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1 Screening for chemical modulators for LRRK2

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4 Abstract

5 After the discovery of LRRK2 as a risk factor for sporadic Parkinson's disease (PD) and mutations in
6 LRRK2 as a cause of some forms of familial PD there has been substantial interest in finding chemical
7 modulators of LRRK2 function. Most of the pathogenic mutations in LRRK2 are within the enzymatic
8 cores of the protein; therefore many screens have focused on finding chemical modulators of this
9 enzymatic activity. There are alternative screening approaches which could be taken to investigate
10 compounds which modulate LRRK2 cellular functions. These screens are more often phenotypic
11 screens. The preparation for a screen has to be rigorous and enable high throughout accurate
12 assessment of a compounds activity. The pipeline to beginning a drug screen and some LRRK2
13 inhibitor and phenotypic screens will be discussed.

14 Keywords

15 LRRK2, high throughput drug screening, phenotypic screening, mitochondria

16 Abbreviations

17 PD: Parkinson's disease, HTS: high through put screening, iPSC's: induced pluripotent stem cells, TR-
18 FRET: time resolved fluorescence resonance transfer, TMRM: tetramethylrhodamine, OTCA: L-2-
19 oxothiazolidine-4-carboxylic acid, PRDX3: Peroxiredoxin 3, UDCA: usrodeoxycholic acid

20 Introduction

21 Mutations in the LRRK2 gene are pathogenic causing late onset autosomal dominant PD. The LRRK2
22 genotype is also associated with sporadic PD [1]. The exact cellular function of LRRK2 remains to be
23 elucidated with much evidence linking LRRK2 to several cellular pathways. Most of the known
24 pathogenic mutations in LRRK2 occur in the enzymatic domains with the most common mutation
25 *LRRK2*^{G2019S} leading to increased LRRK2 kinase activity [2, 3] and other mutations resulting in
26 decreased GTPase activity [4-6]. How the two active domains interact and control one another is still
27 an area of active and intense research. The penetrance of *LRRK2*^{G2019S} is age dependent and varies
28 between different ethnic populations [reviewed by 7]. The increased kinase activity of *LRRK2*^{G2019S}
29 mutation coupled with the reduced penetrance of this mutation suggests biological rescue or
30 protective mechanisms are present in some individuals. This has led to LRRK2 and in particular
31 LRRK2 kinase activity being the target of intense drug screening activity over the past decade. The
32 hope being a drug may be found which could reduce the risk of asymptomatic *LRRK2*^{G2019S} carriers
33 developing clinically manifest PD. In addition as LRRK2 is a susceptibility factor for developing
34 sporadic PD; could a LRRK2 targeted treatment be beneficial for a large sporadic patient group. This
35 review will outline the drug screening process up to lead selection, in the context of LRRK2 as a
36 target for PD therapies.

37 Drug screening

38 There are many stages to successfully setting up, running and completing a drug screen of small
39 molecules using high throughput screening (HTS). HTS is the mainstay of the drug discovery process
40 both in industry and increasingly academic laboratories. HTS requires each step to be considered
41 carefully, tested and the appropriate decision made; with the input of scientists from several

42 disciplines. The decision tree is often not straightforward but requires consideration of the aim and
43 desired outcome of this screen. In general the drug screening process (using HTS) can be broken
44 down into five sections; target choice, assay development, compound high throughput screening
45 (HTS), lead selection and optimization; and toxicity testing. This review will focus on the stages up to
46 lead selection.

47 Target choice

48 When selecting a target for HTS the major concern is disease relevance. The target may be a well
49 described enzyme or receptor which is well characterized in a particular disease in which case the
50 rationale for screening this specific target is high. The target could also be a relatively novel target
51 for a disease where the knowledge of how this target links to disease pathogenesis is less clear but
52 the novelty of resulting compounds is extremely high. Regarding LRRK2, this is a specific target which
53 has been well described and linked to PD for several years. The focus of LRRK2 HTS has been to find
54 novel, specific LRRK2 kinase inhibitors with large scale screens having been undertaken by several
55 large pharmaceutical companies and academic laboratories. Several have been publically disclosed
56 now and some are in pre-clinical development [8-27]. These screens have included several assays to
57 interrogate LRRK2 kinase function, in addition to *in silico* and chemoproteomic approaches [17, 26].
58 In addition screening to identify modulators of the GTPase domain has been performed using *in vitro*
59 and computer aided screening assays [28, 29]. LRRK2 biology also holds other opportunities for drug
60 discovery by investigating chemical modulators of other LRRK2 cellular functions. So far this type of
61 assay have been mainly limited in the LRRK2 field to small scale assessment of a compounds ability
62 to rescue cell/neuron death seen in LRRK2 models [30-33] and to date does not include a large scale
63 screen focusing on LRRK2 modulators. In addition to disease relevance, the screenability of a target
64 is an important consideration for any target. Screenability means the ease with which a target can
65 be screened using HTS and small molecules. For LRRK2 kinase inhibitors the screenability is very high
66 with specific screens being designed to assess a compounds ability to inhibit specifically LRRK2
67 kinase activity; these screens are often designed around specific phosphorylation assays using
68 recombinant LRRK2 or LRRK2 peptide. For other chemical modulators of LRRK2 this may vary and
69 depend on the ability to screen a particular receptor or enzyme linked to LRRK2. Many successful
70 screens are carried out against a specific known molecular target, however in recent years there has
71 been an increase in phenotypic or cell based HTS. This is sometimes referred to as a 'black box'
72 approach but is gaining favor again for complex diseases; as it is being recognized that more than
73 one target will have to be modulated and by using phenotypic screening this can be achieved [34].
74 This type of phenotypic screen would be applicable to screening for modulators of other LRRK2
75 functions within the cell and has proven to be a successful approach in other diseases [35].

76 Assay development and optimization

77 The first step to assay development is deciding which type of assay, a biochemical assay or a cell
78 based assay. Here we mean a biochemical assay to be against a specific target – this is the approach
79 taken for most LRRK2 kinase inhibitor screens. For example Henderson et al, undertook a FRET based
80 screen using tagged truncated LRRK2 protein and LRRK2 peptide as substrates, a similar strategy has
81 been employed by others using MBP and LRRKtide phosphorylation of G2019S LRRK2 [26, 36].
82 Alternatively the cell-based approach can be used to assess a phenotypic read out in a whole cell
83 where the specific target the compound is interacting with is unknown. So far there is no study using
84 this approach specifically for LRRK2 published, however we and others have used it as an approach
85 for PD and other neurodegenerative diseases [30, 35]. The situation often arises where both types of
86 assay are used in the primary and then secondary screening assays; so if a cell based assay is used as
87 a primary screen, then a biochemical assay will be used in the secondary screening phase to narrow

88 the target identification and vice versa. Commonly used modalities for assays are using absorbance,
89 luminescence and fluorescence assays with high content imaging becoming increasingly popular to
90 use with the advent of more advanced high content imaging systems and the return to cell-based
91 screens. The types of assays which are most commonly used for each type of screen are discussed in
92 details elsewhere [37, 38]. Many of the LRRK2 kinase inhibitor screens have used a biochemical
93 assay based upon phosphorylation by either WT, truncated or mutant LRRK2 of MBP or LRRKtide
94 substrates and have yielded promising candidates; some of which have been taken forward into pre-
95 clinical testing [15]. One example of a biochemical assay which was developed for screening of
96 LRRK2 kinase inhibitors was the one by Lui et al [10]. This group developed a HTS assay using full
97 length LRRK2 purified from mouse brain. The primary screen identified compounds which modified
98 LRRK2 kinase activity by directly interacting with the kinase domain of LRRK2 and compounds which
99 modified kinase activity allosterically by interacting with the other domains of LRRK2. Another
100 example of a primary screen used which has identified a very promising LRRK2 inhibitor MLI-2
101 utilized Lanthascreen technology using a tagged truncated human mutant G2019S LRRK2 and a
102 fluorescently labelled LRRKtide substrate [15].

103 Regardless of the type of assay used there are several validation criteria which should be fulfilled by
104 the assay to ensure it is suitable for HTS. Optimizing of the screening assay for statistical robustness
105 is a critical step in the HTS pathway. There are several considerations for a biochemical assay such as
106 ligand concentration and incubation time which need to be optimized for each assay (many of these
107 are discussed in details elsewhere, 37). For cell based assays the major consideration is the quality,
108 amount and stability of the cells to be used. Common cell types used are tumor cell lines however it
109 is becoming more common to carry out HTS using primary patient cells such as fibroblasts [30, 35] or
110 iPSC's [38, 39]. Once established the assay is assessed for robustness and reproducibility. The Z'
111 score is generally used for this and the accepted criteria are $Z' > 0.4$ (cell based HTS) whereas $Z' > 0.6$
112 (biochemical screen). The difference here relates to the fact that cell based screen are inherently
113 more variable.

114 The LRRK2 screen optimized and used by Lui et al, referred to above; used a TR-FRET assay in which
115 first the enzyme amount and GTP content were optimized. Subsequently the Z' score was calculated
116 on 3 separate plates resulting in a mean Z' score of 0.83 which is at an acceptable level to continue
117 and use the assay for HTS [10]. Lovitt et al also optimized the conditions for a screen using the
118 phosphorylation of MBP and LRRKtide by G2019S LRRK2 [36]. These authors explored the various
119 parameters in the assay to fully to establish the conditions used by Chen et al and Henderson et al
120 for their LRRK2 inhibitor screens [36, 40]. Both of these screens found lead compounds which were
121 not very selective for LRRK2, therefore they employed different approaches, Chen et al, undertook a
122 computational screen using homology modelling and ATP binding site analysis whereas Henderson
123 et al used kinase selectivity panel, ligand efficiency, lipophilic efficiency and CNS desirability scores.
124 In terms of cell based phenotypic screens, we have developed a mitochondrial screen in patient
125 fibroblasts [30]. This screen was performed in fibroblasts from patients with *parkin* mutations and
126 we calculated a Z' score of 0.72 using a positive control of L-2-oxothiazolidine-4-carboxylic acid
127 (OTCA) [30]. Subsequently the hits from this screen were used in fibroblasts from patients with
128 LRRK2 G2019S and a Drosophila G2019S LRRK2 model and were shown to be effective [30, 31].

129 Mitochondria and LRRK2

130 As outlined above the functions of LRRK2 are numerous and depend on the state of the cell. LRRK2 is
131 widely expressed in many tissues and cell types. Most is present in the cytosol with a proportion
132 found in organelles such as the mitochondria, Golgi, endosomes and lysosomes. There are multiple
133 strands of evidence indicating that LRRK2 mutations cause mitochondrial dysfunction [41-47],

134 however how this happens is not clear. In particular our work has shown fibroblasts from patients
135 with the LRRK2^{G2019S} mutation have identified mitochondrial functional abnormalities including
136 reduced mitochondrial membrane potential, a specific reduction in complexes III and IV of the
137 respiratory chain rather than complex I which is seen in *parkin* mutant patient cells and this has an
138 overall effect of reducing total cellular ATP levels [30]. Furthermore we have shown some defects
139 are also present in fibroblasts from LRRK2^{G2019S} mutation carriers who do not have Parkinson's
140 symptoms; however the reduction in mitochondrial membrane potential, complex III and IV activity
141 and changes in mitochondrial morphology are less severe in non-manifesting LRRK2^{G2019S} mutation
142 carriers [41]. Several studies have proposed ways in which LRRK2 interacts with mitochondria in
143 various cell and *in vivo* models. Studies by others have implicated a potential role of mitochondrial
144 uncoupling in LRRK2 patient fibroblasts [42]. Peroxiredoxin 3 (PRDX3) a mitochondrial antioxidant
145 protein; interacts with in a yeast 2 hybrid screen and neuroblastoma cells, this indicates reduced
146 potential of mitochondria to scavenge reactive oxygen species may be linked to the mitochondrial
147 dysfunction seen in LRRK2 linked PD [44]. In addition pathogenic mutations in LRRK2 increase
148 inhibition of PDRX3 by phosphorylation thereby promoting oxidative damage to the mitochondria.
149 Evidence is growing for an interaction between Drp1 and LRRK2. Drp1 is involved in mitochondrial
150 morphology. Drp1 has been shown to interact and partially co-localise with LRRK2 in cortical
151 neurons; indicating mutant LRRK2 could disrupt mitochondrial dynamics via this interaction [46, 47],
152 this links with our own data showing the mitochondrial network is more branched in G2019S mutant
153 LRRK2 patient fibroblasts [31]. All of this data suggests using a mitochondrial cell based HTS to
154 identify modulators of mitochondrial function could discover useful compounds for the treatment of
155 PD. Indeed our own work following on from the mitochondrial cell based screen first reported in
156 *parkin* mutant patient fibroblasts [30] resulted in the identification of usrodeoxycholic acid (UDCA)
157 which we have also shown to increase mitochondrial function in LRRK2^{G2019S} mutant manifesting [30]
158 and non-manifesting [41] patient fibroblasts. Another potential avenue to explore using phenotypic
159 screening for LRRK2 would be furthering the work showing that treating both *in vitro* and *in vivo*
160 models with a microtubule deacetylase inhibitor, such as trichostatin A, rescues the axonal transport
161 defects seen in these models [32]. One of the areas which has hampered clinical development of
162 LRRK2 modulators is no clear consensus on LRRK2 substrates; an advancement in this area was made
163 recently when a subset of Rab GTPases were identified as key LRRK2 substrates acting both *in vitro*
164 and *in vivo* at a conserved residue in the switch II domain [48]. In addition this group also found that
165 pathogenic mutations in LRRK2 increase phosphorylation of Rabs which in turn decreases their
166 affinity to regulatory proteins [48]. This exciting discovery opens new opportunities for developing
167 new screening assays to find novel modulators of LRRK2 function.

168 As discussed above phenotypic screens are often used as secondary screens when biochemical
169 screens have been used as primary screens. This has been the case for several LRRK2 kinase
170 inhibitors; PF-06447475 inhibitor has been shown to be protective in a mitochondrial dysfunction
171 induced model (by rotenone treatment) in nerve-like cells [33]. GW5074 and indurubin-3'-
172 monooxime were shown to be protective *in vitro* in neurons overexpressing WT or G2019S LRRK2
173 and *in vivo* in a mouse model of LRRK2 dopaminergic neuron toxicity [49]. Finally any treatment
174 whether found using a biochemical or phenotypic LRRK2 screen needs to be safe to use in man;
175 which means some target engagement should be able to be monitored in patients whilst they are
176 taking the therapy. Studies have shown treatment of rodent models with LRRK2 inhibitors have
177 shown dephosphorylation at S910 and S935 and treatment of peripheral mononuclear cells taken
178 from Parkinson's patients with PF-06447475 and GSK2578215A showed reduced phosphorylation at
179 S910 and S935 [50].

180 Summary

181 In summary LRRK2 is a well characterised target for HTS to find a disease modifying therapy for PD,
182 using either a strategy of screening for LRRK2 kinase inhibitors or other modulators of LRRK2
183 function; the main avenues of screening approaches discussed here are highlighted in Figure 1.
184 There has already been an immense amount of work done in this area already however there is
185 scope for much more.

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353 Figure 1. This diagram shows LRRK2 in the context of HTS screens for small molecules which are
354 discussed in this review. Highlighting which have been done successfully and the scope for new
355 opportunities.