Development of a novel poly bisphosphonate conjugate for treatment of skeletal metastasis and osteoporosis

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Abstract. Advanced stage prostate and breast cancer frequently metastasize to the skeleton (~75%). An additional complication in these patients, that further affects the bones, is that their hormonal treatment, induces osteoporosis. Bisphosphonates (bpns) are standard drugs against osteoporosis and have been shown to have clinically significant anti-tumor effects. This study describes the development of a new polybisphosphonate conjugate (ODX) with enhanced dual efficacy i.e. with anti-bone resorption and anti-tumor properties. Zoledronic acid (Zometa®) was used as a positive control (at equimolar concentrations). Alendronic acid and aminoguanidine were conjugated to oxidized dextran with subsequent reductive amination (on average ~8 alendronate and ~50 guanidine moieties per conjugate). ODX was tested in a bone resorption assay for its capacity to inhibit bone resorbing osteoclasts (bone organ culture from neonatal mice, ^{45}Ca labelled bone mineral). Tumor cell toxicity was studied on prostate (PC3) and breast cancer (MDA231, MDA453) cell cultures. Two methods were employed, a fluorescent cytotoxicity assay (FMCA) and an apoptosis assay (Annexin V assay). In the bone resorption assay, Zometa and ODX showed very similar potency with 50% osteoclast inhibition at ~20 nM and 100% at 0.2 μM. In the FMCA, IC_{50} for ODX was at ~2 μM and 25 μM for Zometa (PC3). In the apoptosis assay, ODX induced ~85-97% apoptosis at 10 μM in both cell lines, while Zometa failed to induce any significant apoptosis in any of the cell lines at the tested concentration range (10 nM-10 μM). ODX appears to be a promising drug candidate with high dual efficacy for the treatment of bone metastasis and osteoporosis. It has both potent osteoclast inhibiting properties and enhanced anti-tumor efficacy.

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Introduction

Advanced stage prostate and breast cancer preferentially develop metastasis to the skeleton. At Post mortem examination, >70% have bone metastasis. The bone microenvironment appears especially favourable for metastatic growth depending on several characteristics, e.g. locally rich vasculature with high permeability surrounding the bone marrow and a constant remodelling of bone. The bone turnover, involving bone cells (osteoclasts and osteoblasts), depends on the release of numerous cytokines, chemokines and growth factors that are favourable for tumor cells. The Paget's seed and soil hypothesis, stating that circulating cancer cells can only grow where the microenvironment is permissive for growth, remains relevant (1,2). The bone metastasis from prostate cancer are mostly osteosclerotic while those from breast cancer are predominantly osteolytic, however, the consequences are the same with severe pain, fractures, nerve root compression due to collapsing vertebra and hypercalcemia. The understanding of the bone cells and tumor cells mutual dependence and influence is of crucial importance for the design of novel and effective treatments or even prevention of metastasis to bone (3).

Bisphosphonates (bpns) are potent antiresorptive drugs that inhibit the action of osteoclasts, the boneresorbing bone cell. Their principal molecular formula is a central carbon with two phosphonates, P-C-P, and two side chains, RI and R2. Nitrogen substituents in R2 increase potency (second and third generation bpns). Once in the circulation, they accumulate in remodelling bone, i.e. areas of active bone metabolism. The accumulation depends on the high affinity of the P-C-P structure to the areas of remodelling bone due to its affinity to hydroxyapatite crystals. During the bone resorption, osteoclasts will absorb bpns locally and be exposed to high concentrations of bpns released from bone tissue which eventually leads to loss of resorptive function and apoptosis. Bpns, have been used for decades in the treatment of osteoporosis (4). Hormonal therapy, anti-estrogens in breast cancer and anti-androgen in prostate cancer, has a number of side effects and among them a cumulative bone loss. Bpns are frequently used in the management of these patients to delay the so-called 'skeletal related events', SRE, and to improve their

bone status (5). Skeletal pain is a common complication of skeletal metastasis and can be severe and debilitating. There are a number of clinical investigations assessing the effect of bpns on metastatic bone pain. Several of the studies find that bpns have a significant positive effect, reducing the experienced pain indicating that bpns could have an important role in the management of pain in these patients (6).

Interestingly, accumulating data show that bpns seem to have a direct anti-tumor effect in addition to the indirect effect through the inhibition of osteoclasts decreasing the concentration of tumor growth promoting molecules. Emerging results indicate significant clinical benefit especially when combined with other drugs e.g. chemotherapeutic agents (7-10). Newer nitrogen containing bpns (second and third generation) act as enzyme inhibitors of proteins in the mevalonate pathway resulting in loss of prenylation and eventually induction of apoptosis of the osteoclasts. Its likely that this mechanism also works in similar way on the tumor cells. This report describes the development of a novel poly bisphosphonate conjugate with potent anti-bone resorption properties and enhanced anti-tumor efficacy.

Materials and methods

Conjugate synthesis. Dextran 40 PhEUR (Pharmacosmos AS, Denmark) was used as conjugate backbone. Sodium metaperiodate (Merck AG, Darmstadt, Germany) was used for dextran oxidation (activation). Aminoguanidine and alendronate (Sigma-Aldrich, Sweden) were used for the conjugation. Sodium borohydride (Chemicon, Stockholm, Sweden) was used for reductive amination. PD-10 disposable Sephadex G-25 columns were used for separation and purification (Pharmacia Amersham Biotech AB).

Activation and conjugation. Briefly, as described previously (11). Conjugation: 30 mg of activated dextran was mixed with 10 mg alendronate in 1 ml 0.2 M borax buffer at pH 8.1 and incubated in the dark for 60 min on a magnetic stirrer. After 60 min, 80 mg aminoguanidine was added and the incubation continued for an additional 3 h. After 4 h of total incubation, 3 mg of sodiumborohydride was added and incubated for 30 min. The solution was then purified on a PD-10 column using 0.5 M NaCl as eluent.

Determination of conjugation yield. The conjugation yield i.e. the number of alendronate and guanidine groups coupled to the dextran backbone was determined by elemental analysis (total nitrogen content, Mikrokemi AB, Uppsala, Sweden).

Fluorimetric cytotoxicity assay (FMCA). The assay was performed as described by Larsson and Nygren (12). Breast carcinoma MDA 453 and prostate adenocarcinoma PC-3 (ATTC, Manassas, USA) were used. Zometa® was from Karolinska Apoteket, Stockholm, Sweden.

Briefly, approximately 10,000 cells/well were seeded (96-well microtiter plates, Falcon, Becton-Dickinson, Meylan, France). Zometa and dextran-conjugate (ODX) were added at equimolar concentrations (0-25 μM). The control wells were given the same volume of PBS. After 72 h incubation the microtiter plates were centrifuged (200 x g for 3 min) and the medium was removed by flicking the plates. The cells were washed in PBS. Fluorescein diacetate (FDA, Sigma, Stockholm, Sweden) was dissolved in DMSO and kept at -20˚C as a stock solution (10 mg/ml). The FDA was diluted in PBS at a concentration of 10 μg/ml and 200 μl was added to each well. The plates were then incubated for 30 min at 37˚C. A 96-well scanning fluorometer (Fluoroscan 2, Labsystems, Helsinki, Finland) was used to count the emitted fluorescence. The data were transferred to a computer and the results were calculated.

Bone resorption assay. Alpha-Minimal Essential Medium (α-MEM) was purchased from Invitrogen, Lidingö, Sweden; bensyl penicillin from AstraZeneca AB, Södertälje, Sweden; essentially acid-free serum bovine albumin, all-trans-retinoic acid (ATRA), gentamycin sulphate and streptomycin sulphate from Sigma Chemicals Co., St Louis, MO, USA; Ready Safe liquid scintillation cocktail from Beckman Coulter Inc., Fullerton, CS, USA. Indomethacin was a kind gift from Merck, Sharp & Dohme, Haarlem, The Netherlands.

Animals. CsA mice from our own inbred colony were used in all experiments. Animal care and experiments were approved and conducted in accordance with accepted standards of humane animal care and use as deemed appropriate by the Animal Care and Use Committee of Umeå University, Umeå, Sweden.

Bone resorption. Neonatal mice were injected with 1.5 μCi ^{45}Ca four days prior to dissection to label the mineral part of the skeleton. At the age of 6-7 days, calvarial bones were micro-dissected and cultured as previously described (13,14). In order to reduce basal rate of bone resorption, the bones were preincubated for 24 h in α-MEM containing 0.1% albumin and 1 μM indomethacin. Bones were then extensively washed and subsequently cultured submerged in 24-wells containing 1 ml of medium in the absence of all-trans-retinoic acid (ATRA) or test substances (controls) or in the presence of ATRA (10^{-7} M) with or without test substances. ATRA was used to induce osteoclastic bone resorption in the calvariae (15). The release of ^{45}Ca induced by ATRA in this bioassay is associated with enhanced release of also type I collagen and is inhibited by calcitonin which demonstrates that ^{45}Ca release is a useful parameter of osteoclastic bone resorption (data not shown). The bone organ cultures were incubated for 120 h in a humidified milieu at 37˚C and in a gas phase of 5% CO_2 in air. At the end of the experiments, the bones were dissolved in HCl and medium and bones were separately analysed for radioactivity by liquid scintillation counting. Mobilization of isotope was expressed as the percentage release of the initial amount of isotope (calculated as the sum of radioactivity in medium and bone after culture). To accumulate data from several experiments, a 100%-transformation was made for each experiment; the ^{45}Ca-release in the ATRA stimulated groups was referred to as maximum release and considered 100%.

Apoptosis assay, cell culture. The prostate cancer cell line PC3 cells and breast cancer MDA-MD 231 cells were obtained from American Type Culture Collection (ATCC), Manassas,

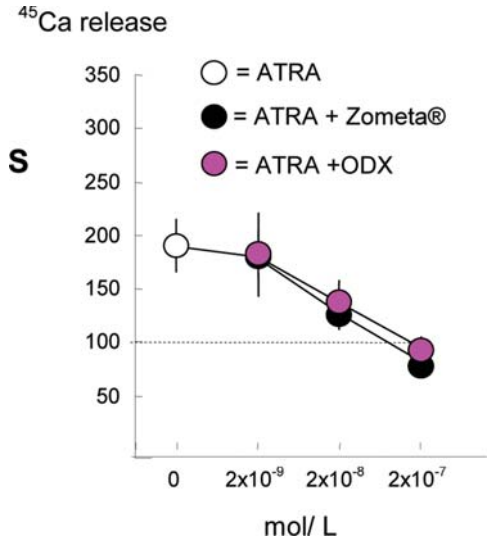

Figure 1. y axes = ^{45}Ca release in % of control, x axes = concentration of test substance, base line = 100, 200 = complete ATRA effect, mean values, n=3, cv = <10%.

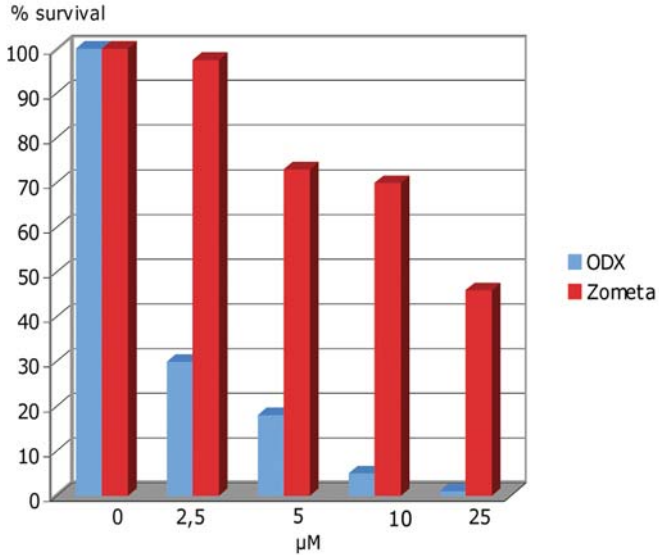

Figure 2. (PC3): y axis = % tumor cell survival, x axes = concentration of test substance, mean values, n=3, cv= <10%.

VA, USA. The PC3 cells were maintained in Dulbecco's modified Eagle's medium Nutrient Mixture F-12 (DMEF/F12) (Invitrogen) supplemented with 10% FBS, 5% horse bovine serum and 1% mixture of AB (Antibiotic Antimycotic). The MDA-MD 231 cells were maintained and grown on RPMI-1640 supplemented with 10% FBS and 1% AB. Cells were allowed to grow in a 60 mm plate to approximately 500,000 cells 24 h before treatment with the specified concentrations of the different compounds except the control cells. Thereafter, the cell plates were incubated at 37˚C in a humidified atmosphere containing 5% CO_2 for 24 and 48 h. Cells were then trypsinated and collected for measurement of apoptosis by flow cytometry analysis.

Apoptosis analysis by Annexin V staining. Cells ($5x10^5$) were cultured in 60 mm plate to reach 50-60% confluence before treatment with the drug samples. The drug samples were first diluted with sterile water to yield the desired drug concentrations (10 nM, 50 nM, 100 nM, 500 nM, 1 μM, 5 μM, 10 μM). Cells were collected at 24 and 48 h, centrifuged and re-suspended in 1 ml; 1X phosphate-buffered saline (PBS). The cells were subsequently stained by using 1.5 μg/ml propidium iodide (PI) incubated in darkness for 45 min before FACS analysis for cell-viability status. Annexin V, Apoptosis Assay kit system, Molecular Probes Eugene, OR, USA, was used for the apoptosis assay according to the manufacturer's instructions. Fluorescence was measured using LSR I FACS flow cytometry system (BD Biosciences San Jose, CA, USA) and flow imaging was analyzed using CellQuest Pro analysis software (BD Biosciences). All the experiments were preformed in duplicates or triplicates for both treated and control cells and the average percentage of apoptotic cells were measured.

Results

Bone resorption assay. Zometa and ODX inhibited the bone resorption (^{45}Ca release) induced by ATRA with similar potency. Different preparations of ODX gave closely resembling results, Fig. 1: ^{45}Ca release in % of control, base line =100, base line x 2 = Full ATRA induced resorption.

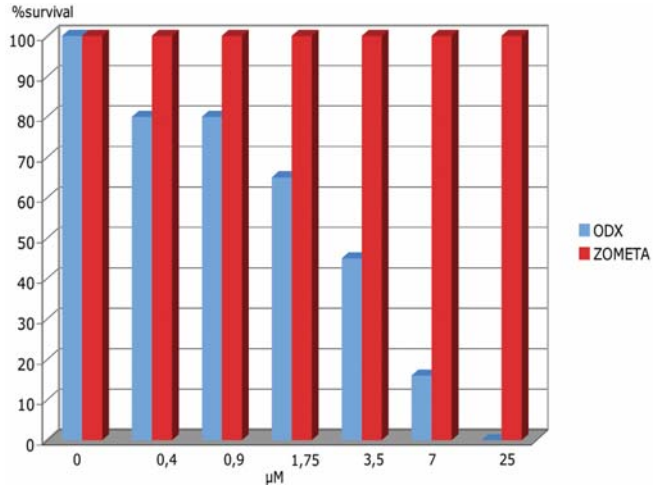

Figure 3. (MDA453): y axis = % tumor cell survival, x axes = concentration of test substance, mean values, n=3, cv = <10%.

FMCA. Both cell lines showed similar sensitivity with almost all cells killed at ODX concentrations >10 μM. IC_{50} for ODX was approximately at 2 μM in both cell lines. IC_{50} for Zometa in the PC3 was at ~25 μM while it failed to induce any significant cell death in the MDA453 cell line (Figs. 2 and 3).

Apoptosis assay. Figs. 3 and 4 show induction of apoptosis after 24 h of incubation of the test samples. Most of the cells (>80%) in both cell lines were in apoptotic state at 10 μM ODX concentration while Zometa failed to induce apoptosis in the tested concentration range. After 48 h incubation, ODX induced apoptosis at sub μM concentrations. When ODX was prepared without the alendronate ligand, no induction of apoptosis was observed (data not shown).

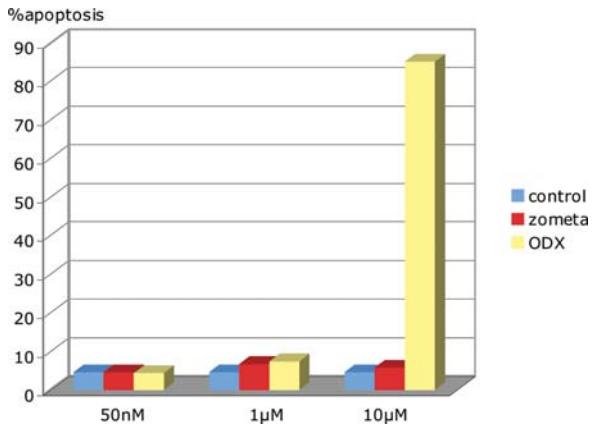

Figure 4. (PC3): y axis = % apoptosis, x axis = concentration of test substance, mean values, n=2-3, cv = <10%.

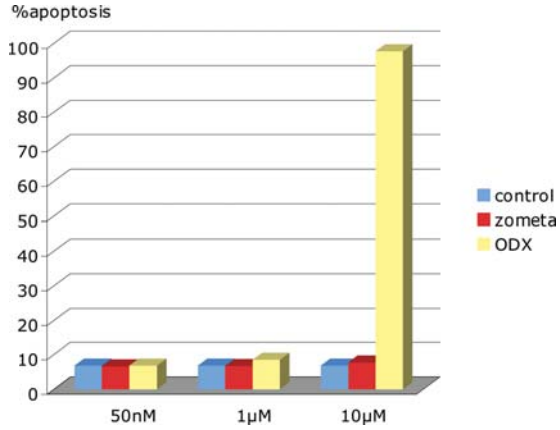

Figure 5. (MDA231): y axis = % apoptosis, x axis = concentration of test substance, mean values, n = 2-3, cv = <10%.

Discussion

Osteoporosis is deemed as a major health problem in more than 50% of individuals over 50 years of age. The vast majority are women (80%). Recent figures from the USA indicate that about 10 million people are affected with a heavy burden on the healthcare system. An estimated annual cost is 18 billion US$ for taking care of ~1.5 million osteoporosis related fractures and the connected consequences (16). The figures are expected to increase in the future.

Bpns are the most frequently prescribed treatment for osteoporosis. The potency of bpns, meaning their capacity to inhibit osteoclastic bone resorption varies depending on their structure, i.e. nitrogen content. Second and especially third generation bpns are much more potent than first generation 'non-nitrogen' containing bpns. Bioavailability is another important issue where 'old' bpns for oral administration have very poor bioavailability (<1%) while late generation of bpns for i.v. administration are excellent in this respect. Zoledronic acid (Zometa) belong to the latest generation bpns and is considered to be the most potent that currently is clinically available. Therefore, it is suitable to use Zometa as reference substance when evaluating new bpn derivatives in order to estimate their relative efficacy.

In the bone resorption assay, ODX typically showed similar anti-resorptive capacity as Zometa and different preparations of ODX, differing in the alendronate substitution level, yielded closely resembling results. Alendronate alone is in comparison to zoledronic acid several orders of magnitude less potent. However, when conjugated to the dextran backbone, on average 8 alendronate per dextran, the resulting 'poly-alendronate' (ODX) exhibits the same anti-resorptive properties as Zometa. Nitrogen containing potency of bpns depends on the ability to inhibit farnesyl diphosphate synthase (FDS) an enzyme in the mevalonate pathway, eventually leading to decreased protein prenylation (e.g. Ras). ODX might acquire this anti-resorptive potency beacuse of its polyvalens in terms of alendronate moieties. An additional possibility for ODX potency is the fact that R2 in alendronate is modified, including a carbohydrate backbone containing additional nitrogen through the guanidine moieties. Its known that modification of the R2 side chain alters the anti resorptive potency by affecting the ability to inhibit FDS (18).

Owing to the high incidence of breast and prostate cancer and the fact that a significant number of these patients will develop bone metastasis, new treatment approaches are of high importance. During the last decade, results are accumulating showing that bpns have certain efficacy against skeletal tumor lesions. The most promising data are on Zometa and a recent phase III trial of breast cancer patients demonstrates significant clinical benefits when combining Zometa with hormone therapy (17). Even a first generation non-nitrogen containing bpn (clodronate) has shown clinical benefits in prostate cancer patients (10).

In the FMCA cytotoxicity assay on PC3, ODX was a factor of 10 more efficient compared to Zometa. The growth inhibition concentrations obtained for Zometa is fairly consistent with earlier results (18), however no effect was seen on the breast cancer cell line and its probable that higher concentrations are necessary (>25 μM).

In the apoptosis assay, Zometa failed to induce any apoptosis in either cell line while ODX was very efficient showing induction at low sub μM concentrations (48 h incubation). Other *in vitro* studies on apoptosis with Zometa indicate that higher concentrations are necessary i.e. 50-100 μM (20).

ODX tumor cell killing efficacy is most likely dependent on several factors such as its capacity to decrease prenylation, activation of apoptosis promoting molecules, and its high nitrogen content dependent on its guanidine moieties. In a recent study (21), it was demonstrated that polymer conjugated guanidine, i.e. a polyguanidine, has strong anti-tumor properties expressed in several different tumor cell cultures. However, in the apoptosis assay employed in this study, ODX without alendronate moieties, i.e. polyguanidine, did not induce apoptosis (i.e. no expression of cell membrane phosphatidylserine) indicating that the alendronate moiety is necessary for this effect. That strongly suggest that the tumor cell killing properties of ODX is dependent on several mechanisms.

In conclusion, ODX has interesting properties, high anti-bone resorption capacity and superior anti-tumor efficacy,

motivating further studies to evaluate its possible clinical usefulness in bone metastasis and osteoporosis.

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References

1. Buijs JT and van der Pluijm G: Osteotropic cancers: from primary tumor to bone. Cancer Lett 273: 177-193, 2009.
2. Paget S: The distribution of secondary growths in cancer of the breast 1889. Cancer Metastasis Rev 8: 98-101, 1989.
3. Bussard KM, Gay CV and Mastro AM: The bone microenvironment in metastasis; what is special about bone? Cancer Metastasis Rev 27: 41-55, 2008.
4. Lambrinoudaki I, Christodoulakos G and Botsis D: Bisphosphonates. Ann N Y Acad Sci 1092: 397-402, 2006.
5. Saad F and Sternberg CN: Multidisciplinary management of bone complications in prostate cancer and optimizing outcomes of bisphosphonate therapy. Nat Clin Prac Urol 4: S3-S13, 2007.
6. Gralow J and Tripathy D: Managing metastatic bone pain: the role of bisphosphonates. J Pain Symptom Manage 33: 462-472, 2007.
7. Hoesl CE and Altwein JE: Bisphosphonates in advanced prostate and renal cell cancer - current status and potential applications. Urol Int 76: 97-105, 2006
8. Brown JE and Coleman RE: The role of bisphosphonates in breast cancer: the present and future role of bisphosphonates in the management of patients with breast cancer. Breast Cancer Res 4: 24-29, 2002.
9. Neville-Webbe HL, Evans CA, Coleman RE and Holen I: Mechanisms of the synergistic interaction between the bisphosphonate zoledronic acid and the chemotherapy agent paclitaxel in breast cancer cells in vitro. Tumor Biol 27: 92-103, 2006.
10. Dearnaley DP, Mason MD, Parmar MKB, Sanders K and Sydes MR: Adjuvant therapy with oral sodium clodronate in locally advanced and metastatic prostate cancer: long-term overall survival results from the MRC PRO4 and PRO5 randomised controlled trials. Lancet Oncol 10: 872-876, 2009.
11. Marquez M, Nilsson S, Lennartsson L, Liu Z, Tammela T, Raitanen M and Holmberg AR: Charge-dependent targeting: results in six tumor cell lines. Anticancer Res 24: 1347-1352, 2004.
12. Larsson R and Nygren P: A rapid fluorometric method for semi-automated determination of cytotoxicity and cellular proliferation of human tumor cell lines in microculture. Anticancer Res 9: 1111-1119, 1989.
13. Lerner UH: Modifications of the mouse calvarial technique improve the responsiveness to stimulators of bone resorption in vitro. J Bone Min Res 2: 375-383, 1987.
14. Ljunggren Ö, Ransjö M and Lerner UH: In vitro studies on bone resorption in neonatal mouse calvariae using a modified dissection technique giving four samples of bone from each calvariae. J Bone Miner Res 6: 543-550, 1991.
15. Conaway HH, Grigorie D and Lerner UH: Differential effects of glucocorticoids on bone resorption in neonatal mouse calvariae stimulated by peptide and steroid-like hormones. J Endocrinol 155: 513-521, 1997.
16. Owens G, Jackson R and Lewiecki M: An integrated approach: bisphosphonate management for the treatment of osteoporosis. Am J Manag Care 13: S290-S308, 2007.
17. Dunford JE, Thompson K, Coxon FP, Luckman SP, Hahn FM, Poulter CD, Ebetino FH and Rogers MJ: Structure-activity relationships for inhibition of farnesyl diphosphate synthase in vitro and inhibition of bone resorption in vivo by nitrogen-containing bisphosphonate. JPET 296: 235-242, 2001.
18. Zhang Y, Cao R, Yin F, Hudock MP, Guo RT, Krysiak K, Mukherjee S, Gao YG, Robinson H, Song Y, No JH, Bergan K, Leon A, Cass L, Goddard A, Chang TK, Lin FY, Van Beek E, Papapoulos S, Wang AHJ, Kubo T, Ochi M, Mukkamala D and Oldfield E: Lipophilic bisphosphonates as dual farnesyl/geranylgeranyl diphosphate synthase inhibitors: an X-ray and NMR investigation. J Am Chem Soc 131: 5153-5162, 2009.
19. Ullén A, Lennartsson L, Hamenberg U, Hjelm-Eriksson M, Kälkner KM, Lennernäs B and Nilsson S: Additive/synergistic antitumoral effects on prostate cancer cells in vitro following treatment with a combination of docetaxel and zoledronic acid. Act Oncol 44: 644-650, 2005.
20. Neville-Webbe H, Rostami-Hodjegan A, Evans CA, Coleman RE and Holen I: Sequence- and schedule-dependent enhancement of zoledronic acid induced apoptosis by doxorubicin in breast and prostate cancer cells. Int J Cancer 113: 364-371, 2005.
21. Meurling L, Márquez M, Nilsson S and Holmberg AR: Polymer-conjugated guanidine is a potentially useful anti-tumor agent. Int J Oncol 35: 281-285, 2009.