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Novel cell line models to study mechanisms and overcoming strategies of proteasome inhibitor resistance in multiple myeloma

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ABSTRACT

Experimental data on resistance mechanisms of multiple myeloma (MM) to ixazomib (IXA), a second-generation proteasome inhibitor (PI), are currently lacking. We generated MM cell lines with a 10-fold higher resistance to IXA as their sensitive counterparts, and observed cross-resistance towards the PIs carfilzomib (CFZ) and bortezomib (BTZ). Analyses of the IXA-binding proteasome subunits PSMB5 and PSMB1 show increased PSMB5 expression and activity in all IXA-resistant MM cells, and upregulated PSMB1 expression in IXA-resistant AMO1 cells. In addition, sequence analysis of *PSMB5* revealed a p.Thr21Ala mutation in IXA-resistant MM1.S cells, and a p.Ala50Val mutation in IXA-resistant L363 cells, whereas IXA-resistant AMO1 cells lack *PSMB5* mutations. IXA-resistant cells retain their sensitivity to therapeutic agents that mediate cytotoxic effects *via* induction of proteotoxic stress. Induction of ER stress and apoptosis by the p97 inhibitor CB-5083 was strongly enhanced in combination with the PI3Kα inhibitor BYL-719 or the HDAC inhibitor panobinostat suggesting potential therapeutic strategies to circumvent IXA resistance in MM. Taken together, our newly established IXA-resistant cell lines provide first insights into resistance mechanisms and overcoming treatment strategies, and represent suitable models to further study IXA resistance in MM.

1. Introduction

Multiple myeloma (MM) is the second most common hematological malignancy and is characterized by production of non-functional immunoglobulins inducing elevated levels of unfolded protein response (UPR) and endoplasmic reticulum (ER) stress [1,2]. Despite various therapy options that are available, patients inevitably relapse after a variable time of therapy. Therefore, the successful clinical introduction of the first-in-class proteasome inhibitor (PI) bortezomib (BTZ), a boronic acid derivative, marked the beginning of a new era of multiple myeloma (MM) treatment [3]. It also triggered the development of second-generation PIs for increased antitumor activity and reduced toxicities, in particular peripheral neuropathy (PN). Thus, ixazomib (IXA), following bortezomib as a boronic acid derivative, has recently been approved as the first orally bioavailable PI showing similar anti-MM potency as BTZ but a minimized PN rate [4]. It could be shown that in high-risk refractory MM patients IXA doubles the overall and progression-free survival when combined with lenalidomide and dexamethasone compared to placebo plus lenalidomide and dexamethasone [5]. However, drug resistance evolving under IXA treatment currently limits its therapeutic success [6], but no resistance mechanisms have been described to date. In contrast, for BTZ, *PSMB5* mutations and PSMB5 upregulation have been described as potential resistance mechanism in various cell lines [7]. Of note, recently, in a BTZ-treated MM patient, *PSMB5* mutations were found by deep sequencing underlining the importance of mutation analysis [8]. Upregulation of ABC transporters as efflux pumps is another common resistance mechanism upon drug exposure. Accordingly, it was recently shown that CFZ, another second-generation PI, induces overexpression of the ABC transporter ABCB1 [9].

Protein homeostasis (proteostasis) is essential for cellular survival and regulated by a complex network to control protein synthesis, folding, trafficking and degradation [10]. Key stress signaling pathways of this network include the heat shock response (HSR) [11], the ER stress response/unfolded protein response (UPR) [12], the mitochondrial UPR [13], histone deacetylases (HDACs) [14], and the ubiquitin-

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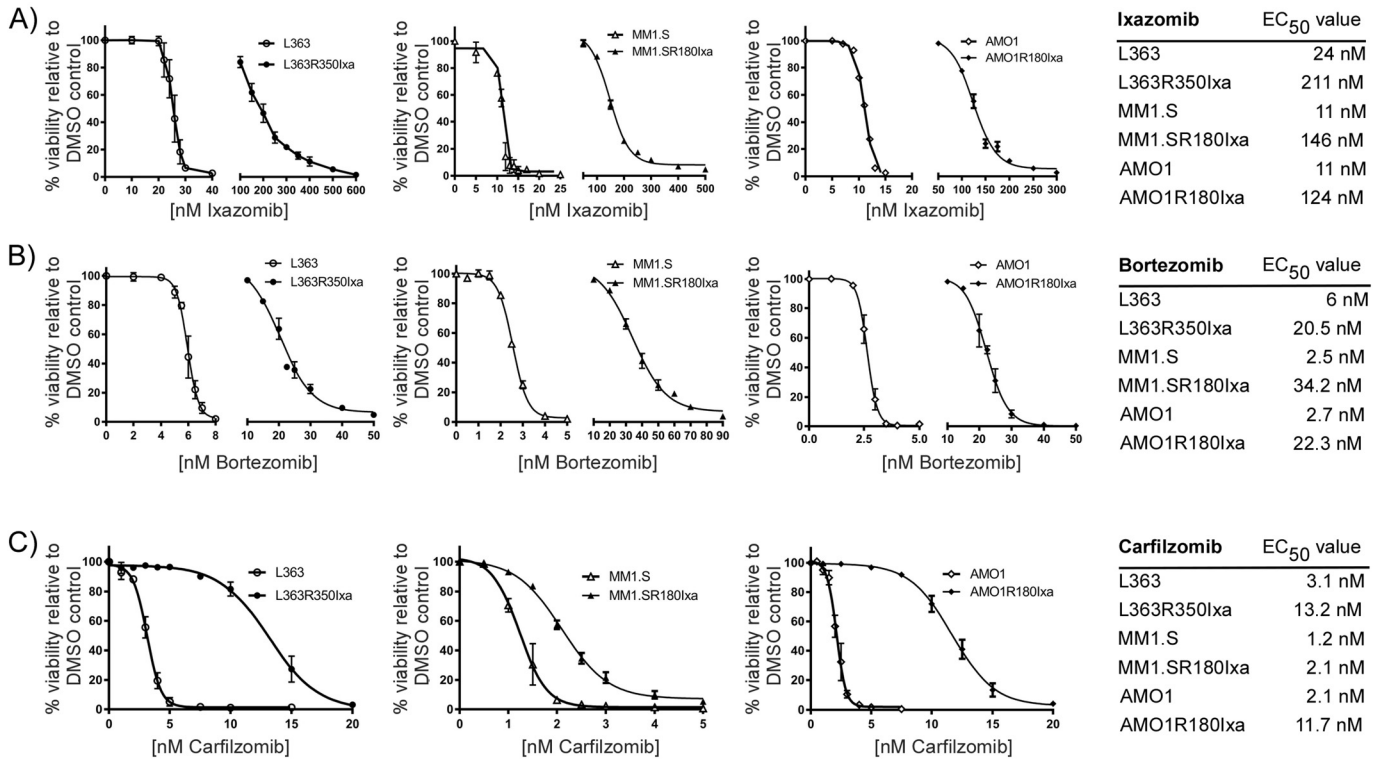

Ixazomib	EC_{50} value
L363	24 nM
L363R350Ixa	211 nM
MM1.S	11 nM
MM1.SR180Ixa	146 nM
AMO1	11 nM
AMO1R180Ixa	124 nM

Bortezomib	EC_{50} value
L363	6 nM
L363R350Ixa	20.5 nM
MM1.S	2.5 nM
MM1.SR180Ixa	34.2 nM
AMO1	2.7 nM
AMO1R180Ixa	22.3 nM

Carfilzomib	EC_{50} value
L363	3.1 nM
L363R350Ixa	13.2 nM
MM1.S	1.2 nM
MM1.SR180Ixa	2.1 nM
AMO1	2.1 nM
AMO1R180Ixa	11.7 nM

Fig. 1. Ixazomib-resistant AMO1, MM1.S and L363 MM cell lines are cross-resistant to other proteasome inhibitors. Comparative cell viability analyses of AMO1/AMO1R180Ixa, MM1.S/MM1.SR180Ixa and L363/L363R350Ixa cells are shown after 3 days of exposure to either (A) ixazomib (IXA), (B) bortezomib (BTZ) or (C) carfilzomib (CFZ) using flow cytometry-based annexin-V/propidium iodide staining. Shown are the means and standard deviations of 3 independent experiments.

proteasome system (UPS). The proteasome degrades the majority of intracellular proteins, and thus significantly contributes to cellular homeostasis affecting specific cellular functions like DNA repair, immune and inflammatory reactions, cell cycle, signal transduction, growth and differentiation [15]. The central catalytic unit of the proteasome of is the 20S core which is composed of 7 different α subunits and 3 types of β subunits with proteolytic 'caspase'- (β1), 'trypsin'- (β2) and 'chymotrypsin'-like (β5) activities. Additionally, active subunits of the interferon-γ inducible immunoproteasome (β1i, β2i and β5i) may be expressed [16,17]. PIs preferentially bind to the 20S β5 subunit (PSMB5) of the proteasome.

In this work, we aimed to generate IXA-resistant MM cell lines as models to study potential resistance mechanisms and evaluate therapeutic interventions targeting protein homeostasis to overcome PI resistance.

2. Material and methods

2.1. Pharmacologic inhibitors

All inhibitors were dissolved in H_2O-free DMSO, and stored at the indicated stock concentrations. Bortezomib (BTZ; 10 mM) was purchased from LC-Labs (Woburn, MA, USA); carfilzomib (CFZ; 20 mM), ixazomib (IXA; 50 mM), CB-5083 (CB; 10 mM), panobinostat (pano; 10 mM) were from Selleck Chemicals (Houston, TX, USA), BYL-719 (20 mM) was from Active Biochemicals (Hong Kong, China) and lenalidomide (LEN) (100 mM) was from Santa Cruz Biotechnology (Dallas, TX, USA). Working solutions were always prepared freshly by further dilution of the stock solutions in cell culture medium.

2.2. Cell culture

The lung cancer cell lines A549 and H460 (both ATCC) were a kind gift from Roswell Park Memorial Institute, and used as a positive control for ABC transporter expression. The human MM cell lines AMO1, L363 (purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ; Braunschweig, Germany) and MM1.S (LGC Biolabs (Wesel, Germany), and their IXA-resistant derivatives were grown in RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% FBS (GE Healthcare), 100 U/ml penicillin, 100 µg/ml streptomycin (Sigma-Aldrich), 2 mM L-glutamine (Sigma-Aldrich) and 1 mM Na-pyruvate (Sigma-Aldrich) in a humidified incubator with 5% CO_2 content at 37 °C. Additionally, for AMO1rCFZ, RPMI-1640 complete medium was supplemented with CFZ.

2.3. Generation of ixazomib-resistant cell lines

For generation of IXA-resistant MM cell lines, we used the long-term PI exposure method as previously described [18,19]. Briefly, kill curves for 3-day-treatment with IXA of AMO1, MM1.S and L363 cells were established by flow cytometry using annexin V/propidium iodide staining, and the respective EC_{50} values calculated. Starting at this concentration, cells were continuously kept in IXA-supplemented culture, and the drug level was stepwise increased over a time period of 6–8 months until aliquots were cryoconserved as respective stock banks. For subsequent experiments fresh aliquots were thawed and used within 3 months. A short tandem repeat (STR) sequencing analysis of 9 different and highly polymorphic short STR loci was performed by DSMZ as described before [20], and could confirm the cell line authenticity of the IXA-resistant sub-cell lines (Supplementary Table 1). In addition, no mitochondrial sequences from mouse, rat or hamster cells could be detected at a detection level of $1:10^5$ confirming human origin of IXA-resistant sub-cell lines.

2.4. Viability assessment by annexin V/propidium iodide staining

Viability was determined by staining with propidium iodide and fluorochrome-labelled annexin V as described before [21]. Briefly,

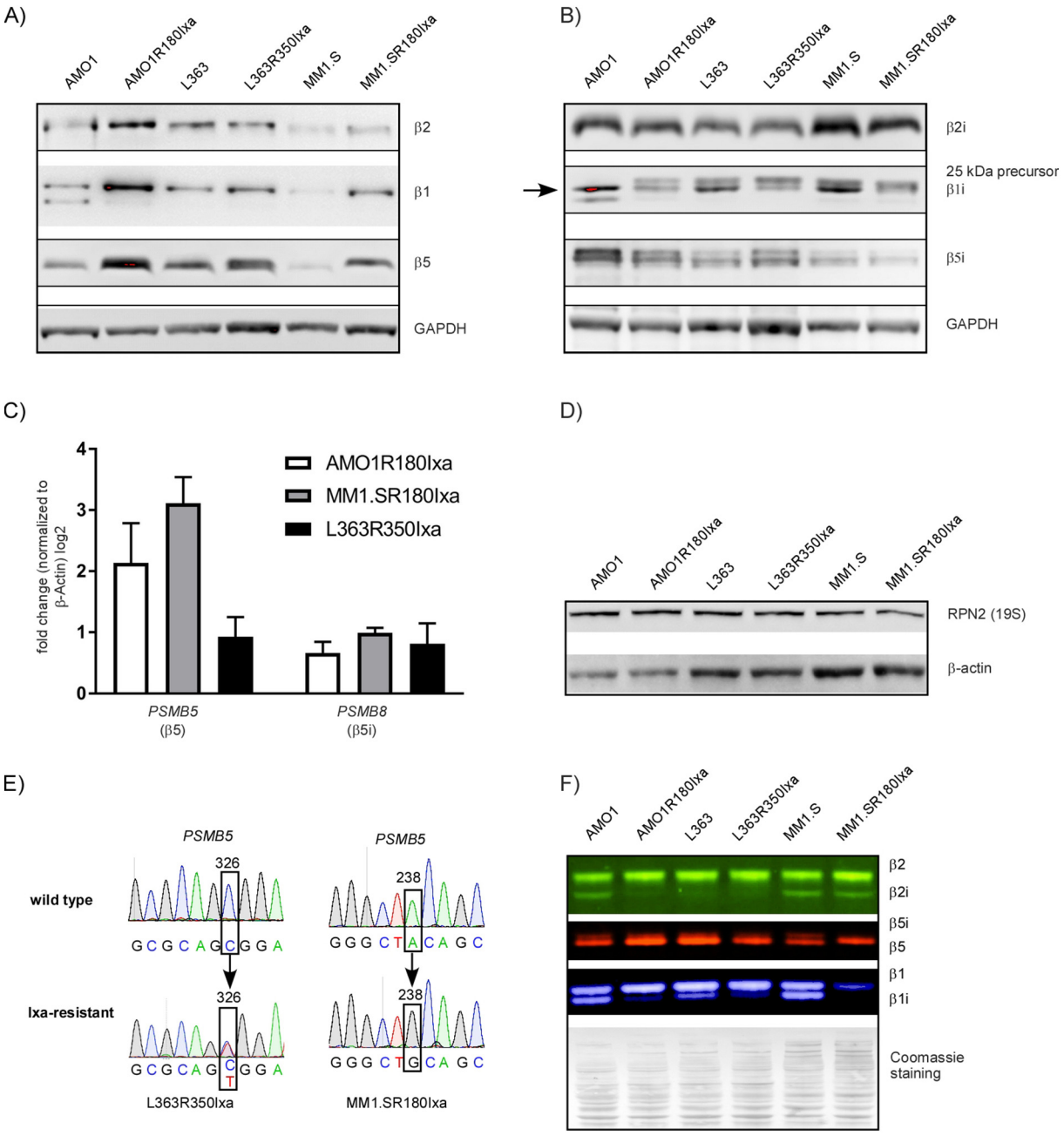

Fig. 2. Characterization of IXA-resistance mechanism in AMO1, MM1.S and L363 MM cell lines. The protein expression of the 20S proteasome subunits β1, β2 and β5 (A) or the 20S immunoproteasome subunits β1i, β2i and β5i (B) was analyzed for parental and IXA-resistant AMO1, L363 and MM1.S cells by Western blotting and GAPDH served as loading control. (C) qPCR-based gene expression changes of the 20S proteasome subunits β5 (*PSMB5*) and β5i (*PSMB8*). (D) Western staining of the 19S proteasome subunit RPN2 in the parental and IXA-resistant cell lines showed no differences. (E) Sequence analysis of the PSMB5 gene revealed a C/T base change at position 326 in one allele in L363R350Ixa cells and an A/T base change at position 238 in the single allele present in MM1.SR180Ixa cells. (F) Activity of the proteasome 20S core subunits (β2, β2i, β5, β5i, β1, β1i) in parental MM cell lines and IXA-resistant sublines was analyzed using an active site labelling assay (ASL). The β1i and β5i sites were less active in the IXA-resistant sub-lines compared to their parental counterparts. Additionally, in AMO1R180Ixa cells, the β2i subunit was less active than in AMO1 cells. Coomassie staining served as loading control.

7500–10,000 cells were seeded and treated with the respective drugs for 3 or 6 days (10 μM LEN) prior to harvesting, staining with annexin V/propidium iodide, and analysis by flow cytometry using a FACSCalibur (BD). Annexin V was prepared following a protocol detailed in [22] and coupled to Promofluor647P-NHS ester (PromoKine, Heidelberg, Germany). Data were interpreted with FlowJo version 8.8.7 (Tree Star, Ashland, USA) and graphs were generated in Prism V 7.04 (GraphPad Software, La Jolla, CA, USA).

2.5. *PSMB5* mutation analysis

For *PSMB5* mutation analysis and real-time PCR, RNA was isolated using the Peqgold RNA isolation kit (Peqlab, Erlangen, Germany), followed by cDNA synthesis using the Reverse transcriptase high capacity kit (Thermo Fisher, Waltham, MA, USA) according to the manufacturer's guidelines. Additionally, DNA was isolated using the NucleoSpin Tissue kit (Macherey-Nagel, Düren, Germany). Amplification of *PSMB5* gene sequence was performed using the Q5-2x-Master Mix (NEB) and the following primers: Fwd: GATATGGCGCTTGCCAGCGTGTT, Rev:

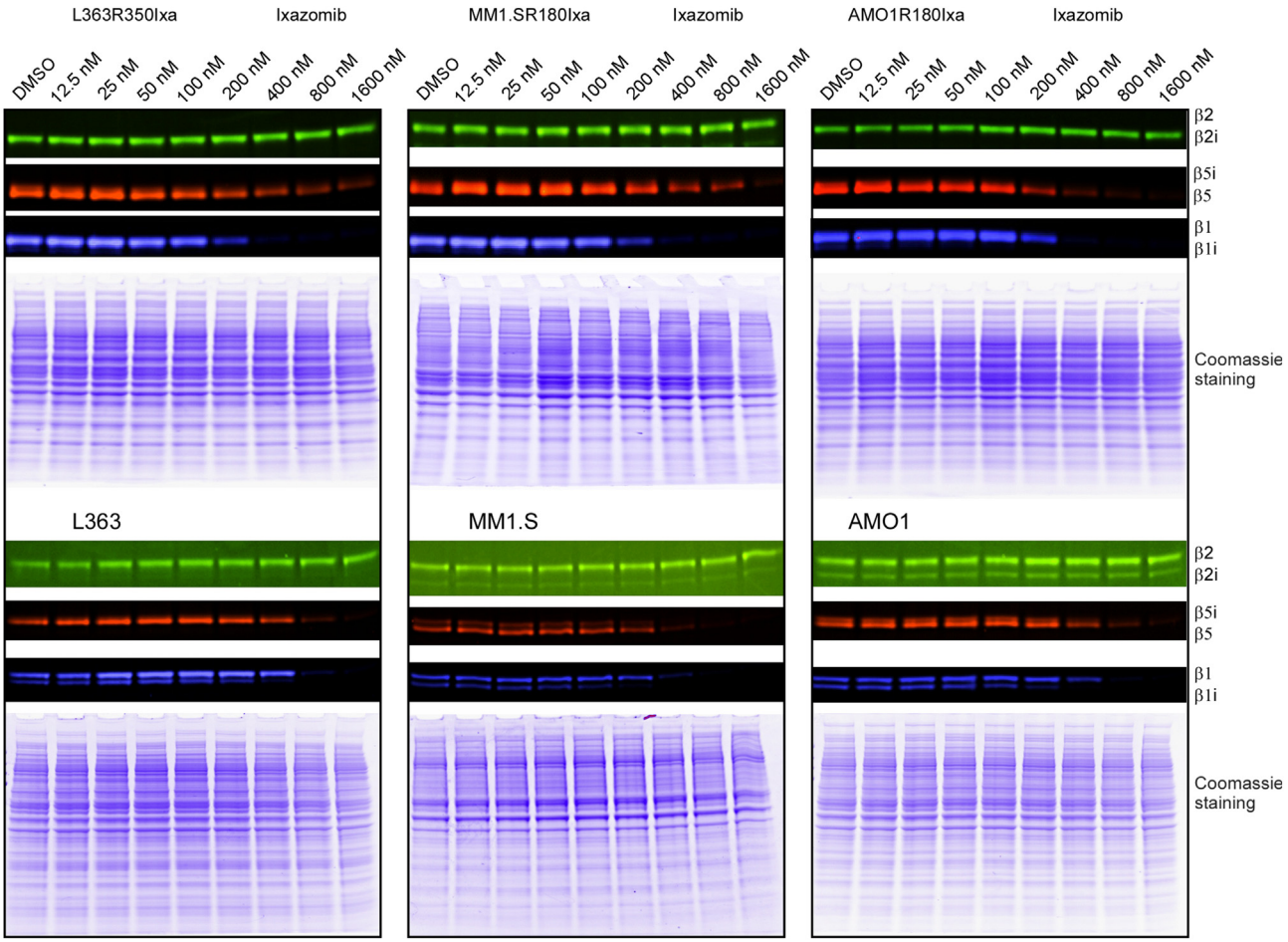

Fig. 3. Proteasomal activity after IXA-pulse labelling of IXA-sensitive and -resistant MM cell lines. Residual activity of proteasome subunits in parental and IXA-resistant sub-lines was visualized by ASL after 1 h pulse treatment with the indicated concentrations of IXA. Whereas the β2 subunit remained unaffected, activity of the β1 and β5 subunits was blocked by higher concentrations of IXA. Interestingly, β5 activity remained stable in the IXA-resistant cell lines at high IXA-concentrations. Of note, in parental cell lines activity of β1 proteasome subunit was diminished at same concentrations and β5 subunit at lower concentration than in their IXA-resistant counterpart. Equal protein load was confirmed by Coomassie staining.

GATTCAGGGGGTAGAGCCACTATACTTCT. The PCR program was as follows: 98 °C 30 s followed by 35 cycles of 98 °C for 10 s, 67 °C for 10 s, 72 °C for 20 s and a final extension at 72 °C for 2 min. In addition, we used the following exon 2-specific PSMB5 primers either for cDNA: exon2-RNA-Fwd: CAGTGATGGTCTGAGCCTGG, exon2-RNA-Rev: TGG CAAGCAGTTTGGAGGC, or for genomic DNA-based mutation analysis exon2-DNA-Fwd: GCTGGGTTTGGATTGATGCA, exon2-DNA-Rev: TCT GTCCATCCAACCCCTCC with the following program 95 °C 2 min followed by 35 cycles of 95 °C for 30 s, 57 °C for 30 s, 72 °C for 1 min and a final extension at 72 °C for 5 min using the PfU Polymerase (Thermo Fisher) PCR products were analyzed on a 1% agarose gel and Sanger sequencing was performed using the PCR primers.

2.6. Real-time qPCR

Real-time qPCR for *PSMB5* (20S β5) and *PSMB8* (20S β5i) was performed on a Roche Lightcycler 96 using the Thermo Dynamo Flash Sybr qPCR kit (Thermo Fisher) with 20 ng/μl cDNA and 5 mM primer stocks. PCR program was as follows: 7 min initial denaturation at 95 °C followed by 40 cycles of 95 °C for 10 s and 60 °C for 30 s and a final melting curve analysis. For verification of amplicon size, samples were loaded on a 1% agarose gel. The following primers were used: PSMB5 (β5) Fwd: GGCCCTGGCCTCTACTACGT, PSMB5 (β5) Rev: GAGAAGG TGGCCCCTGAAA, PSMB8 (β5i) Fwd: GACAGTGGCTATCGGCCTAATC, PSMB8 (β5i) Rev: CCTGCGGCCAAGGTCATA, β-actin Fwd: GCACTCT TCCAGCCTTCCTT, β-actin Rev: AATGCCAGGGTACATGGTGG.

2.7. Western blot analyses

To analyse induction of unfolded protein response (UPR) and HSP72 expression, IXA-resistant cells were treated for 4 h using 1 μM CB-5083 followed by protein isolation and performance of Western blotting as described before [19]. To analyse general protein levels of parental and IXA-resistant cells, equal cell numbers were harvested and processed accordingly. The following antibodies were used in a 1:1000 dilution unless otherwise indicated: α-tubulin (#MCA78G, Bio-Rad, München, Germany, 1:5000), MDR1/ABCB1 (#13978, Cell Signaling Technology (CST), Danvers, MA, USA), ABCC2/MRP2 (#12559, CST), ABCG2 (#42078, CST), ATF4 (#11815, CST), ATF6 (#ab83504, abcam, Cambridge, UK), β-actin (Sigma #A5316, 1:10,000), anti-GAPDH-HRP conjugate (#hrp-60004; Proteintech, IL, USA), SAPK (#9252, CST), HSP72 (ADI-SPA-810, Enzo Life Sciences, Farmingdale, NY, USA) p-SAPK (Thr183/Tyr185, #9255, CST), Proteasome 20S β1 (#BML-PW8140, Enzo Life Sciences,), β1i (#BML-PW8345, Enzo Life Sciences), β2 (#BML-PW8145, Enzo Life Sciences), Proteasome 20S β2i (#BML-PW8350, Enzo Life Sciences), Proteasome 20S β5 (#BML-PW8895, Enzo Life Sciences), Proteasome 20S β5i (#BML-PW8355, Enzo Life Sciences), Proteasome 19S RPN2 (1:5000, #BML-PW9270, Enzo Life Sciences), XBP-1s (#12782, CST), Caspase 3 (#9662, CST), PARP-1 (#sc-8007, Santa Cruz Biotechnology) goat anti-mouse (1:10,000,

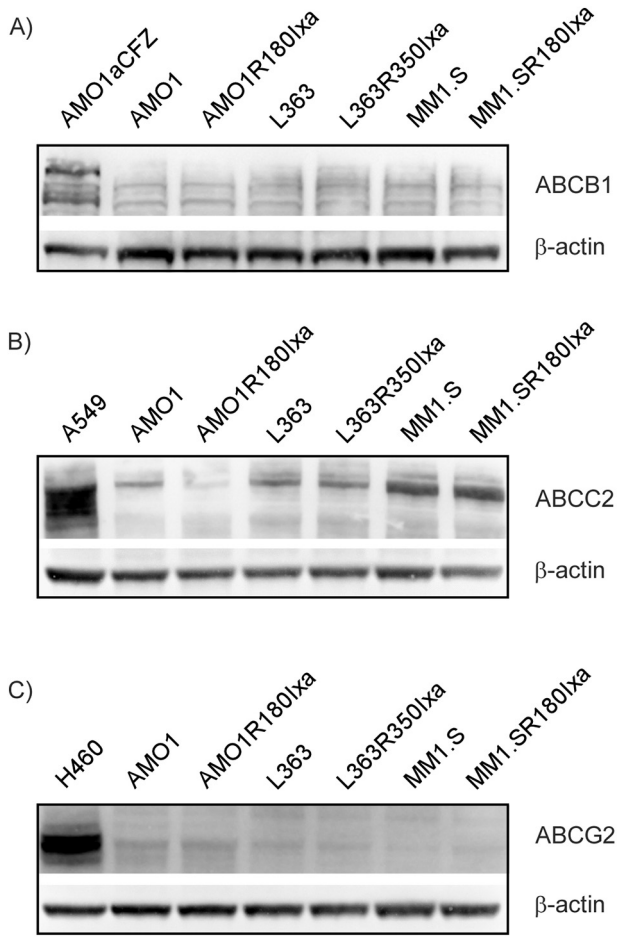

Fig. 4. ABC transporter expression in IXA-resistant cell lines. Western analysis of the ABC transporters (A) ABCB1, (B) ABCC2 and (C) ABCG2 is shown. For the IXA-resistant MM cell lines no changes in the expression of the ABC transporters were detected. Of note, the constitutive ABC protein expression levels appeared to be low. CFZ-resistant AMO1 cells (AMO1aCFZ), and the lung cancer cell lines A549 and H460 were used as positive controls for the respective transporter expression and β-actin served as loading control.

#ab87023, Abcam), goat anti-rabbit (1:20,000, #111036045 Jackson ImmunoResearch Laboratories, West Grove, PA, USA).

2.8. Active site labelling assay

For activity analysis of the 20S β1, β1i, β2, β2i, β5 and β5i proteasome subunits from IXA-resistant or their respective parental cell lines, active site labelling (ASL) was performed as described before [23]. In addition, the activity of the proteasome subunits was determined in all cell lines after 1 h of treatment with a range of IXA concentrations.

2.9. AlamarBlue metabolic activity assay

The AlamarBlue assay measures metabolic activity as a marker for cell proliferation and viability. Cells of the IXA-resistant MM cell lines MM1.SR180Ixa, AMO1R180Ixa or L363R350Ixa cells or their parental cell lines were treated either with 10 μM LEN or DMSO as a solvent control for 6 days prior to AlamarBlue analysis according to the manufacturer's instructions (Bio-Rad, München, Germany).

3. Results

3.1. Long-term exposure of MM cell lines to IXA leads to resistance to IXA and cross-resistance to BTZ and CFZ

After long-term exposure of MM1.S, L363 and AMO1 cells to IXA, the resulting IXA-resistant sub-cell lines termed MM1.SR180Ixa, L363R350Ixa and AMO1R180Ixa were analyzed for their IXA resistance level by treatment with IXA for 3 days followed by cell viability determination using flow cytometry based annexin V/propidium iodide staining. The resulting dose-response curves and the respective EC50 values show that sub-cell lines tolerate an approximately 10-fold higher IXA concentration as compared to their parental cell lines indicating an acquired resistance to IXA (Fig. 1A). In order to test potential cross-resistance of the IXA-resistant cell lines to other PIs, BTZ or CFZ treatment approaches were evaluated. Whereas the IXA-resistant cells exhibit strong increases in resistance to BTZ (3.6-fold in L363R350Ixa, 15-fold in MM1.SR180Ixa, and 8-fold in AMO1R180Ixa cells; Fig. 1B), this effect was less pronounced for CFZ (4.2-fold in L363R350Ixa, 1.7-fold in MM1.SR180Ixa, and 5.6-fold in AMO1R180Ixa; Fig. 1C). These results indicate that the observed resistance is not exclusively directed against IXA but rather broadly against all PIs.

3.2. PSMB5, PSMB1 or PSMB2 expression is increased in IXA-resistant MM cell lines

To analyse potential IXA resistance mechanisms, we asked if the 20S core subunits of the proteasome β5, β1 or β2 (PSMB5, PSMB1 or PSMB2), which have been considered as PI targets, might be differentially expressed. Western analysis of PSMB5 revealed unchanged levels in L363R350Ixa but strong up-regulation in MM1.SR180Ixa and AMO1R180Ixa cells (Fig. 2A). The levels of PSMB8 (immunoproteasome subunit β5i) appeared unchanged in all IXA-resistant cells (Fig. 2B). In addition, real-time quantitative PCR analyses for *PSMB5* or *PSMB8* show a similar gene expression pattern compared to the protein expression pattern indicating transcriptional up-regulation of *PSMB5* in AMO1R180Ixa and MM1.SR180Ixa cells (Fig. 2C). Moreover, we also analyzed expression of other 20S core subunits, and detected up-regulation of PSMB1 and PSMB2 (subunits β1 and 2), although this effect was restricted to AMO1R180Ixa cells (Fig. 2A). Of note, the 19S proteasome subunit is not targeted by PIs including IXA. Accordingly, we found no expression alterations in the respective marker protein RPN2 (Fig. 2D).

3.3. PSMB5 is mutated in IXA-resistant cells

Because mutations of PSMB5, the main target for boronate-based PIs like BTZ or IXA, have previously been described to emerge upon prolonged BTZ treatment, we analyzed the *PSMB5* sequence from IXA-resistant and their parental cells, and found *PSMB5* mutations in the L363- and MM1.S- but not in the AMO1-IXA-resistant sublines. Thus, L363R350Ixa exhibits a DNA base change from cytosine to thymine at position 326 (C326T) in one of the two *PSMB5* alleles, whereas MM1.SR180Ixa harbours an adenine to guanine change at position 238 (A238G) in its single *PSMB5* allele (MM1.S cells display chromosome 14 monosomy) (Fig. 2E). DNA base changes result in replacement of alanine by valine at position 109 (p.Ala109Val) or of threonine by alanine at position 80 (p.Thr80Ala) of the precursor PSMB5 protein. These positions are equivalent to the changes p.Ala50Val and p.Thr21Ala, in the mature functional PSMB5 protein. Both mutations are localized in the PI-binding area of PSMB5 indicating that they compromise binding of IXA and thus contribute to IXA resistance.

3.4. The immunoproteasome activity is decreased in IXA-resistant cell lines

Next, we addressed the question if IXA resistance might cause

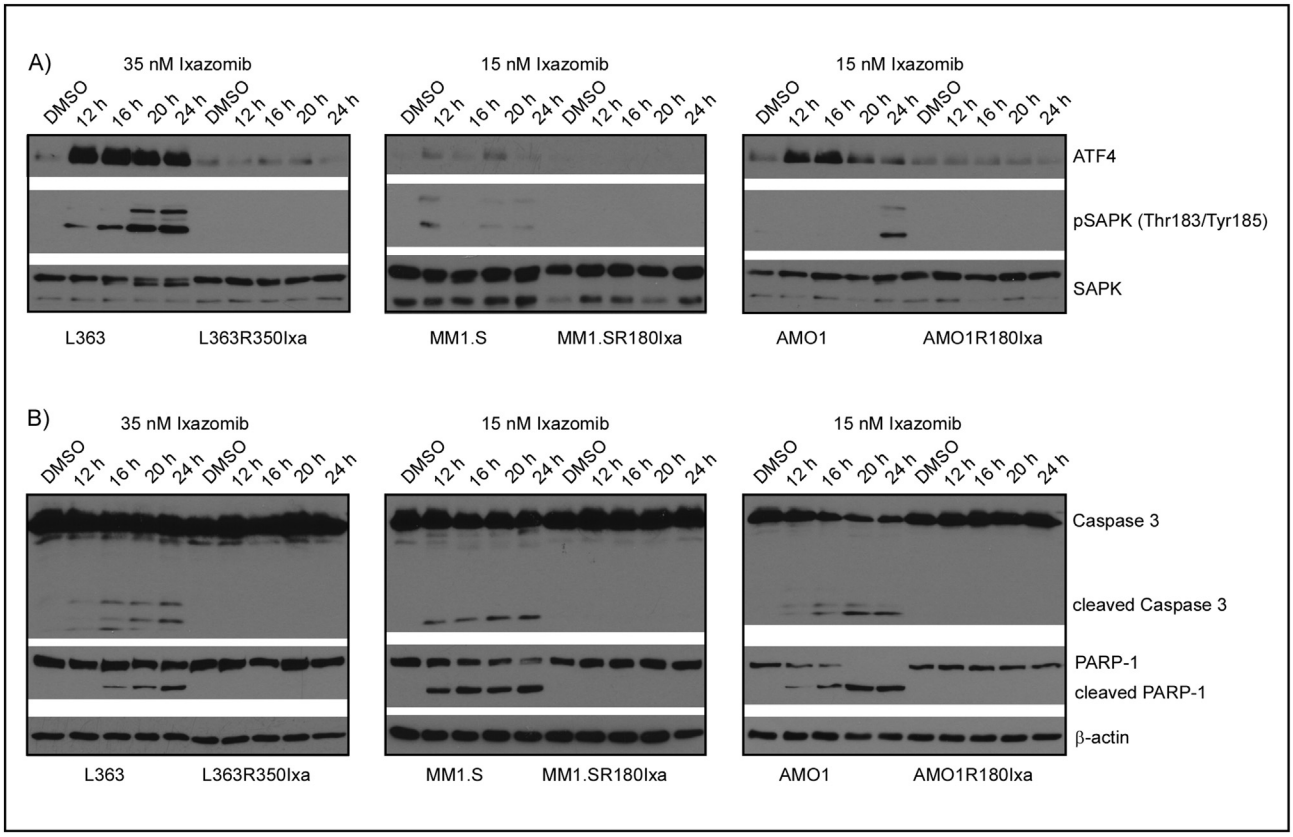

Fig. 5. Lack of UPR and caspase 3-mediated apoptosis induction by IXA in IXA-resistant MM cells. MM1.S, MM1.SR180Ixa, L363, L363R350Ixa, AMO1 and AMO1R180Ixa cells were treated with IXA for 12 h to 24 h followed by cell lysis and Western blot analysis either of the UPR mediators ATF4 and phospho-SAPK/JNK (Thr183/Tyr185) (A), or the Caspase 3 and its substrate PARP-1 (B). β-Actin served as loading control.

changes in the activity of the proteasome and immunoproteasome, and determined activity of the proteasome subunits β1/β1i, β2/β2i or β5/β5i by active site labelling (ASL). We found an increase of β5 activity in AMO1R180Ixa and MM1.SR180Ixa cells, whereas β5 activity was decreased in L363R350Ixa cells as compared to the respective parental cell lines. In addition, we observed a general diminishing of β1i in all three resistant cell lines, decreased β2i activity in AMO1R180Ixa cells and MM1.SR180Ixa cells, and attenuated β5i subunit activity in AMO1R180Ixa cells (Fig. 2F). We could see in various entities that despite irreversible binding of CFZ, the proteasomal activity was even shorter than for BTX (data not shown). This indicates that the chemistry in the active center plays a pivotal role resulting in a higher cross-resistance of BTZ to IXA than to CFZ.

3.5. The proteasome subunits β5 and β1 remain active upon IXA-treatment in IXA-resistant cells

Additionally, we tested the activity of the relevant 20S subunits after 1 h of IXA pulse-treatment, and found that the β2 subunit remained active even at the highest IXA concentration in all parental and resistant sub-cell lines. In contrast, the activity of the β5 and β1 subunits was blocked in a concentration-dependent manner in all cell lines. Upon exposure to high IXA-concentrations, activity of the β5 and β1 subunits thus remained stronger in the IXA-resistant cells as compared to their parental cells (Fig. 3).

3.6. IXA-resistance is independent of ABC transporter up-regulation

Recently, up-regulation of the ABC transporter ABCB1 has been described as mechanism for CFZ resistance [24]. Therefore, we next screened for changes in the levels of ABC transporters ABCB1, ABCC2 and ABCG2 as examples of the huge ABC family. For ABCB1 transporter expression, we used CFZ-resistant AMO1 (AMO1aCFZ) cells as positive control. ABCB1 expression was generally weak and there were no alterations between parental and IXA-resistant sub-cell lines (Fig. 4A). For ABCC2 (Fig. 4B) and ABCG2 (Fig. 4C) transporters, we used the lung cancer cell lines A549 and H460 as positive controls, but also found no substantial differences between the parental and the IXA-resistant sub-lines. Overall, the expression levels of these transporter proteins seemed rather low in the parental and resistant MM cell lines and not involved in IXA resistance.

3.7. The IXA-induced unfolded protein response and apoptosis are suppressed in IXA-resistant MM cells

Next, we investigated potential PI effector mechanisms either in IXA-sensitive or -resistant cells, and analyzed induction of UPR signaling and apoptosis effectors after IXA treatment. Western analyses of the major UPR signaling pathways PERK/ATF4 or IRE1α/SAPK/JNK revealed strong upregulation of ATF4 and increased phosphorylation of SAPK on the residues Thr183/Tyr185 in the IXA-sensitive (parental) but not in the IXA-resistant MM cell lines (Fig. 5A). In the IXA-sensitive MM cell lines, activation of the apoptosis effector Caspase 3 and cleavage of its marker substrate PARP-1 were found by Western analysis indicating apoptosis induction by IXA treatment. In contrast, neither Caspase 3 activation nor the PARP-1 cleavage was observed upon IXA treatment in the IXA-resistant MM cells (Fig. 5B). These findings indicate that the UPR and Caspase 3-mediated apoptosis as PI effector mechanisms are suppressed in the IXA-resistant MM sub-lines.

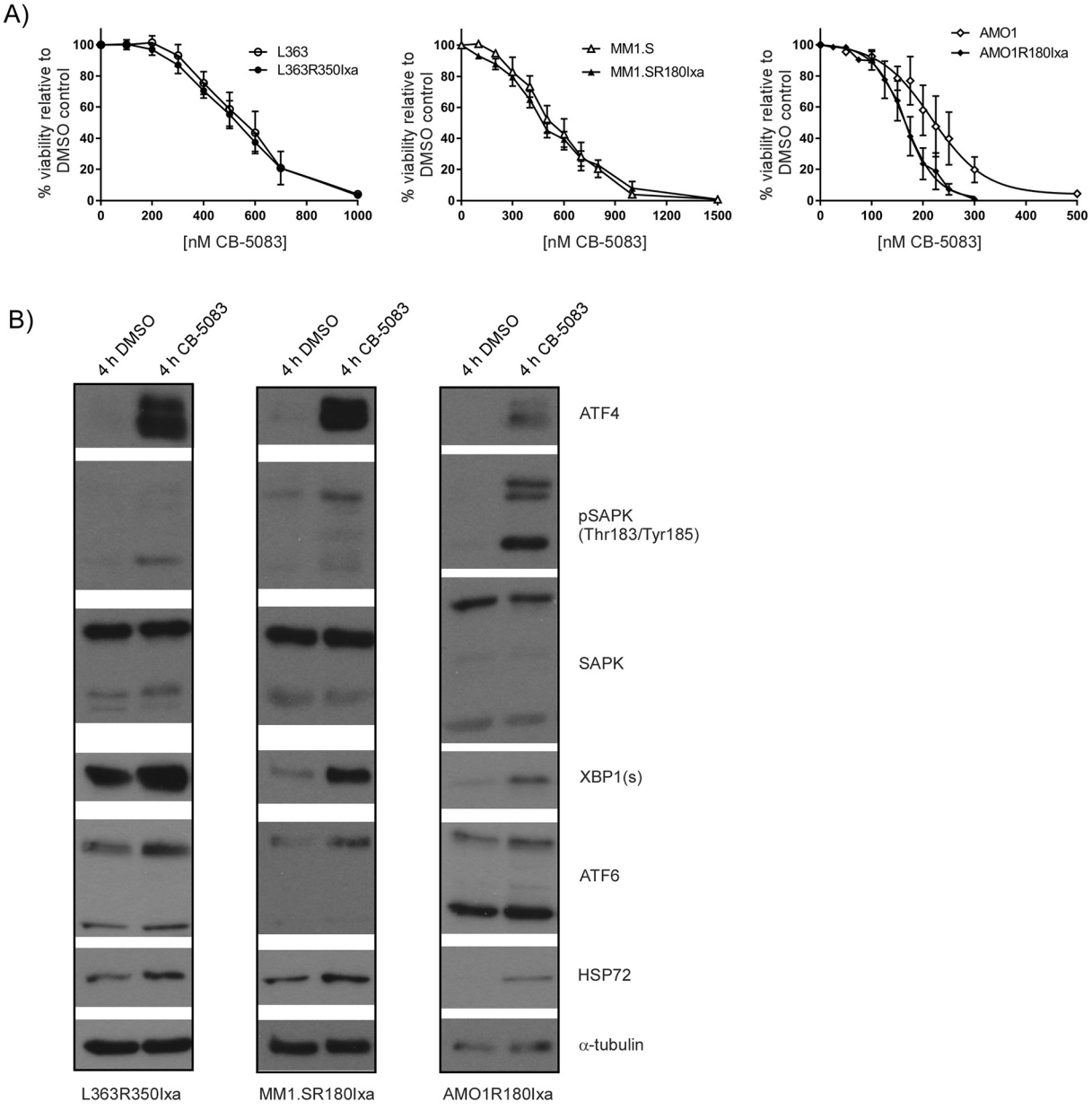

Fig. 6. Proteotoxic stress can be induced by targeting the AAA ATPase p97. (A) Shown are data of the viability assessment of AMO1/AMO1R180Ixa, MM1.S/MM1.SR180Ixa or L363/L363R350Ixa cells after 3 days of treatment with the p97 inhibitor CB-5083 using flow cytometry-based annexin-V/propidium iodide staining. Shown are the means and standard deviations of 3 independent experiments. (B) Stress response as marker for protein homeostasis was analyzed upon treatment with 1 μM CB-5083 for 4 h in L363R350Ixa cells (left panel), MM1.SR180Ixa cells (middle panel) and AMO1R180Ixa cells (right panel). Levels of the UPR mediators ATF4, SAPK/JNK, phosphoSAPK/JNK (Thr183/Tyr185), XBP1(s), ATF6 and as HSP72 as marker for heat shock signaling were analyzed by Western blotting. α-Tubulin served as loading control.

3.8. Inhibition of p97 is effective in IXA-resistant MM cells

MM cell survival is supposed to be dependent on up-regulated adaptation mechanisms that keep the disturbed protein homeostasis in balance, such as the ubiquitin/proteasome system, the HSF1/HSP system, aggresome complex formation or the unfolded protein response (UPR)/autophagy. If the main IXA resistance mechanism is based on PI-binding site mutations, we hypothesize that other protein homeostasis regulators might remain unaffected and therefore still targetable. To test this hypothesis, we evaluated pharmacologic inhibition of p97, an emerging new target of the ubiquitin proteasome system, using the novel p97 inhibitor CB-5083 in the IXA-resistant MM cell line models. We found that sensitivity to CB-5083-induced apoptosis was fully intact in the IXA-resistant cell lines, and, notably, appeared to be even more pronounced in AMO1R180Ixa cells (Fig. 6A). Accordingly, Western blot analyses revealed induction of ER and cytosolic proteotoxic stress markers like induction of ATF4, ATF6, and HSP72 expression, XBP1 splicing and phosphorylation of SAPK/JNK on positions Thr183/Tyr185 upon treatment with 1 μM CB-5083 for 4 h indicating a fatal proteotoxic stress response in IXA-resistant MM cells (Fig. 6B).

3.9. HDAC inhibition remains effective in IXA-resistant MM cells

The pan-HDAC inhibitor panobinostat targets the protein homeostasis machinery by blockade of aggresome formation, and thus represents a reasonable therapy option for MM treatment in combination with PIs. Therefore, we also evaluated the potential of panobinostat for its ability to induce apoptosis in IXA-resistant MM cells and to bypass PI

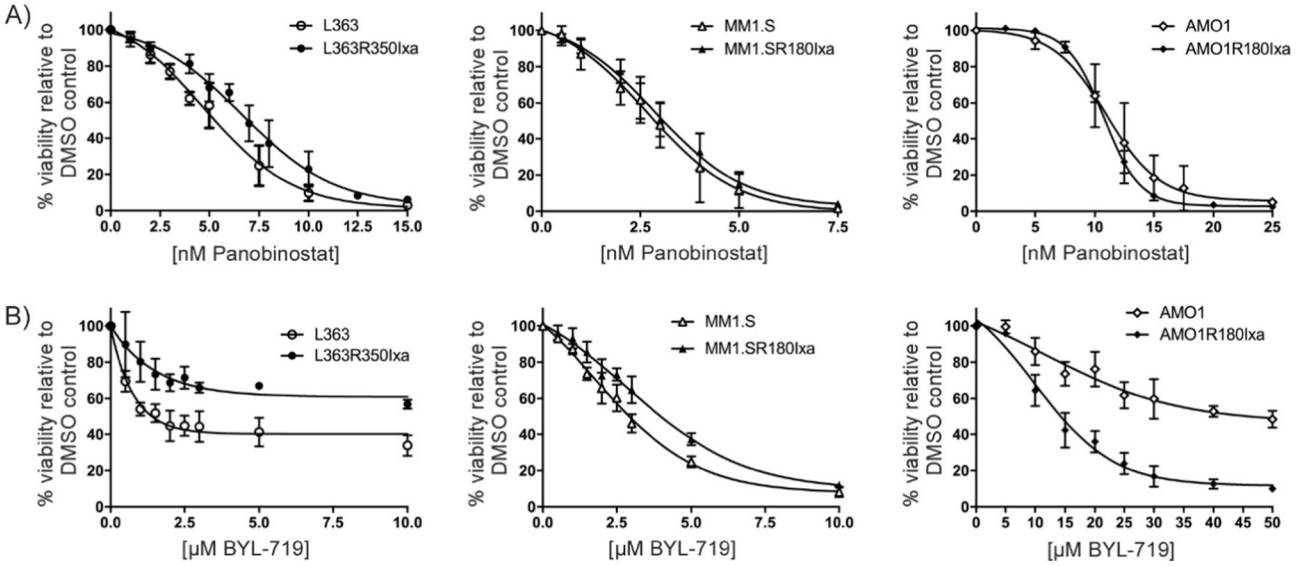

Fig. 7. Viability assessment of the HDAC inhibitor panobinostat and PI3Kα inhibitor BYL-719. Shown are data of the viability assessment of AMO1/AMO1R180Ixa, MM1.S/MM1.SR180Ixa or L363/L363R350Ixa cells after 3 days of treatment with the HDAC inhibitor panobinostat (A) or the PI3K/p110α inhibitor BYL-719 (B) using flow cytometry-based annexin-V/propidium iodide staining. Shown are the means and standard deviations of 3 independent experiments.

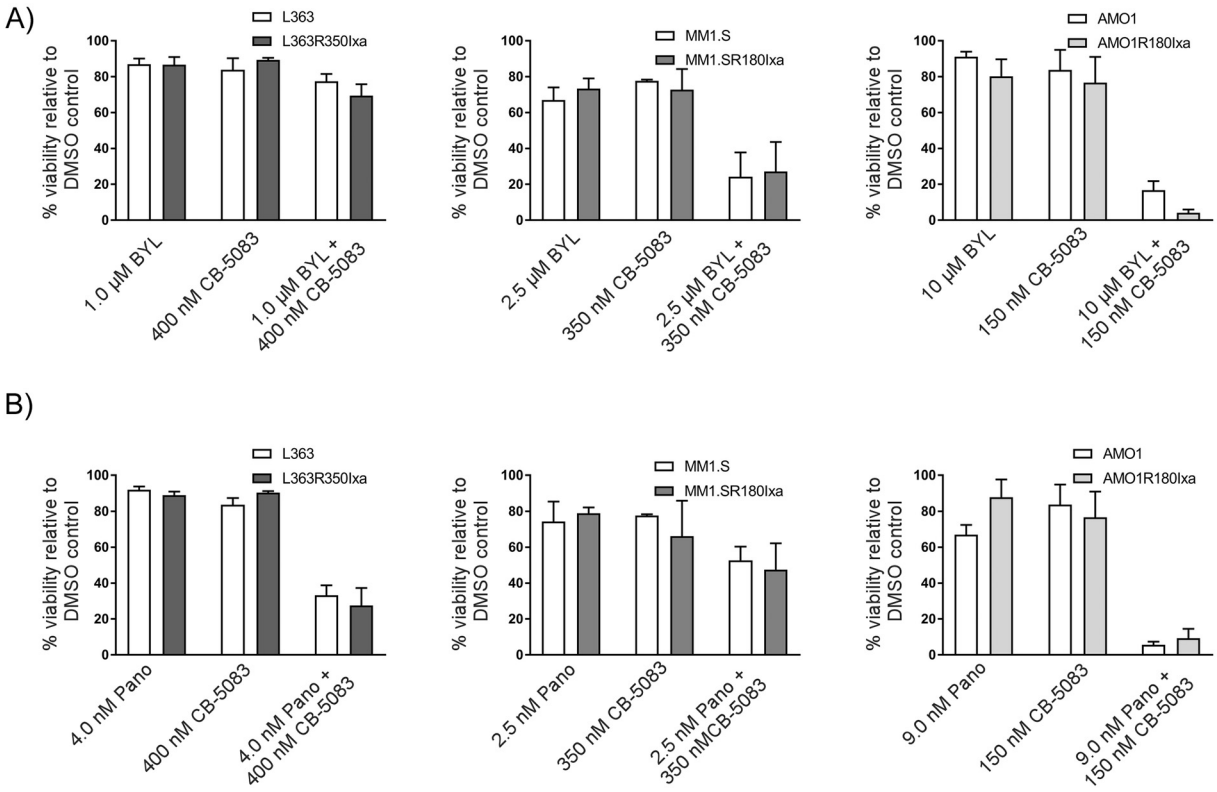

Fig. 8. Viability assessment of the HDAC inhibitor panobinostat and PI3Kα inhibitor BYL-719 in combination approaches with the p97 inhibitor CB-5083. Shown are data of the viability assessment of AMO1/AMO1R180Ixa, MM1.S/MM1.SR180Ixa or L363/L363R350Ixa cells after 3 days of treatment either with (A) CB-5083 and BYL-719 and, or with (B) CB-5083 and panobinostat (Pano) and (bottom row) using flow cytometry-based annexin-V/propidium iodide staining. The means and standard deviations of 3 independent experiments are shown.

resistance. Indeed, apoptosis was induced in IXA-sensitive and -resistant cell lines at low nanomolar concentrations and there was no substantial difference between the sub-cell lines (Fig. 7A) suggesting that targeting HDACs is an alternative therapeutic strategy in targeting the protein homeostasis machinery and overcoming IXA resistance.

3.10. AKT activity determines sensitivity of IXA-resistant cells to PI3K/p110α inhibition

Signaling by the PI3K/AKT pathway is frequently activated in MM, contributes to MM cell survival and has been shown to be involved in the regulation of the HSR and UPR in MM cells. Therefore, we tested inhibition of PI3K/p110α, the dominant isoform in MM, using the

isotype-specific inhibitor BYL-719 (alpelisib) as an alternative therapeutic approach to bypass PI resistance. BYL-719 treatment induced strong apoptotic cell death in both IXA-sensitive and -resistant MM1.S cells, which both exhibit high constitutive AKT signaling activity. In contrast, L363 or AMO1 cells, which both lack strong intrinsic AKT activation, show rather moderate responses to BYL-719 treatment (Fig. 7B). Of note, AMO1R180Ixa cells appeared to be more sensitive towards BYL-719 than their parental counterparts but at high concentrations that target other proteins than PI3Kα. These data indicate that targeting the PI3Kα-AKT pathway might show a potential benefit for MM patients whose cancer cells display strong AKT signaling activity.

3.11. Lenalidomide-induced anti-MM effects remain largely unaffected in IXA-resistant cells

Because immunomodulatory drugs (IMiDs) like LEN play a pivotal role in MM therapy, we also investigated whether anti-MM effects of LEN are affected during development of IXA resistance. We determined metabolic activity as a marker for cell proliferation using the AlamarBlue assay and cellular viability my annexin-V/propidium iodide staining after 6 days exposure of the parental and IXA-resistant cells to 10 μM LEN. We found almost similar effects on cell proliferation and viability in both parental and IXA-resistant cells. LEN induced 40–50% apoptosis in MM1.S and MM1.SR180Ixa cells, whereas in L363/L363R350Ixa cells metabolic activity as indicator for proliferation was reduced by 50–60%. Unlike in parental AMO1 cells, where LEN did not cause any antiproliferative effects, in AMO1R180Ixa cells, LEN treatment reduced proliferation rate by approx. 20% (Supplementary Fig. 1).

3.12. Combined inhibition of p97 and HDAC or PI3K/p110α induces apoptosis in IXA-resistant MM cells

Because single agent treatment is mostly not sufficient for successful MM relapse therapy, the evaluation of clinically suitable combination strategies is warranted. Here, we evaluated two combination approaches using sub-lethal (EC_{25}) concentrations of p97 inhibitor CB-5083 and either panobinostat or BYL-719 in IXA-resistant or -sensitive cells. The BYL-719/CB-5083 combination led to strong and synergistic viability reduction in both MM1.S and MM1.SR180Ixa cells, as well as in the AMO1/AMO1R180Ixa pair, whereas this combination approach was not effective in L363/L363R350Ixa cells (Fig. 8A). The combined approach with CB-5083 and panobinostat led to substantial viability reduction without differences between IXA-resistant and -sensitive cell lines. The strongest apoptotic effects of this combination were observed in AMO1/AMO1R180Ixa cells, intermediate effects in the L363/L363R350Ixa cells, and the weakest effects in MM1.S/MM1.SR180Ixa cells (Fig. 8B).

4. Discussion

Understanding the molecular mechanisms underlying the development of PI resistance is critical for both the subsequent relapse therapy of the individual patient as well as the improvement of current treatment strategies for refractory MM. However, unlike for CFZ or BTZ, no potential resistance mechanisms for IXA have been described so far. Therefore, we generated IXA-resistant cell lines, and found that they also exhibit cross resistance to BTZ and CFZ, the other clinically relevant PIs. In accordance with our finding, it has been demonstrated that all three PIs mainly target the PSMB5 (β5) subunit of the 20S proteasome core unit. However, differences in their chemical structure and the resulting binding mode of the PIs might explain that we observed differences in the extent of cross resistance [25]. Thus, cells resistant to IXA were almost equally resistant to the chemically related BTZ, which like IXA also binds reversibly to the proteasome, whereas resistance to the chemically more different, irreversible proteasome binding CFZ was less pronounced.

Although PIs are not DNA-damaging agents, *PSMB5* mutations have been described as resistance mechanisms against BTZ in various MM cell line models. Most of these mutations were found in the binding pocket of PIs [7]. Accordingly, both *PSMB5* mutations, resulting in p.Thr21Ala and p.Ala50Val, that we found in MM1.SR180Ixa and L363R350Ixa cells, have previously been described as hot-spot mutations affecting the PI-binding site in BTZ-resistant cell lines (p.Thr21Ala in RPMI-8226 cells [26] and p.Ala50Val in THP-1 cells [27]). These data indicate that the p.Thr21Ala and the p.Ala50Val mutations within the PI-binding site of PSMB5 may be instrumental in resistance formation against different PIs, as it has also recently been shown for different PSMB5 mutations in the binding pocket [8]. Spontaneous mutagenesis, might critically contribute to generation of PSMB5 mutations under therapeutic selection pressure. Thus, the spontaneous mutation rate of cell lines has been shown to vary from 2 to 7×10^{-6} depending on the experimental conditions and cell density [28].

Currently, NGS data obtained from IXA-treated patients are still missing mainly for two reasons: i) the recent clinical introduction of IXA, and ii) the low representation of multi-relapsed and refractory MM patient group in the NGS databases. Thus, in the open NGS database CoMMpass covering data from 956 MM sequenced patients, only four PSMB5 mutations (L35F and A45E in exon 1, and E95Q and D76N in exon 2) have been identified so far, all determined in samples from newly diagnosed patients. However, using a local M^3P panel-based sequencing of 161 refractory MM patients, it has been demonstrated that *PSMB5* mutations are mainly acquired mutations under therapy. Their frequency rises from 0.08% at in newly diagnosed patients to 1.2% in multiple refractory patients and thus shows a sub-clonal evolution pattern [8].

In addition to PSMB5 mutations, we observed elevated PSMB5, PSMB1 or PSMB2 protein expression levels and a stronger residual activity of PSMB5 (β5) and PSMB1 (β1) upon IXA pulse treatment in the IXA-resistant MM cells. These findings indicate additional mechanisms that might also contribute to the development of PI resistance. Our data are in a good accordance with previous works showing that IXA predominantly targets PSMB5 (β5) and, although to a much lesser extent, PSMB1 (β1) [25], and that increased expression of PSMB5 is a potential BTZ- and CFZ-induced resistance mechanism [7,9].

When screening for ABC transporters that are known to be upregulated upon PI exposure, we did not find any alterations compared to the parental cells and a generally weak expression in the IXA-resistant cell lines. However, conflicting data exist about the role of ABC transporter up-regulation in PI resistance. In line with our data, BTZ-resistant AMO1 cells have been described to lack ABC transporter upregulation [9]. Yet, for low-level BTZ resistance a correlation with ABCB1 expression has actually been found in K562/A02 cells [7] indicating that such an upregulation might represent an initial and perhaps more generalized BTZ adaption mechanism which is followed and replaced by *PSMB5* mutations and higher PSMB5 expression. In contrast, it has been shown that CFZ is a *bona fide* substrate for ABCB1, thus providing a reasonable explanation for strong and sustained overexpression of this transporter [24].

We observed that IXA-induced UPR and caspase 3 activity is suppressed in IXA-resistant MM cells. Our finding is in line with previous work showing lack of BTZ-induced UPR and caspase 3 activation in BTZ-resistant cells [29]. Both UPR pathways, PERK-ATF4 and IRE1α-SAPK/JNK, are involved in apoptosis induction [30]. Therefore, it seems to be very likely that the apoptotic effects of PIs are mediated by the UPR, and that resistance against PIs often affects UPR-mediated apoptosis [30].

The AAA ATPase p97 plays a key role in the mediation of both ERAD and non-proteasomal protein degradation. In this process, misfolded proteins are targeted by ER chaperones like GRP78 or GRP94 to designated ER membrane sites for a transfer into the cytosol where they

are ubiquitinated, a process which is catalyzed by E3 ligases allowing p97 and its associated cofactors to shuttle the substrates to the proteasome for final degradation [31–33]. Therefore, p97 is an interesting therapeutic target and several attempts to develop specific inhibitors have been made [34–36]. Thereby, CB-5083 is a very promising molecule that has recently been shown to disrupt protein homeostasis and induce apoptotic MM cell death, and is currently in clinical investigation (ClinicalTrials.gov Identifier: NCT02223598) [37]. We found that the IXA-resistant cells are still sensitive to this p97 inhibitor and that UPR is induced upon treatment with CB-5083.

We and others have previously shown that the frequently activated PI3K/AKT signaling affects heat shock and unfolded protein response in MM cells [38,39], and critically contributes to MM cell survival [40,41]. Accordingly, first clinical data also indicates efficacy of AKT inhibition in MM patients [42]. In addition to AKT, the PI3K isoform PI3K/p110α has been described as potential MM target [41]. The inhibitor BYL-719 specifically targets PI3K/p110α and has been tested in various cancer entities like breast cancer, glioblastoma or nasopharyngeal cancer either as single drug or in combination approaches [43–45] and is currently being tested in clinical trials (ClinicalTrials.gov Identifier: NCT021 44038). The sensitivity of MM cells to BYL-719 treatment appears to be correlated with their AKT-status, *i.e.* whether or not this pathway is intrinsically active [41]. The higher sensitivity against BYL-719 in AMO1R180Ixa cells compared to the parental cells that we could observe might arise from a weak AKT activation (phosphorylation at the serine residue 473; data not shown), but at the rather high concentrations of BYL-719 used in these experiments the possibility of off-target effects cannot be excluded.

In addition to epigenetic modification [46], HDACs are also involved in stress response mechanisms like aggresome formation, heat shock response and UPR [47–49]. The pan-HDAC inhibitor panobinostat has already been clinically approved for treatment of refractory MM patients in combination with BTZ [50,51]. Furthermore, it is also being tested as therapeutic agent against lung, breast, ovarian, thyroid and prostate cancer [52–56]. Both the HDAC inhibitor panobinostat and the PI3K/p11a inhibitor BYL (alpelisib) have been described to interfere with the protein stress response in MM [41,50]. Both combinations of CB-5083 with panobinostat or BYL-719 worked cell line dependent to different extent. As the IXA-resistant sub-lines showed no substantial differences in their responses to BYL-719 and panobinostat, these data further support the assumption that in PI resistant cells other components of the protein homeostasis machinery remain therapeutically addressable.

Our data suggest that in contrast to up-regulation of ABC transporters as seen in CFZ-resistant MM cell models [9], therapeutic intervention targeting protein homeostasis is still possible if IXA resistance is mainly based on mutation and elevation of PSMB5. However, revealing *PSMB5* mutations in IXA-resistant patients is currently an unmet task. Given that it took almost 10 years after the first description of *PSMB5* mutations as a major BTZ resistance mechanism *in vitro* to detect the first *PSMB5* mutations in patients [8], we assume that it will only be a matter of time to find the first IXA-resistant patient with an acquired *PSMB5* mutation as consequence of the IXA therapy.

In summary, our data provide first insights into the molecular underpinnings of *in vitro* acquired resistance against IXA in MM, and delineates potential therapeutic strategies against IXA-resistant cells.

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Author contribution

DB, MC planned and designed project; DB, MK, SK, RH, PG performed experiments; DB, MK, RH, TS, PG, MC, CD, RCB analyzed data; DB, MC, TS wrote the manuscript; all authors revised and approved the final manuscript.

Conflicts of interest

The authors have nothing to disclose connected to this manuscript.

Transparency document

The Transparency document associated with this article can be found, in online version.

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