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Nicotine metabolism and urinary elimination in mouse: *in vitro* and *in vivo*

H. RAUNIO[1], N. POKELA[1], K. PUHAKAINEN[1], M. RAHNASTO[1], T. MAURIALA[2], S. AURIOLA[2], & R. O. JUVONEN[1]

[1]*Department of Pharmacology and Toxicology and* [2]*Department of Medicinal Chemistry, University of Kuopio, Kuopio, Finland*

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Abstract

This study aimed at elucidating the *in vivo* metabolism of nicotine both with and without inhibitors of nicotine metabolism. Second, the role of mouse CYP2A5 in nicotine oxidation *in vitro* was studied as such information is needed to assess whether the mouse is a suitable model for studying chemical inhibitors of the human CYP2A6. The oxidation of nicotine to cotinine was measured and the ability of various inhibitors to modify this reaction was determined. Nicotine and various inhibitors were co-administered to CD2F1 mice, and nicotine and urinary levels of nicotine and four metabolites were determined. In mouse liver microsomes anti-CYP2A5 antibody and known chemical inhibitors of the CYP2A5 enzyme blocked cotinine formation by 85–100%, depending on the pre-treatment of the mice. The amount of *trans*-3-hydroxycotine was five times higher than cotinine N-oxide, and ten times higher than nicotine N-1-oxide and cotinine. Methoxsalen, an irreversible inhibitor of CYP2A5, significantly reduced the metabolic elimination of nicotine *in vivo*, but the reversible inhibitors had no effect. It is concluded that the metabolism of nicotine in mouse is very similar to that in man and, therefore, that the mouse is a suitable model for testing novel chemical inhibitors of human CYP2A6.

Keywords: *Nicotine oxidation, CYP2A5, human/mouse correlation*

Introduction

Tobacco is one of the worst threats to human health. It damages the lungs, blood circulation, and heart, and causes cancer (Ezzati et al. 2003). Nicotine is the key compound promoting the use of tobacco due to its highly addictive properties (George and O'Malley 2004) and several lines of evidence indicate that smokers titrate their cigarette consumption to maintain steady levels of nicotine in the brain (Benowitz 1996).

Correspondence: R. O. Juvonen, Department of Pharmacology and Toxicology, University of Kuopio, POB 1627, FIN-70211, Kuopio, Finland. Tel: 358-17-162010. Fax: 358-17-162424. E-mail: risto.juvonen@uku.fi

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Thus, factors that influence nicotine plasma levels, by either intake or elimination, would be anticipated to affect smoking behaviour (Malaiyandi et al. 2005).

In humans, the main elimination route of nicotine is oxidation to cotinine which is catalysed by hepatic CYP2A6 and aldehyde oxidase enzymes (Brandange and Lindblom 1979; Nakajima et al. 1996), reviewed by Hukkanen et al. (2005) (Figure 1), although less is known about the enzymes that catalyse nicotine metabolism in other species. Guinea pig and hamster hepatocytes show the highest total metabolism of nicotine, followed by mouse, rat, and human hepatocytes (Hukkanen et al. 2005). A recent study by Murphy et al. (2005) revealed that mouse CYP2A5 and rat CYP2A3 enzymes are efficient catalysts of cotinine formation *in vitro*. CYP2A5 is an abundant and active enzyme in mouse liver, whereas CYP2A3 is present in minor quantities in extrahepatic tissues in rat (Honkakoski and Negishi 1997; Su and Ding 2004). The mouse has a much higher CYP2A activity *in vivo* than the rat and thus the mouse appears to be the preferred *in vivo* model species for nicotine metabolism studies.

CYP2A6 genetic variations, causing reduced or absent enzyme activity, are associated with a reduced risk for smoking and population studies have indicated that individuals with

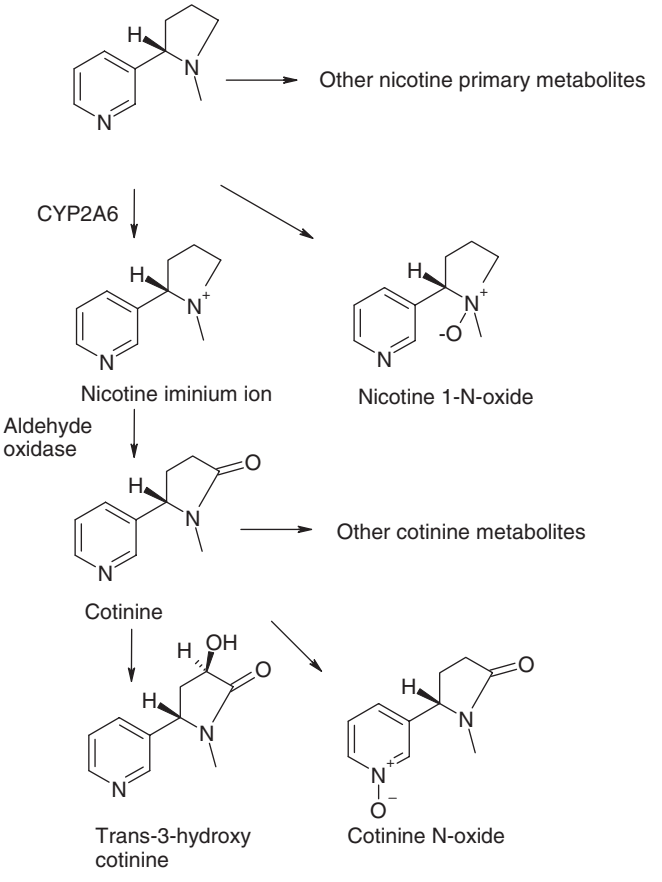

Figure 1. Major metabolic pathways of nicotine in humans. CYP2A6 is the major catalysing enzyme of the oxidation of nicotine to nicotine iminium ion, which is further oxidized to cotinine by aldehyde oxidase.

an inactive CYP2A6 enzyme are less prone to develop cigarette smoking-associated cancers (Oscarson 2001; Raunio et al. 2001; Malaiyandi et al. 2005). This is especially prominent in the Japanese population, which has a high frequency of inactive CYP2A6 alleles (Kamataki et al. 2005). It has been suggested by Sellers and Tyndale (2000) that inhibition of the CYP2A6 enzyme would slow nicotine inactivation, prolong the duration that nicotine remains in the brain, and lead to a reduction in the number of cigarettes smoked. Such inhibition could be achieved by chemical agents that interfere with the catalytic activity of the CYP2A6 enzyme. Pilot studies with CYP2A6 inhibitors suggest that a reduction in cigarette consumption can be achieved (Sellers et al. 2003).

The goals of the current study were (1) to elucidate the metabolic pathways of nicotine in mouse *in vivo*, (2) to study the role of the CYP2A5 enzyme in nicotine-to-cotinine formation in mouse liver microsomes *in vitro*, and (3) to test whether known potent inhibitors of CYP2A5 could reduce the metabolic elimination of nicotine *in vivo*. This kind of information is crucial when deciding which animal model to use in testing novel CYP2A6 inhibitors for potential use in man.

Materials and methods

Reagents

(1′*S*,2′*S*)-nicotine-1′oxide (>98% purity), (*S*)-cotinine N-oxide (98% purity), (+)-cotinine (98% purity), [(4*S*)-(–)-1 methyl-5-(pyridin-3-yl)]-2-pyrroliline (98% purity), *R*-(+)-cotinine, (3*S*,5*S*)-3′-hydroxycotinine (98% purity), and nicotine (96% purity) were from TRC (Toronto Research Chemicals, Toronto, Canada). (–)-Cotinine (98% purity) and (–)-nicotine (99% purity), β-glucuronidase, coumarin, methoxsalen, γ-nonanoic lactone, 2,7-dimethylnaphthalene, 4-methylnaphthalene, 2-bromonaphthalene, and 4-methoxybenzaldehyde were obtained from Sigma/Aldrich (St Louis, MO, USA). NADPH was from Roche (Mannheim, Germany).

Animals

Female DBA/2N/Kuo and CD2F1 mice were obtained from the National Laboratory Animal Centre, Kuopio University. For the *in vitro* experiments, liver microsomes were prepared from livers of DBA/2N and CD2F1 mice (7–12 weeks old, 15–25 g body weight) as described previously (Lang et al. 1981; Rahnasto et al. 2003). The CD2F1 mice used in the *in vivo* experiments were 7 weeks old (17–23 g body weight) at the beginning of the experiments. The mice had unrestricted access to water and standard chow (Lactamin R36, Lactamin AB, Södertälje, Sweden). The Ethics Committee for Animal Experiments, University of Kuopio approved these experiments.

In vitro *experiments*

For the *in vitro* studies, the mice were administered pyrazole (150 mg kg^{-1} intraperitoneally (i.p.) for three consecutive days) or phenobarbital (60 mg kg^{-1} i.p. for four consecutive days). The control animals received vehicle only (0.9% saline). The livers were removed into ice-cold saline and microsomes were prepared, pooled, and stored at –80°C until used (Lang et al. 1981). Pyrazole is known to maximize CYP2A5 oxidizing capacity

(Juvonen et al. 1985), and phenobarbital induces several other CYP forms in mouse liver (Honkakoski and Negishi 1997). Microsomal protein content was determined by the method of Smith et al. (1985). The preparation and characterization of the anti-CYP2A5 antibody has been described earlier by Raunio et al. (1998). This antibody does not inhibit CYP2B, CYP2D, and CYP3A activities in mouse liver microsomes.

In vivo *experiments*

A total of 65 CD2F1 mice were used for the *in vivo* experiments. The test inhibitors were administered as single doses by gavage at $2\,mg\,kg^{-1}$, followed by nicotine at $10\,mg\,kg^{-1}$ (average 1.2 μmol/mouse) 30 min later. The mice were housed in metabolic cages for 24 h for collection of urine. After measuring the volume, the urine was centrifuged and the clear supernatant was stored at –20°C until assayed. The inhibitors (methoxsalen, coumarin, γ-nonanoic lactone, 2,7-dimethylnaphthalene, 2-bromonaphthalene, and 4-methoxybenzaldehyde) were chosen because they are potent CYP2A5 inhibitors *in vitro* (Mäenpää et al. 1993; Juvonen et al. 2000; Rahnasto et al. 2003, 2005).

*High-pressure liquid chromatography (HPLC) assay (*in vitro *experiments)*

Nicotine 5′-oxidation was measured indirectly via the formation of cotinine from nicotine. The reaction mixture consisted of 50 mM potassium phosphate (pH 7.4), 5 mM $MgCl_2$, 200 μM nicotine, 2 mg liver cytosol (as aldehyde oxidase source), and 33–215 μg microsomes in a total volume of 0.5 ml. The reaction was initiated by adding NADPH (1 mM final concentration) and allowed to proceed for 20 min at 37°C. The reaction was terminated by adding 100 μl of 20% Na_2CO_3 and mixing on a Vortex. The samples were extracted with 2 ml of 2-propanol and dichloromethane (1:2 v/v) and shaken vigorously. A total of 1 ml of the organic phase was transferred to another tube, evaporated by centrifugation, and the residual was dissolved in 250 μl of the mobile phase solution for HPLC analysis. Nicotine-to-cotinine formation was determined with a modification of the HPLC method published by Messina et al. (1997). The HPLC system (Shimadzu) consisted of a LC-10AD VP pump, a SIL-9A injector, and an SPD-10A VP multi-wavelength detector. The stationary phase was a reverse phase Supelcosil LC-18-DB column (C_{18}, 250 × 4.6 mm). The mobile phase was a mixture of 27% acetonitrile, 73% 20 mM KH_2PO_4, and 1 mM Na-1-heptane sulfonate. The isocratic flow rate was $0.5\,ml\,min^{-1}$ at 60–70 kgf pressure; the sample volume was 50 μl. Based on spectral analysis of nicotine and cotinine, 261 nm was chosen as the detection wavelength. For the preparation of standard curves, cotinine (0.125–50 μM) was mixed with the reaction mixture, extracted, and dissolved in 250 μl of the HPLC mobile phase solution. The standard curve was linear up to 50 μM cotinine. Cotinine was measured as peak height and compared with the standard curve, enabling peak heights to be converted to cotinine concentrations. Nicotine-to-cotinine kinetic studies were carried out by incubating 10, 20, 40, 100, 20, 400, and 1000 μM nicotine with microsomes from the variously treated mice. Inhibition studies consisted of incubation of 200 μM nicotine with various concentrations of the anti-CYP2A5 antibody and the chemical inhibitors γ-nonanoic lactone and coumarin.

*Mass spectrometry assay for nicotine metabolism (*in vivo *experiments)*

Liquid chromatography-atmospheric chemical ionization mass spectrometer (LC-APCI-MS) consisted of a Thermo Finnigan LTQ (USA) linear ion-trap mass spectrometer coupled with a Surveyor LC pump. The instrument was operated in positive mode using multiple reaction monitoring (MRM). A Phenomenex Gemini (C_{18}, 250 × 2 mm) column was used. Flow rate was 200 µl min^{-1} and injection volume was 10 µl. Solvent A was 0.01% ammonia solution and solvent B was methanol. A linear gradient from 2% B to 100% B in 20 min was used. The MS settings were: APCI vaporizer temperature, 300°C; capillary temperature, 200°C; source voltage, 6000 V; collision energy, 46; sheath gas flow, 48 arbitrary units; and AUX gas flow, 10 arbitrary units. XCalibur software was used to control LC-MS system.

The calibration curves covered the range from 0.01 to 10 µM and their correlation coefficients ranged from 0.990 to 0.999. All of results were calculated using $1/x$ weighted linear regression. All the peak area ratios, regression coefficients, and parameters were calculated from the peak area data by the LCquan program version 2.0 (Thermo Finnigan).

Stock solutions of all six analytes were prepared in methanol (100 µM). Working solutions (10 µM) was prepared by mixing stock solutions of all six analytes and further diluting with water. Calibration curve samples were prepared at concentrations of 0.01, 0.05, 0.1, 0.5, 1.0, 5.0, and 10 µM by diluting the working solution with water. A total of 10 µl of 100 µM acetanilide solution were added as an internal standard before analysis. The pH of a 250 µl aliquot of urine was adjusted to pH 5 with phosphate and incubated with 0.1 U of β-glucuronidase (Sigma G7017, contains sulfatase activity) for 24 h at room temperature, centrifuged at 3000*g* for 20 min, and diluted at 1:15 with water for subsequent analysis.

Calculation of kinetic parameters

Non-linear (Michaelis–Menten) kinetic parameters for nicotine-to-cotinine formation were calculated using Prism 3.0 software (San Diego, CA, USA).

Results

In vitro *metabolism and inhibition of nicotine metabolism*

DBA/2N mice were used in the *in vitro* experiments because they have a high basal hepatic CYP2A5 activity which is further inducible by chemicals such as pyrazole and phenobarbital (Juvonen et al. 1985) and CD2F1 mice possess somewhat lower basal CYP2A5 activity (Wood and Conney 1974; Honkakoski and Negishi 1997). The *in vitro* nicotine-to-cotinine formation conditions were optimized with respect to the amount of microsomes in the reaction mixture and incubation time (data not shown). In addition, different HPLC conditions were examined to obtain an optimal separation of nicotine and cotinine.

The oxidation of nicotine to cotinine followed Michaelis–Menten kinetics, as a hyperbolic curve was obtained (Figure 2). The V_{max} of the reaction was markedly increased in microsomes from pyrazole (7.5-fold) and phenobarbital (5.1-fold) pre-treated mice. The V_{max} in CDF1 control mouse microsomes was 63% of the V_{max} in DBA/2 control mouse microsomes (Table I). These results suggest that CYP2A5 is an efficient catalyst of the reaction since pyrazole and phenobarbital increase the V_{max} of the oxidation reaction.

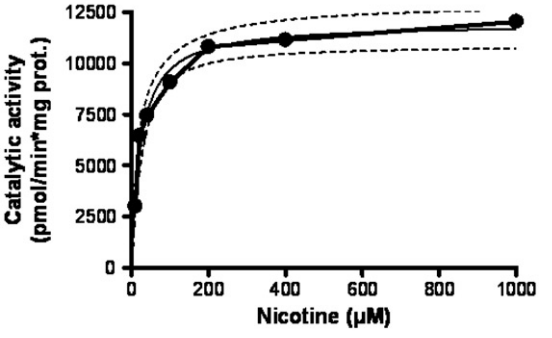

Figure 2. Michaelis–Menten kinetics of oxidation of nicotine to cotinine. The experiment was undertaken with microsomes from pyrazole-treated DBA/2 mice. Data are examples of one determination; the dotted lines represent the 95% confidence limits.

Table I. Kinetic parameters (Michaelis–Menten) of nicotine-to-cotinine formation by mouse liver microsomes. Oxidation of nicotine to cotinine was measured at 0–1 mM nicotine concentrations with 30–250 μg microsomes, liver cytosol as a source for aldehyde oxidase, and 1 mM NADPH at 50 mM phosphate buffer pH 7.4. Non-linear one binding site analysis was used to determine K_m and V_{max} values.

Microsomes	K_m μM (95% CI)	V_{max} (95% CI) nmol mg^{-1} protein min^{-1}
DBA/2 control	33 (6.8–59)	1.6 (1.3–1.9)
DBA/2 pyrazole	23 (14–33)	12 (11–13)
DBA/2 phenobarbital	38 (0.0–80)	8.1 (5.8–10)
CD2F1 control	68 (37–99)	1.0 (0.9–1.1)

Note: Data are the average of two determinations.

The role of CYP2A5 in nicotine-to-cotinine formation *in vitro* was further assessed by adding anti-CYP2A5 antibody to the reaction mixture. As illustrated in Figure 3, the antibody efficiently blocked cotinine formation, the inhibition being more than 90% in microsomes obtained from pyrazole-treated mice. The inhibition was less efficient in microsomes from phenobarbital-treated mice (70%). The potent *in vitro* chemical inhibitor of CYP2A5, γ-nonanoic lactone (Juvonen et al. 2000), was also efficient in reducing cotinine formation. As shown in Figure 4, up to 100% inhibition of the reaction was obtained with this latter compound in microsomes from control and pyrazole-treated DBA/2 mice. In addition, another CYP2A5 substrate, coumarin, completely abolished the reaction, but a concentration as high as 400 μM was required for 100% inhibition (data not shown). These inhibition results confirm that CYP2A5 is the major enzyme involved in the oxidation of nicotine to cotinine in mouse liver microsomes.

In vivo *inhibition of nicotine metabolism*

Nicotine was administered orally to CD2F1 mice, urine collected for 24 h and nicotine and its metabolites cotinine, cotinine N-oxide, 3-hydroxycotinine, and nicotine 1-N-oxide

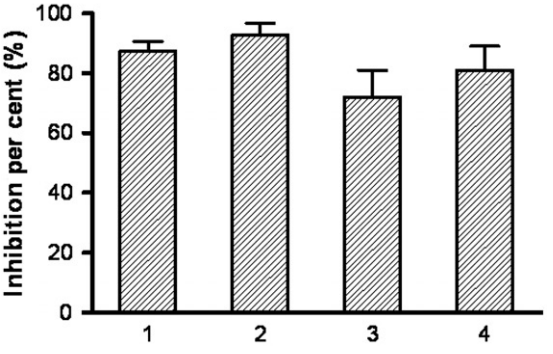

Figure 3. Inhibition of cotinine formation by anti-CYP2A5 antibody in mouse liver microsomes. Microsomes were from D2 mice treated with 0.9% NaCl (1), pyrazole (2), phenobarbital (3), and untreated CD2F1 (4) mice. Data are the mean ± standard deviation derived from three determinations. The absolute value of the 100% uninhibited activity is 1.4 pmol/(min × mg protein) for NaCl D2, 11.8 pmol/(min × mg protein) for pyrazole D2, 7.1 pmol/(min × mg protein) for phenobarbital D2 and 0.86 pmol/(min × mg protein) for CDF1 mice.

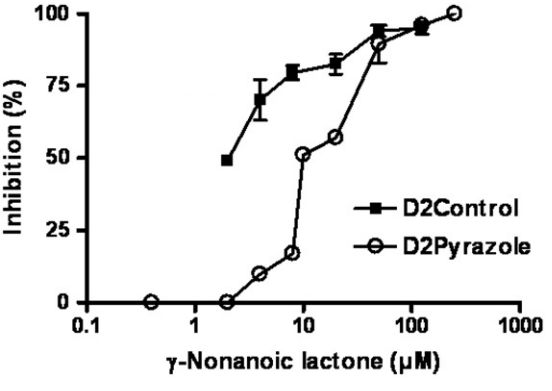

Figure 4. Concentration-dependent inhibition of cotinine formation by γ-nonanoic lactone. Data are the mean ± standard deviation derived from two determinations. The absolute value of the 100% uninhibited activity is 1.3 pmol/(min × mg protein) for D2 control and 12.9 pmol/(min × mg protein) for D2 pyrazole mice.

determined by the APCI-LC-MS analysis. All these analytes were well ionized and yielded a protonated molecule $M+H^+$. To increase the specificity of the assay, the precursor ions were fragmented by colliding them with helium and monitoring the fragment ions created. MRM chromatograms of all analytes are shown in Figure 5. No significant interfering peaks were observed on the ion channels used and a good chromatographic separation was achieved. Cotinine N-oxide and 3-hydroxycotinine were separated by chromatography and had different fragmentation patterns.

To determine the effect of CYP2A5 inhibitors on nicotine elimination, CD2F1 mice were administered orally with nicotine ($10\,mg\,kg^{-1}$) alone or together with the inhibitors methoxsalen, 2,7-dimethylnaphthalene, 2-bromonaphthalene, and 4-methylnaphthalene (each $2\,mg\,kg^{-1}$). Urine was collected for 24 h in metabolic cages.

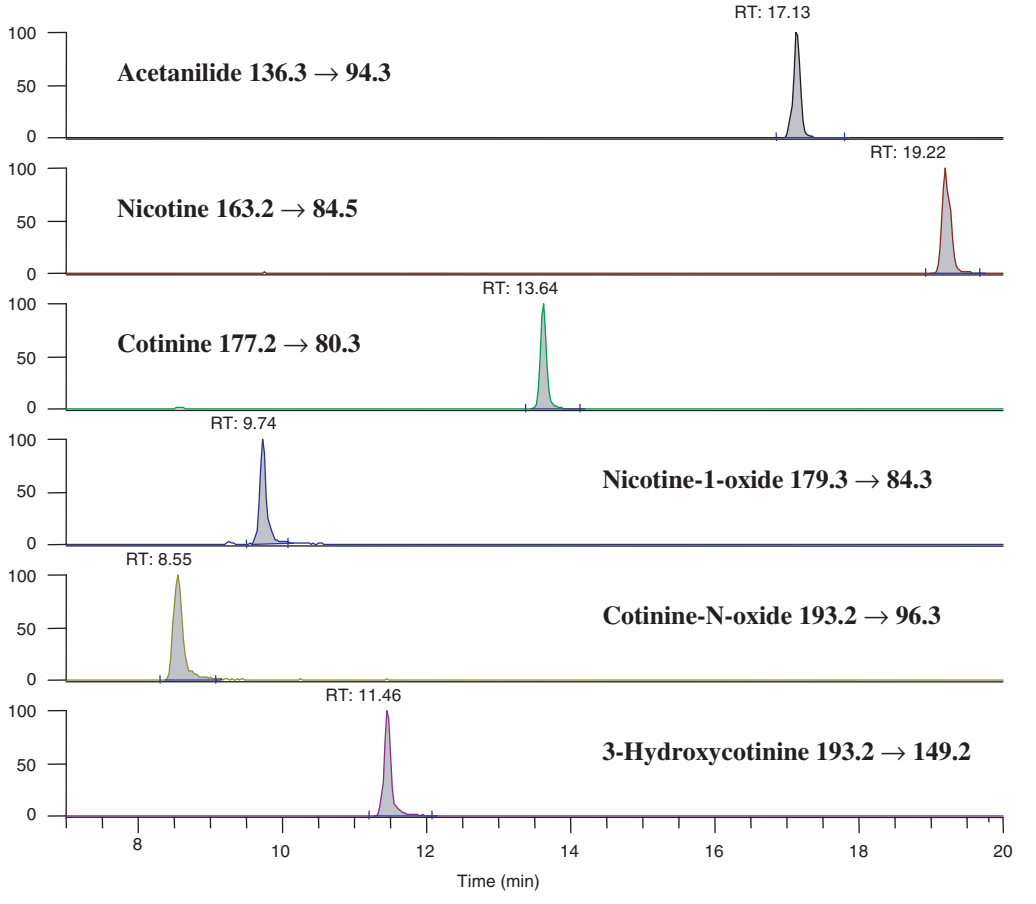

Figure 5. APCI-LC-MS MRM chromatograms of nicotine and its metabolites and MRM transitions monitored. Acetanilide was used as the internal standard.

There was a considerable variation in the total amount of urine excreted during 24 h among individual animals (mean volume = 0.64 ml, range = 0.25–3.1 ml). The average yield of nicotine and four major metabolites from the total nicotine dose (1.2 μmol/mouse) was 39% (range = 6–81%). On average, less than 0.6% of the administered nicotine dose was excreted unchanged in urine (Figure 6). The major measured metabolite in urine was *trans*-3-hydroxycotinine, which represented 77% of the total amount of nicotine-related material. Cotinine N-oxide, nicotine 1-N-oxide, and cotinine represented 16, 3–5, and 3–4% of the administered dose, respectively. These results confirm earlier findings (Hukkanen et al. 2005) that in the mouse metabolism is the major elimination route for nicotine with the main metabolite being *trans*-3-hydroxycotinine.

Methoxsalen was the only inhibitor that increased the amount of nicotine and nicotine N-oxide and reduced the amount of cotinine derived metabolites in the urine. In contrast, 2,7-dimethylnaphthalene had no effect (Figure 7). In addition, 2-bromonaphthalene and 4-methylnaphthalene did not affect the levels of nicotine and its metabolites in urine (results not shown). Methoxsalen increased the amount of nicotine in urine from 1–3 to 21 ± 11 nmol and the amount of nicotine 1-N-oxide to

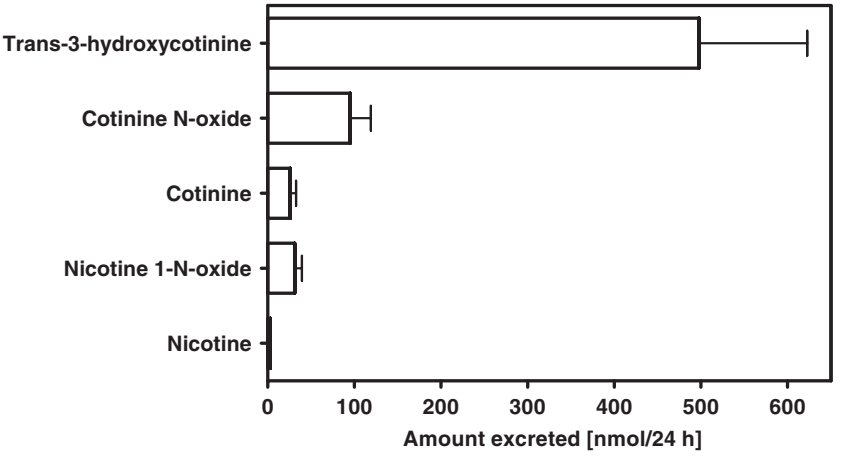

Figure 6. Excretion of nicotine and its metabolites into urine. A total of 10 mg kg^{-1} (average 1.21 μmol/mouse) nicotine was administered per orally to CD2F1 mice, urine was collected for 24 h, and nicotine and its major metabolites were determined by HPLC-MS. Data are the mean ± standard deviation derived from four determinations.

40 ± 18 nmol from the control level of 14–31 nmol. The cotinine level was decreased by methoxsalen co-administration from 26 ± 12 to 13 ± 6 nmol, cotinine N-oxide from 95 ± 42 to 11 ± 8 nmol, and *trans*-3-hydroxycotinine from 500 ± 220 to 35 ± 26 nmol. The yield of nicotine and the four metabolites in urine was markedly decreased by methoxsalen co-administration as it was only 10% ± 4% in mice given methoxsalen and 58% ± 25% in mice treated with the other inhibitors or without inhibitor treatment.

To analyse further the elimination pattern of nicotine, different metabolite/nicotine ratios were calculated to assess if they could be used as an indicator of inhibitory effects (Table II). All these ratios varied between 0.2 and 4 in mice given methoxsalen and between 2 and 840 in control mice. Methoxsalen had the most prominent effect on the cotinine N-oxide/nicotine and *trans*-3-hydroxy cotinine/nicotine ratios but only a minor effect on the nicotine 1-N-oxide/nicotine ratio. In summary, methoxsalen efficiently inhibited the metabolic elimination of nicotine and metabolite excretion into urine whereas 2,7-dimethylnaphthalene, 2-bromonaphthalene and 4-methylanaphthalene failed to do so.

Discussion

The main aim of this study was to assess if the mouse could be used as a model for the testing of chemical inhibitors of CYP2A6. To achieve this aim, three different types of experiments were carried out: (1) *in vivo* metabolism of nicotine, (2) *in vitro* oxidation of nicotine to cotinine, and (3) *in vivo* inhibition of nicotine metabolism by CYP2A5 specific inhibitors. The results of this study show that: (1) nicotine metabolism is very similar between mouse and human; (2) CYP2A5 is the main catalyst of nicotine-to-cotinine formation in mouse liver microsomes *in vitro*; and (3) orally administered methoxsalen, an irreversible inhibitor of CYP2A5, reduced the metabolic elimination of nicotine in mice *in vivo* and, therefore, leads to a prolongation of the presence of nicotine in the body.

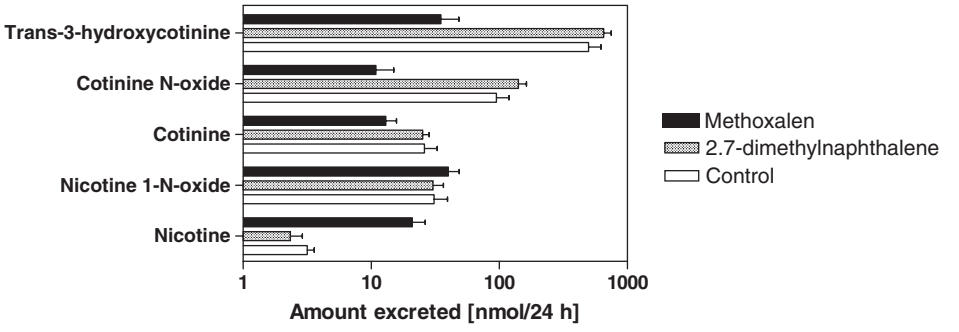

Figure 7. Effect of CYP2A5 inhibitors on the excretion of nicotine and its metabolites into urine. Methoxsalen and 2,7-dimethylnaphthalene (2 mg kg^{-1}) were administered per orally to CD2F1 mice and 10 mg kg^{-1} nicotine were given 30 min later. Urine was collected for 24 h, and nicotine and its major metabolites were determined by HPLC-MS. Data are shown as the mean ± standard deviation derived from four to six determinations.

Table II. Effect of methoxsalen on the metabolite/nicotine ratios in urine.

	Metabolite/nicotine ratio	
Metabolite	Control	Methoxsalen
Nicotine 1-N-oxide	11.5 ± 6.2	2.0 ± 0.4
Cotinine	11.2 ± 5.6	0.7 ± 0.1
Cotinine N-oxide	62.8 ± 28	0.6 ± 0.5
Trans-3-hydroxy cotinine	297 ± 120	2.0 ± 1.6

Note: Data are the mean ± standard deviation derived from four to six determinations.

This is the first study to assess nicotine metabolism in detail in the intact mouse. Nicotine and four major metabolites were assayed in this study and the metabolic pattern in mice resembled that in humans. Cotinine was the major primary metabolite in mouse as it is also in humans, and *trans*-3-hydroxy cotinine is the major overall metabolite in urine (Hukkanen et al. 2005). A total of 40–60% of nicotine is eliminated as *trans*-3-hydroxycotine in human smokers, whereas in mouse it represented 75% of nicotine elimination. In mouse, the second most abundant metabolite was cotinine N-oxide (16% of total), whereas in humans who smoke, cotinine together with cotinine glucuronide constitute 22–35% of nicotine elimination. In mice, cotinine and nicotine N-oxide were excreted in urine in equal amounts. Interestingly, urinary nicotine constitutes less than 1% of its own elimination in mice whereas in smokers, 8–10% of nicotine is eliminated as such in urine. Three main reasons may explain this difference. First, smokers are exposed via the lung whereas mice were exposed orally. Second, smokers are exposed continuously to low nicotine doses and the mice obtained one high, bolus dose, resulting in different blood and tissue concentrations. Third, mice seem to be more efficient nicotine metabolizers than humans. In conclusion, the present study confirms that nicotine oxidation to cotinine is the major primary metabolic pathway in mouse as it is in humans.

In humans CYP2A6 is the key enzyme in elimination of nicotine (Tyndale and Sellers 2001). Although CYP2A6 shares only 82% similarity in its amino acid sequence with

CYP2A5, both enzymes efficiently catalyse many common substrates and are inhibited by the same inhibitors. For example, both enzymes efficiently catalyse coumarin 7-hydroxylation, and methoxsalen is a potent inhibitor of both enzymes (Pelkonen et al. 2000). The current study expands previous knowledge on nicotine-to-cotinine formation in the mouse and confirms the key role of CYP2A5 in this reaction in the mouse both *in vitro* and *in vivo*. Siu and Tyndale (2007) recently showed that CYP2A5 works equally efficiently in oxidation of nicotine to cotinine in DBA/2N and C57BL/6 mouse strains, but C57BL/6 is more efficient in oxidation of cotinine. Mouse liver microsomes were used as the source of the CYP2A5 enzyme. This approach has the clear advantage that the relative contribution of CYP2A5 to cotinine formation can be estimated. According to the results obtained with the specific anti-CYP2A5 antibody, 80% of cotinine formation was attributable to CYP2A5 in control DBA/2 mouse microsomes. In pyrazole-induced microsomes, the contribution of CYP2A5 was 90%. As expected, the reduction in cotinine formation was least efficient in microsomes from phenobarbital-treated mice. This is due to the fact that phenobarbital induces several CYP forms simultaneously, including CYP2A, CYP2B, and CYP3A forms (Honkakoski and Negishi 1997). Some of these forms appear to be able to contribute to cotinine formation in mouse liver. Up to 100% reduction in cotinine formation was obtained with the chemical inhibitor γ-nonanoic lactone (Juvonen et al. 2000) and the CYP2A5 substrate coumarin. Previous studies have shown that methoxsalen blocks CYP2A5 completely (Mäenpää et al. 1993, 1994; Damaj et al. 2007). These data strongly argue for the crucial contribution of CYP2A5 in nicotine-to-cotinine conversion in mouse liver.

The observed kinetic parameters of nicotine-to-cotinine formation in CD2F1 mouse liver microsomes are strikingly similar to those in human liver microsomes. The apparent K_m and V_{max} values for cotinine formation in human liver microsomes are approximately 65 μM and 470 pmol mg^{-1} protein min^{-1}, respectively, with substantial inter-individual variation (Messina et al. 1997). Coumarin can inhibit up to 80% of nicotine-to-cotinine formation in human liver microsomes (Messina et al. 1997). Recently, Murphy et al. (2005) showed that CYP2A5 is an efficient catalyst of nicotine 5́-oxidation, with a $K_m = 7.7$ μM and a $V_{max} = 1.5$ pmol product min^{-1} $pmol^{-1}$ P450; and Siu and Tyndale (2007) showed that there exist the high- and low-affinity active site for this reaction in mouse liver microsomes. The catalysing efficiency expressed as V_{max}/K_m is much higher for mouse CYP2A5 (0.199 l min^{-1}) than the human CYP2A6 (0.009 l min^{-1}). These latter parameters were obtained using heterologously expressed enzymes (Murphy et al. 2005). Thus, with regard to nicotine-to-cotinine reaction kinetics, the CD2F1 mice resemble humans more than the high activity DBA/2 mice. A recent study (Siu et al. 2006) indicated that increased nicotine self-administration in mice was associated with increased rates of nicotine metabolism as a result of greater hepatic CYP2A5 protein levels.

In our *in vivo* experiments with CD2F1 mice showed that only orally administered methoxsalen significantly reduced the conversion of nicotine to cotinine. The lack of effect by the other inhibitors used is not altogether surprising since methoxsalen is an irreversible inhibitor capable of destroying the CYP2A enzyme (Pelkonen et al. 2000). In contrast, 1,7-dimethylnaphthalene, 2-bromonaphthalene, and 4-methylnaphthalene are thought to be competitive and reversible inhibitors based on their structural similarity to naphthalene (Asikainen et al. 2003) and, therefore, they have a transient mode of action. Higher doses could be more efficient *in vivo*. However, as they are competitive inhibitors, it is possible that they are rapidly metabolized and therefore do not have prolonged inhibitory effects on CYP2A5.

There is considerable interest in the development of an inhibitor of the human CYP2A6 enzyme. Inhibition of CYP2A6 and slower nicotine metabolism have been proposed to

decrease (1) the risk of smoking initiation and dependence, (2) the number of cigarettes smoked, and (3) the risk of tobacco-related cancers and mutations. Proof-of-concept studies have been carried out with the CYP2A6 inhibitors methoxsalen (used in the treatment of psoriasis) and tranylcypromine (a monoamine oxidase inhibitor used in the treatment of depression) (Sellers et al. 2000; Tyndale and Sellers 2001; Zhang et al. 2001). A major disadvantage of these CYP2A6 inhibitors is that CYP enzymes are not the only target molecules, the other targets being monoamino oxidase for tranylcypromine (Frieling and Bleich 2006) and methoxsalen being photoactive and hepatotoxic (McNeely and Goa 1998). Secondly, they are not selective for the various CYP forms and thus inhibit several other CYPs, especially CYP2E1 and CYP3A4 (Mäenpää et al. 1994; Pelkonen et al. 1998; Taavitsainen et al. 2001) in addition to CYP2A6.

The present authors are currently developing potent and selective CYP2A6 inhibitors (Poso et al. 1995, 2001; Rahnasto et al. 2005). Usually an inhibitor of the human CYP2A6 is also an inhibitor of the mouse CYP2A5, but there are exceptions such as 2-phenylethylamine, which is a much more potent inhibitor of CYP2A6 (Rahnasto et al. 2003). Initially, the candidate inhibitors are tested with *in vitro* methods, typically using heterologously expressed CYP2A6 enzyme. Since it is not possible to test these compounds in humans (unless they are registered drugs), an animal model is needed. The crucial prerequisite for such a model is that the conversion of nicotine to cotinine occurs in a similar fashion as is the case in humans. The mouse *in vivo* model characterized in the current study appears to fulfil this basic tenet. The effect of inhibitors on the elimination of nicotine can well be evaluated by determining nicotine and its metabolites in urine and the ratio of *trans*-3-hydroxycotine and cotinine N-oxide to nicotine are the most sensitive indicators for inhibitory effects.

In conclusion, robust and simple HPLC and HPLC/MS methods have been developed for the quantitation of nicotine-to-cotinine biotransformation by mouse liver, both *in vitro* and *in vivo*. The relative contribution of liver CYP2A5 enzyme to this reaction was found to be large, up to 100% after CYP2A5 induction by pyrazole. The main features of urine metabolic pattern of nicotine are similar in mouse and human. Therefore this *in vivo* model can be used to test the performance of chemical inhibitors of CYP2A5/CYP2A6.

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