Science Translational Medicine
AAAS

stm.sciencemag.org/cgi/content/full/11/514/eaau6870/DC1

Supplementary Materials for

A modulator of wild-type glucocerebrosidase improves pathogenic phenotypes in dopaminergic neuronal models of Parkinson's disease

Lena F. Burbulla, Sohee Jeon, Jianbin Zheng, Pingping Song, Richard B. Silverman, Dimitri Krainc*

*Corresponding author. Email: dkrainc@nm.org

Published 16 October 2019, *Sci. Transl. Med.* **11**, eaau6870 (2019)
DOI: 10.1126/scitranslmed.aau6870

The PDF file includes:

Materials and Methods
Fig. S1. Characterization of heterozygous 84GG (c.84dupG) *GBA1* mutant iPSCs, CRISPR-Cas9–corrected isogenic iPSCs and control iPSCs, and the midbrain dopaminergic neurons differentiated from them.
Fig. S2. CRISPR-Cas9 editing of heterozygous 84GG (c.84dupG) *GBA1* mutant iPSCs.
Fig. S3. Cell-free in vitro enzyme activity assays of acid α-glucosidase and α-galactosidase A after treatment with S-181.
Fig. S4. Measurement of *GBA1* expression by qPCR in *GBA1* mutant iPSC-derived dopaminergic neurons treated with S-181 or vehicle for 10 days.
Fig. S5. Structure and in vitro enzyme activity of a nonactivating control compound.
Fig. S6. Brain weights of wild-type ($Gba1^{+/+}$) and $Gba1^{D409V/+}$ mice treated with a dose of S-181 (50 mg/kg) or 5% dextrose (vehicle) intraperitoneally twice daily (n = 5 to 8 per group).
Table S1. List of human iPSC lines used in the study including summary of clinical and genotype information for patients with PD.
Table S2. Pharmacokinetics (PK) for S-181 determined in mouse plasma and brain tissue after a single intraperitoneal dose (50 mg/kg) in wild-type mice.
Table S3. Protein binding of S-181 and control compound in mouse brain homogenate.
Table S4. Protein binding of S-181 and control compound in mouse plasma.

Other Supplementary Material for this manuscript includes the following:

(available at stm.sciencemag.org/cgi/content/full/11/514/eaau6870/DC1)

Data file S1 (Microsoft Excel format). Individual level data for all figures.

Materials and Methods

Preparation of (*S*)-*N*-((2,3-dihydrobenzo[b][1,4]dioxin-2-yl)methyl)-*N*-methyl-2-(pyridin-3-yl) quinazolin-4-amine (S-181)

a) Preparation of (*S*)-*N*-((2,3-dihydrobenzo[b][1,4]dioxin-2-yl)methyl)-2-(pyridin-3-yl)quinazolin-4-amine:

A mixture of 4-chloro-2-(pyridin-3-yl)quinazoline (*12*) (114 mg, 0.47 mmol), (*S*)-(2,3-dihydrobenzo[b][1,4]dioxin-2-yl)methanamine (83 mg, 0.5 mmol), and potassium carbonate (69 mg, 0.5 mmol) in DMF (2 mL) was stirred at 70 ^{o}C for 2 hours. Water (20 mL) was added, and the formed solid was filtered, washed with water, and dried *in vacuo* to give 150 mg (yield: 86%) pale-yellow powder, which was used directly in the next step.

b) Preparation of (*S*)-*N*-((2,3-dihydrobenzo[b][1,4]dioxin-2-yl)methyl)-*N*-methyl-2-(pyridin-3-yl) quinazolin-4-amine (S-181): To a solution of (*S*)-*N*-((2,3-dihydrobenzo[b][1,4]dioxin-2-yl)methyl)-2-(pyridin-3-yl)quinazolin-4-amine (60 mg, 0.16 mmol) in dry DMF (2 mL), NaH (60% in mineral oil) (6.5 mg, 0.16 mmol) was added under an argon atmosphere at room temperature, and the mixture was stirred for 15 min. Iodomethane (10 μL, 0.16 mmol) was added dropwise to the mixture, and the mixture was allowed to stir at room temperature overnight. Water (15 mL) was added, and the formed solid was filtered, washed with water, dried *in vacuo* and purified by flash chromatography to give 52 mg (yield: 85%) pale-yellow powder as the product.

^{1}H NMR (500 MHz, $CDCl_3$) δ 9.65 (s, 1H), 8.72 (d, *J* = 7.9 Hz, 1H), 8.69 – 8.64 (m, 1H), 8.13 (d, *J* = 8.4 Hz, 1H), 7.93 (d, *J* = 8.4 Hz, 1H), 7.76 – 7.68 (m, 1H), 7.43 – 7.34 (m, 2H), 6.90 – 6.78 (m, 4H), 4.84 – 4.76 (m, 1H), 4.40 – 4.27 (m, 2H), 4.17 – 4.08 (m, 2H), 3.66 (s, 3H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 163.0, 157.1, 153.1, 150.8, 150.0, 143.1, 142.6, 135.5, 134.1, 132.5,

128.9, 125.5, 124.7, 123.2, 121.8, 121.5, 117.4, 117.2, 115.0, 71.8, 66.0, 53.0, 43.1. HRMS (ESI): calcd for $C_{23}H_{21}N_4O_2$ $[M+H]^+$, 385.1659; found, 385.1657. S-181 was dissolved in dioxane, and treated with 2 equivalent of hydrochloride (4M in dioxane). The formed solid was filtered *in vacuo* and washed with dioxane, and dried to give S-181 hydrochloride salt.

Preparation of *N*-(2-phenoxyethyl)-*N*-phenyl-2-(pyridin-3-yl)quinazolin-4-amine (non-activating control)

a) Preparation of *N*-(2-phenoxyethyl)aniline: A mixture of (2-bromoethoxy)benzene (402 mg, 2 mmol), aniline (182 μL, 2 mmol), and potassium carbonate (276 mg, 2 mmol) in DMF (4 mL) was stirred at room temperature or 60 ^oC for 2 days. Water (20 mL) was added, and the mixture was extracted with ethyl acetate (30 mL). The organic layer was washed with brine, dried (Na_2SO_4), filtered, and concentrated to give 350 mg (yield: 82%) pale-yellow liquid, which was used directly in the next step.

b) Preparation of *N*-(2-phenoxyethyl)-*N*-phenyl-2-(pyridin-3-yl)quinazolin-4-amine: To a solution of *N*-(2-phenoxyethyl)aniline (107 mg, 0.5 mmol) in dry DMF (2 mL) was added NaH (60% in mineral oil) (20 mg, 0.5 mmol) under an argon atmosphere at room temperature, and the mixture was stirred for 15 min. 4-chloro-2-(pyridin-3-yl)quinazoline (120 mg, 0.5 mmol) was added to the mixture, and the mixture was allowed to stir at 50 ^oC overnight. Water (15 mL) was added, and the mixture was extracted with ethyl acetate (15 mL x 2). The combined organic layer was washed with brine, dried (Na_2SO_4), filtered, concentrated and purified by flash chromatography to give 10 mg (yield: 5 %) pale-yellow solid.

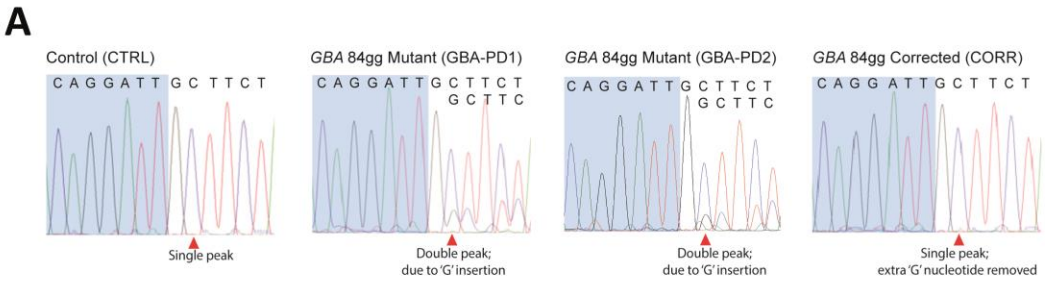
^1H NMR (500 MHz, $CDCl_3$) δ 9.75 (d, *J* = 2.2 Hz, 1H), 8.80 (dt, *J* = 8.0, 1.9 Hz, 1H), 8.70 (dd, *J* = 4.8, 1.9 Hz, 1H), 7.88 (d, *J* = 8.4 Hz, 1H), 7.61 – 7.55 (m, 1H), 7.44 – 7.35 (m, 3H), 7.35 –

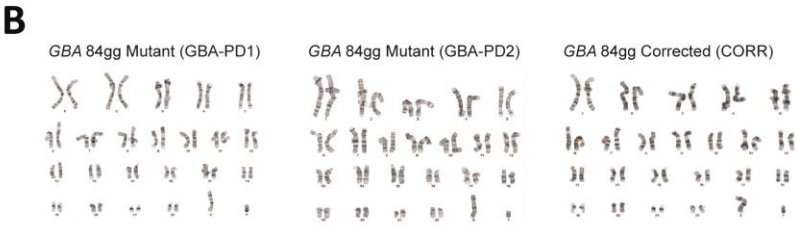
7.26 (m, 3H), 7.23 – 7.17 (m, 2H), 7.03 – 6.98 (m, 2H), 6.89 (td, *J* = 7.3, 1.2 Hz, 1H), 6.87 – 6.81 (m, 2H), 4.65 (t, *J* = 5.7 Hz, 2H), 4.48 (t, *J* = 5.7, 2H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 161.1, 158.6, 157.6, 152.7, 150.8, 150.1, 147.5, 135.6, 132.2, 130.0, 129.4, 128.8, 126.6, 126.5, 126.3, 124.9, 123.3, 120.9, 114.5, 64.9, 53.7. HRMS (ESI): calcd for $C_{23}H_{21}N_4O_2$ $[M+H]^+$, 419.1866; found, 419.1862.

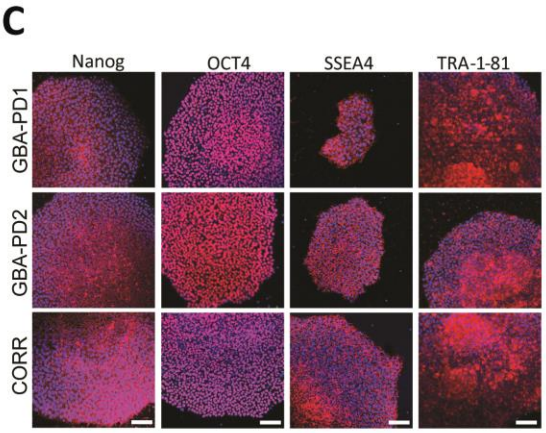
Western blot analysis

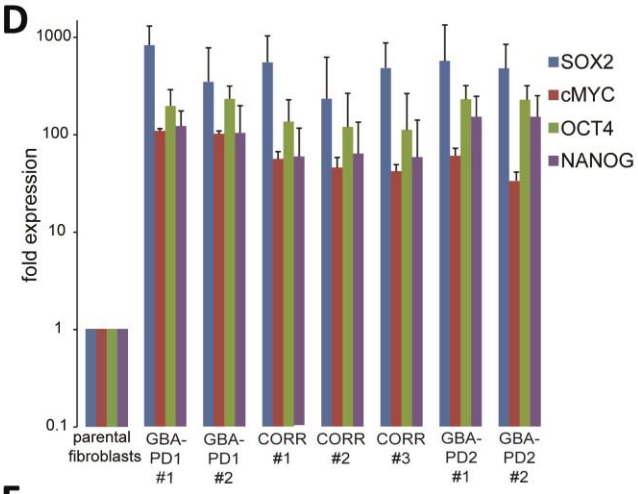
Neurons were scraped in cold PBS and centrifuged at 400x g for 5 min. PBS was removed and the cell pellet was homogenized in 1% Triton X-100 lysis buffer (containing 10% glycerol, 150mM NaCl, 25mM Hepes pH7.4, 1mM EDTA, 1.5mM $MgCl_2$, proteinase inhibitor cocktail) as previously described (*8*). For western blot analysis of brain tissue from Gba1$^{D409V/+}$ mice, freshly dissected hippocampal bran tissue was homogenized in 1% Triton X-100 lysis buffer according to tissue weight. Insoluble pellets from a 100,000 x g spin were further extracted in 2% SDS/50mM Tris pH 7.4 by boiling and sonication. Lysates were analyzed by western blot, blocked in Odyssey Blocking Buffer (LI-COR Biosciences), and incubated with the following primary antibodies overnight at 4^{0}C: GCase (Sigma, #G4171, 1:1000), α-synuclein (C-20, Santa Cruz #SC-7011-R, 1:1000; syn303, BioLegend #MMS-5085, 1:500; syn202, #MMS-529R, 1:1000), synapsin (Santa Cruz, #sc-398849, 1:500), Tyrosine Hydroxylase (Millipore, #657012, 1:1000), β-III-tubulin (BioLegend, #801202, 1:5000), GAPDH (Millipore, #MAB374, 1:5000), β-actin (abcam, #ab6276, 1:5000). Membranes were scanned using Odyssey Infrared imaging System (Li-Cor).

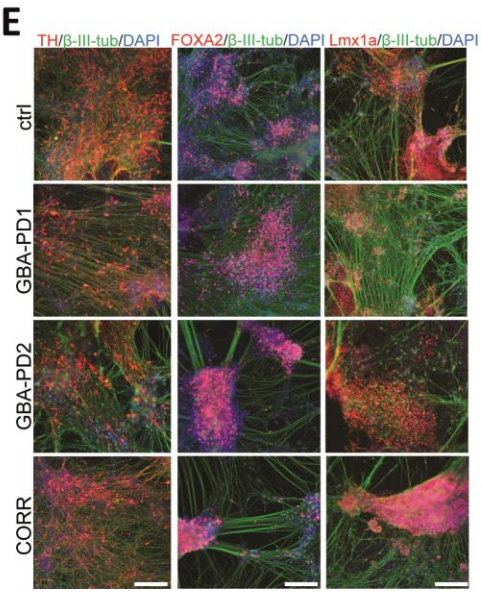
Supplementary Figures and Figure Legends











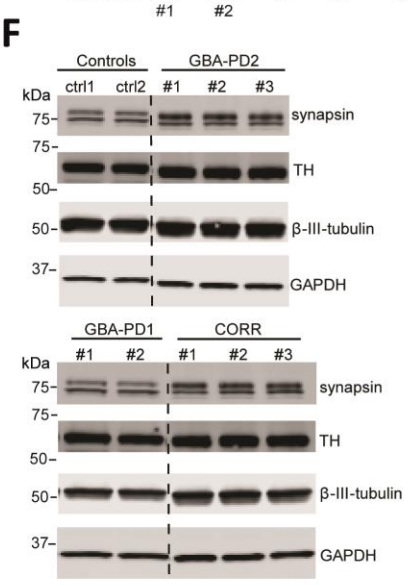


Fig. S1. Characterization of heterozygous 84GG (c.84dupG) GBA1 mutant iPSCs, CRISPR-Cas9–corrected isogenic iPSCs and control iPSCs, and the midbrain dopaminergic neurons differentiated from them. (A) Direct sequencing confirmed the c.84dupG *GBA1* mutation in two heterozygous *GBA1* mutant iPSC lines (GBA-PD1, GBA-PD2). Sequencing of the gene-edited isogenic iPSC line (CORR) confirmed correction of the c.84dupG *GBA1* mutation. **(B)** Karyotype analysis of GBA-PD1 and GBA-PD2 mutant, and gene-edited *GBA1* corrected isogenic (CORR) iPSC lines. **(C)** Immunocytochemistry for pluripotency markers NANOG, OCT4, SSEA4 and TRA-1-81 and nuclear counterstaining with DAPI in iPSCs from GBA-PD1, GBA-PD2 mutant as well as gene-edited *GBA1* corrected isogenic (CORR) iPSC lines. Scale bar, 100μm. **(D)** qRT-PCR analysis for the expression of the indicated pluripotency markers (fold expression) compared to levels in human parental skin fibroblasts. Relative expression was calculated using β-actin as housekeeping gene. **(E)** Immunocytochemistry of dopamine, midbrain and neuronal markers in iPSC-derived dopaminergic neurons from GBA-PD1, GBA-PD2 mutant iPSC lines as well as gene-edited *GBA1* corrected isogenic (CORR) and control iPSC lines at d30. Antibodies against TH, FOXA2, LMX1a and β-III-tubulin were used. Scale bar, 20μm. **(F)** Western blot analysis of TH, synapsin and β-III-tubulin levels in T-soluble neuronal lysates from GBA-PD1, GBA-PD2 mutant neurons as well as gene-edited *GBA1* corrected isogenic (CORR) and control neurons at d70. GAPDH was used as a loading control.

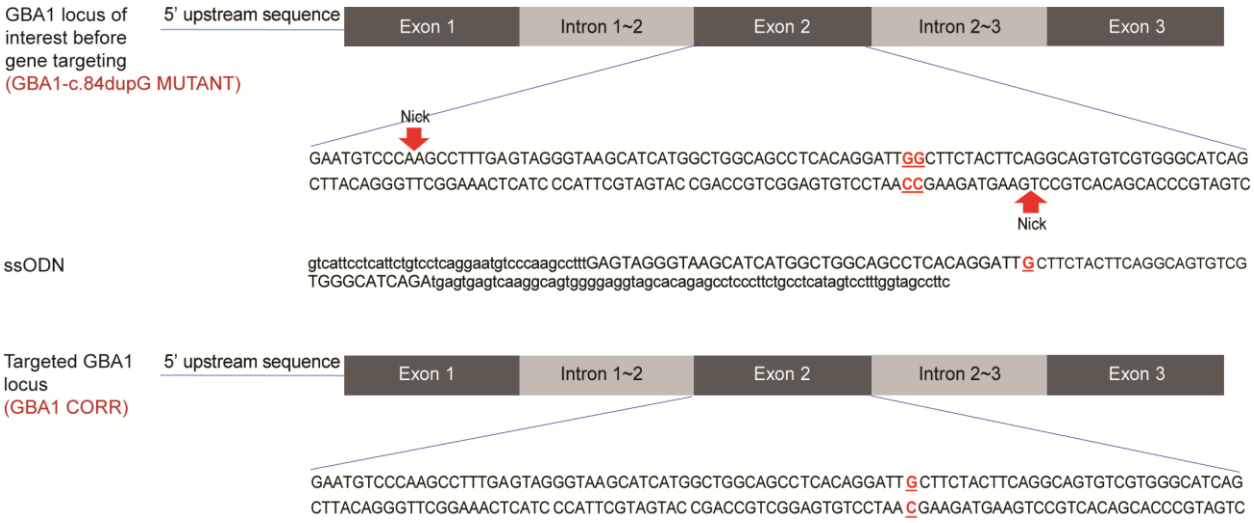


Fig. S2. CRISPR-Cas9 editing of heterozygous 84GG (c.84dupG) GBA1 mutant iPSCs.

Structure of the *GBA1* locus and schematic overview of gene targeting strategy to reverse c.84dupG *GBA1* mutation in *GBA1* mutant iPSCs.

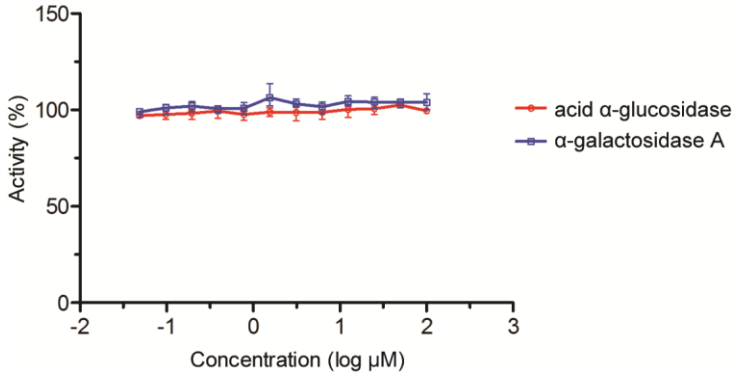


Fig. S3. Cell-free in vitro enzyme activity assays of acid α-glucosidase and α-galactosidase A after treatment with S-181.

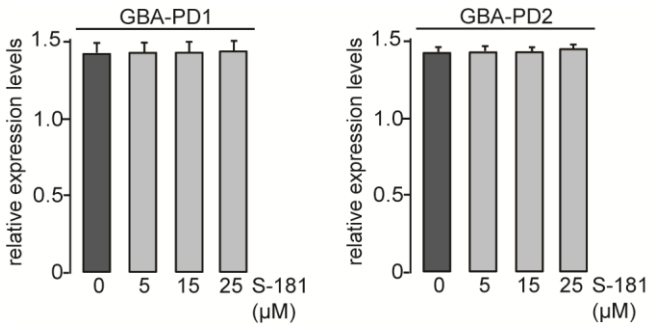


Fig. S4. Measurement of *GBA1* expression by qPCR in *GBA1* mutant iPSC-derived dopaminergic neurons treated with S-181 or vehicle for 10 days.

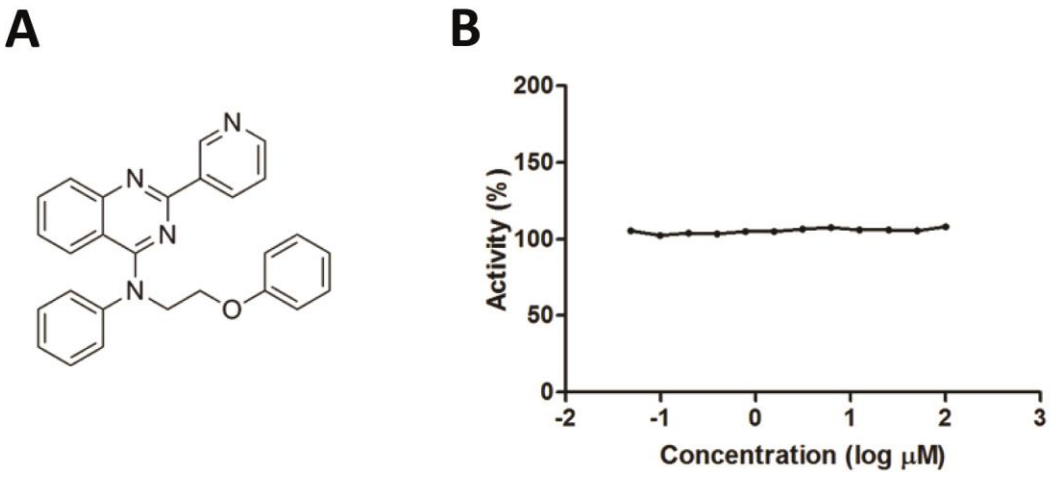


Fig. S5. Structure and in vitro enzyme activity of a nonactivating control compound. (A) Structure of non-activating control compound. **(B)** *In vitro* enzyme activity of a non-activating control compound.

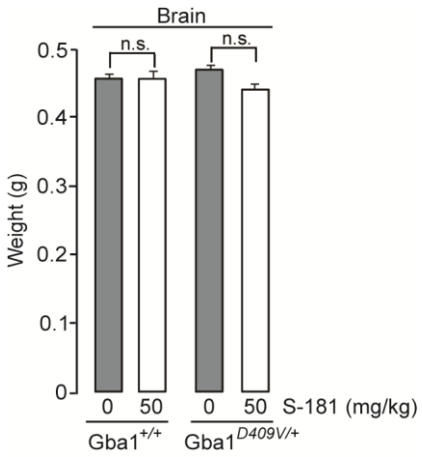


Fig. S6. Brain weights of wild-type (Gba1$^{+/+}$) and Gba1$^{D409V/+}$ mice treated with a dose of S-181 (50 mg/kg) or 5% dextrose (vehicle) intraperitoneally twice daily (*n* = 5 to 8 per group).

Supplementary Tables

Table S1. List of human iPSC lines used in the study including summary of clinical and genotype information for patients with PD.

Cell lines	Description	Sampling age (y)	Gender	Gene	Genotype
Ctrl-1	Control	59	male	---	WT/WT
Ctrl-2	Control	40	male	---	WT/WT
GBA-PD1, #1	PD	57	male	GBA1	c.84GG/WT
GBA-PD1, #2	PD	57	male	GBA1	c.84GG/WT
GBA-PD2, #1	PD	46	male	GBA1	c.84GG/WT
GBA-PD2, #2	PD	46	male	GBA1	c.84GG/WT
CORR, #1	isogenic control of GBA-PD1	57	male	---	WT/WT
CORR, #2	isogenic control of GBA-PD1	57	male	---	WT/WT
CORR, #3	isogenic control of GBA-PD1	57	male	---	WT/WT
LRRK2-PD1	PD	60	male	LRRK2	p.R1441C/WT
LRRK2-PD2	PD	79	male	LRRK2	p.G2019S/WT
Parkin-PD	PD	79	male	Parkin	p.A324fsX110 + p.S245fsX8, compound heterozygous
DJ-1 KO	CRISPR/Cas9-generated DJ-1 null	59	male	DJ-1	null
iPD-1	PD	77	male	---	WT/WT
iPD-2	PD	54	female	---	WT/WT

Ctrl, healthy control; PD, Parkinson's disease; CORR, CRISPR-Cas9-generated isogenic control iPSCs; iPD, idiopathic PD

Table S2. Pharmacokinetics (PK) for S-181 determined in mouse plasma and brain tissue after a single intraperitoneal dose (50 mg/kg) in wild-type mice.

PK parameter	unit	plasma	brain
T_{max}	h	0.5	0.5
C_{max}	ng/mL	1737	1120
		(4.52 μM)	(2.92 μM)
$T_{1/2}$	h	3.77	20.69
AUC_{last}	h·ng/mL	2667	5163
AUC_{INF}	h·ng/mL	2760	12795
AUC_{brain}/AUC_{plasma}	%		194

Table S3. Protein binding of S-181 and control compound in mouse brain homogenate.

Compound ID	Replicate	% Bound	% Unbound	LogK	% Recovery	% Remaining at 6 hr
Telmisartan	Replicate 1	98.69	1.31	1.88	86.00	88.78
	Replicate 2	98.92	1.08	1.96	92.75	95.28
	Mean	98.81	1.19	1.92	89.38	92.03
S-181	Replicate 1	99.85	0.15	2.83	97.41	104.25
	Replicate 2	99.85	0.15	2.81	84.90	89.79
	Mean	99.85	0.15	2.82	91.15	97.02

Table S4. Protein binding of S-181 and control compound in mouse plasma.

Compound ID	Replicate	% Bound	% Unbound	LogK	% Recovery	% Remaining at 6 hr
Warfarin	Replicate 1	91.64	8.36	1.04	98.26	92.55
	Replicate 2	92.17	7.83	1.07	96.98	101.26
	Mean	91.91	8.09	1.06	97.62	96.90
S-181	Replicate 1	99.79	0.21	2.68	96.09	94.06
	Replicate 2	99.81	0.19	2.72	101.09	97.77
	Mean	99.80	0.20	2.70	98.59	95.92