NOVEL STRATEGIES TO THE DESIGN OF PROTEIN DEGRADERS

By

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ABSTRACT

Apoptosis, or programmed cell death, is a biological process in which multicellular organisms are able to eliminate damaged or unwanted cells. It is regulated in part by a myriad of pro- and anti-apoptotic regulatory proteins, which can either promote or inhibit the process of apoptosis in healthy cells. In cancer cells, however, these regulatory proteins are often imbalanced, causing the inhibition of apoptosis and the uncontrolled growth of tumor cells (Baggio et al., 2018).

Researchers theorize that the inhibition of anti-apoptotic proteins is key in the development of novel cancer treatments. Most modern therapies against cancer act as cell death stimuli, which leaves them vulnerable to the development of resistance in the cell through the overexpression of anti-apoptotic proteins (Sun et al., 2019). Because of this, researchers have turned to attempting to inhibit the anti-apoptotic proteins themselves by engineering specific antagonists to be used in conjunction with modern treatments in order to induce cell death in cancerous tissues (Baggio et al., 2018; Bolomsky et al., 2020).

ACKNOWLEDGEMENTS

First and foremost, I would like to extend my utmost gratitude to my faculty mentor, Dr. Maurizio Pellecchia. Thank you for welcoming me into your lab as an inexperienced undergraduate student and for continually extending me your support and encouragement in my endeavors for the past two years. I am so grateful for your steady patience and your excellent guidance throughout my undergraduate career.

I would also like to wholeheartedly thank all of the members of the lab, especially graduate student Giulia Alboreggia, project scientist, Dr. Carlo Baggio, and postdoctoral scholar, Dr. Parima Udompholkul, for closely mentoring me and teaching me virtually everything I know about experimental techniques.

Finally, I would like to acknowledge my family and peers for their unconditional support of my academic and personal goals.

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INTRODUCTION

Proteolysis ***ta***rgeting ***c***himeras, or PROTACs, are small heterobifunctional molecules that are able to induce targeted protein degradation. They are made of two active chemical moieties, one ligand targeting the protein of interest (POI) and the other ligand targeting an E3 ubiquitin ligase, connected by an optimal linker (Chen & Jin, 2020). The name "proteolysis targeting chimera" is derived from the Chimera, a fire-breathing monster from Greek mythology which was composed of a lion, a goat, and a serpent, referencing the two different ligands that PROTACs have on each end.

The Ubiquitin Proteasome System (UPS) is a highly regulated cellular mechanism in which cells are able to mark damaged or misfolded proteins for degradation. In this system, unwanted proteins are tagged with a small signaling protein called ubiquitin when a protein called E3 ubiquitin ligase comes in close enough proximity to catalyze the formation of a peptide bond between a lysine residue on the protein of interest (POI) and the C-terminus of ubiquitin (Sun et al., 2019). The ubiquitin acts as a signal for a specialized multi-protein complex called the 26S proteasome to recognize and bind to the POI, breaking it down into small, reusable peptides (Almond & Cohen, 2002).

By hijacking the already existing UPS, PROTACs are able to destroy specific target proteins according to the discretion of researchers (Wang et al., 2020). The bi-valent composition of PROTACs allows for the formation of a ternary complex containing the PROTAC, an E3 ligase, and the POI. This places the E3 ligase in close vicinity to the POI, facilitating the placing of ubiquitin onto the POI by the E3 ligase and marking the POI for degradation by the proteasome (Sun et al., 2019). This process is catalytic, allowing the PROTAC to remain

unchanged and able to go on to facilitate more ternary structures with other POI and E3 ligases (Sun et al., 2019).

One particular family of proteins called Inhibitor of Apoptosis Proteins (IAPs) exhibit anti-apoptotic activity and are key to the downregulation of apoptosis. They work by inhibiting caspases 3, 7, and 9, which are specialized enzymes that break down proteins and peptides, ultimately killing the cell (Silke & Meier, 2013). Consequently, IAPs have been found to be overexpressed in tumor tissue, where they prevent apoptosis and allow for continued growth and replication of cancer cells. One particular IAP, the X-linked IAP (XIAP), has been shown to be able to directly bind to caspases. Along with XIAP, cellular IAP1 (cIAP1) and cellular IAP2 (cIAP2) are among the most overexpressed IAPs in human tumor tissues, causing them to become potent targets for anti-cancer research (Baggio et al., 2018).

Importantly, IAPs possess an active ubiquitin ligase domain that can be used in an approach similar to that of PROTACs (Baggio et al., 2019). In this process, chimeric molecules termed "***s***pecific and ***n***on-genetic ***i***nhibitor of apoptosis protein (IAP)-dependent ***p***rotein ***er***asers" or SNIPERs can recruit IAPs to effect targeted POI degradation through ternary complex formation and ubiquitination (Naito et al., 2019).

It has also been recently indicated that small molecules possess the ability to facilitate degradation of a target protein through the mimicking of a Lysine residue. These molecules are composed of general binding ligands coupled with nucleophilic primary amines, which act as Lysine mimetics. In a recent example targeting XIAP itself with this single Lys-mimetic approach, the formation of a peptide bond between the Lysine mimetic and the C-terminus of ubiquitin was able to mark the attached XIAP for degradation by the proteasome (den Besten et al., 2021).

Recently, the Pellecchia laboratory has developed new XIAP targeting agents, namely compounds 155A1 and 155A2, that are being investigated as anti-cancer agents (Baggio et al., 2019). These XIAP antagonists are mimetics of a protein called SMAC or Diablo, which is an IAP inhibitor (Liu et al., 2000). As such, they are able to bind to XIAP and are able to be employed in further research on IAPs.

The project discussed in this report is two-fold. In Specific Aim 1, we propose to derivatize 155A1 and 155A2 to be used as Lys-mimetics to degrade XIAP. To accomplish this Aim, we first have to identify the portion of the molecule that is amenable to derivatization with a Lys-mimetic without altering its ability to potently bind to XIAP. These studies will entail molecular modeling, solid phase synthetic chemistry, and ligand binding studies with the BIR3 domain of XIAP using a biophysical (isothermal titration calorimetry) assay. Subsequently, the most active derivatized agents will be tested by western blotting with a human lung cancer cell line, A549, to monitor their ability to cause XIAP degradation.

In Specific Aim 2, we will try to use our derivatized agent to act as a novel SNIPER molecule targeting a POI. This will entail the same steps that have been delineated above and the verification that the POI is effectively degraded by the new agent. Our derivatized agent will be utilized as the IAP ligand in this novel SNIPER and will be connected to a specific ligand, (+)-JQ-1, that binds potently to bromodomain-containing protein 4 (BRD4), a protein involved in the development of cancer (Ohoka et al., 2019).

Hence, the purpose of this Capstone Project is to use an innovative XIAP antagonist to either create a chimeric Lys-mimetic molecule that can trigger self-degradation of XIAP, or to create a bi-valent molecule with (+)-JQ-1 that can cause the degradation of BRD4 (Ohoka et al., 2019). Our studies provide novel insight on possible protein degradation strategies and

approaches that could be eventually translated into therapeutic treatments to complement current therapies such as chemotherapy and radiotherapy in the fight against cancer.

METHODS

Solid State Peptide Synthesis

In this project, XIAP antagonists, 155B2 and 155B3, were synthesized via solid-state peptide synthesis. Solid-state peptide synthesis is a highly efficient method in which numerous peptides can be prepared at once. It first begins with a resin matrix, on which the growing peptide will be attached. This allows for the solution to be filtered without losing the desired product. Amino acids, along with coupling reagents, are added to the solution and allowed to react so that the amino acid is added to the end of the peptide chain (Stawikowski & Fields, 2002). Once the desired product is achieved, the peptide is cleaved from the matrix and deprotected in one step. It can then be purified and utilized for further experimentation.

Each XIAP antagonist was synthesized on 0.05 mmol BAL resin, which was swelled in 10 mL of dichloromethane (DCM), then washed with dimethylformamide (DMF) (3 x 5 mL). After washing, 3 equivalents of 4-fluoro-1-aminoindane in 1 mL DMF were added to the resin and shaken for 15 minutes, at which point 3 equivalents of the reducing agent sodium triacetoxyborohydride ($NaBH(OAc)_3$) were added to the reaction and shaken overnight. The next day, the reaction was washed with DMF (3 x 5 mL), DCM (3 x 5 mL), then DMF (3 x 5 mL), and 3 equivalents of Fmoc-protected Proline dissolved in a coupling solution consisting of 1 mL DMF with 3 equivalents of HATU, 3 equivalents of ethyl (hydroxyimino)cyanoacetate (OximaPure), and 5 equivalents of diisopropylethylamine (DIPEA) were added to the resin and shaken for 2 hours. After the coupling reaction concluded, the Fmoc-protected Proline was

washed and deprotected using 1 mL of a 20% piperidine deprotection solution for 5 minutes, then 15 minutes with washing between each step. Each of the following coupling reactions involved 3 equivalents of Fmoc-protected amino acid dissolved in 1 mL coupling solution added to the resin and shaken for 1 hour, then deprotected with 1 mL 20% piperidine solution. After each step, a Kaiser test was performed to determine the completion of each amino acid coupling and to detect a free primary amine.

The ivDde protecting group on the side-chain amino on the diaminopropionic acid (Dap) and diaminobutyric acid (Dab) residues was then removed in order to couple the aminooctanoic acid Lysine mimetic. The ivDde deprotection is performed by adding a 5 mL solution of 4% hydrazine in DMF to the reaction and shaken 5 minutes 3 times. After ivDde deprotection, 3 equivalents of Fmoc-protected aminooctanoic acid dissolved in 1 mL coupling solution was added to the resin and shaken for 1 hour, then deprotected and Kaiser tested.

After a final washing step, the peptide was cleaved from the BAL resin using a cleavage cocktail consisting of a 94:3:3 solution of trifluoroacetic acid (TFA), triisopropylsilane (TIPS), and water for 3 hours. The peptide, now suspended in the cleavage solution, was filtered from the resin, evaporated using a rotovap, and freeze-dried in the lyophilizer. The crude product was dissolved in 1 mL dimethyl sulfoxide (DMSO) and purified using high performance liquid chromatography (HPLC). Once purified, the peptide products were characterized and confirmed via mass spectrometry and 1D ^{1}H NMR experiments. Finally, 100 mM mother stock solutions of each peptide were created and stored in a -20°C refrigerator.

Isothermal Titration Calorimetry

Isothermal titration calorimetry (ITC) experiments were performed in order to obtain the thermodynamics of binding of the XIAP antagonists to XIAP itself. An isothermal titration

calorimeter contains a sample cell and a reference cell with a constant power supply to each of these cells. The reference cell is kept at a sustained temperature while a syringe injects small amounts of target protein into the agent-containing sample cell. When the ligand and proteins meet and bind within the sample cell, they will undergo a reaction which will either release or absorb some measure of heat, depending on the nature of the reaction. The ITC will detect these small changes in heat and will change the amount of power supplied to the heater located on the sample cell accordingly in order to maintain a constant and uniform temperature between the reference and sample cells (Duff, et al., 2011). These time dependent changes in power input are measured and reported in the form of the change in kJ of energy per mol of injectant.

The ITC is able to directly measure the enthalpy of binding (ΔH) of the reaction occurring in the sample cell via measurements of the power input required to minimize the changes in temperature. It is also able to obtain a value for the dissociation constant (K_d) of the protein-ligand binding equilibrium. Thus, due to the relationship between the dissociation constant (K_d) and the Gibbs free energy (ΔG) of a reaction, expressed by the equation: $-RT\ln(1/K_d) = \Delta G$, the ITC is also able to provide a measurement for ΔG. Once the enthalpy of binding (ΔH) and the Gibbs free energy (ΔG) have been obtained, the Gibbs free energy equation, expressed as: $\Delta G = \Delta H - T\Delta S$, can be used to indirectly determine the loss of entropy upon binding (ΔS). All of these factors can be utilized to determine the stability of binding and the potency of the ligand, as lower K_d values represent a lower rate of dissociation between the protein and the ligand, resulting in a more potent binding.

A buffer composed of 25 mM Tris pH = 7.5, 150 mM NaCl, 50 μM $Zn(Ac)_2$, and 1 mM dithiothreitol (DTT) was first produced, then a 200 μM solution of XIAP BIR3 domain and a 25 μM solution of the respective compounds were made. The cell samples were then composed of

1% deuterated DMSO containing the compounds and 99% buffer. The syringe sample was composed of 1% deuterated DMSO, 200 μM protein, and the remaining volume was made up of buffer. These samples were loaded into their respective wells and 15 x 2.5 μL titrations with stirring were performed in order to obtain optimized binding curves for the XIAP antagonists. ITC measurements were performed using the Affinity ITC equipped with an Autosampler from TA Instruments.

Western Blotting

Western blot experiments on 155A1, 155B2, and 155B3 were performed under the close supervision of Dr. Parima Udompholkul in order to test the cellular efficiency of XIAP antagonists, 155B2 and 155B3 to facilitate the degradation of XIAP. Degradation was tested by first plating and treating A549 lung cancer cell line (also known as NucLight Red Cells) purchased from Essen Biosciences Sartorius with 10 μM 155A1, 155B2, and 155B3 for 6 hours and 24 hours. A bicinchoninic acid (BCA) protein assay was performed in order to quantify the total amount of XIAP protein within the A549 cells.

Once this was determined, the A549 cells were then lysed with a lysis buffer and the resulting cell lysates were collected and used to run a sodium dodecyl sulfate (SDS) polyacrylamide gel (PAGE) in order to separate the proteins by size. These proteins were then transferred from the gel onto a membrane. The resulting membrane was washed with a 1X Tris-Buffered Saline, 0.1% Tween 20 Detergent (TBST) buffer (3 x 10 mL x 5 minutes) and treated with 5% milk for 1 hour in order to prevent non-specific binding of antibodies. After this, the membrane underwent another washing step with 1X TBST buffer (3 x 10 mL x 5 minutes) and was treated with antibodies in order to probe for XIAP and actin respectively. An

electrochemiluminescence system was used to detect the presence of the respective proteins and analysis of the resulting data was performed by Dr. Parima Udompholkul.

RESULTS AND DISCUSSION

Specific Aim 1

Based on molecular modeling design strategies, the most promising route of derivatizing 155A1 and 155A2 to be used as Lysine mimetics was through the P2 position. This position contained either a diaminopropionic acid (Dap) or a diaminobutyric acid (Dab). These amino acids are non-natural amino acids which possess a primary amine that could be used to couple our Lys-mimetic in order to create the XIAP self-degrader. We began by synthesizing 155A1 and 155A2 and recording their thermodynamics of binding via ITC in order to ensure that they still worked as antagonists against XIAP (**Figure 1 B-C**). The thermodynamics of binding of these peptides and all future peptides were measured side by side with 139A1, a previously designed peptide containing the sequence AVPF, as a control (**Figure 1 A**).

Once 155A1 and 155A2 were synthesized, we began the process of derivatizing a Lys-mimetic off of the Dap and Dab residues (**Tables 1-2**). Three different mimetics were originally used: polyethylene glycol (PEG)-4, 6-aminohexanoic acid (6-Ahx), and aminooctanoic acid. It was later determined that we would not move forward with the PEG-4 mimetic, as the other two mimetics more closely resembled a Lysine residue. The resulting peptides 155A4, 155A8, 155A9, and 155A10 were tested via ITC and it was determined that they still possessed an affinity for XIAP.

Due to time constraints, it was decided that the project would move forward with only two of these four remaining agents. It was theorized that the slightly longer carbon chain of the

aminooctanoic acid would provide a slight advantage when attempting to mimic a Lysine residue, as the nucleophilic primary amine would act as a sort of lure to be recognized by an E3 ligase and ubiquitinated. Thus, peptides 155B2 and 155B3 were synthesized with 4-fluoro-1-aminoindane, aminooctanoic acid, and Dap & Dab respectively. 155B2 was then tested via ITC to confirm binding with XIAP (**Figure 1 D**).

Following this, agents 155B2 and 155B3 were tested via Western blot with human lung cancer cell line A549 to test their ability to induce the degradation of XIAP. Two Western blots were run with treatment times of 6 hours and 24 hours. If the Western blot revealed a statistically significant decrease in the amount of cellular XIAP, it would indicate that the synthesized agents were successfully facilitating XIAP degradation. After a one-way analysis of variance (ANOVA) with Dunnett's Multiple Comparisons test, an adjusted p-value of less than 0.05 indicates statistically significant data. Unfortunately, after analysis by Dr. Parima Udompholkul, it was determined that the adjusted p-values for each of the agents after both experiments were above the cutoff of 0.05, indicating no demonstrable degradation of XIAP (**Figure 2**).

Specific Aim 2

After the XIAP antagonists, 155B2 and 155B3, were obtained, the secondary aim of this project was recently begun by graduate student, Giulia Alboreggia. Our XIAP antagonists were synthesized with BRD4 ligand, (+)-JQ-1, coupled to the aminooctanoic acid via peptide bond (**Figure 3**). (+)-JQ-1 was first prepared by Giulia Alboreggia for coupling via a hydrolysis reaction in which the ester group on (+)-JQ-1 was converted to an acid which could couple to form a peptide bond (**Figure 4**). Once synthesized, the compound was analyzed and identified via thin layer chromatography (TLC) and mass spectrometry. This bi-valent molecule, 156B9, is currently being tested by western blotting to verify its ability to effectively degrade the POI.

FIGURES AND TABLES

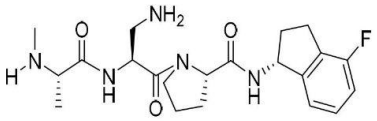
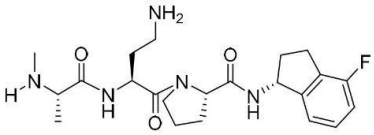
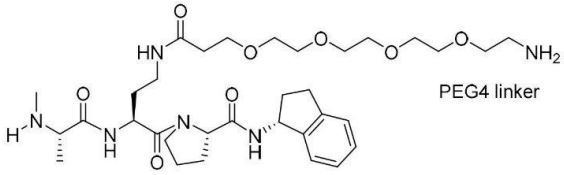
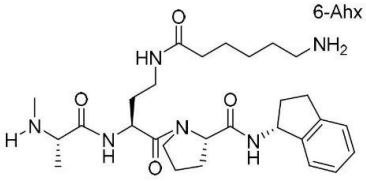
ID	Structure
155A1	Chemical Formula: $C_{21}H_{30}FN_5O_3$ Molecular Weight: 419.50  Me-A Dap P 4-fluoro-1-aminoindane
155A2	Chemical Formula: $C_{22}H_{32}FN_5O_3$ Molecular Weight: 433.53  Me-A Dab P 4-fluoro-1-aminoindane
155A3	Chemical Formula: $C_{33}H_{54}N_6O_8$ Molecular Weight: 662.83  PEG4 linker Me-A Dab P 1-aminoindane
155A4	Chemical Formula: $C_{28}H_{44}N_6O_4$ Molecular Weight: 528.70  6-Ahx Me-A Dab P 1-aminoindane

Table 1. Tested Agents' Names, Structures, Chemical Formulas, and Molecular Weights. Amino acid and linker names are listed underneath/nearby their respective structures.

6-Ahx, 6-aminohexanoic acid.

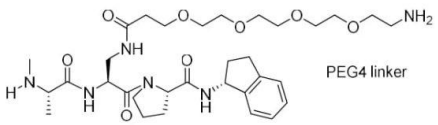
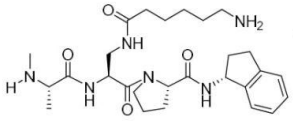
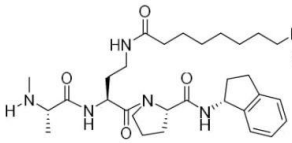
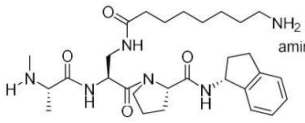
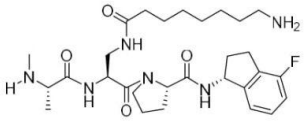
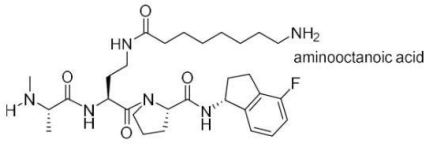
ID	Structure
155A7	Chemical Formula: $C_{32}H_{52}N_6O_8$ Molecular Weight: 648.80  PEG4 linker Me-A Dap P 1-aminoindane
155A8	Chemical Formula: $C_{27}H_{42}N_6O_4$ Molecular Weight: 514.67  6-Ahx Me-A Dap P 1-aminoindane
155A9	Chemical Formula: $C_{30}H_{48}N_6O_4$ Molecular Weight: 556.75  aminooctanoic acid Me-A Dab P 1-aminoindane
155A10	Chemical Formula: $C_{29}H_{46}N_6O_4$ Molecular Weight: 542.73  aminooctanoic acid Me-A Dap P 1-aminoindane
155B2	Chemical Formula: $C_{29}H_{45}FN_6O_4$ Molecular Weight: 560.72  aminooctanoic acid Me-A Dap P 4-fluoro-1-aminoindane
155B3	Chemical Formula: $C_{30}H_{47}FN_6O_4$ Molecular Weight: 574.74  aminooctanoic acid Me-A Dab P 4-fluoro-1-aminoindane

Table 2. Tested Agents' Names, Structures, Chemical Formulas, and Molecular Weights. Amino acid and linker names are listed underneath/nearby their respective structures. 6-Ahx, 6-aminohexanoic acid.

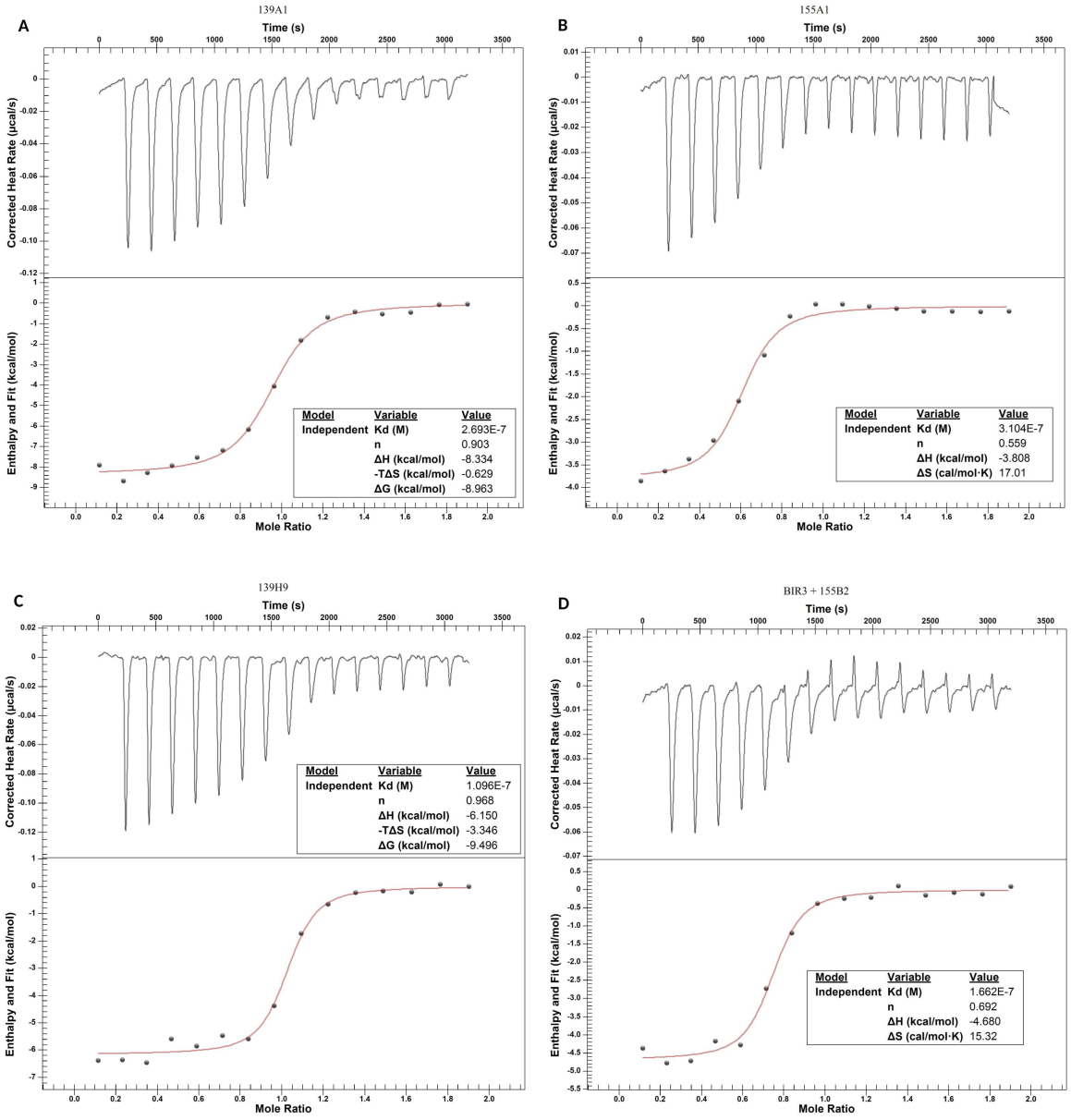


Figure 1. ITC Curves for Binding Between XIAP BIR 3 and (A) 139A1 (K_d = 2.693E-7 M, n = 0.903, ΔH = -8.334 kcal/mol), **(B)** 155A1 (K_d = 3.104E-7 M, n = 0.559, ΔH = -3.808 kcal/mol, **(C)** 139H9 *equivalent to 155A2* (K_d = 1.096E-7 M, n = 0.968, ΔH = -6.150 kcal/mol), **(D)** 155B2 (K_d = 1.662E-7 M, n = 0.692, ΔH = -4.680 kcal/mol)

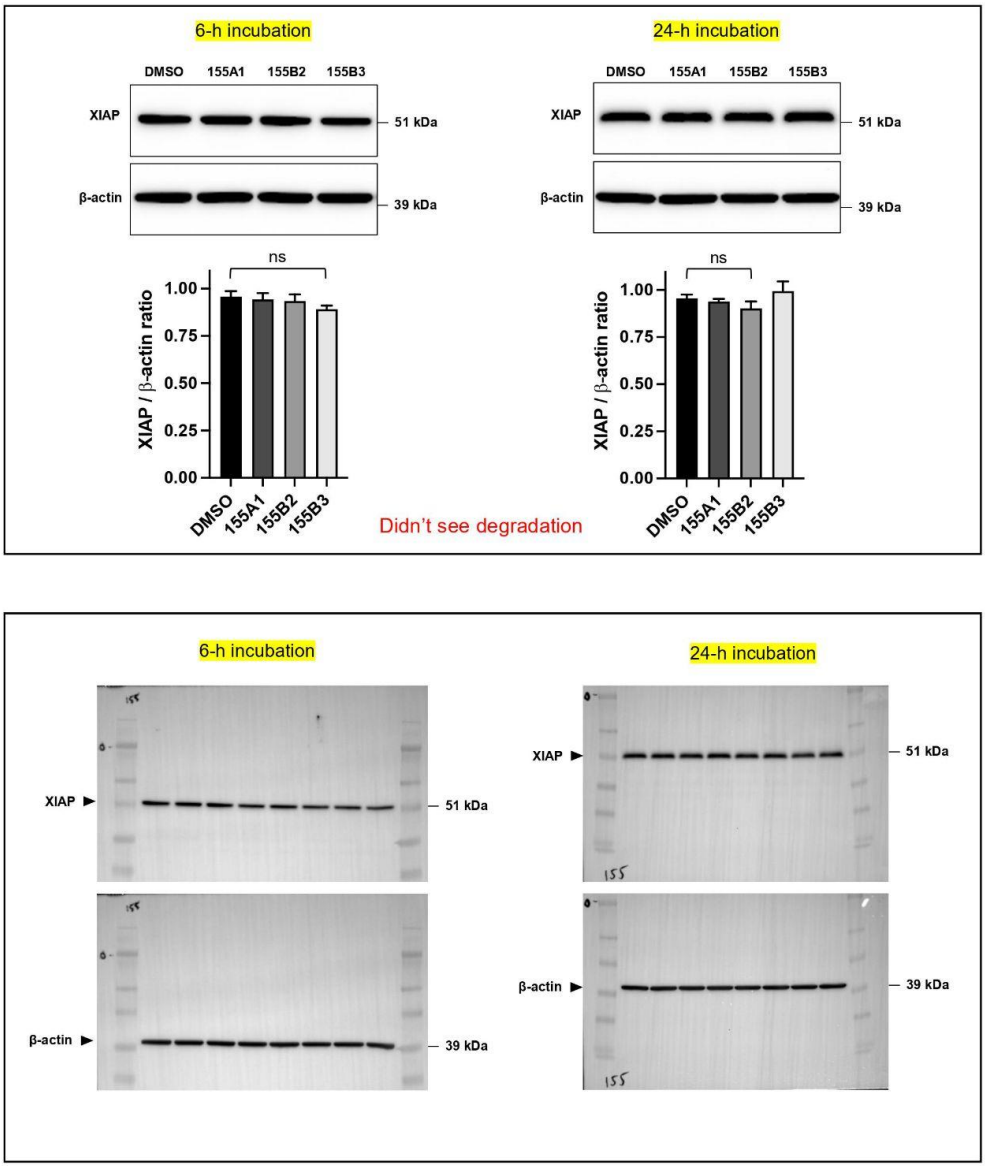


Figure 2. Western Blot Data for 155A1, 155B2, and 155B3. Lanes 6-9 are replicates of Lanes 2-5.

(A) Dec 20, 2021: : Treated A549 with 1% DMSO or 10 µM 155A1, 155B2, & 155B3 for 6h

DMSO vs 155A1 adjusted p value = 0.9683
DMSO vs 155B2 adjusted p value = 0.9049
DMSO vs 155B3 adjusted p value = 0.3817

(B) Dec 22, 2021: Treated A549 with 1% DMSO or 10 µM 155A1, 155B2, & 155B3 for 24 h

DMSO vs 155A1 adjusted p value = 0.9670
DMSO vs 155B2 adjusted p value = 0.5919
DcMSO vs 155B3 adjusted p value = 0.7745

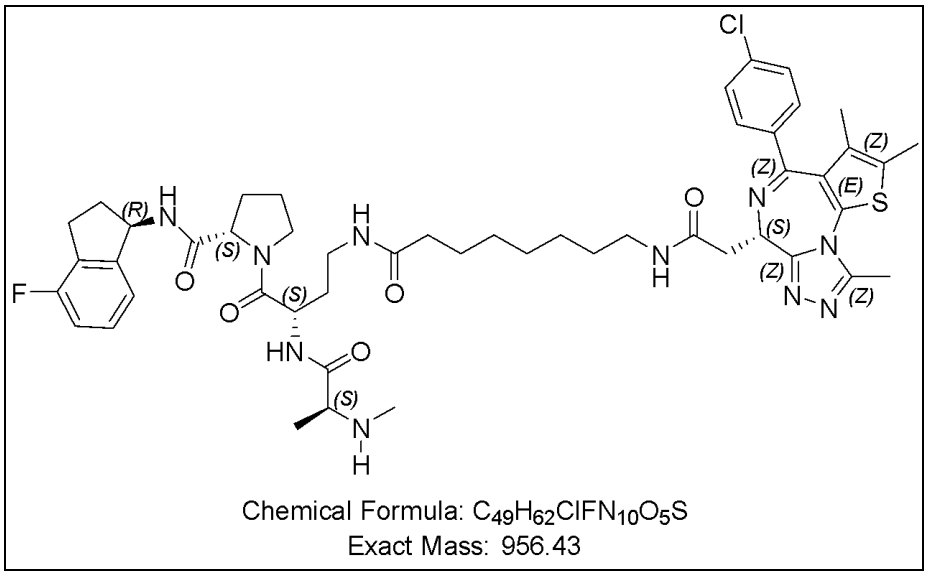


Figure 3. Structure of 156B9 (+)-JQ-1 bidentate.

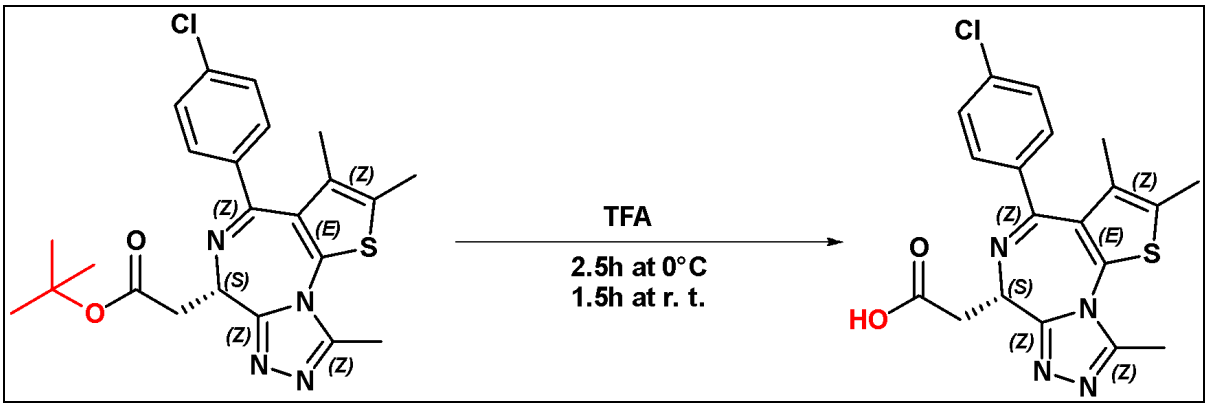


Figure 4. Hydrolysis Reaction of (+)-JQ-1.

CONCLUSIONS

Although 155B2 and 155B3 do not demonstrate effective targeted degradation of XIAP, more molecular modeling can be done in the future in order to improve their ability to induce degradation. Nevertheless, two new antagonistic agents for XIAP coupled with a Lysine mimetic aminooctanoic acid have been successfully produced. Both 155B2 and 155B3 are able to successfully bind directly to XIAP, as evidenced by the ITC data. Additionally, the synthesis and production of these XIAP antagonists have been optimized. 155B2 and 155B3, along with other similar molecules depicted in the 155 library above, are now able to be produced with relatively high yield. Furthermore, our (+)-JQ-1 degrader shows promising potential in degrading both BRD4 and XIAP. Inhibition of anti-apoptotic proteins and cancer promoting proteins in general is a highly sought-after endeavor within the drug discovery industry ((Bolomsky et al., 2020). Thus, we hope that the design of 155B2 and 155B3 may be utilized as a novel strategy for protein degradation as either a Lys-mimetic molecule or as a novel bi-valent molecule with (+)-JQ-1.

RESOURCES

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