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REVIEW

Drug discovery by targeting the protein–protein interactions involved in autophagy

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Abstract Autophagy is a cellular process in which proteins and organelles are engulfed in autophagosomal vesicles and transported to the lysosome/vacuole for degradation. Protein–protein interactions (PPIs) play a crucial role at many stages of autophagy, which present formidable but attainable targets for autophagy regulation. Moreover, selective regulation of PPIs tends to have a lower risk in causing undesired off-target effects in the context of a complicated biological network. Thus, small-molecule regulators, including peptides and peptidomimetics, targeting the critical PPIs involved in autophagy provide a new opportunity for innovative drug discovery. This article provides general background knowledge of the critical PPIs involved in autophagy and reviews a range of successful attempts on discovering regulators targeting those PPIs. Successful strategies and existing limitations in this field are also discussed.

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1. Introduction

It has been known that proteins rarely act alone but interact with each other stably or transiently to function and allow regulation[1]. Till now, the estimated number of protein−protein interactions (PPIs) in the human interactome is about 130,000−650,000[2,3], which greatly out-numbers that of known protein targets[4]. PPIs are pivotal regulatory events in biological systems, providing the structural and functional basis for cellular processes, while abnormal PPIs have been extensively implicated in many human diseases[5−7]. For example, mixed-lineage leukemia (MLL) fusion proteins directly interact with menin protein, driving the leukemogenesis in MLL leukemia[8]. As a result, PPIs represent an important and attractive target class for pharmacological intervention[9−14]. Encouragingly, decades of efforts into molecular biology, biochemistry, structural biology and medicinal chemistry have expanded the cases in the field of PPI drug discovery[15,16]. Though PPIs were once thought of as almost entirely undruggable, it has become clear that many PPIs can be successfully targeted with the right set of tools and sustained efforts[17]. In particular, Venetoclax[18], a selective BCL-2 inhibitor as the first FDA-approved drug, stands as a milestone in PPI drug development.

Autophagy serves as the most important scavenging process of protein aggregates and damaged organelles among eukaryotes[19]. There are three major types of autophagy: macroautophagy, chaperone-mediated autophagy (CMA), and microautophagy[20] (Fig. 1). During macroautophagy, cytoplasmic components are segregated into a double-membrane vesicle called autophagosome, which is then trafficked to lysosomal membrane and fuses along with an available lysosome. Eventually, the contents of the vesicle, namely autolysosome, are degraded and recycled[21]. CMA is characterized by the chaperone-dependent targeting of specific cytosolic proteins for proteolysis[22]. HSC70 chaperone binds to proteins containing KFERQ-like sequence and then associates with the lysosome-associated membrane protein type 2A (LAMP-2A) receptor. This triggers LAMP-2A oligomerization and leads to the translocation of the bound protein into the lysosome. As for microautophagy, it refers to a non-selective process that recruits targeted components around the lysosomal membrane and degrades them by lysosomes[23]. Among these three types of autophagy, macroautophagy is most extensively studied and often referred to as autophagy in literature if not otherwise specified. Notably, the critical role of autophagy in cell biology and its potential therapeutic prospect have gained the most important recognition in science as Professor Yoshinori Ohsumi was awarded the 2016 Nobel Prize in Physiology or Medicine for his achievements[24].

Autophagy is fundamental to the preservation of organismal fitness, and the link between autophagy and human diseases has been well discussed in previous publications[25,26]. Autophagy regulation has been considered a potential therapeutic strategy for various human diseases including cancer[27,28], neurodegenerative disorders[29,30], infectious diseases[31], and autoimmune diseases[32,33]. Currently, small-molecule drugs (or other forms of drug) that regulate certain autophagy process have not reached market. Approved drugs like rapamycin, chloroquine, and hydroxychloroquine were able to regulate autophagy, but they were not originally developed for this purpose. To achieve this goal, more efforts are required for translating preclinical findings on autophagy regulation into practical therapies.

It should be noted that the entire autophagic process from signal transduction to cargo degradation was orchestrated by plenty of proteins and the interaction network between them[34,35]. Thus, multiple targets for autophagy regulation may exist among them. Decades of research into medical chemistry have attempted to achieve precise intervention of a single autophagic molecular machine, such as ULK1 and PI3K kinase[36−38], providing new

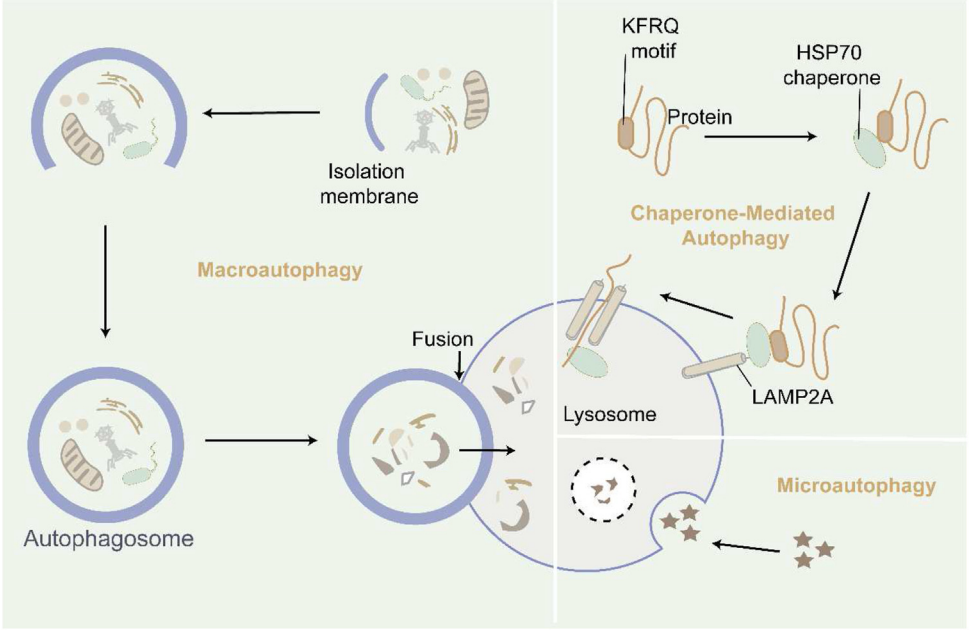


Figure 1 Illustration of three major types of autophagy. Macroautophagy delivers cellular contents to lysosome *via* the formation of autophagosomes. Chaperone-mediated autophagy is characterized by the chaperone-dependent targeting of specific cytosolic proteins containing the KFRQ motif to LAMP-2A on the lysosomal membrane for proteolysis. Microautophagy refers to a non-selective process that recruits and degrades targeted components by lysosomes.

understanding of autophagy regulation and its therapeutical potential. However, proteins often play multiple roles in different pathways rather than executing a single function. Thus, direct intervention of protein activity is prone to bring unwanted side effects. In contrast, intervention of PPI may avoid interfering with irrelevant pathways and allows precise manipulation of the autophagy process with minimized perturbation[39].

In this review article, we will describe the current understanding of autophagy regulation by PPI intervention and the emerging approaches applicable to this goal. Then, we will summarize the well-known drug discovery efforts on targeting autophagy-related PPIs as well as the discovery strategies from some successful examples. We will discuss the valuable lessons learned from these drug discovery efforts.

2. Protein−protein interactions involved in autophagy

Up to now, over 40 autophagy-related genes/proteins (ATG) have been identified in yeast[40], most of which are conserved in mammals and essential in the progress of autophagy. These ATG proteins and related PPIs govern autophagy pathways in an intricate and controlled manner. A few protein complexes formed by critical PPIs are well described in literature[41−45]: (1) The ULK1 complex (or called ATG1 complex in yeast)[46]. This complex consists of four members, *i.e.*, ULK1, ATG13, ATG101, and RB1CC1/FIP200, which is required for autophagy initiation in response to starvation. (2) The ATG9 system[47], that serves as a direct source of autophagosome. (3) The PI3KC3−C1 complex (VPS15-VPS34-ATG14L-Beclin 1 complex)[48], responsible for generating PI3P as a platform for autophagosome biogenesis. (4) The ATG2−WIPI complex (WD-repeat domain phosphoinositide-interacting proteins)[49], which acts as a membrane tether and also has lipid transfer activity. (5) The ATG12−ATG5−ATG16L1 complex[50], which is responsible for lipidation of LC3 to form LC3-II. (6) The ATG8-family proteins conjugation system[51], which participates in phagophore expansion and cargo recruitment.

In particular, the interactions between ATG8 and its partners have offered quite a few targets for drug discovery. Thus, more details should be given here about the ATG8 family proteins. So far, seven ATG8 homologues have been identified in mammals, including MAP1LC3A (2 splice variants), MAP1LC3B, MAP1LC3C (also LC3A-C), GABARAP, GABARAPL1, and GABARAPL2[52]. These ATG8 family proteins are central coordinators of phagophore initiation and elongation, autophagosome assembly, maturation, and lysosomal fusion[53]. In the process of autophagy, ATG8/LC3 undergoes C-terminal lipidation with phosphatidylethanolamine (PE) *via* an ATG7-ATG3 activation and an ATG12-ATG5-ATG16L1 transfer cascade. After that, ATG8 protein is incorporated into the autophagosomal membrane, promoting the cargo recruitment. Here, selective autophagy receptors simultaneously bind the target and ATG8-family proteins (or other components of the autophagosome) and result in sequestering and degrading specific types of cargo in selective autophagy[54,55].

In aspect of structure, topology of the ATG8 family proteins resembles that of ubiquitin, but along with two additional characteristic N-terminal α-helices, which vary significantly among the ATG8 family (Fig. 2A). And the N-terminal α-helices aligned to the ubiquitin-like core form a deep hydrophobic pocket (HP1, also termed W site). Another hydrophobic pocket, HP2 (also termed L site), is built by hydrophobic residues of the central α-helix α3 and β-strand 2 of the UBL core (Fig. 2B). A so-called

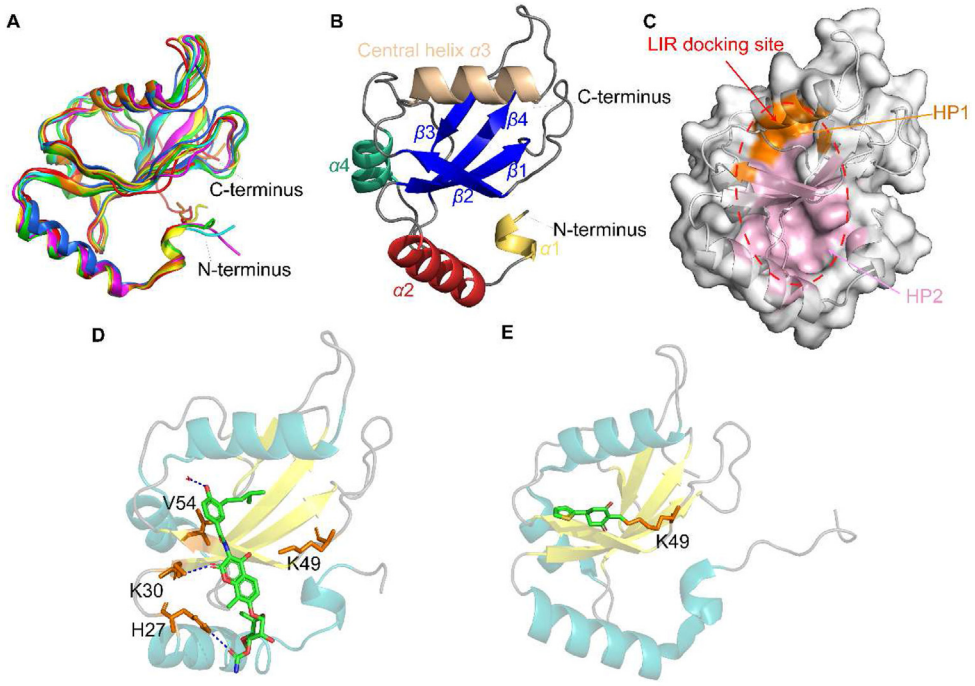


Figure 2 Structural features of ATG8 family proteins and two examples of LC3 inhibitors. (A) Structural alignment of yeast Atg8 and human LC3A, LC3B, LC3C, GABARAP, GABARAPL1, and GABARAPL2. All protein backbones are shown as ribbon models in rainbow color scheme. (B) The crystal structure of LC3B (PDB entry 1UGM). (C) Illustration of the LIR-docking site (LDS) on LC3B, which comprises two hydrophobic pockets HP1 (orange) and HP2 (pink). (D) The crystal structure of LC3A in complex with dihydronovobiocin (PDB entry 6TBE). (E) The crystal structure of LC3B in complex with compound a4 (PDB entry 7ELG).

LIR-docking site (LDS) formed by these two hydrophobic pockets mediates a majority of known interactions between ATG8 family proteins and their interacting partners such as p62 (Fig. 2C). These interactions were extensively investigated, resulting in the concept of LC3-Interacting Region (LIR) motif (also known as ATG8-Interacting Motif, AIM)[56]. Usually, the LIR motif is described as W/F-X-X-L/I/V, and X is any type of amino acid. It is exactly the critical role played by the interaction between ATG8/LC3 and LIR that has drawn an increasing amount of drug discovery efforts on it. Computational methods generally require crystal structural information of high quality to guide screening efforts. Up to date, over 50 structures of LC3/ATG8 with various LIR motifs are available in the Protein Data Bank. Furthermore, Wang et al.[57] revealed LIR peptide specificity among ATG8 family proteins. The interactions between a total of 14 representative LIRs of different selective autophagy receptors and ATG8 proteins were quantitatively characterized by competitive time-resolved FRET analysis. Such studies have laid the foundation for discovering drugs that selectively disrupt specific ATG8−LIR interactions.

Continuous advances in proteomics study have expanded our knowledge of other autophagy-related PPIs[58−60]. For example, Giampietri's group[61] described a novel molecular mechanism of c-FLIP (cellular FLICE-like inhibitory protein) protein in autophagy regulation in 2021. They demonstrated that c-FLIP interacts with Beclin 1 and enhances Beclin 1 stability by preventing its ubiquitination and degradation. Interactions among proteins exist in the form of network, and thus network analysis[62−65] is commonly used for depicting the complicated biological activities in cell and mining of new potential targets for drug discovery. In recent years, many efforts are dedicated to deciphering global PPI networks[66−70]. For example, Harper et al.[71] reported in 2010 a systematic proteomic analysis of the human autophagy system and revealed a network of 751 interactions among 409 candidate proteins, providing a global view over the mammalian autophagy landscape. All such interactions were detectable by mass spectrometry (LC−MS/MS). Large amounts of the interactions not identified before this study were demonstrated to play roles in vesicle trafficking, protein ubiquitination, phosphorylation, and lipid phosphorylation.

Nevertheless, at present only a few databases provide information about the PPIs in autophagy. The Autophagy Database (ADB)[72] currently includes the information of over 7400 genes/proteins in 82 eukaryotes. It records 114 interactions between 31 proteins involved in autophagy, but the sources of such information are often not indicated. Autophagy Regulatory Network (ARN)[73], a systems-level bioinformatics resource for studying the mechanism and regulation of autophagy, contains manually curated, imported, and predicted interactions of autophagy components (1485 proteins with 4013 interactions) in human. Among them are 238 interactions formed by 38 core autophagy proteins. Apparently, more serious efforts on the collection, storage, and analysis of autophagy-related data are still desired to fully unleash the pharmaceutical potential in this field.

3. Drug discovery by targeting autophagy-related protein−protein interactions

Regulation of PPIs has been considered challenging in the history of drug discovery[74]. First, the interface of a PPI is relatively flat and wide (average 1500−3000 Å^2)[75−77], which lacks a well-defined site for small-molecule compounds to bind. Second, strong interactions existing on a PPI interface, formed between continuous or discontinuous amino acid residues on both sides, is difficult to be disrupted by small-molecule compounds[78]. Third, for conventional drug targets such as enzymes and receptors, certain endogenous substances may serve as a good starting point for drug discovery. But in the case of PPI, such convenience does not exist[78]. Last but not the least, due to the size of a PPI interface and its hydrophobic nature, regulators of PPI usually violate the Lipinski's 'rule of five'[79], particularly in terms of molecular weight and lipophilicity.

Despite all these difficulties, an increasing amount of effort has been devoted to this field over the past two decades, which has led to promising outputs[80,81]. The following parts of this article will focus on reviewing the drug discovery efforts by targeting autophagy-related PPIs (Fig. 3). Those candidate drugs mentioned in this article include small-molecule compounds (Table 1) as well as peptides (Table 2), and they mostly function as either autophagy inhibitors or inducers.

3.1. *Inhibitors of the ATG8−ATG3 interaction*

Reynisson et al.[82] aimed at the human ATG8−ATG3 interaction to carry out a docking-based virtual screening of the InterBioScreen Ltd. collection of 9050 natural product entities. The targeted binding site on ATG8 was centered at where the Leu343 on ATG3 was bound. The resultant 30 hits were selected and subsequently tested in the MCF7 breast cancer cell line stably expressing LC3-eGFP. The IC_{50} value derived from the reduction of LC3-eGFP puncta formation was used to evaluate the activity of the tested compounds. Compounds AT109 (IC_{50} = 2.3 ± 0.1 μmol/L) and AT110 (IC_{50} = 1.2 ± 0.6 μmol/L), both of which share the same molecular scaffold, exhibited the best potency (Table 1). Next, a similarity search of AT109 and AT110 led to the discovery of another potent hit AT130 (IC_{50} = 2.8 ± 0.1 μmol/L). All three compounds caused the accumulation of p62-eGFP puncta in the HEK293 human embryonic kidney cell line. In addition, AT110 exhibited little or modest cytotoxicity in nine human cancer cell lines, suggesting that those cancer cells perhaps do not rely on the autophagy mechanism to survive. In an *in vivo* assay conducted in zebrafish, AT110 was observed to block autophagy flux at low concentrations. Nevertheless, this work did not provide any result from an appropriate biophysical or biochemical assay to directly measure the interruption of the ATG8−ATG3 interaction by those active compounds.

3.2. *Inhibitors of the PfATG8−PfATG3 interaction*

Plasmodium falciparum malaria poses a major public health threat among developing countries, and the continuous development of drug resistance has put a high demand on the discovery of novel therapies[83]. Accumulated evidence has indicated that certain proteins involved in autophagy, for example, ATG8 are necessary for the survival and development of *Plasmodium*[84]. Thus, autophagy intervention by small molecules may hold promise in *Plasmodium* treatment through retarding the blood- and liver-stages of parasite growth. In 2012, Hain's group[85] reported the co-crystal structure of *Pf*ATG8 (*P. falciparum* ATG8) with an AIM motif of *Pf*ATG3 (*P. falciparum* ATG3), conferring the structural biology basis for subsequent drug discovery efforts. The same group screened 400 drug-like and probe-like compounds from the Malaria Box Library by employing a surface plasmon resonance (SPR)-based binding assay. In this way, they discovered several

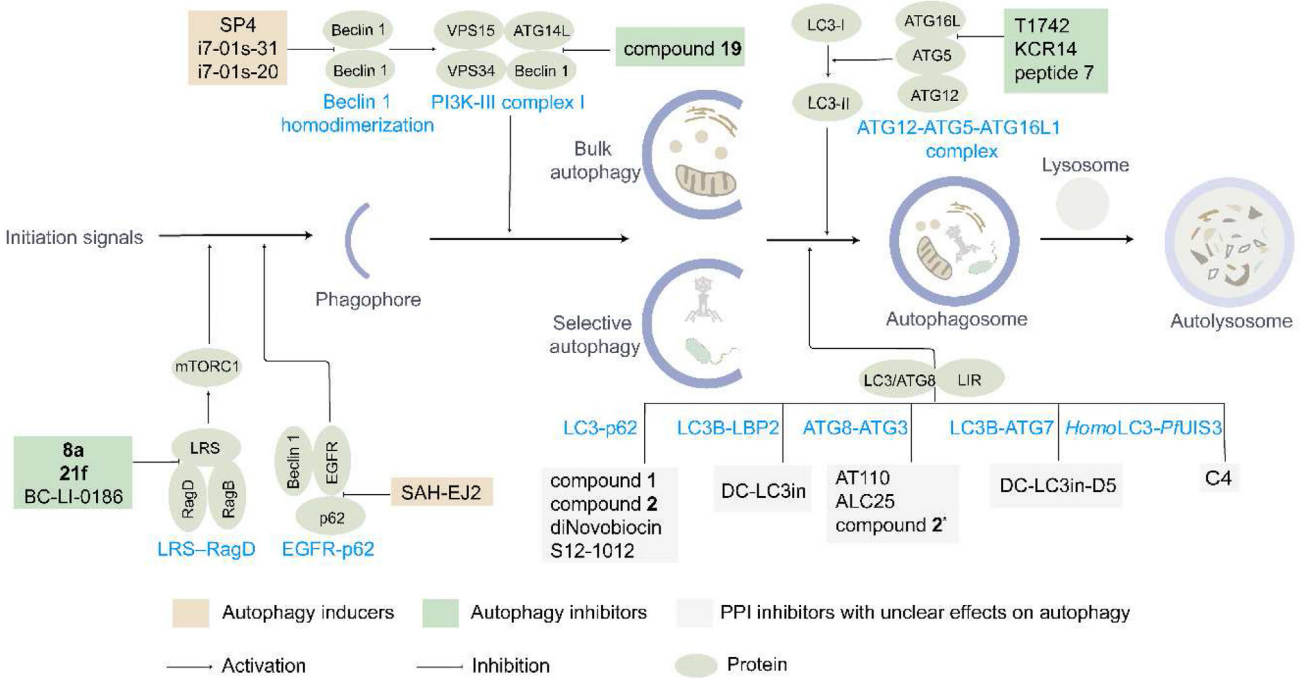


Figure 3 Autophagic processes that are known to be amenable to pharmacological intervention by PPI regulation.

active compounds sharing a common 4-pyridin-2-yl-1,3-thiazol-2-amine scaffold as inhibitors of the *Pf*ATG8−*Pf*ATG3 interaction[86]. Molecular docking suggested that these compounds bound to the W- and L-sites on *Pf*ATG8. Moreover, these small-molecule inhibitors exhibited inhibitory effects against both blood- and liver-stages of infection by *P. falciparum*, presumably through prevention of *Pf*Atg8 lipidation.

Sequence alignment and mutagenesis studies revealed that besides the W- and L-sites on *Pf*ATG8, the *Pf*ATG8−*Pf*ATG3 interaction requires an additional region on *Pf*ATG8 (*i.e.*, residues 67−76) called the A-loop, which is conserved in apicomplexans but absent in human homologues[85]. This finding provides a possibility for the discovery of compounds that inhibit the ATG8−ATG3 interaction in apicomplexans without affecting the homologous interaction in human. In this context, Bosch et al.[87] performed a virtual screening of 369,632 molecules in the ChemBridge library against the A-loop region on *Pf*ATG8. Fourteen selected hits were then tested in an SPR binding assay, leading to the discovery of the most potent compound ALC25 (IC_{50} = 18.5 μmol/L, Table 1). Additional SPR and ITC binding assays confirmed that ALC25 bound predominantly to the A-loop region on *Pf*ATG8 rather than the W-site or L-site. Furthermore, ALC25 gave rise to a shift in lipidation state of *Pf*ATG8 and inhibition of *P. falciparum* growth in both blood- and liver-stage cultures.

Another case study of ATG8−ATG3 interaction inhibitor was reported by Stefania Villa and coworkers[88]. Peptidomimetic can retain key molecular features of the template peptide by replacing peptide bonds with enzymatically more stable bonds and thus is a common strategy for developing PPI inhibitors. In Villa's study, the template peptide to be mimicked is the *Pf*ATG3 interaction motif. Molecular dynamics simulations on the *Pf*ATG8−*Pf*ATG3 complex structure were performed to identify the hot spots on the *Pf*ATG3−*Pf*ATG8 binding interface and the minimal active segment of *Pf*ATG3. As result, the WLLP sequence of *Pf*ATG3 was chosen as the most suitable segment to be mimicked. Heterocyclic building blocks, *i.e.*, 1,2,3-triazoles, were chosen to reproduce peptide primary and secondary structures because they are bioisostere of the amide group and are also resistant to enzymatic degradation. Besides, 1,4-disubstituted 1,2,3-triazoles can be conveniently synthesized through click chemistry, *i.e.*, 1,3-dipolar cycloaddition reaction between azides and alkynes under copper salts catalysis. Other structural optimization included replacement of the isopropyl sidechain of *Pf*ATG3−L106 by a phenyl ring to form additional cation−π or π−π interaction with the phenyl sidechain of *Pf*ATG8−F49 and replacement of *Pf*ATG3−P108 by a pyridine or a pyridazine ring to create suitable H-bonds with the guanidine sidechain of *Pf*ATG8−R28. *T*he optimal length of the ethylene spacer linking those heterocycles and the 1,2,3-triazole ring was also explored and determined. A total of four peptidomimetics were actually synthesized following molecular design. Compound **2*** in the (*S*,*S*) configuration (Table 1) exhibited the most prominent activity in an SPR displacement assay (IC_{50} = 3.8 μmol/L) (Table 1), but showed low potency in preventing *P. falciparum* growth in red blood cell cultures (IC_{50} ≈ 560 μmol/L). However, its corresponding (*R*,*S*)-enantiomer, *i.e.*, compound **1***, was approximately ten-fold more potent in *P. falciparum* growth inhibition assay (IC^{50} ≈ 40 μmol/L), probably due to higher transport efficiency across the red blood cell membrane or lower degradation rate in a cellular context. Therefore, compound **1*** was regarded as a more promising lead for further structural optimization.

3.3. *Inhibitors of the UIS3−LC3 interaction*

Autophagy sometimes emerges as a remarkable intracellular defense tool to confront upon host cell invasion by parasites. To infect the host cell successfully, intracellular pathogens have evolved various molecular-level mechanisms to subvert the host autophagy response, thus preventing pathogen elimination from

Table 1 Some small-molecule inhibitors of autophagy-related PPIs.

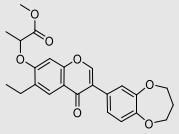
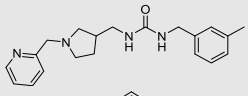
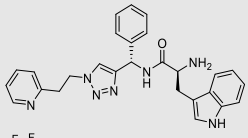
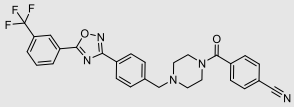
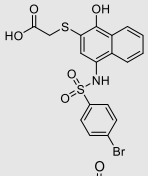
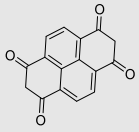
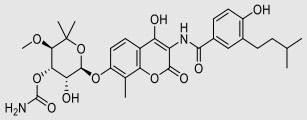
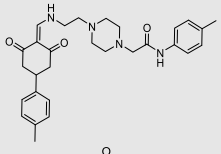
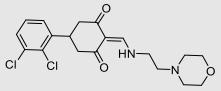
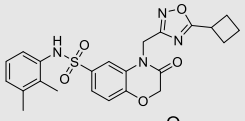
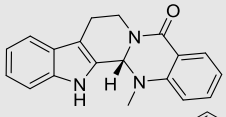
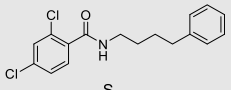
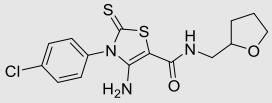
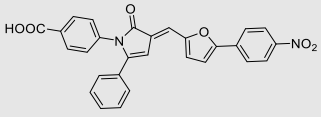
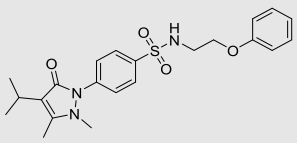
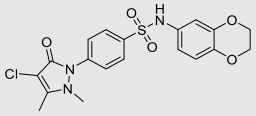
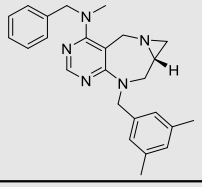
Compd.	PPI target	Chemical structure	IC_{50}/K_D	Assay	Ref.
AT110	ATG8−ATG3		1.2 ± 0.6 μmol/L	eGFP tagged LC3 in MCF7 cells	82
ALC25	*Pf*ATG8−*Pf*ATG3		1.59 μmol/L	SPR	87
Compd **2***	*Pf*ATG8−*Pf*ATG3		3.8 μmol/L	SPR	88
C4	*Homo*LC3−*Pf*UIS3		0.241 nmol/L	ITC	90
Compd **1**	LC3−p62		0.9 μmol/L	SPR	91
Compd **2**	LC3−p62		2.0 μmol/L	SPR	91
Dihydronovobiocin	LC3A−p62 LC3B−p62		16.0 ± 1.0 μmol/L 72.3 ± 7.4 μmol/L	HTRF	92
DC-LC3in	LC3B−p62		3.06 μmol/L	FP	95
DC-LC3in-D5	LC3B−ATG7		200 nmol/L	FP	95
Compd **19**	Beclin1−ATG14L		33.9 μmol/L	BRET	98
Evodiamine	ATG5−ATG16L1		Unknown	BiFC-FRET	103
KCR14	ATG5−TG16L1		Unknown	NanoBIT	104,105
H3	ATG5−ATG16L1		18.4 μmol/L	FP	106
T1742	ATG5−ATG16L1 ATG5−TECAIR		1.1 μmol/L 1.7 μmol/L	HTRF	107

Table 1 (*continued*)

Compd.	PPI target	Chemical structure	IC_{50}/K_D	Assay	Ref.
BC-LI-0186	LRS−RagD		0.109 μmol/L	S6K phosphorylation assay	123
8a	LRS−RagD		0.216 μmol/L	S6K phosphorylation assay	124
21f	LRS−RagD		4.8 ± 0.46 μmol/L	SPR	125

the host cell. Mota's group[89] showed that *Plasmodium*'s parasitophorous vacuole membrane (PVM) transmembrane protein UIS3 (Upregulated in infective sporozoites 3) could directly bind to the LC3 protein in host through a non-canonical LIR motif at the early stage of infection. This event sequestered LC3 on PVM, prevented parasite elimination by the autophagy machinery in host, and supported parasite survival in the hepatocytes. Therefore, the interaction between *Plasmodium* UIS3 and host LC3 represents a potential target for anti-malarial drug development. Later, Mota et al.[90] made a successful effort to discover small-molecule inhibitors of this PPI system. They virtually screened over 20 million compounds in the ZINC library to identify inhibitors targeting the LC3-binding pocket on *Plasmodium* UIS3. All selected hits were then tested in a phenotypic screen (PHS). Consequently, compound C4, which has a core structure of phenyloxadiazole (Table 1), was identified as the best hit. It impaired *Plasmodium* infection by disturbing parasite survival during development inside the host cell (IC_{50} = 176.3 and 121.9 nmol/L for *Plasmodium berghei* infected Huh7 cells and *Pf*UIS3@Pb infected Huh7 cells, respectively). It was noted that administration of C4 barely interfered with normal autophagy in the host cell. Additionally, isothermal titration calorimetry (ITC) showed that the binding affinity of C4 to the UIS3−LC3 complex (K_D = 0.241 ± 0.0011 μmol/L) was about ten-fold higher than that of the UIS3−LC3 direct binding, indicating that C4 was able to compete with the UIS3−LC3 interaction. Overall, this study revealed the disruption of a key host−parasite interaction as an effective strategy for preventing malaria infection in human host cells without affecting the intrinsic host functions.

3.4. *Inhibitors of the LC3−p62/ATG7 interaction*

p62/SQSTM1 is one of the most widely applied markers to monitor autophagy. It also interacts with the LC3 protein, thereby mediating degradation of ubiquitin-decorated cargo like misfolded proteins and aggregates. The LC3−p62 interaction is viewed as a potential PPI target for autophagy intervention. Tanaka et al.[91] performed a screening of ~10,000 compounds for LC3−p62 PPI inhibitors, which employed a fluorescence correlation spectroscopy (FCS) binding assay using a TAMRA-labeled p62-derived probe. Compared to fluorescent polarization (FP)-based assay, FCS-based assay is more sensitive, but may still yield false positives. To reduce false positives, the obtained hits were subsequently evaluated *via* a second FCS-based assay using an Alexa647-labeled p62-derived probe. Finally, an SPR binding assay identified two inhibitors (compounds **1** and **2**, Table 1) with IC_{50} < 2 μmol/L. Note that if the screened compound library scales up, the FCS assay may not be suitable anymore for screening due to its efficiency in signal detection and data acquisition.

In another work, Proschak and coworkers[92] described the discovery of the first drug-like small-molecule inhibitor of proteins LC3A and LC3B. A robust AlphaScreen interaction assay between biotin-LIRtide (*i.e.*, a polypeptide including the LIR sequence from p62) and GST−LC3B was firstly set up for a medium-throughput screening of 1280 approved drugs in the Prestwick Chemicals library. As a result, novobiocin, a natural product antibiotic, was identified as the most promising hit, and was selected for further characterization. In the ITC assay, it was

Table 2 Peptide inhibitors of autophagy-related PPIs.

Peptide	PPI target	Sequence	IC_{50}/K_D	Assay	Ref.
S12-1012	LC3−p62	DDDWTHL	0.108 μmol/L	SPR	93
C-19	ATG5−ATG16L1	KRHISEQLRRRDRLQRQAFS	0.4 μmol/L	SPR	101
peptide 7	ATG5−ATG16L1	Ac-WKRHISEQLR(S_5)RDR(S_5)QRQAFE	0.032 μmol/L	ITC	102
Tat-SP4	Beclin 1 coiled-coil domain	Ac-[Tat]-RLISEL(R_8)DREKQR(S_5)A	6.8 μmol/L	ITC	118
i7-01s-20	Beclin 1 coiled-coil domain	Ac-[Tat]-RVIQ(R_8)LVIIEK(S_5)RDV	0.10 ± 0.05 μmol/L	ITC	119
i7-01s-31	Beclin 1 coiled-coil domain	Ac-[Tat]-VLFN(R_8)LVDVIK(S_5)RKV	0.33 ± 0.28 μmol/L	ITC	119
SAH-EJ2	EGFR−p62 interaction	RHI(S_5)RKR(S_5)LRRLLQE	53.7 nmol/L	SPR	121

observed to bind LC3A (K_D = 1.3 μmol/L) and LC3B (K_D = 32 μmol/L) rather than LC3C and three GABARAP subfamily proteins. In the homogeneous time-resolved fluorescence (HTRF) displacement assay, it exhibited moderate activity in displacement of p62-LIR peptide from LC3A/LC3B (IC_{50} = 42.9 ± 7.1 and 172.0 ± 17.4 μmol/L for LC3A and LC3B, respectively). NMR titration experiments revealed that novobiocin occupies the HP2 pocket on LC3B. SAR study of novobiocin derivatives identified dihydronovobiocin, a reduction product of novobiocin (Table 1) with improved potency in HTRF displacement assay (IC_{50} = 16.0 ± 1.0 and 72.3 ± 7.4 μmol/L for LC3A and LC3B, respectively). Notably, the crystal structure of LC3A in complex with dihydronovobiocin demonstrated that this compound bound to the LC3A−p62 interface (Fig. 2D). In contrast to typical LIR motifs that occupy both HP1 and HP2 of the LIR-docking site, dihydronovobiocin occupied HP2 but insufficiently occupied HP1. Since the LIR motif widely exists in a vast majority of selective autophagy receptors and adaptors, it is necessary to further develop novobiocin derivatives targeting a broad set of interactions between various LIR motifs and LC3/GABARAP subfamily members, not merely the p62−LC3A/LC3B interaction. Toward this direction, synthetic efforts were made to generate a series of novobiocin derivatives, *e.g.*, compounds with enhanced hydrophobic interactions in HP1. However, no substantial increase in the inhibitory activity toward p62−LC3A/LC3B interaction was achieved by those derivatives. Anyway, this work has demonstrated the possibility of discovering small-molecule compounds targeting the LC3A and LC3B adapter interface.

Computational methods have shown their power in PPI-targeted drug discovery as well. Recently, Sun et al.[93] used computational method to design peptide binders of LC3−p62 interaction. The first-round design started from a 10-mer peptide (*i.e.*, GGDDDWTHLS) referring to the LIR motif on p62. To explore the minimal active length and the importance of individual residues, sets of N- or C-terminally truncated versions and alanine-mutated analogs were designed. Conventional MD and steered molecular dynamics (SMD) simulations were performed to predict their binding affinity towards LC3, where only S12-1002 and S12-1006 were predicted to have lower binding energy (*i.e.,* stronger binding) than the original peptide. Their binding modes and key contributors to target binding were further analyzed. Based on the first round of design, a seven-mer peptide S12-1012 (Table 2) was derived by eliminating the residues that were off the binding interface or detrimental to target binding. This peptide showed a decent binding to LC3 (K_D = 0.108 μmol/L by SPR), and thus was set as the reference for the second round of design. Unfortunately, both computational prediction and experimental measurement revealed that the binding affinity of most mutated peptides derived from the second round of design decreased more or less as compared to S12-1012. Nevertheless, the authors' MD stimulations provided some tips for further optimization, for example, the peptide should consist of seven residues to maintain the desired stretched binding conformation to occupy all of the three pockets (P1−P3) on LC3. Besides, Trp125 may deserve more attention for its critical role in occupying the P2 pocket.

Another feasible approach for disrupting the LC3−LIR interaction is to covalently modify the lysine residues on LC3B surface with small molecules. In particular, Lys49 and Lys51 on LC3B are reported to undergo endogenous post-translational modifications, such as acetylation, in living cells. As the location of these two residues is close to the LIR-docking site, their acetylation may cause steric hindrance that prevents LC3B from binding its LIR-containing partners and eventually affects autophagy[94]. Besides, as compared to conventional non-covalent inhibitors, covalent inhibitors are prone to target PPI interface, and also have advantages of sustained inhibition and longer residence time. Along this approach, Luo et al.[95] set out to discover covalent probes targeting the LC3−p62 interaction. They established an FP-based high-throughput screening assay using the LC3B and LIR peptides derived from p62, and then screened a library containing diverse probes with lysine-targeting covalent warheads. DC-LC3in (Table 1) was identified as the most potent inhibitor (IC_{50} = 3.06 μmol/L). Interaction between LC3B and DC-LC3in was validated by differential scanning fluorimetry (DSF) and 2D nuclear resonance experiment. The covalent modification on residue Lys49 rather than Lys51 was determined through mass spectrometry. Cooperatively, the crystal structure of LC3B in complex with a4, a DC-LC3in analog, verified the formation of a covalent bond between the ε-amino group on the side chain of Lys49 and the vinyl group of a4 (Fig. 2E). Structural optimization of DC-LC3in led to DC-LC3in-D5 (Table 1), which achieved a 13-fold increase in binding affinity (IC_{50} = 200 nmol/L) and exhibited a high cellular selectivity for LC3A/LC3B in activity-based protein profiling (ABPP) assay. Besides, DC-LC3in-D5 could block the LC3B−ATG7 interaction and impair LC3B-ATG7 thioester bond formation *in vitro*. In conclusion, DC-LC3in-D5 represents a new type of autophagy inhibitor, which can block autophagy by attenuating LC3B lipidation and thus impair autophagosome formation.

3.5. *Inhibitors of the Beclin 1−ATG14L interaction*

During the initial phase of autophagy, VPS34 engages in VPS34 Complex I (VPS15-VPS34-ATG14L-Beclin 1) to promote autophagosome formation. However, VPS34 may not be an appealing target for autophagy inhibition as direct VPS34 inhibition may cause undesirable defects within endolysosomal pathway due to impact on the vesicle trafficking role of VPS34 Complex II (VPS15-VPS34-UVRAG-Beclin 1)[96]. Instead, Beclin 1−ATG14L interaction is a more promising target. Deletion studies have shown that blocking this interaction could prevent the formation of VPS34 complex I[97]. Pavlinov et al.[98] carried out a study to explore the possibility of Beclin 1−ATG14L interaction as a druggable target for selective autophagy inhibition. They set up a high-throughput cellular NanoBRET assay for detecting the Beclin 1−ATG14L interaction in live cells, and employed it to screen a library of 2560 molecules with properties favorable for PPI inhibition. As a result, compound **19** (Table 1) was selected as a high-priority hit (IC_{50} = 33.9 μmol/L by NanoBRET) that was able to disrupt VPS34 Complex I formation and inhibit LC3-II accumulation. Further study confirmed its specific disruption of the Beclin 1−ATG14L interaction over the Beclin 1−UVRAG interaction. Importantly, compound **19** could selectively inhibit autophagy without affecting vesicle trafficking, which VPS34 inhibitors are not competent for. This work provides an excellent example showcasing that pharmacological regulation of critical PPIs within the autophagy pathway provides an opportunity to regulate specific functions in ways that cannot be conveniently achieved by inhibitors of certain protein targets alone[39]. In this

sense, one may decipher autophagy from a different but delicate perspective.

3.6. Inhibitors of the ATG5−ATG16L1 interaction

The ATG5−ATG12−ATG16L1 complex serves as a ubiquitin-like conjugation system that facilitates ATG8/LC3-PE conjugation and promotes the elongation of autophagosomal membrane. As revealed in the crystal structures of the ATG5−ATG16 complex in yeast and mammals[99,100], the N-terminal region of ATG16 in yeast (or ATG16L1 in mammals) adopts an α-helical structure upon binding to a groove on ATG5. Such α-helix-mediated PPI represents a viable potential target for intervention. Meanwhile, mutation studies proved that disruption of the ATG5−ATG16L1 interaction has a negative impact on the ATG8-PE formation, accumulation of autophagic bodies, and localization of ATG16L1 to the pre-autophagosomal structure[99]. Therefore, intervention of the ATG5−ATG16L1 interaction presents an alternative approach to the regulation of autophagy.

Our group made the first attempt to develop peptide inhibitors targeting the ATG5−ATG16L1 interaction[101]. Since crystal structure of the human ATG5−ATG16L1 complex was not available by then, we first derived a structural model of this complex through homology modeling by using the crystal structure of the yeast ATG5−ATG16 complex (PDB entry 2DYO and 2DYM) as the template. In fact, this structure model generally agreed with the crystal structure of the human ATG12−ATG5−ATG16L1 complex (PDB entry 4GDK) reported later. Guided by this structural model, several residues on the N-terminal helix of ATG16L1 (*i.e.*, Ile17, Leu21, Arg24, Asp25, and Gln28) were determined to be critical for binding to ATG5. Then, 11 different truncations of the N-terminal helix of ATG16L1, but all containing those key residues, were synthesized and tested in binding assay. Among them, a 20-mer peptide (B-1) exhibited micromolar-level binding affinity toward ATG5 (K_D = 21.9 and 1.3 μmol/L by FP and SPR, respectively). It was thereby chosen as the template for the second-round design, and as a result, a total of 19 new peptides were obtained by conducting single-point mutation on B-1. Binding assay results revealed that mutations at Ile17, Leu21, and Arg24 severely attenuated the binding affinity toward ATG5. In particular, the E33S mutant (C-19, Table 2) displayed about a three-fold improvement in binding affinity (K_D = 5.8 μmol/L by FP and 0.4 μmol/L by SPR, respectively) as compared to B-1. In conclusion, this preliminary work proved that peptides derived from ATG16L1 could bind to ATG5 and also confirmed the key residues on the ATG5−ATG16L1 binding interface with biophysical evidence.

Another notable work of developing peptide inhibitors of the ATG5−ATG16L1 interaction was published by T. Watanabe's group in 2022[102]. They also selected the critical AFIM motif (W-x3-I-x3-L-x2-R-x3-Q region) on ATG16L1 as the starting point to design a series of peptides. Then, a hydrocarbon staple was added to various positions on those peptides where amino acid residues were not in direct contact with ATG5. Some stapled peptides obtained by them exhibited very high binding affinity to ATG5 at the nano-molar range, *e.g.*, K_D as low as 3 nmol/L by ITC. In particular, they successfully resolved a crystal structure of the ATG5 structure in complex with peptide 7 (Table 2, PDB entry 7W36). This structure reveals that peptide 7 indeed binds to ATG5 in a helical conformation, which nicely mimics the way of ATG16L1. Thus, installation of the staple has stabilized the desired helical conformation and increased the binding affinity to ATG5. Moreover, those stapled peptides achieved generally improved proteolytic stability against trypsin and chymotrypsin. However, those stapled peptides were observed to cause autophagy inhibition in the MEF cells only at a dose of around 100 μmol/L. Most likely, those stapled peptides still have problem in cell permeability, which hampers their pharmaceutical potential to be fully released.

As in the case of other PPIs, small-molecule compound may represent a more "drug-like" solution to the regulation of the ATG5−ATG16L1 interaction. To the best of our knowledge, the first attempt along this path was published by Li et al. back in 2012[103]. By employing a bimolecular fluorescence complementation-fluorescence resonance energy transfer (BiFC-FRET) assay and a co-immunoprecipitation assay in transfected A549 cells, they discovered that evodiamine (Table 1), a major component of *Evodia rutaecarpa* Benth, could inhibit the formation of the ATG12−ATG5−ATG16L1 heterotrimer at a dose around 40 μmol/L. They also demonstrated that evodiamine could prevent the accumulation of LC3-II and p62 and cause a decrease in dot-like aggregation of EGFP-LC3 at the same dose. In a work published by Reynisson et al. in 2015[104], virtual screening was carried out in an attempt to discover inhibitors of the ATG5−ATG16L1 interaction. Two hit compounds, **14** and **62**, as well as one derivative compound **38**, were observed to decrease the LC3-II level and increase the p62 level in western blot assay, and reduced autophagosome formation in the MCF-7 cells. However, those compounds were not very potent since their effective concentrations in the cellular assays were well over 20 μmol/L. Besides, the authors did not provide biophysical or biochemical evidence that those compounds actually disrupted the ATG5−ATG16L1 interaction. In a follow-up work published in 2020[105], the same research group presented a novel assay, called NanoLuc Binary Technology (NanoBIT), to monitor the ATG5−ATG16L1 interaction in living cells. They fused the cDNAs of ATG5 and ATG16L1 respectively with the coding sequences of SmBIT and LgBIT, both of which were components of the NanoLuc luciferase. The resulting pair could form a functional luciferase and generated a luminescent signal in the event of ATG5−ATG16L1 interaction. In such a NanoBIT assay, compound KCR14 (Table 1) was observed to cause a dose-dependent decrease of luminescence in HEK293 cells after autophagy was stimulated by EBSS. In 2021, another work of discovering of small-molecule inhibitor of the ATG5−ATG16L1 interaction was reported by Aldrich et al. In their work[106], a cost-effective and robust FP-based high-throughput assay using full-length human ATG5 and a red-shifted rhodamine-labeled ATG16L1 peptide was developed to screen some 4800 compounds. Compound H3 (Table 1) was identified as the most potent hit that disrupted the ATG5−ATG16L1 interaction (IC_{50} = 18.4 μmol/L by FP). Nevertheless, this compound was reported to fail in lowering the LC3-II level or increasing the p62 level in living cells.

Recently, our group also discovered a new class of small-molecule inhibitor of the ATG5−ATG16L1 interaction[107]. First, we employed a homogeneous time-resolved fluorescence (HTRF) binding assay to screen an in-house collection of ~1500 compounds, leading to the identification of two hits with modest binding affinity toward ATG5. These two hits were then used as query to perform a structural similarity search throughout several chemical databases combining ~660,000 compounds. A total of 30 compounds were finally selected, and their samples were purchased and tested in our binding assay. Among them, TargetMol T1742 exhibited micro-molar inhibition against both ATG5−ATG16L1 and ATG5−TECAIR interactions (IC_{50} = 1.1 and 1.7 μmol/L, respectively, Table 1). The outcomes of Western

blot assay revealed that T1742 treatment caused an obvious decrease in the LC3-II protein level in several cell lines, including COS-7, U-937 and THP-1 cells. In the flow cytometry assay, T1742 induced autophagy inhibition in a dose-dependent manner in COS-7 cells. All these results suggest that T1742 can down-regulate autophagy *via* inhibition of autophagosome formation. This compound was thus chosen as the lead compound for structural modification. The possible binding mode of T1742 to ATG5 was derived through sophisticated MD simulations, and a total of 56 derivatives of T1742 were synthesized and tested. Although none of the newly synthesized compounds was significantly more potent than T1742, a preliminary structure−activity relationship for the (*E*)-3-(2-furanylmethylene)-2-pyrrolidinone scaffold was deduced. In this work, we have discovered the first class of small-molecule ATG5 inhibitor achieving low micromolar binding affinity and also exhibiting autophagy inhibition in living cells. It provides more convincing proofs that small-molecule inhibitors of ATG5 can block autophagy effectively.

3.7. *Inhibitors of Beclin 1 homodimerization*

As it comes to Beclin 1, Prof. Beth Levine' contributions to the field of autophagy need to be mentioned first. Prof. Levine is best known for discovering the first mammalian autophagy gene *BECN1*[108]. Moreover, she noticed that the *BECN1* gene was often deleted monoallelically in tumor cells, indicating a link between autophagy and tumor suppression[109]. This discovery has been standing as a true landmark in autophagy research. Since then, a number of works by Prof. Levine and coworkers have revealed various functions and roles of autophagy in cell survival, longevity, immunity, as well as cancer[110−113]. Those findings made Beclin 1 perhaps the most extensively studied protein in this field by then. In 2013, Prof. Levine et al.[114] described Tat-BECN1, a peptide derived from an evolutionarily conserved domain of Beclin 1, which was shown to be a potent autophagy inducer. Tat-BECN1 interacts with GAPR-1 (Mammalian Golgi-associated plant pathogenesis-related protein 1) that negatively regulates autophagy. It may function through detethering Beclin 1 from Golgi and thus promote early autophagosome formation. In a subsequent study[115], Prof. Levine et al. further optimized Tat-BECN1 and obtained several cell-penetrating peptides. Encouragingly, those peptides were able to clear aggregates in a cellular model of the Huntington's disease and also induce autophagy *in vivo*. However, the action mechanism of those peptides still needs to be fully revealed.

Beclin 1 self-associates at its coiled-coil domain in an anti-parallel manner to form a homodimer[116]. However, the homodimer form of Beclin 1 is rendered metastable owing to a series of imperfect pairings that destabilize the dimerization interface. This allows ATG14L/UVRAG to readily disrupt Beclin 1 homodimer and form the highly stable Beclin 1-ATG14L/UVRAG heterodimer responsible for VPS34-related autophagy and endolysosomal trafficking[117]. These findings suggest that disruption of Beclin 1 homodimerization may facilitate the transition of endogenous Beclin 1 from functionally inactive self-association to ATG14L/UVRAG-containing heterodimeric complex, and can be utilized as a strategy to regulate VPS34-dependent processes including autophagy.

Working with this concept, Zhao et al.[118] aimed at designing stapled peptides as inhibitor of Beclin 1 homodimerization. Based on the crystal structure of Beclin 1, a 15-mer peptide (Native-P1) corresponding to the segment of residues 191−205 on the Beclin 1 coiled-coil domain was selected as the template for further modification. It was expected to target the Beclin 1 coiled-coil domain surface and thus disrupt its homodimerization, but not compete with the binding of ATG14L/UVRAG. A set of 12 peptides were then computationally designed by introducing multiple-point mutations at the non-critical residues on the template peptide. A hydrocarbon staple was incorporated into each peptide to connect residues 197 and 204 to from an (*i*, *i*+7) linkage, resulting in a panel of stapled peptides (*i.e.*, SP1−SP12) with enforced α-helical conformation (Fig. 4A). In addition, the Tat peptide (YGRKKRRQRRR) was added to the N terminus of each peptide to improve its cell permeability. Among them, peptide SP4 (Table 2) was predicted by molecular modeling to achieve a more favorable binding to Beclin 1 than the template peptide. In fact, SP4 exhibited micromolar binding affinity to Beclin 1 (K_D = 6.8 μmol/L by ITC) and was able to reduce Beclin 1 homodimerization and promoting Beclin 1-ATG14L/UVRAG interaction. Moreover, it could increase autophagic flux and significantly enhance the endolysosomal degradation of epidermal growth factor receptor (EGFR) *in vivo*.

In a follow-up work by the same group[119], Zhao et al. explored various stapled positions along the sequence of the template peptide Native-P1 by predicting its binding mode to Beclin-1 through extensive molecular dynamics simulations. A total of six scaffolds were examined. Among them, the i7-01s scaffold with a staple between Glu195 and Asn202 (Fig. 4B) was considered as the best option because in this case, the hydrocarbon staple located close to the surface of Beclin 1 coiled-coil domain and thus may strengthen peptide binding by supplementary hydrophobic interaction. Then, systematic single-point and multiple-point mutations were performed computationally on the Native-P1

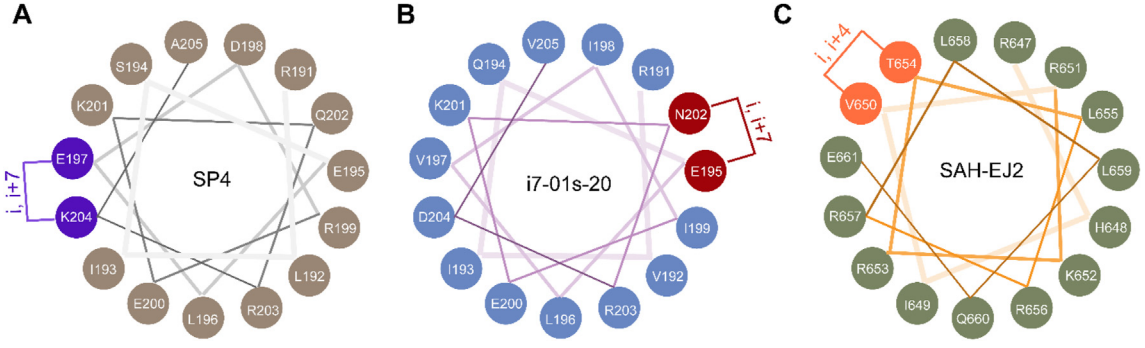


Figure 4 Top-view of the stapled peptides (A) SP4, (B) i7-01s-20, and (C) SAH-EJ2. A hydrocarbon staple was added in each case to form an (i, i + 7) or (i, i + 4) linkage to enforce the helical structure of the peptide.

sequence with the i7-01s scaffold, leading to a total of 75 designed stapled peptides. Seventeen of them were selected for subsequent chemical synthesis by considering their predicted binding affinity and physicochemical properties. ITC assay results revealed that 12 of those newly synthesized peptides exhibited comparable or stronger binding affinity to Beclin 1 as compared to SP4. In particular, i7-01s-20 (K_D = 0.10 ± 0.05 μmol/L) (Fig. 4B) and i7-01s-31 (K_D = 0.33 ± 0.28 μmol/L) displayed ~10-30-fold increase in affinity (Table 2). Similar to SP4, i7-01s-31 reduced Beclin 1 self-association and enhanced Beclin 1-ATG14L/UVRAG interaction. Besides, both i7-01s-20 and i7-01s-31 had comparable autophagy-inducing efficacy as SP4, while promoting endolysosomal trafficking of HER2 and EGFR with enhanced potency *in vivo*.

3.8. *Inhibitors of the EGFR−p62 interaction*

As a cargo receptor in selective autophagy, p62 is able to bind to ubiquitinated proteins *via* its ubiquitin-associated (UBA) domain and deliver them to autophagosomes for degradation[120]. Hua et al. identified EGFR as a binding partner and a negative regulator of p62[121]. To be more specific, EGFR-induced phosphorylation of p62 triggered UBA domain dimerization, which hindered cargo recognition of p62 and led to autophagic flux blocking. Thus, disturbing the EGFR−p62 interaction was expected to rescue p62 cargo function. Herein, an α-helical peptide derived from the EGFR juxtamembrane region (residues 645−663) that is critical for p62 binding was chosen as the starting point to develop peptide-based PPI inhibitors. This template peptide was then fused with a cell-penetrating peptide Pep2 (HLYVSPW) to improve its pharmaceutical properties. It was also structurally modified to add an (i, i + 4) staple between Val650 and Thr654 to obtain a stapled peptide SAH-EJ2 (Fig. 4C). Compared with the unstapled form, SAH-EJ2 had a much stronger binding to p62 (Table 2, K_D = 53.7 nmol/L by SPR), better cell permeability, and a prolonged clearance rate. Further experiments conducted on A549 cells showed that treatment with SAH-EJ2 interrupted the association of EGFR with p62, restored the ubiquitin-binding capacity of p62, and induced activation of autophagy flux.

3.9. *Inhibitors of the LRS−RagD interaction*

Leucyl-tRNA synthetase (LRS) is the main sensor for intracellular leucine levels. It interacts with leucine and directly binds to RagD, one of four mammalian GTPases, and thus regulates the mechanistic target of rapamycin (mTOR) signaling pathway[122]. Blocking the LRS−RagD interaction is an effective strategy to overcome the resistance to mTOR inhibitors, which currently stands as an obstacle in chemotherapy. Kim's group[123] compiled a library of roughly 5000 compounds based on structural similarity to leucenol and leucine analogs. According to their potency in inhibiting leucine-dependent S6K phosphorylation, 12 compounds were selected as the lead compounds for further synthesis of 174 derivatives, among which 21 active compounds were finally identified. By considering the efficacy on mTORC1 activity, cell growth and death, water solubility, and predicted pharmacological behavior, BC-LI-0186 was the most promising one (Table 1). This compound bound to the VC domain of LRS (K_D = 42.1 nmol/L by SPR) and prevented the mTORC1-activating interaction of LRS and RagD, without affecting the catalytic and editing activities of LRS and the kinase activity of mTOR. In the HCT116 cells expressing the rapamycin-resistant mTOR S2035I mutation, BC-LI-0186 inhibited leucine-dependent mTORC1 activity effectively (IC_{50} = 0.109 μmol/L) and the growth of cancer cells. BC-LI-0186 also exhibited the potency of autophagy induction, as indicated by the increase in the LC3-II level. In order to overcome the low solubility and microsomal stability of BC-LI-0186, structural optimization was performed in a follow-up study[124]. As shown in Table 1, BC-LI-0186 consists of two parts: a N-substituted sulfonamide group and a pyrazolone core. For the first part, different N-substituent aromatic and hetero-aromatic groups were introduced to improve physicochemical properties, where 3,4-ethylenedioxyphenyl substitution (**5j**) turned out to be the best choice. Then, the isopropyl group on the pyrazolone core was replaced with chlorine (**8a**) to enhance microsomal stability. Although **8a** had a marginally worse IC_{50} value of 0.216 μmol/L than BC-LI-0186, it had good pharmacokinetic profiles and thus still could be a highly potent mTOR inhibitor.

In another work, Park et al.[125] conducted an ELISA-based screening for potential LRS−RagD interaction inhibitors. Notably, the compounds screened by them were derived from the so-called diversity-oriented synthesis (DOS). Park et al. identified pyrimidine as a new privileged substructure to navigate through bioactive chemical space. A total of 16 distinct pyrimidodiazepine- or pyrimidine-containing polyheterocycles were obtained by following the DOS strategy. A preferred compound **21f** was identified with K_D = 4.8 ± 0.46 μmol/L in SPR assay (Table 1). This compound was shown to regulate the mTORC1 activity through specific inhibition of the LRS−RagD interaction. Moreover, Western blotting and living-cell imaging showed that **21f** could stimulate cellular autophagy.

3.10. *Summary: Analysis of methodological rigor*

A total of 21 drug discovery studies have been reviewed in this article. Obviously, studies that provide both autophagy characterization and pharmacological and functional evidence would be more appealing. Here, we adopt the Autophagy Modulator Scoring System (AMSS) proposed by Dong et al.[126] to assess the methodological rigor of those 21 studies. This scoring system consists of nine indicators, addressing autophagy characterization (AMSS 1−4) and pharmacological and functional features (AMSS 5−9). Analysis results of those 21 studies are given in Fig. 5. Those studies have received AMSS scores ranging from 1 to 7, where half of them have received a fair score (≥4). It is encouraging to observe that 20 out of all 21 studies provide certain evidence of target identification (AMSS_6). However, only five studies provide some kind of *in vitro* data to confirm autophagy modulation, and only one study provides *in vivo* data. Our analysis reveals that current drug discovery studies that aim at autophagy regulation are still less mature, leaving a significant gap in possible therapeutic application.

4. General strategies for obtaining protein−protein interaction regulators

In the above section, we have presented a number of successful drug discovery efforts by targeting autophagy-related PPIs. Many of those efforts are featured with a combination of screening technique, molecular design and synthesis, and biological characterization. Besides, an in-depth understanding of the structural and functional mechanism of the target PPI is certainly helpful. In this section, we will discuss several general strategies for obtaining

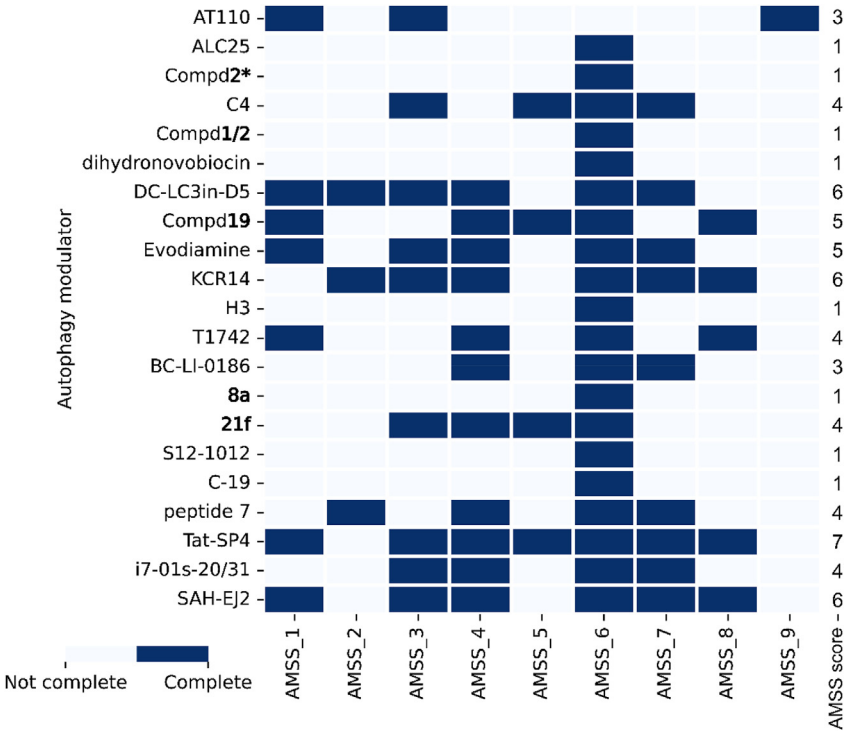


Figure 5 Assessment of the 21 drug discovery studies by targeting the PPIs in autophagy, where the AMSS score of each study is given in the last column. AMSS_1: Autophagosome quantification *via* microscopy analysis; AMSS_2: Autophagosome formation-related biochemical changes; AMSS_3: Autophagy substrate degradation; AMSS_4: Autophagic flux; AMSS_5: Lysosome function-related assays; AMSS_6: Target identification for chemical autophagy modulators; AMSS_7: Autophagy-dependent pharmacological effects; AMSS_8: Autophagy modulation confirmed *in vitro*; AMSS_9: Autophagy modulation confirmed *in vivo*.

regulators of PPIs and lessons learned from those efforts. Apparently, those strategies are not limited to autophagy-related PPIs but applicable to a wider range of PPI systems (Fig. 6).

4.1. *Screening techniques*

High-throughput screening (HTS) has been extensively applied to various targets including PPIs[127,128]. HTS is employed routinely for discovering small-molecule regulators of PPI, especially when structure-based drug design is hindered by lack of a high-quality structure of the PPI of interest. However, HTS is confronted with certain challenges, *e.g.*, low hit rate, weak potency, and false positives[129]. Rognan et al. conducted a systematic comparison of PPI interfaces and the binding pockets on protein-ligand complexes and came to the conclusion that even the most druggable PPI interfaces are often not the suitable target for conventional libraries containing "drug-like" compounds[130]. This implies that the chemical space of PPI regulators may not be sufficiently covered by those conventional chemical libraries, which may account for the low hit rates in HTS. Rather than simply scaling up the chemical libraries subjected to HTS, a few research groups have attempted to construct customized libraries enriched by PPI regulator-like compounds and have gained some benefits[98,131]. Nevertheless, accessibility to such libraries is another problem in practice. Next, as seen in many works described earlier in this article, potency of the active hits identified in HTS is often relatively low, typically at the micromolar or sub-micromolar range. Therefore, structural optimizations are generally needed to obtain more promising drug candidates. Due to the intrinsic limitation in experimental setting, HTS also tends to produce a significant number of false positives. Therefore, the active hits identified in HTS should be verified by additional assays, ideally orthogonal in principle, to reduce false positives.

Compared to experimental HTS, virtual screening (Fig. 6A) provides an alternative, cost-effective approach to hit identification[132,133]. However, due to the lack of druggable binding site, application of virtual screening to a target PPI system is more challenging than a conventional drug target. This difficulty may be overcome if knowledge of the hot spots on the targeted PPI interface is available[134–136]. A notable new trend in recent years is that virtual screening of ultra-large libraries with open-source platforms, such as VirtualFlow, has become feasible. This approach has already demonstrated its potential in accessing a much larger chemical space, discovering novel chemotypes, and identifying PPI regulators with high affinity[137,138].

4.2. *Covalent modification*

As the binding interface of PPI is relatively flat and thus unfriendly for a small-molecule binder, covalent modification of certain residues on the PPI interface is an effective strategy for targeting the PPI of interest[139,140]. Residues possible for covalent modification include serine, lysine, cysteine, histidine, as well as other residues that contain a nucleophilic functional group (Fig. 6B) (*e.g.*, $-OH$, $-SH$ or $-NH_2$) on its side chain. In fact, a significant number of post-translational modifications (PTMs) occur in autophagy regulation[141–145]. Those PTMs on the PPI interface of interest imply that chemical modifications on those

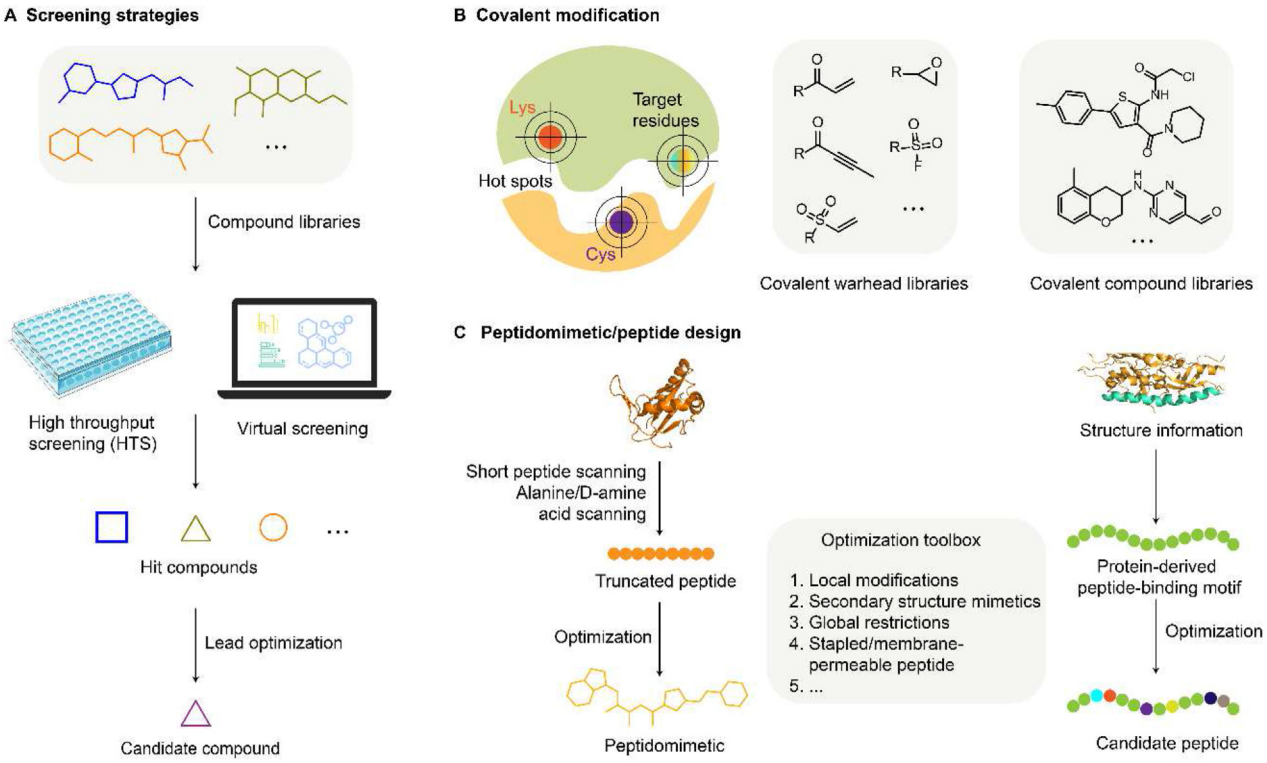


Figure 6 Several general strategies for obtaining PPI regulators. (A) Identification of active hits through screening, including high-throughput screening and virtual screening. (B) Covalent modification of certain residues on the binding interface of the target PPI. (C) Design of peptide/peptidomimetic binders based on the structural information of the target PPI.

relevant residues are potentially favored. For instance, Luo et al.[95] noticed the possible covalent modification of a specific lysine on the LIR-LC3 binding interface, leading to the development of LC3 covalent inhibitors (Section 3.4). Nevertheless, how to achieve the desired selectivity in covalent modification is a critical issue because the same type of residue does exist in other surface regions of the targeted PPI as well as other protein molecules. Low selectivity may cause serious side effects.

4.3. Design of peptide/peptidomimetic binders

Binding of two protein molecules is typically restricted to a limited region on their surface. If one of the two interacting protein molecules is regarded as the "target" and the other as the "ligand", in principle the "ligand" can be simplified into a relatively short peptide that keeps the essential elements needed for its binding to the "target". Thus, truncating a proper segment on the "ligand" into a peptide binder provides a shortcut for obtaining effective PPI regulators[146].

In order to design such peptide rationally, knowledge of the key features in the targeted PPI is crucial. An unambiguous picture of this is normally provided by an experimentally determined structure of the relevant protein−protein complex. If an experimental structure is not available, molecular modeling can be employed to derive a structural model. With the aid of the latest deep-learning techniques, the accuracy of such computational methods has been significantly elevated[147,148]. The peptides directly truncated from the "ligand" often need further optimization because the wild-type sequence may not be able to achieve the desired level of binding affinity to the target protein. Peptide binders with higher affinity can be obtained after a few rounds of single-point or multiple-point mutation and even incorporation of unnatural amino acids[149−151]. During such a process, computation methods again can be employed to depict the possible interaction mode between the designed peptides and the target protein and calculate the binding energy to guide selection or optimization[152,153]. A remarkable issue here is that the desired peptide binders often need to mimic the structure of the template protein, especially in the form of α-helices[154,155]. Macrocyclization enables pre-organization of the structure of a peptide into the desired form, and thus may enhance binding affinity, cell permeability, as well as metabolic stability[156]. In particular, installation of a "staple" at proper sites on a peptide sequence to form the so-called "stapled peptide" can help the reformation of the helical structure. Grafting the designed peptide onto well-characterized cell-permeable peptide vehicles, such as Tat (YGRKKRRQRRR), is another popular way to improve cell permeability[157,158].

Despite all of their advantages, peptide molecules are generally confronted with issues such as poor pharmacokinetic properties and metabolic stability. In order to overcome those intrinsic limitations, peptidomimetics are appealing alternatives for targeting PPIs[159,160]. Peptidomimetics can be described as compounds with essential elements that mimic peptide structure and retain the ability to interact with the target protein and produce the same biological effect. Design of peptidomimetics is adjusted in action according to the knowledge of structure and function of the targeted PPI and the hot spots on its binding interface. The design process is normally divided into several consecutive steps (Fig. 6C). The first step is to reveal the minimal peptide sequence required for the PPI of interest through peptide scanning of an array of short overlapping peptides. It resembles the alanine or D-amino acid scanning in peptide design for identifying the key

residues responsible for bioactivity. The next step requires chemical synthetic works on optimization of the initial peptidomimetics to gain improved biological activities and pharmacokinetic properties. A range of methods have been well applied to the modification of peptidomimetics structures, including replacement of peptide bonds with isosteres, modification of amino acid side chains, incorporation of secondary structure mimetics, and global restrictions such as macrocyclization[161].

5. Perspectives

Years of basic research have expanded our understanding of the biological processes of autophagy. Regulation of autophagy has its attractive potential in treating cancer and other diseases, which apparently relies on the discovery of more potent and selective autophagy regulators[162]. As described earlier in this article, various stages of the autophagy process are regulated by certain PPIs. Moreover, selective regulation of PPIs tends to have a lower risk in causing undesired off-target effects in the context of a complicated regulatory network. Thus, small-molecule regulators, including peptides and peptidomimetics, targeting the critical PPIs involved in autophagy provide a new opportunity for innovative drug discovery. Even though PPIs are considered as a relatively difficult class of drug target, significant advance has been made in this field, and some of the successful attempts are reviewed in this article.

Nevertheless, one should also be aware of the current limitations in developing autophagy regulators by targeting PPIs. Technically, in a multi-component complex system such as the VPS34 complex I discussed in Section 3.5, those protein components may represent more than one PPI as the target for small-molecule regulators. If so, it is not straightforward to determine the most critical pair of PPI in the relevant biological process. Besides, setting up the appropriate binding assay for such a system also needs more careful consideration because one pair of PPI may be affected by other PPIs in the same system. Another issue is that most of the successful attempts made in this field are on discovering PPI inhibitors. In principle, stabilizers of PPIs can also achieve effective regulation of autophagy, but this type of work, is still under-represented in the scientific literature. Special drug discovery strategies, such as molecular glue[163–165], may lead to a notable change along this direction.

Importantly, in most cases the active hits discovered through high-throughput screening, virtual screening or covalent screening only exhibit modest potency to the PPI of interest. The chemical structures of those hits need to be optimized in order to improve their potency, but this task is particularly challenging due to the structural nature of PPI. Besides, a promising drug candidate also requires proper pharmacodynamics and pharmacokinetics properties. However, most of the works reviewed in this article, if not all, do not provide experimental data in this aspect. As a matter of fact, according to the information on *ClinicalTrials.gov*, a database complied by the US National Library of Medicine of clinical studies around the world, so far none of the small-molecule or peptide autophagy inhibitors reviewed in this article has entered clinical trial. Apparently, a tremendous amount of efforts are still needed to explore the real pharmaceutical potential of those compounds.

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Author contributions

Honggang Xiang wrote most parts of the manuscript. Mi Zhou and Yan Li helped to prepare some material and wrote certain parts of the manuscript. Renxiao Wang and Lu Zhou supervised the whole process and also revised the manuscript.

Conflicts of interest

The authors declare no competing financial interest.

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