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A Subset of Genes Can Distinguish between Bortezomib Responsive Versus Bortezomib Resistant Myeloma

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Authors' contributions

This work was carried out in collaboration between all authors. Author YC designed the experiments, made the analyses and wrote the paper. All other authors assisted in patient recruitment and logistic support. All authors read and approved the final manuscript.

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ABSTRACT

Despite the widespread use of proteasome inhibitors in the treatment of multiple myeloma, the mechanisms of the anti-myeloma activity and the molecular pathways that execute the tumor cell killing are still unknown. In the present work we compared gene expression profile changes in response to bortezomib treatment of cultured bone marrow samples from patients with bortezomib-sensitive versus bortezomib-resistant myeloma. The results showed a pronounced induction of>70 genes including>30 heat shock protein transcripts in both patient groups and therefore debate the anti-tumor action, attributed to the unfolded protein response. In contrast, a subset of 7 genes (*MMP12, IL7R, MGST1, C3, CYP27A1, MIR148A* and *CXXC4*) changed only in the samples from

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the bortezomib-sensitive cases and therefore these tumor-associated genes might serve as predictors of the treatment efficacy, as well as for making of further insights onto the mechanism of action of proteasome inhibitors. In summary, we identified a subset of 7 genes which distinguished in our small series betweensensitive versus resistant tumor cells to bortezomib, which requires further assessment in a larger cohort of patients.

Keywords: Multiple myeloma; gene expression profile; MMP12; heat shock proteins; IL7R.

1. INTRODUCTION

Although the novel drug bortezomib (velcade) has become one of the most used agents for treatment of multiple myeloma (MM), the particular mechanism which executes its cytotoxic effect remains elusive due to the diversity of molecular changes induced by proteasome inhibition. One of the earliest hypotheses concerning the mechanism of tumor cell killing induced by bortezomib was derived from the role of the proteasome in degradation of IκB, the inhibitor ofNFκB [1-3]. However, because of the proved markedly less active reduction of the MM cells' proliferation on the influence of IκB inhibitors in comparison with bortezomib [4,5], this explanation seemed incomplete. Bortezomib also caused 20-to-60-fold induction of the proapoptotic gene *NOXA* in various cancer cells [6] whereas in other models it impaired tumor growth via inhibition of HIF-1α and repression of *HIF-1* transcriptional activity with attenuation of the release of vascular endothelial growth factor (VEGF) [7]. Other studies suggested that the general accumulation of misfolded proteins in the endoplasmic reticulum (ER) is the major mechanism responsible for the antitumural activity of bortezomib [8,9]. The latter insult initiates the UPR signaling, which in turn stimulates splicing of inactive XBP1 [10] whereas spliced XBP1/XBP1s regulate(s) genes, which are responsible for the ER-associated degradation (ERAD) (e.g. EDEM), ending-up within the proteasome. XBP1s also induces genes that are responsible for protein folding such as p58IPK and a variety of ER chaperones [11]. Although UPR activation can regenerate protein homeostasis anditis also essential for plasma cell differentiation and survival by induction of various ER chaperones and folding enzymes [12-15], under prolonged and uncompensated ER stress the UPR promotes cellular apoptosis, known as terminal UPR [15-17]. The latter occurrence is mediated via the pro-apoptotic transcription factor CHOP (also known as GADD153 and DDIT3), which is induced via PERK and ATF6 pathways. CHOP causes down-regulation of BCL2, thereby leading to caspase-dependent apoptosis [18,19]. In HNSCC cells, bortezomib induced apoptosis through induction of ER stress along with the generation of reactive oxygen species (ROS) that led to caspase activation whereas inhibition of NFκB was not sufficient to initiate apoptosis [20]. Consistent with the ER stress concept of bortezomib anti myeloma activity, it was found a correlation between the levels of immunoglobulin chain production and the sensitivity to proteasome inhibitors in sub-clones of both human IgG-secreting myeloma cell line JK-6L and murine myeloma cell line Ag8, transfected with expression plasmid, encoding the μ heavy (H) chain [21]. Moreover, pro-apoptotic factors of the ER stress response were induced to a greater extent in sub-clones producing high levels of μH-chains than in those producing no μH-chains. Conversely, MM cells became bortezomib-resistant through inhibition of unfolded protein accumulation by acquired mutations of the PSMB5 gene which prevented the catastrophic ER stress [22].

The present work deals with the cytotoxic mechanisms offered in the context of authentic MM cells from patients whose clinical response to bortezomib regimens was followed for years.

2. METHODS

2.1 Tissue Culture

Bone marrow (BM) aspirates were collected into heparinized syringes and transferred into vertical tubes for ≥30 minutes; then the fluid layer above the red blood cell (RBC) sediment was collected and ~ 10^7 cells (50-300 μl aspiration fluid) were seeded into 24-well plates (BD Falcon™24-well Multi well Plate). After ≥ 15 minute incubation (RT) 1 mlRPMI 1640- containing 2 mM L-glutamine (Sigma, Rehovot, Israel), 50U/mlpenicillin, 50μg/mlstreptomycin (Sigma) and 20% (v/v) heat-inactivated fetal bovine serum (Biological Industries, Kibbutz Beit Haemek, Israel) was added to wells. Cells were maintained at 37℃ in a humidified 5%

CO_2incubator. Cultures were fed two times per week by replacing 70% of the medium with fresh supplement. The bortezomib used was VELCADE® for injection (Janssen-Cilag Ltd). For RNA extraction most of the medium was removed (leaving 200-300 µl) and after a vigorous pippetation the released cells were collected and fixed with liquid nitrogen in ~70 µl fractions.

2.2 RNA Extraction

Frozen samples were lysed by adding 300µllysis buffer to tubes. RNA was isolated by MagNA Pure Compact RNA Isolation procedure using MagNA Pure Compact instrument (Roche Diagnostics, Ltd, Israel). Integrity of RNA was examined by Agilent 2100 Bioanalyzer.

2.3 Gene Expression Profile (GEP)

Biotin-labeled cRNA was generated from 200 ng total RNA, hybridized onto GeneChip Human Gene1.0 ST Array (Affymetrix, Santa Clara, CA, USA) and the data were processed with the Affymetrix GeneChip Scanner 3000 and Affymetrix Expression Console. Normalization was done by the RMA method and fold change results were calculated relative to the fresh BM sample of each case. The microarray data were deposited on the public gene expression ominibus (GEO) accession number GSE51940 http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE51940

2.4 Patients

The MM cases studied were selected according to their clinical fitness to the study design including extreme BM infiltration with tumor cells (> 90%), which eliminated the need for cell separation procedures known to bias the authentic GEP records [23-24] and long-term follow-up. The study was approved by the local institute review boards. We analysed BM samples from an overall group of five MM patients, two of which were newly diagnosed (cases A, B) and responded to velcade/dexamethasone (Vel/Dex) induction, another patient (case C) responded with nearly complete response to retreatment with Vel/Dex combination after long term remission of 30 months following initial treatment with bortezomib, dexamethasone and melphalan (VMP) as opposed to the other two patients who failed velcade regimens, one with newly diagnosed MM (case D) showing primary resistance to Vel/Dex combination as reflected by increase in his paraprotein levels and persistence of > 95% plasma cells in repeated BM examination after 6 injections of velcade (days 1, 4, 8, 11, 29, 32, 36, 39); the second refractory case (case E) was heavily pre-treated and initially responded to Vel/Dex with VGPR (very good partial response) followed by long-term remission after consolidation stem cell transplantation (SCT) with melphalan 200 mg/m^2. However, after almost 3 years the patient progressed and received second line treatment with lenalidomide/dexamethasone (Len/Dex) but was refractory to this regimen, then he partially responded to retreatment with Vel/Dex and continued with 2 salvage cycles of VD-PACE (velcade combined with dexamethasone, platinol, adriamicin, etoposide) and underwent second SCT while in VGPR (case D). Once again, after 6 months the patient progressed but now he became completely resistant to bortezomib with no response to Vel/Dex and to VD-PACE as evident by the development of chest wall plasmacytomas, pancytopenia, hypercalcemia, renal failure, sharp increase in his urine paraprotein levels as well as > 95% plasma cells in the BM aspirate and biopsy under treatment. At this stage his BM sample was examined in our present study and the patient entered a clinical trial with carfilzomib, pomalidomide and dexamethasone after which he achieved a PR (partial response), with drop in his paraprotein levels to almost 1/3 of his pre-treatment levels and improvement in all of his clinical and laboratory tests including repeated BM biopsy. The five patient's characteristics are summarized in Table 1. Velcade was added to the cultured BM samples at a concentration of 2 µg/ml, which was found in our preliminary assessment to kill cultured primary MM cells within 24 hours (data not shown). In one of the cases (case C), velcade was also used at a concentration of 0.2 µg/ml, which showed almost the same GEP changes as the higher concentration. After 6-8 hours the treated and control cells were released from the bottom wells and they were then fixed with liquid nitrogen.

RESULTS

As compared with the control samples, the GEPs analysed from the bortezomib-treated samples showed dramatic up-regulation of a large subset of genes, many of which encoding heat shock proteins (HSPs) and this observation was common to the entire group of 5 patients

Table 1. Patient characteristics and response to treatment

	Age Sex	Paraprotein CRAB*, FISH	ISS	Velcade regimen	Response to velcade regimen	Salvage regimen (if given)/response
Case A	85♂	Non-secreting C, R, A, B t(4;14)	III	Vel/Dex	VGPR* after 16 injections of velcade	-
Case B	60♀	IgAk 3.0 g/dl C, R, A, B t(4;14)	III	VCD	CR* after 16 injections of velcade	-
Case C	75♀	IgAk 3.5 g/dl A, B N/A	III	Vel/Dex	VGPR* after 8 injections of velcade retreatment	NR** to lenalidomide
Case D	55♂	IgAk 3.1g/dl A, B; del 13q	II	Vel/Dex	NR** after 8 injections of velcade retreatment	R+D (PR) → SCT (VGPR)
Case E	63♂	IgAλ 3 g/dl, 16.6 g/day C,R,B; del P53	III	Vel/dex	NR** after 6 injections of velcade retreatment	Pomalidomide + Carfilzomib (PR)

examined independently of their clinical response to bortezomib regimens (Fig. 1). The pronounced induction of HSP transcripts in the samples from our bortezomib resistant cases raises debates concerning the anti-malignancy role attributed to UPR activation [8-10, 17-19, 25-29] and excludes the possibility that the clinical inactivity of the drug in those patients resulted from inaccessible binding site or un blcokable proteasome in the tumor cells otherwise no induction of HSP transcripts could be elicited in vitro. Accordingly, the models of impaired drug binding used to explain the mechanism of bortezomib resistance on the basis of acquired resistance in mutant sub-clones [30-34] might be irrelevant to primary tumor cells as already noticed [35]. In addition, neither *NOX*A nor *HIF1A, GADD45A, GADD45B, GADD45G, TNFRSF10B, FAS, FASLG, DAP3, CASP8, CASP7* or *CASP1*, which were reported to promote apoptosis in bortezomib treated cells [36,37], were induced markedly in any of the BM samples examined in our series and no *BCL2* repression could be recognized. Furthermore, although the pro-apoptotic signaling molecule CHOP (DDIT3), which is considered to be induced and activated by ER stress [18,19], was induced to somewhat greater extent in our sensitive versus resistant to bortezomib cases, the differences in expression were too smallto explain the clinical differences seen. In contrast, a distinct subset of 7 genes (e.g., *MMP12, IL7R, MGST1, C3, CYP27A1, MIR148A* and *CXXC4)* was modulated exclusively in the BM samples from our bortezomib-responsive cases (Fig. 2), though the predictive value of this observation requires further assessment in a larger cohort of samples.

Within the limitations of our results, the first issue to be considered is the differences in the drug exposure in vitro versus in vivo. In the current study, bortezomib was added to cultures in concentration much higher (~20 folds) than the usual peak plasma levels (89-120 ng/ml). However, bortezomib is rapidly and widely distributed to tissues and the mean 24-hour total radioactivity levels (TR) of ^{14}C-bortezomib was found to be 43.5, 30.5 and 27.8 folds higher in the BM versus plasma of Sprague-Dawley rats after the first, third and fourth dose of the drug in a biweekly schedule, respectively [38]. Likewise, the area under the concentration-time curve (AUC; μg-eq. h/g) of TR between 0 and 72 hours post ^{14}C-bortezomib injection ($AUC_{0\text{-}72\ h}$) was 31.7 and 24.1 folds higher in the BM versus plasma after the first and the fourth dose, respectively. Therefore, it seems reasonable that the bortezomib concentration, applied in vitro in the presented paper, is also reachable in the BM. In addition, in case C which was also examined with velcade concentration of 0.2 μg/ml the changes in GEP were almost the same as with 2 μg/ml.

4. DISCUSSION

The comparative GEP presented in the current manuscript, which was analysed from the BM samples of patients with sensitive/responsive ersus resistant to bortezomib myeloma adds complementary information to prior GEP series; the latter provided response and survival classifiers [39]. Our main finding was the observation that preserved proteasome inhibition capacity (evident from the potent UPR induction) is by itself not sufficient neither predictive for the anti-myeloma activity of bortezomib. Thus, it seems possible that the clinical resistance to bortezomib regimens may involve post-proteasome blockade failure to activate the optional pro-apoptotic pathway, e.g., p38 and ATF6 [40], Bax and Bak [41], generation of reactive oxygen species (ROS) [42,43], disregulation of the intra-cellular calcium [44],

Case Treatment	A Vel	B Vel	C Vel	D Vel	E Vel	C Len	D Len	D Thal
HSPA6	113.6	149.2	18.4	49.3	47.5	1.1	2.0	1.2
ZFAND2A	46.0	14.2	14.8	5.9	6.7	1.1	0.9	0.2
HSPB1	21.0	12.5	5.5	5.0	20.4	1.0	0.8	0.5
SERPINH1	20.3	15.0	13.5	9.0	12.8	1.4	1.1	0.6
HSPA7	19.1	93.4	12.9	9.4	11.3	1.3	0.6	0.5
SNORD14E	19.1	10.6	3.1	1.9	3.0	1.0	1.0	0.1
BAG3	18.7	36.1	7.9	6.9	11.4	0.9	1.0	0.6
HSPA1A	16.4	55.1	3.7	14.4	11.3	1.2	1.3	2.2
HSPA1A	16.4	58.0	2.9	15.0	11.0	1.2	1.3	2.0
DNAJB1	15.4	44.0	8.4	19.2	15.3	1.0	1.0	0.6
HSPA1B	14.2	72.3	4.3	8.8	11.7	1.2	0.8	1.6
HSPA1B	14.2	72.3	4.3	8.8	11.7	1.2	0.8	1.6
HSPA1B	13.9	70.3	4.1	10.7	11.5	1.2	1.0	2.1
HSPA4L	13.0	19.1	10.7	17.3	3.0	0.7	1.1	0.6
SERPINB2	11.6	33.3	2.3	3.2	2.8	0.9	1.5	0.7
HSPH1	11.1	23.9	9.4	17.8	10.3	0.9	0.9	1.9
7981333	8.0	79.5	21.0	3.3	15.3	1.0	0.9	0.6
8104625	5.9	13.0	6.2	10.7	8.9	1.0	0.8	0.8
HMOX1	5.5	5.1	16.6	2.4	1.9	1.0	0.6	0.4
HSP90AA6P	5.3	9.6	10.6	5.9	5.0	1.2	1.3	0.1
PRRG4	4.6	5.2	2.9	1.4	3.1	1.7	1.1	0.6
SLC7A11	4.4	6.8	8.4	4.1	2.3	1.2	2.1	1.4
HSP90AA4P	3.8	9.1	5.7	8.6	7.0	0.8	1.0	0.8
SLC5A3	3.8	7.9	2.1	2.5	3.3	1.0	1.0	0.8
PIR	3.6	2.3	2.0	1.4	2.7	0.8	0.9	0.6
HSP90AA5P	3.6	3.2	2.9	2.5	1.7	1.0	1.0	0.6
SLC5A3	3.5	7.9	2.0	1.7	3.7	1.0	1.1	0.7
ABHD3	3.5	3.3	3.8	2.8	3.8	0.7	0.8	0.9
LOC10013234€	3.3	9.5	5.4	4.3	3.7	1.3	0.8	1.0
RBM11	3.3	2.1	4.4	2.7	3.3	1.0	1.0	0.5
CHRNA5	3.2	7.6	8.0	5.9	22.2	0.8	1.3	1.1
FLJ16734	3.1	2.5	2.3	3.3	3.4	1.0	1.3	0.8
8112196	3.1	5.6	4.8	2.6	3.8	0.9	0.7	1.1
MLLT11	3.1	3.2	4.4	3.3	2.9	1.1	0.8	0.2
FCGR2A	3.0	2.4	3.1	2.1	0.8	1.6	2.0	2.4
CHAC1	3.0	3.3	2.0	2.1	2.4	1.4	1.1	1.1
P4HA2	3.0	8.5	4.1	2.2	4.7	1.0	0.9	1.0
HSP90AA2	2.9	5.1	4.5	5.9	3.6	0.9	0.7	0.5
HSPD1	2.9	9.6	4.6	2.7	4.3	1.0	0.5	2.3
TSEN15	2.8	3.6	2.4	2.6	3.2	0.8	1.2	0.7
SNAP23	2.8	2.9	3.1	2.3	2.6	0.9	1.0	0.7
PPID	2.8	4.8	2.4	1.8	2.4	1.1	1.0	1.2
MORC4	2.8	2.9	2.7	3.9	4.4	1.6	0.6	0.7
PTGES3	2.7	3.1	3.0	1.5	2.9	0.9	1.1	0.8
CHORDC1	2.7	9.6	8.6	3.8	5.5	0.9	0.9	1.1
DNAJA4	2.6	15.9	12.1	3.2	5.8	0.9	0.9	0.5
DNAJB4	2.5	9.6	5.9	8.0	3.6	0.8	1.0	1.5
HSP90AA1	2.5	4.4	5.5	4.1	3.1	0.9	1.0	0.8
CRYZ	2.4	2.5	2.2	2.1	2.3	1.0	0.9	0.1
FKBP4	2.3	14.8	4.1	2.7	6.1	0.9	0.7	0.9
DNAJA1	2.3	5.5	4.3	3.6	3.3	1.0	1.6	2.0
CLIC2	2.2	6.0	3.5	3.6	3.6	0.9	1.1	0.4
HSPE1	2.2	7.3	2.6	2.7	2.6	1.1	0.6	2.1
CACYBP	2.1	9.3	4.3	4.7	4.6	0.8	0.7	1.7
SNORD14C	34.7	10.6	1.6	2.1	2.9	1.0	0.9	0.5
7917514	13.7	2.3	1.7	3.9	6.7	0.9	2.6	0.8
CYB5R2	3.6	2.3	1.5	2.4	4.3	0.8	0.7	0.5
CTH	3.4	8.2	1.8	3.1	5.2	1.1	1.1	0.4
CLDN12	3.4	4.0	0.8	2.7	2.9	1.9	3.0	0.6
BLNK	3.1	1.5	2.2	2.4	6.1	0.9	1.1	0.6
BOLL	2.0	2.1	1.2	2.1	2.4	1.2	1.1	0.4
MRPL18	1.9	5.2	4.4	3.3	2.1	0.9	0.8	0.9
7923582	1.9	11.6	5.6	5.0	4.1	1.0	0.7	0.8
MORC4	1.9	2.7	2.6	2.3	3.5	1.3	0.5	0.4
8180310	1.7	3.4	2.8	2.6	2.6	1.0	1.0	0.9
HSP90AB1	1.6	4.2	3.0	3.4	2.6	0.9	1.0	2.3
STIP1	1.6	7.8	3.3	3.0	3.1	0.9	0.9	1.1
CACYBP	1.6	2.6	2.2	2.6	3.2	1.1	0.9	1.0
MLKL	1.6	2.5	2.8	2.9	3.6	1.0	1.2	2.2
HSP90AB2P	1.5	5.2	3.7	3.3	2.7	1.2	1.1	1.2
DNAJB6	1.5	3.5	2.5	2.1	2.2	1.2	1.0	0.4
HSP90AB3P	1.5	5.8	5.1	3.3	3.6	0.9	1.0	2.4
FAM10A4	1.3	2.4	2.0	2.1	2.1	1.0	1.3	0.5
LOC729992	1.2	2.7	2.3	2.5	2.2	1.1	1.0	2.0
SRXN1	1.2	6.7	2.6	2.8	3.2	1.3	1.0	0.6
ME1	1.2	2.3	4.6	2.2	13.4	0.7	0.6	0.3
HSPA4	1.1	4.2	3.2	2.6	2.4	0.8	1.0	1.3
SOD1	0.9	2.9	3.5	2.9	2.2	0.9	0.8	0.5
AHSA1	0.9	6.1	3.8	2.8	2.7	0.9	1.3	1.4
CRYAB	0.6	2.4	6.3	10.5	3.5	0.9	1.0	0.3
FAM72D	1.3	0.2	0.5	0.4	0.5	0.7	0.6	2.9
LOC643332	1.1	0.3	0.4	0.4	0.3	0.9	0.8	0.3
hCG_1990547	0.3	0.7	0.4	0.3	0.5	0.8	0.8	1.1

<0.5 <0.75 >1.5 >2

Fig. 1. Modulated similarly in bortezomib-responsive and resistant cases

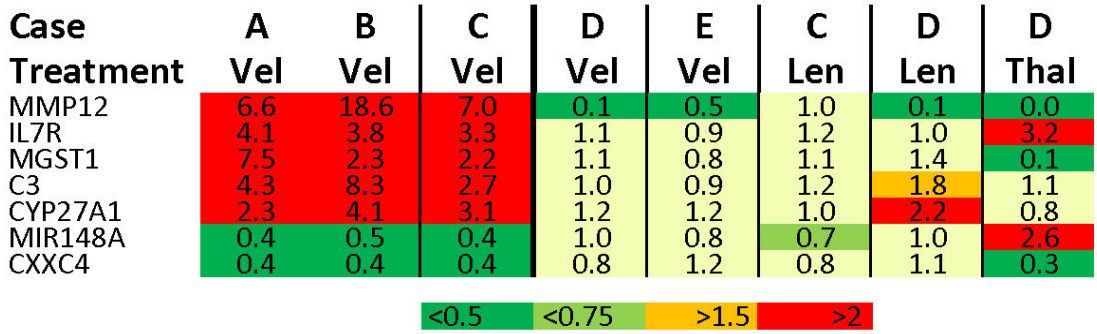

Fig. 2. Genes modulated exclusively in bortezomib-responsive cases

or as yet unrecognized proteasome-related pro-apoptotic pathway. On the other hand, the excellent clinical response to bortezomib regimens despite the marked induction of numerous HSP transcripts in the BM samples from our bortezomib-responsive cases raises debates, connected with the role, attributed to HSPs on bortezomib resistance and concerning the expectations from HSP inhibitors to overcome drug resistance [45-47], though direct ERAD inhibitors might synergized with bortezomib [48]. The second important finding was the subset of 7 genes which distinguished responsive/sensitive versus resistant to bortezomib myeloma. In considering the known roles of these genes, it seems reasonable that the induction of some of which, like *MMP12* and *C3*was originated from contaminated macrophages which sensed the initial injury to adjacent tumor cells. For instance, MMP12 (macrophage metalloelastase) is a matrix metallopeptidase, which predominantly expressed by mature tissue macrophages and is implicated in pathological processes [49]. In contrast, *IL7R* was implicated in leukemogenesis [50]. Currently, we expand the study group in order to test the applicability of the findings in prediction of the clinical response to proteasome inhibitors on the basis of the in vitro results.

CONSENT

Not applicable.

ETHICAL APPROVAL

All authors hereby declare that all experiments have been examined and approved by the appropriate ethics committee and have therefore been performed in accordance with the Ethical standards laid down in the 1964 declaration of Helsinki.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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