and metabolism. The measured concentration of phenacetin or acetaminophen was normalized to a 1.0 mg/kg dose. Data points from the normalized data were fitted to a one-compartment open model with an infusion input function using the PHARM pharmacokinetic parameter estimation program.

Toxicity evaluation. Rats were screened for health and status (physical exams and clinical pathology profiles) on day 8 before ABT treatment. Blood was collected via the tail vein. Thirty-two rats with acceptable clinical pathology profiles were randomly assigned to one of four groups. The first three rats in each group served as controls; the remaining were treated once with ABT (50 mg/kg, i.v.) on day 1. On days 2, 3, 4, and 10, a group (three controls, five treated) was killed (ether inhalation), and blood was collected from the posterior vena cava for hematology and clinical chemistry determinations. The following clinical pathology parameters were evaluated at all time intervals; hemoglobin, packed corpuscular volume, red blood cell count, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentrations, white blood cell count, leukocyte differential, platelet counts, ALT, AST, alkaline phosphatase, creatinine, urea nitrogen, sodium, potassium, chloride, carbon dioxide, glucose, total protein, albumin, total bilirubin, and gamma-glutamyltransferase.

A complete post-mortem examination was conducted on each animal. In each case, the following tissues were examined microscopically for abnormalities: adrenal glands, brain, heart, kidney, liver (left lateral lobe and median lobe), pancreas, pituitary, spleen, sternebrae, bone marrow, skeletal muscle, testis, epididymis, thymus, thyroid, parathyroid, urinary bladder, tail injection site, and macroscopic lesions.

RESULTS

Effect of ABT pretreatment on phenacetin pharmacokinetics and metabolism in rats. As previously reported, following a 1-hr pretreatment of rats with 50 mg/kg of ABT, the clearance of intravenously

Table 1. Inhibition of phenacetin elimination 1 hr after intravenous pretreatment with ABT in rats

Drug	Route	Pretreatment with ABT	$T_{1/2}$ (min)	CL/F (ml/min/kg)	VD (ml/kg)	% Inhibition*
Phenacetin (40 mg/kg)	i.v.†	Vehicle	34	17.7	876	0
	i.v.†	50 mg/kg	237	2.70	914	85
Phenacetin (100 mg/kg)	p.o.	Vehicle	65	42.1		0
	p.o.	50 mg/kg	288	5.14		88

Abbreviations: $T_{1/2}$, half-life; CL/F, total systemic clearance divided by bioavailability (oral clearance); and VD, steady-state volume of distribution.

* Inhibition is defined as the ratio of CL/F values for treatment and control groups.

† Previously reported [14].

Table 2. Time-course of ABT-mediated (50 mg/kg, i.v.) inhibition of intravenous phenacetin elimination

Pretreatment with ABT (mg/kg)	Time* (hr)	$T_{1/2}$ (min)	CL (ml/min/kg)	VD (ml/kg)	% Inhibition†
Vehicle	1	34	17.7	876	0
50	1	237	2.70	914	85
50	12	324	2.05	961	88
50	24	184	3.44	862	81
50	36	60	9.36	812	48

Abbreviations: $T_{1/2}$, half-life; CL, total systemic clearance; and VD, steady-state volume of distribution.

* Time between ABT pretreatment and phenacetin administration.

† Inhibition is defined as the ratio of CL values for treatment and control groups.

administered phenacetin was decreased 7-fold (Table 1). Acetaminophen was readily detectable in control animals treated with phenacetin but was not detectable in ABT-pretreated animals. ABT pretreatment had little effect on the volume of distribution of phenacetin. ABT pretreatment (1 hr, 50 mg/kg, i.v.) also had a profound effect on phenacetin pharmacokinetics when phenacetin was administered orally. The oral clearance was decreased 88%, and the terminal half-life of elimination also increased.

Time-course of ABT inhibition of phenacetin metabolism. The duration of ABT mediated inhibition of phenacetin metabolism was examined by increasing the time interval between ABT pretreatment and measurement of phenacetin pharmacokinetics. As with the 1-hr pretreatment, profound inhibition of phenacetin clearance was observed 12 hr (88% inhibition, Table 2) and 24 hr (81% inhibition) after rats were pretreated with ABT. Decreased rates of phenacetin elimination were still detectable 36 hr after intravenous pretreatment with ABT (48% inhibition).

Dose-dependence of ABT inhibition of phenacetin metabolism. The dose-dependency of inhibition of phenacetin metabolism was examined 12 hr after intraperitoneal pretreatment with 0.3 to 50 mg/kg of ABT. Previous studies demonstrated that the intraperitoneal route is as effective as intravenous ABT administration [14]. A 12-hr pretreatment was chosen because the maximum effect was observed at 12 hr and synthesis of new cytochrome P-450 was expected to require at least 12 hr [16]. As shown in Table 3, inhibition of phenacetin metabolism, as measured by phenacetin clearance rates, was dose dependent. Assuming an 80% maximal effect, the ED_{50} for inhibition of phenacetin metabolism is approximately 0.3 mg/kg.

Effect of ABT pretreatment on antipyrine pharmacokinetics in rats. As with phenacetin, 12 hr after intraperitoneal pretreatment of rats with 10 or 50 mg/kg of ABT, the clearance of antipyrine was decreased from 5.6 ml/min/kg to 3.5 or 1.8 ml/min/kg (Fig. 1, Table 4). No effect on the volume of distribution of antipyrine was observed. The half-life of antipyrine was increased 60 and 194% by 10 and 50 mg/kg ABT pretreatment.

Toxicity evaluation of intravenous administration of 50 mg/kg ABT. No macroscopic or microscopic lesions were associated with the intravenous administration of a single dose (50 mg/kg) of ABT in male Sprague-Dawley rats 1, 2, 3 or 9 days after ABT

Table 3. Dose-dependent inhibition of intravenous phenacetin clearance 12 hr after intraperitoneal pretreatment of rats with ABT

Pretreatment with ABT (mg/kg)	Clearance (ml/min/kg)	% Inhibition*
Vehicle	13.5	0
0.3	8.2	39
1.0	7.2	47
5.0	5.4	60
20	3.4	75
50	2.9	79

* Inhibition is defined as the ratio of clearance values for treatment and control groups.

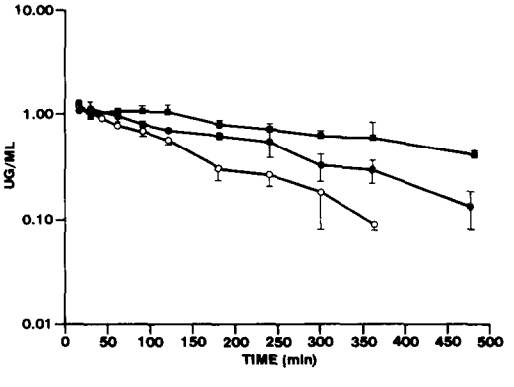

Fig. 1. Plasma concentration versus time profile of antipyrine in control rats (○), rats pretreated with 10 mg/kg of ABT 12 hr before antipyrine administration (●) and rats pretreated with 50 mg/kg of ABT 12 hr before antipyrine administration (■). Concentrations were normalized to a 1 mg/kg dose of antipyrine. Each datum point represents the mean ± SD of data from three individual rats.

Table 4. Antipyrine pharmacokinetics in control rats and 12 hr after intraperitoneal pretreatment with ABT

Pretreatment with ABT (mg/kg)	$T_{1/2}$ (min)	CL (ml/min/kg)	VD (ml/kg)	% Inhibition*
Vehicle	99	5.6	804	0
10	159	3.5	804	38
50	293	1.8	779	68

Abbreviations: $T_{1/2}$, half-life; CL, total systemic clearance; and VD, steady-state volume of distribution.
* Inhibition is defined as the ratio of CL values for treatment and control groups.

Table 5. Effect of ABT on phenacetin clearance in male beagles

	Clearance of phenacetin		
	Control	ABT-treated	% Inhibition*
Dog 1	24.4	11.6	53
Dog 2	31.3	15.8	49
Dog 3	19.7	11.8	40

The dogs were pretreated for 1 hr with ABT (20 mg/kg).
* Inhibition is defined as the ratio of clearance values for treatment and control groups.

treatment. The compound was not irritating at the injection site. Hematological and clinical chemistry abnormalities were not detected.

Effect of ABT pretreatment on phenacetin pharmacokinetics and metabolism in dogs. Pretreatment of male beagles with 20 mg/kg of ABT (1 hr) decreased phenacetin clearance 47 ± 7% (Table 5) from control values. Acetaminophen formed *in vivo* from phenacetin was observed in control experiments (Fig. 2), but only trace quantities of acetaminophen were observed in ABT-pretreated animals.

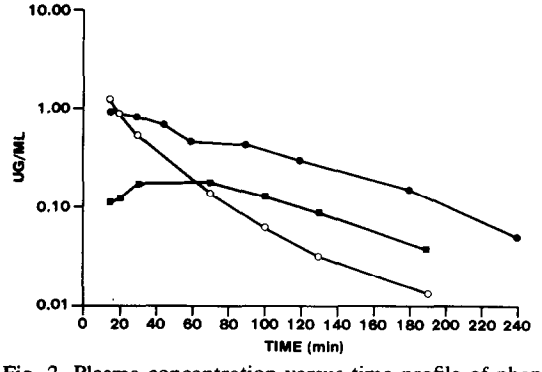

Fig. 2. Plasma concentration versus time profile of phenacetin (○, ●) and acetaminophen formed from phenacetin (■) after intravenous administration of phenacetin to a representative beagle without (○, ■) and with (●) a 1-hr intravenous pretreatment with 20 mg/kg ABT. Only trace concentrations of acetaminophen formed from phenacetin were detectable after pretreatment with ABT. Phenacetin (20 mg/kg) was given intravenously in propylene glycol. Plotted concentrations of phenacetin and acetaminophen are normalized to a 1 mg/kg dose.

DISCUSSION

Inhibitors of mixed-function oxidases have been valuable tools for mechanistic studies of drug action and toxicity [16–18]. An ideal inhibitor of cytochromes P-450 would effectively inhibit the majority of cytochrome P-450 isozymes and act specifically on cytochromes P-450 without other negative physiologic effects. Substrates of cytochromes P-450 which irreversibly inactivate the enzyme following catalysis-dependent liberation of an alkylating (arylating) species would likely be specific for cytochromes P-450 and produce long-lasting effects because resynthesis of cytochromes P-450 is required to restore activity. In all aspects examined to date, 1-aminobenzotriazole (ABT) meets the criteria as an excellent *in vivo* inhibitor of cytochromes P-450. ABT effectively inhibits both phenobarbital- and 3-methylcholanthrane-inducible hepatic cytochromes P-450 [6] as well as the forms of the isozyme responsible for lauric acid hydroxylation [9, 10]. Pretreatment of rats and dogs with ABT decreased the clearance of phenacetin and essentially blocked oxidative metabolism of phenacetin as measured by acetaminophen formation. The residual clearance of phenacetin in ABT-treated animals was probably due to amidase activity. The rates of elimination of antipyrine were also profoundly suppressed by ABT pretreatment.

In rats, the inhibition of phenacetin elimination was profound and detectable for at least 36 hr after ABT pretreatment. Inhibition of phenacetin metabolism by ABT was dose dependent with an ED_{50} of approximately 0.3 mg/kg. Our findings of profound and long-lasting inhibition of *in vivo* drug oxidation with 50 mg/kg of ABT agree closely with *in vivo* studies of the time–course and dose-dependence effects of ABT on hepatic microsomal cytochrome P-450 concentration and activities [16].

An estimate of the *in vivo* efficiency of suicidal inactivation of cytochrome P-450-mediated phenacetin metabolism can be obtained by calculating the *in vivo* partition ratio for ABT metabolism and cytochrome P-450 inactivation. A partition ratio for suicide substrates is a statistical average of the number of molecules metabolized by the enzyme for each molecule of enzyme to be inactivated (alkylated). If it assumed that (a) a virus-free rat has approximately 600 nmol of cytochrome P-450 per kg (all organs included), (b) ABT is metabolized completely within 12 hr of drug administration, (c) no cytochrome P-

450 resynthesis occurs for at least 12 hr after ABT treatment, and (d) half of the molecules of cytochrome P-450 must be inactivated to reduce phenacetin oxidative metabolism by 50%, then approximately 10 molecules of ABT are needed for every molecule of cytochrome P-450 destroyed by ABT. Although this calculation ignores the possibility of isozyme selectivity in phenacetin metabolism and ABT-mediated *in vivo* inactivation, this *in vivo* partition ratio is similar to that observed in *in vitro* experiments [2].

Previous studies demonstrate that ABT has no overt toxicity after single doses up to 200 mg/kg, no effect on rates of drug conjugation as reflected in acetaminophen pharmacokinetics [14], and no effect on cytochrome b_5 [2], serum transaminases [3], or flavin monooxygenase activity [5]. In our study no macroscopic or microscopic lesions were associated with the intravenous administration of a single dose (50 mg/kg) of ABT in male Sprague–Dawley rats 1, 2, 3, and 9 days after drug administration, and no hematological and clinical chemistry abnormalities were observed. The combination of these findings and the profound, long-lasting but selective inhibition of oxidative drug metabolism make ABT an attractive experimental tool in biochemical pharmacology and toxicology studies that examine the role of cytochromes P-450 in drug action.

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