

Activation of $\gamma\delta$ T Cells by Bisphosphonates

Keith Thompson, Anke J. Roelofs, Marjo Jauhiainen, Hannu Mönkkönen, Jukka Mönkkönen, and Michael J. Rogers

Abstract After decades of successful clinical use, the exact molecular mechanisms by which the anti-resorptive bisphosphonate drugs (BPs) exert their effects are now being revealed. In addition to their anti-resorptive effects, it is now apparent that nitrogen-containing BPs (N-BPs) have immunomodulatory properties. Specifically, these drugs activate immune cells called gamma, delta T lymphocytes. In this chapter we discuss the mechanism of gamma, delta T cell activation by N-BPs and propose that N-BPs may provide a safe and effective means for manipulating gamma,delta T cell activity in future immunotherapeutic approaches.

Keywords Bisphosphonate · Gamma, delta T cell · Monocytes · Osteoporosis · Mevalonate pathway

1 Bisphosphonates

Bisphosphonates (BPs) are a class of drugs successfully used to treat a wide variety of diseases characterized by excessive osteoclast-mediated bone resorption, such as tumour-associated osteolysis, Paget's disease of bone and post-menopausal osteoporosis. BPs consist of a common geminal carbon atom linked to two phosphonate groups, to yield a P–C–P structure and, as such, BPs are considered to be analogues of naturally occurring pyrophosphate (P–O–P). However, unlike pyrophosphate, the P–C–P backbone of BPs confers a remarkable resilience to enzymatic hydrolysis. The incorporation of a carbon, rather than oxygen, atom in the P–C–P structure also allows the addition of two further side chains, thus allowing a great number of possible variations in molecular structure (Fig. 1), which influence both the potency and pharmacology of these drugs.

K. Thompson (✉)

Bone and Musculoskeletal Research Programme, Institute of Medical Sciences, University of Aberdeen, Foresterhill, Aberdeen AB25 2ZD, UK
e-mail: k.thompson@abdn.ac.uk

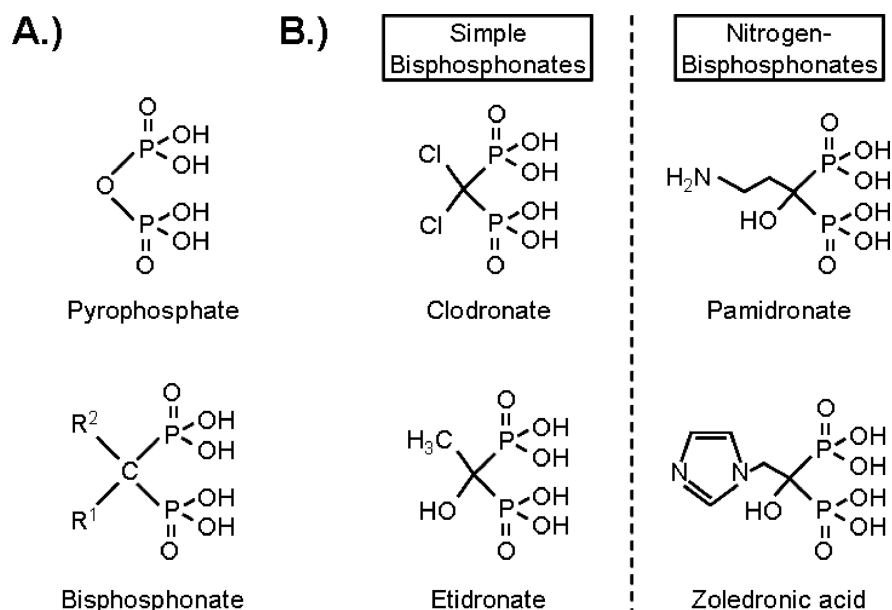


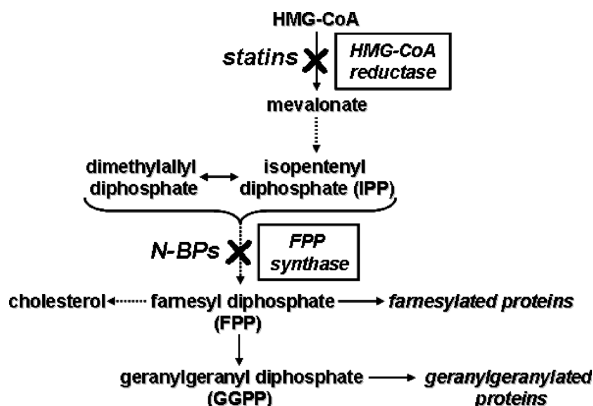
Fig. 1 (A) The structure of pyrophosphate and a geminal bisphosphonate. R^1 and R^2 indicate the positions of the variable side chains. (B) Structures of bisphosphonates (shown as the protonated bisphosphonic acid forms)

The two phosphonate groups present in BPs also endow these compounds with a high affinity for divalent ions, such as calcium. Due to the abundance of calcium in bone mineral, one of the key pharmacological features of these drugs is rapid sequestration by the skeleton. Binding of BPs to bone mineral is crucial for the relatively selective targeting of osteoclasts by these drugs, since these cells are the only cell type currently known that can liberate bound BPs from the bone surface during the resorptive process [6]. During the process of bone resorption, osteoclasts secrete protons to acidify the resorption pit, which decreases the affinity of BPs for calcium ions and causes release of the BP from the bone surface. BPs are then internalized by the osteoclast during a process known as fluid-phase endocytosis [30]. Once BPs gain entry into the osteoclast, they inhibit osteoclast activity by one of two mechanisms, based on the molecular structure of the BP.

2 Mechanism of Action of Bisphosphonates

Simple BPs lacking a nitrogen moiety, such as clodronate (CLO) and etidronate (ETI), are metabolized intracellularly into non-hydrolyzable cytotoxic analogs of ATP, which accumulate in the osteoclast and trigger apoptosis [11]. In contrast, the more potent nitrogen-BPs (N-BPs), such as pamidronate (PAM) and zoledronic acid (ZOL), inhibit osteoclast function by acting as potent inhibitors of the enzyme

Fig. 2 Schematic representation of the cholesterol biosynthetic pathway. The sites of inhibition by statins and nitrogen-bisphosphonates (N-BPs) are indicated



farnesyl diphosphate (FPP) synthase [2, 9, 33] in the cholesterol (or mevalonate) biosynthetic pathway (Fig. 2). FPP synthase activity is essential for the maintenance of an intracellular pool of isoprenoid lipids such as FPP and geranylgeranyl diphosphate (GGPP), which are required for the post-translational modification (isoprenylation) of small GTP-binding proteins such as Rho, Rac and Cdc42. Isoprenylation of these signalling proteins is crucial for the correct sub-cellular localization and function of these GTPases [35]. As a result, inhibition of FPP synthase by N-BPs results in defective regulation of processes reliant on GTPase activity such as cytoskeletal rearrangement [23, 26] and vesicular trafficking [36] in osteoclasts (for review see [5]).

Oral administration of BPs is generally well tolerated, and their potent inhibitory effect on excessive osteoclast activity has resulted in the successful widespread clinical use of these compounds for over 30 years. The profound anti-resorptive effect of a once-yearly infusion of zoledronic acid [3], or more frequent infusions of ibandronate [8], has demonstrated that the intravenous administration of BPs may be 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 self-limiting, flu-like syndrome called the acute-phase reaction (APR) [1, 25], which typically occurs in one-third of patients. Despite this syndrome being first reported over 20 years ago, the molecular events triggering this adverse event of N-BPs are only now becoming apparent.

3 $\gamma\delta$ T Cells and the Acute Phase Response to Nitrogen-Bisphosphonates

The molecular mechanism underlying the acute-phase response (APR) was first revealed by Kunzmann et al. in 1999. Their seminal study revealed that in multiple myeloma patients treated with PAM, the patients who developed an APR had

increased circulating levels of $\gamma\delta$ T cells up to 28 days following the infusion [16]. Furthermore, the severity of the APR, as assessed by increased body temperature, correlated to the extent of the increase in $\gamma\delta$ T cell numbers observed in these patients. Subsequent studies revealed that only the predominant subset of $\gamma\delta$ T cells in peripheral blood, V γ 9V δ 2 T cells, were activated by N-BPs [15]. Thus, it was apparent that the N-BP-induced V γ 9V δ 2 T cell activation, and subsequent release of pro-inflammatory cytokines such as TNF α , IL-6 and IFN γ [25, 24, 29], played a crucial role in the APR; however, the exact mechanism by which N-BPs activated V γ 9V δ 2 T cells remained to be elucidated.

$\gamma\delta$ T cells are non-conventional T cells that recognize non-peptide antigens without the need for conventional MHC class presentation, but they also exhibit characteristics of natural killer (NK) cells and cytotoxic T cells, since they can recognize and kill target cells through Fas-ligand- and perforin/granzyme B-dependent mechanisms. In humans, $\gamma\delta$ T cells comprise only a minor proportion (1–10%) of CD3⁺ T cells in peripheral blood, and the majority (50–90%) belong to the V γ 9V δ 2 subset (also termed V γ 2V δ 2) [13]. While the exact roles of $\gamma\delta$ T cells in humans have yet to be determined, these cells are thought to play an important role in tumour surveillance [17]. Furthermore, they have potent anti-microbial actions, and due to their presence in various epithelial tissues, they may present an important first line of defence to invading pathogens [13].

Until recently, N-BPs were thought to activate V γ 9V δ 2 T cells by acting as agonists for the V γ 9V δ 2-TCR [7]. However, using a Burkitt's lymphoma cell line (Daudi), Gober et al. demonstrated that ZOL required internalization by Daudi cells to induce activation of $\gamma\delta$ T cell clones, thereby arguing against a direct agonistic effect of ZOL on the V γ 9V δ 2-TCR [12]. It is now apparent that N-BPs *indirectly* activate V γ 9V δ 2 T cells through the intracellular inhibition of the molecular target of the N-BPs, FPP synthase. FPP synthase inhibition, as well as causing a depletion of the downstream metabolites FPP and GGPP (and the consequent inhibition of protein prenylation), also induces the accumulation of the substrates of this enzyme, IPP and dimethylallyl diphosphate (DMAPP) [32] (see Fig. 2), which are both agonists for the V γ 9V δ 2-TCR [28]. Gober et al. demonstrated that the stimulatory effect of ZOL on V γ 9V δ 2 T cell activation by tumor cells was due to the accumulation of these upstream mevalonate pathway intermediates in the tumor cells [12]. At the same time, our own parallel studies using peripheral blood mononuclear cell (PBMC) cultures, revealed that the stimulatory capacity of N-BPs for inducing V γ 9V δ 2 T cell activation was determined by the potency of the N-BP for inhibiting FPP synthase [31] (Fig. 3). Furthermore, N-BP-induced V γ 9V δ 2 T cell activation could be blocked by a statin, which inhibits the upstream enzyme HMG-CoA reductase, through a mechanism most likely involving inhibition of IPP/DMAPP synthesis [12, 14, 31]. We also demonstrated that this inhibitory effect of statins was selective for N-BP-induced V γ 9V δ 2 T cell activation, since statins did not inhibit V γ 9V δ 2 T cell activation induced by well characterized V γ 9V δ 2-TCR agonists such as IPP (unpublished observations), or the synthetic V γ 9V δ 2-TCR agonist, bromohydrin pyrophosphate [10, 31].

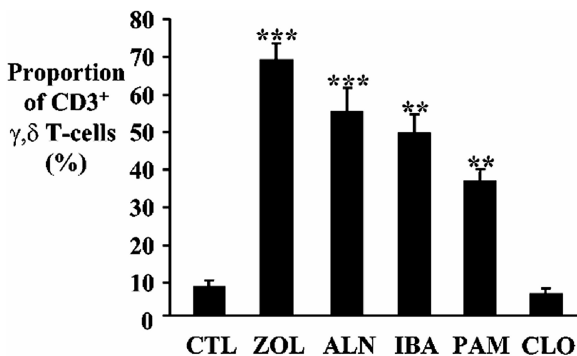


Fig. 3 The effect of N-BPs on $\gamma\delta$ T cell proliferation. PBMCs were cultured for 7 days with 1 μ M ZOL, ALN, IBA, PAM, or CLO in the presence of rhIL-2. PBMCs were dual-stained with anti-CD3-FITC and anti-pan- $\gamma\delta$ -TCR-PC5 antibodies before FACS analysis of the T cell-gated population. Data shown are the mean of experiments with PBMCs from four independent donors \pm S.E.M. *** p < 0.001; ** p < 0.01. Reproduced from Thompson and Rogers [31], with permission of the American Society for Bone and Mineral Research

4 Triggering of the Acute-Phase Response to Nitrogen-Bisphosphonates In Vivo

In N-BP-treated PBMC cultures IPP/DMAPP accumulation undoubtedly plays a crucial role in triggering V γ 9V δ 2 T cell activation, but it was unknown which cell types present in peripheral blood were directly targeted by N-BPs (and hence, accumulate IPP/DMAPP). Following a typical intravenous infusion of ZOL, the peak plasma concentration reaches \sim 1 μ M and is only maintained in the peripheral circulation for \sim 2 h [4], due to a combination of both rapid renal excretion and sequestration by the skeleton. Thus, in order to trigger an APR, ZOL must be internalized rapidly by peripheral blood cells.

We have previously shown that highly endocytic cells such as macrophages are relatively sensitive to N-BPs in vitro [6]. By using a fluorescently labelled N-BP analog, we revealed that N-BPs are internalized into macrophages and osteoclasts predominantly by the process of fluid-phase endocytosis [30] (Fig. 4). Therefore, it is likely that the triggering of an APR results from the rapid uptake of N-BP by a cell population that has high endocytic activity present in peripheral blood. Monocytes (present in PBMC cultures) have previously been shown to be crucial for N-BP-induced V γ 9V δ 2 T cell activation [20], although the role of monocytes in this process was at that time thought to involve presentation of N-BP as antigen to V γ 9V δ 2 T cells and/or provision of a crucial co-stimulatory role, thereby decreasing the threshold of V γ 9V δ 2 T cell activation.

Our recent studies have clarified the role of monocytes in V γ 9V δ 2 T cell activation in vitro. Using our fluorescent N-BP analog-based approach [6, 30], we have shown that N-BPs are selectively internalized by peripheral blood monocytes in human PBMC cultures, with no detectable uptake into B- or T-lymphocytes, at

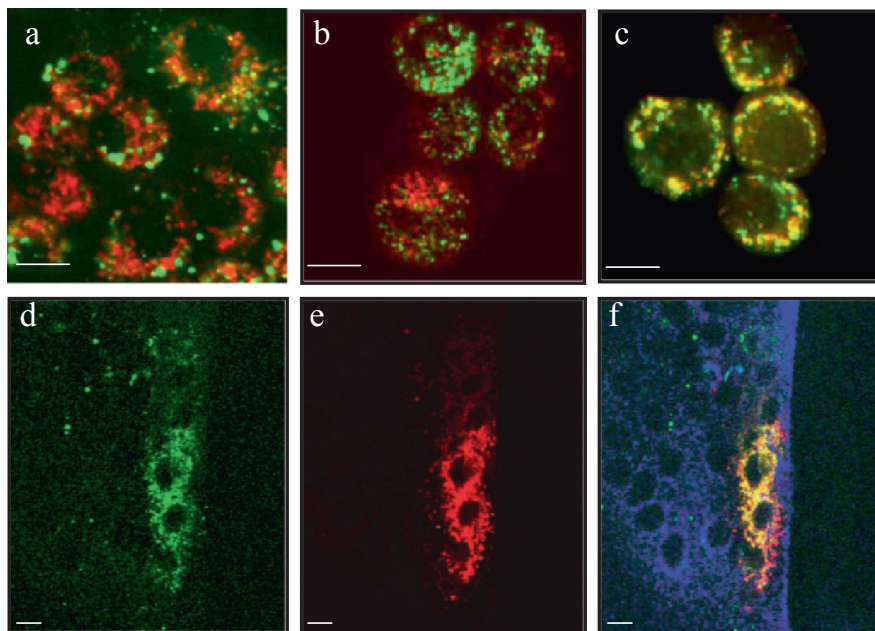


Fig. 4 Fluorescently labelled bisphosphonate (alendronate-Alexa Fluor 488: AF-ALN) is internalized by fluid-phase endocytosis in J774.2 monocyte-macrophage cells and in rabbit osteoclasts. J774.2 cells (**a–c**) were incubated with (**a**) 100 μ M AF-ALN + 1 μ g/ml wheat germ agglutinin-Alexa Fluor 633 (a marker of adsorptive endocytosis); (**b**) 100 μ M AF-ALN + 20 μ g/ml transferrin-Alexa Fluor 633 (a marker of receptor-mediated endocytosis); or (**c**) 100 μ M AF-ALN + 250 μ g/ml TAMRA-dextran (a marker of fluid-phase endocytosis). Rabbit osteoclasts (**d–f**) were treated with (**d**) 100 μ M AF-ALN (green); (**e**) 250 μ g/ml TAMRA-dextran (red); or (**f**) 100 μ M AF-ALN + TAMRA-dextran + transferrin-Alexa Fluor 633 (blue), for 6 h then fixed and analyzed by confocal microscopy (scale bar, 10 μ m). In both J774.2 cells and rabbit osteoclasts co-localization of AF-ALN is only observed with fluorescently-labelled dextran, indicating fluid-phase endocytosis is the major route by which AF-ALN gains entry into these cell types. Reproduced from Thompson et al. [30], with permission of the American Society for Pharmacology and Experimental Therapeutics

low and clinically relevant concentrations [22]. In addition, only CD14⁺ monocytes in these cultures efficiently internalized FITC-dextran, a marker of fluid-phase endocytosis, and N-BP uptake co-localized with uptake of FITC-dextran, suggesting that fluid-phase endocytosis is the major mechanism by which monocytes internalize N-BPs. Furthermore, using magnetic bead-separation approaches, combined with HPLC-ESI-MS, we have also revealed that treatment of human PBMC cultures with a pharmacologically relevant concentration (1 μ M) and duration (2 h) of ZOL induced a detectable accumulation of IPP/DMAPP only in the CD14⁺ fraction. Importantly, we also demonstrated that statins prevent ZOL-induced IPP/DMAPP accumulation [21]. This raises the intriguing possibility that statins may potentially diminish or even prevent the APR to N-BPs *in vivo*.

5 Further Questions

Currently, one aspect of the APR to N-BPs that is poorly understood is the development of symptoms following the initial infusion, but the subsequent lack of symptoms with repeated N-BP infusions. Similarly, decreased responsiveness of V γ 9V δ 2 T cells to repeated treatment with the synthetic V γ 9V δ 2-TCR agonist BrHPP has been reported in cynomolgus monkeys [27]. This suggests that there is some inherent regulatory mechanism(s) that limit(s) the response following repeated challenges with V γ 9V δ 2 T cell activators. Furthermore, it is at present still unclear why only approximately one-third of patients develop flu-like symptoms of an APR following the first infusion, since the majority ($\geq 90\%$) of blood samples isolated from healthy donors demonstrate reactive V γ 9V δ 2 T cells when stimulated with clinically relevant concentrations of N-BPs in vitro (our unpublished observations and [19, 34]). The mechanism underlying this diminished responsiveness of V γ 9V δ 2 T cells to N-BPs in vivo remains to be clarified, but may involve regulatory T cells (T_{regs}), CD4⁺/CD25^{hi}/FoxP3⁺ T cells, which have recently been demonstrated to suppress IFN γ production by antigen-stimulated V γ 9V δ 2 T cells in vitro [18]. Should T_{regs} be found to inhibit V γ 9V δ 2 T cell activation in vivo, strategies to minimize the inhibitory effects of T_{regs} on V γ 9V δ 2 T cells may allow for more effective deployment of $\gamma\delta$ T cell-based therapies for the treatment of lymphoid malignancies and other types of cancer, as well as bacterial and viral infections (Fig. 5).

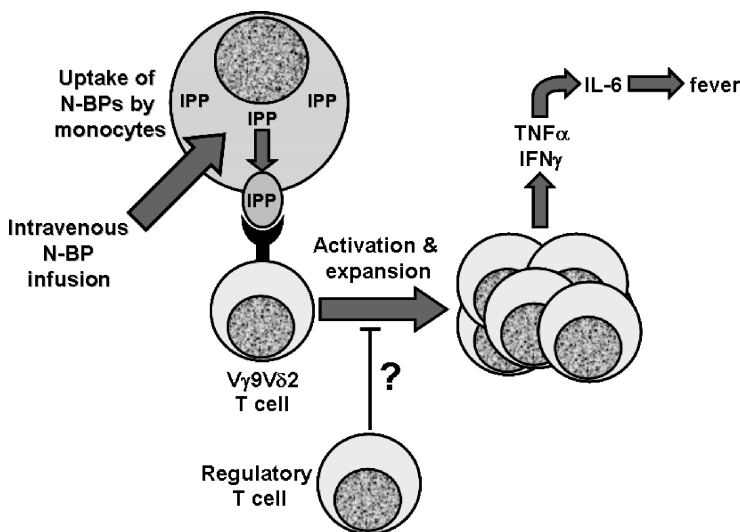


Fig. 5 The acute-phase reaction to nitrogen-bisphosphonates (N-BPs). Following an intravenous infusion, transient uptake of N-BP into peripheral blood monocytes results in intracellular accumulation of IPP due to FPP synthase inhibition. Recognition of IPP by V γ 9V δ 2 T cells triggers their activation and expansion, resulting in the release of pro-inflammatory cytokines that cause the flu-like symptoms of the acute-phase reaction. The relevance of regulatory T cell-mediated inhibitory effects on N-BP-induced V γ 9V δ 2 T cell activation in vivo is currently unknown.

6 Conclusion

The molecular mechanisms responsible for activation of V γ 9V δ 2 T cells by N-BPs, and their role in the APR, have only recently become clear, despite the identification of this syndrome more than 20 years ago. Crucially, this adverse event of intravenous N-BP therapy has unwittingly revealed the potent stimulatory effects of N-BPs on V γ 9V δ 2 T cells. This may allow the use of these compounds in future $\gamma\delta$ T cell-based immunomodulatory strategies to elicit anti-tumor effects, particularly in conditions such as lymphoid malignancies, which are often associated with osteolytic disease. Due to the relative safety, potency and long-term stability of N-BPs such as ZOL, these agents represent ideal candidates for activating V γ 9V δ 2 T cells in the clinical setting, particularly where anti-resorptive effects are beneficial. While many questions remain about $\gamma\delta$ T cell biology, such as their possible regulation by T_{regs}, the therapeutic manipulation of $\gamma\delta$ T cells has great potential and may form the basis of novel anti-tumor, anti-bacterial and anti-viral treatment strategies in the future.

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