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Research Paper

The second generation mixed lineage kinase-3 (MLK3) inhibitor CLFB-1134 protects against neurotoxin-induced nigral dopaminergic neuron loss

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ABSTRACT

Dopaminergic neurons express mixed lineage kinases which regulate the expression of cell death genes. In Parkinson's disease, cell death *via* apoptosis is prevalent, and previous work testing mixed lineage kinase inhibitors in animal models suggested the inhibitors had some neuroprotective potential. CLFB-1134 is a new, brain-penetrant inhibitor specific for MLK3, tested here in a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) model of dopaminergic depletion and nigral neuron death in mice. After ensuring that treatment with CLFB-1134 did not alter conversion of MPTP to MPP+, we demonstrated CLFB-1134's inhibition of MLK3 and neuroprotective efficacy. Specifically we evaluated the integrity of the nigrostriatal dopamine system following MPTP by assessing protein expression, high performance liquid chromatography, and immunohistology with stereology. We found that CLFB-1134 achieves protection of striatal dopaminergic terminals and nigral cell bodies when dosed simultaneously or following MPTP treatment. By preventing phosphorylation of JNK and other downstream targets of MLK3, CLFB-1134 protects against the neurotoxin MPTP. Inhibition of MLK3 may be a valid target for future work investigating treatment of Parkinson's disease.

1. Background

Parkinson's disease (PD) is the second most common neurodegenerative disease and the most common neurodegenerative movement disorder (Hirtz et al., 2007). It is estimated that by 2030, over 1.2 million people in North America will be living with PD (Marras et al., 2018). Depletion of striatal dopamine (DA) due to death of substantia nigra DA neurons has been known for decades to be the cause of PD motor symptoms, yet no disease modifying therapies exist. The drivers of dopaminergic degeneration in PD are not fully understood, and many cellular processes have been implicated. Mixed lineage kinases (MLKs) in neurons are part of a signaling cascade thought to activate cell death machinery in PD. In PD, distinctions between apoptosis, necroptosis, and other forms of programmed cell death have been difficult to establish. Thus, while there is extensive death of DA and norepinephrine neurons in PD, the mechanistic interplay between programmed cell death pathways and cell-non-autonomous killing events remains to be fully elucidated. Molecular markers of apoptosis, such as increased expression of p53, CD95, caspases-3, −8 and − 9, and activity of caspases-1 and -3 have been reported in human PD post-mortem brain (de la Monte et al., 1998; Hartmann et al., 2000; Andersen, 2001; Mogi et al., 2000). The consensus is that there is a proapoptotic environment in the substantia nigra, as reviewed in (Lev et al., 2003). In the 1-methyl-4phenyl-1,3,4,6-tetrahydropyridine (MPTP) mouse model of PD specifically, MPTP's neurotoxic derivative MPP+ kills cells by mitochondrial complex I inhibition which initiates apoptosis (Fall and Bennett Jr, 1999). MLKs belong to the mitogen-activated kinase kinase kinase (MAP3K) family of serine/threonine kinases. Phosphorylation of c-Jun N-terminal kinase (JNK), downstream of MLKs, activates the transcription factor c-Jun which regulates expression of cell death genes. Phosphorylated JNK (pJNK) inhibits pro-survival factors and activates Bad and Bim, apoptotic signaling molecules. In genetic and neurotoxin-based models of PD, JNK activation has been observed (Burke, 2007; Cha et al., 2005), and neuroprotection has been achieved

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with inhibition of JNK (Zhang et al., 2004). Furthermore, pJNK has been observed in cytoplasmic granules adjacent to intranuclear inclusions (Lewy bodies) in post-mortem PD brain (Ferrer et al., 2001).

Mixed lineage kinase-3 (MLK3), encoded by the *MAP3K11* gene, preferentially activates MAPK8/JNK and functions as a positive regulator of the JNK signaling pathway. MLK3 can directly phosphorylate and activate JNK and p38MAPK and is involved in the transcriptional activity of AP1 mediated by Rho family GTPases and Cdc42 (Kant et al., 2011). Though redundancy exists in MLK pathways, specific inhibition of MLK3 has been associated with neuronal health and protection in multiple *in vitro* and *in vivo* systems (Handley et al., 2007). MLK3 has been implicated in apoptosis after nerve growth factor withdrawal in rat sympathetic neurons (Mota et al., 2001). In 2005, the first generation MLK3 inhibitor CEP-1347 was shown to mediate neuroprotection against methamphetamine-exposed human mesencephalic-derived neurons, *in vitro* (Lotharius et al., 2005). CEP-1347 also prevented motor deficits and neuronal degeneration in a mouse MPTP model of nigral degeneration (Hudkins et al., 2008). While results from preclinical models were promising and CEP-1347 was found to be safe and well tolerated over 4 weeks in subjects with PD, clinical trials with CEP-1347 failed in a Phase II trial of 806 PD patients due to futility (Parkinson Study Group, 2007). This raised questions about the central nervous system (CNS) pharmacokinetic properties and target selectivity of CEP-1347. Specifically, CEP-1347 likely failed due to its poor brain penetrance (brain/plasma ratio < 0.2 in mice) (Parkinson Study Group, 2007). Furthermore, interpreting the efficacy of MLK3 inhibition as a therapeutic stategy was complicated by off-target effects of CEP-1347 (50% inhibition of 185 other kinases at 1uM) (Goodfellow et al., 2013).

The objective of the current study was to test a more potent and highly selective MLK3 inhibitor called CLFB-1134 with improved pharmacokinetic properties (brain/plasma ratio ~1.0) in a sub-acute mouse model of MPTP intoxication that induces nigrostriatal dopaminergic pathology. The structure of CLFB-1134 is presented in Fig. 1.

2. Methods

2.1. Screening PK (IV, PO, and IP dosing) in Mice and Rats

Four mice or rats were used for each time point. Male C57/BL/6 mice or Sprague-Dawley rats were dosed IV, PO, and IP at the doses indicated (Table 1A–1C). Total concentrations of the compound were determined by liquid chromatography–tandem mass spectrometry (LC-MS/MS), following plasma protein precipitation with acetonitrile and injection of the supernatant onto the column (XTerra®MS C18, 5 µm, 4.6 × 50 mm). The LC system comprised an Agilent (Agilent Technologies Inc., USA) 1100 series liquid chromatography equipped with G1379A degasser, G1311A Quantpump, G1313A autosampler and G1316A Column Oven. Mass spectrometric analysis was performed using an API4000 (triple-quadrupole) instrument from AB Inc. (Canada) with an ESI interface. Brains were collected at the time points indicated, rinsed with ice-cold saline, weighed, and stored at −80 °C until analysis. For compound quantitation, brains were homogenized in 5 volumes of water. The homogenates were extracted by protein precipitation with acetonitrile. LC-MS/MS analysis was conducted as described for the plasma. Brain homogenate concentrations were converted to brain concentrations for the calculations of brain to plasma ratios.

Table 1A
CLFB-1134 displays a favorable pharmacokinetic profile in mice with high brain penetrance. CLFB-1134's concentration in plasma and in brain following intravenous (IV), oral (PO), or intraperitoneal (IP) dosing, as indicated. AUC = area under curve, T ½ (hr) = half life time in hours. V2 = compartment volume.

	IV Dose 10 mg/kg	PO Dose 30 mg/kg
		*30% solutol in saline
PLASMA		
AUC	5575	7854
T ½ (hr)	2.6	3.3
V2 (mL/kg)	6745	1255
CLZ (mL/h/kg)	1793	2.0
%F		47
BRAIN		
AUC	7286	7185
T ½ (hr)	1.4	3.3
V2 (mL/kg)	2858	765
CLZ (mL/h/kg)	1372	2.0

Table 1B
CLFB-1134 (Single-dose) Pharmacokinetic Properties in Sprague-Dawley Rats.

	IP Dose 10 mg/kg
	*30% solutol in saline
PLASMA	
AUC	5630
T ½ (hr)	2.5
CLZ (mL/h/kg)	1775
Cmax	2135
Tmax	0.25
BRAIN	
AUC	2870
T ½ (hr)	3.6
Cmax	517
Tmax	2

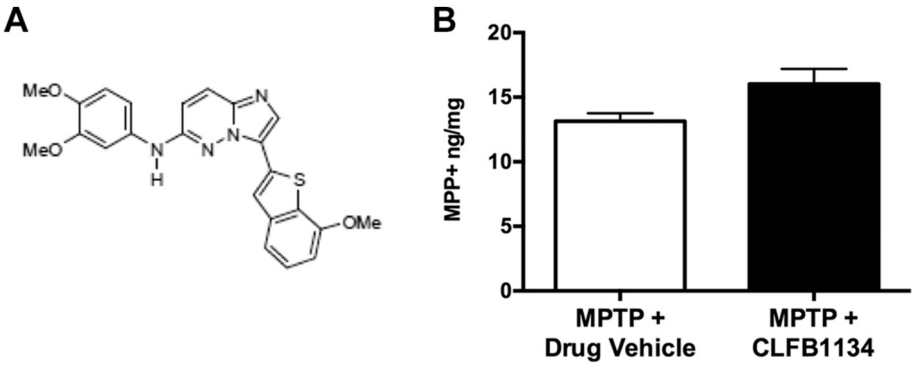

Fig. 1. CLFB-1134 does not interfere with MPTP metabolism. A. Structure of CLFB-1134. CLFB-1134 (Imidazo[1,2-*b*]pyridazine). IC_{50} = 42 nM. B. CLFB-1134 does not interfere with MPTP metabolism. Following 5 days of MPTP + CLFB-1134 or MPTP + drug vehicle treatment, striatal MPP+ levels were quantified using HPLC. MPP+ levels did not differ between mice concurrently dosed with MPTP + drug vehicle and those that received MPTP + CLFB-1134. MPTP + drug vehicle group mean ± SEM: 13.14 ng/mg ± 0.6258, (n = 9). MPTP + CLFB-1134 group mean ± SEM: 16.02 ± 1.175 (n = 12). p = 0.0648, by unpaired, two-tailed t test.

Table 1C
CLFB-1134 chronic dosing pharmacokinetic properties in Sprague-Dawley rats.

Male	Day	Plasma (ng/mL)	Brain (ng/g)	B/P
7 Day 10 mg/kg	Day 1	714	881	1.2
	Day 7	255	446	1.7
	% of Day 1	35%	51%	
7 Day 20 mg/kg	Day 1	1496	1695	1.1
	Day 7	453	543	1.2
	% of Day 1	30%	32%	
35 Day 20 mg/kg	Day 1	1496	1695	1.2
	Day 35	292	359	1.2
	% of Day 1	20%	21%	

Female	Day	Plasma (ng/mL)	Brain (ng/g)	B/P
7 Day 10 mg/kg	Day 1	240	494	2
	Day 7	104	157	1.5
	% of Day 1	43%	31%	
7 Day 20 mg/kg	Day 1	1051	640	0.61
	Day 7	234	441	1.9
	% of Day 1	22%	68%	

2.2. MPTP studies (Table 2)

Eight-week old male C57BL/6J mice purchased from Charles River (Ashland, OH) were used in all studies. Mice were maintained on a 12:12 light/dark cycle and standard rodent chow and tap water were available *ad libitum*. Animals were sacrificed by live decapitation. All procedures were conducted in accordance with the Guide for Care and Use of Laboratory Animals (National Institutes of Health) and have been approved by the Institutional Animal Care and Use Committee at Emory University.

CLFB-1134 was formulated in 5% DMSO, 40% PEG-400 at 2 mg/mL and administered intraperitoneally except where otherwise noted (pharmacokinetic study). 5% DMSO, 40% PEG-400 vehicle solution is referred to throughout as drug vehicle and was also administered intraperitoneally. MPTP was formulated in sterile saline and administered subcutaneously. Sterile saline is referred to throughout at neurotoxin vehicle and was also administered subcutaneously. MPTP was purchased from Sigma-Aldrich (St. Louis, MO). CLFB-1134 was synthesized by Califia Bio, Inc. (Fig. 1).

In the target-engagement study, mice simultaneously treated with the neurotoxin vehicle and CLFB-1134 were compared to those treated with neurotoxin vehicle and drug vehicle. CLFB-1134 (30 mg/kg) or the drug vehicle was administered by intraperitoneal (i.p.) injection 30 min prior to subcutaneous (s.c.) neurotoxin vehicle injection.

To investigate the neuroprotective effect of MLK3 inhibition against MPTP-induced nigrostriatal pathology (Figs. 3–5), mice were divided into the treatment groups described in Table 2, with the addition of untreated animals housed under identical conditions for the high performance liquid chromatography experiment described in the next section. CLFB-1134 was formulated as described above. CLFB-1134 or drug vehicle was administered *via* i.p. injection twice daily (8:00 am and 8:00 pm). MPTP or the neurotoxin vehicle was administered *via* subcutaneous s.c. injection once per day (1:00 pm).

2.3. High performance liquid chromatography (HPLC) of striatal neurochemistry

HPLC analysis of neurochemistry was performed as previously described (Caudle et al., 2007). Monoamine standards for DA, dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), and norepinephrine (NE) were purchased from Sigma-Aldrich (St. Louis, MO). Briefly, dissected striata were sonicated in 0.1 M perchloric acid. Homogenates were centrifuged at 15,000 ×*g* and the supernatant filtered through a 0.22 µm filter by centrifugation at 15,000 ×*g*. The supernatants were analyzed for levels of DA, DOPAC, HVA, and NE. Quantification was made by reference to calibration curves made with individual standards.

MPP+ levels in the striatum were evaluated as previously described (Richardson et al., 2008). Mice were sacrificed 3 h following the last dose of MPTP. Bilateral striata were sonicated in 5% trichloroacetic acid and centrifuged for 10 min at 14,000 ×g. MPP^+ levels were determined in the supernatants by HPLC with UV detection at 290 nm using a reverse-phase Altima C18 column (Alltech Associates Inc., Deerfield, Illinois, USA) and a mobile phase consisting of 89% 50 mM KH_2PO_4 and 11% acetonitrile. MPP^+ was identified and quantified by comparison of retention time with known standards.

2.4. pJNK and Total JNK immunoassay

The levels of JNK phosphorylation at Thr183 and Tyr185 in midbrain tissue was measured using the Phospho-JNK (Thr183/Tyr185) whole cell lysate assay kit (Meso Scale Discovery, Gaithersburg, MD).

Table 2
Experimental groups for MPTP studies.

Group	Treatment description	Figures
MPTP + CLFB-1134 (Co-dosed)	Mice received 20 mg/kg MPTP once per day (s.c.; 1:00 pm) and 30 mg/kg CLFB-1134 twice per day (i.p.; 8:00 am and 8:00 pm) for 5 consecutive days. Sacrifice was 3 h after the final MPTP dose.	Fig. 1
MPTP + drug vehicle (Co-dosed)	Mice received 20 mg/kg MPTP once per day (s.c.; 1:00 pm) and DMSO/PEG vehicle twice per day (i.p.; 8:00 am and 8:00 pm) for five consecutive days. Sacrifice was 3 h after the final MPTP dose.	
Neurotoxin vehicle + CLFB-1134	Mice received a single i.p. injection of CLFB-1134 and were given s.c. saline 30 min later.	Fig. 2
Neurotoxin vehicle + drug vehicle	Mice received a single i.p. injection of DMSO/PEG vehicle and were given s.c. saline 30 min later.	
MPTP + drug vehicle	Mice received a single i.p. injection of DMSO/PEG vehicle and were given s.c. MPTP 30 min later.	
MPTP + CLFB-1134	Mice received a single i.p. injection of CLFB-1134 and were given s.c. MPTP 30 min later.	
Neurotoxin vehicle	Mice received a single daily injection of saline (s.c.) for 5 consecutive days. Mice were sacrificed 3 weeks following the last injection.	Figs. 3 and 4
MPTP	Mice received a single daily injection of 20 mg/kg MPTP (s.c.) for 5 days in a row. Mice were sacrificed 3 weeks following the last injection.	Figs. 3–5
MPTP + CLFB-1134 (Co-dosed)	Mice were treated with a single daily injection of 20 mg/kg MPTP (s.c.) and two daily injections of CLFB-1134 (i.p.) for 5 days. CLFB-1134 injections continued on the same schedule for 3 more weeks following MPTP completion. Mice were sacrificed following the last CLFB-1134 injection.	Figs. 3–5
MPTP + drug vehicle (Co-dosed)	Mice were concurrently treated with a single daily injection of 20 mg/kg MPTP (s.c.) and two daily injections of DMSO/PEG (i.p.) for 5 days. DMSO/PEG injections continued on the same schedule for 3 more weeks. Mice were sacrificed following the last injection.	Figs. 3 and 4
MPTP + CLFB-1134 (Post MPTP)	Mice received a single daily injection of 20 mg/kg MPTP (s.c.) for 5 consecutive days. Mice then received two daily injections of CLFB-1134 (i.p.) for 3 weeks. Mice were sacrificed following the last injection.	Figs. 3–5

The assay was performed according to the manufacturer's instructions and all samples were analyzed in duplicate. In brief, dissected mouse midbrain tissues were homogenized in 1× complete lysis buffer. Lysates were centrifuged at 10,000 ×*g*, at 4 °C for 10 min and supernatant was collected. Total protein concentration in the lysate was determined using a BCA protein assay (Thermo Scientific). The Meso Scale Discovery plate was prepared according to the manufacturer's protocol, using lysate at a concentration of 0.2 μg/μL. The Meso Scale Discovery plate was read on the SECTOR Imager 2400 instrument (Meso Scale Discovery). Data represent background-subtracted chemiluminescent signal units.

2.5. Western blot analysis

Western blots were used to quantify the expression of tyrosine hydroxlase (TH) present in samples of striatal tissue from treated and control mice. Analysis was performed as previously described (Caudle et al., 2006). Briefly, unilateral striatum samples were homogenized, a BCA protein assay kit from Pierce (Rockford, IL) was used, and 5 μg (in 5 μL) of striatal protein were subjected to polyacrylamide gel electrophoresis. Following ectrophoretic transfer to polyvinylidene difluoride membranes, nonspecific sites were blocked in 7.5% nonfat dry milk in Tris-buffered saline. Membranes were incubated overnight in a polyclonal antibody to TH (Immunostar, Hudson, WI, 1:1000). TH antibody binding was detected using a goat anti-rabbit horseradish peroxidase secondary antibody (1:10,000) and enhanced chemiluminescence. The luminescence signal was captured on an Alpha Innotech Fluorochem imaging system and stored as a digital image. Membranes were stripped for 15 min at room temperature with Pierce Stripping Buffer and sequentially reprobed with β-actin (1:3000) antibody. Immunoreactivity of TH was normalized to β-actin levels to ensure equal protein loading across samples. Monoclonal mouse anti-β-actin antibodies were purchased from Sigma-Aldrich (St. Louis, MO). Secondary antibodies conjugated to horseradish peroxidase were obtained from Jackson Immunoresearch Laboratories (West Grove, PA). SuperSignal West Dura Extended duration substrate and stripping buffer were obtained from Pierce (Rockford, IL).

2.6. Histology and stereology

Tissue staining and cell counts were performed as described previously (Caudle et al., 2007). Briefly, midbrain blocks from control and treated mice were immersion fixed in 4% paraformaldehyde for 24 h at 4 °C and equilibrated in 30% sucrose in PBS at 4 °C before being cryosectioned (40 μm, coronal slice). Sections were incubated with a polyclonal anti-TH antibody overnight (EMD Millipore, Billerica, MA). TH signal was amplified with an ABC Elite kit (Vector Laboratories, Burlingame, CA) and then sections were incubated in a biotinylated goat anti-rabbit secondary antibody for 1 h at 4 °C. Visualization was performed using 3,3′-diaminobenzedine with nickel enhancement (Sigma-Aldrich, St. Louis, MO) for 3 minutess at room temperature. Sections were then incubated with monoclonal anti-NeuN antibody overnight (EMD Millipore, Billerica, MA). NeuN signal was amplified with ABC Elite kit (Vector Laboratories) and sections were incubated with biotinylated goat anti-mouse secondary antibody for 1 h at 4 °C. Visualization was performed using 3,3-diaminobenzidine for 3 minutes at room temperature. For cresyl violet stained tissue, a 0.1% aqueous cresyl violet solution (from Poly Scientific R&D Corp, New York) was used followed by destain and dehydration in ethanol and xylenes.

Unbiased stereological estimates of TH+ and total (NeuN+ or cresyl violet) neurons were performed using the optical fractionator method in StereoInvestigator analysis software with sampling parameters chosen to maintain a coefficient of error under 0.12 (MicroBrightField, Williston, VT). The substantia nigra pars compacta (SNpc) was delineated using previously described criteria (West et al., 1991). After delineation at low magnification, every sixth section was sampled at 40× oil-immersion objective on a Nikon 90i microscope (Nikon Melville, NY).

2.7. Statistics

GraphPad Prism (version 6) was used. When comparing only two groups, unpaired, two-tailed *t*-tests were used. Comparison across more than two treatment groups was carried out by one-way analysis of variance (ANOVA), followed by the multiple comparisons q (Newman-Keul's) test. Throughout analysis, $p < .05$ was considered statistically significant.

3. Results

3.1. CLFB-1134 displays a favorable pharmacokinetic profile with high brain penetrance in rats and mice

Studies were performed in mice and rats to determine the half-life (T ½) and brain penetrance of the drug when dosed intravenously (I.V.), orally (P.O.), or intraperitoneally (I.P.). Dosing orally in mice achieved a longer half life compared to i.v. dosing. The brain:plasma ratio is slightly increased when dosed i.v., although both dosing routes gave ratios of nearly one (Table 1A). Acute or subchronic IP dosing in rats achieved similar half-lives as IV dosing in mice but much longer half-lives in brain compared to mice (Tables 1B, 1C). Under all dosing paradigms, the brain:plasma ratios were consistently > 1, indicating excellent brain penetrance. An IP dosing paradigm was selected for the MPTP studies (Table 2).

3.2. MLK3 inhibition in vivo with CLFB-1134 does not interfere with MPTP metabolism to MPP+

The use of MPTP has long been the gold standard for generating a mouse model of the nigrostriatal dopaminergic pathway degeneration characteristic of parkinsonism. Critical to this process is the metabolism of MPTP to its neurotoxic metabolite, MPP+ by astrocytes (Javitch et al., 1985; Ransom et al., 1987) and the selective transport of MPP+ into the DA terminal by the DA transporter (DAT). Given these dynamics, it is imperative to ensure that any intervention, especially one that is viewed as neuroprotective, does not affect the processing of MPP+, leading to an artificial interpretation of neuroprotection. Striatal MPP+ levels were not different in mice concurrently dosed with MPTP + drug vehicle and those that received MPTP + CLFB-1134 (Fig. 1). These data demonstrate that treatment with CLFB-1134 does not impair or enhance the metabolism of MPTP to MPP+, providing a reliable model to evaluate neuroprotection by MLK3 inhibition.

3.3. In vivo administration of CLFB-1134 engages the target MLK3 and results in attenuated JNK phosphorylation

Prior to embarking on neuroprotection experiments, we dosed two cohorts of mice with CLFB-1134 (or drug vehicle as a control) just prior to either an MPTP or neurotoxin vehicle injection as described in the Methods. As a downstream measure of target engagement, we measured pJNK at Thr183/Tyr185 by multiplexed immunoassay. While previous work has shown JNK phosphorylation following MPTP treatment in mice, in our hands, MPTP treatment did not increase pJNK relative to the other treatment groups (Tatton and Kish, 1997). The addition of CLFB-1134 produced a 13% reduction in the background-subtracted pJNK chemiluminescent signal relative to neurotoxin vehicle-treated animals that also receiced drug vehicle (Fig. 2).

3.4. CLFB-1134 protects against MPTP-induced loss of striatal dopaminergic terminals

A hallmark pathological feature of PD is loss of markers of

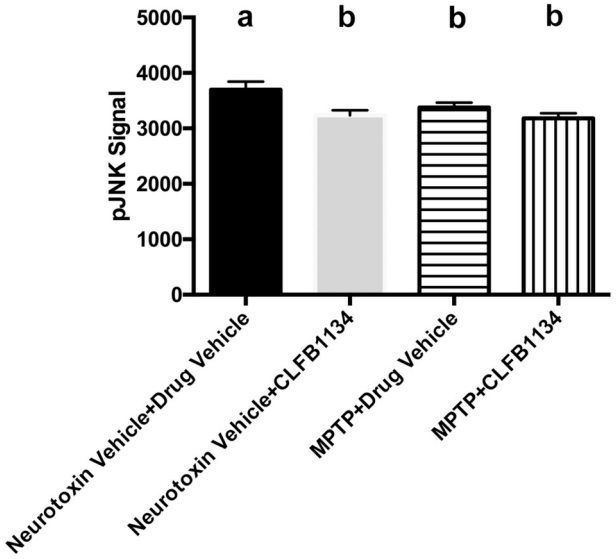

Fig. 2. *In vivo* target engagement by CLFB-1134. As a measure of MLK3 inhibition, JNK phosphorylation was quantified in mouse brain tissue. CLFB-1134 treatment was associated with decreased pJNK signal relative to drug vehicle, although MPTP also was associated with decreased pJNK signal relative to the neurotoxin vehicle condition. Mean ± SEM in background-subtracted chemiluminescent units: Neurotoxin vehicle + drug vehicle: 3699 ± 144.0 (n = 7), Neurotoxin vehicle + CLFB-1134 group: 3234 ± 93.2 (n = 7), MPTP + drug vehicle group: 3378 ± 87.82 (n = 8), MPTP + CLFB-1134 group: 3179 ± 93.78 (n = 8). Different letters indicate that there is a statistically significant difference between groups.

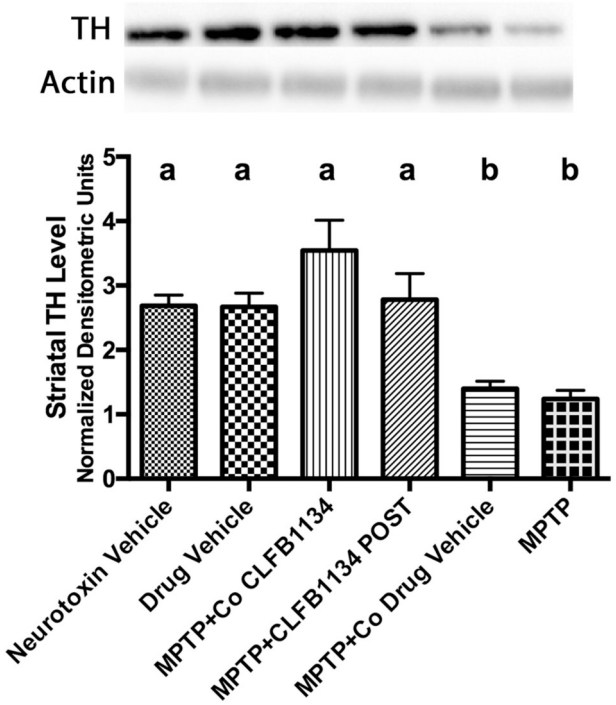

Fig. 3. CLFB-1134 protects against MPTP-induced loss of striatal TH. Immunoblotting analysis of TH (and actin control) in striatum of mice that received MPTP and concurrent or delayed treatment with CLFB-1134. Order of samples in immunoblot matches the groups on the x axis of graph. Ordinary one-way ANOVA with Newman-Keuls multiple comparisons test. Bars represent mean ± SEM. Neurotoxin vehicle n = 7. Drug vehicle n = 6. MPTP + Co-CLFB-1134 n = 7. MPTP + CLFB-1134-Post n = 10. MPTP + Co-Drug Vehicle n = 5. MPTP n = 5. *Co-* indicated co-dosing. *Post-* indicates CLFB-1134 dosing was initiated after MPTP dosing was completed. Different letters indicate that there is a statistically significant difference between groups.

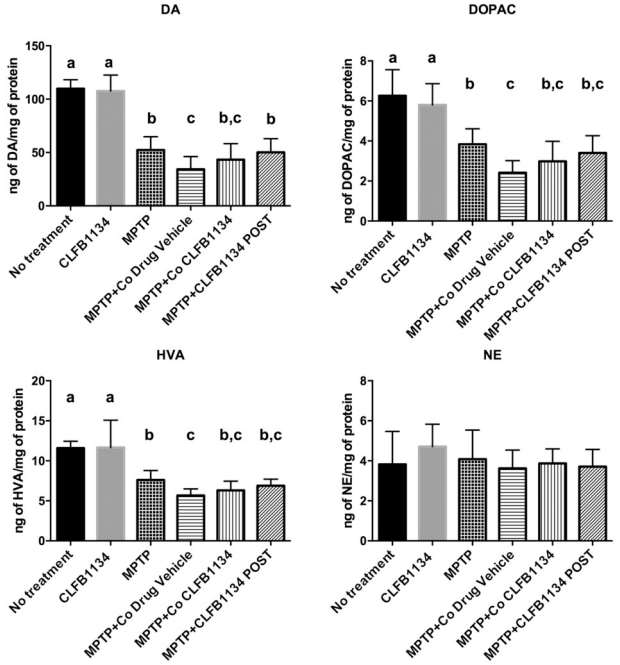

Fig. 4. CLFB-1134 does not protect against MPTP-induced striatal DA depletion. DA (dopamine), DOPAC (3,4-dihydroxy-phenylacetic acid), HVA (homovanillic acid), and NE (norepinephrine) were assessed by high performance liquid chromatography. For each analyte, an ordinary one-way ANOVA with Newman-Keuls multiple comparisons test was used for analysis. Bars represent mean ± SEM. No treatment n = 6. CLFB-1134 n = 8. MPTP n = 12. MPTP + Co-Drug Vehicle n = 10. MPTP + Co-CLFB-1134 n = 9. MPTP + CLFB-1134-Post n = 10. Different letters indicate that there is a statistically significant difference between groups.

dopaminergic terminal integrity and function in the striatum. As previously published (Jackson-Lewis and Przedborski, 2007; Meredith et al., 2008; Tillerson et al., 2002) exposure to 20 mg/kg MPTP for 5 days resulted in an approximately 50% reduction in striatal TH expression, compared with animals that received only neurotoxin vehicle or drug vehicle (Fig. 3). A similar reduction was measured when MPTP was co-administered with the drug vehicle. These reductions were absent when CLFB-1134 was administered *in vivo* simultaneously with MPTP or following the MPTP regimen (Fig. 3).

3.5. MLK3 inhibition in vivo with CLFB-1134 did not protect against MPTP-induced striatal DA depletion

The classic motor deficits observed in PD are attributed to a significant loss of DA in the striatum. MPTP treatment reliably mimics this dramatic DA depletion. Our treatment with MPTP induced an approximately 50% reduction in striatal dopamine, compared with mice treated with neurotoxin vehicle or drug vehicle (Fig. 4). Delayed treatment with CLFB-1134 following MPTP did not protect against loss of striatal DA relative to vehicle, and HVA and DOPAC levels were not restored to untreated levels.

3.6. MLK3 inhibition in vivo with CLFB-1134 protects against MPTP-induced nigral dopaminergic degeneration

In addition to damage to terminals in the striatum, loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) is a classic feature of PD neuropathology. With our MPTP paradigm, we were able to demonstrate a dramatic reduction (approximately 84%) in the number of TH+ neurons in the SNpc of MPTP-treated mice, compared with neurotoxin vehicle or drug vehicle controls (Fig. 5). Consistent with the striatal TH analysis, unbiased stereological estimate of

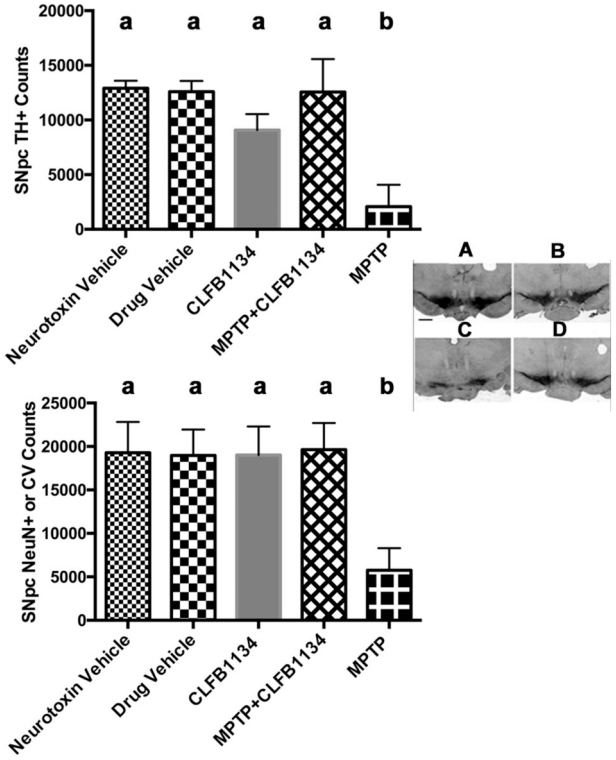

Fig. 5. CLFB-1134 protects against MPTP-induced nigral dopaminergic cell loss. Stereological counts of TH+ and NeuN+ (or cresyl violet for CLFB-1134 group) cells in the substantia nigra. Representative micrographs depict nigral sections double-stained for TH and NeuN (2x). A. Vehicle, B. MPTP + Co-CLFB-1134, C. MPTP, D. MPTP + CLFB-1134-Post. Scale bar = 300 mm. Bars in graphs represent mean ± SEM. Ordinary one-way ANOVA. Neurotoxin vehicle n = 4. Drug vehicle n = 4. CLFB-1134 n = 3. MPTP + CLFB-1134 n = 5. MPTP n = 3. Different letters indicate that there is a statistically significant different between groups.

total neurons in the SNpc confirmed that reductions were a result of loss of TH+ neurons, rather than a reduction or masking of the TH epitope (Fig. 5). In contrast, CLFB-1134 provided robust protection from MPTP-induced nigral dopaminergic cell loss.

4. Discussion

It has long been known that dopaminergic neurodegeneration drives the motor phenotypes of PD. Because of the cell cycle, apoptotic signaling, and immune activation effects of MLK inhibition, several MLK inhibitors have been tested in models of dopaminergic degeneration and in PD patients (Parkinson Study Group, 2007; Saporito et al., 1999; Saporito et al., 2002) (Lotharius et al., 2005; Mathiasen et al., 2004). Using a compound more specific for MLK3 and with better brain penetrance than the earlier inhibitior CEP-1347, we hoped to improve upon previous MLK inhibition work. We found that, indeed, CLFB-1134 does cross the blood-brain barrier (BBB) and achieves physiologically relevant concentrations following oral, intravenous, or intraperitoneal dosing in mice and rats (Table 1A–1C). An IP dosing paradigm was selected for the MPTP/CLFB-1134 studies.

Dosing regimens used in previous work, including once daily dosing (i.p.) have been sufficient to achieve therapeutic effects in other studies with MLK inhibition (Dong et al., 2016; Gnanadhas et al., 2017; Tomita et al., 2017). We then verified that the neuroprotective capacity of CLFB-1134 can be tested in the MPTP-induced dopaminergic degeneration model because it does not interfere with the conversion of MPTP to MPP+ (Fig. 1). While MPTP was not, at the timepoint selected, associated with increases in pJNK, CLFB-1134 was found to engage MLK3 and produce a 13% decrease in phosphorylation of JNK (comparing saline-treated groups, Fig. 2). The lack of MPTP-induced pJNK increase could be due to the transience of MPTP-induced phosphorylation of JNK. The cascade engaging MLK3 and resulting in increased pJNK set off by MPTP may, by time of sacrifice, have been erased by protein turnover and phosphatase activity altering JNK's status. Nevertheless, CLFB-1134, prior to animal sacrifice, had the opportunity to interrupt the cascade and diminish pJNK. An earlier sacrifice, closer to MPTP treatment, may have revealed increased pJNK as in (Huang et al., 2016), where elevated pJNK following MPTP was reported at 6 h post neurotoxin injection.

Whether given simultaneously or after MPTP treatment, CLFB-1134 preserves striatal dopaminergic terminals expressing TH as well as nigral TH+ soma (Fig. 3, Fig. 5). The modest decrease in JNK phosphorylation achieved by MLK3 inhibition is the simplest explanation for the preservation of TH nigral cell bodies following CLFB-1134 treatment. Because MLK3, a MAPKKK, is part of a signaling cascade, MLK3 inhibition has far reaching consequences as pJNK, in turn, acts on many transcription factors and other signaling molecules. Thus, a slight change in MLK3-associated phosphorylation can be amplified and, as we interpret in this case, elicit robust protection of nigral TH+ neurons, and this is similar to what has been previously published with MLK inhibition. Treatment with the broad spectrum MLK inhibitor, URMC-099, in mice was associated with a 21.5, 17.3, and 23.7% decrease in phosphorylation of p38, p46-JNK, and p54-JNK (Dong et al., 2016).

By HPLC, our findings indicate that while there was a significantly greater amount of DA in MPTP + CLFB1134 POST animals than in MPTP + drug vehicle animals, there was no significant difference between concentrations of DA or its metabolites in MPTP, MPTP + Co CLFB1134, or MPTP + CLFB1134 POST groups (Fig. 4). Achieving nigral neuroprotection in the absence of striatal DA restoration has been reported in other studies using MPTP to model PD neuropathology. Others have proposed that the discrepancy between persistent neurotransmitter depletion and the complete preservation of terminals and soma is due to MPTP's rapid effect on dopaminergic terminals and the more gradual, microglia-dependent action in the SNpc (Hu et al., 2008; Liberatore et al., 1999; Zhang et al., 2004). Loss of DA from the striatum in our paradigm may be related to loss of mitochondrial energetics as induced by MPTP directly, rather than the downstream apoptotic and necroptotic signaling that damages terminals and cell bodies. Specificially, TH is activated by phosphorylation, an event which requires ATP (Daubner et al., 2011). The depletion of ATP achieved by MPTP may prevent activation of TH and thus block proper DA synthesis. Neuroprotective interventions given post-MPTP would thus have a greater chance of affecting the SNpc rather than DA synthesis in striatal terminals. Another possible explanation of the discrepancy between terminal rescue in the absence of neurochemical recovery may be that MPTP damages elements of DA synthesis that are not regulated by MLK/JNK signaling pathways and thus cannot be mitigated by MLK inhibition with CLFB-1134. For example, MPTP treatment decreases the activity of aromatic L-amino acid decarboxylase (King et al., 2011) and the activity of this enzyme may not recover following inhibition of MLK3.

Understanding the effect of CLFB-1134 treatment on microglia, infiltrating peripheral immune cells, and astrocytes may provide insight into the role of MLK3 in neuroinflammation. MLK inhibition's effects on microglia have been investigated elsewhere: the broad-spectrum MLK inhibitor URMC-099 was reported to modulate activated microglia in the mouse experimental autoimmune encephalomyelitis (EAE) hippocampus (Bellizzi et al., 2018). While no changes in activated morphology of Iba1+ cells were induced by MLK inhibitor treatment, both URMC-099 and CLFB-1134 prevented increased CD68 expression (a lysosomal marker associated with phagocytosis) (Bellizzi et al., 2018). Furthermore, URMC-099 decreased canonical markers associated with microglial activation: iNOS, immunoglobulin receptor FCγR1/CD64, and the co-stimulatory molecule CD86 (Bellizzi et al., 2018). Overall

the study suggests that MLK inhibition shifts brain microglia toward a less inflammatory and phagocytic phenotype, with broad-spectrum MLK inhibition favoring synaptic preservation in EAE. While the mechanisms of neuroinflammation and the neurons undergoing degeneration obviously differ between EAE and the MPTP model, the capacity for MLK inhibition to engage glia may contribute to the neuroprotective effect of these drugs, in addition to direct neuronal action, and this is an area that merits future study.

In our own work, Fig. 3 suggests that at the height of MPTP toxicity, CLFB-1134 is capable of preserving the dopaminergic phenotype of the vulnerable nigrostriatal terminals. Preservation of tyrosine hydroxlase expression is necessary for DA production, so while DA and its metabolites were decreased in CLFB-1134-treated mice relative to untreated and CLFB-1134-alone groups, the synthetic machinery (TH) necessary for neurotransmitter production remains expressed. Counts of dopaminergic cell bodies in the substantia nigra further support the conclusion that CLFB-1134 protects neuronal potential to produce dopamine, despite DA levels at the time of sacrifice appearing relatively depleted. The endpoint in this study was 3 weeks post-MPTP, and it is possible that more complete neurochemical recovery could have been observed given more time. However, because spontaneous recovery from MPTP has been observed given increased time post-toxin (Mitsumoto et al., 1998), our limited time course is most informative for specifically evaluating CLFB-1134's neuroprotective capacity.

The early findings from pre-clinical models with first generation MLK inhibitors were promising, yet clinical trials with CEP-1347 failed in a Phase II trial for early PD due to futility (Parkinson Study Group, 2007). It was proposed that another way to improve upon the function of MLK3 inhibitors would be to provide additional support from neurotropic factors such as brain-derived neurotrophic factor (BDNF) signaling *via* tropomyosin receptor kinase B (TrkB) receptors (Wang and Johnson, 2008). This recommendation is based on the fact that MLK3 inhibtion increases the expression of Trk receptors. For dopaminergic neurons in SNpc, the Trk receptors expressed are type B and cannot activate in the absence of their ligand. Once activated, as by BDNF, TrkB receptors lead to the activation of the PI3k signaling cascade, engaging Akt and, ultimately, inhibition of GSK3β. The efficacy of GSK3β inhibiton alone as a neuroprotective treatment in an MPTP model of dopaminergic degeneration has been previously described, although the study was limited in translational relevance as a pre-MPTP dosing schedule was used (Wang et al., 2007). Nonetheless, the GSK3β inhibitor did protect dopaminergic soma and terminals. Similar to our own results, the DA concentration in the striatum remained low relative to the control condition, despite the anatomical preservation achieved by GSK3β inhibition. This suggests that targeting the signaling cascade downstream of TrkB may not restore DA levels beyond what was achieved with MLK3 inhibtion. Thus, MLK3 inhibition, even on its own, warrants further investigation.

Here, we demonstrated that CLFB-1134 has a favorable brain penetrance and as evinced from our studies, penetrates the BBB sufficiently to engage its target and dampen the downstream pJNK signal that can be measured by immunoassay in brain lysates from mice treated with drug *in vivo*. This target engagement is then the basis for the robust protection of striatal TH terminals and DA neurons in the SNpc from MPTP-induced damage.

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