Novel LRRK2 GTP-binding inhibitors reduced degeneration in Parkinson’s disease cell and mouse models

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Mutations in the leucine-rich repeat kinase-2 (LRRK2) gene cause autosomal-dominant Parkinson’s disease (PD) and contribute to sporadic PD. LRRK2 contains Guanosine-5′-triphosphate (GTP) binding, GTPase and kinase activities that have been implicated in the neuronal degeneration of PD pathogenesis, making LRRK2, a potential drug target. To date, there is no disease-modifying drug to slow the neuronal degeneration of PD and no published LRRK2 GTP domain inhibitor. Here, the biological functions of two novel GTP-binding inhibitors of LRRK2 were examined in PD cell and mouse models. Through a combination of computer-aided drug design (CADD) and LRRK2 bio-functional screens, two novel compounds, 68 and 70, were shown to reduce LRRK2 GTP binding and to inhibit LRRK2 kinase activity in vitro and in cultured cell assays. Moreover, these two compounds attenuated neuronal degeneration in human SH-SY5Y neuroblastoma cells and mouse primary neurons expressing mutant LRRK2 variants. Although both compounds inhibited LRRK2 kinase activity and reduced neuronal degeneration, solubility problems with 70 prevented further testing in mice. Thus, only 68 was tested in a LRRK2-based lipopolysaccharide (LPS)-induced pre-inflammatory mouse model. 68 reduced LRRK2 GTP-binding activity and kinase activity in brains of LRRK2 transgenic mice after intraperitoneal injection. Moreover, LPS induced LRRK2 upregulation and microglia activation in mouse brains. These findings suggest that disruption of GTP binding to LRRK2 represents a potential novel therapeutic approach for PD intervention and that these novel GTP-binding inhibitors provide both tools and lead compounds for future drug development.

INTRODUCTION

Parkinson’s disease (PD) is a common age-related progressive neurodegenerative disorder resulting from the loss of dopaminergic neurons. Currently, there are no disease-modifying therapeutic agents to slow the neuronal degeneration of PD (1,2). Mutations in the LRRK2 gene have become a major known cause of PD (3–11). There are over 40 reported LRRK2 mutations, seven of which are disease-causing mutations (12). G2019S is the most common pathogenic mutation and is present in 4–8% of genetic and 1–3% of sporadic cases (12). There are 39% of PD patients with the G2019S mutation in North African Arabs and 13% in the US Jewish population. The LRRK2 protein contains multiple functional domains including two functional enzymatic domains: a GTPase domain and a protein kinase domain (5–12). Most disease-causing mutations are in the GTPase and the kinase domains (11–14). The majority of these has abnormally higher kinase activity or disrupted Guanosine-5′-triphosphate (GTP) domain activity compared with wild-type LRRK2 (3–14). Increasing evidence indicates that abnormal LRRK2 kinase and GTP-domain activities likely contribute to the neurodegeneration in LRRK2-linked PD (15–21) suggesting that LRRK2 is a potential target for the development of novel PD medications (12).

Rapid progress has been made towards the development of LRRK2 kinase inhibitors and a number of such inhibitors have been reported (2,15,16,22,23). However, these agents appear to be not clinically viable due to non-specificity or low brain...
penetration (1,2). To date, there are no published reports of GTPase domain inhibitors for LRRK2. Previous studies show that the K1347A genetic alteration abolishes GTP binding and reduces LRRK2 kinase activity, thereby protecting against LRRK2 toxicity in cell culture, suggesting that kinase activity is regulated by GTP-binding activity (18,19,21). Other studies show that the GTP domain exhibits important roles in LRRK2 biological functions (24,25) by regulating neuronal growth and degeneration (24,25). A recent report have shown that PD-linked mutation, R1441H increases 2-fold of GTP binding and kinase activities when compared with wild-type LRRK2 (14). These findings suggest that inhibition of LRRK2 GTP binding may be a novel therapeutic target for PD intervention.

Computer-aided drug design (CADD) has shown utility in the identification of novel inhibitors of target proteins via database screening methods (26) when the targeting protein crystal structure is available. Although the crystal structure of full-length LRRK2 protein is not yet available, the crystal structure of the LRRK2 GTPase domain (ROC) complexed with guanosine diphosphate and Mg^{2+} (PDBID 2zej) (20) provides a structural basis for the identification of novel compounds that target this domain. We performed a CADD virtual database screening of 1.5 million drug-like, low-molecular weight, commercially available compounds (27) targeting the ROC domain to identify compounds that block the GTP-binding site. Selected compounds were then subjected to biological screening using GTP-binding assays. Compounds 68 and 70 were identified from the screens to reduce LRRK2 GTP-binding activity. The biological effects of these two compounds were evaluated in this study using LRRK2-based PD cell and mouse models. Our studies showed that 68 and 70 also reduced LRRK2 kinase activity but did not alter LRRK1 GTP binding and kinase activities. These compounds provide pharmacological tools to further dissect the LRRK2 pathophysiological functions in PD pathogenesis as well as have the potential for development into therapeutic agents for the treatment of PD.

RESULTS

Compound 70 reduced LRRK2 binding GTP and kinase activity

Compound 70 (Fig. 1A) was the first compound that was identified from the combination of CADD and biological screens. The chemical structure of 70 is shown in Figure 1A. Compound

Figure 1. 70 inhibits LRRK2 GTP binding. (A) The chemical structure of compound 70. (B) LRRK2 variants were affinity-purified from lysates of transfected HEK293T cells using GTP-agarose in the absence or presence of 70 or GW5074 at 10 μM with 0.1% DMSO at final concentration. The vehicle control was added as 0.1% DMSO only. Precipitates were subjected to western blot analysis using anti-Flag antibodies. WT: wild-type LRRK2. (C) myc-LRRK1 was affinity-purified from lysates of transfected HEK293T cells using GTP-agarose in the absence or presence of 70 (10 μM). Precipitates were subjected to western blot analysis using anti-myc antibodies. (D and E) LRRK2 was affinity-purified from lysates of transfected HEK293T cells using GTP-agarose in the absence or presence of 70 (0–1000 nM). Precipitates were subjected to western blot analysis using anti-Flag antibodies. (D) Shown is a representative of western blots. (E) Quantification of blots from three separated experiments.
70 [10 μM in 0.1% Dimethyl sulfoxide (DMSO)] reduced both wild-type and PD-linked mutant LRRK2 (G2019S, R1441C and Y1699C) GTP-binding activity (Fig. 1B). The genetic non-GTP-binding control K1347A-LRRK2 did not bind GTP. A LRRK2 kinase inhibitor, GW5074, did not alter the GTP-binding activity of LRRK2 (Fig. 1B) and 70 (10 μM) did not alter LRRK1 (86% homology with LRRK2) GTP-binding activity (Fig. 1C). Moreover, 70 was relatively potent in vitro, at 1 nM reducing up to 85% of GTP binding to LRRK2 (Fig. 1D and E). Further reduction (<1 nM) in the concentration of 70 no longer affected the GTP binding of LRRK2.

Although genetic alteration of the ROC-domain GTP-binding loop at residues 1347 and 1348 reduces LRRK2 kinase activity (18,21), there is no pharmacological approach to verify the relation between GTP binding and kinase activity.

Treatment of HEK 293T cells expressing mutant G2019S-LRRK2 with 70 significantly inhibited LRRK2 phosphorylation at residues S935 and S2032 in a dose-dependent manner (Fig. 2A – B), providing the first pharmacological evidence that disruption of GTP binding regulates LRRK2 kinase activity. Compound 70 at a concentration of 100 nM reduced kinase activity of G2019S-LRRK2 up to 90% (Fig. 2B). Using in vitro autophosphorylation (kinase) assay by incorporation of [γ-32P]ATP, 70 only reduced the kinase activity of full-length LRRK2 protein (Fig. 2C left) but did not alter the kinase activity of the truncated G2019S-kinase domain (Fig. 2C right). Moreover, 70 also did not alter LRRK1 autophosphorylation (kinase activation), determined using an assay that immunoprecipitates LRRK1 from overexpressed cell lysates that were probed with anti-phosphorylation serine antibodies, as described previously (28) (Fig. 2D).

**Compound 68 reduced LRRK2 GTP binding and kinase activities**

Compound 68, another active compound from the CADD screen (Fig. 3A), had a similar effect as 70 in reducing GTP binding LRRK2 in vitro (Fig. 3B). 68 at 10 nM concentration reduced up to 90% of GTP-binding activity. Moreover, treatment of 68 in HEK 293T cells expressing human G2019S-LRRK2 also reduced LRRK2 autophosphorylation (kinase activity) at residue S2032 (Fig. 3C) in a similar fashion to 70. 68 also reduced mutant G2019S- and R1441C-LRRK2 binding GTP (Fig. 3D).

In contrast, a analog of 68, FX2151 had a similar chemical structure but did not alter GTP-binding activity. 68 (10 μM) did not alter LRRK1 GTP binding (Fig. 3E) and kinase activity (Fig. 3F).

**Both 68 and 70 suppress mutant LRRK2-induced neuronal degeneration in cultured neurons**

Expression of mutant G2019S-LRRK2 led to neuronal degeneration in both SH-SY5Y and mouse primary cortical neurons as assessed by cell viability and DNA fragmentation (TUNEL) assays as previously described (18). Treatment of SH-SY5Y cells

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**Figure 2. 70 inhibits LRRK2 kinase activity.** (A and B) HEK-293T cells were transfected with Flag-G2019S-LRRK2 for 36 h and then starved with no serum media for 12 h. Cells were treated with vehicle or 70 (0–1000 nM) for 1 h and cells were harvested for IP using anti-Flag antibodies. The resulting immunoprecipitates were subjected to Western blot analysis using anti-phosphorylation LRRK2 antibodies (S935 or S2032). (A) Representative blots. (B) Quantification of LRRK2 phosphorylation normalized to that of cell lysates of G2019S-LRRK2 treated with vehicle (0.1% DMSO) (three independent experiments). (C) 70 (10 μM) significantly reduced the autophosphorylation of G2019S full-length LRRK2 but did not alter its kinase domain autophosphorylation, as seen by in vitro kinase assays. (D) 70 (10 μM) did not alter serine phosphorylation of LRRK1 using in vitro kinase assays.
with 70 (1 and 5 nM) or 68 (5 and 10 nM) significantly attenuated G2019S-LRRK2-induced neuronal degeneration (Fig. 4A–B). To further confirm these findings, mouse primary cortical neurons were used to assess the effects of 70 and 68. Treatment of 70 (2 and 5 nM) and 68 (5 and 10 nM) protected against mutant LRRK2-induced neuronal degeneration (Fig. 4A and C). Moreover, 70 and 68 both decreased the TUNEL-positive SH-SY5Y cells expressing mutant LRRK2 (Fig. 4D).

**68 attenuated LPS-induced LRRK2 upregulation and microglia activation in a mouse neuroinflammation model**

To assess whether 70 and 68 alter LRRK2 activities in vivo, G2019S-LRRK2-BAC transgenic mice (29,30) were used. 70 had poor solubility in 10% DMSO, 0.9% saline buffer and could not be dissolved at a dose of mg/kg for in vivo testing of LRRK2 functions in mice. Only the effects of 68 on LRRK2 functions were tested in vivo using G2019S-LRRK2-BAC transgenic mice (29,30). Vehicle or 68 at 10 and 20 mg/kg was injected intraperitoneally for 1 h. Mouse brain homogenates were subjected to LRRK2 autophosphorylation and GTP-binding assays. 68 at 10 mg/kg did not alter LRRK2 GTP-binding activity after 1 h injection (Fig. 5A and B). 68 at 20 mg/kg significantly reduced LRRK2 GTP-binding activity in brain homogenates. However, 68 at 20 mg/kg significantly reduced LRRK2 GTP-binding activity after 1 h injection (Fig. 5A and B). 68 at 20 mg/kg also reduced LRRK2 phosphorylation in mouse brains after 1 h injection (Fig. 5C and D). In contrast, FX2151, the ineffective analog of 68, did not alter LRRK2 kinase and GTP-binding activities in mouse brains.
These results indicated that 68 can penetrate BBB with higher dose at 20 mg/kg.

Inflammation is an important factor in PD pathogenesis (31). Microglial activation is one of the early indicators of degeneration and a great contributor to dopaminergic neuron degeneration (31). Chronic microglia-mediated inflammation likely initiates or prolongs neuron degeneration (32). A recent study demonstrated that LRRK2 kinase activation plays an important role in LPS-induced microglia activation in a LRRK2-BAC transgenic mouse model (33). Here, we employed this model to assess the in vivo effects of 68. In white matter tracts in non-injected mouse brain tissue, LRRK2 protein was undetectable,
and there was a weak positive staining in the substantia nigra as described previously (33). Inactive microglia within the substantia nigra in mice that did not receive an LPS injection showed a weak basal level of positive immunostaining with isolectin B4 (microglia marker) antibodies (Fig. 6). However, in LPS-injected sites, there was robust LRRK2 and isolectin B4-positive immunostaining within the substantia nigra compared with vehicle injection groups (Fig. 6). Moreover, there was also a robust anti-phosphorylated LRRK2 immunoactivity, indicating LPS induced microglia activation as well as LRRK2 expression and phosphorylation (Fig. 6). As in a previous report (33), increased LRRK2 protein was predominantly located in the active microglia. Interestingly, treatment with 68 significantly reduced activated microglia numbers and densities of LPS-induced positive immunostaining with anti-isoelectin B4, anti-LRRK2, and anti-phosphorylated LRRK2 antibodies (Fig. 6). In particular, the immunoreactivity of anti-phosphorylated LRRK2 antibodies in the 68 treated group was reduced up to ~54% compared with those in LPS group.

**DISCUSSION**

Two novel GTP-binding inhibitors of LRRK2, 70 and 68, were characterized to reduce kinase activity and attenuate neuronal degeneration. Although the normal function of LRRK2 is not fully understood, the elevated kinase activity in PD-linked mutations (e.g. G2019S) leads to neuronal degeneration (3–11). Thus, the inhibition of this abnormally elevated activity in the mutant LRRK2 PD cases could result in neuroprotection and represent a novel strategy for intervention (12). Even though the exact function of the ROC domain is unknown, our results demonstrated that reduction of the GTP-binding activity inhibited LRRK2 kinase activity. To our knowledge, this study is the first report of LRRK2 GTP-binding inhibitors and provides pharmacological evidence that GTP binding regulates kinase activity. These results further validate the previous genetic alteration findings (18) and indicate that the GTP-binding site in ROC domain is a druggable target.

Only a few proteins (e.g. LRRK1) in mammals are known to have intrinsic GTPase domain activity that regulates kinase domain activity (28). Our results show that 70 and 68 inhibited LRRK2 binding with GTP but did not alter LRRK1 GTP binding nor kinase activity, suggesting 70 and 68 are relatively specific to LRRK2. Moreover, the two inhibitors reduced the kinase activity of full-length LRRK2 but did not alter the kinase activity of the LRRK2 kinase domain, further suggesting that these inhibitors reduce kinase activity via altering GTPase domain function. In vitro, 70 and 68 inhibited LRRK2 GTP binding and kinase activities in the nM range, which is similar
Figure 6. LPS attenuated LPS-induced inflammation and both LRRK2 phosphorylation and expression in mice. LPS (5 μg) was injected unilaterally into the substantia nigra of G2019S-LRRK2 BAC transgenic mice at 6–12 weeks of age. 68 (20 mg/kg) was injected intraperitoneally 1 h prior to the LPS injection and then kept twice daily at the same dose for three days. Immunohistochemistry was performed on serial coronal sections through the substantia nigra. (A) Representative images of immunostaining with anti-LRRK2, anti-isolectin B4 (marker for microglia and endothelial cells), and anti-phosphorylated LRRK2 at S935 antibodies and visualized using DAB. (B) Double immunofluorescent staining using anti-LRRK2 (red) and anti-isolectin (green) antibodies. (C) Quantification of immunofluorescence of panel B: *P < 0.05 by ANOVA compared with vehicle group. #P < 0.05 by ANOVA compared with LPS treated group.
to the effects of known LRRK2 kinase inhibitor, LRRK2-In-1 (34). However, LRRK2-In-1 cannot penetrate the blood brain barrier (BBB), which limits its utility for PD (34). Our results showed that 68 inhibited LRRK2 GTP binding and kinase activities in LRRK2 transgenic mouse brains, suggesting 68 can penetrate the BBB.

One of the critical barriers to developing neuroprotective compounds for PD and other neurodegenerative diseases is that these compounds must cross the BBB to the pathologic sites (1,2). The current reported LRRK2 protein kinase inhibitors either lack specificity or do not cross the BBB (1,2). Our results showed that 68 can readily penetrate the BBB and inhibit LRRK2 GTP binding and kinase activity. Our in vitro characterization studies showed that 70 and 68 inhibited LRRK2 GTP binding at ng concentrations. However, reduction of brain LRRK2 GTP binding and kinase activities required a dose of 20 mg/kg for 68, which, at 10 mg/kg dose, did not have this effect. This suggests that 68 can penetrate the BBB but with a low brain uptake efficiency. Accordingly, these results indicate that 68 may be a lead compound for the further development into compounds that target LRRK2 functions in brain as required for PD intervention.

In LRRK2 PD cases, dopaminergic neuronal degeneration in brains results in locomotor impairment and PD symptoms. In cell culture studies, reduction of LRRK2 GTP binding by a genetic approach reduces its kinase activity, thereby suppressing neuronal degeneration (18). Our data showed that both 70 and 68 at nanomole levels significantly reduced mutant LRRK2-induced neuronal degeneration in SH-SY5Y cells and mouse primary cortical neurons. This is the first proof of principle that GTP-binding inhibitors can suppress LRRK2-linked neuron degeneration.

Microglia activation often occurs in neuronal degenerative diseases, including PD and is one of early pathological hallmark of degeneration (32). Microglia-mediated inflammation triggers the vicious cycle between glial-astrocyte reaction and dopaminergic neuronal loss (32). Recent studies show that LRRK2 is highly expressed in macrophagic and mononuclear cells, and it is expressed in the brain’s immunological cells, microglia (35–38). These findings suggest a potential immunologic function for LRRK2. One report showed that pre-inflammatory stimuli (e.g. LPS) (33) induced LRRK2 expression in peripheral blood mononuclear cells (39). A recent study demonstrated that inflammation increases LRRK2 activity and expression in activated microglia in a mouse model of neuroinflammation (33). Moreover, knockdown of LRRK2 or reduction of LRRK2 kinase activity in primary cultured microglia blocks microglial process outgrowth and TNF-alpha release (33), suggesting that LRRK2 may alter inflammatory responses in neurodegenerative and infectious diseases and may be involved in disease initiation or progression processes. Our data showed that treatment with 68 significantly reduced the LPS-induced microglia activation and LRRK2 expression. 68 reduced LPS-induced LRRK2 phosphorylation in activated microglia cells. Consisting with the previous report (33), there were no changes in anti-TH positive immunostaining between the LPS-injected side and the non-injected side, nor changes in the sham injected vehicle mouse brain tissue, suggesting that there was no dopaminergic neuron degeneration within the substantia nigra under this acute inflammation condition during our testing regimen. However, the inflammation (especially the chronic condition) triggers or accelerates dopaminergic neuronal loss (32). Taken together, these findings not only indicate that LRRK2 GTP binding plays a critical role in the pre-inflammatory response in microglia cells in brains, but also demonstrates that inhibition of LRRK2 GTP binding can attenuate the inflammation-related degenerative pathology.

In conclusion, LRRK2 GTP-binding inhibitors provide a pharmacological tool to further study LRRK2 functions in PD pathogenesis. Our studies provide two lead compounds for further development into potential therapeutic agents for the treatment of PD. The present findings indicate that 70 and 68 reduced GTP-binding LRRK2, thereby decreasing LRRK2 kinase activity, suggesting that the GTPase domain is a novel potential drug target for PD.

MATERIALS AND METHODS

Materials and compounds

Media for cell culture and LipofectAMINE Plus reagent were from Invitrogen (Carlsbad, CA). Anti-Flag, anti-isolectin-biotin, and anti-isolectin-FICT antibodies were from Sigma (St Louis, MO, USA). Anti-tyrosine hydroxylase (TH) was from Millipore (Billerica, MA, USA). Anti-phospho-serine antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-LRRK2 and some anti-phosphorylated LRRK2 antibodies were from Michael J. Fox Foundation. Anti-myc and anti-actin antibodies were from Santa Cruz (Santa Cruz, CA, USA). Some anti-LRRK2 phosphorylation antibodies at S935 and S2032 were kindly provided by Drs Zhenyu Yue and Ted M. Dawson (40,41). GW5074 was purchased from BioMol.

CADD and compounds

CADD database screening was performed targeting the GTP domain as described previously (27,42–45). Briefly, CADD analysis was performed to identify putative inhibitor binding sites on the 3D structure of the ROC homodimer (20) using the Binding Response algorithm (27,42). Docking and simulations were performed with the programs CHARMM (46), NAMD (47) and Dock 4.01 (48) to screen an in silico database of a 1.5 million compound library. Compounds that could potentially bind LRRK2 Roc domain with physicochemical properties that maximize drug-like characteristics were selected for further validation using GTP-binding assays as described below. 70 and 68 were identified from a CADD screen followed by an in vitro GTP-binding assay validation. Details of the CADD screening protocol along with experimental results for all other active compounds will be presented in a subsequent publication. 70 and 68, which are not guanine analogs, were purchased from Chembridge. For in vitro biochemistry analysis and cell culture experiments, 70 and 68 were dissolved in 0.1% DMSO at final concentrations for in vitro experiments. For mouse testing, 68 was dissolved in 10% DMSO/0.9% saline and injected into mice at 20 mg/kg.

Cell culture, LRRK2 constructs transfection

Human HEK293T (human embryonic kidney) and SH-SY5Y (neuroblastoma) cells were from ATCC (Manassas, VA, USA).
and grown in the media as described previously (18). The Flag tagged wild-type, G2019S and G2019S-K1347A constructs were described previously (18). Transient transfections were conducted using Lipofectamine™ and PLUSTM Reagents (Invitrogen) according to the manufacturer’s protocol.

**Immunoprecipitation (IP) and western blot analysis**

Cell lysates or brain homogenates were subjected to IP using anti-FLAG-agarose (Sigma), anti-myc, and anti-LRRK2 antibodies. For western blot analysis, the resulting immunoprecipitates and cell lysates were loaded into 4–12% NuPAGE Bis–Tris gels and transferred onto polyvinylidene difluoride (PVDF) membranes (Invitrogen). The membranes were probed with different antibodies as described previously (18), and then followed by incubation with enhanced chemiluminescence (ECL) reagents to detect proteins.

**LRRK2 autophosphorylation (kinase) and GTP-binding assays**

LRRK2 kinase assay was adapted from previous studies using autophosphorylation of cell lysates or brain homogenates (18,40,41). Briefly, HEK 293T were transiently transfected with various LRRK2 variants or kinase domain fragments for 36 h, followed by no serum starvation for 12 h, and then treated with compound 70 and 68 for 1 h. The cells were harvested using lysis buffer (Cell Signaling). Mouse brains were homogenated using radioimmunoprecipitation assay buffer (Cell Signaling). The resulting cell lysates or brain homogenates were immunoprecipitated using anti-Flag antibodies. The immunoprecipitates were subjected to western blot using anti-phosphorylated LRRK2 S935 and S2032 residues as described previously (40,41). In some cases, immunoprecipitates were incubated with kinase reaction buffer for 90 min at 30°C containing 50 mM MgCl2, 500 μM ATP, and 10 μCi of [γ-32P]ATP (3000 Ci/mmol) followed by 4–12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) separation and blotted onto PVDF membranes. Quantification was performed with a phosphoimager (Bio-Rad Molecular Imager FX). GTP-binding assays were performed as described previously (18). Cell lysates or brain homogenates (100 μg protein/ per reaction) were incubated with vehicle or compounds at 1–50 μM concentration for 1 h followed by addition of GTP-agarose beads (Sigma) for an additional 2 h at 4°C. The resulting beads were washed three times with cell lysis buffer, and bound protein was eluted by adding SDS-PAGE sample buffer and heating for 10 min at 72°C. Precipitates were subjected to western blot analysis using anti-Flag antibodies.

**Mouse Primary cortical neuronal cultures, transfection and cell viability assays**

Mouse primary cortical neurons were derived from CD-1 outbred mice (The Jackson Laboratory) at embryonic Day 16 and cultured on 24-well plates coated with laminin- and poly-L-lysine-coated plates (BD Biosource, San Diego) as described previously (18). Neurons were grown in neurobasal medium containing B-27 supplement, Glutamax, and penicillin/streptomycin. LRRK2 constructs were transfected into mouse primary cortical neurons using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol as described (49). Cell viability assays were conducted as described (18). pcDNA3.1-GFP and FLAG-LRRK2 constructs at 1:15 ratio were cotransfected into SH-SY5Y cells (neurons) in 10% FBS OPTI-MEM 1 media for 24 h. The media were changed to DMEM containing N2 supplement for 24 h. Cell viability was measured by counting GFP-positive viable cells (neurons) from 20 randomly selected fields using fluorescence microscopy. Viable cells (neurons) with one smooth extension (neurite) twice the length of the cell body were counted.

**LPS pre-inflammatory model and compound treatment**

G2019S-LRRK2-BAC (Jackson, Lab. Cat: 012467) transgenic mice (29,30) were food deprived for 4 h before surgery. There were 4–6 mice in each experimental group. Mice were anesthetized with isoflurane. LPS (5 μg containing 15 000 endotoxin units, Sigma) was injected into the substantia nigra pars compacta unilaterally with a 1 μl volume at 0.2 μl/min flow rate as described previously (33). The stereotaxic coordinates were −3.4 anteroposterior (AP), −1.1 mediolateral, and −3.9 dorsoventricular with respect to bregma. After injection, the needle was kept in place for 5 min before withdrawal to avoid LPS leakage up the cannula track. 68 at 20 mg/kg was injected intraperitoneally 1 h prior to LPS injection. Then 68 was injected twice daily for three days. The mice were perfused with 4% paraformaldehyde (PFA) in PBS and the brains were dissected and frozen in isopentane and stored in a −80°C freezer for immunostaining. Animal usage was approved by the University of Maryland School of Pharmacy.

**Immunohistochemical analysis**

Mice were perfused with saline and 4% paraformaldehyde. Frozen mouse brains were sectioned through the substantia nigra at 30 μm and the sections were subjected to immunohistochemical analysis as described previously (50,51). Briefly, frozen sections were placed in 0.6% H2O2 in methanol for 10 min followed by 5% normal goat serum (Sigma) to block non-specific reactions. Sections were then sequentially incubated with primary antibodies and biotinylated anti-rabbit secondary antibodies (Vector Laboratories) and avidin–biotin–peroxidase complex (ABC Elite kit, Vector Laboratories). Anti-LRRK2 (MJFF), anti-phosphorylated LRRK2 S935 (MJFF) and anti-isoleucin antibodies were used as primary antibodies. Isolectin-B4:FITC or Isolectin-B4:Biotin were from Sigma. Some tissue sections were visualized by peroxidase reaction using diaminobenzidine (DAB, Sigma). Some tissue sections were probed with Alexa Fluor 488 goat anti-mouse (rabbit) IgG and Alexa Fluor 568 goat anti-mouse (rabbit) IgG (Invitrogen) as secondary antibodies. The specificity of the immunostaining was checked by incubating adjacent sections with each preabsorbed primary antibody. The images were captured using a Zeiss 250 microscope connected to a Zeiss AxioCam camera. The digital images were captured processed in Adobe Photoshop (v.VII). The density of red and green fluorescence in the six consecutive sections across the LPS injection site of each mouse was quantified using NIH image-J software. Six microscope field images (×20) from one brain section were...
subjected to fluorescence density quantification. Negative controls, omitting primary antibody, were performed and no significant staining was seen.

**Data analysis**

Quantitative data were expressed as arithmetic means ± SEM based on at least three separate experiments. Statistically significant differences among groups were identified by ANOVA using Sigmastart 3.1 statistical software (Aspire Software International, VA). A P-value < 0.05 was considered significant.

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**Conflict of Interest statement**

All authors state that there are no conflicts of interest, and no conflicts with sources of funding. The pharmacological uses of 70 and 68 are included in a provisional patent by the University of Maryland.

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