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ORIGINAL ARTICLE

Nicotine oxidation by genetic variants of CYP2B6 and in human brain microsomes

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Abstract

Common variation in the *CYP2B6* gene, encoding the cytochrome P450 2B6 enzyme, is associated with substrate-specific altered clearance of multiple drugs. *CYP2B6* is a minor contributor to hepatic nicotine metabolism, but the enzyme has been proposed as relevant to nicotine-related behaviors because of reported *CYP2B6* mRNA expression in human brain tissue. Therefore, we hypothesized that *CYP2B6* variants would be associated with altered nicotine oxidation, and that nicotine metabolism by *CYP2B6* would be detected in human brain microsomes. We generated recombinant enzymes in insect cells corresponding to nine common *CYP2B6* haplotypes and demonstrate genetically determined differences in nicotine oxidation to nicotine iminium ion and nornicotine for both (S) and (R)-nicotine. Notably, the *CYP2B6.6* and *CYP2B6.9* variants demonstrated lower intrinsic clearance relative to the reference enzyme, *CYP2B6.1*. In the presence of human brain microsomes, along with nicotine-*N*-oxidation, we also detect nicotine oxidation to nicotine iminium ion. However, unlike *N*-oxidation, this activity is NADPH independent, does not follow Michaelis-Menten kinetics, and is not inhibited by NADP or carbon monoxide. Furthermore, metabolism of common *CYP2B6* probe substrates, methadone and ketamine, is not detected in the presence of brain microsomes. We conclude that *CYP2B6* metabolizes nicotine stereoselectively and common *CYP2B6* variants differ in nicotine metabolism activity, but did not find evidence of *CYP2B6* activity in human brain.

KEYWORDS

brain, *CYP2B6*, microsomes, nicotine

1 | INTRODUCTION

Tobacco consumption and liability to nicotine dependence are heritable traits.^{1,2} One clearly implicated pathway is the C-oxidation of

nicotine to nicotine iminium ion and subsequently to cotinine by *CYP2A6*, one of three nicotine metabolism pathways (Figure 1). Variation in nicotine C-oxidation influences cigarette consumption among dependent smokers and consequent disease risk. *CYP2A6* is responsible for 70%-80% of nicotine metabolism in most people,³ and the mechanism of influence is straightforward: smokers with reduced *CYP2A6* function metabolize nicotine more slowly, delaying the onset of the withdrawal symptoms that compel nicotine addicts

Abbreviations: CL_{int} , intrinsic clearance; CYP, cytochrome P450; FMO, flavin-containing monooxygenase; HBM, human brain microsomes; NADPH, nicotinamide adenine dinucleotide phosphate; POR, P450 oxidoreductase; V_{max} , maximum velocity.

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to reach for the next cigarette. Hence, polymorphisms in *CYP2A6* causing slower nicotine metabolism are also associated with reduced cigarette consumption and disease risk in multiple populations.⁴⁻⁷ Nicotine is also metabolized via two other major pathways: glucuronidation by *UGT2B10*, and oxidation to nicotine-*N*-oxide by the flavin-containing monooxygenases (FMOs). Variation in *FMO3* is associated with altered hepatic nicotine metabolism.⁸

CYP2B6 is a minor contributor to hepatic nicotine metabolism with a K_m approximately 10-fold higher than that of *CYP2A6*.⁹ Genetic variation in the *CYP2B6* locus has a small but clinically insignificant influence on in vivo nicotine clearance in most subjects,^{10,11} but may be important in *CYP2A6* slow metabolizers. *CYP2B6* has also been reported to be expressed in human brain tissue,¹² unlike *CYP2A6*, and it has therefore been proposed that genetic variation affecting the *CYP2B6* enzyme might play a significant role in nicotine-related phenotypes.¹³ *CYP2B6* is highly polymorphic, and common haplotypes, especially *CYP2B6**6 and *9, containing 516G>T (Q172H, minor allele frequency 26% in European Americans, 37% in African Americans (<http://evs.gs.washington.edu/EVS/>)) are associated with clinically significantly altered pharmacokinetics for different substrates, relative to the reference allele.¹⁴⁻¹⁹ Because the effects of *CYP2B6* polymorphisms on *CYP2B6* activity is substrate-specific, activities of different *CYP2B6* isoforms cannot be assumed for untested substrates, such as nicotine. Therefore, we have expressed polymorphic *CYP2B6* enzymes and measured their nicotine metabolism activity in vitro. We further attempted to measure cytochrome P450-mediated nicotine metabolism in human brain microsomes, where FMO activity was previously demonstrated,^{8,20} using nicotine and other *CYP2B6* probe substrates.

2 | MATERIALS AND METHODS

CYP2B6 variants, reference allele P450 oxidoreductase (POR), and reference allele cytochrome *b*₅ vectors were previously generated, and recombinant proteins previously expressed in insect cells, as

described.^{17,21} Haplotype selection was based on the known clinical significance and frequency of the polymorphism, and a desire to inform the mechanistic basis for the influence of these polymorphisms on metabolism. There is growing evidence that the influence of *CYP2B6* polymorphisms varies between substrates, and may vary between enantiomers of those substrates. For example, 516G>T (Q172H) is a canonical single nucleotide polymorphism (SNP) thought to result in diminished metabolism,^{17,21,22} while 785A>G (K262R) alone causes increased activity for some but not all 2B6 substrates.^{21,23} To evaluate contribution from the two major SNPs, we selected variants with various combinations of these mutations (2B6*4, *6, *7, *9, *16, *19, *26) even though some are minor in population frequency. The other three 2B6 variants (*5, *17, *18) have relatively high allele frequency.

Human brain microsomes were prepared as described⁸ from de-identified cerebral cortex flash-frozen postmortem obtained from the National Disease Research Interchange (NDRI), Philadelphia PA, USA. They included four Caucasian males, one Caucasian female, and one African American male with ages ranging from 58 to 90 years. None were reported to be current smokers or substance abusers. In brief, tissues were homogenized with a glass-teflon homogenizer in ice-cold potassium phosphate buffer pH 7.4, and centrifuged 20 minute at 9000g. The S9 fraction was centrifuged 60 minute at 100 000g to obtain a microsomal pellet which was resuspended, further homogenized, centrifuged, and resuspended in buffer containing 20% glycerol^{24,25} for storage at -80°C. Total brain protein concentrations were determined using Bio-Rad Protein Assay Dye Regent Concentrate which is based on Bradford method. Results were expressed as fmol min⁻¹ mg⁻¹ of total brain protein, as *CYP2B6* protein was not detectable.

2.1 | Cytochrome P450 and *b*₅ concentration and POR activity determination

All assays for P450 content, *b*₅ content, and POR activity were carried out using a Synergy MX Microplate Reader (Biotek, Winooski, VT). Total protein concentrations were determined using Bio-Rad

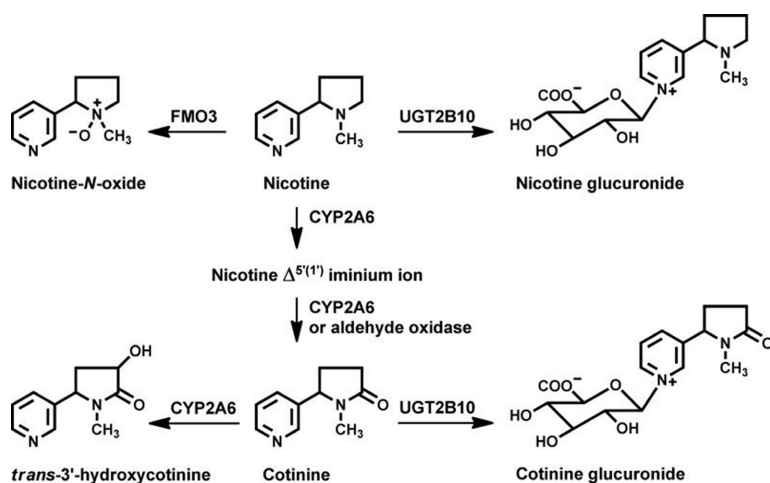


FIGURE 1 Nicotine metabolism pathways

Protein Assay Dye Reagent Concentrate which is based on Bradford method. P450 concentration was determined by difference spectrum of ferrous-carbon monoxide complex in a CO binding assay (reduced vs reduced CO) using an extinction coefficient $De_{450-490\text{ nm}}$ of $91\text{ mmol L}^{-1}\text{ cm}^{-1}$. Cytochrome b_5 content was determined by difference spectrum of NADH-reduced and oxidized b_5 using an extinction coefficient $De_{424-410\text{ nm}}$ of $185\text{ mmol L}^{-1}\text{ cm}^{-1}$.

P450 oxidoreductase activity was measured by NADPH-cytochrome c reductase activity. To $200\text{ }\mu\text{L}$ of diluted cell lysate in 0.3 mol L^{-1} potassium phosphate buffer at pH 7.7 containing $40\text{ }\mu\text{mol L}^{-1}$ cytochrome c, $100\text{ }\mu\text{mol L}^{-1}$ NADPH was added to initiate the reduction of cytochrome c and the reaction was followed at 550 nm and 23°C . The reaction rate was calculated using an extinction coefficient of $e_{550\text{ nm}}$ of $21\text{ mmol L}^{-1}\text{ cm}^{-1}$ for reduced cytochrome c. POR activity was converted to POR content based on the assumption that 3000 nmol of cytochrome c is reduced/min per nmol POR at 23°C .^{17,26}

2.2 | Substrate metabolism

All incubations were carried out in 96-well PCR plates in $50\text{ }\mu\text{L}$ total volume at 37°C , as previously described.⁸ Incubations with recombinant CYP2B6/POR/b5 proceeded for 10 minutes and incubations with human brain microsomes proceeded for 1 hour, unless otherwise noted, following a 2-minute preincubation and initiation with 25, 50, 100, 250, 500, 1000, 1,500, or 2,000 $\mu\text{mol L}^{-1}$ S-nicotine or R-nicotine (Toronto Research Chemicals, Toronto, Canada) in the presence of 1 mmol L^{-1} NADPH (Sigma-Aldrich, St. Louis, MO). Inhibition of metabolite formation was tested at $100\text{ }\mu\text{mol L}^{-1}$ nicotine for convenience to ensure metabolite concentrations remained within the concentration curve after incubation of up to 6 hours. Reactions were quenched by adding $10\text{ }\mu\text{L}$ 15% zinc sulfate containing 200 ng mL^{-1} of the internal standard, (d₃)-nicotine-N-oxide (Toronto Research Chemicals, Toronto, Canada). Samples were centrifuged and supernatants were removed for LC/MS analysis.

Methadone incubations were performed with $10\text{ }\mu\text{mol L}^{-1}$ methadone and processed and analyzed as previously described.^{21,27} In brief, methadone was from the National Institute on Drug Abuse (Bethesda, MD); all other reagents were purchased from Sigma-Aldrich. Reactions were quenched with $40\text{ }\mu\text{L}$ 20% trichloroacetic acid containing internal standard (d₃-EDDP), and centrifuged for 5 minutes at $2500g$; the supernatant was processed by solid phase extraction as previously described,^{28,29} except that for Strata-X-C $33\text{-}\mu\text{m}$, 30 mg/well plates (Phenomenex, Torrance, CA) were used. Analysis was performed on a Sunfire C18 column ($2.1 \times 50\text{ mm}$, $3.5\text{-}\mu\text{m}$) (Waters, Milford, MA) with a $2\text{-}\mu\text{m}$ column filter guard (Supleco Analytical, Bellefonte, PA). Sample injections were $25\text{ }\mu\text{L}$ and the column oven was held at 30°C . Mobile phase A was 4.5 mmol L^{-1} ammonium acetate in Milli-Q water, pH 4.5, and mobile phase B was 4.5 mol L^{-1} ammonium acetate in acetonitrile. The mobile phase gradient (0.4 mL min^{-1}) was 25% B for 0.5 minutes, linear gradient to 75% B between 0.5 and 4.2 minutes, held at

75% B until 5.0 minutes, immediately decreased back to 25% B and re-equilibrated at initial conditions for 3.0 minutes.

Ketamine incubations were performed with $80\text{ }\mu\text{mol L}^{-1}$ ketamine, and processed and analyzed as previously described¹⁷ In brief, the reactions were quenched with 20% trichloroacetic acid containing norketamine-d₄. The plate was centrifuged at $900g$ for 5 minutes and the supernatant was subjected to solid phase extraction.³⁰ Analytes were eluted with methanol and evaporated to dryness under nitrogen. For analysis, samples were resuspended in mobile phase (10 mmol L^{-1} ammonium acetate, pH 7.6). High performance liquid chromatography (HPLC)-mass spectrometry analysis was performed on an ultra-fast liquid chromatography system (Shimadzu Scientific Instruments, Columbia, MD) with a CMB-20A system controller, two LC-20ADXR pumps, DGU-20A3 degasser, SIL-20AC autosampler, FCV-11AL solvent selection module, and CTO-20A column oven (30°C) containing a ChiralPak AGP analytical column ($100 \times 2.0\text{ mm}$, $5\text{ }\mu\text{m}$) and AGP guard column ($10 \times 2.0\text{ mm}$) (Chiral Technologies, West Chester, PA), coupled to an API 4000 QTrap LC-MS/MS linear ion trap triple quadrupole tandem mass spectrometer (Applied Biosystems/MDS Sciex, Foster City, CA). The mobile phase (0.22 mL min^{-1}) was 10 mmol L^{-1} ammonium acetate in water, pH 7.60 (A) and isopropanol (B). The column was equilibrated with 4% B, maintained after injection for 0.1 minute, then a linear gradient to 16.8% B applied over 12 minutes, and then reverted back to 4% B over 0.5 minute and re-equilibrated for 3.5 minutes.

2.3 | Liquid chromatography tandem mass spectrometry of nicotine metabolites

Nicotine metabolites were analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS) based on previous methods⁸ and quantified using the ratio of metabolite to internal standard, subtracting background from control incubations (without NADPH), and using calibration curves. The subtraction of control (NADPH-) incubations was necessary to subtract trace nicotine metabolites present in the substrate, nicotine. Three replicates were performed for each incubation. Further control incubations for brain microsomes included with boiled protein or without protein.

Liquid chromatography tandem mass spectrometry analyses were performed on an API 4000 Q-TRAP triple quadrupole mass spectrometer (Applied Biosystems Sciex, Foster City, CA) equipped with an electrospray source. The HPLC system consisted of two LC 20AC pumps with a CTO-20A column oven, SIL-20A autosampler, DGU-20A3 degasser, FCF-11AL valve, and a CBM 20A controller (Shimadzu, Columbia, MD). The chromatographic separation was performed on an xBridge column (150×2.1 , $3.5\text{ }\mu\text{m}$, Waters, Milford, MA) with an inline precolumn $0.2\text{ }\mu\text{m}$ filter. The injection volume was $5\text{ }\mu\text{L}$ and the oven temperature was 40°C . Mobile phase (0.3 mL min^{-1}) was (A) 4.5 mmol L^{-1} ammonium acetate pH 4.0 and (B) 4.5 mmol L^{-1} ammonium acetate in acetonitrile using the following program: 1% B for 4 minutes, linear gradient to 25% B between 4 and 5 minutes, held at 25% B until 6.0 minutes, and then

re-equilibrated to initial conditions between 6.5 and 8.5 minutes. Under these conditions, the retention times for nicotine iminium and nornicotine (a minor CYP2B6 nicotine metabolite⁹) were 2.5 and 3.7 minutes, respectively; for *cis* and *trans* nicotine-*N*-oxide, they were 4.7 and 5.2 minutes, respectively. The instrument was operated in positive-ion mode at 450°C with an ion spray voltage of 5500 V, entrance potential of 10 V, and exit potential of 22 V. The curtain gas was set at 20, ion source gas 1 at 30, and ion source gas 2 at 40. Transitions monitored for nicotine iminium were *m/z* 161 → 130, for nornicotine, *m/z* 149 → 117, for nicotine-*N*-oxide *m/z* 179 → 132, and for deuterated (d3)-nicotine-*N*-oxide, *m/z* 182 → 132.

2.4 | Statistical analysis

Michaelis-Menten parameters for recombinant enzyme experiments were determined by nonlinear regression using SigmaPlot (Systat Software, San Jose, CA).

3 | RESULTS

3.1 | Nicotine oxidation by variant recombinant CYP2B6 enzymes

Nine recombinant versions of the CYP2B6 enzymes, representing common CYP2B6 haplotypes, were expressed to assess their relative nicotine metabolism activities. Two nicotine metabolites were measured, nicotine iminium ion and nornicotine, with K_m and V_{max} parameters determined for both metabolites for each variant (Table 1). Our results confirm the role of CYP2B6 in nornicotine formation.⁹ As expected, the activity of the most common variant enzyme, CYP2B6.6, was lower than the reference allele enzyme, CYP2B6.1, regarding both nicotine iminium ($Cl_{int} = 1.9$ vs $3.2 \mu\text{L}$

$\text{min}^{-1} \text{nmol}^{-1}$) and nornicotine ($Cl_{int} = 0.5$ vs $1.0 \mu\text{L} \text{min}^{-1} \text{nmol}^{-1}$) (Figure 2).

Variation in CYP2B6 affects enzyme activity differently depending on substrate, which may include differences between isomers of the same molecule. Nicotine occurs in two isomers, though approximately 99% of naturally occurring nicotine derived from tobacco and other plants is the (S) isomer. In order to determine if effects on CYP2B6 nicotine metabolism activity were also enantiomer-specific, incubations with four CYP2B6 haplotypes were repeated with (R)-nicotine (Table 2). K_m and V_{max} parameters were different for (R)-nicotine and (S)-nicotine, but differences between CYP2B6 variants appear consistent, that is, the activity of CYP2B6.6, was again lower than the reference allele enzyme for nicotine iminium ($Cl_{int} = 0.8$ vs $1.8 \mu\text{L}^{-1} \text{min}^{-1} \text{nmol}^{-1}$) and nornicotine ($Cl_{int} = 0.1$ vs $0.2 \mu\text{L}^{-1} \text{min}^{-1} \text{nmol}^{-1}$). Interestingly, the regioselectivity of the enzyme, that is, the metabolite ratio of nicotine iminium ion vs nornicotine, was relatively consistent among different CYP2B6 variants, but differed between (S)- and (R)-nicotine (Tables 1 and 2).

3.2 | CYP2B6 substrate metabolism by human brain microsomes

Microsomes were prepared from the cerebral cortices of six post-mortem individuals. Nicotine-*N*-oxidation by FMO enzymes was used as a positive control for tissue quality because FMO enzyme activity is diminished by heat treatment at room temperature or repeated freeze-thawing. Nicotine-iminium, but not nornicotine, was detected in incubations with all six samples. In the presence of $100 \mu\text{mol L}^{-1}$ nicotine, the average turnover was 264 ± 133 (range 142-498) $\text{fmol min}^{-1} \text{mg}^{-1}$ of microsomal protein. Activity was not significantly correlated with age (mean 72.9 ± 11.6 years) or postmortem interval (mean 11.6 ± 3.1 hours). The activity remained linear for at least 6 hours (Figure 3). Incubation conditions were interrogated to

TABLE 1 Kinetic parameters for (S)-nicotine-iminium and (S)-nornicotine formation by variant CYP2B6 proteins

Allele	Protein variant	(S)-iminium			(S)-nornicotine			
		K_m ($\mu\text{mol L}^{-1}$)	V_{max} (pmol min^{-1} pmol^{-1})	Cl_{int} ($\text{mL}^{-1} \text{min}^{-1}$ nmol^{-1})	K_m ($\mu\text{mol L}^{-1}$)	V_{max} (pmol min^{-1} pmol^{-1})	Cl_{int} (mL min^{-1} nmol^{-1})	Cl_{int} (nornicotine/iminium)
*1		269 ± 37	0.85 ± 0.04	0.0032	184 ± 27	0.19 ± 0.01	0.0010	0.33
*4	K262R	333 ± 32	0.83 ± 0.03	0.0025	167 ± 28	0.21 ± 0.01	0.0013	0.50
*5	R487C	257 ± 22	0.66 ± 0.01	0.0026	132 ± 18	0.09 ± 0.00	0.0007	0.27
*6	Q172H/K262R	307 ± 16	0.58 ± 0.01	0.0019	110 ± 15	0.06 ± 0.00	0.0005	0.29
*7	Q172H/K262R/ R487C	236 ± 21	0.56 ± 0.01	0.0024	108 ± 23	0.09 ± 0.00	0.0008	0.35
*9	Q172H	406 ± 44	0.18 ± 0.01	0.0004	134 ± 21	0.02 ± 0.00	0.0001	0.34
*17	T26S/D28G/R29T	332 ± 22	0.85 ± 0.02	0.0026	130 ± 17	0.11 ± 0.00	0.0008	0.33
*19	Q172H/K262R/ R336C	271 ± 27	0.60 ± 0.02	0.0022	114 ± 26	0.06 ± 0.00	0.0005	0.24
*26	P167A/Q172H/ K262R	189 ± 21	0.41 ± 0.01	0.0022	86 ± 20	0.08 ± 0.00	0.0009	0.43

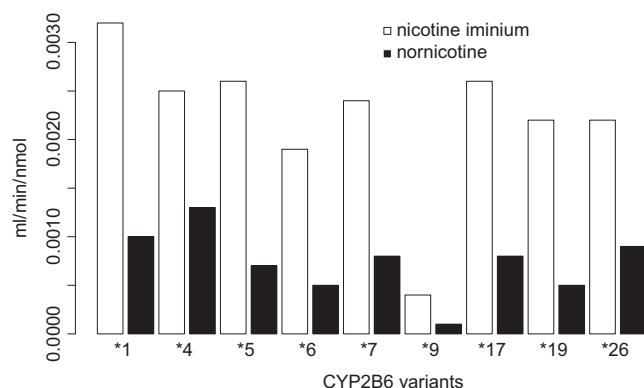


FIGURE 2 Intrinsic clearance (CL_{int}) of (S)-nicotine to nicotine iminium ion and nornicotine by variant recombinant CYP2B6 enzymes

determine whether iminium production was consistent with cytochrome P450 activity. The activity was abolished by heat-treatment (65°C 1 hour), but did not require NADPH, and was not inhibited by NADP (2 mmol L⁻¹) or carbon monoxide treatment. Nicotine-N-oxide, the product of FMO enzymes (Figure 1), was also detected in the microsome incubations, but its formation required NADPH. On further characterizing one brain, we found the NADPH-independent nicotine-iminium formation did not follow Michaelis-Menten kinetics (Figure 4). Cotinine and hydroxycotinine were also not generated in any human brain incubation.

To further investigate possible CYP2B6 activity in human brain microsomes, we incubated them with two additional common CYP2B6 probe substrates, methadone (10 μmol L⁻¹) and ketamine (80 μmol L⁻¹), the approximate K_m for CYP2B6 for each substrate.^{27,31} Neither 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) nor norketamine were detected, respectively.

4 | DISCUSSION

Extensive evidence demonstrates that functional variation in cytochrome P450 genes can significantly affect the metabolism of many small molecules including pharmaceuticals and toxins. Importantly, common functional polymorphisms in CYP2B6 alter enzyme activity in a substrate-dependent manner,^{15,16,19,22,23,32-34} and thus it is important to evaluate these enzyme variants for any CYP2B6

substrate. Here, we demonstrate how these common variants also affect nicotine metabolism in vitro. The differences we report, both for (S)-nicotine and (R)-nicotine, confirm the expectation of altered activities for common variants including CYP2B6.6 and CYP2B6.9, relative to the reference allele enzyme, CYP2B6.1. Because (S)-nicotine is the common naturally occurring form of nicotine relevant to smoking behavior, experiments with (R)-nicotine were only performed to explore substrate specificity and stereoselectivity. The activities of the nine enzyme variants also differed with regard to the relative metabolite ratios of (S)-nicotine iminium and (S)-nornicotine. The differences in this ratio demonstrate altered regioselectivity associated with each polymorphism, that is, changes in the activity of each enzyme isoform depend on which region of the substrate molecule the enzyme oxidizes,³⁵ resulting in the formation of nicotine iminium vs nornicotine.

Of particular interest are the differences in activity related to the two amino acid changes, Q172H and K262R, which individually define the CYP2B6*9 and CYP2B6*4 variants respectively, and together define the very common CYP2B6*6 haplotype. For most substrates, activity of the CYP2B6.9 variant is exceptionally low, activity of the CYP2B6.4 variant is similar or greater than that of the reference allele, while the activity of CYP2B6.6 lies between CYP2B6.4 and CYP2B6.9, depending on substrate.^{14,21,22} This demonstrates that the K262R amino acid change can partially compensate for the Q172H loss-of-function, depending on substrate, and we find this to be the case for nicotine as well. CYP2B6*6 is the most commonly studied variant haplotype because of its high population frequency and has been associated with low activity in vivo.^{16,33} However, it is also important to note that part of this loss of activity may be due to differences in mRNA or protein expression or splicing, and not strictly related to catalytic activity.^{10,36,37} CYP2B6*6 (and CYP2B6*9) have also been reportedly associated with more rapid in vivo nicotine metabolism.³⁴

We previously demonstrated that common CYP2B6 haplotypes, including missense variants and noncoding variants that affect gene splicing, are associated with differences in in vivo nicotine metabolism.¹⁰ However, the small contribution of CYP2B6 to hepatic nicotine metabolism³⁸ is unlikely to influence smoking behaviors. CYP2B6 mRNA expression has been reported in human brain,¹² although direct evidence of CYP2B6 activity in brain tissue was not previously demonstrated. It has therefore been suggested that genetically determined variation in CYP2B6 activity in extrahepatic

TABLE 2 Kinetic parameters for (R)-nicotine-iminium and (R)-nornicotine formation by variant CYP2B6 proteins

Allele	Protein variant	(R)-iminium			(R)-nornicotine			
		K_m (μmol L ⁻¹)	V_{max} (pmol min ⁻¹ pmol ⁻¹)	CL_{int} (mL min ⁻¹ nmol ⁻¹)	K_m (μmol L ⁻¹)	V_{max} (pmol min ⁻¹ pmol ⁻¹)	CL_{int} (mL min ⁻¹ nmol ⁻¹)	CL_{int} (nornicotine/iminium)
*1		1537 ± 352	2.78 ± 0.24	0.0018	1288 ± 289	0.25 ± 0.03	0.0002	0.11
*4	K262R	1602 ± 436	2.11 ± 0.32	0.0013	936 ± 314	0.15 ± 0.02	0.0002	0.11
*6	Q172H/K262R	918 ± 409	0.73 ± 0.15	0.0008	956 ± 584	0.07 ± 0.02	0.0001	0.10
*9	Q172H	5040 ± 2276	0.72 ± 0.25	0.0001	1435 ± 665	0.03 ± 0.01	0.0000	0.13

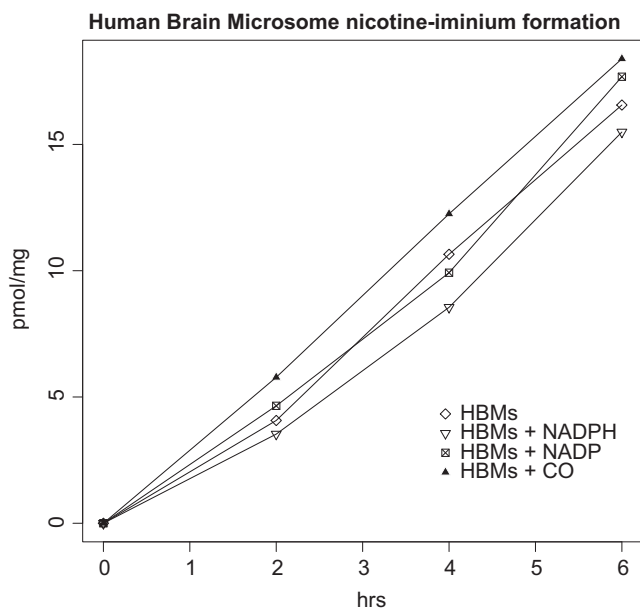


FIGURE 3 Nicotine iminium formation over time in the presence of human brain microsomes (HBM), with and without NADPH, inhibited by NADP or following carbon monoxide (CO) treatment

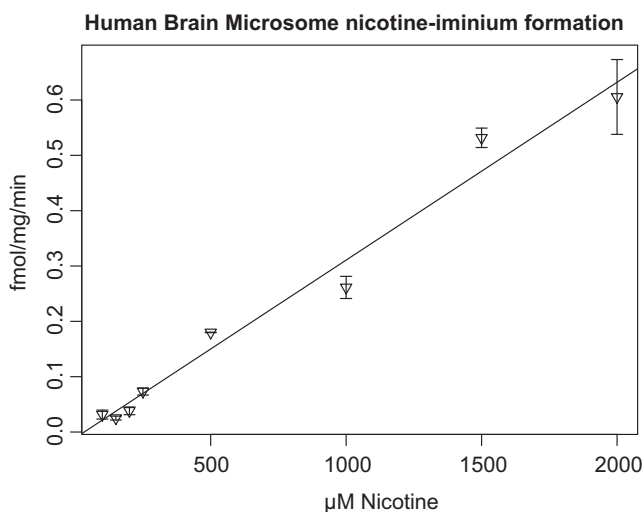


FIGURE 4 Nicotine iminium ion formed per mg human brain microsomal protein per minute

tissues might influence smoking behavior by altering local nicotine levels in the brain.¹³ We recently demonstrated nicotine-*N*-oxidation by flavin-containing monooxygenases in microsomes prepared from human brain.⁸ This provided an opportunity to further investigate possible cytochrome P450-related nicotine metabolism in post-mortem samples known to retain detectable microsomal enzyme activity. Given the difficulty of obtaining postmortem human brain material, it was important to have a positive control for tissue quality. Postmortem intervals before initial freezing were typically 12 hours or more, and FMO activity is also subject to degradation at room temperature and following repeated freeze-thawing.

Interestingly, we found nicotine iminium ion produced in the presence of human brain microsomes (nornicotine was not detected), but

the mechanism of this weak activity was obscure. It is independent of the cofactor NADPH and not inhibited by either carbon monoxide or NADP, though the activity was abolished by heat treatment of the microsomes. Meanwhile, the production of nicotine-*N*-oxide in the same incubations required NADPH and was inhibited by carbon monoxide, NADP, and heat treatment, as expected. We therefore conclude that the iminium ion detected was not due to cytochrome P450s or any typical enzymatic activity. NADP and carbon monoxide are general inhibitors of CYP enzymes. Had either general inhibitor abolished the activity, we would have pursued other specific inhibitors to narrow down the source of the activity to specific enzymes. Given the high K_m of CYP2B6 for nicotine, we further probed human brain microsomes with two common CYP2B6 substrates, ketamine and methadone, and found no evidence of metabolism of either.

Variation in local nicotine metabolism in the brain is ultimately interesting because of the potential for targeting these pathways to aid smoking cessation. Our prior work suggested that differences in nicotine metabolism within the brain might influence nicotine dependence in ways qualitatively different from the influence of hepatic metabolism; namely, common functional variation in an enzyme active in both liver and brain, FMO3, is associated with a significant difference in nicotine dependence independent of cigarette consumption level. Meanwhile, variation in the primary cytochrome-P450-mediated nicotine metabolism pathway affects consumption among dependent smokers but not liability to become dependent.^{8,39} It was therefore important to understand whether the divergent influences of these two pathways are related to inherent differences in their activities—that is, the metabolites produced—or differences in their tissue distributions. If the highly polymorphic CYP enzymes responsible for nicotine C-oxidation were active in brain, it would argue against FMO3's local brain activity being the mechanism of its unique influence on dependence. Unlike CYP2A6, CYP2B6 mRNA expression has been reported in human brain tissue, but aside from its role in bupropion metabolism, genetic variation in CYP2B6 has not been associated with smoking phenotypes. However, given the well-documented substrate specificity of functional variation in CYP2B6, it was also necessary to determine which variants affect nicotine metabolism, a question not previously addressed in vitro. A key limitation of our study was the problem of enzymatic stability in postmortem brain tissue. Nevertheless, if undetected CYP2B6 activity does occur in the human brain, with the potential to influence smoking behavior in a similar manner to FMO3, associations between CYP2B6 genotype and nicotine dependence should be detectable in human genetic studies based on our in vitro activity results together with previously published findings regarding variation in CYP2B6 expression.

In summary, we conclude that nicotine metabolism by CYP2B6 is stereoselective, and common polymorphisms in CYP2B6 significantly influence the oxidation of both (*S*) and (*R*)-nicotine to nicotine iminium and nornicotine. However, the small contribution of CYP2B6 to nicotine metabolism in the liver, and its undetectable activity in human brain tissue, make it highly unlikely that this genetic variation influences complex smoking behaviors.

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AUTHOR CONTRIBUTIONS

Participated in research design: Bloom, Wang, and Kharasch.

Conducted experiments: Bloom.

Contributed new reagents or analytic tools: Bloom, Wang, and Kharasch.

Performed data analysis: Bloom.

Wrote or contributed to the writing of the manuscript: Bloom and Kharasch.

DISCLOSURE

None declared.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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