Effect of Combination Therapy with a Novel Bisphosphonate, Minodronate (YM529), and Docetaxel on a Model of Bone Metastasis by Human Transitional Cell Carcinoma

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Abstract

Purpose: Transitional cell carcinoma (TCC) of the urinary tract is a chemosensitive tumor. Most deaths from TCC of the urinary tract are caused by metastasis, which is resistant to conventional chemotherapy. Frequent sites of metastases from TCC of the urinary tract are regionally lymph nodes, liver, lung, and bone. Of these distant metastases, bone metastasis is consistently resistant to cisplatin-based conventional chemotherapy. Therefore, in this study, we investigated whether or not a newly developed minodronate, YM529, could prevent osteolytic bone metastasis of human TCC and also enhance the effect of docetaxel in a bone tumor model of athymic nude mice.

Experimental Design: In the present study, we evaluated the effect of in vitro treatment with minodronate and/or docetaxel on the proliferation by cell count, the induction of apoptosis by terminal deoxynucleotidyl transferase–mediated nick end labeling (TUNEL) assay, and the biological activity of osteoclast by pit formation assay in human bladder cancer cell line, UMUC-14, and mouse osteoclast cells. In vivo, we examined the effect of minodronate in a bone tumor model of athymic nude mice, in which the percutaneous intraosseous injection in the tibia of UMUC-14, leads to osteolytic bone tumor, as a bone metastasis model. To examine whether or not minodronate could inhibit tumorigenicity and enhance the effect of the chemotherapeutic agent, docetaxel, we gave minodronate i.p. and/or docetaxel i.p. to nude mice 3 days after an intraosseal tumor implantation. Moreover, proliferation and the induction of apoptosis of cancer cells and osteoclasts in bone tumors were determined by immunohistochemistry and the TUNEL assay.

Results: In vitro: In vitro treatment with docetaxel inhibited proliferation and resorption pit-forming activity and induced apoptosis of mouse osteoclast cells and UMUC-14 cells. In vitro treatment with minodronate inhibited proliferation and activity and induced apoptosis of mouse osteoclast cells but not UMUC-14 cells. The treatment with minodronate enhanced the inhibition of proliferation and activity by docetaxel in osteoclasts. In vivo: In vivo combination therapy with docetaxel and minodronate significantly reduced the tumor incidence compared with the control ($P < 0.05$) and also growth of intraosseal TCC in athymic nude mice compared with the control ($P < 0.001$), single therapy with docetaxel ($P < 0.01$), and minodronate ($P < 0.05$). Drug-induced body weight loss was not significantly different in any treatment group. Therapy with minodronate significantly enhanced inhibition of proliferation by docetaxel in osteoclasts of bone tumors compared with the control ($P < 0.01$), single therapy with docetaxel ($P < 0.01$), and minodronate ($P < 0.05$).

Conclusions: These studies indicate that combination therapy with minodronate and docetaxel may be beneficial in patients with bone metastasis of human TCC in the urinary tract.

Transitional cell carcinoma (TCC) of the urinary tract, renal pelvis, ureter, and urinary bladder is the second most common urological malignancy and has a poor prognosis. This malignancy is diagnosed in ~60,000 patients and results in 15,000 deaths annually in the United States (1). Radical operation is the standard treatment for operable invasive TCC urinary tract, whereas a combination of methotrexate, vinblastine, doxorubicin, and cisplatin (M-VAC) chemotherapy offers the only viable therapeutic option for locally advanced cancer, initial and recurrent metastases, and prevention of them.

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Although TCC of the urinary tract is a chemosensitive tumor, most deaths from TCC of the urinary tract are caused by metastasis, which is resistant to conventional chemotherapy (2, 3). It was previously reported that the most frequent sites of metastases were regional lymph nodes (90%), liver (47%), lung (45%), and bone (32%) in autopsies of 367 patients with T2 to T4 bladder cancer (4). Of these distant metastases, bone metastasis is consistently resistant to cisplatin-based conventional chemotherapy (5).

Similar to bone metastasis of breast cancer, and multiple myeloma, bone metastasis due to TCC in the urinary tract is osteolytic. Osteolytic bone metastasis is not only a critical problem in treatment but also the main cause of a decrease in the quality of life of patients with bone metastases. Therefore, the development of novel therapeutic strategies is mandatory if we are to improve the outcome and the quality of life of patients with osteolytic bone metastases of TCC in the urinary tract.

It is well known that bisphosphonate, a specific inhibitor of osteoclastic bone resorption, has been widely used as a beneficial agent in the treatment of osteolytic bone metastases from several cancers (6–8). Minodronate (YM529, [1-hydroxy-2-[(imidazo [1,2] pyridin-3-yl) ethyliden]-bisphosphonic acid monohydrate] is a newly developed third-generation bisphosphonate that has a more potent inhibitory activity toward mouse osteoclastic bone resorption in vitro and in vivo than previously developed bisphosphonates (9), such as pamidronate (10–12), alendronate (13), and ibandronate (14), which are second-generation bisphosphonates, and incadronate (12, 15), risedronate (16), and zoledronate (17), which are third-generation bisphosphonates.

The taxanes, paclitaxel (Taxol) and docetaxel, are effective chemotherapeutic agents used in the treatment of a number of major solid tumors including lung (18), colon (18), pancreatic (18), prostate (19), and bladder (20, 21) cancers. Taxanes bind to the β-subunit of tubulin and interfere with microtubular polymerization by promoting abnormal assembly of microtubules and inhibiting their subsequent disassembly in the mitotic spindle, resulting in arrest of the G2-M phase of the cell cycle, leading to programmed cell death (22, 23). Recently, it has been reported that docetaxel causes apoptosis by inducing phosphorylation of an apoptosis-suppressing oncogene, bcl-2, in prostate (19) and bladder (20, 21) cancers. Moreover, docetaxel was described as the most potent inducer of bcl-2 phosphorylation; docetaxel was >100 times more potent in this effect than paclitaxel (21).

Therefore, we hypothesized that taxane cytotoxicity would be complimentary to the antitumor effect by minodronate and provide additive or synergistic therapeutic effects on tumorigenicity in bone metastasis model.

In the present study described herein, the combination therapy with minodronate (YM529) and docetaxel markedly inhibited tumorigenicity in nonestablished bone tumor models of human TCC compared with therapy with each agent alone. This effect is mediated, at least in part, by the complementary cytotoxicities of cancer cells and osteoclasts.

**Materials and Methods**

**Cell lines and culture conditions.** The highly metastatic human bladder cancer cell line UMMIC-14 (24) was grown as a monolayer in DMEM supplemented with 10% fetal bovine serum (FBS). Mouse osteoclast cells were grown as a monolayer in 25 mL of modified EMEM supplemented with 10 ng/mL of monocyte macrophage/colony-stimulating factor and 10 ng/mL of RANKL from the osteoclast V-2 kit (Mouse; Hokudo, Sapporo Japan).

**Reagents.** Minodronate (YM529) was provided by Yamanouchi Pharmaceutical Co., Ltd. (Tokyo, Japan). Stock solutions of minodronate were prepared in absolute NaOH and suspended in saline. Docetaxel was a kind gift of Chugai Pharmaceutical Co., Ltd. (Tokyo, Japan).

**In vitro cell growth inhibition.** The in vitro dose-dependent antiproliferative effect of minodronate and/or docetaxel was evaluated after incubating 5 × 10^4 UMMIC-14 and 2 × 10^4 osteoclasts for 24 hours in serum-free medium, then exchanging the medium for 10% FBS supplemented with MEM containing an increasing concentration of minodronate (0-10 μg/mL) and/or docetaxel (0-10 μg/mL). Growth inhibition was determined after 48 hours by a cell count using the COULTER Z1 (Beckman Coulter, Inc., Tokyo, Japan) and expressed as the ratio of the number of viable cells in each group treated with minodronate and/or docetaxel to the number in the control group treated with PBS.

**In vitro apoptosis induction.** The in vitro induction of apoptosis of 1 × 10^5 UMMIC-14 cells and 2 × 10^5 osteoclasts by minodronate and/or docetaxel was evaluated after incubating for 24 hours in serum-free medium, then exchanging the medium for 10% FBS supplemented with MEM containing the IC_{50} concentration of minodronate and/or docetaxel. Cells were harvested by centrifugation and incubated at 4°C for 24 hours in 10 × bovine serum supplemented with fresh MEM. Quantification of DNA fragmentation was accomplished using the Apoptosis In situ Detection Kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan). The results were expressed as an average percentage of five highest areas in total of 1,000 cells identified within a single 200 × per field.

**Biological activity of osteoclasts.** The pit formation assay was done using the osteoclast V-2 kit (Mouse, Hokudo). Ivory slices were placed in 96-well plates. Mouse osteoclast progenitor cells (4 × 10^4 cells per well) were seeded to each well with αMEM containing monocyte macrophage/colony-stimulating factor (10 ng/mL) and receptor activator of the necrosis factor α ligand (RANKL, 10 ng/mL) using the osteoclast V-2 kit (Mouse, Hokudo). After incubation for 9 days, fresh medium containing an increasing concentration of minodronate (0-10 μg/mL) and/or docetaxel (0-10 μg/mL) was added. The cells were continuously cultured for 3 days. The ivory slices were soaked in NH_{4}OH (1 mol/L) solution to remove attached cells. The resorption pit on the ivory slices was observed under a scanning electronic microscope (Hitachi, Tokyo, Japan; S-2380N). The results were expressed as an average percentage of the three highest resorption pit areas compared with control as 100 measured by scanning electron microscope and analyzed with a computer analysis system.

**Animals.** Male athymic BALB/c A Jcl-nu nude mice were obtained from Clea Japan, Inc. (Osaka, Japan). The mice were maintained in a laminar-airflow cabinet under pathogen-free conditions and used at 6 to 8 weeks of age. All animal experimental protocols were followed in accordance with the Guide for the Care and Use of Laboratory Animals published by the U.S. NIH (Publication No. 85-23, revised 1996) and the guidelines of our university.

**Ectopic implantation.** Cultured UMMIC-14 cells (60-70% confluent) were prepared as previously described (25). Mice were anesthetized with Nembutal. For ectopic implantation, a percutaneous intraosseal injection was made by drilling a 27-gauge needle into the tibia immediately proximal to the tuberositas tibia. After penetration of the cortical bone, the needle was inserted into the shaft of the tibia and 20 μL of the cell suspension (5 × 10^7 per 20 μL) was deposited in the bone cortex. To prevent leakage of cells into the surrounding muscles, a cotton swab was held for 1 minute over the site of injection. The animals tolerated the surgical procedure well, and no anesthesia-related deaths occurred.
In vivo therapy of human transitional cell carcinoma growing in the tibia of athymic nude mice. Mice were ectopically implanted with viable UMUC-14 cells (5 × 10^5 per 20 μL) into the tibia and randomly separated into four groups. Therapy commenced 3 days after tumor implantation. Groups of mice were given i.p. with control (PBS), docetaxel (10 mg/kg), or minodronate (0.3 mg/kg), once a week for 4 weeks and harvested 38 days after the implantation. Clinically, docetaxel and also minodronate is used once a week in a lot of regimens of chemotherapy. We determined the schedule according to the previous report (26–28).

**Tissue processing.** Mice were killed by cervical dislocation and soft X-ray photographs of the bone tumor were taken to evaluate the antitumoral effects. The estimated volume of bone tumors was calculated by three axes (X, Y, and Z) using the formula of π/6XYZ. The bone tumor were necropsied and fixed in 20% formalin for 24 hours. The bone specimens were decalcified in 10% EDTA solution for 1 week. After decalcification, the specimens were dehydrated with graded ethanols, embedded paraffin, and 4-μm sections were obtained and adhered to ProveOn Plus Microscope Slides (Fisher Scientific, Pittsburgh, PA) for immunohistochemistry, terminal deoxynucleotidyl transferase–mediated nick-end labeling (TUNEL) assay, and tartrate-resistant acid phosphatase (TRAP) staining. Sections (4 μm thick) were also stained with H&E for routine histologic examination.

**Terminal deoxynucleotidyl transferase–mediated nick-end labeling assay.** For the TUNEL assay, tissue sections (5 μm thick) of formalin-fixed, paraffin-embedded specimens were deparaffinized in xylene, rehydrated in graded alcohol, and transferred to PBS. The sections were rinsed twice with distilled water with BRIJ and treated with a 1:500 solution for 15 minutes, and endogenous peroxidase was blocked with 3% hydrogen peroxide in PBS for 12 minutes. The samples were washed thrice with distilled water with BRIJ and incubated for 10 minutes at room temperature with terminal deoxynucleotidyl transferase buffer. Excess terminal deoxynucleotidyl transferase buffer was drained, and the samples were incubated for 18 hours at 4°C with terminal transferase and biotin-16-dUTP. The sections were then rinsed four times with TB buffer and incubated for 30 minutes at 37°C with a 1:400 dilution of peroxidase-conjugated streptavidin. The slides were rinsed with PBS and incubated for 5 minutes with dianinobenzidine (Invitrogen Co., Carlsbad, CA). The sections were then washed thrice with PBS, counterstained with Gill’s hematoxylin (Biogenex Laboratories, San Ramon, CA), and again washed thrice with PBS. The slides were mounted with a Universal Mount (Invitrogen).

**Immunohistochemistry.** For the immunohistochemical analysis, tissue sections (5 μm thick) of formalin-fixed, paraffin-embedded specimens were deparaffinized in xylene, rehydrated in graded alcohol, and transferred to PBS. Endogenous peroxidases were blocked by incubation in 3% hydrogen peroxide in PBS for 12 minutes. The samples were washed thrice with PBS and incubated for 20 minutes at room temperature with a protein-blocking solution containing 5% normal horse serum and 1% normal goat serum in PBS (pH 7.5). Excess blocking solution was drained, and the samples were incubated for 18 hours at 4°C with the appropriate dilution (1:100 of proliferating cell nuclear antigen (PCNA) with monoclonal mouse anti-PCNA Ab (DakoCytomation Co., Ltd., Kyoto, Japan). The samples were then rinsed four times with PBS and incubated for 60 minutes at room temperature with the appropriate dilution of the secondary antibody, peroxidase-conjugated anti-mouse immunoglobulin G (H+L, DakoCytomation). The slides were rinsed with PBS and incubated for 5 minutes with dianinobenzidine (Invitrogen). The

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Fig. 1. A, in vitro growth inhibition of UMUC-14 cells by the treatment of minodronate (YM529) and/or docetaxel. The in vitro dose-dependent antiproliferative effect of minodronate and/or docetaxel was evaluated after incubating 5 × 10^5 UMUC-14 cells for 24 hours in serum-free medium and exchanging the medium for 10% FBS supplemented with MEM containing an increasing concentration of minodronate (0-10 μg/mL) and/or docetaxel (0-10 μg/mL). Proliferation of UMUC-14 was markedly inhibited by in vitro treatment with docetaxel in a dose-dependent manner. There was no significant effect on the proliferation of UMUC-14 by minodronate. B, in vitro growth inhibition of mouse osteoclasts by the treatment of minodronate (YM529) and/or docetaxel. The in vitro dose-dependent antiproliferative effect of minodronate and/or docetaxel was evaluated after incubating 2 × 10^4 mouse osteoclasts for 24 hours in serum-free medium and exchanging the medium for 10% FBS supplemented MEM containing an increasing concentration of minodronate (0-10 μg/mL) and/or docetaxel (0-10 μg/mL). In vitro antiproliferation of osteoclasts was synergistically increased by the combination treatment of minodronate and docetaxel compared with the effect of either agent alone.
sections were then washed thrice with PBS, counterstained with Gill’s hematoxylin (Biogenex Laboratories), and again washed thrice with PBS. The slides were mounted with Universal Mount (Invirotigen).

Quantification of proliferation and apoptosis in cancer cells. Proliferation and apoptosis of cancer cells were determined by immunohistochemical staining of tissue sections with anti-PCNA antibodies and the TUNEL assay. The tissue was recorded using a cooled CCD Optotronics Tec 470 camera (Optotronics Engineering, Goletha, CA) linked to a computer and digital printer (Sony Corp., Tokyo, Japan). The density of proliferative cells and apoptotic cells was expressed as an average percentage of five highest areas in total of 1,000 cells identified within a single 200× per field (26).

Tartrate-resistant acid phosphatase staining. For the detection of osteoclasts, TRAP staining was done using Sigma Diagnosis Acid Phosphatase Kit (Sigma Diagnosis, St. Louis, MO). The number of TRAP-positive osteoclasts at the tumor-bone interface was counted under a microscope within a single 200× per field.

Quantification of proliferation and apoptosis in osteoclasts. Proliferation and apoptosis of osteoclasts were determined by light microscopy after TRAP staining and the TUNEL assay. The tissue sections were recorded using a cooled CCD Optotronics Tec 470 camera (Optotronics Engineering) linked to a computer and digital printer (Sony). TRAP-positive osteoclasts and apoptotic osteoclasts were counted and were expressed as an average percentage of five highest areas in total of 100 cells identified within a single 200× per field (29).

Results

In vitro growth inhibition of UMUC-14 cells by treatment with minodronate and/or docetaxel. The in vitro dose-dependent antiproliferative effect of minodronate and/or docetaxel was evaluated after incubating 5 × 10^4 UMUC-14 cells for 24 hours in serum-free medium, then exchanging the medium for 10% FBS supplemented MEM containing an increasing concentration of minodronate (0-10 μg/mL) and/or docetaxel (0-10 μg/mL). The IC_{50} of UMUC-14 cells treated with docetaxel was <0.001 μg/mL, whereas the IC_{50} of UMUC-14 cells treated with minodronate was >1 μg/mL. Although UMUC-14 cell proliferation was markedly inhibited by in vitro treatment with docetaxel in a dose-dependent manner, there was no significant effect of combination treatment with minodronate on the proliferation of UMUC-14 cells (Fig. 1A).

In vitro growth inhibition of mouse osteoclasts by treatment with minodronate and/or docetaxel. The in vitro dose-dependent antiproliferative effect of minodronate and/or docetaxel was evaluated after incubating 2 × 10^4 mouse osteoclasts for 24 hours in serum-free medium, then exchanging the medium for 10% FBS supplemented MEM containing an increasing concentration of minodronate (0-10 μg/mL) and/or docetaxel (0-10 μg/mL). The IC_{50} of mouse osteoclasts treated with docetaxel and minodronate was ~0.1 μg/mL. Proliferation of osteoclasts was inhibited by in vitro treatment with docetaxel and minodronate in a dose-dependent manner. In vitro antiproliferation of osteoclasts was synergistically increased by the combination treatment of minodronate and docetaxel compared with the effect of either agent alone (Fig. 1B).

In vitro induction of apoptosis by treatment with minodronate and/or docetaxel of human transitional cell carcinoma UMUC-14 cells and mouse osteoclasts. The in vitro induction of apoptosis by treatment with minodronate and/or docetaxel of UMUC-14 cells and mouse osteoclasts was determined by the TUNEL assay using the Apoptosis in situ Detection Kit. The results were expressed as an average percentage of five highest areas in total of 1,000 cells identified within a single 200× per field. *1, P < 0.01; *2, P < 0.005 against control (CTRL) and single therapy with minodronate; *3 and *4, P < 0.005 against control and single therapy with docetaxel (Mann-Whitney statistical comparison).

Statistical analysis. The statistical differences in the amount of cell proliferation and apoptosis within the bone tumors were analyzed with the Mann-Whitney test. The incidence of tumors and estimated tumor volume were statistically analyzed with the χ² test. P < 0.05 was considered significant.

![Fig. 2. In vitro induction of apoptosis by the treatment of minodronate (YM529, MINO) and/or docetaxel (TAX) for human transitional cell carcinoma UMUC-14 cells and mouse osteoclasts. The in vitro induction of apoptosis by treatment with minodronate (1 μg/mL) and/or docetaxel (0.01 μg/mL) of UMUC-14 cells and mouse osteoclasts was determined by the TUNEL assay using the Apoptosis in situ Detection Kit. The results were expressed as an average percentage of five highest areas in total of 1,000 cells identified within a single 200× per field. *1, P < 0.01; *2, P < 0.005 against control (CTRL) and single therapy with minodronate; *3 and *4, P < 0.005 against control and single therapy with docetaxel (Mann-Whitney statistical comparison).](image-url)
In vitro formation of resorption pits by mouse osteoclasts treated with minodronate and/or docetaxel. To determine the biological activity of mouse osteoclasts, the pit formation assay was done using the mouse osteoclast V-2 kit. The resorption pit on ivory slices was observed under a scanning electronic microscope. The resorption pit area determined by the resorption pit assay was expressed as an average percentage of the three highest resorption pit areas compared with control as 100 measured by scanning electronic microscope and analyzed with a computer analysis system.

In vitro formation of resorption pits by mouse osteoclasts treated with docetaxel or minodronate was significantly inhibited in 73% (58-85%) or 44% (36-56%) compared with PBS-treated control (P < 0.05 or P < 0.05), respectively. Combination treatment of docetaxel with minodronate significantly enhanced the inhibition of resorption pit formation in 38% (23-46%) compared with PBS-treated control and single treatment with docetaxel (P < 0.01). There were additive effects of the combination of docetaxel with minodronate on resorption pit formation by osteoclasts (Fig. 3A-B).

In vivo therapy with minodronate and/or docetaxel of human transitional cell carcinoma UMUC-14 cells growing in the tibia of athymic nude mice. To determine whether or not the combination therapy with minodronate would be effective on nonestablished bone tumor of human TCC growing within the tibia of athymic nude mice, therapy was commenced 3 days after tumor implantation. Treated mice were closely monitored for any signs of progressive disease and were sacrificed if they became moribund. The results of the therapy are summarized in Table 1.

The incidence of bone tumor was completely inhibited in seven of nine mice (78%) by the combination of docetaxel with minodronate. In two other mice, bone tumors were detected pathologically, and the tumor volume could not be estimated. In vivo combination therapy with docetaxel and minodronate significantly reduced the tumor growth of nonestablished bone tumor of human TCC in athymic nude mice compared with the controls given PBS [median volume (range), 487.0 cm$^3$ (0.1899.7 cm$^3$); P < 0.001], single therapy with docetaxel [117.7 cm$^3$ (0.502.4 cm$^3$); P < 0.01], and minodronate [63.3 cm$^3$ (0.384.7 cm$^3$); P < 0.05; Fig. 4; Table 1].

Drug-induced body weight loss was not significantly different in any of the therapeutic groups.

In vivo induction of apoptosis and expression of proliferating cell nuclear antigen following the therapy with minodronate and/or docetaxel for cancer cells in the bone tumor of athymic nude mice. We evaluated the effect of the combination therapy with minodronate on apoptosis induction and proliferation of cancer cells by TUNEL assay and immunohistochemistry for PCNA, respectively, in a nonestablished bone tumor model. The density of proliferative cells and apoptotic cells was expressed as an average percentage of five highest areas in total of 500 osteoclasts identified within a single 200× per field (ref. 29; Fig. 5A).

The number of PCNA-positive cancer cells of bone tumors counted per 200× per field was significantly decreased from 27.3% (17.8-32.1%) in control tumors to 8.2% (5.4-10.1%) following the therapy of docetaxel (P < 0.05). However, minodronate did not significantly inhibit proliferation of cancer cells of bone tumors. The combination of docetaxel with minodronate significantly inhibited proliferation of cancer cells of bone tumors in 6.9% (4.5-8.7%) compared with controls and single therapy with minodronate (P < 0.05), whereas not significant to single therapy with docetaxel. There were no additive effects of the combination with minodronate on antiproliferation in cancer cells of bone tumors (Fig. 5A).

The number of apoptotic cancer cells of bone tumors counted per 200× per field was significantly increased from 0.9% (0.3-1.4%) in controls to 4.0% (1.2-6.0%) following the
therapy of docetaxel \( (P < 0.05) \). However, minodronate did not significantly induce apoptosis of cancer cells of bone tumors. The combination of docetaxel with minodronate significantly enhanced apoptosis of cancer cells of bone tumors in 5.1\% \((2.7-7.1\%) \) compared with controls and single therapy with minodronate \( (P < 0.01) \), whereas not significant to single therapy with docetaxel. There were no additive effects of the combination with minodronate on apoptosis induction in cancer cells of bone tumors (Fig. 5A).

In vivo induction of apoptosis and expression of proliferating cell nuclear antigen following the therapy with minodronate and/or docetaxel for osteoclasts in the bone tumor of athymic nude mice. We evaluated the effect of the combination therapy with minodronate on proliferation and apoptosis induction of osteoclasts by TRAP staining and TUNEL assay in a nonestablished bone tumor model. TRAP-positive osteoclasts and apoptotic osteoclasts were counted and expressed as an average percentage of five highest areas in total of 100 osteoclasts identified within a single 200× per field (ref. 29; Fig. 5B). The number of TRAP-positive osteoclasts of bone tumors counted per 200× per field was decreased from 32.8\% \((28.0-44.0\%) \) in control tumors to 22.0\% \((20.0-28.0\%) \) following the therapy of docetaxel but not significantly. Minodronate markedly inhibited proliferation in 17.2\% \((10.0-24.0\%) \) of osteoclasts of bone tumors compared with controls \( (P < 0.05) \). The combination of docetaxel with minodronate additively and significantly inhibited proliferation of osteoclasts of bone tumors in 5.6\% \((4.0-8.0\%) \) compared with controls \( (P < 0.01) \) and single therapy groups \( (P < 0.01 \) with docetaxel and \( P < 0.05 \) with minodronate; Fig. 5B). Although the administration of minodronate markedly inhibited proliferation of osteoclasts in bone tumors, there was no significant difference in the number of apoptotic osteoclasts in bone tumors in any of the therapeutic groups (Fig. 5B).

Discussion

TCC of the urinary tract is a chemosensitive tumor. However, while establishing metastasis, TCC acquires the potential for resistance to cisplatin-based conventional chemotherapy. TCC in the urinary tract frequently metastasizes to the systemic lymph nodes and distant organs, such as the lung, liver, brain, and bone (4). Among the distant metastases, bone metastasis is consistently resistant to conventional chemotherapy (5).

Recently, docetaxel clinically yielded promising results as a key agent in advanced or metastatic TCC of the urothelium, which was resistant to cisplatin. Various docetaxel-containing regimens are also being studied and have resulted in favorable efficacy findings (29–31). On the other hand, a newly developed third-generation bisphosphonate, minodronate (YM539), has been reported to inhibit osteolytic bone metastasis from several cancers (32–34). Therefore, we hypothesized that taxane cytotoxicity would be complimentary to antitumor effect by minodronate and provide additive or synergistic therapeutic effects on tumorigenicity in bone metastasis model. These novel preventive and therapeutic strategies are mandatory if we are to improve the outcome of patients with osteolytic bone metastasis of TCC.

In the present study, we showed that minodronate had a preventive effect on nonestablished osteolytic bone tumor of a human TCC, UMUC-14, which was partial resistant to cisplatin. The IC\(_{50}\) of UMUC-14 cells treated with cisplatin was >1 \( \mu \)g/mL compared with docetaxel (<0.001 \( \mu \)g/mL). Moreover, no additive effect of cisplatin was shown with minodronate (data was not shown). Minodronate alone could completely inhibit tumor growth in 5 of 10 (50\%) bone tumor models of athymic nude mice. In vivo therapy with minodronate resulted in significant regression of bone tumor [median volume \( (range) \), 63.3 cm\(^3\) \((0-384.7 \text{ cm}^3) \)] compared with the controls given PBS [487.0 cm\(^3\) \((0-1899.7) \); \( P < 0.05 \)]. These effects were mainly mediated by growth inhibition of osteoclasts, which in turn inhibited osteoclastic function (osteolytic bone resorption). In vivo therapy with minodronate also inhibited the proliferation of osteoclasts markedly. However, apoptosis was induced in a few residual osteoclasts, similar to the condition in the therapeutic groups without minodronate. From these findings, we infer that minodronate may act on various stages of osteoclast generation. These findings can be assumed to be the last stage of apoptosis. Further studies are being undertaken to determine the exact apoptosis trigger sites. Moreover apoptosis induction and cytostatic effects on cancer cells by some bisphosphonates \((10–15, 17, 33) \) have been reported recently. However, in this study, minodronate did not significantly exhibit apoptosis induction and cytostatic effects and did not directly enhance the cytotoxicity of docetaxel against human TCC, UMUC-14 cells at an adequate biological dose.

On the other hand, the chemotherapeutic agent, docetaxel, completely inhibited tumor growth in 6 of 10 bone tumor models of athymic nude mice, showing an efficacy of a significant 60\%. These effects were observed due to the growth

### Table 1. The combination therapy with minodronate and/or docetaxel for human transitional cell carcinoma UMUC-14 cells growing in the tibia athymic nude mice

<table>
<thead>
<tr>
<th>Therapy*</th>
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<th>Tumor incidence (radial/pathol)</th>
<th>Tumor-free survival</th>
<th>Estimated tumor volume, (^1) median (range), cm(^3)</th>
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<td>CTRL</td>
<td>9</td>
<td>8 (7/1)</td>
<td>11.1</td>
<td>4870.0 (0.0-1,899.7)</td>
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<td>TAX</td>
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<td>60.0</td>
<td>1177.0 (0.0-502.4)</td>
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<td>MINO</td>
<td>10</td>
<td>5 (3/2)</td>
<td>50.0</td>
<td>63.3 (0.0-384.7)</td>
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<tr>
<td>MINO/TAX</td>
<td>9</td>
<td>2 (0/2)(^1)</td>
<td>77.8(^1)</td>
<td>0.0 (0.0-0.0)(^1)</td>
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Abbreviations: CTRL, control; TAX, docetaxel; MINO, minodronate; radial, radiologically detected; pathol, pathologically detected.

*Groups of mice were given i.p. with control (PBS), docetaxel (10 mg/kg), or minodronate (0.3 mg/kg) once a week for 4 wks and harvested 38 days after implantation.

\(^1\) Number of mice in the treated group.

\(^1\) Estimated volume of bone tumor was calculated by three axes \((X, Y, Z)\) measured from radiograph as the formula of \( \sqrt[3]{X \times Y \times Z} \).

\(^1\) \( P < 0.05 \) against single therapy with docetaxel and \( P < 0.01 \) against single therapy with minodronate (Mann-Whitney statistical comparison).

\(^1\) \( P < 0.05 \) against control (Mann-Whitney statistical comparison).

\(^1\) \( P < 0.05 \) against control (\( \chi^2 \) test).

\(^1\) \( P < 0.001 \) against control, \( P < 0.01 \) against single therapy with docetaxel, and \( P < 0.05 \) against single therapy with minodronate (Mann-Whitney statistical comparison).
inhibition and apoptosis induction of cancer cells. Docetaxel also affected the osteoclast population but with much less efficiency than minodronate. In vivo therapy with docetaxel resulted in a few regression of bone tumor [median volume (range), 117.7 cm$^3$ (0-502.4)] but with much less efficiency than minodronate [63.3 cm$^3$ (0-384.7 cm$^3$)]. From the point of view of the result in the in vivo therapy with individual drug, minodronate, and docetaxel, we would conclude that the inhibitory efficiency against bone tumor may be due predominantly to the suppression of osteoclasts proliferation using minodronate.

However, from the present findings, we understand that the inhibition of cancer cells by docetaxel alone or the inhibition of osteoclasts by minodronate alone were not sufficient to show in vivo tumor reduction of bone tumor. This suggests that wholistic antitumor and antosteoclastic actions are essential targets of any potential therapeutic agent. As per our current observations, the combination of minodronate with docetaxel completely inhibited tumor growth in seven of nine (77.8%) bone tumor models of athymic nude mice and complementarily enhanced inhibitory effects on nonestablished osteolytic bone tumor of a human TCC compared with control ($P < 0.001$) and individual drug therapy ($P < 0.01$ with docetaxel and $P < 0.05$ with minodronate). In vivo treatment and also in vivo therapy with both minodronate and docetaxel significantly inhibited proliferation of osteoclasts without an increase in apoptosis compared with minodronate alone. These findings suggested the other cytotoxic effects excluding apoptosis (e.g., cell necrosis, etc.), which is cell death without DNA fragmentation may act on osteoclasts inhibition in the combination treatment.

Previous studies have reported inhibitory effects of minodronate on bone metastasis (33–35). Several reports showed that the indirect antitumor effect of minodronate was additively or synergistically enhanced in combination with cytotoxic agents, such as doxorubicin in prostate cancer (36) and UFT (37) and paclitaxel or docetaxel in breast cancer (38, 39), respectively. Our data, as well as these reports, indicate that docetaxel and minodronate have complementary cytotoxicities, providing a novel and effective biochemotherapy against osteolytic bone metastasis of human TCC. Previously, we showed that paclitaxel and docetaxel enhance the antiangiogenic agent monoclonal antibody C225, which blocks epidermal growth factor receptor function (40), monoclonal antibody DC101, which blocks VEGFR-2 function (41) and angiogenesis inhibitor TNP-470.

Fig. 4. In vivo therapy with minodronate (YM529) and/or docetaxel for human transitional cell carcinoma UMUC-14 cells growing tibia of athymic nude mice. Mice were implanted with $5 \times 10^5$ UMUC-14 cells into the tibia, and treatment with PBS (A), docetaxel (10 mg/kg i.p., B), minodronate (0.3 mg/kg i.p., C), minodronate and docetaxel (D) commenced 3 days later and were harvested 38 days after the implantation. Complete inhibition of tumor growth in seven of nine mice (78%) was shown by the combination therapy with minodronate and docetaxel. The combination therapy, especially with minodronate and docetaxel, significantly reduced tumorigenicity compared with control ($P < 0.001$) and each therapy alone with docetaxel ($P < 0.01$) and minodronate ($P < 0.05$). Abbreviations: Radiol., bone tumor was radiologically detected; Pathol., bone tumor was pathologically detected; N.D., bone tumor was not detected.
In summary, the present study provides evidence that docetaxel enhances the effects of minodronate by increasing its inhibitory effect on bone tumorigenesis of athymic nude mice. Induction of apoptosis in cancer cells by docetaxol and the antosteoclastic activity by minodronate seem to act synergistically to enhance efficacy. These studies indicate that docetaxel and minodronate have complementary cytotoxicities, providing a clear rationale for investigation in future clinical trials.

References

Fig. 5. A, in vivo induction of apoptosis and expression of PCNA following the therapy with minodronate (YM529, MINO) and/or docetaxel (TAX) for cancer cells in the bone tumor of athymic nude mice. We evaluated the effect of the combination therapy with minodronate on apoptosis induction and proliferation of cancer cells by TUNEL assay and immunohistochemistry for PCNA, respectively, in a nonestablished bone tumor model. The density of proliferative cells and apoptotic cells was expressed as an average percentage of five highest areas within a single 200× field. *1 and *2, P < 0.05 against control (CTRL) and single therapy with minodronate; *3, P < 0.05; *4, P < 0.01 against control and single therapy with minodronate (Mann-Whitney statistical comparison). B, in vivo induction of apoptosis and expression of PCNA following the therapy with minodronate (YM529) and/or docetaxel for osteoclasts in the bone tumor of athymic nude mice. We evaluated the effect of the combination therapy with minodronate on proliferation and apoptosis induction of osteoclasts by TRAP staining and TUNEL assay in a nonestablished bone tumor model. TRAP-positive osteoclasts and apoptotic osteoclasts were counted and expressed as an average percentage of five highest areas within a single 200× field. *1, P < 0.05 against control; *2, P < 0.01 against control and single therapy with docetaxel and P < 0.05 against single therapy with minodronate (Mann-Whitney statistical comparison).


Effect of Combination Therapy with a Novel Bisphosphonate, Minodronate (YM529), and Docetaxel on a Model of Bone Metastasis by Human Transitional Cell Carcinoma

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