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BH3-profiling identifies heterogeneous dependency on Bcl-2 family members in Multiple Myeloma and predicts sensitivity to BH3 mimetics

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Despite a significant improvement in the survival of patients with multiple myeloma (MM), the disease remains incurable and innovative strategies are needed. Interactions among BCL-2 family proteins mainly determines cellular fate decision in response to drug therapy. Thus, anti-apoptotic members such as BCL-2, BCL-X_L or MCL-1, represent an attractive target for therapy¹. BH3 profiling is a functional assay that identifies the tumor cell's addiction to these anti-apoptotic members². The oral BCL-2-specific BH3 mimetic ABT-199 demonstrated very promising results in BCL-2 dependent malignancies such as chronic lymphoid leukemia (CLL)³ and mantle cell lymphoma^{4,5}. To date, there has been no determination of what proportion of MM cases are likely to be BCL-2 dependent. The present study demonstrated that MM is a heterogeneous disease with respect to BCL-2, BCL-X_L or MCL-1 dependence. Moreover, identification by BH3 profiling of BCL-2 dependence predicted *in vitro* sensitivity to BH3 mimetics.

Dependence on BCL-2, BCL-X_L or MCL-1 was measured in 8 human myeloma cell lines. After cell permeabilization, mitochondria were exposed to standardized amounts of BAD, HRK or MS1 peptides. The affinity of these peptides for the anti-apoptotic proteins are

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CONFLICT-OF-INTEREST DISCLOSURE

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AUTHORSHIP CONTRIBUTIONS

AL and CT designed research and wrote the manuscript. CT performed experiments. CT, AL, JR, TN analyzed the data. PR, KA, MA, SLG, PM provided myeloma cells. All the authors critically reviewed the manuscript.

summarized in Figure 1a^{2,5,6}. The release of cytochrome c induced by each peptide was quantified by FACS analysis. Dependence on individual anti-apoptotic proteins was found to be very heterogeneous from one cell line to another (Figure 1b). One cell line (LP-1) was found to be relatively purely MCL-1 dependent. XG-5 and SKMM2 were found to be relatively BCL-2 dependent and MM1-S was found to be relatively purely BCL-X_L dependent. The remaining cell lines were characterized by co-dependency on anti-apoptotic proteins: BCL-2 and BCL-X_L (KMS12-PE) or BCL-X_L and MCL-1 (NCI-H929 and RPMI-8226) and BCL-2 and MCL-1 (L-363). We then determined the *in vitro* sensitivity of MM cell lines to the BH3 mimetics ABT-199 and ABT-263 (Figure 1c). The mitochondrial response to BAD peptide predicted the *in vitro* sensitivity to ABT-263 (Figure 1d). The BAD minus HRK mitochondrial response was used to reflect the specific BCL-2 dependency and significantly predicted sensitivity to ABT-199. (Figure 1d) Finally, the cytochrome c release in response to ABT-199 and ABT-263 strongly correlated with the *in vitro* sensitivity to the drug.

We then determined the mitochondrial priming of primary plasma cells obtained from 14 different patients with a diagnosis of de novo or relapsed MM. Patient characteristics are summarized in Figure 2a. As observed with cell lines, patient samples showed a heterogeneous dependence on anti-apoptotic proteins (Figure 2a). Of note, mitochondria of 5 patient samples were found to be relatively independent of BCL-2, BCL-X_L or MCL-1. We also determined the *in vitro* sensitivity to ABT-199 and ABT-263. Three and 5 samples were found sensitive (LD₅₀ 100 nM) to ABT-199 and ABT-263, respectively. As shown in Figure 2b, BH3 profiling using the BAD peptide correlated with *in vitro* sensitivity to ABT-199 and 263.

BH3 profiling is a unique, functional method to measure the dependence to the anti-apoptotic proteins in live cancer cells. The present study demonstrates that Multiple Myeloma is a heterogeneous disease regarding its dependence on anti-apoptotic proteins and cannot be considered as monolithically BCL-2, BCL-X_L or MCL-1 dependent. MCL-1 is expressed in MM cells at levels higher than normal plasma cells and its expression level has been shown to affect clinical outcome^{7,8}. Here, mitochondria from half of MM cell lines (4/8) and from almost one third of primary samples (4/14) were found to be MCL-1 dependent. These results are consistent with the requirement for MCL-1 for survival of many myeloma cells. BH3 profiling also identified a subset of BCL-2 and/or BCL-X_L dependent MM cells with relatively less dependence on MCL-1 and correctly predicted sensitivity to the BH3 mimetics ABT-199 and 263. In our series, one MM cell line (MM1-S) and 2 primary samples (#7 and #11) were found to be sensitive to ABT-263 but insensitive to ABT-199, that linked to their BCL-X_L dependence determined using BH3 profiling. Even if these findings indicate that BCL-X_L could be an attractive target for MM, the BCL-X_L-related platelet toxicity has impaired the clinical development of ABT-263⁹. Previous studies identified MM cells sensitive to BH3 mimetics based on their BCL-2/MCL-1 mRNA ratio^{10,11} or interactions of BCL-X_L and BCL-2 with BIM¹². From a practical point of view, BH3 profiling can be performed in just a three hours with fewer cells (5.10⁴ plasma cells to determine response to the BAD BH3 peptide). This consideration is of importance because bone marrow samples from MM patients usually contain a low percentage of plasma cells (the median percentage of plasma cell in BM aspirate was 9% in our series). It has been

previously reported that sensitivity to ABT-199 was restricted in MM patients with t(11;14) translocation¹¹. Here, among the three ABT-199 sensitive samples, two were found to be t(11;14). By including previous data from Touzeau et al.¹¹, sensitivity to ABT-199 from 29 different primary MM samples was analyzed. Overall, 7 samples (24%) were found to be sensitive to the drug (LD₅₀<100 nM). Interestingly, 6 of these ABT-199 sensitive samples carried the t(11;14) translocation. Of note, the remaining sensitive sample was found to be BCL-2 dependent according to BH3 profiling. Moreover, one sensitive patient sample was found to be negative for the t(11;14) translocation suggesting that BCL-2 dependence may exist beyond this cytogenetic subgroup. The positive (PPV) and negative (NPV) predictive value of BH3 profiling to predict ABT-199 sensitivity were 75 and 100%, respectively (supplemental Table).

Overall, and as previously demonstrated in other hematologic malignancies^{2,13,14}, BH3 profiling identifies BCL-2 dependence in myeloma cells and predicts sensitivity to BH3 mimetics. Phase 1 clinical trials evaluating ABT-199 as a single agent (NCT01794520) or in combination with bortezomib plus dexamethasone (NCT01794507) in relapsed MM patients are ongoing. Preliminary results of these trials demonstrated significant anti myeloma response with a favorable safety profile.^{16,17} The promising predictive value of BH3 profiling needs to be confirmed in the context of prospective clinical studies. Because BCL-2 dependency was found only in a subset of myeloma patients, future clinical trials with an efficacy objective should integrate biomarkers such BH3 profiling to stratify potential candidates for ABT-199 therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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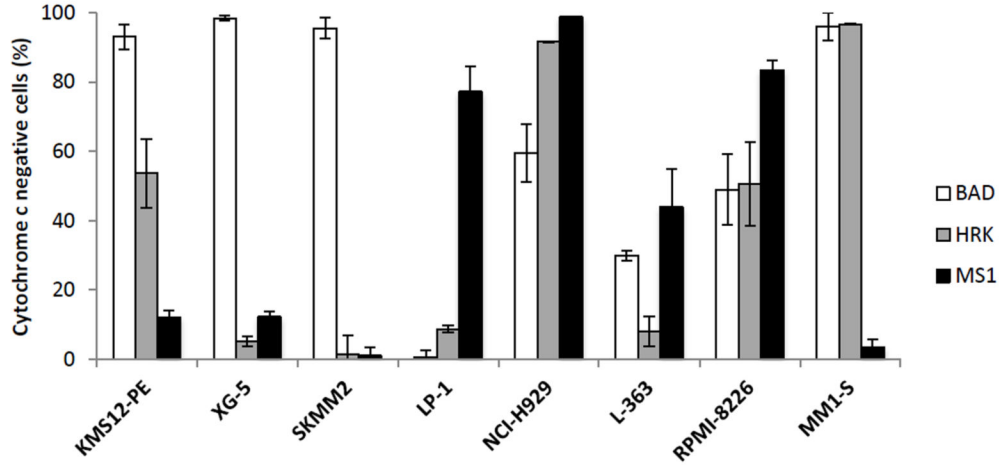
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a

	BAD	HRK	MS1	ABT-199	ABT-263
BCL-2	Red	Green	Green	Red	Red
BCL-X _L	Red	Red	Green	Red	Red
MCL-1	Green	Green	Red	Green	Green

b



c

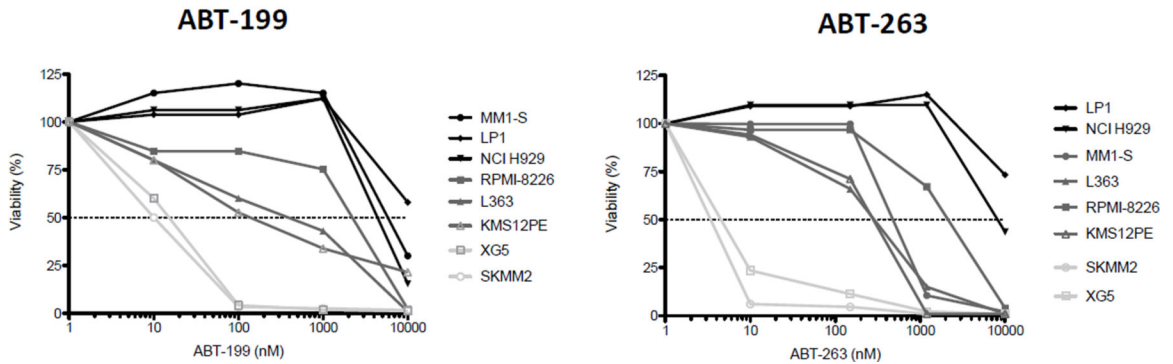


Figure 1. BH3 profiling of Myeloma cell lines demonstrates heterogeneous Bcl-2 dependency and correlates with *in vitro* sensitivity to BH3 mimetics

(a) Binding affinity of BH3 peptides, ABT-199 and ABT-263 for the anti-apoptotic proteins. Green and red colors indicate high and low affinity, respectively^{2,5,6}. (b) KMS12-PE, XG-5, SKMM2, LP-1, L363, NCI-H929, RPMI-8226 and MM1-S identity was confirmed by DNA fingerprinting or HLA typing. Intracellular BH3 (iBH3) profiling was performed using BAD (100µM), HRK (100µM) and MS-1 (10µM) peptides. As previously described¹³, tumor cells were pelleted and resuspended in DTEB buffer with addition of each BH3 peptide

treatment with 0.002% w/v digitonin. BAD, HRK and MS1 peptides sequences have been previously described^{7,8}. Mitochondria in the permeabilized cells were exposed to peptides for 45 min at 26°C before fixation with 2% formaldehyde at room temperature for 15 min. After addition of neutralizing buffer, cells were stained with anti-cytochrome *c*-Alexa488 (#560263, BD Biosciences) 1:100 in 0.1% Saponin/1% BSA/PBS overnight at 4 C. The quantification of cytochrome *c* loss induced by each peptide was analyzed by FACS (LSR Fortessa flow cytometer, BD Bioscience). Values indicate the percentage of cytochrome *c* negative cells. Indicated values are the mean of 3 independent experiments. **(c)** Sensitivity of Myeloma cell lines to the BH3 mimetics ABT-199 and ABT-263 was assessed using Cell Titer Glow® assay. Cell lines were cultured with increasing doses of ABT-199 or ABT-263 during 24 hours. Indicated values are the mean of 3 independent experiments. Light grey line indicates IC₅₀ <100 nM, Dark grey line indicates IC₅₀ between 100 and 5000 nM, black lines indicate IC₅₀ > 5000 nM **(d)** IC₅₀ of myeloma cell lines were correlated with the mitochondrial response to BAD (100µM), BAD (100µM) – HRK (100µM) peptides and with ABT-199 or 263 (1µM). All correlations were tested using a one-tailed Spearman *r* correlation using GraphPad Prism software.

a

#	Sex/Age	Disease Status	Isotype	Cytogenetic Abnormalities ¹	BH3 profiling ²			Sensitivity to BH3 mimetics ³	
					BAD	HRK	MS1	ABT-199	ABT-263
1	M/41	Diag	CL k	t(11;14)	89	90	63	82	75
2	W/59	Rel	CL L	-	5	2	5	11	10
3	W/73	Rel	G k	t(16;14)	2	4	2	6	9
4	W/75	Rel	Ig G k	-	25	30	16	23	36
5	M/73	Diag	Ig G K	t(11;14)	1	5	50	2	8
6	W/81	Diag	Ig G k	HD	7	2	1	26	37
7	M/73	Rel	CL k	t(4;14)	46	76	52	30	55
8	W/57	Rel	Ig G k	HD	7	5	8	11	22
9	W/66	Rel	Ig G k	-	72	1	2	72	70
10	M/44	Rel	Ig G k	17p del	73	34	1	6	46
11	M/49	Diag	Ig G k	HD	44	31	1	27	57
12	W/63	Diag	Ig A k	t(11;14)	2	1	2	14	14
13	W / 60	Diag	Ig G k	-	19	30	61	5	20
14	W / 61	Diag	Ig G k	t(11;14)	51	4	5	70	75

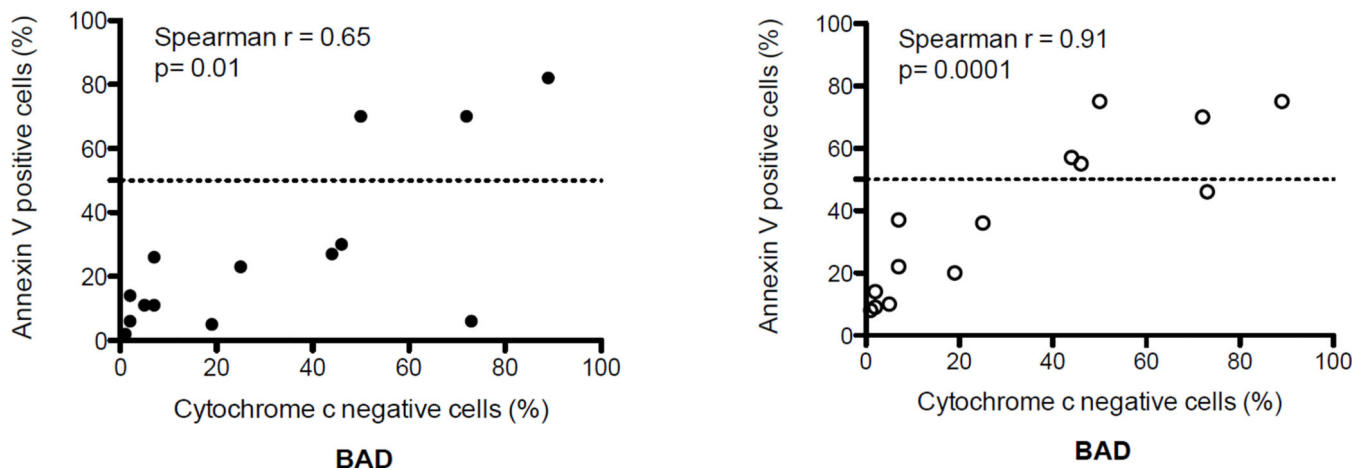
b

Figure 2. BH3 profiling of primary myeloma cells predicts lines in vitro sensitivity to BH3 mimetics

(a) Mitochondrial priming of multiple myeloma primary cells. Primary cells were obtained after informed consent from MM patients treated at the Dana Farber Cancer Institute, Boston, USA. Intracellular BH3 (iBH3) profiling was performed using BAD (100 μ M), HRK (100 μ M) and MS-1 (10 μ M) peptides. Abbreviations: Iso, immunoglobulin isotype; Diag, Newly diagnosed myeloma; Rel, relapsed myeloma. HD: hyperdiploidy. ¹All samples were screened by FISH for the t(11;14), t(4;14), 17p deletion and karyotype was performed for

hyperdiploidy status. ²Values indicate the percentage of cytochrome c negative cell. ³Values indicate the percentage of apoptotic cells. Cells were cultured with/without the drugs during 16 hours. Cell death was assessed by flow cytometry after annexin V staining Dark grey: 50%, Intermediate grey: 20–49, light grey: <20% **(b)** Primary samples were cultured for 16 hours with/without ABT-199 or ABT-263 (100 nM). Cell death was measured by FACS using Annexin V staining. Percentages of apoptotic cells were correlated with the BAD (100µM) peptide. All correlations were tested using a one-tailed Spearman r correlation using GraphPad Prism software.

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