INTRAVENOUS PHARMACOKINETICS AND METABOLISM OF THE REACTIVE OXYGEN SCAVENGER α-PHENYL-*N*-TERT-BUTYL NITRONE (PBN) IN THE CYNOMOLGUS MONKEY

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SUMMARY

The pharmacokinetics and metabolism of the antioxidant and reactive oxygen scavenger α-phenyl-*N*-tert-butyl nitrone (PBN) was examined in the male cynomolgus monkey after intravenous administration. Following an i.v. bolus dose of 5 mg/kg, plasma concentrations of PBN declined in a bi-exponential fashion. PBN demonstrated a moderate plasma clearance (CL_p = 27.02 ± 6.46 ml/min/kg) and a moderate volume of distribution at steady state (Vd_{ss} = 1.70 ± 0.23 l/kg), resulting in a terminal elimination half-life of 0.76 ± 0.25 h. The corresponding area under the curve ($AUC_{0-\infty}$) was 3.20 ± 0.77 μg-h/ml. Scale-up of the *in vitro* microsomal intrinsic clearance data for PBN afforded a blood clearance (CL_b) value of 22 mi/min/kg, which was in reasonable agreement with the observed *in vivo* CL_b. Monkey liver microsomes catalyzed the NADPH-dependent monohydroxylation of PBN to the corresponding α-4-hydroxyphenyl-*N*-tert-butyl-nitrone (4-HOPBN) metabolite. The formation of 4-HOPBN and its corresponding *O*-glucuronide was also discernible upon qualitative analysis of pooled (0-24 h) monkey plasma and urine samples. Less

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than 5% of the administered dose was excreted as unchanged PBN in the urine, suggesting that P450-catalyzed metabolism constituted the major route of PBN clearance in the primate. In conclusion, the pharmacokinetic attributes and the clearance mechanism of PBN in the cynomolgus monkey is similar to that observed in the Sprague-Dawley rat.

KEY WORDS

PBN, pharmacokinetics, monkey, cytochrome P450, antioxidant

INTRODUCTION

A consequence of prolonged life in an aerobic environment is increased oxidative stress with age. Oxidative stress can be defined as a condition in which the production of free radicals and the damage they cause exceed the ability to scavenge free radicals or repair the damage. Radical mediated oxidative tissue damage is often presumed to be the rate-limiting step in the initiation of neurodegenerative processes including stroke. Consequently, reactive oxygen scavengers (antioxidants) such as nitrones or phenolic compounds have received much attention as neuroprotectants in age-associated oxidative tissue damage /1/. α-Phenyl-*N*-tert-butyl nitrone (PBN) (see Fig. 2 for structure) constitutes the parent compound of the nitrone family of spin-trapping agents commonly utilized to trap free radicals. The neuroprotective and the anti-inflammatory effects of PBN in rodent models of neurodegenerative disease, including Huntington's disease and Parkinson's disease, are quite extensive /2-4/. The pharmacological basis for PBN's neuroprotective, anti-aging and anti-inflammatory effects has been attributed to its radical-trapping properties and its ability in mediating the suppression of genes induced by pro-inflammatory cytokines and other mediators associated with enhanced neuroinflammatory processes. Apart from medicinal chemistry efforts /5,6/ tailored to increase the antioxidant potential of PBN, studies devoted to the understanding of the pharmacokinetics/metabolism attributes of PBN in preclinical species have also received much attention /7-9/.

Understanding the pharmacokinetic properties of a drug in the preclinical species used for pharmacological evaluation is of great

importance, especially, for determination of human dosing regimen/ frequency and towards the understanding of pharmacokinetic/ pharmacodynamic (PK/PD) relationships. For example, the *in vivo* observations on the superior neuroprotective properties of PBN in the rat /10,11/, compared to other reactive oxygen scavengers that are 1,000-fold more potent antioxidants than PBN *in vitro* /12/, can be partially explained by comparing their pharmacokinetic properties. In the rat, PBN demonstrates a low plasma clearance (CL_p) of 12 ml/ min/kg and a moderate half-life of 2 h /8/. In comparison, butylated hydroxytoluene (BHT) has an extremely short plasma half-life (12 min) and a overall CL_p (200 ml/min/kg) that exceeds hepatic blood flow in the rat /13/. Thus, in contrast to the earlier hypothesis on free radical trapping activity as the sole criterion for the pharmacological action of PBN in preclinical species, its pharmacokinetic properties (such as clearance, half-life and bioavailability) may also be of equal importance /2/.

Besides the rodent, monkeys have also been extensively used to study neurodegeneration /14,15/. Although the neuroprotective properties of PBN have not been examined in the monkey, we felt that it would be useful to characterize the pharmacokinetic attributes of PBN in this species as a starting point for the characterization of the corresponding pharmacology. Therefore, the pharmacokinetics/clearance pathways of PBN in the cynomolgus monkey were studied and compared to those previously reported in the rat. *In vitro* metabolism studies were also undertaken in monkey liver microsomes towards establishing an *in vitro/in vivo* correlation for CL_p, as well as elucidating major PBN metabolic pathways in the monkey.

MATERIALS AND METHODS

Materials

PBN was purchased from Sigma (St. Louis, MO). Monkey liver microsomes (MKL-121) (liver harvested from two male cynomolgus monkeys) were generated at Pfizer using standard protocols. Protein concentrations were determined using the bicinchoninic assay method (Pierce Chemical, Rockford, IL). Total cytochrome P450 content was measured according to published protocols /16/. The cytochrome P450 content in the microsomal lot # MKL-121 was 0.9 nmol/mg (protein

content = 19 mg/ml). α-4-Hydroxyphenyl-*N*-tert-butylnitrone (4-HOPBN) was synthesized as described previously /8/.

Incubations with monkey liver microsomes

PBN half-life was determined in duplicate following its incubation (1 μM) with monkey liver microsomes (P450 concentration = 0.25 μM) in 0.1 M potassium phosphate buffer (pH = 7.4) at 37°C. The reaction mixture was prewarmed at 37°C for 2 min before adding NADPH (1.2 mM). The final incubation volume was 600 μl. Aliquots (75 μl) of the reaction mixture at t = 0, 2, 5, 15, and 30 min (time period associated with reaction linearity) were added to acetonitrile (200 μl) containing midazolam (0.07 μg/ml) as internal standard and the samples were centrifuged at 2500 *g* for 5 min prior to liquid chromatography/tandem mass spectrometry (LC/MS/MS) analysis. For control experiments, NADPH was omitted from these incubations. The scaling-up of the microsomal half-life ($t_{½}$) data in liver microsomes, reflecting PBN depletion, was performed using the following equation /17/:

Intrinsic clearance (Cl'_{int}) = ($0.693/t_{½}$) × (g liver weight/kg body weight) × (ml incubation/mg of microsomal protein) × (45 mg microsomal protein concentration/g of liver weight) (1)

The average monkey liver and body weights were 32 g/kg body weight and 4 kg, respectively, and the microsomal protein concentration was 0.32 mg/ml. Blood clearance (Cl_b) was estimated using the non-restricted well-stirred model (Equation 2) /18/. The blood to plasma ratio of PBN in the monkey was estimated to be 0.83.

$$Cl_b = Q \times Cl'_{int} / Q + Cl'_{int} \quad (2)$$

where Q is the hepatic blood flow in the monkey (44 ml/min/kg).

For the purposes of metabolite identification, the concentration of PBN was raised to 50 μM and that of P450 in monkey liver microsomes was raised to 0.5 μM. Incubations (final volume = 1 ml) were conducted at 37°C in a shaking water bath for 60 min in the presence or absence of NADPH. Reactions were terminated by the addition of ice-cold acetonitrile (2 ml). The mixture was centrifuged (3,000 *g*, 15 min) and the supernatant was evaporated to dryness under a steady nitrogen stream. The residue was examined for metabolites by LC/MS/MS.

Blood/plasma ratio and plasma protein binding determination

Fresh rat and monkey blood samples were collected in heparinized containers from Sprague-Dawley rats and cynomolgus monkeys, respectively. A portion of the blood samples was centrifuged (3,000 *g*, 5 min) to harvest plasma. Aliquots of rat and monkey blood and plasma (4 ml) were incubated with PBN (10 μM) at 37°C for 15 min (pH 7.4) in a shaking water bath (~100 rpm). The pH of these incubation mixtures was 7.4. The blood samples fortified with PBN were centrifuged (3,000 *g*, 15 min) to harvest plasma. The plasma samples (harvested from blood and those initially fortified with PBN) were treated with two volumes of acetonitrile containing midazolam as internal standard and centrifuged (2,500 *g*, 5 min) prior to LC/MS/MS analysis.

PBN protein binding to rat and monkey plasma was determined by equilibrium dialysis. PBN (5 μM) was added to fresh rat or monkey plasma and the plasma samples were subjected to equilibrium dialysis (n = 3 per concentration), using Spectra Por cellulose dialysis membranes (VWR, West Chester, PA) with a molecular weight cutoff of 12.4 kDa. The resulting plasma samples were subjected to dialysis against isotonic phosphate buffer for 4 h. The plasma samples were treated with 2 volumes of ice-cold acetonitrile containing midazolam as internal standard, centrifuged (2,500 *g*, 5 min) prior to LC/MS/MS analysis. The protein-bound fraction was determined by LC/MS/MS through a comparison of the differences between concentration in the plasma and that in the buffer solution.

Animal studies

The Pfizer Institutional Animal Care and Use Committee approved all procedures. Three fasted male Cynomolgus monkeys (average weight 4 kg) were administered PBN (via the brachial vein) in phosphate buffered saline as an i.v. bolus solution (5 mg/kg). They were restrained in metabolism chairs for 2 h after dosing and then transferred to their primary cages for the remainder of the study. Monkeys were fed 4 h post dose and were allowed full access to water throughout the study. Plasma was prepared from blood samples (600 μl) collected from femoral veins at predosing and at various times after dosing for 24 h. Plasma was thawed, and 100 μl was diluted with 2 volumes of acetonitrile containing midazolam as internal standard.

The samples were centrifuged, and the supernatant was analyzed for PBN concentrations by LC/MS/MS.

LC/MS/MS analysis

PBN depletion was monitored on a Sciex API model 3000 LC/MS/MS triple quadrupole mass spectrometer (Thornhill, Ontario, Canada) or a Micromass Quattro Ultima (Micromass, Beverly, MA). Analytes were chromatographically separated using a Hewlett Packard Series 1100 HPLC system (Phenomenex Primesphere 5 μ C18-HC 30 x 2.0 mm column using a 2 and 3 minute binary gradient consisting of a mixture of 95% water-5% acetonitrile with 0.1% acetic acid [Solvent A] and 95% acetonitrile-5% water with 0.1% acetic acid [Solvent B] and a flow rate of 1.0-1.5 ml/min). Ionization was conducted in the positive ion mode at the ionspray interface temperature of 400°C (Sciex) or 100°C (Micromass) and nitrogen was used as a nebulizing and heating gas. PBN and midazolam were analyzed using multiple reaction monitoring at mass ranges m/z 178→122 and 326→291, respectively. In the case of PBN, this reaction follows the protonated parent mass MH^+ = 178 to its corresponding collision-induced dissociated fragment at m/z 122, which corresponds to the loss of the *tert*-butyl group on PBN. The dynamic range of the assay was 0.001 to 20.0 μg/ml. Calibration curves (0.01-20 μg/ml) were prepared by plotting the appropriate peak area ratios against the concentrations of PBN in plasma using 1/x weighting of PBN/midazolam peak height ratios. The concentration of PBN in the plasma samples was determined by interpolation from the standard curve.

Qualitative assessments of PBN metabolism were conducted on a Sciex API model 2000 LC/MS/MS triple quadrupole mass spectrometer (Thornhill, Ontario, Canada) in conjunction with an LDC Analytical SpectroMonitor 3200 variable wavelength UV detector. PBN and its metabolites were chromatographically separated using a Hewlett Packard Series 1100 HPLC system (Zorbax Eclipse XDB-C8 4.6 x 150 mm column using a binary gradient consisting of a mixture of 10 mM ammonium formate, 0.1% formic acid [Solvent A] and acetonitrile [Solvent B] at a flow rate of 1 ml/min). The LC gradient was programmed as follows: Solvent A to Solvent B ratio was held at 100:0 (v/v) for 3 min and then adjusted from 100:0 (v/v) to 10:90 (v/v) for 20 min and from 10:90 (v/v) to 100:0 (v/v) from 20 to 25 min. Post-column flow was split such that mobile phase was intro-

duced into the mass spectrometer via an ion spray interface at a rate of 50 µl/min. The remaining flow was diverted to the UV detector positioned in-line so as to provide simultaneous UV detection at λ = 254 nm and total ion chromatogram. Ionization was conducted in the positive ion mode at the ion spray interface temperature of 150°C using nitrogen as a nebulizing gas. Ion spray voltage was 4.5 kV, and the orifice voltage was 30 eV. Initial Q1 scans were performed between m/z 50 and 500. Potential PBN metabolites in rat liver microsomes were identified by comparing t = 0 samples to t = 30 min samples (with or without NADPH), and structural information was generated from collision-induced dissociation of the corresponding protonated molecular ions and by comparison with authentic metabolite standards.

Data analysis

Plasma concentration-time profiles were analyzed using the well-established non-compartmental method in WinNonLin v2.1 (Pharsight, Mountain View, CA). The total plasma clearance (CL_p) of i.v. PBN was calculated as the i.v. dose divided by the $AUC_{0-\infty}$ of i.v. PBN and the volume of distribution at steady state (Vd_{ss}) by the method of Benet and Galeazzi /19/. The area under the plasma concentration-time curve from time zero to infinity ($AUC_{0-\infty}$) was calculated by the linear trapezoid rule. The terminal slope of the ln(concentration) versus time plot was calculated by linear least-squares regression and the half-life was calculated as 0.693 divided by the absolute value of the slope.

RESULTS

Intravenous pharmacokinetics of PBN in cynomolgus monkeys

Figure 1 displays the mean plasma PBN concentration-time profile in cynomolgus monkeys after administration of a single i.v. dose of PBN at 5 mg/kg. The resulting pharmacokinetic parameters are summarized in Table 1. For purposes of comparison, the corresponding i.v. pharmacokinetic parameters of PBN in the Sprague-Dawley rat /8/ are also included in Table 1. After i.v. administration, PBN concentrations in plasma decreased steadily in a bi-exponential

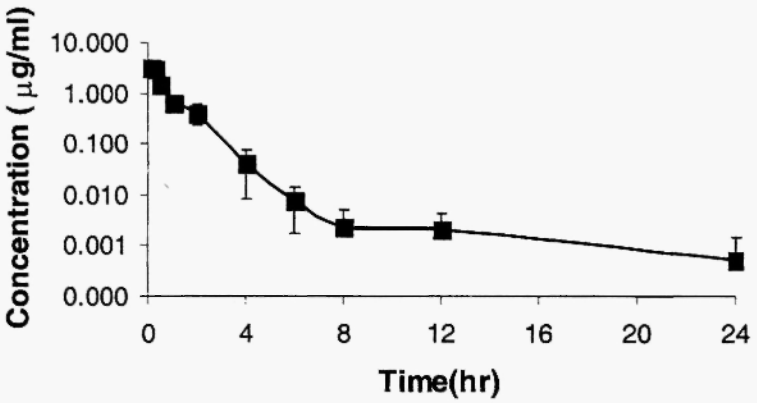

Fig. 1: Plasma concentration-time profiles of PBN after i.v. (5 mg/kg)] administration to male cynomolgus monkeys (mean ± SD; n = 3).

TABLE 1

Pharmacokinetic parameters of PBN (mean ± SD,) after a single i.v. bolus dose (5 mg/kg) in cynomolgus monkeys: comparison with the pharmacokinetic attributes in the Sprague-Dawley rat

Pharmacokinetic parameter	**Monkey** (n = 3)	**Rat** [a]
CL_p (ml/min/kg)	27.0 ± 6.46	12.4 ± 3.82
Vd_{ss} (l/kg)	1.70 ± 0.23	1.74 ± 0.50
$AUC_{0-\infty}$ (μg-h/ml)	3.20 ± 0.77	14.4 ± 4.09
$t_{½}$ (h)	0.76 ± 0.25	2.01 ± 0.35

[a] Reference /8/.

fashion. PBN demonstrated a moderate CL_p (27.02 ± 6.46 ml/min/kg) and a moderate Vd_{ss} (1.70 ± 0.23 l/kg), resulting in a terminal elimination half-life of 0.76 ± 0.25 h. The corresponding area under the curve ($AUC_{0-\infty}$) was 3.20 ± 0.77 μg-h/ml.

In vitro/in vivo correlation for PBN clearance in the cynomolgus monkey

The half-life for PBN depletion in NADPH-supplemented cynomolgus monkey liver microsomes was estimated to be ~77 min. Scale-up of the *in vitro* half-life data afforded a CL_b value of 22 ml/min/kg which was in reasonable agreement (within 1.5-fold) with the observed *in vivo* Cl_b of 32.5 ml/min/kg (blood to plasma ratio of PBN in the cynomolgus monkey was determined to be 0.83) (Table 2). The reasonable *in vitro/in vivo* correlation for the CL_p prediction suggested that the predominant route of PBN clearance in monkey involves its hepatic metabolism. Consistent with this proposal, urinary analysis (pooled monkey urine) revealed that less than 5% of the administered dose was excreted as unchanged PBN after i.v. administration.

Rat and monkey plasma protein binding of PBN

The possibility that differences in the CL_p of PBN in the rat and monkey are due to differences in plasma protein binding affinity was also examined. The average free fraction of PBN (5 μM) in fresh rat and monkey plasma was 0.48 ± 0.03 and 0.64 ± 0.04, respectively.

Metabolite(s) of PBN in the cynomolgus monkey

As had been previously demonstrated in rat liver microsomes /8/, monkey liver microsomes (P450 concentration = 0.5 μM) also catalyzed the NADPH-dependent monohydroxylation of PBN (50 μM) to 4-HOPBN (Fig. 2). No 4-HOPBN formation was discernible when NADPH was omitted from the incubations, implicating the involvement of cytochrome P450 in the oxidation. The formation of 4-HOPBN was also discernible upon qualitative analysis of pooled monkey plasma and urine (0-24 h) after i.v. administration of the spin trap. The presence of 4-HOPBN-glucuronide (see Fig. 2) (MH^+ = 370, m/z = 194, 138, 121) was also evident in monkey urine. The exact regiochemistry of glucuronidation (phenolic glucuronidation versus *N*-oxide glucuronidation) could not be determined from the available mass spectral data.

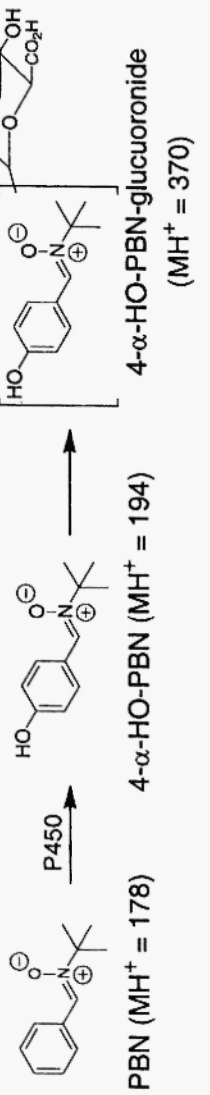

Fig. 2: Biotransformation pathway of PBN in the cynomolgus monkey.

TABLE 2

In vitro/in vivo correlation for PBN clearance prediction in cynomolgus monkey

	Half-life [a] (min)	**Intrinsic clearance** [b] (CL'_{int}, ml/min/kg)	**Predicted blood clearance** [c] (CL_b, ml/min/kg)	**Actual CL,** (ml/min/kg)
Monkey liver microsomes	77.0	44.5	22.1	27.5 (32.5) [d]

[a] PBN concentration used in the incubations was 1 μM.

[b] CL'_{int} in microsomes was calculated from the following equation: $Cl'_{int} = (0.693/t_{½}) \times$ (g liver weight/kg body weight) × (ml incubation/mg of microsomal protein) × (45 mg microsomal protein concentration/g of liver weight).

[c] Intrinsic clearance was converted to blood clearance (CL_b) by the following equation:

$Cl_b = Q \times Cl'_{int} / Q + Cl'_{int}$, where Q is hepatic blood flow

[d] The blood to plasma ratio of PBN in the monkey was determined to be 0.83.

DISCUSSION

The present investigation constitutes the first report on the characterization of the pharmacokinetic properties and clearance mechanism of the reactive oxygen scavenger PBN in the monkey. The pharmacokinetic properties of PBN following i.v. administration in rats have been previously published by us /8/. In the rat, PBN demonstrates a low CL_p (12.4 ml/min/kg) and a moderate Vd_{ss} (1.74 l/kg) that results in a moderate terminal elimination half-life of ~2.01 h. Assuming an average hepatic blood flow in rats of 70 ml/min/kg, the *in vivo* hepatic extraction ratio (CL_p/Q) of PBN is ~0.17, suggesting that PBN is a low clearance agent in the rat. In comparison, based on the observed CL_p of 27.02 ml/min/kg and an average hepatic blood flow in monkeys of 44 ml/min/kg, the *in vivo* hepatic extraction ratio of PBN in the monkey is ~0.6, indicating that PBN demonstrates moderate to high clearance in the monkey. Thus, despite the similarity in the Vd_{ss} of PBN in the rat and the monkey, the lower clearance of PBN in the rat translates into a terminal half-life that is approximately three times longer than that observed in the monkey. Furthermore, as was previously demonstrated in the rat, a reasonable *in vitro/in vivo* correlation for clearance predictions was also observed in the monkey. The increased CL_p of PBN in the primate cannot be explained on the basis of an increased free fraction since the binding of PBN to rat and monkey plasma proteins was similar. Considering that metabolism constitutes the rate-limiting step in PBN clearance in the monkey, a plausible explanation for increased PBN CL_p in the primate may be the increased affinity (low K_m and/or higher V_{max}) of PBN towards monkey P450 isoform(s) that catalyze its oxidation.

The principal metabolic fate of PBN in the monkey was identical to its metabolic fate in the rat and involved the cytochrome P450 mediated oxidation on its phenyl ring to 4-HOPBN. In rats, this biotransformation pathway is catalyzed exclusively by male rat specific P450 2C11 enzyme, thus, it is tempting to speculate the involvement of a related P450 2C isozyme /20/ in PBN metabolism in the monkey. Furthermore, the free phenolic metabolite has been shown to retain the antioxidant properties of PBN /21/ and may provide a additive effect to the pharmacological effects of PBN *in vivo*, a feature that may not be possible with other reactive oxygen scavengers/antioxidants. Based on the similarities in the pharmacokinetic/metabolism attributes of

PBN in the rat and monkey, we speculate that PBN will be an equally effective neuroprotectant in the monkey.

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