EXPERIMENTAL and MOLECULAR MEDICINE, Vol. 43, No. 3, 138-145, March 2011

Bisphosphonate enhances TRAIL sensitivity to human osteosarcoma cells *via* death receptor 5 upregulation

Myung-Hee Moon[1], Jae-Kyo Jeong[1], Jae-Suk Seo[1], Jae-Won Seol[1], You-Jin Lee[1], Meilang Xue[2], Christopher J. Jackson[2] and Sang-Youel Park[1,3]

[1]Center for Healthcare Technology Development
Bio-Safety Research Institute
College of Veterinary Medicine
Chonbuk National University
Jeonju 561-756, Korea
[2]Sutton Arthritis Research Laboratories
Institute of Bone and Joint Research
Kolling Institute
University of Sydney at Royal North Shore Hospital
St. Leonards, New South Wales, Australia
[3]Corresponding author: Tel, 82-63-270-3886;
Fax, 82-63-270-3780; E-mail, sypark@chonbuk.ac.kr
DOI 10.3858/emm.2011.43.3.016

Accepted 3 January 2011
Available Online 7 February 2011

Abbreviations: BP, bisphosphonate; DR5, death receptor 5; OS, osteosarcoma; TRAIL, tumor necrosis factor factor-related apoptosis-inducing ligand

Abstract

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), a member of the TNF superfamily of cytokines, is one of the most promising candidates for cancer therapeutics. However, many osteosarcomas are resistant to TRAIL. Bisphosphonates are very effective in the treatment of bone problems associated with malignancies; the antitumor effects are due to the inhibition of protein prenylation that is essential for cell function and survival. The purpose of this study was to determine the effects of bisphosphonates on TRAIL-resistant MG 63 human osteosarcoma cells. The cells showed no response to TRAIL alone; however, pre-treatment with bisphosphonates significantly increased TRAIL-mediated apoptosis and cellular activation of caspase-3. Bisphosphonates significantly induced mRNA and protein expression of the TRAIL receptor, DR5. Bisphosphonates induced protein unprenylation in MG 63 cells; in addition, co-treatment with TRAIL also significantly increased protein unprenylation. Blocking of protein unprenylation using geranylgeraniol attenuated the cellular responses, including cell apoptosis and protein unprenylation induced by bisphosphonates and TRAIL. This is the first study to demonstrate that bisphosphonates markedly enhanced TRAIL-induced apoptosis in human osteosarcoma cells. These findings suggest that bisphosphonates may be a new and effective anticancer treatment with TRAIL proteins for TRAIL-resistant cancer cells.

Keywords: apoptosis; diphosphonates; protein prenylation; receptors, TNF-related apoptosis-inducing ligand; TNF-Related apoptosis-inducing ligand

Introduction

Osteosarcoma (OS) is the most common malignant bone tumor in both children and adolescents (Lee *et al.*, 2007). The estimated incidence of OS is 4-5 per million in the population (Hori *et al.*, 2010). OS is characterized by the proliferation of neoplastic cells directly from bone or osteoid tissue (Cabral *et al.*, 2009). For the 40% of patients that develop disease progression of OS, after first line therapy, further treatment with additional chemotherapy is palliative and toxic (Dean *et al.*, 2005). The major problems associated with chemotherapy are present includeing the cytotoxic effects of chemotherapy on normal tissues and organs; in addition, there is frequent development of drug-resistant phenotypes. Both are significant drawbacks to the effectiveness of chemotherapy (Chou and Gorlick, 2006). Thus, novel, safe and more effective anti-cancer treatments are needed for patients with chemotherapy- resistant OS.

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand or Apo2 ligand (TRAIL/Apo2L) is a member of the TNF superfamily of cytokines (Shin *et al.*, 2002). TRAIL preferentially induces apoptosis in cancer cells, both *in vitro* and *in vivo*, while exhibiting little or no toxicity in normal cells and tissues (Mahalingam *et al.*, 2009). TRAIL induces apoptosis upon binding to its death domain-containing receptors, TRAIL receptor 1 (DR4) and TRAIL receptor 2 (DR5). Upon binding to DR4 or DR5, TRAIL induces receptor trimerization to form a structure known as the death-inducing signaling complex (DISC), which activates caspase-8, leading directly to the activation of caspase-3 and subse-

quent apoptosis (Mahalingam *et al.*, 2009; Hori *et al.*, 2010). TRAIL sensitivity is also regulated by antiapoptotic genes, such as the cellular FLICE-like inhibitory protein (c-FLIP) and inhibitor of apoptosis (IAP). c-FLIP prevents apoptosis by blocking the association of caspase-8 with DISC. IAP can bind and inactivate caspases-3, -7, and -9 (Mahalingam *et al.*, 2009; Hori *et al.*, 2010).

Most human cancer cell types that have been tested are sensitive to the apoptotic effects of TRAIL. However, many OS cells respond poorly to the cytotoxic effects of TRAIL alone; induction of apoptosis requires additional treatment with other chemotherapeutic agents that damage normal cells and tissues (Evdokiou *et al.*, 2002). Thus, safe and more effective adjuvant treatments for OS other than DNA-damaging agents are needed.

Bisphosphonates (BPs) suppress osteoclastic bone resorption and are currently the most important class of antiresorptive drugs used for the treatment of metabolic bone diseases; they are frequently used in oncology to treat bone complications of malignancies (Roux and Dougados, 1999; Kavanagh *et al.*, 2006). The nitrogen-containing bisphosphonates (N-BPs) are very effective for the treatment of bone problems associated with malignancy, including hypercalcemia and/or increased bone destruction (Ross *et al.*, 2004; Russell *et al.*, 2008). N-BPs reduce the survival, proliferation, adhesion, migration, and invasion of tumor cells *in vitro* (Clezardin *et al.*, 2005). Most, if not all, of these antitumor effects of N-BPs *in vitro* are due to inhibition of farnesyl diphosphate synthase (FPPS) in the 3-Hydroxy-3-methylglutaryl coenzyme A) (HMG-CoA) reductase pathway; this is because the effects of N-BPs can be largely overcome by replenishing cells with isoprenoid substrates (farnesol or geranylgeraniol) essential for connecting some small proteins to the cell membrane. The addition of either of these two metabolites to proteins is known as prenylation, which is important for the proper sub-cellular protein trafficking as well as cell survival and function (Coxon *et al.*, 2004). However, potential enhancement of the effects of the apoptotic pathway, by co-treatment with N-BPs and TRAIL, and determination of the effects on the death receptor pathway with N-BPs, has not yet been studied.

The demonstration that treatment with N-BPs enhances TRAIL-induced apoptosis of cancer cells prompted us to postulate that enhanced DR5 expression and inhibition of protein prenylation, by treatment with N-BPs followed by TRAIL, may be an ideal treatment approach for improved killing of cells without adversely affecting normal tissues in patients with OS. This study was undertaken to examine the combined effects of N-BPs and TRAIL treatment on apoptosis, and to elucidate the molecular mechanisms of apoptosis in human OS cell lines.

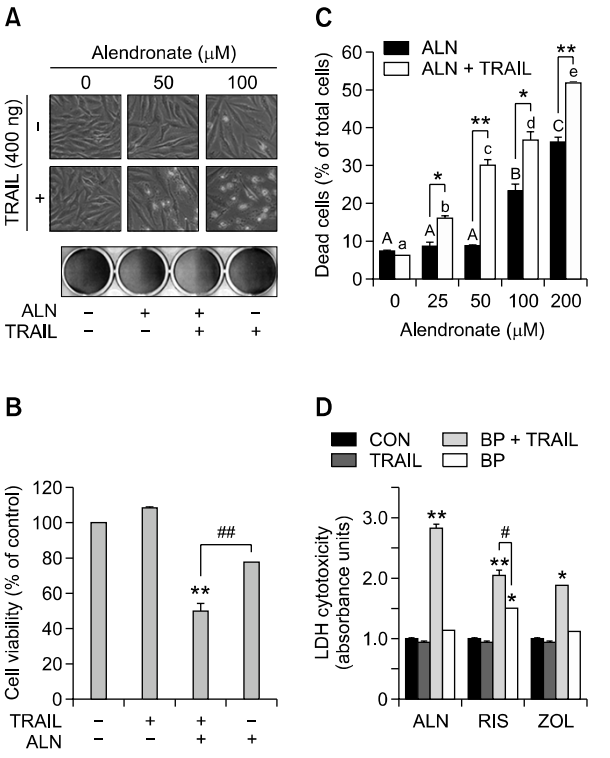

Figure 1. N-BPs sensitize TRAIL-induced cytotoxicity of TRAIL-resistant MG 63 resistant cells. Alendronate significantly increased TRAIL-induced MG 63 cell death as assessed by examinations of cell morphology (A, ×200 magnification) and cell number (B, as determined using crystal violet staining). MG 63 cells were treated with alendronate at the indicated concentrations (A, C) or with 50 μM for 24 h (B, D) and/or with 400 ng/ml of TRAIL for an additional 12 h. Before adding 1% sodium dodecyl sulfate into sample-containing wells, the result of crystal violet staining of each well was monitored (B). Viability of control cells was set at 100%, and viability relative to the control is presented (B). (C) Alendronate dose-dependently enhanced TRAIL-induced MG 63 cell death. Cell death was assessed by the trypan blue exclusion method and is expressed as the percentage ratio of dead cells to live cells. (D) Various kinds of N-BPs had a similar effect of increasing TRAIL-induced cytotoxicity of MG 63. Alendronate was the most effective among the tested N-BPs. Cytotoxicity was calculated by measuring the amount of LDH released from the cytosol of N-BPs damaged cells. The data were analyzed using analysis of variance (ANOVA) and Duncan multiple range test ($P < 0.05$). *Indicates a significant difference from control (*$P < 0.05$, **$P < 0.01$). The experiments were repeatedly performed to confirm the results. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$.

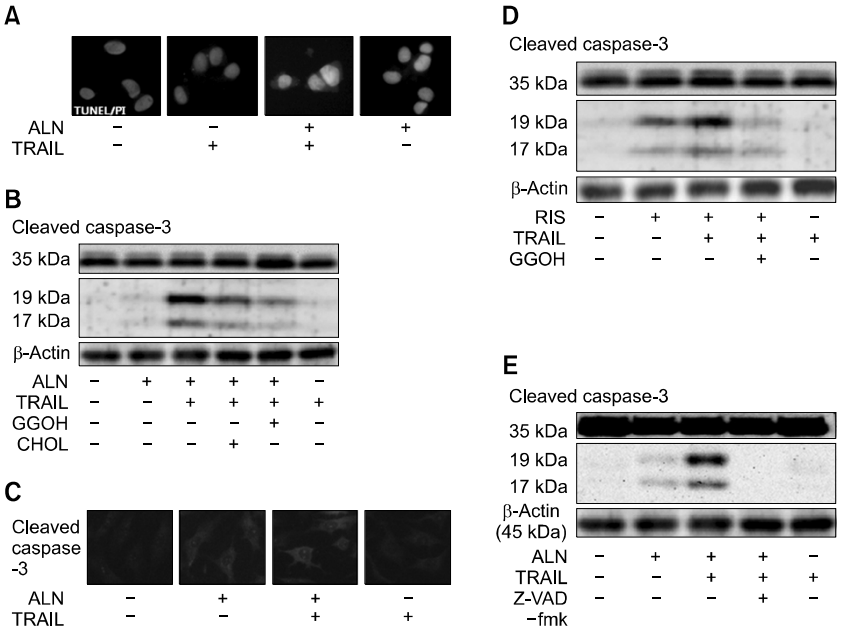

Figure 2. N-BPs enhance TRAIL-induced apoptosis of MG 63 cells. (A) MG 63 cells were co-treated with 50 μM alendronate for 24 h with/without 400 ng/ml TRAIL for an additional 12 h. Following treatment, cells were fixed and immunostained with anti-BrdU antibody to observe the extent of DNA damage and counterstained with propidium iodide. The level of staining indicated the degree of DNA damage induced by treatment, where more positively stained cells were in the final stages of apoptosis. The magnification is ×200. (B-E) MG 63 cells were pretreated with 50 μM alendronate (B) or 100 μM risedronate (D) 10 μM GGOH and/or cholesterol and/or 100 μM Z-VAD-fmk (E) for 24 h with/without 400 ng/ml of TRAIL for an additional 4 h. Cell lysates were analyzed by immunoblotting using antibodies for cleaved caspase-3. (C) MG 63 cells were treated as described in Figure 2A and the cells were fixed and stained with cleaved caspase-3 and Alexa flour 546-conjugated secondary antibody, followed by fluorescence microscope analysis.

Results

Combined treatment with N-BPs and TRAIL significantly induced cell death in TRAIL-resistant OS cells

MG 63 cells were tested for their susceptibility to N-BPs and/or TRAIL as assessed by cell morphology using light microscopy, crystal violet staining, trypan blue exclusion and a lactate dehydrogenase cytotoxicity assay. Only TRAIL alone had no cytotoxic effect on the MG 63 cells (Figures 1A-1D). The N-BPs were only weakly cytotoxic (Figures 1A-1D). Separate treatments with N-BPs or TRAIL induced cell death in only 8% and 6% of MG 63 cell populations, respectively. However, their combined use markedly enhanced MG 63 cell death (46%) (Figures 1A-1D). In addition, risedronate and zoledronate enhanced TRAIL-induced cytotoxicity of the TRAIL-resistant MG 63 cells. Of the three tested compounds, alendronate most effectively increased the cytotoxic effect of TRAIL (Figure 1D). These results provide evidence that combined treatment with N-BPs and TRAIL damages OS cells.

Treatment with N-BPs significantly sensitizes OS cells to TRAIL-induced apoptosis

To explore whether N-BPs could enhance TRAIL-induced apoptosis in MG 63 OS cells, the cells were pretreated with 50 μM alendronate for 24 h, and then exposed to 400 ng/mL TRAIL for 12 h. The apoptotic index was assessed by a TUNEL assay and detection of the active form of caspase-3. Few TUNEL-positive cells were evident in MG 63 OS cells treated with either TRAIL or N-BPs (Figure 2A). However, after combination treatment with N-BPs and TRAIL, the number of TUNEL-positive cells increased (Figure 2A). Next, Western blot examination and immunofluorescence staining were used to confirm that N-BPs markedly sensitized MG 63 cells to caspase-3 activation in response to TRAIL. Treatment with each drug alone scarcely activated caspase-3, but combination treatment significantly activated caspase-3 (Figures 2B-2D). These results indicate that, although the separate treatment with N-BPs or TRAIL had little effect on MG 63 cell apoptosis, the two combined were highly effective in causing OS cell apoptosis. These results provide evidence that N-BPs can

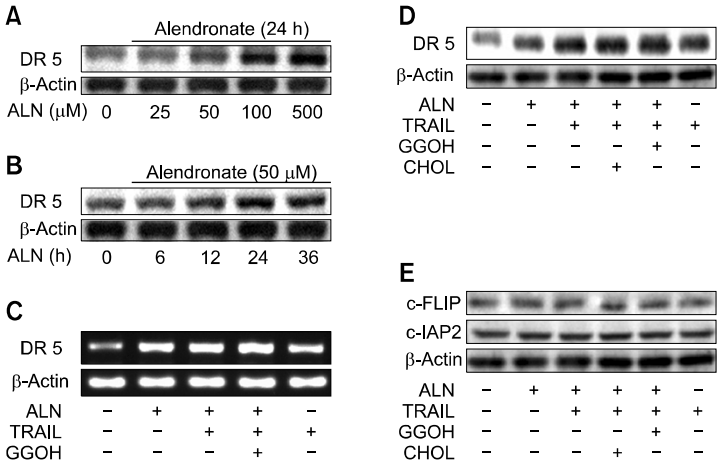

Figure 3. N-BPs increased the expression of DR5 in MG 63 cells and did not affect expression levels of c-IAP2 and c-FLIP. (A, B) MG 63 cells were treated with 25-500 μM alendronate for 24 h (A) or with 50 μM alendronate for 6-36 h (B), followed by Western blotting using antibodies specific for DR5. (C) MG 63 cells were treated as described in Fig. 2B and DR5 mRNA expression was analysed by RT-PCR using DR5 primers. (D) MG 63 cells were treated as described in Fig. 2B and cell lysates were analyzed by Western blotting using antibodies for DR5. (E) MG 63 cells were co-treated with 50 μM alendronate, 10 μM GGOH and/or cholesterol for 24 h with/without 400 ng/mL TRAIL for an additional 4 h. Following treatment, MG 63 cell lysates were collected and immunoblotted with antibodies specific for c-IAP2, c-FLIP and β-actin.

enhance TRAIL-induced apoptosis.

N-BPs up-regulate DR5 proteins in a dose- and time-dependent manner

To determine how N-BPs potentiate TRAIL-induced apoptosis, an experiment investigated the influence of N-BPs on the TRAIL receptor DR5. MG 63 cells were treated with various concentrations of alendronate [(0-500 μM) for 24 hours], or with 50 μM alendronate for various times (0-36 hours). Alendronate increased DR5 protein expression in the MG 63 cells in a dose-dependent manner (Figure 3A) and a time-dependent manner (Figure 3B). In addition, to investigate whether induction of DR5 by N-BPs occurred at the transcriptional level, DR5 mRNA expression was ascertained after the cells were treated with alendronate. Alendronate induced the appearance of the DR5 transcript (Figure 3C), suggesting action at the transcriptional level. In cells treated with alendronate, the expression of the DR5 protein was increased by about two fold compared to the control cells, and co-treatment with alendronate and TRAIL significantly increased the DR5 protein expression (Figure 3D). Treatment with geranylgeraniol (GGOH), which blocks N-BPs-induced protein unprenylation, did not affect the increment of DR5 protein induced by alendronate and TRAIL treatment (Figure 3D). These results were consistent with the suggestion that protein unprenylation does not affect DR5 protein expression. The expression level of c-FLIP and cIAP2 proteins, involved in apoptosis inhibition, showed no change in cells treated with alendronate and/or TRAIL (Figure 3E). These results indicated that N-BPs up-regulated the expression of DR5 protein and suggest that N-BPs may enhance TRAIL-induced MG 63 cell death.

Inhibition of protein prenylation contributes to enhancement of TRAIL-induced apoptosis

To determine the mechanisms underlying sensitization of TRAIL-induced apoptosis by N-BPs, an experiment to measure Rap 1A, a geranylgeranylation marker that binds to the non-geranylgeranylated form, was performed. As expected, 50 μM alendronate inhibited geranylgeranylation and the combination of alendronate and TRAIL increased the unprenylated form of Rap 1A protein compared to treatment with alendronate alone (Figure 4A). A similar result was observed with treatment of 100 μM risedronate (Figure 4B). To investigate whether the inhibition of protein prenylation affected cell survival, MG 63 cell viability was measured following exposure to 50 μM alendronate and/or 400 ng/ml TRAIL and/or 10 μM GGOH. Inhibition of protein prenylation by N-BPs and TRAIL was reversed by GGOH, and cell viability was recovered (Figures 4A-4C), indicating that the inhibition of protein

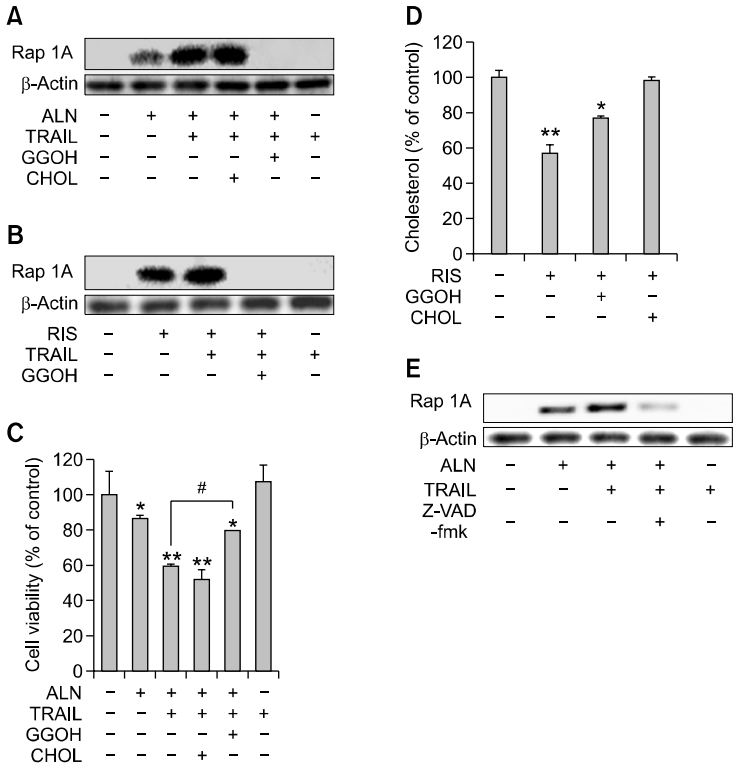

Figure 4. Co-treatment of N-BPs and TRAIL affects prenylation in MG 63 cells. (A, B, E) MG 63 cells were pretreated with 50 μM alendronate (A) or 100 μM risedronate (B) 10 μM GGOH and/or cholesterol and/or 100 μM Z-VAD-fmk (E) for 24 h with/without 400 ng/ml of TRAIL for an additional 4 h. Following treatment, MG 63 cell lysates were collected and immunoblotted with antibodies specific for unprenylated Rap 1A and β-actin. (C) MG 63 cells were pretreated with 50 μM alendronate, 10 μM GGOH and/or cholesterol for 24 h with/without 400 ng/ml TRAIL for an additional 24 h. Cell viability was determined by crystal violet staining. Viablility of control cells was set at 100%, and viability relative to the control is presented. (D) MG 63 cells were treated as described in Figure 2D and total cholesterol levels were measured and normalized to protein concentration. *Indicates a significant difference from control (*$P < 0.05$, **$P < 0.01$). $^{\#}P < 0.05$. The experiments were repeatedly performed to confirm the results.

prenylation by N-BPs contributes to N-BPs-mediated enhancement of TRAIL-induced apoptosis.

N-BPs can block the enzyme FPPS in the HMG-CoA reductase pathway, which is related to cholesterol synthesis (Coxon *et al.*, 2000). Therefore, an experiment to assess the differences in cholesterol levels between N-BPs and/or TRAIL treatment alone, and the addition of cholesterol, was carried out. In addition, whether the different amounts of cholesterol affected N-BPs-mediated TRAIL-induced apoptosis was examined. Treatment with risedronate decreased the cholesterol levels; the levels were recovered by the addition of cholesterol (Figure 4D). However, the cholesterol recovery did not reverse the N-BPs-mediated and TRAIL-induced death of MG 63 cells. These results suggest that N-BPs sensitization to TRAIL-induced apoptosis does not appear to be related to the inhibition of cholesterol synthesis.

Discussion

The main goal of this study was to demonstrate apoptosis of TRAIL-resistant cancer cells using N-BPs. The results show that resistance of OS cells to TRAIL can be overcome by the use of N-BPs; this finding supports the potential use of N-BPs for therapeutic intervention strategies in conditions that have been very difficult to treat. Despite recent progress in surgery and chemotherapy, the outcome of patients with OS remains poor (Lamoureux *et al.*, 2009). TRAIL offers great promise for the pharmacological treatment of cancer. However, many OS cells acquire resistance to TRAIL alone and induction of apoptosis requires additional treatment with other chemotherapeutic agents (Chou and Gorlick, 2006).

The beneficial effects of BPs on bone metastasis of different solid tumors have been demon-

strated (Coleman, 2005). Therefore, N-BPs are currently used as adjuvants and sensitizing agents for TRAIL-induced apoptosis. The results of this study showed that MG-63 OS cells were resistant to the apoptotic effect of TRAIL (Figures 1 and 2). However, the MG-63 cells underwent apoptosis after pretreatment with N-BPs (Figures 1 and 2). Treatment with N-BPs alone failed to cause apoptosis of the MG 63 cells; however, co-treatment with TRAIL rapidly increased MG 63 cell apoptosis (Figures 1 and 2).

Adjuvant agents that lower resistance of cancer cells to TRAIL alter the expression of apoptosis inhibitors such as c-FLIP and IAP family members (Mirandola *et al*., 2006). In this study, the results showed that treatment with N-BPs did not change the expression of c-FLIP and cIAP2 proteins; the findings indicated that treatment with N-BPs do not appear to be able to regulate anti-apoptotic signals; however, they appear to be able to regulate pro-apoptotic signals (Figure 3). The pro-apoptotic signals of TRAIL-related molecular mechanisms were investigated by the expression of DR5. The N-BPs increased DR5 protein expression in a dose- and time-dependent manner (Figure 3); these results provide strong evidence that up-regulation of DR5 can render OS cells sensitive to TRAIL-induced apoptosis. The expression of death receptors has been correlated to the TRAIL response of various tumor cells (Locklin *et al*., 2007). These results indicate that N-BPs-mediated enhancement of TRAIL-induced apoptosis was due to the up-regulation of DR5 expression.

However, the results that only TRAIL treatment increased DR5 protein expression without affecting cell viability and that the addition of GGOH did not affect DR5 expression cloud the issue of how N-BPs can overcome TRAIL resistance. The anti-tumor action of N-BPs in a variety of human cancer cells is due to the inhibition of protein prenylation (Coxon *et al*., 2000). This point was the focus of this study; the unprenylated form of Rap1A proteins in OS cells treated with N-BPs, TRAIL and/or GGOH were examined. N-BPs increased protein unprenylation as expected. The protein prenylation inhibition effect of N-BPs could be completely overcome by adding GGOH, required for protein prenylation (Figure 4). These results demonstrate that blocking protein prenylation caused cell apoptosis. Use of the pancaspase blocker z-vad-fmk partially blocked the unprenylation effect of N-BPs (Figure 4E), suggesting that prenylation is dependent on the activation of caspase-3. This is contrary to the widely held idea that inhibition of protein prenylation causes activation of caspase-3, thereby leading to cell apoptosis. These results suggest that protein prenylation and the activation of caspase-3 interact with each other.

In summary, the results of the present study indicate that N-BPs induce DR5 expression and protein unprenylation, which contributes to circumvented resistance of OS cells to TRAIL-induced apoptosis and enhanced TRAIL-induced apoptosis. This is the first study to identify an effective anti-cancer action of the combined treatment of N-BPs and TRAIL for TRAIL-resistant OS cells. These results support the therapeutic potential of N-BPs as an effective adjuvant for TRAIL-related anti-cancer drug therapy in patients with OS.

Methods

Materials

Human MG 63 osteoblast-like osteosarcoma cells were grown in DMEM with 10% fetal bovine serum to 70% confluence. Cells were treated for 24 or 48 hours with BPs at concentrations ranging from 10^{-3} to 10^{-5} M. Before treatment, cells were preincubated for 2 hours in DMEM with 1% FBS (basal medium). Cells were treated with alendronate sodium trihydrate (Sigma), risedronate sodium (Actonel®; Procter and Gamble Pharmaceuticals) and zoledronic acid (Zometa®; Norvartis).

Cell viability test

Direct microscopic observation. The hallmarks of cell degeneration were assessed by morphological criteria. After the treatment, the cells were photographed with a light microscope (ECLIPSE TS100; Nikon, Japan).

Crystal violet staining. The cells were incubated with N-BPs for additional experimental hours. Cell viability was determined by the crystal violet staining method as previously described (Seol *et al*., 2007).

Trypan blue exclusion test. Cells were incubated with N-BPs for additional experimental hours. The cell viability was determined by trypan blue exclusion method as described (Patterson, 1979; Brining, 1997).

LDH cytotoxicity test. Lactate dehydrogenase (LDH) activity in the previously frozen supernatants was measured using a cytotoxicity detection kit (Takara Bio Inc., Shiga, Japan), according to the manufacturer's instructions.

TUNEL assay

TUNEL analysis was performed to measure the degree of cellular apoptosis using an *in situ* ApoBrdU DNA fragmentation assay kit (BioVision, CA), following the manufacturer's instructions. Cells were fixed by suspending them in 70% (v/v) ethanol. The sample was then incubated with DNA-labeling solution for 1 h at 25°C. Each sample was then exposed to an antibody solution and allowed to react for 20 min; pictures were taken at 20× objective using a fluorescent microscope (Nikon ECLIPSE 80i, Nikon Corporation).

Western blotting

MG 63 cell was lysed in a lysis buffer (25 mM HEPES; pH 7.4, 100 mM NaCl, 1 mM EDTA, 5 mM $MgCl_2$, 0.1 mM DTT, and protease inhibitor mixture). Proteins were electrophoretically resolved on an 8-15% SDS (sodium dodecyl sulfate) gel, and immunoblotting was performed as previously described (Seol *et al*., 2007). The antibodies used for immunoblotting were Rap 1A, caspase-3, DR 5, phospho-p38 and β-actin (Santa Cruz, CA).

Immunofluorescent staining

MG 63 cells cultured on glass slides were fixed with cold acetone and blocked by 5% FBS in TBST and incubated with rabbit active caspase-3 antibody (R&D systems) overnight at 4°C. After washing with TBST, cells were incubated with anti-rabbit IgG conjugated with Alexa Fluor® 546 (red). Cells were washed with TBST, mounted with florescence mounting medium (Dako) and observed under a fluorescence microscope (Nikon ECLIPSE 80i, Nikon Corporation).

Statistical evaluation

All data are expressed as means ± standard deviation (SD), and mean's compared using Student's *t*-test and the ANOVA Duncan test with the SAS statistical package. The results were considered significant for values of $*P < 0.05$, $**P < 0.01$.

Acknowledgements

This work was supported by Regional Research Universities Program and the Center for Healthcare Technology Development and the National Research Foundation of the Korea Grant funded by the Korean Government (2010-E00019)

References

Brining SK. Predicting the *in vitro* toxicity of synthetic beta-amyloid (1-40). Neurobiol Aging 1997;18:581-9

Cabral LA, Werkman C, Brandao AA, Almeida JD. Imprint cytology of osteosarcoma of the jaw: a case report. J Med Case Reports 2009;3:9327

Chou AJ, Gorlick R. Chemotherapy resistance in osteosarcoma: current challenges and future directions. Expert Rev Anticancer Ther 2006;6:1075-85

Clezardin P, Ebetino FH, Fournier PG. Bisphosphonates and cancer-induced bone disease: beyond their antiresorptive activity. Cancer Res 2005;65:4971-4

Coleman RE. Bisphosphonates in breast cancer. Ann Oncol 2005;16:687-95

Coxon FP, Helfrich MH, Van't Hof R, Sebti S, Ralston SH, Hamilton A, Rogers MJ. Protein geranylgeranylation is required for osteoclast formation, function, and survival: inhibition by bisphosphonates and GGTI-298. J Bone Miner Res 2000;15:1467-76

Coxon JP, Oades GM, Kirby RS, Colston KW. Zoledronic acid induces apoptosis and inhibits adhesion to mineralized matrix in prostate cancer cells via inhibition of protein prenylation. BJU Int 2004;94:164-70

Dean M, Fojo T, Bates S. Tumour stem cells and drug resistance. Nat Rev Cancer 2005;5:275-84

Evdokiou A, Bouralexis S, Atkins GJ, Chai F, Hay S, Clayer M, Findlay DM. Chemotherapeutic agents sensitize osteogenic sarcoma cells, but not normal human bone cells, to Apo2L/TRAIL-induced apoptosis. Int J Cancer 2002;99: 491-504

Hori T, Kondo T, Kanamori M, Tabuchi Y, Ogawa R, Zhao QL, Ahmed K, Yasuda T, Seki S, Suzuki K, Kimura T. Ionizing radiation enhances tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis through up-regulations of death receptor 4 (DR4) and death receptor 5 (DR5) in human osteosarcoma cells. J Orthop Res 2010;28: 739-45

Kavanagh K, Guo K, Dunford J, Wu X, Knapp S, Ebetino F, Rogers M, Russell R, Oppermann U. The molecular mechanism of nitrogen-containing bisphosphonates as anti-osteoporosis drugs. Proc Natl Acad Sci USA 2006;103:7829

Lamoureux F, Picarda G, Garrigue-Antar L, Baud'huin M, Trichet V, Vidal A, Miot-Noirault E, Pitard B, Heymann D, Redini F. Glycosaminoglycans as potential regulators of osteoprotegerin therapeutic activity in osteosarcoma. Cancer Res 2009;69:526-36

Lee EJ, Choi EM, Kim SR, Park JH, Kim H, Ha KS, Kim YM, Kim SS, Choe M, Kim JI, Han JA. Cyclooxygenase-2 promotes cell proliferation, migration and invasion in U2OS human osteosarcoma cells. Exp Mol Med 2007;39:469-76

Locklin RM, Federici E, Espina B, Hulley PA, Russell RG, Edwards CM. Selective targeting of death receptor 5 circumvents resistance of MG-63 osteosarcoma cells to TRAIL-induced apoptosis. Mol Cancer Ther 2007;6:3219-28

Mahalingam D, Szegezdi E, Keane M, Jong S, Samali A. TRAIL receptor signalling and modulation: Are we on the right TRAIL? Cancer Treat Rev 2009;35:280-8

Mirandola P, Sponzilli I, Gobbi G, Marmiroli S, Rinaldi L, Binazzi R, Piccari GG, Ramazzotti G, Gaboardi GC, Cocco L, Vitale M. Anticancer agents sensitize osteosarcoma cells to TNF-related apoptosis-inducing ligand downmodulating IAP family proteins. Int J Oncol 2006;28:127-33

Patterson MK Jr. Measurement of growth and viability of cells in culture. Methods Enzymol 1979;58:141-52

Ross JR, Saunders Y, Edmonds PM, Patel S, Wonderling D, Normand C, Broadley K. A systematic review of the role of bisphosphonates in metastatic disease. Health Technol Assess 2004;8:1-176

Roux C, Dougados M. Treatment of Patients with Pagets Disease of Bone. Drugs 1999;58:823-30

Russell RG, Watts NB, Ebetino FH, Rogers MJ. Mechanisms of action of bisphosphonates: similarities and differences and their potential influence on clinical efficacy. Osteoporos Int 2008;19:733-59

Seol JW, Chaudhari AA, Lee YJ, Kang HS, Kim IS, Kim NS, Park JH, Kim TH, Seol DW, Park SY. Regulation of DR-5 protein and mitochondrial transmembrane potential by gemcitabine, a possible mechanism of gemcitabine-enhanced TRAIL-induced apoptosis. Oncol Rep 2007;18: 523-9

Shin EC, Seong YR, Kim CH, Kim H, Ahn YS, Kim K, Kim SJ, Hong SS, Park JH. Human hepatocellular carcinoma cells resist to TRAIL-induced apoptosis, and the resistance is abolished by cisplatin. Exp Mol Med 2002;34:114-22