Supplement to:

Pharmacologic Targeting of Plasma Cell Endoplasmic Reticulum Proteostasis to Reduce Amyloidogenic Light Chain Secretion

Bibiana Rius[1], Jaleh S. Mesgarzadeh[1], Isabelle C. Romine[1], Ryan J. Paxman[2], Jeffery W. Kelly[2,3], and R. Luke Wiseman[1,*]

[1]Department of Molecular Medicine, The Scripps Research Institute, La Jolla, CA 92037, [2]Department of Chemistry, The Scripps Research Institute, La Jolla, CA 92037, [3]The Skaggs Institute for Chemical Biology, The Scripps Research Institute, La Jolla, CA 92037

*Corresponding Author:
R. Luke Wiseman
Department of Molecular Medicine
The Scripps Research Institute
La Jolla, CA 92037
Email: wiseman@scripps.edu
Phone: (858) 784-8820

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Supplemental Methods

Chemicals

Compound **147**, **147-alkyne**, and all **147** analogs were synthesized in house and are described in (1). RB-11-ca and KSC-34 were both kind gifts from Eranthie Weerapana at Boston College (2, 3). Ceapin-7 (CP7) and Ceapin-5 (CP5) were kind gifts from Peter Walter at UCSF (4, 5). Cycloheximide (Fisher), resveratrol (SelleckChem), ISRIB (Sigma), MG132 (Selleckchem), chloroquine (Sigma), PF429242 (S1Pi; Tocris), β-mercaptoethanol (BME; Thermo Fisher), and bortezomib (MilliporeSigma) were all purchased commercially.

Antibodies

All antibodies were commercially purchased. Antibodies were used for immunoblotting at the indicated dilution: mouse human lambda light chain antibody (Novus Biological, NBP2-29462, 1:250); rabbit PDIA1 (PDI) antibody (GeneTex, GTX101468, 1:1000), rabbit PDIA4 (ERP72) antibody (ProteinTech, 14712-1-AP, 1:1000) and mouse PDIA6 antibody (ProteinTech 66669-1-Ig, 1:1000).

Quantitative RT-PCR

The mRNA levels of target genes were measured using quantitative RT-PCR on an ABI 7900HT Fast Real Time PCR machine using the same primers and identical approach to that published in (6).

Detailed ELISA Protocol.

Briefly, ALMC-2 or KAS-6/1 plasma cells were plated in 96-well MultiScreen$_{HTS}$ filtration plates (EMD Millipore). Cells were treated with DMSO or compounds at the indicated concentrations and incubated for 18 hr. Media was removed by filtration using a QIAvac 96 vacuum manifold (Qiagen) and wells were washed two times with media. Wells were then incubated with 150 µL of fresh media for 2 hr and the conditioned media was harvested into a 96-well plate using the vacuum manifold. Whole lysates were obtained by adding RIPA buffer to the cells in the filtration plates. Free LC and IgG concentrations were determined by ELISA in 96-well plates (Immulon 4HBX, Thermo Fisher). Wells were coated overnight at 37 ºC with sheep polyclonal free λ LC antibody (Bethyl Laboratories, A80-127A) or human IgG-heavy and light chain antibody (Bethyl Laboratories, A80-118A) in sodium carbonate (pH 9.6). In between all incubation steps, the plates were rinsed extensively with Tris-buffered

saline containing 0.05% Tween-20 (TBST). Plates were blocked with 5% non-fat dry milk in TBST for 1 hr at 37ºC. Media analytes were diluted in 5% non-fat dry milk in TBST and 100 µL of each sample was added to individual wells. Light chain or IgG standards were prepared from purified human Bence Jones λ light chain or human reference serum (Bethyl Laboratories, P80-127 and RS10-110). Plates were incubated at 37 ºC for 1.5 hr while shaking. Finally, HRP-conjugated goat anti-human λ light chain antibody (Bethyl Laboratories, A80-116P) was added or HRP-conjugate IgG-Fc fragment cross-adsorbed antibody (Bethyl Laboratories, A80-304P, was added in 5% non-fat dry milk in TBST, followed by a 1.5 hr incubation of the plates at 37 ºC. The detection was carried out with 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS, 0.18 mg/mL) and 0.03% hydrogen peroxide in 100 mM sodium citrate pH 4.0. Detection solution (100 µL) was added to each well and the plates were incubated at room temperature. The absorbance was recorded at 405 nm and the values for the LC standards were fitted to a 4-parameter logistic function.

Cell Viability and Apoptosis Assays.

ALMC-2 cells were plated at 33,000 cells/well in a translucent, flat-bottomed 96 well plate, and treated for 24 hr with vehicle, 147 (10 µM) or bortezomib (at the indicated doses). After incubation, cell metabolic activity or caspase 3/7 activity was measured using the CellTiter-Glo assay (Promega) and Caspase-Glo 3/7 assay (Promega), respectively, according to the manufacturer's instructions. Briefly, the plates containing cells were removed from the incubator and allowed to equilibrate to room temperature for 30 min. CellTiter-Glo or Caspase-Glo reagent was added to each well at a 1:1 v/v ratio and incubated for 2 min on an orbital shaker to induce cell lysis. The plate was then incubated at room temperature for 10 min to stabilize the luminescent signal and read on a Tecan F200 Pro microplate reader.

[^{35}S] Metabolic Labeling

For metabolic labeling experiments, ALMC-2 cells were plated at a density of 1x10^6 cells/well on a 6 well plate. Cells were then treated with **147** (10 µM), RB-11-ca (30 µM), and/or resveratrol (10 µM), as indicated. Cells were transferred to microfuge tubes, washed with PBS, and metabolically labeled in DMEM-Cys/-Met (Corning) supplemented with glutamine, penicillin/streptomycin, dialyzed fetal bovine serum, and EasyTag EXPRESS [^{35}S] Protein Labeling Mix (Perkin Elmer) for 30 min in a tissue culture incubator. Cells were washed twice with PBS

and lysates were harvested using RIPA buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.1 % SDS, 1% Triton X-100, 0.5% deoxycholate) and protease inhibitor cocktail (Roche). ALLC was immunopurified using human lambda LC coupled to Protein A Sepharose beads as described above. Lysates were incubated with the beads overnight at 4 degrees. Beads were washed 5 times with RIPA buffer. ALLC was then isolated by boiling in Laemmli buffer + 100 mM DTT and separated on SDS-PAGE. For whole cell lysates, cells were lysed in RIPA buffer. Laemmli buffer + 100 mM DTT was then added to the lysates and separated by SDS-PAGE. All gels were dried, exposed to phosphorimager plates (GE Healthcare), and imaged with a Typhoon imager. Band intensities were quantified by densitometry in ImageQuant.

Profiling of Targets for 147 and PDI Inhibitors

Proteins labeled by treatment with **147-alkyne**, RB-11-ca, or KSC34 in were performed as previously described (1). Briefly, ALMC-2 cells in 6-well plates were treated for 18 h with **147-alkyne** (10uM), RB-11-ca (30 μM) or KSC-34 (30 μM). Lysates were then prepared in RIPA buffer as described above. Protein concentrations were then quantified by Bradford. Lysates containing 50 μg of protein were then incubated for 2 h with shaking at 37ºC with 100 μM TAMRA azide (Click Chemistry Tools Scottsdale, Az), 800 μM copper(II) sulfate, 1.6 mM BTTAA ligand (2-(4-((bis((1-tert-butyl-1H-1,2,3-triazol-4-yl)methyl)amino)methyl)−1 H-1,2,3-triazol-1-yl)acetic acid) (Click Chemistry Tools Scottsdale, Az), and 5 mM sodium ascorbate. Samples were then boiled with Laemmli Buffer and 100mM DTT and separated by SDS-PAGE. TAMRA-labeled proteins were then imaged using a Bio-Rad ChemiDoc using the rhodamine channel.

Preparation of Protein A beads coupled to λ LC antibody

For immunopurification, protein A Sepharose 4B beads (101041, Thermo Fisher) were coupled to human lambda light chain antibody (A80-112A, Bethyl laboratories) (6). Briefly, beads were washed twice with RIPA buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.1 % SDS, 1% Triton X-100, 0.5% deoxycholate) and resuspended in 1 mL of PBS. Beads were then incubated with primary rabbit human lambda light chain antibody (40 uL of antibody for 70-90- μL of beads) and rocked for 1 hour at room temperature. Beads were washed 3 times with 0.2M borate buffer pH = 9. The antibody was crosslinked to the beads by incubating with 20 mM of the crosslinker dimethyl pimelimidate (DMP, Thermo Scientific, 20 μM) for 30 min at room temperature. The crosslinking reaction was

stopped by incubation with 0.2 M ethanolamine pH 8 for 2 h at room temperature. Beads were then washed with Glycine buffer (0.1M, pH=3) to remove uncrosslinked antibody, followed by two washes with PBS. Finally, beads were resuspended in storage buffer (PBS, Azide (0.01%)) and stored at 4ºC.

Supplemental Figure 1.

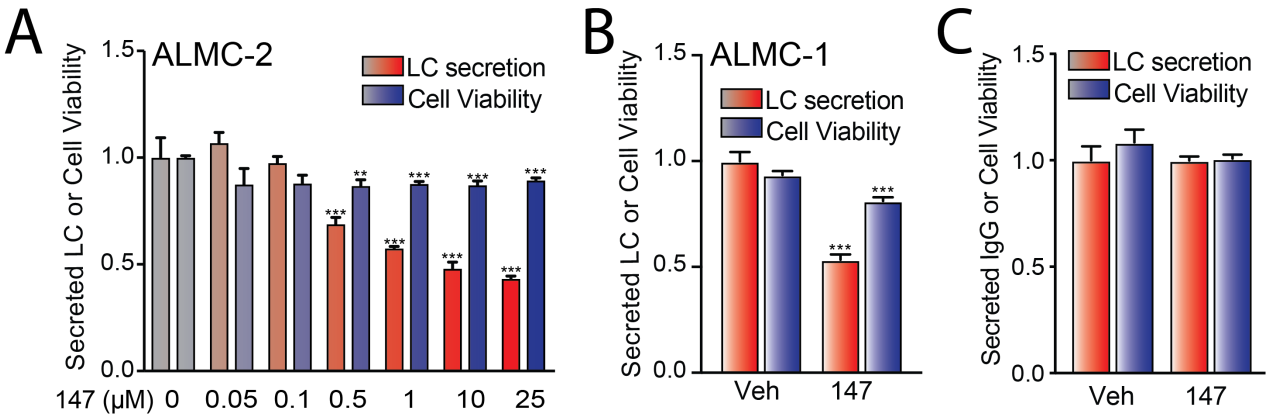

Figure S1 (Supplement to Figure 1). Compound 147 reduces secretion of ALLC from AL patient derived ALMC-2 cells.

- **A.** Graphs showing relative ALLC in conditioned media measured by ELISA (red) and cellular viability measured by CellTiter-Glo (blue) in ALMC-2 cells treated for 18 hr with vehicle or **147** (10 μM). Error bars show SEM for n= 4 replicates. ***p<0.005 vs Veh from an unpaired t-test.
- **B.** Graph showing relative ALLC in conditioned media measured by ELISA (red) and cellular viability measured by CellTiter-Glo (blue) in ALMC-1 cells treated for 18 hr with vehicle or **147** (10 μM). Error bars show SEM for n= 21 replicates across 4 independent experiments. ***p<0.005 vs Veh from an unpaired t-test.
- **C.** Graph showing relative media levels of IgG measured by ELISA (red) and cellular viability measured by CellTiter-Glo (blue) in KAS-6/1 cells treated with vehicle or **147** (10 μM) for 18 hr. Error bars show SEM for n = 5 replicates. ***p<0.005 vs Veh from an unpaired t-test.

Supplemental Figure 2

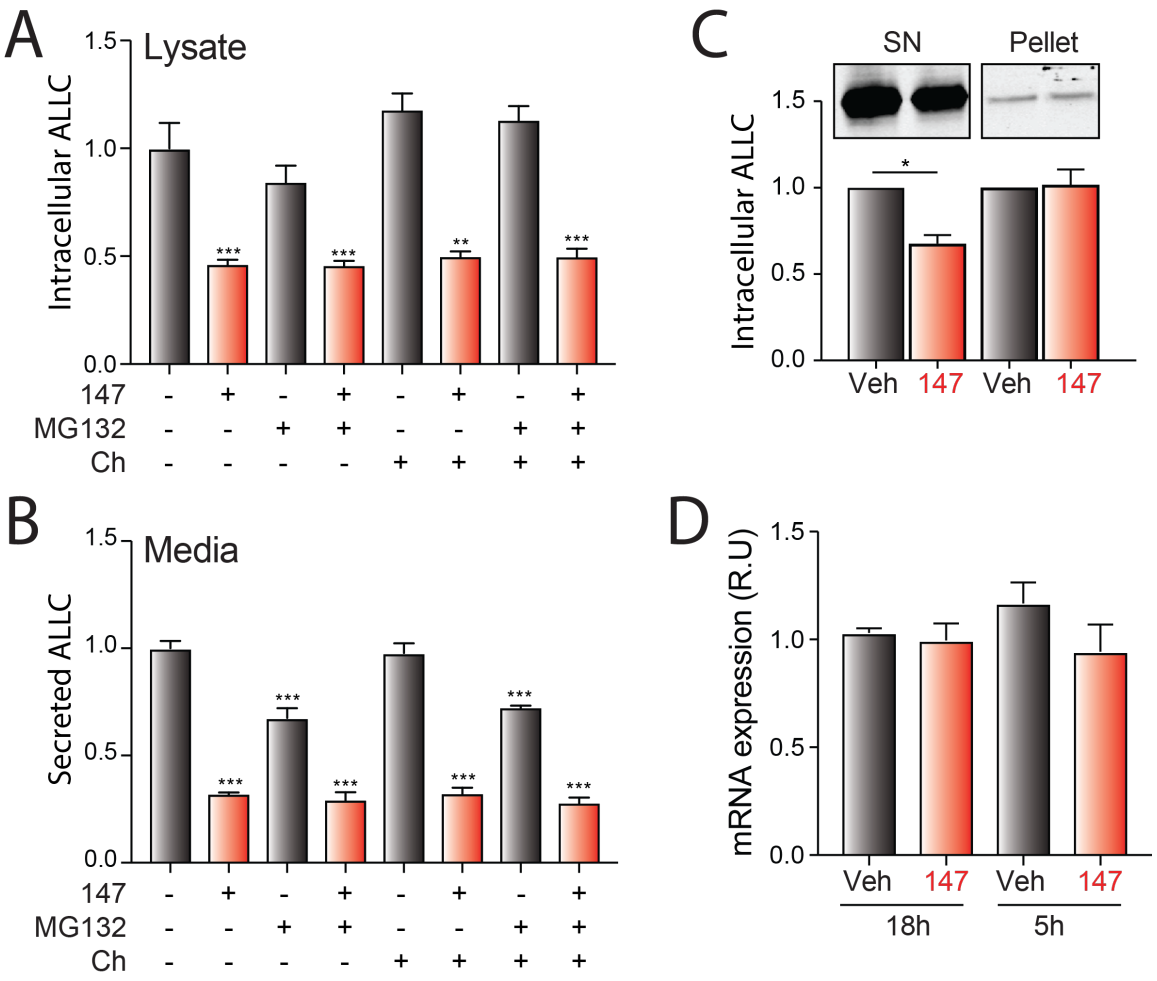

Figure S2 (supplement to Figure 2). Compound 147 reduces lysate levels of ALLC in ALMC-2 cells.

A. Bar graphs showing normalized amounts of ALLC in lysates prepared from ALMC-2 cells treated for 18 hr with vehicle or **147** (10 μM). MG132 (10 μM) or chloroquine (Ch, 20 μM) were added as indicated for the last 5 hours of this incubation and during the 2 hours of media conditioning. ALLC was quantified by ELISA. Error bars show SEM for n=5 replicates. *** $p<0.005$ vs Veh from an unpaired t-test.

B. Bar graphs showing normalized amounts of ALLC in conditioned media prepared from ALMC-2 cells treated for 18 hr with Veh, **147** (10 μM). MG132 (10 μM) or chloroquine (20 μM) were added for the last 5 hours of this incubation and during the 2 hours of media conditioning. ALLC was quantified by ELISA. Error bars show SEM for n=5 replicates. *** $p<0.005$ vs Veh from an unpaired t-test.

C. Representative immunoblot and normalized quantification of ALLC in fractionated lysates from ALMC-2 cells treated with Veh or **147** (10 μM) for 18 hr. The supernatant fraction (SN) is the soluble protein fraction obtained after lysing with RIPA buffer (no DTT). Pellets derived from the first lysis were then solubilized by treatment with SDS (2%), DTT and sonication. Error bars show SEM for n=3 independent replicates. *$p<0.05$ from a paired t-test

D. Bar graphs showing ALLC mRNA measured by qPCR in ALMC-2 cells treated with vehicle or **147** for 18 hr or 5 hr. Error bars show 95% confident intervals for n=3 replicates.

Supplemental Figure 3.

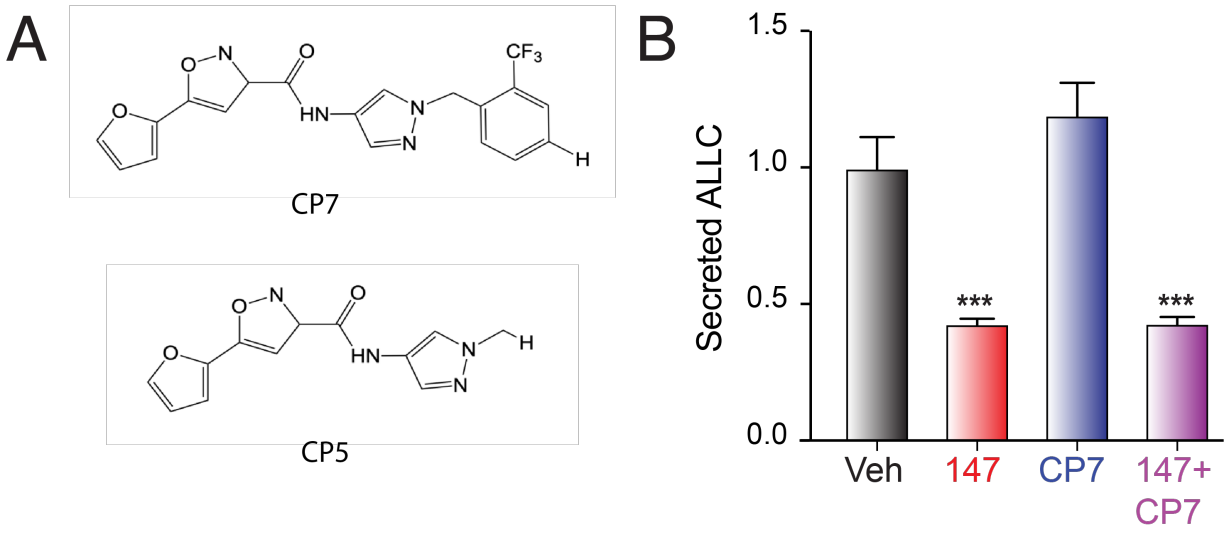

Figure S3 (Supplement to Figure 3). 147-Dependent Reductions in ALLC Secretion are Independent of ATF6 Activation.

A. Chemical structures of the ATF6 inhibitor CP7 and its inactive analog CP5 (4, 5).

B. Graph showing normalized ALLC in conditioned media prepared from ALMC-1 cells treated for 18 hr with vehicle, **147** (10 μM) and/or CP7 (10 μM), as indicated. ALLC was quantified by ELISA. Error bars show SEM for n=7 replicates. ***p<0.005 from an unpaired t-test.

Supplemental Figure 4

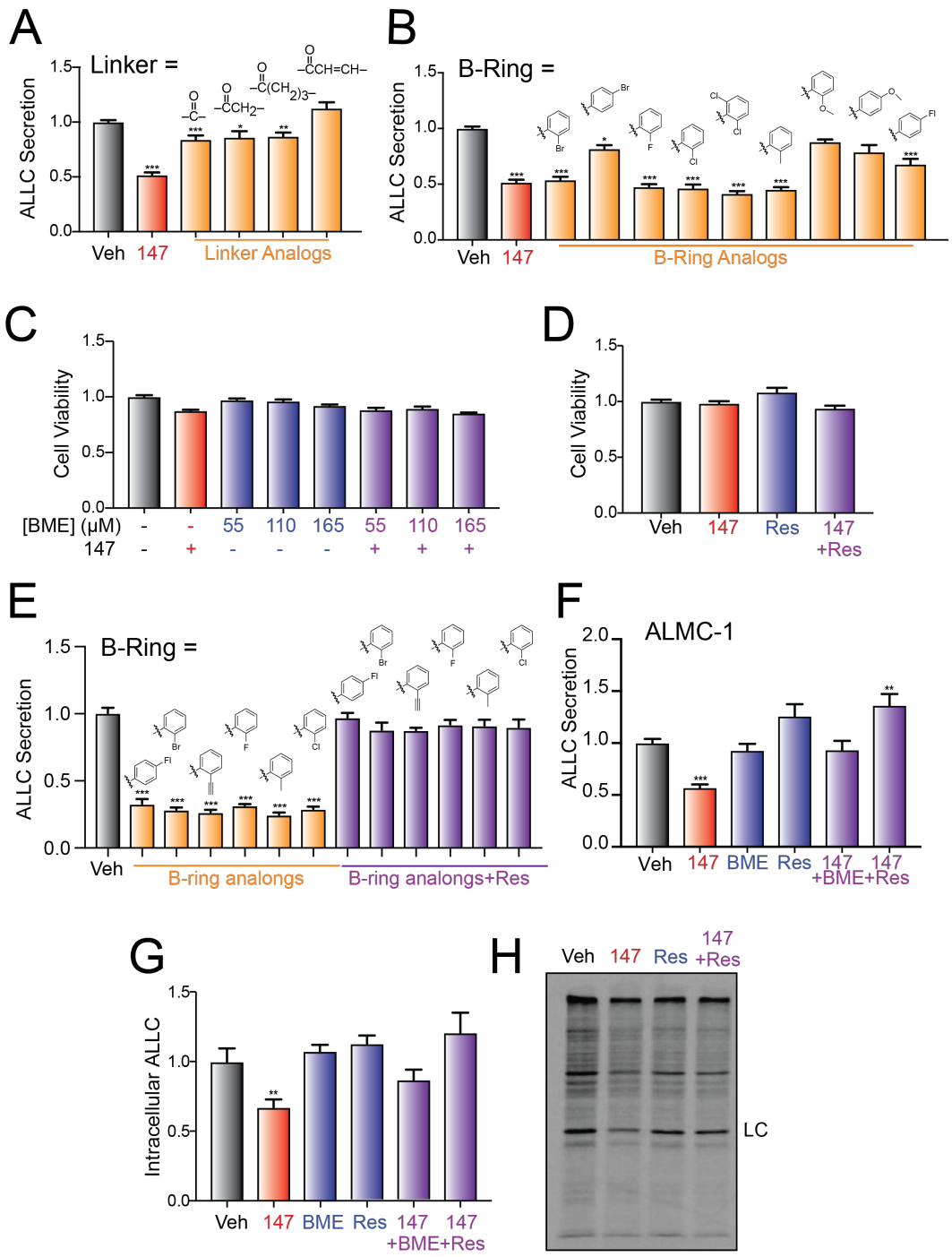

Figure S4 (Supplement to Figure 4). Compound 147 reduces ALLC secretion through a mechanism involving compound metabolic activation and covalent protein modification.

A. Graph showing normalized ALLC in conditioned media from ALMC-2 cells treated for 18 hr with vehicle, **147** (10 μM) or the indicated **147** 'Linker'-analog (10 μM). ALLC was quantified by ELISA. Error bars show SEM for n=10 replicates across two independent experiments. *p< 0.05, **p<0.01, ***p<0.005 vs Veh from an unpaired t-test.

B. Graph showing normalized ALLC in conditioned media from ALMC-2 cells treated for 18 hr with vehicle, **147** (10 μM) or the indicated **147** 'B' ring'-analog (10 μM). ALLC was quantified by ELISA. Error bars show SEM for n=10 replicates across two independent experiments. *p< 0.05, **p<0.01, ***p<0.005 vs Veh from an unpaired t-test.

C. Graph showing normalized quantification of cell viability measured with CellTiter-Glo of ALMC-2 cells treated with vehicle, **147** (10 μM) and/or β-mercaptoethanol (BME) for 18 hr. Error bars show SEM for n=5 replicates.

D. Graph showing normalized quantification of cell viability measured with CellTiter-Glo of ALMC-2 cells treated with vehicle, **147** (10 μM) and/or resveratrol (Res: 10 μM) for 18 hr. Error bars show SEM for n=10 replicates across two independent experiments.

E. Graph showing normalized ALLC in conditioned media from ALMC-2 cells treated for 18 hr with vehicle, **147** (10 μM) or 'B ring'-analog (10 μM) with or without resveratrol (10 μM). Error bars show SEM for n>3 replicates. ***p<0.005 vs Veh from an unpaired t-test.

F. Graph showing normalized ALLC in conditioned media from ALMC-1 cells treated for 18 hr with vehicle, **147** (10 μM), resveratrol (10 μM), or β-mercaptoethanol (BME: 165 μM), as indicated. ALLC was quantified by ELISA. Error bars show SEM shows n=10 replicates across two independent experiments. **p<0.01, ***p<0.005 vs Veh from an unpaired t-test.

G. Graph showing the normalized amounts of ALLC in lysates prepared from ALMC-2 cells treated for 18 hr with vehicle, **147** (10 μM), resveratrol (10 μM), or β-mercaptoethanol (BME: 165 μM), as indicated. ALLC was quantified by ELISA. Error bars show SEM shows n=5 replicates. **p<0.01 vs Veh from an unpaired t-test.

H. Representative autoradiogram of whole cell lysates prepared from ALMC-2 cells treated for 18 hr with vehicle, **147** and/or resveratrol (10 μM) and then metabolically labeled with [^{35}S] for 30 min.

Supplemental Figure 5

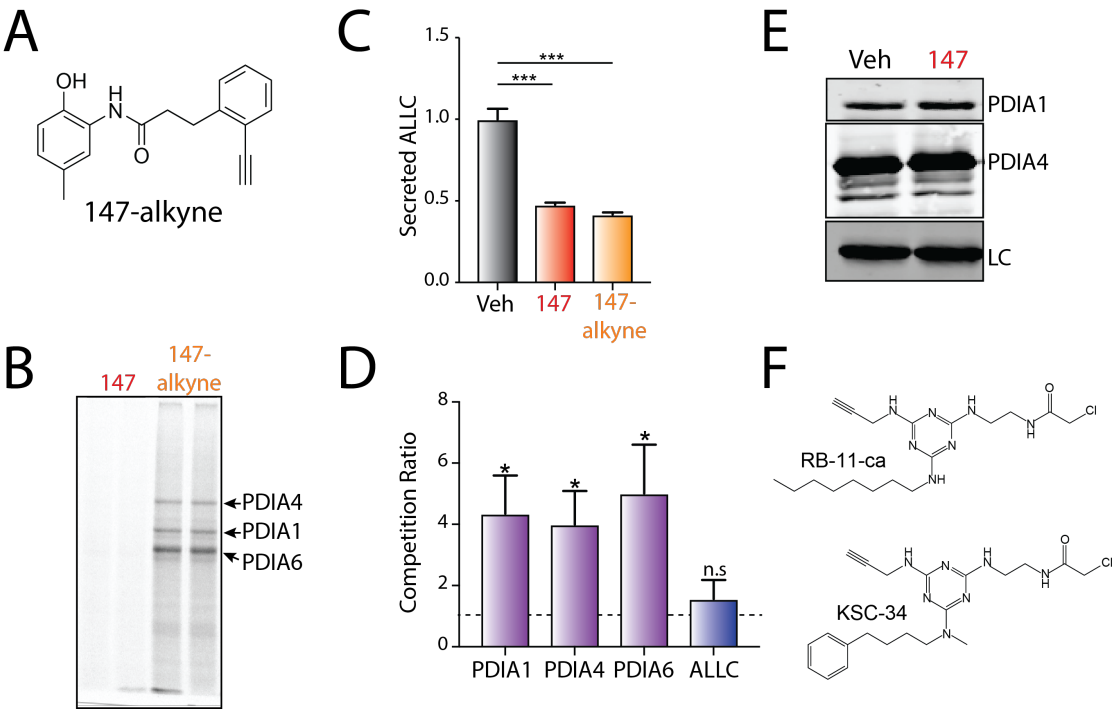

Figure S5 (Supplement to Figure 5). PDI interactions with ALLC are disrupted by 147 treatment.

- **A.** Chemical structure of **147-alkyne**.
- **B.** Representative gel showing the covalent modification of proteins in ALMC-2 cells treated for 18 hr with **147** (10 μM) or **147-alkyne** (10 μM). Click chemistry was used to incorporate a TAMRA fluorophore onto the alkyne contained in each of these molecules. Cells treated with **147** are shown as a control. The PDI bands were assigned based on previous mass spectrometric analysis of SDS-PAGE bands excised from identical gels of **147-alkyne** treated ALMC-2 cell lysates (1).
- **C.** Graph showing ALLC secreted from ALMC-2 cells treated for 18 hr with vehicle, **147** (10 μM) or **147**-alkyne (10 μM). Error bars show SEM for n=10 replicates across two independent experiments. ***p<0.005 from an unpaired t-test.
- **D.** Graph showing competition ratio of the indicated PDI or ALLC in affinity purifications isolated from ALMC-2 cells treated for 18 h with **147-alkyne** (10 μM) or **147-alkyne** (10 μM) and an excess of **147** (50 μM). These data are from experiments described in (1). Briefly, after incubation with ALMC-2 cells, **147-alkyne** was modified with a biotin allowing isolation of the proteins covalently modified by **147-alkyne** using streptavidin affinity purification. The recovery of different proteins across conditions was then quantified using tandem mass tag (TMT) labeling and multi-dimensional protein identification technology (MuDPIT). The competition ratio was calculated as previously described using the equation: competition ratio = protein signal from cells treated with **147-alkyne** treated / protein signal from cells treated with **147-alkyne** and excess **147** (1). Error bars show SEM for n=4 paired replicates across two independent experiments. *p<0.05 from a paired t-test. (n.s), not significant.
- **E.** Immunoblot showing lysate levels of PDIA1, PDIA4 and ALLC in ALMC-2 cells treated for 5 hr with vehicle or **147** (10 μM). These are inputs from the immunopurifications shown in **Figure 5A**.
- **F.** Chemical structures of the PDI inhibitors RB-11-ca and KSC-34.

Supplemental Figure 6

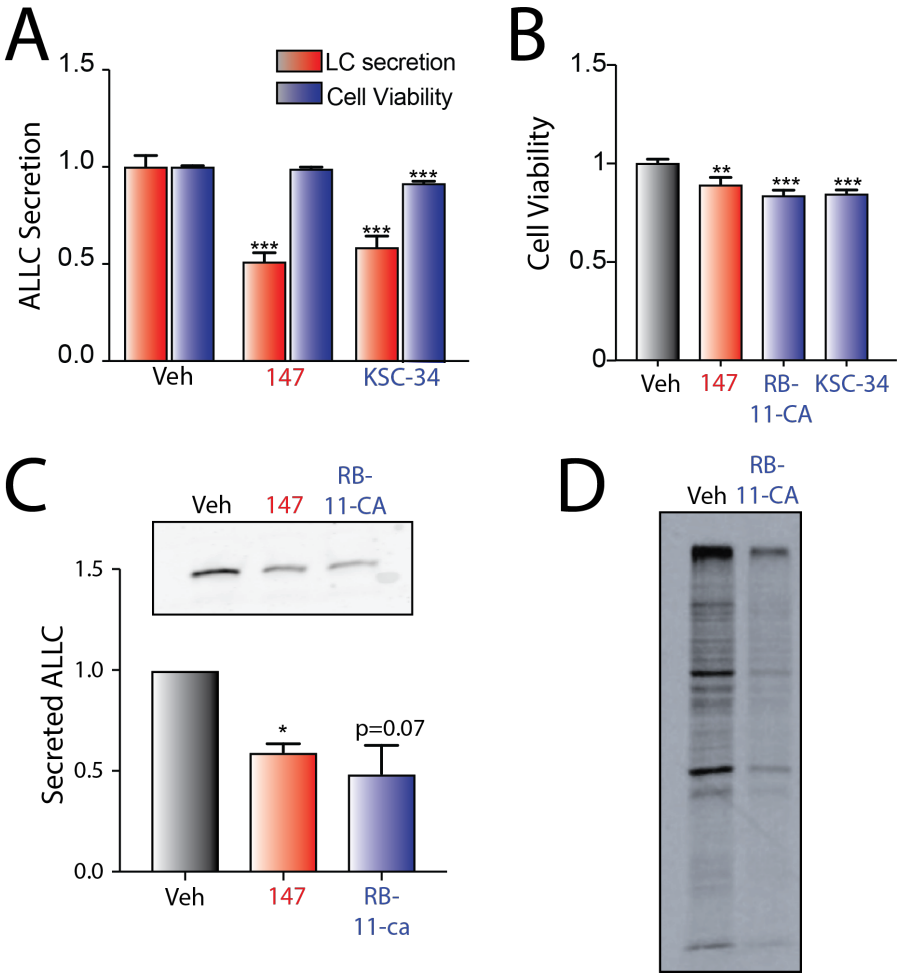

Figure S6 (Supplement to Figure 6). Pharmacologic targeting of PDIs reduces ALLC secretion from ALMC-2 cells

- **A.** Graph showing relative ALLC in conditioned media measured by ELISA (red) and cellular viability measured by CellTiter-Glo (blue) in ALMC-1 cells treated for 8 hr with vehicle, **147** (10 μM), or KSC-34 (30 μM). Error bars show SEM for n= 8 replicates across 2 independent experiments. ***p<0.005 vs Veh from an unpaired t-test.
- **B.** Graph showing the viability measured by CellTiter Glo for ALMC-2 cells treated for 18 hr with vehicle, **147** (10 μM), RB-11-ca (30 μM), or KSC-34 (30 μM). ALLC was quantified by ELISA. Error bars show SEM for n=11 replicates across 2 independent experiments. ***p<0.005 vs Veh from an unpaired t-test.
- **C.** Representative immunoblot and quantification of ALLC in conditioned media prepared from ALMC-2 cells treated with vehicle, **147** (10 μM) or Rb-11-ca (30 μM) for 18 h. Error bars show SEM for n=3 independent experiments. *p<0.05 vs Veh from a one-tailed paired t-test.
- **D.** Representative autoradiogram of whole cell lysates prepared from ALMC-2 cells treated for 18 h with vehicle, **147** (10 μM) or Rb-11-ca (30 μM) and then metabolically labeled with [^{35}S] for 30 min.

Supplemental Figure 7

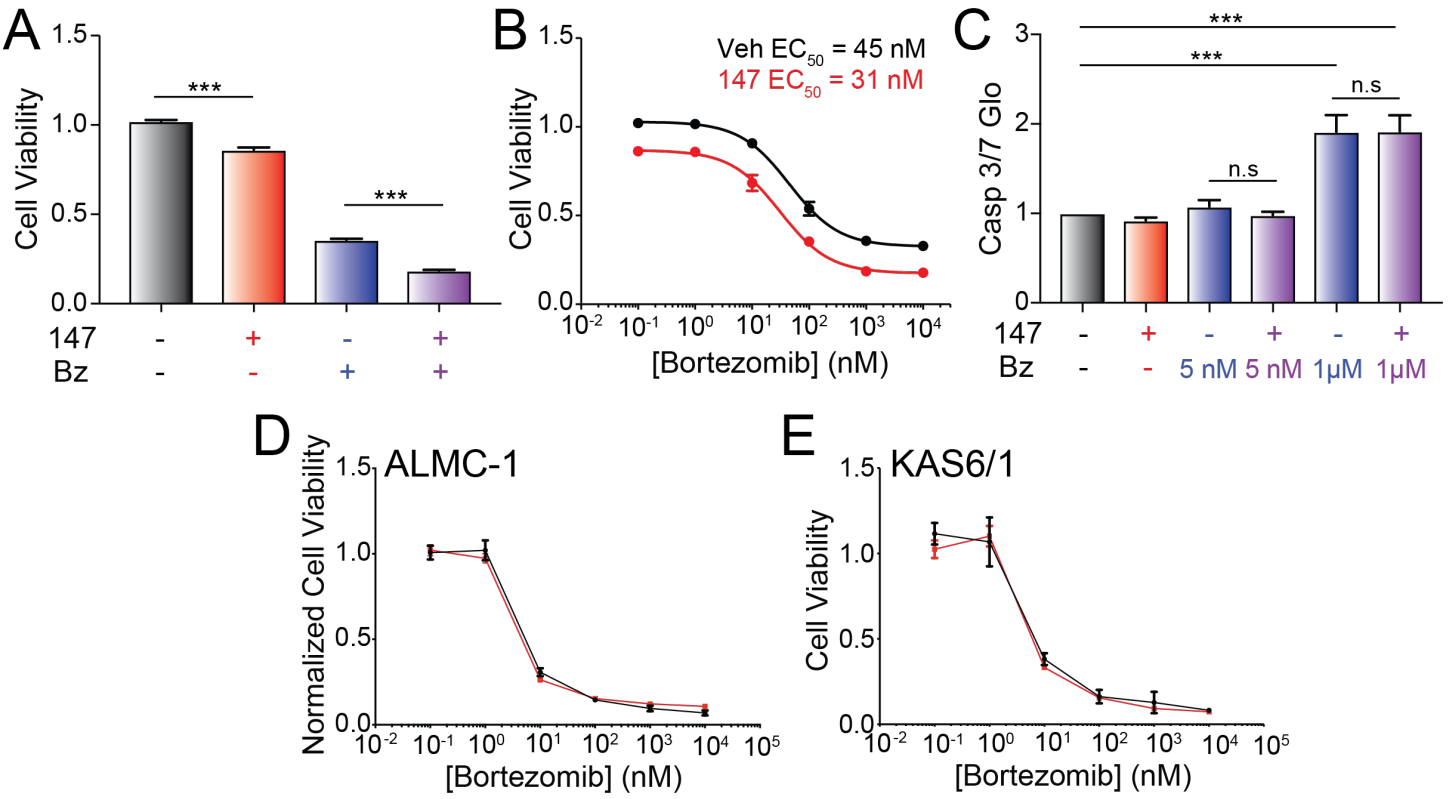

Figure S7 (Supplement to Figure 7). Compound 147 does not influence bortezomib toxicity in ALMC-2 plasma cells

- **A.** Graph showing normalized quantification of viability measured by CellTiter-Glo of ALMC-2 cells treated with vehicle, **147** (10 μM) and/or bortezomib (1 μM) for 24h. Error bars show SEM for n=5 replicates. ***p<0.05 from an unpaired t-test.
- **B.** Graph showing the viability measured by CellTiter-Glo of ALMC-2 cells treated with **147** (10 μM) and/or increasing concentrations of bortezomib (0-10 μM) for 24 h. Error bars show SEM for n=5 replicates.
- **C.** Graph showing normalized caspase 3/7 activation in ALMC-2 cells treated with **147** (10 μM) and/or the indicated concentration of bortezomib (Bz) for 24 h. Error bars show SEM for n=6 replicates across two independent experiments. ***p<0.005 from an unpaired t-test. (n.s), not significant
- **D.** Graph showing the normalized viability measured by CellTiter-Glo of ALMC-1 cells treated with **147** (10 μM) and/or increasing concentrations of bortezomib (0-10 μM) for 24 h. Error bars show SEM for n=10 replicates across 2 independent experiments.
- **E.** Graph showing the viability measured by CellTiter-Glo of KAS6/1cells treated with **147** (10 μM) and/or increasing concentrations of bortezomib (0-10 μM) for 24 h. Error bars show SEM for n=5 replicates

Supplemental References

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