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Review

In Vitro and *In Vivo* Antiresorptive Effects of Bisphosphonates in Metastatic Bone Disease

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Abstract. *Bone metastases commonly occur in the course of malignant tumor disease. For many years, attempts have been made to identify factors for the management of cancer-induced skeletal complications. Nowadays, synthetic antiresorptive agents are considered to be indispensable for the treatment of cancer-related skeletal events, such as bone metastasis. The most common of these drugs are the bisphosphonates, which represent one of the most significant advances over the last 10 years in the field of supportive care and cancer. They are used for the treatment of cancer-induced hypercalcemia, for the prevention and treatment of postmenopausal osteoporosis, for patients with bone metastases secondary to breast cancer and multiple myeloma. A third-generation bisphosphonate, zolendronate, has been shown to minimize the destructive consequences of bone metastases and to exert a profound effect on tumor-induced osteolysis and tumor growth in bone. Zoledronate is already used for the treatment of hypercalcemia of malignancy, multiple myeloma-related osteolytic events and for patients with documented bone metastases from solid tumors in conjunction with standard antineoplastic therapy. The structure-function activity of the three generations of bisphosphonates developed to date, the in vitro models used for studying their effects on osteoclasts and osteoblasts, as well as the results of clinical trials obtained by the third generation bisphosphonate, zoledronic acid, are presented.*

Bone constitutes the third most common site of distant metastases, following lungs and liver, in patients with different solid tumors, particularly breast (*ca.* 73%), prostate (*ca.* 68%) and lung (*ca.* 36%) cancers, and often is the only metastatic site (1). The resulting skeletal events, including bone pain, pathologic fractures, myelosuppression and spinal cord compression, undermine the patients' quality of life. Metastasis is the process by which tumor cells travel from the primary tumor to a distant site *via* the circulatory system and establish a secondary tumor. The process of metastasis is a complex cascade of organized, sequential and interrelated steps, including angiogenesis, local invasion, intravasation and extravasation and degradation of the extracellular matrix by proteolytic enzymes such as metalloproteinases. The bone mineral matrix contains numerous growth factors and cytokines that are released during the bone remodeling cycle, providing a fertile microenvironment for tumor cell colonization and proliferation (2). Tumor cells then release a variety of growth factors that promote bone resorption or formation and increase the risk of skeletal complications of malignancy. The metastatic lesions can be either osteolytic, when resorption by osteoclasts exceeds formation and leads to bone loss, as in the case of osseous metastases by breast cancer, or osteoblastic, where the increased activity of osteoblasts leads to the stimulation of osteoclasts, as in the case of metastatic prostate cancer (3).

Many therapeutic strategies have been developed for the management of cancer-induced skeletal complications. These trials include either antineoplastic intervention, such as hormonal therapies, chemotherapy, radiotherapy and even topical surgeries or bone supportive treatment such as analgesics, calcium and vitamin D supplements. Currently, synthetic antiresorptive agents are considered to be indispensable for the treatment of malignant bone metastasis (4, 5). Bisphosphonates (BPs) are the most potent of these drugs. BPs have an established role as palliative therapy in patients with skeletal metastases, since they increase the inhibition of cancer cell invasion and adhesion to the bone matrix and induce apoptosis of osteoclasts (6).

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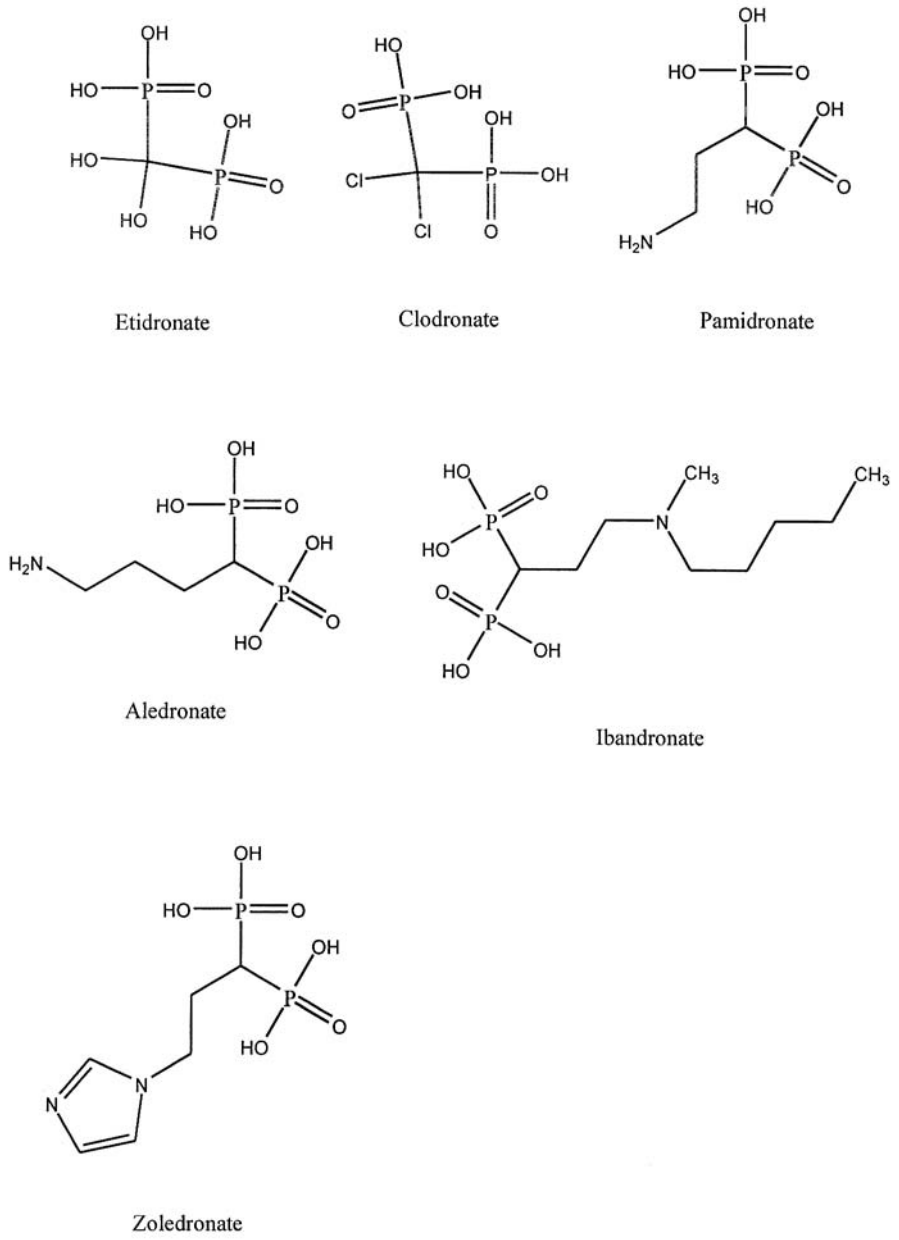

Figure 1. *The chemical structure of the best known bisphosphonates. First generation: etidronate and clodronate, second generation: pamidronate, alendronate and ibandronate, third generation: zoledronate.*

Bisphosphonates: structure and mechanisms of action

BPs are compounds with a chemical structure similar to that of inorganic pyrophosphate (PPi). Although PPi is comprised of two phosphate groups linked by phosphoanhydride bonds (a P-O-P structure), which are extremely unstable, BPs are comprised of two phosphonate groups linked by highly hydrolysis-resistant phosphoether bonds to a central (geminal) carbon atom (a P-C-P structure). Two additional covalent bonds to the geminal carbon atom of BPs can be formed with carbon, oxygen, halogen, sulfur, or nitrogen atoms, giving rise to an enormous range of possible structures. The two covalently bonded groups (side chains) attached to the geminal carbon are usually referred to as R1 and R2 (7, 8). When the R1 side chain is a hydroxyl group, such compounds are able to chelate calcium ions more effectively, by tridentate rather

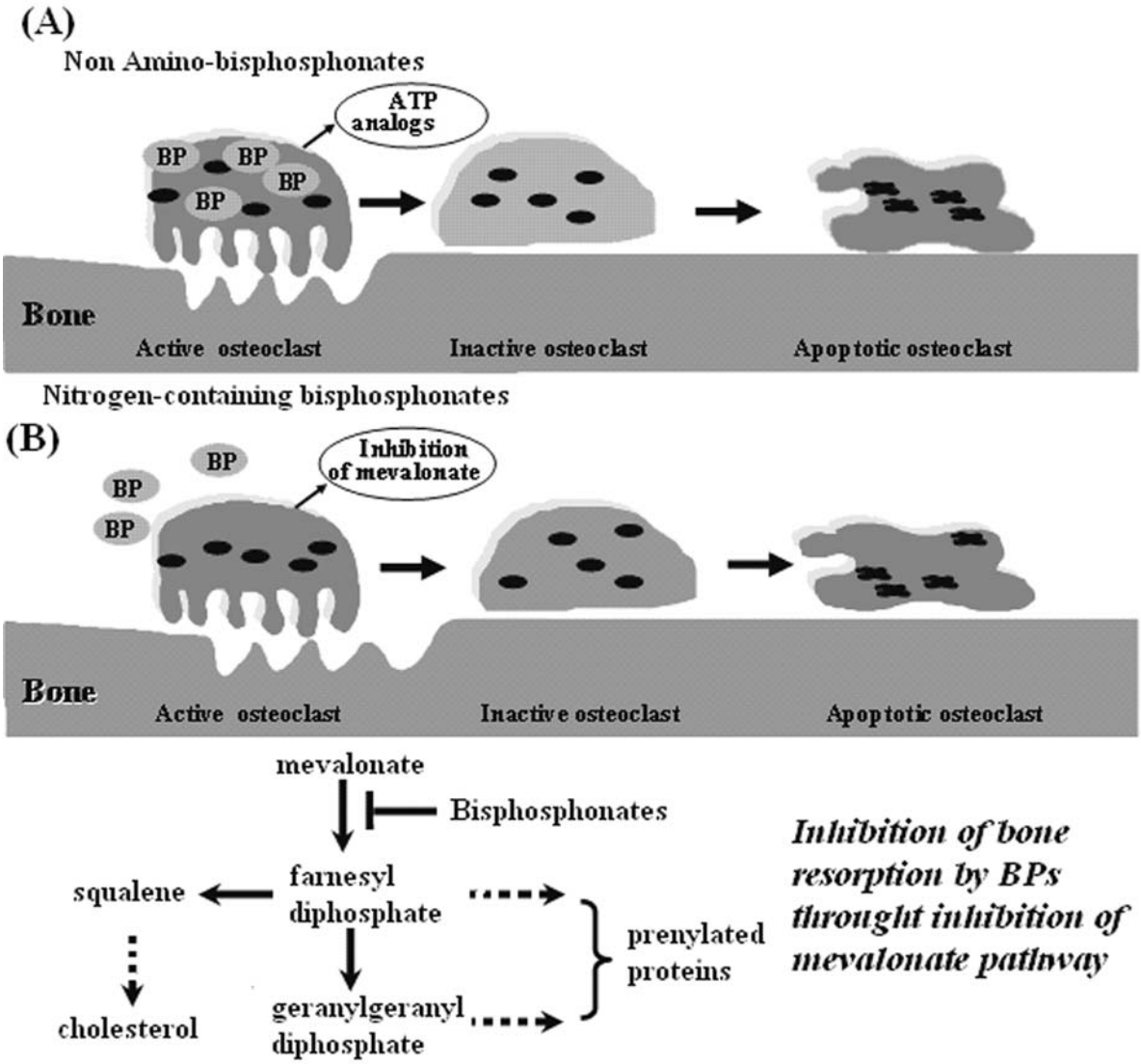

Figure 2. *The apoptotic effect of bisphosphonates on osteoclasts depends on their chemical structure. (A) Non-amino BPs act through the production of non-hydrolyzable, cytotoxic ATP-analogs. (B) Amino-BPs induce cell death through the inhibition of the mevalonate pathway.*

than bidentate binding. When the length of the R2 side chain is increased from a simple methyl group to longer alkyl chains, significantly more potent compounds were obtained (9).

Three generations of BPs have been developed during the last three decades (Figure 1). Etidronate and clodronate were the earliest products used clinically. They continue to be marketed for malignant and nonmalignant bone disorders, which are characterized by increased bone resorption. In recent years, several new BPs, each with a unique side chain, have been developed. Each new generation of BPs has increased bone antiresorptive activity as compared to the previous one (10). Etidronate and clodronate have the lowest potency, while the second-generation BPs, such as pamidronate and alendronate, are of median potency. A new compound, *i.e.*, zoledronate (CGP 42446), has shown to be the most potent *in vitro* and *in vivo* bone antiresorptive agent as compared to currently available BPs (10, 11).

BPs represent one of the most significant advances in the field of supportive care and cancer. They are used for the treatment of cancer hypercalcemia and reduce bone pain in at least 50% of patients (11). They are well established as successful agents for the prevention and treatment of postmenopausal osteoporosis (12, 13) and they are also utilized in corticosteroid-induced bone loss (14). BPs have

a widely recognized role for patients with multiple myeloma and bone metastases secondary to breast cancer (15-17). Intravenous or oral administration of BPs can reduce the overall amount of skeletal events in patients with myeloma and breast cancer by ~50% (18). It should be noticed that, although all of the BPs can be administered either intravenously or orally, the oral bioavailability of any bisphosphonate is extremely limited. Generally, only a small percentage of an oral dose is absorbed from the gastrointestinal tract and the intake of any food or beverage further diminishes absorption to negligible levels (19, 20).

Although all BPs selectively target bone mineral and inhibit osteoclast-mediated bone resorption, the molecular mechanism of their action can be grouped into two classes. The nitrogen-containing BPs inhibit farnesyl diphosphate (FPP) synthase, a key enzyme in the mevalonate pathway in osteoclasts, thereby reducing the prenylation of small guanosine triphosphate (GTP)-binding proteins that are essential for osteoclast function (Figure 2). On the other hand, BPs that lack a nitrogen atom in the chemical structure do not inhibit protein prenylation. They have a different mode of action that may involve the formation of cytotoxic metabolites in osteoclasts or inhibition of protein tyrosine phosphatases (Figure 2). These agents are incorporated into nonhydrolyzable analogs of adenosine triphosphate (ATP) and accumulate intracellularly in osteoclasts, thus interfering with osteoclast function through inhibition of ATP-dependent enzymes (21-23).

Zolendronate or zoledronic acid (ZA) [(1-hydroxyl-2-imidazole-1-yl-phosphonoethyl) phosphonic acid monohydrate, $C_5H_{10}N_2O_7P_2 \bullet H_2O$, molecular weight 290.11 g/mol] is a new third-generation bisphosphonate used for the treatment of cancer-induced hypercalcemia. It has the usual P-C-P bisphosphonate structure with a hydroxyl group and a heterocyclic imidazole ring as additive groups appropriate for its effectiveness. ZA has been shown to be at least 100-fold more potent than pamidronate with respect to bone resorption and exerts no effect on bone mineralization (24-26). It has a direct effect on osteoclasts and is considered to be a potent inhibitor of bone resorption. The mechanisms by which ZA interferes with osteoclastic activity involve: i) the inhibition of osteoclast formation and osteoclast bone resorptive activities, and ii) the induction of osteoclast apoptotic cell death (27-29). Furthermore, ZA has a high affinity for hydroxyapatite, binds directly to mineralized bone and enables the bone to be resistant to endogenous phosphatases (30). On osteoclast stimulation of bone resorption, the bisphosphonate is released and internalized by the osteoclasts, interfering with osteoclast formation, function and survival. ZA and the other nitrogen-containing agents (*i.e.* alendronate, ibandronate, pamidronate, risedronate) are more potent inhibitors of bone resorption as compared to those agents that lack a nitrogen group (*i.e.* clodronate, etidronate). ZA can have a profound effect on tumor-induced osteolysis and on tumor growth in bone, thereby minimizing the destructive consequences of bone metastases (31). The US Food and Drug Administration approved ZA (Zometa™, Novartis Pharma AG, Basel, Switzerland) for the treatment of hypercalcemia of malignancy (HCM), multiple myeloma-related osteolytic events and for patients with documented bone metastases from solid tumors in conjunction with standard antineoplastic therapy.

In vitro models for studying the action of bisphosphonates

The *in vitro* study of osteoclasts is a quite difficult issue, because of difficulty in isolating and culturing large numbers of pure osteoclasts. For this reason, many studies characterizing the pharmacologic properties of BPs *in vitro* have been performed with osteoclast surrogates, in particular macrophages. Osteoclasts are derived from monocyte macrophage cell lineage (32) and, like osteoclasts, they are highly endocytic and capable of demineralizing bone particles. The origin of these cells is from the same haematopoietic colony-forming unit–granulocyte macrophage lineage as osteoclasts and the effect of BPs on macrophages *in vitro* matches their effect on osteoclasts. Rogers *et al.* (7) reported that BPs can be internalized by cells, despite their negative charge. The particular sensitivity of macrophages and osteoclasts to BPs most probably is a reflection of the high endocytic capacity and the enhanced ability of these cells to internalize the drugs. The structure-activity relationship of BPs for inhibiting macrophage proliferation (33) and causing apoptosis of macrophages-like cells J774 (34) matches that for inhibiting bone resorption. This could suggest that macrophage apoptosis is caused by the interaction of BPs with the same molecular target that affects osteoclasts. It is likely that BPs interact with intracellular protein targets that are common to most, if not all, eukaryotic cells, and the ability of these drugs to affect cells is determined by the degree of cellular uptake (7).

Even though the action of BPs has been widely studied on osteoclastic models, many studies refer to the impact of BPs on osteoblastic-like cells (osteosarcoma). *In vitro*, BPs can both inhibit and stimulate the proliferation of osteoblast-like cells and other connective tissue cells (35, 36). Although, the antiproliferative effect of BPs has been observed with a variety of other cell types *in vitro*, this effect is not of physiologic significance, since BPs do not inhibit bone formation. BPs cause the release of a factor from osteoblasts that subsequently inhibits osteoclast activity or osteoclast formation. Thus, when osteoclasts were cultured with conditioned medium taken from the bisphosphonate-treated osteoblast-like cells bone resorption was inhibited, suggesting that the osteoblasts released a soluble factor that inhibits bone resorption. Since BPs act intracellularly, it is

Table I. *Phase I, II and III clinical trials with cancer patients under zoledronic acid treatment.*

	Number of patients	Primary malignancy	Reference
Phase I trials	59	Osteolytic bone lesions from several tumors	Berenson *et al.* (62)
Phase II trials	280	Breast cancer and multiple myeloma	Berenson *et al.* (63)
Phase III trials	1648	Breast cancer and multiple myeloma	Rosen *et al.* (64)
	643	Prostate cancer	Saad *et al.* (65)
	773	Lung cancer and other solid tumors	Rosen *et al.* (68)

remarkable that concentrations of BPs as low as 10^{-11} M had an effect on osteoblasts (7).

Cellular uptake of BPs

During the bone resorption process, osteoclasts appear highly endocytic. For this reason, bisphosphonate present in the resorption lacuna are likely to be internalized by osteoclasts into endocytic vacuoles. BPs affect osteoclast-mediated bone resorption in a variety of ways, which include effects on osteoclast formation, resorptive activity and viability (37-39). Osteoclasts are the bone cells most likely to be exposed to high concentrations of drug; experimental studies support the hypothesis that they are able to internalize BPs by endocytosis (40). *In vivo* administration of radiolabelled bisphosphonate into vacuoles, other organelles and the cytoplasm of rats, visualized the BPs within resorbing osteoclasts (41). In other studies where the influence of bone mineral on the ability of BPs to inhibit osteoclast activity was also examined, it was shown that BPs are just as potent or even more effective when bound to bone mineral (42, 43), because this concentrates the drug and allows it to be released during resorption and be taken up selectively by osteoclasts. Selander *et al.* (44) also observed that the inhibitory effect of BPs on osteoclasts occurred once resorption had begun. The ability of osteoclasts to internalize BPs suggests that the mechanism of action may be intracellular. Felix *et al.* (45) used radiolabelled BPs to study uptake by calvarial cells *in vitro* and confirmed that the BPs could enter the cytoplasm as well as mitochondria and other organelles. After cellular uptake, bisphosphonate-treated osteoclasts show important changes in morphology, both *in vitro* (39, 46) and *in vivo* (47-49).

BPs have been shown to inhibit tumor cell proliferation and to induce apoptosis *in vitro* (50-52). ZA has an antiproliferative effect on cells accumulating in the G0-, G1- and S- phases. An extensive study with three breast cancer cell lines (MCF-7, T47D and MDA-MB-231) incubated for various time periods with four BPs revealed several differences in growth inhibition according to the cell line and the bisphosphonate used (53). Tumor cell invasion requires digestion of the basement membrane by various proteases. Matrix metalloproteinases are the main enzymes in this process. BPs inhibit the activity of several matrix metalloproteinase enzymes (MMP-1, -2, -3, -7, -8, -9, -12, -13, -14) *in vitro* with IC_{50} values in the range of 50-150 μM (54, 55). The mechanism appears to be chelation of divalent cations from the enzyme active site, since it has been shown that addition of excess zinc reverses the inhibition (56).

Clinical trials in cancer patients under zoledronic acid treatment

Numerous clinical studies have proved the efficacy of ZA in the management of cancer patients with skeletal-related events (Table I). Preclinical models of osteoclast-mediated bone resorption demonstrated the improved potency of ZA compared to pamidronate, clodronate and other BPs (57-59). For the follow-up of patients, the most commonly used biochemical tools are markers that reflect either bone formation or resorption (60, 61). These are products that are released into the circulation or excreted in urine during the bone metabolism cycle and can be detected and determined quantitatively in biological samples. Berenson *et al.* (62, 63) evaluated the dose range of ZA in clinical phase I studies with patients with bone metastases from a variety of primary malignancies. These studies primarily assessed the effect of ZA across a range of doses from 1 to 16 mg (single infusion) or from 0.1 to 8 mg (multiple infusions). The results demonstrated a dose-response relationship for doses up to 8 mg; doses > 0.8 mg effectively reduced markers of bone resorption, such as type I collagen N-telopeptide (NTx) and urinary deoxypyridoline (Dpd), whereas doses < 0.8 mg were less effective. A large, randomized, double-blind, phase II trial was subsequently conducted to evaluate several doses (0.4, 2.0, or 4.0 mg) of ZA as compared to the standard 90 mg pamidronate dose for the treatment of osteolytic bone lesions in patients (n=280) with breast cancer and multiple myeloma (6). This study confirmed the dose-response relationship originally observed in the phase I trials. Based on the observed clinical activity and the apparent dose-response data for ZA in these phase I and phase II studies, the 4 and 8 mg doses were chosen for further clinical development. ZA seems not to be potent enough in reducing the need for radiation therapy or reducing the frequency of other skeletal complications. Over 3000 patients with bone

metastases were enrolled in three randomized, double-blind, phase III trials. Each trial was designed to estimate the efficacy and safety of 4 and 8 mg ZA (*via* 5-min intravenous infusion) compared with the current standard bisphosphonate treatment (90 mg pamidronate *via* 2-h infusion) in patients with breast cancer and multiple myeloma, or placebo for the other cancer types. However, the infusion time was increased to 15 minutes to ensure safety of renal function. The first trial enrolled 1648 patients with either stage III multiple myeloma or stage IV breast cancer (64). At the end of the 13-month study, analysis of individual skeletal-related events showed that 4 mg ZA seemed to significantly reduce the need for radiation therapy to bone compared with pamidronate (15% for ZA *vs* 20% for pamidronate; $p=0.031$), and that this difference was more obvious among patients with breast cancer receiving hormonal therapy (16% for ZA *vs* 25% for pamidronate; $p=0.022$). This study also confirmed that ZA effectively inhibited bone resorption. NTx, pyridinoline and Dpd/creatinine ratios and serum levels of bone-specific alkaline phosphatase, a bone formation marker, were all significantly reduced from baseline.

Two placebo-controlled trials were conducted in patients with prostate cancer and patients with other solid tumors, who had newly diagnosed radiologic evidence of bone metastasis. In the prostate cancer trial by Saad *et al.* (65), 643 men were randomized to either 4 or 8 mg ZA or placebo every 3 weeks for 15 months. The results demonstrated that 4 mg ZA significantly reduced the proportion of patients with a skeletal-related event (33% for ZA *vs* 44% with placebo; $p=0.021$) and extended the time to the first skeletal-related event. Additionally, there was a significant reduction in pain scores for patients treated with 4 mg ZA compared with placebo at all measured time points. In contrast, clodronate, whether given orally or intravenously, as well as pamidronate (90 mg) failed to establish a significant clinical profit in trials in patients with prostate cancer (66, 67). In the other trial involving patients with other solid tumors, approximately 45% had non-small cell lung cancer, while the remaining patients had renal cancer, bladder cancer, or a variety of other cancers. In total, 773 patients were randomized to either 4 mg or 8 mg/4 mg ZA or placebo every 3 weeks for 9 months (68). The time to the first pathologic fracture was delayed. Multiple event analysis also concluded that patients who received 4 mg ZA had a 30% reduction in the risk of skeletal events compared with placebo ($p=0.006$). Similar benefits were observed in the approximately 50% of patients in the study who had lung cancer.

Conclusion

Bisphoshonates are synthetic compounds that are able to inhibit osteoclast-induced bone destruction. BPs have been used successfully for many years in the treatment of osteoporosis, hypercalcemia and to reduce the skeletal complications of malignancy. Zoledronic acid, a third-generation nitrogen-containing BP, has been recognized as one of the most potent BPs. *In vitro* studies showed that BPs inhibit proliferation and differentiation of osteoclasts and induce apoptosis of these bone cells. Clinical trials proved the efficacy of ZA in cancer patients with bone metastases, since it decreases bone pain and osteoclasts' activity and, therefore, increases the survival of patients.

References

1 Brown HK and Healey JH: Metastatic cancer to the bone. *In*: Principles and Practice of Oncology (De Vita VT, Hellman S, Rosenberg SA, eds). 6th edition. Lippincott Williams and Wilkins 2001, pp 2713-2729.

2 Rosol TJ: Pathogenesis of bone metastases: role of tumor-related proteins. J Bone Miner Res *15(5)*: 844-850, 2000.

3 Ashcroft AJ, Davies FE and Morgan GJ: Aetiology of bone disease and the role of bisphosphonates in multiple myeloma. Lancet Oncol *4(5)*: 284-292, 2003.

4 Fleisch HA: Bisphosphonates: preclinical aspects and use in osteoporosis. Ann Med *29(1)*: 55-62, 1997.

5 Body JJ, Bartl R, Burckhardt P, Delmas PD, Diel IJ, Fleisch H, Kanis JA, Kyle RA, Mundy GR, Paterson AH and Rubens RD: Current use of bisphosphonates in oncology. International Bone and Cancer Study Group. J Clin Oncol *16*: 3890-3899, 1998.

6 Berenson JR, Rosen LS, Howell A, Porter L, Coleman RE, Morley W, Dreicer R, Kuross SA, Lipton A and Seaman JJ: Zoledronic acid reduces skeletal-related events in patients with osteolytic metastases. A double blind, randomized dose-response study. Cancer *91*: 1191-1200, 2001.

7 Rogers MJ, Gordon S, Benford HL, Coxon FP, Luckman SP, Monkkonen J and Frith JC: Cellular and molecular mechanisms of action of bisphosphonates. Cancer *88*: 2961-2978, 2000.

8 Santini D, Vespasiani Gentilucci U, Vincenzi B, Picardi A, Vasaturo F, La Cesa A, Onori N, Scarpa S and Tonini G: The antineoplastic role of bisphosphonates: from basic research to clinical evidence. Ann Oncol *14(10)*: 1468-76, 2003.

9 Shinoda H, Adamek G, Felix R, Fleisch H, Schenk R and Hagan P: Structure–activity relationship of various bisphosphonates. Calcif Tissue Int *35*: 87-99, 1983.

10 Fleisch H: Disphosphonates. Calcif Tissue Int 27: 91-94, 1997.

11 Body JJ: Clinical research update-zoledronate. Cancer *80(Suppl 8)*: 1699-701, 1997.

12 Watts NB, Harris ST, Genant HK, Wasnich RD, Miller PD, Jackson RD, Licata AA, Ross P and Woodson GC: Intermittent cyclical etidronate treatment of postmenopausal osteoporosis. N Engl J Med *323*: 73-79, 1990.

13 Harris ST, Watts NB, Jackson RD, Genant HK, Wasnich RD, Ross P, Miller PD, Licata AA and Chesnut CH: Four-year study of intermittent cyclic etidronate treatment of postmenopausal osteoporosis: three years of blinded therapy followed by one year of open therapy. Am J Med *95*: 557-567, 1993.

14 Reid IR, King AR, Alexander CJ and Ibbertson HK: Prevention of steroidinduced osteoporosis with (3-amino-1-hydroxypropylidene)-1,1-bisphosphonate (APD). Lancet *1*: 143–146, 1988.

15 Hortobagyi GN, Theriault RL, Porter L, Blayney D, Lipton A, Sinoff C, Wheeler H, Simeone JF, Seaman J and Knight RD: Efficacy of pamidronate in reducing skeletal complications in patients with breast cancer and lytic bone metastases. Protocol 19, Aredia Breast Cancer Study Group. N Engl J Med *335*: 1785-1791, 1996.

16 Diel IJ, Solomayer EF, Costa SD, Gollan C, Goerner R, Wallwiener D, Kaufmann M and Bastert G: Reduction in new metastases in breast cancer with adjuvant clodronate treatment. N Engl J Med *339*: 357-363, 1998.

17 Theriault RL, Lipton A, Hortobagyi GN, Leff R, Gluck S, Stewart JF, Costello S, Kennedy I, Simeone J, Seaman JJ, Knight RD, Mellars K, Heffernan M and Reitsma DJ: Pamidronate reduces skeletal morbidity in women with advanced breast cancer and lytic bone lesions: a randomized, placebo-controlled trial. Protocol 18, Aredia Breast Cancer Study Group. J Clin Oncol *17*: 846-854, 1999.

18 Berenson JR, Lichtenstein A, Porter L, Dimopoulos MA, Bordoni R, George S, Lipton A, Keller A, Ballester O, Kovacs MJ, Blacklock HA, Bell R, Simeone J, Reitsma DJ, Heffernan M, Seaman J and Knight RD: Efficacy of pamidronate in reducing skeletal events in patients with advanced multiple myeloma. Myeloma Aredia Study Group. N Engl J Med *334*: 488-493, 1996.

19 Pentikainen PJ, Elomaa I, Nurmi AK and Karkkainen S: Pharmacokinetics of clodronate in patients with metastatic breast cancer. Int J Clin Pharmacol Ther Toxicol *27(5)*: 222- 228, 1989.

20 Daley-Yates PT, Dodwell DJ, Pongchaidecha M, Coleman RE and Howell A.: The clearance and bioavailability of pamidronate in patients with breast cancer and bone metastases. Calcif Tissue Int *49*: 433-435, 1991.

21 Body JJ, Bartl R, Burckhardt P, Delmas PD, Diel IJ, Fleisch H, Kanis JA, Kyle RA, Mundy GR, Paterson AH and Rubens RD: for the International Bone and Cancer study group. Current use of biphosphonates in oncology. J Clin Oncol *16*: 3890-3899, 1998.

22 Green JR: Antitumor effects of bisphosphonates. Cancer *97*: 840-847, 2003.

23 Edward CL: Zoledronic acid: a new parenteral bisphosphonate. Clin Therapeut *25*: 2669-2708, 2003.

24 Green JR, Müller K and Jaeggi KA: Preclinical pharmacology of CGP 42'446, a new, potent, heterocyclic bisphosphonate compound. J Bone Miner Res *9(5)*: 745-751, 1994.

25 Benford HL, McGowan NW, Helfrich MH, Nuttall ME and Rogers MJ: Visualization of bisphosphonate-induced caspase-3 activity in apoptotic osteoclasts *in vitro*. Bone *28(5)*: 465-73, 2001.

26 Binkley N, Kimmel D, Bruner J, Haffa A, Davidowitz B, Meng C, Schaffer V and Green J: Zoledronate prevents the development of absolute osteopenia following ovariectomy in adult rhesus monkeys. J Bone Miner Res *13(11)*: 1775-1782, 1998.

27 Coxon FP, Helfrich MH, Van't Hof R, Sebti S, Ralston SH, Hamilton A and Rogers MJ: Protein geranylgeranylation is required for osteoclast formation, function, and survival: Inhibition by bisphosphonates and GGTI-298. J Bone Miner Res *15*: 1467-1476, 2000.

28 Nielson OS, Munro AJ and Tannock IF: Bone metastasis: pathophysiology and management policy. J Clin Oncol *9*: 509-524, 1991.

29 Benford HL, McGowan NW, Helfrich MH, Nuttall ME and Rogers MJ: Visualization of bisphosphonate induced caspase-3 activity in apoptotic osteoclasts *in vitro*. Bone *28*: 465-473, 2001.

30 Lipton A: Bisphosphonates and metastatic breast carcinoma. Cancer *97*: 848-853, 2003

31 Cameron D: Proven efficacy of zoledronic acid in the treatment of bone metastases in patients with breast cancer and other malignancies. The Breast *2*: S22-S29, 2003.

32 Roodman GD: Cell biology of the osteoclast. Exp Hematol *27*: 1229-1241, 1999.

33 Cecchini MG and Fleisch H: Bisphosphonates *in vitro* specifically inhibit, among the hematopoietic series, the development of the mouse mononuclear phagocyte lineage. J Bone Miner Res *5*: 1019-1027, 1990.

34 Rogers MJ, Chilton KM, Coxon FP, Lawry J, Smith MO, Suri S, *et al.*: Bisphosphonates induce apoptosis in mouse macrophage-like cells *in vitro* by a nitric oxide-independent mechanism. J Bone Miner Res *11*: 1482-1491, 1996.

35 Fast DK, Felix R, Dowse C, Neuman WF and Fleisch H: The effects of diphosphonates on the growth and glycolysis of connective-tissue cells in culture. Biochem J *172*: 97-107, 1978.

36 Endo N, Rutledge SJ, Opas EE, Vogel R, Rodan GA and Schmidt A: Human protein tyrosine phosphatase-sigma: alternative splicing and inhibition by bisphosphonates. J Bone Miner Res *11*: 535-543, 1996.

37 Hughes DE, Mac Donald B, Russell RGG and Gowen M: Inhibition of osteoclast-like cell formation by bisphosphonates in long term cultures of human bone marrow. J Clin Invest *83*: 1930-1935, 1989.

38 Hughes DE, Wright KR, Uy HL, Sasaki A, Yoneda T, Roodman GD, Mundy GR and Boyce BF: Bisphosphonates promote apoptosis in murine osteoclasts *in vitro* and *in vivo*. J Bone Miner Res *10*: 1478-1487, 1995.

39 Sato M and Grasser W: Effects of bisphosphonates on isolated rat osteoclasts as examined by reflected light microscopy. J Bone Miner Res *5*: 31-40, 1990.

40 Fisher JE, Rogers MJ, Halasy JM, Luckman SP, Hughes DE, Masarachia PJ, Wesolowski G, Russell RG, Rodan GA and Reszka AA: Alendronate mechanism of action: geranylgeraniol, an intermediate in the mevalonate pathway, prevents inhibition of osteoclast formation, bone resorption and kinase activation *in vitro*. Proc Natl Acad Sci USA *96*: 133-138, 1999.

41 Sato M, Grasser W, Endo N, Akins R, Simmons H, Thompson DD, Golub E and Rodan GA: Bisphosphonate action. Alendronate localization in rat bone and effects on osteoclast ultrastructure. J Clin Invest *88*: 2095-2105, 1991.

42 Flanagan AM and Chambers TJ: Inhibition of bone resorption by bisphosphonates: interactions between bisphosphonates, osteoclasts, and bone. Calcif Tissue Int *49*: 407-415, 1991.

43 Carano A, Teitelbaum SL, Konsek JD, Schlesinger PH and Blair HC: Bisphosphonates directly inhibit the bone resorption activity of isolated avian osteoclasts *in vitro*. J Clin Invest *85*: 456-461, 1990.

44 Selander K, Lehenkari P and Vaananen HK: The effects of bisphosphonates on the resorption cycle of isolated osteoclasts. Calcif Tissue Int *55*: 368-375, 1994.

45 Felix R, Guenther HL and Fleisch H: The subcellular distribution of [14C] dichloromethylenebisphosphonate and [14C]1-hydroxyethylidene- 1,1-bisphosphonate in cultured calvaria cells. Calcif Tissue Int *36*: 108-113, 1984.

46 Endo Y, Nakamura M, Kikuchi T, Shinoda H, Takeda Y, Nitta Y and Kumagai K: Aminoalkylbisphosphonates, potent inhibitors of bone resorption, induce a prolonged stimulation of histamine synthesis and increase macrophages, granulocytes, and osteoclasts *in vivo*. Calcif Tissue Int *52*: 248–254, 1993.

47 Schenk R, Merz WA, Muhlbauer R, Russell RG and Fleisch H: Effect of ethane-1-hydroxy-1,1-diphosphonate (EHDP) and dichloro methylene diphosphonate (Cl2[scap]mDP) on the calcification and resorption of cartilage and bone in the tibial epiphysis and metaphysis of rats. Calcif Tissue Res *11*: 196–214, 1973.

48 Plasmans CM, Jap PHK, Kuijpers W and Slooff TJ: Influence of a diphosphonate on the cellular aspect of young bone tissue. Calcif Tissue Int *32*: 247-266, 1980.

49 Miller SC and Jee WS: The effect of dichloromethylene-diphosphonate, a pyrophosphate analog, on bone and bone cell structure in the growing rat. Anat Rec *193*: 439-462, 1979.

50 Apxicio A, Gxdner A, Tu Y, Savage A, Berenson J and Lichtenstein A: *In vitro* cytoreductive effects on multiple myeloma cells induced by bisphosphonates. Leukemia *12*: 220-229, 1998.

51 Derenne S, Amiot M, Barille S, Collette M, Robillard N, Berthaud P, Harousseau JL and Bataille R: Zoledronate is a potent inhibitor of myeloma cell growth and secretion of IL-6 and MMP-1 by the tumoral environment. J Bone Miner Res *14*: 2048-2056, 1999.

52 Hiraga T, Williams PJ, Mundy GR and Yoneda T: The bisphosphonate ibandronate promotes apoptosis in MDA-MB-231 human breast cancer cells in bone metastases. Cancer Res *61*: 4418-4424, 2001.

53 Fromigue O, Lagneaux L and Body JJ: Bisphosphonates induce breast cancer cell death *in vitro*. J Bone Miner Res *15*: 2211-2221, 2000.

54 Teronen O, Konttinen YT, Lindqvist C, Salo T, Ingman T, Lauhio A, Ding Y, Santavirta S, Valleala H and Sorsa T: Inhibition of metalloproteinase-1 by dichloromethylene bisphosphonate (clodronate). Calcif Tissue Int *61*: 59-61, 1997.

55 Teronen O, Heikkila P, Konttinen YT, Laitinen M, Salo T, Hanemaaijer R, Teronen A, Maisi P and Sorsa T: MMP inhibition and down-regulation by bisphosphonates. Ann N Y Acad Sci *878*: 453-465, 1999.

56 Boissier S, Ferreras M, Peyruchaud O, Magnetto S, Ebetino FH, Colombel M, Delmas P, Delaisse JM and Clezardin P: Bisphosphonates inhibit breast and prostate carcinoma cell invasion, an early event in the formation of bone metastases. Cancer Res *60*: 2949-2954, 2000.

57 Hultborn R, Gundersen S, Ryden S, Holmberg E, Carstensen J, Wallgren UB, Killany S, Andreassen L, Carlsson G, Fahl N, Hatschek T, Sommer HH, Hessman Y, Hornmark-Stenstam B, Johnsborg S, Klepp R, Laino R, Niklasson LG, Rudenstam CM, Sundbeck A, Soderberg M and Tejler G: Efficacy of pamidronate in breast cancer with bone metastases: a randomized, double-blind placebo-controlled multicenter study. Anticancer Res *19(4C)*: 3383-3392, 1999.

58 Strang P, Nilsson S, Brandstedt S, Sehlin J, Borghede G, Varenhorst E, Bandman U, Borck L, Englund G and Selin L: The analgesic efficacy of clodronate compared with placebo in patients with painful bone metastases from prostatic cancer. Anticancer Res *17(6D)*: 4717-4721, 1997.

59 Syrigos KN, Michalaki V, Mitromaras A, Pliarchopoulou F, Katirtzoglou N, Antonaki E and Roussou P: Safety and efficacy of the new bisphosphonate ibandronate in the management of bone metastasis following rapid infusion. In Vivo *16(5)*: 361-363, 2002.

60 Kanakis I, Nikolaou M, Pectasides D, Kiamouris C and Karamanos NK: Determination and biological relevance of serum cross-linked type I collagen N-telopeptide and bone-specific alkaline phosphatase in breast metastatic cancer. J Pharm Biomed Anal *34(4)*: 827-832, 2004.

61 Pectasides D, Farmakis D, Nikolaou M, Kanakis I, Kostopoulou V, Papaconstantinou I, Karamanos NK, Economopoulos T and Raptis S: Diagnostic value of bone remodeling markers in the diagnosis of bone metastases in patients with breast cancer. J Pharm Biomed Anal, *37 (1)*: 171-176, 2005.

62 Berenson JR, Vescio RA, Rosen LS, VonTeichert JM, Woo M, Swift R, Savage A, Givant E, Hupkes M, Harvey H and Lipton A: A phase I dose-ranging trial of monthly infusions of zoledronic acid for the treatment of osteolytic bone metastases. Clin Cancer Res *7(3)*: 478-485, 2001.

63 Berenson JR, Vescio R, Henick K, Nishikubo C, Rettig M, Swift RA, Conde F and Von Teichert JM: A Phase I, open label, dose ranging trial of intravenous bolus zoledronic acid, a novel bisphosphonate, in cancer patients with metastatic bone disease. Cancer *91(1)*: 144-54, 2001.

64 Rosen LS, Gordon D, Kaminski M, Howell A, Belch A, Mackey J, Apffelstaedt J, Hussein M, Coleman RE, Reitsma DJ, Seaman JJ, Chen BL and Ambros Y: Zoledronic acid *versus* pamidronate in the treatment of skeletal metastases in patients with breast cancer or osteolytic lesions of multiple myeloma: a phase III, double-blind, comparative trial. Cancer J *7(5)*: 377-87, 2001.

65 Saad F, Gleason DM, Murray R, Tchekmedyian S, Venner P, Lacombe L, Chin JL, Vinholes JJ, Goas JA and Chen B: A randomized, placebo-controlled trial of zoledronic acid in patients with hormone-refractory metastatic prostate carcinoma. J Natl Cancer Inst *94(19)*: 1458-1468, 2002.

66 Small EJ, Smith MR, Seaman JJ, Petrone S and Kowalski MO: Combined analysis of two multicenter, randomized, placebo-controlled studies of pamidronate disodium for the palliation of bone pain in men with metastatic prostate cancer. J Clin Oncol *21(23)*: 4277-4284,2003.

67 Elomaa I, Kylmala T, Tammela T, Viitanen J, Ottelin J, Ruutu M, Jauhiainen K, Ala-Opas M and Seppanen J: Effect of oral clodronate on bone pain. A controlled study in patients with metastic prostatic cancer. Int Urol Nephrol *24(2)*: 159-166, 1992.

68 Rosen L, Gordon D and Tchekmedyian S: Zoledronic acid significantly reduced the occurrence of skeletal related events (SREs) in patients with osteolytic bone metastases from non-small cell lung significantly reduced the occurrence of skeletal related events (SREs) in patients with osteolytic bone metastases from non-small cell lung cancer and other solid tumors. Presented at 3rd International Congress on Prevention and Early Detection of Lung Cancer, Reykjavik, Iceland, 9-12 August, 2001 [abstract].

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