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Review Article

The anti-tumour effects of zoledronic acid

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ABSTRACT

Bone is the most common site for metastasis in patients with solid tumours. Bisphosphonates are an effective treatment for preventing skeletal related events and preserving quality of life in these patients. Zoledronic acid (ZA) is the most potent osteoclast inhibitor and is licensed for the treatment of bone metastases. Clodronate and pamidronate are also licensed for this indication.

In addition, ZA has been demonstrated to exhibit antitumour effect. Direct and indirect mechanisms of anti-tumour effect have been postulated and at many times proven. Evidence exists that ZA antitumour effect is mediated through inhibition of tumour cells proliferation, induction of apoptosis, synergistic/additive to inhibitory effect of cytotoxic agents, inhibition of angiogenesis, decrease tumour cells adhesion to bone, decrease tumour cells invasion and migration, disorganization of cell cytoskeleton and activation of specific cellular antitumour immune response. There is also clinical evidence from clinical trials that ZA improved long term survival outcome in cancer patients with and without bone metastases. In this review we highlight the preclinical and clinical studies investigating the antitumour effect of bisphosphonates with particular reference to ZA.

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1. Introduction

Bisphosphonates are proven to be effective in preventing/delaying skeletal-related events in patients with bone metastases and potentially preserving functional independence and quality of life. This effect is mediated by the inhibitory effect of bisphosphonates on osteoclasts.

Recently, it has been reported that bisphosphonates may have anti-tumour effect as well.

There are two classes of bisphosphonates that differ with regard to structure and mechanism of action [1]. The first one includes pyrophosphate-resembling bisphosphonates, such as clodronate and etidronate, which are metabolically incorporated into nonhydrolyzable adenosine tri-phosphate (ATP) analogues that act as inhibitors of ATP-dependent enzymes. The second class which is more recent and potent includes nitrogen-containing bisphosphonates (N-BPs), such as alendronate, pamidronate, risedronate, ibandronate and zoledronic acid (ZA).

N-BPs inhibit a key enzyme, farnesyl diphosphonate (FPP) synthase, in the biosynthetic mevalonate pathway. As a result, these compounds interfere with a variety of cellular functions essential for the bone-resorbing activity and survival of osteoclasts. Several

intermediates in this pathway (Fig. 1), including farnesyl pyrophosphate and geranylgeranyl pyrophosphate, are required for the post-translational modification (i.e., prenylation) of guanosine triphosphate-binding proteins such as Ras, Rho, and Rac. These signalling molecules are involved in the regulation of cell proliferation, cell survival, and cytoskeletal organization [2,3].

ZA is reported to be more potent inhibitor of farnesyl diphosphonate synthase than the other bisphosphonates risedronate, ibandronate, incadronate, alendronate, and pamidronate [4].

Preclinical findings provide insight into possible mechanisms of action of bisphosphonates that may explain their ability to inhibit tumour cells. This report reviews the preclinical and clinical data investigating the anti-tumour effects of ZA.

1.1. Preclinical rationale for potential anticancer effects of ZA

Preclinical data indicate that possible anti-cancer mechanisms of ZA (and other bisphosphonates) may include (Fig. 2):

- Inhibition of tumour cell proliferation and induction of apoptosis.
- Augmentation of inhibitory effect of cytotoxic agents (additive and synergistic effect).
- Inhibition of angiogenesis.
- Decrease in tumour cell adhesion to bone.
- Decrease in tumour cells invasion and migration and disorganization of cell cytoskeleton.

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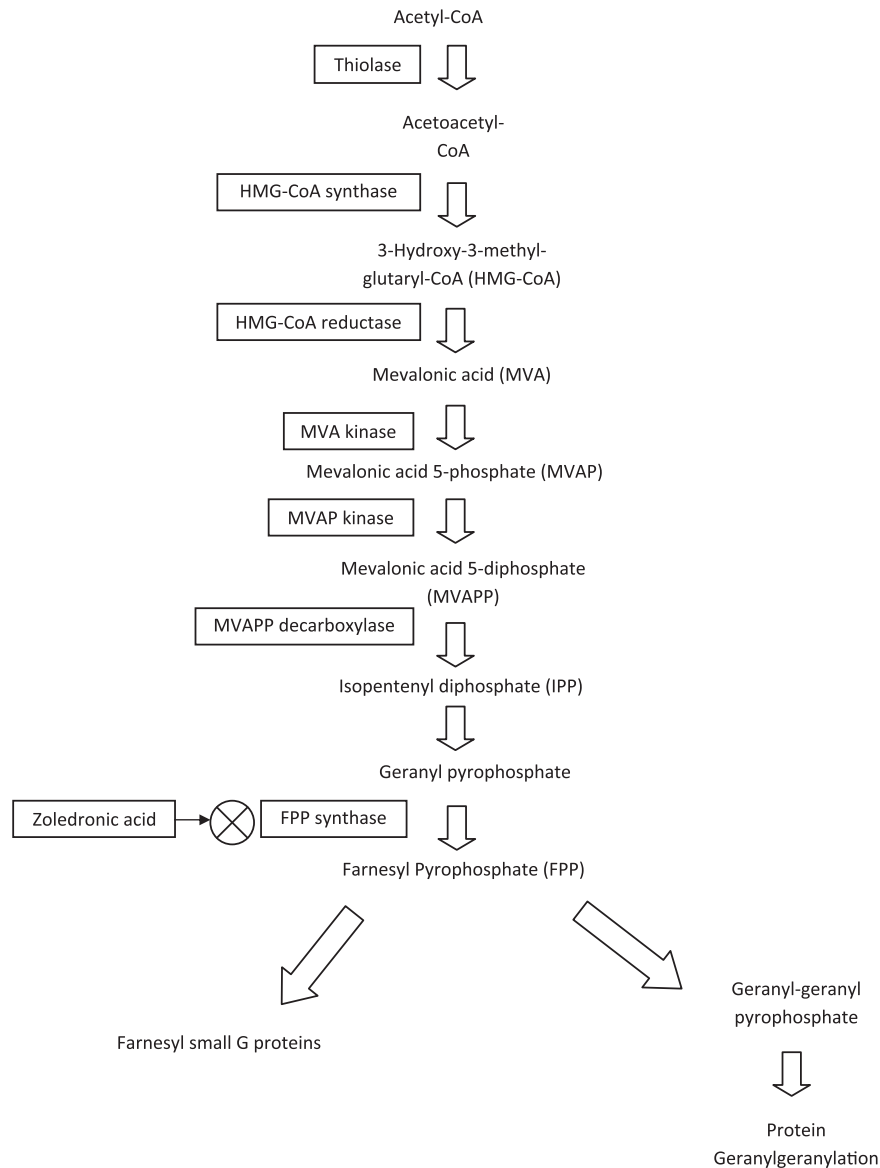


Fig. 1. Flowchart showing the mevalonate pathway.

- Activation of $\gamma\delta$ T cells.
- Effects on tumour macrophage infiltration.

Preclinical studies investigating these possible mechanisms of action are presented below and summarized in [Table 1](#).

1.1.1. Inhibition of tumour cell proliferation and induction of apoptosis

ZA inhibits a key enzyme of the mevalonate pathway, farnesyl diphosphonate synthase.

Inhibition of this enzyme prohibits formation of isoprenoids, such as farnesyl diphosphate (FPP) and geranylgeranyl diphosphate (GGPP), which are required for regular prenylation of small GTPbinding proteins, like Rho and Ras ([Fig. 1](#)) [5].

There is significant preclinical evidence to support the direct antitumour effect of ZA. In a preclinical study, ZA strongly inhibited in vitro proliferation, arrested cell cycle between S and G2/M phases, and induced the apoptosis of human fibrosarcoma cells [6]. The same group of investigators reported inhibition of growth

of osteosarcoma cells at the primary and secondary sites in a murine model [7].

In another study, Zwolak et al. showed that ZA can be released from bone cement (formed with increasing concentrations of ZA, up to 1 mg/1.5 cm³ of bone cement) and the proliferation assay showed ZA to have significant dose dependent cytotoxicity in cultures of stromal giant cell tumour, multiple myeloma, and renal cell carcinoma cells [8]. In a separate study, ZA showed direct antitumor effects against four oral carcinoma cell lines at concentrations ranging from 10 to 100 μ M. ZA activated the potent pro-apoptotic pathways caspase-3, -8 and -9 and induced cellular apoptosis and increased the number of cells in apoptosis. Western blot analysis showed that ZA increased cleaved anti-human poly(ADP-ribose) polymerase expression and decreased Bcl-2 and Bid expression [9]. In renal cancer cell lines (ACHN, A-498 and CAKI-2), a significant reduction in viable cells was seen for all three cell lines following treatment with ZA (at concentrations of 6.25–100 μ M), compared with untreated controls. A concomitant increase in the apoptosis significant caspase-dependent M30 antigen was demonstrated. This effect could be blocked by the pan-caspase inhibitor Z-VAD [10]. Similar apoptotic effect of ZA (in concentrations of 5–40 μ M)

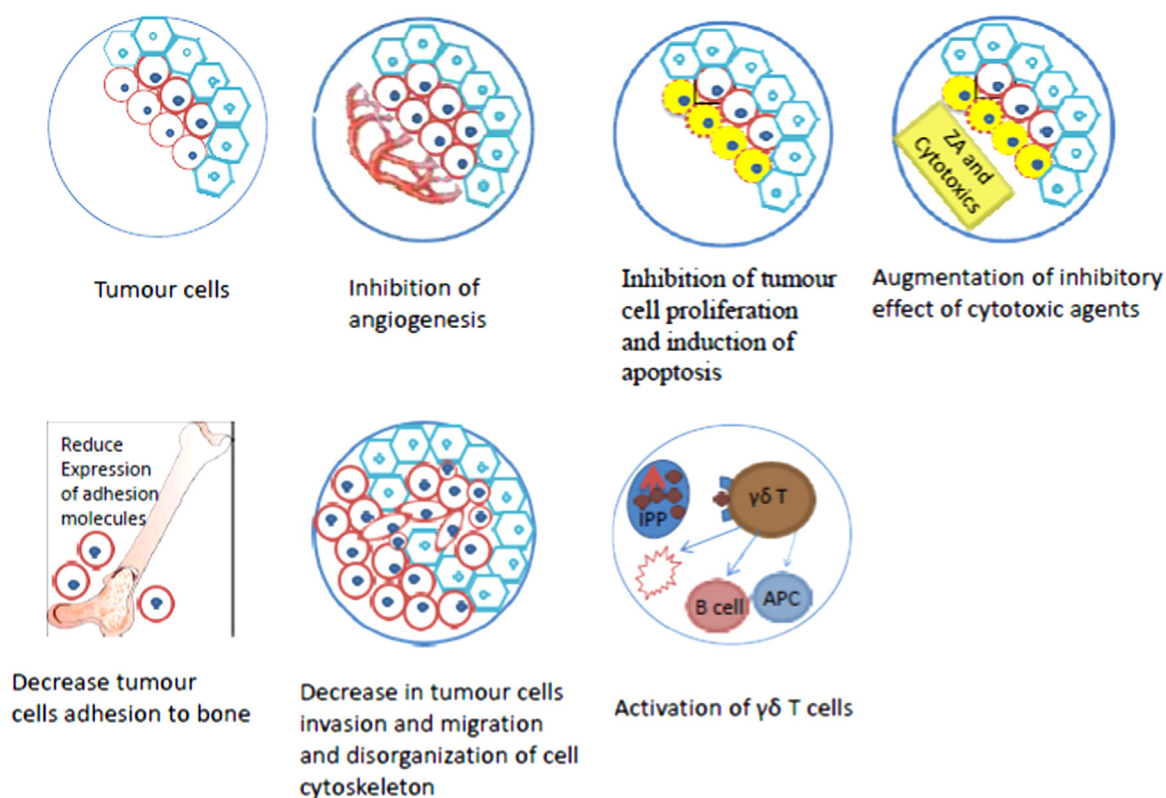


Fig. 2. Possible mechanisms of anti-tumour effect of ZA.

was seen in dexamethasone resistant multiple myeloma (MM) cells [11].

The preclinical evidence of antitumour effect is well documented for other solid tumour models as lung, breast and prostate [12,13].

It seems that at least in the preclinical setting, there is substantial evidence supporting the anti-proliferative effect of ZA mainly by inducing apoptosis. This mechanism is amenable to be studied in clinical setting. Although a 15 min intravenous infusion of 4 mg ZA achieves a maximum concentration 0.97 μM (Product Information: RECLAST(R) IV injection, zoledronic acid IV injection. Novartis Pharma Stein AG, Stein, Switzerland, 2008), some preclinical experiments in mouse models with metastatic breast cancer to bone have demonstrated in-vivo that alternative dosage regimens with ZA (daily and weekly administration in divided doses) can cause decrease in tumour burden in the bone (PMID: 17312309). Therefore daily or weekly treatment regimens of ZA (in divided doses) may be amenable to further investigation in clinical trials.

1.1.2. Augmentation of inhibitory effect of cytotoxic agents (additive and synergistic effect)

Enhancing the cytotoxic effect of anti-cancer drugs on tumour cells has always been an active area of research. In the clinical setting, cancer patients on systemic anti-cancer therapy (chemotherapy or hormonal therapy) may receive ZA for reasons other than its possible anti-tumour effect. It seems attractive to investigate potential additive/synergistic effect of ZA to chemotherapy and/or hormonal therapy. The available literature confirms this effect in pre-clinical studies.

In one study, hormone-refractory prostate cancer cell lines (PC3 and DU145) were treated with increasing concentrations of ZA in the absence or presence of docetaxel. After 72 h incubation, ZA at concentration of 25 μM reduced the viable cell number to 68% and

98% for PC3 and DU145 cells respectively. Docetaxel, on the other hand, at a concentration of 0.1 ng/ml, had no effect on the viability. However, a combination of ZA and docetaxel reduced the cell number to 60% and 81% respectively. ZA in the concentration range 12.0–50 μM enhanced the antitumoral effects of docetaxel (0.01–1 ng/ml) in an additive and/or synergistic manner for both cell lines [14].

These results were repeated by another group of investigators using the same agents and cell lines [15]. Additive/synergistic inhibitory effect of ZA is also documented with other chemotherapy agents as paclitaxel, docetaxel, doxorubicin, etoposide, 5-fluorouracil, gemcitabine, and cisplatin [6,16,17].

The additive and/or synergistic effect of ZA may be dependent on the sequence of treatment. Studies suggest that Sequential treatment with chemotherapy followed by ZA elicit substantial antitumor effects compared to the reverse sequence. Female MF1 nude mice were inoculated human breast cancer MDA-MB-436 cells. On day 7, the mice were injected weekly for 6 weeks with saline, doxorubicin or ZA alone, combination of both, ZA followed 24 h later by doxorubicin, or doxorubicin followed 24 h later by ZA. Treatment with doxorubicin followed by ZA almost completely abolished tumour growth. Tumours from mice in this group had significantly more caspase-3-positive cells than tumours from mice treated with saline, with ZA alone or with ZA followed by doxorubicin. This increase in the number of caspase-3-positive cells was mirrored by a decrease in the number of tumour cells positive for the proliferation marker Ki-67. The authors concluded that sequential treatment with doxorubicin followed by ZA elicited substantial antitumor effect [18].

Synergism is not only limited to chemotherapy. There is evidence that ZA can augment inhibitory effect of hormonal therapy. Aromatase-expressing breast cancer cells were treated with letrozole (100 nM) and ZA (10 μM) either simultaneously or in sequence, each for 24 h. Letrozole and ZA induce levels of apoptosis in breast cancer cells in vitro that are significantly greater compared with

Table 1
Summary of pre-clinical and early clinical [52,56,57] studies and publications describing various mechanisms of anti-tumour activity of nitrogen-containing bisphosphonates.

Reference number	Bisphosphonate(s) used	Concentrations/ doses studied	Cell lines	Results
Proliferation inhibition and apoptosis induction				
[6]	ZA	Up to 10 μ M	Fibrosarcoma	Inhibition, cell cycle arrest
[7]	ZA	80 μ g/kg	Osteosarcoma	Inhibition of growth
[8]	ZA	1.55–4.48 μ g/ml	Giant cell tumour, myeloma, renal cell carcinoma	Dose dependent cytotoxicity
[9]	ZA	10–100 μ M	Oral carcinoma: 4 cell lines	Induction of apoptosis (concentration dependent)
[10]	ZA	6.25–100 μ M	Renal cell carcinoma: 3 cell lines	Induction of apoptosis
[11]	ZA	Up to 20 μ M	Multiple myeloma	Induction of apoptosis
[13]	ZA	Up to 10 μ M	Prostate and breast cancer	Induction of apoptosis (concentration dependent)
[12]	ZA	100 μ M	Non-small cell lung cancer (NSCLC)	Cell cycle arrest, apoptosis induction
Additive and synergistic effect with cytotoxic agents				
[14,15]	ZA	12.5–50 μ M 5–30 μ M	Hormone resistant prostate cancer	Additive and synergistic effect with docetaxel
[16]	ZA	10–100 μ M	Breast cancer	Additive effect with paclitaxel
[17]	ZA	100 μ M	NSCLC	Additive effect with cisplatin
[6]	ZA	Up to 10 μ M	Fibrosarcoma	Additive effect with various chemotherapy agents
[18]	ZA	100 μ g/kg	Breast cancer	Additive effect with doxorubicin (when used with or after doxorubicin)
[19]	ZA	Up to 25 μ M	Breast cancer	Synergistic effect with letrozole
Inhibition of angiogenesis				
[12]	ZA	10–100 μ M	Non-small cell lung cancer	Reduction of VEGF secretion
[28]	ZA	Up to 200 μ M	Endothelial progenitor cells	Reduction of viable cells
Decrease in tumour cell adhesion to bone				
[37]	ZA	100–1000 μ M	Multiple myeloma	Decrease in bone marrow stromal cells, decreased expression of adhesion molecules
[39]	Ibandronate	1000 nM	Breast and prostate cancer	Inhibition of tumour cell binding to bone matrices
[40]	Clodronate, pamidronate, olpedronate, etidronate and ibandronate	1–100 μ M		
[38]	Ibandronate Pamidronate Clodronate	5 picoM 0.1 μ M 100 μ M		
Inhibition of tumour cell invasion and migration				
[41,43]	Reviews		Breast and prostate cancer	Inhibition of visceral metastasis
[42]	ZA	1 μ M		
[44]	ZA	0.5–5 μ mg/ mouse		
[45]	Alendronate	30 μ M	Ovarian cancer	Inhibition of cell migration
Activation of $\gamma\delta$ T cells				
[51]	Alendronate, ibandronate and pamidronate	0.9–4 μ M	Multiple myeloma	Reduced plasma cell survival due to activation of $\gamma\delta$ T cells
[52]	ZA	4 mg every 3 weeks (4 doses)	Metastatic bone disease	Maturation of $\gamma\delta$ T cells (in vivo)
[53]	ZA	1 μ M (with IL-2)	Multiple myeloma	Increase in number of $\gamma\delta$ T cells
[55]	ZA	1 μ M (with IL-2)	Small cell lung cancer and fibrosarcoma	Marked increase in sensitivity to lysis by $\gamma\delta$ T cells
[54]	ZA	3 μ M	Pancreatic cancer	Increased cancer cell cytotoxicity by $\gamma\delta$ T cells
[56]	ZA	Unknown	Prostate cancer	Activation and increase in number of $\gamma\delta$ T cells
[57]	ZA	4 mg (1 dose)	Breast cancer	Activation of $\gamma\delta$ T cells

ZA=zoledronic acid; μ M=micromolar; mg=milligrams; picoM=picomolar; $\gamma\delta$ =gamma delta.

treatment with each drug alone. However, this potentially synergistic relationship was drug-sequence dependent, occurring only when cells were treated with letrozole, followed by ZA [19].

These studies suggest the additive and synergistic effect of ZA with other anti-cancer agents. The exact mechanisms remain unknown but down-regulation of anti-apoptotic genes has been suggested [15]. Preclinical data showed increased anti-tumour efficacy if ZA is sequenced after chemotherapy compared to the opposite sequence [20]. Based on this observation, most clinical trials investigating the role of ZA in the neo-adjuvant setting including NEOZOTAC [21], ANZAC [22] and NeoAZURE [23] administered ZA after chemotherapy. The adjuvant phase III AZURE study is also an example where investigators used ZA after chemotherapy [24].

The sequence of administration may need to be addressed in future clinical trials. One clinical investigational approach will be

to randomize patients in the neoadjuvant or metastatic setting to different sequences of ZA and specific anti-cancer therapy. Pre-treatment and post-treatment biopsies to study molecular changes may help elucidate mechanisms of this additive/synergistic effect.

1.1.3. Inhibition of angiogenesis

Angiogenesis is a prerequisite for the progressive growth of solid tumours and their metastasis [25]. In malignant tumours, the development of new vessels is directed and regulated by a complex network of endogenous pro-angiogenic factors, e.g. vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) and by factors secreted by the tumour itself. Angiogenesis enables the tumour to metastasize to various sites. Thus, inhibition of angiogenesis is a promising strategy in the treatment of malignant tumours.

There is evidence that ZA inhibits differentiation of cultured human peripheral blood mononuclear cells into endothelial progenitor cells [26]. ZA inhibits proliferation of human endothelial cells in vitro and reduces vessel sprouting in cultured aortic rings and chicken egg chorioallantoic membrane assay [27]. This study also provided the first in-vivo evidence that ZA affects angiogenesis occurring in non-mineralized tissue. However, ZA dose of 100 µg/kg was required to achieve any significant antiangiogenic effect.

In one study, ZA at concentration of 10–100 µM induced dose dependent reduction both of mRNA and protein expression of VEGF in A549 NSCLC cells associated with parallel decrease in VEGF secretion in the culture medium after 2 days of treatment [12].

In another study, the viability of human umbilicord vein endothelial cells (HUVEC) and endothelial progenitor cells (EPC) was significantly reduced after incubation with bisphosphonates compared with the non-treated control groups. The nitrogen-containing bisphosphonates pamidronate and ZA had the greatest impact on the cells, whereas the inhibitory effect of clodronate and ibandronate on these cells was less distinct [28].

An in vivo studies show that ZA (20 µg/mouse) for 3 times a week for 3 consecutive weeks inhibited angiogenesis in Matrigel plugs and inhibited the growth and neo-angiogenesis of CG5 xenografts in athymic nude mice [29]. Another in vivo study documented that capillary densities were significantly lower in mice pre-treated with low dose (30 µg/kg) and high dose ZA (100 µg/kg) than in control mice in response to surgically induced hypoxia. Ischaemic tissue from ZA pre-treated mice also showed impaired mobilization of endothelial progenitor-like cells and lower levels of the active form of MMP-9 and VEGF compared to ischaemic tissue from control mice [30].

Another in-vivo supporting evidence that ZA uptake occurs in non-mineralized tissue is provided by Stresing et al. ZA inhibited the revascularization of the prostate gland in testosterone-stimulated castrated rats. It also induced intracellular accumulation of isopentenyl pyrophosphate (IPP) in endothelial cells by blocking the activity of the IPP-consuming enzyme FPPS. Thus, these results indicated that N-BPs inhibited angiogenesis in a FPPS-dependent manner [31].

The more potent antiangiogenic effect of nitrogen-containing bisphosphonates compared to other bisphosphonates in preclinical studies mirrors their superior efficacy seen in clinical setting.

Recent in-vivo evidence showed that bone marrow endothelial progenitor cells (BM EPCs) are affected after ZA administration. On flow cytometry, BM EPCs increased in response to acute parathyroid hormone therapy but not when treatment was combined with ZA. This observation provides indirect suggestion to uptake of ZA by bone endothelial cells [32].

Accumulating reports have shown that cancer patients who have received nitrogen-containing bisphosphonates such as ZA occasionally manifest bisphosphonate-related osteonecrosis of the jaw (BRONJ) following dental treatments including tooth extraction. Little is known about the pathogenesis of BRONJ to date. The results of Kobayashi Y and his colleagues' work showed that ZA alters oral bacterial behaviours and delays wound healing of the tooth extraction socket by inhibiting osteogenesis and angiogenesis. They conclude that these actions of ZA may be relevant to the pathogenesis of ONJ [33]. This conclusion is supported by clinical observation of higher incidence of ONJ in cancer patients treated with ZA and anti-angiogenic agents [34,35].

The available data not only demonstrate the importance of anti-angiogenic properties of ZA but also elucidates the potential mechanism and molecular targets of this property.

Most of preclinical supporting evidence for the anti-angiogenic properties of ZA comes from in vitro models and therefore further evaluation in more animal models and humans is needed. One

challenge in clinical studies will be the need for higher concentrations which seem to be needed to achieve anti-angiogenesis.

1.1.4. Decrease in tumour cell adhesion to bone

Recent studies have shown the importance of the microenvironment in the pathophysiology of metastatic bone disease. Adhesive interactions of tumour cells play an important role in up-regulating the production of cytokines and growth factors by bone marrow stromal cells, which consequently enhance tumour growth, bone destruction, and tumour survival [36]. Multiple myeloma (MM) tumour cells naturally home at bone marrow (BM) and provide a model for studying the effect of bisphosphonates on behaviour of tumour cells in this environment. Adhesion molecules facilitate binding of MM cells to the BM stroma. There is rising laboratory evidence that ZA decrease tumour cells adhesion to bone micro-environment.

In one study, BM stromal cells, obtained from BM mononucleated cells of eight patients with MM, were treated with increasing concentrations of ZA, ranging from 10^{-4} to 10^{-5} M. After 3 days of exposure ZA induced decrease in proliferation and increase in apoptosis. Among the adhesion molecules, CD106, CD54, CD49d, and CD40, which were strongly expressed at baseline, showed a statistically significant reduction compared with controls after exposure to higher concentrations of ZA [37].

Bisphosphonate treatment in particular ZA and ibandronate inhibit the binding of human breast and prostate cancer cells to mineralized and unmineralized matrices [38–40].

Among possible anti-tumour mechanisms of ZA, the above mechanism seems to be the one least studied in pre-clinical setting. If this mechanism is confirmed in clinical setting, it may demonstrate an impact on development of bone metastases rather than direct anti-tumour effect.

1.1.5. Decrease in tumour cell invasion and migration, and disorganization of cell cytoskeleton

Tumour cell invasion is intrinsically linked to localized cell surface proteolytic activity driven by matrix metalloproteinases (MMPs), which facilitates cell detachment from matrix proteins, thereby promoting cell migration. Nitrogen-containing bisphosphonates (NBPs) inhibit breast and prostate cancer cell invasion [41–45]. High concentrations of bisphosphonates inhibit the zinc-dependent proteolytic activity of MMPs, whereas submicromolar concentrations are sufficient to inhibit tumour cell invasion [41]. Indeed, alendronate and ZA inhibit ovarian and breast cancer cell migration, by attenuating the geranylgeranylation of RhoA [42,44,45,37–39], a key player in cell adhesion dynamics that drive cell motility. ZA also inhibits the chemokine CXCL12-induced breast cancer cell migration by decreasing the cell surface expression of CXCR4, the receptor for CXCL-12 [42,37]. Therefore, the anti-invasive properties of NBPs may be the result of the inhibition of distinct molecular pathways that mediate cancer cell invasion in a coordinated fashion. These compounds may eventually also inhibit MMP activity if high local bisphosphonate concentrations are achieved in the tumour microenvironment [46].

Denoyelle et al. studied the effect of ZA on the invasiveness and morphology of MDA-MB-231 breast cancer cells. Treatment by ZA for 18 h induced significant inhibition of cell invasion starting at concentration of 1 µM with higher concentrations (10–100 µM) inducing more inhibition. One micromolar concentration of ZA was chosen to study the effect on cell morphology. Untreated cells were flat and well spread. In contrast ZA induced dramatic morphological changes characterized by a cell rounding and a disorganization of actin cytoskeleton accompanied by a loss of stress fibres formation [42]. The investigators found that the observed inhibition of cell

invasion by ZA was associated with decreased translocation of RhoA from cytoplasm to the cell membrane.

Later work by different group of investigators demonstrated that ZA inhibits HUVEC adhesion, survival, migration and actin stress fibre formation by interfering with protein prenylation and has identified ERK1/2, JNK, Rock (RhoA target mediating actin fibre formation), FAK and PKB as kinases affected by ZA in a prenylation-dependent manner [47].

It remains to be seen if these anti-invasion and anti-migration effects of ZA combined with other anti-tumour effects can be translated into managing early stage breast cancer to prevent relapse and metastases.

1.1.6. Activation of $\gamma\delta$ T cells

T cells bearing the T-cell receptor (TCR)- $\gamma\delta$ represent a minor subset of human peripheral T cells (1–10%). The physiologic function of $\gamma\delta$ T cells remains elusive, though some evidence suggests that $\gamma\delta$ T cells play a role in the “first line of defence” against a broad spectrum of invasive microorganisms such as mycobacteria. In addition, certain hematopoietic tumour cells (e.g., Burkitt lymphoma cell line Daudi or myeloma cell line RPMI 8226) are specifically recognized and lysed by these T cells in vitro [48,49]. The recognition of ubiquitous nonpeptide antigens by $\gamma\delta$ T cells suggests a surveillance function of these T cells for infected or transformed cells [50].

One of the early studies examining the stimulatory capacity of bisphosphonates to $\gamma\delta$ T cells was carried out by Kunzmann et al. In their work alendronate, ibandronate, and pamidronate induced significant expansion of $\gamma\delta$ T cells (V γ 9V δ 2 subset) in peripheral blood mononuclear cell cultures of healthy donors. Pamidronate-activated $\gamma\delta$ T cells produced cytokines (i.e., interferon [IFN]- γ) and exhibited specific cytotoxicity against lymphoma (Daudi) and myeloma cell lines (RPMI 8226, U266). Pamidronate-treated BM cultures of 24 patients with MM showed significantly reduced plasma cell survival compared with untreated cultures [51].

In another study examining the stimulatory capacity of ZA to $\gamma\delta$ T cells, nine patients with metastatic bone disease from breast and prostate cancer were injected with ZA. Peripheral blood mononuclear cells were collected before treatment, and 1 month and 3 months after the first administration. The objective of this study was to evaluate the in vivo effect of ZA on subsets of V γ 9V δ 2 cells. The final conclusion was that in vivo treatment with ZA induces V γ 9V δ 2 cells to mature toward an IFN γ -producing effector phenotype, which may induce more effective antitumor responses [52].

Significant numbers of reports followed and confirmed the stimulatory effect of ZA on $\gamma\delta$ T cells. ZA induced proliferation of $\gamma\delta$ T cells from normal and MM patients. The cells produced IFN- γ and exerted direct cell-to-cell antimyeloma activity. ZA plus IL-2 increased the absolute number of $\gamma\delta$ T cells 298–768 fold after 14 days incubation [53].

Several studies showed that ZA not only induced marked increase in sensitivity of tumour cells to lysis by $\gamma\delta$ T cells [54, 55] but also increased the number of $\gamma\delta$ T cell when IL-2 was co-administered [56]. The activating effect of ZA on $\gamma\delta$ T cells was also observed in vivo in disease-free breast cancer patients after a single-dose of ZA [57]. This observation may serve as a rationale for clinical evaluation of ZA in the adjuvant setting. However, recent evidence suggests that repeated treatment with ZA in vivo reduces V γ 2V δ 2 T cell numbers and their responsiveness to stimulation [58]. This may explain the inconsistent results seen in large clinical trials investigating the role of ZA in adjuvant setting (as discussed below).

1.1.7. Effects on tumour macrophage infiltration

Tumour cells interact with the surrounding microenvironment to grow and develop. Macrophages are a major component of this

microenvironment, and are of particular interest as potential therapeutic targets due to their central role in tumour progression.

It is accepted that tumour associated macrophages have an important role in several stages of oncogenesis and tumour progression including tumour cell growth, angiogenesis, migration, invasion, and metastasis [59]. Higher level of macrophage infiltration is associated with poor prognosis in several tumour types [60]. There is evidence that tumour macrophages play a role in regulating angiogenesis and thus may promote tumour cells proliferation [61].

There is in vivo and in vitro evidence that tumour macrophages uptake ZA. Exposure of J774 macrophages to 5 μ M ZOL for 24 h caused increased uptake of ZA. Higher doses and longer exposure induced apoptotic cell death [62].

There is pre-clinical evidence that ZA is more potent macrophage inhibitor than other bisphosphonates. J774A.1 cells were cultured in a standard culture medium for 2-days. Bisphosphonates (alendronate, pamidronate, etidronate, risedronate and ZA) were added in the medium at concentration of 10^{-6} – 10^{-4} M during 3 days. Etidronate did not cause significant apoptosis or necrosis, at any concentration. Alendronate and pamidronate caused apoptosis and death only at very high concentration [10^{-4} M]. On the contrary, apoptotic and necrotic cells were evidenced with risedronate or ZA at lower concentrations [63].

Enhanced expression of Matrix Metalloproteinase-9 (MMP-9) in macrophages is induced by interaction with tumour cells. This plays a major role in tumour cells invasion and metastasis. In vivo, treatment with amino-bisphosphonates was shown to impair tumour growth, decrease MMP-9 expression and decrease the number of macrophages in tumour stroma [64].

ZA has been found to reverse the polarity of peritoneal and tumour-associated macrophages from M2 to M1. This is a very important finding, as M1 macrophages possess tumoricidal activity, supporting that TAMs are a potential immune target of ZOL therapy [65].

The macrophage mediated anti-tumour effect of amino-bisphosphonates is well established in pre-clinical studies. However, there is lack of clinical studies confirming this effect in cancer patients.

1.2. Early clinical (phase I/II) data regarding anticancer effects of zoledronic acid

1.2.1. Direct cytotoxic effect of ZA

As discussed above, preclinical evidence suggests that bisphosphonates (with special reference to nitrogen containing bisphosphonates including ZA) possess inherent anti-tumour activity. These data suggest that adding ZA to other systemic cancer therapies may provide additional antitumor and anti-metastatic activity.

In the clinical setting, the results of large clinical trials investigating bisphosphonates less potent than ZA (clodronate and pamidronate) were inconsistent [66,67]. Because of these potential antitumor effects, ZA which is more potent than clodronate and pamidronate has been investigated in early clinical studies in cancer patients with and without metastases.

In one study, 40 patients with recurrent or metastatic advanced cancer, without bone metastases, were randomized to receive ZA or no treatment. Patients were followed up until bone metastases were established. At 1 year, 60% of patients in ZA arm compared to 10% in the control group were bone metastases free ($p < 0.0005$). At 18 months, the percentages were 20% and 5% ($p = 0.0002$) [68].

In a phase II trial, 120 women with clinical stage II–III breast cancer planned for four cycles of neoadjuvant chemotherapy were randomised to receive 4 mg ZA intravenously every 3 weeks or no ZA for 1 year. The primary endpoint was the number of patients with detectable disseminated tumour cells (DTCs) at 3 months. At baseline, DTCs were detected in 43% of patients in the ZA group and 48% of patients in the control group. At 3 months, 30% patients

receiving ZA versus 47% of patients who did not receive ZA had detectable DTCs ($p=0.054$) [69]. The antitumour effect of ZA on bone marrow DTC in women with early breast cancer was also shown by other investigators [70,71].

1.2.2. Inhibition of angiogenesis

In an Italian study, 42 breast cancer patients with bone metastases were treated with a single infusion of 4 mg ZA before anticancer chemotherapy. The patients were prospectively evaluated for circulating levels of VEGF just before and at 1, 2, 7, and 21 days after ZA infusion. Serum VEGF median levels were significantly decreased at each time point after treatment, but the major reduction was recorded 21 days after the infusion. Twenty-five patients (59.5%) experienced a reduction of at least 25% in the VEGF circulating levels. The analysis of survival showed that patients with a reduction in VEGF circulating levels had longer time to first skeletal-related event ($p=0.0002$), time to bone progression disease ($p=0.0024$), and time to performance status worse ($p=0.0352$) than those without the VEGF reduction. No statistically significant differences were recorded in terms of overall survival and time to visceral progression [72]. This study confirms that ZA has an *in vivo* antiangiogenic property and that VEGF modifications may represent a surrogate marker to outcome.

These results were repeated by the same group in another 24 patients. Moreover, there was significant correlation between median VEGF and the bone resorption marker betaCTx [73].

Interestingly, in both studies [72,73] serum VEGF levels showed sustained suppression after a single dose of ZA. This is in parallel to the finding that repeated treatment with ZA *in vivo* reduces V γ 2V δ 2 T cell numbers and their responsiveness to stimulation [58]. These findings raise the question of the number of ZA doses that should be considered optimal for future clinical trials.

Reduction in circulating VEGF appeared to be long lasting and persisted as long as patients were receiving ZA [74]. In a study of 60 breast cancer patients with bone metastases, metronomic ZA was more effective than the conventional regimen and generated sustained reductions in circulating VEGF and bone resorption marker levels, as well as stabilization of serum CA 15-3 levels [75,61]. It is interesting that metronomic dose of ZA (1 mg weekly) also reduced serum level of VEGF in cancer patients in other studies [74,75]. Other early clinical studies showed different results. In a study with 18 breast cancer patients with bone metastases, serum VEGF level showed a statistically significant decrease 48 h after ZA infusion but rose above the basal level at 7 days [76]. In another study of 30 patients with metastatic bone disease from breast and lung cancer ZA acid did not exert significant reduction of VEGF and bFGF circulating levels 7 days after treatment [77]. These studies further bring to question the optimal dose and frequency of ZA that should be used in future clinical trials.

1.2.3. Activation of $\gamma\delta$ T cells

A phase I study showed that administration of ZA (with IL-2) to patients with metastatic cancer promoted effector maturation of $\gamma\delta$ T cells. Three patients who showed sustained robust peripheral $\gamma\delta$ T cell populations had decrease in their tumour marker levels, with one patient achieving partial response and two achieving stable disease. In contrast, seven other patients who failed to sustain peripheral $\gamma\delta$ T cells showed clinical deterioration [78].

In a Japanese study, peripheral blood mononuclear cells from 15 patients with advanced non-small cell lung cancer (NSCLC) were stimulated with ZA (5 μ M) and IL-2 (1000 IU/ml) for 14 days. Many of these cells expanded to $\gamma\delta$ T cells. Administration of these *ex vivo* expanded $\gamma\delta$ T cells to patients with recurrent or advanced lung cancer was well tolerated. The number of peripheral $\gamma\delta$ T cells gradually increased. All patients remained alive during the study period with a median overall survival of 589 (range 202–1505)

days, and median progression free survival of 126 (range 34–285) days [79]. According to the Response Evaluation Criteria in Solid Tumours (RECIST), there were no objective responses. Six patients had stable disease, whereas the remaining six evaluable patients experienced progressive disease 4 weeks after the sixth transfer.

Although all of these patients had Eastern Cooperative Group (ECOG) performance status 0 or 1 with minimal co-morbidities, such a long period of survival is very encouraging for recurrent or advanced lung cancer. The reported median survival in this study is about 1.5 times longer than that reported in large phase III lung cancer trial.

Results from other studies were less promising. For example, in a study of 12 patients with renal cell carcinoma, ZA induced only modest increase in $\gamma\delta$ T cell frequency but not to the magnitude anticipated from preclinical models [80].

Recently, investigators have shed some light on possible reason for the lack objective responses of solid tumours to $\gamma\delta$ T cells expansion therapy. Using similar expansion technique, Kunzmann et al. treated 21 adults with advanced malignancies (renal cell carcinoma [RCC], malignant melanoma, and acute myeloid leukaemia). No objective responses were observed in both cohorts of solid tumours (RCC and malignant melanoma), whereas two patients with acute myeloid leukaemia (25%) achieved objective tumour responses. Pharmacodynamic analyses showed significant *in vivo* activation (interferon- γ production) and expansion of $\gamma\delta$ T cells in all evaluable patients. High pretreatment serum VEGF levels and an unexpected increase in VEGF induced ZA plus low-dose interleukin-2 were correlated with the lack of a clinical response. This study indicates that immunotherapy-induced VEGF can limit clinical innate tumour immune responses, especially for angiogenesis-dependent solid tumours. Adoptive immunotherapy in this context is still at its infancy and further early clinical studies may elucidate its potential role.

1.3. Phase III trials of zoledronic acid in early breast cancer

Based on early clinical data suggesting its anti-tumour effect, ZA has been studied in multiple large randomized clinical trials in early breast cancer.

In an Austrian study (ABCSG-12) [81], 1803 endocrine responsive early breast cancer patients on adjuvant goserelin were randomly assigned to receive, tamoxifen or anastrozole, with or without ZA. The dose of ZA was 4 mg given intravenously every 6 months for 3 years. There was a 36% decrease in the risk of disease progression ($p=0.01$) in the ZA arms. This corresponded to a 5 year disease free survival of 94% versus 90.8%, favouring ZA. There were also fewer deaths in the ZA arms (16 versus 26, hazard ratio 0.60, $p=0.11$). Interestingly, patients on the ZA arms had fewer events in all examined categories including loco regional recurrence (10 versus 20), distant recurrence (29 versus 41), and contra lateral breast cancer (6 versus 10) as compared to treatment without ZA.

An integrated analysis [82] of two large randomized controlled trials (Z-FAST and Zo-FAST) treating a total of 1667 patients with early breast cancer was performed. The patients on these studies were randomized to receive adjuvant hormonal therapy (letrozole), with either upfront ZA or delayed ZA (at time of decreased bone mineral density). The analysis was aimed primarily to assess the protective effect of ZA in prevention of bone density loss. One of the secondary objectives of the analysis was time to disease recurrence. The results showed that patients treated with upfront ZA had significantly less recurrences at 12 months as compared to the patients who received delayed ZA (0.84% versus 1.9%, $p=0.04$). An updated analysis of the ZO-FAST study at 36 months showed continued benefit of upfront ZA in prolonging disease free survival (41% relative risk reduction, $p=0.04$) as compared to delayed ZA

[83]. A more recent analysis at 60 months showed continued benefit of prolonged disease free survival (HR0.66, $p=0.0375$) in the upfront ZA group [84].

A third study (AZURE) [24] randomized 3360 patients with early breast cancer to receive standard adjuvant therapy with or without ZA. ZA was administered immediately after each cycle of adjuvant chemotherapy in line with pre-clinical data supporting this sequence as discussed above and was continued for a total of 5 years.

At a median follow up of 59 months, the investigators found no significant difference between the two arms in the primary end-point which was disease free survival (77% in each group, $p=0.79$). Prospective subgroup analyses of the study showed that ZA significantly increased disease free survival (HR=0.76, $p < 0.05$) in women who were at least 5 years past menopause at study entry ($n=1041$). ZA also improved overall survival (HR=0.71, $p=0.017$, $n=1101$) in this patient population along with women of unknown postmenopausal status but age greater than 60 years [85]. Of note, about 45.8% of patients in this study were premenopausal, 95% of whom received chemotherapy, and only 0.2% of these patients received goserelin. This is in contrast to the premenopausal patient cohort in the ABCSG-12 trial, who all received goserelin.

Apparently the results of the AZURE study are in contradiction to the ABCSG-12 results. However, some authorities have argued that in both studies, disease free and overall survival was seen in patients who were either rendered menopausal (with goserelin in ABCSG-12 trial), or who achieved menopause naturally (as seen in the subset analyses of the AZURE trial), suggesting a possible relationship of a menopausal or low oestrogen state with the clinical benefit achieved from addition of ZA [86].

205 Patients in the AZURE study received neo-adjuvant treatment including ZA. A retrospective analysis of pathologic response in these patients [23] showed that the mean residual tumour size in the ZA plus chemotherapy arm was significantly less (15.5 mm) as compared to 27.4 mm in the chemotherapy only arm. The pathologic complete response rate was 11.7% in the ZA arm versus

6.9% in the chemotherapy only arm, but this was not statistically significant ($p=0.146$).

Although the ABCSG-12 and AZURE studies have provided provocative and interesting conclusions, the partly contradicting results of these trials has delayed the incorporation of ZA in the adjuvant treatment of women with early breast cancer.

A prospective study in postmenopausal patients is required to validate and confirm the results of the ABCSG-12 and AZURE trials. This will also be an opportunity to study specific biomarkers that likely explain anti-tumour mechanism of ZA. As discussed above, anti-angiogenesis and anti-proliferative properties of ZA seem to be the most relevant mechanisms from early preclinical and small clinical studies.

Several other large randomized controlled trials are evaluating the anti-tumour efficacy of ZA in the adjuvant setting (Table 2). Most of these studies have completed accrual and are awaiting maturation of the data.

1.4. Trials showing lack of benefit from zoledronic acid

Although the above mentioned trials have shown some clinical benefit from the use of ZA in solid tumours, results from other trials do not show clear benefit from use of ZA. In a recent Italian study [87], patients with controlled stage IIIA/B NSCLC were randomized to receive ZA or no treatment. Progression free survival (PFS), which was the primary endpoint of this trial, was 9 months for ZA versus 11.3 months for control (statistically non-significant difference). Rate of development of bone metastases was also similar in both arms (6.6% in ZA arm and 9% in control arm, no significant difference).

In an open label randomized phase II study, 119 female patients with resectable stage II/III breast cancer [88], patients were randomised to either receive ZA or no ZA starting with first cycle of chemotherapy. At 61.9 months of follow up, there was no difference between the two arms in terms of disease free survival (secondary end-point) or overall survival (tertiary end-point). A subset analysis did show benefit of ZA in improving disease free survival (Hazard ratio 0.361, 95% confidence interval 0.148–0.880)

Table 2

A summary of ongoing large clinical trials evaluating the use of Zoledronic acid in different cancers.

Study name	Patient population	Study design and treatment	Primary endpoints	References
NATAN	654 BC patients	Standard therapy +/-	EFS at 5 y	ClinicalTrials.gov No. NCT00512993
SUCCESS	3754 BC patients (stages I, II, and IIIA)	ZA (4 mg IV q1mo; q3mo; q6mo) FEC/DOC +/- GEM, then endocrine therapy plus ZA for 2 y or 5 y	DFS at 5 y	German Breast Group The SUCCESS Study Group 2009 www.Success-studie.de
SWOG 0307	5400 BC patients (stages I, II, and IIIA)	ZA (4 mg q1mo; q3mo); CLO (1600 mg/d); IBN (50 mg/d)	DFS and OS up to 10 y	ClinicalTrials.gov No. NCT00127205
ZEUS	1498 PC patients (high-risk, early)	Standard therapy +/- ZA (4 mg IV q3mo)	EFS at 4 y	Southwest Oncology Group Register no. 66626762 (http://www.controlled-trials.com/)
RADAR	1071 PC (stage T2b-4)	ADT for 6 mo or 18 mo +/- ZA (4 mg IV q3mo)	PSA-RFS at 18 mo	European Association of Urology ClinicalTrials.gov No. NCT00193856
STAMPEDE	3300 PC patients (high risk)	ADT and (1) no additional therapy; (2) DOC (q3wk); (3) celecoxib (BID); (4) ZA (q3wk; q4wk); (5) DOC+ZOL; (6) celecoxib+ZA	FFS and OS (multiple phases)	Trans-Tasman Radiation Oncology Group ClinicalTrials.gov Identifier, NCT00268476
Study 2419	446 NSCLC patients (stage IIIA or IIIB)	ZA (4 mg IV q1mo)	TTP	Medical Research Council ClinicalTrials.gov Identifier NCT00172042
CALGB 90202	680 pts with met PrCa on ADT	ZA 4 mg IV every month versus placebo	PFS and OS	Novartis 2009 ClinicalTrials.gov Identifier: NCT00079001

BC indicates breast cancer; IBN, ibandronate; IV, intravenous; q4wk, every 4 weeks; +/-, with or without; DFS, disease-free survival; FEC, combined 5-fluorouracil, epirubicin, and cyclophosphamide; DOC, docetaxel; ZA, zoledronic acid; EFS, event-free survival; q1mo, monthly; q3mo, every 3 months; GEM, gemcitabine; OS, overall survival; CLO, clodronate; PC, prostate cancer; ADT, androgen-deprivation therapy; PSA-RFS, prostate-specific antigen recurrence-free survival; BID, twice daily; FFS, failure free survival; NSCLC, non-small cell lung cancer; TTP, time to progression.

and overall survival (Hazard ratio 0.375, 95% confidence interval 0.143–0.985) in patients whose tumours were oestrogen receptor negative as opposed to oestrogen receptor positive, however the study was not powered for this comparison. The authors suggest that in oestrogen receptor negative breast cancer, ZA may have a direct anti-tumour effect which may not be dependent on a low oestrogen environment. The sample size in these two clinical studies was too small to address the question of effect of ZA on disease relapse in early cancer setting.

1.5. Other bone modifying agents in oncology

In addition to ZA and other bisphosphonates, there is a host of other bone modifying agents in different phases of development. These include (but are not limited to) Denosumab (a RANK-ligand antibody), $^{223}\text{RaCl}_2$ or Alpharadin (an alpha particle emitting agent), and antibodies to sclerostin (which is an inhibitor of osteoblastogenesis) [89]. The detailed mechanisms of action of these agents and whether they have anti-tumour effects are beyond the scope of this review.

2. Conclusion

ZA has become an important component in treatment of cancer patients. Apart from its role in bone preservation and reduction of skeletal related events, there is preclinical and also clinical evidence suggesting a direct anti-cancer effect of ZA. Several mechanisms of action for this anti-tumour effect have been studied. The most profound clinical evidence of this effect comes from a number of large adjuvant breast cancer studies, although the results have not been consistently in favour of antitumour effect of ZA. Other adjuvant studies incorporating ZA are currently underway and those results are awaited.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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