Review

Antineoplastic Activity of Zoledronic Acid and Denosumab

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Abstract. *Cancer patients suffer from cancer-induced bone pain, hypercalcemia, and reduced quality of life caused by pathological fractures. Many of these complications related to cancer can be treated, or at least controlled, using new anticancer agents. Recently, two agents used initially to treat osteoporosis demonstrated direct and indirect anticancer activity. In this review, we summarize current knowledge about direct and indirect anticancer activity of zoledronic acid (a third-generation bisphosphonate), and denosumab antibody against RANKL. Zoledronic acid influences the proliferation and viability of tumor cells in vitro, and effectively reduces tumor burden, tumor-induced pain, and tumor growth in vivo. Denosumab is a fully human monoclonal antibody preventing the binding of RANKL to its receptor on osteoclasts' membrane, and through this mechanism inhibits the resorption of the bone. Furthermore, this agent demonstrates direct anticancer activity through the RANKL signaling pathway. Because of these features both drugs may gain broader application for the treatment of cancer patients. However, further pre-clinical and clinical evaluation is needed for both agents to fully assess the antineoplastic mechanisms of activity of both agents.*

Long bones are the most common site of spread of tumors such as breast, prostate, and kidney cancer (1-5) and hematological cancers such as multiple myeloma (6, 7). Patients suffering from these types of cancers experience cancer-induced bone pain, hypercalcemia, and loss of function from pathological fractures (8-10). All these complications related to cancer have a major influence on a patient's quality of life. There is, therefore, an emerging need to develop and introduce into clinical practice agents which could more effectively control tumor growth, and its burden in bone and distant sites. In recent years, two agents were introduced to the therapy of skeletal metastases. Besides inhibition of osteoclastogenesis and bone resorption, these two agents possess a direct antineoplastic activity. In this review we describe the direct and indirect effect of the *bis*phosphonate zoledrenic acid (ZA) and the (RANKL)-binding antibody denosumab on tumor growth.

ZA, a third-generation *bis*phosphonate, has been widely used to treat osteoporosis (11, 12), and skeletal metastases; recent reports also suggest direct activity against cancer cells (13-16). *Bis*phosphonates directly inhibit osteoclast activity through selective affinity to the site of increased bone turnover *e.g.* fracture sites. In addition, the latest studies demonstrated the induction of apoptosis of cancer cells *in vitro* in prostate cancer, breast cancer and multiple myeloma cell lines (17-20). Moreover, ZA successfully inhibits angiogenesis in the tumor environment, and reduces the invasive ability of cancer cells (21, 22), including the one of soft tissue tumors (13, 17, 23). ZA influences the proliferation and viability of tumor cells *in vitro*, and effectively reduces tumor burden, tumor-induced pain, and tumor growth *in vivo*. ZA may decrease tumor growth indirectly by inhibition of tumor-induced osteolysis in bone, and down-regulation of expression and secretion of cytokines and other growth factors in the tumor environment (24, 25). In addition, it reduces new blood vessel formation at the tumor site (26). ZA also exhibits a direct effect on cancer cells through induction of apoptosis, and reduction of migration and adhesion ability (17, 19).

Denosumab is an inhibitor of the receptor activator of nuclear factor-KB (RANK)/RANKL signaling pathway. It was developed using XenoMouse transgenic mouse technology (27). It is a fully human monoclonal antibody which prevents the binding of RANKL to its receptor on osteoclast membrane, and through this mechanism it inhibits the resorption of bone. Recent reports suggest the direct antitumor activity of this antibody (28, 29).

Mechanism of Action of Zoledronic Acid

*Bis*phosphonates accumulate in the mineralized bone matrix and are released during bone resorption. Bone has a very high affinity for *bis*phosphonates, this is possible only

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because of very specific uptake of these agents by activated osteoclasts (30). The nitrogen-containing *bis*phosphonates (N-BPs), which include ZA affect osteoclast activity and survival through inhibition of farnesyl diphosphonate (FPP) synthase, in the biosynthetic mevalonate pathway. This disrupts many cellular functions which are of great importance for osteolytic activity of the osteoclasts and their survival (31-33). Furthermore, ZA affects several signaling proteins such as: Rat sarcoma (RAS), Ras homology (RHO), and Ras-related C3 botulinum toxin substrate (RAC) involved in cytoskeleton and cellular motility (34-36). Selective inhibition of RAS signaling within osteoclasts causes disruption of intracellular vesicle transport, which reduces the ability of the cells to migrate and aggregate at the tumor bone border (30, 37). In addition, ZA may induce intracellular production of triphosphoric acid 1-adenosin-5'-yl ester 3-[3-methylbut-3-enyl] ester (ApppI), which can directly induce apoptosis (38, 39). This is induced by ZA by inhibition of farnesyl pyrophosphate synthase (FPPS), which causes down regulation of the mevalonate pathway, and secondary accumulation of isopentenyl pyrophosphate (IPP), which in turn, is conjugated to AMP to form a novel ATP analog (ApppI). ZA has a high potency at inhibiting FPPS activity and increase synthesis of ApppI in osteoclasts. Induction of synthesis of ApppI directly influences the proliferation, migration and apoptosis of osteoclasts, as well as tumor cells (39-42). Correlation between the osteolytic activity of osteoclasts and increased inhibition of FPPS was demonstrated *in vitro* and *in vivo* by Dunford *et al*. (43). Moreover, inhibition of signaling protein prenylation in cancer cells may produce the direct antineoplastic effect of ZA (Figure 1 a). In addition, ZA may directly influence the survival of cancer cells, by activation of caspases. ZA may directly enhance production of ATP analogs which interfere with mitochondrial ATP/ADP translocase. Treatment of MCF-7 breast cancer cells with ZA increased the release of cytochrome *c* from mitochondria and activation of caspase-3 which was associated with increased apoptosis (14, 44, 45). Furthermore, regulation of expression of B-cell lymphoma 2 (BCL-2) through ZA induces release of cytochrome *c* (46). Inhibition of activation of RAS signaling pathway may modulate both apoptotic pathways (47). ZA significantly inhibits the ability of tumor cells to invade and migrate into surrounding tissue. In *in vitro* experiments, ZA demonstrated dose-dependent reduction of invasion of tumor cells through the extracellular matrix. The inhibition of matrix metalloproteinase (MMP) activity may be one reason for this reduction in invasion ability of tumor cells (24, 48, 49). In addition, the induction of caspase activity and inhibition of RAS signaling pathway are very important mechanisms inhibiting tumor cell adhesion to extracellular matrix and affecting invasiveness. In some pre-clinical studies, ZA was found to have potent antiangiogenic effect. ZA exhibited *in vitro* dose-dependent inhibition of proliferation of human umbilical vein endothelial cells (HUVECs) (50, 51). Similar results have been observed in animal studies: in the 5T2 myeloma model, ZA significantly reduced tumor-associated angiogenesis. The antiangiogenic effect of ZA is mediated at least in part by modulation of $\alpha v\beta 3$ and $\alpha v\beta 5$ integrins, which are also required for osteoclasts for attachment to the bone at the resorption site (52). The integrins ($\alpha v\beta 3$) also play an important role in the metastatic ability of tumor cells (53-55). This suggests that ZA directly affects the ability of tumor cells to spread, invade and migrate, as well as directly inhibit adhesion and resorption ability of osteoclasts. Moreover, ZA down regulates the release of some pro-angiogenic growth factors, such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (BFGF) in patients with cancer (24, 56).

Mechanism of Action of Denosumab

Denosumab is a human monoclonal antibody possessing a very high affinity for RANKL. The monoclonal antibody can directly modulate the RANK/RANKL signaling pathway (57). This pathway is very important for maturation, activation, and survival of osteoclasts and tumor cells (Figure 1 b), and tumor cell ability to spread to distant sites (58-60). Specific binding of denosumab to RANKL prevents stimulation of RANK receptor, which is expressed on the surface of osteoclasts (61, 62). The balance between osteoclastic and osteoblastic activity is directly related to remodeling of bone and its resorption. The osteoclasts differentiate from monocyte-macrophage lineage, after maturation and final differentiation; they resorb bone in physiological and pathological states. Furthermore, some cytokines and growth factors such as RANKL modulate this process. RANKL is a tumor necrosis factor (TNF) family member released by activated T-cells (63). Binding of RANKL to RANK, expressed on osteoclasts and their precursors, increases their activity, migration and adhesion, and reduces apoptosis. This activation of osteoclasts causes osteolysis in bone. T-Cells and osteoblast lineage cells produce RANKL. The soluble and membrane-bound forms are produced by activated T-cells. The osteoblast lineage cells express RANKL on their membrane surface. Many cytokines, growth factors and hormones such 1,25(OH)2 vitamin D3, parathyroid hormone (PTH), TNF, and corticosteroids are involved in the modulation of the RANKL/RANK pathway (64-66). By binding to RANKL, denosumab neutralizes its action. Similar activity to denosumab is demonstrated by osteoprotegerin (OPG) (a decoy receptor for RANKL). Because of this, OPG has been widely used in animal models to study inhibition of RANKL. The reports showed that OPG can significantly reduce osteolysis in the bone (67-69).

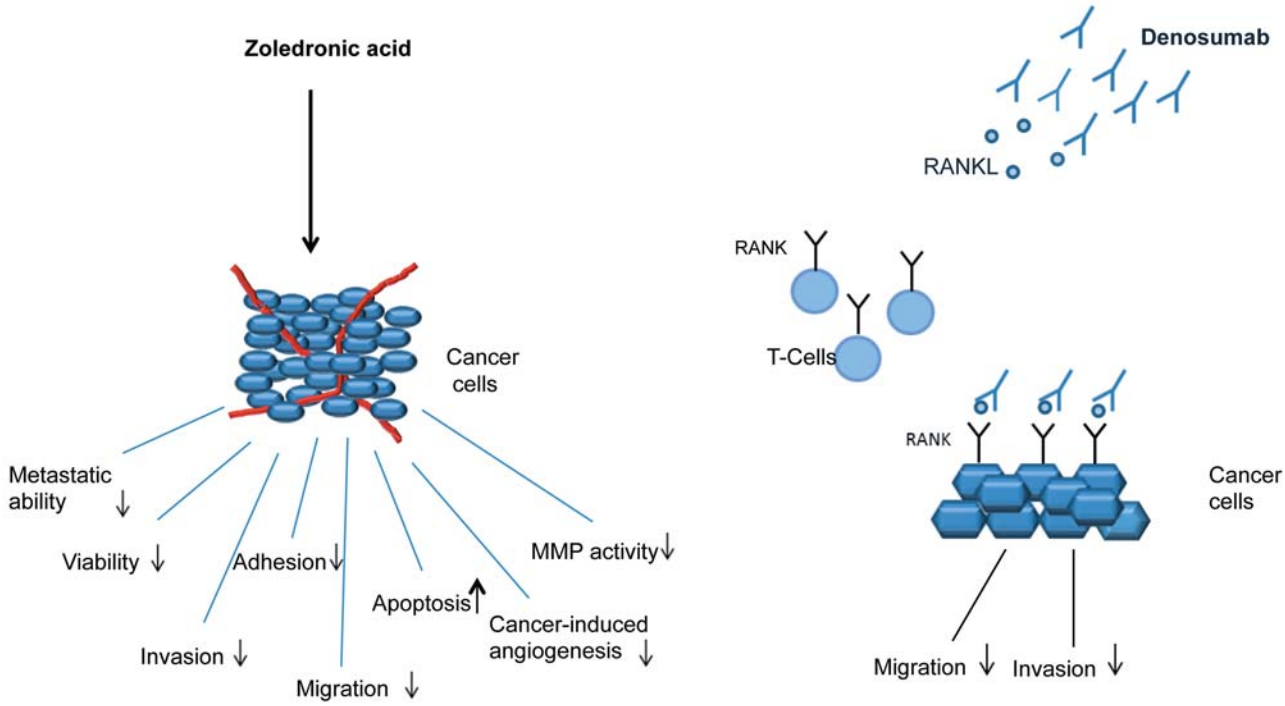

Figure 1. *Direct anticancer activity of zoledronic acid (a) and denosumab (b).*

In Vitro Studies of ZA

Recent reports suggest a direct antineoplastic activity of ZA on variety of tumor cells *e.g.* multiple myeloma, lung cancer, and renal cancer. Pre-clinical studies with MDA-MB-231 and MCF-7 breast cell lines demonstrated dose-dependent reduction of proliferation, and survival by treatment with ZA (14, 47). Moreover, breast cancer cells treated with ZA exhibited an increased apoptosis rate, which could, however, be reversed by geranylgeraniol, which suggests that inhibition of prenylation in cancer cells may induce apoptosis (70, 71). In addition, PC-3 prostate cancer cells treated with ZA exhibited significantly increased apoptosis and reduced proliferation *in vitro* (72). This suggests the direct antitumor activity of ZA against tumor cells in *in vitro* studies. In addition, many preclinical studies demonstrated additive or synergistic effect of ZA with other anticancer drugs *e.g.* paclitaxel, and doxorubicin (73-76).

In Vivo Models – Studying ZA

Pre-clinical data from animal studies support the antineoplastic effect of ZA observed in many *in vitro* studies. N-BPs very efficiently reduce osteolytic lesions in bone caused by tumor. In addition, they reduce formation of metastases and tumor-induced bone pain, and induce apoptosis of tumor cells (77-81). Some antineoplastic effect has also been observed in soft tissue and visceral models (13, 17, 82). Most of these experiments have been carried out with breast and prostate cancer. The activity of ZA on tumor cells was documented indirectly by reduction of tumor-induced pain, or by reduced bone osteolysis around tumor. Direct antitumor activity of ZA on tumor cells has been assessed using pathohistological analysis. A breast cancer model with MDA-MB-231 cells treated with ZA showed significantly reduced osteolytic lesions as compared to non-treated animals (83). Another study with 4T1 murine breast cancer cells treated with ZA at the time of inoculation of the tumor cells demonstrated significantly lower metastatic ability of the cells (84). In addition, the histomorphorogical analysis showed increased apoptosis of osteoclasts and tumor cells. Similarly to *in vitro* experiments, ZA demonstrated additive effect in combination with conventional anti-neoplastic drugs, *e.g.*, doxorubicin. Animal models carried out with prostate cancer PC-3 and LuCaP 23.1 reported an anti-neoplastic effect on tumor burden (85). After injection of these cells into tibia of mice and treatment with ZA either at the time of inoculation of tumor cells or after establishing the tumors, mice demonstrated fewer osteolytic lesions of tibia by x-ray analysis. Moreover, prostate-specific antigen in serum of LuCaP 23.1-bearing mice significantly decreased after treatment with ZA, which provides additional evidence of the direct antitumor activity of ZA. In some animal models ZA had a preventive effect in terms of metastatic ability of tumor

cells. Mice injected with PC-3 cells and treated with ZA had a significantly lower incidence of bone metastases (86). Some animal models suggested that ZA can inhibit development of visceral metastases. Animals injected with 4T1 murine breast cancer and treated with ZA demonstrated a lower tumor burden in bone and reduced the number of lung and liver metatstases (84). The presented data suggest that ZA can significantly reduce osteolytic lesions in bone, tumor burden in soft tissue, and also reduce development of metastases through direct and indirect antitumor activity.

In Vitro Activity of Denosumab

Recent reports interestingly demonstrated no antiangiogenic activity of denosumab in *in vitro* assays as compared to ZA (21). HUVECs treated with 0.31 to 160 μM of denosumab demonstrated no decrease in viability and no effect on invasion and tubule formation. In addition, the treatment did not affect the viability of MDA-MB-436 and CG5 breast cancer cells. Mice treated with 10 mg/kg of denosumab twice a week for four consecutive weeks did not show reduced angiogenesis or any effect on growth of xenograft in that model. This clearly demonstrates that ZA has an additional antitumor effect through antiangiogenic activity as compared to denosumab. There are some data available suggesting that RANKL can modulate the migration of different tumor cells (87, 88). Human breast cell lines (MDA-MB-231, MCF-7 and Hs578T) treated with RANKL demonstrated dose-dependent increased migration. This increase in migration was significantly reduced after treatment with OPG. In the same study, OPG treatment of colon cancer cells (Colo205), which did not express RANK, did not affect migration. Migration of B16F10 melanoma cells increased in a dose-dependent manner after stimulation with RANKL and was again significantly inhibited with OPG (87). In addition, colony-stimulating factor-1 (CSF-1) did not stimulate B16F10 migration in the presence of RANKL. This additionally provides evidence that RANK stimulation has a direct effect on tumor cells in the context of the significant role of CSF-1 in oncogenesis.

In Vivo Studies of Denosumab

In another study, high doses of OPG and ZA were used to inhibit the progression of the MDA-231-B/LUC + breast cancer cells (80). Histological and radiographic analysis demonstrated a significant decrease of osteolysis in bone. Interestingly, no (TRAP)-positive osteoclasts were identified in animals treated with Fc-OPG. However, OPG did not significantly affect tumor growth itself. Osteolysis of the bone caused by tumor growth is modulated by dysregulation of the RANK/RANKL pathway. Mice bearing MDA-MB-231 breast cancer cells expressed higher levels of RANKL than control mice (59). In addition, mice treated with increasing doses of OPG-Fc demonstrated significantly lower osteolysis of the tumor bone border. Furthermore, this treatment significantly inhibited growth of tumor cells in preventive as well as therapeutic modes. The OPG-increased activation of caspase-3, through this apoptosis mechanism, reduced tumor burden and tumor growth. Osteoclastogenesis induced by prostatic cancer CaP in an intratibial model treated with OPG (89), was significantly inhibited. In this study, the authors showed that CaP cells produce a soluble form of RANKL which can modulate osteoclastogenesis. The number of osteoclasts at the tumor bone border decreased after treatment with OPG, however, there was no effect on viability, and proliferation of CaP cells. This demonstrates how important the role of osteoclastogenesis and osteoclast activity is in the development and growth of skeletal metastasis. This study also presented evidence that OPG has no direct activity on tumor cells themself. CaP cells treated with OPG *in vitro*, as well as in subcutaneous models, did not demonstrate increased apoptosis or reduced viability. There is no correlation of data of RANKL level in blood and its impact on tumor growth in bone. This may be due to local release of RANKL at the tumor bone site, or by limitation of detection by currently used assays. Furthermore, RANK receptor is present on many epithelial tissues and epithelial cells, however, not much is known about its role in migration and development of bone metastases. *In vivo*, intracardiac injection of B16F10 cells into C57BL/6 mice resulted in rapid development of multiorgan metastases, including, of long bones. Treatment of the mice with OPG significantly reduced the tumor burden in bone; however, it did not affect tumor burden and metastases in ovaries, adrenal glands, and brain (87).

Clinical Trials

Denosumab in a double-blind, randomized study, phase III trial in breast cancer, prostate and multiple myeloma showed significantly better control of first skeletal events compared to ZA treatment. Furthermore, denosumab more frequently caused hypocalcemia, and did not demonstrate any advantages in terms of tumor progression and overall survival (90). Patients with giant cell tumors of the bone had significantly reduced tumor size. The histopathological analysis demonstrated a reduced number of RANK-positive giant tumor cells. Moreover, the treatment reduced the density of proliferative stromal tumor cells. In a phase II clinical trial, patients with recurrent or unresectable giant cell tumor in bone were treated with 120 mg every four weeks. The histopathological analysis demonstrated 90% decrease in the number of giant cells and significant reduction of tumor stromal cells (91). Male patients with bone metastases from castration-resistant prostate cancer were treated with denosumab at a dose of 120 mg on a month, and compared to a ZA treatment group. Denosumab resulted in delay or

prevention of skeletal-related events in patients with advanced prostate cancer (92). Similarly, in female patients with advanced breast cancer treated either with denosumab (120 mg) or ZA (4 mg), there was a longer delay of time to diagnosis of the first bone metastases with use of denosumab (93). Another preventive study compared the efficacy of denosumab in preventing development of skeletal metastases in patients with advanced cancer, bone tumor or myeloma, excluding breast and prostate cancer. The patients were treated with subcutaneous denosumab or intravenous ZA. The study demonstated that treatment with denosumab was equally good compared to that with ZA in delaying the diagnosis of the first metastasis, with fewer side-effects. There was also no difference between groups in terms of disease progression or survival (94). A randomized, double-blind, double-dummy phase III study, also compared denosumab with ZA for pain alleviation in patients with advanced breast cancer and bone metastases. The denosumab treatment group had significantly improved pain prevention, with similar pain alleviation to that of ZA; in addition, fewer patients in the denosumab group switched to opioid therapy (95).

Conclusion

Nowadays, patients with advanced cancer have longer survival, but have increased risk of bone-related events, such as pathological fractures, reduced ambulation and cancer-induced pain. Because of this, there is increasing need to develop new agents to reduce these complications and improve control of cancer growth in this population. In the past decade, ZA has been introduced into clinical practice as an antiosteoclastic agent. Many patients and physicians may prefer to use denosumab, because of its subcutaneous application compared to intravenous injections of ZA. Furthermore, aninitial evaluation showed that denosumab causes fewer complications related to treatment *e.g.* osteonecrosis of the jaw, and hypocalcemia. In addition, there are no limitations in the use of denosumab for patients with renal impairment. On the other hand, ZA demonstrates superiority in terms of its direct antineoplastic activity demonstrated both *in vitro* and *in vivo* compared to denosumab. Further pre-clinical and clinical evaluation is needed for both agents to fully assess the antineoplastic mechanisms of activity of both agents.

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