Imidazolium Salt (DBZIM) Reduces Gliosis in Mice Treated with Neurotoxicant 2'-CH₃-MPTP

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We have recently identified a class of imidazolium salts (IMSSs) with antioxidative property and can function as scavengers for radical oxygen species (ROS) [18]. Here, we investigate one of the IMSSs, 1,3-bisbenzylimidazolium bromide (DBZIM), for its possible role in attenuating neurotoxicity and gliosis in the retina and the brain induced by a Parkinsonian neurotoxicant, methyl-4(2'-methylphenyl)-1,2,3,6-tetrahydropyridine (2'-CH₃-MPTP), which is a free radical generating agent. In this study, we employ a molecular retinal imaging method, which we recently developed in a transgenic mouse model expressing green fluorescent protein (GFP) under the control of glial fibrillary acidic protein (GFAP) promoter [14], to assess the efficacy of DBZIM, since currently no *in vitro* system with a sufficient complexity is available for accurately assessing a compound's efficacy. The longitudinal imaging results showed DBZIM can effectively suppress the neurotoxicant-induced retinal gliosis. Immunohistochemistry performed on the postmodern mouse brain confirmed that DBZIM also reduced striatal gliosis, and concomitantly attenuated the loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc). These findings suggest that DBZIM could be a useful small molecular compound for studying neurotoxicity and neuroprotection in the retina and the brain.

The retina is composed of several layers of neurons and glia (astrocytes and Müller cells), and is known to be affected in a number of neurodegenerative disorders, including Parkinson's disease (PD), Alzheimer's disease (AD), and multiple sclerosis (MS). Therefore, the health status of the central nervous system (CNS) can be indirectly assessed by a retinal scan in patients. In mouse model of disease, the retina can also be used as a window to facilitate the study of neurological disorders and the evaluation of therapeutic agents, for example, for Huntington's disease [1].

Currently PD is regarded as the second most common degenerative disorder of the aging brain, with AD being the most common. PD is characterized by symptoms of tremor at rest, slowness of voluntary movements, rigidity and postural instability. At the cellular level, a preferential loss of dopaminergic (DA) neurons in the nigrostriatal dopaminergic pathway is a hallmark pathology, resulting in a dopamine deficit in the brain. Since early 1980s, 1-methyl-4-phenyl 1,2,3,6-tetrahydropyridine (MPTP)

has been widely used to generate animal models of PD for both mechanistic study and therapeutic development [2,3]. It was found that the MPTP-induced PD models closely mimic the DA neuronal loss in the substantia nigra pars compacta (SNpc) and reactive gliosis in the striatum as observed in human PD patients [4].

PD is also associated with the neurodegeneration of the visual system in PD patients. Considerable psychophysical and electrophysiological evidence suggests that visual dysfunction in PD patients results from DA deficiency, possibly in the retina [5]. Since its identification in the rabbit retina (amacrine neurons) and choroid [6], dopamine has been recognized as a neurotransmitter playing a critical role in visual perception [7]. Wong et al. [8] showed that systemic administration of MPTP into rabbit impaired the DA metabolism, and consequently reduced the amplitude of the "b-wave" of the electroretinogram (ERG) and light signal transmission through the optic nerve. Subsequently, systemic injection of MPTP was also found to damage the DA amacrine neurons

in the retina of mice [9,10] and monkey [11]. In all cases, the MPTP neurotoxicity was inevitably accompanied by gliosis, exemplified by an increase in the astrocytic marker glial fibrillary acidic protein (GFAP) in the retinal glia [12]. These early findings suggested that retina represents a good venue for studying MPTP neurotoxicity in Parkinsonism using molecular optical imaging technique.

Based on an early proposal by O'Callaghan [13] that GFAP could be used as a reliable marker for profiling neurotoxicity and gliosis, we have successfully established a noninvasive molecular retinal imaging system for longitudinal monitoring and quantifying kainic acid-induced neurotoxicity and gliosis in the retina of a transgenic mouse model [14–16], without using an exogenous dye [17]. In the current study, we noninvasively measured retinal toxicity and evaluated a synthetic antioxidative imidazolium salt, 1,3-bisbenzylimidazolium bromide (DBZIM) [18] for its ability to suppress neurotoxicity and to attenuate DA neuronal loss in the MPTP-induced mouse PD model.

Experimental Procedures

Transgenic GFAP-GFP Mice

The generation and genotyping of the transgenic glial fibrillary acidic protein-green fluorescent protein (GFAP-GFP) mice were performed as previously described [16]. Adult mice (8–10 weeks old) of FVB/N background were used in this study. Animal husbandry was provided by the Biological Resource Center at Biopolis, Singapore. The experimental protocol for this study was approved by the Institutional Animal Care and Use Committee.

Compound Dosing

The synthesis of DBZIM (Fig. 1) is described separately [18]. The neurotoxicant 2'-CH₃-MPTP, which is a more potent analog of MPTP for inducing PD in the adult brain [19], was purchased from Sigma-Aldrich (M103; St. Louis, MO, USA). The dosing and imaging regime is illustrated in Fig. 2. Mice ($n = 5$) in the neurotoxicant-treated group were injected with 2'-CH₃-MPTP only (4 i.p. injections at 15 mg/kg in saline, once every 2 h). Mice ($n = 5$) in the DBZIM/neurotoxicant-treated group received an intraperitoneal (i.p.) injection of DBZIM (20 mg/kg in saline) 24 h before and after the 2'-CH₃-MPTP administration (4 i.p. injections at 15 mg/kg in saline, once every 2 h). Mice ($n = 6$) injected with saline were used as a vehicle control group. Retinal imaging was performed before and after treatment. For assays on TUNEL staining and superoxide dismutase (SOD) activ-

Figure 1 Chemical structure of 1,3-bisbenzylimidazolium bromide (DBZIM).

ity, additional twenty mice (five for each of the saline, 2'-CH₃-MPTP, DBZIM/2'-CH₃-MPTP, and DBZIM groups) were similarly dosed according to the scheme in Fig. 2.

Mouse Preparation and Scanning Laser Ophthalmoscope (SLO) Imaging

Mouse was anesthetized with i.p. injection (0.15 mL/10 g body weight) of Avertin (1.5% 2,2,2-tribromoethanol, T48402, Sigma-Aldrich St. Louis, MO, USA). The mouse pupil was dilated with a drop of 0.5% Cyclogyl[®] sterile ophthalmic solution (cyclopentolate hydrochloride, Alcon[®], Puurs, Belgium). Custom-made polymethylmethacrylate (PMMA) hard contact lenses (Cantor & Nissel, Northamptonshire, UK) were used to correct spherical optical aberration and to minimize dehydration of the cornea during imaging. Careful examination by an eye specialist before SLO imaging ruled out the presence of any corneal or lens opacity. For imaging hardware, the second version of Heidelberg Retina Angiograph (HRA II) SLO (Heidelberg Engineering, Dossenheim, Germany) was modified for use on mice. Detailed procedures for imaging and signal quantification were described in our recent publication [14]. The quantified signal values were expressed as mean $\pm$ SEM, and statistically tested using Student's two-tailed *t*-test.

Retinal Whole-Mount and Brain Immunohistochemistry

After a cardiac perfusion with 4% paraformaldehyde (in 1 $\times$ PBS, pH 7.4), the mouse brain and eye were harvested for fixation in 4% paraformaldehyde overnight at 4°C. The fixed retina, free of the choroid and sclera tissues, was whole-mounted on a glass slide using Vectorshield mounting medium for fluorescence (Vector Laboratories, Inc., Burlingame, CA, USA; H1000). After fixation in 4% paraformaldehyde, the fixed brain was soaked in 30% sucrose at 4°C overnight, and subsequently embedded in OTC freezing medium (Fisher Scientific, Pittsburgh, PA, USA). Coronal cryosections (5 μ m) of the SNpc (bregma -3.16 mm, interaural

	Saline	2'-CH ₃ -MPTP	DBZIM/ 2'-CH ₃ -MPTP	DBZIM
0 h	Pre-treatment imaging	Pre-treatment imaging	Pre-treatment imaging	Pre-treatment imaging
24 h	Saline	Saline	DBZIM (1 × 20 mg/kg)	DBZIM (1 × 20 mg/kg)
48 h	Saline	2'-CH ₃ -MPTP (4 × 15 mg/kg, once every 2 h)		Saline
72 h	Saline	Saline	DBZIM (1 × 20 mg/kg)	DBZIM (1 × 20 mg/kg)
96 h	Post-treatment imaging	Post-treatment imaging	Post-treatment imaging	Post-treatment imaging

Figure 2 Compound dosing and retinal imaging scheme.

0.64 mm) and striatum (bregma 0.62 mm, interaural 4.42 mm), according to the Atlas of Mouse Brain [20], were prepared on a cryostat (Leica Microsystems, Nussloch GmbH; CM-3050S). For retina whole-mount immunohistochemistry (IHC), a rabbit polyclonal antibody against GFAP (Z0334; Dako, Carpinteria, CA, USA) in 1:200 dilution was used. For brain IHC, a rabbit polyclonal antibody against tyrosine hydroxylase (TH) (Chemicon, CA, USA; Ab-152) in 1:200 dilution was used. The two bound primary antibodies on the retinal and brain sections were stained separately with a goat IgG conjugated to Texas-red (Abcam, Ab7088) at 1:100 dilution at room temperature for 2 h, and visualized using confocal microscopy (LSM 510 META, Carl Zeiss Microimaging GmbH, Jena, Germany). The total GFP intensity from the retina as well as the number of astrocyte cell bodies in the striatum are quantified using MATLAB[®] and its Image Processing Toolbox[™].

TUNEL Staining of SNpc

Sagittal hemispheres of the mouse brains were collected and fixed overnight in 4% paraformaldehyde (in 1 × PBS, pH 7.4). After fixation, they were embedded in paraffin and sectioned in 6 μm thickness. The SNc region was lo-

cated such that the hippocampal structure in the paraffin section is comparable to that of the reference section at Interaural and Bregma coordinates of around 0.72 mm and −3.08 mm, respectively (The Mouse Brain Atlas by George Paxinos and Keith B.J. Franklin). TUNEL staining was performed using ApopTag Peroxidase *In Situ* Apoptosis Detection Kit (CHEMICON). Tissue sections were deparaffinized with xylene, rehydrated in a series of descending ethanol concentrations. The procedure of TUNEL staining is essentially the same as manufacturer's instructions except that the pretreatment of tissue with proteinase K was replaced with microwaving in antigen retrieval buffer (Tris-EDTA, pH 9.0), followed by 0.5% Triton X-100 incubation. The color was developed using DAB substrate kit (SK-4100, VECTOR). After washing with PBS for 3 times, immunostaining was then performed using a rabbit antibody against TH (1:200, AB152, CHEMICON), followed with a donkey anti-rabbit Alexa Fluor 488 (1:250, Molecular Probes).

SOD Activity of Striatum

One half of a striatum (from one hemisphere) from each mouse was homogenized in 1X RIPA Buffer containing 0.5 M Tris-HCl, pH 7.4, 1.5 M NaCl, 2.5% deoxycholic

acid, 10% NP-40 and 10 mM EDTA. SOD enzymatic activity in the tissue was determined using SOD assay kit from Cayman Chemical (MI, USA) according to manufacturer's instruction. The levels of SOD activity (U/mg of protein) from different groups were compared to the saline control (normalized to 1).

Results

In Vivo Detection and Quantification of Retinal Gliosis

For easy illustration, the compound dosing and imaging scheme is depicted in Fig. 2. Representative retinal images for mice from different groups (saline, 2'-CH₃-MPTP, and DBZIM/2'-CH₃-MPTP) are shown in Fig. 3. The GFP-labeled astrocytes (Fig. 3A, arrows) of the optic disc can be reliably identified in all treatment groups. A remarkably consistent pattern of retinal images can

be appreciated for the same eye, even acquired at different time points, for example, at day 0 (Fig. 3A) and day 4 (Fig. 3D). Furthermore the vasculature such as retinal vein (arrow head, Figs. 3B and E) and blood vessels (empty arrow heads, Fig. 3B and E) are clearly discernable. The ability to qualitatively identify these landmark features *in vivo* prompted us to employ this imaging method to evaluate the effect of compounds in the retina. As expected, saline alone had no influence on the GFP signal (or gliosis) level over the course of 4 days (Figs. 3A and D). However, a systemic (i.p.) administration of 2'-CH₃-MPTP caused a drastic increase in GFP fluorescence on the mouse optic disc 2 days post-dosing (comparing Figs. 3B and E). Regions with the highest GFP fluorescence were mainly located in the fringe of the optic disc where axon fibers and retinal vasculature are congregated with retinal glia. Most interestingly, mice received two doses of the DBZIM (i.p., 20 mg/kg) 24 h before and after the 2'-CH₃-MPTP

Figure 3 Longitudinal imaging of GFP-labeled retinal glia in live mice. Representative retinal images acquired from the same eye of a saline-treated mouse at 0 h (A) and 96 h (D). Retinal images from the same eye of a 2'-CH₃-MPTP-treated mouse at 0 h (B) and 96 h (E). Retinal images from the same eye of a mouse treated with DBZIM and 2'-CH₃-MPTP, respectively at 0 h (C) and 96 h (F). The retinal vein (arrow head) and blood vessels (empty arrow heads) are clearly recognizable for the same eye acquired

at different times (0 vs. 96 h in B and E). It is noted that the maximal GFP fluorescence is seen in astrocytes (arrows) around the fringe of the optic disc (A). Treatment with 2'-CH₃-MPTP triggered a significant increase in the GFP signal (E) at 96 h (or 48 h after the neurotoxicant dosing), when compared to the basal level (B). Co-treatment of the mouse with DBZIM abolished the 2'-CH₃-MPTP-induced toxicity (F). Scale bar = 100 μ m.

Figure 4 Quantification of retinal GFP fluorescence intensity (FI). Raw retinal images were further processed and quantified for transgenic mice treated with saline ($n=6$), 2'-CH₃-MPTP ($n=5$), or DBZIM plus 2'-CH₃-MPTP ($n=5$). The neurotoxicant 2'-CH₃-MPTP induced a significant increase in GFP signal (* $P < 0.05$), whereas DBZIM abolished the 2'-CH₃-MPTP-induced retinal gliosis at 96 h (or 48 h after the neurotoxicant dosing).

injection showed no apparent gliosis (comparing Figs. 3C and F), suggesting a neurotoxicity-suppressing effect by DBZIM. To ensure that DBZIM itself would not cause any gliosis/neurotoxicity, we also injected a group of mice with two doses of DBZIM (i.p., 20 mg/kg) alone at 24 and 72 h, respectively (see scheme in Fig. 2), and confirmed the lack of reactive gliosis in the retina, striatum, SNpc, and cerebellum of the brain (data not shown).

To achieve authentic signal quantification of the retinal gliosis, multiple raw images acquired from the same eye of a living mouse need to be properly processed further

prior to signal quantification using our proprietary algorithm [15], since each of the 45 individual raw images in a stack (from a single retina) has a distinct X-Y-Z position, primarily due to the continuous motion of breathing and heartbeat of the anesthetized mouse. The quantification revealed a 36% increase ($P < 0.05$) in the retinal GFP signal from the 2'-CH₃-MPTP-treated mice and a lack of signal increase in the DBZIM/2'-CH₃-MPTP-treated mice (Fig. 4).

Retinal IHC Confirmed *In Vivo* Imaging Observations

To prove that the GFP signal noninvasively collected from the retina originated from the retinal glia, but not from other retinal cell types, we performed IHC on the retinal tissues *ex vivo*. Merged confocal images of retinal whole-mount stained for GFAP and GFP showed a basal signal intensity (set a 100%) in the saline group (Fig. 5A), whereas the whole-mount from the 2'-CH₃-MPTP group had a much higher staining intensity (Fig. 5B), that is, about 190.7% by signal. In line with the *in vivo* observation, the gliosis (neurotoxicity)-suppressing effect of the DBZIM was also observed in the retinal whole-mount (Fig. 5C), as judged by the dim staining intensity comparable to that in the saline control (Fig. 5A). When the area of interest (ROI) encompassing mostly the optic disc in the *ex vivo* retinal tissues (Figs. 5A-C), similar to the *in vivo* images (Fig. 3), was quantified using the MATLAB program, the intensity readings obtained were 100%, 190.7%, and 98.8%

Figure 5 Retinal whole-mount staining (merged green and red) from mice with different treatments. (A) In mouse treated with saline, retinal astrocytes and Müller cells expressed basal level of GFP and GFAP. (B) In the 2'-CH₃-MPTP-treated mouse, the retinal glia expressed significantly higher

level of GFP and GFAP, when compared the saline control. (C) Treatment with DBZIM abolished the 2'-CH₃-MPTP-induced signal increase. Scale bar = 200 μ m. Circle centering the optic disc denotes the ROI for GFP fluorescence quantification by MATLAB software.

Figure 6 Transverse view of retinas from mice with different treatments. (A) Retina from saline control mouse showed basal expression for GFP and GFAP in Müller cells at the inner nuclear layer (INL) and astrocytes at the ganglion cell layer (GCL). (B) Treatment with 2'-CH₃-MPTP significantly induced both GFP and GFAP proteins in the two glial cell types. (C) Treat-

ment with DBZIM significantly reduced GFP closer to the basal level. It was noted the GFAP level in the DBZIM group was still quite significant at 96 h after the compound dosing, which is probably due to the highly stable GFAP fiber with a longer half life. Scale bar = 50 μ m.

for Figs. 5A–C, respectively. The staining of transverse retinal sections further confirmed the induction of gliosis by 2'-CH₃-MPTP (Fig. 6B) and the suppression of gliosis by DBZIM (Fig. 6C), specifically in the retinal astrocytes and Müller cells (Fig. 6A). Similarly, the MATLAB fluorescence readings at the transverse images were 100%, 213.6%, and 130.7% for Figs. 6A–C, respectively.

DBZIM Reduced 2'-CH₃-MPTP-Induced Gliosis and DA Neuronal Loss in the Brain

It was well documented that MPTP or its potent analog 2'-CH₃-MPTP, can trigger severe gliosis in the striatum and cause DA neuronal loss in the SNpc. To investigate the effects of the compounds in the brain, mice of all groups (saline, 2'-CH₃-MPTP, and DBZIM/2'-CH₃-MPTP) were sacrificed after the final *in vivo* imaging at 96 h for brain IHC. A moderate number (set at 100% by MATLAB) of astrocytes with basal GFP expression (green) were found in the striatum of the saline group (Fig. 7A), and in contrast an increased number (541.6% by MATLAB counting) of reactive astrocytes with thickened processes and elevated GFP expression were seen in the striatum of the 2'-CH₃-MPTP group (Fig. 7B). In line with the observation for retina, DBZIM treatment suppressed the 2'-CH₃-MPTP-induced gliosis in striatum (Fig. 7C), and kept the number of astrocytes unchanged in this case (Fig. 7C, 100.0% by MATLAB) as in the saline group (Fig. 7A). Finally we observed the protection of DA neurons (stained for TH) by DBZIM in the SNpc (Fig. 8B), placing the number of DA neurons between

the saline group (Fig. 8A) and the 2'-CH₃-MPTP group (Fig. 8C).

Discussion

Currently several drugs are available for PD management. However all these drugs are symptomatic by nature and associated with significant side effects. This situation underlines the fact that an unmet medical need persists for this major neurological disorder. With recent technological advancement, a number of noninvasive methodologies have been developed to aid the study and therapeutic development of PD. Notably, the advanced three-dimensional imaging tools, such as MRI and PET, have proven to be rather effective for studying neurotoxicity and developing therapeutics in models of PD [21]. However, the requirement for using a contrast agent or a labeled ligand and the cost associated with these intricate instruments limit their wide-spread and routine use in an independent laboratory setting.

In search for an alternative noninvasive imaging method to investigate the ocular consequence in Parkinsonism or other neurological disorders, we developed a molecular retinal imaging system, comprising of a GFAP-GFP mouse [16], a confocal scanning laser ophthalmoscope, and an image processing algorithm [14,15]. Results from the current study showed that this imaging system can be applied to study retinopathy in a neurotoxicant (2'-CH₃-MPTP)-induced mouse PD model. In particular, we are able to longitudinally image and quantify gliosis in the retinal glia in response to a known Parkinsonian

Figure 7 DBZIM attenuates 2'-CH₃-MPTP-induced gliosis in striatum. (A) The striatal astrocytes (arrow heads) in the saline control group express a basal level of GFP, indicating a lack of toxicity. The DA axons, labeled by an antibody against TH, appear as dots (arrows). (B) 2'-CH₃-MPTP induces

severe gliosis in the striatum, as indicated by the dramatic increase in GFP signal and astrocyte number. (C) DBZIM significantly suppresses the 2'-CH₃-MPTP neurotoxicity. Scale bar = 50 μ m.

Figure 8 DBZIM attenuates DA neuron depletion in SNpc. (A) Staining with an antibody against TH reveals a normal DA neuronal cluster in the SNpc. The resting astrocytes are labeled by the GFP. (B) A significant number of the DA neurons have been acutely ablated from the SNpc by the neurotoxic treatment, judging from the thinning of DA neuronal cluster, and accompanied by gliosis (an increase in the number of astrocytes in the region). (C) A significant portion of the DA neurons is protected by DBZIM from 2'-CH₃-MPTP-induced depletion. Scale bar = 200 μ m.

toxin 2'-CH₃-MPTP. This technical achievement conceptually validates our method of primarily screening compounds with neuroprotective property by simply measuring the transgenic GFP signal, which is an established surrogate for the endogenous pathological marker GFAP. The theoretical basis for this approach is based on an early concept by O'Callaghan [22] and a large body of literature by others in subsequent years that GFAP can be used as an early and sensitive biomarker for neurotoxicity.

Oxidative stress by the reactive oxygen species (ROS) is believed to play an important role in the pathogenesis of neurodegenerative diseases including PD. Chronic oxidative stress and inflammatory response have been observed particularly in the SNpc of Parkinsonian brains and in the animal models of PD [23–26]. This includes alterations in the activities of the antioxidative enzymes, SOD, and catalase [27], a decrease in the level of glutathione (GSH) [28], a proliferation of reactive microglia with the production and release of a number of pro-inflammatory cytokines, such as tumor necrosis factor- α (TNF- α), interleukins [29], along with an increase in the intracellular iron within the reactive microglia and the melanin-containing DA neurons [30]. In recognition of the oxidation theory in PD etiology, antioxidative [31,32] and anti-inflammatory [26,33] approaches have been devised as therapeutic strategies to combat PD in MPTP-induced animal models.

Imidazolium salts (IMs) can exist in an equilibrium between IMs and *N*-heterocyclic carbines (NHCs) or bis-imidazolidine especially in aqueous phase. The bis-imidazolidine is known as a super-electron-donor

Figure 9 DBZIM moderately rescues striatal SOD activity. 2'-CH₃-MPTP decreased the SOD activity to 80% of the saline control level ($***P < 0.001$), whereas the SOD activity in the DBZIM/2'-CH₃-MPTP group was rescued to 86% of the control ($**P < 0.01$). No difference in the SOD activity between the saline and the DBZIM groups.

(S.E.D.) reagent. We have recently characterized a group of IMSs and found them to be antioxidative [18] in cultured hepatic stellate cells (HSCs). Based on these preliminary *in vitro* observations, we hypothesized that these small antioxidative molecules (<500 daltons) may easily pass through the blood-brain-barrier (BBB) and blood-retina-barrier (BRB) and exert neuroprotective properties *in vivo*. To test this postulate, we directly administered one of the IMSs, DBZIM (Fig. 1) to the 2'-CH₃-MPTP-induced mouse PD model to assess its ability to attenuate neurotoxicity and protect DA neuronal loss, since there is currently no suitable *in vitro* cell-based system to predict the neurotoxicity or neuroprotection of a compound. Our findings clearly showed that the DBZIM not only significantly inhibited 2'-CH₃-MPTP-induced neurotoxicity (gliosis) in the retina as seen by *in vivo* imaging (Fig. 3), but also attenuated striatal gliosis (Fig. 7) and protected DA neurons in the SNpc from degeneration (Fig. 8), as verified by IHC staining *ex vivo*.

Multiple antioxidative mechanisms may contribute to the observed attenuation in striatal gliosis (Fig. 7). To determine whether the activity for one of the major antioxidative enzymes (SOD) was changed by 2'-CH₃-MPTP and/or DBZIM, we measured the striatal SOD activity in tissue extracts from all four treatment groups at 96 h (Fig. 2). The results showed that the SOD activity, which was decreased by 20% in the 2'-CH₃-MPTP group, was moderately rescued by DBZIM treatment (Fig. 9). As to possible mechanism(s) for DBZIM-mediated DA neuron protection (Fig. 8), we attempted TUNEL staining, along with IHC using the TH antibody for marking the DA neurons, on tissue sections prepared from the SNpc. The re-

Figure 10 TUNEL staining of DA neurons in the SNpc at 96 h. IHC (fluorescent images on the left column) using TH antibody clearly identified many DA neurons in the SNpc. On contrast, the number of DA neurons was greatly decreased in the 2'-CH₃-MPTP-treated SNpc, whereas the number of DA neurons in the DBZIM/2'-CH₃-MPTP group was between the saline and the 2'-CH₃-MPTP group, confirming observation in Fig. 8. For TUNEL staining (right column), the number of TH-positive neuron (arrow) and TH-negative cell (arrow head) going through apoptosis was rather limited at 96 h, at which time point, probably most of the neurotoxicant-induced apoptosis already completed.

sults showed that there was little or no TUNEL staining in DA neurons from the saline-treated SNpc (Fig. 10). However there were few DA neurons displaying TUNEL staining (Fig. 10, arrow), though some TH-negative cells were also positive for TUNEL staining (Fig. 10, arrow head). Interestingly, little or no TUNEL staining was found in the DBZIM/2'-CH₃-MPTP or in the DBZIM alone group (data not shown). In retrospect, examining SOD activity and apoptosis at 96 h (or 72 h after the 2'-CH₃-MPTP treatment) may not be the best timing to catch the response dynamics. To sum up the two preliminary experiments here, DBZIM may attenuate neurotoxicity and protect DA neuron by up-regulating SOD activity and by inhibiting apoptosis. However, more effort is needed to confirm this hypothesis.

With the establishment of our molecular retinal imaging method, several aspects of the drug development could be improved. First, the success of the 2'-CH₃-MPTP-induced toxicity and DA neuronal loss in the brain could be indirectly estimated from the real-time live retinal imaging, since the retinal neurotoxicity (or gliosis) was

tightly coupled to neurotoxicity in the striatum and DA neuronal loss in the SNpc. In other words, the Parkinsonian neurotoxicant 2'-CH₃-MPTP not only caused damages in the brain, but also imprinted a pathological signature on the retina. Only mice that have been successfully induced as a PD model with a correct dosing scheme in an appropriate genetic background among other factors (sex and age, etc.) were further used to test anti-PD candidate compounds. In this way, false positive results and data variation were minimized. Second, the potential neurotoxicity of a candidate compound could also be independently assessed. Drug compounds (intended for nervous or nonnervous system) ought to be systematically tested for potential neurotoxicity *in vivo*. Under the dosing scheme used in this study, DBZIM itself showed no neurotoxicity in the retina and the brain. Third, the efficacy of the candidate compounds could be concurrently evaluated using retinal imaging as well. There seemed to be, in the current study, a reasonable correlation between the retinal (Fig. 3), striatal (Fig. 7) gliosis, and the DA neuronal loss in the SNpc (Fig. 8).

In summary, we have established a molecular imaging method for noninvasively measuring retinal gliosis induced by the Parkinsonian neurotoxicant 2'-CH₃-MPTP. Broadly speaking, the imaging method could be seen as an *in vivo* version of the high-throughput neurotoxicity screening for drug development. We used this system to evaluate an imidazolium salt, 1,3-bisbenzylimidazolium bromide (DBZIM) for its ability to inhibit neurotoxicity *in vivo*. Our results showed that DBZIM is a neuroprotectant. However future mechanistic studies are required to elucidate additional molecular mechanism(s) to account for the neurotoxicity-suppressing and the neuroprotective properties. Insights gained from such studies would facilitate the development of novel IMS-based therapeutics for PD.

Conflict of Interest

The authors have no Conflict of Interest.

Acknowledgments

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