High throughput screening of small molecules that inhibit LRRK2 phosphorylation on Serine 935 reveals novel inhibitors and cellular pathways that affect LRRK2

Spencer B. Hermanson[1], Coby B. Carlson[1], Steven M. Riddle[1], Jing Zhao[2], Kun Bi[1] and R. Jeremy Nichols[2]

[1]Life Technologies Corporation, Madison, WI U.S.A; [2]The Parkinson's Institute, Sunnyvale, CA U.S.A.

Abstract

LRRK2 is phosphorylated on Serines 910, 935, 955 and 973, which has been shown to be likely regulated by cellular kinases. These sites are modified in an apparent feedback mechanism where LRRK2 kinase activity potentiates the kinase activity against these sites or perhaps negatively regulates the phosphatase of these sites (**Figure 1**). Acute inhibition of LRRK2 leads to rapid dephosphorylation of the cellular phosphosites. Inhibition of Serine 935 may indicate perturbation of the signaling pathway upstream or direct inhibition of LRRK2. Immunological detection of Serine 935 phosphorylation has been adapted to a homogenous TR-FRET based high-throughput cellular assay for LRRK2. This assay was applied to a small compound library to assess applicability of the assay to screening against a larger compound library. We identified ~20 compounds that led to the cellular dephosphorylation of LRRK2 Serine 935 in SH-SY5Y cells. We counter screened these hits against LRRK2 in vitro and indeed some of these compounds had direct inhibitory effects on LRRK2 and interestingly, others did not. We confirmed a selection of the cellular pathways indicated by these hits with further pharmacological and reverse genetic approaches. We provide evidence that cell based screening for phosphorylation of Serine 935 (and potentially other phosphosites) can yield novel therapeutic targets for Parkinson's disease as well as new potential direct inhibitors of LRRK2.

Figure 1. Schematic of LRRK2 regulation at Serines 910/935/955& 973

LRRK2 is found to be phosphorylated on Serines 910/935/955/973. This model shows that LRRK2 kinase activity potentiates a kinase activity towards these cellular phosphosites. Conversely, LRRK2 may negatively regulate a phosphatase that dephosphorylates LRRK2.

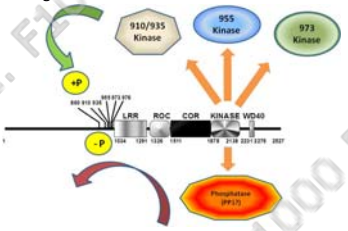
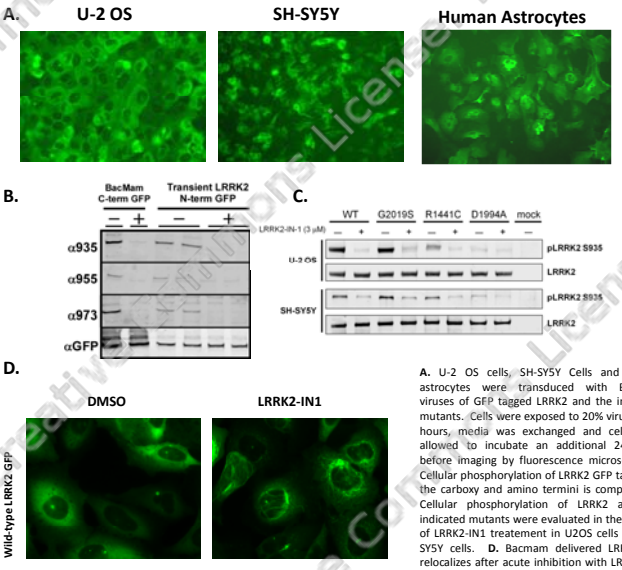
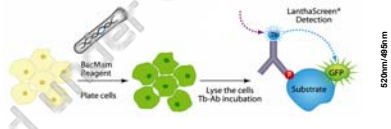


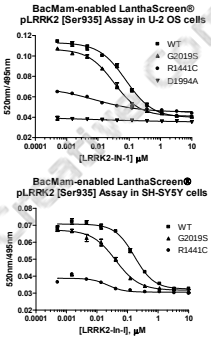
Figure 2. BacMam Enabled Expression of LRRK2 in multiple cell lines



A. U-2 OS cells, SH-SY5Y Cells and human astrocytes were transduced with BacMam viruses of GFP tagged LRRK2 and the indicated mutants. Cells were exposed to 20% virus for 24 hours, media was exchanged and cells were allowed to incubate an additional 24 hours before imaging by fluorescence microscopy. **B.** Cellular phosphorylation of LRRK2 GFP tagged at the carboxy and amino termini is compared. **C.** Cellular phosphorylation of LRRK2 and the indicated mutants were evaluated in the context of LRRK2-IN1 treatement in U2OS cells and SH-SY5Y cells. **D.** Bacmam delivered LRRK2-GFP relocalizes after acute inhibition with LRRK2-IN1 in U2OS cells.

Figure 3. HTS Amenable Cellular Assay for Monitoring the Phosphorylation of LRRK2 Ser935

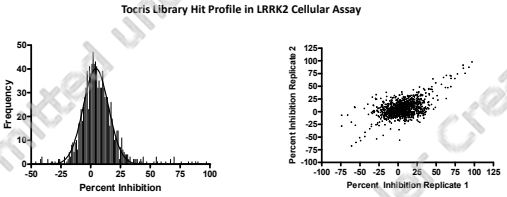




IC50 (μM)	U-2 OS		SHSY5Y		HEK293T	
Compound Name	WT	G2019S	WT	G2019S	WT	G2019S
LRRK2-IN-I	0.08	0.06	0.22	0.094	0.03	0.01
JAK3 Inh. VI	0.24	0.18	1.2	0.73	1.17	0.31
Indirubin-3-oxine	5.12	2.54	>10	3.8*	5.96	3.55
Sunitinib	0.27	0.15	0.23	0.11	0.60	0.48
GW5074	>20	0.03*	>20	>20	>20	0.28*
H-1152	2.80	0.85	1.7	0.62	3.88	1.43
H-89	>20	>20	>20	>20	nd	nd
Staurosporine	0.001	0.001	nd	nd	0.001	0.002

Using Time-Resolved Förster Resonance Energy Transfer (TR-FRET) technology, we developed a high-throughput compatible homogenous cellular assay for monitoring LRRK2 phosphorylation at Ser935. LRRK2-GFP fusion proteins were transiently expressed in a variety of cell backgrounds via BacMam gene delivery system, and were then plated in 384-well assay plates (Schematic in **A**). The phosphorylation at Ser935 in these cells was detected using a terbium labeled anti-Ser935 phosphorylation specific antibody that generates TR-FRET signals between terbium and GFP. **B**. A variety of LRRK2 expression constructs were evaluated in U2OS and SH-SY5Y cells against a titration of the selective LRRK2 inhibitor LRRK2-IN1. Consistent with previous reports and our western blot results, wild type and G2019S LRRK2 are constitutively phosphorylated at Ser935 in cells measured by TR-FRET. The phosphorylation level was reduced for R1441C mutant and little could be detected for the kinase activity-dead mutant D1994A*. **TABLE 1.** The TR-FRET cellular assay was further validated with reported LRRK2 inhibitors including LRRK2-IN1 in U2Os, SH-SY5Y cells and HEK293 cells demonstrating an applicability to multiple systems. Cellular IC50 values are reported for each inhibitor. nd=not determined.

Figure 4. Tocris Mini Library Screen Using LRRK2 pSer935 Cellular Assay



To demonstrate the utility of the LRRK2 cellular assay in HTS applications a compound screen was performed using the Tocris mini library – a collection of 1120 biologically active compounds. **Table 2.** Percent inhibition of some top hits from the screen with their biological activity shown and the maximal inhibition observed. Titrations of these compounds were evaluated to derive cellular IC50 values. Since 935 inhibition can be indicitive of direct inhibition of LRRK2, hese hits were tested in vitro against recombinant LRRK2 for inhibition (potent hits in Red).

% Inhibition (at 20 μM)	Compound Name	Known Compound Activity (Tocris description)	*In vivo* Cellular TR-FRET assay LRRK2 G2019S		Cytotoxicity Assay	*In vitro* Biochemical LRRK2 assay
			Max % Inhibition	IC_{50} (μM)	IC_{50} (μM)	IC_{50} (μM)
97.5	Bay 11-7085	Irreversible inhibitor of TNFa-induced IkB phosphorylation	96.6	8.3	>20	>100
85.5	Bay 11-7821	Irreversible inhibitor of TNFa-induced IkB phosphorylation	94.2	5.2	20	>100
81.7	IKK 16	Inhibitor of IKK	82.5	2.1	>20	0.75
69.8	Ro 106-9920	Inhibitor of NFkB activation	76.4	7.6	20	>100
54.8	TPCA-1	Inhibitor of IKK-2	46.2	9.2	>20	ND
76.1	NSC 95397	Selective Cdc25 dual specificity phosphatase inhibitor	95.9	2.2	7.8	1.9
67.3	PD 407824	Inhibitor of Chk1 and Wee1	65.3	0.6	15	0.69
59.1	NSC 663284	Cdc25 phosphatase inhibitor	45.6	4.9	2	0.13
55.3	SB 218078	Inhibitor of checkpoint kinase 1 (Chk1)	58.9	0.1	>20	6
88.3	SP 600125	JNK and other kinase inhibitor	103.1	0.9	>20	0.4
69.7	GW-441756	TrkA inhibitor	72.2	2.3	>20	0.65
67.3	Y-27632	p160ROCK inhibitor	68.8	14.5	>20	0.5
100	LRRK2 IN-1	Positive Control Compound	100.2	0.1	>20	0.06

Figure 5. Testing Library hits against LRRK2 and the Inhibitor desensitized mutant A2016T in HEK293 cells and against endogenous LRRK2

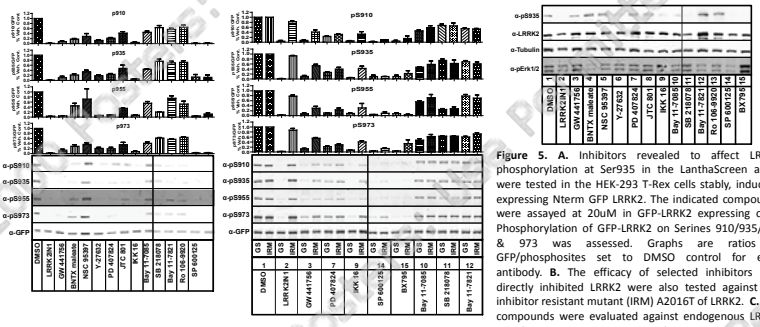


Figure 5. A. Inhibitors revealed to affect LRRK2 phosphorylation at Ser935 in the LanthaScreen assay were tested in the HEK-293 T-Rex cells stably, inducibly expressing Nterm GFP LRRK2. The indicated compounds were assayed at 20uM in GFP-LRRK2 expressing cells. Phosphorylation of GFP-LRRK2 on Serines 910/935/955 & 973 was assessed. Graphs are ratios of GFP/phosphosites set to DMSO control for each antibody. **B.** The efficacy of selected inhibitors that directly inhibited LRRK2 were also tested against the inhibitor resistant mutant (IRM) A2016T of LRRK2. **C.** The compounds were evaluated against endogenous LRRK2 in the mouse Raw Macrophages. GS=G2019S, IRM=G2019S/A2016T

Figure 6. In Vitro Evaluation of Kinase Activity against LRRK2 Ser910/935/973

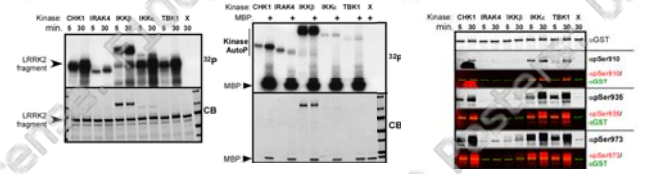


Figure 6. GST-LRRK2 (aa882-1300) **(A)** or MBP **(B)** were subjected to in vitro kinase assay with the indicated kinases in the presence of 32P-ATP. Reaction products were visualized by autoradiography and colloidal blue staining (A) and immunoblot with anti-GST (Green) and anti-phosphoserine 910, 935 & 973 antibodies (Red) (C). Similar units of kinase activity were used as assessed against the non specific substrate myelin basic protein (MBP) shown in B. AutoP=autophospohrylation

Figure 7. In Vivo Evaluation of Kinase Activity against LRRK2 Ser910/935/973

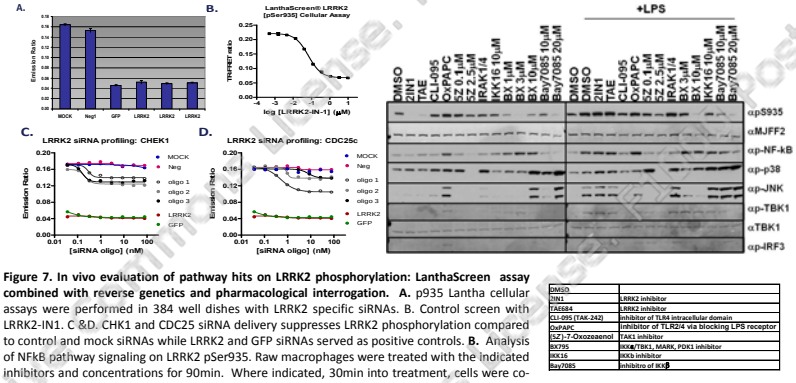


Figure 7. In vivo evaluation of pathway hits on LRRK2 phosphorylation: LanthaScreen assay combined with reverse genetics and pharmacological interrogation. A. p935 Lantha cellular assays were performed in 384 well dishes with LRRK2 specific siRNAs. B. Control screen with LRRK2-IN1. C &D. CHK1 and CDC25 siRNA delivery suppresses LRRK2 phosphorylation compared to control and mock siRNAs while LRRK2 and GFP siRNAs served as positive controls. **B.** Analysis of NFkB pathway signaling on LRRK2 pSer935. Raw macrophages were treated with the indicated inhibitors and concentrations for 90min. Where indicated, 30min into treatment, cells were co-treated with LPS treatment for 1hr.

DMSO	
2IN1	LRRK2 inhibitor
TAE684	LRRK2 inhibitor
CL-095 (TAK-242)	inhibitor of TLR4 intracellular domain
OxPAPC	inhibitor of TLR2/4 via blocking LPS receptor
SZ( )-7-Oxozeaenol	TAK1 inhibitor
BX795	IKKε/TBK1, MARK, PDK1 inhibitor
IKK16	IKKb inhibitor
Bay7085	inhibitro of IKKβ

•Conclusions:

- •BacMam gene delivery systems efficiently express LRRK2 in multiple cell lines.
- •TR-FRET based detection of phospho935 LRRK2 with a Terbium labeled antibody-GFP pair was successfully adapted to multiwell assays to create a LanthaScreen cellular assay for LRRK2
- •The LanthaScreen cellular assay is amenable to high throughput screening for direct inhibitors as well as for biological inputs.
- •Factors that activate NFκB also induce LRRK2 phosphorylation at Ser935, revealing that LRRK2 is not fully phosphorylated in these cells.
- •Repression of the CHK1 & CDC25 expression results in a decrease in LRRK2 phosphorylation.

This work was supported by LifeTechnologies™, and grants from the Michael J Fox Foundation and the benevolence of the Brin/Wojicki Foundation to RJN.