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Inhibitor screening and enzymatic activity determination for autophagy target Atg4B using a gel electrophoresis-based assay.

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ABSTRACT

Atg4B is a cysteine hydrolase that plays a key role in autophagy. Although it has been proposed as an attractive drug target, inhibitor discovery has proven highly challenging. The absence of a standardized, easily implementable enzyme activity/inhibition assay for Atg4B most likely contributes to this situation. Therefore, three different assay types for Atg4B activity/inhibition quantification were first compared: (1) an approach using fluorogenic Atg4B-substrates, (2) an in-gel densitometric quantification assay and (3) a thermal shift protocol. The gel-based approach showed the most promising results and was validated for screening of potential Atg4B inhibitors. A set of 8 literature inhibitors was included. Remarkably, in our hands only 2 literature references were found to have measurable Atg4B affinity. Furthermore, a fragment library (n=182) was tested for Atg4B inhibition. One library member showed inhibition at high micromolar concentration and was found fit for further, fragment-based inhibitor design.

1. INTRODUCTION

Autophagy is an intracellular recycling process for bulk degradation of long-lived cytosolic proteins and organelles. The process is characterized by the formation of double-walled vesicles (autophagosomes) that sequestrate cytoplasm contents and ensure breakdown of their contents by fusing with a lysosome. Autophagy only occurs at basal levels under normal conditions, although the process is much more abundant during crucial phases of cell development (e.g., differentiation or metamorphosis). In addition, exposure to several types of cellular stress, including nutrient starvation, induces autophagy. The latter is regarded as a cellular survival mechanism. Furthermore, multiple disease types (e.g. cancer, neurodegenerative afflictions, cardiovascular disease) are known to be characterized by relevant degrees of autophagy in the tissues they affect [1,2]. To date however, the potential therapeutic benefit of pharmacologically blocking or modulating autophagic processes in disease, has been poorly assessed. Several autophagy modulators (e.g. 3-methyladenine, wortmannin) are known to act upstream of autophagosome formation and target a variety of mechanisms [3,4]. Others like bafilomycin A1 and chloroquine inhibit autophagy nonspecifically by preventing lysosomal acidification, hereby hindering fusion of lysosomes with autophagosomes [5,6]. Furthermore, the required therapeutic doses are, except for

chloroquine and its derivatives, unworkably high in translational settings. The availability of specific autophagy modulators would therefore be most desirable.

A promising target for specific autophagy inhibitors is the cysteine protease Atg4B (autophagin-1). The enzyme proteolytically activates human homologues of the Atg8 family, the so-called ubiquitin-like light chain proteins (LCPs). Atg4B cleaves proLCPs to yield the corresponding LCP-Is. The latter in turn are conjugated to phosphatidylethanolamine, forming LCP-IIs and these function as critical chaperones of autophagosome formation [7,8]. Atg4B also recycles LCP-IIs by again deconjugating them from phosphatidylethanolamine. So far, out of 4 occurring Atg4-homologues (Atg4A-D), Atg4B is the only enzyme in the autophagy cascade shown to execute these conversions in a highly efficient way, indicating its attractiveness as a target for pharmacologic autophagy modulation. It deserves mentioning however that the activities and physiological functions of the other family members remain to be fully addressed. In spite of this, very few inhibitors of Atg4B have been described to date. Furthermore, these compounds generally have micromolar potencies, and so far none of these structures have been reconfirmed as Atg4B-inhibitors by other groups. A factor that most likely hampers progress of the domain, is the lack of a robust, broadly implementable, quantitative read-out system for the enzyme's catalytic activity or alternatively, the potency of its inhibitors.

So far, only a limited number of reports have claimed successful development of Atg4B activity assays. Only two groups have demonstrated that their activity assays are also compatible with Atg4B inhibitor screening. (1) Reed *et al.* [9] have applied an indirect approach relying on a fusion protein of an LCP (LC3B) and phospholipase A_2 (PLA_2). PLA_2 is an assayable enzyme that is released in an active form by Atg4B-mediated cleavage of the fusion protein, and its activity is used as a surrogate for Atg4B-activity. (2) More recently, Young *et al.* [10,11] have published two manuscripts describing direct FRET- and fluorogenic substrate-based assays. Most interestingly, both groups independently report screening results of the commercial LOPAC1280^{TM} library. Both however obtain different, structurally orthogonal Atg4B-inhibitor "hit"-sets. A number of inherent methodological limitations can be discerned with all mentioned approaches, that might account for this discrepancy. The [LC3B-PLA_2]-based screening setup could, e.g., produce false positive results with compounds that influence PLA_2 activity or interfere with the PLA_2-substrate in the assay (the fluorescent phosphatidylcholine derivative NBD-C_6-HPC). Likewise, the tags used for FRET assessment were found to be sensitive to rapid photobleaching and photoconversion. In addition, the internally quenched fluorescent protein pairs used, showed a large degree of donor-acceptor emission overlap, making quantification difficult: spectral imaging and linear unmixing were found to be indispensable, potentially complicating the observation [12-16]. Interestingly, in Young et al.'s second published approach, the LOPAC1280^{TM} library was screened again, yielding four additional "hits" that were not identified earlier using either their own FRET-based approach or Reed's [LC3B-PLA2] screen of the same library [11].

Given our recent interest in Atg4B inhibitor discovery, we decided to initiate our activities in this domain with an experimental comparison of the potency of Atg4B inhibitors published in literature. As this effort required a reliable screening assay, preferably distinctive from the approaches used by the teams of Reed and Young, attention was first devoted to Atg4B assay development. Three approaches were evaluated that rely, respectively, on (1) fluorogenic Atg4B-substrates, (2) in-gel densitometric quantification of Atg4B-mediated cleavage of an LC3B-fusion protein and (3) thermal shift-based determination of Atg4B-affinity. Obtained results with each approach will be discussed separately in the next part.

2. RESULTS & DISCUSSION

2.1 Fluorogenic substrate-based assay

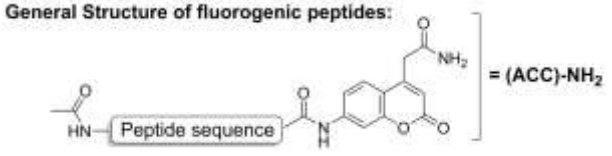
For many protease targets, peptide substrates that release a fluorophore or a chromophore upon enzymatic cleavage, have been reported. Generally available at low cost, they allow the quantification of target activity in real time, with high accuracy and sensitivity and without the need for advanced instrumentation. Several groups have so far explored the possibility of identifying fluorogenic substrates of Atg4B, mostly based on the sequence of the amino acids preceding the scissile peptide bond in Atg4B's endogenic substrate

proLC3B. Although proLC3B-derived peptides and analogues thereof with lengths varying between dimeric and nonameric were reported, no representatives were identified that are cleaved with sufficient efficiency to allow practical assessment of Atg4B activity [17,18]. Crystallographic data have been used to rationalize these remarkable results, by suggesting that only full-length proLCPs or LCP-IIs can be processed by Atg4B: in the crystal lattice, an inhibitory loop blocks the enzyme's active site, necessating conformational change prior to substrate binding [17]. Only full-length substrate proteins were hypothesized to induce this conformational change through interaction with an allosteric switch distant from the active center. In spite of this, Young et al. recently were able to produce fluorogenic dipeptide derivatives that display measurable, though still modest, substrate properties. Crucial to their approach seemed to be the presence of a so-called self-immolative linker between the scissile peptide and the fluorophore, and the nature of the *N*-terminal capping group of the peptide [11].

Our own effort in this domain consisted of evaluating significantly longer proLC3B-derived fluorogenic peptides than hitherto evaluated by other groups. We hypothesized that longer peptides could have an increased affinity for the active center: this could result in a decreased K_M-value, and hence, in an increased catalytic efficiency. Second, increased affinity could compensate for the energy penalty required to remove the inhibitory loop from the active center's entrance: in this way, the need to control substrate binding via allosteric switching, could be reduced.

Evaluated peptides consisted of a sequence of 8, 10, 12 or 16 amino acids, corresponding to the region *N*-terminal of the scissile Gly120 present in pro-LC3B, ranging from Glu105 to Gly120. (**Table 1**) These peptides were capped *C*-terminally with a fluorogenic aminocarboxycoumarine (ACC) tag. The assessment took place using similar conditions as mentioned in the fluorescence-based substrate profiling assay described by Harris *et al.* (see supporting information for full protocol) [19]. However, no cleavage of any of the tested synthetic substrates could be observed, even after prolonged incubation (20 hs) and using sufficiently high enzyme concentration (appr. 20 nM). These data did not encourage us to invest additional effort in the identification of fluorogenic peptides. Alternatively, an Atg4B activity assay was pursued that relies on in-gel densitometric quantification of activity.

Table 1: Sequences of the fluorogenic LC3B-derived peptides evaluated as potential substrates of Atg4B

	(1) Ac-YASQETFG-(ACC)-NH_2
	(2) Ac-MVYASQETFG-(ACC)-NH_2
	(3) Ac-LYMVYASQETFG-(ACC)-NH_2
	(4) Ac-EDGFLYMVYASQETFG-(ACC)-NH_2

2.2 In-gel densitometric quantification of Atg4B activity

In this approach, a fusion protein of LC3B and glutathione-S-transferase ([LC3B-GST]), is incubated with Atg4B in Tris-HCl buffer. After enzymatic processing during a fixed time window, residual fusion protein and cleavage products are separated using SDS-PAGE. Dye-staining of the spots is then followed by in-gel quantification of both the intact fusion protein and its cleavage products, using optical densitometry. The % of uncleaved [LC3B-GST] is used as a measure for Atg4B activity, and is calculated using the following formula (OD= Optical Density):

$$\%\ uncleaved\ [LC3B-GST] = \frac{OD([LC3B-GST])}{OD([LC3B-GST])+OD(GST)+OD(LC3B)}\ \text{x}\ 100\%$$

The presented methodology is characterized by low-cost, low enzyme and substrate consumption and requires limited expertise. It was optimized starting from earlier, seminal work of Li and coworkers, who used in-gel densitometric quantification of Atg4B-activity to identify [LC3B-GST] as a highly efficiently processed substrate of Atg4B (k_{cat}/K_m 8.96 x 10^4 M^{-1}/sec^{-1}) [20]. In this study, we replace the read-out

method based on Coomassie Brilliant Blue staining by detection with Oriole fluorescent gel stain. This modification in our hands produced more reproducible staining and, owing to Oriole's fluorophoric properties, could allow for quantification of smaller protein concentrations. Furthermore, we also demonstrate that the assay can be modified to screen significant numbers of potential Atg4B inhibitors within a reasonable timeframe. It deserves mentioning that Akin *et al.* recently used a comparable approach to identify an Atg4B inhibitor, but no assay conditions, validation or optimization steps were described, complicating repeatability of the reported results [21]. Next to detailed assay protocols, we describe here the results of linearity and reproducibility validation of the assay. In addition, several experimental parameters that are relevant in inhibitor screening settings are investigated: sensitivity of enzymatic activity to DMSO, the influence of pre-incubating enzyme and an inhibitor and the possibility to discriminate reversible and irreversible competitive inhibition profiles. Results of these experiments are summarized in **Figure 1**.

Three different types of detection linearity were controlled first. (1) Linearity of densitometric quantification was evaluated using GST concentrations between 0 and 18000 units, and found to be satisfactory (correlation coefficient R^2 = 0.988, **Fig. 1A**). (2) Next, the timeframe during which substrate processing occurs in a linear fashion, was determined. Atg4B concentrations of 0.3125 ng/µL and 0.625 ng/µL were assessed, using incubation times of 2-10 minutes. A concentration of 0.625 ng/µL and an incubation interval of 6 minutes was found suitable, yielding approximately 50% of substrate truncation (**Fig. 1B**). (3) Finally, substrate processing was also found to increase in a linear manner with Atg4B concentrations increasing from 0 to 0.750 ng/µL (R^2 = 0.990, **Fig. 1C**) Readout reproducibility was verified by incubating [LC3B-GST] (0.4 µg/µL) with Atg4B (0.625 ng/µL) for 6 minutes in ten parallel experiments. The intra-assay variation (samples run on the same gel) on the percentage of uncleaved substrate was determined, yielding a standard deviation of 1.7 % on individual measurements (SD) and 0.6 % on the mean (SEM) for a sample size of n = 10. The coefficient of variation (CV) was 6.1 % (**Fig. 1D**). Furthermore, the inter-assay variability (samples run on different gels) on the percentage of uncleaved substrate was evaluated. For a sample size of n = 15, an SD of 6.0 % and SEM of 1.5 % was found. The CV was 11.4 % (**Fig. 1E**). For verifying assay compatibility with inhibitor screening, Atg4B activity was first tested in the presence of DMSO concentrations ranging from 0 to 20%. Even at a concentration of 20% DMSO, enzyme activity was not significantly affected (**Fig. 1F**). Based upon these results, we decided that 5% of DMSO can be regarded as a safe standard for inhibitor screening. Next, the possibility to discriminate between irreversible and reversible competitive inhibition profiles was checked using two typical, low-affinity, covalent labels for cysteine hydrolases: iodoacetamide (IAA) or N-ethylmaleimide (NAM). Atg4B (0.625 ng/µL) was preincubated with each label separately for 15 minutes. Then, [LC3B-GST] (0.4 µg/µL) was added and incubated for 6 minutes. Complete enzyme inhibition could be observed using a 4 mM concentration of IAA or a 5 mM concentration of NAM. Tests were repeated using the same inhibitor/enzyme concentrations but without preincubation (**Fig. 1G**). Under these conditions, it can be expected that the slow, irreversible binding step of the inhibitor has not taken place yet and that mainly affinity-driven, reversible inhibition is present. In this case, NAM only yielded complete inhibition at 20 mM, while IAA still did not give complete inhibition at 40 mM. When applying increasing preincubation intervals of 0 - 20 minutes, a grossly incremental increase in enzyme inhibition was observed. (**Fig. 1H**) These results indicate that reversible, irreversible and slow tight-binding inhibition can indeed be distinguished.

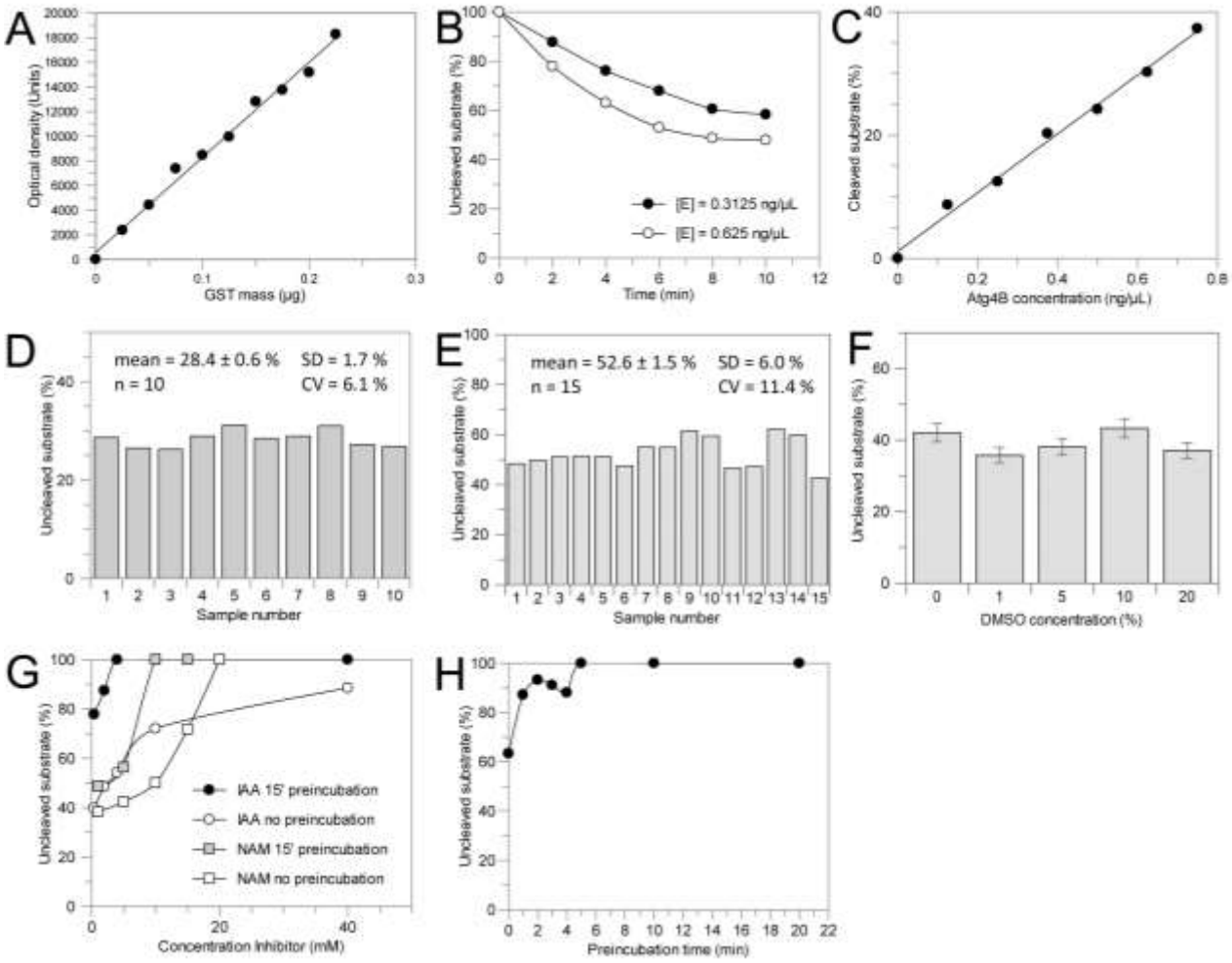


FIGURE 1. **SDS-PAGE assay development and validation.** A) From a commercial 1mg/mL GST stock solution, GST dilutions were prepared and the diluted samples were analyzed by SDS-PAGE. Corresponding optical densities were quantified. B) Atg4B concentrations of 0.3125 ng/µL and 0.625 ng/µL were prepared and added to a 0.4 µg/µL LC3B-GST solution. Mixtures were incubated during 0 - 10 minutes and were resolved by SDS-PAGE. The percentage of uncleaved substrate was quantified and plotted against the incubation time. C) Atg4B concentrations between 0 - 0.8 ng/µL were prepared and added to a 0.4 µg/µL LC3B-GST solution. The mixture was resolved by SDS-PAGE and the percentage of cleaved substrate was plotted against the enzyme concentration. D) A 0.4 µg/µL LC3B-GST solution was prepared in tenfold and coincubated with a 0.625 ng/µL Atg4B dilution as mentioned earlier. Uncleaved substrate percentages were assessed. E) Uncleaved substrate percentages obtained from 15 samples run on 15 different gels were plotted. F) Five different DMSO concentrations ranging 0 - 20% DMSO (in assay) were prepared and added to a diluted LC3B-GST solution. After addition of a diluted Atg4B solution, mixtures were analyzed by SDS-PAGE and optical densities were quantified. Error bars are based upon the % CV of panel 1D. F) IAA and NAM dilutions were prepared between respectively 0 - 40 and 0 - 20 mM. Preincubation of Atg4B samples with inhibitor was either executed during 15 minutes or not executed at all. Mixtures were resolved by SDS-PAGE and uncleaved substrate was quantified. G) A 4 mM IAA concentration was prepared and added to a 0.625 ng/µL Atg4B dilution. Preincubation was executed during 0 - 20 min and the amount of uncleaved substrate was measured. Validation tests were executed as a single experiment as representative.

2.3 Thermal shift-based determination of Atg4B-affinity

The thermal shift assay (TSA) technique is based on protein denaturation and unfolding by heating. It is monitored by addition of a fluorescent dye that adheres to both hydrophilic and hydrophobic regions of the protein. When protein unfolding initiates, the hydrophobic region is being exposed, rendering an increased fluorescence signal [22]. TSA relies on determination of the so-called transition temperature T_m (also known as unfolding temperature) at which protein denaturation occurs. In the presence of an inhibitor, change in unfolding temperature (ΔT_m) can be used as a measure for ligand-binding affinity: a rise in T_m can be observed when the ligand interacts and complexes to the protein, making unfolding more difficult. Contrary to the two former assay types, TSA on enzymatic targets does not offer information on catalytic activity status. This however also implies that verifying whether competitive binding or allosteric inhibition is present, can be challenging. Nonetheless, the technique's sensitivity and the fact that it requires only basic expertise, makes it an interesting approach [23]. It has not been applied earlier to Atg4B inhibitors.

Initial effort therefore focused on devicing a workable TSA protocol for Atg4B. Several enzyme

concentrations ranging between 0.05 and 0.4 µg/µL were added to 0.001% SYPRO-orange in TRIS-buffer (50 mM, pH = 7.4) in a 96-well microtiter plate. A temperature progress curve of +1 °C/min was applied to the samples over a timelapse of 70 minutes (detailed protocols can be retrieved in the Supporting Information part). After 70 minutes, a sigmoidal melting curve was obtained and T_m was assessed. Steepest melting curves were obtained for 0.2 and 0.4 µg/µL enzyme concentrations. However, the obtained baseline was too high for optimal curve fitting. An enzyme concentration of 0.3 or 0.4 µg/µL was chosen and further optimization of the experimental protocol was pursued to overcome the baseline problem.

Next, the impact of dithiothreitol (2 mM) and Tween 20 (0.1%) in the TSA medium were tested. The effect on obtained baselines for each of the individual melting curves, was found to be insufficient to allow satisfactory fitting. Continuing, buffer pH was modified to either 7.0 or 7.2. Although the melting curve profile obtained with an enzyme concentration of 0.4 mg/mL and a buffer pH of 7.2 were optimal among the evaluated conditions (Tm_{Atg4B} = 46.6 °C), the quality-of-fit was still deemed unsufficient to allow reliable inhibitor screening. In addition, reproducibility of the methodology was found to be limited and its outcome to be heavily depending on the presence of eventual interfering factors like air bubbles or dust particles in the medium. Finally, the relatively high enzyme consumption of this technique makes it less appealing for screening of large numbers of compounds.

2.4 Assay selection

As no suitable fluorogenic Atg4B substrates could be identified, this approach was deemed unamenable to further development. Analogously, the TSA-based model was found not to be sufficiently performant and robust to allow for inhibitor screening. On the other hand, the in-gel densitometric quantification methodology was shown to combine satisfactory linearity and reproducibility and to offer potential for Atg4B inhibitor screening. Based on all these findings, the latter methodology was selected for all further experiments.

2.5 Screening of literature Atg4B inhibitors

First, a set of seven literature Atg4B inhibitors was assessed in the SDS-PAGE-based assay. For maximal clarity, conditions of the different assay types reported in literature, are listed in Table 2 and inhibitory screening results obtained under our conditions, were compared with published compound potencies (Table 3). To apply the Atg4B-activity assay for inhibitor screening, [LC3B-GST] was incubated with a test compound and Atg4B in buffer solution for 6 minutes. The experiment was then stopped and the incubation mixture was transferred onto gel. Electrophoretic separation was followed by staining and densitometry as described earlier. The percentage of uncleaved substrate was then used to express the amount of enzyme inhibition as percent inhibition. Herefore, we set the percentage of uncleaved substrate in the control sample as zero percent of inhibition, while 100% of uncleaved substrate corresponded to complete enzyme inhibition. For this conversion, we applied the following formula:

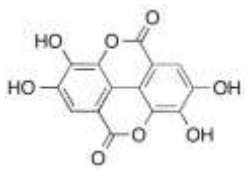
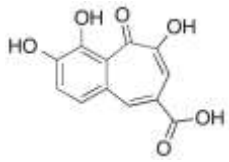
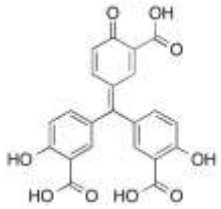
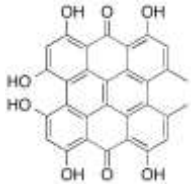
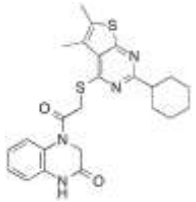
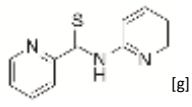
$$enzyme\ inhibition\ (\%) = \frac{\%\ uncleaved([LC3B{-}GST])_{test\ sample} - \%\ uncleaved([LC3B{-}GST])_{control\ sample}}{100\ \% - \%\ uncleaved([LC3B{-}GST])_{control\ sample}} \text{ x } 100\%$$

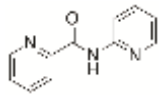
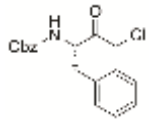
Table 2. Comparison of assay conditions.

Parameter	SDS-PAGE assay Cleenewerck (this study)	Indirect assay (phopholipase A2 activity) Reed et al. [9]	FRET-based assay Young et al. [10]	SDS-PAGE assay Akin et al. [21]
Substrate concentration in assay (nM)	2.4 x 10^3	25	3.5 x 10^3	-[(a)]

Enzyme concentration in assay (nM)	0.0142	0.1	48	-
Compound concentration in assay (μM)	500	≤100	7	-
Buffer type	TRIS	TRIS	TRIS	-
Incubation time (min)	6	60	40	-
[a]"-" means not reported in this article				

Table 3. Benchmark screening of reported Atg4B inhibitors and comparison with published data

	Structure	SDS-PAGE assay Cleenewerck % inhibition at 500 μM[a]	Indirect assay (phopholipase A2 activity) Reed et al. IC_{50} (μM) [9]	FRET-based assay Young et al. IC_{50} (μM) [10]	SDS-PAGE assay Akin et al. IC_{50} (μM) [21]
1		N.I.[b]	5.5	N.R.[c]	N.R.
2		52.3 ± 3.7 %[d] (n = 4)	2.3	N.R.	N.R.
3		No substrate cleavage IC_{50} = ~38 μM[e] (n = 1)	N.R.	1.3	N.R.
4		N.A[f]	N.R.	57	N.R.
5		N.I.	0.316	N.R.	N.R.
6	 [g]	N.I.	N.R.	N.R.	51

7		N.I.	N.R.	N.R.	N.R.
8		N.I.	N.R.	0.63	N.R.

[(a)] Percentage Atg4B inhibition at a 500 µM compound concentration
[(b)] "N.I.": No Inhibition at 500 µM compound concentration
[(c)] "N.R.": Compound was not reported in this publication
[(d)] Percentage inhibition is represented as mean ± standard error of the mean
[(e)] Estimated IC_{50}-value from concentration dependence in single experiment
[(f)] "N.A.": Not Analyzable: hypericin smeared out on gel during electrophoretic analysis
[(g)] Reported as compound "NSC185058"

All compounds in **Table 3** for which Atg4B-inhibition was observed, showed reversible inhibition since no significant differences in enzyme inhibition could be reported by varying preincubation times. Considering the sometimes significant differences in enzyme, substrate and compound concentrations between our own and the literature assays, comparison of compound potency remains difficult. Another factor that might complicate comparison, is the enzyme source used. While we used a recombinant HsAtg4B enzyme expressed in E. coli, other groups have used different sources, His-tagged enzyme or do not even mention the origin of the used enzyme batch [9,10]. Nonetheless, even when taking into account these considerations, the data in **Table 3** point out that caution might be advisable when studying published data on Atg4B inhibitors, or in cases where a compound is selected for use in biological studies. When taking a closer look at the chemical characteristics of reported reference hit structures, bulky polyphenolics are clearly represented. These compound classes are known for their broad nonspecific activity against a wide range of biological targets. Hence, this knowledge should be taken into account when choosing candidates for hit-to-lead inhibitor optimization. Isolated hit structures detected in the fluorescence-based assay were structure **1** and **5**. Hypericine (**4**), an active polyphenolic molecule reported by the FRET-based assay, could not be confirmed by us as an Atg4B inhibitor due to erratic behavior ("smearing") of the compound during electrophoresis. In this way, the intrinsic fluorescence of hypericin rendered reliable densitometric protein quantification impossible. Hypericine has also not been reported as an Atg4B inhibitor by other literature sources. Intrinsic fluorescence has been reported for structures **1** and **4**, and seems likely for structure **5**, potentially compromising fluorescence-based read-outs [24,25]. This possibility is however not verified in the corresponding manuscripts (for example by measuring and correcting for background fluorescence of these molecules). Furthermore, discrepancies in hit detection and compound activity between the indirect (PLA_2-activity) assay and our gel-based assay could be explained by the difference in method sensitivity, as the indirect assay allows for detection of smaller substrate concentrations. However, this suggests interference with PLA_2-activity could easily generate false positive hits. Interference at the compound concentration during the assay was assessed for structures **1** and **2**, and a decrease of 10-15% in PLA_2-activity was reported compared to blank measurements [9]. No counterscreen results were published for structure **5** and one hit compound (not determined in this work) even showed complete abrogation of PLA_2-activity. These results leave open the possibility of an overestimation of compound potency for Atg4B inhibitors reported for the indirect screening approach. Z-L-Phe-CMK (structure **8**) and two related chloromethyl ketones were also reported as covalent inhibitors by the group of Young et al., relying on their FRET-based and/or fluorogenic substrate methodologies. Nonetheless, as this molecule contains an electrophilic chloromethylketone group, the possibility of non-specific covalent bond formation with nucleophilic residues cannot be excluded. Moreover, based on Atg4B's strict preference for a P1-glycine-residue, specific covalent interaction of these compounds with Atg4B's active site cysteine seems unlikely. NSC185058 (structure **6**) reported as a modestly active compound (IC_{50} = 51 µM) by Akin *et al.* showed good results in phenotypic autophagy inhibition screening [21]. However, in our hands, no measurable affinity was observed for this compound. Furthermore, NSC185058 and the corresponding amide (structure **7**) were tested in our SDS-PAGE screening assay and could not be reconfirmed as inhibitors at compound concentrations as high as 500 µM in assay. Aurintricarboxylic acid (structure **3**) was, together with structure

2 the only reference structure that was reconfirmed as an active compound by our SDS-PAGE screening assay, with an estimated IC_{50} of 45 µM, compared to an IC_{50} of 1,3 µM reported by Young *et al*. (see supporting information for IC_{50}-determination and SDS-PAGE panel of active compounds) [10]. However, as aurintricarboxylic acid is known for its ability to potently inhibit different types of topoisomerases and nucleases by polymerizing in aqueous solution and forming a stable free radical, it inhibits cell functionality at a very fundamental level [26,27]. Therefore, it is seen as an unattractive compound for hit-to-lead optimization steps. Structure **2**, a compound containing a benzotropolone core structure, was mentioned as hit structure in the fluorescence-based assay. As it comprises multiple phenolic groups and enzyme inhibition was only observed at a compound concentration of 500 µM in assay, interaction with the enzyme is likely to occur in a nonspecific manner. In conclusion, no published hits could be used as a good reference for inhibitor screening validation in our hands.

2.6 MSAS approach to Atg4B inhibitor detection and optimization

A 182-member Substrate Activity Screening (SAS) library of fragment-sized *N*-7-amino-4-methylcoumarines was synthesized and evaluated using the Modified Structure Activity Screening (MSAS) protocol reported by Gladysz *et al.*[28]. Chemical structures and characterization data for all library members can be retrieved in the supporting information file associated with this manuscript. According to the MSAS-protocol, library members were first screened as potential inhibitors of Atg4B, relying on the in-gel densitometric quantification of Atg4B activity. One fragment, S069 was identified and confirmed *in quadruplo* as a weak, competitive Atg4B inhibiting fragment, causing 83.4 ± 3.0 % inhibition of enzymatic activity at 500 µM compound concentration. (**Figure 2**) Nonetheless, satisfactory sigmoidal fitting of inhibitory activity *vs.* compound concentration to determine an IC_{50}-value, was found to be impossible for S069. As imposed by the MSAS-protocol, the identified "hit" was then screened as a substrate of Atg4B-mediated hydrolysis by measuring release of 7-amino-4-methylcoumarin. No cleavage by Atg4B could be observed. Our own previous results have clearly demonstrated that inhibitory potency and substrate properties are not necessarily correlated for SAS-"hits"[28]. Additionally, sluggish processing of small substrates by Atg4B (as also observed with the fluorogenic peptides, *cfr. supra*) could be invoked to explain the absence of substrate properties of S069. Summarizing, these data suggest that the 2-methylaminopyrazine part of S069, could be used as an S1-region binding fragment with potential value for fragment-based Atg4B inhibitor discovery.

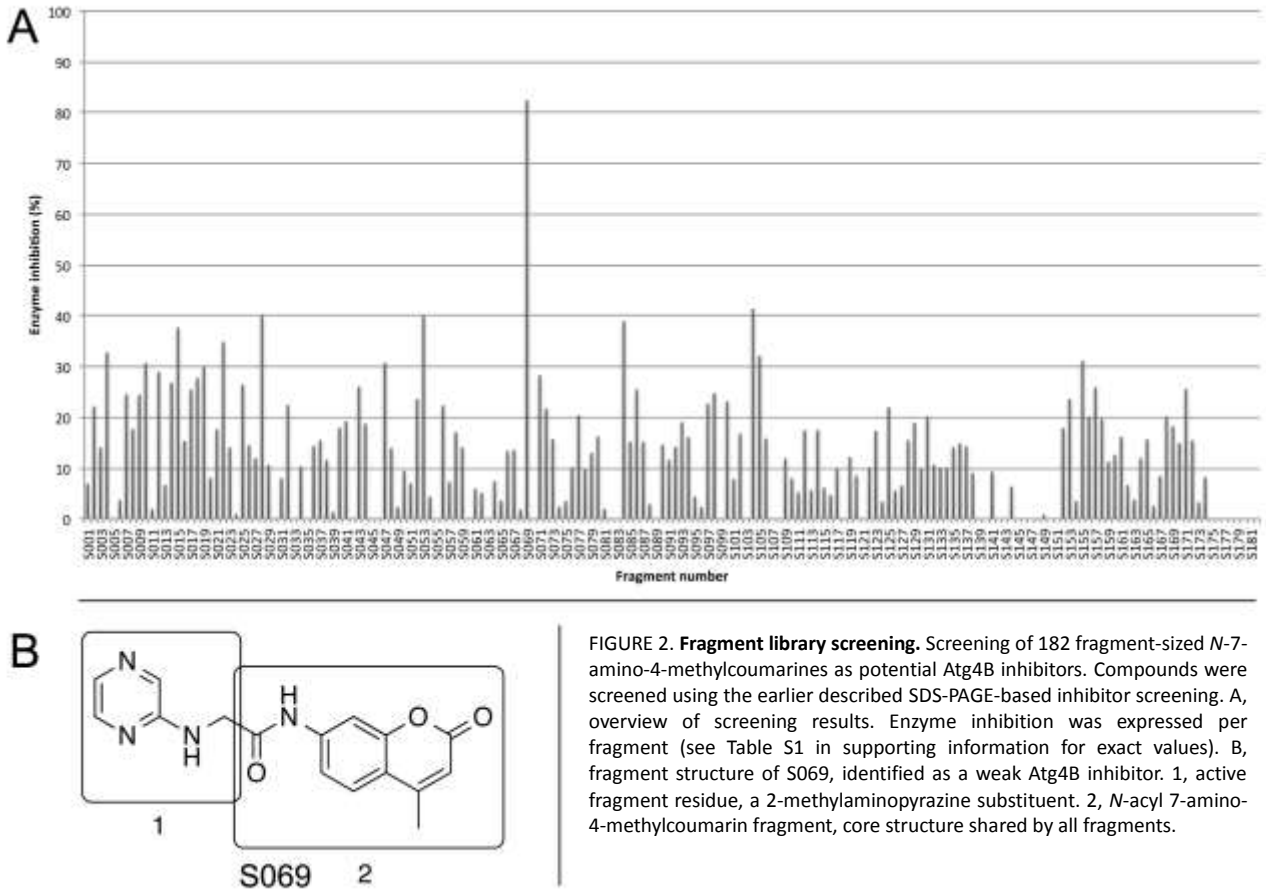


FIGURE 2. **Fragment library screening.** Screening of 182 fragment-sized *N*-7-amino-4-methylcoumarines as potential Atg4B inhibitors. Compounds were screened using the earlier described SDS-PAGE-based inhibitor screening. A, overview of screening results. Enzyme inhibition was expressed per fragment (see Table S1 in supporting information for exact values). B, fragment structure of S069, identified as a weak Atg4B inhibitor. 1, active fragment residue, a 2-methylaminopyrazine substituent. 2, *N*-acyl 7-amino-4-methylcoumarin fragment, core structure shared by all fragments.

3. CONCLUSIONS

Different methodologies for quantitative Atg4B activity/inhibition readout have been investigated in recent literature. However, none of these methodologies have been instrumental for the identification of a reliable, standardized experimental protocol. This is most likely one of the factors contributing to the limited numbers of Atg4B inhibitors reported so far in the literature. In response to current deficiencies, we have investigated an SDS-PAGE-based screening assay and methodologies relying on fluorogenic substrates and thermal shift analysis, of which the gel-based method was found optimal. Comparable orders of magnitude were found for the inter- and intra-assay variabilities of the assay, indicating its robustness and potential for Atg4B inhibitor screening. A set of reference literature inhibitors and a library of 182 fragment-sized molecules were subsequently screened. Two reference compounds, structure **2** and **3**, and one fragment, S069, showed modest inhibitory activity in our hands. Altogether, our own screening data call for a cautious interpretation of inhibitory potencies of Atg4B-inhibitors reported in literature. Although we did not perform a side-by-side comparison of the sensitivity of our own approach and other reported types, it might be anticipated that the intrinsic sensitivity of a gel-based read-out is lower than for the published procedures. This limitation should however not have an influence on the potency ranges that were determined under our conditions.

4. SUPPORTING INFORMATION

Detailed experimental protocols for all assay types reported are provided as Supporting Information. In addition, structural information and characterization data for all members of the fragment library are provided.

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