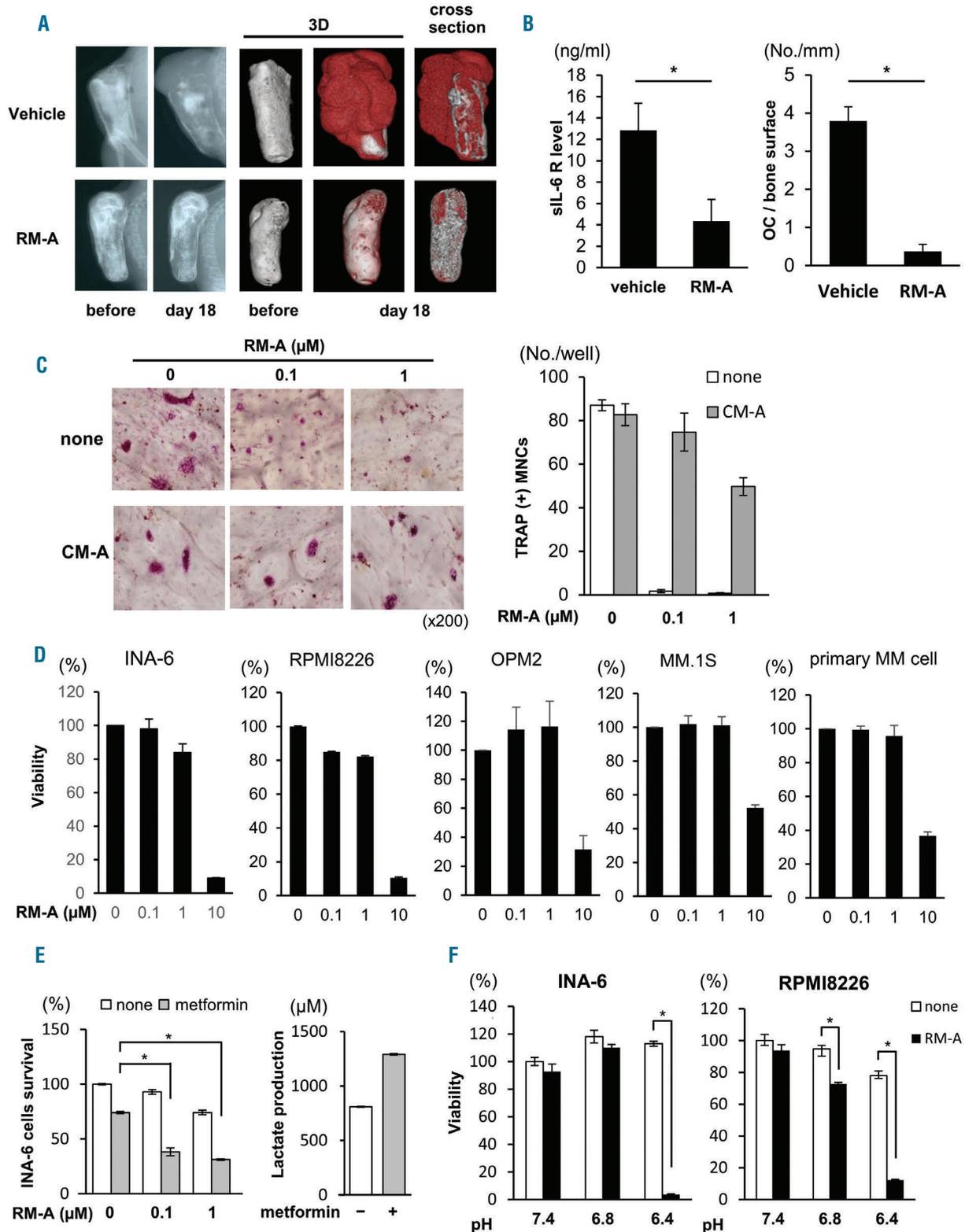


**Reveromycin A, a novel acid-seeking agent, ameliorates bone destruction and tumor growth in multiple myeloma**

Along with the progression of bone disease, the bone marrow (BM) microenvironment is skewed in multiple myeloma (MM). This underlies the unique pathophysiology of MM, and confers aggressiveness and drug resist-

ance. Receptor activator of NF- $\kappa$ B ligand (RANKL), a critical mediator of osteoclastogenesis, is upregulated to extensively enhance osteoclastogenesis and bone resorption in MM. Importantly, activated osteoclasts (OC) in turn enhance glycolysis in MM cells and thereby MM cell proliferation, leading to the formation of a vicious cycle between MM tumor expansion and osteoclastic bone destruction.<sup>1-3</sup> OC should therefore be targeted to improve treatment efficacy, especially in MM cells



**Figure 1. Effects of reveromycin A (RM-A) on osteoclasts (OC) and multiple myeloma (MM) cell viability.** (A) Effects of RM-A on MM cell-bearing SCID-rab models. To prepare SCID-rab mice, rabbit femurs were cut into two and implanted subcutaneously in SCID mice. A month later the human MM cell line INA6 was inoculated directly into the bone marrow (BM) cavity in the rabbit bones implanted in SCID mice. After confirming the MM cell growth at 4 weeks after the MM cell inoculation, we started to inject RM-A at 4 mg/kg or a vehicle (saline) to the mice (n=5 for each treatment) intraperitoneally twice daily for 18 days. Soft X-ray and micro-computed tomography ( $\mu$ CT) images of the implanted rabbit femurs were taken before and after the treatment with RM-A or a vehicle. Representative images of soft X-ray (left panels) and  $\mu$ CT (right panels) are shown. MM tumor lesions are shown in red in 3D and cross sections of the rabbit bones in  $\mu$ CT images. (B) INA-6 cell-derived human soluble IL-6 receptor (sIL-6R) levels in mouse sera were measured as a marker for MM tumor burden after the treatment for 18 days with RM-A or a vehicle. The rabbit bones were taken out and analyzed to count the numbers of OC over bone surface (OC/bone surface). Data are expressed as the mean  $\pm$  standard error (SE). (C) Rabbit BM cells were cultured on the bovine bone slice in 96-well culture plates in RPMI1640 containing 10% fetal bovine serum with 20 ng/mL soluble receptor activator of NF- $\kappa$ B ligand (RANKL) for 4 days. After washing, RM-A at the indicated concentrations was added in triplicate for 24 hours (h) in the presence or absence of concanamycin A (CM-A) at 100 nM. The cells were then stained with tartrate-resistant acid phosphatase (TRAP), and photos were taken (original magnitude, x200) (left). The numbers of TRAP-positive multinucleated cells (MNC) with three and more nuclei were counted (right). Data were expressed as % changes from the baseline (mean  $\pm$  SE). (D) INA-6, RPMI8226, OPM2 and MM.1S MM cell lines and primary MM cells were cultured at  $2 \times 10^5$ /mL for 24 h at the indicated concentrations of RM-A. Viable cell numbers were counted with a WST-8 assay. The data were expressed as % changes from the baseline (mean  $\pm$  SE). (E) INA-6 cells were cultured in triplicate for 24 h in the presence of RM-A at the indicated concentrations with or without 5 mM metformin. Cell viability was analyzed by a WST-8 assay (left). The results were expressed as % changes from the baseline without any treatment (mean  $\pm$  SE). \* $P < 0.05$ . Lactate levels in the culture supernatants were measured after the treatment with metformin for 24 h. (F) INA-6 and RPMI8226 MM cells were cultured in triplicate in the media whose pH values were adjusted by sodium hydroxide or lactic acid. RM-A was added at 1.0  $\mu$ M. After culturing for 24 h, cell viability was analyzed by a WST-8 assay. Results were expressed as mean  $\pm$  SE. \* $P < 0.05$ .

expanding in the BM with enhanced osteoclastogenesis.

Under low  $O_2$  conditions, and as a consequence of glycolysis (the Warburg effect), cancer cells highly produce protons and lactate, leading to an extracellular acidification to pH 6.4-7.0, while pH values are 7.2-7.4 in normal tissues.<sup>4</sup> Activated OC on the bone surface abundantly secrete protons into excavated pits ( $\sim$ pH 4-5) to resorb bone while acidifying their close vicinity.<sup>5</sup> In osteolytic bone lesions in MM, therefore, the MM cell-OC interaction appears to create a highly acidic milieu by protons produced by OC and lactate by proliferating glycolytic MM cells. We reported that acid activates the PI3K-Akt signaling to upregulate the acid sensor TRPV1 in MM cells, thereby forming a positive feedback loop between acid sensing and the PI3K-Akt survival signaling.<sup>6</sup> In addition, tumor acidity has been demonstrated to blunt cytotoxic effects of various chemotherapeutic agents as well as the activity of immune effector cells.<sup>7,8</sup> Therefore, acidic conditions should be targeted to improve the therapeutic efficacy against MM.

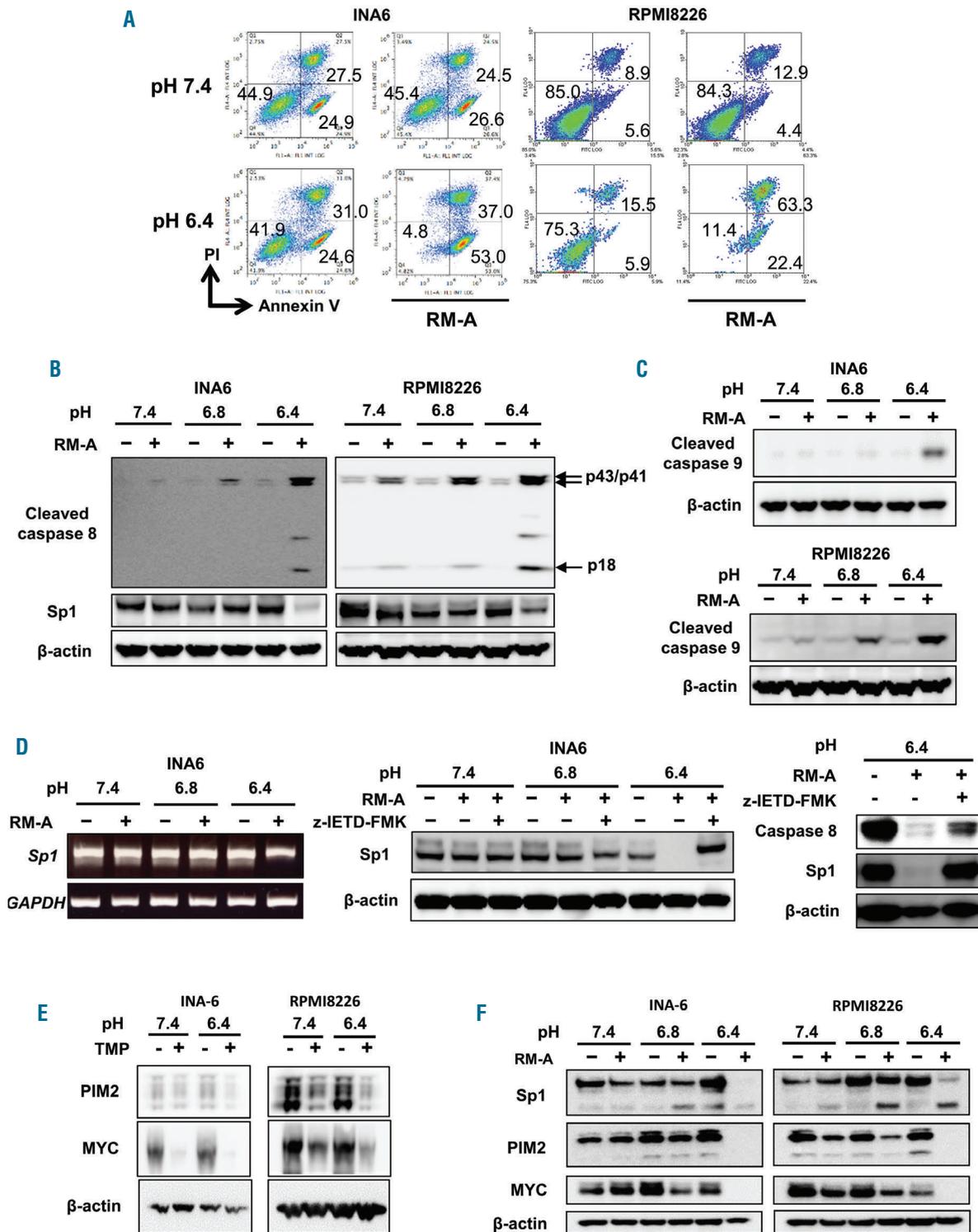
Reveromycin A (RM-A) is a small microbial metabolite with three carboxylic groups, isolated from *Streptomyces* sp. SN-593.<sup>9,10</sup> In an acidic microenvironment, RM-A becomes a non-polar form, which is able to permeate a cell membrane and induce apoptosis by inhibiting isoleucine tRNA synthesis.<sup>9,10</sup> As such, RM-A has been demonstrated to preferentially induce apoptosis in acid-producing OC but not in other types of normal cells.<sup>9-11</sup> In the present study, we explored whether RM-A targets an acidic condition induced by the MM cell-OC interaction to alleviate tumor expansion and bone destruction in MM.

To clarify anti-tumor activity of RM-A against MM, we examined the *in vivo* effects of RM-A in animal models mimicking MM bone lesions. The human MM cell line INA6 was inoculated into rabbit femurs subcutaneously implanted in SCID mice (SCID-rab), as previously reported.<sup>12</sup> SCID-rab mice have been demonstrated to allow human MM cells to grow within the rabbit bones and induce bone destructive lesions as in patients with MM. In vehicle-treated mice, marked radiolucent osteolytic lesions were observed in the implanted rabbit bones on X-ray and micro-computed tomography ( $\mu$ CT) images, and MM tumor was packed in the BM cavity and expanded outside the rabbit bones (Figure 1A). However, in RM-A-treated mice, MM tumor markedly decreased in size without apparent bone destruction in rabbit bones. The levels of human soluble IL-6 receptor in mouse sera, a marker of human MM tumor burden, were also substantially reduced in the RM-A-treated mice (Figure 1B). OC numbers were increased in bone specimens from

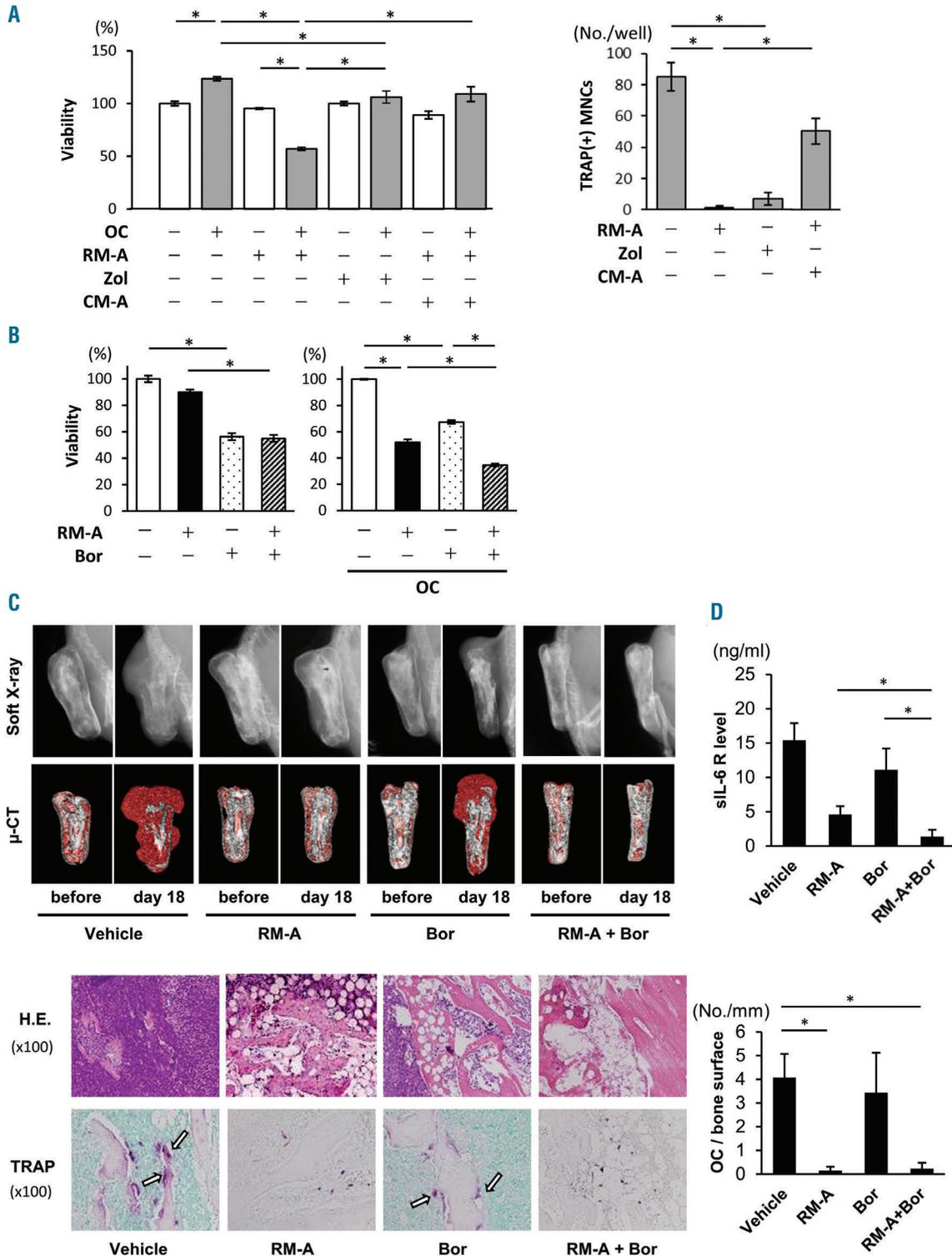
vehicle-treated SCID-rab mice; however, they were markedly reduced in RM-A-treated mice (Figure 1B). These results suggest that RM-A can suppress MM cell growth in the BM along with preventing bone destruction and loss *in vivo*.

To further investigate the effects of RM-A, we first generated OC on bone slices from whole rabbit BM cells, and then treated them with RM-A. Large multinucleated tartrate-resistant acid phosphatase (TRAP)-positive mature OC almost completely disappeared upon treatment with RM-A at 100 nM for 12 hours (h) (Figure 1C). Interestingly, blockade of acid release from OC by the proton pump inhibitor concanamycin A abolished the cytotoxic effect of RM-A on OC, indicating the critical role of acid released from OC in triggering the cytotoxic activity of RM-A. In contrast to OC, RM-A did not affect the viability of MM cell lines and primary MM cells even at higher concentrations up to 1  $\mu$ M at 24 h (Figure 1D). However, RM-A was able to induce MM cell death even at concentrations as low as 100 nM when lactate production from MM cells was enhanced by metformin (Figure 1E). Furthermore, RM-A at 1  $\mu$ M was able to induce cell death in MM cells when culture media were acidified to be at pH6.4 with exogenously added lactic acid (Figure 1F). These results suggest that acid-producing OC are highly susceptible to RM-A, and that an acidic milieu with lactate can trigger the cytotoxic effects of RM-A against MM cells.

We next dissected the mechanisms of the MM cell death in acidic conditions by RM-A. RM-A induced apoptosis in MM cells at pH6.4 but not at pH7.4, as indicated with annexin V-propidium iodide dual staining (Figure 2A). RM-A activated caspase-8 as well as caspase-9 in MM cells at pH6.4 (Figure 2B and C), indicating the induction of caspase-dependent apoptosis. The transcription factor Sp1 has been demonstrated to be overexpressed and to act as a critical pro-survival mediator in MM cells.<sup>13,14</sup> In parallel with the caspase-8 activation, the protein levels of Sp1 were reduced in MM cells at pH6.4 but not at pH6.8 nor pH7.4 (Figure 2B). However, Sp1 mRNA was not decreased in MM cells even at pH6.4. We previously reported that Sp1 protein is subject to enzymatic degradation by caspase-8, thereby inducing MM cell death.<sup>14</sup> Consistent with the previous observation,<sup>14</sup> treatment with the caspase-8 inhibitor z-IETD-FMK abolished the reduction of Sp1 protein at pH6.4 (Figure 2D), indicating caspase-8-mediated degradation of Sp1 protein. Furthermore, treatment with the Sp1 inhibitor terameprocol was able to reduce its target molecules critical for MM cell growth and survival, PIM2<sup>15</sup> and MYC<sup>14,15</sup> (Figure 2E). Consistently, PIM2 and MYC levels were decreased



**Figure 2. Induction of multiple myeloma (MM) cell apoptosis by reveromycin A (RM-A).** (A) INA-6 and RPMI8226 MM cells were cultured for 24 hours (h) at pH 7.4 or 6.4 in the presence or absence of RM-A at 1  $\mu$ M. Apoptotic cells were evaluated with annexin V and propidium iodide staining by flow cytometry. Distributions (%) of cells in each column are indicated. (B and C) INA-6 and RPMI8226 MM cells were cultured for 24 h at different pH values as indicated in the presence or absence of RM-A at 1  $\mu$ M. The protein levels of cleaved caspase-8 and Sp1 (B) and cleaved caspase-9 (C) were analyzed by western blotting.  $\beta$ -actin was used as a protein loading control. (D) INA-6 cells were cultured for 24 h at the indicated pH values in the presence or absence of RM-A at 1  $\mu$ M. Sp1 mRNA levels were analyzed by reverse transcription-polymerase chain reaction (RT-PCR) (left). GAPDH served as an internal control. The caspase-8 inhibitor z-IETD-FMK at 100  $\mu$ M was added together with RM-A as indicated. Sp1 and caspase 8 protein levels were analyzed by western blotting (right).  $\beta$ -actin was used as a protein loading control. (E) INA-6 and RPMI8226 MM cells were cultured for 24 h at pH 7.4 or 6.4 in the presence or absence of the Sp1 inhibitor terameprocol (TMP) at 50  $\mu$ M. PIM2 and MYC protein levels were analyzed by western blotting.  $\beta$ -actin was used as a protein loading control. (F) INA-6 and RPMI8226 cells were cultured for 24 h at the indicated pH values in the presence or absence of RM-A at 1  $\mu$ M. The protein levels of Sp1, PIM2 and MYC were analyzed by western blotting.  $\beta$ -actin was used as a protein loading control.



**Figure 3. Cytotoxic effects of reveromycin A (RM-A) on multiple myeloma (MM) cells in combination with bortezomib.** (A) Rabbit bone marrow cells were cultured on bone slices in the presence of soluble receptor activator of NF- $\kappa$ B ligand (RANKL) to generate osteoclasts (OC). INA-6 cells labeled with the fluorescein dye PKH26 at  $1 \times 10^6$ /mL were co-cultured with the OC generated on bone slices or cultured alone on bone slices. RM-A (1  $\mu$ M), zoledronic acid (Zol) (5  $\mu$ M), or CM-A (100 nM) were added as indicated. After culturing for 12 hours (h), 7-AAD was added to stain dead cells. (B) RM-A at 1  $\mu$ M and/or bortezomib (Bor) at 5 nM were added as indicated. After culturing for 24 h, 7-AAD was added to stain dead cells. The distribution of 7-AAD-negative alive cells was counted within PKH-labeled MM cells in flow cytometry. Results were expressed as % changes from the baselines. (C) INA6 cell-bearing SCID-rab models as described in Figure 1A were prepared. RM-A (4 mg/kg, twice a day) and/or bortezomib (Bor) (0.5 mg/kg, twice a week) were intraperitoneally injected for 18 days ( $n=5$  for each treatment). Saline was injected as a vehicle. Soft X-ray and micro-computed tomography ( $\mu$ CT) images were taken before and after the treatment. Representative images of soft X-ray (upper panels) and  $\mu$ CT (lower panels) are shown. Soft tissue area is shown in red in cross sections of the rabbit bones in  $\mu$ CT images. (D) INA-6 cell-derived sIL-6R levels in mouse sera were measured after the treatment. (E) Hematoxylin and eosin (H&E) (upper) and tartrate-resistant acid phosphatase (TRAP) (lower) staining was performed in the rabbit bones resected from SCID-rab mice. White arrows indicate TRAP-positive OC. The rabbit bones were further analyzed to count the numbers of OC over bone surface (OC/bone surface). Data are expressed as the mean  $\pm$  standard error.

in MM cells preferentially at pH6.4 in parallel with the reduction of Sp1 protein by RM-A.

Because an extracellular acidification makes RM-A permeate cell membrane to induce apoptosis, it is plausible that an acidic milieu created by the OC-MM cell interaction rather induces cytotoxic activity by RM-A against MM cells as well as acid-producing OC. To clarify whether RM-A affects MM cell viability in the presence of OC, we next examined the cytotoxic effects of RM-A on MM cells in co-cultures with OC on bone slices generated from rabbit BM cells. RM-A at 1  $\mu$ M was able to decrease the MM cell viability in the co-cultures with OC, although RM-A at this concentration did not affect MM cell viability when MM cells were cultured alone (Figure 3A, left). When the bisphosphonate zoledronic acid was added to deplete mature OC, viable INA6 cells were decreased in number in co-cultures with OC to the levels observed in the cultures of INA6 cells alone. RM-A reduced the viability of INA6 cells more potently than zoledronic acid in the presence of OC, although RM-A and zoledronic acid similarly reduced the numbers of TRAP-positive multinucleated OC (Figure 3A, right), suggesting that the anti-MM effects of RM-A is not merely due to depletion of mature OC. Blockade of acid release by the proton pump inhibitor concanamycin A abolished the cytotoxic effects of RM-A on MM cells in the co-cultures with OC. These results suggest that RM-A not only impairs OC but also disrupts the OC-MM cell interaction.

We next examined the combinatory effects of RM-A with the proteasome inhibitor bortezomib. Although bortezomib was able to induce MM cell death, the cytotoxic effects of bortezomib on MM cells were mitigated in co-cultures with OC (Figure 3B), indicating drug resistance by OC. However, RM-A impaired the viability of MM cells cultured in the presence of OC; and further potentiated the cytotoxic effects on MM cells in combination with bortezomib, suggesting that RM-A overcomes the drug resistance induced by OC. Finally, we validated the combinatory therapeutic effects of RM-A and bortezomib *in vivo*, using human MM cell-bearing SCID-rab models. Treatment with RM-A suppressed bone destruction and MM tumor growth; importantly, the suppressive effects of RM-A on MM tumor growth and bone destruction was further enhanced in combination with bortezomib, as shown in X-ray and  $\mu$ CT images and the levels of human soluble IL-6 receptor in mouse sera, a marker of MM tumor burden (Figure 3D). In histological analyses, MM cells were tightly packed in the BM cavity of the rabbit bones while bone trabeculae decreased in size with the appearance of multinucleated OC on the surfaces of the remaining bone (Figure 3E). RM-A, but not bortezomib, markedly reduced the number of OC in the SCID-rab mouse MM lesions (Figure 3E). However, treatment with RM-A and bortezomib cooperatively reduced MM tumors along with the disappearance of TRAP-positive large OC on the bone surface.

These results collectively suggest that the acidic microenvironment produced by the MM-OC interaction enhances MM tumor progression but can trigger the cytotoxic effects of RM-A on MM cells as well as acid-producing OC. Given that an acidic condition makes MM cells resistant to chemotherapeutic agents, RM-A could be a candidate to target MM cells at acidic bone lesions, and augment the therapeutic efficacy of currently available anti-MM agents which are active at non-acidic sites.

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## References

- Abe M, Hiura K, Wilde J, et al. Osteoclasts enhance myeloma cell growth and survival via cell-cell contact: a vicious cycle between bone destruction and myeloma expansion. *Blood*. 2004;104(8):2484-2491.
- Lawson MA, McDonald MM, Kovacic N, et al. Osteoclasts control reactivation of dormant myeloma cells by remodelling the endosteal niche. *Nat Commun*. 2015;6:8983.
- Nakano A, Miki H, Nakamura S, et al. Up-regulation of hexokinase II in myeloma cells: targeting myeloma cells with 3-bromopyruvate. *J Bioenerg Biomembr*. 2012;44(1):31-38.
- Ji K, Mayemik L, Moin K, Sloane BF. Acidosis and proteolysis in the tumor microenvironment. *Cancer Metastasis Rev*. 2019;38(1-2):103-112.
- Teitelbaum SL. Bone resorption by osteoclasts. *Science*. 2000; 289(5484):1504-1508.
- Amachi R, Hiasa M, Teramachi J, et al. A vicious cycle between acid sensing and survival signaling in myeloma cells: acid-induced epigenetic alteration. *Oncotarget*. 2016;7(43):70447-70461.
- Gerweck LE, Vijayappa S, Kozin S. Tumor pH controls the in vivo efficacy of weak acid and base chemotherapeutics. *Mol Cancer Ther*. 2006;5(5):1275-1279.
- Tannock IF, Rotin D. Acid pH in tumors and its potential for therapeutic exploitation. *Cancer Res*. 1989;49(16):4373-4384.
- Kawatani M, Osada H. Osteoclast-targeting small molecules for the

- treatment of neoplastic bone metastases. *Cancer Sci.* 2009; 100(11): 1999-2005.
10. Woo JT, Kawatani M, Kato M, et al. Reveromycin A, an agent for osteoporosis, inhibits bone resorption by inducing apoptosis specifically in osteoclasts. *Proc Natl Acad Sci U S A.* 2006;103(12):4729-4734.
  11. Muguruma H, Yano S, Kakiuchi S, et al. Reveromycin A inhibits osteolytic bone metastasis of small-cell lung cancer cells, SBC-5, through an antiosteoclastic activity. *Clin Cancer Res.* 2005;11(24 Pt 1):8822-8828.
  12. Takeuchi K, Abe M, Hiasa M, et al. Tgf-Beta inhibition restores terminal osteoblast differentiation to suppress myeloma growth. *PLoS One.* 2010;5(3):e9870.
  13. Fulciniti M, Amin S, Nanjappa P, et al. Significant biological role of sp1 transactivation in multiple myeloma. *Clin Cancer Res.* 2011; 17(20):6500-6509.
  14. Bat-Erdene A, Miki H, Oda A, et al. Synergistic targeting of Sp1, a critical transcription factor for myeloma cell growth and survival, by panobinostat and proteasome inhibitors. *Oncotarget.* 2016;7(48): 79064-79075.
  15. Asano J, Nakano A, Oda A, et al. The serine/threonine kinase Pim-2 is a novel anti-apoptotic mediator in myeloma cells. *Leukemia.* 2011;25(7):1182-1188.