Pamidronate and 1,24(S)-Dihydroxyvitamin D2 Synergistically Inhibit the Growth of Myeloma, Breast and Prostate Cancer Cells

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Abstract. Background: Bisphosphonates have proven to be effective in the management of multiple myeloma and bone metastases secondary to breast and prostate carcinoma. Vitamin D compounds are important modulators of cellular proliferation and differentiation. 1,24(S)-dihydroxyvitamin D2 [1,24(OH)2D2] is a naturally occurring active vitamin D compound with high antiproliferative activity and low calcemic response. Materials and Methods: We examined the antiproliferative effects of 1,24(OH)2D2 in combination with the bisphosphate pamidronate on multiple myeloma (H929), prostate (LNCaP) and breast (MCF-7) cancer cell lines. Drug-drug interactions were analyzed using the median-effect/isobologram method to characterize the interactions as synergistic, additive, or antagonistic. Results: Pamidronate and 1,24(OH)2D2 were found independently to inhibit cancer cell growth in a dose-dependent manner. Combinations of these compounds produced marked synergistic growth-inhibitory effects at several clinically relevant concentrations. Conclusion: Combined dosing of pamidronate and 1,24(OH)2D2 may have therapeutic value for the treatment of multiple myeloma, prostate and breast cancers.

Osteolysis, fractures, bone pain and hypercalcemia of malignancy are all tumor-associated skeletal complications resulting from cancers of breast, prostate and multiple myeloma. Although the molecular mechanisms of these complications are not completely understood, it is believed that tumor cells adhere to bone extracellular matrix or bone marrow microenvironments, and release soluble factors that stimulate abnormal osteoblast activity and osteoclast-mediated bone resorption at these adhesion sites (1-3).

High morbidity and mortality rates indicate that new and more effective treatment strategies are essential to improve patient outcomes in these cancer populations (4).

Bisphosphonates are synthetic analogs of pyrophosphate in which the central oxygen is replaced by a carbon atom. The highly charged bisphosphonates exhibit a high binding affinity for calcified matrices such as hydroxyapatite in the bone, where they act directly on osteoclasts and are potent inhibitors of bone resorption. (5). Bisphosphonates act in part by inhibiting the recruitment, proliferation and differentiation of pre-osteoclasts, and by shortening the lifespan of mature osteoclasts through induction of apoptosis (6, 7).

Recent evidence indicates that bisphosphonates affect cell types in addition to osteoclasts, including osteoblasts and bone marrow stromal cells. Several studies have shown that bisphosphonates exert direct cytostatic and pro-apoptotic effects on a variety human cancer cell lines (8-11). While a limited amount of evidence exists for direct antitumor effects in vivo (12, 13), several studies have demonstrated that bisphosphonates reduce tumor-induced osteolysis in animal models of breast and prostate cancer (14-17), as well as multiple myeloma (18-20).

Bisphosphonates have proven to be effective for the management of multiple myeloma and bone metastases secondary to breast and prostate carcinoma (21-25), and constitute the standard therapy for hypercalcemia of malignancy associated with metastatic bone disease (26, 27). Multiple myeloma is characterized by the clonal expansion of malignant plasma cells within the bone marrow, where secreted factors stimulate osteoclastic bone resorption. Malignant breast and prostate cancers frequently spread to bone through metastasis, and such metastases are always associated with osteolysis. By inhibiting osteoclast-mediated bone resorption, bisphosphonates decrease the release of tumor-promoting growth factors from bone and delay the further progression of bone disease.

Vitamin D compounds are significant modulators of cellular proliferation and differentiation and have potential
utility as anticancer drugs (28, 29). 1,25-dihydroxyvitamin D$_3$ (1,25(OH)$_2$D$_3$), the active metabolite of vitamin D$_3$, is a seco-steroid whose primary target tissues include intestine, kidney and bone (30). The classical functions of 1,25(OH)$_2$D$_3$ include regulation of calcium absorption in the intestine, maintenance of mineral homeostasis in the kidney and regulation of bone resorption. The hormonal effects of vitamin D analogs and derivatives are mediated through specific interaction with the vitamin D receptor (VDR), a ligand-dependent transcription factor belonging to the nuclear receptor superfamily (31). In addition to the classical target tissues, VDR has been identified in a wide array of tissues that do not participate in mineral and bone metabolism (32). 1,25(OH)$_2$D$_3$ and vitamin D analogs have shown antiproliferative activity in vitro and in vivo against a variety of human cancer cell lines including breast, prostate and colon, as well as multiple myeloma and myeloid leukemia (33, 34).

1,24(S)-dihydroxyvitamin D$_2$ (1,24(OH)$_2$D$_2$) is a naturally occurring compound that has been isolated from serum of several species, including humans (35), and can be generated from both vitamin D$_2$ (ergocalciferol) and 1α-hydroxyvitamin D$_2$ (doxercalciferol, Hectorol$^\text{®}$) (36, 37). Previous studies have demonstrated that the binding affinity of 1,24(OH)$_2$D$_2$ to VDR approaches that of 1,25(OH)$_2$D$_3$, and that 1,24(OH)$_2$D$_2$ exhibits transcriptional activity equivalent to that of 1,25(OH)$_2$D$_3$ in gene reporter assays (38). The antiproliferative activity of 1,24(OH)$_2$D$_2$ has been demonstrated in several human cancer cell lines including prostate (39, 40) and breast (41, 42). Despite its similarities to 1,25(OH)$_2$D$_3$ in vitro, metabolic studies in rats have shown that 1,24(OH)$_2$D$_2$ increases serum and urinary calcium to a much lesser degree than 1,25(OH)$_2$D$_3$ (43). Animal models with 1,24(OH)$_2$D$_2$ demonstrated significant ability to inhibit the growth of MCF-7 human breast cancer xenografts in nude mice, with complete absence of hypercalcemia or toxicity-related weight loss (44).

Combination chemotherapy has become an essential and effective treatment option for the management of cancer. Combining two drugs with different mechanisms of action can elicit a synergistic response greater than the sum of the individual drug effects. Pronounced synergistic effects have been observed when bisphosphonates are combined with other common antineoplastic drugs both in vitro and in vivo. The antiproliferative and apoptotic effects of zoledronate are enhanced several fold when combined with low concentrations of either paclitaxel or tamoxifen in breast cancer cells (45, 46), or with dexamethasone in human multiple myeloma cells (47). Co-administration of taxol with the bisphosphonate alegrostone is more effective than either compound given individually in preventing the growth of bone and non-bone metastases in mice injected with prostate carcinoma cells (48). Combination treatments with bisphosphonates and common chemotherapeutic drugs have also proven effective in human cancer patients. The addition of pamidronate or clodronate to standard chemotherapy or to hormonal therapy in breast carcinoma patients with lytic bone disease produced a sustained reduction in new skeletal complications (26), and decreased the number of incidences while reducing the number of bone and non-bone metastases (49).

As seen with bisphosphonates, combining vitamin D analogs with chemotherapeutic drugs also has pronounced synergistic effects on cell growth and apoptosis. Platinum drugs are reported to interact synergistically with 1,25(OH)$_2$D$_3$ to inhibit the growth of several cell lines including breast, prostate and squamous cell carcinoma (SCC) (50-52). 1,25(OH)$_2$D$_3$ and the vitamin D analog EB1089 enhance the antiproliferative and apoptotic effects of doxorubicin in MCF-7 breast cancer cells (40, 53, 54), while paclitaxel was shown to interact synergistically with 1,25(OH)$_2$D$_3$ in breast and SCC cells (55). Clinical studies have begun investigating the effects of combined treatment of 1,25(OH)$_2$D$_3$ with standard chemotherapeutics (56, 57). A study investigating the effects of pamidronate and 1,25(OH)$_2$D$_3$ on bone disease in stage-III B multiple myeloma patients identified a dramatic improvement in cancer cell activity and bone healing (58).

We examined the antiproliferative effects of 1,24(OH)$_2$D$_2$ alone and in combination with the bisphosphonate pamidronate on the growth of H929 multiple myeloma, LNCaP prostate and MCF-7 breast cancer cell lines. Combination drug effects were characterized using the median-effect/combination index isobologram method of multiple drug effect analysis. We report a marked synergistic interaction between pamidronate and 1,24(OH)$_2$D$_2$, producing enhanced antiproliferation activity in all of the cell lines tested.

Materials and Methods

Cell lines and culture. Human cancer cell lines and all cell culture reagents were obtained from the American Type Culture Collection (Rockville, MD, USA). MCF-7 breast cancer cells were maintained in DMEM medium; RPMI-1640 was used for both LNCaP prostate cancer and H929 multiple myeloma cells. All media were supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin. H929 cells were further supplemented with 50 µM 2-mercaptoethanol. Cell cultures were maintained in 100-mm$^2$ dishes and incubated at 37°C in a humidified atmosphere of 5% CO$_2$.

Drugs. 1,24(OH)$_2$D$_2$, (Bone Care International, WI, USA) was dissolved in 100% ethanol (vehicle) and stored, protected from light, as a concentrated solution at –70°C. Pamidronate (Bedford Laboratories) was obtained as a stock 10 mg/ml solution. Working stock solutions of pamidronate were made in sterile distilled water and stored at –20°C. Each compound was diluted in growth medium on the day of the experiment with ethanol concentrations never exceeding 0.1%.
Growth inhibition assays. LNCaP or MCF-7 cells were harvested at confluence and seeded in a volume of 0.15 ml culture medium into 96-well tissue culture plates at 2x10^3 cells/well and 5x10^3 cells/well, respectively. Cells were allowed to grow for 24 hours and then the plating medium was replaced with 0.1 ml medium containing the vehicle control, 1,24(OH)2D2, pamidronate, or a combination of 1,24(OH)2D2 and pamidronate.

Suspension cultures of H929 cells were directly harvested and transferred into 96-well tissue culture plates at 3x10^4 cells/well in a volume of 0.2 ml medium containing the vehicle control, 1,24(OH)2D2, pamidronate, or a combination of 1,24(OH)2D2 and pamidronate.

Cells were cultured for an additional 6 days. Cell viability was quantitated with the addition of CellTiter 96 Aqueous One Solution Assay Reagent (Promega Corp., Madison, WI, USA), incubated 1-2 hours at 37°C in a humidified atmosphere of 5% CO2, and absorbance read at a wavelength of 490 nm with an ELISA plate reader (Bio-Tek Instruments, Winooski, VT, USA).

Growth inhibition was calculated as the percent difference between drug-treated cells and vehicle control cells. Each experiment was carried out in triplicate.

**Isobologram analysis of interactions between 1,24(OH)2D2 and pamidronate.** Dose-effect relationships between 1,24(OH)2D2 and pamidronate were analyzed with the software program CalcuSyn (Biosoft, Cambridge, UK). This program performs multiple drug dose-effect calculations using the median-effect methods developed by Chou (59). Unlike empirical or statistical approaches to dose-effect analysis in biology, CalcuSyn utilizes calculations that are derived from common enzyme kinetic equations of mass-action law. The derivations have rigorous mathematical proofs and are based on well-defined models. As a result, Chou’s dose-effect calculations are widely used for multiple drug-effect analysis.

Data from growth inhibition assays for individual drugs, as well as combinations of the two drugs, were entered into CalcuSyn to produce median-effect plots from which isobolograms were constructed. Isobolograms are graphs indicating the equipotent combinations of two or more doses to illustrate additive, synergistic, or antagonistic drug interactions. For a given dose-effect level (i.e. 50% growth inhibition), the required individual doses (i.e. of drug 1 and drug 2) to produce that effect level are plotted on the x- and y-axis respectively, with a diagonal line drawn between them. Combinations of drugs producing the same effect level are then plotted coordinately on the graph with data-points falling on the diagonal representing additivity, while those plotted to the lower left or upper right of the diagonal line indicate synergism or antagonism respectively. When a constant dose ratio is used for multiple drug-effect analysis, isobolograms are constructed with the x- and y-axis representing the concentration of each individually dosed drug that produced a given effect level. However, when a non-constant dose ratio is used, as performed in this study, the dose concentrations of the two drugs on the coordinates of the isobologram are normalized by dividing all dose values, both individual and combinations, by the corresponding doses or concentrations of the individual respective doses. This produces a "normalized" isobologram that does not show the actual dose or concentration levels, yet can still be utilized to illustrate degrees of synergism or antagonism.

The combination index (CI) equation is a method of analysis based on the multiple drug-effect equation (60), which permits a quantitative measure of the degree of interaction between drug combinations. The formula \( CI = d_1 / D_1 + d_2 / D_2 \), where \( d_1 \) and \( d_2 \) represent the doses of drug 1 and drug 2 that, when given individually, produce the same effect. Combination index values equal to 1 (CI = 1) indicate an additive response and describe the combined effect predicted by the mass-action law principle. This value would be illustrated on an isobologram as a data-point plotted on, or close to, the diagonal line of additivity. Combination index values less than 1 (CI < 1) describe synergistic combinations with decreasing CI values indicating greater levels of synergism, while CI > 1 represents antagonistic combinations with increasing CI values indicating greater levels of antagonism. It is recommended in CalcuSyn that the degree of interaction be reported based on CI values and represented by symbols ranging from ++ + + + + for very strong synergism to -- -- -- -- -- for very strong antagonism.

The dose-reduction index (DRI) is a measure of how much the dose of each drug in a synergistic combination can be reduced and still produce an effect level comparable to that for each drug alone (61). The DRIs for two drugs are derived from combination index values such that \( DRI_1 = D_1 / d_1 \) and \( DRI_2 = D_2 / d_2 \).

**Results**

Dose responses of pamidronate and 1,24(OH)2D2 in multiple myeloma (H929), prostate (LNCaP) and breast cancer (MCF-7) cell lines. Both pamidronate and 1,24(OH)2D2 inhibited cell proliferation dose-dependently in H929, LNCaP and MCF-7 cell lines (Figure 1). The % inhibition data for single doses of 1,24(OH)2D2 are plotted on the far left of each graph above the "0 µM Pamidronate" coordinate, while single doses of pamidronate are plotted as "EtOH" identified in the figure inset. IC50 values in H929, LNCaP and MCF-7 cells were 1.5 nM, 6.2 nM and 144 nM, respectively for 1,24(OH)2D2, 239 µM, 47.9 µM and 21.2 µM, respectively for pamidronate (data not shown). These % inhibition data and IC50 values indicate that, while both compounds possess antiproliferative ability, the effectiveness of each compound is highly cell type-specific.

Whereas lower doses of 1,24(OH)2D2 were required to reach an equivalent IC50 in H929 and LNCaP cells when compared to MCF-7 cells, this pattern was reversed with pamidronate treatments in which the IC50 value was highest in H929 cells.

Combination dosing of pamidronate and 1,24(OH)2D2 in H929, LNCaP and MCF-7 cells. The effect of combinations of pamidronate and 1,24(OH)2D2 on growth inhibition in three cell lines was evaluated over a range of concentrations. With H929 cells (Figure 1A), the concentrations of pamidronate used in the combination experiment were identical to those studied using pamidronate alone (20 – 120 µM), but with 1,24(OH)2D2 added at a constant concentration. This combination experiment was repeated with three different concentrations...
of 1,24(OH)_{2}D_{2} (0.15, 0.5, or 1.5 nM), thus producing 3 independent growth curves for each combination of 1,24(OH)_{2}D_{2} and pamidronate. Similar protocols were followed with LNCaP (Figure 1B) and MCF-7 (Figure 1C) cells. The combinations consistently showed the increased antiproliferative effect of 1,24(OH)_{2}D_{2} and pamidronate over that of either drug alone.

Pamidronate and 1,24(OH)_{2}D_{2} are synergistic in H929, LNCaP and MCF-7 cells. The accurate prediction of drug interactions is not possible from the simple addition of individual drug effects because of the non-linearity of the response curve for each drug (62). The isobologram method of analysis alleviates this problem by evaluating drug interaction independent of the shape of the dose-response curve, and allows for concise classification of the degree of interaction between two drugs (63, 64). Therefore, we further assessed the interactions of pamidronate and 1,24(OH)_{2}D_{2} by constructing isobolograms from the growth inhibition data. Because we used differing molar ratios of 1,24(OH)_{2}D_{2} to pamidronate for the combination experiments, single dose concentrations of pamidronate and 1,24(OH)_{2}D_{2} were normalized by their corresponding IC_{50} values for the coordinates of the isobolograms (Materials and Methods). Individual data-points, reflective of a specific dose ratio, are indicated in Figure 2. All plotted combination data-points but one lay below the line that represents an additive effect, thus indicating synergistic growth inhibition for all ratios tested in H929 and LNCaP cells, and 2 of 3 drug combinations in MCF-7 cells.

While isobolograms pictorially illustrate the synergistic, additive, or antagonistic responses of two molecules, combination index (CI) values quantitate those interactions. Table I lists the drug concentrations, the % cell growth inhibited by the doses, and the CI values for all the tested combinations between pamidronate and 1,24(OH)_{2}D_{2} which were shown in Figure 2. The calculated CI values for drug combinations in H929 and LNCaP cells were all well under 1, indicating a high degree of synergy between pamidronate and 1,24(OH)_{2}D_{2} in these two cell types. The strongest synergy in H929 cells occurred at the two highest doses of pamidronate, while similarly strong synergistic interactions of the drugs were observed with all drug concentrations in LNCaP cells. Pamidronate and 1,24(OH)_{2}D_{2} were synergistic in MCF-7 cells at 2 of 3 combinations, with an overall lower degree of synergy when compared to the H929 and LNCaP cell lines.

The Dose Reduction Index (DRI) values presented in Table I quantitate the advantage synergism provides for dose reduction when the two drugs are used in combination. For example, the combination of 20 µM pamidronate and 0.5 nM 1,24(OH)_{2}D_{2} in H929 cells produced 38% cell growth inhibition and a CI = 0.60 indicating synergy.
DRI value of 8.7 for pamidronate indicates that this drug would need an 8.7-fold higher concentration to produce the same inhibition effect in the absence of 1,24(OH)2D2. In other words, the addition of 0.5nM 1,24(OH)2D2 allowed for an 8.7-fold reduction in the concentration of pamidronate to achieve the same inhibition effect. DRI values are listed for all doses of pamidronate and 1,24(OH)2D2 used in isobologram experiments that identified synergism.

**Discussion**

Bisphosphonates are a primary treatment method for hypercalcemia of malignancy and have been shown to be highly effective in the management of osteolytic lesions associated with multiple myeloma and cancers of the breast and prostate (65). The addition of bisphosphonates to standard chemotherapy and hormone therapy is becoming increasingly common for the reduction of skeletal complications and enhanced quality of life.

We chose to investigate 1,24(OH)2D2, a vitamin D analog with low calcemic activity, in combination with the bisphosphonate pamidronate for possible enhanced antiproliferative effects in multiple myeloma, breast, and prostate cancer cell lines. All three cell lines are representative of cancers associated with osteolytic lesions in human cancers and have previously been shown to possess the potential for direct antitumor response to one or both of the investigated compounds.

We identified synergistic responses for 1,24(OH)2D2 and pamidronate in all three cell lines tested. It is likely that these findings have clinical relevance. Several concentrations of 1,24(OH)2D2 that were synergistic with pamidronate in our experiments (0.5 nM in H929 cells, and 3 nM in LNCaP) are probably achievable in the clinic; for example, peak blood levels of the active vitamin D metabolite 1,25(OH)2D3 of 0.7 nM (66) and 3.7 - 6.0 nM (with minimal patient toxicity) have been reported in clinical trials (56). It is likely that a vitamin D analog such as 1,24(OH)2D2, with lower calcemic activity than 1,25(OH)2D3, can be administered at higher doses, and attain tissue and plasma levels greater than that observed with 1,25(OH)2D3 without serious side-effects. The lowest concentrations of pamidronate found to be synergistic with 1,24(OH)2D2 (3, 5 and 20 μM in LNCaP, MCF-7 and H929 cells, respectively) are slightly above the serum levels of pamidronate (0.5 to 8 μM) reported in clinical trials (67, 68); however, the concentrations of bisphosphonates in the bone microenvironment are likely to be higher than serum levels due to the affinity of bisphosphonates for bone.

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<th>Combination Index for Experimental Values</th>
<th>Dose Reduction Index</th>
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<tr>
<td>Pamidronate (μM)</td>
<td>1,24(OH)2D2 (nM)</td>
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<tr>
<td>(A) H929</td>
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<tr>
<td>20</td>
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<td>40</td>
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<td>60</td>
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<td>(B) LNCaP</td>
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the synergistic concentrations found in these in vitro experiments may be achievable in the clinic, and may alleviate the side-effects produced by higher levels of drug required with individual drug therapy.

While the current study shows that 1,24(OH)2D2 potentiates the growth-inhibitory effect of pamidronate, to our knowledge the mechanisms by which vitamin D compounds interact with bisphosphonates to produce this synergism are unknown. The antiproliferative activity of vitamin D compounds has been associated with G0/G1 cell cycle arrest and induction of apoptosis, yet the exact mechanisms of action remain only partly understood. 1,25(OH)2D3 (the most studied active vitamin D) has been shown to reduce the expression of cyclin D1 and associated cyclin-dependent kinase 4 (cdk 4), while increasing expression of the cdk inhibitors p21 and p27 in several cancer cell lines (69-71). Several cell survival/death signaling pathways have been reported to be influenced by 1,25(OH)2D3. Mitogen-activated protein kinase kinase kinase (MEKK-1), a stress signaling molecule known to generate apoptotic responses in several cancer cell lines, is up-regulated in squamous cell carcinoma cells after 1,25(OH)2D3 dosing (52). This increased expression of MEKK-1 may result in sensitizing the cells to apoptosis, while combination dosing with bisphosphonates may further enhance the cell death pathway. 1,25(OH)2D3-mediated apoptosis is also associated with down-regulation of survival signaling through the extracellular signal-regulated kinase (Erk) pathway as a result of caspase-dependent cleavage of MEK (Erk kinase) (72). Erk is central to a critical signaling pathway that regulates proliferation and is frequently increased in cancer through dysregulation of the oncogene Ras. Bisphosphonates are known to inhibit prenylation and subsequent membrane localization of small GTP proteins including Ras (73-75). Therefore, one possible mechanism for the observed synergistic activity of 1,24(OH)2D2 and pamidronate may be an amplified disruption of the Ras/MEK/Erk pathway.

In summary, we established that the combined treatment of pamidronate and the vitamin D analog 1,24(OH)2D2 produces highly synergistic antiproliferative activity in H929, LNCaP and MCF-7 cancer cell lines. Such combination therapy potentially offers a greater therapeutic effect than a standard dose of a single drug, thereby enhancing the effectiveness of the therapy and/or reducing the number of treatments required, thus limiting toxic side-effects.

References


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