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Identification of chemicals to inhibit the kinase activity of leucine-rich repeat kinase 2 (LRRK2), a Parkinson's disease-associated protein

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ABSTRACT

Parkinson's disease (PD) is a late-onset neurodegenerative disease which occurs at more than 1% in populations aging 65-years and over. Recently, leucine-rich repeat kinase 2 (LRRK2) has been identified as a causative gene for autosomal dominantly inherited familial PD cases. LRRK2 G2019S which is a prevalent mutant found in familial PD patients with LRRK2 mutations, exhibited kinase activity stronger than that of the wild type, suggesting the LRRK2 kinase inhibitor as a potential PD therapeutics. To develop such therapeutics, we initially screened a small chemical library and selected compound **1**, whose IC_{50} is about 13.2 μM. To develop better inhibitors, we tested five of the compound **1** derivatives and found a slightly better inhibitor, compound **4**, whose IC_{50} is 4.1 μM. The cell-based assay showed that these two chemicals inhibited oxidative stress-induced neurotoxicity caused by over-expression of a PD-specific LRRK2 mutant, G2019S. In addition, the structural analysis of compound **4** suggested hydrogen bond interactions between compound **4** and Ala 1950 residue in the backbone of the ATP binding pocket of LRRK2 kinas domain. Therefore, compound **4** may be a promising lead compound to further develop a PD therapeutics based on LRRK2 kinase inhibition.

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Parkinson's disease (PD) is a neurodegenerative movement disorder whose symptoms are tremors, bradykinesia, and rigidity.[1,2] PD is a late-onset disease and approximately 10% of the cases are an inherited form called familial PD (FPD). Intensive study of samples obtained from these FPD cases so far revealed 16 PARK loci.[3] Leucine-rich repeat kinase 2 (LRRK2) was originally identified as a gene corresponding to PARK8, which is inherited in an autosomal dominant manner.[4–6] LRRK2 mutations occur in not only 5–10% of FPD cases, but also 1–2% of sporadic PD cases.[7,8] LRRK2 contains two functional enzyme activities, a kinase and a small GTPase. G2019S is the most prevalent mutation, which occurs in the 85% of the PD cases containing LRRK2 mutations.[9] Interestingly, the G2019S mutant exhibited a 3–10-fold increase of its kinase activity in comparison with the wild type.[10,11] These observations led to the hypothesis that chemicals to inhibit LRRK2 kinase activity can be, or can at least lead to, a potential PD therapeutics. In fact, there are already several reports that have screened novel LRRK2 kinase inhibitors[12] or tested non-selective kinase inhibitors against LRRK2 kinase activity.[13–15]

To screen compounds inhibiting LRRK2 kinase activity, we employed in vitro kinase assay with radiolabeled [γ^{32}-P]ATP and purified N-terminal truncated LRRK2 G2019S mutant protein fused to glutathione S-transferase (GST, Invitrogen Co.).[16] We screened a small in-house chemical library of 160 putative ATP-competitive inhibitors at 25 μM in in vitro LRRK2 kinase assay and measured their inhibitory effects on LRRK2's autophosphorylation activity (data not shown). Compound **1**, (*E*)-4-((2-(4-methylquinolin-2-yl)hydrazono)methyl)benzene-1,2-diol, was selected as a hit compound, and its IC_{50} was calculated as approximately 13.2 μM[17] (Fig. 1a lanes 1 vs 3 & Fig. 1b). We used staurosporine (St) as a positive control since it was reported as a strong non-selective LRRK2 kinase inhibitor (Fig. 1a lanes 1 and 2).[16] For a kinase inhibitor to be developed as a therapeutics, its specific inhibitor activity is vital because many kinases share several conserved active domains that can be common target sites for the inhibitory compounds. As an initial step to assess the specificity of compound **1**, we tested its kinase inhibitory effect against bacterially expressed DYRK1A protein [Dual specificity tyrosine(Y) phosphorylation Regulated Kinase 1A].[17,18] DYRK1A is one of autophosphorylated kinases, like LRRK2, which is useful to assay kinases whose substrates are unknown. Figure 1a shows that compound **1** inhibited the autophosphorylation of LRRK2, but not of DYRK1A (lanes 1 vs 3 and lanes 4 vs 5). In order to validate the activity of the chemical scaffold of compound **1** and to further

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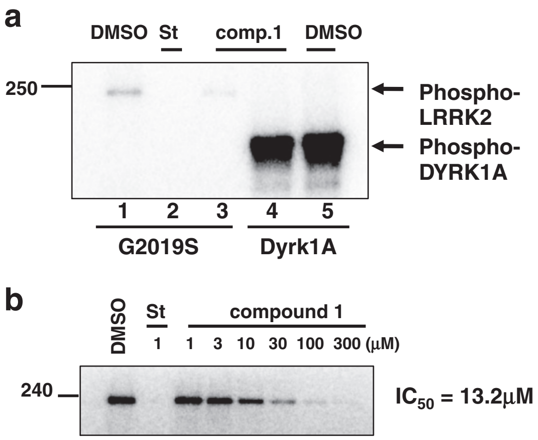


Figure 1. The inhibitory effect of the compound **1** on LRRK2 kinase activity. (a) Compound **1** (compd **1**)at 25 μM inhibited LRRK2, but not DYRK1A kinase activity. LRRK2 fusion protein, GST-LRRK2 G2019S with N-terminal truncation (Invitrogen), or bacterially expressed and purified DYRK1A protein were used for in vitro kinase assay with radiolabelled [γ^{32}-P]ATP. Staurosporine at 1 μM was also added to compare its inhibitory activity to that of compound (compd) **1**. Both phosphorylated LRRK2 and DYRK1A were indicated by arrows. (b) The inhibitory effect of compound **1** at various concentrations from 1 μM to 300 μM on LRRK2 kinase. The treatment of the indicated concentration of compound **1** or 1 μM of staurosporine (St) was compared to the reaction treated with DMSO only (DMSO).

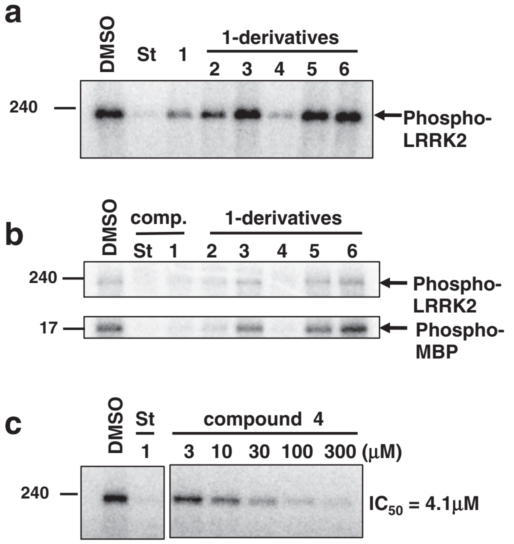


Figure 2. The inhibitory effect of compound **4** on LRRK2 kinase activity. (a) Five compound **1** derivatives and compound **1** were tested at 25 μM for their inhibitory effects on LRRK2 kinase. (b) MBP was included as general nonspecific substrate for in vitro LRRK2 kinase assay. All other procedures were performed as (a). (c) The inhibitory effect of compound **4** at various concentrations from 3 μM to 300 μM on LRRK2 kinase. The treatment of either 1 μM of staurosporine (St) or the same volume of DMSO is also shown.

identify compounds showing better activity, we purchased five of compound **1** derivatives and retested again their inhibitory activity against LRRK2 kinase. Table 1 shows their structures and molecular weights along with those of compound **1**. In this assay, we included myelin basic protein (MBP) which was often used as a general, nonspecific kinase substrate for LRRK2.[21] Figure 2a and 2b clearly shows that only compound **4** [(*E*)-4-methyl-2-(2-(pyridin-4-ylmethylene)hydrazinyl)quinoline], out of all of the compounds tested, is slightly better than compound **1** although the compound **2** also showed some inhibitory effect, but no better than compound **1**. Addition of MBP as LRRK2 kinase substrate made autophosphorylation of LRRK2 weaker (Fig. 2a vs 2b), although the compounds' inhibitory pattern was similar. Compound **4**'s IC_{50} was calculated as approximately 4.1 μM,[17] based on data shown in Figure 2c.

In addition, to test inhibitory specificity of compound **4** against LRRK2, in vitro kinase assays of several MAPKKKs and kinases other than LRRK2 were performed[22] and the results were compared to compound **4**'s inhibitory activities for LRRK2 which were calculated from Figure 2a and 2c. Table 2 showed that compound **4** had much weaker inhibitory effect on kinases other than LRRK2. A recent inhibitor study showed that GW5074, a compound to inhibit RAF kinase, also strongly inhibited LRRK2.[21] However, our data showed that compound **4** contained very weak inhibitory effect on RAF, suggesting its relative specificity for LRRK2, unlike GW5074. Instead, compound **4** exhibited inhibition on the mixed-lineage kinase (MLK), as strong as LRRK2 (Table 2). This is

Table 1
The structures of the compounds tested in this study

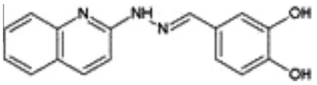
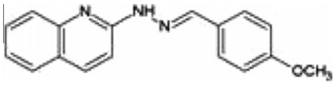
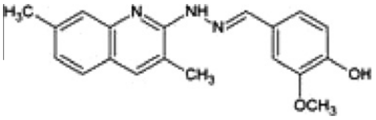
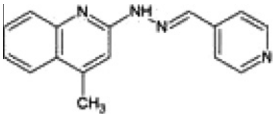
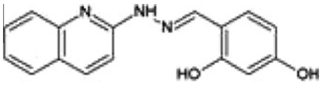
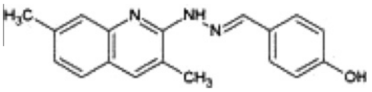
Compound no.	Structure	Mol. weight	Cat. no.
1		293.32	BAS 02379154 (ASINEX)
2		277.32	5554268 (ChemBridge)
3		321.37	5559663 (ChemBridge)
4		262.31	5773896 (ChemBridge)
5		279.29	5564319 (ChemBridge)
6		291.35	5511947 (ChemBridge)

Table 2
The inhibitory effect of compound **4** on various MAPKKKs and other kinases

Kinase	Protein identity	Remained kinase activity (%) after treatment of compound **4** at	
		10 μM	25 μM
LRRK2*	NP_940980	28	11
ASK1*	NP_005914	94	68
c-RAF*	P04049	92	86
CDK5/p25	NP_004926 (CDK5)/ NP_003876 (p25)	97	81
CK1	NP_001020276	77	59
GSK3 β	NP_001139628	99	94
MLK1*	NP_149132	20	12
mTOR	NP_004949	103	99
p70S6 K	NP_003152	67	50
TAK1*	NP_001122390	68	48
TAO1*	NP_065842	88	84
TAO2*	NP_057235	89	84
TAO3*	NP_057365	97	81

[*] MAPKKK proteins.

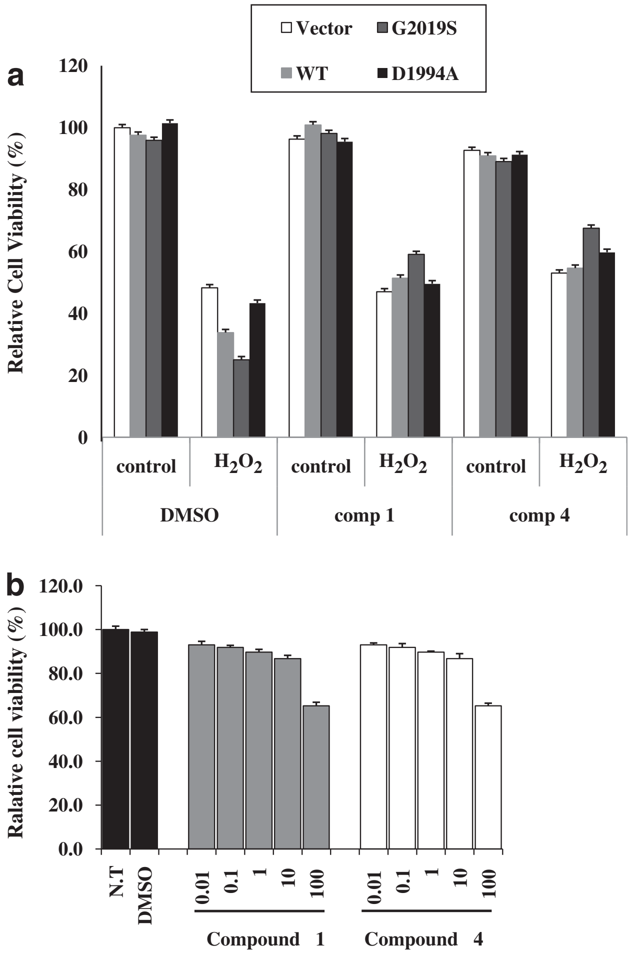


Figure 3. (a) The oxidative stress-induced neurotoxicity of SN4741 cells transiently expressing LRRK2 WT, G2019S or kinase dead D1994A mutants with the treatment of compounds **1** and **4** at 10 μM. Dopaminergic SN4741 cells were transfected with plasmids containing LRRK2 WT G2019S or D1994A gene, incubated for two days, and treated with 100 μM of hydrogen peroxide for one day. The crystal violet assay was applied to measure the cell survival rate.[27] The compounds (compd **1** and **4** at 10 μM) were treated at the indicated concentrations at 1 h before hydrogen peroxide treatment. The data are shown as means with SEM calculated from experiments performed in triplicate. Statistical analysis was performed by ANOVA with Turkey's *post hoc* test. Control means no treatment. (b) The neurotoxicity of compounds **1** and **4** on the primary rat cortex neuronal cell cultures. The chemicals were applied to the cells at the indicated concentrations and, after incubation for one day, the surviving cells were measured by the MTT assay.[28] The numbers above the line of each compound indicated the concentration of the compounds (μM) applied to the cells.

somewhat expected because MLK kinase domain has been reported to be the most similar to that of LRRK2.[23] In summary, Table 2 suggested that compound **4** contains moderately specific inhibitory effect on LRRK2 kinase and more screening and/or refining of compound **4** is necessary to identify a more specific inhibitor for LRRK2.

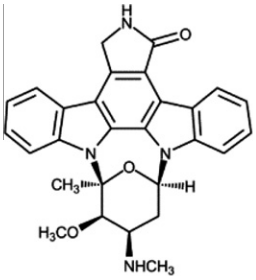
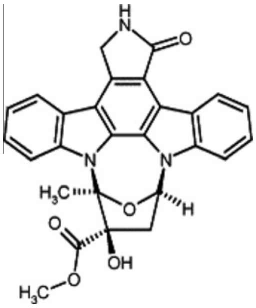
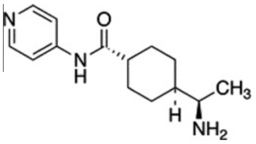
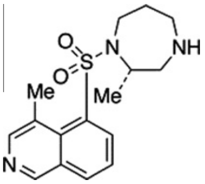
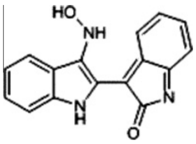
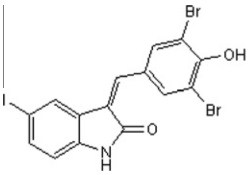
Next, we tested the inhibitory effect of compounds **1** and **4** in a cell-based assay system. Previously, we have reported that the over-expression of LRRK2 wild type (WT) and G2019S in murine dopaminergic SN4741 cells enhanced oxidative stress-induced neurotoxicity in the order of G2019S>WT and the enhancement was also caused by the expression of the LRRK2 kinase domain alone, but not by the expression of the LRRK2 kinase dead mutant, D1994A.[24,25] The result suggested that the LRRK2 kinase activity was responsible for the oxidative stress-induced neurotoxicity. Therefore, we applied this cell-based assay to confirm whether compounds **1** and **4** could inhibit LRRK2 kinase activity.[19] The result showed that the transient expression of LRRK2 WT or G2019S exhibited about 35% or 25% cell survival rate in the presence of hydrogen peroxide, respectively (Fig. 3a). However, the treatment of compound **1** or **4** at 10 μM to the SN4741 cells that were transfected with LRRK2 WT or G2019S and treated with hydrogen peroxide, restored their cell survival rates to approximately 50% which was the level of the survival rate of the control cells which were transfected with empty vector alone or plasmids expressing D1994A and treated with hydrogen peroxide. Therefore, Figure 3a suggested that compound **1** and **4** could inhibit LRRK2's kinase activity in intracellular environment.

The treatment of compounds **1** and **4** to SN4741 cells exhibited similar toxicity each other, which were slightly more toxic than the cells treated with DMSO (white bars in Fig. 3a in the control samples). To assess neurotoxicity of compounds, primary rat cortical neuronal cells were cultured under normal conditions with treatment of various concentrations of compound **1** and **4** and cell survival rates were measured. Figure 3b showed that both compounds exhibited similar cell survival rates, approximately 85% at 10 μM concentration, although their neurotoxicities were significantly increased at 100 μM (Fig. 3b).

Several well-known ATP-competitive inhibitors of kinases such as staurosporine, K-252a, Y-27632, H-1152, indirubin-3′-monoxime, and GW5074 have been reported to inhibit LRRK2 kinase activity.[13–16,20] Though we originally screened putative ATP-competitive inhibitors for their activity as LRRK2 kinase inhibitors, the structures of compounds **1** and **4** are considerably different from these known chemicals (Table 3). Therefore, we tested in silico whether compound **4** is a putative ATP-competitive inhibitor for LRRK2 kinase. A molecular docking study with compound **4** which exhibited inhibitory activity stronger than compound **1**, was performed. First, we constructed a homology model structure of the LRRK2 kinase domain.[26] Using this refined model of the LRRK2 kinase domain, we investigated the plausible docking model of compound **4**. Analysis of the result from the docking studies suggested hydrogen bond interactions between compound **4** and Ala 1950 residue in the backbone of LRRK2 kinase (Fig. 4a). In addition, we also found that the 4-methyl quinoline group of compound **4** structurally overlapped with the adenine rings of ATP, and a 4-pyridine ring was located in the solvent exposed region of the LRRK2 kinase domain (Fig. 4b). Therefore, the binding of compound **4** on the LRRK2 ATP binding pocket is highly capable of inhibiting the binding of ATP in this site.

We also constructed LRRK2 kinase binding model with staurosporine and compared it to the LRRK2 kinase binding model with compound **4** or ATP, as shown in Figure 4c or d, respectively. The

Table 3
The structures of other nonspecific LRRK2 kinase inhibitors which were previously reported

Chemical (reference)	Staurosporine (18)	k-252a (18)	Y-27632 (14)
Structure			
Chemical (reference)	H-1152 (14)	Indirubin-3′-monoxime (22)	GW5074 (22)
Structure			

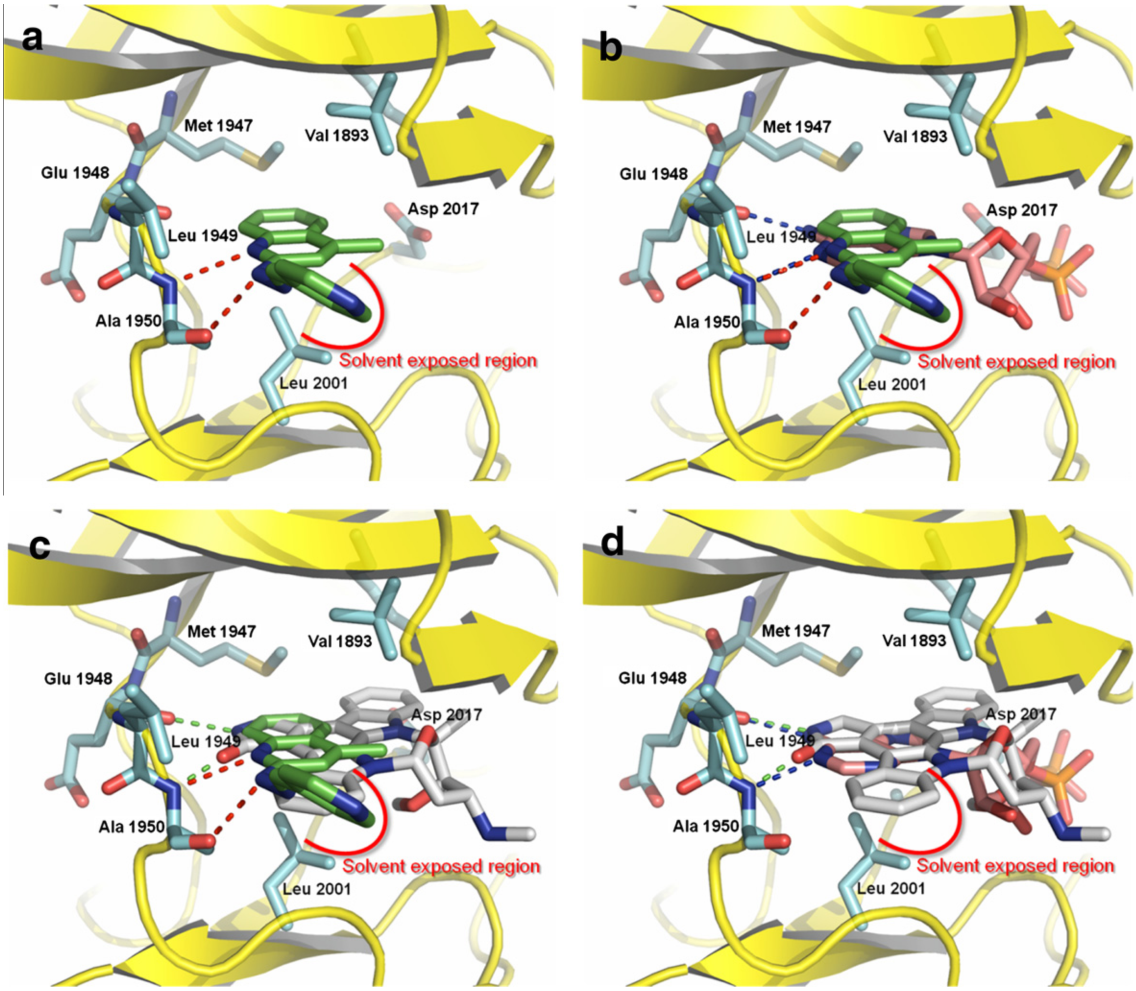


Figure 4. (a) Refined docking model of compound **4** (green color) with LRRK2 kinase. The red dotted lines denote hydrogen bonding interactions of compound **4** with LRRK2. (b) Comparison of the proposed binding model of compound **4** and ATP with LRRK2. The ATP is presented in pink. The blue dotted lines denote hydrogen bonding interactions of ATP with LRRK2. The refined docking model of compound **4** into ATP binding pocket of LRRK2 was performed using the AutoDock4 program.[29] (c) Comparison of the proposed binding model of staurosporine (white color) and compound **4** (green color) with the LRRK2 kinase domain. The right blue and red dotted lines denote hydrogen bonding interactions of staurosporine and compound **4** with LRRK2, respectively.(d) Comparison of the proposed binding model of staurosporine (white color) and ATP (pink color)with the LRRK2 kinase domain. The right blue and blue dotted lines denote hydrogen bonding interactions of staurosporine and ATP with LRRK2, respectively.

model showed that staurosporine formed hydrogen bond between Glu 1948 and Ala 1950 residues in the backbone of LRRK2 hinge contact region. Staurosporine extensively occupied LRRK2 kinase domain whereas compound **4** partially occupied the ATP binding pocket and 4-pyridine ring located in solvent exposed region (compare Figure 4a and 4d).

In summary, our data showed that compounds **1** and **4**, (*E*)-4-((2-(4-methylquinolin-2-yl)hydrazono)methyl)benzene-1,2-diol and (*E*)-4-methyl-2-(2-(pyridin-4-ylmethylene)hydrazinyl)quinoline, exhibited reasonable LRRK2 kinase inhibitor activities which may provide useful information for the development of new chemical entities of PD therapeutic agents.

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16. In vitro kinase assay was performed by mixing 30 ng of GST-LRRK2 G2019S protein (Invitrogen) with 28 nM of $[\gamma^{32}\text{-P}]$ATP (IZOTOP, SBP-501) in 15 μl of the kinase assay buffer [20 mM Tris (pH 7.5), 0.02% Tween20, 10 mM $MgCl_2$, 1 mM EGTA, 5 mM β-glycerophosphate, 1 mM Na_3Vo_4, 2 mM DTT] and incubating the mixture at 30 °C for 10 min. When indicated, 1 μg of MBP (myelin basic protein) was added as general kinase substrate. To test the compounds' inhibitory effects on LRRK2 kinase activity, each chemical was prepared at 10 mM stock in dimethyl sulfoxide (DMSO) and added to the reaction mixture at the indicated concentration. Then, the whole mixture was subjected to the protein gel electrophoresis, and levels of the LRRK2 autophosphorylation or MBP phosphorylation were analyzed by an image analyzer (Typhoon 9200, GE healthcare).
17. *IC_{50} was calculated as follows*: density of each phosphorylated band in Figures 1b and 2b was measured using densitometer program (Image Gause version 4.0) and plotted them against each inhibitor's concentration using MS office Excel program. A fitting formula was obtained from each compound and compounds' IC_{50}s were calculated from the formula.
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25. SN4741 cells were transfected with empty vectors or vectors expressing LRRK2 WT or G2019S gene and, after 48 h of incubation, 100 μM of hydrogen peroxide was treated for 24 h. To measure the cell survival rate, crystal violet assay was carried out[27] and relative cell survival rates were calculated. To test compounds' inhibitory activities, the indicated concentration of chemicals or an equal volume of dimethyl sulfoxide (DMSO) were treated at 1 h before the treatment of hydrogen peroxide.
26. To construct a homology model structure of the LRRK2 kinase domain, we used a crystal structure of transforming growth factor-beta (TGF-β) activated kinase 1 (TAK1) (PDB accession code: 2EVA). The sequence alignment of LRRK2 and template proteins was generated using the FASTA program (http://www.ebi.ac.uk/Tools/fasta33). A 3D model structure of LRRK2 was built by using the HOMOLOGY module of Insight II program (Accelrys Software Inc., San Diego, USA) and was further refined by using the Discover 2.98 of the Insight II.
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