Leucine-Rich Repeat Kinase 2 (LRRK2) Inhibitors

Paul Galatsis, Jaclyn L. Henderson, Bethany L. Kormos, and Warren D. Hirst

Abstract Mutations in the leucine-rich repeat kinase 2 (LRRK2) are the most common known cause of autosomal dominant Parkinson's disease (PD), accounting for approximately 1% of "sporadic" and 4% of familial cases. These mutations either lead directly to an increased kinase activity (G2019S and I2020T are in the kinase activation loop) or to a reduced GTPase activity (R1441C/G and Y1699C), that in turn positively regulate kinase activity. The physiological substrate of the LRRK2 kinase has yet to be definitively identified, yet autophosphorylation is emerging as a relatively robust measure of its activity. LRRK2 has been implicated in a number of diverse cellular processes such as vesicular trafficking, microtubule dynamics, protein translation control, inflammation, and immune function, all of which have been linked to PD. LRRK2 is a large, multi-domain protein; a thorough understanding of the protein domain organization and identification of interacting partners is important to determine the underlying mechanism of LRRK2. Substantial recent effort has been directed towards identifying potent LRRK2 kinase inhibitors, from the repurposed kinase inhibitors to the first through third generation of LRRK2-focused kinase inhibitors, from a range of chemotypes, which are now providing researchers with new tools to better interrogate LRRK2 function.

Keywords Inhibitors, Kinase, Leucine-rich repeat kinase 2, LRRK2

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Abbreviations

6-OHDA	6-Hydroxydopamine
ANK/AR	Ankyrin repeats
ARM	Armadillo
BA	Brain availability
BBB	Blood brain barrier
BI	Brain impairment
CNS	Central nervous system
COR	C-terminal of Roc
GPCR	G-protein coupled receptor
GWAS	Genome-wide association study
HB	hydrogen bond
HBD	hydrogen bond donor count
hERG	Human ether-a-go-go-related gene
HLM	Human liver microsome
KO	Knockout
LE	Ligand efficiency
LipE	Lipophilic ligand efficiency
logD	Natural logarithm of the distribution coefficient
logP	Natural logarithm of the water/octanol partition coefficient
LPS	Lipopolysaccharide
LRR	Leucine-rich repeats
LRRK2	Leucine-rich repeat kinase 2
MDR1	Multidrug resistance
MPO	Multiparameter optimization
MPTP	1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MW	Molecular weight

NHP   Nonhuman primate
PBMC   Peripheral blood mononuclear cell
PD   Parkinson's disease
PSA   Polar surface area
RLM   Rat liver microsome
Roc   Ras of complex proteins
ROS   Reactive oxygen species
RRCK   Ralph Russ Canine Kidney
SAR   Structure–activity relationship
SN   Substantia nigra
THLE   Transformed human liver epithelial
VDW   Van der Waals
WD40   WD40 repeats
α-syn   α-Synuclein

1   Introduction

Parkinson's disease (PD) is the most common movement disorder and the second most common neurodegenerative disorder. The etiology of PD is complex but the most common phenotype is the loss of dopaminergic neurons of the substantia nigra (SN) leading to the clinical symptoms of bradykinesia, resting tremors, rigidity, and postural instability. Historically, at a pathological level, PD is characterized by inclusions containing the synaptic protein α-synuclein (α-syn) in the cell bodies and processes of surviving neurons (known as Lewy bodies and Lewy neurites, respectively). However, more recent observations describe not only a wider distribution of α-syn pathology, but also accumulation of tau and Aβ aggregates, suggesting a more complex pathology [1]. In addition, inflammation, including reactive gliosis, is observed in the striatum and substantia nigra of PD patients [2, 3].

The penetrance of the disease increases with age with 1% of the population over 65 being affected, rising to 5% by age 85 [4]. The past 17 years has led to considerable progress in both the identification of mutations that cause disease and in the mapping of common variants that alter risk for PD [5]. It is now clearly established that many, if not all, forms of Parkinson's disease (PD) contain a genetic component [5], and mutations in the leucine-rich repeat kinase 2 (LRRK2) are the most common known cause of autosomal dominant PD, accounting for approximately 1% of "sporadic" and 4% of familial cases [5–7]. In specific populations, notably Ashkenazi Jews and North African Berber Arabs, the prevalence of LRRK2 mutations can be as high as 40% [8]. Of the LRRK2 mutations, the most common is G2019S with penetrance ranging from ~30 to 70% by the age of 80 [8–10]. This mutation occurs in the kinase domain of the protein, increasing the kinase activity, and consequently, a great deal of effort has been directed towards identifying potent LRRK2 kinase inhibitors, with a biopharmaceutical profile congruent with clinical evaluation, as novel therapeutics for PD. This chapter describes the current state of the art in developing appropriate kinase inhibitors.

2 LRRK2 Biology and Pharmacology

2.1 LRRK2 Genetics and Human Biology

The LRRK2 gene has 51 exons, with multiple potential splice sites, which encodes a large, 2,527 amino acid protein containing two predicted enzymatic domains (GTPase and kinase) and multiple protein–protein interaction domains (Fig. 1). See below for a more detailed account of LRRK2 structural biology (Sect. 3). A number of novel variants have been identified in this gene in PD patients, but only seven of these (N1437H, R1441C, R1441G, S1761R, Y1699C, G2019S, and I2020T) can be considered as definitively disease causing, on the basis of co-segregation with disease in families, and an absence in controls [5, 11–13]. These mutations either lead to an increased kinase activity (G2019S and I2020T) or to a reduced GTPase activity (R1441C/G and Y1699C), which in turn regulates kinase activity [14–16] (*vide infra*).

The G2019S mutation is relatively frequent in some populations from Southern Europe and in certain populations, such as the Ashkenazi Jews and North African Berber Arabs, where the prevalence can be as high as 40% [8]. However, there is an incomplete but age-related penetrance, ranging from ~30 to 70% by 80 years old in different studies, that has been estimated for carriers of the G2019S mutation, and the associated range of PD onset age is broad, including patients with early and late disease onset [8–10]. Dopaminergic neuronal loss and gliosis in the substantia nigra are the common pathological features in patients with LRRK2 mutations, and classical Lewy bodies are found in the majority of them. However, in some cases α-synuclein-positive inclusions are not observed, and only tau-positive or ubiquitin-positive inclusions are seen [7]. Overall, the clinical characteristics of patients with LRRK2 mutations, particularly those with the more common G2019S mutation, are very similar to those with sporadic PD [8, 17].

The incomplete penetrance clearly indicates that other factors are involved in the pathogenesis (Fig. 1); these may include other genetic contributions, both known (e.g., α-synuclein, Tau, RAB7L1, GAK) [5, 18] and yet to be defined, and/or "environmental" factors such as inflammation or oxidative/nitrative/unfolded and/or misfolded protein stress [19–21], many of which are actively being investigated. For example, LRRK2 kinase levels are increased by inflammatory mediators, such as IFNγ and LPS, in vitro [22, 23] and in vivo [23], and two recent reports suggest that LRRK2 levels are increased in sporadic PD brains [24, 25], potentially regulated by microRNA-205 [24]. LRRK2 has been shown to exist as a dimeric protein [26–29] with this form having greater kinase activity [28, 30]. These data have led to a proposed model in which LRRK2 cycles from a cytoplasmic, low-activity monomer to a higher-activity plasma membrane-associated dimer driven by, for example, LPS [28, 31, 32]. However, such biochemical experiments, investigating these potentially more active conformations of LRRK2, have not yet been performed in postmortem patient brains. Furthermore, to date, there is no direct evidence of increased LRRK2 kinase activity in postmortem patient brains from either G2019S mutation carriers or patients with sporadic disease. This is partly due to the challenges of immunoprecipitating sufficient active LRRK2 for in vitro phosphorylation assays, compounded by
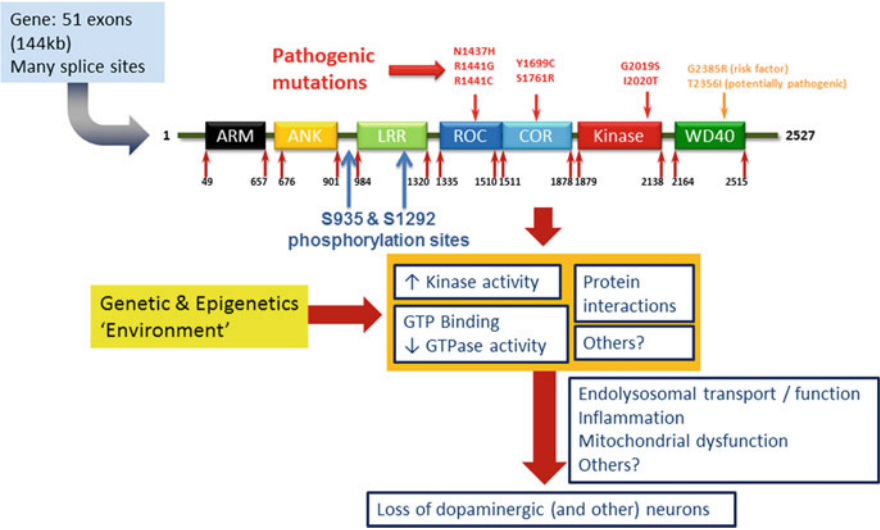
Fig. 1 LRRK2 protein domain structure and putative PD pathways

the potential effects of postmortem delay on the activity [33]. The lack of a validated substrate (vide infra) and the reliance on surrogate substrate, e.g., LRRKtide and NICtide [34, 35], also hampered such efforts. Despite this we, and others, are currently investigating these assays, and other end-points, to provide a critical link, which is currently missing, between LRRK2 and sporadic disease.

2.2   LRRK2 Substrates

The physiological substrate of the LRRK2 kinase has yet to be definitively identified. A number of potential substrates, other than LRRK2 (see next section), have been proposed (Table 1). However, full validation of these, including a clear demonstration of an increase in human PD patient cells and tissues and reduction in phosphorylation with multiple, structurally diverse inhibitors and by knocking out LRRK2, remains to be demonstrated.

2.3   Effects of Mutations on Kinase and GTPase Activity

The G2019S mutation, in the activation loop, has been consistently shown to result in increased kinase activity of LRRK2 [57, 58]. However, the functional consequences of the other mutations reported in the literature are conflicting. The other kinase domain mutation (I2020T) has been reported to either increase [59] or decrease kinase activity [34, 60]. Similarly, mutations in the GTPase domain have been demonstrated

Table 1 Putative LRRK2 kinase substrates

Protein	Phosphorylation site(s) – if determined	Identification and validation	References
4E-BP	T37/T46, T70	In vitro assay, drosophila, and LRRK2 overexpression in HEK293 cells	[36]
		Note: follow-up publications did not show robust phosphorylation	[37, 38]
α-Synuclein	S129	In vitro kinase assay (G2019S)	[39]
Akt1 (PKB), ARHGEF7, ARHGEF11 and others	S473, S153, S176 and S150	SH-SY5Y cells (endogenous and overexpressed LRRK2) +/− LRRK2-IN-1. Pathway analysis, microglial inflammatory responses and neurite outgrowth demonstrated off-target effects of LRRK2-IN-1	[40]
Akt1 (PKB)	S473	In vitro kinase assay and LRRK2 knockdown	[41]
ArfGAP1	S155, S246, S284, T189, T216, T292	Interaction studies and in vitro kinase assay	[42, 43]
β-tubulin	T107	In vitro assay and LRRK2 overexpression in HEK293 cells	[44]
Endophilin A	S75	In vitro assay and effects on synaptic vesicle trafficking	[45]
Ezrin/radixin/moesin proteins	Not determined	Primary neurons pERM increased with G2019S	[46]
FoxO	S259 (drosophila, corresponding human residue: S319)	In vitro assay and drosophila	[47]
MAP2	T1433	Consensus phosphorylation motif and peptide phosphorylation	[48]
MARKK	Not determined	In vitro kinase assay (G2019S)	[49]
MKK3/4/6/7	K149/S207 (MKK4 and 7)	In vitro kinase assay (G2019S)	[50, 51]
MKK3/6/7	Not determined	In vitro kinase assay	[52]
Moesin	T558	KESTREL screen and in vitro kinase assay	[34]
Praja	T74	Consensus phosphorylation motif and peptide phosphorylation	[48]
Ribosomal protein s15	T136	Interacting phosphoproteins identified by tandem affinity purification, LRRK2 overexpression in neurons	[53]
RIPK2	Not determined	Protein array with G2019S	[49]

Table 1 (continued)

Protein	Phosphorylation site(s) – if determined	Identification and validation	References
Serine–protein kinase ATM (ataxia telangiectasia mutated)	T1769	Consensus phosphorylation motif and peptide phosphorylation	[48]
Snapin	T117	Interaction identified by yeast-2-hybrid. In vitro kinase assays with purified recombinant Snapin and LRRK2	[54]
Tau (tubulin associated)	T181	In vitro kinase assay and LRRK2 knockdown and overexpression in SH-SY5Y cells	[55]
	T149, T153, T205, and S199/S202/T205	In vitro tau phosphorylation; LRRK2/Tau$_{P301L}$ transgenic mice	[56]
ULK1	T456	Consensus phosphorylation motif and peptide phosphorylation	[48]

to increase kinase activity [57, 61], whereas in other studies, they have had little or no effect [34, 58]. More recent data have shown that the R1441C/G and Y1699C mutations reduced the GTPase activity that in turn regulates kinase activity [14, 15]. The majority of studies have used either recombinantly expressed and purified LRRK [58] or immunoprecipitated LRRK2 from recombinant mammalian expression systems [34, 57, 61] and investigated autophosphorylation or the use of surrogate substrates (myelin basic protein, LRRKtide, NICtide), all which are relatively poor substrates under those assay conditions.

LRRK2's autophosphorylation has been characterized [30, 62, 63], and given the caveats of the validation of the exogenous substrate, perhaps this end-point is currently the most relevant. This is supported by recent data that demonstrated an increase in phosphorylation at S1292 by the pathological mutations [16]. Importantly, and in contrast to most of the previous results using purified enzyme, the magnitude of increase in phosphorylation of S1292 by, for example, G2019S is over 10-fold, when compared to wild-type LRRK2 [16] versus the 2–3-fold typically observed with the isolated enzyme [58]. This is clearly suggesting that the microenvironment of LRRK2 within the cell, including accessory proteins, interaction partners, and membrane association, is critical for the optimal kinase activity and that such a measure of kinase activity is a potentially valuable biomarker if it can be applied to patient samples. This will be a challenge as the stoichiometry of phosphorylation at S1292 is very low (<1%) ([16], unpublished observations), which may also reflect the tight control of LRRK2's activity in the cellular context.

There are additional phosphorylation sites on LRRK2, including S910 and S935 [64], which have been shown to interact with 14-3-3 proteins and regulate the subcellular localization of LRRK [65]. While not autophosphorylation sites, they

are clearly sensitive to LRRK2 kinase inhibitors, potentially due to conformational changes [66] exposing these residues to phosphatases, and have been used extensively in both cellular and in vivo studies (vide infra).

2.4   Role of LRRK2 in Normal and Pathological Biological Pathways

The understanding of LRRK2's role in normal and pathological biological pathways is still at a relatively early stage, with a number of key questions that remain to be answered. For example, the endogenous substrate for the kinase domain is not known (vide supra and Table 1), and despite recent progress identifying interaction partners [67, 68], the full understanding of LRRK2's function and interactome in different cell types remains an area of active research. The signaling pathways, through which LRRK2 elicits its actions and others that it potentially modulates, are emerging [69, 70] and its role in PD pathophysiology, while not definitive, is also developing.

LRRK2 has been implicated in a number of diverse cellular processes [19, 71, 72] including autophagy, vesicular trafficking, microtubule dynamics, neurite outgrowth, endosomal/synaptic dysregulation, protein translation control, mitochondrial pathology/ROS, WNT and MAPK/MEK/ERK/EIF2 signaling pathways, inflammation, and immune function, all of which have been linked to PD.

2.5   Animal Models to Understand LRRK2 Function

Studies in *Drosophila melanogaster* [73] and *Caenorhabditis elegans* [74] have provided important insights into LRRK2 toxicity in these species. The LRRK2 transgenic mouse models created to date do not completely recapitulate the hallmarks of PD (i.e., dopaminergic neuronal loss, α-synuclein accumulation, the development of Lewy bodies, and behavioral phenotype) [75–77], and with the exception of pharmacodynamic end-points, measuring reduction of LRRK2 phosphorylation at S935 and S1292 has not been used, to date, for any long-term LRRK2 inhibitor studies [16]. In contrast, viral overexpression of LRRK2 does result in dopaminergic neuronal loss [78, 79], but these models need to be fully validated with the more selective, brain-penetrant compounds described herein.

Toxins such as 6-hydroxydopamine (6-OHDA) or 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) have been effective in acutely inducing DA cell loss [80]. However, the LRRK2 knockout mouse displays the same sensitivity to MPTP as wild-type mice [81] suggesting that LRRK2 may not be involved in the events downstream of the mitochondrial toxicity in the mouse brain. There are no published reports on 6-OHDA in in vivo models, with LRRK2 overexpression or with endogenous LRRK2. The use of other toxins, such as LPS, may provide a more relevant model as LRRK2 kinase levels are increased by LPS in vitro [22, 23] and in vivo [23].

The lack of validated mammalian preclinical models of LRRK2 has been an impediment to the development of LRRK2 kinase inhibitors. This has also been hampered by the lack of suitable tool compounds that possess the appropriate pharmacokinetic properties and safety profiles for long-term dosing studies that are likely to be required.

2.6 LRRK2 and Potential Safety Concerns

LRRK2 is widely expressed, with highest expression in kidney, lung, and peripheral blood monocytes and lower levels in the brain [82]. LRRK2 functions in the peripheral tissues are also not well understood, and transgenic animals, knock-ins and knockouts, have been generated in an attempt to gain greater insight into LRRK2 biology. In all cases, the animals are viable and exhibit normal life spans. Initial studies with LRRK2 knockout (KO) mice exhibited phenotypes which showed a lack of hypersensitivity to MPTP, caused impairment of protein degradation pathways, resulted in accumulation of α-synuclein in kidney, not brain, and resulted in apoptotic cell death [76, 80, 83]. A single report with conditional LRRK2 G2019S overexpression in rats showed impaired dopamine reuptake and improved locomotor activity without loss of SN dopaminergic neurons [84]. LRRK2 KO rats are now commercially available [85]. While these animals appear to not have functional impairment, they do exhibit two distinct peripheral phenotypes, similar to the LRRK2 KO mice: (1) the kidneys present with a dark color that show inclusion bodies upon microscopic examination, and (2) type II pneumocytes of the lungs show changes in lamellar body morphology with altered surfactant secretion, measured in vitro [86–89]. The changes in type 2 pneumocyte vacuolation/lamellar body enlargement have recently been observed with 7- and 29-day NHP toxicology studies [90]. It remains to be determined whether KO phenotypes are a consequence of a total lack of LRRK2 from the embryonic stage to adult and if the drug-mediated phenotypes are chemotype or mechanism based.

3 LRRK2 Structural Biology

3.1 LRRK2 Protein Domain Structure

The LRRK2 gene encodes a 286 kDa protein comprising 2,527 amino acids [6, 91]. LRRK2 protein belongs to the Roco family of proteins whose members contain multiple domains including a tandem Roc (Ras of complex proteins)–COR (C-terminal of Roc) motif [92, 93]. As with the other members of the Roco family, LRRK2 contains multiple domains (Fig. 1): the enzymatic Roc–COR and kinase domains, which are flanked by putative protein–protein interaction domains, including the N-terminal armadillo (ARM) repeats, ankyrin repeats (AR or ANK)

and leucine-rich repeats (LRR), and the C-terminal WD40 repeats [94, 95]. Currently, there is no unanimous agreement on the identity and number of armadillo, ankyrin, leucine-rich, and WD40 repeats in the LRRK2 protein [94]. A thorough understanding of the protein domain organization and identification of interacting partners is important to understand the function(s) of LRRK2.

3.1.1 Protein–Protein Interaction Domains

No X-ray crystal structures have yet been reported for any of the LRRK2 putative protein–protein interaction domains; therefore, its domain identification relies on bioinformatic analysis of the primary sequence to predict domain composition and boundaries.

The N-terminal LRRK2-specific repeats characterized by Marín [94] have been predicted to adopt the folds of 13 armadillo-type repeats from residues E49 to K657 [95]. Each ARM repeat is made up of ~42 amino acids that form three α-helices; tandem ARM repeats form a super helical structure that is involved in protein–protein interactions in other ARM repeat proteins [96]. This domain is predicted to be the largest LRRK2 domain containing ~600 amino acids.

Seven ANKs have been predicted to comprise the subsequent LRRK2 domain, made up of residues F676–S901 [95, 97]. Each AR is made up of 33 amino acids that form two antiparallel α-helices followed by a β-hairpin or a long loop, assembling to form a curved structure [98]. AR proteins are involved in a wide range of functions and typically mediate protein–protein interactions [99].

The next domain in sequence is the LRR domain, from where LRRK2 gets its name. As many as 14 LRRs have been predicted from residues I984 to R1320 [95, 100], though fewer have also been suggested [92, 94, 97, 101]. Each LRR is typically made up of 20–30 amino acids (~24 in LRRK2) and contains an unusually high number of leucine residues that form a β-strand followed by an α-helix or extended chain [102]. Tandem LRRs assemble into a horseshoe- or solenoid-type shape whose curvature depends on the specifics of the secondary structure. The framework of LRR domains makes them well suited for protein–protein interactions, and the concave surface of the LRR structure is usually, though not always, the site of macromolecular recognition or dimerization [103].

The C-terminal domain has been predicted to contain one [94], two [92], or seven [95, 97] WD40 repeats; the seven repeats are predicted to occur between residues N2164 and K2515 [95]. Each WD40 repeat contains a four-stranded, antiparallel β-sheet made up of 40 residues [97]. They usually occur in multiples of six or seven and assemble to form a propeller-like structure that serves as a rigid scaffold for protein–protein interactions [104].

3.1.2 Enzymatic Domains

The Roc domain of LRRK2 is a Ras-like GTPase domain that is followed immediately by the COR domain as in all members of the ROCO protein family

[93]. These domains approximately span residues M1335–N1510 and F1511–E1878, respectively [97]. Ras family GTPases act as molecular switches between an active GTP-bound state and an inactive GDP-bound state [105, 106]. Evolutionary analysis indicates that not all ROCO proteins contain a kinase domain; as such, it has been suggested that GTPase activity is the primary function of ROCO proteins and those with kinase domains evolved independently to regulate that function [92, 93, 95, 107].

Two X-ray crystal structures have been solved providing the ability to form hypotheses about the LRRK2 Roc and COR domains and their functions based on structural information. One is a structure of the human LRRK2 Roc domain bound to GDP-Mg^{2+}, which reveals a domain-swapped homodimer [108]. This structure suggests the Roc dimer acts as one functional unit with each nucleotide binding site formed by contributions from both monomers. The other relevant X-ray crystal structure contains both the Roc and COR domains from the bacteria *Chlorobium tepidum* and does not exhibit the domain swapping configuration [109]. This structure suggests that the COR domain is responsible for dimerization, that the Roc domain is stabilized by the COR domain, and that the Roc–COR domain is stabilized by nucleotide binding. Much discussion of the validity and relevance of these X-ray crystal structures has taken place [105, 107, 110–114]. Regardless, these structures both have implications for LRRK2 GTPase function that have spurred important discussions, and results from hypotheses generated based on these structures will only improve our understanding of the LRRK2 protein.

The kinase domain has the most straightforward therapeutic potential and thus has garnered the most interest of all of the domains in the LRRK2 protein. Characterized as a Ser/Thr kinase in the TKL group of kinases, it has the highest homology to LRRK1 and the receptor-interacting protein (RIP) kinases [94, 115], which are sensors of intracellular and extracellular stresses [116]. The LRRK2 kinase domain spans residues Q1879–V2138. Protein kinases catalyze the transfer of a phosphoryl group from ATP to serine, threonine, or tyrosine residues in protein substrates and mediate most cellular signaling processes [117]. The physiological substrate of the LRRK2 kinase has yet to be definitively identified (vide supra). The X-ray crystal structure of the kinase domain from the slime mold *Dictyostelium discoideum* Roco4 protein may provide insight into the structure of the LRRK2 kinase domain [118]. With a kinase domain that is ~30% identical and ~50% similar to that of LRRK2, as well as a similar domain structure that contains LRRs, Roc–COR, kinase, and WD40 repeat domains, the *D. discoideum* Roco4 kinase domain may be a suitable crystallography surrogate to help build an understanding of LRRK2 kinase structure and mechanism.

3.2 LRRK2 Pathogenic PD Mutations

To date, the proven pathogenic PD mutations (Fig. 1) segregate to the enzymatic Roc–COR and kinase domains of LRRK2 [13, 119, 120], though there are a number of variants that have been identified as PD risk factors that are distributed
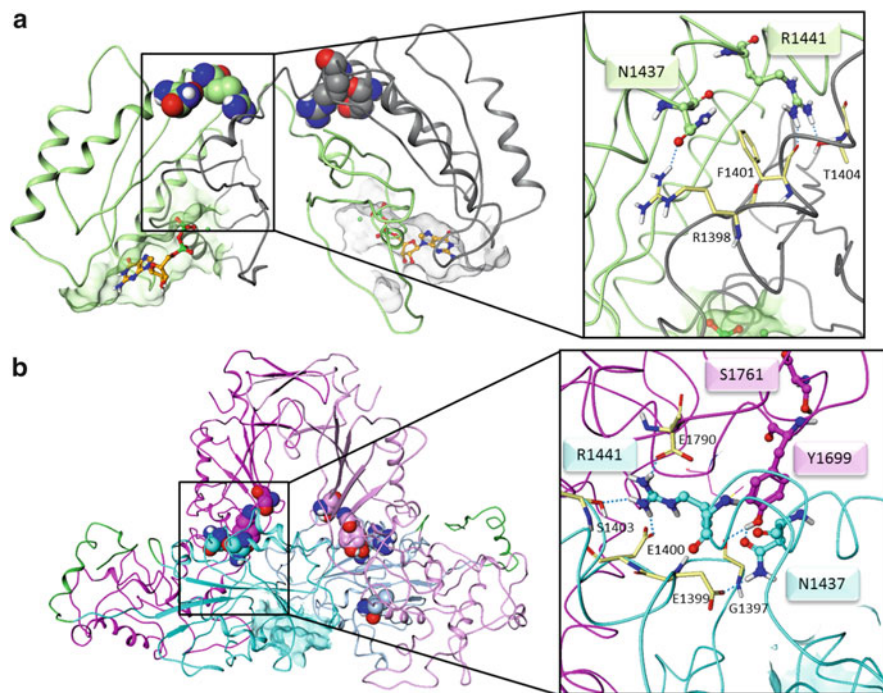
Fig. 2 Roc–COR structures with positions of pathogenic mutations and interacting residues. (**a**) Self-homology model of the domain-swapped LRRK2 Roc–Roc homodimer (PDB ID: 2ZEJ). *Orange*: GDP; *Green*: monomer A; *Gray*: monomer B; *Yellow*: interacting residues. (**b**) Homology model of LRRK2 Roc–COR dimer based on the *C. tepidum* structure (PDB ID: 3DPU). *Cyan*: Roc monomer A; *Pink*: COR monomer A; *Light blue*: Roc monomer B; *Light pink*: COR monomer B; *Yellow*: interacting residues

throughout the entire protein [121, 122]. As such, these mutations have the potential to disrupt not only LRRK2 enzymatic functions but also protein–protein interactions. In the absence of LRRK2 X-ray crystal structures, homology models [123, 124] have been valuable tools to help understand the position of pathogenic mutations and the effect(s) they may have on the function of the LRRK2 protein.

3.2.1 Roc–COR Pathogenic Mutations

The R1441C/G/H and N1437H pathogenic mutations are located in the Roc domain and the Y1699C and S1761R mutations are located in the COR domain. The two current models for the Roc domain suggest different roles for these residues (Fig. 2) [125]. In the domain-swapped homodimer X-ray crystal structure of the human LRRK2 Roc domain proposed by Cookson and coworkers [108], R1441 and N1437 are situated such that they stabilize the Roc–Roc dimer interface through interactions with the other monomer. R1441 forms HB interactions with the backbone carbonyl of

F1401 and the side chain of T1404 and forms a cation-π interaction with the side chain of F1401, while N1437 forms a HB interaction with the side chain of R1398.

Alternatively, in the X-ray crystal structure of the Roc–COR dimer from *C. tepidum* proposed by Wittinghofer and coworkers [109], the residues equivalent to R1441, N1437, and Y1699 (Y558$^{C.\ tepidum}$, H554$^{C.\ tepidum}$, and Y804$^{C.\ tepidum}$, respectively) are clustered together at the interface of the Roc and COR domains, involved in interactions within and between the Roc and COR domains. Based on the Y558$^{C.\ tepidum}$ interactions, it is hypothesized that the aliphatic side chain of R1441 is involved in structurally important hydrophobic interactions with the COR domain. A homology model based on this structure suggests that R1441 may also form HB interactions with the side chains of E1400 and S1403 in the Roc domain and that N1437 forms HB interaction with the side chains of E1399 in the Roc domain and Y1699 in the COR domain [126] (Fig. 2a). Based on the Y804$^{C.\ tepidum}$ interactions, Y1699 may form a hydrogen bond with N1437 (H554$^{C.\ tepidum}$). A homology model based on this structure suggests Y1699 may also form a HB with the N1437 side chain or the G1397 backbone carbonyl and a CH-π interaction with the C$^{\alpha}$ of N1437 [126] (Fig. 2b).

Neither model places these residues close enough to the binding site to interact with GTP or GDP, so the pathogenic mutations do not appear to disrupt nucleotide binding directly. In both models, mutation of R1441 to Cys, Gly, or His and N1437 mutation to His would disrupt HB and van der Waals (VDW) interactions, and in the *C. tepidum* model, mutation of Y1699 to Cys would disrupt a HB between the Roc and COR domains. Therefore, evidence from both models suggests that these residues play an important role in dimer formation or interactions between domains, affecting LRRK2 protein function (vide supra). In addition, these three residues form interactions with residues in the Switch II region of the Roc domain [109], a conserved region in Ras proteins that is important for GTPase function [127, 128]. This suggests that mutation of these residues could also affect LRRK2 GTPase function by altering interactions with the Switch II region.

The S1761 residue in the COR domain corresponds to S852$^{C.\ tepidum}$ in the *C. tepidum* structure. This residue is farther away from the Roc–COR interface and Switch II than the other pathogenic mutations and is situated on the loop between β9 and β10. As such, it may be involved in protein–protein interactions with other LRRK2 domains or other scaffolding proteins. Mutation to Arg could affect these protein–protein interactions or the flexibility of the loop.

3.2.2 Kinase Domain Pathogenic Mutations

The G2019S and I2020T pathogenic mutations are located in the kinase domain. In the absence of an X-ray crystal structure of the LRRK2 kinase domain, a number of homology models have been reported and used to understand the position of these residues and the implications of the pathogenic mutations. There is no consensus on a "best" template to build LRRK2 kinase domain homology models due to the fact that the kinases for which X-ray crystal structures have been published have at most ~30% sequence identity to LRRK2. The most common templates have been from
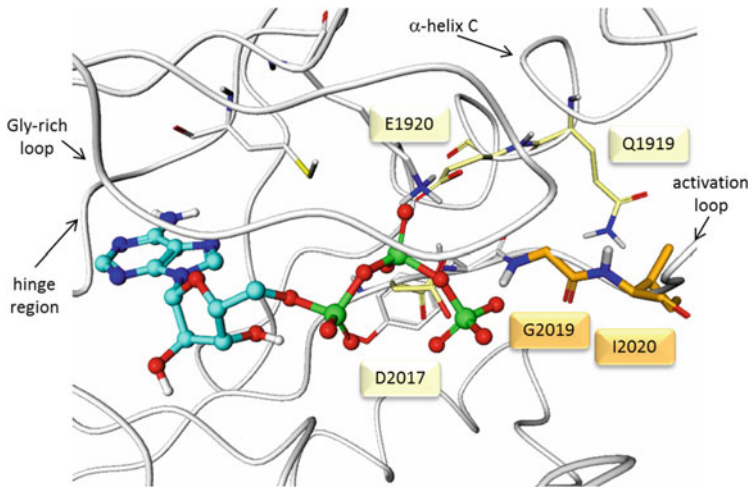
Fig. 3 LRRK2 ATP-binding site. ATP (*cyan*) is shown docked into a LRRK2 homology model based on the X-ray crystal structure of JAK3 with CP-690550 (PDB ID: 3LXK). *Orange*: location of the G2019S and I2020T mutations; *Yellow*: hypothesized interacting residues

B-Raf [94, 101, 129–131], JAK-2 [132–138], MLK1 [132, 136–139], ROCO4 [131, 140], and TAK1 [132, 141, 142] kinases, chosen based on overall kinase sequence identity, ATP-binding site identity, and/or crossover of inhibitor activity. Consensus from these alignments is that G2019 is the Gly in the conserved DFG motif (DYG in LRRK2) and I2020 is the subsequent residue in the activation loop (Fig. 3) [125].

Modeling of the G2019S mutation suggests that the change from a flexible Gly residue to a Ser causes the activation loop to be less flexible due to an increase in HB interactions with D2017, E1920, or other nearby residues, increasing the population of conformations in the active state [114, 131, 136, 141]. The X-ray crystal structure of Roco4 with the mutation that corresponds to LRRK2 G2019S, G1179S^{Roco4}, was solved to 2.04 Å resolution and reveals a HB interaction between G1179S^{Roco4} and R1077^{Roco4} in the αC-helix, which corresponds to Q1919 in LRRK2 [118]. This construct was shown to have increased kinase activity compared to WT. In addition, the double mutants G1179S^{Roco4}/R1077A^{Roco4} in Roco4 and G2019S/Q1919A in LRRK2, in which the Ser is unable to make a HB interaction to the αC-helix to stabilize the active conformation, have nearly wild-type activity. Metadynamic simulations combined with kinetics studies suggested that the energy barrier to achieve the inactive DYG-out conformation in LRRK2 is much higher for the G2019S mutant than for WT [131]. Together, this evidence suggests that an additional hydrogen bond in the G2019S mutant stabilizes the active kinase conformation, causing an increase in kinase activity.

Modeling of the I2020T mutation, on the other hand, does not suggest a clear hypothesis for its pathogenicity. The X-ray crystal structure of Roco4 kinase with the corresponding mutation, L1180T ^{Roco4}, was solved to 2.3 Å resolution and shows the side chain of the Thr interacting with solvent [118]. It was suggested that
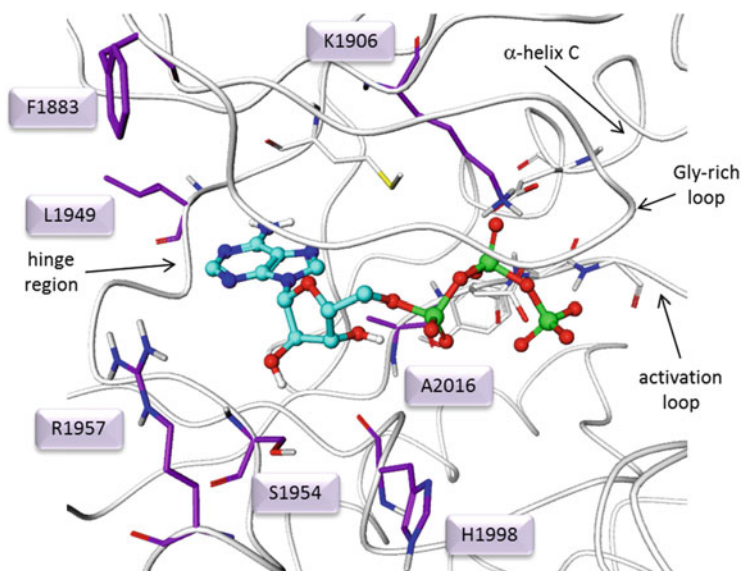
Fig. 4 LRRK2 ATP-binding site. ATP (*cyan*) is shown docked into a LRRK2 homology model based on the X-ray crystal structure of JAK3 with CP-690550 (PDB ID: 3LXK). *Purple*: residues that have been targeted for LRRK2 specificity

the effect of the mutation may only be evident upon LRRK2 dimerization or interactions with other domains, which cannot be discerned from the X-ray crystal structure of the kinase domain alone. Alternatively, molecular dynamics simulations suggest the Thr mutation may form a HB interaction with the backbone carbonyl of D2017, which is involved in substrate binding [60, 114]. Metadynamic simulations combined with kinetic studies suggested that the I2020T mutant stabilized the active DYG-in conformation in LRRK2 compared to WT [60].

3.3 Using LRRK2 Structure to Guide Kinase Inhibitor Design

Most of the LRRK2 kinase inhibitors that have been published to date are ATP-competitive inhibitors that contain hinge-binding motifs common to many kinase inhibitors (vide infra). Through the use of structure–activity relationships (SAR), X-ray crystal structures, and homology models, specific residues in the LRRK2 ATP-binding pocket have been targeted for inhibitor interactions to improve potency and selectivity for LRRK2 kinase (Fig. 4). Hydrophobic interactions with A2016 have been shown to be important for potency and selectivity of some LRRK2 kinase inhibitors [35, 140, 143, 144]. Selectivity of these compounds is especially improved over kinases that have a more polar residue at that position – 28% of kinases have a Ser or Thr at that position. Mutation of A2016 to Thr

abolishes activity in some inhibitors [35, 140, 143], but not others [145–147], which is indicative of the inhibitor binding modes.

The non-conserved residues L1949, S1954, R1957, and F1883 were identified as potential selectivity handles in the LRRK2 kinase domain, due to their proximity to the ATP-binding pocket for a series of diaminopyrimidine compounds [132–135]. L1949 near the hinge region was targeted for selectivity since it is a smaller residue than Phe or Tyr, which is found in this position in approximately 60% of the kinome. Inhibitors with a nitrogen lone pair or substituents or that extend into this region can be accommodated with the Leu residue, but sterically clash with the larger Phe or Tyr. This strategy was used to obtain selectivity for LRRK2 over the JAK family of kinases and JAK2 was found to be a useful surrogate for predicting general kinome selectivity. Interactions with S1954 were targeted to achieve both general kinase selectivity and to overcome specific selectivity issues with the kinase TTK. Approximately 55% of the kinome has a larger group at this position, and approximately 50% has a negatively charged Asp or Glu residue in this position. Strategies to mitigate crossover to TTK, which has an Asp residue in this position, have included the introduction of small groups that would cause unfavorable steric and/or electrostatic interactions with the Asp side chain [132, 133, 135]. Selectivity of a series of 7-aryl-substituted quinoline derivatives may also be due to the placement of polar groups in the vicinity of the unconserved S1954 and R1957 residues [138]. Interactions with the unconserved R1957 and H1998 LRRK2 residues may also contribute to selectivity in a series of indolinone compounds [144]. Optimizing interactions with H1998 and the catalytic K1906 in this series of compounds was hypothesized to improve kinase selectivity, especially over RET kinase.

Alternative strategies used to obtain selectivity in kinases are through Type II inhibitors, which stabilize an inactive DFG-out conformation, or Type III inhibitors, which are not ATP-competitive. Only a handful of LRRK2 inhibitors have been reported that fall into these categories [131, 148]. These inhibitors were shown to be more potent against WT LRRK2 kinase compared to G2019S, supporting the hypothesis that the pathogenic G2019S mutation stabilizes the active conformation of LRRK2 [60, 114, 131, 136, 141]. This suggests a Type II kinase inhibitor may not be the optimal approach for the treatment of PD due to the LRRK2 G2019S mutation. The X-ray crystal structure of Roco4 kinase domain co-crystallized with the inhibitor H1152 revealed two binding sites for this inhibitor: one in the expected ATP-binding site and the other close to the αC-helix [118]. The implications of this second binding site for LRRK2 are unclear, but suggest not completely ruling out the possibility of developing Type III inhibitors for LRRK2 kinase.

4 Medicinal Chemistry: LRRK2 Kinase Inhibition

Historically, kinase inhibitors have been a key target for oncology indications. The critical role of kinases in cell cycle or apoptotic signaling pathways made them a logical target. From a drug discovery perspective for non-oncology indications, pharmacologically promiscuous kinase inhibitors can potentially give rise to safety

Table 2 Physicochemical property comparison of CNS marketed drugs and Pfizer CNS clinical candidates (CANs) with marketed kinase drugs

	MW	clogP	tPSA	clogD	HBD	pK_a	CNS MPO
CNS drugs[a] (121 cmpds)	298.7	2.72	47.6	1.70	1	6.2	4.61
CNS CANs[a] (108 cmpds)	357.4	3.44	53.6	2.16	1	7.5	4.25
Kinases[a] (21 cmpds)	461.2	4.24	85.6	2.63	2	5.6	3.56

[a]Average values

concerns due to their varied roles in signaling pathways. These safety issues are particularly acute for the treatment of chronic diseases that require long-term treatment, typically experienced with neurodegenerative disorders. Recently, kinase inhibitors have been developed for non-oncology indications. Xeljanz® (tofacitinib), a JAK3 inhibitor, is a good example and was approved in 2012 for moderate to severe rheumatoid arthritis (http://www.xeljanz.com/).

For a central nervous system (CNS) indication, in addition to the off-target selectivity, one needs to contend with the blood–brain barrier (BBB) and designing compounds that have access to the central compartment. The medicinal chemistry challenge is readily apparent when one analyzes the current marketed kinase chemical space relative to the historical CNS chemical space. From a kinase inhibitor perspective, the potential pharmaceutical agent must contend with equilibria of active, inactive, and intermediate conformations (e.g., DFG-in vs. DFG-out). Most of the initially marketed kinases targeted the DFG-out conformation which generates a much larger active site pocket, thus giving rise to compounds with MW, logP, or PSA parameters outside the range of typical CNS space. Comparison of these physicochemical parameters is presented in Table 2. These data were generated from an analysis of marketed kinases relative to the marketed CNS drugs and a set of preclinical CNS candidates from the Pfizer pipeline. Additionally, employing the CNS multiparameter optimization (MPO) score [149], as a guide to the likelihood the compound may be CNS drug like, clearly shows the current chemical matter is outside this space. Furthermore, as a consequence to the high concentration of ATP present in the cell, any potential kinase inhibitor will need to have high potency to be able to compete at a mass balance level.

For a CNS indication, such as PD, clearly having free drug in the brain at the site of action is an absolute requirement. Moreover, to minimize the potential for off-target toxicology, the requisite concentration of free drug in the brain should be achieved with minimal exposure in the periphery. Combining all these attributes generates a CNS kinase design strategy that incorporates the selection of chemical matter that has low MW and is polar and neutral with high brain availability. These compounds additionally will need to have high ligand binding efficiencies (LE), lipophilic efficiencies (LipE), and low efficacious concentration (C_{eff}), all the while having high kinome selectivity.

The current public domain LRRK2 kinase inhibitor chemical matter will be summarized with an eye to how they compare to the design strategy articulated (vide supra).

4.1 LRRK2 Patent Space Analysis

As a target, LRRK2 was first identified in 2004 and its connection to PD was confirmed by genome-wide associate studies (GWAS) in 2010 (For a recent PD genetics review: [150]). This short-time frame coupled with the lack of its (patho) physiological role(s) (vide supra) has presented a challenge to the medicinal chemist as witnessed by a relatively small compound set compared to targets with a more rich medicinal chemistry history. Conversely, the highly conserved nature of the ATP-binding site of kinases does give rise to the great potential for crossing-over of chemical scaffolds from inhibitors designed for alternate kinases. Indeed, the first set of LRRK2 kinase inhibitors investigated were compounds repurposed from other kinase programs (vide infra). A handful of LRRK2 selective kinase inhibitors have been published in the primary literature; however, they are typically specific examples from a greater set of compounds claimed in the LRRK2 patent literature. The Markush structures for this set of LRRK2 kinase inhibitors are presented in Fig. 5. In general terms, the LRRK2 ATP-binding site can accommodate known core scaffolds that incorporate 1-, 2-, and potentially 3-point hinge-binding motifs. Addressing the safety, CNS design features, and chemical novelty will arise from how these scaffolds are adorned with requisite substituents.

The attributes that the medicinal chemist has the greatest design control over are the physicochemical properties. All subsequently measured compound properties are embedded once the structure has been fixed. Figure 6 provides an overview of the LRRK2 patented chemical matter as it relates to physicochemical space. The box plots provide a summary of each property showing the data distribution with the average (red line), the CNS drug set average (green line), and the marketed kinase set (black dashed line). MW shows a normal distribution with the average between those for the kinase and CNS drug sets. This tendency towards the CNS drug set is not observed for PSA with the LRRK2 and kinase sets being essentially the same. Measures of lipophilicity (clogP and logD) again show a normal distribution with the average closer to the CNS drug set. HB donor count (HBD) for the LRRK2 compounds will be skewed to the kinase set due to the required hinge interactions, and this was observed. The distribution for pK_a appears to be bimodal for the LRRK2 set, but the average tends closer to the CNS drug set.

Figure 7 provides an overview of the probability of in vivo toxicology findings (tox plot) [151]. The upper left quadrant (clogP > 3 and PSA < 75) was determined to have the greatest likelihood of in vivo safety findings. Conversely, the lower right quadrant (clogP < 3 and PSA > 75) was found to have the lowest probability of a safety finding. The pie charts are scaled to the number of compounds in each quadrant, and Fig. 7 is color coded for CNS MPO desirability. Clearly, most of the compounds reside in good CNS space (MPO > 4), but a significant number of these compounds reside in physicochemical space expected to give rise to safety findings.

Figure 8 illustrates the breakdown of chemical matter by the patent assignee. Again, most organizations are working in chemical space that is congruent with CNS drugs, and in several instances, the companies have leveraged existing chemotypes from marketed kinases (Fig. 5). John Hopkins University has patented the use of existing kinase inhibitors, e.g., staurosporine, damnacanthal, SP600125,
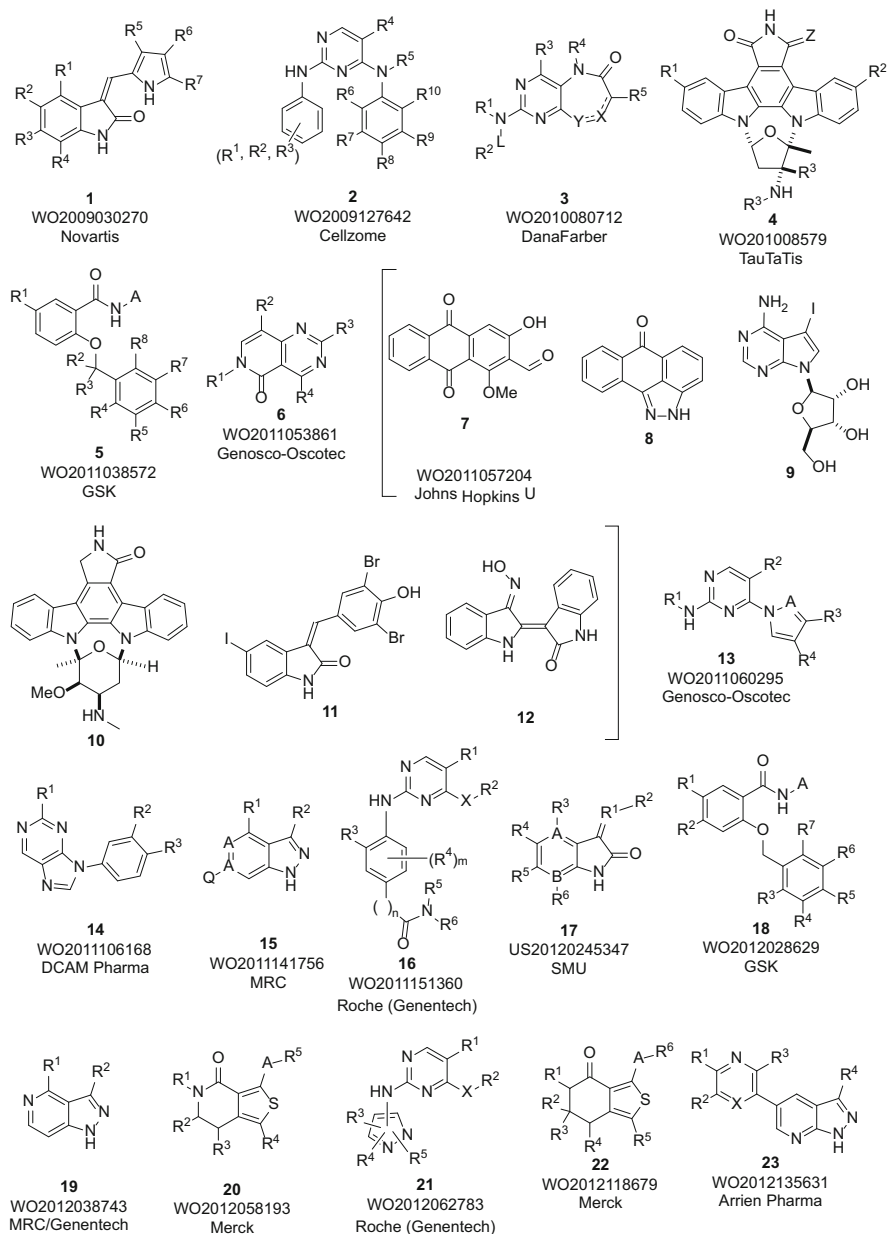
1
WO2009030270
Novartis

2
WO2009127642
Cellzome

3
WO2010080712
DanaFarber

4
WO201008579
TauTaTis

5
WO2011038572
GSK

6
WO2011053861
Genosco-Oscotec

7

8

9

WO2011057204
Johns Hopkins U

10

11

12

13
WO2011060295
Genosco-Oscotec

14
WO2011106168
DCAM Pharma

15
WO2011141756
MRC

16
WO2011151360
Roche (Genentech)

17
US20120245347
SMU

18
WO2012028629
GSK

19
WO2012038743
MRC/Genentech

20
WO2012058193
Merck

21
WO2012062783
Roche (Genentech)

22
WO2012118679
Merck

23
WO2012135631
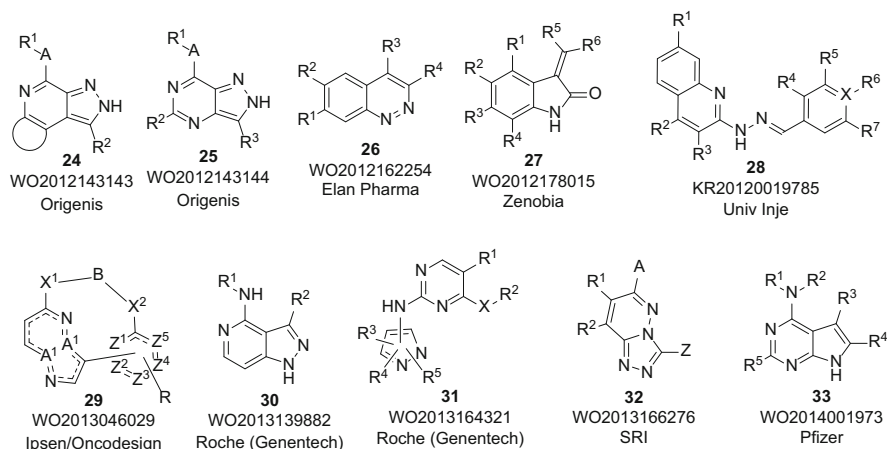Arrien Pharma

Fig. 5 (continued)

Fig. 5 LRRK2 kinase inhibitor Markush structures (chronological order)

5-iodotubercidin, GW5074, and indirubin-3′-monoxime as LRRK2 inhibitors, whereas Tautatis focused on modified versions of staurosporine analogues. Novartis, Southern Methodist University, and Zenobia focused on the oxindole framework as exemplified by sunitinib (Sutent®). The diaminopyrimidine core can be found in patents from Cellzome, Dana Farber, and Roche (Genentech). The Medical Research Council alone and in collaboration with Genentech, DCAM Pharma, Merck, Arrien, Origenis, Pfizer, and Southern Research Institute have all generated variations on a 6,5-fused bicyclic framework. Ipsen/Oncodesign extended the use of this scaffold by exemplifying macrocyclic variants of this core. GSK has disclosed a novel LRRK2 kinase inhibitor scaffold in the form of an aryl ether amide.

Comparing this set of compounds, as a whole, to the data for CNS drugs and marketed kinases (vide supra) provides potential insight as to the position in chemical space these compounds have relative to the space currently defined by the CNS drug and marketed kinase sets. All these compounds are ATP-competitive inhibitors presumably targeting the DYG-in conformation as the equilibrium distribution between the DYG-in and DYG-out (inactive conformation) appears to be skewed towards the active conformation by 5–6 kcal/mol [131]. Despite the lack of a LRRK2 crystal structure, one can leverage the knowledge from the kinase literature and predict that these compounds will interact with the hinge motif in a 1- or 2-point manner. Thus, to drive selectivity and ultimately safety, the substitution pattern from the core is crucial to accomplish this requisite attribute. From homology models, Genentech has identified two critical residues necessary to drive LRRK2 selectivity. These include the hinge residue Leu[1949] and the activation loop residue Arg[1957]. In the case of the diaminopyrimidines, Genentech was able to increase selectivity to off-targets (e.g., MST2 and JAK1) with substituents on the pendant aniline or aminopyrazole moieties.
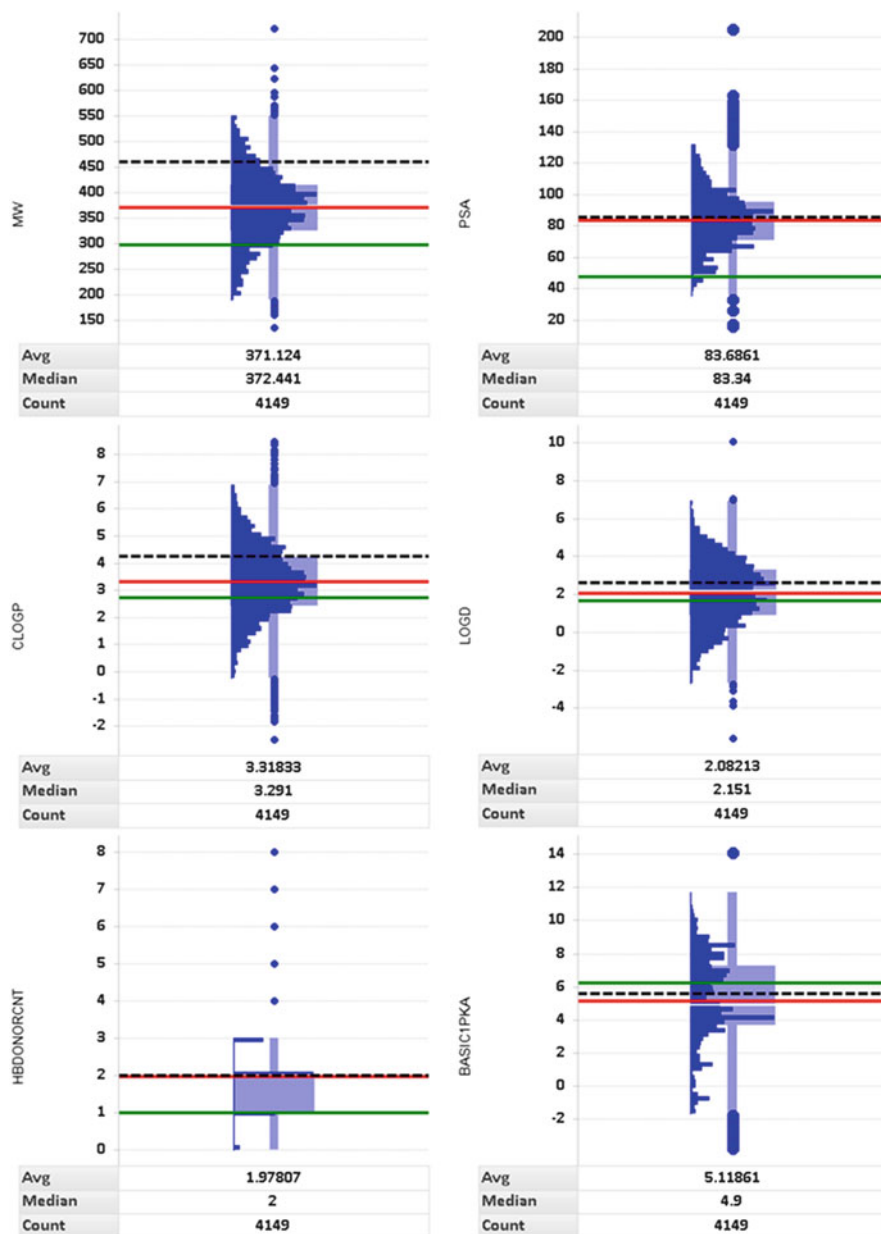
Fig. 6 Physicochemical analysis of LRRK2 patent chemical matter. Each box overlays the corresponding distribution for the descriptor on its box plots. The *dots* represent outliers and the *lines* denote LRRK2 data set average (*red*), CNS drug set average (*green*), and marketed kinase drugs average (*black dashed*)
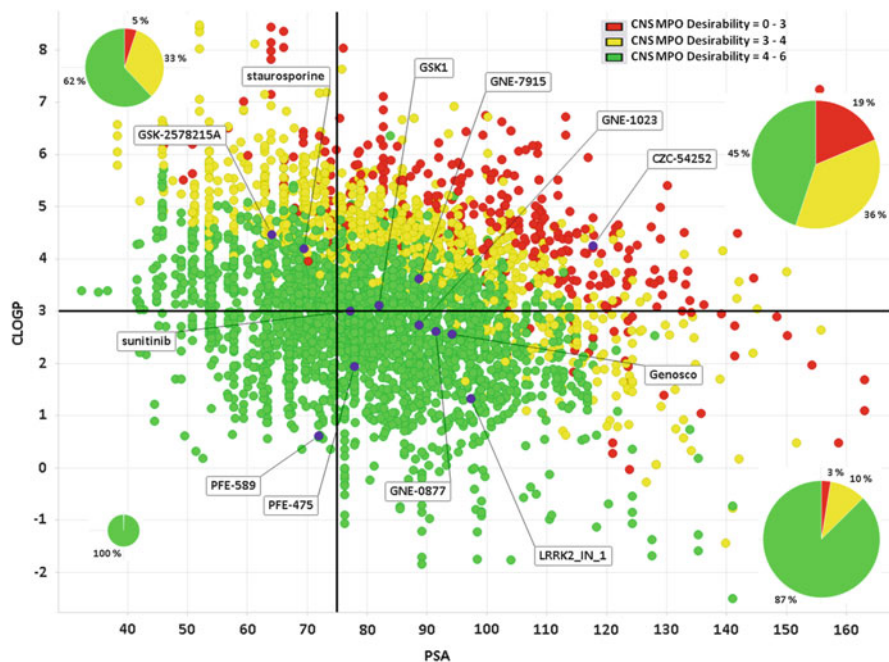
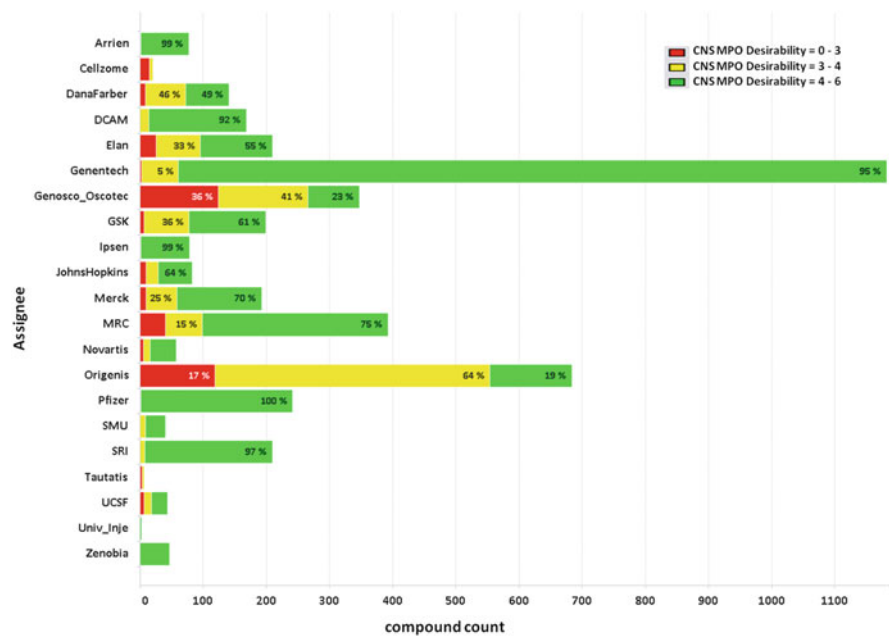**Fig. 7** Toxicity plot for LRRK2 patent chemical space (2009–2014)

**Fig. 8** CNS MPO desirability analysis for LRRK2 patents (2009–2014)

4.2 LRRK2 Chemical Matter Overview

It has been suggested that the ability to study a protein's function is enhanced when small molecule tools for the target exist [152]. Ideally a "compound toolbox" would be available, covering different chemical scaffolds and physicochemical and ADMET properties, and these compounds would be well characterized in terms of on- and off-target effects. With distinct scaffolds, overlap of off-target effects, for example, between the compounds is likely to be reduced, and consistent findings across the compounds would therefore be indicative of activity at the target in question.

The 28 patent applications of small molecules (Fig. 5), to date, represent roughly 10 unique chemical scaffolds. Through the use of SAR, X-ray crystal structures (surrogate crystallography), and homology models, specific residues in the LRRK2 ATP-binding pocket have been targeted for inhibitor interactions to improve potency and selectivity for LRRK2 kinase. Initial activities focused on LRRK2 potency and selectivity. As the compounds achieved these goals, focus shifted to address ADME and safety issues that were revealed as these compounds were used to expand LRRK2 biology. The diversity of the current chemical matter provides a toolbox of compounds that should enable the detailed elaboration of LRRK2 kinase function.

4.2.1 Repurposed Kinase Inhibitors

Early work in the field of LRRK2 kinase inhibitor development sought to compliment the range of biochemical tools that were becoming available [153] with small molecule kinase inhibitors. By screening panels of commercial compounds, groups began to identify known kinase inhibitors that also displayed activity against LRRK2 [35, 78, 154]. Initially identified compounds, using LanthaScreen detection and a surrogate peptide substrate (LRRKtide), included **7–9** and **11–12**. Staurosporine, **10**, was one of the most potent LRRK2 inhibitors reported (wt $IC_{50} = 6$ nM); however, it is also highly promiscuous across the kinome [155]. Capitalizing on this observation, **4** illustrates how modifications to the bis-indole core were pursued to potentially address the selectivity issue. Sunitinib, **34**, is a potent LRRK2 inhibitor (wt $IC_{50} = 37$ nM), but also displays similar promiscuity. Related indolidinones (**1**, **17**, and **27**) have been disclosed. Screening of a number of Rho-kinase inhibitors led to the identification of **35** [35]. Although it has a number of off-target effects, selectivity is much improved over **10** and **34**, although potency against LRRK2 is also reduced (wt $IC_{50} = 244$ nM).

34   **35**
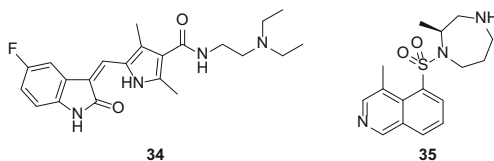
Although these early inhibitors are structurally different, they have in common potent activity across a range of kinases, and thus it is difficult to separate effects at LRRK2 from off-target effects, especially when examined in isolation. These compounds proved useful for initial study of the function of LRRK2; however, it was clear that the field could benefit from more selective compounds.
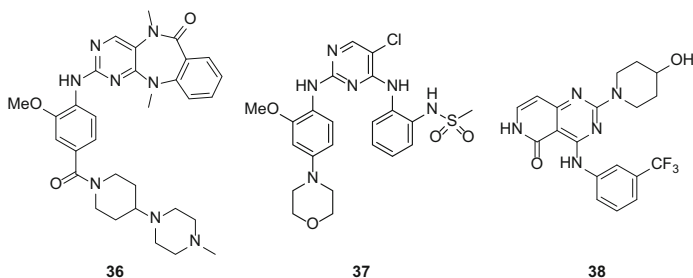
### 4.2.2 First-Generation LRRK2-Focused Kinase Inhibitors

In 2011, the first LRRK2-optimized kinase inhibitor LRRK2-IN-1, **36**, was reported (derived from **3**) [143]. Due to its high potency against both wild-type and mutant LRRK2 (IC_{50} values of 13 and 6 nM, respectively, measured in cell-free enzymatic assays), and its improved selectivity across a range of kinases, it was clearly a vast improvement on previous compounds and was initially employed throughout the field as a selective LRRK2 inhibitor. While **36** is potent against both LRRK2 WT and G2019S mutant enzymes, it has been demonstrated to display moderate potency in a whole cell assay, 200–600 nM [40, 146]. Further, high doses are required to observe any in vivo effects due to its low permeability and high levels of plasma protein binding. Additionally, no CNS effects were observed as **36** had low brain availability (unbound C_{brain}/unbound C_{plasma}). Although **36** demonstrated improved selectivity in comparison to previous LRRK2 inhibitors, it has some significant off-target potency, most notably displaying equipotent activity against ERK5 (BMK1, MAPK7), DCAMKL, PLK1, and PLK4. This off-target activity confounded some of the biological assays in which the effects of **36** are attributed to LRRK2, in particular when assessing effects on neurite outgrowth and inflammation end-points [40]; consequently, use of **36** has diminished.

4.2.3 Second-Generation LRRK2-Focused Kinase Inhibitors

As intensive research into the function and dysfunction of LRRK2 and its mutants continued, a number of other tool compounds and inhibitor series have been published from both academic and industrial laboratories. The most potent of these derived from **2**, in both enzyme (5–7 nM in WT and G2019S, respectively)

and cell-based assays (attenuation of G2019S and R1441C-induced neuronal injury and death in a concentration-dependent manner with an EC_{50} of <10 nM), was CZC-54252 (**37**) reported in 2011 [156]. Unfortunately, **37** had disappointing selectivity across the kinome. Although CZC-54252 may prove a useful tool for cell-based assays, its poor CNS MPO score (2.12) suggested it was outside of CNS space which will limit its application in in vivo models (confirmed with a reported brain availability of 0.05).

The diaminopyrimidine core has been extensively examined by several groups. In addition to compounds derived from **2** and **3** (vide infra), variations including analogues from **6**, **13**, **16**, **21**, and **31** have been reported. An early example is exemplified by **38** (CNS MPO = 3.97), but more advanced analogues successfully balanced good physicochemical properties with high degrees of LRRK2 selectivity [157, 158]. Focusing on diaminopyrimidine HTS hits, due to high ligand efficiency and reasonable PK properties, optimization of the hits using a LRRK2 homology model to identify both key interactions for LRRK2 potency and sites that may offer enhanced kinase selectivity yielded **39** (MPO = 5.37) with improved kinase selectivity over their initial hit, suitable in vivo exposures, and similar cellular activity (IC_{50} = 389 nM) to **36**. While disclosed in a patent the previous year (WO2012062783; see Fig. 5, above), **39** was subsequently described as HG-10-102-01 and reported to exhibit in vivo mouse brain activity [132].


39 **40** **41**
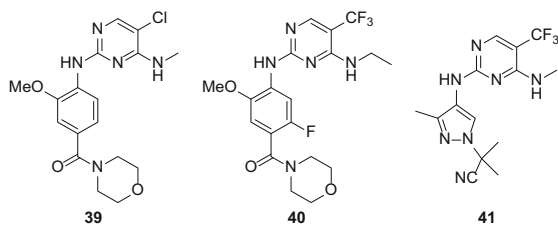
Having identified TTK (MPS1) as the major off-target interaction of this series, and significant safety concern, continued optimization by Genentech scientists, using structure-based drug design initially arrived at **40** (CNS MPO = 4.60), a compound having excellent selectivity for LRRK2 [145]. The in vivo activity of this compound and related analogues was shown using dephosphorylation of S1292 as a measure of LRRK2 kinase activity. Although **40** is an excellent tool compound for probing the effect of LRRK2 inhibition in vivo, it has some major liabilities, thus further optimization was needed for progression towards a potential clinical candidate. As the diaminopyrimidine forms the key hinge interactions with LRRK2, optimization was focused around the side chain. The aniline motif, which could lead to idiosyncratic toxicity findings, was replaced with an aminopyrazole, and side chains to enhance aqueous solubility were incorporated [133–135]. This optimization lead to GNE-0877, **41** (CNS MPO = 5.45), which has been progressed to preclinical safety studies (see Sect. 2.6, above).

4.2.4 Third-Generation LRRK2-Focused Kinase Inhibitors

Moving away from diaminopyrimidines, several other chemotypes have been reported. A series defined by **5** and **18**, while likely to be ATP-competitive, do not contain a common hinge-binding motif [135]. This attribute may be responsible for the excellent kinase selectivity. GSK2578215A, **42**, is a representative analogue from this series and showed reasonable potency ($IC_{50} = 47$ nM). However, it has poor rodent PK, with a relatively low oral bioavailability ($\%F = 12$) and half life of 1.1 h [159]. The lack of LRRK2 inhibition observed in the brain (potentially predicted by its CNS MPO score of 3.82), in comparison to kidney and spleen, suggested low levels of unbound compound in brain potentially limiting the use of this series for in vivo models.

Quinoline derivatives, **26** and **28**, are known single-point hinge binders and have been found to be good LRRK2 kinase inhibitors [139, 147]. Although the cinnoline variants [141] were potent LRRK2 inhibitors (wt $IC_{50} = 7$ nM), they were found to be promiscuous in a small kinase panel. By focusing the core to quinoline [138], e.g., **43**, kinase selectivity was significantly improved and in vivo activity was demonstrated (CNS MPO = 5.05).
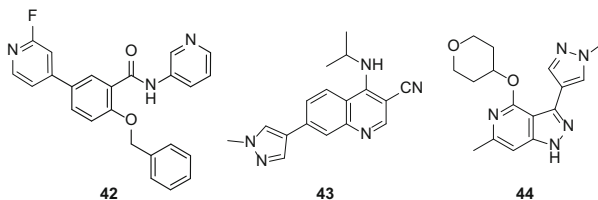
Pyrazolopyri(mi)dines, **15**, **19**, **23–25**, and **30**, have been well represented in the patent literature. For analogues, such as **44** (CNS MPO = 5.83), potency is comparable to **36** with overall good ADME [159]. Alternate 6,5-fused ring systems, **14**, **20**, and **22**, have been reported; however, the pyrrolopyrimidine **33** appears to have the best alignment of properties. Specifically, **45** (CNS MPO = 5.83) is one of the most active LRRK2 inhibitors in vivo (brain free drug wt $IC_{50} = 15$ nM) along with high kinase selectivity [160].
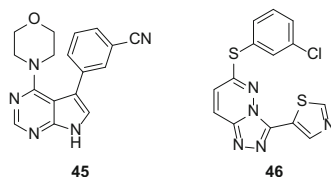
One final variation of the 6,5-fused ring system is illustrated by **32**. These compounds are also postulated to be single-point hinge binders, interacting through one of the triazole nitrogens. A representative compound from this series, **46** (CNS MPO = 4.88), displayed excellent in vitro potency (wt $IC_{50} = 31$ nM; G2019S $IC_{50} = 8$ nM) against LRRK2, but this did not translate to cellular assays resulting in a 100-fold right

shift [137]. As witnessed with other scaffolds (vide supra), compounds from this chemotype would make good tool compounds for probing LRRK2 in vitro biology.

4.2.5 Pharmacological Profiles for Key LRRK2-Focused Kinase Inhibitors

As the relatively young LRRK2 field of research has evolved, there have been frequent advances in the biochemical reagents available, as the biology has developed. In part, this has been facilitated by the improvement of the chemical tools (vide supra). However, the literature data documenting the pharmacological profiles of the tool compounds is often not consistent in terms of the assays or conditions used in reporting their potencies. Thus, Table 3 presents a head-to-head comparison of several of the top compounds, from our internal assessments, with the intention of providing LRRK2 researchers with additional data to better enable selecting of the best compound(s) for their studies.

The LRRK2 inhibitors described below have been characterized in a number of in vitro and in vivo models. Activity against LRRK2 wild-type and G2019S mutant was assayed using truncated enzyme from Invitrogen in a LanthaScreen format at 50 μM and 1 mM ATP concentrations [58]. The whole cell potency is measured in HEK293 cells transfected with WT LRRK2, using the antibody against pS935 [64]. The RRCK (Ralph Russ Canine Kidney) assay is a measure of passive permeability, using a canine kidney cell line which expresses low levels of efflux transporters [161]. MDR1 is a measure of efflux by human P-gp transporter and is used to assess the likelihood of brain impairment [162]. HLM and RLM are an assessment of metabolism in human and rat liver microsomes, respectively. Several safety assays are performed; "dof" is a measure of the ability of compounds to displace [^{3}H]-labeled dofetilide from the hERG K$^+$-channel, important for cardiac safety in clinical candidates [163]. General cytotoxicity is measured using the THLE assay [164]. Effects on cell health are followed up with a panel of assays performed in HepG2 cells, measuring mitochondrial function [165]. Kinome selectivity is reported from a Dundee Panel [166] using recombinant enzymes at the ATP K_M or ActivX data [167], which used lysed PBMC cells to assay selectivity in a more native system.

Table 3 Pharmacological profile of key LRRK2 kinase inhibitors

	Assay	36	37	42	40	45
LRRK2 (in vitro)	1 mM ATP LRRK2 IC_{50} (nM)	68	22	150	37	8
	1 mM ATP LRRK2 (G2019S) IC_{50} (nM)	25	13	247	23	34
	pS935 WCA LRRK2 IC_{50} (nM)	233	4.95	992	194	53
	ActivX huPBMC LRRK2 IC_{50} (nM)	> 300 [142]	15	400	nt	15
Physico-chem	LE	0.23	0.31	0.31	0.33	0.48
	LipE	5	4.1	2.5	3.5	5.5
	CNS MPO	4.52	2.12	3.82	4.6	5.83
ADME	RRCK AB (x 10^{-6} cm/sec)	3.88	14.7	2.87	12.7	27.2
	MDR1 ER	22	3.67	2.24	1.07	1.02
	HLM $CL_{IA,S}$ (mL/min/kg)	22	17	< 8	< 8	36
	RLM $CL_{IA,S}$ (mL/min/kg)	39	101	nt	nt	89
Safety	Dof IC_{50} (µM)	32	16	> 100	50	44
	HepG2 (24h) Gal IC_{50} (µM)	90	> 300	> 300	103	> 300
	HepG2 (24h) Glu IC_{50} (µM)	103	> 300	> 300	159	> 300
	HepG2 (72h) Glu IC_{50} (µM)	26	112	> 300	42	> 300
	THLE Cv IC_{50} (µM)	18	17	> 300	75	> 223
Rat BA	C_{bu}/C_{pu} (AUC)	< 0.1	nt	nt	0.5	1
KSS	Dundee Panel 1 µM (hits\total)	2/121	15/131	2/131	20/140	7/159[a]
LRRK2 (in vivo)	Mouse brain free drug pS935 IC_{50} (nM)	7,798[b]	nt	nt	75	<20

Colors represent a stop-light analysis of the data based on Pfizer cut-offs (green low; yellow moderate; red high)
[a] ActivX data at 1 µM
[b] Kidney data. *nt*, not tested. See text for description of assays

5 Summary, Conclusions, and Outlook

The answers to some key questions are important for a more thorough understanding of LRRK2 structure, function, and the development of inhibitors, including how the Roc domain regulates the kinase domain, what the role of the COR domain is, and how the pathogenic mutations alter the function of the LRRK2 protein. X-ray crystal structures of the LRRK2 enzymatic domains, including both WT and the pathogenic mutations, could contribute to an understanding of these questions and would be a key accomplishment to help drive inhibitor design.

The strength of research in the field of LRRK2, both academic and industrial, has generated a wealth of chemical tools for the study of LRRK2. Where the initially reported compounds had activity across multiple kinase targets, new inhibitors have a high degree of kinome selectivity along with improved physicochemical properties, providing opportunities to study inhibition of LRRK2 kinase activity

from the isolated enzyme through to in vivo assays. Ultimately, this collection of inhibitors should allow researchers the flexibility to design experiments to probe LRRK2 function, knowing that they have the right tools for the job.

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