

Synthesis and Purification of Cyclic Peptide Autophagy Inhibitor 4B1W and Its Target LC3a

Vucic I¹, Engel BJ¹, Gray JP¹, Millward SW¹.

¹Department of Cancer Systems Imaging, The University of Texas MD Anderson Cancer Center

Introduction

- Autophagy is an energy producing mechanism that helps some cancer cells build resistance to chemotherapeutics.
- Inhibiting autophagy leads to decreased treatment resistance.
- The goals of this work are to:

- Purify a key autophagy protein to be used to develop future peptide inhibitors
- Produce a cyclic peptide inhibitor of the protein target that will be used to inhibit autophagy *in vitro* and *in vivo*

Autophagy is a Cell Survival

Mechanism that Leads to Treatment Resistance

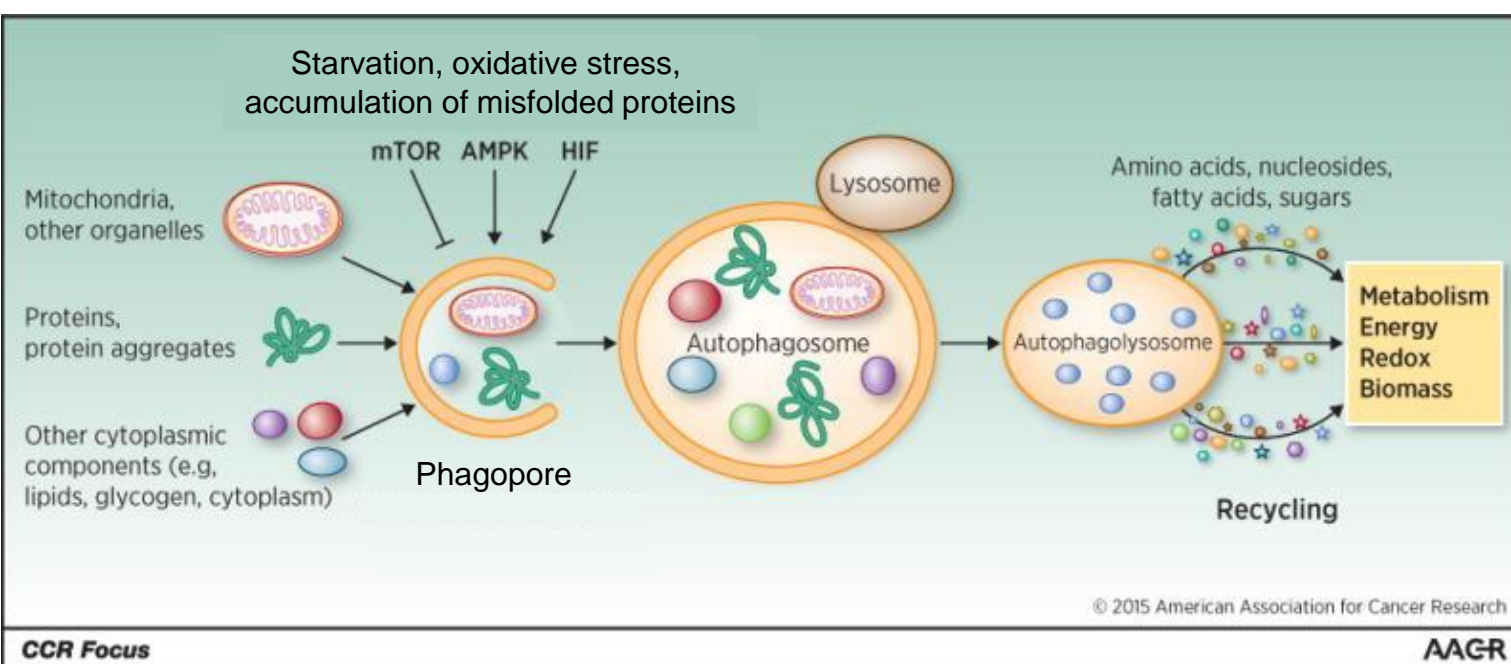


Figure 1. General Mechanism of Autophagy. Autophagy is a cellular process to recycle old or misfolded proteins and organelles for reuse in cellular biogenesis. First, cargo is enveloped around a double-membrane structure called the phagopore, which fully closes into a vesicle called the autophagosome. This then fuses with the lysosome to form an autophagolysosome. Cargo is broken down into substituent biomolecules to be reused. Induction of autophagy occurs after treatment in some cancers as an escape mechanism. Inhibition of autophagy, therefore, would be a suitable target for combating treatment resistance. Figure adapted from ¹.

LC3a Loads Cargo into Autophagosomes

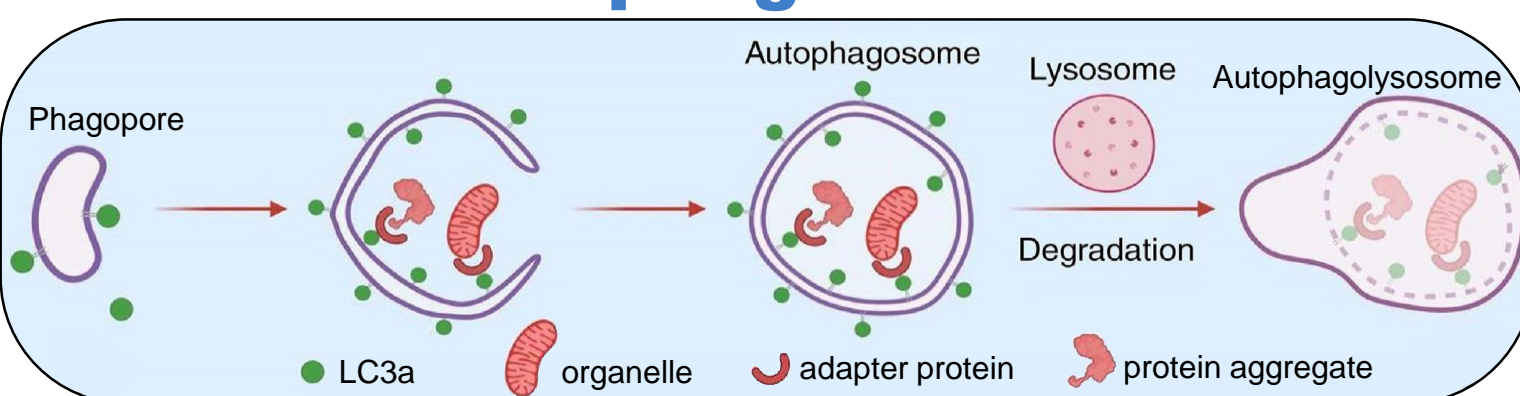


Figure 2. Cargo is loaded into autophagosomes by LC3a. LC3a aids in the elongation of the phagopore and formation of a fully closed autophagosome. As the phagopore grows, LC3a molecules bind to cargo (e.g. organelles and protein aggregates) through adapter proteins. These are sequestered to the inner phagopore membrane and are enclosed inside the autophagosome and degraded upon fusion with the lysosome. Figure adapted from ².

4B1W is a Cyclic Peptide Inhibitor of LC3a

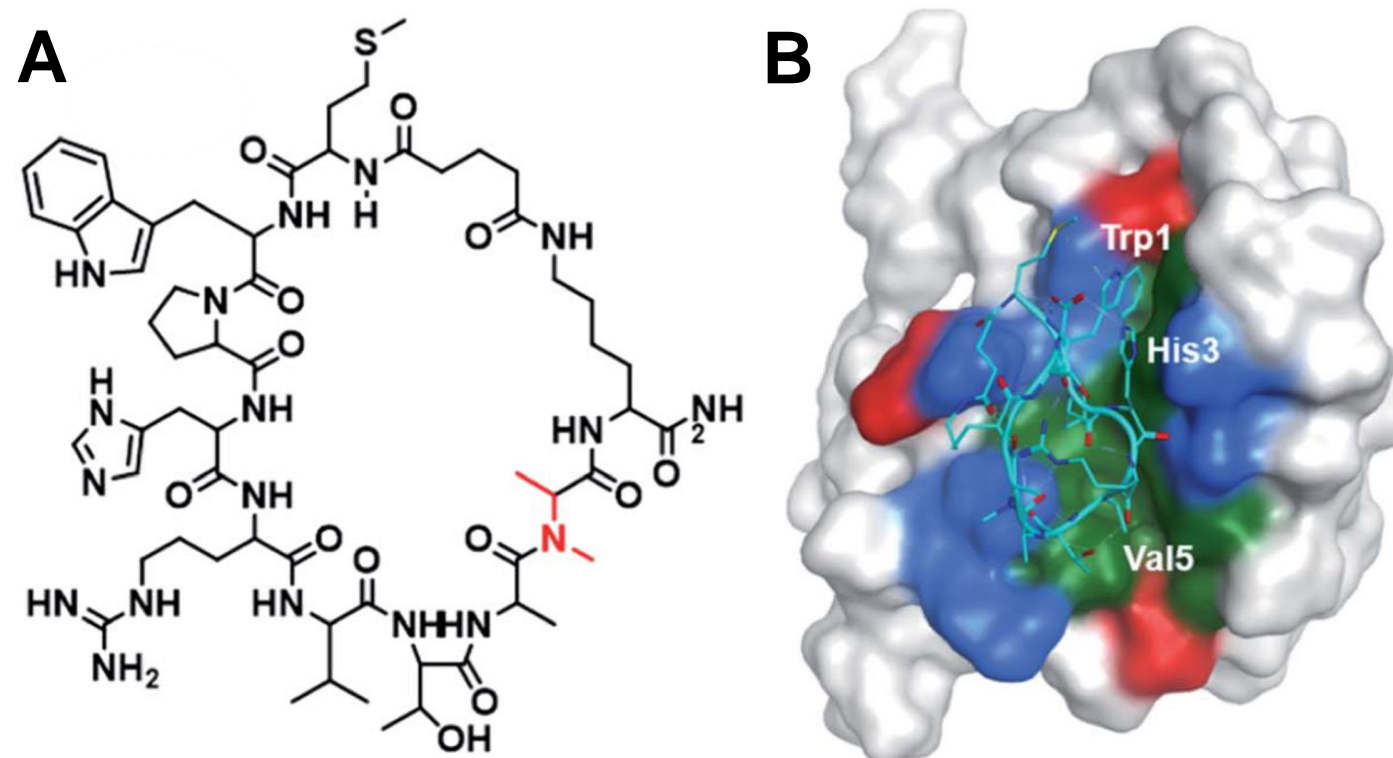


Figure 3. Cyclic 4B1W structure and computational model of 4B1W:LC3a interaction. (A) Chemical structure of cyclic 4B1W. Cyclization occurs between the lysine side chain and the N-terminus of methionine. (B) Cyclic 4B1W binds strongly to the LC3a interacting motif, blocking interaction with other autophagy proteins. As a result, cyclic 4B1W is an inhibitor of cellular autophagy which sensitizes cancer cells to chemotherapeutics. Figure adapted from ³.

Expression & Purification of Recombinant LC3a Protein

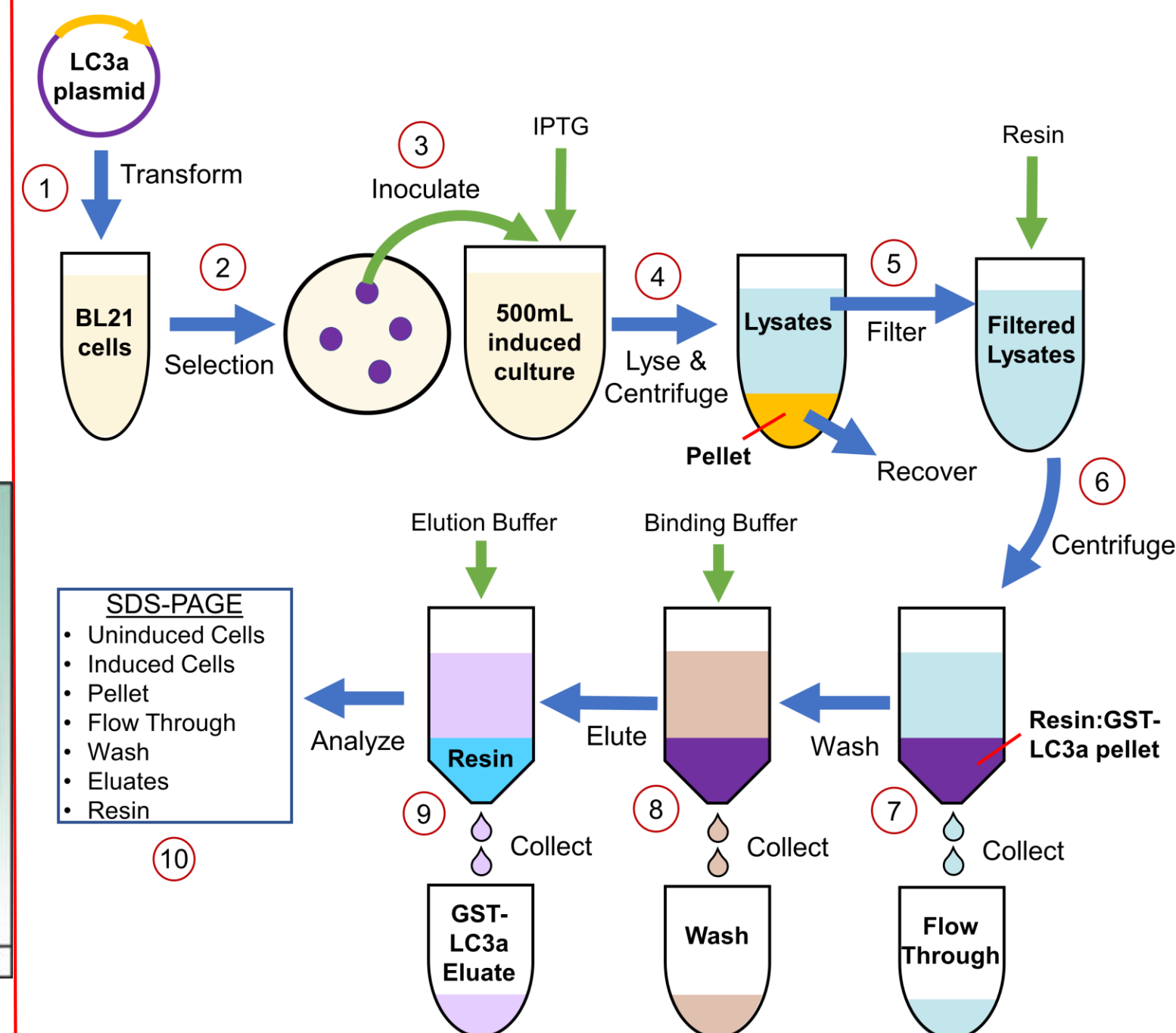


Figure 5. General Protocol for GST-LC3a expression and purification. (1) BL21 *E. coli* are transformed with GST-LC3a expression plasmid. (2) Select transformed colonies with ampicillin agar plates. (3) Inoculate 500 mL culture with colony and induce protein expression with IPTG. (4) Lyse cells with lysozyme and tip-sonication then centrifuge. (5) Filter lysate for affinity purification. (6) Add glutathione agarose resin to lysates to bind GST-LC3a and purify by gravity column. (7) Recover flow-through containing unbound proteins. (8) Wash resin with buffer. (9) Elute GST-LC3a glutathione buffer solution. (10) Run SDS-PAGE of all steps to analyze expression and purity.

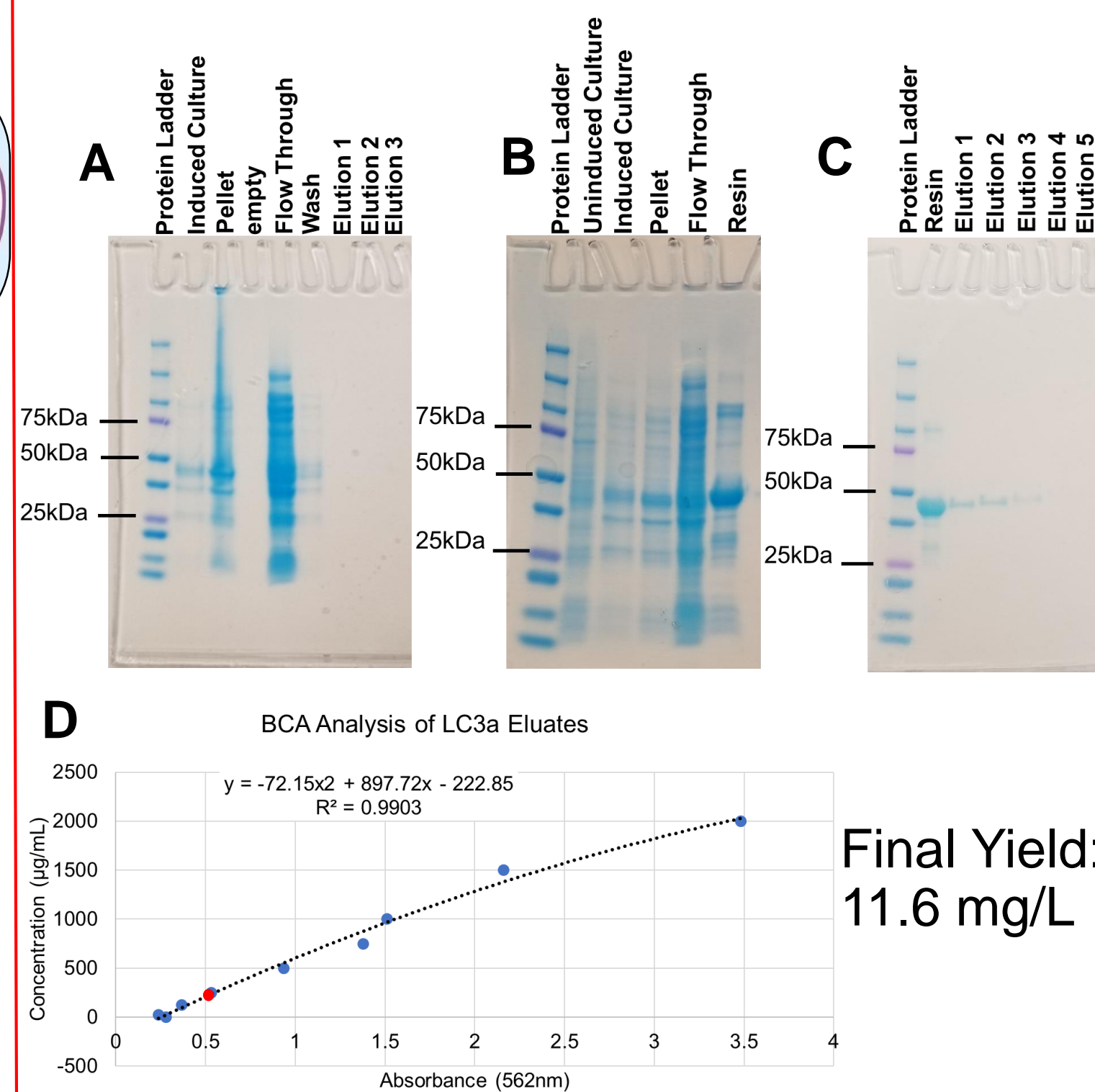


Figure 5. SDS-PAGE and BCA assay of recombinant GST-LC3a. (A) SDS-PAGE of initial purification with 10 mM glutathione did not elute GST-LC3a, however expression was detected as a protein band near 49 kDa. (B) A follow-up SDS-PAGE of the resin showed GST-LC3a was still bound. (C) The resin was incubated with 50 mM glutathione to elute GST-LC3a from the resin. SDS-PAGE analysis shows characteristic 49 kDa bands in each elution fraction, indicating GST-LC3a was properly eluted. (D) BCA assay standard curve (blue dots) and pooled, dialyzed elution fractions (red dot) revealed an undiluted concentration of 1.16 mg/mL with final yield of 11.6 mg GST-LC3a/L *E. coli*.

Solid Phase Peptide Synthesis of 4B1W

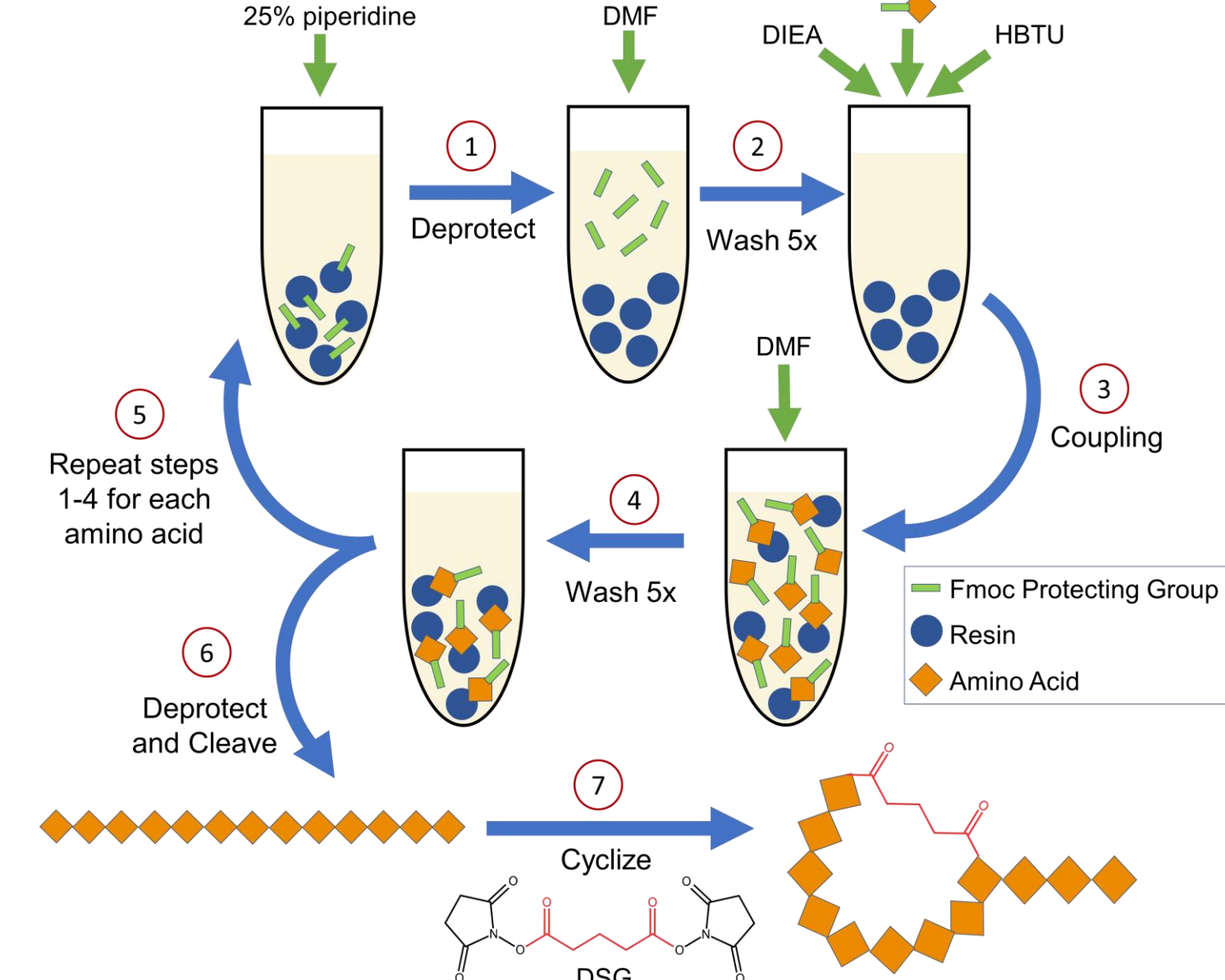
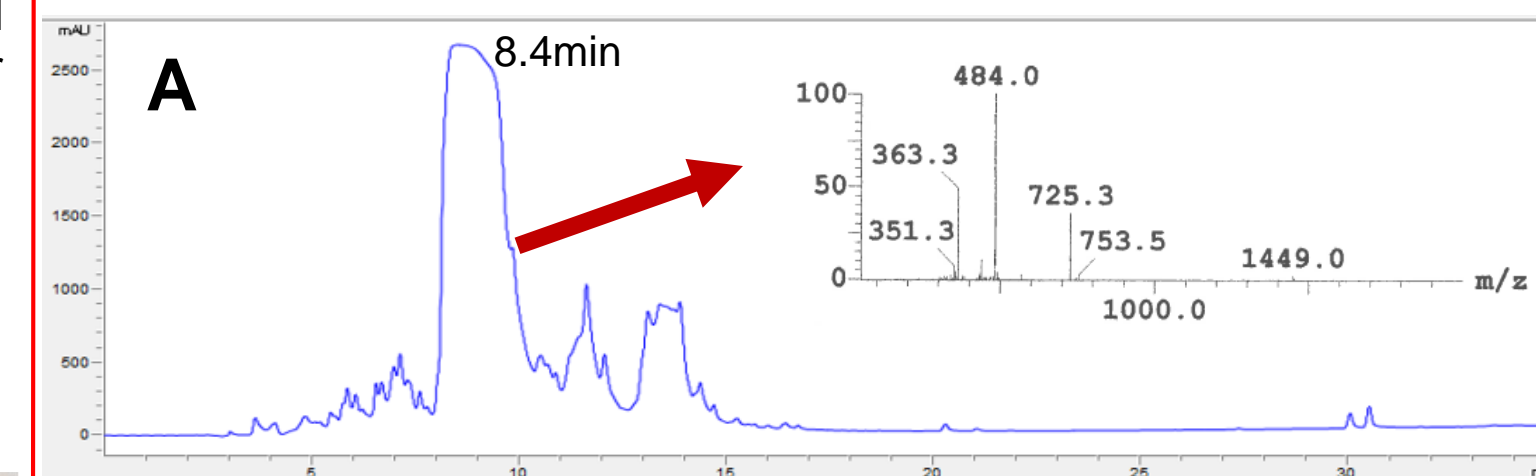


Figure 6. General solid phase peptide synthesis protocol. (1) Deprotect resin using 25% piperidine to remove Fmoc protecting groups. (2) Wash resin 5x with DMF to remove released Fmoc groups. (3) Couple Fmoc-amino acid to resin. HBTU stabilizes carboxylic acid groups and DIEA initiates the coupling reaction. (4) Wash 5x with DMF to remove excess Fmoc-amino acid and reagents. (5) Repeat steps 1-4 for each amino acid to build a peptide chain. (6) After synthesis is complete, deprotect with 25% piperidine and cleave peptide off the resin with TFA. (7) Cyclize the peptide using DSG.

4B1W Purification & Final Yields



Synthesis #	4B1W Present?	4B1W Mass Detected (expected 725.3[M+2H] ²⁺)	Yield mg(%)
1	Yes	725.0	14.2(9.951%)
2	No	--	--
3	No	--	--
4	Yes	725.3	39.7(49.63%)
5	Yes	725.3	40.39(28.71%)
6	Yes	725.3	41.57(30.17%)
7	Yes	725.3	47.9(34.29%)
8	Yes	725.4	47.27(33.57%)
9	Yes	725.7	46.57(33.34%)
10	Yes	725.3	50.33(35.64%)
11	Yes	725.2	48.84(35.06%)
12	Yes	725.5	40.6(29.34%)
13	Yes	725.3	43.26(31.42%)
14	Yes	725.4	45.18(32.13%)

Total Yield:
505.81 mg

Average
Percent Yield:
31.94%

Figure 7. Purification, identification, and final yields of linear 4B1W. (A) HPLC of a gradient from 10%-95% acetonitrile+0.1% TFA over 35 minutes revealed a large peak near 8.4 min for each sample. Mass spectrometry (inset) confirmed product identity as linear 4B1W. (B) Summary of fourteen 126 μmol scale linear 4B1W syntheses yielded of 507.11 mg peptide (average percent yield 32.01%). Successful syntheses have mass spectra similar to the inset of (A), only the [M+2H]²⁺ adduct is shown for simplicity.

Conclusion & Future Work

- Linear 4B1W peptide and GST-LC3a produced at high yield and purity
- Cyclization of 4B1W is ongoing
- Cyclic 4B1W will be used in *in vitro* cell assays & mouse models to evaluate the efficacy of autophagy inhibition in different contexts
- Recombinant GST-LC3a will be used as a target for further improvement of peptide inhibitors of autophagy

References

- White et al. Clin. Cancer Res. 2015;21(22);5037-5046
- Nieto-Torres et al. Trends Biochem. Sci. 2021;46(8);673-686
- Gray et al. Chem. Sci., 2021;12;3526-35434