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MPP⁺ Scaffold Containing Lipophilic Compounds are Potent Complex I Inhibitors and Selective Dopaminergic Toxins

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Abstract

Although the exact cause(s) of Parkinson's disease (PD) is not fully understood, it is believed that environmental factors play a major role. The discovery that the synthetic chemical, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-derived N-methyl-4-phenylpyridinium (MPP⁺), recapitulates major pathophysiological characteristics of PD in humans, has provided the strongest support for this possibility. While the mechanism of the selective dopaminergic toxicity of MPP⁺ has been extensively studied and is in most respects well accepted, several key aspects of the mechanism are still debatable. In the present study, we use a series of structurally related, novel, and lipophilic MPP⁺ derivatives [N-(2-phenyl-1-propene)-4-phenyl-pyridinium (PP-PP⁺)] to probe the mechanism of action of MPP⁺ using dopaminergic MN9D and non-neuronal HepG2 cells *in vitro*. Here we show that effective mitochondrial complex I inhibition is necessary and that the specific uptake through DAT is not essential for dopaminergic toxicity of MPP⁺ and related toxins. We also provide strong evidence to support our previous proposal that the selective vulnerability of dopaminergic cells to MPP⁺ and similar toxins is likely due to the high inherent propensity of these cells to produce excessive ROS as a downstream effect of complex I inhibition. Based on the current and previous findings, we propose that MPP⁺ is the simplest of a larger group of unidentified environmental dopaminergic toxins, a possibility that may have major public health implications.

Graphical Abstract

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Keywords

Parkinson's disease (PD); Dopaminergic Toxins; Complex I Inhibitors; Environmental Toxins; Reactive Oxygen Species (ROS); MPP⁺-derivatives

Introduction

Parkinson's disease (PD), the second most common neurodegenerative disorder, is associated with the loss of dopaminergic neurons in the substantia nigra of the midbrain. PD is a chronic and progressive disorder in mid to late ages and is usually diagnosed by characteristic motor impairments and autonomic dysfunctions (1–3). Although the exact causes of PD are not fully understood, environmental factors are believed to contribute to the etiology of PD (4). The accidental discovery that the synthetic chemical, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), recapitulates major pathophysiological characteristics of PD in humans and other mammals has provided the strongest support for this possibility (1, 5). Numerous previous studies have demonstrated that lipophilic MPTP crosses the blood brain barrier, enters the brain, and is metabolized to the oxidized product, 1-methyl-4-phenylpyridinium (MPP⁺) by monoamine oxidase-B in glial cells. It is the oxidized product, MPP⁺, not MPTP, which selectively destroys dopaminergic neurons (1, 5, 6). Thus, MPTP/MPP⁺ has been widely used as a model to examine the mechanisms of specific dopaminergic neuronal death in PD, and to develop therapeutics and preventive strategies for the disease (7, 8).

The currently accepted mechanism for the dopaminergic toxicity of MPP⁺ consists of several key steps including: uptake of extracellular MPP⁺ selectively into dopaminergic neurons through the plasma membrane dopamine transporter (DAT) (9, 10), mitochondrial accumulation of cytosolic MPP⁺, inhibition of complex-I leading to increased reactive oxygen species (ROS) production, intracellular ATP depletion, and perturbation of the cellular Ca²⁺ homeostasis etc., causing apoptotic cell death (6, 11–14). Although many aspects of this mechanism have been widely tested and verified, several key steps of the mechanism including whether (a) DAT-mediated selective uptake is essential for selective dopaminergic toxicity (9, 10); (b) the structure of MPP⁺ is unique and optimal for the toxicity (6, 11–13), (c) the inhibition of mitochondrial complex I is required for the toxicity

(15, 16), remain uncertain. Thus, the mechanism of the selective dopaminergic toxicity of MPTP/MPP⁺ is not a settled issue and its full description is vital in assessing the environmental contributions to the etiology of PD. A comprehensive understanding of the mechanism could also aid in the development of preventive and/or therapeutic strategies to protect the aging population from this disease.

In the present study, a series of novel, structurally related, and lipophilic MPP⁺ derivatives [N-(2-phenyl-1-propene)-4-phenyl-pyridinium (PP-PP⁺) (17, 18)] have been used to test the above described key aspects of this mechanism, using dopaminergic MN9D and non-neuronal HepG2 cells *in vitro*. Here we show that effective mitochondrial complex I inhibition is obligatory and that the specific uptake through DAT is not essential for the selective dopaminergic toxicity of MPP⁺ and related toxins. Our findings strongly support the notion that the high vulnerability of dopaminergic cells to these toxins is likely a consequence of their high inherent propensity to produce excessive ROS in response to effective complex I inhibition and associated downstream effects, in comparison to non-dopaminergic neurons (6, 13). Perhaps the most significant implication of these and our previous findings is the indication that MPP⁺ is the simplest of a larger group of unidentified environmental dopaminergic toxins [for example cationic cyanines (11–13)], a possibility that may have great public health consequences related to the environmental causes of PD.

Results and Discussion

The primary aim of the present study was to investigate several uncertain aspects of the mechanism of selective dopaminergic toxicity of MPP⁺ and related toxins including: (1) is the selective uptake of MPP⁺ into dopaminergic neurons responsible for the selective vulnerability of these cells?, (2) is MPP⁺ structurally unique and optimal for the observed dopaminergic toxicity?, (3) are mitochondrial accumulation and complex I inhibition required for the toxicity?, (4) does MPP⁺-mediated selective ROS production in dopaminergic neurons contribute to their selective vulnerability? Accordingly, a series of structurally related, novel, and lipophilic MPP⁺ derivatives [Scheme; PP-PP⁺; N-(2-phenyl-1-propene)-4-phenyl-pyridinium] have been synthesized (18, 19) and their toxicological characteristics have been studied, compared, and contrasted at the molecular level using dopaminergic MN9D in reference to non-neuronal HepG2 cells *in vitro*.

All PP-PP⁺ derivatives are selectively toxic to dopaminergic MN9D cells similar to MPP⁺.

To evaluate the dopaminergic selectivity and structure activity relationship of the toxicity of PP-PP⁺ derivatives relative to MPP⁺, a series of standard *in vitro* toxicity experiments were carried out with dopaminergic MN9D and SH-SH5Y, and liver HepG2 (control) cells. These experiments show that the parent PP-PP⁺ derivative [1] (Scheme) is toxic to dopaminergic MN9D and SH-SH5Y cells with IC₅₀s of ~23 and ~50 μM (Table 1), respectively and not significantly toxic to HepG2 cells under the same experimental conditions (Fig 1A), parallel to the previously reported behavior of MPP⁺, 4'-I-MPP⁺, and cationic cyanines (11–14). In addition, these studies reveal that lipophilic substituents in the phenyl rings of the parent derivative [1] significantly increase the MN9D toxicity (e.g. [2]-[4]) with IC₅₀s in the range of 4–5 μM (Figs 1B & C and Table 1). Furthermore, mimicking the behavior of [1] and MPP

⁺, none of these substituted derivatives were measurably toxic to HepG2 cells at the same concentrations (Fig 1D), though they did show moderate toxicities to HepG2 cells at higher concentrations (data not shown). The IC₅₀ parameters estimated for the MN9D toxicities of these derivatives were in the order [2] > [3] > [4] > [1] > 4I'MPP⁺ > [5] > [6] > MPP⁺ (Table 1) and appear parallel to their relative lipophilicities [estimated from the relative RP-HPLC retention times (Table 2)], with some notable exceptions e.g. [3] & [4] vs [5] & [6]. In addition, most of these derivatives were significantly more toxic to MN9D cells in comparison to MPP⁺ (~10–20 fold), suggesting that the structure of MPP⁺ is not unique in its selective dopaminergic toxicity, as generally believed (11, 12).

PP-PP⁺ derivatives accumulate in both MN9D and HepG2 cells by simple diffusion with similar efficiencies.

The cellular uptake characteristics of a subset of PP-PP⁺ derivatives were examined to determine whether their marked difference in dopaminergic toxicity was due to differences in cellular uptake efficiency. These studies show that the time course of MN9D cell uptake of the parent PP-PP⁺ derivative, [1], is linear (at 50 μM; up to 1 h; Fig 2A), and the concentration dependence of the uptake was non-saturable up to 100 μM under standard incubation conditions (data not shown). Additionally, the uptake efficiencies of PP-PP⁺ derivatives with differing toxicities i.e. [3] & [4] vs [5] were not significantly different, and were also concentration dependent and non-saturable in the concentration range tested (0–100 μM; for 45 min; Fig 2B). Similarly, the uptake of these derivatives into HepG2 cells was also concentration dependent and non-saturable with a lower efficiency than that of MN9D cells, especially at higher concentrations (Fig 2C). These findings suggest that PP-PP⁺ derivatives accumulate in both MN9D and HepG2 cells through a non-specific, simple diffusion process with similar efficiency, most likely due to their high lipophilicity in comparison to MPP⁺ or 4I'MPP⁺. Thus, the marked differences of MN9D toxicities of [3] & [4] vs [5] cannot simply be explained by their cellular uptake differences. Therefore, a comprehensive comparative toxicological study of these derivatives was carried out to gain further insight into their mechanism of action relative to MPP⁺.

PP-PP⁺ derivatives accumulate in the mitochondria of both MN9D and HepG2 cells.

Our previous studies have shown that dopaminergic toxins, cationic cyanines and 4I'MPP⁺ actively accumulate in the mitochondria of both MN9D and HepG2 cells (11, 12, 14). Thus, the possibility that PP-PP⁺ derivatives also accumulate in the mitochondria of these cells was investigated. Because the PP-PP⁺ derivatives [3], [4], & [5] were intrinsically moderately fluorescent (Fig 3A), a series of dual fluorescence imaging experiments with the mitochondrial dye Mitotracker Red and these toxins were carried out to determine their intracellular localizations in live MN9D and HepG2 cells (12, 14). The results indicate that both [3] and [5] are primarily localized in the mitochondria of both MN9D and HepG2 cells (Figs 3B & C) as was previously reported for cationic cyanines and 4I'MPP⁺ (11, 12, 14). In addition, the relative mitochondrial uptake efficiencies of [3] and [5] were determined using intact mitochondria isolated from MN9D cells as previously described (12, 14) to test whether the toxicity difference between these two derivatives was a consequence of a difference in their mitochondrial uptake efficiencies. The results show that both derivatives accumulate efficiently in intact mitochondria with comparable efficiency (Fig 4). These

observations suggest that the observed difference in toxicity in MN9D cells of [3] & [5] cannot be a consequence of the differences in their intracellular localizations (i.e. preferential mitochondrial accumulation) cellular or mitochondrial uptake efficiency.

[3] Depolarizes the mitochondrial membrane potential in both MN9D and HepG2 cells strongly in comparison to [5].

Because cationic cyanines, MPP^+ , and its derivatives are known to depolarize the mitochondrial membrane potential in live cells, the effects of [3], [4], and [5] on the mitochondrial membrane potential were examined using the fluorescent probe tetramethylrhodamine methyl ester (TMRM) as previously reported (11, 12, 14, 20). As shown in Fig 5A, [3] depolarizes MN9D cell mitochondrial membrane potential drastically, [4] moderately and, [5] very weakly at 500 μM concentrations (1 h of incubation; Fig 5A), a trend that is parallel to the observed MN9D cell toxicity profiles of these derivatives. However, a similar set of experiments, shows that a parallel HepG2 cell mitochondrial membrane potential depolarization is also observable with these derivatives, (Fig 5B) again mimicking the behavior of cationic cyanines and MPP^+ . Qualitative fluorescence imaging experiments with MN9D and HepG2 cells with the above PP-PP⁺ derivatives further confirmed the results of the quantitative experiments (Fig 5C). These findings suggest that the mitochondrial membrane depolarization alone could not be a specific indicator of the dopaminergic toxicities of PP-PP⁺ derivatives.

PP-PP⁺ derivative [3] strongly inhibits rat brain mitochondrial complex I relative to [5].

To explore whether the difference toxicities of PP-PP⁺ derivatives are related to their relative complex I inhibition potencies, a series of complex I inhibition experiments were carried out with a selected group of toxins. Specifically, the effects of PP-PP⁺ derivatives [2], [3], [5], and [4], rotenone, and MPP^+ on the NADH-ubiquinone oxidoreductase activity of complex I were determined using isolated rat brain mitochondrial membranes as described by Diwakar *et al.* (21). Specific complex I inhibition activity was measured observing the decrease in absorbance due to the ubiquinone dependent oxidation of NADH at 340 nm as a function of time, using the specific mitochondrial complex I inhibitor, rotenone, as a positive control (Fig 6). The data shows that rotenone inhibits more than 85% of ubiquinone dependent NADH oxidation activity of complex I at 10 μM compared to control (Fig 6). The relative percent inhibition determined for [2], [3], [4], MPP^+ , and [5] were 84%, 65.6%, 51.6% , 12.5%, and 10% , respectively at 50 μM (or 100 μM for MPP^+). These results suggest that the relative toxicities of PP-PP⁺ derivatives correlate well with their complex I inhibition potencies.

The above results strongly suggest that in addition to efficient cellular and mitochondrial accumulation, effective mitochondrial complex I inhibition is obligatory for the dopaminergic toxicity of MPP^+ and related PP-PP⁺ derivatives. This view is especially supported by the strong correlation of the toxicities of the structurally similar [5] vs [3] & [4] to their relative mitochondrial complex I inhibition potencies. For example, [3] & [4] were 5 to 7 times stronger mitochondrial complex I inhibitors in comparison to [5], and their relative toxicity differences are also in the same order. The MN9D cell toxicities of most of the other PP-PP⁺ and MPP^+ derivatives are also well correlated with their complex I

inhibition potencies. The finding that PP-PP⁺ derivatives are both significantly more potent complex I inhibitors and also dopaminergic toxins in comparison to MPP⁺, is consistent with previous reports showing that lipophilic phenyl ring substituents increase complex I inhibition potency as well as the dopaminergic toxicity of MPP⁺ derivatives (22–24). Thus, these results are in disagreement with several previous reports suggesting that mitochondrial complex I inhibition is not required for the dopaminergic toxicity of MPP⁺ (15, 25). The observation that potent complex I inhibitors are stronger mitochondrial membrane potential depolarizers (with similar mitochondrial uptake characteristics; for example [3] & [5]), further suggests that complex I inhibition could be the primary cause of mitochondrial membrane depolarization mediated by MPP⁺ and related toxins. Although effective mitochondrial accumulation of these cationic toxins could also lead to the depolarization of the negative interior of the mitochondrial membrane potential independent of the complex I inhibition, this cationic effect appears to be modulated either by transient stimulation of complex I activity or by other electrogenic mechanisms, as we have proposed previously for 4I'MPP⁺ (14).

PP-PP⁺ derivatives [3] and [4] increase intracellular ROS levels selectively in MN9D cells, but [5] has little or no effect.

Although the relative toxicities of PP-PP⁺ derivatives are intimately associated with mitochondrial complex I inhibition, they do not explain the selective susceptibility of dopaminergic cells to these toxins. For example, we have previously shown that MPP⁺, 4I'MPP⁺, and various cationic cyanines actively accumulate in the mitochondria, inhibit complex I, and depolarize the mitochondrial membrane in both MN9D and HepG2 cells. However increased ROS production and cell death was observed only in MN9D cells (6, 11–13). To test whether a similar behavior is operative with PP-PP⁺ toxins, the effects of [3], [4], and [5] on intracellular ROS levels of MN9D and HepG2 cells were examined using the ROS sensitive fluorescence probe DCFH-DA. These studies showed a concentration dependent increase of intracellular ROS level (~ 2.5–3.0 fold increase from the baseline levels at 100 μM at 1 h; Fig 7A) upon treatment of MN9D cells with [3] or [4]. On the other hand, the relatively less toxic PP-PP⁺ derivative, [5], did not significantly increase intracellular ROS levels at these concentrations (Fig 7B). Parallel experiments also showed that treatment with [3], [4], or [5] did not increase intracellular ROS levels in HepG2 cells (Figure 7B). These findings were further confirmed by a series of imaging experiments which were carried out with [3] or [5]-treated MN9D cells. In agreement with the quantitative data, these experiments showed increased ROS production only in MN9D cells treated with [3], but not in cells treated with [5] (Fig 7C). These findings support the hypothesis that mitochondrial complex I inhibition associated, selective ROS production in dopaminergic cells may be the origin of their high susceptibility to some PP-PP⁺ derivatives. To further confirm that mitochondria was the origin of the PP-PP⁺-mediated ROS production, dual fluorescence images of DCF and Mitotracker Red were simultaneously recorded for [1] or [3] treated MN9D cells (Fig 8). The co-localization of the DCF and Mitotracker Red signals (Fig 8) suggest that ROS predominantly originates from the mitochondria of the cell, further supporting the hypothesis that mitochondrial toxin induced, selective ROS production may be the origin of the specific dopaminergic toxicity of MPP⁺, PP-PP⁺ and related toxins.

Ascorbate (Asc) reduces ROS production and protects MN9D cells from PP-PP⁺-mediated cell death.

To further confirm that selective ROS production in MN9D cells induced by PP-PP⁺ toxins is the cause of cell death, we sought to reduce the intracellular ROS levels and therefore protect against toxicity using ascorbate (Asc), an antioxidant. Accordingly, a series of experiments was carried out with varying concentrations of Asc (0-2 mM) to determine the effect of the antioxidant on PP-PP⁺-mediated ROS production and MN9D cell death. Quantitative experiments with the ROS probe DCFH-DA show that Asc effectively reduces [1]-mediated ROS production in MN9D cells in a concentration dependent manner (Fig 9A). Similarly, these experiments further confirmed that Asc (2 mM) pretreatment of MN9D cells abolishes [1] or [3]-mediated (200 and 100 μ M, respectively; 5 h and 2h incubation) intracellular ROS production and protects MN9D cells from [1] and [3]-mediated cell death in a concentration dependent manner (Figs 9 B–D). These findings further support the conclusion that mitochondrial complex I inhibition associated increased ROS production is responsible for dopaminergic cell death caused by these toxins (6, 11–14).

PP-PP⁺ derivatives cause apoptotic cell death in MN9D cells.

PP-PP⁺-mediated MN9D cell death was associated with drastic cell shrinkage (Fig 10), suggesting that cell death could be due to apoptosis induced by ROS, as observed in MPP⁺-mediated cell death. To confirm this possibility, a chromatin condensation diagnostic test for apoptotic cell death was performed using the fluorescence probe DAPI (26). As shown in Fig 10, parent PP-PP⁺ derivative [1]-mediated MN9D cell death is associated with chromatin condensation as indicated by the significant increase of nuclear DAPI fluorescence in toxin treated cells, in comparison to untreated controls. These findings suggest that PP-PP⁺ induces apoptosis of MN9D cells subsequent to an increase in ROS.

Our study provides comprehensive evidence to suggest that increased ROS production associated with the inhibition of mitochondrial complex I in dopaminergic cells makes them selectively vulnerable to PP-PP⁺, MPP⁺, and related toxins. This is in strong support of previous work showing that the inherent high propensity of dopaminergic cells to produce high levels of ROS relative to other cell types, especially when exposed to mitochondrial toxins, is responsible for the selective dopaminergic toxicity of MPP⁺ and related toxins (6, 11–13). Previous studies have also shown that the presence of high concentrations of oxidatively labile dopamine in these cells at least partially contributes to this high vulnerability (11, 12), especially under the conditions in which mitochondrial oxidative energy metabolism, Ca²⁺ homeostasis, or other vital regulatory physiological mechanisms are perturbed. On the other hand, the relative resistance of liver HepG2 and other non-dopaminergic cell types to produce increased ROS in response to these mitochondrial toxins, could be due to a number of reasons including the presence of robust antioxidant systems, absence of oxidatively labile radical promoters such as dopamine, and other distinct physiological characteristics in these cells (11–13).

The above findings strongly suggest that, in addition to effective cellular and mitochondrial accumulation, effective mitochondrial complex I inhibition is necessary for the dopaminergic toxicity of MPP⁺ and related toxins. More importantly, the high vulnerability

of dopaminergic cells to these toxins in comparison to other cell types is partly due to their high inherent propensity to produce excessive ROS levels in response to complex I inhibition by these toxins. Furthermore, the death of these cells is caused by ROS-mediated induction of the apoptotic pathway. Although the molecular details of the selective vulnerability of dopaminergic cells to these mitochondrial toxins have not been fully understood, a combination of unique characteristics of these cells including the presence of oxidatively highly labile DA and its metabolites, presence of relatively low levels of antioxidant enzymes, the hypersensitivity to perturbation of intracellular Ca^{2+} levels, relatively high energy demand, and stress induced alpha-synuclein associated toxic effect may play a role. The identification of PP-PP⁺ derivatives with similar structural properties, but highly contrasting complex I inhibition and toxicological profiles could be useful in further defining the key functional elements required for the selective dopaminergic toxicity of MPP⁺ and related toxins. Additionally, the efficient passive DAT independent electrogenic cellular and mitochondrial accumulation of more lipophilic MPP⁺ related toxins such as cyanines and PP-PP⁺, together with their more potent inhibition of complex I may make them more dangerous environmental dopaminergic toxins than MPP⁺, suggesting that MPP⁺ is the simplest of a larger group of unidentified environmental dopaminergic toxins (11–13).

Although the exact causes of PD are not fully understood, the general consensus is that the etiology of PD is complex and consist of multiple origins. A large number of epidemiological and experimental model studies suggest that exposure to environmental toxins including some agricultural, industrial and household chemicals may increase the risk of PD (27). Since the natural death of dopaminergic neurons in the CNS is not effectively replenished, the risk of developing PD is significantly increased with the age of healthy individuals. Accordingly, the chronic exposures to even low levels of environmental dopaminergic toxins could certainly accelerate the dopaminergic neuronal death resulting in the increase risk of developing PD at an early age. Thus, identification of potential environmental dopaminergic toxins, to develop preventive strategies, should certainly be a major challenge in the public health field. Our finding that there could be a large group of MPP⁺-related unidentified environmental dopaminergic toxins that could contribute to the etiology of PD may clearly have major public health implications, if confirmed *in vivo*. Currently, these *in vitro* findings are being further tested in *C. elegans* neurodegenerative *in vivo* models (28–30) in our laboratory to further validate the significance of the above findings.

Materials and Methods

Cell lines and material

The mouse hybridoma cell line MN9D [PRID: CVCL_M067] (31, 32) was graciously provided by Dr. Alfred Heller, University of Chicago, IL. Human hepatocellular liver carcinoma cell line [ATCC Cat# HB-8065, RRID: CVCL_0027; (HepG2)] (33–36) was a kind gift from Dr. Tom Wiese, Fort Hays University, KS. Fresh rat (12 months old, Sprague Dawley) brains for mitochondrial isolations were kindly provided by Drs. Li Yao and Yang, Shang-You of Wichita State University. The animals used in this study were treated in

accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health with the approval of the Wichita State University Institutional Animal Care and Use Committee.

Chemicals, reagents, and instrumentation.

All reagents and supplies were purchased from Fisher Scientific (Pittsburg, PA, USA), Sigma-Aldrich (Milwaukee, WI, USA) or Tocris Bioscience (Bristol, United Kingdom) unless otherwise noted. Krebs-Ringer Buffer-HEPES (KRB-HEPES) contained 109.5 mM NaCl, 5.34 mM KCl, 0.77 mM NaH₂PO₄, 1.3 mM CaCl₂, 0.81 mM MgSO₄, 5.55 mM dextrose, and 25 mM HEPES at pH 7.4. Dulbecco's Modified Eagles Medium (DMEM) contained 109.5 mM NaCl, 5.34 mM KCl, 0.77 mM NaH₂PO₄, 1.8 mM CaCl₂, 0.81 mM MgSO₄, 44 mM NaHCO₃, and 5.55 mM dextrose. Mitochondrial uptake medium (KCl buffer) contained 0.12 M KCl, 50.0 μM EGTA, 20.0 mM MOPS at pH 7.4. Stock solutions of all PP-PP⁺ derivatives, rotenone, 4', 6-diamidino-2-phenylindole, dilactate (DAPI), tetramethylrhodamine, methyl ester (TMRM), and 2', 7'-dichlorofluorescein diacetate (DCFH-DA) were prepared in 100% dimethyl sulfoxide (DMSO) or a fix mixture of DMSO and water. The final DMSO concentrations were kept to a minimum in all experiments usually < 0.05% v/v.

UV-visible spectra were recorded on a Cary Bio 300 UV-visible spectrophotometer (Varian Inc., Palo Alto, CA). Fluorescence emission spectra were recorded on a Jobin Yvon-Spex Tau-3 spectrophotometer (USA Instruments, Inc., Aurora, OH). The cellular and mitochondrial uptakes of all PP-PP⁺ derivatives were quantified by HPLC-UV using a solvent system of 48% aqueous buffer containing 20 mM Na₃PO₄, 20 mM CH₃CO₂Na, 30 mM triethylamine, 1.7 mM 1-octanesulfonic acid sodium salt, pH 7.0, and 52% CH₃CN at a flow rate of 0.8 mL/min with the UV detection at appropriate wavelengths (typically in the range of 290 to 340 nm). All light fluorescence microscopic experiments were carried out using a Nikon ECLIPSE Ti microscope equipped with a Nikon S FLURO 40X objective (Nikon Instrument Inc., Melville, NY). Intracellular and mitochondrial accumulation of various toxins, ROS levels in live cells were monitored using a Leica TCS SP5 II (37) confocal fluorescence microscope equipped with a 40X and 63X objectives (Leica Microsystems Inc., Buffalo Grove, IL).

Syntheses.

Synthesis and spectroscopic characterizations of PP-PP⁺ derivatives [1], [2], [4], and [6] have been previously reported (19). Similarly, ring substituted 3-bromophenylpropene and 4-phenylpyridine precursors used in the syntheses of [3] and [5] have also been previously reported (19).

Synthesis of PP-PP⁺ derivatives [3] and [5].—A mixture of 4'-methoxy-4-phenylpyridine (6.5 millimoles) and corresponding 3-bromo-2-phenylpropene (4'-chloro- or 4'-fluoro-3-bromo-2-phenylpropene; 6.5 millimoles) (19) were stirred with excess NaHCO₃ in THF (50 mL) at room temperature for 5 h. The products were concentrated under a vacuum and the resultant solids were suspended in absolute ethanol and insoluble NaHCO₃ and other salts were removed by filtration. The filtrates were evaporated and the products

were crystallized with absolute ethanol and ether. The products were characterized by standard ^1H - ^{13}C -NMR, MS, and elemental analyses.

4-(4'-methoxyphenyl)-1-[2-(4-chlorophenyl)-propyl] pyridinium bromide (3): Yield 74%; mp 238-239 °C ^1H NMR (D_2O) δ 4.79 (s, 3H), 5.42 (s, 1H), 5.65 (s, 2H), 5.71 (s, 1H), 7.14 (d, 2H), 7.28 (d, 2H), 7.44 (d, 2H), 7.46 (d, 2H), 8.21 (d, 2H), 8.76 (d, 2H); ^{13}C δ 55.7, 63.9, 115.1, 119.3, 122.4, 125.8, 127, 129, 130.7, 134.8, 135.2, 141.3, 144.2, 156.5, 164, 166.4 MS (ESI) m/z 336.82 (M^+).

4-(4'-methoxyphenyl)-1-[2-(4-fluorophenyl)-propyl]pyridinium bromide (5): Yield 74%; mp 237-239 °C ^1H NMR (D_2O) δ 4.78 (s, 3H), 5.43 (s, 1H), 5.62 (s, 2H), 5.73 (s, 1H), 7.14 (t, 2H), 7.28 (d, 2H), 7.44 (q, 2H), 7.89 (d, 2H), 8.20 (d, 2H), 8.75 (d, 2H); ^{13}C δ 55, 62.9, 115.2, 119.8, 123.4, 125.5, 128, 129, 130, 134.8, 135.2, 141.3, 144.2, 158.5, 164, MS (ESI) m/z 320.37 (M^+).

Methods

Cell culture.—MN9D and HepG2 cells were cultured in 100 mm^2 Falcon tissue culture plates in DMEM- HCO_3^- media supplemented with 10% fetal bovine serum, 50 $\mu\text{g/mL}$ streptomycin, and 50 IU/mL penicillin at 37 °C in a humidified atmosphere of 7% CO_2 . Cells were cultured to about 70-80% confluence and then seeded into glass-bottomed culture plates (for imaging studies) or 12- or 96-well plates (for uptake or toxicity studies, respectively) depending on the experiment and grown to about 70-80% confluence (usually two days) unless otherwise stated.

Measurement of cellular uptakes of PP-PP⁺ derivatives.—Cells were seeded into 12-well plates at 0.25×10^6 cells/well and grown 2-3 days to about 70-80% confluency. The media was removed and a solution containing the desired concentration of the corresponding PP-PP⁺ in warm KRB-HEPES was added to each well and incubated at 37 °C for the desired period of time (see the corresponding figure legends for further details). After the incubations, the media were removed and cells were washed with ice-cold KRB-HEPES. Washed cells were suspended in 1.0 mL of ice-cold KRB-HEPES and 50 μL aliquots were withdrawn for protein determination. The remaining cell suspensions were centrifuged at $5,000 \times g$ at 4 °C for 6 min and the cell pellets were treated with 75 μL of 0.1 M HClO_4 . The coagulated proteins were pelleted by centrifugation at $13,200 \times g$ at 4 °C for 8-10 min and the PP-PP⁺ contents of the supernatants were determined by C_{18} -reversed-phase HPLC-UV (HPLC-UV) using standard curves constructed using authentic samples of the corresponding PP-PP⁺ derivative. All PP-PP⁺ levels were normalized to the respective protein concentration of each sample and were corrected for non-specific membrane binding by subtracting the corresponding time zero readings for each PP-PP⁺ concentration (normally <1% of the intra-cellular concentration).

Measurement of cell viability.—Cell viability was determined by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (38, 39). Briefly, cells were seeded on 96-well plates and allowed to grow to ~70–80% confluence. The culture media was replaced with DMEM containing the desired concentrations of PP-PP⁺ derivative and

incubated for 18 h (or desired time) at 37 °C. After the incubation, 10 μ L of 5 mg/mL MTT (in sterile water) was added to each well and incubated for 2 h at 37 °C. The resulting formazan was solubilized by the addition of 210 μ L of detergent solution [50% DMF/H₂O (v/v), 20% SDS (w/v)] followed by incubation at 37 °C for 12 h. The cell viabilities were determined by quantifying solubilized formazan by measuring the difference in the absorbance at 570 nm and 650 nm (38, 39). Results were expressed as % viability of toxin treated cells with respect to control cells, which were treated under the same conditions except in the absence of the toxin.

In the experiments where ascorbate was used as an antioxidant agent, cells were pre-treated with the desired concentration of ascorbate (0-500 μ M) in Ca-HCO₃ buffer for 30 min and then the cells were incubated with the desired concentration of the toxin for 18 h at 37 °C and cell viabilities were determined as above.

Isolation of Rat Brain Mitochondria.—Mitochondria from rat brains were isolated according to the protocol of *Diwakar et al* (21). Briefly, after animals were sacrificed, the brains were removed, dissected and homogenized using a Potter-Elvehjem homogenizer in ice cold isolation media containing 100 mM K₃PO₄, 0.25 mM sucrose, 1 mM EDTA, and a cocktail of protease inhibitors at pH 7.4. The homogenate was centrifuged at 1,000 g for 10 min, and the pellet was discarded. The supernatant was re-centrifuged at 14,000 g for 30 min and the supernatant was discarded. The white fluffy layer containing lipid and lysosomes was carefully removed and the brown/red mitochondrial fraction of the pellet was collected. The crude intact mitochondrial fraction was re-suspended in the homogenization buffer and used immediately in uptake experiments or stored at –80°C for complex I assays.

Measurement of mitochondrial complex-I inhibition.—Isolated rat brain intact mitochondria were lysed by freeze-thawing in homogenization buffer containing 100 mM K₃PO₄, 0.25 mM Sucrose, 1 mM EDTA, and a cocktail of protease inhibitors, pH 7.4. The complex-I (i.e. NADH: ubiquinone oxidoreductase) activity of mitochondrial membrane fragments were determined according to the procedure of *Diwakar et al.*(21). Briefly, mitochondrial membranes (~ 500 μ g protein) were incubated with 130 μ M NADH (final concentration) and antimycin A (2 μ g/mL) in a 1.0 mL assay solution containing 35 mM K₃PO₄, 1 mM EDTA, 1 mg/mL bovine serum albumin, 2.65 mM potassium cyanide, 5 mM MgCl₂, pH 7.2, for 2 min at 37 °C with or without rotenone or toxin. The complex-I mediated ubiquinone dependent NADH oxidation was initiated by adding 65 μ M ubiquinone-1 (final concentration) to the test cuvette. The reaction rates were monitored by following the decrease in absorbance at 340 nm for 5 min against the ubiquinone-1 absent control.

Measurement of PP-PP⁺ uptake into isolated MN9D cell mitochondria.—The isolated intact mitochondrial preparations were suspended in KCl buffer and aliquots (5-10 μ L) were withdrawn for protein determination. Desired amounts of mitochondrial suspensions were incubated with 50 μ M [3] or [5] in KCl buffer for 45 min at 37 °C. After the incubation, samples were diluted 16-fold (to terminate the uptake) and centrifuged at 5,000 g at 4 °C for 6 min. The mitochondrial pellets were then treated with 75 μ L of 0.1 M HClO₄. The coagulated proteins were removed by centrifugation at 13,200 g for 8–10 min at

4 °C and [3] and [5] contents in HClO₄ extracts were quantified by HPLC-UV as detailed above. All intracellular [3] and [5] levels were normalized to the respective protein concentration of each sample and were corrected for non-specific membrane binding by subtracting the corresponding time zero readings for each [3] and [5] concentration (normally <5-10% of the intracellular concentration).

Measurement of intracellular Reactive Oxygen Species (ROS).—Cells grown in 12-well plates were incubated with 10 μM DCFH-DA in KRB-HEPES for 1 h. DCFH-DA-loaded cells were washed with ice-cold KRB-HEPES and treated with the desired concentration of PP-PP⁺ derivative in the same buffer for 1 h at 37 °C. After the incubations, cells were washed, harvested, solubilized with 0.1 M Tris buffer (pH 7.5) containing 1% Triton X-100, and cell debris were removed by centrifugation at 13,200 *g* for 8 min at 4 °C. The content of ROS-oxidized DCFH product, 2',7'-dichlorofluorescein (DCF) in the supernatants was quantified by fluorescence (Ex/Em 488/524 nm) (40). The data were normalized to the protein contents of individual samples. ROS production in DCFH-DA (50 μM) loaded PP-PP⁺ treated MN9D and HepG2 cells was visualized by confocal fluorescence microscopy at Ex/Em 488/524 nm using a Leica TCS SP5II microscope.

Mitochondrial localization of intracellular PP-PP⁺.—Cells were grown in glass-bottomed plates and incubated with 10-50 μM [3] or [5] and 100 nM Mito Tracker Red (Life Technologies, Grand Island NY, USA) in KRB-HEPES at 37 °C for 15 min. Then, cells were washed, reconstituted with KRB-HEPES (1.0 mL), pH 7.4, and dual fluorescence of intracellular [3] or [5] (Ex/Em 340/420-530 nm) and MitoTracker Red (Ex/Em 644/665 nm) was recorded using a Nikon ECLIPSE Ti-S inverted fluorescence microscope fitted with a 63X objective.

Effects of PP-PP⁺ on the mitochondrial membrane potentials.—MN9D or HepG2 cells grown in glass-bottomed plates were treated with 50 nM TMRM (AnaSpec, Fremont CA, USA) in KRB-HEPES for 45 min in the dark (20). After mounting on the stage of a Nikon ECLIPSE Ti-S inverted fluorescence microscope, 15-20 ROIs with uniform fluorescence were selected and TMRM fluorescence at Ex/Em at 543/573 nm was recorded at 5 sec time intervals for 3 min. Then, desired concentrations of [3], [4] or [5] were added and TMRM fluorescence measurements were continued for an additional 1h at 15 sec time intervals. The background fluorescence was subtracted from the ROI fluorescence and averages of corrected data were used to estimate the magnitude of the mitochondrial membrane potential. At the end of the time measurements the images of all samples were recorded for visualization purposes.

Effect of Ascorbate (Asc) on the PP-PP⁺ induced ROS production and MN9D cell death.—To determine the effect of antioxidant Asc on the PP-PP⁺-mediated ROS production in MN9D cells, cells were pretreated with 0-2 mM Asc in KRB for 1 h at 37 °C and then incubated with 200 μM [1] containing the same concentration of Asc for additional 5 h. After the incubation, the intracellular ROS levels were determined by DCFH-DA method. In addition, the effect of Asc on [1] and [3]-mediated ROS productions in MN9D cells were visualized by confocal fluorescence microscopy as detailed above. The effect of

varying concentrations of Asc (0-500 μ M) on [1] and [3] mediated MN9D cell death was determined at fixed concentrations of the toxin ([3] and [1] 40 μ M) using a standard MTT cell viability assay as detailed above. To correct for the slow chemical reduction of MTT by Asc, a series of parallel control experiments were carried out for each Asc concentration under the same conditions except that toxins were excluded from the incubation and readings were subtracted from the corresponding test readings.

Chromatin condensation test for apoptosis.—MN9D cells grown in glass-bottomed plates were treated with 100 μ M [1] for 12 h and then with 300 nM DAPI in KRB-HEPES for 15 min in the dark at 37 °C (26, 41). Then cells were rinsed and KRB was added to cover the cell layer. Increase in nuclear DAPI fluorescence (Ex/Em at 358/461 nm) due to chromatin condensation was observed using a Nikon ECLIPSE Ti-S inverted fluorescence microscope. The controls were treated similarly except that the toxin was omitted from the initial incubation medium.

Protein determination.—Protein contents of various cell preparations were determined by the Bradford protein assay using bovine serum albumin (BSA) as the standard (42). Aliquots of (50 μ L) of cell suspensions were incubated with Bradford protein reagents (950 μ L) for 10 min and absorbance at 595 nm were recorded. The absorbance readings were converted to the corresponding protein concentrations using a standard curve constructed employing BSA as the standard.

Statistical analyses.

All data are averages of three or more replicates of a single experiment and presented as mean $\pm$ SD (all the experiments were carried out multiple times to test the reproducibility and the representative data from a single experiment are shown). To correct for the minor variations in color development in the MTT assay, all absorbance values were converted to percentages of controls. All quantitative data i.e. uptake, and ROS levels were normalized to the protein content of individual samples to correct for the cell density variations. Statistical analyses of the data were carryout by one-way ANOVA or Student t-Test and * $p < 0.0025$ was considered statistically significant.

Technical statement.

Due to the high toxicity and obvious health hazards of MPP⁺ and PP-PP⁺ derivatives extreme caution was used in their handling in accordance with published procedures (43).

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Abbreviations used:

DAT   plasma membrane dopamine transporter

DAPI	4',6-Diamidino-2-phenylindole dilactate
DCF	2',7'-dichlorofluorescein
DCFH-DA	2',7'-dichlorofluorescein diacetate
DMEM	Dulbecco's Modified Eagles Medium
KRB	Krebs ringer buffer
MPP⁺	1-methyl-4-phenylpyridinium
PP-PP⁺	N-(2-phenyl-1-propene)-4-phenyl-pyridinium
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PD	Parkinson's disease
ROS	Reactive oxygen species
TMRM	Tetramethylrhodamine methyl ester

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Fig. 1.

Comparative MN9D, SH-SY5Y, and HepG2 cell toxicities of [1]-[5]: Cells were grown in 96 well plates and treated with the toxin in DMEM for 12 (Fig. 1A) and 18 h (Figs 1B-D) at 37° C. Cell viabilities were determined by 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as detailed in the Material and Methods. Results are expressed as % cell viability with respect to parallel untreated controls. Data are presented as mean $\pm$ SD (n=6). *p< 0.0025 (one-way ANOVA). (A) MN9D, SH-SY5Y, and HepG2, cell toxicities of [1]; (B) MN9D, cell toxicity of [2]; (C) MN9D, cell toxicities of [3], [4], and [5]; (D) HepG2, cell toxicities of [3], [4], and [5].

Fig. 2.

Characteristics of cellular uptake of [1], [3], [4], and [5]: (A) Time course of the uptake of [1] into MN9D cells. Cells were treated with 50 μ M toxin from 0–60 min at 37°C and intracellular compound levels were determined as described in Materials and Methods. All uptake levels were normalized to their respective protein concentrations. Concentration dependence of the uptake of [3], [4] and [5] into MN9D (B) and HepG2 (C) cells: Cells were treated with varying concentrations of toxin for 45 min at 37°C and intracellular levels were determined as described in Materials and Methods. All uptake data were normalized to respective protein concentrations. All data are presented as mean $\pm$ SD (n=3) *p< 0.0025 (one-way ANOVA).

Fig. 3.

Intracellular localization of [3] and [5] in MN9D cells: (A) Cells were grown on glass bottomed plates. After adding the toxin at the desired concentration, the light and fluorescence images (intrinsic fluorescence of [3]) were recorded at t = 0 and 60 min. (B) Cells were incubated with 100 nM MitoTracker Red for 15 min, followed by 10 μM [3] for 30 min or 50 μM [5] for 1 h. Fluorescence images of [3] or [5] [Ex 340(or 405 (confocal)); 3B&C]/Em 420-530 nm) and MitoTracker Red (Ex/Em 644/665 nm) were recorded. (C)

HepG2 cells were grown, treated with 25 μM [3] or 50 μM [5] for 1 h, and fluorescence images were recorded.

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Fig. 4.

The relative mitochondrial uptakes efficiencies of [3] and [5]. Isolated intact MN9D mitochondria were incubated with 50 μ M [3] and [5] for 45 min and intercellular levels were determined by HPLC-UV as described in the Material and Methods. All toxin levels were normalized to the protein concentrations and corrected for non-specific membrane binding. Data are presented as mean $\pm$ SD (n=3) *p< 0.0025 versus zero time control (Student t-Test).

Fig. 5.

The effects of [3], [4], and [5] on the mitochondrial membrane potentials of (A) MN9D and (B) HepG2 cells. Cells were treated with 500 μ M of toxin and the mitochondrial membrane potentials were monitored as a function of time using the fluorescent probe tetramethylrhodamine, methyl ester (TMRM) (Ex/Em 543/573 nm) as detailed in the Material and Methods. (C) Effect of [3], [4], and [5] on mitochondrial membrane potential as visualized by living cells. Images of MN9D and HepG2 cells were recorded at $t = 0$ and 60 min after adding toxin.

Fig. 6. PP-PP⁺ derivatives are potent mitochondrial complex I inhibitors. (A) Isolated rat brain mitochondrial membranes were incubated with 50 μ M concentration of [2], [3], [4], and [5], 10 μ M rotenone, or 100 μ M MPP^+ for 2 min and then 10 mM NADH was added and ubiquinone dependent oxidation of NADH was initiated with ubiquinone-1 (67 μ M) and followed at 340 nm for 5 min, as described in the Materials and Methods. Data are presented as mean $\pm$ SD (n=3).

Fig. 7.

The effect of PP-PP⁺ derivatives [3], [4], and [5] on intracellular ROS levels in MN9D and HepG2 cells: Cells were incubated with varying concentrations of [3], [4], and [5] for 1 h (or desired period) at 37°C and the intracellular ROS levels were determined by the DCF method as detailed in the Materials and Methods. ROS levels in (A) MN9D and (B) HepG2 cells treated with [3], [4], or [5] * $p < 0.0025$ (one-way ANOVA); (C) ROS levels in [3] and [5] treated MN9D cells as visualized by live cell DCF imaging: MN9D cells were grown in glass bottomed plates, treated with 50 μM DCF-DA for 30 min, followed by 100 μM [3] or [5] or without toxin (control) for 2 h at 37°C, and DCF (Ex/Em 488/525) or light images were recorded.

Fig. 8. PP-PP⁺ mediated ROS production is initially localized into the mitochondria of MN9D cells. MN9D cells were grown in glass bottomed plates and treated with 50 μM DCFH-DA for 30 min at 37°C and then with 100 nM MitoTracker Red for 15 min. Cells were then incubated with 200 μM [1] for 5h or 100 μM [3] for 1 h, and DCF (Ex/Em 488/525 nm) and MitoTracker Red (Ex/Em 644/665 nm) fluorescence images were simultaneously recorded.

Fig. 9.

(A) PP-PP⁺-mediated ROS production in MN9D cells is attenuated by ascorbate (Asc). MN9D cells were pre-incubated with the desired concentration of Asc for 1 h, followed by 200 μ M [1] for an additional 5 h at 37°C and intracellular ROS levels were determined as described in the Materials and Methods. (B) Asc modulates [1] and [3]-mediated ROS production in MN9D cells as visualized by live cell imaging. MN9D cells were incubated with or without 2 mM Asc for 1 h, treated with 50 μ M DCF-DA for 30 min, and incubated with 200 μ M [1] or 100 μ M [3] in the presence or absence of 2 mM Asc for 5 h or 2 h, respectively at 37 °C. Light images and DCF-DA fluorescence (Ex/Em 488/525) images of cells were recorded. (C) & (D) Asc protects MN9D cells from [3] and [1] toxicity: MN9D cells were pre-treated with the desired concentration of Asc for 30 min at 37°C and then incubated with 8 μ M [3] or 40 μ M [1] in the presence of the same respective concentrations of Asc for 18 h at 37°C, and cell viabilities were determined by MTT assay. All data are represented as mean $\pm$ SD (n=6). *p< 0.0025 (one-way ANOVA).

Fig. 10.

PP-PP⁺ causes apoptotic MN9D cell death: Cells were grown in glass bottomed plates and incubated with 200 μM [1] in DMEM for 12 h at 37°C and then treated with 300 nM DAPI for 15 min at 37°C and apoptotic chromatin condensation was visualized by fluorescence microscopy (Ex/Em 358/461 nm). Control cells were treated in the same manner except that [1] was excluded from the incubation medium.

Scheme.

Table 1:

Comparison of the relative IC₅₀ values for various PP-PP⁺ derivatives, MPP⁺, 4'IMPP⁺: IC₅₀ values were estimated from the linear portions of the cell viability data which were determined as detailed in Materials and Methods by fitting to the linear equation $IC_{50} = (0.5 - b)/a$. The MN9D cell viabilities of PP-PP⁺ derivatives were determined at 18 h incubations, MPP⁺, and 4'IMPP⁺ at 16h incubations. (r^2 = values for fits were given in parenthesis).

Toxin	IC ₅₀ μ M	Toxin	IC ₅₀ μ M
[2]	3.9 (0.96)	4'IMPP ⁺	46.6 (0.99), 16h
[3]	4.6 (0.96)	[5]	> 60
[4]	5.4 (0.98)	[6]	96.4 (0.92)
[1]	23.0 (0.99)	MPP ⁺	117.8 (0.99), 16 h

Table 2:

Relative lipophilicities of PP-PP⁺ derivatives as estimated by reverse phase HPLC retention times (RT).

Toxin	RT (min)	Toxin	RT (min)
[2]	11.95	[5]	6.97
[3]	8.39	[4]	6.88
[6]	7.02	[1]	6.33