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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: tetramethlyrhodamine, 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 LRKK2 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.

186   Acknowledgements

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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.