The use of activity based protein profiling to study proteasome biology

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Chapter 5: Proteasome Inhibitor-Adapted Myeloma Cells Show Proteomic Alterations That Suggest Complex Changes in Metabolic Pathways

Introduction

Proteasome inhibition is an important therapeutic concept in the treatment of multiple myeloma (MM), the most frequent hematologic malignancy [1]. Proteasome inhibitors are also increasingly used to treat lymphoma and acute leukemia, mostly in clinical trials. Next to the first in class, reversible, boronate-type proteasome inhibitor bortezomib and the irreversible, epoxyketone-type inhibitor carfilzomib that are currently in clinical use, several boronate- and epoxyketone-type proteasome inhibitors are in advanced clinical development [2, 3]. The mechanism of action of proteasome inhibition for MM treatment exploits the highly developed protein biosynthesis machinery in B-cell derived malignancies, including MM. This extraordinarily active biosynthetic route critically relies on timely disposal of misfolded and dysfunctional newly synthesized proteins through the ER-associated degradation machinery, of which the proteasome is the rate-limiting factor. Effective proteasome inhibition disturbs the equilibrium between production and disposal of non-functional or misfolded protein, which results in proteotoxic stress and excess activation of the unfolded protein response, which triggers apoptosis [4-6].

Current proteasome inhibitor-based myeloma treatments offer reliable control of the disease during early stages. However, MM treatment is not curative and a majority of MM patients will still die from relapsed refractory MM [7]. Understanding the biology of proteasome inhibitor resistance in MM, and also in other hematologic malignancies, and finding potential therapeutic strategies to overcome this resistance, are key challenges towards a more effective use of proteasome inhibition in MM and cancer treatment.

The proteasome is composed of two pairs of three proteolytically active sites (β1, β2, β5), which differ with respect to their substrate specificity. Immune cells, including myeloma, may replace these by respective active sites of the immunoproteasome (β1i, β2i, β5i) [8]. The β5 activity is the quantitatively most important, rate-limiting individual protease in the proteasome, and consequently bortezomib and also carfilzomib were designed to target the active center of the β5 subunit.

Proteasome inhibitor-resistant cells have been generated *in situ* by continuous exposure of cell lines to proteasome inhibiting drugs, and such proteasome inhibitor-adapted cells

served as models to understand and potentially overcome proteasome inhibitor resistance. Several groups have reported mutations in the PSMB5 gene leading to amino acid changes in the β5 active site or the bortezomib-binding pocket, currently considered as the most likely mechanism of proteasome inhibitor resistance [9]. However the functional relevance of these mutations to impair bortezomib binding and hence proteasome inhibition has never been directly demonstrated. In addition, it is unknown whether such mutations would also provide resistance against irreversible, epoxyketone-type next generation inhibitors.

Extensive analysis of material derived from patients with relapsed-refractory myeloma has so far failed to confirm the presence of such PSMB5 mutations [10, 11]. Instead, an alternative model for the biological basis of bortezomib resistance in MM has been put forward, based on findings from MM cells of bortezomib-resistant patients. In this model, bortezomib resistance was the result of complex changes in the activation status of the UPR, which were initiated by decreased activity of the IRE1/XBP-1 axis, one of the three main regulatory switches that control UPR activity, which in turn is closely connected to MM cell differentiation [12, 13].

Recently, selective probes for active proteasome subunits that for the first time allow to address directly the activity states of all subunits of the constitutive proteasome and the immunoproteasome have been developed [14-18]. Using these tools, the functional relevance of the most common PSMB5 mutation for proteasome inhibition by bortezomib and carfilzomib in resistant MM cells were examined as reported in this chapter. Because the here presented results suggest that PSMB5 mutations are dispensable to mediate proteasome inhibitor resistance in myeloma cells, a global proteomics analysis is carried out to compare IRE1/XBP-1-high, proteasome inhibitor-sensitive MM cells to IRE1/XBP-1 low, bortezomib- or carfilzomib-resistant subclones to map the complex changes in functional protein networks of proteasome inhibitor resistant myeloma cells to ultimately suggest new potential therapeutic targets.

Results

Bortezomib- or carfilzomib-adapted sub-cultures were established from the AMO-1 myeloma cell line by continuous drug exposure, as described [19]. Because such sub-cultures do not exhibit uniform cell morphology, as determined by light microscopy, limiting dilution experiments were performed to derive single cell-derived, bortezomib- or carfilzomib-adapted subclones (AMO-BTZ and AMO-CFZ) as a uniform and reliable basis

for further studies. Sequencing of all six proteasome active subunits revealed the presence of an A310G mutation leading to a Met45Val change in the S1 pocket of the PSMB5 active site in bortezomib-resistant bulk cultures as well as in all single cell-derived clones from these cultures (data not shown). In contrast, genetic changes in the PSMB1, PSMB2 and PSMB5 genes could be excluded in carfilzomib-adapted AMO cells, as well as in all respective single-cell derived clones. The absence of a PSMB5 mutation in AMO-CFZ cells demonstrates that point mutations in proteasome genes are not required in proteasome inhibitor resistant myeloma cells.

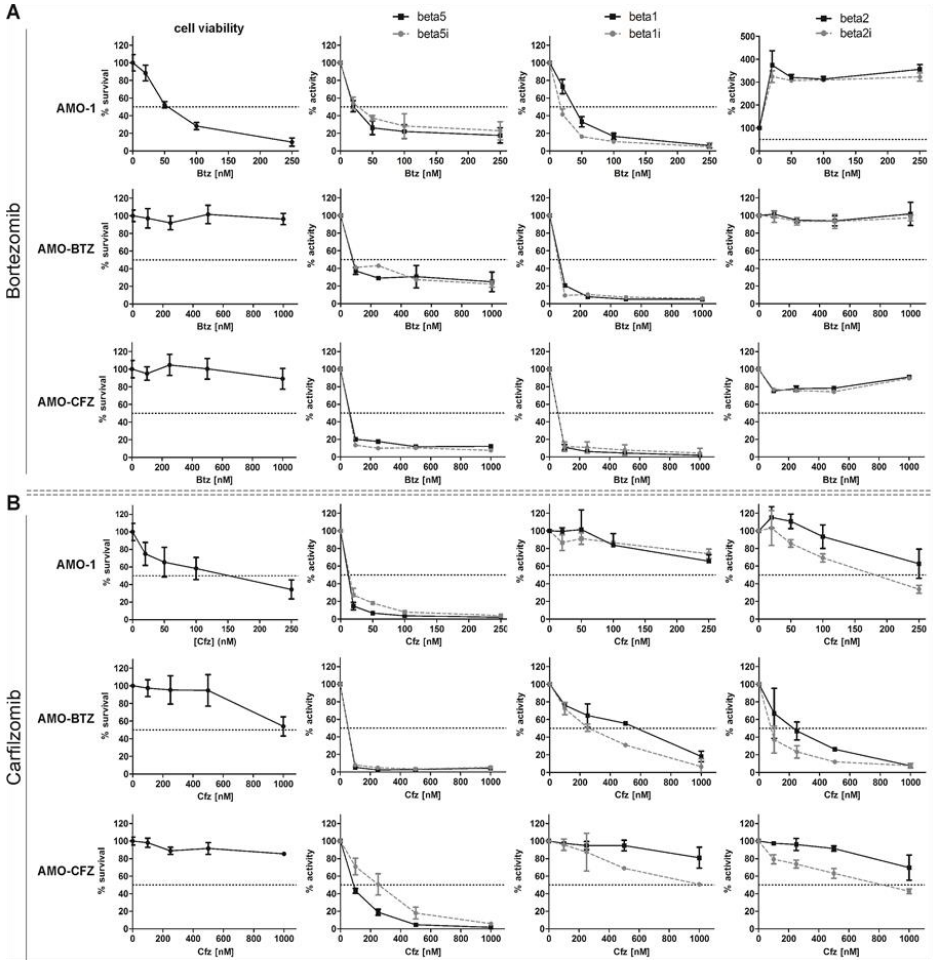

***Figure 1**. Activity-based protein profiling for all proteasome subunits in every cell line after 1 h exposure to bortezomib (A) or carfilzomib (B) and their proliferation rate after 48 h.*

Next a set of newly developed activity-based probes (described in Chapter 4 in more detail) is used to directly visualize changes in the proteasome-inhibiting on-target activity of bortezomib in the respective proteasome inhibitor-adapted MM cells (figure 1a). To this end, the proteasome inhibitor-adapted AMO-BTZ and AMO-CFZ cells were grown in the absence of proteasome inhibitors for 14 days and consecutively exposed to increasing concentrations of either bortezomib or carfilzomib for one hour, followed by removal of the drugs, mimicking the pharmacokinetic situation after intravenous (i.v.) administration of proteasome inhibitors in the clinic. Cells were subsequently analyzed with proteasome activity-specific chemical probes. The IC_{50} values for β1c and β1i inhibition was found to be comparable in AMO-1 and AMO-BTZ cells (table 1), while the IC_{50} value for β5c was approximately threefold lower in AMO-BTZ cells, compared to AMO-1 These data are consistent with a PSMB5 mutation hampering binding of bortezomib at the β5c, but not at the β1 subunits. Incubation with 250 nM bortezomib, a concentration that matches peak bortezomib plasma levels in myeloma patients minutes after i.v. bolus administration, resulted in approximately 75% inhibition of β5c/β5i in AMO-1 cells, and only moderately less effective inhibition (60-70%) in AMO-BTZ cells. These moderate quantitative differences in inhibition of β1/β5-type proteasome activity contrasted with the fundamentally different dose response for bortezomib-induced cytotoxicity between both cell lines, where the IC_{50} was 50 nM in AMO-1 cells, while evidence for toxicity was essentially lacking even at 1000 nM bortezomib in AMO-BTZ cells.

Bortezomib inhibited β5/β1 activity in AMO-CFZ cells with similar efficacy, compared to AMO-1 control cells, consistent with the absence of mutations in the proteolytically active proteasome subunits in AMO-CFZ cells. Strikingly, bortezomib did not induce cytotoxicity despite >80% inhibition of active β1c/β1i and β5c/β5i proteasome activities in AMO-CFZ cells. The activity of β2c/β2i proteasome subunits is not targeted by bortezomib, and a substantial upregulation of β2/β2i activity was seen in AMO-1 cells after bortezomib treatment consistent with earlier work, while AMO-BTZ or AMO-CFZ myeloma cells or HL-60 leukemia cells lacked such β2-activation by bortezomib.

		Proteasome subunit	AMO-1	AMO-BTZ	AMO-CFZ
BTZ	IC_{50} (nM) (50% functional proteasome inhibition)	β2	N.A.	N.A.	N.A.
		β2i	N.A.	N.A.	N.A.
		β1	40	60	50-60
		β1i	20-25	50	50-60
		β5	25	80-90	50-60
		β5i	30	80-90	50-60
	LD_{50} (nM) (50% cell viability)		50	> 1000	> 1000
CFZ	IC_{50} (nM) (50% functional proteasome inhibition)	β2	300	220	>1000
		β2i	180	90	800
		β1	> 500	600	> 1000
		β1i	>250	250	1000
		β5	10	50	90
		β5i	10	50	250
	LD_{50} (nM) (50% cell viability)		150	> 1000	> 1000

***Table 1**. IC_{50} values obtained for Bortezomib and Carfilzomib in each cell line after 1 hour exposure*.

The effect of carfilzomib on proteasome activity in adapted and non-adapted AMO cells was analyzed in the same way (figure 1b). Carfilzomib was much more selective for β5c/5i over β1c/1i and in addition had some β2c/2i-inhibiting activity, both in contrast to bortezomib. The pattern and the dose response of inhibition of β2c/2i and β1c/1i were identical between AMO-1 and AMO-BTZ cells during carfilzomib treatment, while the IC50 for β5c/5i was approximately 5 fold higher (10 nM vs. 50 nM) in AMO-1 vs. AMO-BTZ cells, suggesting that the PSMB5 A310G mutation affects β5 binding of carfilzomib in a fashion comparable to bortezomib. Carfilzomib treatment was significantly less effective in proteasome inhibition in AMO-CFZ cells, in contrast to bortezomib in the same cells. However, this was not a subunit-selective feature and had the same order of magnitude (approximately 5-10 fold lower IC50) also for β1c/1i and β2c/2i proteasome activity, suggesting that active drug export may be involved. If equally effective proteasome inhibition was achieved in AMO-1 and AMO-CFZ cells (>90% inhibition of β5c/5i, 20% inhibition of β1/1i, β2/2i), this resulted in marked (>50%) cytotoxicity in AMO-1 cells, whereas no cytotoxicity was observed in AMO-CFZ cells with the same degree of proteasome inhibition.

Together, these results indicate that adaptive resistance to proteasome inhibitors *in situ* can render myeloma cells largely independent from activity of both the constitutive proteasome and the immunoproteasome, that this is not specific for a given type of proteasome inhibitor, and that also active site mutations are not required to reach

adaptive proteasome inhibitor resistance. Complex biological adaptations outside the proteasome pathway are thus likely to be involved.

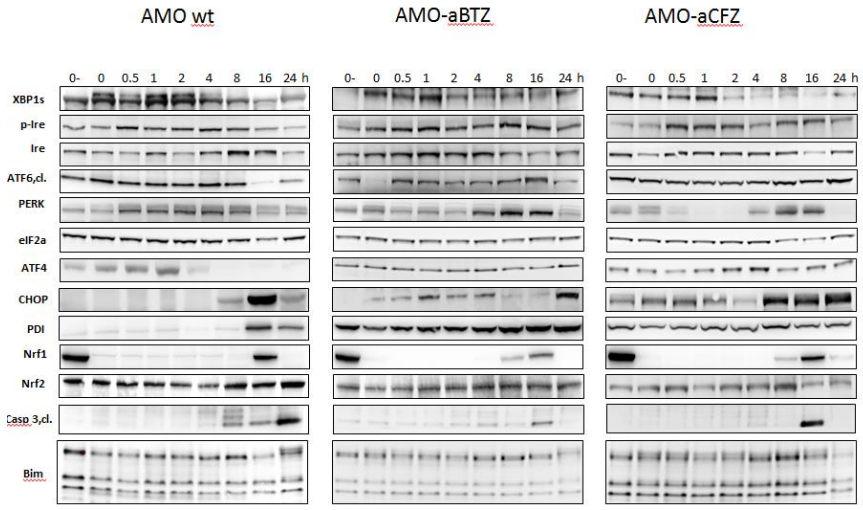

Figure 2. *Western blot analysis of UPR-induced apoptotic machinery*.

Next the effects of bortezomib on the UPR-induced apoptotic machinery in AMO-1, AMO-CFZ and AMO-BTZ cells were compared by Western blot (figure 2). The active version of the Nrf1 transcription factor is generated by partial proteolysis through the proteasome [20]. Exposure of AMO-1, AMO-BTZ and AMO-CFZ cells to bortezomib abolished production of active Nrf1 for approximately 8 hours, functionally confirming that proteasome inhibition and blockade of its protein turnover has been achieved in all three cell types, independent from the PSMB5 mutation status. Of the three major UPR-controlling transmembrane regulators IRE-1, PERK and ATF6, it was found that upon bortezomib challenge phosphorylation of IRE-1 was triggered within 1-2 hours in all cell types, leading to a consecutive increase in the spliced version of XBP1 also in all three cell types. Increased expression of PERK is initiated at later time points. Triggering of the UPR-related apoptotic machinery via ATF4 and CHOP is observed in AMO-1 and AMO-CFZ cells, but only poorly in AMO-BTZ cells, and downstream activation of caspase 3 is again less prominent in AMO-BTZ cells. Interestingly, bortezomib treatment led to a marked increase in PDI expression in AMO-1 cells, as expected, no change in PDI expression was observed in AMO-BTZ or AMO-CFZ cells, suggesting adaptive changes in the reducing and protein folding machinery of the endoplasmatic reticulum. In summary the data shows that

functionally efficient proteasome inhibition is being achieved with bortezomib in proteasome inhibitor-adapted cells, independently from the presence or absence of a mutation in the PSMB5 gene, and that comparable downstream signaling along the UPR apoptotic pathway is initiated, albeit less efficient in AMO-BTZ cells, while no cytotoxicity is achieved in proteasome inhibitor-adapted cells.

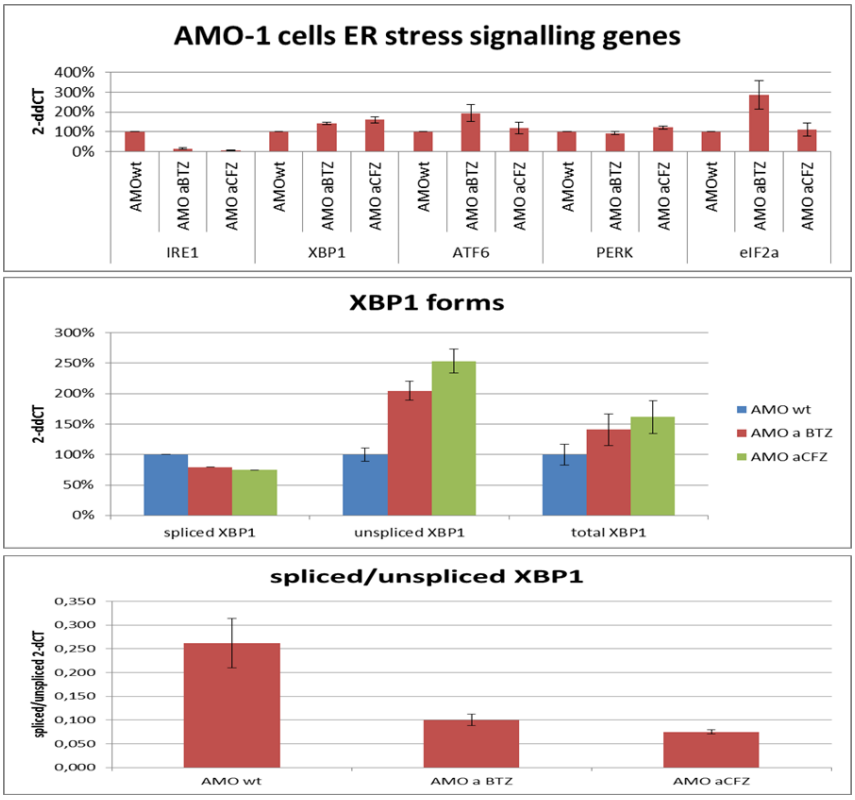

Figure 3. *UPR sensors screen. RNA quantification of the different UPR sensors and screen on the different XBP1 isoforms.*

Quantitative changes in the regulatory machinery of the UPR, namely IRE-1 and the product of its activity, sXBP1, result in bortezomib resistance in genetically engineered myeloma cells and have also been found in myeloma cells from BTZ-resistant patients. Whether such downregulation of the IRE1/XBP1 regulatory axis evolves during the adaptation of myeloma cells to proteasome inhibitor treatment was analyzed next. Only IRE-1 showed a quantitatively significant difference on mRNA levels in AMO-1 vs. AMO-BTZ and AMO-CFZ cells, in contrast to ATF6, PERK and eIF2a (figure 3). Consistent with this, significantly reduced levels of spliced XBP-1 mRNA, the functional result of IRE-1

activity, were found. On protein level, IRE1 and sXBP1 were uniformly strongly reduced in AMO-BTZ and AMO-CFZ, in contrast to elF2a, ATF6 and PERK.

In order to unravel the biological complexity of adaptation to PIs, a proteomics approach was used to identify proteins with significant changes in expression levels in the adapted vs. non-adapted cells. In each experiment, from around 3500 different identified proteins, 2000 were quantified of which more than 600 were differentially expressed in proteasome inhibitor-adapted cell lines, com-pared to the non-adapted controls (using as cut-off a statistically significant two-fold change in expression over 3 replicates, figure 4). Interestingly, in the bortezomib-adapted leukemia cell line HL60-BTZ, only 300 proteins showed significantly changed expression levels, although a similar total number of proteins as in the myeloma cell lines was identified and quantified, suggesting a more complex adaptation pathway of myeloma cells, compared to leukemia cells, consistent with our experience that the adaptation process *in situ* takes considerably longer in myeloma cells.

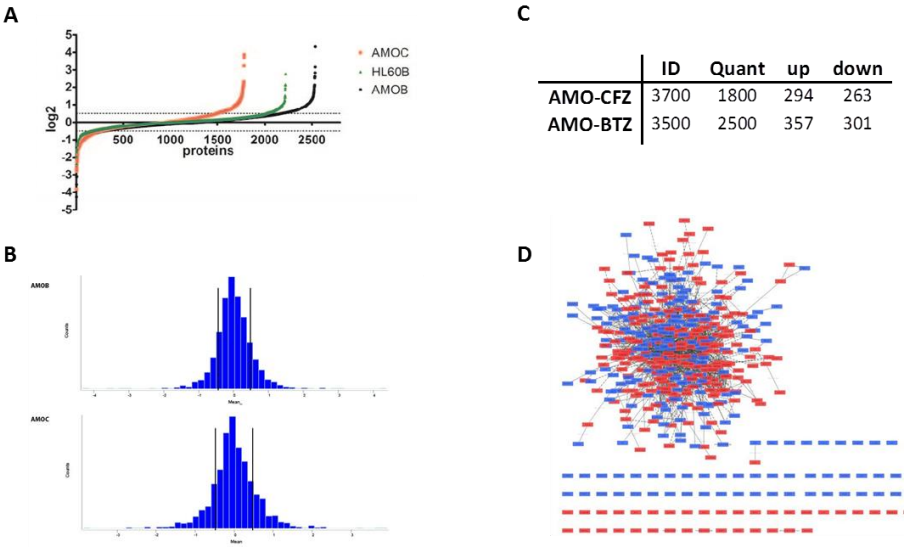

Figure 4*. Global proteome analysis for differentially expressed proteins. A)* Distribution of proteins according to their log2 value. B) Histogram plot of the log2 distribution for AMO-BTZ and AMO-CFZ of the differentially expressed genes; the solid vertical lines indicate the log2 cut-off. C) Table 1 showing the average values obtained in each analysis. (ID= found in either of the 3 replicates. Q=found in at least 2 out of 3 replicates. Light and heavy peptides are found in each replicate. Difference between ID and Q is due to absence of light or heavy peptide in the analysis) D) Protein-Protein interaction network where red boxes are proteins upregulated and blue boxes are downregulated.

The differentially expressed proteins were subjected to a protein-protein interaction (PPI) search to build a PPI network. This resulted in a highly complex adaptive net-work in proteasome inhibitor-resistant myeloma cells that cannot easily be reconciled with a single point mutation mediating proteasome inhibitor resistance by interfering with the on target activity of the inhibitor. Differentially expressed proteins were subjected to a Gene Ontology (GO) analysis, resulting in an average of 30 GO terms for the overexpressed proteins and 10 for the downregulated species in all three adapted clones. GO terms involving the proteasome were highly enriched in all samples, as expected, in agreement with proteasome over-expression. GO terms were then manually grouped into functionally related clusters resulting in 5-6 functionally connected groups of differentially expressed proteins (table 1). This supervised clustering showed high concordance between the different adapted clones and therefore suggested that acquired resistance to PI is characterized by a typical, complex pattern of changes in protein expression.

Change	GO term	p-value range	number of proteins	mean fold change (range)
AMO-BTZ				
upregulated	protein catabolism	10^{-8}	19	1.646 (2,30-1,41)
	redox homeostasis	$10^{-7} – 10^{-6}$	25	1.875 (6,06-1,41)
	folding and complex formation	$10^{-3} – 10^{-2}$	53	1.955 (19,70-1,41)
	transport/signaling	$10^{-3} – 10^{-2}$	116	1.730 (4,29-1,41)
	metabolic regulation	$10^{-3} – 10^{-2}$	114	1.805 (9,19-1,41)
downregulated	transcription/translation	10^{-2}	63	0.559 (0,71-0,20)
	differentiation	10^{-2}	13	0.557 (0,71-0,38)
	cytoskeleton organization	10^{-2}	22	0.478 (0,71-0,08)
	metabolic regulation	10^{-2}	45	0.589 (0,71-0,38)
	transport/signaling	10^{-2}	89	0.518 (0,71-0,05)
	apoptosis	10^{-1}	8	0.547 (0,71-0,26)
AMO-CFZ				
upregulated	protein catabolism	10^{-8}	22	1.741 (2,64-1,41)
	transport/signaling	$10^{-6} – 10^{-2}$	85	1.891 (12,13-1,41)
	metabolic regulation	$10^{-6} – 10^{-2}$	110	1.795 (4,59-1,41)
	folding and complex formation	$10^{-3} – 10^{-2}$	39	2.148 (13,93-1,41)
	redox homeostasis	$10^{-3} – 10^{-2}$	15	2.201 (4,29-1,41)
downregulated	apoptosis	10^{-3}	7	0.468 (0,71-0,16)
	metabolic regulation	$10^{-3} – 10^{-2}$	58	0.536 (0,71-0,22)
	transport/signaling	10^{-2}	72	0.509 (0,71-0,14)
	transcription/translation	10^{-2}	58	0.555 (0,71-0,19)
	differentiation	10^{-2}	10	0.479 (0,71-0,23)
	cytoskeleton organization	10^{-2}	24	0.451 (0,71-0,07)

***Table 2**. Manually annotated GO (gene ontology) terms. P-values indicate the range of the clustered GO terms. Mean fold change is representative for the proteins grouped in each term.*

The functional protein clusters found overexpressed in this analysis were proteins involved in protein catabolism, redox control and protein folding, consistent with proteotoxic stress induced by proteasome inhibition. Almost all proteasome alpha (PSMA

1,2,3,4,7) and beta (PSMB 1,2,3,4,5,7) subunits were individually detected as significantly overexpressed polypeptide species in in both adapted myeloma cells, as expected from previous studies, corroborating the sensitivity and specificity of our analysis. Interestingly, only the constitutive proteasome active subunits were found upregulated, while the β2i subunits were found down-modulated in the bortezomib-adapted cells.

The adapted clones had higher concentrations of antioxidant and ROS scavenging proteins, like NQO1, PRDX1 or SOD1, illustrating an increased importance of redox cycling in adapted cells. Adapted cells also expressed increased amounts of proteins involved in glutathione regulation, one of the most potent cellular antioxidants. Variations were found in the individual proteins up-regulated in a given functional pathway between bortezomib-adapted and carfilzomib-adapted clones (e.g., GPX1, one of the major enzymes responsible for glutathione peroxidation and thus removal of oxygen peroxide, was only overexpressed in AMO-BTZ, but not in AMO-CFZ, while glutathione transferase 1 (MGST1), which acts by conjugating reduced glutathione to a wide number of exogenous and endogenous hydrophobic electrophiles, was only found upregulated in AMO-CFZ, of which a variant (MGST3) was found upregulated in AMO-BTZ). However, the upregulation of proteins involved in redox pathways overall showed a very consistent pattern between AMO-CFZ and AMO-BTZ cells. Of the 16 proteins significantly upregulated in this cluster in AMO-CFZ cells, 12 were likewise found upregulated in AMO-BTZ cells, suggesting a central role of the redox equilibrium in proteasome inhibitor-adapted myeloma cells.

The protein folding/chaperoning capacity of the adapted cells was also markedly increased, compared to non-resistant control cells. The heat shock proteins HSP70, HSP90 and HSP105 were consistently among the top 15 quantitatively most strongly upregulated proteins (2.3 fold to 13 fold increase) in the group of proteins involved in folding/chaperoning in both adapted cell clones.

A pattern of uniform downregulation was observed for protein clusters involved in transcription/translation, differentiation, apoptosis and structural/cytoskeletal functions. Transcription and translation regulation proteins comprised about one fourth of the total downregulated protein species. In this group, transcription factors (e.g. GTF2I), histone subunits (e.g. HIST1H1B) or DNA/RNA processing enzymes (e.g. TOP2A or DCPS) were found, as well as the Ki67 antigen that is involved in RNA biosynthesis and is commonly used as a marker for a proliferative cell fraction. The apoptosis protein cluster comprised the lowest number of proteins. Both adapted cells markedly downregulated individual key proteins involved in apoptosis: AMO-CFZ had decreased expression of BAX, while the AMO-BTZ had a lower expression of the CASP10 and DIABLO proteins. BCLAF2, a transcriptional repressor, which promotes cell death, was the only protein shared in this

group by both adapted clones. Downmodulation of cytoskeleton proteins were indicated by CORO1A or CAPG, both modulators of the cytosolic or nuclear structure. The last biological cluster found downregulated in this model was the group of proteins involved in cell differentiation, which included for example VAV1, involved in the activation of Rho/Rac GTPases, or IKZF3, a transcription factor implicated in lymphocyte differentiation.

The quantitatively largest group of proteins with significantly altered expression levels in adapted vs. control cells was the one involved in metabolic regulation. This big cluster comprised close to 50 % of all polypeptide species with significant quantitative changes identified, suggesting that metabolic homeostasis is a major challenge for myeloma cells adapted to proteasome inhibitor treatment. This group of upregulated proteins consisted mainly of proteins involved in the respiratory chain (e.g. CYC1 or UQCR1), the generation of metabolites (e.g. BLVRA or APOA1BP), glycolysis (e.g. PFKP or PKM) and amino acid (e.g. GOT1 or EEFSEC) or nucleic acids metabolism (e.g. UMPS or BOP1). On the other hand, the metabolic regulation category also comprised a significant fraction of proteins (30%) that were significantly downregulated. These were in particular proteins involved in lipid and cofactor metabolism (e.g. ACST2 or ACLY) as well as proteins involved in glycolysis events (e.g. B4GALT3 or NAGK). Interestingly, of the top 25 quantitatively downregulated proteins in the metabolism protein cluster, 12 proteins were mitochondrial proteins and mostly involved in the biosynthesis of fatty acids (ACSF2, ACSF3) in carfilzomib-adapted cells, and likewise 5 respective mitochondrial proteins were found among the top 25 downregulated in bortezomib-adapted cells.

Likewise, in the transport and signaling protein cluster upregulated and down regulated proteins were found, however, there was quantitatively a rough balance between upregulated and down-regulated species. The upregulated protein/ion transport and signaling group was characterized by upregulation of ion transporters (e.g. ATP1A1 or ATP2B4), which allow cations to cross the plasma membrane, and which transfer small charged molecules through the mitochondrial outer membrane (STIM1 or VDAC2). Amino acid pumps like SLC1A4 or SLC7A5 were also found in this cluster as well as protein transporters, for example SEC23A which promotes the transport of proteins from the ER to the Golgi complex. Proteins species with decreased protein expression were pumps involved in ion transport into the ER (e.g. STIM1 or ATP13A1) and also proteins involved in ER signaling (e.g. ERP29 or SRPR), consistent with changes in the ER homeostasis. Other signaling molecules with lower expression in the adapted cells included some G-proteins (GNAI3 or GNG7) involved in cell division and other signaling pathways or proteins implicated in cell adhesion (e.g. CD44, one of the top hits in the quantitative ranking of downregulated proteins).

A manual search was performed for individual proteins which showed quantitatively prominent changes in protein expression levels, at the same time have key functions in their pathways that can rationally be explained as compensation mechanism for a lack of proteasome function, and that are definitively or at least potentially druggable. The quantitatively most significantly upregulated protein was the multidrug resistance protein 1 (ABCB1, 12 fold upregulated in AMO-ACFZ), suggesting that carfilzomib, a predicted substrate of MDR proteins, is quantitatively exported from the extracellular space, limiting the intracellular efficacy of proteasome inhibition. ABCB can be targeted with calcium channel blockers such as verapamil. Of note, ABCB was not upregulated in AMO-BTZ cells. The second most prominent quantitative hit in AMO-CFZ cells was the N-myc downstream-regulated gene NDRG1 (>9 fold upregulated in AMO-CFZ, 4.4 fold up-regulated in AMO-BTZ), a protein that is known as the molecular cause of Charcot–Marie–Tooth type 4D disease. NDRG1 is a hydrolase related to cell stress and cancer conditions, and is strongly upregulated under hypoxic conditions. NADPH dehydrogenase is the quantitatively most important reducing enzyme in eukaryotic cells. It was overexpressed 4-6 fold in AMO-BTZ and AMO-CFZ, respectively, and in addition the enzymes that generate NADPH (malate dehydrogenase and enzymes of the pentose phosphate pathway) were also overexpressed, high-lighting the crucial functional role of maintaining reducing conditions under the selective pressure of proteasome inhibitor treatment. The anti-diabetes drug metformin has been shown to suppress the activity of NADPH dehydrogenase. The transcription factor IKZF3 is known as an essential transcription factor in myeloma, which is targeted by treatment with lenalidomide. IKZF3 was the top downregulated protein in the cluster of differentiation-related proteins in AMO-BTZ cells it and was also found significantly reduced in AMO-BTZ cells.

Discussion

It has been postulated that point mutations in the proteasome β5 pocket are sufficient to confer proteasome inhibitor resistance. This feature though, was only observed in PI adapted cell lines but not in relapsed patients, suggesting that a different adaptation mechanism apart from mutations in the β5 subunit might be triggering the resistance against proteasome inhibitor induced cytotoxicity. In the adapted cell line AMO-BTZ a single point mutation was found in the β5 subunit pocket, and indeed this was causing a lower binding affinity for both inhibitors tested in this study, needing higher inhibitor concentrations to reach a comparable inhibition rate as the one obtained in the wild type AMO-1 cells. In AMO-CFZ cells no mutations were found in any of the active proteasome β-subunits. The proteasome inhibition rates for bortezomib and carfilzomib in these cells

were comparable with those observed in AMO-1 cells. At high inhibitor concentrations the β5/5i subunit activity was almost fully inhibited in all three cell lines, showing that proteasome inhibitors are still efficiently blocking proteasome activity even in resistant cells and that a comparable downstream signaling among the UPR pathway is initiated. However no cytotoxicity is induced in the adapted cell lines even at high inhibitor concentrations (1000 nM) while the wild type AMO-1 cells on the other hand experience high proteotoxic stress already at 250 nM which induces apoptosis in these cells. When analyzing the mRNA levels of the UPR sensors and downstream effectors in all three cell lines, it was found that the mRNA levels of the IRE1/XBP1 branch had substantially changed which is in concordance with previous observations in patients. This data shows that proteasome inhibitor resistance is independent of the type of proteasome inhibitor used and that site mutations are not required for adaptation.

The comparison of protein levels between AMO-1 cells and the adapted subclones shows high concordance between the found biological clusters in both resistant cell lines. Most of the proteins grouped in each cluster are shared between both resistant clones and the ones that are not have a comparable biological function suggesting that resistance is characterized by a typical pattern of changes in protein expression. Among the upregulated clusters, the proteasome machinery, the redox apparatus and the protein folding capacity are in concordance with PI induced proteotoxicity, thus an overexpression of these cellular mechanisms is in agreement with a lower sensitivity towards proteasome inhibition due to the cellular capacity to couple proteolytic stress. Taking this together with the fact that the protein synthesis machinery is downmodulated might explain why proteasome inhibition is not a suitable treatment for adapted cells. Adapted cells seem to have a lower basal ER-stress due to their lower production of proteins and an increased folding and degradation army. The fact that a cluster comprising apoptosis signaling was found to be downregulated in adapted cells does support this hypothesis. Having a downmodulated cluster covering differentiation proteins gives an additional indication towards this theory since plasma cells are fully differentiated B-cells, whose main function is to produce immunoglobulins for secretion. This high protein production is increased in neoplastic plasma cells and is what makes this cancer suitable for proteasome inhibition treatment. Therefore de-differentiation might be beneficial for adaption towards PI. This is in agreement with the loss of IRE1/XBP1 expression, which overexpression is fundamental for full differentiation into functional antibody producing plasma cells. This phenomenon was observed in patients with relapsed or refractory myeloma, where a subpopulation of progenitor cells was found to have lower expression levels of IRE1 and XBP1 [13] and was conferring resistance towards proteasome inhibitors.

Finding that the ion and protein transport into the ER and between this organelle and the Golgi apparatus is hampered in the resistant subclones does support the hypothesis of a rearrangement in the cellular secretion machinery since secreted proteins are mainly synthetized and folded inside the ER and translocated to the Golgi for their transport to the extracellular matrix. While this specific transport signaling is downmodulated in resistant cells, some ion pumps and amino acid transporters are overexpressed. Having enhanced the ion import/export machinery may be beneficial for redox homeostasis being able to quickly exchange protons or ions between organelles or with the extracellular matrix when dealing with redox stress. Overexpressing pumps to export potentially dangerous compounds may influence the intracellular drug efficiency and thus be favorable for adaptation.

About 50% of the proteins found in this study were involved in metabolic regulation indicating that metabolic homeostasis is a major challenge for adapted cells. Even having in this cluster proteins up- and downregulated, their biological functions are different. In the downmodulated group mainly proteins involved in the lipid and cofactor metabolism were found while the overexpressed proteins could be grouped into metabolites, amino and nucleic acids metabolism, glycolysis and into the respiratory chain machinery. This is indicating that tight regulation of the cellular metabolism is necessary for adaptation against proteasome inhibition.

From all the different protein clusters found in this study the most potentially druggable target groups might be the ion transporters and the redox machinery. It gives the impression that the antioxidant capacity of the cells plays a central role in resistant cells thus obstructing these mechanisms by blocking ions/proton channels or inhibition of reducing enzymes, or even by inducing reactive oxygen species, might be a valuable option as alternative or supplementary treatment.

Conclusion

It has been proven that proteasome inhibitor acquired resistance is independent of point mutations in the active proteasome subunits and of the type of proteasome inhibitor used and that efficient proteasome blockade is achieved in adapted cells in a comparable manner as in wild type cells without experiencing proteotoxic stress. This indicates that the adaptation mechanism might be a complex cellular rearrangement rather than a simple mutation that disrupts an efficient proteasome inhibition. Having comparable results in the complex protein expression changes and also in the pathways affected by

these adjustments in this proteomic study for both resistant cell lines, does support this hypothesis. This is also in accordance with the recent literature reports with patient material were no point mutations have been found and an imbalance in the ER homeostasis has been observed.

The here presented data shows that a complex regulation of cellular homeostasis has taken place to couple proteasome inhibition. From all the different pathways found affected by this rearrangement, the antioxidant machinery and the ion transporters seem to be the best targets for supplementary treatments. It might be wise combining proteasome inhibitors with ion channel blockers alone or together with oxidative agents or inhibitors of reducing enzymes. This combination of drugs might be a useful tool to fight proteasome inhibitor acquired resistance.

Experimental procedures

Activity-based protein profiling (ABPP) and survival

Adapted cells were grown for two weeks without the presence of inhibitor before performing the experiments. Cells were seeded to an end concentration of $0.5x10^6$ cells/mL and treated with the indicated inhibitor concentration. After 1 h of pulse treatment, medium was refreshed and 25000 cells were seeded in a new dish and grown for extra 48 h to measure the cell proliferation rate. Rest of the cells were harvested immediately and subjected to ABPP. Three different activity-based probes (ABPs) were used to independently quantify each active subunit of the proteasome. Each experiment was performed in triplicate. Fluorescent bands were measured with the ChemiDoc™ MP System and quantified using Image Lab software (BioRad). Untreated cells were used as control and its band intensity was defined as 100, the rest of the samples were normalized to the control. Proliferation assay was performed using the CellTiter-Glo Luminescent Cell Viability Assay kit (Promega) and performed as indicated by manufacturer. Each experiment was performed in triplicate.

Global Proteomics

Whole cell lysate was first digested with trypsin and then dimethyl labeled light (wildtype cells) or heavy (resistant cells). After labeling the samples were pooled together, subjected to SCX fractionation (strong cation exchange) and analyzed by LC/MS. Identification and quantification was done by MaxQuant software. A cut off of log2=0.5 was used to identify differentially expressed proteins. Each analysis was performed at least in triplicate. Only proteins being identified and quantified in at least 2 out of 3 replicates were used for further analysis. The differentially expressed

proteins were subset to a protein-protein interaction analysis by the Cytoscape software. The Cytoscape app BINGO was used to perform a gene ontology (GO) analysis looking for biological processes being overrepresented in the network. The search gave around 20-30 biological processes being either up- or downregulated in the resistant cell lines. From these 20-30 terms, a manual curation was done in order to cluster these GO terms in more general groups. P-values indicate the range of the clustered GO terms. Proteins not classified by the software were manually assigned to the different clusters.

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