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

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Abstract

**Background.** Bisphosphonates are well established in 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, we examined the effects of minodronate on prostatic cancer cell growth and the expression of apoptosis related proteins and osteoclastogenic factors.

**Methods.** The amino-bisphosphonate minodronate was added to PC-3, DU145 and LN-CaP. Cell proliferation, apoptosis and expression of bcl-2, bax, PARP, caspase-3, RANKL, OPG, MMP-2 and PTHrP was assessed.

**Results.** The cell proliferation of LN-CaP, DU145 and PC-3 cells were inhibited by minodronate. DNA fragmentation and TUNEL positive nuclei were observed in minodronate treated PC-3 cells. Bcl-2/bax ratio, expression of poly (ADP)-ribose polymerase (PARP), and caspase-3 were regulated by minodronate in DU145 and PC-3 cells. Furthermore, decreased expression of RANKL, MMP-2 and PTHrP mRNA and protein were observed in PC-3 cells.

**Conclusions.** Our results suggest that bisphosphonate not only promotes apoptosis directly but also regulates expression of genes associated with osteolysis in prostatic cancer cells.
Introduction

Early staged prostate cancer can be cured completely because of recent development of hormone, radiation therapy, or surgical operation. However, only hormone therapies are available for advanced prostate cancer (1) but those effects are limited because character of prostate cancer with hormone therapies changes to androgen-independent phenotype in several years (2-4). About 80% of metastatic prostatic cancer occurs in multiple bones (pelvis or vertebra, femur, etc) (5). Management of bone metastases which cause pathological fracture, nerve paralysis and movement restrictions is very important for improvement and maintenance of patient’s quality of life and prognosis (6-8). However, the effective treatment for hormone refractory prostate cancer with bone metastasis has not been established yet.

Bisphosphonates are analogues of endogeneous pyrophosphate in which a carbon atom replaces the central atom of oxygen. In vivo, bisphosphonate binds strongly to hydroxyapatite on the bone surface and are preferentially delivered sites of increased bone formation or resorption. They are potent inhibitors of osteoclast-mediated bone resorption and are effective in lowering serum calcium concentration. Bisphosphonates have direct effects on osteoclasts, inducing apoptosis through mevalonate pathway and preventing the isoprenylation of small GTP-binding proteins such as Ras and Rho. These effects results in inhibition of bone absorption (9-11). Recently, 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 some kinds of cancer cells (14-16). In clinical study of prostate cancer, bisphosphonate treatment of painful osseous metastases due to hormone refractory prostate cancer results in a significant pain decrease and a significant decrease in the daily consumption of analgesics in 75% of patients (17). However, mechanism of these in vivo effects of bisphosphonate has not been fully elucidated yet.

In this study, in order to clarify the mechanisms for therapeutic effects of bisphosphonates on hormone refractory prostate cancer, we evaluated the effects of bisphosphonate on the regulation of proliferation and apoptosis and cytokine or growth factor expression in prostate cancer cell lines using new third generation amino-bisphosphonate: minodronate (YM529).
Materials and methods

Reagents.

Minodronate (MIN, also known as YM-529) and incadronate (INC, also known as YM175) were gifted from Yamanouchi (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 line PC-3, DU145 and LNCaP were maintained in RPMI or DMEM medium supplemented with antibiotics and 10% fetal bovine serum (FBS). Cells were passaged by trypsinization and cultured at 37°C in 5% CO2. All of the medium and reagents were from Sigma-aldrich, Missouri, USA.

Cell proliferation assay

Prostate cancer cell line PC-3, DU145 and LNCaP were plated at a density of 5×10^5 cells onto 6 well plates with DMEM or RPMI-10% FBS and allowed to adhere and proliferate for 24 h. Then cells were incubated with fresh medium containing increasing concentrations of minodronate or incadronate for 72 h, or cells were incubated with fresh media containing 10 μM of YM529 for 24, 48, 72 h, respectively. In each experiment, cells were
harvested and cell number was determined using a haemocytometer.

**Detection of Apoptosis using DNA fragmentation assay.**

5×10⁴ PC-3 cells were plated onto 6 wells plates, allowed to adhere overnight and treated with minodronate at the indicated concentrations, or estramustine phosphate (20 μM) as positive control for 24 h. Cells were harvested and DNA was extracted and 10 μg of DNA samples were electrophoreosed on an 1.5 % agarose gel using 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 Yellow Gelatin Filter was used.

**Detection of Apoptosis using TUNEL assay.**

After 5×10⁴ PC-3 and DU145 cells were plated, cells were treated with 10 μM minodronate for 24 h. A TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling) technique was performed to detect apoptotic cells using In situ Apoptosis Detection Kit (TAKARA BIO INC, Shiga, Japan) according to the manufacturer instruction. Fluorescene microscopy was used to confirm the existence of positively stained cells.
Measurement of activity of caspase-3.

After $3 \times 10^5$ PC-3 and DU145 cells were plated onto 6 wells plates, and cells were treated with 30 μM minodronate in the absence or presence of 20 μM of broad caspase-3 inhibitor: Z-VAD-FMK. The caspase-3 specific activity in PC-3 and DU145 was determined using CaspACE™ ASSAY System, Colorimetric (Promega, Wisconsin, USA) according to manufacturer instructions. Then, cells were harvested and cell number was determined using a haemocytometer.

RT-PCR.

Total RNA was extracted using ISOGEN (Nippon Gene, Tokyo, Japan). To make complementary DNA from 1 μg total RNA, 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™ (R&D systems, Minneapolis, USA) for RANKL, human/mouse OPG PCR Primer Pair™ (R&D systems) for OPG, human/mouse MMP-2 PCR Primer Pair™ (R&D systems) for MMP-2. RT-PCR condition for RANKL was 94°C for 4 min followed by 35 cycles of 94°C for 45s/56.5°C for 45s/72°C for 45s, for OPG 94°C for 4 min followed by 30 cycles of 94°C for 45s/55.5°C for 45s/72°C for 45s and for MMP-2 94°C for 4 min followed by 33 cycles of 94°C for 30s/55°C for 30s/72°C for 30s, for PTHrP 94°C for 4
min followed by 20 cycles of 94 ℃ for 30s/55 ℃ for 30s/72 ℃ for 30s respectively. Rt-PCR products were electrophoresed on an 1.5% agarose gel and visualized by ethidium bromide staining under UV light.

**OPG protein measurement.**

After 5×10⁴ PC-3 cells were plated onto 6 wells plates, cells were treated with 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 manufacturer instruction.

**Western Blotting**

After 5×10⁵ PC-3 cells were plated, cells were treated with minodronate for 24 h. Then, cells were lysed with lysis buffer composed of 50 mM Tris-HCl, 150 mM Sodium chloride (NaCl) pH8.0, 0.1% Triton X-100, 0.01 mg/ml aprototinin 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 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 (Santa Cruz, California, USA), PARP (Santa Cruz) and rabbit
monoclonal antibody against bax (Santa Cruz), MMP-2 (Santa Cruz) and RANKL (Santa Cruz) and goat monoclonal antibody against PTHrP (Santa Cruz). Horse radish 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, 2 tailed t test, assuming unequal variance.
Results

Cell proliferation study.

To evaluate the effect of bisphosphonates on proliferation of prostate cancer, we examined the number of minodronate treated prostatic cancer cells. Exposure of 0-100 μM minodronate for 72 h significantly inhibited the cell proliferation of PC-3, DU145 and LNCaP in a dose dependent manner. Compared with incadronate which is ordinal third generation bisphosphonate, minodronate significantly showed more potent effect than incadronate in inhibition of growth of prostate cancer cells (Fig. 1A). To evaluate whether effect of minodronate is time dependent, we examined the number of minodronate treated prostatic cancer cells every 24 h. Exposure of 10 μM minodronate significantly inhibited proliferation of PC-3, DU145 and LNCaP cells in a time dependent manner, compared to control (Fig. 1B). IC50 value for incadronate in LNCaP, DU145 and PC-3 was 31.6 μM, 17.0 μM and 37.1 μM, whereas that for minodronate was 2.62 μM, 0.92 μM and 18.8 μM, respectively (Table.1).

Detection of apoptosis.

To investigate whether minodronate induce apoptosis in inhibition of cell growth of prostate cancer, we performed DNA fragmentation assay and TUNEL assay. 10-100 μM of minodronate exposure for 24 h induced DNA fragmentation in PC-3 cells in a dose dependent manner (Fig. 2A). In
TUNEL assay, apoptotic nuclei were observed in PC-3 cells with 10 μM of minodronate treatment (Fig.2B). In DU145 cells, the same phenomenon was observed (date not shown). These results suggest that minodronate directly caused apoptosis in PC-3 cells at the concentration of at least 10 μM of minodronate.

**Effects of bisphosphonate on expression of bcl-2 and bax.**

A number of genes and 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, whilst its homologue, bax, promotes apoptosis (18,19). In order to investigate mechanism of apoptosis by bisphosphonate in prostate cancer cells, expression of bcl-2 and bax were evaluated by western blotting. Decreased expression of bcl-2 was observed at the concentration of 30 μM of minodronate (Fig.3A). In contrast, there was no remarkable regulation in expression of Bax (Fig.3B). Yin et al. reported that the susceptibility of a cell to undergo apoptosis depends in part on the ratio of bcl-2 to bax (18). When bcl2/bax expression ratio was calculated from results of western blotting, minodronate treatment reduced the ratio in PC-3 and DU145 in a dose dependent manner (Fig.3C).

**Effects of minodronate on expression of caspase-3 and poly (ADP)-ribose polymerase (PARP).**
Caspase-3 is the most prevalent caspase and it is responsible for the majority of apoptotic effects. To investigate the effect of minodronate on caspase-3 activity, we assessed the activity of caspase-3 in PC-3 and DU145. Compared to control, caspase-3 activity was significantly increased with 30 μM of minodronate treatment for 24 h (Fig.4A). In contrast, treatment of minodronate with additional Z-VAD-FMK resulted in decreased caspase-3 activity in PC-3 and DU145, compared with treatment of minodronate alone. In addition, when minodronate was added with Z-VAD-FMK, the number of the survived cell was partially recovered in PC-3 and DU145 (Fig.4B). These results suggest that effect of minodronate on apoptosis induction is mainly caspase-dependent. Another key feature of apoptosis is proteolytic cleavage of PARP as a consequence of activation of caspases. It has been suggested that PARP inhibits DNA fragmentation through ribosylation of DNA. 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 (20). In order to determine the effect of minodronate on PARP activity, expression of full-length PARP in PC-3 and DU145 was examined by western blotting. Decreased expression of PARP was observed at the concentration of 30 μM of minodronate in both cell lines (Fig.5). This implies that decreased expression of PARP followed by caspase-3 activation cause DNA fragmentation.
Effect of minodronate on expression of growth factors and cytokines derived from prostatic cancer cells.

Various cancer cells secrete a number of growth factors and cytokines that stimulate osteoclast which cause bone resorption. It has been suggested that prostate cancer cells also release various kinds of growth factors and cytokine, which promote osteoclastogenesis in bone tissue (21). To determine the effect of bisphosphonate on secretion of promoter proteins for osteoclastogenesis, expression of RANKL and OPG was assessed by RT-PCR, ELISA or western blotting and expression of MMP-2 and PTHrP were examined by RT-PCR and western blotting.

In RT-PCR, addition of minodronate to PC-3 cells for 24 h decreased RANKL mRNA level in a dose dependent manner. In western blotting, expression of RANKL also decreased in a dose dependent manner (Fig.6A). In contrast, mRNA and protein expression of OPG was not regulated with minodronate treatment in PCR or ELISA (Fig.6B).

Next, we examined MMP-2 expression in minodronate treated PC-3. In RT-PCR, mRNA expression of MMP-2 decreased in a dose dependent manner with minodronate treatment. In addition, protein expression of MMP-2 also decreased in a dose dependent manner in minodronate treated PC-3 cells (Fig.6C).

Expression of PTHrP protein was also decreased in minodronate treated PC-3. However, mRNA expression of PTHrP was not altered with
minodronate treatment (Fig. 6D).
Discussion

It has been suggested that bisphosphonate indirectly inhibited growth of cancer cells by inhibition of bone absorption mediating through inducing apoptosis of osteoclast (15,22). However, there is increasing evidence that bisphosphonates may have direct inhibitory effects on cancer cells. In breast cancer, Senaratne et al. demonstrated that pamidronate inhibited cell proliferation of MDA-MB-231 by inducing proteolysis of PARP through bcl-2/bax ratio regulation (14). In prostate cancer, Lee et al. demonstrated that pamidronate inhibited cell proliferation of PC-3 and DU145 and LN-CaP in vitro (23). Moreover, Virtanen et al. reported that alendronate induced apoptosis and prevented invasion of prostate cancer cells by inhibiting mevalonate pathway (24). In the present study, minodronate, which is a new bisphosphonate of the third generation significantly, decreased the number of prostate cancer cell in dose and time dependent manner, showing the direct effect in LNCaP, DU145 and PC-3. This is the same result with the previously reported by others. In addition, minodronate treatment induced DNA fragmentation in a dose dependent manner, and TUNEL assay showed the appearance of apoptotic nucleus in PC-3 with minodronate treatment. These findings suggest that bisphosphonates have direct effect on prostate cancer cells by inducing apoptosis. In recent study, Oades et al. demonstrated that 10 μM of zoledronate directly induced apoptosis in prostatic cancer cell lines (25). As
compared with IC50 value of incadronate, which is the conventional bisphosphonate of third generation, the IC50 of minodronate was significantly lower than of incadronate in each prostate cancer cell lines and this suggests that minodronate had more potent effect than incadronate.

In the present study, we clearly demonstrated that minodronate decreased PARP activity following bcl-2/bax regulation and caspase-3 activation. These results suggest that direct effect of bisphosphonate on cancer cells might be caspase-3 dependent. However, in our study, addition of caspase inhibitor: Z-VAD-FMK to minodronate partially recovered cell survival rate compared with minodronate alone in PC-3 and DU145 cells. This finding raises possibility that other caspases like caspase-8 or -9 also might been involved in the apoptosis signaling pathway induced by bisphosphonate since these caspase-3 family members are substrates for upstream caspases. As another mechanism of apoptosis induction by bisphosphonate, a recent study has suggested that amino-bisphosphonate inhibit farnesyl pyrophosphate synthase in the cholesterol biosynthetic pathway in osteoclasts (10). In study of macrophage like cell line J774, Benford et al. demonstrated that amino-bisphosphonates blocks mevalonate pathway of J774 in inducing apoptosis (11). Mevalonate pathway is a part of cholesterol biosynthesis pathway, which mediates isoprenylation of small GTP binding proteins such as Rac, Rho, Rab and Ras family. Oades et al. demonstrated that zoledronic acid impair membrane localization of isoprenylated Ras also in apoptotic
prostate cancer cells (25). However, it still remains unclear whether inhibited isoprenylation of small GTP binding proteins can regulate the expression of bcl-2/bax or activation of caspases.

In the present study, we have found decreased expression of RANKL mRNA and protein but stable expression of OPG in PC-3 with minodronate treatment. This is the first report that demonstrated down-regulation of RANKL by bisphosphonate in prostate cancer although down-regulation of RANKL was shown in study of multiple myeloma (26). RANKL is a member of the TNF family, which is initially expressed by bone marrow stromal cells, osteoblasts and activated T cells (27). RANKL is mainly a membrane-anchored molecule. However, small fraction of RANKL is released through proteolytic cleavage 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 (28). Prostate cancer is known to express membrane anchored and soluble RANKL (21,29). In contrast, OPG acts as a decoy receptor against RANKL and expression of OPG by osteoblasts under hormone regulation is thought to inhibit osteoclastogenesis by binding the differentiating factor (30). 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 (31). In addition, it has been demonstrated that the induction of osteoclastogenesis is dependent
upon the relative abundance of RANKL compared with the level of OPG (32). Therefore, our data suggests that down regulation of RANKL with stable OPG levels from cancer cells with minodronate treatment might cause reduction of local osteoclast formation and activation in prostate cancer.

Various cancer cells secrete matrix metalloproteinases (MMPs) and MMPs is essential factor for invasion and metastasis in cancer cells. MMPs have an important role also in bone remodeling. In study of mouse calvaria, 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, suggesting that MMP-dependent degradation of bone matrix plays a key role in bone resorption (33). Prostate cancer secretes a large number of MMPs (34-38) and recently, Liao et al demonstrated that androgen stimulation increased expression of MMP-2 in prostate cancer (39). In the present study, we demonstrated that high concentration of minodronate inhibited mRNA and protein expression of MMP-2 in PC-3. In support for our observation, Stearns et al. demonstrated that decreased activity of MMP-2 and -9 in zymography of alendronate treated PC-3 injected in femur of SCID mice (40). These data suggest that the anti-bone resorptive effect of bisphosphonate in prostate cancer might be attributable for inhibiting MMP-2 secretion from prostate cancer cells by bisphosphonate, in part.

Another important osteoclastgenic factor is parathyroid hormone related protein (PTHrP), which increases bone resorption and renal tubular
resorption of calcium in solid tumors (41). Overexpression of PTHrP in prostate cancer cells has been shown to induce osteolytic lesions in the bone of rats (42). We demonstrated that high concentration of minodronate inhibited protein expression of PTHrP in PC-3 and this is the first report. 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 (43). 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.

In the present study, we demonstrated that bisphosphonate treatment could regulate expression of some osteoclastogenic proteins such as RANKL, MMPs, or PTHrP as well as apoptosis related proteins in prostatic cancer cell lines in vitro. However, the mechanism for the regulation of the expression of those osteoclastogenic factors by bisphosphonate is still unclear. In study of lovastatin which inhibits the synthesis of cholesterol and affects posttranslational modification or isoprenylation of several proteins including Ras in the signal transduction pathway, western blotting showed lovastatin-induced decrement of MMP-9 expression as well as membrane localization of Ras proteins in fibroblast (44). In another study of fibroblast, Aklilu et al. reported that inhibition of Ras decreased mRNA and protein expression of PTHrP (45). These results by others raises the
possibility that the inhibitory effect of bisphosphonate on isoprenylation of small GTP-binding protein such as Rac, Ras, Rho through mevalonate pathway might link to decreased production of some kinds of growth factors or cytokines in cancer cells. However, in our study, mRNA and protein expression of MMP-2 and RANKL was decreased while the mRNA expression of PTHrP was not altered in spite of decreased protein expression of PTHrP. In addition, OPG expression was stable with bisphosphonate treatment. These results imply that molecular action of bisphosphonate on the protein regulation in cancer cells involves multiple signaling pathways other than mevalonate pathway.

In study of other cell lines, Mackie et al. demonstrated down-regulation of osteopontin (OPN) mRNA as well as RANKL in multiple myeloma (26). OPN has been shown to mediate cell attachment and migration of malignant cells. Moreover, Viereck et al. demonstrated that bisphosphonate treatment increased OPG expression from osteoblasts in vitro (46). Taken together, bisphosphonates might have possibilities to influence on prostatic cancer cells and to alter secretion of various kinds of protein in vitro. It may be possible that the sum activity of bisphosphonate action in the bone microenvironment will reflect alterations of these interactions in a dynamic fashion. 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 (47).
The preclinical data including ours raises the intriguing possibility that the observed clinical utility of bisphosphonates in managing skeletal metastases may in part derive from direct inhibition of prostate cell growth and regulation of protein secretion from tumor cells in the bone microenvironment. Additional studies are required to determine if such effects of bisphosphonates are actually translated into the in vivo setting to functionally alter activity of tumor cells and osteoclasts in bone microenvironment.
Conclusions

We have demonstrated a direct inhibitory effect of minodronate on proliferation of prostatic cancer cell lines. Induction of apoptosis by minodronate is caspase-3 dependent and activation of caspase-3 cascades including PARP is a main stream to cause DNA fragmentation by minodronate. Furthermore we have demonstrated an alteration in mRNA and protein expression of RANKL and MMP-2 and protein expression of PTHrP. Thus local decrease of such growth factor or cytokines levels in bone microenviroment may be an important components of the paracrine or autocrine mechanism by which bisphosphonate reduce bone resorption and clinical skeletal events in prostate cancer.
FIG. 1. Effects of Minodronate on PC-3, DU145 and LN-CaP.

Cells were plated at a density of $5 \times 10^5$ cells in 6 well plates with DMEM or RPMI medium containing 10% FBS and treated with treated with 0-100 μM of minodronate or incadronate for 72 hours (A) or 10 μM of minodronate for 0-72 hours (B). With minodronate treatment, the number of cells was decreased in a time and dose dependent manner. Error bars indicate mean ± 1 SD. Single asterisk indicates p<0.05.

FIG.2. Detection of apoptosis.

With minodronate treatment in PC-3, DNA fragmentation was detected in a dose dependent manner (A). Apoptotic nuclei was seen in minodronate treated PC-3 in TUNEL staining, magnification x200 (B).

FIG. 3. Expression of bcl-2 and bax in Minodronate-treated prostate cancer cell lines.
The expression of bcl-2 was decreased in 30 μM of minodronate but expression of bax was not regulated with minodronate treatment in DU145 and PC-3 (A). Corresponding densitometric analysis of bcl-2 and bax level expression was shown as bcl2/bax ratio according to dose of minodronate (B).

**FIG. 4. Measurement of Caspase-3 activity.**

PC-3 and DU145 cells were treated for 24 hours with 0 or 30-μM minodronate with or without 20 μM of Z-VAD-FMK before incubating with caspase-3 substrate. Caspase-3 activity was increased with 30 μM of minodronate (A). Cytotoxic effect of minodronate was partially inhibited with Z-VAD-FMK treatment. Error bars indicate mean ± 1 SD.

**FIG. 5. Expression of PARP (Poli-ADP ribose polymerase) in minodronate-treated prostate cancer cell lines.**

The expression of full length PARP was decreased in 30 μM of minodronate in DU145 and PC-3.

**FIG. 6. Expression of proteins and cytokines in minodronate treated PC-3.**
PC-3 cells were treated with 0-30 μM minodronate for 24 hours prior to RT-PCR and western blotting or ELISA. Equal amounts of protein were loaded per lane for western blotting.

(A) Expression of RANKL was assessed by PCR and western blotting.

(B) Expression of OPG was assessed by PCR and ELISA.

(C) Expression of MMP-2 was assessed by PCR and western blotting.

(D) Expression of PTHrP was assessed by western blotting.
References


