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Combination with third-generation bisphosphonate (YM529) and interferon-alpha can inhibit the progression of established bone renal cell carcinoma

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The aim of this study was to investigate whether the third-generation nitrogen-containing bisphosphonate (YM529) can inhibit the progression of established bone renal cell carcinoma (RCC) and to elucidate its mechanism. Antiproliferative effect and apoptosis induction of RCC cells and mouse osteoclasts by YM529 and/or interferon-alpha (IFN-α) were evaluated *in vitro* using cell counting and *in vivo* using soft X-ray, the TUNEL method and tartrate-resistant acid phosphatase stain. For the in *vivo* study, male athymic BALB/cA Jc1-nu nude mice bearing human RCC cell line RBM1-IT4 cells were treated with YM529 and/or IFN-α. The biological activity of osteoclasts was evaluated using the pit formation assay. The antiangiogenetic effect by YM529 and/or IFN-α was analyzed using micro-vessel density and *in situ* mRNA hybridization. Osteoclast number in bone tumors was decreased in YM529-treated mouse. YM529 also inhibited osteoclast activity and proliferation *in vitro*, whereas basic fibroblast growth factor expressions and micro-vessel density within tumors were inhibited by IFN-α. Neither YM529 nor IFN-α alone significantly inhibited the growth of established bone metastatic tumors. Combined treatment with YM529 and IFN-α may be beneficial in patients with human RCC bone metastasis. Their effects are mediated by osteoclast recruitment inhibition and inactivation by YM529 and antiangiogenesis by IFN-α.

Renal cell carcinoma (RCC) is the most common malignant tumor arising from the kidney and accounts for approximately 3% of adult malignancies.[1] The prognosis of patients with metastatic RCC is improving with the introduction of molecular-targeted therapy. Motzer *et al.*[2] report that median overall survival was greater in a sunitinib group than in an interferon-α (IFN-α) group (26.4 *vs* 21.9 months, respectively). However, the prognosis of metastatic RCC is still poor. Moreover, RCC is characterized by high-degree resistance to chemotherapy. IFN-α and interleukin (IL)-2 have been used as immunotherapeutic agents to treat metastatic RCC. However, each achieves complete or partial response in only 10–20% of patients.[3–7]

Bone metastasis from RCC is common. With disease progression, approximately 30% of RCC patients develop bone metastasis.[8] Similar to bone metastasis of breast cancer[9] and multiple myeloma,[10] RCC bone metastasis is osteolytic.[11] Such bone metastases are associated with considerable skeletal morbidity, including severe bone pain and spinal cord or nerve root compression. Osteolytic bone metastasis is not only a critical problem in treatment but also the main reason for the reduced quality of life of patients. Therefore, the development of novel therapeutic strategies is required to improve the outcome and quality of life of patients with RCC bone metastasis.

Bisphosphonates, specific inhibitors of osteoclastic bone resorption, have been widely used as beneficial agents for treating osteolytic bone metastases from several cancer types. Minodronate, YM529 [1-hydroxy-2-(imidazo [1,2-a] pyridin-3-yl) ethylidene]-bisphosphonic acid monohydrate, is a newly developed third-generation bisphosphonate that has more potent inhibitory activity against mouse osteoclastic bone resorption *in vitro* and *in vivo* than that of previously developed bisphosphonates, including pamidronate, alendronate, risedronate and incadronate.[12,13]

We therefore hypothesized that IFN-α would be complimentary to the antitumor effect by YM529 and that it provides an additive or synergistic therapeutic effect against RCC bone metastasis. In this study, combined administration of YM529 and IFN-α, *in vivo*, inhibited tumor growth in established human RCC bone tumor models compared with the treatment with each agent alone.

Materials and Methods

Cell lines and culture conditions. The RCC cell line RBM1-IT4, developed from a bone lesion in a patient with metastatic RCC, was grown as a monolayer in DMEM supplemented with 10% FBS. We obtained the mouse osteoclast commercially. Mouse osteoclast cells using V-2 kit (Mouse; Hokudo, Sapporo, Japan), were grown as a monolayer in 25-mL modified MEM supplemented with 10-ng/mL monocyte macrophage-colony-stimulating factor (M-CSF) and receptor

activator of necrosis factor-κB ligand (RANKL). These cells were maintained at 37°C in a 5%-CO_2 environment.

Reagents. YM529 was provided by Yamanouchi Pharmaceutical (Tokyo, Japan). YM529-stock solutions were prepared in absolute NaOH and suspended in saline. Natural INF-α (OIF) was a kind gift from Otsuka Pharmaceutical, Tokyo, Japan.

***In vitro* cell growth inhibition and apoptosis induction.** The dose-dependent antiproliferative effect and the apoptosis induction by YM529 and/or IFN-α were evaluated after incubating 5×10^4-RBM1-IT4 cells and 2×10^4-osteoclasts in 10%-FBS-supplemented MEM containing increasing YM529 concentration (0–10 μg/mL) or IFN-α (0–10 IU/mL). The effects of combination therapy with YM529 and IFN-α were evaluated after incubating cells with increasing IFN-α concentration (0–10 IU/mL) and YM529 (1 μg/mL). The effects on mouse osteoclasts by mouse IFN-α (mIFN-α) were also evaluated after their incubation with increasing mIFN-α concentration (0–10 IU/mL; PBL Biomedical laboratories, Piscataway, NJ, USA). Growth inhibition was determined after 48 h by cell counting using COULTER Z1 (Beckman Coulter, Tokyo, Japan) and expressed as the ratio of the number of viable cells in each group treated with YM529 and/or IFN-α to the number in the control group treated with PBS. DNA fragmentation quantification was accomplished using the Apoptosis *in situ* Detection Kit (Wako Pure Chemical Industries, Osaka, Japan) after incubation for 48 h.

Biological activity of osteoclasts. The pit formation assay was performed using the osteoclast V-2 kit (mouse; Hokudo), as described previously.[14] The ivory slices were placed in 96-well plates. The mouse osteoclast progenitor cells (4×10^4 cells /well) were seeded in each well. After incubation for 9 days with M-CSF and RANKL, fresh medium containing increasing YM529 concentration (0–10 μg/mL) or IFN-α (0–10 IU/mL) and mIFN-α (0–10 IU/mL) were added. The resorption pit area was expressed as an average percentage of the three highest resorption pit areas compared with the control group (100%) measured using scanning electronic microscopy (SEM; HITACHI, S-2380N, Tokyo, Japan) and analyzed using a computer analysis system.

Animals. Male athymic BALB/cA Jc1-nu nude mice were obtained from Clea Japan (Osaka, Japan). The mice were maintained in a laminar-airflow cabinet in pathogen-free conditions and used at 6–8 weeks of age.

Ectopic implantation and therapy for RBM1-IT4 cells in the tibia of nude mouse. All animal experiments were conducted with care in a manner approved by the Guide for Animal Care and Use Committee of Kochi Medical School. Mice were anesthetized with Nembutal. For ectopic implantation, a percutaneous intraosseal injection was made by drilling a 27-gauge needle into the proximal side of the tibia. After penetration of the cortical bone, RBM1-IT4 cells (2×10^6 cells/20-μL medium) were injected. After 2 months, mice with tumor growth demonstrated on soft X-ray images were randomly separated into four groups. Mice in each group were treated for 4 weeks with i.p. injections of either physiological saline (control) or YM529 (0.3 mg/kg/week), and/or s.c. injections of physiological saline and IFN-α (100 IU/day), according to the schedule shown in Figure 1. Tumors were harvested on day 88 after implantation.

Tissue processing. Mice were killed by cervical dislocation; soft X-ray images of the bone tumors were obtained to evaluate antitumoral effects. The estimated volume of each bone tumor was calculated using three axes (X, Y, Z) using the formula π/6 XYZ. The bone tumors were necropsied and fixed in 20% formalin for 24 h. The bone specimens were decalcified in 10%-ethylene diamine tetra acetic acid solution for 1 week. Bone tissues were processed for routine paraffin-wax histology; sections were adhered to ProveOn Plus Microscope Slides (Fisher Scientific, Pittsburgh, PA, USA) for *in situ* mRNA hybridization (ISH), TUNEL assay and tartrate-resistant acid phosphatase (TRAP) staining. Sections were also stained with HE for routine histological examination.

TUNEL assay. *In vivo* apoptotic tumor cells and bodies were visualized using the Apoptosis *in situ* Detection Kit (Wako Pure Chemical), as described in the kit manual. In agreement with a previous study,[15] apoptotic tumor cells were counted in high-frequency areas under 10 high-power fields. More than 1000 tumor cells were counted to calculate the apoptotic index (AI). AI values were expressed as percentages of TUNEL-positive cells. Apoptotic cells were not evaluated in the vicinity of necrotic areas.

Tartrate-resistant acid phosphatase staining. For osteoclasts detection, TRAP staining was performed using the Sigma Diagnosis Acid Phosphatase Kit (Sigma Diagnosis, St. Louis, MO, USA). The number of TRAP-positive osteoclasts at the tumor–bone interface was counted under a microscope in 10 random high-power fields.

***In situ* mRNA hybridization analysis.** *In situ* mRNA hybridization of basic fibroblast growth factor (bFGF), vascular

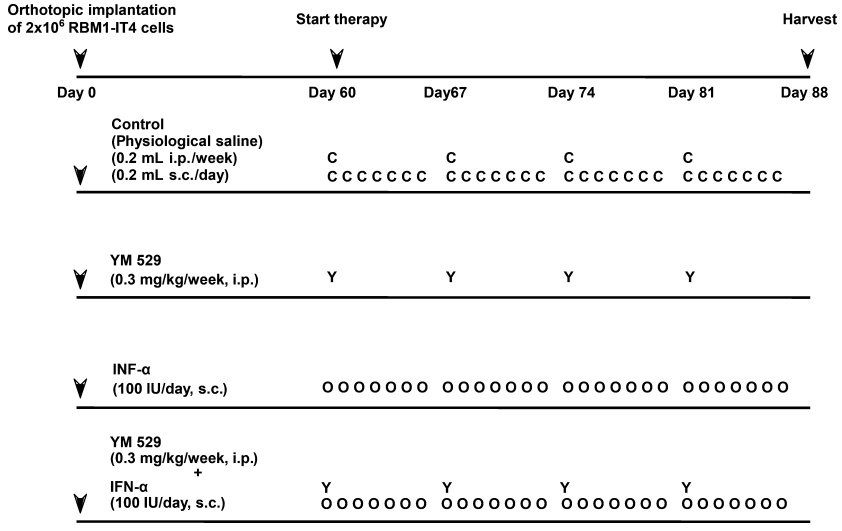

Fig. 1. Treatment schedule. Therapy began 60 days after tumor inoculation. The mice were randomly assigned to one of the four treatment groups and treated for 4 weeks according to schedule. The control and experimental mice were killed and necropsied 4 weeks later.

endothelial cell growth factor (VEGF), Interleukin-8 (IL-8), matrix metalloproteinase type 9 (MMP-9) and 2 (MMP-2), E-cadherin and epidermal growth factor receptor (EGFR) was performed as described previously.[16] ISH was carried out using the Microprobe Manual Staining System (Fisher Scientific). A positive reaction in this assay appears red stained. The control for endogenous alkaline phosphatase activity included sample treatment in the absence of the biotinylated probe and using chromogen alone.

Immunohistochemistry. Immunohistochemical staining was performed with a Ventana Nexus automated immunohistochemistry system (Discovery TM [Ventana Medical Systems, Tucson, AZ, USA]). We used an antihuman factor VIII protein polyclonal antibody (dilution 1:200; DAKO, Kyoto, Japan).

Micro-vessel density quantification. Micro-vessel density (MVD) was determined by microscopy immediately after immunostaining of the section with anti-Factor VIII antibody according to the procedure used in a previous study. The MVD is expressed as the average number in the five most dense areas identified within a single 200× field.[16]

Statistical analysis. The statistical differences in the amount of cell proliferation and apoptosis within the bone tumors were analyzed using the Mann–Whitney test. The incidence of tumors and estimated tumor volume were statistically analyzed ;using the χ^2-test. A P-value <0.05 was considered significant.

Results

***In vitro* inhibition of mouse osteoclast growth by YM529, interferon-alpha and mouse interferon-alpha.** The *in vitro* dose-dependent antiproliferative effects of YM529, IFN-α and mIFN-α for mouse osteoclasts are summarized in Figure 2a. For IFN-α and mIFN-α, no significant antiproliferative effect was observed at any concentration. Osteoclast proliferation was inhibited by treatment with YM529 in a dose-dependent manner. Significant antiproliferative effects were observed with 1 and 10 μg/mL of YM529 (P = 0.0058 and P = 0.0068, respectively). Combined treatment with YM529 (1 μg/mL) and IFN-α (0–10 IU/mL) had significant antiproliferative effects in each IFN-α concentration group compared with the control group (P = 0.0399, 0.001 IU/mL; P = 0.0209, 0.01 IU/mL; P = 0.0140, 0.1 IU/mL; P = 0.0204, 1.0 IU/mL; P = 0.0016, 10.0 IU/mL).

***In vitro* inhibition of RBM1-IT4 cell growth by YM529 and/or interferon-alpha.** The *in vitro* dose-dependent antiproliferative effects of YM529 and/or IFN-α for RBM1-IT4 cells are summarized in Figure 2b. No significant antiproliferative effect was observed in any treatment group.

***In vitro* apoptosis induction in RBM1-IT4 cells and mouse osteoclasts by YM529 and/or interferon-alpha.** The *in vitro* apoptosis induction in RBM1-IT4 cells or mouse osteoclasts with the single-agent treatment groups, that is, YM529 (0–10 μg/mL), IFN-α (0–10 IU/mL) and mIFN-α (0–10 IU/mL), and the combined treatment group with YM529 (1 μg/mL) and IFN-α (0–10 IU/mL) was determined by TUNEL assay. For both mouse osteoclasts and RBM1-IT4 cells, no significant relationship between the AI and drug concentration was observed in any of the treatment groups, including mouse osteoclasts treated with 1 and 10 μg/mL of YM529, in which significant antiproliferative effects were observed (data not shown). There were no additive effects of combined treatment with YM529 for apoptosis induction in mouse osteoclasts or RBM1-IT4 cells.

***In vitro* resorption pit formation by mouse osteoclasts treated with YM529 and/or interferon-alpha.** *In vitro* resorption pit

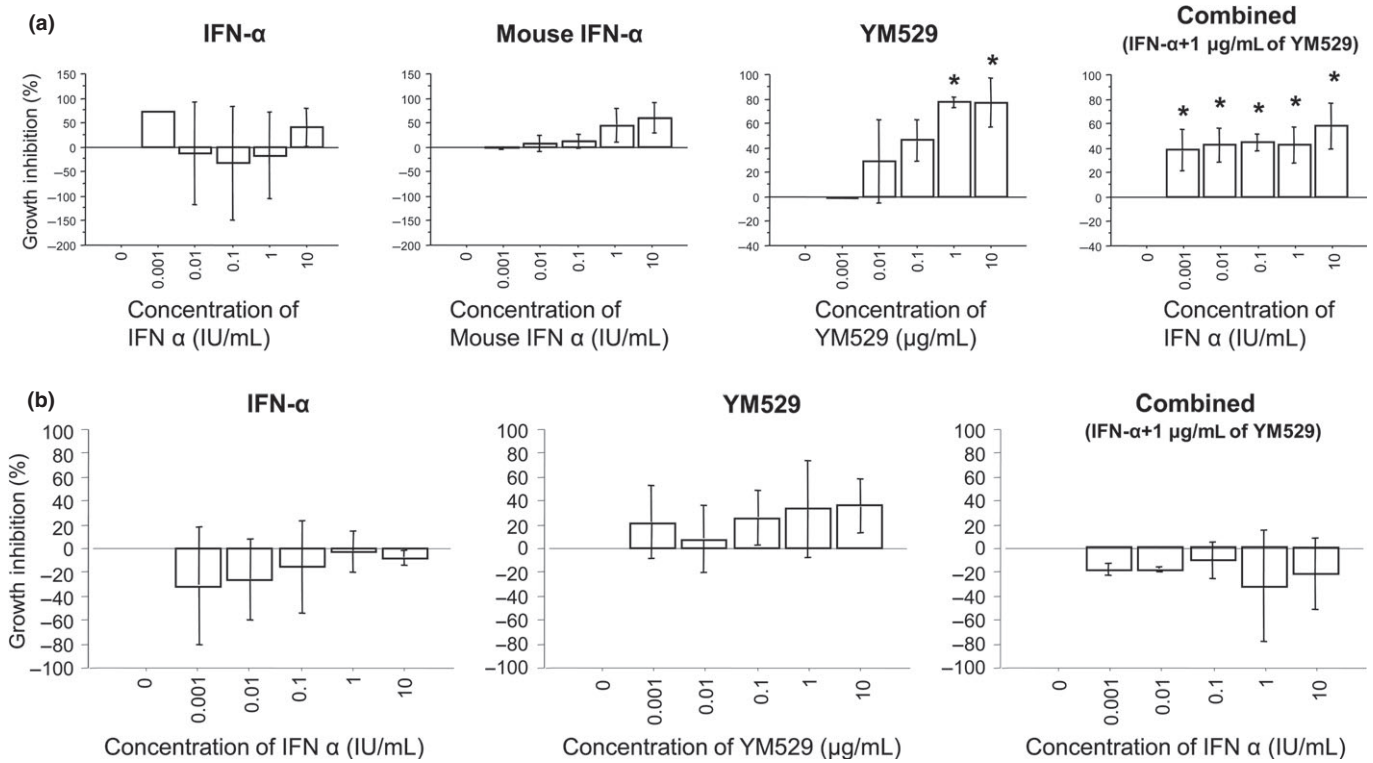

Fig. 2. *In vitro* cell growth inhibition by treatment with single-agents; that is, YM529, IFN-α and mINF-α, and combined treatment with YM529 and IFN-α in mouse osteoclast cells (a) and human RCC RBM1-IT4 cells (b). YM529, both alone and in combination with IFN-α, inhibited mouse osteoclast proliferation in a dose-dependent manner. However, no treatment group significantly inhibited RBM1-IT4 cell proliferation in a dose-dependent manner. (The column upward [+%]mean growth inhibition. Bars represent SD; *P < 0.05.)

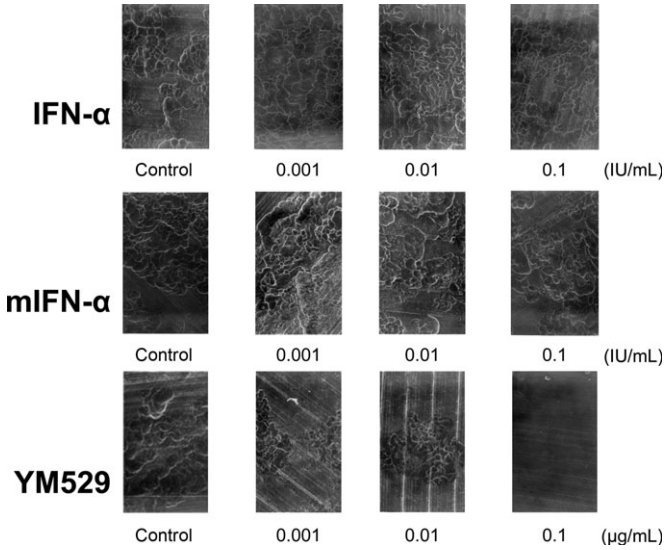

Fig. 3. *In vitro* resorption pit formation by mouse osteoclasts treated with YM529, IFN-α and mIFN-α. *In vitro* resorption pit formation by mouse osteoclasts treated with YM529 was completely inhibited in a dose-dependent manner. Both IFN-α-treatment and mIFN-α-treatment groups did not inhibit resorption pit formation as compared with that of the PBS-treatment control group.

formation by mouse osteoclasts treated with YM529 was inhibited in a dose-dependent manner to 88.2% ± 16.4% (67.3–100%) compared with the PBS-treatment control group. Neither the IFN-α-treatment group nor the mIFN-α-treatment group exhibited inhibition of resorption pit formation at −14.2 ± 4.0% (−19.5 to −8.4%) for the IFN-α-treatment group and –9.5 ± 10.7% (−22.8 to −4.4%) for the mIFN-α-treatment group, when compared with the PBS-treatment control group (Fig. 3).

***In vivo* growth and volume of RBM1-IT4 cell tumors in the tibia of nude mice after treatment with YM529 and/or interferon-alpha.** To determine whether combined treatment with YM529 is effective against established bone RCC growing within the tibia of athymic nude mice, treatment with YM529 and/or IFN-α was started 2 months after tumor implantation. Soft X-ray images of bone tumors before and after treatment are shown in Figure 4a. Substantial bone erosion was observed in the control group. In contrast, the extent of bone destruction in the group treated with YM529 in combination with IFN-α was markedly less. The treatment results are summarized in Table 1 and Figure 4b. The median percentage increase in tumor volume in mice after treatment was 196.64% (99.54–361.83%) when treated with IFN-α alone, 322.20% (151.69–490.57%) when treated with YM529 alone, and 138.56% (86.32–235.40%) when treated with YM529 and IFN-α. *In vivo*

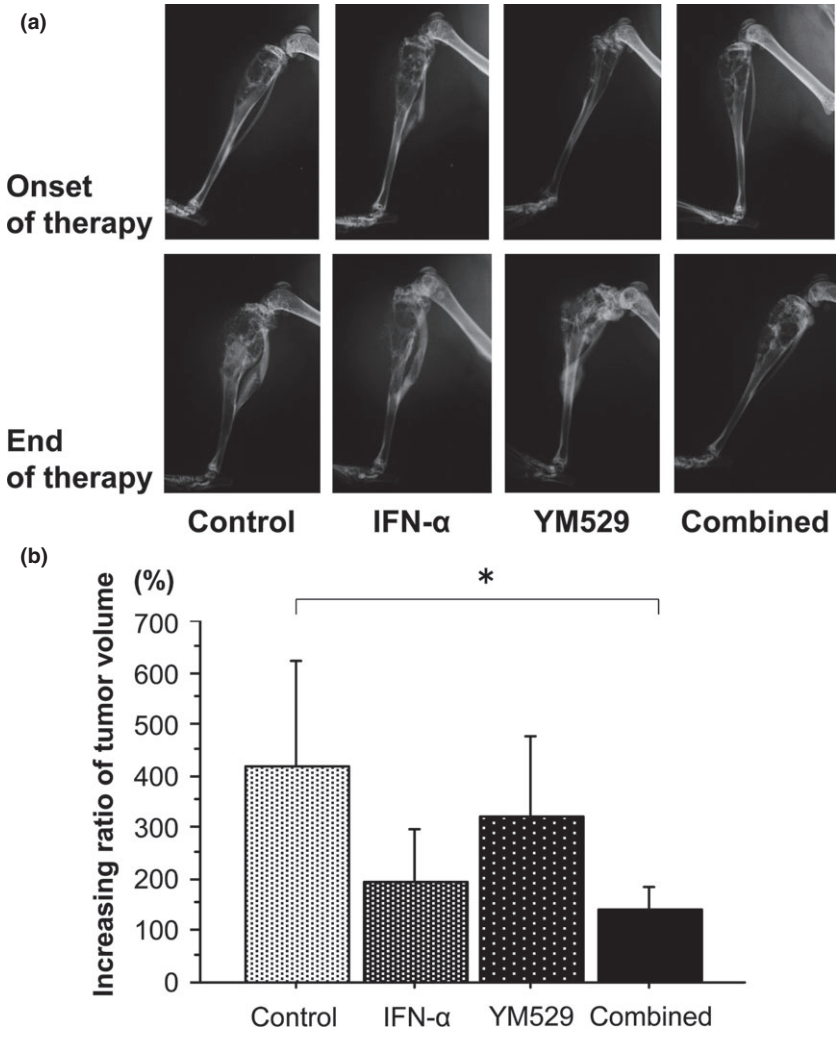

Fig. 4. Viable RBM1-IT4 cells (2×10^6/20 μL) were ectopically implanted into mice tibias, and therapy was started 2 months after tumor implantation. (a) Serial tubial X-rays were performed at the onset of therapy (day 60) and at the end of therapy (day 87) in mice bearing RBM1-IT4 tumors. In the control group, there were substantial bony erosions. In contrast, the extent of bony destruction in the group treated with YM529 in combination with IFN-α was markedly less. (b) The median percentage increase in tumor volume after treatment. Only combined treatment with YM529 and IFN-α resulted in significant regression of established human RCC bone tumors as compared with that of the control group (*P = 0.0074).

Table 1. ***In vivo*** **growth and volume of RBM1-IT4 cells in the tibia of athymic nude mice after treatment with YM529 and/or IFN-α**

Therapy	Number of mice	Tumorigenicity		
		Median tumor volume (range, mm^3)		Increasing ratio† (range, %)
		The onset of therapy	The end of therapy	
Control (physiological saline)	5	82.39 (26.26–137.48)	282.11 (191.28–379.74)	416.37 (222.96–728.45)
IFN-α (100 IU/day s.c.)	7	105.74 (41.74–202.52)	179.32 (45.02–295.81)	196.64 (99.54–361.83)
YM529 (0.3 mg/kg/week i.p)	7	89.19 (22.57–200.39)	267.91 (42.05–493.19)	322.2 (151.69–490.57)
Combined	7	128.15 (68.01–178.30)	172.11 (191.59–164.44)	138.56 (86.32–235.40)‡§

†Increasing ratio (*P*, Mann–Whitney statistical comparison). ‡$P = 0.0074$ against control group. §$P = 0.0064$ against YM529 group.

treatment with single-agent-YM529 or single-agent-IFN-α yielded no significant antiproliferative effect on established bone RCC as compared with the control group ($P = 0.4649$ and $P = 0.0882$, respectively), whereas combined treatment with YM529 and IFN-α resulted in a significant antiproliferative effect as compared with the control group ($P = 0.0074$).

Apoptosis induction by YM-529 and/or interferon-alpha in RBM1-IT4 cells growing in the tibia of nude mice. We evaluated the effects of treatment on apoptosis induction by TUNEL assay in an established bone RCC growing within the tibia of athymic nude mice (Table 2). The mean AI was 4.9 ± 1.0% (3.4–5.8%) in the control group, 4.1 ± 1.2% (2.8–6.2%) in the single-agent-IFN-α group, 5.0 ± 1.9% (3.1–7.6%) in the single-agent-YM529 group and 5.3 ± 1.2% (3.4–6.8%) in the combined treatment group. Neither YM529 nor IFN-α significantly induced cancer cell apoptosis in bone tumors. In addition, no additive effects were observed for the combination of YM529 and IFN-α.

Effects of YM529 and/or interferon-alpha on number of osteoclasts in RBM1-IT4 cell tumors in nude mice. Histological analysis of untreated mice revealed that osteolytic bone lesions comprised cancer cells and that numerous osteoclasts stained for TRAP were observed along the trabecular bone surface surrounded by RBM1-IT4 cells (Fig. 5a). The mean number of TRAP-positive osteoclasts in bone tumors counted under a microscope in 10 random microscopy fields at 400×/field was 7.1 ± 2.3 (5.8–10.6) in the control group, 6.1 ± 1.8 (2.9–8.5) in the single-agent-IFN-α group, 2.8 ± 0.7 (2.0–3.5) in the single-agent-YM529 group, and 2.7 ± 0.6 (2.5–3.5) in the combined treatment group (Fig. 5b). The number of osteoclasts was significantly lower in bone lesions of mice treated with either YM529 alone or in combination with IFN-α than in those of control mice or in those treated with IFN-α alone (single-agent-YM529 *vs* control: $P = 0.0105$; single-agent-YM529 *vs* single-agent-INF-α: $P = 0.0101$; combined treatment *vs* control: $P = 0.0082$; combined treatment *vs* single-agent-INF-α: $P = 0.0073$). There were additive antiproliferative effects of YM529 on osteoclasts in bone tumors.

Table 2. Apoptosis induction by YM-529 and/or IFN-α in RBM1-IT4 cells growing in the tibia of nude mice

Therapy	Number of mice	Apoptosis index (%)† Median ± SD (range)
Control (Physiological saline)	5	4.9 ± 1.0 (3.4–5.8)
IFN-α(100 IU/day s.c.)	7	4.1 ± 1.2 (2.8–6.2)
YM529 (0.3 mg/kg/week i.p.)	7	5.0 ± 1.9 (3.1–7.6)
Combined	7	5.3 ± 1.2 (3.4–6.8)

†There was no significant relationship between apoptotic index (AI) and treatment group.

Effects of YM529 and/or interferon-alpha on mRNA expression and micro-vessel density in RBM1-IT4 cells growing in the tibia of nude mice. mRNA expression of bFGF, VEGF, IL-8, MMP-9, MMP-2, E-cadherin and EGFR was analyzed by ISH (Table 3, Fig. 6), and MVD was determined by IHC (Fig. 7). In RBM1-IT4 cells growing in the tibia of athymic nude mice, bFGF mRNA expression within the tumors of mice treated with IFN-α alone or in combination with YM529 was significantly reduced by 84% ($P = 0.0330$) and 82% ($P = 0.0275$), respectively, compared with that in control tumors. Moreover, MVD was significantly lower in tumors treated with IFN-α alone (27.2 ± 5.5) or in combination with YM529 (25.7 ± 9.3) than in control tumors (44.8 ± 11.1; $P = 0.0252$ and $P = 0.0275$, respectively) and in those of the single-agent YM529-treated group (39.4 ± 8.2; $P = 0.0202$ and $P = 0.0285$, respectively; Fig. 7).

Discussion

The antiproliferative effects of YM529 on human RCC osteolytic bone metastasis are unclear. Therefore, we examined the effects of YM529 in the bone metastatic RCC model. At the cellular level, the principal site of action of bisphosphonates in the normal bone is at the osteoclasts. Tumor cells, including those of RCC,[17] release parathyroid hormone-related peptide, stimulating osteoclasts to resorb bone. Transforming growth factor-β and other peptides are then released from bone, enhancing tumor cell proliferation. Bisphosphonates may interrupt this cycle by inhibiting osteoclastic bone resorption.[18] *In vitro* and *in vivo* evidence suggests at least four ways in which bisphosphonates can inhibit osteoclast activity: inhibition of osteoclast formation and recruitment, inhibition of osteoclast activation, inhibition of mature osteoclasts, and reduction of osteoclast lifespan by apoptosis induction.[19]

Bisphosphonate-induced inhibition of the mevalonate pathway increases the levels of the middle metabolic products of isopentenyl pyrophosphate, which stimulate gamma delta (γδ) T lymphocyte.[20] γδ-T lymphocyte exhibited marked cytotoxicity against various tumor cells including RCC.[21] However, Yuasa *et al.*[22] report that using the *in vivo* mouse model bearing murine RCC cells into the subcutaneous tissue, the YM529-treated mice (with or without IFN) did not alter the γδ-T lymphocyte numbers. In this study, we also found that YM529 significantly inhibited proliferation and activation (as measured by resorption pit formation) of mouse osteoclasts *in vitro*, although no significant relationship was observed between AI and treatment. Moreover, in the *in vivo* model, the number of osteoclasts was also significantly reduced in bone lesions treated with YM529. In recent years, it has been

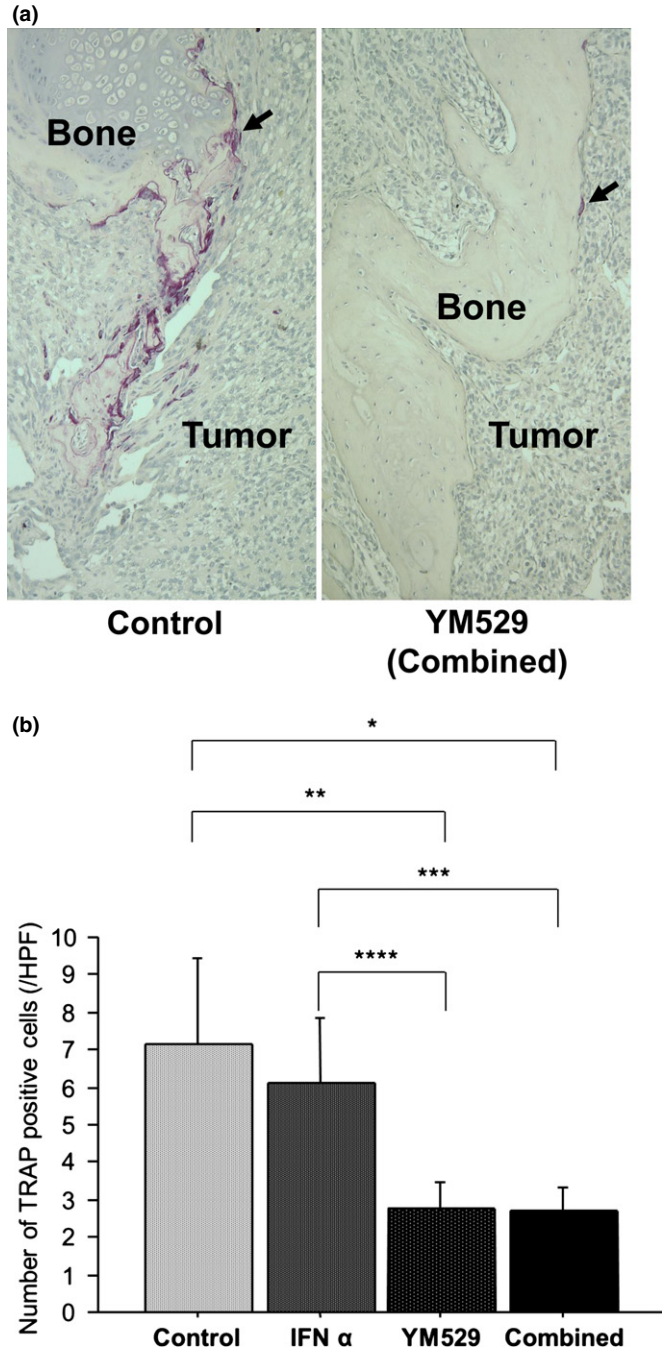

Fig. 5. Effect of YM529 on the number of osteoclasts in bone lesions. (a) At the end of therapy (day 88), bone lesions were harvested and osteoclasts were stained for TRAP. (b) The number of osteoclasts was significantly less in bone lesions of mice treated with YM529 alone or in combination with IFN-α than in control mice or in mice treated with IFN-α alone (*P = 0.0082, **P = 0.0105, ***P = 0.0073, ****P = 0.0101).

Table 3. Effects of YM529 and/or IFN-α on mRNA expression in RBM1-IT4 cells growing in the tibia of nude mice

Therapy	mRNA expression index†						
	b-FGF	VEGF	IL-8	EGFR	E-cadherin	MMP-2	MMP-9
Control (physiological saline)	100	100	100	100	100	100	100
IFN-α(100 IU /day s.c.)	84‡	91	91	93	100	92	94
YM529 (0.3 mg /kg/week i.p.)	95	94	94	100	95	95	99
Combined	82§	90	91	93	89	95	93

†The intensity of the cytoplasmic color reaction was measured using an image analyzer. The intensity was determined by comparison with the integrated absorbance of poly d(T)20. The results for each treatment group were presented relative to the control, which was set to 100. ‡P = 0.0330 against control group (P, Mann-Whitney statistical comparison). §P = 0.0275 against control group (P, Mann–Whitney statistical comparison).

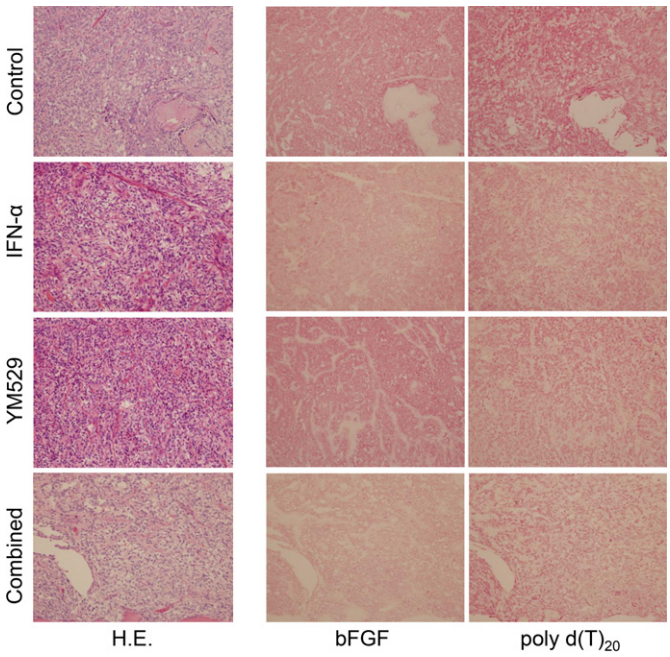

Fig. 6. Expression of bFGF, VEGF, IL-8, MMP-9, MMP-2, E-cadherin and EGFR mRNA analyzed by ISH. In RBM1-IT4 cells, bFGF mRNA expression within the mice tumors treated with IFN-α alone or in combination with YM529 was significantly reduced by 84% (P = 0.0330) and 82% (P = 0.0275), respectively, as compared with that in control tumors. These results are summarized in Table 3.

reported that bisphosphonates inhibit the myeloma cell cycle to inhibit cell proliferation directly, and it also has been reported that bisphosphonates induce myeloma cells apoptosis[23–25] and breast cancer cell apoptosis[26] *in vitro*. However, YM529 did not significantly inhibit RBM1-IT4 cell proliferation directly and did not significantly induce tumor cell apoptosis. These findings suggest that the therapeutic effect of YM529 on bone metastatic RCC might be predominantly due to osteoclast generation and/or function inhibition, rather than direct tumor cell proliferation inhibition.

Interferon has been considered an antiangiogenic agent, and some of its activity in the RCC treatment might result from the prevention of blood vessel growth.[27] Recently, in many cases, bone metastatic RCC patients have not been clinically treated with IFN-α. With the introduction of molecular targeted drugs, the treatment of metastatic RCC has dramatically changed. However, a complete response is rarely observed, and a change of drugs is usually needed. Both the incidence and severity of adverse events associated with the use of these agents in Japanese patients appear to be higher than in Western

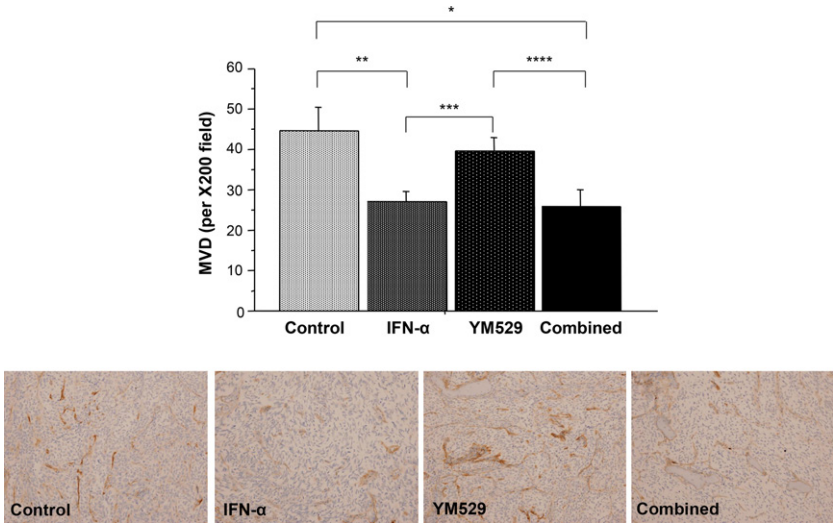

Fig. 7. Micro-vessel density was significantly lower in tumors treated with IFN-α alone or in combination with YM529 than in control tumors (*P = 0.0275, **P = 0.0252) and in tumors of the single-agent YM 529-treatment group (***P = 0.0202, ****P = 0.0285).

populations.[28,29] In addition, two clinical Japanese studies involving a large number of patients indicated that overall survival was markedly longer in cytokine-treated patients than in the European and American series.[30,31] Therefore, we have focused on IFN-α as a therapeutic partner for YM529. In our *in vivo* tumor model, bFGF mRNA expression and MVD within tumors treated with IFN-α were also significantly reduced. A previous study suggested that the IFN-α antitumor activity could still involve unexplored mechanisms based on post-translational and translational control of the expression of proteins that regulate cell proliferation and apoptosis.[32] However, in our study, IFN-α did not significantly inhibit the proliferation of RBM1-IT4 cells or mouse osteoclasts. mIFN-α seems to have a slight growth inhibitory effect on mouse osteoclasts in a dose-dependent manner *in vitro*. However, no significant antiproliferative effect was observed at any concentration compared with the control group. In addition, no significant relationship was found between apoptosis and treatment with IFN-α. YM529 did not affect the proangiogenic factor production and angiogenesis. On the antiproliferative effects for mouse osteoclasts, combination of 1μg/mL of YM529 with several concentrations of IFN-α seems to be less effective than 1μg/mL of YM529 alone. However, a significant antiproliferative effect was observed in each IFN-α concentration compared with the control group. Moreover, in the *in vivo* study, there was no significant effect of treatment with IFN-α on the number of osteoclasts. Although more careful and extensive analysis *in vitro* may be needed, our results suggest that the therapeutic effects of IFN-α against bone-metastatic RCC might be predominantly a result of antiangiogenic effects exerted via the reduction of bFGF expression within tumors rather than direct cytotoxicity against tumor cells and osteoclasts.

In bone tumors, therapy with IFN-α did not enhance decrease of osteoclast count by YM529 compared with single therapy with YM529. Conversely, therapy with YM529 did not enhance inhibition of bFGF expression and decrease of MVD by IFN-α compared with single therapy with IFN-α. These results suggest that YM529 and IFN-α act to inhibit tumor growth independently, and that combining YM529 and IFN-α does not reduce their respective antitumor effects. In the *in vivo* study, treatment with single-agent-YM529 or single-agent-IFN-α yielded no significant antiproliferative effect on bone RCC, whereas combined treatment with YM529 and IFN-α resulted in a significant antiproliferative effect. Thus, although YM529 and IFN-α act to inhibit tumor growth independently, antiosteoclastic activity by YM529 and angiogenesis inhibition by IFN-α seem to act additively to enhance efficacy.

In summary, in established human RCC bone tumors growing within the tibia of nude mice, YM529 alone did not markedly inhibit RCC cell growth. However, combining YM529 with IFN-α appeared to have significant antiproliferative effects. These effects are mediated by osteoclast recruitment inhibition and inactivation by YM529 and antiangiogenesis by IFN-α. This study yielded evidence that the combination of YM529 with IFN-α was sufficient to suppress the established human RCC bone tumor growth. Therefore, combined treatment with a bisphosphonate and IFN-α may be useful in treating RCC patients with bone metastasis.

Disclosure Statement

The authors have no conflict of interest to declare.

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