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Structure-Activity Study of the Mechanism of 1-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine (MPTP)-Induced Neurotoxicity. II. Evaluation of the Biological Activity of the Pyridinium Metabolites Formed from the Monoamine Oxidase-Catalyzed Oxidation of MPTP Analogs[1]

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ABSTRACT

In the accompanying paper, several tetrahydropyridine analogs of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) were screened for their abilities to be oxidized by monoamine oxidase (MAO) to pyridiniums and to produce neurotoxicity in mice. We reported that most of the analogs were oxidized by MAO to pyridiniums and some of the analogs were neurotoxic. We concluded that the capacity of a tetrahydropyridine MPTP analog to be oxidized by MAO to a pyridinium was a necessary, but not sufficient, condition for the compound to be a neurotoxin. In the present paper we attempt to explain further the neurotoxicity or lack of neurotoxicity of these analogs by evaluating the abilities of the pyridinium compounds to serve as substrates for the neostriatal dopamine (DA) transport system and as inhibitors of mitochondrial respiration. We now report that all of the neurotoxic MPTP analogs are oxidized to pyridiniums that are good substrates for the neostriatal DA carrier and good inhibitors of mitochondrial respiration. The results are consistent with an important role for both uptake of the pyridiniums by the DA carrier and inhibition by the pyridiniums of mitochondrial respiration in the neurotoxicity induced by MPTP and its analogs.

The systemic administration of MPTP causes a Parkinsonian syndrome in humans (Langston *et al.*, 1983) and destroys nigrostriatal dopaminergic neurons in monkeys (Burns *et al.*, 1983; Langston *et al.*, 1984a) and mice (Heikkila *et al.*, 1984a; Hallman *et al.*, 1985; Sundstrom *et al.*, 1987; Gupta *et al.*, 1984). MPTP is oxidized to the $MPDP^+$ species by MAO-B (Chiba *et al.*, 1984; Castagnoli *et al.*, 1985), and $MPDP^+$ is further oxidized to the MPP^+ species. This latter species is the major metabolite of MPTP found in the brains of experimental animals including monkeys and mice. MPTP-induced neurotoxicity is prevented and MPP^+ formation is decreased substantially in experimental animals by inhibitors of MAO-B, suggesting that the enzyme-catalyzed oxidation of MPTP is a necessary step in the neurotoxic process (Heikkila *et al.*, 1984b; Langston *et al.*, 1984b; Markey *et al.*, 1984). MPP^+ is actively accumulated *via* the DA transport system into the dopaminergic neuron and this may explain, in part, the relative selectivity of MPTP for the dopaminergic nigrostriatal tract (Javitch *et al.*, 1985). MPTP-induced neurotoxicity can be prevented in experimental animals by pretreating them with inhibitors of DA uptake (Javitch *et al.*, 1985; Mayer *et al.*, 1986). This suggests that the active uptake of MPP^+ by dopaminergic neurons is a necessary step in the neurotoxic process. MPP^+ is also accumulated into mitochondria (Ramsay *et al.*, 1986), where it inhibits the oxidation of NADH-linked substrates by Complex

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ABBREVIATIONS: MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; $MPDP^+$, 1-methyl-4-phenyl-2,3-dihydropyridinium; MAO, monoamine oxidase; MPP^+, 1-methyl-4-phenylpyridinium; DA, dopamine; DOPAC, dihydroxyphenylacetic acid; HVA, homovanillic acid; MOPS, 3-(N-morpholino)propanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N′-tetraacetic acid; 2′Me-MPP^+, 1-methyl-4-(2′-methylphenyl)pyridinium; 2′Cl-MPP^+, 1-methyl-4-(2′-chlorophenyl)pyridinium; 3′Br-MPP^+, 1-methyl-4-(3′-bromophenyl)pyridinium; 2′6diMe-MPP^+, 1-methyl-4-(2′6′-dimethylphenyl)pyridinium; M4tBuP^+, 1-methyl-4-(tert-butyl)pyridinium; 2′Cl-MPTP, 1-methyl-4-(2′-chlorophenyl)-1,2,3,6-tetrahydropyridine; 3′OMe-MPTP, 1-methyl-4-(3′-methoxyphenyl)-1,2,3,6-tetrahydropyridine; 2′Et-MPTP, 1-methyl-4-(2′-ethylphenyl)-1,2,3,6-tetrahydropyridine; MCP^+; 2′ET-MPP^+, 1-methyl-4-(2′-ethylphenyl)pyridinium; 4′F-MPP^+, 1-methyl-4-(4-fluorophenyl)pyridinium; 2′OMe-MPP^+, 1-methyl-4-(2′-methoxyphenyl)pyridinium; 4′Me-MPP^+, 1-methyl-4-(4′-methylphenyl)pyridinium; 2′Me-MPTP, 1-methyl-4-(2′-methylphenyl)-1,2,3,6-tetrahydropyridine; M4BzTP, 1-methyl-4-benzyl-1,2,3,6-tetrahydropyridine; 2′6′diMeMPTP, 1-methyl-4-(2′6′-dimethylphenyl)-1,2,3,6-tetrahydropyridine; M4tBuTP, 1-methyl-4-(tert-butyl)-1,2,3,6-tetrahydropyridine; 4′Me-MPTP, 1-methyl-4-(4′-methylphenyl)-1,2,3,6-tetrahydropyridine; EPTP, 1-ethyl-4-phenyl-1,2,3,6-tetrahydropyridine; 4′F-MPTP, 1-methyl-4-(4′-fluorophenyl)-1,2,3,6-tetrahydropyridine.

I (Nicklas *et al.*, 1985; Vyas *et al.*, 1986; Ramsay *et al.*, 1986. It has been suggested that the decrement in ATP levels within the DA neurons resulting from the inhibition of mitochondrial respiration by MPP^+ is responsible for the death of the DA neurons (Nicklas *et al.*, 1985; Vyas *et al.*, 1986; Ramsay *et al.*, 1986).

In this and the accompanying paper, a structure-activity approach has been used in an attempt to evaluate whether or not the following three steps are important features in the mechanism of the neurotoxicity induced by MPTP and its analogs: 1) MAO, either the A-form, the B-form or both, catalyzes the oxidation of the tetrahydropyridines to dihydropyridinium intermediates which then form pyridinium species; 2) the pyridinium species is actively accumulated into dopaminergic neurons by the DA transport system; and 3) the pyridinium inhibits mitochondrial respiration within the dopaminergic neuron, thereby killing the cells.

We have synthesized several analogs of MPTP and in the accompanying paper describe both the neurotoxic potentials and capacities of the analogs to be oxidized by MAO-A and/or MAO-B. All of the neurotoxic MPTP analogs were oxidized by MAO and formed pyridiniums whereas several non-neurotoxic analogs were not substrates for MAO and one was oxidized by MAO to a product other than the expected pyridinium compound. However, several non-neurotoxic MPTP analogs were also oxidized by MAO and formed pyridinium compounds. It seems that the capacity of an MPTP analog to be metabolized by MAO and the formation of a pyridinium species is a necessary, but not sufficient, condition for the analog to be neurotoxic. In the present paper we have attempted to explain the neurotoxicity, or lack of neurotoxicity, of the MPTP analogs which were metabolized by MAO to pyridiniums by evaluating the abilities of the pyridiniums to be transported by the DA carrier and to inhibit mitochondrial respiration.

Materials and Methods

Materials. Swiss-Webster (CF-W) mice were obtained from Charles River Laboratories (Wilmington, MA). MPP^+ iodide was purchased from Research Biochemicals (Natick, MA); DOPAC, HVA, dihydroxybenzylamine hydrobromide and DA hydrochloride were purchased from Sigma Chemical Co. (St. Louis, MO); [^{3}H]DA (29.7 Ci/mmol) was obtained from New England Nuclear (Boston, MA). 5-(4-chlorophenyl)-2,5-dihydro-3H-imidazo [2,1-*a*]isoindol-5-ol (mazindol) was obtained from Dr. William J. Houlihan (Sandoz Pharmaceuticals, E. Hanover, NJ). 1-{2-{bis(4-fluorophenyl) methoxy}ethyl-4-(3-phenyl-2-propenyl)piperazine dimethane sulfonate} (GBR 13069) was obtained from Dr. W. Hespe (Gist-Brocades, Haarlem, The Netherlands). All other MPTP and MPP^+ analogs were synthesized in our laboratories and their structures were confirmed variously by nuclear magnetic resonance spectra, mass spectra and elemental analyses, the details of which have been published elsewhere (Youngster, 1988).

Statistical analysis. All results were analyzed by the Student's two-tailed *t* test for unpaired samples. A probability value of .05 or less was considered significant.

Drug treatment. Details for each of the experiments are described in the table legends. All of the drugs were administered i.p. at the indicated doses.

Measurement of DA and its metabolites. The method used has been described previously (Sonsalla *et al.*, 1987). Briefly, the mice were sacrificed, the neostriata were dissected and levels of DA, DOPAC and HVA were determined using high-performance liquid chromatography with electrochemical detection.

Release of [^{3}H]DA from neostriatal synaptosomal preparations. The methods used for the preparation of mouse neostriatal synaptosomes and the uptake of [^{3}H]DA and measurement of release have been described previously (Sonsalla *et al.*, 1987). Briefly, the neostriata from mice were dissected, homogenized in 0.32 M sucrose and centrifuged at 700 × *g* for 10 min at 5°C. The supernatant was then centrifuged at 27,000 × *g* for 30 min and the resulting pellet was resuspended in a Krebs-Ringer phosphate buffer [composed of (millimolar): sodium chloride, 118; sodium phosphate, 15.8; potassium chloride, 4.7; calcium chloride, 1.8; magnesium chloride, 1.2; glucose, 5.6; EDTA, 1.3; ascorbic acid, 1.7; and pargyline, 0.08, pH 7.4] to a tissue concentration of 5 mg of original wet wt. tissue per ml. The suspension was incubated in a shaking water bath at 37°C for 5 min and then [^{3}H]DA was added to a concentration of 20 nM. After a 10-min uptake, the suspension was centrifuged for 30 min at 27,000 × *g*. The pellet was rinsed twice with ice-cold buffer and then resuspended in buffer to 5 mg of original wet wt. tissue per ml. One milliliter of this suspension was added to 9 ml of buffer and the samples were incubated at 37°C for 5 min. The compounds to be tested for releasing ability were then added and the incubation was continued for an additional 10 min. Mazindol (10 μM) was added before the addition of the releasing agents in some samples. The samples were then poured into centrifuge tubes, kept on ice and centrifuged for 30 min at 27,000 × *g*. The resulting pellets were washed twice with 5 ml of ice-cold saline and 3 ml of ethanol was added to quantitatively extract the radioactivity. The samples were vortexed and, after 15 min, 1 ml of the extract was added to 9.5 ml of scintillation fluid and the radioactivity was measured. The amount of release was determined by subtracting the radioactivity remaining in the tissue of those samples to which the compounds had been added from those samples to which no compounds had been added. The data were calculated as a percentage of control release.

Preparation of mitochondria. Brain mitochondria were prepared from mice by the Ficoll-gradient procedure of Clark and Nicklas (1970). In a typical experiment, the brains of 20 mice were removed and rinsed in ice-cold isolation medium consisting of (molar): mannitol, 0.225; sucrose, 0.075; MOPS, .005; and EGTA, .0001 (pH 7.4). The tissue was chopped finely with scissors, washed with isolation medium and then homogenized manually with a Dounce homogenizer to make approximately a 10% homogenate (approximately 8 g of tissue in 80 ml of isolation medium). The homogenate was centrifuged at 5°C for 3 min at 2000 × *g* and the supernatant from this spin was then centrifuged for 8 min at 12,500 × *g*. The pellet was resuspended in a 3% Ficoll medium (3% Ficoll, 0.12 M mannitol, 0.03 M sucrose, 0.025 mM EGTA and 1.25 mM MOPS, pH 7.4) to a final volume of 20 ml. This suspension was divided in half and each aliquot was layered onto 20 ml of a 6% Ficoll medium (6% Ficoll, 0.25 M mannitol, 0.06 M sucrose, 0.05 mM EGTA and 2.5 mM MOPS, pH 7.4) in two separate centrifuge tubes. The suspensions were centrifuged for 30 min at 11,500 × *g* and the pellets were washed with isolation medium. The pellets were then combined and resuspended in 40 ml of isolation medium and centrifuged for 10 min at 12,500 × *g*. The mitochondrial pellet was then resuspended in approximately 1 ml of isolation medium to a concentration of about 10 mg of protein per ml. Protein was measured by the method of Lowry *et al.* (1951).

Mitochondrial incubation conditions. The method used has been described previously (Vyas *et al.*, 1986). An aliquot of the mitochondrial preparation was incubated in the following medium: 0.095 M KCl, 0.075 M mannitol, 0.025 M sucrose, 0.005 M potassium phosphate, 1 mM EGTA and 1.05 mM Tris, pH 7.4. Oxygen uptake was measured polarographically using a Clark-type electrode (Yellow Springs Instruments, Yellow Springs, OH) modified to fit a 1.5-ml water-jacketed incubation chamber. All incubations were done at 24°C. In the usual preparation, 0.2 to 0.25 mg of mitochondrial protein was incubated in 1.0 ml of incubation medium. The respiratory control ratio (the ratio of state 3 oxygen uptake to state 4 oxygen uptake) was greater than six for each preparation tested using malate/pyruvate as substrate.

Results

The abilities of the pyridiniums to cause the release of previously accumulated [^{3}H]DA from neostriatal synaptosomes. The pyridinium compounds were tested for their

capacities to be transported by the DA carrier into mouse neostriatal synaptosomes by a [^{3}H]DA release assay. The assay involves measuring the abilities of the compounds to cause release of preaccumulated [^{3}H]DA from synaptosomes, with release mediated by the DA carrier being defined as release which could be blocked in synaptosomes coincubated with the DA uptake blocker mazindol (10 μM). The carrier-mediated release caused by the compounds is a reflection of the capacity of the compounds to be transported into the DA neurons by the DA carrier. The EC_{50} values, which are the concentrations required to cause the carrier-mediated release of 50% of the preaccumulated [^{3}H]DA, for each of the pyridinium compounds are shown in table 1. In other experiments (Sonsalla *et al.*, 1987) we found that the K_m values for the uptake of [^{3}H]MPP$^+$ and [^{3}H]2′Me-MPP$^+$ were very similar to the EC_{50} values for the release of [^{3}H]DA caused by the same compounds in nonradioactive form. The EC_{50} value for MPP$^+$ is 0.83 μM (relative releasing ability = 100); 2′Cl-MPP$^+$ (112) and 3′Br-MPP$^+$ (108) were similar in potency. Only one compound, 2′Me-MPP$^+$ (156), was a significantly better releasing agent than was MPP$^+$. The remainder of the compounds had significantly lower releasing abilities than MPP$^+$ (relative releasing abilities of less than 100). The compounds 2′6′diMe-MPP$^+$ and M4tBuP$^+$ were very weak; at 25 μM, each compound caused the mazindol-inhibitable release of less than 20% of the previously accumulated [^{3}H]DA.

Effects of pretreatment with DA uptake inhibitors on the neurotoxicity induced by the administration of MPTP and its analogs. Originally, we attempted to block the neurotoxicity induced by the MPTP analogs in tables 2 and 3 with mazindol; however, all of the mice given the combination of mazindol and the MPTP analog died. We then tried the highly potent and selective DA uptake inhibitor GBR 13069 (Heikkila and Manzino, 1984) and found that mortality was not a problem. GBR 13069 pretreatment fully protected against the 1-methyl-4-(3′-fluorophenyl)-1,2,3,6-tetrahydropyridine (table 2) and 2′Cl-MPTP-induced (table 3) depletion of neostriatal DA (53 and 64%, respectively), DOPAC (35 and 53%, respectively) and HVA (27 and 43%, respectively). GBR 13069 pretreatment attenuated the 1-methyl-4-(3′-bromophenyl)-1,2,3,6-tetrahydropyridine- and 1-methyl-4-(3′-chlorophenyl)-1,2,3,6-tetrahydropyridine-induced depletion of DA from 47 to 19% and 40 to 14%, respectively, and completely prevented the depletion of DOPAC, which was 35% in each case, and HVA, which was 27% in each case (table 2). Pretreatment with GBR

TABLE 1

Comparative potencies of pyridiniums in causing release of previously accumulated [^{3}H]DA from mouse neostriatal synaptosomes

Data represent the EC_{50} ± S.D. values for three to five separate experiments for each compound. EC_{50} values are the concentrations of the pyridinium compounds that cause the release of 50% of the previously accumulated [^{3}H]DA from a mouse (male Swiss-Webster CF-W) neostriatal synaptosomal preparation. The EC_{50} values were obtained by linear regression of a plot of the log of the concentration *vs.* the percentage of release (based on control) using three to five concentrations of pyridiniums which produced between 20 and 80% release. The release caused by each compound was blocked by 10 μM mazindol. The EC_{50} for release of [^{3}H]DA by DA itself was 0.15 ± 0.02 μM. 3′Me-MPP$^+$, 1-methyl-4-(3′-methylphenyl)pyridinium; 3′Cl-MPP$^+$, 1-methyl-4-(3′-chlorophenyl)pyridinium; 3′F-MPP$^+$, 1-methyl-4-(3′-fluorophenyl)pyridinium; 3′-OME-MPP$^+$, 1-methyl-4-(3′-methoxyphenyl)pyridinium; EPP$^+$, 1-ethyl-4-phenylpyridinium.

Compound	EC_{50}	Relative [^{3}H]DA Releasing Ability
	μM	
MPP$^+$	0.83 ± 0.03	100
2′Me-MPP$^+$	0.53 ± 0.05*	156
2′Cl-MPP$^+$	0.74 ± 0.13	112
2′OMe-MPP$^+$	8.53 ± 0.76*	10
2′Et-MPP$^+$	0.97 ± 0.08	86
2′6′diMe-MPP$^+$	>25*	<3
3′Me-MPP$^+$	2.24 ± 0.33*	37
3′Cl-MPP$^+$	1.39 ± 0.16*	59
3′F-MPP$^+$	1.76 ± 0.28*	47
3′OMe-MPP$^+$	1.48 ± 0.21*	56
3′Br-MPP$^+$	0.76 ± 0.11*	108
4′F-MPP$^+$	8.93 ± 1.12*	9
4′Me-MPP$^+$	9.31 ± 1.20*	9
EPP$^+$	4.99 ± 0.04*	17
M4tBuP$^+$	>25*	<3

* Significantly different from MPP$^+$.

TABLE 2

Effects of pretreatment with the DA uptake inhibitor GBR-13069 on the *in vivo* neurotoxicity induced by various MPTP analogs

Male Swiss-Webster (CF-W) mice were injected i.p. 4 times at 2-hr intervals with the analogs at the following doses in micromoles per kilogram/injection: 1-methyl-4-(3′-fluorophenyl)-1,2,3,6-tetrahydropyridine (3′F-MPTP), 226; 1-methyl-4-(3′-bromophenyl)-1,2,3,6-tetrahydropyridine (3′Br-MPTP), 150; and 3′OMe-MPTP, 140. 2′Et-MPTP was injected (100 μmol/kg i.p.) 2 times with a 2-hr interval between injections. GBR 13069 (GBR) was injected (3 mg/kg i.p.) 30 min before the first and third injections of the neurotoxins (only before the first dose in the case of 2′Et-MPTP). Mice were sacrificed 7 days later and levels of DA, DOPAC and HVA were determined. Data are the mean values ± S.D. Numbers in parentheses in the left column indicate the number of mice in each group, and in the other columns indicate percentage of decrements from appropriate controls for statistically significant effects. 3′Cl-MPTP, 1-methyl-4-(3′-chlorophenyl)-1,2,3,6-tetrahydropyridine.

Pretreatment	Treatment	Neostriatal Levels		
		DA	DOPAC	HVA
		μg/g tissue		
None (4)	None	12.9 ± 1.0	1.7 ± 0.3	1.1 ± 0.2
GBR (4)	None	13.4 ± 0.7	1.3 ± 0.1	1.4 ± 0.1
None (6)	3′F-MPTP	6.1 ± 2.6 (−53%)	1.1 ± 0.2 (−35%)	0.8 ± 0.1 (−27%)
GBR (4)	3′F-MPTP	13.2 ± 0.5	1.4 ± 0.1	1.2 ± 0.1
None (4)	3′Br-MPTP	6.9 ± 0.4 (−47%)	1.1 ± 0.2 (−35%)	0.8 ± 0.1 (−27%)
GBR (4)	3′Br-MPTP	10.9 ± 1.6 (−19%) (−19%)	1.4 ± 0.1	0.9 ± 0.1
None (7)	3′Cl-MPTP	6.6 ± 2.0 (−49%)	1.1 ± 0.2 (−35%)	0.8 ± 0.1 (−27%)
GBR (4)	3′Cl-MPTP	11.5 ± 0.8 (−14%)	1.4 ± 0.2	1.0 ± 0.1
None (6)	3′OMe-MPTP	7.9 ± 2.2 (−39%)	1.1 ± 0.1 (−35%)	0.9 ± 0.1
GBR (4)	3′OMe-MPTP	12.6 ± 0.5	1.4 ± 0.1	1.1 ± 0.1
None (6)	2′Et-MPTP	7.8 ± 1.4 (−40%)	1.3 ± 0.2 (−24%)	0.8 ± 0.1 (−27%)
GBR (4)	2′Et-MPTP	12.0 ± 0.5 (−10%)	1.4 ± 0.2	1.4 ± 0.2

TABLE 3

Effects of pretreatment with GBR 13069 on the *in vivo* neurotoxicity induced by 2′Cl-MPTP

Male Swiss-Webster (CF-W) mice were injected with 2′Cl-MPTP (113 μmol/kg i.p.) twice with a 2-hr interval between injections. GBR 13069 (GBR) was injected (3 mg/kg i.p.) 30 min before the first injection of 2′Cl-MPTP. Mice were sacrificed 6 days later and neostriatal levels of DA, DOPAC and HVA were determined. Data are the mean values ± S.D. Numbers in parentheses in the left column indicate the number of mice per group and in the other columns indicate percentage of decrements from the appropriate control values for statistically significant effects.

Pretreatment	Treatment	Neostriatal Levels		
		DA	DOPAC	HVA
		μg/g tissue		
None (5)	None	15.1 ± 1.1	1.9 ± 0.2	1.4 ± 0.2
GBR (4)	None	13.0 ± 0.9	1.4 ± 0.2	1.1 ± 0.1
None (7)	2′Cl-MPTP	5.4 ± 1.4 (−64%)	0.9 ± 0.3 (−53%)	0.8 ± 0.2 (−43%)
GBR (6)	2′Cl-MPTP	11.7 ± 0.9	1.4 ± 0.3	1.1 ± 0.2

13069 prevented the 3'OMe-MPTP-induced depletion of DA (39%) and DOPAC (35%) and attenuated the 2'Et-MPTP-induced depletion of DA (from 40 to 10%) while completely preventing the depletion in DOPAC (24%) and HVA (27%) induced by 2'Et-MPTP (table 2).

Inhibition of mitochondrial respiration by pyridiniums. The relative potencies of the pyridinium compounds as inhibitors of respiration in mouse brain mitochondria are presented in table 4. In order to take into account both the time- and concentration-dependence of the inhibition of oxidation of malate/pyruvate by intact mouse brain mitochondria, we determined apparent second-order rate constants (Kitz and Wilson, 1962). First-order rate constants were determined from slopes obtained by linear regression of plots of log (percentage of remaining activity) *vs.* time of incubation at three concentrations of the pyridinium compounds. Double reciprocal plots of the first-order rate constants *vs.* pyridinium concentrations gave straight lines for each compound, which is consistent with a bimolecular mechanism. The reciprocals of the slopes (obtained by linear regression) of these lines were equivalent to the apparent second-order rate constants shown in table 4. The rate constant for inhibition of respiration by MPP^+ was found to be 824 M^{-1} min^{-1} (relative inhibitory potency = 100). 2'Me-MPP^+ (79), 1-methyl-4-(3-fluorophenyl)pyridinium (71), 1-methyl-4-(3'-methoxyphenyl)pyridinium (115), 3'Br-MPP^+ (86) and 1-ethyl-4-phenylpyridinium (73) had rate constants which were not significantly different from that of MPP^+. 2'Cl-MPP^+ (37), 2'Et-MPP^+ (49), 2'6'diMe-MPP^+ (23), 1-methyl-4-(3'-chlorophenyl)pyridinium (60), 4'F-MPP^+ (25) and M4tBuP^+ (4) had rate constants significantly less than that of MPP^+. Only three compounds, 2'OMe-MPP^+ (233), 1-methyl-4-(3'-methylphenyl)pyridinium (137) and 4'Me-MPP^+ (378), were significantly more potent inhibitors of mitochondrial respiration than was MPP^+.

TABLE 4

Comparative potencies of pyridiniums in inhibiting mitochondrial respiration

Compounds were incubated with mouse (male Swiss-Webster CF-W) brain mitochondria at three concentrations (100–2000 μM) for three periods of time (1.5–9 min) (see "Materials and Methods"). The concentrations and incubation times were chosen for each compound so as to give a range of inhibition from 25 to 75%. Respiration in the presence of pyruvate/malate and ADP was then measured. Apparent second-order rate constants were calculated as described by Kitz and Wilson (1962). 3'Me-MPP^+, 1-methyl-4-(3'-methylphenyl)pyridinium; 3'Cl-MPP^+, 1-methyl-4-(3'-chlorophenyl)pyridinium; 3'F-MPP;sp, 1-methyl-4-(3'-fluorophenyl) pyridinium; 3'-OMe-MPP^+, 1-methyl-4-(3'-methoxyphenyl)pyridinium; EPP^+, 1-ethyl-4-phenylpyridinium.

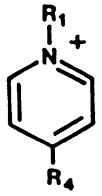
Compound	K	Relative Inhibitory Rate
	$M \times min^{-1}$	
MPP^+	824 ± 121	100
2'Me-MPP^+	651 ± 181	79
2'Cl-MPP^+	301 ± 9*	37
2'OMe-MPP^+	1916 ± 487*	233
2'Et-MPP^+	402 ± 38*	49
2'6'diMe-MPP^+	188 ± 64*	23
3'Me-MPP^+	1131 ± 200*	137
3'Cl-MPP^+	497 ± 45*	60
3'F-MPP^+	589 ± 252*	71
3'OMe-MPP^+	947 ± 209	115
3'Br-MPP^+	711 ± 381	86
4'F-MPP^+	209 ± 61*	25
4'Me-MPP^+	3117 ± 1378*	378
EPP^+	603 ± 188	73
M4tBuP^+	34 ± 15*	4

* Significantly different from MPP^+.

Discussion

MPP^+ has been shown to be a good substrate for the DA transport system *in vitro* (Javitch *et al.*, 1985; Chiba *et al.*, 1985; Mayer *et al.*, 1986; Shen *et al.*, 1986). In contrast, both $MPDP^+$ and MPTP are poor substrates. The active accumulation of MPP^+ by dopaminergic neurons may explain, in part, the relative selectivity of MPTP-induced neurotoxicity for nigrostriatal dopaminergic neurons. The observation that mice can be protected from the neurotoxic effects of MPTP by pretreatment with DA uptake inhibitors, such as mazindol, supports the important role of the uptake process in the *in vivo* neurotoxicity induced by MPTP (Javitch *et al.*, 1985; Mayer *et al.*, 1986). In view of these findings, we tested the capacities of the various pyridinium analogs shown in figure 1 to be transported by the DA carrier. The release of previously accumulated [^{3}H] DA caused by the compounds was considered to be due to the carrier-mediated uptake of the compounds, inasmuch as the release was blocked by mazindol, which presumably does so by blocking the active uptake of the compounds. Therefore, the releasing abilities of the compounds are taken as indications of the abilities of the compounds to be transported into the DA neurons *via* the DA carrier. Ideally, uptake experiments would have been done directly with each compound, but this would have necessitated that each compound be radioactively labeled. Where we have studied both the carrier-mediated DA release and direct uptake of ^{3}H-labeled pyridiniums, the EC_{50} values obtained for each compound have been very close to their respective K_m values for uptake (Sonsalla *et al.*, 1987).

The data in the present study confirm that MPP^+ is a good substrate for the DA carrier, although not quite as good as DA itself (see legend of table 1). The 2'-substituted analogs, with the exception of 2'OMe-MPP^+, are comparable to MPP^+ as



Abbreviation	R_1	R_4
MPP^+	methyl	phenyl
2'Me-MPP^+	methyl	2'methylphenyl
2'OMe-MPP^+	methyl	2'methoxyphenyl
2'Cl-MPP^+	methyl	2'chlorophenyl
2'Et-MPP^+	methyl	2'ethylphenyl
2'6'diMe-MPP^+	methyl	2'6'dimethylphenyl
3'Me-MPP^+	methyl	3'methylphenyl
3'OMe-MPP^+	methyl	3'methoxyphenyl
3'F-MPP^+	methyl	3'fluorophenyl
3'Br-MPP^+	methyl	3'bromophenyl
3'Cl-MPP^+	methyl	3'chlorophenyl
4'Me-MPP^+	methyl	4'methylphenyl
4'F-MPP^+	methyl	4'fluorophenyl
M4tBuP^+	methyl	tert-butyl
M4BzP$^+$	methyl	benzyl
EPP^+	ethyl	phenyl

Fig. 1. Structure of the pyridinium nucleus and the MPP^+ analogs used in these studies. 3'Me-MPP^+, 1-methyl-4-(3'-methylphenyl)pyridinium; 3'OMe-MPP^+, 1-methyl-4-(3'-methoxyphenyl)pyridinium; 3'F-MPP^+, 1-methyl-4-(3'-fluorophenyl)pyridinium; 3'Cl-MPP^+, 1-methyl-4-(3'-chlorophenyl)pyridinium; M4BzP$^+$, 1-methyl-4-benzylpyridinium; MCP$^+$; EPP^+, 1-ethyl-4-phenylpyridinium.

substrates for the DA carrier. Perhaps the strong resonance electron-donating effect of the methoxy substituent is responsible for the diminished activity of 2′OMe-MPP+. It is interesting and perhaps surprising that 2′6′diMe-MPP+ is not transported by the DA carrier at all since 2′Me-MPP+ is such a good substrate. The 3′-substituted compounds are transported similarly to MPP+ whereas both 4′-substituted analogs are substantially weaker substrates than MPP+. In other experiments (Youngster *et al.*, 1987) we have shown that MCP+, which has a cyclohexyl group in place of the phenyl group of MPP+, is also transported by the DA carrier, although not nearly as well as MPP+. The results with MCP+ indicate that an aromatic substituent in position 4 of the pyridinium ring is not a requirement for transport. However, the compound with a tert-butyl substituent in this position is not transported effectively. EPP+, which has an ethyl group rather than a methyl group in position 1, is transported, although not nearly as well as MPP+. In other experiments (not shown), we have found that 1-propyl-4-phenylpyridinium ion is not a substrate for the DA carrier. The progressive decrease in activity as the size of the 1-substituent increases suggests a steric effect in this region of the molecule.

In order to assess the importance of the DA carrier in mediating the neurotoxicity of some of the MPTP analogs, we treated the mice with DA uptake blockers before administration of the neurotoxicants. Pretreatment with the uptake inhibitors afforded either full or partial protection against the neurotoxicity induced by administration of all of the compounds that were tested (tables 2 and 3). In other studies we have shown that DA uptake blockers also protect against the neurotoxicity of 1-methyl-4-cyclohexyl-1,2,3,6-tetrahydropyridine (Youngster *et al.*, 1987) and 2′Me-MPTP (Sonsalla *et al.*, 1987). Thus, it appears as if transport of the pyridinium species by the DA carrier plays a critical role in the neurotoxic process of the MPTP analogs, as it does in the case of MPTP-induced neurotoxicity.

MPP+ has been shown to inhibit the ADP-stimulated mitochondrial oxidation of NAD(H)-linked substrates (Complex I) while leaving the oxidation of succinate and ascorbate relatively unaffected (Nicklas *et al.*, 1985; Ramsay *et al.*, 1986; Mizuno *et al.*, 1988)). The inhibition is both time- and concentration-dependent and consists of at least two steps, the active accumulation of MPP+ by the mitochondria and the subsequent inhibition of NADH dehydrogenase (Ramsay *et al.*, 1986). It has been proposed that inhibition of Complex I by MPP+, which would result in a decrease in cellular ATP levels as well as acidosis due to lactate accumulation, might be the mechanism whereby MPTP ultimately kills cells (Nicklas *et al.*, 1985; Vyas *et al.*, 1986; Ramsay *et al.*, 1986). Consistent with this hypothesis is the finding that the stereotaxic injection of the well-known Complex I inhibitor rotenone into the median forebrain bundle of rats results in striatal dopaminergic neurotoxicity similar, but not identical, to that resulting from the similar administration of MPP+ (Heikkila *et al.*, 1985). We studied the effects of the pyridinium compounds structurally related to MPP+ on mitochondrial respiration in order to determine some of the structure-activity relationships for inhibition of Complex I and to see if a correlation existed between Complex I inhibition by the pyridiniums and neurotoxicity of the parent tetrahydropyridines (table 4).

MPP+, being a quarternary compound, would not be expected to readily enter mitochondria by passive diffusion. However, MPP+ inhibits NADH-linked respiration in intact mitochondria at lower concentrations than it inhibits isolated NADH dehydrogenase (Ramsay *et al.*, 1986). This suggests that the energy-dependent accumulation of MPP+ results in a concentration of MPP+ at the NADH dehydrogenase site and effectively leads to inhibition in intact mitochondria. Because of their charge, the pyridinium compounds studied in this paper would not be expected to enter the mitochondria by passive diffusion, and are presumably inhibiting mitochondrial respiration after they have been actively accumulated. Therefore, the structure-activity relationships elucidated in this study are most likely a composite of the capacity of the pyridinium species to be accumulated and their capacities to inhibit NADH dehydrogenase.

The results of the present study show how alteration of the structure of MPP+ affects the abilities of the resulting compounds to inhibit state 3 oxidation of malate/pyruvate by intact mitochondria (table 4). Substituting a tert-butyl group in position 4 results in a compound (M4tBuP+) with drastically lowered activity. Increasing the size of the substituent at the 2′ position from H (MPP+) to methyl (2′Me-MPP+) to ethyl (2′Et-MPP+) results in a progressive decrease in activity of the resulting pyridiniums (table 4). 2′6′diMe-MPP+, with two methyl groups ortho to the pyridinium ring, is about one-fifth as potent as MPP+ whereas 2′OMe-MPP+ is about twice as potent as MPP+. It appears that electronic as well as steric effects are important in determining the activity of the 2′-substituted compounds. Substitutions in the 4′-position give rise to compounds with drastically different activities, 4′F-MPP+ having one-fourth the activity and 4′Me-MPP+ having nearly 4 times the activity of MPP+. In contrast to the 4′-substituted compounds, the 3′-substituted compounds have activities relatively close to that of MPP+ (table 4).

In Table 5 we have summarized the relationships between the dopaminergic neurotoxicity of the MPTP analogs, the MAO activities of the MPTP analogs (both determined in the accompanying paper) and the capacities of the corresponding pyridiniums to be transported by the DA carrier (table 1) and to inhibit mitochondrial respiration (table 4). All of the compounds which were found to be neurotoxic were also found to be substrates for MAO and, furthermore, the MAO-catalyzed oxidation of these compounds resulted in the formation of pyridinium metabolites (see accompanying paper). M4BzTP, which is a good substrate for MAO, did not form the corresponding pyridinium compound as a result of its MAO-catalyzed oxidation and was not neurotoxic. Also, all of the compounds which were not substrates for MAO were not neurotoxic. These results are consistent with the importance of the role of MAO in catalyzing the formation of pyridinium compounds from MPTP and its analogs in the neurotoxic process.

It can also be seen in table 5 that all of the neurotoxic MPTP analogs are metabolized by MAO and form pyridiniums which are substrates for the DA transport system. Also, the nigrostriatal dopaminergic neurotoxicity of all of the compounds tested was either prevented completely or attenuated in mice by pretreatment with inhibitors of DA uptake (tables 2 and 3). These data, taken together, are consistent with the importance of uptake of the pyridinium metabolites by the DA neurons in the neurotoxicity induced by the tetrahydropyridines. The lack of neurotoxic potential of the compounds 2′6′diMeMPTP and M4tBuTP may simply be explained by the lack of capacity of their respective pyridinium metabolites, 2′6′diMeMPP+ and M4tBuP+, to be transported effectively and accumulated within the dopaminergic neuron by the DA transport system.

TABLE 5

Summary

Neurotoxicity was determined in the accompanying paper. Numbers under "MAO Activity" are also from the accompanying paper and represent the MAO activity (both MAO-A and MAO-B together) for each compound relative to MPTP, which was assigned an activity of 100. Numbers under "Transport Capacity" are from table 1 and represent the relative abilities of the corresponding pyridiniums to be transported by the DA carrier. Numbers under "Mitochondrial Inhibition Capacity" are from table 4 and represent the relative abilities of the corresponding pyridiniums to inhibit the mitochondrial oxidation of pyruvate/malate. NF, pyridinium not formed; 2'-OMe-MPTP, 1-methyl-4-(2'-methoxyphenyl)-1,2,3,6-tetrahydropyridine; 3'F-MPTP, 1-methyl-4-(3'-fluorophenyl)-1,2,3,6-tetrahydropyridine; 3'Br-MPTP, 1-methyl-4-(3'-bromophenyl)-1,2,3,6-tetrahydropyridine; 3'Cl-MPTP, 1-methyl-4-(3'-chlorophenyl)-1,2,3,4-tetrahydropyridine; PPTP, 1-*n*-propyl-4-phenyl-1,2,3,6-tetrahydropyridine; M2BzTP, 1-methyl-2-benzyl-1,2,3,6-tetrahydropyridine; M5BzTP, 1-methyl-5-benzyl-1,2,3,6-tetrahydropyridine; M5PTP, 1-methyl-5-phenyl-1,2,3,6-tetrahydropyridine; M2TTP, 1-methyl-2-(4'-methylphenyl)-1,2,3,6-tetrahydropyridine.

Compound	MAO Activity	Transport Capacity	Mitochondrial Inhibition Capacity
Neurotoxic Compounds			
MPTP	100	100	100
2'Me-MPTP	357	156	79
2'-OMe-MPTP	110	10	233
2'Cl-MPTP	98	112	37
2'Et-MPTP	182	86	49
3'Me-MPTP	108	37	137
3'OMe-MPTP	25	56	115
3'F-MPTP	79	47	71
3'Br-MPTP	101	108	86
3'Cl-MPTP	75	59	60
Non-Neurotoxic Compounds			
2'6'-diMe-MPTP	172	0	23
4'Me-MPTP	9	9	378
4'F-MPTP	22	9	25
M4tBuTP	40	0	4
EPTP	9	17	73
PPTP	0	NF	NF
M2BzTP	0	NF	NF
M5BzTP	0	NF	NF
M5PTP	0	NF	NF
N2TTP	0	NF	NF
M4BzTP	219	NF	NF

All of the neurotoxic MPTP analogs are metabolized by MAO to pyridinium compounds which are capable of inhibiting mitochondrial respiration (see table 5). Although this does not constitute proof for the role of this property in mediating the neurotoxicity of MPTP and its analogs, it is consistent with the hypothesis that such mitochondrial inhibition might be important.

Three non-neurotoxic compounds (4'Me-MPTP, EPTP and 4'F-MPTP) were found to be metabolized to pyridiniums which were both substrates for the DA carrier and inhibitors of mitochondrial respiration (see table 5). However, 4'Me-MPTP and EPTP are both relatively poor substrates for MAO and, furthermore, their pyridinium metabolites are relatively poor substrates for the DA carrier. These two factors, taken together, may account for the lack of neurotoxicity, as they would result in a low concentration of the pyridinium ions within the DA neurons, presumably below the threshold concentration required for neurotoxicity. 4'F-MPTP is a somewhat better substrate for MAO, but its pyridinium metabolite is both a relatively poor substrate for the DA carrier and a weak inhibitor of mitochondrial respiration. It is possible that these factors may account for the lack of neurotoxic potential of the parent compound. A comparison of the parameters for the neurotoxic compounds with the non-neurotoxic compounds is helpful in this regard (table 5). The neurotoxic compound with the lowest MAO activity (3'OMe-MPTP) is metabolized to a pyridinium which is about half as good as a substrate for the DA carrier and is equivalent to MPP^+ as an inhibitor of mitochondrial respiration. The non-neurotoxic compound 4'F-MPTP is comparable to 3'OMe-MPTP as a substrate for MAO but the pyridinium metabolite of 4'F-MPTP has a rather low capacity to use the DA transport system and to inhibit mitochondrial respiration. 3'OMe-MPTP is neurotoxic but much less potent than MPTP, possibly due to its relative deficiencies both to be oxidized by MAO and the capacity of its pyridinium to utilize the DA transport system. Apparently, the maximal dose of 3'OMe-MPTP that can be tolerated by the mice is sufficient to produce neurotoxicity whereas the maximally tolerated dose of 4'F-MPTP results in an inadequate intraneuronal concentration of 4'F-MPP^+ for neurotoxicity. The possibility exists that if 4'F-MPTP were administered under conditions such that more reached or stayed in the brain or if it were tested in a more susceptible animal species (possibly the monkey), it could be neurotoxic. The same can be said for 4'Me-MPTP and EPTP.

Other factors which may have relevance include the effects that structural alterations have on the metabolic susceptibility (other than MAO activity of the MPTP analogs) of both the parent compounds and the pyridinium metabolites. Metabolic studies (Kindt *et al.*, 1988; Heikkila *et al.*, 1988) have shown that high brain levels of the pyridinium metabolite persist for a much longer time after the peripheral administration of 2'Me-MPTP than they do following the similar administration of MPTP. 2'Me-MPTP, as well as four other 2'substituted compounds (2'Cl-MPTP and 2'Et-MPTP, see accompanying paper; 1-methyl-4-(2'-fluorophenyl)-1,2,3,6-tetrahydropyridine and 1-methyl-4-(2'-trifluoromethylphenyl)-1,2,3,6-tetrahydropyridine-MPTP Riachi *et al.*, 1988), are more potent neurotoxins than MPTP. Perhaps the 2' substitution reduces the affinities of the pyridinium compounds (relative to MPP^+) for some, as yet unknown, degradative enzyme or elimination system and this is the primary reason that the compounds are more neurotoxic than MPTP. This could also contribute to the neurotoxicity of 1-methyl-4-(2'-methoxyphenyl)-1,2,3,6-tetrahydropyridine which is neurotoxic despite the relatively low capacity of its pyridinium metabolite to be transported by the DA carrier (table 5). Similarly, the possibility exists that the 3'- and 4'-substituted compounds are eliminated or metabolized more rapidly than MPTP (or MPP^+), making them less neurotoxic or non-neurotoxic, respectively. Other factors which may differentially affect the relative neurotoxic potentials of the MPTP analogs include the peripheral metabolism of the compounds, binding of the compounds to plasma amine oxidase (Bhatti *et al.*, 1988) and uptake of the compounds by the catecholamine storage vesicles (Reinhard *et al.*, 1987). Obviously, the neurotoxicity of MPTP and its analogs is a multifaceted process and no single explanation can account for the differential effects of these analogs.

In summary, we feel that the data in this and the accompanying study supports the premise that three steps are essential in the neurotoxic mechanism of MPTP and its analogs: first, MAO-A and/or MAO-B catalyze the oxidation of the tetrahydropyridine to a pyridinium ion; second, the pyridinium is accumulated by DA neurons *via* the DA transport system; third, the pyridinium inhibits mitochondrial respiration within the DA neurons, resulting in their death. The results of the present study are consistent with the importance of these three steps as all of the neurotoxic tetrahydropyridines are capable of being metabolized by MAO to pyridiniums which are substrates for the DA carrier and inhibitors of mitochondrial respiration.

Other indications of the validity of the hypothesis were obtained: compounds which were not substrates for MAO were not neurotoxic, supporting the role of MAO; the compound metabolized by MAO, but not forming a pyridinium metabolite (M4BzTP), was not neurotoxic, indicating the importance of pyridinium metabolite formation; and the compounds (M4tBuTP and 2'6'diMeMPTP) metabolized by MAO to pyridiniums which were not substrates for the DA carrier were not neurotoxic, supporting the role of the DA carrier. Moreover, compounds which were weak in two of the three categories (relative activities less than 25) possessed no dopaminergic neurotoxicity. One strength of the arguments derived from this study lies in the large number of compounds which have been studied. We are currently in the process of applying this same approach to new compounds which we have synthesized as well as to compounds which have been reported on previously by other groups. By doing so, we hope to contribute to establishing the validity of the above hypothetical mechanism for the neurotoxicity induced by MPTP and its analogs.

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