Supporting Information

Discovery of Highly Potent, Selective and Brain Penetrable Leucine-Rich Repeat Kinase 2 (LRRK2) Small Molecule Inhibitors

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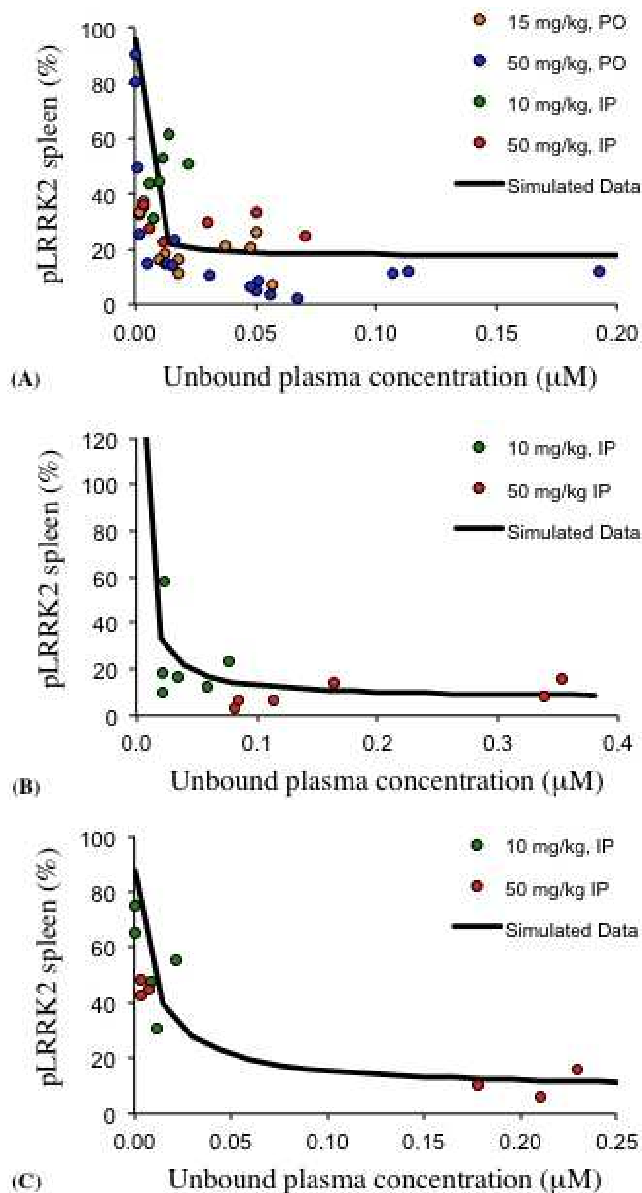

Supplementary Figure 1. In vivo G2019S LRRK2 transgenic mouse PK/PD results measuring spleen pSer1292 autophosphorylation. The closed circles represent the observed data and the line represents the predicted data from a direct inhibition model. Percent inhibition is normalized to pSer1292 levels observed in mice dosed with vehicle alone (n=3). Plotted data is shown for mice treated with (A) **18** [15 mg/kg, PO at 1, 3 and 6 h (n =4/dose); 50 mg/kg, PO at 1, 3, 6 and 12 h (n=4/dose) and 24 h (n=2); 10 mg/kg, IP, at 1 and 2.5 h (n=3/dose); 50 mg/kg, IP at 1 and 6 h (n=3/dose)]; (B) **19** [10 and 50 mg/kg IP at 1, and 6 h (n=3/dose)]; (C) **14** [10 and 50 mg/kg IP at 1 and 6 h (n=3/dose)].

<u>In vitro hepatocyte metabolic stability experiments were carried out as follows:</u>

The metabolic stability of test compounds (1 μM test concentration) was determined in human, Sprague–Dawley rat, and cynomolgus monkey, cryopreserved primary hepatocytes (CellzDirect Invitrogen Corporation, Durham, NC, USA, pooled donors). Incubations (final incubation volume = 250 μL) were performed at 37 °C under an atmosphere of 95%/5% CO_2/O_2 using 0.5 million cells mL^{-1} in hepatocyte incubation media (In Vitro Technologies, Baltimore, MD, USA). 50-μL

aliquots (cells and media) were taken at zero, 1, 2 and 3 h following the start of the incubation and quenched using 100 μL of ice-cold acetonitrile. The resulting samples were centrifuged at 3000g for 10 min. Supernatant (90 μL) was diluted in water (180 μL) and the percent remaining test compound was assessed by LC/MS/MS. All incubations were performed in quadruplicate.

Time-dependent CYP inhibition experiments were carried out as described in:

(a) Mukadam, S.; Tay, S.; Tran, D.; Wang, L.; Delarosa, E. M.; Khojasteh, S. C.; Halladay, J.; Kenny, J. R. Evaluation of time-dependent cytochrome P450 inhibition in a high-throughput, automated assay: Introducing a novel area under the curve shift approach. *Drug Metab. Lett.* **2012**, *6*, 45–53.

(b) Obach, R. S.; Walsky, R. L.; Venkatakrishnan, K. Mechanism-based inactivation of human cytochrome P450 enzymes and the prediction of drug-drug interactions. *Drug Metab. Dispos.* **2007**, *35*, 246–255.

In vitro MDCK-MDR1 experiments were carried out as follows:

MDCKI cells transfected with the human MDR1 gene (MDCKI–MDR1) were maintained at 37 °C, 95% humidity, and 5% CO_2 in culture with Eagle's minimum essential medium Earle's BSS (0.1% nonessential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine, 1.5 g/L sodium bicarbonate) supplemented with 10% fetal bovine serum. The MDCKI–MDR1 cell medium was further supplemented with 80 ng/mL colchicine to sustain MDR1 expression. The monolayers were equilibrated for 30 min in transport buffer (HBSS with 10 mM HEPES, pH 7.4) at 37 °C with 5% CO_2 and 95% humidity prior to the experiment. The transport of LRRK2 inhibitors (5 μM) were examined in the apical-to-basolateral (AB) and basolateral-to-apical (BA) directions. The apparent permeability (Papp) was calculated using the following equation:

$$P_{app} = \frac{dQ}{dt} \bullet \frac{1}{C_0} \bullet \frac{1}{A}$$

where dQ/dt is the rate of compound appearance in the receiver compartment, C_0 is the concentration in the donor compartment at time = 0, and A is the surface area of the insert.

In vitro protein binding experiments were carried out as follows:

Binding in plasma or brain homogenate was determined using a 48-well rapid equilibrium dialysis (RED) device using a dialysis membrane with molecule weight cut-off value of 8000 Da (Pierce Biotechnology, Rockford, IL). Blank plasma or brain homogenate was spiked with testing compound for a concentration of 5 μM. 300 μL plasma or brain homogenate containing compound was added to the donor side and 500 μL buffer was added to the receiver side. The plate was placed on a rocking platform at 400 rpm with 1 mm radius agitation for 4 hr at 37 °C. 20 μL of buffer, plasma, or brain homogenate samples were transferred to a 96-well plate and 20 μL of opposite blank matrix was added. Plasma or brain homogenate proteins were precipitated using 65% acetonitrile containing internal standard (0.1 μM propranolol) and the unbound fraction (f_u) was calculated as the ratio of the buffer concentration versus the plasma or brain homogenate concentration. The unbound fraction in brain was estimated by a nonlinear dilution equation as reported in:

(c) Liu, X.; Vilenski, O.; Kwan, J.; Apparsundaram, S.; Weikert, R. Unbound brain concentration determines receptor occupancy: a correlation of drug concentration and brain serotonin and dopamine reuptake transporter occupancy for eighteen compounds in rats. *Drug Metab. Dispos*. **2009**, *37*, 1548–1556.

In vivo animal experiments were carried out as follows:

Male Sprague-Dawley rats with dual cannulation of the jugular and femoral vein were obtained from Charles River Laboratories (Hollister, CA). They were housed at controlled temperature and humidity in an alternating 12 h light/dark cycle with free access to food and water. Rats were dosed at 0.5 mg/kg intravenously at jugular vein. Dosing solutions of each drug were prepared in 100% *N*-Methyl-2-pyrrolidone and were dosed at 1 mL/kg. Whole blood was collected from femoral vein into Microtainer tubes containing K2EDTA and stored on ice until centrifuged for the preparation of plasma. Rats were euthanized via IV Euthasol solution at 0.25, 1, and 2 h post dose and approximately 50 μL CSF samples were collected via cisterna magna puncture and were stored on dry ice at -70 °C before analysis. Whole brains were collected by decapitation, rinsed in phosphate-buffered saline, weighed, and immediately frozen on dry ice. All studies were conducted in accordance with approved Genentech Animal Care and Use Procedures.

In vivo sample analyses were carried out as follows:

Standard curves and quality control samples were prepared by spiking a known amount of compound into a blank mixed matrix of rat plasma, brain homogenate, and CSF (1:1:1, v/v/v). The brain tissue of each rat was homogenized in four volumes (w/v) of water. 25 μL of plasma sample was mixed with 25 μL of blank brain homogenate and 25 μL of buffer. 25 μL of brain homogenate sample was mixed with 25 μL of blank plasma and buffer and 25 μL of CSF was mixed with 25 μL of blank plasma and 25 μL of blank brain homogenate. Total 50 μL of samples, 50 μL of calibration standards, or 50 μL of quality controls was mixed with 15 μL of internal standard (amprenavir-D4) and 150 μL acetonitrile. Following vortexing and centrifugation at 1500 x g for 10–15 minutes, 150 μL of supernatant was transferred to a 96-well plate, and diluted with 50 μL water prior to analysis by high performance liquid chromatography combined with tandem mass spectrometry. The system consisted of an Accela pump (Thermo Scientific, Waltham, MA), an HTS-PAL autosampler (Leap Technologies, Switzerland), and an AB Sciex API 5000 (AB Sciex, Foster City, CA) mass spectrometer with a turbo ion spray interface. A 20 μL aliquot of each sample was injected onto a reverse-phase HALO C18 column. The response from the mass spectrometry was used to calculated the brain/plasma and CSF/plasma concentration ratio based on their AUC as described in:

(d) Liu, X.; Ding, X.; Deshmukh, G.; Liederer, B. M.; Hop, C. E. Use of the cassette-dosing approach to assess brain penetration in drug discovery. *Drug Metab. Dispos.* **2012**, *40*, 963–969.

In vivo PK/PD formulation and analysis:

Mice were dosed with LRRK2 inhibitor suspended in 1% Avicel® RC-591 (microcrystalline cellulose vehicle) via intraperitoneal (IP) or oral (PO) routes. Dosing volumes did not exceed 0.4 mL for IP doses, or 0.3 mL for PO doses. At euthanasia, mice were anesthetized with avertin (0.5 mL/25 g body weight IP) and blood was harvested by cardiac puncture for isolation of serum. Mice were euthanized and the spleen and brain were harvested. The brain was bisected and the hippocampus and cerebellum were sub-dissected out of the right hemi-brain.

Serum and hemi-cerebellum were homogenized for analysis of drug content (pharmacokinetic analysis). Spleen and hemi-hippocampus were homogenized for analysis of LRRK2 Ser1292 phosphorylation state (pharmacodynamic analysis).

The in vivo pLRRK2 inhibition versus unbound plasma and brain concentration data were fitted with the following equation using WinNonlin (Pharsight):

$$E = E_{max} – (E_{max} – E_0)*IC_{50}/(IC_{50} + C)$$

where E represents the remaining brain pLRRK2 activity as compared to the vehicle treated group following the treatment, E_{max} represents maximal activity of pLRRK2, E_0 represents residual activity, IC_{50} represents unbound plasma or brain concentrations that inhibit 50% of pLRRK2 in the brain, C represents unbound plasma or brain concentration.