Brain Penetrant LRRK2 Inhibitor.

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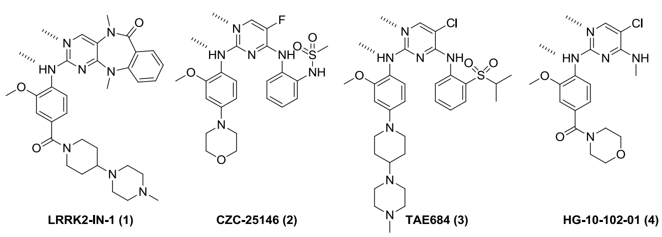
KEYWORDS. LRRK2, Blood-brain barrier, Brain Penetrant Inhibitor, 2,4-diaminopyrimidine

Supporting Information Placeholder

ABSTRACT: Activating mutations in leucine-rich repeat kinase 2 (LRRK2) are present in a subset of Parkinson's disease (PD) patients and may represent an attractive therapeutic target. Here we report a 2-anilino-4-methylamino-5-chloropyrimidine, HG-10-102-01(**4**) is a potent and selective inhibitor of wild-type LRRK2 and the G2019S mutant. Compound **4** substantially inhibits Ser910 and Ser935 phosphorylation of both wild-type LRRK2 and G2019S mutant at a concentration of 0.1─0.3 µM in cells and is the first compound reported to be capable of inhibiting Ser910 and Ser935 phosphorylation in mouse brain following intraperitoneal delivery of doses as low as 50 mg/kg.

Parkinson's disease (PD) is the second most common neurodegenerative disease in the world. It affects over one million Americans and more than 60,000 patients are newly diagnosed each year.[1,2] Recent genetic studies have revealed an underlying genetic cause in at least 10% of all PD cases,[3] which provides new opportunities for the discovery of molecularly targeted therapeutics that may ameliorate neurodegeneration. Among the genes associated with PD, leucine-rich repeat kinase 2 (LRRK2) is unique because a missense mutation, G2019S, is frequently found in both familial and sporadic Parkinson's disease cases.[4-9] The G2019S mutation increases kinase activity which may result in activation of the neuronal death signal pathway, suggesting that small molecule LRRK2 kinase inhibitors may be able to serve as a new class of therapeutics for the treatment of Parkinson's disease.[10-13] Transgenic G2019S LRRK2 mice aged to 12 to 16 months display progressive degeneration of the substantia nigra pars compacta (SNpc) dopaminergic neurons and Parkinson's phenotypes of motor dysfunction suggesting that this mutation may be functionally relevant to the disease.[14]

LRRK2 kinase inhibitors are being actively pursued both as 'tools' to pharmacologically interrogate normal and pathological LRRK2 biology and as experimental therapeutic agents. For example, LRRK2-IN-1 (**1**)[15] and CZC-25146 (**2**)[16] have been reported as the first-generation 'tool' inhibitors that exhibit excellent potency and selectivity for LRRK2. However, none of these compounds are able to efficiently cross the mouse blood-brain barrier (BBB) and inhibit LRRK2 kinase activity which limits their utility in murine PD models and eventual clinical development.[15,16] Here we report that a lower molecular weight 2,4-diaminopyrimidine, HG-10-102-01 (**4**), maintains highly potent and selective inhibition of LRRK2 and is, to our knowledge, the first compound reported to be capable of inhibiting LRRK2 phosphorylation in mouse brain.



LRRK2-IN-1 (1) CZC-25146 (2) TAE684 (3) HG-10-102-01 (4)

Compound ID	IC_{50} (nM)[a]			
	wild-type LRRK2	LRRK2-G2019S	LRRK2-A2016T	LRRK2-G2019S+A2016T
LRRK2-IN-1 (1)	13	6.0	2450	3080
TAE684 (3)	7.8	6.1	93.3	21.9
HG-10-102-01 (4)	20.3	3.2	153.7	95.9

Figure 1. LRRK2 inhibitors. a. Biochemical inhibition of GST-LRRK2 (1,326-2,527), GST-LRRK2[G2019S] (1,326-2,527), GST-LRRKT[A2016T] (1,326-2,527) and GST-LRRK2[G2019S+A2016T] (1,326-2,527) was assayed using 20 µM Nictide in the presence of 100 µM ATP. Results are average of triplicate experiments.

Several 2,4-diaminopyrimidine based inhibitors of LRRK2 have been reported including LRRK2-IN-1 (**1**)[15], CZC-25146 (**2**)[16] and TAE684 (**3**)[17], but none of these compounds are capable of effectively inhibiting phosphorylation of Ser910 and Ser935 of LRRK2 in mouse brain at intraperitoneal doses of up to 100 mg/Kg. Analysis of predicted docked conformations of these compounds to homology models of LRRK2 suggest that the 4-anilino moiety of each compound occupies quite distinct regions of the ATP-binding site. In an attempt to lower the molecular weight and remove possible disfavorable interactions with the protein, we explored compounds where the 4-anilino moiety was removed. We and others[18,19] discovered that simplified structures such as **4** maintain the ability to potently inhibit the biochemical activity of wild-type and G2019S mutant LRRK2. Compound **4** exhibited biochemical IC_{50}s of 20.3 and 3.2 nM against wild-type LRRK2 and LRRK2[G2019S], respectively (**Figure 1**). The biochemical potency of **4** for inhibition of wild-type LRRK2 and LRRK2[G2019S] is similar to that observed for LRRK2-IN-1 (**1**), however, **4** maintains inhibition of the A2016T mutation which induces dramatic resistance to LRRK2-IN-1 (**1**) (**Figure 1**). Although both LRRK2-IN-1 (**1**) and **4** share the aminopyrimidine pharmacophore, a molecular model of **4** docked to a homology model of LRRK2 built based on a previously published crystallographic structure of ALK,[20] suggests that there is less possibility for steric hindrance with the A2016T mutation (**Figures 2a** and **2b**).

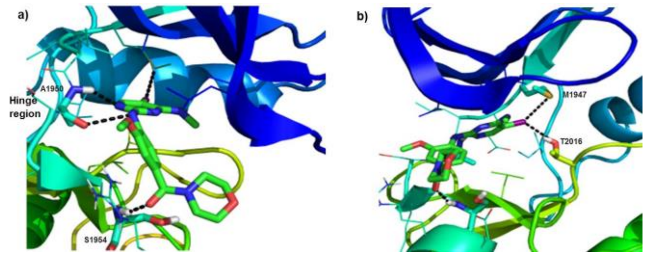
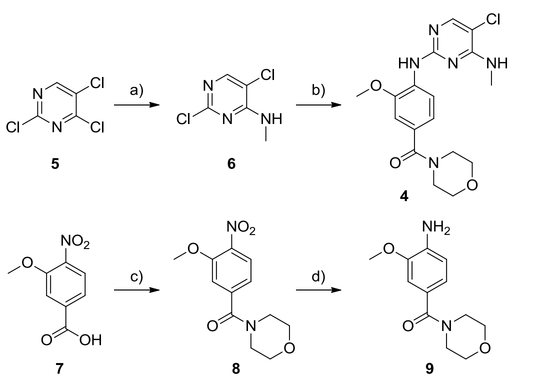


Figure 2. Molecular model of HG-10-102-01 (4) with LRRK2[T2016]. a) Two hydrogen bonds are predicted between the hinge region A1950 and the aminopyrimidine motif of the inhibitor. One hydrogen bond is predicted between the backbone amide carbonyl of S1954 with the amide carbonyl of the inhibitor. b) Two potential interactions exist between M1947 and T2016 with the 5-chloro group on the pyrimidine of the inhibitor.



Scheme 1. Reagents and conditions: a) 2.0 M $MeNH_2$ in THF, THF, 0 °C to RT, 6 h, 85%, b) **9**, TFA, 2-BuOH, 110 °C, 12 h, 76%, c) i. thionyl chloride, toluene, 120 °C, 2 h, ii. morpholine, DIEA, THF, 0 °C to RT, 1 h, 92%, d) 10% Pd/C, MeOH, RT, 12 h, 98%.

Compound **4** was prepared from commercially available 2,4,5-trichloropyrimidine and 3-methoxy-4-nitrobenzoic acid (**Scheme 1**). The 3-methoxy-4-nitrobenzoic acid **7** was subjected to chlorination with thionyl chloride followed by reaction with morpholine to form the corresponding amide **8** which was reduced by hydrogenation to yield aniline **9**. 2,4,5-trichloropyrimidine **5** was regioselectively aminated with methylamine to afford to 2,5-dichloro-N-methylpyrimidin-4-amine **6.** Compound **6** was aminated with aniline **9** under acidic conditions to furnish the desired compound **4**.

We next examined the ability of **4** to inhibit LRRK2 in a cellular context in comparison to LRRK2-IN-1 (**1**). As there are no validated direct phosphorylation substrates of LRRK2, we monitored phosphorylation of Ser910 and Ser935, two residues whose phosphorylation is known to be dependent upon LRRK2 kinase activity[21] (**Figure 3**). Compound **4** induced a dose-dependent inhibition of Ser910 and Ser935 phosphorylation in both wild-type LRRK2 and LRRK2[G2019S] stably transfected into HEK293 cells (**Figure 3a**). Substantial dephosphorylation of Ser910 and Ser935 was observed at approximately 1 μM concentrations of **4** for wild-type LRRK2 and at a slightly lower dose of 0.3 μM for LRRK2[G2019S] (**Figure 3b**), which is a similar potency to that observed for LRRK2-IN-1 (**1**). Consistent with the biochemical results, **4** also induced dephosphorylation of Ser910 and Ser935 at a concentration of 1─3 μM in the drug-resistant LRRK2[A2016T+G2019S] and LRRK2[A2016T] mutants, revealing that the A2016T mutation is not an effective way to induce resistance to **4**.

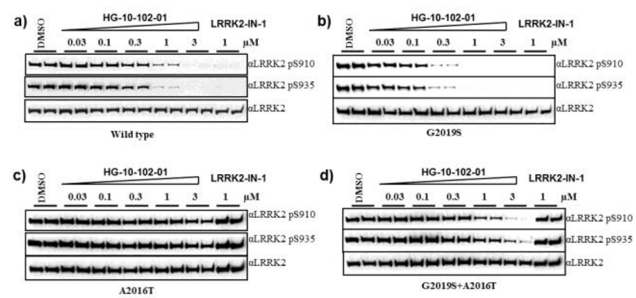


Figure 3. Compound HG-10-102-01 (4) inhibits LRRK2 in cells. HEK293 cells stably expressing a) wild-type GFP-LRRK2, b) GFP-LRRK2[G2019S], c) GFP-LRRK2[A2016T] and d) GFP-LRRK2[G2019S+A2016T] were treated with DMSO or increasing concentrations of **4** for 90 min (1 μM of LRRK2-IN-1 was used as a control). Cell lysates were subjected to immunoblotting for detection of LRRK2 phosphorylated at Ser910 and Ser935 and for total LRRK2.

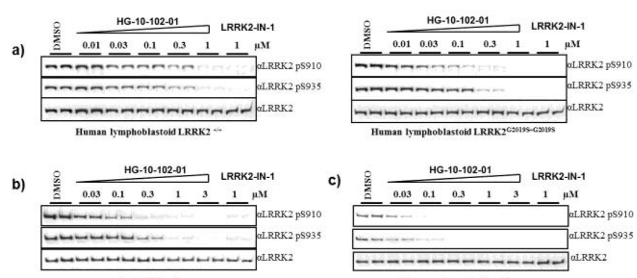


Figure 4. Compound 4 inhibits endogenously expressed LRRK2 a) Endogenous LRRK2 from EBV immortalized human lymphoblastoid cells from a control subject ($LRRK2^{+/+}$) a Parkinson's disease patient homozygous for the LRRK2[G2019S] mutation. After treatment of the cells with DMSO or the indicated concentration of **4** (or LRRK2-IN-1 (**1**)) for 90 min, cell lysates were subjected to immunoblot analysis with the indicated antibody for western analysis. Immunoblots were performed in duplicate, and results were representative of at least two independent experiments. b) As in **a** except mouse Swiss 3T3 cells were used. c) As in **a** except mouse embryonic fibroblast cells were used.

We next examined the effect of **4** on endogenously expressed LRRK2 in human lymphoblastoid cells derived from a control and Parkinson's patient homozygous for the LRRK2[G2019S] mutation (**Figure 4a**). We found that increasing doses of **4** led to similar dephosphorylation of endogenous LRRK2 at Ser910 and Ser935, as was observed in HEK293 cells stably expressing wild-type LRRK2 or LRRK2[G2019S] (compare **Figure 3a** to **Figure 4a**). Moreover, endogenous LRRK2 was also more sensitive to **4** than LRRK2-IN-1 (**1**), which is consistent with the trend we observed in HEK293 cells. We also found that **4** induced similar dose-dependent Ser910 and Ser935 dephosphorylation of endogenous LRRK2 in mouse Swiss 3T3 cells and mouse embryonic fibroblast cells (**Figures 4b** and **4c**).

The mouse pharmacokinetic profile of **4** demonstrated good oral bioavailability (67 %F), a short half-life of 0.13 hours and low plasma exposure (502 hr*ng/mL, AUC_{last}) (Complete PK parameters in the supplementary data). The short half-life is likely the consequence of rapid first-pass metabolism as incubation with mouse liver microsomes also revealed a short $T_{1/2}$ of 13 minutes.[22] We next investigated the pharmacodynamic properties of **4** by monitoring inhibition of LRRK2 Ser910/Ser935 phosphorylation in kidney, spleen and brain following intraperitoneal delivery of 100 mg/kg of **4**. We observed near complete dephosphorylation of Ser910 and Ser935 of LRRK2 in all tissues including brain at this dose. We then repeated the study at lower doses of 50, 30 and 10 mg/kg where we observed near complete inhibition in all tissues at 50 mg/kg but only partial inhibition in brain at the 30 and 10 mg/kg doses. These results indicate that **4** is a promising chemo-type for achieving dephosphorylation of Ser910 and Ser935 in the brain.

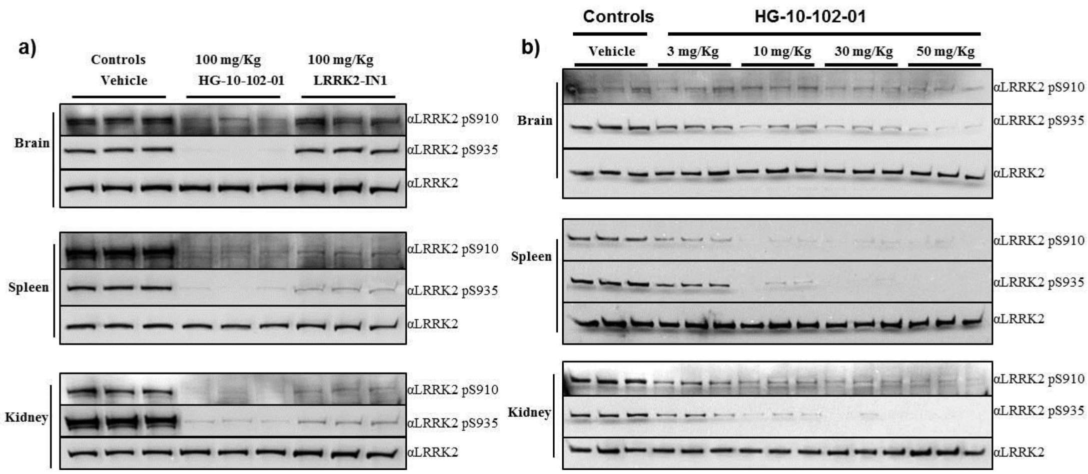


Figure 5. Pharmacodynamic analysis for HG-10-102-01 (4). Pharmacodynamic study of HG-10-102-01 (**4**) from brain, spleen and kidney following intraperitoneal administration at the indicated doses. Tissues were collected and endogenous LRRK2 was resolved by SDS-PAGE and blotted with a phospho-specific antibody directed against Ser910, Ser935 and total LRRK2. (The quantitative analysis is included in supporting information)

To further explore the ability of **4** to inhibit LRRK2 *in vivo*, we used a chemical proteomics approach, KiNativ, to monitor kinase inhibition in tissues from the inhibitor treated animals. Brains and spleens from animals treated with **4** at 3, 10, 30, 50 and 100 mg/kg, and LRRK2-IN-1 (**1**) at 100 mg/kg were evaluated and compared to vehicle treated animal brains. Briefly, the brains were lysed in a 5X volume of buffer (no detergent) and labeled with an ADP-acylphosphate probe. Following sample workup and trypsin digestion, kinase labeling by the acylphosphate probe was quantitatively determined by mass spectrometry. Data was collected for >150 protein and lipid kinases (supplemental data). Of the kinases profiled, only LRRK2 showed a clear and significant dose-related increase in engagement with **4**. LRRK2 inhibition was ~40% at 30 mg/kg and ~70% at 50 and 100 mg/kg in the brain. Greater LRRK2 target engagement was observed in the spleen: ~40% at 3 mg/kg, ~80-90% at 10 mg/kg and greater than 90% in the 30-100 mg/kg treated animals. In contrast, no LRRK2 inhibition was observed in brains of mice treated with 100 mg/kg LRRK2-IN-1 (**1**), consistent with the inability of the compound to induce dephosphosphorylation of the Ser910 and Ser935 sites of LRRK2. Considering these inhibition values correspond to a 5X dilution of material relative to the intact brain, these engagement values correlate very well with the observed inhibition of p-LRRK2. No other kinases showed >50% inhibition at any dose of **4**, and only JNK1/2/3 and TBK1 showed potential dose related inhibition (~35-40% at 50 and 100 mg/kg) suggesting that compound **4** is a highly selective inhibitor of LRRK2.

The kinase selectivity of **4** was further assessed using standard radioactivity-base enzymatic assays against a panel of 138 kinases (Dundee profiling).[23] At a concentration of 10 µM, compound **4** only inhibited the kinase activities of MLK1 and MNK2 to greater than 80% of the DMSO control (Complete results presented in the supplementary data). Dose-response analysis revealed inhibition of MLK1 with an IC_{50} 2.1 μM and MNK2 with an IC_{50} 0.6 μM. KinomeScan analysis against a near comprehensive panel of 451 kinases at a concentration of 1 μM resulted in no interactions detected with kinases other than G2019S LRRK2 with the exception of one mutant form of c-Kit (L576P) demonstrating the outstanding selectivity of this inhibitor (complete profiling results provided in the supplementary data).[24] These results suggest that **4** is a highly selective LRRK2 inhibitor however further profiling of additional kinases and other ATP-dependent enzymes is still warranted.

In summary, we have discovered that **4** is a potent biochemical and cellular inhibitor of LRRK2 kinase activity. Detailed characterization of **4** using LRRK2-IN-1 as a bench mark revealed that **4** significantly inhibited phosphorylation of wild-type LRRK2 and LRRK2[G2019S] mutant at Ser910 and

Ser935 at 0.3-1.0 µM in cell culture, which is approximately the same potency as LRRK2-IN-1 (**1**). Compound **4** is relatively insensitive to the A2016T mutation which suggests that this mutant will not be useful to validate whether the pharmacological effects of the compound are LRRK2-dependent. Compound **4** exhibits excellent kinase selectivity as assessed by recombinant kinases (124), KinomeScan (451 kinases) and KiNativ profiling. Compound **4** can inhibit phosphorylation of Ser910 and Ser935 of LRRK2 in brain and peripheral tissues following intraperitoneal doses of 50 mg/kg. Further optimization of this chemo-type especially in regards to *in vivo* half-life will be reported in due course.

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Contributions

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ABBREVIATIONS

LRRK2, leucine-rich repeat kinase 2; PD, Parkinson's disease; ATP, adenosine triphosphate; ALK, anaplastic lymphoma kinase; THF, tetrahydrofuran**;** RT, room temperature; TFA, trifluoroacetic acid; 2-BuOH, 2-butanol; DIEA, *N*,*N*-diisopropylethylamine; PK, pharmacokinetics**;** F, bioavailability; AUC, area under the concentration time curve; CL, clearance; C_{last}, last measured concentration; C_{max}, maximum concentration observed; $T_{1/2}$, elimination half-life**;** Vss, volume in steady state; MLK1, mixed-lineage Kinase 1; MNK2, MAP kinase-interacting serine/threonine-protein kinase 2; DMSO, dimethyl sulfoxide.

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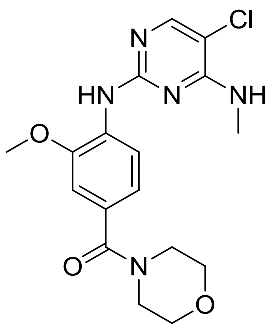
Table of Contents Graphic

Brain Penetrant LRRK2 Inhibitors



HG-10-102-01 (4)

	IC_{50} (nM)
LRRK2-wild-type	20.3
LRRK2-[G2019S]	3.2
LRRK2-[A2016T]	153.7
LRRK2-[G2019S+A2016T]	95.9