

The novel β 2-selective proteasome inhibitor LU-102 synergizes with bortezomib and carfilzomib to overcome proteasome inhibitor resistance of myeloma cells

Marianne Kraus,¹ Juergen Bader,¹ Paul P. Geurink,² Emily S. Weyburne,³ Anne C. Mirabella,³ Tobias Silzle,¹ Tamer B. Shabaneh,³ Wouter A. van der Linden,² Gerjan de Bruin,² Sarah R. Haile,^{4,5} Eva van Rooden,³ Christina Appenzeller,¹ Nan Li,² Alexei F. Kisselev,³ Herman Overkleeft,² and Christoph Driessen^{1,2}

¹Experimental Oncology and Hematology, Department of Oncology and Hematology, Kantonsspital St.Gallen, Switzerland; ²Gorlaeus Laboratories, Leiden Institute of Chemistry and Netherlands Proteomics Centre, the Netherlands; ³Department of Pharmacology and Toxicology, Norris Cotton Cancer Center, Geisel School of Medicine at Dartmouth, Lebanon, NH, USA; ⁴Clinical Trials Unit, Kantonsspital St. Gallen, Switzerland; and ⁵Department of Hematology, Division of Biostatistics, Institute for Social and Preventive Medicine, University of Zurich, Switzerland

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Correspondence: christoph.driessen@kssg.ch

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Supplemental material

Supplemental methods:

MTT assays and Antibodies

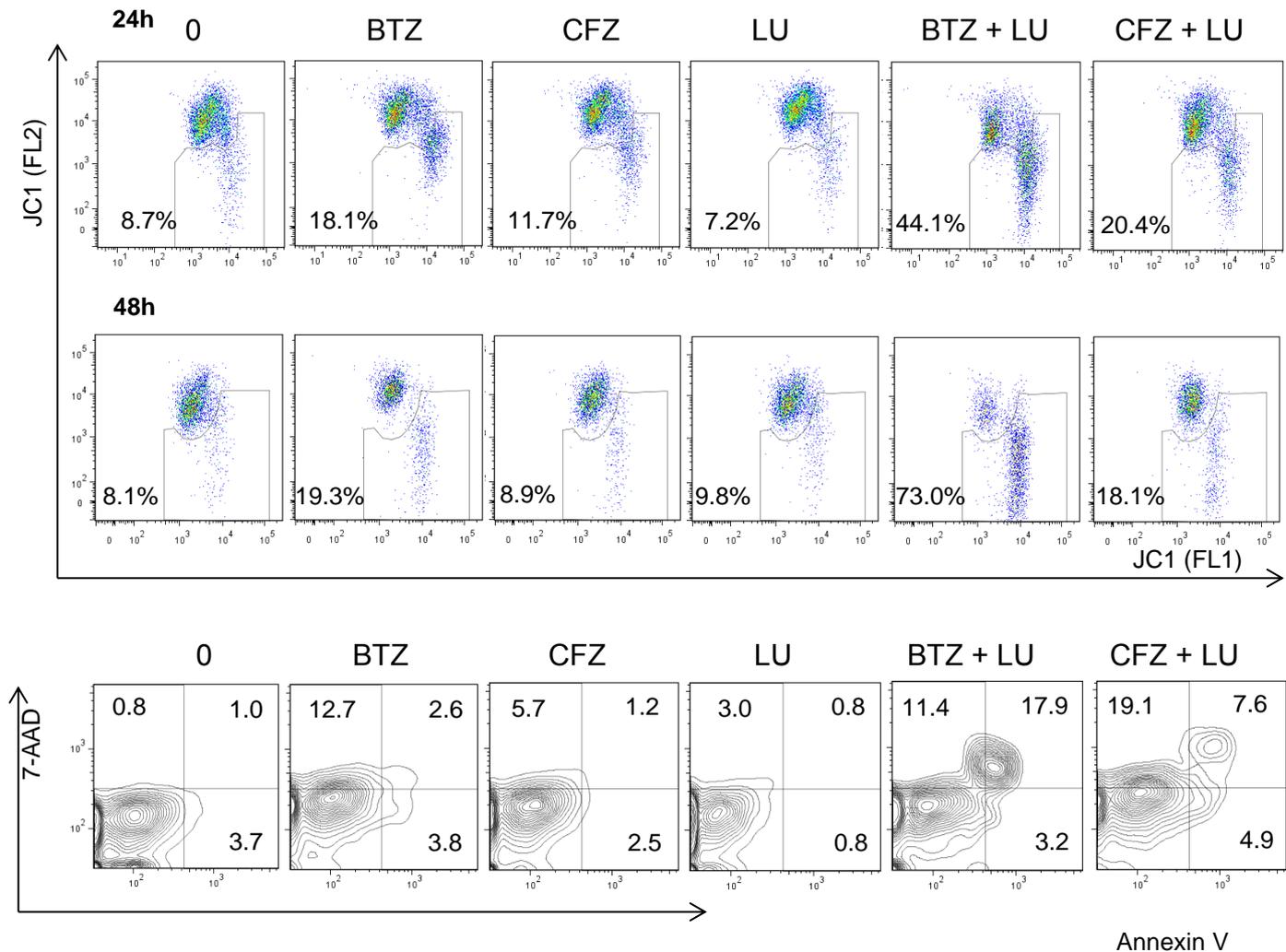
For the determination of cytotoxicity, we used CellTiter 96® AQueous One Solution cell proliferation assay (Promega) for cell lines, and CellTiter-Glo® 2.0 (CTG, Promega) for primary cells, because of the superior sensitivity of the CTG assay, according to the manufacturer's technical manual. We here use the generic term MTT assay when referring to these assays in the respective cell types throughout the manuscript. Mean values from quadruplicate samples representing at least three independent experiments are presented.

Anti-CHOP (Gadd 153, Santa Cruz, USA), anti-BiP (Grp78, Becton Dickinson, Heidelberg, Germany), anti-ATF4 (Proteintech, Chicago, USA), anti-IRE1 α , anti-cleaved caspase 9 and anti-caspase 3 and 7 (Cell Signalling Technology, Boston, USA), anti-cleaved PARPp85 (Promega, Madison, USA) and anti-polyubiquitin (Viva Bioscience, Exeter, UK), anti-p38, anti-pT180/pY182-p38, anti-ERK1/2, anti-pT202/pY204-ERK1/2, anti-JNK/SAPK, anti-pT183/pY185-JNK/SAPK (Transduction Laboratories), anti-HSP70 (Dianova, Hamburg, Germany), anti-GAPDH and anti- β -actin (Sigma-Aldrich, St Louis, MO, USA) antibodies were used.

Animal Experiments

MM1.S cells were suspended in unsupplemented RPMI-1640 media and injected subcutaneously in the flank of seven-week old female SCID mice (3×10^6 cells in 100 μ L/mouse). Twice-weekly intraperitoneal treatments with LU-102 (30mg/kg) were started on day 4 after cell inoculation. Treatments with escalating doses of Cfz (as indicated on Fig. 6) were added on day 10. The dose-escalating strategy was designed to acclimate mice to the combination treatment and minimize toxicity. Cfz was administered via tail vein injections, simultaneously with LU-102 injections. Tumor sizes were measured twice weekly with a caliper, tumor volumes were calculated using the following formula for a sphere: $(4/3) \cdot (22/7) \cdot (\text{length}/2)^2 \cdot (\text{width}/2)$. Mice were euthanized when tumors reached 3000mm³. All procedures were performed in accordance with the protocol approved by the Dartmouth Institutional Animal Care and Use Committee (IACUC).

Supplemental figure 1



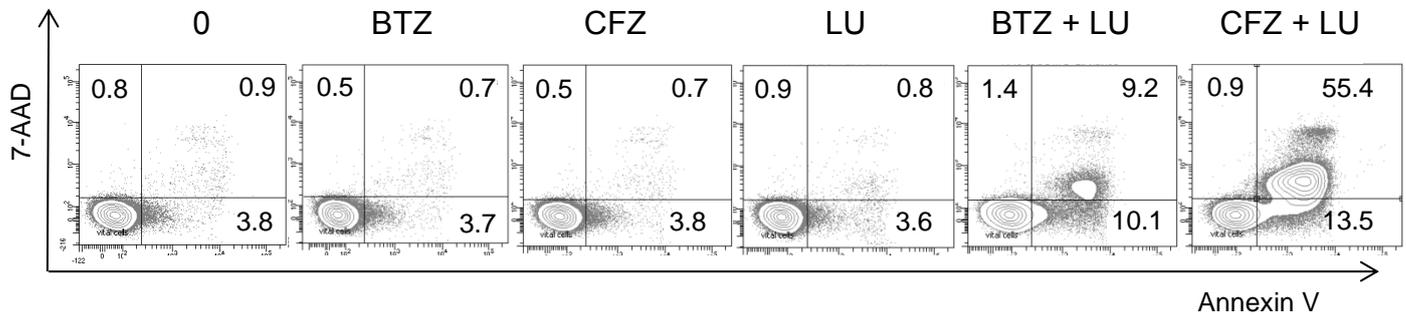
Loss of mitochondrial membrane potential and myeloma cell apoptosis in RPMI 8226 cells

Upper panel: The membrane-permeant JC-1 dye was used to monitor loss of mitochondrial membrane potential. JC-1 dye exhibits potential-dependent accumulation in mitochondria, indicated by a fluorescence emission shift from green (~529 nm) to red (~590 nm). RPMI 8226 cells were incubated with proteasome inhibitors as indicated (BTZ: bortezomib 20 nM; CFZ: carfilzomib 20 nM, LU: LU-102 3 μ M) for 24 and 48h, respectively, and the JC-1 membrane potential assay kit MitoProbe (Life Technologies Europe, Zug, Switzerland) was used in conjunction with flow cytometry according to the manufacturer's instructions. The relative amount of cells with loss of the FL2 signal, indicating loss of mitochondrial integrity, is indicated.

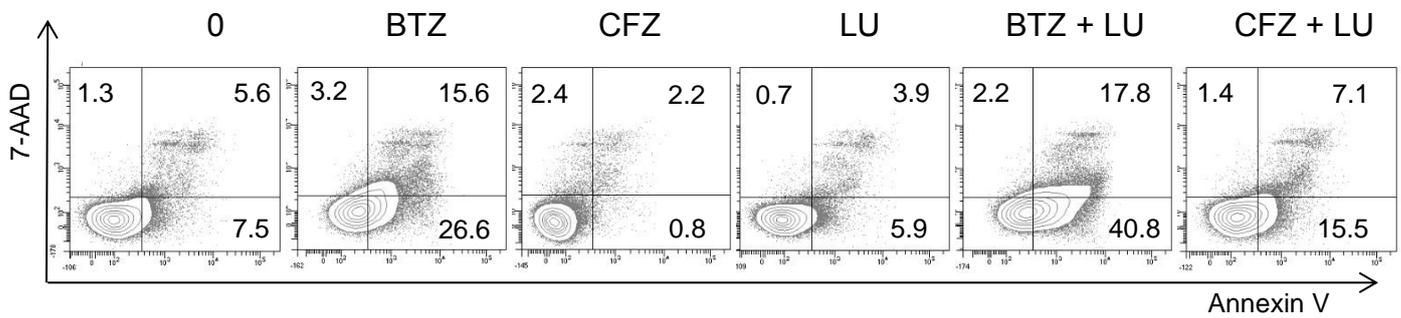
Lower panel: For measurement of apoptosis, RPMI 8226 cells treated with proteasome inhibitors as indicated above were assessed for surface annexin V and 7-AAD permeability using the BD Bioscience Apoptosis kit in conjunction with flow cytometry. The relative fraction of events in the different quadrants is indicated in percentage of total cells.

Supplemental figure 2

AMO-abtz



AMO-acfz



Myeloma cell apoptosis in proteasome inhibitor-resistant myeloma cell lines

Proteasome inhibitor resistant myeloma cell lines (AMO-abtz, AMO-acfz) were incubated with proteasome inhibitors as indicated (BTZ: bortezomib 20 nM; CFZ: carfilzomib 20 nM, LU: LU-102 1 μ M) for 24 h (AMO-abtz) and 48 h (AMO-acfz), and assessed for surface annexin V and 7-AAD permeability using the BD Bioscience Apoptosis kit in conjunction with flow cytometry. The relative fraction of events in the different quadrants is indicated in percentage of total cells. One representative experiment out of three is shown.