Review

Direct effects of bisphosphonates on breast cancer cells

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Abstract

In addition to inhibiting bone resorption, bisphosphonates have also been shown to exhibit antitumour effects. *In vitro*, bisphosphonates inhibit proliferation and induce apoptosis in cultured human breast cancer cells. In addition, bisphosphonate treatment interferes with breast cancer cell adhesion to bone matrix, and inhibits cell migration and invasion. The combination of bisphosphonates with other anticancer drugs such as the taxoids markedly enhances these effects. These newly recognized direct actions of bisphosphonates on breast cancer cells indicate that these agents may have a greater role to play in treatment of patients suffering from cancers with a propensity to metastasize to bone.

Keywords: apoptosis, bisphosphonates, breast cancer, cell adhesion, invasion

Introduction

Over 80% of women with advanced breast cancer ultimately develop bone metastases that result in significant morbidity and mortality. Breast cancer metastases in bone can cause intractable pain, bone fracture, spinal cord compression and hypercalcaemia [1–3]. From the moment breast cancer cells arrive in the bone microenvironment, however, they stimulate bone resorption with subsequent selective increase in the attraction and growth of new cancer cells to bone [4]. Therefore, any treatment aimed at palliation or perhaps even prevention of bone metastases should focus on disrupting this attraction and growth, which are involved in the initiation and amplification of the metastatic process.

Bisphosphonates are widely used for the treatment of bone metastases, and an increasing body of evidence suggests that these compounds provide benefit to breast cancer patients with secondary cancers in bone [5]. Bisphosphonates are analogues of endogenous pyrophosphates in which a carbon atom replaces the central atom of oxygen. *In vivo*, bisphosphonates bind strongly to hydroxyapatite on the bone surface and are preferentially delivered to sites of increased bone formation or resorption. They are potent inhibitors of osteoclast-mediated bone resorption [6] and are effective in lowering serum calcium concentrations in patients with hypercalcaemia of malignancy [7,8]. Treatment with bisphosphonates has also been shown to reduce skeletal morbidity significantly and to improve quality of life in breast cancer patients with bone metastases [7].

The mechanisms by which bisphosphonates inhibit osteoclast-mediated bone resorption appear to involve an inhibition of formation of osteoclasts from immature precursor cells [6,9,10] or direct inhibition of resorption via induction of apoptosis in mature osteoclasts [9,11,12]. Furthermore, as outlined elsewhere, bisphosphonate treatment has been shown to inhibit the progression and development of bone metastases in a mouse model of breast cancer [13,14]. Such a beneficial effect of bisphosphonates on tumour burden in bone may result from a direct antitumour effect on breast cancer cells. Evidence is now emerging that this is indeed the case and that treatment of cultured human

MMP = matrix metalloproteinase.

breast cancer cells treated with bisphosphonates induces inhibitory effects on adhesion, invasion and cell survival.

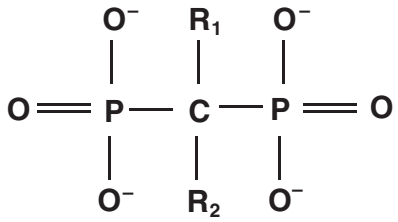
Effects of bisphosphonates on cell adhesion to and invasion of bone

It is well accepted that adhesion of cancer cells to bone matrix is a vital step in the bone metastasis process, and it has been suggested that exposure of bone to bisphosphonates could alter properties of the bone matrix that are required for adhesion of breast cancer cells. In this regard, previous studies [6,9,15] have indicated that exposure of calcified matrix of bone to bisphosphonates *in vitro* alters the properties of the bone matrix that are required for attachment of the osteoclast.

Initially, the ability of human breast cancer cells to adhere to bone matrices that had been pretreated with bisphosphonates was investigated by van der Pluijm *et al*. [16]. In those experiments adhesion of MDA-MB-231 human breast cancer cells to bovine cortical bone slices and sections of developing trabecular bone from neonatal mouse tail were assessed. Those studies showed that pretreatment of bone matrices with certain bisphosphonates at concentrations of 1–100 μmol/l not only prevented adhesion of breast cancer cells to bone matrix, but also inhibited cell spreading. However, of the bisphosphonates tested only pretreatment of matrices with nitrogen-containing bisphosphonates (pamidronate, olpandronate, alendronate and ibandronate) led to these inhibitory effects. Ibandronate was found to be the most potent compound. Pretreatment with clodronate or etidronate did not affect adhesion to bone matrix or cell spreading, and the order of potency of the six bisphosphonates corresponded to their ranking in bone resorption assays [6,9]. No effects on cell viability were observed over the 3 h period during which the cells were allowed to adhere to bone matrices.

Subsequently, Boissier *et al.* [17] evaluated the effect of direct treatment of breast cancer cells with bisphosphonates on their ability to adhere to unmineralized and mineralized bone extracellular matrices. Using mineralized bovine cortical bone slices and unmineralized extracellular matrices produced by cultured osteoblastic cells, those investigators found that pretreatment of MCF-7 and MDA-MB-231 breast cancer cells for 24 h with bisphosphonates inhibited cell adhesion. Similar effects were found with cultured prostatic carcinoma cells. Of the bisphosphonates used, only ibandronate, NE-10244 (antiresorptive active pyridinium analogue of risedronate) and pamidronate inhibited cell adhesion at low concentrations, with half-maximal inhibitions at 5 pmol/l, 0.1 nmol/l and 10 nmol/l, respectively. Clodronate achieved the same inhibitory effects at a high concentration, with half-maximal inhibition at 10 μmol/l. At concentrations that inhibited cell adhesion after 24 h of treatment, no effects of bisphosphonates on cell viability or integrin expression were detected.

Figure 1



Structure of bisphosphonates.

The mechanism by which the various bisphosphonates inhibit cell adhesion when coated onto mineralized or unmineralized matrix does not relate to their direct inhibitory action when incubated with cells *in vitro*. Thus, the analogue NE-58051 (inactive pyridylpropylidene analogue of risedronate, which lacks a methyl group in the R2 chain of the molecule; Fig. 1) was effective in preventing cell adhesion when cortical bone slices were coated with this compound [17]. However, pretreatment of cultured breast cancer cells *in vitro* with NE-58051, before seeding onto uncoated bone slices, did not affect adhesion.

The taxoids taxol and taxotere are effective antitumour compounds that are currently used routinely in the treatment of metastatic breast carcinoma. Magnetto *et al.* [18] determined whether there could be additive or synergistic effects of bisphosphonates in combination with taxoids on adhesion of breast cancer cells to bone. Using cortical bone slices, it was shown that exposure of MDA-MB-231 cells to taxoids for 1 h inhibited adhesion to mineralized bone matrices in a dose-dependent manner, with half-maximal inhibition seen with approximately 80 nmol/l for both taxoids. However, the concentrations of taxoids used to inhibit cell invasion (up to 500 nmol/l) were 25-fold higher than the concentrations required to induce apoptosis. In combination experiments the inhibitory effect of ibandronate on cell adhesion was additive to that of taxoids. Studies done using matrigel invasion assays revealed that exposure of breast cancer cells to ibandronate for 23 h followed by 1 h exposure to taxoids increased inhibitory effects on cell invasion by 70–78% as compared with taxoids alone.

Because breast cancer cell invasion requires both cell migration and digestion of the basement membrane by matrix metalloproteinases (MMPs), bisphosphonates could affect one or the other of these mechanisms. Because ibandronate treatment did not inhibit cell migration in the study of Magnetto *et al*. [18], it is possible that the bisphosphonate mediates effects on cell invasion by decreasing production of MMPs or by inhibiting their activity. Using similar methods, those investigators determined the order of potency of four bisphosphonates in the inva-

sion assay [19]. The order of potency was found to be as follows: zoledronic acid > ibandronate > NE-10244 (active analogue) > clodronate. The half-maximal inhibition values were found to be <1 pmol/l, 1 pmol/l, 0.5 nmol/l and 50 μmol/l, respectively.

NE-58051 had no inhibitory effects on cell invasion [19], which is in accord with its ineffectiveness in preventing cell adhesion, as outlined above. This indicates that the direct inhibitory action of bisphosphonates on breast cancer cells involves the R2 group of the molecule (Fig. 1). On the other hand, NE-10790 (a phosphonocarboxylate analogue of risedronate, in which one of the phosphonate groups is substituted by a carboxyl group) had inhibitory effects on cell invasion to an extent similar to that observed with NE-10244, even though NE-10790 has little effect on antiresorptive activity as compared with NE-10244 on bone. This suggests that the pharmacological mechanism of action of bisphosphonates on tumour cell invasion is distinct from the mechanism of action on bone.

The results from that study [19] also lend support to the suggestion that the inhibitory effects of bisphosphonates on cell invasion are related to the inhibition of the proteolytic activity of MMPs rather than to modulation of their expression. At high concentrations (~100 μmol/l), bisphosphonate treatment inhibited the activity of MMP-2, -9 and -12. Excess of zinc completely reversed bisphosphonate-induced inhibition of cell invasion. In addition, NE-10790 did not inhibit MMP activity. Those findings suggest that the phosphonate groups of bisphosphonates are responsible for the chelation of zinc and the subsequent inhibition of MMP activity. However, although treatment with NE-10790 did not decrease MMP activity, it inhibited breast cancer cell invasion to an extent similar to that observed with NE-10244; this suggests that inhibition of MMP activity is not the sole mechanism by which bisphosphonates inhibit invasion.

Effects of bisphosphonates on breast cancer cell growth and apoptosis

Previous studies have shown that bisphosphonates reduce metastatic tumour burden in bone with increased apoptosis in osteoclasts [20]. In addition, a number of *in vitro* studies have indicated that bisphosphonate treatment of myeloma cells leads to growth inhibition and induction of apoptosis [21]. Clinical findings have suggested that clodronate treatment may reduce incidence of bone metastases, although these results are not yet conclusive [22]. Taken together, these results indicate that bisphosphonates may exert direct growth inhibitory effects on breast cancer cells, leading to reduced metastatic tumour burden in bone.

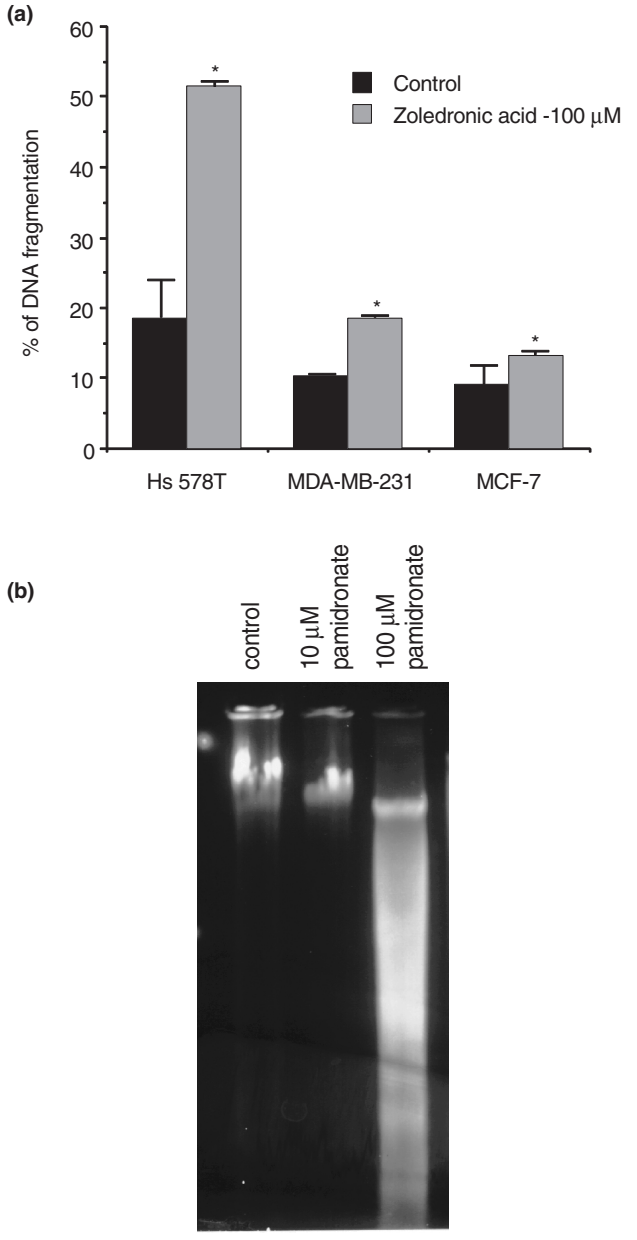
This suggestion was confirmed by our group with the demonstration that treatment of cultured breast cancer cells with bisphosphonates leads to growth inhibition and induction of apoptosis [23]. In those initial studies, a non-reversible inhibition of cell growth and viability of three human breast cancer cell lines was seen, together with morphological and biochemical changes consistent with apoptosis. Four structurally different bisphosphonates (zoldedronic acid, pamidronate, clodronate and EB-1053) induced apoptosis in a time- and dose-dependent manner. Zoledronic acid was found to be the most potent bisphosphonate, with half-maximal inhibition values in MDA-MB-231 cells of 15 μmol/l; the corresponding values for pamidronate, EB-1053 and clodronate were 40, 1000 and 700 μmol/l, respectively. Furthermore, the order of potency of the bisphosphonates was similar in all three cell lines tested. This finding is in contrast to that of Busch *et al*. [24], who reported that clodronate is able to reduce survival of MDA-MB-435S but not that of MCF-7 cells.

Our findings indicate that the order of bisphosphonate potency on bone resorption is not equivalent to that for inhibiting cell viability in breast cancer cells [23]; it is cell-type-specific. Zoledronic acid has been reported to be 100 times more potent than pamidronate in reducing bone resorption [25], and our study suggests it is approximately threefold more potent than pamidronate in reducing cell viability in breast cancer cells. EB-1053, which is 100 times more potent than pamidronate in inhibiting bone resorption in rats [25], was substantially less effective on breast cancer cells.

We found evidence for fragmentation of chromosomal DNA, a key feature of apoptosis, in MCF-7, MDA-MB-231 and Hs578T breast cancer cells after 2–3 days of treatment with bisphosphonates (Fig. 2) [23]. Induction of apoptosis in MDA-MB-231 cells by pamidronate was accompanied by decreased expression of the antiapoptotic protein bcl-2 as well as cleavage of poly (ADP-ribose) polymerase, thus implicating the activation of a caspase-dependent pathway. This was later confirmed by Fromigue *et al*. [26], who showed that inhibition of MCF-7 cell proliferation by four bisphosphonates (zoledronic acid, ibandronate, pamidronate and clodronate) could be abrogated by cotreatment with z-VAD-fmk, a broad-spectrum caspase inhibitor. We similarly found that z-VAD-fmk attenuates loss of MDA-MB-231 breast cancer cell viability in response to pamidronate (Fig. 3) and identified caspase-3 as one of the cell death proteases that are activated by zoledronic acid treatment in MDA-MB-231 cells [27]. Furthermore, Hiraga *et al*. [28] demonstrated that a selective caspase-3 inhibitor is capable of blocking ibandronate-induced DNA fragmentation in these breast cancer cells.

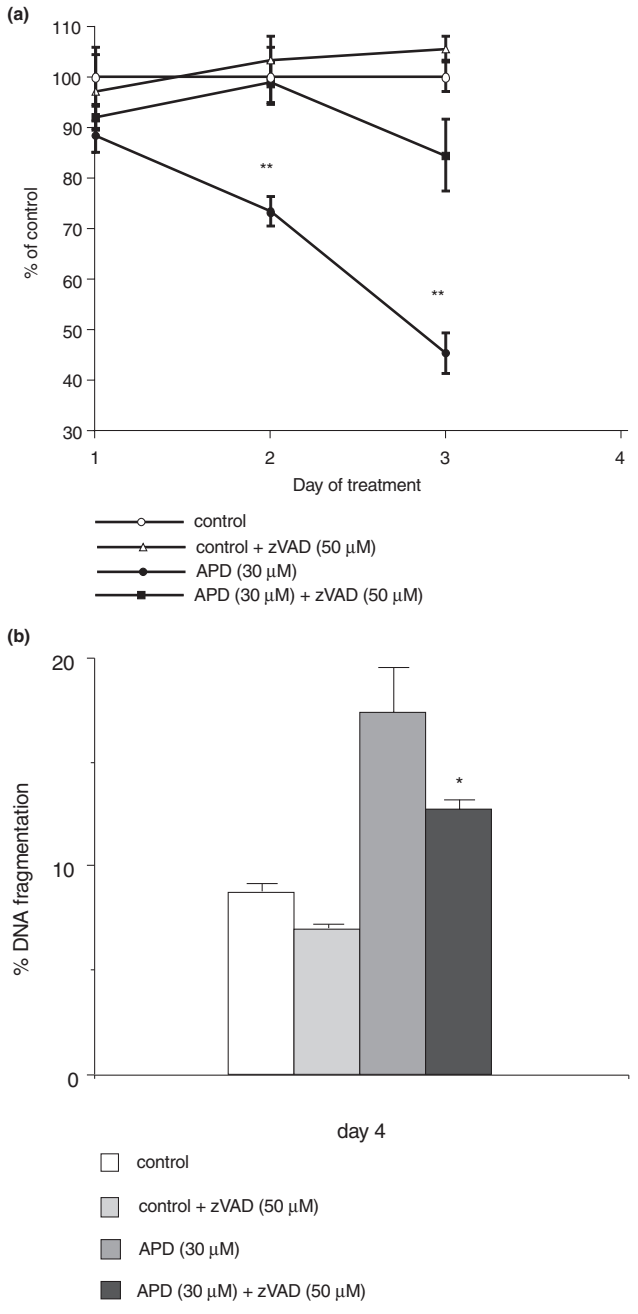
A recent report by Jagdev *et al.* [29] presents evidence for synergistic effects of zoledronic acid and paclitaxel on induction of apoptosis in MCF-7 and MDA-MB-231 breast

Figure 2



(a) Hs 578T, MDA-MB-231 and MCF-7 breast cancer cells were treated for 2 days with 100 μmol/l zoledronic acid. The percentage of fragmented chromosomal DNA was measured in cells treated with zoledronic acid and compared with that of control (vehicle-treated) cells as previously described [23]. *$P < 0.0005$ versus control. **(b)** Apoptosis was examined by electrophoretic analysis of internucleosomal DNA fragmentation following treatment of MDA-MB-231 cells with 10 and 100 μmol/l pamidronate for 3 days.

Figure 3



(a) Attenuation by broad-spectrum caspase inhibitor z-VAD-fmk (zVAD) of the effects of pamidronate (APD) on cell viability in MDA-MB-231 cells. Cells were plated in 96-well plates (1×10^3 cells/well) and treated with 50 μmol/l zVAD 1 h before addition of 30 μmol/l APD for 3 days. On days 1, 2 and 3, cell viability was quantitated using MTS dye reduction assay. Results are shown as mean ± SD. **$P < 0.0001$ versus APD + zVAD treatment. **(b)** Effects of zVAD on APD-induced DNA fragmentation in MDA-MB-231. Cells were plated in 6-well plates at a density of 1×10^6/well and treated with 50 μmol/l of zVAD for 1 h before addition of 30 μmol/l APD for 4 days without renewal of medium. Cotreatment with zVAD significantly reduced APD-induced DNA fragmentation. *$P < 0.0005$ versus APD treatment alone.

cancer cells. Those investigators found a fourfold to fivefold increase in induction of apoptosis in MCF-7 cells when zoledronic acid was used in combination with paclitaxel. This finding is in accord with previous findings of additive effects of bisphosphonates and taxoids on inhibi-

tion of breast cancer cell invasion and adhesion. In addition, Hiraga *et al*. [28] reported that bisphosphonates directly induce apoptosis in breast cancer cells that metastasize to bone. Ibandronate (4 μg/mouse per day subcutaneously) was administered after bone metastases had been established by intracardiac inoculation of MDA-MB-231 cells. Inhibition of progression of established osteolytic bone metastases was demonstrated by radiological analysis. Ibandronate significantly decreased tumour burden and increased MDA-MB-231 cell apoptosis in bone metastases. Ibandronate treatment was not able to induce apoptosis in tumours developed by inoculation of MDA-MB-231 cells in the orthotopic mammary fat pads, however, indicating that effects of ibandronate on breast cancer cell apoptosis are restricted to bone in which ibandronate selectively deposits.

The mechanisms by which bisphosphonates promote breast cancer cell apoptosis remain to be established. In osteoclasts and myeloma cells it has been suggested that nitrogen-containing bisphosphonates induce apoptosis by inhibiting enzymes in the mevalonate pathway, preventing the generation of isoprenoid moieties and thereby impairing the isoprenylation (farnesylation and geranylgeranylation) of small GTP proteins such as Ras, Rho and Rac [11,30,31]. Jagdev *et al*. [29] recently presented evidence that loss of MCF-7 cell viability induced by zoledronic acid could be prevented by coincubation with geranylgeraniol, suggesting a role for impaired protein geranylgeranylation in the effects of the bisphosphonate. The identity of the protein that is affected by zoledronic acid treatment remains to be determined, however. Our own studies have shown that treatment of both MCF-7 and MDA-MB-231 cells with zoledronic acid leads to impaired membrane localization of Ras that is consistent with impaired farnesylation [27]. Further studies are required to determine the signal transduction pathways that are modulated by the alterations in protein isoprenylation induced by bisphosphonates.

Conclusion

Laboratory studies increasingly suggest that bisphosphonates can induce important antitumour effects in breast cancer cells *in vitro* by promoting apoptosis, and inhibiting cell adhesion and invasive potential. It is therefore possible that the beneficial effects reported in patients receiving treatment with bisphosphonates may involve direct effects on tumour cells in bone as well as inhibition of osteoclast-mediated bone resorption. It has been noted, however, that the *in vitro* concentrations of bisphosphonates required to induce breast cancer cell apoptosis are higher than those required for osteoclast apoptosis. At the present time the concentrations of bisphosphonates to which tumour cells in bone are exposed are unclear.

Bisphosphonates bind to hydroxyapatite by virtue of their carbon-substituted pyrophosphate structure, and this accounts for their selective action on the skeleton. The local concentrations of bisphosphonate released from the hydroxyapatite surface into the resorption space are probably considerably higher than the circulating concentration, and have been suggested to approach 800 μmol/l [32]. If this is the case then cancer cells may be exposed to concentrations that are sufficient to induce apoptosis.

In summary, the newly recognized direct actions of bisphosphonates on breast cancer cell adhesion, invasion and cell viability indicate that these agents may have a wider role to play in prophylactic treatment of patients suffering from cancers with a propensity to metastasize to bone.

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