

Antibody-catalyzed oxidative degradation of nicotine using riboflavin

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Abstract—Tobacco abuse remains a major cause of death worldwide despite ample evidence linking nicotine to various disease states. Consequently, immunopharmacotherapeutic approaches for the treatment of nicotine abuse have received increasing attention. Although a number of nicotine-binding antibodies have been disclosed, no antibody catalysts exist which efficiently degrade nicotine into pharmacologically inactive substances. Herein, we report the first catalytic antibodies which can oxidatively degrade nicotine. These biocatalysts use the micronutrient riboflavin and visible light as a source of singlet oxygen for the production of reactive oxygen species. Along with various known nicotine metabolites, antibody-catalyzed nicotine oxidations produce two novel nicotine oxidation products that were also detected in control ozonation reactions of nicotine. The reaction is efficient, with multiple turnovers of catalyst observed and total consumption of nicotine attained. These results demonstrate the potential of harnessing riboflavin as an endogenous sensitizer for antibody-catalyzed oxidations and demonstrate a new approach for the development of an active vaccine for the treatment of nicotine addiction using in vivo catalytically active antibodies.

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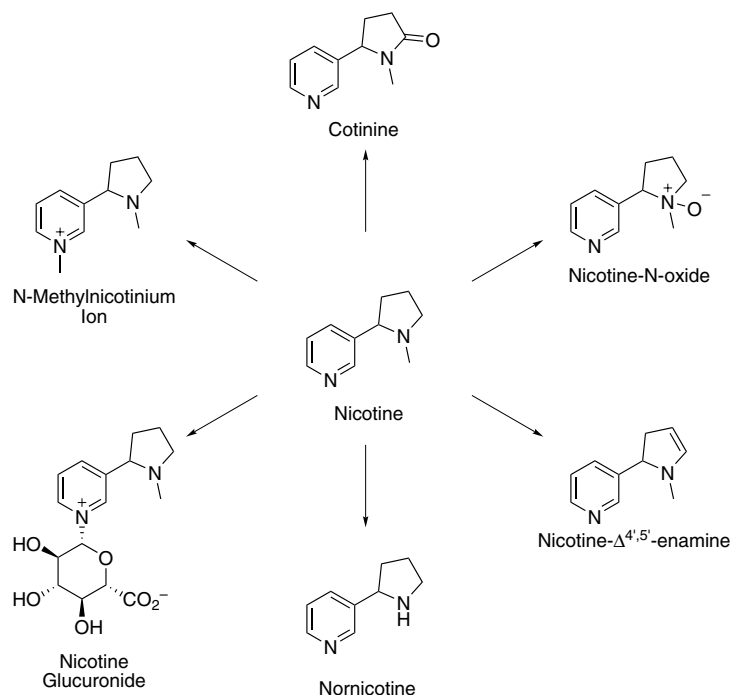
1. Introduction

Nicotine is the most widely used addictive drug in the world. The drug itself has been intimately linked with cigarette smoking, the leading preventable cause of death in the United States,^{1,2} hence, extensive studies have been performed into both the psychoactive properties and metabolism of nicotine.³ The metabolic fate of nicotine in mammals comes mainly from P-450 oxidation and six main metabolites of nicotine are known (Scheme 1). The exact mechanisms of formation of all metabolites are not well understood; however, nicotine is primarily converted to the pharmacologically inactive and long-lived metabolite, cotinine or one of its corresponding secondary metabolites, such as 3-hydroxycotinine.⁴ In certain bacteria, tobacco alkaloids can serve as a carbon and nitrogen source through catabolism by various biochemical processes. Yet, there are no known biological catalysts that specifically and efficiently degrade nicotine under physiologically relevant conditions.

Efficient de novo catalysts for the degradation of drugs of abuse have been highly sought after, especially in the context of catalytic antibodies capable of cocaine hydrolysis.⁵ However, no antibody catalysts have been described in the context of nicotine addiction, although numerous nicotine-binding antibodies are known.⁵ Our interest in nicotine and the oxidative biochemical pathways utilized in its processing are grounded upon an immunological viewpoint; we have focused efforts on methods to procure tailored antibodies that not only recognize nicotine, but catalyze its degradation. The latter of these two goals has proven significantly more demanding as to obtain catalytic antibodies with the energetic capabilities to degrade nicotine via an oxidative mechanism, we were faced with a hapten design challenge wherein neither a transition-state analogue,⁶ nor reactive immunization⁷ approach would be applicable.

Clues for the design of antibody catalysts for the oxidative degradation of nicotine come from both natural metabolic pathways and work from Lerner and co-workers whom have provided the first direct evidence that all antibodies, regardless of specificity or source, have the innate ability to produce reactive oxygen species.^{8–12} The importance of these studies to our antibody

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Scheme 1. The six major metabolites of nicotine in mammals. The major metabolites, cotinine, and nicotine-*N*-oxide, are clearly derived from oxidation processes, but all are synthesized via formal oxidations.

catalyst efforts include the observation that for the generation of these oxidants to occur, a source of singlet oxygen ($^1\text{O}_2$) is necessary. Given a suitable source of $^1\text{O}_2$, an antibody oxidative pathway for nicotine degradation could parallel many of the oxidative products found in natural biochemical systems, albeit from a completely different source.

To this end, an efficient endogenous photosensitizing chromophore was required. Many sensitizers, such as porphyrins, could have been investigated, however, we chose riboflavin, or vitamin B₂. Our reasoning for examining riboflavin stems from reports, which describe the interaction of riboflavin with immunoglobulins in serum, however, the functional significance of this interaction has, to date, not been understood.¹³ Based upon the inherent ability of riboflavin to sensitize molecular oxygen, coupled with its proximity to circulating antibodies, and the antibody oxidative cycle detailed by Lerner and co-workers, we have sought to investigate whether the immune system could contain a set of catalytic antibodies that utilize riboflavin as a source of $^1\text{O}_2$. Thus, the fundamental nature of an antibody–riboflavin chemical interaction could provide a functional oxidation system in vivo for the catalytic destruction of nicotine and provide a powerful tool in the treatment of nicotine addiction by passive, or possibly active, immunization.

2. Results and discussion

In light of the countless deaths attributable to tobacco abuse each year and the low success rates of current nicotine cessation therapies, extensive efforts have been devoted to the production of novel treatments for nico-

tine addiction.⁵ Recently, antibodies that specifically recognize nicotine have emerged as a promising method for attenuating the psychoactive effects of nicotine.^{5,14} Because of its addictive properties we viewed nicotine as an attractive target for the development of novel antibody-based treatments, however, as there are no catalysts either enzyme or antibody based that can specifically and efficiently degrade this molecule, it also represented a formidable energetic challenge for antibody catalysis.

In an effort to identify systems where antibody-catalyzed oxidation of nicotine could be feasible, we examined the ability of the micronutrient riboflavin to assist in this energetically demanding oxidation. Antibodies are known to interact with riboflavin in vivo, however, the significance of antibody–riboflavin union is still cryptic since its discovery 20 years ago.¹³ In light of the recent discovery that all antibodies can produce reactive oxygen species when given a source of singlet oxygen,^{8–12} we hypothesized that a subset of antibodies could use riboflavin as an innate source of singlet oxygen, thereby unifying foreign particle recognition and destruction into one molecular complex. In this context, the oxidation of a bound protein or organic molecule could occur catalytically through the ternary interaction between antibody–antigen and riboflavin.

To characterize potential catalytic antibodies for oxidative nicotine degradation, we first examined several control oxidation reactions for both product identity and relative distribution. Because antibodies have been shown to produce H_2O_2 ,^{8,9} we first treated nicotine with this oxidant. As previously reported, this reaction yields nicotine-*N*-oxide as the major product.¹⁵ Our second

oxidant examined was ozone, again, based on the work of Lerner and co-workers.^{10–12} Treatment of nicotine with ozone provided seven major products as identified by cation-exchange HPLC. Five of these products, nor-nicotine, anabasine, β -nicotyrine, cotinine, and nicotine-*N*-oxide have been reported previously to be formed with ozone,¹⁶ while two new products, **1** and **2**, have not (Scheme 2). We have isolated these compound and surprisingly, neither corresponded to common nicotine metabolites. Purification and analytical characterization revealed the structures of these compounds to be 3-(1-hydroxy-2-pyrrolidinyl)pyridine **1** and polyhydroxylated cotinine derivative **2** (Fig. 2). Lastly, the reaction of nicotine with potassium superoxide was studied, however presumably due to its instability in water, only hydrogen peroxide was generated, yielding nicotine-*N*-oxide.

To elicit antibody catalysts for nicotine degradation, a traditional transition-state analogue approach was abandoned for a hapten–substrate tact. Our reasoning here was similar to previous reports from our group on photochemical antibody-catalyzed reactions where an external source of energy was present.^{17,18} We initially screened a panel of monoclonal antibodies previously elicited against a hapten **3**, which is highly congruent to nicotine (Fig. 1).¹⁹ Antibodies in this panel bound nicotine in the range of nanomolar to micromolar affinity. However, no catalysts were observed after either irradiation with UV light, or irradiation with white light in the presence of riboflavin. Interestingly, some of these antibodies actually protected nicotine from oxidation relative to control reactions containing nicotine and riboflavin alone.

In light of these findings, we envisioned that antibodies that bound nicotine weakly would provide a reasonable local concentration of nicotine in the vicinity of the antibody without protecting the nicotine molecule from oxidation by generated reactive oxygen species. To this end,

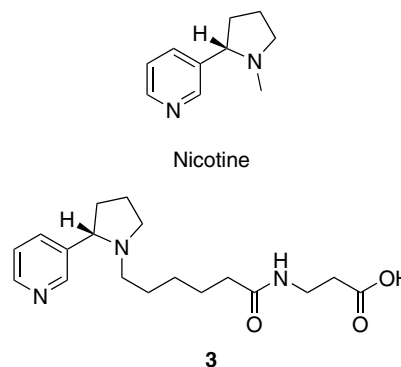
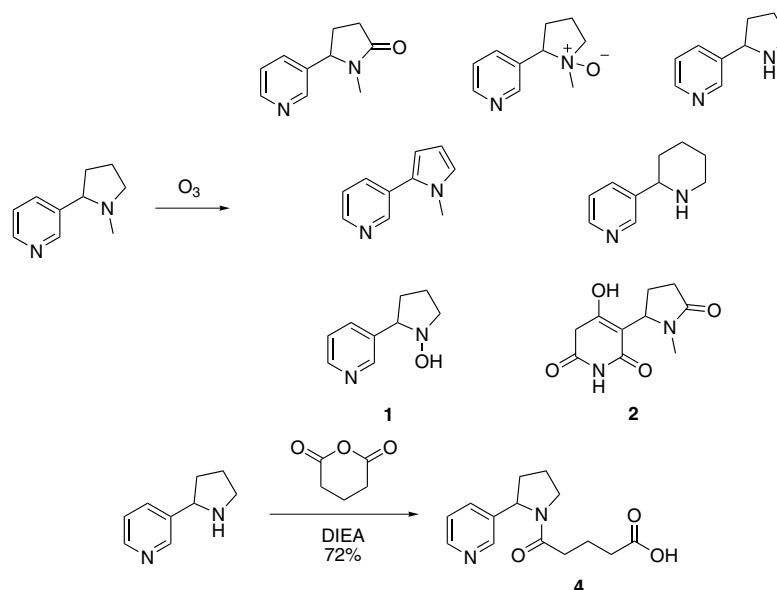


Figure 1. Structures of nicotine and structurally congruent hapten **3** used to elicit tight binding nicotine antibodies.

hapten **4** (hapten code: TD1) was synthesized from nor-nicotine and glutaric anhydride to elicit nicotine-binding antibodies (Scheme 2). Our design principle was thus grounded upon two aspects: (1) Substrate recognition by antibodies to **4** would be sufficient, but not tight as seen previously with hapten **3**; thus, **4** represents the global scaffold of nicotine less the charge on the pyrrolidine nitrogen. (2) The oxidation of nicotine dramatically changes the charge distribution within the molecule (e.g., formation of *N*-oxides), and as such, product inhibition using antibodies raised against **4** was expected to be minimal. Hapten **4** was conjugated to keyhole limpet hemocyanin (KLH) and bovine serum albumin (BSA) using standard protocols and mice were immunized with the **4**-KLH conjugate. The titer against **4**-KLH was measured to be $\sim 25,000$, indicating that the immunogenicity of hapten **4** was comparable to titers observed in our active cocaine vaccine studies²⁰ and significantly greater than our previous nicotine hapten **3**.¹⁹ The resulting panel of 26 monoclonal antibodies was purified²¹ and screened for nicotine oxidation in the presence of riboflavin. The range of binding of these antibodies to



Scheme 2. Chemical ozonation of nicotine and hapten **4** used to elicit nicotine-binding antibodies.

nicotine was much weaker as expected from our hapten design and was found to be between 0.2 and 50 mM as determined by competition ELISA. Using riboflavin (60 μ M) and a source of white light, selected monoclonal antibodies from the panel raised against 4-KLH were able to catalyze the oxidation of nicotine to products strikingly similar to the ozone positive control reaction. Of the 26 antibodies screened, the most proficient were TD1-10E8 ($K_{d,app}$ = 6.3 mM) and TD1-36H10 ($K_{d,app}$ = 6.3 mM), which were selected for further study.

The products that were observed in both the uncatalyzed synthetic ozone control reaction as well as with TD1-10E8 and TD1-36H10 oxidations were identical with regard to the seven major products. In essence, synthetically produced ozone provided an accurate 'blueprint' for the antibody-catalyzed oxidations. Of the seven products observed with antibodies TD1-10E8 and TD1-36H10, the greatest overall rate acceleration was seen with compounds **1** and **2**. Both of these compounds were formed at approximately 10-fold rate enhancements over the uncatalyzed reactions of nicotine and riboflavin alone (Fig. 2). Control reactions in which visible light or oxygen were omitted showed no production of compounds **1**, **2**, or degradation of nicotine. The presence of these two new products is interesting since a powerful oxidant must be invoked in the formation of **1** as it has only previously been observed in metal-catalyzed oxidation systems.²² Furthermore, compound **2** also implicates potent oxidants, potentially hydroxyl radical or a more stable version of this oxidant,²³ capable of heavily oxidizing bound antigens.

A more detailed analysis of the formation of **1** and **2** was performed to glean additional information into the specific nature of this reaction. The kinetic profile of the reaction revealed that approximately 50% of the total nicotine was converted to products **1** and **2** (Fig. 2). In the presence of TD1-10E8, the initial rates of formation of **1** and **2** were 0.54 and 0.67 μ M/min, respectively, and the relative formation of **1**:**2** was found to be 1.05:1 (Fig. 2). In contrast, TD1-36H10 formed **1** and **2** at initial rates of 0.67 and 0.49 μ M/min, respectively, and produced these compounds in a 1:1.75 ratio (Fig. 2). In comparison, the uncatalyzed control reaction in which nicotine is treated with ozone produces these two products in a ratio of 1.62:1. These differences are remarkable, and presumably, these two antibodies have unique combining site architectures that allows for altered product distributions and a disfavored reaction ratio.²⁴

To inhibit the reaction, hapten **4** could not be used as its structural congruency is too close to the substrate and would presumably undergo oxidation, thus we explored the use of various reactive oxygen species scavengers including methionine and thiosulfate.²⁵ Neither compound eliminated the catalytic activity of TD1-10E8; indeed, methionine had no effect on the product ratio (1.02:1, **1**:**2**), while a drastically different ratio of compounds was observed in antibody-catalyzed reactions in the presence of thiosulfate (2.02:1, **1**:**2**). Furthermore, other methods for quenching reactive oxygen species,

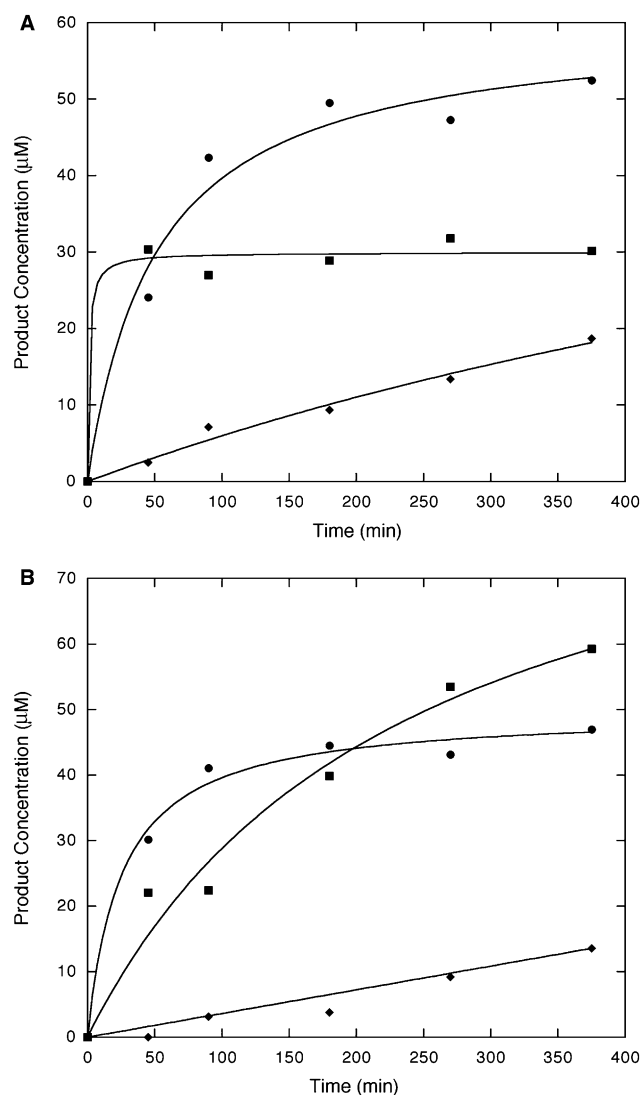


Figure 2. Time course plots of the formation of **1** (A) and **2** (B). Filled circles: TD1-10E8, riboflavin, nicotine, and visible light; Filled squares: TD1-36H10, riboflavin, nicotine, and visible light; Filled diamonds: riboflavin, nicotine, and visible light. Data was fit using Kaleidagraph 3.0.5 (Abelbeck software) using nonlinear regression analysis.

such as the use of superoxide dismutase to destroy any superoxide, also did not diminish catalytic activity. In the presence of SOD (150 U/mL), no effect was observed on the product ratio (1:1.03, **1**:**2**). In total, this data indicates that although the identity of the antibody-produced reactive oxygen species is unknown, this oxidant is likely protected from bulk solvent, yet accessible to the antibody combining site.

It is critical to note that independent of the identity of the reactive oxygen species, after only 6 h of white light irradiation in the presence of either antibody (20 μ M), no detectable nicotine remains in the reaction mixtures (starting nicotine concentration = 200 μ M), implying multiple turnover events by the antibody. As a further control, nicotine was also irradiated in the presence of riboflavin and BSA, a protein known to bind small molecules and accelerate some reactions.²⁶ Product ratios in this reaction greatly differed from our antibody-

catalyzed reactions (1:4, 1:2). Furthermore, in contrast to our antibody-catalyzed reactions, following a short burst of reactive oxidant production (<1 turnover), a drastic decrease in rate due to photooxidation of the protein was observed.⁹

A subset of immunoglobulins has been previously reported to bind riboflavin,¹³ and as such, we tested the most efficient antibody catalysts for binding by equilibrium dialysis to determine if riboflavin binding was essential for efficient transfer of singlet oxygen to the antibody. We observed no specific riboflavin binding ($K_d \geq 1$ mM), thus it is clear that binding is not a prerequisite for antibody-catalyzed oxidation processes. Indeed, these results demonstrate that antibodies, independent of intimate riboflavin-binding ability, can utilize riboflavin as an innate sensitizer and source of singlet oxygen for the production of powerful oxidants.

3. Conclusion

In summary we have demonstrated that antibody-catalyzed oxidation of nicotine is feasible, and may be an unrecognized method for the destruction of nicotine in biological systems. The concentrations of riboflavin (~10 nM)²⁷ and specific antibody (~1 μ M)²⁸ *in vivo* are reasonable for riboflavin-assisted antibody oxidations to have biological relevance. We emphasize that the oxidation and product distribution seen for nicotine degradation would not have previously been envisioned to be in the repertoire of antibody catalysis based on traditional hapten design using transition-state or reactive immunization theory.

Our active vaccine approach does not require the presence of tight-binding antibodies, and in fact, these results suggest that antibodies with modest affinity may serve as more efficient catalysts for nicotine destruction. Furthermore, a weaker binding constant may also allow for the binding of various pharmacologically and chemically active nicotine metabolites, such as cotinine²⁹ and nornicotine,^{30,31} and their subsequent oxidation and clearance. Hapten **4** is highly immunogenic, as is evidenced by the high circulating titer in mice, and could lead to the production of a novel active vaccine in which multiple catalytic antibodies could be elicited *in vivo* against a given drug of abuse. We envision that this strategy is applicable to the destruction of other drugs of abuse containing bonds that can be oxidized (T.J.D and K.D.J., unpublished results).

4. Experimental

4.1. General synthetic methods

Unless otherwise stated, all reactions were performed under an inert atmosphere with dry reagents and solvents and flame-dried glassware. Analytical thin-layer chromatography (TLC) was performed using 0.25 mm precoated silica gel Kieselgel 60 F₂₅₄ plates. Visualization of the chromatogram was by UV absorbance,

iodine, dinitrophenylhydrazine, ceric ammonium molybdate, ninhydrin, or potassium permanganate as appropriate. ¹H NMR spectra were recorded on either a Varian INOVA-400 or Bruker DRX-600 spectrometer at 400 and 600 MHz, respectively. ¹³C NMR spectra were recorded on a Varian INOVA-400 spectrometer at 100 MHz. Matrix-assisted laser desorption/ionization (MALDI) FTMS experiments were performed on an IonSpec FTMS mass spectrometer. Electrospray ionization (ESI) mass spectrometry experiments were performed on an API 100 Perkin Elmer SCIEX single quadrupole mass spectrometer. Analytical high pressure liquid chromatography (HPLC) was performed on a Hitachi D-7000 instrument equipped with a Phenomenex Partisil 10 SCX cation-exchange analytical column, a Hitachi L-7100 pump, Hitachi L-7200 autosampler, a Hitachi L-7455 diode array detector, and mobile phases comprised of 0.3 M sodium acetate/methanol (95:5, v/v, pH 4.5), at a flow rate of 1 mL/min. The L-7100 pump was controlled using Hitachi-HSM software on a Dell Optiplex GX110 PC computer.

4.2. Synthesis of TD1 Hapten **4**

Glutaric anhydride (77 mg, 0.68 mmol), *N,N*-dimethylaminopyridine (8 mg, 0.07 mmol), and diisopropylethylamine (235 μ L, 1.35 mmol) were added to a solution of nornicotine (100 mg, 0.68 mmol) in CH₂Cl₂ (6 mL), at room temperature and the reaction mixture allowed to stir for 18 h. The crude mixture was concentrated, and then purified by preparative TLC using CHCl₃/MeOH (3:2) as the eluent. The desired band was triturated with CHCl₃/MeOH (3:2) and concentrated to obtain the TD1 hapten **4** as a clear oil (152 mg, 86%). Hapten conjugation to BSA and KLH and monoclonal antibody production was performed. Approximate loadings of the TD1-BSA conjugate were determined by MALDI-TOF mass spectrometry and revealed that approximately 19 copies of the hapten were present per BSA molecule.

4.3. Nicotine competition ELISA

An ELISA plate (CoStar; 96 well) was coated uncovered overnight at 37 °C with 25 μ L of a nicotine-BSA conjugate previously reported to resemble free nicotine²² diluted to 5 μ g/mL. To this, methanol (50 μ L/well) was added and allowed to sit 5 min at room temperature. The methanol was then removed from the wells and the plates allowed to dry. Wells were then blocked with 50 μ L of Blotto (5% skim milk powder in PBS) for 5 min at 37 °C. Typically, 25 μ L of a solution containing a 1:100 dilution of mAb in Blotto along with varied serial dilutions of nicotine (50 μ M–50 mM) was then added and incubated 1 h at 37 °C. After washing, 25 μ L of a 1:1000 dilution of a goat-antimouse/horseradish peroxidase conjugate in Blotto was added and incubated for 30 min. The plate was developed with the colorimetric reagent ABTS for 30 min and the absorbance measured on an ELISA plate reader at 405 nm.

4.4. Riboflavin equilibrium dialysis

Equilibrium dialysis was performed using [^3H]-riboflavin as ligand and the following antibodies: TD1-10E8 and TD1-36H10. The antibodies were serially diluted in PBS (pH 7.4), starting from 50 μM , and added to wells in a 96-well microtiter plate (150 μL /well). Wells in a second microtiter plate were filled with [^3H]-riboflavin (150 μL , 5 nM, 0.5 μCi) in PBS (pH 7.4). The two plates were tightly connected with filled wells facing each other and separated with a dialysis membrane (molecular weight cutoff 6000–8000 Da). The plates were attached vertically to a shaker and were shaken at 24 h at room temperature, after which they were carefully separated. The membrane was discarded and from each well, 100 μL was transferred to a scintillation vial. To each solution, scintillation fluid (5 mL) was added and the radiation counted for 5 min/sample. The experiment was repeated twice for each serum sample. The average in differences in DPM (disintegrations per minute) between opposite wells was determined for each mAb concentration.

4.5. Oxidation of nicotine by chemically supplied ozone

Nicotine (50 mg, 0.31 mmol) was dissolved in CH_2Cl_2 (10 mL). The reaction mixture was then cooled to -78°C and treated with ozone for 30 min. After this time, the reaction mixture was concentrated to yield 68 mg of a brown thick tar. A sample (~ 1 mg) of the tar was removed, dissolved in PBS (2 mL, 10 mM, pH 7.4) and analyzed by HPLC using the system described above.

4.6. Isolation and spectral characterization of hydroxylamine 1

Nicotine ozonation reactions were performed with chemically supplied ozone as described above (vide supra). A sample from the resulting product mixture (~ 4 mg) was dissolved in phosphate buffer (10 mM, pH 7.4) and injected onto the ion-exchange HPLC system described above. The peak with retention time 4.2 min was isolated and the solvent removed by lyophilization. ^1H NMR (D_2O): δ 1.92 (m, 2H), 2.03 (m, 1H), 2.34 (m, 1H), 3.20 (m, 1H), 3.48 (m, 1H), 3.77 (d, $J=6.7$ Hz, 1H), 7.19 (m, 1H), 7.84 (m, 1H), 8.48 (m, 1H), 8.55 (m, 1H). ^{13}C NMR (D_2O): δ 24.3, 31.5, 59.0, 71.9, 123.5, 134.5, 137.1, 148.0, 149.2. ESI-MS: Found 187 ($\text{M}+\text{Na}^+$). MALDI-FTMS for $\text{C}_9\text{H}_{13}\text{N}_2\text{O}$ ($\text{M}+\text{H}^+$) calculated 165.1028, found 165.1027.

4.7. Isolation and spectral characterization of polyhydroxylated cotinine 2

Nicotine ozonation reactions were performed with chemically supplied ozone as described above (vide supra). A sample of the resulting product mixture (~ 4 mg) was dissolved in phosphate buffer (10 mM, pH 7.4) and injected onto the ion-exchange HPLC system described above. The peak with retention time 5.2 min was isolated and the solvent removed by lyophilization. ^1H NMR (D_2O): δ 2.09 (m, 2H), 2.68 (m, 2H),

3.06 (s, 3H), 3.22 (m, 2H), 3.97 (d, $J=6.2$ Hz, 1H), 5.26 (m, 1H). ^{13}C NMR (D_2O): δ 27.2, 29.8, 34.4, 44.3, 62.9, 73.4, 167.0, 171.9, 176.5, 207.7. ESI-MS: Found 247 ($\text{M}+\text{Na}^+$). MALDI-FTMS for $\text{C}_{10}\text{H}_{13}\text{N}_2\text{O}_4$ ($\text{M}+\text{H}^+$) calculated 224.0797, found 224.0799.

4.8. Nicotine oxidation using ultraviolet light

Antibody stock concentrations were determined by UV-Vis spectroscopy using 1 OD = 1.25 mg/mL at 280 nm and 1 mg/mL = 6.7 μM . High-intensity photolysis was performed in a Rayonet photochemical reactor with RPR-3500 bulbs. The assay was initiated by the addition of a solution of antibody (20 μM in PBS) to a solution of nicotine (200 μM) in buffer (10 mM PBS, pH 7.4) in 4 mL glass vials. High-intensity photolysis was performed for 10 min intervals, and at times throughout the assay, aliquots (50 μL) were removed and directly injected onto the analytical HPLC system described above.

4.9. Nicotine oxidation using riboflavin as a visible light sensitizer

A solution of nicotine (200 μM), riboflavin (60 μM), and antibody (20 μM) in PBS (pH 7.4) was irradiated on a white light transilluminator (2.8 mW/cm 2) at 4°C in duplicate in a standard polystyrene ELISA plate. The final volume of each reaction was 200 μL . At given time points throughout the assay, aliquots (50 μL) were removed and directly injected onto the analytical HPLC system described above. The following control reactions were also performed: nicotine, riboflavin, and antibody in the dark; nicotine, riboflavin, and visible light; nicotine and riboflavin in the dark; antibody, riboflavin, nicotine, and visible light in degassed PBS. In all cases, no product was observed in any control reaction.

4.10. Reactive oxygen inhibition experiments

A solution of nicotine (200 μM), riboflavin (60 μM), antibody (20 μM), and methionine (1 mM) or superoxide dismutase (150 U/mL) in PBS (pH 7.4) was irradiated on a white light transilluminator (2.8 mW/cm 2) at 4°C in duplicate in a standard polystyrene ELISA plate. The final volume of each reaction was 200 μL . At given time points throughout the assay, aliquots (50 μL) were removed and directly injected onto the analytical HPLC system described above.

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