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Intensification Therapy with Anti-Parathyroid Hormone-related Protein Antibody Plus Zoledronic Acid for Bone Metastases of Small Cell Lung Cancer Cells in SCID Mice

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Running title: Anti-PTHrP antibody and ZOL suppress bone metastasis of SCLC.
**Key words:** bone metastasis; combination therapy; small cell lung cancer

**Abbreviations list:**

Bps, bisphosphonates; SCLC, small cell lung cancer; ZOL, zoledronic acid; SRE, skeletal-related event; PTHrP, parathyroid hormone-related protein; HHM, humoral hypercalcemia of malignancy; NK, natural killer; SCID, severe combined immunodeficient; PTHrP Ab, anti-PTHrP neutralizing antibody; TUNEL, TdT-mediated dUTP-biotin nick end labeling.


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Abstract

Bone metastases occur in more than one-third of patients with advanced lung cancer and are difficult to treat. We previously demonstrated the therapeutic effect of a third generation bisphosphonate (BP), minodronate, and anti-parathyroid hormone-related protein (PTHrP) neutralizing antibody (PTHrP Ab) on bone metastases induced by the human small cell lung cancer (SCLC) cell line, SBC-5, in natural killer (NK)-cell depleted SCID mice. The purpose of our current study was to examine the effect of the combination of PTHrP Ab and zoledronic acid (ZOL), which has been approved to treat bone metastases, against bone metastases produced by SBC-5 cells expressing PTHrP. Treatment with PTHrP Ab and/or ZOL did not affect the proliferation of SBC-5 cells in vitro. Repeated treatments with either PTHrP Ab or ZOL inhibited the formation of osteolytic bone metastases of SBC-5 cells, but had no effect on metastases to visceral organs. Importantly, combined treatment with PTHrP Ab and ZOL further inhibited the formation of bone metastases. Histological assays showed that, compared with either PTHrP Ab or ZOL alone, their combination decreased the number of tumor associated osteoclasts and increased the number of apoptotic tumor cells. These findings suggest that this novel dual targeting therapy may be useful for controlling bone metastases in a subpopulation of SCLC patients.
Introduction

Lung cancer is the most common cause of cancer deaths in the world, with more than 60,000 patients newly diagnosed per year in Japan. Lung cancer frequently metastasizes to systemic lymph nodes and distant organs, and >90% of deaths from lung cancer can be attributed to metastases (1). Bone is the third most common metastatic organ in lung cancer patients, with bone metastases occurring in more than one-third of patients with advanced lung cancer. These metastases can cause bone pain, hypercalcemia, nerve compression syndromes, and even fractures, and can decrease patient quality of life (2). Although skeletal complications can be managed locally by surgery or radiotherapy or systemically with chemotherapy and analgesics, these treatments are not sufficient for improving patient prognosis.

The formation of bone metastases is a multi-step event, regulated not only by cancer cells but also by host microenvironments. Of cells in the host microenvironment, osteoclasts are regarded as playing critical roles. Osteoclasts cause bone resorption, which provides the spaces in which cancer cells grow, as well as releasing various growth factors from bone matrix (3, 4). These findings suggest that osteoclasts may be ideal therapeutic targets for the inhibition of osteolytic bone metastases. Bisphosphonates (BPs) are hydrolysis-resistant PP1 derivatives that have a high affinity for bone and block the
mevalonate pathway, resulting in apoptosis of osteoclasts and inhibiting osteoclastic bone resorption (5). Several BPs have been used recently to treat osteoporosis and hypercalcemia (6). In addition, we have shown that a third generation nitrogen-containing bisphosphonate, minodronate (YM529), could inhibit the growth of bone metastases produced by the SBC-5 human small cell lung cancer (SCLC) cell line in severe combined immunodeficient (SCID) mice (7). Zoledronic acid (ZOL) is another third-generation BP that has demonstrated superior efficacy, compared with pamidronate, in the treatment of hypercalcemia of malignancy (8). A phase III, randomized, placebo-controlled trial has shown that ZOL reduced the proportion of lung cancer patients with skeletal-related events (SREs) (9). Although ZOL has been approved for the treatment of bone metastases in patients with multiple myeloma and other solid tumors, including breast and lung cancer (10), ZOL delayed SREs only by 2 months when combined with conventional chemotherapy, and it could not improve the survival of advanced lung cancer patients with bone metastases (9, 11, 12).

Parathyroid hormone-related protein (PTHrP) has a 70% homology to the first 13 amino acids of the N-terminal protein of PTH (13). PTHrP, which was originally identified as a 17-kDa PTH-like adenylate cyclase-stimulating protein from a tumor associated with humoral hypercalcemia of malignancy (HHM) (14), has been shown to enhance osteoclast
formation and bone destruction in malignant diseases. Moreover, this protein is overexpressed by many tumor cell types, including those of breast, prostate and lung cancer (15). The importance of PTHrP to the development and progression of bone metastases has been shown in several rodent models of bone metastasis, including those from breast, prostate, and lung cancer (14, 16, 17). We previously established a bone metastasis model with multiple-organ dissemination using the human SCLC cell line, SBC-5, which overexpresses PTHrP, in natural killer (NK) cell-depleted SCID mice (18). Using this model, we found that anti-PTHrP neutralizing antibody (PTHrP Ab) successfully inhibited the production of osteolytic bone metastases of SBC-5 cells (14). The goal of our research is to establish more effective therapeutic modalities against lung cancer bone metastases. We therefore investigated the effect of the combination of PTHrP Ab (targeting PTHrP) and ZOL (targeting osteoclasts) in our bone metastasis model of SBC-5 cells in NK-cell depleted SCID mice.
Materials and methods

Cell lines and culture conditions

The SBC-5 human small cell lung cancer cell line was the kind gift of Drs. M. Tanimoto and K. Kiura (Okayama University, Okayama, Japan) (18). These cells were cultured in RPMI 1640 medium, supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (50 μg/ml), in a humidified CO₂ incubator at 37°C.

Reagents

Anti-mouse IL-2 receptor β chain monoclonal Ab, TM- β1 (IgG2b), was kindly supplied by Drs. M. Miyasaka and T. Tanaka (Osaka University, Osaka, Japan) (19). A murine monoclonal Ab directed against PTHrP-(1-34) was kindly supplied by Chugai Pharmaceutical Co. (Shizuoka, Japan) (20), and ZOL was purchased from Novartis Pharmaceuticals (East Hanover, NJ).

In Vitro Effect of anti-PTHrP Ab and/or ZOL on proliferation of SBC-5 Cells

SBC-5 cells at 80% confluence were harvested, seeded at 2 x 10³ cells per well in 96-well plates, and incubated in RPMI 1640 for 24 h. Various concentrations of anti-PTHrP Ab and/or ZOL were added, the cultures were incubated for 72 h at 37°C, a 50 μL aliquot of
3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide solution (2 mg/mL; Sigma, St. Louis, MO) was added to each well and the cells were incubated for 2 h at 37°C (21). The media were removed and the dark blue crystals in each well were dissolved in 100 μL DMSO. Absorbance was measured with an MTP-120 microplate reader (Corona Electric, Ibaraki, Japan) at test and reference wavelengths of 550 nm and 630 nm, respectively. Data shown are representative of five independent experiments.

**Animals**

Male SCID mice, 6-8 weeks old, were obtained from CLEA Japan (Osaka, Japan) and maintained under specific pathogen-free conditions. All experiments were performed according to the guidelines established by the Tokushima University Committee on Animal Care and Use.

**Model of multiple-organ metastasis by SBC-5 cells and anti-metastatic effect of anti-PTHrP Ab and/or ZOL**

To facilitate the metastasis of SBC-5 cells, SCID mice were depleted of NK cells (22). Briefly, each mouse was injected i.p. with TM-β1 monoclonal antibody (300 µg/300 µl PBS/mouse) 2 days before tumor cell inoculation. Subconfluent SBC-5 cells were harvested and washed
with Ca\(^{2+}\)- and Mg\(^{2+}\)-free PBS. Cell viability was determined by the trypan blue exclusion test, and only single cell suspensions of >90% viability were used. Cells (1 x 10\(^6\) /300 µl) were injected into the lateral tail vein of mice on day 0. To determine the optimum timing and dosage of ZOL, tumor-bearing mice were treated with i.p. control IgG (300 µg) on days 7, 11, 14, 18, 21, and 25, i.p. ZOL (2 µg) on days 7, 14, and 21, and intravenous (i.v.) anti-PTHrP Ab (200 µg) on days 7, 14, and 21 (14).

Five weeks after tumor cell inoculation, the mice were anesthetized by i.p. injection of pentobarbital (0.5 mg/body), and X-ray photographs of the mice were taken to evaluate osteolytic bone metastases; the numbers of osteolytic bone metastases on the X-ray photographs were evaluated independently by two investigators (T. Y. and K. I.). The mice were sacrificed by cutting the subclavian artery, and the liver and lung were removed. The lungs were fixed in Bouin’s solution for 24 h. The number of macroscopic metastatic lesions larger than 0.5 mm in diameter in the liver and lung was counted.

**Histology and Immunohistochemical-immunofluorescent analysis**

The hind limbs of the mice were fixed in 10% formalin. Bone specimens were decalcified in 10% EDTA solution for 1 week and embedded in paraffin. Tissue sections (4 µm thick) were processed. For detection of osteoclasts, TRAP staining was performed using a Sigma
Diagnostics Acid Phosphatase Kit (Sigma Diagnostics, St. Louis, MO). *In vivo* cell PTHrP production was quantitated using mouse anti-human PTHrP monoclonal antibody (Santa Cruz Biotechnology, CA), cell proliferation was quantitated using mouse anti-human Ki-67 monoclonal antibody (MIB1, Pharmingen, San Diego, CA), and apoptosis was quantitated using the TdT-mediated dUTP-biotin nick end labeling (TUNEL) method. For Ki-67 staining, antigen retrieval was performed by boiling in a microwave for 10 min in 0.01 M citrate buffer (pH 6.0). The TUNEL assay was performed using the Apoptosis Detection System (Promega, Madison, WI) according to the manufacturer’s instructions (23), and in situ programmed cell death was assessed by specific labeling of nuclear DNA fragmentation as described (24). All sections were also stained with H&E for routine histological examinations.

**Quantification of immunohistochemistry and immunofluorescence**

The five areas containing the highest numbers of stained cells within a section were selected for histologic quantitation by light or fluorescent microscopy with a 200-fold magnification. All results were independently evaluated by two investigators (T.Y. and K.I.).

**Statistical analysis**
All data, expressed as means ± SE, were analyzed by one-way analysis of variance. Between group differences in the number of metastases to different organs (e.g. bones, lungs, liver) were assessed by the Fisher protected least-significant difference test. Proliferation index and the numbers of TRAP-positive and apoptotic cells were compared using Student's t test (two-tailed). P values less than 0.05 were considered statistically significant. All statistical analyses were performed using StatView ver.5.0.
Results

In vitro effects of anti-PTHrP Ab and/or ZOL on SBC-5 cell proliferation

We first tested the direct effect of anti-PTHrP Ab and/or ZOL against SBC-5 cells in vitro. As previously reported, we again found that anti-PTHrP Ab had no effect on the proliferation of SBC-5 cells (14). We also found that neither ZOL alone, at < 10 μg/ml, nor the combination of anti-PTHrP Ab nor ZOL at various doses, significantly affected SBC-5 proliferation (Figure 1).

Effects of ZOL monotherapy on the production of bone metastases in NK-cell depleted SCID mice

SBC-5 cells inoculated intravenously into NK-cell depleted SCID mice produced osteolytic bone metastases in the vertebral bone, pelvis scapulae, and hind limbs, as well as in the lungs and liver, consistent with our previous reports (14, 18). The mice had micrometastases in the bone by 7 days after inoculation (data not shown), experienced paralysis four weeks after inoculation, and 30-50% experienced paralysis five weeks after inoculation. We found that a single treatment with ZOL on day 7 significantly reduced the formation of bone metastases in a dose-dependent manner, but had no effect on the production of metastases to visceral organs, such as the lungs and liver (Table 1-A). Administration of up to 2 μg ZOL
did not cause a reduction in body weight, suggesting the feasibility of treatment with this drug. Based on these results, we used a dose of 2 µg ZOL per mouse in the following experiments.

When we examined the effect of the timing of ZOL monotherapy against bone metastasis, we found that earlier administration (i.e., on day 0, 7, or 14) suppressed bone metastases (Table 1-B). In contrast, ZOL administered on day 21 (i.e., after the development of macroscopic bone metastases) (14) did not reduce the number of bone metastases, suggesting that ZOL may suppress the growth of micrometastatic tumor cells in the bone. Moreover, ZOL did not inhibit production of visceral metastases, suggesting that this drug has limitations as monotherapy against SCLC bone metastasis with multiple organ dissemination. Since preliminary experiments showed that repeated treatments with ZOL were more effective than a single treatment in inhibiting bone metastases (data not shown), we commenced administering ZOL once weekly for three weeks (i.e., on days 7, 14, and 21).

Effects of combined therapy with anti-PTHrP Ab and ZOL on the production of bone metastases in NK-cell depleted SCID mice

We found that three treatments with ZOL (2 µg) on days 7, 14, and 21 significantly reduced
the formation of bone metastases (p<0.05), but again had no effect on metastases to visceral organs, such as the lungs and liver. As reported previously (14), three treatments with anti-PTHrP Ab (200 μg) on days 7, 14, and 21 also significantly reduced the formation of bone metastases (p<0.05), while having no effect on the development of visceral metastases. Importantly, three treatments each with PTHrP Ab plus ZOL further inhibited the production of bone metastases, while having no effect on visceral metastases (Fig. 2, Table 2). These results indicate that PTHrP Ab and ZOL each have bone-specific antimetastatic effects and that these therapeutic effects were intensified when the two agents are combined.

**Immunohistochemical and immunofluorescence staining to clarify the anti-bone metastatic mechanism of PTHrP Ab and ZOL**

To assess the mechanism by which PTHrP Ab and/or ZOL inhibits bone metastases, we performed immunohistochemical and immunofluorescence staining of the bone lesions induced by SBC-5 cells. We found that treatment with PTHrP Ab and/or ZOL did not affect the production of PTHrP by SBC-5 cells (data not shown) or the number of Ki-67-positive proliferating tumor cells (Fig. 3 and 4A). However, treatment with either PTHrP or ZOL trended to decrease the number of TRAP-positive cells (osteoclasts), compared with control
or control IgG-treated mice. The combination of PTHrP and ZOL further decreased the number of osteoclastic cells in bone lesions compared with either agent alone, although the differences were not significant (Fig. 3 and 4B). In contrast, the number of apoptotic tumor cells (positive for TUNEL) was dramatically increased in the lesions of mice treated with either PTHrP Ab or ZOL, and combined treatment increased significantly the number of apoptotic tumor cells in bone metastases compared with either agent alone (Fig. 3, 4C, and 4D). These results suggest that PTHrP Ab and/or ZOL decreased the number of tumor-associated osteoclasts and hence induced the apoptosis of tumor cells in bone metastases.
Discussion

Molecular interactions between tumor cells and their microenvironments play pivotal roles throughout the multiple steps of bone metastasis (25, 26). Once tumor cells adhere to the bone microenvironment, they can survive and grow, as well as promote bone destruction.

Tumor cells produce various factors that increase osteoclast formation, including PTHrP, interleukin-6, prostaglandin E₂, and tumor necrosis factor. In addition, these cells produce various bone resorption-releasing factors, including transforming growth factor-β, insulin-like growth factors, bone morphogenetic proteins, platelet-derived growth factor, and fibroblast growth factors, which in turn stimulate tumor cells to proliferate and secrete more of the factors that increase osteoclast formation (27). PTHrP has prominent effects on bone via its interaction with the PTH-1 receptor on osteoblasts. For example, PTHrP has been shown to directly regulate the proliferation and differentiation of osteoblasts and to indirectly support osteoclastogenesis by upregulating the receptor activator of the NFκB ligand RANKL in osteoblasts (27). These findings have suggested that osteoclasts and PTHrP may be attractive therapeutic targets to shut off this vicious cycle and hence inhibit bone metastasis.

We have shown here that the combination of PTHrP Ab and the third generation BP, ZOL, inhibit the production of bone metastasis of SCLC to a greater extent than either agent alone. The therapeutic effect of these agents, whether as monotherapy or combined, may
be predominantly due to the inhibition of osteoclast activation and/or accumulation in bone lesions, followed by suppression of bone resorption and induction of tumor-cell apoptosis. This is supported by our findings, showing that PTHrP Ab and/or ZOL did not directly inhibit the proliferation of SBC-5 cells \textit{in vitro} and \textit{in vivo}, and that treatment with PTHrP Ab and/or ZOL decreased the number of osteoclasts and increased the number of apoptotic tumor cells in bone lesions. Similar results have been observed by treatment of bone metastatic lesions with reveromycin A, an inhibitor of isoleucyl-tRNA synthesis that efficiently induces the apoptosis of osteoclasts (28). The mechanism by which these agents induce tumor cell apoptosis without affecting the number of proliferating tumor cells is unknown at present. Further experiments are required to clarify their underlying mechanism.

Several studies have reported that PTHrP is involved in the resistance to BPs on HHM. For example, the rate of response to pamidronate was higher in patients with lower (2-12 pg/mL) than higher (> 12 pg/mL) blood PTHrP concentrations (29). In addition, the emergence of alendronate-refractory HHM was associated with high levels of circulating PTHrP (30), further suggesting that PTHrP may play a critical role in intrinsic and/or acquired resistance to BPs in bone metastases. Thus, combined treatment with of PTHrP Ab and ZOL may control the progression of bone metastases.

We found, however, that combined therapy with PTHrP Ab and ZOL did not reduce the
SBC-5 metastasis to visceral organs, such as the lungs and liver. This finding is consistent with our previous reports on PTHrP Ab alone (14) and reveromycin A (28). Using a breast cancer model, however, we found that ZOL suppressed lung and liver metastases and prolonged overall survival (31), and a recent clinical trial demonstrated that ZOL inhibited visceral metastases and prolonged survival of patients with breast cancer (32). While the reasons for these discrepancies are unclear, the effects of ZOL may be dependent on the types of cancer cells as well as on organ microenvironments. If visceral metastases of lung cancer are refractory to ZOL monotherapy as shown here, a combination with other agents, such as conventional chemotherapy, may suppress the progression of visceral metastases and hence prolong survival.

In conclusion, we have shown here that the combination of PTHrP Ab and ZOL successfully inhibited the production of bone metastases of human SCLC SBC-5 cells expressing PTHrP in NK-cell depleted SCID mice, suggesting that this novel dual targeting therapy may be useful in controlling bone metastases in a subpopulation of SCLC patients. In contrast, this combination therapy did not inhibit the progression of visceral metastases. Combination with additional agents, including conventional chemotherapy, may therefore be required to suppress visceral metastases and prolong survival.
Acknowledgements

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Table 1. Effect of ZOL on multiple organ metastases produced by SBC-5 cells in NK-cell depleted SCID mice.

<table>
<thead>
<tr>
<th>A</th>
<th>ZOL (µg)</th>
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<th>Number of Metastases †</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Bone</td>
</tr>
<tr>
<td>Control</td>
<td>5</td>
<td>7 (4-11)</td>
<td>47 (20-56)</td>
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<tr>
<td>0.2</td>
<td>5</td>
<td>6 (4-7)</td>
<td>37 (25-65)</td>
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<tr>
<td>0.6</td>
<td>5</td>
<td>4 (0-3) **</td>
<td>42 (28-70)</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>3 (0-3) ***</td>
<td>44 (14-63)</td>
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<table>
<thead>
<tr>
<th>B</th>
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<th>Number of mice</th>
<th>Number of metastases †</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Bone</td>
</tr>
<tr>
<td>Control</td>
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<td>3 (0-6)</td>
<td>11 (5-18)</td>
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<tr>
<td>Day 0</td>
<td>5</td>
<td>1 (0-2) **</td>
<td>10 (2-20)</td>
</tr>
<tr>
<td>Day 7</td>
<td>5</td>
<td>1 (0-2) *</td>
<td>10 (2-16)</td>
</tr>
<tr>
<td>Day 14</td>
<td>5</td>
<td>2 (0-2) *</td>
<td>11 (6-24)</td>
</tr>
<tr>
<td>Day 21</td>
<td>5</td>
<td>3 (0-5) *</td>
<td>10 (3-24)</td>
</tr>
</tbody>
</table>

A: SBC-5 cells (1 x 10⁶ cells/mouse) were injected into the lateral tail veins of NK cell-depleted SCID mice on day 0, and various doses of ZOL were injected intraperitoneally on day 7. The mice were sacrificed on day 35 and the production of metastasis was evaluated. B: SBC-5 cells (1 x 10⁶ cells/mouse) were injected into the lateral tail veins of NK cell-depleted SCID mice on day 0, and ZOL (2µg) was injected intraperitoneally on the indicated day. The mice were sacrificed on day 35 and the production of metastasis was evaluated. † Values are the median (minimum-maximum). Data shown are representative of two independent experiments with similar results. * p<0.05, ** p<0.01, *** p<0.001, compared with the control group (Mann-Whitney U test).
Table 2. Therapeutic effect of injection with PTHrP Ab and ZOL on multiple organ metastases by SBC-5 cells in NK cell-depleted SCID mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of mice</th>
<th>Number of Metastases†</th>
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<td></td>
<td></td>
<td>Bone</td>
</tr>
<tr>
<td>Control</td>
<td>5</td>
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</tr>
<tr>
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<td>5</td>
<td>10 (7-11)</td>
</tr>
<tr>
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<td>5</td>
<td>6 (5-7) *</td>
</tr>
<tr>
<td>ZOL</td>
<td>5</td>
<td>3 (2-5) *</td>
</tr>
<tr>
<td>PTHrP Ab+ ZOL</td>
<td>5</td>
<td>1 (0-2) **</td>
</tr>
</tbody>
</table>

SBC-5 cells (1 x 10^6 cells/mouse) were injected into the lateral tail veins of NK cell-depleted SCID mice on day 0. Mice were injected intraperitoneally with control IgG (300 µg) on days 7, 11, 14, 18, 21, and 25, and with ZOL (2µg) on days 7, 14, and 21, and intravenously with PTHrP Ab (200µg) on days 7, 14, and 21. The mice were sacrificed on day 35 and the production of metastases was evaluated. † Values are the median (minimum-maximum). Data shown are representative of three independent experiments with similar results. * p<0.05, compared with the control group, ** p<0.05, compared with the PTHrP Ab or ZOL group (Mann-Whitney U test).
Figure Legends

**Figure 1.** Effect of Anti-PTHrP antibody (PTHrP Ab) and zoledronic acid (ZOL) on proliferation of the SBC-5 cell line.

SBC-5 cells (2 x 10^3/well) plated in 96-well plates were incubated overnight in the appropriate medium. The cultures were treated with PTHrP Ab and/or ZOL at the indicated concentrations for 72h. Proliferation of SBC-5 cells was determined by the MTT dye reduction method. Values are the mean ± SDs (error bars) of triplicate cultures. Data shown are representative of five independent experiments with similar results.

**Figure 2.** Inhibition of bone metastasis by treatment with PTHrP Ab and/or ZOL.

SBC-5 cells (1 x 10^6 cells/mouse) were injected into the lateral tail veins of NK cell-depleted SCID mice on day 0. Tumor-bearing mice were treated i.p. with control IgG (300 µg) or ZOL (2 µg) or i.v. with PTHrP Ab (200 µg) at the indicated times. Five weeks after tumor cell inoculation, bone metastases were assessed by X-ray photograph. Arrows, osteolytic bone metastases.

**Figure 3.** Histologic examination of bone metastatic lesions.
SBC-5 cells (1 x 10^6 cells/mouse) were intravenously inoculated into NK cell-depleted SCID mice on day 0. Tumor-bearing mice were treated i.p. with control IgG (300 µg) or ZOL (2 µg) or i.v. with PTHrP Ab (200 µg) at the indicated times. The mice were sacrificed on day 35, and their bone metastatic lesions were harvested and histologically examined. Sections were stained with H&E, anti-human PTHrP monoclonal antibody, antibody to Ki-67, TRAP and TUNEL.

**Figure 4.** Quantification of osteoclasts and apoptotic cells in bone lesions.

(A) Tumor cell proliferation was determined by Ki-67-positive proliferation index (percentage of Ki-67-positive cells). (B) Osteoclasts were determined by TRAP staining. (C,D) Apoptotic cells were determined by immunofluorescence (C) and by TUNEL staining (D). Columns, mean of five areas; bars, SD. *, P < 0.01 compared with the control group; **, P < 0.05 compared with the PTHrP Ab or ZOL group (Mann-Whitney U test).
Fig 2. Tadaaki Yamada et al.
Fig 1. Tadaaki Yamada et.al

- **PTHrP Ab**
- **PTHrP Ab+ZOL 1μg/ml**
- **ZOL**
- **ZOL+PTHrP Ab 10μg/ml**

Growth (%) vs. Concentration of PTHrP Ab and ZOL
**Fig 3.**

<table>
<thead>
<tr>
<th></th>
<th>HE</th>
<th>Ki67</th>
<th>TRAP</th>
<th>TUNEL</th>
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<tr>
<td><strong>Control</strong></td>
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<td>![PTHrP Ab + ZOL HE](436x70 to 537x199)</td>
<td>![PTHrP Ab + ZOL Ki67](435x205 to 537x334)</td>
<td>![PTHrP Ab + ZOL TRAP](435x475 to 537x604)</td>
<td>![PTHrP Ab + ZOL TUNEL](435x340 to 537x469)</td>
</tr>
</tbody>
</table>
Fig 4.

A

Ki-67 proliferation index (%)

B

No. of TRAP+ cells/HPF

C

No. of Apoptotic cells/HPF

D

No. of Apoptotic tumor cells/HPF

Control Control PTHrP Ab ZOL PTHrP Ab +ZOL

Control Control PTHrP Ab ZOL PTHrP Ab +ZOL

Control Control PTHrP Ab ZOL PTHrP Ab +ZOL

Control Control PTHrP Ab ZOL PTHrP Ab +ZOL

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