Small molecule enhancers of autophagy for neurodegenerative diseases

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Neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, prion diseases and polyglutamine disorders, including Huntington's disease and various spinocerebellar ataxias, are associated with the formation of protein aggregates. These aggregates and/or their precursors are thought to be toxic disease-causing species. Autophagy is a major degradation pathway for intracytosolic aggregate-prone proteins, including those associated with neurodegeneration. It is a constitutive self-degradative process involved both in the basal turnover of cellular components and in response to nutrient starvation in eukaryotes. Enhancing autophagy may be a possible therapeutic strategy for neurodegenerative disorders where the mutant proteins are autophagy substrates. In cell and animal models, chemical induction of autophagy protects against the toxic insults of these mutant aggregate-prone proteins by enhancing their clearance. We will discuss various autophagy-inducing small molecules that have emerged in the past few years that may be leads towards the treatment of such devastating diseases.

Neurodegenerative diseases

The functions of normal proteins are generally determined by their precise three-dimensional structures, which are determined by their amino acid sequences during the process of protein folding. Some proteins achieve their proper functional conformations by themselves, while others require the help of a class of proteins called molecular chaperones. Failure to reach or maintain the correct folded structure can lead to serious disease consequences.[1] Mutations in the primary amino acid sequences can cause proteins to misfold and aggregate, and such mutant protein aggregates are characteristics of protein conformational disorders (PCDs) or proteinopathies.

PCDs encompass a family of diverse human neurodegenerative disorders, such as Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), prion diseases and polyglutamine (polyQ) disorders, including Huntington's disease (HD) and various spinocerebellar ataxias (SCA).[2–4] The hallmark of this group of diseases is the presence of mutant protein aggregates (also referred to as inclusions). In most neurodegenerative diseases such as HD, PD, SCA, ALS and tauopathy, the aggregates are intracellular. Extracellular amyloid plaques are seen in AD. However, prion aggregation can occur both intracellularly and extracellularly.[3] The mutant protein aggregates usually comprise fibrils containing the misfolded proteins in a β-sheet conformation.[3] In principle, this conformational change may promote the disease by either a toxic gain-of-function or by diminished activity. However, in Mendelian neurodegenerative

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diseases, the mutant aggregate-prone proteins generally cause disease by gain-of-function mechanisms.

Autophagic clearance of mutant aggregate-prone proteins associated with neurodegenerative disorders

Autophagy is a major degradation pathway for various aggregate-prone disease-causing proteins, such as mutant huntingtin (both N-terminal fragments and full length forms) associated with HD, the A53T or A30P point mutants of α-synuclein causing familial PD, ataxin 3 involved in SCA3, and mutant forms of tau causing fronto-temporal dementias.[5–10] While the ubiquitin proteasome system (UPS) degrades short-lived nuclear or cytosolic proteins, autophagy substrates are generally long-lived cytoplasmic proteins, protein complexes or damaged organelles.[11] Although the aggregate-prone proteins can be cleared by the UPS, the narrow pore of the proteasome precludes entry of oligomers and larger structures. Thus, the UPS can only clear soluble monomeric species.[11]

Under physiological conditions, autophagy is a constitutive self-degradative process involved both in basal turnover of cellular components and as an induced response to nutrient starvation in eukaryotes.[12] During autophagy, portions of the cytoplasm are sequestered by elongation of double-membrane structures called phagophores, which form vesicles called autophagosomes. These vesicles then fuse with lysosomes to form autolysosomes, where their contents are degraded by acidic lysosomal hydrolases for subsequent recycling (Fig. 1). The only known mammalian protein that specifically associates with the autophagosome membrane is LC3 [microtubule-associated protein 1 (MAP1) light chain 3]. There are two forms of LC3: LC3-I and LC3-II. After autophagy induction, the cytosolic LC3-I form is conjugated with phosphatidylethanolamine, resulting in autophagosome-associated LC3-II.[13] As autophagic substrates can include oligomers and organelles, like mitochondria, it is likely that this system can clear intracytosolic monomeric and oligomeric species of aggregate-prone proteins. However, the inclusions in HD and PD that are visible by light microscopy are not membrane-bound, and are larger than typical autophagosomes.[11] Thus, it is likely that autophagy does not clear the large inclusions themselves, but removes the oligomeric and monomeric precursors.

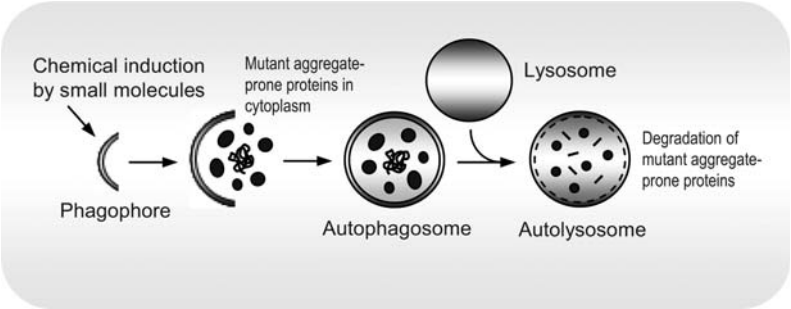

Fig. 1 Autophagy–lysosomal pathway for degradation of aggregate-prone proteins. Autophagy is the major degradation pathway for various intracytosolic, mutant, aggregate-prone proteins associated with neurodegenerative disorders. Induction of autophagy involves formation of double-membrane structures in the cytoplasm called phagophores. During the elongation process, portions of the cytoplasm containing the mutant proteins are sequestered into vesicular structures called autophagosomes. These vesicles then fuse with lysosomes to form autolysosomes, where their contents are degraded by acidic lysosomal hydrolases. Autophagy-inducing small molecules show protective effects in various models of neurodegenerative diseases by enhancing the clearance of disease-associated, aggregate-prone proteins.

Recent studies have highlighted the role of autophagy in neurodegeneration, since loss of basal autophagy in mouse neuronal cells resulted in neurodegeneration.[14,15] Enhancing autophagy may be a possible therapeutic strategy for neurodegenerative disorders where the mutant proteins are autophagy substrates. Thus, understanding the pathways regulating mammalian autophagy will help us enable to identify better drug targets for these devastating conditions.

Small molecule enhancers of autophagy

Chemical induction of autophagy protects against the toxic insults of disease-causing proteins associated with neurodegeneration by enhancing the clearance of the aggregate-prone proteins. Various compounds or strategies for inducing autophagy in mammalian systems that have emerged in the past few years are discussed in this review (Table 1). These were identified by cell-based assays, either by measuring the clearance of autophagy substrates or autophagic flux.

Inhibitors of mTOR

The mammalian target of rapamycin (mTOR), which is a key regulator of cell growth and proliferation, negatively regulates autophagy. The mTOR pathway involves two functional complexes: mTORC1 comprising mTOR, raptor (regulatory associated protein of mTOR) and GβL (G-protein β-subunit like protein), and mTORC2 consisting of mTOR, rictor and GβL.[16] Rapamycin (sirolimus), a lipophilic macrolide antibiotic first isolated from *Streptomyces hygroscopicus*, is a well-established inducer of autophagy (Fig. 2).[17] Rapamycin forms a complex with the immunophilin FK506-binding protein 12 (FKBP12), which then stabilizes the raptor-mTOR association and inhibits the kinase activity of mTOR.[18] The binding of GβL to mTOR stimulates its kinase activity, and GβL is necessary for the formation of a rapamycin-sensitive interaction between raptor and mTOR.[19] Inhibition of mTOR by rapamycin induces autophagy, but the exact downstream mechanism of action is still unknown in mammalian cells.[17]

We have previously shown that rapamycin enhanced the autophagic degradation of various aggregate-prone proteins, such as mutant forms of huntingtin, α-synucleins, ataxin 3, and tau in mammalian cell culture models.[5,6,9] This effect of rapamycin was associated with a reduced number of mutant protein aggregates and toxicity.[5,6] Consistent with these findings, we showed that autophagy induction by rapamycin, or its analogue CCI-779, was protective in *Drosophila* and mouse models of HD, and in *Drosophila* models of

Table 1 Autophagy-inducing small molecules and their effects in neurodegenerative diseases

Autophagy-inducing compounds	Target	Protective effects in neurodegenerative diseases	References
Rapamycin, CCI-779, glucose, glucose-6-phosphate	Inhibit mammalian target of rapamycin (mTOR)	Enhance clearance of mutant huntingtin, α-synuclein, tau, ataxin 3; protect cell, fly and mouse models of HD; protect fly models of tauopathy	5–7,9,20
Lithium, L-690,330	Inhibit inositol monophosphatase, and lower inositol and IP_3 levels	Enhance clearance of mutant huntingtin and α-synuclein; protect cell and fly models of HD; protect ALS mouse models and patients	23,36,44
Carbamazepine, sodium valproate	Lower inositol and IP_3 levels	Enhance clearance of mutant huntingtin and α-synuclein; protect cell models of HD	23
Trehalose	Unknown, mTOR-independent	Enhances clearance of mutant huntingtin and α-synuclein; protects cell and mouse models of HD	52,53
SMER10, SMER18, SMER28, 18 analogs of SMERs	Unknown, mTOR-independent	Enhance clearance of mutant huntingtin and α-synuclein; protect cell and fly models of HD	45
Loperamide, niguldipine, amiodarone, pimozide	Ca^{2+} channel blocker	Reduce expanded polyQ aggregates in cells	46
Fluspiriline, trifluoperazine	Dopamine antagonist	Reduce expanded polyQ aggregates in cells	46
Penitrem A	Inhibits high conductance Ca^{2+}-activated K^+ channel	Reduces expanded polyQ aggregates in cells	46

tauopathy.[5,7] Raised intracellular glucose or glucose-6-phosphate induced autophagy by inhibiting mTOR, and also reduced mutant huntingtin aggregates and toxicity by enhancing its clearance in HD cell models.[20]

Interestingly, mTOR was found to be sequestered in mutant huntingtin aggregates in HD cell models, transgenic mice and patient brains.[7] This sequestration impaired its kinase activity, and thereby would induce autophagy. This study identified a new protective role of mutant huntingtin aggregates in inducing autophagy, thus enhancing their own clearance.

Rapamycin also protected cells against pro-apoptotic insults by clearing mitochondria, which are endogenous autophagy substrates.[21] As a consequence of reduced mitochondrial load due to rapamycin pretreatment, the intrinsic apoptotic pathway was inhibited because of decreased cytochrome c release, leading to lower activities of caspases 9 and 3.[21] This additional protective effect of the autophagy-inducing property of rapamycin may be beneficial in the context of neurodegenerative disorders where there are secondary apoptotic insults.

Inositol-lowering agents

We have previously shown that mood-stabilizing drugs, like lithium, carbamazepine and valproic acid, which all lower intracellular inositol levels,[22] also induce autophagy.[23] Lithium (Li^+) is a monovalent cation with a hydrated ionic radius similar to that of magnesium (Mg^{2+}), which allows it to inhibit some enzymes by competing with the cofactor Mg^{2+}. It inhibits a small group of Mg^{2+}-dependent phosphomonoesterase enzymes in the phospho-inositol signaling pathway.[24] This pathway is stimulated by G-protein coupled receptor-mediated activation of phospholipase C (PLC), which hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP_2) to form inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG).[25] IP_3 functions as a second messenger and binds to its receptors (IP_3R) on the endoplasmic reticulum (ER), thereby releasing the stored ER Ca^{2+} into the cytoplasm, which then elicits a range of cellular responses.[26] IP_3 is degraded by a 5′-phosphatase and inositol polyphosphate 1-phosphatase (IPPase) to form inositol monophosphate (IP_1).[27] Inositol monophosphatase (IMPase) catalyses the hydrolysis of IP_1 into free inositol, which is required for the phospho-inositol signalling pathway (Fig. 2).[28]

Lithium inhibits IMPase and IPPase in the phospho-inositol pathway,[29,30] and its inhibitory effect primarily on IMPase prevents inositol recycling, leading to depletion of cellular inositol and a run-down of the phospho-inositol cycle (Fig. 2).[31] Lithium is a noncompetitve inhibitor of IMPase, as Li^+ occupies the second Mg^{2+}-binding site, leading to trapping of the phosphate group of the substrate.[32] Atack and co-workers synthesized bisphosphonate-containing inhibitors of IMPase. L-690,330 is one such specific competitive IMPase inhibitor, whose effects *in vitro* and *in vivo* resulted in increased IP_1 levels, thus mimicking the effects of lithium.[33] On the other hand, valproic acid reduces inositol levels by inhibiting myo-inositol-1-phosphate (MIP) synthase that catalyses the rate-limiting step of inositol biosynthesis.[34]

We have shown that lithium and L-690,330 induced autophagy by inhibiting IMPase.[23] Carbamazepine and valproic acid also induced autophagy by reducing inositol levels (Fig. 2). Induction of autophagy by these agents enhanced the clearance of soluble aggregate-prone proteins, and reduced mutant huntingtin aggregates and toxicity. These effects of the inositol-lowering agents were most likely mediated at the level of, or downstream of, lowered IP_3, as they were abolished by pharmacological treatments that increased IP_3. Furthermore, IP_3-regulated autophagy was mTOR-independent.[23] It is tempting to speculate whether the beneficial effects of inositol-lowering agents used in the treatment of bipolar disorders are mediated by the clearance of long-lived autophagy substrates, since these compounds have acute effects on free inositol levels before showing clinical effects.[35] This also opens an interesting speculation whether other autophagy inducers like rapamycin can be used in such conditions.

We have recently shown that glycogen synthase kinase-3β (GSK-3β), another intracellular target of lithium, has opposing effects on autophagy in an

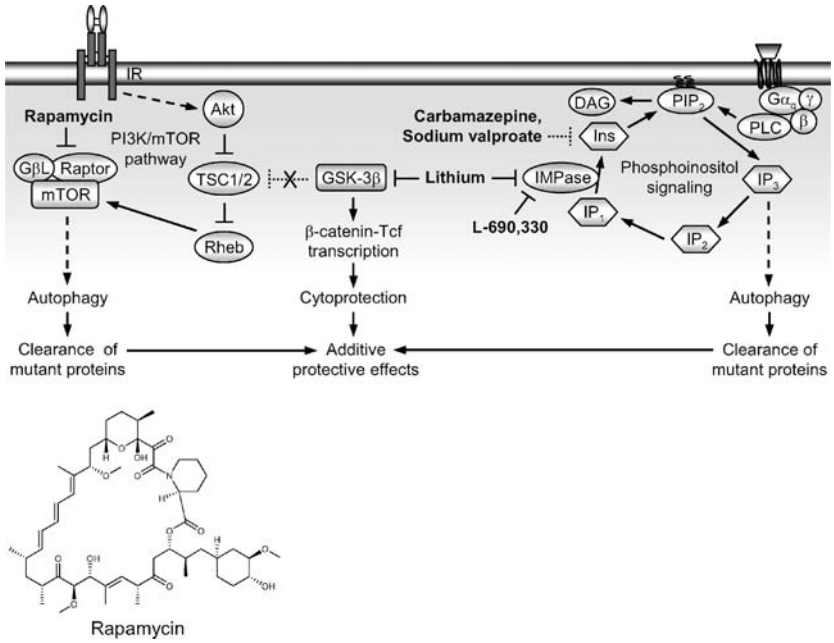

Fig. 2 Autophagy-inducing mTOR-dependent and mTOR-independent pathways for the clearance of aggregate-prone proteins. The mammalian target of rapamycin (mTOR), which is a downstream target in the phosphatidylinositol 3-kinase (PI3K) pathway, is a negative regulator of autophagy. Many diverse signals, such as growth factors and amino acids, regulate the raptor–mTOR pathway by inhibiting tuberous sclerosis complex (TSC1/2). This relieves the inhibitory effect of TSC1/2 on rheb, thereby activating the raptor–mTOR complex. Several kinases, such as Akt, signal to raptor–mTOR by phosphorylating TSC2 and inhibiting the activity of the TSC1/2 heterodimer. Rapamycin forms a complex with the immunophilin FK506-binding protein 12 (FKBP12), which then stabilizes the raptor–mTOR association and inhibits the kinase activity of mTOR. Inhibition of mTOR by rapamycin enhances the clearance of aggregate-prone proteins by inducing autophagy, but the exact downstream mechanism of action is currently unknown in mammalian cells. Autophagy can also be induced with drugs that decrease inositol or inositol 1,4,5-trisphosphate (IP_3) levels in the phospho-inositol signaling pathway in an mTOR-independent fashion. These drugs include lithium and L-690,330 by inhibiting inositol monophosphatase (IMPase), and carbamazepine and sodium valproate by inhibiting inositol (Ins) synthesis. However, lithium also inhibits glycogen synthase kinase-3β (GSK-3β) that activates mTOR by inhibiting TSC1/2, thereby impairing autophagy. Combination treatment with lithium and rapamycin alleviates the block in autophagy by GSK-3β inhibition (shown by a cross, X), and hence additively enhances autophagy and facilitates greater clearance of aggregate-prone proteins. Furthermore, GSK-3β inhibition by lithium increases β-catenin-Tcf-mediated transcription, which is cytoprotective and may contribute to additional protective effects of this combination treatment for neurodegenerative disorders.

mTOR-dependent manner.[36] GSK-3β is active in its non-phosphorylated form, whereas site-specific phosphorylation by lithium at Ser9 inhibits its activity.[37] Lithium is an uncompetitive inhibitor of GSK-3β with respect to substrate,[38] but competes with Mg^{2+} for binding.[39] Impairment of autophagy by GSK-3β inhibition was independent of its target, β-catenin, but was due to mTOR activation through phosphorylation of the tuberous sclerosis complex (TSC) protein TSC2 (Fig. 2).[36,40] Interestingly, lithium or L-690,330 reduced mutant huntingtin aggregates in GSK-3β null cells where mTOR is activated, implying that induction of autophagy due to IMPase inhibition occurred even in the absence of GSK-3β in an mTOR-activated state.[36] We have further investigated the benefits of combination treatment with lithium and rapamycin, where rapamycin was used to counteract the autophagy-inhibitory, mTOR-dependent effects of GSK-3β, caused by lithium.[36] Enhancing autophagy by mTOR-independent (with lithium) and mTOR-dependent (with rapamycin) pathways had additive protective effects in increasing autophagy and reducing mutant huntingtin aggregation and toxicity in HD cell and *Drosophila* models, compared to either drug alone (Fig. 2).[23,36]

Lithium also has likely anti-apoptotic beneficial effects in HD models by inhibiting GSK-3β and upregulating β-catenin.[41,42] The autophagy-inhibitory effects of GSK-3β may explain the moderate effects of lithium in an HD mouse model.[43] A recent study showed a promising effect of lithium in significantly attenuating the disease progression in ALS patients and mouse models.[44] Apart from lithium's neuroprotective effects, this fascinating result was suggested to be due to induction of autophagy.

Small molecule enhancers of rapamycin

We have recently described a chemical screening approach for identifying small molecule enhancers of mammalian autophagy (Fig. 3).[45] From 50 729 compounds, we screened for the enhancers [small-molecule enhancers of rapamycin (SMERs)] and suppressors [small-molecule inhibitors of rapamycin (SMIRs)] of the cytostatic effects of rapamycin in yeast. Such a screen would reveal the enhancers and suppressors of the physiological state induced by rapamycin in yeast, and we thought that the activities of at least some of these modifiers as single agents would be conserved in mammalian systems. The SMERs and SMIRs were then re-tested by themselves (without rapamycin) in mammalian cells, where three SMERs and thirteen SMIRs increased or decreased the clearance of the autophagy substrate A53T α-synuclein, respectively.[45] Of the three SMERs, SMER10 is an aminopyrimidone, SMER18 is a vinylogous amide and SMER28 is a bromo-substituted quinazoline. These SMERs induced mTOR-independent autophagy and reduced mutant huntingtin aggregates/toxicity in HD cell models, where they also showed additive protective effects with rapamycin. These SMERs also protected against mutant huntingtin fragment toxicity in *Drosophila*.[45]

Further screening of structural analogs of these three SMERs identified eighteen additional candidates that enhanced the clearance of mutant proteins and reduced the number of mutant huntingtin aggregates.[45] Analyses of limited

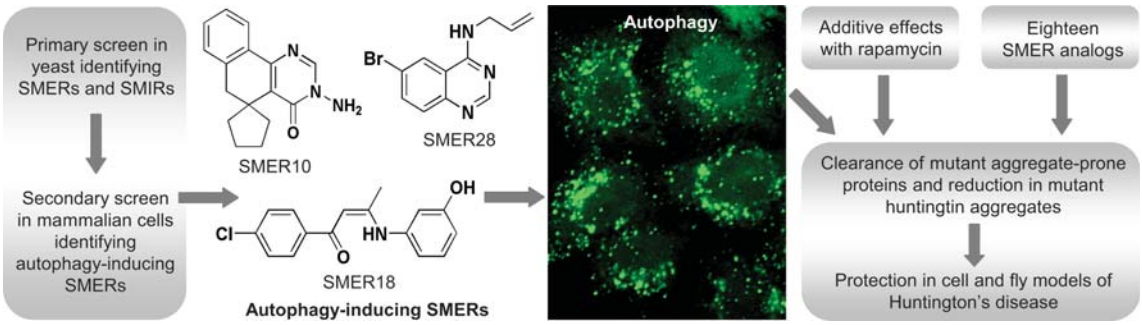

Fig. 3 A chemical screening approach for identifying autophagy-inducing small molecules. Schematic representation of a chemical screening approach for identifying small molecule regulators of mammalian autophagy, in which a primary screen in yeast identified small molecule enhancers (SMERs) and inhibitors (SMIRs) of the cytostatic effects of rapamycin. A secondary screen in mammalian cells assaying the clearance of autophagy substrates revealed three autophagy-inducing SMERs (SMERs 10, 18 and 28) acting independently of rapamycin and mTOR. These SMERs showed protective effects in cells and fly models of HD and had additive effects with rapamycin. Screening of the SMER analogs further identified eighteen candidates that showed protective effects in HD cell models.

structure–activity relationship (SAR) data revealed the effects of various chemical substitutions on the three parent SMERs. The pyrimidone functionality of SMER10 is essential for its autophagy-inducing effects, since substitution of a bulky phenyl group or creating a fused tetrazole abolished its activity. The hydroxyl group at the *meta* position in SMER18 is important, because removal of this group, but not changing it to *para* or *ortho* positions, abolished its autophagy-inducing activity. None of the substitutions in SMER28 were more potent than its own effects, and most of them were well tolerated. This was the first large-scale chemical screen for discovering autophagy modulators, where the SMERs identified in yeast act as autophagy enhancers by themselves in mammalian system.[45]

FDA-approved drugs

Recently, an image-based screen for autophagy inducers was carried out by Yuan and colleagues with 480 bioactive compounds in a stable human glioblastoma cell line expressing the autophagy marker, GFP-LC3.[46] Analysis was done by scoring GFP-LC3 punctate structures with high-throughput fluorescent microscopy, and the screen hits were classified into three groups depending on the number, size and intensity of the GFP-LC3 vesicles. Seven compounds were identified that induced mTOR-independent autophagic degradation without notable cellular damage, and also reduced expanded polyQ aggregates in a cell-based assay. These compounds were fluspirilene, trifluoperazine (dopamine antagonist), pimozide, niguldipine, amiodarone, loperamide (Ca^{2+} channel blockers) and penitrem A (inhibitor of high conductance Ca^{2+}-activated K^+ channels). Except penitrem A, all of them were FDA-approved drugs used for treating various disease conditions. This opens a new perspective for the treatment of neurodegenerative disorders, as six of the final hits were FDA-approved drugs.[46]

Some of the drugs identified in the screen by Yuan and colleagues may have undesirable side effects precluding their long-term use. However, they may be informative about relevant autophagy-regulating pathways for future study. Recently, we reported another drug screen where we discovered certain safe drugs that induce autophagy and protect in HD models, in addition to some overlapping hits with those found by Yuan and colleagues.[47] For example, we found that verapamil, an L-type Ca^{2+} channel antagonist used to treat hypertension with minimal side effects, induced autophagy and reduced toxicity in cell, fly and zebrafish models of HD.[47]

Trehalose

Trehalose is a disaccharide found in many non-mammalian species, including bacteria, yeast, fungi, insects, invertebrates and plants, in which two glucose molecules are linked together in an α,α-1,1-glycosidic linkage (α-D-glucopyranosyl-1,1-α′-D-glucopyranoside). Being a non-reducing sugar, it cannot be easily hydrolysed by acids, nor can the glycosidic bond be cleaved by glucosidase. The physical properties that make trehalose unique are its high degree of optical rotation and its melting behaviour. The combination of molecular structure and physico-chemical properties of trehalose results in a very stable disaccharide.[48]

Trehalose has been termed as 'chemical chaperone' as it influences protein folding through direct protein–trehalose interactions, and thus it protects cells against various environmental stresses by preventing protein denaturation.[48,49] It inhibits amyloid formation of insulin *in vitro*,[50] and has been recently shown to prevent aggregation of beta-amyloid (Aβ) associated with Alzheimer's disease.[51] Trehalose has also been shown to alleviate polyQ-induced pathology in HD mouse model, and this protective effect was suggested to be mediated by trehalose binding to the expanded polyQs, thus stabilizing the partially unfolded mutant protein.[52]

We have recently shown a novel property of trehalose in inducing mTOR-independent autophagy. Trehalose enhanced the clearance of mutant huntingtin (and of A53T and A30P mutants of α-synuclein), thereby reducing mutant huntingtin aggregation/toxicity.[53] Trehalose treatment increased autophagic flux in a variety of mammalian cells, and we showed that these effects were mediated by intracellular trehalose.[53] Furthermore, trehalose pre-treatment protected against pro-apoptotic insults by reducing mitochondrial load, as seen with rapamycin.[21,53] The dual protective properties of trehalose ('autophagy induction' for enhancing clearance of aggregate-prone proteins and 'chemical chaperone' for inhibiting aggregation), coupled with its

lack of toxicity, make it a possible candidate for the treatment of neurodegenerative disorders.

Future prospects

The neurodegenerative diseases that are the most obvious targets for autophagy upregulation are those that manifest intracytosolic mutant protein aggregates, such as HD, PD caused due to mutant α-synucleins, tauopathies and SOD1 ALS. It is currently unclear if this strategy would be of benefit to AD that has extracellular aggregates. In principle, one would like to treat patients with autophagy enhancers from the earliest possible age to maximize the reduction of the toxic species. In cases with a positive family history, mutations can be diagnosed and the patients who are at risk may undergo pre-symptomatic treatment. Furthermore, a recent study has highlighted the protective effects of post-symptomatic lithium treatment in ALS patients.[44]

Autophagy has emerged as an important field in recent years with implications in various human pathologies, such as neurodegeneration, infections, cancer, heart diseases and longevity.[12,54] Although a variety of autophagy enhancers and inhibitors have been identified of late, therapy for different diseases may require different drugs and pathways—increasing *versus* decreasing autophagy, mTOR-independent *versus* mTOR-dependent pathways, and different drugs depending on the target tissue. Understanding the molecular pathways and the role of autophagy is of pivotal importance in each of the disease contexts. One way to further understand the regulation of autophagy is through further studies of the pathways influenced by autophagy-modulating small molecules and drugs.

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