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CONCISE ARTICLE

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

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Herein we report the development of a new versatile chemical tool for the rapid identification of LRRK2-targeting probes as potential anti-Parkinson's agents. Based on the structure of recently identified inhibitors, we decided to develop a new multicomponent approach to explore the biologically relevant space around their key pharmacophore-fragment. The combination of organo/metal catalysis and microwave assisted technology, allowed to quickly generate highly functionalized heteroaryl-hydrazone derivatives for biological investigation. Enzymatic studies on the synthesized compounds allowed to identify promising compounds endowed with a good LRRK2 specificity index (wt/G2019S activity ratio), low affinity towards a small panel of selected kinases and a mixed type inhibition against the pathogenic G2019S mutant. These results show how a diversity-oriented approach based on a privileged pharmacophore fragment may play a key role in the identification of novel biologically relevant chemical probes.

The discovery of new first-in-class medicines to improve current treatment regimens or to treat unmet medical needs is one of the most challenging tasks for a Medicinal Chemist, both in Academia and Big Pharma. Despite the recent advancements in molecular biology and gene-to-medicine approaches, 37% of new small molecule first-in-class drugs approved in the last 10 years came from phenotypic approaches while only 23% were discovered by hypothesis driven target-based approaches.[1,2] In this scenario, repositioning the classical chemistry-based phenotypic approaches at the center of an integrated multidisciplinary drug-discovery platform could provide an important contribution to the discovery of new medicines. The recently launched Open Antimicrobial Drug Discovery initiative is an important example of how chemical diversity is currently considered as a key resource to discover new antibiotics.[3] The availability of versatile chemical approaches to rapidly produce highly functionalized molecules represent therefore an essential medicinal chemistry tool for different drug-discovery campaigns: diversity-oriented synthesis (DOS), combinatorial chemistry and biology-oriented synthesis (BIOS) are widely used to explore the biologically relevant chemical space.[4-6] In particular, exploring the chemical space around a privileged scaffold (privileged-substructure-based diversity-oriented synthesis; pDOS) has been considered as a suitable strategy for the systematic enhancement of molecular diversity to discover new probes for chemical biology and drug discovery.[7] In analogy with the pDOS approach and as a continuation of our interest in the development of microwave-assisted and multicomponent strategies for the synthesis of new chemical probes,[8-12] we decided to build a fast and versatile diversity-oriented synthesis around a key pharmacophore fragment. This pharmacophore fragment-decoration approach could be considered as a good compromise between a target-based and a phenotypic drug discovery approach. Specifically, we became interested in the biological versatility of the heteroaryl-hydrazone pharmacophore moiety and in its potential for the discovery of new anti-Parkinson agents.[13] Among heteroaryl-hydrazones, 2-quinoline-derivatives have been studied as antineoplastic drugs (**1**, **2**, Figure 1),[14,15] while 2-pyridine derivatives displayed interesting anti-inflammatory (**3**, Figure 1), anticancer (**4**, Figure 1) and broad-spectrum antimicrobial activities (**5**, Figure 1).[16-18] Interestingly, a recent study identified (E)-2-(2-arylidenehydrazinyl)quinolone derivatives (**6**, Figure 1) as selective inhibitors of leucine-rich repeat kinase 2 (LRRK2) that has been associated with both autosomal-dominant and late-onset sporadic Parkinson's disease (PD) cases.[19] Inhibition of LRRK2 kinase activity, and in particular of the pathogenic mutant G2019S, has been proposed as an attractive therapeutic strategy for the treatment of PD.[20,21] Despite a few LRRK2 inhibitors have been published in the last years,[22-30] to the best of our knowledge, only a few reports have been published on the selective inhibition of the G2019S mutant over wild type LRRK2.[31-33] Considering that the physiological and pathological functions of this kinase remain poorly understood, it would be desirable to identify novel chemical probes able to selectively

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Electronic Supplementary Information (ESI) available: [experimental procedures and analytical data for compounds **9** and **10**, in vitro LRK2 inhibition assays, inhibition mechanism and kinase panel data]. See DOI: 10.1039/x0xx00000x

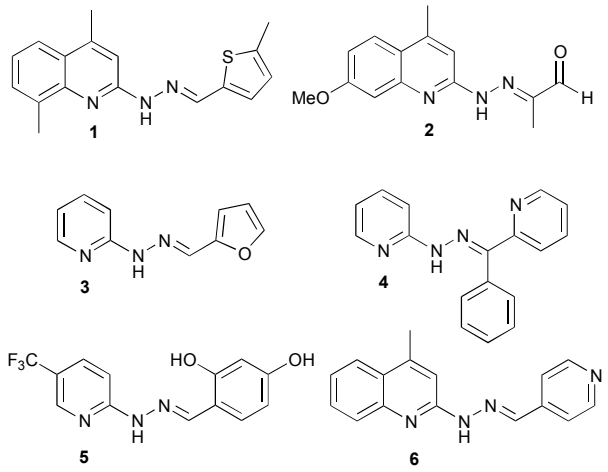
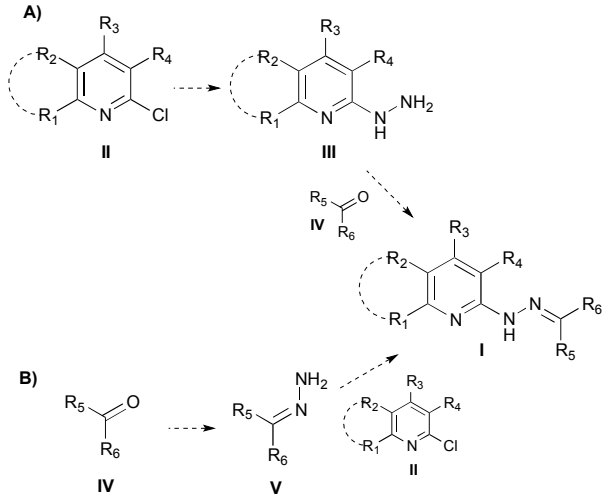


Figure 1. Representative biologically relevant hetroaryl-hydrazones **1-6**.

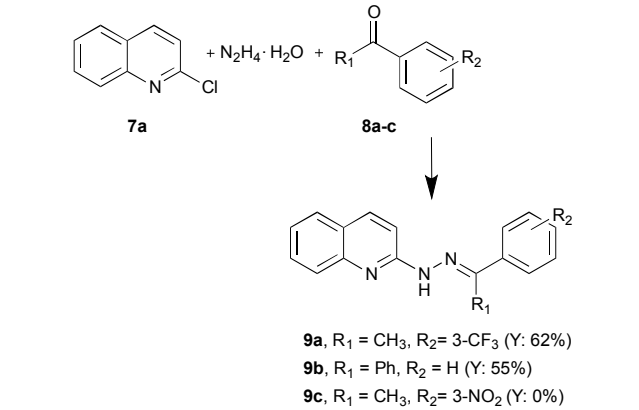
inhibit the pathogenic G2019S LRRK2 mutant. Herein we report the development of a microwave-assisted proline/Pd catalysed multicomponent approach for the rapid synthesis of substituted heteroaryl-hydrazones and their biological evaluation as LRRK2 inhibitors, both WT and G2019S mutant. The classical approach for the synthesis of substituted heteroaryl-hydrazones **I**, requires the conversion of heteroaryl halides **II** into hydrazino derivatives **III** by heating in an excess of hydrazine hydrate (approach **A**, Scheme 1).[34] Heteroaryl-hydrazones are finally obtained by coupling hydrazino derivatives **III** with the appropriate aldehydes or ketones **IV** in refluxing ethanol using acetic acid as catalyst.[35,36] Alternatively, the less explored approach **B** (Scheme 1) requires the preliminary conversion of aldehydes or ketones **IV** into the corresponding hydrazones **V** followed by Pd-catalyzed coupling with heteroaryl halides **II** to give the desired heteroaryl-hydrazones **I**. Only a few examples on the application of the latter approach have been reported in the literature while, to the best of our knowledge, no multicomponent strategies for the synthesis of heteroaryl-hydrazones **I** have been reported so far.[37-39]



Scheme 1. Approaches for the synthesis of heteroaryl-hydrazones **I**.

Multicomponent approaches display the advantage of atom- and cost-efficiency that, coupled with microwave irradiation, could permit to quickly generate compound libraries with high chemical variability for drug discovery purposes.[40] A new microwave-assisted multicomponent approach for the direct synthesis of substituted heteroaryl-hydrazones **I** could therefore represent a valuable tool to further explore the structure-activity-relationships of quinolone-based LRRK2 inhibitors and to discover new active scaffolds.

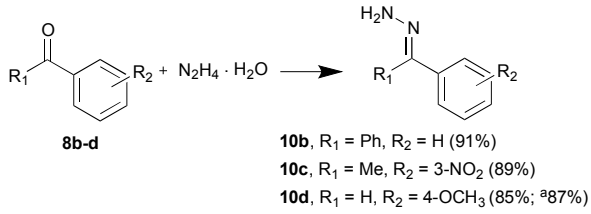
We initially focused our attention on the development of a single-step multicomponent procedure following the synthetic approach **A**: a mixture of 2-chloroquinoline **7a**, hydrazine monohydrate and ketones **8a-c** were irradiated in a sealed microwave tube at 120 °C (Scheme 2). Compounds **9a,b** were thus obtained in good yields after 15 min while compound **9c** was never obtained even under harsh reaction conditions. The same protocol also failed when we replaced the ketones **8a-c** with different aldehydes, giving a complex mixture of many side-products. Given the poor versatility of the above approach, we decided to investigate the possibility of developing a microwave-assisted multicomponent strategy based on the synthetic approach **B**. As far as we know, the microwave-accelerated catalytic amination of heteroaryl halides with hydrazone derivatives has never been addressed before and could be exploited to quickly generate highly functionalized heteroaryl-hydrazones starting from commercially available aldehydes/ketones, hydrazine and heteroaryl halides (see Scheme 5). We decided to optimize the first step of this new protocol in toluene because the following Pd-catalyzed amination of heteroaryl halides frequently requires this solvent.[41] Different temperatures, reaction times and catalysts (sodium tert-butoxide, acetic acid and L-proline) were used in order to improve the conversion of aldehydes or ketones **IV** into the corresponding hydrazones **V**. After several attempts, the best reaction conditions were found to be the irradiation of ketones with hydrazine monohydrate in a sealed microwave tube at 300 Watt for 15 minutes in the presence of a catalytic amount of L-proline to give the hydrazones **10b-d** in good yields (Scheme 3). For the second step of the protocol we extensively evaluated the Pd-catalyzed coupling of aryl halides with benzophenone hydrazone reported by different research groups.[42-44] In order to quickly obtain highly funzionalized heteroaryl-hydrazones our preliminary experiments



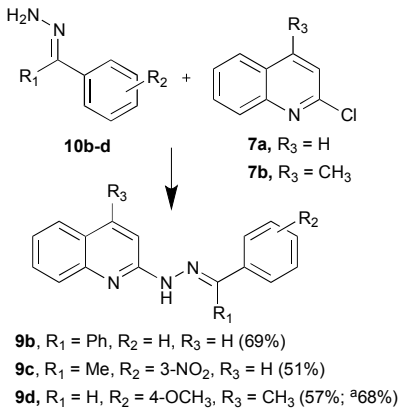
Scheme 2. Three component synthesis of heteroaryl-hydrazones **9a-c**. [Reaction conditions: EtOH, MW, 120 °C, 15 min.

were inspired by the works published by Maes *et al*., who developed a fast microwave-assisted Pd-catalyzed amination of aryl chlorides with aromatic and aliphatic amines.[45,46] After different pivotal studies to choose the best catalyst system, we selected 2-dicyclohexylphosphino-2'-(*N*,*N*-dimethylamino)biphenyl (DavePhos) as the ligand and $Pd(OAc)_2$ as the Pd(0) source. We applied the procedure proposed by Maes *et al.* to the coupling of hydrazones **10b-d** and 2-chloroquinolines **7a,b** using *t*-BuONa as base in combination with a solution of 2% $Pd(OAc)_2$/4% DavePhos in toluene. The substituted heteroaryl-hydrazones **9b-d** were obtained by microwave heating at 150 °C in only 10 minutes (Scheme 4). Finally we combined the optimized Step I and II in a single one-pot two-step protocol: this new organo/metal catalyzed strategy links the advantages of the multicomponent procedures with those of microwave-assisted reactions (Scheme 5). In the optimized protocol, a mixture of substituted aldehydes or ketones **8b-n** and hydrazine monohydrate in toluene was irradiated in a sealed microwave tube at 300 Watt for 15 minutes in the presence of a catalytic amount of L-proline. After that time, heteroaryl chlorides and *t*-BuONa were added to the same reaction vessel and the mixture was flushed under Argon atmosphere for few minutes before adding a toluene solution of 2% $Pd(OAc)_2$/4% DavePhos previously prepared. The desired heteroaryl-hydrazones **9b-n** were quickly obtained in moderate to good yields after microwave irradiation at 150-180 °C for 10-20 minutes (Table 1). To demonstrate the efficiency of our protocol, the synthesis of compound **9d** was also conducted step-by-step under conventional heating (CH) conditions: as reported in Schemes 3 and 4, the yield of each step is comparable with those obtained under microwave assisted conditions but, in the latter case, compounds **9d** was obtained in only 25 minutes (18 h under CH) after a single chromatographic purification. This protocol was efficiently applied to a series of cheap and commercially available building blocks, allowing us to easily decorate the aryl-hydrazone pharmacophore fragment. Different heteroaryl chlorides provided a different core scaffold to the final compounds: 2-chloroquinoline (compounds **9b,c,e,f,m,n**), 2-chloro-4-methylquinoline (compounds **9d,g**), substituted 2-chloropyridines (compounds **9h-j**) and substituted 2-chloro-5,6,7,8-tetrahydroquinoline (compounds **9k,l**). On the other hands, different aldehydes and ketones projected different functional groups in the surrounding space of the final compounds: substituted ketones (compounds **9b,c,e,f**) and benzaldehydes (compounds **9d,g-n**). Overall, the final compounds **9b-n** were obtained after only 25-35 min each followed by a single chromatographic purification, instead of multiple synthetic steps/purifications. In addition, aryl/heteroaryl-hydrazones can be also used as versatile key intermediates to increase their molecular complexity by easy conversion into different heterocycles, such as indoles, pyrazoles and triazolopyridines.[37-39] We attempt this additional molecular complication on compounds **9i**, since the chlorine in position C3 could be further displaced by different nucleophiles (Scheme 6). Copper-catalyzed oxidation[47] of **9i** yielded the corresponding [1,2,4]triazolo[4,3-*a*]pyridine intermediate **11**, which was then submitted to a microwave-assisted Buchwald amination with 4-(2-aminoethyl)morpholine to give the desired compound **12** in good overall yields. Finally, we also decided to modify one of the quinoline detivatives (**9g**) on the base of the docking studies reported for similar compounds (**6**) on a LRRK2 homology model.[19] Since the 4-pyridyl substituent of compound **6** was proposed to occupy a solvent exposed region just outside the ATP-binding pocket, we decided to attach a polar methylpiperazine moiety, common to different kinase inhibitors, to the hydroxyl group of compound **9g**. Reacting the latter compound with 4-(4-methylpiperazin-1-yl)benzoic acid under standard coupling conditions, afforded the desired derivative **13** in high yields, as described in Scheme 7.

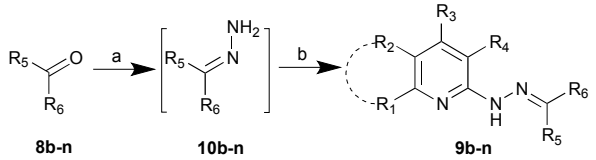
The inhibition potency of the synthesized compounds (**9c-l**, **12** and **13**) towards both LRRK2 wild type (wt) and G2019S mutated-form was evaluated in cell-free assays: a preliminary screening was initially conducted to select the most promising compounds for additional biological studies (Figure 2). Among the quinoline derivatives, the most interesting compounds were found to be those bearing a hydroxyl or methoxy substituent on the right part of the molecule (**9d,f,g**) while pyridine derivatives (**9h-j**) generally showed poor inhibitory potency. Despite its high micromolar activity, the novel tetrahydroquinoline-3-carbonitrile derivative **9k**



Scheme 3. First step optimization. [Reaction conditions: L-proline, toluene, MW, 300 W, 15 min; [a]conventional heating, toluene, reflux, 90 min].

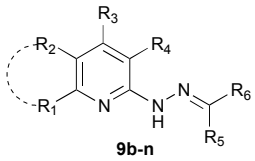


Scheme 4. Second step optimization. [Reaction conditions: $Pd(OAc)_2$, DavePhos, *t*-BuONa, toluene, MW, 150 °C, 10 min; [a]conventional heating, toluene, 100°C, 16 h].

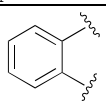
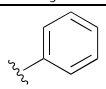
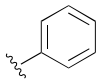
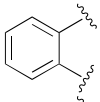
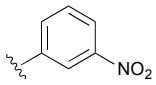
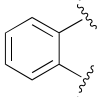
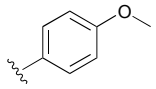
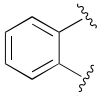
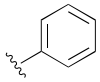
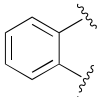
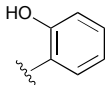
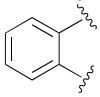
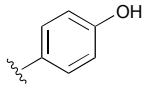
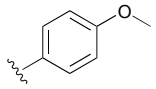
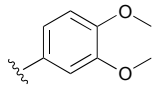
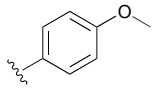
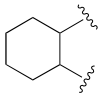
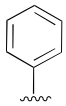
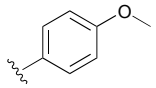
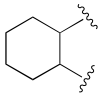
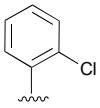
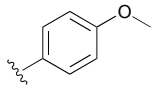
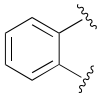
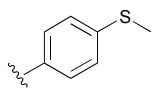
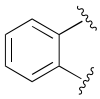
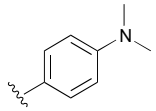


Scheme 5. Optimized one-pot two-step protocol. [Reaction conditions: (a) hydrazine monohydrate, L-proline, toluene, MW, 300 W, 15 min; (b) heteroaryl chloride, *t*-BuONa, $Pd(OAc)_2$, DavePhos, toluene, MW, 150-180 °C, 10-20 min].

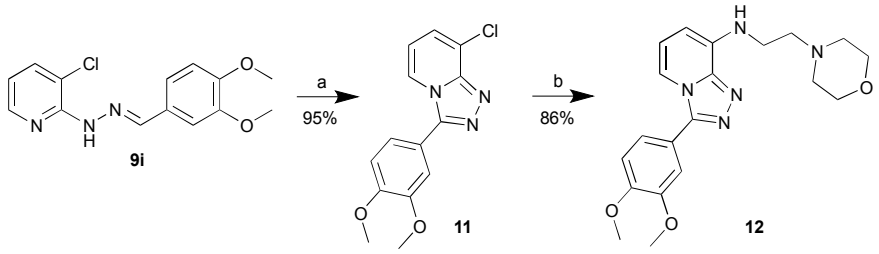
Table 1. Heteroaryl-hydrazone derivatives



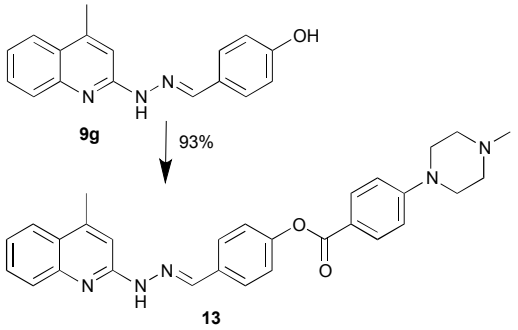
9b-n

Cpd	R_1	R_2	R_3	R_4	R_5	R_6	Yield (%)[a]
9b			H	H			53
9c			H	H	CH_3		33
9d			CH_3	H	H		39
9e			H	H	CH_3		32
9f			H	H	CH_3		41
9g			CH_3	H	H		42
9h	H	H	H	H	H		45
9i	H	H	H	Cl	H		40
9j	CH_3	H	CH_3	CN	H		48
9k				CN	H		44
9l				CN	H		51
9m			H	H	H		38
9n			H	H	H		41

[a] Isolated yield.



Scheme 6. Synthesis of compound **12**. [Reaction conditions: (a) $CuCl_2$, DMF, 90 °C, 1h; (b) 4-(2-Aminoethyl)morpholine, *t*-OBuNa, $Pd(OAC)_2$, rac-BINAP, toluene, MW, 120 °C, 10 min]



Scheme 7. Synthesis of compound **13**. [Reaction conditions: 4-(4-methylpiperazin-1-yl)benzoic acid, EDC HCl, DMAP, dry CH_2Cl_2, 25 °C, 12 h].

could be also considered as an interesting starting point for future chemical exploration. Another promising result was obtained from the screening of the triazolo[4,3-*a*]pyridine derivative **12** while the complete loss of activity converting compound **9g** into **13** may provide useful indications to refine the docking models. In the next phase, inhibition potencies of compounds **9d,f,g** and **12** were further investigated in dose dependent experiments and ID_{50} values towards LRRK2 wt and G2019S were determined. Furthermore, the specificity of the compounds was tested in a panel of selected kinases (Table 2). Two well known kinase-targeting drugs (Bosutinib and Sorafenib), recently investigate as LRRK2 inhibitors,[32] were also included in Table 2 for comparison purpose. It was interesting to notice that, compared to Bosutinib and Sorafenib, our compounds showed a better selectivity profile as indicated by the higher wt/G2019S activity ratio (LRRK2 specificity index) and by the very low inhibitory potency against all the tested kinases. Despite Bosutinib and Sorafenib were not developed as LRRK2 inhibitors, their good inhibitory efficacy against a few of the kinases reported in Table 2 is representative of the poor selectivity that characterizes most of the common kinase-targeting drugs. We than investigated the mechanism of inhibition of compounds **9d,f,g** and **12** toward the pathogenic G2019S mutant**.** LRRK2 G2019S kinase reactions were conducted titrating ATP at different fixed doses of the

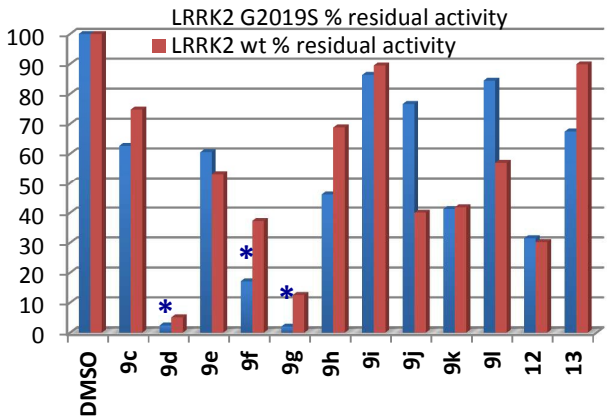


Figure 2. Inhibitory effect of synthesized compounds against LRRK2 wt (red bars) and G2019S (blue bars) expressed as % residual activity at 100μM concentration of the inhibitors. *% residual activity for compounds **9d,f,g** was reported at 50μM.

Table 2. The potency of compounds **9d,f,g** and **12** against LRRK2 (wt and G2019S mutant) and their inhibitory effect on selected kinases.

kinase	ID_{50} (μM)[a]				Ki (μM)[b]	
	9d	**9f**	**9g**	**12**	**Bosutinib**	**Sorafenib**
LRRK2 *wt*	3.0 (4)[c]	32.6 (7.5)[c]	2.7 (2.4)[c]	32.0 (3.5)[c]	0.3 (1.5)[c]	0.7 (0.07)[c]
LRRK2 G2019S	0.9	4.8	1.3	9.1	0.2	9.7
kinase	% inhibition at 100μM				Kd (nM)[d]	
	9d	**9f**	**9g**	**12**	**Bosutinib**	**Sorafenib**
Src	31	5	21	40	1	NA
GSK3β	0	1	0	14	NA[e]	NA
Hck	0	0	0	0	3.4	8500
FAK	11	1	13	8	570	NA
DYRK1A	0	9	22	26	NA	NA
ABL	43	6	24	19	0.1	130
FLT3	50	3	62	0	2200	13
CDK2/cycA	9	3	12	22	NA	8700
CDK9/cycT	0	1	0	24	NA	NA
CDK9/cycK	53	2	24	38	NA	NA
CDK6/cycD	42	1	40	18	ND[f]	ND
CDK4/cycD	9	7	16	10	NA	NA
Pi4Kβ	32	0	31	3	NA	NA
Pim1	20	3	43	29	NA	NA

[a]ID_{50} values are the mean of at least three independent experiments. Standard errors were all within 10% of the mean. [b]Ki values were extrapolated from Ref. 25. [c]wt/G2019S activity ratio (LRRK2 specificity index). [d]Kd values were extrapolated from Ref. 48. [e]NA = not active. [f]ND = not determined

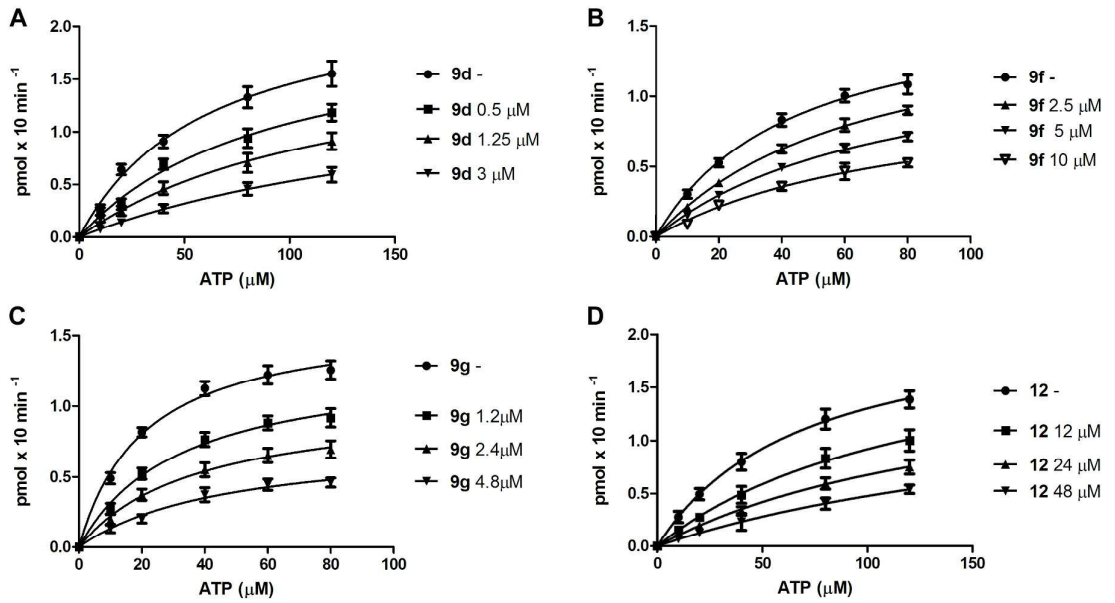


Figure 3. Kinetic analysis of the Kinase reaction in the presence of different concentration of LRRK2 G2019S inhibitors **9d,f,g** and **12**. Variation of the reaction velocity of LRRK2 G2019S as function of ATP concentration at different fixed concentrations of **9d** (A), **9f** (B), **9g** (C) and **12** (D). Each reaction was performed as described in the supporting information. Values are the means of three independent experiments. Error bars represent ± S.D.

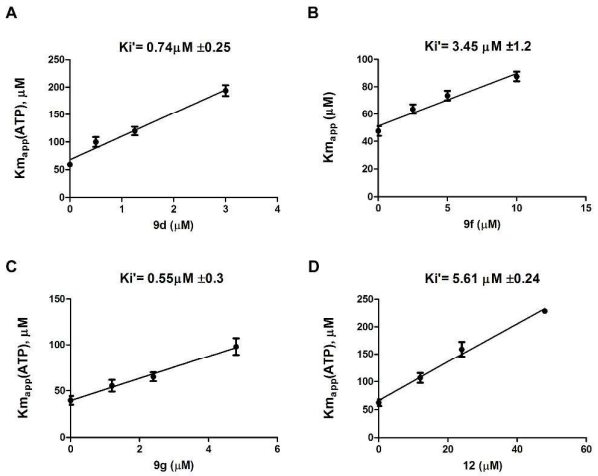


Figure 4. Determination of inhibitors affinities toward LRRK2 G2019S free enzyme complex. Variation of the Km_{app} values of LRRK2 G2019S determined as shown in Figure 3 as function of **9d** (A), **9f** (B), **9g** (C) and **12** (D) concentration. Ki' values are reported at the top of each graph. Data analysis was performed as described in supporting information section. Values are the means of three independent experiments. Error bars represent ± S.D.

compounds (Figure 3, supporting information Eq. (1)). Trend of the apparent affinities (Km_{app}) and apparent maximal velocities ($Vmax_{app}$) obtained from this analysis were then studied as function of inhibitors concentration (Figure 4 and 5). For all these compounds, the Km_{app} values increased at increasing dose of inhibitors, underlying a competitive mechanism of action toward ATP. In particular, compound **12** inhibited G2019S reaction without affecting the $Vmax_{app}$ parameter (Figure 5D), thus resulting in a pure ATP-competitive mechanism of inhibition. On the other hand, increasing the concentration of compounds **9d, f** and **g** resulted in a decrease of $Vmax_{app}$ values, revealing these compounds as mix-type inhibitors against G2019, being able to compete towards ATP to form a complex with the free enzyme ([E]->[E:I]), and to bind also to the enzyme in complex with ATP ([E:ATP]->[E:ATP:I]). The affinity for the free enzyme (Ki') was calculated for each compound, according to Eq. (2) (see supporting information), and reported in

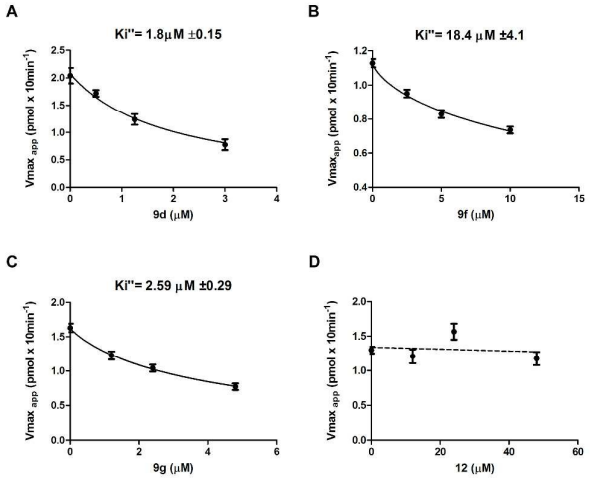


Figure 5. Determination of inhibitors affinities toward [LRRK2 G2019S:ATP] complex. Variation of the $Vmax_{app}$ values of LRRK2 G2019S determined as shown in Figure 3 as function of **9d** (A), **9f** (B), **9g** (C) and **12** (D) concentration. Ki'' values are reported at the top of each graph. Dotted line in panel D was obtained from data linear interpolation. Data analysis was performed as described in supporting information section. Values are the means of three independent experiments. Error bars represent ± S.D.

Figure 4; the affinity for the binary complex was calculated according to Eq. (3) (see supporting information) and reported in Figure 5. The mix-type inhibitors **9f** and **9g** showed a higher affinity toward the free enzyme than toward the [E:ATP] complex, with Ki' values about 5-fold lower than their Ki''. Interestingly, compound **9d**, which exhibited the highest affinity toward the [E:ATP] complex among the tested compounds, showed also comparable Ki' and Ki'' values. This is of particular interest since the intracellular ATP concentration is quite higher than that used in the *in vitro* assays, and this could affect the activity of purely ATP-competitive inhibitors. The mixed-type inhibitor **9d** (and in a lesser extent **9f** and **9g**) showed the ability to inhibit the catalytic activity of the LRRK2 G2019S mutant at low micromolar concentrations even when the enzyme is already bound to the ATP. This peculiar mechanism of action makes **9d** a promising candidate for further biological investigations and an interesting tool to identify a possible allosteric pocket on the LRRK2 kinase.

Conclusions

We herein presented the application of a new pharmacophore fragment-decoration strategy for the identification of new LRRK2-targeting chemical probes as potential anti-Parkinson agents. Starting from the knowledge of a key pharmacophore substructure, common to a few LRRK2 specific inhibitors, we develop a versatile microwave-assisted proline/Pd catalyzed multicomponent approach for the rapid synthesis of substituted heteroaryl-hydrazones. This novel synthetic protocol was efficiently applied to a series of cheap and commercially available building blocks, allowing to easily decorate the heteroaryl-hydrazone pharmacophore fragment. Additional molecular complication was also generated by simple modifications of the final compounds. *In vitro* biological evaluation of the synthesized compounds allowed to quickly identify a few promising LRRK2 inhibitors (**9d,f,g** and **12**) endowed with a low micromolar activity, good LRRK2 specificity index (wt/G2019S activity ratio), low affinity towards a small panel of selected kinases and a mixed type inhibition against the pathogenic G2019S mutant. Among these compounds, **9d, 9f** and **9g** resulted in a mixed-type inhibition towards G2019S, being able to bind also toward the enzyme in complex with the ATP substrate. This is of particular interest since, differently from purely ATP-competitive inhibitors, the inhibitory activity of these compounds should not be affected by the high intracellular ATP concentration. In summary, our results demonstrate how a diversity-oriented approach based on a key pharmacophore fragment could represent an efficient strategy for the identification of new chemical probes of biological interest. Further studies on the optimization of the identified compounds and on the comprehensive elucidation of their mechanism of action will be reported in due course.

Experimental

General. All commercially available chemicals were purchased from both Sigma - Aldrich and Alfa Aesar and, unless otherwise noted, used without any previous purification. Solvents used for work-up and purification procedures were of technical grade. TLC was carried out using Sigma-Aldrich TLC plates (silica gel on Al foils, SUPELCO Analytical). Where indicated, products were purified by silica gel flash chromatography on columns packed with Merck Geduran Si 60 (40-63 µm). ^{1}H and ^{13}C NMR spectra were recorded on BRUKER AVANCE 300 MHz and BRUKER AVANCE 400 MHz spectrometers. Chemical shifts (δ scale) are reported in parts per million (ppm) relative to TMS. ^{1}H-NMR spectra are reported in this order: multiplicity and number of protons; signals were characterized as: *s* (singlet), *d* (doublet), *dd* (doublet of doublets), *ddd* (doublet of doublet of doublets), *t* (triplet), *m* (multiplet), *bs* (broad signal). HPLC/MS analyses were conducted on an Agilent 1100 series, equipped with a Waters Symmetry C18 column (3.5 µm, 4.6 x 75 mm) and a MS detector (Applied Biosystem/MDS SCIEX, with API 150EX ion source). Elemental analyses were performed on a Perkin-Elmer PE 2004 elemental analyzer, and the data for C, H, and N were all within 0.4% of the theoretical values.

Microwave Irradiation Experiments. Microwave reactions were conducted using a CEM Discover Synthesis Unit (CEM Corp., Matthews, NC). The machine consists of a continuous focused microwave power delivery system with operator-selectable power output from 0 to 300 W. The temperature inside the reaction vessel was monitored using a calibrated infrared temperature control mounted under the reaction vessel. All experiments were performed using a stirring option whereby the reaction mixtures are stirred by means of a rotating magnetic plate located below the floor of the microwave cavity and a Teflon-coated magnetic stir bar in the vessel.

General procedure for the one-pot two-step synthesis of functionalized heteroaryl-hydrazone derivatives (9b-n). Substituted ketone/aldehyde (0.73 mmol), hydrazine monohydrate (0.73 mmol), L-proline (0.15 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 colorless 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). The solution became light yellow and the TLC, eluting with petroleum ether/ethyl acetate, 6/4 (vol/vol), showed the complete conversion of ketone/aldehyde into the corresponding hydrazone. Subsequently heteroaryl chloride (0.61 mmol) and *t*-BuONa (0.98 mmol) were added and the tube was flushed with Argon for 1 minute. Then, 1 mL of a stock solution of the catalyst [$Pd(OAc)_2$ (27.0 mg, 0.12 mmol) plus DavePhos (96 mg, 0.24 mmol) in anhydrous toluene (10 mL) stored under Ar atmosphere] was added and the resulting mixture was stirred and flushed with Argon for an additional 2 minutes. Next, the tube was heated under microwave irradiation at 150 °C for 10 minutes (for compounds **9b-g,j,m,n**) or for 20 minutes (for compounds **9k,l**) or at 180 °C for 20 minutes (for compounds **9h,i**) (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 resulting solution was evaporated under reduced pressure. The residue was purified by silica gel flash chromatography (petroleum ether/ethyl acetate, from 9/1 to 7/3), affording pure, target heteroaryl-hydrazones (**9b-n**).

2-(2-(Diphenylmethylene)hydrazinyl)quinoline (9b). Starting from benzophenone and 2-Chloroquinoline, the title compound was obtained as a yellow solid (53% yield). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.59 (s, 1H), 8.11 (d, *J* = 8.9 Hz, 1H), 7.91 (d, *J* = 8.9 Hz, 1H), 7.69-7.66 (m, 4H), 7.62-7.59 (m, 3H), 7.44- 7.30 (m, 7H). ^{13}C NMR (75

MHz, $CDCl_3$) δ 160.6, 155.5, 138.5, 137.9, 132.4, 129.9 (2C), 129.6, 129.1, 129.0 (2C), 128.8, 128.5, 128.3 (2C), 128.1, 127.9, 127.8, 126.9 (2C), 123.3, 110.1. MS (ESI) *m/z* 324.2 $[M + H]^+$, 346.3 $[M + Na]^+$.

(*E*)-2-(2-(1-(3-Nitrophenyl)ethylidene)hydrazinyl)quinoline (9c). Starting from 3'-Nitroacetophenone and 2-Chloroquinoline, the title compound was obtained as a yellow solid (33% yield). ^1H NMR (400 MHz, $CDCl_3$) δ 8.67 (s, 1H), 8.21-8.12 (m, 3H), 7.75-7.64 (m, 5H), 7.58 (t, *J* = 7.6 Hz, 1H), 7.36 (bs, 1H), 2.38 (s, 3H). ^{13}C NMR (100.6 MHz, $CDCl_3$) δ 155.3, 148.6, 146.1, 140.4, 138.7, 136.2, 131.4, 130.2, 129.3, 128.8, 127.9, 126.2, 123.6, 122.9, 120.6, 109.9, 29.7. MS (ESI) *m/z* 307.2 $[M + H]^+$, 329.3 $[M + Na]^+$.

(*E*)-2-(2-(4-Methoxybenzylidene)hydrazinyl)-4-methylquinoline (9d). Starting from 4-Methoxybenzaldehyde and 2-Chloro-4-methylquinoline, the title compound was obtained as a yellow solid (39% yield). ^1H NMR (400 MHz, DMSO-d_6) δ 11.12 (s, 1H), 8.03 (s, 1H), 7.88 (d, *J* = 7.4 Hz, 1H), 7.67 (d, *J* = 8.4 Hz, 2H), 7.61-7.55 (m, 2H), 7.49 (s, 1H), 7.31 (t, *J* = 6.8 Hz, 1H), 7.00 (d, *J* = 8.8 Hz, 2H), 3.81 (s, 3H), 2.65 (s, 3H). ^{13}C NMR (100.6 MHz, DMSO-d_6) δ 160.3, 156.0, 147.4, 145.9, 139.8, 129.9, 128.5, 128.1 (2C), 126.6, 126.1, 124.6, 122.6, 114.7 (2C), 110.3, 55.7, 19.1. MS (ESI) *m/z* 292.1 $[M + H]^+$, 314.3 $[M + Na]^+$.

(*E*)-2-(2-(1-Phenylethylidene)hydrazinyl)quinoline (9e). Starting from acetophenone and 2-Chloroquinoline, the title compound was obtained as a yellow solid (32% yield). ^1H NMR (400 MHz, $CDCl_3$) δ 8.11 (d, *J* = 9.0 Hz, 1H), 7.86 (d, *J* = 7.6 Hz, 2H), 7.81 (d, *J* = 8.9 Hz, 1H), 7.75 (t, *J* = 7.5 Hz, 2H), 7.64 (t, *J* = 7.4 Hz, 1H), 7.46-7.28 (m, 5H), 2.37 (s, 3H). ^{13}C NMR (100.6 MHz, $CDCl_3$) δ 155.3, 148.6, 138.7, 137.9, 132.1, 130.1, 129.8, 128.6, 128.4 (2C), 127.8 (2C), 125.9, 124.7, 115.7, 110.2, 29.7. MS (ESI) *m/z* 262.2 $[M + H]^+$, 284.1 $[M + Na]^+$.

(*E*)-2-(1-(2-(Quinolin-2-yl)hydrazono)ethyl)phenol (9f). Starting from 2'-Hydroxyacetophenone and 2-Chloroquinoline, the title compound was obtained as a yellow solid (41% yield). ^1H NMR (300 MHz, $CDCl_3$) δ 12.35 (bs, 1H), 8.09 (d, *J* = 6.9 Hz, 1H), 7.73-7.63 (m, 3H), 7.48 (d, *J* = 7.4 Hz, 1H), 7.37-7.27 (m, 4H), 7.04 (d, *J* = 8.2 Hz, 1H), 6.92 (m, 1H), 2.41 (s, 3H). ^{13}C NMR (100.6 MHz, $CDCl_3$) δ 158.1, 154.4, 149.6, 146.8, 139.2, 130.6, 130.4, 127.8, 127.2, 126.1, 124.9, 123.8, 119.8, 119.1, 117.5, 108.9, 29.7. MS (ESI) *m/z* 278.4 $[M + H]^+$, 300.4 $[M + Na]^+$.

(*E*)-4-((2-(4-Methylquinolin-2-yl)hydrazono)methyl)phenol (9g) Starting from 4-Hydroxybenzaldehyde and 2-Chloro-4-methylquinoline, the title compound was obtained as a yellow solid (42% yield).^1H NMR (400 MHz, DMSO-d_6) δ 11.05 (bs, 1H), 9.79 (s, 1H), 7.99 (s, 1H), 7.86 (d, *J* = 7.9 Hz, 1H), 7.60-7.58 (m, 2H), 7.55 (d, *J* = 8.4 Hz, 2H), 7.48 (s, 1H), 7.27 (t, *J* = 7.4 Hz, 1H), 6.83 (d, *J* = 8.3 Hz, 2H), 2.64 (s, 3H). ^{13}C NMR (100.6 MHz, DMSO-d_6) δ 158.7, 156.1, 147.8, 146.0, 140.3, 129.8, 128.2 (2C), 126.8, 126.6, 124.6, 124.4, 122.5, 116.1 (2C), 109.8, 19.1. MS (ESI) *m/z* 278.2 $[M + H]^+$, 300.1 $[M + Na]^+$.

(*E*)-2-(2-(4-Methoxybenzylidene)hydrazinyl)pyridine (9h). Starting from 4-Methoxybenzaldehyde and 2-Chloropyridine, the title compound was obtained as a light yellow solid (45% yield). ^1H NMR (400 MHz, $CDCl_3$) δ 8.90 (s, 1H), 8.16 (d, *J* = 4.2 Hz, 1H), 7.76 (s, 1H), 7.64 (d, *J* = 8.7 Hz, 2H), 7.62-7.60 (m , 1H), 7.38 (d, *J* = 8.4 Hz, 1H), 6.94 (d, *J* = 8.7 Hz, 2H), 6.78 (dd, *J* = 6.4, 5.3 Hz, 1H), 3.86 (s, 3H). ^{13}C NMR (100.6 MHz, $CDCl_3$) δ 160.3, 157.2, 147.3, 139.2, 138.2, 127.9 (2C), 127.8, 115.3, 114.2 (2C), 107.5, 55.4. MS (ESI) *m/z* 228.2 $[M + H]^+$, 250.3 $[M + Na]^+$.

(*E*)-3-Chloro-2-(2-(3,4-dimethoxybenzylidene)hydrazinyl)pyridine (9i). Starting from 3,4-Dimethoxybenzaldehyde and 2,3-Dichloropyridine, the title compound was obtained as a yellow solid (40% yield). ^1H NMR (300 MHz, $CDCl_3$) δ 8.48 (s, 1H), 8.26-8.25 (m, 1H), 8.00 (s, 1H), 7.57 (d, *J* = 7.5 Hz, 1H), 7.49 (s, 1H), 7.15 (d, *J* = 7.9 Hz, 1H), 6.87 (d, *J* = 8.0 Hz, 1H), 6.76-6.75 (m, 1H), 3.97 (s, 3H), 3.92 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 150.6, 150.2, 149.3, 146.9, 144.2, 137.1, 127.2, 121.9, 115.6, 114.2, 110.6, 108.5, 56.1, 55.9. MS (ESI) *m/z* 292.2 $[M + H]^+$, 314.1 $[M + Na]^+$.

(*E*)-2-(2-(4-Methoxybenzylidene)hydrazinyl)-4,6-dimethylnicotinonitrile (9j). Starting from 4-Methoxybenzaldehyde and 2-Chloro-4,6-dimethylpyridine-3-carbonitrile, the title compound was obtained as a yellow solid (48% yield). ^1H NMR (400 MHz, $CDCl_3$) δ 8.49 (s, 1H), 7.76 (d, *J* = 8.2 Hz, 2H), 7.73 (s, 1H), 6.95 (d, *J* = 8.2 Hz, 2H), 6.60 (s, 1H), 3.85 (s, 3H), 2.50 (s, 3H), 2.43 (s, 3H). ^{13}C NMR (100.6 MHz, $CDCl_3$) δ 161.3, 160.8, 156.2, 154.7, 141.5, 130.1, 128.8 (2C), 127.1, 116.7, 116.5, 114.2 (2C), 55.3, 24.5, 20.8. MS (ESI) *m/z* 281.4 $[M + H]^+$, 303.3 $[M + Na]^+$.

(*E*)-2-(2-(4-Methoxybenzylidene)hydrazinyl)-4-phenyl-5,6,7,8-tetrahydroquinoline-3-carbonitrile (9k). Starting from 4-Methoxybenzaldehyde and 2-Chloro-4-phenyl-5,6,7,8-tetrahydroquinoline-3-carbonitrile, the title compound was obtained as an orange solid (44% yield). 2-Chloro-4-phenyl-5,6,7,8-tetrahydroquinoline-3-carbonitrile was prepared from 2-Oxo-4-phenyl-1,2,5,6,7,8-hexahydroquinoline-3-carbonitrile, as previously described by our research group.[8] ^1H NMR (400 MHz, $CDCl_3$) δ 8.51 (s, 1H), 7.77 (s, 1H), 7.73 (d, *J* = 8.2 Hz, 2H), 7.53-7.46 (m, 3H), 7.30-7.28 (m, 2H), 6.91 (d, *J* = 8.2 Hz, 2H), 3.83 (s, 3H), 2.93-2.90 (m, 2H), 2.39-2.36 (m, 2H), 1.87-1.86 (m, 2H), 1.72-1.70 (m, 2H). ^{13}C NMR (100.6 MHz, $CDCl_3$) δ 161.3, 160.7, 156.6, 153.4, 141.6, 136.4, 132.4, 128.8 (2C), 128.7 (2C), 128.1, 127.2 (2C), 122.6, 116.8, 114.2 (2C), 113.6, 55.3, 33.3, 26.7, 22.8, 22.5. MS (ESI) *m/z* 383.3 $[M + H]^+$, 405.4 $[M + Na]^+$.

(*E*)-4-(2-Chlorophenyl)-2-(2-(4-methoxybenzylidene)hydrazinyl)-5,6,7,8-tetrahydroquinoline-3-carbonitrile (9l). Starting from 4-Methoxybenzaldehyde and 2-Chloro-4-(2-chlorophenyl)-5,6,7,8-tetrahydroquinoline-3-carbonitrile, the title compound was obtained as an orange solid (51% yield). 2-Chloro-4-(2-chlorophenyl)-5,6,7,8-tetrahydroquinoline-3-carbonitrile was prepared from 4-(2-Chlorophenyl)-2-oxo-1,2,5,6,7,8-hexahydroquinoline-3-carbonitrile, as previously described by our research group.[8] ^1H NMR (400 MHz, $CDCl_3$) δ 8.57 (s, 1H), 7.75 (d, *J* = 8.4 Hz, 2H), 7.71 (s, 1H), 7.56-7.53 (m, 1H), 7.45-7.39 (m, 2H), 7.25-7.21 (m, 1H), 6.91 (d, *J* = 8.4 Hz, 2H), 3.83 (s, 3H), 2.92-2.88 (m, 2H), 2.33-2.29 (m, 2H), 1.89-1.88 (m, 2H), 1.71-1.69 (m, 2H). ^{13}C NMR (100.6 MHz, $CDCl_3$) δ 161.2, 160.8, 156.4, 153.5, 141.8, 135.4, 132.2, 130.3, 129.9, 129.5, 128.8 (2C), 127.4, 127.2, 122.9, 116.4, 114.3 (2C), 114.1, 55.3, 33.1, 29.7, 25.9, 22.5. MS (ESI) *m/z* 417.2 $[M + H]^+$, 439.3 $[M + Na]^+$.

(*E*)-2-(2-(4-(methylthio)benzylidene)hydrazinyl)quinoline (9m). Starting from 4-(Methylthio)benzaldehyde and 2-Chloroquinoline, the title compound was obtained as a yellow solid (38% yield). ^1H NMR (400 MHz, $CDCl_3$) δ 8.60 (s, 1H), 8.25-8.23 (m, 1H), 7.83-7.77 (m, 3H), 7.60-7.56 (m, 4H), 7.48 (t, *J* = 7.6 Hz, 1H), 7.21 (d, *J* = 8.1 Hz, 2H), 2.49 (s, 3H). ^{13}C NMR (100.6 MHz, $CDCl_3$) δ 154.5, 147.4, 139.3,

138.4, 130.1, 129.6, 128.7, 128.5, 127.9 (2C), 127.6, 127.3, 126.3 (2C), 126.2, 117.5, 15.6. MS (ESI) *m/z* 294.1 $[M + H]^+$, 316.2 $[M + Na]^+$.

(*E*)-*N*,*N*-dimethyl-4-((2-(quinolin-2-yl)hydrazono)methyl)aniline (9n). Starting from 4-(Dimethylamino)benzaldehyde and 2-Chloroquinoline, the title compound was obtained as a yellow solid (41% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.55 (s, 1H), 8.20 (d, *J* = 8.2 Hz, 2H), 7.83-7.77 (m, 3H), 7.61-7.55 (m, 3H), 7.45 (t, *J* = 7.6 Hz, 1H), 6.67 (d, *J* = 8.0 Hz, 2H), 2.98 (s, 6H), ^{13}C NMR (100.6 MHz, $CDCl_3$) δ 154.9, 151.2, 147.5, 142.3, 138.1, 129.4, 128.7, 128.3 (2C), 127.5, 126.3, 125.2, 123.5, 117.7 (2C), 112.0, 40.4 (2C). MS (ESI) *m/z* 291.2 $[M + H]^+$, 313.2 $[M + Na]^+$.

Synthesis of 8-Chloro-3-(3,4-dimethoxyphenyl)-[1,2,4]triazolo[4,3-*a*]pyridine (11). A solution of $CuCl_2$ (369 mg, 2.74 mmol) in DMF (4.5 mL) is added to a stirred solution of **9i** (400 mg, 1.37 mmol) in DMF (4.5 mL) and the mixture is heated at 90 °C for an hour. After cooling to room temperature, 20 mL of water and 0.5 mL of ammonia solution 30% are added, followed by extraction with ethyl acetate (3x20 mL). The collected organic phases were washed with an aqueous solution of LiCl (5%), brine, dried over Na_2SO_4 and evaporated under vacuum to give the product as a beige solid (95% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.17 (d, *J* = 6.8 Hz, 1H), 7.30 (s, 1H), 7.25 (d, *J* = 8.4 Hz, 1H), 7.22 (d, *J* = 8.4 Hz, 1H), 6.97 (d, *J* = 7.2 Hz, 1H), 6.79 (t, *J* = 7.0 Hz, 1H), 3.91 (s, 3H), 3.88 (s, 3H). MS (ESI) *m/z* 290.2 $[M + H]^+$, 312.3 $[M + Na]^+$.

Synthesis of 3-(3,4-Dimethoxyphenyl)-*N*-(2-morpholinoethyl)-[1,2,4]triazolo[4,3-*a*]pyridin-8-amine (12) In a microwave tube a mixture of **11** (50 mg, 0.17 mmol), 4-(2-Aminoethyl)morpholine (68 µL, 0.51 mmol), *t*-OBuNa (33 mg, 0.35 mmol), rac-BINAP (32 mg, 0.051 mmol), $Pd(OAc)_2$ (8 mg, 0.034 mmol) and toluene dry (1 mL) were heated at 120 °C for 10 min in the microwave apparatus (maximum power input: 300 W; maximum pressure: 250 PSI; 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 Na_2SO_4 and evaporated under vacuum. The residue was purified by silica gel flash chromatography (dichloromethane/methanol 96/4) to give the desired compound as a white solid (86% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.63 (d, *J* = 6.9 Hz, 1H), 7.40 (s, 1H), 7.33 (d, *J* = 8.3 Hz, 1H), 7.02 (d, *J* = 8.3 Hz, 1H), 6.70 (t, *J* = 7.0 Hz, 1H), 6.04 (d, *J* = 7.3 Hz, 1H), 5.88-5.87 (m, 1H), 3.95 (s, 6H), 3.76-3.73 (m, 4H), 3.36 (dd, *J* = 11, 5.5 Hz, 2H), 2.74 (t, *J* = 6.0 Hz, 2H), 2.54-2.52 (m, 4H). ^{13}C NMR (100.6 MHz, $CDCl_3$) δ 150.4, 149.6, 147.6, 145.5, 136.3, 120.5, 119.8, 115.9, 111.8, 111.3, 110.5, 97.3, 66.9 (2C), 56.6, 56.1, 56.0, 53.4 (2C), 39.4. MS (ESI) *m/z* 384.1 $[M + H]^+$,406.2 $[M + Na]^+$.

Synthesis of (*E*)-4-((2-(4-methylquinolin-2-yl)hydrazono)methyl)phenyl 4-(4-methylpiperazin-1-yl)benzoate (13). A solution of 4-(4-Methylpiperazin-1-yl)benzoic acid (44 mg, 0.20 mmol), (*E*)-4-((2-(4-Methylquinolin-2-yl)hydrazono)methyl)phenol **9g** (50 mg, 0.18 mmol), EDC HCl (63 mg, 0.33 mmol) and DMAP (37 mg, 0.30 mmol) in dry CH_2Cl_2 is stirred at room temperature overnight. At the end of the reaction water was added, followed by extraction with CH_2Cl_2. The collected organic phases were washed with brine, dried over Na_2SO_4 and evaporated under vacuum. The residue was purified by silica gel flash chromatography (dichloromethane/methanol 95/5) to give the desired compound as a yellow solid (93% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.34 (s, 1H), 8.10 (s, 1H), 7.96 (d, *J* = 8.4 Hz, 2H), 7.91 (d, *J* = 8.0 Hz, 1H), 7.80 (d, *J* = 8.0 Hz, 2H), 7.64-7.55 (m, 3H), 7.34-7.29 (m, 3H), 7.06 (d, *J* = 8.0 Hz, 2H), 3.37-3.35 (m, 4H), 2.67 (s, 3H), 2.47-2.45 8s, 4H), 2.23 (s, 3H). ^{13}C NMR (100.6 MHz, DMSO-d_6) δ 164.8, 156.0, 154.9, 151.6, 147.6, 146.3, 139.0, 133.3, 132.0 (2C), 129.9, 127.6 (2C), 126.8, 126.6, 124.8, 124.6, 122.9 (2C), 117.1, 113.8 (2C), 109.8, 54.7 (2C), 46.9 (2C), 46.2, 19.1. MS (ESI) *m/z* 480.2 $[M + H]^+$, 502.1 $[M + Na]^+$.

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