Differential Effects of Nicotine Treatment and Ethanol Self-administration on CYP2A6, CYP2B6 and Nicotine Pharmacokinetics in African Green Monkeys

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ABSTRACT

In primates, nicotine is metabolically inactivated in the liver by CYP2A6 and possibly CYP2B6. Changes in the levels of these two enzymes may impact nicotine pharmacokinetics and influence smoking behaviors. This study investigated the independent and combined effects of ethanol self-administration and nicotine treatment (0.5 mg/kg bid s.c.) on hepatic CYP2A6 and CYP2B6 levels (mRNA, protein and enzymatic activity), in vitro nicotine metabolism and in vivo nicotine pharmacokinetics in monkeys. CYP2A6 mRNA and protein levels, and in vitro coumarin (selective CYP2A6 substrate) and nicotine metabolism were decreased by nicotine treatment but unaffected by ethanol. CYP2B6 protein levels and in vitro bupropion (selective CYP2B6 substrate) metabolism were increased by ethanol but unaffected by nicotine treatment; CYP2B6 mRNA levels were unaltered by either treatment. Combined ethanol and nicotine exposure decreased CYP2A6 mRNA and protein levels, as well as in vitro coumarin and nicotine metabolism, and increased CYP2B6 protein levels and in vitro bupropion metabolism, with no change in CYP2B6 mRNA levels. Chronic nicotine resulted in higher nicotine plasma levels achieved after nicotine administration, consistent with decreased CYP2A6. Ethanol alone, or combined ethanol and nicotine, resulted in lower nicotine plasma levels by a mechanism independent of the change in these enzymes. Thus, nicotine can decrease hepatic CYP2A6 reducing the metabolism of its substrates, including nicotine, while ethanol can increase hepatic CYP2B6 increasing the metabolism of CYP2B6 substrates. In vivo nicotine pharmacokinetics are differentially affected by ethanol and nicotine, but when both drugs are used in combination the effect more closely resembles ethanol alone.
INTRODUCTION

Nicotine is responsible for the reinforcing effects of cigarette smoking and dependent smokers will adjust their smoking behavior (i.e., cigarettes/day, inhalation volume, puff frequency) to maintain desired plasma nicotine levels (Scherer, 1999). Therefore, factors that influence nicotine pharmacokinetics, either genetic or environmental (Swan et al., 2009), can have an impact on smoking behaviors and ultimately the risk for certain smoking-related diseases (Liu et al., 2011; Strasser et al., 2011; Wassenaar et al., 2011). Identifying factors that modify nicotine pharmacokinetics is an important step toward understanding smoking behaviors.

In humans, nicotine is mainly eliminated via metabolic inactivation to cotinine in the liver (Benowitz and Jacob, 1994). This reaction is mediated primarily by CYP2A6, with some contribution from CYP2B6 (Messina et al., 1997; Yamazaki et al., 1999; Yamanaka et al., 2005). Cotinine is further metabolized to trans-3-hydroxycotinine via a reaction mediated exclusively by CYP2A6 (Nakajima et al., 1996; Mwenifumbo et al., 2008). Cigarette smoking decreases nicotine metabolism, which is in contrast to the well-known effect of cigarette smoking on accelerating the metabolism of many other drugs (Zevin and Benowitz, 1999). This paradox is presumably due to the downregulation of hepatic CYP2A6 by nicotine, since chronic nicotine treatment decreased hepatic CYP2A6 mRNA, protein and activity levels in monkeys (Schoedel et al., 2003). CYP2B6 levels and activity are unaffected by smoking and nicotine treatment does not affect hepatic CYP2B6 mRNA, protein or activity levels in monkeys (Schoedel et al., 2003; Hesse et al., 2004). Elevated levels of CYP2A6 and CYP2B6 protein have been reported in livers from alcoholics (Niemelä et al., 2000; Hesse et al., 2004). Chronic ethanol administration induces protein levels of hepatic CYP2A5 (orthologue to human CYP2A6) in mice (Lu et al., 2011) and hepatic CYP2B1/2 (orthologue to human CYP2B6) in rats (Howard et al., 2001),
suggesting that ethanol exposure may be responsible for the higher levels of CYP2A6 and CYP2B6 in alcoholics; however, the induction of these hepatic CYPs by ethanol has not yet been demonstrated in primates.

Despite the large proportion of smokers who regularly consume alcohol, there is limited information about the combined effects of smoking and alcohol consumption on CYP2A6, CYP2B6 and nicotine pharmacokinetics. There have been, however, many studies showing that alcohol consumption can modify smoking behaviors (Shiffman and Balabanis, 1995). For example, smokers consume a greater number of cigarettes and inhale more deeply while drinking alcohol (Mintz et al., 1985; Witkiewitz et al., 2011). The association between smoking and alcohol consumption may be due in part to ethanol-induced changes in nicotine pharmacokinetics, possibly involving the induction of CYP2A6 and/or CYP2B6.

The African green monkey (AGM) is an ideal animal model for studying hepatic CYP2A6 and CYP2B6 regulation, nicotine metabolism and nicotine pharmacokinetics. The expression, substrate specificity and regulation of AGM CYP2A6 (also referred to as CYP2A26) and CYP2B6 are similar to their respective human orthogues (Schoedel et al., 2003; Uno et al., 2011). Like humans, AGM eliminate nicotine mainly via metabolic inactivation to cotinine in the liver, with CYP2A6 having a major role in mediating this reaction and CYP2B6 having a minor contribution (Schoedel et al., 2003). The apparent km value (mean ± SD) for in vitro nicotine metabolism in AGM hepatic microsomes is 29.1 ± 8.6 μM (n= 6) (Schoedel et al., 2003), which is comparable to the apparent km value of 64 ± 32.7 μM (n = 31) in human hepatic microsomes (Messina et al., 1997). Consistent with these similarities, the plasma half-life for nicotine ranges from 1-4 hrs in both AGM and humans (Hukkanen et al., 2005; Lee et al., 2006). AGM are also an important model for complex human behaviors and are routinely used in
alcohol research because they will voluntarily self-administer alcohol at levels comparable to human consumption (Ervin et al., 1990).

In this study we characterized the effects of chronic nicotine treatment and ethanol self-administration, alone and in combination, on hepatic CYP2A6 and CYP2B6 levels, in vitro nicotine metabolism and in vivo nicotine pharmacokinetics in AGM. First, we showed that nicotine treatment can decrease hepatic CYP2A6 levels and that ethanol self-administration can increase hepatic CYP2B6 levels. Second, we demonstrated that the metabolism of nicotine to cotinine in monkey liver microsomes is mediated primarily by CYP2A6 and is reduced by nicotine treatment but unaffected by ethanol exposure. Lastly, we showed that exposure to ethanol and nicotine, either alone or in combination, can modify in vivo nicotine pharmacokinetics.
METHODS

Animals. Adult male African green monkeys (vervets, Chlorocebus sabeus) were housed outdoors in social groups at the Behavioral Sciences Foundation (St. Kitts). The study timeline is shown in Figure 1 and has been previously described in detail (Ferguson et al., 2011). Briefly, the first 14 days of the study consisted of an ethanol preference screening phase, where monkeys were allowed to self-administer 10% v/v ethanol in 0.5% w/v sucrose solution for 4 h/day. Forty monkeys that voluntarily consumed more than 1 g of ethanol/kg per day were randomized into four groups based on daily ethanol consumption (n = 10/group): a no-drug control group, an ethanol-only group, a nicotine-only group and an ethanol + nicotine group. The following 14 days (days 15–28) consisted of a washout period, during which monkeys were not exposed to any ethanol or nicotine. From days 29 to 42, monkeys in the ethanol-only and ethanol + nicotine groups were allowed to self-administer 10% ethanol in 0.5% sucrose solution for 4 h/day, whereas the other groups consumed 0.5% sucrose solution on the same schedule. From days 43 to 63, in addition to ethanol (or sucrose), monkeys in the nicotine-only and ethanol + nicotine groups were given subcutaneous injections of nicotine bitartrate (milligram base in saline, pH 7.0, Sigma-Aldrich Canada Ltd, Oakville, ON, Canada) twice daily at a dose of 0.05 mg/kg on day 43, 0.1 mg/kg on day 44, 0.25 mg/kg on day 45, and 0.5 mg/kg for subsequent days. The first injection was given 30 min before the ethanol (or sucrose) access period and the second injection was given 10 h later. Monkeys in the ethanol-only and control groups were given saline injections (as a vehicle control for nicotine bitartrate) on the same schedule. On day 50, nicotine treatment and ethanol access were suspended to conduct pharmacokinetic testing. Throughout the study, monkeys were fed standard rations of Purina monkey chow supplemented with fresh fruit and vegetables twice a day and drinking water was available ad libitum. Body weights at the
start of the study (average = 5.9 ± 0.5 kg) were not significantly different from body weights at
sacrifice (average = 5.7 ± 0.5 kg) (paired t test, \( p > 0.05 \)). There were no significant differences
in body weights among groups at the start of the study (one-way ANOVA, \( F[3, 36] = 0.3784, p \)
> 0.05) or at sacrifice (one-way ANOVA, \( F[3, 36] = 1.860, p > 0.05 \)). Animals were sacrificed
by exsanguination via the femoral artery under ketamine anesthesia, and livers 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 the
Behavioral Sciences Foundation and the University of Toronto Animal Care Committee. All the
procedures were conducted in accordance with the guidelines of Declaration of Helsinki and the
Canadian Council on Animal Care

**Microsomal membrane preparation.** Monkey liver tissue was homogenized in 100 mM Tris,
0.1 mM EDTA, 0.1 mM dithiothreitol, and 0.32 M sucrose (pH 7.4) for immunoblotting or in
1.15% w/v KCl for in vitro metabolism assays and then centrifuged at 12,500g for 30 min at
4°C. The supernatant was then centrifuged at 110,000 g for 90 min at 4°C, and the pellet was
resuspended in 100 mM Tris, 0.1 mM EDTA, 0.1 mM dithiothreitol, 1.15% w/v KCl, and 20%
v/v glycerol for immunoblotting or 1.15% w/v KCl for in vitro metabolism assays. The protein
content of liver microsomes was assayed with the Bradford (1976) technique using a Bio-Rad
Protein Assay kit (Bio-Rad Laboratories, Hercules, CA, USA). Microsomes were stored at -80°C
until further use.

**Immunoblotting.** Monkey liver microsomal protein was serially diluted to generate a standard
curve and to establish the linear detection range for the immunoblotting assays (See
Supplemental Figure 1). Liver microsomal proteins (10 μg for the CYP2A6 assay and 5 μg for the CYP2B6 assay) were separated by SDS-polyacrylamide gel electrophoresis (4% stacking and 8% separating gels). A portion of each gel was stained with Coomassie Blue R-250 to ensure equal loading of protein among lanes and the remaining gel was transferred overnight onto nitrocellulose membranes (Pall Corporation, Pensacola, FL, USA). To detect CYP2A6 and CYP2B6 protein, the membranes were first blocked with 1% skim milk in 50 mM Tris-buffered saline (TBS) containing 0.1% w/v bovine serum albumin and 0.1% v/v Triton X-100 for 1 h. Membranes were then incubated with either polyclonal anti-human CYP2A6 antibody (Fitzgerald Industries, Acton, MA, USA) or anti-human CYP2B6 antibody (Fitzgerald Industries, Acton, MA, USA) diluted 1:2000 for 2 h, followed by three 5 min washes with TBS containing 0.1% v/v Triton X-100. The membranes were then blocked again with the initial blocking solution for 1 h and incubated with peroxidase-conjugated rabbit anti-sheep antibody (Millipore Corporation, Bilerica, MA, USA) diluted 1:5000 for 1 h, followed by three 5 min washes with TBS containing 0.1% v/v Triton X-100. Proteins were visualized using chemiluminescence (Thermo Fisher Scientific, Mississauga, ON, Canada) followed by exposure to autoradiographic film (Ultident, St Laurent, QC, Canada).

**In vitro nicotine c-oxidation assay.** Nicotine c-oxidation (NCO) was assayed as previously described (Schoedel et al., 2003). For the NCO chemical inhibition assays, pooled samples containing equal amounts of microsomal protein from all monkeys in a study group were used. The reaction mixtures were pre-incubated for 15 min with chemical inhibitors. The inhibitor concentrations were approximately equal to Ki and 10-times higher than Ki for the target cytochromes P450 in human liver microsomes: Pilocarpine (CYP2A6, 4 and 40 μM, Sigma-
Aldrich Canada Ltd, Oakville, ON, Canada) (Zhang et al., 2001), C8-Xanthate (CYP2B6, 1 and 10 μM, Toronto research chemicals, Toronto, ON, Canada) (Bourrié et al., 1996) and quinidine (CYP2D6, 0.4 and 4 μM, Sigma-Aldrich Canada Ltd, Oakville, ON, Canada) (Bourrié et al., 1996). Chemically inhibited NCO was compared with reactions performed in the presence of appropriate vehicle controls but not chemical inhibitors. Samples were then analyzed for nicotine and cotinine concentrations.

In vivo nicotine and cotinine plasma assessments. To determine in vivo kinetic parameters for nicotine and cotinine, on days 22 and 50 monkeys were anesthetized with ketamine and injected with 0.1 mg/kg nicotine s.c. (milligram base in saline, pH 7.0, Sigma-Aldrich Canada Ltd). Blood samples (6 ml) were drawn at baseline and at 10, 20, 30, 60, 120, 240 and 360 min after the injection; due to the need for continuous anesthesia, latter sample collection times were not feasible. The blood samples were centrifuged, and the plasma was removed and frozen at -20°C for subsequent drug analyses. Total nicotine and cotinine was measured after deconjugation by an overnight incubation with β-glucuronidase (15 mg/ml, 250 μl per 0.5 ml of plasma) at 37°C.

Nicotine and cotinine measurements. The method for nicotine and cotinine measurement was adapted from a previous study (Siu et al., 2006). Briefly, samples (either plasma or reaction mixtures from the in vitro NCO assays) were extracted with dichloromethane and the organic phase was dried under nitrogen. Samples were reconstituted with 105 μl of 0.01 M HCl and 90 μl of each sample was analyzed by HPLC with UV detection (260 nm). Separation of nicotine and cotinine was achieved using ZORBAX Bonus-RP column (5 μm, 150x4.6 mm; Agilent Technologies, Mississauga, ON, Canada) and a mobile phase consisting of acetonitrile/potassium
phosphate buffer (10:90 v/v, pH 5.07) containing 3.3 mM heptane sulfonic acid and 0.5% triethylamine. The separation was performed with a flow rate of 0.9 ml/min. Nicotine and cotinine sample concentrations were determined from standard curves. The quantitation limits were 5 ng/ml for nicotine and 12.5 ng/ml for cotinine. Cotinine concentrations were adjusted to account for baseline plasma cotinine present in monkeys from the nicotine-only and the ethanol + nicotine groups using the following equation: $C_{\text{adjusted}} = C_t - (C_0 \times e^{Kt})$, where $C_{\text{adjusted}} = $ baseline adjusted cotinine concentration, $C_t = $ the actual cotinine plasma concentration measured at time t, $C_0 = $ concentration of cotinine immediately prior to nicotine dosing, $k = $ elimination rate constant, and $t = $ the post-dosing interval at which $C_t$ was sampled.

**In vitro coumarin hydroxylation assay and 7-OH-coumarin measurement.** The methods used for the in vitro coumarin hydroxylation assays and for the measurement of coumarin and 7-OH-coumarin were adapted from previous studies (O'Kennedy, 1996; Li et al., 1997). Monkey hepatic microsomal protein (0.25 mg/ml) was mixed with 50 mM Tris buffer (pH 7.6) and 1.0 mM NADPH. After a 2 min pre-incubation, reactions were initiated by the addition of coumarin (30 μl M, Sigma-Aldrich Canada Ltd, Oakville, ON, Canada) making the final volume 200 μl. Incubations were for 5 min at 37°C and 50 μl acetonitrile was added to stop the reaction. Trichloroaetic acid (10 μl, 20% v/v) and the internal standard 4-hydroxycoumarin (20 μl, 1 mg/ml) were added to the reaction mixture, which was then vortexed and spun at 16500 g for 10 min. A 100 μl aliquot of each sample was analyzed by HPLC with UV detection (315 nm). Separation of 7-OH-coumarin was achieved using ZORBAX SB C18 column (5 μm 250 x 4.6 mm, Agilent Technologies, Mississauga, ON, Canada) and a mobile phase consisting of acetonitrile, water and acetic acid (25:75:0.1, v/v). The separation was performed with a flow
rate of 1 ml/min. The concentration of 7-OH-coumarin was determined from a standard curve. The quantitation limit for 7-OH-coumarin was 25 ng/ml.

**In vitro bupropion hydroxylation assay and hydroxybupropion measurement.** The methods used for the in vitro bupropion hydroxylation assays and for the measurement of hydroxybupropion were adapted from a previous study (Hesse et al., 2000; Loboz et al., 2005). Monkey hepatic microsomal protein (0.25 mg/ml) was mixed with 50 mM Tris buffer (pH 7.6) and 1.0 mM NADPH. After pre-incubation for 2 min, the reactions were initiated by the addition of bupropion (300 μM, Sigma-Aldrich Canada Ltd, Oakville, ON, Canada) making the final volume 250 μl. Incubations were for 20 min at 37°C and sodium carbonate (100 μl, 20% v/v) was added to stop the reaction. Timolol (25 μl, 100 μg/ml) was added as an internal standard and the mixture was vortexed and spun at 16500 g for 20 min. A 100 μl aliquot of each sample was analyzed by HPLC with UV detection (210 nm). Separation of hydroxybupropion was achieved using ZORBAX Bonus-RP column (250 x 4.6 mm, 5 μM, Agilent Technologies, Mississauga, ON) and a mobile phase consisting methanol and 0.05 M phosphate buffer (pH = 5.8, 45:55 v/v). The separation was performed with a flow rate of 1 ml/min. The concentration of hydroxybupropion was determined from a standard curve. The quantitation limit for hydroxybupropion was 10 ng/ml.

**Isolation, cDNA synthesis, and mRNA quantification.** Liver tissue (50-100 mg) was homogenized in TRIzol reagent (Invitrogen, Carlsbad, CA), and total RNA was isolated according to the TRIzol reagent protocol. RNA concentrations were determined spectrophotometrically and total RNA integrity was confirmed by electrophoresis on a 1.2%
agarose gel (Onbio, Inc., Richmond Hill, ON, Canada) stained with ethidium bromide. Total mRNA from one animal from the nicotine-only group was not included in our analysis due to poor RNA integrity. cDNA was synthesized using 1 μg of total RNA, random hexamers (Invitrogen), Ribolock RNase inhibitor (Fermentas, Burlington, ON, Canada) and MMLV Reverse Transcriptase (Invitrogen) according to protocols provided by Invitrogen. Primers for real-time PCR amplification of CYP2A6, CYP2B6 and β-actin were as follows: CYP2A6 forward primer (CYP2A6ex1), 5’-TGGCAGCAGAGGACAGCAA-3’; CYP2A6 reverse primer (CYP2A6ex2), 5’-GCCTCCCTGTACGGCAGCATATA-3’; CYP2B6 forward primer (CYP2B6ex2), 5’-TATGGGGACGTCTTCACA-3’; CYP2B6 reverse primer (CYP2B6ex3), 5’-ATCAGACACTGAGCCTCC-3’; β-actin forward primer (ACTBex3), 5’-CAGAGCAGAGAAGGCTCCT-3’; and β-actin reverse primer (ACTBex4), 5’-GGTCTCCTCAATGATGCTGCTG-3’. The sequences for AGM CYP2A6 and CYP2B6 are not known; primer specificity was based on the CYP2A6 and CYP2B6 sequences from human and rhesus macaque (Macaca mulatta). Amplification and fluorescence detection were performed using the Applied Biosystems Vii6A7 Real-Time PCR system (Invitrogen). The real-time PCR amplification mixture (20 μl) contained 1 μl of synthesized cDNA, 10 μl of 2X Fast SYBR-Green Mix (Invitrogen), and 0.3 μM concentrations of each primer. Cycling conditions consisted of an initial activation of AmpliTaq Fast DNA polymerase followed by 40 cycles of denaturing (95°C for 15 s) and annealing/extension (58°C for 20 s). Dilutions of monkey and human cDNA were used to determine the range of log-linear detection. CYP2A6 and CYP2B6 mRNA levels were normalized to β-actin mRNA using the comparative CT method for relative quantification as described by the manufacturer (Real-Time PCR Chemistry Guide; Invitrogen).
Data analysis. In vivo pharmacokinetics were derived using non-compartmental analysis. Elimination half-life was estimated by the terminal slope of the concentration vs. time curve. The area under the concentration time curve from 0-6h (AUC$_{0-6h}$) was calculated using the trapezoidal rule and the area under the concentration curve from 0-infinity (AUC$_{0-\infty}$) was calculated using the equation AUC$_{0-\infty}$ = AUC$_{0-6h}$ + C$_{6h}$/k, where C$_{6h}$ is the concentration at 6 hr post-nicotine dosing and k is the elimination rate constant. Apparent volume of distribution was estimated using the equation: [Nicotine dose/AUC$_{0-\infty}$] x 1/half-life. The effect of nicotine and ethanol on hepatic CYP2A6 and CYP2B6 protein, in vitro activity and mRNA were assessed using a two-way analysis of variance (ANOVA) (nicotine treatment X ethanol self-administration), followed by a one-way ANOVA and Least Significant Difference post hoc test. Paired Student’s t tests were used to assess differences in in vivo nicotine and cotinine pharmacokinetic parameters measured pre- and post-drug administration. Statistical significance was set at p < 0.05.
RESULTS

Monkeys voluntarily self-administered alcohol

The amount of alcohol consumed by the monkeys in this study has previously been described (Ferguson et al., 2011). Briefly, average daily alcohol consumption during the 4hr alcohol access session was 38.12 ± 7.8 ml/kg (3.0 g ethanol/kg). There was no significant difference in mean ethanol consumption between the ethanol-only and the ethanol + nicotine groups, indicating no effect of nicotine treatment on voluntary alcohol consumption (Ferguson et al., 2011).

Nicotine and ethanol alter hepatic levels of CYP2A6 and CYP2B6 respectively

There was a significant main effect of nicotine treatment on hepatic CYP2A6 protein (F[1, 36] = 23.73; p < 0.001; Figure 2A), in vitro activity (F[1, 36] = 27.18; p < 0.001; Figure 2C) and mRNA levels (F[1, 35] = 13.2; p < 0.001; Figure 2E). Compared to the control group, monkeys in the nicotine-only and nicotine + ethanol groups had a 40% (p < 0.01) and 47% (p < 0.05) decrease in hepatic CYP2A6 protein, respectively (Figure 2A). CYP2A6 protein levels in the two nicotine-treated groups were also significantly lower compared to the ethanol-only group (Figure 2A). Hepatic CYP2A6 in vitro activity, measured by the rate of coumarin metabolism to 7-OH-coumarin in monkey liver microsomes, showed a pattern of reduction similar to the CYP2A6 protein levels, with a 55% (p < 0.05) and 35% (p < 0.05) decrease in activity in the nicotine-only and ethanol + nicotine groups, respectively (Figure 2C). Hepatic CYP2A6 mRNA was reduced by 46% (p < 0.01) in the nicotine-only group and 37% (p < 0.05) in the nicotine + ethanol group compared to the control group (Figure 2E). Ethanol self-administration did not
significantly affect hepatic CYP2A6 protein (F[1, 36] = 0.02; p = 0.9; Figure 2A), activity (F[1, 36] = 0.16; p = 0.76; Figure 2C) or mRNA levels (F[1, 35] = 0.16; p = 0.17; Figure 2E). There was no interaction effect between ethanol and nicotine on hepatic CYP2A6 protein (F[1, 36] = 0.39; p = 0.89; Figure 2A) or mRNA levels (F[1, 35] = 1.93; p = 0.17; Figure 2E). However, a significant drug interaction on CYP2A6 in vitro activity was observed (F[1, 36] = 13.02; p < 0.01; Figure 2C). This may be a spurious finding, since post-hoc testing revealed no significant difference in in vitro CYP2A6 activity between the nicotine-only group and the ethanol + nicotine group.

There was also significant main effect of ethanol self-administration on hepatic CYP2B6 protein (F[1, 36] = 18.81; p < 0.001; Figure 2B) and in vitro activity (F[1, 36] = 12.16; p = 0.001; Figure 2D). Compared to the control group, monkeys in the ethanol-only and the ethanol + nicotine groups had a 1.96-fold (p < 0.01) and 1.73-fold (p < 0.05) increase in hepatic CYP2B6 protein, respectively. CYP2B6 protein levels in the ethanol-consuming groups were also significantly higher compared to the nicotine-only group (Figure 2B). Hepatic CYP2B activity, measured by the rate of bupropion metabolism to hydroxybupropion in monkey liver microsomes, showed a pattern of increase similar to the CYP2B6 protein levels, and with a 1.26-fold (p > 0.05) and 1.51-fold (p < 0.05) increase in activity in the ethanol-only and ethanol + nicotine groups, respectively. Ethanol-self administration did not significantly affect CYP2B6 mRNA levels in the liver (F[1, 35] = 0.13, p = 0.7; Figure 2F). As a positive control for transcriptional induction of CYP2B6, we used hepatic mRNA from AGMs treated with phenobarbital, a known inducer of CYP2B6 mRNA and protein (Lee et al., 2006). A 6.5-fold increased in mRNA was detected in the livers from phenobarbital-treated monkeys compared to untreated control monkeys, indicating that phenobarbital, but not ethanol, induces CYP2B6 via a
transcriptional mechanism. Nicotine treatment did not significantly affect hepatic CYP2B6 protein ($F[1, 36] = 0.0003, p = 0.99$, Figure 2B), in vitro activity ($F[1, 36] = 0.95; p = 0.4$; Figure 2D) or mRNA levels ($F[1, 35] = 2.86; p = 0.1$; Figure 2F). There was no interaction effect between ethanol and nicotine on hepatic CYP2B6 protein ($F[1, 36] = 2.10; p = 0.99$; Figure 2B), in vitro activity ($F[1, 36] = 1.54; p = 0.2$; Figure 2D) or mRNA ($F[1, 35] = 0.15; p = 0.70$; Figure 2F).

*In vitro nicotine c-oxidation is decreased by nicotine treatment but unaffected by ethanol self-administration*

The rate of NCO in monkey liver microsomes was assessed at two nicotine concentrations: 30 μM ($\approx K_m$ for NCO) and 300 μM ($\approx V_{max}$ for NCO) (Schoedel et al., 2003). At the 30 μM concentration, there was a significant main effect of nicotine treatment on the rate of in vitro NCO ($F[1, 36] = 14.08; p < 0.001$), with a 47% ($p < 0.05$) decrease in the nicotine-only group and a 32% ($p > 0.05$) decrease in the nicotine + ethanol group compared to the control group (Figure 3A). Similarly, at the 300 μM substrate concentration, there was also a significant effect of nicotine on the rate of in vitro NCO ($F[1, 36] = 11.12; p < 0.001$), with a 42% ($p < 0.05$) decrease in the nicotine-only group and a 20% ($p > 0.05$) decrease in the nicotine + ethanol group compared to the control group (Figure 3B). Ethanol did not have a significant effect on NCO at either the 30 μM ($F[1, 36] = 2.25; p = 0.14$; Figure 3A) or 300 μM nicotine concentrations ($F[1, 36] = 3.13; p = 0.085$; Figure 3B). There was no interaction effect between ethanol and nicotine on the rate of NCO at 30 μM ($F[1, 36] < 0.001; p = 0.99$) or at 300 μM ($F[1, 36] = 0.05; p = 0.82$).
Nicotine c-oxidation is primarily mediated by CYP2A6 in monkey liver

Pilocarpine, a selective chemical inhibitor of CYP2A6, strongly inhibited NCO in monkey liver microsomes from all four study groups to a similar extent. At 30 μM nicotine (= Km for NCO), a concentration of pilocarpine equivalent to Kᵢ decreased NCO by 68-74% and a concentration 10-times higher than Kᵢ, decreased NCO by approximately 94% (Figure 4). Results were similar at 300 μM nicotine (= Vmax for NCO) with 36-44% inhibition at a concentration of pilocarpine equivalent to Kᵢ and with 75-79% NCO inhibition at 10-times Kᵢ (Supplemental Figure 2). The selective CYP2B6 inhibitor C8-xanthate used at a concentration equivalent to Kᵢ had little effect on NCO at 30μM or 300 μM nicotine in monkey liver microsomes from all four groups. At 10-times Kᵢ, C8-xanthate decreased NCO by 35-38% at 30 μM nicotine (Figure 4) and 18-27% at 300 μM nicotine (Supplemental Figure 2). Quinidine, a selective CYP2D6 inhibitor, was used as a negative control and did not inhibit NCO at any concentration of nicotine or inhibitor (Figure 4 and Supplemental Figure 2).

There was a significant positive correlation between hepatic CYP2A6 protein levels and in vitro NCO at both 30 μM (R = 0.64, p<0.001; Supplemental Figure 3A) and 300 μM nicotine concentrations (R = 0.52, p < 0.001; Supplemental Figure 3C). Hepatic CYP2B6 protein levels did not correlate with NCO at either 30 μM (R = -0.20, p = 0.13; Supplemental Figure 3B) or 300 μM (R = -0.09, p = 0.3; Supplemental Figure 3D) nicotine concentrations.

Ethanol and nicotine modify in vivo nicotine pharmacokinetics

Like humans, monkeys have substantial interindividual variation in nicotine pharmacokinetics (see Figure 5, control day 22) hence a within-animal design was used to assess changes in in vivo nicotine disposition. After chronic nicotine treatment, monkeys in the nicotine-only group had a
1.39-fold increase in nicotine half-life (p < 0.01), a 1.50-fold increase in nicotine area under the curve from 0-6 h (AUC$_{0-6h}$) (p < 0.05), and a 1.66 increased in nicotine area under the curve extrapolated to infinity (AUC$_{0-infinity}$) (p < 0.05), (Table I, Figures 5 and 7). After chronic ethanol self-administration, monkeys in the ethanol-only group had a 72% reduction in the maximal concentration of nicotine (C$_{max}$) (p < 0.05), a 52% reduction in nicotine AUC$_{0-6h}$ (p < 0.05) and 50% reduction in nicotine AUC$_{0-infinity}$ (p < 0.05), (Table I, Figures 5 and 7). Combined ethanol self-administration and nicotine treatment increased nicotine half-life by 1.26-fold, decreased the nicotine C$_{max}$ by 76% (p < 0.05), AUC$_{0-6h}$ by 62% (p < 0.01) and AUC$_{0-infinity}$ by 54% (p < 0.01) (Table I, Figures 5 and 7). Therefore combined ethanol self-administration and nicotine treatment increased nicotine half-life, an effect that is presumably mediated by nicotine treatment, and decreased nicotine AUC and C$_{max}$, an effect that appears to be mediated by ethanol. No significant changes in nicotine kinetic parameters were observed in the control group between pharmacokinetic challenge days (Table I, Figures 5 and 7).

After chronic nicotine treatment, monkeys in the nicotine-only group had a 2.48-fold increase in cotinine AUC (p < 0.01) and a 2.52-fold increase in cotinine C$_{max}$ (p < 0.01) (Table II, Figure 6). Similarly, combined ethanol self-administration and nicotine treatment increased cotinine AUC by 1.89-fold (p < 0.001) and increased cotinine C$_{max}$ by 1.82-fold (p < 0.01) (Table II, Figure 6). No significant changes in cotinine kinetic parameters were observed in the control or ethanol-only groups between pharmacokinetic challenge days (Table II, Figure 6). Therefore, the effect of combined ethanol and nicotine treatment on cotinine pharmacokinetics appears to be an effect mediated by nicotine treatment, not by ethanol.
DISCUSSION

Nicotine treatment and/or ethanol exposure did not significantly change the relative proportion of NCO mediated by hepatic CYP2A6 and CYP2B6. For example, monkeys exposed to nicotine and ethanol in combination had approximately a 50% reduction in hepatic CYP2A6 protein and 2-fold increase in hepatic CYP2B6 protein. However NCO in these animals was still mediated primarily by CYP2A6 with very little contribution from CYP2B6. These results argue against the concept that CYP2B6 plays a greater role in nicotine metabolism among individuals with reduced CYP2A6 activity (Ring et al., 2007). CYP2A6 protein was significantly correlated with NCO activity, whereas variability in CYP2B6 was not associated with changes in NCO. Similar results have also been shown in human liver microsomes (Al Koudsi and Tyndale, 2010; Al Koudsi et al., 2010), suggesting that CYP2A6, but not CYP2B6, hepatic activity is an important factor influencing nicotine metabolism.

Nicotine treatment increased the plasma half-life of nicotine by approximately 1.3-fold in monkeys, suggesting that nicotine is the agent in cigarette smoke responsible for the quantitatively similar decrease in nicotine clearance (12-27%) during smoking compared to non-smoking (Benowitz and Jacob, 1993; 2000). The total daily dose of nicotine administered to the monkeys (1.0 mg/kg/day) is similar to the average amount of nicotine acquired from smoking (0.2 to 1.1 mg/kg) (Benowitz and Jacob, 1984). This dose produced mean nicotine plasma levels of approximately 25 ng/mL for over 16 hrs/day in AGM, which is within the range of nicotine plasma levels achieved by a smoker during waking hours (10-50 ng/mL) (Benowitz NL, 1990). Our nicotine treatment paradigm was shown to downregulate CYP2A6 leading to slower nicotine metabolism and decreased nicotine clearance in vivo. In contrast, the administration of a 42-mg
transdermal nicotine patch twice a day for 10 days did not affect nicotine clearance in humans (Hukkanen et al., 2009). In this human study, the measurement of hepatic CYP2A6 mRNA and protein was not feasible and the impact of nicotine on in vivo CYP2A6 activity was not assessed. Our study, however, was specifically designed to concurrently investigate the impact of nicotine treatment on CYP2A6 levels, nicotine metabolism and in vivo nicotine disposition.

Nicotine treatment also had a significant impact on cotinine pharmacokinetics, resulting in an increased plasma cotinine AUC after nicotine administration. Cotinine is metabolized to its major metabolite trans-3-OH-cotinine in a reaction mediated exclusively by CYP2A6 (Nakajima et al., 1996). While nicotine and cotinine are both metabolized by hepatic CYP2A6, cotinine has a much lower hepatic extraction ratio and thus changes in the level of hepatic CYP2A6 will have a greater impact on cotinine clearance compared to nicotine clearance (Hukkanen et al., 2005). Therefore it was expected that the downregulation of hepatic CYP2A6 would decrease the rate of cotinine metabolism to a greater extent that the rate of cotinine formation, resulting in an overall increase in cotinine levels.

The daily amount of alcohol self-administered by the monkeys is comparable to moderate human alcohol consumption (Ferguson et al., 2011). At this level of intake, ethanol did not affect hepatic CYP2A6 protein levels or activity. Our findings are consistent with a human study that showed no association between level of alcohol intake and in vivo CYP2A6 activity in a population of moderate alcohol consumers (Mwenifumbo et al., 2007). It has been proposed that hepatic CYP2A6 may be induced in response to ethanol-mediated oxidative stress and/or liver damage (Lu et al., 2011). Treatment with 100 mM ethanol induced CYP2A6 in a human monocytic cell line and this induction was blocked by pre-treatment with the antioxidant Vitamin C, suggesting a role for oxidative stress in the regulation of CYP2A6 (Jin et al., 2012). This
mechanism may contribute to the elevated levels of CYP2A6 found in the livers of alcoholics compared to non-alcoholics (Niemelä et al., 2000). However, moderate alcohol consumption, which was modeled in our study, did not induce CYP2A6 mRNA, protein levels or activity.

Ethanol induced hepatic CYP2B6 protein and activity in AGM, suggesting that ethanol exposure is responsible, at least in part, for the higher levels of CYP2B6 in livers from alcoholics compared to non-alcoholics (Hesse et al., 2004). Hepatic CYP2B6 mRNA was not significantly altered by ethanol, indicating a non-transcriptional mechanism of induction at this dose and duration of ethanol exposure. In rats, both transcriptional and non-transcriptional mechanism for the induction of CYP2B6 by ethanol have been reported (Nanji et al., 1994; Schoedel et al., 2001).

The induction of CYP2B6 by ethanol did not significantly alter in vitro NCO, however chronic ethanol self-administration substantially decreased nicotine Cmax and AUC by more than 50%. Post-ethanol in vivo nicotine pharmacokinetic testing was performed 24 hrs after the last ethanol self-administration session, at which point blood ethanol levels would be negligible, based on an average ethanol elimination rate of 40-62 mg%/h in AGM (Grant and Bennett, 2003). Therefore, the changes in nicotine pharmacokinetic parameters observed in the ethanol-exposed monkeys are not likely to be due to the acute effects of ethanol. This is consistent with human studies showing no effect of short term ethanol exposure on nicotine pharmacokinetics (Benowitz et al., 1986). We speculate that chronic ethanol exposure may be affecting the distribution kinetics of nicotine. Monkeys that self-administered ethanol had a noticeable but non-significant increase in the apparent volume of distribution (Vd) of nicotine, whereas monkeys in the control and nicotine-only groups showed no change in nicotine Vd over the course of the study. Our estimation of Vd is based on the assumption of 100% nicotine
bioavailability from a subcutaneous injection and pseudo-equilibrium conditions (Le Houezec et al., 1993), however these assumptions may not be correct. In rats, chronic administration of ethanol increased the Vd of procainamide, a drug that shares structural and chemical similarities with nicotine (Gole and Nagwekar, 1991), consistent with the possibility that ethanol can increase nicotine Vd. The potential effect of ethanol on nicotine Vd may be explained by ethanol’s effect on cell membrane structure and permeability, and epithelial barrier function (Nanji et al., 1994; Bor et al., 1998; 1998; Carrasco et al., 2006; 2007). An increase in nicotine Vd can explain the decrease in nicotine C\text{max} observed in the ethanol-exposed animals, but cannot account for the observed decreased in nicotine AUC\text{0-\infty}. This suggests that ethanol is also affecting nicotine clearance, possibility by increasing renal clearance or the efficiency of in vivo metabolic clearance in the liver. Monkeys in the ethanol + nicotine group had a reduction in nicotine AUC after treatment, which was similar to the ethanol-only group but opposite to the nicotine-only group. Therefore, the influence of ethanol on nicotine plasma levels, which is potentially mediated by an increase in nicotine Vd, seems to outweigh the effect of nicotine treatment, which is mediated by a an increase in nicotine metabolism.

A limitation of our study was the duration (6 hours) of pharmacokinetic sampling, which was restricted due to the need for continuous anesthesia. For most animals the ratio of AUC\text{0-6h} / AUC\text{0-\infty} was greater than 0.8, indicating an acceptable proportion of the AUC\text{0-\infty} is captured by the AUC\text{0-6h}. However, chronic nicotine treatment significantly increased the plasma half-life of nicotine, which decreased the ratio of AUC\text{0-6h} / AUC\text{0-\infty} to approximately 0.7 for the nicotine-treated animals, potentially reducing the reliability of the estimates of nicotine AUC and half-life for the nicotine-only and nicotine + ethanol groups.
In this study nicotine exposure and ethanol consumption were identified as factors that modify nicotine pharmacokinetics. Nicotine downregulated its own CYP2A6-mediated metabolism and decreased in vivo nicotine clearance, a paradoxical effect. Chronic ethanol consumption substantially decreased the nicotine plasma levels achieved after nicotine administration, an effect that cannot be attributed to altered CYP2A6, CYP2B6 or nicotine metabolism. The lower nicotine plasma levels achieved after ethanol exposure may contribute to the greater number of cigarettes/day smoked by individuals who regularly consume alcohol compared to those who do not (Witkiewitz et al., 2011). Nicotine and ethanol seem to have opposing effects on nicotine pharmacokinetics, but when nicotine and ethanol are presented in combination, the effect of ethanol on nicotine plasma levels dominates. Thus, it is important that researchers and clinician are aware that smokers who regularly consume alcohol may have lower plasma nicotine levels compared to smokers who have the same nicotine intake but rarely consume alcohol. This may also have implications for the efficacy of nicotine replacement therapies.

Both CYP2A6 and CYP2B6 metabolize a variety of compounds other than nicotine; these include clinical drugs, toxicants, procarcinogens, drugs of abuse and endogenous molecules (Honkakoski and Negishi, 1997; Mo et al., 2009). The downregulation of CYP2A6 by nicotine, and the induction of CYP2B6 by ethanol in humans could potentially alter the efficacy of clinical drugs, the susceptibility to chemical toxicity and carcinogenesis, and vulnerability to drug abuse.

In conclusion, our findings demonstrate that nicotine treatment can decrease hepatic CYP2A6 levels, ethanol self-administration can induce hepatic CYP2B6 levels, and combined nicotine and ethanol exposure can alter levels of both enzymes respectively. In addition, our
results suggest that chronic exposure to nicotine and ethanol, either alone or in combination, can modify in vivo nicotine pharmacokinetics in humans.

ACKNOWLEDGMENTS

We thank Dr Bin Zhao, Ewa Hoffman and Joel Keshwa for excellent technical assistance, and the staff of Behavioural Science Foundation, St Kitts, for their dedication and care in conducting all aspects of the animal experiments.
AUTHORSHIP CONTRIBUTIONS

Participated in research design: Miksys, Palmour, Tyndale

Conducted experiments: Ferguson, Miksys, Palmour

Performed data analysis: Ferguson

Wrote the manuscript: Ferguson, Tyndale
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FOOTNOTES

This study was supported by the Centre for Addiction and Mental Health; Canadian Institute of Health Research [MOP97751]; Canadian Foundation for Innovation [20289 and 16014]; Ontario Ministry of Research and Innovation; Canada Research Chair in Pharmacogenetic to Dr. R.F. Tyndale; Canadian Liver Foundation and Scholarship Program for Interdisciplinary Capacity Enhancement. Dr. R.F. Tyndale has participated in one day workshops with Novartis and McNeil. Dr Palmour is Scientific Director of Behavioural Science Foundation, St Kitts, a not-for-profit research foundation registered in St Kitts and the State of Delaware, and receives some travel support from this enterprise.

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FIGURE LEGENDS

**Figure 1.** Overview of the study timeline. Monkeys were assessed for ethanol preference and then randomized into four study groups (n =10/group) based on daily ethanol consumption. The groups consisted of a no-drug control group, an ethanol-only group, a nicotine-only group and an ethanol + nicotine group. In vivo nicotine pharmacokinetic (PK) testing was performed pre-drug administration on day 22 (during the washout period) and again post-drug administration on day 50. Nicotine treatment and alcohol access were suspended on day 50 to allow for pharmacokinetic testing. Monkeys were sacrificed on day 63.

**Figure 2.** CYP2A6 and CYP2B6 are altered by nicotine and ethanol, respectively. Mean CYP2A6 (A) and CYP2B6 (B) hepatic protein levels (n=10/group). Representative blots for CYP2A6 and CYP2B6 are shown with Coomassie blue staining to indicate equal protein loading among lanes (n=4 shown of 10/group analyzed). C, mean velocity of 7-OH-coumarin formation in monkey liver microsomes (n=10/group). D, mean velocity of hydroxybupropion formation in monkey liver microsomes (n=10/group). Mean hepatic CYP2A6 (E) and CYP2B6 (F) mRNA levels normalized to β-actin (n=9 for nicotine-only group and n = 10 for all other groups). For all graphs: *p<0.05 compared to control group, †p<0.05 compared to ethanol-only group, #p<0.05 compared to nicotine-only group.
Figure 3. NCO is decreased by nicotine treatment but unaffected by ethanol self-administration. In vitro NCO in liver microsomes (mean ± SE, n = 10/group) at two nicotine concentrations: (A) 30 μM (≈ K_m for NCO) or (B) 300 μM (≈ V_max for NCO). For all graphs: *p<0.05 compared to control group, †p<0.05 compared to ethanol-only group.

Figure 4. NCO is mediated primarily by CYP2A6 in monkey liver. Percent NCO formation with chemical inhibitors at concentrations equivalent to human K_i or 10-times K_i [pilocarpine (4 μM and 40 μM), C8-Xanthate (1 μM and 10 μM) and quinidine (0.5 μM and 5 μM)]. Values are expressed as percent of vehicle control. Assays were performed in duplicate at 30 μM nicotine (n=10 per pooled sample). See Figure S3 for data at 300 μM nicotine.

Figure 5. The nicotine concentration vs. time curves after a 0.1 mg/kg s.c. nicotine injection for monkeys pre-treatment (day 22) and post-treatment (day 50). For the control group each point represents the mean nicotine concentration for 9 monkeys; for all other groups each point represent the mean for 10 monkeys.

Figure 6. The cotinine concentration vs. time curves after a 0.1 mg/kg s.c. nicotine injection for monkeys pre-treatment (day 22) and post-treatment (day 50). Each point represents the mean cotinine concentration for 10 monkeys.

Figure 7. Ethanol self-administration and nicotine treatment alter in vivo nicotine disposition. Lines represent the change in plasma nicotine AUC_0-6hr for individual monkeys from pre-drug administration (day 22) to post-drug administration (day 50). Diamonds with error bars represent
mean AUC₀-₆hr ± SE for the group pre- and post-drug administration. *p<0.05 compared to same group of monkeys pre-drug administration.
Table I. Nicotine pharmacokinetic parameters (mean ± SE, n = 10 per group)

<table>
<thead>
<tr>
<th>Nicotine Parameter</th>
<th>Control</th>
<th>Ethanol-only</th>
<th>Nicotine-only</th>
<th>Ethanol + Nicotine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 22</td>
<td>Day 50</td>
<td>Pre-</td>
<td>Post-</td>
</tr>
<tr>
<td><strong>Cmax (ng/ml)</strong></td>
<td>36.0 ± 15.0</td>
<td>36.9 ± 15.3</td>
<td>51.9 ± 17.1</td>
<td>14.6 ± 2.4*</td>
</tr>
<tr>
<td><strong>Elimination half-life (h)</strong></td>
<td>3.6 ± 0.9</td>
<td>3.2 ± 0.4</td>
<td>2.09 ± 0.3</td>
<td>2.1 ± 0.3</td>
</tr>
<tr>
<td><strong>AUC_0-6h (ng/ml.h)</strong></td>
<td>79.5 ± 29.0</td>
<td>95.3 ± 26.8</td>
<td>66.1 ± 15.5</td>
<td>31.8 ± 4.2*</td>
</tr>
<tr>
<td><strong>AUC_0-∞ (ng/ml.h)</strong></td>
<td>99.2 ± 31.9</td>
<td>121.9 ± 27.3</td>
<td>73.7 ± 16.5</td>
<td>36.9 ± 6.4*</td>
</tr>
</tbody>
</table>

No significant difference in pre-drug administration parameters between groups

Pharmacokinetic parameters were calculated for each animal individually and then average

*p<0.05, compared to pre-drug administration from the same group

AUC; Area under the curve, Cmax; Maximal concentration
Table II. Cotinine pharmacokinetic parameters (mean ± SE, n = 10 per group)

<table>
<thead>
<tr>
<th>Cotinine Parameter</th>
<th>Control Day 22</th>
<th>Control Day 50</th>
<th>Ethanol-only Pre-</th>
<th>Ethanol-only Post-</th>
<th>Nicotine-only Pre-</th>
<th>Nicotine-only *Post-</th>
<th>Ethanol + Nicotine Pre-</th>
<th>Ethanol + Nicotine *Post-</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC_{0-6h} (ng/ml.h)</td>
<td>240 ± 56.6</td>
<td>196.7 ± 44.3</td>
<td>256.5 ± 22.4</td>
<td>269.2 ± 58.7</td>
<td>162.9 ± 24.4</td>
<td>404.8 ± 5.5*</td>
<td>185.8 ± 28.7</td>
<td>351.1 ± 2.0*</td>
</tr>
<tr>
<td>Cmax (ng/ml)</td>
<td>57.4 ± 10.9</td>
<td>47.4 ± 8.0</td>
<td>53.8 ± 4.5</td>
<td>59.9 ± 12.6</td>
<td>41.8 ± 4.5</td>
<td>105.2 ± 16.4*</td>
<td>42.5 ± 6.7</td>
<td>77.2 ± 10.8*</td>
</tr>
</tbody>
</table>

No significant difference in pre-drug administration parameters between groups

Pharmacokinetic parameters were calculated for each animal individually and then average

*p<0.05, compared to pre-drug administration from the same group

*aCotinine measurements were adjusted to account for baseline plasma cotinine

AUC; Area under the curve, Cmax; Maximal concentration
Figure 1

Days:

Day 1-14: Ethanol preference screening
Day 15-28: Washout
Day 29-42: Ethanol self-administration
Day 42-63: Ethanol self-administration and nicotine treatment
Day 50: Post-drug administration nicotine PK testing
Day 63: Sacrifice

Study groups:

Control
Ethanol-only
Nicotine-only
Ethanol + Nicotine

☐ No ethanol or nicotine ☐ Ethanol self-administration ☐ Nicotine (0.5 mg/kg bid) ☒ Ethanol self-administration and nicotine
Figure 2

A

CYP2A6 protein (pmol/μg protein, mean + SE)

B

CYP2B6 protein (pmol/μg protein, mean + SE)

C

Velocity of 7-OH-coumarin formation (nmol/min/mg, mean + SE)

D

Velocity of hydroxybupropion formation (nmol/min/mg, mean + SE)

E

Relative CYP2A6 mRNA (mean + SE)

F

Relative CYP2B6 mRNA (mean + SE)
Figure 3

A

NCO at 30 μM nicotine (nmol cotinine formed/min/mg protein)

Control
Ethanol-only
Nicotine-only
Ethanol + Nicotine

B

NCO at 300 μM nicotine (nmol cotinine formed/min/mg protein)

*†
Figure 4

Legend:
- Hexagon line with circles: Pilocarpine (CYP2A6)
- Square line: C8 xanthate (CYP2B6)
- Triangle line: Quinidine (CYP2D6)

Graphs showing the effect of inhibitors on various conditions:
- Control
- Ethanol-only
- Nicotine-only
- Ethanol + Nicotine
Figure 5
Figure 6

![Graph showing plasma cotinine levels over time for different conditions.

- Open squares represent Pre-ethanol (Day 22).
- Filled circles represent Post-ethanol (Day 50).
- Open circles with a dashed line represent Post-nicotine (Day 22).
- Filled circles with a solid line represent Pre-nicotine (Day 22).
- Open squares with a dashed line represent Pre-ethanol + nicotine (Day 22).
- Filled circles with a solid line represent Post-ethanol + nicotine (Day 22).

Y-axis: Plasma cotinine (ng/ml), mean ± SE
X-axis: Time (min) from 0 to 360 minutes in increments of 60 minutes.

Legend:
- Open squares - Day 22
- Filled circles - Day 50
- Open squares with a dashed line - Pre-ethanol (Day 22)
- Filled circles with a solid line - Pre-nicotine (Day 22)
- Open squares with a dashed line - Post-ethanol (Day 50)
- Filled circles with a solid line - Post-nicotine (Day 22)
- Open squares with a dashed line - Pre-ethanol + nicotine (Day 22)
- Filled circles with a solid line - Post-ethanol + nicotine (Day 22)
Figure 7

Nicotine AUC0-6h (ng/ml.hr)

Control Ethanol-only Nicotine-only Ethanol + Nicotine

Control (Day 22) Pre-ethanol (Day 22) Post-ethanol (Day 50) Pre-nicotine (Day 22) Post-nicotine (Day 50) Pre-ethanol + nicotine (Day 22) Post-ethanol + nicotine (Day 50)