Exploring Arylidene-Indolinone Ligands of Autophagy Proteins LC3B and GABARAP

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ABSTRACT: Herein we report the first structure-activity studies of compound **GW5074** which has demonstrated binding affinity to autophagy-related proteins LC3B and GABARAP. The literature has conflicting information on the binding affinities of this compound to LC3B and GABARAP, and there is some debate regarding its use as a component of autophagy-targeting chimeras (AUTACs) or autophagosome-tethering chimeras (ATTECs). We developed an AlphaScreen binding assay to compare the potencies of these compounds for inhibiting binding of known peptide ligands to LC3B and GABARAP. 38 analogs were synthesized and tested against both proteins. Inhibitory potencies were found to be mid to high micromolar, and 2D-NMR data revealed the binding site as hydrophobic pocket 1, where the native peptide ligands bind with an aromatic side chain. Our results suggest that **GW5074** does bind LC3B and GABARAP in the micromolar range, but it may not be a good candidate for potent autophagy inhibition. These affinities could support further exploration in targeted protein degradation, but only if off-target effects can be appropriately controlled for.

Macroautophagy is a process by which intracellular cargo, including proteins, protein aggerates, and organelles, is recruited to and degraded by the lysosome. Macroautophagy (referred to here as simply "autophagy") is essential for maintaining cellular homeostasis. In advanced cancers, autophagy contributes to disease progression through multiple mechanisms including immune evasion,[1,2] metabolic adaptation,[3] and accelerated metastasis.[4,5] Additionally, autophagy is typically upregulated in response to DNA-damaging agents and genetic studies have shown that inhibiting autophagy re-sensitizes late-stage cancers to cisplatin treatment.[6–8] Therefore, autophagy inhibitors are a promising area for novel combination therapies.[9] Unfortunately, commonly used autophagy inhibitors such as hydroxychloroquine are not specific to autophagy, they have many side effects, and they can have dose-limiting toxicity.[10,11] Targeting protein-protein interactions involved in autophagy is a good strategy for developing selective inhibitors.[9] Specifically, LC3/GABARAP family proteins mediate protein-protein interactions at every step of the autophagy pathway.[12] Genetic knockdowns and knockouts of LC3/GABARAP proteins inhibit autophagy selectively, demonstrating that this family of autophagy proteins is a promising drug target.[13–16] At high concentrations, ligands for LC3/GABARAP proteins should be effective autophagy inhibitors, but at lower concentrations they could also be used as components of targeted degrader compounds. Such degrader compounds have been termed autophagy-targeted chimeras (AUTACs) or autophagosome-tethering chimeras (ATTECs), among other terms. These compounds tether proteins or cellular components of interest to the autophagosome, leading to the degradation of the liganded protein, organelle, or cellular component.[17–29]

GW5074 (Fig. 1, hereafter called compound **1**) was one of the earliest small molecules reported to bind LC3B, the most well-studied LC3/GABARAP protein. It was discovered in 2019 by Li and coworkers as a compound that induced degradation of aggregated 72Q-huntingtin.[19] Since that report, **1** has been used for several applications. For example, in 2021, Li and coworkers attached **1** to a lipid droplet-binding compound to produce a chimeric compound that degraded lipid droplets.[20] Also in 2021, Fu et. al attached **1** to the BRD4 ligand JQ1,

producing a chimeric compound that degraded BRD4.[17] More recently, Gu and coworkers reported an ATTEC that incorporated **1** to degrade the protein PCSK9 as a potential atherosclerosis therapy.[22] Similarly, Dong and coworkers recently described an ATTEC using **1** to degrade PDEδ, an emerging target for pancreatic cancer therapy.[29]

These findings suggested that **1** could be used in selective and modular fashion to bind LC3/GABARAP proteins and induce the degradation of other proteins and cellular components via autophagy. However, other work has questioned the mechanism of degradation for ATTECs incorporating **1**. Recent findings by Winter, Waldmann, and coworkers found that **1** is a selective, covalent ligand of the E3 ligase DCAF11.[30] They further showed that its ability to direct targeted protein degradation is dependent on the proteasome and not lysosomal function, consistent with DCAF11 engagement in cells. Similarly, a recent preprint by Hong, Wang, Tian, Li, and coworkers found that a heterobifunctional molecule of JQ1 and a **1** derivative degraded BRD4 through recruitment of the E3 complex CRL4 which contains DCAF11.[31] Thus, degradation by chimeric compounds incorporating **1** may not occur via an autophagy-dependent mechanism. Notably, while the report from Winter, Waldmann, and coworkers tested many analogs of **1** for DCAF11 engagement, none were tested for LC3/GABARAP binding.

Compound **1** was derived from a screen of known bioactive compounds and it has not undergone any reported optimization or structure-activity relationship studies for LC3/GABARAP binding. This lack of information contributes to the questions surrounding the ability of **1** to bind LC3/GABARAP proteins. Even the published data on **1** binding to LC3/GABARAP proteins have been contradictory (Fig. 1). The binding affinity of **1** for recombinant LC3B was reported in different papers as 0.468 μM (measured by small molecule microarray with a scanning oblique-incidence reflectivity difference microscope),[19] 8.9 μM (measured by surface plasmon resonance),[17] and greater than 200 μM (measured by 2D-NMR titration).[32] Overall, these contradictory findings called into question the actual binding affinity of **1** for LC3B and other family members. A recent report by Knapp, Rogov, and coworkers more directly addressed this question using competition fluorescence polarization, NMR titration, and NanoBRET assays. They found that **1** binds weakly to GABARAP-L2 and has weak, if any, binding to LC3B.[32] In that work, the authors suggested that these activities were too weak to account for the compound's ability to mediate targeted degradation.

Concurrently with these more recent studies, we took up the question of whether **1** binds to recombinant LC3B and GABARAP and whether it inhibits their interactions with representative ligands derived from native binding partners. We also sought to uncover structure-activity relationships for these inhibitory activities. We began by developing more reliable binding assays for recombinantly expressed LC3B and GABARAP. We first tried to test **1** in competitive fluorescence polarization assays that were previously developed in the Kritzer lab.[33] However, the compounds had background fluorescence which interfered with the assay. As a convenient alternative to biolayer interferometry assays developed by us and others,[33–35] we developed a solution-phase competition assay using AlphaScreen. The AlphaScreen assay (Fig. 2) reports on the inhibitor's ability to block binding of known peptide ligands of LC3B or GABARAP which bind in the canonical protein-protein interaction site responsible for these proteins' functions. For GABARAP, the ligand was biotin-labeled K1 peptide, which has a K_d of 55 ± 8 nM as measured by biolayer interferometry.[34,36] For LC3B, the ligand was a modified, biotin-labeled FYCO1 peptide, FYCO1S which has a K_d of 330 ± 30 nM as measured by biolayer interferometry.[33] Using untagged versions of these tracer peptides as positive controls, we measured dose dependent inhibition in AlphaScreen with IC_{50} values of 55 ± 3 nM for K1 binding to GABARAP and inhibiting its interaction with biotinylated K1, and 56 ± 4 nM for FYCO1S binding to LC3B and inhibiting its interaction with biotinylated FYCO1S. These controls demonstrate that this assay measures the dose-dependent inhibition of the protein-peptide interactions relevant for LC3/GABARAP functions in autophagy. These new assays are similar to the AlphaScreen assay reported in 2021 by Proschak and coworkers, who measured the disruption of binding between GST-LC3B and biotin-LIRtide, a short peptide derived from LC3-Interacting Region (LIR) of p62.[37]

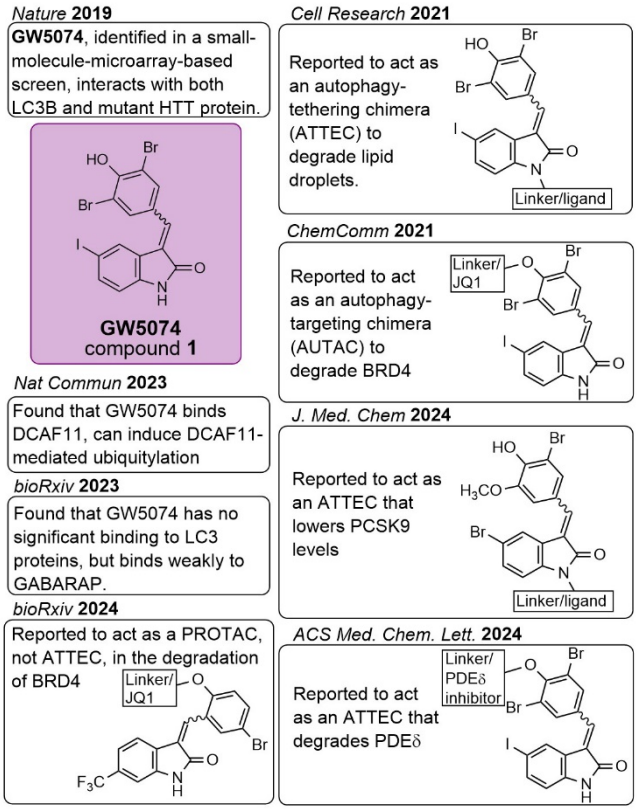

Figure 1. Summary of findings related to compound **1**, also known as **GW5074**, in prior literature. Most arylidene-indolinones analogous to compound **1** are interconverting diastereomers with respect to the double bond in the arylidene.

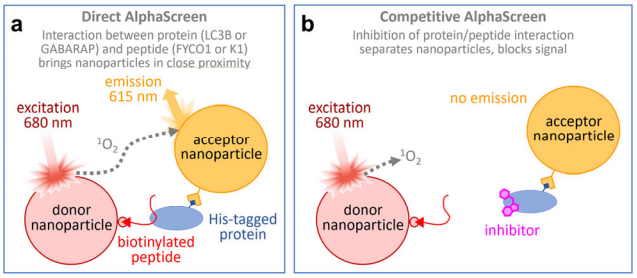

Figure 2. Newly developed AlphaScreen assays. One assay was used to measure inhibitory potency for inhibition of LC3B binding to the dye-labeled peptide ligand FYCO1S, and a similar assay was used to measure inhibitory potenct for inhibition of GABARAP binding to the dye-labeled peptide ligand K1. Peptide ligands were immobilized on streptavidin-functionalized donor beads and recombinant LC3B or GABARAP was immobilized on Ni-NTA-functionalized acceptor beads. Additional assay details provided in Supporting Information.

Using the AlphaScreen assays, we measured the IC_{50} of **1** at 4.75 µM for GABARAP and 4.52 µM for LC3B. These results encouraged us to develop structure-activity relationships by altering one functional group at a time on **1** (Fig. 3). Commercially available oxindoles and aldehydes readily underwent an aldol condensation in the presence of a base and ethanol, allowing us to produce a library of 38 analogs in total. Considering alterations of the arylidene portion of **1**, we observed that removal of all functional groups from the arylidene resulted in complete loss of binding to both proteins (**2a**). Removal of just the phenolic hydroxyl group at C11 (**2b**) resulted in similar binding to GABARAP but five-fold poorer binding to LC3B. By contrast, removing the bromo groups at C10 and C12 while retaining the hydroxyl group at C11 (**2c**) resulted in only mild decrease in binding to LC3B, but nearly 10-fold poorer binding to GABARAP. Methylation of the phenolic hydroxyl group (**2d**) resulted in complete loss of binding to both proteins. Retaining one bromo group *ortho* to the hydroxy (**2e**) resulted in a decrease in binding to both proteins while moving the hydroxy group to the C9/C13 position (**2f**) resulted in decreased binding to GABARAP but comparable binding to LC3B compared to **1**.

Another series of compounds explored substitutions on the oxindole portion of **1** (Fig. 4). Methylation of the amide (**2g**) led to a loss of detectable binding to both proteins. Removal of iodine from oxindole position C5 (**2h**) also led to a loss of detectable binding to both proteins. Movement of the iodine from the C5 to the C6 position of the oxindole (**2i**) resulted in a slight decrease in binding to LC3B, but roughly 3-fold poorer binding to GABARAP. Replacement of iodine with fluorine at C6 (**2j**) resulted in complete loss of binding to both proteins, while replacement with a methyl group at C6 (**2k**) decreased binding to LC3B but increased binding to GABARAP relative to iodine at this position (**2i**). Moving the substituent back to the C5 position on the oxindole, replacement of the iodine with bromine (**2l**) resulted in slightly decreased binding to both proteins, while replacement with a trifluoromethyl group (**2m**) resulted in no detectable binding to either protein. A methyl group at the C5 position (**2n**) also resulted in slight decreases in binding to GABARAP and LC3B. Substituting a chlorine at C6 and a choroethyl at C5 position (**2o**) also resulted in a slight decrease in binding to both proteins relative to **1**. Replacement at the C6 with a methyl acetate (**2p**) resulted in no observed binding to either protein.

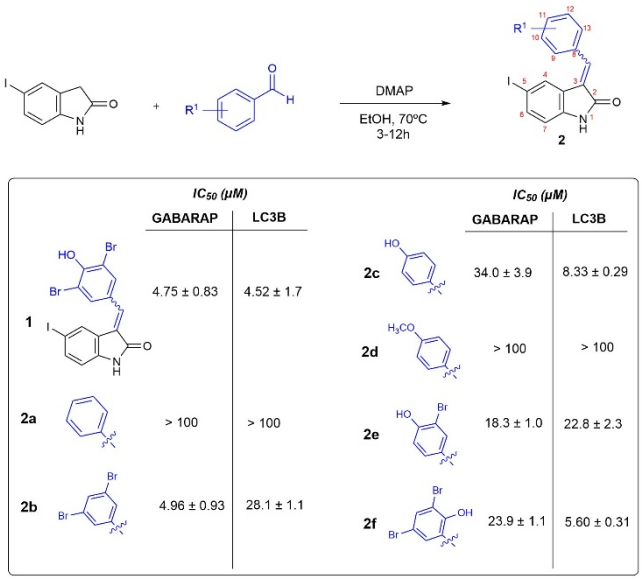

Figure 3. Series 1, exploring alterations to the arylidene portion of compound **1**. Complete compound characterization and assay data are provided in Supporting Information.

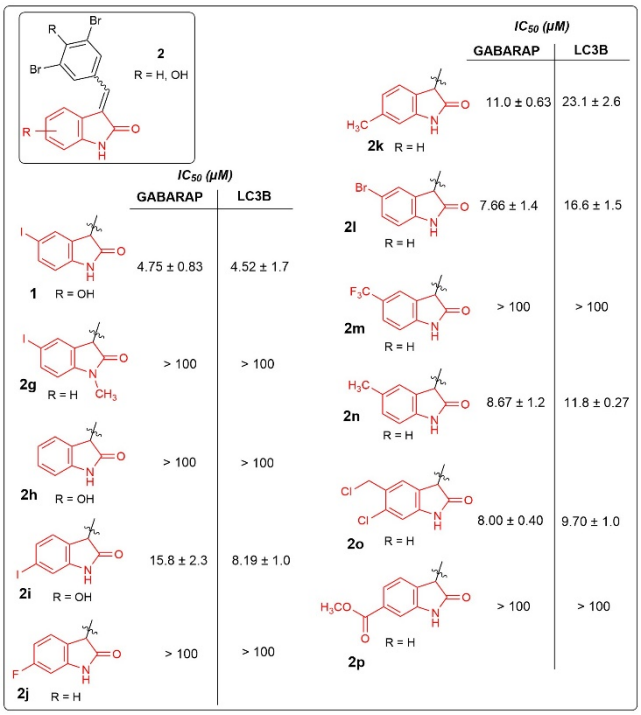

Figure 4. Series 2, exploring alterations to the oxindole portion of compound **1**.

After acquiring these initial structure-activity relationships, we next sought to alter the scaffold of **1** starting with substituting the arylidene with various heterocycles (Fig. 5). Replacement of the phenyl group with a benzofuran (**2q**) or pyrrole (**2s**) resulted in no binding for either protein. However, replacement of the phenyl group with a *para*-trifluoro-biphenyl (**2r**), imidazole (**2t**), oxazole (**2u**), or a thiazole (**2v**) resulted in decreased binding relative to **1**, but still measurable binding to both proteins. Shifting the position of the nitrogen within the

thiazole ring (**2w**) resulted in a slight decrease in binding to GABARAP, but a large decrease in binding to LC3B relative to **2v**. Adding a methyl to the C4 position of the thiazole (**2x**) resulted in a improved binding to GABARAP and LC3 relative to the thiazole without the methyl group (**2x**), potencies similar to the oxazole (**2u**).

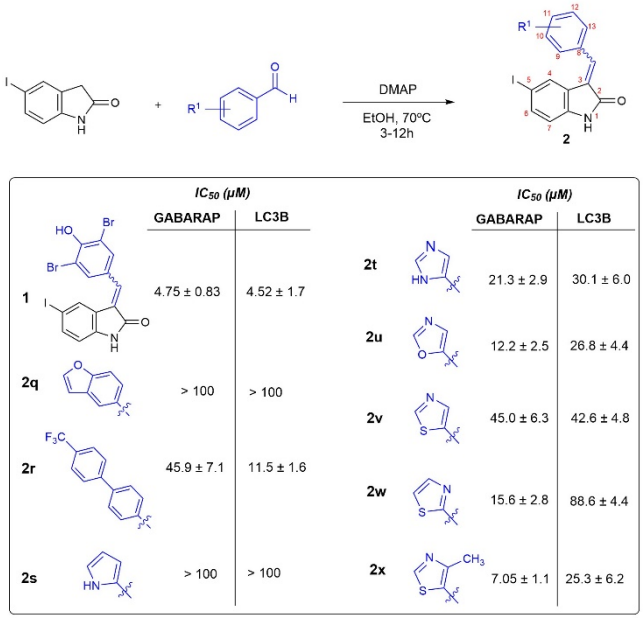

Figure 5. Series 3, exploring substitutions of heterocycles within the arylidene group.

The selectivity determinants of peptide and protein binding to LC3B and GABARAP have been extensively explored,[33–35,38–41] but few studies have explored selectivity of small molecule binding.[32,37,42] Compound **1** has similar apparent affinity for both proteins, but several analogs had selectivity for either LC3B or GABARAP (Fig. 6).

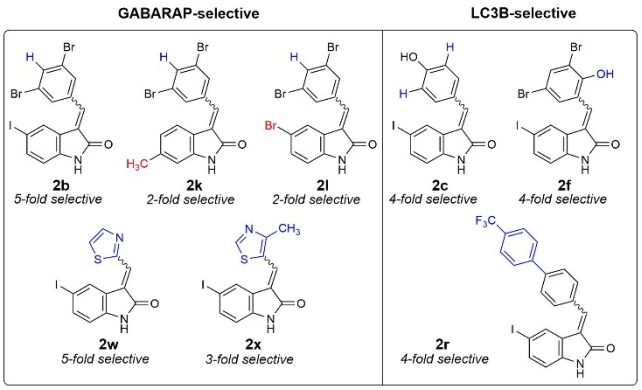

Figure 6. Summary of compounds with selectivity for GABARAP over LC3B, or selectivity for LC3B over GABARAP.

In recent findings, Winter, Waldmann, and coworkers demonstrated that **1** covalently reacts with the E3 ligase DCAF11.[30] Saturation of the arylidene double bond was shown to prevent reaction with DCAF11, consistent with a cysteine of DCAF11 reacting with the Michael acceptor on **1**. Guided by this SAR for DCAF11, we produced saturated versions of selected analogs to explore whether they would still bind LC3B or GABARAP. Saturation was achieved via reduction with sodium borohydride (Fig. 7). Unfortunately, binding was diminished for all compounds after saturation with only a few still exhibiting any detectable binding (Fig. 7). Compounds **3a** and **3c** showed relatively similar, weak IC_{50} values for both GABARAP (46.7 and 49.8 μM, respectively) and LC3B (28.9 and 34.8 μM, respectively). Weak binding was detected for both proteins for the saturated *para*-hydroxyl analog as well (**3b**). Very weak binding was detected for LC3B for the C4 methyl-thiazole (**3e**) but no binding was detected for GABARAP. Binding was lost to both proteins when the nitrogen position was moved within the thiazole (**3d**) and when *para*-trifluoro-biphenyl was substituted (**3f**) within the saturated scaffold.

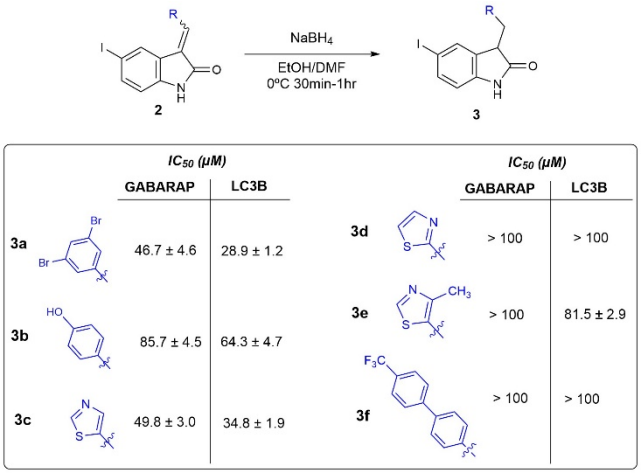

Figure 7. Series 4, selected compounds with a saturated arylidene.

To rule out an entirely nonspecific mode of action, we also tested selected analogs in an orthogonal assay for inhibition of beta-lactamase activity. If the mode of inhibition for LC3/GABARAP proteins was nonspecific, for instance via colloidal aggregation of the compounds leading to protein denaturation,[43,44] we would expect to see inhibition of an unrelated protein at similar concentrations. We observed no effect on beta-lactamase activity for compounds **1**, **2b**, and **3a** at concentrations up to 25 μM (Fig. S1). These results, along with the differential selectivity for LC3B versus GABARAP among different analogs (Fig. 6) and the SAR gathered to date (Fig. 8), provide additional evidence that these compounds inhibit LC3/GABARAP via selective binding to the LIR-domain-binding pocket.

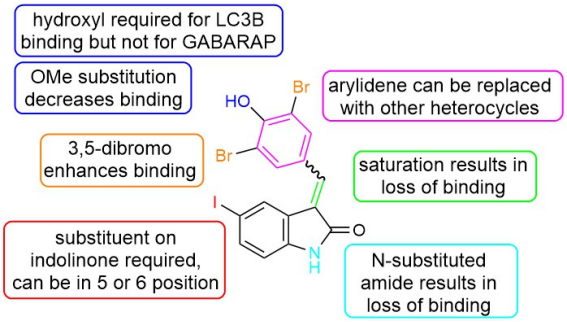

Figure 8. Summary of Structure-Activity Relationships of **1**.

To date, all compounds using **1** for targeted protein degradation (AUTACs and ATTECs) have attached a linker either to the C11 hydroxyl[17,29] or the oxindole amide,[20,22] suggesting that LC3/GABARAP binding is relatively insensitive to modifications at these positions. Additionally, **1** was first discovered to bind LC3B in a microarray in which it was linked to the array by the C11 hydroxyl.[19] These prior results contrast to our present work, which finds that methylation of the C11 hydroxyl (**2d**) results in loss of binding to both LC3B and GABARAP, removing the C11 hydroxyl (**2b**) results in loss of binding to LC3B but not GABARAP, and that N-methylating the amide amide on the oxindole (**2h**) results in a loss of binding to both proteins. No other group has directly reported on the effect of these conservative substitutions on the binding of **1** to LC3/GABARAP proteins. Within the context of a heterobifunctional ATTEC, it is clear from previous reports that the linker affects LC3/GABARAP binding. For example, Wang, Ouyang, and coworkers measured **1** binding LC3B with a K_d of 8.9 μM, but when attached to JQ1 by linkers of various lengths and structures, binding to LC3B varied from 1.3 μM to 73.2 μM.[17] Similarly, Zhao, Guo, Gu, and coworkers found that changes in the linker structure within their PCSK9 ATTEC compounds resulted in an increase in binding to LC3B from 9.88 μM to 5.69 μM.[22] Given our results, it is possible that positive contributions to binding by the linker attached to the C11 hydroxyl or oxindole amide compensates for the decrease in binding to LC3/GABARAP proteins caused by substitution at these positions.

The binding site of compound **1** on GABARAP was investigated in more detail by titrating [U-^{15}N] GABARAP with increasing amounts of **1** and monitoring GABARAP using 2D [^{1}H,^{15}N] HSQC NMR (Fig. 9a-b). Ligand binding led to sizeable chemical shift perturbations (CSPs), several of which (E8, E17, E19, R22, K23, Y25, V29, V31, I32, V33, K48, Y49, L50, V51, S53, H70, F103, F104, Y106) systematically exceeded twice the root-mean-square CSP (Fig. 9a). Y49 was affected by strong line broadening in addition to a particularly large CSP. Mapping the chemical shift perturbations onto the molecular surface of GABARAP clearly shows that **1** binds to the N-terminal hydrophobic pocket of the two-pocket LIR binding site (Fig. 9c). This hydrophobic pocket, commonly referred to as HP1, is where aromatic residues from peptide ligands bind. This finding makes sense since the scaffold of **1** is made up of two aromatic ring systems. Also, this binding site may explain our SAR that the double bond of the arylidene must be unsaturated for low-micromolar binding, if both unsaturated rings must be coplanar to fit in the pocket. Given that residues lining HP1 are highly conserved across LC3/GABARAP subfamily members,[40] the binding of **1** in this pocket is consistent with the mild selectivity for LC3B or GABARAP found for the majority of compounds tested (Fig. 6).

Despite the growing interest in autophagy inhibitors for cancer therapy and for autophagy-mediated degradation, few small molecules with potent and selective LC3/GABARAP binding have been reported. Here, we further investigated the structure-activity relationships of compound **1** against both LC3B and GABARAP. The best compounds have low micromolar IC_{50} values for inhibiting the interactions of these proteins with known peptide ligands. Because LC3/GABARAP proteins are highly conserved and expressed in every tissue at moderate to high levels,[45–47] we anticipate that these compounds are likely not potent enough to make good candidates for autophagy inhibitors. Still, at micromolar potency they would be usable for autophagy-mediated targeted protein degradation if not for the reported off-target covalent binding of DCAF11, which was not directly explored in this work. While the double bond of the arylidene was required for low micromolar affinity for LC3B and GABARAP, the mechanism for the LC3B/GABARAP binding is unlikely to be covalent because these proteins lack cysteines. Instead, we infer that the planarity or other feature of the double bond is likely required for higher-affinity binding within the established binding pocket HP1. Overall, the structure-activity relationships uncovered here suggest that the LC3/GABARAP binding may yet be separable from the Michael acceptor, either by substituting the Michael acceptor with a planar isostere or by scaffold hopping to remove the Michael acceptor.

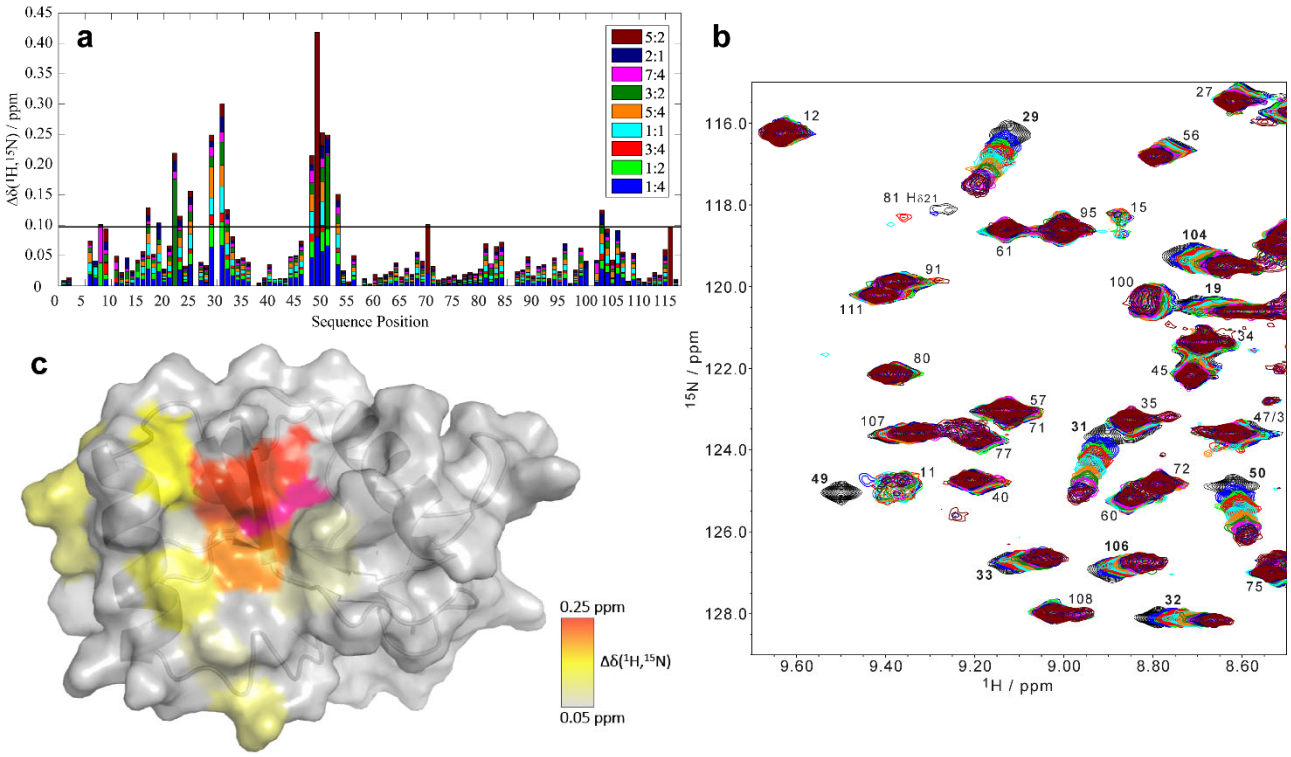

Figure 9. Titration of GABARAP with compound **1** as monitored by NMR spectroscopy. a) Chemical shift perturbations (CSP) for backbone amide resonances of GABARAP incubated with the indicated stoichiometric ratios of **1**:GABARAP. The horizontal line indicates the position of twice the root-mean-square CSP variation (0.0972 ppm at 5:2 excess of **1**). b) Section of the [^{1}H,^{15}N] HSQC showing chemical shift perturbations with increasing concentrations of **1**. Spectra are color-coded to match the ratios shown in panel a, and the spectrum for GABARAP in the absence of **1** is shown in black. c) Molecular surface of GABARAP (PDB 1KOT)[48] with the residues with CSPs observed at 5:2 **1**:GABARAP shown in color. Y49, which is affected by strong line broadening in addition to a particularly large CSP, is highlighted in magenta.

Overall, we provide definitive evidence that **1** binds LC3B and GABARAP and inhibits their interactions with peptide ligands. We also provide the first structure-activity relationships which provide some insight into the selective recognition of LC3B and GABARAP by these compounds. Despite the ongoing controversy surrounding these compounds, our work suggests that compounds with similar scaffolds or similar pharmacophores could be viable LC3/GABARAP ligands for applications in cancer chemotherapy and targeted degradation of proteins, organelles, and protein aggregates. The similarity of compound **1**-induced CSP patterns observed for GABARAPL2 (ref. 32) and GABARAP (this study) attests to the high conservation of the core LIR docking site and to the challenges of devising small-molecule ligands targeting individual family members. More broadly, this work adds to the growing evidence that these protein-protein interactions are highly likely to be druggable using orally bioavailable small molecules.

ASSOCIATED CONTENT

Supporting Information

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

Full experimental details, full compound characterization, complete binding assay data, and additional supporting data, PDF

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Author Contributions

A. L., H. B., and J.A.K. designed experiments. A.L., J.P., T.S., and B.T. prepared and characterized compounds. A. L. and H. B. measured binding affinities. P.N., A.Ü., O. H. W. and D. W. produced and interpreted NMR data and wrote the summary of the NMR results. A.L., H.B., and J.A.K. wrote the rest of the manuscript.

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ABBREVIATIONS

SPR, surface plasmon resonance; DMAP, 4-dimethylaminopyridine; EtOH, ethanol; $NaBH_4$, sodium borohydride; DMF, dimethylformamide; LIR, LC3-interacting region.

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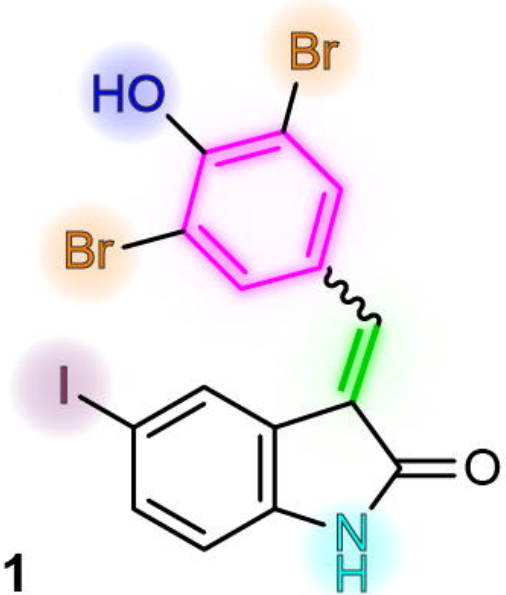
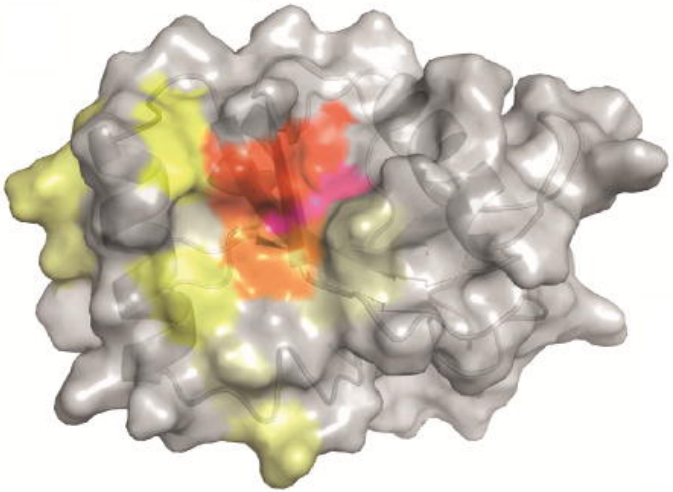
1 was reported as a LC3/GABARAP ligand and component for autophagy-mediated targeted protein degradation.

We report the first SAR for **1** binding LC3B and GABARAP...



1



...and the identification of its binding site on GABARAP.