Title:

Selegiline (L-deprenyl) is a mechanism-based inactivator of CYP2A6 inhibiting nicotine

metabolism in humans and mice

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Running Title:

Inhibition of nicotine metabolism by selegiline

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Nonstandard abbreviations: CYP – cytochrome P450, MAO – monoamine oxidase, NIC – nicotine, SEL – selegiline, DES – desmethylselegiline, L-AMP – L-amphetamine, L-MAMP – L-methamphetamine, MBI – mechanism-based inhibitor, AUC – area under the concentration time curve, $t_{1/2}$ – elimination half-life, CL – clearance, F – bioavailability

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. Pre-treatment 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; $k_{inact} = 0.34±0.04$ 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., 1996) 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. 1) (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., 2005; Siu and Tyndale, 2007). 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 replacement therapies, or it could be combined with nicotine as an oral combination therapy with nicotine for smoking cessation.

Methods

Animals

Adult male DBA/2 mice, previously characterized in nicotine metabolism studies (Siu and Tyndale, 2007), 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^oC 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^oC shaking at 150 rpm. Following incubation the culture was centrifuged at 2800xg at 4^oC for 20 min and the pellet was resuspended in $1/20^{th}$ 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 $1/20^{th}$ 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^oC for 1 hr followed by centrifugation at 2800xg at 4^oC for 20 min. After centrifugation the pellet was resuspended in $1/25^{th}$ 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 b_5 (Invitrogen, Carlsbad, CA) with an incubation time of 15

min. For expressed CYP2A6 (containing P450 reductase and cytochrome b_5) (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

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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 Na_2CO_3. After incubation, 5-methylcotinine (70 μg) was added as the internal standard

and the samples were prepared and analyzed for nicotine and metabolites by HPLC system 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 (0 to 100 μM) 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

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nicotine or nicotine plus selegiline (both at 1 mg/kg). The nicotine dose was chosen based on

previous pharmacokinetic studies (Siu and Tyndale, 2007) 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 $v = V_{max} [S] / (K_m + [S])$ (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, 2007).

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. 2a). Selegiline also inhibited CYP2A5 to a great extent while desmethylselegiline, L-methamphetamine, and L-amphetamine had similar inhibitory effects (Fig. 2b). In human liver microsomes (HLMs), selegiline and L-amphetamine appeared to show the greatest inhibitory activities on nicotine metabolism closely followed by desmethylselegiline (Fig. 2c), while L-methamphetamine did not inhibit cotinine formation (Fig. 2c). As with the three previous enzyme sources, selegiline caused the greatest inhibition on nicotine metabolism in CYP2A6 (Fig. 2d). Desmethylselegiline and L-amphetamine showed almost similar inhibition whereas L-methamphetamine did not alter nicotine metabolism (Fig. 2d), as was seen with HLMs (Fig. 2c).

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 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. 3a & b). Desmethylselegiline also dose-dependently inhibited nicotine metabolism in MLMs but its effect was much weaker in expressed CYP2A5 (Figs. 3c & d). As with selegiline, pre-incubation with desmethylselegiline enhanced inhibition in MLMs (6.1-fold change in IC_{50}) but not in CYP2A5 (Figs. 3c & 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. 4, Table I). 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 CYP2A6 (Figs. 5a & 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. 5a & b). Desmethylselegiline also dose-dependently inhibited nicotine metabolism in HLMs (Fig. 5c) but seemed to be a weaker inhibitor of CYP2A6 (Fig. 5d). Pre-incubation with desmethylselegiline did not enhance inhibition in HLMs but may modestly increase inhibition in CYP2A6 (Figs. 5c & 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 6a).

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 6b 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_{inact} were estimated to be 15.6 ± 2.7 μM and 0.34 ± 0.04 min^{-1}, respectively (Fig. 6c).

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. 6d), indicating a requirement for the cofactor NADPH. In

contrast, in the presence of NADPH 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. 6d). 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 Center for Health

Statistics, 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% *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., 2005; Siu and Tyndale, 2007). 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., 2007), 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_I 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., 1991). 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., 2003), 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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Footnotes

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Legends for Figures

Figure 1. Selegiline and its metabolites. Selegiline is metabolized to desmethyselegiline and L-methamphetamine, both of which can be metabolized to L-amphetamine (Shin, 1997; Valoti et al., 2000).

Figure 2. 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.

Figure 3. 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_{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.

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Figure 4. 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 ($\pm$ S.D.) of 3 to 6 animals for

each treatment; statistical comparisons are listed in Table 1.

Figure 5. 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 6. 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

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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.

Table 1. 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.

	-SEL	+SEL
	(mean $\pm$ SD)	(mean $\pm$ SD)
$AUC_{0\text{-}40}$ (ng • hr/ml)	57.4 ± 5.3	90.7 ± 5.8*
$t_{1/2}$ (min)	6.6 ± 1.4	12.5 ± 6.3*
CL/F (ml/min)	7.3 ± 0.3	4.6 ± 0.4*

*$p < 0.05$ compared to –SEL

Figure 1
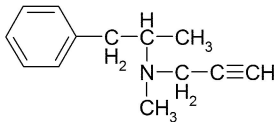
Selegiline (L-deprenyl)
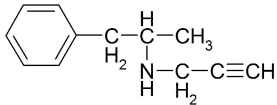
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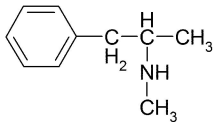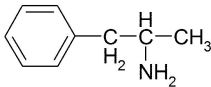
L-methamphetamine

L-amphetamine

Figure 2

Figure 3

Figure 4

Figure 5

A **HLMs**

$IC_{50} = 66.5\pm13.5 \ \mu M$

$IC_{50} = 18.1\pm3.1 \ \mu M$ *

-PRE
+PRE

% Activity Remaining ± SEM

[SEL] μM

B **CYP2A6**

$IC_{50} = 50.3\pm13.0 \ \mu M$

$IC_{50} = 3.4\pm1.0 \ \mu M$ *

-PRE
+PRE

% Activity Remaining ± SEM

[SEL] μM

C **HLMs**

-PRE
+PRE

% Activity Remaining ± SEM

[DES] μM

D **CYP2A6**

$IC_{50} = 72.6\pm3.6 \ \mu M$

-PRE
+PRE

% Activity Remaining ± SEM

[DES] μM

Figure 6