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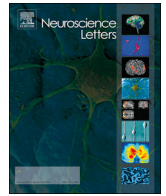
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Review article

Therapeutic approaches to enhance PINK1/Parkin mediated mitophagy for the treatment of Parkinson's disease

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ABSTRACT

The discovery of rare familial monogenic forms of early-onset Parkinson's disease has led to the identification of a mitochondrial quality control process as a key player in this disease. Loss-of-function mutations in the genes encoding PINK1 or Parkin result in insufficient removal of dysfunctional mitochondria through autophagy, a process termed mitophagy. Understanding the mechanism of this process and the function of its two key players, PINK1 and Parkin, has led to the discovery of new therapeutic approaches. Small molecule activators of mitophagy, either activating PINK1 or Parkin directly or inhibiting Parkin's counterplayer, the ubiquitin-specific protease USP30, are in preclinical development. To enable clinical success of future small molecule mitophagy enhancers, biomarkers for mitochondrial integrity and mitophagy are being developed. Only a few years after the discovery of mitophagy deficits in Parkinson's disease, research of the underlying mechanisms, drug discovery of modulators for this mechanism and identification of biomarkers provide new avenues towards the development of disease-modifying therapies.

1. Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disorder and is characterized clinically by tremor, rigidity, bradykinesia and postural instability. Pathologically, the disease is associated with loss of dopaminergic neurons in the substantia nigra, decreased dopamine neurotransmission and the presence of neuronal intracellular Lewy body inclusions [64]. Current treatments for PD alleviate the symptoms of parkinsonism in the first years after diagnosis, but become less effective as the neurodegeneration advances and there are no therapies currently available that prevent the onset or progression of the disease. Therefore, it is of great importance to understand the molecular basis of PD so that therapeutic breakthroughs can be made in the near future. Several lines of evidence suggest that mitochondrial dysfunction is central to the disease [39] including the observations that mitochondrial toxins cause parkinsonism in humans and animal models, that PD patients have systemic mitochondrial complex I deficits, and that dopaminergic neurons in elderly individuals accumulate high levels of mitochondrial DNA (mtDNA) mutations relative to other neuronal subtypes [5,18,22,56,98]. Discovery of rare familial monogenic forms of PD has provided insights into the pathogenic mechanisms of mitochondrial dysfunction in PD and provided new targets to pursue for drug development [103]: Early-onset

autosomal recessive Parkinson's disease can be caused by mutations in the ubiquitin ligase Parkin and protein kinase, PTEN-induced kinase 1 (PINK1) [50,111], both of which maintain a healthy mitochondrial network by stimulating removal of dysfunctional parts through selective autophagy, a process termed mitophagy [76]. The current model is that in functional mitochondria, *i.e.* those with an intact membrane potential, PINK1 is imported into mitochondria and subsequently cleaved by the protease, presenilins-associated rhomboid-like protein (PARL) [47], leading to its degradation *via* the N-end rule pathway [38]. However, in response to mitochondrial membrane depolarisation, PINK1 [111] is not cleaved and accumulates on the outer membrane of mitochondria where it phosphorylates ubiquitin on outer mitochondrial membrane (OMM) proteins at Serine 65 (Ser65) [48,49,55]. Phosphorylated ubiquitin at Ser65 (pS65-Ub) triggers the recruitment of Parkin to the OMM [47], whereupon it is phosphorylated by PINK1 at an equivalent Ser65 residue within its N-terminal ubiquitin-like domain [54]. This stimulates the ubiquitin-ligase activity of Parkin resulting in ubiquitination of multiple OMM proteins, such as the voltage-dependent anion channel 1 and mitofusins 2 [81,95]. This creates a feed-forward mechanism where newly assembled ubiquitin is phosphorylated by PINK1, which in turn promotes further Parkin recruitment [82]. The accumulation of pS65-Ub chains on the OMM is reported to stimulate the recruitment of ubiquitin adaptor proteins including optineurin and

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nuclear dot protein 52 kDa (NDP52), and TAXBP1 which promote engulfment of the depolarized mitochondria by autophagosomes [40,62,118]. However, recent studies have suggested that pS65-Ub chains inhibit adaptor protein recruitment [80]. Furthermore, conflicting findings have been reported for p62/sequestosome 1 (SQSTM1) as the adaptor for ubiquitinated mitochondria [26,62,75,78,118].

2. Evidence for mitophagy *in vivo*

Most of the current understanding of PINK1 and Parkin mediated mitophagy stems from studies in cell lines overexpressing Parkin that are treated with high concentrations of mitochondrial uncouplers such as carbonyl cyanide m-chlorophenyl hydrazine (CCCP) or depolarizing agents such as antimycin alone or in combination with oligomycin. Evidence that the PINK1/Parkin pathway is active *in vivo* was first provided by studies in *Drosophila* showing that deficiency of PINK1 or Parkin triggers the accumulation of enlarged, dysfunctional mitochondria in sperm, flight muscle and dopaminergic neurons [10,35,85,120]. Moreover, a proteomics approach revealed that the half-life of several mitochondrial proteins was increased in Parkin mutant flies in a pattern similar to that of autophagy-defective *Atg7* mutants, thus supporting a role for Parkin in mitophagy [113]. Different reporters have been used to image mitophagy directly in flies, but yielded discrepant results [12]. In one study, flies expressing the mitochondrial reporter mtKeima whose fluorescence changes when mitochondria are degraded in the lysosome, indicated a potential role for PINK1 and Parkin in age-dependent mitophagy [12]. In contrast, in another study, flies expressing mtKeima or the alternate mitoQC reporter, a mCherry-GFP tandem construct fused to the mitochondrial fission protein (Fis), in which GFP is quenched in the lysosome, did not demonstrate PINK1-dependent mitophagy [63]. Studies in rodents have also produced equivocal results. While mice expressing the mitoQC reporter show robust levels of basal mitophagy in dopaminergic neurons in the midbrain, crossing these mice with PINK1 knockout (KO) or Parkin^{S65A/S65A} mutant mice did not significantly alter basal mitophagy levels [70,71]. However, KO mice lacking PINK1 or Parkin genes show subtle behavioral and neurochemical abnormalities and no overt dopaminergic neuron degeneration [2,31,34,51,86] and therefore have been unable to address the contribution of PINK1 and Parkin to stress-evoked mitophagy *in vivo*. PINK1, but not Parkin KO rats showed progressive nigral neurodegeneration with about 50% dopaminergic cell loss observed at 8 months of age [15] and the reasons for this difference are not known. Crossing mouse models of mitochondrial dysfunction with Parkin KO mice has also produced mixed results: Mice with KO of the mitochondrial biogenesis transcription factor A (TFAM) in dopaminergic neurons did not show Parkin-dependent dopaminergic cell loss [106]. However, enhanced parkinsonian-like neurodegeneration has been observed by crossing Parkin KO mice with mouse models of mitochondrial DNA (mtDNA) mutations, including mutator mice that lack the mitochondrial DNA polymerase gamma subunit (POLG) and accumulate mtDNA mutations [87], Twinkle (an essential mitochondrial helicase) transgenic mice in which mtDNA deletions are increased specifically in substantia nigra [105] and PD-mito-*PstI* mice, where mtDNA undergoes double-strand breaks only in dopaminergic neurons [88]. Mt-Keima mice have not yet been employed to assess whether mitophagy is elevated in these mice lines [57]. However, recently it was observed that exhaustive exercise led to an elevation of mitophagy in heart muscle using the mt-Keima reporter line and critically, this was prevented in mice lacking PINK1 [104]. This represents the strongest evidence to date in mammalian models for an essential role of PINK1 in stress-evoked mitophagy *in vivo* [104]. Relatively modest morphological or functional changes in mitochondria have been observed in iPSC-derived neurons with PINK1 [72,93,99] or Parkin mutations [9,43,46,100] and analysis of mitophagy in these neuronal systems using either mt-Keima or mitoQC reporter assays is awaited.

Finally, accumulation of pS65-Ub was demonstrated in postmortem

brains from elderly human subjects [23] and in subjects with Lewy body disease [24], supporting the notion that the PINK1/Parkin pathway is active in human brain during aging and disease states.

3. Role of PINK1/ Parkin mediated mitophagy in inflammation

How can we reconcile the modest phenotypes caused by loss of PINK1 or Parkin in rodent models or human iPSC-derived dopaminergic neurons with the development of early onset Parkinson's disease in humans? A recent study suggests that a more systemic view beyond dopaminergic neurodegeneration may be required to fully understand the consequences of dysfunctional mitophagy [104]: Using an exhaustive exercise experimental paradigm the authors show that PINK1/Parkin mediated mitophagy restrains innate immunity *in vivo* and that dysfunction in mitophagy leads to upregulation of the stimulator of interferon genes (STING) pathway causing an inflammatory phenotype that ultimately may contribute to the loss of dopaminergic neurons [104]. Importantly, components of innate immunity activation are also found in patients with Parkin mutations [104]. Supporting a role for PINK1 and Parkin in immune responses, PINK1 and Parkin were shown to mediate mitochondrial antigen presentation and trigger adaptive immune responses in a mouse macrophage cell line through mitochondrial-derived vesicles [69]. PINK1 KO mice show changes in innate immunity and inflammation early during the progression of pathology [107]. Parkin KO increases vulnerability to inflammation-related nigral degeneration by lipopolysaccharide injections [25]. A vicious cycle has been proposed whereby inflammation leads to more mitochondrial damage and that promotes further inflammatory processes [112]. It may therefore be that synergy between mitochondrial dysfunction and immune responses is required for Parkinson's disease to develop and that many rodent models only reproduce part of the systemic dysfunction.

4. Therapeutic approaches to enhance PINK1/Parkin mediated mitophagy

To ameliorate the consequences of mitochondrial dysfunction leading PD pathology, several therapeutic approaches are being explored (Fig. 1).

4.1. PINK1 activators

PINK1 is highly conserved across species and this had led to identification of insect PINK1 homologues including *Pediculus humanus corporis* PhPINK1 [33] and *Tribolium castaneum* TcPINK1 [58,79,119], that can be expressed in sufficient quantities in *E.coli* to study the structure and substrate recognition mechanisms of PINK1 and understand the impact of the autosomal recessive mutations in PD. PINK1 is distinct from other protein kinases due to the presence of three unique insertions within the kinase domain and a C-terminal region of unknown function with no homology to any known protein [66]. Almost 30 missense and nonsense human PINK1 (hPINK1) mutations have been reported in patients [17] that are located predominantly within the kinase domain or predicted to truncate the C-terminal region [33,58,79]. PINK1 rapidly accumulates on the OMM when mitochondria become depolarized and forms a large 700 kDa multimeric complex with the translocase of the outer membrane (TOM) complex [4,61], which in turn triggers autophosphorylation and activation of PINK1 [77,80]. Three autophosphorylation sites have been confirmed for hPINK1, Thr257 [54], Ser228 and Ser402, of which the latter two influence Parkin and subsequent ubiquitin phosphorylation [1]. Ser402 is located in the conserved activation loop of hPINK1, which is predicted to maximize kinase activity upon autophosphorylation through interaction with residues in the catalytic motif and stabilization of the active conformation [102]. Ser402 autophosphorylation and activation of hPINK1 increases Parkin recruitment and conversely, lack of

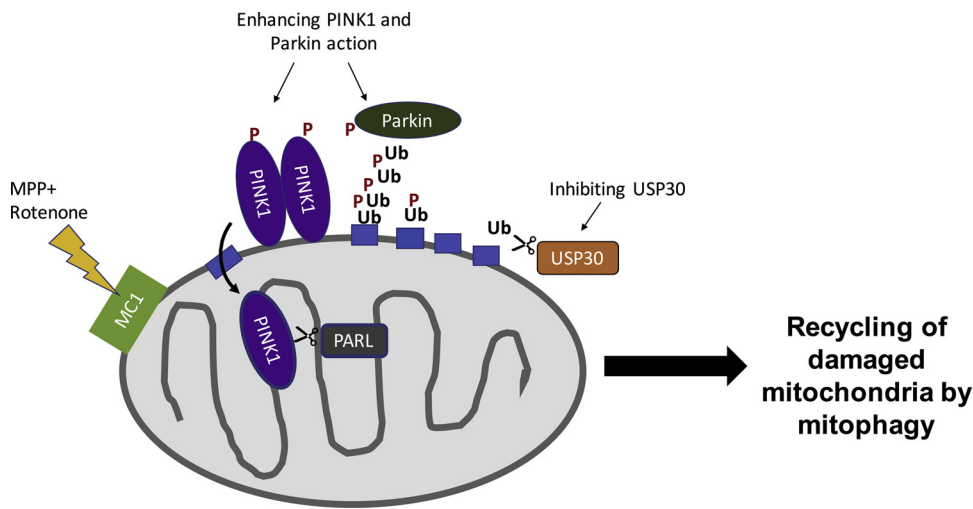


Fig. 1. Therapeutic Strategies to Enhance Mitophagy.

In healthy mitochondria PINK1 is imported and cleaved by the protease PARL. Ubiquitin residues are removed from OMM proteins by USP30. Mitochondrial damage can occur through natural processes and experimental conditions, including mitochondrial complex I (MCI) inhibition with MPP+ (the active metabolite of MPTP) and rotenone. Upon mitochondrial damage PINK1 localizes to the OMM, is activated by autophosphorylation and recruits Parkin by phosphorylation on Ser65. Phosphorylated Parkin ubiquitinates several OMM proteins and the ubiquitin residues in turn are phosphorylated on Ser65 by PINK1 creating a positive feed forward loop. The long phosphorylated ubiquitin chains on OMM proteins cannot be removed by USP30 culminating in engulfment of the mitochondrion by autophagosomal membranes and recycling in lysosomes. Mitophagy enhancing therapies are aimed at increasing PINK1 and Parkin activity and/or inhibiting USP30.

autophosphorylation attenuates Parkin recruitment [102]. Ser228 autophosphorylation increases substrate phosphorylation *in vitro*, but it is dispensable for Parkin recruitment and the initiation of mitophagy [1,80]. Instead, structural data of insect PINK1 suggest that phosphorylation in the N-lobe, in particular of Ser202 (phPINK) or Ser205 (TcPINK1), respectively (corresponding to human Ser228), is crucial for kinase integrity and activity, as well as for ubiquitin binding to the N-lobe [33,58,79]. Small-molecule activation of PINK1 could be accomplished by increasing dimerization or enhancing autophosphorylation through binding to allosteric regulatory sites. Alternatively, ubiquitin conformational modulators are proposed to increase ubiquitin phosphorylation levels [28]. A third approach to increase in PINK1 activity *in vitro* is achieved using a neo-substrate, *i.e.* the ATP analogue kinetin triphosphate [41,84], WO-2015/123365. No effects of kinetin were detectable in PINK1 KO rats after oral dosing for up to 6 months, but also no dopaminergic neurodegeneration was observed in this study leaving the question open whether kinetin could mitigate pathology induced by PINK1 KO [83]. Future studies will show whether any of these approaches can yield efficacious small molecules for translation in animal models and ultimately, in humans.

4.2. Parkin Activators

Parkin belongs to the RBR [RING– in-between-RING (IBR)–RING] family of E3 ligases, which contain a RING1 domain for E2 recruitment, a catalytic domain (RING2) through which transfer of ubiquitin is mediated, and a linking IBR domain [116]. Parkin comprises five domains: ubiquitin-like domain (Ubl), RING0, RING1, IBR, and RING2. The Ubl and RING0 domains are unique to Parkin, but the RBR module is common to all RBR ligases. In Parkin-associated PD more than hundred different mutations have been identified that result in deletions, truncations and exon duplications, all of which lead to a loss of function of Parkin [13,54]. PD associated Parkin mutations are distributed across all five domains and are also found within interfaces between domains and in linker regions suggesting that the structural integrity of all domains is important in PD pathogenesis [115]. Structures of Parkin show that under basal conditions it adopts an autoinhibited conformation mediated by its N-terminal Ubl domain through three mechanisms [8,57,109]: First, the RING0 domain partially occludes the catalytic cysteine (Cys431). Second, a small helical repressor element of Parkin (REP) together with the Ubl domain blocks the predicted E2 binding site on RING1. Third, the distance between the RING2 catalytic

cysteine and predicted E2 catalytic cysteine is over 30 Å, which is too far to allow transfer of ubiquitin in the autoinhibited conformation of Parkin [8,57,109]. Recent structural studies have revealed the mechanisms of Parkin activation and shown that the phosphorylated Ubl binds to a site on the RING0 domain [32,97]. Furthermore, a conserved linker region between the Ubl and RING0 domains functions as an activating element to facilitate the release of the RING2 domain [32,97]. These new structures provide atomic insights into how to design Parkin activators to disrupt the autoinhibitory mechanisms and stimulate catalytic activity. It is currently not known whether ubiquitin transfer occurs through conformational change or through cooperative binding of multiple Parkin molecules [3]. Towards the development of small molecule activators, an UbFluor probe has been described to precisely quantify changes in the activity of Parkin in response to phosphorylation, allosteric activation by pUb, protein substrates, and activating structural mutations [24]. Small molecule Parkin activators active in *in vitro* assays have been described in WO-2018/023029 in addition to assays to identify such molecules in WO-2010/011839, but no cellular or *in vivo* data with Parkin activators are available to date.

4.3. Ubiquitin-specific protease (USP) 30 inhibitors

The actions of E3 ubiquitin ligases such as Parkin are regulated by deubiquitinating enzymes (DUBs), which catalyze the removal of ubiquitin from substrates [53]. Due to the challenges with increasing PINK1 or Parkin activity as outlined above, modulating DUBs involved in mitophagy is an appealing approach for developing small molecule activators of mitophagy. Two DUBs, USP15 and USP30 were shown to oppose Parkin-mediated mitochondrial ubiquitination and mitophagy in human cells *in vitro* and in *Drosophila* [6,11,12]. Another DUB, USP8, was shown to activate mitophagy by removing ubiquitin residues from Parkin and preventing its auto-inhibition [21]. USP30 is a particularly attractive drug target to selectively increase mitochondrial ubiquitination, since it is localized at the OMM [74], where it preferentially hydrolyses Lys6 and Lys11-linked ubiquitin chains [14]. Original work by Bingol et al. showed that USP30 KO increases mitophagy in human cell lines overexpressing Parkin and rescues parkinsonian phenotypes in *Drosophila* expressing PINK1 or Parkin mutations [6]. Several structures of USP30 have been solved and provide further insights in its function [28,96]. USP30 is thought to fulfill a homeostatic role by removing and recycling ubiquitin residues at the OMM [14]. When mitochondrial damage occurs, USP30 may help to limit Parkin action and mitophagy to the site of damage [28]. Whether USP30 acts

upstream or downstream of PINK1 activation is not fully resolved. Using both, mtKeima and mitoQC reporter systems it was shown in cells *in vitro* that depletion of USP30 increases mitophagy, but not in the absence of PINK1 [67]. These findings suggest that USP30 may act upstream of PINK1 and prevent tonically active mitophagy [67]. The same authors showed that USP30 depletion also increases the removal of peroxisomes from cells, a process termed pexophagy, pointing to a previously unknown regulatory role of USP30 in pexophagy [67]. Effects of USP30 KO in mice have not yet been published. To further understand the therapeutic potential of USP30 inhibition for ameliorating mitophagy deficits in Parkinson's disease, several USP30 small molecule inhibitors are under development by Mission Therapeutics (11 patents describing selective small molecule inhibitors of USP30) and Mitobridge LLC [52]. A natural molecule inhibiting USP30 has also been described [36].

4.4. Novel targets

How can we find novel targets in the PINK1/ Parkin pathway for future drug development? Several high-content imaging based phenotypic screens have been published. Genome-wide siRNA screens for modulators of CCCP-induced mitophagy in HeLa cells overexpressing Parkin identified upstream regulators of Parkin [38] and ATPase inhibitory factor 1 as key players in this pathway [65]. A similar siRNA screening paradigm in *Drosophila* cells discovered several targets in the sterol regulatory element binding transcription factor (SREBF) lipogenesis pathway and showed that exogenous lipids can promote mitophagy [44]. A sophisticated chemogenomic approach able to detect subtle increases in Parkin expression discovered epigenetic agents, drugs controlling cholesterol biosynthesis and c-Jun N-terminal kinase inhibitors that can enhance Parkin expression [37]. Finally, a genome-wide CRISPR screen in SH-SY5Y neuroblastoma cells revealed a role for the transcriptional repressor THAP domain-containing protein 11 in regulating cellular Parkin and pUb levels [91]. Future research is necessary to validate these novel targets in neuronal cells *in vitro* and *in vivo*.

5. Efficacy and safety considerations for mitophagy enhancers

Activators of mitophagy may have a therapeutic benefit for PD patients beyond those with genetic mutations in the PINK1/ Parkin pathway. Inactivation of Parkin due to post-translational modifications has been reported in sporadic, late-onset PD [16,59,60,117]. In addition, mitochondrial dysfunction and mitochondrial complex I deficiency has been widely observed in PD [98,108]. And even if the mitophagy pathway is not impaired in these patients, further boosting its function may be beneficial to clear dysfunctional mitochondria and ameliorate pathology. However, removal of too many mitochondria may aggravate pathology rather than mitigating it. Since mitophagy is a complex pathway that is intricately regulated by other pathways, such as lysosomal function and inflammation [89,112], unwanted side-effects, such as overwhelming the lysosomal system could also be anticipated. PINK1, Parkin and USP30 serve cellular functions beyond mitophagy and enhancing their function could cause dysregulation of other cellular pathways (for recent reviews, see [67,94,101,114]). Due to these potential safety concerns it is necessary to identify and stratify Parkinson's disease patients with mitochondrial dysfunction that may most benefit from mitophagy enhancing treatments and to carefully monitor responses to therapeutic agents using biomarkers.

6. Biomarkers for mitochondrial dysfunction and mitophagy

As a direct measure of PINK1 and Parkin activity, pS65-Ub is a promising biomarker [23,27]. A specific pS65-Ub antibody shows accumulation of pS65-Ub on mitochondria in brains from elderly human subjects [23] and in patients with Lewy body disease [24] and could

potentially be used for enzyme-linked immunosorbent assay in human biofluids including cerebrospinal fluid [23]. Alternatively, pS65-Ub can be detected by mass spectrometry [104] and phosphoproteomics approaches may prove successful for detecting pS65-Ub in human biofluids [30,81]. A low abundance of pS65-Ub in cerebrospinal fluid or blood could be used to identify patients with defective mitophagy and an increase in pS65-Ub in response to treatment with a mitophagy enhancer could provide a pharmacodynamic read-out. Furthermore, several biomarkers are being developed to monitor mitochondrial health: A novel positron emission tomography (PET) tracer binds selectively to mitochondrial complex one in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated monkeys [110] and is currently in explorative clinical research by a pre-competitive consortium called MIND-MAPS (<https://goo.gl/PI7H7C>). If successful, this PET tracer may help stratify PD patients with mitochondrial complex I deficiency and indirectly monitor treatment response to mitophagy enhancing small molecules by measuring the desired outcome of these therapies, *i.e.* healthy mitochondria with normal mitochondrial complex I levels. Multiple fluid biomarkers of mitochondrial DNA integrity are also in discovery, such as mtDNA copy number, methylation, deletions and point mutations, and damage (for review, see [29,73]). A significant decrease in mtDNA copy number is observed in PD patient blood cells independent of age and gender [36,92] correlating with changes observed in postmortem brain [36,92], but see [20]. Reduced cell-free circulating mtDNA levels in the cerebrospinal fluid are detected in early-stage Parkinson's disease [92]. However, elevated levels of cell-free circulating mtDNA levels were found in symptomatic PD patients carrying LRRK2 mutations compared to asymptomatic LRRK2 mutation carriers, idiopathic PD patients and controls [90] suggesting that more studies are necessary to fully understand the time course of changes in cell-free circulating mtDNA levels for biomarker purposes. Methylated mitochondrial DNA (mtDNA) was reported to be reduced in the cerebrospinal fluid of PD patients, but with no association between age, gender or clinical presentation and severity [42]. In postmortem PD brains, the D-loop region of mtDNA, which is important for mitochondrial transcription and replication, showed a loss of methylation in nearly all CpG and non-CpG sites relative to control samples [7], but further research is necessary to confirm concordance between blood and brain [45]. MtDNA sequence analysis has revealed an increasing number of mutations associated with diverse clinical phenotypes, however, specific PD-linked mutations have not been found [19]. Other potential biomarkers of mitochondrial integrity are being researched in animal models of PD, but will require further and independent validation in human postmortem brain tissue and biofluids: Accumulation of mono-oxygenated, mitochondria-specific cardiolipins is observed in the substantia nigra and elevated levels of polyunsaturated cardiolipins are detected in plasma of rats exposed to rotenone, a toxin-based animal model of PD [68]. Taken together, on-target PINK1/Parkin activity markers, such as pS65-Ub in combination with PET imaging and/or biofluid measures of mitochondrial integrity may enable clinical assessment of small molecule mitophagy enhancers.

7. Conclusions

Targeting PINK1/Parkin mediated mitophagy is an emerging therapeutic approach for the treatment of PD. Significant progress has been made since the discovery of the PINK1/Parkin pathway less than a decade ago: Several small molecule drugs to enhance mitophagy are in preclinical development as are exploratory biomarkers for mitochondrial dysfunction and mitophagy activation. Many questions remain open with regards to selecting the most effective and feasible targets in the mitophagy pathway, possible side effects of mitophagy enhancement and the PD patient population that will most likely benefit from such treatments. Until these questions can be answered, mitophagy research remains a promising new avenue for drug and biomarker development.

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