AN INVESTIGATION OF NICOTINE METABOLISM IN MICE:
THE IMPACT OF PHARMACOLOGICAL INHIBITION AND GENETIC INFLUENCES
ON NICOTINE PHARMACOLOGY

By

Eric Chun Kit Siu

A thesis submitted in conformity with the requirements for the degree of
Doctor of Philosophy
Graduate Department of Pharmacology
University of Toronto

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ABSTRACT

INTRODUCTION: Smoking is one of the single greatest causes of numerous preventable diseases. We were interested in developing an animal model of nicotine metabolism that can be used to examine the effects of potential CYP2A6 inhibitors on nicotine metabolism and nicotine-mediated behaviours. Pharmacogenetic studies have demonstrated that in humans, smoking behaviour is associated with rates of nicotine metabolism by the CYP2A6 enzyme. Mouse CYP2A5 shares structural and functional similarities to human CYP2A6 and has been implicated in nicotine self-administration behaviours in mice, therefore the mouse represents a potential animal model for studying nicotine metabolism. METHODS: We characterized nicotine and cotinine metabolism in two commonly used mouse strains (DBA/2 and C57Bl/6). We also examined the association between nicotine self-administration behaviours and nicotine metabolism, and the impact of direct manipulation (i.e. inhibition) of nicotine metabolism on nicotine pharmacodynamics (hot-plate and tail-flick tests) in mice. Finally, we studied the effect of selegiline (a known cytochrome P450 mechanism-based inhibitor) on nicotine metabolism in mice and in human CYP2A6. RESULTS: Nicotine metabolism in mice in vitro was mediated by CYP2A5, and this enzyme was responsible for over 70% and 90% of the metabolism of nicotine to cotinine and cotinine to 3-hydroxycotinine as shown by immuno-inhibition studies, respectively. A polymorphism in CYP2A5 between mouse strains, known to alter the probe substrate coumarin’s metabolism, did not affect
nicotine metabolism but dramatically altered cotinine metabolism. Nicotine self-administration behaviour in mice was associated with level of hepatic CYP2A5 proteins and rates of nicotine metabolism in male mice. In inhibition studies, the CYP2A5/6 inhibitor methoxsalen inhibited both \textit{in vitro} and \textit{in vivo} nicotine metabolism in mice and substantially increased the anti-nociceptive effect of nicotine. Finally, selegiline was found to be an inhibitor of CYP2A5 decreasing nicotine metabolism \textit{in vitro} and \textit{in vivo} in mice. Moreover, we showed that selegiline is a mechanism-based inhibitor of CYP2A6 inhibiting nicotine metabolism irreversibly. CONCLUSION: The above data suggested that the mouse model may be suitable for examining the impact of inhibition (and genetic variation) on nicotine metabolism and nicotine-mediated behaviours and may potentially be used to screen for novel inhibitors of nicotine metabolism.
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SUMMARY OF ABBREVIATIONS

%MPE  maximum possible effect
β-PEA  phenylethylamine
3-HC  3'-hydroxycotinine
8-MOP  8-methoxypsoralen
Ala  alanine
AOX  aldehyde oxidase
AUC  area under the concentration
CI  confidence interval
CL  clearance
C_{\text{max}}  maximum concentration
COT  cotinine
COU  coumarin
CYP  cytochrome P450
DES  desmethylselegiline
ED_{50}  effective dose 50%
F  bioavailability
FAD  flavin adenine dinucleotide
FMO  flavin-containing monooxidase
HLM  human liver microsome
HNC  high nicotine consumption
HNF-4α  hepatic nuclear factor-4 alpha
IC_{50}  inhibition concentration 50%
L-AMP  L-amphetamine
LC  liquid chromatography
L-MAMP  L-methamphetamine
LNC  low nicotine consumption
MAOI  monoamine oxidase inhibitor
MBI  mechanism-based inhibitor
MLM  mouse liver microsome
NADPH  nicotinamide adenine dinucleotide phosphate
NCO  nicotine C-oxidation
NIC  nicotine
NNAL  4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol
NNK  4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone
NRT  nicotine replacement therapy
NSA  nicotine self-administration
OR  odds ratio
QTL  quantitative trait loci
SEL  selegiline
SNP  single nucleotide polymorphism
SSRI  selective serotonin reuptake inhibitor
T_{1/2}  half-life
T_{\text{max}}  time at maximum concentration
UGT  uridine diphosphate-glucuronosyl-transferase
UTR  untranslated region
Val  valine
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STATEMENT OF RESEARCH PROBLEM

According to the World Health Organization, over one billion men and 250 million women smoke daily (WHO, 2007). Smoking is the leading preventable cause of morbidity and mortality worldwide and accounts for almost five million deaths annually (Ezzati and Lopez, 2004). The primary causes of mortality from smoking are cardiovascular disease, lung cancer, and chronic obstructive pulmonary disease (Ezzati and Lopez, 2004). Currently there are several approved therapies available for smoking cessation that include nicotine replacement products (patch, gum, nasal spray, lozenges), bupropion, and the recently marketed varenicline (Cahill et al., 2007; Siu and Tyndale, 2007b; Stead et al., 2008). We have been interested in understanding the effects of compounds that inhibit nicotine metabolism, and ultimately their therapeutic roles in smoking cessation. The human cytochrome P450 2A6 (CYP2A6) is responsible for the primary metabolism of nicotine and it is genetically and functionally variable (Mwenifumbo and Tyndale, 2007; Nakajima et al., 1996b). Pharmacogenetic studies have demonstrated that among smokers, slow metabolizers of nicotine (reduced CYP2A6 function) smoke fewer cigarettes and have higher rates of quitting compared to normal nicotine metabolizers (Malaiyandi et al., 2006; Patterson et al., 2008; Schoedel et al., 2004). More importantly, preliminary studies have shown that inhibitors of CYP2A6 are effective in decreasing nicotine metabolism as well as reducing smoking behaviours (Sellers et al., 2002; Sellers et al., 2000; Tyndale et al., 1999). Considering the potential of CYP2A6 inhibitors in smoking cessation, development and characterization of an animal model of nicotine pharmacokinetics and pharmacodynamics will be important in studying these novel inhibitors and their specificities, efficacies, toxicity profiles, and effects on nicotine pharmacokinetics and pharmacodynamics. Furthermore, an animal model of nicotine metabolism may be useful to examine the impact of genetic variation (e.g. the effects of
increased or decreased nicotine metabolism) on nicotine pharmacology and or nicotine-induced behaviours.

In this thesis we investigated the mouse as an experimental model on the basis that the mouse CYP2A5 is highly identical in amino acid sequence to the human CYP2A6 and a preliminary quantitative trait loci analysis has suggested the possible involvement of CYP2A5 in oral nicotine self-administration behaviours in mice (Wildenauer et al., 2005). Furthermore, the rat CYP2A enzymes do not appreciately metabolize nicotine (Nakayama et al., 1993), therefore, the mouse may represent a useful small animal model of nicotine metabolism and pharmacodynamics.
CHAPTER 1  INTRODUCTION

Section 1  Nicotine Metabolism

Section 1.1  Nicotine Metabolism in Mice

Early in vitro mouse studies focused on the metabolism of nicotine and the distribution of metabolites in tissue preparations using thin-layer chromatography but did not provide the identification of various nicotine metabolites other than cotinine (Hansson et al., 1964; Stalhandske, 1970; Stalhandske et al., 1969). Later, it was found using mouse (DBA/Jax) hepatocytes, nicotine is metabolized primarily to cotinine, as well as nornicotine and nicotine N-oxide (cotinine – 26.4%, nornicotine – 10.3%, and nicotine N-oxide 8.9% of measurable metabolites formed) (Kyerematen et al., 1990), suggesting the involvement of cytochromes P450 (C-oxidation) and flavin monooxygenases (N-oxidation). One study found that neither nicotine nor cotinine were N-glucuronidated in vitro (Ghosheh and Hawes, 2002), suggesting a potential lack of involvement of UGTs in nicotine metabolism in mice.

Section 1.2  Nicotine Pharmacokinetics in Mice

The majority of in vivo nicotine disposition kinetic studies in mice were limited in details (Hatchell and Collins, 1980; Mansner and Mattila, 1977). Nicotine and its metabolites are mainly found in the liver and kidney, but they are also distributed in other tissues such as bronchi, nasal mucosa, salivary gland, stomach, spleen, pancreas, intestine, bone, gallbladder, and adrenal medulla (Waddell and Marlowe, 1976). A more comprehensive study examining nicotine pharmacokinetics in three strains of mice (C3H/1bg [C3H], DBA/21bg [DBA/2], C57BL/61bg [C57BL/6]) showed that following a single intraperitoneal injection, plasma nicotine levels peaked at around 5 minutes followed by rapid (~6-7 min)
and monophasic elimination (Petersen et al., 1984). This elimination half-life is considerably faster than rats (~0.92-1.1 hr) (Lesage et al., 2007; Miller et al., 1977) and humans (~1.6-2.8 hr) (Benowitz and Jacob, 1993; Benowitz et al., 2006b). In mice, nicotine is present in the brain and liver, and its elimination from these organs is similar (~6-9 min) compared to plasma (Petersen et al., 1984). Cotinine is the major nicotine metabolite in plasma. It is formed rapidly but has a significantly slower elimination half-life compared to nicotine. Its rate of elimination differs between strains (e.g. 39.8±2.0 min for DBA/2, 20.1±2.3 min for C57Bl/6 and 24.8±2.6 min for C3H) (Petersen et al., 1984). Similar to nicotine, the rate of cotinine elimination in the brain and liver approximate that of the plasma. Nicotine N-oxide is a minor metabolite compared to cotinine; however, its plasma elimination half-life is longer than nicotine but shorter than cotinine (Petersen et al., 1984).

Section 1.3  Mouse Nicotine Metabolizing Enzymes

Section 1.3.1 Mouse Cytochrome P450 2A5 (CYP2A5; P450Coh; Coumarin Hydroxylase)

CYP2A5 was originally identified as coumarin hydroxylase due to its exclusive capability to hydrolyze coumarin to 7-hydroxycoumarin in mice (Lang et al., 1989). Recently it was reported that the initial step in nicotine to cotinine conversion in mice is the 5'-oxidation of the pyrrolidine ring of nicotine to the $\Delta^{\text{5'}}(\text{1}')$-iminium ion which is mediated by CYP2A5; this is followed by its conversion to cotinine by cytosolic aldehyde oxidase (Gorrod and Hibberd, 1982; Murphy et al., 2005b). The affinity for nicotine, as measured by the total formation of $\Delta^{\text{5'}}(\text{1}')$-iminium ion and cotinine, by cDNA-expressed CYP2A5 is ~8 µM (Murphy et al., 2005b). In humans, cotinine 3'-hydroxylation is thought to be exclusively
mediated by CYP2A6 (Nakajima et al., 1996a; Yamanaka et al., 2004); however, the enzyme(s) responsible for this pathway in mice is unknown.

The mouse Cyp2a5 transcript is found in several tissues including liver, kidney, lung, olfactory mucosa, brain, small intestines, but not in the heart or spleen (Su and Ding, 2004). The tissue-specificity of the protein is not well characterized but coumarin hydroxylase activity was found to be the highest in the liver followed by kidney and lung (Kaipainen and Lang, 1985). In terms of sex differences, females have higher coumarin 7-hydroxyase activities (specific to CYP2A5) in some strains examined (e.g. DBA/2, 129, CBA/Ca) but not in others (e.g. C3H/He, BALB/c, A/J) (van Iersel et al., 1994).

The 494-amino acid protein encoded by the Cyp2a5 gene is located on chromosome 7 (6.5 cM; 9 exons) (Mouse Genome Informatics 4.01) and shares 86% amino acid sequence identity with human CYP2A6. Mouse coumarin hydroxylase activity is genetically variable between mouse strains (Wood and Taylor, 1979). Thus far there are 38 identified single nucleotide polymorphisms (SNPs) that exist in Cyp2a5. Two of the identified SNPs are in the coding region and result in amino acid changes (non-synonymous): Valine117Alanine (V117A) and Threonine486Asparagine (T486N). Twenty-nine of the SNPs are located in the introns; one is 1853-bp downstream of the coding sequence, three are in the 3'-UTR of the mRNA, two are located in coding sequence but are synonymous changes and one is located in the 5'-flanking region. The full updated list of SNPs is found at Mouse Genome Informatics (MGI 4.01).

Polymorphisms in Cyp2a5 can have functional consequences; for example, the DBA/2J strain expresses the high coumarin hydroxylase activity variant (valine at position 117) whereas the C57BL/6J strain expresses the low activity variant (alanine at position 117). The reported $K_m$ for coumarin 7-hydroxylase activities of the fractional purified CYP2A5 from the high (DBA/2) and low (C57Bl/6) activity variants are 0.35 µM and 4.0 µM, respectively (Kaipainen et al., 1984).
Section 1.3.2  Mouse Flavin-containing Monoxidase 1 (FMO1)

In general, FMOs are endoplasmic reticulum enzymes responsible for the oxidation of nucleophilic oxygen, nitrogen, sulphur, phosphorus, or selenium atoms from a wide range of substrates such as amines, amides, thiols, and sulfides (Eswaramoorthy et al., 2006). In mice, the FMO1 enzyme appears to be responsible for the conversion of nicotine to nicotine N-oxide (Itoh et al., 1997), although the contributions of polymorphisms and other FMO isoforms to this process have not been determined. In vitro, the metabolism of nicotine in mouse hepatocytes (DBA/Jax) showed that nicotine N-oxide constitutes ~26% of all metabolites formed from nicotine (Kyerematen et al., 1990). It is unclear if nicotine N-oxide mediates any pharmacological actions in mice.

In mice (129/Sv), the Fmo1 mRNA is found in similar levels in the liver, lung, and kidney, while the brain has over 10-fold lower levels of the transcript (Janmohamed et al., 2004). The relative levels of this protein in various tissues have not been examined. In terms of sex, the mRNA is found at 10-20% higher in male liver, lung, and brain compared to females. In contrast, the Fmo1 mRNA is two times higher in the kidneys of female compared to male mice (Janmohamed et al., 2004).

The mouse Fmo1 gene is located on chromosome 1 (92.6 cM) and encodes a 532-amino acid protein (Mouse Genome Informatics 4.01). Bioinformatic searches indicated there are 152 polymorphisms in this gene: eight upstream, nine downstream, two synonymous, 25 in the 3'-UTR of mRNA, and the remaining SNPs are found in the intron (MGI 4.01). Between DBA/2J and C57BL/6J mice, there are 50 SNPs: four upstream, two non-synonymous, nine 3'-UTR, four downstream, and the remaining SNPs are found in introns (MGI 4.01). Despite the large number of polymorphisms present in Fmo1, there is little difference in the mRNA transcript levels and catalytic
activities between mouse strains (Falls et al., 1995; Siddens et al., 2007), suggesting a small impact of these SNPs on enzyme function.

Section 1.3.3 Mouse Aldehyde Oxidase 1 (AOX1)

Aldehyde oxidase is a member of the molybdo-flavoenzymes that require both flavin adenine dinucleotide and molybdenum cofactor for its catalytic activity (Garattini et al., 2003). The substrate specificity of mouse AOX1 is not well studied (Vila et al., 2004). Aldehyde oxidase is responsible for the conversion of the nicotine-$\Delta^5(1')$-iminium ion to cotinine (Brandange and Lindblom, 1979). In mouse hepatic microsomes (plus cytosol) nicotine-$\Delta^5(1')$-iminium ion is metabolized to cotinine, although the enzyme responsible for this reaction was not identified (Gorrod and Hibberd, 1982). However, it is likely that mouse AOX1 is involved in this process due to its amino acid sequence similarity with the human counterpart (83% identity, NCBI Blast). It is thought that, at least in humans, the conversion of the nicotine-$\Delta^5(1')$-iminium ion to cotinine is not rate limiting in this two-step process of cotinine formation since the $K_m$ of aldehyde oxidase for the iminium ion ($K_m = 0.85 \mu M$, $V_{max} = 5300 \text{ pmol/min/mg}$; step 2) is much lower than the $K_m$ of nicotine C-oxidation (step 1) (Fig. 1) (Murphy et al., 2005b; Obach, 2004). In addition, in our in vitro studies we used sufficient amount of cytosol to ensure that aldehyde oxidase is not rate limiting (Fig. 10d).

In mice, the expression of AOX1 is tissue- and sex-dependent. In CD-1 mice, Aox1 mRNA is found in the liver, heart, lung, and esophagus; in contrast, the protein is found in liver, lung, brain, and spinal cord, suggesting transcriptional or post-translational control of enzyme expression (Kurosaki et al., 1999). With regards to sex, the AOX1 protein levels are higher in the liver but lower in the lung of males compared to females (Kurosaki et al., 1999). Such sex difference may be determined by levels of sex
hormones as hepatic AOX1 protein increases in both sexes upon treatment with testosterone (Vila et al., 2004).

The mouse Aox1 gene is located on chromosome 1 (23.2 cM) and encodes a 300-kDa homo-dimer (Kurosaki et al., 1999). Based on mouse genome informatic searches, there are 81 polymorphisms in the mouse Aox1 gene: 14 insertion-deletion and/or SNPs in the upstream locus; three synonymous; one non-synonymous; 55 intron polymorphisms; and eight in the 3’-UTR region (MGI 4.01). Between DBA/2J and C57BL/6J mice, Aox1 has 56 SNPs: two intronic and one upstream deletion with the remaining found in the introns. The functional consequences of these SNPs have not been determined; however, DBA/2 mice have significantly less hepatic AOX1 protein compared to C57Bl/6 (Vila et al., 2004), suggesting possible influence of these polymorphisms on protein levels.

Section 1.4 Nicotine Metabolism in Humans

The in vivo metabolism and disposition of nicotine and its metabolites in humans have been examined in detail. A summary of the nicotine metabolic pathway and major nicotine metabolites are shown in figure 1A (a comparison of known enzymes responsible for nicotine metabolism between human and mice is shown in figure 1B). In addition to these metabolites, there are other minor metabolites that are present at extremely low levels (Hecht et al., 1999).

Section 1.5 Nicotine Pharmacokinetics in Humans

Nicotine is distributed widely but is found mainly in blood, brain, lung, liver, kidney, spleen, muscle, but are very low in adipose tissues (Urakawa et al., 1994). During
Figure 1a. Metabolism and in vivo disposition of nicotine and metabolites in humans. Approximately 80% of nicotine is metabolized (5’ C-oxidation) to the nicotine \( \Delta^{5(1)} \)-iminium ion by CYP2A6, this is followed by the conversion of the iminium ion to cotinine by aldehyde oxidase. A significant portion (~60%) of cotinine is further metabolized to trans-3’-hydroxycotinine by CYP2A6. Numbers in the brackets represent the approximate % recovery of each compound in urine from a systemic dose of nicotine. Structures of nicotine, nicotine-\( \Delta^{1(5)} \)-iminium ion, cotinine, and trans-3’-hydroxycotinine are shown in insets. References (Benowitz et al 1994, Benowitz and Jacob 2001, Chen et al. 2007, Hukkanen et al 2005, Kaivorsaari et al 2007, Kyerematen et al 1990, Murphy et al 1999, Yamanaka et al 2005a, Yamanaka et al 2005b).
smoking, nicotine is rapidly absorbed via the lung and reaches the brain within 20 seconds (Benowitz, 1990) and its plasma levels peak within five minutes (Armitage et al., 1975). However, unlike mice, the elimination of nicotine follows a two compartmental model with an initial rapid distribution (half-life 8.1±6.4 min) followed by elimination (half-life 140±16 min) (Benowitz and Jacob, 1993; Benowitz et al., 1991b). *In vivo*, ~80% of nicotine is metabolized to cotinine and 98% of a given nicotine dose can be recovered in the urine as parent and metabolites within 24 hours (Benowitz et al., 1994).

Cotinine is the major metabolite of nicotine and it represents ~26% (free and conjugated) of an intravenously infused dose of nicotine (Benowitz and Jacob, 1994). Cotinine has a half-life of ~17.5 hr, although there is considerable variation between subjects (range 8.1 to 29.3 hr) (Benowitz et al., 1983). The primary metabolite of cotinine is trans 3'-hydroxycotinine (3-HC), and it represents ~40% (free and conjugated) of an intravenously infused dose of nicotine (Benowitz and Jacob, 2001). The plasma half-life of a single dose of 3-HC is ~6.6 hr (range 4.6 to 8.3 hr) (Benowitz and Jacob, 2001), but the elimination of 3-HC is generation-limited (Dempsey et al., 2004). The primary metabolite of 3-HC is *trans* 3'-hydroxycotinine glucuronide (Neurath and Pein, 1987).

Section 1.6  Human Nicotine Metabolizing Enzymes

Section 1.6.1  Human Cytochrome P450 2A6 (CYP2A6; Coumarin 7-hydroxylase)

Similar to mouse CYP2A5, human CYP2A6 was originally named coumarin 7-hydroxylase due to its exclusive ability to catalyze the formation of 7'-hydroxycoumarin from coumarin (Pelkonen et al., 2000; Yamano et al., 1990). CYP2A6 is the primary enzyme responsible for the C-oxidation of nicotine (Messina et al., 1997; Nakajima et al., 1996b). It also contributes to the metabolism of a variety of compounds such as the
anesthetic halothane (Spracklin et al., 1996), the platelet activator factor inhibitor SM-12502 (Nunoya et al., 1996), the anti-convulsants losigamone (Torchin et al., 1996) and valproic acid (Sadeque et al., 1997), the anti-neoplastic agent tegafur (Ikeda et al., 2000), and it also activates the tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butane (NNK), which is further described in section 2.3.5 (Tiano et al., 1993). CYP2A6 also metabolizes several endogenous substrates such as trans-retinoic acid, testosterone, estradiol, and progesterone although its contribution to their metabolism is minor (Di et al., 2009).

In humans, the two-step inactivation pathway of nicotine to cotinine is the same as mice: nicotine is C-oxidized by CYP2A6 to form the $\Delta^{5(1)}$-iminium ion, which is subsequently converted to cotinine by aldehyde oxidase (Gorrod and Hibberd, 1982). In the cDNA-expressed system, CYP2A6 has an apparent $K_m$ of 11 to 58 $\mu$M for nicotine (Ho et al., 2008; Messina et al., 1997; Nakajima et al., 1996b; Yamazaki et al., 1999). In human liver microsomes, the $K_m$ of cotinine formation also varies greatly (range 13-131 $\mu$M) (Messina et al., 1997; Nakajima et al., 1996b). Similarly, in microsomes the $V_{max}$ of cotinine formation from nicotine ranges from 4.2 to 120 nmol/mg protein/min (Messina et al., 1997). These variations may be due to the genetic (e.g. CYP2A6 or transcription factor variants) and/or environmental (e.g. dietary) influences. Chemical inhibitors and antibodies were used to determine that the contribution of hepatic microsomal CYP2A6 to the metabolism of nicotine to cotinine to be $\sim$90% (Messina et al., 1997; Nakajima et al., 1996b). Consistent with this, individuals genetically lacking CYP2A6 produce little cotinine (Kitagawa et al., 1999; Mwenifumbo and Tyndale, 2007; Yamanaka et al., 2004).

In addition to nicotine, CYP2A6 also metabolizes cotinine to 3-HC (Nakajima et al., 1996a). In vitro, hepatic microsomes produce 3-HC from cotinine with a $K_m$ of $\sim$234.5 $\mu$M (range 201.9 to 288 $\mu$M). This activity is highly correlated with coumarin 7-
hydroxylase activity and is inhibited by both coumarin and a CYP2A6 inhibitory antibody (Nakajima et al., 1996a). In vivo, individuals lacking CYP2A6 do not produce 3-HC (Dempsey et al., 2004; Yamanaka et al., 2004). Finally, CYP2A6 can also metabolize nicotine to nornicotine, and cotinine to 5'-hydroxycotinine and norcotinine (Fig. 1) (Murphy et al., 1999; Yamanaka et al., 2005a).

CYP2A6 mRNA is found predominantly in the liver, but is also found in lung, trachea, nasal mucosa, and skin (Ding and Kaminsky, 2003; Janmohamed et al., 2001). The transcript is not found in the kidney, duodenum, peripheral lymphocytes, or uterine endometrium (Koskela et al., 1999). CYP2A6 protein is expressed primarily in the liver (Ding and Kaminsky, 2003; Yamano et al., 1990) and is also found in the lungs (Zhang et al., 2007). Its protein levels in other tissues have not been described. CYP2A6 protein is found at higher levels in females than males (Al Koudsi et al., 2006b). This finding is consistent with pharmacokinetic studies showing females have significantly faster nicotine to cotinine metabolism (~20%) (Benowitz et al., 2006a).

CYP2A6 is 494 amino acids in length and is found on chromosome 19 (19q13.2; 9 exons) (Ensembl Genome Browser). CYP2A6 is highly polymorphic – currently there are 34 named alleles (*1 to *34). Variant alleles contain non-synonymous SNP including deletion, duplication, frameshift, and gene conversion, and promoter region alleles. The full updated list of CYP2A6 polymorphisms can be found at http://www.cypalleles.ki.se/cyp2a6.htm. The functional aspects of the alleles vary ranging from a significant drop in nicotine metabolic activity (*2, *4, *7, *10, *17, *19, *20, *23, *26, and *27) (Benowitz et al., 2001; Fukami et al., 2005a; Fukami et al., 2005b; Fukami et al., 2004; Ho et al., 2008; Mwenifumbo et al., 2008a; Xu et al., 2002), a moderate drop in activity (*9 and *12) (Benowitz et al., 2006b; Oscarson et al., 2002; Yoshida et al., 2003), no change in activity (*8, *14, *16, *18, *21, *24, *25, and *28) (Al Koudsi et al., 2006a; Fukami et al., 2005b; Ho et al., 2008; Mwenifumbo et al., 2008a;
Nakajima et al., 2006a; Xu et al., 2002), to increased activity (*1B, *1X2A, and *1X2B) (Fukami et al., 2007; Mwenifumbo et al., 2008b; Rao et al., 2000; Wang et al., 2006a). The functional impact of several other SNPs (*5, *6, *11, *13, *15, and *22) are predicted to have reduced activities (Daigo et al., 2002; Haberl et al., 2005; Kitagawa et al., 2001; Kiyotani et al., 2002; Oscarson et al., 1999) while others (*29, *30, *32, *33, and *34) remain to be published. The impact of reduced nicotine metabolism on smoking behaviour is briefly discussed in section 2.3.4.

Section 1.6.2 Human Cytochrome P450 2A13 (CYP2A13)

CYP2A13 is 93.5% identical in amino acid sequence with CYP2A6 (NCBI Pubmed). Despite this homology, CYP2A13 has only approximately one-tenth the coumarin 7-hydroxylase activity ($V_{\text{max}}/K_m$) of CYP2A6 (He et al., 2004b). CYP2A13 also metabolizes tobacco alkaloids such as nicotine, cotinine (Bao et al., 2005), and NNK (Su et al., 2000).

CYP2A13 exhibits a similar affinity ($K_m$) for cotinine formation from nicotine as CYP2A6 (20 vs. 26 $\mu$M, respectively) in a heterologous expression system (Bao et al., 2005). However, the enzyme is much more efficient in metabolizing cotinine to 3HC compared to CYP2A6 (45 vs. 265 $\mu$M, respectively) (Bao et al., 2005). However, due to the absence of CYP2A13 protein in the liver (Zhu et al., 2006), CYP2A6 remains the main hepatic nicotine and cotinine metabolizing enzyme.

CYP2A13 mRNA is primarily found in the respiratory tract (Su et al., 2000). The transcript is also found in liver, brain, mammary gland, prostate, testis, and uterus but not in kidney, heart, small intestine, spleen, and stomach (Koskela et al., 1999; Su et al., 2000). Using a CYP2A13-specific antibody the protein was found to be expressed in the
lung but not the liver (Zhu et al., 2006). Thus far, sex differences in CYP2A13 expression have not been examined.

CYP2A13 is a CYP2A6 homologue located in the same gene cluster on chromosome 19 (19q13.2) with the same number (nine) of exons encoding the same number (464) of amino acids. As with CYP2A6, CYP2A13 is polymorphic. Currently there are nine named alleles (*1 to *9) which include one wild-type allele, seven alleles that contain at least one non-synonymous SNPs, and one frame-shift allele. The full updated list of CYP2A13 polymorphisms can be found at http://www.cypalleles.ki.se/cyp2a13.htm. The functional consequences of the variation range from decrease in function (*2, *5, *6, *8, and *9) (Schlicht et al., 2007; Zhang et al., 2002) to loss-of-function (*4 and *7) (Cauffiez et al., 2004; Wang et al., 2006b). The contribution of CYP2A13 to systemic nicotine and cotinine levels is likely to be minor considering that the protein is not expressed in the liver (Zhu et al., 2006) and individuals who are poor metabolizers of CYP2A6 do not produce 3'-hydroxycotinine (Yamanaka et al., 2004). However, it may contribute to local metabolism of xenobiotics such as nicotine and NNK, particularly in the lung where the protein is predominantly expressed (Bao et al., 2005; Su et al., 2000). There is no identified structural/functional homologue of this enzyme in mice.

Section 1.6.3 Human Cytochrome P450 2B6 (CYP2B6)

CYP2B6 was originally named as the phenobarbital-inducible cytochrome P450IIIB; CYP2B6 metabolizes a wide range of important compounds including nicotine (Yamazaki et al., 1999), the smoking cessation drug/anti-depressant bupropion (Faucette et al., 2000), the HIV treatment drug efavirenz (Ward et al., 2003), and various
endogenous substrates such as testosterone and estrone (Imaoka et al., 1996; Shou et al., 1997).

In an expression system, CYP2B6 has a low affinity for nicotine C-oxidation ($K_m$ of 105±25 µM) (Yamazaki et al., 1999). Therefore, the contribution of CYP2B6 in the metabolism of nicotine to cotinine is expected to be negligible at low concentrations, but it may increase significantly when the nicotine concentration is high (Nakajima et al., 1996b). The in vivo involvement of CYP2B6 in cotinine formation is debatable. For instance, in a study of individuals deficient in CYP2A6 (CYP2A6*4), the metabolism of nicotine was faster in those who had a CYP2B6 functional genetic variant (CYP2B6*6) (Jinno et al., 2003), while this variant had no impact in the individuals with wild-type CYP2A6 (Ring et al., 2007). In contrast, in another study, the same CYP2B6*6 variant had no impact on in vivo nicotine plasma concentrations (obtained from nicotine patch) regardless of the activity of CYP2A6 (Lee et al., 2007). Considering that a recent study found CYP2B6*6 to be transcriptionally unstable resulting in a significant decrease in protein production (Hofmann et al., 2008), additional studies will be required to reconcile the observed differences. Nonetheless, a role for CYP2B6 may underlie the observation that individuals lacking CYP2A6 still produce cotinine (~5% of normal level) following nicotine administration, but the contribution of other enzymes has not been ruled out (Yamanaka et al., 2004).

The contribution of CYP2B6 to nicotine metabolism may also be important under situations of CYP2B6 enzyme induction. For example, in non-human primates phenobarbital treatment led to greater than 6-fold increase in CYP2B6agm protein and doubled its contribution to nicotine metabolism (Schoedel et al., 2003). CYP2B6 also takes part in the N-demethylation of nicotine to form nornicotine although its involvement is secondary to CYP2A6 (Yamanaka et al., 2005a).
CYP2B6 mRNA and protein are found at low levels in the liver and are also found in the brain, lung, and kidney, and intestine (Gervot et al., 1999; Miksys et al., 2003). Sex difference exists for CYP2B6 as females tend to have higher levels of mRNA, protein, and activity (S-mephenytoin N-demethylation) (Lamba et al., 2003; Stresser and Kupfer, 1999).

CYP2B6 is located in the same gene cluster as CYP2A6 and CYP2A13 on chromosome 19 (19q13.3; 9 exons) and the protein is 491 amino acids in length (Ensembl Genome Browser). CYP2B6 is also polymorphic and currently there are 29 named alleles for CYP2B6 (*1 to *29). There are at least 25 alleles; the effects of many of these variants on nicotine metabolism have not been studied. Mouse Cyp2b10 is the most similar to human CYP2B6 in nucleic acid sequence similarity (~75%; NCBI Blast); however, whether CYP2B10 metabolizes nicotine or its metabolites in mice has not been studied.

Section 1.6.4 Human Aldehyde Oxidase 1 (AOX1)

AOX1 is responsible for the metabolism of a large number of nitrogen-containing xenobiotics including the antiviral famcyclovir, the antineoplastics methotrexate and 6-mercaptopurine, and the ethanol metabolite acetaldehyde (Garattini et al., 2003). And as discussed in the previous section (1.3.3), AOX1 metabolizes the nicotine-\(\Delta\)\(^{5(1)}\)-iminium ion to cotinine (Brandange and Lindblom, 1979).

In humans, AOX1 mRNA is found in highest level in the liver followed by the kidney and adrenal gland, and it is found in trace levels in various peripheral tissues (Nishimura and Naito, 2006). There are no apparent sex differences in aldehyde oxidase activity (Al-Salmy, 2001).
**AOX1** is located on chromosome 2 (2q33; 35 exons) and encodes a 1338 amino-acid protein (Ensemble Genome Browser). The human **AOX1** is highly polymorphic with 125 polymorphisms identified thus far, but only one non-synonymous SNP is predicted to have any functional consequence (Steinberg et al., 2007). Despite the lack of the impact of genetic variation, there is considerable variation in the activity of hepatic AOX1 activity (up to 50-fold depending on substrate tested) (Al-Salmy, 2001; Sugihara et al., 1997). AOX1 variation is not expected to affect the formation of cotinine because up to 90% of variability in cotinine formation can be explained by CYP2A6 immunoreactivity (Messina et al., 1997; Mwenifumbo and Tyndale, 2009)

### Section 1.6.5 Human Flavin-containing Monooxygenase 3 (FMO3)

FMO3 metabolizes various compounds such as the pesticide fenthion (Furnes and Schlenk, 2004), the anti-thyroid drug methimazole (Krueger and Williams, 2005), triethylamine, as well as the conversion of nicotine to nicotine N-oxide (Cashman et al., 1992). In humans, ~4-7% of administered nicotine is excreted in the urine as nicotine N-oxide (Fig. 1) (Benowitz et al., 1994; Meger et al., 2002) and this percentage is three- to four-fold higher in individuals lacking CYP2A6 (Yamanaka et al., 2004).

Like many other drug metabolizing enzymes the **FMO3** transcript is mainly found in the liver with minor transcript levels in both kidney and the lung (Zhang and Cashman, 2006), consistent with its protein expression (Krause et al., 2003; Overby et al., 1997). There is marked inter-individual variation (over 9-fold) in the protein’s expression (Krause et al., 2003; Overby et al., 1997). On the other hand, FMO3 expression and nicotine N-oxide activity is uninfluenced by gender (Cashman et al., 1992; Krause et al., 2003).
The FMO3 gene is located on chromosome 1 (1q23; 8 coding exons and 1 non-coding exon) and encodes a 532 amino-acid protein (Ensemble Genome Browser). There are currently 40 identified FMO3 alleles with the majority being non-synonymous SNPs. In addition, there are two deletion alleles and one truncation allele. The list of alleles can be found at http://human-fmo3.biochem.ucl.ac.uk/Human_FMO3/Tables/tableall.html. Thus far there have been no studies examining the direct impact of FMO3 polymorphism on nicotine disposition kinetics; however, in rare cases (<1%) individuals with loss-of-function FMO variants there may be minor re-routing of nicotine metabolism.

Section 1.6.6 Human Uridine Diphosphate (UDP)-Glucuronosyltransferase (UGT)

UGTs are phase II enzymes responsible for the transfer of glucuronic acid from UDP-glucuronic acid to various endogenous and exogenous substrates which increases their water-solubility and subsequent excretion (Kiang et al., 2005). In humans, nicotine N-glucuronide represents approximately 3-5% of total urinary metabolite of a dose of nicotine (Benowitz et al., 1994). Individuals deficient for CYP2A6 may excrete up to ~45% of nicotine as the glucuronidated product over 24 hours due to metabolic re-routing (Yamanaka et al., 2004). Cotinine and trans 3’-hydroxycotinine are also found as glucuronidated products in the urine (12-17% and 7-9%, respectively) (Benowitz et al., 1994).

The enzymes responsible for the N-glucuronidation of nicotine was thought to be UGT1A4 with some contribution by UGT1A9 (Kuehl and Murphy, 2003; Nakajima et al., 2002). Similarly, cotinine was thought to be N-glucuronidated by UGT1A4 (Kuehl and Murphy, 2003; Nakajima et al., 2002). However, with improved detection method, it was
later identified that UGT2B10 to be the primary UGT enzyme that catalyzes the N-glucuronidation of nicotine and cotinine (Kaivosaari et al., 2007). This was confirmed by pharmacogenetic studies showing that hepatic microsomes from individuals with the loss-of-function UGT2B10 enzyme (UGT2B10*2) showed a 5-fold reduction in nicotine N-glucuronide production (Chen et al., 2007). This isoform also plays a predominant role in the N-glucuronidation of cotinine (Kaivosaari et al., 2007) and UGT2B10*2 individuals showed a 16-fold reduction in cotinine N-glucuronide production (Chen et al., 2007). Trans 3’-hydroxycotinine is mainly O-glucuronidated and the primary enzyme involved in this reaction is UGT2B7 (Yamanaka et al., 2005b).

Both UGT2B10 and UGT2B7 transcripts are found at high levels in the liver and kidney (Turgeon et al., 2001). However, the variation in the expression of these two genes between males and females has not been examined. Numerous UGT2B variants have been identified in recent years; a current list of the variants can be found at http://galien.pha.ulaval.ca. Both the UGT2B10 and UGT2B7 genes are located on chromosome 4 (4q13; both are encoded by 6 exons) and are 528 and 529 amino acids in length, respectively (Ensemble Genome Browser). Currently there are two identified UGT2B10 and four UGT2B7 alleles with the variants coding for non-synonymous SNPs. In mice neither nicotine and cotinine are glucuronidated (3-HC was not examined) (Ghosheh and Hawes, 2002), suggesting a lack of involvement of UGT enzymes in these metabolic pathways.
Nicotine replacement products are the first line treatments for nicotine dependence (Siu and Tyndale, 2007b). Since nicotine is the primary addictive component of cigarettes (Russel, 1987), NRTs promote smoking cessation by delivering nicotine to replace nicotine from cigarettes. The potential use of nicotine to alleviate withdrawal symptoms was recognized in the 1970s (Schneider et al., 1977). Currently there are several nicotine delivery devices available that include gum (2 and 4 mg), transdermal patch (7, 14, and 21 mg), vapor inhaler, nasal spray, sublingual tablet (2 mg), and lozenge (2 and 4 mg).

The general feature of NRTs is that the nicotine is absorbed through the skin or the nasal/buccal mucosal membrane in order to avoid the extensive hepatic first-pass metabolism (~80%; unclear in mice) associated with oral ingestion (Benowitz et al., 1991a; Benowitz et al., 1991b; Zins et al., 1997). However, in practice, a large portion of the nicotine delivered via the gum (Benowitz et al., 1987), tablets (Molander and Lunell, 2001), and lozenges (Choi et al., 2003) is swallowed and metabolized before reaching systemic circulation. After a single cigarette puff, nicotine reaches peak plasma concentration in 5 to 8 min (Hukkanen et al., 2005b). On the other hand, NRTs in general have slower nicotine delivery kinetics with slower peak times after administration (gum – 30 min; patch – 3 to 12 hr; inhaler – 30 min; nasal spray – 11 to 18 min; tablet and lozenges – 1 hr) (Hukkanen et al., 2005b). The slower delivery and absorption kinetics of nicotine from NRTs significantly reduces their abuse liabilities (Henningfield and Keenan, 1993; West et al., 2000) but may also reduce their long term success as smoking cessation treatments.
A systemic review of 111 studies examining the efficacies of various NRT products found that the relative risk (RR) of smoking abstinence after six months of follow up with NRTs was 1.58 (95% CI 1.50 to 1.66) (Stead et al., 2008). Between NRTs, nasal spray appeared to be the most effective [RR 2.02 (95% CI 1.49 to 3.73)] while the gum appeared to be the least effective [RR 1.43 (95% CI 1.33 to 1.53)]. The RRs for patch, inhaler, and lozenges are 1.66 (95% CI 1.53 to 1.81), 1.90 (95% CI 1.36 to 2.67), and 2.00 (95% CI 1.63 to 2.45), respectively. It should be noted that not all studies in each of the NRT forms showed definitive benefits over smoking cessation. There appeared to be no overall effect of the type of NRTs on success of treatment outcome (Stead et al., 2008). Behavioural support (e.g. advice, counseling) plus NRT pharmacotherapy provided only minor advantage compared to pharmacotherapy alone (Stead et al., 2008). There are conflicting evidence as to whether higher dose of nicotine provided better outcome. For instance, 4 mg gum has been found to be provide better cessation rates compared to 2 mg gum in some studies but not others (Garvey et al., 2000; Herrera et al., 1995; Kornitzer et al., 1987). Nicotine patch studies provided less evidence of dose-dependent efficacies (Killen et al., 1999; Tonnesen et al., 1999). The primary adverse affects of nicotine replacement therapies depend on the type of products used (e.g. skin irritation for patches), but common side effects include dizziness, nausea, and headache (Stead et al., 2008).

**Section 2.1.2 Bupropion**

Smoking and depression are strongly associated perhaps because both are influenced by dopamine levels (Paperwalla et al., 2004; Quattrocki et al., 2000). Bupropion is an atypical antidepressant and was the first non-nicotine product approved as a first-line therapy for smoking cessation. At therapeutic doses, bupropion binds to striatal
dopamine transporters (~20% occupancy). This potentially prevents dopamine reuptake (Argyelan et al., 2005; Learned-Coughlin et al., 2003) which contribute to the mechanism for reducing withdrawal symptoms. The noradrenergic system is also implicated in the pharmacological effects of bupropion, possibly via noradrenaline reuptake inhibition (Foley et al., 2006). The above mechanisms may explain, in part, bupropion’s ability to reduce cravings in abstinent smokers and alleviate certain withdrawal symptoms which may explain its usefulness in smoking cessation (Durcan et al., 2002; Jorenby et al., 1999; Shiffman et al., 2000; Tashkin et al., 2001). Finally, bupropion can also act as a nicotinic receptor antagonist via a non-competitive inhibition mechanism, suggesting that it may attenuate the rewarding effect of nicotine (Alkondon and Albuquerque, 2005; Fryer and Lukas, 1999; Slemmer et al., 2000).

A Cochrane systemic review of 31 studies found that the odds ratio of smoking abstinence after six months of follow up with bupropion alone was 1.94 (95% CI 1.72 to 2.19) (Hughes et al., 2007). As with NRT, not all studies showed significant benefit of bupropion over placebo. The intensity of behavioural support did not alter the cessation rate although it is not known if counseling plus bupropion had significant advantages over bupropion alone (Hughes et al., 2007). The analysis also found no effect of dose on cessation rate from two studies (150 mg vs 300 mg at 12 months after target quit date). However, a study directly comparing different dose of bupropion (100, 150 and 300 mg/day) found that bupropion was effective in smoking cessation in a dose-dependent manner, although statistical comparisons between dosing groups were not made (Hurt et al., 1997). Bupropion was not advantageous over NRTs for relapse prevention (Hughes et al., 2007). The major adverse reactions associated with bupropion are insomnia, nausea/vomiting, and dizziness (Boshier et al., 2003).

Section 2.1.3 Varenicline
Varenicline is a partial agonist of the $\alpha 4\beta 2$ nicotinic cholinergic receptor (Coe et al., 2005). In mouse studies, the $\beta 2$ receptor subunit is required for nicotine reward, as measured by conditioned place preference, and the $\alpha 4$ receptor subunit is important for reward and development of tolerance to nicotine (McCallum et al., 2006; Tapper et al., 2004). Varenicline is hypothesized to reduce activation of the dopaminergic system via partial blockade of nicotinic receptors stimulated by high levels of nicotine during smoking, thus lowering the rewarding effects of nicotine. In addition, as a weak agonist, varenicline may also stimulate dopamine release by the mesolimbic neurons and reduce craving as well as withdrawal during abstinence (Coe et al., 2005; Papke and Heinemann, 1994).

Four studies that examined the 52-week continuous abstinence with varenicline compared to placebo have all found varenicline to provide significantly better quit rate compared to placebo (Gonzales et al., 2006; Jorenby et al., 2006; Nides et al., 2006; Oncken et al., 2006). When analyzed together the odds of quitting were 3.22 (95% CI 2.43 to 4.27) favouring varenicline (Cahill et al., 2007). Two out of three studies that compared the efficacy of varenicline to bupropion have found varenicline to be superior (statistically significant) in maintaining 52-week continuous abstinence rate compared to bupropion (Gonzales et al., 2006; Jorenby et al., 2006; Nides et al., 2006). Furthermore, varenicline was more effective in reducing withdrawal symptoms compared to bupropion (West et al., 2008). Varenicline was significantly more effective in preventing relapse compared to placebo [198 (95% CI 159-260) days vs 87 (95% CI 58-143) days, respectively; log-rank $P<0.001$] (Tonstad et al., 2006). The main adverse events associated with varenicline are relatively mild and includes nausea, abnormal dreams, and insomnia (Oncken et al., 2005). However, recent evidence indicated that varenicline
may induce depression and suicidal ideation (Kuehn, 2008) and a public health advisory had been placed on this drug by the FDA (http://www.fda.gov/bbs/topics/NEWS/2008/NEW01788.html).

Section 2.2 Secondary and Investigative (Non-approved) Smoking Cessation Therapies

Section 2.2.1 Nortriptyline

Nortriptyline is a second-line therapy for smoking cessation but has not yet been approved for this indication by the US Federal Drug Administration. Nortriptyline is a tricyclic antidepressant that inhibits the reuptake of serotonin and noradrenalin. A meta-analysis comparing the efficacy of nortriptyline vs. placebo found the pooled odds ratio to be 2.34 (95% CI 1.61 to 3.41) (Hughes et al., 2007). The smoking cessation rate achieved with nortriptyline was comparable to those achieved with bupropion [pooled ORs: 2.1 (1.5 – 3.1) vs. 2.0 (1.7 – 3.4) for nortriptyline and bupropion, respectively] (Hughes et al., 2005). However, nortriptyline appears to cause greater adverse events compared to bupropion (Hall et al., 2002; Wagena et al., 2005) and may increase abnormal cardiac rhythms (QTc interval) and increased adverse cardiac events in ischemic heart disease patients (Roose et al., 1998; van Noord et al., 2009), thus preventing its wide use.

Section 2.2.2 Clonidine

The α2-adrenergic receptor agonist clonidine is primarily used for the treatment of hypertension; however, it has also been used for treatment of opiate and alcohol withdrawal (Gossop, 1988; Manhem et al., 1985). Clonidine can alleviate smoking
withdrawal symptoms (Glassman et al., 1984; Ornish et al., 1988). A Cochrane analysis of six studies found that clonidine treatment was associated with increased smoking cessation [pooled OR: 1.89 (1.30 – 2.74)]; however, only one study showed significant improvement in smoking cessation when the studies were analyzed separately (Gourlay et al., 2004). Finally, adverse effects such as dry mouth, sedation, and postural hypotension limit its general use.

Section 2.2.3  Serotonergic Agents

Section 2.2.3.1  Buspirone

The serotonergic system is responsible for the regulation of mood and appetite. Buspirone is serotonin receptor (5-HT₁₅) partial agonist used for the treatment of generalized anxiety disorder. The drug appears to function via the inhibition of the release of serotonin and may potentially increase noradrenergic and dopaminergic activity (Eison and Temple, 1986). Buspirone appears to benefit smokers with high level of anxiety. For instance, in an eight-week treatment with maximum dose of 60 mg of buspirone, the one-month abstinence in the high-anxiety group was 88% (treatment) compared to 61% (placebo) but in the low-anxiety group the abstinence was 60% (treatment) compared to 89% (placebo). At 12 months, there was no difference between treatment and placebo in both anxiety group (Cinciripini et al., 1995). However, a subsequent study revealed that buspione (six-week treatment; maximum dose of 60 mg) was ineffective in smoking cessation regardless of degree of anxiety (Schneider et al., 1996). In another study, buspirone (up to 40 mg for four weeks) was found to be equally as effective as nicotine patch (fixed: 21/22 mg for six weeks; tapered: 21/22 mg for four weeks, 14 mg for 4 weeks, and 7 mg for 4 weeks) (Hilleman et al., 1994). While the drug
appears to be safe, with little adverse effects, the overall usefulness of buspirone in smoking cessation is inconclusive.

Section 2.2.3.2 Selective Serotonin Reuptake Inhibitors (SSRIs)

SSRIs are used for the treatment of depression and anxiety (Nutt et al., 1999). Fluoxetine has not been demonstrated to be effective for smoking cessation but may decrease the negative and increase the positive affects after quitting (Blondal et al., 1999; Cook et al., 2004; Niaura et al., 2002; Saules et al., 2004). Paroxetine was not effective beyond four weeks (during a 9-week treatment protocol) (Killen et al., 2000) and sertraline did not increase abstinence compared to placebo (Covey et al., 2002). SSRIs do not appear to be effective smoking cessation aides.

Section 2.2.4 Naltrexone

Endogenous opioids (endorphins, enkephalins, dynorphins), together with their receptors (mu, kappa, delta) are responsible for various biological processes which include autonomic, neuroendocrine, hedonic, and emotional responses (Kieffer and Evans, 2009). There are several lines of evidence that suggested the involvement of the opioid system in nicotine addiction. First, animals exhibit similar somatic withdrawal signs from both nicotine and opiates (e.g. tremors, scratches, chewing, etc.) (Malin and Goyarzu, 2009). Second, in rats nicotine administration led to increased turnover of Tyr-Gly-Gly, a marker of enkephalin release (Houdi et al., 1991), and smoking cigarettes containing more nicotine led to greater plasma levels of β-endorphin compared to cigarettes with low nicotine (Gilbert et al., 1992). Third, mice with the preproenkephalin or the mu-opioid receptor genes knocked-out do not respond to the rewarding properties of nicotine as
demonstrated by lack of nicotine-induced place preference (Berrendero et al., 2002; Berrendero et al., 2005). Finally, in rats the opioid antagonist naloxone precipitated nicotine withdrawal as well as prevented nicotine alleviation of withdrawal (Malin et al., 1993; Malin et al., 1996) further suggesting the involvement of the opioid system in nicotine dependence.

Naltrexone is a non-specific antagonist of the opioid receptors used for the treatment of opioid-dependence (Modesto-Lowe and Van Kirk, 2002). Due to the connection between nicotine dependence and opioid system, naltrexone has been investigated as a potential therapy for smoking cessation. However, it should be noted that several studies have found that naltrexone, when administered alone, led to significant adverse effects due to precipitation of nicotine withdrawal (Covey et al., 1999; Wong et al., 1999). On the contrary, studies of naltrexone with nicotine replacement have found the drug combination to be more effective. For example, in a preliminary study naltrexone (50 mg) plus nicotine patch (21 mg) resulted in higher 2-week continuous abstinence rate (weeks 3-4 in a 4 week study) compared to placebo plus patch (56.3% vs. 31.3%) (Krishnan-Sarin et al., 2003). In a larger study examining naltrexone (0, 25, 50, and 100 mg/d) plus nicotine patch (21 mg) for smoking cessation, it was found that naltrexone only at 100 mg/d (but not 25 and 50 mg/day) plus nicotine patch led to significantly better 6-week continuous abstinence rate compared to placebo plus patch. However, subsequent exploratory analysis of 7-day point-prevalence abstinence at 3-, 6-, and 12-months did not find any difference in cessation between naltrexone vs placebo (O'Malley et al., 2006). As mentioned previously, naltrexone causes withdrawal symptoms such as nausea, vomiting, diarrhea, depression, and insomnia which may limit its utility (O'Malley et al., 2006).

Section 2.2.5  Rimonabant (SR141716)
The involvement of the endocannabinoid system with nicotine dependence was established when it was observed that chronic nicotine administration increased the level of the endogenous cannabinoid receptor agonists anandamide (AEA) and 2-arachidonoyl-glycerol (2-AG) (for CB₁ and CB₂ receptors, respectively). The increases in these agonists are localized to the brainstem, a region involved in drug withdrawal (Gonzalez et al., 2002; Shoemaker et al., 2005; Steffens et al., 2005). In rats, administration of the CB₁ receptor antagonist SR141716 (rimonabant) blocked nicotine-induced dopamine release in the nucleus accumbens (Cohen et al., 2002). Rimonabant also prevented IV nicotine self-administration, conditioned stimuli-induced nicotine-seeking behaviors and nicotine-conditioned place preference in rats (Cohen et al., 2005; Cohen et al., 2002; Le Foll and Goldberg, 2004). The precise mechanism of actions of CB₁ receptor antagonism on nicotine reward/reinforcement remains unclear.

A recent Cochrane review found that the pooled odds ratio (from STRATUS-US and STRATUS-EUROPE) of quitting smoking at one year with 20 mg rimonabant to be 1.61 (95% CI 1.12 to 2.30) with the 5 mg showing no benefit over placebo (Cahill and Ussher, 2007). When analyzed separately, only the STRATUA-US study showed a significant benefit in cessation. Rimonabant showed potential benefit in relapse prevention (STRATUS-Worldwide); both 5 mg and 20 mg treatment provided approximately 1.5-fold increase in remaining abstinent (Cahill and Ussher, 2007). The most common side effects were nausea and upper respiratory tract infection (Anthenelli, 2005). Due to the inconclusive evidence showing rimonabant to be effective in smoking cessation, the drug is currently not approved for the indication in the United States. As of 2008, the European Medicines Agency has suspended the use of rimonabant in the treatment of obesity due to increased risk of psychiatry disorders in overweight or obese patients (Allchurch, 2008).
Section 2.2.6 Nicotine Vaccines

The nicotine vaccine is another potential treatment for smoking. The mechanism behind this therapeutic strategy is to prevent nicotine from entering the brain via binding to nicotine-specific antibodies (Hieda et al., 2000; Pentel et al., 2000). An advantage of nicotine vaccines is that only occasional booster shots, rather than daily administration, should be needed for therapeutic effect. In a 38-week study it was found that during *ad libitum* smoking, nicotine vaccine, at all doses tested (50, 100, and 200 µg; 1 primary challenge shot and 3 booster shots at each dose), did not cause compensatory smoking behaviors or precipitate withdrawal (Hatsukami et al., 2005). In addition, the 30-day continuous abstinence rates were higher in patients receiving the highest vaccine dose (~38%) compared with placebo (~10%) (Hatsukami et al., 2005). Careful interpretation of these results is needed as the sample size was small and the study was primarily designed to evaluate the safety and immunogenicity of the vaccine. In a subsequent study with larger sample size (N=341) and a different vaccine, it was found that the four-month continuous abstinent rate was significantly higher in individuals with the highest antibody titer compared to placebo (56.6% [N=30 out of 53] vs. 31.3% [N=25 out of 80]; OR = 2.9; p<0.01) (Cornuz et al., 2008). The vaccine is associated with greater adverse events such as flu-like symptoms and pyrexia (Cornuz et al., 2008). Currently nicotine vaccine is still being investigated for efficacy in Phase III clinical trials (Clinicaltrial.gov).

Section 2.2.7 Monoamine Oxidase Inhibitors (MAOIs)

MAO enzymes are responsible for the metabolism of various endogenous substrates (e.g. adrenaline, noradrenaline, dopamine, 5-hydroxytryptamine, 2-phenylethylamine,
tryptamine, tyramine) and thus they can play an important role in smoking behaviours (Youdim et al., 2006). The relationship between MAO activities and smoking was first observed almost three decades ago (Oreland et al., 1981). In smokers, the brain MAO-A and MAO-B activities are ~30 and ~40% lower compared to non-smokers, respectively (Fowler et al., 1996a; Fowler et al., 1996b). MAO-B is the major isoform of the monoamine oxidase in the brain (~75-80%) (Saura Marti et al., 1990; Squires, 1972). The inhibition of MAO-B is reversed after long-term abstinence from smoking, likely due to the synthesis of new enzymes (Gilbert et al., 2003). These findings suggest MAO-B inhibitor(s) may be present in tobacco smoke (Khalil et al., 2000) that may inhibit MAO-mediated dopamine metabolism, thereby enhancing the rewarding effects of nicotine during smoking (Lewis et al., 2007). However, the specific tobacco smoke compounds that lead to long term inhibition of MAO-B have not been identified.

Through inhibition of MAO-A and MAO-B, the metabolism of dopamine can potentially be reduced and this may enhance the rewarding effects of nicotine during smoking. In a preliminary study, the reversible MAO-A inhibitor moclobemide significantly reduced self-reported smoking rates, although at one year there was no difference in abstinence compared with placebo (Berlin et al., 1995). The reversible inhibitor of MAO-B lazabemide has also been evaluated for smoking cessation, but the study was terminated due to concerns of hepatotoxicity (Berlin et al., 2002).

The irreversible MAO-B inhibitor selegiline has also demonstrated some efficacy in several small studies. In one study, oral selegiline (10 mg) decreased physical symptoms (e.g. hand shakes, increased heart rate) and craving during abstinence as well as reducing smoking satisfaction during smoking (Houtsmuller et al., 2002). In another study, 5 mg b.i.d. oral selegiline increased the trial end point (8-week) 7-day point prevalent abstinence compared with placebo [OR 4.64 (95% CI: 1.02-21.00 p<0.05)]; however at 6 months the 7-day point prevalent abstinence was not significant
even though the OR was 4.75. A limitation of the study was the small sample size (N=40) (George et al., 2003). In a third study that used a combination of oral selegiline and nicotine patch, selegiline plus nicotine patch doubled the 52-week continuous abstinence rate compared to nicotine patch alone (25% vs. 11%), although the difference was not significant possibly due to small number of subjects (N=38) (Biberman et al., 2003).

Common adverse effects associated with selegiline monotherapy (5 mg b.i.d.) are nausea and dizziness (Heinonen and Myllyla, 1998). Despite the dopamine potentiating effect of selegiline, as well as the production of L-methamphetamine and L-amphetamine metabolites (instead of the more potent D-isomers) from selegiline, there is no indication that selegiline is addictive (Schneider et al., 1994).

Section 2.2.7.1 Selegiline (L-deprenyl; Eldepryl®)

Section 2.2.7.1.1 Selegiline Pharmacodynamics

Selegiline is used clinically in conjunction with levodopa for the treatment of Parkinson’s disease (Gerlach et al., 1996). The recommended dose of selegiline for Parkinson’s disease is 10 mg (5 mg p.o. b.i.d.) (Gerlach et al., 1996). This is the same dose of selegiline that was used in several smoking cessation trials. At these clinical doses, selegiline is a selective and irreversible inhibitor of MAO-B (Gerlach et al., 1996). The drug inhibits MAO-B by covalently binding to its flavine-adenine dinucleotide (FAD) co-factor (Youdim, 1978), effectively decreasing the amount of enzyme available to metabolize dopamine. At higher doses the selectivity of selegiline decreases and it also inhibits MAO-A (Gerlach et al., 1992).

In addition to enhancing dopamine availability by inhibiting MAO-B, selegiline may have another mechanism of action that modulates dopaminergic activities that may be important to smoking cessation. Phenylethylamine (β-PEA), an endogenous
substrate of MAO-B, exists in trace concentrations in the brain and was found to be significantly higher (up to 10-fold) in selegiline-treated Parkinson’s patients compared to non-treated patients (Reynolds et al., 1978). In monkeys, selegiline increased brain $\beta$-PEA levels (Paterson et al., 1995). In rats, selegiline increased $\beta$-PEA levels in the striatum and enhanced the neuronal response to dopamine stimulation (Berry et al., 1994; Paterson et al., 1991). In addition, direct administration of $\beta$-PEA into the rat striatum caused the release of dopamine (Bailey et al., 1987; Kuroki et al., 1990). $\beta$-PEA has been proposed to be a neuromodulator of dopaminergic transmission although the mechanism by which this occurs has not been determined (Paterson et al., 1990). Therefore, the selegiline-mediated increase in $\beta$-PEA may enhance neuronal stimulation by dopamine. The selegiline metabolite desmethylselegiline can also inhibit MAO-B activity in vivo in humans although its contribution may be minor (Heinonen et al., 1997).

Section 2.2.7.1.2 Selegiline Metabolism

In humans, selegiline is extensively metabolized; less than 1% of an oral dose is excreted unchanged in the urine (Shin, 1997). In correlation analyses, CYP2B6 accounts for the largest variation in the metabolism of selegiline to its primary metabolites desmethylselegiline (77%) and L-methamphetamine (79%) (Benetton et al., 2007). Both CYP2A6 and CYP3A4 contribute to these metabolic pathways to some extent (14-15% for both desmethylselegiline and L-methamphetamine by CYP2A6 and 33-35% for both desmethylselegiline and L-methamphetamine by CYP3A4) (Benetton et al., 2007). The plasma half-life of 10 mg of oral selegiline is $1.0 \pm 0.9$ hr; however, this increased to $2.7 \pm 1.8$ hr following chronic (8 days) administration, indicating the metabolism slows with chronic selegiline treatment. In addition, the observed accumulation ratio of the drug is
higher at 2.7 compared to the estimated accumulation ratio of 1.0 based on elimination half-life (although not statistically significant), indicating possibly that selegiline, or a metabolite, down-regulates the enzyme(s) responsible for its metabolism or inhibits selegiline’s clearance (Laine et al., 2000). Age and sex were not found to contribute to differences in selegiline pharmacokinetics (Barrett et al., 1996a; Barrett et al., 1996b).

The plasma elimination half-lives of desmethylselegiline and L-methamphetamine are 31 ± 8 and 161 ± 38 hr, respectively. L-amphetamine, the major metabolite of desmethylselegiline, has a half-life of 40 ± 7 hr (Laine et al., 2000). Similar to selegiline, the three metabolites showed accumulation over eight days to some extent. L-methamphetamine and L-amphetamine are further metabolized to various p-hydroxylation and β-hydroxylation products whose pharmacokinetic profiles have not been examined in detail (Shin, 1997).

In mice, an inhibitor study showed that CYP2E1 is responsible for more than 50% of the metabolism of selegiline to all three of its metabolites (desmethylselegiline, methamphetamine, and amphetamine) with a minor contribution from mouse CYP3A enzyme(s) (Valoti et al., 2000). The contribution of CYP2A5 to the metabolism of selegiline was not examined (Valoti et al., 2000). Similar to humans, the plasma half-life of selegiline in mice is between 0.9 to 1.8 hr (Magyar et al., 2007).

The observation that CYP2A6 is involved in selegiline metabolism and chronic administration of this drug decreases its own metabolism suggests that selegiline may inhibit CYP2A6. These findings have implications for selegiline in the smoking cessation and will be further described in sections 2.3.3.2 and 5.3.2.

Section 2.3  The Inhibition of Nicotine Metabolism and its Application to Smoking Cessation
Section 2.3.1  Inhibitors of Human CYP2A6
CYP2A6 has a compact, narrow, hydrophobic active site (Yano et al., 2005); therefore, compounds that are good inhibitors of CYP2A6 are small and planar (e.g. structurally related to nicotine) (Denton et al., 2005; Rahnasto et al., 2005; Yano et al., 2006). A variety of compounds can inhibit CYP2A6 \textit{in vitro} including pharmaceuticals [e.g. methoxsalen (Maenpaa et al., 1993), pilocarpine (Kimonen et al., 1995), clotrimazole, miconazole (Draper et al., 1997), tranylcypromine, tryptamine (Zhang et al., 2001), amphetamine (Rahnasto et al., 2005)], naturally occurring substances [furanocoumarins (Maenpaa et al., 1993), various nicotine-related alkaloids (\textit{in vitro}) (Denton et al., 2004; Denton et al., 2005), soy isoflavones (\textit{in vivo}) (Nakajima et al., 2006b), grapefruit juice (\textit{in vivo}) (Hukkanen et al., 2006), star fruit juice (\textit{in vitro}) (Yang, 2007)], and endogenous substances (\textit{in vitro}) [e.g. $\beta$-PEA (Rahnasto et al., 2003), dopamine (Higashi et al., 2007b)]. Of the above, methoxsalen can also inhibit nicotine metabolism \textit{in vivo} and may be clinically significant in its application in smoking cessation (further discussed in sections 2.3.3.1 and 5.3.1).

**Section 2.3.2 Inhibitor of Mouse CYP2A5**

Some CYP2A6 inhibitors also appear to be inhibitors of CYP2A5. Examples are furanocoumarins-derivatives such methoxsalen (Maenpaa et al., 1993), pilocarpine (Kimonen et al., 1995), and amphetamine (Rahnasto et al., 2005). Other sample compounds that inhibit CYP2A5 include, various lactone (coumarin-like) compounds (Juvonen et al., 2000), as well as etomidate, metomidate and metyrapone (Kojo et al., 1989). These results suggest a similar inhibitor profile between the human CYP2A6 and mouse CYP2A5 and the mouse may be useful in the evaluation of novel CYP2A6 inhibitors.
Section 2.3.3 Mechanism-Based Inhibition

Mechanism-based (suicide) inhibitors (MBIs) are compounds that inhibit enzyme activities by covalent modification. As a result, the enzyme is no longer functional, and its activity can only be recovered via synthesis of new enzyme. There are several ways in which mechanism-based inhibition can occur (Osawa and Pohl, 1989). First is the covalent modification of the prosthetic heme in which the reactive metabolite binds to the heme and together they dissociate from the enzyme. Another is the covalent binding of a reactive metabolite to the holoenzyme. Third is the covalent binding of the heme to the protein mediated by the reactive metabolite. In addition, the reactive metabolite can also covalently bind to the enzyme co-factor [e.g. binding of selegiline reactive metabolite to the flavin adenine dinucleotide (FAD) of MAO-B (Youdim, 1978)].

There are seven experimental criteria that can be used to establish whether a compound is a MBI (Silverman, 1995). First, there is time-dependent loss of activity due to reduction in active enzyme. Second, the inhibition is saturable – the rate of inactivation of enzyme is proportional to the number of inactivator molecules added until all enzyme molecules are saturated. Third, a substrate or a competitive (reversible) inhibitor should slow down inactivation by the potential MBI (substrate protection). Fourth, the inhibition is irreversible such that dialysis or filtration (gel or membrane) does not restore enzyme activity. Fifth, there should be inactivator stoichiometry in which only one inactivator molecule is attached to each enzymatic site. Sixth, the involvement of a catalytic step – the enzyme needs to convert the inhibitor to an active species that actually carries out the inactivation step and therefore an enzymatic reaction is required. Finally, the active species formed from enzymatic activity must inactivate the enzyme prior to its release from the active site.
**Section 2.3.3.1 Methoxsalen (8-methoxypsoralen; 8-MOP): a CYP2A5 and CYP2A6 MBI**

Methoxsalen is a naturally occurring psoralen compound that is used for the treatment of psoriasis and cutaneous T-cell lymphoma in conjunction with UV exposure (Bond et al., 1981; Young, 1993). Methoxsalen is a competitive ($K_i$), non-competitive ($K_{iu}$), and a mechanism-based ($K_i$ and $k_{inact}$) inhibitor of CYP2A6 inhibiting both coumarin ($K_i =1.5 \ \mu M$ [human liver microsomes]; $K_{iu }=0.5 \ \mu M$; $K_{i} =0.3-0.8 \ \mu M$ [$k_{inact} =0.5 \ \text{min}^{-1}$]) and nicotine ($K_i =0.8 \ \mu M$) metabolism (Draper et al., 1997; Koenigs et al., 1997; Maenpaa et al., 1994; Zhang et al., 2001). In mice, methoxsalen is a competitive ($K_i$ not determined), non-competitive ($K_{iu }=1.7 \ \mu M$), and mechanism-based ($k_{inact} =0.17 \ \text{min}^{-1}$) inhibitor of microsomal CYP2A5-mediated coumarin metabolism (Visoni et al., 2008). Methoxsalen also inhibits human CYP1A2, CYP2B6, CYP2A13, and to some extent several other P450 enzymes (von Weymarn et al., 2005; Zhang et al., 2001) in addition to human CYP2A6 and mouse CYP2A5.

**Section 2.3.3.2 Selegiline as a MBI**

As mentioned in section 2.2.7.1, selegiline is an irreversible MBI of MAO-B. Selegiline belongs to the acetylene group of chemicals that contain a carbon-carbon triple bond, which are known to be potent MBIs (Correia and Ortiz de Montellano, 2005). Selegiline is a MBI of CYP2B1, the rat nicotine metabolizing enzyme, although the precise mechanism of inactivation is unclear (Sharma et al., 1996). It is unknown what other additional rodent P450 enzymes selegiline may inhibit; however, in humans selegiline
has little to no inhibitory effects on CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A in vitro (Polasek et al., 2006). Considering that CYP2A6 is involved in the metabolism of selegiline in vitro (Benetton et al., 2007) and chronic selegiline treatment reduces its own metabolism (Laine et al., 2000), it is possible that selegiline is a MBI of CYP2A6.

**Section 2.3.4  Improving Smoking Cessation through CYP2A6 Inhibition**

The impact of CYP2A6 genotype on nicotine metabolism and smoking behaviors has been examined in detail (Ho and Tyndale, 2007; Malaiyandi et al., 2005; Mwenifumbo and Tyndale, 2007). The most striking observation of the influence of CYP2A6 genetics on smoking behaviours comes from slow metabolizers of nicotine (50% or less predicted CYP2A6 activity). For instance, slow metabolizers are less likely to be current smokers (OR 0.52 [95% CI: 0.29-0.95], p=0.03) (Schoedel et al., 2004), smoke significantly fewer (20-25%) cigarettes (Malaiyandi et al., 2006; Schoedel et al., 2004), and have significantly reduced (~30%) total inhalation volume (Strasser et al., 2007). In addition, when smokers are ranked according to rates of nicotine metabolism (as measured by the 3-hydroxycotinine to cotinine ratio), individuals in slowest quartile are more likely to quit smoking after counseling (32%) compared to those in the fastest quartile (10%) (Patterson et al., 2008). Furthermore, with nicotine patch treatment, individuals in the upper quartiles (faster nicotine metabolism) have lower chance of quitting smoking compared to those in the lowest quartile at the end of treatment and 6 months’ follow-up (OR 0.72 [95% CI: 0.83-1.33], P=0.005) (Lerman et al., 2006). Similar observation is also seen in light-smokers treated with nicotine gum (Ho et al., 2009).

Based on the above findings, it was hypothesized that one could pharmacologically mimic impairments in the CYP2A6 enzyme (phenocopying) to increase nicotine levels and decrease the number of cigarettes smoked (Sellers et al.,...
2000; Tyndale and Sellers, 2002). By inhibiting nicotine metabolism, the systemic
availability of nicotine is increased; therefore, the concomitant use of CYP2A6 inhibitors
with NRT products should enhance smoking cessation. For example, some of the
nicotine released from nicotine gum is absorbed by the buccal cavity, while a significant
portion is swallowed (Benowitz et al., 1987). Of the nicotine swallowed (from nicotine
gum, lozenges, or pills), approximately 80% is metabolized via first-pass metabolism
before entering systemic circulation (Benowitz et al., 1991b). Inhibition of CYP2A6 would
decrease first-pass metabolism and increase the bioavailability of nicotine as well as
other CYP2A6 substrates (see Section 1.6.1). In a preliminary study, after three days of
treatment with methoxsalen (10 mg t.i.d.) plus nicotine gum (4 mg), the mean plasma
nicotine levels were significantly higher compared to placebo plus nicotine gum (15.3 vs.
10.1 ng/ml; p<0.01) (Sellers et al., 2002). Similarly, the pill form of nicotine for smoking
cessation may also benefit from the co-administration of a CYP2A6 inhibitor. This is due
to the fact that higher doses of oral nicotine that are required to overcome first-pass
metabolism can cause gastrointestinal irritation (Benowitz et al., 1991b). Compared with
oral nicotine alone, the addition of methoxsalen (10 and 30 mg) to 4 mg of oral nicotine
significantly increased mean plasma nicotine levels (>70%) and decreased the desire to
smoke (p<0.05) (Tyndale et al., 1999). In another study, methoxsalen (30 mg) plus oral
nicotine (4 mg) also significantly altered smoking behaviours. Participants on this
treatment smoked fewer cigarettes (3.1 vs. 4.1; p<0.01) during a 90 minute free smoking
session, had lower levels of breath carbon monoxide (4.6 vs 8.7 ppm; p<0.01), lower
numbers of puffs (~28 vs. ~38; p<0.01) and inhalation intensity compared with placebo.
The subjects also had increased latency to lighting the second cigarette (~43 vs. ~32
mins; p<0.01) on methoxsalen plus nicotine compared to placebo plus nicotine (Sellers
et al., 2000). Finally, CYP2A6 inhibitor alone may be able to reduce smoking and
increase quitting, similar to the pharmacogenetic findings in slow metabolizers (Malaiyandi et al., 2006; Patterson et al., 2008; Rao et al., 2000; Schoedel et al., 2004); however, this remains to be tested. An advantage of this strategy is that the long-term behavioral effects of mimicking a defect in nicotine metabolism can be predicted based on available pharmacogenetic and behavioral studies.

Section 2.3.5 Reduction of Tobacco-Specific Nitrosamine Activation through Human CYP2A6 Inhibition

One goal of smoking cessation is to reduce smoking-associated morbidity and mortality. The tobacco-specific nitrosamine NNK is a potent procarcinogen found in tobacco smoke (Hecht and Hoffmann, 1988). NNK is primarily detoxified by conversion to 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), followed by conjugation to NNAL-glucuronide (Hecht et al., 1993; Maser et al., 1996). Alternatively, NNK can be metabolically activated to a reactive metabolite capable of forming DNA adducts via $\alpha$-hydroxylation (Hecht et al., 1988; Peterson et al., 1990); one enzyme responsible for this process is CYP2A6 (Nishikawa et al., 2004). The other enzyme that exhibits the highest in vitro activity towards NNK activity is CYP2A13 (with at least 100-fold greater activity than CYP2A6) and it is primarily expressed in the lung and may be one of the enzyme responsible for NNK activation in situ (Jalas et al., 2005). However, CYP2A13 level in lung tissues is variable between individuals and thus CYP2A6, as well as other enzymes with lower in vitro NNK activation activities, such as CYP1A1/2, CYP2B6, CYP2D6, CYP2E1, and CYP3A4, may contribute to NNK activation in those lacking CYP2A13 (Jalas et al., 2005; Zhang et al., 2007). The involvement of CYP2A6 in lung carcinogenesis is supported in a recent epidemiology study: individuals that were CYP2A6-poor metabolizers had significantly less risk for developing lung cancer, even
after adjusting for the number of cigarettes smoked (Fujieda et al., 2004). Furthermore, the expression of CYP2A6 is higher in large lung adenocarcinoma tissues and tumors that have metastasized compared to smaller tumor and non-metastasized tissues, respectively, suggesting the involvement of CYP2A6 in lung carcinogenesis (Matsuda et al., 2007).

In mice, NNK exposure causes the formation of lung tumors (Hecht et al., 1978; Hecht et al., 1994; Okubo et al., 2005), and administration of the mouse CYP2A5 inhibitor, methoxsalen, reduced lung malignancies (Miyazaki et al., 2005; Takeuchi et al., 2003). The finding is corroborated with findings in humans in which methoxsalen administration in smokers re-routed excretion of NNK to NNAL and NNAL-glucuronide, suggesting the α-hydroxylation of NNK was inhibited (Sellers et al., 2003a).

CYP2A13 also contributes to the α-hydroxylation of NNK (He et al., 2004a). Due to the high level of CYP2A13 in the respiratory tract (Su et al., 2000), it is thought that CYP2A13 plays a significant role in the bioactivation of NNK in the lung. This is supported by the finding that NNK activation is significantly correlated with CYP2A13 levels in lung microsomes (Zhang et al., 2007). In contrast, the association between CYP2A13 levels/activities with NNK metabolism was only found in a subgroup of lung cancer biopsy samples (Brown et al., 2007). It is possible that inhibition of CYP2A enzymes (CYP2A6 and CYP2A13) may decrease the risk of development of NNK-induced lung cancer.
Section 3 Nicotine Pharmacological Behavioural Models

Section 3.1 Nicotine Self-Administration

Nicotine has reinforcing properties in mice as demonstrated through the use of self-administration paradigms. Intravenous nicotine self-administration (NSA) is a widely used method of testing nicotine’s reinforcing properties in rats (Corrigall and Coen, 1991; Corrigall et al., 1994) but is more technically demanding in mice due to their small size (Sparks and Pauly, 1999). Oral NSA, whilst not modeling smoking behaviours directly, has been studied extensively in mice (Adriani et al., 2002; Aschhoff et al., 1999; Meliska et al., 1995; Robinson et al., 1996; Stolerman et al., 1999) and has some similar features to smoking, which may make it a suitable method of studying nicotine pharmacology. For instance, oral NSA occurs intermittently primarily during the wake period. There exhibits a wide spectrum of preference for oral nicotine between mouse strains (Aschhoff et al., 1999; Robinson et al., 1996), suggesting a role of genetic influences in NSA, similar to smoking behaviours in humans (Ho and Tyndale, 2007). Chronic oral NSA produces pharmacologically relevant levels of nicotine in the plasma of mice. When nicotine is provided in a free-access two-bottle choice paradigm, mice consume an amount of nicotine that results in a plasma concentration (~35 ng/ml) similar to those seen in humans during *ad libitum* smoking (10 to 50 ng/ml) (Hukkanen et al., 2005b; Rowell et al., 1983). Chronic oral NSA can also lead to physical dependence as measured by somatic signs (paw tremors, backing, and head shakes) although this occurs at high nicotine levels (200 µg/ml) (Grabus et al., 2005). In addition, oral NSA up-regulates both α4 and α7 nicotinic acetylcholine receptors in mouse brain (Sparks and Pauly, 1999); this is consistent with findings that chronic nicotine and smoking increase nicotinic receptors in baboons and smokers, respectively (Kassiou et al., 2001; Perry et al., 1999).
Oral nicotine also alters the activities of enzymes (e.g. CREB, pERK) in pathways thought to mediate nicotine reward (Brunzell et al., 2003). Behaviourally, in mice oral nicotine is rewarding at an optimal range: too low a dose and nicotine is not rewarding and at high doses it is aversive (Robinson et al., 1996). This finding is in line with human and other animal studies (Rose and Corrigall, 1997). Together, these data suggest that oral NSA in mice may be a suitable method of studying some aspects of the pharmacology of nicotine.

**Section 3.2 Analgesic Effect of Nicotine and Application in Nicotine Studies**

Nicotine’s analgesic properties have long been documented (Meyer et al., 2000). The mechanism of action is thought to be mediated indirectly by the activation of the opioid system. In a study examining the anti-nociceptive effect of nicotine on tail-pinches in mice, nicotine induced the release of the endogenous opioid peptide, Met-enkephalin (agonist at both µ- and δ-opioid receptors), but not dynorphins and endorphins, in the spinal cord. The analgesic action was mediated in part by the nicotinic acetylcholine receptors as mecamylamine ameliorated the effect (Kiguchi et al., 2008). In addition to analgesia, nicotine also has both hypothermic and ataxic effects (Kita et al., 1988; Marks et al., 1984).

The tail-flick and hot-plate tests are used to study nicotine’s analgesic effects (Marubio et al., 1999; Rogers and Iwamoto, 1993). The tail-flick response is a spinal reflex (Caggiula et al., 1995); therefore, the ataxic-properties of nicotine are not thought to be confounding variables in this behavioural test (Rogers and Iwamoto, 1993). On the other hand, the hot-plate response is thought to be mediated by supra-spinal (i.e. brain) processes, thus it is conceivable that ataxia induced by nicotine may confound
interpretation of results from this method (Caggiula et al., 1995). A recent study has found that the tail-flick reflex requires $\alpha_4\beta_2$ receptors whereas response to hot-plate requires $\alpha_4\beta_2$ and possibly $\alpha_7$ or other receptor subsets (Damaj et al., 2007a), both indicating the direct involvement of nicotinic receptors in nicotine-induced analgesia.
Section 4 Outline of Chapters

Section 4.1 Chapter 2: Characterization and Comparison of Nicotine and Cotinine Metabolism \textit{In Vitro} and \textit{In Vivo} in DBA/2 and C57BL/6 Mice

\textbf{Rationale}

In order to determine whether the mouse is a suitable model of nicotine metabolism, the disposition kinetics of nicotine and its metabolites, as well as the enzyme responsible for the primary metabolism of nicotine, need to be assessed. Second, to examine whether interstrain differences in nicotine pharmacology could be contributed to by differences in nicotine metabolism, nicotine pharmacokinetics between mouse strains also need to be studied. We hypothesized that the mouse CYP2A5 is responsible for the primary metabolism of nicotine to cotinine and that strain differences (in CYP2A5) could result in differences in nicotine pharmacokinetics.

\textbf{Outline}

This chapter describes the studies undertaken to examine the pharmacokinetics of nicotine and cotinine in mice. Immuno-inhibition studies indicated that, similar to human CYP2A6, the mouse CYP2A5 is responsible for the primary metabolism of nicotine to cotinine and cotinine to 3-hydroxycotinine \textit{in vitro}. The metabolism of nicotine and cotinine, both \textit{in vitro} and \textit{in vivo}, between two commonly studied mouse strains (DBA/2 and C57BL/6) were also described. Pharmacokinetic studies and \textit{in vitro} assays indicated that the metabolism of cotinine, but not nicotine, is different between these two mouse strains.

\textbf{Contribution to Thesis}
This chapter contributes to the overall thesis by first describing the pharmacokinetics of nicotine and cotinine in mice, and second, by defining the contribution of a specific enzyme (i.e. CYP2A5) responsible for the (in vitro) metabolism of these compounds. The latter observation provided a line of evidence that the mouse could be a suitable model for studying inhibitors of human nicotine metabolism. The chapter also suggests that interstrain genetic variations may affect xenobiotic metabolism in a substrate-dependent manner.

Section 4.2 Chapter 3: Nicotine Self-Administration in Mice is Associated with Rates of Nicotine Inactivation by CYP2A5

Rationale
In humans, smoking (nicotine self-administration) is significantly affected by CYP2A6 variations (structure/function). We have previously demonstrated that CYP2A5 is responsible for the primary metabolism of nicotine to cotinine in mice. However, the association of nicotine self-administration behaviours with CYP2A5 variations has not been examined. We hypothesized that oral nicotine self-administration in mice is associated with CYP2A5 levels and rates of nicotine inactivation.

Outline
This chapter describes the study carried out to determine the amount of CYP2A5 protein as well as the rates of nicotine metabolism in a group of F2 mice. The F2 mice were previously generated by breeding a high nicotine consumption strain with a low nicotine consumption strain. The F2 mice were also tested for nicotine self-administration in a 2-bottle choice paradigm. In male mice, the amount of nicotine self-administered was
significantly associated with the amount of CYP2A5 protein as well as rates of *in vitro* nicotine metabolism.

**Contribution to Thesis**

The above findings further demonstrated that: 1) nicotine metabolism is associated with CYP2A5; 2) genetic variation in CYP2A5 between DBA/2 and C57Bl/6 mice can alter nicotine metabolism; and 3) CYP2A5 (and nicotine metabolism) is associated with, and potentially influencing, nicotine pharmacology including, importantly, nicotine self-administration. This chapter contributes to the overall thesis by demonstrating that CYP2A5 influences nicotine metabolism through its expression/activity. In addition, CYP2A5 can potentially alter the behavioural pharmacology of nicotine. These observations strengthen the application of the mouse model of nicotine metabolism for both inhibitor studies as well as genetic studies.

**Section 4.3 Chapter 4: Inhibition of Nicotine Metabolism by Methoxysalen: Pharmacokinetic and Pharmacological Studies in Mice**

**Rationale**

We have previously demonstrated that CYP2A5 levels/activities are significantly associated with nicotine behavioural pharmacology (i.e. oral nicotine self-administration behaviour) in mice. This finding is analogous to human studies in which CYP2A6 function is significantly correlated with levels of smoking. We are interested in determining whether direct manipulation (i.e. inhibition) of CYP2A5, can alter nicotine metabolism as well as the resulting behavioural response to nicotine. We hypothesized that inhibition of CYP2A5 would significantly reduce nicotine metabolism and enhance the pharmacological effects of nicotine.
Outline

This chapter describes the use of the CYP2A6 inhibitor, methoxsalen, to determine whether it can inhibit nicotine metabolism in mice. Initially the examination of the inhibition of nicotine metabolism in mouse hepatic microsomes was performed to determine whether this inhibitor is effective in mice (specifically in inhibiting CYP2A5-mediated nicotine metabolism). This was followed by the in vivo administration of methoxsalen and the characterization on its impact on nicotine pharmacokinetics and resulting behavioural pharmacology. The anti-nociception effect of nicotine on tail-flick and hot-plate procedures was measured as the indicator of the pharmacological effects of nicotine. Methoxsalen significantly inhibited nicotine metabolism both in vitro and in vivo. More importantly, the reduction in clearance of nicotine was associated with a dramatic prolongation in its anti-nociception effect as measured by tail-flick and hot-plate procedures.

Contribution to Thesis

This chapter contributes to the thesis by demonstrating that: 1) Mice may be suitable for studying CYP2A6 inhibitors; and 2) inhibition of nicotine metabolism can directly alter the behavioural pharmacological effects of nicotine. These observations further support the use of the mouse for the in vivo screenings of novel inhibitors of nicotine metabolism, especially for the purpose of smoking cessation.

Section 4.4  Chapter 5: Selegiline (L-Deprenyl) is a Mechanism-based Inactivator of CYP2A6 Inhibiting Nicotine Metabolism in Humans and Mice
Rationale

Selegiline is an anti-Parkinsonian drug currently being investigated as a potential therapy for smoking cessation due to its ability to decrease dopamine metabolism through inhibition of MAO-B. In humans, chronic treatment with selegiline decreases its own metabolism. Selegiline belongs to the acetylene group of compounds that are known mechanism-based inhibitors. Since CYP2A6 has been demonstrated to metabolize selegiline, we investigated whether selegiline could also inhibit mouse and human nicotine metabolism. We hypothesized that selegiline can inhibit nicotine metabolism by mouse CYP2A5 and human CYP2A6. This may be an additional mechanism by which selegiline mediates smoking cessation.

Outline

This chapter described the various studies performed to characterize the inhibitory effect of selegiline on nicotine metabolism in mouse and human hepatic microsomes, in cDNA-expressed CYP2A5 and CYP2A6, and in mouse in vivo. Inhibition of nicotine metabolism in vitro with major selegiline metabolites (desmethylselegline, L-methamphetamine, and L-amphetamine) was also performed. Selegiline significantly inhibited nicotine metabolism in all in vitro enzyme systems studied. Selegiline also inhibited nicotine metabolism in mice in vivo. Most importantly, selegiline was found to be a potent mechanism-based inhibitor of CYP2A6-mediated nicotine metabolism.

Contribution to Thesis

This chapter contributes to the thesis by demonstrating that in vitro, and especially in vivo, the mouse model of nicotine metabolism is a potentially useful for the screening of novel inhibitors of CYP2A6.
CHAPTER 2: CHARACTERIZATION AND COMPARISON OF NICOTINE AND COTININE METABOLISM IN VITRO AND IN VIVO IN DBA/2 AND C57BI/6 MICE

Eric C. K. Siu and Rachel F. Tyndale

All of the experiments, data analyses, and writing of the manuscript were performed by ECK Siu except for the mass-spectrometry analysis which was performed at the Proteomic and Mass Spectrometry Centre at the University of Toronto. The study was designed by ECK Siu and RF Tyndale.
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.
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., 2004b).

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 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). This may be due to hormonal influences (mouse estrous cycle is approximately 3-7 days) as estrogen-only oral contraceptives increased nicotine metabolism in human females (Benowitz et al., 2006a). In addition a second CYP2A enzyme, CYP2A4, is present in female mice that may metabolize nicotine (Murphy et al., 2005b) 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 effects 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 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°C in a final volume of 0.5 ml. The reaction was stopped with a final concentration of 4% v/v Na$_2$CO$_3$. 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 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°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 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 $K_m$ and $V_{max}$ for one and two enzymatic sites were $v = V_{max} [S] / (K_m + [S])$ and $v = [V_{max1} [S] /$
\( (K_{m1} + [S]) + \frac{[V_{max2} [S]]}{(K_{m2} + [S])} \), respectively, where [S] denotes substrate concentration.

The \textit{in vivo} pharmacokinetic parameters were determined using non-compartmental analysis: \( AUC_{0-480} \), peak plasma concentration \( (C_{max}) \), maximum plasma concentration \( (T_{max}) \). \( AUC_{0-480} \) 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 following subcutaneous injection in mice, \( CL \) (clearance) was determined as a hybrid parameter \( CL/F \) and was calculated as \( \frac{Dose}{AUC_{0-480}} \). The average weights of the animals of the strains were similar \((24.8 \pm 1.7 \text{ vs. } 25.5 \pm 1.1 \text{ g for DBA/2 and C57Bl/6, respectively, n=50 for each strain})\), therefore the dose of 25 \( \mu g \) (1 mg/kg) was used for the calculation of \( CL/F \) for nicotine.

\textit{Statistical Analyses}

Statistical analyses of \textit{in vitro} kinetic parameters were tested by Mann-Whitney U test. Assessment of \textit{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. 2A) and Eadie-Hofstee plotting (Fig. 2B), 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 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. 3A; Table 2). The overall $AUC_{0-480}$ 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.
Figure 2. *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) Eadie-Hofstee plots of the kinetic data for DBA and C57Bl/6 mice. See Table 1 for values of kinetics parameters. V: velocity; S: substrate
Table 1. *In vitro* nicotine metabolism (C-oxidation) parameters.

<table>
<thead>
<tr>
<th></th>
<th>DBA/2 (n=5 mice) mean ± S.D.</th>
<th>C57Bl/6 (n=4 mice) mean ± S.D.</th>
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<tbody>
<tr>
<td><strong>High Affinity</strong></td>
<td></td>
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<tr>
<td>$K_m$ ($\mu$M)</td>
<td>10.7 ± 4.8</td>
<td>11.4 ± 3.6</td>
</tr>
<tr>
<td>$V_{max}$ (nmol/min/mg)</td>
<td>0.58 ± 0.18</td>
<td>0.50 ± 0.07</td>
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<td>$V_{max}/K_m$</td>
<td>0.05 ± 0.03</td>
<td>0.04 ± 0.02</td>
</tr>
<tr>
<td><strong>Low Affinity</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$K_m$ ($\mu$M)</td>
<td>234 ± 77</td>
<td>306 ± 126</td>
</tr>
<tr>
<td>$V_{max}$ (nmol/min/mg)</td>
<td>1.60 ± 0.63</td>
<td>1.18 ± 0.36</td>
</tr>
<tr>
<td>$V_{max}/K_m$</td>
<td>0.007 ± 0.001*</td>
<td>0.004 ± 0.001</td>
</tr>
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</table>

*p < 0.05 compared to C57Bl/6 mice*
Figure 3. 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 $AUC_{0-480}$ 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 $AUC_{0-480}$ 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.
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.

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<thead>
<tr>
<th></th>
<th>Nicotine</th>
<th>Cotinine</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>DBA/2</td>
<td>C57Bl/6</td>
</tr>
<tr>
<td>$AUC_{0-480}$ (ng•hr/ml)</td>
<td>92.9 ± 2.9*</td>
<td>80.8 ± 3.2</td>
</tr>
<tr>
<td>$T_{1/2}$ (min)</td>
<td>8.6 ± 0.4</td>
<td>9.2 ± 1.6</td>
</tr>
<tr>
<td>$CL/F$ (ml/min)</td>
<td>4.5 ± 0.1*</td>
<td>5.2 ± 0.2</td>
</tr>
<tr>
<td>$T_{max}$ (min)</td>
<td>10.0 ± 2.2</td>
<td>10.0 ± 4.7</td>
</tr>
<tr>
<td>$C_{max}$ (ng/min)</td>
<td>201 ± 15*</td>
<td>160 ± 15</td>
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</tbody>
</table>

*p < 0.05 compared to C57Bl/6 mice, ND – not determined
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 3B). In contrast, compared to C57Bl/6 mice, DBA/2 mice showed a significantly larger $AUC_{0-480}$ 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 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. 4A). To confirm the identity of the putative trans-3'-hydroxycotinine 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. 4B).

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. 4C) 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. 4D). Fragmentation of the trans-3'-hydroxycotinine standard ion ($m/z$ 193) gave two fragments of $m/z$ 80 and $m/z$ 134 (Fig. 4E). Fragmentation of the cotinine metabolite at $m/z$ 193 also gave two major ions of $m/z$ 80 and $m/z$ 134 (Fig. 4F). 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 $m/z$
Figure 4. 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 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.
80 and the $m/z$ 134 fragments corresponded to $(C_8H_5N)H^+$ 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. 5A, Table 3). Similar to cotinine derived from nicotine injection, following cotinine injection the cotinine $AUC_{0-180}$ was much higher in the DBA/2 mice compared to the C57Bl/6 mice. The clearance of 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 $AUC_{0-180}$ of 3′-hydroxycotinine was higher in the DBA/2 mice compared to the C57Bl/6 mice (Fig. 5B, 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. 6A), mediated by a single enzymatic site in both strains (Fig. 6B). 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.
Figure 5. 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 $AUC_{0-180}$ of cotinine (COT) was 37% higher and its elimination half-life was 34% longer in DBA/2 mice compared to C57Bl/6 mice. B) The $AUC_{0-180}$ 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.
### 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.

<table>
<thead>
<tr>
<th></th>
<th>Cotinine</th>
<th>3'-hydroxycotinine</th>
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<tbody>
<tr>
<td></td>
<td>DBA/2</td>
<td>C57Bl/6</td>
</tr>
<tr>
<td>$AUC_{0-180}$ (ng•hr/ml)</td>
<td>1087 ± 33*</td>
<td>796 ± 33</td>
</tr>
<tr>
<td>$T_{1/2}$ (min)</td>
<td>50.2 ± 4.7*</td>
<td>37.5 ± 9.6</td>
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<tr>
<td>$CL/F$ (ml/min)</td>
<td>0.38 ± 0.01*</td>
<td>0.52 ± 0.02</td>
</tr>
<tr>
<td>$T_{max}$ (min)</td>
<td>15.0 ± 7.5</td>
<td>15.0 ± 0.0</td>
</tr>
<tr>
<td>$C_{max}$ (ng/ml)</td>
<td>748 ± 35</td>
<td>759 ± 39</td>
</tr>
</tbody>
</table>

*p < 0.05 compared to C57Bl/6, ND – not determined
Figure 6. *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
Table 4. *In vitro* cotinine hydroxylation parameters.

<table>
<thead>
<tr>
<th></th>
<th>DBA/2 (n=4) mean ± S.D.</th>
<th>C57Bl/6 (n=4) mean ± S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_m$ (µM)</td>
<td>51.0 ± 15.6*</td>
<td>9.5 ± 2.1</td>
</tr>
<tr>
<td>$V_{max}$ (nmol/min/mg)</td>
<td>0.10 ± 0.01*</td>
<td>0.04 ± 0.01</td>
</tr>
<tr>
<td>$V_{max}/K_m$</td>
<td>0.002 ± 0.000*</td>
<td>0.005 ± 0.002</td>
</tr>
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</table>

*p < 0.05 compared to C57Bl/6 mice
Previously the mouse CYP2A5 was identified as the enzyme responsible for the high-affinity metabolism of nicotine using cDNA-expressed CYP2A5 (Murphy et al., 2005b). 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. 7A, filled symbols). Similar results were seen in hepatic microsomes from C57Bl/6 mice, tested at 80 µl antibody / mg protein (Fig. 7A, 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. 7A).

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. 7B, 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. 7B, open symbols). Inhibitory antibodies against CYP2B6 and CYP2D6 had no effect on cotinine metabolism (Fig. 7B).
Figure 7. 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) 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.
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 its pharmacology. CYP2A5 metabolizes nicotine (Murphy et al., 2005b) 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., 2005b) 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 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\text{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_{0-48h}.

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., 2005b). 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 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\(_{0-180}\) 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., 2005b). 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., 2005b); however, no functional polymorphisms for the mouse aldehyde oxidase genes have been reported (Mouse Genome Informatics), and our in vivo data was consistent with our in vitro data suggesting the variations 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 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.

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 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 a 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

We would like to thank Linda Liu for assistance in HPLC and Dr. Sharon Miksys and Jill Mwenifumbo for critical review of this manuscript. RFT is a shareholder and chief scientific 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.

Footnote

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SIGNIFICANCE OF CHAPTER

This study described the in vitro and in vivo metabolism of both the nicotine and cotinine in mice. Similar to human CYP2A6, the mouse CYP2A5 is responsible for the majority of metabolism of nicotine to cotinine, and it appears to be exclusively responsible for the metabolism of cotinine to 3-HC. This provided a line of evidence that the mouse may be a suitable model for studying inhibitors of nicotine metabolism. In addition, the study also showed that polymorphism in CYP2A5 can affect cotinine metabolism differentially compared with nicotine. This suggested that careful interpretations are required in nicotine pharmacology studies as well as in studies where cotinine are used as biomarkers.
CHAPTER 3: NICOTINE SELF-ADMINISTRATION IN MICE IS ASSOCIATED WITH RATES OF NICOTINE INACTIVATION BY CYP2A5

Eric C. K. Siu, Dieter B. Wildenauer, and Rachel F. Tyndale
As published in: Psychopharmacology, 2006, Mar; 184(3-4):401-408.

The immunoblotting and in vitro enzyme activities assays, data analyses, and writing of the manuscript were performed by ECKS. The nicotine self-administration study was performed by DB Wildenauer. The study was designed by ECK Siu and RF Tyndale.
Abstract

Rationale: Cyp2a5, the mouse homologue of human CYP2A6, encodes for the enzyme responsible for the primary metabolism of nicotine. Variation in human CYP2A6 activity can alter the amount smoked such as numbers of cigarettes smoked per day and smoking intensity. Different mouse strains self-administer different amounts of oral nicotine and quantitative trait loci analyses in mice suggested that Cyp2a5 may be involved in differential nicotine consumption behaviours. Objectives: The goal of this study was to examine whether in vivo nicotine consumption levels were associated with CYP2A5 protein levels and in vitro nicotine metabolism in mice. Methods: F2 mice propagated from high (C57Bl/6) and low (St/bJ) nicotine consuming mice were analyzed for CYP2A5 hepatic protein levels and in vitro nicotine metabolizing activity. Results: We found that F2 male high nicotine (n=8; 25.1±1.2 µg nicotine/day) consumers had more CYP2A5 protein compared to low (n=11; 3.8±1.4 µg nicotine/day) consumers (10.2±1.0 vs. 6.5±1.3 CYP2A5 units). High consumers also metabolized nicotine faster than the low consumers (6 µM: 0.18±0.04 vs. 0.14±0.07; 30 µM: 0.36±0.06 vs. 0.26±0.13; 60 µM: 0.49±0.05 vs. 0.32±0.17 nmol/min/mg). In contrast, female high (25.1±2.1 µg nicotine/day) and low (4.7±1.4 µg nicotine/day) nicotine consumers did not show pronounced differences in nicotine metabolism or CYP2A4/5 protein levels; this is consistent with other studies of sex differences in response to nicotine. Conclusions: These data suggested that among male F2 mice, increased nicotine self-administration is associated with increased rates of nicotine metabolism, most likely as a result of greater CYP2A5 protein levels.
Introduction

Nicotine is the main constituent of tobacco responsible for the addictive property of cigarettes (Stolerman and Jarvis, 1995). Once dependent, smokers adjust their smoking behaviours (e.g. number of cigarettes smoked, puff intensity) to maintain constant nicotine plasma levels (McMorrow and Foxx, 1983; Russel, 1987). As a consequence, factors affecting nicotine clearance alter an individual’s smoking behaviours. In humans approximately 80% of nicotine is metabolically inactivated to cotinine by the liver and approximately 90% of this reaction is carried out by CYP2A6 (Benowitz et al., 1994; Messina et al., 1997; Nakajima et al., 1996a). Individuals with different reduced activity variants of CYP2A6 smoke lower numbers of cigarettes; for instance, smokers who are slow metabolic inactivators of nicotine smoke fewer cigarettes compared to those who are normal or faster metabolizers (Rao et al., 2000; Schoedel et al., 2004). To further investigate the impact of nicotine metabolism on nicotine consumption, a genetic and kinetic study in mice was initiated.

The mouse is a better suited model for the study of nicotine metabolism and behaviours compared to rats for several reasons. First, the predominant enzymes responsible for the metabolism of nicotine in rats belong to the CYP2B family (Nakayama et al., 1993; Schoedel et al., 2001). Second, CYP2A5 is approximately 84% identical to CYP2A6 in amino acid sequence, and cDNA-expressed CYP2A5 metabolizes nicotine with similar efficiency to human CYP2A6 (Murphy et al., 2005a). Finally, the close structural similarity between CYP2A5 and CYP2A6 allows for the development of inhibitors and in vivo screening of these compounds in mice. In addition, the behavioural and physiological effects of nicotine in mice have been well documented (Collins et al., 1988; Faraday et al., 2005; Grabus et al., 2005; Marks et al., 1985).
With respect to nicotine intake, it has been shown that different mouse strains self-administer different amounts of oral nicotine (Aschhoff et al., 1999; Robinson et al., 1996), and a variety of mechanisms may explain the differential preferences. These include differences in the rewarding properties of nicotine and taste preference for bitter compounds (i.e. nicotine) (Lush and Holland, 1988; Merlo Pich et al., 1999). Acetylcholine receptor density and receptor affinity for nicotine could also potentially alter nicotine preference (Marks et al., 1989; Marks et al., 1986). However, there are no studies examining the effect of differing rates of nicotine metabolism on oral nicotine self-administration in mice. Recently, a quantitative trait loci study revealed that the Cyp2a5 gene is situated close to a marker on Chromosome 7 associated with differential nicotine self-administration behaviours, suggesting a possible involvement of Cyp2a5 in nicotine consumption behaviours in mice (Aschhoff et al., 1999; Wildenauer et al., 2005). The goal of this study was to determine whether CYP2A5 protein levels, and resulting nicotine metabolism, differed between the high and low nicotine consuming mice examined in the QTL study. The mice were selected from the F2 generation and would allow us to address the relationship between gene function and behaviour.
Materials and Methods

Animals

The F2 mice were established by crossing the F1 mice generated from a high nicotine-consuming strain (C57Bl/6) and a low-nicotine consuming strain (St/bJ) (Aschhoff et al., 1999). The mice were housed individually in polycarbonate cages with wood-shaving bedding. Each cage contained two graduated bottles with one on the left and one on the right side of each cage cover. The bottles were filled with tap water and total consumption from each bottle was estimated from the decrease of the fluid cylinder along the graduation of the tube every day during a 10 day acclimation period. On day 11 one bottle was filled with tap water, containing 5 µg/ml nicotine tartrate, while the other one contained tap water only. Subsequently, nicotine concentrations were raised to 10, 25, 50, 75 and 100 µg/ml at 5-day intervals. To minimize problems associated with side preference, the position of the bottles at the right or left side of the cage was changed every day after measuring consumption. During the experiment, animals were fed ad libitum (24-hour free-access). There were a total of 485 F2 mice, of which the 46 lowest nicotine-consuming mice and the 47 highest nicotine-consuming mice were selected for genotyping and QTL analyses (Wildenauer et al., 2005). From these we analyzed the highest and lowest nicotine-consuming mice (males and females). High nicotine consumers (HNC, n=22) and low nicotine consumers (LNC, n=23) were selected based on the highest and lowest average amount of nicotine consumed over the testing period, respectively. Animals were sacrificed after the last exposure at the beginning of the light cycle at the end of the test. Following sacrifice the livers were removed and frozen in liquid nitrogen and transferred to -80°C for storage.
Membrane Preparations

Microsomal membranes were prepared from mouse livers for the *in vitro* nicotine metabolism assay and stored at −80°C in 1.15% KCl as previously described (Messina et al., 1997). Briefly, the livers were homogenized in 1.15% KCl buffer and centrifuged at 9,000 g for 20 minutes. The supernatant fractions were collected and centrifuged again at 100,000 g for 90 minutes. The resultant microsomal pellets were resuspended in 1.15% KCl buffer for *in vitro* NCO assay. The cytosolic fractions (supernatant) were also collected from each of the membrane preparations and were pooled for used as the common source of aldehyde oxidase. For immunoblotting, microsomal membranes were prepared as above with the use of 100 mM Tris, pH 7.4, 0.1 mM EDTA, 0.1 mM dithiothreitol, 1.15% (w/v) KCl, and 20% (v/v) glycerol. Protein concentrations were determined with the Bradford reagent according to manufacturer's protocol (Bio-Rad Labs Ltd., Mississauga, ON).

Immunoblotting

Immunoblotting was performed essentially as previously described (Schoedel et al., 2003). To determine the linear range of CYP2A5 detection for the immunoblotting assay, mouse liver microsomes were serially diluted and used to construct standard curves (0.05 to 12.8 µg protein). Membrane proteins from livers (4.5 µg) were separated by SDS-polyacrylamide gel electrophoresis (4% stacking and 8% separating) and transferred overnight onto nitrocellulose membranes. Human lymphoblastoid cDNA-expressed CYP2A6 (BD Biosciences, San Jose, CA) was used as a positive control. For the detection of CYP2A proteins, nitrocellulose membranes were preincubated for 1 h in a blocking solution containing 1% skim milk powder (w/v), and 0.1% bovine serum albumin (w/v) in Tris-buffered saline-Triton X-100 [50 mM Tris, pH 7.4, 150 mM NaCl,
0.1% (v/v) Triton X-100]. Membranes were probed with a monoclonal antibody to human CYP2A6 (1:3000 dilution) (BD Biosciences), a horseradish peroxidase-conjugated anti-mouse secondary antibody (1:20000 dilution) (Pierce Biotechnology, Rockfort, IL), followed by detection using enhanced chemiluminescence (Pierce Biotechnology). Controls included immunoblots that were incubated without primary antibody as well as immunoblots loaded with several other cDNA-expressed CYPs for the assessment of cross-reactivity. Nitrocellulose membranes were exposed to Progene autoradiographic film (Ultident Scientific, St. Laurent, P.Q.) for 0.5 to 2 min. Immunoblots were analyzed using the MCID imaging system (Imaging Research Inc., St. Catherines, ON, Canada).

**Nicotine C-Oxidation (NCO) Assay**

To establish assay linearity, increasing concentrations of hepatic microsomal proteins (0, 0.25, 0.5, 1, 1.5, 2, 2.5, 3 mg/ml) were incubated with 65 and 650 µM of nicotine for 30 minutes; microsomal protein (0.5 mg/ml) were incubated for various durations (0, 5, 10, 15, 30, 45, 60 mins) with 65 and 650 µM of nicotine. These concentrations of nicotine were originally examined based on human pharmacokinetic variables (Messina et al., 1997), and then refined as data on the mice was generated. Varying concentrations of cytosolic protein (0.3, 0.6, 0.75, 0.9, 1.1, 1.2 mg/ml) were incubated with 0.5 mg/ml of microsomal protein and with 30 and 300 µM of nicotine for 15 minutes to ensure that aldehyde oxidase was not rate limiting. This is important since the conversion of nicotine to cotinine follows two steps: the first of which is the conversion of nicotine to nicotine-$\Delta^{1(5)}$-iminium ion by CYP2A5. The iminium ion is then converted to cotinine by aldehyde oxidase which is present in the cytosolic fraction (Brandange and Lindblom, 1979). From these studies a protein concentration of 0.5 mg/ml and an incubation time of 15 min was selected for all subsequent assays. Incubation mixtures also 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°C in a final volume of 0.5 ml. The reaction was stopped with 4% Na₂CO₃, and 5-methylcotinine (70 µg) was added as the internal standard. Nicotine metabolizing activity was analyzed as previously described (Messina et al., 1997) with the modification of the solid phase extraction procedure for assessment of 3-hydroxycotinine. Briefly, samples were extracted using ISOLUTE HM-N columns (Argonaut Technologies, Redwood City, CA) with 13 ml of dichloromethane and were 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, cotinine, and 3-hydroxycotinine was achieved using a ZORBAX Bonus-RP column (5 µm, 150 × 4.6 mm; Agilent Technologies Inc., Mississauga, ON) 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 isocratic elution at a flow rate of 0.9 ml/min. Nicotine, cotinine, and 3-hydroxycotinine sample concentrations were determined from standard curves. The quantitation limits were 5 ng/ml for nicotine, 12.5 ng/ml for cotinine, and 10 ng/ml for 3-hydroxycotinine.

**Statistical Analyses**

Data were analyzed by Graphpad Prism (version 4.00, San Diego, CA). Statistical significance was calculated using Mann-Whitney U test and the significance level was set at \( p=0.05 \). Correlation analyses were performed using Pearson’s method.
**Results**

**CYP2A5 detection in mouse liver**

An immunoblotting assay was developed in order to detect CYP2A5 in male mouse liver microsomes. The CYP2A5 signal was linear between 2.5 to 10 µg of microsomal protein (Fig. 8a & b). Selectivity was confirmed by incubating the immunoblots without the primary antibody (Fig. 8c). The primary antibody did not react with several alternative cDNA-expressed CYPs (Fig. 8d).

**Increased oral nicotine self-administration in male mice is associated with higher CYP2A5 protein levels**

Male mice in the high nicotine consumption (HNC) group self-administered significantly higher amounts of oral nicotine compared to the low nicotine consumption (LNC) group (Table 5; Fig. 9a). We first determined if the increased consumption of nicotine in the male mice was associated with elevated levels of hepatic CYP2A5 protein. HNC mice had ~60% more CYP2A5 protein compared to the LNC mice (10.2 ± 1.0 vs. 6.5 ± 1.3 units, respectively, $p=0.03$, Fig. 9a). Figure 9b shows a representative immunoblot of CYP2A5 expression in both the high and the low consumption mouse groups.

**In vitro NCO rate is faster in high nicotine consuming male mice**

In addition to affecting levels of protein expression, genetic variation in CYP2A5 may also affect protein structure altering the interaction with nicotine ($K_m$). We compared the kinetic parameters of *in vitro* NCO activities between the HNC and LNC mice. We first optimized assay conditions and linearity for mouse NCO reactions. We established good separation of peaks (Fig. 10a) and identified appropriate concentration of cytosol (1
Figure 8. Selectivity and linearity of CYP2A5 immunoblotting for mouse liver microsomes. a) A dilution curve of mouse liver microsomal proteins detected by immunoblotting (± SEM, 3 experiments). Representative immunoblots incubated with (b) and without (c) primary antibody. d) cDNA-expressed CYP proteins as well as mouse liver microsomal protein detected (lanes 1:CYP1A1, 2:CYP1A2, 3:CYP2A2, 4:CYP3A2, 5:CYP1B1, 6:CYP2C11, 7:CYP2D, 8:CYP2E1, 9:CYP2A6; 0.2 pmol each; lane 10:mouse liver microsomal protein, 2 µg).
Table 5. Average nicotine consumption (µg/day; mean ± SD) in F2 male and female mice in a two-bottle choice paradigm.

<table>
<thead>
<tr>
<th></th>
<th>LNC Group</th>
<th>HNC Group</th>
</tr>
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<tbody>
<tr>
<td>Male</td>
<td>3.8 ± 1.4 (n=11)</td>
<td>25.1 ± 1.2* (n=8)</td>
</tr>
<tr>
<td>Female</td>
<td>4.7 ± 1.4 (n=12)</td>
<td>25.1 ± 2.1* (n=14)</td>
</tr>
</tbody>
</table>

*p<0.05 compared to the LNC group
**Figure 9.** CYP2A5 levels are higher in male high nicotine consuming mice. 

a) Microsomal proteins from both male LNC (n=11) and HNC (n=8) mice were analyzed for CYP2A5 protein levels. For each animal, CYP2A5 protein levels were plotted against nicotine (NIC) consumption. Protein data are from 4 independent immunoblots for each animal. 

b) A representative immunoblot of CYP2A5 in LNC and HNC mice. *p=0.03
Figure 10. Optimization of nicotine-C-oxidation assay. a) HPLC separation of a blank sample containing the standards nicotine (NIC), cotinine (COT), 3-hydroxycotinine (3HC), and the internal standard 5-methylcotinine (5MC). Assay conditions were optimized for: b) hepatic microsomal protein concentration; c) incubation time and; d) cytosolic protein concentration. Arrows at the X-axis indicate conditions used for subsequent experiments.
mg/ml, Fig. 10b) and microsomes (0.5 mg/ml, Fig. 10c), as well as the incubation time (15 mins, Fig. 10d) for the assay.

Next, based on the recent publication of the mouse nicotine kinetics using expressed cDNAs, the $K_m$ value (7.7 µM) from the cDNA-expressed CYP2A5 (Murphy et al., 2005a) we examined nicotine at 6, 30, and 60 µM - concentrations predicted to allow for the separation of structural ($K_m$) and expression ($V_{max}$) differences between the HNC and LNC groups. We found that the NCO activities were significantly higher in the male HNC group compared to the LNC group at all three nicotine concentrations (6 µM: 0.18±0.04 vs. 0.14±0.07 nmol/min/mg, $p=0.02$; 30 µM: 0.36±0.06 vs. 0.26±0.13 nmol/min/mg, $p=0.01$; 60 µM: 0.49±0.05 vs. 0.32±0.17 nmol/min/mg, $p=0.01$; Figs. 11a & b), consistent with the higher levels of CYP2A5 protein. There was also a relatively good association ($R^2=0.28; p=0.02$; Fig. 11c) between NCO activities (60 µM of nicotine) and CYP2A5 protein levels. We found that while the predicted $V_{max}$ was significantly higher in the HNC mice, the apparent $K_m$ was not different between the two groups (Table 6). The concentrations of nicotine, cotinine, and 3-hydroxycotinine, as measured by HPLC at 30 µM nicotine, are listed in Table 7.

**Nicotine self-administration in female mice was not associated with CYP2A5 protein levels or *in vitro* NCO activity**

Similar to males, the female mice in the HNC group consumed significantly more nicotine compared to the LNC group (Table 5; Fig. 12a & b). When we measured CYP2A protein, no differences in CYP2A levels between the female HNC and LNC mice were observed (Fig. 12a). When we assessed NCO activities, we found that the female HNC (n=14) mice tended to metabolize nicotine (30 µM) at higher rates compared to the LNC (n=12) but this did not reach significant difference (0.43 ± 0.18 vs. 0.39 ± 0.30 nmol/min/mg; $p=0.12$; Fig. 12b). There were also no differences in activities at 60 µM.
Figure 11. Nicotine-C-oxidation activity in male high nicotine consuming mice is higher compared to the low nicotine consuming mice. a) Liver microsomes from male LNC and HNC mice were tested for velocity (V) of cotinine formation (mean ± SD). b) Velocities at 30 µM nicotine were plotted against averaged daily nicotine consumption. c) Correlation between CYP2A5 protein levels and velocity at 60 µM nicotine. *p=0.02 and **p=0.01 compared to the LNC group.
Table 6. Estimated *in vitro* NCO kinetic parameters of male LNC and HNC mice (mean ± SD)

<table>
<thead>
<tr>
<th></th>
<th>$K_m$ (µM)</th>
<th>$V_{max}$ (nmol/min/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LNC (n=10)</td>
<td>7.8 ± 2.0</td>
<td>0.30 ± 0.16</td>
</tr>
<tr>
<td>HNC (n=8)</td>
<td>10.7 ± 4.7</td>
<td>0.48 ± 0.05*</td>
</tr>
</tbody>
</table>

* $p=0.003$ compared to the LNC group
Table 7. Concentrations (mean ± SD) of nicotine, cotinine, and 3-hydroxycotinine (ng/mL) from the in vitro NCO activity assays (at 30 µM nicotine)

<table>
<thead>
<tr>
<th></th>
<th>Nicotine (µg/mL)</th>
<th>Cotinine (µg/mL)</th>
<th>3-hydroxycotinine (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male LNC (n=11)</td>
<td>4.63 ± 0.42</td>
<td>0.17 ± 0.09</td>
<td>ND</td>
</tr>
<tr>
<td>Male HNC (n=8 )</td>
<td>4.58 ± 0.41</td>
<td>0.24 ± 0.04</td>
<td>ND</td>
</tr>
<tr>
<td>Female LNC (n=12)</td>
<td>4.41 ± 0.36</td>
<td>0.26 ± 0.20</td>
<td>ND</td>
</tr>
<tr>
<td>Female HNC (n=14)</td>
<td>4.37 ± 0.34</td>
<td>0.28 ± 0.12</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND: not detected
Figure 12. CYP2A4/5 levels and *in vitro* NCO activities do not differ between female LNC and HNC mice. Microsomal proteins from female LNC and HNC mice were analyzed for a) CYP2A4/5 protein levels and b) NCO activities at 30 µM of nicotine. Protein data are from 4 independent immunoblots for each animal.
nicotine between LNC (n=7) and HNC (n=8) mice (0.61 ± 0.48 vs. 0.59 ± 0.17 nmol/min/mg; p=0.40).
Discussion

One determinant of the amount of smoking is the rate of nicotine metabolic inactivation, whereby the systemic availability of nicotine influences the number of cigarettes smoked and the intensity of inhalation (McMorrow and Foxx, 1983; Russel, 1987; Schoedel et al., 2004). Here we showed that nicotine self-administration in male mice is associated with the amount of nicotine metabolizing enzyme CYP2A5 as well as the rate at which nicotine is metabolized in vitro. CYP2A5 protein levels were correlated with NCO activities. Results from the current study are consistent with human data in which cyp2a6 genotype and/or phenotype can have a significant impact on cigarette consumption behaviours (Rao et al., 2000; Schoedel et al., 2004) where reducing or increasing nicotine clearance can decrease smoking (Sellers et al., 2000; Sellers et al., 2003b) or increase smoking (Benowitz and Jacob, 1985), respectively.

There are several interpretations for the presence of higher CYP2A5 levels and NCO activities in the HNC mice. Quantitative trait loci analyses have implicated the potential involvement of CYP2A5 in differential nicotine consumption levels (Wildenauer et al., 2005); therefore, genetic variation in CYP2A5 may contribute to the observed nicotine intake behaviours. For instance, CYP2A5 levels may be related to polymorphisms in cis- and/or trans-regulatory factors that influence transcriptional efficiency, translational efficiency, as well as message stability. This is plausible since the human CYP2A6 contains multiple polymorphisms that affect its expression and activity (Nakajima and Yokoi, 2005). For example, the CYP2A6*9 polymorphism encodes a TATA box mutation which reduces its mRNA production by 40-70% (Kiyotani et al., 2003; Pitarque et al., 2001; Yoshida et al., 2003). Smokers who are homozygous for CYP2A6*9 (slow metabolizers) have reduced nicotine metabolism by approximately 50% (Schoedel et al., 2004; Yoshida et al., 2003); and slow metabolizers smoke ~20%
fewer cigarettes compared to individuals who are normal metabolizers (Schoedel et al., 2004). Polymorphism in CYP2A6 may also affect the structural stability of the enzyme. The CYP2A6*2 variant codes for a protein with a single amino-acid substitution (L160H) that results in an unstable and inactive enzyme (Yamano et al., 1990). In mice a single nucleotide polymorphism causing a change in amino acid (V117A) in CYP2A5, and the resultant coumarin-hydroxylase activity, has also been described (Lindberg et al., 1992).

Another potential explanation for the greater CYP2A5 levels in male HNC mice is that mice that consume more nicotine may have induced CYP2A5, thus further increasing nicotine consumption. However, studies have shown that chronic nicotine administration decreases both the plasma clearance of nicotine in humans and CYP2A6 protein expression in monkeys (Benowitz and Jacob, 1993; Lee et al., 1987; Schoedel et al., 2003). Therefore it is unlikely that the higher CYP2A5 levels in the male HLC mice are the result of induction by nicotine consumption.

In addition to the nicotine metabolic pathway, other components (and their respective genetic variations) involved in nicotine sensitivity and reward properties could also affect nicotine intake, and many of these candidate genes have been investigated in humans (Tyndale, 2003). In inbred mice there are marked differences in response to nicotine across strains (Collins et al., 1988; Crawley et al., 1997; Marks et al., 1983). It is therefore possible that nicotine self-administration in mice may also be related to factors such as fluid intake and taste preference. Water consumption between the high (C57Bl/6) and the low (St/bJ) nicotine consumption parental strains do not differ, indicating that the difference in nicotine consumption is not related to fluid intake (Aschhoff et al., 1999). Lush and Holland (1998) found that the C57Bl/6 mice have a greater preference for the bitter compound copper glycinate compared to the St/bJ mice. However, this may be difficult to interpret with regards to nicotine since the ranking of copper glycinate
preference does not match that of nicotine preference in several other strains (Aschhoff et al., 1999; Lush and Holland, 1988).

In females, the higher overall CYP2A protein levels compared to males and lack of difference in CYP2A protein between consumption groups is likely due to the presence of the female specific CYP2A4 protein (Burkhart et al., 1985; Harada and Negish, 1984). The CYP2A4 protein is approximately 98% identical to CYP2A5 in amino acid sequence and they are indistinguishable from one another using available antibodies (Su et al., 1998). CYP2A4 metabolizes nicotine only at very high concentrations (more than 15-fold lower in affinity) and should not contribute to metabolism at the nicotine concentrations consumed (Murphy et al., 2005a). We therefore investigated the in vitro NCO activities between the two female groups. We observed no significant difference between the female high and low consumers in in vitro nicotine metabolism; however, the distribution of nicotine metabolism within each group is such that the majority of NCO activities were higher in the high consumption group compared to the low consumption group.

It appears that components in addition to nicotine metabolism have a greater contribution to nicotine consumption in females compared to males. The apparent lack of association between nicotine consumption levels and nicotine metabolism in females may be related to effects of steroids (Dempsey et al., 2002). For example, in humans, pregnancy increases plasma nicotine and cotinine clearances, but nicotine intake from cigarettes are similar between pregnant and post-partum females (Dempsey et al., 2002). On the other hand, in ICR mice, administration of exogenous progesterone or 17β-estradiol to female mice dose-dependently decreased the antinociceptive effect of nicotine, but administration of testosterone to male mice did not alter pain sensitivity (Damaj, 2001). The differential effects of these hormones were attributed to the ability of progesterone and 17β-estradiol to antagonize the binding of nicotine to the α4β2
nicotinic-receptors (Damaj, 2001). Therefore, female sex hormones may modulate nicotine-mediated behaviours (e.g. consumption) reducing the relative impact of nicotine metabolism in these mice.

In conclusion, we found that varying levels of nicotine self-administration behaviour in male mice were associated with different rates of nicotine metabolism, most likely mediated by genetic controls over the differing expression levels of the CYP2A5 protein. This observation is similar to that observed in smokers in which the amount smoked is related to the functional levels of CYP2A6 (Rao et al., 2000; Schoedel et al., 2004). Using inducers and inhibitors, as well as various mouse strains, we can model the influence of nicotine metabolism on various stages of smoking seen in humans such as initiation, acquisition, maintenance, and cessation.
Acknowledgements

We would like to thank Amir Boutrous for his assistance in protein analysis; Sharon Miksys for guidance and critical review of the manuscript; Helma Nolte and Raj Sharma for their assistance with HPLC assessment.

Support

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SIGNIFICANCE OF CHAPTER

This is the first study to demonstrate the association between CYP2A5 and the pharmacology of nicotine in mice. Specifically, we found that nicotine metabolism is associated with the amount of CYP2A5 (expression/activity) as contributed by genetic variations. More importantly, CYP2A5-mediated nicotine metabolism is associated with NSA in which mice exhibiting faster *in vitro* nicotine metabolism self-administered more nicotine. These findings are in line with humans in which CYP2A6 phenotype can have a significant impact on cigarette consumption behaviours.
CHAPTER 4: INHIBITION OF NICOTINE METABOLISM BY METHOXSALLEN: PHARMACOKINETIC AND PHARMACOLOGICAL STUDIES IN MICE

M. Imad Damaj, Eric C. K. Siu, Edward M. Sellers, Rachel F. Tyndale, and Billy R. Martin

The in vitro enzyme activities assays, analyses of the pharmacokinetic data, writing of the enzyme kinetics and pharmacokinetics section of the manuscript were performed by ECKS. The behavioural work were performed by MI Damaj. The study was designed by MI Damaj and RF Tyndale.

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Abstract

Studies were undertaken to examine whether methoxsalen, a specific and relatively selective inhibitor of human CYP2A6, inhibited CYP2A5-mediated nicotine metabolism in vitro. Furthermore, studies were performed in vivo to determine whether methoxsalen would modulate acute nicotine pharmacokinetics and pharmacological effects (antinociception and hypothermia) in ICR mouse. Our results demonstrated that methoxsalen competitively inhibits in vitro nicotine metabolism in mice. The inhibition was potent, as seen in human inhibition studies, with a $K_i$ of 0.32 µM. In addition, we found that administration of methoxsalen significantly increased the plasma half-life of nicotine (approximately doubled) and increased its AUC compared to saline treatment. There was a dose-dependent enhancement in the pharmacological effects of nicotine (body temperature and analgesia) after methoxsalen treatment. Methoxsalen prolonged the duration of nicotine-induced antinociception and hypothermia (2.5 mg/kg) for periods up to 180 min post-nicotine administration. Furthermore, this prolongation in nicotine’s effects after methoxsalen was associated with a parallel prolongation of nicotine plasma levels in mice. These data strongly suggest that variation in the rates of nicotine metabolic inactivation substantially alter nicotine’s pharmacological effects. In conclusion, these results confirmed that methoxsalen did indeed inhibit the conversion of nicotine to cotinine both in vitro and in vivo. They also suggest that mice may represent a suitable model for studying variation in nicotine metabolism and its impact on mechanisms of nicotine dependence, including the use of inhibitors to reduce nicotine metabolism.
INTRODUCTION

Nicotine is known to induce several physiological and pharmacological effects and to produce subjective feelings of reward and pleasure in humans and animals. Several well-characterized behavioral models and tests are currently used for investigating the biological and pharmacological mechanisms underlying nicotine dependence. These models reveal a high level of variability among nicotine behavioral responses due to factors such as differing genetic background, species, sex, age, initial basal and behavioral state, route and regimen of administration. In addition, the narrow effective dose range for many pharmacological and behavioral effects of nicotine further compounds the variability (see review by (Picciotto, 2003)). Species differences also represent an important consideration regarding in vivo responses to either acute or chronic nicotine exposure, particularly mice versus rats. In general, mice are less sensitive to the acute pharmacological effects of nicotine than rats. For example, nicotine’s potency in the tail-flick and hot-plate tests after systemic administration in mice is 5-10 fold lower than that reported for rats (Tripathi et al., 1982). One of the most striking differences concerns nicotine’s locomotor effects. Although nicotine induces locomotor hyperactivity in rats, it generally fails to induce locomotor hyperactivity in mice at any dose (Castane et al., 2002; Damaj and Martin, 1993; Freeman et al., 1987; Gaddnas et al., 2002; Itzhak and Martin, 1999; Kita et al., 1992; Marks et al., 1983; Smolen et al., 1994). In addition, even though i.v. nicotine self-administration can be accomplished in mice, a model using drug-naïve mice not restricted in their movement and a limited access schedule similar to that frequently used in rats is not reported. This rat-mouse variability can be attributed to various factors, such as pharmacokinetics, stress-induced by behavioral manipulations, physiological status, and/or psychological context. Given the very rapid metabolism of nicotine in mice (plasma and brain $t_{1/2} = 5.9-6.9$ min) (Petersen et al., 1984) compared to that of the rat (45 min) (Hwa Jung et al.,
distribution and kinetic factors appear to play an important role in the behavioral response to nicotine in mice. A recent study reported that nicotine oral self-administration in male mice is associated with the amount of nicotine metabolizing enzyme CYP2A5 as well as the rate at which nicotine is metabolized in vitro (Siu et al., 2006).

The main purpose of this study was to elucidate the impact of nicotine metabolism on nicotine’s acute pharmacological effects in mice. The mouse is a better-suited model for the study of nicotine metabolism and behaviors, compared to rats, since the predominant enzymes responsible for the metabolism of nicotine in rats belong to the CYP2B family (Nakayama et al., 1993; Schoedel et al., 2001; Siu et al., 2006). In contrast to rats, human CYP2A6 and mouse CYP2A5 (mouse orthologous form of human CYP2A6) are the main enzymes involved in nicotine metabolism (Messina et al., 1997; Murphy et al., 2005b). There is close structural (84% amino acid sequence similarity) and functional (CYP2A5 metabolizes nicotine with similar efficiency to human CYP2A6) similarity between CYP2A5 and CYP2A6 (Murphy et al., 2005b; Siu et al., 2006). In addition a similarly large portion of nicotine is metabolized to cotinine in humans and mice, relative to the rat, making the mouse a good model for the pharmacological impacts of manipulating nicotine metabolism in vivo. Accordingly, studies were undertaken to examine whether methoxsalen [a specific and relatively selective inhibitor of human CYP2A6, (Zhang et al., 2001)] inhibited CYP2A5-mediated nicotine metabolism in vitro. Subsequently studies were performed in vivo to determine whether methoxsalen would modulate acute nicotine pharmacokinetics and pharmacological effects (antinociception and hypothermia) in the mouse.
Materials and Methods

Animals
Male ICR mice (20-25g) obtained from Harlan Laboratories (Indianapolis, IN) were used throughout the study. Animals were housed in groups of six and had free access to food and water. Animals were housed in an AAALAC approved facility and the study was approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University.

Drugs
Mecamylamine hydrochloride was supplied as a gift from Merck, Sharp and Dohme & Co. (West Point, PA). (-)-Nicotine hydrogen tartrate was obtained from Aldrich Chemical Company, Inc. (Milwaukee, WI) and converted to the ditartrate salt as described by (Aceto et al., 1979) (1979). Methoxsalen was purchased from Sigma-Aldrich (St. Louis, MO). All drugs were dissolved in physiological saline (0.9% sodium chloride). All doses are expressed as the free base of the drug.

Membrane Preparations
Microsomal membranes were prepared from mouse livers for the in vitro nicotine metabolism assay as previously described (Messina et al., 1997; Siu et al., 2006) and stored at 80°C in 1.15% KCl. The cytosolic fractions were also acquired during the membrane preparation and were used as a source of aldehyde oxidase.

Nicotine C-Oxidation Assay
Assay conditions were optimized for ICR mouse microsomes as previously described (Siu et al., 2006). Linear formation of cotinine from nicotine was obtained under assay
conditions of 0.5 mg/ml protein concentration with an incubation time of 15 min. Incubation mixtures contained 0.5 mg/ml microsomal protein, 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°C in a final volume of 0.5 ml. The reaction was stopped with 4% Na₂CO₃, and 5-methylcotinine (70 µg) was added as the internal standard. \textit{In vitro} kinetic parameters (\(K_m\) and \(V_{max}\)) of nicotine metabolism in ICR mice were characterized using substrate concentrations of 0, 3.0 to 1000 \(\mu\text{M}\) nicotine. Nicotine metabolizing activity was analyzed by HPLC as previously described (Messina et al., 1997) with the modification of the solid phase extraction procedure for assessment of 3-hydroxycotinine (Siu et al., 2006). Separation of nicotine and cotinine was achieved using a ZORBAX Bonus-RP column (5 mm, 150 x 4.6 mm; Agilent Technologies Inc., Mississauga, ON) 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 isocratic elution at a flow rate of 0.9 ml/min. Nicotine, cotinine, and 3-hydroxycotinine sample concentrations were determined from standard curves. The limits of quantification were 5 ng/ml for nicotine and 12.5 ng/ml for cotinine.

\textit{Methoxsalen Inhibition of Cotinine Formation from Nicotine}

To determine the apparent \(K_i\) value of inhibition of cotinine formation by methoxsalen in the ICR mouse microsomes, 0, 0.04, 0.1, 0.2, and 0.4 \(\mu\text{M}\) methoxsalen were used. The substrate (nicotine) concentrations were 10, 20 and 40 \(\mu\text{M}\), which were approximately equal to \(1/2K_m\), \(K_m\), and \(2K_m\).

\textit{Plasma nicotine and cotinine levels measurement}

To determine plasma nicotine and cotinine levels, blood samples were drawn by cardiac puncture at 5, 15, 30, 45, 60, 120, and 180 minutes after nicotine administration (2.5
mg/kg, s.c.). Animals were pretreated with saline or methoxsalen for a variety of times before nicotine administration. Immediately afterwards the plasma samples were prepared by centrifugation at 3000 x g for 10 min and frozen at –20˚C until analysis. To measure total nicotine and cotinine levels (free and glucuronides) the samples were incubated with β-glucuronidase at a final concentration of 5 mg/ml in 0.2 M acetate buffer, pH 5.0, at 37°C overnight. After incubation the samples were processed and analyzed for nicotine and metabolite levels by HPLC as described above.

Behavioral tests

1. **Tail-flick test.** Antinociception was assessed by the tail-flick method of D'Amour and Smith (d'Amour and Smith, 1941) as modified by Dewey et al. (Dewey et al., 1970). A control response (2-4 sec) was determined for each mouse before treatment, and a test latency was determined after drug administration. In order to minimize tissue damage, a maximum latency of 10 sec was imposed. Antinociceptive response was calculated as percent maximum possible effect (% MPE), where % MPE = [(test-control)/(10-control)] x 100.

2. **Hot-plate Test.** Mice were placed into a 10 cm wide glass cylinder on a hot plate (Thermojust Apparatus) maintained at 55.0°C. Two control latencies at least ten min apart were determined for each mouse. The normal latency (reaction time) was 8 to 12 seconds. Antinociceptive response was calculated as percent maximum possible effect (% MPE), where % MPE = [(test-control)/(40-control) x 100]. The reaction time was scored when the animal jumped or licked its paws. In order to minimize tissue damage, a maximum latency of 40 sec was imposed.

Groups of eight to twelve animals were used for each dose and for each treatment. Studies were carried out by pretreating the mice with either saline or
methoxsalen 30 min before nicotine. The animals were tested 5 min after administration of nicotine.

3. **Body temperature.** Rectal temperature was measured by a thermistor probe (inserted 24 mm) and digital thermometer (Yellow Springs Instrument Co., Yellow Springs, OH). Readings were taken just before and at 30 min after the s.c. injection of either saline or nicotine. The difference in rectal temperature before and after treatment was calculated for each mouse. The ambient temperature of the laboratory varied from 21-24°C from day to day.

**Statistical analysis**

Kinetic (i.e. apparent $K_m$, $V_{max}$, $K_i$) parameters were calculated using Graphpad Prism (Graphpad Software Inc., San Diego, CA) and were verified by the Eadee-Hofstee plots. The type of inhibition by methoxsalen was further assessed by the Dixon method. Assessment of *in vivo* nicotine and cotinine plasma levels for the entire time course from individual animals was not possible due to limited blood volume; therefore, each time point represents data from individual mice. Due to this restriction to the experimental design, statistical parameters (i.e. half life) were estimated by resampling methods using the PKR and Test software (H. L. Kaplan, Toronto, ON). Statistical analysis of all analgesic and behavioral studies was performed using either t-test or analysis of variance (ANOVA) with Tukey’s test post hoc test when appropriate. All differences were considered significant if at $p < 0.05$. ED$_{50}$ values with 95% CL for behavioral data were calculated by unweighted least-squares linear regression as described by Tallarida and Murray (Tallarida and Murray, 1987).
Results

*In vitro nicotine C-oxidation activity of mice*

We first established the kinetic parameters apparent $K_m$ and $V_{max}$ for *in vitro* nicotine metabolism in ICR mice. Nicotine metabolism followed two-site Michaelis-Menten kinetics as illustrated by the Eadee-Hofstee plot (Fig. 13 & inset). The estimated kinetic parameters for the *in vitro* nicotine metabolism, derived from five animals, for both sites can be found in Table 8. One site exhibited high affinity for nicotine with an apparent $K_m$ of 18.6 ± 5.9 µM. The second site had a much lower affinity for nicotine with an apparent $K_m$ of 215.2 ± 60.0 µM. The overall catalytic efficiency ($V_{max}/K_m$) was greater for the high-affinity site compared to the low-affinity site (0.013 ± 0.007 vs. 0.003 ± 0.001).

*Inhibition of in vitro nicotine C-oxidation in mouse microsomes by methoxsalen*

Previous studies indicated that CYP2A5 is the primary enzyme responsible for nicotine metabolism in mice (Murphy et al., 2005b). We tested whether the human CYP2A6 inhibitor methoxsalen (Zhang et al., 2001) was also an inhibitor of nicotine metabolism in ICR mouse microsomes. Methoxsalen inhibited nicotine metabolism with a $K_i$ of 0.32 ± 0.03 µM (Fig. 14 & Table 8). Methoxsalen was a competitive inhibitor with some indication of a mixed-type of inhibition consistent with it also being a mechanism-based inhibitor *in vitro*.

*Effects of methoxsalen on nicotine and cotinine plasma levels after in vivo treatment*

Having established that methoxsalen can inhibit *in vitro* nicotine metabolism in the ICR mice, we tested the effect of the inhibitor on *in vivo* plasma nicotine and cotinine levels.
Figure 13. *In vitro* nicotine C-oxidation activity of ICR mice. Microsomal proteins (0.5 mg/ml) from adult male ICR mice were incubated with increasing concentrations of nicotine for 15 minutes. The plot shows the average cotinine formation velocity ± SD values for 5 individual animals. Inset: a representative Eadie-Hofstee plot indicating the presence of two enzymatic sites responsible for the metabolism of nicotine.
Table 8. Kinetic parameters of *in vitro* nicotine C-oxidation activities of adult male ICR mouse liver microsomes. $K_m$, $V_{max}$ and $K_i$ values were determined independently for each animal and the average ± SD values are shown.

<table>
<thead>
<tr>
<th></th>
<th>ICR (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>High Affinity</strong></td>
<td></td>
</tr>
<tr>
<td>$K_m$ (µM)</td>
<td>18.6 ± 5.9</td>
</tr>
<tr>
<td>$V_{max}$ (nmol/min/mg)</td>
<td>0.22 ± 0.05</td>
</tr>
<tr>
<td>$V_{max}/K_m$</td>
<td>0.013 ± 0.007</td>
</tr>
<tr>
<td><strong>Low Affinity</strong></td>
<td></td>
</tr>
<tr>
<td>$K_m$ (µM)</td>
<td>215.2 ± 60.0</td>
</tr>
<tr>
<td>$V_{max}$ (nmol/min/mg)</td>
<td>0.65 ± 0.08</td>
</tr>
<tr>
<td>$V_{max}/K_m$</td>
<td>0.003 ± 0.001</td>
</tr>
<tr>
<td><strong>Methoxsalen Inhibition</strong></td>
<td></td>
</tr>
<tr>
<td>$K_i$ (µM)</td>
<td>0.32 ± 0.03</td>
</tr>
</tbody>
</table>
Figure 14. A representative Dixon plot of inhibition of cotinine formation by ICR mouse microsomes in presence of varying concentrations of methoxsalen. In such plots, the abscissa of the point where the regression lines intersect is the negative of the estimated value of $K_i$. Three animals were used to establish $K_i$ value. Velocity is expressed as nmol/min/mg.
Administration of methosalen significantly increased the plasma half-life of nicotine and increased its AUC compared to saline treatment (Fig. 15A & Table 9). Methosalen had minimal effect on the maximum nicotine concentration (Table 9) consistent with the inhibition of nicotine given by the subcutaneous route. The plasma levels of the primary CYP2A5-mediated metabolite of nicotine, cotinine, were also reduced as a result of inhibition of this nicotine metabolic pathway by methosalen (Fig 15B).

*Effects of methosalen on nicotine acute pharmacological effects (antinociception and hypothermia): Time-course, potency and selectivity studies*

Methosalen given s.c. was evaluated for its ability to enhance a 2.5 mg/kg dose (an ED$_{84}$ dose) of nicotine-induced antinociception (tail-flick and hot-plate procedures) and hypothermia after nicotine s.c. injection of the drug. We first carried out a time-course study of methosalen (10 mg/kg) enhancement of nicotine’s effects to determine an optimal pretreatment time. Mice were given methosalen at different times and then received nicotine (2.5 mg/kg s.c.). The mice were tested 45 min after nicotine administration. As illustrated in Fig. 16A, the potentiation of nicotine’s antinociceptive effects by methosalen pretreatment in the tail-flick and hot-plate tests was time-dependent with maximum enhancement occurring between 30 and 60 min. A similar time-course was seen with nicotine-induced hypothermia (Fig. 16B) with maximum enhancement occurring between 30 and 120 min. Based on the time-course results, subsequent studies were carried out by pretreating the mice with methosalen 30 min before nicotine. We then determined its potency of enhancing nicotine’s effects at this pretreatment time. As showed in Fig. 17, methosalen dose-dependently enhanced nicotine-induced antinociception in the tail-flick (Fig. 17A), hot-plate (Fig. 17B) assays and hypothermia (Fig. 17C).
Figure 15. Time course of nicotine and cotinine plasma concentration in mice pretreated with methoxsalen. Nicotine was administered s.c. (2.5 mg/kg) 30 min after pretreatment with (---) saline or (----) methoxsalen. Each time point represents the mean ± SD of 4 to 6 animals. For the saline pretreatment nicotine plasma levels, five of seven values at 120 min, and all values at 180 min, were below the limits of detection. They were included in the plot as 1 ng/ml for visualization of the data.
Table 9. Pharmacokinetic parameters of nicotine in adult male ICR mouse after pretreatment with methoxsalen. Values are shown as mean ± standard deviation.

<table>
<thead>
<tr>
<th></th>
<th>$C_{\text{max}}$ (ng/ml)</th>
<th>$AUC$ (ng*hr/ml)</th>
<th>$T_{1/2}$ (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline/Nicotine</td>
<td>314 ± 170</td>
<td>132 ± 14</td>
<td>14.3 ± 8.1</td>
</tr>
<tr>
<td>Methoxsalen/Nicotine</td>
<td>354 ± 26</td>
<td>334 ± 24*</td>
<td>30.1 ± 13.5*</td>
</tr>
</tbody>
</table>

* $p < 0.05$ compared to Saline/Nicotine
Figure 16. Time-course of methoxsalen effects on nicotine-induced (A) antinociception and (B) hypothermia after s.c. administration of 10 mg/kg in mice. Nicotine was given at the dose of (2.5 mg/kg, s.c.) and animals were tested 45 min after injection. Each point represents the mean ± SE of 8 to 12 mice. *P <0.05 compared to correspondent zero time point.
Figure 17. Dose-response enhancement of nicotine by methoxsalen after s.c. administration in mice. Methoxsalen at different doses was administered s.c. 15 min before nicotine (2.5 mg/kg, s.c.) and mice were tested 45 min later in (A) the tail-flick and (B) the hot-plate tests. Each point represents the mean ± SE of 8 to 12 mice. *P <0.05 compared to saline (zero dose) treatment.
By itself, methoxsalen did not significantly change tail-flick or hot-plate basal latencies, or body temperature at the indicated doses and times. Furthermore, the antinociceptive and hypothermic effects of methoxsalen/nicotine combination were totally blocked by a pretreatment with mecamylamine, a non-competitive nicotinic antagonist, at 2 mg/kg (Fig. 18 & Table 10).

To determine whether methoxsalen produced similar effects on other analgesic substances, it was given at a dose of 10 mg/kg before the administration of an inactive dose of morphine. Pretreatment with methoxsalen failed to significantly enhance the effect of morphine in the tail-flick test [Saline/Saline = 7 ± 5 % MPE; Saline/Morphine (0.5 mg/kg, s.c.) = 20 ± 7 % MPE; Methoxsalen (10 mg/kg)/Morphine (0.5 mg/kg, s.c.) = 25 ± 8 % MPE]. Thus, the effect of methoxsalen appears not to be generalized to other analgesic substances since it did not enhance the effects of morphine after systemic administration.

Effects of methoxsalen on the time-course of nicotine’s pharmacological effects

The onset of action for nicotine (2.5 mg/kg, s.c.) in the tail-flick test was rapid with maximum antinociception occurring between 0 and 5 min. The duration of antinociception was relatively brief in that the effect had disappeared completely within 45 min after nicotine administration in mice (Fig. 19A). A similar time-course pattern was observed in the hot-plate test (Fig. 19B). The duration of nicotine-induced hypothermia was however longer compared to that of antinociception. Indeed, nicotine’s effect disappeared completely within 180 min after s.c. administration in mice (Fig. 19C). When animals were pretreated with methoxsalen (10 mg/kg, s.c.), the effects of nicotine in both analgesic tests were significantly prolonged. Nicotine-induced antinociception did not disappear completely till 180 min after nicotine administration in mice (Fig. 19A &
Figure 18. Blockade of methoxsalen’s effects on nicotine-induced antinociception in the tail-flick test after s.c. administration in mice. Animals were pretreated with either saline or mecamylamine (2 mg/kg, s.c.) followed by methoxsalen (10 mg/kg, s.c.). Mice received 30 min later nicotine at a dose of 2.5 mg/kg and were tested 45 min after injection. Each point represents the mean ± SE of 8 to 12 mice. MOP (10) = methoxsalen at 10 mg/kg; Sal: Saline; Nic (2.5): Nicotine at 2.5 mg/kg; Meca (2): Mecamylamine at 2 mg/kg. *P <0.05 compared to control.
Table 10. Blockade of methoxsalen’s effects on nicotine acute pharmacological responses after s.c. administration in mice. Animals were pretreated with either saline or mecamylamine (2 mg/kg, s.c.) followed by methoxsalen (10 mg/kg, s.c). Mice received 30 min later nicotine at a dose of 2.5 mg/kg and were tested 45 min after injection. Methoxsalen (10) = methoxsalen at 10 mg/kg; Meca (2): Mecamylamine at 2 mg/kg. Each point represents the mean ± SE of 8 to 12 mice.

<table>
<thead>
<tr>
<th>Treatment (mg/kg)</th>
<th>Tail-flick Test % MPE (Mean ± SEM)</th>
<th>Hot-plate Test % MPE (Mean ± SEM)</th>
<th>Body temperature Δ°C (Mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline/Saline/Saline</td>
<td>7 ± 2</td>
<td>8 ± 2</td>
<td>-0.1 ± 0.2</td>
</tr>
<tr>
<td>Saline/Methoxsalen (10)/Saline</td>
<td>10 ± 3</td>
<td>12 ± 5</td>
<td>-1.5 ± 0.2</td>
</tr>
<tr>
<td>Meca (2)/Methoxsalen (10)/Saline</td>
<td>15 ± 7</td>
<td>10 ± 5</td>
<td>-1.8 ± 0.5</td>
</tr>
<tr>
<td>Meca (2)/Saline/Saline</td>
<td>8 ± 3</td>
<td>6 ± 4</td>
<td>-0.5 ± 0.3</td>
</tr>
<tr>
<td>Meca (2)/Saline/Nicotine (2.5)</td>
<td>5 ± 2</td>
<td>6 ± 2</td>
<td>-1.9 ± 0.6*</td>
</tr>
<tr>
<td>Saline/Saline/Nicotine (2.5)</td>
<td>10 ± 5</td>
<td>15 ± 5</td>
<td>-4.1 ± 0.6</td>
</tr>
<tr>
<td>Saline/Methoxsalen (10)/Nicotine (2.5)</td>
<td>80 ± 10*</td>
<td>83 ± 8*</td>
<td>-6.8 ± 0.4*</td>
</tr>
<tr>
<td>Meca (2)/Methoxsalen (10)/Nicotine (2.5)</td>
<td>15 ± 8</td>
<td>23 ± 10</td>
<td>-2.2 ± 0.5*</td>
</tr>
</tbody>
</table>

*P <0.05 compared to control.
Figure 19. Effects of methoxsalen on the time-course of nicotine’s effects in (A) the tail-flick test, (B) the hot-plate assay and (C) body temperature in mice. Animals were pretreated with either (---) saline or (-----) methoxsalen (10 mg/kg, s.c) and 30 min later received nicotine at a dose of 2.5 mg/kg. Mice were tested at different times after injection. Each point represents the mean ± SE of 8 to 12 mice. *P <0.05 compared to control.
B). On the other hand, the effects of nicotine on body temperature were still significant beyond the 180 min time point (Fig. 19C). Further times were not evaluated.

The prolongation of nicotine’s acute pharmacological effects correlated well with the prolongation of nicotine plasma concentrations after methoxsalen administration in mice. Indeed, as shown in Figure 20, there was a corresponding increase in nicotine's sensitivity in the tail-flick test to the increased plasma concentration of nicotine after methoxsalen pretreatment as measured by the time-course curves. A comparable profile can also be seen in the hot-plate test and body temperature changes (data not shown).
Figure 20. Relationship between nicotine-induced antinociception in the tail-flick test and plasma levels of nicotine in the ICR mice after methoxsalen treatment. MOP = methoxsalen; Sal: Saline; TF = Tail-flick effect; Conc: plasma concentration of nicotine.
Discussion

A previous study examining nicotine metabolism in C57Bl/6 and DBA/2 mice found that nicotine is metabolized by two enzymatic sites with the high affinity sites having $K_m$'s of $\sim 10 \mu M$ (Siu et al., unpublished) consistent with the nicotine metabolism mediated by cDNA-expressed CYP2A5 ($K_m = 7.7 \pm 0.8 \mu M$; 129/J mouse strain) (Murphy et al., 2005b). This current study indicates that ICR mice microsomes also contain two enzymatic sites; the high affinity site had a modestly lower affinity for nicotine ($\sim 18 \mu M$) compared to that observed in C57Bl/6 and DBA/2 mice. Such small differences may be attributed to unidentified polymorphisms in the ICR mouse strain altering the structure of the enzyme to a minor extent (Lindberg et al., 1992). The low affinity enzyme site in mice may be a member of the CYP2B family consistent with the low affinity site in human and monkey hepatic nicotine metabolism studies (Messina et al., 1997; Schoedel et al., 2003); the rat CYP2B can also metabolize nicotine (Schoedel et al., 2001).

The present study was the first to demonstrate that the human CYP2A6 inhibitor methoxsalen (Zhang et al., 2001) can inhibit \textit{in vitro} nicotine metabolism in mice. The inhibition was competitive with some indication of mixed inhibition. The inhibition was potent, as seen in human inhibition studies, with a $K_i$ of 0.32 $\mu M$, indicating a 60-fold higher affinity for the mouse enzyme compared to nicotine ($K_m = 19 \mu M$, Table 8). These pharmacokinetic values are similar to human where the $K_m$ is estimated to be 65 $\mu M$ in human liver microsomes (Messina et al., 1997) and the $K_i$ was found to be 0.01 $\mu M$ (Zhang et al., 2001). As methoxsalen may be able to inhibit both CYP2A5 and CYP2B enzymes in mice (Maenpaa et al., 1994), it is possible that methoxsalen reduces nicotine metabolism by inhibiting both the high affinity CYP2A5 enzyme site (Murphy et al., 2005b; Siu et al., 2006) as well as the second low affinity site. The \textit{in vitro} mouse pharmacokinetic data (Table 8) suggest that even at the very high concentrations initially
achieved following 2.5 mg/kg s.c. nicotine injections (100-300 ng/ml) that approximately 80% of the metabolism is mediated by the high affinity site kinetic site (Table 8).

Several studies have suggested that methoxsalen acts as a competitive, non-competitive and/or mechanism-based inhibitor of human CYP2A6 in vitro (Draper et al., 1997; Koenigs et al., 1997; Maenpaa et al., 1994; Zhang et al., 2001). Methoxsalen potently inhibited CYP2A6-mediated nicotine metabolism in vivo in humans however a clear indication of mechanism-based inhibition was not seen using either coumarin (Kharasch et al., 2000) or nicotine (Sellers et al., 2000) as a substrate.

This is the first study to examine the pharmacokinetic impact of methoxsalen in vivo in mice. We found that the half-life of plasma nicotine in mice treated with methoxsalen was significantly longer (approximately doubled) compared to the saline inhibition group. In addition, as evident in Fig. 16, methoxsalen’s effects increased with pretreatment times from zero to 60 minutes suggesting that this pretreatment time enhanced methoxsalen’s ability to inhibit nicotine metabolism. Together with the in vitro data this suggests that methoxsalen may be acting in vivo in mice as a competitive inhibitor and through an additional mechanism, possibly mechanism-based inhibition. It has been shown in vitro that mouse CYP2A5 can metabolize methoxsalen (Maenpaa et al., 1994). Mechanism-based inhibitors, also known as suicide substrates, are compounds which are metabolically activated by the enzyme and the metabolite binds irreversibly to the enzyme inactivating it. Therefore, it is possible that metabolites of methoxsalen, even if produced by other enzymes such as CYP2B, may inhibit the CYP2A5 through a variety of mechanisms including mechanism-based inhibition, competitive inhibition and/or binding to the heme moiety of the enzyme. Together this suggests that preinhibition with methoxsalen, prior to administration of a substrate such as nicotine, may lead to enhanced metabolic inhibition via one of these mechanisms. The similar maximal plasma nicotine levels seen with and without pretreatment with
methoxsalen are consistent with the subcutaneous route of nicotine’s administration, but predicts a large increase in $C_{\text{max}}$ if nicotine is given by an oral route where the inhibition would also block a first pass metabolism.

Our results confirmed that methoxsalen did indeed inhibit the conversion of nicotine to cotinine both \textit{in vitro} and \textit{in vivo}. Pretreatment with methoxsalen increased the AUC 2.5 fold and prolonged the nicotine plasma levels; it was also associated with a 2-fold increase in the half-life of the drug (Table 9). The nicotine plasma levels remained above 10 ng/ml in the saline pretreatment for approximately 90 minutes but with pretreatment with methoxsalen the nicotine plasma levels were still above 10 ng/ml at 180 min. Similar to the marked effects of CYP2A5 inhibition on nicotine metabolism, there were dramatic enhancement in the pharmacological effects of nicotine (body temperature and analgesia) after methoxsalen treatment. The effects of methoxsalen on nicotine were dose-dependent and reached maximal enhancement at a dose of 15 mg/kg. Furthermore, the duration of methoxsalen-induced potentiation of nicotine’s antinociceptive and hypothermic lasted close to 180 min after drug pretreatment.

Pharmacogenetic studies in humans have showed that smoking behaviours are affected by polymorphisms in CYP2A6 that alter nicotine metabolism (Ariyoshi et al., 2002; Malaiyandi et al., 2006; Minematsu et al., 2006; Rao et al., 2000; Schoedel et al., 2004). For instance, individuals who are slow metabolizers of nicotine (e.g. those with 50% of less activity due to having inactive and/or decreased function alleles) smoked significantly fewer cigarettes compared to individuals that are normal nicotine metabolizers (those without variants) (Ariyoshi et al., 2002; Malaiyandi et al., 2006; Rao et al., 2000; Schoedel et al., 2004). Furthermore, inhibition of orally delivered nicotine in smokers using methoxsalen (to phenocopy slower metabolism found in those with defects in CYP2A6) led to increases in plasma nicotine levels, reduction of cravings for nicotine, and fewer numbers of cigarettes smoked relative to nicotine alone;
methoxsalen alone, in smokers, also increased the latency between lighting the first and second cigarette (Sellers et al., 2000). Consistent with human data, we have shown that CYP2A5 levels and \textit{in vitro} nicotine metabolism rates are associated with nicotine oral self-administration in male mice (Siu et al., 2006). These findings suggest that mice may represent a suitable model for studying variation in nicotine metabolism, including the use of inhibitors to reduce nicotine metabolism.

The main purpose of the current study was to determine the impact of nicotine metabolism (i.e. inhibition) on nicotine’s acute pharmacological effects in mice. Methoxsalen prolonged the duration of nicotine-induced antinociception and hypothermia (2.5 mg/kg) for periods up to 180 min post-nicotine administration. Although this increase in nicotine’s time course was seen in all responses measured, it was more dramatic in antinociceptive tests (an additional 150 min compared to the control group). This differential effect is probably due to the fact that nicotine-induced antinociception time-course is much shorter than the effects on body temperature. Furthermore, this prolongation in nicotine’s effects after methoxsalen was associated with a parallel prolongation of nicotine plasma levels in mice (Figure 20). Consistent with these present results, we have shown that CYP2A5 levels and \textit{in vitro} nicotine metabolism rates are associated with nicotine oral self-administration in male mice (Siu et al., 2006). These findings suggest that mice may represent a suitable model for studying variation in nicotine metabolism and its impact on mechanisms of nicotine dependence, including the use of inhibitors to reduce nicotine metabolism. Although the study of nicotine’s acute effects is important to the understanding of nicotine dependence, one must also consider responses after chronic exposure of the drug in animals. Animal models studying the different aspects of dependence such as tolerance, withdrawal and reward involve a chronic/repeated exposure of the animals to nicotine. Assessing the impact of nicotine metabolism in these behavioral models will
enhance our understanding of the molecular mechanisms involved in nicotine dependence and will facilitate the development of new strategies for smoking cessation therapies.

These data strongly suggest that variation in the rates of nicotine metabolic inactivation substantially alter nicotine’s pharmacological effects. These data suggest that in mice, with very rapid rates of metabolism, small differences in levels or function of CYP2A5 between different mouse strains may result in substantially differing nicotine pharmacological effects. Thus pharmacokinetic differences likely contribute to differences in effects seen between studies employing the use of different mouse strains.

In conclusion we have shown that the human CYP2A6 inhibitor methoxsalen is also a potent inhibitor of mouse CYP2A5, both in vitro and in vivo. We have shown that the pharmacological effects of inhibiting nicotine’s metabolism are profound, illustrating the differences in resulting effects of nicotine that will occur between individuals and also between animal strains that differ in levels of CYP2A-mediated nicotine metabolism.
Acknowledgments

The authors greatly appreciate the technical assistance of Tie Han and W Zhang.

Support

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Disclosures

EMS and RFT are shareholders in Nicogen, a company focused on novel treatment approaches involving inhibition of hepatic CYP2A6. No support from Nicogen was used and no benefit to the company was obtained.
SIGNIFICANCE OF CHAPTER

This chapter demonstrated the application of the inhibition of nicotine metabolism on nicotine pharmacology in a mouse model. Specifically, the human CYP2A6 inhibitor methoxsalen significantly inhibited the CYP2A5-mediated nicotine metabolism in mice both in vitro and in vivo. The inhibition of nicotine metabolism in mice led to a substantial increase in nicotine’s pharmacological effect (i.e. tail-flick and hot-plate tests).
CHAPTER 5: SELEGILINE (L-DEPRENYL) IS A MECHANISM-BASED INACTIVATOR OF CYP2A6 INHIBITING NICOTINE METABOLISM IN HUMANS AND MICE

Eric C. K. Siu and Rachel F. Tyndale
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All of the experiments, data analyses, and writing of the manuscript were performed by ECK Siu. The study was designed by ECK Siu and RF Tyndale.
Abstract

Selegiline (L-deprenyl) is in clinical treatment trials as a potential smoking cessation drug. We investigated the impact of selegiline and its metabolites on nicotine metabolism. In mice, selegiline was a potent inhibitor of nicotine metabolism in hepatic microsomes and cDNA-expressed CYP2A5; the selegiline metabolites desmethylselegiline, L-methamphetamine, and L-amphetamine, also inhibited nicotine metabolism. Pretreatment with selegiline and desmethylselegiline increased inhibition (IC$_{50}$) in microsomes by 3.3- and 6.1-fold, respectively. In mice in vivo, selegiline increased AUC (90.7±5.8 vs. 57.4±5.3 ng • hr/ml, $p < 0.05$), decreased clearance (4.6±0.4 vs. 7.3±0.3 ml/min, $p < 0.05$), and increased elimination half-life (12.5±6.3 vs. 6.6±1.4 min, $p < 0.05$) of nicotine. In vitro, selegiline was a potent inhibitor of human nicotine metabolism in hepatic microsomes and cDNA-expressed CYP2A6; desmethylselegiline and L-amphetamine also inhibited nicotine metabolism. Selegiline pre-incubation increased inhibition in microsomes (3.7-fold) and CYP2A6 (14.8-fold); the $K_i$ for CYP2A6 was 4.2 µM. Selegiline dose- and time-dependently inhibited nicotine metabolism by CYP2A6 ($K_i = 15.6±2.7$ µM; $k_{\text{inact}} = 0.34±0.04$ min$^{-1}$) and the inhibition was irreversible in the presence of NADPH, indicating that it is a mechanism-based inhibitor of CYP2A6. Thus, inhibition of mouse nicotine metabolism by selegiline was competitive in vitro, and significantly increased plasma nicotine in vivo. In humans where selegiline is both a competitive and mechanism-based inhibitor, it is likely to have even greater effects on in vivo nicotine metabolism. Our findings suggest that an additional potential mechanism of selegiline in smoking cessation is through inhibition of nicotine metabolism.
Introduction

Nicotine is the primary psychoactive component in tobacco responsible for the addictive properties of cigarettes (Henningfield and Keenan, 1993). One action of nicotine is the binding to nicotinic receptors stimulating dopamine release in the nucleus accumbens, an area of the brain responsible for reward (Balfour, 2004). In the brain, dopamine is metabolized by monoamine oxidases A and B (MAO-A and MAO-B) (Youdim et al., 2006) although MAO-B appears to be the major form (Fowler et al., 1987). The amount of MAO-B activity in the brain of smokers is ~40% lower compared to non-smokers (Fowler et al., 1996a) and this inhibition of MAO-B is reversed during long-term smoking abstinence (Gilbert et al., 2003), suggesting the presence of a MAO-B inhibitor in tobacco smoke (Khalil et al., 2000). These findings suggest that the reduced metabolism of dopamine, as mediated by the inhibition of MAO, may enhance the rewarding effects of nicotine during smoking (Lewis et al., 2007).

Selegiline (L-deprenyl) is a selective and irreversible MAO-B inhibitor used in conjunction with L-dopa to alleviate symptoms associated with Parkinson’s disease (Gerlach et al., 1996). Due to its ability to reduce dopamine metabolism, selegiline has been investigated as a potential therapy for smoking cessation. Several small-scale studies have shown that selegiline is effective in reducing withdrawal symptoms and increasing abstinence compared to placebo. For instance, in one study 10 mg of oral selegiline decreased craving during abstinence and reduced smoking satisfaction during smoking (Houtsmuller et al., 2002). In another study, 5 mg b.i.d. oral selegiline increased the trial end point (8-week) 7-day point prevalent abstinence compared with placebo by three-fold (George et al., 2003). In a third study that used a combination of oral selegiline and nicotine patch, selegiline plus nicotine patch doubled the 52-week continuous abstinence rate compared to nicotine patch alone although the difference was not
significant due to small subject numbers (Biberman et al., 2003). Other MAO inhibitors have also been investigated for their effects on smoking cessation, but due to poor efficacy (moclobemide) or toxicity (lazabemide) they are no longer studied (McRobbie et al., 2005).

Selegiline is metabolized to desmethylselegiline and L-methamphetamine, both of which can be further metabolized to L-amphetamine as well as other minor metabolites (Fig. 21) (Shin, 1997; Valoti et al., 2000); despite this, there is no evidence indicating that selegiline is addictive (Schneider et al., 1994). In humans, chronic treatment with selegiline reduces the metabolism of selegiline and its metabolites, suggesting that selegiline or its metabolites may inhibit or down-regulate its own metabolic enzymes (Laine et al., 2000). Selegiline belongs to the acetylene group of compounds that contain a carbon-carbon triple bond, which are known to be potent mechanism-based inhibitors (Correia and Ortiz de Montellano, 2005). Since the main human nicotine metabolizing enzyme CYP2A6 appears to play a role in the metabolism of selegiline in vitro (Benetton et al., 2007), we examined whether selegiline and its metabolites (desmethylselegiline, L-methamphetamine, and L-amphetamine) (Shin, 1997) could inhibit nicotine metabolism in vitro in human and mouse hepatic microsomes, as well by both cDNA-expressed major human nicotine metabolizing enzyme CYP2A6 and mouse CYP2A5 (Murphy et al., 2005b; Siu and Tyndale, 2007a). Genetically slow CYP2A6 metabolizers have a greater likelihood (1.75-fold) of quitting smoking (Gu et al., 2000) suggesting that if selegiline inhibits nicotine metabolism, this may be an additional mechanism through which it reduces smoking. In addition, selegiline could potentially be used to enhance the efficacies of current nicotine
Figure 21. Selegiline and its metabolites. Selegiline is metabolized to desmethyloselegiline and L-methamphetamine, both of which can be metabolized to L-amphetamine (Shin, 1997; Valoti et al., 2000).
replacement therapies, or it could be combined with nicotine as an oral combination therapy with nicotine for smoking cessation.
Materials and Methods

Animals

Adult male DBA/2 mice, previously characterized in nicotine metabolism studies (Siu and Tyndale, 2007a), 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.

Reagents

(-)-Nicotine hydrogen tartrate, (-)-cotinine, selegiline (R-(-)-deprenyl hydrochloride), R-(-)-desmethylselegiline, thiamine hydrochloride, and 5-aminolevulinic acid (ALA) were purchased from Sigma-Aldrich (St. Louis, MO). L-methamphetamine and L-amphetamine were purchased from Research Biochemicals Inc. (Natwick, MA). The internal standard 5-methylcotinine was custom-made by Toronto Research Chemicals (Toronto, ON). All dosed drugs are expressed as the free base of the drug. Ampicillin and lysozyme were purchased from BioShop Canada (Burlington, ON). IPTG (Isopropyl-beta-D-thiogalactopyranoside) was purchased from MBI Fermentas (Burlington, ON). The monoclonal antibody to human CYP2A6 was purchased from BD Biosciences (Mississauga, ON). Horseradish peroxidase-conjugated anti-mouse secondary antibody and enhanced chemiluminescence were purchased from Pierce Biotechnology (Rockfort, IL). Nitrocellulose membrane was purchased from Bio-Rad Labs (Mississauga, ON). Autoradiographic film was purchased from Ultident Scientific (St. Laurent, PQ).

Membrane Preparations

Microsomal membranes were prepared from mouse and human livers for in vitro nicotine metabolism assays as previously described (Siu et al., 2006) and stored at -80°C in
1.15% KCl. The human liver is from the previously characterized K-series liver bank (Messina et al., 1997) and was a generous gift from Dr. T. Inaba at the University of Toronto. The cytosolic fractions were acquired during membrane preparation and were used as a source of aldehyde oxidase. All mouse livers and plasma samples were collected and frozen prior to 3 pm to minimize circadian effects on CYP2A5 expression. Membrane protein concentrations were determined with Bradford reagent according to manufacturer's protocol (Bio-Rad Labs Ltd., Mississauga, ON).

Expression of CYP2A5

Cyp2a5 cDNA vector in *Escherichia coli* was a generous gift from Dr. Xinxin Ding (Wadsworth Center, New York State Department of Health, NY) and prepared as previously described with modifications (Gu et al., 1998). Briefly, *E. coli* colonies from ampicillin plates were inoculated in 2 ml of LB broth with 100 μg/ml of ampicillin and incubated overnight (no more than 16 hours) at 37°C shaking at 200 rpm. The culture was then diluted (1:100) in TB broth, with final concentrations of 100 μg/ml of ampicillin, 1 mM thiamine, 0.5 ml of ALA, and 1 mM IPTG, and incubated for 48 hours at 25°C shaking at 150 rpm. Following incubation the culture was centrifuged at 2800xg at 4°C for 20 min and the pellet was resuspended in 1/20th culture volume of ice-cold TSE buffer (100 mM Tris, pH 7.4, 0.1 mM EDTA, 0.1 mM dithiothreitol, 1.15% KCl, 110 g/L sucrose) and 1/20th culture volume of ice-cold water. Lysozyme was then added to a final concentration of 0.25 mg/ml and this was shaken gently at 4°C for 1 hr followed by centrifugation at 2800xg at 4°C for 20 min. After centrifugation the pellet was resuspended in 1/25th culture volume of ice-cold TE buffer. The resuspension was placed on ice and sonicated in 3 x 30 sec bursts with a Branson Digital Sonifier (Model S-450D; Branson Ultrasonics, Markham, ON) set at 30% output. The suspension was
spun at 12,000xg at 4°C for 12 min and the supernatant was re-spun at 180,000xg at 4°C for 60 min. The pellet containing the bacterial membrane fraction was resuspended in 1.15% KCl and stored at -80°C.

Quantification by Immunoblotting of CYP2A5
Immunoblotting was performed for CYP2A5 and the reference lymphoblastoid cDNA-expressed human CYP2A6 (BD Biosciences, San Jose, CA) essentially as previously described (Siu et al., 2006). To determine the linear ranges of detection of CYP2A5 and CYP2A6, in order to quantify the amount of CYP2A5 in the bacterial membrane, the bacterial membrane fraction and CYP2A6 were serially diluted from 0.25 to 2 µg protein and from 0.06 to 1.5 pmol, respectively.

In Vitro Nicotine C-Oxidation Assay
The linear conditions for nicotine C-oxidation to cotinine (nicotine metabolism) in DBA mouse liver microsomes were previously described (Siu et al., 2006) (0.5 mg/ml protein; 15 min). In human liver microsomes, the linear conditions of nicotine metabolism were obtained under assay conditions of 0.5 mg/ml protein with an incubation time of 20 min. For CYP2A5, the linear conditions of nicotine metabolism were obtained under assay conditions of 60 pmol P450/ml, 60 pmol of reductase and cytochrome b₅ (Invitrogen, Carlsbad, CA) with an incubation time of 15 min. For expressed CYP2A6 (containing P450 reductase and cytochrome b₅) (BD Gentest, Woburn, MA), the linear conditions of nicotine metabolism were obtained under assay conditions of 20 pmol P450/ml with an incubation time of 15 min. All incubation mixtures contained 1 mM NADPH and 1 mg/ml of liver cytosol in 50 mM Tris-HCl buffer, pH 7.4 and were performed at 37°C in a final volume of 0.5 ml. Unless specified, nicotine concentrations used were 10 µM (mouse liver microsomes), 60 µM (CYP2A5), 20 µM (human liver microsomes), and 100 µM
(CYP2A6). The reactions were stopped with a final concentration of 4% v/v Na₂CO₃. 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 as described previously (Siu et al., 2006). The limits of quantification were 5 ng/ml for nicotine and 12.5 ng/ml for cotinine.

In Vitro Inhibition of Nicotine Metabolism

In all in vitro inhibition experiments assays were conducted as above with the addition of the inhibitors at the same time as nicotine. In experiments with the pre-incubation step, reactions containing increasing concentrations of the inhibitor were initiated by pre-warming the mixture for two minutes prior to the addition of NADPH and pre-incubation of 15 minutes at 37°C prior to addition of nicotine and incubation.

For the NADPH-dependent inactivation experiment, 0 or 100 µM of selegiline was added to the mixture in the presence or absence of NADPH. Following pre-incubation, samples were loaded into the Microcon YM-30 centrifuge membrane filters (Dyck and Davis, 2001) (Millipore, Etobicoke, ON) and centrifuged for at least 30 min at 4°C. Retentates (typically 40-50 µl) were added to fresh reaction mixtures containing nicotine and the reactions were allowed to proceed.

In Vivo Inhibition of Nicotine Metabolism

Nicotine and selegiline were dissolved in physiological saline (0.9% sodium chloride) and adjusted to pH 7.4 for use in in vivo studies. All animals were injected intraperitoneally with nicotine or nicotine plus selegiline (both at 1 mg/kg). The nicotine dose was chosen based on previous pharmacokinetic studies (Siu and Tyndale, 2007a) and relevance in behavioural models for nicotine in mice (Marks et al., 1985). The selegiline dose was based on its relevance in the mouse MPTP-model of Parkinson’s
disease (Fredriksson and Archer, 1995). Blood samples were drawn by cardiac puncture at various times after the injection. Immediately after collection plasma was prepared by centrifugation at 3000xg for 10 min and kept at –20˚C until analysis. Total plasma nicotine 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˚C overnight. Samples were then analyzed by HPLC.

In Vitro Kinetic and Pharmacokinetic Parameters Analyses and Statistical Analyses

The Michaelis-Menten kinetic parameters $K_m$ and $V_{max}$ were calculated using Graphpad Prism (Graphpad Software Inc., San Diego, CA) and were verified by the Eadie-Hofstee method. The equation used to determine $K_m$ and $V_{max}$ was $v = V_{max} \frac{[S]}{K_m + [S]}$ (Eqn. 1) where [S] denotes substrate concentration. These kinetic parameters were used to determine nicotine concentrations used. Statistical analyses of in vitro kinetic parameters were tested by student's t-test.

The in vivo pharmacokinetic parameters were determined using non-compartmental analysis: $AUC_{0-40}$ was calculated using the trapezoidal rule. Elimination half-life ($t_{1/2}$) was estimated by the terminal slope. Since the bioavailability ($F$) of nicotine was unknown following intraperitoneal injection in mice, $CL$ (clearance) was determined as a hybrid parameter $CL/F$ and was calculated as $Dose/AUC_{0-40}$. The average weights of the animals were similar (24.4 ± 0.7 g, n=24), therefore an estimated dose of 25 µg was used for the calculation of $CL/F$ for nicotine. Assessment of in vivo nicotine 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) (Siu and Tyndale, 2007a).
Results

Inhibition of Nicotine Metabolism by Selegiline and Metabolites

Selegiline and desmethylselegiline had the highest inhibitory activities on nicotine metabolism in mouse liver microsomes (MLMs) while L-methamphetamine and L-amphetamine had smaller effects (Fig. 22a). Selegiline also inhibited CYP2A5 to a great extent while desmethylselegiline, L-methamphetamine, and L-amphetamine had similar inhibitory effects (Fig. 22b). In human liver microsomes (HLMs), selegiline and L-amphetamine appeared to show the greatest inhibitory activities on nicotine metabolism closely followed by desmethylselegiline (Fig. 22c), while L-methamphetamine did not inhibit cotinine formation (Fig. 22c). As with the three previous enzyme sources, selegiline caused the greatest inhibition on nicotine metabolism in CYP2A6 (Fig. 22d). Desmethylselegiline and L-amphetamine showed almost similar inhibition whereas L-methamphetamine did not alter nicotine metabolism (Fig. 22d), as was seen with HLMs (Fig. 22c).

The above findings suggested that selegiline had the greatest inhibitory effect on CYP2A5-mediated nicotine metabolism in vitro in mice followed by selegiline’s three metabolites. Likewise, selegiline, desmethylselegiline, and L-amphetamine could inhibit CYP2A6-mediated human nicotine metabolism in vitro whereas L-methamphetamine did not.

Effects of Selegiline and Desmethylselegiline Pre-incubation on Nicotine Metabolism in Mouse Liver Microsomes and CYP2A5

As selegiline and desmethylselegiline contain a carbon-carbon triple bond found in mechanism-based inhibitors (MBI), the effects of pre-incubation of these compounds on
Figure 22. Selegiline and its metabolites inhibited nicotine metabolism. a) MLMs, b) CYP2A5, c) HLMs, and d) CYP2A6 were incubated with 0 (control), 10, and 100 µM of selegiline (SEL), desmethylselegiline (DES), L-methamphetamine (L-MAMP), or L-amphetamine (L-AMP) in the presence of nicotine and analyzed for cotinine formation. Activity remaining for each compound was compared to the corresponding control treatment. Each data point represented the average of 2 to 5 independent experiments. * $p < 0.05$ compared to control.
nicotine metabolism in MLMs and CYP2A5 were investigated. If pre-treatment increased inhibition, this could indicate that the inhibitor is either acting as a MBI or is metabolized to a more potent inhibitor during the pre-incubation. Selegiline dose-dependently inhibited cotinine formation in both MLMs and CYP2A5; pre-incubation with selegiline decreased the IC$_{50}$ in MLMs (3.3-fold) but not in CYP2A5 (Figs. 23a & b). Desmethylselegiline also dose-dependently inhibited nicotine metabolism in MLMs but its effect was much weaker in expressed CYP2A5 (Figs. 23c & d). As with selegiline, pre-incubation with desmethylselegiline enhanced inhibition in MLMs (6.1-fold change in IC$_{50}$) but not in CYP2A5 (Figs. 23c & d). This indicated that selegiline and desmethylselegiline were competitive inhibitors, but not MBIs, of CYP2A5 in vitro; and other enzymes in MLMs can metabolize selegiline and desmethylselegiline to inhibitors of greater potency.

**Effect of Selegiline on Nicotine Metabolism in vivo in DBA/2 Mice**

To determine if selegiline could also inhibit nicotine metabolism in vivo, we treated DBA/2 mice with selegiline and nicotine. Selegiline co-administration decreased the clearance of nicotine by ~40%, resulting in 58% greater AUC and almost doubling the elimination half-life (Fig. 24, Table 11). These results demonstrated that selegiline is an effective inhibitor of nicotine metabolism in vivo in mice.

**Effects of Selegiline and Desmethylselegiline Pre-incubation on Nicotine Metabolism in Human Liver Microsomes and CYP2A6**

To further investigate the effect of selegiline and desmethylselegiline on nicotine metabolism in humans, HLMs and CYP2A6 were pre-incubated with the inhibitors. Selegiline dose-dependently inhibited cotinine formation in both HLMs and expressed
Figure 23. Pre-incubation of MLMs, but not CYP2A5, with selegiline increased inhibition of nicotine metabolism. MLMs (a & c) and CYP2A5 (b & d) were pre-incubated (+PRE) for 15 minutes with increasing concentrations (0, 0.3, 1, 3, 10, 30, and 100 µM) of selegiline (a & b) or desmethylselegiline (c & d) prior to addition of nicotine and an additional incubation of 15 minutes. Samples were also incubated with the inhibitors and nicotine without pre-treatment (-PRE). Activity remaining was calculated as % control (no inhibitor, same incubation conditions) within each treatment group. IC<sub>50</sub> was determined as the concentration of inhibitor required to decrease nicotine metabolism by 50%. Each data point represented the average of 2 to 5 independent experiments. * p < 0.05 compared to no pre-incubation.
Figure 24. Selegiline decreased nicotine metabolism in vivo in DBA/2 mice. DBA/2 mice were injected with nicotine (1 mg/kg, i.p.) (-SEL) or with nicotine plus selegiline (1 mg/kg, i.p.) (+SEL). Following nicotine injection plasma samples were collected at the indicated time points and analyzed for nicotine levels. Each time point represents mean (± S.D.) of 3 to 6 animals for each treatment; statistical comparisons are listed in Table 1.
Table 11. Pharmacokinetic parameters of plasma nicotine in mice treated intraperitoneally with nicotine (1 mg/kg) or nicotine plus selegiline (1 mg/kg). Results were derived using data (Fig. 3) from three to six animals at each time point.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>-SEL (mean ± SD)</th>
<th>+SEL (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$AUC_{0-40}$ (ng • hr/ml)</td>
<td>57.4 ± 5.3</td>
<td>90.7 ± 5.8*</td>
</tr>
<tr>
<td>$t_{1/2}$ (min)</td>
<td>6.6 ± 1.4</td>
<td>12.5 ± 6.3*</td>
</tr>
<tr>
<td>$CL/F$ (ml/min)</td>
<td>7.3 ± 0.3</td>
<td>4.6 ± 0.4*</td>
</tr>
</tbody>
</table>

*p < 0.05 compared to –SEL
CYP2A6 (Figs. 25a & b). In contrast to the mouse enzymes, pre-incubation of both HLMs and CYP2A6 with selegiline decreased the IC$_{50}$ by 3.7- and 14.8-fold, respectively (Figs. 25a & b). Desmethylselegiline also dose-dependently inhibited nicotine metabolism in HLMs (Fig. 25c) but seemed to be a weaker inhibitor of CYP2A6 (Fig. 25d). Pre-incubation with desmethylselegiline did not enhance inhibition in HLMs but may modestly increase inhibition in CYP2A6 (Figs. 25c & d). The above findings suggested that selegiline is acting as a MBI of CYP2A6 or that selegiline could be metabolized by HLMs and CYP2A6 to metabolites that are even more potent inhibitors than selegiline. Following preincubation, a $K_i$ of 4.2 µM for the competitive inhibition of CYP2A6 was observed (Fig 26a).

**Time-, Concentration-, and NADPH-Dependent Irreversible Inhibition of CYP2A6-Mediated Nicotine Metabolism**

In addition, to determine if the inhibition of CYP2A6-mediated nicotine metabolism by selegiline is mechanism-based, we first carried out time- and concentration-dependent inactivation assays. Figure 26b indicates that the cotinine formation decreases with increasing pre-incubation time and the decreases were dose-dependent. Using a double-reciprocal plot, the $K_i$ and $k_{\text{inact}}$ were estimated to be 15.6 ± 2.7 µM and 0.34 ± 0.04 min$^{-1}$, respectively (Fig. 26c).

To determine if the decrease in nicotine metabolism was due to covalent modification of CYP2A6 by the metabolically activated selegiline, we used a centrifuge filtering system that would allow for the removal of the unbound inhibitors from the pre-incubation mixture (Dyck and Davis, 2001). In the absence of NADPH in the pre-incubation mixture with selegiline, filter removal of unbound selegiline (-N+I) did not lead to a significant decrease in enzyme activity compared to control (-N-I) (Fig. 26d), indicating a requirement for the cofactor NADPH. In contrast, in the presence of NADPH
Figure 25. Pre-incubation of human hepatic microsomes and CYP2A6 with selegiline increased inhibition of nicotine metabolism. HLMs (a & c) and CYP2A6 (b & d) were pre-incubated (+PRE) for 15 minutes with increasing concentrations (0, 1, 3, 10, 30, and 100 µM) of selegiline (a & b) or desmethylselegiline (c & d) prior to addition of nicotine and an additional incubation of 15 or 20 (HLMs) minutes. Samples were also incubated with the inhibitors and nicotine without pre-treatment (-PRE). Activity remaining was calculated as % control (no inhibitor, same incubation conditions) within each treatment group. IC$_{50}$ was determined as the concentration of inhibitor required to decrease nicotine metabolism by 50%. Each data point represented the average of 2 to 5 independent experiments. * $p < 0.05$ compared to no pre-incubation.
Figure 26. Selegiline inhibited CYP2A6-mediated nicotine metabolism in a time-, concentration-, and NADPH-dependent manner. 

a) A Dixon plot of competitive inhibition: CYP2A6 were pre-incubated with increasing concentrations of selegiline (0, 0.5, 1, 2, and 4 µM) for 15 minutes. Following pre-incubation 50, 100, or 200 µM of nicotine was added to each of the samples and reactions were carried out for an additional 15 min. 

b) CYP2A6 was pre-incubated with increasing concentrations of selegiline (0, 1, 2.5, 10, 20, 40 µM) for 0, 1, 3, and 5 minutes. Following pre-incubation nicotine was added to each sample and the reactions were carried out for an additional 15 minutes. Data was plotted as the natural log (Ln) of % activity remaining against the pre-incubation time. Regression lines represent the initial slope of the inhibition curve (Ghanbari et al., 2006).

c) Double reciprocal plot of the rate of inactivation ($k_{obs}$) of cotinine formation as a function of the concentration of selegiline. Data represents duplicate experiments. 

d) CYP2A6 was pre-incubated without NADPH or inhibitors (-N-I), without NADPH but with 100 µM of selegiline or desmethylselegiline (-N+I), or with NADPH and 100 µM of selegiline or desmethylselegiline (+N+I) for 15 minutes in the absence of nicotine. This was followed by removal of the unbound inhibitors through centrifugal filtration. Retentates were added to fresh reaction mixture containing nicotine and incubated for an additional 15 minutes. Data were averaged from 2 to 3 experiments. * $p < 0.05$ compared to the -N+I treatment group.
during pre-incubation (+N+I), filter removal of unbound selegiline did not lead to significant recovery of cotinine formation activity, suggesting that CYP2A6 was inactivated by metabolic activation of selegiline. Similar findings were also seen with desmethylselegiline (Fig. 26d). The above findings demonstrated that the inhibition of CYP2A6-mediated nicotine metabolism by selegiline is irreversible in the presence of NADPH and that selegiline is a MBI of CYP2A6.
Discussion

It is estimated that 20% of adults in the US are current smokers (National, 2007). Previous studies demonstrated that selegiline was an effective aide in smoking cessation (Biberman et al., 2003; George et al., 2003). At least four clinical trials are currently underway to evaluate the effectiveness of selegiline as a potential therapy in smoking cessation (www.clinicaltrial.gov). Selegiline can prevent dopamine metabolism by irreversibly inhibiting MAO-B (Youdim, 1978). Selegiline also appeared to be able to inhibit its own metabolism in vivo (Laine et al., 2000), possibly via inhibition of CYP2A6. In the present study we showed that nicotine metabolism in both mice and human liver microsomes and expressed enzymes could be inhibited by selegiline, desmethylselegiline, L-methamphetamine (only in mice), and L-amphetamine. In addition, we also demonstrated that selegiline inhibited nicotine metabolism in vivo in mice. More importantly, we showed that selegiline is a mechanism-based inhibitor of human CYP2A6.

In all in vitro systems tested in our study, selegiline (and desmethylselegiline in MLMs) inhibited nicotine metabolism with the greatest potency compared to its metabolites. In mice, a greater level of inhibition was seen in MLMs compared to the expressed CYP2A5. However, the enhanced inhibition of nicotine metabolism following selegiline pre-incubation in MLMs, not observed with CYP2A5, suggested that selegiline is unlikely to be a MBI of CYP2A5. The greater inhibition in MLMs (compared to expressed CYP2A5; and with pre-incubation in MLMs) may be due to the formation of more potent inhibitory metabolite(s) of selegiline. None of the tested selegiline metabolites inhibited CYP2A5 with greater potency compared to selegiline. However, another metabolite that is produced in mice is selegiline-\(N\)-oxide (Levai et al., 2004). It is possible that in addition to the compounds tested here, that selegiline-\(N\)-oxide, produced by other enzymes in MLMs, can also inhibit CYP2A5-mediated nicotine metabolism.
For both HLMs and CYP2A6, pre-incubation with selegiline led to an increase in inhibition potency. This enhancement in inhibition was more dramatic in CYP2A6 compared to HLMs, which might reflect the metabolism of selegiline in HLMs by other CYPs such as CYP2B6 and CYP2C8 (Benetton et al., 2007), thus reducing its availability to inhibit CYP2A6. More importantly, the inhibition of CYP2A6, in addition to being competitive, was likely mechanism-based. This was supported by the findings that in the time- and concentration-dependent inhibition study, the decrease in nicotine metabolism was enhanced with increasing inhibitor pre-incubation time and with increasing inhibitor concentration. Furthermore, the inhibition of CYP2A6 by selegiline was irreversible in the presence of NADPH, suggesting that selegiline was metabolically activated by CYP2A6 and the reactive intermediate formed a covalent linkage with the holoenzyme. The general mechanism of acetylene-mediated destruction of P450s appears to be the direct N-alkylation of the porphyrin (Correia and Ortiz de Montellano, 2005). However the precise mechanism by which selegiline irreversibly inhibited CYP2A6 activity was not determined. The minor inhibition seen in the presence of selegiline without NADPH was likely due to residual inhibitor left in the retentate transferred to the fresh nicotine-containing reaction mixture.

Since desmethylselegiline also contains the carbon-carbon triple bond and irreversibly inhibited MAO-B and decreased its activity by up to 65% \textit{in vivo} (Heinonen et al., 1997), we also characterized its effects on nicotine metabolism in both species. In mice in the absence of pre-incubation, desmethylselegiline inhibited nicotine metabolism in MLMs to the same extent as selegiline. The greater inhibition by desmethylselegiline in MLMs (compared to CYP2A5), and the potentiation of inhibition with pre-incubation, is likely due to the production of more potent metabolite(s) of desmethylselegiline by other enzymes in MLMs. The only known metabolites of desmethylselegiline are L-amphetamine and its hydroxylated products (Shin, 1997); however, L-amphetamine had
weaker inhibitory activities. Thus the specific desmethylselegiline metabolite (e.g. potentially N-oxides) that may be contributing to the inhibition in MLMs, but not in CYP2A5, is unknown.

Interestingly in HLMs pre-incubation with desmethylselegiline did not increase the inhibition potency but it appeared to show minor effects on CYP2A6. One possibility is that desmethylselegiline had weaker affinity for CYP2A6 compared to other enzymes present in HLMs (i.e. inhibiting or metabolized by other enzymes) and thus was less available to inhibit nicotine metabolism by CYP2A6. The inhibition of CYP2A6 by desmethylselegiline could potentially be mechanism-based since in the presence of NADPH the inhibition of nicotine metabolism was irreversible.

In our study L-methamphetamine inhibited nicotine metabolism in mice but not in humans. L-amphetamine inhibited nicotine metabolism almost to the same extent as selegiline in CYP2A5 and HLMs but had the weakest effect in CYP2A6. Again, it is possible that L-amphetamine was metabolized to more potent inhibitors of nicotine metabolism by other enzymes in HLMs. The effects of the D-isomers of methamphetamine and amphetamine on CYP2A5 or CYP2A6 activities are unknown; however, one study found that racemic (D,L-) amphetamine was a weak inhibitor of CYP2A6 and a much weaker inhibitor of CYP2A5 (Rahnasto et al., 2003).

We and others have previously demonstrated that nicotine, at relevant pharmacological concentrations, is metabolized essentially exclusively by CYP2A5 in mice (Murphy et al., 2005b; Siu and Tyndale, 2007a). We have also shown that subcutaneous administration of the CYP2A5/6 inhibitor methoxsalen significantly inhibited nicotine metabolism when nicotine was given subcutaneously and this increase in nicotine plasma levels subsequently increased the pharmacological actions of nicotine (Damaj et al., 2007b), demonstrating the value of mice in studying nicotine metabolism and pharmacology. Since selegiline inhibited CYP2A5-mediated nicotine metabolism in
MLMs and CYP2A5, we determined its effect on nicotine clearance in mice in vivo. We treated mice with nicotine and selegiline intraperitoneally as this route mimics oral delivery of both drugs in animals, the route of choice for a novel smoking cessation product. Administration of selegiline together with nicotine caused almost a 40% decrease in clearance of nicotine and doubled its elimination half-life. These data suggested that selegiline and its metabolites can act as competitive inhibitors of nicotine in vivo even though selegiline is not a MBI of CYP2A5. It is likely that in humans, selegiline can decrease the first-pass metabolism and elimination half-life of nicotine via mechanism-based inactivation as well as competitive inhibition of CYP2A6. Genetically slow CYP2A6 metabolizers have an increased likelihood of quitting smoking (Gu et al., 2000) suggesting that inhibition of CYP2A6 by selegiline may contribute to selegiline’s ability as a smoking cessation therapeutic agent.

Our findings suggest that oral selegiline may also be combined with oral nicotine to create an orally bioavailable and clinically effective form of nicotine due to the high hepatic extraction of selegiline (Heinonen et al., 1994) (suggesting that selegiline will be able to rapidly inhibit CYP2A6 prior to reaching the systemic circulation) and the relatively smaller $K_i$ and $K_f$ of selegiline towards nicotine by CYP2A6 (4.2 and 15.6 µM respectively) compared to the $K_m$ for nicotine (~65 µM) (Messina et al., 1997). Currently there is no pill form of nicotine replacement therapy as the higher doses required to overcome first-pass metabolism may cause nausea and diarrhea due to gastrointestinal irritation (Benowitz et al., 1991b). Oral administration of even low doses (4 mg) of nicotine to those genetically deficient in CYP2A6 increased nicotine AUC by over 3-fold (Xu et al., 2002), suggesting that inhibition of nicotine metabolism can make nicotine orally bioavailable. We have also shown that the combination of the CYP2A6 inhibitor methoxsalen and nicotine significantly increased the mean plasma level of nicotine in humans as well as decreased the number of cigarettes smoked, reduced craving, the
number of puffs, the inhalation intensity, and breath carbon monoxide levels, a biomarker of smoke inhalation (Sellers et al., 2000). Furthermore, inhibition of CYP2A6 by methoxsalen also re-routed the tobacco-specific pro-carcinogen NNK to NNAL and glucuronidation pathways (Sellers et al., 2003a), indicating the additional potential benefit for harm-reduction.

Here we have shown that selegiline is a mechanism-based inhibitor of human CYP2A6, the major enzyme responsible for nicotine metabolism. In mice, where selegiline and its metabolites are competitive inhibitors, selegiline substantially reduced nicotine metabolism in vivo resulting in prolonged elevated nicotine plasma levels. These findings suggest that in humans, where selegiline is a competitive and MBI of CYP2A6-mediated nicotine metabolism, selegiline can potentially be an effective inhibitor of nicotine metabolism.
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SIGNIFICANCE OF CHAPTER

In this study we found selegiline to be an inhibitor of CYP2A5-mediated \textit{in vitro} nicotine metabolism. Selegiline also inhibited \textit{in vivo} nicotine metabolism in mice. More importantly, in humans the pre-incubation of hepatic microsomes and cDNA-expressed CYP2A6 with selegiline enhanced the inhibition nicotine metabolism. This with additional studies showing that selegiline irreversibly inhibited CYP2A6 under condition of metabolic activation demonstrated that selegiline to be a MBI of CYP2A6-mediated nicotine metabolism. The latter may represent an additional mechanism by which selegiline mediates smoking cessation.
Prior to the approval of a candidate drug for use in humans, the compound must first be tested for its pharmacological and toxicological effects \textit{in vivo} in animal models. In this thesis I examined the mouse as a potential model of nicotine metabolism and its application in studying inhibitors and the consequence of genetic variation in nicotine metabolism and pharmacology. In order to determine the suitability of the mouse as an experimental model, a fundamental requirement is that the metabolism of nicotine to cotinine occurs similarly to that seen in humans (e.g. the reaction is primarily catalyzed by a homologous enzyme with similar activities). We were interested in the mouse as an experimental model to study nicotine metabolism based on several reasons. First, a preliminary quantitative trait loci study in mice suggested the possible involvement of \textbf{Cyp2a5} in nicotine self-administration (Wildenauer et al., 2005). Second, mouse CYP2A5 shares high amino acid sequence similarity to human CYP2A6. Third, the pharmacodynamics of nicotine in several inbred mouse strains have been well established (Collins et al., 1988; Faraday et al., 2005; Grabus et al., 2005; Marks et al., 1985) and this may be combined with nicotine metabolism data to study the contribution of genetic variation to nicotine pharmacology. Fourth, mice can be genetically modified (e.g. introducing human cytochrome P450 genes). Finally, mice are small, easily maintained, and are economical compared to other animal species.

\textbf{Section 5} \hfill \textbf{Metabolism Topics}

\textbf{Section 5.1} \hfill \textit{In Vitro} Metabolism

\textbf{Section 5.1.1} \hfill \textit{In Vitro} Nicotine Metabolism
The *in vitro* nicotine metabolism data from our studies are in accordance with similar studies published during this period. The apparent $K_m$s of cotinine formation in the hepatic microsomes from DBA/2, C57BL/6 (Siu and Tyndale, 2007a), and the F2 hybrid (C57BL/6 x ST/bJ) mice (Siu et al., 2006) are similar to the $K_m$ of the cDNA-expressed CYP2A5 (from the 129/J strain) (range 8 to 11 µM) (Murphy et al., 2005b). The $K_m$s observed here are lower compared to the outbred ICR mice (~19 µM) (Damaj et al., 2007b). Another study found the $K_m$ for cotinine formation to be much higher in the CD2F1 mice (~68 µM) (Raunio et al., 2008). It is likely that polymorphisms in CYP2A5 account for the variation in the observed $K_m$s. For example, substitution of amino acid 207 from glycine (polar, uncharged) to proline (non-polar, hydrophobic) substantially increased the $K_m$ (~100-fold) of CYP2A5 for coumarin 7-hydroxylation, possibly through structural alteration increasing the size of the substrate binding pocket (Juvonen et al., 1993). The Cyp2a5 nucleic acid sequences of ICR and CD2F1 mice and further kinetic studies are required to confirm this hypothesis. Also, one cannot rule out the contribution of differences in experimental procedure. Nevertheless, compared to humans, the $K_m$s of cotinine formation in the examined mouse strains (cDNA-expressed CYP2A5 and hepatic microsomes; range 8 to 68 µM) largely overlap with those seen in humans (cDNA-expressed CYP2A6 and hepatic microsomes; range 13 to 162 µM) (Messina et al., 1997; Nakajima et al., 1996b; Yamazaki et al., 1999), suggesting similar enzymatic activities, at least for cotinine formation.

The DBA/2 and C57BL/6 mice also have higher $V_{max}$s for nicotine metabolism compared to the CD2F1 mice (Raunio et al., 2008) and this could be due to several reasons. First, between-strain variation in the *cis*-acting regulatory elements and/or *trans*-acting regulatory factors can affect both Cyp2a5 transcriptional and post-transcriptional processes, thereby altering protein levels. For example, the human
CYP2A6*9 contains a polymorphism in the TATA box resulting in decreased transcription (Kiyotani et al., 2003; Pitarque et al., 2001). Hepatic nuclear factor-4α (HNF-4α) is a major transcriptional factor for CYP2A6 and Cyp2a5 (Kamiyama et al., 2007; Ulvila et al., 2004) and bioinformatic database showed that both human and mouse HNF-4α are highly polymorphic (MGI 4.12, Ensembl). Other regulatory factors that can influence CYP2A6 expression include PXR, PPARγ, ER, C/EBPα, and hnRNP-A1 (Christian et al., 2004; Di et al., 2009; Higashi et al., 2007a; Pitarque et al., 2005). Genetic variations in HNF-4α or other regulatory elements could potentially alter the expression of Cyp2a5. Second, polymorphisms affecting various steps in the P450 catalytic cycle and the interaction of the P450 oxidase with heme or oxidoreductase may also affect the rate of substrate metabolism. However, such a change would likely alter the $K_m$ to some extent as well since $K_m$ is defined as $\frac{k_{-1} + k_2}{k_1}$ where $k_2$ is the turnover number (i.e. the catalytic step) that is dependent on the overall efficiency of the catalytic cycle (after taking into consideration the total number of catalytic sites). For instance, CYP2A6*6 contains an amino acid substitution at position 128 where arginine (polar, basic) is replaced with glutamine (polar, uncharged). The substitution resulted in a 10-fold decrease in $V_{max}$ and 5-fold increase in $K_m$ for coumarin metabolism, and this was due to decreased heme binding and disruption of the holoprotein structure (Kitagawa et al., 2001). Random mutagenesis in CYP2A6 found that substitution of residue 476 from lysine (polar, basic) to glutamine acid (polar, acidic) caused a 20-fold decrease in $k_{cat}$ (=k, and by extension $V_{max}$) but only a 25% decrease in $K_m$ for coumarin hydroxylation, a result thought to be due to reduced electron transfer efficiency (Kim et al., 2005).

Our in vitro studies demonstrated that, as in humans, there exists a second nicotine metabolizing enzyme in mice. However, the catalytic efficiency of this enzyme for cotinine formation is at least 10-fold lower compared to CYP2A5, suggesting that this
enzyme does not contribute significantly to the overall metabolism of nicotine under normal pharmacological levels/exposures. At the moment it is difficult to speculate which cytochrome P450 is contributing to the minor formation of cotinine as inhibitory antibodies against human CYP2B6 and CYP2D6 did not alter nicotine metabolism in mice hepatic microsomes (Fig. 7A).

Section 5.1.2  **In Vitro Cotinine Metabolism**

We have shown, from *in vitro* kinetic and inhibitory antibody studies that cotinine is metabolized to 3-HC and this process appeared to be mediated almost entirely by CYP2A5 (Figs. 6 & 7B). This finding parallels those in humans in which CYP2A6 is solely responsible for the formation of 3-HC (Dempsey et al., 2004; Nakajima et al., 1996a). The affinity for 3-hydroxylation of cotinine is much higher in mice (~10 to ~50 µM) hepatic microsomes compared to humans (~235 µM) (Nakajima et al., 1996a; Yamanaka et al., 2004). Genetic variation may contribute to the difference in the affinity between mouse strains. Overall, our findings show that mice exhibit similar characteristics in the metabolism of nicotine and its primary metabolite cotinine, as observed in humans.

Section 5.1.3  **Effect of a CYP2A5 Polymorphism on Substrate Metabolism**

It is interesting to note that despite the reported amino acid and coumarin hydroxylase activity differences in CYP2A5 between DBA/2 (Val<sup>117</sup>; high COH activity) and C57BL/6 (Ala<sup>117</sup>; low COH activity) (both amino acids are non-polar and hydrophobic) (Lindberg et al., 1992), there is no difference in their cotinine formation capacity as measured by $K_m$.
in our study. In contrast, the $K_m$ for 3-HC formation is five-fold higher in DBA/2 mice. Previous studies have also demonstrated that amino acid 117 determines whether CYP2A5 possesses $7\beta$- or $2\alpha$-hydroxylase activity for dehydroepiandrosterone (Uno et al., 1997). In CYP2A6, mutagenesis of residue 117 from valine to alanine in the cDNA-expressed protein did not affect its $K_m$ but resulted in a substantial drop in $V_{\text{max}}$ for coumarin hydroxylation (He et al., 2004b; Yano et al., 2005). Although crystallography studies of the CYP2A6 crystal structure coupled with coumarin showed that amino acid 117 is in close proximity with coumarin (He et al., 2004b; Yano et al., 2005), the aforementioned effect was hypothesized to be an indirect role of residue 117 in coumarin hydroxylation through modulation of the function of surrounding amino acids potentially involved in proton/electron transfer and reaction stabilization (He et al., 2004b). It may be that these observations are restricted only to the effect of valine and alanine on coumarin. As yet this particular mouse polymorphism has not been found in the general human population and the impact of this alteration on nicotine and cotinine metabolism in CYP2A6 has not been studied. The high sequence similarity of CYP2A5 with CYP2A6 suggests that residue 117 of CYP2A5 should also be located in the substrate binding pocket and potentially be in contact with the substrate. It is possible that the changes in amino acid 117 in mouse CYP2A5 may affect binding and catalytic activity depending on the substrate.

**Section 5.2 In Vivo Metabolism**

Our data demonstrated that the *in vivo* clearance of nicotine in mice following subcutaneous administration is rapid ($t_{1/2} \sim 8$ to $9$ min) consistent with findings in these mice following intraperitoneal injection ($\sim 6$ to $7$ min) (Petersen et al., 1984). The half-life of plasma nicotine is longer at 14 minutes in the ICR strain. This is consistent with *in*
vitro data showing lower nicotine metabolic efficiency ($V_{max}/K_m$) in the ICR (0.01) mice compared to the DBA/2 (0.05) and C57BL/6 (0.04) mice. The elimination of plasma nicotine in mice is monophasic compared to the biphasic elimination in humans; this could be due to the much more rapid rate of metabolism compared to distribution of nicotine following absorption in mice. The percentage recoveries of nicotine and its metabolites in the urine of these mice were not measured. However, urinary analysis in the CD2F1 strain indicated that nicotine represented <1% of the systemic dose 24 hours following its oral administration (Raunio et al., 2008), whereas in humans (normal CYP2A6 activity), the recovery is higher (~8 to 10%) (Benowitz and Jacob, 1994; Meger et al., 2002). The difference could be due to the high activity of CYP2A5 as well as the rapid hepatic blood flow in mice (four-times faster) compared to humans (Bischoff et al., 1971; Boxenbaum, 1980), resulting in the rapid metabolic clearance of nicotine.

The results from our study, and from others, indicate that in mice cotinine is the primary metabolite of nicotine in plasma and has a significantly longer half-life (e.g. 24 to 51 min) compared to nicotine (DBA/2; C57BL/6; C3H) (Figs. 3 & 5A) (Petersen et al., 1984). In the urine of CD2F1 mice, cotinine represented ~3-4% of the systemic nicotine dose (Raunio et al., 2008). The above findings are compared with humans in which the half-life of cotinine is much longer (>10 hours) (Benowitz and Jacob, 1994) and its urinary recovery is ~10-15% of an oral dose of nicotine (Meger et al., 2002).

In mice (and in humans) unconjugated 3-HC is the predominate metabolite of nicotine that is accumulated in the urine (77% in CD2F1 mice; 33-40% in humans) (Benowitz and Jacob, 1994; Meger et al., 2002; Raunio et al., 2008). We found that in the DBA/2 and C57BL/6 mice the clearance of 3-HC is slower compared to cotinine whereas in humans, 3-HC clearance is formation dependent (Dempsey et al., 2004). This may be due to differences in the speed of formation (e.g. slower in mice) and renal clearance of 3-HC considering metabolic clearance (i.e. glucuronidation) does not
contribute to the removal 3-HC in mice (Ghosheh and Hawes, 2002) and only plays a small role (up to ~25%) in the clearance of 3-HC in humans (Nakajima and Yokoi, 2005). One possibility is that the renal clearance of 3-HC is influenced by genetic differences. For instance, a human twin study found the genetic component accounted for up to 61% of the variance in the renal clearance of nicotine and cotinine (clearance of 3-HC not examined) (Benowitz et al., 2008). The genetic variation was suggested to be contributed by the variation in the active secretory or reabsorptive components for both nicotine and cotinine, although this remains to be determined for 3-HC. Another possibility is that CYP2A5 and CYP2A6 produce different isomers of 3-HC which have different affinities for renal excretion, but this remains to be verified.

In our studies we did not examine the kinetics of nicotine N-oxide and cotinine N-oxide formation. Raunio et al (Raunio et al., 2008) found that cotinine N-oxide was the second most abundant mouse urinary metabolite (~16%) followed by nicotine N-oxide, which was found at lower levels (~3-5%). In humans, the urinary recovery of nicotine N-oxide and cotinine N-oxide are 4-7% and 2-5%, respectively.

**Section 5.2.1  Biomarker of Nicotine Metabolism**

Many studies in humans have used ratios of nicotine metabolites as indicators of CYP2A6 activity as majority of nicotine is metabolized by CYP2A6 (Nakajima et al., 1996a), and it is the only enzyme that metabolizes cotinine to 3-HC (Nakajima et al., 1996a). For example, salivary and plasma 3-HC/cotinine ratios have been found to be highly correlated with oral nicotine clearance (first-pass metabolism and systemic clearance) (Dempsey et al., 2004). The 3-HC/cotinine ratio following systemic (intravenous) administration of nicotine is also indicative of CYP2A6 genotype as grouped by activity (Benowitz et al., 2006b). In addition, the urinary and plasma 3-
HC/cotinine ratios are highly correlated within subjects and also with the clearance of nicotine by the cotinine pathway, suggesting that urinary 3-HC/cotinine ratio may be used as a marker of CYP2A6 activity (Benowitz et al., 2006b). In addition, those without CYP2A6 have no 3-HC production making the ratio both selective and sensitive.

Levels of nicotine metabolites (particularly in urine) in mice can also potentially be used as indicators of CYP2A5 activity in inhibition studies; this may minimize between-animal variations when studying multiple inhibitors and reduce the number of animals needed. We showed that administration of methoxsalen to the ICR mice significantly increased nicotine and decreased cotinine plasma levels (Fig. 15) although metabolite ratios were not measured in either plasma or urine. In the CD2F1 mice (Raunio et al., 2008), urinary metabolite ratios were examined as a measure of CYP2A5 activity. Following *in vivo* administration of methoxsalen, 24-hour total urinary 3-HC and cotinine N-oxide levels (the two most abundant metabolites) decreased and nicotine increased leading to significant decreases in 3-HC/nicotine and cotinine N-oxide/nicotine ratios (Raunio et al., 2008). However, since the production of both 3-HC and cotinine N-oxide requires another metabolic step after cotinine formation, the use of nicotine as the denominator in the ratio may increase variability. Furthermore, due to the high variability in the level of urinary metabolites (Raunio et al., 2008), the metabolite ratio may be less sensitive to detecting the effect of weaker inhibitors.

**Section 5.3 Mice (CYP2A5) as a Model to Study CYP2A6 Inhibition**

Apart from nicotine metabolism, with respect to the structural/functional differences between CYP2A5 and CYP2A6, inhibitor and modeling studies showed that CYP2A5 possess a larger active site compared to CYP2A6 (Juvonen et al., 2000; Poso et al., 2001) and this has been suggested to be important for the relative potency of inhibitors
For instance, compounds with the bulkier lactone ring structure were demonstrated to be better inhibitors of CYP2A5 than CYP2A6 (Juvonen et al., 2000). However, subsequent experiments showed substantial differences in the inhibition efficacies between compounds of similar sizes, suggesting that the size of the active site may play a secondary role to the chemical properties of the inhibitors and their interactions with the binding site (Rahnasto et al., 2003).

Section 5.3.1  Methoxsalen

Methoxsalen is one of the most thoroughly studied inhibitors of CYP2A5 and CYP2A6. In mice, methoxsalen decreased NNK-induced lung tumor formation (Section 2.3.5) (Miyazaki et al., 2005; Takeuchi et al., 2003). In humans, methoxsalen increased plasma nicotine level and decreased smoking behaviours (Section 2.3.3.1) (Sellers et al., 2002; Sellers et al., 2000; Tyndale et al., 1999) as well as decreased NNK activation (Section 2.3.5) (Sellers et al., 2003a).

Our data showed that \textit{in vitro} methoxsalen acted as a competitive and potentially a mixed-type inhibitor of nicotine metabolism (Fig. 14). This is consistent with the compound acting as a mixed-type (competitive and non-competitive) inhibitor of CYP2A5-mediated coumarin metabolism (Visoni et al., 2008). Methoxsalen is also a MBI of CYP2A5 but the mechanism through which this occurs has not been studied (Visoni et al., 2008). Results from another study suggested that the active metabolite(s) of methoxsalen covalently modifies the apo-protein of CYP2A5 (Mays et al., 1990). During \textit{in vivo} behavioural tests, pre-treatment of methoxsalen 30 to 60 minutes prior to subcutaneous nicotine injection in the ICR mice led to the greatest change in the anti-nociceptive and hypothermic effects of nicotine (tested 45 minutes after nicotine injection; Fig. 16). This also led to a substantial change in nicotine pharmacokinetics as
demonstrated by a ~30% decrease in its half-life (Fig. 15). However, since we only examined nicotine pharmacokinetics following 30 minutes (but not 60 minutes) pretreatment with methoxsalen, one cannot be definitive regarding the direct correlation between behavioural changes and plasma nicotine levels across time. The pharmacokinetics of methoxsalen in the ICR mice has not been studied and therefore it is uncertain which inhibition mechanism (i.e. competitive and/or mechanism-based inhibition) is responsible for the \textit{in vivo} inhibition (Visoni et al., 2008). It is also unknown if metabolites of methoxsalen in mice are capable of inhibiting CYP2A5.

In humans, methoxsalen acts as both a competitive and a MBI of CYP2A6 \textit{in vitro} (Section 2.3.3.1) (Draper et al., 1997; Koenigs et al., 1997; Maenpaa et al., 1994; Zhang et al., 2001). The mechanism through which methoxsalen inactivates CYP2A6 is unclear but it may be due to denaturation of the apo-protein, heme modification, or covalent binding of methoxsalen to the active site of CYP2A6 (Koenigs et al., 1997). \textit{In vivo}, pre-administration of methoxsalen at clinical doses resulted in a statistically significant decrease in the $C_{\text{max}}$ of the coumarin metabolite 7-hydroxycoumarin and its AUC (Kharasch et al., 2000) suggesting inhibition of coumarin metabolism; however, the pharmacokinetics of coumarin was not determined.

\textbf{Section 5.3.2 Selegiline}

We showed for the first time that selegiline, and to some extent, its metabolites, are capable of inhibiting CYP2A6- and CYP2A5-mediated nicotine metabolism. The inhibition seemed to be slightly more potent for CYP2A6 than CYP2A5. However, the mechanisms through which selegiline inhibits these enzymes differ as selegiline is an \textit{in vitro} MBI of CYP2A6 but not of CYP2A5. \textit{In vivo} in mice, co-administration of selegiline with nicotine led to a significant increase in the plasma half-life of nicotine but had no
effect on $C_{\text{max}}$ (Fig. 24). It is likely that both selegiline and its metabolites contribute to the in vivo inhibition observed in mice. A recent study of the disposition kinetics of selegiline and its metabolites in NMRI mice showed that following intraperitoneal injection of selegiline, selegiline was the primary compound found in the plasma (Magyar et al., 2007). In contrast, when the drug was administered orally, the bioavailability of selegiline was substantially lowered (by more than three-fold) (Magyar et al., 2007). The reduced selegiline bioavailability was not thought to be due to incomplete oral absorption, suggesting instead that the majority of selegiline has reached hepatic circulation and the liver (i.e. inhibiting CYP2A5) prior to reaching the systemic circulation, in contrast to intraperitoneal administration. Thus, these data suggests that intraperitoneal administration (in our study) may be underestimating the impact of selegiline on the in vivo inhibition of CYP2A5-mediated nicotine metabolism in mice compared to oral administration. Nevertheless, confirmation is needed as our study was done in the DBA/2 mice and strain differences may alter the pharmacokinetics of selegiline. Ideally in clinical setting both the nicotine and inhibitor would be given orally and these data suggest that an even greater inhibition of nicotine metabolism may be seen with oral selegiline administration alone.

In humans, the effect of selegiline on nicotine metabolism in vivo has not been examined. The fold-reduction in intrinsic clearance by the MBI can be roughly estimated by the equation: fold reduction in $CL_{\text{int}} = \frac{k_{\text{deg}} + [I] \times k_{\text{inact}}}{k_{\text{deg}} [I] + K_I}$, where $k_{\text{deg}}$ is the degradation rate constant of the enzyme (CYP2A6) and $[I]$ is the concentration of the inhibitor at the active site, the latter of which can be determined from total and free concentrations in the plasma (Rostami-Hodjegan and Tucker, 2004). The $K_I$ value needs to be corrected for non-specific binding of the inhibitor to microsomes. The effect of the inhibitor in vivo
also depends on several factors. These include the properties of the substrate co-administered, the relative fraction of the substrate metabolized by the enzyme being inhibited (Ghanbari et al., 2006), and the potential inhibition caused by different metabolites (e.g. desmethylselegiline, L-amphetamine, and L-methamphetamine). For example, the plasma $AUC_s'$ of metabolites following oral selegiline administration are substantially greater than selegiline (demethylselegiline: 31-fold; L-methamphetamine: 161-fold; L-amphetamine: 50-fold) as well as having longer elimination half-lives (Laine et al., 2000). Since we showed that these metabolites are capable of inhibiting CYP2A6 $\textit{in vitro}$ (Fig. 22), they may potentially contribute to the inhibition of $\textit{in vivo}$ nicotine metabolism. By determining the effect of the inhibitor on nicotine clearance $\textit{in vivo}$, the relative contribution of the inhibitory effect of selegiline on CYP2A6 vs. MAO-B inhibition in smoking cessation can potentially be estimated. For instance, by comparing the nicotine pharmacokinetics resulting from selegiline-mediated CYP2A6 inhibition to individuals with a particular CYP2A6 genotype that produces similar nicotine pharmacokinetics, the extent of smoking cessation contributed by CYP2A6 and MAO-B inhibition can be inferred from smoking behaviours of individuals with the CYP2A6 genotype being compared. Alternatively, if a compound exhibits similar MAO-B inhibition characteristics but does not interact with CYP2A6 or other nicotine metabolizing enzymes, its effect on smoking cessation may be compared with and estimate the contribution of inhibition of MAO-B or CYP2A6 by selegiline. Practically speaking, the concomitant inhibition of nicotine and dopamine metabolism provides selegiline an advantage by targeting two distinct aspects important in nicotine dependence. As mentioned in Section 2.3.4., individuals with normal CYP2A6 activity are less likely to quit smoking with nicotine replacement therapy (transdermal and gum) or with counseling compared to individuals with impaired CYP2A6 activity (Ho et al., 2009; Lerman et al., 2006; Patterson et al., 2008); therefore, normal metabolizers may obtain
the greatest benefit from inhibition of CYP2A6 by selegiline. Selegiline at 10 mg daily
dose plus oral nicotine may be particularly useful for smokers with Parkinson’s disease
although this remain to be tested.

Section 6 Factors Affecting Nicotine Intake
Section 6.1 Impact of Genetic Variations
Section 6.1.1 Variations in Nicotine Metabolism

We showed that oral nicotine consumption is associated with CYP2A5 levels and in vitro
nicotine metabolism in male mice. This is consistent with observations in humans where
CYP2A6 activity is associated with smoking behaviours (Section 2.3.4). In our study we
examined in vitro nicotine metabolism in mice from two extreme ends of the
consumption spectrum rather than from across the entire consumption range; therefore,
we were unable to assess the contribution of nicotine metabolism (and Cyp2a5 genotype)
to the variability in oral NSA. Since one of the parental strains (ST/bJ) self-administrated
significantly less amount of oral nicotine compared to the C57BL/6 mice, it would be
advantageous to have the Cyp2a5 sequence data and hepatic microsomes from the
ST/bJ mice to verify that they have a different Cyp2a5 sequence and slower nicotine
metabolism than the C57BL/6 mice. Similarly, the lower affinity of the ICR hepatic
microsomes for nicotine C-oxidation may be due to a polymorphism in Cyp2a5 which
may contribute to differences in oral NSA (and other nicotine behavioural responses),
but this remains to be verified. A recent genetic study in mice using the F2 offspring of
C57BL/6J (high nicotine consumption strain) and C3H/HeJ (low nicotine consumption
strain) has revealed that at least four QTLs in the mouse genome are associated with
oral NSA (LOD score of >2.0 [suggestive association]), and these QTLs contributed to
~62% of the variance in this behaviour (Li et al., 2007). One of the loci is located on
chromosome 7 and the highest combined LOD score found on this loci is 5.9 (highly significant association). Overall, the QTL on chromosome 7 was estimated to contribute ~13% of the variance in oral NSA. However, the study only reported data derived from 10 cM and onward, and Cyp2a5 is located at ~6.5 cM, thus it is unclear what the contribution of this region is (i.e. Cyp2a5) to the overall variance in oral NSA (Li et al., 2007). It is also possible that other genes influencing nicotine consumption co-segregate with Cyp2a5 but this remains to be investigated. In order to directly test the contribution of CYP2A5-mediated nicotine metabolism on NSA, inhibitors of CYP2A5 could be used.

Similarly in humans, genetics play a significant role in various aspects of smoking behaviour which include initiation (Li et al., 2003; Vink et al., 2005), persistence to regular smoking (Maes et al., 2004), number of cigarettes smoked (Koopmans et al., 1999), degree of nicotine dependence (Vink et al., 2005), withdrawal severity (Xian et al., 2003), and smoking cessation (Broms et al., 2006). The heritability estimates for the amount of cigarettes smoked (once initiated) was shown to be up to 86% (Koopmans et al., 1999), which is similar to the results from the above mouse study. Vast numbers of pharmacogenetic studies have demonstrated that the CYP2A6 genotype to be highly associated with many aspects of smoking (Section 2.3.4). The heritability estimates of CYP2A6 to different dimensions of smoking behaviour have not been specifically defined but a genome-wide linkage scan found this gene to be situated near a locus associated with smoking frequency (Swan et al., 2006).

Section 6.1.2 Non-Nicotine Metabolism Variations

In addition to nicotine metabolism, other genetic factors may contribute to variations in oral NSA among mouse strains. One particular striking feature is that a significant association exists between the amount of nicotine self-administered and the threshold of
nicotine-induced seizures observed between 19 strains of mice (r=0.89, P<0.01) (Crawley et al., 1997); although this may be related to the sensitivity of mice to nicotine which is in part controlled by the bioavailability of oral nicotine following hepatic first-pass metabolism. An earlier study conducted by Hatchell and Collins showed a lack of clear-cut association between brain nicotine levels or hepatic nicotine clearance and motor depression between mouse strains, suggesting differential sensitivity to the pharmacological effects of nicotine (Hatchell and Collins, 1980). Nicotine sensitivity may be influenced by polymorphisms in genes of the neurotransmitter system involved in nicotine reward, which include the dopaminergic (David et al., 2006), serotonergic (Villegier et al., 2006), cholinergic (Levin et al., 2008), GABAergic (Markou et al., 2004), opiate (Berrendero et al., 2002), and cannabinoid (Merritt et al., 2008) systems. Although currently there are no known studies demonstrating the association between polymorphisms in these genes with nicotine sensitivity in mice, studies using inhibitors and knock-out animals have revealed their direct role in nicotine pharmacology. For instance, administration of the dopamine D1 receptor antagonist SCH 23390, or the nicotinic receptor antagonist DHβE, disrupted intra-cranial self-administration of nicotine to the ventral tegmental area, a brain region important for drug reward (David et al., 2006). Mice with the β2 nicotinic acetylcholine receptors knocked out showed reduced oral NSA in the first weeks of testing but nicotine consumption level returned to the wild-type levels within the first month. In contrast, α7 nicotinic acetylcholine receptors knockout mice showed normal oral NSA at the beginning of test but their nicotine consumption level declined after several weeks (Levin et al., 2008). In humans, evidence indicates that polymorphisms in the neurotransmitter systems of the nicotine reward pathways potentially influence smoking behaviours. These include dopamine receptors (e.g. DRD2) (Comings et al., 1996), transporter (DAT) (Ling et al., 2004), and
metabolism (e.g. \textit{TH}) (Olsson et al., 2004); serotonin transport (\textit{5-HTT}) (Gerra et al., 2005) and metabolism (\textit{TPH1}) (Sullivan et al., 2001); nicotinic acetylcholine receptors (e.g. $\alpha_4$) (Li et al., 2005); GABA receptor (\textit{GABAB2}) (Beuten et al., 2005a); opiate receptor (\textit{OPRM1}) (Swan et al., 2006); and brain-derived neurotrophic factor (Beuten et al., 2005b).

**Section 6.2 Non-Genetic Factors**

Although in general oral nicotine self-administration appears to mimic nicotine consumption via smoking (Section 3.1), the two behaviours may be influenced by different factors. For instance, in addition to nicotine, cigarette smoke contains numerous chemicals and additives that may affect smoking behaviour through their interactions with various cranial nerves, such as the olfactory nerve (smell), trigeminal nerve (irritation), as well as facial, glossopharyngeal, vagal nerves (taste) (Megerdichian et al., 2007). Studies have shown that application of anesthetic to the airway significantly reduced satisfaction and craving from smoking (Rose et al., 1985; Rose et al., 1984) and blockade of olfactory/taste stimuli from smoking reduced self-administration of cigarette puffs (Perkins et al., 2001). In addition, studies have also shown that neither denicotinized cigarettes nor pure nicotine alone completely abolish cravings or provide the subjective satisfaction of smoking regular cigarettes (Rose et al., 2003; Rose et al., 2000). This evidence suggests the importance of non-nicotinic factors in contributing to smoking behaviours and they may be different to oral NSA.

There appears to be another important distinction between nicotine self-administration in mice and smoking in humans. Stress is considered to be an important factor for the amount of cigarettes smoked in humans (Baker et al., 2004; Todd, 2004).
However, a genetic study using F2 mice comprised of low, average, and high stress responders revealed that despite showing different response to stress, the mice did not show different level of NSA. A caveat of this study was that the degree of NSA was not measured against the degree of stress induced within the parental strain (or within the stress-responder groups), thus the potential influence of genetic components (e.g. nicotine metabolism) in NSA cannot be ruled out (Bilkei-Gorzo et al., 2008).

Section 7 Sex-Dependent Association between Nicotine Metabolism and NSA

Even though our study chose male and female mice that were matched for their NSA level, on average the female mice tend to metabolize nicotine slightly faster compared to males. Similar observations are also seen in humans (Benowitz et al., 2006a). It has been reported that on average, both men and women smoke a similar number of cigarettes (Benowitz and Hatsukami, 1998; Benowitz et al., 2006a; Zeman et al., 2002). However, women in general have a faster rate of nicotine metabolism (e.g. fractional clearance of nicotine to cotinine [>15%] and 3-HC/cotinine ratios [>20%]) compared to men (Benowitz et al., 2006a; Zeman et al., 2002). This is supported by our previous findings that human females expressed a greater amount of CYP2A6 protein and have faster in vitro nicotine metabolism compared to males (Al Koudsi et al., 2006b).

We also noted that female mice tend to have more variable rates of nicotine metabolism compared to males. One potential explanation is the fluctuation in the level of steroid hormones in females altering the level of CYP2A5 (as the mouse oestrous cycle is less than one week). $17\beta$-estradiol has been shown to induce a significant rise in CYP2A6 mRNA in human hepatocytes (likely via the estrogen receptor-α by interacting
with the estrogen response element [AGGTCAnnnCAACCT]), although protein levels were not measured (Higashi et al., 2007a). Immunostaining also revealed that CYP2A6 protein levels tend to be higher during the follicular phase compared to the luteal phase in endometrial tissues (Higashi et al., 2007a). The effect of sex steroids on nicotine metabolism in mice has not been examined; however, there also appears to be an estrogen response element in mice as well [AGGGCAnnnTGACCT] although it is unclear if it is involved in the regulation of mouse Cyp2a5 (Orimo et al., 1995). It is possible that sex hormones may induce other nicotine-metabolizing hepatic enzymes in mice.

In contrast to the above *in vitro* study, pharmacokinetic studies in humans have revealed some discrepancies on the role of female sex hormones on nicotine metabolism. One study found that the pharmacokinetics of nicotine or cotinine are not affected by the menstrual cycle, as measured during the mid-follicular and mid-luteal phases (Hukkanen et al., 2005a). In contrast, other studies have shown that pregnancy and the use of estrogen-containing oral contraceptives significantly increases the clearance of nicotine via the cotinine pathway (~25% and ~40%, respectively) (Benowitz et al., 2006a; Dempsey et al., 2002). This inconsistency may be due to the varying amount and the duration of exposure to estrogen during these two distinctive physiological phases. Other available evidence indicates that smoking behaviour is influenced by menstrual cycle (increased during late luteal phase) but does not change during pregnancy (DeBon et al., 1995; Dempsey et al., 2002; Snively et al., 2000), suggesting the role of other influences on nicotine intake in females. 17β-estradiol and progesterone have been shown to decrease nicotine’s antinociceptive effect in female mice, through antagonism of α4β2 nicotinic receptor, whereas testosterone had no effect in males (Damaj, 2001). These findings suggest a balance exists between nicotine
metabolism and nicotine pharmacodynamics as influenced by female steroids (both appeared to be, in part, affected by 17β-estradiol) in controlling nicotine intake, especially in females. This may also underlie our observation that nicotine metabolism is less correlated with NSA in female mice compared to the male mice. The complex interactions between nicotine metabolism and sex may also contribute to the conflicting evidence showing reduced efficacy of NRTs in females compared to males in some studies but not others (Bjornson et al., 1995; Perkins and Scott, 2008; Schnoll et al., 2009; Wetter et al., 1999).

Section 8 Effect of Chronic Nicotine Treatment on Nicotine Metabolism

In our studies we examined nicotine metabolism in mice in acute settings (i.e. following a single nicotine administration); however, in clinical settings, nicotine metabolism and in particular, nicotine-mediated behavioural effects, take place during chronic nicotine exposure. Therefore, nicotine metabolism during chronic exposure is of particular interest.

In monkeys, chronic nicotine treatment (0.3 mg/kg b.i.d.) for three weeks led to a ~60% decrease in CYP2A6agm protein levels and ~40% decrease in \textit{in vitro} nicotine metabolism (Schoedel et al., 2003). Cigarette smoke exposure in human led to only a small reduction in the fractional clearance of nicotine to cotinine (Benowitz and Jacob, 2000). However, 7-day tobacco abstinence led to a 39% increase in non-renal nicotine clearance (Lee et al., 1987) and a recent study showed that the clearance of oral nicotine is reduced by up to 25% in smokers compared to non-smokers (Mwenifumbo et al., 2007). Therefore, chronic nicotine exposure would likely decrease the metabolism of CYP2A6 substrates, in particular nicotine. We have previously examined the effect of nicotine metabolism in mice following chronic nicotine treatments. In a first study, DBA/2
mice were treated with a single injection of nicotine (1 mg/kg s.c.) once daily for seven days. On the last day plasma concentrations of nicotine were measured but no differences were found in its elimination kinetics compared to those of acute treatment (unpublished observations). This may be due to the rapid clearance of nicotine in the mice. In a subsequent study osmotic mini-pumps providing 1 mg/kg/hr or 5 mg/kg/hr of nicotine for seven days were implanted in DBA/2 mice. On day seven the mice were sacrificed and livers were removed to examine hepatic CYP2A5 protein levels. Compared to saline, 1 mg/kg/hr nicotine did not caused significant changes in CYP2A5 levels. In contrast, 5 mg/kg/hr nicotine caused a drop in CYP2A5 levels although nicotine at this level were toxic to mice, therefore we felt that nicotine at this level were of little pharmacological relevance (unpublished observations). A recent study found that chronic nicotine exposure in DBA/2 and C57Bl/6 mice, while leading to changes in various behavioural measures, not all the changes were congruent in terms of direction of change. For example, during withdrawal both strains showed decreases in somatic withdrawal signs between day 1 and day 3; in contrast, in the plus-maze test, the DBA/2 mice increased time spent whereas C57Bl/6 mice decreased time spent in the opening. Therefore, the contribution of chronic nicotine exposure to behavioural changes by potential differences in nicotine metabolism could be secondary to and/or confounded by the impact of other physiological/neurological changes (Jackson et al., 2009). The above data suggested while in both humans and monkeys chronic nicotine exposure could lead to reduction in CYP2A6-mediated nicotine metabolism, which may play a role in altered nicotine pharmacology in chronic settings; its impact on mouse nicotine metabolism has not yet been demonstrated or is minimal, possibly due to rapid elimination of the drug in this species.

Section 9  Future Directions
Section 9.1  Selegiline

Previous study indicated that mouse deficient for MAO-B showed no changes in brain dopamine levels, suggesting its lack of involvement in dopamine metabolism (Grimsby et al., 1997). In contrast, dopamine metabolism in mice appeared to be mediated in part by MAO-A (Shih et al., 1999). Thus, although selegiline is an irreversible inhibitor of MAO-B, it would have no impact on dopamine metabolism in mice. Consequently, the effect of selegiline on nicotine pharmacology can be tested and the changes in nicotine-mediated behaviour can be attributed to the CYP2A5-inhibition (i.e. inhibition of nicotine metabolism) aspect of selegiline. On the other hand, this also excludes the use of mice for studying the impact of selegiline on dopamine metabolism and its subsequent effects on nicotine pharmacodynamics.

Based on our observation that selegiline is an MBI of CYP2A6, it is reasonable to assume that it will exert its greatest impact on the first-pass metabolism (i.e. inhibition) of orally delivered nicotine. Considering existing clinical trials only examine selegiline either alone (capsule or patch) or in combination (capsule plus nicotine patch) for smoking cessation (Clinicaltrials.gov), it would also be important to examine the effect of oral selegiline in combination with oral nicotine capsule/pill. It is likely that the smoking cessation effect may be greater when oral selegiline is used in combination with oral nicotine due to inhibition of dopamine metabolism and decreased nicotine metabolism. Consider the involvement of various non-nicotine factors in nicotine-dependence, the above therapeutic combination may be particularly useful as an initial treatment in conjunction with behavioural therapies for smokers consider quitting.

Section 9.2  Mouse Model of Nicotine Metabolism
In this thesis we demonstrated the usefulness of the mouse model in studying nicotine metabolism in the context of pharmacological inhibition. However, extrapolation of pharmacokinetic data from animals to humans has limitations due to species differences in physiology and conservation of drug metabolism enzymes. The former can be addressed by allometric scaling which proposed that certain pharmacokinetic information (e.g. plasma-concentration-time profile, clearance, and distribution) can be estimated between species as a function of body weight (Boxenbaum, 1984; Martignoni et al., 2006). It has also been suggested that, in general, small animals have a higher hepatic P450 content per unit body weight due to a larger liver to body weight ratio as well as faster hepatic blood flow (Davies and Morris, 1993). As a consequence, smaller animals have a faster metabolic clearance of xenobiotics compared to humans (Davies and Morris, 1993). However, species variations in P450 represents a major factor in drug metabolism difference between species, particularly for compounds that are extensively metabolized (Martignoni et al., 2006). Therefore in these circumstances pharmacokinetic data from animals may not be reasonably applied to humans. For instance, while existing evidence, and results from our study, indicate that both human CYP2A6 and mouse CYP2A5 exhibit similar nicotine metabolizing properties, studies with selegiline showed that this drug to be a MBI of CYP2A6 but not CYP2A5. The endogenous compound 2-phenylethyamine is a far better inhibitor of CYP2A6 than CYP2A5 in vitro (IC$_{50}$ ratio of <0.04) (Rahnasto et al., 2003) and other experimental compounds also exhibit different inhibition potencies for these enzymes (Juvonen et al., 2000). Thus, evaluating the impact of CYP2A6 inhibitors in mice in vivo may yield different results. Furthermore, potential pharmacokinetic differences in the metabolism of the inhibitors between species can also alter their inhibitory properties. While the mouse model may be imperfect, it represents the best small animal model we have to date and may prove useful for the first line in vivo animal studies considering that the main nicotine
metabolizing enzymes in rats, the other well studied small animal species, belong to the CYP2B family (Nakayama et al., 1993).

In the past few years humanized mice have been developed using several strategies. One method is to introduce human P450 cDNA with tissue-specific promoter or the entire gene (with regulatory elements) into the pronuclei of the mice (Cheung and Gonzalez, 2008). With this method, however, the presence of endogenous counterparts (homologous or orthologous genes) may interfere with, and confound the effects of, the introduced genes. Therefore mice with the corresponding gene knocked out should be used. Another method is to introduce cDNA into the site of the endogenous gene thereby disrupting its function (Cheung and Gonzalez, 2008). An advantage of this model is that once generated, the transgenic line can be maintained indefinitely. Conceivably this model is ideal for studying the impact of various Cyp2a5 and CYP2A6 genetic polymorphisms on nicotine-mediated behaviours. In the case of inhibitor studies, (human) enzymes involved in the metabolism of the inhibitor should ideally be introduced to increase the predictability of the animal model; however, this may not be feasible since different inhibitors will likely be metabolized by different enzymes. We are aware that humanized CYP2A6 mice (Cyp2a5-null background) are currently being developed and this model may be invaluable in studying CYP2A6 inhibitors in vivo.

Another method that has been developed is the transplantation of human hepatocytes into mice (Katoh et al., 2008). This model employs a urokinase-type plasminogen activator+/−/ severe combined immunodeficient mouse strain in which human hepatocytes are directly injected into the spleen of the mice and the resultant mice have between 80-90% of their hepatocytes replaced (Tateno et al., 2004). This model may provide a much more accurate picture of in vivo metabolism (and inhibition) as all human hepatic enzymes and their regulatory factors are present (Katoh et al., 2008). This model may also indicate whether the target compound is inducing the
expression of other enzymes through human (rather than mice) xenobiotic receptors, which is particularly important for drug-drug interactions (Katoh et al., 2008). A drawback of this particular model is that the chimera mice have a shorter life-span due to organ failure caused by graft vs. host disease and administration of protease inhibitor is also required to prolong survival (Tateno et al., 2004). The mice also need to be constantly generated and thus costly and time-consuming. Their short life-span and inability to generate offspring carrying human hepatocytes can limit their use in behavioural studies.

Clearly, each of the above mouse models has its advantages and drawbacks in studying nicotine metabolism. Further development of these models, either through creating mice with multiple human transgenes, or refinement of the hepatocyte transplant model, are needed to increase their applicability to humans; however, a disadvantage associated with the humanized mouse model is that the generation of a panel of mice each with different CYP2A6 genotype is both time and resource consuming. Continual functional characterization of CYP2A5 (and in vivo nicotine metabolism) from different mouse strains may also be useful in correlating their metabolism phenotype with that of CYP2A6 and its functional variants. Generation of mutations in mouse Cyp2a5 that mimic various CYP2A6 genotypes may also be useful in understanding the contribution of these genotypes in altering nicotine pharmacology. Finally, even though nicotine is largely metabolized by the liver in mice, the contribution of other pharmacokinetic factors such as absorption, distribution, and extrahepatic metabolism need to be considered.

**Section 10 Conclusion**

Based on our findings that: 1) CYP2A5, as with CYP2A6, is the primary enzyme responsible for the metabolism of nicotine to cotinine and cotinine to 3-HC; 2) genetic
variations in CYP2A5 may alter nicotine metabolism; 3) inhibition of CYP2A5-mediated nicotine metabolism led to change in nicotine pharmacokinetics and pharmacodynamics; and 4) identification of an existing drug (selegiline) as an inhibitor of CYP2A5 and CYP2A6 suggested the mouse may be a suitable model for studying novel CYP2A6 inhibitory compounds. Overall, the use of CYP2A5 inhibitors in the mouse model may be suitable for studying the direct effect of nicotine metabolism on nicotine pharmacology. On the other hand, genetic studies across strains may be useful for dissecting the genetic architecture of nicotine-mediated behaviours, in this case oral NSA.
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