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BL-1023 IMPROVES BEHAVIOR AND NEURONAL SURVIVAL IN 1-METHYL-4-PHENYL-1,2,3,6-TETRAHYDROPYRIDINE-INTOXICATED MICE

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Abstract

The therapeutic potential of BL-1023, a chemical combination of L-3,4-dihydroxyphenylalanine (L-DOPA) and γ -amino butyric acid (GABA), was investigated in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) intoxicated mice. Such animals exhibit nigrostriatal degeneration, characteristic of human Parkinson's disease. Drug was administered during and after the development of MPTP-induced nigrostriatal lesions followed by measures of motor function and behaviors, nigrostriatal dopaminergic neurons and termini, and striatal dopamine levels. When administered after lesion development, BL-1023 improved motor function of MPTP-mice as measured by rotarod, total floor and vertical planes, and stereotypic movements in open field activity tests when compared to MPTP-mice without treatment. This also paralleled modest nigral dopaminergic neuronal protection. Such significant improvements in motor function, behaviors, and dopaminergic neuronal numbers were not seen when BL-1023 was administered during MPTP-induced lesion development. The data demonstrate select abilities of BL-1023 to increase dopaminergic neuronal survival and improve motor function in MPTP-mice.

Keywords

Parkinson's disease; neuroprotection; dopamine; motor function; behavior; 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

Parkinson's disease (PD) is the second most common neurodegenerative disease. Current treatments are solely palliative with noted secondary side effects. These are often difficult to tolerate with limited improvements in clinical status. Thus, improved treatments are a significant and unmet need. PD is characterized by the progressive degeneration of dopaminergic neurons in the substantia nigra pars compacta (SNpc) that leads to decreased amounts of dopamine in the dorsolateral putamen of the striatum (Bernheimer et al., 1973).

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The loss of dopamine in the striatum manifests as motor disabilities that are characteristic of PD, including bradykinesia, resting tremor, muscular rigidity, and gait abnormalities. Most of these are attenuated, in part, with the administration of L-3,4-dihydroxyphenylalanine (L-DOPA), a precursor to dopamine that increases levels of dopamine in the striatum. While L-DOPA (known also as levodopa) is the gold standard for the treatment of PD (Agid, 1998), long-term treatment can lead to secondary symptoms. These include L-DOPA-induced dyskinesia and drug-refractory behaviors (Melamed et al., 1998, Dauer and Przedborski, 2003). Furthermore, L-DOPA treatment was found to decrease the activity of dopamine transporters in the nigrostriatal nerve terminals of the striatum. This suggested that while L-DOPA treatment may improve clinical motor symptoms, it may also speed the neurodegenerative process. Such data was reported in a 2- β -carbomethoxy-3- β -(4-iodophenyl) tropane (β -CIT) SPECT sub-study of early versus late L-DOPA (ELLDOPA) use (Fahn, 2005). While most PD therapies are aimed at restoring striatal dopamine levels, γ -amino butyric acid (GABA) may also serve as a therapeutic target to ameliorate disease symptoms. Reduced levels of GABA (Gerlach et al., 1996) and glutamic acid decarboxylase (Rinne et al., 1974) as well as reductions in GABA_A receptor density (Nishino et al., 1988) are found in PD brains, and cerebral spinal fluid GABA levels are reduced in PD patients (Manyam, 1982, Kuroda, 1983). Furthermore, GABA therapy reduces motor asymmetry in rats with excitotoxically lesioned striata (Rozas et al., 1996), and GABA receptor agonists result in suppression of limb tremor in PD patients (Levy et al., 2001). Together, these data suggests that GABAergic neuronal function in the basal ganglia may be contributing to PD motor dysfunction and that restoration of GABA levels in the CNS may relieve motor symptoms. However, GABA comprises many hydrophilic functional groups that limit its ability to cross the blood brain barrier.

The acute 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model is commonly used to study nigrostriatal degeneration, a pathological hallmark of PD (Jackson-Lewis and Przedborski, 2007). Herein, the acute MPTP-mouse model was used to test the efficacy of BL-1023, a small molecule that chemically combines L-DOPA and GABA, for potential PD clinical use. The combination of L-DOPA with GABA increases the transport of GABA across the blood brain barrier (International Publication Number: WO/2009/101616A1). As such, the drug could alleviate disease-linked symptoms associated with PD while decreasing secondary side effects associated with other drug regimens. To assess the potential therapeutic efficacy of BL-1023 we administered the drug during and after MPTP-induced lesion development. We assessed effects on behavior and dopaminergic nigral neuronal protection. These results raise the potential for BL-1023 therapeutic development.

2. EXPERIMENTAL PROCEDURES

2.1. Animals

Male C57BL/6J mice, 10–16 weeks of age were purchased from Jackson Laboratories (Bar Harbor, ME). The study was conducted in accordance with the animal care guidelines issued by the National Institutes of Health and approved by the Institutional Animal Care and Use Committee of the University of Nebraska Medical Center.

2.3. MPTP intoxication and drug treatments

Mice were randomized into treatment groups (Table 1) and on day 0, four subcutaneous injections were administered, one every 2 or 3 hours of either vehicle (PBS, 10 ml/kg, groups 1, 7, 8, and C1) or MPTP-HCl (20 mg/kg free base in PBS, unless otherwise stated, groups 2–6, and C2–C4). MPTP handling and safety measures were in accordance with published guidelines (Jackson-Lewis and Przedborski, 2007). L-DOPA (Sigma, USA) was reconstituted in water, pH 2.7, to a concentration of 2.48 mg/ml, which is equimolar to the

L-DOPA in the BL-1023 compound. BL-1023 (BioLineRx, Ltd., Jerusalem, Israel) was reconstituted in water, pH 7, to a concentration of 4 mg/ml and was synthesized as previously described (International Publication Number: WO/2009/101616A1). For each group (Table 1) of mice treated with MPTP or PBS on day 0, drug regimens comprised daily intraperitoneal administration of PBS at 10 ml/kg (groups 1 and 2); BL-1023 at 40 mg/kg on the day before MPTP injections (day -1), and days 1–6 (groups 3 and 7); L-DOPA at 24.8 mg/kg on day -1 and days 1–6 post-MPTP (groups 4 and 8); BL-1023 at 40 mg/kg on days 7–34 (group 5); and L-DOPA at 24.8 mg/kg on days 7–34 post MPTP (group 6) (Table 1 and Fig. 1A). Drug regimens that were initiated on day -1 and continued on days 1–6 post-MPTP (*i.e.*, before and during MPTP lesion development) are hereafter referred to as “per-lesion,” while regimens administered on days 6–34 post-MPTP (*i.e.*, after lesion development) are designated hereafter as “post-lesion”. To preclude interference with dopamine transporter-mediated MPP⁺ uptake, drug was not administered on day 0 of the perlesion regimen. To inhibit aromatic-L-amino-acid decarboxylase (AADC), mice were administered carbidopa (2.48 mg/kg, *i.p.*) 20 minutes before treatment with PBS (groups C1 and C2); BL-1023 (40 mg/kg) (group C3); and L-DOPA (24.8 mg/kg) (group C4) (Table 1 and Fig. 1B).

2.4. Motor function and behavior tests

The ability to traverse a rotating road (rotarod test) was used to evaluate motor performance of MPTP-intoxicated mice treated with L-DOPA or BL-1023 over time. Ninety seconds was the maximum time for each trial. The apparatus was fitted with a 7-cm diameter rod and was interfaced with automatic timing instrumentation (Rotamex, Columbus Instruments, Inc. Columbus, OH). Mice were habituated and trained to perform on the accelerating rotarod at 2–12 rpm for 5 minutes $\times$ 4 daily sessions for three consecutive days prior to the administration of MPTP (Fig. 1A). One day after training and one day before MPTP-intoxication (groups 2–6) or PBS treatment (group 1), mice were evaluated at 10 rpm for a maximum of 90 seconds per trial to obtain a pre-treatment baseline performance for each mouse. After MPTP- or PBS-treatment, mice were re-evaluated on the rotarod at weeks 1, 2, 3, 4 and 5 post-MPTP. Each post-treatment rotarod performance was calculated as a ratio relative to each animal's baseline performance, and then scores from MPTP mice treated with or without L-DOPA or BL-1023 were normalized to the mean performance of the PBS control group.

Analysis of general activity and natural behaviors were assessed using an automated open field activity system (Tru Scan 2.0, Coulbourn Instruments, Whitehall, PA) to measure different types of movement including the total number of movements within the floor plane or vertical plane (*i.e.*, rearing) and stereotypic movements (*i.e.* grooming). Mice were habituated to the open field arena (25.4 cm $\times$ 25.4 cm) for 10 minutes per day for three consecutive days. After the three-day habituation, baseline measurements were recorded over a 25-minute observation period for each animal prior to PBS- or MPTP-treatment. Each animal was re-tested at 1, 2, 3, 4, and 5 weeks post PBS- or MPTP-treatment. Similar to rotarod, post-MPTP intoxication open field measurements were represented as a relative ratio to each animal's baseline activity, which was then normalized to the mean ratio of PBS controls. For rotarod and open field analyses, repeated measures on one set of animals were longitudinally evaluated over the 5 week study term.

2.5. Immunohistochemistry

At time points indicated on the timeline (Fig. 1A and 1B) following MPTP-intoxication, mice were terminally-anesthetized with pentobarbital and transcardially perfused with PBS then 4% paraformaldehyde in PBS (PFA, Sigma-Aldrich, St. Louis, MO). Brains were removed, postfixed in PFA overnight, cryopreserved in 30% sucrose/PBS for two days, snap

frozen in 2-methylbutane, embedded in OCT compound (Sakura Finetek USA, Inc., Torrance, CA) and 30 μ m frozen sections were cut from the midbrain and basal ganglia using a cryostat (CM1900, Leica, Nussloch, Germany). Sections were processed free-floating in 48-well plates. Tissue sections were probed using rabbit anti-tyrosine hydroxylase (TH) as the primary antibody (1:2000 for substantia nigra, 1:1000 for striatum, Calbiochem/EMD Chemicals, Gibbstown, NJ), and biotinylated goat anti-rabbit IgG as the secondary antibody (1:400, Vector Laboratories, Inc.). Sections were incubated in streptavidin-horseradish peroxidase (HRP) solution (ABC Elite vector kit, Vector Laboratories, Burlingame, CA). Immunohistochemical staining was visualized using 3,3'-Diaminobenzidine (Sigma-Aldrich) as the chromogen and mounted on slides. Nissl substance was counter-stained by thionin. Photomicrographs were taken with a Nikon TE300. The contrast of photomicrographs was adjusted in Adobe Photoshop CS3 Extended version 10.0.1 using the Auto Contrast function.

2.6. Quantification of Neuronal Survival

TH- and Nissl-stained neurons in the substantia nigra were counted in a blinded-fashion using design-based stereology software (StereoInvestigator, MicroBrightField, Inc., Williston, VT) and the Fractionator Probe module as previously described (Benner, 2004). The densities of dopaminergic neuron axonal termini in the striatum were determined by digital image analysis of TH-stained striata as previously described (Benner et al., 2004).

2.7. Dopamine levels and its metabolites

Mice were euthanized by CO₂ hypoxia, brains were removed and striatal tissue dissected on an ice cold platform, weighed, and frozen at -80°C until processed and analyzed as previously described (Przedborski et al., 1996, Liberatore et al., 1999, Benner et al., 2004, Boska et al., 2005). Briefly, striatal tissue was sonicated in 10 volumes (wt/vol) of 0.1 M perchloric acid/ 10^{-7} M ascorbic acid, clarified by centrifugation at 14,000 g for 15 minutes, and triplicate 20 μ l supernatant samples were injected onto a C18-reverse-phase HR-80 catecholamine column (ESA, Bedford, MA) at 25°C . Mobile phase consisted of 93.5% 0.15 M monochloroacetic acid/0.1 mM EDTA/0.86 mM sodium octyl sulfate (pH 3.0), 4% acetonitrile, and 2.5% tetrahydrofuran and was pumped at a flow rate of 0.5 ml/min. Peaks were detected at +650 mV with a 0.1 Hz filter using an electrochemical detector (BAS, West Lafayette, IN) equipped with a glassy carbon working electrode and a Ag/AgCL reference electrode. Dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), and homovanillic acid (HVA) levels were quantitated by comparison of peak areas to those of known standards (0.015–1.5 $\mu\text{g/ml}$ DA and DOPAC and 0.1–10 $\mu\text{g/ml}$ HVA). Stock solutions of catecholamines were prepared in 100% methanol at a concentration of 2.0 mg/ml and stored for up to 3 months at -20°C . Catecholamine standard curves were prepared in 0.1 M perchloric acid/ 10^{-7} M ascorbic acid.

2.8. Statistical analysis

Data were expressed as the mean $\pm$ standard error of the mean (SEM). All statistical tests were performed using Statistica (StatSoft, Inc. Tulsa, OK). Statistical significance was evaluated by one-way or two-way ANOVA followed by *post-hoc* paired comparisons using Bonferroni correction or Fisher's LSD, respectively. In later experiments, C57BL/6J mouse behavior and motor function were analyzed by comparing post-MPTP intoxication test results to baseline functions before MPTP intoxication.

3. RESULTS

3.1. BL-1023 improves MPTP-induced motor deficits

Diagnosis of PD is based on clinical symptoms, which become evident only after loss of 50–60% of the dopaminergic neurons or 60–80% of striatal dopaminergic termini (Bernheimer et al., 1973). Thus an MPTP regimen was utilized that is sufficient to induce a level of dopaminergic destruction detectable by motor function and behavior testing. To determine the effect of BL-1023 treatment on motor function, we assessed weekly rotarod performances and open field activity of MPTP-intoxicated mice treated with BL1023 administered before and during or after MPTP lesion development; hereafter referred to as per-lesion and post-lesion regimens, respectively. Mice treated with equivalent amounts of L-DOPA served as controls for the active moiety of BL1023, as well as a comparable drug utilized in PD. MPTP-intoxication diminished rotarod performance by weeks 1, 2, and 3, yet those effects were no longer significant by weeks 4 or 5 (Fig. 2A and 2B) suggesting that MPTP-induced motor impairments improve over time. Treatment of intoxicated mice with BL-1023 or L-DOPA either during or after lesion development yielded no significant week-to-week effects, however, overall trends suggested that treatment with L-DOPA during lesion development and BL-1023 post-lesion development had beneficial locomotor effects. Indeed, taken together over the entire study as the mean-weekly activity and compared to MPTP-treated mice, L-DOPA per-lesion treatment and BL-1023 post-lesion treatment increased rotarod performance (Fig. 2C). Moreover, cumulative rotarod performance over the entire study, increased with BL-1023 post-lesion treatment compared to BL-1023 per-lesion treatment. This suggests that daily administration of BL-1023 after the development of the initial lesion increases motor function, as measured by rotarod, in MPTP-treated mice.

With open field activity measures of MPTP mice over 5 weeks, we found decreased relative floor plane movements and vertical plane entries (*i.e.*, rearing) by BL-1023 and L-DOPA per-lesion-treated mice compared to PBS controls (Fig. 3A and 3B). Post-lesion treatment with either drug resulted in increased activities compared to per-lesion treatment of the same drug. Furthermore, per- and post-L-DOPA-treated mice showed significant increases in rearing compared to those treated with BL-1023.

Similarly, compared to per-lesion treatment, post-lesion treatment of either drug increased mean cumulative stereotypic movements (Fig. 4C), which are believed to be associated with dopaminergic mechanisms within the basal ganglia (Creese and Iversen, 1973, Graybiel et al., 2000, Chartoff et al., 2001, Zhuang et al., 2001, Kitanaka et al., 2005, Luchtman et al., 2009, Oksman et al., 2009). Assessment for interactions of treatment and time revealed that by one week post-MPTP intoxication, regardless of drug treatment regimen, and compared to PBS-treated controls, stereotypic movements were diminished in all MPTP-treated groups, which improved weekly until week 3 (Fig. 4A and 4B). At times thereafter, post-lesion drug treatment with daily regimens supported improved stereotypic movements, while per-lesion-treated mice exhibited diminishing trends of stereotypy that ended at week 5 with significantly lower activities than those shown for PBS or MPTP controls.

3.2. BL-1023 effects on the nigrostriatum

To assess the effects of per-lesion treatment on nigrostriatal dopaminergic survival, we assessed the number and densities of TH immunoreactive (TH+) nigral neurons and striatal termini from MPTP-intoxicated mice treated with BL-1023 or L-DOPA during lesion development. Immunohistochemical analysis showed substantive losses of TH+ nigral neurons and striatal termini of all MPTP-intoxicated mice compared to PBS controls at weeks 1 and 5 post-MPTP (Fig. 5A). Per-lesion treatment of MPTP mice with PBS or either drug yielded 50%–55% losses of nigral TH+ dopaminergic neurons one week following

MPTP intoxication (Fig. 5B) and by week 5, MPTP mice treated with L-DOPA showed a 20% increase in nigral neuron numbers compared to week 1. Similar to neuronal bodies, densitometric analysis of TH+ striatal termini by week 1 showed a 68%–70% total loss in MPTP mice regardless of drug treatment (Fig. 5C). By week 5, striatal termini densities were increased by 32% (36% total loss) in drug-naïve MPTP mice, however treatment with either BL-1023 or L-DOPA only increased striatal densities by 19%–20% (47%–48% total loss), which were lower than those from untreated MPTP mice.

To determine if striatal histological effects of BL-1023 or L-DOPA treatment were correlated with catecholamine levels, we measured dopamine, DOPAC, and HVA levels by HPLC analyses of striatal tissue extracts from PBS control mice or MPTP mice treated with PBS, BL-1023, or L-DOPA on days -1 and 1–6 (Table 1, Fig. 1A). Striatal dopamine and DOPAC levels from MPTP-treated mice regardless of drug regimen were reduced by greater than 92% of levels from PBS control striata, but not significantly different from one another (Fig. 6A and 6B). Significant differences in HVA levels were not detected between groups regardless of treatment (data not shown). We also determined that treatment of PBS control animals with BL-1023 or L-DOPA on days -1 and 1–6 (Table 1, groups 7 and 8, respectively) increased striatal dopamine levels by 18% and 24%, respectively, and striatal DOPAC levels by 25% and 40%, respectively. Although these levels did not reach significance compared to untreated PBS controls, the results demonstrated that neither BL-1023 nor L-DOPA treatment regimens diminished catecholamine levels and suggested that treatments under non-intoxicating conditions augment those levels.

In studies to assess chronic treatment with BL-1023 or L-DOPA after MPTP lesion development, immunohistochemistry at week 5 post-MPTP showed more increased numbers of TH+ nigral neurons from drug-treated MPTP mice compared to drug-naïve MPTP mice, while intensities of striatal TH+ termini were comparable regardless of drug treatment (Fig. 7A). Stereological and densitometric analyses of nigrostriatal tissues from mice treated over the 5-week study confirmed these impressions. At each weekly time point, BL-1023 treatment of MPTP mice consistently afforded increased numbers of nigral neurons on weeks 2, 3, 4 and 5 post-MPTP (week 1–4 of drug treatment) compared to that of drug-naïve MPTP mice. L-DOPA treatment initially exhibited increased TH+ neuron numbers only at week 3, which diminished to levels of drug-naïve MPTP mice by weeks 4 and 5 that were lower than neuron numbers of BL-1023 treated mice (Fig. 7B). Together these data suggested cumulative beneficial effects of BL-1023 and L-DOPA treatments after lesion development over the 5-week study period. Indeed, this was confirmed by numbers of nigral TH+ neurons that increased by 24% and 13% in MPTP mice treated with BL-1023 or L-DOPA, respectively compared to those of drug-naïve MPTP mice (Fig. 7C). Moreover, BL-1023 post-lesion treatment significantly increased TH+ nigral neuron numbers in MPTP mice compared to those of L-DOPA-treated mice.

Densitometric analysis of striatal tissues revealed diminished beneficial effects of either drug treatment on dopaminergic termini in contrast to the effects observed on nigral neurons. These disadvantageous effects were clearly evident in MPTP mice treated with BL-1023 where striatal TH+ densities were consistently below those of drug-naïve mice; the latter showing spontaneous and progressive improvement from a nadir of 33% of PBS controls on week 1 to 62% by week 5 (Fig. 7D). Moreover, L-DOPA treatment yielded even lower striatal densities at weeks 2 and 5 suggesting this treatment also provided little cumulative support for striatal dopaminergic termini despite increased densities at week 3. Indeed, over the entire study period, mean relative striatal TH densities of MPTP mice treated with BL-1023 or L-DOPA were significantly lower than those of drug-naïve MPTP mice (Fig. 7E). Striatal dopamine, DOPAC, and HVA levels at weeks 1, 2, 3, 4 and 5 post-

MPTP intoxication, regardless of drug treatment regimen were consistently diminished from those of PBS control mice, but not significantly different from one another (data not shown).

3.3. BL-1023 administered with carbidopa attenuates MPTP-induced neurodegeneration

As peripheral L-DOPA can be converted into dopamine by aromatic L-amino acid decarboxylase (AADC), which limits drug availability for CNS entry, we hypothesized that the efficacy of post-lesion drug treatment may be improved by inhibition of AADC. Thus, to increase levels of peripheral L-DOPA capable of entering the CNS, carbidopa, an AADC inhibitor, was administered to all mice in a three-week study of daily administration of BL-1023 and L-DOPA initiated after MPTP lesion development (Table 1, Fig. 1B). Stereological analyses of TH+ nigral tissues from carbidopa/drug-treated MPTP mice were similar to those of carbidopa-naïve, drug-treated mice. Compared to their respective MPTP controls, treatment with either BL1023 or L-DOPA afforded increased dopaminergic neuronal survival at each weekly time point and over the entire study period regardless of carbidopa treatment (Fig. 7B, 7C, 8A, and 8B). Moreover, BL-1023 treatment without or with carbidopa increased mean numbers of TH+ nigral neurons over the entire study period by similar levels of 24% and 26%, respectively, compared to MPTP controls (Fig. 7C and 8B). This is congruent with the notions that BL-1023 is protected from AADC metabolism in the periphery and that carbidopa-mediated inhibition of AADC would have little effect on BL-1023-mediated survival. In contrast, L-DOPA treatment in the absence of adjunctive carbidopa yielded only an insignificant 13% increase in TH+ nigral neuron number compared to drug-naïve MPTP mice (Fig. 7C), whereas L-DOPA treatment with carbidopa significantly increased survival of TH+ nigral numbers by 29% over drug-naïve MPTP mice (Fig. 8B), suggesting that carbidopa-mediated inhibition of AADC increases support for dopaminergic survival with chronic L-DOPA post-lesion treatment.

Densitometric analysis of striatal TH+ terminal fibers in MPTP mice showed those densities were significantly diminished with BL-1023 treatment with or without adjunctive carbidopa therapy compared to respective MPTP controls (Fig. 7D, 7E, 8C, and 8D). Treatment of MPTP mice with carbidopa/L-DOPA increased the mean terminal densities to levels that were within the 95% confidence interval of MPTP controls and were 7% above striatal densities of carbidopa/BL-1023 treated MPTP mice, though not significantly different (Fig. 8D). This is in contrast to MPTP mice treated in the absence of carbidopa wherein the mean terminal striatal densities after post-lesion treatment with L-DOPA or BL-1023 were comparable (Fig. 7E). To assess the effects of carbidopa/drug treatment on catecholamine levels in MPTP mice, we evaluated striatal tissue extracts by HPLC analysis. Over the entire 3-week study, mean striatal dopamine, DOPAC, and HVA levels from MPTP mice regardless of drug treatment regimen were consistently diminished below those of PBS control mice, but were not significantly different from one another (data not shown). Taken together, increased survival of nigral neurons and striatal fibers suggest that carbidopa inhibition of AADC augments post-lesion treatment with L-DOPA, while providing little adjunctive support for BL-1023 treatment.

4. DISCUSSION AND CONCLUSIONS

Over the past decade, a substantive resurgence in drugs serving as neuroprotectants have been tested for their effects in ameliorating the signs and symptoms of PD. Nonetheless, the means to protect the nigrostriatal pathway and improve clinical symptoms have remained elusive. The importance of developing treatment strategies, from symptom-alleviating therapies to disease-modifying modalities, is becoming increasingly clear (Obeso et al., 2010). In the current report, we sought to compare the efficacy of a novel PD therapeutic, BL-1023, to L-DOPA using the MPTP mouse model of nigrostriatal degeneration. BL-1023 chemically combines L-DOPA and GABA to increase dopamine levels in the striatum for

improved motor function and increase GABA levels for decreased L-DOPA-induced dyskinesia (International Publication Number: WO/2009/101616A1). L-DOPA is metabolized to dopamine, which is greatly reduced in the striatum of PD patients (Bernheimer et al., 1973). L-DOPA was first introduced in 1967 and is the most effective pharmacological agent for managing PD symptoms, prolonging life expectancy and improving quality of life (Shin et al., 2009). While initial evidence suggested that L-DOPA may increase free radicals in the CNS (Melamed et al., 1998), recent reports suggest that L-DOPA may be neuroprotective; capable of improving neuronal survival in MPTP-intoxicated mice (Shin et al., 2009). GABA is the most abundant inhibitory neurotransmitter in the CNS (Owens and Kriegstein, 2002), and it too is reduced in PD brains (Gerlach et al., 1996). GABA is synthesized from glutamic acid by glutamic acid decarboxylase (GAD), which is also decreased in PD brains (Rinne et al., 1974).

To assess the potential therapeutic role of BL-1023 in MPTP-intoxicated mice, we developed two treatment paradigms. In the first, we administered BL-1023 during MPTP-induced lesion development. In the second paradigm, we administered BL-1023 after the MPTP-induced lesion had developed. Here, we demonstrated significant improvements in motor function, some behaviors, and neuronal survival in MPTP-intoxicated mice treated daily with BL-1023 starting after lesion development. These improvements were similar to or better than those seen in mice treated with L-DOPA post-lesion development. In contrast, BL-1023 administered for the 6 days during lesion development showed no significant improvements over MPTP-intoxicated controls or L-DOPA-treated mice. Furthermore, L-DOPA per-lesion treatment showed improved rotarod performance, increased total number of rears and increased neuronal counts at week 5 post intoxication compared to both MPTP-intoxicated controls and BL-1023 per-lesion mice. Taken together, these data suggest that while BL-1023 may not inhibit MPTP-induced lesion development, it is able to reverse MPTP-induced neuronal loss and improve normal motor function. This supports a potential therapeutic role for BL-1023 in the treatment of dopaminergic neuronal loss in PD and warrants further investigation.

Interestingly, compared to drug-naïve MPTP mice, activity on the floor plane increased with BL-1023 post-lesion treatment, while vertical plane entries (rearing) and stereotypic movements were not increased. These data suggest an impairment of dopamine-mediated circuitry in the basal ganglia. Indeed, stereotypic movements, such as syntactic grooming, require skilled forepaw and digit-use, are under supraspinal control and are sensitive to the loss of dopamine in the striatum (Tillerson et al., 2002, Meredith and Kang, 2006). In MPTP mice, striatal dopamine and its metabolites were profoundly diminished compared to PBS control mice, and notably, dopamine levels were unaffected by treatments with BL-1023 or L-DOPA in MPTP-treated mice. Immunohistochemical analysis showed a severe loss of dopaminergic neuronal termini in the striatum of MPTP-intoxicated mice treated with BL-1023 post-lesion development. Normally, dopamine is stored in vesicles within the pre-synaptic axonal termini in the striatum. With the MPTP-induced ablation of the neuronal termini in the striatum, dopamine acquired through dopamine replacement therapies may not be stored in this region of the brain without significant sprouting or termini regeneration. Thus, the absence of an increase in dopamine levels with L-DOPA and BL-1023 treatments may be due, in part, to the loss of neuronal termini in the striatum. This is supportive of the “distal axonopathy” or “dying back” theory of PD nigrostriatal degeneration, which hypothesizes that neuronal degeneration in PD begins in the striatum and affects neuronal soma during the later stages of the disease. Another factor that may have contributed to the lack of dopamine and its metabolites in the striatum after BL-1023 or L-DOPA treatment is the peripheral metabolism of L-DOPA. L-DOPA is often prescribed to PD patients in combination with an AADC inhibitor such as carbidopa or benserazide. Without an AADC inhibitor, up to 95% of L-DOPA is metabolized in the periphery with less than 1%-10%

finally crossing the blood brain barrier (Nutt and Fellman, 1984, Fariello, 1998, Khor and Hsu, 2007, Miller et al., 2009, Contin and Martinelli, 2010). Thus, these data led us to hypothesize that the therapeutic effects of BL-1023 could be increased with the administration of carbidopa before administration of BL-1023. Due to the severe ablation of dopaminergic neurons and termini, and the profound decrease in dopamine with a 20 mg/kg/dose of MPTP, in the carbidopa sub-study, the MPTP dose was reduced to 16 mg/kg/dose. Carbidopa was administered before BL-1023, L-DOPA or PBS, and HPLC analysis for dopamine and immunohistochemistry for dopaminergic neurons and termini were repeated. While significant increases in the levels of striatal dopamine and its metabolites were appreciated, neuronal survival increased significantly in the carbidopa/BL-1023-treated mice, when compared to MPTP controls and is supportive of data from BL-1023-treated mice that did not receive carbidopa and confirmatory that BL-1023 is seemingly protected from AADC metabolism. Evidence that adjunctive carbidopa therapy affords beneficial outcomes is provided by increased neuronal survival in post-lesion MPTP mice treated with carbidopa/L-DOPA compared to those treated with only L-DOPA.

Indeed, one week after beginning BL-1023 post-lesion treatment, MPTP mice exhibited significantly increased neuronal counts, which were maintained through week 5 compared to drug-naïve MPTP controls. The increase in neuronal survival with BL-1023 treatment was not observed when the drug was administered during lesion development.

Neuroinflammation and microglial activation significantly contribute to the acute degeneration of the nigrostriatal pathway in the MPTP mouse model, and anti-inflammatory modalities have been efficacious in this model (Kurkowska-Jastrzebska et al., 1999, Vijitruth et al., 2006, Pattarini et al., 2007, Carbone et al., 2008, Yokoyama et al., 2008, Miller et al., 2009). However, few studies have shown L-DOPA, an active component of BL-1023, to be neuroprotective (Shin et al., 2009). No known anti-inflammatory properties in MPTP-intoxicated mice have been ascribed to either L-DOPA or GABA, the second active component of BL-1023. However, here, we showed that in combination, L-DOPA and GABA, administered in the form of BL-1023 after lesion development, was able to increase neuronal numbers in the SNpc compared to MPTP controls.

Taken together, these data suggest that BL-1023 may be suitable for the treatment of motor function deficits resulting from the degeneration of the nigrostriatal pathway. BL-1023 restores motor and behavior function and has neuroprotective properties as observed by increased neuronal counts in the SNpc of BL-1023-treated mice. However, while the MPTP mouse model yields a disease that is pathologically similar to PD, nigrostriatal degeneration in the MPTP mouse model is rapid, occurring within days of intoxication, whereas degeneration in PD may progress over decades. Thus, while BL-1023 holds promise, the current study suggests that further investigation into the efficacy of BL-1023 in the treatment of PD is needed as well as studies to determine the mechanism of neuroprotection.

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Abbreviations

(L-DOPA)	L-3,4-dihydroxyphenylalanine
(GABA)	γ -amino butyric acid
(MPTP)	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

(PD)	Parkinson's disease
(SNpc)	substantia nigra pars compacta
(PBS)	phosphate buffered saline
(PFA)	paraformaldehyde
(TH)	tyrosine hydroxylase
(HRP)	horseradish peroxidase
(DA)	Dopamine
(DOPAC)	3,4-dihydroxyphenylacetic acid
(HVA)	homovanillic acid
(SEM)	standard error of the mean
(ANOVA)	analysis of variance
(AADC)	aromatic L-amino acid decarboxylase
(CNS)	central nervous system

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Figure 1. Experimental timelines

(A) A timeline detailing training and habituation, pretesting and testing of motor function and behavior as well as drug treatments and time points for sacrifice. (B) A timeline detailing the carbidopa experiment, in which mice were treated with carbidopa 20 minutes prior to treatment with PBS, BL-1023 or L-DOPA.

Figure 2. Motor function is improved by BL-1023 treatment

Starting 7 days after MPTP-intoxication, mice were tested weekly for forced motor function using the rotarod motor function test. (A) Weekly performance values for mice treated with PBS daily (black, circles) or MPTP mice treated with PBS daily (blue, squares), BL-1023 daily (red, diamonds), or L-DOPA daily (green, triangles) during lesion development (days -1 and 1–6, per-lesion). (B) Weekly performance values for mice treated with PBS daily (black, circles) or MPTP mice treated with PBS daily (blue, squares), BL-1023 daily (red, diamonds), or L-DOPA daily (green, triangles) after lesion development (days 7–34, post-lesion). (C) Weekly performance values were averaged to assess overall rotarod performance for mice treated with PBS or MPTP and PBS, BL-1023 or L-DOPA administered either during (per-lesion) or after (post-lesion) development. (A, B, and C) Data represent means $\pm$ SEM of activities normalized to baseline activity for each mouse and the PBS control performance means. Means for 9–16 mice per group were compared by factorial (A and B) or one-way ANOVA (C) with pair-wise comparison using Fisher's or Bonferroni *post-hoc* tests, respectively. Significant differences at $P \leq 0.05$ are denoted by letters. Each group is compared to mice treated with ^aPBS, ^bMPTP/PBS, ^cMPTP/L-DOPA within regimen, or ^dsame drug between regimens (per- versus post-lesion).

Figure 3. Movements are increased by BL-1023 after MPTP-induced lesion development

Starting 7 days post MPTP-intoxication, mice were tested weekly for unforced movement and behavior using the open field activity test to evaluate number of floor plane moves (A) and vertical plane entries (rearing) (B). Mice were treated with PBS or MPTP (day 0) and PBS, BL-1023, or L-DOPA either per-lesion (days -1 and 1–6) or post-lesion (days 7–34) development. Data represent means $\pm$ SEM of weekly performance values for activities normalized to pre-inoculation baseline activity for each mouse and the PBS control mean. Means for 12–16 mice per group were compared by one way ANOVA with pair-wise comparison using Fisher's *post-hoc* tests. Significant differences at $P \leq 0.05$ are denoted by letters. Each group is compared to mice treated with ^aPBS, ^bMPTP/PBS, ^cMPTP/L-DOPA within regimen, or ^dsame drug between regimens (per- versus post-lesion).

Figure 4. Stereotypic moves are increased over time in BL-1023-treated MPTP-intoxicated mice Starting 7 days post MPTP-intoxication, mice were tested weekly for stereotypic movement and behavior using the open field activity test. (A) Weekly stereotypic movement values for mice treated with PBS (black, circles) or MPTP (day 0) and PBS (blue, squares), BL-1023 (red, diamonds), or L-DOPA (green, triangles) during lesion development (days -1 and 1–6, perlesion). (B) Weekly stereotypic movement values for mice treated with PBS (black, circles) or MPTP and PBS (blue, squares), BL-1023 (red, diamonds), or L-DOPA (green, triangles) after lesion development (days 6–34, post-lesion). (C) Weekly performance values were averaged to assess overall rotarod performance for mice treated with PBS or MPTP (day 0) and PBS, BL-1023, or L-DOPA administered either during (per-lesion) or after (post-lesion) development. (A, B, and C) Data represent means $\pm$ SEM of activities normalized to baseline activity for each mouse and the PBS control performance means. Means for 12–16 mice per group were compared by factorial (A and B) or one-way ANOVA (C) with pair-wise comparison using Fisher's or Bonferroni *post-hoc* tests, respectively. Significant differences at $P \leq 0.05$ are denoted by letters. Each group is compared to mice treated with ^aPBS, ^bMPTP/PBS, ^cMPTP/LDOPA within regimen, or ^dsame drug between regimens (per- versus post-lesion).

Figure 5. BL-1023 does not alter dopaminergic neuronal numbers when administered during MPTP-induced lesion development

(A) Photomicrographs of substantia nigra (SN) and striatum (Str) of mice at 1 and 5 weeks post-treatment with PBS (black) or MPTP (day 0) and then daily with PBS, BL-1023, or L-DOPA before and during lesion development on days -1 and 1–6. Scale bars are 200 μ m for SN and 2 $\times$ original magnification for Str. (B) TH immunoreactive (TH+) dopaminergic neuronal counts from mice at 1 week and 5 weeks post-PBS treatment (black) or MPTP-intoxication and treated with PBS, BL-1023, or L-DOPA on days -1, and 1–6 during lesion development. (C) Striatal TH+ densities of dopaminergic terminal fibers from mice at 1 week and 5 weeks post-PBS treatment or MPTP-intoxication and treated with PBS, BL-1023, or L-DOPA on days -1, and 1–6 during lesion development. Data represent means $\pm$ SEM of neuronal counts (B) or striatal densities (C) normalized to mean of PBS controls. Means for 6–7 mice per group animals were compared by factorial ANOVA with pair-wise comparison using Fisher's *post-hoc* tests. Significant differences at $P \leq 0.05$ are denoted by letters. Each group is compared to mice treated with ^aPBS, ^bMPTP/PBS, ^cMPTP/L-DOPA within regimen, or dsame drug between regimens (per- verses post-lesion).

Figure 6. Dopamine and DOPAC levels in the striatum of per-lesion treated MPTP mice
Dopamine (A) and DOPAC (B) concentrations per mg of wet striatal tissue were determined by HPLC for mice treated with PBS or MPTP on day 0 and then PBS, BL-1023, or L-DOPA daily on days -1 and 1–6 during lesion development (Table 1). Striatal tissues were harvested on day 7 post-MPTP treatment. Comparisons of mean concentrations $\pm$ SEM for 4–9 mice per group were determined by one-way ANOVA with pair-wise comparison using Fisher's *post-hoc* tests. Significant differences at $P \leq 0.05$ are denoted by letters. Each group is compared to mice treated with ^aPBS, ^bMPTP/PBS, ^cMPTP/L-DOPA. Significant differences in HVA concentrations between groups regardless of treatment were not detected (data not shown).

Figure 7. BL-1023 treatment is associated with increased dopaminergic neuronal numbers when administered after MPTP-induced lesion development

(A) Photomicrographs of SN and striata (Str) at 5 weeks post PBS treatment or MPTP-intoxication and treatment with PBS, BL-1023, or L-DOPA after lesion development (days 7–34). Scale bars are 200 μ m for SN and 2 $\times$ original magnification for Str. (B) Weekly TH+ (dopaminergic) nigral neuron counts, (C) mean of cumulative weekly TH+ nigral neuron counts, (D) weekly striatal TH+ densities, and (E) mean of cumulative striatal TH+ densities from mice treated with PBS (black, circles) or MPTP (day 0) and treated with PBS (blue, squares), BL-1023 (red, diamonds), or L-DOPA (green, triangles) after development of MPTP lesion (days 7–34). Data represent means $\pm$ SEM of nigral TH+ neuronal counts (B and C) or striatal TH+ densities (D and E) normalized to mean of PBS controls. Means for 6–7 mice per group were compared by factorial (B and D) or one-way (C and E) ANOVA with pair-wise comparison using Fisher's or Bonferroni *post-hoc* tests, respectively. Significant differences at $P \leq 0.05$ are denoted by letters. Each group is compared to mice treated with ^aPBS, ^bMPTP/PBS, ^cMPTP/L-DOPA within regimen, or ^dsame drug between regimens (per- versus post-lesion).

Figure 8. Dopaminergic neuronal numbers and striatal densities following carbidopa and BL-1023 or L-DOPA treatments after MPTP-induced lesion development

(A) Weekly TH+ (dopaminergic) nigral neuron counts, (B) mean of cumulative weekly TH+ nigral neuron counts, (C) weekly striatal TH+ densities, and (D) mean of cumulative striatal TH+ densities from mice treated with 4 i.p. injections of PBS or 16 mg MPTP/kg on day 0. On days 6–20, all mice were treated with carbidopa 20 minutes prior to treatment of PBS control mice with PBS (black, circles) and MPTP mice with PBS (blue, squares), BL-1023 (red, diamonds), or L-DOPA (green, triangles) after development of MPTP lesion. Data represent means ± SEM of nigral TH+ neuronal counts (A and B) or striatal TH+ densities (C and D) normalized to mean of PBS controls. Means for 4–7 mice per group animals were compared by factorial (A and C) or one-way (B and D) ANOVA with pair-wise comparison using Fisher's or Bonferroni *post-hoc* tests, respectively. Significant differences at $P \leq 0.05$ are denoted by letters. Each group is compared to mice treated with ^aPBS, ^bMPTP/PBS, ^cMPTP/L-DOPA within regimen, or ^dsame drug between regimens (per- versus post-lesion).

Table 1

Treatment arms

Treatment Group Number	MPTP or PBS Treatment (Day 0)	Drug	Days treated with drug (relative to Day 0)
1	PBS	PBS	-1, 1-34
2	MPTP	PBS	-1, 1-34
3	MPTP	BL-1023	-1, 1-6
4	MPTP	L-DOPA	-1, 1-6
5	MPTP	BL-1023	7-34
6	MPTP	L-DOPA	7-34
7	PBS	BL-1023	-1, 1-6
8	PBS	L-DOPA	-1, 1-6
C1	PBS	Carbidopa + PBS	6-20
C2	MPTP	Carbidopa + PBS	6-20
C3	MPTP	Carbidopa + BL-1023	6-20
C4	MPTP	Carbidopa + L-DOPA	6-20