Synthesis and Structural Optimization of ATG4B Inhibitors for the Attenuation of Treatment-Induced Autophagy in Glioblastoma

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ABSTRACT: Glioblastoma, a prevalent malignant CNS tumor, presents a therapeutic challenge because of resistance to standard treatments including radiation therapy and temozolomide. Both modalities induce autophagy, paradoxically promoting tumor survival. The cysteine protease ATG4B is implicated in this cellular process, highlighting the enzyme as a viable therapeutic target for glioblastoma. We have developed streamlined syntheses for ATG4B inhibitor NSC185058, its derivatives, and fluorogenic ATG4B substrate im-FG-PABA-AMC. We leveraged these findings to rapidly identify new compound MJO445, which demonstrates markedly greater potency biochemically and in cells.

Glioblastoma (GBM) is a common and malignant tumor of the central nervous system (CNS) carrying an extremely poor prognosis1-2 and a median survival of less than 2 years.3 Approximately 23,000 adults were diagnosed with a primary brain or other nervous system cancer in 2015 in the United States, with GBM accounting for over half of these cases.4 Treatment of GBM is complicated by both intrinsic and acquired resistance to the standard of care, including radiation therapy (RT) and temozolomide (TMZ).5 RT and TMZ both induce autophagy, a degradative process which maintains homeostasis in the context of cellular stress.6 TMZ was shown to induce autophagy through the action of O6-methylguanine-DNA methyltransferase (MGMT), the DNA mismatch repair system (MMR), and RadS1-mediated homologous recombination.7 Radiation can cause an autophagic response by the PI3K/AKT/mTOR pathway, reactive oxygen species (ROS), and MST4 kinase-ATG4B signaling.8-11 Through these processes, RT and TMZ indirectly and paradoxically promote tumor cell survival.12 Cysteine protease ATG4B plays a critical role in the formation of autophagosomes by cleaving autophagy-related protein 8 (ATG8) to reveal a glycine residue necessary for conjugation to plasma membrane-bound phosphatidylethanolamine (PE, Figure 1).13 The suppression of autophagy by inhibiting ATG4B is a feasible strategy to augment the efficacy of GBM.14

The central role of ATG4B in autophagy is well described,15 and ATG4B has been recognized as an attractive therapeutic target for cancers.16-17 Inhibition of ATG4B by small molecule inhibitors has been demonstrated in multiple contexts,17 including GBM,11 colorectal cancer,18 and osteosarcoma.19 As we (Cheng) have previously demonstrated,11 shRNA knockdown of endogenous ATG4B in glioma stem-like cell (GSC) 83 and 1123 cells results in decreased sphere-forming frequency and cell proliferation. Furthermore, we demonstrated that ATG4B knockdown cells exhibit reduced tumorigenicity and are associated with prolonged animal survival when injected into the brains of athymic nude mice. Further, treatment with ATG4B inhibitor NSC185058 at high concentrations13 (1, Figure 2) attenuates the tumor-initiating ability of GSCs and sensitizes GBM to RT.11 Immunoblot (IB) analyses demonstrate that 1 significantly reduces the conversion of LC3-I to the lipided, autophagosome-associated form known as LC3-II and attenuates degradation of the autophagy substrate p62/SQSTM1. Treatment with 1 sensitizes GSCs to RT by suppressing lipidation of LC3-I isoform LC3B and increasing the cytosolic concentration of substrate p62.11

Figure 1: Simplified depiction of the elongation phase of cellular autophagy, highlighting the roles of ATG4 and ATG8.
Other small molecule compounds exhibiting modest inhibitory activity against ATG4B beyond 1 have been identified (Figure 2). This group includes highly reactive \( \alpha \)-haloketones, radical precursors in tricarboxylic acid (ATA), as well as polyphenolic compounds (i.e., 201508 and UAMC-2526). Additionally, recent studies have revealed natural product azalomycin F4a and thiocetate LV-320 as potent ATG4B inhibitors. Demonstrating the growing interest in this field, in 2019, Agrotis and Ketteler identified several grams of material. The prohibitive pricing of this compound or its analogues. Commercial sources of 1 remain scarce, and the material is prohibitively expensive. For instance, as of November 2023, Cayman Chemical prices 1 mg of 1 at $55, which negatively impacts the viability of mouse model studies of this compound that often require several grams of material. The prohibitive pricing of 1 presumably stems from a dearth of known synthetic routes to prepare this particular compound or its analogues. Traditionally, 1 has been prepared by heating 2-aminopyridine, elemental sulfur at 180°C for up to two days, yielding the compound of interest in 22% yield (Scheme 1). These harsh conditions and protracted reaction times are not amenable for diversification.

To support a variety of different cellular and animal experiments, we designed a convergent approach to the synthesis of 1 and its derivatives employing the corresponding isothiocyanate and 2-metallopypyrimidine. Lithiation of the ortho position of pyridine can be facilitated by complex formation with a Lewis acid such as boron trifluoride (BF₃). Another method for preparing 2-lithiopyridine involves lithium halogen (Li-X) exchange with 2-bromopyridine; however, 2-lithiopyridine is not stable at –78°C, and must be formed and maintained as low as –98°C. Early preparations of 1 (Scheme 1) in our laboratories employed the latter Li-X exchange strategy, affording 1 in roughly 50% yield. Magnesium-halogen exchange was found subsequently to perform better at scale. Towards this end, 2-bromopyridine was refluxed with isopropylmagnesium chloride for several hours before cooling to 22°C for reaction with the corresponding isothiocyanate. This process yields grams of the coveted material in hours (70%), employing economical and readily available reagents (Scheme 1). A third approach to the synthesis of 1 and its derivatives was developed as synthetic focus shifted to diversity generation. Leveraging the kinetics of Li-X exchange, \( n \)-BuLi was added directly to a –78°C ethereal solution containing both the heteroaryl bromide and the isothiocyanate, affording the desired product upon aqueous workup and chromatographic isolation.

Figure 2: Some compounds known to demonstrate inhibitory capacity against ATG4B.

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ingly, the activity of compound 1 compared to a literature cellular potency of ~50 μM, compared to a literature route to this compound is the instability of various intermediates in the previous approach.

In addition, we employed computational modeling and molecular docking studies to gain insights into the possible binding orientation(s) of proposed compounds in the active site and attempted to optimize the potential for π-stacking with the Trp142 residue in the ATG4B active site and novel compounds (see below). The various aromatic rings in the synthesized thioamides were selected to align favorably with the indole ring of Trp142, thus promoting strong and specific π-stacking interactions.

Our in vitro investigation commenced with a reported fluorometric ATG4B enzymatic assay. Activated recombinant ATG4B proteins prepared as described before were incubated with fluorescent peptide substrate (pim-FG-PABA-AMC, 5, Scheme 2) and inhibitor at various concentrations. The fluorogenic peptide was prepared by a modified route of the literature report necessitated by the instability of various intermediates in the previous approach (Scheme 2). Our revised synthesis cleanly and reproducibly afforded the desired compound in 7 steps from Boc-phenylalanine. The key deviation from the literature route to this compound is the formation of the pimelic amide through the aminolysis of pimelic anhydride. Briefly, known compound 2 was coupled with 4-aminobenzyl alcohol to afford 3. The corresponding benzyl mesylate was displaced with 7-hydroxycoumarin 6 to provide 4 in 54% yield. Finally, the N-Boc-phenylalanine was deprotected to unmask a primary amine which can add into pimelic acid anhydride to afford fluorogenic substrate 5. This strategic modification decreases step count and avoids a problematic deprotection of an allyl ester in the published synthesis.

**Scheme 1** The traditional synthetic approach towards 1 compared to methods developed in our laboratories.

After this brief optimization activity, we next sought to evaluate our hypothesis of π-stacking between compound 1 and Trp142 in ATG4B as a potential beneficial interaction. To explore the chemical space adjacent to 1, we initiated a preliminary survey through the parallel synthesis of several thioamides utilizing the 3rd-generation synthetic approach developed above. In addition, we employed computational modeling and molecular docking studies to gain insights into the possible binding orientation(s) of proposed compounds in the active site and attempted to optimize the potential for π-stacking with the Trp142 residue in the ATG4B active site and novel compounds (see below). The various aromatic rings in the synthesized thioamides were selected to align favorably with the indole ring of Trp142, thus promoting strong and specific π-stacking interactions.

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**Scheme 2** Modified synthesis of pim-FG-PABA-AMC (5)

In our evaluation, compound 1 exhibited an in vitro IC<sub>50</sub> of >100 μM, compared to a literature cellular potency of ~50 μM. Our newly measured in vitro potency is not in contrast to the known inhibitory activity of 1 as measured by autophagy in GSC cells, which may be aided by feedback loops and other complexities of the cellular milieu. Despite the promising in vitro activity of 1, our studies are the first to quantify the in vitro activity of 1 towards ATG4B to date. Gratifyingly, the activity of new compound 7 was qualitatively and quantitatively superior to that of 1 (IC<sub>50</sub> = 12.7 μM, Figure 4).

**Figure 3**: Synthetic strategy towards novel towards derivatives of 1.

**Figure 4**: Head-to-head in vitro potency comparison of lead compound and 7.

With this ~10 fold improvement through slight structural modification, we then sought out to demonstrate the effects of 1 and 7 on the attenuation of GSC growth and self-renewal, as well as the ability of these compounds to inhibit autophagic activity in GSC cells. Thus, in GSC cells with autophagic activity, compared to compound 1 (NSC), compound 7 (MJO445) displayed a significantly improved effects on inhibiting ATG4B-induced conversion of LC3-I ( uncleaved human ATG8) to cleaved and lipidated LC3-II and the degradation of autophagy substrate p62/SQSTM1 p62 (an autophagosomal cargo protein that targets other proteins for autophagy) (Figure 5A,B). Notably, compound 7 effectively inhibited LC3 conversion and promoted accumulation of p62 even at greatly diminished concentrations. Furthermore, compound 7 was found to significantly decrease relative cell viability in GSC83 as well as GSC576 cells when compared to 1 as a positive control (Figure...
Sphere formation essays were then performed to investigate the impact of compound 7 and compound one of the ability of GSC83 and GSC576 cells to form spheres as a metric of autophagic activity (Figure 5E,F). Again, compound 7 demonstrated superior potency over 1 for the inhibition of sphere-forming activity of both GSC cell lines.

We performed molecular docking studies to gain insight into the improved potency of 7 compared to 1 (Figure 6). We focused on the putative NSC binding site containing Asp278 and His280, two residues of the canonical cysteine protease catalytic triad. An inhibitor engaging this binding pocket present in the inactive form of ATG4B, with the active site masked by a loop comprising residues 259 through 262, could prevent substrate binding and cleavage. The structure of inactive ATG4B (PDB: 2CY7) with overlaid binding poses of 1 and 7 is depicted in Figure 6A. Our docked pose of 1 in this pocket was similar to those previously reported with a similarly modest docking score. In our model, 1 participates in a single hydrogen bonding interaction with His264 and otherwise engages with the binding pocket through van der Waals interactions (Figure 6B). By contrast, 7 is positioned deeper in the pocket, with hydrogen bonds with Asp278, Cys306, and Thr10 as well as a π-stacking interaction with His264 (Figure 6C). This more favorable binding pose, including direct engagement with key catalytic residue Asp278, could account for the superior potency of 7 as compared to 1.
In summary, we have developed efficient syntheses of ATG4B inhibitor NSC185058 (1), new derivatives based on this compound, and fluorogenic ATG4B substrate pim-FG-PABA-AMC. We leveraged these insights towards the design, synthesis, and evaluation of a focused set of NSC185058 derivatives. Biochemical and cellular evaluation of new derivative 7 identified it as markedly enhanced potency against ATG4B as a promoter of cellular autophagy. These findings will expedite further investigations of ATG4B inhibitors as therapy potentiators in the context of glioblastoma. Our postulated binding mode of NSC185058 is supported by the expedient development of 7, which displays greater potency in biochemical and cellular models.

Figure 6: Computational studies of ATG4B. (A) Structure of ATG4B (PDB: 2CY7) with 1 and 7 docked. (B) Docked pose of 1 (orange). (C) Docked pose of 7 (blue). Orange dashed lines represent hydrogen bonds, and light blue dashed line represent π-stacking interactions.

ASSOCIATED CONTENT
Supporting Information
The Supporting Information is available free of charge on the ACS Publications website.

Additional in vitro results, experimental details for in vitro and cellular experiments, modeling protocols, compound synthesis, and characterization.

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Notes
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ABBREVIATIONS
AKT, protein kinase B (aktivtät); ATG, Autophagy Related Protein; CNS, Central Nervous System; GBM, Glioblastoma Multiforme; GSC, Glioma Stem-like Cell; IB, Immunoblot; LC3-I, Microtubule-associated protein 1A/1B-light chain 3; LC3-II, Lipidated form of LC3-I; MGMT, O6-methylguanine-DNA methyltransferase; MMR, DNA mismatch repair system; MST4, Mammanlike STE20-like Cell Death Peptidase; PDB, Protein Data Bank; PE, Phosphatidylethanolamine; PI3K, Phosphatidylinositol 3-kinase; ROS, Reactive Oxygen Species; RT, Radiation Therapy; SQSTM1, Sequestosome 1; TMZ, Temozolomide

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