In vivo and in vitro characterization of chlorzoxazone metabolism and hepatic CYP2E1 levels in African Green monkeys; induction by chronic nicotine treatment.

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Nicotine induces monkey in vivo chlorzoxazone disposition

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Non-standard abbreviations
CYP2E1 (cytochrome P450 2E1), CYP2D6 (cytochrome P450 2D6), CZN (chlorzoxazone), 6OHCZN (6-hydroxychlorzoxazone), AUC_T, (area under the curve to the last quantifiable concentration) AUC_inf, (area under the curve extrapolated to infinity), C_max (maximum observed concentration), T_max (time at which the observed C_max occurred), K_el (terminal disposition rate constant calculated from last 3 to 4 points on the log-linear end of the concentration versus time curve), t_1/2 (half-life).
Abstract

CYP2E1 metabolizes compounds including clinical drugs, organic solvents and tobacco-specific carcinogens. Chlorzoxazone is a probe drug used to phenotype for CYP2E1 activity. Smokers have increased chlorzoxazone clearance during smoking compared with non-smoking periods; however, it is unclear which cigarette smoke component is causing the increased activity. The relationships between in vivo chlorzoxazone disposition, in vitro chlorzoxazone metabolism, and hepatic CYP2E1 have not been investigated in a within-animal design. In control-treated monkeys (Cercopithecus aethiops), the in vivo chlorzoxazone AUC$_{\text{inf}}$ was 19.7 ± 4.5 µg×h/ml, t$_{1/2}$ was 0.57 ± 0.07 h and K$_{\text{el}}$ was 1.2 ± 0.2 /h. In vitro, the apparent V$_{\text{max}}$ was 3.48 ± 0.02 pmol/min/µg microsomal protein and the K$_{\text{m}}$ was 95.4 ± 1.8 µM. Chronic nicotine treatment increased in vivo chlorzoxazone disposition, as indicated by a 52% decrease in AUC$_{\text{inf}}$ (p<0.01) and 52% decrease in T$_{\text{max}}$ (p<0.05) compared to control-treated monkeys. The log metabolic ratios at 0.5, 1, 2 and 4 h significantly negatively correlated with chlorzoxazone AUC$_{\text{inf}}$ (p=0.01-0.0001). Monkey hepatic CYP2E1 levels significantly correlated with both in vivo AUC$_{\text{inf}}$ (p=0.03) and to in vitro (p=0.004) chlorzoxazone metabolism. Together the data indicated that nicotine induction of in vivo chlorzoxazone disposition is related to the rates of in vitro chlorzoxazone metabolism and to hepatic microsomal CYP2E1 protein levels. Nicotine is one component in cigarette smoke that can increase in vivo chlorzoxazone metabolism via induction of hepatic CYP2E1 levels. Thus nicotine exposure may affect the metabolism of CYP2E1 substrates such as acetaminophen, ethanol and benzene.
CYP2E1 is a drug-metabolizing enzyme that biotransforms many compounds including clinical drugs such as acetaminophen and halothane (Caro and Cederbaum, 2004). CYP2E1 also metabolizes ethanol (Lieber, 1999) and can be induced by ethanol in rats (Howard et al., 2001), rabbits (Lieber, 1999) and humans (Oneta et al., 2002). CYP2E1 contributes to ethanol metabolism at high blood alcohol concentrations in humans (Salaspuro and Lieber, 1978) and rats (Matsumoto et al., 1996). Many low molecular weight compounds such as benzene and carbon tetrachloride are also CYP2E1 substrates (Caro and Cederbaum, 2004). CYP2E1 produces a high level of reactive oxygen species, without need of a ligand, that can result in cell damage from lipid peroxidation and DNA strand breaks (Caro and Cederbaum, 2004).

Chlorzoxazone (CZN), a clinically used muscle relaxant, is metabolized by CYP2E1 to 6-hydroxychlorzoxazone (6OHCZN) and this reaction has been established as a phenotypic measure of CYP2E1 activity in vivo in humans (Lucas et al., 1999), and in vitro in rats (Kobayashi et al., 2002) and cynomolgus monkeys (Amato et al., 1998). No studies linking in vivo CZN disposition, in vitro hepatic CZN metabolism, and hepatic CYP2E1 protein levels have been published in any species. The in vivo CZN disposition, in vitro CZN metabolism and hepatic CYP2E1 protein levels have not yet been investigated in African Green monkeys (vervets, Cercopithecus aethiops). This monkey is a useful model of human metabolism; it has been established as a model of CYP2A6 (Schoedel et al., 2003) and CYP2B6 (Lee et al., 2006) isozyme metabolism. Our model provides an opportunity to investigate the relationship between these three variables in a non-human primate. African Green monkey CYP2E1 has not yet been sequenced but it is expected to have similar amino acid homology to that of human CYP2E1.
Other monkeys such as the cynomolgus monkey (*Macaca fascicularis*) and the rhesus monkey (*Macaca mulatta*) have 92% and 95% amino acid homology to human CYP2E1, respectively (cynomolgus accession number: **P33266, GI:461827** (Komori et al., 1992), rhesus accession number: **AAT49269.1, GI:49066335** (Carr et al., 2004)).

Cigarette smoking can alter the activity of CYP2E1. In humans, periods of cigarette smoking increase the *in vivo* CZN clearance compared to non-smoking periods (Benowitz et al., 2003). In mice, exposure to cigarette smoke increases CYP2E1 protein, mRNA and activity in lung, kidney and liver (Seree et al., 1996; Villard et al., 1994; Villard et al., 1998). CZN metabolism is not affected by carbon monoxide (Benowitz et al., 2003), which is produced by burning cigarettes, nor by cotinine, the primary metabolite of nicotine (Micu et al., 2003). The increase in CYP2E1 is important because it may contribute to the harmful effects of smoking. CYP2E1 activates many pro-carcinogens found in cigarette smoke such as 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), N-nitrosodimethylamine (NDMA) and N-nitrosodiethylamine (NDEA) (Kushida et al., 2000), and is associated with the development of some types of cancer (Caro and Cederbaum, 2004). Nicotine could be the inducing agent responsible for the increase in CZN clearance in smokers. Nicotine has been shown to increase CYP2E1 protein and *in vitro* activity in rats (Anandatheerthavarada et al., 1993; Bhagwat et al., 1998; Howard et al., 2001; Micu et al., 2003). The effect of nicotine on CYP2E1 levels is of interest as current and former smokers may be exposed to nicotine via smoking or as a smoking cessation drug.

We hypothesize that there will be a significant relationship between *in vivo* CZN disposition, *in vitro* CZN metabolism and hepatic CYP2E1 levels in monkeys. In addition, we hypothesize that chronic nicotine treatment will increase the rate of *in vivo* CZN disposition and
the rate of *in vitro* CZN metabolism in hepatic microsomes, via an increase in hepatic CYP2E1 protein levels in monkeys.
Methods

Materials.
Nicotine bitartrate, chlorzoxazone and 2-benzoxazolinone were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). All other chemical reagents were obtained from standard commercial sources. The protein assay dye reagent was purchased from Bio-Rad Laboratories (Hercules, CA). Pre-stained molecular weight protein markers were purchased from MBI Fermentas (Flamborough, ON, Canada). Hybond nitrocellulose membrane was purchased from Amersham Biosciences (Toronto, ON, Canada). Human CYP2A6, CYP2B6, CYP2C19, CYP2D6, CYP2E1 and CYP3A4 expressed from cDNA, polyclonal anti-human CYP2E1 antibody for immunoblotting, and polyclonal anti-rat CYP2E1 antibody for immunohistochemistry were purchased from BD Gentest (Woburn, MA). Horseradish peroxidase-conjugated anti-goat secondary antibody was purchased from Chemicon International, Inc. (Temecula, CA). Avidin-biotin complex with peroxidase kit and DAB were purchased from Vector Laboratories (Burlington, ON, Canada). Chemiluminescent substrate was purchased from Pierce Chemical Company (Rockford, IL). Autoradiographic film was purchased from Ultident (St. Laurent, PQ, Canada). Human liver was kindly provided by Dr. Ted Inaba (University of Toronto, ON, Canada). Three freshly dissected livers from untreated, drug-free, male African Green monkeys were generously donated by Aventis Pasteur Ltd. (formerly Connaught Laboratories, Ltd.), Toronto, Canada. Freshly dissected tissues were immediately frozen and transported to the laboratory.

Animals.
The study consisted of 12 male adult African Green monkeys ( vervets, *Cercopithecus aethiops* ) housed at Caribbean Primates Ltd. (St. Kitts) and 3 untreated adult African Green monkey livers. The 12 African Green monkeys were drawn from a large, isolated and non-endangered Caribbean population and housed outdoors in social groups (Ervin et al., 1990). Monkeys were given standard rations of Purina monkey chow supplemented with fresh fruit and vegetables. Drinking water was available ad libitum. The nicotine-treated monkeys (n=6) were given nicotine bitartrate (mg base in saline, pH 7.0) at 0.05 mg/kg (all injections subcutaneous, twice daily) for 2 days, then 0.15 mg/kg for 2 days, followed by 0.3 mg/kg for 18 days. The control-treated monkeys (n=6) received sham nicotine injections (saline subcutaneous, twice daily). On day 14, all monkeys received 7 mg/kg chlorzoxazone intragastrically under ketamine anaesthesia instead of nicotine or saline treatment and a 6 ml blood sample was drawn at baseline, 10 min, 20 min, 30 min, 60 min, 120 min, 240 min and 360 min after chlorzoxazone administration. Blood samples were centrifuged and the plasma removed and frozen for drug analyses. All animals were sacrificed on day 22, 6 hours after A.M. drug treatments under ketamine anaesthesia. At the time of sacrifice, nicotine levels are estimated to be below 5 ng/ml (Lee et al., 2006) and we have shown that nicotine does not interact with CYP2E1 (Howard et al., 2001) suggesting no interference in subsequent assays. Body weights of the monkeys did not decrease as a result of treatment. Organs were immediately dissected and flash frozen in liquid nitrogen and stored at –80°C until further use. The experimental protocol was reviewed and approved by the Institutional Review Board of Behavioural Sciences Foundation and the University of Toronto Animal Care Committee. All procedures were conducted in accordance with the guidelines of the Canadian Council on Animal Care.
In vivo chlorzoxazone and 6-hydroxychlorzoxazone plasma assessments.

Plasma CZN and 6OHCZN levels were assayed based on the methods in Howard et al (2001) with minor modifications. Briefly, plasma was centrifuged at 3000g for 10 minutes and 0.5 ml of the supernatant removed for analysis. Internal standard of 2.0 mM 2-benzoxazolinone (25 µl) and β-glucuronidase (10 mg/ml, 250 µl) were added to 0.2 M acetate buffer (pH 5.0, 1.0 ml) and incubated overnight at 37°C. Following incubation, 10% perchloric acid (600 µl) and ethylacetate:hexane (5 ml) were added, the sample was shaken for 30 minutes, centrifuged at 3500g for 15 minutes and the organic phase evaporated to dryness at 37°C. The sample was reconstituted into 200 µl of mobile phase consisting of 19% acetonitrile in 10 mM sodium acetate buffer (pH 4.5). CZN and 6OHCZN were measured by HPLC with UV detection at 295 nm. A Waters Spherisorb S5 ODS2 column (4.6 x 150 mm; Waters, Bedford, MA) was used to separate CZN, 6OHCZN and 2-benzoxazolinone using a mobile phase performed with isocratic elution at a flow rate of 1 ml/min. The retention times for CZN, 6OHCZN and 2-benzoxazolinone were 19.6, 4.7 and 6.5 minutes, respectively.

Microsomal membrane preparation and protein assay.

Monkey or human liver tissue was homogenized in 100 mM Tris, 0.1 mM EDTA, 0.1 mM DDT and 0.32 M sucrose (pH 7.4) for immunoblotting, or in 1.15% w/v KCl for in vitro metabolism assessments, then centrifuged at 12 500g for 30 minutes at 4°C. The supernatant was then centrifuged at 110 000g for 90 minutes at 4°C and the pellet re-suspended in 100 mM Tris, 0.1 mM EDTA, 0.1 mM DDT, 1.15% w/v KCl and 20% v/v glycerol for immunoblotting or 1.15% w/v KCl for in vitro metabolism assays. Microsomes were aliquoted and stored at –80°C. The
protein content of liver microsomes was assayed with the Bradford technique using a BioRad Protein Assay kit.

*In vitro* chlorzoxazone and 6-hydroxychlorzoxazone assessments.

Chlorzoxazone 6-hydroxylation was assayed according to the method of Leclercq *et al* (1998) for rat liver microsomes with minor modifications for use with monkey tissues (Leclercq *et al.*, 1998). African Green monkey hepatic microsomes were mixed with 0.1 M Tris buffer at pH 7.6 containing 10 mM magnesium chloride and 5 mM NADPH to a final volume of 500 µl. Incubations were carried out with CZN in a shaking water bath at 37°C. Different protein concentrations and incubation times were tested to establish the linear incubation conditions for CZN metabolism. Final reactions included 0.4 mg of protein incubated for 20 min. Zinc sulphate (15% w/v, 0.2 ml) was added to stop the reaction and 6.4 µg of the internal standard, 2-benzoxazolinone in Tris buffer, was added per reaction. Following centrifugation for 10 min at 12700 g, the supernatant was injected onto an Agilent Zorbax SB-C18 column (5 µm, 4.6×250 mm; Agilent, USA) with UV detection at 287 nm. Separation of peaks was performed using a mobile phase of 50 mM ammonium acetate (adjusted to pH to 4.0 with 1 M glacial acetic acid)-acetonitrile (65:35) at a flow-rate of 0.7 ml/min. The retention times for CZN, 6OHCZN, and 2-benzoxazolinone were 18.5, 7.5, and 10.1 min, respectively. Kinetic parameters (Km and Vmax) in untreated monkey liver microsomes were obtained by incubating with CZN at various concentrations.

Immunohistochemistry.
Frozen monkey liver, fixed with 4% paraformaldehyde, was cut into 16 µm sections. Sections were washed twice for 5 minutes with phosphate buffered saline (PBS, 10 mM sodium phosphate buffer, 0.9% sodium chloride, pH 7.4) and incubated for 1 hour in a blocking solution (PBS with 1% w/v skim milk powder, 0.1% BSA, 0.01% Triton X-100 and 2% normal horse serum). Tissue sections were then incubated for 48 hours at 4°C with polyclonal goat anti-rat CYP2E1 antibody diluted 1:1000 with 0.1% BSA, 0.01% Triton X-100 and 2% normal horse serum in PBS. Tissue sections were washed 3 times for 5 minutes each with 0.01% Triton X-100 in PBS then reblocked for 30 minutes with 0.1% BSA, 2% normal horse serum, 0.01% Triton X-100 in PBS. Sections were then incubated for 1 hour with biotinylated anti-goat secondary antibody diluted 1:1500 with 0.1% BSA, 2% normal horse serum, 0.01% Triton X-100 in PBS followed by 3 washes of 5 minutes. Tissue sections were then quenched for 10 minutes with 0.3% hydrogen peroxide in PBS followed by 2 washes of 5 minutes each with 0.01% Triton X-100 in PBS. The antigen-antibody complex was visualized using the avidin-biotin complex followed by a reaction with 3,3’-diaminobenzidine and hydrogen peroxide. Tissue sections were then dehydrated and mounted onto silane-coated slides with Permount.

Immunohistochemical control sections were incubated without primary antibody.

Immunoblotting.

Monkey liver microsomes and cDNA expressed CYP2E1 were serially diluted to generate standard curves and establish the linear detection range for the assays. Liver microsomal proteins (3 µg) were separated with SDS-polyacrylamide gel electrophoresis using a 10% separating gel and a 4% stacking gel. Proteins were transferred overnight onto nitrocellulose membranes. To detect CYP2E1, membranes were incubated for 1 hour in a blocking solution of...
1% w/v skim milk powder, 0.1% BSA, 0.1% Triton X-100 and Tris buffered saline (TBS, 50 mM Tris, 150 mM NaCl, pH 7.4). Membranes were then incubated for 1 hour with polyclonal goat anti-human CYP2E1 antibody diluted 1:3000 in 0.1% BSA, 0.1% Triton X-100 and TBS followed by 3 washes of 5 minutes each with TBS and 0.1% Triton X-100. Membranes were re-blocked with the same initial blocking solution for 45 minutes. To detect CYP2E1, membranes were then incubated for 1 hour with horseradish peroxidase conjugated anti-goat secondary antibody diluted 1:2500 in 0.1% BSA, 0.1% Triton X-100 and TBS followed by 3 washes for 5 minutes each with 0.1% Triton X-100 and TBS. CYP2E1 proteins were visualized on immunoblots using chemiluminescence followed by exposure to autoradiographic film for 1 to 5 minutes. MCID Elite imaging software (Imaging Research, Inc., St. Catherines, ON, Canada) was used to analyse films. Baseline corrections for band intensities were made by subtracting the film background from the band intensity. Using a standard curve of cDNA expressed CYP2E1, band intensities were calculated and expressed as amount of CYP2E1.

Statistical Analysis

In vivo CZN and 6OHCZN pharmacokinetic parameters were calculated for each treatment group. Pharmacokinetic results are expressed as mean ± S.D. (range). Parameters measured were AUCT, (area under the curve to the last quantifiable concentration) AUCinf, (measurement of area under the curve up to the last quantifiable concentration plus additional area extrapolated to infinity calculated using Ke), Cmax (maximum observed concentration), Tmax (time at which the observed Cmax occurred), Ke (terminal disposition rate constant calculated from last 3 to 4 points on the log-linear end of the concentration versus time curve) and t1/2 (half-life calculated from the natural log of 2 divided by Ke). All pharmacokinetic statistical analyses were carried
out using SAS software version 8.2. Differences between groups were evaluated using a one-tailed Student t-test and were deemed significant if \( p \leq 0.05 \). Prior to statistical analysis, plasma levels were corrected for any baseline values by subtraction of time zero values from all subsequent time points.

For *in vitro* CZN metabolism, the calibration curves were constructed by using peak area ratios (6OHCZN/internal standard) and least square linear regression analysis was used to determine slope, intercept and correlation coefficients. The absolute recovery was calculated by comparing the peak area obtained with the standards in the Tris-HCl buffer and standards with the microsomes, and relative recovery was obtained by calculating the ratio of the additional amount to the value calibrated by the curve. The apparent \( V_{\text{max}} \) and \( K_m \) were calculated using non-linear regression. The 6OHCZN formation activities between the treatment groups were evaluated using unpaired, one-tailed Student t-tests. *In vitro* pharmacokinetics and the Hill coefficient were calculated using GraphPad Prism version 4.0 (GraphPad Software, Inc., San Diego, CA). CYP2E1 fmol protein levels per \( \mu \)g microsomal protein were extrapolated from expressed cDNA CYP2E1 and control treated monkey microsomal protein dilution curves. CYP2E1 content of microsomes prepared from livers of control- and nicotine-treated monkeys were compared using unpaired one-tailed Student t-tests. For *in vitro* kinetics and CYP2E1 protein measurements, results are expressed as mean ± S.D., which represents the average of the 6 animals per group from at least 4 different experiments. Groups were deemed significantly different if \( p \leq 0.05 \). Correlations between groups were calculated with Pearson correlation coefficients, correlations were deemed significant if \( p \leq 0.05 \).
Results

**In vivo chlorzoxazone disposition and in vitro chlorzoxazone metabolism in control-treated monkeys**

Control-treated monkeys (n=6) had a CZN AUC$_{\text{inf}}$ of $19.7 \pm 4.5 \, \mu g \times h/ml$, a $C_{\text{max}}$ of $9.2 \pm 2.1 \, \mu g/ml$, a $t_{1/2}$ of $0.57 \pm 0.07 \, h$, and a $K_{\text{el}}$ of $1.2 \pm 0.2 \, /h$. The parameters for 6OHCZN included an AUC$_{\text{inf}}$ of $6.6 \pm 0.7 \, \mu g \times h/ml$, a $t_{1/2}$ of $0.68 \pm 0.26 \, h$ and a $K_{\text{el}}$ of $1.2 \pm 0.4 \, /h$. Concentration-time curves are presented in Figure 1A and 1B, and other pharmacokinetic values for control-treated monkeys are listed in Table 1.

The *in vitro* pharmacokinetic parameters of CZN metabolism were determined in hepatic microsomes prepared from 3 untreated African Green monkey livers. The absolute and relative recoveries of both CZN and 6OHCZN were superior when centrifugation was used compared to liquid-liquid extraction with ethyl acetate and filtration. The average absolute and relative recoveries of 6OHCZN were 90.4% and 99.4%, respectively. The linear detection range for 6OHCZN formation was 0.5 to 16 $\mu g/ml$, and intra-day and inter-day standard deviations were all less than 1%. During assay optimization, we established that the formation of 6OHCZN was linear up to microsomal protein concentrations of 800 $\mu g/ml$ (Fig. 2A) and up to an incubation time of 40 minutes (Fig. 2B). Incubations lacking NADPH, or using heat denatured monkey microsomes, produced no detectable 6OHCZN (data not shown).

The formation of 6OHCZN displayed monophasic Michaelis-Menten kinetics (Fig. 3). The apparent $V_{\text{max}}$ and $K_{m}$ for the formation of 6OHCZN in the three untreated monkeys were $3.48 \pm 0.02 \, \text{pmol/min}/\mu g \, \text{microsomal protein}$ and $95.4 \pm 1.8 \, \mu M$, respectively (Fig. 3). The average $V_{\text{max}}/K_{m}$ is $34.2 \pm 2.3 \, \mu l/min/\mu g \, \text{microsomal protein}$. The Hill coefficient was 0.99,
suggesting the involvement of one catalytic site on one enzyme, although a second enzyme with identical catalytic characteristics cannot be ruled out.

**Hepatic CYP2E1 expression and protein levels in control-treated monkeys**

CYP2E1 immunoreactivity was primarily found around the central hepatic vein with lower CYP2E1 immunoreactivity in mid-zonal areas and surrounding the hepatic portal vein (Fig. 4A). No immunoreactivity was found in sections incubated without primary antibody (Fig. 4C).

An immunoblotting assay was established to measure CYP2E1 protein levels (Fig. 5). Serially diluted control-treated monkey liver CYP2E1 co-migrated with both cDNA expressed human CYP2E1 and serially diluted human liver microsomal CYP2E1 (Fig. 5B). Serially diluted cDNA expressed human CYP2E1 and control-treated monkey liver microsomes indicated that signal detection was linear up to 12 µg of monkey microsomal protein (Fig. 5A) and all immunoblots were subsequently loaded at 3 µg of microsomal protein. Assuming equal detection of human and monkey CYP2E1 by the human CYP2E1 antibody, control-treated monkey CYP2E1 levels were 46 ± 11 fmol/µg microsomal protein, and control-treated monkey liver microsomes had similar concentrations of CYP2E1 levels per µg microsomal protein as human liver (Fig. 5B). The anti-human CYP2E1 antibody did not cross-react with other expressed human CYPs (Fig. 5C).

Nicotine treatment increased *in vivo* chlorzoxazone disposition, which was related to induction of *in vitro* chlorzoxazone metabolism and hepatic CYP2E1 protein levels.
Nicotine treatment increased in vivo CZN disposition in monkeys. Nicotine-treated monkeys had a 52% lower CZN AUC_{inf} (p<0.01) and a 52% decrease in T_{max} compared to control-treated monkeys (p<0.05) (Fig. 1A). Other CZN pharmacokinetic parameters were not significantly different between treatments (Table 1).

Monkey CZN AUC_{inf} values correlated significantly with the log plasma metabolic ratio [6OHCZN/CZN] calculated at 0.5, 1, 2 and 4 hours (Table 2, Fig. 1C), indicating a strong relationship between CZN AUC_{inf} and the metabolic ratio. The in vivo CZN disposition and hepatic CYP2E1 protein levels in control- and nicotine-treated monkeys were significantly correlated (r=-0.62, p=0.03, Fig. 6A), with higher CYP2E1 protein levels associating with lower CZN AUC_{inf}. Nicotine treatment resulted in a 1.35-fold increase in CYP2E1 levels per µg of microsomal protein compared to control treatment (62 ± 21 vs. 46 ± 11 fmol/µg microsomal protein, p=0.07) although this was not significant (Fig. 6B). There was large inter-animal variation in CYP2E1 levels within the treatment groups shown in a representative immunoblot (Fig. 5D).

The log plasma metabolic ratio at 1 hour approached a significant correlation with hepatic CYP2E1 levels (p=0.07) and 6OHCZN AUC_{inf} (r=0.53, p=0.17). The 6OHCZN clearance increased in nicotine-treated monkeys, indicated by a 29% decrease in 6OHCZN AUC_{inf} (p<0.01) (Fig. 1B, Table 1). One nicotine- and one control-treated monkey were outliers (greater than two standard deviations away from the mean of their group). When their values were omitted (n=10), significant correlations existed between both the log plasma metabolic ratio at 0.5 and 1 hour with 6OHCZN AUC_{inf} (r=-0.61, p=0.03; r=-0.83, p<0.001, respectively) and hepatic CYP2E1 protein content (r=0.88, p<0.0001; r=0.58, p=0.05, respectively). This data
indicated a significant relationship existed between the log plasma metabolic ratio, hepatic CYP2E1 protein levels, and *in vivo* CZN and 6OHCZN disposition in most monkeys.

The *in vitro* CZN velocity at 950 µM CZN (approximate $V_{\text{max}}$) and hepatic CYP2E1 protein levels in control- and nicotine-treated monkeys were significantly correlated ($r=0.74$, $p=0.004$, Fig. 7A), with higher CYP2E1 protein levels associated with higher rates of CZN oxidation *in vitro*. Nicotine treatment resulted in a 1.27-fold increase in velocity at 950 µM CZN (approximate $V_{\text{max}}$) compared to control treatment ($2.13 \pm 0.83$ vs. $1.67 \pm 0.04$ pmol/min/µg microsomal protein, $p=0.14$), but this did not reach significance (Fig. 7B), likely due to the large variation within the treatment groups. There was also no significant difference in velocity at 95 µM CZN (approximate $K_m$) between nicotine- and control-treated groups ($0.95 \pm 0.37$ vs. $1.20 \pm 0.47$ pmol/min/µg, $p=0.34$), suggesting that nicotine increased the amount of CYP2E1 rather than altered the affinity of the interaction of CZN with the enzyme. The increases in CYP2E1 protein levels and velocity at 950 µM CZN (approximate $V_{\text{max}}$) in the nicotine-treated monkeys were similar (1.35-fold increase in CYP2E1 levels and 1.27-fold increase in CZN metabolism, Fig. 6B and 7B).

For hepatic CYP2E1 protein, chronic nicotine treatment did not alter the location of CYP2E1 immunoreactivity in monkey liver slices (Fig. 4B). CYP2E1 immunoreactivity appeared to be more intense in nicotine-treated monkey liver compared to controls (Fig. 4A).
Discussion

This was the first study to evaluate the within-animal *in vivo* CZN disposition, *in vitro* CZN metabolism, and hepatic CYP2E1 protein content in non-human primates. African Green monkeys are a good model of human CYP2E1 activity and protein. Control-treated monkeys had similar *in vivo* CZN disposition, *in vitro* CZN metabolism, and CYP2E1 protein expression and levels, compared to humans. In humans, doses of 4 to 7 mg/kg CZN are used to phenotype for CYP2E1 activity (Dreisbach et al., 1995; Frye et al., 1998; Girre et al., 1994). The African Green monkeys were treated with the higher dose of 7 mg/kg due to the faster *in vitro* CZN metabolism previously observed for the cynomolgus monkey (Court et al., 1997).

Control-treated African Green monkeys had similar CZN disposition compared to humans. Studies that administered 500 mg CZN doses (approximately 7 mg/kg for an average 70 kg person) to healthy human volunteers reported AUCs_{inf} from 25 to 34 µg×h/ml and a C_{max} of 11 µg/ml (Dreisbach et al., 1995; Girre et al., 1994). Control-treated monkeys given a 7 mg/kg CZN dose had an average AUC_{inf} of 19.7 ± 4.5 µg×h/ml and an average C_{max} of 9.2 ± 2.1 µg/ml. Monkey CZN t_{1/2} (0.57 ± 0.07 h) was faster than humans (1.45 ± 0.44 h) (Dreisbach et al., 1995) and monkey CZN and 6OHCZN T_{max} values were 2-fold smaller than values reported for humans (Frye et al., 1998). The African Green monkeys studied here have a 1.5-fold higher turnover rate for CYP2E1-mediated CZN metabolism compared to humans (Court et al., 1997), which can contribute to the faster half-life and T_{max}. In humans, the metabolic ratio [6OHCZN/CZN] is optimal for detection of CZN metabolism at 2 through 4 hours post-CZN (Frye et al., 1998). In African Green monkeys, we observed significant correlations between the
log metabolic ratio calculated at 0.5, 1, 2 and 4 hours with CZN AUC_{inf} (p=0.01-p=0.0001, Table 2) further indicating that these monkeys are a good model of human CZN metabolism.

The Hill coefficient of 0.99 for \textit{in vitro} CZN metabolism suggested that only one catalytic site was involved in CZN metabolism, providing further support for the specificity of CYP2E1-mediated CZN metabolism in these monkeys. \textit{In vitro} CZN metabolism is mediated by CYP2E1 in humans (Lucas et al., 1999), rats (Kobayashi et al., 2002) and cynomolgus monkeys (Amato et al., 1998), consistent with the single site kinetics observed in these African Green monkeys and further supporting the role of CYP2E1 as the sole enzyme importantly involved in CZN metabolism.

The characteristics of \textit{in vitro} CZN metabolism in African Green monkeys are similar to humans. In humans, the \(K_{m}\) ranges from 77 to 149 \(\mu\text{M}\) (Court et al., 1997; Lejus et al., 2002) and in African Green monkeys the \(K_{m}\) was 95.4 \(\mu\text{M}\). In humans, the \(V_{\text{max}}\) ranges from 1.43 to 3.19 pmol/min/µg (Court et al., 1997; Lejus et al., 2002; Muzeed et al., 2005); the \(V_{\text{max}}\) in African Green monkeys was 3.5 pmol/min/µg and in cynomolgus monkeys it ranges from 0.52 to 3.38 pmol/min/µg (Court et al., 1997; Tanaka et al., 2000). The amount of CYP2E1 protein detected in microsomes from African Green monkeys is similar to the levels of CYP2E1 in human microsomes, assuming equal detection of the two proteins by the antibody, consistent with the similar \(V_{\text{max}}\) values for CZN metabolism between African Green monkeys and humans (Court et al., 1997; Lejus et al., 2002; Muzeed et al., 2005). Monkey CYP2E1 is mainly expressed around the central veins in liver tissue (Fig. 4), similar to the expression in humans (Cohen et al., 1997).

There were significant correlations between \textit{in vitro} CYP2E1 protein content with \textit{in vivo} CZN AUC_{inf} (p=0.03) (Figure 6), and between \textit{in vitro} CYP2E1 protein content with velocity at
950 µM CZN (approximate V_{max}) (p=0.004, Figure 7), indicating a consistent relationship between \textit{in vivo} CZN AUC_{inf}, \textit{in vitro} CZN velocity and CYP2E1 protein levels among the monkeys, and strongly suggested that the increase in \textit{in vivo} CZN disposition is mediated by an increase in hepatic CYP2E1 protein, which is also reflected by the increased \textit{in vitro} CZN metabolism.

Nicotine-treated monkeys had significantly increased \textit{in vivo} CZN disposition. The amount of nicotine a 70 kg smoker receives per day is estimated to range from 0.2 mg/kg to 1.1 mg/kg (Benowitz and Jacob, 1984). The total daily dose of nicotine administered to the monkeys (0.6 mg/kg/day) is similar to the average amount received by a smoker. Although the frequency of administration differs from smoking, this dosing regime has previously been shown to alter other cytochromes P450 to a similar extent to that seen in smokers (Schoedel et al., 2003).

In humans, periods of smoking increase \textit{in vivo} CZN clearance compared to non-smoking periods (Benowitz et al., 2003). Previous studies have shown that carbon monoxide does not increase CZN metabolism (Benowitz et al., 2003). During cigarette smoking, the CZN clearance increases by 24\% (p<0.05), while the half-life does not change, suggesting that cigarette smoking increases CZN first-pass metabolism (Benowitz et al., 2003). Chronic nicotine treatment in monkeys resulted in similar kinetic changes to those seen in humans with cigarette smoking, with a 52\% decrease in CZN AUC_{inf}, a decreased time to T_{max} and no change in half-life (Table 1). These data suggested that nicotine increased the first-pass metabolism and decreased the oral bioavailability of CZN, as seen in human smoking, where CZN clearance increased without a
change in half-life. The decrease in CZN $T_{\text{max}}$ and non-significant decrease in $C_{\text{max}}$ are consistent with a larger first pass metabolism.

Differences in hepatic enzyme levels have been previously shown to alter these pharmacokinetic aspects of orally administered drugs. For example, subjects with higher hepatic CYP2D6 metabolism showed a decrease in debrisoquine AUC$_{\text{inf}}$ but no change in half-life compared to subjects with lower CYP2D6 metabolism (Dalen et al., 1999; Sloan et al., 1983). This indicates that having more functional CYP2D6 increased debrisoquine first-pass metabolism after oral administration without altering systemic metabolism. The increases in velocity at 950 µM CZN (approximate $V_{\text{max}}$) and CYP2E1 protein levels in the nicotine-treated monkeys were similar to each other (1.27-fold compared to 1.35-fold, respectively) and to the increase in CZN clearance seen in smokers (1.24-fold) (Benowitz et al., 2003). There was no difference in velocity at 95 µM CZN (approximate $K_{\text{m}}$) between control- and nicotine-treated monkeys, suggesting that nicotine treatment does not alter the affinity of the enzyme for CZN, but rather acts via an increase in hepatic CYP2E1 levels. The removal of nicotine during smoking cessation may reverse the induction of CYP2E1 protein and activity such that CYP2E1-metabolized drugs, such as acetaminophen, may require dose adjustments due to the decreased level of hepatic CYP2E1 (Frishman et al., 2006).

It is possible that the decrease in CZN AUC$_{\text{inf}}$ may be due to nicotine’s effect on hepatic blood flow, but this is unlikely. In rats, the decreased hepatic blood flow after intraportal nicotine administration returns to baseline after 15 minutes of ceasing treatment (Hashimoto et al., 2004). The monkeys were administered CZN more than 12 hours after the last nicotine treatment; therefore, any effect on hepatic blood flow should have ceased.
In this study, we did not investigate the mechanism of CYP2E1 induction by nicotine in monkeys. Our laboratory has shown that in rats, nicotine does not increase hepatic CYP2E1 by increasing transcription (Howard et al., 2001), by protein stabilization, or by regulation via cotinine, the primary metabolite of nicotine (Micu et al., 2003). Kocarek and colleagues have shown that translational efficiency of CYP2E1 mRNA is increased if the 5’ UTR region of the CYP2E1 mRNA is deleted (Kocarek et al., 2000). It is possible that chronic nicotine treatment results in a direct or indirect interaction with the 5’ UTR region of CYP2E1 mRNA to increase translation.

Nicotine-treated monkeys also had a decrease in 6OHCZN AUC_{inf} (Table 1) suggesting an increase in 6OHCZN disposition. 6OHCZN is glucuronidated and excreted quickly compared to its rate of formation (Dreisbach et al., 1995). Cigarette smoking can increase glucuronidation in humans (Benowitz and Jacob, 2000) suggesting that nicotine may increase 6OHCZN glucuronidation leading to the observed increase in 6OHCZN disposition.

Nicotine induction of CYP2E1 is of interest because it could result in metabolic cross-tolerance via increased metabolism of alcohol by CYP2E1. In humans, smokers have faster alcohol metabolism than non-smokers (Kopun and Propping, 1977; Morabia et al., 1999), and smoking increases alcohol consumption (Barrett et al., 2006). In rats, nicotine increases ethanol consumption and can result in reinstatement of alcohol seeking behaviour (Le et al., 2003). This suggests that nicotine, for example via cigarette smoking, can increase CYP2E1 levels which may result in increased alcohol metabolism, possibly leading to increased consumption of alcohol.

In conclusion, we assessed in vivo CZN disposition, in vitro CZN metabolism, and hepatic CYP2E1 protein levels within 12 monkeys. Monkeys are good models of human
CYP2E1 activity and protein as the *in vivo* CZN disposition, *in vitro* CZN metabolism, and CYP2E1 expression are highly related to each other and similar to that seen in humans. Chronic nicotine treatment increased *in vivo* CZN clearance by increasing first-pass metabolism, likely via increased hepatic CYP2E1, which is reflected in the increased *in vitro* metabolism. These effects of nicotine may be importantly increasing CYP2E1 metabolism of drugs and toxins such as alcohol, acetaminophen and nitrosamines.
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Footnotes

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Conflict of Interest Statement
RFT is a shareholder in Nicogen, a company focused on novel treatment approaches. No support from Nicogen was used and no benefit to the company was obtained.
Legends for figures

Fig 1. Plasma concentration-time curves and correlations between CZN AUC$_{\text{inf}}$ and log metabolic ratio, in control- and nicotine-treated monkeys. (A) CZN and (B) 6OHCZN plasma concentration over time (mean ± SD, 6 per treatment group) after a 7 mg/kg CZN dose. Nicotine-treated monkeys had a 52% decrease in CZN AUC$_{\text{inf}}$ (p<0.001) and a 29% decrease in 6OHCZN AUC$_{\text{inf}}$ (p<0.001). (C) CZN AUC$_{\text{inf}}$ and log metabolic ratio at 1 hour were significantly correlated (p<0.0001) in control- and nicotine-treated monkeys.

Fig 2. Establishment of linear protein and time conditions for the in vitro formation of 6OHCZN. Hepatic microsomes were incubated with 20, 80 or 800 µM CZN. The formation of 6OHCZN in untreated African Green monkey liver microsomes was linear (A) for liver microsomal protein concentrations at 20 minutes and (B) for incubation times at 400 µg of protein. Reactions were linear up to 800 µg of protein and up to 40 minutes of incubation.

Fig 3. Investigation of in vitro $K_m$ and $V_{\text{max}}$ in untreated African Green monkeys. CZN was metabolized to 6OHCZN with a $K_m$ of 95.4 ± 1.8 µM and $V_{\text{max}}$ of 3.5 ± 0.02 pmol/min/µg protein in liver microsomes from three untreated African Green monkeys (mean ± SD). Inset is an Eadie-Hofstee plot.

Fig 4. Monkey hepatic CYP2E1 protein expression detected by immunohistochemistry in liver slices. (A) Hepatic CYP2E1 is primarily distributed around the central and portal veins in liver slices from control- and (B) nicotine-treated monkeys. (C) No detection is seen in a section
incubated without primary antibody. Bars represent 500 µm, (v) indicates central veins, (p) indicates portal veins.

Fig 5. CYP2E1 protein detection in immunoblots of monkey liver microsomes. (A) Standard curve for CYP2E1 immunoblotting in monkey liver microsomes in one control-treated monkey. CYP2E1 detection was linear up to 12 µg of protein. (B) Monkey CYP2E1 co-migrates with both human microsomal CYP2E1 and cDNA expressed human CYP2E1 protein. Human liver (HL, lane 1) loaded at 10 µg; expressed human CYP2E1 loaded at 0.1, 0.2, 0.3, 0.4, 0.5 pmol (lane 2-6); monkey CYP2E1 at 1, 3, 5, 7.5, 10, 12.5 µg microsomal protein (lane 7-12). Migration of CYP2E1 is increased with increasing amount of protein loaded. (C) Anti-human CYP2E1 antibody did not cross-react with other human CYP isozymes. Each CYP is loaded in pairs of lanes at 0.3 pmol and 3.0 pmol. CYP2E1 is indicated with an arrow. (D) Representative immunoblot of CYP2E1 in control treated and nicotine treated monkeys (6 per group). Monkey CYP2E1 is indicated with an arrow.

Fig 6. Relationship between in vivo CZN AUC_{inf} and hepatic CYP2E1 protein in control- and nicotine-treated monkeys (6 per group). (A) There was a significant negative correlation between in vivo CZN AUC_{inf} and hepatic CYP2E1 protein, indicating a relationship between lower in vivo CZN AUC_{inf} and higher CYP2E1 protein levels. (B) Nicotine treatment increased CYP2E1 protein levels by 1.35-fold (non-significant, p=0.07).

Fig 7. Relationship between 6OHCZN formation velocity at 950 µM CZN (approximate V_{max}) and hepatic CYP2E1 protein. (A) There was a significant positive correlation between the
6OHCZN formation velocity at 950 µM CZN (approximate \( V_{\text{max}} \)) and hepatic CYP2E1 protein content, indicating a relationship between higher \textit{in vitro} velocity and higher CYP2E1 protein levels. (B) Nicotine treatment increased 6OHCZN formation velocity at 950 µM CZN (approximate \( V_{\text{max}} \)) by 1.27-fold (non-significant, \( p=0.14 \)), 6 per group.
Table 1. Kinetic parameters for CZN and 6OHCZN in control- and nicotine-treated monkeys, n=6 per treatment group; expressed as mean ± SD (range).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>CZN Control</th>
<th>Nicotine</th>
<th>6OHCZN Control</th>
<th>Nicotine</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC_T (µg×h/ml)</td>
<td>19.7 ± 4.5 (14.4-27.1)</td>
<td>9.5 ± 3.2** (5.9-14.3)</td>
<td>6.6 ± 0.7 (6.0-7.6)</td>
<td>4.7 ± 1.2* (3.3-6.1)</td>
</tr>
<tr>
<td>AUC_{inf} (µg×h/ml)</td>
<td>19.7 ± 4.5 (14.4-27.2)</td>
<td>9.5 ± 3.2** (5.9-14.3)</td>
<td>6.6 ± 0.7 (6.0-7.6)</td>
<td>4.8 ± 1.2* (3.3-6.2)</td>
</tr>
<tr>
<td>C_{max} (µg/ml)</td>
<td>9.2 ± 2.1 (7.0-11.8)</td>
<td>7.8 ± 2.8 (5.9-11.1)</td>
<td>2.7 ± 0.5 (2.0-3.2)</td>
<td>2.8 ± 1.4 (1.6-5.6)</td>
</tr>
<tr>
<td>T_{max} (h)</td>
<td>0.75 ± 0.27 (0.50-1.00)</td>
<td>0.36 ± 0.07* (0.33-0.50)</td>
<td>1.08 ± 0.49 (0.50-2.00)</td>
<td>0.61 ± 0.31 (0.33-1.00)</td>
</tr>
<tr>
<td>t_{1/2} (h)</td>
<td>0.57 ± 0.07 (0.59-0.66)</td>
<td>0.60 ± 0.08 (0.47-0.70)</td>
<td>0.68 ± 0.26 (0.38-1.03)</td>
<td>0.76 ± 0.23 (0.32-0.93)</td>
</tr>
<tr>
<td>$K_{el}$ (1/h)</td>
<td>1.22 ± 0.17</td>
<td>1.18 ± 0.16</td>
<td>1.16 ± 0.45</td>
<td>1.05 ± 0.54</td>
</tr>
<tr>
<td>---------------</td>
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</tr>
<tr>
<td></td>
<td>(1.06-1.55)</td>
<td>(0.99-1.49)</td>
<td>(0.68-1.81)</td>
<td>(0.74-2.13)</td>
</tr>
</tbody>
</table>

Significantly different from the relative control, *$p \leq 0.01$, **$p \leq 0.001$.}
Table 2: Correlations between log [6OHCZN/CZN] plasma levels and *in vivo* kinetics and *in vitro* hepatic CYP2E1 levels (n=12).

<table>
<thead>
<tr>
<th>Correlation variables</th>
<th>r</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CZN AUC&lt;sub&gt;inf&lt;/sub&gt; vs. log [6OHCZN/CZN] plasma at</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5 h</td>
<td>-0.78</td>
<td>0.002</td>
</tr>
<tr>
<td>1 h</td>
<td>-0.91</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>2 h</td>
<td>-0.80</td>
<td>0.001</td>
</tr>
<tr>
<td>4 h</td>
<td>-0.70</td>
<td>0.01</td>
</tr>
<tr>
<td>6 h</td>
<td>0.18</td>
<td>0.58</td>
</tr>
</tbody>
</table>
$V_{\text{max}} = 3.5 \text{ pmol/min/} \mu \text{g protein}$

$K_m = 95.4 \mu \text{M}$
Figure 4

A Control

B Nicotine

C No primary
Figure 5

A

CYP2E1 protein, arbitrary units (mean ± SE)

0
1
2
3

0
5
10
15

Microsomal protein (µg)

B

HL

Expressed CYP2E1

Monkey CYP2E1

C

CYP2E1

CYP2A6

CYP2B6

CYP2C19

CYP2D6

CYP3A4

D

Control liver

Nicotine liver
Figure 7

A

![Graph showing CYP2E1 activity with control and nicotine conditions.

- **r = 0.74**
- **p = 0.004**

Velocity at 950 μM CZN (pmol/min/μg protein)

- Control
- Nicotine

B

![Bar graph comparing velocity at 950 μM CZN between control and nicotine conditions.

- **p = 0.14**

Velocity at 950 μM CZN (pmol/min/μg protein, mean ± SD)

- Control
- Nicotine