Published in final edited form as:

Chem Res Toxicol. 2016 September 19; 29(9): 1468–1479. doi:10.1021/acs.chemrestox.6b00138.

Lipophilic Cationic Cyanines are Potent Complex I Inhibitors and Specific *in vitro* Dopaminergic Toxins with Mechanistic Similarities to both Rotenone and MPP⁺

Chamila C. Kadigamuwa^{†,‡}, Sumudu T. Mapa[†], and Kandatege Wimalasena^{†,*}

[†]Department of Chemistry, Wichita State University, Wichita, KS 67260

[‡]Current address: Center for Cancer Research, National Cancer institute, Building 37, Room 1130, Bethesda, MD 20892

Abstract

We have recently reported that simple lipophilic cationic cyanines are specific and potent dopaminergic toxins with a mechanism of toxicity similar to the Parkinsonian toxin MPP⁺. In the present study a group of fluorescent lipophilic cyanines have been used to further exploit the structure-activity relationship of the specific dopaminergic toxicity of cyanines. Here we report that all cyanines tested were highly toxic to dopaminergic MN9D cells with IC₅₀s in the range of 60–100 nM and not toxic to non-neuronal HepG2 cells parallel to the previously reported for 2,2'- and 4,4'-cyanines. All cyanines non-specifically accumulate in the mitochondria of both MN9D and HepG2 cells at high concentrations, inhibit the mitochondrial complex-I with the inhibition potencies similar to the potent complex I inhibitor, rotenone. They increase the reactive oxygen species (ROS) production specifically in dopaminergic cells causing apoptotic cell death. These and other findings suggest that the complex-I inhibition, the expression of low levels of antioxidant enzymes, and presence of high levels of oxidatively labile radical propagator, dopamine, could be responsible for the specific increase in ROS production in dopaminergic cells. Thus, the predisposition of dopaminergic cells to produce high levels of ROS in response to mitochondrial toxins together with their inherent greater demand for energy may contribute to their specific vulnerability towards these toxins. The novel finding that cyanines are an unusual class of potent mitochondrial toxins with specific dopaminergic toxicity suggest that their presence in the environment could contribute to the etiology of PD similar to MPP⁺ and rotenone.

Graphical Abstract

*To whom correspondence should be addressed: Tel (316)-978-7386; Fax (316)-978-3431; kandatege.wimalasena@wichita.edu.

Notes

The authors have no conflict of interest to declare

Keywords

Parkinson's disease; MPP^+ ; cyanine dyes; neurotoxins; reactive oxygen species; mitochondrial stress; complex I inhibition

INTRODUCTION

1-Methyl-4-phenylpyridinium (MPP^+), a specific dopaminergic toxin which causes Parkinson's (PD) like symptoms in humans and other primates, has been widely used to model the environmental contributions to the etiology of PD.^{1, 2} Numerous previous studies have suggested that the specific dopaminergic toxicity of MPP^+ is primarily due to its ability to specifically enter into dopaminergic neurons through the presynaptic dopamine transporter (DAT) followed by the inhibition of the mitochondrial electron transport chain complex I.^{3–5} However, a number of recent studies show that MPP^+ is efficiently taken up not only into dopaminergic cells, but also into many other cell types through a number of transporters other than DAT, including organic cation transporters (OCT), plasma membrane monoamine transporters (PMAT) and through other non-specific transporters^{6–10} suggesting that the specific vulnerability of dopaminergic cells towards MPP^+ and similar toxins could not solely be due to the specific uptake through DAT.

In a previous study, we have used two simple lipophilic cyanines, 1'-diethyl-2,2'-cyanine (2,2'-cyanine) and 1,1'-diethyl-4,4'-cyanine (4,4'-cyanine) in a comparative study with MPP^+ to address the above possibility.¹¹ These studies have shown that both cyanines freely and non-specifically accumulate in all cell types, but they are also specifically toxic to dopaminergic cells similar to MPP^+ . More importantly, they are about 1000 fold more toxic to dopaminergic cells in comparison to MPP^+ .¹¹ In the present study we have extended our studies to a structurally diverse group of fluorescent, lipophilic cationic cyanines to further exploit the structure-activity relationship of their specific dopaminergic toxicity. Here we report that all cationic cyanines tested i.e. 3,3'-diethyloxacarbocyanine [DiOC2(3)], 3,3'-dipropylthiacarbocyanine [DiSC3(3)] and 3,3'-dipropylthiadibenzocarbocyanine [DiSC3(5)]

(Figure 1), are highly toxic to dopaminergic MN9D cells with IC₅₀s in the range of 60–100 nM and not significantly toxic to non-neuronal HepG2 cells parallel to the behavior of 2,2'- and 4,4'-cyanines. In contrast, an anionic fluorescent dye, bis-(1,3-diethylthiobarbituric acid) trimethine oxonol [DiSBAC2(3)] (Figure 1) was not significantly toxic to either MN9D or HepG2 cells. All cationic cyanines electrogenically accumulate in the mitochondria of both MN9D and HepG2 cells at high concentrations causing drastic mitochondrial membrane depolarization in both cell types. More importantly, they also found to inhibit rat brain mitochondrial complex I with the inhibition potencies in the same range as the best known complex I inhibitor, rotenone.¹² All cationic cyanines induce the over production of reactive oxygen species (ROS) specifically in dopaminergic cells, leading to apoptotic cell death, again parallel to MPP⁺. Based on these and other findings we propose that the predisposition of dopaminergic cells to produce high levels of ROS in response to mitochondrial toxins together with other inherent characteristics such as greater demand for energy may contribute to their specific vulnerability towards these toxins.^{13,14} Our findings that cationic cyanines are an unusual new class of potent mitochondrial toxins with specific dopaminergic toxicity further suggest that their presence in the environment could contribute to the etiology of PD similar to MPP⁺ and rotenone.^{1,2,15–17}

EXPERIMENTAL PROCEDURES

Chemicals and Reagents.

All reagents and solvents were obtained from various commercial sources with the highest purity available and used without further purification. Krebs-Ringer Buffer-HEPES (KRB-HEPES) contained 125 mM NaCl, 5.34 mM KCl, 0.81 mM MgSO₄, 1.3 mM CaCl₂, 0.77 mM NaH₂PO₄, 25 mM HEPES and 5.55 mM glucose, pH 7.4. Dulbecco's Modified Eagles Medium (DMEM-HCO₃⁻) contained 109.5 mM NaCl, 5.34 mM KCl, 0.81 mM MgSO₄, 1.8 mM CaCl₂, 0.77 mM NaH₂PO₄, 44 mM NaHCO₃ and 5.55 mM glucose. Stock solutions of 3,3'-diethyloxycarbocyanine iodide [DiOC2(3)], 3,3'-dipropylthiacarbocyanine iodide [DiSC3(3)], 3,3'-dipropylthiadibocarbocyanine iodide [DiSC3(5)], bis-(1,3-diethylthiobarbituric acid) trimethine oxonol [DiSBAC2(3)], rotenone, 4', 6-diamidino-2-phenylindole, dilactate (DAPI), tetramethylrhodamine, methyl ester (TMRM), and 2', 7'-dichlorofluorescein diacetate (DCFH-DA) were prepared in dimethyl sulfoxide. In all experiments, final dimethyl sulfoxide concentration was kept to a minimum, usually < 0.05% v/v.

Cell Lines.

The mouse hybridoma cell line MN9D was graciously provided by Dr. Alfred Heller, University of Chicago.¹⁸ Human hepatocellular liver carcinoma (HepG2) cell line was obtained from Dr. Tom Wiese (Fort Hays University, Hays KS).¹⁹ Fresh rat brains (from 12 months old, male, Sprague Dawley rats) for mitochondrial isolations were kindly provided by Dr. Li Yao (Wichita State University, Wichita, KS).

Instrumentation.

Images of DAPI fluorescence of toxin treated cells were captured using a Nikon ECLIPS-Ti inverted fluorescence microscope equipped with a Nikon S FLURO 40X objective (Nikon

Instrument Inc., Melville, NY, USA). Mitochondrial accumulation of various toxins and intracellular ROS production in live cells were monitored using a Leica TCS SP5II (Leica Microsystems Inc., Buffalo Grove, IL, USA) confocal fluorescence microscope equipped with 40X objective.

Cell Culture.

MN9D and HepG2 cells were cultured in 100 mm² Falcon tissue culture plates in DMEM with high glucose (4500 mg/L) supplemented with 10% fetal bovine serum, 50 µg/ml streptomycin and 50 IU/ml penicillin at 37 °C in an humidified atmosphere with 7% CO₂. Cells were cultured to about 70–80% confluence and then, seeded into glass bottom culture plates, 12-well plates, or 96-well plates depending on the nature of the experiment and grown to 70–80% confluence for 2–3 days unless otherwise stated.

Measurement of Cellular Uptake of Cyanines.

Cells were grown in 12-well plates, washed with KRB-HEPES, incubated with a desired concentration of cyanine in the same buffer for 1 h at 37 °C. After the incubation, cells were washed, gently scraped, and collected in 1.0 mL of ice-cold KRB-HEPES. Aliquots (50 µL) were removed for protein assay, the remainder was centrifuged for 3 min at 3,500 g, and the cell pellet was solubilized with 0.1 M Tris-chloride buffer (pH 7.5) containing 1% Triton X-100 and centrifuged at 16,000 g for 8 min at 4 °C to remove cell debris. The cyanine content of the cell extracts were quantified by fluorescence using standard curves constructed with standard concentrations of the corresponding cyanine. All uptake readings were normalized to the protein content of each sample and corrected for non-specific binding by subtracting the corresponding zero time readings (usually the non-specific bindings were small in all cases and were about < 5% of the corresponding uptakes).

Measurement of Cell Viability.

Cell viabilities were determined by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] cell viability assay.⁽²⁰⁾ Briefly, cells were seeded on 96-well plates and allowed to grow to about 70–80% confluence, the growth media were replaced with KRB-HEPES containing a desired concentration of cyanine or other reagents, and incubated 12 h at 37 °C. After the incubation, 10 µL of 5 mg/mL MTT solution was added to each well and the incubation was continued for additional 2 h at 37 °C. The resulting formazan was solubilized by the addition of 210 µL detergent solution (50% DMF, 20% SDS) followed by incubation for 4 h at 37 °C. The cell viabilities were estimated by quantifying solubilized formazan by measuring the difference in the absorbance at 570 nm and 650 nm.²¹ Results were expressed as % viability of toxin treated cells with respect to control cells which were treated under the same conditions except that the toxin was omitted from the incubation media.

In experiments where tyrosine hydroxylase inhibitor was used, cells were treated with the desired concentration of tyrosine hydroxylase inhibitor, L- α -methyl-*p*-tyrosine (THI), 37 °C in regular growth media for 24 h and then the media was removed and the toxin was added with the same concentration of THI in KRB-HEPES. Cells were incubated for 12 h at 37 °C and cell viabilities were determined as above. In experiments where ascorbate was used,

cells were pre-incubated with the desired concentration of sodium ascorbate for 1 h and then the toxin was added with the same concentration of ascorbate in KRB-HEPES. Cells were incubated for 12 h at 37 °C and the cell viabilities were determined as above.

Mitochondrial Localization of Cyanines.

MN9D and HepG2 cells grown in glass bottom plates were treated with 100 nM DiSC3(5) or DiSBAC2(3) in KRB-HEPES for 15 min at 37 °C and washed with KRB-HEPES. Then, cells were treated with 100 nM mitotracker green or red for 15 min at 37 °C and washed 3 times with KRB-HEPES and the fluorescence images of cells were recorded at the corresponding excitation and emission wavelengths using a confocal microscope equipped with 40X objective (*see* corresponding figure legends for further detail).

Measurement of the Mitochondrial Membrane Potential.

MN9D and HepG2 cells grown in glass bottom plates were treated with 50 nM TMRM in KRB-HEPES for 45 min in the dark.²² After mounting on a Nikon eclipse Ti-S fluorescence microscope stage, regions of interest (ROIs) were selected (20–30) and TMRM fluorescence (Ex/Em 543/573 nm) was measured in 5 sec intervals for 2 min. After 2 min, the toxin was added to a final concentration of 2.5 µM and the fluorescence measurement was continued for an additional 6 min. A parallel controls were carried out using an identical protocol except that the toxin was omitted from the incubation media. The background fluorescence was subtracted from the ROI fluorescence of both test and controls and averages of background corrected, control subtracted test data were used to estimate the mitochondrial membrane potential.

Measurement of Mitochondrial Complex I Inhibition.

Rat brain mitochondria were isolated according to the published procedure of Iglesias-Gonzalez *et al.*²³ Isolated mitochondria were lysed by freeze-thawing three times in hypotonic media containing 25 mM potassium phosphate (pH 7.2), 5 mM MgCl₂.²⁴ The complex I (i.e. NADH: ubiquinone oxidoreductase) activity of mitochondrial membranes were determined according to the procedure of Birch-Machin & Turnbull.²⁵ Briefly, mitochondrial membranes (50 µg protein) were incubated with 10 mM NADH and antimycin A (2 µg/mL) in a 1.0 mL assay solution containing 25 mM potassium phosphate, 5 mM MgCl₂, 2 mM KCN, 2.5 mg/mL bovine serum albumin, pH 7.2 for 2 min at 37 °C with or without the toxin. The Complex I mediated ubiquinone dependent NADH oxidation reaction was initiated by adding ubiquinone₁ (65 µM final concentration) and the initial rates of the reactions were measured by following the decrease in absorbance at 340 nm with respect to 425 nm reference for 5 min as a function of time.

Measurement of Intracellular ATP Levels.

Cells grown in 12-well plates were treated with the desired concentration of cyanine or rotenone for 6 h in KRB-HEPES buffer. After the treatments, cells were collected in 1 mL of KRB-HEPES and a 50 µL samples were withdrawn for the protein determination. The remaining cell suspensions were centrifuged for 3 min at 3,500 g and the cell pellets were used for the measurement of intracellular ATP levels. Briefly, the cell pellets were treated

with 1 mL of lysis buffer (0.1 M Tris, 1 % Triton X-100, pH 7.5) for 2 min and centrifuged at 3,500 g for 3 min to remove the cell debris. ATP content of the supernatants were determined using a BioVision fluorometric ATP assay kit following the manufactures guidelines. All ATP levels were normalized to the protein content of each sample.

Measurement of Reactive Oxygen Species (ROS).

Cells grown in 12-well plates were rinsed with KRB-HEPES and incubated with 10 μ M 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 cyanine in the same buffer for 1 h at 37 °C. Toxin treated (or control) 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. The content of ROS-oxidized DCFH-DA product, 2',7'-dichlorofluorescein, in the supernatants were quantified by fluorescence [Ex/Em 504/526 nm].⁽²⁶⁾ The data were normalized to the protein content of individual samples. ROS productions in live DCFH-DA (10 μ M) loaded toxin treated (1 h) MN9D cells were visualized by confocal fluorescence microscopy using appropriate instrumental settings (Ex/Em 488/524 nm).

Mitochondrial Localization of Cyanines Mediated-ROS Production in MN9D Cells.

MN9D cells grown in glass bottom plates were treated with 10 μ M DCFH-DA in KRB-HEPES for 1 h. DCFH-DA-loaded cells were washed with the same buffer and treated with 1.0 μ M DiSC3(5) in KRB-HEPES for 1 h at 37 °C and washed with KRB-HEPES. The intracellular localization of DiSC3(5) and DCFH-DA were visualized by confocal fluorescence microscopy at appropriate excitation and emission wavelengths.²⁷

Chromatin Condensation Test for Apoptosis.

MN9D cells grown in glass bottom plates were treated with the desired concentration of the toxin in KRB-HEPES for 12 h. Then, cells were treated with 300 nM 4',6-diamidino-2-phenylindole dilactate (DAPI) for 15 minutes in the dark rinsed and reconstituted with KRB-HEPES.²⁸ The increase of DAPI fluorescence in the nuclei due to chromatin condensation was observed at Ex/Em 358/461 nm using a Nikon eclipse Ti-S inverted fluorescence microscope. The controls were treated similarly except that the toxin was omitted from the incubation medium.

Protein Determination.

Protein contents of various cell preparations were determined by the method of Bradford.²⁹ Samples of cell suspensions (50 μ L) in KRB-HEPES were incubated with 950 μ L of Bradford protein reagents for 10 min and absorbance at 595 nm was recorded. The absorbance readings were converted to the corresponding protein concentrations using a standard curve constructed employing bovine serum albumin as a standard.

Data Analyses.

To correct for minor variations in color development in the MTT assay of cell viability, all the absorbance values were converted to a percentages of controls which were treated identically except that the toxins were excluded from the incubations. All data are averages

of three to six determinations and presented as means $\pm$ SD. The error bars represent the SD of the data from the mean. All quantitative uptake, ATP and ROS data were normalized to protein content of individual incubations to correct the results for the variations of cell densities between individual experiments. P values for pair-wise comparisons were calculated by two-tailed Student's t test. For all the data a level of $p < 0.05$ was considered statistically significant.

Technical Statement.

Due to the high toxicity and obvious health hazards of MPP^+ , cyanines (handled with the same precautions as for MPP^+) and rotenone extreme caution was used in their handling in accordance with published procedures.^{30,31}

RESULTS

Cyanines are Highly Toxic to Dopaminergic Cells and the Cell Specificity of Toxicities is parallel to that of MPP^+ and Rotenone.

We have recently reported that the commonly used Parkinsonian toxin MPP^+ is toxic to mouse dopaminergic MN9D cells with an IC_{50} in the range of 100–125 μM and not significantly toxic to liver HepG2 cells under similar experimental conditions.^{11,18} We have further shown that commonly used two simple cyanine dyes, 1,1'-diethyl-2,2'-cyanine (2,2'-cyanine) and 1,1'-diethyl-4,4'-cyanine (4,4'-cyanine) (Figure 1), are highly toxic to MN9D cells with an IC_{50} in the range of 100–125 nM and not significantly toxic to HepG2 cells under similar experimental conditions. In the present study, we found that all commonly used cyanine dyes tested, i.e. DiOC2(3), DiSC3(3) and DiSC3(5) (Figure 1) are also highly toxic to MN9D cells with IC_{50} s in the range of 75–100 nM and not significantly toxic to HepG2 cells under similar conditions (Figures 2A and B). In sharp contrast, a negatively charged dye, DiSBAC2(3), is not toxic to both MN9D and HepG2 cells (Figures 2A and B). We also found that the well-characterized mitochondrial complex I inhibitor, rotenone, is also highly toxic to MN9D cells with an IC_{50} in the range of 25–30 nM, but only very weakly toxic to HepG2 cells under similar experiment conditions similar to cyanines and MPP^+ (Figure 2C).³²

All Cationic Cyanines and the Anionic DiSBAC2(3) are Taken into MN9D and HepG2 Cells by Simple Diffusion and all Cationic Cyanines Actively Accumulate in the Mitochondria of all Cells.

Cationic cyanines DiOC2(3), DiSC3(3) and DiSC3(5) as well as the anionic DiSBAC2(3) taken up into MN9D cells in a concentration dependent manner in the concentration range of 0–1.0 μM during 1 h incubation period (Figure 3A). Similarly, both cyanine and the negatively charged DiSBAC2(3) dyes also accumulate in HepG2 cells in a concentration dependent manner with an efficiency that is similar to that of MN9D cells (Figure 3A). The live cell imaging experiments with mitochondrial labeling dye Mitotracker Green show that all cationic cyanines actively accumulate in the mitochondria of both MN9D and HepG2 cells [data shown only for DiSC3(5), but other cyanines were also found to behave the same way] (Figure 3B). However, as shown from the data in Figure 3C the anionic dye,

DiSBAC2(3), is distributed throughout the cell without specifically localizing into the mitochondria of MN9D and HepG2 cells.

Cationic Cyanines and Rotenone Depolarize the Mitochondrial Membrane in both MN9D and HepG2 Cells but not the Anionic Dye DiSBAC2(3).

The effects of cyanines and rotenone on the mitochondrial membrane potential was determined using tetramethylrhodamine methyl ester (TMRM) according to the well worked out literature procedures.²² The data presented in Figure 4A show that 2.5 μ M cyanines i.e. DiOC2(3), DiSC3(3) and DiSC3(5) depolarize the mitochondrial membrane potentials of MN9D cells to a similar extent (by about 30–40 %) within the first 8 min of incubation. The data also show that 2.5 μ M rotenone also depolarize the mitochondrial membrane potential of MN9D cells, but less effectively in comparison to the cyanines under similar experimental conditions (about 10–20 % during first 8 min of incubation). In contrast to cationic cyanines, the anionic DiSBAC2(3), was ineffective in depolarizing the mitochondrial membrane of MN9D cells (Figure 4A). A parallel and similar effects on HepG2 cells mitochondrial membrane potentials were also observed with the above cyanines, rotenone, and DiSBAC2(3) (Figure 4A).

Cationic Cyanines Inhibit the Mitochondrial Complex I with a Potency Similar to Rotenone but the Anionic DiSBAC2(3) is Ineffective.

The effect of cyanines, rotenone, MPP⁺, and the negatively charged DiSBAC2(3) on the NADH-ubiquinone oxidoreductase activity of complex I was determined using isolated rat brain mitochondrial membranes according to the well established ubiquinone dependent NADH-oxidation assay.²⁵ Specific complex I inhibition activity was measured by following the decrease in absorbance due to the ubiquinone dependent oxidation of NADH at 340 nm as a function of time, using the well characterized specific mitochondrial complex I inhibitor, rotenone as a standard. Rotenone showed 88% inhibition of the ubiquinone dependent NADH oxidation activity of complex I at 10 μ M concentration under the standard assay conditions confirming the suitability of the assay for specific complex I activity measurements (Figure 4B). As shown from the data in Figure 4B, DiSC3(3), DiSC3(5), and DiOC2(3) were found to inhibit 83%, 83%, and 79% the complex I activity at 10 μ M concentrations under the standard assay conditions. In addition, previously reported 2,2'-cyanine and 4,4'-cyanine, were also found to be potent inhibitors of the complex-I with 70.8% and 60.8% inhibition of complex I at 10 μ M concentrations, respectively.¹¹ Interestingly, MPP⁺ was found to be a much weaker inhibitor with 12.5% inhibition of complex I at 200 μ M concentration under similar experimental conditions. The anionic DiSBAC2(3) showed very weak or no complex I inhibition at 10 μ M concentration (Figure 4B).

Cyanines and Rotenone Cause the Depletion of Intracellular ATP Levels in both MN9D and HepG2 Cells but not the Anionic DiSBAC2(3).

The effect of cyanines, rotenone and DiSBAC2(3) on the intracellular ATP levels in MN9D and HepG2 cells were determined using a commercial ATP assay kit (Sigma) as detailed in Experimental Procedures. As shown in Figure 4C, treatment of MN9D or HepG2 cells with 100 nM DiSC3(5) caused the depletion intracellular ATP levels by ~40% and ~20%,

respectively with respect to control cells. Similarly, rotenone treatments cause the depletion of ATP levels in MN9D and HepG2 cells by about 25% and 50%, respectively. However, the anionic DiSBAC2(3) showed no significant effect on the intracellular ATP levels in both MN9D and HepG2 cells even at 250 nM concentration (Figure 4C).

Both Cyanines and Rotenone Cause Increase of Intracellular and Mitochondrial ROS Levels Specifically in MN9D Cells, but DiSBAC2(3) has no Effect.

The effect of cyanines and rotenone on the intracellular ROS levels were examined by using the fluorescent ROS probe DCFH-DA. Treatment of MN9D cells with increasing concentrations of (0–1000 nM) cyanines, DiOC2(3), DiSC3(3) or DiSC3(5) for 1 h showed a concentration dependent increase of intracellular ROS levels. For example, all three cyanines increase the intracellular ROS by about 3–4 fold from the baseline levels at 1000 nM concentration within 1 h (Figure 5A). Interestingly, only a moderate increase of ROS levels were observed upon the treatment of MN9D cells with the same concentrations of rotenone under similar experimental conditions (about 2 fold at 1000 nM concentration). On the other hand, the anionic dye, DiSBAC2(3) did not increase the intracellular ROS levels significantly even at maximum 1000 nM concentration under similar experimental conditions (Figure 5A). Parallel experiments show that cyanines, rotenone or DiSBAC2(3) treatments do not increase the intracellular ROS levels in HepG2 cells (Figure 5A). To determine whether the mitochondria is the origin of the cyanine-mediated ROS production in MN9D cells, a series of imaging experiments were carried out with cyanine treated live cells. In these experiments fluorescence of DiSC3(5) and ROS probe DCFH-DA were simultaneously recorded and images were overlaid (Figure 5B). The excellent overlap of the fluorescence images of DiSC3(5) which accumulates in the mitochondria of the cell (Figure 3B) with Mitotracker Green (Figure 5B) show that ROS is predominantly localized into the mitochondria of the cell.

Intracellular Dopamine (DA) Contributes to the ROS Production MN9D Cells upon Cyanines and Rotenone Treatments.

Our previous studies have shown that intracellular DA amplify the 2,2'-cyanine mediated production of ROS in MN9D cells.¹¹ Thus, to determine the effect of intracellular DA on the ROS production in response to these novel cyanines and rotenone treatments, MN9D cells were initially incubated with the increasing concentration of (1–6 mM) tyrosine hydroxylase inhibitor (THI) L- α -methyl-p-tyrosine for 24 h to deplete intracellular DA.³³ Then, DA depleted and control cells were incubated with 500 nM cyanines or rotenone for 1 h and intracellular ROS levels were determined by DCFH-DA method. The data presented in Figure 6A show that the ROS production in DA depleted cells is significantly reduced in comparison to the control cells grown under normal conditions in the absence of THI. The data further show that reduction of ROS production is proportional to the THI concentration and 6 mM THI treatment causes about 35% reduction of ROS production relative to control cells for all cyanines i.e. DiOC2(3), DiSC3(3), DiSC3(5) as well as rotenone.

Ascorbate (Asc) Reduces the ROS Production upon Cyanines and Rotenone Treatments in MN9D Cells.

To determine the effect of anti-oxidant Asc on the DiOC2(3), DiSC3(3) and DiSC3(5) cyanines and rotenone mediated ROS production, MN9D cells were pretreated with 0–2 mM concentrations of Asc in KRB for 1 h at 37 °C and then incubated with 500 nM cyanines or rotenone containing the same concentration of Asc for additional 1 h. After the incubation, the intracellular ROS levels were determined by DCFH-DA method. As shown in Figure 6B, Asc treatment effectively reduces the DiOC2(3), DiSC3(3) and DiSC3(5) and rotenone mediated ROS production in MN9D cells in a concentration dependent manner.

DA Depletion and Asc Pretreatment Protect MN9D Cells from Cyanine and Rotenone Toxicities.

The effect of the depletion of intracellular DA in MN9D cells through the inhibition of tyrosine hydroxylase by THI on the toxicities of DiOC2(3), DiSC3(3) and DiSC3(5) is shown in Figure 7A. The data clearly show that THI pre-treatment significantly reduces the DiOC2(3), DiSC3(3) and DiSC3(5) toxicities in a concentration dependent manner parallel to that is observed with the reduction of ROS production under similar conditions (Figure 6A). However, the data also show that the even the drastic DA depletion only protect MN9D cells partially from cyanine toxicities (about 40%; at 6 mM THI ~90% DA is depleted). As expected, the anti-oxidant, Asc, treatment also protects MN9D cells from the cyanine toxicities. The data presented in Figure 7C show that 500 μ M Asc effectively protect MN9D cells from DiOC2(3), DiSC3(3) and DiSC3(5) toxicities again parallel to the effect on the intracellular ROS levels (Figure 6B). Parallel to the effects on the cyanine toxicities, DA depletion as well as the Asc treatment significantly protect MN9D cells from rotenone toxicity as well (Figures 7B and D).

Cyanines, Rotenone and MPP⁺ Cause Apoptotic cell Death in MN9D Cells.

The cyanine, rotenone and MPP⁺ mediated MN9D cell death was associated with drastic cell shrinkage (Figure 8A), suggesting that cell death could be due to the apoptosis. To further confirm this possibility the diagnostic chromatin condensation test for apoptotic cell death was carried out using the fluorescence probe DAPI. As shown from the data in Figure 8B, DiOC2(3), DiSC3(3), DiSC3(5), rotenone and MPP⁺ mediated MN9D cell death is associated with chromatin condensation as indicated by the significant increase of the nuclear DAPI fluorescence in all toxin treated cells in comparison to untreated controls. Furthermore, as expected the non-toxic anionic, DiSBAC2(3), treated cells showed no significant cell shrinkage or increase in nuclear DAPI fluorescence (data not shown).

DISCUSSION

In a recent study we have shown that simple 2,2'- and 4,4'-cyanines are specifically toxic to dopaminergic MN9D cells with IC₅₀s in the range of 100–125 nM, suggesting that they are about 1000 fold more toxic than well-known Parkinsonian toxin MPP⁺ (IC₅₀ 100 μ M) under similar experimental conditions.¹¹ In the present study we have extended our studies to a new diverse group of cyanines (Figure 1) to further exploit the structure-activity relationship of the specific dopaminergic toxicity of this group of compounds. Here we show that all

that they could also be inhibitors of the mitochondrial complex I and that may also contribute to their drastic effects on the mitochondrial membrane potential.

Based on the above arguments, the effect of cyanines on the ubiquinone dependent oxidoreductase activity of rat brain mitochondrial electron transport chain complex I was investigated against the well-characterized potent complex I inhibitor, rotenone. Remarkably, all cyanines tested (including 2,2'- and 4,4'-) were found to be potent complex I inhibitors with the inhibition potencies in the same range as rotenone (Figure 4B). These studies further show that MPP⁺ is a much weaker inhibitor and the anionic DiSBAC2(3) is not an inhibitor of the complex I. Therefore, in addition to the permanent positive charge of cyanines, their ability to effectively inhibit mitochondrial complex I may also contribute to their drastic mitochondrial membrane potential depolarization effects. According to this model, the permanent positive charge of cyanine causes their active mitochondrial accumulation and efficient complex I inhibition leading to the drastic depolarization of the mitochondrial membrane. This notion is further supported by the observation that most potent, but uncharged complex I inhibitor, rotenone, depolarizes the mitochondrial membrane potential to a lesser extent in comparison to all cyanines under similar conditions (Figure 4A). Parallel to the depolarization of the mitochondrial membrane potential and complex I inhibition, both cyanines and rotenone cause the reduction of intracellular ATP levels without significant cell specificity, but the anionic dye has no effect (Figure 4C). Therefore, cyanines possess the most vital and destructive characteristics of the widely used both Parkinsonian toxin models, MPP⁺ and rotenone.^{1,2,16}

As previously reported for 2,2'- and 4,4'-cyanines, all cyanine treatments cause the increase of intracellular ROS levels specifically in MN9D cells, but not in HepG2 cells. Cell imaging studies show that the intracellular ROS is initially concentrated in the mitochondria of the cell suggesting that the complex I inhibition could be the major cause of ROS production. Parallel experiments with rotenone also show significant increase in the intracellular ROS production in specifically MN9D cells, but the overall efficiency is somewhat less than that of cyanines. This minor difference could be due to the ability of cyanines to accumulate in relatively high concentration in the mitochondria of the cell electrogenically relative to rotenone, due to their permanent positive charges as argued above. As expected, the anionic DiSBAC2(3), had no effect on the intracellular ROS levels in MN9D cells. Based on the parallel behaviors of cyanins, MPP⁺, and rotenone with respect to mitochondrial membrane potential depolarization, complex I inhibition and intracellular ROS production, we expected that rotenone also to be specifically toxic to MN9D and but not to HepG2 cells. In good agreement, rotenone was found to be highly toxic to MN9D cells in comparison to HepG2 cells with an IC₅₀ in the range of 30 nM. These findings suggest that the dopaminergic toxicities of all three classes of toxins, cyanines, rotenone and MPP⁺, are closely associated with their ability to increase the intracellular ROS production specifically in dopaminergic cells, as we proposed previously for MPP⁺, 2,2'- and 4,4'-cyanines.^{11,14} A further strong support for this proposal is provided by the observation that water soluble anti-oxidant, Asc, reduces the production of ROS in MN9D cells and at the same time effectively protect cells from the toxicities of all three classes of toxins. Thus, as indicated by the extensive cell shrinkage, chromatin condensation, and DNA laddering (data not shown; previously shown

for 2,2-cyanine),¹¹ the cell death caused by all three classes of toxins could be due to the ROS mediated activation of the apoptotic pathway.

The depletion of intracellular DA levels by inhibiting the rate limiting catecholamine biosynthetic enzyme, tyrosine hydroxylase, reduces the ROS production and protect the cells from the toxicities of both cyanines and rotenone [as well as MPP⁺; see ref. 11]. This finding support the notion that the excessive ROS production in MN9D cells in response to cyanine, rotenone, or MPP⁺ treatments is specifically amplified by the presence of high levels of oxidatively sensitive DA in MN9D cells.^{38,39} Our previous studies have also shown that the vital antioxidant enzymes catalase, glutathione peroxidase and superoxide dismutase levels in MN9D cells are much lower in comparison to liver HepG2 cells and that may also contribute to the specific increased ROS production in MN9D cells.¹¹ Therefore, the specific susceptibility of dopaminergic cells towards mitochondrial toxins such as rotenone, cyanine, and MPP⁺ must at least be partly due to their inherent predisposition to produce high levels of ROS in comparison to other cell types as a consequence of the presence of the high levels of oxidatively sensitive DA and the expression of relatively low levels of antioxidant enzymes.^{11,14} In addition, the observed depletion of intracellular ATP levels by all three classes of toxins could cause the release of synaptic stores of catecholamines into the cytosol due the dissipation of the V-ATPase generated intra-granular pH gradient further augmenting the catecholamine mediated ROS production.^{11,14,40}

Taken together, the above findings show that cationic lipophilic cyanines accumulate non-specifically and electrogenically in the mitochondria of both MN9D and HepG2 cells in large quantities. More importantly, cyanines were found to be an unanticipated new class of potent mitochondrial complex I inhibitors as effective as the best known complex I inhibitor, rotenone. Cyanines, MPP⁺ and rotenone all depolarize the mitochondrial membrane potential in both HepG2 and MN9D cells, but cause high levels of ROS production specifically in MN9D cells. All three groups of toxins show varying degrees of specific MN9D toxicities and the efficacies of toxicities are parallel to the extent of toxin-mediated over production of ROS. The presence of high levels of DA and the expression of low levels of antioxidant enzymes, catalase, superoxide dismutase and glutathione peroxidase in MN9D relative to HepG2 cells may contribute to the increased ROS production specifically in MN9D cells as proposed earlier.¹¹ As expected, the mitochondria appears to be the primary source of initial ROS production and the inhibition of the mitochondrial electron transport chain complex I may play a central role in the toxicities of all cases. The depletion of the cytosolic ATP levels by these toxins could also lead to the release of synaptic stores of DA and other catecholamine into the cytosol further augmenting the cytosolic catecholamine mediated ROS production specifically in dopaminergic cells. The observed dopaminergic cell death appears to be due to the ROS induced activation of the apoptotic pathway.

Finally, cyanines are a family of lipophilic cationic dyes that are commonly used in industry and scientific research. For example, they are used in solar cells, photographic films, computer storage devices, optical disks and video recording media.^{41,42} In biological research, cyanines are frequently used as inhibitors of extra-neuronal noradrenaline,⁴³ plasma membrane monoamine, and organic cation transporters and their fluorescent derivatives are commonly used in cellular imaging studies.^{44–48} In spite of the wide usage,

structural resemblance to MPP⁺ (Figure 1), the capacity to freely accumulate in the mitochondria of all cell types, and the ability to effectively inhibit the mitochondrial electron transport chain complex I (present study), neurotoxicological properties of cyanines and related compounds have not been reported to our knowledge. Based on the above findings, especially the remarkable mechanistic similarities of the toxicities of cyanines to that of well characterized both PD models MPP⁺ and rotenone, we propose that cyanines could be potential environmental neurotoxins that may contribute to the etiology of PD. However, obviously these *in vitro* findings must be confirmed with appropriate *in vivo* models to further advance these highly critical proposals.

Acknowledgments

Funding source. Funds to purchase an inverted fluorescent microscope in part is provided by the National Institutes of Health National Center for Research Resources INBRE Program [Grant P20 RR016475] (to KW).

Abbreviations used:

2,2'-Cyanine	1,1'-diethyl-2,2'-cyanine
4,4'-cyanine	1,1'-diethyl-4,4'-cyanine
DiOC2(3)	3,3'-diethyloxacarbocyanine
DiSC3(3)	3,3'-dipropylthiacarbocyanine
DiSC3(5)	3,3'-dipropylthiadibenzocarbocyanine
DiSBAC2(3)	bis-(1,3-diethylthiobarbituric acid) trimethine oxonol
DA	Dopamine
DAT	Dopamine transporter
DAPI	4',6-Diamidino-2-phenylindole diacetate
DCFH-DA	2',7'-dichlorofluorescein diacetate
DMEM	Dulbecco's Modified Eagles Medium
KRB	Krebs ringier buffer
MPP⁺	1-methyl-4-phenylpyridinium
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
PD	Parkinson's disease
ROI	Regions of interest
ROS	Reactive oxygen species
THI	tyrosine hydroxylase inhibitor L- α -methyltyrosine
TMRM	Tetramethylrhodamine, methyl ester

REFERENCES

- (1). Bove J, Prou D, Perier C, and Przedborski S (2005) Toxin-induced models of Parkinson's disease. *NeuroRx* 2, 484–494. [PubMed: 16389312]
- (2). Javitch JA, D'Amato RJ, Strittmatter SM, and Snyder SH (1985) Parkinsonism-inducing neurotoxin, N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine: uptake of the metabolite N-methyl-4-phenylpyridine by dopamine neurons explains selective toxicity. *Proc. Natl. Acad. Sci. U S A* 82, 2173–2177. [PubMed: 3872460]
- (3). Gainetdinov RR, Fumagalli F, Jones SR, and Caron MG (1997) Dopamine transporter is required for in vivo MPTP neurotoxicity: evidence from mice lacking the transporter. *J. Neurochem* 69, 1322–1325. [PubMed: 9282960]
- (4). Sotnikova TD, Beaulieu JM, Gainetdinov RR, and Caron MG (2006) Molecular biology, pharmacology and functional role of the plasma membrane dopamine transporter. *CNS Neurol. Disord. Drug Targets* 5, 45–56. [PubMed: 16613553]
- (5). Schapira AH, Cooper JM, Dexter D, Clark JB, Jenner P, and Marsden CD (1990) Mitochondrial complex I deficiency in Parkinson's disease. *J. Neurochem* 54, 823–827. [PubMed: 2154550]
- (6). Calhau C, Martel F, Hipolito-Reis C, and Azevedo I (2003) Effect of thiamine on 3H-MPP+ uptake by Caco-2 cells. *Pharmacol. Res* 48, 579–584. [PubMed: 14527822]
- (7). Engel K, and Wang J (2005) Interaction of organic cations with a newly identified plasma membrane monoamine transporter. *Mol. Pharmacol* 68, 1397–1407. [PubMed: 16099839]
- (8). Martel F, Ribeiro L, Calhau C, and Azevedo I (1999) Characterization of the efflux of the organic cation MPP+ in cultured rat hepatocytes. *Eur. J. Pharmacol* 379, 211–218. [PubMed: 10497908]
- (9). Zwart R, Verhaagh S, Buitelaar M, Popp-Snijders C, and Barlow DP (2001) Impaired activity of the extraneuronal monoamine transporter system known as uptake-2 in Orct3/Slc22a3-deficient mice. *Mol. Cell Biol* 21, 4188–4196. [PubMed: 11390648]
- (10). Goldman SM (2014) Environmental toxins and Parkinson's disease. *Annu. Rev. Pharmacol. Toxicol* 54, 141–164. [PubMed: 24050700]
- (11). Kadigamuwa CC, Le VQ, and Wimalasena K (2015) 2, 2'- and 4, 4'-Cyanines are transporter-independent in vitro dopaminergic toxins with the specificity and mechanism of toxicity similar to MPP(+). *J. Neurochem* 135, 755–767. [PubMed: 26094622]
- (12). Li N, Ragheb K, Lawler G, Sturgis J, Rajwa B, Melendez JA, and Robinson JP (2003) Mitochondrial complex I inhibitor rotenone induces apoptosis through enhancing mitochondrial reactive oxygen species production. *J. Biol. Chem* 278, 8516–8525. [PubMed: 12496265]
- (13). Bolam JP, and Pissadaki EK (2012) Living on the edge with too many mouths to feed: why dopamine neurons die. *Mov. Disord* 27, 1478–1483. [PubMed: 23008164]
- (14). Wimalasena K (2016) The inherent high vulnerability of dopaminergic neurons toward mitochondrial toxins may contribute to the etiology of Parkinson's disease. *Neural. Regener. Res*
- (15). Uversky VN (2004) Neurotoxicant-induced animal models of Parkinson's disease: understanding the role of rotenone, maneb and paraquat in neurodegeneration. *Cell Tissue Res* 318, 225–241. [PubMed: 15258850]
- (16). Betarbet R, Sherer TB, MacKenzie G, Garcia-Osuna M, Panov AV, and Greenamyre JT (2000) Chronic systemic pesticide exposure reproduces features of Parkinson's disease. *Nat. Neurosci* 3, 1301–1306. [PubMed: 11100151]
- (17). Hauser DN, and Hastings TG (2013) Mitochondrial dysfunction and oxidative stress in Parkinson's disease and monogenic parkinsonism. *Neurobiol. Dis* 51, 35–42. [PubMed: 23064436]
- (18). Choi HK, Won LA, Kontur PJ, Hammond DN, Fox AP, Wainer BH, Hoffmann PC, and Heller A (1991) immortalization of embryonic mesencephalic dopaminergic neurons by somatic cell fusion. *Brain Res* 552, 67–76. [PubMed: 1913182]
- (19). Wilkening S, Stahl F, and Bader A (2003) Comparison of primary human hepatocytes and hepatoma cell line Hepg2 with regard to their biotransformation properties. *Drug Metab. Dispos* 31, 1035–1042. [PubMed: 12867492]

- (20). Denizot F, and Lang R (1986) Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. *J. Immunol. Methods* 89, 271–277. [PubMed: 3486233]
- (21). Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods* 65, 55–63. [PubMed: 6606682]
- (22). Joshi DC, and Bakowska JC (2011) Determination of mitochondrial membrane potential and reactive oxygen species in live rat cortical neurons. *J. Vis. Exp.*
- (23). Iglesias-Gonzalez J, Sanchez-Iglesias S, Beiras-Iglesias A, Soto-Otero R, and Mendez-Alvarez E (2013) A simple method for isolating rat brain mitochondria with high metabolic activity: effects of EDTA and EGTA. *J. Neurosci. Methods* 213, 39–42. [PubMed: 23261657]
- (24). Zheng XX, Shoffner JM, Voljavec AS, and Wallace DC (1990) Evaluation of procedures for assaying oxidative phosphorylation enzyme activities in mitochondrial myopathy muscle biopsies. *Biochim. Biophys. Acta* 1019, 1–10. [PubMed: 2168748]
- (25). Birch-Machin MA, and Turnbull DM (2001) Assaying mitochondrial respiratory complex activity in mitochondria isolated from human cells and tissues. *Methods Cell Biol.* 65, 97–117. [PubMed: 11381612]
- (26). Oubrahim H, Stadtman ER, and Chock PB (2001) Mitochondria play no roles in Mn(II)-induced apoptosis in HeLa cells. *Proc. Natl. Acad. Sci. U S A* 98, 9505–9510. [PubMed: 11493712]
- (27). Adachi M, Higuchi H, Miura S, Azuma T, Inokuchi S, Saito H, Kato S, and Ishii H (2004) Bax interacts with the voltage-dependent anion channel and mediates ethanol-induced apoptosis in rat hepatocytes. *Am. J. Physiol. Gastrointest Liver Physiol* 287, G695–705.
- (28). Luo Y, Umegaki H, Wang X, Abe R, and Roth GS (1998) Dopamine induces apoptosis through an oxidation-involved SAPK/JNK activation pathway. *J. Biol. Chem* 273, 3756–3764. [PubMed: 9452508]
- (29). Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem* 72, 248–254. [PubMed: 942051]
- (30). Przedborski S, Jackson-Lewis V, Naini AB, Jakowec M, Petzinger G, Miller R, and Akram M (2001) The parkinsonian toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP): a technical review of its utility and safety. *J. Neurochem* 76, 1265–1274. [PubMed: 11238711]
- (31). WorldHealthOrganization. (1992) Rotenone health and safety guide, W. H. O., Geneva.
- (32). Alam M, and Schmidt WJ (2002) Rotenone destroys dopaminergic neurons and induces parkinsonian symptoms in rats. *Behav. Brain Res* 136, 317–324. [PubMed: 12385818]
- (33). McCormack AL, Thiruchelvam M, Manning-Bog AB, Thiffault C, Langston JW, Cory-Slechta DA, and Di Monte DA (2002) Environmental risk factors and Parkinson's disease: selective degeneration of nigral dopaminergic neurons caused by the herbicide paraquat. *Neurobiol. Dis* 10, 119–127. [PubMed: 12127150]
- (34). Joshi SG, Cooper M, Yost A, Paff M, Ercan UK, Fridman G, Friedman G, Fridman A, and Brooks AD (2011) Nonthermal dielectric-barrier discharge plasma-induced inactivation involves oxidative DNA damage and membrane lipid peroxidation in *Escherichia coli*. *Antimicrob Agents Chemother.* 55, 1053–1062. [PubMed: 21199923]
- (35). Klapperstuck T, Glanz D, Klapperstuck M, and Wohlrab J (2009) Methodological aspects of measuring absolute values of membrane potential in human cells by flow cytometry. *Cytometry A* 75, 593–608. [PubMed: 19504578]
- (36). McNaught KS, Thull U, Carrupt PA, Altomare C, Cellamare S, Carotti A, Testa B, Jenner P, and Marsden CD (1995) Inhibition of complex I by isoquinoline derivatives structurally related to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). *Biochem. Pharmacol* 50, 1903–1911. [PubMed: 8615871]
- (37). Gluck MR, Krueger MJ, Ramsay RR, Sablin SO, Singer TP, and Nicklas WJ (1994) Characterization of the inhibitory mechanism of 1-methyl-4-phenylpyridinium and 4-phenylpyridine analogs in inner membrane preparations. *J. Biol. Chem* 269, 3167–3174. [PubMed: 8106350]

- (38). Vermeer LM, Florang VR, and Doorn JA (2012) Catechol and aldehyde moieties of 3,4-dihydroxyphenylacetaldehyde contribute to tyrosine hydroxylase inhibition and neurotoxicity. *Brain Res.* 1474, 100–109. [PubMed: 22877852]
- (39). Linsenbardt AJ, Breckenridge JM, Wilken GH, and Macarthur H (2012) Dopaminochrome induces caspase-independent apoptosis in the mesencephalic cell line, MN9D. *J. Neurochem* 122, 175–184. [PubMed: 22486217]
- (40). Wimalasena DS, Wiese TJ, and Wimalasena K (2007) Copper ions disrupt dopamine metabolism via inhibition of V-H⁺-ATPase: a possible contributing factor to neurotoxicity. *J. Neurochem* 101, 313–326. [PubMed: 17217412]
- (41). Mustroph H, Stollenwerk M, and Bressau V (2006) Current developments in optical data storage with organic dyes. *Angew Chem. Int. Ed. Engl* 45, 2016–2035. [PubMed: 16518782]
- (42). Wurthner F, Kaiser TE, and Saha-Moller CR (2011) J-aggregates: from serendipitous discovery to supramolecular engineering of functional dye materials. *Angew Chem. Int. Ed. Engl* 50, 3376–3410. [PubMed: 21442690]
- (43). Russ H, Sonna J, Keppler K, Baunach S, and Schomig E (1993) Cyanine-related compounds: a novel class of potent inhibitors of extraneuronal noradrenaline transport. *Naunyn Schmiedebergs Arch. Pharmacol* 348, 458–465. [PubMed: 8114944]
- (44). Park J (2000) AM1 semiempirical calculated potential energy surfaces for the isomerization of symmetrical carbocyanines. *Dyes and Pigments* 46, 155–161.
- (45). Schomig E, Babin-Ebell J, and Russ H (1993) 1,1'-diethyl-2,2'-cyanine (decynium22) potently inhibits the renal transport of organic cations. *Naunyn Schmiedebergs Arch. Pharmacol* 347, 379–383. [PubMed: 8510766]
- (46). Chen Y, Gryshuk A, Achilefu S, Ohulchansky T, Potter W, Zhong T, Morgan J, Chance B, Prasad PN, Henderson BW, Oseroff A, and Pandey RK (2005) A novel approach to a bifunctional photosensitizer for tumor imaging and phototherapy. *Bioconjug. Chem* 16, 1264–1274. [PubMed: 16173807]
- (47). Uchida S, Endo S, Akita K, Ohta T, and Fukuda S (2012) The cyanine Dye NK-4 improves scopolamine-induced memory impairments in mice. *Biol. Pharm. Bull* 35, 1831–1835. [PubMed: 23037173]
- (48). Ohta H, Arai S, Akita K, Ohta T, and Fukuda S (2011) Neurotrophic effects of a cyanine dye via the PI3K-Akt pathway: attenuation of motor discoordination and neurodegeneration in an ataxic animal model. *PLoS One* 6, e17137. [PubMed: 21347252]

Figure 1.

Structures of toxins. A, 1-methyl-4-phenylpyridinium (MPP^+); B, 1,1-diethyl-2,2'-cyanine (2,2' - cyanine); C, 1,1-diethyl-4,4'-cyanine (4,4' - cyanine); D, 3,3'-diethyloxacarbocyanine [DiOC2(3)]; E, 3,3'-dipropylthiacarbocyanine [DiSC3(5)]; F, 3,3'-dipropylthiadibenzocyanine [DiSC3(5)]; G, bis-(1,3-diethylthioberbituric acid trimethine oxonol [DiSBAC2(3)]; H, rotenone

Figure 2.

Relative MN9D and HepG2 cell toxicities of cationic cyanines, anionic DiSBAC2(3), and rotenone. Cells were grown in 96-well plates and were treated with desired concentrations of toxin in KRB-HEPES for 12 h at 37 °C. Cell viabilities were determined by MTT assay as detailed in Experimental Procedures. Results are expressed as % cell viabilities with respect to parallel controls which were carried out under similar experimental conditions except that the toxins were excluded from the incubations. Data are presented as mean $\pm$ SD (n=6). *p<0.005 and #p 0.025 versus controls without toxins. (A) MN9D and (B) HepG2 cell toxicities of cyanines and anionic DiSBAC2(3); (C) MN9D and HepG2 cell toxicities of rotenone under similar experimental conditions.

Figure 3.

Characteristics of the cellular uptake and localization of cyanines and anionic DiSBAC2(3). (A) Concentration dependency of the cellular uptake into MN9D and HepG2 cells: MN9D cells were grown in 12-well plates and incubated with desired concentration of the dye in KRB-HEPES at 37 °C for 1 h and the intracellular dye levels were quantified by fluorescence as detailed in Experimental Procedures. The data were normalized to respective protein concentrations of each sample and presented as mean $\pm$ S.D (n=3). *p < 0.005 and #p < 0.003 versus the background fluorescence of controls without cyanine (B) Mitochondrial localization of cationic cyanine DiSC3(5) in MN9D and HepG2 cells: Cells grown in glass bottomed plates were incubated with 100 nM DiSC3(5) in KRB-HEPES for 15 min at 37 °C. The incubation media was removed, cells were washed with the same buffer and incubated with 100 nM of Mitotracker Green in KRB-HEPES for 15 min at 37 °C. Then, cells were washed, reconstituted with KRB-HEPES and the confocal fluorescence images of cells were captured at corresponding wavelengths of various dyes as detailed in Experimental Procedures. (C) Anionic DiSBAC2(3) is not localized into the mitochondria of MN9D or HepG2 cells: Cell were treated identically to that of DiSC3(5) above, except that DiSC3(5) was replaced with the same concentrations of DiSBAC2(3) and Mitotracker Green was replaced with Mitotracker Red.

Figure 4.

The effects of cyanines, anionic DiSBAC2(3), and rotenone on the mitochondrial membrane potential, mitochondrial complex I inhibition and intracellular ATP levels. (A) The effects on the mitochondrial membrane potential of MN9D and HepG2 cells: Cells were treated with 2.5 μ M cyanines, negative dye or rotenone and mitochondrial membrane potentials were determined by using the fluorescent probe TMRM (Ex/Em 543/573 nm) as detailed in Experimental Procedures (arrows indicate the time points of the toxin addition). (B) Inhibition of the rat brain mitochondrial complex I: Isolated rat brain mitochondria were osmotically disrupted and incubated with cyanines, MPP⁺, or rotenone and 10 mM NADH for 2 min at 37 °C, and the rate of ubiquinone dependent oxidation of NADH was measured by following the decrease of the absorbance at 340 nm for a period of 5 min as described in Experimental Procedures. In all experiments, 10 μ M fixed concentration of cyanines, DiSBAC2(3) or rotenone were used. In the case of MPP⁺ a 200 μ M concentration was used. (C) Effects on the intracellular ATP levels in MN9D and HepG2 cells: Cells were grown to 70–80 % confluence in 12 well plates and treated with 100 nM DiSC3(5), 250 nM DiSBAC2(3) or 25 nM rotenone for 6 h at 37 °C and intracellular ATP levels were quantified as detailed in Experimental Procedures. All the intracellular ATP levels were normalized to respective protein concentrations and data presented as mean $\pm$ S.D (n = 3). *p 0.0002, #p 0.002 and **p 0.01 versus respective controls.

Figure 5. Effect of cyanines, rotenone and anionic DiSBAC2(3) on the intracellular and mitochondrial ROS levels. (A) Effect on the intracellular ROS levels of MN9D and HepG2 cells: Cells were grown in 12-well plates and treated with varying concentrations of dye or rotenone for 1 h at 37 °C in KRB-HEPES and intracellular ROS levels were quantified by the 2',7'-dichlorofluorescein diacetate (DCFH-DA) method as detailed in Experimental Procedures. The data were normalized to respective protein concentrations of each samples and represent as mean $\pm$ S.D (n=3). Significance of difference was determined with t-test * p < 0.0001, # p < 0.0005, ** p < 0.003 and + p < 0.01 compared with controls; (B) Effect of cationic cyanine DiSC3(5) on the mitochondrial ROS levels: MN9D cells were grown in glass bottomed plates and treated with 10 μ M DCFH-DA for 1 h at 37 °C in KRB-HEPES. Then, cells were washed and incubated with 1 μ M DiSC3(5) for 1 h at 37 °C in with KRB-HEPES, washed, reconstituted with KRB-HEPES and fluorescence images of cells were recorded at the corresponding wavelengths. The bottom row show the selected enlarged single cell images (for clarity).

Figure 6.

The effects of the DA depletion or ascorbate pre-incubation on the cyanine and rotenone mediated ROS productions in MN9D cells. (A) The effect of DA depletion on the cyanine and rotenone mediated ROS productions in MN9D cells: MN9D cells were pre-incubated with the desired concentration of THI in KRB-HEPES for 24 h at 37 °C. Then, cells were incubated in a medium containing 500 nM cyanine or rotenone and the same concentrations of THI for an additional 1 h at 37 °C in KRB-HEPES and intracellular ROS levels were quantified by the DCFH-DA method as detailed in Experimental Procedures. Data were normalized to the protein concentration of the respective samples and presented as mean $\pm$ S.D (n=3). Significance of difference was determined with t-test * p 0.005, # p 0.01 and ** p 0.005 compared with controls; (B) The effect of the anti-oxidant, ascorbate, on the cyanine or rotenone mediated ROS productions in MN9D cells: MN9D cells were pre-incubated with the desired concentration of sodium ascorbate (Asc) for 1 h at 37 °C in KRB-HEPES. Then, cells were incubated in a medium containing 500 nM cyanine or rotenone and the same concentrations of Asc for an additional 1 h at 37 °C in KRB-HEPES and intracellular ROS levels were quantified by the DCFH-DA method as detailed in Experimental Procedures. Data were normalized to the protein concentration of the respective samples and presented as mean $\pm$ S.D (n=3). Significance of difference was determined with t-test * p 0.002, # p 0.005, ** p 0.01 and + p 0.05 compared with controls.

Figure 7.

The effect of DA depletion or ascorbate pre-treatment on the MN9D toxicities of cyanines and rotenone. (A and B) The effect of DA depletion: MN9D cells were pre-incubated with 6 mM THI for 24 h at 37 °C in KRB-HEPES to deplete intracellular DA levels. Then, DA depleted cells or regular cells were incubated with (A) 0–1000 nM cyanines or (B) 0–100 nM rotenone in the presence of the same respective concentration of THI in KRB-HEPES for 12 h at 37 °C, and cell viabilities were determined by MTT assay as detailed in Experimental Procedures. (C and D) The effect of anti-oxidant, ascorbate pre-treatment: MN9D cells were pre-incubated with 500 μ M Asc for 1 h at 37 °C in KRB-HEPES. Then, ascorbate treated cells were incubated with (C) 0–1000 nM cyanines or (D) 0–100 nM rotenone in the presence of the same respective concentration of ascorbate in KRB for 12 h at 37 °C, and cell viabilities were determined by MTT assay. Data represent as mean $\pm$ S.D (n=6). Significance of difference was determined with t-test * p 0.0001, # p 0.001 and + p 0.005 compared with controls.

Figure 8.

Cyanines, rotenone and MPP⁺ toxicities cause MN9D cell shrinkage and nuclear chromatin condensation. Cells were grown in glass bottomed plates and incubated with cyanines (all 1 μ M), rotenone (1 μ M) or MPP⁺ (200 μ M) in KRB-HEPES for 12 h at 37 °C. Then, cells were washed and incubated with 300 nM DAPI for 15 min at 37 °C and cell shrinkage was visualized by light microscopy. Apoptotic chromatin condensation was visualized by monitoring the typical increase in nuclear DAPI fluorescence (Ex/Em 358/461 nm) as detailed in Experimental Procedures. Control cells were treated similarly except that the toxins were excluded from the initial incubation medium. (A) Morphology (B) DAPI Fluorescence of control and toxin treated MN9D cells.