Combined Therapy with a New Bisphosphonate, Minodronate (YM529), and Chemotherapy for Multiple Organ Metastases of Small Cell Lung Cancer Cells in Severe Combined Immunodeficient Mice

Seiji Yano,1,2 Helong Zhang,1 Masaki Hanibuchi, Toyokazu Miki, Hisatsugu Goto, Hisanori Uehara, and Saburo Sone

Departments of Internal Medicine and Molecular Therapeutics [S. Y., H. Z., M. H., T. M., H. G., S. S.] and Environmental and Molecular Pathology [H. U.], Course of Bioregulation and Medical Oncology, University of Tokushima School of Medicine, Tokushima, Japan

ABSTRACT

Purpose: Lung cancer in the advanced stage frequently metastasizes to multiple organs, including the liver, lungs, lymph nodes, and bone. Bisphosphonates have been widely used to treat osteolytic bone metastasis in the past years; however, many studies have implicated that a single use of bisphosphonates could not prolong the survival of patients. In the present study, using a multiple-organ metastasis model of human lung cancer cells, we examined the effect of combined therapy with a new bisphosphonate (YM529) and etoposide (VP-16).

Experimental Design: Human small cell lung cancer (SBC-5) cells i.v. inoculated into natural killer cell-depleted severe combined immunodeficient mice metastasized to multiple organs, including the lungs, liver, kidneys, lymph nodes, and bone. SBC-5-bearing mice were treated with YM529 and/or VP-16 and sacrificed 5 weeks after tumor cell inoculation. Bone metastasis was assessed by X-ray photographs, and visceral metastasis was evaluated macroscopically. The number of osteoclasts in the bone lesions was examined by tartrate-resistant acid phosphatase staining.

Results: Monotherapy with YM529 suppressed the production of bone metastases, but not visceral metastasis. Histological analyses revealed that the number of osteoclasts in bone lesions was lower in YM526-treated mice, compared with control mice. VP-16 inhibited both bone metastasis and visceral (lung and liver) metastasis. However, neither YM529 alone nor VP-16 alone significantly prolonged the survival of SBC-5-bearing mice. Combined use of YM529 and VP-16 further inhibited the production of bone metastasis and significantly prolonged survival.

Conclusions: Combined therapy with bisphosphonate and chemotherapy may be useful for small cell lung cancer patients with multiple organ metastases including bone metastasis.

INTRODUCTION

Lung cancer is the most common cause of cancer deaths in the world. Lung cancer frequently metastasizes to the systemic lymph nodes and distant organs, including the liver, lung, kidney, and bone, and >90% of deaths from lung cancer can be attributed to metastases (1). Therefore, metastasis is a critical problem in the treatment of lung cancer patients. More than one-third of patients with advanced lung cancer manifest osteolytic bone metastases that can cause bone pain, hypercalcemia, nerve compression syndromes, and even fractures (2). These events might be the main causes of the decrease in quality of life in lung cancer patients with bone metastases. Therefore, the prevention and treatment of osteolytic bone metastases are clinically important.

Bisphosphonates are potent inhibitors of osteoclastic bone resorption and have been widely used in the treatment of hypercalcemia (3). In addition, several bisphosphonate products, for example, clodronate (4), etidronate (5), alendronate (6), ibandronate (7), pamidronate (8), and zoledronic acid (9), have been developed and used in the treatment of cancer patients with bone metastases. These bisphosphonates, however, could improve the quality of life but could not improve the survival of advanced cancer patients with bone metastases (10).

We recently established a model of multiple-organ metastasis with a small cell lung cancer cell line, SBC-5, in NK1 cell-depleted SCID mice (11). In this model, SBC-5 cells metastasize into multiple organs, such as the lung, liver, kidneys, systemic lymph nodes, and bone, resembling characteristics of small cell lung cancer in humans. We found that a new bisphosphonate, minodronate (YM529), inhibited osteolytic bone metastasis via the inhibition of bone resorption; however, it could not prolong the survival of tumor-bearing mice because of visceral metastasis (12).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received 4/16/03; revised 7/18/03; accepted 7/22/03.

The abbreviations used are: NK, natural killer; SCID, severe combined immunodeficient; PTHrP, parathyroid hormone-related protein; VEGF, vascular endothelial growth factor; TRAP, tartrate-resistant acid phosphatase; IL, interleukin.
In the present study, we examined the therapeutic efficacy of YM529 combined with etoposide (VP-16), which is commonly used for small cell lung cancer patients, in terms of metastasis formation and survival.

MATERIALS AND METHODS

Cell Culture. The human small cell lung cancer cell line SBC-5 was provided by Dr. K. Hiraki (Okayama University, Okayama, Japan; Ref. 11). The SBC-5 cells were maintained in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (CRPMI-1640) and 1% gentamicin. The cells were incubated at 37°C in a humidified atmosphere of 5% CO₂ in air.

Reagents. Antimouse IL-2 receptor β chain monoclonal antibody, TM-B1 (IgG2b), was supplied by Drs. M. Miyasaka and T. Tanaka (Osaka University, Osaka, Japan; Ref. 13). Minodronate (YM529) was provided by Yamanouchi Pharmaceutical Co. (Ibaragi, Japan; Ref. 14). Etoposide (VP-16) was obtained from Nippon Kayaku Co. (Tokyo, Japan).

Animals. Male S EB-17/Icr-scid mice, age 6–8 weeks, were obtained from CLEA (Osaka, Japan) and maintained under specific pathogen-free conditions throughout the experiment. Experiments were performed according to the guidelines of our university.

In Vitro Effect of YM529 on Proliferation of SBC-5 Cells. SBC-5 cells at 80% confluency were harvested, plated into 96-well tissue culture plates (5000 cells/100 μl/well), and incubated for 24 h at 37°C in 5% CO₂. Then, various concentrations of YM529 were added to the cultures. After a 72-h incubation at 37°C, 50 μl of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide stock solution (2 mg/ml) were added to each well, and the cells were further incubated for 2 h at 37°C (15). Then, the culture media were removed, and 100 μl of DMSO were added to dissolve the dark blue crystals. Absorbance was measured with a MTP-32 Microplate Reader (Corona Electric, Ibaragi, Japan) at test and reference wavelengths of 550 and 630 nm, respectively.

Effect of YM529 on Production of PTHrP and VEGF of SBC-5 Cells. SBC-5 cells at 80% confluency were harvested, plated into 6-well tissue culture plates (1 × 10⁵ cells/2 ml/well), and incubated for 24 h at 37°C and 5% CO₂. Then, the cultures were washed, and various concentrations of YM529 were added. After a 72-h incubation at 37°C, culture supernatants were collected and were concentrated by the PTHrP and VEGF were determined using radioimmunoassay (Otsuka Assay, Tokushima, Japan) and ELISA (R&D Systems, Minneapolis, MN), respectively.

Model of Multiple-Organ Metastasis by SBC-5 Cells and Antimetastatic Effect of YM529. To facilitate the metastasis of SBC-5 small cell lung cancer cells, NK cells were depleted in SCID mice (16). For NK cell depletion, TM-B1 monoclonal antibody (300 μg/300 μl PBS/mouse) was injected i.p. into SCID mice 2 days before tumor cell inoculation. SBC-5 cells at the subconfluent condition were harvested and washed with Ca²⁺- and Mg²⁺-free PBS (CMF-PBS). Cell viability was determined by the trypan blue exclusion test, and only single cell suspensions of >90% viability were used. SBC-5 cells (1 × 10⁶ cells/300 μl) were injected into the lateral tail vein of mice on day 0. At the indicated periods, tumor-bearing mice were treated with i.v. administration of YM529 (0.2 μg) on day 7 and/or VP-16 (200 μg) on days 2, 3, 9, and 10, based on the previous report (17).

Five weeks after the 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 bone metastasis. Then, mice were sacrificed by cutting the subclavian artery, and all major organs were removed. The lungs were fixed in Bouin’s solution for 24 h. The number of metastatic lesions larger than 0.5 mm in diameter in the major organs was counted macroscopically.

For evaluation of survival, tumor-bearing mice were sacrificed when the mice became moribund.

Histology and Immunohistochemistry. The hind limbs of the mice were taken and fixed in 10% formalin. The bone specimens were decalcified in 10% EDTA solution for 1 week and then embedded in paraffin. Tissue sections (4-μm thick) of the major organs were cut and stained with H&E for routine histological examination.

Statistical Analysis. The Mann-Whitney U test was used to determine the significance of difference in the number of metastases between YM529 and/or VP-16-treated groups and the untreated group. The significance of differences in the number of TRAP-positive osteoclasts and the survival rate of the mice were analyzed by Student’s t test (two-tailed) and the log-rank test, respectively. P < 0.05 was considered significant in all experiments.

RESULTS

In Vitro Effects of YM529 and VP-16 on Proliferation of SBC-5 Cells. YM529 at <1 μg/ml did not affect proliferation of SBC-5 cells, whereas it did inhibit proliferation of SBC-5 cells at higher concentrations. VP-16 at >100 ng/ml suppressed proliferation of SBC-5 cells. YM529 at 1 μg/ml did not affect the susceptibility of SBC-5 cells to VP-16 (Fig. 1).

Effect of YM529 and VP-16 on Production of PTHrP and VEGF by SBC-5 Cells. We reported previously (11) that SBC-5 cells produced PTHrP and VEGF, which are thought to be crucial regulatory molecules for bone resorption and angiogenesis, respectively. We next examined the effect of YM529 and VP-16 on the production of these two molecules by SBC-5 cells. Nontoxic concentrations of YM529 or VP-16 did not affect the production of these two molecules (Fig. 2). In addition, combined treatment with YM529 and VP-16 had no effect on the production of PTHrP or VEGF (data not shown).

Effects of YM529 and VP-16 on Metastases in SCID Mice. SBC-5 cells injected into NK cell-depleted SCID mice developed osteolytic bone metastatic lesions in the limbs, vertebral bone, pelvis scapulae, and the hind limbs. These lesions were detected on day 28 by radiography (11). Several mice experienced paralysis (probably asso-
ciated with spinal cord compression and bone metastases in the hind limbs) 4 weeks after SBC-5 cell inoculation, and the incidence of mice with paralysis became 30–50% five weeks after inoculation. In addition, SBC-5 cells produced macroscopically detectable metastasis in the visceral organs, such as the liver, lungs, kidneys, and lymph nodes by day 28. A single injection of 0.2 g/H9262 of YM529 significantly reduced the formation of bone metastasis; however, it had no effect on the development of metastasis to other organs, such as the liver, lungs, kidneys, or systemic lymph nodes (Fig. 3; Table 1). Chemotherapy with VP-16 significantly inhibited the development of metastasis to the liver, lungs, and bone, but not to the kidneys or lymph nodes. The combined use of YM529 and VP-16 further inhibited bone metastasis, compared with treatment with YM529 alone or VP-16 alone. These results suggested that both YM529 and VP-16 show promise in treating bone metastasis, and their combined use is necessary for control of multiorgan metastasis.

**Effect of YM529 and VP-16 on the Number of Osteoclasts in Bone Lesions.** Histological analysis of the untreated mice revealed that osteolytic bone lesions consisted of cancer cells. Numerous osteoclasts stained by TRAP staining were observed along the trabecular bone surface surrounded by SBC-5 cells. The number of osteoclasts was significantly lower in bone lesions of mice treated with either YM529 or VP-16, compared with control mice (Fig. 4). In addition, combined use of YM529 and VP-16 tended to decrease the number of osteoclasts, compared with single use of YM529 or VP-16, although the difference was not significant.

**Effect of YM529 Administration on Survival of Mice Inoculated with SBC-5 Cells.** We finally evaluated the therapeutic effect of YM529 on survival. The SCID mice inoculated with SBC-5 cells became moribund on days 35–42 (Fig. 5). Treatment with YM529 or VP-16 marginally prolonged the survival of SBC-5-bearing SCID mice, but the difference was not significant. However, combined treatment significantly prolonged the survival of these mice (P < 0.05).

**DISCUSSION**

Metastasis to multiple organs is a critical problem for lung cancer patients. In particular, small cell lung cancer metastasizes to various distant organs, including the liver, systemic lymph nodes, and bone. In the present study, we demonstrated that a new nitrogen-containing bisphosphonate (YM529) had a therapeutic effect against osteolytic bone metastasis, but not visceral metastasis, produced by SBC-5 cells. On the other hand, VP-16 suppressed the metastasis to the liver, lungs, and bone, but not to the kidneys or lymph nodes. Combined use of these two agents could produce a better effect in terms of survival and development of metastasis.
A large number of studies have reported the inhibitory effects of bisphosphonates against bone metastasis; however, the survival benefit of bisphosphonates is still controversial in experimental animal models (18–20). In the present study, treatment with YM529 alone could not significantly prolong survival, although it inhibited bone metastasis remarkably, suggesting that selective inhibition of bone metastasis is not sufficient for prolonging survival. Our results are consistent with most of the clinical studies showing that bisphosphonates can improve the quality of the life but not the survival of advanced cancer patients with bone metastases (21, 22).

On the other hand, a chemotherapeutic agent, VP-16, suppressed metastasis to some visceral organs (the liver and lungs). It also suppressed bone metastasis in our protocol, but with much less efficiency than YM529. In addition, VP-16 alone could not prolong survival. Because the mice with severe bone metastases became paralyzed and could not obtain enough food or water, the poor prognosis of the mice treated with VP-16 alone may be due not only to the progression of visceral metastasis but also to restricted quality of life because of bone metastasis. Collectively, the control of both bone metastasis and visceral metastases by multiple modalities may be necessary to prolong the survival of cancer patients with multiple organ metastases. To further prolong survival, experiments to evaluate the effect of continuing treatment with YM529 and VP-16 are ongoing.

![Fig. 3](image)

**Table 1**  Effect of YM529 and/or VP-16 on multiorgan metastasis of SBC-5 cells in NK cell-depleted SCID mice

SBC-5 cells (1 × 10^6 cells/mouse) were i.v. injected into the NK cell-depleted SCID mice on day 0. The mice were treated with i.v. administration of YM529 (0.2 μg) on day 7 and/or VP-16 (200 μg) on days 2, 3, 9, and 10. Metastasis was evaluated on day 35, as described in “Materials and Methods.” Data shown are representative of two independent experiments with similar results.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Bone Incidence</th>
<th>Liver Incidence</th>
<th>Lung Incidence</th>
<th>Kidney Incidence</th>
<th>Lymph node Incidence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5/5 14 (11–18)</td>
<td>5/5 72 (61–84)</td>
<td>5/5 27 (19–41)</td>
<td>5/5 5 (3–7)</td>
<td>5/5 5 (3–11)</td>
</tr>
<tr>
<td>YM529</td>
<td>4/5 3 (0–7)</td>
<td>5/5 66 (58–88)</td>
<td>5/5 23 (12–31)</td>
<td>5/5 4 (3–4)</td>
<td>5/5 3 (2–4)</td>
</tr>
<tr>
<td>VP-16</td>
<td>5/5 7 (3–11)</td>
<td>5/5 41 (14–83)</td>
<td>5/5 13 (8–21)</td>
<td>5/5 4 (2–5)</td>
<td>5/5 4 (2–5)</td>
</tr>
<tr>
<td>YM529 + VP-16</td>
<td>3/5 1 (0–3)</td>
<td>5/5 43 (25–73)</td>
<td>5/5 12 (9–23)</td>
<td>5/5 3 (2–7)</td>
<td>5/5 3 (2–4)</td>
</tr>
</tbody>
</table>

* Number of metastatic nodules [median (range)].

b P < 0.01 compared with control.
c P < 0.05 compared with control.
d P < 0.05 compared with VP-16 alone.
metastasis by SBC-5 cells in NK cell-depleted SCID mice, indicating that PTHrP is responsible for the production of bone metastasis by SBC-5 cells (25).

A topoisomerase II inhibitor, VP-16, is commonly used for patients with small cell lung cancer. We reported previously (17) that 200 μg (8 mg/kg) of VP-16 i.v. injected twice a week for 2 weeks was feasible and efficiently inhibited production of multiple organ metastases by SBC-3 (human small cell lung cancer cell line) in NK cell-depleted SCID mice. Therefore, we used the same protocol in the present study. Although it is not clear why lymph node or kidney metastasis was not inhibited by VP-16 with this protocol, it is possible that the dose of VP-16 was not sufficient to suppress metastasis to these two organs.

Recent studies (26, 27) have reported that some bisphosphonates (pamidronate and zoledronate) directly induced apoptosis or had cytostatic effects against cancer cells at relatively high concentrations. Moreover, YM529 was also reported to cause apoptosis of myeloma cell lines (28). Therefore, one possible explanation for the therapeutic effect of YM529 on bone metastasis was due to direct cytotoxicity. However, this is not the case, because YM529 did not have a direct cytotoxic effect and did not enhance the cytotoxicity of VP-16 against SBC-5 cells at physiologically achievable concentrations (<1 μg/ml; Ref. 12). Furthermore, YM529 did not affect the production of PTHrP or VEGF, even in combination with VP-16. Therefore, the augmented therapeutic efficiency of YM529 with VP-16 against bone metastasis may be predominantly due to inhibition of osteoclast generation and/or function by YM529 plus suppression of cell proliferation by VP-16 in the bone lesions.

In conclusion, use of bisphosphonate (YM529) alone inhibited bone metastasis but was not sufficient to prolong the survival of mice with bone metastasis and visceral dissemination. Combined use of YM529 with VP-16 to suppress visceral metastasis successfully suppressed multiorgan metastases and prolonged survival. Therefore, a combined modality with bisphosphonate and chemotherapy may be useful for lung cancer patients who have bone metastasis and visceral dissemination.

REFERENCES


Combined Therapy with a New Bisphosphonate, Minodronate (YM529), and Chemotherapy for Multiple Organ Metastases of Small Cell Lung Cancer Cells in Severe Combined Immunodeficient Mice

Seiji Yano, Helong Zhang, Masaki Hanibuchi, et al.