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PERSPECTIVES

Bisphosphonates and γδ T-Cells: New Insights into Old Drugs

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Abstract

Bisphosphonates (BPs) are now well established as front-line treatments for post-menopausal osteoporosis, Paget's disease and tumor-associated osteolysis and hypercalcemia. Since the basic pharmacology and mechanisms of action of these bone-targeting agents have been reviewed in detail elsewhere (1), we summarize new insights into their mode of inhibition of FPP synthase, concepts of their anti-resorptive effects on osteoclasts and, in particular, discuss their recently-discovered effects on γδ T-cells and prospects for their future use as immune therapies. *BoneKEy-Osteovision*. 2006 August;3(8):5-14.

Current Concepts of Cellular Uptake and Molecular Actions in Osteoclasts

Because of the ability of BPs to bind avidly to bone mineral, approximately 50% of a circulating dose of BP binds to the skeleton. Recent studies with fluorescently labelled BPs have shown that after release of BPs into the acidic environment of the osteoclastic resorption lacuna, osteoclasts internalize BPs, probably as microprecipitates with Ca^{2+}, into membrane-bound vesicles by fluid-phase endocytosis (2;3). In addition to enabling release from the mineral surface, the acidifying activity of H^+-ATPases is also essential for the uptake of BPs into the cytosol of osteoclasts (2;3), probably since acidification of endocytic vesicles reduces the negative charge on the phosphonate groups of BPs, allowing dissociation from Ca^{2+} ions and subsequent diffusion or transport of BPs across the vesicular membrane (2).

Once internalized by osteoclasts, BPs can be separated into two groups according to their intracellular molecular mechanism of action. The simple BPs that most closely resemble pyrophosphate, such as clodronate and etidronate, are metabolized into methylene-containing analogues of ATP by cytosolic aminoacyl-tRNA synthetase enzymes, with the non-hydrolyzable P-C-P group of the BP replacing P-O-P (i.e. the β,γ-phosphate groups) in ATP (4). These AppCp-type metabolites accumulate in the osteoclast cytosol and induce apoptosis, thereby inhibiting bone resorption (1). Nitrogen-containing bisphosphonates (N-BPs), however, are not metabolized. Rather, N-BPs act by inhibiting farnesyl diphosphate synthase (FPP synthase), a peroxisomal enzyme of the mevalonate pathway (1). The recent generation of X-ray crystal structures of human FPP synthase co-crystallized with N-BPs (5;6), together with detailed kinetic analysis of the recombinant human enzyme, indicates that the interaction with N-BPs is highly complex and characteristic of 'slow-tight binding' inhibition (5). Initially, N-BPs appear to compete directly for binding with the isoprenoid lipid substrates DMAPP or GPP, forming interactions between the nitrogen moiety of the N-BP and a conserved threonine and lysine residue in the enzyme. This is associated with complex conformational changes in the enzyme that promote the binding of the second substrate, IPP, which itself stabilizes the final ternary, "locked" complex of N-BP-bound enzyme, helping to explain why some N-BPs, such as zoledronate, are such potent inhibitors of this enzyme (5;7).

Inhibition or Activation of Small GTPases?

Inhibition of FPP synthase prevents the synthesis of the isoprenoid lipids FPP and geranylgeranyl diphosphate (GGPP), which are required for the post-translational prenylation of small GTPases such as those

of the Ras, Rho and Rab families. Since prenylation is required for the localization of these GTPases to subcellular membranes, N-BPs inhibit resorption by disrupting the normal localization and function of small GTPases that are essential for osteoclast activity and survival (1). It has been generally assumed that N-BPs, by preventing protein prenylation, disrupt the membrane interaction of small GTPases and thereby inhibit downstream signaling pathways. Surprisingly, we demonstrated recently that the unprenylated forms of Rho, Rac and Cdc42 GTPases that accumulate in osteoclasts and macrophages following treatment with N-BPs are in the active GTP-bound form (8). Although, in the absence of prenylation, unprenylated small GTPases are mislocalized and would therefore not be expected to be able to activate downstream signaling pathways, N-BPs cause sustained activation of p38 downstream of Rac, at least in macrophages (8). These observations suggest that N-BPs, rather than causing the loss of pre-existing, prenylated small GTPases and hence loss of downstream signaling in osteoclasts, may inhibit osteoclast function at least in part by causing the accumulation of the unprenylated form of small GTPases. This accumulation would result in inappropriate activation of some downstream signaling pathways, and/or a dominant negative effect on small GTPase signaling due to the formation of non-productive, cytoplasmic complexes of unprenylated GTPases with effector proteins. Further studies are required to determine which mechanisms occur in osteoclasts and in other cell types that might be affected by N-BPs *in vivo*, such as tumor cells (9).

The Acute Phase Reaction to Bisphosphonates

The profound anti-resorptive effect of a once-yearly infusion of zoledronic acid (10), or more frequent infusions of ibandronate (11), indicates that the intravenous administration of BPs is an attractive therapeutic alternative to oral administration. The most common adverse-effect of intravenous N-BP administration, which is not generally observed with oral dosing, is the development of a flu-like syndrome called the acute-phase reaction (12) that typically occurs in one-third of patients receiving intravenous N-BPs for the first time. While this syndrome was first described nearly 20 years ago, the exact molecular mechanism underlying this adverse effect has only recently been elucidated. In a seminal study by Kunzmann *et al.* in 1999, the authors reported that patients that suffered an acute-phase reaction to pamidronate had increased levels of circulating γδ T-cells up to 28 days following the initial infusion (13). γδ T-cells are non-conventional T-cells that, unlike conventional αβ T-cells, can recognize antigen without the need for presentation by MHC-class molecules. In humans, γδ T-cells comprise only a minor proportion (1-10%) of $CD3^+$ T-cells in peripheral blood, and the majority (50-90%) of these γδ T-cells belong to the $V\gamma9V\delta2^+$ subset (also termed $V\gamma2V\delta2^+$). $V\gamma9V\delta2^+$ T-cells are activated by a diverse array of non-peptide molecules, such as pyrophosphomonoesters (IPP, DMAPP) (14) and the synthetic agonist bromohydrin pyrophosphate (BrHPP) (15), alkylamines (16), and N-BPs (13;17). Since N-BPs closely resemble isoprenoid pyrophosphates such as IPP, DMAPP and GPP (which is why N-BPs such as pamidronate and zoledronate inhibit FPP synthase) (5;6;18), this activation of $V\gamma9V\delta2^+$ T-cells by N-BPs was initially thought to occur by a direct agonistic effect of the N-BP on the $V\gamma9V\delta2^+$ T-cell receptor (TCR). However, it is now clear that inhibition of FPP synthase is responsible for the activation of $V\gamma9V\delta2^+$ T-cells by N-BPs.

Following an intravenous infusion of N-BP, the plasma concentration (approximately 10^{-6}M for zoledronic acid) (19) is probably sufficiently high to allow the internalization of N-BP into endocytic cells such as monocytes (20). The subsequent inhibition of FPP synthase causes intracellular accumulation of the substrates of this enzyme (IPP and DMAPP) (21-23), which are known agonists of the $V\gamma9V\delta2^+$ TCR (14). This intracellular IPP is then somehow 'presented' to $V\gamma9V\delta2^+$ T-cells in the peripheral circulation (by an unknown mechanism), thereby causing their activation (13;17) and the release of the pro-inflammatory cytokines TNFα and IFNγ characteristic of the acute-phase reaction (24-26) (See Figure 1). This hypothesis is supported by the finding that N-BPs must be internalized in order to stimulate $V\gamma9V\delta2^+$ T-cells *in vitro* (21), and that the ability of N-

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http://www.bonekey-ibms.org/cgi/content/full/ibmske;3/8/5
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BPs to activate Vγ9Vδ2$^+$ T-cells closely matches the potency of the N-BP for inhibiting FPP synthase (22). We have recently shown that dietary alkylamines, which are found in numerous edible plants and plant products, also *indirectly* activate Vγ9Vδ2$^+$ T-cells through a similar mechanism to N-BPs (i.e. inhibition of FPP synthase and the accumulation of IPP) (27). Furthermore, statins can completely prevent the stimulatory effect of N-BPs on Vγ9Vδ2$^+$ T-cells *in vitro* (21-23) by preventing the accumulation of IPP through inhibition of HMG-CoA reductase, an enzyme upstream of IPP synthesis. This raises the intriguing possibility that statins could be used to prevent the acute-phase reaction to N-BPs *in vivo* (22;23).

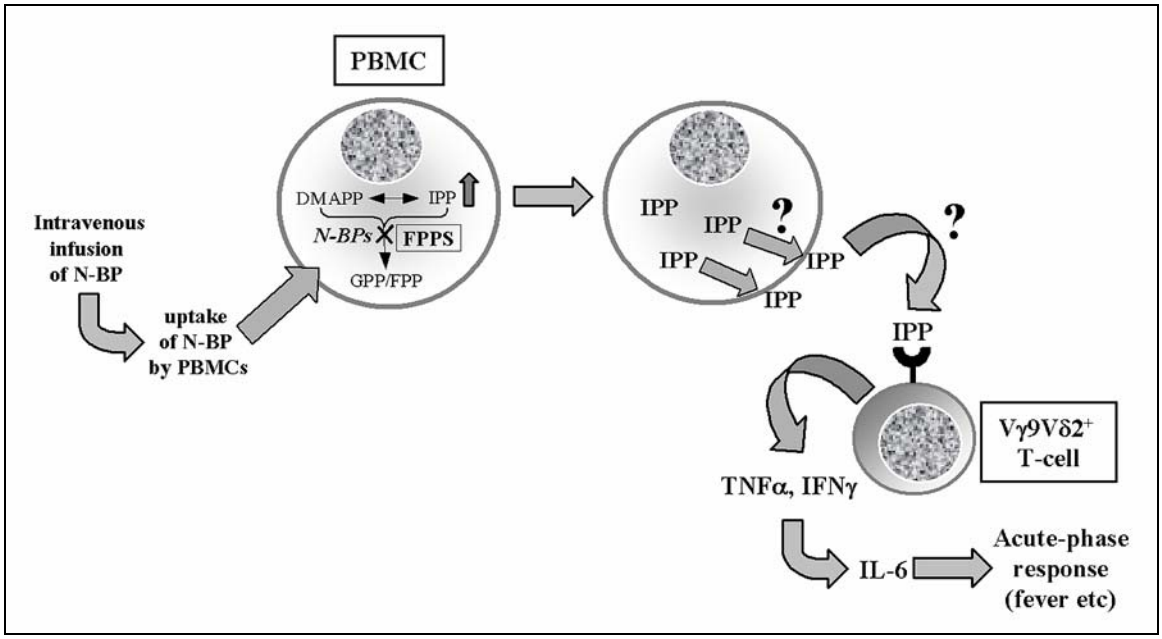

Figure 1. Current model of the molecular mechanism underlying the acute-phase reaction to N-BPs *in vivo*. Following an intravenous administration of N-BP, endocytic cells such as monocytes internalise N-BP, resulting in the inhibition of FPP synthase and the accumulation of IPP/DMAPP. IPP/DMAPP is then presented to Vγ9Vδ2$^+$ T-cells by an as yet unidentified mechanism, causing their activation and the release of pro-inflammatory cytokines such as TNFα and IFNγ, characteristic of the acute-phase reaction *in vivo*.

In accord with the above observations that N-BPs activate Vγ9Vδ2$^+$ T-cells indirectly, a recent report by Hewitt *et al.* suggests that the acute-phase reaction to N-BPs differs from a classical acute-phase response in that the Vγ9Vδ2$^+$ T-cells, rather than CD14$^+$ monocytes and macrophages, are the primary source of the pro-inflammatory cytokines TNFα and IL-6 (23). Thus, although CD14$^+$ cells are necessary for uptake of N-BP, IPP accumulation and the subsequent presentation of IPP to Vγ9Vδ2$^+$ T-cells, the activated Vγ9Vδ2$^+$ T-cells themselves are ultimately responsible for the characteristic cytokine profile observed in the acute-phase reaction to N-BPs *in vivo.*

One poorly understood aspect of the acute-phase reaction to N-BPs is that it usually only manifests itself following the initial infusion, with subsequent infusions failing to trigger symptoms of comparable severity. Since these subsequent infusions should attain similar plasma concentrations of N-BP *in vivo*, there appears to be some form of inherent regulatory mechanism(s) that limits further Vγ9Vδ2$^+$ T-cell activation in response to subsequent N-BP infusions. Similar *in vivo* Vγ9Vδ2$^+$ T-cell activation and subsequent failure to respond has also been observed with repeated treatments of cynomolgus monkeys with the synthetic Vγ9Vδ2-TCR agonist BrHPP (28). This *in vivo* regulatory mechanism may also explain why only about one-third of patients appear to develop clinical symptoms of an acute-phase reaction, whereas the majority (90-100%) of blood samples taken from healthy

volunteers demonstrate reactive $V\gamma9V\delta2^+$ T-cells when stimulated with clinically relevant concentrations of N-BPs *in vitro* (our unpublished observations and (29;30)). The mechanism responsible for limiting the further stimulatory effects of N-BPs on $V\gamma9V\delta2^+$ T-cells *in vivo* remains to be clarified, but may involve regulatory T-cells (T_{regs}), for example, $CD4^+/CD25^{hi}/FOXP3^+$ self-reactive T-cells capable of suppressing T-cell function. Their defective regulatory nature is thought to underlie a variety of autoimmune diseases, and has been suggested to play a role in tumor growth and infection (reviewed in (31)). However, further investigation is necessary to clarify the exact role of these T_{regs} in the acute-phase reaction to N-BPs *in vivo*.

γδ T-cells in Human Health and Disease

The germline-encoded TCR repertoire of γδ T-cells is strikingly small compared to that of conventional αβ T-cells, and the usage of the few available gene segments is further restricted within different anatomical sites in the body. In the peripheral blood of healthy adults $V\gamma9V\delta2^+$ T-cells are predominant (50-90% of all γδ T-cells), whilst $V\delta1^+$ cells co-expressing different Vγ genes are primarily localized to epithelial surfaces such as the gut, skin, esophagus, trachea, lungs and genital epithelia (32). In the early 1990s, $V\gamma9V\delta2^+$ T-cells in PBMC cultures were demonstrated to proliferate dramatically *in vitro* following exposure to extracts of *Mycobacterium tuberculosis* (33). Subsequent studies revealed that the most potent ligands for $V\gamma9V\delta2^+$ T-cells are phosphorylated intermediates of the non-mevalonate (1-deoxy-D-xylulose-5-phosphate/DOXP) pathway of isoprenoid biosynthesis, which is utilized by many bacteria including *M. tuberculosis* but not eukaryotic cells (34;35). Therefore, γδ T-cells have the innate ability to recognize a diverse array of microbial pathogens containing these phosphorylated isoprenoid intermediates.

In addition to these anti-bacterial effects, γδ T-cells, and other immune cells such as NK cells, seem to play an important role in tumor surveillance, since mice deficient in these cell types show increased incidence of tumors (36;37). Whilst the study of $V\gamma9V\delta2^+$ T-cells has been hampered by the lack of a comparable, analogous subset of phosphoantigen-reactive γδ T-cells in murine models, clinical evidence is mounting for a similar anti-tumor role for γδ T-cells in humans. γδ T-cells kill autologous tumor cells and have been isolated from a variety of tumors such as renal cell carcinoma (38), colorectal cancer (39) and lung carcinoma (40). *In vitro,* γδ T-cells also demonstrate broad cytotoxic activity towards many different tumour cells types (reviewed in (41)).

$V\gamma9V\delta2^+$ T-cells are also capable of anti-viral responses, since treatment of PBMC cultures with IPP induces the release of a wide variety of molecules with anti-viral or immunomodulatory properties such as the cytokines IFNγ, TNFα, IL-1α, IL-6 and GM-CSF, and chemokines such as MIP-1α and MIP-1β, RANTES and SDF-1 (reviewed in (42)). Therefore, through the release of this diverse array of cytokines and chemokines, activation of innate γδ T-cells induces further humoral and cellular adaptive immune responses to co-ordinate a sustained anti-viral response to pathogens.

$V\gamma9V\delta2^+$ T-cells have been reported to proliferate in response to HIV-infected cells, exert powerful cytotoxic activity against HIV-infected targets (43), and produce HIV-inhibitory β-chemokines and α-defensins (44;45). Due to the poorly co-ordinated immune response in HIV-infected patients, the *ex vivo* expansion of $V\gamma9V\delta2^+$ T-cells with N-BPs (or synthetic agonists such as BrHPP) presents a tantalizing approach to restoring a more effective immune response in these patients.

Potential of Bisphosphonates for Immunotherapy

Due to the potent stimulatory effects of N-BPs on $V\gamma9V\delta2^+$ T-cells, this blockbuster class of drugs, developed originally for the treatment of osteoporosis, Paget's disease and metastatic bone disease, may offer a novel approach for selective activation of $V\gamma9V\delta2^+$ T-cells *in vivo*. Since N-BPs are already licensed for clinical use, these drugs are suitable candidates for development as vaccines to boost innate immune responses mediated by $V\gamma9V\delta2^+$ T-cells. Despite their relative scarcity in peripheral blood

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(compared to conventional $CD4^+$ αβ T-cells), γδ T-cells can orchestrate a co-ordinated immune response to pathogens, mediated through both cytokine release and also by direct cytotoxic effects on target cells (See Figure 2). Upon activation by pamidronate, $V\gamma9V\delta2^+$ T-cells release IFNγ (17;46), a pro-inflammatory cytokine with multiple anti-tumor effects, including direct inhibition of tumor cell growth, inhibition of angiogenesis and stimulation of macrophage activity (47). Thus, IFNγ may be a key cytokine involved in γδ T-cell-mediated anti-tumor responses. Furthermore, *in vivo* treatment of cancer patients with zoledronic acid causes $V\gamma9V\delta2^+$ T-cells to mature towards an IFNγ-producing effector phenotype, suggesting that zoledronic acid treatment may lead to more effective anti-tumor responses (48). $V\gamma9V\delta2^+$ T-cells can recognize and lyse tumor cells pre-treated with N-BPs (21). This effect is due to the intracellular accumulation of IPP in the tumor cells, which in turn activates $V\gamma9V\delta2^+$ T-cells. γδ T-cells have also been found to form stable conjugates with N-BP-pulsed tumor cells (49) and ZOL treatment increases sensitivity of small cell lung cancer and fibrosarcoma cell lines to lysis by γδ T-cells, an effect mediated through direct cell to cell contact and perforin-dependent cytotoxicity (50).

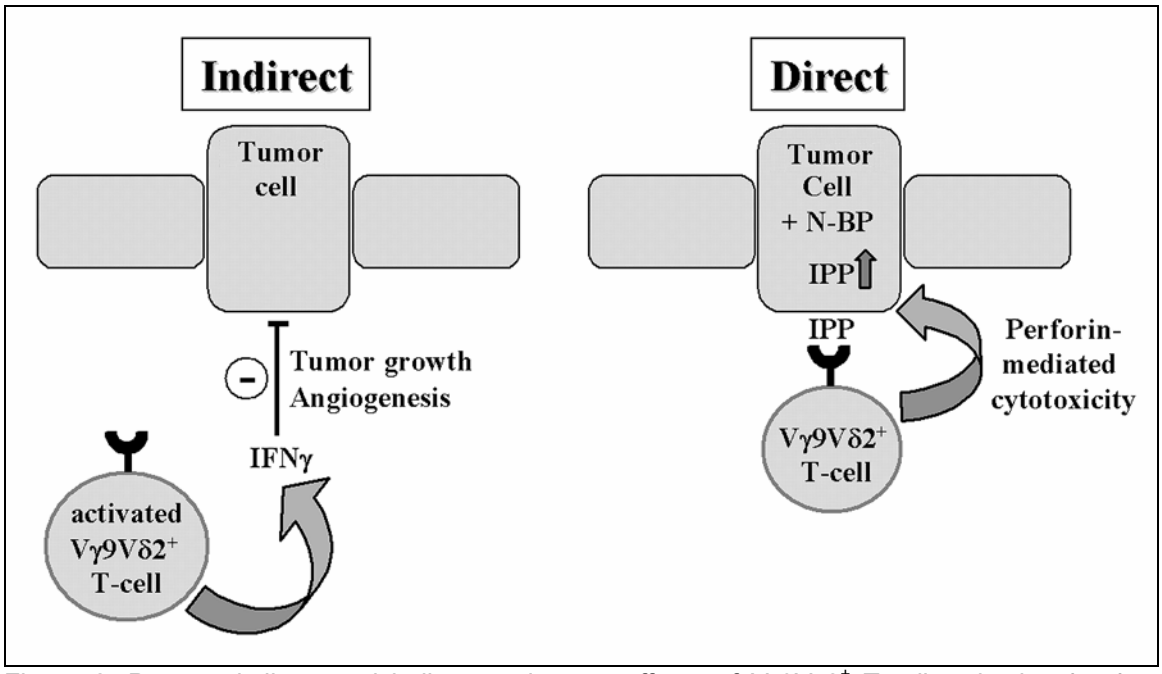

Figure 2. Proposed direct and indirect anti-tumor effects of $V\gamma9V\delta2^+$ T-cell activation *in vivo*. Following activation, $V\gamma9V\delta2^+$ T-cells release IFNγ (left panel) that is capable of inhibitory effects on tumor cell growth and angiogenesis. Activated $V\gamma9V\delta2^+$ T-cells can also recognize IPP on the surface of N-BP-pulsed tumor cells (right panel), and have cytotoxic effects on tumor cells through the release of the cytolytic protein perforin.

The anti-lymphoma activity of $V\gamma9V\delta2^+$ T-cells (in combination with the T-cell survival factor IL-2) has been investigated in patients with lymphoid malignancies and has demonstrated some efficacy *in vivo,* with $V\gamma9V\delta2^+$ T-cell expansion being a pre-requisite for tumour regression (29). While the evidence for N-BPs themselves having direct effects on tumor cells *in vivo* remains inconclusive, the potent stimulatory effects of N-BPs on $V\gamma9V\delta2^+$ T-cells provides strong justification for incorporating N-BPs in future treatment regimens for lymphoid malignancies (reviewed in (51)). The use of N-BPs in this setting will most likely require combination with factors such as IL-2, since γδ T-cells themselves do not produce IL-2 in sufficient quantities to maintain a strong proliferative response (52).

Finally, it is tempting to speculate that activation of γδ T-cells with anti-tumor activity could explain the striking anti-tumor effects of N-BPs that have recently been described in animal models (9). However, rodents do not appear to possess a

population of γδ T-cells analogous to the Vγ9Vδ2$^+$ T-cells, i.e. those activated by IPP (32), therefore the exact mechanism underlying the anti-tumor effects of N-BPs in mouse models remains to be determined.

Conclusion

Due to the relative safety, potency and long-term stability of N-BPs such as zoledronic acid, these agents represent ideal candidates for activating Vγ9Vδ2$^+$ T-cells in the clinical setting, particularly in conditions such as lymphoid malignancies, which are often associated with osteolytic disease. However, inhibition of bone resorption would be a notable side-effect in patients lacking such metastatic bone disease and thus warrants consideration. Although the therapeutic manipulation of γδ T-cells is currently still in its infancy, it has enormous potential and could form the basis of numerous anti-bacterial, anti-viral and anti-tumor treatment strategies in the future.

Conflict of Interest: Dr. Thompson reports that no conflict of interest exists. Dr. Rogers reports that he receives research funding from Procter & Gamble, Novartis and Roche, and that he is a consultant for Procter & Gamble and Novartis.

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