

## *Supporting Information.*

### **A multicomponent pharmacophore fragment-decoration approach to identify selective LRRK2-targeting probes**

Sabrina Tassini,<sup>a</sup> Daniele Castagnolo,<sup>bc</sup> Nicolò Scalacci,<sup>bc</sup> Miroslava Kissova,<sup>d</sup> Jorge I. Armijos-Rivera,<sup>d</sup> Federica Giagnorio,<sup>ac</sup> Giovanni Maga,<sup>d</sup> Gabriele Costantino,<sup>a</sup> Emmanuele Crespan,<sup>d†</sup> and Marco Radi<sup>\*a†</sup>

<sup>a</sup>P4T Group, Dipartimento di Farmacia, Università degli Studi di Parma, Viale delle Scienze, 27/A, 43124 Parma, Italy. E-mail: marco.radi@unipr.it. <sup>b</sup>Institute of Pharmaceutical Science, King's College London, 150 Stamford Street, SE1 9NH London, United Kingdom. <sup>c</sup>Northumbria University Newcastle, Department of Applied Sciences, Ellison Building, Ellison Place, NE1 8ST Newcastle upon Tyne, United Kingdom. <sup>d</sup>Istituto di Genetica Molecolare, IGM-CNR, Via Abbiategrasso 207, 27100 Pavia, Italy  
† Co-last authors

#### **Table of Contents:**

<b>CHEMISTRY</b> .....	S2
<b>BIOLOGY</b> .....	S4

## CHEMISTRY:

### Synthetic procedures

**General procedure for the three component synthesis of hetheroaryl-hydrazones (9a,b).** In a microwave tube to a stirred solution of 2-Chloroquinoline **7a** (0.10 g, 0.61 mmol) in EtOH (1 mL), hydrazine monohydrate (35.6  $\mu$ L, 0.73 mmol) and 3'-(Trifluoromethyl)acetophenone **8a** (111  $\mu$ L, 0.73 mmol, for **9a**) or benzophenone **8b** (133 mg, 0.73 mmol, for **9b**) or were added and the mixture was heated at 120 °C for 15 minutes (maximum power input: 300 W; maximum pressure: 250 PSI; power max: OFF; stirring: ON). After cooling to room temperature, the solvent of reaction was evaporated under reduced pressure and the residue was purified by silica gel flash chromatography (petroleum ether/ethyl acetate, from 8/2 to 7/3), to afford pure compounds as yellow solids.

**(E)-2-(2-(1-(3-(Trifluoromethyl)phenyl)ethylidene)hydrazinyl)quinoline (9a).** (62% yield).  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$  8.68 (bs, 1H), 8.11-8.10 (m, 2H), 8.02 (d,  $J = 7.9$  Hz, 1H), 7.78-7.75 (m, 4H), 7.63 (d,  $J = 7.7$  Hz, 1H), 7.55 (t,  $J = 7.8$  Hz, 1H), 7.36 (s, 1H), 2.38 (s, 3H).  $^{13}\text{C}$  NMR (100.6 MHz,  $\text{CDCl}_3$ )  $\delta$  157.7, 147.1, 141.6, 139.4, 138.5, 130.6, 130.0, 128.8, 127.8, 126.3, 126.0, 125.1, 124.8, 124.6, 123.4, 122.4, 109.9, 29.3. MS (ESI)  $m/z$  330.2  $[\text{M} + \text{H}]^+$ , 352.3  $[\text{M} + \text{Na}]^+$ .

**2-(2-(Diphenylmethylene)hydrazinyl)quinoline (9b).** (55% yield). The characterisation was in line with the same product obtained with our optimized one-pot two-step protocol.

**General procedure for the first step optimization: synthesis of hydrazones (10b-d).** Benzophenone **8b** (100 mg, 0.55 mmol, for **10b**) or 3'-Nitroacetophenone **8c** (91 mg, 0.55 mmol, for **10c**) or 4-Methoxybenzaldehyde **8d** (66.9  $\mu$ L, 0.55 mmol, for **10d**), hydrazine monohydrate (26.7  $\mu$ L, 0.55 mmol), L-proline (13 mg, 0.11 mmol) and 1 mL of anhydrous toluene were placed in a dried 10 mL microwave tube, equipped with magnetic stir bar and septum, and the colourless mixture was irradiated at 300 Watt for 15 minutes in the microwave apparatus (maximum pressure: 250 PSI; maximum temperature: 200 °C; power max: OFF; stirring: ON). After cooling to room temperature, water was added, followed by extraction with ethyl acetate. The collected organic phases were washed with brine, dried over  $\text{Na}_2\text{SO}_4$  and evaporated under vacuum. The residue was purified by silica gel flash chromatography (dichloromethane/methanol from 97/3 to 98/2) to give the desired compounds (**10b-d**) as yellow solids.

**(Diphenylmethylene)hydrazine (10b).** (91% yield).  $^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ )  $\delta$  7.68-7.62 (m, 4H), 7.42-7.23 (m, 6H), 5.55 (s, 2H). MS (ESI)  $m/z$  197.2  $[\text{M} + \text{H}]^+$ , 219.3  $[\text{M} + \text{Na}]^+$ .

**(E)-(1-(3-nitrophenyl)ethylidene)hydrazine (10c).** (89% yield). <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>) δ 8.72 (s, 1H), 8.42 (m, 1H), 8.26 (m, 1H), 7.67 (t, *J* = 8.0 Hz, 1H), 5.53 (s, 2H), 2.67 (s, 3H). MS (ESI) *m/z* 180.2 [M + H]<sup>+</sup>, 202.3 [M + Na]<sup>+</sup>.

**(E)-(4-methoxybenzylidene)hydrazine (10d).** (85% yield). <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>) δ 8.05 (s, 1H), 7.65 (d, *J* = 8.4 Hz, 2H), 6.99 (d, *J* = 8.6 Hz, 2H), 5.49 (s, 2H), 3.81 (s, 3H). MS (ESI) *m/z* 151.2 [M + H]<sup>+</sup>, 173.2 [M + Na]<sup>+</sup>.

**General procedure for the second step optimization: synthesis of heteroaryl-hydrazones (9b-d).**

(Diphenylmethylene)hydrazine **10b** (143 mg, 0.73 mmol, for **9b**) or (E)-(1-(3-nitrophenyl)ethylidene)hydrazine **10c** (131 mg, 0.73 mmol, for **9c**) or (E)-(4-methoxybenzylidene)hydrazine **10d** (110 mg, 0.73 mmol, for **9d**), 2-Chloroquinoline **7a** (100 mg, 0.61 mmol, for **9b,c**) or 2-Chloro-4-methylquinoline **7b** (108 mg, 0.61 mmol, for **9d**), *t*-BuONa (94 mg, 0.98 mmol) and 1.0 mL of anhydrous toluene were added in a dried 10 mL microwave tube, equipped with magnetic stir bar and septum and flushed with Argon for 1 minute. Then, 1 mL of a stock solution of catalyst<sup>1</sup> was added and the resulting mixture was stirred and flushed with Argon for an additional 2 minutes. Next, the tube was heated by microwave irradiation at 150 °C for 10 minutes (maximum power input: 300 W; maximum pressure: 250 PSI; power max: OFF; stirring: ON). After cooling to room temperature the dark red reaction mixture was filtered over Celite and the filtrate was evaporated under reduced pressure. The residue was purified by silica gel flash chromatography (petroleum ether/ethyl acetate, from 9/1 to 8/2), affording heteroaryl-hydrazones (**9b-d**) as yellow solids.

<sup>1</sup> The stock solution of catalyst was prepared using Pd(OAc)<sub>2</sub> (27.0 mg, 0.12 mmol), DavePhos (96 mg, 0.24 mmol) and anhydrous toluene (10 mL) and stored under an Ar atmosphere.

**2-(2-(Diphenylmethylene)hydrazinyl)quinoline (9b).** (69% yield). The characterisation was in line with the same product obtained with our optimized one-pot two-step protocol.

**(E)-2-(2-(1-(3-Nitrophenyl)ethylidene)hydrazinyl)quinolone (9c).** (51% yield). The characterisation was in line with the same product obtained with our optimized one-pot two-step protocol.

**(E)-2-(2-(4-Methoxybenzylidene)hydrazinyl)-4-methylquinoline (9d).** (57% yield). The characterisation was in line with the same product obtained with our optimized one-pot two-step protocol.

## BIOLOGY:

### *In vitro* LRRK2 inhibition assay

***In vitro* LRRK2 inhibition assay.** Recombinant LRRK2 wt (968-end) was purchased from Signalchem, recombinant G2019S mutant (970-end) was from Life Technologies. Assay conditions: LRRK2 reaction were performed in 10 mM MOPS/NaOH pH 7.2, 0.8 mM EDTA, 2mM EGTA, 50 ng/μl BSA, 0,0013% NP40, 3 μM Na-orthovanadate, 10% DMSO, 10 mM MgCl<sub>2</sub>, 12.5 μM ATP/[g-33P]ATP, 100 μM LRRKtide (Signalchem) RLGRDKYKTLRQIRQ, 10ng active enzyme. To avoid plastic adsorption of enzymes and peptide, all reactions were performed using protein low-binding tubes (LoBind, Eppendorf). All reaction were performed in 10 μl at 30 °C for 10 min. Reactions were stopped by adding 5μl of phosphoric acid 0.8%. Aliquots (10 μL) were then transfer into a P30 Filtermat (PerkinElmer), washed five times with 75 mM phosphoric acid and once with acetone for 5 min. The filter was dried and transferred to a sealable plastic bag, and scintillation cocktail (4 mL) was added. Spotted reactions were read in a scintillation counter (Trilux, Perkinelmer). Data were plotted using GraphPad Prism 5.0. ID<sub>50</sub> values were obtained according to Equation (1), where  $v$  is the measured reaction velocity,  $V$  is the apparent maximal velocity in the absence of inhibitor,  $I$  is the inhibitor concentration, and ID<sub>50</sub> is the 50% inhibitory dose.

$$v = V / \{1 + (I / ID_{50})\} \quad (1)$$

**Inhibition mechanism.** G2019S kinase activity assays were performed in the presence of different fixed amounts of inhibitor with varying ATP substrate concentrations. Data were plotted using GraphPad Prism 5.0. Data were analyzed according to Michaelis–Menten equation and  $K_{m_{app}}$  and  $V_{max_{app}}$  were derived.  $K_i'$  and  $K_i''$  values were determined using equation (2) and (3) respectively.

$$K_{m_{app}} = K_m (1 + [I] / K_i') \quad (2)$$

$$V_{max_{app}} = V_{max} / (1 + [I] / K_i'') \quad (3)$$

All experiments were repeated three times

### Kinase Panel

All Tyrosine- and Serine/Threonine kinases reaction were performed according to manufacturer's instructions, using 10-50 ng of enzyme. The nature of the substrates and their concentration are reported in Table S1. For some kinases, NP-40 or BSA was added. All reactions were performed in 10 μl at 30 °C for 10 min using protein low-binding tubes. Reactions were stopped, transferred to

filter and counted as in LRRK2 *in vitro* assay. PI4K $\beta$  was purchased from Proqinase. Reaction was performed according to manufacturer's instruction and detected using ADP-Glo™ Lipid Kinase Assay (Promega).

**Table S1. Kinase panel. Additional reaction condition and substrates.**

Kinase	Peptide substrate	Peptide substrate [ $\mu$ M]	ATP [ $\mu$ M]	NP-40 %	BSA [mg/ml]
Src (Millipore)	KVEKIGEGTYGVVYK	250	100	0.00087	
GSK3 $\beta$ (Signalchem)	GSK3 Substrate (Signalchem)	4	1	0.0013	
Hck (Proqinase)	KVEKIGEGTYGVVYK	50	2		
FAK (Proqinase)	KVEKIGEGTYGVVYK	50	1	0.0013	
DYRK1A (Millipore)	KKISGRLSPIMTEQ-NH2	50	170		
ABL (Promega)	KKGEAIYAAPFA-NH2	38	20	0.0013	
FLT3 (Proqinase)	KVEKIGEGTYGVVYK	50	2	0.013	
CDK2/CycA2 (Proqinase)	Histone H1	0.02mg/ml	2		1
CDK9/CycT1 (Proqinase)	CDKtide (Signalchem)	20	1		1
CDK9/CycK (Signalchem)	CDKtide (Signalchem)	20	1		1
CDK6/CycD1 (Signalchem)	RB-CTF (Proqinase)	1.22	1		1
CDK4/CycD1 (Signalchem)	RB-CTF (Proqinase)	1.525	1		1
Pim1 (Millipore)	S6 Kinase/Rsk2 Substrate Peptide 2 (Millipore)	50	1	0.013	
Pi4K $\beta$ (Proqinase)	PI:PS (1:3)	200	20		