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Discovery of novel indolinone-based, potent, selective and brain penetrant inhibitors of LRRK2

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ABSTRACT

Mutations in leucine-rich repeat kinase-2 (LRRK2) are the most common genetic cause of Parkinson's disease (PD). The most frequent kinase-enhancing mutation is the G2019S residing in the kinase activation domain. This opens up a promising therapeutic avenue for drug discovery targeting the kinase activity of LRRK2 in PD. Several LRRK2 inhibitors have been reported to date. Here, we report a selective, brain penetrant LRRK2 inhibitor and demonstrate by a competition pulldown assay in vivo target engagement in mice.

Parkinson's disease (PD) is the second most common progressive neurodegenerative movement disorder affecting 1% of people over the age of 60. Currently, no disease-modifying therapies are available. Human genetics have identified manifold causes of PD that have opened up new possibilities for therapeutic targets. Mutations in leucine-rich repeat kinase-2 (LRRK2) cause late-onset autosomal dominant PD that is clinically indistinguishable from idiopathic PD and account for approx. 5% of familial and 1–2% of sporadic PD.[1–8]

LRRK2 is a large multi-domain protein containing several potential protein–protein interaction domains, as well as a GTPase and a serine/threonine kinase domain. The most prominent mutation associated with PD resides in the activation loop of the catalytic kinase domain and encodes for a glycine-to-serine substitution (G2019S). Several studies have shown that this mutation increases kinase activity[9,10] and induces toxicity,[11–13] hence opening the possibility for therapeutic kinase inhibition. Several putative substrates have been put forward,[14–17] but a robust physiological substrate for LRRK2 is still missing.[18–20] In addition, despite the genetic link for the causal role of LRRK2 mutations in late-onset PD the underlying patho-mechanism and molecular pathways are still uncertain and remain to be elucidated.[21] In the brain, LRRK2 expression is not restricted to the nigro-striatal pathway. Outside the nervous system, several peripheral organs and cell types express LRRK2 including kidney, lung and B cells suggesting that LRRK2 mutations may impact physiology and exert disease-relevant roles.[22–29]

Several phosphorylation sites in LRRK2 have been discovered and extensively analyzed, like the serine at position 935 and 910 before the LRR domain[30–37] and as well as others.[38,39] Importantly, all of these sites are sensitive to LRRK2 inhibitors and can thus be used to monitor LRRK2 activity. These discoveries are vital in drug discovery as they provide the basis for cellular assays and in vivo pharmacodynamics to find suitable candidates for further development. To date, several groups have reported potent and brain penetrant LRRK2 inhibitors.[30,39–44] Here, we report a LRRK2 inhibitor with high potency and good PK properties and demonstrate its target engagement in vivo.

The first low molecular weight compounds reported to inhibit LRRK2 kinase activity (mainly staurosporine-related compounds, maleimides or indolinones)[45,46] typically had low potency and selectivity, and were tool-like in structure. Improved second

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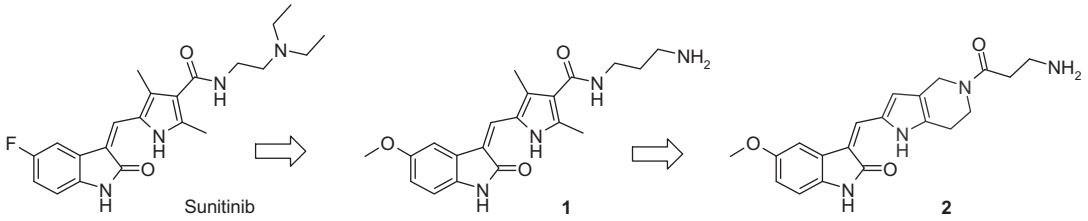


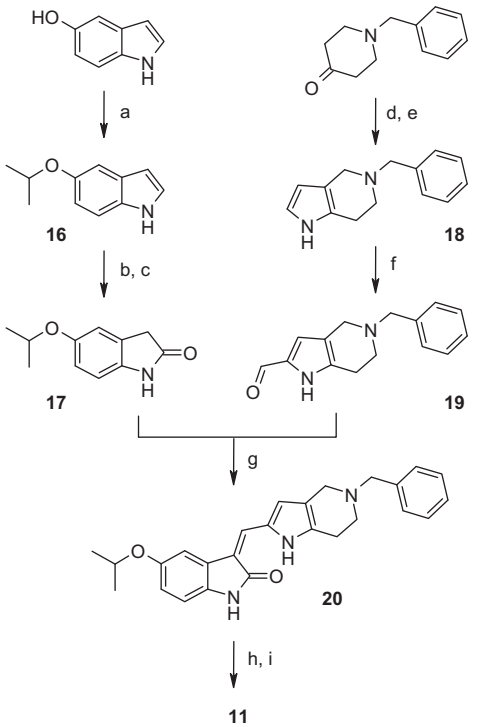
Figure 1. Discovery of 1,4,6,7-tetrahydro-pyrrolo[3,2-*c*]pyridine containing LRRK2 inhibitors.

generation inhibitors like the 2,4-diaminopyrimidines LRRK2-IN-1[47] or CZC-25146[48] showed excellent potency and selectivity for LRRK2, but lacked sufficient brain exposure to be used in rodent models of PD. In contrast to this, the dual ALK/LRRK2 inhibitor TAE684 has been shown to achieve significant mouse brain exposure, but failed to inhibit LRRK2 phosphorylation in the brain after oral dosing.[36] More recently, further optimized 2,4-diaminopyrimidines with improved brain penetration have been disclosed by several groups.[30,40,41,44] Some of them were shown to significantly reduce LRRK2 autophosphorylation in the brain of G2019S LRRK2 transgenic mice after oral dosing.[30,44] In addition to these 2,4-diaminopyrimidines based compounds, cinnolines,[42] triazolopyridazines[49] and 3-cyanoquinolines[43] have been published that potently inhibit wild-type and mutant LRRK2 in vitro.

Early on in our LRRK2 kinase inhibitors program, a medicinal chemistry effort was initiated with two main goals: (i) identification of a potent, reasonably selective LRRK2 kinase inhibitor with an attachment point (preferably a primary or secondary amine) remote from the kinase interaction part that allows for crosslinking to a solid support for pulldown experiments,[50] and (ii) identification of a proprietary starting point for a derivation program with the potential for high potency, selectivity and brain penetration. For the first goal, we decided to start from the indolinone Sunitinib, a broad-band kinase inhibitor that was found to also inhibit LRRK2[51] (Fig. 1). In our biochemical assay, Sunitinib inhibited LRRK2 kinase with an IC_{50} of 0.028 μM, and, as expected, was found to be highly unselective in a kinase selectivity panel (inhibition of 20 out of 54 kinases with an IC_{50} <1 μM). A limited derivation program around Sunitinib revealed that the ethylene linker at the amide can be extended in length, and the tertiary amine at its end be replaced with a primary amine without loss of activity in the biochemical assay. In addition, replacement of the fluorine in position 5 of the indolinone core with a methoxy considerably improved kinase selectivity, while retaining full activity on LRRK2. Hence, derivative **1** inhibited LRRK2 with an IC_{50} of 0.046 μM, and blocked only 5 out of 36 other kinases in the selectivity panel with an IC_{50} <1 μM. This cross-linkable compound could successfully be coupled to sepharose solid support (see Supplementary data), and turned out to be a highly versatile tool for pull-down experiments.

In order to obtain proprietary starting points for a drug development program, options were considered on how to morph the 2,4-dimethyl-3-carboxamide substituted pyrrole into a novel, hitherto unknown moiety. Docking studies of **1** in a LRRK2 homology model (vide infra) suggested that forming an additional ring between the 2-methyl group and the amide nitrogen, with concomitant inversion of the amide, should be tolerated by the kinase. Indeed, 4,5,6,7-tetrahydro-1*H*-pyrrolo[3,2-*c*]pyridine derivative **2**, the corresponding analog of **1**, showed an IC_{50} of 0.011 μM on LRRK2, nicely confirming this hypothesis. Although some of the kinase selectivity of **1** was lost by incorporation of this new moiety (20 out of 32 other kinases inhibited with IC_{50} <1 μM), it was decided, based on the high initial potency and the favorable IP position for this novel moiety, to initiate a derivation program around **2** with the main goals of improving kinase selectivity and demonstrating favorable PK properties, including brain penetration.

Derivatives **2**–**14** were prepared by condensation of an appropriately substituted indolinone (as exemplified by **17**) with the benzyl protected tetrahydropyrrolo-pyridine-2-carbaldehyde building block **19**, followed by debenzylation and introduction of the final substituent at the tetrahydropyridine nitrogen (e.g., acetyl for derivative **11**, Scheme 1). 5-substituted indolinones were either commercially available, or were prepared from the corresponding indole by a bromination/hydrolysis sequence (e.g., **16**–**17**, Scheme 1). Aldehyde building block **19** was prepared as described by Voskressensky et al.[52] from 1-benzyl-piperid-4-one oxime (KOH, acetylene gas, DMSO), followed by selective formylation in position 2 of the pyrrole derivative **18** ($POCl_3$, DMF/Et_2O) (Scheme 1). Derivative **7** bearing a chloro substituent in position 3 of the pyrrole moiety was prepared starting from **18** by de-benzylation (H_2, Pd/C), acetylation (AcCl, Et_3N, DCM), formylation in position 2 ($POCl_3$, DMF/Et_2O) and chlorination in position 3 (NCS, benzoylperoxide, CCl_4), followed by condensation with 5-methoxyindolinone (EtOH, cat. piperidine, reflux). Compound **15** (Fig. 2) carrying a methyl



Scheme 1. Synthesis of LRRK2 inhibitor **11**. Reagents and conditions: (a) *i*-PrOH, PPh_3, DIAD, THF, rt, 16 h (51%); (b) Br_2, DMF, 0°, 30′ (quant.); (c) phosphoric acid, $MeOCH_2CH_2OH$, 100°, 2 h (28%); (d) $NH_2OH{\cdot}HCl{\cdot}K_2CO_3$, EtOH, 80°, 1 h (98%); (e) acetylene gas, KOH, DMSO, 90°, 6 h (36%); (f) $POCl_3$, DMF, Et_2O, rt, 1.5 h (71%); (g) EtOH, cat. piperidine, 95°, 4 h (92%); (h) ammonium formate, Pd/C, MeOH, rflx, 2 h (97%); (i) ClCOMe, DIEA, DCM, rt, 1 h (75%).

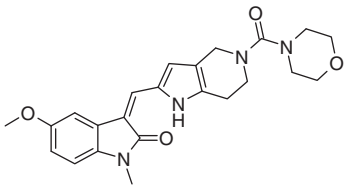


Figure 2. Structure of N-methylated indolinone derivative **15**.

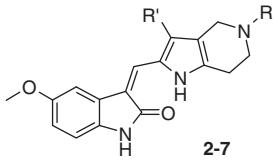
group at the indolinone nitrogen was obtained by methylation (MeI, Cs_2CO_3, DMF, rt, 16 h) of derivative **6**.

A considerable variety of substituents R1 was tolerated at the tetrahydropyridine nitrogen of the bicyclic pyrrole moiety (**3**–**6**, Table 1): Acetamide, an isoxazole amide as well as *N*-Me-piperazinyl and morpholinyl ureas all retained low nanomolar activity on LRRK2, without much influence on selectivity versus the main kinase off-targets. This tolerability for different functional groups turns this position into a convenient handle to adjust physicochemical parameters and PK properties. Acetamide **3** showed a somewhat improved selectivity as compared to **2** and **4**–**6**, especially over KDR, LCK and RET. Introduction of a chlorine atom on the pyrrole moiety (**7**) also fully retained LRRK2 potency, and knocked out activity on ALK.

A first small set of substituents R was introduced in position 5 of the indolinone core while keeping the morpholinyl urea of **2** on the pyrrole moiety constant (**8**–**10**, Table 2). All new derivatives showed very high LRRK2 activity. Substituted amines **9** and **10** turned out to be remarkably unselective inhibitors of a number of kinases, whereas the trifluoromethyl analog **8** showed some improved selectivity versus the methoxy analog **6**, especially versus ALK, KDR and RET.

In a second round of derivation in position 5, the acetamide group that was shown earlier to impart best selectivity (compound **3**) was kept constant at the pyrrole moiety (**11**–**14**, Table 3). Increasing the size of methoxy to isopropoxy (**11**) led to improved

Table 1
SAR at 1,4,6,7-tetrahydro-pyrrolo[3,2-*c*]pyridine moiety: Inhibition of LRRK2 and main off-target kinases for derivatives **2**–**7**



2-7

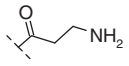
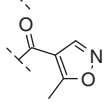
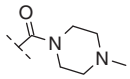
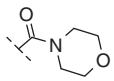
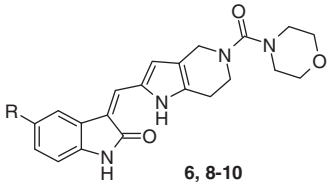
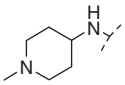
Cmpd	R	R′	IC_{50} [μM]					
			LRRK2	ALK	KDR	LCK	PDGFRa	RET
2		H	0.011	0.37	0.008	0.14	0.12	0.004
3		H	0.014	1.5	0.21	6.05	0.25	0.054
4		H	0.007	1.4	0.016	0.39	0.54	0.075
5		H	0.009	0.55	0.006	0.05	0.06	0.006
6		H	0.003	0.66	0.005	0.12	0.15	0.025
7		Cl	0.008	>10	0.087	1.1	—	0.034

Table 2
SAR (I) at position 5 of indolinone moiety: Inhibition of LRRK2 and main off-target kinases for derivatives **6**, **8**–**10**



6, 8-10

Cmpd	R	IC_{50} [μM]					
		LRRK2	ALK	KDR	LCK	PDGFRa	RET
6		0.003	0.66	0.005	0.12	0.15	0.025
8		<0.003	>10	0.083	0.18	0.046	0.14
9		<0.003	0.076	0.003	0.007	0.013	0.005
10		0.004	0.21	0.007	0.33	0.091	0.025

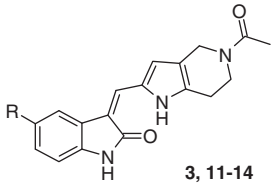
LRRK2 activity as well as better selectivity against ALK, KDR, PDGFRa and RET. Introduction of more electron withdrawing groups (–CF_3, –CN, –NO_2) in this position was well tolerated by LRRK2, but did not lead to more selective derivatives (**12**–**14**).

Methylation of the indolinone nitrogen led to a 50-fold drop in LRRK2 kinase activity (**15**: IC_{50} LRRK2 = 0.15 μM, Fig. 2), thus confirming the expected binding mode that postulates a crucial interaction of the indolinone lactam with the kinase hinge (vide infra).

Several homology models of LRRK2 have recently been disclosed.[36,40,43,49,51,53] Chen et al.[40] discussed the different factors that can influence the choice of structural template for homology model building. Psi-blast[54] search results indicate that MLK1, TIE2, BRAF and TAK1 have the highest sequence identity to LRRK2 (29–32%). They rejected models based on MLK1, TIE2, and TAK1, but instead chose JAK2 as a suitable template based on biochemical activity profiles of LRRK2 inhibitors. Liu et al.[53] used B-raf kinase as their template, whereas in the Franzini et al.[49] and Garofalo et al.[43] publications the homology model is based on MLK1. Zhang et al.[36] used anaplastic lymphoma kinase (ALK) to construct their homology model, whilst Nichols et al.[51] used ROCK1. Our LRRK2 homology model was constructed with IRAK4 which in the kinase domain has 26% sequence identity, and 46% similar residues.

We used our homology model to manually dock chemical classes of interest, one of these being the indolinone series. The postulated binding mode of compound **11** is shown in Figure 3. Hydrogen bonds are made from the indolinone lactam to the hinge region, and the carbonyl of the tetrahydropyridine amide interacts with R1957, a residue identified by Chen et al.[40] to be one of the four least conserved residues in potential contact distance with ATP-competitive inhibitors. Of the other mentioned residues, S1954 is in close proximity, but not in vdW contact with the inhibitor, and according to our model F1883 is relatively distant from the inhibitor. The closest contact between L1949 and our inhibitor is 4.2 Å. This residue is predominantly a Phe or Tyr side-chain in other kinases[55,56] and its' small size has been exploited by other chemotypes to achieve significant selectivity against a range of kinases. However, for this indolinone series though the potency is impressive, the kinase selectivity profile is less so. The selectivity against RET is particularly poor, and this is unsurprising based on the crystal structure that exists for the indolinone core structure co-crystallized with RET-kinase (PDB ID 2X2M).[57] The binding

Table 3
SAR (II) at position 5 of indolinone moiety: Inhibition of LRRK2 and main off-target kinases for derivatives **3**, **11**–**14**



3, 11-14

Cmpd	R	IC_{50} [μM]					
		LRRK2	ALK	KDR	LCK	PDGFRa	RET
3		0.014	1.5	0.21	6.05	0.25	0.054
11		0.004	3.3	0.9	1.1	1.7	0.15
12		0.031	9.5	0.32	0.36	0.06	0.3
13		0.007	1.6	0.02	0.12	<0.003	0.004
14		<0.003	>10	0.06	1	0.031	0.073

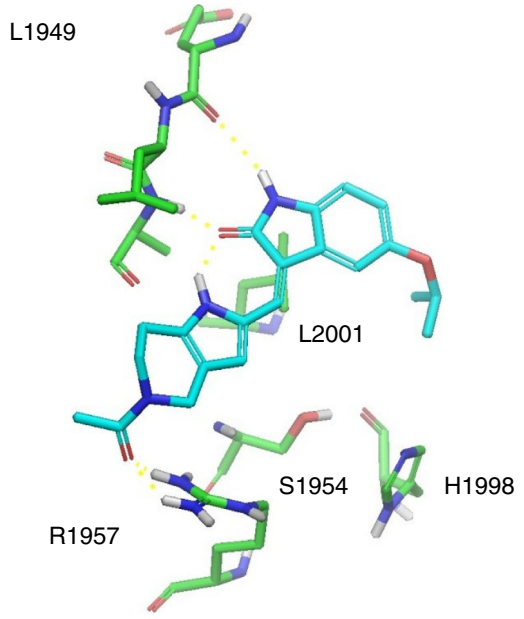


Figure 3. Putative binding mode of compound **11** in LRRK2 homology model. Hydrogen bonds are shown as dashed yellow lines. The image was made using PyMOL (The PyMOL Molecular Graphics System, Version 1.2.r3pre Schrödinger, LLC).

mode is exactly as modeled for our inhibitors. We do however show that with an optimized substituent in position 5 of the indolinone, the selectivity window can be widened.

H1998 makes favorable hydrophobic interactions with the O–iPr group of compound **11**, likely contributing to its better affinity as compared to compound **3**. In contrast, H1998 is an arginine in RET-kinase, pointing away from the binding site and not in contact with the inhibitor. S891 (RET-kinase) is an alanine (A2016) in LRRK2. This more extended side-chain probably interacts unfavorably with the O–iPr moiety of compound **11** in RET-kinase, leading to a reduction of binding affinity of this inhibitor, as compared to compound **3**. The nitro group of compound **14** forms a hydrogen bond with the catalytic lysine in both LRRK2 and RET-kinase. Our tentative interpretation of the data and our modeling is that this salt bridge is likely to be at the expense of unfavorable electrostatic interactions with D892 in RET-kinase, but not so in the case of LRRK2. Clearly, the trifluoro methyl group (**12**) is well tolerated by LRRK2. It is in the vicinity of the catalytic lysine (K1906), and $-CF_3$ is often used as a mimic for electronegative groups. However, in RET-kinase, as was the case for the nitro group (**14**), unfavorable electrostatic interactions are penalized. These assertions are consistent with those of Mologni et al. They performed structure–activity relationship and crystallographic studies on 3-substituted indolin-2-one RET inhibitors and concluded that bulky substituents or electronegative groups are not favored in this position.

Sun et al.[58] have evaluated 3-substituted indolin-2-ones against a number of receptor tyrosine kinases, and have also found that C-5 substituents with different steric and electronic properties can be used to modulate binding affinity.

Two derivatives were selected for a preliminary assessment of pharmacokinetic (PK) properties in mice, namely the *N*-methyl piperazine derivative **5** as its solubility is acceptable (15 mg/L at pH 6.8), and the acetyl derivative **11** (solubility <1 mg/L at pH 6.8) as the most kinase selective compound (Table 4). In a mouse PK cassette study, both compounds showed moderate to good absolute oral bioavailability, and good exposures to blood and brain. The total blood clearance was lower for **5** than for **11** (blood CL of 28 and 50 mL/min/kg, respectively). Overall, both compounds showed attractive PK properties in mice. Based on its higher solubility, and more favorable bioavailability and blood clearance, piperazine **5** was chosen for further assessment in animal studies (this study and Herzig et al.[24]).

To measure binding of piperazine derivative **5** in vitro we performed competition pulldown experiments (Fig. 4). For this, we used indolinone derivative **1** that was cross-linked to sepharose solid support that could compete with unlabeled **5** with the binding to LRRK2 in vitro (Fig. 4A). For these in vitro pulldown assays, mouse brain extracts from C57BL/6 animals were mixed with either **5** or negative control compound **21** (structure not shown), the latter being a LRRK2 inhibitor from a different chemical class, with an enzymatic IC_{50} on LRRK2 comparable to **5**, but with very poor cell and brain penetration properties. Both compounds showed substantial binding to LRRK2 at 1 μM and higher. The low cell penetration of **21** could be observed in ex cell pulldown experiments, where C2C12 cells were incubated either with **5** or **21** and subsequently were subjected to the competition pulldown assay (Fig. 4A). There again a significant binding of **5** at 1 μM and higher could be observed whereas for **21** no binding was obvious as the compound did not penetrate the cells. To show target engagement in vivo we treated C57BL/6 mice with both compounds (Fig. 4B), using p.o. application for **5** at different doses and i.v. application for **21** (the i.v. route had to be chosen for **21** due to the physicochemical properties of this particular compound). A clear dose-dependent in vivo brain binding to LRRK2 of **5** was observed. A moderate binding at the dose of 10 mg/kg

Table 4
Brain penetration and PK properties of indolinones **5** and **11** in mice

		5	**11**
p.o. Dose 3 mg/kg	C_{max} Blood@1 h (pmol/mL)	410 ± 122	854 ± 366
	C_{max} Brain@1 h (pmol/g)	734 ± 195	1839 ± 655
	AUC_{inf} blood (pmol h/mL)	3744	1567
	F (%)	88	57
i.v. Dose 1 mg/kg	Apparent terminal $t_{1/2}$ (h)	2.2	0.4
	Total blood CL (mL/min/kg)	28	50
	V_{dss} (L/kg)	4.9	1.7

Cassette dosing (6 compounds in total) to male C57BL/6 mice, *n* = 3/time point (see method paragraph).

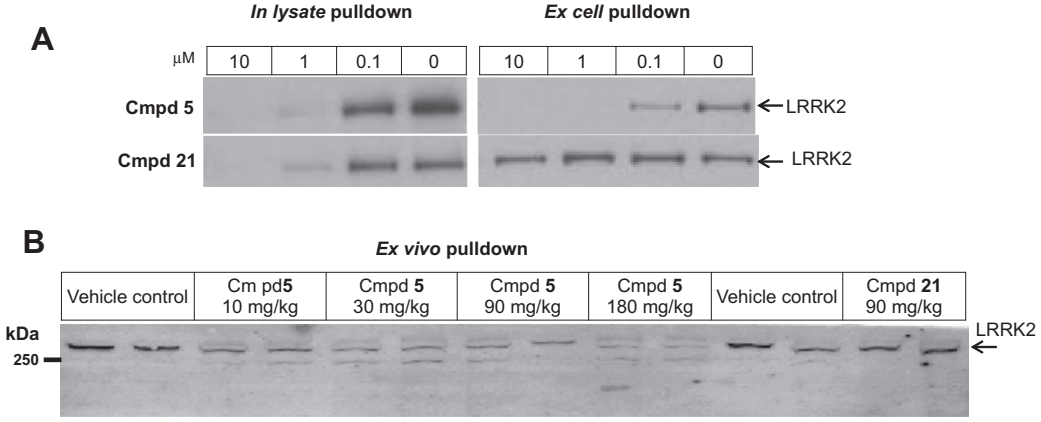


Figure 4. LRRK2 pulldown experiments with compounds **5** and **21**. (A) LRRK2 pulldown in lysates from naive mouse brains and in C2C12 cells after compound treatment; (B) LRRK2 pulldown from mouse brains after compound treatment at different doses as indicated.

was visible and was maximal at the highest tested dose of 180 mg/kg compared to vehicle treated animals. **21** on the other hand showed no binding to LRRK2 in the brain as expected, as it does not cross the blood brain barrier. Thus, this ex vivo competition pulldown assay can be used to demonstrate target engagement of LRRK2 in vivo in the mouse brain.

In summary, we have identified novel, highly potent, reasonably selective inhibitors of LRRK2 kinase with promising pharmacokinetic properties in mice. Target engagement of one of these derivatives in the mouse brain could be demonstrated using an ex vivo competition pulldown assay.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2013.05.054.

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