Purpose: The clinical use of the potent bisphosphonate zoledronic acid has increased recently, especially for the treatment of bone metastases. Synergistic effects with chemotherapeutic agents (e.g., doxorubicin, paclitaxel) have been shown. It is not known whether similar synergistic effects exist with radiation.

Methods and Materials: IM-9 myeloma cells and C4-2 prostate cancer cells were treated with up to 200 μM concentrations of zoledronic acid, irradiated with single doses of up to 1,000 cGy, or exposed to combinations of both treatments. Cell viability was then determined via yellow dye 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide assay and the affected fractions analyzed using the median effect principal, a method developed and validated by Chou and Talalay.

Results: A statistically significant synergistic cytotoxic effect of the combination of zoledronic acid and radiation was documented. The extent of the effect was cell type–dependent, with the C4-2 cells showing a greater synergistic effect than the IM-9 cells.

Conclusions: The combined use of zoledronic acid and radiotherapy shows enhanced in vitro cytotoxicity for two human prostate and myeloma cancer cell lines over that expected for a simple additive effect from each treatment alone. A clinical trial is under way to test this combination therapy. © 2005 Elsevier Inc.

INTRODUCTION

Bisphosphonates (BP) are a family of pyrophosphate analogs characterized by a phosphorus-carbon-phosphorus backbone (Fig. 1). Their chemical structure is responsible for the BP affinity to bone tissue. In the early 1980s, their potential in the treatment of metastatic bone disease was recognized (1). In the last few years, a large number of clinical studies have confirmed that BP effectively inhibits the progression of bone metastases of breast cancer, prostate cancer, and multiple myeloma (2–7). The different BP differ in potency in part because of the structure of their side chains, which can be grouped into the older and less active class of non-nitrogen-containing BP and the more recent, more potent nitrogen-containing BP (amino-BP) (3, 8).

Bisphosphonates inhibit both bone resorption and inflammatory processes. Although the reasons for these effects are still under investigation, it seems that BP bind with high affinity to bone minerals (hydroxyapatite crystals), especially in areas of bone destruction (9, 10). The accumulated high concentrations of BP are taken up by osteoclasts, specifically inhibit adenosine triphosphate (ATP)-dependent enzyme systems, induce apoptosis of osteoclasts by inhibiting mevalonic acid pathway (11), and thus disrupt the biochemical processes that lead to bone destruction (12).

One of the most potent nitrogen-containing BP is zoledronic acid (Fig. 1) with the chemical formula (1-hydroxy-2-imidazol-1-yl-phosphonoethyl) phosphonic acid monohydrate (Zometa; Novartis Pharmaceuticals Corp., East Hanover, NJ). If the potency of the first non-nitrogen-containing BP etidronate is set at 1, then the relative potency of zoledronic acid is 100,000 (8). Its use in the treatment of multiple myeloma and bone metastases of breast carcinoma was approved by the Food and Drug Administration in 2002 and it has now become an integral part of the treatment of multiple myeloma (13). Zometa is also approved in the United States and Europe for the treatment of bone metastases associated with breast, prostate, lung, and renal cancer, as well as other solid tumors (14). The long-term efficacy and safety of zoledronic acid has been shown in a clinical trial (15). Zoledronic acid is superior to pamidronate for the treatment of bone metastases in breast carcinoma patients with at least one osteolytic lesion (16). It delays the onset of
Zoledronic acid solutions

Zoledronic acid was obtained as pure research compound in the form of the disodium salt from Novartis Pharma AG, Basel, Switzerland. A 10-mM stock solution of zoledronic acid was prepared and sterile filtrated through a 0.22-μm filter. Zoledronic acid dilutions of 10, 20, 50, 100, and 200 μM were prepared with RPMI-1640 medium (Lerner Media Laboratory, Cleveland Clinic, Cleveland, OH). Zoledronic acid was obtained as pure research compound in the form of the disodium salt from Novartis Pharma AG, Basel, Switzerland. A 10-mM stock solution of zoledronic acid was prepared and sterile filtrated through a 0.22-μm filter. Zoledronic acid dilutions of 10, 20, 50, 100, and 200 μM were prepared with RPMI-1640 medium (Lerner Media Laboratory, Cleveland Clinic, Cleveland, OH).

Cell lines

C4-2 cells are a subline of LNCaP, an androgen-dependent human prostate cancer cell line, and were obtained from Dr. W. Heston (Cleveland Clinic) (24). C4-2 cells are androgen-independent and highly tumorigenic (25, 26). IM-9 cells (ATCC, Manassas, VA) are a human B-lymphoblastoid cell line, established from the bone marrow of a female patient with multiple myeloma in 1967 (27). Both cell lines were cultured in RPMI-1640 plus 10% fetal bovine serum (dialyzed at 10,000 molecular weight cutoff, Sigma) supplemented with 2 mM glutamine, 100 μg/mL streptomycin, 100 units/mL penicillin, and 250 μg/mL Fungizone (BioWhittaker, Walkersville, MD).

MTT assay

To each well of a 96-well plate coated with collagen (Biocoat; Becton Dickinson Labware, UK), 100 μL of a 10^5 cells/mL cell suspension was added. To each column of eight wells, increasing amounts of zoledronic acid were added to yield a concentration of 0, 10, 20, 50, 100, and 200 μM. Thirty minutes later, the 96-well plates were irradiated with doses of 100, 200, 500, or 1,000 cGy. All experiments were repeated five times. In one additional experiment, IM-9 cells were irradiated with smaller doses of 20, 40, 60, or 80 cGy. Irradiations at a dose rate of 300 cGy/min were performed in a calibrated Mark I 137Cs γ-iradiator (JL Shepherd, San Francisco, CA).

After irradiation or addition of the zoledronic acid, all plates were incubated at 37°C in a 5% CO2 atmosphere for either 48 (IM-9 cells) or 72 h (C4-2 cells) with the zoledronic acid still present. No medium changes were performed. After incubation, cell viability was measured with a colorimetric assay based on the yellow dye 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) (Sigma Diagnostics), which turns blue in the mitochondria of healthy cells (28). For the assay, 20 μL of a 0.5 mg/mL MTT solution was added to each well, the plates shaken for 3 min at 350 rpm and 37°C in an Eppendorf Thermomixer (Brinkmann, Westbury, NY) and incubated for 3 h. The medium was then aspirated from each well either directly (C4-2 cells) or after centrifugation for 5 min at 1,000 rpm (IM-9 cells), followed by the addition of 150 μL of DMSO to each well. The blue crystals and the cell pellet from the centrifugation dissolved within 30 min on the Thermomixer at 37°C shaking at 400 rpm; the color was then read in a 96-well plate spectrophotometer at a wavelength of 540 nm.

The average absorbance reading of the experimental eight wells was subtracted from that of the eight wells containing no cells (background) and the resulting difference divided by the background-corrected average of the control tumor cells. For each MTT assay, the control tumor cell viability was thus by definition 100%. The results were graphed in Origin (OriginLab Corp., Northampton, MA).

Analysis of the multiple drug effects

The cell viability and effects from multiple drugs were analyzed according to the computerized method of Chou and Talalay (29, 30). This analysis of drug interactions is based on the median effect principle (31). The analysis involves plotting dose–effect curves for each agent and for multiple diluted, fixed ratio combinations of agents using the median effect equation:

\[
\frac{f_a}{f_u} = \left(\frac{D}{D_m}\right)^m
\]

where \(D\) is the dose, \(D_m\) is the dose required to reach a 50% effect (e.g., 50% inhibition of cell growth), \(f_u\) is the fraction affected by \(D\) (e.g., 0.9 if cell growth is inhibited by 90%), \(f_a\) is the unaffected fraction (1– \(f_u\)), and \(m\) is the coefficient of sigmoidicity of the dose–effect curve. For \(m = 1\), the dose–effect curve is hyperbolic; for \(m > 1\), it is sigmoidal; and for \(m < 1\), it is negative sigmoidal.
The dose–effect curve is plotted using a logarithmic conversion of this equation to

\[
\log(f_a/f_u) = m \log(D) - m \log(D_m) \tag{2}
\]

for the median effect plot \( x = \log(D) \) versus \( y = \log(f_a/f_u) \), which determines \( m \) (slope) and \( D_m \) (antilog of x intercept) values.

A combination index (CI) is then determined with the classic isobologram equation of Chou-Talalay (29, 30)

\[
CI = (D)_{1}\/(Dx)_{1} + (D)_{2}\/(Dx)_{2} \tag{3}
\]

where \( (Dx)_{1} \) is the dose of agent 1 (radiation) required to produce \( x \) percentage effect alone and \( (D)_{1} \) is the dose of agent 1 required to produce the same \( x \) percentage effect in combination with \( (Dx)_{2} \). Similarly, \( (Dx)_{2} \) is the dose of agent 2 (zoledronic acid) required to produce \( x \) percentage effect alone, and \( (D)_{2} \) is the dose required to produce the same \( x \) percentage effect in combination with \( (Dx)_{1} \). The denominators of the CI equation, \( (Dx)_{1} > (Dx)_{2} \), can be determined by \( Dx = Dx(1-f_a)/(1-f_u) \). Different values of CI may be obtained by solving the equation for different values of \( f_a \) (e.g., different degrees of inhibition of cell growth). CI values of \(<1 \) indicate synergy, CI values \( >1 \) indicate antagonism, and a CI of \( 1 \) indicates additive effects. The program CalcuSyn ( Biosoft, Ferguson, MO) was used to calculate the dose effect parameters \((m, D_m, r)\) and linear correlation coefficient \( r \) for each agent alone and for their fixed ratio combinations and for the quantification of CI values.

RESULTS

Treatment of C4-2 and IM-9 cells with increasing concentrations of zoledronic acid caused a dose-dependent decrease in cell number (Fig. 1). The toxic effects were similar for both cell lines with an effective dose required to produce a specified effect in 50% of the population (ED\(_{50}\)) of 320.4 cGy for the C4-2 cells and 32.0 \( \mu \)M for the IM-9 cells. Irradiation also caused a dose-dependent decrease in cell number (Fig. 2). The C4-2 prostate cancer cells with an ED\(_{50}\) of 1,314.6 cGy, however, were significantly more radiosensitive than the IM-9 cells with an ED\(_{50}\) of 91.1 cGy.

Combining radiation with zoledronic acid caused a greater reduction in cell viability than did either treatment on its own. Figure 3 shows that higher radiation doses result in successively lower cancer cell survival. The synergistic effect of zoledronic acid with radiation is most obvious when lower doses are used, as illustrated by the results seen especially between 20 and 200 cGy in the IM-9 myeloma cells (Fig. 3B).

Graphing the same data with the radiation dose on the x axis further highlights the added toxicity of the combination. Figure 4A shows the added cytotoxic effect of zoledronic acid when it is combined with radiotherapy in the C4-2 prostate cancer cells. As an example, 400 cGy without zoledronic acid (0 \( \mu \)M) result in a cell viability of almost 25%. The same cell viability is achieved with 150 cGy combined with 50 \( \mu \)M zoledronic acid.

The synergistic effect is especially prominent at lower radiation dosages. Both cell lines achieved their maximum cytoreduction level at a zoledronic acid concentration of about 100 \( \mu \)M.

Because our data suggest that zoledronic acid might have a synergistic effect with radiation, a detailed analysis of multiple drug effects was undertaken according to Chou and Talalay’s method (29, 31). The calculated dose effect parameters \( m, D_m \), and the linear correlation coefficient \( r \) for each agent alone and for their fixed ratio combinations are given in Table 1. The dose–effect relationship for zoledronic acid, radiation therapy, and their combination is clearly sigmoidal and indicates, on cursory inspection, an effect greater than would be expected by simple addition of the doses (Figs. 5A and 5B). This can be clearly seen in both cell lines. With C4-2 cells, for example, the \( D_m \) for radiation alone was 1,314.6 cGy and that for zoledronic acid 82.0 \( \mu \)M, whereas the same effect only required the combination of 320.4 cGy of radiation and 32.0 \( \mu \)M of zoledronic acid, which is less than half of each their \( D_m \).

Figure 6 demonstrates the synergistic effect of zoledronic acid and radiation quantitatively. The combination index is well below 1 over the entire range of \( f_a \) values, suggesting synergism at all effect levels. According to Chou and Talalay (29), the effect on the IM-9 cells is moderately synergistic, because the combination effect is between 0.7 and 0.85. For the C4-2 cells, the combination effect is between 0.3 and 0.7, which means it is clearly synergistic.

DISCUSSION

Our study substantiates the cytotoxic effects of zoledronic acid on both prostate cancer and lymphoma cells.
These results agree with those reported by Oades et al. (32), except that their ED_{50} using three different prostate cell lines was about five times lower (10 μM) than the one determined in our study with C4-2 cells. Another article by Lee et al. reported cell growth inhibition levels closer to our results, between 10–50 μM (19). It is not clear why such differences are seen, although cell type and media differences are likely to be involved in the discrepancy. Assay time and type might also play a role in these differences. Brown and Wouters, for example, have shown that results from short-term MTT assay can be quite different from those of a long-term clonogenic assay (33). Our results with IM-9 cells, on the other hand, are close to the ones previously reported (34, 35).

The radiation sensitivities we measured are typical results for both prostate cancer and lymphoma cell lines (36, 37). The results for the combination of zoledronic acid and radiation, however, are novel and intriguing because they immediately suggest the clinical utility of using combined zoledronic acid and radiation in the clinic. The combination proved to be more active than simple additive effects would let us expect, as shown clearly by the combination index calculated with the median effect analysis (Table 1 and Fig. 6). This synergistic effect was statistically significant and may have been even greater if the concentration and kinetics of the drugs had been further optimized. For example, in our experiment, zoledronic acid was added to the cells approximately 30 min before gamma irradiation with a^{137}Cs source. Increasing this incubation time to yield the optimal BP concentration during irradiation might have enhanced the synergistic effect of BP and radiation.

Treatments of bone metastases with combinations of BP and radiation have been described in the literature both in animal experiments and clinical trials. None, however, has mentioned the potential of BP, or specifically of zoledronic acid, for synergistically enhancing the treatment response in
the lesion. The BP are generally thought to be agents for increasing remineralization and bone density, as shown by Krempien et al., who treated rats with Walker carcinosarcomas in the proximal tibia with a combination of daily clodronate (20 mg intraperitoneally) and 17 Gy of irradiation (38). Kouloulias et al. reported similar significant increases in bone mass and bone formation in a clinical study of 33 breast cancer patients with bone metastases (39). The patients received long-term BP (pamidronate 180 mg every month for 2 years), together with radiation therapy (30 Gy in daily local doses of 3 Gy). The reported outcomes were a high 88% complete response with no significant toxicity of long-term pamidronate in conjunction with radiotherapy.

Although no other studies published to date have specifically looked at the concomitant combination therapy of zoledronic acid and radiation, many patients receive BP and at some later point also receive concomitant radiation therapy, as documented in a recent Phase III trial by Rosen (14) and a recent review by Hoskin (40). The increase in bone density and decrease in pain (41) are excellent reasons for pursuing this combination therapy.

In addition, it might be possible to further enhance tumor cell eradication and treatment success by defining the ideal doses and application schedules for optimal synergistic effects of radiation and BP.

Zoledronic acid has been shown to act synergistically with other chemical substances, a notion that further supports the combined use of BP and radiotherapy. Tassone was the first to show, in several different myeloma cell lines, that zoledronic acid and dexamethasone inhibit cell growth and synergistically induce apoptosis (35). Jagdev et al. then demonstrated that zoledronic acid and paclitaxel have synergistic effects, suggesting that BP not only inhibit bone resorption, but also are directly active as antitumor agents (42). Other preclinical studies with combinations of BP and chemotherapeutic agents have also shown the benefits of the combination regimen (43–45). A most exciting recent development is that sequential treatment of two drugs is even better than simultaneous treatment (46). Neville-Webbe orally recently presented (IVth International Conference on Cancer-Induced Bone Diseases, December 7–9, 2003; San Antonio, TX) new data that showed good synergy between sequential treatment of breast cancer cells with doxorubicin followed by zoledronic acid (1 h at 1 μM, a clinically achievable exposure).

Some cell culture and animal studies suggest that BP promote apoptosis in cancer cells, such as myeloma, breast, and prostate cancer cells (6, 20, 34, 47, 48). This effect, however, was often only seen with high BP concentrations. It is not feasible to reach such high concentrations in nonosseous metastases in vivo, which was probably the reason that studies in nonosseous animal tumor models have generally not shown a

<table>
<thead>
<tr>
<th></th>
<th>C4-2 cells</th>
<th>IM-9 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zoledronic acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>m</td>
<td>1.450</td>
<td>1.407</td>
</tr>
<tr>
<td>(D_m (\mu M))</td>
<td>82.0</td>
<td>58.6</td>
</tr>
<tr>
<td>r</td>
<td>0.961</td>
<td>0.962</td>
</tr>
<tr>
<td>Radiation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>m</td>
<td>0.970</td>
<td>1.125</td>
</tr>
<tr>
<td>(D_m (cGy))</td>
<td>1314.6</td>
<td>91.2</td>
</tr>
<tr>
<td>r</td>
<td>0.979</td>
<td>0.958</td>
</tr>
<tr>
<td>Zoledronic acid and radiation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>m</td>
<td>1.356</td>
<td>1.274</td>
</tr>
<tr>
<td>(D_m (\mu M/cGy))</td>
<td>32.0/320.4</td>
<td>20.6/41.2</td>
</tr>
<tr>
<td>r</td>
<td>0.998</td>
<td>0.981</td>
</tr>
</tbody>
</table>

The dose required to reach a 50% effect \(D_m (ED_{50})\) is the antilog of x-intercept in μM and cGy; m is the slope of the median effect plot signifying the shape of the dose–effect curve (\(m = 1\) indicates a hyperbolic, \(m > 1\) a sigmoidal, and \(m < 1\) a negative sigmoidal curve) and r is the linear correlation coefficient of the median effect plot.

![Fig. 5. Cytotoxic effects of zoledronic acid and radiation to (A) C4-2 cells and (B) IM-9 cells. The lowest effective dose for zoledronic acid was 10 μM for both cell lines. Because the lowest effective radiation doses for C4-2 and IM-9 cells were different (100 cGy and 20 cGy, respectively), a different ratio was chosen for the multiple drug effect analysis (1:10 and 1:2, respectively).](https://example.com/image-url)
Concentrations up to 1,000/\text{H9262} areas of bone destruction after a short time (within 1 h) (9). Venously, very high concentrations of BP have been found in acid would result in a blood concentration of about 2 using the common clinical dosing level of 4 mg of zoledronic tumor reduction (49, 50). Biodistribution studies performed using the common clinical dosing level of 4 mg of zoledronic acid would result in a blood concentration of about 2 \mu{M}, which our experiments have shown to be ineffective under the experimental conditions used. However, the requisite high concentrations of BP should be reached in the bone destruction regions, because their chemical structure makes them selectively accumulate zoledronic acid (9, 10). When given intravenously, very high concentrations of BP have been found in areas of bone destruction after a short time (within 1 h) (9). Concentrations up to 1,000 \mu{M} have been reported in the space beneath resorbing osteoclasts (6). Because metastatic cells are found in close association with these sites of active bone resorption in myeloma and bone metastasis of prostate cancer (51, 52), the synergistic effects of a combination of BP with local radiotherapy would be expected to be most evident at these sites.

Radiotherapy is accepted for the local treatment of bone metastases (53), although different dose schedules, such as a single 8-Gy dose or 30 Gy delivered in 10 fractions, are used depending on the cancer type, patient situation, and institutional treatment protocol (54–56). The goal of the radiotherapy is to eradicate malignant cells without damaging surrounding normal cells. In multiple myeloma, radiotherapy is indicated in solitary lesions and lytic, painful bone lesions. Radiotherapy is usually delivered only to the immediate lesion area to spare as much bone marrow and normal soft tissue as possible. In bone metastases, the endpoint of clinical success is the pain palliation and enhancement of bone strength. In patients presenting with widespread bone metastases, retreatment of the same bone lesion is a factor of diminishing life quality. Adding zoledronic acid to the standard palliative radiotherapy might improve its effectiveness. The outcomes can be monitored in terms of mineralization, which represents the healing of a particular metastasis. For patients with minimal bone involvement, there might even be a survival advantage from the concomitant treatment. For myeloma and prostate carcinoma, combining standard radiation treatments with zoledronic acid might produce the same effect with a lower radiation dose, thus allowing for fewer side effects, lower fraction doses, or lower total doses. There is more than one way to translate our \textit{in vitro} results into a clinical trial. One possibility is intravenous infusion of 4 mg zoledronic acid 1 day before starting radiation therapy (e.g., 30 Gy in 10 fractions) followed by further zoledronic acid infusions every 3 weeks.

Our results suggest that it is important for a radiation oncologist to know if a patient is taking or has been treated previously with BP. If a patient simultaneously is under treatment with zoledronic acid during radiotherapy, the treatment response might be accelerated. If the patient took BP some time before radiotherapy, then possible interactions are still unknown. After BP bind to bone, the newly formed bone covers the BP molecules, embedding them deep beneath the bone surface (9). Such bound BP lose their inhibitory effect on bone destruction and BP doses then have to be repeated for maximal effect (4, 11). It is unknown, however, if BP would lose their radiosynergistic interaction potential under these circumstances.

Newer Food and Drug Administration–approved radiation treatments with the same aim of eradicating malignant bone metastases without damaging normal cells include the radiopharmaceuticals $^{89}$Sr chloride (Metastron) and $^{153}$Sm lexidronam (Quadramet). These radiopharmaceuticals might also be advantageous in combination with BP because they seem to accumulate in the same bone lesions as the BP, irradiate areas of high BP concentrations, and thus might synergistically increase the treatment effects. We are implementing additional laboratory experiments designed to explore this hypothesis as well as a clinical trial of concomitant external beam radiotherapy and zoledronic acid for patients with symptomatic bone metastases.

\textbf{CONCLUSION}

Zoledronic acid combined with radiotherapy can induce cytotoxicity in a synergistic manner in prostate cancer and myeloma cells. This \textit{in vitro} finding agrees with previous studies showing a synergistic effect of zoledronic acid with different chemotherapeutic drugs. The interaction of BP-induced apoptosis and radiation-induced cell death, however, is not clear yet and requires more fundamental studies. Transferred to the clinic, the combination of zoledronic acid and radiotherapy might allow a reduction in radiation fraction doses or fraction numbers with the same therapeutic or palliative effect. In addition, the use of high-dose precision radiotherapy delivered via stereotactic localization methodologies may allow long-term control of large focal lesions in patients with just one or (at most) a few metastatic sites (“oligometastases”) (57).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{image.png}
\caption{The combination index (CI) for the cell treatments with zoledronic acid and radiation at a ratio of 1:10 for C4-2 cells and of 1:2 for IM-9 cells. A CI of 1.0 indicates that the effects are additive, whereas a CI lower than 1.0 clearly points to synergistic effects.}
\end{figure}
REFERENCES


52. Mundy GR, Yoneda T, Hiraga T. Preclinical studies with zoledronic acid and other bisphosphonates: impact on the bone microenvironment. Semin Oncol 2001;28:35–44.


