Electronic Supplementary Material (ESI) for RSC Chemical Biology.


# Caged Aminoluciferin Probe for Bioluminescent Immunoproteasome Activity Analysis

Cody A. Loy[1] & Darci J. Trader[1]

[1]Department of Pharmaceutical Sciences, University of California, Irvine, California 92617

Supporting Information content:

***General Methods and Materials***

All chemical, reagents, and solvents were purchased from commercial vendors and were used as such. Reactions were monitored by LCMS on an Agilent 1260 Infinity II system with Single Quadrupole LC/MS system. HRMS was performed by UCI's Mass Spectrometry Core facility. Mass of synthesized molecule was recorded using Agilent 1260 series LC couple to ESI-MS detector. Purification was performed on Agilent 1260 Infinity II Analytical HPLC reverse phase with scalar (c18) column. Suc-LLVY-Aminoluciferin (Cat. #. 13452) was purchased from AAT Bioquest. 20S Human Immunoproteasome (SBB-PP0004) and 20S Human Proteasome (SBB-PP0005) was purchased from South Bay Bio. ONX-0914 (HY-13207) immunoproteasome inhibitor was purchased from MedChemExpress. Ramos (ATCC® CRL-1596™) was grown in RPMI-1640 (ATCC® 30-2001™) supplemented with 10% FBS. 4T1-Luc cells were gifted from Prof. Jennifer Prescher (UCI) and were grown in DMEM (Dulbecco's Modified Eagle's Medium, ATCC® 30-2002™). For suspension cells (Ramos) the cell media was pelleted @ 800 xg for 5 min. For adherent cells (4T1-Luc) the media was aspirated, and the flask rinsed with sterile PBS before treating cells with 0.25% (w/v) Trypsin-0.53 mM EDTA solution. The flasks were returned to the incubator for 5 min. Trypsin was quenched with supplemented media, suspension was collected and cells pelleted @ 1000 rpm for 5 min. After aspirating media, the cell pellet was resuspended in fresh media with 10% FBS. Cell concentration was determined by Trypan Blue, a hemocytometer and plated accordingly.

***General Solid Phase Synthesis of m-ATMW (1)***

Peptide m-ATMW was synthesized by solid phase peptide synthesis (SPPS). 2-Chlorotrityl chloride resin (1.0 – 1.2 meq/g, 200-400 mesh, Chem-Impex Int'l Inc.) was swelled in dry dichloromethane (DCM) for 60 min at room temperature (RT). Fmoc-Trp(boc)-OH (2 equiv relative to resin) and diisopropylethylamine (DIPEA) (4 eq. relative to resin) was dissolved in dry DCM (10 mL/g of resin) and added to the resin. Resin was agitated with this mixture for 60 min at RT. Then, a DCM/methanol/DIPEA (17:1:2) mixture was added to the resin and agitated for 60 min at RT to cap any unreacted trityl sites. Resin was washed with DCM (3x) and then dimethylformamide (DMF) (3x). Fmoc deprotection was done with 20% piperidine in DMF for 15 min at RT. Fmoc-Met-OH (5 equiv), HBTU (4.5 equiv), and DIPEA (10 equiv) in DMF was added to the resin. Resin was agitated with this reaction mixture for 60 min at RT. Fmoc deprotection was done with 20% piperidine in DMF for 15 min at RT. Fmoc-Thr(tBu)-OH (5equiv), HBTU (4.5 equiv), and DIPEA (10 equiv) in DMF was added to the resin. Resin was agitated with this reaction mixture for 60 min at RT. Lastly, Fmoc-Ala-OH (5 equiv), HBTU (4.5 equiv), and DIPEA (10 equiv) in DMF was added to the resin. Fmoc deprotection was done with 20% piperidine in DMF for 15 min at RT. The resin was washed with DMF (3x) and then dry DMF (3x). Lastly, the *N*-terminus was capped with a morpholine group. Bromoacetic acid (2M) and DIC (1M) were prepared in dry DMF, and allowed to react for 5 mins at 37ºC. The activated mixture was added to resin and reacted for 20 mins at 37ºC. The solution was drained and subsequently added morpholine (0.5 M) in dry DMF and allowed to react for 1 hr at 37ºC. Completion of morpholine cap was checked by Kaiser test. m-AT(tBu)MW(Boc) resin was washed with DCM, suspended in strong cleavage cocktail (95% TFA, 2.5% DCM, and 2.5% triisopropylsilane) and agitated for 60 min at RT. Cleavage mixture was then collected and evaporated with argon. Crude m-ATMW was precipitated with cold ether and centrifuged. Ether supernatant was decanted, and crude peptide was washed with ether (2x). Powder was dried under vacuum overnight.

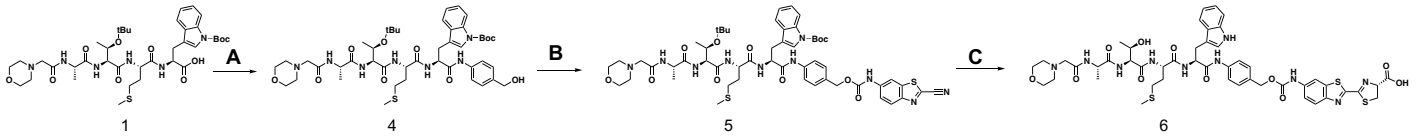
***Synthesis of m-ATMW-aminocyanobenzothiazole (2)***

m-ATMW-aminocyanobenzothiazole (**2**) was synthesized with a one-pot synthesis. First, m-ATMW (1 equiv.) is dissolved in dry DMF (0.9 mL) and cooled to 0 °C. *tert*-Butyldimethylsilyl chloride (TBDMS-Cl, 0.9 equiv.) and imidazole (1.8 equiv.) was added to the reaction. Mixture was stirred overnight and allowed to come to room temperature. Second, TCFH (0.9 equiv.) and n-methyl imidazole (2 equiv,) was added to the reaction at RT, and mixture stirred for 2 hours to activate the free carboxylic acid. A solution of 6-amino-2-cyanobenzothiazole (2 equiv.) and NMI (3 equiv) in DMF (0.45 mL) was added dropwise to the reaction. After 24 hours at RT, water was added to the reaction mixture and extracted with ethyl acetate 3x. The organic layer was washed with sat. sodium bicarbonate 3x, brine 3x, then dried with $MgSO_4$. The solvent was removed under vacuo and redissolved in 50/50 Water/ACN (0.1% TFA) for HPLC purification. (Yield: 31%)

***m-ATMW-aLuc (3)***

m-ATMW-aminocyanobenzothiazole (**2**) was dissolved in MeOH/THF (1 equiv.). D-Cysteine (1.5 equiv.) was dissolved in $H_2O$ and added to the solution of **2** under argon starting at pH ~1.6. After 20 mins at RT, 5% (m/m) $NaHCO_3$ was added dropwise over an hour, continuously checking pH until pH~7.4. The reaction was left to stir for an additional 20 mins at RT. The organic solvents were removed in vacou and water added and adjusted to

pH~5. Ethyl Acetate was used to extract 3x and washed with brine and dried with $MgSO_4$. Solvent removed in vacuo and redissolved in 50/50 Water/ACN (0.1% TFA) for HPLC purification. (Yield: 72%)




**Scheme 1:** Synthesis of M-ATMW-Linker-aLuc (6) achieved through solid phase synthesis of **(1)**, followed by coupling of para-aminobenzyl alcohol (4) , carbamate formation with triphosgene and amino-cyanobenzothiazole, and cyclization of D-Cysteine to give **(6)**

***m-ATMW-para-aminobenzylaclohol synthesis (4)***

m-AT(tbu)MW(boc) is prepared by solid phase peptide synthesis as previously mentioned and cleaved from resin with 5% TFA in DCM to retain protecting groups for further synthesis. m-AT(tbu)MW(boc) (1 equiv.), 4-aminobenzyl alcohol (1.2 equiv.) and n-Methyl Imidazole (3.1 eq) were dissolved in DCM before addition of Chloro-*N*,*N*,*N*′,*N*′-tetramethylformamidinium hexafluorophosphate **(**TCFH) (1.1 equiv). The reaction was stirred at r.t. overnight. Solvent removed under vacuo, water added and extracted with ethyl acetate 3x. The organic layer was washed with sat. sodium bicarbonate 3x, brine 3x, then dried with $MgSO_4$ and solvent removed under vacuo. Crude Product moved forward without purification.

***m-ATMW-linker-aminocyanobenzothiazole synthesis (5)***

A solution of triphosgene (1 equiv.) in dry toluene at r.t. was added dropwise to a solution of amino-cyanobenzothiazole (1 equiv.) and DMAP (1 equiv.) in dry toluene under nitrogen atmosphere. The reaction mixture was warmed to 120ºC and stirred at reflux for 3 hr. After 3 hr, solvent was removed under reduced pressure and redissolved in DCM. To this, a solution of **4 (**1.2 equiv.) and DMAP (1 equiv.) in DCM was added dropwise at 0ºC. The reaction mixture was stirred at room temperature for 5 hr under nitrogen atmosphere. Reaction washed with water, brine, and solvent removed under reduced pressure. Crude moved forward without purification.

***m-ATMW-linker-aLuc (6)***

m-ATMW-linker-aminocyanobenzothiazole (**5**) was dissolved in MeOH/THF (1 equiv.). D-Cysteine (1.5 equiv.) was dissolved in $H_2O$ and added to the solution of **5** under argon starting at pH ~1.6. After 20 mins at RT, 5% (m/m) $NaHCO_3$ was added dropwise over an hour, continuously checking pH until pH~7.4. The reaction was left to stir for an additional 20 mins at RT. The organic solvents were removed in vacou and water added and adjusted to pH~5. Ethyl Acetate was used to extract 3x and washed with brine and dried with $MgSO_4$. Protecting groups removed by 95% TFA in DCM for 1hr. Resdissolved in 50/50 Water/ACN (0.1% TFA) for HPLC purification.
**HRMS** m/z: $[M+H]^+$ : Calcd for $C_{48}H_{56}N_{10}O_{11}S_3$ 1045.3370; Found 1045.3358 (-1.2 mDa). (Yield: 28% overall)

***LCMS Cleavage Studies***

Each compound was initially dissolved in DMSO and further diluted in 50 mM Tris HCl, pH 7.5, at a proper stock concentration to achieve a final DMSO concentration of 2% in the 50 µL biochemical assay. In a black flat bottom 96-well plate, compound was incubated with either buffer, 5 nM human 20S proteasome or 5 nM of human 20S immunoproteasome, at 37 °C. After the designated time point, the 20S cleavage reaction was quenched by adding 50 µL acetonitrile. Samples were dried with a speed vacuum and then reconstituted in 105 µL of 50/50 water/acetonitrile with 0.1% formic acid. 100 µL of each sample was run on an Agilent 1260 Infinity II with a Zorbax StableBond- C18 1.8 micron, 2.1x50mm, column attached to an Agilent 6129 quadrupole mass spectrometer. The column was held at 30 °C. Flow rate was set at 0.450 mL/min. Mobile solution A was 0.1% formic acid in water, and mobile phase B was 0.1% formic acid in acetonitrile. The gradient used was held at 5% B for 2 min, increased linearly to 100% B for 8 min, and then held at 95% B for 2 min. The mass data was scanned in positive ion mode and collected at a range of 300 - 900 m/z starting 2 minutes after injection. MSD's spray chamber was set to API-ES (atmospheric pressure ionization – electrospray ionization), with drying gas flow at 12.0 L/min, nebulizer pressure at 35 psi, drying gas temperature at 300 °C, and positive capillary voltage at 3000 V. For each 20S treated sample, the mass of free Aminoluciferin was extracted (279.3 ± 0.5 Da) and the area under the curve was measured. To calculate percent release of Aminoluciferin, area under extracted ion for samples incubated with iCP were compared to mass of intact probe 3 without iCP or sCP as 100% intact.. The

solutions were diluted by adding 50 µL acetonitrile. Samples were dried with a speed vacuum and then reconstituted in 105 µL of 50/50 water/acetonitrile with 0.1% formic acid. For probe 6 (with linker) method was altered to flow rate was set at 0.7 mL/min. Mobile solution A was 0.1% formic acid in water, and mobile phase B was 0.1% formic acid in acetonitrile. The gradient used was held at 5% B for 2 min, increased linearly to 100% B for 8 min, and then held at 95% B for 2 min. The mass data was scanned in positive ion mode and collected at a range of 300 - 900 m/z starting 2 minutes after injection. MSD's spray chamber was set to API-ES (atmospheric pressure ionization – electrospray ionization), with drying gas flow at 12.0 L/min, nebulizer pressure at 35 psi, drying gas temperature at 300 °C, and positive capillary voltage at 3000 V. For each 20S treated sample, the mass of free Aminoluciferin was extracted (279.3 ± 0.5 Da) and the area under the curve was measured. To calculate percent release of Aminoluciferin, area under extracted ion for samples incubated with iCP were compared to mass of intact probe 6 without iCP or sCP as 100% intact.

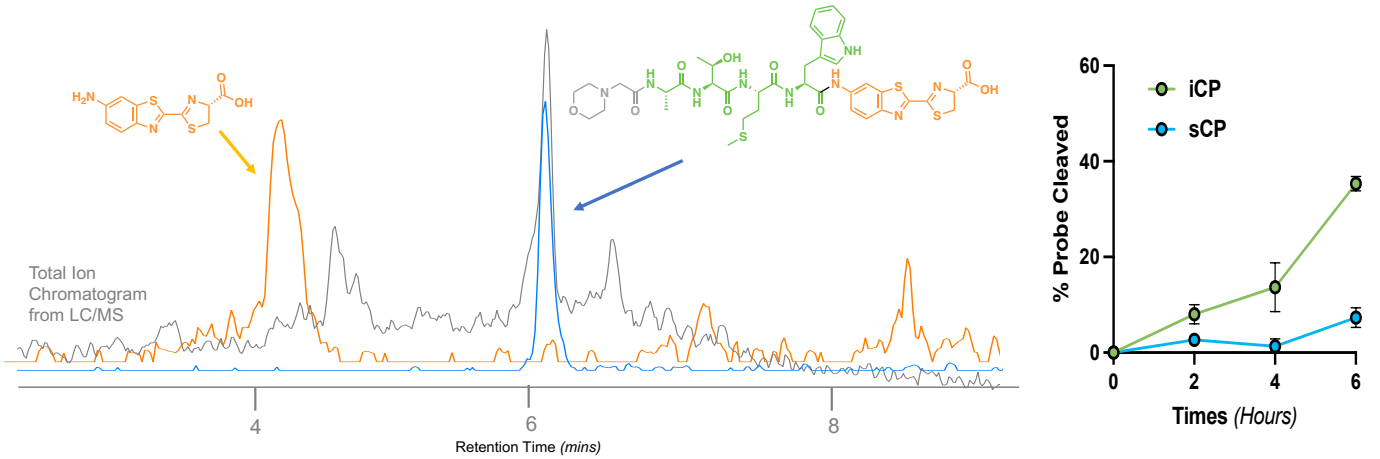



**Figure S1:** LCMS Cleavage Assay of **3** incubated with purified iCP (green) or sCP (blue) over 2 hours. From the total ion chromatogram (grey), masses for free aLuc (orange) and intact probe (blue) could be extracted. Probe **3** shows selectivity for the iCP over the sCP for the course of 6 hours.

### *Biochemical Luminescence Detection Assays*

**1.** *Dose-Dependent Probe Dosing:* 20 mM stocks of **6** and **3** were diluted to corresponding concentrations in 50 mM Tris-HCl buffer at pH~7.5. 45 µL of solution added to white 96 well plates in triplicate for each concentration. Purified Human Immunoproteasome (25 µg) or Standard Proteasome (50 µg) was prepared at 10X the desired concentration so that upon addition of 5 µL, sample will be diluted to 1x. 5µL added to each well excluding the buffer control wells. Plate was spun down gently and placed in a 37 ºC incubator for 1 hr. After 1 hr, plate was removed and 50 µL of Luciferin Detection Reagent (Promega) was added to each well. Plate was imaged on a BioTek Synergy™ Neo2 Multimode Microplate Reader with a gain of 105. All luminescent values were normalized to probe in buffer control to obtain normalized RLU units. These values were plotted in GraphPad Prism 10.

**2.** *ONX-0914 Inhibition:* Probes **6** and **3** were prepared at the corresponding concentrations as mentioned above. ONX-0914 (HY-13207) was prepared at desired concentration by initially preparing a 50X stock in DMSO. The stock is then diluted in probe/buffer solution to give 1X ONX at 2% DMSO. The solution is added to a white 96 well plate in triplicate for each concentration. 5 nM immunoproteasome prepared as previously mentioned and added to all wells except buffer control wells. Plate was spun down gently and placed in a 37 ºC incubator for 1 hr. After 1 hr, plate was removed and 50 µL of Luciferin Detection Reagent (Promega) was added to each well. Plate was imaged on a BioTek Synergy™ Neo2 Multimode Microplate Reader with a gain of 105. All luminescent values were normalized to probe in buffer control to obtain normalized RLU units. These values were plotted in GraphPad Prism 10.

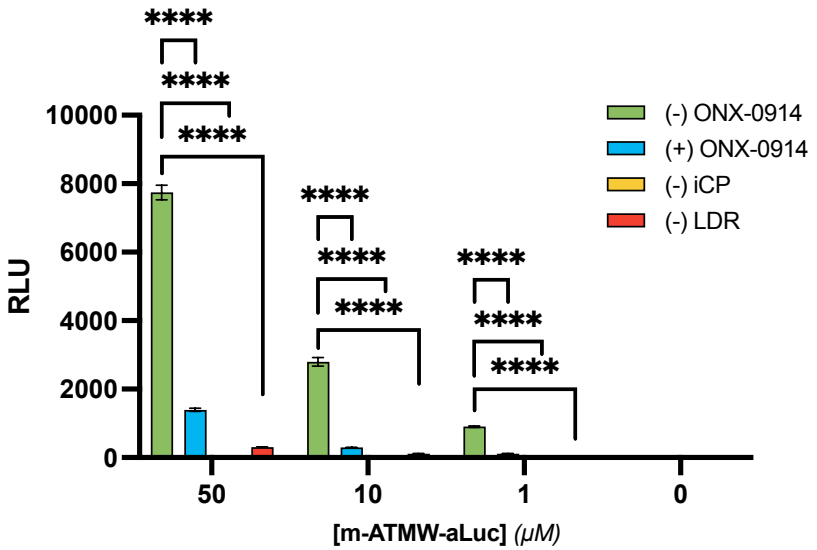



**Figure S2:** m-ATMW-aLuc probe incubated with and without iCP inhibitor ONX-0914 (25 µM), or without iCP for 1 hr, followed by addition of LDR or no LDR (red) to demonstrate signal is selective for iCP and signal can only be achieved through interaction with iCP and luciferase enzyme.

***General Cell Culture***

Ramos (ATCC® CRL-1596™) were grown in RPMI-1640 (ATCC® 30-2001™) supplemented with 10% FBS. 4T1-Luc-mRFP (gift from Prescher Lab) were grown in DMEM (Dulbecco's Modified Eagle's Medium, ATCC® 30-2002™) supplemented with 10% FBS. All cells were grown in a humidified 37 ºC incubator at 5% $CO_2$. For suspension cells (Ramos) the cell media was pelleted @ 1,000 xg for 5 min. For adherent cells (4T1-Luc) the media was aspirated, and the flask rinsed with sterile PBS before treating cells with 0.25% (w/v) Trypsin-0.53 mM EDTA solution. The flasks were returned to the incubator for 5 min. Trypsin was quenched with supplemented media, suspension was collected and cells pelleted @ 1000 xg for 5 min. After aspirating media, the cell pellet was resuspended in fresh media with 1% FBS. Cell concentration was determined by Trypan Blue, a hemocytometer and plated accordingly.

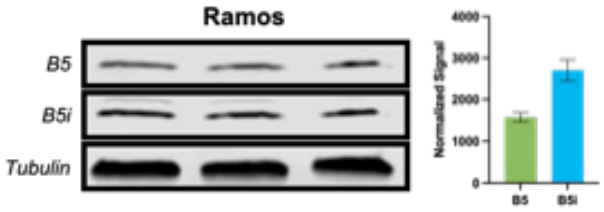



**Figure S3:** Ramos cells subjected to western blot for beta-5 and beta-5i (right). Normalized signal (left) shows relatively high amounts of beta-5i without treatment.

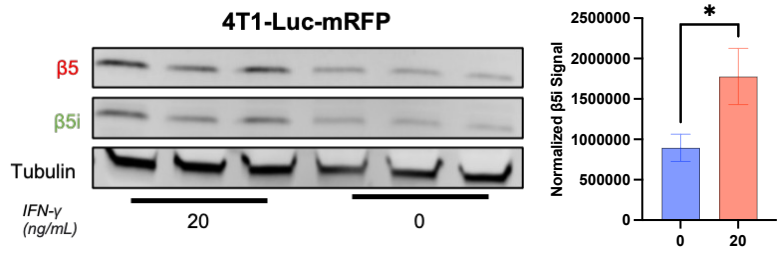



**Figure S4:** 4T1-Luc cells after treatment with IFN-gamma or DMSO for 72 hr followed by western blot analysis of Beta-5 and Beta-5i (left). Increase in Beta-5i upon treatment of 20 ng/mL IFN-gamma (right).

### *Cell Viability Assay (Adherent)*

4T1-Luc cells were plated in 96 well opaque plates at a density of 10,000 cells/well (100 µL per well of a 100,000 cells/mL stock). 200 µL of PBS was added to surrounding wells. Cells were left to adhere overnight in cell incubator. Probe was prepared at 1X target concentration at 0.5 % DMSO in cell media (1% FBS). 100 uL of media removed from each well and 100 uL of compound/media was added to the corresponding wells. Cell plate was placed into the incubator for 24 hours. After 24 hours, cell plate was removed and brought to r.t. for 30 mins. 90 µL of Cell Titer-Glo® luminescent reagent was added to each well. Plate was gently shaken for 2 minutes. Plate was then protected from light for 10 mins prior to being measured. Luminescence was recorded using BioTek Synergy™ Neo2 Multimode Microplate Reader with a gain of 105. Data was plotted in GraphPad Prism 10.

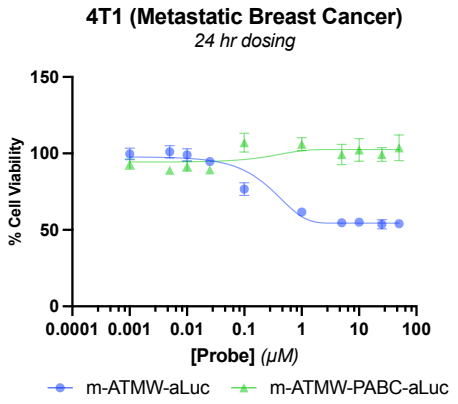



**Figure S5:** Cell viability of m-ATMW-aLuc probe in 4T1-Luc cells. *No cell death at the relevant concentrations or time points.

### *Cell Viability Assay (Suspension Cells)*

Ramos cells were plated in 96 well opaque plates at a density of 10,000 cells/well (50 µL per well of a 200,000 cells/mL stock). 200 µL of PBS was added to surrounding wells. Cells were then treated with 10 µL of 2X target concentration of compound stocks prepared at an initial 1% DMSO solution in cell media (1% FBS), in triplicate. This dilution makes the final 100 µL solution 0.5% DMSO overall at 1x the target concentration of compound. Cell plate was placed into the incubator for 24 hours. After 24 hours, cell plate was removed and brought to r.t. for 30 mins. 90 µL of Cell Titer-Glo® luminescent reagent was added to each well. Plate was gently shaken for 2 minutes. Plate was then protected from light for 10 mins prior to being measured. Luminescence was recorded using BioTek Synergy™ Neo2 Multimode Microplate Reader with a gain of 105. Data was plotted in GraphPad Prism 10.

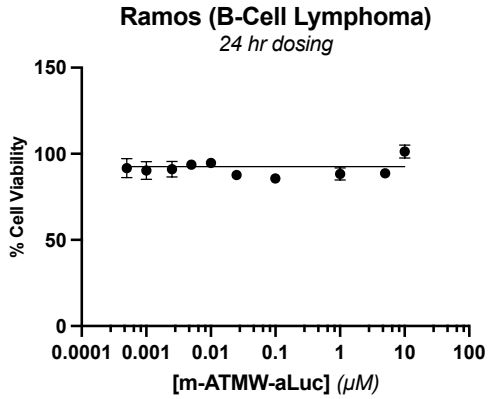



**Figure S6:** Cell viability of m-ATMW-aLuc probe in Ramos cells. *No cell death at the relevant concentrations or time points.

### *Cell-based Assay (Suspension Cells)*

Ramos cells were plated in 96 well white plate at a density of 20,000 cells/well (50 µL of 400,000 cells/mL stock). Probe was prepared at 2x concentration desired so that once addition of 50 µL probe in media was added to 50 µL of cell (total 100 µL in assay) the final concentration is at 1x with 0.5% DMSO. Cells were allowed to incubate with probe for 1 hr. After 1 hr, cells were supplemented with Luciferin Detection Reagent and luminescence read on Synergy™ Neo2 Multimode Microplate Reader with a gain of 105. Data was plotted in GraphPad Prism 10.

### *Cell-based Assay (Adherent Cells)*

1. *Dose-Dependent Probe Dosing:* 4T1-Luc cells were plated in a 96 well white plate at 20,000 cell/well (100 µL from a 200,000 cell/mL stock) and allowed to adhere overnight at 37ºC w/ 5% $CO_2$. The following day, probes were prepared at desired concentration by diluting in media at 1X desired concentration. Cell media was removed, and probe/media was added to each well (100 µL). Control well with no cells, just probe and media was used for normalization later. Cells were incubated with probes for 4 hr before reading luminescence on Synergy Neo™ 2 Multimode Microplate Reader with gain of 105. Cells were also supplemented with Luciferin Detection Reagent and read again to determine if adding the reagent caused any additional effect. Probes were normalized to their buffer control and plotted in GraphPad Prism 10.

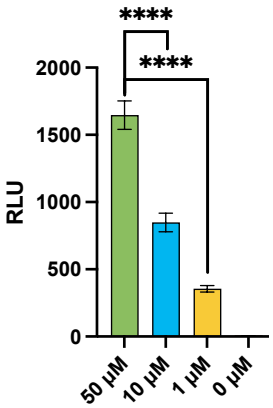



**Figure S7:** Dose dependent probe analysis with Ramos cells. Cells dosed with probe at various concentrations for 1 hr, followed by addition of Luciferin Detection Reagent. Signals are normalized to just probe in media control and plotted in GraphPad Prism.

2. *ONX-0914 Inhibitor Dosing:* 4T1-Luc cells were plated as mentioned before. After cells had adhered, 25 µM ONX-0914 was prepared. Media was removed from the desired wells and ONX-0914/media added (100 µL) and incubated for 1 hr. After 1 hr, media removed, and probe prepared at desired concentrations added to corresponding cells and allowed to incubate for 4 hrs. After 4 hrs, luminescence read on Synergy Neo™ 2 Multimode Microplate Reader with gain of 105. Cells were also supplemented with Luciferin Detection Reagent and read again to determine if adding the reagent caused any additional effect. Probes were normalized to their buffer control and plotted in GraphPad Prism 10.
3. *Cellular Limit of Detection*: 4T1-Luc cells were plated at 5000, 10,000, 20,000, and 40,000 cells/well (100µL of 50,000, 100,000, 200,000, 400,000 cell/mL stock respectively) and allowed to adhere overnight at 37ºC w/ 5% $CO_2$. The following day, media removed, and probe/media added at the desired concentration. Probe allowed to incubate with cells for 1 hr. After 1 hr, luminescence read on Synergy Neo™ 2 Multimode Microplate Reader with gain of 105. Cells were also supplemented with Luciferin Detection Reagent and read again to determine if adding the reagent caused any additional effect. Probes were normalized to their buffer control and plotted in GraphPad Prism 10.

***Biochemical Comparison Assay (sCP probe vs iCP probe)***

*Limit of Detection:* Probes **6** and **3** and **Suc-LLVY-aLuC** were prepared at 10 µM following method described above. 45 µL of solution added to white 96 well plates in triplicate for each concentration of proteasome. Purified Human Immunoproteasome (25 µg) or Standard Proteasome (50 µg) was prepared at 10X the desired concentration so that upon addition of 5 µL, sample will be diluted to 1x. This was done for concentrations from 0.1 pM – 5000 pM. 5µL was added to wells corresponding to probes, excluding buffer control wells. Plate was spun down gently and placed in a 37 ºC incubator for 1 hr. After 1 hr, plate was removed and 50 µL of Luciferin Detection Reagent (Promega) was added to each well. Plate was imaged on a BioTek Synergy™ Neo2 Multimode Microplate Reader with a gain of 105. All luminescent values were normalized to probe in buffer control to obtain normalized RLU units. These values were plotted in GraphPad Prism 10.

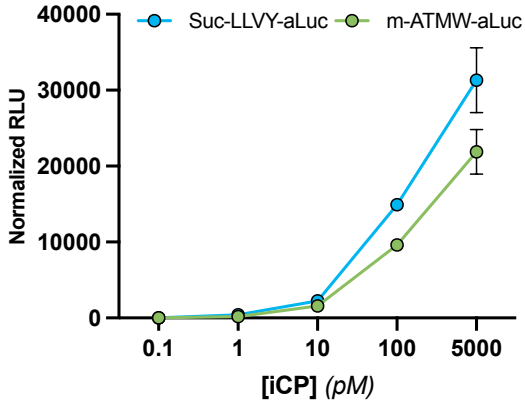



**Figure S8:** iCP probe (m-ATMW-aLuc) compared to sCP probe (Suc-LLVY-aLuc) with purified iCP. Signals from both probes are similar, demonstrating similar sensitivity for the iCP, however the Suc-LLVY-aLuc is not selective making it not ideal in cellular or *in vivo* settings.

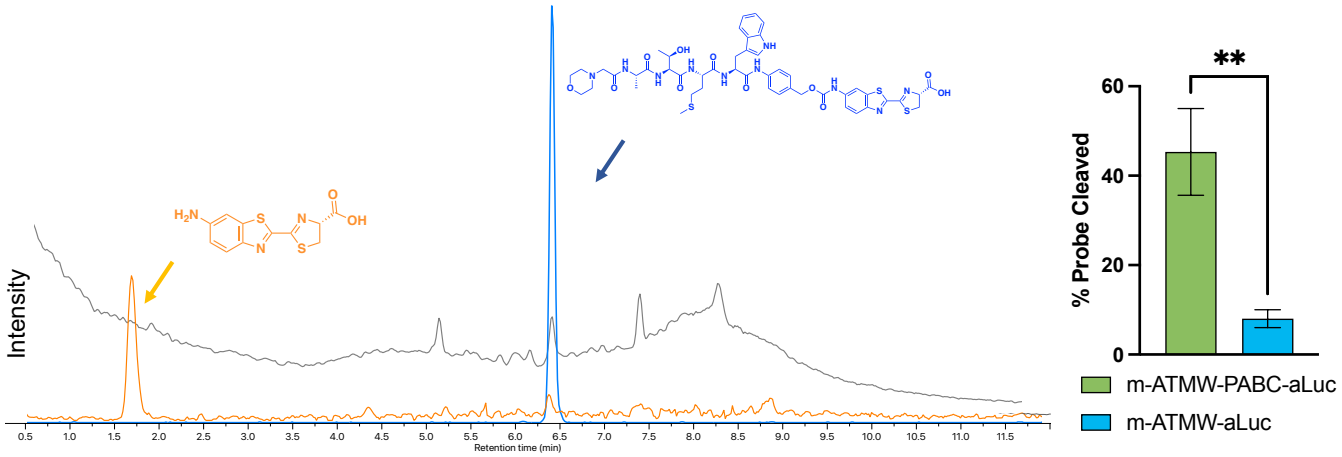



**Figure S9:** LCMS Cleavage Assay of **6** incubated with purified iCP . From the total ion chromatogram (grey), masses for free aLuc (orange) and intact probe (blue) could be extracted (Left). Probe **6** shows significantly more cleavage at 2 hrs compared to probe **3** at the same time point (right) indicating the linker allows for more probe to be cleaved by the iCP as hypothesized.

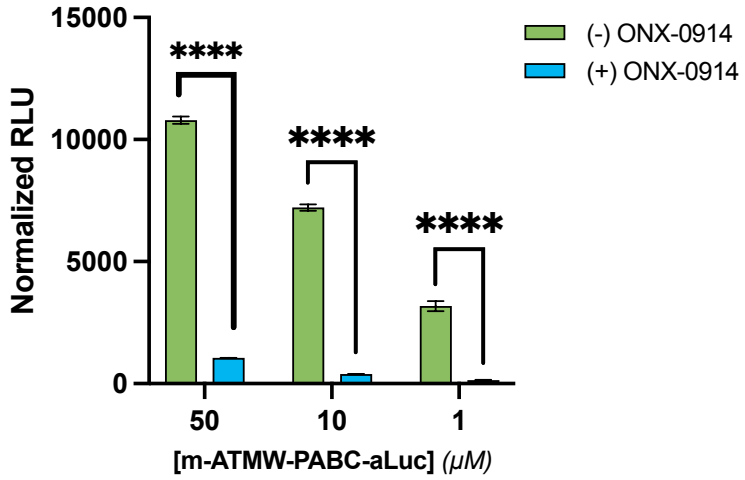



**Figure S10**: Biochemical evaluation of **6** with iCP inhibitor ONX-0914 to demonstrate selectivity for Beta-5i. ONX-0914 (25 μM) was dosed at the same time as probe at various concentrations. Almost complete shut down of luminescent signal in the ONX-0914 treated samples indicate our probe is working by cleavage by the Beta-5i subunit.

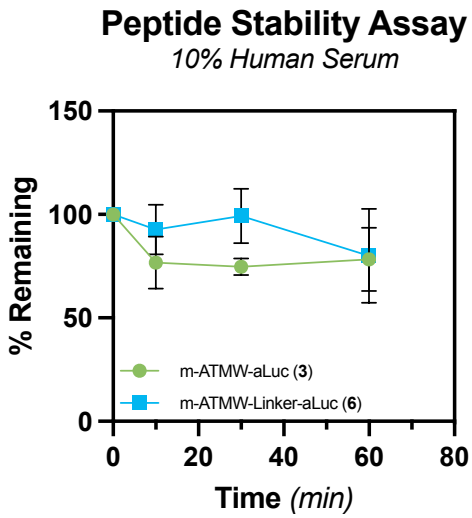



**Figure S11**: Serum Stability of Probes. Probe incubated with 10 % Human Serum Albumin or Buffer for desired time points then precipitated with chilled acetonitrile. After removal of precipitated protein sample injected on LCMS and extracted ion for intact probe compared to buffer control to determine % remaining.

***Serum Stability of Probes*** Each compound is initially dissolved in DMSO and further diluted in 50 mM Tris HCl, pH 7.5, at a stock concentration to achieve a final DMSO concentration of 2% in the 50 µL biochemical assay. In a polypropylene 96-well plate, compound was incubated with buffer or 10% human serum at 37ºC. After desired time points, proteins from human serum were precipitated by adding chilled acetonitrile (to make final amount of acetonitrile ~85%). Samples vortexed and stored at -20 ºC for at least 2 hrs. Samples centrifuged at 21,000 xg for 30 mins at 4ºC. 300 µL of supernatant was carefully removed and transferred to fresh eppindorf tube. Samples dried with a speed vacuum and then reconstituted in 100 uL of 50/50 water/acetonitrile with 0.1% formic acid and injected into LCMS.

***Bioluminescence Imaging***

Images were captured on an Andor iXon Ultra 888 EMCCD camera (Oxford Instruments) equipped with an F 0.95 lens (Schneider), with an EM gain of 1000. Images were acquired (300 s acquisitions) over 10 min. Images were Z-stacked in ImageJ and densitometry was used to measure peak intensity values. 4T-1 cells plated in black 96 well plates and allowed to adhere overnight. Media removed the following day and supplemented with fresh media +ONX-0914 or DMSO for 1 hr before dosing with probe at various concentrations for another hour. Plate was then transferred to dark room where images were taken on camera described above. Images were transferred to ImageStudio and values normalized to probe in media (no cell) control.

***Turkey Bacon (Tissue Mimic)***

Cells were plated and dosed following the same method previously described. Before imaging, thin slices of Oscar Mayer Original Turkey Bacon (2 mm thick) were cut to fit the wells of a black 96 well plate. The cells were imaged following the same method previously described with an acquisition time of 5 mins. Normalization performed by subtracting amount of signal from probe with LDR with cells to no cell background. % signal that passes is calculated by taking the normalized luminescence with bacon and dividing by the normalized luminescence without x100. (~5% signal passes barrier). Images were plotted in GraphPad Prism.

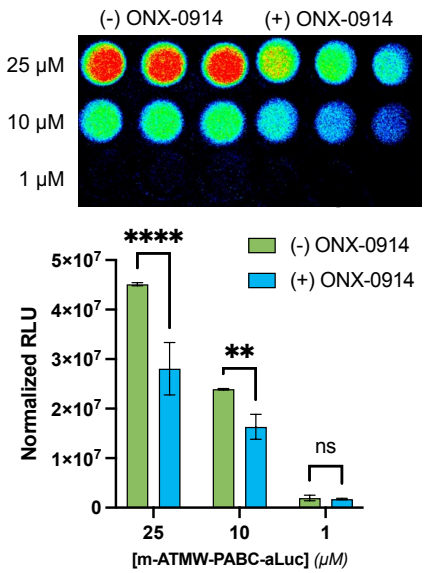
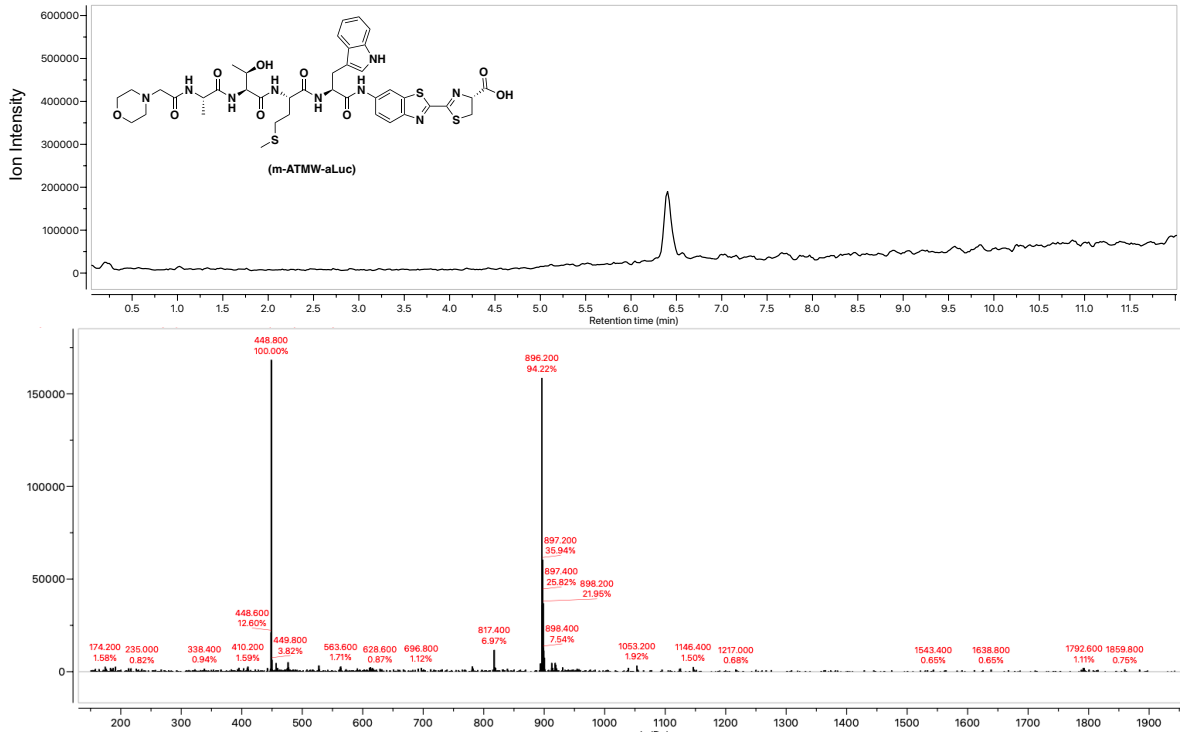
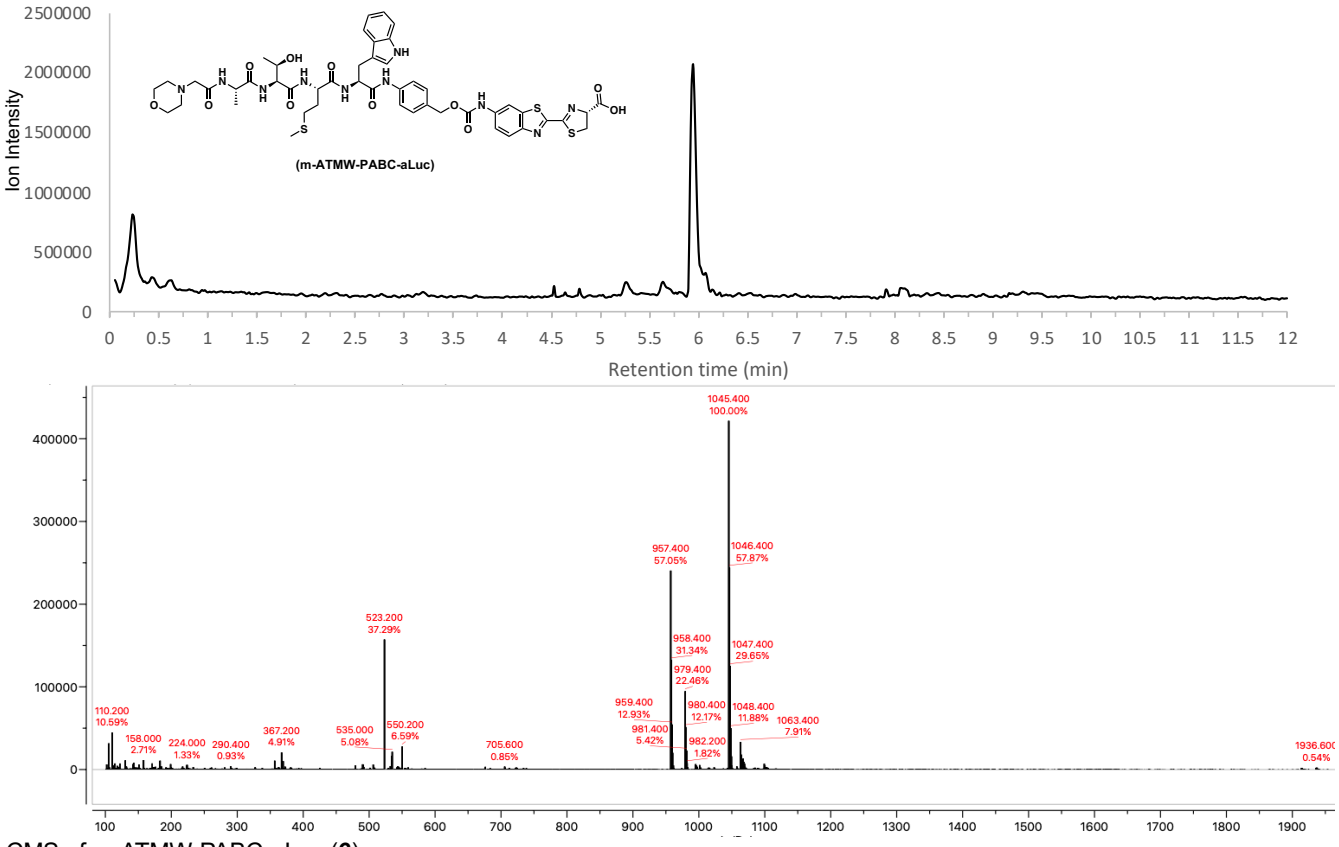



**Figure S12**: Bioluminescent imaging with Andor iXon Ultra 888 EMCCD camera. Cells treated with ONX-0914 show less signal than the DMSO control demonstrating the probe is being activated by the Beta-5i subunit.

## *Appendix*




LCMS of m-ATMW-aLuc (**3**).




LCMS of m-ATMW-PABC-aLuc (**6**).