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Inhibition and inactivation of cytochrome P450 2A6 and cytochrome P450 2A13 by menthofuran, β -nicotyrine and menthol

Valerie M. Kramlinger, Linda B. von Weymarn, and Sharon E. Murphy

Department of Biochemistry, Molecular Biology, and Biophysics and Masonic Cancer Center, University of Minnesota, Minneapolis, MN 55455, USA

Abstract

Nicotine is the primary addictive agent in tobacco products and is metabolized in humans by CYP2A6. Decreased CYP2A6 activity has been associated with decreased smoking. The extrahepatic enzyme, CYP2A13 (94% identical to CYP2A6) also catalyzes the metabolism of nicotine, but is most noted for its role in the metabolic activation of the tobacco specific lung carcinogen, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK). In this study, the inhibition and potential inactivation of CYP2A6 and CYP2A13 by two tobacco constituents, 1-methyl-4-(3-pyridinyl) pyrrole (β -nicotyrine) and (–)-menthol were characterized and compared to the potent mechanism based inactivator of CYP2A6, menthofuran. The effect of these compounds on CYP2A6 and CYP2A13 activity was significantly different. (–)-Menthol was a more efficient inhibitor of CYP2A13 than of CYP2A6 (K_i , 8.2 μ M and 110 μ M, respectively). β -nicotyrine was a potent inhibitor of CYP2A13 (K_i , 0.17 μ M). Neither menthol nor β -nicotyrine were inactivators of CYP2A13. Whereas, β -nicotyrine was a mechanism based inactivator of CYP2A6 ($K_{I(inact)}$, 106 μ M, k_{inact} was 0.61 min^{–1}). Similarly, menthofuran, a potent mechanism based inactivator of CYP2A6 did not inactivate CYP2A13. Menthofuran was an inhibitor of CYP2A13 (K_i , 1.24 μ M). The inactivation of CYP2A6 by either β -nicotyrine or menthofuran was not due to modification of the heme and was likely due to modification of the apo-protein. These studies suggest that β -nicotyrine, but not menthol may influence nicotine and NNK metabolism in smokers.

Keywords

Cytochrome P450; Inhibition; Inactivation; CYP2A6; CYP2A13; menthol; menthofuran; β -nicotyrine

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Corresponding author: Sharon E. Murphy, Masonic Cancer Center, University of Minnesota, Mayo Mail Code 806, 420 Delaware Street SE, Minneapolis, MN 55455, USA, Phone: 612-624-7633; Fax: 612-626-5135; murph062@umn.edu.

Requests for reprints: Sharon E. Murphy, Masonic Cancer Center, University of Minnesota, Mayo Mail Code 806, 420 Delaware Street SE, Minneapolis, MN 55455, USA, Phone: 612-624-7633; Fax: 612-626-5135; murph062@umn.edu

Conflict of Interest

The authors declare no financial or commercial conflict of interest.

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1. Introduction

Tobacco-use is driven by the addictive nature of nicotine [1]. However, the use of tobacco products results in the exposure to a diverse array of compounds. Some, like nicotine are naturally occurring. Others, for example menthol, are additives and some, such as the tobacco specific carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) [2], form during the curing of the tobacco (Fig 1). Menthol is reported to modify nicotine metabolism and thus has been the focus of recent FDA regulation [3–5]. The primary route of nicotine elimination is CYP2A6-catalyzed metabolism [6], and a key pathway of NNK activation is CYP2A13 catalyzed hydroxylation [7]. Pharmacologically blocking nicotine metabolism has been reported to reduce the extent of smoking [8;9]. Similarly, the inhibition of NNK metabolism could mitigate exposure to activated NNK. Therefore, the characterization of compounds that modify CYP2A6 and CYP2A13 activity is critical to understanding the influence of these enzymes on tobacco consumption and carcinogenesis.

CYP2A6 is primarily a hepatic enzyme [10] and CYP2A13 is expressed in the respiratory tract [11;12]. Both enzymes efficiently catalyze nicotine 5'-oxidation, the first step in the conversion of nicotine to cotinine, the primary nicotine metabolism pathway. However, due to its presence in the liver, CYP2A6 plays the major role in nicotine elimination in smokers [6;13]. NNK requires metabolic activation to exert its carcinogenic potential and CYP2A13 catalyzed activation likely plays a significant role in the induction of lung cancer in smokers [7;12].

CYP2A6 and CYP2A13 share 94% sequence identity and catalyze the metabolism of many common substrates. However, they often have different catalytic efficiencies and generate unique metabolites. One common substrate, coumarin is frequently used to probe CYP2A activity. CYP2A6 selectively catalyzes the 7-hydroxylation of coumarin but, CYP2A13 catalyzes both the 7-hydroxylation and 3,4 epoxidation of coumarin [14]. Both CYP2A6 and CYP2A13 catalyze the metabolic activation of NNK however, CYP2A13 is a 200-fold more efficient catalyst (k_{cat}/K_m) than CYP 2A6 [7]. CYP2A13 is also a somewhat superior catalyst of nicotine metabolism; catalyzing nicotine 5'-oxidation at a 5- to 20-fold higher catalytic efficiency than CYP2A6 [15;16].

While mechanism-based inactivation of CYP2A6 and CYP2A13 has been reported for several compounds it is fully characterized for only a few. To be classified as a mechanism-based inactivator, a compound must be metabolically activated to an intermediate that covalently modifies the enzyme and renders it inactive [17]. Menthofuran has been identified as a potent mechanism-based inactivator of CYP2A6. Menthofuran-mediated loss of CYP2A6 activity is dependent on catalytic turnover and immunohistochemistry analysis has provided evidence that the apo-protein is modified [18]. Inactivation of both CYP2A6 and CYP2A13 occurs during nicotine metabolism [16]. Nicotine, however, is not the agent of inactivation; a secondary or even tertiary nicotine metabolite is likely the inactivator. The species responsible for enzyme inactivation has yet to be identified, however one possible contender is β -nicotyrine. It has been suggested that β -nicotyrine, which differs from nicotine in the presence of a pyrrole instead of a pyrrolidine ring (Fig 1), is a mechanism-based inactivator of CYP2A6 [19]. β -Nicotyrine is a urinary metabolite of nicotine in dogs and rats but has not been identified as a nicotine metabolite in humans [20]. Regardless, smokers are exposed to β -nicotyrine, since it is present in tobacco [21].

The importance of understanding the effects of tobacco constituents on CYP2A6 and CYP2A13 activity is underscored by their potential impact on smoking behavior and NNK activation. In addition, the characterization of potential mechanism-based inactivators may aid in the design of specific inactivators of these enzymes. The objectives of the current

study were to determine the relative inhibition of CYP2A6 and CYP2A13 activity by β -nicotyrine, menthol and menthofuran and to assess inactivation of CYP2A6 and CYP2A13 by each of these compounds.

2. Materials and Methods

2.1 Chemicals and Enzymes

Menthofuran, menthol, 7-hydroxycoumarin, coumarin, dilauroyl-l- α -phosphatidylcholine (DLPC), NADPH, bovine serum albumin, catalase, and all other biochemical reagents were obtained from Sigma-Aldrich (St. Louis, MO) and were of analytical grade. β -Nicotyrine (99% pure) was obtained from Toronto Research Chemicals. Trifluoroacetic acid (TFA) was obtained from Pierce Chemical (Rockford, IL). The enzymes used in this study were heterologously expressed in *E. coli* and purified according to previously published methods [22–24]. CYP2A13 and his-tagged CYP2A6 are full-length enzymes. At saturating concentrations of coumarin (40 or 100 μ M) the rate of coumarin 7-hydroxylation for both CYP2A13 and CYP2A6 were comparable with that reported previously [25]

2.2 Reconstitution

CYP2As were reconstituted with rat NADPH-P450 oxidoreductase (reductase) in a 1 to 2 ratio with lipid (DLPC, 0.2 μ g/pmol P450) and incubated for 45 min at 4 °C. Then, 50 mM Tris buffer, pH 7.4 and catalase were added to give an final concentrations of 1 pmol/ μ l P450 2A, 2 pmol/ μ l reductase, 0.2 μ g/ μ l lipid, and 60 U/ μ l catalase.

2.3 CYP2A Coumarin 7-hydroxylation Activity

The reaction mixtures contained reconstituted enzyme solution (5 pmol CYP2A), coumarin (0.4 – 20 μ M), NADPH-generating system (0.4 mM NADP, 10 mM glucose 6-phosphate, and 0.4 units/ml glucose phosphate dehydrogenase) and 40 μ g/ml bovine serum albumin in 300 μ L 50 mM Tris buffer, pH 7.4. The reaction was allowed to proceed for 10 min at 37 °C prior to termination by the addition of 30 μ l of 15% trichloroacetic acid. To investigate inhibition, CYP2A6 or CYP2A13 activity was measured in the presence of menthofuran, menthol or β -nicotyrine. Experiments were carried out within the linear range of product formation. CYP2A6 reactions contained 0, 1, 2 and 3 μ M menthofuran, 0, 1, 3 and 5 μ M β -nicotyrine, or 0, 50, 100 and 200 μ M menthol. CYP2A13 reactions contained 0, 5, 10 and 25 μ M menthofuran, 0, 1, 5 and 10 μ M β -nicotyrine, or 0, 50 100 and 200 μ M menthol. 7-Hydroxycoumarin was quantified by HPLC with fluorescence detection [25]. K_m , V_{max} and K_i values were determined using the Sigma Plot kinetics program from Systat Software Inc. (Chicago, IL). K_i estimates were determined using nonlinear regression analysis. All data were fit to a competitive inhibition model (eq 1) and tested using the Runs test of residuals to determine statistically whether experimental data are randomly distributed around the curve with 95% confidence.

$$(V_o = V_{max}[S]/K_m(1 + ([I]/K_i)) + [S]) \quad (\text{eq 1})$$

Additionally, global R^2 values to assess the goodness of fit confirmed that the competitive model fit the data sets well ($R^2 = 0.92$) except for the data set from menthofuran inhibition of CYP2A13 ($R^2 = 0.84$). All data sets passed the Runs test except for the data set from β -nicotyrine inhibition of CYP2A6.

2.4 Inactivation

Primary reaction mixtures containing menthofuran, menthol or β -nicotyrine and the reconstituted enzyme mixture described in section 2.2 were pre-incubated for 5 min at 30 °C

prior to the addition of 1mM NADPH. At various times aliquots (5 μ l) were removed and added to a secondary reaction mixture (20 μ M coumarin, the NADPH-generating system and 40 μ g/ml bovine serum albumin in 50 mM Tris buffer, pH 7.4; 300 μ l total volume) and incubated for 10 minutes at 30 °C, then 7-hydroxycoumarin formation was quantified as described in section 2.3. Inactivation experiments were carried out at 30 °C in order to minimize the loss of CYP2A6 activity in the presence of NADPH and no inactivator and to be comparable to previous literature [16;23].

2.4.1 Effect of trapping agents—Effect of trapping agents on inactivation were determined by co-incubating menthofuran (5 μ M) or β -nicotyrine (20 μ M) in the primary reaction mixture (as described in 2.4) with 10 mM glutathione or semicarbazide. The primary reaction mixtures were incubated for ten minutes before aliquots were added to the secondary reaction mixture (as in section 2.4).

2.4.2 Inactivation kinetics—In the initial analyses of menthofuran, menthol or β -nicotyrine, 20 – 400 μ M of the potential inactivator was included in the primary reaction, prepared as in 2.4. After 10–30 min at 30 °C, 5 μ l (5 pmol CYP) aliquots were removed and added to the secondary reactions. To determine the kinetic parameters for the inactivation of CYP2A6 either menthofuran (0 – 10 μ M) or β -nicotyrine (0 – 80 μ M) were incubated with the primary reaction mixture for the indicated times (0 – 5 min), after which aliquots (5 μ l, 5 pmol CYP) were removed and added to the secondary reaction mixture. Kinetic rate constants were determined from the slopes of the lines when the logarithm of the percent activity remaining was plotted against time. To determine partition ratio, the primary reaction mixtures containing menthofuran (0 to 100 μ M) or β -nicotyrine (0 to 100 μ M) were incubated for 30 min at 30 °C in the presence of 1 mM NADPH. Aliquots containing 5 pmol of CYP2A6 were removed at 0 and 30 min and added to the secondary reaction mixture. The partition coefficient was estimated from the intercept of the regression line obtained at low [inhibitor]/[2A6] ratios and the line obtained at saturating inhibitor concentrations [17].

2.4.3 Irreversibility—The determination of irreversibility was carried out at 30°C using primary reaction mixtures (300 μ l) containing 300 pmol CYP2A6 and either 20 μ M menthofuran or 50 μ M β -nicotyrine, with or without (exposed control) 1 mM NADPH. At 0 and 10 minute 5 μ l of the primary reaction mixture (5 pmol CYP) was transferred (in duplicate) to a secondary reaction mixture and coumarin 7-hydroxylation activity determined (as described in section 2.4.2). Portions of the control and inactivated samples (200–250 pmol enzyme) were applied to a G50 Sephadex spin-column to remove small molecules which was processed as previously described [26]. The spin column-treated samples were reconstituted with 0.2 μ g lipid and 30pmol reductase (30 min at 4 °C) and then analyzed for coumarin 7-hydroxylase activity and compared to non-spin column treated sample.

2.4.4 Heme loss and CO spectra—The amount of native heme remaining after inactivation was determined using the experimental conditions as described in the irreversibility (2.4.3) experiment, except that CYP2A6 (250 pmol) was reconstituted with 250 pmol of reductase. After 0 and 10 min, aliquots of the samples were added to a secondary reaction and the coumarin 7-hydroxylation activity was determined. The remaining sample (100 pmol in duplicate) was analyzed for native heme by HPLC with UV detection at 405 nm as previously described [26]. This experiment was carried out in triplicate for menthofuran and β -nicotyrine. The mobile phase consisted of 0.1% TFA in water (A) and 0.05% TFA in acetonitrile (B). The column VYDAC C4 (250 $\times$ 4.6mm; Chromtech, Apple Valley, MN) was eluted isocratically at 70% A: 30% B for 10 min

followed by a linear gradient to 80% B in 15 min and then to 95% B in 10 min. The flow rate was 1.0 ml/min. In an independent experiment, carried out in triplicate, the amount of spectrally active CYP2A6 remaining relative to the amount of coumarin 7-hydroxylation activity after was quantified. The experimental conditions were identical to those used to measure native heme, but the reduced CO spectrum of remaining sample (100 pmol) was measured as described previously [16;27].

3. Results

3. 1 Inhibition

The relative inhibition of CYP2A6 and CYP2A13-catalyzed coumarin 7-hydroxylation by menthol, β -nicotyrine and menthofuran was determined and the kinetic of CYP2A13-catalyzed constants are presented in Table 1. As previously reported [28], the K_m coumarin 7-hydroxylation was lower than the K_m for CYP2A6 (data not shown). Both menthofuran and β -nicotyrine were relatively potent inhibitors of CYP2A6 activity, with K_I values of 0.29 μ M and 1.07 μ M, respectively. Inhibition of CYP2A6 by menthol was 100 times less potent (K_I of 110 μ M). The K_I value for menthol inhibition of CYP2A13, 8.17 μ M, was 13 times less than that of CYP2A6. β -Nicotyrine was also a more potent inhibitor of CYP2A13 than of CYP2A6. The inhibition data were analyzed according to several kinetic inhibition models, however, no model was determined to be a superior fit to the data as indicated by the goodness of fit (R^2) values. Therefore, the reported K_I values were determined using the competitive inhibition model and should be considered the apparent K_I .

3. 2 Inactivation

To determine the relative potential of menthofuran, β -nicotyrine or menthol to inactivate either CYP2A6 or CYP2A13, an initial experiment was carried out at a single inhibitor concentration (20 μ M). Incubation of CYP2A6 in the presence of NADPH with either menthofuran or β -nicotyrine resulted in the loss of coumarin 7-hydroxylation activity, 95% and 51% respectively (Table 2). In contrast, there was no significant loss of CYP2A13 activity with either menthofuran or β -nicotyrine. At higher concentrations of menthofuran (200 μ M) or β -nicotyrine (100 μ M) or with 30 min incubations, no loss of CYP2A13-catalyzed coumarin 7-hydroxylation was observed (data not shown). Therefore, menthofuran- and β -nicotyrine-mediated inactivation of CYP2A13 does not occur or it is too minor to detect. There was no indication that menthol inactivated CYP2A6 or CYP2A13 (Table 2).

To trap reactive intermediates and potentially protect CYP2A6 from inactivation glutathione or semicarbazide were included in the reaction mixture. Menthofuran and β -nicotyrine concentrations were chosen so that an equal loss of enzyme activity occurred for each compound, about 60% (Table 3). Neither trapping agent protected CYP2A6 from inactivation by menthofuran or β -nicotyrine. However, under the conditions of these experiments (10 min) the extent of β -nicotyrine-mediated inactivation in the presence of glutathione or semicarbazide was greater than in the absence of these agents (Table 3). After 30 min, the extent of β -nicotyrine mediated CYP2A6 inactivation in the samples with and without glutathione were similar. Therefore, glutathione and semicarbazide appear to accelerate the rate of inactivation by β -nicotyrine.

The kinetic parameters of menthofuran and β -nicotyrine-mediated CYP2A6 inactivation were determined (Fig 2). The loss in CYP2A6 activity was dependent on the concentration of menthofuran or β -nicotyrine and increased with time. The K_I for the inactivation of CYP2A6 by menthofuran, 2.2 μ M, was almost 50 -fold lower than that of β -nicotyrine, 106 μ M. The maximum rate constants for menthofuran- and β -nicotyrine-mediated inactivation, k_{inact} , were 1 and 0.61 min^{-1} , respectively; the $t_{1/2}$ values were 0.7 and 1.1 minutes,

respectively. In contrast, the partition ratio, defined as the number of molecules of the inactivator metabolized per molecule of CYP2A6 inactivated, was more than 3 –fold lower for β -nicotyrine compared to menthofuran (Fig. 3).

Inactivation of CYP2A6 by menthofuran and by β -nicotyrine was not reversible upon removal of unbound small molecules. In three experiments, the CYP2A6-catalyzed coumarin 7-hydroxylation activity that remained following menthofuran inactivation ($8 \pm 5\%$ relative to a minus NADPH control) was unchanged after the sample was passed through a size exclusion column ($5 \pm 3\%$). Similarly, the coumarin 7-hydroxylation activity of β -nicotyrine-inactivated CYP2A6 ($34 \pm 2\%$) was not recovered when the sample was filtered through the column ($29 \pm 3\%$).

To assess the potential modification of the heme and apoprotein after inactivation of CYP2A6 by menthofuran or β -nicotyrine, two experiments were carried out. In the first, inactivated CYP2A6 was quantified by a reduced CO difference spectra and the loss in spectrally active enzyme was compared to the loss of 7-hydroxycoumarin activity relative to a non-NADPH-treated control. After menthofuran treatment, the 7-hydroxycoumarin activity remaining was $28.5 \pm 1.6\%$, comparable to the amount of spectrally active CYP2A6 enzyme remaining $36 \pm 4.6\%$ (Table 4). Likewise, after β -nicotyrine treatment the enzyme activity remaining was the same as the amount of spectrally active CYP2A6 remaining (Table 4). The second experiment quantified the amount of native heme by HPLC analysis. For both compounds, the loss in activity after incubation in the presence of NADPH was not accompanied by a loss in native heme (data not shown), and no modified heme was detected. These results together with the observed loss in the spectrally quantifiable protein, suggest that the inactivation of CYP2A6 by both menthofuran and β -nicotyrine was due to modification of the apo-protein.

4. Discussion

CYP2A6 and CYP2A13 are critical catalysts of nicotine and NNK metabolism in smokers [6;12]. Therefore, inhibitors of these enzymes may influence both nicotine addiction and tobacco-induced carcinogenesis. In the study presented here, the inhibition and inactivation of CYP2A6 and CYP2A13 by the tobacco constituents, β -nicotyrine and menthol, were characterized and compared to the potent CYP2A6 inactivator, menthofuran [18]. Both compounds inhibited the coumarin7-hydroxylation activity of these enzymes. Yet, despite the high degree of similarity between CYP2A6 and CYP2A13, the effect of menthol, β -nicotyrine and menthofuran on each of these enzymes was quite different. Menthol did not inactivate either enzyme, but was a significantly better inhibitor of CYP2A13 than CYP2A6. β -Nicotyrine was also a more potent inhibitor of CYP2A13 than CYP2A6. In addition, β -nicotyrine was a mechanism based inactivator of CYP2A6 but not CYP2A13. Similarly, menthofuran was not an inactivator of CYP2A13. This observed enzyme specific inactivation is consistent with the previously reported unique substrate specificity for these enzymes, and is likely due to the larger active site of CYP2A13 compared to CYP2A6[29;30].

Menthol cigarettes are used by the majority of African American smokers (85%), but only 10–20% of European Americans [31]. The prevalence of menthol cigarettes among African American smokers was hypothesized to contribute to their higher incidence rate of lung cancer compared to European Americans [32]. However, the proposed link of menthol cigarette use to lung cancer was strongly refuted by the results of a recent prospective epidemiology study [33]. The conclusion of that study was that menthol cigarettes are potentially less harmful, not more, than non-menthol cigarettes. Part of what was driving the original hypothesis were studies that suggested that the use of menthol cigarettes effected

cigarette smoking behavior and nicotine metabolism [3]. In the study reported here, menthol was found to be a modest inhibitor of CYP2A13 and a poor inhibitor of CYP2A6. In addition, menthol did not inactivate either enzyme. MacDougall et al [4] also reported that menthol is a weak inhibitor of CYP2A6. Inhibition of CYP2A13 by menthol, which in tobacco smoke is present at much higher levels than NNK, might reduce NNK activation by CYP2A13 present in the lung, potentially decreasing lung cancer risk. Additionally, lower nicotine metabolism would result in higher bioavailability and might lessen tobacco use, but Benowitz and co-workers [3] found no evidence of modified nicotine intake when smokers used menthol cigarette.

β -Nicotyrine, a minor tobacco alkaloid, has previously been reported to inhibit nicotine metabolism in rodents. The pretreatment of mice with β -nicotyrine resulted in a significant increase in tissue and blood levels of nicotine [34]. In addition, there is evidence in rabbit lung cells and microsomes that the metabolism of β -nicotyrine results in the formation of reactive and potentially toxic intermediates [35;36]. More recently, β -nicotyrine has been reported to be the most potent inhibitor of CYP2A6 activity among a series of nicotine related alkaloids and metabolites with a K_i of 0.37 μ M [19]. This study also suggested that β -nicotyrine was a mechanism based inactivator and time dependent inactivation was reported, however no further characterize of the observed inactivation was provided. Here we have confirmed that β -nicotyrine is an irreversible inactivator of CYP2A6 and provide evidence that supports the formation of an apoprotein adduct. Alternatively, the observed inactivation could be the result of a tightly bound metabolite not removed by dialysis.

Both CYP2A6 and CYP2A13 are inactivated during nicotine metabolism [16]. The observed inactivation of both enzymes appears to be mediated by a metabolite of the nicotine 5'-iminium ion, that is, nicotine-mediated inactivation requires at least two sequential oxidation of nicotine. β -Nicotyrine is a urinary metabolite of nicotine in some species, and forms from the nicotine 5'-iminium ion *in vitro* [20;35]. We report here that β -nicotyrine is an inactivator of CYP2A6 and suggest that inactivation of CYP2A6 by nicotine may be mediated by a metabolite of β -nicotyrine. The metabolism of β -nicotyrine by either CYP2A6 or CYP2A13 has not been studied, however, in the rabbit the P450-catalyzed metabolism results in the formation of two pyrrolinones that likely form by way of an unstable epoxide of the pyrrole ring. This epoxide is one possible candidate for the metabolite that inactivates CYP2A6. Experiments are on-going to characterize both CYP2A6 and CYP2A13-catalyzed β -nicotyrine metabolism.

Menthofuran, and β -nicotyrine irreversibly inactivated CYP2A6 but not CYP2A13. Consistent with earlier studies of menthofuran-inactivated CYP2A6 [18] our data supported the conclusion that modification of the apoprotein, not the heme moiety, is the likely mechanism of inactivation. Like β -nicotyrine, menthofuran is metabolized to a reactive epoxide that may contribute to protein adduct formation [37]. Menthofuran is a more potent inactivator of CYP2A6 than β -nicotyrine. However, the calculated partition ratio for menthofuran-mediated inactivation was ~3.5 times higher than for β -nicotyrine-mediated inactivation. Therefore, despite the relatively high $K_{I(inact)}$ for β -nicotyrine inactivation of CYP2A6, its oxidation more frequently generates a reactive metabolite that inactivates CYP2A6.

The presence of neither glutathione nor semicarbazide protected P450 2A6 from inactivation by menthofuran or by β -nicotyrine. Therefore, the metabolite responsible for loss of CYP2A6 activity must react with the enzyme without exiting the active site. It was curious however, that P450 2A6 was more quickly inactivated by β -nicotyrine when glutathione or semicarbazide was present.

In summary, β -nicotyrine but not menthol is likely to impact nicotine metabolism and possibly NNK metabolism in smokers. Menthol was a modest inhibitor of CYP2A6 and CYP2A13. Whereas, β -nicotyrine, which is present in tobacco or generated as a metabolite of nicotine, was an effective inhibitor of both CYP2A6 and CYP2A13. More importantly, β -nicotyrine inactivated CYP2A6, the primary catalyst of nicotine metabolism. Interestingly, neither β -nicotyrine nor menthofuran, the potent mechanism based inactivator of CYP2A6, are inactivators of CYP2A13. Experiments are on-going to characterize the mechanistic basis for this, since it may be useful in the design of CYP2A6 inhibitors and help to elucidate the pathway responsible for the inactivation of CYP2A6 by nicotine.

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Abbreviations

Menthol	(-)-Menthol or (1R,2S,5R)- 2- isopropyl-5-methylcyclohexanol
Menthofuran	(R)-(+)-Menthofuran or 3,6R-dimethyl-4,5,6,7-tetrahydro-1-benzofuran
NNK	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone
β-nicotyrine	1-methyl-4-(3-pyridinyl) pyrrole
CYP	cytochrome P450

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Highlights

β -Nicotyrine, menthol and menthofuran inhibit CYP2A6 and CYP2A13 activities in a time and dose dependent manner.

β -Nicotyrine and menthofuran are much more potent inhibitors of both CYP2A6 and CYP2A13 activity than menthol.

Like menthofuran, β -nicotyrine is an inactivator of CYP2A6 but not CYP2A13.

Glutathione and semicarbazide do not protect CYP2A6 from either menthofuran or β -nicotyrine-mediated inactivation.

Figure 1.
Structures of nicotine, β -nicotyrine, (-)- menthol, and menthofuran.

Figure 2. Time and concentration-dependent inactivation of CYP2A6 by menthofuran (A) and β -nicotyrine (B)

Activity remaining refers to coumarin 7-hydroxylation activity determined in a secondary reaction. Details of the experiments are as described in *Materials and Methods*. The menthofuran concentrations used were 0(●), 1(○), 2.5(▼), 5(▽) and 10(■) μ M and the β -nicotyrine concentrations used were 0(●), 5(○), 10(▼), 40(▽) and 80(■) μ M. Values are the mean $\pm$ S.D. from three independent experiments. The insets represent the double-reciprocal plot generated from the slopes of the lines at the various concentrations.

Figure 3. Partition ratio determination for CYP2A6 with β -nicotyrine (A) and menthofuran (B)
 The inactivation was allowed to go to completion, and then coumarin 7-hydroxylation activity was determined in a secondary reaction as described under *Materials and Methods*. Values are the mean $\pm$ S.D. of three independent experiments performed in duplicate. The arrow indicates the intercept used to determine the partition ratio.

Table 1Calculated inhibition constants for inhibition of CYP2A activity by menthofuran, β -nicotyrine and menthol^a

Inhibitor	K_I (μM)	
	CYP2A6	CYP2A13
Menthofuran	0.29 ± 0.05	1.24 ± 0.26
β -nicotyrine	1.07 ± 0.02	0.17 ± 0.003
Menthol	110 ± 10.7	8.17 ± 0.86

^a Values are means $\pm$ S.D. from three experiments carried out in duplicate. The inhibition of CYP2A6 and CYP2A13-mediated coumarin 7-hydroxylation was determined as described in the materials and methods. Curves were generated using non-linear regression analysis.

Table 2Loss of CYP2A activity following incubation with menthofuran, β -nicotyrine or menthol^a

Inhibitor (20 μ M)	% Activity Remaining ^b	
	CYP2A6	CYP2A13
Menthofuran	5 $\pm$ 1	91 $\pm$ 5
β -nicotyrine	49 $\pm$ 10	87 $\pm$ 2
Menthol	97 $\pm$ 6	87 $\pm$ 11

^aSamples were prepared as described in the Materials and methods. Reconstituted enzyme was incubated for 10 minutes at 30 °C in the presence of 20 μ M Menthofuran, β -nicotyrine or menthol.

^bValues are means $\pm$ S.D. from three experiments. Percent activity remaining is compared to control samples that contained no NADPH.

Table 3Effect of NADPH and trapping agents on the inhibition of CYP2A6 activity by menthofuran and β -nicotyrine^a

Components	Percent Activity ^b		Percent Activity Remaining ^c
	0 min	10 min	
Menthofuran (5 μ M)	100	100	100
NADPH	91 $\pm$ 11	81 $\pm$ 3	90
Menthofuran, NADPH	107 $\pm$ 9	46 $\pm$ 14	43
Menthofuran, NADPH, glutathione (10mM)	115 $\pm$ 13	56 $\pm$ 3	48
Menthofuran, NADPH, semicarbazide (10mM)	104 $\pm$ 3	42 $\pm$ 3	40
β -nicotyrine (20 μ M)	100	100	100
NADPH	90 $\pm$ 12	83 $\pm$ 3	86
β -nicotyrine, NADPH	89 $\pm$ 19	32 $\pm$ 2	35 ^d
β -Nicotyrine, NADPH, glutathione (10mM)	96 $\pm$ 25	13 $\pm$ 7	14 ^d
β -Nicotyrine, NADPH, semicarbazide (10mM)	83 $\pm$ 14	10 $\pm$ 1	13 ^d

^aReconstituted enzyme was incubated at 30 °C with the components indicated.^bValues are percent coumarin 7-hydroxylation activity relative to samples that contained menthofuran but no NADPH, means $\pm$ S.D. from three independent experiments.^cValues are the percent of the mean activity after a 10 min reaction relative to the activity at time 0 min.^dCYP2A6 activity remaining after 30 minute incubation with 20 μ M β -nicotyrine and NADPH was 10% in the presence or absence of glutathione or semicarbazide.

Table 4Relationship of CYP2A6 inactivation on to spectrally active enzyme levels^a

Inactivator	Activity Remaining	Spectrally active P450
Menthofuran	28.5±1.6 ^b	36±4.6 ^b
β-nicotyrine	55.5±0.7 ^c	48±7.3 ^c

^aReconstituted enzyme was incubated for 10 minutes at 30 °C in the presence of 20 μM menthofuran or 50 μM β-nicotyrine. Control samples contained no NADPH. The samples were analyzed for the coumarin 7-hydroxylation activity and the spectrally active enzyme quantified by reduced CO spectra. Values are means ± S.D. of percent coumarin 7-hydroxylation activity and percent spectrally active CYP2A6 remaining from three experiments.

^bValues were not significantly different, unpaired T-test p=0.068.

^cValues were not significantly different, unpaired T-test p=0.15.