Inhibition of nicotine metabolism by methoxysalen: pharmacokinetic and pharmacological studies in mice


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Abbreviations

%MPE, maximum possible effect; s.c., subcutaneous injection; CL, confidence limit; ED50, effective dose 50%; ED84, effective dose 84%.
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 t1/2 = 5.9-6.9 min) (Petersen et al., 1984) compared to that of the rat (45 min) (Hwa Jung et al., 2001), 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). 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., 2005). 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., 2005; 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 \textit{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 \textit{in vitro}. Subsequently studies were performed \textit{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 was obtained from Aldrich Chemical Company, Inc. (Milwaukee, WI) and converted to the ditartrate salt as described by Aceto et al. (1979). Methoxsalen (9-Methoxyfuro[3,2-g][1]benzopyran-7-one) 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. In vitro kinetic parameters (Km and Vmax) of nicotine metabolism in ICR mice were characterized using substrate concentrations of 0, 3.0 to 1000 µM 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.

**Methoxsalen Inhibition of Cotinine Formation from Nicotine**

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

**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. For each time point, 4 to 6 mice were used. 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 (1941) as modified by 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. Groups of eight to twelve animals were used for each dose and for each treatment.

**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 (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 1. & inset). The estimated kinetic parameters for the in vitro nicotine metabolism, derived from five animals, for both sites can be found in Table I. 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 ($K_m/V_{max}$) 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., 2005). 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 2 & Table 1). 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 plasma nicotine and cotinine levels after systemic administration in mice. Administration of methoxsalen significantly increased the plasma half-life of nicotine and increased its AUC compared to saline treatment (Fig 3A & Table 2). Methoxsalen had minimal effect on the maximum nicotine concentration (Table 2) 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 methoxsalen (Fig 3B).

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

Methoxsalen 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 methoxsalen (10 mg/kg) enhancement of nicotine’s effects to determine an optimal pretreatment time. Mice were given methoxsalen 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. 4A, the potentiation of nicotine’s antinociceptive effects by methoxsalen 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. 4B) 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 methoxsalen 30 min before
nicotine. We then determined its potency of enhancing nicotine’s effects at this pretreatment time. As showed in
Fig. 5, methoxsalen dose-dependently enhanced nicotine-induced antinociception in the tail-flick (Fig. 5A), hot-plate
(Fig. 5B) assays and hypothermia (Fig. 5C).

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. 6 & Table 3).

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. 7A). A similar time-course pattern
was observed in the hot-plate test (Fig. 7B). 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. 7C). 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. 7A & B). On the other hand, the effects of
nicotine on body temperature were still significant beyond the 180 min time point (Fig. 7C). 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 8, 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 hypothermic responses (data not shown).

**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 ($K_m \sim 7.7 \pm 0.8 \mu M$; 129/J mouse strain) (Murphy et al., 2005). 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 *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 1). 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 (Siu et al., 2006; Murphy et al., 2005) as well as the second low affinity site. The *in vitro* mouse pharmacokinetic data (Table 1) 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 1).

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; 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 4, 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 possibly mechanism-based inhibition. It has been shown in vitro that mouse CYP2A5 can metabolize methoxsalen (Maepaa et al., 1994). Mechanism-based inhibitors, also known as suicide substrates, 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_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 in vitro and 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 2). 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 (Rao et al., 2000; Ariyoshi et al., 2002; Schoedel et al., 2004; Malaiyandi}
et al., 2006; Minematsu et al., 2006). 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) (Rao et al., 2000; Ariyoshi et al., 2002; Schoedel et al., 2004; Malaiyandi et al., 2006). 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 8). 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 if 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.

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FOOTNOTES

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LEGENDS FOR FIGURES

Figure. 1  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 Eadee-Hofstee plot indicating the presence of two enzymatic sites responsible for the metabolism of nicotine. NIC = Nicotine.

Figure. 2  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 Ki. Three animals were used to establish Ki value. Velocity is expressed as nmol/min/mg. 8-MOP = methoxsalen.

Figure. 3  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.

Figure. 4  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. 5  Dose-response enhancement of 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. 8-MOP = methoxsalen.

Figure. 6  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.

**Figure 7** 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.

**Figure 8** 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.
**Table 1:** Kinetic parameters of in vitro nicotine C-oxidation activities of adult male ICR mouse liver microsomes.

*Kₘ, Vₘₐₓ and Kᵢ values were determined independently for each animal and the average ± SD values are shown.*

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<td><em>Kₘ (µM)</em></td>
<td>18.6 ± 5.9</td>
</tr>
<tr>
<td><em>Vₘₐₓ (nmol/min/mg)</em></td>
<td>0.22 ± 0.05</td>
</tr>
<tr>
<td><em>Vₘₐₓ/Kₘ</em></td>
<td>0.013 ± 0.007</td>
</tr>
<tr>
<td><strong>Low Affinity</strong></td>
<td></td>
</tr>
<tr>
<td><em>Kₘ (µM)</em></td>
<td>215.2 ± 60.0</td>
</tr>
<tr>
<td><em>Vₘₐₓ (nmol/min/mg)</em></td>
<td>0.65 ± 0.08</td>
</tr>
<tr>
<td><em>Vₘₐₓ/Kₘ</em></td>
<td>0.003 ± 0.001</td>
</tr>
<tr>
<td><strong>Methoxsalen Inhibition</strong></td>
<td></td>
</tr>
<tr>
<td><em>Kᵢ (µM)</em></td>
<td>0.32 ± 0.03</td>
</tr>
</tbody>
</table>
**Table 2:** 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&lt;sub&gt;max&lt;/sub&gt; (ng/ml)</th>
<th>AUC (ng*hr/ml)</th>
<th>T&lt;sub&gt;1/2&lt;/sub&gt; (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>
**Table 3:** 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</th>
<th>Tail-flick Test</th>
<th>Hot-plate Test</th>
<th>Body temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% MPE (Mean ± SEM)</td>
<td>% MPE (Mean ± SEM)</td>
<td>∆℃ (Mean ± SEM)</td>
</tr>
<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 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
Figure 7
Figure 8