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Publication details:
Future Neurology
v. 8
Chapter No. 3
pp. 347-357
1479-6708 (ISSN)

Publication Date:
2013

Publisher DOI:
http://dx.doi.org/10.2217/fnl.13.9

Unlocking the secrets of LRRK2 function with selective kinase inhibitors.

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Abstract/Summary:

Leucine rich repeat kinase 2 is currently considered a potential therapeutic target for the treatment of Parkinson's disease. A number of pathological mutations, all of which lie in the dual catalytic domains of LRRK2, segregate with Parkinson's disease in an autosomal dominant fashion. The most common mutation, G2019S, results in an increase in the kinase activity of LRRK2 and much work has therefore gone into the development of potent and specific inhibitors of LRRK2 kinase activity. A number of LRRK2 kinase inhibitors have now been employed in the search for the physiological function of LRRK2 and the targets of LRRK2 kinase activity.

Keywords:

Parkinson's, kinase, LRRK2-IN1, CZC25146, TLR, inflammation, WNT, mitochondria, neurodegeneration, endocytosis

Introduction:

Parkinson's disease (PD) is the second most common neurodegenerative movement disorder worldwide. It presents clinically with motor symptoms such as bradykinesia

and resting tremor resultant from a significant loss of dopaminergic neurons from the substania nigra. PD is defined postmortem by the presence of alpha-synuclein rich protein inclusion bodies, termed Lewy bodies, in the brain. Once considered unlikely, it is now clear that in a growing number of cases PD can be inherited. Advances in DNA sequencing technology has allowed a number of PD associated genes and the proteins they encode to be identified by linkage studies (*1*).

Discovered in 2004 as the PARK8 loci segregating with familial PD (*2, 3*), leucine-rich repeat kinase 2 (LRRK2) has become a major focus for drug development. Inherited LRRK2-related PD is largely clinically indistinguishable from the more common sporadic form of PD (*4*), suggesting a possible role for LRRK2 in both forms of the disease. Indeed, LRRK2 risk variants have also been uncovered in sporadic PD genome wide association studies (*5-7*). LRRK2 contains both an active GTPase domain and a serine/threonine protein kinase domain, with a complex interplay between the two domains evident. Importantly, the most common LRRK2 pathogenic mutation, G2019S, is located in the kinase domain and results in a constitutive 2-3 fold increase in kinase enzymatic activity (*8, 9*). Due to the success of kinase inhibitors in the oncology field, platforms and processes for developing and screening kinase inhibitors are well established and the generation of specific and potent inhibitors of LRRK2 has been underway for some time. The literature shows a number of inhibitors published by major pharmaceutical companies, revealing the hope that these compounds may become PD therapeutics. However, there is still a need to generate greater evidence that pharmacological inhibition of LRRK2 will be of benefit to PD patients, with or without the activating G2019S mutation. A current problem with obtaining this evidence lies with the cryptic nature of LRRK2

physiological function, in particular it remains largely unknown what LRRK2 kinase activity actually does. The increasing availability and use of LRRK2 kinase inhibitors will likely help deduce LRRK2 function. Emerging results thus far suggest diverse roles for LRRK2 in signal transduction, inflammation, neurogenesis and synaptic vesicle localization.

Early non-selective LRRK2 inhibitors:

Although their selectivity profiles essentially rendered them unsuitable for studies of LRRK2 function, the first described LRRK2 kinase inhibitors found utility *in vitro* for the establishment of biochemical screening assays for future LRRK2 inhibitors. This collection, including the staurosporine analogue K252a, PKR inhibitor, LDN-22684, the multi tyrosine kinase inhibitor sunitinib and the rho kinase (ROCK) inhibitors hydroxyfasudil, Y-27632 and H-1152, could inhibit LRRK2 with IC50's for wildtype LRRK2 ranging from 0.02 μM (Sunitinib) to 7 μM (hydroxyfasudil) (*10-12*). Interestingly a number of inhibitors had a greater effect on G2019S mutant LRRK2 over wild-type, a property that can potentially be exploited to generate compounds that will preferentially target mutant G2019S LRRK2 (*10*).

As well as for *in vitro* screening, the first generation LRRK2 kinase inhibitors were also instrumental in establishing cell based *in vivo* screens. The most common screen described to date involves assessing the phosphorylation status of LRRK2 at two serine residues (ser910 and ser935). These residues are constitutively phosphorylated and are required for interaction of LRRK2 with members of the 14-3-3 adaptor protein family (*13*). This cell-based screen arose following observations that treatment of cells with either H-1152 or sunitinib resulted in a dose-dependent decrease in the

phosphorylation of endogenous LRRK2 at these residues, concomitant with dissociation of 14-3-3 from LRRK2 (*14*). Dephosphorylation of LRRK2 at these residues has subsequently been observed with a number of more selective LRRK2 kinase inhibitors (*15*). Furthermore, other LRRK2 residues including Ser955 and Ser973 are also dephosphorylated when cells are treated with LRRK2 kinase inhibitors (*16*). Although their phosphorylation is controlled by LRRK2 kinase activity, none of these residues appear to be bona fide LRRK2 autophosphorylation sites (*14, 16*), suggesting a more complex feedback loop involving other protein kinases is in operation. Using GFP-LRRK2 expressing cell lines it was also discovered that dephosphorylation of LRRK2 at ser910 and ser935, either by point mutation or pharmacologically with a kinase inhibitor, resulted in an altered localization of LRRK2 (*13, 14*). The GFP-LRRK2 in inhibitor treated cells co-localized to microtubules (*14*), a cytoskeletal component that has been shown by other investigators to interact with LRRK2 (*17*). The altered localization of GFP-LRRK2 was not seen when an inhibitor-resistant mutant form of LRRK2 (A2016T) was expressed (*14*), suggesting that the regulation of LRRK2 phosphorylation, 14-3-3 binding and subcellular localization are dependent on LRRK2 kinase activity. To what extent the localization phenomenon occurs outside of GFP-LRRK2 overexpressing cells however, is currently unknown.

With increased screening, potent inhibition of LRRK2 kinase activity has now been observed as an off target effect for a number of additional compounds including the Raf kinase inhibitors GW5074 and sorafenib, and the anaplastic lymphoma kinase (ALK) inhibitor TAE684 (*18, 19*). In experiments employing overexpression models of LRRK2 G2019S induced neurodegeneration in *C. elegans* and *Drosophila*, both

GW5074 and sorafenib enhanced neuron survival whereas another Raf kinase inhibitor ZM336372 could not (*20*). Both GW5074 and sorafenib, like sunitinib, target a number of receptor tyrosine kinases, however, this work has been recently reproduced using more selective inhibitors (*21*). These studies in lower model organisms have been a large step forward in the progression of LRRK2 inhibitors to PD therapeutics.

First generation selective LRRK2 inhibitors: LRRK2-IN1 and CZC25146

Screening for LRRK2 kinase inhibitors inevitably moved from readily available known compounds to screening for novel LRRK2 specific compounds. The first in the field was LRRK2-IN1, a potent inhibitor of LRRK2 with an IC50 for wild-type LRRK2 of 0.013 μM (*15*). Although the selectivity profile of LRRK2-IN1 was a marked improvement over current inhibitors, LRRK2-IN1 is still an equipotent inhibitor of LRRK2 and ERK5, with DCLK1 another off target kinase (*15*). The even more potent CZC25146 (IC50 for wild-type LRRK2 = 0.005 μM) soon followed (*22*). CZC25146 also has reasonable selectivity, inhibiting a reported five additional kinases (PLK4, GAK, TNK1, CAMKK2 and PIP4K2C). This compound was subsequently able to protect against neurite retraction and cellular toxicity resultant from overexpression of mutant LRRK2 in primary cortical neurons (*22*). Both of these compounds fail to cross the blood brain barrier limiting usefulness for assessing effects of LRRK2 inhibition in whole animal models of neurodegeneration. Nonetheless, LRRK2-IN1 and CZC25146 have become important tool compounds for assessing LRRK2 function in cellular models.

LRRK2 inhibition and the immune system

The first publication utilizing LRRK2-IN1 to explore LRRK2 function identified that this compound (along with K252A and sunitinib) regulated interferon-gamma (IFNγ) induced expression of monocyte cluster of differentiation markers CD14 and CD16 (*23*). The expression of these cell surface markers is robustly increased by IFNγ, a cytokine traditionally secreted by natural killer cells and T-lymphocytes in response to infection as a part of the monocyte activation process. Treatment of human monocytes with LRRK2-IN1 substantially impaired the IFNγ induced expression of CD14 and CD16 (*23*). The authors also found that LRRK2 expression was higher in the more mature, pro-inflammatory $CD14^{+}CD16^{+}$ monocyte population than in $CD14^{+}CD16^{-}$monocytes leading to the conclusion that LRRK2 may be important for monocyte maturation. LRRK2 expression itself can be additionally increased in these cell types by IFNγ, and possibly agonists of innate immune toll-like receptor (TLR) signaling such as the bacterial lipoprotein LPS (*24, 25*). Furthermore, LPS stimulation results in increased phosphorylation of LRRK2 on serines 910 and 935 in mouse bone marrow derived macrophages (*26*). The phosphorylation of LRRK2 on the two 14-3-3 binding residues is mediated by IKK family kinases when activated by MYD88 dependent toll-like receptor (TLR) signaling, potentially implicating LRRK2 as a direct component of the inflammatory TLR signaling pathway. Intriguingly, treatment with LRRK2-IN1 significantly reduced expression of the inflammatory cytokine TNFα following activation of primary rat neonatal microglia with LPS (*27*). Although requiring further confirmation using additional selective LRRK2 inhibitors, this is of substantial interest as inflammation is increasingly being implicated in the pathogenesis, and even arguably the onset, of PD. Emerging evidence suggests that PD may begin following an immune event in the gut (*28*) whilst a number of publications have shown increased inflammatory cytokines and chemokines in serum

and CSF of PD patients (for further reviews see (*29, 30*)). These studies suggest a role for LRRK2 kinase activity to regulate aspects of monocyte/macrophage/microglia biology in relation to innate immune inflammatory signaling. Potential mechanisms by which LRRK2 kinase activity may mediate these effects are currently unknown and the identification of LRRK2 substrates in immune pathways would be of importance to identify.

LRRK2 inhibition and mitochondrial depolarization

Mitochondrial dysfunction has long been implicated in PD pathogenesis with the mitochondrial associated genes that cause familial PD in a recessive manner, PINK1 and Parkin, particularly involved (*31*). Although predominantly a cytoplasmic protein, a small subset of LRRK2 seems to localize to membrane structures that include mitochondria (*32*). Intriguingly, fibroblasts derived from PD patients harboring the G2019S mutation have increased oxygen consumption and reduced cellular ATP levels compared to control fibroblasts (*33, 34*). A similar phenomenon was observed when G2019S mutant LRRK2, but not wild type LRRK2, was overexpressed in SHSY5Y cells with both LRRK2-IN1 and CZC25146 treatments able to block the G2019S LRRK2 mediated increased oxygen consumption (*34*). Proton leakage resulting from upregulation of uncoupling proteins (UCP) was deduced as the reason for increased oxygen consumption in G2019S LRRK2 transfected cells, with the increased expression of UCP being blocked by LRRK2-IN1 (*34*). Interestingly, increased kinase activity induced by overexpression of wild-type LRRK2 had no effect on oxygen consumption compared to untransfected cells, suggesting that this mechanism is dependent on a particular property of G2019S LRRK2. Intriguingly, the opposite result of decreased oxygen consumption has been reported in LRRK2

G2019S iPSC-derived neural cells (*35*). No evidence of proton leakage was detected but rather G2019S neural cells had mitochondria that were more motile. Moreover, G2019S iPSC-derived neural cells were more susceptible to membrane stress induced by the potassium ionophore valinomycin, an effect that was reduced by the treatment of cells with GW5074 (*35*). Reconciling these studies is difficult. The use of patient derived cells presumably requires analysis of multiple lines and/or gene correction studies to determine the predominant phenotype. Nonetheless, the overall outcome suggests inhibition of LRRK2 may be beneficial for rescuing defects associated with altered mitochondrial membrane potential.

LRRK2 inhibition and WNT signaling

The complex WNT/β-catenin signal transduction pathway regulates a number of important processes, particularly during development. Mutations, which can be either loss or gain of function, in components of this signaling cascade result in a number of developmental conditions and are also implicated in cancer and diabetes (*36*). To a lesser extent this pathway has also been implicated in neurodegenerative disease. Under basal conditions, β-catenin is a component of a multi-protein complex where it is phosphorylated by GSK3β, resulting in its being targeted for degradation. Canonical activation of the WNT signaling pathway by binding of WNT proteins to their receptors, termed frizzled proteins, results in the release of the transcription factor β-catenin from this complex. No longer phosphorylated by GSK3β and therefore not targeted for degradation, β-catenin enters the nucleus to induce TCF/LEF dependent gene transcription. In Alzheimer's disease (AD) recent data suggests that the natural WNT inhibitor protein Dickkopf 1 (dkk1) is upregulated in both human postmortem brain and in an AD animal model (*37, 38*). Subsequent

inhibition of WNT signaling results in increased GS3Kβ activity and phosphorylation of β-catenin and may contribute to the hyper-phosphorylation of tau seen in AD (*39*). In PD however the situation is less clear. Treatment with dkk1 to inhibit WNT signaling potentiated 6-hydroxy-dopamine-mediated neuronal loss in rats (*40*), whilst increased WNT signaling has been implicated in neuronal cell loss in Parkin null mice (*41*). LRRK2 has been shown to interact with a number of components of the multi-protein β-catenin-destruction complex including disheveled (DVL), GSK3β, β-catenin, Axin1 and low-density lipoprotein receptor related 6 (LRP6) (*42, 43*). Co-expression of LRRK2 with DVL proteins, but not overexpression of LRRK2 itself, increased canonical WNT signaling as determined by luciferase assay (*43*). Increased WNT signaling was dependent on LRRK2 kinase activity as increased WNT signaling was blocked by either LRRK2-IN1 treatment or the use of kinase inactive LRRK2 constructs. Intriguingly, pathogenic LRRK2 mutations R1441C, Y1699C and G2019S also blunted DVL-stimulated WNT activity. This data suggests that LRRK2 mutations and indeed LRRK2 kinase inhibitors may decrease WNT stimulated signaling in PD, comparable to AD, resulting in increased GSK3β activity. Further work is required to extend these largely *in vitro* luciferase based assays to *in vivo* models of PD. Further work is also required to define the role of LRRK2 kinase activity in this pathway. Studies investigating the substrate specificity of LRRK2 show that this kinase is unlikely to phosphorylate the same residues as the proline directed GSK3β (*10, 44*).

LRRK2 and neurogenesis

A number of recent publications have taken advantage of induced pluripotent stem cells derived from PD patients with the G2019S mutation. In particular a recent study

further differentiated these cells to neural stem cells and discovered that neural stem cells with G2019S LRRK2 failed to maintain differential capacity after 14 passages (*45*). This was associated with decreased nuclear architecture leading to enlarged compartmentalized nuclei (*45*). This phenomenon appeared restricted to neural stem cells suggesting a specific role for LRRK2 in this cell type. Importantly gene correction of G2019S back to wild type reversed the effects of the G2019S mutation on neural stem cell nuclear architecture. Furthermore inhibition of G2019S LRRK2 with LRRK2-IN1 also rescued the aberrant cellular phenotype of late passage neural stem cells (*45*). Intriguingly overexpression of G2019S LRRK2 in mice has also been associated with impaired neurogenesis (*46*) further suggesting a potentially exciting role for LRRK2 in this area.

LRRK2 inhibition and synaptic vesicle localization

A very promising area of research surrounds a role for LRRK2 kinase activity in mediating synaptic vesicle endocytosis. Beginning with a yeast 2-hybrid screen that identified the vesicular transport regulating GTPase, rab5b, as a potential LRRK2 interactor, it was confirmed that LRRK2 was associated with synaptic vesicles (*47*). Subsequent overexpression of wild-type or mutant LRRK2 in neuronal cell lines impaired synaptic vesicle endocytosis but not exocytosis (*47*). A similar result on synaptic vesicle transport has also been reported for overexpression of LRRK2 in Saccharomyces cerevisiae (*48*). Further characterization of LRRK2 in the synaptasome uncovered a number of additional LRRK2 interacting proteins, including syntaxin 1 and vesicle fusing ATPase (NSF), associated with presynaptic vesicles (*49*). Most recently, evidence has been presented that LRRK2 phosphorylates an additional synaptic vesicle associated protein, endophilin A (EndoA), and that this

phosphorylation event is critical for regulating synaptic vesicle endocytosis (*50*). In this study LRRK2 was able to phosphorylate EndoA *in vitro* at serine 75, a site well conserved across species. In *Drosophila* models mimicking EndoA phosphorylation (S97D mutation) or dephosphorylation (S97A) reduced synaptic vesicle endocytosis was observed, clearly highlighting the importance of this residue. Synaptic endocytosis was also impaired in control *Drosophila* treated with the LRRK2-IN1, suggesting that LRRK2 regulates EndoA S97 phosphorylation *in vivo*. How well these finding translate to mammalian systems remains to be demonstrated. A lack of antibody to phosphorylated mammalian EndoA complicates important control experiments such as measuring reduced Endo S97A phosphorylation in LRRK2 null cells. A potential promising LRRK2 substrate previously identified in *Drosophila*, 4EBP1 (*51*), appears to not be conserved in mammalian systems (*52, 53*).

Second generation brain penetrant LRRK2 inhibitors:

At the time of writing, second generation brain penetrant LRRK2 inhibitors have begun to appear in the literature. The first HG-10-102-01 is a lower molecular weight 2,4-diaminopyrimidine based compound in the same class as LRRK2-IN1, CZC25146 and TAE684 (*54, 55*). It demonstrates similar potency to LRRK2-IN1 and maintains high selectivity with MLK1, the only known off target kinase. When administered into the peritoneal cavity in mice, HG-10-102-01 was able to cross the blood brain barrier and inhibit LRRK2 as assessed by reduced phosphorylation of brain LRRK2 at the serine 910 and serine 935 residues (*55*). Substantial optimization of this lead compound has resulted in GNE7915-18, a highly potent LRRK2 kinase inhibitor with an IC50 of 9 nM when assessed by a cell based assay reportedly examining the autophosphorylation of LRRK2 at serine1292 (*56*). The high selectivity of previously

described LRRK2 inhibitors is maintained with only TKK and ALK reported as modest off target kinases (*56*). The brain penetrance and bioavailability of GNE7915-18 is much improved and clearly acceptable for future use of this compound for *in vivo* preclinical studies.

Future challenges and potential for LRRK2 kinase inhibitors as PD therapeutics.

Is LRRK2 a therapeutic target for sporadic PD?

The majority of results to date, based largely on overexpression studies, suggest that pathogenic LRRK2 mutations impart a toxic gain of function. In an increasing number of instances this toxic gain of function is kinase activity dependent. Overexpression of mutant, but not wild-type or kinase inactive LRRK2 in cell lines, such as neuronal SHSY5Y cells, results in toxicity (*8, 21, 57*). Although generally insufficient to promote neuronal loss, bacterial artificial chromosome (BAC) overexpression of mutant human LRRK2 in mice produces age dependent decreases in dopamine transmission (*58-60*) and movement dysfunction (*59, 61*). Herpes simplex virus-mediated overexpression of LRRK2 G2019S, injected stereotactically into mouse striatum results in a loss of substantia nigra neurons, whereas overexpression of wild type and kinase dead LRRK2 do not (*19*). Microglia from R1441G BAC transgenic mice have increased LPS stimulated inflammatory cytokine release compared to wild type overexpression (*62*) and over expression of human mutant G2019S LRRK2 but not wild-type causes toxicity in *C. elegans* and *Drosophila* models (*20, 21*). Although phenotypes based on overexpression will be greatly exacerbated compared to patients heterozygous for endogenous pathogenic LRRK2 mutations, it seems plausible that LRRK2 kinase inhibitors could be of benefit for this group of PD patients. In particular, G2019S carriers may benefit

greatly from the LRRK2 inhibitors showing more potency against the G2019S mutation compared with wild-type LRRK2, as side effects are more likely to be circumvented with reduced dosages. Outside of familial LRRK2 however, the usefulness of LRRK2 inhibitors becomes less clear. Overexpression of wild type LRRK2 enhanced alpha-synuclein accumulation in transgenic A53T alpha-synuclein mice (*61*). However, this effect could also be mediated by overexpression of kinase inactive LRRK2 (*61*), and the extent of the effect itself may be mouse model dependent (*63*). Indeed, very little is known about endogenous wild-type LRRK2 in sporadic PD patients. The expression of LRRK2 and importantly kinase activity are yet to be thoroughly investigated. If LRRK2 inhibition is to comprise a frontline treatment for sporadic PD, much more needs to be known about the physiological function of wild-type LRRK2 in humans with and without PD.

What are the PD relevant LRRK2 functions?

It is evident that a growing number of roles are being prescribed to LRRK2. It is not uncommon for protein kinases to have a number of substrates and to sit at important crossroads in cellular signal transduction. Delineating the functions of LRRK2 that are most applicable to PD will be a challenge. Whether a unifying hypothesis surrounding LRRK2 function emerges or whether some functions of LRRK2 are redundant for the understanding of PD remains to be seen. Complicating matters is that PD is a heterogeneous disease. A number of clinical studies have suggested subtypes of PD that likely differ in etiology and pathogenesis (*64*). Of the potential roles described to date for LRRK2 function, it is inflammatory signaling that is seemingly of particular interest. Synonymous LRRK2 mutations, albeit different to familial PD causing mutations or sporadic PD risk factors, also comprise a risk factor

for Crohn's inflammatory bowel disease (*65*, *66*). Furthermore, loss of LRRK2 protein increases the susceptibility of mice to the widely used dextran sulfate sodium model of inflammatory bowel disease (*66*). That LRRK2 may comprise a regulatory protein in innate immune inflammatory signaling is interesting in that loss of function mutations, or loss of LRRK2 protein, could increase susceptibility to inflammatory disease whilst gain of function mutations, or increased LRRK2 protein, could stimulate the immune system mimicking inflammatory disease. The availability of pharmaceutical quality inhibitors will allow much needed work to progress to determine LRRK2 substrates, interacting proteins and physiological functions to enable a better understanding of whether inhibition of LRRK2 will be of benefit to PD patients, and if so for how many.

Is central inhibition of LRRK2 essential?

As a cause of neurodegenerative disease it has generally been assumed that it is a neurological defect in LRRK2 signaling that should be targeted. Evidence for this stems largely from the transgenic mouse studies described above where pathological phenotypes have been discovered using promoters that have been chosen to drive LRRK2 over expression in the CNS. However, constitutive expression of G2019S LRRK2 has no reported effect on the nigrostriatal pathway in knockin mice (*67*). Endogenous LRRK2 is also very robustly expressed in a number of tissues, particularly peripheral immune cells and kidney. In humans premotor symptoms, such as olfactory deficits and constipation, are often the first signs of PD and pre-motor symptoms have recently been linked to the secretion of inflammatory IL-6 (*68*). This has lead to hypotheses such as the dual hit hypothesis that suggests peripheral immune events may precede neuronal loss (*28*). In mice peripheral LPS exacerbates

alpha-synuclein mediated neuronal loss (*69*). Without robust disease biomarkers an overriding role for peripheral inflammation in human PD remains inconclusive. If however, LRRK2 mediated peripheral events do precede neuroinflammation and neuronal loss, it remains controversially possible that LRRK2 inhibitors may not need to cross the blood brain barrier, at least early in the development and progression of the CNS manifestations of PD.

Will there be long-term consequences of LRRK2 inhibition?

Encouragingly a number of studies utilizing LRRK2 knockout mice have demonstrated that these mice have normal viability and lifespan. The only commonly observed phenotype relates to the kidney, a tissue with particularly high LRRK2 expression in mouse. In the initial report, kidneys from LRRK2 knockout mice were reported as substantially smaller and darker than wild type controls (*70*). LRRK2 null kidneys displayed increased markers of apoptosis and inflammation as well as defects in lysomosal mediated protein degradation all of which were only observed in aged mice (*70*). Most recently this has been attributed to LRRK2 kinase activity as kinase inactive knockin mice also display the darker kidney phenotype, albeit with kidney hypertrophy rather than the originally described kidney atrophy (*67*). Darker hypertrophic kidneys have also been seen in a third group of LRRK2 null mice (*71*). Both the causes and physiological consequences of this kidney phenotype and abnormal lysosomal homeostasis remain unknown. Importantly, the reported phenotypes are not observed in heterozygous knockout or heterozygous kinase inactive mice (*67, 71*), which more likely mimic the reduced activity seen with pharmacological inhibition rather than mice with lifelong homozygous kinase inactive mutations or LRRK2 nulls. LRRK2 knockout mice have also been shown to be more

sensitive to experimental ulcerative colitis (*66*). This was due to a reduced ability of LRRK2 to sequester the transcription factor NFAT into the cytoplasm, resulting in increased secretion of certain inflammatory cytokines. The ability of LRRK2 to negatively regulate NFAT appears to be related to LRRK2 scaffolding function rather than its enzymatic activity (*66*). It therefore seems unlikely that kinase inhibition of LRRK2 would predispose to inflammatory bowel disease. One report however has suggested that LRRK2 kinase activity is required for LRRK2 protein stability (*67*). If this holds true then prolonged use of LRRK2 kinase inhibitors may reduce steady state LRRK2 protein levels, potentially rendering cells susceptible to inflammatory insult or defects in lysosomal homeostasis. To date this is not a common observation however, with a number of ongoing studies utilizing LRRK2 inhibitors the results should become clearer.

Future Perspective

It is likely that highly potent and selective inhibitors of LRRK2 will be generated. If results from pre-clinical models continue to provide proof of concept that inhibition of LRRK2 is of therapeutic benefit in pre-clinical models of PD. If acceptable safety profiles are maintained, then it is possible that the best compounds may continue to clinical trial. Such trials would likely involve PD patients harboring pathogenic LRRK2 mutations, particularly G2019S, as evidence that wild-type LRRK2 kinase activity plays a role in sporadic PD is currently insufficient. The discovery of substrates or the optimization of current pharmacodynamic biomarkers will allow for LRRK2 inhibitor efficacy to be monitored using peripheral blood cells. Like the majority of clinical trials for neurodegenerative diseases, timing and dosing will be critical to estimate. In addition to LRRK2 itself, the inevitable identification of future

LRRK2 substrates and interacting proteins will provide new areas of investigation for drug development.

Executive summary

Introduction:

- Mutations in LRRK2 are a cause of autosomal inherited Parkinson's disease.
- The most common LRRK2 mutation, G2019S, increases LRRK2 kinase activity.
- LRRK2 kinase inhibitors are being developed as potential PD therapeutics.

Early non-selective LRRK2 inhibitors:

- The first LRRK2 kinase inhibitors were non-selective.
- They were however essential for assay development and optimization
- Robust *in vitro* and *in vivo* screens now exist for evaluating LRRK2 inhibitors.

First generation selective LRRK2 inhibitors: LRRK2-IN1 and CZC25146

- LRRK2-IN1 was the first described selective LRRK2 kinase inhibitor.
- CZC25146 was the second described selective LRRK2 kinase inhibitor.
- These compounds have been used to probe LRRK2 function in a number of systems.

LRRK2 inhibition and the immune system

- LRRK2 kinase activity regulates monocyte maturation.
- LRRK2 is a component of the inflammatory TLR signaling pathway.
- LRRK2 kinase activity may regulate inflammatory cytokine secretion.

LRRK2 inhibition and mitochondrial depolarization

- Mitochondrial dysfunction is commonly observed in Parkinson's disease
- LRRK2 inhibitors can block the toxic mitochondrial effects observed with pathogenic LRRK2 mutations.

LRRK2 inhibition and WNT signaling

- LRRK2 interacts with numerous members of the WNT/β-catenin signaling pathway.
- Both LRRK2 kinase inhibitors and pathogenic LRRK2 mutations impair WNT signaling.

LRRK2 and neurogenesis

- Neural stem cells with the G2019S LRRK2 mutation have reduced differentiation capacity and disturbed nuclear architecture.
- These effect can be rescued with LRRK2-IN1 inhibitor treatment.

LRRK2 inhibition and synaptic vesicle localization

- LRRK2 protein is present at synaptic vesicles.
- LRRK2 may phosphorylate endophilin A to regulate synaptic vesicle endocytosis.

Second generation brain penetrant LRRK2 inhibitors:

- Brain penetrant LRRK2 inhibitors such as GNE7915-18 have been developed.

Future challenges - Is LRRK2 a therapeutic target for sporadic PD?

- Most studies demonstrate a toxic gain of function for LRRK2 mutations.
- There is little evidence that LRRK2 inhibitors will be of benefit to sporadic PD patients.

Future challenges - What are the PD relevant LRRK2 functions?

- A number of different functions are being attributed to LRRK2.
- Identifying the functions relevant to different forms of PD is challenging.
- LRRK2 is also a risk factor for Crohn’s disease

Future challenges - Is central inhibition of LRRK2 essential?

- Peripheral non-motor symptoms precede neuronal loss in PD
- Blocking peripheral LRRK2 may have a beneficial effect.

Future challenges - Will there be long-term consequences of LRRK2 inhibition?

• LRRK2 knockout mice are viable and live a normal life span.

• LRRK2 knockout and kinase inactive knockin mice develop kidney inflammation and have impaired kidney lysosmal function.

• Heterozygous mice do not develop kidney phenotypes

• LRRK2 knockout mice are susceptible to inflammatory bowel disease.

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Financial disclosure/Acknowledgements

N. Dzamko is supported by a national health and medical research (NHMRC) postdoctoral fellowship. G. Halliday is a NHMRC supported senior research principal fellow. Our work on Parkinson's disease is funded by the Michael J Fox Foundation and Shake It Up Australia. The authors declare no conflicts of interest.