IBMS BoneKEy. 2010 June;7(6):208-217
http://www.bonekey-ibms.org/cgi/content/full/ibmske;7/6/208
doi: 10.1138/20100450

PERSPECTIVES

Nitrogen-Containing Bisphosphonates and Human γδ T Cells

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Abstract

Bisphosphonates, especially nitrogen-containing bisphosphonates (N-BPs), are widely used to block bone destruction associated with bone metastasis because they are effective inhibitors of osteoclast-mediated bone resorption. In addition to their antiresorptive activity, growing preclinical evidence shows that N-BPs have direct and indirect anti-tumor activities. Some of the anti-tumor activities of N-BPs are associated with human γδ T cells that are key players in the interface between innate and adaptive immunity. This review examines the molecular and cellular mechanisms through which N-BPs stimulate the expansion and anti-tumor activity of human γδ T cells, suggesting a new role for N-BPs in cancer immunotherapy. *IBMS BoneKEy*. 2010 June;7(6):208-217.

Keywords: bisphosphonate; γδ T cell; immunotherapy; bone metastasis; IPP

Introduction

Bisphosphonates are synthetic analogues of the naturally occurring pyrophosphate molecule that have the ability to bind to bone mineral and inhibit osteoclast-mediated bone resorption (1). Bisphosphonates may be broadly classified on the basis of whether or not they contain a nitrogen atom, with nitrogen-containing bisphosphonates (N-BPs) being more potent than non N-BPs at inhibiting osteoclast activity (1). Bisphosphonates that lack a nitrogen atom are metabolized into non-hydrolyzable pro-apoptotic ATP analogues that accumulate in the cytosol of osteoclasts (1). By contrast, N-BPs interfere with a specific enzyme in the mevalonate pathway, farnesyl pyrophosphate synthase (FPPS), thereby depleting the osteoclasts of isoprenoid lipids (2-5). More specifically, FPPS inhibition by N-BPs blocks the covalent attachment of isoprenyl chains to small GTPases (*e.g.*, Ras, Rac, Rho, and CDC42), which is crucial for their intracellular localization and functions in osteoclasts. In addition to the effects on the function of small GTPases, the disruption of the mevalonate pathway by N-BPs results in the accumulation of isopentenyl pyrophosphate (IPP), which is then converted (most probably via aminoacyl tRNA-synthase) to a cytotoxic adenosine triphosphate analogue (ApppI) that can directly induce osteoclast apoptosis (6,7). Thus, N-BPs may exert their pharmacological effects on osteoclasts through the formation of ApppI or via the inhibition of protein prenylation, particularly of small GTPases.

N-BPs also exert indirect and direct anticancer activities by interacting with monocytes, macrophages, endothelial and tumor cells (8). In addition, they specifically stimulate the expansion and antitumor activity of a subset of human γδ T cells (referred to as Vγ9Vδ2 or Vγ2Vδ2 T cells), which are strongly activated by natural phosphoantigens from bacteria, parasites and eukaryotic cells (8;9). In this review, we focus on the cellular and molecular mechanisms through which N-BPs stimulate the expansion and cytotoxic activity of human Vγ9Vδ2 T cells and discuss the preclinical evidence that N-BPs may have a role in cancer immunotherapy.

Activation of Human Vγ9Vδ2 T Cells by Phosphoantigens

The immune system can be divided into innate and adaptive immunity. The innate immune system serves as the first line of host defense against infection and involves non-specific guardians (*e.g.*, macrophages, dendritic cells, natural killer cells, γδ T cells) (9). Then, the adaptive immune system, mediated by $CD4^+$ and $CD8^+$ αβ T cells and B cells, provides antigen-specific lasting immunity (*e.g.*, antibody-producing and antigen-targeted cytotoxic cells) (9).

Human γδ T cells are cytotoxic $CD3^+CD4^-CD8^-$ lymphocytes that are at the interface between innate and adaptive immunity (9;10). They exhibit features of the innate immune system because, unlike αβ T cells, antigen recognition by the γδ T cell receptor (TCR) is not constrained by the requirement to bind major histocompatibility complex (MHC), thereby allowing γδ T cells to recognize a wide range of nonpeptide phosphoantigens (9;10). Conversely, the ability of γδ T cells to undergo major clonal expansion in primary infection and to mount rapid memory-type expansion upon reinfection is a feature of the adaptive immune system (10). The expression of TCR variable segments in γδ T cells is associated with tissue prevalence. For example, 70% of the human γδ T cells that circulate in the blood and reside in lymphoid organs express the Vγ9Vδ2 TCR, whereas γδ T cells residing in the skin, gut and lungs express the Vδ1 segment and different Vγ segments (9;10).

Circulating Vγ9Vδ2 T cells represent 1 to 5% of whole peripheral blood T cells and they exist only in primates and humans. They recognize non-peptide phosphorylated intermediates of bacterial isoprenoid biosynthesis (*i.e.*, (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate [HMBPP]) and phosphorylated metabolites of the mevalonate pathway in eukaryotic cells (IPP, dimethylallyl pyrophosphate [DMAPP], farnesyl pyrophosphate [FPP] and geranylgeranyl pyrophosphate [GGPP]) (9;11). Synthetic antigens such as Phosphostim (BrHPP) are also recognized by Vγ9Vδ2 T cells (11). HMBPP is very similar in structure to IPP, but is much more potent in stimulating proliferation of Vγ9Vδ2 T cells (half-maximal concentration for stimulation of proliferation are 0.00032 and 1 μM, respectively) (11). Other mevalonate metabolites (DMAPP, FPP, GGPP) have considerably lower potency (30- to 300-fold less than IPP) (11). The mevalonate metabolite Apppl has little stimulatory activity on Vγ9Vδ2 T cells; it could represent an inactive storage form of phosphoantigen that would require conversion to IPP to activate γδ T cells (12). Thus, phosphorylated mevalonate metabolites activate Vγ9Vδ2 T cells only at high concentrations. However, certain tumors do produce, under basal conditions, elevated endogenous concentrations of IPP (*e.g.*, the B cell lymphoma cell line Daudi), which can then be sensed by Vγ9Vδ2 T cells as a tumor antigen (9;11).

The exact mechanisms through which Vγ9Vδ2 T cells become activated by IPP are still unclear, but are most likely γδ TCR-mediated and require cell-cell contacts. Following activation by IPP or other phosphoantigens, Vγ9Vδ2 T cells produce pro-inflammatory chemokines (*e.g.*, MIP1α, RANTES), interleukins (*e.g.*, GM-CSF, TGF-β), Th1 (*e.g.*, INF-γ, TNF-α) but not Th2 cytokines (*e.g.*, IL4, IL5) (13;14), and finally proliferate in the presence of IL2 (9-11). In addition, these cytokines secreted from activated γδ T cells are able to regulate and stimulate other immune cells. For example, Ismaili *et al.* (15) demonstrated that γδ T cell-derived TNF-α induced the maturation of dendritic cells from monocytes. Human γδ T cells could also function to help initiate adaptive αβ T cell responses by antigen presentation (11) and B cells for antibody production (13). Moreover, Vγ9Vδ2 T cells activated by the synthetic phosphoantigen Picostim antagonize IL2-induced expansion of Foxp3+ T regulatory cells (Tregs) (16). These findings (16) are of importance because Tregs play a role in mediating a balance between immunity and tolerance; they suppress the activation, proliferation

and functions of various immune cells (17). More specifically, Tregs inhibit anticancer immune responses (17) and, for example, the proliferation of human γδ T cells induced by Phosphostim plus IL-2 can be suppressed by Tregs (18). It remains to be determined whether N-BPs, by promoting activation of Vγ9Vδ2 T cells through IPP production, could counteract Tregs' activity, thereby facilitating cancer immunity.

Activation of Human Vγ9Vδ2 T Cells by N-BPs

Evidence for the stimulation of Vγ9Vδ2 T cells by N-BPs was first found when increased numbers of γδ T cells were observed in patients who had flu-like acute-phase reactions after their first intravenous infusion of pamidronate (19). N-BPs might induce or activate Vγ9Vδ2 T cells either by mimicking phosphoantigens and/or by increasing phosphoantigen levels. For example, N-BPs (pamidronate, risedronate, zoledronate), in the presence of low doses of IL-2, can activate and stimulate the proliferation of human Vγ9Vδ2 T cells *in vitro* and *in vivo* (11;20). Furthermore, zoledronate induces functional changes in Vγ9Vδ2 T cell subsets (8;21). *In vivo*, it promotes the differentiation of Vγ9Vδ2 T cells toward $CD45RA^{-}CD27^{-}$ γδ T cells, which produce interferon-γ and exert cytotoxicity, while decreasing $CD45RA^{+}$ $CD27^{+}$ naive and $CD45RA^{-}CD27^{+}$ memory γδ T cells (8;21). This effect is specific to Vγ9Vδ2 T cells. Neither human γδ T cells expressing the Vγ9Vδ1 TCR, nor human αβ T cells, monocytes, NK or B cells are responsive to N-BPs (11;21). These results suggested a direct binding of N-BPs to the TCR expressed by human Vγ9Vδ2 T cells. However, it has since become clear that the activation of human γδ T cells by N-BPs requires both antigen-presenting cells (monocytes, dendritic cells) and the inhibition of the mevalonate pathway (11;21). For instance, zoledronic acid promotes the immunostimulatory properties of human dendritic cells by enhancing their ability to activate γδ T cells (22). Similarly, pamidronate-treated, but not untreated, THP-1 monocytic cells are capable of activating purified γδ T cells to produce interferon-γ (23). In addition, pamidronate-treated THP-1 cells activate TCR-defective Jurkat cells only when these cells are stably transfected to express the Vγ9Vδ2 TCR (23;24). These results (23;24) suggest therefore that N-BP-treated antigen-presenting cells (as exemplified by THP-1 cells) could activate γδ T cells in a TCR-dependent manner. Because N-BPs specifically inhibit the IPP-consuming enzyme FPPS, IPP could then be directly recognized by γδ T cells. For example, zoledronic acid induces the accumulation of IPP in monocytes from human peripheral blood mononuclear cells (PBMCs) which, in turn, activates the expansion of γδ T cells in a cell contact-dependent manner (25). In addition, the expansion of γδ T cells from PBMCs treated with a N-BP is prevented by statins, which inhibit HMG-CoA reductase upstream of FPP synthase and prevent the synthesis of IPP (26). Thus, the internalization of N-BPs by monocytes and dendritic cells, which are highly endocytic cells, leads to the inhibition of the mevalonate pathway and subsequent intracellular accumulation of IPP which, in turn, activates γδ T cells. Mechanisms through which mevalonate metabolites (such as IPP) are cell-surface exposed and recognized by the Vγ9Vδ2 TCR are, however, still unknown. Attempts to co-crystallize IPP (or HMBPP) with the Vγ9Vδ2 TCR have not succeeded (27). However, it has been shown that there exists a protein-associated membrane component on the antigen-presenting cell surface that presents HMBPP to the Vγ9Vδ2 TCR for immune recognition (28). It is therefore most conceivable that IPP may also be complexed with a cell surface antigen-presenting molecule in order to be recognized by the Vγ9Vδ2 TCR.

Preclinical Evidence for a Role of N-BPs in Promoting Cancer Immunotherapy

Most of the human tumor cell lines treated with a N-BP can efficiently activate human γδ T cells to proliferate and lyse tumor cells in a γδ TCR-dependent manner (24). In

contrast, tumor cell lines of nonhuman origins treated with a N-BP fail to activate human Vγ9Vδ2 T cells, indicating species-specific cell-cell interactions (29). These findings (29) are in line with the observation that nonhuman antigen-presenting cells do not have a protein-associated membrane component that presents HMBPP for recognition by the Vγ9Vδ2 TCR (28).

N-BPs induce intracellular accumulation of IPP/ApppI in a wide variety of human tumor cell lines (30;31) and these mevalonate metabolites could be sensed by Vγ9Vδ2 T cells as tumor phosphoantigens. This contention is supported by the observation that the silencing of FPPS protein expression by a shRNA in Raji and HepG2 tumor cells converts these cells into Vγ9Vδ2 T cell activators (32), presumably because of the higher intracellular IPP levels. In addition, mevastatin and lovastatin (HMG-CoA reductase inhibitors that prevent the synthesis of IPP) completely abolish Vγ9Vδ2 T cell activation induced by zoledronate- or pamidronate-treated tumor cells (Daudi lymphoma, K562 leukemia, KMM1 myeloma and colon carcinoma cell lines) (12;33-35). Thus, tumor cells that are treated with N-BPs overproduce mevalonate metabolites (such as IPP) that are somehow sensed by Vγ9Vδ2 T cells as tumor antigens, causing their activation and then the efficient killing of bisphosphonate-treated tumor cells. As aforementioned for HMBPP (28), it is most unlikely that IPP directly binds to the Vγ9Vδ2 TCR. Instead, IPP (or ApppI) may be complexed with an antigen-presenting molecule in order to be recognized by the Vγ9Vδ2 TCR (11). In this respect, the Vγ9Vδ2 TCR binds to a complex formed between apolipoprotein A1 and F1-ATP synthase (AS), which is a mitochondrial enzyme that is translocated to the cell surface of tumor cells (36). Interestingly, ApppI inhibits a mitochondrial ADP/ATP translocase (6), suggesting that ApppI might also bind to AS in order to be recognized by the Vγ9Vδ2 TCR (36). It has also been proposed that ApppI could represent an inactive storage form of phosphoantigen that, when exposed at the tumor cell surface, would require hydrolysis by some ecto-nucleotide pyrophosphatases for conversion into IPP (12). Thus, upon treatment of tumor cells with a N-BP, ApppI could be translocated to the tumor cell surface as a complex with a putative antigen-presenting molecule and then processed into IPP for its subsequent recognition by the Vγ9Vδ2 TCR. Although highly speculative, this hypothesis clearly warrants further investigation.

Other cell surface receptors have been involved in mediating cell-cell interactions between Vγ9Vδ2 T cells and N-BP-treated tumor cells (Table 1). For example, LFA1 expressed on γδ T cells mediates a stable interaction with pamidronate- or zoledronate-treated tumor cells expressing ICAM-1 (29;37). However, relatively limited types of tumor cells express these molecules. Vγ9Vδ2 T cells also express the NKG2D receptor, a type II C-lectin-like protein that is expressed by NK cells. NKG2D interacts with MHC class I-related chains A/B (MICA/MICB) and the UL-16 binding proteins 1 to 4 (ULBP1-4) that are frequently expressed by tumor cells (colon and renal carcinomas, myelomas, lymphomas) and thus can contribute to the efficient killing of N-BP-treated tumor cells by activated Vγ9Vδ2 T cells (8;11). The engagement of CD6 on human γδ T cells by CD166 on human tumor cells also seems to play an important role in bisphosphonate-mediated γδ T-cell activation (38). Expression of CD166 has been described in malignant melanoma and various carcinomas (breast, prostate, lung, colon, and bladder) (8). Its *de novo* expression in CD166-negative K562 leukemia cells markedly enhances the activation of γδ T cells following pamidronate treatment of CD166-expressing K562 cells (38). Conversely, the silencing of CD166 in LK-2 lung carcinoma cells treated with pamidronate decreases γδ T cell activation (38). Thus, upon treatment of tumor cells with N-BPs, CD6/CD166 and NKG2D/MICA/B interactions could provide costimulatory signals for TCR-mediated γδ T cell activation (Table 1). In addition, it has been reported that only IPP-activated γδ T cells expressing CD56 can efficiently kill

Table 1. Contribution of cell surface receptors in activated human Vγ9Vδ2 T cells to recognition of bisphosphonate-treated tumor cells.

γδ T cell surface receptor	Tumor cell surface ligand(s)	Ref.
γ9δ2 TCR	F1-ATP synthase, apolipoprotein A1	(36)
NKG2D	MICA, MICB, ULBP 1-4	(8;11)
LFA1	ICAM-1	(29;37)
CD6	CD166	(38)
CD56	unidentified	(39)

tumor cells because they secrete increased amounts of cytolytic granules (granzyme B, perforin) when compared to CD56-negative γδ T cells (39) (Table 1).

In vivo experimental studies demonstrate that zoledronate significantly enhances the anti-tumor activity of purified human Vγ9Vδ2 T cells, which have been transferred into immunodeficient mice xenografted with SBC-5 small cell lung carcinoma cells (40), UM-UC-3 bladder cancer cells (41) or MM1 chronic myelogenous leukemic cells (42). Similarly, upon transfer into SCID mice, purified human Vγ9Vδ2 T cells given together with alendronate plus IL-2 significantly prolong the survival of animals bearing MeWo melanoma cells or PancTu1 pancreatic carcinoma cells (43). Importantly, Vγ9Vδ2 T cells can be expanded from PBMCs of patients with cancer (chronic myeloid leukemia, multiple myeloma, breast and prostate carcinomas), following treatment with zoledronate (or Phosphostim) plus IL-2 (34;42;44-47). These cells exhibit potent anti-tumor activity *in vitro* against human cancer cell lines (33;34;44-46). In addition, Vγ9Vδ2 T cells expanded from PBMCs of patients with chronic myeloid leukemia also exhibit antitumor activity *in vitro* against autologous or allogeneic, zoledronate-treated leukemia cells isolated from patients with chronic myeloid leukemia (42).

Furthermore, *in vitro* experiments showed that zoledronate enhances the chemotherapy-induced sensitization of different tumor cell lines to Vγ9Vδ2 T cell cytotoxicity (44). It also sensitizes human colon cancer stem cells (which are more resistant to chemotherapy) and imatinib-resistant chronic myeloid leukemic cell lines to Vγ9Vδ2 T cell-mediated killing *in vitro* (35;42).

Concluding Comments and Future Directions

Studies on the effect of N-BPs on human γδT cells have revealed a previously unappreciated approach to exploit the antitumor potential of bisphosphonates. These studies have shown that by blocking FPPS activity with N-BPs it is possible to cause intracellular accumulation of IPP/ApppI both in tumor cells and antigen-presenting cells (monocytes, dendritic cells), thereby leading to Vγ9Vδ2 T cell antitumor activity. Future work should aim at characterizing molecular mechanisms responsible for the cell surface exposure and recognition of IPP/ApppI by the Vγ9Vδ2 TCR. However, these preclinical studies already suggest that N-BPs could be used as a promising immunotherapeutic approach for Vγ9Vδ2 T cell activation. Two strategies for the potential usage of γδT cells in cancer immunotherapy are currently under clinical investigation: (1) the adoptive transfer of *ex vivo* expanded autologous Vγ9Vδ2 T cells and (2) the *in vivo* activation of Vγ9Vδ2 T cells. Abe *et al*. (48) conducted a phase I clinical trial in multiple myeloma patients using adoptive transfer of autologous

Table 2. Therapeutic activation of Vγ9Vδ2 T cells by bisphosphonates in early clinical trials

Cancer type	Treatment	Observations	Ref.
Multiple myeloma	zoledronate + IL-2	Study in 52 patients. Induction of γδ T cell effector functions in half of the patients.	(34)
Low-grade non-Hodgkin lymphoma	pamidronate + IL-2	Antitumor activity was noted when patients who responded to pamidronate *in vitro* were treated (50% of patients). In this respect, a significant *in vivo* activation of γδ T cells and objective clinical responses in 5 out of 9 patients were reported.	(50)
Breast carcinoma	zoledronate	Study in 23 patients. A single dose of zoledronate induces a long-lasting activation of γδ T cells.	(49)
Prostate carcinoma	zoledronate + IL-2	Study in 18 patients. Induction of γδ T cell effector functions and improvement of clinical responses.	(47)

Vγ9Vδ2 T cells generated from PBMCs after *ex vivo* incubation with zoledronate plus IL-2. The authors reported an increased number of CD45RA-CD27- effector memory cells in the blood and bone marrow from these patients, 4 weeks after initiation of treatment (48). Regarding the second immunotherapeutic strategy that consists of the *in vivo* activation of γδ T cells, a study showed that treatment of breast cancer patients with zoledronate without IL-2 led to an increase in the percentage of effector Vγ9Vδ2 T cells in blood (49). Moreover, early clinical trials with N-BPs (pamidronate, zoledronate) plus IL-2 have been performed in patients with multiple myeloma (34), prostate carcinoma (47) and lymphoma (50), and data showed a significant expansion of Vγ9Vδ2 T cells in several cancer patients and even good clinical responses in some prostate cancer patients (Table 2). These results suggest, therefore, that there is an interest in (and rationale for) using N-BPs for *ex vivo* or *in vivo* activation of Vγ9Vδ2 T cells. However, about half of the patients enrolled in these clinical trials failed to expand their Vγ9Vδ2 T cells after treatment with a N-BP plus IL-2 (Table 2). This kind of γδ T cell anergy is frequently observed in cancer patients (34;50). It might be related to the negative effect of Tregs on Vγ9Vδ2 T cell expansion (9;17;18). Alternatively, there might be a progressive deterioration of Vγ9Vδ2 T cell immunity along with disease progression. Future challenges will be, therefore, to optimize immunotherapeutic protocols that both target Vγ9Vδ2 T cell expansion and overcome γδ T cell anergy. Combination approaches that associate Vγ9Vδ2 T cell-based immunotherapy with chemotherapy might be another promising therapeutic strategy to sensitize tumor cells to Vγ9Vδ2 T cell cytotoxicity.

Conflict of Interest: Dr. Clézardin reports that he has served on advisory boards for Novartis, and has received lecture fees and research support from Novartis and Procter & Gamble Pharmaceuticals. Dr. Benzaid: none reported.

Peer Review: This article has been peer-reviewed.

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