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

by Keiichiro Watanabe, Ariunzaya Bat-Erdene, Hirofumi Tenshin, Qu Cui, Jumpei Teramachi, Masahiro Hiasa, Asuka Oda, Takeshi Harada, Hirokazu Miki, Kimiko Sogabe, Masahiro Oura, Ryohei Sumitani, Yukari Mitsui, Itsuro Endo, Eiji Tanaka, Makoto Kawatani, Hiroyuki Osada, Toshio Matsumoto, and Masahiro Abe

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Along with the progression of bone disease, the bone marrow microenvironment is skewed in multiple myeloma (MM), which underlies the unique pathophysiology of MM and confers aggressiveness and drug resistance. Receptor activator of NF-κB ligand (RANKL), a critical mediator of osteoclastogenesis, is upregulated to extensively enhance osteoclastogenesis and bone resorption in MM. Importantly, activated osteoclasts (OCs) in turn enhance glycolysis in MM cells and thereby MM cell proliferation, leading to formation of a vicious cycle between MM tumor expansion and osteoclastic bone destruction\(^1\)\(^3\). Therefore, OCs should be targeted to improve treatment efficacy especially in MM cells expanding in the bone marrow 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\(^4\). Activated OCs on the bone surface abundantly secrete protons into excavated pits (~pH 4-5) to resorb bone while acidifying their close vicinity\(^5\). In osteolytic bone lesions in MM, therefore, the MM cell-OC interaction appears to create a highly acidic milieu by protons produced by OCs 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\(^6\). In addition, tumor acidity has been demonstrated to blunt cytotoxic effects of various chemotherapeutic agents as well as the activity of immune effector cells\(^7\)\(^8\). 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 \textit{streptomyces} sp. SN-593\(^9\)\(^10\). 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\(^9\)\(^10\). As such, RM-A has been demonstrated to preferentially induce apoptosis in acid-producing OCs but not in other types of normal cells\(^9\)\(^11\). 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 \textit{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 rabbit bones in SCID mice (SCID-rab), as previously reported\(^12\). 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 \(\mu\)CT images, and MM tumor was packed in the bone marrow 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 bone marrow along with preventing bone destruction and loss in vivo.

To further investigate the effects of RM-A, we first generated OCs on bone slices from whole rabbit bone marrow cells, and then treated them with RM-A. Large multinucleated tartrate-resistant acid phosphatase (TRAP)-positive mature OCs were almost completely disappeared upon treatment with RM-A at 100 nM for 12 hours (Figure 1C). Interestingly, blockade of acid release from OCs by the proton pump inhibitor concanamycin A abolished the cytotoxic effect of RM-A on OCs, indicating the critical role of acid released from OCs in triggering the cytotoxic activity of RM-A. In contrast to OCs, RM-A did not affect the viability of MM cell lines and primary MM cells even at higher concentrations up to 1 µM at 24 hours (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 µM 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 OCs are highly susceptible to RM-A, and that an acidic milieu with lactate can trigger the cytoidal 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 iodine dual staining (Figure 2A). RM-A activated caspase-8 as well as caspase-9 in MM cells at pH6.4 (Figures 2 B-C), indicating the induction of caspase-dependent apoptosis. The transcription factor Sp1 has been demonstrated to be overexpressed and act as a critical pro-survival mediator in MM cells13, 14. 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 death14. Consistent with the previous observation14, treatment with the caspase8 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, PIM215 and MYC14, 15 (Figure 2E). Consistently, PIM2 and MYC levels were decreased 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 induce cytotoxic activity by RM-A against MM cells as well as acid-producing OCs. To clarify whether RM-A affects MM cell viability in the presence of OCs, we next examined the cytotoxic effects of RM-A on MM cells in cocultures with OCs on bone slices generated from rabbit bone marrow cells. RM-A at 1 µM was able to decrease the MM cell viability in the cocultures with OCs, 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 OCs, viable INA6 cells were decreased in number in cocultures with OCs 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 OCs, although RM-A and zoledronic acid similarly reduced the numbers of TRAP-positive multinucleated OCs (Figure 3A, right), suggesting that the anti-MM effects of RM-A is not merely due to depletion of mature OCs. Blockade of acid release by the proton pump inhibitor concanamycin A abolished the cytotoxic effects of RM-A on MM cells in the cocultures with OCs. These results suggest that RM-A not only impairs OCs 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 bortezomib’s cytotoxic effects on MM cells were mitigated in cocultures with OCs (Figure 3B), indicating drug resistance by OCs. However, RM-A impaired the viability of MM cells cultured in the presence of OCs; and further potentiated the cytotoxic effects on MM cells in combination with bortezomib, suggesting that RM-A overcomes the drug resistance induced by OCs. 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 µCT 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 bone marrow cavity of the rabbit bones while bone trabeculae decreased in size with appearance of multinucleated OCs on the surfaces of the remaining bone (Figure 3E). RM-A but not bortezomib markedly reduced the number of OCs in the SCID-rab mouse MM lesions (Figure 3E). However, treatment with RM-A and bortezomib cooperatively reduced MM tumors along with disappearance of TRAP-positive large OCs on the bone surface.

These results collectively suggest that 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 OCs. Given that an acidic condition confers the resistance of MM cells to chemotherapeutic agents, RM-A can 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.

References


Figure legends

Figure 1. Effects of RM-A on OC and 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 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 µCT 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-hand panels) and µCT (right-hand panels) are shown. MM tumor lesions are shown in red in 3D and cross sections of the rabbit bones in µCT 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 osteoclasts over bone surface (OC/bone surface). Data are expressed as the mean ± SE. (C) Rabbit bone marrow cells were cultured on the bovine bone slice in 96-well culture plates in RPMI1640 containing 10%FBS with 20 ng/ml soluble RANKL for 4 days. After washing, RM-A at the indicated concentrations were added in triplicate for 24 hours in the presence or absence of concanamycin A (CM-A) at 100 nM. The cells were then stained with TRAP, and photos were taken (original magnitude, x200) (left). The numbers of TRAP-positive multinucleated cells with 3 and more nuclei were counted (right). The data were expressed as % changes from the baseline (the mean ± SE). (D) INA-6, RPMI8226, OPM2 and MM.1S MM cell lines and primary MM cells were cultured at 2x10^5/ml for 24 hours 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 (the mean ± SE). (E) INA-6 cells were cultured in triplicate for 24 hours 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 (the mean ± SE. *p <0.05). Lactate levels in the culture supernatants were measured after the treatment with metformin for 24 hours. (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 μM. After culturing for 24 hours, cell viability was analyzed by a WST-8 assay. Results were expressed as the mean ± SE. *p <0.05.

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

**Figure 3.** Cytotoxic effects of RM-A on MM cells in combination with bortezomib. (A) Rabbit bone marrow cells were cultured on bone slices in the presence of soluble RANKL to generate OCs. INA-6 cells labeled with the fluorescein dye PKH26 at 1×10⁶/ml were cocultured with the OCs generated on bone slices or cultured alone on bone slices. RM-A (1 µM), zoledronic acid (Zol) (5 µM), or CM-A (100 nM) were added as indicated. After culturing for 12 hours, 7-AAD was added to stain dead cells. (B) RM-A at 1 µM and/or bortezomib (Bor) at 5 nM were added as indicated. After culturing for 24 hours, 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. The results were expressed as % changes from the baselines. The results were expressed as percent 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 µCT images were taken before and after the treatment. Representative images of soft X-ray (left-hand panels) and µCT (right-hand panels) are shown. Soft tissue area is shown in red in cross sections of the rabbit bones in µCT 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 TRAP (lower) staining was performed in the rabbit bones resected from SCID-rab mice. White arrows indicate TRAP-positive OCs. The rabbit bones were further analyzed to count the numbers of osteoclasts over bone surface (OC/bone surface). Data are expressed as the mean ± SE.
Figure 1
Figure 2
Figure 3
Supplementary information

Materials and Methods

Ethics Statement
All experiments with animals were performed according to the guidelines for animal protection in University of Tokushima, and approved by the Institutional Review Board for animal protection. All procedures involving human samples from patients were performed with written informed consent in accordance with the Declaration of Helsinki and a protocol approved by the Institutional Review Board for human protection at University of Tokushima (Permission number: 240).

Reagents
The following reagents were purchased from the indicated manufacturers: concanamycin A from Funakoshi (Tokyo, Japan); rabbit anti-cleaved caspase-8 antibody, rabbit anti-cleaved caspase-9 antibody, rabbit polyclonal anti-Sp1 antibody, rabbit anti-Pim-2 antibody, rabbit anti- antibody, rabbit anti-cMyc antibody, horseradish-peroxidase-conjugated goat anti-rabbit IgG, horseradish-peroxidase-conjugated goat anti-mouse IgG and bortezomib from Cell Signaling Technology (Beverly, MA, USA); mouse anti-β-actin antibody from Sigma (St Louis, MO, USA); Z-Ile-Glu(O-ME)-Thr-Asp(O-Me) fluoromethyl ketone (Z-IETD-FMK) from TONBO biosciences (San Diego, CA, USA); and metformin hydrochloride from Sigma (St Louis, MO, USA).

Cells and cultures
Human MM cell lines, RPMI8226, OPM2 and MM.1S were obtained from the American Type Culture Collection (Rockville, MD). The MM cell line INA6 was kindly provided by Dr. Renate Burger (University of Kiel, Kiel, Germany). Primary MM cells were purified from bone marrow mononuclear cells from patients with MM by positive selection using anti-CD138 microbeads and Miltenyi magnetic cell sorting system (Miltenyi Biotec, Auburn, CA), in accordance with the manufacturer's instructions. MM cells were cultured in RPMI1640 supplemented with 5% fetal bovine serum, 2 mmol/L L-glutamine (Sigma), 100 U/mL penicillin G and 100 μg/mL streptomycin (Sigma). The pH levels in culture media were adjusted by adding lactic acid (Wako, Osaka,
Japan).

**Osteoclast (OC) formation**

Whole bone marrow cells were harvested from the femur of 10-day-old Japanese white rabbit (CLEA, Tokyo, Japan). The cells were cultured on bovine bone slices in Eagle's minimal essential medium alpha modification (α-MEM; Sigma-Aldrich) supplemented with 10% FBS, L-glutamine and 50 μg/mL penicillin/streptomycin. Soluble RANKL (R&D Systems, Minneapolis, MN) was added at 20 ng/mL for 4 days to generate OCs. After culturing for 4 days, non-adherent cells were removed by washing the well with PBS. TRAP-positive cells were detected with a Leukocyte Acid Phosphatase Assay kit (Sigma-Aldrich). TRAP-positive cells containing three or more nuclei were counted as mature OCs under a light microscope. (BX50, Olympus, Tokyo, Japan).

**Cell viability**

Viable cell numbers were measured by cell proliferation assay using 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium (WST-8; Kishida Chemical, Osaka, Japan). After the incubation, the absorbance of each well was measured at 450-655 nm with iMarkTM microplate reader (Bio-Rad Laboratories, Hercules, CA). To assess apoptotic cells, cells were stained with an annexin V-FITC and propidium iodide labeling kit (MEBCYTO Apoptosis Kit; MBL, Nagano, Japan) according to the manufacturer’s instruction, and analyzed by flow cytometry. PKH26 labeled MM cell death was also assessed by flow cytometry using PKH26 Red Fluorescent Cell Linker Kits and 7-AAD ready made solution (Sigma-Aldrich).

**Lactate production assay**

After cultured MM cell lines 24 hours in RPMI1640, the cell culture supernatant was assessed for lactate content by L-lactate assay Kit (Colorimetric) (Abcam, Cambridge, MA).

**Western blot analysis**

Whole cell lysate was lysed in RIPA buffer, and nuclear extract was lysed by NE-PER nuclear and cytoplasmic extraction reagent kit (Thermo Fisher Scientific, Rockford, IL, USA). These lysates were supplemented with 1 mmol/L phenylmethylsulfonyl fluoride
and protease inhibitor cocktail solution (Sigma). Cell lysates were electrophoresed in a 10% SDS-PAGE gel and blotted onto polyvinylidene difluoride membranes (Millipore, Bedford, MA). After blocking with 5% non-fat dry milk, the membranes were incubated with primary antibodies overnight at 4°C, followed by washing and addition of a horseradish-conjugated secondary antibody for 1 hour. The protein bands were visualized with the Enhanced Chemiluminescence Plus Western Blotting Detection System (Amersham Biosciences, Piscataway, NJ).

**RT-PCR**

Total RNA was extracted from cells using TRIZOL reagent (Gibco BRL, Rockville, MD). For reverse transcription-polymerase chain reaction (RT-PCR), 2 µg of total RNA was reverse-transcribed with Superscript II (Gibco) in a 20-µL reaction solution. One tenth of the RT-PCR products were used for subsequent PCR analysis with 24–30 cycles of 95°C for 30 seconds, 58°C for 30 seconds and 72°C for 30 seconds. The following primers were used: The primers used for RT-PCR were as follows: human SP1 sense 5'-TTGAAAAAGGAGTTGGTGGC -3' and anti-sense 5'-TGCTGGTTCTGTAAGTTGGG -3'; human GAPDH sense 5'-TGTTTCACCACCATGGAGAAGG-3' and anti-sense 5'-GTGGATGCAGGGATGATGTTCTG-3'.

**MM mouse model and measurement of serum soluble human IL-6 receptor levels**

The SCID-rab MM mouse model was prepared as previously described. Briefly, femora and tibiae from 4-week-old Japanese white rabbits (Kitayama Labes, Nagano, Japan) were implanted subcutaneously into six-week-old male CB-17 SCID mice (CLEA Japan, Tokyo, Japan). After allowing bone engraftment for 4 weeks following the implantation, 1 x 10⁶ INA-6 cells in 50 µL of phosphate-buffered saline (PBS) was inoculated directly into the bone marrow cavity of the rabbit bones. The treatment with RM-A or bortezomib was initiated 4 weeks after the inoculation of INA6 cells. Saline was injected as a vehicle. Serum levels of soluble human IL-6 receptor derived from INA6 cells were used as a marker for tumor burden as previously described. Mouse sera were collected and serum levels of soluble human IL-6 receptor were measured with an enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN). X-ray photographs and µCT images of the rabbit bones were taken before and after the treatment. µCT images were
taken with Latheta LCT-200 (Hitachi, Tokyo, Japan). The rabbit bones were then collected, fixed in 10% phosphate-buffered formalin, and decalcified with 10% EDTA. The samples were further embedded in paraffin, and sectioned. The sections were stained with hematoxylin and eosin (H.E.) and TRAP for histopathologic examination.

**Statistical analysis**

Statistical significance was determined by a one-way analysis of variance (ANOVA) with Scheffe post hoc tests. The minimal level of significance was a p value equal to 0.05.

**Reference**