# Molecular mechanism of bone metastasis in prostate cancer



# 前立腺癌骨転移の分子機構の解明：破骨細胞抑制因子（osteoprotegerin：OPG）の関わりについて

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研究代表者　越田　潔
（金沢大学大学院医学系研究科・助教授）

研究課題

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研究組織


研究代表者　越田　潔（金沢大学大学院医学系研究科・助教授）

研究分担者　江川雅之（金沢大学大学院医学系研究科・助手）

小川　智（金沢大学大学院医学系研究科・教授）

並木幹夫（金沢大学大学院医学系研究科・教授）


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研究発表

1）論文発表

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Bisphopsphonate therapy for hormone refractory prostate cancer with bone metastasis.

J Urol 169: 281-82, 2003.

Asahi H, Mizokami A, Keller ET, Koshida K, Namiki M.

Bisphosphonate induces spoptosis and inhibits pro-osteoclastic gene expression in prostate cancer cells .

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2）学会発表

第６１回日本癌学会総会（２００２）

前立腺癌における Bisphosphonate(YM529)による抗腫瘍効果の検討

朝日秀樹、溝上敦、越田潔、並木幹夫

第５３回日本泌尿器科学会中部総会（２００３）

前立腺癌におけるビスフォスフォネートの作用

朝日秀樹、溝上敦、高島博、越田潔、並木幹夫

研究成果

ホルモン不応性の LNCaP の樹立

ホルモン不応性の LNCaP の樹立を目指してマトリゲルを用いて LNCaP 細胞を SCID マウス皮下へ移植した。マウスの精巣を摘除後、増殖を来した移植腫瘍を摘出し、再度予め精巣を摘除されたマウスに移植、これを３回繰り返した。ここで腫瘍を形成したものを LNCaP-Lec 4 とし、この腫瘍組織より細胞株を樹立した。いっぽう LNCaP を低アンドロゲン環境下に６ヶ月間継代したものを LNCaP-SF とした。

ホルモン不応性の LNCaP の特性

LNCaP-Lec 4、および LNCaP-SF は親株である LNCaP に比べ、castrated mice において明らかに増殖速度は速く（ **Figure 1** ）、アンドロゲン非依存性の獲得が示された。また LNCaP-SF をマウス骨（ tibia ）に注入したところ、約１０週で osteoblastic change を主体とした骨腫瘍を形成した （ **Figure 2** ）。組織学的には骨梁形成が著明であり osteoblast の活性化が示唆された（ **Figure 3** ）。以上の結果から、in vitro, in vivo における低アンドロゲン環境が、ホルモン抵抗性の獲得、ならびに骨組織での造骨性変化を伴う増殖に関わることが示唆された。これらのモデルは、ヒト前立腺癌におけるホルモン不応性の獲得ならびにホルモン不応性前立腺癌の造骨性骨転移巣形成の分子機構を研究するうえで有用であると考えられた。

ビスフォスフォネートの抗腫瘍効果

破骨細胞の活性を抑制するビスフォスフォネートは癌の骨転移による骨痛を抑制する作用が報告されている。乳癌においては、in vitro における殺細胞効果、さらに骨転移巣で腫瘍増殖抑制効果が報告され、癌治療薬としての役割が新しい世代のビスフォスフォネートに期待されるようになった。そこで我々は第３世代のビスフォスフォネートであるインカドロネートあるいはミドロノネートを３種のヒト前立腺癌細胞に加え、その殺細胞効果を検討した。濃度依存的、時間依存的に細胞増殖

抑制効果が認められた（ **Figure 4** ）。ついで細胞増殖抑制効果にアポトーシスの関与があるか否かを検討した。ヒト前立腺癌由来培養細胞： PC-3 cell における DNA fragmentation、および核の凝縮像（タネル染色陽性）が認められ、アポトーシスの関与が示された（ **Figure 5** ）。この分子機構を明らかにする目的に、アポトーシス関連因子の変化を検討した。Bcl2 の抑制および Bax の発現増強が認められ、濃度依存的に bcl-2/bax ratio の低下が認められた（ **Figure 6** ）。さらにカスパーゼ３の活性化が認められ、カスパーゼ３の inhibitor を投与すると、殺細胞効果は抑制され、ある程度アポトーシスが回避されることが示唆された（ **Figure 7** ）。ついで DNA の修復に促進的な作用を持つ poly(ADP)-ribose polymerase (PARP)の変化を検討したところ、ビスフォスフォネートの濃度依存的に分解されることが示され（ **Figure 8** ）、アポトーシス関連因子の一連の動きが部分的ではあるが解明された。さらにビスフォスフォネートの投与により、receptor activator of nuclear factor κB ligand (RANKL), parathyroid hormone related protein (PTHrP), matrix metalloprotease-2 (MMP-2)の抑制が認められ（ **Figure 9** ）、これらの変化は破骨細胞の活性化の抑制、骨基質への細胞浸潤の抑制につながることが考えられ、いずれも転移巣の成立、進展を阻止するメカニズムを説明しうる変化と考えられた。

ついで in vivo におけるビスフォスフォネートの抗腫瘍効果を先に示したマウス骨腫瘍のモデルを用いて検討した。LNCaP-SF 細胞をマウス骨（ tibia ）に移植し、１週目より第３世代のビスフォスフォネートである YM-529 を週１回１０週間に渡り腹腔内に投与し腫瘍増殖抑制効果を検討した。投与量依存的に抗腫瘍効果が確認された（ **Table 1** ）。組織学的には、コントロールでは骨基質の形成を伴った腫瘍細胞の増殖が認められるのに対して、YM—529 投与群では、骨髄内には腫瘍細胞の存在は認められるものの骨基質に浸潤する像は認められず、増殖および骨基質への浸潤は抑制されたと考えられる（ **Figure 10** ）。

以上の結果から、新世代のビスフォスフォネートは破骨細胞の活性抑制ばかりでなく、腫瘍細胞に対する直接的な殺細胞効果、さらには腫瘍細胞から産生される骨吸収促進を促す因子に対する抑制効果を通して、骨転移巣に対する治療効果を発揮することが期待される。

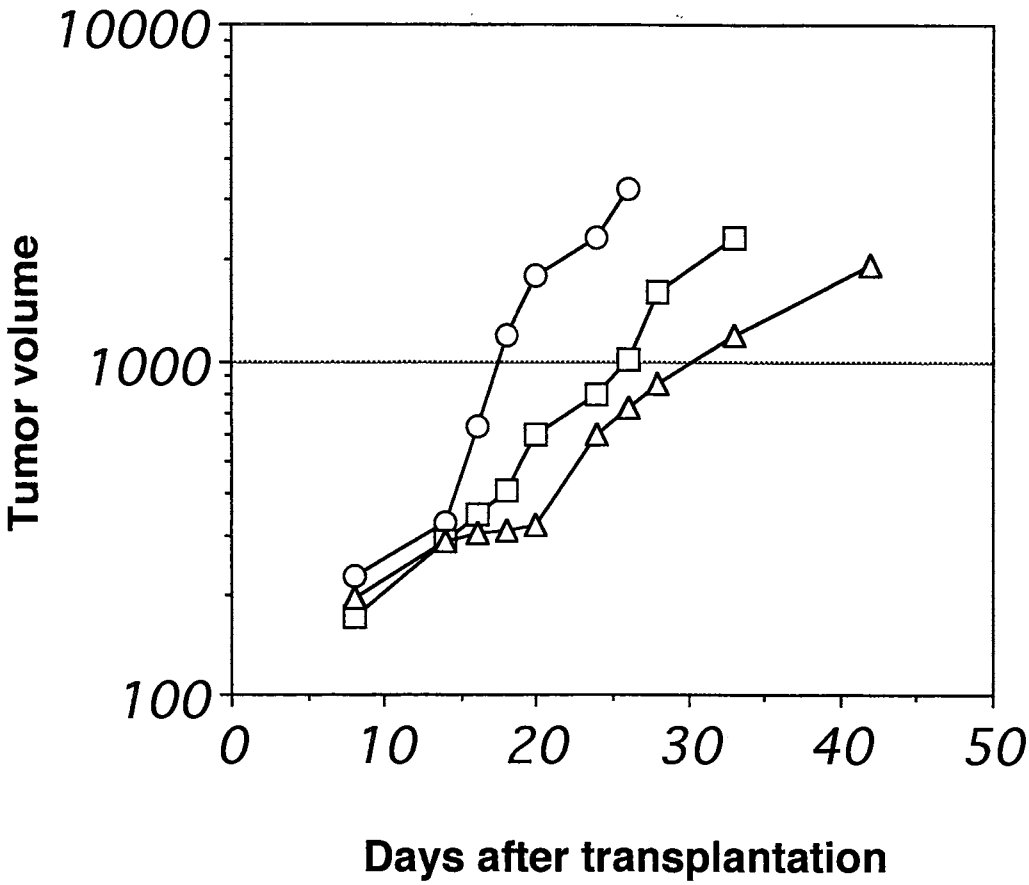


**Figure 1. Growth curves of three different xenografts.**

They were subcutaneously transplanted into the back of SCID mice; LNCaP-Lac 4 (circle), LNCaP-SF (square), LNCaP (triangle).

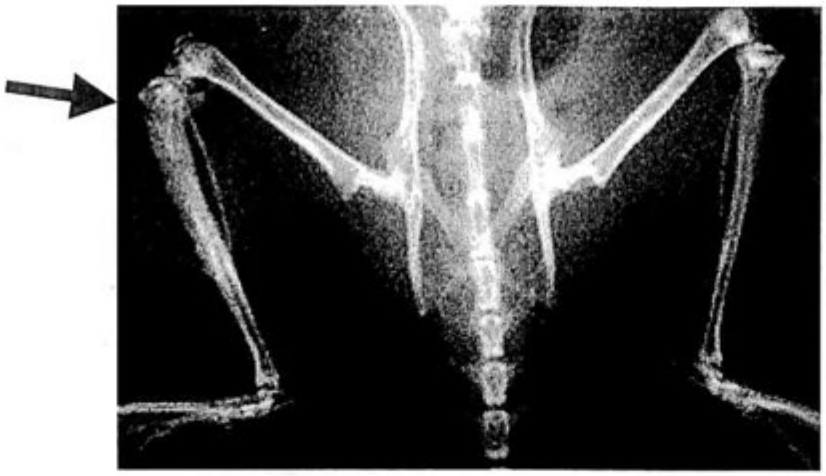

**Figure 2. X-ray of mouse tibia injected with LNCaP-SF.**

Osteoblastic change was evident as compared with normal counter part.

**Figure 3. Histological appearance of the lesion.**

Tumor cell proliferation associated with new osteoid deposition is shown.

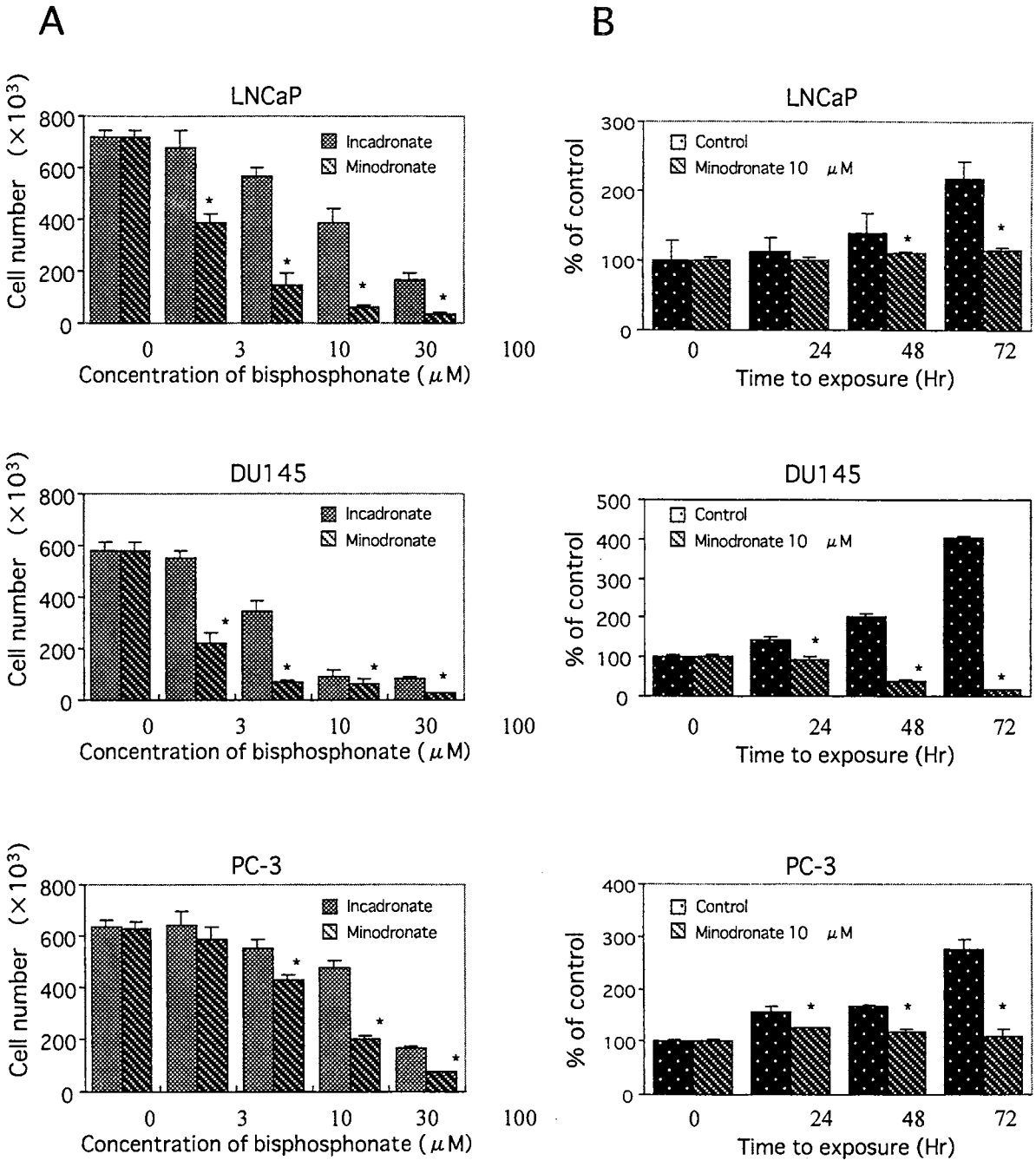


**Figure 4. Effects of Minodronate on prostate cancer cell lines.**

LNCaP, DU145 and PC-3 cells were treated with 0-100 uM of minodronate or incadronate for 72 h before harvesting. The viable cells were counted using a hematocytometer (A). LNCaP, DU145 and PC-3 cells were treated with 10 uM of minodronate for 0-72 h before harvesting and the viable cell number was counted (B). Error bars indicate mean ± 1 SD. *$p<0.05$.

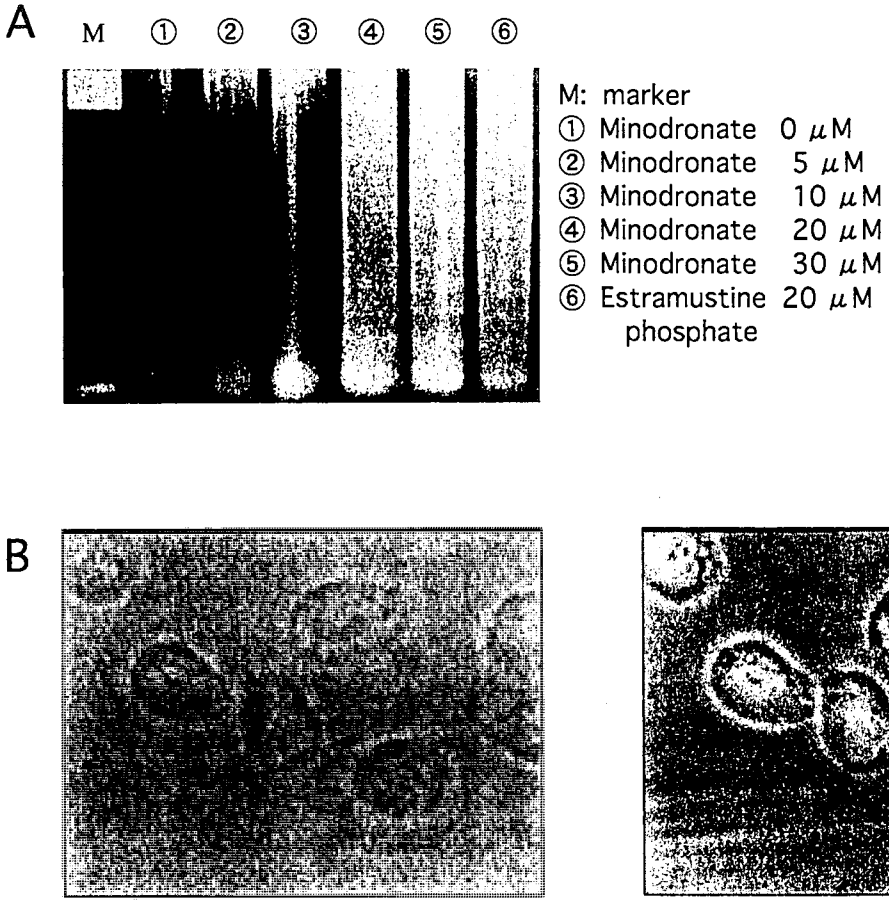


**Figure 5. Minodronate induces apoptosis in PC-3 cells.**

PC-3 cells were treated with 0-30 uM minodronate or with 20 uM estramustine phosphate as a positive control for 24 h. Cells were harvested total DNA was extracted and electrophoresed on an 1.5 % agarose gel (A). PC-3 cells were treated with 10 uM minodronate for 24 h and TUNEL was performed to detect apoptotic cells. Fluorescence microscopy was used to confirm the existence of positively stained cells, magnification x200 (B).

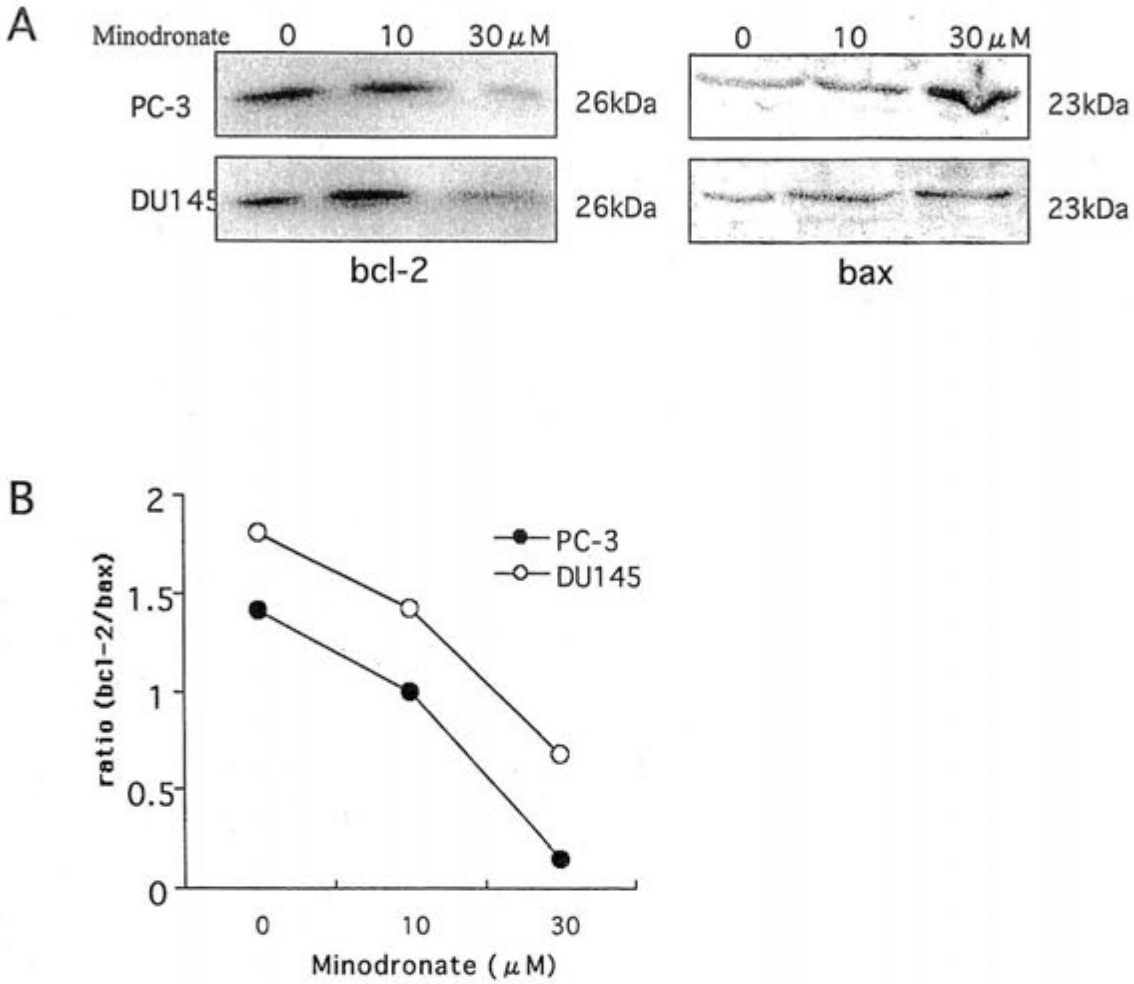


**Figure 6. Expression of bcl-2 and bax in minodronate-treated prostate cancer cell lines.**

PC-3 and DU145 cells were treated with 0-30 uM minodronate for 24 h. Total cell protein was collected, equal amounts of protein were loaded per lane and subjected to western blot for bcl-2 and bax (A). Corresponding densitometric analysis of bcl-2 and bax expression level is shown as the ration of bcl2/bax (B).

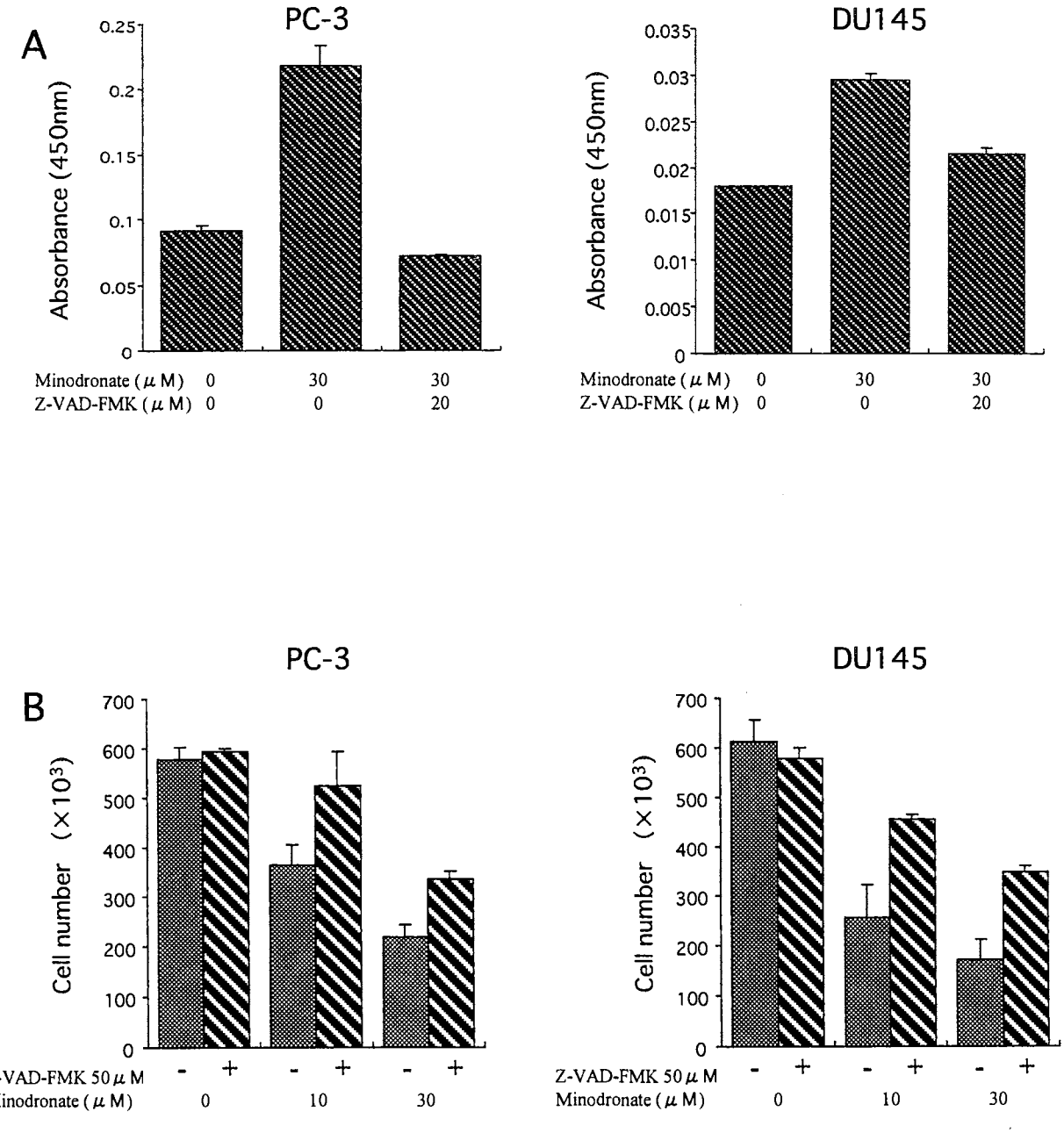


**Figure 7. Minodronate induces caspase-3 activity in prostate cancer cell lines.**

PC-3 and DU145 cells were treated with 0 or 30 uM minodronate with or without the addition of 20 uM of Z-VAD-FMK for 24 h. Total cell extracts were tested for caspase-3 activity using the CaspACE assay system. Results are reported as absorbance at 450 nM (A). PC-3 and DU145 cells were treated with 0-100 uM with or without 50uM of Z-VAD-FMK for 72 h. The viable cells were counted using hematocytometer (B). Error bars indicate mean ± 1 SD.

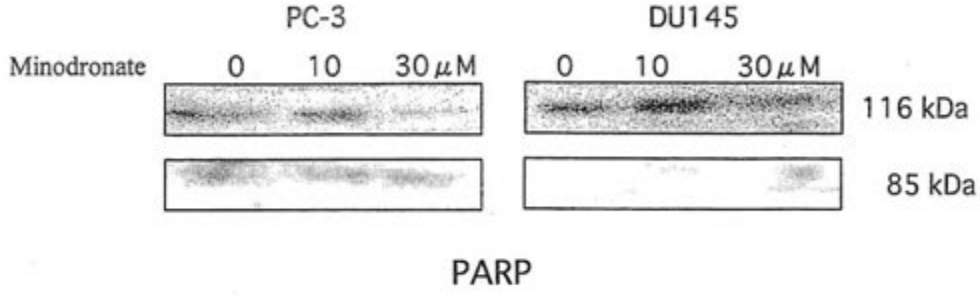


**Figure 8. Minodronate induces degradation of PARP (Poli-ADP ribose polymerase) prostate cancer cell lines.**

PC-3 and DU145 cells were treated with 0-30 uM minodronate for 24 h. Total cells extracts were obtained, equal amounts of protein loaded per lane and then subjected to western blotting for full-length PARP (116 kDa) and the PARP cleavage fragment (85 kDa).

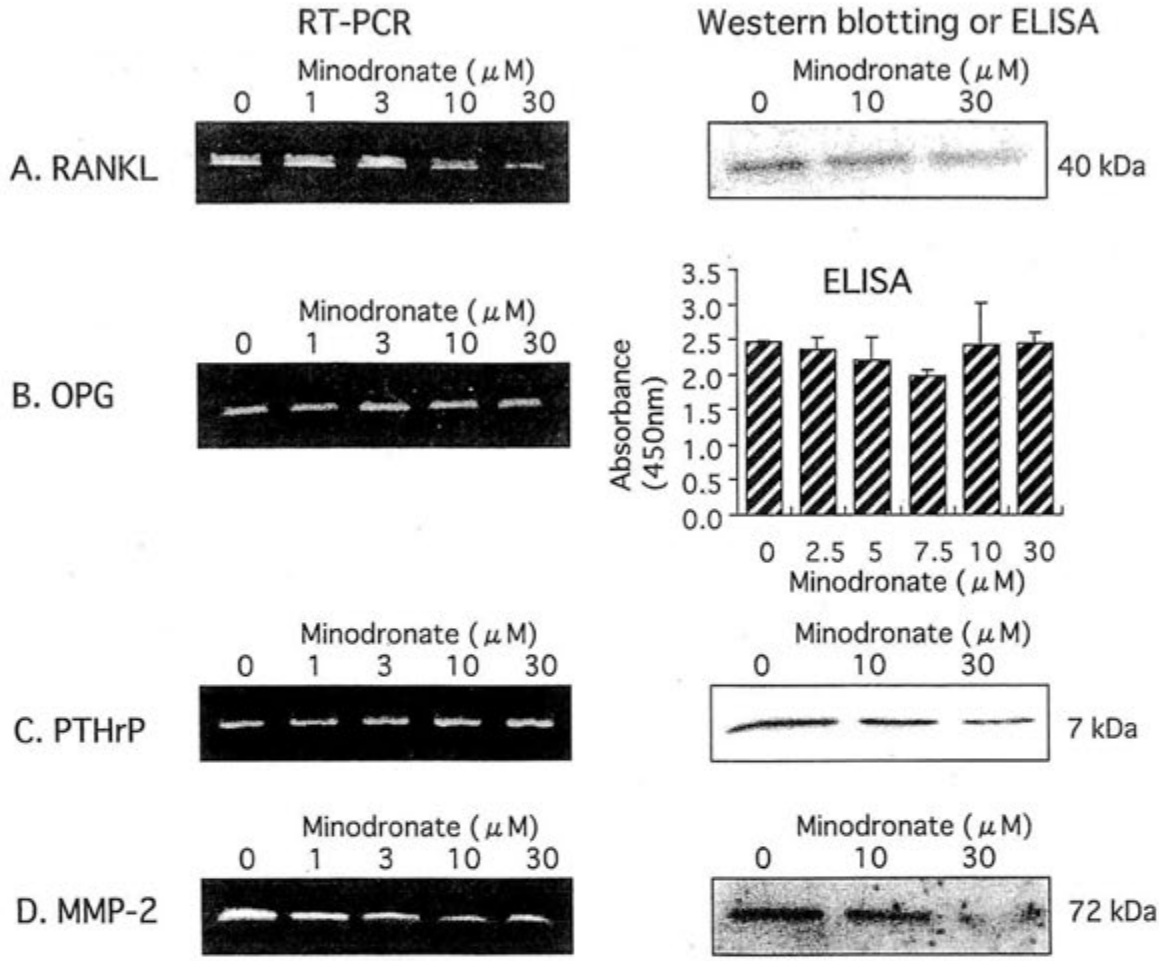


**Figure 9. Expression of proteins and cytokines in minodronate-treated PC-3.**

PC-3 cells were treated with 0-30 uM minodronate for 24 h. Total RNA and total protein were collected. RT-PCR and western blotting or ELISA was carried out to assess the expression of osteoclastogenic cytokines and MMP-2 in PC-3 cells. Equal amounts of cDNA and protein were loaded per lane for RT-PCR and western blotting. As for ELISA, absorbance at 450 nm was measured to assess the OPG protein level. Expression of RANKL was assessed by RT-PCR and western blotting (A). Expression of OPG was assessed by RT-PCR and ELISA (B). Expression of PTHrP was assessed by RT-PCR and western blotting (C). Expression of MMP-2 was assessed by RT-PCR and western blotting (D).

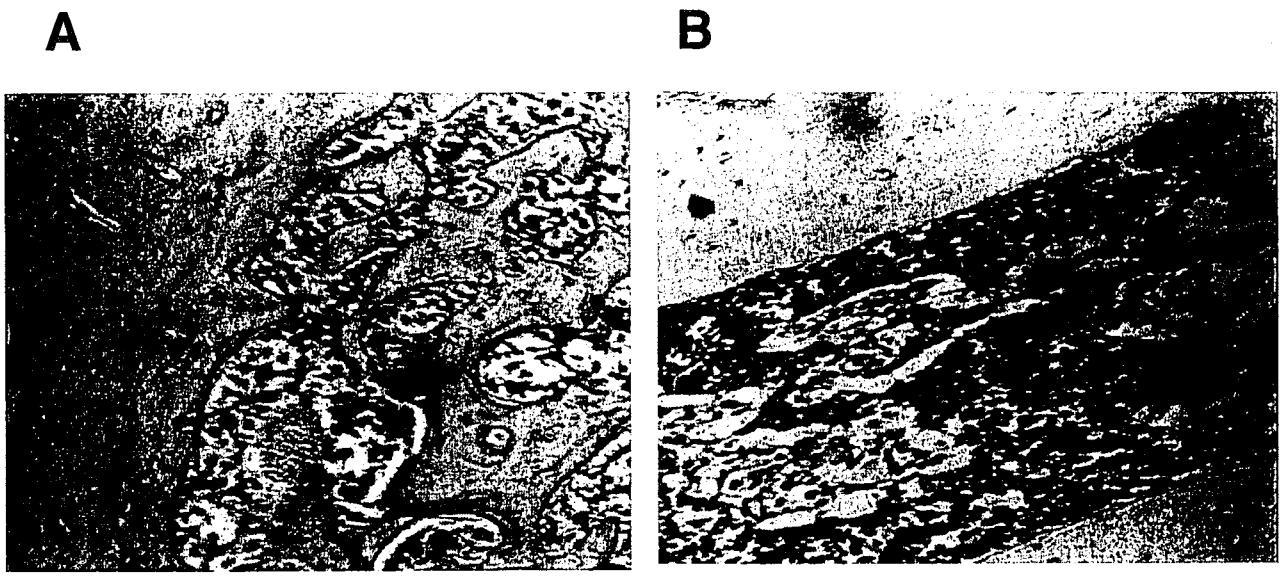


**Figure 10. Histological features of tumors formed in bone from untreated (A) and YM-529 treated (B) mouse.**

Tumor cells exist in bone marrow without new osteoid formation in a mouse treated with YM-529, while tumor cell invasion into bone matrix was remarkable in a control mouse.

**Table 1**

**Antitumor effect of YM-529 on bone tumor formation**

| YM-529 | Tumor formation | |
|---|---|---|
| 0 mg/kg | 5/6 | (83 %) |
| 0.1 mg/kg | 5/10 | (50 %) |
| 0.2 mg/kg | 2/7 | (29 %) |

**Bisphosphonate induces apoptosis and inhibits pro-osteoclastic gene expression in prostate cancer cells**

Hideki Asahi, Atsushi Mizokami, Evan T. Keller, Kiyoshi Koshida and Mikio Namiki

Department of Urology, School of Medicine, Kanazawa University; Department of Urology, University of Michigan, Ann Arbor, MI

Running title: Effects of bisphosphonate on prostate cancer
Key words: bisphosphonate, minodronate, prostate cancer

Correspondence:
Atsushi Mizokami, M.D., M.M.Sc.
Address: Takara-machi, 13-1, Kanazawa, Ishikawa prefecture,
Japan
Zip code 920-8640
Telephone: 81-76-265-2393
Fax: 81-76-222-6726
E-mail: syume@fki.fitweb.or.jp

**ABSTRACT**

Bisphosphonates are well established for the management of cancer-induced skeletal complications. Recent studies suggest that bisphosphonates promote apoptosis of cancer cells as well as osteoclasts in bone metastatic sites. In the present study, to determine the direct effects of bisphosphonate on prostate cancer cells,
we examined the effects of minodronate on prostatic cancer cell growth and the expression of apoptosis related proteins and osteoclastogenic factors. PC-3, DU145 and LNCaP cells were treated with amino-bisphosphonate minodronate. Then proliferation, apoptosis and expression of bcl-2, bax, poly (ADP)-ribose polymerase (PARP), caspase-3, receptor activator of nuclear factor-ÎB ligand (RANKL), osteoprotegerin (OPG), matrix metalloproteinases-2 (MMP-2), and parathyroid hormone related protein (PTHrP) were assessed. The proliferation of LNCaP, DU145 and PC-3 cells were inhibited by minodronate. DNA fragmentation and TUNEL positive nuclei were observed in minodronate treated PC-3 cells. Minodronate decreased bax expression and induced bcl-2 expression, caspase-3 activity and degradation of PARP in DU145 and PC-3 cells. Minodronate decreased expression of RANKL, PTHrP and MMP-2 in PC-3 cells. Our results suggest that bisphosphonate not only directly promotes prostate cancer cell apoptosis but also decreases pro-osteoclastic gene expression in prostate cancer cells.

**INTRODUCTION**

Recent developments in hormone therapy, radiation therapy, and surgical methods allow for complete cure of early stage prostate cancer. In contrast, there are no durable therapies for advance prostate cancer (1). Hormonal therapy is useful but its effects are limited because the prostate cancer changes to androgen-independent phenotype over several years of therapy (2-4). As prostate cancer progress, bone metastases develop in over 80% of the patients (5). Management of bone metastases, which cause pathological fracture, nerve paralysis, bone pain and limited mobility, is very important for improvement and maintenance of the patient's quality of life and prognosis (6-8). However, an effective treatment for hormone refractory prostate cancer with bone metastases has not been established.

Bisphosphonates are analogues of endogenous pyrophosphate. In vivo, bisphosphonates bind to hydroxyapatite on the bone surface and are preferentially delivered to sites of increased bone remodeling. They are potent inhibitors of osteoclast-mediated bone resorption and are effective in lowering serum calcium concentration. Bisphosphonates directly induce apoptosis of osteoclasts through the mevalonate pathway and preventing the isoprenylation of small GTP-binding proteins such as Ras and Rho. These osteoclast inhibitory effects result in inhibition of bone absorption (9-11). Bisphosphonates have been shown to reduce bone pain, improve quality of life, and to delay skeletal event in patients with breast cancer or multiple myeloma (12, 13). In addition, bisphosphonates have been shown to exhibit direct anti-tumor effects in multiple types of cancer cells (14-16). In a clinical study, it was shown that administration of bisphosphonates to patients with painful prostate cancer bone metastases resulted in a significant pain decrease and a significant decrease in the daily consumption of analgesics (17). Moreover, we previously reported a case of hormone refractory prostate cancer with bone metastasis in which a

bisphosphonate decreased not only bone pain, but also serum PSA levels during clinical treatment for prostate cancer (18). The reduction in PSA levels suggests that bisphosphonates decreases tumor burden. However, the mechanisms of the in vivo effects of bisphosphonates have not been fully elucidated.

The goal of this study was to clarify the mechanisms through which bisphosphonates achieve their therapeutic effects on hormone refractory prostate cancer. Accordingly, we evaluated the effects of bisphosphonates on the proliferation, apoptosis and cytokine and growth factor expression in prostate cancer cell lines, using the new third generation bisphosphonates minodronate and incadronate.

## MATERIALS AND METHODS

**Reagents.** Minodronate and incadronate were a gift from Yamanouchi pharmaceutical (Tokyo, Japan). Minodronate was dissolved in 1N NaOH then the pH adjusted to 7.4. Bisphosphonates were prepared in phosphate buffered saline and then diluted prior to addition to cell cultures.

**Cell line and cell culture.** The human prostatic cancer cell lines PC-3, DU145 and LNCaP were maintained in RPMI or DMEM medium supplemented with antibiotics and 10% fetal bovine serum (Sigma-aldrich, Missouri, USA). Cells were passaged by trypsinization and cultured at 37℃ in 5% $CO_2$.

**Cell proliferation assay.** PC-3, DU145 and LNCaP prostate cancer cells were plated at a density of 5_$10^4$ cells onto 6 well plates with DMEM or RPMI-10% FBS and allowed to adhere and proliferate for 24 h. Cells were then incubated with fresh medium containing indicated concentrations of minodronate or incadronate for 72 h, or cells were incubated with fresh media containing 10 ÌM of minodronate for 24, 48, and 72 h. In each experiment, cells were harvested and cell number was counted using a haemocytometer.

**Detection of Apoptosis using DNA fragmentation assay.** 5_$10^4$ PC-3 cells were plated onto 6 wells plates, allowed to adhere overnight and treated with minodronate at the indicated concentrations or with estramustine phosphate (20 ÌM) as a positive control for 24 h. Cells were harvested and DNA was extracted and 10 Ìg of DNA samples were electrophoresed on an 1.5 % agarose gel using the Apoptosis ladder detection kit (WAKO, Osaka, Japan). After electrophoresis, staining in SYBR™ Green I working solution was performed. To visualize bands, an UV transilluminator of wave length 254 nm with a yellow gelatin filter was used.

**Detection of Apoptosis using TUNEL assay.** After 5_$10^4$ PC-3 and DU145 cells were plated, cells were treated with 10 ÌM minodronate for 24 h. A TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling) technique was performed to detect apoptotic cells using the *in situ* Apoptosis Detection Kit (TAKARA BIO INC, Shiga, Japan) according to the manufacturer's instruction. Fluorescene microscopy was used to confirm the ex istence of positively stained cells.

**Measurement of activity of caspase-3.** After 3_$10^5$ PC-3 and DU145 cells were plated onto 6 wells plates and cells were treated with 30 ÌM minodronate in the absence or presence of 20 ÌM

of broad caspase-3 inhibitor, Z-VAD-FMK, for 24 h. The caspase-3 specific activity in PC-3 and DU145 was determined using CaspACE™ ASSAY System, Colorimetric (Promega, Wisconsin, USA) according to manufacturer's instructions. In another experiment, 5_10$^4$ PC-3 and DU145 cells were plated onto 6 wells plates, and cells were treated with 0-30 ìM minodronate in the absence or presence of 50 ìM Z-VAD-FMK for 72 h. Viable cells were then counted using a haemocytometer.

**RT-PCR.** Total RNA was extracted using ISOGEN (Nippon Gene, Tokyo, Japan). To make complementary DNA, 1 ìg total RNA was subjected to reverse transcription was performed using ThermoScript™RT (Invitrogen, California, USA). RT-PCR was performed using TaKaRa Ex Taq™ Hot Start Version (TAKARA BIO INC, Shiga, Japan) and the following primers: human/mouse TRANCE PCR Primer Pair™ for RANKL, human/mouse OPG PCR Primer Pair™ for OPG, human/mouse MMP-2 PCR Primer Pair™ for MMP-2 (R&D systems, Minneapolis, USA), and PTHrP primer for PTHrP (Sigma-genosys Japan, Hokkaido, Japan). RT-PCR condition for RANKL was 94℃ for 4 min followed by 35 cycles of 94℃ for 45s/56.5℃ for 45s/72 ℃ for 45s, for OPG 94℃ for 4 min followed by 30 cycles of 94℃ for 45s/55.5℃ for 45s/72℃ for 45s, for MMP-2 94℃ for 4 min followed by 33 cycles of 94℃ for 30s/55℃ for 30s/72℃ for 30s, and for PTHrP 94℃ for 4 min followed by 20 cycles of 94℃ for 30s/55℃ for 30s/72℃ for 30s. RT-PCR products were electrophoresed on an 1.5% agarose gel and visualized by ethidium bromide staining under UV light.

**OPG protein measurement.** After 5_10$^4$ PC-3 cells were plated onto 6 wells plates, cells were treated with 0-30 ìM minodronate for 24 h. Conditioned medium was harvested and centrifuged to remove debris. Samples were stored at –80℃ until used. OPG protein secretion was determined using Osteoprotegerin ELISA kit (BIOMEDICA, Wien, Austria) according to the manufacturer's instruction.

**Western Blotting.** After 5_10$^5$ PC-3 cells were plated, cells were treated with 0-30 ìM minodronate for 24 h. Then, cells were lysed with lysis buffer composed of 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.1% Triton X-100, 0.01 mg/ml aprotinin and 0.05 mg/ml phenylmethylsulphonyl fluoride. Protein was quantified by methods of Bradford and equal amount of protein were loaded and electrophoresed on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis mini-gel. Proteins were transferred to PVDF membrane (Bio-rad, California, USA) and preblocked with casein PBS and 0.05% Tween-20 for 1 h at room temperature. Membranes were incubated with mouse monoclonal antibody against bcl-2, PARP, and rabbit monoclonal antibody against bax, MMP-2, RANKL, and goat monoclonal antibody against PTHrP (Santa Cruz, California, USA). Horseradish peroxidase-conjugrated secondary antibody against anti-mouse or rabbit or goat monoclonal antibody was used and protein bands were visualised with enhanced chemiluminescence reagent.

**Statistics**. Experiments were performed in triplicate unless otherwise stated. All statistical comparisons were made using unpaired, two-tailed Student's t test, assuming unequal variance.

## RESULTS

**Growth inhibition by minodronate.** We first evaluated the effect of minodronate and incadronate on proliferation of prostate cancer cells. Both bisphosphonates inhibited proliferation of PC-3, DU145 and LNCaP cells in a dose-dependent manner (Fig. 1A). Minodronate was a

more potent inhibitor of proliferation than incadronate. We next evaluated if the effect of minodronate was time dependent. Minodronate significantly inhibited proliferation of PC-3, DU145 and LNCaP cells in a time dependent manner (Fig. 1B). The $IC_{50}$ value for incadronate in LNCaP, DU145 and PC-3 was 34.0 ìM, 16.3 ìM and 57.2 ìM, respectively, whereas that for minodronate was 6.32 ìM, 5.23 ìM and 18.7 ìM, respectively (Table.1).

**Detection of apoptosis.** To investigate whether minodronate induced apoptosis, we performed DNA fragmentation and TUNEL assays. Minodronate induced DNA fragmentation in PC-3 cells in a dose-dependent manner (Fig. 2A) and nuclear apoptosis (Fig.2B). In DU145 cells, the same phenomenon was observed (date not shown). These results suggest that minodronate directly cause apoptosis in prostate cancer cells.

**Effects of bisphosphonate on expression of bcl-2 and bax.** A number of proteins are implicated in the regulation of apoptosis. It has been demonstrated that the bcl-2 gene product confers resistance to apoptosis induced by a numbers of stimuli, while its homologue, bax, promotes apoptosis (19, 20). In order to investigate the mechanism through which bisphosphonates induce apoptosis in prostate cancer cells, the effects of minodronate on bcl-2 and bax expression were evaluated. Minodronate reduced bcl-2 expression (Fig.3A). In contrast, minodronate induced expression of bax in a dose-dependent manner (Fig.3A). Yin et al. reported that the susceptibility of a cell to undergo apoptosis depends in part on the ratio of bcl-2 to bax, with a lower ratio favoring of apoptosis (19). Minodronate reduced the bcl2/bax ratio in both PC-3 and DU145 cells in a dose-dependent manner (Fig.3B), suggesting that minodronate induces apoptosis through down-regulation of the bcl2/bax ratio in prostate cancer cells.

**Effects of minodronate on expression of caspase-3 and poly (ADP)-ribose polymerase (PARP).** Caspase-3 is the most prevalent caspase and is responsible for a majority of apoptotic effects. Thus, we investigated the effect of minodronate on caspase-3 activity in PC-3 and DU145 cells. Minodronate induced caspase-3 activity (Fig.4A). Addition of the caspase-3 inhibitor Z-VAD-FMK resulted in inhibition of minodronate-induced caspase-3 in both PC-3 and DU145 cells. In addition, Z-VAD-FMK blocked minodronate cell death in both PC-3 and DU145 cells (Fig.4B). These results suggest that minodronate induces apoptosis through a caspase-3-dependent mechanism.

Another key feature of apoptosis is proteolytic cleavage of PARP as a consequence of activation of caspases. It has been suggested that PARP is associated with breaking and rejoining of DNA strands, and plays a pivotal role in DNA damage repair. Full-length active PARP is a 116 kDa molecule which is cleaved to fragments of 85 kDa and 30 kDa by the action of caspase-3 and related caspases (21). Caspase-mediated proteolysis of PARP is an early event of programmed cell death in a variety of cells (22). In order to determine the effect of minodronate on PARP, expression of PARP in PC-3 and DU145 was examined. Minodronate treatment decreased expression of intact PARP and increased expression of the 85 kDa fragment in both cell lines (Fig.5). These findings suggest that minodronate induced proteolysis of PARP through caspase-3 activation.

**Effect of minodronate on expression of growth factors and cytokines derived from prostatic cancer cells.** Various cancer cells, including prostate cancer cells, secrete a number of growth factors and cytokines which stimulate osteoclast-mediated

bone resorption (23). Accordingly, we determined if minodronate modulated expression of pro-osteoclastogenic proteins. Minodronate decreased both RANKL mRNA and protein levels in a dose-dependent manner in PC-3 cells in a dose-dependent manner (Fig.6A). In contrast, minodronate had no effect on OPG mRNA and protein expression (Fig.6B). Minodronate had no effect on mRNA expression but decreased PTHrP protein level in PC-3 cells (Fig.6C). Finally, expression of minodronate decreased MMP-2 mRNA and protein expression in a dose-dependent manner PC-3 cells (Fig.6D).

## DISCUSSION

In the present study, minodronate, which is a new third generation bisphosphonate, directly inhibited total cell accumulation of LNCaP, DU145 and PC-3 prostate cancer cells in vitro through induction of apoptosis. In addition, minodronate treatment induced PARP degradation and apoptosis through caspase-3 in PC-3 cells. It has been suggested that bisphosphonates indirectly inhibit tumor growth through inhibition of bone absorption (15, 24). However, there is increasing evidence that bisphosphonates may directly inhibit tumor growth. For example, the bisphosphonate pamidronate inhibited proliferation of the MDA-MB-231 breast cancer cell accumulation through induction of apoptosis that was associated with a decreased bcl-2/bax ratio and PARP proteolysis (14). Additionally, pamidronate inhibited cell proliferation of PC-3, DU145 and LNCaP prostate cancer cells in vitro (25). Moreover, another bisphosphonate, alendronate, induced apoptosis and prevented invasion of prostate cancer cells by inhibiting the mevalonate pathway (26). Finally, it was demonstrated that 10 μM of zoledronate, which is also a new third generation of bisphosphonate, directly induced apoptosis in prostatic cancer cell lines (27). These previous reports, taken together with our results, suggest that bisphosphonates directly induce prostate cancer cell apoptosis.

In the present study, we demonstrated that minodronate decreased bcl-2 expression, increased bax expression and induced proteolysis of PARP following caspase-3 activation. These results suggest that the direct effect of bisphosphonate on cancer cells is caspase-3 dependent. However, addition of the caspase-3 inhibitor Z-VAD-FMK only partially protected prostate cancer cells from minodronate induced-apoptosis. This finding implies that other caspases may be involved in bisphosphonate-induced apoptosis. Alternatively, other mechanisms may play a role in the induction of apoptosis. For example, another mechanism through which bisphosphonates may induce apoptosis in cancer cells is suggested by the report that amino-bisphosphonates inhibit farnesyl pyrophosphate synthase in the cholesterol biosynthetic pathway in osteoclasts (10). Specifically, amino-bisphosphonates block the mevalonate pathway that induces apoptosis in the J774 macrophage-like cell line (11). The mevalonate pathway mediates cholesterol biosynthesis which is important for isoprenylation of small GTP binding proteins such as Rac, Rho, Rab and Ras. It has been demonstrated that the bisphosphonate zoledronic acid impairs membrane localization of isoprenylated Ras, which results in promoting apoptosis of prostate cancer cells (27). Although these pathways induce apoptosis, it is unknown whether inhibition of isoprenylation of small GTP binding proteins induces apoptosis through regulation of bcl-2 or

bax or activation of caspases.

In addition to apoptosis, bisphosphonates may regulate cancer cells through effecting growth factors and cytokines that promote cancer cell progression. Several important molecules that effect cancer phenotype include osteoprotegerin (OPG) and RANKL (28). RANKL is a member of the TNF family, which is initially expressed by bone marrow stromal cells, osteoblasts and activated T cells (30). RANKL is mainly a membrane-anchored molecule. However, small fraction of RANKL is released through proteolytic cleavage on cell surface as a soluble homotrimeric molecule and both kinds of RANKL promote osteoclast formation and activation by binding to RANK on the osteoclast precursor membrane (31). Prostate cancer is known to express membrane anchored and soluble RANKL (23, 32). In contrast, OPG acts as a decoy receptor against RANKL and expression of OPG by osteoblasts under hormone regulation is thought to inhibit osteoclastgenesis by binding the differentiating factor (33). In immunohistochemical study, bone metastases of prostate cancer were consistently immunoreactive for both RANKL and OPG compared with non-osseous metastases or primary prostate cancer (34). It has been demonstrated that the induction of osteoclastgenesis is dependent upon the relative abundance of RANKL compared with the level of OPG (35). Bisphosphonates have been shown to increase OPG expression from osteoblasts in vitro (29). The increased OPG diminishes osteolytic activity. We observed that minodronate caused down-regulation of the expression of two pro-osteolytic molecules, RANKL, in cancer cells. Thus, similar to the previously reported effect on OPG, minodronate diminishes osteolytic activity through inhibition of these proteins.

Another important osteoclastogenic factor is parathyroid hormone related protein (PTHrP), which increases bone resorption in solid tumors (36). Overexpression of PTHrP in prostate cancer cells has been shown to induce osteolytic lesions in the bone of rats (37). PTHrP production by primary prostatic tumors is associated with increased tumor size and rate of growth in an animal model, suggesting that PTHrP acts in an autocrine or intracrine mechanism to promote tumor growth (38). We found that minodronate decreased PTHrP expression, thus favoring a decrease in osteolytic activity. These findings suggest that minodronate might prevent osteoclastogenesis and growth of prostatic cancer cells in bone microenvironment by inhibiting paracrine and autocrine secretion of PTHrP from cancer cells.

MMPs are essential factors for invasion and metastasis in cancer cells. MMPs also have an important role in bone remodeling. Kusano et al. demonstrated that induction of MMP-2, -3, -9 and -13 by IL-1 and IL-6 is closely linked to the respective bone-resorbing activity in mouse calvaria, suggesting that MMP-dependent degradation of bone matrix plays a key role in bone resorption (39). Prostate cancer secretes a large number of MMPs (40-44) suggesting that they may play a role in prostate cancer-mediated bone resorption. We identified that minodronate decreased MMP-2 expression from prostate cancer cells. These results are similar to a previous report in which it was shown that alendronate decreased MMP-2 and -9 activity in PC-3 cells injected into the femur of SCID mice (45). These data suggest that inhibition of MMPs may contribute to bisphosphonate-mediated inhibition of osteolytic activity in prostate cancer.

In conclusion, in addition to inducing osteoclast apoptosis,

minodronate induces apoptosis of prostate cancer cells directly via caspase-3. Additionally, bisphosphonates may decrease osteoclastogenesis through inhibiting expression of pro-osteoclastic protein production from prostate cancer cells. Taken together, these results imply that some bisphosphonates have a dual role in therapy of prostate cancer bone metastases; namely, inhibiting prostate cancer-induced bone resorption and directly inhibiting prostate tumor growth through promotion of apoptosis.

**Acknowledgments**

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## FIGURE LEGEND

**Figure 1. Effects of Minodronate on prostate cancer cell lines.**

LNCaP, DU145 and PC-3 cells were treated with 0-100 ìM of minodronate or incadronate for 72 h before harvesting. The viable cells were counted using a hematocytometer (A). LNCaP, DU145 and PC-3 cells were treated with 10 ìM of minodronate for 0-72 h before harvesting and the viable cell number was counted (B). Error bars indicate mean ± 1 SD. *p<0.05.

**Figure 2. Minodronate induces apoptosis in PC-3 cells.**

PC-3 cells were treated with 0-30 ìM minodronate or with 20 ìM estramustine phosphate as a positive control for 24 h. Cells were harvested total DNA was extracted and electrophoresed on an 1.5 % agarose gel (A). PC-3 cells were treated with 10 ìM minodronate for 24 h and TUNEL was performed to detect apoptotic cells. Fluorescence microscopy was used to confirm the existence of positively stained cells, magnification x200 (B).

**Figure 3. Expression of bcl-2 and bax in minodronate-treated prostate cancer cell lines.**

PC-3 and DU145 cells were treated with 0-30 ìM minodronate for 24 h. Total cell protein was collected, equal amounts of protein were loaded per lane and subjected to western blot for bcl-2 and bax (A). Corresponding densitometric analysis of bcl-2 and bax expression level is shown as the ration of bcl2/bax (B).

**Figure 4. Minodronate induces caspase-3 activity in prostate cancer cell lines.**

PC-3 and DU145 cells were treated with 0 or 30 ìM minodronate with or without the addition of 20 ìM of Z-VAD-FMK for 24 h. Total cell extracts were tested for caspase-3 activity using the CaspACE assay system. Results are reported as absorbance at 450 nM (A). PC-3 and DU145 cells were treated with 0-100 ìM with or without 50ìM of Z-VAD-FMK for 72 h. The viable cells were counted using hematocytometer (B). Error bars indicate mean ± 1 SD.

**Figure 5. Minodronate induces degradation of PARP (Poli-ADP ribose polymerase) prostate cancer cell lines.**

PC-3 and DU145 cells were treated with 0-30 ìM minodronate for 24 h. Total cells extracts were obtained, equal amounts of protein loaded per lane and then subjected to western blotting for full-length PARP (116 kDa) and the PARP cleavage fragment ( 85 kDa).

**Figure 6. Expression of proteins and cytokines in minodronate-treated PC-3.**

PC-3 cells were treated with 0-30 ìM minodronate for 24 h. Total RNA and total protein were collected. RT-PCR and western blotting or ELISA was carried out to assess the expression of osteoclastogenic cytokines and MMP-2 in PC-3 cells. Equal amounts of cDNA and protein were loaded per lane for RT-PCR and western blotting. As for ELISA, absorbance at 450 nm was measured to assess the OPG protein level. Expression of RANKL

was assessed by RT-PCR and western blotting (A). Expression of OPG was assessed by RT-PCR and ELISA (B). Expression of PTHrP was assessed by RT-PCR and western blotting **(C). Expression of MMP-2 was assessed by RT-PCR and western blotting (D).**

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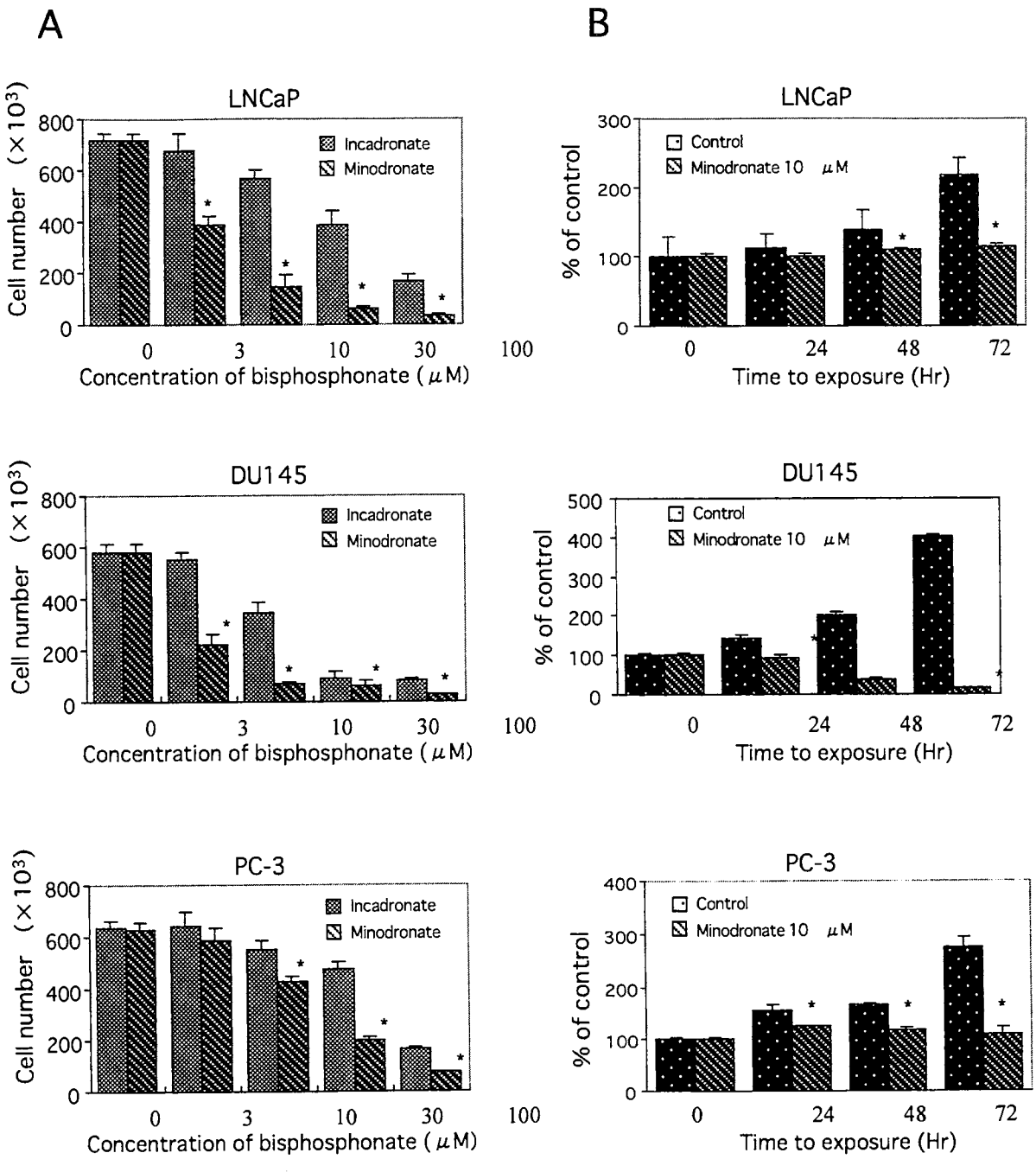
Figure. 1. H. Asahi et al.



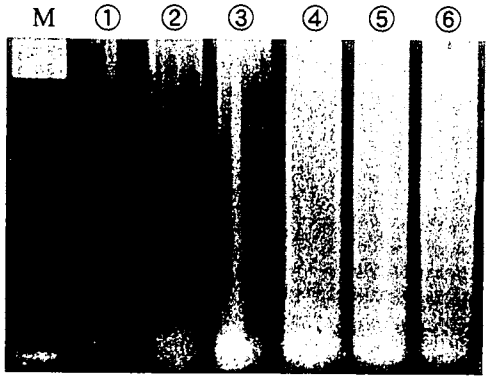
# Table. 1. H. Asahi et al.

Table. 1. IC50 of bisphosphonate s for prostate cancer cell lines

| | Concentration ($\mu$ M) | |
|---|---|---|
| | Minodronate | Incadronate |
| LNCaP | 6.32 | 34.0 |
| DU145 | 5.23 | 16.3 |
| PC-3 | 18.7 | 57.2 |

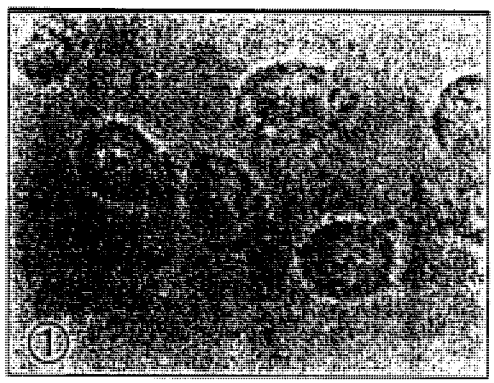
Figure. 2. H. Asahi et al.

A

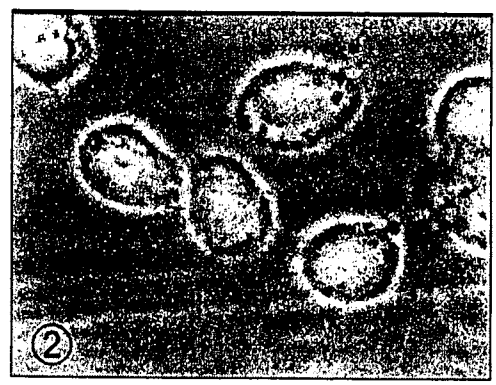



M: marker
① Minodronate 0 μM
② Minodronate 5 μM
③ Minodronate 10 μM
④ Minodronate 20 μM
⑤ Minodronate 30 μM
⑥ Estramustine phosphate 20 μM

B



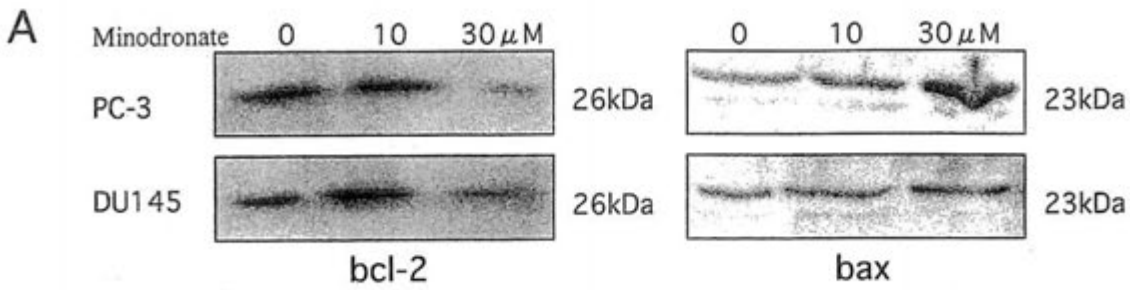

①Non fluorescene micrograph of PC-3.




② Fluorescene micrograph of PC-3.

(×200 magnification)

Figure . 3. H. Asahi et al.



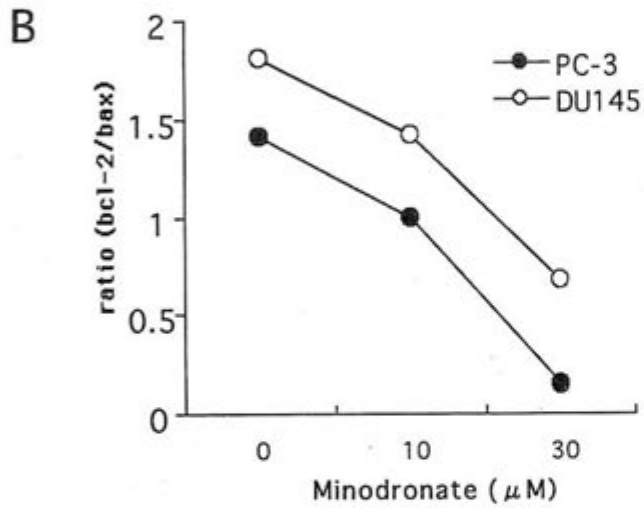
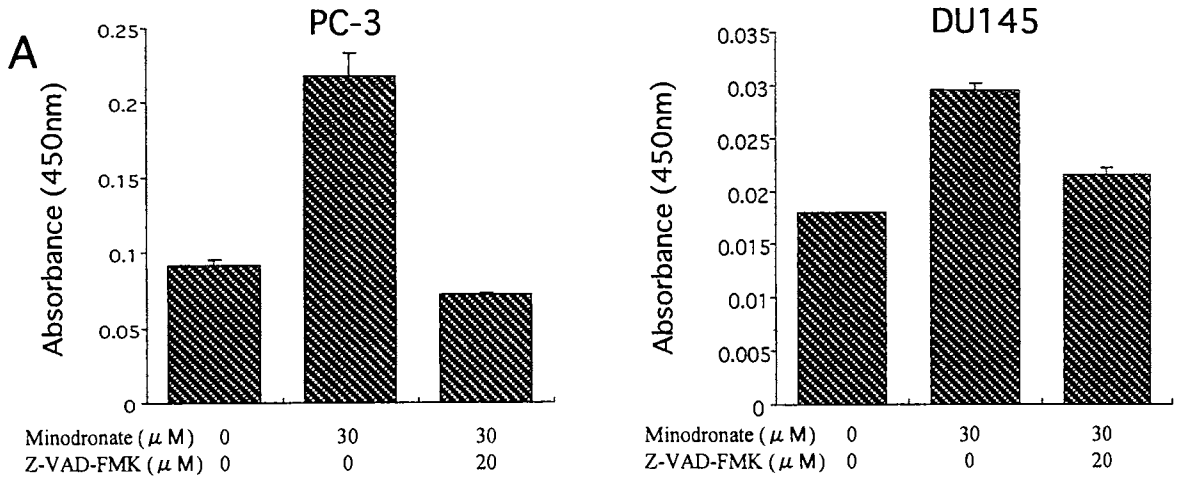



Figure . 4. H. Asahi et al.



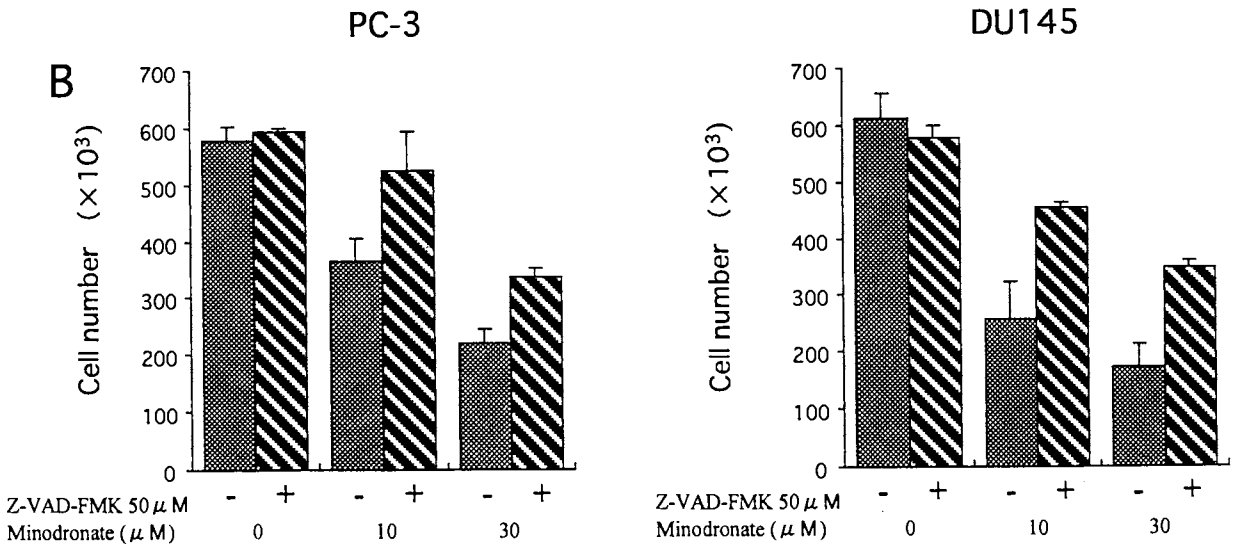



Figure . 5. H. Asahi et al.

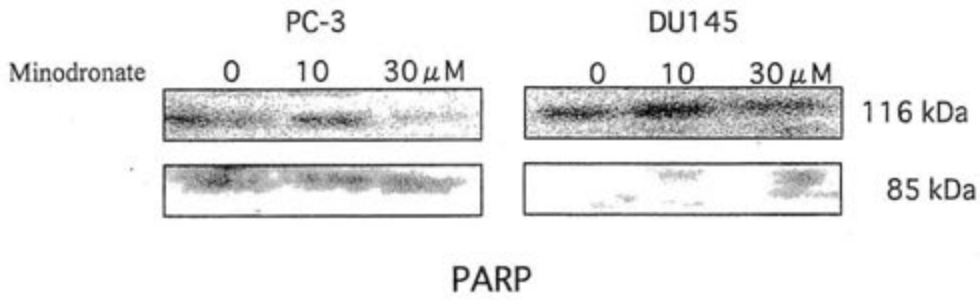



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Figure . 6. H. Asahi et al.