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A New Strategy for Smoking Cessation: Characterization of a Bacterial Enzyme for the Degradation of Nicotine

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Supporting Information

ABSTRACT: Smoking is the leading cause of preventable diseases; thus, effective smoking cessation aids are crucial for reducing the prevalence of cigarette smoking and smoking-related illnesses. In our current campaign we offer a nicotine-degrading enzyme from *Pseudomonas putida*, NicA2, a flavin-containing protein. To explore its potential, a kinetic evaluation of the enzyme was conducted, which included determination of K_m, k_{cat}, buffer/serum half-life, and thermostability. Additionally, the catabolism profile of NicA2 was elucidated to assess the potential toxicity of the nicotine-derived products. In characterizing the enzyme, a favorable biochemical profile of the enzyme was discovered, making NicA2 a prospective therapeutic candidate. This approach provides a new avenue for the field of nicotine addiction therapy.

Tobacco use continues to be one of the leading causes of preventable death; indeed, approximately 6 million mortalities are attributed to nicotine use per year worldwide.[1] Most smokers are aware of the health consequences of smoking, and while they want to quit, abstinence is usually difficult to maintain.[2] The current pharmacological aids used in smoking cessation can have significant clinical effects. Representative examples include nicotine replacement therapies,[3] the antidepressant drug bupropion,[4] and the recently introduced varenicline, which have all shown success in increasing abstinence rates compared to placebo.[5] Still, even with these pharmacological aids, for the majority of the smokers, their long-term success rates remain low, as only 15−30% of smokers remain abstinent for at least 1 year after treatment. Therefore, alternative therapies are needed.[6]

We and others have pursued a pharmacokinetic (antibody-based) as opposed to a pharmacodynamic (drug-based) strategy to aid in smoking abstinence.[7] Nicotine vaccines have been proposed to provide long-lasting protection, and to date multiple nicotine vaccines have advanced into clinical trials.[8] However, these vaccines have also presented limited efficacy or failed to achieve their primary end-point of increased smoking cessation rates compared to placebo.[8,9] Thus, while past studies on antibody sequestration of nicotine delivered a proof-of-concept that a pharmacokinetic strategy can enhance smoking cessation rates,[10] it is also apparent that higher concentrations of antibody are needed to make an effective vaccine.[10b] In contrast to antibody-mediated blockade of nicotine, an alternative strategy in reducing the effects of nicotine would be its degradation. Simply stated, the goal here would be to decrease nicotine's circulation through its destruction so that an effective concentration is not reached or maintained in the brain. To our knowledge, cocaine is the only drug where this strategy has been actively pursued.[11] Herein, we report the first steps in the development of an enzyme as a potential therapeutic for the degradation of the critically addictive component in tobacco, nicotine.

In humans nicotine is absorbed rapidly from cigarette smoke, from which it enters the arterial circulation through the oral mucosa and lungs and is rapidly distributed to body tissues.[12] It takes approximately 20 s for nicotine to pass through the brain,[13] while the elimination half-life that is relevant to the accumulation of nicotine during the use of tobacco averages 2−3 h.[12] Thus, nicotine levels accrue over 6−8 h during regular smoking, and there is a long terminal half-life, 20 h or more, presumably reflecting the slow release of nicotine from tissue.[12] Moreover, smoking represents a multiple dosing situation with considerable accumulation while smoking and persistent levels for 24 h of each day.

The metabolism of nicotine in mammals is complex; in Figure 1 we adumbrate an annotated representation. Of interest

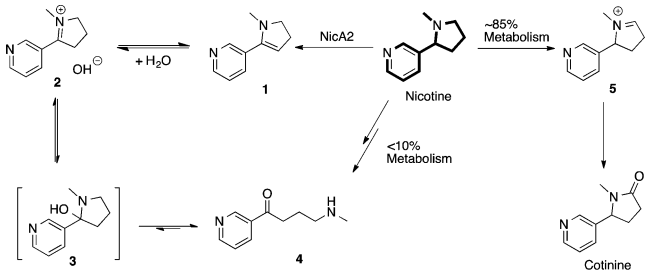

Figure 1. Annotated pathway of nicotine metabolism in mammals (continine pathway) and bacteria/mammals (aminoketone **4** pathway).

are two unique pathways, one producing cotinine and the other 4-(methylamino)-1-(pyridin-3-yl) butan-1-one (**4**). Cotinine and its metabolites account for 70−80% of nicotine metabolism in humans, while aminoketone **4** has been found as a minor component in humans.[12] It is this latter pathway that raised the tantalizing possibility of an alternative to nicotine immunotherapy (Figure 1).[14]

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Since the 1950s, tobacco alkaloids and in particular the bacterial decomposition of nicotine have been known;[15] however, these earlier studies, while informative, were also rather cryptic from the standpoint of both the microbes involved and the components produced from nicotine's degradation. More recently, there has been renewed interest in nicotine-degrading microorganisms, with research initiatives spanning the quality of tobacco products to the treatment of tobacco waste.[16] Our interest stemmed from reports of bacteria that could use nicotine as their sole carbon and nitrogen source, as we posit these bacteria would possess the most efficient enzymes for therapeutic strategies. In particular we were drawn to strains belonging to *Pseudomonas putida*, a non-pathogenic member of the genus *Pseudomonas*, which engenders a series of enzymes capable of metabolizing nicotine to fumaric acid.[14]

P. putida S16 was originally isolated from a field under continuous tobacco cropping in Shandong, People's Republic of China.[17] This S16 strain was found to be effective in degrading nicotine, and it has been shown that S16's metabolism of nicotine follows the pyrrolidine pathway.[14] The enzyme found in the first committed step of S16's degradation of nicotine is NicA2 (PPS_4081). Although, NicA2 is an essential enzyme within the purview of *P. putida*'s degradation of nicotine, it was unclear if the abstraction of a sole enzyme from its metabolic cascade could translate to a potential cessation therapeutic. As a means to gauge the "risks versus rewards" of this nicotine-degrading enzyme, we performed biochemical studies, including the determination of NicA2's K_m, k_{cat}, thermostability, and half-life in buffer and serum, as well as its product toxicity profile.

NicA2 was expressed in BL21(DE3) cells and purified by affinity chromatography. Under these conditions, 21 mg/L of the 52.5 kDa NicA2 protein was obtained. Once pure NicA2 was attained (Figure S1), kinetics assays were initiated to determine catalytic parameters. However, unlike most enzymatic systems, the reaction of nicotine with NicA2 gave a complex mixture of interconverting products.

As presented in Figure 1, NicA2 has evolved so as to catalyze the oxidation of nicotine to *N*-methylmyosmine (**1**).[14] This 4,5-dihydropyrrole can then undergo non-enzymatic ring tautomerism and hydrolysis to ultimately form pseudooxynicotine, **4**.[18] The tautomerism/hydrolysis of **1** occurs spontaneously, and its equilibration is pH dependent.[18] We observed three products by LC-MS: one with m/z 179 (**4**) and two inseparable nicotine metabolites with m/z 161 (**1** and **2**, Figure S2). Due to the limitation of instrument sensitivity and the dynamic equilibration of the products, direct quantification of the enzyme's efficiency was challenging. To compensate for these shortcomings, our strategy was to integrate product peaks from **1**, **2**, and **4** in order to back-calculate the amount of nicotine consumed by NicA2. In doing so, we could accurately determine kinetic parameters of the enzyme.

Standard curves were generated wherein nicotine was fully oxidized by NicA2 and m/z 161/179 signals were plotted against a change in nicotine concentration, providing a direct linear relationship (Figure S3). To determine kinetic parameters of the enzyme, curves were generated at varying nicotine concentrations utilizing 10 nM NicA2 at room temperature. Samples were analyzed by LC-MS using nicotine (methyl-D3) as an internal standard. Target m/z values (161, 179, and 166) were extracted, integrated, and fit to obtain the velocity, v_0. The v_0 was plotted against a series of nicotine concentrations and fit to the Michaelis–Menten equation

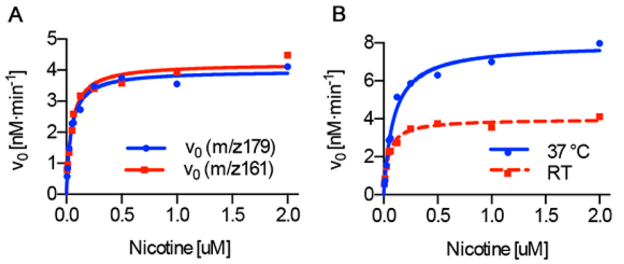

Figure 2. (A) Michaelis–Menten curve of NicA2 based on m/z 179 or 161. (B) Michaelis–Menten curve of NicA2 at 37 °C; K_m = 91.9 ± 10.4 nM, k_{cat} = (1.32 ± 0.04) × 10^{-2} s^{-1}, and k_{cat}/K_m = 1.44 × 10^5 s^{-1}·M^{-1} (see Table 1 for comparison to room temperature).

Table 1. Michaelis–Menten Parameters of NicA2 at Room Temperature

	m/z 179	m/z 161
K_m [nM]	43.5 ± 4.7	46.2 ± 6.4
k_{cat} [s^{-1}]	(6.64 ± 0.17) × 10^{-3}	(7.02 ± 0.23) × 10^{-3}
k_{cat}/K_m [s^{-1}·M^{-1}]	1.53 × 10^5	1.52 × 10^5

(Figure 2A). The K_m and k_{cat} were derived from integrating the two different m/z peaks shown in Table 1; the values derived from either peak are almost identical, indicating good accuracy of our method. As m/z 179 gave a single peak and was integrated more accurately, we used the results from m/z 179 for further studies.

In general, enzyme activity can be highly sensitive to temperature. Thus, as the temperature increases, the expected increase in velocity resulting from increased enzyme–substrate collisions can be offset by denaturation. At room temperature, NicA2 showed excellent activity. From a therapeutic vantage, the same assay was run at 37 °C. As anticipated at this temperature, both K_m and k_{cat} were increased, yet the specificity constant k_{cat}/K_m remained virtually unchanged (Figure 2B). We also examined the effect of temperature on the enzyme's stability. It was unanticipated, and quite remarkable, that the enzyme has an "optimum temperature" of 70 °C, implying outstanding thermostability (Figure 3A).

Furthermore, to be a candidate for nicotine addiction therapy, the enzyme should possess longevity in buffer and ultimately serum. To test for these metrics, NicA2 was incubated at 37 °C in HEPES buffer, and enzyme activity was examined at different time points. Again, unexpectedly, and impressively, the enzyme showed excellent stability and activity over 3 weeks (Figure 3B) and a half-life of 3 days in mice serum (Figure 3C). This is in contrast to the cocaine bacterial esterase CocE, which has a half-life of 11 min in aqueous milieu[11] and 13 min in serum.[19]

Smoking one cigarette provides an absorbed nicotine dose of about 1–2 mg and results in a peak concentration of 20–60 ng/mL (162–370 nM) in blood.[12] Our results reveal NicA2 to have a K_m of 44 nM (92 nM at 37 °C), which is well below the concentration range of nicotine in serum; in theory this would equate to the enzyme working at saturating conditions. As a means to test NicA2's efficiency "*in vivo*", we doped nicotine with or without enzyme in serum (Figure 3D). The enzyme in a 30 min window consumed all nicotine, whereas in the background reaction nicotine remained fully stable. To further substantiate this point, we simulated (Figure S4) NicA2's catabolism of nicotine based upon the determined kinetic constants. NicA2 specificity constant is approximately 10^5 M^{-1}

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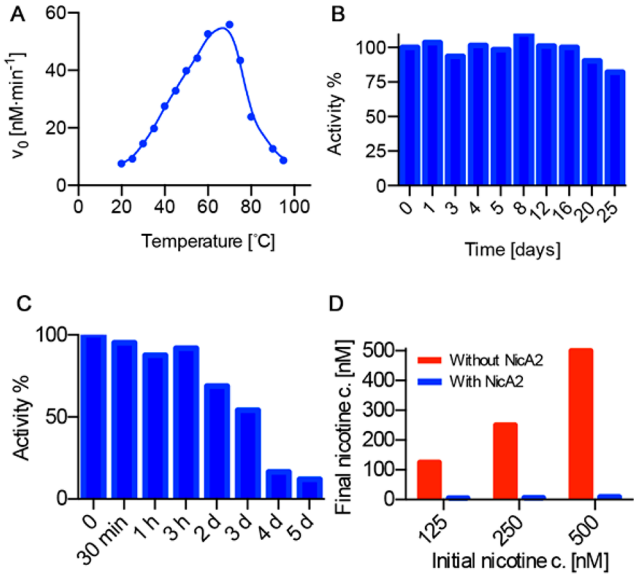

Figure 3. (A) NicA2's thermostability. (B) Long-term stability of NicA2 (37 °C in HEPES buffer, pH 7.4). Note the v_0 remained virtually constant over the duration of the study. (C) Stability of NicA2 in mouse serum at 37 °C. (D) The ability of NicA2 to degrade nicotine in mouse serum. Concentrations of 125, 250, and 500 nM nicotine with and without enzyme (20 nM NicA2) were incubated in serum for 30 min. Residual nicotine left after this time period was measured.

s^{-1}, and while clearly far from a perfect enzyme (10^8–10^9 M^{-1} s^{-1}), NicA2 at 20 nM still possesses enough "catalytic power" to readily decrease nicotine's half-life from 2–3 h for a single cigarette to 9–15 min (5.0 mg of NicA2 for a 70 kg person).

NicA2's remarkable catalytic ability comes from its cofactor, a flavin.[14] Biochemical evidence for the flavin was furnished through UV–vis spectrum analysis (Figure S5). We believe the flavin is either covalent or tightly bound as the yellow tint purified NicA2 when examined with an excess of either FMN or FAD; neither altered its activity (Figure S6). This is important from the context of a therapeutic, as co-administration of either cofactor will not be necessary.

For an enzyme to be relevant at the clinical level, one must further consider the toxicity/addictive properties of the reaction products. Pseudooxynicotine (**4**) has not been reported to possess addictive properties. However, it has been reported as a likely precursor to 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), a potential carcinogen.[20] To investigate any harmful effects of **4**, we performed toxicology studies in which mice were administered **4**. Here, we dosed mice with 35, 70, or 140 ng of **4** daily [representative of the typical range of nicotine amounts (4–72 ng/mL) found in human smokers,[19] if fully converted to **4**]. Under a 5-day administration regimen, none of the mice showed any evidence of health or behavioral problems at any of the listed dosages. Additionally, a long-term exposure (5 weeks) of **4** was undertaken, dosing every other day at 200 ng/dose. All mice remained healthy, and autopsies did not reveal any sign of neoplasia or organ damage. Thus, we believe the enzyme degradation products from nicotine should not incur safety issues.

A variety of microsomal strains that can degrade nicotine have been known for well over 50 years.[21] However, their characterization has been limited to green strategies wherein essential genes are stratified for the bioremediation of nicotine.[16] We have examined the enzyme NicA2 that can catabolize nicotine to a non-addictive substance, 4-(methylamino)-1-(pyridin-3-yl) butan-1-one. We have conducted a kinetic profile on the enzyme and have found it has many qualities that would be desirable for a therapeutic utilization in smoking cessation, or nicotine intoxication.

A goal of contemporary immunotherapy is the generation of sufficient pharmacokinetic capacity to substantially reduce free drug concentrations regardless of body burden of drug. The degree of reduction of free drug concentration that is needed to achieve efficacy will vary with the pharmacodynamics of the drug. The daunting constraints placed upon current biologic strategies for treating nicotine addiction, i.e., antibody affinity and titers, have sobered enthusiasm for immunopharmacotherapy. NicA2 offers an alternative pharmacokinetic strategy to that of immunotherapeutics, and while its potential immune interaction is still unknown, it represents a first step in using a non-native nicotine metabolizer for smoking cessation strategies. With enzyme engineering we envision a highly active, stable, immunologically silent entity that will be able to degrade nicotine under physiological conditions for the effective treatment of nicotine addiction.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.5b06605.

Materials, experimental details, data analysis, supplementary data, and simulation (PDF)

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Notes

The authors declare no competing financial interest.

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