Preclinical evidence for the effect of bisphosphonates and cytotoxic drugs on tumor cell invasion

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Bisphosphonates (BPs) are stable pyrophosphate analogs currently used in the treatment of patients with metastatic bone disease, known to affect bone resorption by reducing osteoclast activity. Use of these drugs in adjuvant therapy is currently under investigation following reports of an effect of BPs on tumor cell apoptosis in preclinical models. Recent evidence has suggested that BPs might also affect tumor cell invasion *in vitro*, and the component processes of adhesion, migration and degradation, through mechanisms including inhibition of prenylation of intracellular small GTPases such as Ras and Rho. The effects potentially may be enhanced through co-administration with chemotherapy agents, as both synergistic and additive effects have been described *in vitro*. This review discusses the preclinical evidence for the potential use of BPs and cytotoxic drugs for inhibiting tumor cell invasion, a key process in cancer progression. *Anti-Cancer Drugs* 16:11–19
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Introduction

Despite unprecedented advances in tumor therapy, the main cause of treatment failure and death from cancer is the development of metastasis [1]; a process involving the detachment of malignant cells from the primary tumor mass, followed by dissemination and establishment at a distant site [2]. For successful invasion, tumor cells from the primary tumor must undergo a series of adhesion and dissociation steps, proceeded by movement through the complex network of proteins and polysaccharides comprising the extracellular matrix (ECM) and basement membrane, and ultimately the endothelium (Fig. 1) [3]. During migration through the ECM, the leading edge of the cell adheres to new substrates requiring altered expression of cell adhesion molecules such as integrins to guide the cells and provide the necessary traction required to pull the cell forward. The trailing edge of the cell retracts from the matrix proteins, involving dissociation of cell adhesion molecules from their ligands. To assist motility, degradation of the individual components of the ECM and basement membrane is essential, facilitated by secretion and activation of a number of proteolytic enzymes, including the matrix metalloproteinase (MMP) and serine protease families, producing protein fragments for cells to migrate towards and adhere.

The migration and invasion of tumor cells of epithelial origin such as breast, prostate and colorectal cancer cells is controlled intracellularly by members of the Ras superfamily of small GTPases such as Ras, Rho, Rac and Rab [4,5]. Upon extracellular stimulation a cascade of events is initiated, and through activation of proteins such as integrins on the cell surface and down-stream tyrosine kinases, the transfer of inactive Ras-GDP to active Ras-GTP is regulated. Once activated, Ras-GTP molecules further stimulate a multitude of cytosolic effector molecules to control many intracellular processes including cytoskeletal organization to determine cell motility, cell adhesion and matrix degradation. For many tumors, dysregulation of Ras activity (or receptors coupled to Ras proteins), through over-expression or mutation of the GTPase or of the down-stream signaling pathways can lead to a more motile, invasive and thus aggressive phenotype, as, for example, has been shown in the breast cancer cell lines MDA-MB 435 for Rac 1 and MCF-7 and MDA-MB 231 for Rho-A and Rho-C [6–11] and the prostate cancer cell line LNCaP [12].

Breast and prostate cancer metastasis to the bone

For many cancers, tumor cell dissemination is an early event of malignancy, often going undetected at the time of initial diagnosis. Growth of micrometastases at distant sites is thought to occur in many cases, with tumor cells often targeting specific organs. Of such tumors, breast and prostate cancers are good examples, frequently targeting the bone and often resulting in skeletal complications including pain, fracture and spinal cord compression. Under normal circumstances, tight regulation of bone formation by osteoblasts and bone

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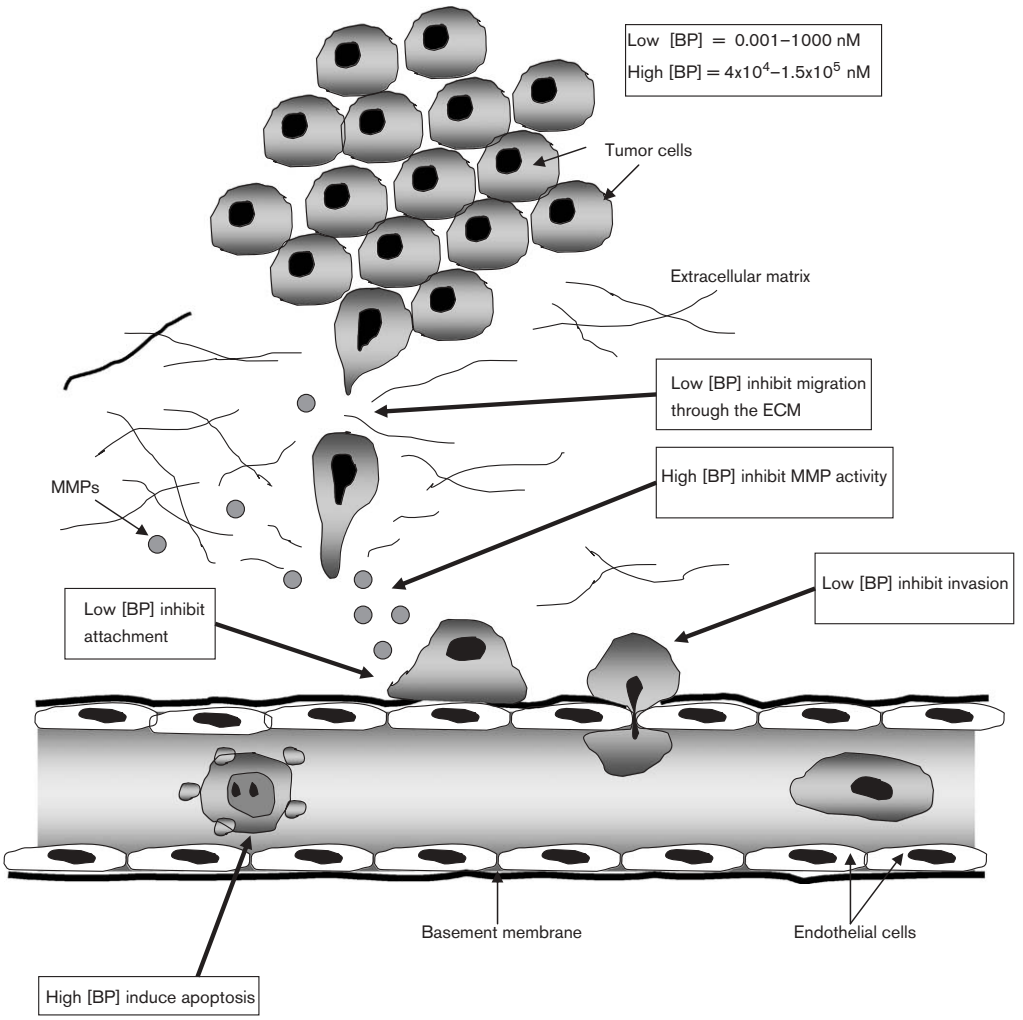
Fig. 1



Schematic illustration of the effects of BPs on tumor cell invasion. Nitrogen-containing BPs have been shown to inhibit migration through the extracellular matrix [43], MMP activity, adhesion to the extracellular matrix and invasion [31,36] and induce apoptosis [19], by preventing prenylation of a wide variety of intracellular GTPases, including Ras and Rho. Low bisphosphonate concentrations are reported as 0.001–1000 nM and high bisphosphonate concentrations are 4×10^4–1.5×10^5 nM.

breakdown by osteoclasts exists. This fine-tuned balance is disrupted by the development of bone metastases facilitating either increased bone resorption by osteoclasts or bone formation by osteoblasts, ultimately leading to osteolytic and osteoblastic lesions in breast and prostate cancers, respectively.

At present a number of therapies are offered for breast and prostate cancer patients often following surgical intervention, dependent upon the individual prognosis, relating to tumor grade, hormonal status and other biological considerations. In the case of breast cancer patients, in addition to radiotherapy, cytotoxic drugs such as anthracyclines and taxoids are commonly given as early treatments, often in combination with hormonal therapy. For many patients, however, metastatic bone disease will still develop. Once metastasized, treatment is primarily palliative, aiming to reduce the associated complications of bone destruction, reducing pain and morbidity. Median survival of patients with metastatic disease is 2–3 years [13], and thus improvement in quality of life for this period is of primary importance. At this stage use of bisphosphonates (BPs), a family of drugs commonly used to treat osteoporosis sufferers, has been found to reduce the frequency of skeletal-related events and hence improve their quality of life.

BPs are stable pyrophosphate analogs, potently inhibiting osteoclast-mediated bone resorption. Two classes of BPs exist, defined by the presence or absence of a nitrogen

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atom in the second (R_2) side-chain from the central carbon atom. Once administered, these drugs rapidly distribute to the bone, binding strongly to hydroxyapatite bone mineral surfaces. The precise concentration of BPs in the resorption lacunae is currently unknown, but values of 0.1–1 mM have been approximated [14]. In contrast, peak plasma levels are significantly lower with estimated values of 1–3 μM, which are maintained for a few hours only [14,15]. Following administration, osteoclasts internalize BPs, and through either inhibition of the mevalonate pathway by nitrogen-containing BPs (N-BPs) (e.g. pamidronate, ibandronate, alendronate, risedronate and zoledronate) or by incorporation as non-hydrolysable ATP analogs by non-nitrogen containing BPs (non-N-BPs) (such as clodronate and etidronate), their resorptive activity is inhibited through disruption of processes including cytoskeletal organization, vesicular trafficking and ruffled border formation. Of the two BP classes, N-BPs are more potent than non-N-BPs, with zoledronate proving to be the most potent at reducing bone resorption. By inhibiting a key enzyme in the mevalonate pathway [farnesyl diphosphate (FPP) synthase], N-BPs prevent protein prenylation (geranylgeranylation and farnesylation) of a wide variety of small intracellular G-proteins including Ras and Rho (Fig. 2). As a consequence, loss of prenylation alters the subcellular distribution of small GTPases, preventing them from acting at the correct spatial location within the cell, thus ultimately affecting normal cell function. RhoC mutant cells treated with farnsesyl transferase inhibitors (FTIs) to directly prevent farnesylation (Fig. 2) are, for example, less invasive and motile than their non-transfected counterparts [8]. In response to a decrease in osteoclast-mediated resorption, fewer growth factors are released from the bone, affecting attraction of tumor cells to the bone matrix, their invasive phenotype and subsequent growth [16]. A recent study by Fromigue *et al.* has, for example, shown that at 1 μM concentrations, ibandronate, pamidronate, zoledronate and clodronate all decrease IGF-I, IGF-II, FGF-2 and EGF release from bone, thereby reducing MCF-7 and T47D breast cancer cell survival by limiting the supply of bone-derived growth factors [17]. In support using a rat model, Kostenuik *et al.* demonstrated growth of metastatic Walker 256 carcinosarcoma tumor cells in bone is increased following stimulation of bone resorption, implying that the factors released affected cell growth [18].

Similar to the effects on osteoclasts, extensive evidence has now also shown a pro-apoptotic effect of BPs on tumor cells including breast and prostate cancer cells, proposing involvement of inhibition of the mevalonate pathway [19], reduction of anti-apoptotic gene bcl-2 [20,21], and activation of caspases in response to cytochrome *c* release into the cytosol [20,22,23]; all of which are associated with Ras inactivation in response to decreased prenylation [21,23]. Moreover, through studies carried out *in vitro* it is now becoming evident that treatment of tumor cells with BPs also affects a number of other cellular processes including those involved with invasion, potentially mediated through loss of function of Ras family members. Such effects may be enhanced by co-administration of chemotherapy drugs [19,24,25], as will be discussed below.

Effect of BPs on tumor invasion

Effect on adhesion and invasion

The specific spread of breast and prostate cancers to the bone would suggest that properties of both the tumor cells and the bone microenvironment are beneficial to their growth and progression. Following dissemination, once located in the bone microenvironment tumor cells adhere to mineralized bone matrix, exposed upon osteoclast-mediated bone resorption. Initial studies on the effect of BPs on bone resorption reported that in addition to their effect on apoptosis, exposure of calcified bone matrix to BPs altered the properties of the matrix required for osteoclast adhesion [26–28]. Using slices of cortical and trabecular bone, Van der Pluijm *et al.* further demonstrated that pre-treatment of bone for 18 h with pamidronate, olpadronate, alendronate and ibandronate at concentrations of 1–100 μM, also reduced MDA-MB 231 breast cancer cell adhesion and spreading on the bone matrices [29], potentially interfering with binding via modulation of bone-specific sialoproteins peptides [30].

In addition to the effect on bone matrix proteins, pre-treatment of tumor cells with BPs has also been shown to alter their adhesion, affecting cell attachment via a different mechanism. Incubation of the breast cancer cell lines MCF-7 and MDA-MB 231 and the PC3 prostate cancer cell line with N-BPs and non-N-BPs for 24 h decreased their adhesion to both mineralized and unmineralized matrices, with the level of inhibition corresponding to their anti-resorptive potencies *in vivo* [31] (Table 1). Pamidronate, the active NE-10244 analog of risedronate (capable of inhibiting bone resorption) and ibandronate had a greater effect on adhesion than clodronate, with inhibitory IC_{50} values of 10, 0.1, 0.005 and 100 μM, respectively. In contrast incubation with the inactive risedronate analog NE-58051 (lacking a methyl group from the R_2 side-chain), a poor inhibitor of FPP synthase (Fig. 2) and a weak inhibitor of bone resorption, had no effect on adhesion, suggesting that the methyl side group is important in adhesion these tumor cells to bone matrix. Treatment of the cells therefore also required lower concentrations of BPs to trigger an inhibitory response in comparison to modulation of the bone matrix [29]. Analysis of integrin expression after 24 h at these concentrations, however, identified that expression was unaffected. As N-BPs inhibit prenylation

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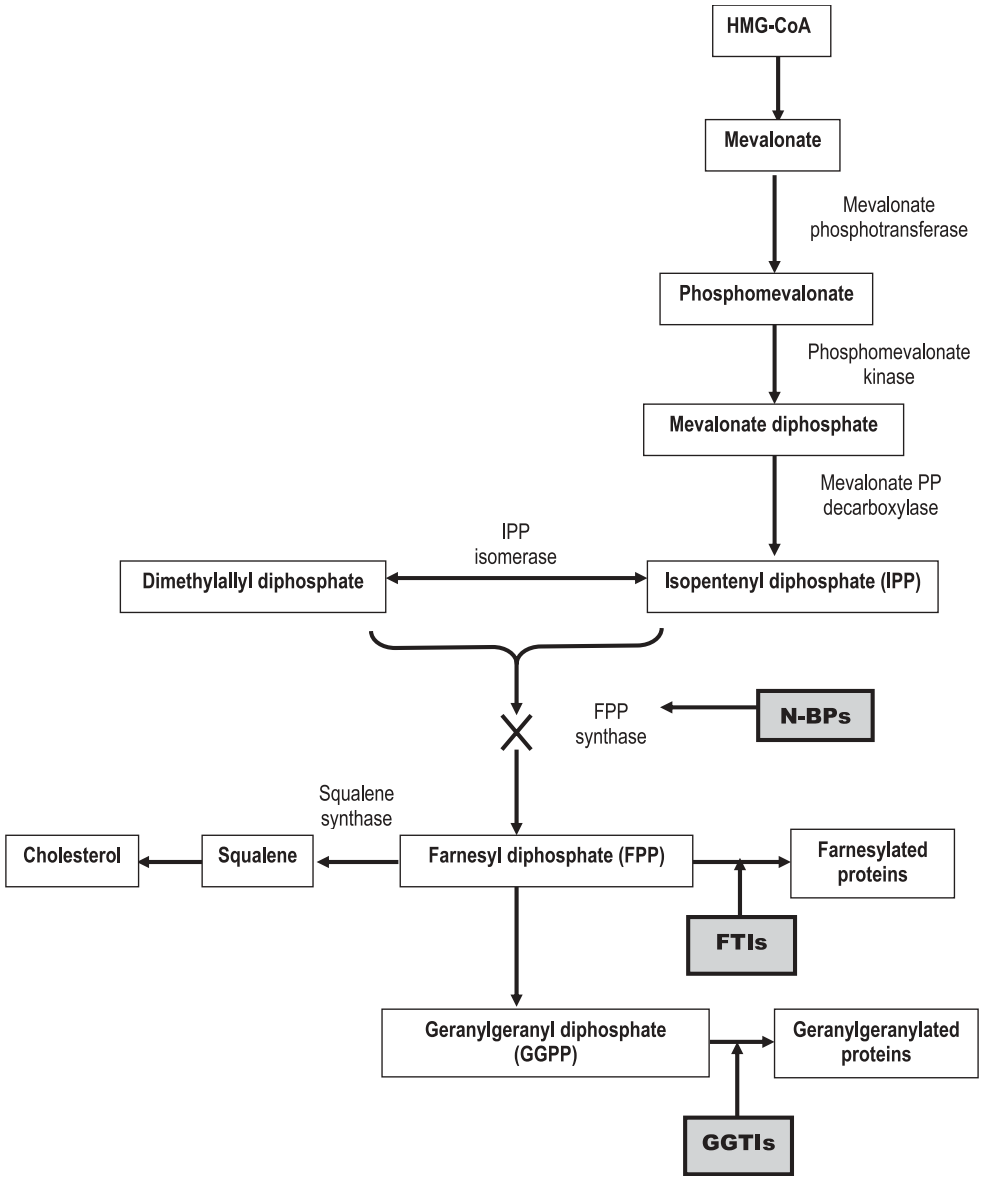
Fig. 2



Schematic diagram of the mevalonate pathway. Nitrogen-containing BPs inhibit FPP synthase, ultimately preventing protein prenylation (farnesylation and geranylgeranylation), thereby inhibiting normal cellular processes, potentially including invasion and migration. Farnesyl transferase inhibitors (FTIs) and geranylgeranyl transfer inhibitors (GGTIs) directly inhibit farnesylation and geranylgeranylation.

of small G-proteins such as Ras and Rho, which are involved in integrin signaling [6,32], activation of these receptors rather than expression of integrins themselves could have been affected by BP treatment. In support, a recent study by Bezzi *et al.* reported that treatment of human umbilical vein endothelial cells (HUVECs) for 24 h with 100 μM zoledronate decreased $\alpha_v\beta_3$-mediated adhesion to vitronectin but did not affect $\alpha_v\beta_3$- or β_1-integrin expression or affinity [33]. Instead, zoledronate disrupted focal adhesions and stress fiber formation in adherent cells both of which were associated with suppression of focal adhesion kinase phosphorylation and required for $\alpha_v\beta_3$ function. As studies on skeletal metastases in breast cancer patients have also revealed an increase in β_3-integrin expression when compared with controls [34], whilst tumor cells expressing $\alpha_v\beta_3$-integrins have a greater predilection of metastasizing to the bone [35], BPs could potentially regulate breast cancer adhesion to bone via a similar mechanism.

A number of other *in vitro* studies have also now reported changes in adhesive properties of tumor cells following

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Table 1 Inhibitory effect of BPs on bone resorption, adhesion, migration, protease activity and invasion of tumor cells *in vitro*

Compound	Bone resorption activity *in vivo*	Adhesion IC_{50} (nM)	Migration IC_{50} (nM)	Protease activity IC_{50} (nM)	Invasion IC_{50} (nM)	Cell types (and lines affected)	Reference
Clodronate	active	100	NE	4×10^4–1.5×10^5	100–8×10^4	breast cancer (MCF-7 and MDA-MB 231); prostate cancer (PC3); osteosarcoma (MG-63)	[31,36,40,41,43,44]
Pamidronate	active	10	NE	4×10^4–1.5×10^5	8×10^4	breast cancer (MCF-7 and MDA-MB 231); prostate cancer (PC3)	[31,36,40,44]
Alendronate	active	10^4	1×103–3×10^4	4×10^4–1.5×10^5/NE	0.001–8×10^4	breast cancer (MDA-MB 231); prostate cancer (PC3 and Du-145); fibrosarcoma (HT1080); melanoma (C8161); ovarian cancer (Caov-3); osteosarcoma (SaOS-2 and U2OS)	[40,43,44,48]
Ibandronate	active	0.005	NE	10^5	0.001	breast cancer (MCF-7 and MDA-MB 231); prostate cancer (PC3)	[25,31,36]
NE-10244 risedronate analog	active	0.1	NE	10^5	0.5	breast cancer (MCF-7 and MDA-MB 231); prostate cancer (PC3)	[31,36]
NE-58051 risedronate analog	inactive	>1000	NE	10^5	>1000	breast cancer (MCF-7 and MDA-MB 231)	[31,36]
NE-10790 risedronate analog	active	ND	NE	NE	1	breast cancer (MCF-7 and MDA-MB 231); prostate cancer (PC3)	[31,36]
Zoledronate	active	1×10^2–1×10^5	NE	4×10^4–1.5×10^5	0.001–1000	breast cancer (MDA-MB 231); prostate cancer (PC3)	[11,31,36,40,44,63]

ND, not done; NE, no effect.

BP treatment, often in the context of studying tumor invasion (Table 1 and Fig. 1). Following their previous work [31], Boissier *et al.* assessed the effect of a number of BPs on metastatic breast (MDA-MB 231) and prostate (PmPC3) cancer cells *in vitro* invasion through an artificial basement membrane (Matrigel) [36]. In agreement with the effect on adhesion [29,31], after incubation with the BP for 24 h, zoledronate, ibandronate and the active analog of risedronate NE-10244 had a greater inhibitory effect on invasion than clodronate, with IC_{50} values of < 1 pM, 1 pM, 0.5 nM and 50 μM, respectively. The phosphonocarboxylate risedronate analog NE-10790 (in which a phosphate group of the R_2 side-chain has been substituted with a carboxyl group) also reduced invasion to a similar extent as NE-10244, whilst the inactive NE-58051 analog, lacking the methyl side group from the R_2 side-chain, had no effect, implying that properties of the R_2 side-chain are also involved with invasion, potentially through inhibition of adhesion to the matrix as previously described [31]. Moreover, as NE-10790 specifically inhibits Rab geranylgeranyl transferase, and has been shown to prevent prenylation of Rab GTPases in osteoclasts and macrophages [37], whilst transient expression of an inhibitor of all Rab G proteins prevents stress fiber and focal adhesion assembly [38] required for cell adhesion and motility, Rab G proteins could be involved with regulation of adhesion in this instance.

Effect on migration and matrix degradation

In the study by Boissier *et al.*, parallel apoptosis and migration assays revealed that viability and motility of the breast (MDA-MB 231) and prostate (PmPC3) cancer cells (after 6 h) were unaffected at concentrations less than 50 μM [36] (Table 1). Further investigation identified that in addition to the effect on adhesion to matrix proteins previously reported [31], treatment with high BP concentrations (100 μM) for 24 h inhibited the activity of MMP-2, MMP-9 and MMP-12 secreted by MDA-MB 231 breast cancer cells. Incubation of cells with BPs at 1 μM did not, however, affect enzyme secretion, whilst production was unaffected at any concentration. Interestingly, with the exception of the phosphonocarboxylate analog of risedronate NE-10790, all BPs inhibited MMP activity in equimolar concentrations, despite small differences in structure. NE-10790 did not, however, affect enzyme activity, suggesting an involvement of the phosphate group in MMP inhibition. As addition of EDTA to chelate Zn^{2+} and Ca^{2+} (required for MMP activity) reversed the inhibitory effect, this would suggest that at increased concentrations BPs are reducing the degradative activity of these cells through reducing the access of binding sites to divalent cations, potentially regulated by the phosphate group of the R_2 side-chain.

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The inhibitory effect of BPs on MMP activity is now well documented (Table 1). Proteolytic enzymes are required for a number of processes relating to tumor growth and invasion. To aid tumor cell migration, degradation of the ECM facilitates the production of chemotactic protein fragments and the release of growth factors to both attract tumor cells and stimulate their growth. Endothelial cells also secrete proteolytic enzymes, promoting angiogenesis and subsequent tumor growth. Inhibition of proteolytic activity will thus have a dramatic effect on tumor growth and metastasis, as demonstrated by Stearns and Wang [39]. Using the SCID mouse model these authors reported that treatment with alendronate at a concentration of 0.1 mg/kg twice a week, for 3 weeks, reduced MMP-2 and MMP-9 levels and subsequent collagen solubilization by metastatic prostate cancer cells, decreasing their degradative activity and tumor progression. More recently, Heikkila *et al.* [40,41] have shown that concentrations of clodronate, alendronate, pamidronate and zoledronate (40–70 μM) inhibit the activity of a number of purified and recombinant MMPs. Comparable concentrations reduced HT1080 fibrosarcoma and C8161 melanoma cell invasion *in vitro*, and interestingly also migration of both these tumor cell types and endothelial cells (HUVECs), thus in fact differing from the results presented by Boissier *et al.* [36]. In support, using the Matrigel invasion assay, real-time PCR and ELISAs, Cheng *et al.* have also reported that treatment of SaOS-2 and U_2OS osteosarcoma cells with 50–150 mM alendronate for 24 and 48 h inhibits invasion and MMP-2 expression and secretion [42]. The relevance of these results *in vivo* are nevertheless uncertain as local BP concentrations around the periphery of the tumor may not be sufficiently high to achieve these effects, whilst naturally occurring protease inhibitors in the ECM are likely to affect the response.

Virtanen *et al.* have more recently described a similar inhibitory effect of BPs on cell migration [43]. Although treatment of PC3 prostate cancer cells with 10 μM alendronate for 24 h significantly reduced ECM adhesion, gelatinase secretion and activity was unaffected by both 0.001 and 10 μM alendronate after the 72-h incubation in the invasion assay. This result differs from previous reports [36,44], but as greater BP concentrations (50–150 μM) were described to inhibit gelatinase activity, it is possible that concentrations of alendronate greater than 10 μM would be required to induce an inhibitory effect. Cell migration was, however, reduced by pre-treatment with 10 μM alendronate for 24 h. Likewise pre-treatment of PC3 cells for 24 h with the lower dose of 1 pM also reduced *in vitro* invasion through Matrigel. Interestingly when cells were not pretreated, and instead alendronate was included with the cells in the assay, the concentration had to be increased to 10 μM to have a comparable effect. Reproducible results were also seen with MDA-MB 231 breast cancer cells and the prostatic cell line Du-145 (an IC_{50} of 1 nM). Parallel experiments treating cells with the mevalonate pathway inhibitor mevastatin similarly reduced invasion, whilst in experiments in which mevalonate pathway intermediates geranylgeraniol and *trans-trans*-farnesol (Fig. 2) were included, the effect was reversed suggesting involvement of the mevalonate pathway in PC3 invasion, as corroborated by Andela [45]. Although pre-incubation with clodronate also reduced cell invasion, the effect was less potent (an IC_{50} of 0.1 μM), and was instead accentuated by the addition of geranylgeraniol and *trans-trans*-farnesol, thus implying a different mechanism of inhibition. As growth of these cells was again unaffected by concentrations of less than 100 μM, concentrations of alendronate of 10 μM and below were thought to inhibit invasion rather than induce apoptosis.

Although BPs have been shown to affect tumor invasion *in vitro*, the precise mechanisms described are unclear. The reduced concentrations required to inhibit invasion when compared with the individual processes of adhesion, migration and degradation would suggest that the inhibitory action is due to the culmination of effects (Fig. 1). In a recent review, it was proposed that BPs inhibit invasion by two mechanisms, dependent upon the concentration [46]. At high concentrations, BPs potentially act by inhibiting MMP activity, with all BPs working at equimolar concentrations. In contrast, at lower concentrations, inhibition of the mevalonate pathway induces their effect. In support, a recent study by Valleala *et al.* [47] has reported that MMP-9 protein, but not mRNA, levels in freshly extracted monocytes are significantly reduced following incubation for 20–24 h with clodronate and pamidronate. In these cells, high levels of BP (100–300 μM) inhibited MMP-9 secretion, yet interestingly low concentrations of 30 μM enhanced secretion. Work by Teronen *et al.* has also demonstrated that concentrations of BPs (including clodronate, alendronate and zoledronate) of 50–150 μM are required to inhibit the activity of purified and recombinant MMPs, whilst at these and lower concentrations, *in vitro* invasion of osteoclasts, melanoma and fibrosarcoma cells was inhibited [44].

Involvement of intracellular GTPases

Further insight into the effect of BPs on the intracellular mechanism of invasion has been gained by investigators recently using MDA-MB 231 breast cancer cells [11]. As both Ras and RhoA proteins are reported to be involved in breast cancer migration and invasion [6,9], and N-BPs inhibit post-translational prenylation of such proteins (Fig. 2), these authors have investigated the effect of zoledronate on Ras and RhoA prenylation. MDA-MB 231 breast cancer cells were pretreated with zoledronate (10 nM–100 μM) for 18 h and the subsequent effect on

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invasion through Matrigel assessed after a further 18 h. After treatment with 1 μM zoledronate, invasion was reduced by 62%, a result that could be reversed by inclusion of 10 μM of the artificial isoprenoid geranylgeraniol (GGOH) in the assay to restore geranylgeranylation (Fig. 2). Replacement of zoledronate with an inhibitor of geranylgeranylation reduced invasion to a similar extent as zoledronate, further implying prenylation involvement in this process. Analysis of the protein content of the cytosol identified that treatment with 1 μM zoledronate for 18 h was inhibiting membrane localization, and hence function of RhoA, but not Ras, proteins, ultimately leading to cytoskeletal disorganization. This effect was again reversed by the incorporation of GGOH, but not by the addition of the mevalonate pathway intermediate farnesol (FOH), further identifying the potential importance of geranylgeranylation, but not farnesylation (Fig. 2). Concentrations of zoledronate above 100 μM also inhibited expression of the serine protease uPA, whilst lower concentrations (1–100 μM) dose-dependently decreased expression of its receptor uPAR, required for plasminogen degradation to plasmin. In agreement with previous reports [36,46] it was thus suggested that the effect of zoledronate on invasion was mediated by disorganization of the actin cytoskeleton through inhibition of RhoA function; an observation also reported in Caov-3 ovarian cancer cells following alendronate treatment [45,48]. As BPs inhibit growth factor release by decreasing bone resorption, through addition of stromal-derived factor-1 (SDF-1) as a chemoattractant, Denoyelle *et al.* also demonstrated that 1 μM zoledronate reduced invasion to a comparable level as inclusion of a monoclonal antibody against the SDF-1 receptor CXCR-4 [11]. As malignant breast cancer cell lines express high levels of CXCR-4 [49] and the SDF-1/CXCR-4 interaction is thought to contribute to bone-specific metastasis [50], zoledronate may also be affecting metastatic development through down-regulation of CXCR-4 receptors.

Co-administration of BPs with cytotoxic drugs

At present BPs are used clinically once a patient has presented with metastatic bone disease. As preclinical evidence has suggested that in addition to their effect on bone resorption, BPs have anti-tumor effects, the use of BPs as an adjuvant therapy (in combination with chemotherapy drugs) is under investigation to target tumor cells systemically. Clinical trials to date assessing potential adjuvant use of BPs have nevertheless reported conflicting results, with studies monitoring survival and development of bone metastases of patients with primary operable breast cancers after treatment with clodronate [51–53]. Thus, despite much evidence supporting an anti-tumor effect of BPs in pre-clinical studies, the effect *in vivo* still remains unclear.

Data generated from *in vitro* and *in vivo* models has nevertheless provided promising results regarding an enhanced anti-tumor effect of co-administration of BPs and chemotherapy drugs. Combined treatment with both alendronate and the taxane paclitaxel has, for example, a greater effect on bone and non-bone metastatic development than treatment with each alone [24], whilst the apoptotic effect of zoledronate (10 μM) on MCF-7 breast cancer cells *in vitro* is synergized with co-administration of paclitaxel (2 μM) [19]. Following orthotopic injection of the 4T1 mouse mammary tumor cells, co-administration of doxorubicin and ibandronate has also been shown to reduce the occurrence of adrenal metastases [54].

Few studies have investigated the combined effect of BPs and cytotoxic drugs on the invasion process, but limited evidence supports an effect of cytotoxic drugs alone on invasion. Using PC3-ML prostate cancer cells in a SCID mouse model, initial studies by Stearns and Wang demonstrated that treatment with paclitaxel at 0.5–1.0 μM for 6 h could reduce the establishment, growth and survival of the tumor cells [55]. By investigating the individual processes *in vitro*, the authors also reported that paclitaxel at these concentrations affected microtubule formation, thereby inhibiting MMP-2 and MMP-9 secretion by PC3-ML cells and their invasion through Matrigel. These observations have now been supported by a number of studies to suggest that paclitaxel affects cell attachment, migration, degradation, and subsequent invasion of both tumor and endothelial cells [56]. For example, paclitaxel treatment of glioma cells prevents *in vitro* invasion and migration, whilst treatment of B16F10 melanoma cells also inhibits both invasion and migration through collagen, together with adhesion to fibronectin and laminin, and gelatinase activity [57,58]. Pre-treatment of the ovarian cell line Ovcar-3 with 1.0 μM paclitaxel for 6 h decreased attachment to type IV collagen and laminin and significantly inhibited migration and invasion by affecting the actin cytoskeleton, but in contrast to the study by Stearns and Wang [55], had no effect on MMP-2 secretion [59]. The discrepancies may suggest that in common with BPs, effects of paclitaxel on MMP activity and secretion depend upon the concentration used, or alternatively the cell type studied.

Other cytotoxic drugs commonly used for treatment of breast cancer patients, such as the anthracycline doxorubicin, have also been shown to affect the invasion process at clinically relevant concentrations in breast and other tumor cells. These drugs affect the cells through a number of different mechanisms dependent upon the concentration, but primarily act as DNA-damaging agents, inhibiting topoisomerases. An effect of doxorubicin on tumor invasion has nevertheless also been reported, as treatment of the aggressive melanoma cell line A2058 with 0.5–1.5 μM doxorubicin for 1 h reduced collagen I invasion and inhibited collagenase I secretion,

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without causing apoptosis [60]. More recently, Sliva *et al.* have shown that pretreatment of MDA-MB 231 breast cancer cells with up to 0.5 μg/ml doxorubicin for 1 h dose-dependently inhibited migration [61]. At 0.05 μg/ml, doxorubicin induced apoptosis by blocking the cells in G_2 phase of mitosis, damaging the DNA and altering the cell membrane structure, suggesting that the inhibitory effect on migration could be attributed to a cytotoxic effect in contrast to actively inhibiting motility.

In addition to a synergistic effect of BPs and cytotoxic drugs *in vivo* [24], limited evidence has described an additive effect of BPs and cytotoxic drugs on breast cancer cell invasion *in vitro* [25]. In this study, cells of the breast cancer cell line MDA-MB 231 were treated with either paclitaxel (or the taxane docetaxel) for 1 h or ibandronate for 23 h, or alternatively sequentially treated with paclitaxel followed by ibandronate. Assessment of attachment to bone matrices and invasion revealed an additional decrease of 38–59% in adhesion and 70–80% in invasion when compared with single treatments. Analysis of migration, however, identified that although paclitaxel and docetaxel dose-dependently inhibited migration; ibandronate exerted no additional effect on migration, thus reflecting previous work of the effects of BPs on migration [36]. In contrast, although ibandronate affects MMP activity [36], the taxoids had no affect on gelatinase expression. As taxoids have been previously reported to reduce both tumor adhesion and migration [55,62], the additive effect of taxoids and ibandronate was therefore proposed to relate to their affect on migration and degradation, respectively, in combination with a dual effect on attachment.

Conclusions

In summary, both BPs and drugs commonly administered during chemotherapy are capable of affecting the invasion of tumor cells *in vitro*, potentially through direct anti-tumor effects and indirectly by affecting bone resorption and hence reducing attraction of tumor cells to the bone. Further *in vivo* research is nevertheless required to qualify these interesting findings. The more potent N-BPs such as ibandronate and zoledronate mediate their anti-resorptive effect through processes including inhibition of prenylation and hence activity of a large number of small GTPases. As these proteins could influence the intracellular functions involved with invasion, including regulation of integrin activity and cell motility, inhibition of small GTPase function is likely to affect the process as an entirety. Interestingly, the concentrations of BPs required to inhibit invasion are much reduced when compared with the individual steps of this process, suggesting that the culmination of effects are required to bring about most effective inhibition. As the serum concentration of BPs following administration is thought to be much lower than doses reported to affect cell survival, research into the dose and treatment time required to bring about the synergistic action of chemotherapy drugs on the anti-invasive effect of BPs will help to target metastatic cells both located in the bone and systemically.

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