

## **Inhibitors of LRRK2 kinase attenuate neurodegeneration and Parkinson-like phenotypes in *C. elegans* and *Drosophila* Parkinson's disease models**

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**ABSTRACT**

Mutations in *LRRK2* have been identified as a genetic cause of familial Parkinson's disease (PD), and mutations in *LRRK2* have also been found in the more common sporadic form of PD, thus positioning *LRRK2* as important in the pathogenesis of PD. Biochemical studies of the disease causing mutants of *LRRK2* implicates an enhancement of kinase activity as the basis of neuronal toxicity and thus possibly the pathogenesis of PD due to *LRRK2* mutations. Previously, a chemical library screen identified inhibitors of *LRRK2* kinase activity. Here, two of these inhibitors, GW5074 and sorafenib are shown to protect against G2019S *LRRK2*-induced neurodegeneration *in vivo* in *C. elegans* and in *Drosophila*. These findings indicate that increased kinase activity of *LRRK2* is neurotoxic and that inhibition of *LRRK2* activity can have a disease modifying effect. This suggests that inhibition of *LRRK2* holds promise as a treatment for PD.

## INTRODUCTION

Parkinson's disease (PD) is a complex neurodegenerative disorder that is both sporadic and familial. It is currently thought that PD results from a combination of genetic and environmental susceptibility factors. Gene mutations in the *leucine-rich repeat kinase 2 (LRRK2)* have recently been shown to result in autosomal dominant PD. A high prevalence of these mutations in unrelated PD patients strongly suggests that mutant LRRK2 may play a key role in sporadic PD as well. The clinical and pathological phenotypes of LRRK2 PD patients are similar to classic late-onset PD, further emphasizing the potential importance of LRRK2 (1, 2).

Biochemical evidence suggests that disease-causing mutations are linked to aberrant GTPase and kinase activities, and that these modifications may be at the basis of neuronal toxicity and pathogenesis of PD. Studies in cultured rodent primary cortical neurons have revealed that expression of disease causing full-length human LRRK2 mutants (G2019S, R1441C and Y1699C) induces marked neuronal toxicity relative to the wild type (WT) protein (3-5). LRRK2-induced toxicity is dependent on intact GTP binding and kinase activity. Biochemical characterization of LRRK2 and its disease-causing mutants, including R1441C, R1441G, Y1699C, G2019S and I2020T, reveal that the vast majority of disease-associated mutations enhance kinase activity *in vitro* (6, 7). Most importantly, this enhancement is particularly marked for the G2019S variant (3-5).

*Drosophila melanogaster* and *Caenorhabditis elegans* are excellent organisms to model neuronal degenerative diseases and have been used to study the mechanism of degeneration in models of PD and other neurodegenerative diseases (3-5). The LRRK2 gene is highly conserved across species. *C. elegans* and *Drosophila* each have a single

ortholog of the human LRRK2 (8). Transgenic expression of LRRK2 and the disease causing mutations of LRRK2 in *C. elegans* or in *Drosophila* results in loss of dopaminergic neurons and behavioral deficits (9-13). These transgenic models recapitulated several key features of human parkinsonism, indicating that over-expression of LRRK2 in *C. elegans* or *Drosophila* can provide a valuable model for preclinical testing of compounds for PD.

Currently there is no therapeutic treatment to slow or ameliorate dopaminergic neuron degeneration in PD. Recently, a chemical library screen revealed a panel of inhibitors of LRRK2 kinase activity (14). GW5074, an indoline compound, and sorafenib, a Raf kinase inhibitor, were both shown to strongly inhibit autophosphorylation of LRRK2 and G2019S LRRK2 (14). GW5074 demonstrates slightly more inhibitory effects on LRRK2 kinase activity than does sorafenib (14). In this study, we evaluate the ability of inhibitors of LRRK2 kinase, GW5074 and sorafenib, to rescue dopaminergic cell loss in a *C. elegans* model of LRRK2 dopaminergic cell loss and in a *Drosophila* model of dopaminergic cell loss and behavioral deficits. Both GW5074 and sorafenib increased survival and reduced dopaminergic neuron degeneration in G2019S-LRRK2 transgenic *C. elegans* and *Drosophila*. GW5074 was determined to be slightly more potent than sorafenib in both *in vivo* model systems. Moreover, GW5074 and sorafenib significantly reduced mutant LRRK2 protein kinase activity and attenuated locomotor impairment in transgenic LRRK2-G2019S *Drosophila*. These results further demonstrate that LRRK2 protein kinase activity significantly contributes to development of PD-like phenotypes and suggest that GW5074 and sorafenib represent potential inhibitors with promise for therapeutic intervention in PD.

## RESULTS

### **GW5074 and sorafenib increased dopamine neuron survival in a *C. elegans* LRRK2 G2019S-induced neurodegeneration model**

Previously, the screening of 84 commercially available kinase inhibitors revealed 8 compounds that inhibited LRRK2 kinase activity. Among the positive hits, GW5074 and sorafenib strongly inhibited LRRK2 WT and G2019S kinase activity (14). ZM336372, a Raf kinase inhibitor, had no effect on LRRK2 kinase activity and served as a negative control. To evaluate the role of GW5074, sorafenib and ZM336372 in protection against dopamine (DA) neuron loss in *C. elegans*, we generated a transgenic *C. elegans* model of LRRK2 G2019S-induced neurodegeneration. As previously described (15, 16), the loss of GFP serves as an indicator of DA neurodegeneration. Since the nematode is transparent and its development is tightly regulated, it allows for rapid quantitative assessment of morphological changes in the six anterior DA neurons.

Using this model, expression of LRRK2 G2019S and GFP under the control of DA transporter promoter ( $P_{dat-1}$ ) resulted in an age-dependent degeneration of DA neurons whereby there was 83% and 90% loss of neurons at 7 and 10 days, respectively (Fig. S1A). We were curious to determine if DMSO (at 1%), the solvent required for examination of GW5074, sorafenib and ZM336372, would reduce the amount of DA neurodegeneration observed in *C. elegans*. Treatment with 0.5% and 2% DMSO has been documented to extend lifespan in a *sir-2.1*- and *daf-16*-dependent manner (17). In this regard, DA neuronal health could be artificially enhanced following DMSO exposure by itself. We discovered partial neuroprotection following exposure to 1% DMSO; the amount of DA neurodegeneration observed was 52% and 65% at 7 and 10 days,

respectively (n=90 worms). However, despite DMSO exposure, the amount of DA neurodegeneration observed remained significant and increased as the animals aged.

Within the *C. elegans* population expressing LRRK2 G2019S, at least one DA neuron was degenerated in 52% of 7-day-old animals following 1% DMSO treatment; thus, the remaining 48% of the population displayed all 6 intact anterior DA neurons (Fig. 1A, B). Treatments with 25  $\mu$ M and 10  $\mu$ M GW5074 and 25  $\mu$ M sorafenib significantly enhanced DA neuron survival whereby 67%, 66%, and 62% of the same staged animals were rescued (n=90 for each treatment) (Fig. 1B). ZM336372 failed to suppress DA neurodegeneration (Fig. 1B). We also quantified the number of healthy DA neurons in each worm population analyzed in 7-day old animals. While 88% of total DA neurons (n=540) remained normal in the 7-day old LRRK2 G2019S worms, treatments with 25  $\mu$ M, 10  $\mu$ M and 1  $\mu$ M GW5074, as well as 25  $\mu$ M sorafenib, significantly rescued 94%, 93%, 92%, and 92% of the neurons, respectively (n=540 neurons for each treatment) (Fig. 1C).

When *C. elegans* expressing LRRK2 G2019S specifically in DA neurons were analyzed at 10 days, only 35% of the population displayed normal DA neurons following 1% DMSO treatment. We found that treatment with 25  $\mu$ M and 10  $\mu$ M GW5074, as well as 25  $\mu$ M sorafenib significantly rescued the neurons to 49%, 48%, and 49%, respectively (Fig. S1B). Collectively, these findings indicate that the *C. elegans* model recapitulates the suppression of LRRK2 G2019S-induced neurodegeneration by GW5074 and sorafenib in the cultured rodent primary cortical neurons (14), and suggest GW5074 as the most potent compound for ameliorating the toxicity.

We next sought to examine the timing of larval stages and lifespan since these kinase inhibitors (or DMSO) may modulate development and general cellular stress response. Specifically, since the neurodegeneration is age-dependent, it is conceivable that the compounds may delay aging, inaccurately demonstrating neuroprotection. The worms treated with the kinase inhibitors or DMSO alone (n=150 for each treatment) demonstrated comparable larval development to control worms treated with water (Fig. S2A). Furthermore, the compound-treated worms exhibited indistinguishable lifespan (Fig. S2B). These results illustrate that, while the kinase inhibitors may still modulate cellular processes associated with aging, these effects were maintained to a minimum in our studies. Semi-quantitative RT-PCR also demonstrated that GW5074, sorafenib, and ZM336372 did not modify the expression level of LRRK2 G2019S mRNA (Fig. 1D). Our data suggest that enhanced DA neuron survival after the kinase inhibitor treatment is not a consequence of decreased growth rate, delayed aging, nor reduced expression of the transgene.

### **GW5074 and sorafenib do not protect DA neurons from $\alpha$ -synuclein- and 6-OHDA-induced degeneration**

While mutations in LRRK2 are the most prevalent genetic form of PD, we sought to determine if the neuroprotective kinase inhibitors could ameliorate cellular toxicity caused by another genetic form of PD. In this regard, multiplication of the wildtype  $\alpha$ -synuclein ( $\alpha$ -syn) allele can result in PD (18). The  $\alpha$ -syn protein has a high propensity for misfolding and forming protein aggregates. We have established that overexpression of wildtype human  $\alpha$ -syn under control of a DA-specific promoter [ $P_{dat-1}$ ] results in age-

and dose-dependent neurodegeneration (Fig. 1E). This transgenic *C. elegans*  $\alpha$ -syn neurodegeneration model has proved predictive; it has facilitated the identification of multiple neuroprotective genetic and chemical factors that are conserved across species (19-21). Using this model, expression of  $\alpha$ -syn and GFP in the DA neurons resulted in the loss of neurons in 64% of 7 day-old worms (n=90) after 1% DMSO treatment, indicating that 36% of the population displayed all 6 intact anterior DA neurons (Fig. 1E). Notably, following exposure to the kinase inhibitors, the DA neurons were not rescued from  $\alpha$ -syn-induced neurodegeneration. For example, treatment with the most potent inhibitor, GW5074, resulted in 34% normal neurons for concentrations of both 25  $\mu$ M and 10  $\mu$ M (Fig. 1E).

Since multiple genetic and environmental factors have been postulated to be associated with the underlying molecular and cellular mechanisms involved in DA neuron loss, we also tested the kinase inhibitors in worms exposed to the neurotoxin, 6-OHDA. This toxin, which selectively kills DA neurons through the formation of reactive oxygen species, causes a characteristic dose-dependent pattern of cell death in *C. elegans* DA neurons (15, 22). This degeneration can be monitored using a GFP marker expressed in DA neurons. Following a one-hour exposure to 6-OHDA, 63% of control worms displayed all 6 intact anterior DA neurons (Fig. 1F). Exposure to the kinase inhibitors did not rescue toxicity induced by 6-OHDA, as there was an insignificant change in the percentage of worms with intact anterior DA neurons following treatment with GW5074 (58% for 25  $\mu$ M and 65% for 10  $\mu$ M).



### **Enhanced kinase activity is required in LRRK2 G2019S-induced neurodegeneration**

Introduction of D1994A mutation abolishes enhanced kinase activity of LRRK2 G2019S (23, 24). Since multiple pathways are evolutionary conserved from worms to mammals, we wished to further verify the requirement of kinase activity in LRRK2 G2019S-induced neurodegeneration in the *C. elegans* model by generating transgenic *C. elegans* expressing LRRK2 G2019S/D1994A in DA neurons (Fig. 2A, B). In this experiment, while GFP was expressed in all DA neurons, the transgenic nematode lines expressed the LRRK2 G2019S or LRRK2 G2019S/D1994A transgenes as extrachromosomal arrays, resulting in mosaic expression. It should be noted that the LRRK2 G2019S strain used in this experiment is unlike the aforementioned LRRK2 G2019S strain used in the experiments described in Fig 1, S1, and S2, where the construct was chromosomally integrated and all animals had consistent and equivalent expression. Hence, the experimental strategy for extrachromosomal arrays involved the comparison of 3 independent lines of LRRK2 G2019S/D1994A + GFP to three equally independent lines of LRRK2 G2019S + GFP and GFP alone to distinguish the loss of GFP due to neurodegeneration or incomplete transmission of transgenes. In 7 and 10 day-old animals, expression of GFP alone resulted in 100% of the worm population (n=90 per line) with all 6 anterior DA neurons (Fig. 2A, B). As expected from our previous data with the integrated LRRK2 G2019S transgene, all three worm strains expressing extrachromosomal arrays of LRRK2 G2019S demonstrated a progressive degeneration of DA neurons. The population exhibiting a full complement of normal neurons went from 65% to 50%, at 7 and 10 days, respectively (Fig. 2A, B). This age-dependent degeneration induced by LRRK2 G2019S was significantly rescued in

transgenic worms expressing LRRK2 G2019S/D1994A. Here, the worm population with a full complement of anterior DA neurons at days 7 and 10 was 85% and 70%, respectively (Fig. 2A, B). These results demonstrate that an age-associated loss of DA neurons in *C. elegans* is dependent on enhanced LRRK2 kinase activity.

### **GW5074 and sorafenib increased survival and reduced locomotor dysfunction in G2019S-LRRK2 transgenic flies.**

The activity of GW5074, sorafenib and ZM336372 were next evaluated in the G2019S-LRRK2 *Drosophila* model in which the UAS-G2019S-LRRK2 transgene is driven by the dihydroxyphenylalanine decarboxylase (ddc)-GAL4 driver. The ddc-GAL4 or UAS-LRRK2 flies that had no transgene expression served as controls. The flies including ddc-GAL4;UAS-G2019S-LRRK2, ddc-GAL4 and UAS-LRRK2, were treated with 1 nM, 1  $\mu$ M, 10  $\mu$ M or 100  $\mu$ M of ZM336372, GW5074 or sorafenib immediately after eclosion by inclusion of these compounds in the fly food. Fly food with compounds were refreshed every three days throughout the lifetime of the flies. Survival and climbing assays (measuring the locomotor activity) were measured weekly. ZM336372 did not alter survival or locomotor activity in ddc-GAL4;UAS-G2019S-LRRK2 flies (Fig. 3A and 3B). However, GW5074 and sorafenib at 1  $\mu$ M and 10  $\mu$ M increases survival and reduces locomotor impairment in ddc-GAL4;UAS-G2019S-LRRK2 flies (Fig. 3C-H). GW5074 appears to be slightly more potent than sorafenib (Fig. 3G-H). None of the concentrations of ZM336372, GW5074 or sorafenib alters the life span and locomotor activity of non-transgenic control flies, ddc-GAL4 or UAS-LRRK2, which live a normal 12-13 week lifespan (Fig. S3). A high dose of GW5074 (100  $\mu$ M) or sorafenib (100  $\mu$ M)

did not affect mutant G2019S-LRRK2-induced Parkinsonian-like phenotypes for unclear reasons.

### **GW5074 and sorafenib reduced G2019S-LRRK2-induced dopaminergic neuron degeneration.**

There are six dopaminergic neuronal clusters present in each *Drosophila* adult brain hemisphere that express tyrosine hydroxylase (TH). Expression of mutant *G2019S-LRRK2* results in degeneration of DA neurons (9, 10). To test whether LRRK2 inhibitors reduce dopaminergic neuron degeneration, fluorescent immunostaining was performed on whole-mount dissected adult fly brains. Brains from *LRRK2-G2019S* or control (*ddc-GAL4* or *UAS-LRRK2*) flies treated with vehicle, GW5074, sorafenib or ZM336372 each at 10  $\mu$ M were dissected and immunostained with anti-TH antibodies at 42 days after eclosion. The numbers of DA neurons were counted under confocal microscopy. We found that ZM336372 did not alter the DA neuron degeneration. However, both GW5074 and sorafenib attenuated mutant G2019S-LRRK2-induced dopaminergic neuron degeneration (Fig. 4A-C). GW5074 appears to be slightly more effective than sorafenib.

### **GW5074 and sorafenib reduced mutant G2019S-LRRK2 protein kinase activity in flies.**

To test whether GW5074 and sorafenib inhibit LRRK2 protein kinase *in vivo*, we performed LRRK2 autophosphorylation (kinase) assays using fly head homogenates from *LRRK2-G2019S* or control (*ddc-GAL4* or *UAS-LRRK2*) flies treated with vehicle, 10  $\mu$ M

GW5074, sorafenib or ZM336372. We found that both GW5074 and sorafenib reduced mutant G019S-LRRK2 kinase activity by 89% and 84% respectively (Fig. 5A, B).

## DISCUSSION

LRRK2 is a large, multi-domain protein including a kinase and GTPase domain that has potential involvement in several cellular processes including protein translational control, cytoskeletal dynamics, MAPK pathways, and apoptosis (25-28). Mutations in *LRRK2* are thought to be the most common genetic cause of PD producing phenotypes similar to those typical for late-onset of the disease. Disease causing mutations in *LRRK2* have variable effects on kinase activity although increased kinase activity is a consistent observation for the most common disease-associated mutation, G2019S (2, 6, 7). Expression of LRRK2 G2019S in cultured rodent primary cortical neurons (24, 29, 30), dopamine neurons of *C. elegans* (11, 13) and neurons in *Drosophila* (9, 10, 12) causes neuronal degeneration. In the current study two inhibitors of LRRK2, GW5074 and sorafenib ameliorate LRRK2 G2019S-induced neurodegeneration of DA neurons in *C. elegans* and *Drosophila*. Both GW5074 and sorafenib, reversed locomotor defects in the *Drosophila* model through suppression of LRRK2 G2019S kinase activity. GW5074 is slightly more potent than sorafenib in both *in vivo* model systems consistent with their potency in inhibiting LRRK2. Taken together, these data suggest that LRRK2-induced neurodegeneration is associated with the kinase activity of LRRK2, indicating that LRRK2 kinase activity may be tractable target for PD intervention.

The two LRRK2 kinase inhibitors described here are promising starting points for designing drugs to halt the progressive neurodegeneration associated with PD. GW5074

is a cell-permeable synthetic drug known for its Raf kinase inhibiting activity (31, 32). Sorafenib is currently used in the clinic for treatment of advanced renal cell carcinoma and hepatocellular carcinoma (33). Sorafenib inhibits several tyrosine protein kinases including Raf kinase, platelet derived growth factor, vascular endothelial growth factor receptor 2 and 3 kinases, and cKit (34). Given the homologous kinase domain sequences shared by LRRK2 and Raf1, the neuroprotective capacities of GW5074 and sorafenib may be linked via direct inhibition of LRRK2. ZM336372 is also a potent Raf kinase inhibitor with broad selectivity, but ZM336372 does not inhibit LRRK2 kinase activity and is not protective. Thus, GW5074 and Sorafenib have structures that interfere with LRRK2 kinase activity, but ZM336372 does not. These data suggest that the protective effects of GW5074 and Sorafenib are due to LRRK2 inhibition rather than Raf inhibition although it is conceivable that these compounds rescue DA neurons through unexplored pathways parallel or downstream of LRRK2.

There has been great interest in defining classes of drugs that could inhibit LRRK2 kinase activity. Others have found similar or different compounds using a variety of approaches to identify LRRK2 inhibitors. LDN-22684 was identified from a chemical library screen using a TR-FRET assay with LRRK2 and a peptide derived from polo-like kinase, a serine/threonine kinase as the phosphoryl acceptor. LDN-22684 is non-competitive with ATP in inhibiting both WT and G2019S LRRK2 suggesting that LDN-22684 has an alternative binding site outside the ATP pocket (35). However, there is no information yet regarding the specificity of LDN-22684 in inhibiting other kinases including LRRK1, nor what its biologic activity might be. Other investigators measuring autophosphorylation, phosphorylation of a general phosphoryl acceptor myelin basic

protein or the putative LRRK2 substrate moesin, have found that general kinase inhibitors with indolocarbazole moieties, GO6976, K-252a, staurosporine, effectively inhibit LRRK2 kinase activity (36-38). The Rho kinase inhibitors H-1152, and Y-27632 are effective in inhibiting LRRK2 while another Rho kinase inhibitor GSK429286A did not. Sunitinib, a receptor tyrosine kinase inhibitor, also inhibits LRRK2 (37, 39). The action of these inhibitors on limiting LRRK2 neurotoxicity is not yet known. The findings that some inhibitors in a class, such as the Rho kinase inhibitors H-1152, and Y-27632 can inhibit LRRK2 but other Rho kinase inhibitors such as GSK429286A cannot raises the possibility that drugs selective for LRRK2 can be developed.

Our findings confirm the neuropathological link between enhanced LRRK2 kinase activity and neurodegeneration, and we further demonstrate the capacity of select compounds to rescue DA neurons from degeneration through the inhibition of LRRK2 kinase activity. In this regard, the statistically robust and reproducible neuroprotection observed across multiple model systems not only provides a proof-of-principle that LRRK2 kinase is a therapeutic target, but also suggest that compounds similar to GW5074 and sorafenib may have therapeutic potential for LRRK2-linked PD.

## **MATERIALS AND METHODS**

### **Generation of Transgenic *C. elegans* Strains**

Nematodes were maintained following standard procedures (40). LRRK2 G2019S cDNA (gift from Matthew Farrer, Mayo Clinic) and LRRK2 G2019S/D1994A were cloned into pDEST-*dat-1* via Gateway technology (Invitrogen, Carlsbad, CA) to generate expression plasmids  $P_{dat-1}::LRRK2$  G2019S and  $P_{dat-1}::LRRK2$  G2019S/D1994A.

Dopamine transporter promoter ( $P_{dat-1}$ ) allows selective gene expression in the DA neurons. A concentration of 50  $\mu\text{g/ml}$   $P_{dat-1}::\text{LRRK2 G2019S}$  or  $P_{dat-1}::\text{LRRK2 G2019S/D1994A}$  was co-injected into the gonads of strain BY250 [ $vtIs7$ ;  $P_{dat-1}::\text{GFP}$ ] (a gift from Randy Blakely, Vanderbilt University) to generate UA215 [ $baEx128$ ;  $P_{dat-1}::\text{LRRK2 G2019S}$ ; BY250], and UA216 [ $baEx129$ ;  $P_{dat-1}::\text{LRRK2 G2019S/D1994A}$ ; BY250]. Strain UA118 [ $baIn20$ ;  $P_{dat-1}::\text{LRRK2 G2019S}$ ;  $P_{dat-1}::\text{GFP}$ ] is the integrated version of UA136 which was generated by coinjecting 50  $\mu\text{g/ml}$   $P_{dat-1}::\text{LRRK2 G2019S}$  into the gonads of N2 Bristol strain along with 50  $\mu\text{g/ml}$   $P_{dat-1}::\text{GFP}$ . The transgene was integrated via gamma irradiation resulting in lines with 100 percent GFP expression. Strain UA44 [ $baIn11$ ;  $P_{dat-1}::\alpha\text{-syn}$ ,  $P_{dat-1}::\text{GFP}$ ], which co-expresses human  $\alpha\text{-syn}$  and GFP in the DA neurons, and BY250 ( $vtIs7$ ;  $P_{dat-1}::\text{GFP}$ ) to visualize GFP in DA neurons, were used in these studies.

### ***C. elegans* DA Neurodegeneration Assays**

Age-synchronized worms were obtained by treating gravid adults with 2% sodium hypochlorite and 0.5 M NaOH to isolate embryos (41). These embryos were treated with or without indicated concentrations of LRRK2 kinase inhibitors, which were dissolved in 1% DMSO, for 24 h at 20°C with gentle shaking. The L1 (larval stage L1) worms were then washed and transferred onto NGM plates seeded with OP50 bacteria, and incubated at 20°C. Subsequent larval stages and lifespan were analyzed as described previously (3 trials of 50 animals/trial) (42, 43). The six anterior DA neurons of 30 animals/trial were examined for neurodegeneration when the animals were 7 and 10 days old (15, 16). A total of 90 animals for each treatment were analyzed (3 trials of 30 animals/trial). If a

worm displayed at least one degenerative change (dendrite or axon loss, cell body loss), the animal was scored as exhibiting degenerating neurons (15, 16). Conditions used for 6-OHDA treatment and scoring were performed as described previously (15, 16). Likewise, the methods for analyzing worms expressing  $\alpha$ -synuclein in DA neurons were performed as previously described (15, 16).

For statistical analysis of the *C. elegans* experiments, quantitative data were displayed as arithmetic means  $\pm$  SD in triplicate, and the Student's *t*-test ( $p < 0.05$ ) was used to examine significance.

### **Semi-quantitative RT-PCR**

Semi-quantitative RT-PCR was performed as described previously (16). Briefly, worm embryos were treated with 25  $\mu$ M kinase inhibitors for 24 h at 20°C with gentle shaking, and subsequent L1 worms were washed and transferred onto NGM plates seeded with OP50 bacteria. These worms were incubated at 20°C for additional 48 h, and total RNAs were isolated from fifty 3 day-old worms. Primers (*LRRK2*: 5' CAA-CTG-TTT-TCT-TAT-GCA-GCT-TTC 3' and 5' CAT-GAC-ATT-TTT-AAG-GCT-TCC-TAG 3'; *dat-1*: 5' GTC-TGC-GAC-AAT-TTG-TTC-ATG 3' and 5' CTC-AGA-TAT-CGT-GTT-CCC-CC 3') were used to detect mRNA levels of *LRRK2 G2019S* and *dat-1*.

### **LRRK2 Transgenic Fly Stock and Survival Curve**

The *ddc*-GAL4 was used to express UAS-LRRK2-G2019S in DA neurons as previously described (9). *Drosophila* were grown on standard cornmeal medium supplemented with different concentrations of GW5074, sorafenib or ZM336372 at 25°C.



Fresh media with various compounds were changed every 3–4 days. Cohorts of 60 flies from each experimental group were monitored for survival weekly. Mortality was analyzed by Kaplan–Meier survival curves.

### **Climbing Assay**

A climbing assay (negative geotaxis assay) was used to assess locomotor ability as described previously (9). Cohorts of 60 flies from each experimental group were subjected to the assay weekly from 1 week to the time of death. The tested flies were placed in a vertical plastic column (length, 25 cm; diameter, 1.5 cm). Flies were tapped to the bottom of the column. We counted and calculated the percentage of flies that could climb to or above the median line of the cylinder in 10 seconds. Each week, the assay was repeated three times.

### **Immunoprecipitation and *in vitro* Autophosphorylation (Kinase) Assays**

Immunoprecipitation (IP) experiments from fly head homogenates were performed with anti-FLAG-agarose (Sigma). Precipitates were subjected to LRRK2 *in vitro* autophosphorylation assay as described previously (9). Briefly, kinase reactions were carried out for 90 min at 30°C in the kinase assay buffer containing 50 mM MgCl<sub>2</sub>, 500 μM ATP, and 10 μCi of [ $\gamma$ -<sup>32</sup>P]ATP (3,000 Ci/mmol). Reactions were stopped by the addition of Laemmli sample buffer and boiling for 5 min. Samples were separated on 4–12% SDS/PAGE and blotted onto PVDF membranes. Quantification was performed with a phosphoimager (Bio-Rad Molecular Imager FX).

## **Immunostaining of Whole-mount Dissected Brains and Cell Counting**

Fluorescent immunostaining was performed on whole-mount dissected adult brains (9) at 6 weeks of age. Cohorts of six to eight flies per experimental group were used for immunostaining. Rabbit polyclonal anti-TH (Chemicon), mouse monoclonal anti-TH (Immunostar) antibodies were used as the primary antibodies. Alexa Fluor 488 goat anti-mouse IgG and Alexa Fluor 568 goat anti-mouse IgG (Invitrogen) were used as secondary antibodies. The numbers of DA were scored under fluorescent (Zeiss LSM 250) and/or confocal microscopy (Zeiss LSM 510).

## **Drosophila Data Analysis**

Quantitative data were expressed as arithmetic means  $\pm$  SEM based on at least three separate experiments. Statistically significant differences among groups were analyzed by ANOVA. A  $p$  value  $<0.05$  was considered significant.

## **FUNDING**

This work was supported by grants from the NIH/NINDS P50NS38377 (TMD, VLD), Michael J. Fox Foundation (WWS, VLD, SH, GAC, KAC), and the Howard Hughes Medical Institute (SH, GAC, KAC).

## **ACKNOWLEDGEMENTS**

T.M.D. is the Leonard and Madlyn Abramson Professor in Neurodegenerative Diseases.

*Conflict of Interest Statement.* None declared.

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**FIGURE LEGENDS**

**Figure 1. Exposure to GW5074 and sorafenib are protective in a *C. elegans* LRRK2 G2019S-induced neurodegeneration model.** (A) *C. elegans* strain expressing LRRK2 G2019S in DA neurons displays neurodegeneration. In the worm exposed to the solvent DMSO, note that only 4 of 6 anterior DA neurons remain (cell bodies indicated with arrows). The arrowheads show areas where DA neurons have degenerated. Scale bar = 50  $\mu$ m. Treatment with one of the LRRK2 kinase inhibitors protects worms from neurodegeneration whereby worms display all 6 anterior DA neurons (arrows depict cell bodies). (B) Worm population analysis of 7-day old animals reveals that GW5074 and sorafenib have protective effects on degeneration of DA neurons in this isogenic model of LRRK2 G2019S-induced neurodegeneration. Data represent the mean  $\pm$  S.D. (three independent experiments, n=30 per independent groups). \*p < 0.05 by Student's *t*-test where populations of normal worms with 6 DA neurons in 1% DMSO without compound are compared to treatment. (C) Worm neuron analysis further demonstrates neuroprotective effects of GW5074 and sorafenib in 7-day old adults. Data represent the mean  $\pm$  S.D. (three independent experiments, n=30 per independent groups). \*p < 0.05 by Student's *t*-test, where populations of normal DA neurons in 1% DMSO without compound are compared to populations of neurons treated with compound. (D) Semi-Quantitative RT-PCR images of LRRK2 G2019S after treatment of LRRK2 kinase inhibitors (GW5074 and sorafenib) and negative control compound (ZM336372). *dat-1* was used as a loading control. (E) Worm population analysis reveals that the kinase inhibitors do not have a protective effect against  $\alpha$ -syn-induced toxicity. These worms were analyzed as 7-day adults. Data represent the mean  $\pm$  S.D. (three independent

experiments, n=30 per independent groups). **(F)** Worm population analysis demonstrates that the kinase inhibitors do not have a protective effect against 6-OHDA-induced toxicity. Toxicity was examined 24 hours following exposure to 6-OHDA. Data represent the mean  $\pm$  S.D. (three independent experiment, n=30 per independent groups).

**Figure 2. Dopamine neuron degeneration in LRRK2 transgenic *C. elegans*.** **(A)** Three independent lines of transgenic worms were analyzed where the transgenes (LRRK2 G2019S, and LRRK2 G2019S/D1994A cDNA) remain as extrachromosomal arrays. The GFP transgenic line is integrated into the worm chromosomal DNA and was used as the genetic background for injecting the LRRK2 transgenes examined in this experiment. GFP expression in 7 and 10 day old worms was monitored for each type of transgenic worm. Worms expressing GFP alone exhibit all 6 anterior DA neurons. As anticipated, worms expressing LRRK2 G2019S as an extrachromosomal array displayed significant DA neurodegeneration that was rescued by LRRK2 G2019S/D1994A. Data represent the mean  $\pm$  S.D. (n=90 per independent transgenic line). \*p < 0.05 by Student's *t*-test. **(B)** Representative photographs of worms expressing GFP alone displaying all 6 anterior DA neurons, or LRRK2 transgenic *C. elegans*. The LRRK2 G2019S worm displays only 4 DA neurons (arrows); arrowheads indicate areas where DA neurons have degenerated. The LRRK2 G2019S/D1994A worm displayed in this image depicts DA neuron rescue where all 6 anterior neurons are present (arrows). Scale bar = 50  $\mu$ m.

**Fig. 3. Dose-response studies of GW 5074 and sorafenib in LRRK2 transgenic *Drosophila*.** The ddc-GAL4;G2019S flies, in which the mutant G2019S-LRRK2 protein is expressed in dopaminergic neurons by the ddc-GAL4 driver, were treated with



ZM336372, GW5074, or sorafenib at 0, 1 nM, 1  $\mu$ M, 10  $\mu$ M, or 100  $\mu$ M concentrations from 1 day post-eclosion through lifetime. Cohorts of 60 flies (20 flies, 3 vials) from each experimental group were assayed weekly for (A, C, E, G) survival and (B, D, F, H) climbing. A and B: ZM336372; C and D: GW5074; E and F: sorafenib. (G, H) To allow comparison between the 10  $\mu$ M drug treatments were plotted. Survival data were analyzed by Kaplan-Meier log Rank survival analysis. In C and E,  $p < 0.05$ , statistically significant differences between between vehicle and 1  $\mu$ M kinase inhibitor (GW4074 or sorafenib) treated transgenic flies; between vehicle and 10  $\mu$ M kinase inhibitor (GW4074 or sorafenib) treated transgenic flies. The data of climbing assays were analyzed by ANOVA. In D and F,  $p < 0.05$ , between vehicle and 1  $\mu$ M kinase inhibitor (GW4074 or sorafenib) treated transgenic flies;  $p < 0.05$  by ANOVA, between vehicle and 10  $\mu$ M kinase inhibitor (GW4074 or sorafenib) treated transgenic flies. In G,  $p < 0.05$ , statistically significant differences between vehicle and kinase inhibitor (GW5074 or sorafenib) treated transgenic flies; between GW5074 and sorafenib treated transgenic flies. The data of climbing assays were analyzed by ANOVA. In H, \*  $p < 0.05$ , statistically significant differences between vehicle and kinase inhibitor (GW4074 or sorafenib) treated transgenic flies; between ZM336372 and kinase inhibitor (GW4074 or sorafenib) treated transgenic flies. #  $p < 0.05$ , significant differences between GW5074 and sorafenib treated groups.

**Fig. 4. GW5074 and sorafenib reduced G2019S-LRRK2-induced dopaminergic neuron degeneration.** The ddc-GAL4;G2019S flies were treated with ZM336372, GW5074, or sorafenib at 0 and 10  $\mu$ M concentrations from 1 day post-eclosion to 6

weeks of age. Flies were sacrificed and the brains were dissected. The whole brains were subjected to anti-TH immunofluorescent staining. The dopaminergic neurons were counted in each cluster except the paired anterolateral medial (PAM) neurons. **(A)** Quantification of total dopaminergic neurons in all clusters except PAM. **(B)** Quantification of TH-positive neurons in protocerebral posterior medial (PPM) 1 and PPM2 clusters. **(C)** Representative images of anti-TH staining in PPM1 and PPM2 clusters from 6-week-old flies of the indicated experimental group. Arrows indicate an example of a TH-positive neuron in each condition. Statistically significant differences between the non-transgenic flies are indicated: \*  $p < 0.05$  by ANOVA vs. non-transgenic flies with vehicle; #  $p < 0.05$  by ANOVA vs. 10  $\mu\text{M}$  ZM336372 treatment group. \*\* $p < 0.05$  by ANOVA, between GW5072 and sorafenib treatment groups.

**Fig. 5. GW5074 and sorafenib reduced mutant G2019S-LRRK2 protein kinase activity.** The *ddc-GAL4;G2019S* flies were treated with ZM336372, GW5074, or sorafenib at 0 and 10  $\mu\text{M}$  concentrations from 1 day post-eclosion to 2 weeks of age. Flies were sacrificed and fly head homogenates were subjected to LRRK2 autophosphorylation (kinase) analysis. Anti-Flag-LRRK2 immunoprecipitated samples from fly head homogenates were incubated with [ $\gamma$ - $^{32}\text{P}$ ] ATP, subjected to SDS/PAGE, and blotted onto PVDF membranes. The samples were then visualized using a phosphoimaging system. **(A)** Shown are representative images from three independent experiments. **(B)** Quantitation of LRRK2 autophosphorylation. Statistically significant differences between the non-transgenic flies are indicated: \*  $p < 0.05$  by ANOVA vs. non-transgenic flies with vehicle; #  $p < 0.05$  by ANOVA vs. 10  $\mu\text{M}$  ZM336372 treatment group.

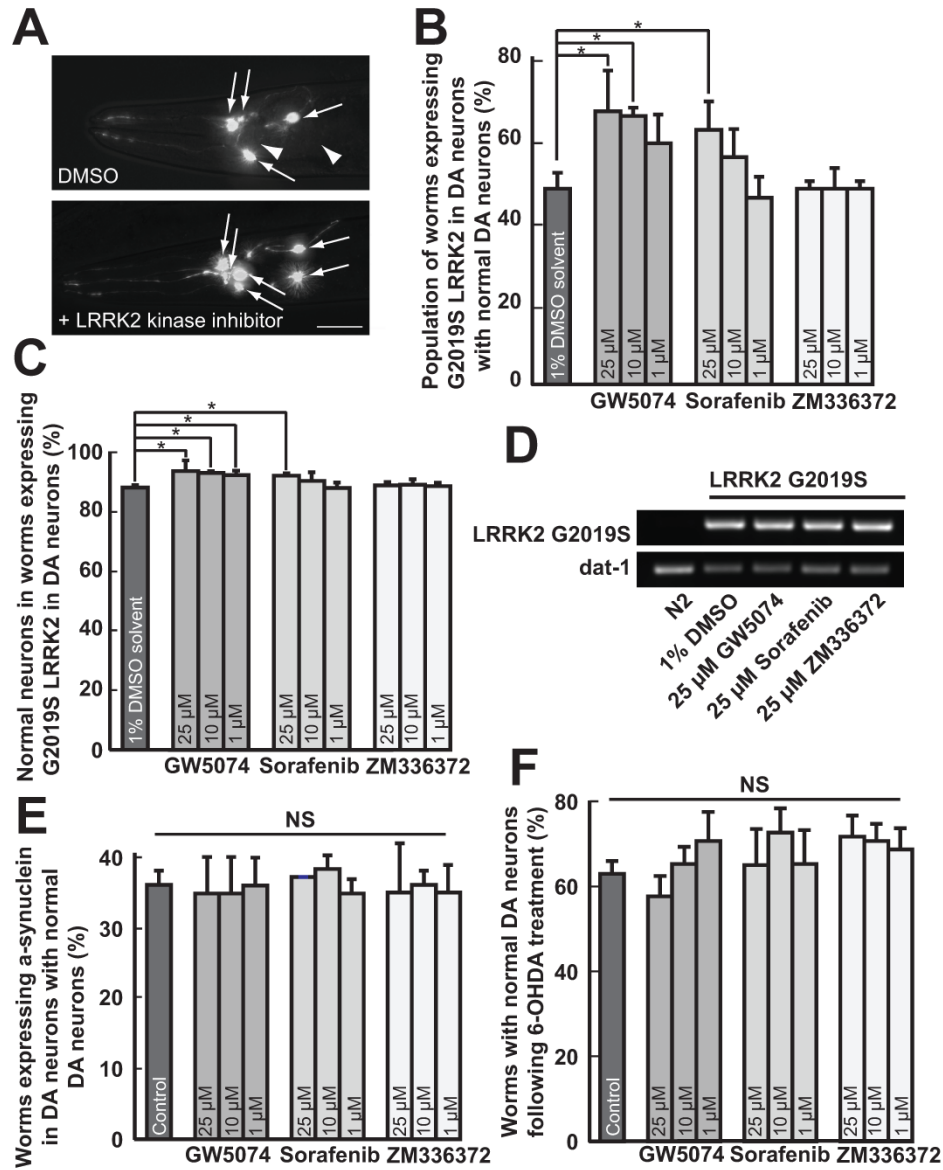
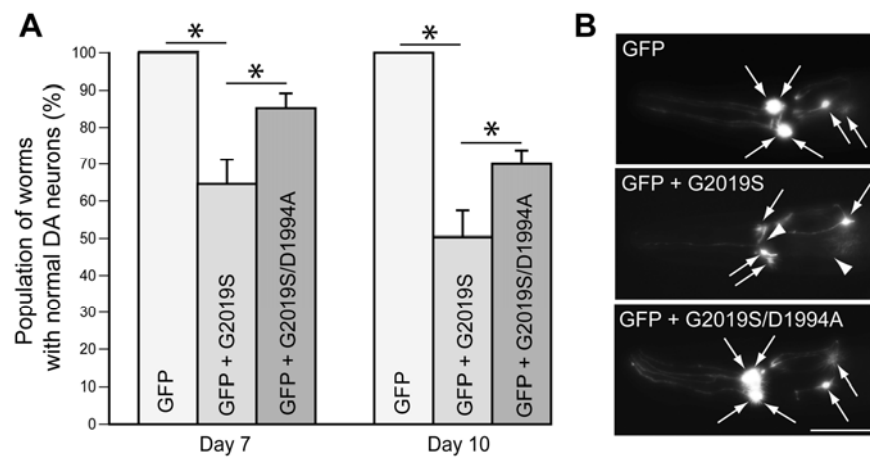


Figure 1



**Figure 2**

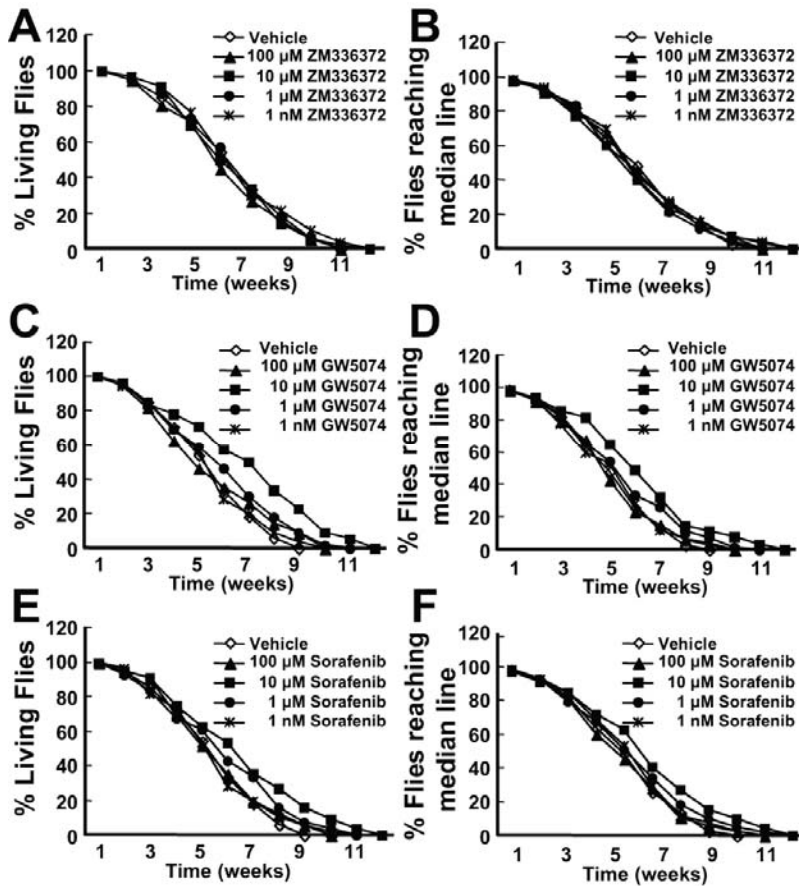


Figure 3

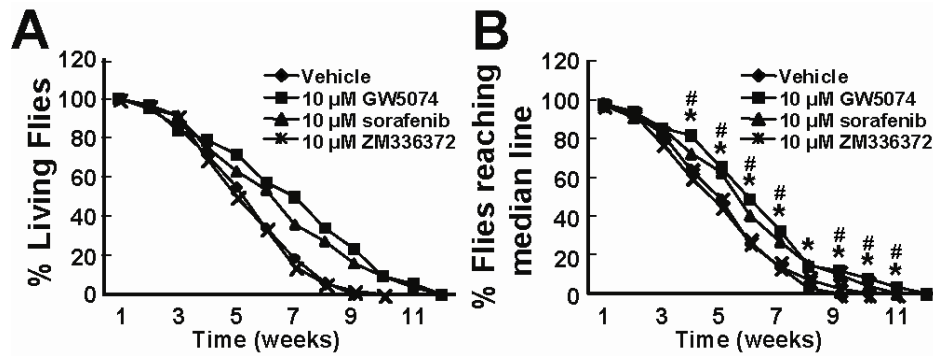
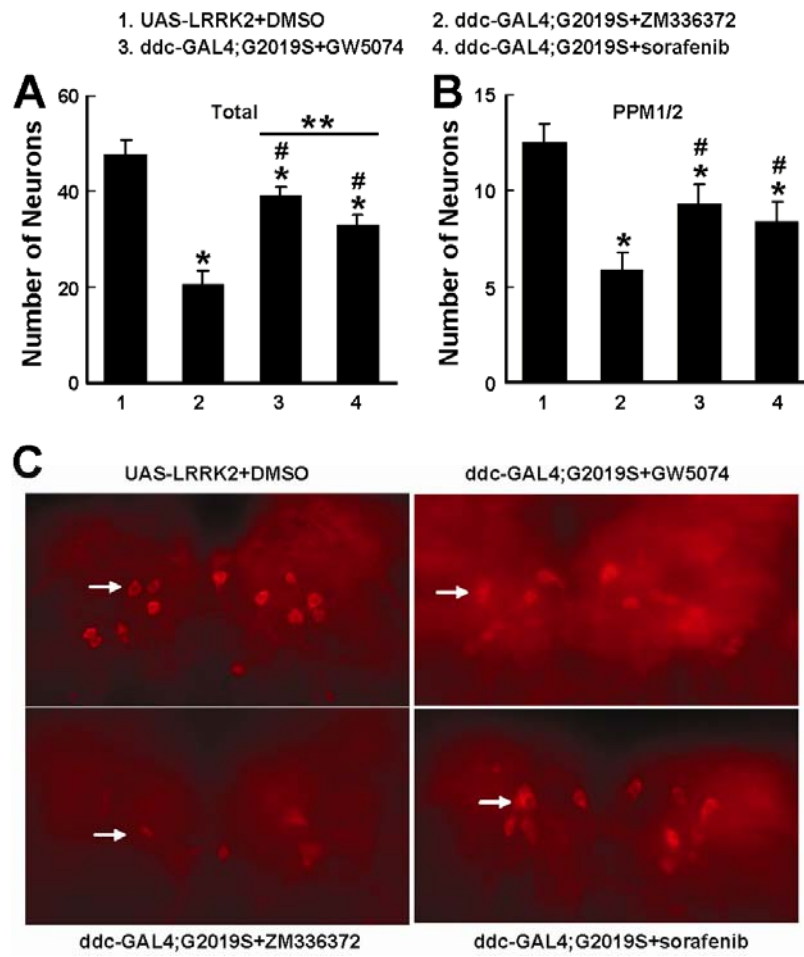


Figure 4



**Figure 5**

## ABBREVIATIONS:

Parkinson's disease (PD)

*leucine-rich repeat kinase 2 (LRRK2)*

dopamine (DA)

$\alpha$ -synuclein ( $\alpha$ -syn)

dihydroxyphenylalanine decarboxylase (ddc)

tyrosine hydroxylase (TH)

Immunoprecipitation (IP)

paired anterolateral medial (PAM) neurons

protocerebral posterior medial (PPM)