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**Mapping the Proteasome Inhibitor
Resistance in Multiple Myeloma**

Habilitation Thesis

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Abstract

Multiple myeloma is a plasma cell malignancy that, despite two decades of notable therapeutic progress, remains difficult to treat. Proteasome inhibitors were the first biologically driven therapy introduced for myeloma and remain a cornerstone of nearly every treatment regimen, yet nearly all patients ultimately relapse and develop resistance. The biology underlying this resistance is one of the central unresolved questions in the field and also the overarching theme of this thesis. The presented habilitation thesis summarizes the current understanding of multiple myeloma biology, the proteostasis network that malignant plasma cells depend on, and the mechanisms by which resistance to proteasome inhibitors develops, highlighting key contributions from my research group and collaborations. Together the contributions support a unifying view, that resistance to proteasome inhibitors emerges as a proteostasis network plasticity rather than as a single fixed genetic lesion. Consequently, durable sensitization to proteasome inhibitors requires overwhelming the proteostasis network. This can be achieved directly, by broader-subunit, higher-dose proteasome inhibition, or by targeting additional vulnerabilities of therapy-resistant plasma cells such as membrane biology or upstream signaling, which collectively disrupt proteostasis from different angle, preventing the compensable node.

The proposed habilitation thesis is written as a collection of previously published scholarly works, while the relevant parts of the theoretical introduction are always supplemented by a commentary on published contributions within the given topic.

Declaration

I hereby declare that this thesis with title Mapping the Proteasome Inhibitor Resistance in Multiple Myeloma that I submit for assessment is entirely my own work and has not been taken from the work of others save to the extent that such work has been cited and acknowledged within the text of my thesis.

In accordance with the standards of proper scientific practice, I declare that an AI-based language tool was used during the preparation of this thesis solely to assist with language editing and stylistic refinement of the text.

Brno, August, 2026

.....
Lenka Bešše

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Table of Contents

List of Figures	9
Glossary	10
1 Introduction	15
2 Theoretical Background	17
2.1 Multiple Myeloma.....	17
2.2 Protein Homeostasis in MM	19
2.3 Proteasome Inhibition as a Therapeutic Strategy in MM	25
2.4 Mapping Proteasome Inhibitor Resistance in MM	31
2.5 The Future of Proteasome Inhibitor-Based Therapy in MM	44
3 Commentary to Published Work	46
3.1 Commentary to Published Work – Annex 1	46
3.2 Commentary to Published Work – Annex 2	49
3.3 Commentary to Published Work – Annex 3	52
3.4 Commentary to Published Work – Annex 4	55
3.5 Commentary to Published Work – Annex 5	58
4 Conclusion	61
Bibliography	63
5 Supplementary Material	78
5.1 Annex 1.....	78
5.2 Annex 2.....	95

TABLE OF CONTENTS

5.3 Annex 3..... 108

5.4 Annex 4..... 119

5.5 Annex 5..... 125

List of Figures

Figure 1: Proteostasis in MM.....	20
Figure 2: Proteasome in MM. Types of core 20S units and caps present in MM PCs	22
Figure 3: UPR sensing in MM PCs	24
Figure 4: Cellular effects of proteasome inhibition	26
Figure 5: Proteasome-related mechanisms of resistance to PI in MM.....	34
Figure 6: Cellular adaptations to proteasome inhibition in MM: mechanisms and therapeutic strategies	42
Figure 7: Graphical abstract representing the main methodology and findings of Annex 1.....	48
Figure 8: Graphical illustration of the main methodology presented in Annex 2	51
Figure 9: Summary of the proposed model of adaptation presented in Annex 3	54
Figure 10: Graphical summary of the proposed metabolic rewiring presented in Annex 4	57
Figure 11: Graphical summary of the proposed mechanism of action of nelfinavir in MM, presented in Annex 5	60

Glossary

AAG (17-AAG)	– see Tanespimycin
ABCB1	– ATP-binding cassette subfamily B member 1
AKT	– Protein kinase B
ALK	– Anaplastic lymphoma kinase
AMO-BTZ	– AMO-1 myeloma cell line adapted to bortezomib
AMO-CFZ	– AMO-1 myeloma cell line adapted to carfilzomib
APEX	– Phase III bortezomib clinical trial
ATF6	– Activating transcription factor 6
ATP	– Adenosine triphosphate
BCMA	– B-cell maturation antigen
BiP	– Binding immunoglobulin protein
BM	– Bone marrow
BTZ	– Bortezomib
CAR	– Chimeric antigen receptor
CB-5083	– p97 inhibitor
CD3	– Cluster of differentiation 3
CD38	– Cluster of differentiation 38
CDK5	– Cyclin-dependent kinase 5
CFZ	– Carfilzomib
CHOP	– C/EBP homologous protein
COBRA	– KRd vs. VRd phase III clinical trial

CRAB	– Calcium elevation, Renal insufficiency, Anemia, Bone lesions (diagnostic criteria)
CREST	– Phase II bortezomib clinical trial
CRISPR/Cas9	– Clustered Regularly Interspaced Short Palindromic Repeats / CRISPR-associated protein 9
D-VRd	– Daratumumab, bortezomib, lenalidomide, dexamethasone (drug combination)
D609	– Tricyclodecan-9-yl xanthogenate (sphingomyelin synthase inhibitor)
DRiPs	– Defective ribosomal products
ER	– Endoplasmic reticulum
ERAD	– Endoplasmic reticulum-associated degradation
FDA	– Food and Drug Administration
Ub-G76V-GFP	– Ubiquitin-G76V-green fluorescent protein degradation reporter
GADD34	– Growth arrest and DNA damage-inducible protein 34
GFP	– Green fluorescent protein
GPRC5D	– G protein-coupled receptor, class C, group 5, member D
GRIFFIN	– Phase II daratumumab-VRd clinical trial
GRP78	– 78 kDa glucose-regulated protein
HIV	– Human immunodeficiency virus
HSF1	– Heat shock factor 1
HSP70 / HSP90	– Heat shock protein 70 / 90

GLOSSARY

HSPA5	– Heat shock protein family A member 5 (gene encoding GRP78/BiP)
HSR	– Heat shock response
IC50	– Half-maximal inhibitory concentration
IDPs	– Intrinsically disordered proteins
IGF-1R	– Insulin-like growth factor 1 receptor
IMiD(s)	– Immunomodulatory drug(s)
IMROZ	– Isatuximab-VRd phase III clinical trial
INSR	– Insulin receptor
IRE1	– Inositol-requiring enzyme 1
Isa-VRd	– Isatuximab, bortezomib, lenalidomide, dexamethasone (drug combination)
i.v.	– Intravenous
KRd	– Carfilzomib, lenalidomide, dexamethasone (drug combination)
LMP2	– Low molecular mass polypeptide 2 (immunoproteasome subunit β 1i)
LMP7	– Low molecular mass polypeptide 7 (immunoproteasome subunit β 5i)
mAb(s)	– Monoclonal antibody/antibodies
MagnetisMM	– Elranatamab clinical trial series
MDR1	– Multidrug resistance protein 1

MECL-1	– Multicatalytic endopeptidase complex-like 1 (immunoproteasome subunit $\beta 2i$)
MGUS	– Monoclonal gammopathy of undetermined significance
MM	– Multiple myeloma
MonumenTAL	– Talquetamab clinical trial series
mTORC1	– Mechanistic target of rapamycin complex 1
NAD(P)H / NADP / NADPH	– Nicotinamide adenine dinucleotide (phosphate) (hydrogen)
NDMM	– Newly diagnosed multiple myeloma
NRF2	– Nuclear factor erythroid 2-related factor 2
OXPHOS	– Oxidative phosphorylation
PC(s)	– Plasma cell(s)
PERK	– Protein kinase RNA-like endoplasmic reticulum kinase
PERSEUS	– Phase III daratumumab-VRd clinical trial
PI(s)	– Proteasome inhibitor(s)
PI3K	– Phosphoinositide 3-kinase
PSMB5 / PSMB6 / PSMB7	– Proteasome subunit beta type-5 / -6 / -7 (genes encoding $\beta 5$, $\beta 1$, $\beta 2$)
PSMD1 / PSMD4	– Proteasome 26S subunit, non-ATPase 1 / 4
ROS	– Reactive oxygen species
RP	– Regulatory particle (19S RP)
RRMM	– Relapsed/refractory multiple myeloma
SAKK	– Swiss Group for Clinical Cancer Research

GLOSSARY

SLiM	– Sixty percent bone marrow plasma cells, Light chain ratio ≥ 100 , MRI with >1 focal lesion (biomarkers of imminent progression)
SUMMIT	– Phase II bortezomib clinical trial
TCA	– Tricarboxylic acid (cycle)
TNFRSF17	– Tumor necrosis factor receptor superfamily member 17 (gene encoding BCMA)
UPR	– Unfolded protein response
UPS	– Ubiquitin-proteasome system
VCP	– Valosin-containing protein (p97)
VRd	– Bortezomib, lenalidomide, dexamethasone (drug combination)
XBP1	– X-box binding protein 1

1 Introduction

Multiple myeloma remains, despite two decades of notable therapeutic progress, a disease that is difficult to treat. The introduction of proteasome inhibitors, immunomodulatory drugs, and more recently monoclonal antibodies and T-cell-redirecting immunotherapies has transformed what was once a rapidly fatal malignancy into a disease with substantially extended survival. Yet nearly all patients eventually relapse and become progressively more difficult to treat with each successive line of therapy. Understanding why myeloma cells escape effective treatment, particularly proteasome inhibitor-based therapy, which has remained a cornerstone of nearly every treatment regimen since its introduction, is therefore one of the central unresolved questions in the field. This thesis introduces the biology of multiple myeloma, with particular emphasis on the plasma cell's dependence on protein homeostasis, followed by a detailed account of proteasome inhibition as a therapeutic strategy and the mechanisms by which resistance to this class of drugs develops. Its aim is to bring together the current understanding of the mechanisms underlying proteasome inhibitor resistance in myeloma. Further, it documents my contributions to this field, which our group and collaborators have approached from multiple complementary directions: through direct comparison of how individual proteasome inhibitors engage their target, through unbiased proteomic and metabolomic characterization of resistant disease models, and through the identification and preclinical-to-clinical validation of combination strategies capable of restoring drug sensitivity. The following chapters provide the theoretical background necessary to place these contributions in context. At the relevant points, the

text is supplemented by chapters documenting and commenting on my contribution to this field, based on the published works that form the annexes of this thesis. These published contributions are then presented individually, followed by a concluding synthesis of the gained knowledge and future perspectives for MM therapy.

2 Theoretical Background

2.1 Multiple Myeloma

Multiple myeloma (MM) is the second most common hematological malignancy, arising from the malignant transformation of terminally differentiated, antibody-secreting plasma cells (PCs) within the bone marrow. Its incidence has risen substantially over recent decades, in part due to an aging population and improved diagnostic detection, and it mainly affects older adults, with a median age at diagnosis in the late sixties, men, and individuals of African descent, who are diagnosed at more than double the rate of other populations¹.

Almost all cases of MM evolve from an asymptomatic precursor state termed monoclonal gammopathy of undetermined significance (MGUS), present in roughly 5% of the population over age 50, which progresses to multiple myeloma or a related malignancy at a rate of approximately 1% per year. This stepwise clinical progression reflects the gradual accumulation of genomic alterations and the selection of increasingly proliferative and microenvironment-independent plasma cell clones, culminating in symptomatic disease².

The distinction between these precursor states and active MM requiring treatment is defined clinically by the CRAB criteria (hypercalcemia, renal insufficiency, anemia, and lytic bone lesions), which reflect biomarker evidence that the disease has progressed to the point of causing organ damage, together with additional myeloma-defining biomarkers (the SLiM criteria) that identify patients at imminent risk of such damage even before it becomes clinically

manifest³. Diagnosis typically combines serum and urine protein electrophoresis with free light chain assays and cross-sectional or whole-body imaging to detect osteolytic lesions⁴.

The excessive production and secretion of monoclonal immunoglobulins, together with multiple interactions with other components of the bone marrow microenvironment, underlies the pathological complications characteristic of MM at diagnosis. Despite the development of novel, biologically driven anti-MM drugs over the past two decades, disease heterogeneity, early relapse, and treatment resistance remain major challenges in MM therapy. Moreover, the subclonal heterogeneity of plasma cells continues to evolve alongside disease progression, through the selection of subclones that are increasingly drug-resistant as well as genetically and metabolically adapted^{5,6}. Today, immunotherapies based on monoclonal antibodies, immunomodulatory drugs, and proteasome inhibitors form an integral part of MM treatment regimens and have considerably improved patient prognosis⁷. However, patients who become triple-class refractory to these three drug classes have a median overall survival of only 5.6 months⁸, underscoring the need to understand the mechanisms driving (multi-)drug resistance in MM.

The reliance on an extraordinarily high secretory burden, and the corresponding dependence of malignant PCs on a tightly regulated proteostasis network to sustain it, provides the central rationale for proteasome inhibitor therapy in MM, and the starting point for the mechanistic work presented in this thesis.

2.2 Protein Homeostasis in MM

MM PCs secrete vast amounts of monoclonal immunoglobulin, therefore they heavily rely on tightly regulated proteostasis, where the endoplasmic reticulum (ER) oversees protein synthesis, quality control, folding, and the secretion of proteins, as well as stringent degradation of unwanted, and misfolded proteins. The biosynthetic part of ER proteostasis ensures that proteins are folded into their unique three-dimensional shapes via the ER-resident protein-folding and modification machinery comprising a network of chaperones, glycosylating enzymes, and oxido-reductases⁹. Molecular chaperones catalyze the 3D conformation acquisition of synthesized proteins, with binding immunoglobulin protein (BiP, also known as 78 kDa glucose-regulated protein, GRP78), a member of the heat shock protein HSP70 family, being the most prominent. BiP innately displays high affinity for unfolded peptides and proteins, therefore facilitating their shape rearrangement. Proteins dissociate from BiP once they acquire the correct 3D structure, rendering BiP a central mediator of global protein folding¹⁰. Under physiological conditions, the mechanisms that govern peptide folding are prone to errors, leading to unfolded or misfolded proteins. It is estimated that up to 30% of nascent proteins never reach a properly folded, functional state¹¹. These incompletely folded proteins, termed defective ribosomal products, are not tolerated by the cell and are removed by stringent quality control systems, such as the ER-associated degradation (ERAD) machinery¹². Through ERAD, and specifically via valosin-containing protein (VCP)/p97, a multifunctional ATPase, the unfolded proteins are returned to the cytosol for subsequent ubiquitylation and degradation by the ubiquitin-proteasome system (UPS)⁹. To ensure that the proteostasis is

balanced, MM PCs constantly monitor the number of misfolded proteins in the ER lumen. When misfolded proteins accumulate above a critical threshold, this causes ER stress and triggers a signal transduction pathway called the unfolded protein response (UPR) within the cell to restore homeostasis (Figure 1).

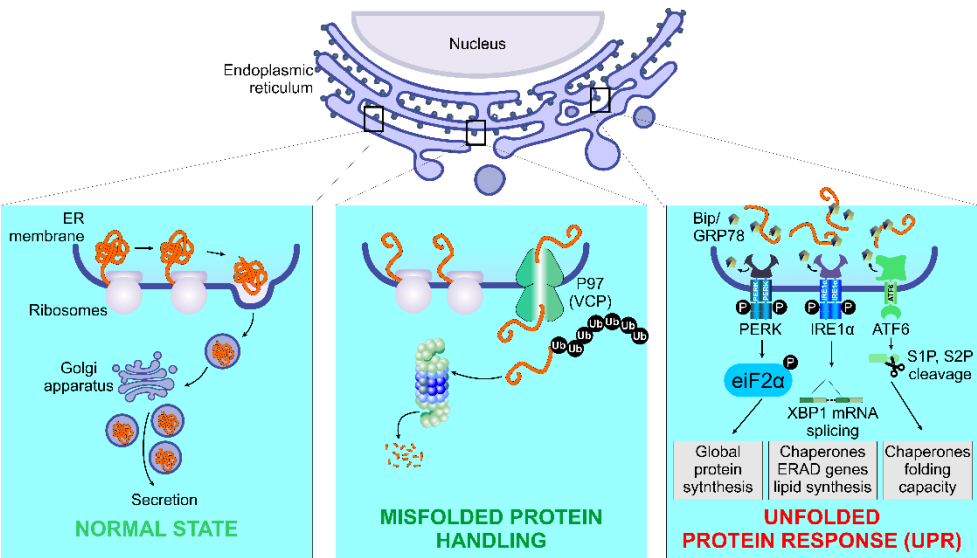


Figure 1: Proteostasis in MM

2.2.1 The Ubiquitin-Proteasome System

The UPS ensures and regulates the turnover and recycling of the majority of intracellular proteins in eukaryotic cells, including MM PCs. While the UPS accounts for roughly 80-90% of total protein degradation¹³, the remaining 10-20% are degraded via the alternative proteolytic mechanisms that

cooperate with the UPS in guaranteeing proteostasis, such as the macroautophagy (autophagy-lysosome) system and the aggresome-autophagy pathway¹⁴.

In the well-characterized UPS a protein substrate is targeted to the 26S proteasome following conjugation of a poly-ubiquitin chain¹⁵. The 26S proteasome consists of the 20S catalytic domain assembled with either one or two 19S regulatory particles (RP)^{16,17}. The polyubiquitinated substrate is recognized by specific subunits of the 19S RP of the 26S proteasome where it is deubiquitinated, unfolded by the ATPases and translocated into the 20S catalytic chamber for degradation¹⁸. The 20S barrel-shaped core chamber contains 28 subunits organized in 4 layers of stacked rings with 7 subunits per layer, which subsequently form the barrel-shaped structure of two inner β -rings and two outer α -rings^{19,20}. The α -rings allow the unfolded proteins to enter the 20S core, while β -rings ensure the breakdown of the proteins in the proteasome. The proteolytic site with substrate specificity in the β -rings is made of 3 β -subunits, β 1 with caspase-like activity (encoded by *PSMB6*), β 2 with trypsin-like activity (encoded by *PSMB7*) and β 5 with chymotrypsin-like activity (encoded by *PSMB5*) (Figure 4). While all of the active β -subunits participate in protein degradation, the β 5-subunit is the most important and thus also the rate limiting for the activity of the whole 20S proteasome²¹. In addition to the constitutive proteasome, immune cells can be equipped with immunoproteasome, typically in the setting of infection or pro-inflammatory stimuli²². The immunoproteasome subunits β 1i (LMP2), β 2i (MECL-1), and β 5i (LMP7) replace the constitutive catalytic subunits²². Our data, and those of others have shown that MM cells are equipped with large amounts of immunoproteasome, and selective inhibition of these catalytic activities is an interesting therapeutic strategy for MM and potentially other hematologic malignancies²³⁻²⁷.

The 20S proteasome is usually coupled with one or two 19S RP to ensure ubiquitin-dependent degradation. Over the years, an alternative, ubiquitin-independent proteasome degradation pathway has been described whereby intrinsically disordered proteins (IDPs) such as p53 and others are degraded by the 20S proteasome in a process that does not involve active ubiquitin tagging^{28,29}. In these cases of ubiquitin-independent degradation, 20S proteasome can be capped with alternative caps, such as 11S, which serves as a gate opener of the barrel and allows for degradation of IDPs or defective ribosomal products, often recognized by intrinsically disordered regions. MM PCs show a variety of proteasomes/immunoproteasomes, capped with different caps 19S/11S³⁰ (Figure 2).

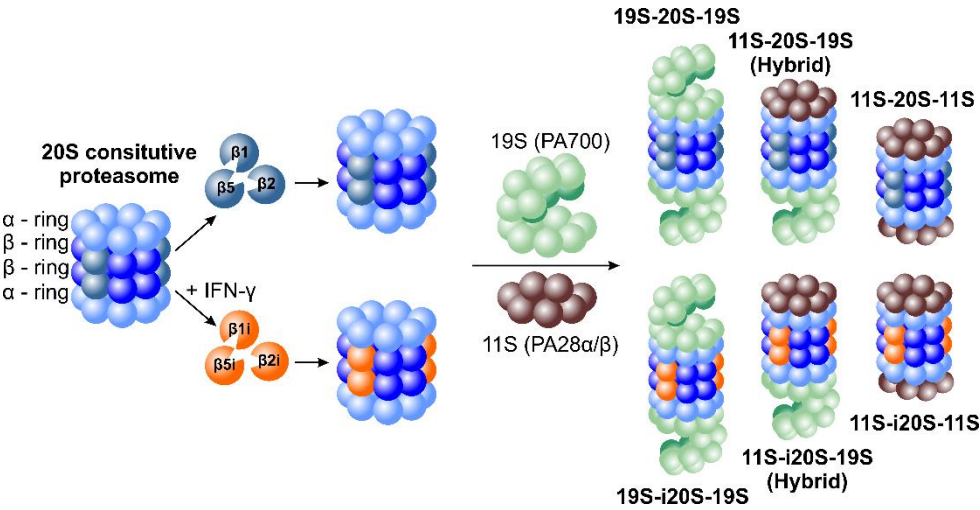


Figure 2: Proteasome in MM. Types of core 20S units and caps present in MM PCs

2.2.2 Sensing of Proteotoxic Stress in MM – UPR and HSR

The accumulation of unfolded or misfolded proteins in the ER lumen that exceeds the ERAD is sensed as toxic by cells and leads to ER stress. The highly conserved adaptive responses to ER stress are the UPR and the heat shock response (HSR), which are triggered with a goal of restoring protein homeostasis^{31,32}.

The UPR consists of three signaling axes that start in the ER lumen at the ER membrane with three transmembrane proteins: IRE1 (Inositol Requiring Enzyme 1) ATF6 (Activating, Transcription Factor 6) and PERK (Protein Kinase RNA-like Endoplasmic Reticulum Kinase). Central role in UPR activation plays ER-luminal BiP/GRP78. Under ER stress-free conditions it is bound to the luminal part of IRE1, ATF6 and PERK surrounded by high calcium concentration; however, when unfolded or misfolded proteins start to accumulate in the ER, calcium is being released from ER, BiP unbinds and binds to the misfolded proteins, thus releasing IRE1, ATF6 and PERK from the inactive state, leading to the activation of these three UPR branches³³ (Figure 3). The ultimate goal of the UPR is to reduce protein toxicity by decreasing total protein synthesis while selectively upregulating chaperone transcription and translation³². However, if homeostasis cannot be restored in rather short time, the UPR activates a terminal pathway, triggering C/EBP homologous protein (CHOP) and GADD34 transcription and ultimately apoptosis. At the same time, the HSR induces up-regulation of chaperones via the master-regulator heat shock factor 1 (HSF1) to counteract protein misfolding and stabilize proteasome-client proteins in the cytosol³⁴.

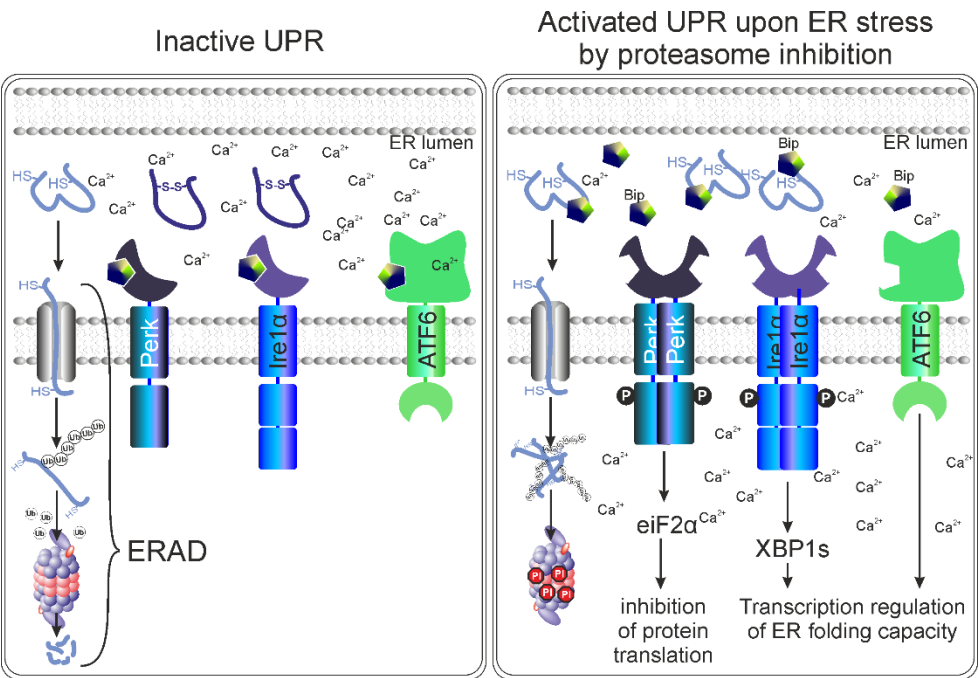


Figure 3: UPR sensing in MM PCs

MM PCs have a lower threshold for UPR induction and ER stress-induced apoptosis because they constitutively and highly express ER stress survival factors to function as secretory cells³⁵. Not only are they strongly dependent on ERAD and the UPS for protein degradation due to high levels of ER stress, but they are also highly sensitive to proteasome inhibition³⁶.

2.3 Proteasome Inhibition as a Therapeutic Strategy in MM

2.3.1 Development of Proteasome Inhibitors in MM

Proteasome inhibitors (PIs) were initially developed in the mid-1990s as a research tool to investigate proteasome-mediated proteolysis mechanisms and as agents with potential benefit in preventing cancer-related cachexia, owing to the role of the UPS in protein turnover³⁷. Many preclinical studies then emerged showing that small-molecule proteasome inhibitors could induce apoptosis in cultured cell lines and murine models of cancer, at which point their utility as chemotherapeutics was postulated. Their remarkable activity in preclinical MM models moved them rapidly forward to clinical testing. In fact, the clinical use of PI has radically changed the natural history of MM and for a long time, along with immunomodulatory agents and monoclonal antibodies, these drugs have formed the backbone of MM treatment.

The currently clinically available PI were designed to primarily inhibit the $\beta 5$ proteasome subunit. Bortezomib (Velcade®, Takeda Oncology, MA, USA), as the first-in-class proteasome inhibitor received an accelerated approval by the U.S. Food and Drug Administration (FDA) in 2003 after two successful phase II clinical trials (SUMMIT)³⁸ and (CREST)³⁹ and randomized phase III trial (APEX)⁴⁰ that confirmed its superior activity over standard treatment at that time. Bortezomib is a peptide-based boronate PI designed to bind reversibly to the active site of the proteasome $\beta 5$ subunit, the rate-limiting protease. By inhibiting proteasome activity, bortezomib disturbs the protein homeostasis leading to accumulation of proteasome-client proteins, which triggers the UPR an ER-stress-mediated apoptosis of MM PCs³⁵. Moreover, it affects

multiple signaling pathways by interfering with the proteasomal proteolytic degradation of numerous signaling molecules^{41,42} (Figure 4).

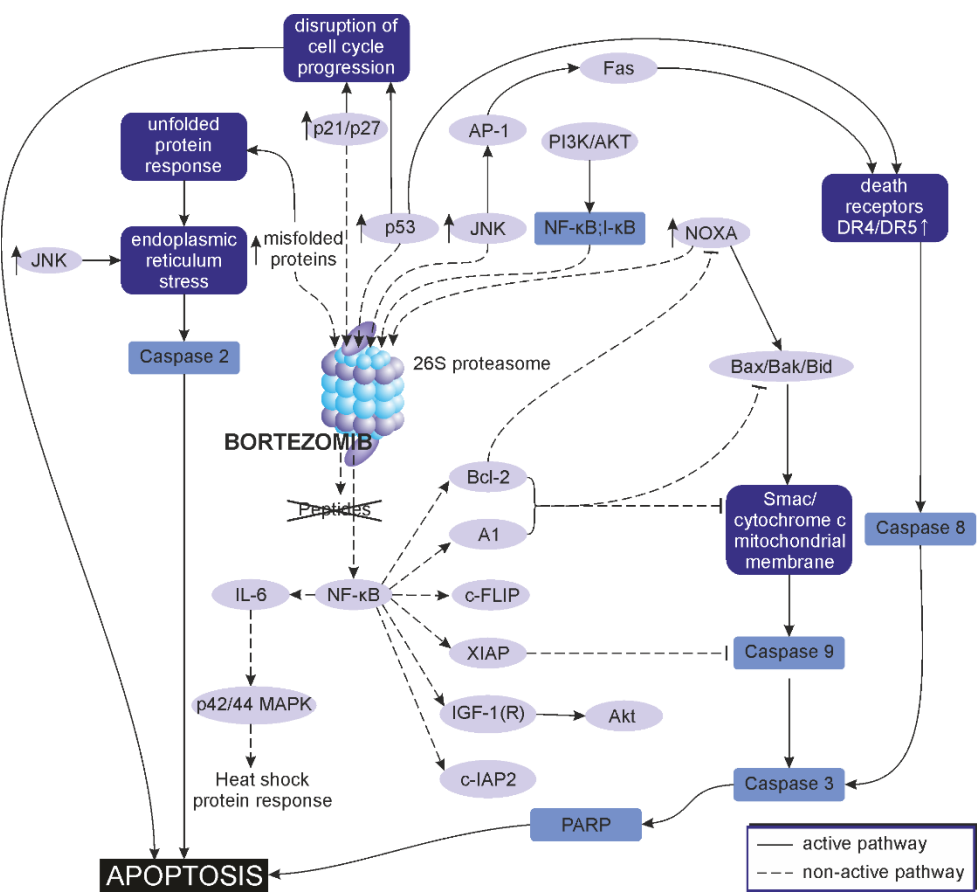


Figure 4: Cellular effects of proteasome inhibition

Bortezomib treatment is linked to side effects, in particular peripheral neuropathy experienced in more than 30% of patients, which is likely due to off-target inhibition of other proteases and warrants dose reductions in spite of efficacy^{43,44}. The change in bortezomib application route from i.v. bolus to subcutaneous significantly reduced the polyneuropathy rate while efficacy was preserved⁴⁵. Epoxyketones were identified as a class of compounds that targets the proteasome active sites with higher selectivity and in a covalent fashion, compared to the reversibly-binding boronates. The epoxyketone-type proteasome inhibitor carfilzomib (Kyprolis®, Onyx Pharmaceuticals, Inc., CA, USA) was subsequently developed and clinically approved in 2012 as a second-generation PI, mainly to overcome bortezomib-resistance and reduce side-effects associated with bortezomib treatment. Carfilzomib is peptide-epoxyketone PI that binds irreversibly to the active site of proteasome $\beta 5$ subunit with increased selectivity, and stronger anti-myeloma activity *in vitro* and *in vivo*^{46,47}. Therefore, it can be administered at higher doses, compared to bortezomib, until significant side-effects occur. While its use is not associated with neuropathies, at higher doses its side effects involve cardiotoxicity^{48,49}. The third clinically approved PI in 2015 is ixazomib (Ninlaro®, Takeda Pharmaceuticals, Osaka, Japan), the only peptide-boronate-based orally administered PI that is currently approved in a relapsed/refractory setting⁵⁰.

Although all PI by design inhibit the $\beta 5$ proteasome subunit, in higher doses they co-inhibit other proteasome subunits. Our data show that this differential inhibition patterns of proteasome subunits $\beta 1$, $\beta 2$, and $\beta 5$ by clinically approved PI significantly influence their cytotoxic effects on MM cells²⁴. In contrast to bortezomib and ixazomib, which target the proteasome subunits $\beta 5$ and $\beta 1$ at higher doses, carfilzomib is the only approved PI that co-targets $\beta 2$

subunit, at higher doses, besides $\beta 1$ co-targeting^{24,51}. This results in stronger functional proteasome inhibition, leading to different transcriptional and metabolic responses in MM cells, increased proteotoxic stress, and cytotoxicity as well as in strong proteasome inhibition in the heart, suggesting the basis of cardiotoxicity⁵². Other PIs such as marizomib, oprozomib, and delanzomib are on hold in further clinical investigation⁵³, with unlikely approval.

2.3.2 Standard Drug Combinations with PIs in MM

Although PIs reflect biology-driven drug development in MM, they are essentially never used as monotherapy in current MM practice due to emergence of resistance. Their efficacy, tolerability, and durability of response can be substantially improved when combined with other agents from different drug classes, an approach that has become the major feature of modern MM treatment.

In newly diagnosed MM (NDMM), combinations with the anti-CD38 monoclonal antibodies become a new standard of care, based on the recent clinical evaluation. In transplant-eligible NDMM, the current standard of care is a triplet or quadruplet regimen built around a PI-dexamethasone backbone, most commonly bortezomib combined with the immunomodulatory drug (IMiD) lenalidomide and dexamethasone (VRd combination), with the anti-CD38 monoclonal antibody daratumumab, increasingly added as a fourth agent (D-VRd combination)^{54,55}. The initial phase II GRIFFIN study showed the improved depth of response and progression-free survival of D-VRd⁵⁴, while following phase III PERSEUS study confirmed the clinical benefits of adding

daratumumab to standard VRd therapy⁵⁵. D-VRd is now the only FDA-approved quadruplet regimen for newly diagnosed disease irrespective of transplant eligibility. In transplant-ineligible NDMM patients, VRd combination with isatuximab (Isa-VRd), another anti-CD38 monoclonal antibody, has similarly shown deepened responses over VRd alone⁵⁶. Such quadruplet regimen is currently supported as a new standard of care for the frontline treatment of transplant-ineligible patients with NDMM.

Combination with carfilzomib, lenalidomide and dexamethasone (KRd) represents an alternative PI-based triplet for NDMM patients. Although data from phase III clinical trials about its superiority to VRd are rather conflicting, it is generally used and reserved for higher-risk disease^{57,58}.

In the relapsed/refractory (RR) setting, PIs are similarly deployed as combination partners rather than as single agents, in various combinations, mostly with IMiDs and anti-CD38 antibodies⁵⁹. The choice of appropriate therapy for a specific patient with RR disease depends on the context of the clinical relapse, such as prior treatment and duration of response. However, because daratumumab-, bortezomib-, and lenalidomide-containing regimens (D-VR/D-VRd) are often given as induction therapy, it is likely that the disease may not be sensitive to one or more of these agents and therefore, regimens that do not include previously used agents are preferred⁵⁹.

Together, these regimens illustrate a broader therapeutic concept: better disease control is achieved by simultaneously targeting the multiple, partially redundant mechanisms MM PCs use to buffer proteotoxic stress. Durable responses are thus best achieved not by proteasome inhibition alone, but by exploiting several non-overlapping vulnerabilities in parallel. As such, the immunomodulatory, immune-directed, and proteostatic approach has driven the

field's shift from doublet, to triplet, and now to quadruplet regimens as the standard of first-line MM therapy.

2.3.3 Modern Immunotherapy and Combinations with PI in MM

The most recent extension of the therapeutic concept in MM comes from a class of agents that engage the immune cells, namely T cells, to fight MM PCs. T-cell engaging immunotherapy is a major breakthrough for the RRMM disease. RRMM patients refractory to conventional regimens can now be treated with chimeric antigen receptor (CAR)-T cell therapy targeting B-cell maturation antigen (BCMA)⁶⁰ — idecabtagene vicleucel (ide-cel; bb2121) and ciltacabtagene autoleucel (cilta-cel) — or with BCMA-directed T-cell engaging bispecific antibodies such as teclistamab and elranatamab, which bridge the BCMA antigen on plasma cells to CD3 on T cells^{61,62}. BCMA, also known as TNFRSF17, is a cell-surface receptor selectively expressed on normal and malignant plasma cells⁶³. BCMA CAR-T cells, now FDA approved for RRMM, achieve significantly higher overall response rates from the third line of therapy onward compared with carfilzomib-based regimens⁶¹.

More recently, GPRC5D has emerged as a second validated immunotherapeutic target in MM. Unlike BCMA, its expression is largely confined to malignant plasma cells and is frequently retained after BCMA-directed therapy, making it a rational option for overcoming BCMA antigen escape. Talquetamab, a GPRC5D×CD3 bispecific antibody, received FDA accelerated approval in 2023 for RRMM patients with at least four prior lines of therapy⁶⁴. Talquetamab remains, for now, the only regulator-approved GPRC5D-targeted therapy, though GPRC5D CAR-T constructs, trispecific antibodies, and antibody-drug

conjugates are already in active clinical development and are expected to broaden this therapeutic class in the coming years⁶⁵.

Yet none of these agents alone reliably produces a complete response in RRMM, underscoring again the need for rational combination strategies built on currently approved drugs. Our recent data suggest that BCMA is ubiquitinated for proteasome-mediated degradation in MM PCs, and that proteasome inhibition with carfilzomib reduces its turn-over and stabilizes it on the cell surface, providing more durable antigen presentation to BCMA CAR-T cells and translating into an improved therapeutic response⁶⁶.

Thus, despite the rise of novel therapeutic approaches, PI remain an integral part of most MM treatment regimens, in both the newly diagnosed and RRMM settings, and continue to considerably improve the lives of patients, often in combination with other approved drugs, which they help potentiate. Therefore, understanding the underlying mechanisms of resistance to PI is still considered as unmet medical need.

2.4 Mapping Proteasome Inhibitor Resistance in MM

The rapid progression of disease in the relapsed/refractory setting, driven by continuously evolving drug resistance, remains a long-standing clinical challenge in MM. The underlying biology and mechanisms of PI resistance have been studied for the past two decades, and while still not fully understood, they are now being dissected in more detail⁶⁷. The progress was possible largely by parallel advances in experimental technology. The development of robust *in vitro* and *in vivo* MM models of PI resistance⁶⁸⁻⁷¹, alongside activity-based probes²³ capable of resolving individual proteasome subunit function

within living cells, has allowed resistance to be studied not merely as a static endpoint but as a dynamic, functional process. This has been complemented by substantial improvements in proteomic and metabolomic profiling and by increasingly deep, unbiased functional loss of function studies and sequencing approaches, which together have enabled the field to move beyond single-gene or single-pathway hypotheses toward a systems-level view of how MM cells adapt to proteasome inhibition^{72,73}. These advances allowed for identification of mechanistic insights driving evolution of PI resistance, as well as therapeutic vulnerabilities of PI resistant cells, which are summarized in the following sections.

2.4.1 Proteasome-Related Changes as a Mechanism of PI Resistance in MM

Initially, PI resistance was dissected in *in vitro* cell line models, in which different hematologic and MM cell lines were exposed to increasing concentrations of PIs over long periods of time. The aim was to identify specific adaptive changes in MM cell lines that survived the initial exposure to PIs such as bortezomib and carfilzomib. Early studies of bortezomib-exposed cell lines revealed increased proteasome $\beta 5$ subunit level and mutations in the *PSMB5*, which were thought to underlie PI resistance in the clinical setting by preventing the drug from binding its target and thus retaining cleavage activity⁷⁴⁻⁷⁷. However, these *PSMB5* mutations were not confirmed in PI-resistant cells derived from the broader MM patient population, suggesting they do not fully account for clinical resistance⁷⁸. Although *PSMB5* mutations were eventually identified in MM patients, their introduction into cell line models conferred only a partial

resistance phenotype, suggesting that other adaptive changes underlie clinical resistance⁷⁹.

Further analyses focused on global changes in the UPS and proteasome in PI-resistant MM *in vitro* models. Using proteasome activity-based probes to map not only the presence but also the catalytic activity of individual proteasome subunits within the assembled proteasome, these studies revealed increased overall proteasome activity in bortezomib-resistant cells, driven in particular by increased $\beta 2$ subunit^{68,74}. This finding was consistent with the fact that, at higher doses, bortezomib co-inhibits the $\beta 1$ and $\beta 5$ subunits but spares the $\beta 2$ subunit²⁴, allowing $\beta 2$ activity to partly compensate for the inhibited, rate-limiting $\beta 5$ activity. Building on this observation, the $\beta 2$ -selective proteasome inhibitor LU-102⁸⁰ showed strong synergistic activity with bortezomib and carfilzomib, both in bortezomib-resistant cell lines generated *in vitro* and in plasma cells from RRMM patients⁸¹. Although further clinical development of LU-102 proved difficult, we built on this biological rationale and showed that carfilzomib, which is the only clinically approved PI that co-inhibits the $\beta 2$ subunit at higher doses²⁴, is able to overcome bortezomib resistance in the clinical setting⁵¹.

Resistance mechanisms to PIs in MM extend beyond the core 20S proteasome (Figure 5). Later studies suggested that the composition of the whole 26S proteasome also plays an important role, with loss of the 19S regulatory subunit associated with increased resistance in MM and across various tumor types⁸²⁻⁸⁴. Although loss of 19S did not alter $\beta 5$ subunit activity or its susceptibility to carfilzomib, it significantly induced alternative degradative pathways, such as the aggresomal-autophagy pathway, which was observed in patient derived PCs as well and associated with a worse response to treatment⁸².

Recently, PSMD1 or PSMD4, subunits of the 19S have been identified as a therapeutic target in MM^{85,86}.

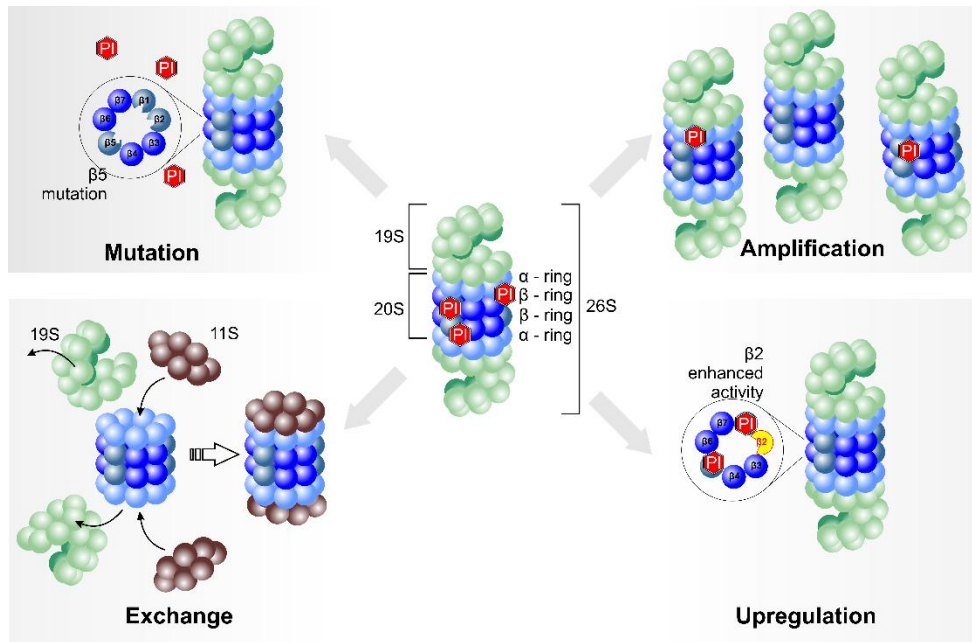


Figure 5: Proteasome-related mechanisms of resistance to PI in MM

Our contribution to this topic.

Our study²⁴, "**Proteasome Inhibition in Multiple Myeloma: Head-to-Head Comparison of Currently Available Proteasome Inhibitors**," was the first to directly compare the activity of all clinically available or investigational proteasome inhibitors across individual proteasome subunits. Using proteasome-selective activity-based probes²³, we showed that at low doses, PIs selectively inhibit the rate-limiting β5 subunit, while at higher doses they lose this selectivity and co-inhibit other subunits. Bortezomib and ixazomib lose β5 selectivity already at only slightly higher doses, beginning to co-inhibit the β1

subunit, whereas carfilzomib remains $\beta 5$ -selective over a considerably wider concentration range and only co-inhibits $\beta 2$ activity at higher doses. This combination of $\beta 5$ and $\beta 2$ inhibition produces stronger total proteasome inhibition and greater accumulation of ubiquitinated substrates than $\beta 5/\beta 1$ co-inhibition, and is associated with stronger cytotoxicity even in the setting of bortezomib resistance. This unique activity profile positions carfilzomib as the most effective clinically available proteasome inhibitor, capable of overcoming proteasome inhibitor resistance.

Building on these findings, we performed a clinically oriented study with carfilzomib⁵¹: **"High-Dose Carfilzomib Achieves Superior Anti-Tumor Activity over Low-Dose and Recaptures Response in Relapsed/Refractory Multiple Myeloma Resistant to Low-Dose Carfilzomib by Co-Inhibiting the $\beta 2$ and $\beta 1$ Subunits of the Proteasome Complex."** Using activity-based proteasome probes on immune cells obtained directly from peripheral blood of MM patients following carfilzomib administration at different doses, we showed that low-dose carfilzomib (20-27 mg/m²) fully inhibits the proteasome $\beta 5$ subunit while only partially inhibiting the other subunits, whereas higher doses (>36 mg/m²) inhibit the proteasome more potently, achieving over 50% inhibition across all subunits. Consistent with these findings, carfilzomib dose escalation recaptured clinical response in RRMM patients who were resistant to low-dose carfilzomib. Together, these data indicate that carfilzomib's superior clinical activity over bortezomib is not, as previously proposed, due to co-targeting of non-proteasomal off-targets, but rather reflects a unique and highly effective mechanism of proteasome inhibition involving $\beta 5/\beta 2$ coinhibition.

2.4.2 ER, UPR and Metabolic Changes as an Adaptive Way to Proteasome Inhibition

In parallel to modulation of proteasome itself, other proposed mechanisms of PI resistance extend more to protein homeostasis pathways, representing the broader adaptive changes induced in MM PCs by proteasome inhibition. As noted earlier, disruption of protein homeostasis by proteasome inhibition leads to accumulation of proteasome-client proteins in the ER and cytosol, triggering the pro-survival UPR and heat shock response and, when stress remains unresolved, apoptosis. A logical consequence of chronic ER stress from protein accumulation is an increased capacity to buffer that stress. One of the first adaptive mechanisms described, over a decade ago, was decreased activity of the IRE1/X-box binding protein 1 (XBP1) pathway, a branch of the UPR⁸⁷. This finding was initially surprising, since XBP1 is not merely an executor of the UPR but also a transcription factor required for plasma cell differentiation, and was thought to be indispensable for viability of PCs. The data nonetheless showed that IRE1/XBP1 signaling is required for bortezomib-induced cytotoxicity *in vitro* and is suppressed in bortezomib-refractory primary MM PCs, suggesting that loss of IRE1/XBP1 signaling blocks commitment to PC differentiation and thereby lowers basal ER stress⁸⁷. Although subsequent studies, including our own work, found that direct silencing of XBP1 in MM PCs resistant to bortezomib has only a modest effect on bortezomib sensitivity⁶⁹, low levels of spliced XBP1 have been repeatedly confirmed in primary MM patient-derived cells as a surrogate marker of bortezomib resistance^{26,88-90}. This prompted further investigation into other adaptive changes, and consistently broader changes to protein-folding chaperones such as HSP70, HSP90, and the chaperone master transcriptional regulator HSF1, and compensation of proteasomal

blockade by increasing protein degradation capacity via the autophagy–lysosome pathway were identified by us and others^{69,91,92}. As an example, the glucose-regulated protein GRP78 (HSPA5), is required for the efficient delivery of bortezomib-induced aggresomes to autophagosomes, thereby contributing to resistance⁹¹.

Notably, along with the chaperon activity, major adaptive mechanism of resistance to PIs are associated with tumor metabolism. The increased oxidative capacity and oxidative phosphorylation (OXPHOS) in PI-resistant cells has been identified, suggesting that metabolic adaptation is key to withstanding PI-based therapy⁶⁹. In line with these findings, rewired glycolysis resulting in higher flux through the pentose phosphate and serine synthesis pathways, and ultimately increased antioxidant capacity, was identified as an additional adaptive mechanism in bortezomib-resistant cells⁹³. Broader shifts in glucose metabolism toward OXPHOS and mitochondrial respiration — generating more antioxidants and supporting more effective protein folding to reduce dependency on proteasomal degradation — have since been identified across multiple reports as a strong driver of PI resistance in MM⁹⁴⁻⁹⁷. In conclusion, resistance to PI in MM is currently viewed as multifactorial, with changes related to UPS itself as well as the adaptive mechanisms in protein folding and mitochondria aiming at lowering the proteotoxic stress to decrease the proteasomal protein burden, preventing terminal UPR and apoptosis induction.

Our contribution to this topic.

Our global proteomic profiling of PI-sensitive and PI-resistant cells, published as⁶⁹: **"Proteasome inhibitor-adapted myeloma cells are largely**

independent from proteasome activity and show complex proteomic changes, in particular in redox and energy metabolism", was one of the first comprehensive proteomic characterizations of the PI-resistance model. Combining quantitative and functional proteomic approaches on bortezomib- and carfilzomib-adapted cell lines (AMO-BTZ, AMO-CFZ), we identified over 600 differentially expressed proteins shared between the two resistant lines. The differences were mainly in increased metabolic regulation, redox homeostasis, and protein folding and degradation, while proteins involved in apoptosis and transcription/translation were downregulated. The study shed light not only on proteasome-related changes in the adapted cell lines, but, more importantly, identified extensive metabolic reprogramming as a defining feature of PI resistance. Together, these adaptations allowed PI-resistant MM cells to tolerate subtotal proteasome inhibition and survive with markedly reduced proteasome activity, by lowering the overall proteasomal protein-folding burden rather than by restoring proteasome function itself.

Building on this knowledge, we validated key metabolic changes using mass spectrometry-based whole-metabolome profiling, published as: "**A metabolic switch in proteasome inhibitor-resistant multiple myeloma ensures higher mitochondrial metabolism, protein folding and sphingomyelin synthesis**"⁹⁷. This analysis confirmed that bortezomib- and carfilzomib-resistant cells share similar patterns of metabolic rewiring, with pathways feeding the synthesis and regeneration of glutathione, NAD(P)H, and the tricarboxylic acid cycle prominently upregulated. Together, these data suggest that PI resistance in MM is sustained by a coordinated metabolic switch that

collectively lowers the proteasomal burden of misfolded protein and identifies exploitable metabolic vulnerabilities specific to the PI-resistant state.

2.4.3 Overcoming PI Resistance: Mechanism-Driven Therapeutic Strategies

Initially, it was thought that new classes of PIs, which can more comprehensively target the multiple proteolytic activities of the 20S proteasome, may be more effective than bortezomib or carfilzomib. However, despite many years of investigation, these strategies have not yet been successful and the only clinically approved inhibitor significantly inhibiting various subunits remains carfilzomib^{24,51}. In parallel, a focus shifted to strategies inhibiting the 19S subunits or deubiquitinases, enzymes deubiquitinating the ubiquitin-tagged substrates, which are often associated with the proteasome^{85,86,98}, with no therapeutic implications so far.

Stemming from this broad body of knowledge on PI resistance in MM, derived from both clinical observations and comparative analyses of PI-sensitive and PI-resistant MM cells *in vitro*, researchers have identified several molecular determinants of PI sensitivity that represent promising therapeutic targets for overcoming resistance^{67,99} (Figure 6). Within the context of protein homeostasis, inhibition of the ERAD pathway, particularly through targeting VCP/p97 with selective inhibitor CB-5083, has emerged as a promising strategy¹⁰⁰. Although the clinical development of CB-5083 has been placed on hold due to dose-limiting off-target toxicities, pharmacological inhibition of VCP/p97 by disulfiram has been proposed as an alternative approach and has demonstrated activity against PI-resistant myeloma cells¹⁰¹. Further, the

adaptive low IRE1/XBP1 branch has brought the attention due to being associated with low response to PIs⁸⁷⁻⁹⁰. Although clinical development focused more on the UPR inhibitors, a more biologically grounded alternative in MM is to intentionally amplify ER stress, pushing the cell past its adaptive threshold and into a pro-apoptotic "terminal UPR." Several such UPR inducers have shown synergistic anti-MM activity and preclinical efficacy even in PI-resistant MM, yet none have been pursued further clinically^{102,103}. Building on the generally accepted biological rationale underlying PI resistance, we identified nelfinavir, a first-generation HIV protease inhibitor, as a potent UPR inducer that, in combination with bortezomib or carfilzomib, produced a strong cytotoxic effect in the PI-resistant setting¹⁰⁴. This activity was successfully translated into the clinic: the combination of nelfinavir and bortezomib showed tolerable toxicity in a phase I trial¹⁰⁵, and subsequent clinical development of nelfinavir with bortezomib and dexamethasone in RRMM achieved an overall response rate exceeding 65%— a notably strong result in this heavily pre-treated patient population¹⁰⁶. Mechanistically, nelfinavir's potent UPR induction arises from its effects on membrane fluidity, which in turn disrupts protein transport and mitochondrial activity, the two adaptive processes central to PI-resistant MM¹⁰⁷. Moreover, nelfinavir has been shown to inhibit multidrug resistance protein 1 (MDR1/P-glycoprotein), an ATP-binding cassette transporter encoded by the *ABCB1* gene. MDR1 upregulation or activity has been implicated in carfilzomib resistance *in vitro* and has been observed in patients evolving toward plasma cell leukemia, suggesting a potential role for drug efflux mechanisms in advanced disease¹⁰⁸.

Beyond characterizing adaptive changes identified in PI-resistant MM cells *in vitro*, other approaches used unbiased identification of novel targets in

PI-resistant MM via loss-of-function screenings. Initial genome-wide loss-of-function screens for genes sensitizing MM cells to bortezomib or carfilzomib identified subunits of the 20S proteasome core particle, as well as the heat shock transcription factor HSF1 and stress-response factors such as nuclear factor erythroid 2-related factor 2 (NRF2), as major genes whose depletion sensitizes cells to proteasome inhibition^{82,109}. Targeting the heat shock pathway and molecular chaperones has been extensively explored in MM as well, given the strong anti-MM effect of HSP inhibition and its ability to overcome PI-resistance^{110,111}. Tanespimycin (17-AAG), the first HSP90 inhibitor to reach the clinic, showed tolerable toxicity¹¹² and preclinical synergy with bortezomib, with the combination inducing stronger accumulation of ubiquitinated proteins than either agent alone in RRMM¹¹³. Although promising, clinical development was ultimately halted in 2010, owing to a combination of modest efficacy, formulation and tolerability challenges, and a fundamental mechanistic limitation: HSP90 inhibition activates HSF1, which drives compensatory up-regulation of other chaperones, particularly HSP70, thereby blunting the cytotoxic effect of the inhibitor itself¹¹³. Our own work has since shown that this pro-survival chaperone induction can instead be suppressed by drug repurposing, using the ALK inhibitor ceritinib¹¹⁴. Ceritinib, designed to inhibit anaplastic lymphoma kinase (ALK) and approved for the treatment of non-small cell lung cancer^{115,116}, exhibits potent off-target activity against the insulin receptor (INSR) and insulin-like growth factor-1 receptor (IGF-1R)¹¹⁷ signaling. This signaling, when active, fuels high chaperone production in MM. The combination of carfilzomib and ceritinib showed a strong synergistic effect in preclinical MM models, notably without inducing HSP70, supporting its potential for further clinical development¹¹⁴. In line with the data about interplay between

metabolism and protein folding, targeting mitochondria represents an important biology-driven strategy in MM, with mitochondrial HSP70 analogs¹¹⁸, superoxide dismutase 1¹¹⁹, and mitochondrial pyruvate carrier 1¹²⁰ proteins proposed as therapeutic targets to overcome PI-resistance in MM. The summary of adaptive changes and potential therapeutic interventions are presented in Figure 6.

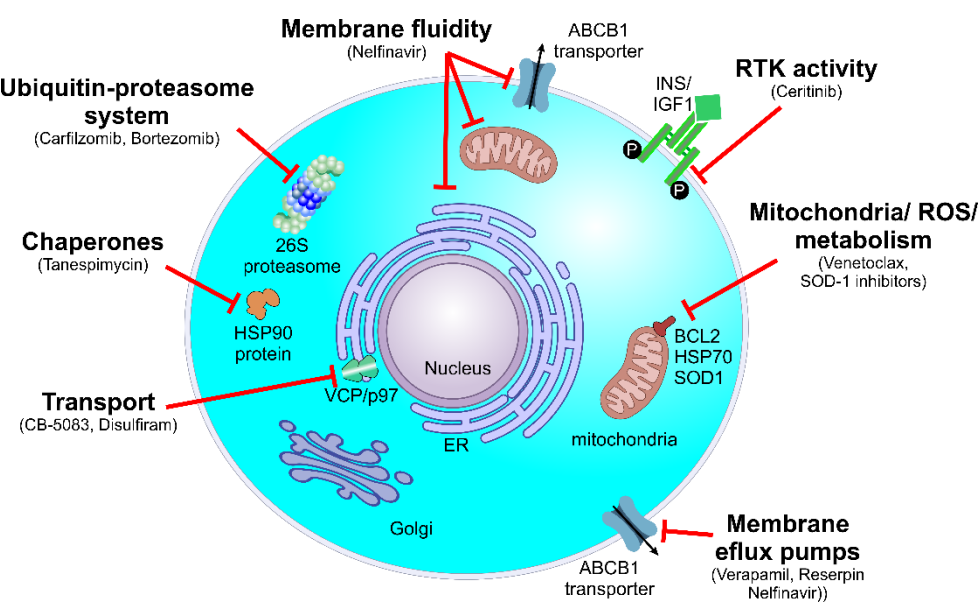


Figure 6: Cellular adaptations to proteasome inhibition in MM: mechanisms and therapeutic strategies

Own contribution to this topic

We identified an actionable vulnerability in PI resistant MM within the INSR/IGF-1R-driven proteostasis axis in our study¹¹⁴: **"Ceritinib overcomes proteasome inhibitor resistance in multiple myeloma by suppressing the protein folding response"**. MM cells show a pronounced dependence on insulin and insulin-like growth factor-1 signaling through their cognate receptors, IGF-1R and INSR, to sustain the protein-folding capacity and high chaperone production required to withstand proteasomal load. We identified ceritinib, a clinically approved ALK inhibitor, as a drug capable of inhibiting IGF-1R/INSR activity and its downstream PI3K/AKT/mTORC1 as well as HSF signaling in MM cells. In combination with carfilzomib, ceritinib was able to overcome PI resistance by suppressing this protein-folding response. These findings position ceritinib as a promising drug-repurposing candidate to resensitize PI-resistant MM to carfilzomib, and, together with the earlier chaperone-targeting data, further underscore the central role of the protein-folding machinery as a therapeutic vulnerability in PI-resistant disease.

We further dissected the mechanism underlying nelfinavir's UPR-inducing activity¹⁰⁷, published as: **"Treatment with HIV-Protease Inhibitor Nelfinavir Identifies Membrane Lipid Composition and Fluidity as a Therapeutic Target in Advanced Multiple Myeloma"**. Combining proteome-wide affinity purification of nelfinavir-interacting proteins with genome-wide CRISPR/Cas9 loss-of-function screening, we identified multiple activity-specific nelfinavir-binding partners embedded within lipid bilayers of the mitochondria and endoplasmic reticulum. Based on these findings, we postulated and further validated that nelfinavir induces lipid bilayer stress by rigidifying

lipid-rich membranes, altering their fluidity and composition. This membrane-level perturbation disrupts mitochondrial respiration and vesicular transport, and triggers UPR induction, thereby identifying an unconventional route by which nelfinavir overcomes PI resistance in combination with bortezomib or carfilzomib in RRMM.

2.5 The Future of Proteasome Inhibitor-Based Therapy in MM

Despite recent, biology-driven strategies to overcome PI resistance described in more detail above and elsewhere⁹⁹, these focused approaches may be surpassed by quickly emerging immunotherapy strategies that do not selectively target PI resistance mechanisms but are highly efficacious in PI-resistant disease, nonetheless. Still, proteasome inhibition remains an active area of investigation, with continued potential to improve outcomes when combined thoughtfully with the therapies now reshaping the field.

In NDMM, PIs have a strong position in triplet/quadruplet therapeutic combinations with bortezomib. In RRMM setting, carfilzomib in particular as the most effective clinically available PI, still holds a strong position as a backbone therapy in a wave of novel combination trials, reflecting a rational, mechanism-driven integration of proteasome inhibition with the newer immunotherapeutic modalities rather than their replacement. Several ongoing trials illustrate this trend directly. MonumenTAL-3 is evaluating the GPRC5D-directed bispecific talquetamab in combination with the immunomodulatory drug mezigdomide, carfilzomib, and dexamethasone in RRMM (ClinicalTrials.gov

identifier NCT05572515). Data presented at the EHA Congress 2026 showed dynamic results for patients with MM assigned to the GPRC5D-targeting bispecific antibody talquetamab and mezigdomide plus carfilzomib and dexamethasone, extending the earlier rationale that BCMA CAR-T efficacy is enhanced by carfilzomib-mediated stabilization of surface BCMA⁶⁶ to a GPRC5D-directed platform where PI addition may similarly modulate the tumor and immune microenvironment. Separately, MagnetisMM-20 (ClinicalTrials.gov identifier NCT05675449) is testing the BCMA-directed bispecific antibody elranatamab in combination with carfilzomib and dexamethasone in RRMM, directly testing whether this combination is safe and whether proteasome inhibition potentiates a BCMA-bispecific in the same way it has been shown to potentiate BCMA CAR-T therapy⁶⁶.

3 Commentary to Published Work

3.1 Commentary to Published Work – Annex 1

Proteasome Inhibition in Multiple Myeloma: Head-to-Head Comparison of Currently Available Proteasome Inhibitors

Original article published in Cell Chemical Biology, Q1, IF (2019): 7.739

*Andrej Besse**, ***Lenka Besse*\$***, Marianne Kraus, Max Mendez-Lopez, Juergen Bader, Bo-Tao Xin, Gerjan de Bruin, Elmer Maurits, Herman S. Overkleeft, Christoph Driessen

* equal contribution

\$ corresponding author

From the beginning, PI therapy in MM has been built around a single design principle: block the chymotrypsin-like $\beta 5$ subunit, the proteolytic activity regarded as rate-limiting for proteasomal degradation. Bortezomib, carfilzomib, and ixazomib were all developed on this premise. What had never been formally tested was whether $\beta 5$ inhibition alone is sufficient to produce the functional, cell-wide proteasome blockade required for cytotoxicity, or whether the clinical superiority observed for carfilzomib actually derives from a distinct, previously overlooked mechanism.

We addressed this directly, using newly developed subunit-selective activity-based probes²³ to visualize the activity/occupancy of all six catalytic proteasome subunits ($\beta 1$, $\beta 2$, $\beta 5$, constitutive and immuno-) simultaneously in living MM cells, combined with a proteasome-targeted Ub-G^{76V}-GFP reporter of

functional proteasomal flux¹²¹ and viability analysis. This approach allowed, for the first time, a head-to-head comparison of every clinically available or clinically-investigated PI (bortezomib, carfilzomib, ixazomib, oprozomib, delanzomib, and marizomib) at pharmacologically relevant exposure times and concentrations, rather than relying on isolated 20S biochemical IC₅₀ values that had previously guided the field's understanding of PI selectivity.

The central finding modifies a long-standing assumption and elucidates that short-term, selective $\beta 5$ inhibition alone, even when complete, is not sufficient to produce meaningful cytotoxicity in MM cells. Only when $\beta 5$ inhibition is combined with co-inhibition of a second catalytic subunit, either $\beta 1$ or $\beta 2$, the proteasome complex becomes functionally blocked enough to trigger substrate accumulation and cell death. Critically, not all co-inhibition patterns are equal: $\beta 5/\beta 2$ co-inhibition proved substantially more effective than $\beta 5/\beta 1$ co-inhibition, both in PI-sensitive MM cells and, more importantly, in bortezomib- and carfilzomib-resistant MM cell lines and primary patient PCs. Among the tested drugs, this favorable $\beta 5/\beta 2$ inhibition profile is achieved only by high-dose carfilzomib and by marizomib, not by bortezomib, ixazomib, or low-dose carfilzomib, which remain restricted to $\beta 5$ ($\pm \beta 1$) inhibition (Figure 7).

This finding shed more light on the clinical discussion around carfilzomib's superiority over bortezomib. Rather than reflecting the superiority to $\beta 5$ inhibition¹²², the data indicate that carfilzomib's advantage is a direct, dose-dependent consequence of its unique subunit co-inhibition profile. Our data provided a mechanistic explanation with immediate translational implications, since they predict that dose intensity, not just drug identity, determines whether a patient receives the more effective $\beta 5/\beta 2$ inhibition pattern. The

findings were provided a commentary opinion in Science Translational Medicine (as Proteasome Inhibitors Refined by Derek Lowe).

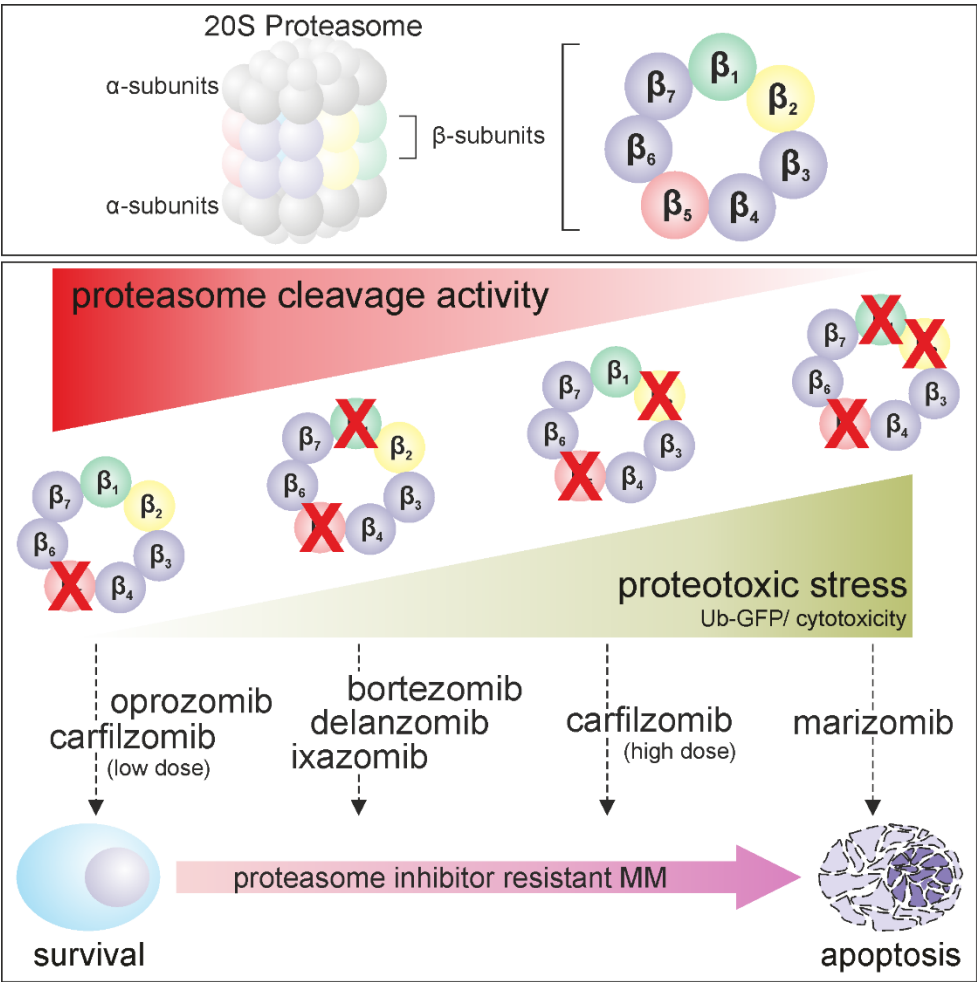


Figure 7: Graphical abstract representing the main methodology and findings of Annex 1

3.2 Commentary to Published Work – Annex 2

High-dose carfilzomib achieves superior anti-tumor activity over low-dose and recaptures response in relapsed/refractory multiple myeloma resistant to low-dose carfilzomib by co-inhibiting the $\beta 2$ and $\beta 1$ subunits of the proteasome complex

Original article published in *Haematologica*, Q1, IF (2023): 8.2

*Xiang Zhou, Andrej Besse, Jessica Peter, Maximilian Johannes Steinhardt, Cornelia Vogt, Silvia Nerreter, Eva Teufel, Emilia Stanojkovska, Xianghui Xiao, Hannah Hornburger, Larissa Haertle, Max Mendez-Lopez, Umair Munawar, Angela Riedel, Seungbin Han, Elmer Maurits, Herman S Overkleeft, Bogdan Florea, Hermann Einsele, K Martin Kortüm, Christoph Driessen, **Lenka Besse***, Leo Rasche**

* equal contribution

The published head-to-head comparison of proteasome inhibitors and established unique mechanism of proteasome inhibition by carfilzomib raised an immediate clinical question: Is this dose-dependency actually present in patients, and can it be exploited to recapture response in MM patients once resistance to standard-dose carfilzomib develops?

We answered this question directly by analyzing subunit-specific proteasome inhibition profiles in 103 paired peripheral blood mononuclear cell samples from RRMM patients receiving either low-dose (20-27 mg/m²) or high-dose (≥ 36 mg/m²) carfilzomib (Figure 8). The results confirmed the cell-line-derived model at the patient level with notable precision: $\beta 5$ activity was already substantially inhibited at the lowest clinical dose, but $\beta 2$ and $\beta 1$ co-

inhibition emerged only at ≥ 36 mg/m² and ≥ 56 mg/m², respectively. Residual $\beta 2$ activity dropped from a median of 82% at low dose to 46% at high dose, and residual $\beta 1$ activity from 93% to 51%, while $\beta 5$ inhibition itself did not differ significantly between dose levels. The data demonstrated that the clinical benefit of dose escalation cannot be attributed to "just inhibiting the same target harder," but specifically to recruiting additional catalytic subunits into the inhibited state, exactly as predicted by the earlier mechanistic work.

This molecular dose-response was mirrored in clinical outcomes. First, in a retrospective cohort of 114 patients treated with carfilzomib-based combinations, high-dose carfilzomib produced a significantly higher overall response rate and longer progression-free survival than low-dose carfilzomib. More importantly, in the prospective validation in 16 patients who had progressed on low-dose carfilzomib-containing regimens, solely the dose escalation of carfilzomib to ≥ 36 mg/m², recaptured a response ($\geq$ partial remission) in 56% of patients. This is an example of a resistance mechanism identified at the bench level and being translated into an actionable clinical intervention. Beyond its direct clinical value, the study argues that "carfilzomib resistance" is, at least in a subset of patients, not a fixed biological property of the tumor but a consequence of insufficient target engagement at conventional dosing.

The main limitation acknowledged by us is the retrospective, uncontrolled nature of the dose-escalation cohort and the need to balance this efficacy gain against the known dose-dependent toxicity of carfilzomib (cardiovascular and renal adverse events), meaning the ≥ 36 mg/m² threshold identified here should inform, but not replace, individualized clinical judgment on tolerability.

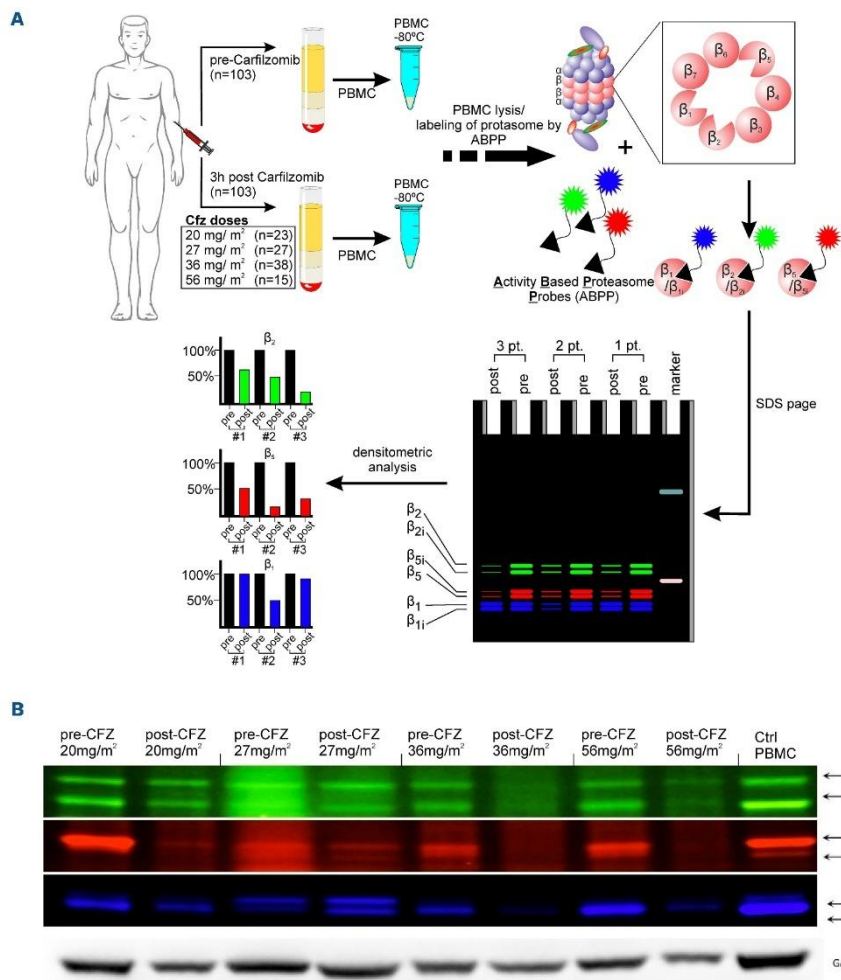


Figure 8: Graphical illustration of the main methodology presented in Annex

3.3 Commentary to Published Work – Annex 3

Proteasome inhibitor-adapted myeloma cells are largely independent from proteasome activity and show complex proteomic changes, in particular in redox and energy metabolism

Original article published in *Leukemia*, Q1, IF (2016): 11.702

*Guillem Paniagua Soriano**, ***Lenka Besse****, *Nan Li, Marianne Kraus, Andrej Besse, Nico Meeuwenoord, Juergen Bader 2, Bart Everts, Hans den Dulk, Herman S Overkleeft, Bogdan I Florea, Christoph Driessen*

* equal contribution

Prior to this study, PI resistance in MM was explained mainly by two models: acquired *PSMB5* mutations preventing drug binding, or a low IRE1 α /XBP1 signature reducing basal ER stress. Neither had been tested systematically in an unbiased fashion in PI resistant cells, nor had been asked how much residual proteasome inhibition such cells can actually tolerate.

We addressed these questions using bortezomib- and carfilzomib-adapted MM clones (AMO-BTZ, AMO-CFZ) and a combined quantitative/functional proteomic approach. The key finding is that PI-adapted cells tolerate substantial, subtotal proteasome inhibition irrespective of proteasome mutation status. They adapt to impaired proteolysis, challenging *PSMB5* mutation as a general resistance mechanism. The IRE1/XBP1-low signature was confirmed in both clones but shown to be correlative rather than functional, since restoring IRE1/XBP1 signaling did not sensitize the cells to PIs.

At the global protein level, over 600 differentially expressed proteins were shared between the two independently adapted cell lines to bortezomib and carfilzomib, with consistent upregulation of metabolic regulation, redox homeostasis, and protein-folding/degradation machinery, and downregulation of apoptosis and translation components, suggesting that PI resistance converges on a common, drug-agnostic adaptive proteome.

The paper's central contribution is its proposed model: PI resistant MM cells shift their metabolism toward OXPHOS, upregulating tricarboxylic acid (TCA or citrate)-cycle-associated NAD(P)H-generating reactions, such as the malate-pyruvate shuttle and mitochondrial isocitrate dehydrogenase, to produce more reducing equivalents. These reducing equivalents serve a dual purpose: buffering the excess reactive oxygen species (ROS) generated under chronic proteotoxic stress, and, critically, supporting more effective oxidative protein folding within the ER. These adaptations allow the cells to tolerate short term the subtotal proteasome inhibition (Figure 9).

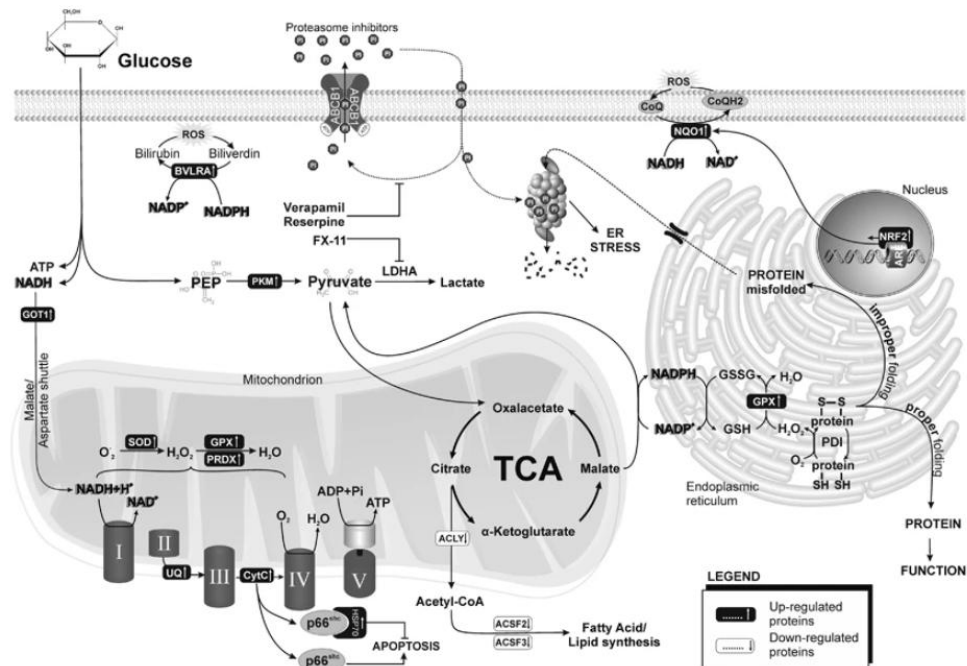


Figure 9: Summary of the proposed model of adaptation presented in Annex 3

3.4 Commentary to Published Work – Annex 4

A metabolic switch in proteasome inhibitor-resistant multiple myeloma ensures higher mitochondrial metabolism, protein folding and sphingomyelin synthesis

Original article published in *Haematologica*, Q1, IF (2019): 7.116

Lenka Besse*^{\$}, Andrej Besse*, Max Mendez-Lopez, Katerina Vasickova, Miroslava Sedlackova, Petr Vanhara, Marianne Kraus, Jürgen Bader, Renan B Ferreira, Ronald K Castellano, Brian K Law, Christoph Driessen

* equal contribution

^{\$} corresponding author

The earlier proteomic study proposed that PI-adapted MM cells tolerate subtotal proteasome inhibition through a metabolic shift favoring reducing-equivalent generation, but this model was based on deduction from proteomic data rather than on direct metabolite measurement. This follow-up study is its direct confirmation using mass spectrometry-based whole-metabolome profiling and functional assays of the same bortezomib and carfilzomib-adapted cell lines.

The data confirmed that the adapted cells share a convergent metabolic signature, with coordinated upregulation of pathways feeding glutathione synthesis and NAD(P)/NAD(P)H regeneration, alongside increased TCA-cycle flux and coenzyme A levels supporting ATP production. Individual assays validated the proteomic-level predictions directly, confirming elevated glutathione, NADP, and NADPH in resistant cells. Functionally, this translated into

demonstrably more efficient protein folding in the ER of PI-adapted cells, linked mechanistically to a redistribution of ATP toward the ER lumen at the expense of cytosolic ATP, providing a direct functional link between redox adaptation and the folding capacity needed to tolerate reduced proteasomal clearance. These adaptations are directly linked to structural mitochondrial changes.

Besides these findings, the study's novel contribution is the identification of a substantial lipid-class switch in resistant cells, from lysolipids toward sphingomyelins. This shifted the resistance model beyond redox/energy metabolism into lipid biology, and proved therapeutically exploitable: the sphingomyelin synthase inhibitor D609 was selectively more cytotoxic in PI-resistant than PI-sensitive cells and synergized with both bortezomib and carfilzomib in primary MM cells — translating a descriptive metabolomic finding into an actionable combination strategy.

Together, this work reframes PI resistance as a coordinated, multi-layered metabolic adaptation, spanning redox buffering, mitochondrial bioenergetics, and membrane lipid remodeling, that collectively lowers the cell's dependence on proteasomal proteolysis, rather than a single point-mutation or signaling escape (Figure 10).

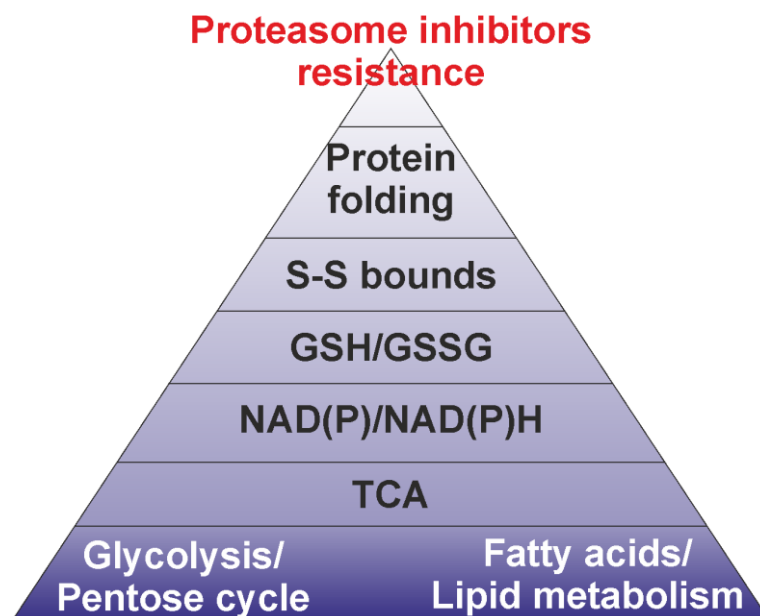


Figure 10: Graphical summary of the proposed metabolic rewiring presented in Annex 4

3.5 Commentary to Published Work – Annex 5

Treatment with HIV-Protease Inhibitor Nelfinavir Identifies Membrane Lipid Composition and Fluidity as a Therapeutic Target in Advanced Multiple Myeloma

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Nelfinavir's anti-MM activity, including its ability to overcome PI resistance in combination with bortezomib or carfilzomib, had by this point been established clinically, with a phase I trial confirming tolerable toxicity and subsequent phase II trial testing combination therapy, achieving an overall response rate exceeding 65% in RRMM. Nelfinavir is a potent UPR inducer, but the molecular basis of this activity, and therefore strong synergy with PI-induced proteotoxic stress, was unclear.

To elucidate nelfinavir targets in cancer in general, and specifically in MM, we used an unbiased combination of proteome-wide affinity purification to identify nelfinavir-interacting proteins, together with genome-wide CRISPR/Cas9 screening. This dual approach allowed to determine what

nelfinavir actually binds and what genes its cytotoxicity depends on. The result was unexpected: rather than converging on a discrete protein target, both approaches pointed toward lipid-embedded proteins in mitochondria and ER and pathways associated with fatty acid oxidation, redirecting the mechanistic question from "what does nelfinavir bind" to "what does nelfinavir do to membranes."

The functional data confirmed this reframing directly. Nelfinavir was shown to induce lipid bilayer stress by rigidifying lipid-rich membranes, altering both their fluidity and composition. This membrane-level perturbation had broad downstream consequences: disrupted mitochondrial respiration, and impaired vesicular transport from the ER, causing induction of the UPR through a route entirely independent of proteasome-mediated proteotoxic stress (Figure 11). This is the key conceptual contribution of the paper: it establishes that nelfinavir's synergy with bortezomib and carfilzomib arises because the two agents converge on the same terminal UPR/apoptotic endpoint via mechanistically non-overlapping routes. The first route is through misfolded-protein accumulation following proteasome inhibition, the second is through direct membrane perturbation, making the combination genuinely synergistic rather than redundant.

This study established membrane lipid composition as a therapeutic target in cancer cells. A considerable weakness is that it remains a relatively diffuse therapeutic target compared to a discrete protein, which makes rational optimization or biomarker-based patient selection for nelfinavir-based combinations considerably harder.

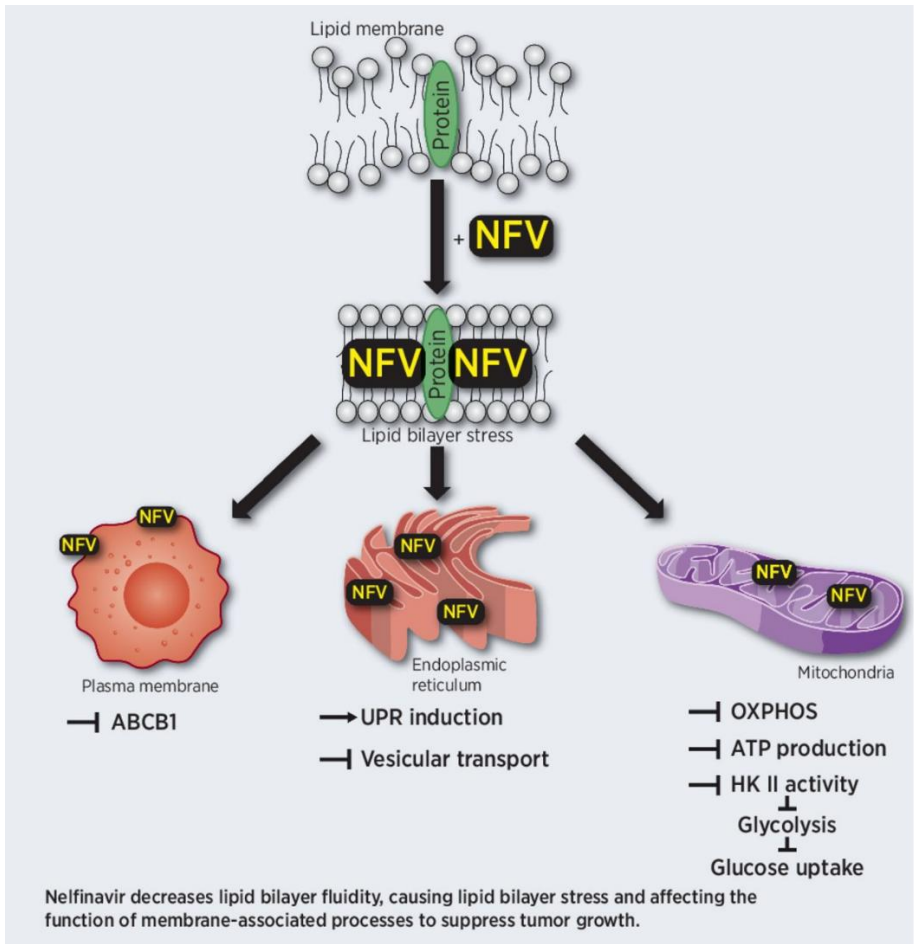


Figure 11: Graphical summary of the proposed mechanism of action of nelfinavir in MM, presented in Annex 5

4 Conclusion

Proteasome inhibitors have fundamentally transformed the treatment of multiple myeloma, turning a rapidly fatal disease into one with meaningfully extended survival. Yet resistance to these agents remains virtually universal over the course of the disease, and understanding why it develops, and more importantly, how it might be delayed or reversed, remains one of the central open questions in the field.

The work summarized in this thesis addresses this question from several complementary directions, each corresponding to one of the annexed contributions. At the level of the drug-target interaction itself, our head-to-head comparison of all clinically available proteasome inhibitors (Annex 1) showed that selective $\beta 5$ inhibition alone is not sufficient for cytotoxicity. The meaningful toxicity is reached via the combined $\beta 5/\beta 2$ subunit inhibition, a pattern uniquely achieved by carfilzomib at sufficient dose. This finding was directly validated in patients (Annex 2): profiling of active proteasome subunits in RRMM patients confirmed that carfilzomib dose escalation recruits $\beta 2$ co-inhibition and recaptures clinical response in patients resistant to standard-dose carfilzomib, translating a biochemical mechanism into an immediately actionable clinical intervention.

At the level of cellular adaptation, unbiased proteomic and metabolomic profiling of PI-resistant myeloma models (Annexes 3 and 4) revealed that resistant cells tolerate subtotal proteasome inhibition not by restoring proteasome function, but through a coordinated metabolic switch, favoring redox buffering, mitochondrial oxidative metabolism, and altered sphingolipid

biosynthesis, that collectively lowers the proteasomal burden of misfolded protein. This extended PI resistance understanding to proteostasis network plasticity rather than to a single fixed genetic lesion.

At the level of therapeutic intervention, the adaptation to proteasome inhibition provided therapeutic strategies. Our mechanistic dissection of nelfinavir (Annex 5) identified membrane lipid composition and fluidity as an unconventional, non-proteotoxic therapeutic vulnerability that triggers the UPR through a route entirely independent of proteasome inhibition itself, explaining its clinical synergy with bortezomib and carfilzomib in PI-refractory patients. Our identification of ceritinib (Annex 6) resolved a mechanistic dead-end that had stalled chaperone-targeting strategies for over a decade. By suppressing chaperone induction upstream, via IGF-1R/INSR signaling, rather than inhibiting chaperones directly, ceritinib, an already-approved drug with a well-characterized safety profile, avoids the compensatory heat-shock response that limited earlier HSP90 inhibitors.

Together, these six contributions support a unifying view: durable resensitization to proteasome inhibitors requires overwhelming the proteostasis network. This can be achieved directly, by broader-subunit, higher-dose proteasome inhibition, or by targeting additional PCs vulnerabilities such as membrane biology or compensatory signaling, which collectively disrupt proteostasis from different angle, preventing the compensable node. They highlight also future perspectives of MM therapy, which stem from better understanding of PC plasticity, that may drive resistance to other conventional therapies and immunotherapy.

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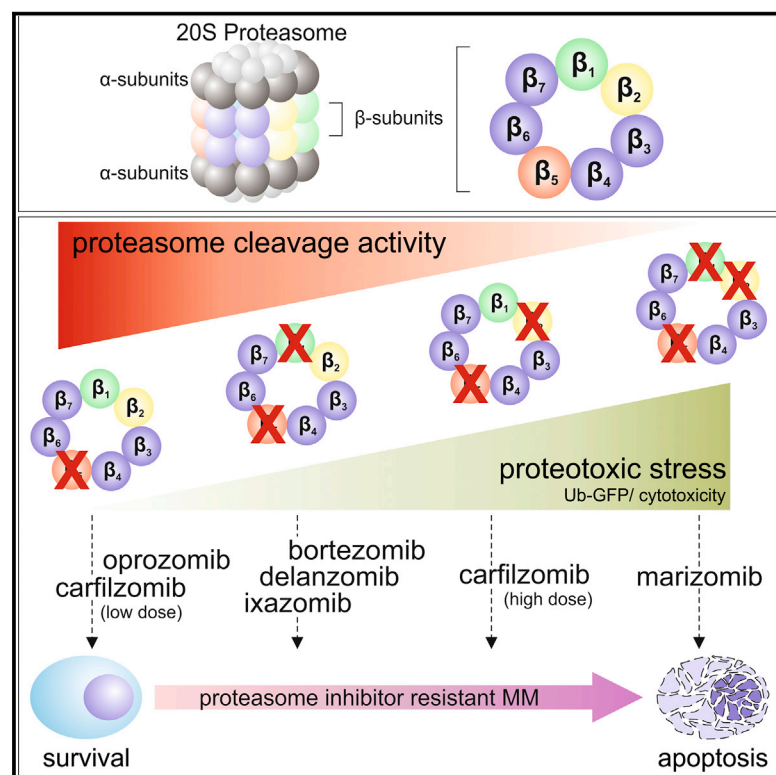
5 Supplementary Material

5.1 Annex 1

Cell Chemical Biology

Proteasome Inhibition in Multiple Myeloma: Head-to-Head Comparison of Currently Available Proteasome Inhibitors

Graphical Abstract



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In Brief

Besse et al. demonstrate that clinically available proteasome inhibitors designed to target β_5 subunit are cytotoxic only when another proteasome subunit (β_1 or β_2) is co-inhibited. The co-inhibition of subunits is not equal and the most effective is $\beta_5 + \beta_2$ proteasome inhibition, effective also against proteasome inhibitor-resistant multiple myeloma.

Highlights

- Direct comparison of proteasome inhibitors by activity-based probes and Ub^{-G76V}-GFP
- Short-term β_5 inhibition alone is not cytotoxic for MM cells
- β_5/β_2 co-inhibition is the most effective in PI-sensitive and PI-resistant MM
- From the available PI, only high-dose carfilzomib provides β_5/β_2 co-inhibition



Proteasome Inhibition in Multiple Myeloma: Head-to-Head Comparison of Currently Available Proteasome Inhibitors

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SUMMARY

Proteasome inhibitors (PIs) are a backbone of multiple myeloma (MM) therapy. The proteasome harbors six proteolytically active subunits ($\beta 1$, $\beta 2$, $\beta 5$), while $\beta 5$ was identified as rate-limiting and is a primary target of clinically available PIs. The most effective pattern of subunit inhibition provided by these PIs for cytotoxic activity in MM is unknown. A head-to-head comparison of clinically available PIs shows that in the clinically relevant setting only the co-inhibition of $\beta 1$ or $\beta 2$ with $\beta 5$ activity achieves meaningful functional proteasome inhibition and cytotoxicity, while the selective $\beta 2/\beta 5$ inhibition of both constitutive and immunoproteasome is the most cytotoxic. In the long-term setting, selective inhibition of $\beta 5$ subunit is sufficient to induce cytotoxicity in PI-sensitive, but not in PI-resistant MM, and the $\beta 5/\beta 2$ co-inhibition is the most cytotoxic in PI-resistant MM. These results give a rational basis for selecting individual PIs for the treatment of MM.

INTRODUCTION

Proteasome inhibitors (PIs) have improved the survival of patients with multiple myeloma (MM) and represent a backbone of current MM therapy in all treatment phases (Moreau et al., 2012). The first-in-class, reversibly binding peptide boronate-based PI bortezomib (Velcade) (BTZ) has been initially approved for relapsed/refractory MM (Kane et al., 2003) and soon after moved also to the frontline setting (Kouroukis et al., 2014). The second-generation, irreversibly binding peptide epoxyketone-based PI carfilzomib (Kyprolis) (CFZ) has been developed mainly to overcome intrinsic or acquired resistance and off-target toxicity of BTZ (Arastu-Kapur et al., 2011). Currently a third PI, the boronate-based, orally available ixazomib (Ninlaro), is approved in relapsed/refractory MM (Al-Salama et al., 2017). Additional PIs are under clinical investigation, such as

the epoxyketone-based PI oprozomib, the boronate-based PI delanzomib, and the irreversibly proteasome binding β -lactone PI marizomib (Rajan and Kumar, 2016; Vogl et al., 2017).

MM cells produce vast amounts of secretory protein and therefore heavily rely on proper proteasome function (Laubach et al., 2011). Proteasome inhibition in MM disrupts the equilibrium between protein production and disposal of misfolded or non-functional protein, which leads to proteotoxic stress, excess activation of the unfolded protein response, and ultimately apoptosis (Obeng et al., 2006). The 26S proteasome is a large multi-catalytic protein complex composed of the 19S regulatory particle and 20S core particle; the core particle is made of two outer α rings and two inner β rings (Levine and Kroemer, 2008). Each of the β rings contains three individual proteolytic subunits with distinct substrate specificity and activity ($\beta 5$: chymotrypsin-like; $\beta 2$: trypsin-like; $\beta 1$: caspase-like) (Ciechanover, 2005). These subunits of the so-called constitutive (c) proteasome can be replaced by respective immunoproteasome (i) subunits $\beta 1i$, $\beta 2i$, and $\beta 5i$ in immune cells (Huber et al., 2012), where they shape the repertoire of antigenic peptides presented by major histocompatibility complex class I (Rock et al., 2002).

The biological and functional significance of the six different individual proteasome subunits is poorly understood. The $\beta 5$ subunit of the proteasome was initially identified as the rate-limiting protease for proteasomal protein turnover based on the individual genetic knockdown of the proteolytically active protein domains of the constitutive proteasome in yeast (Heinemeyer et al., 1997; Arendt and Hochstrasser, 1997; Groll et al., 1999). Consequently, all PIs clinically available for MM therapy by design target the $\beta 5$ subunit of the constitutive proteasome and immunoproteasome (Kubiczkova et al., 2014; Kisselev et al., 2012). Further work showed that the co-inhibition of $\beta 1$ or $\beta 2$ subunits sensitizes MM cells for treatment with CFZ or BTZ (Mirabella et al., 2011; Kraus et al., 2015). Only recently chemical tools became available that allow selective manipulation and monitoring of the activity of each individual proteolytic subunit (de Bruin et al., 2016). Using these tools, it became evident that all $\beta 5$ -targeted PIs in fact lose their subunit selectivity at higher concentrations and that they co-inhibit either $\beta 1$ and/or $\beta 2$ type of proteasome subunits. These co-inhibition patterns differ between the individual PIs (de Bruin et al., 2016;



Kraus et al., 2015). The functional and biological significance of the different co-inhibitory patterns of the available PI drugs is unknown.

Understanding the functional significance of the different co-inhibitory patterns of PIs may help to explain differential activity and toxicity observed during treatment with different entities or different doses of PIs in head-to-head comparisons. The latter at present yields puzzling results: CFZ is superior or not to BTZ in clinical trials ENDEAVOR versus CLARION (Dimopoulos et al., 2017a; Facon et al., 2017), a once-weekly high dose of CFZ is more active, but not more toxic than a twice-weekly low dose (A.R.R.O.W. trial) (Moreau et al., 2018), with a similar cumulative dose delivered, while a once-weekly dose of BTZ is similarly active and less toxic than a twice-weekly dose (Brinchen et al., 2010). Comparative clinical trials with ixazomib and CFZ or BTZ are entirely lacking, and we have likewise no direct information about the comparative activity of ixazomib, oprozomib, delanzomib, and marizomib.

In this study we performed a head-to-head comparison of the clinically approved and tested PIs in a setup (1-hr pulse treatment) that resembles the pharmacokinetics of clinical intravenous (i.v.) dosing of PIs (Shabane et al., 2013). We determined the inhibition profile of the constitutive proteasome and immunoproteasome under increasing concentrations of PIs and also assessed the duration of the inhibition *in vitro*. Using highly selective inhibitors of individual proteasome subunits, we directly linked the inhibition profile of individual β subunits to functional proteasome inhibition and cytotoxicity in MM cells, and demonstrate the $\beta 5c/i$ and $\beta 2c/i$ co-inhibition, which is exclusively achieved by CFZ, at high dose levels is the most effective proteasome inhibition profile in MM, which is also able to overcome BTZ and CFZ resistance. The *in vitro* data are complemented by the proteasome subunit-inhibition profile of boronates and epoxyketone-based drugs after i.v. and intraperitoneal (i.p.) injection *in vivo*.

RESULTS

Side-by-Side Comparison of Clinically Available Proteasome Inhibitors in Viable MM Cells

We performed side-by-side comparison of the clinically available PIs by simultaneous assessment of proteasome activity, functional evaluation of proteasomal proteolysis, and cell death in MM cells. To directly account for functional proteasomal proteolysis we quantified the accumulation of proteasome substrate protein upon proteasome inhibition, using AMO-1 cells equipped with Ub^{-G76V}-GFP, a ubiquitin-fusion degradation substrate that builds up an intracellular fluorescence signal upon functional inhibition of the proteasomal proteolysis pathway. This was complemented by the use of fluorescence-labeled, proteasome activity-based chemical probes (ABP) that allow individual visualization of the activity state of the proteolytic $\beta 1$, $\beta 2$, and $\beta 5$ subunits, as well as a cytotoxicity assay. Cells were treated with increasing doses of proteasome-inhibiting drugs for a 1-hr pulse, followed by removal of the drug, which resembles pharmacokinetic exposure of the drugs during clinical i.v. dosing (Shabane et al., 2013). We observed that a 50% inhibition of $\beta 5c/i$ activity was already achieved with BTZ and marizomib at 10 nM concentration, whereas delanzomib required 30 nM, CFZ 100 nM, and

ixazomib and oprozomib 1,000 nM (Figures 1 and S1). More strikingly, we observed qualitative differences in the co-inhibition of $\beta 1$ and $\beta 2$ subunits by the different drugs. Marizomib, as expected, already inhibited the complete set of proteasome subunits at 100 nM. Bortezomib showed complete co-inhibition of $\beta 1c/i$ activity at doses inhibiting $\beta 5c/i$; likewise delanzomib co-inhibits $\beta 1c/i$, although at slightly higher doses than $\beta 5c/i$, while ixazomib displays a slightly higher preference for $\beta 1c/i$ over $\beta 5c/i$ subunit. In contrast to BTZ and ixazomib, CFZ is the only approved proteasome inhibitor with significant $\beta 2$ -inhibiting activity; however, this is only achieved at higher doses of the drug. Furthermore, oprozomib, a next-generation orally available epoxyketone-based drug, prevents increase of $\beta 2c/i$ activity at high dose, which only then is connected with cytotoxicity (Figures 1 and S1). Thus for the PIs, significant or even subtotal inhibition of $\beta 5c/i$ activity alone did not affect the degradation rate of Ub^{-G76V}-GFP or cell viability (e.g., BTZ 30 nM, marizomib 10 nM, CFZ 300 nM) or is possible only when there is no increase in the activity of the other proteasome β subunit (oprozomib, 10 μ M). This suggested that inhibition of $\beta 5c/i$ proteasome activity alone is not sufficient for MM cytotoxicity. By contrast, functional inhibition of proteolysis (as observed from the accumulation of Ub^{-G76V}-GFP) occurred only when significant co-inhibition of $\beta 5$ and at least one additional proteasome subunit (either $\beta 1$ or $\beta 2$) was achieved. While for BTZ 100–1,000 nM the functional proteasome inhibition assessed by Ub^{-G76V}-GFP accumulation and cytotoxicity was achieved through combined $\beta 5/\beta 1$ inhibition (without quantitatively affecting $\beta 2$ activity), a similar effect was reached with CFZ 300–3,000 nM through combined $\beta 5/\beta 2$ inhibition (without affecting $\beta 1$ activity). Ixazomib had functionally by far the weakest on-target activity during pulse exposure. With ixazomib, co-inhibition of $\beta 5/\beta 1$ was observed above 1,000 nM, which resulted only in a weak functional inhibition of proteasomal proteolysis at 10,000 nM, insufficient to translate into cytotoxicity.

The Duration of Proteasome Subunit Inhibition Does Not Differ Based on the Chemistry of the Inhibitor

By their chemical nature, epoxyketone-type PIs (CFZ and oprozomib) provide irreversible proteasome inhibition, while peptide boronates (e.g., BTZ and ixazomib) bind to the proteasome in a reversible fashion. This is taken as an argument to predict a superior duration of functional proteasome inhibition for irreversible inhibitors. However, this issue has not been directly addressed in a head-to-head comparison. Toward this aim, we directly compared the duration of $\beta 5c/i$ inhibition after a pulse treatment with the available PIs (Figure 2). To rule out effects of the differential cytotoxicity of the drugs, we compared the drugs at concentrations that lead to a selective inhibition of $\beta 5c/i$ activity in a 1-hr pulse treatment setting. Ixazomib also co-inhibited $\beta 1c/i$, due to its substrate selectivity mentioned above. The recovery of $\beta 5c/i$ was followed over time for 72 hr using ABP. All PIs showed complete inhibition of $\beta 5c/i$ after 1 hr of treatment (median 10% of residual $\beta 5c/i$ activity, minimum 3% for oprozomib, maximum 13% for BTZ, Figure S2). Within 24 hr, the cells in all settings recovered proteasome $\beta 5c/i$ activity to 50% (median 53% of residual $\beta 5c/i$ activity, minimum 44% for oprozomib and maximum 71% for BTZ). Therefore, there is no significant

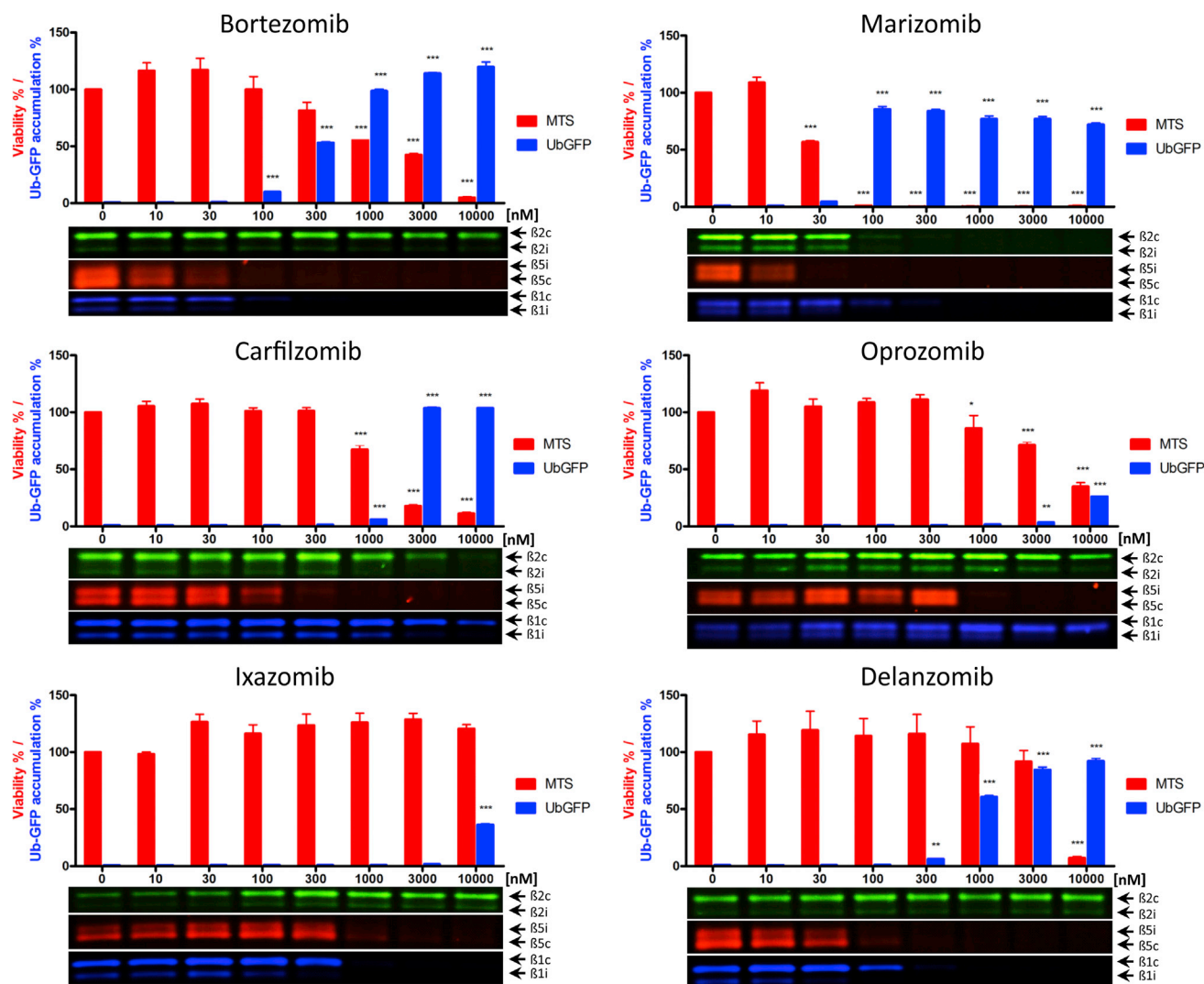


Figure 1. Side-by-Side Comparison of Clinically Approved and Tested Proteasome Inhibitors in AMO-1 Cells Equipped with Ub^{G76V}-GFP Construct

The cells were treated with increasing doses of indicated proteasome inhibitors for 1 hr and then suspended in drug-free medium. Part of the cells was directly lysed for ABP labeling, a second part was incubated for 8 hr for the Ub^{G76V}-GFP fluorescence determination by flow cytometry and third part was seeded for viability determination for 48 hr by MTS assay. For each proteasome inhibitor, subsequent data are represented: gel images of the activity of $\beta 1c/i$ (blue bands), $\beta 2c/i$ (green bands), and $\beta 5c/i$ (red bands) subunits evaluated by ABP labeling and SDS-PAGE, then accumulation of Ub^{G76V}-GFP, represented as blue bars in the graphs, and corresponding cell viability, represented as red bars in the graphs. The Ub^{G76V}-GFP fluorescence and viability data are presented as a mean $\pm$ SD of at least 3 independent experiments, and statistical significance is presented as * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. Gel image is representative of 3 experiments, and quantification of the intensity of the bands is represented in Figure S1.

difference in the duration of proteasome inhibition achieved with the different available drugs.

$\beta 5/\beta 2$ Co-inhibition Is the Most Effective Pattern of Proteasome Inhibition

Given that co-inhibition of non- $\beta 5$ subunits is essential for MM cytotoxicity and that the patterns of this co-inhibition differ between the available PIs, we next aimed to define the most effective pattern of proteasome subunit inhibition to reach maximum functional proteasome inhibition and cytotoxicity in MM cells. Using AMO-Ub^{G76V}-GFP in conjunction with β -subunit-selective PIs of the constitutive proteasome and immuno-

proteasome (described in STAR Methods), we explored the relationship between the different patterns of subunit-selective functional proteasome inhibition, the functional effect on proteasomal protein degradation, and cytotoxicity. This was performed in a similar 1-hr pulse exposure setup as previously with Ub^{G76V}-GFP-AMO-1 cells. Single inhibition of β subunits of constitutive proteasome and immunoproteasome ($\beta 1c/i$, $\beta 2c/i$, $\beta 5c/i$) did not functionally inhibit proteasomal protein turnover and likewise did not lead to cytotoxicity, confirming our observation above. Dual inhibition of $\beta 5c/i$ with $\beta 1c/i$ inhibited proteasome functionally and led to significant cell toxicity. Even more significant functional proteasome inhibition and cytotoxicity

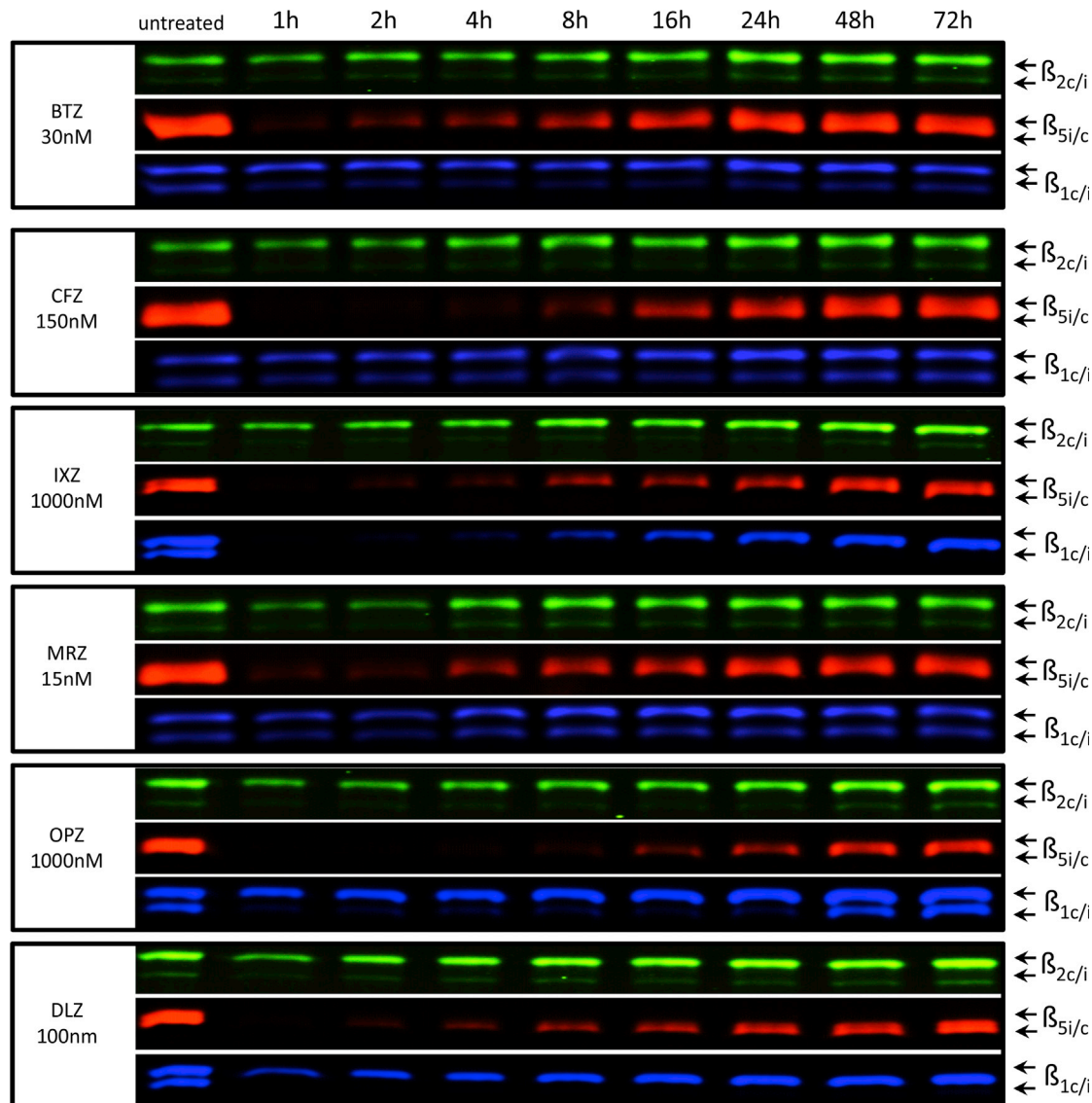


Figure 2. Recovery of Proteasome $\beta 5c/i$ Activity after 1 hr of Pulse Treatment with Indicated Proteasome Inhibitors in AMO-1 Cells

The cells were treated with indicated dose of indicated proteasome inhibitors for 1 hr and then suspended in drug-free medium. Gel images represent the activity of $\beta 1c/i$ (blue bands), $\beta 2c/i$ (green bands), and $\beta 5c/i$ (red bands) subunits after 1 hr of exposure to indicated drug concentration and follow-up of proteasome activity recovery at the indicated time points, evaluated by ABP labeling and SDS-PAGE. BTZ, bortezomib; CFZ, carfilzomib; DLZ, delanzomib; IXZ, ixazomib; MRZ, marizomib; OPZ, oprozomib. The quantification of the intensity of the bands is presented in [Figure S2](#).

were observed when $\beta 5c/i$ with $\beta 2c/i$ subunits were co-inhibited. This combination functionally inhibited proteasomal proteolysis almost as strongly as total proteasome inhibition with $\beta 5c/i$ -, $\beta 2c/i$ -, and $\beta 1c/i$ -selective inhibitors, as deferred from the accumulation of Ub^{-G76V}-GFP ([Figure 3A](#)). Next we analyzed whether selective co-inhibition $\beta 5/\beta 2$ or $\beta 5/\beta 1$ subunits of either the constitutive proteasome or the immunoproteasome (combination of $\beta 5i + \beta 2i$, $\beta 5i + \beta 2c$, $\beta 5c + \beta 2i$, $\beta 5c + \beta 2c$) would likewise induce Ub^{-G76V}-GFP accumulation and cytotoxicity. The results demonstrate that complete inhibition of both species of a subunit (constitutive proteasome and immunoproteasome) is required to achieve a functional effect on protein degradation and cytotoxicity. Thus subunits of the constitutive proteasomes and immu-

noproteasomes are able to take over the protein degradation once the respective corresponding complementary subunit is inhibited ([Figure 3B](#)). Next, we addressed whether the activity of $\beta 5c/i$ and $\beta 2c/i$ subunits is independent from each other or whether the inhibition of one subunit alters the sensitivity of the other subunit for inhibition. Indeed, 5 μM $\beta 2c/i$ -selective inhibitor LU102 was required to induce a sizable reduction in the amount of $\beta 2c/i$ proteasome activity detected in the absence of $\beta 5c/i$ inhibition, while in the presence of $\beta 5c/i$ inhibition a similar effect on $\beta 2c/i$ activity was already reached at 1.25 μM ([Figure 3C](#)). This suggests a better accessibility of the $\beta 2c/i$ subunit for PIs in the presence of $\beta 5c/i$ inhibition, possibly by conformational changes that are induced upon $\beta 5c/i$ inhibition.

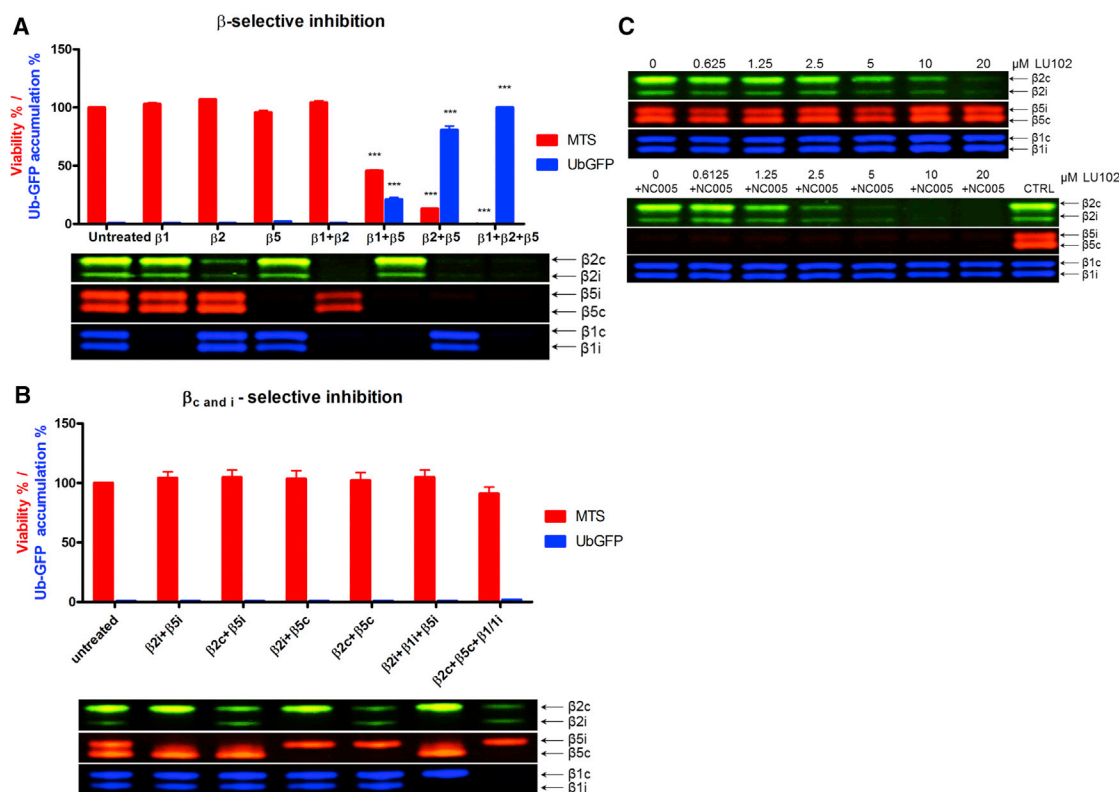


Figure 3. The Most Effective Profile of Proteasome Inhibition Determined in AMO-1 Cells Equipped with Ub^{G76V}-GFP Construct

(A and B) AMO-1 cells were treated with (A) indicated β-subunit-selective inhibitors of different proteasome β subunits of both constitutive and immunoproteasome (c/i) (NC001: β1c/i inhibitor, 10 μM; LU102: β2c/i inhibitor, 3 μM; NC005: β5c/i inhibitor, 5 μM) or (B) combination of the selective inhibitors for the β subunits of either constitutive (c) or immunoproteasome (i) (LU001i: β1i inhibitor, 2 μM; NC001: β1c/i inhibitor, 10 μM; LU002i: β2i inhibitor, 2 μM; LU002c: β2c inhibitor, 6 μM; LU015i: β5i inhibitor, 3 μM; LU025c: β5c inhibitor, 3 μM) for 1 hr, then suspended in drug-free medium. Part of the cells was directly lysed for ABP labeling and SDS-PAGE, a second part was incubated for 8 hr for the Ub^{G76V}-GFP fluorescence determination by flow cytometry, and a third part was seeded for viability determination after 48 hr by MTS assay. The Ub^{G76V}-GFP fluorescence and viability data are presented as a mean ± SD of 3 independent experiments, statistical significance is presented as ***p < 0.0001.

(C) AMO-1 cells were treated with NC005 (β5c/i) and LU102 (β2c/i) selective inhibitors. In the upper panel, increasing concentrations of LU102 are used to inhibit β2c/i when β5c/i is active, whereas in the lower panel increasing concentrations of LU102 are used to inhibit β2c/i when β5c/i is inhibited with 10 μM NC005. The cells were lysed after 1 hr of exposure to the inhibitors for ABP labeling and SDS-PAGE.

Selective Inhibition of β2 + β5, but Not β1 + β5, Overcomes Bortezomib and Carfilzomib Resistance in PI-Resistant MM

We next addressed whether combined inhibition of β5/β2 subunits is able to overcome PI resistance. Toward this aim, we used BTZ-resistant and CFZ-resistant AMO-1 described earlier (Besse et al., 2018), RPMI8226, L363 cells (aBTZ, aCFZ) (Table 1), in conjunction with 48-hr continuous treatment with PIs *in vitro*. In PI-sensitive cells, already β5c/i inhibition alone led to significant cellular toxicity, while selective β2 inhibition had no effect. BTZ-resistant cells are not sensitive to β5c/i-selective proteasome inhibition, consistent with the inhibition profile of BTZ; however, the resistance can be overcome by co-inhibition of β5 and β2c/i (Figure 4A), which induces strong synergistic cytotoxicity. Likewise, BTZ resistance of primary malignant plasma cells derived from MM patients could be overcome by combining BTZ with the β2-inhibitor LU-102. By contrast, carfilzomib resistant MM cell lines were not sensitive to combined β5/β2 proteasome inhibition, although CFZ-resistant primary malignant plasma cells were again significantly sensitive to β5c/i + β2c/i proteasome inhibition (Figures 4B and

4C, additional patients were already shown; Kraus et al., 2015). CFZ-resistant MM cell lines express high activity of ABCB-type drug export proteins (Figure 4D; Besse et al., 2018; Soriano et al., 2016). Epoxyketones, including the selective β5c/i inhibitors used by us, are strong substrates of ABCB-type pumps (Besse et al., 2018). This suggested that the lack of cytotoxic activity of the β5c/i + β2c/i inhibitor combination in CFZ-resistant cells may be due to ABCB1 mediated export of PI, as already published by us (Besse et al., 2018). Indeed, inhibition of ABCB-type efflux pumps by reserpine restored the sensitivity of CFZ-resistant MM cell lines to β5c/i + β2c/i inhibition (Figure 4E). Furthermore, additive β2 proteasome inhibition was able to increase the cytotoxicity of all currently available PIs (Figure 5 and Table S1) in cells without strong ABCB-type pump expression (AMOWt, AMOaBTZ), in contrast to cells with high drug efflux activity (AMOaCFZ).

The Proteasome-Inhibiting Activity *In Vivo* Differs between Boronates and Epoxyketones

To address the tissue distribution of the different active subunits of the constitutive proteasome versus immunoproteasome, we

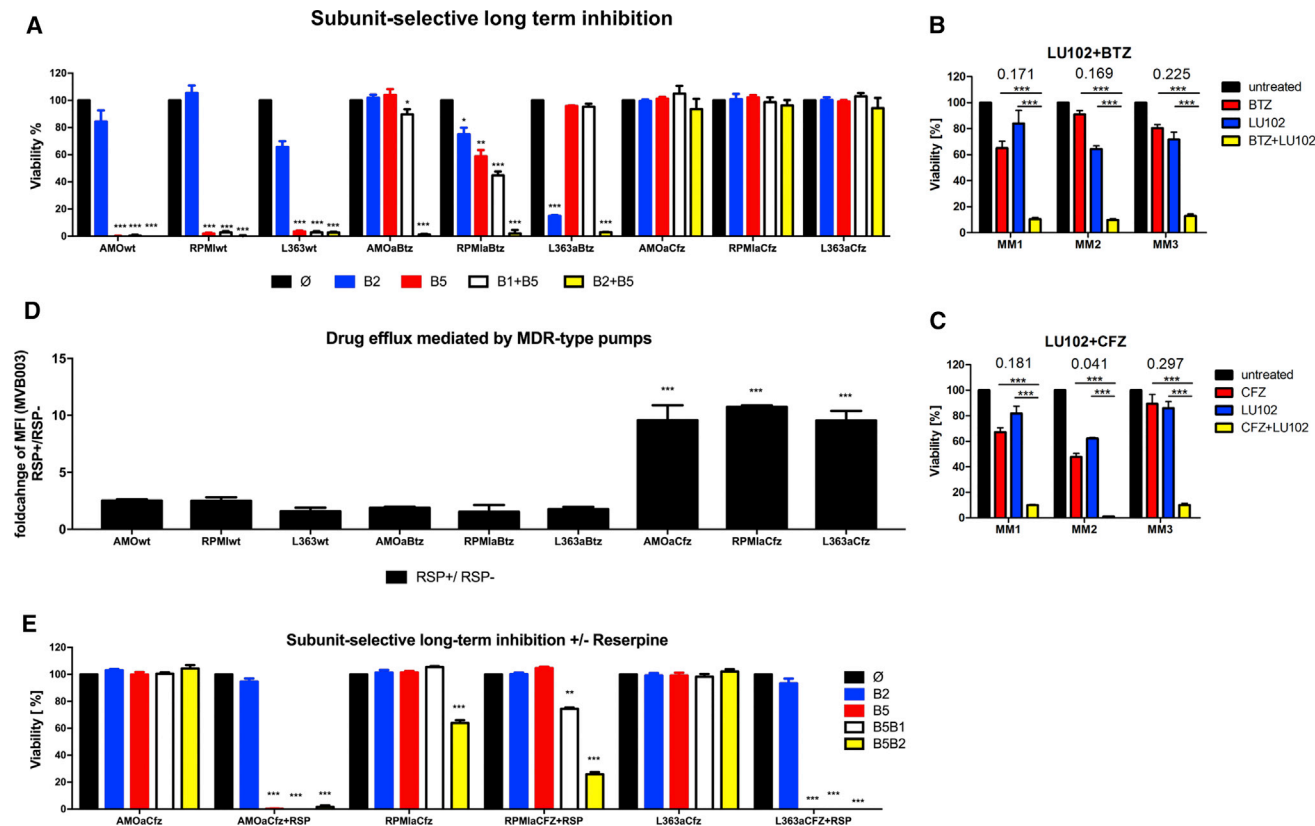


Figure 4. $\beta 5/\beta 2$ Co-inhibition without or with ABCB Type of Drug Exporters Inhibitors Overcomes Bortezomib and Carfilzomib Resistance in MM

(A) PI-sensitive (wt) and PI-resistant (aBTZ or aCFZ) cell lines were exposed for 48 hr continuously to β -subunit-selective PI or their combination, with subsequent viability determination by MTS. The following concentrations were used: NC001 ($\beta 1c/i$ inhibitor; 10 μ M), LU102 ($\beta 2c/i$ inhibitor; 3 μ M), NC005 ($\beta 5c/i$ inhibitor; 5 μ M).

(B and C) Primary malignant plasma cells from 3 MM patients were exposed for 48 hr to (B) bortezomib (BTZ; MM1: 5 nM, MM2: 2.5 nM, MM3: 5 nM) and (C) carfilzomib (CFZ; MM1 and MM2: 5 nM, MM3: 2.5 nM) alone or in combination with $\beta 2$ -selective inhibitor LU102 (MM1 and MM2: 10 μ M, MM3: 3.3 μ M). Statistical significance is presented as *** p < 0.001 of respective comparisons obtained by one-way ANOVA with Tukey's post test for each individual MM patient. The coefficient of drug interaction (CDI) is indicated. CDI < 1 indicates a synergistic effect; CDI = 1 indicates an additive effect; CDI > 1 indicates an antagonistic effect.

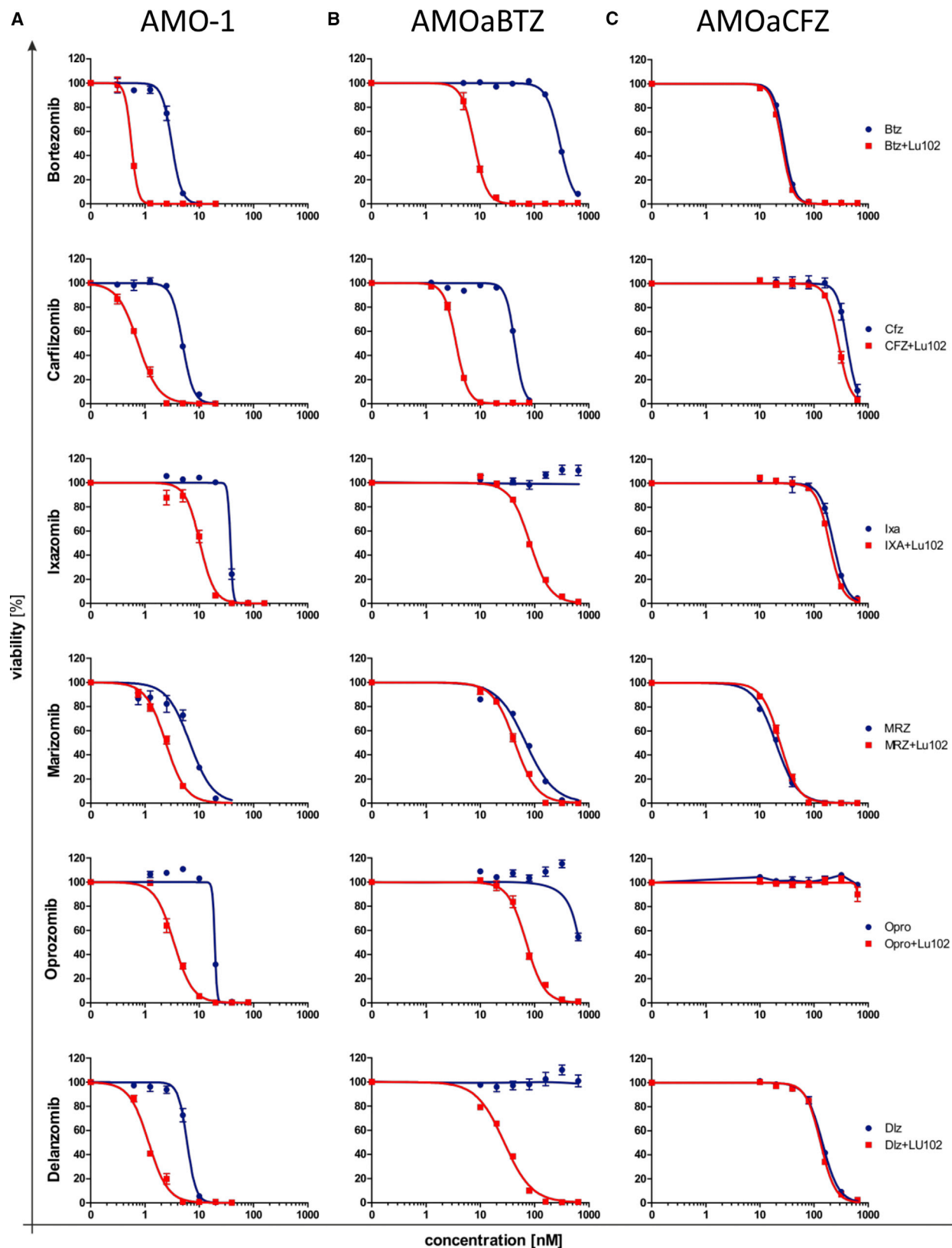
(D) PI-sensitive (wt) and PI-resistant cell lines (aBTZ or aCFZ) were incubated for 3 hr with/without 10 μ M ABCB-type efflux pump inhibitor 10 μ M reserpine (RSP), with subsequent incubation with 1 μ M MVB003, an ABCB1 substrate, for ABCB type of pump activity determination. Median fluorescence intensity (MFI) was measured by flow cytometry and is depicted as a ratio between MVB003 (ABCB1 substrate) labeled and RSP⁺/RSP⁻ (ABCB1 inhibitor) treated cells.

(E) CFZ-resistant cell lines were exposed for 48 hr continuously to β -subunit-selective PIs (drugs and concentrations are indicated in A) or their combination, $\pm$ reserpine, with subsequent viability determination.

The viability and flow-cytometry data for cell lines are presented as a mean $\pm$ SD of at least 3 independent experiments, and statistical significance is presented as * p < 0.05, ** p < 0.01, *** p < 0.001. The IC₅₀ values of PI-resistant cell lines to bortezomib and carfilzomib are presented in Table 1.

performed ABP labeling of extracts from murine organs that have been normalized for equal amounts of total protein (Figures 6A and S3A). The contributions of constitutive proteasome and immunoproteasome markedly differed per organ. While there was an equal distribution between both types in peripheral blood mononuclear cells and bone marrow, immunoproteasome was the dominant species in spleen and lung tissue, while liver and kidney contained mostly constitutive proteasome activity. Immunoproteasome activity was absent from brain lysates. Strikingly, heart tissue contained significantly lower active proteasome per total protein than any other type of organ tested here. This may reflect in part the cardiac vulnerability for high doses of carfilzomib observed in the clinic.

We next asked whether the different inhibitory patterns toward proteasome subunits of BTZ and CFZ would translate into similar differences in the various tissues *in vivo*, either after application that results in high peak plasma levels (i.v., like CFZ) or in an application mode leading to lower peak plasma levels and prolonged exposure (like BTZ, subcutaneous [s.c.] in the clinic and i.p. in our experimental setup). We therefore treated mice with BTZ (1 mg/kg) and CFZ (4 mg/kg) both i.v. and i.p. and evaluated proteasome activity 2 hr post treatment. After i.v. administration, BTZ significantly inhibited $\beta 5c/i$ and $\beta 1c/i$ activity in all studied organs except the brain, whereas CFZ significantly inhibited $\beta 5c/i$ activity, except for brain and lungs (Figure 6B and S3B). Interestingly, after i.v. administration BTZ provided slightly



(legend on next page)

stronger $\beta 5$ -inhibiting activity, despite its lower total dose, compared with CFZ in our setup in all peripheral organs tested, except for the heart. In heart tissue after i.v. injection, CFZ showed a significant reduction of both $\beta 5$ and $\beta 2$ proteasome activity. Clear differences in proteasome inhibition were observed after i.p. administration, where BTZ was as effective as in the i.v. setting, while CFZ failed to inhibit $\beta 5c/i$ to more than 50% in all studied organs apart from the liver (Figures 6C and S3C). Thus, CFZ and BTZ show their different patterns of $\beta 5/\beta 1$ and $\beta 5/\beta 2$ co-inhibition also *in vivo* after systemic administration i.v. or i.p. The pharmacodynamic activity of CFZ after i.p. injection is poor in comparison with BTZ.

DISCUSSION

After the initial approval of BTZ (Kane et al., 2003), new PIs with improved proteasome selectivity, differential subunit-inhibition profiles, and different toxicity profiles are being used and developed (Herndon et al., 2013; Shirley, 2016; Moreau et al., 2012; Kubiczekova et al., 2014). However, the clinical choice between different PIs and their different dosing strategies is based on data from very few clinical head-to-head comparisons in selected settings and/or doses that give partly conflicting results (Brighen et al., 2010; Dimopoulos et al., 2017a; Facon et al., 2017; Moreau et al., 2018).

We here provide a systematic overview of the proteasome inhibition profiles and key functional pharmacodynamic properties of current PI drugs or drug candidates. Our data are consistent with the major clinical results observed to date and may therefore help to guide the future use and dosing of PIs in the next generation of clinical trials.

All PIs by design target the $\beta 5$ proteasome subunit, the key molecular target. Our data show clearly that $\beta 5c/i$ inhibition alone is insufficient to decrease protein breakdown in viable MM cells *in vitro*, after short 1-hr pulse treatment, and lacks cytotoxic activity, in agreement with earlier data (Kisselev et al., 2006). Functional inhibition of proteasomal protein destruction and, hence, cytotoxic activity is only achieved upon co-inhibition of a second β subunit ($\beta 1$ or $\beta 2$), in addition to $\beta 5$ inhibition (either $\beta 5/\beta 1$ or $\beta 5/\beta 2$ co-inhibition) (Weyburne et al., 2017; Kraus et al., 2015). We here show that $\beta 5/\beta 2$ co-inhibition is more effective compared with a $\beta 5/\beta 1$ inhibition profile. While both profiles, $\beta 5/\beta 1$ and $\beta 5/\beta 2$, induce potent cytotoxicity in therapy-sensitive MM cells, $\beta 5/\beta 2$ co-inhibition was more potent and the only inhibition profile that was cytotoxic to proteasome inhibitor-refractory MM cell lines or primary cells. The co-inhibition of $\beta 2$ activity improved the cytotoxic activity of all available proteasome-inhibiting drugs and drug candidates. Interestingly, the chemistry of the PI warhead (expolyketone, β -lactone, or boronate) did not significantly affect the duration of functional proteasome inhibition, consistent with the very slow off-rate of, e.g., BTZ (Kisselev et al., 2012), so that differences observed in the clinical efficacy of

different drugs are hardly explained by reversible versus irreversible proteasome binding.

The cytotoxicity and half maximal inhibitory concentration (IC_{50}) values of PIs vary depending on the treatment schedule used *in vitro*. Here, we used a 1-hr pulse treatment setting because it largely resembles the pharmacokinetic profile after i.v. treatment with PIs in the clinic. This pattern does not directly mirror the situation with the oral inhibitors oprozomib and ixazomib as well as with s.c. BTZ. However, the identical setup used here for each drug allows their direct comparison.

Peptide boronates (BTZ, ixazomib, delanzomib) show a $\beta 5/\beta 1$ proteasome inhibition pattern. CFZ achieves $\beta 5$ inhibition at relatively low concentrations (100 nM in a 1-hr pulse experiment), but is unique in that it is the only PI that induces a dose-dependent co-inhibition of $\beta 2$ activity over a relatively wide concentration range (from 1 μ M to 10 μ M). The approved dose of 27 mg/m² CFZ results in a 1.79 μ M C_{max} , whereas the 56-mg/m² dose given as 30-min infusion translates to a 2.89 μ M concentration (Papadopoulos et al., 2015). Thus it is plausible from our data that a 27-mg/m² dosing of CFZ has only very weak $\beta 2$ co-inhibiting activity, because it is at the low end of the concentration range that induces $\beta 2$ co-inhibition (insufficient $\beta 2$ co-inhibition). This is consistent with an inhibitory effect on protein destruction and cytotoxic activity that is comparable with BTZ. High doses of CFZ (56 mg/m² and higher), however, lead to significant $\beta 5/\beta 2$ co-inhibition, which is more potent than $\beta 5/\beta 1$ co-inhibition (Figures 1 and 3). The results from clinical head-to-head comparisons of BTZ and CFZ in MM are consistent with the interpretation that high doses of CFZ are superior to BTZ (ENDEAVOR study, $\beta 5/\beta 2$ inhibition superior to $\beta 5/\beta 1$ inhibition) (Dimopoulos et al., 2016, 2017a), and once-weekly high doses of CFZ are superior to low doses of CFZ (sufficient $\beta 2$ co-inhibition superior to insufficient $\beta 2$ co-inhibition, A.R.R.O.W. trial) (Moreau et al., 2018). On the other hand the standard-dose twice-weekly CFZ, is not more effective than BTZ ($\beta 5/\beta 1$ co-inhibition versus $\beta 5$ inhibition with insufficient $\beta 2$ co-inhibition; CLARION trial) (Facon et al., 2017).

Given that the degree of $\beta 2$ co-inhibition largely determines effective inhibition of protein turnover and cell death (Figure 3A), it appears plausible that it also determines on-target toxicity in non-multiple myeloma cells *in vivo*. The relatively wide dose range over which the increasing $\beta 2$ inhibition activity of CFZ is observed makes it easier to safely titrate the $\beta 2$ -inhibition level (and CFZ dose) to the maximum tolerated level in the clinic. By contrast, marizomib, the only other drug with significant $\beta 2$ -inhibiting activity, shows $\beta 2$ co-inhibition only in a very narrow concentration window between 30 and 100 nM. This feature may make it more difficult to reach a safe $\beta 5/\beta 2$ co-inhibiting dosing with this drug.

Our data show that the heart contains exceptionally low amounts of active proteasome species per microgram of protein (Figure 6), and the i.v. application of CFZ *in vivo* results in a particularly potent inhibition of cardiac $\beta 5/\beta 2$ compared with, e.g., lung tissue, i.v. application of BTZ, or i.p. application of CFZ. This special combination of highly effective inhibitory

Figure 5. Dose-Response Curves of Proteasome Inhibitor-Sensitive and -Resistant Cells to Different Proteasome Inhibitors in Monotherapy or in Combination with $\beta 2$ -Selective Inhibitor

(A–C) (A) AMO-1 PI-sensitive, (B) AMOaBTZ, and (C) AMOaCFZ were exposed for 48 hr to increasing concentrations of clinically available proteasome inhibitors alone or in combination with 3 μ M $\beta 2c/i$ -selective inhibitor LU102. BTZ, bortezomib; CFZ, carfilzomib; DLZ, delanzomib; IXZ, ixazomib; MRZ, marizomib; OPZ, oprozomib. IC_{50} values for all proteasome inhibitors as single drugs or in combination with LU102 are presented in Table S1.

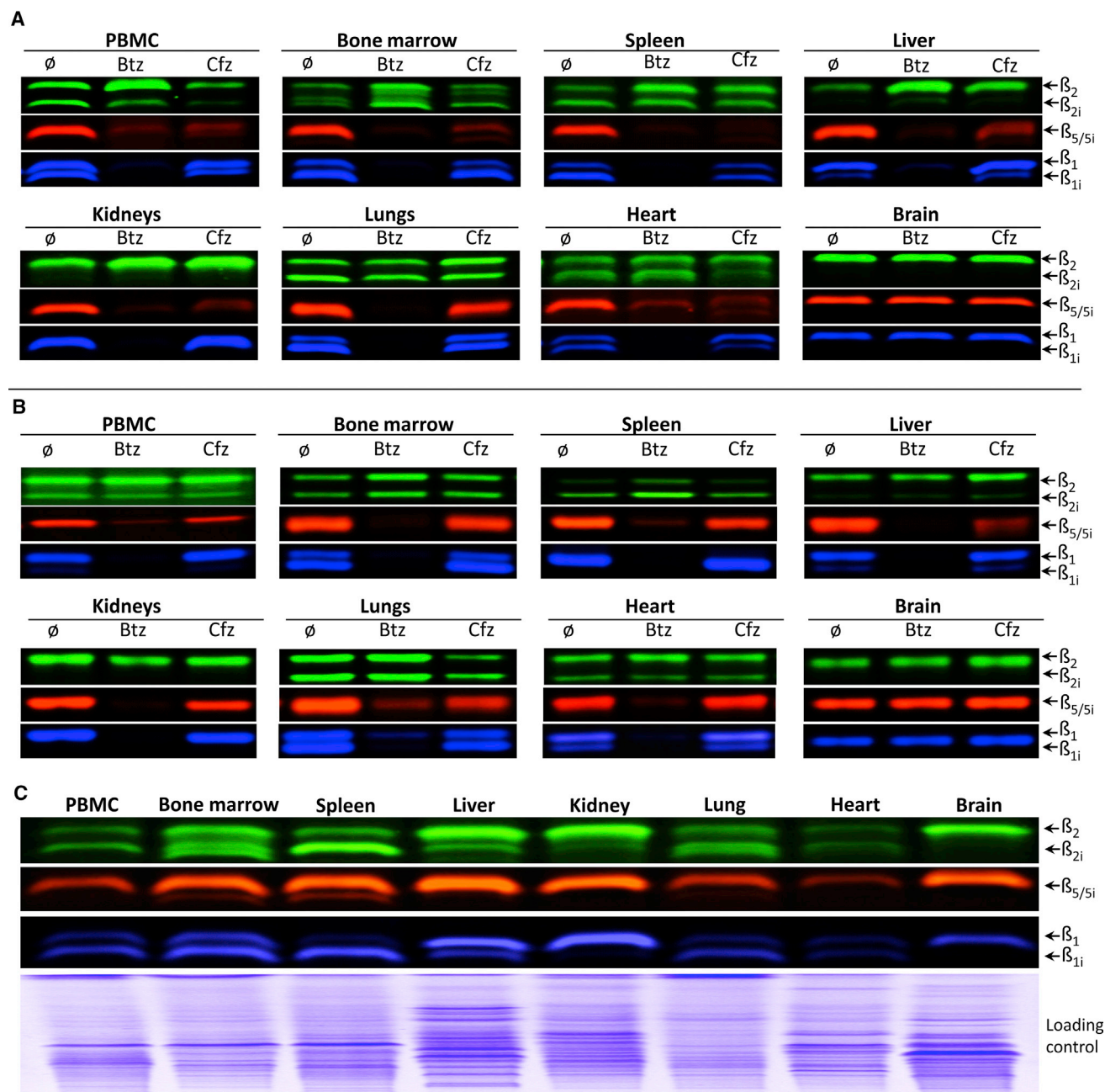


Figure 6. Pharmacodynamic Activity of Bortezomib and Carfilzomib after i.v. and i.p. Administration

(A and B) Basal proteasome activity determined in different mouse organs by ABP labeling and SDS-PAGE (A). Organ processing is described in STAR Methods. (B) Biodistribution and inhibition profile of bortezomib (1 mg/kg) and carfilzomib (4 mg/kg) in different organs from mice 2 hr after i.v. administration of the drugs. (C) Biodistribution and inhibition profile of bortezomib (1 mg/kg) and carfilzomib (4 mg/kg) in different organs from mice 2 hr after i.p. administration of the drugs. The organs were processed; cells were lysed, labeled with ABP, and proteasome subunits were separated by SDS-PAGE. Green bands indicate β2c/i subunit activity, red bands indicate β5c/i subunit activity, and blue bands indicate β1c/i subunit activity. The quantification of the intensity of the bands for each panel is presented in Figure S3.

pattern (β5/β2), tissue distribution, and low cardiac proteasomal capacity may serve as a mechanistic basis for the cardiac toxicity of PIs, and especially CFZ (Dimopoulos et al., 2017b).

β5/β2 co-inhibition with boronate-based drugs was effective in resistant cell lines and primary patient samples, despite the presence of PSMB5 mutations in the cell lines, which has

recently been confirmed also in patient samples (Barrio et al., 2018). This co-inhibition with epoxyketone-based inhibitors, which are strong substrates for ABCB-type drug transporters, was not effective in CFZ-resistant cell lines *in vitro* due to high functional expression of ABCB-type transporters (Besse et al., 2018; Soriano et al., 2016). Likewise, solid tumors or circulating

Table 1. Characteristics of Proteasome Inhibitor-Sensitive and -Resistant Cell Lines

Cell Line	IC ₅₀ BTZ (nM)	IC ₅₀ CFZ (nM)	PSMB5 Status
AMO-1	4.009	4.798	wt
RPMI-8226	5.259	10.81	wt
L363	5.595	5.156	wt
AMObaBTZ	178.2	25.34	A310G (Met45Val) heterozygous
RPMIaBTZ	147.4	38.33	G322A (Ala49Thr)
L363aBTZ	151.3	31.78	G322A (Ala49Thr)
AMObaCFZ	18.52	195.7	wt
RPMIaCFZ	167.4	531.5	wt
L363aCFZ	17.21	253.6	wt

IC₅₀ values for proteasome inhibitors bortezomib (BTZ) and carfilzomib (CFZ) in proteasome inhibitor-sensitive (AMO-1, RPMI-8226 and L363) and proteasome inhibitor-resistant cells (cells resistant to bortezomib: AMObaBTZ, RPMIaBTZ, L363aBTZ; cells resistant to carfilzomib: AMObaCFZ, RPMIaCFZ, L363aCFZ) used in Figures 4A, 4D, 4E, and 5; and mutation status within the active site of PSMB5 subunit. wt, wild-type. Mutations are presented as respective substitutions on gene and protein level (in parentheses).

MM plasma cells with high expression of ABCB1 are not sensitive to CFZ even in high doses (Abt et al., 2018; Pilarski et al., 1997). Inhibition of ABCB-type transporters is able to sensitize the CFZ-resistant cells to $\beta 5/\beta 2$ inhibition and likewise to CFZ (Besse et al., 2018; Abt et al., 2018; Soriano et al., 2016). Several reports showed effective $\beta 5/\beta 2$ proteasome inhibition by two independent drugs, or by one single molecule, such as CFZ or syringolin analog syringolog-1 (Kraus et al., 2015; Weyburne et al., 2017; Yoshida et al., 2018). We here show that $\beta 5/\beta 2$ dual inhibition using two selective inhibitors is more effective than one bispecific inhibitor, as lower doses of $\beta 2$ inhibitor are needed to fully inhibit $\beta 2$ activity when the $\beta 5$ site is inactive, compared with the setting when $\beta 5$ is active. This is likely possible due to allosteric regulation of active proteasome subunits (Kisselev et al., 1999). The use of two selective inhibitors allows for lower dose of the inhibitors to be used with the same effect on proteasome subunit inhibition, which may translate into fewer side effects; moreover, it allows a sequential dosing of the therapy, which may minimize the acute toxicity (Dimopoulos et al., 2016).

Next, our data (Figure 3B) largely rule out that selective immunoproteasome inhibitors can be used in monotherapy to achieve proteotoxic stress and cytotoxicity in cell types that co-express the immunoproteasome together with the constitutive proteasome, such as myeloma. Selective immunoproteasome $\beta 5$ inhibitors may, however, have a cytotoxic potential to eliminate cells that exclusively express the immunoproteasome, such as pediatric acute lymphoblastic leukemia or autoimmune diseases, or even in MM when combined with other subunits inhibitors such as $\beta 2$ (Niewerth et al., 2016; Basler et al., 2015; Downey-Kopy-scinski et al., 2018).

SIGNIFICANCE

Proteasome inhibitors are a backbone of multiple myeloma therapy. We herein provide comprehensive side-by-side

direct comparison of the pharmacodynamic activity of the proteasome inhibitors that are clinically available. Our results show that selective inhibition of the primary pharmaceutical target, the $\beta 5$ subunit, has no functional effect in myeloma in a short-term exposure setting. Proteotoxic stress and cytotoxicity is set only upon co-inhibition of either $\beta 5/\beta 1$ species or $\beta 5/\beta 2$ species. The $\beta 5/\beta 2$ inhibitory pattern has higher functionally proteasome-inhibiting and cytotoxic activity than the $\beta 5/\beta 1$ pattern. Proteasome inhibitor resistance could be overcome exclusively by $\beta 5/\beta 2$ inhibition. Full $\beta 5/\beta 2$ -type of proteasome inhibition is reached by carfilzomib only at higher concentrations, and not by bortezomib or low doses of carfilzomib, consistent with the results from clinical head-to-head comparisons. We conclude that co-inhibition of $\beta 5/\beta 2$ is a functionally more efficient mode of proteasome inhibition than co-inhibition of $\beta 5/\beta 1$. This is likely to translate into a higher clinical effectiveness of $\beta 5/\beta 2$ inhibition with, e.g., carfilzomib versus bortezomib in situations where maximum functional proteasome inhibition is the aim, and supports the use of carfilzomib at doses of 56 mg/m² or 70 mg/m² as opposed to lower doses. However, on-target toxicity in nonrelated organs, in particular cardiac toxicity, may also well be more closely associated with this more effective mode of proteasome inhibition, and needs to be addressed and better understood.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- CONTACT FOR REAGENT AND RESOURCE SHARING
- EXPERIMENTAL MODEL AND SUBJECT DETAILS
 - Cell Lines
 - Primary Cells
 - Balb/c Mice
- METHOD DETAILS
 - Proteasome Subunit Selective Inhibitors
 - Activity-Based Probes Labelling
 - In Vivo Drug Distribution
 - Organs and Single Cells Isolation
 - Generation of AMO-1 Ub^{-G76V}-GFP Cells
 - Flow Cytometry
 - Assessment of Cell Viability
- QUANTIFICATION AND STATISTICAL ANALYSIS

SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures and one table and can be found with this article online at <https://doi.org/10.1016/j.chembiol.2018.11.007>.

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AUTHOR CONTRIBUTIONS

A.B. and L.B. performed the experiments, analyzed the data, and designed and wrote the manuscript; M.K. was responsible for primary MM experiments and data; M.M.-L. helped with *in vivo* experiments; J.B. adapted the cells to proteasome inhibitors; B.-T.X., G.d.B., and E.M. synthesized the ABP and selective inhibitors; H.S.O. provided the chemicals; C.D. designed and critically revised the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, Peptides, and Recombinant Proteins		
bortezomib (PS-341)	Selleck Chemicals	S1013
carfilzomib (PR-171)	Selleck Chemicals	S2853
marizomib (NPI-0052)	Adipogen	AG-CN2-0444-C100
delanzomib (CEP-18700)	Selleck Chemicals	S1157
oprozomib (ONX0912)	Selleck Chemicals	S7049
ixazomib (MLN9708)	Selleck Chemicals	S2181
LU-001i (β 1i selective inhibitor)	(de Bruin et al., 2014) Leiden University	N/A
NC001 (β 1c/i selective inhibitor)	(van der Linden et al., 2012) Leiden University	N/A
LU-002i (β 2i selective inhibitor)	(de Bruin et al., 2016) Leiden University	N/A
LU-002c (β 2c selective inhibitor)	(de Bruin et al., 2016) Leiden University	N/A
LU-025c (β 5c selective inhibitor)	(de Bruin et al., 2014) Leiden University	N/A
LU-015i (β 5i selective inhibitor)	(de Bruin et al., 2016) Leiden University	N/A
LU-102 (β 2c/i selective inhibitor)	(Geurink et al., 2013) Leiden University	N/A
NC005 (β 5c/i selective inhibitor)	(Britton et al., 2009) Leiden University	N/A
Activity based probes	(de Bruin et al., 2016) Leiden University	N/A
MVB003	(Kolodziejek et al., 2011) Leiden University	N/A
Experimental Models: Cell Lines		
AMO-1	DSMZ	ACC538
RPMI-8226	ATCC	CCL-155
L363	DSMZ	ACC 49
Experimental Model: Organisms/Strains		
Balb/cJ	Jackson laboratories	000651
Critical Commercial Assays		
CellTiter 96® AQueous One Solution	Promega	G3581
MethoCult	StemCell Technologies	H4434
Recombinant DNA		
Ub ^{G76V} -GFP	(Dantuma et al., 2000)	Addgene plasmid #11941
Software and Algorithms		
FlowJo v10 Software	FlowJo Company	www.flowjo.com
BD FACSDIVA Software	BD Biosciences	www.bdbiosciences.com/us/instruments/research/software/flow-cytometry-acquisition/bd-facsdiva-software/m/111112/overview
Fiji package of Image J	(Schindelin et al., 2012)	fiji.sc
GraphPad Prism v.5	GraphPad Software	www.graphpad.com/scientific-software/prism
Fusion Solo S Western Blot and Chemi Imaging System	Vilber Lourmat	www.vilber.com/fusion-solo-s

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Lenka Besse (lenka.besse@kssg.ch).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Cell Lines

Cell lines RPMI-8226, AMO-1 and L363 were obtained from commercial sources (American Type Culture Collection, ATCC, Wesel, Germany; Deutsche Sammlung von Mikroorganismen und Zellkulturen, DSMZ, Braunschweig, Germany) and were maintained under standard conditions in RPMI-1640 medium (Sigma-Aldrich, MO, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 µg/ml streptomycin and 100 U/ml penicillin (Sigma-Aldrich, MO, USA). Cells were adapted to bortezomib (AMO-BTZ, RPMI-BTZ and L363-BTZ) and carfilzomib (AMO-CFZ, RPMI-CFZ and L363-CFZ) by continuous exposure to increasing drug concentrations as previously described (Soriano et al., 2016). Cell lines were routinely tested for mycoplasma contamination using the MycoAlert™ Mycoplasma Detection Kit (Lonza, Switzerland) and STR-typed to confirm the authenticity of the derived cell line with parental cell lines (Braunschweig, Germany).

Primary Cells

Primary cells were obtained from relapsed, pretreated MM patients during routine diagnostic procedures after approval by the independent cantonal ethical committee and after obtaining written informed consent form. Where necessary, primary cells were enriched by Ficoll density gradient centrifugation. Primary cell preparations were analyzed microscopically after routine staining and only preparations with > 80% malignant cells were used for experiments described here. Patients' baseline characteristic is as follows.

Sample	Age	Sex	Number of Previous Treatment Lines	PCs Site	Previously Refractory to BTZ	Previously Refractory to CFZ	Extramedullary Manifestation	Primary PCL	Secondary PCL
MM1	51	F	3	Peripheral blood	yes	yes	yes	no	yes
MM2	74	F	5	Bone marrow	yes	yes	no	no	no
MM3	61	M	0	Pleural effusion	no	no	yes	no	no

Balb/c Mice

Age-matched (8–10 weeks old) female Balb/c mice were obtained from Charles River, Germany, and kept in isolated ventilated cages with food *ad libitum*. The study was carried out in accordance with the 3Rs principle. Experiments were approved by the Committee for Animal Experiments (St Gallen, Switzerland), application no. 26311.

METHOD DETAILS

Proteasome Subunit Selective Inhibitors

For subunit-selective proteasome inhibition, following concentration of chemicals synthesized at the Leiden Institute of Chemistry, Leiden University, were used:

Selective Inhibitor	Target	Concentration
LU001i	β1i	2µM
NC001	β1c/i	10µM
LU002i	β2i	2µM
LU002c	β2c	6µM
LU025c	β5c	3µM
LU015i	β5i	3µM
LU102	β2c/i	3µM
NC005	β5c/i	5µM

Activity-Based Probes Labelling

Activity of proteasome subunits 1h after treatment with proteasome inhibitors was assessed using the recently developed set of subunit-selective activity based probes (ABP) that differentially visualize individual activities of β1, β2 and β5 subunits of the constitutive and immunoproteasome (de Bruin et al., 2016). Briefly, cellular pellets were lysed with a lysis buffer (1 mM DTT, 5 mM MgCl₂, 10% glycerol, 50 mM Tris-HCl, 2 mM ATP, 0.2% NP-40, 0.5% digitonin, adjusted pH for 7.4) for 10 min/4°C for protein extraction.

Protein content of each sample was measured using Bradford method (Roti-Nanoquant, Carl Roth, Germany) and proteins were adjusted for 30 μ g/9.5 μ l per sample with dilution buffer (1 mM DTT, 5 mM $MgCl_2$, 10% glycerol, 50 mM Tris-HCl, 2 mM ATP) and incubated with 0.5 μ l 20x ABP cocktail (final concentration of compounds is: 100 nM Cy5-NC001, 100 nM BODIPY(TMR)-NC005-VS and 30 nM BODIPY(FL)-LU112) for 1h at 37°C. Then, proteins were denatured by 2 min incubation at 95°C, loaded on a gel, separated by SDS-PAGE and visualized by Fusion Solo S Western Blot and Chemi Imaging System (Vilber Lourmat, France).

In Vivo Drug Distribution

Balb/c mice were injected i.v. or i.p. with bortezomib (1 mg/kg) and carfilzomib (4 mg/kg) or with vehicle. Two hours after the drug administration, mice were sacrificed by cervical dislocation and the organs (brain, heart, lungs, spleen, liver, kidneys, bone marrow and PBMC) were isolated.

Bortezomib

Bortezomib was dissolved in DMSO to achieve 2.6 mM stock solution. Out of the stock solution we used 10 μ l for 10g of mouse body weight, which was diluted in PBS to achieve 200 μ l of final volume per 1 mouse.

Carfilzomib

Carfilzomib was dissolved in DMSO to achieve 5.6 mM stock solution. Out of the stock solution we used 10 μ l for 10g of mouse body weight, which was diluted in the vehicle to achieve 200 μ l of final volume per 1 mouse.

Vehicle: 10 % Captisol (sulfobutylether- β -cyclodextrin; Cydex pharmaceuticals, Lenexa, Kansas, USA) in 10 mM sodium citrate in PBS (pH 3.5).

Organs and Single Cells Isolation

Mice organs were isolated into cold PBS supplemented with 2mM EDTA. The organs were cut into pieces and passed through the mash/BD cell strainer (40 μ m) into 50 ml falcon tubes. Cell suspension was centrifuged at 250g/2min, then suspended in cold Red Blood Cell lysis buffer (BD Biosciences, CA, USA) and incubated for 3 min at RT. This was followed by next round of centrifugation at 250g/2 min until the cell pellet was white, without residual red blood cells. Pellet was washed with cold PBS and then processed for protein extraction and ABP labelling as described above. Bone marrow was isolated into the Eppendorf tubes as described following the protocol (Amend et al., 2016). Next steps from the lysis of red blood cells were the same as described above.

Generation of AMO-1 Ub^{-G76V}-GFP Cells

AMO-1 cells were electroporated with a vector containing Ub^{-G76V}-GFP (obtained from Nico Dantuma; Addgene plasmid #11941) using Gene Pulser Electroporator system (Bio-Rad, CA, USA) and selected using G418 (500ng/ml, Gibco/Invitrogen, MA, USA). The polyclonal culture was subcloned to obtain a single cell derived population using MethoCult (StemCell Technologies, Köln, Germany) and the colony with highest accumulation of fluorescence after PI treatment was chosen for further analyses. Briefly, single-cell derived cell populations were incubated with 100 nM bortezomib for 8h and compared for the intensity of Ub^{-G76V}-GFP by flow cytometry, the population with the highest mean fluorescence intensity was chosen for further experiments.

Flow Cytometry

The functional evaluation of ATP binding cassette ABCB-type activity was performed as described previously (Besse et al., 2018). Briefly, 3×10^5 /ml cells were seeded and treated with ABCB-type inhibitor reserpine (10 μ M) for 3h, then the cells were incubated with 1 μ M MVB003 (an epoxyketone-based pan-reactive probe that was used previously as an ABCB1 substrate) for 30 min/37°C, followed by washing with PBS and analysis by flow cytometer BD Fortessa (BD Biosciences, CA, USA).

For the functional proteasome inhibition, accumulation of GFP fluorescence in AMO-1-Ub^{-G76V}-GFP cells after the treatment was assessed. Cells were treated as indicated for 1h with respective proteasome inhibitors, and GFP fluorescence was acquired after 8h incubation in drug-free media by BD Fortessa flow cytometer (BD Biosciences, CA, USA).

Assessment of Cell Viability

Viability of cells was determined after 48h of treatment by MTS tetrazolium compound using CellTiter 96® Aqueous One Solution (Promega, WI, USA) according to manufactures protocol. For the experiments 1×10^4 cells/well in 96-well plates were seeded.

QUANTIFICATION AND STATISTICAL ANALYSIS

Flow cytometry data were evaluated using FlowJo v10 Software (FlowJo Company, Ashland, OR, USA) and are presented as a mean and $\pm$ SD of median fluorescence intensity (MFI) of at least 3 independent experiments. Gel images were analyzed by Fiji (open source image processing package based on ImageJ) (Schindelin et al., 2012) and are presented as a mean $\pm$ SD of three replicates.

Statistical evaluation was performed in GraphPad Prism v.5 (GraphPad Software, La Jolla, CA, USA). For comparison of three or more groups, one-way ANOVA was used with Tukey post-test, for comparison of two groups unpaired *t*-test was used, values $p < 0.05$ were considered as statistically significant. The coefficient of drug interaction (CDI) was determined as described previously (Li et al., 2016).

5.2 Annex 2

High-dose carfilzomib achieves superior anti-tumor activity over low-dose and recaptures response in relapsed/refractory multiple myeloma resistant to low-dose carfilzomib by co-inhibiting the $\beta 2$ and $\beta 1$ subunits of the proteasome complex

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Abstract

Optimal carfilzomib dosing is a matter of debate. We analyzed the inhibition profiles of proteolytic proteasome subunits $\beta 5$, $\beta 2$ and $\beta 1$ after low-dose (20/27 mg/m²) versus high-dose (≥ 36 mg/m²) carfilzomib in 103 pairs of peripheral blood mononuclear cells from patients with relapsed/refractory (RR) multiple myeloma (MM). $\beta 5$ activity was inhibited (median inhibition $>50\%$) *in vivo* by 20 mg/m², whereas $\beta 2$ and $\beta 1$ were co-inhibited only by 36 and 56 mg/m², respectively. Co-inhibition of $\beta 2$ ($P=0.0001$) and $\beta 1$ activity ($P=0.0005$) differed significantly between high-dose and low-dose carfilzomib. Subsequently, high-dose carfilzomib showed significantly more effective proteasome inhibition than low-dose carfilzomib *in vivo* ($P=0.0003$). We investigated the clinical data of 114 patients treated with carfilzomib combinations. High-dose carfilzomib demonstrated a higher overall response rate ($P=0.03$) and longer progression-free survival (PFS) ($P=0.007$) than low-dose carfilzomib. Therefore, we escalated the carfilzomib dose to ≥ 36 mg/m² in 16 patients who progressed during low-dose carfilzomib-containing therapies. High-dose carfilzomib recaptured response ($\geq$ partial remission) in nine (56%) patients with a median PFS of 4.4 months. Altogether, we provide the first *in vivo* evidence in RRMM patients that the molecular activity of high-dose carfilzomib differs from that of low-dose carfilzomib by co-inhibition of $\beta 2$ and $\beta 1$ proteasome subunits and, consequently, high-dose carfilzomib achieves a superior anti-MM effect than low-dose carfilzomib and recaptures the response in RRMM resistant to low-dose carfilzomib. The optimal carfilzomib dose should be ≥ 36 mg/m² to reach a sufficient anti-tumor activity, while the balance between efficacy and tolerability should be considered in each patient.

Introduction

The proteasome is a multi-subunit complex that is responsible for intracellular protein degradation. Only three proteasome subunits harbor proteolytic activity, $\beta 1$ (caspase-like), $\beta 2$ (trypsin-like), and $\beta 5$ (chymotrypsin-like), which cleave peptide bonds C-terminally of acidic, basic,

and hydrophobic amino acid residues, respectively.¹ Currently, proteasome inhibition is a major treatment strategy for multiple myeloma (MM).² All currently approved proteasome inhibitors (PI) primarily target the rate-limiting $\beta 5$ subunit.³ Carfilzomib (CFZ), a second-generation epoxyketone-based PI selectively targets the $\beta 5$ subunit at low concentrations but co-inhibits the $\beta 2$ and $\beta 1$ subunits

only at high concentrations, which subsequently enhances the cytotoxic activity against MM *in vitro*.⁴ CFZ-containing therapies have shown outstanding anti-MM activity in patients with relapsed/refractory (RR) MM.⁵⁻¹⁰ As of August 2022, CFZ has been approved for RRMM in various combination regimens, such as Kd (CFZ and dexamethasone), KRD (CFZ, lenalidomide, and dexamethasone), and D-Kd (daratumumab, CFZ, and dexamethasone).¹¹ However, with the approved dose ranging from low-dose (20-27 mg/m²) to high-dose (up to 70 mg/m² once weekly or twice weekly), optimal CFZ dose is still a matter of debate. In clinical practice, the relationship between CFZ dose and inhibition profiles of proteasome subunits is largely unknown. Moreover, it remains to be explored whether CFZ dose escalation may recapture the clinical response in RRMM patients progressing under low-dose CFZ-containing treatments. Furthermore, real-world data on high-dose CFZ are still very limited for the currently approved Kd, KRD, and D-Kd combination regimens. Therefore, the aim of the current study was to address these issues by analyzing the inhibition profiles of proteolytic proteasome subunits β 1, β 2, and β 5 in RRMM treated with different CFZ doses and to investigate the clinical efficacy and safety of high-dose CFZ in RRMM treated with Kd, KRD, and D-Kd combinations. In addition, we aimed to evaluate CFZ dose escalation treatment in patients with RRMM resistant to low-dose CFZ-containing treatments.

Methods

Patients

First, 103 pairs of peripheral blood mononuclear cell (PBMC) samples were collected before and 3 hours after CFZ treatment from RRMM patients (defined by the current International Myeloma Working Group guidelines¹²) and were included in the study. The patient cohort characteristics are presented in Table 1. Second, the clinical data of 114 RRMM patients treated with CFZ combination regimens (Kd, KRD, and D-Kd) were investigated. The patient characteristics are summarized in Table 2. Third, the clinical data of 16 heavily pretreated RRMM patients, in whom we escalated CFZ dosing due to progression during low-dose CFZ-containing therapy, were analyzed. The characteristics of these patients and their CFZ-containing treatment regimens are shown in Table 3 and the *Online Supplementary Table S1*. Patient demographics, MM-related data, therapy responses, adverse events (AE), and survival outcomes were investigated. AE during treatment were classified according to the Common Terminology Criteria for Adverse Events (CTCAE) version 5.0. All procedures were performed in accordance with the Declaration of Helsinki and the national ethical standards. Informed consent was obtained from all patients included in the study.

Sampling and sample preparation

Proteasome inhibition in PBMC largely mirrors proteasome inhibition in plasma cells *in vivo*.^{4,13} Therefore, we analyzed proteasome inhibition in PBMC of RRMM patients in our study. The detailed description of sampling and sample preparation is provided in the *Online Supplementary Appendix*.

Table 1. Characteristics of 103 relapsed/refractory multiple myeloma patients treated with respective carfilzomib doses.

Parameter	
Patients, N	103
Sex, N (%)	
Male	55 (53.4)
Female	48 (46.6)
Age in years at sampling (range)	64 (41-83)
Subtype, N (%)	
IgG	61 (59.2)
Non-IgG	30 (29.1)
LC	12 (11.7)
EMD, N (%)	47 (45.6)
EMD adjacent to bone	13 (12.6)
EMD without bone contact	34 (33.0)
Cytogenetics, N (%)	
High risk [#]	59 (57.3)
Standard-risk	44 (42.7)
Prior lines of therapies at sampling, N(%)	
1-3	50 (48.6)
4-6	30 (29.1)
>6	23 (22.3)
Drug exposure at sampling, N (%)	
IMiDs	
Lenalidomide	100 (97.1)
Pomalidomide	72 (69.9)
PI	
Bortezomib	89 (86.4)
Carfilzomib	97 (94.2)
Monoclonal antibody	
Elotuzumab	37 (35.9)
Daratumumab	87 (84.5)
Prior SCT	
Autologous SCT	85 (82.5)
Allogeneic SCT	14 (13.6)
BCMA-directed therapy	
ADC	3 (2.9)
CAR T cell	5 (4.9)
Bispecific antibody	2 (1.9)
CFZ dosing, N (%)	
20 mg/m ²	23 (22.3)
27 mg/m ²	27 (26.2)
36 mg/m ²	38 (36.9)
56 mg/m ²	15 (14.6)

ADC: antibody drug conjugate; BCMA: B-cell maturation antigen; CAR T cell: chimeric antigen receptor modified T cell; CFZ: carfilzomib; EMD: extramedullary disease; ImiD: immunomodulatory drug; LC: light chain; PI: proteasome inhibitor; RRMM: relapsed/refractory multiple myeloma; SCT: stem cell transplant; [#]defined as the presence of at least one of the following: t(4;14), t(14;16), t(14;20), del17p and amp1q21.

Table 2. Characteristics of 114 relapsed/refractory multiple myeloma patients treated with Kd, KRD or D-Kd.

Basic characteristics					
Parameter	All	Kd	KRD	D-Kd	P
Patients, N	114	33	71	10	
Sex, N (%)					0.31
Male	76 (66.7)	17 (51.5)	51 (71.8)	8 (80.0)	
Female	38 (33.3)	16 (48.5)	20 (28.2)	2 (20.0)	
Age in years at diagnosis of MM, median (range)	58 (34-81)	58 (45-73)	57 (34-81)	56 (44-71)	0.69
Age in years at Kd/KRD/D-Kd, median (range)	63 (39-83)	65 (54-76)	62 (39-83)	63 (52-80)	0.33
Subtype, N (%)					0.12
IgG	68 (59.6)	22 (66.7)	41 (57.8)	5 (50.0)	
non-IgG	32 (28.1)	8 (24.2)	19 (26.8)	5 (50.0)	
LC	14 (12.3)	3 (9.1)	11 (15.5)	0 (0.0)	
Cytogenetics, N (%)					0.21
High-risk [#]	63 (55.2)	18 (54.5)	28 (39.4)	7 (70.0)	
Standard-risk	51 (44.8)	15 (45.5)	43 (60.6)	3 (30.0)	
Prior lines of therapy, N (%)					0.22
1-3	72 (63.1)	17 (51.5)	48 (67.6)	7 (70.0)	
4-6	28 (24.6)	9 (27.3)	17 (23.9)	2 (20.0)	
>6	14 (12.3)	7 (21.2)	6 (8.5)	1 (10.0)	
Pretreatment prior to the current line of therapy, N (%)	Exposed/Refractory	Exposed/Refractory	Exposed/Refractory	Exposed/Refractory	
IMiDs	80 (70.2)/ 50 (43.9)	28 (84.8)/19 (57.6)	43 (60.6)/26 (36.6)	9 (90.0)/5 (50.0)	0.15/0.25
Lenalidomide	80 (70.2)/40 (35.1)	28 (84.8)/13 (39.4)	43 (60.6)/23 (32.4)	9 (90.0)/4 (40.0)	0.15/0.24
Pomalidomide	38 (33.3)/34 (29.9)	16 (48.5)/14 (42.4)	17 (23.9)/15 (21.1)	5 (50.0)/5 (50.0)	0.38/0.39
PI	106 (92.9)/53 (46.5)	28 (84.8)/17 (51.5)	68 (95.8)/32 (45.1)	10 (100.0)/4 (40.0)	0.48/0.08
Bortezomib	104 (91.2)/49 (42.9)	27 (81.8)/16 (48.5)	67 (94.4)/30 (42.3)	10 (100.0)/3 (30.0)	0.47/0.11
Carfilzomib	18 (15.8)/12 (10.5)	6 (18.2)/3 (9.1)	8 (11.3)/5 (7.0)	4 (40.0)/4 (40.0)	0.46/0.49
Monoclonal antibodies					
Daratumumab	32 (28.1)/30 (26.3)	9 (27.3)/9 (27.3)	17 (23.9)/16 (22.5)	6 (60.0)/5 (60.0)	0.36/0.36
Elotuzumab	18 (15.8)/12 (10.5)	7 (21.2)/4 (12.1)	10 (14.1)/7 (9.9)	1 (10.0)/1 (10.0)	0.40/0.43
Prior SCT, N(%)					
Autologous SCT	100 (87.7)	29 (87.9)	62 (87.3)	9 (90.0)	0.40
Allogeneic SCT	6 (5.3)	0 (0.0)	6 (8.5)	0 (0.0)	0.43
Treatment response and toxicity					
Parameter	All	Kd	KRD	D-Kd	P
Number of cycles, median (range)		3 (1-10)	3 (1-20)	3 (1-5)	0.11
Maximum CFZ dose, N (%)					0.35
15 mg/m ²	5 (4.4)	2 (6.1)	3 (4.2)	0 (0.0)	
20 mg/m ²	8 (7.0)	4 (12.1)	3 (4.2)	1 (10.0)	
27 mg/m ²	64 (56.1)	7 (21.2)	54 (76.1)	3 (30.0)	
36 mg/m ²	9 (7.9)	1 (3.0)	7 (9.9)	1 (10.0)	
45 mg/m ²	1 (0.9)	0 (0.0)	1 (1.4)	0 (0.0)	
56 mg/m ²	27 (23.7)	19 (57.6)	3 (4.2)	5 (50.0)	
Maximum LEN dose (QD), N (%)					
5 mg		/	8 (11.3)	/	
10 mg		/	10 (14.1)	/	
15 mg		/	13 (18.3)	/	
20 mg		/	2 (2.8)	/	
25 mg		/	38 (53.5)	/	
Best response, N (%)					0.04
CR	7 (6.1)	1 (3.0)	4 (5.6)	2 (20.0)	
VGPR	29 (25.4)	4 (12.1)	24 (33.8)	1 (10.0)	
PR	36 (31.6)	11 (33.3)	22 (31.0)	3 (30.0)	
MR	19 (16.7)	8 (24.3)	10 (14.1)	1 (10.0)	
PD	21 (18.4)	8 (24.3)	10 (14.1)	3 (30.0)	
na	2 (1.8)	1 (3.0)	1 (1.4)	0 (0.0)	

Continued on following page.

Treatment response and toxicity					
Parameter	All	Kd	KRD	D-Kd	P
Non-hematologic toxicities grade ≥3, N (%)					
Cardiotoxicity	11 (9.6)	5 (15.2)	6 (8.5)	/	
Pneumonia	5 (4.4)	/	5 (7.0)	/	
Fever of unknown origin	3 (2.6)	3 (9.1)	/	/	
Respiratory infection	11 (9.6)	5 (15.1)	6 (8.5)	/	
Fatigue	2 (1.8)	2 (6.1)	/	/	
Renal failure	4 (3.5)	1 (3.0)	3 (4.2)	/	
Thromboembolic events	1 (0.9)	1 (3.0)	/	/	
Catheter associated infection	1 (0.9)	1 (3.0)	/	/	
Liver enzyme elevated	2 (1.8)	/	2 (2.8)	/	
Bacterial meningitis	1 (0.9)	/	1 (1.4)	/	
Delirium	1 (0.9)	/	1 (1.4)	/	
Infection of urinary tract	2 (1.8)	/	1 (1.4)	1 (10.0)	
Herpes zoster	1 (0.9)	/	1 (1.4)	/	
Soft tissue infection	1 (0.9)	/	/	1 (10.0)	
Hematologic toxicities grade ≥3, N (%)					
Anemia	29 (25.4)	6 (18.2)	19 (26.8)	4 (40.0)	0.29
Leukopenia	34 (29.8)	7 (21.2)	21 (29.6)	6 (60.0)	0.30
Thrombocytopenia	37 (32.4)	14 (42.4)	18 (25.4)	5 (50.0)	0.35
Neutropenia	30 (26.3)	2 (6.1)	23 (32.4)	5 (50.0)	0.30

CFZ: carfilzomib; CR: complete remission; D-Kd: daratumumab, carfilzomib, dexamethasone; ImiDs: immunomodulatory drugs; Kd: carfilzomib, dexamethasone; KRD: carfilzomib, lenalidomide, dexamethasone; LC: light chain; LEN: lenalidomide; MM: multiple myeloma; MR: minor response; na: not available; PD: progressive disease; PI: proteasome inhibitors; PR: partial remission; SCT: stem cell transplant; VGPR: very good partial remission; #defined as presence of at least one of the following: del(17p), amp1q21, t(4;14), t(14;16) and t(14;20). IgG: immunoglobulin G.

Proteasome β-subunits profiling with activity-based proteasome probes labeling

Proteasome subunit activity was assessed using protein lysate from PBMC. Briefly, PBMC pellets were lysed with a lysis buffer. The lysates were then labeled for 1 hour at 37°C with subunit-selective, fluorescent, activity-based proteasome probes (ABPP) that visualized β1, β2, and β5 activity of the constitutive proteasome and immune proteasomes as previously described by de Bruin *et al.*¹⁴ (*Online Supplementary Figure S1*), and proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). In order to limit variability, samples from respective patients were always run on the same gel and to minimize differences in gel exposure, a sample prepared from a pool of PBMC obtained from healthy donors was run on each gel. After SDS-PAGE, gel images were acquired using Fusion Solo S Western Blot and Chemi Imaging System (Vilber Lourmat, Collégien, France). Active proteasome subunits were quantitatively assessed in each sample by densitometry using Fiji (an open-source image processing package based on ImageJ)¹⁵ and normalized to a fluorescence intensity obtained from the PBMC sample on each gel. For each sample, the activity of each proteolytically active β subunit was calculated by summarizing the normalized band fluorescence intensity of the respective constitutive (c) proteasome subunit and the corresponding subunit of the immuno (i) proteasomes (i.e., β1c+i, β2c+i, and β5c+i). The

inhibition of subunit activity after CFZ exposure in relation to a paired sample before CFZ exposure was calculated for each individual patient (Figure 1A, B). Total proteasome activity was defined as the average activity of the β1c+i, β2c+i, and β5c+i proteasome subunits. For further analysis, we dichotomized the patients into two groups: low-dose (≤27 mg/m²) and high-dose (≥36 mg/m²) CFZ.

Kd, KRD, and D-Kd regimens

A detailed description of the drug administrations and treatment regimens is provided in the *Online Supplementary Appendix*.

Statistical evaluation

Statistical analyses were performed using GraphPad Prism 9.0 (GraphPad Software Inc., San Diego, CA, USA). Statistical significance was set at 0.05 (*P* value <0.05). A more specific description of the statistical evaluation used in this study is provided in *Online Supplementary Appendix*.

Results

Inhibition profile of proteolytic proteasome subunits at different carfilzomib doses in relapsed/refractory multiple myeloma patients

CFZ has a very short half-life of <30 minutes (min) and

reaches full proteasome inhibition within 60-90 min after CFZ treatment in peripheral tissues *in vivo*.¹⁶ Additionally, *in vitro*, proteasome activity recovers to baseline after 24 hours from CFZ treatment and remains largely constant at later time points.⁴ Therefore, we investigated proteolytic proteasome subunit activity in PBMC before and 3 hours after CFZ treatment, and we combined the activity of constitutive (c) and immuno-proteasome (i), i.e., β 1c+i, β 2c+i, and β 5c+i. In our cohort, 23, 27, 38, and 15 patients received 20, 27, 36, and 56 mg/m² of CFZ, respectively (Table 1). All 103 patients were treated with twice-weekly CFZ regimen, and the PBMC samples were collected on day 1 before and 3 hours after CFZ infusion. Generally, proteasome subunit activity decreased with increasing CFZ dose, with the strongest inhibition of β 5c+i, followed by β 2c+i, and β 1c+i at all dose levels (Figure 1B). Biologically meaningful inhibition (median residual activity <50% of baseline) of β 5c+i was already achieved with 20 mg/m², whereas β 2c+i and β 1c+i activity remained largely unchanged at this dosing level. Meaningful co-inhibition of β 2c+i and β 1c+i was observed only at higher doses of 36 and 56 mg/m², respectively. Interestingly, the active β 2c+i and β 1c+i subunits were moderately upregulated upon β 5c+i inhibition by CFZ only at 20 mg/m², possibly contributing to compensatory activity. In patients treated with 56 mg/m², all β 1c+i, β 2c+i, and β 5c+i were inhibited by >50% compared to baseline before CFZ. We noticed a significant difference in β 2c+i and β 1c+i subunit inhibition between the groups treated with 20 mg/m² versus 36 mg/m² with a median residual activity of β 2c+i of 120.1% (95% confidence interval [CI]: 76.8-174.6) versus 47.2% (95% CI: 31.2-53.8; *P*<0.0001); median residual activity of β 1c+i of 132.8% (95% CI: 62.4-188.0) versus 60.7% (95% CI: 39.3-77.1; *P*=0.0009). The same held true for the groups CFZ treated with 27 mg/m² versus 56 mg/m² with a median residual activity of β 2c+i of 65.9% (95% CI: 32.5-88.4) versus 39.5% (95% CI: 17.8-54.9; *P*=0.035); median residual activity of β 1c+i of 81.2% (95% CI: 53.8-116.9) versus 42.1% (95% CI: 23.8-69.5; *P*=0.017). In contrast, β 5c+i inhibition did not differ significantly between the groups at 20 mg/m² versus 36 mg/m² or 27 mg/m² versus 56 mg/m² (*P*>0.05). In terms of total proteasome inhibition (average of β 1c+i, β 2c+i, and β 5c+i), we observed a significant difference between the groups treated with 20 mg/m² versus 36 mg/m² with a median residual total proteasome activity of 91.7% (95% CI: 56.1-133.1) versus 41.7% (95% CI: 28.2-52.7; *P*=0.0003). Similarly, CFZ treatment with 56 mg/m² showed significantly superior total proteasome inhibition over 27 mg/m² with a median residual total proteasome activity of 55.0% (95% CI: 37.8-86.7) versus 33.2% (95% CI: 23.8-35.7; *P*=0.019). We then dichotomized the CFZ dosing into two groups: low-dose ($\leq$ 27 mg/m²) and high-dose ($\geq$ 36 mg/m²) CFZ. Between both groups, β 2c+i and β 1c+i inhibition significantly differed

Table 3. Characteristics of patients with relapsed/refractory multiple myeloma progressing from low-dose carfilzomib and treated with carfilzomib dose escalation.

Parameter	
Patients, N	16
Sex, N (%)	
Male	10 (62)
Female	6 (38)
Age in years at diagnosis of MM, median (range)	65 (41-79)
Age in years at CFZ dose escalation, median (range)	71 (45-83)
Time in months between diagnosis of MM and CFZ dose escalation, median (range)	61 (22-144)
Subtype, N (%)	
IgG	11 (69)
non-IgG	5 (31)
Cytogenetics, N (%)	
High-risk [#]	11 (69)
Standard-risk	5 (31)
EMD, N (%)	
EMD without bone contact	6 (38)
no EMD	10 (62)
Prior lines of therapy, N (%)	
2-4	5 (31)
5-7	5 (31)
$\geq$ 8	6 (38)
Pretreatment prior to the current line of therapy, N (%)	Exposed/Refractory
IMiDs	15 (94)/13 (81)
Lenalidomide	15 (94)/11 (69)
Pomalidomide	14 (88)/13 (81)
PI	16 (100)/15 (94)
Bortezomib	16 (100)/14 (88)
Carfilzomib (low-dose)	8 (50)/8 (50)
Monoclonal antibodies	
Daratumumab	15 (94)/15 (94)
Elotuzumab	3 (19)/3 (19)
Penta-refractory [†]	6 (38)
Prior SCT	
Autologous SCT	12 (75)
BCMA-directed therapy	
ADC	2 (13)
CAR T cell	2 (13)
BITE	1 (6)
CFZ dose escalation, N(%)	
15→36 mg/m ²	1 (6)
20→56 mg/m ²	1 (6)
27→36 mg/m ²	6 (38)
27→56 mg/m ²	8 (50)
Best response to CFZ dose escalation, N (%)	
VGPR	5 (31)
PR	4 (25)
MR	6 (38)
PD	1 (6)
Non-hematologic toxicities grade $\geq$ 3 after CFZ dose escalation, N(%)	
Pneumonia	4 (25)
Fever of unknown origin	2 (13)
Fatigue	1 (6)
Cardiotoxicity	1 (6)
Corona virus infection HKU1	1 (6)
Renal failure	1 (6)
Hematologic toxicities grade $\geq$ 3 after CFZ dose escalation, N(%)	
Anemia	10 (63)
Leukopenia	10 (63)
Thrombocytopenia	8 (50)
Neutropenia	4 (25)

ADC: antibody drug conjugate; BCMA: B-cell maturation antigen; BITE: bispecific T-cell engager; CAR T cell: chimeric antigen modified T cell; CFZ: carfilzomib; EMD: extramedullary disease; IMiDs: immunomodulatory drugs; MM: multiple myeloma; MR: minor response; PD: progressive disease; PI: proteasome inhibitors; PR: partial remission; RR: relapsed/refractory; SCT: stem cell transplant; VGPR: very good partial remission; IgG: immunoglobulin G; [#]defined as presence of at least one of the following: del(17p), amp1q21, t(4;14), t(14;16) and t(14;20); [†]defined as refractory to bortezomib, carfilzomib, lenalidomide, pomalidomide and daratumumab.

with a median residual activity of β_{2c+i} of 81.9% (95% CI: 63.3-104.6) *versus* 45.5% (95% CI: 26.8-52.8; $P=0.0001$; median residual activity of β_{1c+i} of 92.8% (95% CI: 65.7-127.6) *versus* 51.0% (95% CI: 39.3-69.5; $P=0.0005$). However, high-dose CFZ did not show significantly superior β_{5c+i} inhibition ($P>0.05$) compared to low-dose CFZ. Taken together, high-dose CFZ demonstrated superior total proteasome inhibition compared to low-dose CFZ through

the co-inhibition of β_{2c+i} and β_{1c+i} proteasome subunits activity with a median residual total proteasome activity of 65.8% (95% CI: 47.7-91.8) *versus* 35.7% (95% CI 28.2-43.7; $P=0.0003$) (Figure 2A-E).

High-dose carfilzomib showed more effective anti-multiple myeloma activity than low-dose
In order to address the issue of whether high-dose CFZ

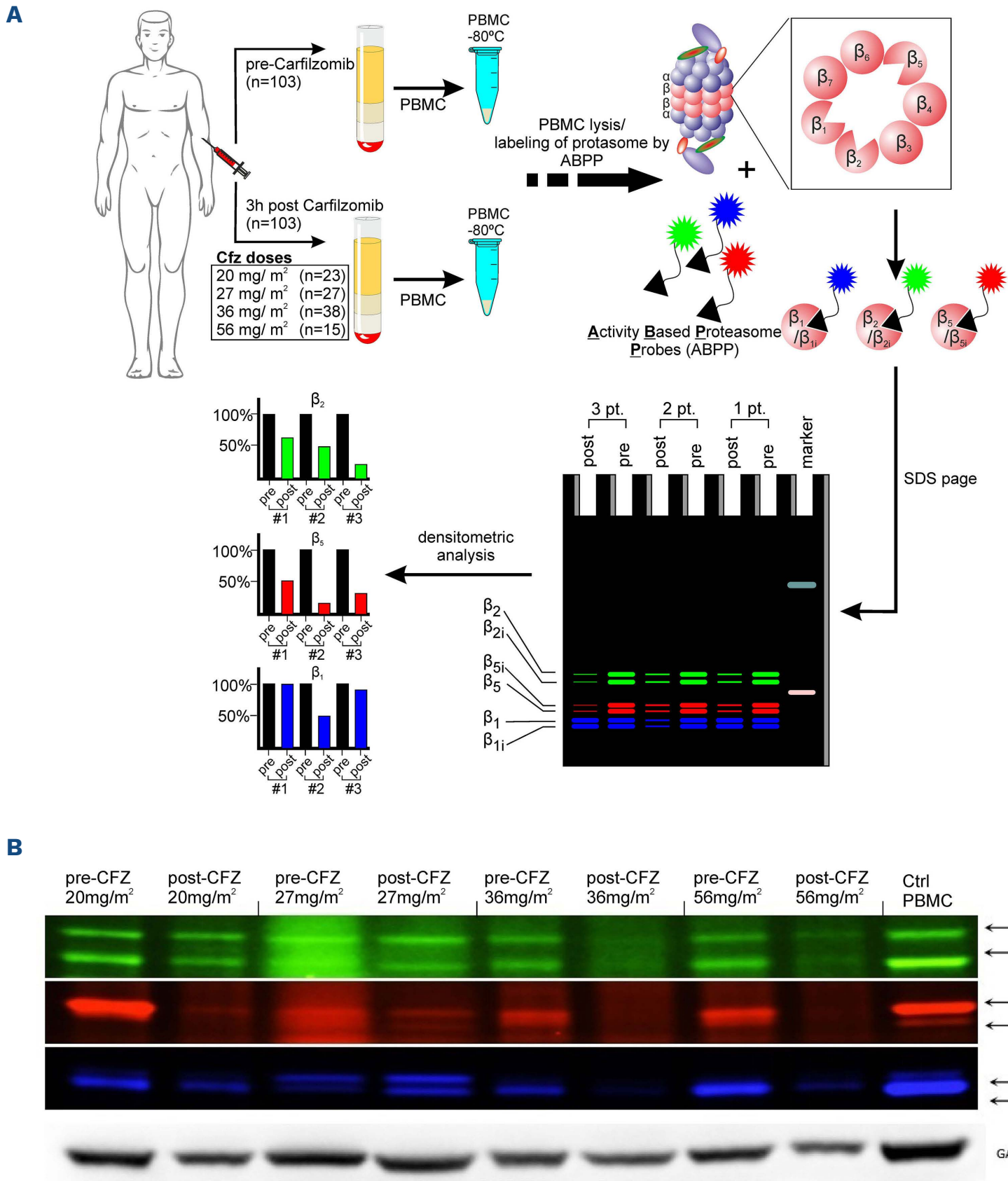


Figure 1. Experimental design and a representative gel image. In (A) the experimental design of our study is depicted. We collected 103 paired peripheral blood mononuclear cell (PBMC) samples (before and 3 hours [h] after carfilzomib [CFZ]) from patients with RRMM. CFZ was given at different doses. PBMC were lysed and then proteolytically active constitutive (c) proteasome and immuno (i) proteasome subunits, β_{1c+i} , β_{2c+i} , and β_{5c+i} were labeled using activity-based proteasome probes. Subsequently, proteins and labeled proteasome subunits were separated with SDS-PAGE, and the β_{1c+i} , β_{2c+i} , and β_{5c+i} activity was evaluated using densitometric analysis. Panel (B) shows a representative gel (SDS-PAGE) with proteasome subunits activity before and 3 h after CFZ.

could achieve more effective anti-MM efficacy and superior progression-free survival (PFS) compared to low-dose CFZ in routine clinical practice, we investigated the real-world data of 114 RRMM patients who were treated with three currently approved CFZ-containing combinations Kd, KRd, and D-Kd. The median age at therapy initiation was 63 years (range, 39-83 years). In our study, 33, 71, and ten patients received Kd, KRd, and D-Kd combinations, respectively. In total, 20, 11 and five patients were treated with high-dose CFZ in subgroups, Kd, KRd, and D-Kd, respectively. The remaining 78 patients received low-dose CFZ in three different combinations. CFZ and/or LEN dosing was individually determined by the treating physician based on the expected tolerability of each pa-

tient. The median number of prior therapies was two (range, 1-12). Patients received a median of three (range, 1-20) cycles of CFZ combinations. Patient characteristics are summarized in Table 2. The median follow-up time was 15.6 months in this cohort. Overall, 72 patients achieved partial remission (PR) or better, yielding an overall response rate (ORR) of 64.3% in 112 patients with response data (Table 2). Notably, in patients treated with the Kd combination, high-dose CFZ showed a significantly higher ORR compared to low-dose CFZ (ORR: 73.8% vs. 15.4%; $P=0.003$) (*Online Supplementary Figure S2*). We then analyzed survival outcome in the entire group and found that patients who had received high-dose CFZ showed significantly superior PFS compared to

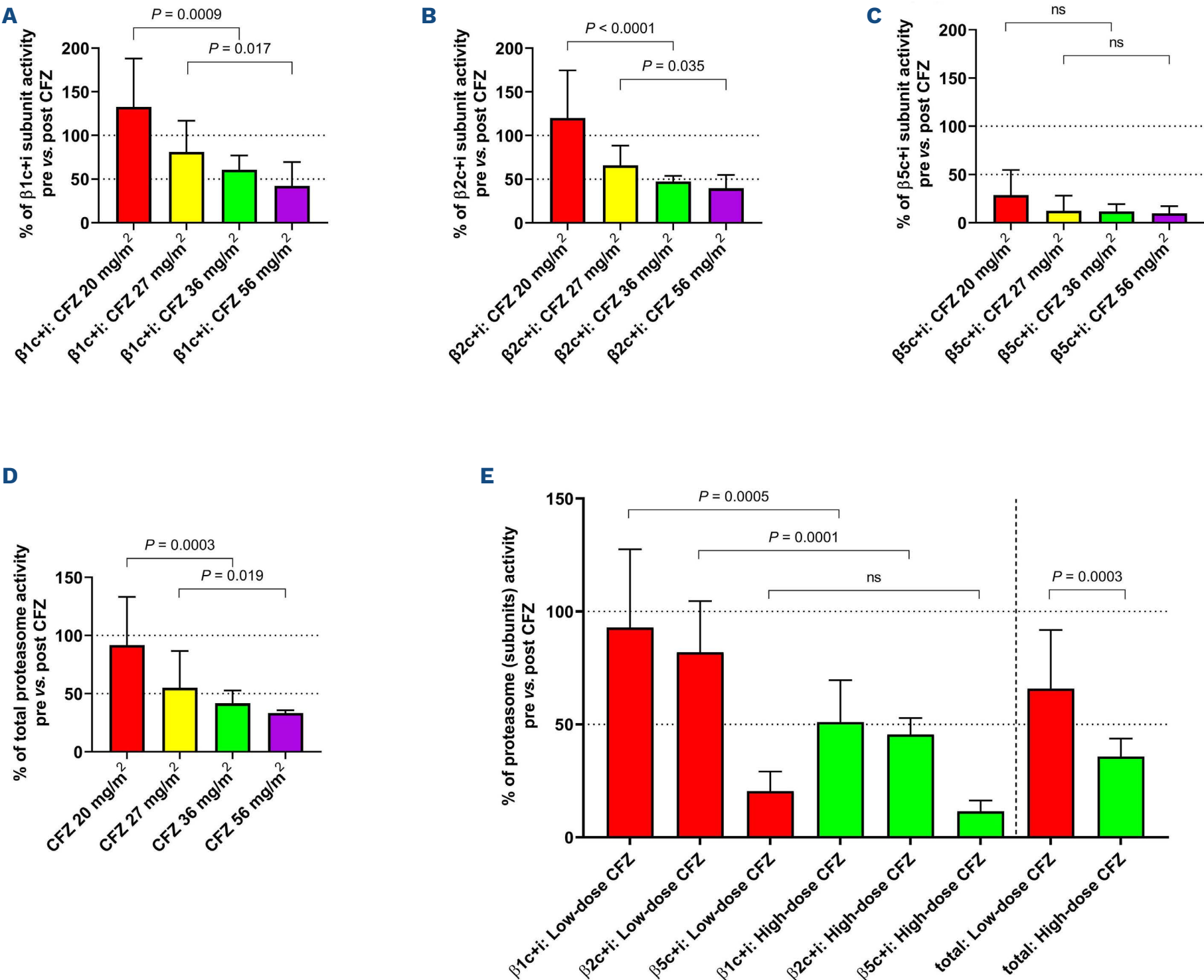


Figure 2. Proteasome subunit activity evaluated in peripheral blood mononuclear cells of multiple myeloma patients undergoing carfilzomib treatment and their outcome upon dose escalation. Panels (A-C) display proteasome subunit activity of β1c+i, β2c+i, and β5c+i pre-carfilzomib (CFZ) vs. 3 hours (h) post CFZ. Panel (D) demonstrates the whole proteasome activity at respective doses prior CFZ vs. 3 h post CFZ treatment. In (E), residual proteasome (subunit) activity 3 h after CFZ infusion is illustrated. ns: not significant; P value of two-tailed unpaired Student's t -test. c: constitutive; i: immuno.

low-dose (median PFS: 11.7 vs. 4.5 months; $P=0.007$) (*Online Supplementary Figure S3*). In the subgroup analysis of Kd, high-dose CFZ likewise demonstrated improved PFS over low-dose (median PFS: 11.7 vs. 2.1 months; $P=0.0006$) (Figure 3A, B). In the D-Kd subgroup, we also observed superior PFS in the high-dose CFZ group than in the low-dose CFZ group. However, owing to the low number of cases in this subgroup, the difference in PFS was not statistically significant. (Figure 3C, D). In patients treated with KRD, PFS was significantly longer in patients who had received high-dose CFZ than low-dose (median PFS: 13.2 vs. 5.6 months; $P=0.02$), while LEN dose did not affect PFS in our cohort. (Figure 4). Overall, the most common non-hematologic AE grade ≥ 3 included cardiotoxicity ($n=11$, 9.6%) and respiratory infections ($n=11$, 9.6%). Importantly, among the 11 patients who suffered from cardiotoxicity ≥ 3 , only two of them received high-dose CFZ, while the remaining nine patients were treated with low-dose CFZ. Regarding hematologic AE, 29 (25.4%), 34 (29.8%), 37 (32.4%) and 30 (26.3%) patients developed anemia, leukopenia, thrombocytopenia, and neutropenia grade ≥ 3 , respectively. Of note, in the entire group, the frequencies of hematologic AE grade ≥ 3 were not significantly higher in patients who received high-dose compared to low-dose CFZ (*Online Supplementary Figure S4*).

Carfilzomib dose escalation recaptured clinical response in relapsed/refractory multiple myeloma patients who were resistant to low-dose carfilzomib

Considering the afore-mentioned findings, we treated 16 patients with RRMM who progressed during low-dose CFZ-containing therapy by escalating CFZ dosing as a personalized treatment approach. The median age of the patients was 71 years (range, 45-83 years), and high-risk cytogenetics was present in 11 (69%) patients. The patients were heavily pretreated with a median of six (range, 2-13) lines of therapies. In prior lines of therapies, all 16 patients received at least one PI, and the vast majority ($n=15$, 94%) was pretreated with at least one immuno-modulatory drug (IMiD). Daratumumab was administered to 15 (94%) patients in prior treatments. All 16 patients were refractory to their last treatment line. Eleven (69%) patients were refractory to LEN, 13 (81%) to pomalidomide, 14 (88%) to bortezomib (BTZ), eight (50%) to low-dose CFZ, and 15 (94%) patients were refractory to daratumumab in prior lines of therapy. Six (38%) patients were penta-refractory (daratumumab, BTZ, low-dose CFZ, LEN, and pomalidomide). One patient had relapsed MM after treatment with a B-cell maturation antigen (BCMA)-targeted bi-specific antibody, and two patients relapsed after chimeric antigen receptor-modified T-cell (CAR T)

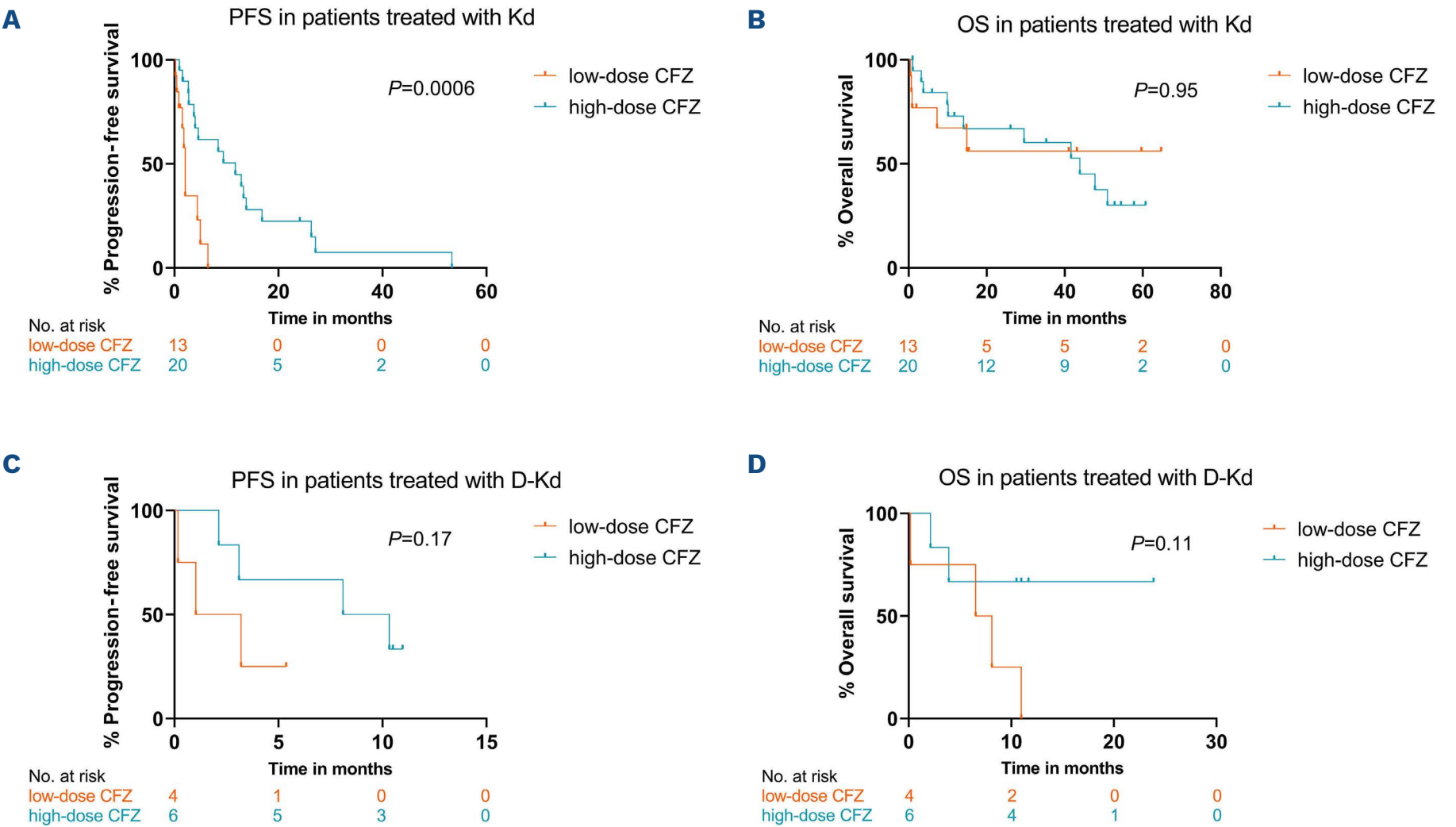


Figure 3. Survival outcome in multiple myeloma patients that received immunomodulatory imide drug-free carfilzomib-containing regimens: Kd and D-Kd. Panels demonstrate progression-free survival (PFS) and overall survival (OS) of patients treated with high-dose carfilzomib (CFZ) vs. low-dose CFZ in the group of patients who (A, B) received Kd (carfilzomib, dexamethasone) and (C, D) received D-Kd (carfilzomib, daratumumab, dexamethasone).

therapy. In the current line of therapy, all 16 patients showed progression during low-dose CFZ-containing combination regimens (range of CFZ dose, 15-27 mg/m² twice weekly), and six patients presented true extramedullary disease (EMD) without bone contact. Therefore, we escalated the CFZ dose in these patients to high-dose (36 or 56 mg/m²), while the doses and schedules of all other anti-MM drugs remained the same (Table 3; *Online Supplementary Table S1*). After a median time to response of 0.7 (range, 0.3-1.1) months, high-dose CFZ recaptured response in nine (56%) patients, including five and four patients with very good partial remission (VGPR) and PR, respectively. Additionally, high-dose CFZ controlled disease progression (minor response) in six (38%) patients, yielding a clinical benefit rate of 94%. Importantly, four of six patients with true EMD achieved a PR (n=2) or VGPR (n=2), and one patient showed a minor response after CFZ dose escalation. This finding underlined that even high-risk RRMM patients with EMD might benefit from CFZ dose escalation. The only patient who progressed after CFZ dose escalation harbored multiple high-risk features, such as high-risk cytogenetics (amp1q21, t(4;14)) and EMD,¹⁷⁻¹⁹ which were potentially associated with aggressive disease and drug resistance, suggesting that CFZ resistance may be related to factors other than CFZ dosing.²⁰

Serial PBMC samples before and 3 hours after CFZ administration at different dose levels were evaluated in a patient with CFZ dose escalation (patient #1 in the *Online Supplementary Table S1*). As expected, the β 2c+i proteasome subunit was inhibited more effectively at 36 mg/m² (residual β 2c+i activity: 52.8%) than at 20 mg/m² (residual β 2c+i activity: 76.5%), whereas the β 5c+i subunit was already meaningfully inhibited at 20 mg/m² (residual β 5c+i activity: 29.1%) (*Online Supplementary Figure S5*). After a median follow-up time of 13.0 months, high-dose CFZ achieved a median PFS of 4.4 (95% CI: 4.0-4.8) months and a median overall survival (OS) of 8.9 (95% CI: 6.0-11.7) months in our cohort of patients progressing under low dose CFZ therapy (Figure 5; *Online Supplementary Figure S6*). However, increased doses of CFZ may cause more severe side effects. Indeed, non-hematologic AE grade $\geq$ 3 were present in six (38%) patients after CFZ dose escalation to high-dose, while only four (25%) patients showed non-hematologic AE grade $\geq$ 3 during low-dose CFZ-containing treatments. Pneumonia (n=4, 25%) was the most common non-hematologic AE observed after CFZ dose escalation. Interestingly, cardiotoxicity grade $\geq$ 3 was observed in only one (6%) patient after CFZ dose escalation. However, two (12%) patients experienced cardiotoxicity grade $\geq$ 3 during the low-dose CFZ phase before dose es-

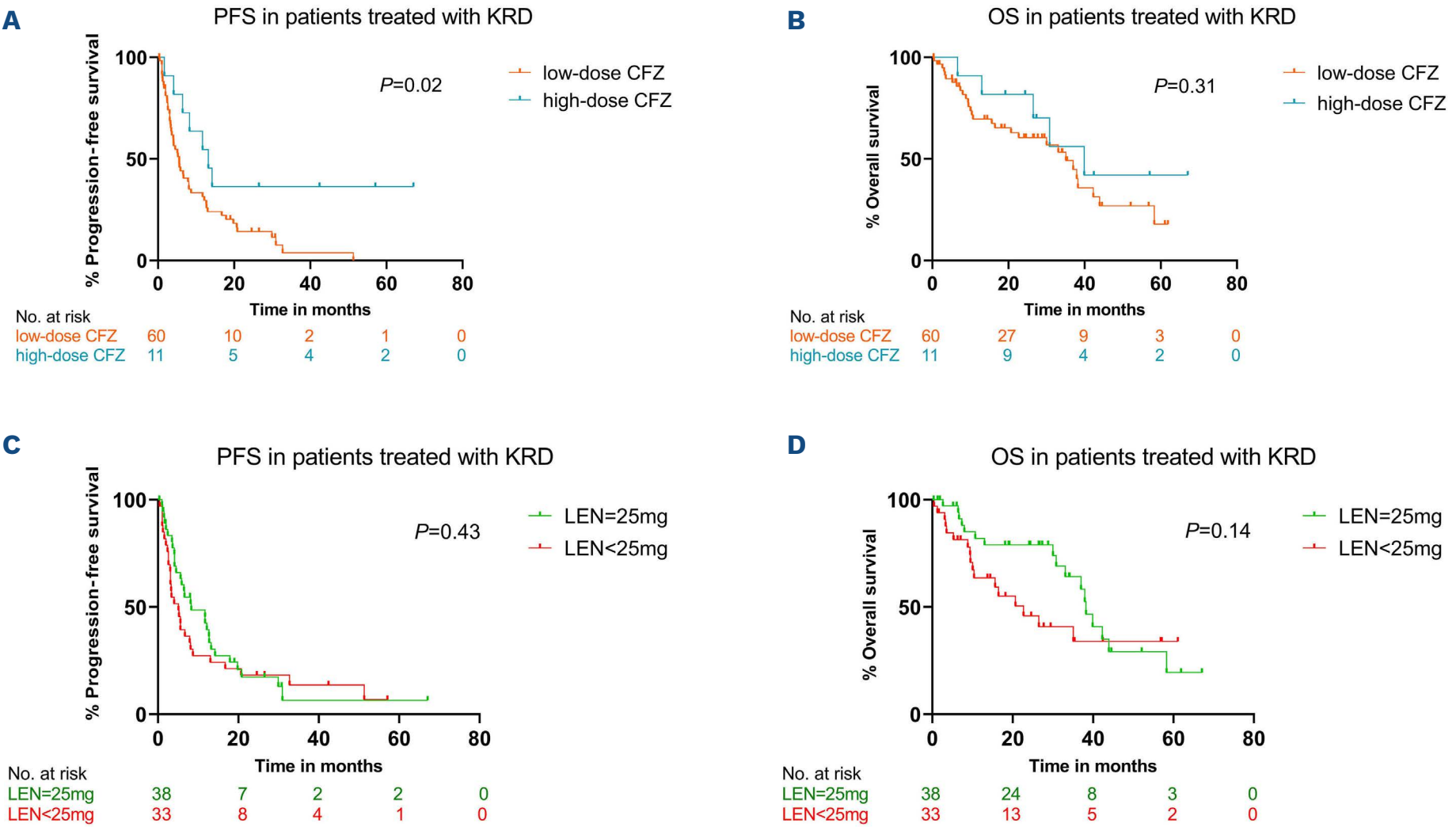


Figure 4. Survival outcome in multiple myeloma patients that received immunomodulatory imide drug-containing carfilzomib-containing regimen. (A) Progression-free survival (PFS) and (B) overall survival (OS) of patients treated with high-dose carfilzomib (CFZ) vs. low-dose CFZ in patients who received KRD (carfilzomib, lenalidomide, dexamethasone). (C, D) PFS and OS of patients who received lenalidomide (LEN) 25 mg vs. < 25 mg.

calation, and both patients tolerated high-dose CFZ without cardiotoxicity. Importantly, two patients who achieved PR with high-dose CFZ required CFZ dose reduction to 27 and 20 mg/m² due to cardiotoxicity and fatigue, respectively. Notable, both patients showed prompt disease progression after the CFZ dose reduction (Online Supplementary Table S1).

Discussion

In our study, high-dose CFZ showed significantly more effective proteasome inhibition than low-dose CFZ *in vivo* by co-inhibiting the β 2 and β 1 proteasome subunits, suggesting that patients resistant to low-dose CFZ-containing therapies could recapture the clinical response by escalating the CFZ dose. Moreover, high-dose CFZ resulted in longer PFS in RRMM patients treated with CFZ-containing combinations. In the last few years, CFZ has been evaluated in different dosing regimens within clinical trials. In the phase III ENDEAVOR study evaluating the Kd combination (CFZ 56 mg/m² twice weekly), CFZ showed a significantly longer PFS compared to BTZ (median: 18.7 months in the Kd group vs. 9.4 months in the BTZ group) in RRMM patients.⁶ Moreover, additional use of daratumumab to the Kd regimen (D-Kd) further improved PFS in RRMM (median: not reached in the D-Kd group vs. 15.8 months in the Kd group), as suggested by the phase III CANDOR trial.⁵ In terms of immunomodulatory imide drugs (IMiD)-containing combinations, the KRD regimen demonstrated superior PFS compared with the control group RD (lenalidomide, dexamethasone) (median: 26.3 months in the KRD group vs. 17.6 months in the RD group). However, only low-dose CFZ (27 mg/m² twice weekly) was administered to avoid severe AE in the KRD combination.⁷ Indeed, at present, the optimal CFZ dose remains controversial in clinical practice.

High-dose once weekly CFZ is being developed as a mainstream regimen to improve patients' compliance with a more convenient proteasome inhibition.^{21,22} In the Kd combination, Moreau *et al.* reported that CFZ 70 mg/m² once weekly significantly improved PFS and ORR compared with 27 mg/m² twice weekly in RRMM.²¹ Moreover, in real-world data, high-dose CFZ (56 mg/m² twice weekly or 70 mg/m² once weekly) likewise showed significantly superior patients survival outcomes when compared to low-dose CFZ (20-27 mg/m² twice weekly) in patients treated with Kd combination.²³ In contrast, Ailawadhi *et al.* did not observe a significant ORR or PFS benefit with twice-weekly CFZ 56 mg/m² over 27 mg/m² in the Kd regimen.¹⁷ Regarding the IMiD-containing CFZ combination, that is, the KRD regimen, our results demonstrate that high-dose CFZ can significantly improve PFS, while the LEN dose does not show any significant impact on patient outcome. Importantly, in different data sets, the safety profile of high-dose CFZ appears similar to that of low-dose CFZ.^{17,21,24,25} Here, we provide the first *in vivo* evidence in a clinical setting that the molecular activity of high-dose CFZ (≥ 36 mg/m²) differs from that of low-dose CFZ (≤ 27 mg/m²) by means of β 2 and β 1 proteasome subunit co-inhibition, which may be a potential mechanism to overcome low-dose drug resistance in RRMM patients. Although the patients in the current study were relatively heterogeneous, including patients treated with Kd, KRD and D-Kd regimens, high-dose CFZ showed improved survival outcome compared with low-dose in the entire cohort as well as in each subgroup Kd, KRD and D-Kd. This is in line with our previous *in vitro* findings⁴ and may explain the superior anti-MM activity of higher doses of CFZ compared to lower doses. PI-resistant cells change the level of the β 2 and β 1 proteasome subunits and become insensitive to sole β 5 inhibition. At the same time, co-inhibition of other subunits, especially the β 2 subunit, is able to overcome PI resistance.^{4,26,27} However, in our study, there were also patients who did not respond

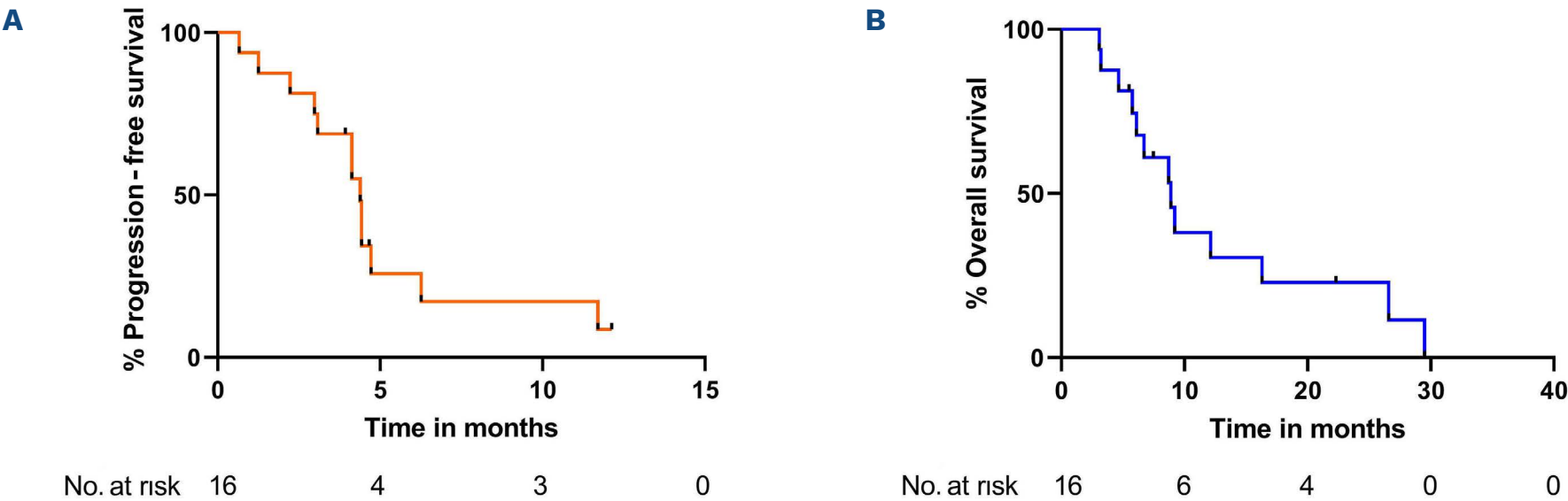


Figure 5. Survival outcome in multiple myeloma patients that received carfilzomib dose escalation. (A) Progression-free survival (PFS) and (B) overall survival (OS) of 16 relapsed/refractory multiple myeloma patients treated with carfilzomib (CFZ) dose escalation.

to CFZ dose-escalation, meaning that high-dose CFZ was not a “game changer” in all patients with heavily pre-treated RRMM. In contrast, disease progression during CFZ-containing treatment may be related to mechanisms other than proteasome inhibition, such as high-risk cytogenetics, EMD, and epigenetic changes.^{18,19,28} The underlying resistance mechanisms should be further investigated. In addition, it could not be excluded that low-dose CFZ might potentially achieve a cumulative proteasome inhibition effect on the second day in twice-weekly regimens, and this issue should be addressed in future studies. In recent years, marizomib, a third generation β -lactone- γ -lactam PI, has been developed for the treatment of RRMM, and this novel agent is characterized by its irreversible inhibition of all three proteolytic subunits β 5, β 2, and β 1 of the proteasome complex.^{29,30} Marizomib has shown high anti-MM activity in RRMM patients and may overcome BTZ and CFZ resistance.^{31,32} Therefore, marizomib-containing regimens might be a further option for patients resistant to low-dose CFZ.

Taken together, high-dose CFZ demonstrates superior anti-MM effect to low-dose CFZ by co-inhibiting β 2 and β 1 proteasome subunits, and resistance to low-dose CFZ does not exclude sensitivity to high-dose CFZ. The optimal CFZ dose should be ≥ 36 mg/m² to achieve sufficient anti-MM activity, while the balance between efficacy and tolerability should be taken into account during treatment decision-making in each patient. In patients with RRMM refractory to low-dose CFZ, dose escalation to ≥ 36 mg/m² may be worthwhile, as suggested by our data. Our findings provide a rationale for selecting high-dose CFZ to achieve

maximum proteasome inhibition, an optimal anti-MM effect, and to avoid adaptive resistance. The mechanisms of toxicity, particularly cardiotoxicity, of high-dose CFZ remain to be addressed. In addition, future work is required to compare the effects of high-dose CFZ between once-weekly and twice-weekly schedules.

Disclosures

No conflicts of interest to disclose.

Contributions

XZ, AB, KMK, CD, LB and LR designed the research. XZ, AB, CV, SN, ET, ES, LH, MML, UM, SH and LB performed the experiments. XZ, JP, MJS, XX, HH, AR, HE, KMK and LR provided patient samples and clinical data. EM, BF and HSO provided the proteasome activity based probes. XZ, AB, CD, LB and LR wrote the manuscript which was approved by all authors and all authors analyzed and interpreted the data.

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Data-sharing statement

The data generated in this study are available upon request from the corresponding author.

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5.3 Annex 3

ORIGINAL ARTICLE

Proteasome inhibitor-adapted myeloma cells are largely independent from proteasome activity and show complex proteomic changes, in particular in redox and energy metabolism

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Adaptive resistance of myeloma to proteasome inhibition represents a clinical challenge, whose biology is poorly understood. Proteasome mutations were implicated as underlying mechanism, while an alternative hypothesis based on low activation status of the unfolded protein response was recently suggested (IRE1/XBP1-low model). We generated bortezomib- and carfilzomib-adapted, highly resistant multiple myeloma cell clones (AMO-BTZ, AMO-CFZ), which we analyzed in a combined quantitative and functional proteomic approach. We demonstrate that proteasome inhibitor-adapted myeloma cells tolerate subtotal proteasome inhibition, irrespective of a proteasome mutation, and uniformly show an 'IRE1/XBP1-low' signature. Adaptation of myeloma cells to proteasome inhibitors involved quantitative changes in > 600 protein species with similar patterns in AMO-BTZ and AMO-CFZ cells: proteins involved in metabolic regulation, redox homeostasis, and protein folding and destruction were upregulated, while apoptosis and transcription/translation were downregulated. The quantitatively most upregulated protein in AMO-CFZ cells was the multidrug resistance protein (MDR1) protein ABCB1, and carfilzomib resistance could be overcome by MDR1 inhibition. We propose a model where proteasome inhibitor-adapted myeloma cells tolerate subtotal proteasome inhibition owing to metabolic adaptations that favor the generation of reducing equivalents, such as NADPH, which is supported by oxidative glycolysis. Proteasome inhibitor resistance may thus be targeted by manipulating the energy and redox metabolism.

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INTRODUCTION

Proteasome inhibition is highly active for the treatment of multiple myeloma (MM).¹ Current proteasome-inhibiting drugs comprise the first-in-class, reversible, boronate-type proteasome inhibitor bortezomib and its oral permutation ixazomib and the approved, irreversible, epoxyketone-type inhibitor carfilzomib, as well as next-generation boronate-, epoxyketone- or β -lactone-type of inhibitors.² Their mechanism of action exploits the highly developed protein biosynthesis machinery of myeloma.³ This extraordinarily active biosynthetic route is controlled by the unfolded protein response (UPR), a complex transcriptional network that balances protein transcription, folding and destruction.⁴ The IRE1/XBP1 pathway, one of the three key regulatory switches to control UPR activity, also guides plasma cell differentiation.^{5,6} MM cells critically rely on timely disposal of misfolded and dysfunctional newly synthesized protein through the endoplasmic reticulum (ER)-associated degradation machinery, of which the proteasome is the rate limiting protease.⁷ Functional proteasome inhibition disrupts the equilibrium between production and disposal of such protein, which leads

to proteotoxic stress and excess activation of the UPR, triggering apoptosis.³

The constitutive proteasome is composed of three pairs of proteolytically active sites (β 1c, β 2c, β 5c) with different substrate specificity.⁸ Immune cells, including myeloma, may replace these by respective active sites of the immunoproteasome (β 1i, β 2i, β 5i).^{9,10} The β 5 activity is rate-limiting, and consequently bortezomib and carfilzomib, as well as all synthetic proteasome inhibitors in clinical development, are designed to target β 5.^{2,11–13}

Proteasome inhibitor resistance of MM is an emerging clinical problem whose biology is poorly understood. Proteasome inhibitor-resistant cell lines generated *in vitro* by continuous exposure to proteasome-inhibiting drugs serve as models to understand and potentially overcome proteasome inhibitor resistance.^{14–16} Mutations in *PSMB5* (encoding for β 5c) were predicted to lead to impaired inhibitor binding owing to changes in the β 5c active site or the inhibitor-binding pocket.^{14,17,18} However, the functional relevance of such mutations on the active site binding of bortezomib or carfilzomib in MM cells has not been demonstrated, and extensive analysis in MM cells derived from patients resistant to proteasome inhibitor therapy failed to

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identify such *PSMB5* mutations.¹⁹ Moreover, artificial introduction of mutant *PSMB5* in MM cells did not confer bortezomib resistance comparable to bortezomib-selected tumor cells.²⁰ Recently, an alternative biological model for proteasome inhibitor resistance was put forward, supported by respective findings from MM cells of bortezomib-resistant patients. It suggests that bortezomib resistance is the result of changes in the activation status of the UPR, in particular decreased activity of the IRE1/XBP1 axis,²¹ consistent with high XBP1 being a biomarker for bortezomib sensitivity in the clinic.²²

We here dissect the relationship between *PSMB5* mutation, proteasome inhibitor target inhibition and resistance to proteasome inhibitor-induced cell death of MM cells. Because our results suggest a complex mechanism of proteasome inhibitor resistance largely independent from either *PSMB5* mutations or even significant $\beta 5c$ proteasome activity, we provide a global proteomic comparison of proteasome inhibitor-sensitive vs bortezomib- and carfilzomib-adapted myeloma cells to identify novel potential therapeutic strategies beyond the ubiquitin proteasome pathway.

METHODS

Cell culture

The AMO-1 proteasome inhibitor-resistant cell lines (AMO-BTZ and AMO-CFZ) as well as their single clone-derived derivatives were established and maintained from the AMO-1 myeloma cell line by continuous drug exposure for >12 months.¹⁵ Additional information is provided in Supplementary Methods.

Relationship between proteasome inhibition and cytotoxicity

Measurement of proteasome activity was performed as described previously.²³ Additional information is provided in Supplementary Methods.

Proteome analysis

Briefly, full-cell lysates were digested with trypsin labeled with light (sensitive cells AMO-1) or intermediate (adapted cells) stable formaldehyde isotopes,²⁴ mixed, fractionated by SCX and analyzed by liquid chromatography-tandem mass spectrometry. Each analysis was performed in triplicate. The MaxQuant (Max Planck Institute of Biochemistry, Martinsried, Germany) was used for identification and quantification of the proteins. A cutoff of $\log_2 = 0.5$ was chosen to categorize differentially expressed proteins. The differentially expressed proteins were subjected to a protein–protein interaction analysis with the Cytoscape (The Cytoscape Consortium, San Diego, CA, USA). Gene ontology (GO) analysis was performed with the BinGo (The Cytoscape Consortium).²⁵ Manual curation was carried out to cluster the GO terms in more general groups (see Supplementary Materials for full protocol).

Statistical analysis

If not stated otherwise, results represent means and s.d. from at least three independent experiments. Additional information is provided in Supplementary Methods.

RESULTS

Subtotal functional proteasome inhibition lacks cytotoxicity in proteasome inhibitor-adapted myeloma cells

Sequencing of the active subunits of the constitutive proteasome revealed a heterozygous A310G mutation leading to a Met45Val change in the S1 pocket of the *PSMB5* active site in AMO-BTZ bulk cultures as well as in all single cell-derived clones from these cultures (data not shown). Genetic changes in the *PSMB5* ($\beta 5c$) *PSMB6* ($\beta 1c$) and *PSMB7* ($\beta 2c$) genes could be excluded in AMO-CFZ cells. Therefore, all experiments with AMO-BTZ cells reported here were performed with the Met45Val mutation present, while all experiments in AMO-CFZ were performed in the absence of a mutation. The IC₅₀ for $\beta 5c$ during bortezomib treatment was higher in AMO-BTZ, compared with AMO-1 and

AMO-CFZ, while the IC₅₀ for $\beta 1c$ was comparable (Figure 1a; Table 1). Bortezomib increased $\beta 2$ activity in AMO-1 cells but not in AMO-BTZ or AMO-CFZ. Treatment with 250 nM bortezomib, a concentration in the range of peak bortezomib plasma levels in myeloma patients minutes after intravenous bolus administration, induced approximately 70–80% inhibition of active $\beta 5c$ in AMO-1, AMO-BTZ or AMO-CFZ cells. This resulted in >90% cytotoxicity in AMO-1 cells, while viability in AMO-BTZ or AMO-CFZ remained >90%. Apoptosis was confirmed by propidium iodide/Annexin V staining (Supplementary Figure S1). HL60 acute myeloid leukemia cells similarly adapted to bortezomib (HL60-BTZ) and lacking a *PSMB5* mutation showed not only similarly effective proteasome $\beta 5c/\beta 5i$ inhibition by bortezomib, compared with non-adapted control cells, but also a lower sensitivity to bortezomib-induced cytotoxicity (data not shown).

Carfilzomib was more selective for $\beta 5c/\beta 5i$ activity over $\beta 1c/\beta 1i$ activity and, in addition, had some $\beta 2c/\beta 2i$ -inhibiting activity, both in contrast to bortezomib (Figure 1b; Table 1, Supplementary Figure S2). The IC₅₀ values of $\beta 2c/\beta 2i$ and $\beta 1c/\beta 1i$ were in the same order of magnitude for carfilzomib treatment in AMO-1 and AMO-BTZ. By contrast, the IC₅₀ for $\beta 5c$ was approximately five-fold lower in AMO-1 vs AMO-BTZ cells (10 vs 50 nM), suggesting that the *PSMB5* A310G mutation also affects $\beta 5$ binding of carfilzomib, comparable to bortezomib. Inhibition of $\beta 5c$ by carfilzomib was 9× less effective in AMO-CFZ, compared with AMO-1, while this difference had a factor of only two for bortezomib. Likewise, inhibition of $\beta 1/\beta 1i$ and $\beta 2/\beta 2i$ by carfilzomib was less effective in AMO-CFZ than in AMO-1. When approximately equally effective proteasome inhibition was achieved by carfilzomib in AMO-1, AMO-BTZ and AMO-CFZ (>90% inhibition of $\beta 5c/\beta 5i$, 20% inhibition of $\beta 1/\beta 1i$ and $\beta 2/\beta 2i$, achieved by 250 nM carfilzomib in AMO-1 and AMO-BTZ, and 1000 nM carfilzomib in AMO-CFZ), this resulted in >60% cytotoxicity in AMO-1 cells, in contrast to AMO-BTZ or AMO-CFZ.

Proteasome inhibitor-adapted myeloma cells show low IRE1/XBP1 expression

Protein and mRNA expression for IRE1 was uniformly decreased in AMO-BTZ and AMO-CFZ, (Figure 2). Consistent with this, spliced XBP1 was reduced on the mRNA and protein level, while total XBP1 and unspliced XBP1 were increased. Thus AMO-BTZ and AMO-CFZ both match the 'IRE1/sXBP1-low' expression pattern that was observed in myeloma cells from bortezomib-refractory patients. We did not find changes in the expression of protein or mRNA indicative of an activation of autophagy in AMO-BTZ or AMO-CFZ (Supplementary Figure S3).

We compared the effects of equi-efficient proteasome inhibition on the activation of the UPR-induced apoptotic machinery (Figure 3). AMO-1, AMO-BTZ and AMO-CFZ cells were pulse-treated for 1 h with bortezomib, followed by western blotting analysis of UPR-related proteins. Loss of expression of the active version of the NRF1 transcription factor that is generated by partial proteolysis through the proteasome²⁶ confirmed functional proteasome inhibition for 8 h in all treated cells, independent from the *PSMB5* mutation status. Phosphorylation of IRE1 and expression of spliced XBP1 was triggered within 1–2 h upon bortezomib challenge in all cell lines, and increased expression of PKR-like ER kinase was initiated at later time points. Triggering of the UPR-associated apoptotic machinery was observed in all cells, based on the expression of ATF4 and CHOP and activation of caspase 3, albeit with lower signal intensity in AMO-BTZ. By contrast, protein disulfide isomerase (PDI), a key enzyme for the formation of disulfide bonds and protein folding in the ER, showed low expression in AMO-1 at baseline and was strongly induced by bortezomib, while it was constitutively expressed at considerably higher levels in AMO-BTZ or AMO-CFZ cells without significant

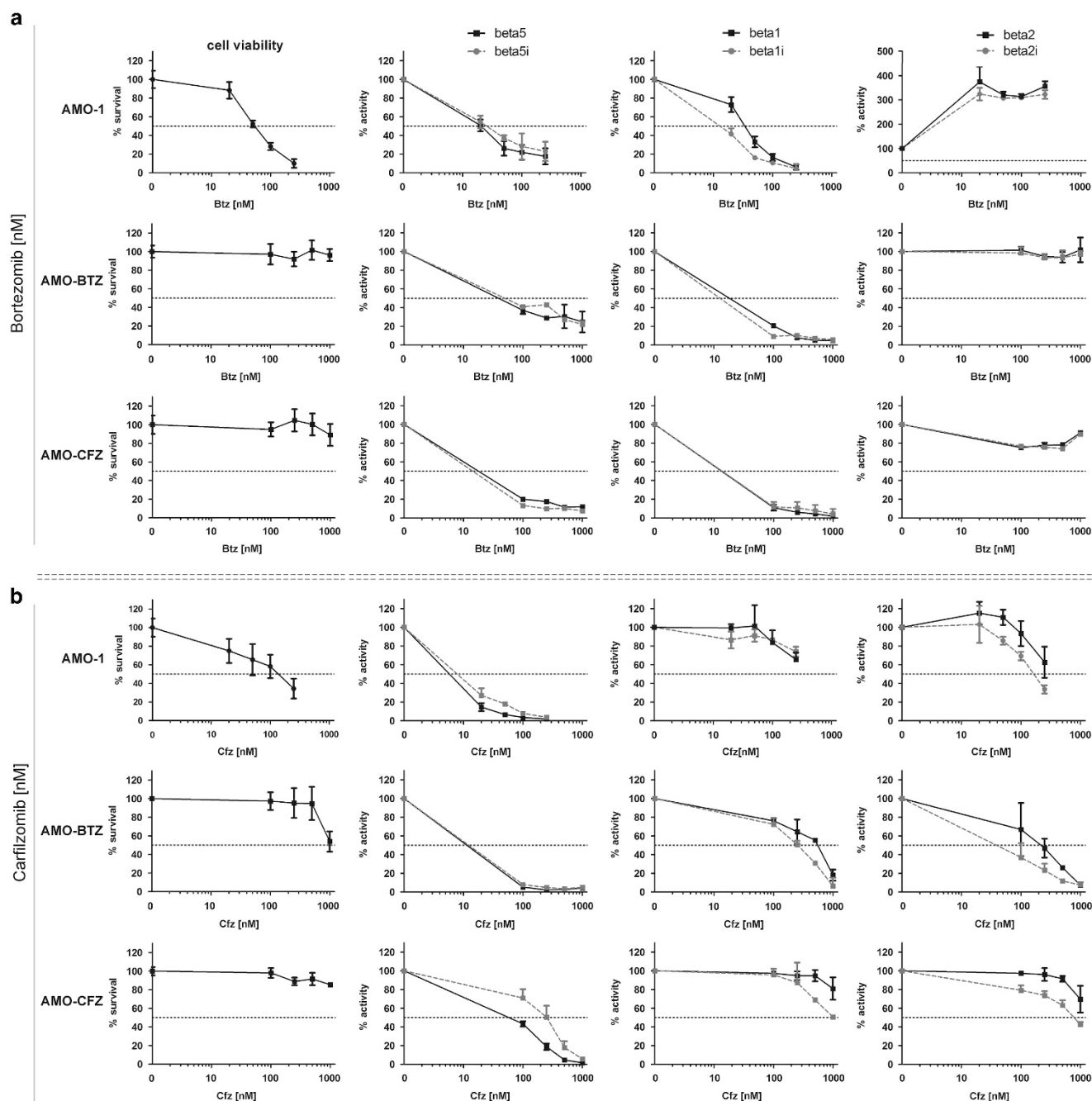


Figure 1. Relationship between cytotoxicity and proteasome β -subunit activity. AMO-1 cells and its derivatives adapted to bortezomib (AMO-BTZ) and carfilzomib (AMO-CFZ) have been cultured for 2 weeks without proteasome inhibitors. Then AMO-1, AMO-BTZ and AMO-CFZ were treated for a 1-h pulse with different concentrations of (a) bortezomib (Btz) or (b) carfilzomib (Cfz). Proteasome activity after drug exposure was measured directly using activity-specific, fluorescent chemical probes.²³ Cell viability was determined after cells were grown in fresh medium for 48 h following the 1-h drug exposure. Panels represent (in the order from left to right): cell viability determined by MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium), activity of the proteasome β 5c/5i, β 1c/1i and β 2c/2i subunits, respectively, as assessed by activity-based labeling, for each cell type (AMO-1, AMO-BTZ, AMO-CFZ, horizontal rows) and for each proteasome inhibitor (bortezomib, carfilzomib, panels a and b). Data are presented as a mean $\pm$ s.d. of three independent replicates.

bortezomib-induced change. This suggested adaptive changes in the reducing and protein-folding machinery of the ER.

Global quantitative protein expression in proteasome inhibitor-adapted myeloma cells

We chose a proteomic approach to identify the full pattern of proteins with significant changes in the expression levels in

AMO-1 vs AMO-BTZ and AMO-CFZ cells. In each experiment, >3500 different proteins were identified, of which >2000 proteins showed consistent peptide identification for both labels and in each triplicate, and were thus used for quantification, resulting in >600 protein species identified with statistically significant changes in the expression levels in proteasome inhibitor-adapted cells, compared with the non-adapted controls (three independent replicates, $P < 0.01$, Table 2, Supplementary

Table 1. IC50 values obtained for each β -subunit

	Proteasome subunit	AMO-1	AMO-BTZ	AMO-CFZ
BTZ				
IC50 (nM) (50% functional proteasome inhibition)	β 2c	NA	NA	NA
	β 2i	NA	NA	NA
	β 1c	40	60	50–60
	β 1i	20–25	50	50–60
	β 5c	25	80–90	50–60
	β 5i	30	80–90	50–60
LD50 (nM) (50% cell viability)		50	> 1000	> 1000
CFZ				
IC50 (nM) (50% functional proteasome inhibition)	β 2c	300	220	> 1000
	β 2i	180	90	800
	β 1c	> 500	600	> 1000
	β 1i	> 250	250	1000
	β 5c	10	50	90
	β 5i	10	50	250
LD50 (nM) (50% cell viability)		150	> 1000	> 1000

IC50 values for the inhibition of proteasome β -subunits in AMO-1-sensitive cells and AMO cells adapted to bortezomib (AMO-BTZ) and carfilzomib (AMO-CFZ) during treatment with bortezomib (Btz) and carfilzomib (Cfz), as deferred from subunit-selective activity-based fluorescent labeling, in relation to the LD50 (50% cell viability, determined by MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)). NA, not applicable or > 1000 nM indicate no significant effect on the IC50 value up to 1000 nM.

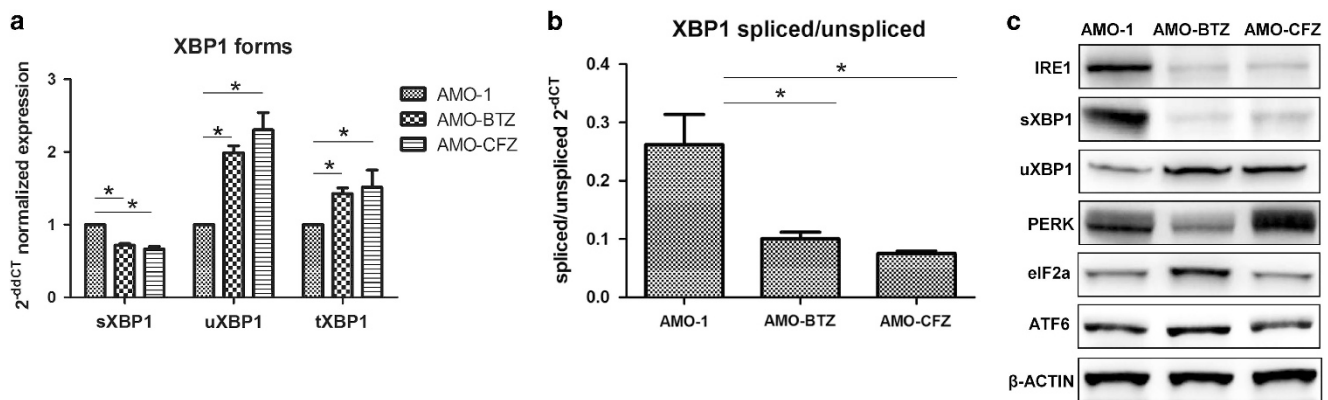


Figure 2. Protein and RNA expression for UPR signaling pathway. AMO-1 and its derivatives adapted to bortezomib (AMO-BTZ) and carfilzomib (AMO-CFZ) have been cultured for 2 weeks without proteasome inhibitors before RNA and protein was collected. **(a)** Level of spliced (s), unspliced (u) and total (t) XBP1 in the cell lines. **(b)** Presence of activated XBP1 depicted as a ratio of spliced XBP1 vs unspliced XBP1 in the cell lines. **(c)** Representative western blotting bands for protein levels of UPR signaling proteins in the cell lines. For panels **a** and **b**, data are presented as mean $\pm$ s.d. of three independent replicates. Statistical significance < 0.05 is marked by asterisks.

Figure S4). 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 was identified and quantified as in the myeloma cell lines.

The differentially expressed proteins were subjected to a protein–protein interaction search to build a protein–protein interaction network (Supplementary Figure S5). This resulted in a complex network of changes in proteasome inhibitor-adapted cells. Differentially expressed proteins were therefore subjected to a Gene Ontology (GO) analysis, resulting in an average of 30 GO terms for the overexpressed proteins and 10 for the down-regulated species in all the three adapted clones (Supplementary Table S1). GO terms involving the ubiquitin proteasome pathway were enriched in all samples, in agreement with proteasome overexpression. GO terms were manually grouped into functionally related clusters resulting in 5–6 groups of differentially expressed proteins (Table 2; see also Supplementary Tables S2–S7 for individual upregulated/downregulated proteins per GO

pathway). This supervised clustering showed some general concordance between the adapted cells, suggesting that acquired resistance to proteasome inhibitors is characterized by a complex, but characteristic pattern, of changes in protein expression.

The quantitatively largest group with altered expression levels was a group of proteins involved in metabolic regulation, comprising close to 50% of all polypeptide species with expression changes identified. This suggested that metabolic homeostasis is a major challenge for myeloma cells adapted to proteasome inhibitor treatment. Upregulated proteins included proteins involved in the respiratory chain (for example, CYC1 or UQCRI), the generation of metabolites (for example, BLVRA or APOA1BP), glycolysis (for example, PFKP or PKM) and amino acid (for example, GOT1 or EEFSEC) or nucleic acid metabolism (for example, UMP5 or BOP1). The metabolic regulation category also contained proteins (30%) that were significantly down-regulated, in particular proteins involved in lipid and cofactor metabolism (for example, ASCT2 or ACLY). Of the top 25

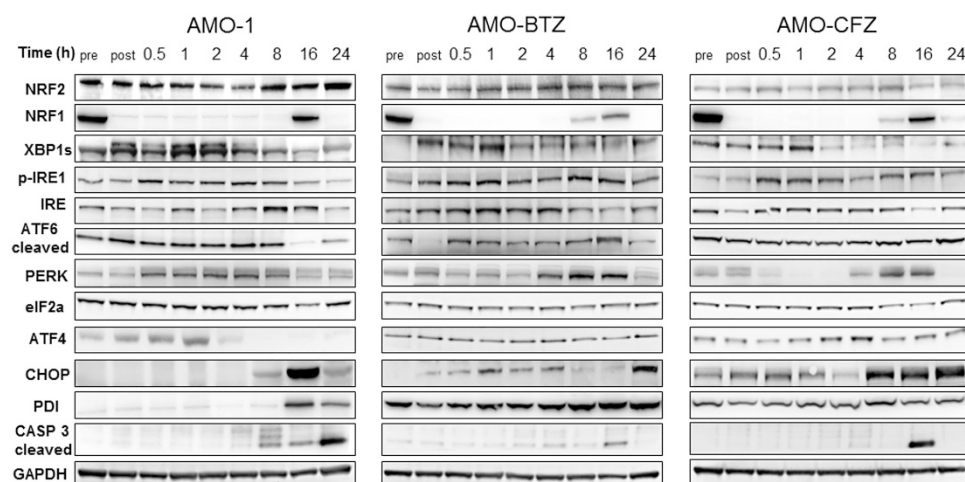


Figure 3. Activation of the UPR-induced apoptotic machinery. AMO-1 derivatives adapted to bortezomib (AMO-BTZ) and carfilzomib (AMO-CFZ) have been cultured for 2 weeks without proteasome inhibitors. Then, together with AMO-1 sensitive cell line, they were treated with 500 nM bortezomib for a 1-h pulse. After the pulse, cells were grown in fresh medium for different chase time points (0.5, 1, 2, 4, 8, 16, 24 h). Directly before the addition of the proteasome inhibitor (presample) and immediately after the pulse (postsample), as well as at each chase time point indicated, an aliquot was harvested for protein isolation, and equal amounts of protein from each sample were subsequently processed for western blotting. Data are presented as representative bands of three independent experiments.

Table 2. Proteomic comparison of AMO-1 cells with AMO-BTZ and AMO-CFZ

Change	GO term	P-value range	Number of proteins	Mean fold change (range)
AMO-BTZ				
Upregulated	Protein catabolism	10^{-8}	19	1.7 (2.3–1.4)
	Redox homeostasis	10^{-7} – 10^{-6}	25	1.9 (6.1–1.4)
	Folding and complex formation	10^{-3} – 10^{-2}	53	2.0 (19.7–1.4)
	Transport/signaling	10^{-3} – 10^{-2}	116	1.7 (4.3–1.4)
	Metabolic regulation	10^{-3} – 10^{-2}	114	1.8 (9.2–1.4)
Downregulated	Transcription/translation	10^{-2}	63	0.6 (0.7–0.2)
	Differentiation	10^{-2}	13	0.6 (0.7–0.4)
	Cytoskeleton organization	10^{-2}	22	0.5 (0.7–0.1)
	Metabolic regulation	10^{-2}	45	0.6 (0.7–0.4)
	Transport/signaling	10^{-2}	89	0.5 (0.7–0.1)
	Apoptosis	10^{-1}	8	0.5 (0.7–0.3)
AMO-CFZ				
Upregulated	Protein catabolism	10^{-8}	22	1.7 (2.7–1.4)
	Transport/signaling	10^{-6} – 10^{-2}	85	1.9 (12.1–1.4)
	Metabolic regulation	10^{-6} – 10^{-2}	110	1.8 (4.6–1.4)
	Folding and complex formation	10^{-3} – 10^{-2}	39	2.2 (13.9–1.4)
	Redox homeostasis	10^{-3} – 10^{-2}	15	2.2 (4.3–1.4)
Downregulated	Apoptosis	10^{-3}	7	0.5 (0.7–0.2)
	Metabolic regulation	10^{-3} – 10^{-2}	58	0.5 (0.7–0.2)
	Transport/signaling	10^{-2}	72	0.5 (0.7–0.1)
	Transcription/translation	10^{-2}	58	0.6 (0.7–0.2)
	Differentiation	10^{-2}	10	0.5 (0.7–0.2)
	Cytoskeleton organization	10^{-2}	24	0.5 (0.7–0.1)
HL60-BTZ				
Upregulated	Protein catabolism	10^{-15}	23	1.7 (2.7–1.4)
	Transport/signaling	10^{-10} – 10^{-2}	80	1.8 (6.8–1.4)
	Metabolic regulation	10^{-3} – 10^{-2}	65	1.8 (4.5–1.4)
	Folding and complex formation	10^{-2}	28	1.8 (3.9–1.4)
	Redox homeostasis	10^{-2}	20	1.9 (4.4–1.4)
Downregulated	Transport/signaling	10^{-5}	40	0.6 (0.7–0.3)
	Metabolic regulation	10^{-3} – 10^{-2}	34	0.6 (0.7–0.3)
	Transcription/translation	10^{-2}	64	0.6 (0.7–0.2)
	Redox homeostasis	10^{-2}	16	0.6 (0.7–0.2)
	Cytoskeleton organization	10^{-2}	12	0.6 (0.7–0.4)

Abbreviation: GO, gene ontology. Supervised clustering of GO terms into functionally related clusters of biological processes found for the upregulated and downregulated proteins in the cell lines adapted to bortezomib (AMO-BTZ, HL60-BTZ) and carfilzomib (AMO-CFZ). P-values range is obtained from the unsupervised clusters of different GO terms (see Supplementary Table S1) clustered in each category.

quantitatively downregulated proteins in this cluster, 12 were mitochondrial proteins involved in fatty acid biosynthesis (ACSF2, ACSF3) in AMO-CFZ, and likewise five mitochondrial proteins with similar function were among the top 25 downregulated in AMO-BTZ. Proteins involved in protein catabolism, redox control and protein folding were also overexpressed. This included all proteasome alpha (PSMA 1,2,3,4&7) and beta (PSMB 1,2,3,4,5&7) subunits and corroborated the validity of our analysis. Proteins involved in antioxidant and reactive oxygen species functions (NQO1, PRDX1, SOD1) were also increased in adapted cells, as well as proteins involved in glutathione regulation. Different individual proteins were upregulated in a given functional pathway between AMO-BTZ and AMO-CFZ (for example, 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. The protein folding/chaperoning capacity of the adapted cells was also markedly increased: the heat-shock proteins HSP70, HSP90 and HSP105 were consistently among the top 15 most strongly upregulated proteins (2.3–13-fold increase) among proteins involved in folding/chaperoning in both adapted cell clones.

A pattern of 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. The apoptosis protein cluster comprised the lowest number of proteins, of which both adapted cells markedly downregulated individual key proteins (decreased BAX in AMO-CFZ, decreased CASP10 and DIABLO in AMO-BTZ). Downmodulation of cytoskeleton and cell differentiation included

IKZF3, a transcription factor implicated in lymphocyte differentiation and activity of immunomodulatory myeloma drugs. AMO-BTZ cells were significantly more sensitive to lenalidomide, consistent with a role of IKZF3 levels, while AMO-CFZ were lenalidomide refractory, potentially explained by their high ABCB1 expression (Supplementary Figure S6A).

Upregulation of MDR1 and oxidative glycolysis functionally contribute to resistance

ABCB1, the MDR1, was 12-fold upregulated only in AMO-CFZ on the protein and 120-fold by gene expression analysis (data not shown). Likewise, we observed $>100\times$ increased ABCB1 gene expression in carfilzomib-adapted ARH-77 plasma cell leukemia cells (ARH-CFZ) and primary malignant plasma cell samples from a myeloma patient who initially responded to carfilzomib treatment and later showed progressive disease under carfilzomib. The primary patient sample showed more than four-fold upregulated ABCB1 expression upon progression, compared with the baseline (Supplementary Figure S7). The addition of the functional MDR1 inhibitors verapamil and reserpine significantly decreased the LD50 for CFZ-treated AMO-CFZ cells, in contrast to AMO-BTZ cells or AMO-CFZ cells treated with bortezomib, suggesting a high functional significance of ABCB1 overexpression for CFZ resistance (Figure 4 and Supplementary Figure S8). The sensitivity of AMO-CFZ against cyclophosphamide or daunorubicin was unchanged (Supplementary Figures S6C and D), while AMO-BTZ and AMO-CFZ showed decreased sensitivity for panobinostat, consistent with the role of HDAC6 in non-proteasomal protein degradation (Supplementary Figure S6B).

Oxidative glycolysis is upregulated in proteasome inhibitor-resistant myeloma cells

NADPH dehydrogenase, the quantitatively most important oxidoreductase in eukaryotic cells, 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 oxidative branch of the pentose phosphate pathway (PPP)) were found overexpressed, implicating that increased production of

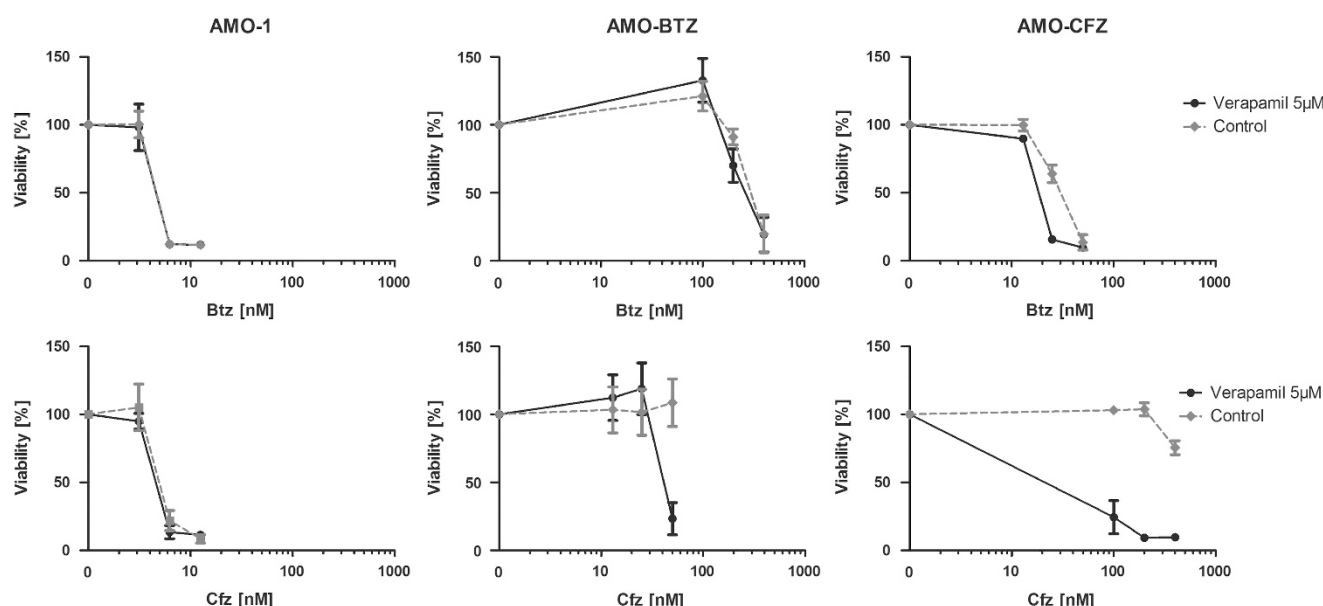


Figure 4. Cytotoxic effect of specific ABCB1 inhibitor verapamil. The AMO-1 cell line and its derivatives adapted to bortezomib (AMO-BTZ) and carfilzomib (AMO-CFZ) have been cultured in the presence of 5 μM verapamil and bortezomib/carfilzomib (Btz/Cfz) for 5 days and viability was assessed by MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) test. Absorbance values of treated cells were normalized to the controls without specific inhibitors. Data are presented as a mean $\pm$ s.d. of three independent replicates.

reducing equivalents may serve as a metabolic adaptive response to render cells more proteasome inhibition resistant. Glycolysis can contribute significantly to the synthesis of reducing equivalents via NADPH (via PPP and mitochondrial pyruvate–malate cycle) and NADH (glycolysis and TCA cycle), which are crucial for maintenance of redox balance and neutralization of reactive oxygen species. Indeed, we observed that the AMO-BTZ and AMO-CFZ cells displayed higher baseline and maximum glycolytic rates than their parental cell line (Figure 5a) as determined by real-time metabolic flux analysis, a finding that is supported by the increased expression of several glycolytic enzymes (Supplementary Tables S2 and S4). Consistent with these findings, high glucose medium decreases the proteasome inhibitor sensitivity of AMO-1 cells, in contrast to AMO-CFZ or AMO-BTZ (Supplementary Figure S9). Malignant cells often display increased glycolytic rates with lactate as end product despite the availability of ample oxygen (Warburg Effect), a phenomenon thought to reflect a metabolic adaptation to maintain cellular ATP levels in the face of often dysfunctional mitochondria. In agreement with the increased expression of several proteins of the electron transport chain in AMO-BTZ and AMO-CFZ cells (Supplementary Tables S2 and S4), we found, however, that these cells showed higher baseline and maximum mitochondrial respiration than the AMO-1 cell line (Figure 5b), indicative of enhanced mitochondrial activity and health. This suggests that the enhanced glycolytic flux in these cells is unlikely merely a bioenergetic adaptation to impaired ox-phos but may have a role in fueling the PPP and TCA

to, among other things, aid in the generation of reducing equivalents. To further test this concept, we used FX11, a specific inhibitor of lactate dehydrogenase A that catalyzes the conversion of pyruvate into lactate (Figure 5c), thereby instead promoting the oxidation of pyruvate in the mitochondria. Indeed, this selective block of aerobic glycolysis considerably increased the LD50 for bortezomib in AMO-1, AMO-BTZ and AMO-CFZ cells. This would be in support of the notion that the increased glycolysis in AMO-BTZ and AMO-CFZ cells is not ATP-driven but may rather serve to fuel mitochondrial metabolism and the PPP that could help in the generation of reducing power to mitigate the deleterious effects of proteasome inhibition.

DISCUSSION

Proteasome *PSMB5* gene mutations are believed to explain clinical proteasome inhibitor resistance, although attempts have failed to identify them in patient-derived material. This 'mutation model' was challenged when downmodulation of IRE1/XBP1 was shown to result in proteasome inhibitor resistance while 'IRE1/XBP1-low' myeloma cells accumulate in bortezomib-resistant patients. We demonstrate that proteasome inhibitor-adapted myeloma cells tolerate near-complete inhibition of proteasome activity, irrespective of the presence of a *PSMB5* mutation. Furthermore, AMO-BTZ and AMO-CFZ showed an 'IRE1/XBP1-low' pattern of UPR activation, independently from proteasome mutations. These data argue against individual *PSMB5* mutations as key factor for

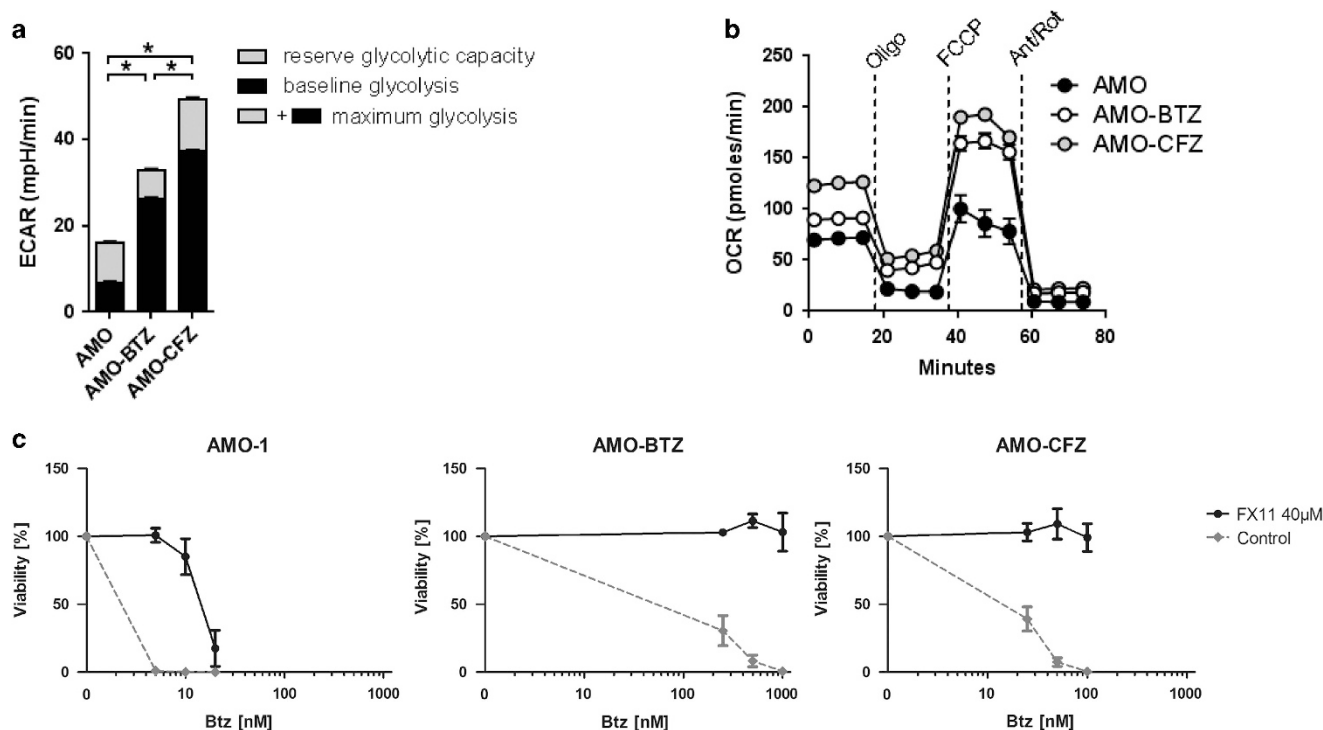


Figure 5. Role of glycolysis in proteasome inhibitor resistance. (a, b) The AMO-1 cell line and its derivatives adapted to bortezomib (AMO-BTZ) and carfilzomib (AMO-CFZ) were seeded in a 96-well XF Extracellular Flux Analyzer to simultaneously quantify (a) extracellular acidification rates (ECAR) and (b) oxygen consumption rates (OCR) as measures of glycolysis and mitochondrial respiration, respectively. (a) Real-time baseline glycolytic rate of the different cell lines was determined by subtracting the difference in ECAR before and after injection of 10 mM glucose. Reserve glycolytic capacity was calculated by subtracting baseline ECAR readings from ECAR values following treatment with ETC blockers antimycin A/rotenone. (b) Real-time OCR was determined during sequential treatments with oligomycin (ATP-synthase inhibitor), FCCP (uncoupler) and antimycin A/rotenone (ETC inhibitors). Maximum mitochondrial respiration is reached following treatment with FCCP that leads to uncoupling of the mitochondria. Data represent means $\pm$ s.e.m. of eight replicates. One representative experiment of three is shown. (c) The AMO-1 cell line and its derivatives adapted to bortezomib (AMO-BTZ) and carfilzomib (AMO-CFZ) have been cultured in the presence of 40 μ M FX11, and bortezomib (Btz) for 5 days, and viability was assessed by MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) test. Absorbance values of treated cells were normalized to the controls without specific inhibitors. Data are presented as a mean $\pm$ s.d. of three independent replicates.

proteasome inhibitor resistance and support the 'IRE1/XBP1-low' model. They also suggest that proteasomal proteolysis is no longer the 'Achilles' heel' of proteasome inhibitor-resistant myeloma but that its functionality is almost dispensable.

Because our findings suggested a complex biology of proteasome inhibitor resistance, we undertook the first quantitative proteome analysis of proteasome inhibitor-resistant myeloma. Our results show that adaptation of myeloma to proteasome inhibitors involves complex changes in hundreds of proteins. The gross pattern observed was similar between AMO-BTZ and AMO-CFZ cells and may be understood as characteristic adaptive response to proteasome inhibition, balancing the equilibrium between proteasomal load and capacity at a new functional level of very

low proteasome activity. Although different proteins were involved in the adaptation process between both cell lines, the pattern of changes in particular included proteins involved in energy homeostasis or the production, folding or destruction of protein. Our data are consistent with the observation that proteasome overexpression mediated by nuclear factor 2 (NRF2) can contribute to adaptive resistance to proteasome inhibitors,²⁷ although in our model NRF2 was not upregulated (data not shown).

To our knowledge, while all bortezomib-adapted myeloma cells with a biologically meaningful resistance factor $>5\times$ that tolerate high (100 nM) bortezomib concentrations contain a *PSMB5* mutation,²⁸ carfilzomib-resistant myeloma cells have not yet been

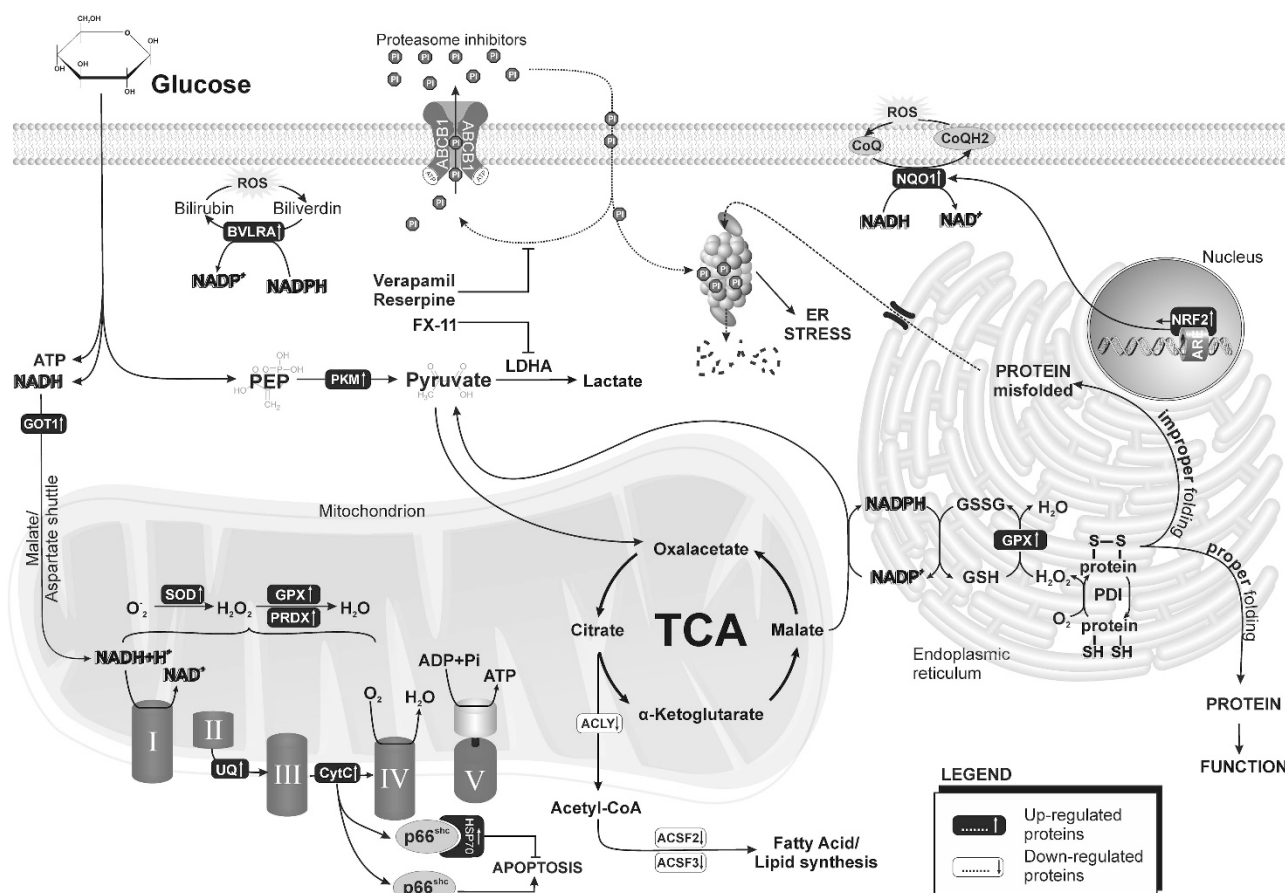


Figure 6. Model of redox homeostasis and metabolic changes during the adaptation to proteasome inhibitors. Excessive proteotoxic stress is the major mechanism of cytotoxicity of proteasome inhibitors in myeloma. The level of proteotoxic stress results from the degree of imbalance between proteasomal load and proteasomal proteolytic capacity. The proteasomal load of misfolded protein is tightly connected to the protein-folding capacity of the ER, which in turn relies on proper redox conditions. NAD(P)H is critical for providing oxidizing equivalents and thus for minimizing the proteasomal load in proteasome inhibitor-adapted myeloma cells that are viable with extremely low proteasome activity while producing high paraprotein amounts. NAD(P)H is mostly provided from intermediates of the TCA (Krebs Cycle), for example, malate–pyruvate and mitochondrial isocitrate dehydrogenase reactions, which we found consistently upregulated in proteasome inhibitor-adapted myeloma cells. In line with this, multiple other enzymes that are involved in NAD(P)H generation were upregulated, while enzymes mediating lipid biosynthesis from Acetyl-CoA, which removes tricarboxylic acid intermediates from the Krebs Cycle and thus decreases NAD(P)H generation, are downregulated. This way, we hypothesize that maximum NAD(P)H-producing intermediates may be provided, limiting proteotoxic stress in adapted/resistant cells. Cancer cells often use the faster, but less efficient, pathway for ATP production to generate ATP via pyruvate–lactate conversion (Warburg effect). When this is inhibited using the specific inhibitor of LDHA, FX-11, the sensitivity of myeloma cells to proteasome inhibitors decreases, presumably because glucose metabolism entirely switches to the TCA cycle, increasing NAD(P)H production and reducing proteotoxic stress. Upregulation of ABCB1 decreases proteotoxic stress via reduced intracellular concentrations of proteasome inhibitors, which can be reversed by specific ABCB1 inhibitors Verapamil or Reserpine. ABCB1, ATP-binding cassette, sub-family B (MDR/TAP) member 1; ACLY, ATP Citrate Lyase; ACSF2, Acyl-CoA synthetase family member 2; ACSF3, Acyl-CoA synthetase family member 3; ARE, antioxidant response elements; BVLRA, biliverdin reductase; CoQ, coenzyme Q; CoQH2, coenzyme Q-reduced; CytC, cytochrome C; GSSG, oxidized glutathione; GSH, reduced glutathione; GOT1, glutamic-oxaloacetic transaminase 1; GPX, glutathione peroxidase; LDHA, lactate dehydrogenase; NQO1, NAD(P)H dehydrogenase, quinone1; NRF2, nuclear factor (erythroid-derived 2)-like 2; PDI, protein disulfide isomerase; PEP, phosphoenolpyruvate; PI, proteasome inhibitors; PKM, pyruvate kinase, muscle; PRDX, peroxiredoxin; ROS, reactive oxygen species; SOD, superoxide dismutase; UQ, ubiquinone.

reported. AMO-CFZ cells described here lack a mutation in the *PSMB5*, *PSMB6* or *PSMB7* genes, have a resistance factor of approximately $>20\times$ and $>7\times$ for carfilzomib and bortezomib, respectively, and tolerate very high concentrations of each of the proteasome-inhibiting drugs, so that they represent the first comprehensive cell line model for proteasome inhibitor resistance and at the same time mirror the mutation status as well as the 'IRE1/XBP1-low' biology found in resistant patients. The A310G mutation at Met45Val directly affects the S1-binding pocket in the $\beta 5$ substrate channel and significantly impairs chymotryptic proteasome activity and binding to bortezomib or carfilzomib.¹⁷ We demonstrate that the heterozygous Met45Val mutation results in 3–5-fold lower $\beta 5$ -inhibiting activity of bortezomib and carfilzomib in human myeloma cells.

The MDR ABCB1 was the most significantly upregulated protein in AMO-CFZ cells. MDR-mediated resistance to carfilzomib has been observed in solid tumor cells,²⁹ in contrast to bortezomib, and we did not observe ABCB1 upregulation in AMO-BTZ cells. The increased IC₅₀ for inhibition of all individual proteasome subunits in AMO-CFZ cells after carfilzomib treatment is consistent with drug export. Indeed, co-treatment of AMO-CFZ with carfilzomib and MDR inhibitors decreased carfilzomib resistance.

Given the redundancy of signaling and the genetic architecture of advanced myeloma, targeting individual signaling proteins may lack significant activity in proteasome inhibitor-refractory myeloma. However, most of these patients still have measurable paraprotein levels, indicating that bortezomib-adapted myeloma cells *in vivo* usually continue to produce high amounts of secretory protein. Our results suggest that such myeloma cells may secure continued paraprotein production by entertaining a highly optimized machinery of protein folding via increased chaperone expression and stringent redox conditions, thus minimizing defective folding that would otherwise increase proteasomal load. Indeed, AMO-CFZ maintained fully functional IgA secretion levels, whereas paraprotein secretion was absent from AMO-BTZ (Supplementary Figure S10). Adequate supply of energy and oxidizing equivalents are key requisites toward proper redox conditions. Tumor cells often use aerobic glycolysis instead of oxidative generation of ATP, called 'Warburg Effect'. However, the oxidative glucose turnover is also one of the major providers of NADPH, the most important oxidizing agent, regenerated from NADP when malate from the Krebs cycle is converted into pyruvate. Indeed, several of our results suggest that the metabolism of proteasome inhibitor-resistant myeloma cells has been geared to provide NADPH for reducing activity with high efficiency: NADPH dehydrogenase, the most important oxidoreductase in eukaryotes was overexpressed 4–6-fold in AMO-BTZ and AMO-CFZ cells, similarly to malate dehydrogenase and enzymes of the PPP, the most important providers of NADPH. At the same time, several enzymes involved in synthesis of fatty acids and lipids from pyruvate/citrate backbones in the mitochondria via Acetyl-CoA were among the top downregulated proteins in both AMO-CFZ and AMO-BTZ cells, so that mitochondrial pyruvate is, to a smaller fraction, used as building blocks for lipid biosynthesis and can, to a larger fraction, undergo oxidative glycolysis. These characteristics suggest a quantitative adaptation in the flow of metabolic intermediates in proteasome inhibitor-resistant myeloma cells, optimizing for the provision of energy and oxidizing equivalents via oxidative glycolysis (see proposed metabolic model of proteasome inhibitor-adapted myeloma cells, Figure 6). Proteasome inhibitor-resistant myeloma may thus be sensitive to the manipulation key metabolic processes, that is, oxidation of glucose, or the generation of NADPH and/or ATP. A comparison of the metabolomes between AMO-1, AMO-BTZ and AMO-CFZ is currently underway.

We document that proteasome activity is largely dispensable in bortezomib- or carfilzomib-adapted myeloma cells, suggesting that nonproteasomal targets are required to overcome

proteasome inhibitor resistance. *PSMB5* mutations are likewise not required for high-level proteasome inhibitor resistance of myeloma, while an important role of the 'IRE1/XBP1-low' activation status of the UPR is supported. Our full proteomic analysis of proteasome inhibitor-resistant myeloma cells reveals a complex adaptation pattern that in particular involves changes in glycolysis, protein folding and redox function. Our data suggest exploring these metabolic pathways in proteasome inhibitor-resistant myeloma.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies this paper on the Leukemia website (<http://www.nature.com/leu>)

5.4 Annex 4

A metabolic switch in proteasome inhibitor-resistant multiple myeloma ensures higher mitochondrial metabolism, protein folding and sphingomyelin synthesis

Proteasome inhibitors (PI) have evolved as the central backbone of treatment for multiple myeloma (MM), with first-in-class bortezomib and second- and third-generation PI, carfilzomib and ixazomib, having been approved for this indication.¹ Proteasome inhibition disrupts the unfolded protein response to resolve excessive endoplasmic reticulum stress, but also leads to massive metabolic changes manifested by the induction of amino acid biosynthesis, an anti-oxidant response, lipogenesis and an increase in protein folding.^{2,3} In this sense, PI represent a unique class of drugs targeting cancer cell metabolism by affecting the balance between protein biosynthesis,

folding and destruction. However, the adaptive changes in MM cell metabolism may provide the basis of resistance to PI, as high glycolytic activity or rewiring glucose metabolism is associated with bortezomib resistance in MM.^{4,5}

The biology of PI resistance in MM is poorly understood and established treatment options for PI-refractory MM are lacking. Our recent quantitative proteomic analysis of the AMO-1 MM cell line and its derivatives resistant to bortezomib (AMO-BTZ) and carfilzomib (AMO-CFZ) suggested that the PI-resistance phenotype is characterized in particular by massive alterations in cellular metabolism and high oxidative phosphorylation.⁶ Herein we directly substantiated this by mass spectrometry-based, whole metabolome profiling demonstrating that AMO-BTZ and AMO-CFZ show similar patterns of metabolic changes in global pathways that fuel synthesis and regeneration of glutathione, NAD(P)H and the tricar-

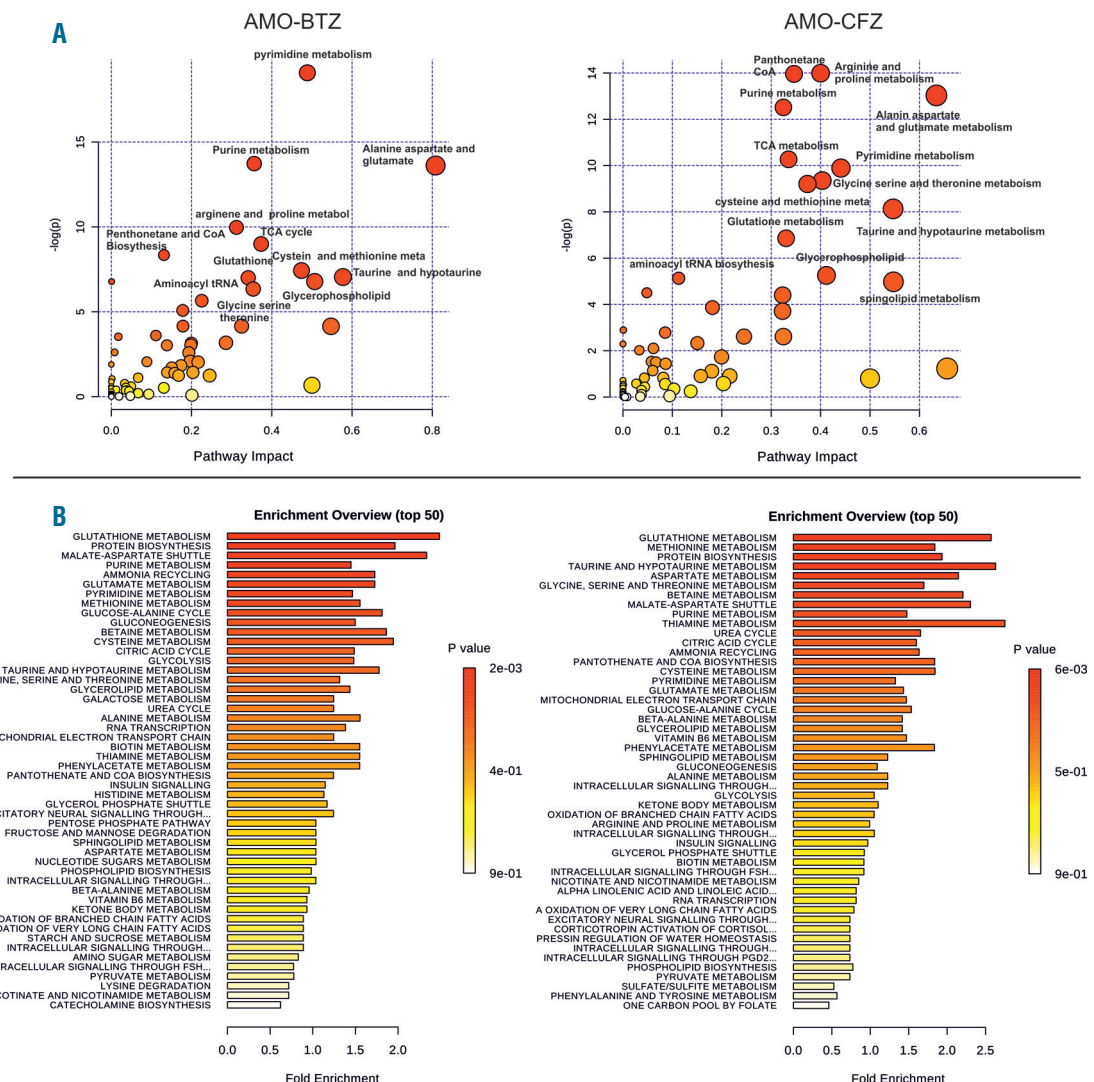


Figure 1. Pathway analysis and metabolite set enrichment analysis of proteasome inhibitor-adapted multiple myeloma cells (AMO-BTZ and AMO-CFZ). (A) Metabolite pathway analysis of deregulated metabolites in AMO-BTZ (aBTZ) and AMO-CFZ (aCFZ) cells compared to AMO-1 cells. The metabolic pathways are represented as circles, according to their scores from enrichment (y axis) and topology analyses (pathway impact, x axis) using MetaboAnalyst 3.0. Darker red colors indicate more significant changes of metabolites in the corresponding pathway. The size of the circle corresponds to the pathway impact score and is correlated with the centrality of the involved metabolites. (B) Metabolite set enrichment analysis pathway overview in AMO-BTZ and AMO-CFZ, in comparison with AMO-1 proteasome inhibitor-sensitive cells obtained by MetaboAnalyst 3.0. Over-representation analysis was implemented to evaluate whether a particular metabolite set was represented (fold enrichment) more than expected by chance within the given compound list. One-tailed *P* values are provided after adjusting for multiple testing. TCA: tricarboxylic acid; CoA: coenzyme A.

boxylic acid cycle. These changes functionally lead to increased antioxidant capacity and preferential oxidative phosphorylation, which ultimately supports more effective protein folding that results in a decreased proteasome load of misfolded proteins in PI-resistant cells. Moreover, PI-resistant cells show structurally altered mitochondria and a massive lipid class switch from lysolipids to sphingomyelins. Based on the highly specialized metabolism of bortezomib- and carfilzomib-resistant MM, we propose that these unique metabolic characteristics may offer new “vulnerable spots” that can be therapeutically exploited, e.g. with agents that target protein folding, energy supply, mitochondria biology or lipid homeostasis, to overcome PI resistance.

The targeted metabolomic profiling was performed in PI-resistant AMO-BTZ and AMO-CFZ cells, bulk culture and single-cell derived colonies, as well as in PI-sensitive AMO-1 control cells as described elsewhere,⁶ and revealed 534 metabolic intermediates of known identity

that were quantitatively significantly changed in PI-resistant cells (*Online Supplementary Table S1*). Principal component analysis, supported by hierarchical cluster analysis, revealed strong segregation of the three cell lines based on global biochemical profiles of each cell line, suggesting distinct metabolic changes in bortezomib and carfilzomib adaptation (*Online Supplementary Figure S1A,B*). We observed strong concordance in the metabolic changes between bulk-cultures of PI-resistant cells and single cell-derived populations, confirming consistency of the metabolic changes. Metabolic set enrichment analysis revealed a strong concordance between AMO-BTZ and AMO-CFZ in overall deregulated processes and metabolites involved in protein biosynthesis, glutathione metabolism, the malate/aspartate shuttle, metabolism of purines, pyrimidines, amino acids and the tricarboxylic acid cycle (Figure 1A). Comparative pathway analysis confirmed the results of the metabolic set enrichment analysis, indicating highly deregulated metabolism of

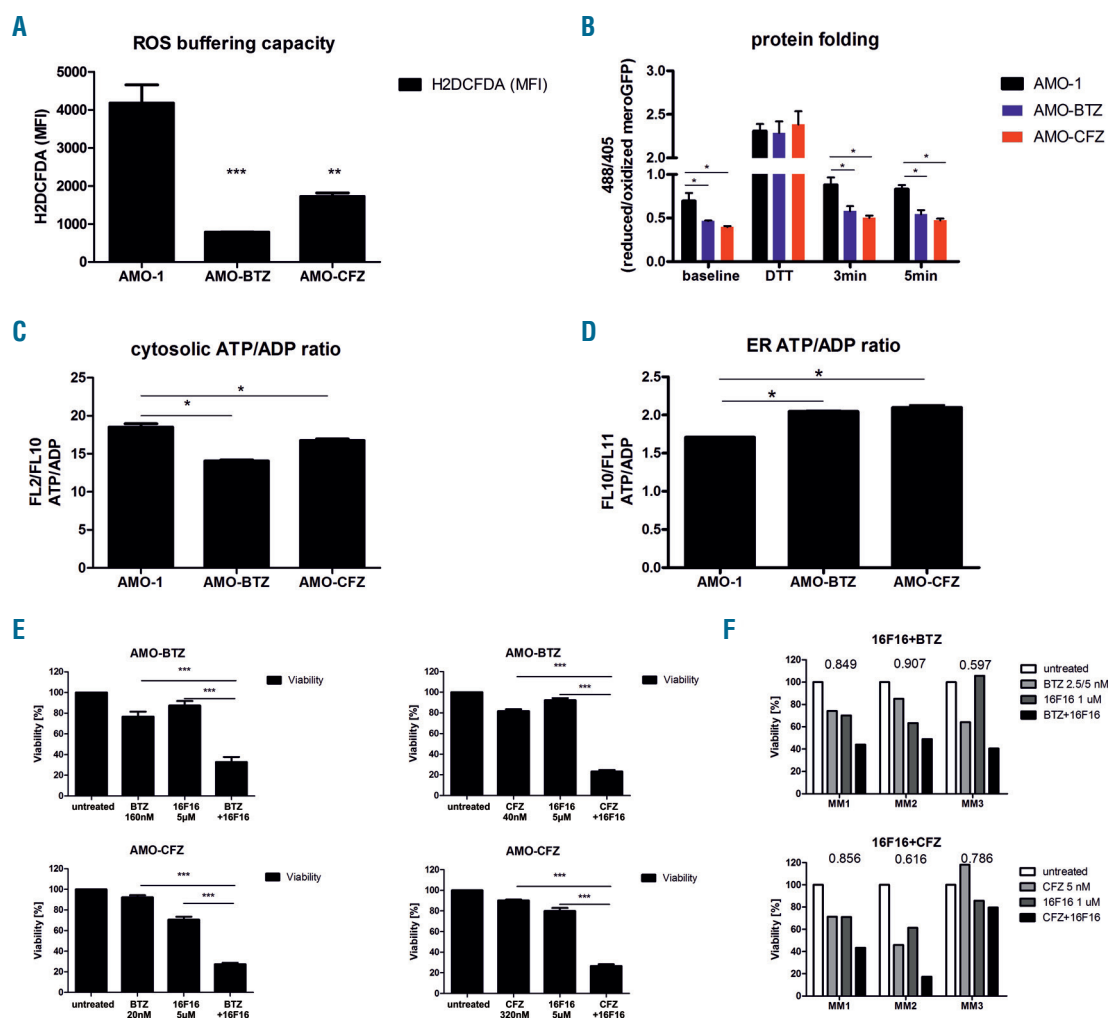


Figure 2. Functional changes in antioxidant capacity, protein folding and ATP capacity in the endoplasmic reticulum of proteasome inhibitor-resistant cells.

(A) Reactive oxygen species (ROS) buffering capacity in proteasome inhibitor (PI)-sensitive AMO-1 and PI-resistant AMO-BTZ and AMO-CFZ cells evaluated by short-term exposure of cells to 0.08% H₂O₂ and subsequent ROS measurement. (B) Protein folding in PI-sensitive AMO-1 and PI-resistant AMO-BTZ and AMO-CFZ cells evaluated by disulfide bond formation of the endoplasmic reticulum (ER)-localized mero-GFP construct. (C, D) ATP/ADP ratio in the cytosol (C) and ER (D) of AMO-1, AMO-BTZ and AMO-CFZ cells evaluated by specific ratiometric constructs localized in the cytoplasm or ER. (E) Cytotoxicity of 16F16, bortezomib (BTZ) or carfilzomib (CFZ) and the combination of 16F16 with BTZ or CFZ in AMO-BTZ and AMO-CFZ cells. Data represent the mean $\pm$ standard error of mean of three independent measurements. (F) Cytotoxicity of BTZ (MM1: 5 nM, MM2: 2.5 nM, MM3: 5 nM), CFZ (all 5 nM), 1 μ M 16F16 and their combinations in primary malignant plasma cells. The coefficient of drug interaction (CDI) is indicated. A CDI < 1 indicates a synergistic effect, a CDI = 1 indicates an additive effect, and a CDI > 1 indicates an antagonistic effect. Statistical significance is depicted with asterisks: **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

purines/pyrimidines, alanine/aspartate/glutamate, arginine/proline, pantothenate/coenzyme A, and cysteine, along with significant changes in the tricarboxylic acid cycle in the PI-resistant cell lines (Figure 1B). The most prominent changes in glutathione and NAD(P)⁺/NAD(P)H in AMO-BTZ and AMO-CFZ were further validated using individual assays that confirmed increased glutathione, NADP⁺ and NADPH in the PI-resistant cells, whereas the NADP⁺/NADPH ratio in the cells remained constant (Online Supplementary Figure S2). Graphical visualization of the most important metabolic changes in AMO-BTZ and AMO-CFZ in the key pathways driving glutathione synthesis and NAD(P)⁺/NAD(P)H production as well as increased levels of coenzyme A to fuel the tricarboxylic acid cycle and ATP production are summarized in Online Supplementary Figure S3. The data highlight increased antioxidant capacity, higher redox homeostasis and NAD⁺ levels as the key metabolic changes in PI-resistance and are consistent with findings from earlier models including our own proteomic data.⁵⁻¹³

We functionally validated the increased capacity of PI-resistant cells to buffer the formation of reactive oxygen species (ROS), demonstrating 2- to 4-fold lower ROS levels in these cells than in PI-sensitive cells, confirming a significantly higher antioxidant capacity (Figure 2A). Consistent with this, PI-resistant cells are more resistant than PI-sensitive cells to H₂O₂-induced cytotoxicity (Online Supplementary Figure S4, Online Supplementary

Table S2). In order to compare protein folding capacity directly between PI-resistant and PI-sensitive viable MM cells, the cells were equipped with mero-GFP, which enables quantitative real-time monitoring of functional protein folding based on the formation of disulfide bonds in the endoplasmic reticulum.¹⁴ PI-adapted cells showed significantly higher amounts of the folded, oxidized form of mero-GFP already at steady state level, reflecting a higher protein folding capacity through more effective disulfide bond formation. Upon short 5 min pulse treatment with the reducing agent dithiothreitol, mero-GFP is converted into its unfolded, fully reduced state in all types of cells. The reconstitution of the folded, fully oxidized state of mero-GFP was observed already 3 min after wash-out of dithiothreitol in PI-adapted MM, in contrast to PI-sensitive MM, directly demonstrating the increased activity of the protein folding machinery in PI-resistant MM cells (Figure 2B). Chaperones involved in protein folding are highly ATP-dependent. We therefore addressed whether metabolically adapted, PI-resistant MM show a higher rate of ATP production in the endoplasmic reticulum, which could drive more effective protein folding. To this end the cells were equipped with an ATP/ADP ratiometric construct that is selectively targeted to the cytosol or the endoplasmic reticulum. This construct allows assessment of changes in the ATP/ADP ratio in live cells in the endoplasmic reticulum *versus* the cytosol through quantitative fluorescence imaging. Both AMO-BTZ and AMO-CFZ showed a significantly

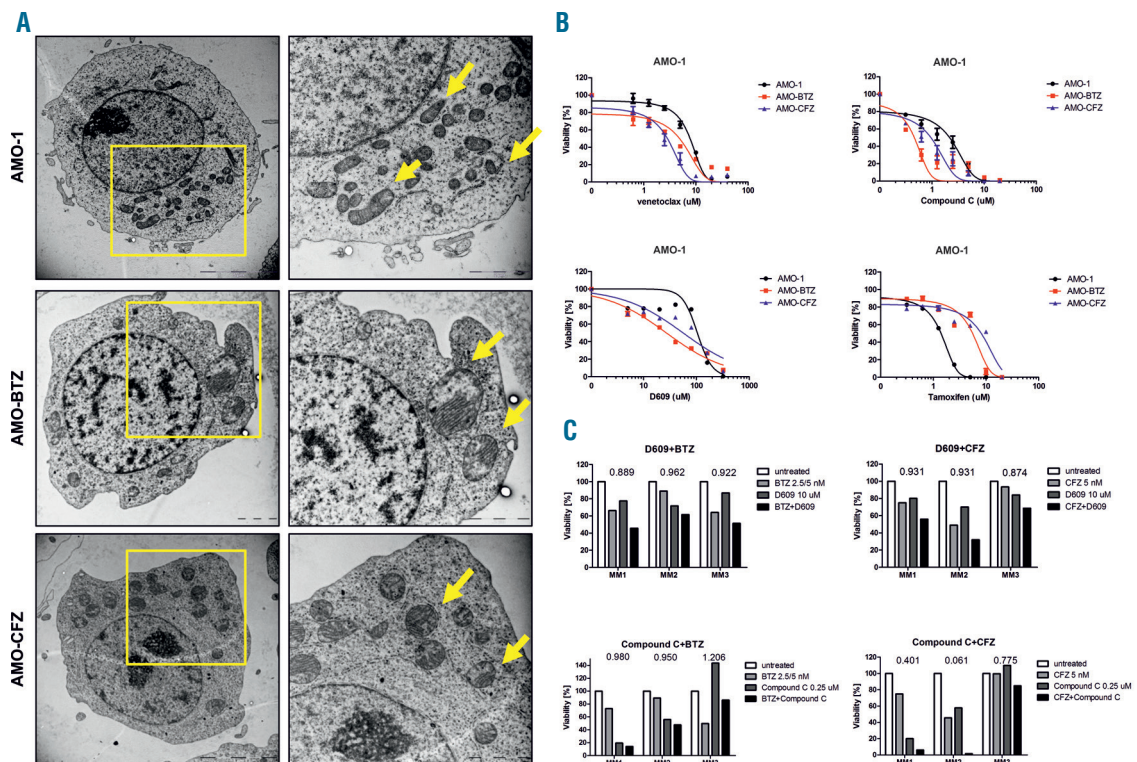


Figure 3. Changes in mitochondria morphology, energy demand and lipid composition in proteasome inhibitor-resistant cells. (A) The mitochondria in proteasome inhibitor (PI)-resistant AMO cells show prominent changes. The panels on the left show transmission electron microscopy images of whole cells, whereas those on the right are zoomed-in areas of the cell in the left panel with mitochondria indicated by the arrows. (B) Dose-response curves of PI-sensitive and PI-resistant AMO-1 cells (BTZ=bortezomib, CFZ=carfilzomib) to drugs targeting mitochondria, energy metabolism and lipid homeostasis: a BCL2 inhibitor (venetoclax), an AMPK inhibitor (compound C), a sphingomyelin synthase inhibitor (D609) and a sphingolipid metabolism inhibitor (tamoxifen). The half maximal inhibitory concentration (IC₅₀) values are presented in Online Supplementary Table S3. Data represent the mean ± standard error of mean of three independent measurements. (C) Primary malignant plasma cells were exposed for 48 h to bortezomib (BTZ; MM1: 5 nM, MM2: 2.5 nM, MM3: 5 nM) and carfilzomib (CFZ; all 5 nM) alone or in combination with 0.25 μM compound C or 10 μM D609.

reduced ATP/ADP ratio in the cytosol, compared to that of PI-sensitive cells, but significantly increased ATP/ADP ratio in the endoplasmic reticulum (Figure 2C,D). These results directly indicated a selective accumulation of ATP in the endoplasmic reticulum of PI-resistant MM cells, consistent with the high ATP demand for chaperone-driven protein folding.

The functional importance of protein folding for the PI-resistant phenotype was validated using specific inhibitors: the protein disulfide isomerase inhibitor 16F16 and the disulfide bond-disrupting agent TCyDTDO (*for more information see the Online Supplement*), which both, in combination with PI, significantly sensitized PI-adapted MM cell lines and primary MM cells derived from patients resistant to bortezomib or carfilzomib (Figure 2E,F, *Online Supplementary Figure S5*; the patients' characteristics are included in *Online Supplementary Table S3*). Targeting protein disulfide isomerase in MM *in vitro* and *in vivo* has been suggested as a promising therapeutic strategy in combination with bortezomib.¹⁵

Mitochondria serve as a major source of ATP through oxidative phosphorylation (OXPHOS). Increased ATP levels in PI-resistant cells are consistent with increased activity of the tricarboxylic acid cycle and the metabolic shift towards OXPHOS observed previously in these cells.⁶ To further highlight the key role of ATP supply and mitochondria in PI-resistant MM we conducted a morphological analysis using transmission electron microscopy. While mitochondria in PI-sensitive AMO-1 cells were a normal shape and size, delimited by double membranes that enclosed the matrix compartment, mitochondria in PI-resistant AMO-BTZ and AMO-CFZ cells were enlarged, round, and contained a much larger matrix compartment (Figure 3A). To our knowledge, this is the first demonstration of morphological changes in drug-resistant MM. The functional importance of these changes was assessed using mitochondria-targeting drugs: venetoclax (a BCL2 inhibitor) and compound C (an AMPK inhibitor). Strikingly, compared to PI-sensitive cells, PI-resistant cells were more sensitive to venetoclax and compound C (Figure 3B, *Online Supplementary Figure S6*, *Online Supplementary Table S2*). Moreover, compound C showed synergistic effects with carfilzomib in primary cells from MM patients (Figure 3C, *Online Supplementary Figure S6*). This suggests that the highly adapted metabolic machinery in PI-resistant MM may, in turn, render these cells particularly sensitive to drugs that target mitochondria function or metabolic activity.

Consistent with the increased energy production described above and changes in the metabolism of coenzyme A, we observed a lipid class switch from lysolipids to sphingomyelins, accompanied by accumulation of mono-acylglycerols in PI-resistant AMO-BTZ and AMO-CFZ cells (*Online Supplementary Table S4*). The role of sphingolipid synthesis in PI-resistance was evaluated using D609 (an inhibitor of sphingomyelin synthase) and tamoxifen (a sphingomyelin transferase inhibitor). D609 was more cytotoxic in PI-resistant cells than in PI-sensitive cells, in contrast to tamoxifen, which showed the opposite effect, supporting the functional importance of sphingolipid synthesis in PI resistance (Figure 3B, *Online Supplementary Table S2*) and likewise identifying the sphingomyelin biosynthesis pathway as a potential therapeutic target in PI-resistant MM. Consistently, the combination of D609 with bortezomib or carfilzomib showed synergistic effects in primary MM cells (Figure 3C). In conclusion, we present here a global metabolic analysis of bortezomib- and carfilzomib-resistant MM cells and elucidate the most prominent changes in two

PI-resistant MM cell lines on the functional level, especially in redox and energy homeostasis. Although the cell lines differed in some aspects, they both contained a highly complex signature of metabolic changes that were consistent with the predictions made from proteomic profiling,⁶ implicating a general pattern of adaptation to proteasome inhibiting drugs as being likely involved in cross-resistance between individual drugs. We suggest targeting the downstream pathways of high energy supply driven by mitochondria in PI-resistant cells, such as more effective protein folding or lipid homeostasis, as a promising strategy to selectively target the adaptive features of these PI-resistant MM cells. Our data support previous reports about high energy demand in PI-resistant cells¹⁶ and highlight global metabolic mitochondrial changes leading to increased protein folding and lipid rewiring as major drivers of adaptation towards PI, and at the same time identify vulnerable spots in cellular homeostasis specifically in PI-resistant MM. The key findings are summarized in the hierarchical model for the metabolic adaptation of PI-resistant MM (*Online Supplementary Figure S7*).

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5.5 Annex 5

Treatment with HIV-Protease Inhibitor Nelfinavir Identifies Membrane Lipid Composition and Fluidity as a Therapeutic Target in Advanced Multiple Myeloma

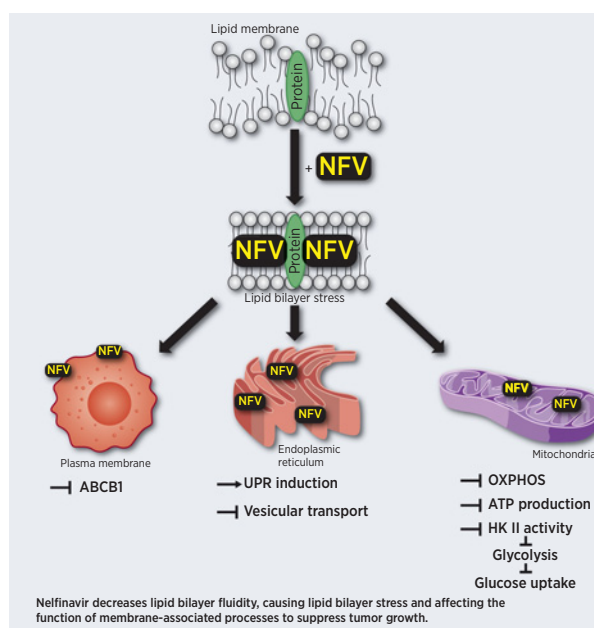


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ABSTRACT

The HIV-protease inhibitor nelfinavir has shown broad anticancer activity in various preclinical and clinical contexts. In patients with advanced, proteasome inhibitor (PI)-refractory multiple myeloma, nelfinavir-based therapy resulted in 65% partial response or better, suggesting that this may be a highly active chemotherapeutic option in this setting. The broad anticancer mechanism of action of nelfinavir implies that it interferes with fundamental aspects of cancer cell biology. We combined proteome-wide affinity-purification of nelfinavir-interacting proteins with genome-wide CRISPR/Cas9-based screening to identify protein partners that interact with nelfinavir in an activity-dependent manner alongside candidate genetic contributors affecting nelfinavir cytotoxicity. Nelfinavir had multiple activity-specific binding partners embedded in lipid bilayers of mitochondria and the endoplasmic reticulum. Nelfinavir affected the fluidity and composition of lipid-rich membranes, disrupted mitochondrial respiration, blocked vesicular transport, and affected the function of membrane-embedded drug efflux transporter ABCB1, triggering the integrated stress response. Sensitivity to nelfinavir was dependent on ADIPOR2, which maintains membrane fluidity by promoting fatty acid desaturation and incorporation into phospholipids. Supplementation with fatty acids prevented the nelfinavir-induced effect on mitochondrial metabolism, drug-efflux transporters, and stress-response activation. Conversely, depletion of fatty acids/cholesterol pools by the FDA-approved drug ezetimibe showed a synergistic anticancer activity with nelfinavir *in vitro*. These results identify the modification of lipid-rich membranes by nelfinavir as a novel mechanism of action to achieve broad anticancer activity, which may be suitable for the treatment of PI-refractory multiple myeloma.

Significance: Nelfinavir induces lipid bilayer stress in cellular organelles that disrupts mitochondrial respiration and transmembrane protein transport, resulting in broad anticancer activity via metabolic rewiring and activation of the unfolded protein response.



Introduction

The repurposing of established drugs is evolving as a promising, sustainable, cost- and time-saving approach to improve success rate, speed, and cost effectiveness of anticancer drug development (1). Nel-

finavir is a first generation HIV-protease inhibitor approved for HIV treatment that by design binds to the viral protease in a competitive manner, based on high enthalpy and entropy (2). To date, nelfinavir has largely been replaced for HIV treatment by next-generation HIV-protease inhibitors (HIV-PI)

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with increased specificity and efficacy (3). Meanwhile, nelfinavir has shown strong anticancer activity in multiple preclinical models and clinical trials, both as monotherapy (4, 5) and in combination with established antineoplastic drugs and treatment modalities (6, 7).

In particular, nelfinavir sensitizes cancer cells to proteasome inhibitor (PI) treatment, a backbone therapy for multiple myeloma (8, 9). The combination of nelfinavir with the PI bortezomib (BTZ) and carfilzomib (CFZ) overcomes PI resistance in preclinical models of multiple myeloma (10, 11) and has significant activity against solid tumors and hematological malignancies (8, 12, 13). In patients with bortezomib-refractory multiple myeloma, the combination of nelfinavir yielded an overall response rate (ORR, partial response or better) >65% in phase II clinical trial (14), scoring among the highest ORR observed in PI-refractory multiple myeloma in phase II/III trials.

A plethora of individual molecular effects of nelfinavir has been described previously to date: Induction of the unfolded protein response (UPR) through IRE1/XBP1, PERK/eIF2a, and ATF6 signaling (11, 15) inhibition of proteasomal protein degradation (11, 16, 17), inhibition of proteolysis and nuclear translocation of ATF6 and SREBP-1 (18, 19), fatty acid (FA) and cholesterol biosynthesis induction (20), STAT3 and PI3K/Akt signaling inhibition (21–23), and transmembrane multidrug transporter protein ABCB1 inhibition (10). It is unclear, however, whether such diverse effects are mediated through direct interaction of nelfinavir with different targets in different cell types, or if they represent downstream responses to a primary effect of nelfinavir on one, so far unknown, target. This uncertainty hampers both, a rational clinical repurposing development of nelfinavir as antineoplastic drug, as well as the design, synthesis and testing of next-generation nelfinavir-like compounds with optimized antineoplastic activity, and improved specificity or pharmacologic properties. Therefore, we aimed to identify direct targets of nelfinavir across different human malignant cell lines and link them with cell biological processes and mechanisms mediating sensitivity or resistance to nelfinavir treatment in cancer.

Materials and Methods

Cell lines and chemicals

Across the study, following cell lines were used: Multiple myeloma cell lines AMO-1 (DSMZ, German Collection of Micro-organisms and Cell Cultures GmbH) and its derivatives resistant to proteasome inhibitors bortezomib: AMO-BTZ and carfilzomib: AMO-CFZ. Further following cell lines were used: MDA-MB-231 (DSMZ), BT-474 (DSMZ), U-2 OS (ATCC) K562 (ATCC), HeLa (ATCC), HEK293 (ATCC), HEK293T (ATCC), and Caki2 (DSMZ). The cells were authenticated by STR-typing and routinely tested

for *Mycoplasma* contamination (MycoAlert Mycoplasma Detection Kit). For detailed information about cell lines maintenance, see Supplementary Methods. For the complete list of chemicals used across the study, see Supplementary Table S1.

Functionalized photoreactive nelfinavir-mimetics probes and chemical pull-down

To identify proteins that interact with nelfinavir in intact cells, a set of functionalized photoreactive nelfinavir-mimetics probes was synthesized: A functional ether modification of nelfinavir with a linker molecule containing the diazirine as photolabel and the alkyne as click handle (SC-441) and a nonfunctional modification of nelfinavir in the putative active site with diazirine and alkyne (SC-451). To validate functionality of probes, a modification of SC-441 without the diazirine (dummy probe, SC-454) was synthesized. Chemical synthesis of the probes is described previously in Supplementary Methods.

The pull-down experiments were carried out in triplicate in multiple myeloma cells (AMO-1 and AMO-CFZ) and breast cancer cells (MDA-MB-231, BT-474). The general experimental layout is shown in Supplementary Table S2. The whole procedure of chemical pull-down is in detail presented in Supplementary Methods.

CRISPR/Cas9 pooled library screen

For the genome-wide CRISPR/Cas9 screen, human Brunello CRISPR/Cas9 knockout pooled library was used. Detailed description of the whole procedure is presented in Supplementary Methods, for the complete list of primers used to amplify the library, see Supplementary Table S3.

Isotope tracing

¹³C tracer experiments upon nelfinavir treatment were performed as described before (24) and are in detail presented in Supplementary Methods.

Lipidomics

Lipidomic experiments with nelfinavir were performed in HEK293 and AMO-1 cells, global analysis of lipids was performed in AMO-1, MDA-MB-231, and Caki2 cells. For a detailed description, see Supplementary Methods.

Nelfinavir intracellular quantification

LC-MS-based quantification of nelfinavir was performed in AMO-1 cells. For a detailed description, see Supplementary Methods.

Fluorescence recovery after photobleaching experiments

Fluorescence recovery after photobleaching (FRAP) experiments upon nelfinavir treatment were performed with CFP-tagged Rab1A in HeLa cells and with C1-BODIPY-C12 in HEK293 cells. For a detailed description, see Supplementary Methods.

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Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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Laurdan dye staining to assess membrane fluidity

Live HEK293 and U-2 OS cells were stained with Laurdan dye at 15 $\mu\text{mol/L}$ in serum-free media for 45 minutes at 37°C. For a detailed description, see Supplementary Methods.

RUSH system and protein secretion assessment

Retention Using Selective Hooks (RUSH) system was used in U-2 OS cells for the visualization of protein trafficking upon nelfinavir or control treatment (25). For a detailed description, see Supplementary Methods.

Generation of cells with various reporter systems

U-2 OS cells were equipped with full length HKII and truncated HKII constructs, AMO-1 and MDA-MB-231 cells were equipped with ratiometric ATP/ADP constructs and AMO-1 and MDA-MB-231 cells were equipped with shRNA constructs that allowed for a decreased ADIPOR2 expression. For a detailed description of generation of respective cell lines, see Supplementary Methods.

Single gene knockout using CRISPR/Cas9

The specific knockout of a gene was performed using two-vector CRISPR/Cas9 system in AMO-1 cells and is described previously in Supplementary Methods. For a detailed information about the sequences of sgRNA used, see Supplementary Table S3.

Mitochondria metabolic activity analysis

For real-time analysis of extracellular acidification rates (ECAR) and oxygen consumption rates (OCR), AMO-1 cells were analyzed using an XF-96 Extracellular Flux Analyzer (Seahorse

Bioscience/Agilent Santa Clara) as described previously in detail elsewhere (26, 27).

Flow cytometry

Flow cytometry was used to assess the rate of apoptosis, ABCB1 efflux, glucose flux, and MHC class I expression in AMO-1 cell lines. For a detailed description, see Supplementary Methods.

Quantification and statistical analysis

Statistical evaluation was performed in GraphPad Prism v.5 (GraphPad Software). For group comparison, two-way ANOVA was used with Bonferroni post-test, for comparison of two groups unpaired *t* test was used, values $P < 0.05$ were considered as statistically significant. Specific statistical analysis for CRISPR/Cas9 screening, chemical pull-down analysis, lipidomics is presented in respective sections in Supplementary Methods.

Flow cytometry data were evaluated using FlowJo v10 Software (FlowJo Company) and are presented as a mean and $\pm$ SD of median fluorescence intensity of at least 3 independent experiments.

Results

Conserved binding partners of nelfinavir across different cell types are enriched in mitochondria and the ER membranes

To identify proteins that interact with the active site of nelfinavir in intact living cells, we synthesized photoreactive nelfinavir-mimetics: The nelfinavir active probe (SC-441), the nelfinavir inactive probe with a substitution in the putative active site (SC-451), and the dummy probe (SC-454; Fig. 1A; Supplementary Fig. S1). For the synthesis of

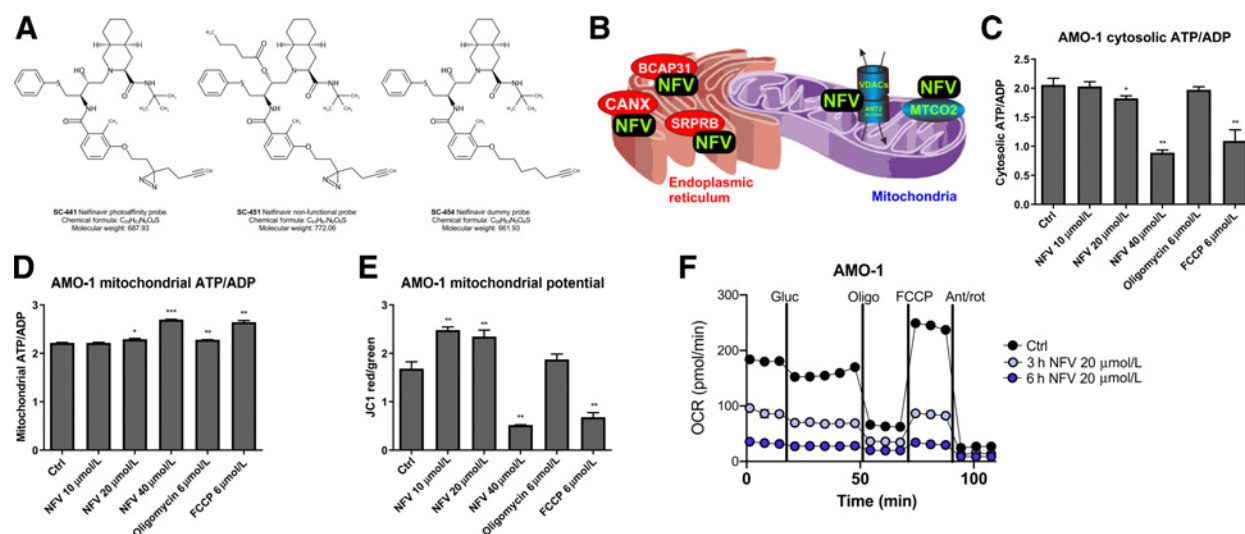


Figure 1.

Nelfinavir binds to targets in organellar membranes and affects ATP transport from mitochondria. **A**, A set of photoreactive nelfinavir-mimetic probes to identify nelfinavir targets in an activity-dependent fashion. For a detailed scheme illustrating synthesis of the probes and their cytotoxic activity in combination with carfilzomib, see also Supplementary Figs. S1 and S2. The experimental outline to identify candidate proteins binding to the active site of nelfinavir and the identified protein candidates are presented in Supplementary Tables S1 and S4. **B**, Schematic visualization of the localization of the conserved nelfinavir-binding partners across four different cell lines. **C**, Assessment of cytosolic ATP/ADP ratio in AMO-1 multiple myeloma cells upon treatment for 6 hours with increasing doses of nelfinavir, oligomycin, and FCCP as a positive control. For the analysis in MDA-MB-231 cells, see Supplementary Fig. S3. **D**, Assessment of mitochondrial ATP/ADP ratio in AMO-1 multiple myeloma cells upon treatment for 6 hours with increasing doses of nelfinavir, oligomycin, and FCCP. **E**, Assessment of the JC1 ratio in AMO-1 multiple myeloma cells upon treatment for 6 hours with increasing doses of nelfinavir, oligomycin, and FCCP. **F**, OCR as a function of mitochondria respiration assessed in AMO-1 cells after incubation with 20 $\mu\text{mol/L}$ nelfinavir for 3 and 6 hours. In all experiments, data represent a mean $\pm$ SD from three replicates and statistically significant differences are marked. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

SC-451, the available data were analyzed (28) and it was concluded that a modification of C(18) of nelfinavir with a hydrophobic residue could serve to inactivate the molecule. We used a short aliphatic moiety to change the molecular structure as little as possible. A crystal structure of nelfinavir with HIV protease (29) shows the hydroxyl group in the center of the binding pocket, thus a modification of the central hydroxyl may cause enough steric clash to disfavor binding of the inhibitor to the active site. The loss of activity of SC-451 was assessed as the loss of PI-sensitizing activity, in contrast with retained activity of SC-441, so that the probes differentiate between activity-dependent (specific), and activity-independent (nonspecific) interaction partners of nelfinavir. Moreover, a “dummy probe” that carries a terminal alkyne tail similar to the photo-reactive probe just without the photo-active diazirine moiety has been synthesized to confirm that the photo-active moiety has no effect on the SC-441 probe activity (Supplementary Fig. S2A). Three independent sets of experiments were performed (Supplementary Methods and Supplementary Table S1) to identify nelfinavir target proteins, and to verify and validate the hits in multiple myeloma cells (AMO-1), carfilzomib-resistant multiple myeloma cells (AMO-CFZ), as well as the breast cancer cell lines MDA-MB-231 and BT474, which are comparably sensitive to nelfinavir in a low micromolar range (NFV IC₅₀ values: AMO-1 = 10.5 μmol/L, AMO-CFZ = 11.7 μmol/L, MDA-MB-231 = 14.4 μmol/L, BT-474 = 14.9 μmol/L; Supplementary Fig. S2B).

Our approach identified 83 binding partners in four tested cell lines, the complete list of identified targets is provided in Supplementary Table S4. The functional impact of nelfinavir, based on all identified proteins, was further investigated by the search for Gene Ontology term enrichment using Enrichr software (30). The most significant GO terms for categories Biological Process, Cellular Component and Molecular Function are included in Supplementary Table S5. On the basis of these, the identified proteins are significantly enriched in lipid droplets, mitochondria, and ER organelles and are associated with processes related to mitochondria and the ER function, protein transport or Ras/Rab-related vesicular transport.

The eight overlapping activity-specific targets of nelfinavir identified in at least three out of four cell lines (Supplementary Table S6) are intramembrane-resident proteins with lipid- and cholesterol-interacting domains (31, 32), embedded predominantly in mitochondria, ER or cellular vesicles, consistent with the identified GO terms. The mitochondrial membrane-embedded proteins are proteins involved in the formation of the multiprotein mitochondria permeability transition pore [mPTP; such as voltage dependent anion channel proteins, VDACs, and adenine nucleotide translocator (ANT) proteins, known as ADP/ATP translocase proteins]. The ER membrane-resident proteins are involved in protein folding (calnexin, CALX), quality control, and export of newly synthesized proteins from the ER to Golgi (B-cell-associated protein, BAP31) or co-translational targeting of secretory and membrane proteins to the ER membrane (Signal recognition particle receptor subunit beta, SRPRB; Fig. 1B). Together, these results suggest that the interacting partners of nelfinavir are partially conserved across different cell types. These conserved binding partners are intra-membrane proteins, suggesting further that irrespectively of the cell type, nelfinavir localizes predominantly to membranous systems of the cells.

Shutdown of mitochondrial respiration and ATP transmembrane transport by nelfinavir

HIV-PIs have been suggested to suppress apoptosis by preserving mitochondrial function via their ability to prevent formation or

opening of the mPTP (33, 34). Our data show that nelfinavir directly interacts with several key proteins involved in mPTP formation, such as VDACs and ANT (Fig. 1B). The mPTP has been proposed to form F-ATP synthase dimers in the lipid region that generate ATP during oxidative phosphorylation (35, 36), whereas ANT proteins transport ATP synthesized from oxidative phosphorylation into the cytoplasm (37). To directly assess whether nelfinavir affects ATP generation or transport along the mitochondrial membrane to the cytosol, we determined ATP/ADP ratio using ratiometric ATP/ADP probes located in the cytosol and mitochondria of the cells. Nelfinavir dose-dependently decreased cytosolic ATP/ADP in two independent cell lines (Fig. 1C; Supplementary Fig. S3), whereas it increased mitochondrial ATP/ADP ratio (Fig. 1D), suggesting that it interferes with mitochondrial ATP transport. At the same time, nelfinavir changed mitochondria potential, which was observed by an initial accumulation, followed by consecutive strong dose-dependent decrease of the fluorescence of JC1, a cationic dye that accumulates in energized mitochondria (Fig. 1E). Next, to analyze the functional effect of nelfinavir on mitochondrial respiration (a proxy of oxidative phosphorylation), we measured the OCR. Nelfinavir inhibited mitochondrial respiration in a time-dependent manner (Fig. 1F), confirming the inability of mitochondria to perform oxidative phosphorylation in the presence of nelfinavir. In conclusion, nelfinavir disturbs ATP transport from mitochondria to the cytosol by affecting the function of mitochondria membrane-resident proteins, and thus impairs mitochondria metabolism.

Nelfinavir affects glycolysis by interfering with the VDAC-bound HKII-mediated glucose phosphorylation

HIV-PIs impair glycolysis and cause insulin resistance (38, 39). ATP is critical for the initial step of glycolysis in which glucose is phosphorylated by VDAC-bound hexokinase II (HK II). We hypothesized that nelfinavir may reduce the supply of ATP for VDAC-bound HKII by impairing the ATP translocation from mitochondria. By measuring uptake of fluorescent glucose analogue (2-NDBG) in AMO-1 cells we confirm that nelfinavir inhibits glucose flux in a dose- and time-dependent fashion (Fig. 2A). This finding is accompanied by decreased glycolysis, as determined by a significantly decreased ECAR as a proxy for lactic acid production (Fig. 2B). To further analyze this hypothesis, we followed the metabolism of ¹³C-glucose in AMO-1 multiple myeloma cells over time (8 and 24 hours) upon nelfinavir treatment. In addition, changes in levels of extracellular glucose metabolites were analyzed. Nelfinavir increased ¹³C-glucose levels in culture media, consistent with a decreased uptake of extracellular ¹³C-glucose and with lower glycolytic activity (Supplementary Fig. S4A). At the same time, nelfinavir-treated cells consistently showed a significantly reduced incorporation of ¹³C into downstream glucose metabolites: Glucose-6 phosphate, pyruvate, and lactate (Fig. 2C and D) and lower lactate production (Supplementary Fig. S4B). This block in downstream glucose metabolites persisted over 24 hours, and the respective metabolites were more significantly reduced over time in nelfinavir-treated cells. This work demonstrates that nelfinavir impairs intracellular glucose metabolism at the level of HKII processing into both the oxidative and non-oxidative pathways, consistent with reduced glycolysis and oxidative phosphorylation (Fig. 2E; Supplementary Fig. S5). Consequently, metabolites of the tricarboxylic acid cycle were also consistently decreased after nelfinavir treatment (Supplementary Fig. S5, relative data).

To independently confirm that nelfinavir affects glycolysis via ATP depletion and subsequent impairment of ATP supply to VDAC-bound HKII, rather than VDAC-free HKII, we used U-2 OS cells containing

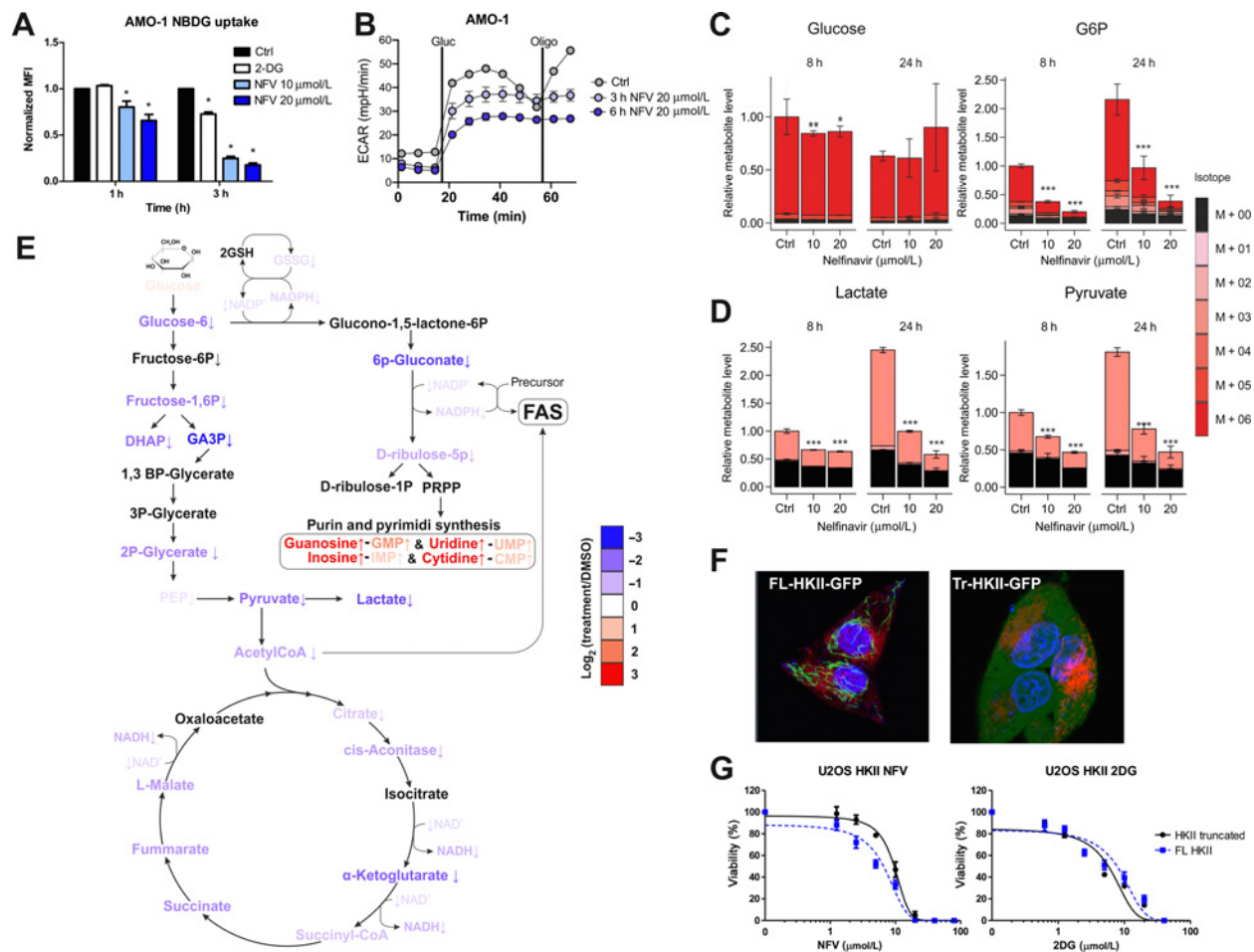


Figure 2.

Nelfinavir affects glycolysis by interfering with glucose phosphorylation mediated by HKII bound to VDACs. **A**, Glucose flux in AMO-1 cells was estimated by measuring the uptake of 2-NBDG upon nelfinavir treatment; 10 mmol/L 2-deoxyglucose (2-DG) served as a positive control of glucose flux inhibition. **B**, ECAR was assessed in AMO-1 cells after incubation with 20 $\mu\text{mol/L}$ nelfinavir for 3 and 6 hours. **C**, Relative levels of intracellular glucose and glucose-6-phosphate (G6P) after the treatment with nelfinavir. For relative levels of glucose in the cell culture media 8 hours after the treatment, see also Supplementary Fig. S4. **D**, Relative levels of intracellular levels of lactate and pyruvate after the treatment with nelfinavir. The legend for **C** and **D** represents the fractional abundance of ^{13}C isomers in the metabolites. **E**, A scheme illustrating change in level of metabolites from ^{13}C glucose after 24 hours incubation with 20 $\mu\text{mol/L}$ nelfinavir or DMSO only in AMO-1 cells. The color scale indicates $\log_2$ -fold change between the metabolites. For a detailed heat map illustrating the changes after 8 and 24 hours with 10 and 20 $\mu\text{mol/L}$ nelfinavir, see also Supplementary Fig. S5. **F**, Live imaging of single-cell-derived colonies from the U-2 OS cells equipped with FL-HKII (left) and Tr-HKII (right) constructs. ER is visualized with mCherry-ER-3 vector and nuclei by Hoechst staining. **G**, Dose response curves of U-2 OS cells equipped with FL-HKII and Tr-HKII exposed to increasing concentrations of nelfinavir or 2-deoxyglucose. Data represent a mean $\pm$ SD from three replicates and statistically significant differences are marked. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

either a full-length HKII (FL-HKII) or a truncated HKII (Tr-HKII), lacking the VDAC-binding sites (40). The FL-HKII localized strictly to rod-type structures in the cells, consistent with the interpretation that it is bound to mitochondria at the VDAC sites, whereas Tr-HKII is dispersed over the entire cytoplasm (Fig. 2F). Nelfinavir did not outcompete the FL-HKII from the mitochondria over the time, suggesting again that it does not impair its VDAC binding, but that it may affect its ATP supply. The FL-HKII-equipped cells were more sensitive to the cytotoxic effect of nelfinavir than the Tr-HKII cells, but equally sensitive to cytotoxicity induced by 2-deoxyglucose, a glucose analog that blocks HKII irrespective of its subcellular location (Fig. 2G). Altogether, these results implicate that nelfinavir causes reduced ATP availability for VDAC-bound HKII that impairs glycolysis and oxidative phosphorylation at the glucose phosphorylation level.

Nelfinavir inhibits ER to Golgi protein trafficking

As nelfinavir targets ER membrane-resident proteins (BAP31, CALX, SRPRB) and proteins required for vesicular protein transport (Rab proteins) between the ER and Golgi compartment, we hypothesized that apart from its known effects on protein homeostasis and induction of the UPR (8, 15), it also affects ER membrane dynamics and protein trafficking from the ER. To test this hypothesis, we used FRAP microscopy of CFP-tagged Rab1A, a GTPase required for vesicular protein transport from the ER to the Golgi compartment. Pretreatment of cells with increasing doses of nelfinavir for 3 hours before FRAP microscopy delayed the recovery of the bleached area in the Golgi starting at the 10 $\mu\text{mol/L}$ dose (Fig. 3A). The alteration of Rab1A dynamics at the Golgi opens the possibility that nelfinavir alters secretory trafficking in the early secretory pathway.

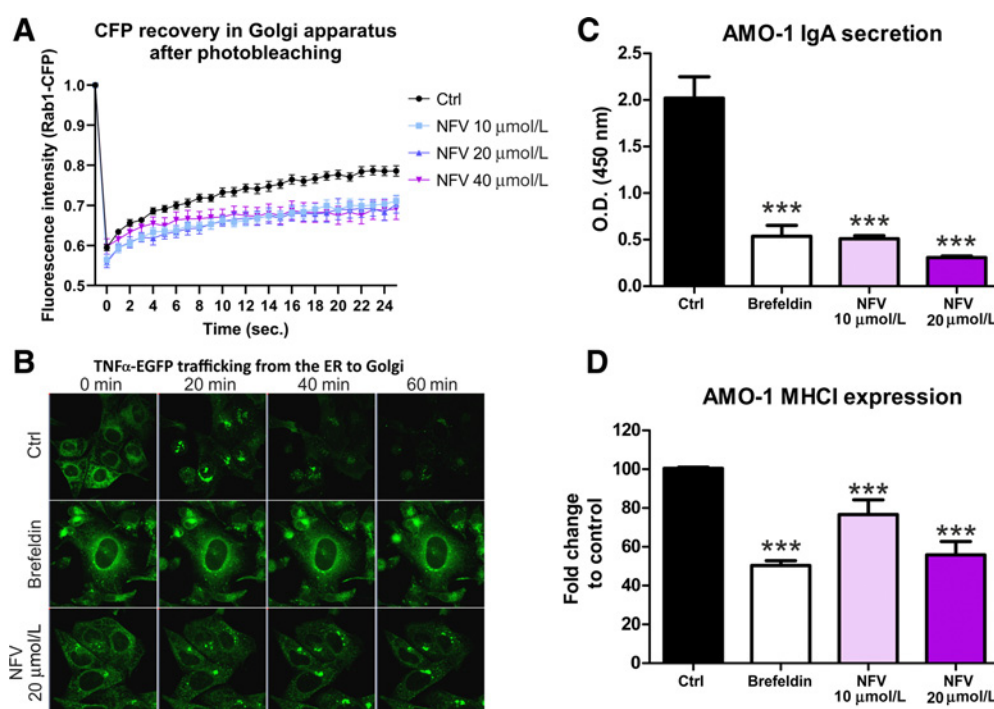


Figure 3.

Nelfinavir impairs intracellular trafficking, plasma membrane deposition, and secretion of ER-resident proteins. **A**, FRAP of CFP-Rab1A protein in the Golgi of control, untreated cells, or cells pretreated for 3 hours with increasing doses of nelfinavir. **B**, Representative picture of TNF α -eGFP retained in the ER of the U-2 OS cells after 3 hours treatment with 10 μ mol/L brefeldin A or 20 μ mol/L nelfinavir. For the movies showing trafficking of TNF α -EGFP after the treatment, see Supplementary Movie S1A–S1C. For the quantification of TNF α -EGFP signal retained in the cell after exposure to nelfinavir and other drugs, see Supplementary Fig. S6. **C**, IgA secretion in AMO-1 multiple myeloma after treatment for 3 hours with 10 μ mol/L brefeldin A or 10 and 20 μ mol/L nelfinavir. **D**, Surface expression of MHC class I on AMO-1 cells after treatment for 3 hours with brefeldin A or 10 and 20 μ mol/L nelfinavir. Data for **C** and **D** represent means $\pm$ SD from three independent replicates, statistically significant differences are marked. ***, $P < 0.001$.

To test this hypothesis, we generated U-2 OS cells equipped with RUSH system (Str-KDEL-TNF-SBP-EGFP; ref. 25). In this system, the TNF α -EGFP protein, which is initially bound to streptavidin (Str) via a streptavidin-binding peptide (SBP) and thus retained in the ER via the KDEL motif, is released upon biotin treatment, trafficks from the ER to the Golgi, and ultimately to the extracellular space. One hour after biotin treatment, we observed accumulation of the EGFP-tagged TNF α in the ER of the cells pretreated with brefeldin A (positive control) and nelfinavir (Fig. 3B, Movie S1A–C). Importantly, nelfinavir did not completely prevent trafficking of TNF α -EGFP from the ER, in contrast with brefeldin A, but rather delayed it. Flow cytometry-based quantification of the relative amount of EGFP-tagged TNF α that was retained in the cell upon treatment with different drugs (at $t = 0$ and $t = 60$ minutes after biotin treatment) showed that only nelfinavir and SC-441 caused retention of TNF α -EGFP in the cell, in contrast with SC-451, other HIV-PIs, the PIs bortezomib and carfilzomib or other known UPR-inducing drugs (tunicamycin and thapsigargin; Supplementary Fig. S6). We subsequently evaluated, whether nelfinavir affects protein transport of secretory and membrane proteins along the secretory pathway, such as MHC class I surface expression or immunoglobulin A secretion in multiple myeloma cells. Nelfinavir significantly decreased both IgA secretion and MHC class I surface expression 3 hours after treatment (Fig. 3C and D). Together, these results show that via interaction with several ER and vesicle membrane-resident proteins, nelfinavir functionally impinges on ER to Golgi vesicular protein trafficking and protein secretion.

Genes involved in vesicular transport and lipid metabolism modulate sensitivity/resistance toward nelfinavir

Genetic knockout of single direct interaction proteins of nelfinavir (BAP31 or MTDH) in AMO-1 cells did not affect nelfinavir cytotoxicity (Supplementary Fig. S7A–S7C), suggesting that either direct interaction of nelfinavir with several of the identified nelfinavir-binding proteins may be critical for its cytotoxicity, or that integration of active nelfinavir into cellular membranes results in its interference with a plethora of intramembrane proteins. To further identify the key functional pathways involved in nelfinavir cytotoxicity in cancer cells, we performed genome-wide CRISPR/Cas9 screening using the Brunello library in the K562 cell line. Both, negative and positive-selection screen with 5 and 10 μ mol/L nelfinavir were used to identify genes whose loss sensitizes the cells to a low concentration of nelfinavir or that allow cell survival in the presence of higher concentrations of nelfinavir decreasing the viability to 50%. Overall, we identified 7 candidate sensitivity genes (*ACACA*, *ATG9A*, *CLUH*, *MYLIP*, *VAPA*, *CSTB*, and *GOSR2*) at an FDR < 0.01 , with highest negative fold change, relative to control ($\log FC < -0.8$) and 1 candidate resistance gene at FDR < 0.01 and $\log FC > 2$ (Fig. 4A; Supplementary Table S7A and S7B). The candidate sensitivity genes are particularly involved in FA and cholesterol metabolism, vesicular formation and trafficking and mitochondria biogenesis, whereas the only identified resistance gene, *ADIPOR2*, encodes a member of the PAQR (Progesterone and AdipoQ Receptor) protein family. *ADIPOR2* is an integral component of cellular membranes that maintains membrane fluidity and cell viability in the presence of exogenously added

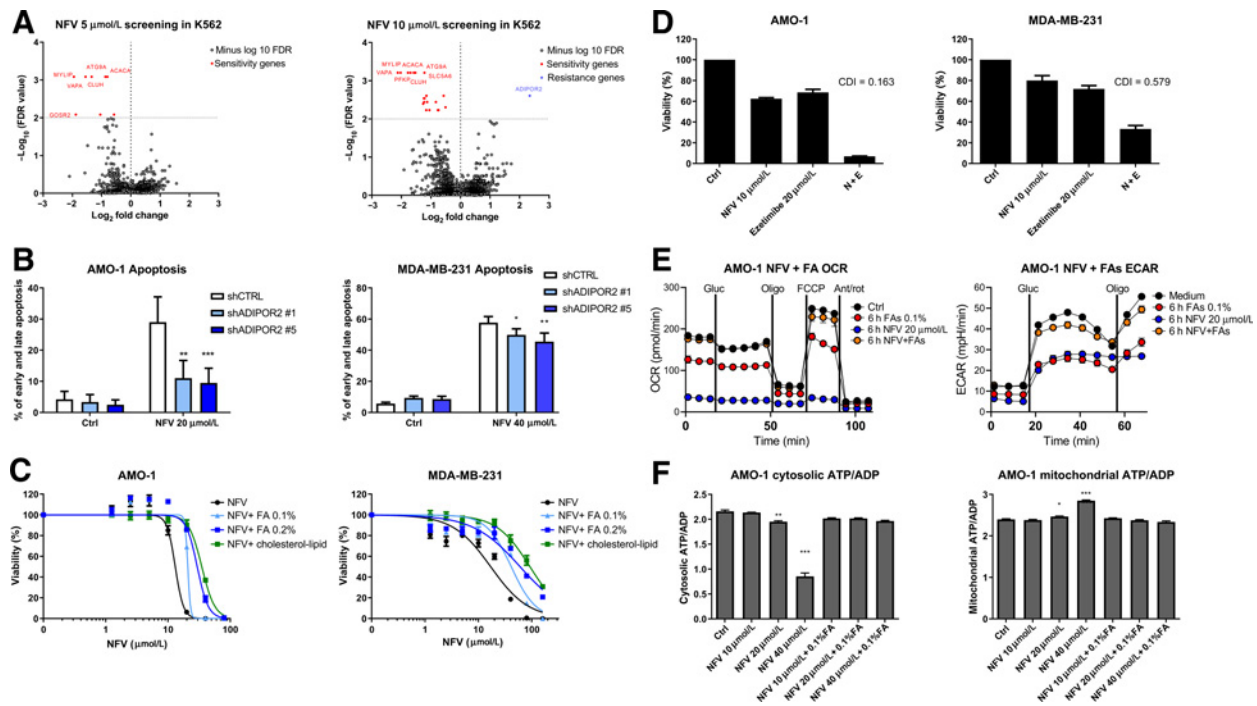


Figure 4.

CRISPR/Cas9 library screening suggests involvement of *ADIPOR2* and fatty acids in the resistance to nelfinavir and consequently modulation of fatty acids changes nelfinavir-induced effects on cell viability and energetics. **A**, Genome-wide CRISPR/Cas9 library screening in K562 cells with 5 and 10 $\mu\text{mol/L}$ nelfinavir identified candidate genes involved in nelfinavir sensitivity (red) or in nelfinavir resistance (blue) at the cutoff value of $-\log_{10} \text{FDR} = 2$. For a detailed list of the sensitivity and resistance candidate genes, their log-fold change over the DMSO-treated cells, and FDR value, see Supplementary Table S7A and S7B. **B**, Apoptosis rate evaluated 24 hours after treatment with 20 $\mu\text{mol/L}$ nelfinavir in the AMO-1 and MDA-MB-231 cells with decreased *ADIPOR2* expression. For the efficacy of *ADIPOR2* silencing in the two cell lines, see Supplementary Fig. S8. **C**, Dose response curves of cell lines exposed to increasing doses of nelfinavir alone or in combination with fatty acid (FA) supplement or cholesterol-lipid concentrate. For the cytotoxicity of increasing doses of FA supplement alone, see Supplementary Fig. S9A. **D**, Cytotoxicity of nelfinavir (N), ezetimibe (E), and their combination (N+E) in the presence of 0.1% FA supplement or cholesterol-lipid concentrate, see Supplementary Fig. S9B and S9C. **E**, OCR (left) and ECAR (right) in AMO-1 cells 6 hours after treatment with 20 $\mu\text{mol/L}$ nelfinavir, 0.1% FA supplement, or their combination. **F**, Assessment of the cytoplasmic (left) and mitochondrial (right) ATP/ADP ratio in AMO-1 cells 6 hours after treatment with increasing concentrations of nelfinavir (10, 20, and 40 $\mu\text{mol/L}$) alone or in combination with 0.1% FA supplement. In all experiments, viability was assessed 48 hours after continuous treatment. For the drug combinations, coefficient of drug interaction was calculated. Data of viability assays, flow cytometry, and Seahorse analysis represent a mean $\pm$ SD from three replicates and statistically significant differences are marked. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

saturated FAs, it acts primarily by promoting FAs desaturation and their incorporation into phospholipids, which helps to restore membrane fluidity (41).

To validate the screening data with an independent approach, we silenced the expression of *ADIPOR2* with shRNA in AMO-1 and MDA-MB-231 cells (Supplementary Fig. S8A and S8B). Decreased *ADIPOR2* level significantly protected the cells from nelfinavir-induced apoptosis (Fig. 4B). Together, these results suggest that functional pathways involved in nelfinavir's cytotoxicity are conserved across different cell types and center around FA metabolism and membrane fluidity.

Fatty acids modulate sensitivity toward nelfinavir and prevent nelfinavir-induced mitochondria shut down

To directly address the role of FAs in nelfinavir-induced cytotoxicity, we exposed nelfinavir-treated AMO-1 and MDA-MB-231 cells to increasing doses of FA supplement, an aqueous mixture of cholesterol-free saturated and unsaturated fatty acids. Initially, we tested the cytotoxicity of FA supplement alone and set the doses of 0.1% and 0.2% to have minimal effect on cell viability (Supplementary Fig. S9A).

FA supplement rescued the cells from the cytotoxic activity of nelfinavir in a dose-dependent fashion (Fig. 4C). Moreover, co-treatment of the cells with a cholesterol-lipid concentrate (in a dilution of the commercial product at 1:250, in agreement with the manufacturer's recommendation for cell culture supplementation) prevented toxicity even more effectively (Fig. 4C), suggesting that increasing the supply of membrane components (FA and cholesterol) protects against nelfinavir. In contrast, depletion of FA/cholesterol by ezetimibe, an FDA approved drug reducing lipid and cholesterol uptake, resulted in a highly synergistic cytotoxic effect in combination with nelfinavir against both cell lines (Fig. 4D). This synergistic cytotoxicity could likewise be abolished by the presence of 0.1% FA supplement or the cholesterol-lipid concentrate in both cell lines (Supplementary Fig. S9B and S9C).

Next, we addressed whether the prevention of nelfinavir-induced cell death by FA would likewise restore mitochondrial respiration and glycolysis. Treatment with 0.1% FA supplement abolished the nelfinavir-induced block in mitochondrial respiration (OCR) and glycolysis (ECAR) in AMO-1 cells (Fig. 4E). Likewise, 0.1% FA supplement restored the nelfinavir-induced cytosolic ATP/ADP decrease and

mitochondrial ATP/ADP increase (Fig. 4F). Therefore, FA and cholesterol significantly antagonize nelfinavir-induced cytotoxicity and reverse the nelfinavir-induced metabolic shut-down caused by impaired ATP transport through the mitochondrial membranes.

The incorporation of nelfinavir into cellular lipid membranes impairs membrane fluidity

The uniform pattern of intramembrane protein interaction partners of nelfinavir, its highly lipophilic nature (42) and the modulation of the downstream effects of nelfinavir by FA supplementation together suggest that nelfinavir integrates into biomembranes of eukaryotic cells, where it may affect the composition and physical properties of such membranes. To directly test this hypothesis we performed FRAP experiments using C1-BODIPY-C12 recovery. Nelfinavir significantly slows down the recovery of the C1-BODIPY-C12 signal in the bleached area, indicating a significant rigidification of the biomembranes (Fig. 5A and B). This observation was independently confirmed by staining of the cells with laurdan dye, a reporter of membrane penetration by water that correlates with fluidity. Variations in membrane water content cause a shift in the laurdan emission spectrum, which can be quantified by calculating

the generalized polarization (GP) index. Nelfinavir-treated HEK293 and U-2 OS cells had significantly more rigid membranes, presented as an increased GP index, including distinct internal structures with significant rigidity (Fig. 5C and D; Supplementary Fig. S10A and S10B). This effect is nelfinavir-specific and is not observed for other drugs, such as the PIs bortezomib and carfilzomib (Supplementary Fig. S10C). To further dissect if nelfinavir co-treatment with FA can prevent membrane rigidification and to address the effect of saturated versus unsaturated FA, we exposed the cells to nelfinavir in combination with saturated (16:0 palmitic acid) and unsaturated (16:1 palmitoleic acid) FA for 6 hours. Only unsaturated FA were able to prevent nelfinavir-induced changes in GP index, whereas loading the cells with saturated FA had the opposite effect and significantly potentiated the effect of nelfinavir on membrane rigidity (Fig. 5E). Interestingly, co-treatment with FA supplement reduced the amount of intracellular nelfinavir, compared with cells treated with nelfinavir alone (Fig. 5F), suggesting that FA may compete with nelfinavir for membrane uptake and thus prevent membrane rigidification. Overall, the data suggest that nelfinavir integrates into lipid-rich membranes of eukaryotic cells and increases membrane rigidity.

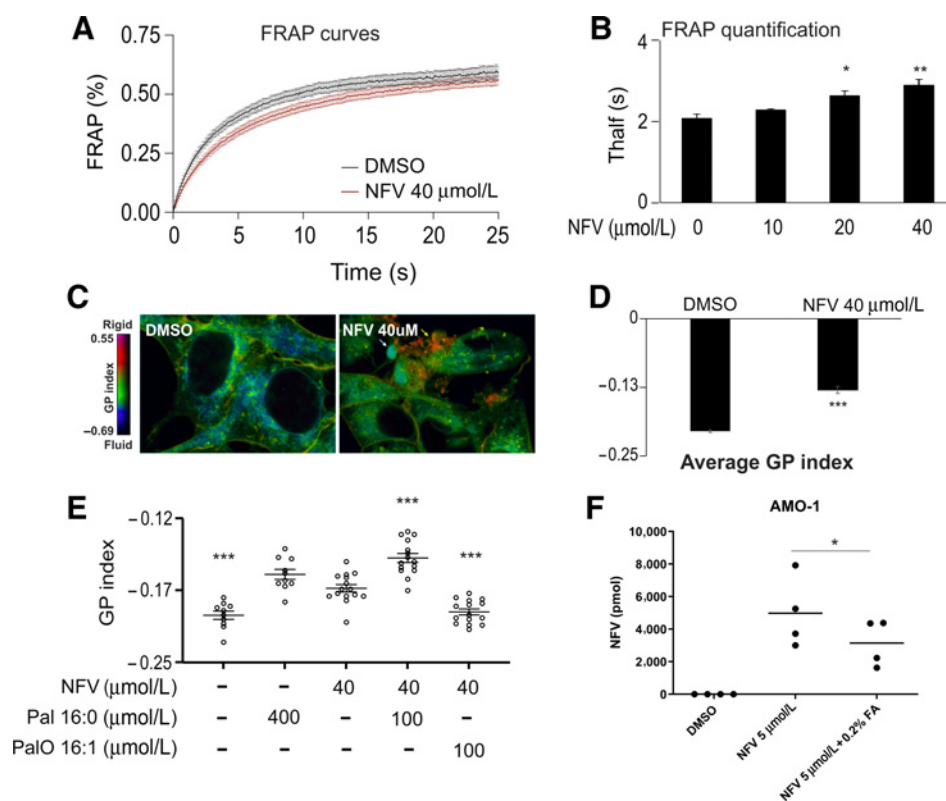


Figure 5.

Nelfinavir increases membrane rigidity, which can be reverted by unsaturated FA. **A**, FRAP results in HEK293 cells challenged with 40 μmol/L nelfinavir for 6 hours. Data of a representative experiment with $n = 15$ –18. **B**, Quantification of a FRAP experiment in HEK293 treated with increasing concentrations of nelfinavir for 6 hours. Average T_{half} values, the time by which half of the maximum fluorescence recovery is reached. Data of a representative experiment with $n = 5$ –15. **C**, Pseudocolor images showing the laurdan dye GP index at each pixel position in HEK293 cells challenged with 40 μmol/L nelfinavir for 6 hours. Yellow arrow, a spot with very strong rigidity. **D**, Average GP index from several images as depicted in C ($n = 15$ –19). **E**, Average GP index from several images of the laurdan dye staining in U-2 OS cells challenged with 40 μmol/L nelfinavir alone or in combination with saturated (Pal) or unsaturated (PalO) FA for 6 hours. For the pseudocolor images showing the laurdan dye staining in U-2 OS cells, see Supplementary Fig. S10A. For the effect of bortezomib and carfilzomib on membrane fluidity, see Supplementary Fig. S10C. **F**, Intracellular nelfinavir assessment upon treatment of AMO-1 cells for 6 hours with 5 μmol/L nelfinavir alone or in combination with 0.2% FA supplement. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

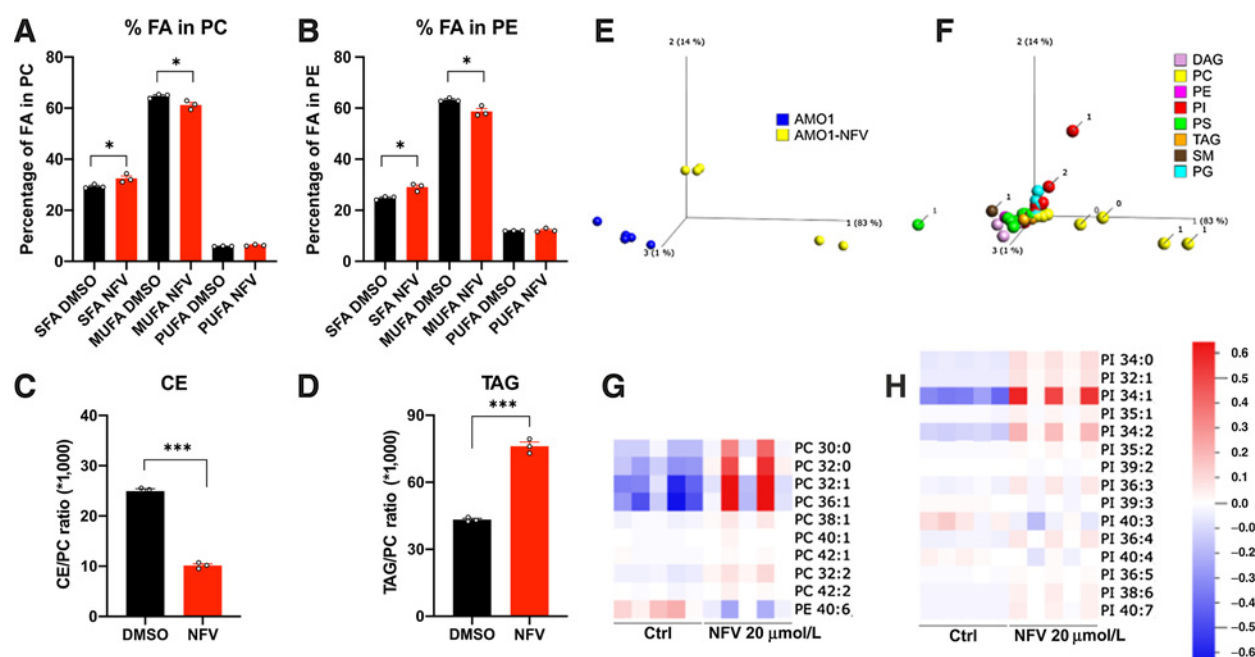


Figure 6.

Nelfinavir impairs the homeostasis of lipid composition in lipid-rich membranes and lipid droplets. **A**, Relative contents of FAs in membrane phosphatidylcholines (PC) upon treatment with 40 μ mol/L nelfinavir for 6 hours. SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids. **B**, Relative content of FA in membrane PEs upon treatment with 40 μ mol/L nelfinavir for 6 hours. **C** and **D**, Cholesterol esters (CE; **C**) and triacylglycerols (TAG; **D**) in lipid droplets upon treatment with 40 μ mol/L nelfinavir for 6 hours expressed as a ratio between cholesterol ester or triacylglycerol to membrane PC. **E**, Principal component analysis plot separating control and 20 μ mol/L nelfinavir-treated AMO-1 cells for 6 hours, based on their lipid composition. **F**, Lipid composition separating untreated and nelfinavir-treated AMO-1 cells, where the number of double bonds is indicated for the most differentiated lipids. Note that the main separators are PC containing 0 or 1 double bonds. **G** and **H**, Heat maps for PC/PE and PI species. Data show results of five replicates. Data show the mean of a representative experiment $\pm$ SEM and statistically significant differences are marked. *, $P < 0.05$; ***, $P < 0.001$. PI, phosphatidylinositol.

Nelfinavir alters composition of lipids predominantly in lipid membranes

The effect of nelfinavir on lipid-rich membranes led us to hypothesize that it affects the composition of cellular lipids. The quantitative and qualitative analysis of the lipid content 6 hours after nelfinavir treatment in HEK293 cells shows that nelfinavir causes a significant increase in saturated FA (SFA) in membrane phospholipids [both phosphatidylcholines (PC) and phosphatidylethanolamines (PE)], whereas monounsaturated FA (MUFA) were decreased (Fig. 6A and B). SFA increases membrane rigidity, whereas MUFA promotes membrane fluidity (41), consistent with the data indicating a significant loss of membrane fluidity upon nelfinavir treatment. Nelfinavir likewise causes significant changes in the composition of lipid droplets, where we observed a strong decrease in the relative fraction of cholesterol esters, whereas triacylglycerols were increased (Fig. 6C and D). An independent global lipidomics analysis performed in AMO-1 cells confirmed the previous data and shows in more detail that nelfinavir predominantly affects PC and phosphatidylinositols (Fig. 6E and F), two lipid species present predominantly in the membranes. Specifically, nelfinavir increases the unsaturated forms of PC and phosphatidylinositols with low numbers of double bonds, whereas PE with high numbers of double bonds are decreased (Fig. 6G and H). Interestingly, relative resistance of the Caki2 cell line to nelfinavir (NFV IC_{50} = 20.7 μ mol/L) compared with AMO-1 or MDA-MB-231 cells (NFV IC_{50} = 11.7 and 14.4 μ mol/L, respectively), is associated with enrichment of unsaturated PC, PE, and phosphatidylinositols in this cell line (Supplementary Fig. S11A–S11E).

Perturbation of membrane lipid homeostasis by nelfinavir or ezetimibe induces the UPR, inhibits efflux by ABCB1, and shows synergistic cytotoxicity with PIs

One of the main cellular responses to the perturbation of lipid and cholesterol homeostasis is the induction of the UPR, mainly through activation of the IRE1/XBP1 and ATF3 signaling (43, 44). Nelfinavir was observed previously to activate the IRE1/XBP1 pathway (8, 11). Moreover, it induced rapid and potent expression of ATF3 and CHOP (Fig. 7A). The induction of the UPR was prevented by the cotreatment with increasing, nontoxic concentrations of FA supplement (0.1% and 0.2%), supporting the interpretation that nelfinavir directly induces the UPR by affecting the lipid composition of biomembranes (Fig. 7A). Interestingly, a similar profile of UPR induction observed for nelfinavir was obtained when cells were treated with 40 μ mol/L ezetimibe (Fig. 7B), a dose resulting in comparable cytotoxicity with 20 μ mol/L nelfinavir (Supplementary Fig. S12A). We previously demonstrated that nelfinavir is a potent modulator of ABCB1 drug export pump (10). We here show that ezetimibe likewise partly inhibits ABCB1 function, and that ABCB1 inhibition by nelfinavir and ezetimibe can be rescued by FA supplement (Supplementary Fig. S12B).

Nelfinavir has synergistic activity with PI against myeloma *in vitro* and in PI-refractory patients with multiple myeloma (11, 14). Myeloma cells adapted to continuous PI treatment *in vitro* are characterized by altered membrane lipid composition (45), suggesting that the specific membrane properties may be important for cell survival under continuous PI pressure. To address whether

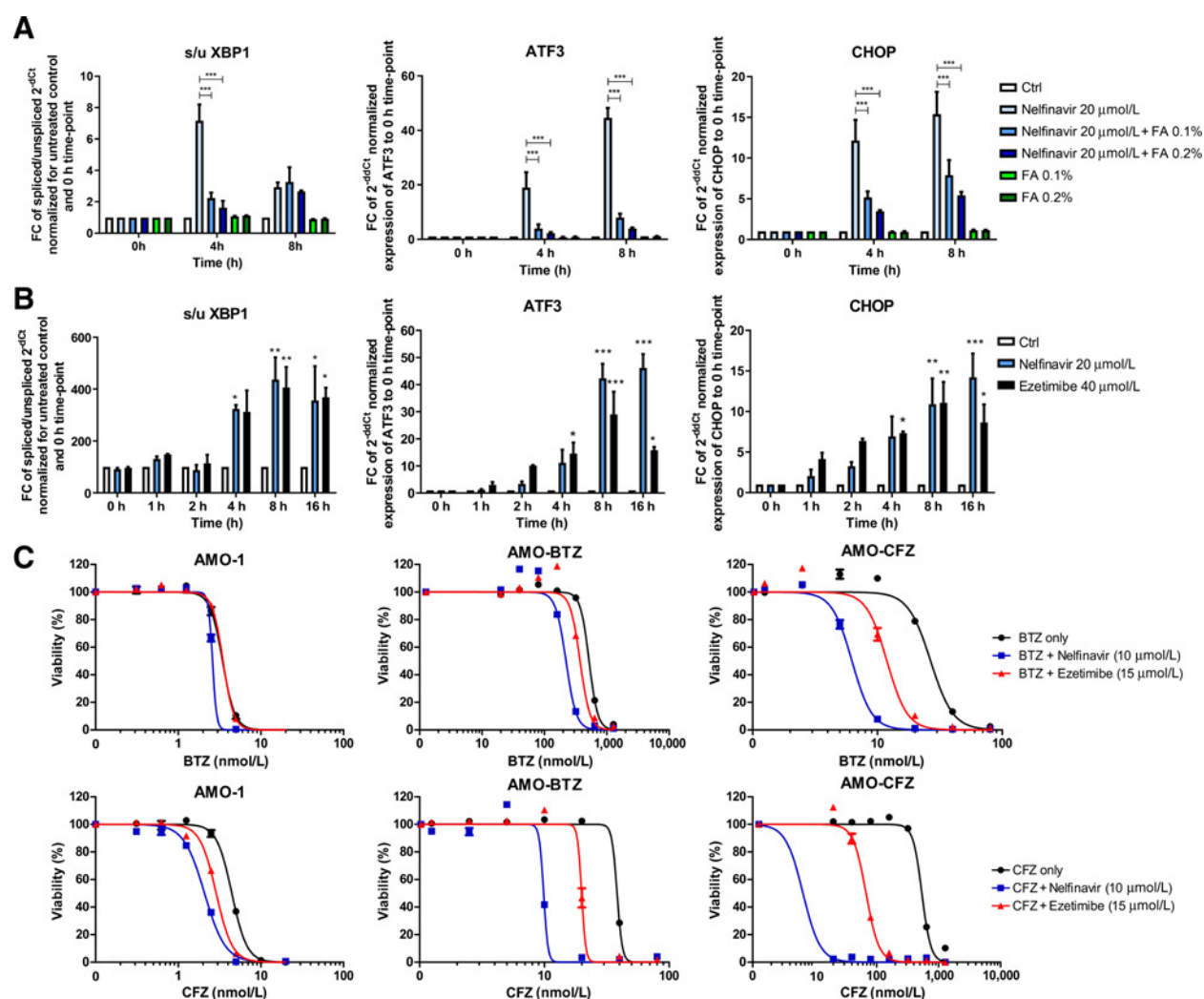


Figure 7.

Nelfinavir induces lipid-bilayer stress by rigidification of lipid-rich membranes, which triggers UPR induction that overcomes proteasome inhibitor (PI) resistance in multiple myeloma in combination with PIs. **A**, Induction of the UPR in AMO-1 cells assessed as the increase in the splicing of the XBP1, ATF3, and CHOP expression by 20 μmol/L nelfinavir alone, 0.1% and 0.2% fatty acids (FA) supplement or their combination. **B**, UPR induction by equally cytotoxic doses of nelfinavir (20 μmol/L) and ezetimibe (40 μmol/L) assessed as the increase in the splicing of the XBP1, ATF3, and CHOP expression. For the dose-response curves of cells to nelfinavir and ezetimibe see Supplementary Fig. S12A. **C**, Dose-response curves of PI-sensitive AMO-1 cells and bortezomib (BTZ) and carfilzomib (CFZ)-resistant cells alone and in combination with 10 μmol/L nelfinavir and 15 μmol/L ezetimibe. For the IC₅₀ values and IC₅₀ fold change between the single drugs and the combination in respective cell lines, see also Supplementary Table S8. The data represent mean ± SD of three independent repeats, statistically significant differences are marked. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

the observed synergistic cytotoxicity of nelfinavir with PI against multiple myeloma, and in particular against PI-resistant multiple myeloma is directly linked to perturbation of lipid homeostasis, we compared the cytotoxicity of nelfinavir with the effect of ezetimibe, both combined with PI. Ezetimibe overcame PI resistance in combination with bortezomib and carfilzomib in AMO-BTZ and AMO-CFZ and showed superior synergistic toxicity in PI-adapted cells in comparison with PI-sensitive cells, closely resembling the synergistic cytotoxic activity of nelfinavir (Fig. 7C; Supplementary Table S8). However, the magnitude of the synergistic cytotoxic activity of ezetimibe was lower compared with nelfinavir. Nevertheless, the combination between nelfinavir and ezetimibe showed a strong synergistic cytotoxic effect in PI-resistant cells (Supplementary Fig. S12C), suggesting that both drugs may differentially affect cellular lipid homeo-

stasis, triggering the same effector cascade for cytotoxicity. The manipulation of lipid homeostasis in conjunction with proteasome inhibition is a promising way to overcome PI resistance of multiple myeloma.

Discussion

We here characterize the molecular target and mechanism of action for the antineoplastic activity of nelfinavir. Nelfinavir binds to proteins embedded in lipid-rich cellular membranes, which subsequently alters membrane composition and reduces membrane fluidity of the cell and cellular organelles. These changes in cellular membranes result in UPR induction, defective subcellular, and transmembrane trafficking and interfere with key components of cellular energy

supply, including glucose metabolism, cellular respiration and ABCB1 activity.

Our model is supported by multiple lines of evidence. First, we identified a common set of nelfinavir-interacting proteins embedded in intracellular membranes and conserved across multiple cancer cell types, supporting general, rather than cell-type-specific interactions. Next, we identified a key regulator of lipid membrane composition and fluidity, *ADIPOR2* (46–48), as a unique genetic driver to mediate nelfinavir-induced cytotoxicity. Subsequently, we directly demonstrated the quantitative changes in membrane lipid composition and the induction of increased membrane rigidity upon nelfinavir treatment. On the basis of this, we hypothesized that nelfinavir integrates into lipid-rich membranes due to its very high lipophilicity (42), thereby affecting membrane fluidity in a structural manner, and competing against intramembrane FA and/or cholesterol. The physico-structural alteration of lipid-rich membranes caused by nelfinavir affects the function of membrane-associated processes.

The accurate composition of lipid membranes allowing high membrane fluidity is crucial for cancer cells (49–51). Pharmacological targeting of membrane lipid composition and fluidity is emerging as a novel field for potential therapeutic intervention (52). Nelfinavir can therefore be viewed as the first clinically active anticancer drug that acts through targeting structural properties of cellular membranes.

Nelfinavir impairs the function of several membrane-associated protein machineries important for tumor cell survival and growth, that is, glucose uptake and metabolism, oxidative phosphorylation, ATP production/transport, protein and vesicle transport and ABCB1 activity. Altered metabolic and glycolytic activity is a basic hallmark of cancer (53, 54) that represents an important target for specific pharmacological intervention. Nelfinavir significantly decreases glucose metabolism at the level of HKII activity, which matches the reduced glucose uptake and expression of GLUT receptors in patient-derived cells upon treatment with anti-retroviral agents (38), as well as reduced glucose flux and insulin resistance leading to hyperglycemia in patients on the anti-retroviral therapy (39, 55). Nelfinavir thus may be used for targeted disruption of glucose metabolism in diverse cancer types.

Our study suggests a comprehensive model of molecular targets and downstream effects that allows to integrate numerous observations that have been made in the past regarding the activity of nelfinavir on cancer cells. Nelfinavir has been shown to bind to ANT and VDAC proteins embedded in mitochondria (33). We show that nelfinavir directly binds to ANT2 or VDACs proteins. Disruption of the nuclear envelope integrity leading to a release of nuclear DNA into the cytoplasm (56) is consistent with the binding of nelfinavir to ZMPSTE24 (FACE1; Supplementary Table S2) embedded in the nuclear envelope. Our genome-wide screening data reveal candidate genes involved in nelfinavir resistance and sensitivity, such as *EIF2AK4* and *PPP1R15B*, respectively, that play a role in eIF2a signaling. eIF2a signaling, as part of the integrated stress response, has been shown to be modulated by nelfinavir, and PPP1R15B has been proposed as a direct nelfinavir target (15). Moreover, earlier observations of nelfinavir inhibiting the processing and nuclear translocation of ER membrane-embedded transcription factors SREBP-1, ATF6 or TCF11/Nrf1 (17–19) may be well explained by our finding that nelfinavir interferes with the functionality of ER membranes and ER-Golgi trafficking.

Clinical activity of nelfinavir-based therapy with PI has yielded a noteworthy >65% ORR in patients with PI-refractory multiple myeloma (14). The cell biology of multiple myeloma cells adapted to PI is highly complementary to the mechanism of action of nelfinavir identified here. PI-resistant multiple myeloma cells show alterations in membrane lipid composition, cellular metabolism, and metabolic reprogramming toward higher oxidative phosphorylation, which leads to increased redox and protein-folding capacity (24, 27, 45). Nelfinavir, as we show here, increases membrane rigidity and decreases the activity of multiple membrane proteins and membrane-associated processes, disrupts ATP transport, and blocks the activity of the ABCB1 transmembrane drug exporter, whose activity is involved in PI resistance, as we have shown previously (10, 12).

In conclusion, we here identify altered lipid homeostasis and membrane lipid composition as the basis for the anticancer activity of nelfinavir. Consequently, drugs that interfere with cellular lipid uptake showed effects similar to nelfinavir and synergized with nelfinavir *in vitro*. Elevated blood lipids are a major side effect of nelfinavir treatment in patients with HIV (57). The high serum lipids induced by nelfinavir may therefore even have antagonized the anti-multiple myeloma activity of nelfinavir in the clinical setting over time, which might partly explain the limited duration of the clinical responses observed in the clinical trial (14). The addition of lipid lowering drugs like ezetimibe to the nelfinavir-containing regimen is likely feasible and may allow to further improve the clinical effectiveness of the nelfinavir-based treatment for PI-resistant multiple myeloma.

Authors' Disclosures

H. Ludwig reports grants from Amgen, Takeda, and personal fees from Amgen, Takeda, Sanofi, Janssen, Celgene-BMS, Seattle Genetics outside the submitted work. B. Everts reports grants from NWO during the conduct of the study. C. Driessen reports grants from Swiss National Fonds during the conduct of the study. No disclosures were reported by the other authors.

Authors' Contributions

L. Besse: Conceptualization, supervision, investigation, visualization, writing—original draft. **A. Besse:** Validation, visualization, methodology. **S.C. Stolze:** Formal analysis, investigation. **A. Sobh:** Formal analysis, supervision. **E.A. Zaai:** Investigation. **A.J. van der Ham:** Investigation. **M. Ruiz:** Investigation. **S. Phuyal:** Investigation. **L. Büchler:** Validation. **M. Sathianathan:** Validation. **B.I. Florea:** Software, supervision. **J. Borén:** Investigation. **M. Stahlman:** Supervision. **J. Huber:** Investigation, methodology. **A. Bolomsky:** Formal analysis, investigation. **H. Ludwig:** Writing—review and editing. **J.T. Hannich:** Investigation, writing—review and editing. **A. Loguinov:** Software. **B. Everts:** Resources, supervision. **C.R. Berkens:** Resources, supervision. **M. Pilon:** Resources, supervision, writing—review and editing. **H. Farhan:** Conceptualization, resources. **C.D. Vulpe:** Resources, supervision, writing—review and editing. **H.S. Overkleeft:** Resources, supervision, methodology. **C. Driessen:** Conceptualization, funding acquisition, project administration, writing—review and editing.

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