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REVIEW

Anti-tumour activity of bisphosphonates in preclinical models of breast cancer

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Abstract

There is increasing evidence of anti-tumour effects of bisphosphonates from pre-clinical studies, supporting a role for these drugs beyond their traditional use in treatment of cancer-induced bone disease. A range of model systems have been used to investigate the effects of different bisphosphonates on tumour growth, both in bone and at peripheral sites. Most of these studies conclude that bisphosphonates cause a reduction in tumour burden, but that early intervention and the use of high and/or repeated dosing is required. Successful eradication of cancer may only be achievable by targeting the tumour cells directly whilst also modifying the tumour microenvironment. In line with this, bisphosphonates are demonstrated to be particularly effective at reducing breast tumour growth when used in combination with agents that directly target cancer cells. Recent studies have shown that the effects of bisphosphonates on breast tumours are not limited to bone, and that prolonged anti-tumour effects may be achieved following their inclusion in combination therapy. This has opened the field to a new strand of bisphosphonate research, focussed on elucidating their effects on cells and components of the local, regional and distal tumour microenvironment. This review highlights the recent developments in relation to proposed anti-tumour effects of bisphosphonates reported from *in vitro* and *in vivo* models, and summarises the data from key breast cancer studies. Evidence for effects on different processes and cell types involved in cancer development and progression is discussed, and the main outstanding issues identified.

Introduction

In addition to the established role as inhibitors of osteoclast activity and bone resorption, bisphosphonates (BPs) also affect tumour cells. Their ability to induce apoptosis, reduce proliferation, and inhibit tumour cell migration and invasion has been demonstrated in numerous *in vitro* studies (reviewed in [1]). As nitrogen-containing BPs (NBPs) act by inhibiting key enzymes in the metabolic pathway responsible for cholesterol synthesis, which is essential for all nucleated cells, the drugs do have the potential to affect any cell type that takes up sufficient quantities of them [2].

The anti-tumour effects reported from *in vitro* systems led to subsequent investigations using *in vivo* models in different tumour types, including breast cancer (reviewed in [3]). Most of these focussed on elucidating the effects of BPs on tumours in bone, and it quickly became clear that BP treatment prevented the development of cancer-induced bone disease, but that tumour growth was only temporarily halted and eventually progressed. Increasing the dosing frequency and/or starting therapy at early stages of the disease process increased the anti-tumour effect, but did not completely eradicate tumours.

Subsequent studies explored the potential of BPs as part of combination therapy schedules. BPs were added to a range of standard chemotherapy agents used to treat breast, prostate and small cell lung cancer, multiple myeloma and osteosarcoma [3,4]. In all reports published to date, addition of a BP to other anti-cancer therapies caused significantly decreased tumour burden compared to that seen when the single agents were used. This has in turn led to clinical trials in breast cancer investigating whether adding BPs to standard treatment translates to additional benefit for patients [5,6]. Although substantial increased anti-tumour effects are demonstrated when BPs are added to a range of therapeutic agents, the underlying molecular and cellular mechanisms remain to be established.

Over the past decade it has become apparent that the tumour microenvironment has a key role in both cancer development and determining the response to therapy. A multitude of cellular and molecular interactions take place between malignant and normal cells during tumour progression, and increasingly the normal cells are considered to be therapeutic targets in their own right. These interactions take place at several different levels;

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thus, tumours are affected by complex networks of cells and molecules that comprise their local, medial and distal microenvironment (Figure 1). BPs are prime examples of agents that modify the normal cells of the bone microenvironment and thereby have profound effects on tumour progression. The potential for these agents to also affect cells distal to bone is currently an area of active research.

Anti-tumour effects of bisphosphonates - direct, indirect or both?

The high affinity BPs have for bone is key to their successful use in the treatment of a number of skeletal disorders [7]. BPs rapidly home to bone following administration, with a half-life in serum of only a few hours [8-10]. BPs can, however, be retained in the skeleton for several years, and during normal bone turnover small amounts of BPs may be released into the circulation and thereby potentially affect peripheral tissues.

There is broad agreement that due to the high concentration of BPs in bone, bone metastases are the tumours most likely to be directly exposed to significant levels of the drugs for prolonged periods of time, and tumour cells residing in bone may be directly affected through uptake of BPs released during normal bone turnover. The current evidence for this proposed direct anti-tumour effect is not compelling, as we are unable to measure the local 'free' concentration of BPs in metastatic foci. In addition, the presence of BPs effectively reduces bone resorption, thereby limiting the amount of drug released to subsequently affect resident tumour cells.

A recent report indicates that there may be alternative explanations for the anti-tumour effects of BPs, not involving osteoclasts. This study investigated the effects of zoledronic acid on B16 melanoma bone tumour burden in irradiated mice that had received a transplant of splenic cells from src-/- mice that lack functional osteoclasts [11]. This elegant approach allowed the researchers to study the effects of zoledronic acid on tumour growth in bone, independent of any effects on bone resorption. Intriguingly, zoledronic acid caused an 88% reduction in bone tumour growth compared to irradiated vehicle-treated controls, strongly indicative of osteoclast-independent effects on tumour cells. The authors suggest that effects on endothelial cells or perhaps direct effects on the tumour cells may cause the reduced tumour growth, but the cellular and molecular mechanisms remain unknown.

Within bone it is likely that a combination of direct and indirect effects of BPs contribute to inhibiting tumour growth and the associated cancer-induced bone disease [12]. In contrast, we know little about how inhibiting bone resorption affects tumours outside the skeleton. Perhaps BPs disrupt the migration of bone marrow precursors that are essential for peripheral tumour growth, thereby indirectly reducing tumour burden. This is an area of great interest, as a recent clinical study has indicated that even 6-monthly administration of the potent BP zoledronic acid improves outcome for breast cancer patients by reducing local recurrence [13]. Zoledronic acid is reported to reduce granulocyte-macrophage colony stimulating factor (GM-CSF)-stimulated tumour growth in bone, and this may partly be due to inhibition of mobilisation of dormant tumour cells during active bone resorption [14].

BPs may also affect disseminated tumour cells in the bone marrow, as demonstrated in a study of women with locally advanced breast cancer [15]. In this study, zoledronic acid added to neo-adjuvant chemotherapy reduced the number of patients with detectable disseminated tumour cells in the bone marrow at 3 months compared to those that received chemotherapy alone. Evidence for a direct effect of zoledronic acid on primary breast tumours has been reported in a separate neo-adjuvant study that was incorporated in the AZURE trial [16]. Patients receiving zoledronic acid in addition to standard therapy had significantly smaller residual invasive tumour size compared to those receiving standard therapy alone. There are thus emerging clinical data to support a wider therapeutic effect of BPs in breast cancer. In breast cancer models, BPs affect a range of cell types contributing to tumour development, including those of the local and distal tumour microenvironment (Figures 2 and 3). The following sections will give some examples of studies investigating the effects of BPs on different cell types *in vitro* and *in vivo*.

Bisphosphonates may modify a range of cell types

From *in vitro* studies we know that BPs may induce apoptosis and reduce proliferation of a range of tumour cells, but high and/or frequent dosing has often been used to generate these effects [1]. In addition, effects on other cell types, including endothelial cells [17], macrophages [18], immune cells [19], osteoblasts [20], fibroblasts and stromal cells [21], have been reported *in vitro*. Subsequent studies using *in vivo* tumour models showed that reduced tumour growth is associated with changes in the tumour microenvironment - for example, reduced vascularisation and macrophage infiltration [22]. As shown in Figure 2, BPs may modify a number of processes and cell types involved in the development and progression of peripheral tumours. In all cases, the question of dose and distribution of BPs following a clinical administration is key. Cells of peripheral tumours are exposed to very low levels of BPs for a short period of time, whereas tumour cells in bone are likely to encounter higher concentrations of BPs. The lack of suitable research tools has hampered studies of the distribution

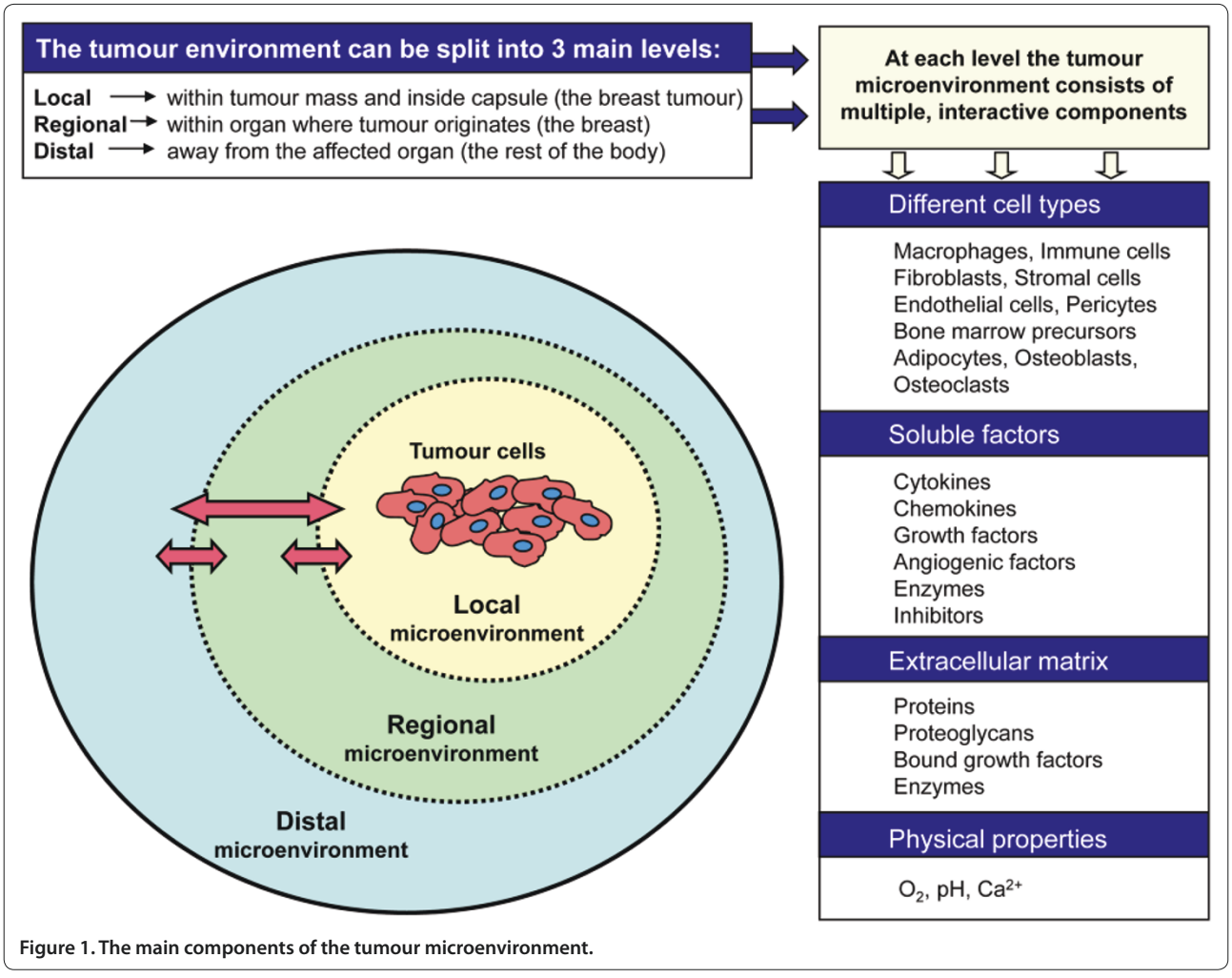

Figure 1. The main components of the tumour microenvironment.

and retention of BPs in tumour models. Similarly, the precise molecular and cellular BP targets within tumours, and the effects of changes in systemic factors remain to be firmly established (Table 1).

Anti-angiogenic effects of BPs potentially contribute to reduced tumour growth

Key to tumour development is the ability to establish a functional blood supply to support the high metabolic activity of a rapidly growing tumour mass; thus, the tumour vasculature represents an attractive but elusive therapeutic target. The potential for BPs to modify tumour angiogenesis has been addressed in several studies, summarised in the following sections.

Effects on cells of the normal vasculature

BPs may elicit their proposed anti-angiogenic effects through inhibiting maturation and/or proliferation of endothelial cells (ECs), by affecting their adhesion, or by reducing their ability to migrate and form functional vessels. Several of these processes are driven by vascular endothelial growth factor (VEGF), and hence may be modified through a BP-mediated reduction in the level of this key pro-angiogenic factor [23]. Interest in this area has been rekindled by the reports of cases of osteonecrosis of the jaw (ONJ) following treatment with zoledronic acid [24]. Reduced vascularisation is suggested to be one of the contributing factors of ONJ, potentially mediated via the reported anti-angiogenic effects of zoledronic acid. However, recent reports of ONJ following treatment with the new anti-resorptive agent denosumab would indicate that effects on osteoclasts are central to ONJ [25].

The anti-angiogenic effects of BPs were first investigated using primary endothelial cells [17]. Human umbilical cord-derived ECs (HUVECs) were treated with increasing doses of zoledronic acid or pamidronate *in vitro*, and the effect on EC apoptosis, proliferation and migration and vessel sprouting were determined. This study clearly demonstrated how cellular processes have

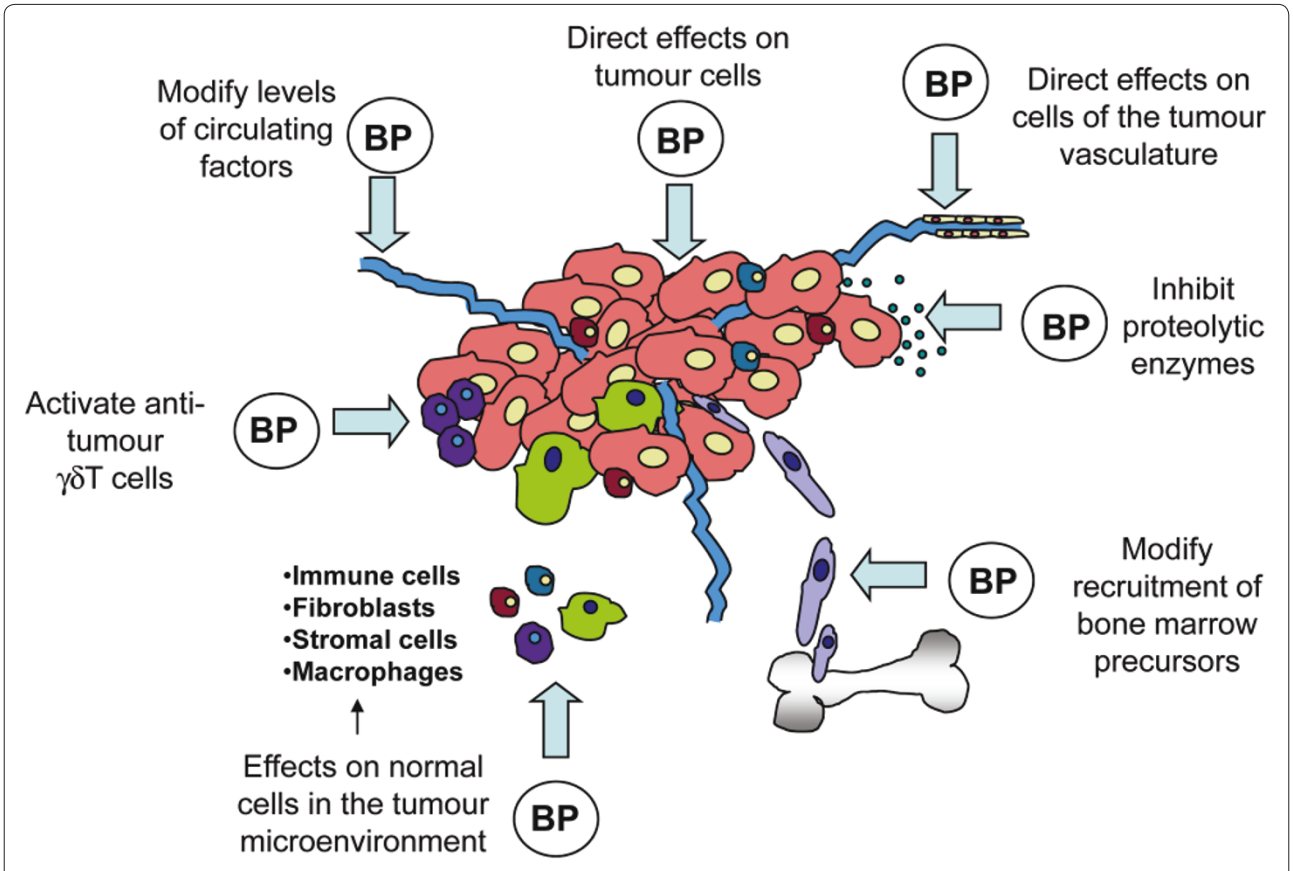

Figure 2. Potential anti-tumour effects of bisphosphonates (BPs) outside the skeleton.

differential sensitivity to BPs. Whereas basic fibroblast growth factor-stimulated HUVEC proliferation was significantly reduced by a low dose of zoledronic acid (3 μM for 24 hours), a reduction in cell adhesion required exposure to 30 μM for 48 hours, and exposure to 100 μM for 48 hours was needed to induce a significant increase in the levels of HUVEC apoptosis. Both BPs were found to reduce angiogenesis in the vessel sprouting assays, but doses as high as 1 mM were applied, thus limiting the clinical relevance of these findings. In an *in vivo* angiogenesis assay, zoledronic acid caused 98.5% and 46% reductions in blood volume of basic fibroblast growth factor and VEGF implants, respectively, compared to control.

In general, endothelial cells are less sensitive to BPs compared to tumour cells. This is probably due to the low endocytic uptake of BPs in these cells, coupled with their long cycling time *in vitro*. Human dermal microvascular endothelial cells (HuDMECs) have been shown to take up BPs, as demonstrated by accumulation of unprenylated Rap1a (a surrogate marker for NBP uptake) [26]. The cells of the normal vasculature appear to be less sensitive to BPs than tumour cells and highly endocytic/phagocytic cells (like osteoclasts and macrophages) [26].

Effects on endothelial progenitor cells

The majority of studies to date have focussed on endothelial cell function, but two recent reports suggest that BPs may perhaps also reduce the viability and maturation of EC precursors. Zeibart and colleagues [27] demonstrated that 48-hour *in vitro* exposure to zoledronic acid, ibandronate, clodronate or pamidronate reduced the viability of human endothelial progenitor cells (EPCs) isolated from peripheral blood mononuclear cells. Zoledronic acid was the most potent compound, reducing EPC numbers by more than 40% following 48-hour incubation with 50 μM. These results suggest that the high concentration of BPs in bone may reduce the viability of resident EPCs, causing a downstream inhibition of angiogenesis.

An independent investigation by Yamada and colleagues [28] addressed whether zoledronic acid can inhibit EPC differentiation from peripheral blood mononuclear cells. The phenotype of the cells was characterised by measuring their expression of VE-cadherin/CD144 and VEGF receptor 2 (VEGFR2), and the functionality assessed through the ability of the cells to form tubules on matrigel. Exposure of the EPCs to the relatively low doses of 1 and 5 μM zoledronic acid for 5 days caused the cells to retain a rounded EPC

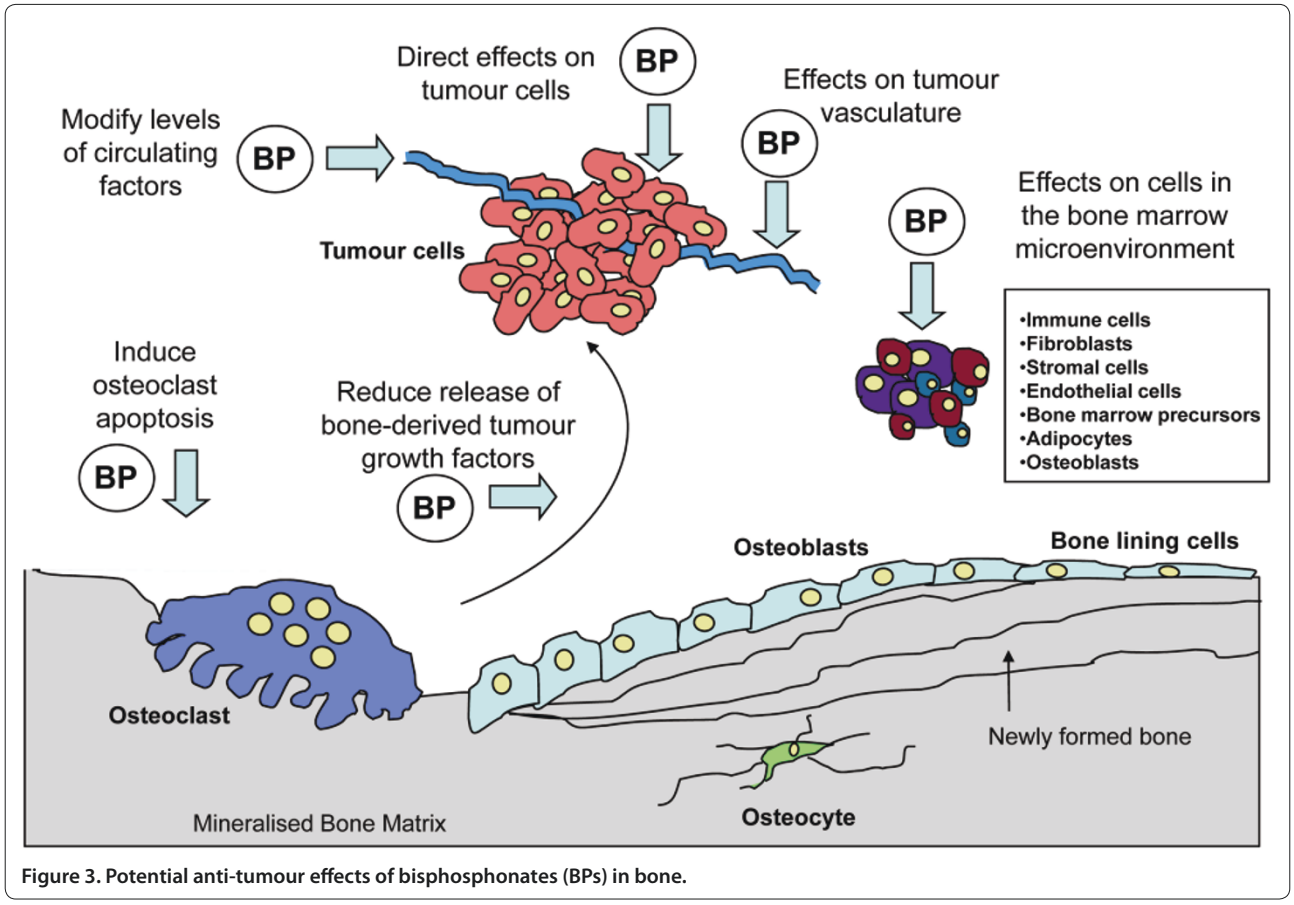

Figure 3. Potential anti-tumour effects of bisphosphonates (BPs) in bone.

Table 1. Overview of processes determining the anti-tumour effects of bisphosphonates outside bone

Factors contributing to anti-tumour effects of BPs in peripheral tumours	Questions still to be resolved
Concentration in tumour	What concentration of BPs reaches the tumour following a clinical dose?
Cellular uptake	How much BP is taken up by the tumour cells and by the cells of the local tumour microenvironment?
Duration and clearance	How long is BP retained in the cells and within the tumour mass?
Molecular and cellular targets	What are the specific molecular targets of BPs in tumour cells and in the cells of the tumour microenvironment?
Systemic effects	BPs may reduce the levels of circulating factors like VEGF, thereby affecting tumour growth indirectly
Effects on bone marrow precursors	BPs may inhibit recruitment of bone marrow precursors essential for primary tumour growth
Activation of γδ T cells	BPs may facilitate tumour killing through activation of anti-tumourigenic γδ T cells
Release of BPs from bone	Does long-term release of low levels of BPs during normal bone turnover reach levels that affect peripheral tumours?

BP, bisphosphonate; VEGF, vascular endothelial growth factor.

morphology, coupled to a downregulation of the endothelial cell markers, as well as a reduced capacity to form tubules in a matrigel assay. These effects were reversed by inclusion of geranylgerinaol, and thus possibly mediated through disrupting the cellular localisation of small GTPases [29].

Effects on tumour angiogenesis

BPs may also reduce tumour vascularisation. However, there have been few studies addressing this in detail due to technical difficulties in establishing reliable model systems. Recent developments in advanced imaging systems mean the biological effects may now be more readily addressed [30,31].

Reports that zoledronic acid causes decreased plasma VEGF levels in advanced cancer patients [23] led to a number of studies of the potential link between anti-tumour and anti-angiogenic effects of BPs [22,32-35]. However, in most of these studies the suggested effects of BPs on tumour angiogenesis are based on observations of apparently reduced levels of micro-vessel density, associated with a decrease in tumour volume. No attempts

have been made to demonstrate a causal link between administration of BPs and reduced tumour micro-vessel density. Whether the decrease in tumour vascularisation directly reduces tumour growth, or vice versa, therefore remains to be established. Changes in the tumour vasculature may precede effects on bone lesions, as indicated by a recent study utilising dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI) to investigate the effects of zoledronic acid and sunitinib malate in experimental breast cancer bone metastases in nude rats [30].

Effects on tumour macrophage infiltration

A high level of macrophage infiltration is associated with poor prognosis in several tumour types [36], and ablation of macrophages in breast cancer models has been shown to reduce tumour growth and progression [37]. Tumour cells release a range of chemotactic factors that attract circulating monocytes, which subsequently mature to become tumour macrophages. In breast cancer models, macrophages have been shown to regulate the angiogenic switch required for tumour vascularisation [38]. Hence, there is considerable evidence that tumour-associated macrophages contribute to driving breast cancer development, and therefore represent a therapeutic target.

Although the main cellular targets of BPs are the bone-resorbing osteoclasts, early work to identify the molecular mechanism of action of BPs was performed using the JJN4 mouse macrophage cell line [39]. BPs induced macrophage apoptosis *in vitro*, and peritoneal macrophages have subsequently been shown to take up zoledronic acid following *in vivo* administration [40]. Whether tumour macrophages also take up BPs *in vivo* is currently unknown, but a recent study demonstrated significantly reduced tumour macrophage infiltration caused by zoledronic acid in a model of spontaneous mammary carcinoma [22]. Zoledronic acid-treated animals displayed fewer and smaller mammary tumours compared to the untreated control animals, and increased survival. The reduced tumour burden following zoledronic acid treatment was associated with decreased levels of circulating VEGF, and reduced tumour vascularisation and number of tumour-associated macrophages. In addition, there was a repolarisation of the macrophages from a M2 to a tumouricidal M1 phenotype in zoledronic acid-treated animals. These data suggest that the anti-tumour effects of zoledronic acid are mediated through depletion of macrophages required for vascularisation of the tumour, rather than through affecting tumour cells directly. One key limitation to the direct transfer of these promising data to human cancer is that zoledronic acid treatment had to be started early in tumour development (at the hyperplastic stage) in order to inhibit tumour growth.

Similar data have been reported in a previous study using the same model to investigate the effects of BPs on bone marrow hematopoiesis [18]. The mammary tumours in BALB-neuT mice produce VEGF, which in turn stimulates production of myeloid-derived suppressor cells. Daily administration of pamidronate (2 mg/kg) or zoledronic acid (100 μg/kg) starting at 4 or 7 weeks (hyperplastic stage) caused significantly reduced tumour growth compared to control, whereas this was less pronounced if treatment started at 12 weeks when numerous mammary carcinomas were established. Zoledronic acid caused a reduction in the levels of circulating pro-matrix metalloproteinase-9 and VEGF, accompanied by decreased infiltration of macrophages in the tumour stroma, and reduced myeloid-derived suppressor cell expansion in both bone marrow and peripheral blood.

The suggestion that early BP treatment may be required to reduce tumour growth is supported by data from a study using established breast cancer xenografts, where animals with palpable, subcutaneous MDA-MB-436 derived tumours were administered up to 6 mg/kg of zoledronic acid once weekly for 6 weeks [41]. In contrast to the data described above, zoledronic acid did not reduce tumour growth, even at the highest concentrations used (60× equivalent of the 4-mg clinical dose). These data highlight the need for caution when interpreting and comparing results reported from *in vivo* studies, as different models representing different stages of tumour development may display variability in terms of sensitivity to anti-cancer agents [42]. In particular, there may be significant differences in therapeutic response recorded between xenograft studies that use immunocompromised mice and studies using murine mammary carcinoma models in immunocompetent mice.

Immunomodulatory effects of BPs may contribute to their anti-tumour effects

Although BPs are generally very well tolerated, around a third of patients receiving intravenous NBPs, such as zoledronic acid, experience a short-term acute phase response, mainly after the initial infusion. The mechanism triggering this response was first identified in patients with multiple myeloma receiving intravenous pamidronate, where it was found that the acute phase response was linked to increased levels of circulating γδ T cells [43]. Subsequent studies revealed that BPs activated a particular subset of γδ T cells (Vγ9Vδ2), leading to increased release of pro-inflammatory cytokines and hence initiating acute phase response. The mechanism behind activation of γδ T cells has been shown to be the accumulation of isopentenyl diphosphate and dimethylallyl pyrophosphate, following inhibition of farnesyl diphosphate synthase by NBPs [19]. In humans, γδ T cells constitute a minor proportion of T cells that are

Table 2. Overview of studies investigating bisphosphonates in models of breast cancer bone metastases

Breast cancer model	Bisphosphonate (dose)	Effect	Reference
MDA-MB-231: intracardiac implantation	Risedronate (0.4, 4 and 40 μg/mouse/day)	Reduced osteolytic lesion volume Reduced intra-osseous tumour volume Increased bone-associated soft tissue tumour burden	[45]
MDA-MB-231: intracardiac implantation	YH529 (0.2, 2 and 20 μg/mouse/day)	Reduced osteolytic lesion volume Reduced intra-osseous tumour volume Increased bone-associated soft tissue tumour burden after 0.2 and 2 μg doses, decreased after 20 μg	[46]
4T1/luc: bone metastases	Zoledronic acid (0.5 and 5 μg/mouse)	Reduced osteolytic lesion volume Increased tumour cell apoptosis	[51]
MDA-MB-231: intracardiac implantation	Ibandronate (4 μg/mouse/day, 7 days)	Reduced osteolytic lesion volume Reduced intra-osseous tumour volume	[49]
MDA-MB-231: injected in femoral artery, nude rats	Ibandronate (10 μg/kg/day)	Reduced osteolytic lesion volume Reduced intra-osseous tumour volume	[50]
MDA-MB-231/luc: intracardiac and intra-osseous implantation	Olpandronate (1.6 μm/kg/day, 18 days/40 days) Pamidronate (1.6 μm/kg/day, 40 days)	Olpandronate: reduced osteolytic lesion volume and reduced intra-osseous tumour volume Pamidronate: reduced lytic lesions and intra-osseous tumour growth after intratibial implantation No effect of BPs on bone-associated soft tissue burden	[47]
B02: generates bone metastases following intravenous injection	Zoledronic acid (3 and7 μg/kg/day; 20 and 50 μg/kg/week; 100 μg/kg 1×) Clodronate (530 μg/kg/day)	Clinically relevant doses used No effect of a single dose of zoledronic acid Weekly and daily administration of zoledronic acid reduced osteolysis and intra-osseous tumour growth Daily clodronate less effective compared to zoledronic acid	[52]

thought to be involved in tumour surveillance. Hence, it has been hypothesised that activation of γδ T cells by NBPs may result in triggering of an anti-tumour immune response leading to tumour cell death. Small-scale clinical feasibility studies have been performed to explore the potential of using NBPs as immuontherapy to trigger an anti-tumour response [44]. However, the clinical significance of γδ T cell activation in the context of potential anti-tumour effects remains to be established.

In addition to the different processes affected by BPs described above, their anti-tumour effect may also involve other elements of the tumour microenvironment - for example, inhibition of proteolytic enzymes required for tumour cell migration, and modification of the capacity of bone marrow precursor cells to migrate to peripheral tissues (Figures 2 and 3).

Anti-tumour effects of BPs in models of breast cancer bone metastases

The effects of BPs on lytic bone disease have been investigated in great detail, confirming that BPs inhibit the development of bone lesions and thereby increase survival [3]. Reduced lesion volume is generally associated with a decrease in skeletal tumour burden, suggesting that BPs have anti-tumour effects in bone. But do BPs reduce tumour growth directly, or is their positive effect mediated exclusively through the protection of bone from further destruction stimulated by tumour cells? The studies discussed in the following section (Table 2) illustrate that it has been difficult to dissect the direct from the indirect anti-tumour effects of BPs in bone metastasis models.

Bisphosphonates used as single agents

Data from *in vitro* and *in vivo* studies have demonstrated that BPs have the capacity to modify a number of cell types and processes involved in the development and progression of bone metastases [3] (Figure 3). Early studies focussed on the ability of BPs to prevent or reduce the extent of breast cancer-induced bone disease were performed by Sasaki and colleagues [45] using MDA-MB-231 human breast cancer cells implanted by intracardiac injection into female BALB/c-nu/nu mice to generate tumour foci in bone. Animals received risedronate either in the setting of established bone metastases, in an early treatment protocol from the day of tumour cell inoculation, or in a prevention protocol. In all cases, risedronate treatment reduced the development or slowed progression of bone lesions, and this was associated with increased numbers of apoptotic osteoclasts at the metastatic sites. The authors noted that risedronate caused a surprising reduction in the intra-osseous tumour burden, whereas tumour growth in bone-associated soft tissues was unaffected. This was the first indication that BPs may have bone-specific anti-tumour effects.

Sasaki and colleagues went on to repeat their study to investigate the effect of minodronic acid (a third generation BP) using the same model and treatment protocols [46]. Daily administration of minodronic acid

from the day of tumour cell inoculation caused a dose-dependent reduction in osteoclast number, as well as in the number and area of osteolytic lesions, and decreased bone tumour burden. Short-term treatment (days 17 to 28) and preventive treatment (7 days before tumour cell inoculation) caused similar effects. Only prophylactic administration caused near complete inhibition of the development of new metastases, indicating that once metastases are established they become less sensitive to drugs targeting osteoclastic bone resorption. One interesting finding was that administration of 0.2 and 2 μg minodronic acid caused an increase in bone-associated soft tissue tumour volume, similar to their earlier finding using risedronate [45]. This indicates that BP-mediated inhibition of bone resorption may cause expansion of extra-osseous tumour growth, a common finding in studies of late stage disease [47,48]. The relevance of this observation for human cancer is currently unknown.

Whether soft tissue tumours are less sensitive to BP therapy compared to tumours in bone was further investigated by Hiraga and colleagues [49]. In this study, MDA-MB-231 cells were implanted by intracardiac injection in female BALB/c-nu/nu mice (to generate bone metastases), or in the mammary fat pad (to mimic extra-skeletal tumour growth). Animals were subsequently treated with ibandronate (4 μg/mouse/day) once bone metastases were established (days 21 to 28), and the same treatment was given to animals with tumours implanted in the mammary fat pad. Ibandronate had profound effects on tumour growth in bone, reducing progression of osteolytic lesions, inducing osteoclast apoptosis, inhibiting formation of new bone metastases, increasing cancer cell apoptosis and reducing tumour burden. In sharp contrast, tumour growth in the mammary fat pad was unaffected, supporting the hypothesis that the anti-tumour effects of ibandronate are restricted to tumours growing within the bone microenvironment. A later study, using MDA-MB-231 human breast tumour cells injected directly into the femoral artery of male athymic rats, also showed that ibandronate (10 μg/kg/day, days 18 to 30) reduced the extent of the osteolytic lesions [50]. This study also provided evidence that once tumours have reached a certain size (>6 mm in this model) they become less dependent on the bone microenvironment for their further expansion, and hence less sensitive to BP therapy.

The first bone metastasis study of the effects of zoledronic acid, the most potent of the BPs, used the 4T1 mouse mammary tumour model [51]. In this model there is spontaneous metastatic spread to bone, lung and liver following implantation of 4T1/luc breast cancer cells in the mammary fat pad of female BALB/c mice. This study clearly demonstrated that zoledronic acid affects both tumour cells and osteoclasts, but did not distinguish between direct effects on tumour cells and indirect effects via reduced bone resorption.

A study by van der Pluijm and colleagues showed that BPs modify tumour growth primarily through effects on bone, rather than through targeting tumour cells directly [47]. MDA-231-B/luc+ breast cancer cells were implanted by intracardiac injection, and olpadronate given as a preventive (subcutaneous 1.6 μmol/kg/day from 2 days before implantation) or a treatment (days 3 to 43) schedule. Effects on the formation of new bone metastases and osteolysis were assessed, as well as tumour burden both inside and outside the bone marrow cavity. As expected, BP treatment reduced the level of cancer-induced bone disease regardless of schedule, with preventive treatment causing a substantial reduction in the number of bone metastases. However, the reduction in tumour growth was only transient and did not affect progression of established tumours. The study also included an intra-osseous model, where daily injections of pamidronate or olpadronate (1.6 μmol/kg/day) were given from day 3 to day 43. In this experiment, both BPs caused a significant reduction of the intra-osseous tumour burden. However, there was an increase of the total tumour burden (including in the bone-associated soft tissues), indicating that tumour growth is shifted from the bone marrow cavity to extra-osseous sites.

The optimal dosing regimen of BPs for inhibition of tumour growth remains to be established, and whether clinically relevant BP doses are sufficient to affect tumour growth is a hotly debated issue. One study has aimed to establish whether low, frequent (daily) dosing with BPs is superior to weekly administration, and how this compares to a single administration of the same total dose [52]. Female BALB/c athymic mice were injected with human B02/GFP.2 breast cancer cells (a bone-homing subclone of MDA-MB-231) and zoledronic acid administered daily (intravenous 3 μg/kg preventive and 7 μg/kg therapeutic), weekly (20 μg/kg preventive and 50 μg/kg therapeutic) or as a single dose schedule (100 μg/kg preventive or therapeutic). The total accumulated concentration of zoledronic acid was 98 to 100 μg/kg/mouse, equivalent to the 4-mg clinical dose. Clodronate was administered daily at 530 μg/kg, equivalent to the clinical dose of 1,600 mg/day. Both preventive and therapeutic administration of clodronate (daily) and zoledronic acid (daily or weekly) caused a significantly reduced bone tumour burden, and there was no evidence of increased bone-associated soft tissue tumour growth. In contrast, the single administration of zoledronic acid had only minimal effect on tumour growth, even when administered prior to tumour cell inoculation (13% reduction compared to control). Importantly, the different BPs and schedules all inhibited bone resorption to a

comparable degree, whereas the effects on tumour growth varied. These intriguing data demonstrate that there is a substantial difference in the outcome depending on the BP schedule used, and that frequent low dose administration has more profound effects on tumour growth in bone compared to giving the same total dose as a single injection.

Bisphosphonates as part of combination therapy

As the above studies demonstrate at best a limited, transient anti-tumour effect of BPs, these agents may hold greater promise when used in combination with therapies that target tumour cells directly. This has been explored in a number of *in vitro* and *in vivo* studies, using a variety of cancer cell types [1,3].

Initial studies of the effects of the chemotherapy regimen UFT (tegafur plus uracil) combined with zoledronic acid used the syngeneic 4T1 model, where female BALB/c mice were injected orthotopically (mammary fat pad) with the murine breast cancer cell line 4T1, resulting in dissemination of the tumour cells to bone [53]. A single injection of zoledronic acid (250 μg/kg, day 7), or oral administration of UFT (20 mg/kg/day, days 14 to 21), significantly reduced the area of bone metastases. Combining both therapies caused an increased reduction in bone lesions compared to that caused by giving the single agents, but crucially there was no reduction in tumour volume at the primary site.

The majority of combination therapy studies in breast cancer have used xenograft models, where human breast cancer cells are implanted in immunocompromised mice via intra-cardiac or intra-tibial injection. Most studies have been done with zoledronic acid, due to its widespread use in the treatment of breast cancer-induced bone disease (Table 3). The effects of combining zoledronic acid with the antibiotic doxycycline have been tested on tumour growth in bone following intracardiac injection of MDA-MB-231 human breast cancer cells in Balb/c-nu/nu mice [54]. Both single treatments and the combination resulted in reduced osteolysis, and in decreased tumour burden in bone and surrounding soft tissues. Intriguingly, administration of zoledronic acid alone resulted in a 93% reduction of bone-associated soft tissue tumour area, but only in a 73% reduction in total tumour burden, suggesting a direct effect on tumours growing outside the bone microenvironment. These promising data need to be confirmed using a treatment protocol, to determine whether the combination of doxycycline and zoledronic acid can also reduce the growth of established breast cancer metastases.

Whether a single administration of a clinically relevant dose of zoledronic acid can increase the anti-tumour effect of doxorubicin has been investigated using female BALB/c-nu/nu mice injected with MDA-MB-231/B02 human breast cancer cells that specifically metastasise to bone [48]. Animals with confirmed tumour growth in bone were treated with saline, doxorubicin (2 mg/kg, days 18 and 25), zoledronic acid (100 μg/kg day 19, equivalent to the 4-mg clinical dose), zoledronic acid and doxorubicin simultaneously, or doxorubicin followed 24 hours later by zoledronic acid. All the treatment schedules that included zoledronic acid caused a significant reduction in osteolytic lesion area compared to control or doxorubicin treatment. The most effective reduction in intra-osseous tumour burden was found in animals that received sequential treatment with doxorubicin followed by zoledronic acid. The reduced tumour burden in this group was associated with increased levels of tumour cell apoptosis and a decrease in tumour cell proliferation. In contrast, extra-osseous tumour burden was unaffected by all of the treatment schedules, suggesting that the tumour microenvironment as well as differential drug concentration in different parts of the tumour may determine the response to treatment.

The molecular processes affected by combination therapy with doxorubicin and zoledronic acid were further elucidated using a model of MDA-MB-436 breast cancer cells directly implanted in bone [55]. A 6-week course of weekly administration of doxorubicin (2 mg/kg), followed 24 hours later by zoledronic acid (100 μg/kg), caused substantial inhibition of tumour burden in bone compared with administration of the single agents. Molecular analysis of the tumours from animals treated sequentially with doxorubicin followed by zoledronic acid showed reduced numbers of proliferating tumour cells, accompanied by decreased levels of expression of cyclins E1, B, D1, and D3, as well as cdk2 and cdk4. Tumours from the sequential treatment group also displayed increased levels of apoptosis, associated with increased expression of the pro-apoptotic molecule bax, decreased expression of the anti-apoptotic molecule bcl-2, and activation of caspases 3, 8, and 9. Doxorubicin had no effect on tumour growth, cell cycle, or apoptosis *in vivo*, but did cause increased accumulation of a BP in MDA-MB-436 cells *in vitro*, suggesting that doxorubicin may affect subsequent uptake of zoledronic acid. In support of this, accumulation of unprenylated Rap1A, a surrogate marker of zoledronic acid, was only detected in tumours following sequential treatment.

Benefits of adding BPs to combination therapy is not limited to zoledronic acid, as demonstrated by a recent study using risedronate [56]. Female BALB/c-nu/nu mice were inoculated intratibially with MDA-231-B/luc+ cells, and treated with risedronate, docetaxel or a combination of both. Risedronate, alone or in combination with docetaxel, prevented osteolytic bone destruction compared to control, whereas administration of

Table 3. Overview of studies investigating bisphosphonates as part of combination therapy in breast cancer

Breast cancer model	Bisphosphonate (dose)	Anti-cancer agent (dose)	Effect compared to single agent	Reference
4TC/luc: spontaneous bone metastases	Zoledronic acid (250 μg/kg single administration)	Uracil, tegaflur (20 mg/kg/day for 7 days)	Reduced area of bone metastases	[53]
MDA-MB-231: intracardiac injection	Zoledronic acid (0.2 μg/mouse every 2 days ×9)	Doxycycline (15 mg/kg/day for 21 days)	Reduced tumour burden in bone and in soft tissue	[54]
B02: generates bone metastases following intravenous injection	Zoledronic acid (100 μg/kg single administration)	Doxorubicin (2 mg/kg weekly for 2 weeks)	Reduced intra-osseous tumour growth and lytic bone disease No effect on extra-osseous parts of the tumour	[48]
MDA-MB-436: subcutaneous tumours	Zoledronic acid (100 μg/kg weekly for 6 weeks)	Doxorubicin (2 mg/kg weekly for 6 weeks)	Maximal reduction of tumour growth when doxorubicin given 24 h prior to zoledronic acid No evidence of tumours in bone	[41]
MDA-MB-231luc: intratibial implantation	Risedronate (150 μg/kg, 5×/week)	Docetaxel (4 mg/kg, 2×/week)	Reduced tumour burden in bone and reduced osteolytic lesions	[56]
MDA-MB-436: intratibial implantation	Zoledronic acid (100 μg/kg weekly for 6 weeks)	Doxorubicin (2 mg/kg weekly for 6 weeks)	Reduced tumour burden in bone and reduced lytic bone disease	[55]
MDA-MB-436: subcutaneous tumours	Zoledronic acid (100 μg/kg weekly for 6 weeks)	Doxorubicin (2 mg/kg weekly for 6 weeks)	Reduced tumour growth and increased survival Sustained inhibition of tumour growth following 6 weeks of treatment	[58]

docetaxel alone had no effect. Tumour growth in bone was undetectable in six out of seven mice following combination treatment, treatment with docetaxel prevented tumour growth in two out of seven mice, and risedronate treatment had no effect.

Anti-tumour effects of bisphosphonates in breast tumours outside bone

A number of different mechanisms contribute to the observed anti-tumour effects (Figure 3), including reduction in tumour macrophage infiltration, decreased tumour angiogenesis, activation of immune cells, reduction in the levels of bone-derived tumour growth factors and effects on bone marrow precursors. But could BPs also reduce tumour growth outside the skeleton? Many of the proposed mechanisms responsible for BPs reducing tumour growth in bone would also apply to tumours growing at peripheral sites (Figure 2), and this has initiated a limited number of studies aimed at determining whether BPs, alone or in combination with chemotherapeutic agents, reduce either the development of visceral metastases or directly reduce the growth of subcutaneously implanted breast tumours.

Bisphosphonates used as single agents

The effects of zoledronic acid on the development of visceral breast cancer metastases have been determined using the 4T1 model [57]. While a single dose of 5 μg zoledronic acid did not affect tumour burden in visceral organs, a repeated dosing regimen significantly reduced the number of metastatic foci in lung and liver. Detailed histological analysis revealed that there was no increase in the levels of apoptotic 4T1/luc cell death in the lung, suggesting that the anti-tumour effect was not mediated through increased tumour cell killing. The authors concluded that the anti-tumour effects induced by zoledronic acid in soft tissues are probably due to inhibition of tumour cell invasion and migration. These results were, however, generated through high and repeated dosing with zoledronic acid, and the clinical relevance of the findings remains to be established.

Bisphosphonates as part of combination therapy

In order to separate the direct anti-tumour effects of BPs from those mediated via bone, Ottewell and colleagues [41] investigated whether sequential or combined treatment with doxorubicin and zoledronic acid can affect subcutaneous breast tumour growth. MDA-G8 human breast cancer cells (a subclone of MDA-MB-436) were injected subcutaneously in the flank of female MF1 nu/nu mice, and once tumours were palpable, animals were treated once per week for 6 weeks with saline, doxorubicin (2 mg/kg), zoledronic acid (100 μg/kg), zoledronic acid and doxorubicin together, doxorubicin followed 24 hours later by zoledronic acid, and vice versa. Administration of the single agents had no significant effect on tumour size compared to saline control, but combined administration of the two agents caused around 50% reduction in tumour size when compared to animals treated with doxorubicin alone. Surprisingly, sequential treatment with doxorubicin followed by zoledronic acid caused almost complete abolition of tumour growth, whereas administration of the reverse drug sequence had no effect.

The anti-tumour effect was associated with increased levels of cancer cell apoptosis and reduced proliferation compared to other treatment groups. Pathway-specific gene array analysis showed that at least 30 genes involved in cell cycle regulation and apoptosis had been specifically changed in the tumours following sequential

treatment. The reduction of tumour growth may also be partly mediated by inhibition of angiogenesis, as both combined and sequential treatment (doxorubicin followed by zoledronic acid) appeared to cause a major reduction in tumour vascularisation. However, the cumulative concentrations of zoledronic acid used, although clinically achievable, still exceed doses used to treat advanced breast cancer.

In a follow-up study, the same group reported that a 6-week course of weekly sequential treatment with doxorubicin and zoledronic acid had a sustained anti-tumour effect, as the tumours did not re-grow in the 5 months following completion of treatment [58]. Detailed molecular analysis of the tumours from the different treatment groups showed that sequential therapy triggered particular molecular pathways, inducing increased apoptosis and reducing tumour cell proliferation. In addition, there was a substantial reduction in the number of F4/80 positive cells (macrophages) infiltrating the tumours following sequential administration of doxorubicin and zoledronic acid.

Clinical perspective

There is increasing clinical evidence to support an 'anti-tumour effect' of BPs in breast cancer and indeed other malignancies. In addition to the benefits of adjuvant zoledronic acid seen in premenopausal oestrogen receptor positive (ER+) breast cancer described earlier [13], other clinical studies [15,16,59,60] in breast cancer have shown intriguing positive results and are reviewed elsewhere in this issue. Furthermore, the incidence of invasive breast cancer appears to be lower in post-menopausal women taking oral BPs for breast cancer [61-63], survival in multiple myeloma is enhanced with zoledronic acid in combination with chemotherapy [64] and sequence-dependent anti-tumour effects with docetaxel followed by zoledronic acid have been observed in prostate cancer [65]. It is becoming increasingly evident that BPs are more than just supportive care drugs.

Conclusion

This review has summarised our current understanding of the anti-tumour effects of BPs in breast cancer, based on data from *in vitro* and *in vivo* model systems, as well as linking these to recent reports from clinical studies. Taken together, there is considerable evidence to show that as long as tumour cells are exposed to sufficient doses of BPs, they will be negatively affected by the drugs. However, whether this is achieved following clinical administration of BPs to a degree that ultimately affects tumour growth remains to be determined. Recent data suggest that we should not focus exclusively on whether BPs target tumour cells directly, but also consider how these potent anti-resorptive agents modify cells in the bone microenvironment that are essential for tumour growth.

This article is part of a review series on Bisphosphonates and other bone-targeted agents in breast cancer, edited by Janine Mansi and Robert Coleman. Other articles in the series can be found online at http://breast-cancer-research.com/series/bisphosphonates

Abbreviations

BP, bisphosphonate; EC, endothelial cell; EPC, endothelial progenitor cell; HUVEC, human umbilical cord-derived endothelial cell; NBP, nitrogen-containing bisphosphonate; ONJ, osteonecrosis of the jaw; VEGF, vascular endothelial growth factor.

Competing interests

The authors declare that they have no competing interests.

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