REVIEW

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Emerging anti-cancer molecular mechanisms of aminobisphosphonates

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Abstract

Bone metastases are common in patients with many types of cancer, especially breast and prostate cancer — in which the incidence is approximately 70% among patients with advanced metastatic disease. Aminobisphosphonates (NBPs) have entered clinical practice in the treatment of bone metastases from several neoplasms, including breast and prostate adenocarcinoma, as a result of their anti-resorption properties. However, evidence has accumulated on the direct anti-tumour effects of NBPs. This review describes the metabolic pathways that are putative molecular targets of NBPs and that are involved in the prenylation processes of several intracellular small GTP-binding proteins (ras family related proteins). The latter regulate the intracellular survival and proliferative pathways of tumour cells and could be the intracellular molecular targets of the NBPs responsible for the direct anti-cancer effects, even if definitive conclusions cannot be drawn at present. Different mechanisms have been reported to account for the anti-neoplastic action of NBPs, including: the induction of apoptosis; cell cycle perturbations; and anti-invasive, anti-migration and anti-angiogenic effects. Moreover, this review describes the most important clinical studies that demonstrate the activity of NBPs in preventing skeletal-related events induced by bone metastases. The main pharmacokinetic pitfalls of NBPs are described, and methods of overcoming these pitfalls through the use of liposome vehicles are proposed. Finally, the principal pre-clinical studies on the interaction between NBPs and other biological agents are also described; these studies may enable reductions in the *in vivo* NBP concentrations required to achieve anti-tumour activity. To date, however, the real molecular targets of NBPs are not completely known and new technological platforms are required in order to detect them and to develop new anti-cancer strategies based on the use of NBPs.

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Introduction

Bone metastases are common in patients with many types of cancer (Coleman 1997, Coleman 2001, Ferlay *et al*. 2001, Zekri *et al*. 2001), especially breast and prostate cancer — in which the incidence is approximately 70% among patients with advanced metastatic disease (Coleman 1997, 2001). Because patients with breast or prostate cancer have a relatively long median survival time after diagnosis of bone metastases, the prevalence of bone metastases is high. These two cancer types probably account for more than 80% of cases of metastatic bone disease. In addition, approximately 40% of patients with advanced lung cancer develop bone metastases.

The high prevalence of bone metastases in patients with advanced metastatic disease contributes substantially to the burden of disease. Bone metastases are associated with considerable skeletal morbidity — including severe bone pain that may require strong narcotics or palliative radiation therapy — pathological fracture, spinal cord or nerve root compression, and hypercalcaemia of malignancy (HCM), which can substantially reduce quality of life. Across all tumour types, patients with breast cancer have the highest incidence of skeletal complications (Berenson *et al*.

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1998, Lipton *et al*. 2000, Rosen *et al*. 2004*b*, Saad *et al*. 2004). After over 2 years of follow-up, nearly 70% of patients treated with placebo had more than one skeletal complication, and approximately 50% experienced a pathological fracture (Lipton *et al*. 2000). Patients with multiple myeloma, prostate cancer, lung cancer or other solid tumours are also at high risk for skeletal complications. The most common events in all tumour types are radiation to bone and pathological fracture. These complications result from excessive bone metabolism, principally bone resorption which characterizes malignant bone lesions and leads to severe bone pain. Therefore, there is a great need for therapies that effectively inhibit bone resorption, thereby reducing the risk of skeletal complications. Consequently, bisphosphonates (BPs) have become the standard treatment for malignant bone disease. BPs effectively inhibit bone resorption, have been shown to significantly reduce the incidence of skeletal complications and have analgesic effects on bone pain (Ross *et al*. 2003).

Traditional approaches for treating patients with bone metastases include standard anti-neoplastic therapies (chemotherapy or biological therapies), which may be administered in conjunction with additional supportive or palliative therapies. Severe bone pain is treated with radiotherapy and/or radionuclides, and many patients also receive systemic analgesic treatment with non-steroidal anti-inflammatory drugs or opioids. Radiotherapy is also used to stabilize bone lesions and may prevent impending fractures. Orthopaedic surgery is used to treat existing fractures or to prevent impending fractures or spinal cord compression. In addition to these strictly palliative interventions, BPs have emerged in recent years as a highly effective therapeutic option for the prevention of skeletal complications secondary to bone metastases. BPs bind preferentially to bone at sites of active bone metabolism, are released from the bone matrix during bone resorption and potently inhibit osteoclast activity and survival, thereby reducing osteoclast-mediated bone resorption (Fleisch 2002). Newer nitrogen-containing bisphosphonates (NBPs) — such as zoledronic acid (ZOL), pamidronate (PAM) and ibandronate (IBA) — have a unique mechanism of action and greater clinical activity than first-generation BPs such as etidronate and clodronate (Green 2003). These newer agents are orders of magnitude more potent than the first-generation compounds. Consequently, they can inhibit bone resorption at micromolar concentrations.

BPs inhibit osteoclast activity at multiple levels; they prevent differentiation of macrophages into osteoclasts, block the activity of mature osteoclasts and induce osteoclast apoptosis (Rodan 1998, Rogers *et al*. 2000). The exact molecular mechanisms of action of BPs are only partially understood and appear to differ among different families of BPs (Fleisch 1998, Rodan 1998). Non-nitrogen-containing BPs (e.g. clodronate and etidronate) can be incorporated into non-hydrolyzable ATP analogues, which accumulate intracellularly and thereby suppress ATP-dependent enzymes (Rogers *et al*. 1996*a*). NBPs inhibit critical enzymes of the mevalonate pathway, in particular farnesyl diphosphate synthase, required for the synthesis of farnesyl diphosphate and geranylgeranyl diphosphate, and thereby suppress prenylation of small GTPases essential for many cellular functions (Luckman *et al*. 1998, van Beek *et al*. 1999*b*, Bergstrom *et al*. 2000, Dunford *et al*. 2001).

Clinical and experimental evidence indicates that NBPs suppress the progression of bone metastases, and recent observations suggest that this effect may be independent of the inhibition of bone resorption (Neville-Webbe *et al*. 2002, Green 2003). Tumour progression and metastasis formation are critically dependent on tumour angiogenesis (Carmeliet & Jain 2000). Anti-angiogenic treatments suppress tumour progression in animal models, and many anti-angiogenic substances are currently being tested in clinical trials for their therapeutic efficacy against human cancer (Carmeliet 2003). Recent evidence indicates that zoledronate possesses anti-angiogenic activities as discussed below.

Biochemical pathways as molecular targets of NBPs: the mevalonate pathway

NBPs are potent inhibitors of the synthesis of both farnesyl and geranylgeranyl lipidic residues and therefore, of protein isoprenylation. The consequences of this inhibition are the disruption of important signal transduction pathways that regulate the proliferation, the invasive properties and the pro-angiogenic activity of human tumour cells. In fact, the addition of a lipidic residue to all the small GTP-binding proteins is essential for their correct location on the inner side of the plasma membrane and for their consequent activation by external signals. In fact, they must co-localize with their effectors that are all placed on the inner side of the plasma membrane, in which location are also found the substrates that are necessary to mediate the different functions of this class of molecules. Among documented farnesylated proteins are: H-, K- and N-Ras GDP/GTP-binding GTPases;

the nuclear lamins; and the kinetechores centromere-associated protein (CENP)-E and -F. Geranylgeranylated proteins include: GTP/GDP-binding GTPases, RhoA, RhoC, Rac1, cdc-42, Rab and R-Ras (Reid *et al*. 2004). RhoB is found both farnesylated and geranylgeranylated in cells (Armstrong *et al*. 1995), whereas K-Ras becomes geranylgeranylated when farnesyltransferase (Ftase) activity is blocked (Lerner *et al*. 1997, Rowell *et al*. 1997, Whyte *et al*. 1997).

In human cells, isoprenoids are derived from the mevalonate pathway that starts from reaction catalyzed by the 3-hydroxy-3-methylglutaryl CoA (HMGCoA) reductase (the rate-limiting reaction in cholesterol biosynthesis) which catalyzes the conversion of HMGCoA to mevalonic acid. The pathway triggered by this reaction can lead to the synthesis of a key isoprenoid molecule: the farnesyl-pyrophosphate (FPP) whose formation is catalyzed by the farnesyl-pyrophosphate synthase (FPPS) (Soma *et al*. 1992). FPP can be: converted by a series of reactions into cholesterol; or transferred on target cellular proteins as FPP itself (reaction catalyzed by farnesyltransferase); or firstly converted into geranylgeranyl-pyrophosphate and then transferred on cellular proteins by type I or type II geranylgeranyltransferase.

The isoprenylation process

The joining of the 15-carbon farnesyl group ($C_{15}H_{25}$) and the 20-carbon geranylgeranyl group ($C_{20}H_{33}$) to protein–cysteines at or near their carboxy-termini is catalyzed by protein FTase and protein geranylgeranyltransferase-I and -II (GGTase-I and GGTase-II) (Zhang & Casey 1996). The prenyltransferases are heterodimers consisting of α- and β-subunits with combined molecular masses ranging from 91 to 98 kDa. The α-subunits of FTase and GGTase-I are the same, and the β-subunits differ. The β-subunits of the three enzymes are homologous to the α-subunits and to each other. The isoprenoid groups become linked to polypeptidic cysteines through thioether (C–S–C) bonds. Conversion of the protein–cysteine acceptor site to protein–serine in oncogenic H-Ras prevents prenylation and abolishes its malignant transforming ability (Lowy & Willumsen 1993). FTase and GGTase-I catalyze the prenylation of substrates with a carboxy-terminal tetrapeptide sequence called a CA_1A_2X box, where C refers to cysteine, A refers to an aliphatic residue and X typically refers to methionine, serine, alanine or glutamine for FTase or to leucine for GGTase-I. Following prenylation of physiological substrates, the terminal three residues (A_1A_2X) are subsequently removed by a CA_1A_2X endoprotease and the carboxyl group of the terminal cysteine is methyl esterified by a methyltransferase (Zhang & Casey 1996). Protein GGTase-II, or Rab GGTase, catalyzes the geranylgeranylation of Rab proteins that terminate in CC or CXC sequences. Rab proteins ending with CXC residues are methyl esterified; those ending with CC are not. FTase and GGTase-I can catalyze the prenylation of tetrapeptides, polypeptides and proteins containing appropriate CA_1A_2X box sequences. GGTase-II, in contrast, cannot catalyze the prenylation of these peptides; it uses a Rab–Rab escort protein heterodimer as substrate (Zhang & Casey 1996). There are a few exceptions to the substrate-specificity rules for FTase and GGTase-I noted above. K-RasB, which has a classical FTase CA_1A_2X box (CVIM), is a substrate for FTase. Following inhibition of cellular FTase, K-RasB becomes a substrate for geranylgeranylation by GGTase-I (Gibbs 2001, Yokayama & Gelb 2001). The latter reaction is made possible by an upstream polybasic sequence that alters GGTase-I substrate specificity. Furthermore, RhoB, which contains a GGTase-I CA_1A_2X box (CKVL), is found in both farnesylated and geranylgeranylated forms in cells. This is due to the ability of GGTase-I to both geranylgeranylate and farnesylate this substrate (Armstrong *et al*. 1995). It appears that upstream sequences (as yet uncharacterized) are responsible for this altered substrate specificity. Moreover, Cdc42, which contains a carboxyterminal CCIF sequence, undergoes geranylgeranylation. Ordinarily GGTase-I substrates contain leucine in the X position of the CA_1A_2X box, but Cdc42 represents an exception to the leucine rule (Roskoski 2003). All three prenyltransferases require Zn^{2+}, and FTase and GGTase-II require Mg^{2+} for activity (Huang *et al*. 1997, Hightower *et al*. 1998, Spence & Casey 2001, Terry *et al*. 2001, Urano *et al*. 2001, Yokayama & Gelb 2001). Both protein geranylgeranylation and farnesylation are followed by the cleavage of the terminal tripeptide A_1A_2X, catalyzed by a specific peptidase, and by the subsequent methylation of the terminal cysteine catalyzed by a methyltransferase. Finally, the protein is ready to be translocated on the cellular membranes to receive extra- or intracellular signals. After methylation, a palmitoylation on the -SH group of the penultimate cysteine residue can occur. This last reaction is reversible and occurs only for Ras proteins with a cysteine residue upstream of the CAAX motif (namely H-Ras, N-Ras and K-Ras4A), whereas the other CAAX-triggered events are irreversible (Clarke *et al*. 1988, Hancock *et al*. 1991, Zhang & Casey 1996) (for a summary see Fig. 1).

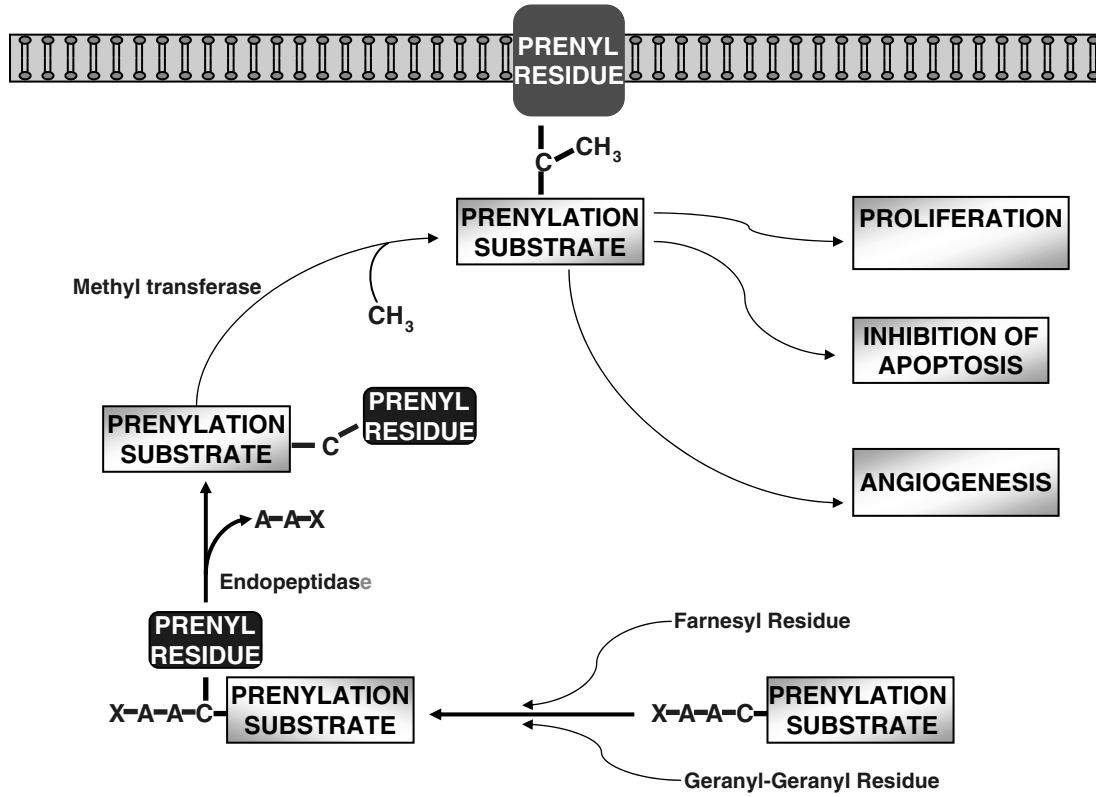

Figure 1 Isoprenylation mechanisms of intracellular substrates. The joining of the 15-carbon farnesyl group ($C_{15}H_{25}$) and the 20-carbon geranylgeranyl group ($C_{20}H_{33}$) to protein–cysteines at or near their carboxy-termini is catalyzed by protein FTase and protein GGTase-I or GGTase-II. FTase and GGTase-I catalyze the prenylation of substrates with a carboxy-terminal tetrapeptide sequence called a CA_1A_2X box; where C refers to cysteine, A refers to an aliphatic residue and X typically refers to methionine, serine, alanine or glutamine for FTase or to leucine for GGTase-I. Following prenylation of physiological substrates, the terminal three residues (A_1A_2X) are subsequently removed by a CA_1A_2X endoprotease and the carboxyl group of the terminal cysteine is methyl esterified by a methyltransferase. At this moment prenyl substrates are ready to be located on the inner side of the biological membranes to receive signals mediated by external factors.

Suggested intracellular molecular targets of NBPs

NBPs are currently used for the treatment of bone metastases and were initially thought to act by inhibition of formation of osteoclasts from immature precursor cells or by direct inhibition of resorption through induction of apoptosis in mature osteoclasts (Hughes *et al*. 1995). Recently, evidence suggests that NBPs including PAM and ZOL are also potent inducers of apoptosis in several cancer cell types such as myeloma (Shipman *et al*. 1997, Aparicio *et al*. 1998, Tassone *et al*. 2000) and breast (Senaratne *et al*. 2002), prostate (Lee *et al*. 2001) and pancreatic cancer (Tassone *et al*. 2003) as well as in macrophage (Rogers *et al*. 1996*b*) and intestinal epithelial cell lines (Twiss *et al*. 1999). These data indicate that the beneficial effect of NBPs on metastatic bone disease may result also from direct anti-cancer activity that may affect a broad range of tumours. The molecular basis of the NBP anti-cancer action relates to their ability to inhibit the farnesyl diphosphate synthase, probably by mimicking the diphosphate moiety (van Beek *et al*. 1999*a*). Therefore, they are also inhibitors of the synthesis of higher isoprenoids such as geranylgeranyl diphosphate. In contrast to farnesyltransferase inhibitors (FTIs) or geranylgeranyltransferase inhibitors (GGTI), it is now emerging that NBPs could inhibit both of the two different mechanisms of isoprenylation of intracellular proteins.

Many solid tumours cause hypercalcaemic bone destruction and the primary cause of this is often due to release of parathyroid hormone-related peptide (PTHrP). This may be released by the primary tumour, or by metatases, and can cause intense general or local bone destruction. Moreover, transforming growth

factor (TGF)-β, one of the most abundant of the bone-derived factors, promotes increased production of PTHrP by tumour cells, establishing a 'vicious cycle' leading to progressive tumour growth and bone destruction. BPs interrupt this cycle by inhibiting osteoclasts, in part by inducing osteoclast apoptosis (Mundy *et al*. 2001).

However, the exact molecular target of NBPs is still unknown and its identification could be useful in potentiating the beneficial effects of these drugs. Several isoprenylated molecules are emerging as potential targets of NBPs and they could be differently implicated in the biological effects induced by these agents. In order to be clear on the identification of the emerging molecular mechanisms responsible for the different biological effects (antiproliferative, anti-invasive and anti-angiogenic), each effect will be discussed separately.

Anti-proliferative effect: apoptosis and cell cycle disruption

Taking into account the relative importance of ras in regulating cell proliferation of several tumours both *in vitro* and *in vivo*, attention has been focused on ras in order to explain the direct anti-proliferative effects of NBPs. Two mechanisms of NBP-induced tumour growth inhibition have been described: cell cycle disruption and apoptosis. In this regard, it has recently been demonstrated *in vitro* that NBPs, such as PAM and ZOL, induce apoptosis and growth inhibition in human epidermoid cancer cells, together with depression of ras signalling and of Erk and Akt survival pathways. These effects occurred together with poly (ADP ribose) polymerase (PARP) fragmentation and the activation of caspase 3. Moreover, the latter seemed to be essential for apoptosis induced by NBPs in this experimental model. The synthesis of isoprenoids appeared largely responsible for the biological and biochemical effects of NBPs since the addition of farnesol (FOH), which restores farnesylation, to tumour cells completely antagonized apoptosis and the inhibition of ras activity in tumour cells exposed to NBPs. These data suggest that the activity of NBPs could be due to the inactivation of the farnesylpyrophosphate synthase activity (Caraglia *et al*. 2004). Moreover, the effects of ZOL on growth inhibition and apoptosis seemed to be p53-independent (Kuroda *et al*. 2004). In fact, Kuroda *et al*. have demonstrated that 40 μM ZOL induced about 40% apoptosis in leukaemia and colon cancer cells. The effect on growth inhibition was not dependent on p53 function since p53-defective colon cancer HCT116/E16 cells were equally as sensitive to ZOL as the parental cells. In these experimental conditions, ZOL decreased rap-1A prenylation and Erk activity, but had no effects on the expression of cyclin-dependent kinase inhibitors p27 and p21, and also increased the expression of M-phase cyclins. These results suggest that ZOL induced p53-independent growth inhibition and apoptosis in these experimental systems. In contrast to the results of Caraglia *et al*. (2004) apoptosis induced by NBPs could also be due to the inhibition of ras geranylgeranylation instead of farnesylation as recently demonstrated by Senaratne *et al*. (2002). These conclusions are further supported by the findings of Coxon *et al*. (2004) who described apoptotic effects after 48 h of exposure to 100 μM ZOL on prostate adenocarcinoma DU145 and PC3 cells. Apoptosis was completely antagonized by both the pan-caspase inhibitor and by the addition of geranyl-geranol (GGOH), which restores geranylgeranylation, but not by FOH. Similar effects on apoptosis induction were also recorded after FTI manumycin A addition; this was, however, aspecific and also acted on GGTase. In this report, the inhibition of Rho A, a geranylgeranylated protein, through the use of C3 exoenzyme, was not able to induce apoptosis. Therefore, the authors failed to detect a putative molecular target of NBPs and it could only be suggested that a ras isoform or another GTP-binding protein that can be geranylgeranylated was involved. On the other hand, other data exclude the involvement of ras in the anti-proliferative effects exerted by ZOL on human prostate adenocarcinoma cells. In fact, Nogawa *et al*. (2005) found that the amount of membrane-anchored ras (the active fraction of ras) was clearly independent of ZOL-mediated growth inhibition of prostate cancer cell lines and concluded that geranylgeranylation of a still unknown substrate could be a therapeutic target in this experimental model. Moreover, it was also shown that PAM, alendronate and risedronate were all able to induce apoptosis in osteoclasts through the caspase cleavage of mammalian sterile 20-like kinase 1 (Mst1) to form the active 34 kDa species associated with apoptosis. Moreover, these effects seemed to be independent on the blockade of geranylgeranylation (Reszka *et al*. 1999). Both incandronate and etidronate accelerate phosphate-primed mineralization of a murine calvaria-derived osteoblastic cell line through the Erk1/2-Cbfa1 signalling pathway again in a ras-independent manner (Fujita *et al*. 2001). In this report, a differential involvement of Erk1/2 and Akt-dependent pathways was also described since risedronate suppressed the phosphorylation of ERK 1/2, affected the intracellular distribution of Bcl-xL and facilitated dephosphorylation

of Bad at Ser112 (an ERK phosphorylation site), but not at Ser136 (an Akt phosphorylation site). All of these apoptosis-related changes induced by risedronate were strongly suppressed by cytochalasin B, an inhibitor of actin filament polymerization, thus excluding ras involvement but not suggesting an alternative substrate (Fujita *et al*. 2005). Moreover, differential biochemical effects within the different NBPs can not be excluded. In fact, it is known that the anti-resorptive effects of PAM are not completely inhibited by the addition of GGOH which, in turn, is able to antagonize completely the effects of alendronate (van Beek *et al*. 2003).

Based on the evidence that NBPs act as inhibitors of geranylgeranylation, a later-generation NBP NE10790 was developed with the specific ability to inhibit the activity of Rab GGTase but with no activity against either FTase or GGTase I (Coxon *et al*. 2001). NE10790 was able to inhibit bone resorption without affecting osteoclast number and causing the formation of intracellular vacuoles and protrusions of the basolateral membrane, thus affecting Rab-mediated intracellular trafficking (Coxon *et al*. 2001). Therefore, it is possible to design NBPs with selective activity against GTP-binding proteins different from ras and rho.

Another way to induce cell growth inhibition is cell cycle perturbation. In this regard, it was reported that ZOL at concentrations higher than 100 μM induced 75% growth inhibition after 4 days of treatment on both androgen-dependent LnCaP and androgen-independent PC3 prostate cancer cell lines (Corey *et al*. 2003). In these experimental conditions G1 accumulation was found, even at only 2 days from the beginning of the treatment. The same authors reported the anti-cancer effects of ZOL on bone PC3 and LnCaP xenografts and the effect was paralleled by a significant reduction in serum prostate-specific antigen (PSA). The ability of ZOL to induce growth inhibition in cancer cells has also been studied in correlation with the reduced expression of metallo-proteinase (MMP)-2 and MMP-9 in PC3 cells, but not LnCaP cells. The effects of NBPs on cell cycle inhibitors was further confirmed by Reszka *et al*. (2001) who described the effects of alendronate and risedronate on normal human epidermal keratinocytes (NHEKs). Alendronate and risedronate induced growth inhibition without apoptosis and with S-phase accumulation in NHEKs. These effects were paralleled by p21 and p27 increased expression and consequent decreased phosphorylation of Rb likely due to the inhibition of geranylgeranylation.

In summary, these findings suggest again that the real targets of NBPs, responsible for growth inhibition, are still undiscovered and that their identification requires further investigations.

Anti-invasive effects

It has been reported that ZOL is also able to inhibit tumour cell invasiveness of breast and prostate cancer at 1 μM concentrations; this makes it 150-fold more potent than other NBPs such as risedronate and PAM, and very different from non-nitrogen BPs such as clodronate (Boissier *et al*. 2000). At these concentrations, ZOL does not induce apoptosis and inhibits the *in vitro* activity of several MMPs. The authors suggest that this inhibition was due not to a decreased synthesis of the enzymes, but to the steric interaction between the bone hook of NBPs and the active site of the proteins, since only at higher concentrations were NBPs able to reduce the secretion of these enzymes. However, Montague *et al*. (2004) have reported that ZOL and PAM reduce prostate cancer PC3 invasion on matrigel chambers (also at a concentration of 5 μM ZOL) and inhibit the binding of PC3 to the marrow stroma. Finally, ZOL was also able to inhibit PC3 colony formation on marrow stroma at the optimal concentration of 10 μM. All these effects were paralleled by the inhibition of MMP7 secretion and tissue inhibitor of matrix metalloproteinase (TIMP)-2 expression. In these experimental conditions, decreasing vascular endothelial growth factor (VEGF) and granulocyte monocyte colony stimulating factor expression was also recorded. Denoyelle *et al*. (2003), in agreement with previous studies (Fromigue *et al*. 2000, Hiraga *et al*. 2001, Jagdev *et al*. 2001), have also shown that ZOL inhibits breast cancer cell proliferation, but only at high concentrations (4100 μM) that are certainly higher than those obtained *in vivo*. The effect of ZOL has also been studied on breast MDA-MB-231 cell invasion. After an 18 h incubation time, ZOL, at low concentrations (from 100 nm), conferred potent anti-invasive properties on MDA-MB-231 cells (62% decrease at 1 μM). As ZOL did not induce apoptosis at these concentrations, the possibility that ZOL interfered with invasion by inducing cell death was excluded. This is also in agreement with the observations of Boissier *et al*. (2000). The same authors have shown that it does not involve proteases involved in tumour invasion by inducing the degradation of the extracellular matrix (ECM). Indeed, neither MMP secretion nor u-PA (urokinase-plasminogen activator) expression was modified at concentrations that inhibit cell invasion. Reduction of the secretion of both MMP-2 and MMP-9 (Boissier *et al*. 2000) and u-PA expression in MDA-MB-231 cells required much higher concentrations. In contrast, u-PA receptors expressed on the cell surface of MDA-MB-231 cells were dramatically reduced by ZOL at low

concentrations. u-PA receptor is a ligand for vitronectin, which is a common protein in the mature bone microenvironment (Cooper *et al*. 2002). Consequently, the decrease of u-PA receptor by ZOL could contribute to the previously reported prevention of breast cancer cell attachment on to bone matrices (van der Pluijm *et al*. 1996, Boissier *et al*. 1997). In this study, it was demonstrated that GGOH, which restores geranylgeranylation, but not FOH, which restores farnesylation, reversed the effect of ZOL, suggesting that the inhibition of protein(s) geranylgeranylation rather than farnesylation seems to account for ZOL anti-invasive action. To test this hypothesis further, the effect of FTI-277 and GGTI-298 (which potently and selectively inhibit FTase and GGTase respectively), was compared with the action of ZOL on breast cancer cell invasiveness (Lerner *et al*. 1995, Vogt *et al*. 1996). The incubation of MDA-MB-231 cells with GGTI-298 mimicked the anti-invasive effect of ZOL, whereas FTI-277 did not. Thus, inhibition of protein geranylgeranylation seems to be important in explaining the anti-invasive action of ZOL. This effect was also mimicked by C3 exoenzyme, which is a specific inhibitor of RhoA, but not other Rho subfamily members, Rac and Cdc42 (Boquet 1999). Therefore, it was suggested that the inhibition of cell invasion by ZOL could be related to the inhibition of RhoA cell signalling. This was also supported by the observation that ZOL at low concentrations prevents the translocation of RhoA from cytoplasm to the cell membrane. In contrast to the effect of ZOL on cell invasion, the inhibition of cell proliferation seemed to be independent of RhoA inactivation because at the concentration for which RhoA, but not ras, was inhibited, cell proliferation and apoptosis were unaltered. Denoyelle *et al*. (2003) reported that ZOL also inhibited the chemotactic effect induced by the chemokine SDF-1 on MDA-MB-231 cells. This observation constitutes an important addition to the mechanistic understanding of how NBPs, given in the adjuvant setting, could prevent the development of bone metastases as shown by two clinical trials (Diel *et al*. 1998, Powles *et al*. 2002). Similar data were obtained with aledronate on the invasiveness and migration of both ovarian and prostate cancer cells (Sawada *et al*. 2002, Virtanen *et al*. 2002). Sawada *et al*. (2002) have also reported that alendronate inhibited lysophosphatidic acid-induced migration of human ovarian cancer cells by attenuating the activation of RhoA. These effects were again antagonized completely by GGOH and partially by FOH. On the other hand, Virtanen *et al*. (2002) have demonstrated that alendronate was able to decrease the migration and invasion of human prostate cancer cells and these effects were completely antagonized by both GGOH and FOH. Possible explanations of the discrepancy of this study with all the other reports could be the following: (a) compensatory conversion of some FOH to geranylgeranyl by farnesyl; (b) since rho can be activated by ras, Ras-activation induced by FOH could still stimulate cell spreading and actin filament assembly through the activation of Rho.

Taken together these data prevent conclusive remarks on the molecular targets of NBPs being given. However, considering the results from the literature it can be suggested that inhibition of the isoprenylation of intracellular proteins could be the mechanism of action of NBPs. Moreover, ras seems to be involved in the induction of apoptosis while rho-A is involved in the regulation of cell invasion (for a summary see Fig. 2).

Anti-angiogenic effects

Both *in vitro* and *in vivo* studies have further demonstrated that NBPs have anti-angiogenic effects. *In vitro* assays with human umbilical vein endothelial cells (HUVECs) have shown that ZOL dose-dependently inhibited the proliferation of HUVECs induced by fetal calf serum and basic fibroblast growth factor (bFGF), and these findings have been confirmed *in vivo*.

Systemic administration of ZOL to mice resulted in potent inhibition of angiogenesis induced by s.c. implants impregnated with bFGF, with a dose of 3 μg/kg producing a 50% efficacy (ED_{50}) (Wood *et al*. 2002). It has also been reported that ZOL can reduce bone-tumour-associated angiogenesis in the murine 5T2 myeloma model (Croucher *et al*. 2003).

In another series of experiments, ZOL, as well as IBA, risedronate and clodronate, inhibited the formation of capillary-like tubules by HUVECs *in vitro*. *In vivo*, ZOL and IBA, but not clodronate, decreased revascularization (as measured by vessel area) of the ventral prostate gland in castrated rats treated with testosterone (Fournier *et al*. 2002).

The inhibitory effect of NBPs on endothelial cell adhesion and migration appears to be mediated, at least in part, by modulation of integrins (e.g. $\alpha_v\beta_3$ and $\alpha_v\beta_5$) that are involved in angiogenesis (Bonjean *et al*. 2001, Bezzi *et al*. 2003). Interestingly, $\alpha_v\beta_3$ integrin is also required for osteoclasts to adhere tightly to the bone and form resorption lacunae during active bone resorption, and $\alpha_v\beta_3$ expression confers on tumour cells a greater propensity to metastasize to bone (Pécheur *et al*. 2002). In fact, a small molecule inhibitor

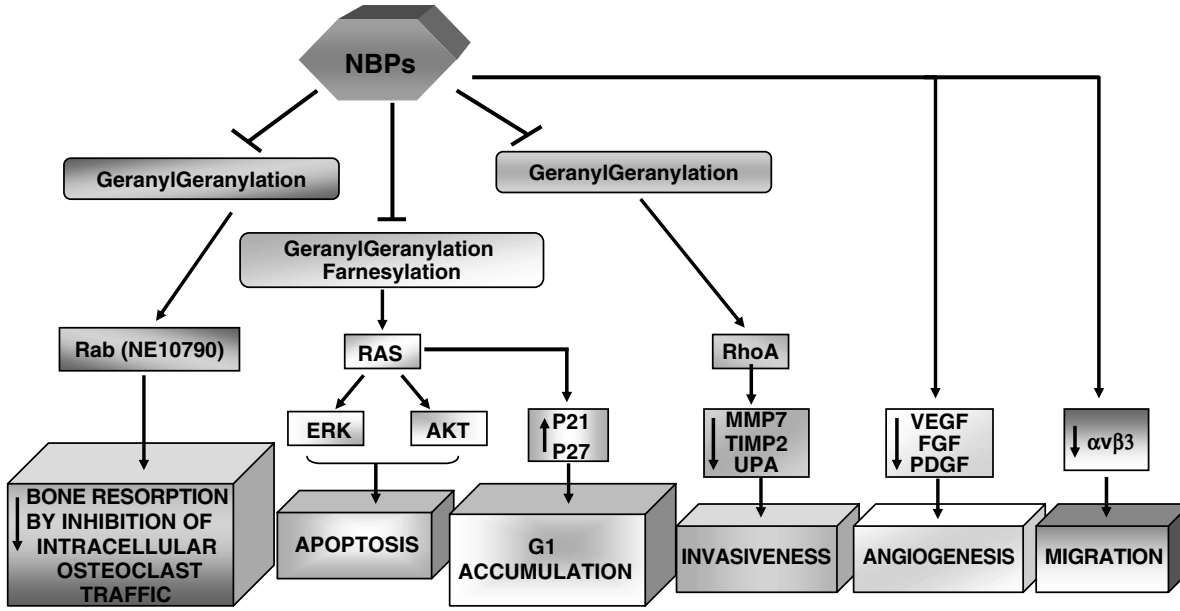

Figure 2 Intracellular molecular targets and modes of action of NBPs. NBPs can block the geranylgeranylation of Rab (reported for the new NBP NE10790), thus affecting bone resorption by inhibition of osteoclast intracellular traffic. NBPs can also antagonize geranylgeranylation and/or farnesylation of Ras family proteins (H-Ras, N-Ras, K-Ras) that, in turn, inhibit Erk and/or Akt activity or increase p21 and p27 expression. These effects can lead to apoptosis or G1 accumulation respectively, in osteoclasts and/or tumour cells. The block of geranylgeranylation of RhoA by NBPs can decrease the expression of molecules involved in invasion such as MMP7, TIMP-2 and u-PA. Finally, NBPs have been reported, in both *in vitro* and *in vivo* models (including humans), to reduce both the expression of angiogenic factors such as VEGF, bFGF and platelet-derived growth factor and to affect cell migration through the decrease of $\alpha_v\beta_3$ integrin expression.

of $\alpha_v\beta_3$ was recently shown to prevent effectively the metastasis of MDA-MB-435 breast cancer cells to bone (Harms & Welch 2003).

Therefore, effects on $\alpha_v\beta_3$ could be pleiotropic on both bone resorption and tumour metastasis. In addition, it has recently been reported that ZOL decreases the survival of HUVECs by sensitizing them to tumour necrosis factor-induced programmed cell death. ZOL also appears to modulate serum levels of pro-angiogenic growth factors such as VEGF and bFGF in cancer patients (Santini *et al.* 2003), as previously demonstrated also for the parent compound PAM (Santini *et al.* 2002). These studies suggest a variety of potential mechanisms to account for the observed anti-angiogenic effects of NBPs (for a summary see Fig. 2).

Clinical evidence of anti-resorptive and anti-cancer activity of NBPs

Skeletal complications from bone metastases remain an important health care problem in patients with advanced cancer. However, BPs provide significant benefits to patients with bone metastases by decreasing skeletal complications and reducing bone pain. In patients with bone metastases from advanced breast cancer, several BPs — including oral clodronate, i.v. PAM, oral and i.v. IBA, and i.v. ZOL — have demonstrated significant clinical benefits over placebo. In this setting, i.v. PAM and ZOL have demonstrated the most consistent clinical benefits across multiple end points that provide both a conservative and comprehensive assessment of skeletal morbidity. ZOL has also been shown to be significantly more effective than PAM in patients with breast cancer. Consequently, i.v. ZOL is the standard of care in patients with breast cancer. In patients with prostate cancer, lung cancer, renal cancer and other solid tumours, the situation is quite different. In these tumour types, ZOL is the only BP that has demonstrated significant clinical benefit. Indeed, ZOL has demonstrated the broadest clinical activity of any BP across multiple tumour types.

Regardless of the cancer type, ZOL resulted in a statistically significant lower cumulative incidence of skeletal-related event (SREs) compared with PAM (in patients with breast cancer) and placebo (in patients with prostate cancer or other solid tumours).

Moreover, the overall safety profile of ZOL was comparable with those of other i.v. BPs (Major *et al*. 2004).

The clinical benefits of BP therapy have been evaluated in many clinical trials designed to capture data on skeletal complications. The majority of these trials used a composite end point defined as a SRE or bone event, which generally includes events such as pathological fracture, radiation to bone, surgery to bone, spinal cord compression, and HCM. Such composite end points capture data on all clinically relevant events and are more likely to detect therapeutic benefits when treatment effects and disease morbidity are multifaceted (Johnson *et al*. 2003).

Using a composite definition of skeletal events, it is possible to assess treatment effect using a variety of outcome analyses. First-event descriptors such as proportion of patients with ≥ 1 SRE or time to first SRE are objective and conservative end points that provide readily assessable estimations of treatment effect. Of these, the US Food and Drug Administration has suggested that time to first event is the preferred end point because it also accounts for the patient's time on the study (Williams *et al*. 2004).

However, first-event analyses only capture information about the first event and ignore data on all subsequent events that occur in any given patient. Skeletal morbidity rates (SMRs) or skeletal morbidity period rates (SMPRs) assess the number of events that occur during a designated time period (e.g. events per year). These analyses account for the occurrence of multiple skeletal events but assume that these events occur at a constant rate. However, clinical evidence suggests that patients with bone metastases exhibit considerable variation in both the number of skeletal events they experience and the rate at which these events occur (Major *et al*. 2004).

Moreover, skeletal events do not demonstrate random distribution but often occur in clusters. Therefore, analyses that assume a linear event rate tend to overestimate the differences between study groups and may unduly overestimate treatment effects (Major & Cook 2002).

Regression analyses such as a Poisson analysis or the Andersen–Gill multiple event analysis are able to model all events, as well as the time between events, and are able to account for inter- and intra-patient variations in event rates (Andersen & Gill 1982).

However, Poisson analyses that do not account for non-constant event rates are subject to the same limitations as those of other analyses, such as analyses using SMR, that do not account for variability in event rate. Therefore, multiple event analysis provides a statistically robust and comprehensive assessment of skeletal morbidity throughout the entire length of follow-up. Andersen–Gill multiple event analysis calculates a hazard ratio (HR) which indicates the extent to which the risk of skeletal events is affected by one specific treatment relative to another. A hazard ratio <1 indicates a favourable treatment effect. Recently, non-parametric methods for multiple event analysis have been described (Cook & Lawless 1996, Ghosh & Lin 2000).

These models calculate the cumulative incidence of skeletal complications and allow for right-censored data, thus accounting for death or study discontinuation for other reasons. Together, all these statistical analyses provide both conservative and comprehensive assessments of the clinical benefit of BPs in patients with bone metastases.

NBPs in breast cancer

ZOL (4 mg in 15-min infusions every 4 weeks for 1 year) was also recently compared with placebo in 227 Japanese women with bone metastases from breast cancer (see Kohno *et al*. (2004) and Theriault *et al*. (1999)). In that trial, the primary end point was the SRE rate ratio adjusted for history of pathological fractures before study entry, which showed a significant 39% lower rate of SREs in the ZOL group (ratio = 0.61; $P = 0.027$). In addition, secondary efficacy analyses showed that ZOL produced a significantly lower percentage of patients with an SRE (31% versus 52% for placebo; $P = 0.001$) and significantly longer time to first SRE (median was not reached in ZOL group versus 360 days for placebo; $P = 0.004$) than placebo. Multiple event analysis demonstrated a 44% lower risk of developing an SRE (HR = 0.56; $P = 0.009$) in the ZOL group. It is noteworthy that the magnitude of the clinical benefit of ZOL observed in that trial appears to be greater than that achieved with PAM or IBA in similar patient populations after 1 year of treatment (Hortobágyi *et al*. 1996, Theriault *et al*. 1999). Similar to the PAM trials, patients enrolled in this trial had predominantly osteolytic lesions. ZOL was well tolerated; the adverse events that occurred more often with ZOL than with placebo included acute-phase, infusion-related symptoms of pyrexia (55% versus 33% for placebo), fatigue (44.5% versus 31.9% for placebo) and arthralgia (21.1% versus 15.9% for placebo). Only 1 of 114 patients experienced an elevated serum creatinine level with ZOL treatment in that study (Table 1).

Owing to the proven efficacy of PAM at the time, the pivotal trial of ZOL in patients with breast cancer

Table 1 Clinical end points of bisphosphonates in placebo-controlled trials for the treatment of bone metastases

	Bisphosphonate vs control									
Study	Dose (mg)	*n*	Patients with an event (%)	*P* value	Median time to 1st event (months)	*P* value	SMR or SMPR	*P* value	Overall risk of SREs	**95% CI of overall risk**
i.v. Pamidronate										
Hortobágyi *et al.* (1998)	90	382	50 vs 70	<0.001	14 vs 7	<0.001	2.4 vs 3.7	<0.001	0.77	(0.69–0.87)
Theriault *et al.* (1999)	90	372	56 vs 67	0.049	10 vs 7	0.049	2.4 vs 3.8	0.008	NR	NR
Hultborn *et al.* (1999)	60	404	NR	—	12 vs 8	0.006	1.0 vs 1.4	<0.01	0.97	0.97–1.07
Conte *et al.* (1996)	45	295	22 vs 16	NR	18 vs 16	NR	135 vs 169	NR	0.97	0.79–1.19
i.v. Zoledronic acid (breast cancer)										
Kohno *et al.* (2005)	4	227	31 vs 52	0.001	ND vs 12	0.004	0.61	0.027	NR	NR
i.v. Zoledronic acid (other cancers)										
Rosen *et al.* (2004)	4/8	773	36 vs 46	0.0127	7.8 vs 5.1	0.009	1.47 vs 2.71	0.012	0.693	NR
Saad F *et al.* (2004)	4	122	38 vs 49	0.028	16.2 vs 10.7	0.009	0.77 vs 1.47	0.005	0.693	NR
Lipton *et al.* (2003)	4/8	74	37 vs 74	0.015	ND vs 2.5	0.015	2.68 vs 3.38	0.014	0.394	NR
Oral ibandronate										
Body *et al.* (2004)	50	564	52 vs 45	0.122	23 vs 16	0.089	1.0 vs 1.2	0.041	NR	NR
Oral clodronate										
Paterson *et al.* (1993)	160	185	NR	—	10 vs 5	0.022	219 vs 305	<0.001	0.83	0.68–1.02
Kristensen *et al.* (1990)	800	100	29 vs 41	NR	NR	0.015	0.4 vs 0.5	NR	0.69	0.40–1.20
Tubiana Hulin *et al.* (2001)	160	144	61 vs 72	NR	8 vs 6	0.05	NR	—	0.92	0.71–1.19

CI, confidence interval; NR, not reported; SMR, skeletal morbidity rate; ND, not determined.

Table 2 Randomized, double-blind, multicentre, comparative trials between ZOL and PAM

	Bisphosphonate vs control									
Study	Dose (mg)	*n*	Patients with an event (%)	*P* value	Median time to 1st event (months)	*P* value	SMR or SMPR	*P* value	Overall risk of SREs	**95% CI of overall risk**
Rosen *et al.* (2001)	4/8 vs 90	1648	Similar	n.s.	12 vs 12	n.s.	lower for ZOL	Significant	NR	NR
Rosen *et al.* (2003)	4/8 vs 90	1648	19 vs 24 (radiotherapy)	0.037	13.8 vs 12.3 (breast cancer)	0.047	1.04 vs 1.39	0.084	0.841	0.719–0.983
Rosen *et al.* (2004*b*)	4/8 vs 90	528 (at least 1 lesion	48 vs 58	0.058	10.3 vs 5.8	0.013	1.16 vs 2.36 (lytic lesions)	0.008	0.801	NR

CI, confidence interval; NR, not reported; SMR, skeletal morbidity rate; ND, not determined.

or multiple myeloma was designed as a non-inferiority trial comparing ZOL (4 mg via a 15-minute infusion) with PAM (90 mg via a 2-h infusion) (Rosen *et al.* 2003). In a stratified subset of patients with breast cancer, 377 patients were randomized to receive 4 mg ZOL and 389 patients were randomized to receive 90 mg PAM (every 3–4 weeks for 25 months). The primary end point (percentage of patients with >1 SRE) showed that ZOL was at least as effective as PAM (46% versus 49% for PAM). Moreover, the prospectively planned Andersen–Gill multiple event analysis demonstrated that ZOL resulted in a 20% significantly lower risk of developing SREs than PAM (risk ratio = 0.799; $P = 0.025$). A similar, significantly lower cumulative incidence of SREs among patients treated with ZOL versus PAM was also demonstrated by the method of Cook & Lawless (1996) ($P = 0.046$) (Major *et al.* 2003) (Table 2). In addition, among patients with predominantly osteolytic lesions, ZOL has been shown, by Andersen–Gill analysis, to result in a 30% significantly lower risk of developing SREs compared with PAM ($P = 0.010$). Therefore, based on the comprehensive assessment of skeletal morbidity provided by two independent multiple event analyses, ZOL appears to be superior to PAM in patients with breast cancer. Based on this trial, ZOL has received broad international approval for the treatment of patients with bone metastases from breast cancer and is becoming the new standard of care worldwide (Rosen *et al.* 2004*b*).

NBPs in prostate cancer

In contrast to clodronate and PAM, ZOL (4 mg via a 15-min infusion every 3 weeks) has demonstrated statistically significant reductions in the incidence of SREs and sustained palliation of bone pain over 2 years in patients with bone metastases from hormone-resistant prostate cancer (HRPC). The results of this multicentre, randomized, placebo-controlled trial enrolling 643 patients were first reported at 15 months and then after 2 years of treatment (Zekri *et al.* 2001, Saad *et al.* 2004).

The primary end point was the percentage of patients with >1 SRE — defined as pathological fracture, radiation or surgery to bone, spinal cord compression, HCM, or change of therapy to treat bone pain. Secondary end points included time to first SRE, SMR, Andersen–Gill multiple event analysis, and Bone pain intensity (BPI) score. After 2 years, treatment with ZOL resulted in a significantly lower percentage of patients with >1 SRE (38% versus 49% for placebo; $P = 0.028$) and consistently lower incidences of all types of SREs, particularly fractures (Saad *et al.* 2003) (Table 1).

ZOL also produced a 36% significantly lower risk of developing a skeletal complication, as compared with placebo by multiple event analysis (HR = 0.640; $P = 0.002$) (Zekri *et al.* 2001) (Table 1).

Recently, Ernst and colleagues (2003) reported the results of a randomized, double-blind, controlled trial comparing the incidence of palliative response in HRPC treated with mitoxantrone and prednisone (MP) plus clodronate, with that of patients treated with MP plus placebo. They failed to demonstrate a significant difference between the arm groups but suggested a possible benefit of adding clodronate in patients with more severe pain.

NBPs in other solid cancers

To date, ZOL is the only BP to be evaluated for the prevention of skeletal complications in patients with bone metastases secondary to solid tumours other than breast or prostate cancer. In the first trial, 773 patients with bone metastases from non-small cell lung cancer (NSCLC) or other advanced-stage solid tumours were randomized to receive either ZOL (4 mg via a 15-min infusion) or placebo every 3 weeks for 21 months (Rosen *et al.* 2004*a*). That trial enrolled a diverse patient population composed of patients with NSCLC (49% of patients) and more than 20 other tumour types including renal cell carcinoma (RCC), colorectal cancer, small cell lung cancer and bladder cancer. However, this was a poor-prognosis group of patients with a median survival of only 6 months, and patients received a median of only four infusions of ZOL. Nevertheless, Andersen–Gill multiple event analysis demonstrated that treatment with ZOL resulted in a 31% significantly lower risk of developing an SRE compared with placebo (HR = 0.693; $P = 0.003$), and ZOL produced a significantly longer median time to first SRE (236 days versus 155 days for placebo; $P = 0.009$) (Table 1). A long-term subset analysis of 46 patients with RCC enrolled in that trial has also been reported (Lipton *et al.* 2004), showing that patients with bone metastases secondary to advanced RCC are at extremely high risk for skeletal complications. Among patients randomized to placebo, 79% had >1 SRE over 21 months of follow-up. Moreover, ZOL provided highly significant clinical benefits in this subset (Table 1). For example, patients treated with 4 mg ZOL had a significantly lower SRE incidence of 41% ($P = 0.011$) and longer time to onset of SREs (by approximately 1 year) than patients treated with placebo (median 424 days versus 72 days for placebo;

$P = 0.007$). In addition, Andersen–Gill multiple event analysis showed that ZOL produced a 58% lower risk for SREs (HR = 0.418; $P = 0.010$). Even more noteworthy is the observation that treatment with ZOL resulted in greater bone lesion response and a significantly longer time to progression of bone lesions by approximately 6 months (median 256 days versus 89 days for placebo; $P = 0.014$). ZOL was well tolerated in patients with RCC; the most common adverse events reported by patients for both 4 mg ZOL and placebo included bone pain, nausea, vomiting, anaemia and fatigue. There was no significant difference between treatment groups in the incidence of renal-related adverse events (Table 1). These results indicate that the predominantly osteolytic bone metastases associated with RCC are clinically aggressive, and these patients are at high risk for skeletal complications, but this risk can be significantly reduced with ZOL.

The clinical results based on the use of either clodronate or IBA are summarized in Table 1 and indicate that they are less promising than ZOL.

Most of the effects on bone are mediated by endocrine changes, either induction of an early menopause by chemotherapy and ovarian ablation, or further suppression of post-menopausal circulating oestrogens by aromatase inhibitors. Several studies have reported that cancer treatment-induced bone loss (CTIBL) can be prevented with BP treatment (Saarto *et al.* 1997, Powles *et al.* 1998). The Z-FAST study is the first trial designed to prevent, with ZOL, the bone loss that occurs with aromatase inhibitors in the typical post-menopausal setting. In this study, patients treated with adjuvant letrozole are randomized to either immediate treatment with ZOL (4 mg every 6 months) or to a delayed strategy. Preliminary 12-month bone mineral density (BMD) data have showed that upfront ZOL preserves bone density. However, further follow-up is required to determine the clinical importance of this over a wait-and-see policy (Brufsky *et al.* 2005).

Predictive value of bone resorption and formation markers in bone metastatic cancer patients receiving the BP ZOL

Coleman *et al.* (2005) have recently investigated the correlation between bone metabolism and clinical outcome during BP therapy. They analysed urinary measurements of N-telopeptide (Ntx) obtained in 1824 BP-treated patients (1462 multiple cancer types with ZOL and 362 with PAM) and estimated the correlation between this bone resorption marker and the relative risks for negative clinical outcomes. Interestingly, Ntx levels correlated with risk of skeletal complications and disease progression and, furthermore, high Ntx levels in each solid tumour category were associated with a 4- to 6-fold increased risk of death on study. This impressive study highlights the role of the bone resorption marker Ntx in providing correct prognostic information in patients with bone metastases receiving BPs. This could lead to the selection of cancer patients who might benefit most from therapy with BPs by evaluating urine or serum markers that predict who is at highest risk for skeletal complications and disease progression.

Translation of the results derived from pre-clinical studies in the treatment of human neoplasms

A series of pre-clinical findings suggest that NBPs have direct anti-tumour effects acting on different phases of tumour growth and progression. Moreover, the molecular targets and mechanisms of these effects are becoming clear. Clinical studies are confirming the activity of NBPs in preventing not only SREs but also skeletal metastases occurrence. The latter *in vivo* effect suggests a putative anti-invasive potential of NBPs. However, due to the intrinsic limitations of current NBPs, further efforts are required in order to allow the clinical translation of experimental results recorded to date and to increase the anti-cancer activity of these drugs. Two possibilities are the improvement of the pharmacokinetic profile and the design of rationale-based drug combinations.

Improvement of the pharmacokinetic profile

One of the most important limits of NBPs, which makes the direct anti-cancer activity difficult to demonstrate *in vivo*, is their pharmacokinetic profile. This issue is demonstrated by pharmacological studies performed on different NBPs. In fact, studies on ZOL pharmacokinetics demonstrate that, after intravenous administration (4 mg over 15 min), an abrupt increase of its concentration in peripheral blood is recorded, as shown by estimations of the early distribution and elimination of the drug, which results in plasma half-lives of the drug of about 15 min ($t_{1/2\alpha}$) and of 105 min ($t_{1/2\beta}$) respectively. The maximum plasma concentration (C_{max}) of ZOL is about 1 μM; that is 10- to 100-fold less than that required in *in vitro* studies to induce apoptosis and growth inhibition in tumour cell lines, while the concentrations required for anti-invasive effects are in the range of those achieved after *in vivo* administration. Moreover, approximately 55% of the initially administered dose of the drug is retained in the skeleton and is slowly released back into circulation,

resulting in a terminal elimination half-life ($t_{1/2\gamma}$) of about 7 days (Chen *et al*. 2002, Skerjanec *et al*. 2003). Other studies performed on alendronate demonstrate that NBP concentration in non-calcified tissues declines rapidly at 1 h (5% of the initial concentration). On the other hand, its concentration in the bone continuously increases, reaching its peak at 1 h, demonstrating that a significant redistribution of the drug from non-calcified tissues to bone occurs. The drug is retained in bone tissue for a long time and is slowly released into plasma, with a terminal half-life of about 200 days (Lin 1996). Similar data were obtained with IBA and ZOL (Chen *et al*. 2002, Barrett *et al*. 2004) demonstrating that long-lasting accumulation in bone is a common feature of NBPs. The rapid redistribution of NBPs results both in a short exposure of non-calcified tissues to the drug but also in a prolonged accumulation in bone where NBPs can also reach higher and tumoricidal concentrations. These considerations explain the relative efficacy of NBPs on tumours placed in bone tissues. A method of increasing the availability of these drugs in extra-bone tissues and increasing their plasma half-lives involves encapsulation in liposome vehicles (Harrington *et al*. 2002). Liposome-encapsulated clodronate, PAM and alendronate have already been produced and used for their ability to accumulate in the reticulo-endothelial system and for their macrophage-depleting properties (van Rooijen & van Kesteren-Hendrikx 2002, Danenberg *et al*. 2003). However, these agents have non-specific uptake in white blood cells and are, therefore, not suitable for anti-cancer therapy. Stealth liposomes (pegylated liposomes or second-generation liposomes) are more germane for anti-tumour clinical practice because they evade interception by the immune system. They are characterized by very long circulation half-lives, favourable pharmacokinetic behaviour and specific accumulation in tumour tissues (Cattel *et al*. 2004). One of the first anti-cancer drugs encapsulated in pegylated liposomes and used in clinical trials was doxorubicin (Gabizon *et al*. 2003). More recently, the ability of doxorubicin encapsulated in stealth liposomes to be active in the treatment of brain tumour metastases has also been reported (Caraglia *et al*. 2005). These favourable pharmacokinetic properties of pegylated liposomes could encourage their use as NBP vehicles in order to increase the uptake of the latter in tumour sites.

Rationale-based combinations between NBPs and other biological agents

On the basis of the emerging mechanisms of action of NBPs, several combinations of NBPs and other biological agents have been designed and their activity on tumour cell proliferation has been evaluated.

ZOL and other NBPs have been combined with different biological agents based on their ability to inhibit crucial processes of protein isoprenylation. In fact, the prenyltransferases are not strictly specific and a small G protein can be the substrate for different enzymes. In fact, K-RasB, which has a classical FTase CA_1A_2X box (CVIM), is a substrate for FTase, but following inhibition of cellular FTase, K-RasB becomes a substrate for geranylgeranylation by GGTase-I (Gibbs 2001). Similarly, RhoB, which contains a GGTase-I CA_1A_2X box (CKVL), is found in both farnesylated and geranylgeranylated forms in cells (Yokayama & Gelb 2001). These events allow the alternative isoprenylation of the substrate of prenyltransferase inhibitors when FTIs are added to tumour cells. Based on the relevance of any farnesylation inhibitory effects on anti-tumour activity of the BPs, the FTI R115777 was used together with PAM or ZOL and the effects of the combination treatment on growth inhibition and apoptosis evaluated. BPs and FTI given in combination were strongly synergistic since a CI_{50} (the combination index of the two drugs calculated for 50% cell survival by isobologram analysis using dedicated software) of less than 0.5 was recorded (Caraglia *et al*. 2004). In fact, CI_{50} values less than 1.0 suggest synergism and values less than 0.5 suggest strong synergism in inducing growth inhibition by drug combinations (Chou & Talalay 1984). Notably, low concentrations of FTI induced a strong increase of ras expression with only a moderate reduction of ras activity that was, on the other hand, significantly reduced by the combined treatment (Caraglia *et al*. 2004). These data suggest that escape mechanisms for the inhibition of isoprenylation of ras might be based on the geranylgeranylation or other prenylating processes (Lee *et al*. 2001). The addition of FOH to cells treated with the combination abolished the effects of the BPs/FTI combination on apoptosis and on the activity of the signalling molecules. These data suggest that the synergistic growth-inhibitory and pro-apoptotic effects produced by the NBP/FTI combination involve the inhibition of both Erk and Akt survival pathways acting in these cells in a ras-dependent fashion (Caraglia *et al*. 2004).

Andela *et al*. (2002) have recently reported that the NBP alendronate and R11577 used alone had no significant effects on the growth inhibition and apoptosis of murine lung alveolar carcinoma, but showed a reduction in *in vitro* invasiveness when the two drugs were combined. Moreover, alendronate

potentiated the *in vivo* anti-metastatic effects of R115777.

Imatinib mesylate, a selective inhibitor of Abl tyrosine kinase, has significant and rapid activity against chronic myelogenous leukaemia (CML) and Philadelphia-positive (Ph^+) acute lymphoblastic leukaemia (ALL). Targeting one of the Bcr-Abl downstream signaling proteins essential for Bcr-Abl-mediated leukaemogenesis, in addition to Bcr-Abl, was intriguing. Several lines of evidence have implicated the Ras/mitogen-activated protein kinase (MAPK) signalling pathway as an important molecular target (Turhan *et al*. 1998, le Coutre *et al*. 2000, Mahon *et al*. 2000, Weisberg & Griffin 2000, Druker *et al*. 2001*a*,*b*, Hofmann *et al*. 2002, Hoover *et al*. 2002, Ottman & Hoelzer 2002, Yu *et al*. 2002). On the basis of these considerations Kuroda *et al*. (2004) have used imatinib in combination with ZOL in imatinib-responsive or -resistant AML cell lines. Interestingly, imatinib-resistant cell lines also displayed sensitivity to the growth inhibitory activity of both ZOL and PAM, and also ZOL induced a decrease of Rap-1A and ras prenylation, G2M-phase accumulation and apoptosis.

It was also reported that ZOL was able to induce growth inhibition of a small cell lung cancer (SCLC) cell line subcutaneously transplanted in nude mice and was synergetic on *in vitro* growth inhibition with several anti-cancer agents and had additive effects if combined with imatinib (Matsumoto *et al*. 2005). Another study examined the effects of combining a cyclooxygenase-2 inhibitor SC236 with ZOL and/or docetaxel in a HER-2/neu-transfected and a control human breast cancer cell line. Enhanced growth inhibition was observed in both cell lines with both the combination of docetaxel and SC236, and of docetaxel and ZOL (Witters *et al*. 2003).

Zhang *et al*. (2004) have demonstrated a differential sensitivity of breast cancer cell lines to PAM based upon differential modulation of ras protein expression induced by the NBPs. They have found that MDA-175 was a PAM-resistant cell line in which the modulation of ras expression by PAM was lost. On the basis of these findings they have used in combination with PAM, imatinib or bortezomib, a proteasome inhibitor or rapamycin, an inhibitor of the mammalian target of rapamycin (m-TOR); all combinations showed additive effects in causing inhibition of proliferation in MDA-175 cells (Zhang *et al*. 2004). It was also reported that another newly synthesized NBP, minodronic acid, synergized with all-trans retinoic acid, thalidomide, or interferon beta on the growth inhibition and apoptosis of myeloma cells (Yata *et al*. 2002). More recently, it was shown that minodronic acid has anti-proliferative activity and reduces the prenylation of Rap-1A in human RCC cells (Yuasa *et al*. 2005).

The findings of synergy of interaction between NBPs and other agents could reduce the NBP concentrations required for anti-tumour activity and could allow the achievement of effective *in vivo* levels.

Conclusions

The discovery of the locus of action of NBPs could have important consequences in the understanding of their anti-cancer activity and may suggest pharmacological modifications that could be made to increase their tumouricidal activity. One example is given by liposomal formulations that could allow distribution to visceral cancer sites rather than NBPs being mainly accumulated in bone tissue.

The body of evidence accumulated on NBP mechanisms of action does suggest some innovative strategies based on the use of rationale-based drug combinations. The existence of a pro-apoptotic effect of NBPs based on the disruption of ras (or ras-like)-mediated pathways supports the combination between NBPs and other isoprenylation inhibitors such as FTIs. However, further pre-clinical investigations, eventually supported by advanced technological platforms, are required for the discovery of molecular targets of NBPs and for the optimization of their mode of administration.

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