The novel β2-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]

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

ABSTRACT

Proteasome inhibitor resistance is a challenge for myeloma therapy. Bortezomib targets the β5 and β1 activity, but not the β2 activity of the proteasome. Bortezomib-resistant myeloma cells down-regulate the activation status of the unfolded protein response, and up-regulate β2 proteasome activity. To improve proteasome inhibition in bortezomib-resistant myeloma and to achieve more efficient UPR activation, we have developed LU-102, a selective inhibitor of the β2 proteasome activity. LU-102 inhibited the β2 activity in intact myeloma cells at low micromolar concentrations without relevant co-inhibition of β1 and β5 proteasome subunits. In protea-some inhibitor-resistant myeloma cells, significantly more potent proteasome inhibition was achieved by bortezomib or carfilzomib in combination with LU-102, compared to bortezomib/carfilzomib alone, resulting in highly synergistic cytotoxic activity of the drug combination *via* endoplasmatic reticulum stress-induced apoptosis. Combining bortezomib/carfilzomib with LU-102 significantly prolonged proteasome inhibition and increased activation of the unfolded protein response and IRE1-α activity. IRE1-α has recently been shown to control myeloma cell differentiation and bortezomib sensitivity (*Leung-Hagesteijn, Cancer Cell 24:3, 289-304*). Thus, β2-selective proteasome inhibition by LU-102 in combination with bortezomib or carfilzomib re-sults in synergistic proteasome inhibition, activation of the unfolded protein response, and cytotoxicity, and overcomes bortezomib/carfilzomib resistance in myeloma cells *in vitro*.

Introduction

Proteasome inhibition is a backbone for multiple myeloma treatment.[1] While control of myeloma can be achieved with first- and second-line therapy utilizing proteasome inhibitors and immunomodulating agents (IMIDs), most myeloma patients die from myeloma that has become refractory to antimyeloma drugs. Partial response rates achieved by next generation drugs like carfilzomib or pomalidomide are only in the 20% range in proteasome inhibitor-resistant myeloma.[2-4] Overcoming proteasome inhibitor resistance is a central unmet clinical need for myeloma patients.[5]

Myeloma cells synthesize large amounts of immunoglobulin protein, accompanied by constitutive activation of the unfolded protein response (UPR),[6] a homeostatic mechanism that limits protein biosynthesis, facilitates protein folding and increases proteolytic destruction of misfolded protein by the proteasome.[7] They, therefore, critically rely on proteasomal disposal and undergo apoptosis *via* a terminal unfolded protein response when proteasomal proteolysis is disturbed.[8,9]

The proteasome carries three proteolytically active sites (β1, β2 and β5), which differ in their substrate preferences.[10] These subunits of the so-called constitutive proteasome can be replaced by respective immunoproteasome subunits β1i, β2i and β5i in some cell types, including myeloma,[11] which results in a total of six different proteolytic enzymes with different substrate preferences in the 20S proteasome core particle. The two approved proteasome-inhibiting drugs, bortezomib and carfilzomib, by design target the β5 subunit of the constitutive proteasome and the immunoproteasome, which mediate the rate-limiting proteolytic proteasome activity.[1,12] Several β5-targeted next generation proteasome inhibiting drugs like delanzomib[13] (CEP-18770), ixazomib[14] (MLN-2238), and oprozomib[15] (ONX-0912) are under development.

Mutations in the bortezomib binding pocket[16,17] have been suggested to provide bortezomib resistance in myeloma, based on *in vitro* studies, but have not been confirmed *in vivo*.[18] Recent data suggest that changes in the activation state of the unfolded protein response allow myeloma cells to resist β5-targeted proteasome inhibitors: bortezomib resistance can be achieved by elimination of IRE-1α,[19] an endonuclease that controls the activation state of the transcription factor XBP-1, which is the key regulator of the UPR and plasma cell maturation at the same time. Consequently, bortezomib-resistance

 doi:10.3324/haematol.2014.109421
The online version of this article has a Supplementary Appendix.
Manuscript received on April 23, 2014. Manuscript accepted on June 5, 2015.
Correspondence: christoph.driessen@kssg.ch

by IRE-1α knockdown is characterized by decreased activation of the UPR and a less mature phenotype with a lack of fully developed ER. Likewise, myeloma cells adapted to bortezomib treatment *in vitro* (bortezomib-adapted cells) have a reduced rate of protein biosynthesis and a low activation state of the UPR.[20] Together, these data support a "low-IRE-1-α/XBP-1-model" of bortezomib resistance, the validity of which is supported by the identification of XBP1-negative, immature myeloma cell populations accumulating in bortezomib-resistant patients.[19]

The degree of cytotoxicity of β5-targeting proteasome inhibitors against myeloma cell lines correlates with their degree of additional inhibition of the β2- or β1 subunits.[21] Conversely, bortezomib-adapted myeloma cells increase β2 proteasome activity, which may allow the bortezomib-mediated proteasome inhibition to be by-passed.[20] We hypothesized that additional inhibition of β2 proteasome activity in bortezomib-resistant myeloma cells would increase the degree of proteasome inhibition achieved by bortezomib alone, and re-sensitize them for bortezomib treatment *via* IRE-1α activation and induction of a terminal UPR. With this aim in view, we have developed the first synthetic proteasome inhibitor to inhibit the β2 activity,[21] and have improved its cell permeability and potency, yielding the compound LU-102,[22] which sensitizes RPMI8226 cells to bortezomib and carfilzomib-induced cytotoxicity. We now address the potential of LU-102 to overcome bortezomib/carfilzomib-resistance.

Methods

Cells and Inhibitors

Human myeloma cell lines RPMI8226, LP-1, AMO-1, U266 (obtained from ATCC), MM1S and MM1R (obtained from ATCC) were maintained in 10% FCS-supplemented RPMI-1640 medium with gentamycin. AMO-abtz/acfz cells were adapted to proteasome inhibitor-containing culture conditions as described.[20] The proteasome inhibitors bortezomib, carfilzomib and LU-102[22] were synthesized at the Leiden Institute of Chemistry. The selective inhibitors NC-001 (inhibiting β1/β1i) and NC-005 (β5/β5i) have been described by Britton *et al.*[21]

MTT assay, western blot, antibodies

Cell viability was determined using the MTT [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] method using commercial kits (details in the *Online Supplementary Appendix*). SDS-PAGE and western blot were performed as described (for antibodies see *Online Supplementary Appendix*).[23]

Determination of proteasome activity by active-site labeling

The proteasome-specific activity-based probes MV-151, BODIPY-NC-005-vs and BODIPY(RED)-NC-001 were used and measured as described;[23-25] the synthesis of BODIPY(RED)-NC-001 will be described elsewhere. Both the constitutive and the immunoproteasome subunits are labeled by MV-151 in intact cells,[26] and resolved after cell lysis by SDS-PAGE.

Patients' samples

Patients' samples were obtained after written informed consent and approval by the independent ethics review board, in accordance with ICH-GCP and local regulations. Malignant plasma cells were retrieved from peripheral blood or bone marrow of patients with multiple myeloma or plasma cell leukemia, and enriched by Ficoll density centrifugation or by CD138 magnetic sorting to a purity of more than 80%, if necessary.

Animal experiments

MM1.S cells were subcutaneously injected in SCID mice and treated with LU-102 (30 mg/kg) *via* intraperitoneal, and carfilzomib *via* intravenous injection twice weekly (Figure 6). Tumors were measured twice weekly and mice were euthanized when tumors reached 3000 mm^3. (*See Online Supplementary Appendix for details.*)

Statistical analysis

Unless stated otherwise, one representative experiment out of at least three independent experiments is shown. For MTT assays mean values from quadruplicate samples are represented and synergism was calculated using R as described by Chou *et al.*[27] A combination Index (CI) <0.8 indicates synergism, >1 indicates antagonism. Statistical significance was calculated using Student's *t*-test.

Results

Selectivity of proteasome inhibition by LU-102 in myeloma cells

To compare target inhibition and specificity of LU-102 with the approved proteasome-inhibiting drugs in viable myeloma cells, we performed activity-based proteasome labeling experiments with the cell permeable proteasome probe MV151 in RPMI8226 cells (Figure 1A). Bortezomib and carfilzomib resulted in a significant decrease in β5/β5i proteasome activity starting in the 0.03-0.1 μM concentra-

Table 1. Patients' characteristics.

ID	#Prior lines of therapy	Refract. to last bortezomib	Refract. to next bortezomib	Extramed. manifest.	Primary PCL	Second. PCL	LU-102 sensitive *in vitro*	Bortezomib sensitive *in vitro*	Carfilzomib sensitive *in vitro*
MM1	7	Yes	Yes	No	No	No	No	Not known	Not known
MM2	1	Yes	N/A	Yes	No	No	No	No	No
MM3	1	No	No	No	Yes	N/A	Yes	Not known	Not known
MM4	4	Yes	N/A	Yes	No	Yes	No	No	No
MM5	0	No	No	Yes	Yes	N/A	Yes	No	Yes
MM6	7	Yes	No	No	No	No	Yes	No	Yes

Clinical characteristics of patients from which primary myeloma/plasma cell leukemia samples were derived. PCL: plasma cell leukemia. Cells were considered sensitive (+) for treatment with the individual proteasome inhibitors in vitro, when more than 50% cytotoxicity was observed during continuous incubation with LU-102 3 μM: bortezomib 10 nM, carfilzomib 5 nM.

tion range while bortezomib provided also inhibition of β1/β1i activity. Interestingly, in contrast to bortezomib, at higher concentrations (>0.1 μM) carfilzomib resulted in additional concentration-dependent inhibition of β2/β2i activity. In contrast to bortezomib and carfilzomib, LU-102 resulted in selective and potent inhibition of β2/β2i proteasome subunits. Compared to these two, LU-102 was less active, so that 0.3-1 μM LU-102 was required for measurable proteasome inhibition.

To confirm selectivity of β2/β2i inhibition, we used advanced affinity-based probes for selective visualization of active β1 and β1i or the β5 and β5i subunits[24] (Figure 1B). Affinity labeling with MV-151 in conjunction with LU-102 showed the expected loss of β2-type of activity signals without affecting β1- and β5-type of activities, while LU-102 had no influence on the intensity of β5/β5i and β1/β1i-selective labeling. Thus, we can exclude quantitatively relevant co-inhibition of β1, β1i, β5 and β5i proteasome activity when myeloma cells are exposed to 3.3 μM LU-102.

Selective inhibition of β2/β2i in RPMI8226 and AMO-1 myeloma cell lines resulted in accumulation of polyubiquitinated (polyUb) protein, suggesting a quantitative role for the β2/β2i proteasome activity in protein breakdown in myeloma cells. The activity and selectivity of LU-102 was confirmed in a panel of myeloma cell lines (*data not shown*).

Cytotoxic effect of β2/β2i proteasome inhibition

To assess the cytotoxic effect of β2 proteasome subunit inhibition on myeloma cells, we incubated myeloma cell lines and primary cells with the respective inhibitors. At the β2/β2i selective concentration of 3.3 μM or less, LU-102 induced moderate cytotoxicity in myeloma cell lines and in 3 of 6 primary myeloma cell samples, while the viability of PBMC was unaffected (Table 1 and Figure 2A). The β2/β2i-selective proteasome inhibition was unable to induce cytotoxicity in carfilzomib-adapted (AMO-acfz) or bortezomib-adapted (AMO-abtz) cells, indicating that inhibition of the trypsin-type proteasome activity alone is not sufficient to overcome bortezomib or carfilzomib resistance (Figure 2B and *Online Supplementary Figures S1 and S2*).

Combination treatment with LU-102 overcomes proteasome inhibitor resistance

The excellent selectivity of LU-102 for the β2/β2i proteasome activity suggested to combine LU-102 with β5-targeted proteasome inhibitors to increase their therapeutic potency (Figure 3A, left panels, and *Online Supplementary Figures S1 and S2*). The combination of bortezomib or carfilzomib with LU-102 showed strong synergistic cytotoxicity (CI between 0.129 and 0.048 for bortezomib or carfilzomib, respectively; values less than 1 indicate synergism) in bortezomib-resistant AMO-abtz cells, while the respective monotherapies had no or a considerably weaker, cytotoxic effect. Likewise, carfilzomib-resistant cells (AMO-acfz) showed highly synergistic cyto-toxicity when either bortezomib or carfilzomib was combined with LU-102 (CI 0.002 each), while the individual drugs lacked relevant cytotoxic monoactivity in this setting. Similar results were obtained with bortezomib- or carfilzomib-adapted versions of the myeloma cell line LP-1 (*data not shown*).

Because interactions of myeloma cells with bone marrow stroma provide resistance against proteasome inhibitors,[28] we co-cultured AMO-1 cells with the human HS5 bone marrow stroma cell line and assessed the effects of proteasome inhibition on myeloma cell viability (Figure 3A, middle panels). As expected, co-culture of myeloma cells with HS5 cells resulted in significantly reduced sensitivity against bortezomib or carfilzomib. The combination of bortezomib/carfilzomib with LU-102 was sufficient to overcome this stroma-mediated resistance against the two approved proteasome inhibitors. To confirm that proteotoxic stress was the major mechanism for this synergistic cytotoxicity, we inhibited protein biosynthesis of myeloma cells by cycloheximide exposure prior to challenging them with proteasome inhibitors (Figure 3A, right panels). The combination treatment of LU-102 with either carfilzomib or bortezomib resulted in less than 10% viable RPMI8226 cells, which increased to 42% (bortezomib) and 47% (carfilzomib) in the presence of cycloheximide, indicating that reduced protein biosynthesis can partly rescue myeloma cells from the synergistic cytotoxicity that is induced by the combined treatment with LU-102 and bortezomib or carfilzomib.

Combination treatment with LU-102 overcomes proteasome inhibitor resistance in primary myeloma cell samples

LU-102 showed highly synergistic cytotoxicity with bortezomib also in all bortezomib-insensitive, malignant primary plasma cell samples tested (CI <0.001, 0.132, 0.008, 0.09 for patients MM2, MM4, MM5, MM6) (Table 1 and Figure 3B), in contrast to PBMC. Likewise, synergistic cytotoxicity of LU-102 with carfilzomib was observed in the same samples, however, with weaker combination indices (CI 0.391 for MM5 and 0.244 for MM6). This corresponded with the finding that MM5 and MM6 were still carfilzomib-sensitive *in vitro* and the fact that all patients were carfilzomib-naïve. By contrast, there was no such synergy in PBMC with BTZ (CI 0.86±0.38) and a borderline synergistic effect if any with CFZ (CI 0.78±0.18). Importantly, the difference in CI between primary tumor cell samples and PBMC was statistically significant for CFZ and BTZ (P=0.01 and P<0.001) demonstrating superior synergy in tumor cells *versus* PBMC. We conclude that LU-102 in combination with bortezomib or carfilzomib overcomes acquired bortezomib or carfilzomib resistance of primary myeloma cells *in vitro*.

Molecular activity of LU-102 in combination with bortezomib/carfilzomib

To visualize to what extent the combination of LU-102 with bortezomib or carfilzomib would improve the degree of proteasome inhibition in bortezomib/carfilzomib-resistant cells, we performed affinity labeling experiments with MV-151 in the AMO-abtz /AMO-acfz myeloma cells (Figure 4, upper panel). In the clinic, the pharmacodynamic conditions during i.v. application of carfilzomib are characterized by a rapid decline of carfilzomib plasma concentrations from 10 μM peak levels directly after the i.v. push, to 2 nM after 60 min, so that 100 nM levels are reached at the 30 min time point.[29] To mimic these conditions, we exposed RPMI8226 cells to the respective proteasome inhibitors for a 1 h pulse, followed by removal of proteasome inhibitors. AMO-abtz and AMO-acfz cells showed higher base-line proteasome activities, and in particular, an increased β2/β2i activity

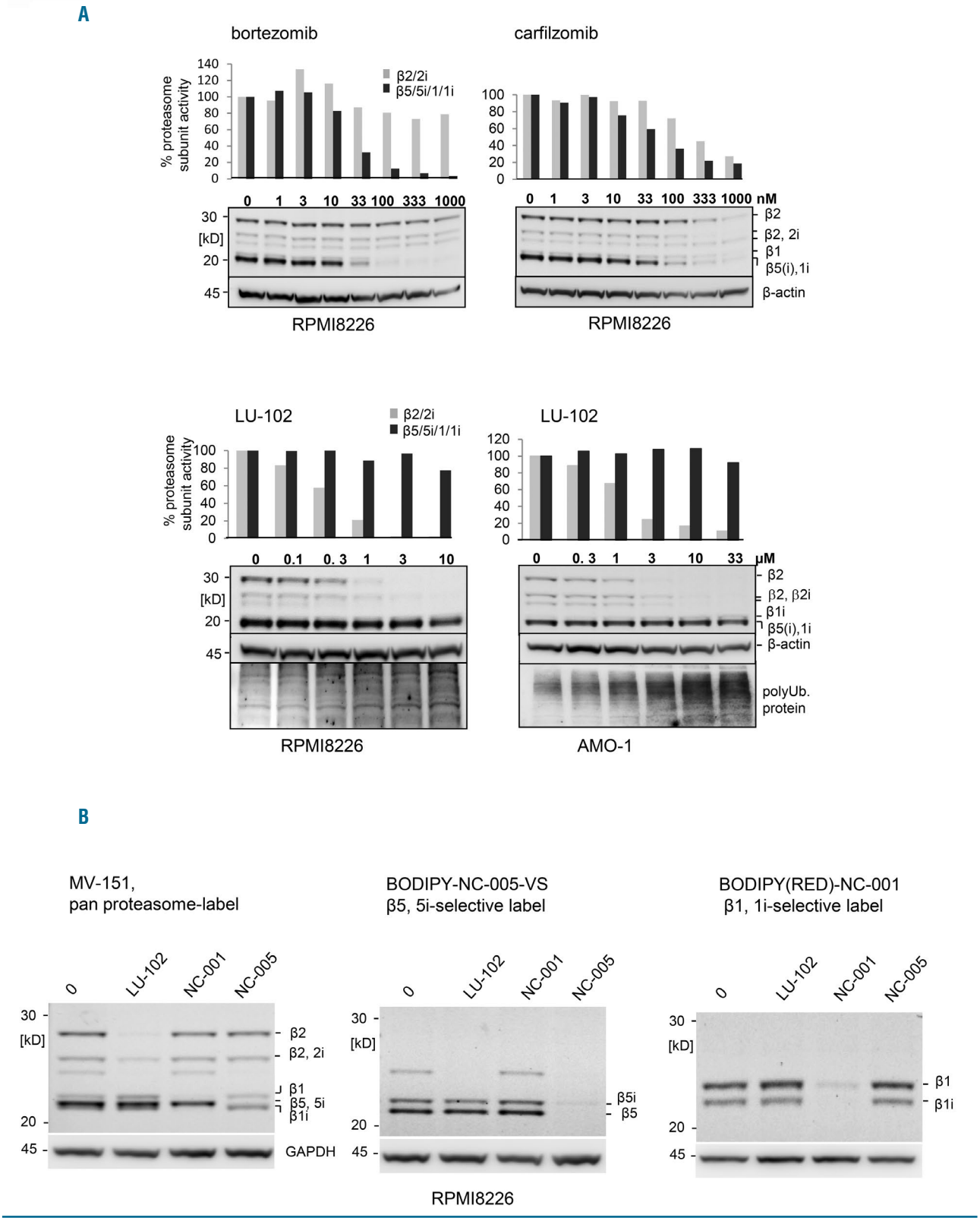

Figure 1. (A). Selectivity of proteasome inhibition in intact myeloma cells RPMI8226 or AMO-1 myeloma cells were incubated with the cell-permeable, proteasome specific, fluorogenic activity-based affinity probe MV151 in the presence or absence of the proteasome inhibitors indicated. Cell lysates were resolved by SDS-PAGE. Specific fluorescence signals for the respective proteasome activities were visualized by fluorescence scanning (upper part of the gels) and resolved quantitatively for the respective activities (β1, β2, β5) in the bar graphs relative to control signals without proteasome inhibitor. Western blot for β-actin and polyubiquitinated protein served as loading control and to visualize the accumulation of proteasome substrates upon inhibition of the β2-like activity. (B) Selectivity of LU-102 in myeloma cells RPMI 8226 myeloma cells were incubated with the pan-reactive proteasome activity-specific affinity probe MV151 in the presence or absence of the subunit-selective inhibitors indicated (LU-102 3.3 μM, NC-001 and NC-005 5 μM). This was compared to cells labeled with the β5/β5i-selective activity probe MVB125 and to a β1/β1i-selective labeling achieved by combining the probe EV-031 with pre-incubation with NC-005. Active proteasome subunits were visualized as before.

compared to AMO-1, consistent with similar observations made earlier in AMO-abtz cells[20] (Figure 4A). We observed complete or near-complete inhibition of proteasome polypeptides when bortezomib or carfilzomib were combined with LU-102 in AMO-1 cells, while some residual active proteasome polypeptide could still be visualized in bortezomib/carfilzomib-adapted cells under these conditions. However, since base-line proteasome activity detected was much higher in the adapted cells, compared to the AMO-1 parental cells, the relative fraction inhibited by the drug in both cell types was very similar, consistent with unaffected interaction between bortezomib/carfilzomib and their target in adapted cells. Importantly, the levels of total residual proteasome activity detected in proteasome inhibitor resistant cells after treatment with carfilzomib or bortezomib alone were consistently higher compared to cells exposed to carfilzomib/bortezomib combined with LU-102. Thus the combination of LU-102 with β5-targeted proteasome inhibitors results in superior proteasome inhibition in bortezomib/carfilzomib-resistant myeloma cells.

We next assessed whether this would translate into increased activation of the unfolded protein response and consecutive apoptosis. RPMI8226 cells were exposed to the respective proteasome inhibitors for a 1 h pulse, followed by washing and a chase period of 4-16 h without proteasome inhibitors, before the expression of proteins indicating UPR activation and UPR-mediated apoptosis was analyzed (Figure 4B). The combination of LU-102 with either bortezomib or carfilzomib resulted in upregulation of pIRE-1α, ATF4, and CHOP expression, compared to samples treated with the individual proteasome inhibitors under the same conditions. This indicated superior triggering of the major molecular regulator of bortezomib-sensitivity (IRE-1α), alongside with the activation of a "terminal" UPR with consecutive ER stress-induced apoptosis by ATF4.[7] Consistent with this, we observed synergistic activation of caspases 9, 3 and 7 by bortezomib/carfilzomib in combination with LU-102, which translated into apoptosis, as shown by expression of cleaved poly(ADP-ribose) polymerase (PARP). This was accompanied by superior activation of the MAPK pathway (p-MEK, p-ERK, p-p38, p-JNK), and increased expression of p53, p27, p-STAT3 and p-cJUN that are all involved

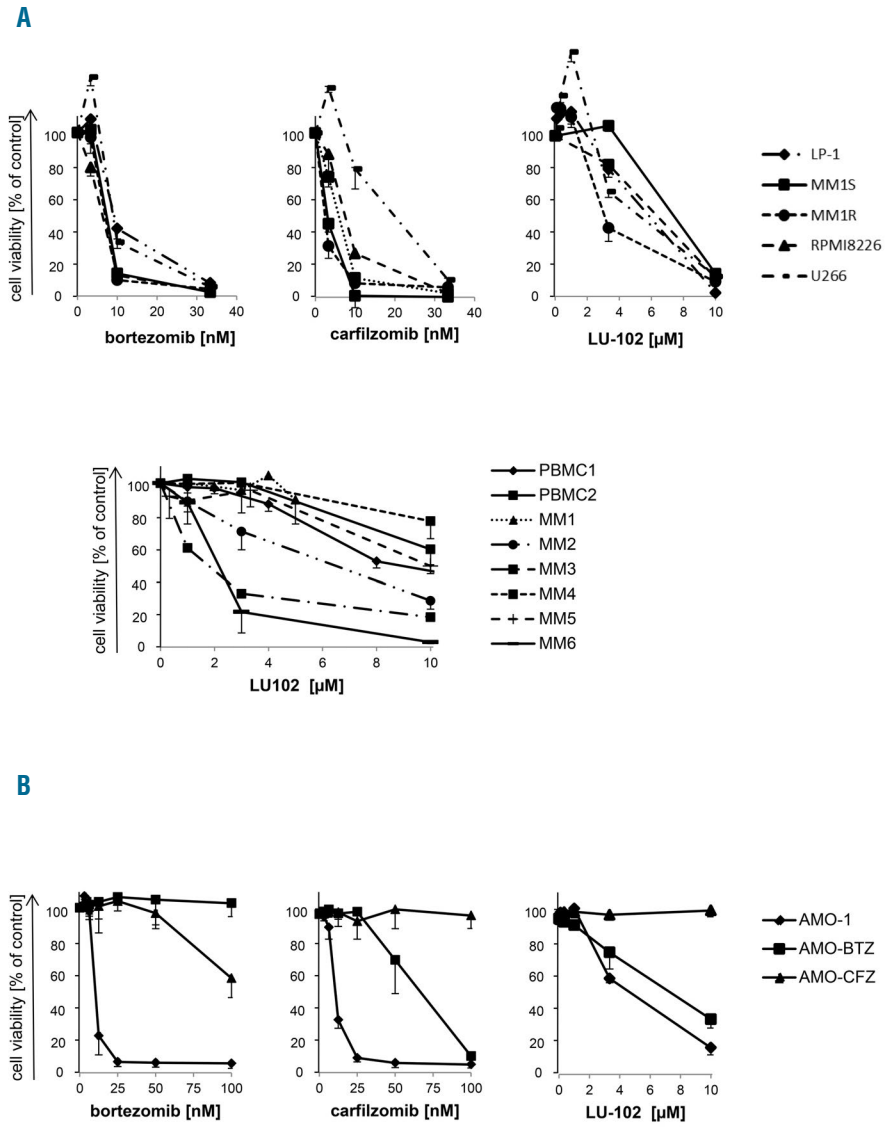

Figure 2. (A). Single agent cytotoxicity of LU-102 against myeloma cells. The standard myeloma cell lines indicated, as well as primary myeloma cells (MM1-MM6, see Table 1) alongside with PBMC from 2 healthy donors, were incubated with the indicated concentrations of LU-102, carfilzomib or bortezomib for 48 h, and cell viability was assessed by MTT test. (B). Cytotoxicity of LU-102 in bortezomib/carfilzomib-resistant myeloma cells. The bortezomib-resistant AMO-abtz and the carfilzomib-resistant AMO-acfz, were incubated with the indicated concentrations of LU-102, carfilzomib or bortezomib for 48 h as above, and cell viability was assessed by MTT test.

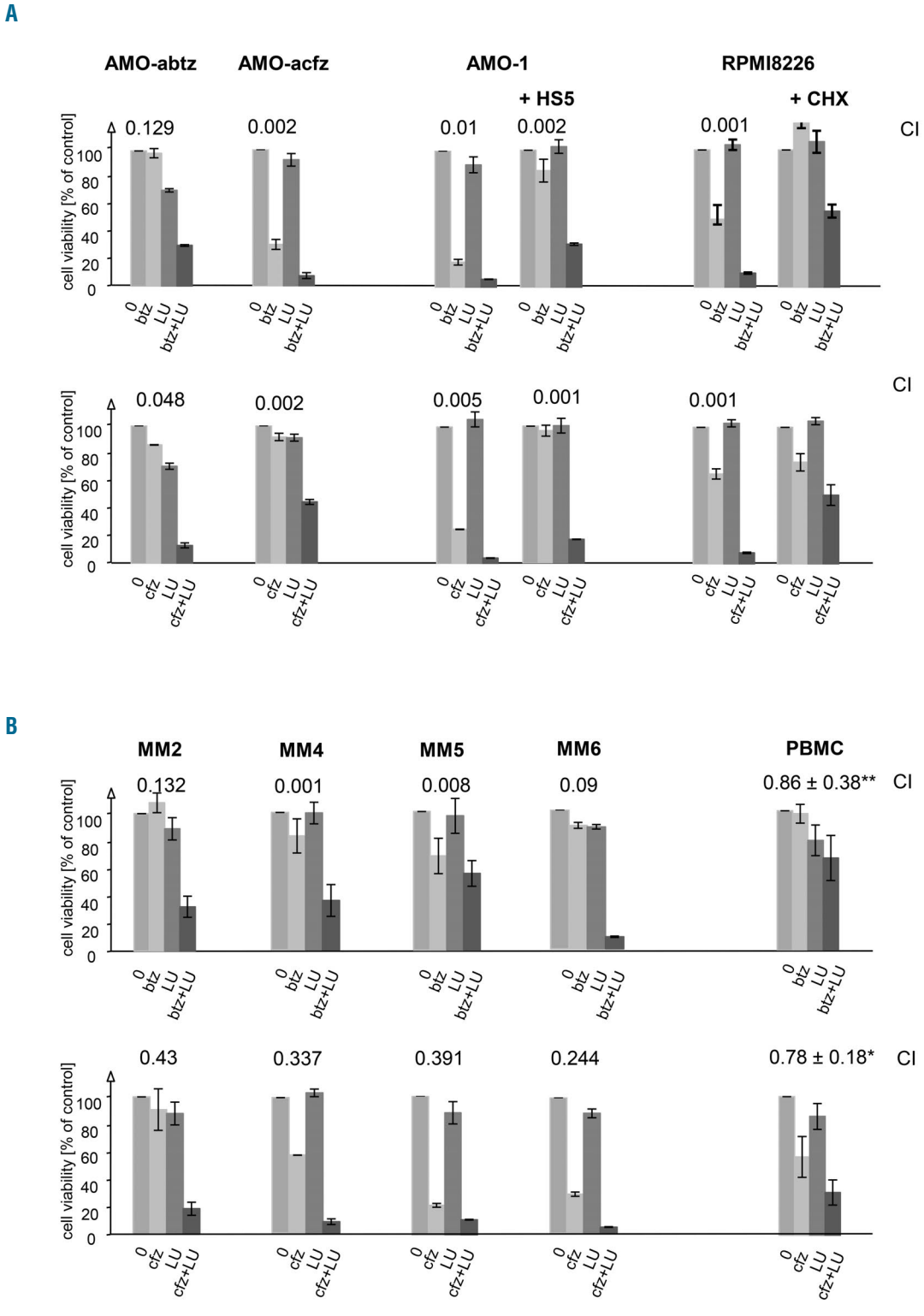

Figure 3. Effect of the combination of carfilzomib/bortezomib with LU-102 on the viability of bortezomib/carfilzomib resistant myeloma cells (A) AMO-abtz and AMO-acfz (left panels) and AMO-1 cells cultured in the presence/absence of the human bone marrow stromal cell line HS5 (middle panels) were incubated with carfilzomib/bortezomib (CFZ/BTZ) and/or LU-102 (LU), followed by assessment of cell viability by MTT test relative to untreated control cells. Right panel: RPMI8226 were cultured in the presence of bortezomib or carfilzomib with/without LU102 (1 μM) for 24 h, with/without treatment with cycloheximide (CHX, 2 μg/ml) for 4 h prior to incubation with proteasome inhibitors. The calculated combination indices (CI) for the respective combinations are indicated above the bar graphs. The following concentrations of inhibitors were used: AMO-acfz: BTZ and CFZ: 33 nM, LU-102: 3 μM; all other cell lines: BTZ and CFZ: 10 nM, LU-102: 1 μM). (B) Primary malignant plasma cells with bortezomib resistance (MM 2, 4, 5, 6, as characterized in Table 1) were assessed for cytotoxicity by MTT test after incubation with the respective proteasome inhibitors alone or in combination (BTZ 10 nM, CFZ 5nM, LU-102 1 μM. For MM4, 20 nM BTZ was used). The calculated combination indices (CI) for the respective combinations are indicated on top of the bar graphs. Control PBMC represent mean values from 7 healthy donors under the same conditions, statistical difference of the CI between PBMC *versus* primary malignant cells is indicated as **P*=0.01, ***P*<0.001.

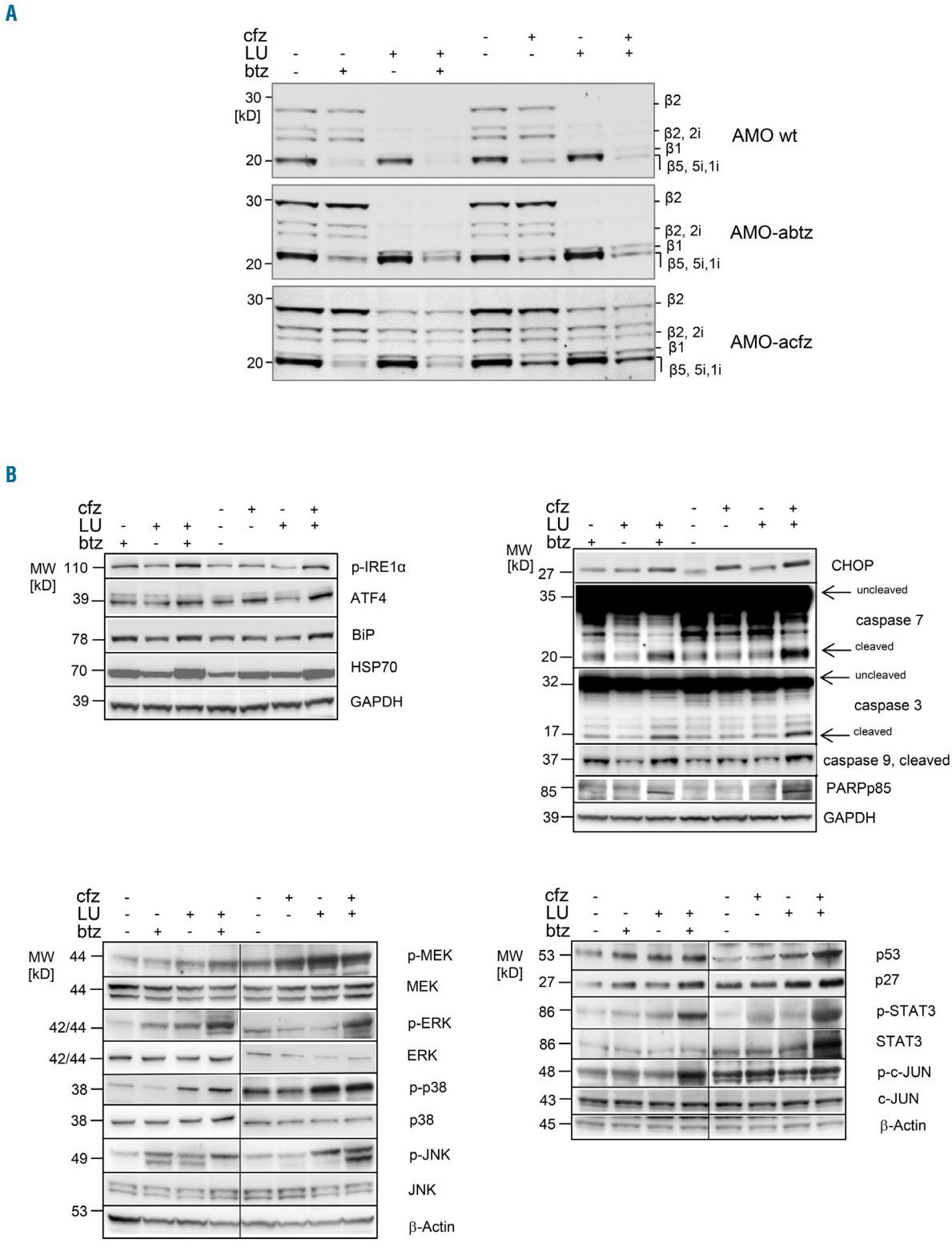

Figure 4. (A) Effect of the combination of carfilzomib/bortezomib with LU-102 on proteasome activity in bortezomib/carfilzomib resistant myeloma cells. AMO-1 myeloma cells as well as AMO-1 derived subclones selected to grow in the continued presence of bortezomib or carfilzomib (AMO-abtz, AMO-acfz, respectively) were pulse treated with proteasome inhibitors alone or in combination (BTZ and CFZ 100 nM each, LU-102 3 μM, 1h) and active proteasome polypeptides in intact cells were visualized after affinity labeling with MV-151, cell lysis and SDS-PAGE, using a fluorescence scanner. (B) Effect of the combination of bortezomib/carfilzomib with LU-102 on UPR-triggered apoptosis RPMI8226 cells were pulse-treated with bortezomib or carfilzomib (BTZ , CFZ,100 nM) in the presence or absence of LU-102 (3 μM) for 1 h, followed by washing and continued cell culture in the absence of proteasome inhibitors for 16 h. The expression of proteins indicative for activation of the unfolded protein response (BIP, CHOP, p-IRE1α, ATF4), of proteins involved in mediating the UPR-triggered apoptotic signal (p-MEK, p-ERK, p-p38, p-JNK, p53, p27, p-STAT3, p-c-JUN) as well as proteins indicating apoptosis (caspases 3, 7, 9, PARPp85) was analyzed by western blot, relative to controls without proteasome inhibition. GAPDH and actin served as a protein loading control.

in apoptotic signaling triggered by the terminal UPR.[30] The apoptotic nature of cell death was demonstrated directly via assessment of changes in mitochondrial membrane potential and 7-AAD/Annexin V staining (*Online Supplementary Figures S1 and S2*). In conclusion, additive inhibition of the β2 proteasome subunit significantly increases proteasome inhibition, activation of the UPR, apoptotic signaling, and the induction of apoptosis in bortezomib or carfilzomib-treated myeloma cells.

Effect of LU-102 on the duration of proteasome inhibition achieved by bortezomib or carfilzomib

Superior activity of carfilzomib against myeloma cells and bortezomib-insensitive tumor cells has been attributed to a longer duration of proteasome inhibition after carfilzomib treatment, compared to bortezomib,[12] based on the irreversible nature of binding of carfilzomib. We compared the impact of LU-102 on the duration of proteasome inhibition, the recovery of proteasome function and the accumulation of polyUb protein and its subsequent recovery in myeloma cells treated with carfilzomib or bortezomib (Figure 5). Pulse-treatment of RPMI 8226 cells with bortezomib/carfilzomib (100 nM) or LU-102 (3 μM) alone for 1 h resulted in inhibition of the individual active proteasome polypeptides. Combination treatments of bortezomib/carfilzomib with LU-102 resulted in true additive proteasome inhibition. The different amounts of polyUb protein were consistent with this pattern of inhibition. After 4 h of chase, the pattern of proteasome inhibition had not changed, in agreement with the slow off rate of bortezomib, and polyUb protein accumulated in bortezomib or carfilzomib treated samples. After 16 h, similar degrees of recovery of proteasome activity were observed in bortezomib and carfilzomib-treated myeloma cells, while poorer recovery of proteasome activity was observed in cells treated with the combination of LU-102 with either of the two approved proteasome inhibitors. Importantly, polyUb protein was still present in sizable amounts 16 h and up to 24 h after treatment with LU-102 in combination with bortezomib or carfilzomib, while it had cleared almost to background levels in samples treated with bortezomib or carfilzomib alone. Near-complete or complete proteasome recovery after bortezomib or carfilzomib treatment was observed

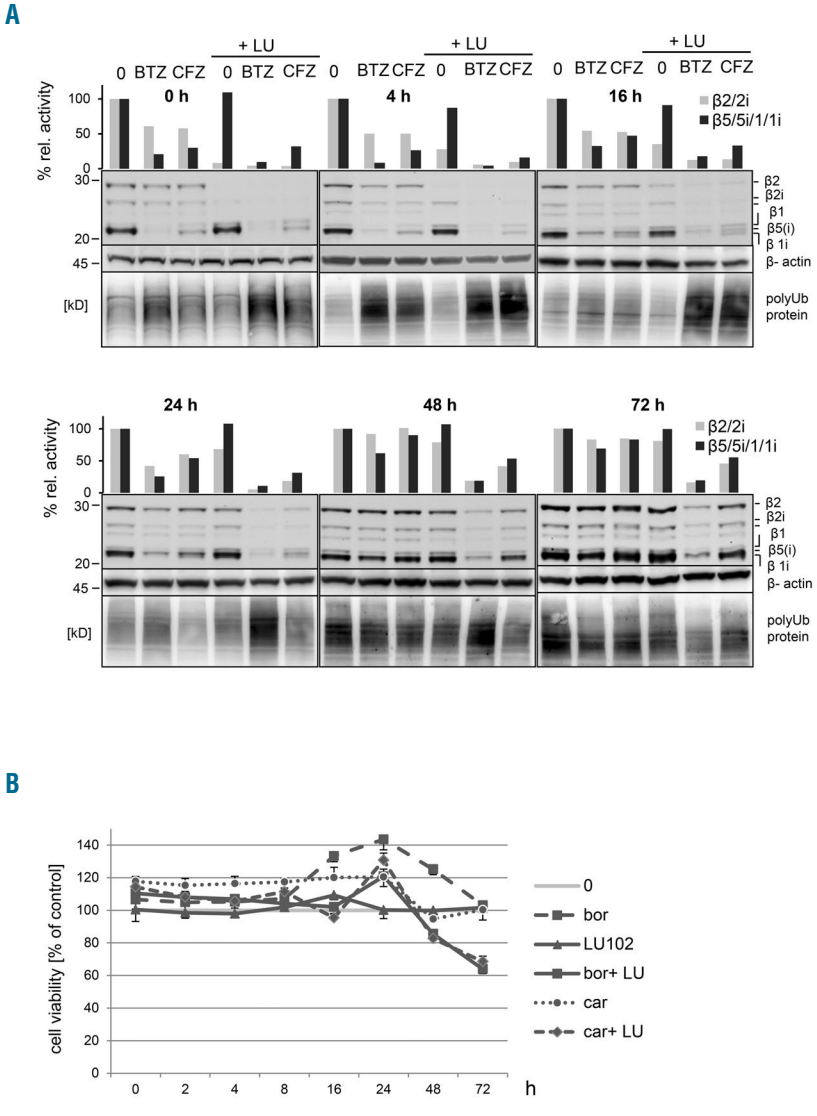

Figure 5. Effect of pulse treatment with carfilzomib/bortezomib with or without LU-102 on proteasome activity and polyUb protein. (A) RPMI8226 cells were exposed to carfilzomib (CFZ) or bortezomib (BTZ, 100 nM each), with or without LU-102 (LU, 3 μM) for 1 h. Following consecutive removal of the proteasome inhibitors by washing, cells were either immediately analyzed (0 h), or cultured in the absence of proteasome inhibitors for up to 72 h. Analysis of proteasome activity (activity-based affinity labeling in intact cells with MV-151, upper parts of the gel panels), protein load (β-actin, western blot, middle part gel panel) and polyubiquitinated protein (polyUb, western blot, bottom parts gel panel) was performed at the indicated chase times after removal of proteasome inhibitors. Bar graphs above the gel panels visualize the relative amounts of β2(i) *versus* β5(i)/β1(i) proteasome activity, as retrieved by quantitative fluorescence scanning from the respective gels below. (B) Cell viability of samples analyzed in (A), as determined by MTT assay. Error bars represent standard deviation between 3 individual samples.

after 48 h, but was not observed even 72 h after exposure to carfilzomib or bortezomib in combination with LU-102. At the 48 h and the 72 h time points, respectively, 82% and 63% of cells treated with LU-102 in combination with bortezomib/carfilzomib were viable (Figure 5B) compared to 100% in the cells treated without LU-102, so that this difference could account only in part for the lack of full proteasome recovery. Similar data were obtained using AMO-1 cells (*data not shown*). The data demonstrate that treatment of myeloma cells with LU-102 in combination with either bortezomib or carfilzomib prolongs the duration of proteasome inhibition, which results in prolonged intracellular accumulation of proteasome substrate protein in myeloma cells.

LU 102 improves the antimyeloma activity of carfilzomib in vivo

MM1S cells were selected for *in vivo* testing as they have only moderate sensitivity for carfilzomib/bortezomib treatment (compare Figure 2B), and injected into SCID mice. After subcutaneous tumor inoculation, mice were treated with carfilzomib at a schedule providing 80% proteasome inhibition *in vivo* (carfilzomib 2 mg/kg on a weekly day 1, 4 schedule for 8 weeks[29]). The LU-102 dose 30 mg/kg was co-administered with carfilzomib as indicated. The dose was extrapolated from *in vitro* activity of LU-102 and inhibited more than 70% of the trypsin-like proteasome activity in bone marrow *in vivo* after 2 h in pilot experiments (*data not shown*). Co-treatment with carfilzomib and LU-102 was tolerated without weight loss or overt clinical toxicity. We observed a significant reduction in mean tumor size in mice treated with carfilzomib+LU-102, compared to mice treated with either single agent: mean difference in tumor volume between carfilzomib-treated *versus* carfilzomib+LU-102 treated mice 353 mm^3 (95%CI: 130-576 mm^3; *P*=0.008); difference for LU-102 treated *versus* carfilzomib+LU102 treated 432 (95%CI: 46-817 mm^3; *P*=0.03) (Table 2). This translated into a survival advantage of mice treated with the carfilzomib+LU-102 combination relative to mice undergoing either monotherapy (median survival 48 days for combination treatment *versus* 38 and 39.5 for LU-102 or carfilzomib mono-therapy, respectively; *P*=0.02 and 0.009 (Figure 6). Co-treatment with bortezomib (1 mg/kg) and LU-102 resulted in hemorrhagic enteritis in the majority of mice and was, therefore, not feasible at the indicated doses (*data not shown*). We conclude that co-treatment with LU-102 is feasible and increases the antimyeloma activity of carfilzomib *in vivo*.

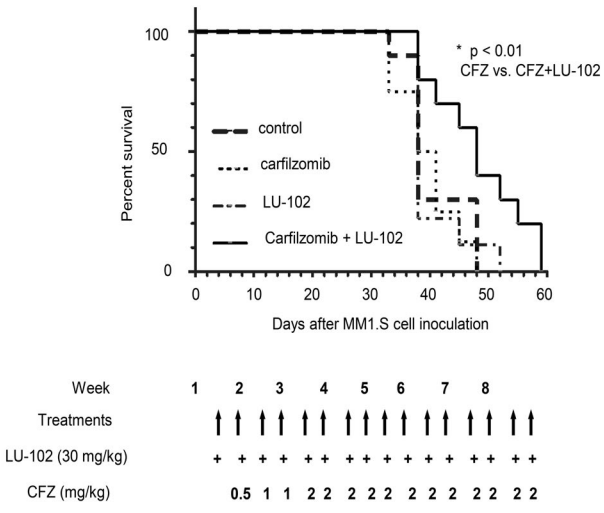

Figure 6. Effect of the combination of carfilzomib with LU-102 on myeloma bearing mice *in vivo* MM1.S cells were injected into SCID mice (3x10^6 cells in 100 μL/mouse). Twice-weekly intraperitoneal treatments with LU-102 (30 mg/kg) combined with intravenous carfilzomib (CFZ) was performed as indicated. Mice were euthanized when tumors reached 3000mm^3.

Discussion

Bortezomib-resistant myeloma cells contain increased proteolytic processing capacity, including upregulation of the β2 proteasome activity not targeted by bortezomib.[20] We hypothesized that β2 proteasome activity contributes to bortezomib resistance and may, therefore, represent a rational target for the treatment of proteasome inhibitor-resistant myeloma.

We here describe the effect of LU-102, the first β2/β2i-selective proteasome inhibitor available for pre-clinical studies in myeloma, on bortezomib- and carfilzomib-resistant myeloma cells. Selective elimination of β2 proteasome activity alone was not sufficient to induce a meaningful cytotoxic effect in bortezomib- or carfilzomib-resistant myeloma cells. However, the combination of LU-102 with bortezomib or carfilzomib was highly potent to overcome bortezomib- or carfilzomib-resistance. This was observed not only in a proteasome inhibitor-adapted myeloma cell line model that mirrors several of the biological features of bortezomib resistance in patients, but also in a model of bone marrow stroma-mediated proteasome inhibitor resistance, and in primary malignant plasma cell samples from bortezomib-resistant patients. Based on our results, we suggest the following mechanism: co-inhibition of the β2/β2i proteasome activity during treatment with the β5-targeted drugs bortezomib or carfilzomib results in more effective accumulation of proteasome substrate proteins due to reduced levels of residual total proteasome activity, as well as due to prolonged proteasome inhibition. The accumulation of proteasome sub-strate protein triggers the UPR, and in particular its major regulatory axis, IRE-1α/XBP-1. Downregulation of IRE1-α/XBP1 is known to mediate bortezomib-resistance of myeloma cells[19] and high XBP-1 is a biomarker for bortezomib sensitivity in the clinic.[9] The synergistic induction of the UPR, and in particular IRE-1α, by LU-102 in combination with bortezomib/carfilzomib results in increased sensitivity of myeloma cells to UPR-associated apoptosis and myeloma cell death. This can in part be rescued when protein biosynthesis and hence the level of ER stress after additional inhibition of β2/β2i is mitigated by cycloheximide, an inhibitor of protein translation.

Recent data suggest that bortezomib resistance in myeloma is an adaptive process in which the myeloma cell population adjusts the homeostasis of its protein biosynthesis apparatus to the selective pressure of proteasome inhibition.[19] This is achieved by decreased activity of the IRE-1α/XBP-1 regulatory axis of the UPR, which results in a more immature differentiation stage of myeloma cells and a reduction of the protein biosynthesis

machinery of the ER. We currently lack appropriate animal models for bortezomib-resistant myeloma that reflect this biology *in vivo*. However, the essential features of this "adaptive bortezomib resistance" in myeloma patients outlined by Leung-Hagesteijn[19] are mirrored well in the *in vitro* model of bortezomib/carfilzomib-adapted myeloma cells that we used here (selective pressure by a proteasome inhibitor, lack of mutations in the inhibitor-binding active site, decreased protein biosynthesis rate of resistant cells, decreased XBP-1 expression of resistant cells). The lack of mutations that would prevent effective binding of the drugs in the proteasome inhibitor binding pockets of our bortezomib/carfilzomib-adapted cells was verified by sequencing, but can also be deduced from Figure 4A, where more than 90% inhibition of the respective baseline bortezomib or carfilzomib-reactive proteasome activities was achieved by either bortezomib or carfilzomib in the bortezomib/carfilzomib resistant adapted cells. This is also in agreement with the lack of evidence for bortezomib resistance-conferring proteasome gene mutations in clinical samples.[18,31] It remains to be assessed whether myeloma with acquired bortezomib-resistance is similarly characterized not only by a reduced load of protein biosynthesis, but also by an increased β2 proteasome activity *in vivo*, as suggested by the *in vitro* model.

The therapeutic potential of targeting the β2/β2i proteasome activity as an addition to β5-targeted drugs is supported by pre-clinical as well as by clinical data: in most myeloma cell lines, the cytotoxicity of proteasome inhibitors does not correlate with the degree of inhibition of the chymotryptic activity, but with loss of subunit selectivity and the onset of inhibition of the tryptic or caspase-like sites.[21] Carfilzomib has a particularly high clinical activity against relapsed and refractory myeloma when delivered at an escalated 20/56 mg/m^2 dose, which results in significant co-inhibition of β2i activity *in vivo* (also in agreement with Figure 1A[32]). Indeed, our data demonstrate that the antimyeloma activity of carfilzomib *in vivo* can be increased by additional β2-inhibition with LU-102. It has been shown that combination of two β5-targeted proteasome inhibitors enhance the anti-myeloma activity of each, supporting the concept of combining proteasome inhibiting drugs.[33]

LU-102 is the first β2-selective proteasome inhibitor available to test a novel treatment concept to overcome bortezomib/carfilzomib resistance *via* simulatenous inhibition of the β2 proteasome subunit. However, LU-102 still has important limitations for potential clinical use, including its micromolar activity and poor tolerability of the vinylsulfone-type inhibitor in combination with bortezomib in the murine model. Replacing the reactive group by an epoxyketone, similar to carfilzomib, and fine-tuning the substituents in particular in the P1 and P3 positions with additional non-natural aminoacids with pKa covering the 5.5-9.0 range are predicted to improve activity, selectivity and cell permeability, and are underway to yield improved β2-selective candidate compounds towards further pre-clinical development of this concept.

In summary, our results demonstrate that the combination of bortezomib or carfilzomib with LU-102, a novel irreversible inhibitor selective for the β2/β2i proteasome subunits, induces superior proteasome inhibition and UPR activation compared to bortezomib or carfilzomib alone. This results in highly synergistic induction of proteotoxic death in bortezomib/carfilzomib-resistant myeloma cells. The combination of β5-targeting proteasome inhibitors with β2-selective proteasome inhibition may in particular offer treatment options for patients with bortezomib/carfilzomib resistant myeloma, a growing patient group with poor therapeutic options. Based on their target profile, β2-selective proteasome inhibitors may be potentially applied also to increase the antimyeloma activity of delanzomib, ixazomib and oprozomib in the future.

Funding

This research was supported by the Swiss National Research Foundation SNF (grant 31003A_143924/1 to C.D).

Authorship and Disclosures

Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at www.haematologica.org.

References

1. Adams J. The proteasome: a suitable antineoplastic target. Nat Rev Cancer. 2004;4(5):349-360.
2. San MJ, Weisel K, Moreau P, et al. Pomalidomide plus low-dose dexamethasone versus high-dose dexamethasone alone for patients with relapsed and refractory multiple myeloma (MM-003): a randomised, open-label, phase 3 trial. Lancet. Oncol 2013;14(11):1055-1066.
3. Siegel DS, Martin T, Wang M, et al. A phase 2 study of single-agent carfilzomib (PX-171-003-A1) in patients with relapsed and refractory multiple myeloma. Blood. 2012; 120(14):2817-2825.
4. Vij R, Siegel DS, Jagannath S, et al. An open-label, single-arm, phase 2 study of single-agent carfilzomib in patients with relapsed and/or refractory multiple myeloma who have been previously treated with bortezomib. Br J Haematol. 2012; 158(6):739-748.
5. Orlowski RZ. Novel agents for multiple myeloma to overcome resistance in phase III clinical trials. Semin Oncol. 2013; 40(5):634-651.
6. Reimold AM, Iwakoshi NN, Manis J, et al. Plasma cell differentiation requires the transcription factor XBP-1. Nature. 2001; 19;412(6844):300-307.
7. Obeng EA, Carlson LM, Gutman DM, et al. Proteasome inhibitors induce a terminal unfolded protein response in multiple myeloma cells. Blood. 2006;107(12):4907-4916.
8. Bianchi G, Oliva L, Cascio P, et al. The proteasome load versus capacity balance determines apoptotic sensitivity of multiple myeloma cells to proteasome inhibition. Blood. 2009;113(13):3040-3049.
9. Ling SC, Lau EK, Al-Shabeeb A, et al. Response of myeloma to the proteasome inhibitor bortezomib is correlated with the unfolded protein response regulator XBP-1. Haematologica. 2012;97(1):64-72.
10. Ciechanover A. Proteolysis: from the lysosome to ubiquitin and the proteasome. Nat Rev Mol.Cell Biol. 2005;6(1):79-87.
11. Singh AV, Bandi M, Aujay MA, et al. PR-924, a selective inhibitor of the immunoproteasome subunit LMP-7, blocks multiple myeloma cell growth both in vitro and in vivo. Br J Haematol. 2011;152(2):155-163.
12. Demo SD, Kirk CJ, Aujay MA, et al. Antitumor activity of PR-171, a novel irreversible inhibitor of the proteasome. Cancer Res. 2007;67(13):6383-6391.
13. Piva R, Ruggeri B, Williams M, et al. CEP-18770: A novel, orally active proteasome inhibitor with a tumor-selective pharmacologic profile competitive with bortezomib. Blood. 2008;111(5):2765-2775.
14. Kupperman E, Lee EC, Cao Y, et al. Evaluation of the proteasome inhibitor MLN9708 in preclinical models of human cancer. Cancer Res. 2010;70(5):1970-1980.
15. Zhou HJ, Aujay MA, Bennett MK, et al. Design and synthesis of an orally bioavailable and selective peptide epoxyketone

proteasome inhibitor (PR-047). J Med Chem. 2009;52(9):3028-3038.
16. Oerlemans R, Franke NE, Assaraf YG, et al. Molecular basis of bortezomib resistance: proteasome subunit beta5 (PSMB5) gene mutation and overexpression of PSMB5 protein. Blood. 2008;112(6):2489-2499.
17. Franke NE, Niewerth D, Assaraf YG, et al. Impaired bortezomib binding to mutant beta5 subunit of the proteasome is the underlying basis for bortezomib resistance in leukemia cells. Leukemia. 2012; 26(4):757-768.
18. Lichter DI, Danaee H, Pickard MD, et al. Sequence analysis of beta-subunit genes of the 20S proteasome in patients with relapsed multiple myeloma treated with bortezomib or dexamethasone. Blood. 2012;120(23):4513-4516.
19. Leung-Hagesteijn C, Erdmann N, Cheung G, et al. Xbp1s-negative tumor B cells and pre-plasmablasts mediate therapeutic proteasome inhibitor resistance in multiple myeloma. Cancer Cell. 2013;24(3):289-304.
20. Ruckrich T, Kraus M, Gogel J, et al. Characterization of the ubiquitin-proteasome system in bortezomib-adapted cells. Leukemia. 2009;23(6):1098-1105.
21. Britton M, Lucas MM, Downey SL, et al. Selective inhibitor of proteasome's caspase-like sites sensitizes cells to specific inhibition of chymotrypsin-like sites. Chem Biol. 2009;16(12):1278-1289.
22. Geurink PP, van der Linden WA, Mirabella AC, et al. Incorporation of Non-natural Amino Acids Improves Cell Permeability and Potency of Specific Inhibitors of Proteasome Trypsin-like Sites. J Med Chem. 2013;56(3):1262-1275.
23. Kraus M, Malenke E, Gogel J, et al. Ritonavir induces endoplasmic reticulum stress and sensitizes sarcoma cells toward bortezomib-induced apoptosis. Mol Cancer Ther. 2008;7(7):1940-1948.
24. Li N, Kuo CL, Paniagua G, et al. Relative quantification of proteasome activity by activity-based protein profiling and LC-MS/MS. Nat Protoc. 2013;8(6):1155-1568.
25. Verdoes M, Florea BI, Menendez-Benito V, et al. A fluorescent broad-spectrum proteasome inhibitor for labeling proteasomes in vitro and in vivo. Chem Biol. 2006; 13(11):1217-1226.
26. Berkers CR, Verdoes M, Lichtman E, et al. Activity probe for in vivo profiling of the specificity of proteasome inhibitor bortezomib. Nat Methods. 2005;2(5):357-362.
27. Chou TC. Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies. Pharmacol Rev. 2006; 58(3):621-681.
28. Hao M, Zhang L, An G, et al. Bone marrow stromal cells protect myeloma cells from bortezomib induced apoptosis by suppressing microRNA-15a expression. Leuk. Lymphoma. 2011;52(9):1787-1794.
29. Wang Z, Yang J, Kirk C, et al. Clinical pharmacokinetics, metabolism, and drug-drug interaction of carfilzomib. Drug Metab Dispos. 2013;41(1):230-237.
30. Tabas I, Ron D. Integrating the mechanisms of apoptosis induced by endoplasmic reticulum stress. Nat Cell Biol. 2011;13(3):184-190.
31. Politou M, Karadimitris A, Terpos E, Kotsianidis I, Apperley JF, Rahemtulla A. No evidence of mutations of the PSMB5 (beta-5 subunit of proteasome) in a case of myeloma with clinical resistance to Bortezomib. Leuk Res. 2006;30(2):240-241.
32. Badros, AZ; Papadopoulos, KP; Zojwalla, N; Lee, JR; Siegel, DS. A phase 1b study of 30-minute infusion Carfilzomib 20/45 and 20/56 mg/m2 plus 40 mg weekly Dexamethasone in patients with relapsed and/or refractory (R/R) multiple myeloma. Blood (ASH Annual Meeting Abstracts) 2012 120: Abstract 4036.
33. Sanchez E, Li M, Steinberg JA, et al. The proteasome inhibitor CEP-18770 enhances the anti-myeloma activity of bortezomib and melphalan. Br J Haematol. 2010; 148(4):569-581.