Neuroprotection in the MPTP Parkinsonian C57BL/6 Mouse Model by a Compound Isolated from Tobacco†

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Epidemiological evidence suggests a lower incidence of Parkinson's disease in smokers than in nonsmokers. This evidence, together with the lower levels of brain monoamine oxidase (MAO) activity in smokers and the potential neuroprotective properties of MAO inhibitors, prompted studies which led to the isolation and characterization of 2,3,6-trimethyl-1,4-naphthoquinone (TMN), an MAO-A and MAO-B inhibitor which is present in tobacco and tobacco smoke. Results of experiments reported here provide evidence that this compound protects against the MPTP-mediated depletion of neostriatal dopamine levels in the C57BL/6 mouse. These results support the hypothesis that the inhibition of MAO by constituents of tobacco smoke may be related to the decreased incidence of Parkinson's disease in smokers.

Introduction

Parkinson's disease (PD)[1] is an age-dependent, neurodegenerative disorder involving the selective loss of dopaminergic nigrostriatal neurons (*1*). Since no strong genetic link has been identified for this disease, other factors, including exposure to environmental neurotoxins, have been proposed to play a critical role in its etiology (*2*). The characterization of the parkinsonian-inducing properties of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine [MPTP (**1**); see Figure 1 for structures] more than 15 years ago (*3*) has lent additional support to the environmental neurotoxin theory and also has led to the development of animal models for studying how such neurotoxins might lead to pathological lesions of the nigrostriatal system. A useful animal model for studying neurodegenerative and neuroprotective processes is the MPTP-treated C57BL/6 mouse (*4*, *5*). In one variation of this model, a single intraperitoneal (ip) dose of MPTP leads to the loss of nigrostriatal dopaminergic cell bodies in the substantia nigra and depletion of neostriatal dopamine (DA) levels (*6*−*8*).

The molecular mechanism by which MPTP selectively damages nigrostriatal neurons has been the subject of extensive research, and much is known (*9*). Critical to its mode of action is the monoamine oxidase B (MAO-B)-catalyzed allylic α-carbon oxidation of the parent tetrahydropyridine, leading to the corresponding dihydropyridinium species $MPDP^+$ (**2**) (*10*). This intermediate undergoes a second two-electron oxidation to generate the pyridinium metabolite MPP^+ (**3**), the ultimate neurotoxin (*10*).

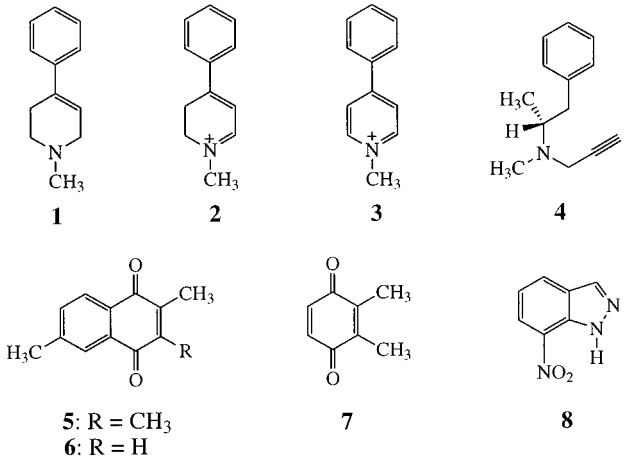

Figure 1. Structures of compounds discussed in the text.

Several compounds have been reported to protect against MPTP's nigrostriatal toxicity in the C57BL/6 mouse. The mechanisms by which most of these compounds act are only poorly understood [for a recent review, see Alexi et al. (*11*)]. One well-documented neuroprotective pathway involves inhibition of the MAO-B-catalyzed bioactivation of MPTP to MPP^+. For example, pretreatment of C57BL/6 mice with (*R*)-deprenyl (**4**), or other potent and selective mechanism-based inactivators of MAO-B, renders animals resistant to the neurotoxic effects of MPTP (*12*−*16*). Also of interest is the use of this MAO-B inhibitor to treat patients diagnosed with PD (*17*).

One of the interesting outcomes of epidemiological studies on PD is the clear evidence of a lower incidence of this disease in tobacco smokers than in nonsmokers

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[1] Abbreviations: MAO, monoamine oxidase; GC/EIMS, gas chromatography/electron ionization mass spectrometry; PD, Parkinson's disease; TCA, trichloroacetic acid; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; $MPDP^+$, 1-methyl-4-phenyl-2,3-dihydropyridinium ion; MPP^+, 1-methyl-4-phenylpyridinium ion; TMN, 2,3,6-trimethyl-1,4-naphthoquinone; 7-NI, 7-nitroindazole; DA, dopamine; MP, mobile phase; IS, internal standard.

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(*18*). Consistent with this antiparkinsonian effect is the report by Carr et al. (*19*) that tobacco smoke attenuates the neurotoxicity of the parkinsonian-inducing drug MPTP in the C57BL/6 mouse model. These results have prompted us to consider the possibility that tobacco and/or tobacco smoke may contain one or more neuroprotective compounds that act via inhibition of MAO. Early studies by Essman (*20*) showed that in mouse skin exposed to cigarette smoke, the level of MAO-catalyzed oxidation of serotonin was decreased. Norman et al. (*21*) claimed that cigarette smoke inhibited MAO activity. Carr et al. (*22*) in mouse brain homogenates and Yu et al. (*23*) in rat lung tissue have shown MAO inhibition with tobacco smoke extracts, although nicotine itself, at physiologically significant levels, was shown not to contribute to this inhibition (*22*). In addition, a number of authors have shown that the human blood platelet MAO activity (MAO-B) is significantly reduced in smokers compared to that in nonsmokers (*24*−*26*). Even more relevant are the recent reports by Fowler et al. (*27*, *28*) using in vivo positron emission tomography that demonstrated lower MAO-A and MAO-B activities in the brains of smokers.

Recent efforts in our laboratory have led to the isolation from tobacco leaf extracts of 2,3,6-trimethyl-1,4-naphthoquinone [TMN (**5**)], a compound which competitively inhibits both MAO-A ($K_I = 3\ \mu M$) and MAO-B ($K_I = 6\ \mu M$) (*29*) and which has been shown to be present in tobacco leaves and tobacco smoke (*30*, *31*). In this paper, we describe the results of studies aimed at assessing the potential in vivo inhibition of brain MAO by TMN and its neuroprotective properties in the C57 parkinsonian mouse model.

Experimental Procedures

Caution: *MPTP is a known nigrostriatal neurotoxin and should be handled following established procedures (32).*

Chemicals, Reagents, and General Methods. Chemicals and reagents not described elsewhere were purchased from Aldrich Chemical Co. (Milwaukee, WI). Milli-Q water was obtained from a Waters Milli-Q UV Plus system (Millipore, Bedford, MA). The following were obtained as indicated: HPLC grade methanol (Burdick & Jackson, Muskegon, MI); trichloroacetic acid (TCA), 50% H_2O_2, enzyme grade Na_2HPO_4 and NaH_2PO_4, Na_2SO_4, and glycerol (Fisher Scientific Products, Fair Lawn, NJ); Hollywood peanut oil (Kroger Inc., Blacksburg, VA); dopamine hydrochloride (DA) and 3,4-dihydroxybenzylamine hydrobromide (Research Biochemicals Inc., Natick, MA); and hydrated salcomine (Sigma Chemical Co., St. Louis, MO). TMN was synthesized as reported previously (*29*) with minor modifications as described below. MPTP·HCl (*33*) was synthesized according to literature methods. Melting points were obtained on a Thomas-Hoover melting point apparatus and are uncorrected. NMR spectra were obtained on a Brucker WP 360 MHz spectrometer. The GC/MS system was as follows: Hewlett-Packard MSD 5870, GC 5890, ChemStation 5970, HP-1 column, 25 m × 0.2 mm × 0.33 μm, split/splitless injector, splitless mode, port temperature of 250 °C; GC oven program, from 60 to 290 °C at a rate of 25 °C/min.

Syntheses. (1) 2,6-Dimethyl-1,4-naphthoquinone (6). This compound**,** which was used as an internal standard (IS) in the assay developed to estimate brain concentrations of TMN, was synthesized following the literature procedure for the synthesis of 2-methyl-1,4-naphthoquinone (*34*) using commercially available 2,6-dimethylnaphthalene as a starting material: mp 133−136 °C [lit. mp 134−135 °C (*35*)].

(2) 2,3,6-Trimethyl-1,4-naphthoquinone [TMN (5)] was prepared as described previously (*29*) except for the preparation of the synthetic intermediate 2,3-dimethyl-1,4-benzoquinone (**7**) which was synthesized as follows. A solution of 2,3-dimethylphenol (3.1 g, 25 mmol) and hydrated salcomine (1.6 g, 5 mmol) (*36*) in 100 mL of *N*,*N*-dimethylformamide was stirred under an oxygen atmosphere for 24 h. After addition of an equal volume of brine, the solution was filtered, the filtrate was extracted with diethyl ether (3 × 300 mL), and the combined organic layers were filtered. The filtrate was washed twice with an equal volume of brine, dried over Mg_2SO_4, and concentrated to a small volume which was purified by passage through a column of neutral, activated alumina (50 g) using 9:1 hexane/ethyl acetate. Fractions (5 mL) containing the desired product (3−25) were combined and evaporated to dryness in vacuo, and the residue was recrystallized from *n*-hexane to give the pure product (1.1 g, 33%): mp 55 °C [lit. mp 56−57 °C (*37*)].

HPLC Assay for TMN. TMN analyses were performed on a Hewlett-Packard 1100 HPLC system equipped with a UV/vis diode array (DA) detector, a Rheodyne 7725I injector, an Altech adsorbosphere C18 analytical column (4.6 mm × 150 mm, 5 μm) preceded by a C18 guard column (Opti Guard, 1 mm; Altech). The mobile phase (MP) consisted of 70% methanol and 30% Milli-Q water with a flow rate of 1 mL/min. The IS (**6**) and TMN were monitored at 265 nm. Calibration curves for the quantitative measurements of TMN were constructed using standards prepared in 65% MeOH and 35% Milli-Q water, containing 5% TCA (w:v) (solution A) and 1.3 μM IS with concentrations of TMN ranging from 0.5 to 9.0 μM. These calibration standards were injected (200 μL) into the HPLC system, and the TMN:IS peak height ratios were plotted versus the micromolar TMN concentration.

Protocols for all animal experiments were reviewed and approved by the Virginia Tech Animal Care Committee and follow the Guide for the Care and Use of Laboratory Animals, 7th ed., 1996, National Research Council.

TMN Recovery from Brain Homogenates. Two sets of samples were utilized to measure the recovery of TMN from mouse brain homogenates. Brain tissues were homogenized in solution A (6 μL/mg of wet tissue). Each sample for group 1 ($n = 3$) consisted of 343 μL of brain homogenate, 100 ng of internal standard in 10 μL of solution A, 280 ng of TMN in 28 μL of solution A, and an additional 19 μL of solution A to give a final volume of 400 μL. For group 2 ($n = 3$), essentially the same procedure was followed except that 28 μL was added to the homogenate instead of the TMN. Each sample was again homogenized and then centrifuged for 10 min at 15000*g*. An aliquot of each supernatant (300 μL) was transferred to a new microcentrifuge tube. Solution A (21 μL) was added to samples in group 1, and TMN (210 ng in 21 μL of solution A) was added to group 2 samples which were then vortexed. An aliquot (200 μL) was injected into the HPLC system. Recoveries were determined by comparing the TMN:IS peak height ratios from group 1 samples to those from group 2.

Bioavailability of TMN in the Brain. Injection solutions of TMN were prepared in peanut oil by heating the mixture for 20 min at 50 °C. Male C57BL/6 mice, 11−13 months of age (Harlan-Sprague Dawley, Dublin, VA), were treated sc with TMN at doses ranging from 200 to 400 mg/kg of body weight in 0.2−0.3 mL. Animals were sacrificed at time points postinjection ranging from 10 to 120 min. Each brain was removed, weighed, and homogenized in 6 μL/mg of the IS solution (1.34 μM in solution A). The resulting homogenate was centrifuged for 10 min at 15000*g*. The supernatant was transferred to a clean tube and recentrifuged for 5 min at 15000*g*. The resulting supernatant was analyzed by the HPLC assay described previously.

Neuroprotection Studies in the C57BL/6 Mouse. Eleven-month-old, retired male breeder C57BL/6 mice (Harlan-Sprague Dawley) were housed one per cage in a temperature-controlled room with free access to food and water on a 12 h light/dark cycle. Injection solutions of TMN (189 mM) in peanut oil were prepared by stirring and heating at 50 °C for 20 min. Control mice ($n = 4$) were treated sc with peanut oil (0.32 mL/30 g of body weight) 25 min prior to the administration of sterile saline

(0.2 mL ip). MPTP-treated mice ($n = 10$) were treated sc with peanut oil (0.32 mL/30 g) 25 min prior to MPTP (35 mg/kg of MPTP·HCl) ip administration. Mice treated with TMN and MPTP ($n = 7$) were administered TMN (400 mg/kg) 25 min prior to MPTP·HCl ip administration (35 mg/kg). The animals were sacrificed 10 days after drug administration, and the striata were dissected free; 10 μL of the IS solution {3,4-dihydroxybenzylamine [6.82 μM in 5% TCA (w:v) in Milli-Q water]} was added per milligram of wet striatal tissue, and the striata were homogenized. The samples were subsequently centrifuged (16000*g*), and 20 μL aliquots of the resulting supernatants were analyzed for DA by HPLC as previously described (*37*, *38*) using a Bioanalytical C4 system with a PM 60 pump (BAS, Indianapolis, IN) and a Kipp and Zonen BD 41 recorder.

Results and Discussion

In addition to the well-characterized effects of irreversible MAO-B inhibitors in the C57 mouse PD model, reversible inhibitors of MAO-B also have been shown to protect against the neurotoxic effects of MPTP in this model by inhibiting the MAO-B-catalyzed bioactivation of this proneurotoxin (*40*−*42*). Our experience is with 7-nitroindazole [7-NI (**8**)], a selective inhibitor of neuronal nitric oxide synthase (*43*) that also is a competitive inhibitor ($K_I = 4$ μM, mouse brain mitochondria) of MAO-B (*44*). Administration of this compound to the C57BL/6 mouse prior to MPTP decreased the extent of conversion of MPTP to MPP^+ in the brain and protected the mice against the MPTP-induced long-lasting depletion of neostriatal DA levels (*6*, *7*, *44*). Using these studies as a guide, we proceeded to examine the neuroprotective properties of TMN which also would provide an indication of its in vivo MAO-B inhibiting properties.

The poor water solubility of TMN led us to administer the compound sc in peanut oil, a procedure which has been used by us and others with 7-NI (*6*, *45*). Estimates of the extent to which TMN distributes to the brain under these conditions were made employing an HPLC diode array assay using 2,6-dimethyl-1,4-naphthoquinone (**6**) as the IS. Calibration curves with good linearity ($r^2 = 0.991$) and coefficients of variation ($n = 3$) between 5.5 and 6.5% were obtained over a concentration range of 0.5−9.0 μM TMN, while recoveries of TMN-spiked whole brain homogenates were found to be 73.7 ± 2.0% (SD) (see Experimental Procedures).

Utilizing this assay, we measured the TMN brain concentrations of mice treated sc with various doses of TMN in peanut oil (1−2 mmol/kg) and at varying postinjection times (10−120 min). The results established that the brain concentrations are maximal 25 min postinjection. Unfortunately, only 0.003−0.023% of the doses that were administered partitioned into the brain. Part of the problem appeared to be due to the tendency of these oily preparations to form saclike structures at the sites of injection. This behavior was not observed with 7-NI. Brain concentrations of ∼40 μmol TMN/kg, or about 6.6 times the K_I value for the inhibition of MAO-B, were achieved 25 min postinjection when a dose of 400 mg/kg was administered with gentle massaging at the site of injection.

In the neuroprotection studies, we chose an ip dose of MPTP·HCl (0.17 mmol/kg, equivalent to 35 mg/kg ip) which, in our experience, leads to an ∼40% decrease in the concentration of DA in the neostriata of C57BL/6 mice. When administered ip, MPTP reaches its maximum brain concentration at approximately 10 min and then

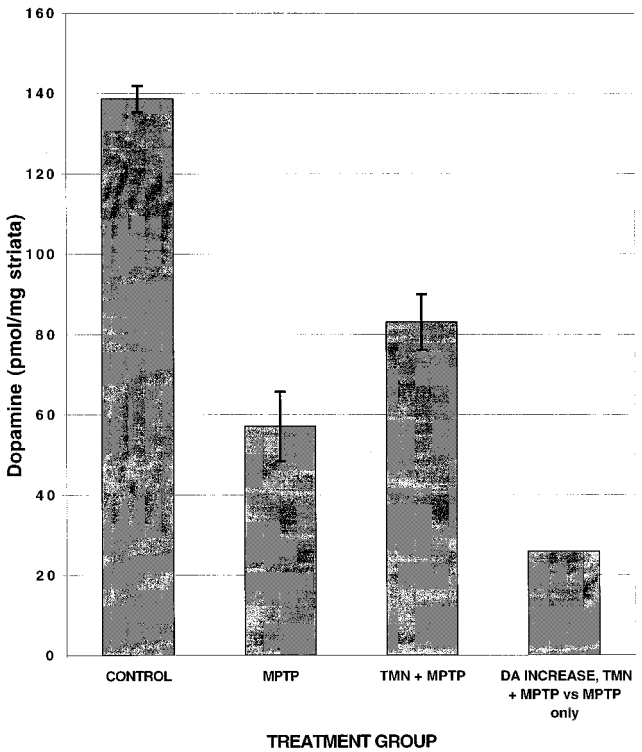

Figure 2. Striatal dopamine levels ± SEM (picomoles per milligram of wet tissue) in C57BL/6 male retired breeder mice in the following treatment groups: control ($n = 4$), no drug treatment; MPTP ($n = 10$), peanut oil sc 25 min prior to MPTP·HCl in sterile saline ip at 35 mg/kg; TMN + MPTP ($n = 7$), TMN in peanut oil at 400 mg/kg sc 25 min prior to MPTP·HCl in sterile saline ip at 35 mg/kg.

it rapidly declines (*44*). Consequently, MPTP was administered 25 min after a sc 400 mg/kg dose of TMN. Since the K_m value for the MAO-B-catalyzed oxidation of MPTP is 40 μM in mouse brain mitochondrial preparations (*44*), we anticipated that the bioactivation reaction would be significantly inhibited by the TMN concentration of 40 μM achieved under these conditions. Similar brain concentrations of 7-NI (20 μmol/kg), which has a K_I value of 4 μM for this reaction, were effective in inhibiting the MAO-B-catalyzed oxidation of MPTP·HCl (0.24 mmol/kg, equivalent to 50 mg/kg).

Three groups of mice were used for the neuroprotection experiments. Control mice ($n = 4$) received no drug treatment; MPTP mice ($n = 10$) received peanut oil sc 25 min prior to MPTP·HCl administration (ip 0.17 mmol/kg = 35 mg/kg in sterile saline), and TMN mice ($n = 7$) received TMN (sc 400 mg/kg in peanut oil) followed 25 min later by MPTP (ip 0.17 mmol/kg in sterile saline). The animals were sacrificed 10 days later; the striata were isolated, and the concentrations of DA were determined using HPLC with electrochemical detection (*38*, *39*). The control mice had neostriatal DA levels of 138.54 ± 3.26 (SEM) pmol/mg of wet tissue (100.0%) (see Figure 2). The corresponding DA levels in mice that received only MPTP were 57.01 ± 8.67 pmol/mg of wet tissue or ∼41.1% of the control levels. The DA levels in the animals treated with TMN and MPTP were 82.96 ± 6.83 pmol/mg of wet tissue or 59.9% of control levels. These striatal DA concentrations in both groups treated with MPTP are significantly different from those in control animals ($p < 0.0001$ for control vs MPTP only, $p < 0.0003$ for control vs TMN and MPTP, Student's t test, Instat for Graphics 4). However, the mice treated with TMN and MPTP

exhibited an increase in DA content, compared to animals treated with only MPTP, of ~50%, and a comparison of these values demonstrates significant difference ($p < 0.05$). These results are quite similar to those obtained when 7-NI was administered prior to MPTP. In those earlier studies, 7-NI pretreatment (sc 50 mg/kg in peanut oil) 25 min prior to MPTP·HCl administration (0.194 mmol/kg = 40 mg/kg) also led to an increase of ~50% in the striatal DA concentrations compared to mice treated with MPTP only. It should also be mentioned that the mice pretreated with TMN appeared to be sluggish prior to treatment with MPTP, while the mice pretreated with vehicle (peanut oil) did not exhibit this behavior. Both groups displayed the early onset characteristics of MPTP treatment, i.e., shakiness, Straub tail, and some difficulty in walking. These symptoms dissipate rapidly after ~10 min.

These studies establish that, under the experimental conditions that were utilized, the tobacco constituent TMN, which we have previously shown to be a reversible inhibitor of human MAO-A and MAO-B, is protective in the C57BL/6 mouse against the neurotoxicity of MPTP. The poor and variable partitioning characteristics of TMN when administered sc in peanut oil required a very high dose of the drug to achieve the brain concentrations observed in these studies. What brain levels might be achieved in smokers, who will be self-dosing with TMN on a continuous basis, is hard to predict. Nevertheless, it may be reasonable to speculate that the well-documented lowered activity of MAO in the brain of smokers may be in part mediated by TMN. Also noteworthy is recent evidence from our laboratory that more than one inhibitor of MAO may be present in the tobacco plant, since significant MAO-B inhibiting activity has been observed in tobacco leaf extracts with widely different chromatographic polarities compared to TMN, and we have evidence that both reversible and irreversible inhibitors of MAO-A and MAO-B are present in tobacco smoke hexane extracts.

In conclusion, although no causative links between attenuated brain MAO activity levels in human smokers and the lowered incidence of PD have been established, these findings, together with results of studies on tobacco smoke exposure itself, provide evidence that constituents of tobacco can provide neuroprotection in a well-characterized PD animal model of nigrostriatal degeneration. These results lead to provocative questions regarding the clearly lowered incidence of PD in human smokers and a possible relationship to components in the tobacco leaf and tobacco smoke which may include reversible and irreversible monoamine oxidase inhibitors.

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