Development of Fluorescent Substrates and Assays for the Key Autophagy-Related Cysteine Protease Enzyme, ATG4B

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ABSTRACT

The cysteine protease ATG4B plays a role in key steps of the autophagy process and is of interest as a potential therapeutic target. At an early step, ATG4B cleaves proLC3 isoforms to form LC3-I for subsequent lipidation to form LC3-II and autophagosome membrane insertion. ATG4B also cleaves phosphatidylethanolamine (PE) from LC3-II to regenerate LC3-I, enabling its recycling for further membrane biogenesis. Here, we report several novel assays for monitoring the enzymatic activity of ATG4B. An assay based on mass spectrometric analysis and quantification of cleavage of the substrate protein LC3-B was developed and, while useful for mechanistic studies, was not suitable for high throughput screening (HTS). A doubly fluorescent fluorescence resonance energy transfer (FRET) ligand YFP-LC3B-EmGFP (FRET-LC3) was constructed and shown to be an excellent substrate for ATG4B with rates of cleavage similar to that for LC3B itself. A HTS assay to identify candidate inhibitors of ATG4B utilizing FRET-LC3 as a substrate was developed and validated with a satisfactory Z' factor and high signal-to-noise ratio suitable for screening small molecule libraries. Pilot screens of the 1,280-member library of pharmacologically active compounds (LOPACTM) and a 3,481-member library of known drugs (KD2) gave hit rates of 0.6% and 0.5% respectively, and subsequent titrations confirmed ATG4B inhibitory activity for three compounds, both in the FRET and mass spectrometry assays. The FRET- and mass spectrometry-based assays we have developed will allow for both HTS for inhibitors of ATG4B and mechanistic approaches to study inhibition of a major component of the autophagy pathway.

INTRODUCTION

Autophagy is a ubiquitous and evolutionarily conserved process whereby cells capture intracellular proteins, lipids, and organelles and deliver them for degradation within lysosomes, ostensibly for the purpose of removing and re-

cycling unwanted or defective cellular contents. Thus, autophagy fulfills an important homeostatic role in cells. In addition to the normal basal level of activity, autophagy is upregulated under a variety of conditions such as nutrient starvation or hypoxia to provide cellular nutrients and energy for survival.¹ Recent reports have linked autophagy with a variety of diseases, including liver disease, neurodegenerative disorders, infectious diseases,² and cancer, where there has been wide-ranging debate as to whether, in treating disease, it might be beneficial to upregulate or to inhibit autophagy.^{3,4} Autophagy has been shown to be “naturally” upregulated in many cancerous cells, and solid tumors may utilize autophagy as a mechanism to survive during periods of rapid growth where microvasculature has not yet had time to be properly established.⁵ The process may also help to protect cancer cells when they are under stress from cytotoxic anticancer drugs or irradiation treatments by removing defective and damaged components and thus imparting a degree of resistance to treatment. In contrast to this survival role, some have suggested that autophagy can serve as a mechanism to support cell death in cancer cells, particularly when primary apoptosis has been disabled.⁶

Some lysosomotropic drugs such as chloroquine and hydroxychloroquine suppress the autophagic process at least in part by basifying the lysosomes and thus impairing the activity of lysosomal proteases that have optimal activity when the pH is acidic. However, these drugs are general inhibitors of the lysosome and would be expected to lead to a build-up of unprocessed proteins and organelles.^{4,7} To date, there are no known selective small molecule pharmacological tools with which to test and evaluate the role of autophagy definitively in cancer or health, but evidence is growing that temporary inhibition of autophagy may serve as a useful primary or adjunctive anticancer therapy.

The process of autophagy begins when double-walled isolation membranes within the cytosol are induced to form vesicles, termed “autophagosomes,” effectively engulfing the offending cellular contents. These autophagosomes then fuse with lysosomes, thus delivering the contents for degradation (Fig. 1).⁸ The genes involved in autophagy have been identified in yeast but are largely conserved in all eukaryotes. In mammalian cells, the process is controlled by proteolytic cleavage of microtubule-associated light chain 3 (LC3), which has three isoforms (A, B, and C). Processing of the proLC3 isoforms exposes a C-terminal glycine residue and provides LC3-I, which is then converted to a lipidated form, LC3-PE (or LC3-II), by conjugation with phosphatidylethanolamine. LC3-PE associates with the preformed membranes, inducing autophagosome formation. During and after autophagosome formation, the exterior membrane-embedded LC3-PE is delipidated, and LC3

Fig. 1. Macroautophagy (otherwise known as autophagy). Reproduced with modifications, with permission from Rubinsztajn.⁸

is removed and recycled. This delipidation may be important for efficient autophagosome biogenesis.^{9–13} In human cells, both the initial proteolytic activation of LC3 and the subsequent delipidation are controlled by a cysteine protease enzyme, autophagin 1, more commonly known as ATG4B.¹⁴ Humans express three other homologous enzymes—ATG4A, ATG4C, and ATG4D—and these (and ATG4B) process at least three other autophagic factors known as GATE-16, GABARAP, and GABARAP-L1, but only ATG4B efficiently processes LC3.¹⁵ Considering its critical role in the macro-autophagy cascade, a selective, cell-penetrating small molecule inhibitor of ATG4B could serve as an excellent pharmacological tool to define better the role of autophagy in health and disease. Such an inhibitor might also serve as a useful adjunctive therapy for the treatment of cancer and other diseases.

Our goal of discovery, design, and evaluation of inhibitors of ATG4B first demanded development of facile assays for monitoring relevant enzyme activity. Ideally, we sought to develop rapid high throughput screening (HTS) assays monitoring the direct cleavage of LC3, but a simpler monitoring of cleavage of an active site-dependent fluorogenic peptide substrate was also considered. On initiation of our studies, there were no published assays for ATG4B suitable for screening, but subsequently a high throughput assay based on a construct of LC3 and phospholipase A2 (PLA2) was described, along with its successful HTS application in conjunction with a PLA2 counterscreen.¹⁶ More recently, a fluorescence resonance energy transfer (FRET)-based assay using a fluorescent LC3 construct has been described,¹⁷ but thus far no application to HTS has been published. Herein, we discuss the development of two useful assays for studying ATG4B activity and inhibition and the optimization of one assay for HTS. This screen has identified a number of interesting active inhibitors that may be suitable for lead optimization.

MATERIALS AND METHODS

General Methods

The high-resolution mass spectra were determined with the Agilent Technologies 1200 Series liquid chromatography (LC) system using a Zorbax[®] 300SB-C8 Analytical Guard Column (4.6 mm × 12.5 mm, 5 μm) connected to an Agilent Technologies 6210 Time-of-

Flight LC-MS in positive ion mode with an ESI ion source. Proteins were analyzed by gradient elution from a mixture of acetonitrile and water with 0.1% formic acid at a flow rate of 1 mL/min. The gradient started at 10% acetonitrile for 0.5 min, increased to 60% at 2 min, and maintaining 60% until 3 min before going back to 10% at 3.1 min. The entire analysis lasted up to 5 min with the first minute diverted to waste. Proteins were eluted between 2 and 2.5 min.

Proteins used were His-tagged proteins proLC3B and ATG4B, which were kind gifts from Aled Edwards (Structural Genomics Consortium [SGC], Toronto, Canada). (Mca)-TFGM-(Dpa), (Mca)-QETFGMKLS-(Dpa), or (Mca)-QETFGTALA-(Dpa) were purchased after custom synthesis from Biomer Technology (Hayward, CA).

Preparation of the ATG4B FRET Ligand, His₆-Tagged-YFP-LC3-EmGFP (FRET-LC3)

Protein expression and purification. DNA coding for human LC3-B (F1, residues 1363–1737) was amplified by polymerase chain reaction (PCR) from pCMV-GFP-LC3 expression vector (Cell Biolabs, Inc.; Product #CBA-401) and engineered to contain an N-terminal BamHI restriction site and a C-terminal SalI restriction site. DNA coding for YFP (F2, residues 1772–2479) was amplified by PCR from pCAG-YFP plasmid (Addgene; Plasmid 11180) and engineered to contain an N-terminal SalI site and C-terminal EcoRI site. The resulting fragments F1 and F2 were ligated into pRSET-EmGFP expression vector (Invitrogen; cat. # V353-20) using BamHI, SalI, and EcoRI restriction sites. The final sequence of the expression vector is YFP-LC3B-EmGFP.

The resulting plasmid was transformed into *Escherichia coli* XL-10 Gold Ultracompetent cells (Stratagene; cat. # 200314). A colony of freshly transformed cells was cultured in lysogeny broth (LB) medium that contained 100 μg/mL ampicillin. Cells were grown at 37°C overnight. The cells were then centrifuged and subjected to a mini-prep protocol (Qiagen QuickLyse[™] Miniprep Kit; cat. # 27405). The resulting plasmid was sent for sequencing to verify the expression vector. Once verified, the plasmid was then transformed into *E. coli* BL-21-CodonPlus[®] (DE-3)-RP cells (Stratagene; cat. # 230245). A colony of freshly transformed cells was cultured in LB medium that contained 100 μg/mL ampicillin. Cells were grown at 37°C until A₆₀₀ of 0.6 was reached, and expressed overnight at 25°C using 0.5 mM IPTG. Harvested cells were resuspended in binding buffer (20 mM Na₂PO₄, 5 mM imidazole, 20 mM NaCl, pH 7.4) that also contained protease inhibitor mix (Roche cOmplete[™] Protease Inhibitor Cocktail EDTA free; cat. # 11697498001). Cell lysis was achieved by sonication, and the lysate was centrifuged for 1 h at 12,000 *g*. The supernatant was then subjected to protein purification using a 5 mL Fast Flow[™] HisTrap (GE Healthcare; cat. # 17-5248-02) that was pre-equilibrated with the same binding buffer. Bound protein was then

washed with 100 mL wash buffer (20 mM Na₂PO₄, 60 mM imidazole, 500 mM NaCl, pH 7.4) and finally eluted in 20 mL using an elution buffer (20 mM Na₂PO₄, 250 mM imidazole, 300 mM NaCl, pH 7.4). The eluted protein was exchanged to a Tris buffer (50 mM Tris, 100 mM NaCl, pH 8) and used without further purification.

DNA ligation. A total of 3.5 μ L of digested F1 and F2 (gel denaturation extracts) were incubated with 1 μ L of digested pRSET-EmGFP expression vector using 1 μ L 100U T4 DNA Ligase (Invitrogen) in a final volume of 10 μ L. After incubating for 30 min at 25°C, an additional 1 μ L of T4 DNA ligase was added to the mixture, and incubation continued for an additional 30 min. The resulting plasmid obtained from transformed XL10-Gold miniprep-ultracompetent cells (QIAPrep[®] Spin Miniprep Kit, QIAGEN; cat. # 27104) was subjected to an insertion check prior to sequencing. Five microliters of the plasmid was incubated for 1 h at 37°C with 1 μ L of BamH1 restriction enzyme and 1 μ L EcoR1 restriction enzyme in a final volume of 10 μ L using a standard 10 $\times$ buffer solution. Gel electrophoresis confirmed the insertion size displaying a band at the appropriate size when compared to a control plasmid that did not contain F1 and F2.

LC-MS Assay—Kinetics of Cleavage of ProLC3B by ATG4B

Stock solutions of LC3B (223 μ M) and Atg4B (213 μ M) in 50 mM of Tris buffer and 500 mM of NaCl at pH 8 were stored in a -80°C freezer in small aliquots. Each assay utilized fresh aliquots of protein and enzyme. Aliquots were flash thawed, then stored over ice at 0°C. A fresh reducing buffer consisting of TCEP (1.0 mM) in a stock buffer solution of 50 mM of Tris and 35 mM of NaCl at pH 8 was prepared daily.

ATG4B was diluted to a concentration of 5 μ M in the reducing buffer and incubated for 1 h at 0°C. With a final working volume of 100 μ L, the assay was run with ATG4B at 12 nM to 48 nM and proLC3B at concentrations varying from 12 μ M to 480 μ M. Two samples of protein plus enzyme were prepared simultaneously by first diluting the proLC3B to the appropriate concentration and then adding the enzyme. The cleavage of proLC3B in the two samples was monitored in tandem using LC-MS.

The analytical systems used included an Agilent Technologies 1200 Series LC using a Zorbax 300SB-C8 Analytical Guard Column (4.6 mm $\times$ 12.5 mm, 5 μ m) connected to an Agilent Technologies 6210 Time-of-Flight LC-MS. Alternating injections from the two samples (1 μ L) were performed every 5 min for 1 h. Elution was performed at 1.0 mL/min using a gradient elution from 90:10 water:acetonitrile + 0.1% formic acid to 40:60 water:acetonitrile + 0.1% formic acid. Data analysis was performed using Agilent Technologies MassHunter Workstation Software Qualitative Analysis Version B.02.00 Build 2.0.197.0. The relative concentrations of proLC3B and cleaved LC3B-I were calculated by measuring the peak height of the two proteins in the deconvoluted mass spectra.

K_M determination via LC-MS was performed in duplicate in 4 mL HPLC vials using 350 μ L glass inserts. The vials were charged with 50 μ L of proLC3B at various concentrations and then diluted with the reducing buffer (50 mM Tris, 35 mM NaCl, 5 mM TCEP, pH 8) to bring the volume up to 90 μ L. Finally, 10 μ L of ATG4B at 0.48 μ M, pre-

incubated with the reducing buffer for 60 min, was added to the vials. Every 5 min, 0.5–5 μ L was injected into a TOF LC-MS (Agilent 2610). The analysis of the m/z distribution signal resulting from the protein substrate (proLC3B) was performed by Agilent MassHunter Workstation software elucidating proteins 10,000–30,000 Da between 700 and 1,800 m/z . Peak intensities were recorded for the parent substrate (16,720 Da) and cleaved protein (16,161 Da). The amount of cleavage was determined by taking the ratio of cleaved and noncleaved peak intensities and comparing with a standard curve derived from known concentrations of proLC3B and fully cleaved LC3B-I. To obtain a standard solution of LC3B-I, proLC3B (100 μ M) in the reducing buffer was incubated with Atg4B (48 nM) overnight at 4°C. Full cleavage was verified by mass spectral analysis. Peak intensities increased linearly with concentration, and ionization potentials did not change for the product peaks over the concentrations tested. K_M and k_{cat} values were determined by nonlinear regression analysis fitting to the equation $Y = E_t \times k_{cat} \times X / (K_M + X)$.

ATG4B Labeling and Inhibition with N-ethylmaleimide

Frozen aliquots of ATG4B (20 μ L, 99 μ M) were freshly thawed and diluted to a concentration of 5 μ M using the reducing buffer (50 mM Tris, 35 mM NaCl, 2 mM TCEP, pH 8). N-ethylmaleimide (NEM) stock solutions were prepared in dimethyl sulfoxide (DMSO) at various concentrations from 20 to 100 mM. Eppendorf tubes were then charged with 5 μ L of NEM solution followed by 85 μ L of the reducing buffer and 10 μ L of ATG4B to ensure a final concentration of either 500 nM or 250 nM of ATG4B with no more than 5% DMSO and a final volume of 100 μ L. Incubation of 500 nM of ATG4B with 1 mM of NEM took place overnight at 4°C. Similarly, 250 nM of ATG4B was incubated with 5 mM of NEM over 5 h at 4°C. The enzyme samples were analyzed by TOF LC-MS (Agilent 2610). The analysis was performed using Agilent MassHunter Workstation software elucidating proteins of 20,000–60,000 Da between 700 and 1,800 m/z . Incubation of 500 nM of ATG4B with 1 mM NEM led to a major peak at 45,513 Da, with the parent peak at 45,013 Da undetected. Incubation of 250 nM ATG4B with 5 mM NEM led to a major peak at 46,639 Da. These enzyme samples were then assayed for residual activity via LC-MS using proLC3B as the substrate using the method described above.

Time Dependence of Inhibition of ATG4B with NEM

A frozen stock of ATG4B (60 μ M) was flash thawed and diluted to a concentration of 48 nM using the reducing buffer (50 mM Tris, 500 mM NaCl, 2 mM TCEP, pH 8). A frozen stock of LC3B (1 mM) substrate was flash thawed and diluted to a concentration of 60 μ M. ATG4B and LC3B were allowed to incubate with the reducing buffer at 0°C for 1 h prior to experiments. Testing solutions of NEM were prepared at various concentrations from a 15 mM stock solution of NEM dissolved in a 9:1 buffer:DMSO solution. The dilutions were performed to ensure no more than 5% DMSO would be present in the final 50 μ L working volume. Incubation of 12.5 μ L of ATG4B (48 nM) with 12.5 μ L of NEM (4 mM) took place for 15 and 60 min, at which point 25 μ L of LC3B (60 μ M) was added to the mixture. Samples were analyzed by TOF LC-MS as described above.

Determination of Optimal FRET Excitation-Emission Wavelength

Spectrophotometric experiments were performed using a BioTek Synergy 4 Fluorometer. An absorbance spectrum was obtained on a 1 mL sample of FRET-LC3 (10 μ M) in order to determine the wavelength of maximum absorption (λ_{max}), which was found at 485 nm (for the EmGFP component) and 512 nm (for the YFP component; *Supplementary Fig. S1*; *Supplementary Data* are available online at www.liebertpub.com/adt). Emission spectra were obtained at various excitation wavelengths (435–475 nm) of both FRET-LC3 and cleaved FRET-LC3, each 10 μ M. Subtraction of the FRET-LC3 spectra from the corresponding cleaved FRET-LC3 spectra gave the difference emissions at each wavelength. The greatest difference in emission between the whole and cleaved FRET-LC3 samples at 510 nm was observed to be from using an excitation wavelength of 465 nm.

K_M Determination—Fluorescent Protein Substrate via Fluorescence

Ten-microliter aliquots of Atg4B were flash thawed and diluted to 0.48 μ M using a reducing buffer (50 mM Tris, 35 mM NaCl, pH 8, 5 mM TCEP). ATG4B was incubated with the reducing buffer at 0°C for 1 h. His₆-YFP-LC3B-EmGFP (FRET-LC3) was also incubated with the reducing buffer for at least 30 min prior to loading, and diluted into aliquots in such a way that 20 μ L of fluorescent protein would be delivered to each well to achieve the desired substrate concentrations. Twenty-five microliters of the reducing buffer was loaded into an all-black 384-well plate (Corning product #3822). Five microliters of ATG4B was then added to all of the wells, except for the negative control well. Finally, 20 μ L of fluorescent protein substrate was added to the wells to bring the final volume to 50 μ L, and the plate was then loaded into a Synergy 4 Fluorometer. Experiments were performed in triplicate at 25°C with slow shaking prior to each reading. Fluorescence was scanned every 2 min for up to 2 h excitation at 450 nm and monitoring the emission at 510 nm. Raw data were replotted and analyzed using GraphPad Prism v5 software fitting to the equation: $Y = E_t \times k_{\text{cat}} \times X / (K_M + X)$.

Calibration Curve—FRET-LC3 Fluorescence

The FRET-LC3 calibration curve was obtained by serial dilution into 384-well all-black plates to achieve four final concentrations between 0.15 μ M and 4.5 μ M and a final volume of 50 μ L. Twenty microliters of the FRET-LC3 at various concentrations were manually pipetted into the plate followed by 30 μ L of the reducing buffer (50 mM Tris, 35 mM NaCl, 5 mM TCEP, pH 8). A similar calibration curve was derived from a stock sample of FRET-LC3 (100 μ M) that had been fully cleaved (verified by mass spectrometry) after incubated in assay buffer with 48 nM ATG4B at 4°C for 18 h. An average RFU difference was calculated for each substrate concentration and then plotted using Prism 5. Differences in fluorescence between intact substrate and cleaved substrate were compared and used as the calibration curve.

Calibration Curve—FRET-LC3 by LC-MS

Various concentrations of substrate (0.15–20 μ M) were prepared in the standard reducing buffer up to a final volume of 100 μ L.

Each concentration was analyzed in terms of peak intensities and was found to increase linearly with concentration. Additionally, known ratios of cleaved and uncleaved substrate were analyzed to determine if ionization potentials differed between the two cleaved peptides. Again, the peak intensities increased linearly with concentration.

K_M Determination—FRET-LC3 substrate via LC-MS Analysis

K_M determination via LC-MS was achieved as described above, except that experiments were performed in duplicate in 4 mL HPLC vials using 350 μ L glass inserts. The vials were charged with 50 μ L of FRET-LC3 at various concentrations and then diluted with the reducing buffer (50 mM Tris, 35 mM NaCl, 5 mM TCEP, pH 8) to bring the volume up to 90 μ L. Finally, 10 μ L of Atg4B at 0.48 μ M pre-incubated with the reducing buffer was added to the vials. Every 5 min, 1 μ L was injected into a TOF LC-MS (Agilent 2610). The analysis of the m/z distribution signal resulting from the protein substrate was performed using Agilent MassHunter Workstation software elucidating proteins of 20,000–80,000 Da between 700 and 1,800 m/z . Peak intensities were recorded for the parent substrate (72,902 Da) and both cleaved peptides (27,885 and 45,036 Da). The amount of cleavage was determined by taking the ratio of cleaved and noncleaved peak intensities and by comparison with a standard curve. The ratio between the 27,884 and 72,902 Da peaks were used for the calculations. The standard curve was obtained by injecting known ratios of cleaved and noncleaved substrate. Peak intensities increased linearly with concentration, and ionization potentials did not change for the product peaks over the concentrations tested. K_M and k_{cat} values were determined by nonlinear regression analysis fitting to the equation: $Y = E_t \times k_{\text{cat}} \times X / (K_M + X)$.

LC-MS Assay for Inhibition of ATG4B

For evaluation of inhibitors, the optimized low throughput LC-MS assay monitoring cleavage of proLC3B or FRET-LC3 by ATG4B is summarized in *Table 1*.

ATG4B was diluted to a concentration of 240 nM in the reducing buffer (50 mM Tris, 35 mM NaCl, 5 mM TCEP, pH 8) and incubated for 1 h at 0°C. With a final working volume of 100 μ L, the assay was run with ATG4B at 48 nM final concentration. Inhibitor at various concentrations (in 5 μ L DMSO or DMSO control) was added to 4 mL HPLC vials using 350 μ L glass inserts and diluted with 25 μ L of the reducing buffer, and then 20 μ L of the above solution of 240 nM ATG4B was added, and the mixture was incubated at room temperature for 30 min. Finally, 50 μ L of the substrate, proLC3B or FRET-LC3 at 5 μ M, was added, and the reaction was allowed to proceed. To obtain triple replicates, samples of the control reaction with inhibitor or two inhibitor samples were injected alternately in parallel for each inhibitor concentration. Every 5 min, 0.5–5 μ L was injected into a TOF LC-MS (Agilent 2610) alternating between the two parallel reactions. The analysis of the m/z distribution signal resulting from the protein substrate (proLC3B or FRET-LC3) was

Table 1. Liquid Chromatography/Mass Spectrometry Assay Protocol Table for ATG4B Activity

Step	Parameter	Value	Description
1	Reduce enzyme	1 h	Incubate enzyme in reducing buffer
2	Load inhibitor	5 μ L	Dispense inhibitor into HPLC vial with 350 μ L glass insert
3	Load buffer	25 μ L	Dispense reducing buffer into HPLC vial
4	Load enzyme	20 μ L	Dispense enzyme into HPLC vial
5	Incubation with enzyme	30 min	Let enzyme and inhibitor incubate for 30 min at room temperature
6	Dispense substrate	50 μ L	Dispense substrate into HPLC vial
7	Assay readout	5 min	Obtain mass spectrum

Step Notes

1. Incubate 240 nM ATG4B in reducing Tris buffer (50 mM Tris, 35 mM NaCl, 5 mM TCEP, pH 8) for 1 h at 0°C.
2. Dispense 5 μ L of inhibitor dissolved in dimethyl sulfoxide (DMSO) into a standard 2 mL HPLC vial with a 350 μ L glass flat-bottom insert, or 5 μ L pure DMSO in the case of a negative control.
3. Dispense 25 μ L reducing buffer into each HPLC vial.
4. Dispense 20 μ L of 240 nM ATG4B (final enzyme concentration in assay: 48 nM). Pipette mixture up and down five times to ensure a well-mixed and homogenous solution.
5. Let enzyme and inhibitor incubate at room temperature for 30 min.
6. Dispense 50 μ L of 6 μ M substrate (final substrate concentration in assay: 3 μ M). Pipette mixture up and down five times to ensure well mixed. Substrate can be pro-LC3B or FRET-LC3B.
7. Inject 2 μ L of assay sample every 5 min alternating between duplicate samples. Mass spectrum between 700 and 1,800 m/z deconvoluted by MassHunter software to elucidate proteins between 20,000 and 100,000 Da. Ratio of peak heights (product peak/[product peak+parent peak]) over time compared to a sample with no inhibitor present to determine percentage of inhibition.

performed using Agilent MassHunter Workstation software elucidating proteins of 10,000–30,000 Da between 700 and 1,800 m/z . Peak intensities were recorded for the parent substrate (16,720 Da) and cleaved protein (16,161 Da) for pro-LC3 and the parent substrate (72,903 Da) and two fragments (27,885 and 45,036 Da) for cleaved FRET-LC3. The amount of cleavage was determined by taking the ratio of cleaved and noncleaved peak intensities and by comparison with a control sample of proLC3 or FRET-LC3 with no inhibitor added.

Development of the High Throughput Assay

Optimization of enzyme and substrate concentrations. Enzyme concentrations of 0, 3, 6, 12, 24, and 48 nM were titrated against various substrate concentrations to find optimal concentrations to be used for screening in a 384-well format. ATG4B stock was incubated on ice for 1 h with reducing Tris buffer (50 mM Tris, pH 8, 1 mM TCEP, 500 mM NaCl) and manually pipetted into a 384-well black-bottom plate. Next, 5 μ L of various Atg4B stock concentrations was added to the wells, and the plate was pre-incubated at 25°C for 30 min. Following that, 20 μ L of various substrate stock concentrations were delivered with an electronic pipettor and mixed by pipetting up and down (final substrate concentrations were 0, 0.4, 0.9, 1.8, 3.5, and 7 μ M), to give a final assay volume of 50 μ L in all wells. Fluorescent kinetic reads were performed on the Synergy 4 (Biotek) at 25°C

(readings taken every 2 min for 2 h, shaken on slow for 1 s before every read, using excitation and emission wavelengths of 465/510 nm).

Optimization of salt concentrations. The 96-well assay employed very high salt concentrations in the assay buffer (500 mM NaCl). For HTS, we wanted to reduce the amount of salt to mimic physiological conditions. Reducing Tris assay buffer containing final NaCl concentrations of 0, 35, 50, 100, and 150 mM were tested. ATG4B stock was incubated on ice for 1 h with reducing Tris buffer (50 mM Tris, pH 8, 1 mM TCEP, 500 mM NaCl). In a 384-well black-bottom plate, reducing Tris buffer with various salt concentrations was manually pipetted to the plate. Next, 2.5 μ L of 960 nM ATG4B stock was added to all wells (final enzyme concentration of 48 nM), and the enzyme was pre-incubated on the plate at 25°C for 30 min before 6.3 μ L of 14.4 μ M FRET-LC3 (final substrate concentration of 1.8 μ M) was dispensed into the appropriate wells using a manual pipettor to give a final assay volume of 50 μ L in all wells. Fluorescent kinetic reads were performed using the

Synergy 4 at 25°C (readings taken every 2 min for 2 h, shaken slowly for 1 s before every reading, using excitation and emission wavelengths of 465/510 nm).

Optimization of reducing agent concentrations. Sufficient reducing agent is required to keep active site thiol groups from being oxidized. Refer to the above procedure (see *Optimization of salt concentrations*). In this case, the salt concentration was held constant at 35 mM NaCl, while the enzyme was incubated with either 1 mM or 5 mM TCEP. The final optimized FRET assay for monitoring cleavage of FRET-LC3 by ATG4B is summarized in Table 2.

LOPAC™ Pilot Screen

The 1,280 compound LOPAC library (Sigma) distributed over 4 $\times$ 384-well plates was screened to test automation parameters of the assay and to assess assay robustness. The library consists of 5 mM compounds dissolved in DMSO. Four control columns were included, one column (16 wells) containing substrate alone (c– controls), and one column containing substrate in the presence of enzyme (c+ controls). A total of 960 nM ATG4B stock was prepared in reducing Tris buffer (50 mM Tris, pH 8, 5 mM TCEP, 500 mM NaCl), and incubated on ice for 1 h. This enzyme stock was then diluted to 80 nM of ATG4B stock in the assay buffer (50 mM Tris, pH 8, 5 mM TCEP, 35 mM NaCl), and 30 μ L was delivered to the assay plates to give a

Table 2. High-Throughput Screening Fluorescence Assay Protocol for ATG4B Activity

Step	Parameter	Value	Description
1	Reduce enzyme	1 h	Incubate enzyme in reducing buffer
2	Plate buffer	25 μ L	Dispense assay buffer to wells to be screened
3	Library compounds	70 nL	Pin library compounds
4	Enzyme dispense	10 μ L	Dispense enzyme to plates
5	Substrate dispense	15 μ L	Dispense fluorescent substrate to plates
6	Assay readout	465 nm/510 nm	Read plate, detect fluorescence

Step Notes

1. Incubate 240 nM ATG4B in reducing Tris buffer for 1 h.
2. Dispense 25 μ L assay buffer to columns, which will contain screening compounds (columns 3–22) and positive control (column 23); dispense 35 μ L assay buffer to negative control (column 2), use solid black 384-well plates.
3. Pin 70 nL of screening library to the plate (columns 3–22) using the PlateMate affixed with the FP3 pin tool.
4. Dispense 10 μ L of 240 nM ATG4B using the WellMate (final enzyme concentration 48 nM).
5. Dispense 15 μ L of 11.7 μ M fluorescent substrate using the Microfill (final substrate concentration 3.5 μ M).
6. Perform fluorescent kinetic reads using the Varioskan (rt), read plate every 2 min for 40 min using excitation and emission wavelengths of 465/510 nm.

final enzyme concentration of 48 nM. The LOPAC library was pinned using the PlateMate (ThermoScientific, Matrix Technologies) and the FP3 pin tool (ThermoScientific, Matrix Technologies). Plates were pre-incubated at 25°C for 30 min before 20 μ L of fluorescent substrate was dispensed using the Microfill™ (Biotek; final substrate concentration was 3.5 μ M). Fluorescent kinetic reads were performed using the Varioskan® (ThermoScientific) at 25°C (readings taken every 2 min for 40 min, using excitation and emission wavelengths of 465/510 nm). Net change in fluorescence was determined by subtracting the raw fluorescence data from control columns containing substrate alone (c–). ATG4B initial velocities were calculated by measuring the slope from 10 to 20 min (a 5 min lag time was observed in some wells before a linear velocity was reached). The percent inhibition was determined by normalizing initial velocities to c– wells (100% inhibition) and to c+ wells (0% inhibition). Assay robustness was assessed by determining the Z' values for all four assay plates using values obtained from the control columns. Frequency distributions were plotted using GraphPad Prism v5.02 software.

Known Drugs Library (KD2) Screen

An additional 3,481 compounds were screened using a Known Drugs Library (a collection of compounds from Prestwick, Microsource, and BIOMOL) over 12 $\times$ 384-well plates. The library consists of 5 mM compounds dissolved in DMSO. A 240 nM ATG4B stock in reducing Tris buffer (50 mM Tris, pH 8, 5 mM TCEP, 500 mM NaCl) was incubated on ice for 1 h. In 384-well black-bottom plates, 35 μ L

of assay buffer (50 mM Tris, pH 8, 5 mM TCEP, 35 mM NaCl) was added to column 2 (c–), and 25 μ L of assay buffer was added to columns 3–22, and column 23 (c+). The Known Drugs Library was delivered into the assay plates using the PlateMate and FP3 pin tool. Next, 10 μ L of 240 nM ATG4B assay buffer was delivered using the WellMate (for a final enzyme concentration of 48 nM). Immediately before reading the plates, 15 μ L of 11.7 μ M fluorescent peptide substrate stock was dispensed using the Microfill (final substrate concentration was 3.5 μ M). Fluorescent kinetic reads were performed using the Varioskan at 25°C (readings taken every 2 min for 40 min, using excitation and emission wavelengths of 465/510 nm). Data were processed as described above for the LOPAC pilot screen.

Concentration Response Curves of Actives Identified from the LOPAC and Known Drugs Libraries

Actives were confirmed by generating concentration–response curves first as single-point curves from library daughter plates. Several selected actives—hypericin (AG Scientific), aurintricarboxylic acid (Sigma), and Z-L-Phe-chloromethyl ketone (Sigma)—were then purchased, and concentration–response curves in triplicate were generated from fresh drug stocks. Serial dilutions of compounds were created, with final concentrations tested ranging from 0.014 to 30 μ M diluted in assay buffer. To each well, 5 μ L of serially diluted compound was added to 25 μ L assay buffer before the addition of the enzyme or substrate. Assay protocol and data processing were performed as described above (see *Known Drugs Library Screen*).

RESULTS**Mass Spectrometry Assay to Measure ATG4B Activity**

To monitor ATG4B activity, we initially developed an assay measuring cleavage of the proLC3B substrate by mass spectrometry. Recombinant human His₆-ATG4B and His₆-proLC3B (proLC3B) were used (*Supplementary Figs. S2 and S3*; proteins and cDNA were kindly supplied by Aled Edwards, SGC), and ATG4B was shown to convert proLC3B effectively, with an observed molecular mass of 16,720.3 Da, to LC3-I with a mass of 16,161.4 Da, in keeping with the predicted loss of 558.9 Da (*Supplementary Figs. S4 and S5*). The kinetics of cleavage of proLC3B was then examined in detail. For the assay, varying concentrations of either ATG4B or proLC3B were mixed, and both the disappearance of the nominal mass for proLC3B and the appearance of the expected mass of the processed protein LC3-I were monitored. Change of peak heights of the respective peaks were used to calculate rate of cleavage according to the formula: Percentage of product formation = $A/(A+B) \times 100$, where A and B are the peak heights of LC3-I and proLC3B respectively. Concentrations were adjusted to allow the reaction to proceed over about a 1 h period, and reaction mixtures were sampled

every 3–5 min to allow duplicate runs to be followed in parallel. The ATG4B was activated by pre-incubation for 1 h with a suitable reducing agent (TCEP). As expected, ATG4B converted proLC3B to LC3B-I in a concentration- and time-dependent manner. Reactions were all run at pH 8.0, which has been previously reported to be optimal.¹⁸ Standard curves for proLC3B and LC3B-I were developed using the purified proLC3B and samples of known concentrations of proLC3B that had been fully cleaved to yield LC3B-I by incubation with ATG4B at 25°C over 12 h. Using the mass spectrometric method for analysis of the enzyme kinetics, the K_M was determined as $12 \pm 4.6 \mu\text{M}$, k_{cat} as $0.98 \pm 0.82 \text{ s}^{-1}$, and k_{cat}/K_M as $81,000 \text{ M/s}^{-1}$ (Fig. 2A).

Inhibition of ATG4B with NEM

NEM is a highly electrophilic compound known to act as an inhibitor of cysteine proteases by irreversible alkylation of the active site thiol moiety. The ATG4B construct we employed has 13 cysteine residues, and so it was not clear if NEM would react preferentially with the active site thiol relative to the other thiol functions in the protein. Incubation of ATG4B (500 nM; predicted and observed mass of 45,013 Da) with NEM (1 mM; 2,000 molar equivalents) resulted in a protein with an observed molecular mass of 45,513 Da. This mass indicates addition of NEM to four nucleophilic sites on the enzyme, but surprisingly, in spite of this multiple alkylation, the modified enzyme showed little reduction in activity (Supplementary Figs. S6A and S7). Pre-incubation with higher relative concentrations of NEM was necessary to achieve significant inhibition of activity. After pre-incubation of ATG4B (250 nM) with 5 mM of NEM (20,000 molar equivalents) inhibition was complete (Supplementary Figs. S6B and S7), and a protein with a mass of 46,639 Da was observed, in keeping with the addition of 13 equivalents of NEM. The inhibition was time dependent, and inactivation was slow. Pre-incubation of ATG4B

(12 nM) with 1 mM NEM followed by the addition of proLC3B (30 μM) showed only about 50% inhibition after 15 min and complete inhibition after 1 h (Fig. 2B).

Development of a Fluorogenic Substrate and Assay for ATG4B

An assay using active site-dependent cleavage of a small molecule fluorogenic substrate was preferred for higher throughput, and we sought to develop such a substrate by investigating the structure–activity relationships for the enzyme on fluorogenic peptides. Studies have indicated that ATG4B requires a glycine at P1 and recognizes the motif phenylalanine-glycine at P2 and P1,¹⁹ but recombinant ATG4B was initially reported to be unable to cleave small peptide fragments (such as QETFGTALA) spanning the murine LC3B cleavage site.²⁰ Recent reports have subsequently indicated the enzyme is capable of cleaving small peptide-amino-trifluoromethylcoumarin (AFC) substrates such as Ac-GTFG-AFC, albeit with very low rates of turnover.¹⁸ Other studies have shown that fluorescent self-quenching peptides such as Mca-TFGM-Dpa are cleaved by the homologous ATG4C,²¹ but in our hands, recombinant ATG4B did not cleave (Mca)-TFGM-(Dpa), (Mca)-QETFGMKLS-(Dpa), or (Mca)-QETFGTALA-(Dpa) at any measureable rate.

To develop an assay suitable for screening at lower enzyme concentrations, we devised a fluorescent analog of proLC3B. We conceptualized that an LC3B analog with appropriately chosen fluorescent proteins appended at the N and C termini might display FRET, which would be lost after cleavage, thus allowing us to monitor the progression of the reaction by changes in fluorescence. A published X-ray structure of LC3B²⁰ indicated that the C and N termini are within about 20 Å of each other, a distance that should be consistent with a robust FRET interaction.²² Also, both the His₆-proLC3B

construct and the LC3B-GST fusion protein²⁰ are substrates for ATG4B, indicating the enzyme is largely indifferent to bulky protein elongations at either the N or C termini. Evaluation of possible candidate fluorescent proteins led to the choice of emerald-green fluorescent protein (Em-GFP) and yellow fluorescent protein (YFP), in that Em-GFP absorbs with maximum at 487 nM and emits at 509 nM, while YFP absorbs at 514 nM and emits at 527 nM.²³ The construct His₆-YFP-LC3-EmGFP (FRET-LC3; Fig. 3A) was prepared (Supplementary Fig. S8), and the UV spectrum of the purified protein had absorption maxima at 485 and 515 nm (Supplementary Fig. S1) and an observed molecular mass of 72,902 Da corresponding to the expected molecular mass (Fig. 3B). When FRET-LC3 was

Fig. 2. Kinetics of cleavage of proLC3B by ATG4B (A), and time-dependent inhibition of ATG4B with N-ethylmaleimide (NEM) (B) as monitored by mass spectrometry. (A) Conditions: Various concentrations of proLC3B in the presence of 48 nM ATG4B, 500 mM NaCl, 1 mM TCEP, 50 mM Tris, pH 8, 50 μL working volume, 1 μL injection volume, 3 min run time per injection, up to 5% dimethyl sulfoxide (DMSO) is tolerable. Only the linear portions of initial rates of product formation were used to determine K_M . Initial rates of product generated are plotted against substrate concentrations. $K_M = 12 \pm 4.6 \mu\text{M}$, $k_{\text{cat}} = 0.98 \pm 0.82 \text{ s}^{-1}$, $k_{\text{cat}}/K_M = 81,000 \text{ M/s}^{-1}$. Error bars denote standard deviations ($n = 3$). (B) Conditions: As for A but 12 nM ATG4B, 30 μM proLC3B. Control (black diamonds); 15 min pre-incubation with 1 mM NEM (gray squares); 1 h pre-incubation with 1 mM NEM (red triangles).

Fig. 3. (A) YFP-LC3-EmGFP as a fluorescence resonance energy transfer (FRET) ligand. (B) Deconvoluted mass spectrum of YFP-LC3-EmGFP. (C) Deconvoluted spectrum of YFP-LC3-EmGFP after partial cleavage by ATG4B.

incubated with ATG4B and the reaction followed by mass spectrometry, it was found to be rapidly and completely cleaved to yield the expected fragments of molecular masses 27,885 Da (for the cleaved EmGFP) and 45,036 Da (for the YFP-LC3 fragment; Fig. 3C). Observation of the fluorescence emission spectrum of FRET-LC3 showed that excitation at 485 nm led to emission with maximum at 527 nm, indicative of the expected FRET response. FRET-LC3 was then incubated with ATG4B, and the fluorescence changes were monitored (excitation at 485 nm). The reaction was also monitored in parallel experiments by mass spectrometry. Emission at 510 nm exhibited the largest relative increase (Fig. 4A), but changes over time were not linear with concentration of

product formed. The absorption spectrum for EmGFP includes a shoulder with significant absorption at about 490 nm,²⁴ and excitation near this wavelength could be expected to lead to significant, non-FRET excitation of GFP that could complicate the fluorescence changes. Thus, in initial studies with FRET-LC3, we decided to excite at a wavelength lower than 485 nm to avoid overlap with this absorption shoulder (Fig. 4B). It was found that monitoring cleavage of FRET-LC3 by ATG4B with excitation at 450 nm and observation of fluorescence changes at 510 nm gave increases in fluorescence that were linear and correlated with respect to concentration of the components of the reaction (Supplementary Fig. S9). Equally, correlation of concentration of

Fig. 4. (A) Fluorescence emission spectrum of YFP-LC3-EmGFP (excitation at 485 nm) before and after incubation with ATG4B. (B) Difference in fluorescence emission spectra of FRET-LC3 (10 μM) and fully cleaved FRET-LC3 (10 μM; MS verified mixture of YFP-LC3 and EmGFP obtained after 24 h of incubation with ATG4B) with excitation at 435 nm (mauve squares), 445 nm (blue crosses), 455 nm (blue diamonds), 465 nm (red squares), and 475 nm (green triangles).

FRET-LC3 and its fragments with mass spectrometric peak heights was also linear (Supplementary Fig. S10).

Next, kinetic analyses of activity of ATG4B using FRET-LC3 substrate were performed comparing the fluorescence and mass spectrometric readout. Following processing of FRET-LC3 by ATG4B with the fluorescence readout (excitation 450 nm; emission 510 nm) gave $K_M = 16 \pm 13 \mu\text{M}$ (with 48 nM enzyme) and $k_{\text{cat}} = 0.52 \pm 0.23 \text{ s}^{-1}$ and k_{cat}/K_M of $33,000 \text{ M/s}^{-1}$ (Fig. 5A). Parallel assays using mass spectrometric analysis gave $K_M = 9 \pm 3 \mu\text{M}$, $k_{\text{cat}} = 0.25 \pm 0.04 \text{ s}^{-1}$, and $k_{\text{cat}}/K_M = 27,000 \text{ M/s}^{-1}$ (with 48 nM enzyme; Fig. 5B). Thus, the FRET

Fig. 5. (A) Kinetics of cleavage of YFP-LC3B-EmGFP by ATG4B monitored by fluorescence change at 510 nm. (B) Cleavage of YFP-LC3B-EmGFP by ATG4B monitored by mass spectrometry. (A) Fluorescence signal (ex 450 nm/em 510 nm) was measured at various concentrations of fluorescent protein substrate (FP). Only the linear portions of initial rates of product formation were used to determine K_M . Initial rates of product generated are plotted against substrate concentrations. $K_M = 16 \pm 13 \mu\text{M}$ (via nonlinear regression analysis), $k_{\text{cat}} = 0.52 \pm 0.23 \text{ s}^{-1}$ (via nonlinear regression analysis, $[E] = 48 \text{ nM}$), $k_{\text{cat}}/K_M = 33,000 \text{ M/s}^{-1}$. Error bars denote standard deviations ($n=3$). (B) Cleavage of YFP-LC3B-EmGFP in the presence of 48 nM ATG4B was monitored by mass spectrometry. Only the linear portions of initial rates of product formation were used to determine K_M . Initial rates of product generated are plotted against substrate concentrations. $K_M = 9 \pm 3 \mu\text{M}$, $k_{\text{cat}} = 0.25 \pm 0.04 \text{ s}^{-1}$, $k_{\text{cat}}/K_M = 27,000 \text{ M/s}^{-1}$ when $[E] = 48 \text{ nM}$. Error bars denote standard deviations ($n=3$).

assay of ATG4B activity using YFP-LC3-EmGFP as substrate was validated and considered suitable for further optimization to support HTS.

Adapting the FRET-LC3 Assay for HTS

A series of experiments was undertaken to optimize the variables of the assay in order to yield the most reliable and responsive results possible. First, a study was undertaken to optimize excitation and observed emission wavelengths for linearity and magnitude of change before and after cleavage. The differences in emission spectra observed for samples of uncleaved FRET-LC3 and fully cleaved substrate were evaluated as excitation wavelengths were scanned in 10 nm steps from 435 to 475 nm. Excitation at 465 nm and observation of fluorescence changes at 510 nm gave differences in fluorescence that were maximal (Fig. 4B) and that correlated with concentration of product in the samples.

Then the FRET-LC3 assay was further optimized to a 384-well format in order to afford robust data with an adequate signal-to-noise ratio for HTS. Enzyme and substrate concentrations were titrated to find optimal activity and to yield acceptable statistical validation for the assay (Supplementary Fig. S11). With 48 nM of enzyme, substrate concentrations $\geq 1.8 \mu\text{M}$ yielded a sufficient rate of cleavage with a sufficient change in fluorescence for adequate separation between positive and negative controls. This concentration also resulted in linear initial velocities spanning 0–30 min.

Next, to mimic physiological conditions better, buffer composition was optimized to reduce the amount of salt. The original FRET-LC3 assay as developed for low throughput contained 500 mM salt. However, intracellular ion concentrations have been reported as 12 mM Na^+ and 139 mM K^+ .²⁵ Tris buffer with varying NaCl concentrations was titrated into the assay wells, and 35 mM NaCl

afforded a sufficient signal yet minimized noise (Supplementary Fig. S12).

Finally, to ensure optimal enzyme activity, two different concentrations (1 and 5 μM) of reducing agent—TCEP—were explored. The higher concentration of reducing agent resulted in increased activity as monitored by fluorescence kinetic reads at all substrate concentrations tested (Supplementary Fig. S13). As a result of increasing the concentration of reducing agent, an improved signal was obtained using a higher concentration of FRET-LC3 (3.5 μM instead of 1.8 μM).

Small Molecule Library Screening Identifies Candidate ATG4B Inhibitors

Using these optimized enzyme, substrate, and buffer conditions, a pilot

screen employing the library of pharmacologically active compounds (LOPAC) library was performed. A total of 1,280 pharmacologically active compounds were screened to assess assay robustness and to ensure the assay was amenable to high throughput automation. Compounds were pinned into assay wells (final concentration of compound was 8 μM), and liquid dispensers were used to deliver reagents to assay plates. The screen was found to be robust, with a Z' of 0.58 and a signal-to-noise ratio of 13.9 (Fig. 6A). Eight compounds were identified with percent inhibition values $>50\%$ (hit rate 0.6%; Supplementary Table S1).

Next, the Known Drugs Library (KD2) containing 3,481 compounds was screened using identical conditions to the LOPAC screen. Again, the screen was robust with a Z' of 0.63 and a signal-to-noise ratio of 17.2 (Fig. 6B). Seventeen compounds were identified with inhibition values $>50\%$ (hit rate 0.5%; Supplementary Table S2).

Actives identified from the LOPAC and KD2 libraries were retested to generate concentration–response curves. Of the eight LOPAC actives, five compounds provided concentration-dependent inhibition on retest, and two—aurintricarboxylic acid and *Z*-*L*-Phe-chloromethyl ketone—were reconfirmed from fresh solid compound reordered from the supplier. Six of 17 KD2 actives reconfirmed in a concentration-dependent manner on retest, and two—aurintricarboxylic acid and hypericin—were reconfirmed from fresh solid compound reordered from the supplier. Thus, aurintricarboxylic acid was found in both screens. The three reconfirmed compounds were retested in triplicate, and concentration–response curves generated in the FRET-LC3 assay (Fig. 7A–C) and also in the LC-MS assay monitoring inhibition of cleavage of FRET-LC3 (Fig. 7D–F). Hypericin only achieved a maximum of about 50% inhibition at 10 μM in the FRET-LC3 assay, but a complete inhibition curve was obtained in the LC-MS assay with $\text{IC}_{50} = 57 \mu\text{M}$ observed.

Fig. 6. Frequency distribution plots of LOPACTM pilot screen of 1,280 pharmacologically active compounds (**A**) and KD2 pilot screen of 3,481 pharmacologically active compounds (**B**). 384-well plates containing 48 nM ATG4B and 3.5 μ M YFP-LC3-EmGFP in Tris reducing buffer were pinned with library compounds (with a final drug concentration of 8 μ M). Percent inhibition values of the compounds (black circles) were normalized to negative controls c- (substrate alone; blue circles) and positive controls c+ (substrate in the presence of enzyme, red circles). The assay of LOPAC compounds yielded a Z' of 0.58 with a signal-to-noise ratio of 13.9. The assay of KD2 compounds yielded a Z' of 0.63 with a signal-to-noise ratio of 17.2.

Fig. 7. Concentration-response curves of select active compounds hypericin (**A**), aurintricarboxylic acid (**B**), and Z-L-Phe-chloromethyl ketone (**C**) identified from the KD2 and LOPAC screening regimes using FRET-LC3 assay. Into a 384-well plate containing 48 nM ATG4B and 3.5 μ M YFP-LC3-EmGFP in Tris reducing buffer, 5 μ L of a serial dilution was dispensed (top final concentration of drug tested was 30 μ M). Error bars denote standard deviations ($n=3$). Concentration-response curves were also generated for hypericin (**D**), aurintricarboxylic acid (**E**), and Z-L-Phe-chloromethyl ketone (**F**) using the LC-MS assay.

Fig. 8. Deconvoluted mass spectrum reconstituting proteins between 20,000 and 60,000 Da. The mass of 45,309.08 Da is seen after incubation of ATG4B (1 μ M) with 10 μ M *Z*-L-Phe-chloromethyl ketone for 5 min. The mass shift corresponds with complete mono-alkylation of parent ATG4B (45,013 Da) by *Z*-L-Phe-chloromethyl ketone.

Aurintricarboxylic acid was consistently potent in both the FRET and LC-MS assays with IC_{50} of 1.3 and 4.4 μ M respectively. The most potent active compound was *Z*-L-Phe-chloromethyl ketone (IC_{50} = 0.63 μ M in FRET-LC3 assay and 0.1 μ M in LC-MS assay). When ATG4B (1 μ M) was incubated with *Z*-L-Phe-chloromethyl ketone (10 μ M) for 5 min, inhibition of the enzyme activity was found to be complete, and analysis of the enzyme by mass spectrometry showed a signal at 45,309 Da, consistent with mono-alkylation (Fig. 8). When ATG4B was similarly incubated with aurintricarboxylic acid, no change in the observed molecular mass of ATG4B was observed.

DISCUSSION

Assay of Enzymatic Activity of ATG4B Using Mass Spectrometry and Inhibition with NEM

Recombinantly expressed proLC3B gave a strong mass spectrometric signal that after deconvolution corresponds to a molecular mass of 16,720.3 Da, as predicted from the calculated molecular weight. Upon processing of proLC3B with recombinant ATG4B, a mass of 16,161.4 Da was observed, corresponding to LC3-I with the predicted loss of 558.9 Da relative to proLC3B. The parent mass signal intensities of proLC3B and LC3-I were linear with respect to concentration, and thus useful to follow enzymatic cleavage over time with an optimized sampling time of one measure every 3 min. Kinetic analysis of the reaction gave a K_M of 12 μ M and k_{cat}/K_M of 81,000 M/s⁻¹ consistent with parameters determined by other modes of assay.¹⁸ Such an assay, while low throughput, gives a direct and sensitive measure of activity and is not prone to interference with fluorescence quenching or photo bleaching, which can complicate fluorescence-monitored assays. In that one can also monitor the mass of the ATG4B enzyme at the same time, this technique allowed the observation of enzyme integrity throughout the reaction. Additionally, chemical modifications of ATG4B can be directly monitored using mass spectrometry-based methods, which may provide mechanistic insights as outlined below.

Alkylation of ATG4B by NEM

Pretreatment of ATG4B with high concentrations (20,000-fold excess) of the nonspecific thiol trapping agent NEM for 5 h led to complete inactivation of the enzyme. Mass spectrometric evaluation of the mixture revealed that the reagent reacted (putatively) with multiple thiol nucleophiles on the enzyme, giving modified ATG4B protein with mass equivalent to the addition of 13 molecules of NEM, one for each of the 13 cysteines present in the protein. Pretreatment of ATG4B for 24 h with a lower, 2,000-fold excess of NEM led to addition of 4 M equivalents of NEM, and this modification had *little effect on the rate of the enzyme*. This indicates that the active site thiol of ATG4B is less accessible than some other peripheral thiols on the protein. This is in keeping with structural observations from the literature²⁰ that indicate that many of the cysteines are at or near the exterior surface of the protein and that access to the active site and its cysteine thiol is limited by a blocking loop. One cannot rule out from these results that some level of structural reorganization or denaturation might occur due to alkylation of the nonactive site cysteine thiol groups that in itself might affect activity or make the active site cysteine more accessible.

Time Dependence of Inhibition of ATG4B with NEM

Pretreatment of ATG4B (12 nM) with 1 mM NEM (a 83,000-fold molar excess) for 50 min led to complete inhibition of activity, while pretreatment under the same conditions but for only 15 min gave partial inhibition (~50%). When the substrate proLC3B (30 μ M) was added after 15 min, the residual rate of activity was linear over the next 45 min of observation (Fig. 2B). This shows that inhibition of ATG4B with NEM is time dependent and that the LC3B substrate effectively protects the active site cysteine from alkylation by competition for the active site.

Development of a FRET Assay for Screening for Inhibitors of ATG4B

While the enzyme activity assay based on mass spectrometric analysis is useful, it is a very low throughput assay and is therefore inappropriate for large-scale chemical library screening. Thus, the protein construct FRET-LC3 (His₆-YFP-LC3-EmGFP) was prepared and was found to be a substrate for ATG4B with kinetic parameters very similar to proLC3B itself. Thus, FRET-LC3 can serve as a "natural" fluorescent substrate more amenable to HTS. The construct has UV absorption maxima at 485 and 512 nm (Supplementary Fig. S1) close to those reported for the individual fluorescent proteins EmGFP and YFP respectively,²³ and thus was evaluated to determine the best conditions for the greatest FRET response and to determine that fluorescence changes correlate with processing of the substrate by ATG4B. While one might expect the maximum FRET response (and therefore sensitivity) would be derived from excitation at 485 nm (with predicted emission of the EmGFP component at 509 nm, almost coincident with the absorption maximum attributed to the YFP component at 512 nm), these conditions gave nonlinear results when monitoring cleavage of FRET-LC3 by ATG4B over time. YFP has a significant absorption shoulder at 490 nm,²⁴ and thus excitation

close to this wavelength can confound the response. After scanning a number of lower excitation frequencies, an optimal excitation wavelength of 465 nm was found. This wavelength provided maximum sensitivity while maintaining linear correlation with changing concentrations of products in the reaction. The enzyme kinetic parameters, determined by mass spectrometric analysis, were found to parallel those derived from the FRET-LC3 assay offering validation of the fidelity of the FRET assay. Optimization of the FRET assay for testing in a 384-well format showed that preferred conditions involved more physiological sodium ion concentration of 35 mM and a higher concentration of reducing agent TCEP (5 mM) with a FRET-LC3 substrate concentration of 3.5 μ M. The FRET-LC3 assay was shown to give robust data after optimization for HTS format in 384-well plates and served to identify a small number of verified hits (>50% inhibition at 8 μ M) from the 4,761 compounds tested (overall hit rate 0.5%). There was no indication that the assay identified a significant number of false positive compounds due to coloration or fluorescence, and compounds such as sennosides A and B or fluorescein, while found in the initial screen, did not confirm on retest for a dose response.

Subsequent to the completion of these studies, another report of a similar FRET assay¹⁷ and also an enzyme-coupled assay have been reported.¹⁶ The enzyme-coupled assay of Shu *et al.*¹⁶ utilizes an LC3 construct with phospholipase A₂ appended at the C-terminus and was used also for screening the commercial LOPAC compound set, as well as the SpectrumTM library, and was subsequently used in a campaign to screen 340,000 compounds.²⁶ This construct has the possibility of finding inhibitors of PLA₂ as false positives, and thus a parallel PLA₂ assay counterscreen was run on identified active hits. Shu's screen of the LOPAC compounds identified three confirmed hits (including aurintricarboxylic acid, also found in our screens), and a low rate of positive PLA₂ actives was found (none of his LOPAC hits were found active in the PLA₂ counterscreen, while only 1 of 14 of the hits in the Spectrum assay was active). The other hits confirmed by Shu in the LOPAC (R2751 and T7822) were not found as hits in our screen. While we did not screen the Spectrum compounds *per se*, several of the actives found by Shu were also found initially in our screen of the KD2 compound library. These included sennosides A and B and gossypol. The sennosides did not confirm in our hands (*Supplementary Table S2*). The large-scale 340,351 compound screen conducted by Shu is reported on PubChem as multiple project iterations.²⁶ It is reported that 1,735 actives were identified with up to 1,085 of these showing significant PLA₂ inhibitory activity and 289 actives identified with IC₅₀ of <20 μ M for ATG4B. No further follow-up is reported, although a second set of 16 compounds screened provided three actives with IC₅₀ <20 μ M.²⁶ The FRET assay of Li *et al.*¹⁷ uses a fluorescent protein construct cyan fluorescent protein-LC3-yellow fluorescent protein (CFP-LC3-YFP) very similar to our FRET-LC3. Their method involves excitation at 435 nm and monitoring of the ratio of emissions at 477 nm (increase) and the emission at 527 nm (decrease). There are no published results where the Li assay has been used in HTS thus far, but our FRET assay may be comple-

mentary, as irradiation is at the higher wavelengths (465–475 nm), and this may offer some advantage to avoid interference from fluorescent test compounds.

From the confirmed active inhibitors, aurintricarboxylic acid is known to be a promiscuous compound active in many screening regimes. It was nonetheless retested as an internal control, and its potency was consistent in multiple assay evaluations. The most potent compound found in the screen was *Z*-*L*-Phe-chloromethyl ketone with an IC₅₀ of 0.63 μ M, but it is a potent electrophile that also shows multiple activities, including inhibition of enzymes such as aldehyde dehydrogenase 1 and thiol proteases such as cruzain.²⁷

Z-*L*-Phe-Chloromethyl Ketone is an Irreversible Inhibitor and Alkylator of ATG4B

Based on its structure, it was thought that *Z*-*L*-Phe-chloromethyl ketone could be an irreversible inhibitor. Indeed, when ATG4B was pre-incubated with *Z*-*L*-Phe-chloromethyl ketone (10-fold molar excess for 5 min), enzymatic activity was completely inhibited, and concomitant mass spectrometric analysis of the ATG4B indicated only mono-alkylation of the inhibited enzyme, presumably at the active site cysteine.

CONCLUSIONS

We have developed two assays to monitor activity of the key autophagy protease enzyme ATG4B: one monitoring reaction progress with the substrate proLC3B by mass spectrometry, and the other employing a fluorescent protein construct EmGFP-LC3-YFP (FRET-LC3). The mass spectrometric analysis methodology allows direct observation of ATG4B-mediated enzymatic cleavage and inhibition thereof, and can allow characterization of inhibitors as reversible or irreversible (if by covalent alkylation) at the molecular level. The second assay, a fluorescent resonance energy transfer (FRET) assay to measure the activity of ATG4B uses a novel protein construct wherein the substrate LC3 is appended with YFP at the N-terminus and EmGFP at the C-terminus. The kinetics of cleavage of FRET-LC3 (His₆-YFP-LC3-EmGFP) were similar when followed by mass spectrometry or fluorescence, and the derived parameters, K_M , k_{cat} , and k_{cat}/K_M were similar to those determined with proLC3 as substrate. The FRET-LC3 assay was readily adapted for HTS in a 384-well format, and a preliminary screen of 4,761 known active compounds yielded a small number of actives (25) as determined by >50% inhibition at the highest tested concentration of 8 μ M. These actives were titrated in the FRET-LC3 assay to determine if they showed a concentration-dependent response (eight reconfirmed), and several were selected to derive IC₅₀ values from fresh stock. Three *bona fide* hits—hypericin (IC₅₀ ca. 10 μ M), aurintricarboxylic acid (IC₅₀ 1.25 μ M), and *Z*-*L*-Phe-chloromethyl ketone (IC₅₀ 0.63 μ M)—were confirmed as inhibitors of ATG4B. These three hits were further confirmed by titration in the LC-MS assay, with FRET-LC3 as substrate, giving IC₅₀ values of 57 μ M for hypericin, 4.4 μ M for aurintricarboxylic acid, and 0.1 μ M for *Z*-*L*-Phe-chloromethyl ketone. *Z*-*L*-Phe-chloromethyl ketone was shown to mono-alkylate Atg4B irreversibly, presumably at the

active suite cysteine, and thus may be a useful tool to study inhibition of ATG4B and its effects on autophagy in cellular models.

The FRET assay identified a low hit rate of actives (initially 0.5%, and 0.17% after confirmation of a dose response), and thus should be useful to scale up for further HTS of large chemical libraries. There was no indication that the FRET assay identified a significant number of false positives due to interference by colored or fluorescent compounds, and subsequent evaluation of a limited number of hits using the lower throughput LC-MS assay allows for definitive identification of *bona fide* inhibitors.

The LC-MS method also gives clear indication if putative inhibitors modify the ATG4B enzyme and helps to identify the mechanism of irreversible covalent inhibitors rapidly.

This assay technology should be useful in advancing the search for a potent, selective, and reversible inhibitor of ATG4B. Such an inhibitor could then be evaluated for its potential to suppress autophagy *in vivo*, as well as to provide proof of concept of ATG4B inhibition as a therapy for the control and treatment of cancer.

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DISCLOSURE STATEMENT

The authors declare they have no institutional or commercial affiliations that might pose a conflict of interest regarding the publication of this article.

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Abbreviations Used

ATG4B = autophagin 1
EmGFP = emerald green fluorescent protein
FRET = fluorescence resonance energy transfer
FRET-LC3 = yellow fluorescent protein-LC3_emerald green fluorescent protein
HTS = high throughput screening

LC3B = microtubule-associated protein 1 A/B light chain 3B
NEM = N-ethylmaleimide
RFU = relative fluorescence unit
PE = phosphatidylethanolamine
TCEP = tris-(2-carboxyethyl)phosphine
Tris = tris(hydroxymethyl)aminomethane
YFP = yellow fluorescent protein