COMMENTARY

LRRK2 and ubiquitination: implications for kinase inhibitor therapy

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Pathogenic mutations and risk variants in LRRK2 (leucine-rich repeat kinase 2) represent the most common genetic cause of familial and sporadic PD (Parkinson's disease). LRRK2 protein is widely expressed throughout the brain and the periphery. Structurally, LRRK2 contains several functional domains, including a dual enzymatic core consisting of a kinase and GTPase domain. Disease-linked variants are found in both these enzymatic domains as well as in the COR [C-terminal of ROC (Ras of complex proteins)] and WD40 protein–protein binding domain. The kinase domain is widely believed to be linked to toxicity, and thus the thrust of pharmaceutical effort has focused on developing LRRK2 kinase inhibitors. However, recent data have suggested that inhibition of LRRK2 activity results in reduced LRRK2 levels and peripheral side effects, which are similar to those observed in homozygous LRRK2-knockout and LRRK2 kinase-dead rodent models. In a recent issue of the *Biochemical Journal*, a study led by Nichols reveals that dephosphorylation of LRRK2 cellular phosphorylation sites (Ser^{910}/Ser^{935}/Ser^{955}/Ser^{973}) triggers its ubiquitination and subsequent degradation and thus may account for the loss of function phenotypes observed in peripheral tissues in LRRK2-knockout/kinase-dead or inhibitor-treated rodents and primates. Albeit negative from a kinase inhibitor standpoint, the data open new avenues for LRRK2 biology and therapeutic approaches to counteract LRRK2 toxicity.

Key words: Parkinson's disease, LRRK2, kinase inhibitor, GNE1023, ubiquitin, phosphorylation site.

Since the discovery of LRRK2 (leucine-rich repeat kinase 2) mutations in 2004 [1,2], a huge academic and pharmaceutical effort has ensued. Variants in LRRK2 are the most common genetic cause of Parkinsonism, accounting for a remarkable 40 % in certain ethnic populations [3–5]. LRRK2 has several features that make it an attractive therapeutic target. First, and most obvious, kinases are druggable targets and the most common pathogenic LRRK2 mutation G2019S happens to reside within the kinase domain. Secondly, although LRRK2 penetrance is age-related, it is variable and aged carriers exist that have escaped disease [6], suggesting that other disease-modifiers must exist and the progression of disease could potentially be modified. Thirdly, within LRRK2 families, patients can present with different pathologies that overlap with other neurodegenerative diseases such as AD (Alzheimer's disease) and progressive supranuclear palsy [2], and, as such, the benefit of a LRRK2-based therapeutic may extend beyond PD (Parkinson's disease).

The physiological and pathological roles of LRRK2 are not yet fully understood, but it is generally accepted that, in the brain, LRRK2 is involved in neurotransmitter release and neuronal maintenance and outgrowth. In a general cellular context, LRRK2 has been linked to vesicular dynamics/trafficking, mitochondria dynamics and autophagy. Outside the central nervous system, it has become apparent that LRRK2 is a mediator of the immune system and has been linked to other diseases including inflammatory bowel disease [7–9], cancer [10–12] and leprosy [13–15]. Knockout of LRRK2 in rodents does not appear to be detrimental to the brain, since no abnormal behaviours, neurochemical alterations or neuropathologies can be found; however, in the periphery, homozygous knockout rodents have kidney and lung abnormalities [16–19 and 20]. Similar phenotypes have also been reported in kinase-dead mice, probably due to the ∼75 % reduction in full-length LRRK2 levels in their kidneys [16]. Interestingly, heterozygous knockout mice do not have kidney abnormalities, possibly indicating that 50 % function is sufficient [17]. Also worth noting is that LRRK2-knockout rodents appear to be protected against inflammatory insult via lipopolysaccharide [22], α-synuclein-induced dopaminergic degeneration [23] and rhabdomyolysis-induced kidney injury [24].

Recent data emerging from studies utilizing LRRK2 inhibitors suggest that LRRK2 toxicity depends on LRRK2 levels rather than kinase activity, and inhibition of kinase activity can also result in a reduction of steady-state LRRK2 levels [16,25,26]. Furthermore, the inhibitor-induced reduction in LRRK2 levels may also lead to loss of function peripheral phenotypes; for example, a recent study testing two distinct LRRK2 inhibitors in non-human primates revealed that both of these kinase inhibitors induced abnormal cytoplasmic accumulation of secretory lysosome-related organelles known as lamellar bodies in type-II pneumocytes of the lung [21]. The potential side effects represent a significant hurdle for LRRK2 inhibitor treatment in humans, and suggest that targeting what is conceived as LRRK2's most promising druggable attribute, its kinase domain, could be a 'double-edged sword'.

The study by Zhao et al. [27] published in a recent issue of the *Biochemical Journal* seeks to understand the events underlying LRRK2 degradation following kinase inhibition by analysing LRRK2 cellular overexpression models, as well as endogenous LRRK2 in cells and mouse tissues after treatment with a recently described LRRK2 inhibitor, GNE1023 [28]. Although the levels of $pSer^{935}$ are reduced as expected, the levels of total LRRK2 (overexpressed and endogenous LRRK2 in lung cells and various

Abbreviations: AD, Alzheimer's disease; DUB, deubiquitinating enzyme; LRRK2, leucine-rich repeat kinase 2; PD, Parkinson's disease; PP1, protein phosphatase 1; UPP, ubiquitin–proteasome pathway; WT, wild-type.

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mouse tissues) are lower after GNE1023 treatment. The effect of GNE1023 was confirmed to be at the protein level since mRNA levels of LRRK2 remained unchanged in lung cells and in lung, kidney and brain from drug-treated mice. Total LRRK2 levels did not change in cells expressing A2016T, an inhibitor-resistant form of LRRK2, suggesting that kinase activity is somehow responsible for reducing LRRK2 levels.

Zhao et al. [27] next asked whether LRRK2 degradation was either via the autophagy–lysosome pathway or via ubiqutination. They found that in HEK (human embryonic kidney)-293 cells overexpressing LRRK2, blocking autophagy induction or progression did not affect GNE1023-induced LRRK2 degradation; however, proteasomal inhibition reversed it. Since proteasomal degradation is driven by ubiquitination, they went on to investigate ubiquitin levels and which potential ubiquitin linkages could be involved. GNE1023 inhibition of both overexpressed and endogenous LRRK2 resulted in increased ubiquitination and linkages to Lys^{48} and Lys^{63}. Lys^{48}-linked ubiquitin chains target proteins to the proteasome, which negatively affect protein stability, whereas Lys^{63} linkages are crucially implicated in several non-proteolytic signalling processes [29]. In human epithelial cells transfected with WT (wild-type) LRRK2, GNE1023 alone or co-expression of mutant Lys^{48} or Lys^{63} linkages drove LRRK2 into cytoplasmic skein-like aggregates. Furthermore, GNE1023 plus either ubiquitin-linkage mutant appeared to synergize this effect. When all of the ubiquitin lysate linkages were mutated, LRRK2 was still ubiquitinated, but this could be reduced by mutating the ubiquitin start methionine to arginine, and likewise when the start methionine mutant was paired with either the mutant Lys^{48} or Lys^{63} linkage, ubiquitination of LRRK2 decreased. Thus the data suggest that at least three linkages are involved in LRRK2 ubiquitination. Since distinct ubiquitin linkage conformations determine decoding at ubiquitin receptors and subsequent targeting to different pathways, these new data deepen the complexity of LRRK2 function and add a whole new dimension to downstream LRRK2 signalling.

A known consequence of LRRK2 dephosphorylation of the cellular sites ($pSer^{910}$/$pSer^{935}$/$pSer^{955}$/$pSer^{973}$) is loss of 14-3-3 protein binding to $pSer^{910}$/$pSer^{935}$ [30,31]; however, whether LRRK2 signals to a phosphatase or an upstream kinase to regulate these sites is unknown. Zhao et al. [27] suspected that dephosphorylation at these upstream kinase sites was linked to ubiquitination. By employing a GFP–difopein (difopein is a 14-3-3 inhibitor) fusion protein that bound 14-3-3 and disrupted phosphorylation of LRRK2, they showed that dephosphorylation of LRRK2 at $pSer^{935}$ (but not $pSer^{1292}$) increased LRRK2 ubiquitination to levels similar to GNE1023, while still leaving LRRK2 kinase activity intact. Moreover, difopein–GFP decreased steady-state protein levels 50 % more than GFP alone when protein synthesis was blocked, indicating that dephosphorylation at LRRK2 $pSer^{953}$ is sufficient for the ubiquitination and degradation of LRRK2. This is curious because the PD mutants N1347H, R1441C/G, Y1699C and I2020T were found previously to be more dephosphorylated than G2019S mutant and WT [31,32] and now these new data show, in fact, that their basal level of ubiquitination is higher. It was recently reported that blocking PP1 (protein phosphatase 1) with Calyculin A restores phosphorylation of the upstream kinase sites following inhibitor treatment [33]. Zhao et al. [27] consequently studied the effects of Calyculin A with regard to the impact on ubiquitination in a series of LRRK2 mutants, and revealed differential responses of the various LRRK2 mutants. In short, kinase inhibition with GNE1023 only increased ubiquitination in the G2019S- and WT-expressing cells, but did not alter the mutants (I2020T, R1441G and Y1699C) that already had enhanced basal ubiquitination levels. On the other hand, PP1 inhibition was able to restore phosphorylation at the upstream sites for all mutants, resulting in a minimally ubiquitinated LRRK2 species.

So how do we put these findings in perspective and what are the implications for the development of LRRK2 inhibitors? From a safety standpoint, the potential for loss-of-function phenotypes will certainly heighten concerns around the potential side effects. Zhao et al. [27] suggest that one solution could be molecules selective for mutant LRRK2 activity or agents that are highly selective, but have low-affinity inhibition. However, data from our laboratory may indicate that G2019S mutant preferring inhibitors may not be effective clinically in heterozygous patients. We have tested HG-11-31-01 (which is structurally almost identical with GNE1023 and has a 30-fold preference for the G2019S mutant) for its rescue potential of dopamine-release deficits in G2019S knockin mice [34]. HG-11-31-01 was only effective at restoring dopamine release in homozygous knockin mice, but had no effect in heterozygous mice [35]. Analysis of both the $pSer^{910}$ and $pSer^{935}$ sites revealed that HG-11-31-01 was significantly more effective at inhibiting phosphorylation in the homozygous mice. The lack of effect in the heterozygous mice may be attributable to mutant-induced alterations in protein folding around the binding site or, alternatively, the inhibitor may confer differential kinase affinity on the phosphatase acting on Ser^{910}/Ser^{935} sites. Assuming the dopamine-release phenotype is relevant to PD, this may have clinical implications since most G2019S patients are heterozygous. Thus we have reasoned that kinase inhibitors that have a similar affinity for WT and mutant LRRK2 may have more therapeutic potential. In the light of the new data by Zhao et al. [27], the considerations for developing LRRK2 inhibitors and their clinical efficacy has intensified further. Future therapies may require different inhibitor types depending on which LRRK2 mutation the patient has.

Interestingly, West's group [36] recently published a systematic study of how small molecules differentially target *trans*- and *cis*-phosphorylation activities and suggested that the multidimensional aspect of LRRK2 kinase activity may strongly influence the success or failure of a particular compound in pre-clinical models. In *trans*-peptide assays, most of the inhibitors had more potency towards G2019S LRRK2 compared with WT LRRK2. It was suggested that the catalytic pocket may adopt different conformations depending on the nature of the substrate, and the small-molecule inhibitors may show different binding affinities for these different conformations (or not bind at all).

It is prudent to point out that much of the data generated by Zhao et al. [27] was performed in cellular/overexpression systems. Currently, there is no genetic evidence for patients with multiplication of LRRK2, and the jury is still out as to whether splice variants influence LRRK2 expression in humans [37–40]. Also, the absence of anti-LRRK2 antibodies that can be used for immunohistochemistry in human brain [41] precludes the assessment of skein-like inclusions of LRRK2 in humans, so it is unclear whether this is a result of overexpression systems, or whether this can also occur in the physiological setting. Certainly aggregation of proteins such as α-synuclein, β-amyloid and tau in PD and AD is well characterized, but then again one school of thought is that protein aggregates are actually a cell-protective/coping mechanism. As Zhao et al. [27] suggest, it will be important to define the ubiquitination linkage types on LRRK2 under various pathogenic conditions, as well as fully determining tissue- or cell-population-specific differences. It will also be informative to study the phosphorylation, ubiquitination and degradation cycle in physiological LRRK2 animal models. Indeed, it will be very interesting to see how this cycle differs

with a range of different LRRK2 inhibitor types in G2019S and R1441C knockin mice [16,34,42]. Lastly, determining the ubiquitination status of the LRRK2 risk factor variants, i.e. R1628P and M1646T in the COR domain and G2385R in the WD40 domain [43–45] could also reveal clues to how they influence PD risk.

The discovery of this pSer^{935}-mediated mechanistic switch which can alter LRRK2 ubiquitination and downstream stability and function could certainly widen the potential for therapeutic intervention. The UPP (ubiquitin–proteasome pathway) is critical for normal nervous system function and is implicated in neurodegenerative disease including PD and AD, where toxin protein aggregates accumulate in certain vulnerable brain regions. Many crucial nervous system pathways are regulated by post-translational conjugation of ubiquitin to target proteins. Equally important is the reversal of ubiquitination, controlled by DUBs (deubiquitinating enzymes) which have important roles in determining neuronal fate, axonal pathfinding, and synaptic connection and plasticity [46]. Several of the 80 known DUBs have already been linked in some manner to PD, including UCHL1, USP24 USP30, USP40 and OTUB1, which, interestingly, is found in Lewy bodies in post-mortem PD brain (reviewed in [47]). Targeting specific DUBs has already been proposed as a potential therapeutic route in some neurodegenerative diseases [48]. A situation can be envisaged whereby LRRK2 inhibitor treatment could be supplemented by co-treatment with drugs that targets specific DUBs to thwart the unwanted signalling outcomes.

Another key component of the UPP is the ubiquitin ligases, the enzymes that selectively recognize and mediate the ubiquitination of substrates, involving the transfer of E2-conjugated ubiquitin to lysine residues of the target substrate. Like DUBs, ubiquitin ligases are also potential PD targets. Parkin functions as an ubiquitin ligase and was shown to be protective in LRRK2 G2019S in *Drosophila* [49]. Several ubiquitin ligases are also implicated in the turnover of α-synuclein, including CHIP, MDM2 and HRD1 [50]. The work of Zhao et al. [27] opens up new avenues for LRRK2 biology, and future work to determine the ubiquitin ligases and DUBs that interact with differentially phosphorylated forms of LRRK2 and possible mechanistic links to other PD genes and pathways are sure to be enlightening for the field.

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