==========REVIEWS==========

AUTOPHAGY IMPAIRMENT IN PARKINSON'S DISEASE: APPROACHES TO THERAPY

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Abstract. Parkinson's disease (PD) is one of the most common neurodegenerative disorders characterized by progressive motor impairment due to the death of dopaminergic neurons in the substantia nigra of the brain. PD affects more than 1% of the population over 60 years of age worldwide. Despite significant progress in understanding the pathogenesis of PD, including genetic and biochemical aspects, current therapies are limited to symptomatic treatment. Recent evidence suggests that impaired autophagy leads to the accumulation of abnormal proteins, particularly α-synuclein, aggregated forms of which are neurotoxic to dopaminergic neurons in the substantia nigra. Notably, PD is predominantly sporadic. However, monogenic forms of the disease have also been described. Among the most common PD forms with known etiology are PD associated with mutations in the *GBA1* gene and PD associated with mutations in the *LRRK2* gene. Leucine-rich repeat kinase 2 (LRRK2), encoded by the *LRRK2* gene, and the lysosomal enzyme glucocerebrosidase (GCase), encoded by the *GBA1* gene, are involved in the same endolysosomal pathway. The LRRK2 and GCase dysfunction reported in PD, especially in the case of mutations in the genes encoding them, can lead to impairment of the endolysosomal pathway, lysosomal function, and possibly autophagy. This review highlights the molecular mechanisms of autophagy and prospects for targeted therapy of PD based on the induction of autophagy by influencing key players in this process.

Keywords: *Parkinson's disease, GBA1, LRRK2, autophagy, mTOR, inducers, therapy*

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INTRODUCTION

Parkinson's disease (PD) is the second most common neurodegenerative disease after Alzheimer's disease, affecting about 1% of the world's population over 60 years of age. Between 1990 and 2016, the number of people with PD doubled to over 6 million worldwide [1]. According to preliminary projections, the number could reach 12 million or more by 2040 [2]. PD is characterised by motor symptoms such as bradykinesia, resting tremor and rigidity, and non-motor symptoms such as cognitive impairment and depression [3, 4]. The symptoms of PD are due to the death of dopaminergic neurons in the substantia nigra (SN) of the brain. The accumulation and aggregation of α-synuclein protein in the CS of the brain is thought to underlie the pathogenesis of PD [5]. PD is mostly sporadic, that is, it occurs spontaneously or of unknown cause or aetiology. Sporadic forms of PD account for about 85% of cases. Familial forms of PD are rare and account for about 10-15% [6]. To date, more than 20 genes have been identified, mutations in which lead to the development of autosomal dominant and autosomal recessive forms of PD (PARK7, LRRK2, APOE, PINK1, PRKN, GBA, VPS35, RAB39B, ATP13A2 (PARK9), WDR45, FBXO7, etc.). [7, 8].

Among the forms of PD with known etiology, two of the most common can be distinguished: one is associated with mutations in the gene*GBA1*(GBA-BP), and the other in the gene*LRRK2*(LRRK2-BP) [9, 10].

The exact molecular mechanisms of PD, both sporadic and monogenic, remain unknown, and as a result, there is currently no therapy aimed at reducing and slowing down the death of neurons in the SN. Thus, the identification of targets for targeted therapy of PD is extremely important.

Recent studies suggest that impaired autophagy, a process that degrades about half of the α-synuclein protein, may underlie the pathogenesis of PD [11, 12]. Understanding the role of autophagy in the pathogenesis of PD is very important for the development of new therapeutic approaches. This review highlights key links in the molecular mechanism of autophagy that may be associated with the development of PD.

MOLECULAR MECHANISMS OF AUTOPHAGY

Autophagy plays a key role in cells by removing misfolded proteins, damaged cellular organelles, renewing organelles for cellular homeostasis, as well as maintaining energy homeostasis during starvation by recycling cytosolic components to compensate for nutrient deficiency. Autophagy can be activated by various cellular stress conditions, such as nutrient deficiency or

starvation, as well as by the accumulation of misfolded proteins [13]. There are three types of autophagy: chaperone-mediated autophagy (CMA), microautophagy, and macroautophagy.

CMA is a selective mechanism for the degradation of specific cytosolic proteins inside lysosomes. In this process, cytosolic proteins containing the pentapeptide KFERQ or its analogues are recognized in the cytosol by the Hsp70 chaperone complex and its co-chaperones: Hsp40, Hsp90aa1 (Hsp90), Dnajb1 (Hsp40), St13 (Hip), Stip1 (Hop), Bag1. After recognition, the proteins bound to chaperones are transported to the lysosomal membrane, where they are translocated into the lumen of lysosomes with the help of lysosome-associated membrane protein type 2A (LAMP2A) [14] (Fig. 1 *a*). It is important to note that LAMP2A, which is an alternatively spliced form encoded by the *LAMP2* gene, is considered the rate-limiting stage of CMA, as the activity of this process directly depends on the level of LAMP2A protein in the lysosomal membrane [15, 16] . Further, a cylindrical pore forms in the lysosomal membrane, substrate proteins finally penetrate into lysosomes, where they undergo degradation [17, 18] . CMA is considered one of the main pathways for intracellular degradation of α synuclein in cells, the accumulation and aggregation of which is currently considered the cause of neurodegeneration in PD [19–21].

Fig. 1. Schematic representation of autophagy processes. *a* - Chaperone-mediated autophagy; *b* - microautophagy; *c* - macroautophagy. Here and below: figures created using BioRender (https://www.biorender.com).

Microautophagy refers to one of the least studied types of autophagy and involves direct engulfment of cytoplasmic substrates by lysosomes through membrane invagination [22] (Fig. 1 *b*).

The main and most represented form of autophagy in the cell is macroautophagy. In this case, cup-shaped or rod-shaped bimembranous sequestering structures called phagophores are first formed inside the cell [23]. The membrane source of phagophores presumably originates from the endoplasmic reticulum (ER), plasma membrane, mitochondria, and contact sites between the ER and mitochondria [24-28]. During elongation, phagophores engulf cytoplasmic "cargo," during which the LC3B protein (microtubule-associated protein 1A/1B light chain 3; MAP1LC3B or ATG8F) is transformed through adenylation of its C-terminus first into LC3B-I. After this, cytosolic LC3B-I covalently binds to phosphatidylethanolamine (PE), forming the lipid-bound form LC3B-II, which is recruited to the phagophore membrane. The ubiquitin signal in tagged proteins binds to the p62 protein (sequestosome-1, or ubiquitin-binding protein p62), which interacts with LC3B-II, leading to the engulfment of ubiquitinated proteins by the phagophore. Once the phagophore completely engulfs its "cargo," it forms an autophagosome. Subsequently, it fuses with late endosomes, forming single-

membrane hybrid organelles called amphisomes, which likely act as a sink for the autophagic and endocytic pathways [29]. The formed amphisomes fuse with lysosomes, creating acidic autolysosomes for subsequent degradation of the contents (Fig. 1 *c*). The regulation of macroautophagy in the cell follows two main pathways: mTOR-dependent and mTOR-independent.

mTOR-dependent and mTOR-independent regulation of autophagy processes

The main pathway that negatively regulates macroautophagy is PI3K /AKT/mTOR [30-32]. This signaling cascade involves phosphoinositide-3-kinase (PI3K), Akt, and the downstream molecule mTOR . mTOR is the catalytic component of two distinct multiprotein complexes: mTORC1 and mTORC2 [33]. The rapamycin-sensitive complex mTORC1 includes mTOR, Raptor, and mLST8 and primarily regulates cell growth and energy metabolism. The DEP domain, part of the mTOR-interacting protein DEPTOR, and the proline-rich 40-kDa AKT substrate PRAS40, are auxiliary components that modulate mTORC1 activity. Additionally, mTORC1 promotes lipid synthesis by phosphorylating lipin-1, thereby increasing the activity of sterol regulatory element-binding protein-1 (SREBP1). mTORC2, consisting of mTOR, Rictor, mSin1, and mLST8, is primarily involved in cytoskeleton reconstruction and cell survival and is not sensitive to rapamycin [34, 35] (Fig. 2 *a*).

Fig. 2. mTOR complexes and regulation of macroautophagy through the mTORC1 pathway. *a* - Structure of mTORC1 and mTORC2 complexes; *b* - activation of mTORC1.

The PI3K subunit, downstream in the signaling cascade of effector G-coupled protein receptors and tyrosine kinase receptors, is a heterodimer consisting of the p110 catalytic subunit and the p85 regulatory subunit with dual activity: serine/threonine (Ser/Thr) kinase and phosphatidylinositol kinase [36]. PI3K plays an important role in growth, survival, differentiation, transport, and glucose metabolism in cells [37].

The PI3K subunit binds to corresponding receptors or receptor-binding proteins on the cell membrane: epidermal growth factor receptor (EGFR), vascular endothelial growth factor receptor (VEGFR), and fibroblast growth factor receptor (FGFR), which leads to activation of its catalytic activity. Activated PI3K, by phosphorylating phosphatidylinositol-4,5-bisphosphate (PI(4,5)P2; PIP2), catalyzes the formation of phosphatidylinositol-3,4,5-trisphosphate (PIP3). In turn, PIP3 serves as a messenger that binds to phosphoinositide-dependent kinase-1 (PDK1) for subsequent phosphorylation of protein kinase B (AKT) at Thr308 for its activation.

AKT can also be regulated by various hormones, including insulin and growth factors [38]. Activated AKT is a key protein of signal transduction that phosphorylates several substrates and downstream effectors, including GSK-3, FOXOs, BAD, caspase-9, nuclear transcription factor kappa-B (NF-κB), p21, and mTOR [39]. Activated AKT phosphorylates mTOR, leading to activation of the mTORC1 complex. AKT can also activate mTORC1 by phosphorylating the TSC2 protein from the tuberous sclerosis complex TSC1/TSC2 (hamartin/tuberin). Phosphorylation of TSC2 leads to inactivation of the functional TSC1/TSC2 complex [40]. When the TSC1/TSC2 complex is in an active, i.e., non-phosphorylated state, TSC2 promotes the hydrolysis of GTPase Rheb (Rheb-GTP) to GDPase, which leads to inactivation of mTORC1. Conversely, activation of AKT through phosphorylation of TSC2 causes dissociation of the TSC1/TSC2 complex and the release of Rheb-GTP, which stimulates mTORC1 activity. AKT can also be activated through its phosphorylation by mTORC2 at position Ser473 [41] (Fig. 2 *b*), and inactivation of TSC2 can occur under the action of 5'AM P-activated protein kinase (AMPK) [42]. The PI3K kinase can also be involved in the activation of mTORC2. However, the exact mechanism remains unclear [43]. Further, inactivation of mTORC1 leads to the induction of autophagy through inhibition of mTORC1-dependent phosphorylation of serine/threonine protein kinase ULK1 at position Ser757, which is known as the anti-autophagy site. Phosphorylation at this position results in the isolation of ULK1 from the AMPK molecule, which activates ULK1 by phosphorylation at position Ser317 (Fig. 2 *b*) [35]. Further, activated ULK1 kinase phosphorylates proteins necessary for initiating the autophagy process and subsequent phagosome formation, as described above [44] .

In addition to the classical mTOR pathway regulating macroautophagy, mTOR-independent pathways have recently been described, which include signaling cascades involving cAMP/EPAC/PLC-IP3, Ca^{2+} /calpain ions, and inositol (Ins) [45-48]. These pathways have an additive effect on mTOR-dependent autophagy.

The increase in intracellular levels of cyclic AMP (cAMP) with enhanced adenylate cyclase (AC) activity leads to the activation of EPAC protein (exchange protein directly activated by cAMP). The pathway is stimulated by a receptor mediated by the α-subunit of the heterotrimeric G-protein (GSA), resulting in the activation of phospholipase C (PLC). PLC hydrolyzes phosphatidyl-4,5-bisphosphate (PIP2) into phosphatidylinositol-1,4,5-trisphosphate (IP3) and diacylglycerol (DAG). IP3 acts as a secondary messenger, binding to its receptors (IP3R) on the ER, which leads to the release of Ca^{2+} ions from the ER into the cytoplasm, inducing a series of cellular responses [49, 50]. The increase in Ca^{2+} ions inside the cytosol activates the family of Ca^{2+} -dependent cysteine proteases called calpains (autophagy inhibitors), which cleave and in turn activate the GSA protein. This leads

to increased AC activity and, consequently, cAMP levels, thereby forming a feedback loop. Phosphatidyl-IP2 is cleaved by 5'-phosphatase and inositol polyphosphate-1-phosphatase (IPPase) to form phosphatidyl-PIP1, which is further hydrolyzed by inositol monophosphatase (IMPase) into free inositol - an important component of the subsequent signaling cascade [51, 52]. Elevation of intracellular levels of inositol or phosphatidyl-IP3 inhibits autophagosome synthesis [53]. Thus, activation of this pathway suppresses autophagy (Fig. 3). To initiate autophagy, it is necessary to reduce the level of inositol or phosphatidyl-IP3. This cyclic pathway has multiple targets for autophagy induction.

Fig. 3. mTOR-independent regulation of autophagy through cAMP/EPAC/PLC-IP3, Ca^{2+}/calpain and inositol (Ins) signaling pathways. Activation of receptors through the α-subunit of the heterotrimeric G-protein (GSA) and adenylate cyclase (AC) leads to an increase in cyclic AMP (cAMP) levels, which activates the EPAC protein. This is followed by activation of phospholipase C (PLC), which hydrolyzes phosphatidylinositol-4,5-bisphosphate (PIP2), forming inositol-1,4,5-trisphosphate (IP3) and diacylglycerol (DAG). IP3 binds to its receptors on the endoplasmic reticulum (ER), causing the release of Ca^{2+} ions into the cytoplasm. This activates a family of Ca^{2+}-dependent cysteine proteases — calpains, which cleave and activate GSA. This, in turn, enhances AC activity, which increases cAMP levels and creates a feedback loop. PIP2 is hydrolyzed by 5'-phosphatase and inositol phosphatase (IPPase) to form inositol-1-phosphate, which is further cleaved by inositol monophosphatase (IMPase) to free inositol, necessary for downstream signaling. An increase in intracellular levels of inositol or IP3 inhibits autophagosome synthesis.

AUTOPHAGY DYSFUNCTION IN THE PATHOGENESIS OF PARKINSON'S DISEASE

Dysfunction of macroautophagy, both mTOR-dependent and mTOR-independent, may contribute to the development of neurodegenerative diseases, especially those associated with protein folding disorders, due to their accumulation and subsequent induction of cellular toxicity [51].

Thus, disruption of both CMA and macroautophagy processes may be associated with PD pathogenesis. The monomeric form of α-synuclein protein is removed from the cell through the CMA process [54-56]. In turn, oligomeric forms of α-synuclein can be degraded by macroautophagy [19, 57].

Disruption of the macroautophagy process has been detected in postmortem brain studies of PD patients, particularly in the SN where elevated levels of LC3B protein were found [54, 58, 59]. Other studies in the SN of PD patients' brains revealed a decrease in key CMA proteins: Hsp70 and

LAMP2A, which correlated with α-synuclein accumulation [54, 60]. Recently, it has been shown that dysregulation of mTOR protein kinase, the main regulator of autophagy, is involved in PD pathogenesis [61, 62], however, the exact mechanism remains unknown. For instance, S. Gaacoppo et al. [63] showed that mTOR levels are significantly reduced in brain tissues of PD patients. At the same time, L. Crews et al. [64] found that mTOR levels positively correlated with α-synuclein accumulation in brain tissues of PD patients. Interestingly, α-synuclein overexpression can inhibit autophagy processes at the autophagosome formation stage [65], as well as through induction of mTOR activity [66]. The existence of a feedback loop between α-synuclein and mTOR levels cannot be excluded.

The current understanding of neurodegeneration mechanisms in PD has significantly advanced due to the discovery of hereditary forms of this disease and the study of molecular mechanisms of their pathogenesis. This review examines genes whose mutations lead to the most common forms of PD with known etiology, and the protein products of these genes that participate in the regulation of autophagy processes.

LRRK2 Gene

The gene *LRRK2* encodes leucine-rich repeat kinase 2 (LRRK2), which is a multifunctional serine/threonine kinase with GTPase activity. Mutations in the *LRRK2* gene lead to the development of autosomal dominant form of PD (LRRK2-PD). Among sporadic cases, the frequency of mutations in the *LRRK* 2 gene is 0.7–1%, and among familial cases up to 7% in various populations, including the Russian population [67, 68]. It is known that mutations located in the GTPase and kinase domains of LRRK2 increase kinase activity. For example, the p.G2019S mutation in the kinase domain leads to a tenfold increase in kinase activity, while mutations in the GTPase domain, such as p.R1441C/G, increase it by 4 times. This suggests that excessive phosphorylation of kinase substrates due to increased LRRK2 activity negatively affects the viability of dopaminergic neurons [69].

The main substrates of LRRK2 are Rab proteins, which belong to the GTPase family and play a key role in various cellular processes, including the maintenance of lysosomal function [70]. Phosphorylation of Rab proteins mediated by LRRK2 is directly related to their function in maintaining lysosomal activity [71, 72]. It has recently been shown that LRRK2 kinase is involved in intracellular transport [73]. Increased LRRK2 kinase activity disrupts vesicle transport and lysosomal function, and also promotes neuroinflammation [74]. LRRK2 is thought to play an important role in regulating autophagy. In particular, the p.G2019S mutation in the LRRK2 protein has been found to be associated with impaired autophagosome maturation. In neurons differentiated from induced

pluripotent stem cells (iPSCs) of patients with LRRK2-PD, a decrease in colocalization of LC3 protein with LAMP1 was revealed [75]. At the same time, in a culture of neurons treated with LRRK2-in-1, an inhibitor of LRRK2 kinase activity, an increase in LC3B-II and p62 protein levels was observed, indicating activation of the autophagy process [45]. Additionally, LRRK2 kinase has recently been identified as an important player in the cellular degradation of α-synuclein, where it affects the key autophagy protein LAMP2A. It is suggested that mutant LRRK2 kinase may disrupt lysosomal protein degradation and contribute to the accumulation and formation of oligomeric forms of α-synuclein in neuronal cells by blocking its translocation to lysosomes [76–78].

It has been found that the cytosolic form of LRRK2 can be degraded under the action of CMA. In turn, the p.G2019S mutation in LRRK2, as well as high concentrations of wild-type LRRK2, can inhibit CMA by blocking the formation of the CMA translocation complex on the lysosomal membrane, leading to LAMP2A accumulation [77]. Similar results were obtained in cultures of astrocytes derived from human iPSCs with LRRK2-G2019S, as well as in embryonic fibroblasts from mice knocked out for LRRK2-R1441C [79, 80]. Recently, it has been shown that in dopaminergic neurons differentiated from iPSCs of patients with LRRK2 p.G2019S, the mutant kinase phosphorylates and thereby activates Leu tRNA tRNA synthetase, which attaches Leu to tRNA, leading to mTORC1 activation and suppression of autophagy processes [81]. Inhibition of LRRK2 kinase activity in macrophages differentiated from patient iPSCs, and in microglia, led to increased levels of lysosomal proteins and enzymes and induction of autophagy processes [82]. Our group and other authors have shown that patients with PD associated with mutations in the *LRRK2* gene that lead to increased kinase activity of the protein, have altered activity of lysosomal hydrolases in blood [83-86]. Additionally, inhibition of LRRK2 kinase activity led to changes in the level of lysosomal proteins and enzyme activity, including GCase [87-89]. GCase is encoded by the *GBA1* gene, mutations in which lead to decreased GCase activity and are considered a high-risk factor for PD.

The GBA1 gene

The gene *GBA1* encodes the lysosomal enzyme GCase, which is involved in glycosphingolipid metabolism. Mutations in the *GBA1* gene are considered high genetic risk factors for PD with known etiology – GBA1-PD – in 10–15% of all cases, with figures varying across different populations [90–95]. In patients with GBA1-PD, the most common mutations are p.N370S and p.L444P in GCase [10]. Biallelic pathogenic variants of *GBA1* lead to the development of a rare autosomal recessive disorder belonging to the class of lysosomal storage diseases – Gaucher disease [90]. One hypothesis regarding the connection between GCase enzyme activity dysfunction and, consequently, impaired lysosomal

function and PD development involves an increase in oligomeric forms of α-synuclein. Previously, we and other researchers have shown that patients with GBA1-PD have reduced GCase enzymatic activity, with accumulation of lysosphingolipids and oligomeric forms of α-synuclein in the blood and brain [96–98]. At the same time, a feedback loop has been identified, involving an increase in concentration and subsequent formation of α-synuclein protein aggregates. These aggregates are thought to impede the transport of GCase protein to lysosomes. This leads to accumulation of its substrates: glucosylceramide and glucosylsphingosine in lysosomes, which promotes accelerated formation of oligomeric forms of α-synuclein [99, 100]. Additionally, in dopaminergic neurons differentiated from human iPSCs carrying a heterozygous mutation in the *GBA1* gene, the addition of GCase to the medium reduced the concentration of glucosylceramide and, consequently, the level of α-synuclein [101]. Accumulation of α-synuclein in GCase dysfunction, including through disruption/inhibition of autophagy processes, has also been demonstrated in other PD models [99, 102, 103]. Thus, it can be assumed that decreased GCase activity and α-synuclein accumulation represent links in a vicious cycle where increased α-synuclein levels reduce GCase activity, which leads to increased α-synuclein concentration in cells [104]. Recently, a study conducted on both primary neurons of mice with GCase dysfunction and fibroblasts obtained from patients with GBA1 *PD* revealed elevated levels of phosphorylated ribosomal protein – protein kinase S6 (phospho-S6K) – which is one of the substrates and markers of mTOR activity [105]. When comparing transcriptomic patterns of primary peripheral blood macrophage cultures from patients with GBA1-PD, asymptomatic carriers of mutations in the *GBA1* gene, and neurologically healthy individuals, we identified [106] disturbances in the PI3K/AKT/mTOR signaling pathway in the GBA1-PD patient group. Moreover, similar disruptions were found when comparing the transcriptome of the SN in the brain of a mouse PD model with GCase dysfunction [107]. This model was created through combined injections of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) – the gold standard for parkinsonism induction – and conduritol-β-epoxide (CBE) – a GCase inhibitor that forms a covalent bond with the enzyme. For comparison, transcriptome data from the SN of mice injected with either MPTP or CBE separately were used (Fig. 4). Disruption of the mTOR pathway was also identified in proteome analysis of dopaminergic neurons differentiated from iPSCs of patients with GBA1-PD [108]. Additionally, in *Drosophila* models with *dGba1* deficiency (ortholog of the *GBA1* gene) *in vivo* , disruption of autophagy processes and decreased mTOR levels were observed [109].

Fig. 4. Scheme of RNA sequencing and data analysis of primary culture of peripheral blood macrophages from patients with GBA1-PD, asymptomatic carriers of mutations in the *GBA1* gene (GBA1-carriers), and neurologically healthy individuals, as well as samples of SN from mice with combined induction of parkinsonism with GCase dysfunction (MPTP+CBE), mice with induction of parkinsonism (MPTP), mice with GCase dysfunction (CBE), and mice with NaCl injection (control).

Thus, the homeostasis of the autolysosomal pathway is closely linked to PD, and disruption of autophagy can cause accumulation of aberrant proteins, thereby inducing neuronal death and accelerating disease progression [110]. At the same time, the commonality of mechanisms leading to impaired activity of enzymes involved in autophagy processes, such as GCase and LRRK2, in both monogenic and sporadic forms of PD, allows us to hope for the identification of common therapeutic targets - regardless of the etiology of the disease. Interestingly, drugs being developed for monogenic forms of PD (LRRK2-PD, GBA1-PD) are also being tested on patients with the sporadic form. Studies of brain autopsies from patients with sporadic PD revealed decreased GCase activity and increased LRRK2 kinase activity [59, 111-113].

Thus, one of the promising potential targets for the development of targeted therapy for both GBA1-PD and LRRK2-PD, as well as sporadic forms of PD, may be genes and proteins involved in autophagy processes, including those participating in the regulation of mTOR activity.

TARGETS AND INDUCERS OF AUTOPHAGY

Targets of mTOR-dependent and mTOR-independent autophagy

As discussed above, autophagy is regulated through two main pathways: mTOR-dependent and mTOR-independent. In the mTOR-dependent pathway, the level of phosphorylated mTOR plays a key role, while in the mTOR-independent pathway, the main regulator is the level of inositol in the cell. Based on these data, drugs that induce autophagy are being developed. These inducers can be categorized by the targets they act on in mTOR-dependent and -independent autophagy .

The main target for inducing mTOR-dependent autophagy is the mTOR protein kinase. The most well-known drug acting on the mTORC1 complex is rapamycin. This antibiotic was isolated from soil bacterium *Streptomyces hygroscopeus* and was used as an antifungal agent. Later, it was discovered to be a potent immunosuppressant with broad antiproliferative effects in mammalian cells [114]. To improve the pharmacokinetics of rapamycin, several derivatives were designed: RAD001, CCI-779, and AP23573, which were named "rapalogs" [115-118]. Rapalogs, like rapamycin, inhibit mTORC1 but with less immunosuppressive effect [119]. Later, ATP-competitive mTOR inhibitors were developed that simultaneously suppress the activity of both mTORC1 and mTORC2. One

example is torin-1 (an mTOR kinase inhibitor), which blocks phosphorylation of all mTORC1 substrates more effectively than rapamycin and induces autophagy in both mouse and human cell lines more efficiently than rapalogs [120]. It's worth noting that second and third-generation mTOR activity inhibitors are also known today. The second generation includes ATP-competitive mTOR protein kinase inhibitors that act on both mTORC1 and mTORC2 (for example, OSI-027) [121]. Third-generation inhibitors exhibit dual specificity - for PI3K and mTOR - and can inhibit PI3K/mTOR (for example, BEZ-235 and omipalisib) [122].

Another target for inducing mTOR-dependent autophagy is the AMPK molecule - an upstream negative regulator of mTORC1. Drugs that directly affect AMPK activity include biguanides, such as metformin, which is used for treating type 2 diabetes. The autophagy-inducing effect of metformin has been demonstrated in various cancer cells *in vitro* , as well as in *in vivo* models [123, 124]. Harmol, an alkaloid of β-carbolines, like metformin, activates AMPK and inhibits mTOR [125]. This drug reduces activation of PI3K and AKT, leading to mTOR inhibition and subsequent induction of autophagy [126]. For the mTOR-dependent autophagy target ULK1, the inducer BL-918 has been developed. It increases phosphorylation of ULK1 at Ser317 and Ser555 and decreases phosphorylation at Ser757. This leads to autophagy induction, as the level of autophagic protein LC3B-II increases and autophagic flux is enhanced, promoting autophagosome formation [127].

It is still unclear whether the cell needs mTOR-independent autophagy and its induction. However, to date, a number of compounds have been identified that activate autophagy independently of mTOR. Inositol can be identified as the main target for inducing mTOR-independent autophagy. Drugs that reduce inositol levels, such as lithium, carbamazepine, or valproic acid [128], induce autophagy and promote the elimination of its substrates without inhibiting mTORC1 activity. Lithium, for example, primarily acts on autophagy induction by inhibiting IMPase , thereby preventing inositol recycling and leading to depletion of its cellular pool [129]. Similarly, valproic acid reduces inositol levels by inhibiting myo-inositol-1-phosphate synthase (MIPS), which catalyzes the rate-limiting step in inositol biosynthesis [130]. The specificity of inositol signaling pathway regulation has been demonstrated using the competitive IMPase inhibitor L-690,330 [131]. This inhibitor prevents inositol dephosphorylation, leading to depletion of its cellular levels and, consequently, stimulation of autophagy. Ca^{2+} -channels are also among the main targets for inducing mTOR-independent autophagy. Drugs such as fluspirilene, trifluoperazine (dopamine antagonist), pimozide, niguldipine, nicardipine, amiodarone, as well as loperamide, penitrem A [132] are used to block them. Another target can be the level of intracellular cAMP. To reduce it, the adenylate cyclase inhibitor 2'5'-

dideoxyadenosine is used, which blocks the conversion of ATP to cAMP, thereby reducing the concentration of the latter [132]. Enhanced autophagy is also observed when using calpain-specific inhibitors, such as calpastatin [133, 134].

Another target of the mTOR-independent pathway includes the autophagy proteins SQSTM1/p62 and LC3B. It has been shown that trehalose—a disaccharide considered a "chemical chaperone"—can specifically induce the expression of two autophagic proteins: SQSTM1/p62 and LC3B, the conversion of LC3B-I to its autophagosome-associated lipid form, LC3B-II, as well as nuclear translocation of TFEB (transcription factor EB, the main regulator of the expression of many autophagolysosomal components) [135, 136].

Using the screening method of SMERs (small molecule enhancers) and SMIRs (small molecule inhibitors), 8 compounds have been identified that can activate autophagy via an mTOR-independent pathway [137]. However, the molecular mechanism of their action is unknown.

Many of the autophagy inducers described above and presented in Table 1 may find therapeutic applications in neurodegenerative diseases. Many of them have been approved by the Food and Drug Administration (FDA) and have already been implemented in the production for therapy of various diseases not related to neurodegeneration processes. For example, metformin is used to treat type 2 diabetes, torin is used in the treatment of depression, verapamil is used for the prevention of angina attacks, arrhythmia, and in the treatment of arterial hypertension, calceptin is used to correct disorders of calcium and other types of mineral metabolism, trehalose is used for weight loss, proper nutrition, as a natural sweetener, etc.

Table 1. Targets of mTOR-dependent and mTOR-independent autophagy

Targets	Drugs	Reference
mTOR-dependent autophagy		
mTORC1	rapamycin, CCI-779, glucagon-like peptide-1, latrepirdine, torin 1	[115–118]
APMK, APK, PI3K	metformin, harmol	[123–125, 138]
ULK1	BL-918	[123, 124]
mTOR-independent autophagy		
IMPase	lithium, L-690,330	[129]
Inositol	valproic acid	[129]
Calpain	calpastatin, calceptin	[115]

$Ca2^{+}$-channels	verapamil, loperamide, amiodarone, nimodipine, nitrendipine, niguldipine, pimozide	[128, 132]
cAMP	clonidine, rilmenidine, 2'5'-dideoxyadenosine	[132]
GSA	NF449	[132]

Currently, the possibility of repositioning these drugs for the treatment of neurodegenerative diseases, including PD, is being discussed. At the same time, it should be noted that autophagy is a regulated process vital for cell functioning, therefore the effect of potential drugs regulating this process should be comprehensively evaluated when used in clinical practice.

Approaches to the Therapy of Parkinson's Disease Through Induction of Autophagy Processes

Recent studies have shown the effectiveness of rapamycin, an inhibitor of mTOR (activator of mTOR-dependent autophagy). It is known that rapamycin, by inhibiting mTOR, induces autophagy, which leads to a decrease in the level of α-synuclein protein and neurodegeneration. This was demonstrated in a mouse model of parkinsonism induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) [139]. Also, inhibition of mTOR led to enhanced clearance of mutant α-synuclein in neuronal cells expressing the *SNCA* gene with the p.A53T mutation [140]. In a study conducted on neurons differentiated from iPSCs obtained from patients with the neuropathic form of Gaucher disease (homozygous or compound carriers of mutations in the *GBA1* gene) and cultivated with the mTOR protein kinase inhibitor Torin-1, an improvement in the processes of lysosome biogenesis and autophagy was shown. "Process improvement" in this context means increasing the efficiency of formation and function of lysosomes, as well as activation of cellular mechanisms for cleaning and processing damaged or unnecessary cell components [141]. At the same time, cell models demonstrated enhanced clearance of mutant α-synuclein under the influence of mTOR-independent chemical inducers such as lithium, carbamazepine, trehalose, SMER, calpastatin, rilmenidine, and Ca^{2+} channel blockers [142, 143].

Based on the above, the main targets that can be used for the development of neuroprotective therapy for PD include the following: 1) direct regulators of the PI3K/AKT/mTOR pathway (AMPK, mTORC1, ULK1, PI3K); 2) regulators that are necessary to maintain the autophagy process from the moment of phagophore formation, including beclin 1 and IMPase, as well as 3) mTOR-independent autophagy, in particular TFEB, which is involved in the formation of autophagosome components. In addition, important targets include the LRRK2 kinase, which indirectly regulates mTORC1 and is involved in the regulation of autophagy at the phagophore formation stage, and the GCase enzyme,

which is critical for the stage of fusion of the lysosome with the amphisome [82, 144]. Targeting these targets can promote autophagy induction and enhance the clearance of misfolded proteins such as α-synuclein, which may slow the progression of PD, as shown in studies on cell lines and model animals [125, 128, 138, 145-149] (Fig. 5). Currently, drugs targeting these targets are in clinical trials: metformin, which affects the induction of mTOR-dependent autophagy via AMPK (phase II clinical trials; https://clinicaltrials.gov/study/NCT05781711); as well as trehalose, targeting the TFEB protein (phase IV clinical trials, which include patients with sporadic PD and LRRK2-PD; https://clinicaltrials.gov/study/NCT05355064).

Fig. 5. Schematic representation of the main potential autophagy targets for Parkinson's disease therapy.

It should be noted that when developing drugs affecting mTOR activity, both excessively high and excessively low mTOR activity can be fatal for neurons. It is necessary to precisely control the balance between activation of mTOR signaling and enhancement of autophagy. The therapeutic potential of mTOR inhibitors, for example, to enhance autophagy, is limited because this complex regulates many other cellular functions besides autophagy [150]. For instance, in the SH-SY5Y neuroblastoma cell line in the presence of the 1-methyl-4-phenylpyridinium ion (MPP^+), it was shown that the combined action of rapamycin and metformin leads to enhanced induction of autophagy, as expected, but is also accompanied by induction of cell death [115]. This indicates that hyperactivation of autophagy can be harmful to the cell. Apparently, the result depends on the concentration and mechanism of action of the drugs used. Thus, both rapamycin and metformin activate mTOR-dependent autophagy, which can lead to hyperactivation of other mTOR-regulated pathways in the cell and thereby cause its death. Based on these data, a promising plan can be built for the simultaneous use of inducers of mTOR-dependent and mTOR-independent autophagy as a promising strategy for the treatment of neurodegenerative diseases.

For example, induction of autophagy by combining mTOR-dependent and -independent pathways has an additive effect on the clearance of mutant α-synuclein in PC12 cells when rapamycin and lithium [48] or rapamycin and calpastatin [151] are used in combination. In a mouse model of PD induced by MPTP, it was shown that activation of both autophagy regulation pathways by rapamycin and trehalose leads to decreased neurodegeneration and restoration of cognitive activity in animals [48]. Positive therapeutic results have been described for the combination of rapamycin with some mTOR-independent agonists, including lithium [115] or calpastatin [48]. This therapeutic strategy

may also be applicable to other proteinopathies, such as Alzheimer's and Huntington's diseases, which are also characterized by abnormal accumulation of aberrant proteins in cells and impaired autophagy, including mTOR-dependent autophagy. For these diseases, it has been shown that induction of autophagy contributes to improved protein clearance [48, 152]. Currently, phase III clinical trials are being conducted to evaluate the effectiveness of metformin as a drug for the treatment of Huntington's disease (NCT04826692) and Alzheimer's disease (NCT04098666).

Further research on autophagy induction in *in vivo* and *in vitro* models with combined use of inducers is needed to determine effective concentrations and combinations of these drugs. A strategy can be proposed in which, simultaneously with inhibition of the mTOR complex, there is suppression of the activation of other pathways regulated by this complex – for example, the immune response, since uncontrolled immune response induces inflammation with mTOR hyperactivation [153]. It is known that increased secretion of pro-inflammatory cytokines is characteristic of PD patients [154]. One such therapeutic target that can be proposed is the STING protein (stimulator of interferon genes) – a key regulator of innate immunity cascades [155]. It is known that in PD, both sporadic and monogenic forms, STING activity is increased, which induces neuroinflammation [156]. Thus, the combined use of STING and mTOR inhibitors can be considered as a promising approach to PD therapy. This is confirmed by the results of a recently published study by J. Hinkle et al. [156], which demonstrated improved clearance of α-synuclein protein and reduced death of dopaminergic neurons in a mouse model of parkinsonism when STING was inhibited. Another direction may consist in using the combined action of mTOR-dependent or -independent autophagy inducers and pharmacological GCase chaperones or inhibitors of LRRK2 kinase activity. Such studies may become the next stage in the search for targets for targeted therapy of PD, both with known etiology and sporadic form.

CONCLUSION

One of the main hypotheses of PD pathogenesis is that disruption of autophagy processes leads to the accumulation of neurotoxic forms of α-synuclein in cells, which, in turn, contributes to disease progression. Conversely, activation of autophagy is considered a potential therapeutic approach that can slow down the development of PD. Understanding the role of autophagy in PD pathogenesis has significantly advanced through the study of hereditary forms of the disease. For example, PD-associated mutations in the genes *LRRK2* and *GBA1* lead to disruption of the autophagy process. Hopefully, understanding the molecular mechanisms of both mTOR-dependent and mTOR-independent autophagy pathways in the cell will eventually lead to the development of a strategy for

combined treatment of PD. It is logical to assume that using two drugs that activate autophagy through different mechanisms will provide a much better effect than monotherapy and slow down disease progression. However, it will be necessary to consider that excessive induction of autophagy can lead to cell death due to accelerated accumulation of degradation products. The effect of complex interactions between autophagy and other intracellular processes raises the question of how to induce autophagy without affecting cell homeostasis. There remains a problem with understanding the mechanisms of autophagy in PD pathogenesis and transforming already known autophagy inducers into clinical practice. Thus, it is necessary to continue research on the role of autophagy in PD pathogenesis and the possibility of using autophagy regulators as targets for targeted therapy. This approach may also be applicable to other human diseases associated with the accumulation of toxic proteins in cells.

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ETHICD DECLARATION

This article does not contain any studies involving humans or animals as research subjects.

CONFLICT OF INTERESTS

The authors declare no conflict of interest.

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