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Title Page

Title:

Characterization and comparison of nicotine and cotinine metabolism *in vitro* and *in vivo* in DBA/2 and C57Bl/6 mice

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Running Title Page

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Nicotine and cotinine metabolism in mice

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Nonstandard abbreviations: CYP – cytochrome P450, NIC – nicotine, COT – cotinine, 3-HC – 3'-hydroxycotinine, Val – valine, Ala – alanine, HPLC – high performance liquid chromatography, MS – mass spectrometry, EMS – enhanced mass scan, EPI – enhanced product ion, *AUC* – area under the concentration, C_{max} – maximum concentration, T_{max} – time at maximum concentration, $T_{1/2}$ – elimination half-life, *CL* – clearance, *F* – bioavailability

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Abstract

DBA/2 and C57Bl/6 are two commonly used mouse strains that differ in response to nicotine. Previous studies have shown that the nicotine metabolizing enzyme CYP2A5 differs in coumarin metabolism between these two strains suggesting differences in nicotine metabolism. Nicotine was metabolized to cotinine *in vitro* by two enzymatic sites. The high-affinity sites exhibited similar parameters (K_m: 10.7 ± 4.8 vs. 11.4 ± 3.6 μM; V_{max}: 0.58 ± 0.18 vs. 0.50 ± 0.07 nmol/min/mg, for DBA/2 and C57Bl/6, respectively). *In vivo*, the elimination half-lives of nicotine (1 mg/kg, s.c.) were also similar between DBA/2 and C57Bl/6 mice (8.6 ± 0.4 vs. 9.2 ± 1.6 min, respectively); however, cotinine levels were much higher in DBA/2 mice. The production and identity of the putative cotinine metabolite 3'-hydroxycotinine in mice was confirmed by LC/MS/MS. The *in vivo* half-life of cotinine (1 mg/kg, s.c.) was significantly longer in the DBA/2 mice compared to the C57Bl/6 mice (50.2 ± 4.7 vs. 37.5 ± 9.6 min, respectively, $p<0.05$). The *in vitro* metabolism of cotinine to 3'-hydroxycotinine was also less efficient in DBA/2 than C57Bl/6 mice (K_m: 51.0 ± 15.6 vs. 9.5 ± 2.1 μM, $p<0.05$; V_{max}: 0.10 ± 0.01 vs. 0.04 ± 0.01 nmol/min/mg, $p<0.05$, respectively). Inhibitory antibody studies demonstrated that the metabolism of both nicotine and cotinine was mediated by CYP2A5. Genetic differences in *Cyp2a5* potentially contributed to similar nicotine but different cotinine metabolism, which may confound interpretation of nicotine pharmacological studies and studies utilizing cotinine as a biomarker.

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Introduction

Nicotine is the primary component of cigarettes that is responsible for the addictive properties of smoking, which include feelings of pleasure and reward (Henningfield and Keenan, 1993). Rodents, in particular mice, have been widely used for studying the pharmacological effects of nicotine (Aschhoff et al., 1999; Stolerman et al., 1999). Two of the most commonly used mouse strains for studying nicotine behavioral effects are the DBA/2 and C57Bl/6. A large number of studies have examined various aspects of nicotine-mediated behaviors such as discrimination, self-administration, tolerance, and withdrawal and the majority of these studies have found some differences in nicotine effects between these strains (Aschhoff et al., 1999; Stolerman et al., 1999).

The amino acid sequence of CYP2A5 is 84% identical to the human CYP2A6, the main enzyme responsible for the metabolic inactivation of nicotine (Messina et al., 1997; Nakajima et al., 1996b). The mouse *Cyp2a5* gene is genetically polymorphic (Lindberg et al., 1992). Specifically, the DBA/2 mice express the amino acid Val^{117} in hepatic CYP2A5 and metabolized coumarin, a selective probe substrate for mouse CYP2A5 and human CYP2A6, much more efficiently than C57Bl/6 mice which express the amino acid Ala^{117} (Lindberg et al., 1992). Similarly, mutagenesis of CYP2A6, substituting the valine with alanine at the same position, also significantly reduced its catalytic efficiency for coumarin (He et al., 2004).

Genetic variation in human CYP2A6 can alter nicotine metabolism resulting in altered smoking behaviors (Malaiyandi et al., 2006; Schoedel et al., 2004). For instance, individuals who are homozygous for the *CYP2A6* deletion variant (*CYP2A6*4*) produce minimal cotinine (Yamanaka et al., 2004). These individuals smoke fewer cigarettes and are less likely to be

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dependent on tobacco (Malaiyandi et al., 2006; Schoedel et al., 2004). Likewise, in male mice we have previously shown that lower nicotine self-administration behaviors were associated with lower CYP2A5 protein levels and rates of nicotine metabolism (Siu et al., 2006). Furthermore, inhibition of CYP2A5-mediated nicotine metabolism significantly enhanced the pharmacological (i.e. anti-nociceptive) effects of nicotine in mice (Damaj et al., 2006). These data together suggest that, as in humans, nicotine metabolism can significantly affect nicotine-mediated behaviors in mice. Therefore, the main objective of the study was to characterize nicotine and cotinine metabolism (both *in vitro* and *in vivo*) in both the DBA/2 and C57Bl/6 mouse strains. Such differences may account for the variations observed in the pharmacological effects of nicotine in these mice.

Materials and Methods

Animals

Adult male C57Bl/6 and DBA/2 mice (22-24g) were obtained from Charles River Laboratories Inc. (Saint-Constant, PQ). Animals were housed in groups of three to four on a 12-hour light cycle and had free access to food and water. We restricted the study to male mice as we have previously found large variation in CYP2A levels and nicotine metabolism among female mice (Siu et al., 2006) which may be due to hormonal influence (mouse estrous cycle is approximately 3-7 days) as estrogen-only oral contraceptives increased nicotine metabolism in human females (Benowitz et al., 2006). In addition a second CYP2A enzyme, CYP2A4, is present in female

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mice that may metabolize nicotine (Murphy et al., 2005) complicating the interpretation of our CYP2A5 studies.

Reagents

(-)-Nicotine hydrogen tartrate and (-)-cotinine were purchased from Sigma-Aldrich (St. Louis, MO). Both nicotine and cotinine were dissolved in physiological saline (0.9% sodium chloride) for use in *in vivo* studies. *Trans*-3'-hydroxycotinine was custom-made by Toronto Research Chemicals Inc. (Toronto, ON). The internal standard 5-methylcotinine was a generous gift from Dr. Peyton Jacob III at UCSF. All doses are expressed as the free base of the drug. Inhibitory antibodies against human CYP2A6, CYP2B6, and CYP2D6 were purchased from BD Biosciences (Mississauga, ON).

Membrane Preparations

Microsomal membranes were prepared from mouse livers for *in vitro* nicotine metabolism assays as previously described (Messina et al., 1997; Siu et al., 2006) and stored at -80°C in 1.15% KCl. The cytosolic fractions were acquired during membrane preparation and were used as a source of aldehyde oxidase. All livers were collected and frozen prior to 3 pm to avoid circadian effect on CYP2A5 expression.

Nicotine C-Oxidation Assay

Prior to determining the *in vitro* kinetic parameters (K_m and V_{max}) for nicotine metabolism in C57Bl/6 and DBA mice, assay conditions were optimized as previously described (Siu et al., 2006). Linear formation of cotinine from nicotine was obtained under assay conditions of 0.5

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mg/ml protein concentration with an incubation time of 15 min. Incubation mixtures contained 1 mM NADPH and 1 mg/ml of mouse liver cytosol in 50 mM Tris-HCl buffer, pH 7.4 and were performed at 37^{o}C in a final volume of 0.5 ml. The reaction was stopped with a final concentration of 4% v/v Na_2CO_3. After incubation 5-methylcotinine (70 µg) was added as the internal standard and the samples were prepared and analyzed for nicotine and metabolites by HPLC system I as described previously (Siu et al., 2006). The limits of quantification were 5 ng/ml for nicotine, 12.5 ng/ml for cotinine, and 10 ng/ml for 3'-hydroxycotinine.

Cotinine Hydroxylation Assay

Prior to determining the *in vitro* kinetic parameters (K_m and V_{max}) of cotinine metabolism in C57Bl/6 and DBA mice, assay conditions were optimized. Linear formation of 3'-hydroxycotinine from cotinine was obtained under assay conditions of 1 mg/ml protein with an incubation time of 20 min. The incubation mixture was the same as above with the exception that aldehyde oxidase was not added as cotinine metabolism to 3'-hydroxycotinine does not require this cytosolic enzyme. Samples were then analyzed by HPLC system I.

In Vivo Nicotine and Cotinine Treatments and Plasma Nicotine, Cotinine, and 3'-Hydroxycotinine Measurements

To determine the *in vivo* kinetic parameters of nicotine and cotinine in C57Bl/6 and DBA/2 mice, animals were injected with nicotine (1 mg/kg, s.c.) or cotinine (1 mg/kg, s.c.). Blood samples were drawn by cardiac puncture at baseline from untreated animals and from treated animals at various times after the injections. Immediately after collection plasmas were prepared by centrifugation at 3000 x g for 10 min and frozen at –20˚C until analysis. Sample collection

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took place prior to 3 pm. Total nicotine, cotinine, and 3'-hydroxycotinine levels (free and glucuronides) were measured following deconjugation by β-glucuronidase at a final concentration of 5 mg/ml in 0.2 M acetate buffer, pH 5.0, at 37^{o}C overnight. Samples were then analyzed by HPLC system I.

LC/MS/MS Analysis of Cotinine Metabolite

An alternative HPLC system (system II) suitable for separation of eluate for mass-spectrometry was used for the characterization of the cotinine metabolite. This system was similar to that previously described with minor modifications (Murphy et al., 1999). Briefly, using the same column as HPLC system I, cotinine and its metabolites were eluted with a linear gradient from 100% A' (10 mM ammonium acetate buffer, pH 6.5) to 70% A' and 30% acetonitrile over the course of 30 minutes at a flow rate of 1 ml/min.

Mass-spectrometry analysis was performed at the Proteomic and Mass Spectrometry Centre at the University of Toronto (Toronto, ON). Data were acquired with the Q TRAP LC/MS/MS System (Applied Biosystems/MDS Sciex, Toronto, ON). The sample was injected into the sample loop and delivered to the mass spectrometer by 65% acetonitrile and 0.1% formic acid in water at 20 μl/min. Liquid chromatography conditions were as described above (system II) except a flow rate of 0.8 ml/min was used. The liquid was introduced to the mass spectrometer directly after 40:1 splitting. Electrospray ionization was performed in enhanced mass scan (EMS) mode with positive ionization. Nitrogen was used as curtain gas (25 psi), nebulizer gas (25 psi), and heater gas (0 psi). The spray needle voltage was set at 5.5KV and collision induced dissociation gas was set at high. The decluster potential was 20V, collision

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energy was 30eV, and entrance potential was 10V. Enhanced product ion (EPI) was performed at a collision energy of 30eV, all other parameters were same as described for EMS.

Antibody Inhibition of Nicotine and Cotinine Metabolism

We have previously demonstrated that the anti-CYP2A6 antibody was able to cross-react with mouse CYP2A5 (Siu et al., 2006). Microsomes were preincubated with anti-human selective P450 antibodies (anti-CYP2A6, anti-CYP2B6, and anti-CYP2D6), at concentrations of 0, 2, 40, and 80 µl antibodies per mg microsomal protein, for 15 minutes on ice according to manufacturer's instruction. Substrate concentrations used represented the high-affinity K_m value concentrations for nicotine and cotinine metabolism, specifically 11 µM for nicotine and 51 µM for cotinine for DBA/2 mice microsomes and 11 µM for nicotine and 9.5 µM for cotinine for C57Bl/6 mice microsomes.

In Vitro Kinetic and Pharmacokinetic Parameters Analyses

The Michaelis-Menten kinetic parameters K_m and V_{max} from *in vitro* metabolism studies were calculated using Graphpad Prism (Graphpad Software Inc., San Diego, CA) and were verified by the Eadee-Hofstee method. The equation used to determine Km and Vmax for one and two enzymatic sites were $v = V_{max}\,[S] / (K_m + [S])$ and $v = [V_{max1}\,[S] / (K_{m1} + [S])] + [V_{max2}\,[S] / (K_{m2} + [S])]$, respectively, where [S] denotes substrate concentration.

The *in vivo* pharmacokinetic parameters were determined using non-compartmental analysis: $AUC_{0\text{-}480}$, peak plasma concentration (C_{max}), maximum plasma concentration (T_{max}). $AUC_{0\text{-}480}$ was calculated using the trapezoidal rule. Elimination half-life ($T_{1/2}$) was estimated by the terminal slope. Since the bioavailabilities (F) of nicotine and cotinine were unknown

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following subcutaneous injection in mice, *CL* (clearance) was determined as a hybrid parameter *CL*/*F* and was calculated as $Dose/AUC_{0\text{-}480}$. The average weights of the animals of the strains were similar (24.8 ± 1.7 vs. 25.5 ± 1.1 g for DBA/2 and C57Bl/6, respectively, n=50 for each strain), therefore the dose of 25 μg (1 mg/kg) was used for the calculation of *CL*/*F* for nicotine.

Statistical Analyses

Statistical analyses of *in vitro* kinetic parameters were tested by Mann-Whitney U test. Assessment of *in vivo* nicotine, cotinine and 3'-hydroxycotinine plasma levels for the entire time course was not possible from individual animals due to limited blood volume, therefore each time point represented data from multiple mice. Due to this experimental design pharmacokinetic parameters (e.g. half life) were estimated by resampling methods using the PKRandTest software (H. L. Kaplan, Toronto, ON) (Damaj et al., 2006).

Results

In Vitro Nicotine C-Oxidation in DBA/2 and C57Bl/6 Mice

We first assessed the *in vitro* kinetic parameters of nicotine C-oxidation in hepatic microsomes prepared from DBA/2 and C57Bl/6 mice. Nicotine metabolism to cotinine, demonstrated with Michaelis-Menten kinetics (Fig. 1A) and Eadee-Hofstee plotting (Fig. 1B), revealed two enzymatic sites in both strains. The high-affinity sites for hepatic microsomes from both DBA/2 and C57Bl/6 showed similar K_m, V_{max}, and V_{max}/K_m values for nicotine (Table 1). In contrast, the low-affinity enzymatic sites exhibited differing, and much lower nicotine metabolic activities

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with a slight non-significantly higher K_m for C57Bl/6 and V_{max} for DBA/2. The V_{max}/K_m value was significantly higher for the DBA/2 microsomes (Table 1).

Characterization of In Vivo Nicotine Metabolism in DBA/2 and C57Bl/6 Mice

Since the rate of drug metabolism *in vitro* does not necessarily reflect drug clearance *in vivo* (e.g. presence of non-hepatic elimination processes), we determined whether the *in vivo* clearance of nicotine was similar between the two mouse strains. Adult male mice from both strains were treated with 1 mg/kg subcutaneous nicotine, a dose used previously in nicotine behavioral studies (Zarrindast et al., 2003). In both mouse strains, nicotine concentrations peaked at 10 minutes with DBA/2 mice having a significantly greater maximum concentration compared to C57Bl/6 mice (Fig. 2A; Table 2). The overall $AUC_{0\text{-}480}$ of nicotine was also modestly higher for DBA/2 than for C57Bl/6 mice. Both strains had similar elimination half-lives for nicotine but the clearance of nicotine was slower in the DBA/2 mice compared to C57Bl/6 mice.

When examining the disposition kinetics of cotinine formed from injected nicotine, we observed that the appearance of the cotinine metabolite was rapid and similar between the two mouse strains and achieving peak concentrations around 15 minutes (Fig 2B). In contrast, compared to C57Bl/6 mice, DBA/2 mice showed a significantly larger $AUC_{0\text{-}480}$ and longer elimination half-life (Table 2).

LC/MS/MS Characterization of the Putative Cotinine Metabolite 3'-Hydroxycotinine

In humans cotinine is metabolized exclusively to *trans*-3'-hydroxycotinine by CYP2A6 (Dempsey et al., 2004; Nakajima et al., 1996a; Yamanaka et al., 2004). To our knowledge no prior studies have examined or confirmed the production of *trans*-3'-hydroxycotinine from

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cotinine in mice, therefore our immediate goal was to determine whether mice metabolize cotinine to 3'-hydroxycotinine. In a preliminary *in vitro* study we identified a cotinine metabolite that displayed the same retention time as the *trans*-3'-hydroxycotinine standard (Fig. 3A). To confirm the identity of the putative *trans*-3'-hydroxcotinine compound, a second HPLC system compatible with MS/MS analysis was used. Both the *trans*-3'-hydroxycotinine standard and the cotinine metabolite eluted with the same retention time (10.4 min) using the new HPLC system (Fig. 3B).

In the LC/MS/MS analyses, EMS indicated that the *trans* 3'-hydroxycotinine standard had a retention time of 11.8 minutes with a *m*/*z* of 193 (Fig. 3C) and when the metabolite of *in vitro* cotinine metabolism was monitored at *m*/*z* 193, a major peak was present at 11.1 minutes (Fig. 3D). Fragmentation of the *trans*-3'-hydroxycotinine standard ion (*m/z* 193) gave two fragments of *m/z* 80 and *m/z* 134 (Fig. 3E). Fragmentation of the cotinine metabolite at *m/z* 193 also gave two major ions of *m/z* 80 and *m/z* 134 (Fig. 3F). The peak area ratios of *m*/*z* 80/134 for the *trans*-3'-hydroxycotinine and the cotinine metabolite were 3.44 and 3.41, respectively. The *m*/*z* 80 and the *m*/*z* 134 fragments corresponded to $(C_5H_5N)H^+$ and pyridyl-$C_3H_4O^+$, respectively (Murphy et al., 1999).

Characterization of In Vivo Cotinine Metabolism in DBA/2 and C57Bl/6 Mice

Having confirmed that 3'-hydroxycotinine is produced from cotinine in mice, we proceeded with *in vivo* injections of cotinine (1 mg/kg, s.c.). Plasma cotinine concentrations were maximal between 5 to 15 minutes and were similar for both DBA/2 and C57Bl/6 mice (Fig. 4A, Table 3). Similar to cotinine derived from nicotine injection, following cotinine injection the cotinine $AUC_{0\text{-}180}$ was much higher in the DBA/2 mice compared to the C57Bl/6 mice. The clearance of

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cotinine was slower in DBA/2 mice, which resulted in longer elimination half-life of cotinine compared to C57Bl/6 mice.

The plasma levels of 3'-hydroxycotinine formed from cotinine injections were also monitored. The plasma $AUC_{0\text{-}180}$ of 3'-hydroxycotinine was higher in the DBA/2 mice compared to the C57Bl/6 mice (Fig. 4B, Table 3).

In Vitro Cotinine Metabolism in DBA/2 and C57Bl/6 Mice

To determine whether cotinine was metabolized to 3'-hydroxycotinine differently between the two mouse strains, accounting for the differences in cotinine plasma concentrations seen *in vivo*, we performed *in vitro* cotinine metabolism studies. We found that cotinine metabolism to 3'-hydroxycotinine was characterized by Michaelis-Menten kinetics (Fig. 5A), mediated by a single enzymatic site in both strains (Fig. 5B). The DBA/2 mice had a significantly higher K_m compared to the C57Bl/6 mice (Table 4), while the V_{max} for cotinine was much greater for DBA/2 than for C57Bl/6 mice. This resulted in an overall lower catalytic efficiency (V_{max}/K_m) for DBA/2 compared to C57Bl/6.

Inhibition of In Vitro Nicotine and Cotinine Metabolism

Previously the mouse CYP2A5 was identified as the enzyme responsible for the high-affinity metabolism of nicotine using cDNA-expressed CYP2A5 (Murphy et al., 2005). To extend these studies characterizing the enzyme involved, we tested the effect of inhibitory antibodies on *in vitro* nicotine metabolism. Anti-CYP2A6 inhibitory antibodies dose-dependently inhibited the formation of cotinine from nicotine in DBA/2 microsomes with maximal inhibition of 70% at 40 µl antibody/mg microsomal protein (Fig. 6A, filled symbols). Similar results were seen in

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hepatic microsomes from C57Bl/6 mice, tested at 80 μl antibody / mg protein (Fig. 6A, open symbols). Inhibitory antibodies against CYP2B6 and CYP2D6, enzymes postulated to be involved in the remaining small percentage of metabolism of nicotine in humans (Messina et al., 1997; Nakajima et al., 1996b; Yamazaki et al., 1999), did not inhibit nicotine metabolism in either mouse strain (Fig. 6A).

In humans CYP2A6 is exclusively responsible for the metabolism of cotinine to trans-3'-hydroxycotinine (Dempsey et al., 2004; Nakajima et al., 1996a). To determine whether mouse CYP2A5 was also responsible for this metabolic pathway we performed inhibition studies on cotinine metabolism. Anti-CYP2A6 inhibitory antibodies dose-dependently inhibited the formation of 3'-hydroxycotinine from cotinine in DBA/2 mouse liver microsomes (Fig. 6B, filled symbols), with a maximal inhibition of 90% at 40 μl antibody / mg microsomal protein. Anti-CYP2A6 inhibitory antibodies also inhibited cotinine metabolism (no detectable metabolite peak) in C57Bl/6 hepatic microsomes (Fig. 6B, open symbols). Inhibitory antibodies against CYP2B6 and CYP2D6 had no effect on cotinine metabolism (Fig. 6B).

Discussion

In the present study we examined both *in vitro* metabolism and in *vivo* pharmacokinetics of nicotine and cotinine in two commonly used inbred mouse strains, DBA/2 and C57Bl/6. Mice have been used extensively for the study of nicotine behaviors; however, the effects of nicotine vary widely between strains (Aschhoff et al., 1999; Stolerman et al., 1999). One potential contributing factor may be differences in nicotine pharmacokinetics which can significantly alter

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its pharmacology. CYP2A5 metabolizes nicotine (Murphy et al., 2005) and previous studies found that DBA/2 and C57Bl/6 mice differed in CYP2A5 structure and function (Lindberg et al., 1992). Therefore the primary goal of this study was to determine whether nicotine and cotinine metabolism differed between these two strains, which may be contributed by the *Cyp2a5* polymorphism between these mice (Lindberg et al., 1992).

The *in vitro* metabolism of nicotine to cotinine was mediated by a high- and a low-affinity enzyme site in both mouse strains. The K_m values for the high-affinity sites reported here are consistent with those seen using cDNA-expressed CYP2A5 (7.7 ± 0.8 μM; 129/J mouse strain) (Murphy et al., 2005) but are modestly higher affinity relative to hepatic microsomes from ICR mice (18.6 ± 5.9 μM) (Damaj et al., 2006). The identity of the high-affinity site was confirmed, as CYP2A5 inhibitory antibodies inhibited up to 70% of nicotine metabolism at K_m for nicotine in both strains. The low-affinity sites in our mice could potentially belong to the 2B family. In humans, cDNA-expressed CYP2B6 metabolizes nicotine but with much lower affinity and activity compared to CYP2A6 (Yamazaki et al., 1999). In monkeys, CYP2B6agm is a minor enzyme compared to CYP2A6agm for the metabolism of nicotine to cotinine (Schoedel et al., 2003). In contrast, rat CYP2B1/2 is the primary enzyme responsible for this process (Nakayama et al., 1993). In our experiments, however, no indication of inhibition of nicotine metabolism was seen at the highest CYP2B antibody concentration tested. Considering that at the highest plasma nicotine concentrations observed (~200 ng/ml ≅ 1.2 μM) following nicotine injection the estimated contribution of the low-affinity enzymes to nicotine metabolism was only ≤10%, the identity of this enzyme was not pursued.

To determine if nicotine was metabolized similarly between DBA/2 and C57Bl/6 mice *in vivo* we administered nicotine subcutaneously as this route kinetically mimics, somewhat, the

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route of nicotine intake from smoking in that it bypasses first-pass metabolism. The *in vivo* kinetics of nicotine in these two strains differed though not dramatically. The higher nicotine C_{max} in DBA/2 mice may be due to a smaller volume of distribution of nicotine: male DBA/2 mice have, on average, 34-40% more body fat and 10% lower lean mass compared to C57Bl/6 mice (Mouse Phenome Database, Jackson Labs) which could result in higher levels of nicotine in the plasma (and other highly perfused organs such as liver, kidneys, and the lung) (Urakawa et al., 1994). Despite similar nicotine clearance, however, we found that cotinine was removed more slowly in DBA/2 mice as demonstrated by the two-fold longer elimination half-life and higher cotinine $AUC_{0\text{-}480}$.

In humans, the main metabolites of cotinine recovered in urine are *trans*-3'-hydroxycotinine and its glucuronide which account for 40-60% of the total administered dose of nicotine (Hukkanen et al., 2005). Initially we demonstrated that mice produced 3'-hydroxycotinine from cotinine with LC/MS/MS. We then confirmed that the metabolism of cotinine to 3'-hydroxycotinine was mediated by CYP2A5 – up to 90% of cotinine metabolism to 3'-hydroxycotinine was inhibited by anti-CYP2A6 inhibitory antibodies. This is consistent with the metabolism of cotinine to 3'-hydroxycotinine being mediated exclusively by human CYP2A6 *in vitro* and *in vivo* (Dempsey et al., 2004; Nakajima et al., 1996a). Following cotinine injections, DBA/2 mice showed slower clearance of cotinine compared to C57Bl/6 mice, which was consistent with the pharmacokinetics of cotinine formed from nicotine injections. It is likely that DBA/2 mice have a slower hepatic (intrinsic) clearance of cotinine to 3'-hydroxycotinine compared to C57Bl/6; this is supported by our *in vitro* findings that cotinine was metabolized to 3'-hydroxycotinine significantly slower in the DBA/2 compared to C57Bl/6 mice. Even at the maximum plasma cotinine concentrations observed (~760 ng/ml ≅ 4.3 μM) our *in vitro* data

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indicated that the DBA/2 mice metabolized cotinine slower than the C57Bl/6 mice (v = 0.008 vs. 0.0012 nmol/min/mg, respectively).

When examining the plasma concentrations of 3'-hydroxycotinine formed from cotinine, we found that DBA/2 mice had a larger $AUC_{0\text{-}180}$ compared to C57Bl/6 mice. The higher level of 3'-hydroxycotinine in DBA/2 mice was likely to be due to reduced elimination of 3'-hydroxycotinine. This could occur through slower rates of conjugation to O-glucuronide and/or slower renal excretion of 3'-hydroxycotinine and its glucuronidated metabolite. In mice, N-glucuronides of nicotine and its proximal metabolites have not been detected nor identified (although O-glucuronides were not measured) (Ghosheh and Hawes, 2002) while in humans ~80% of trans-3'-hydroxycotinine is excreted unchanged (Hukkanen et al., 2005). Thus we believe the higher level of 3'-hydroxycotinine was most likely due to slower renal excretion in the DBA/2 mice.

This study showed the *in vitro* and *in vivo* metabolism of nicotine was similar between DBA/2 and C57Bl/6 mice. In contrast to nicotine, DBA/2 mice metabolized cotinine to 3'-hydroxycotinine with lower efficiency compared to C57Bl/6 mice both *in vitro* and *in vivo*. These data indicate that genetic differences in the structure of CYP2A5 between the two strains can potentially alter the rate of metabolism depending on the specific substrate. The DBA/2 CYP2A5, which has valine at position 117, is more efficient at metabolizing coumarin compared to C57Bl/6, which has an alanine in this position (Lindberg et al., 1992; van Iersel et al., 1994). In contrast, this genetic variant does not appear to alter the metabolism of nicotine but rather, it reduces the catalytic activity for cotinine. Mouse CYP2A5 oxidizes nicotine to $\Delta^{5'(1')}$-iminium ion followed by conversion to cotinine by aldehyde oxidase (Murphy et al., 2005); however, no functional polymorphisms for the mouse aldehyde oxidase genes have been reported (Mouse

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Genome Informatics) and our *in vivo* data was consistent with our *in vitro* data suggesting the variation in aldehyde oxidases were not contributing substantively to our observations. Substrate selective metabolisms by genetic variants of human *CYP2A6* have been observed. For example, the *CYP2A6*7* variant has reduced nicotine metabolic activity but the coumarin metabolism was minimally affected (Ariyoshi et al., 2001). In addition, different levels of cotinine following similar nicotine intake have been observed in smokers (Benowitz et al., 1999) and this may be partly related to genetic variations in *CYP2A6* that have minor impacts on the metabolism of nicotine relative to the impact on cotinine metabolism. Finally, these observations warrant further studies on the metabolic activation of CYP2A5/6 substrates such as NNK, a tobacco-specific nitrosamine known to cause lung cancer (Miyazaki et al., 2005), and the consequence of these genetic variants on NNK activation. Future studies will focus on the expression of variants V117A (found in CYP2A5 [Lindberg et al., 1992] and CYP2A13 [NCBI]) as well as F118L (found in CYP2A6 [NCBI]), in all three enzymes and their impact on multiple substrates including NNK and Tegafur.

The finding that cotinine is differentially metabolized relative to nicotine, between strains, has implications with respect to interpreting nicotine pharmacological studies. Cotinine can pass through the blood brain barrier (Lockman et al., 2005). In rats, cotinine can bind to epibatidine-sensitive nicotinic receptors in frontal cortex and hippocampus tissues, although with lower affinity than nicotine (Vainio and Tuominen, 2001). Furthermore, administration of cotinine in rat striatal tissues evoked dopamine overflow in a dose-dependent manner (Dwoskin et al., 1999). Thus, differing levels of cotinine may result in altered pharmacological effects despite similar nicotine levels between the two mouse strains and the effects may be erroneously attributed to nicotine pharmacology. On the other hand, the effects of cotinine in humans are less

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clear (Buccafusco and Terry, 2003; Crooks and Dwoskin, 1997). Cotinine alone did not show pharmacological effects but it did interfere with the ability of nicotine patch to reduce withdrawal symptoms (Hatsukami et al., 1998b). Cotinine also increased plasma nicotine levels in smokers, possibly through increased smoking to compensate for the interference of nicotine action by cotinine (Hatsukami et al., 1998a). In other studies cotinine appeared to have some effects in reducing withdrawal symptoms (Benowitz et al., 1983; Keenan et al., 1994).

Consideration should also be taken when using cotinine as biomarker for environmental tobacco smoke exposure (Benowitz, 1999) and cigarette intake (de Leon et al., 2002) in humans, or nicotine consumption in mice (Sparks and Pauly, 1999), as cotinine can be metabolized at different rates by human CYP2A6 and mouse CYP2A5. Differing levels of cotinine could be erroneously interpreted as different levels of exposure rather than differing rates of removal.

In conclusion, we have characterized nicotine and cotinine metabolism in two mouse strains which differed in CYP2A5 enzyme structure (Lindberg et al., 1992). While coumarin metabolism differed (Lindberg et al., 1992) we observed no substantial difference in nicotine metabolism. In contrast, CYP2A5-mediated cotinine metabolism to 3'-hydroxycotinine was different between the mouse strains which may confound interpretation of pharmacological and biomarker studies.

Acknowledgments

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officer of Nicogen Inc., a company focused on the development of novel smoking cessation therapies; no funds were received from Nicogen for these studies and this manuscript was not reviewed by other people associated with Nicogen prior to submission or revision.

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Footnotes

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Legends for Figures

Figure 1. *In vitro* metabolism of nicotine to cotinine in DBA/2 and C57Bl/6 mice. *In vitro* kinetic parameters of nicotine metabolism were investigated using hepatic microsomes. A) Rate of formation of cotinine from nicotine (NIC) (mean ± SD of 4 to 5 mice). B) Eadee-Hofstee plots of the kinetic data for DBA and C57Bl/6 mice. See Table 1 for values of kinetics parameters. V: velocity; S: substrate

Figure 2. Plasma nicotine and cotinine concentrations following nicotine treatment. Adult male DBA/2 and C57Bl/6 mice were injected with subcutaneous nicotine (1 mg/kg). A) The $AUC_{0\text{-}480}$ of nicotine (NIC) was 15% higher while the elimination half-life was similar in the DBA/2 mice compared to C57Bl/6 mice. B) The $AUC_{0\text{-}480}$ of cotinine (COT) in the DBA/2 mice was twice that of the C57Bl/6 mice. Each time point represents mean (± SD) of 5 to 7 animals for each strain. Values below the limit of quantification are not shown.

Figure 3. Characterization of 3'-hydroxycotinine in mice by HPLC and LC/MS/MS. A) Cotinine and its metabolite (COT metabolite) from a pilot *in vitro* cotinine metabolism study using mouse liver microsomes (DBA/2) were separated with HPLC system I in the absence (*left*) or presence (*right*) of the *trans*-3'-hydroxycotinine standard (3-HC). B) Cotinine and *trans*-3'-hydroxycotinine standards (*left*) as well as products from a pilot *in vitro* cotinine metabolism study (*right*) were separated with HPLC system II. C) Enhanced mass scan at *m*/*z* 193 showed that the *trans*-3'-hydroxycotinine standard had a retention time of 11.8 minute. D) Liquid chromatography of products from *in vitro* cotinine metabolism monitored at *m*/*z* 193 identified a

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putative 3'-hydroxycotinine compound with a retention time of 11.1 minutes. E) Fragmentation of the *m*/*z* 193 ion (from C) at 30eV produced *m*/*z* 80 and *m*/*z* 134 ions. F) Fragmentation of the *m*/*z* 193 ion (from D) at 11.1 minutes at 30eV produced *m*/*z* 80 and *m*/*z* 134 ions.

Figure 4. Plasma cotinine and 3'-hydroxycotinine concentrations following cotinine injections. Adult male DBA/2 and C57Bl/6 mice were injected with subcutaneous cotinine (1 mg/kg). A) The $AUC_{0\text{-}180}$ of cotinine (COT) was 37% higher and its elimination half-life was 34% longer in DBA/2 mice compared C57Bl/6 mice. B) The $AUC_{0\text{-}180}$ of 3'-hydroxycotinine (3-HC) was 94% higher in the DBA/2 mice compared to C57Bl/6 mice. Each time point represents mean ± SD of 3 to 8 animals for each strain.

Figure 5. *In vitro* metabolism of cotinine to 3'-hydroxycotinine in DBA/2 and C57Bl/6 mice. *In vitro* kinetic parameters of cotinine metabolism were investigated using hepatic microsomes. A) Rate of formation of 3'-hydroxycotinine from cotinine (mean ± SD; n=4 samples per strain). DBA/2 and C57Bl/6 mice. Regression lines and intercepts for both DBA/2 and C57Bl/6 were calculated from K_m and V_{max} values derived from Michaelis-Menten fitting and no data points were censored. See Table 4 for values of kinetic parameters. V: velocity; S: substrate

Figure 6. Antibody inhibits *in vitro* nicotine and cotinine metabolism in DBA/2 and C57Bl/6 mice. A) At K_m (11 μM) nicotine, anti-CYP2A6 inhibitory antibodies inhibited cotinine formation maximally by 70% in hepatic microsomes in DBA/2 mice (filled symbols). At K_m (11 μM) nicotine, anti-CYP2A6 inhibitory antibodies (80 μl antibodies / mg protein) inhibited cotinine formation by 70% in C57Bl/6 hepatic microsomes (open symbols). B) At K_m (51 μM)

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cotinine, anti-CYP2A6 inhibitory antibodies inhibited 3'-hydroxycotinine formation by >90% in hepatic microsomes DBA/2 mice (filled symbols). At K_m (9.5 μM) cotinine, anti-CYP2A6 inhibitory antibodies inhibited 3'-hydroxycotinine formation in C57Bl/6 hepatic microsomes (open symbols). *Activity of cotinine metabolism was below limit of quantification. Anti-CYP2B6 and anti-CYP2D6 antibodies had no effect on nicotine or cotinine metabolism. Values are expressed as a percent of activity in the absence of antibodies and were determined from pooled sample of 2 animals.

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Tables

Table 1. *In vitro* nicotine metabolism (C-oxidation) parameters.

		DBA/2 (n=5 mice) mean ± S.D.	C57Bl/6 (n=4 mice) mean ± S.D.
High Affinity	K_m (μM)	10.7 ± 4.8	11.4 ± 3.6
	V_{max} (nmol/min/mg)	0.58 ± 0.18	0.50 ± 0.07
	V_{max}/K_m	0.05 ± 0.03	0.04 ± 0.02
Low Affinity	K_m (μM)	234 ± 77	306 ±126
	V_{max} (nmol/min/mg)	1.60 ± 0.63	1.18 ± 0.36
	V_{max}/K_m	0.007 ± 0.001*	0.004 ± 0.001

**p < 0.05 compared to C57Bl/6 mice*

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Table 2. Pharmacokinetic parameters of plasma nicotine and cotinine in mice treated with nicotine (1 mg/kg, s.c.). Results are derived using data from 5 to 7 animals at each time point.

	Nicotine		Cotinine	
	DBA/2	C57Bl/6	DBA/2	C57Bl/6
$AUC_{0\text{-}480}$ (ng•hr/ml)	92.9 ± 2.9*	80.8 ± 3.2	245 ± 10*	120 ± 7
$T_{1/2}$ (min)	8.6 ± 0.4	9.2 ± 1.6	51.0 ± 4.1*	23.7 ± 2.0
CL/F (ml/min)	4.5 ± 0.1*	5.2 ± 0.2	ND	ND
T_{max} (min)	10.0 ± 2.2	10.0 ± 4.7	45.0 ± 13.3*	20.0 ± 13.3
C_{max} (ng/min)	201 ± 15*	160 ± 15	141 ± 7*	112 ± 12

**p < 0.05 compared to C57Bl/6 mice, ND – not determined*

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Table 3. Pharmacokinetic parameters of plasma cotinine and 3'-hydroxycotinine in mice treated with cotinine (1 mg/kg, s.c.). Results are derived using data from 3 to 8 animals at each time point.

	Cotinine		3'-hydroxycotinine	
	DBA/2	C57Bl/6	DBA/2	C57Bl/6
$AUC_{0\text{-}180}$ (ng•hr/ml)	1087 ± 33*	796 ± 33	518 ± 30*	268 ± 19
$T_{1/2}$ (min)	50.2 ± 4.7*	37.5 ± 9.6	ND	ND
CL/F (ml/min)	0.38 ± 0.01*	0.52 ± 0.02	ND	ND
T_{max} (min)	15.0 ± 7.5	15.0 ± 0.0	60.0 ± 0.0	60.0 ± 23.6
C_{max} (ng/ml)	748 ± 35	759 ± 39	255 ± 23*	110 ± 10

**p < 0.05 compared to C57Bl/6, ND – not determined*

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Table 4. *In vitro* cotinine hydroxylation parameters.

	DBA/2 (n=4) mean ± S.D.	C57Bl/6 (n=4) mean ± S.D.
K_m (μM)	51.0 ± 15.6*	9.5 ±2.1
V_{max} (nmol/min/mg)	0.10 ± 0.01*	0.04 ± 0.01
V_{max}/K_m	0.002 ± 0.000*	0.005 ± 0.002

**p < 0.05 compared to C57Bl/6 mice*

Figure 1

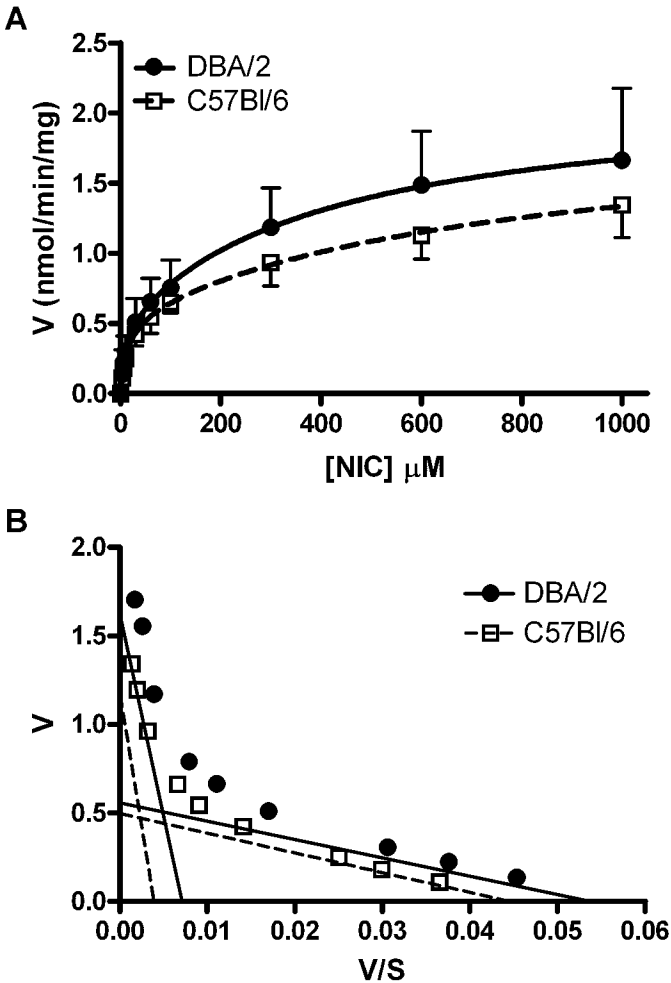

A
DBA/2
C57Bl/6
V (nmol/min/mg)
2.5
2.0
1.5
1.0
0.5
0.0
0
200
400
600
800
1000
[NIC] μM
B
DBA/2
C57Bl/6
V
2.0
1.5
1.0
0.5
0.0
0.00
0.01
0.02
0.03
0.04
0.05
0.06
V/S

Figure 2

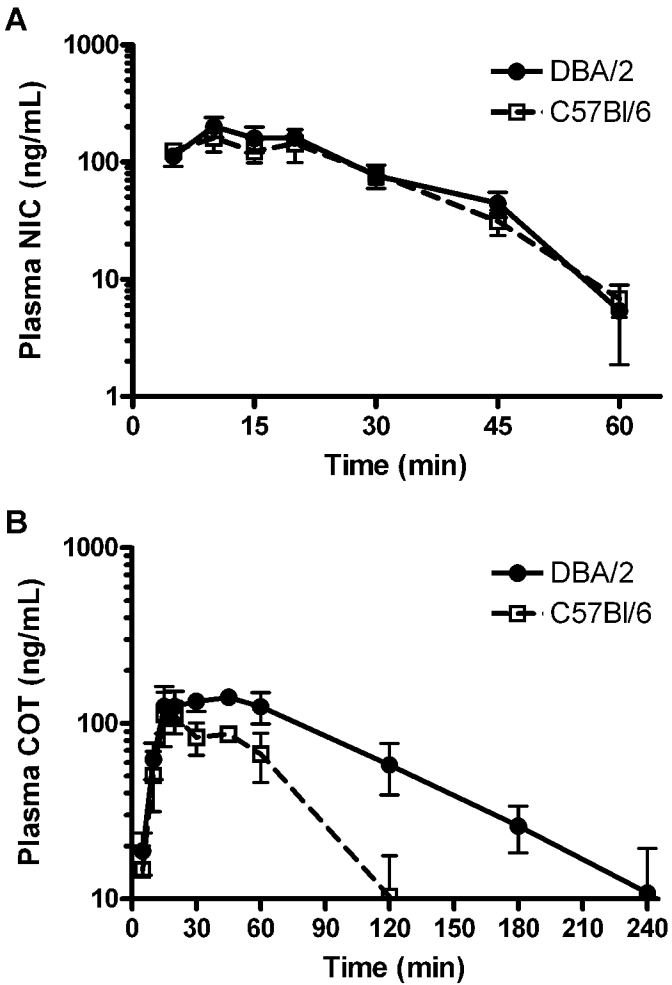

A
1000
100
10
1
Plasma NIC (ng/mL)
DBA/2
C57Bl/6
0
15
30
45
60
Time (min)
B
1000
100
10
Plasma COT (ng/mL)
DBA/2
C57Bl/6
0
30
60
90
120
150
180
210
240
Time (min)

Figure 3

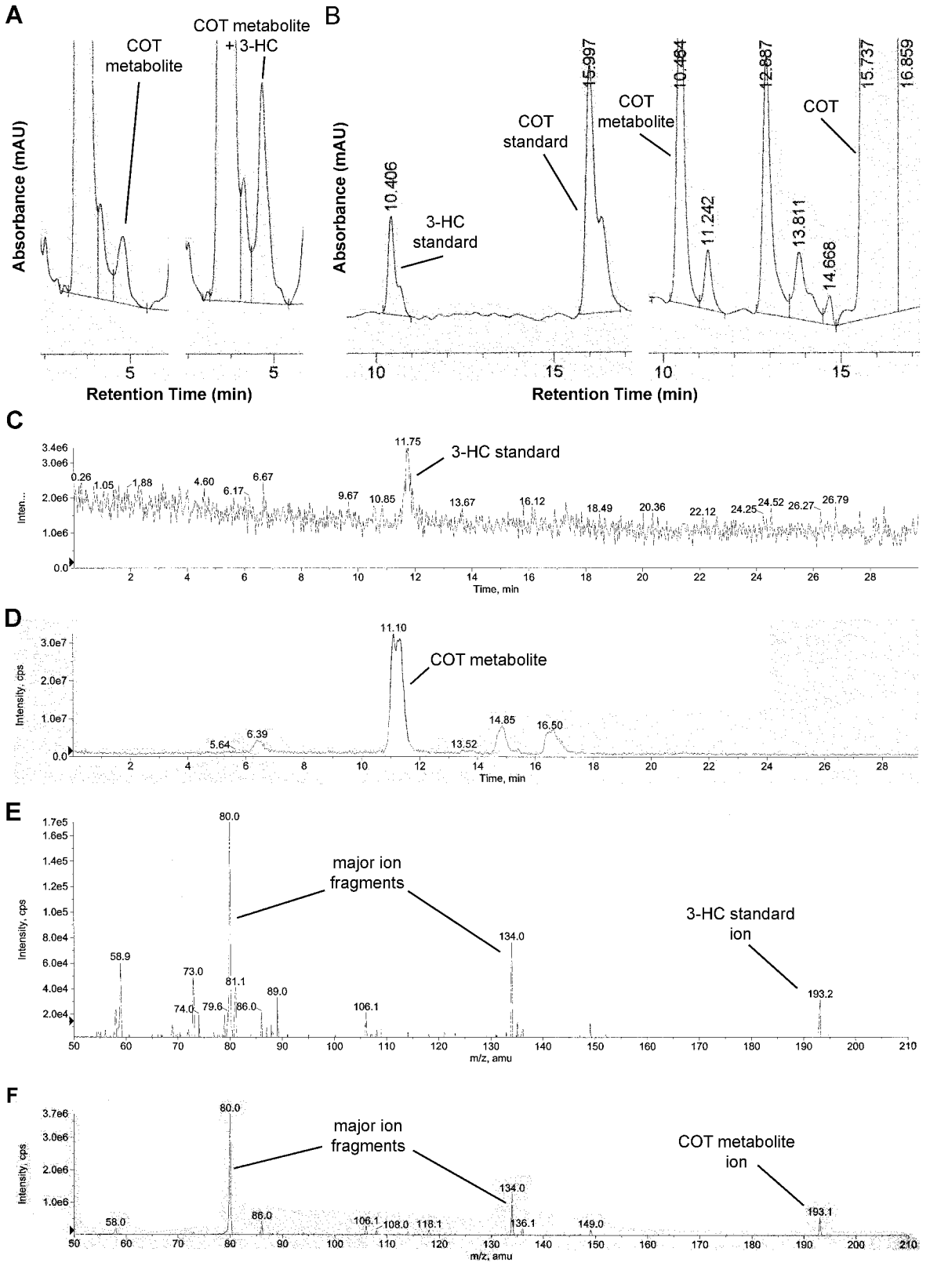

A
COT metabolite
COT metabolite + 3-HC
Absorbance (mAU)
Retention Time (min)
B
10.406
3-HC standard
COT standard
15.997
COT metabolite
10.484
11.242
12.887
13.811
14.668
COT
15.737
16.859
Absorbance (mAU)
Retention Time (min)
C
11.75
3-HC standard
Time, min
D
11.10
COT metabolite
Intensity, cps
Time, min
E
80.0
major ion fragments
134.0
3-HC standard ion
193.2
Intensity, cps
m/z, amu
F
80.0
major ion fragments
134.0
COT metabolite ion
193.1
Intensity, cps
m/z, amu

Figure 4

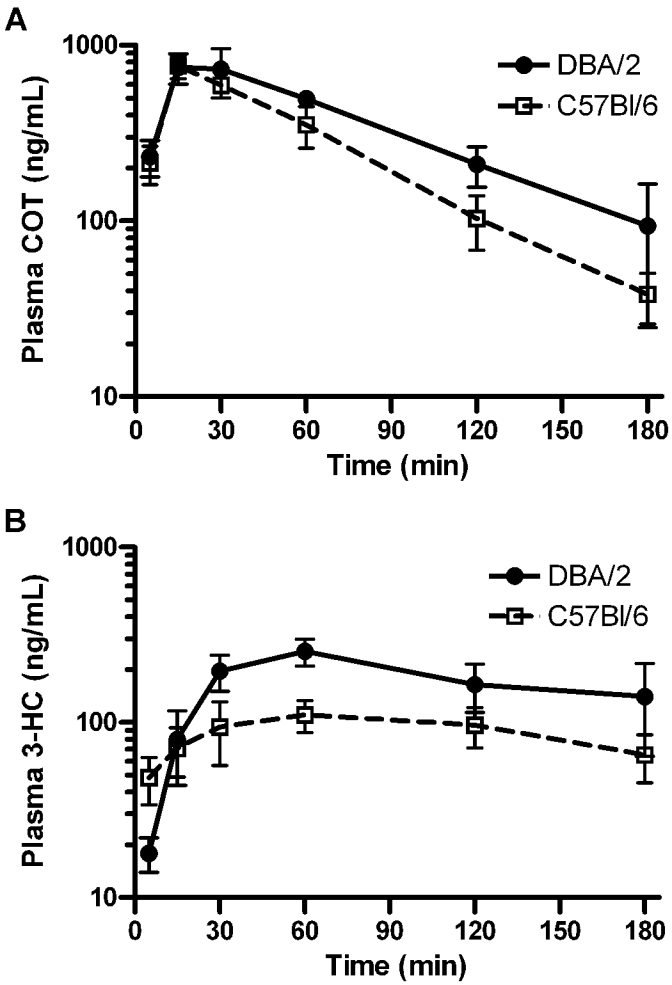

A
1000
100
10
Plasma COT (ng/mL)
DBA/2
C57Bl/6
0
30
60
90
120
150
180
Time (min)
B
1000
100
10
Plasma 3-HC (ng/mL)
DBA/2
C57Bl/6
0
30
60
90
120
150
180
Time (min)

Figure 5

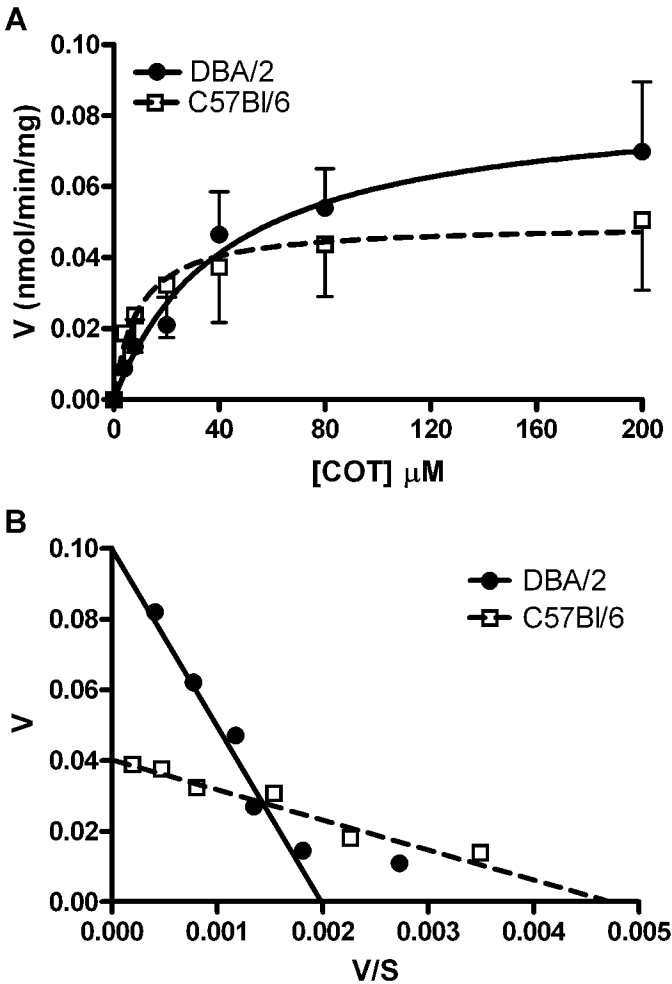
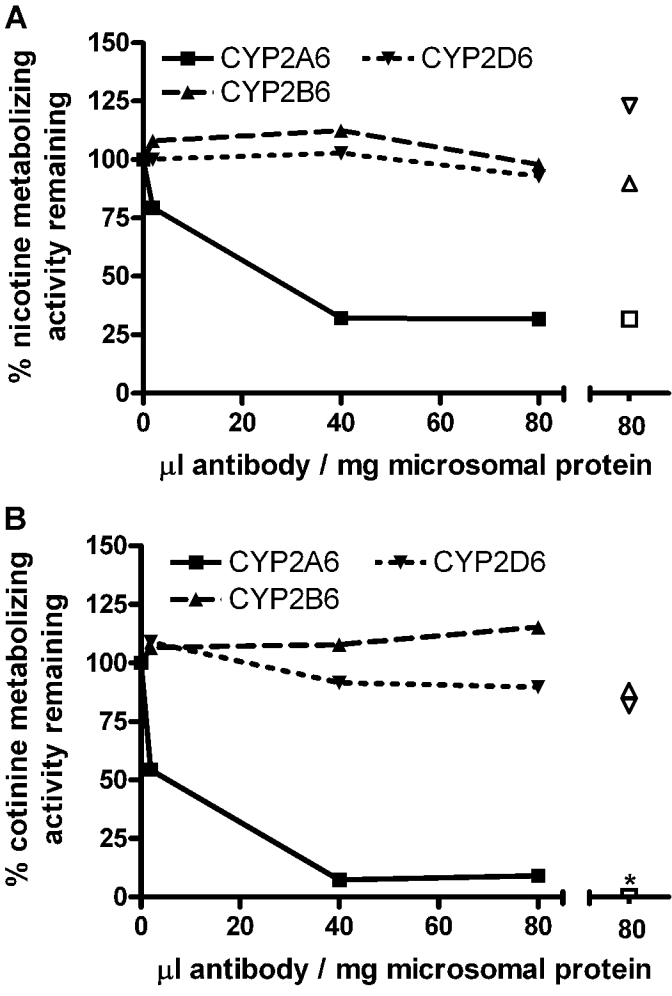

A
0.10
0.08
0.06
0.04
0.02
0.00
V (nmol/min/mg)
DBA/2
C57Bl/6
0
40
80
120
160
200
[COT] μM
B
0.10
0.08
0.06
0.04
0.02
0.00
V
DBA/2
C57Bl/6
0.000
0.001
0.002
0.003
0.004
0.005
V/S

Figure 6


A
% nicotine metabolizing activity remaining
CYP2A6
CYP2D6
CYP2B6
150
125
100
75
50
25
0
0
20
40
60
80
80
μl antibody / mg microsomal protein
B
% cotinine metabolizing activity remaining
CYP2A6
CYP2D6
CYP2B6
150
125
100
75
50
25
0
*
0
20
40
60
80
80
μl antibody / mg microsomal protein