Histone deacetylase inhibition in combination with MEK or BCL-2 inhibition in multiple myeloma

Vijay G. Ramakrishnan,1,* Kevin C. Miller,2* Elaine P. Macon,3 Teresa K. Kimlinger,4 Jessica Haug,5 Sanjay Kumar,1 Wilson I. Gonsalves,1 S. Vincent Rajkumar1 and Shaji K. Kumar1

1Division of Hematology, Department of Medicine, Mayo Clinic, Rochester, MN and 2Mayo Clinic School of Medicine, Rochester, MN, USA
*VGR and KCM contributed equally to this work.

©2019 Ferrata Storti Foundation. This is an open-access paper. doi:10.3324/haematol.2018.211110

Received: November 16, 2018.
Accepted: March 5, 2019.
Pre-published: March 7, 2019.
Correspondence: VIJAY G. RAMAKRISHNAN - ramakrishnan.vijay@mayo.edu
Supplementary Methods

Multiple Myeloma Cell Lines and Patient Cells

MM1S, MM1R, OPM2, RPMI8226 and U266 cell lines were kindly provided by Dr. Jonathan Keats (TGen, Phoenix, AZ, USA). KMS11, KMS18, KMS28BM, OPM-1 and OPM-2 were obtained from Dr. Leif Bergsagel (Mayo Clinic, Scottsdale, AZ, USA). DOX40 was kindly provided by Dr. William Dalton's laboratory (Moffitt Cancer Center, Tampa, FL, USA). H929 was purchased from ATCC (Manassas, VA, USA). All cell lines, except OPM-1 and OPM-2, were cultured in RPMI 1640 media (Mediatech Inc., Manassas, VA, USA) containing 10% fetal bovine serum (FBS) (Mediatech, Inc.), 2 mM L-glutamine (Invitrogen, Grand Island, NY, USA), 100 U/mL penicillin, and 100 µg/mL streptomycin (Invitrogen); OPM-1 and OPM-2 were cultured in the same type of media except instead 20% FBS was used. Likewise, freshly obtained bone marrow aspirates from patients with MM were collected after informed consent, and cultured in RPMI 1640 media that contained 20% fetal bovine serum, 2 mM L-glutamine (GIBCO), 100 U/mL penicillin, and 100 µg/mL streptomycin.
**Supplementary Table 1.** Clinical characteristics of the patients with multiple myeloma (MM), smoldering multiple myeloma (SMM) and monoclonal gammopathy of undetermined significance (MGUS) from which bone marrow plasma cells were sorted, cultured and exposed to drugs of interest at indicated doses (Supplementary Tables 2-4).

| Patient # | Sex | Diagnosis | Age at diagnosis | ISS at diagnosis | Disease status at time of biopsy | Age at time of biopsy | ISS at diagnosis | Cytogenetic/ FISH risk status | Cytogenetics/FISH | # of prior lines of therapy | Prior ASCT? |
|-----------|-----|-----------|------------------|------------------|---------------------------------|----------------------|------------------|--------------------------|----------------|-----------------------------|-------------|
| MC1       | M   | MGUS      | 62               | n/a              | n/a                             | 62                   | Normal           | trisomy 3, 7, 9, 15 and trisomy/tetrasomy 11 | 0             | N                           |             |
| MC2       | M   | MM        | 86               | n/a              | Newly diagnosed                 | 86                   | Normal           | trisomy 3, 7, 9, 11, and 15 | 0             | N                           |             |
| MC3       | F   | MM        | 56               | II               | Relapsed                        | 58                   | High             | TP53 deletion, t(4;14) and monosomy 13 | 1             | Y-1                         |             |
| MC4       | F   | MM        | 70               | n/a              | Day +100 post ASCT, in VGPR     | 71                   | High             | TP53 deletion, 1q duplication, monosomy 13 and 16, and trisomy 7 and 9 | 2             | Y-1                         |             |
| MC5       | M   | MM        | 70               | III              | Newly diagnosed                 | 70                   | High             | TP53 deletion, and monosomy 13 and 14 | 0             | N                           |             |
| MC6       | M   | MM        | 60               | III              | Relapsed                        | 64                   | Normal           | hyperdiploidy, with trisomies 3, 7, 9, 11 and 15 | 4             | Y-1                         |             |
| MC7       | M   | MM        | 61               | III              | Relapsed                        | 62                   | Normal           | t(11;14)                      | 1             | N                           |             |
| MC8       | M   | MM        | 59               | III              | Newly diagnosed                 | 59                   | High             | 1q duplication, trisomy 9 and 15 | 0             | N                           |             |
| MC9       | F   | MM        | 78               | n/a              | Newly diagnosed                 | 78                   | Normal           | t(11;14)                      | 0             | N                           |             |
| MC10      | M   | MM        | 63               | I                | Newly diagnosed                 | 63                   | High             | 1q duplication and monosomy 14 | 0             | N                           |             |
| MC11      | F   | MM        | 65               | III              | Relapsed and refractory         | 76                   | High             | trisomy 3, 9, 11, and trisomy/tetrasomy 15 | 12            | Y-2                         |             |
| MC12      | M   | MM        | 62               | II               | Newly diagnosed                 | 62                   | Normal           | trisomy 3, 9, 11, and trisomy/tetrasomy 15 | 0             | N                           |             |
| MC13      | M   | MM        | 54               | III              | Relapsed                        | 61                   | High             | 1q duplication and trisomies 3, 7, 11 and 17 | 2             | Y-1                         |             |
| MC14      | M   | MM        | 60               | III              | Newly diagnosed                 | 60                   | High             | t(4;14) and 1q duplication | 1             | N                           |             |
| MC15      | M   | MM        | 73               | II               | Newly diagnosed                 | 73                   | Standard         | trisomy 3, 7, 9, 11, 15 | 0             | N                           |             |
| MC16      | F   | MM        | 79               | III              | Newly diagnosed                 | 79                   | High             | 1q duplication 13q deletion; trisomies 5, 9, and 15 | 0             | N                           |             |
| MC17      | M   | SMM       | 58               | n/a              | Stable (evaluated for neuropathy) | 61                   | Standard         | t(11;14)                      | 0             | N                           |             |
| MC18      | M   | MM        | 70               | n/a              | Relapsed and refractory         | 75                   | High             | TP53 deletion, 1q duplication, trisomies 3, 7, 9, 11, and 15, and monosomy 13 | 4             | Y-1                         |             |
| MC19      | M   | MM        | 71               | I                | Newly diagnosed                 | 71                   | Standard         | monosomy 13, and trisomies 3, 7, and 9 | 0             | N                           |             |

ISS: International Staging System. ASCT: Autologous stem cell transplantation. VGPR: Very good partial remission.
Supplementary Table 2. Plasma cells from patients with multiple myeloma were exposed to either AZD6244 or ABT-199 alone, or in combination with either MS275 (entinostat) or FK228 (romidepsin) for 72 hours. The proportion of cells undergoing apoptosis was measured using flow cytometry, and the relative fold change in apoptosis is indicated. The mean fold change in apoptosis, summarizing the results from the n=2, n=2, and n=4 patients is also shown. Few patient samples were available, so choice of particular drug combinations was arbitrary and based on limited availability.

| Drug Dose (nM) | Relative fold change in apoptosis |
|---------------|----------------------------------|
|               | Control | AZD6244 | MS275 | AZD6244+MS275 |
| AZD6244+MS275 |         |         |       |               |
| MC13          | 500 AZD6244 | 200 MS275 | 1   | 2      | 4.25 | 8   |
| MC16          | 500 AZD6244 | 200 MS275 | 1   | 4      | 3.67 | 15.33 |
| Mean fold change in apoptosis | 1   | 3      | 3.96 | 11.67 |

| AZD6244+FK228 |         |         |       |               |
| MC7           | 500 AZD6244 | 2.5 FK228 | 1   | 1.67 | 2.9 | 10.78 |
| MC9           | 500 AZD6244 | 2.5 FK228 | 1   | 1      | 1.77 | 2.62 |
| Mean fold change in apoptosis | 1   | 1.34 | 2.34 | 6.70 |

| ABT-199+MS275 |         |         |       |               |
| MC13          | 500 ABT-199 | 200 MS275 | 1   | 3      | 4.25 | 14 |
| MC16          | 500 ABT-199 | 200 MS275 | 1   | 2.33 | 3.67 | 12.67 |
| MC17          | 500 ABT-199 | 200 MS275 | 1   | 7.71 | 2.71 | 10.57 |
| MC19          | 500 ABT-199 | 200 MS275 | 1   | 2.38 | 2.62 | 6.62 |
| Mean fold change in apoptosis | 1   | 3.86 | 3.31 | 10.97 |
Supplementary Figure Legends

Supplementary Figure 1.

Single-agent MEK inhibition does not effectively induce cell death in MM cell lines. (A) Cellular viability at 72h, assayed with MTT, after single-agent MEK inhibition with AZD6244 in a panel of human MM cell lines, shown as % of control in the Y-axis. (B) Proliferation arrest by 72h in the same cell lines, assayed using ³H-thymidine incorporation into DNA, after single agent AZD6244 treatment, shown as % of control in the Y-axis. (C) Viability of the RAS/RAF mutated human MM cell lines H929 and MM1S, measured as the proportion of annexin (-)/PI (-) cells, assessed using flow cytometry, after 24, 48 and 72h of AZD6244 (5000 nM) treatment, shown as % of control in the Y-axis. (D) Viability of the human MM cell line MM1S, assayed with MTT, after 72h of treatment with AZD6244/LBH589, MEK162/LBH589, or SCH772984/LBH589 at the indicated doses, shown as % of control in the Y-axis. Error bars represent the standard error of the mean (SEM) of triplicate experiments. Differences between groups were calculated with the Student t test, where ** denotes \( P < 0.001 \) and ## denotes \( P < 0.01 \). All experiments were performed in triplicate.

Supplementary Figure 2.

BCL-2+HDAC inhibition induces enhanced apoptosis in BCL-2 primed MM cell lines. (A) Cell viability, assessed using flow cytometry by analyzing the proportion of annexin (-)/PI (-) cells, shown as a percentage, after ABT-199/LBH589 treatment at 72h in KMS18 and OPM2. All experiments were performed in triplicate.

Supplementary Figure 3.

HDAC6 inhibition does not enhance apoptosis induced by MEK inhibition. (A) The HDAC6 inhibitor tubacin and AZD6244 were combined in increasing doses. Cellular viability was assessed using MTT at 72h in the human MM cell lines MM1S and H929. Viability is shown as % of control in the Y-axis. Both experiments were performed in triplicate. (B) MM1S was electroporated with scrambled siRNA or HDAC6 siRNA, then left untreated or treated with 150 nM AZD6244. At 72h, cell viability was assessed using flow cytometry by analyzing the proportion of annexin (-)/PI (-) cells, shown as % of control in the Y-axis. Whole-cell lysates were separated using SDS-PAGE and subject to western blotting for the indicated proteins to confirm silencing. Error bars represent the SEM of triplicate experiments.

Supplementary Figure 4.

Class I HDAC inhibition replicates synergy with MEK or BCL-2 inhibition in MM cell lines. (A) The HDAC1, 2 and 3 inhibitor MS275 (entinostat) was combined with AZD6244 in increasing doses. Cellular viability was assessed using MTT at 72h in the human MM cell lines MM1S, RPMI8226 and MM1R. Viability is shown as % of control in the Y-axis. Combination index (CI) values <1.0, indicating synergy, are shown for each cell line. (B) MS275 was combined with ABT-199 in increasing doses in the human MM cell lines KMS18, KMS28 and OPM2. Cellular viability was assessed using MTT at 72h, shown as % of control in the Y-axis. (C) H929 was treated with 250 nM AZD6244 and 1 nM of the HDAC1 and 2 inhibitor FK228 for 24h, then immunoprecipitates for BIM, BCL-2, MCL-1, BCL-X\( _L \) or whole cell lysates (input) were separated using SDS-PAGE and probed for the indicated proteins. All experiments were performed in triplicate.
Supplementary Figure 2.

A.

KMS18

Control

ANNA

88.8%

ABT-199-48HRS

77.5%

LBH589-48HRS

44.8%

ABT-199 + LBH589-48HRS

8.7%

OPM2

Control

ANNA

87.9%

ABT-199-48HRS

80.4%

LBH589-48HRS

46.2%

ABT-199 + LBH589-48HRS

28.3%

ABT-199-72HRS

70.6%

LBH589-72HRS

9.7%

ABT-199 + LBH589-72HRS

82.9%

ABT-199-72HRS

28.3%

LBH589-72HRS

3.4%

ABT-199 + LBH589-72HRS
Supplementary Figure 3.

A.

Viability (% of control) vs. AZD6244 (nM) and Tubacin (µM) for MM1S and H929 cell lines.

B.

Annexin + PI + (% of scrambled siRNA) for different conditions:
- Scrambled siRNA
- HDAC6 siRNA

Western blot images of HDAC6 and GAPDH for MM1S and H929 cell lines.
Supplementary Figure 4.

A. MCL-1 primed cell lines

B. BCL-2 primed cell lines

C. H929

|        | BIM IP         | Input         |
|--------|----------------|---------------|
|        | BIM (EL)       | MCL-1         |
|        | BIM (L)        | pMCL-1<sup>564</sup> |
|        | BIM (S)        | BCL-2         |
|        |                 | BIM (EL)      |
|        |                 | BIM (L)       |
|        |                 | BIM (S)       |

|        | BCL-2 IP       | MCL-1 IP      |
|--------|----------------|---------------|
|        | BIM (EL)       | MCL-1         |
|        | BIM (L)        | pMCL-1<sup>564</sup> |
|        | BIM (S)        | BCL-2         |
|        |                 | BIM (EL)      |
|        |                 | BIM (L)       |
|        |                 | BIM (S)       |

|        | BCL-X<sub>1</sub> IP | AZD6244 | FK228 |
|--------|-----------------------|---------|-------|
|        | BIM (EL)              | +       | +     |
|        | BIM (L)               | +       | +     |
|        | BIM (S)               | +       | +     |

|        | ABT199 (nM) | MS275 (nM) | Fa | CI |
|--------|-------------|-------------|----|----|
| MM1S   | 250         | 150         | 0.638 | 0.745 |
| MM1R   | 250         | 150         | 0.641 | 0.285 |
| KMS18  | 150         | 125         | 0.726 | 0.325 |
| KMS28  | 250         | 225         | 0.668 | 0.578 |
| OPM2   | 150         | 125         | 0.672 | 0.512 |