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Nicotine-related alkaloids and metabolites as inhibitors of human cytochrome P-450 2A6

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Abstract

S-(−)-Nicotine and 13 of the most prevalent nicotine-related alkaloids and metabolites (i.e., *S*-(−)-nornicotine, myosmine, β-nicotyrine, *S*-cotinine, *S*-norcotinine, *S*-(−)-nicotine *N*-1′-oxide, *S*-(−)-nicotine $\Delta^{1'-5'}$-iminium ion, *S*-(−)-anabasine, *S*-(−)-*N*-methylanabasine, anabaseine, *S*-(−)-anatabine, nicotelline, and 2,3′-bipyridyl) were evaluated as inhibitors of human cDNA-expressed cytochrome P-450 2A6 (CYP2A6) mediated coumarin 7-hydroxylation. Tobacco alkaloids myosmine, *S*-(−)-nornicotine, *S*-cotinine, *S*-norcotinine, *S*-(−)-nicotine *N*-1′-oxide, *S*-(−)-nicotine $\Delta^{1'-5'}$-iminium ion, *S*-(−)-*N*-methylanabasine, anabaseine, and nicotelline had K_i values for inhibition of coumarin 7-hydroxylation ranging from 20 μM to more than 300 μM whereas nicotine and *S*-(−)-anatabine were much more potent (i.e. 4.4 and 3.8 μM, respectively). The tobacco alkaloids 2,3′-bipyridyl (7.7 μM) and *S*-(−)-anabasine (5.4 μM), were somewhat less potent compared with *S*-(−)-nicotine or *S*-(−)-anatabine in inhibition of human CYP2A6. β-Nicotyrine, in which the *N*-methylpyrrolidino moiety of nicotine was replaced by the aromatic *N*-methylpyrrole ring, was shown to inhibit human CYP2A6 with much greater potency ($K_i = 0.37$ μM) compared with *S*-(−)-nicotine. Among the compounds examined, only nicotine and β-nicotyrine were mechanism-based inhibitors of human CYP2A6. The potency of the mechanism-based CYP2A6 inhibitors suggests that, for smokers, modulation of CYP2A6 may be greater than that predicted on the basis of serum concentration of these alkaloids. Our results indicate that the prominent nicotine-related alkaloid β-nicotyrine present after smoking potently inhibits human CYP2A6.

Keywords: Cytochrome P-450 2A6; *S*-(−)-Nicotine; β-Nicotyrine; *S*-(−)-Anatabine; *S*-(−)-Anabasine; 2,3′-Bipyridyl

1. Introduction

Currently, 1.2 billion people worldwide smoke tobacco despite clear evidence that smoking is a leading ''preventable'' cause of death. Smoking has been shown to cause complex central nervous system, behavioral, cardiovascular, endocrine, neuromuscular, and metabolic effects in humans [1–3]. Smoking is also unquestionably linked to lung cancer [4,5]. The addiction liability and pharmacological effects of smoking are primarily mediated by the major tobacco alkaloid *S*-(−)-nicotine (nicotine) [2,6,7]. Nicotine is metabolized to *S*-(−)-nicotine $\Delta^{1'-5'}$-iminium ion by the genetically variable hepatic enzyme CYP2A6 and then to the pharmacologically less active derivative, *S*-cotinine (cotinine), by aldehyde oxidase [8,9]. Thus far, 17 allelic variants of human *CYP*2A6 have been characterized (*1–16 and *1 × 2). *CYP*2A6 variants have been implicated in the ability of people to metabolize nicotine at different rates [10]. Inter-individual variation in nicotine metabolism may play a role in a person's level of smoking as well as effecting the transition from initiation of smoking to the maintenance of a smoking behavior pattern. People apparently smoke to achieve a specific concentration of nicotine in their blood [2] and a deficiency in CYP2A6-mediated metabolism of nicotine may permit longer exposure to nicotine and, in turn, may decrease the number of cigarettes a person needs to smoke to obtain their desired blood nicotine concentration. In contrast, individuals with very efficient nicotine metabolism smoke a greater number of cigarettes each day to maintain a certain nicotine

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Abbreviations: CYP, cytochrome P-450; anatabine, *S*-(−)-anatabine dipicrate; anabasine, *S*-(−)-anabasine dipicrate; nornicotine, *S*-nornicotine; norcotinine, *S*-norcotinine camsylate; cotinine, *S*-cotinine; anabasine, *S*-(−)-*N*-methylanabasine; nicotine *N*-1′-oxide, *S*-(−)-nicotine *N*-1′-oxide; nicotine $\Delta^{1'-5'}$-iminium ion, *S*-(−)-nicotine $\Delta^{1'-5'}$-iminium dipicrate; g, gas; μM, micromolar.

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concentration and, therefore, are much more likely to develop and maintain a smoking pattern and ultimately become dependent. For example, compared to Caucasians, African Americans have a significantly decreased ability to metabolize nicotine to cotinine and a decreased ability to eliminate cotinine from their blood [11,12]. Evidence suggests that inhibition of CYP2A6 with substances other than nicotine decreases nicotine metabolism and, therefore, decreases nicotine-related carcinogen concentration and may be of benefit for smoking cessation [13]. Nicotine replacement therapies used for smoking cessation (i.e. nicotine gum, nicotine patch, inhaler, lozenges, etc.), while removing many of the possible injurious components of tobacco smoke, do not address the issue of decreasing or eliminating tobacco and nicotine consumption [13,14]. The success rate for people using nicotine replacement therapies is only about double that of abstinence. Selective inhibition of CYP2A6 may lead to a decrease in smoking behavior, to a decrease in the amount smoked and a decrease in tobacco-related carcinogenesis and mortality [15]. While the metabolism of nicotine by CYP2A6 has been studied to a considerable extent, relatively little work has been focused on the interaction of the minor tobacco alkaloids with CYP2A6. The objectives of the current study were to (a) determine the extent of inhibition of CYP2A6-catalyzed coumarin 7-hydroxylation by the nicotine-related tobacco alkaloids and metabolites and (b) evaluate the selectivity of the most potent alkaloids or metabolites on CYP inhibition.

2. Materials and methods

2.1. Chemicals and reagents

β-Nicotyrine tartrate, *S*-(−)-anatabine dipicrate (anatabine), *S*-(−)-anabasine dipicrate (anabasine), myosmine, *S*-norcotinine (norcotinine), and *S*-nornicotine camsylate (norcotinine) were a generous gift from Peyton Jacob, III (San Fanscisco General Hospital, UCSF). *S*-(−)-*N*-Methylanabasine (anabaseine), cotinine, and nicotelline were purchased from Toronto Research Chemicals. 2,3′-Bipyridyl, *S*-(−)-nicotine *N*-1′-oxide (nicotine *N*-1′-oxide), and *S*-(−)-nicotine $\Delta^{1'-5'}$-iminium dipicrate (nicotine $\Delta^{1'-5'}$-iminium ion) were synthesized as described below. Microsomes from human lymphoblast cells expressing human CYP2A6 and human liver microsomes were obtained from BD Gentest™. Microsomes from baculovirus-infected cells co-expressing CYPs (2E1 and 2B6), NADPH-CYP reductase and cytochrome b_5 (BACULOSOMES®) were purchased from PanVera LLC. Tetrakis(triphenylphosphine)palladium(0), nicotine, testosterone, 6-hydroxytestosterone, and coumarin were purchased from Sigma-Aldrich. Other buffers, reagents and chemicals were of the highest purity available and were purchased from commercial sources.

2.2. Chemical synthesis

3-Pyridine boronic acid [16], nicotine *N*-1′-oxide [17], and nicotine $\Delta^{1'-5'}$-iminium ion [17] were synthesized as previously reported.

2.2.1. 2,3′-Bipyridyl

To a vial containing a solution of 2-bromopyridine (0.21 g, 1.3 mmol) in deoxygenated dimethoxymethane (1 mL) under an atmosphere of argon (g) was added a slurry of tetrakis(triphenylphosphine)palladium(0) (0.05 g, 0.03 mmol) in deoxygenated dimethoxymethane (4 mL) and a deoxygenated aqueous sodium carbonate solution (2 M, 1.3 mL, 2.6 mmol). The resultant biphasic solution was stirred for 5 min. To the solution was added a slurry of 3-pyridine boronic acid (0.2 g, 1.63 mmol) in deoxygenated ethanol (95%, 2 mL), the solution was purged with argon, the vial was tightly capped, heated to 90° and stirred for 1 hr. The solution was cooled to room temperature (RT) and filtered through a pad of celite. The celite pad was washed with additional dichloromethane and the combined organic fractions were dried over magnesium sulfate, filtered through filter paper and the solvent was removed *in vacuo*. The product was purified by flash chromatography (silica gel; EtOAc/hexane 50:50, v/v) to afford a colorless oil (0.14 g, 70% yield). NMR and mass spectra were consistent with previously reported values [18].

2.3. Enzyme assays

2.3.1. Human CYP2A6 assay

To measure CYP2A6 functional activity, coumarin 7-hydroxylation was determined. Microsomes containing 1 pmol CYP2A6 were added to 0.1 M Tris buffer (pH 7.5) containing 3 μM coumarin (final concentration) and individual inhibitors with final concentrations of 400, 100, 25, 6.3, 1.6, 0.4, 0.1, and 0.02 μM. The reactions were initiated by the addition of an NADPH-generating system consisting of 0.5 mM $NADP^+$, 0.5 mM glucose-6-phosphate, 5 U/mL glucose-6-phosphate dehydrogenase, 1 mg/mL diethylenetriaminepentaacetic acid (DETAPAC), and 7 mM $MgCl_2$ for a final incubation volume of 0.2 mL. Incubations were run for 10 min at 37° and were terminated by the addition of 0.75 mL of CCl_3COOH/CH_3CN (20:80, w/v). After centrifugation at 12,000 *g* for 5 min, 200 μL was transferred to a Packard OptiPlate 96-well plate and the formation of the coumarin metabolite, 7-hydroxycoumarin, was determined fluorometrically using a Wallac Victor^2 1420 Multilabel Counter (Wallac Software Version 2.00 release 9) at excitation and emissions wavelengths of 355 and 460 nm. The amount of product formed was obtained by interpolation from a standard curve of 7-hydroxycoumarin. The IC_{50} values were determined using GraphPad Prism® Version 3.00 and are reported as an average of three experiments ±SD. Using these values, the apparent K_i values were calculated using

the Cheng–Prusoff equation. For each assay, the reaction was a linear function of time for 60 min and of protein concentration from 0.5 to 2 pmol per reaction well.

2.3.2. Human CYP2E1 and CYP2B6 assays

To measure CYP2E1 and CYP2B6 functional activity, isozyme-specific vivid blue substrate *O*-dealkylation was used *via* a modified PanVera Vivid Assay as previously described [19]. β-Nicotyrine concentrations (i.e. 400, 100, 25, 6.3, 1.6, 0.4, 0.1, and 0.02 μM) were added to a 96-well plate (BD Falcon Microtest, Black Flat Bottom) containing Vivid® Substrate (final concentration: CYP2E1 assay, 10 μM; CYP2B6 assay, 5 μM) in 0.2 M potassium phosphate buffer (pH 8.0), followed by the appropriate BACULOSOMES (enzyme final concentration: CYP2E1 assay, 5 nM; CYP2B6 assay, 10 nM). The incubations were initiated by the addition of the NADPH-generating system for a final incubation volume of 0.2 mL. After a 40-min incubation at RT, the formation of the fluorescent, *O*-dealkylated metabolite for each isozyme was determined fluorometrically as above at excitation and emissions wavelengths of 405 and 460 nm, respectively. Apparent K_i values were determined as described above. For each assay, the reaction was a linear function of time for 60 min and of protein concentration from 0.5 to 2 pmol per reaction well.

2.3.3. Human CYP3A4 assay

An HPLC enzyme assay for inhibition of human liver microsomal testosterone 6-hydroxylase was done as previously described [20]. β-Nicotyrine concentrations used were 44, 15, 4.94, 1.65, and 0.55 μM in the presence of testosterone (200 μM) and human liver microsomes (0.4 mg of protein). Organic extracts were injected onto a Hitachi L-7100 system equipped with a Hitachi L-7400 UV detector. Separations were done with an Altex Ultrasphere ODS (4.6 mm × 250 mm, 5 μm) column. The analytes were eluted with an isocratic solvent system consisting of water/acetonitrile/methanol (30:10:60, v/v/v) at a flow rate of 1.0 mL/min. Testosterone and 6-hydroxytestosterone were efficiently separated by this system with retention times of 7.95 and 3.94 min, respectively. Quantification of substrate and metabolite was determined from peak areas of the chromatogram and comparison with standard curves. IC_{50} values were determined as described above. For each assay, the reaction was a linear function of time for 60 min and of protein concentration from 0.2 to 1 mg of protein per reaction well.

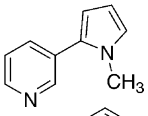
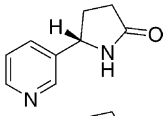
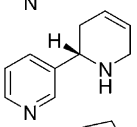
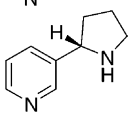
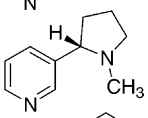
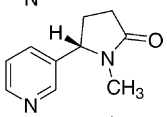
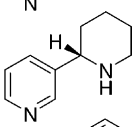
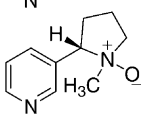
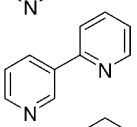
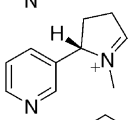
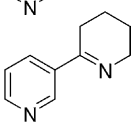
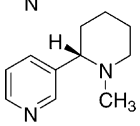
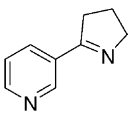
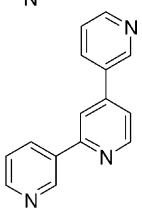
2.4. Determination of human CYP2A6 mechanism-based inhibition

To determine if β-nicotyrine and anatabine are mechanism-based inhibitors of CYP2A6, time-dependent inhibition of coumarin 7-hydroxylation was determined. First, microsomes containing 20 pmol CYP2A6 were added to 0.1 M Tris buffer (pH 7.5). Individual inhibitor studies with final concentrations of 2.5, 4, and 8 μM (β-nicotyrine) and 16, 25, and 50 μM (anatabine) were combined and the incubations were initiated by the addition of an NADPH-generating system as described above for a final incubation volume of 0.2 mL. After incubation of the enzyme for various time periods in the presence of β-nicotyrine (37°) and anatabine (25°), the incubations were terminated by transferring 10 μL of the reaction mixture to vials at −78°. In a second step, the percent remaining coumarin 7-hydroxylation activity was determined after addition of 190 μL of a pre-made solution containing the NADPH-generating system described above and coumarin (i.e. final concentration 3 μM) to the enzyme. After incubation for an appropriate amount of time, the incubations were terminated by addition of 0.75 mL of CCl_3COOH/CH_3CN (20:80, w/v) and the formation of 7-hydroxycoumarin was determined as described above.

3. Results and discussion

To examine the inhibitory potency of minor tobacco alkaloids and nicotine metabolites on human CYP2A6 functional activity, a microtiter plate fluorescence assay monitoring coumarin 7-hydroxylation was employed [21]. The results are listed in Table 1. Among the nicotine-related alkaloids and metabolites tested, nornicotine, cotinine, nicotine *N*-1′-oxide, nicotine $\Delta^{1'-5'}$-iminium ion, *N*′-methylanabasine, and nicotelline failed to inhibit the formation of 7-hydroxycoumarin by 50% at the highest concentration tested (i.e. 400 μM). Thus, these compounds were classified as ''non-inhibitors'' of CYP2A6. Anabaseine, myosmine, and *S*-norcotinine showed modest inhibition of CYP2A6 with K_i values of 20, 31, and 46 μM, respectively. Anabasine and 2,3′-bipyridyl were considerably more potent inhibitors with K_i values of 5.4 and 7.7 μM, respectively, and these K_i values approached that of nicotine itself (4.4 μM). Two tobacco alkaloids, namely, β-nicotyrine (0.37 μM), and anatabine (3.8 μM), had K_i values lower than that of nicotine and β-nicotyrine was the most potent. β-Nicotyrine ($IC_{50} = 2.2$ μM for CYP2A6) was tested for selectivity among biologically relevant CYPs by determining the IC_{50} values for inhibition of CYP3A4, CYP2E1, and CYP2B6 by high throughput fluorometric and HPLC assays. The IC_{50} values for β-nicotyrine-mediated inhibition of CYP2B6, CYP3A4, and CYP2E1 were 145.2 ± 13.0 μM, 18.8 ± 2.8 μM, and 1.8 ± 0.3 μM, respectively. To examine the selectivity of inhibition, the selectivity ratios of CYP2B6/CYP2A6, CYP3A4/CYP2A6, and CYP2E1/CYP2A6 were calculated to be 66.0, 8.5, and 0.8, respectively. The mechanism of CYP2A6 inhibition by the most potent nicotine minor alkaloids and metabolites was examined by determining whether inhibition was time dependent. We evaluated nicotine, β-nicotyrine, and anatabine for time-dependent inactivation of CYP2A6. Only nicotine (i.e. $k_{inactivation}$,

Table 1
Effect of nicotine-related alkaloids and metabolites on the inhibition of human CYP2A6 coumarin 7-hydroxylase activity[a]

Structure	Name	CYP2A6 K_i (μM) ± SD	Structure	Name	CYP2A6 K_i (μM) ± SD
	β-Nicotyrine	0.37 ± 0.04		*S*-Norcotinine	46 ± 6
	*S*-(−)-Anatabine	3.8 ± 0.6		*S*-(−)-Nornicotine	≥300
	*S*-(−)-Nicotine	4.4 ± 0.6		*S*-Cotinine	≥300
	*S*-(−)-Anabasine	5.4 ± 1.2		*S*-(−)-Nicotine *N*-1′-oxide	≥300
	2,3′-Bipyridyl	7.7 ± 1.2		*S*-(−)-Nicotine-iminium ion	≥300
	Anabaseine	20 ± 6		*N*-1′-Methylanabasine	≥300
	Mysomine	31 ± 8		Nicotelline	≥300

[a]Incubations were done as described previously [21]. The K_i values were determined using GraphPad Prism® Version 3.00. K_i values are reported as an average ±SD of three experiments.

71.1 min^{-1}) and β-nicotyrine (i.e. $k_{inactivation}$, 6.25 min^{-1}) showed mechanism-based inactivation of CYP2A6. Because nicotine $\Delta^{1'-5'}$-iminium ion did not show significant CYP2A6 inhibition, we conclude that a species on the reaction path prior to the iminium ion is responsible for nicotine-mediated inhibition of CYP2A6.

In humans, the genetically variable hepatic enzyme CYP2A6 is responsible for conversion of approximately 90% of nicotine metabolism to its pharmacologically less active derivative, cotinine [22]. CYP2A6 also contributes to further cotinine metabolism [23,24]. Smoking initiation, dependence, and amount of cigarettes smoked are directly related to the concentration of nicotine in the blood and possibly CYP2A6 activity. Smokers modulate their blood nicotine concentration by smoking cigarettes. Various populations are genetically predisposed to different rates of nicotine metabolism, and this is largely determined by the level of functional CYP2A6 and other enzymes. Some individuals are genetically predisposed to metabolize nicotine at rates much faster than that of others, which means they can lower their bloodstream concentration of nicotine at a much faster rate than a person who is genetically predisposed to metabolize nicotine more slowly. Thus, "efficient metabolizers" often smoke more cigarettes to keep their blood nicotine concentration within the level their body desires. Nicotine "self-medication" is modulated by metabolism *via* CYP2A6 and, therefore, can be regulated by a CYP2A6 inhibitor. The inhibition of CYP2A6 by a nicotine-related alkaloid or metabolite may decrease the metabolism of nicotine and increase the level of circulating nicotine in the blood and may decrease the craving for a cigarette over a longer period of time than if CYP2A6 was not inhibited.

Recently, we observed that L-menthol inhibits CYP2A6 coumarin 7-hydroxylation and nicotine metabolism [25]. This may be another example of self-medication because individuals with efficient nicotine metabolism tend to smoke mentholated cigarettes.

Due to the structural similarities to nicotine, nicotine-related alkaloids and metabolites have the potential to inhibit CYP2A6. Figure 1 shows the structures of the minor tobacco alkaloids and nicotine metabolites tested that were found to inhibit CYP2A6 with potency similar to or greater than that of nicotine. Of the nicotine minor alkaloids tested, it was observed that β-nicotyrine was the most potent CYP2A6 inhibitor with a K_i value of 0.37 μM.

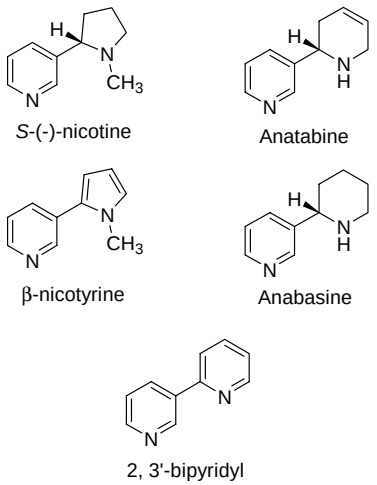


Fig. 1. Minor tobacco alkaloids and nicotine metabolites found to inhibit CYP2A6-mediated coumarin 6-hydroxylation with potency similar or greater than that of *S*-(−)-nicotine.

In β-nicotyrine, the *N*-methylpyrrolidine ring of nicotine is replaced by the isosteric *N*-methylpyrrole ring. The planar structure may be more readily accommodated in the active site of CYP2A6 than nicotine and provide a lower K_i value. Extensive studies on the metabolic fate of β-nicotyrine *in vitro* and *in vivo* have been done [26,27] and previously it was shown that β-nicotyrine inhibited nicotine metabolism *in vivo* [28]. β-Nicotyrine is produced from the pyrolysis of nicotine, but it is unknown whether or not clinically relevant concentrations of β-nicotyrine accumulate in the blood to effectively inhibit human CYP2A6 and modulate nicotine metabolism. However, because β-nicotyrine is a mechanism-based inhibitor, it may be more potent than predicted on the basis of serum β-nicotyrine concentration.

Anatabine, anabasine, and 2,3′-bipyridyl (Fig. 1) are minor tobacco alkaloids containing a six-membered heterocyclic ring in place of the pyrrolidine ring of nicotine that have K_i values of 3.8, 5.4, and 7.7 μM, respectively. Introduction of a methyl group on the piperidine nitrogen of anabasine dramatically decreases its inhibitory potency from 5.4 μM for anabasine to above 300 μM for *N*-1′-methylanabasine. Although the *N*-1′-methyl substituent of nicotine is tolerated by the enzyme, the six-membered ring of *N*-1′-methylanabasine, while adding steric abundance to the overall molecule, may be forcing the *N*-methyl group into a substrate binding region that is detrimental to enzyme inhibition.

When comparing the K_i values of β-nicotyrine and 2,3′-bipyridyl, it is important to point out that, due to the aromatic nature of the pyrrole and pyridine rings, neither alkaloid will be charged at the pH of the assay. The difference in inhibitory potency of β-nicotyrine and 2,3′-bipyridyl is likely due to both the increased ring size of the bipyridyl ring and/or the introduction of the *N*′-methyl group on the β-nicotyrine molecule. The aromaticity of the pyrrole ring may be a contributing element for competitive alternate substrate inhibition as there are a number of sites available for CYP2A6-catalyzed oxidation that are not present in the *N*-methylpyrrolidine ring of nicotine.

Comparison of nicotine ($K_i = 3.8$ μM) with its *N*′-demethylated analogue nornicotine ($K_i > 300$ μM) shows that the methyl group is favored for inhibitor potency. These data suggest that the enzyme active site prefers a five-membered heterocycle, either aromatic or aliphatic, that incorporates a small alkyl group at the 1′ position.

That nicotine is a potent, time-dependent inhibitor of CYP2A6 suggests that its ability to modulate nicotine metabolism in smokers may be greater than that predicted on the basis of serum concentrations alone. The results reported herein are consistent with recently reported research that shows that smoking inhibits nicotine metabolism [29], that smokers metabolize nicotine more slowly than do non-smokers [30] and that low doses of nicotine induce CYP2E1 and chlorzoxazone metabolism in rat liver [31].

In conclusion, 14 naturally occurring tobacco alkaloids and nicotine metabolites were tested for the inhibition of CYP2A6 activity. β-Nicotyrine was found to inhibit CYP2A6-catalyzed coumarin 7-hydroxylation with significantly greater potency than that of *S*-(−)-nicotine. β-Nicotyrine was found to be highly selective for CYP2A6 inhibition vs. CYP2B6 and moderately selective for CYP3A4 but was a potent inhibitor of CYP2E1.

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