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Alkylamines and bisphosphonates: a common mechanism for activation of γδ T-cells

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The major subset of γδ T-cells in humans (Vγ9Vδ2$^+$) are activated by three distinct classes of stimulatory molecules: pyrophosphomonoesters, such as isopentenyl diphosphate (IPP)[1] and bromohydrin diphosphate (BrHPP);[2] nitrogen-containing bisphosphonates (N-BPs),[3,4] such as alendronate (ALN), pamidronate (PAM) and zoledronic acid (ZOL); and alkylamines, such as sec-butylamine (SBA) and iso-butylamine (IBA).[5] Whilst the N-BPs were previously thought to have a direct agonistic effect at the Vγ9Vδ2-TCR,[6] our group and others have recently shown that the activation of Vγ9Vδ2 T-cells by N-BPs is via an indirect effect involving inhibition of FPP synthase and the intracellular accumulation of the pyrophosphomonoester IPP[7,8] (Figure 2). Due to the similarity in structure between the R^2 side-chain of N-BPs and the Vγ9Vδ2 T-cell-stimulatory alkylamines (Figure 1), we decided to investigate whether the alkylamines also induce Vγ9Vδ2 T-cell activation by a similar mechanism to the N-BPs; i.e. inhibition of the mevalonate pathway and the intracellular accumulation of IPP.

Treatment of human PBMCs (isolated from healthy volunteers by density-gradient separation) with 0.5 mM *n*-butylamine (BA), *iso*-propylamine (IPA), IBA or SBA all induced IFNγ release after 48hrs treatment, with an order of potency: IBA∼SBA>BA ∼IPA. Treatment of PBMCs with 0.5mM alkylamines for 7 days increased the proportion of Vγ9Vδ2 T-cells in the CD3$^+$ population, in line with the potency observed for the induction of IFNγ release (IBA∼SBA>IPA∼BA).[9]

To determine whether the alkylamines could inhibit the mevalonate pathway we used J774 macrophage cells, which we have studied extensively during our studies with N-BPs and the mevalonate pathway. Treatment of J774 cells with ≥5mM IBA/SBA or 10mM BA/ IPA caused a marked accumulation of the unprenylated form of the small GTPase Rap1A, indicative of mevalonate pathway inhibition. In accordance with this finding, when J774 cells are metabolically-labelled with ^{14}C-mevalonate, 1-10 mM IBA/SBA/BA/IPA all caused a decrease in the incorporation of ^{14}C-mevalonate into prenylated 21-26kDa small GTPases.[9]

We have previously demonstrated that the anti-resorptive effects of N-BPs (mediated by FPP synthase inhibition) can be overcome by replenishing cells with a cell-permeable form of geranylgeranyl diphosphate (GGPP), geranylgeraniol (GGOH), but not by replenishing cells with a cell-permeable form of farnesyl diphosphate (FPP), farnesol (FOH). In accord with this, the inhibitory effects of ALN or ZOL on Rap1A prenylation could be largely prevented with GGOH but not FOH. Furthermore, when we replenished IBA or SBA-treated J774 cells with FOH or GGOH, as we observed with N-BPs, only GGOH could overcome the inhibitory effects of SBA/IBA on Rap1A prenylation. Despite the potent stimulatory effect of 1 μM ZOL for inducing Vγ9Vδ2 T-cell activation and proliferation,[7] inhibitory effects of ZOL on Rap1A prenylation could only be detected with a ten-fold higher concentration of ZOL (10 μM) in human PBMC cultures.[9] Similarly, treatment of human PBMCs with 10 mM alkylamine for 48hrs induced marked accumulation of unprenylated Rap1A, which could be largely overcome by replenishing cells with GGOH, possibly implicating a shared pharmacological target of N-BPs and alkylamines.

Due to the similarity in the biochemical effects of N-BPs and alkylamines, we then screened the alkylamines for any inhibitory effect on the major pharmacological target of the N-BPs, FPP synthase. All of the alkylamines were found to inhibit recombinant human FPP synthase, with an order of potency identical to that observed for the induction of Vγ9Vδ2 T-cell activation and proliferation, and the inhibitory effects on protein prenylation: SBA∼IBA>IPA∼BA. However, in line with the increased concentrations required for

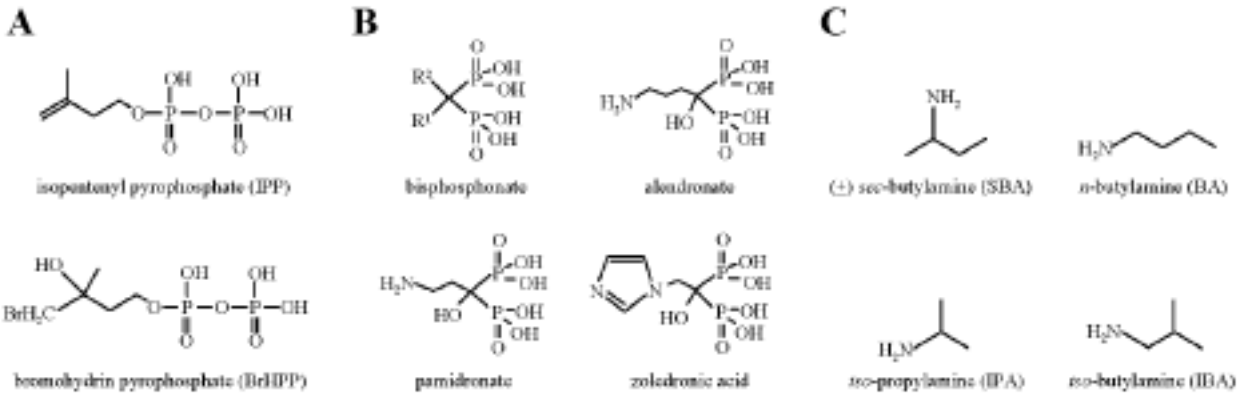

Figure 1. Structures of the three general classes of Vγ9Vδ2 T-cell stimulatory compounds: A – pyrophosphomonoesters; B – nitrogen-containing bisphosphonates; C – alkylamines.

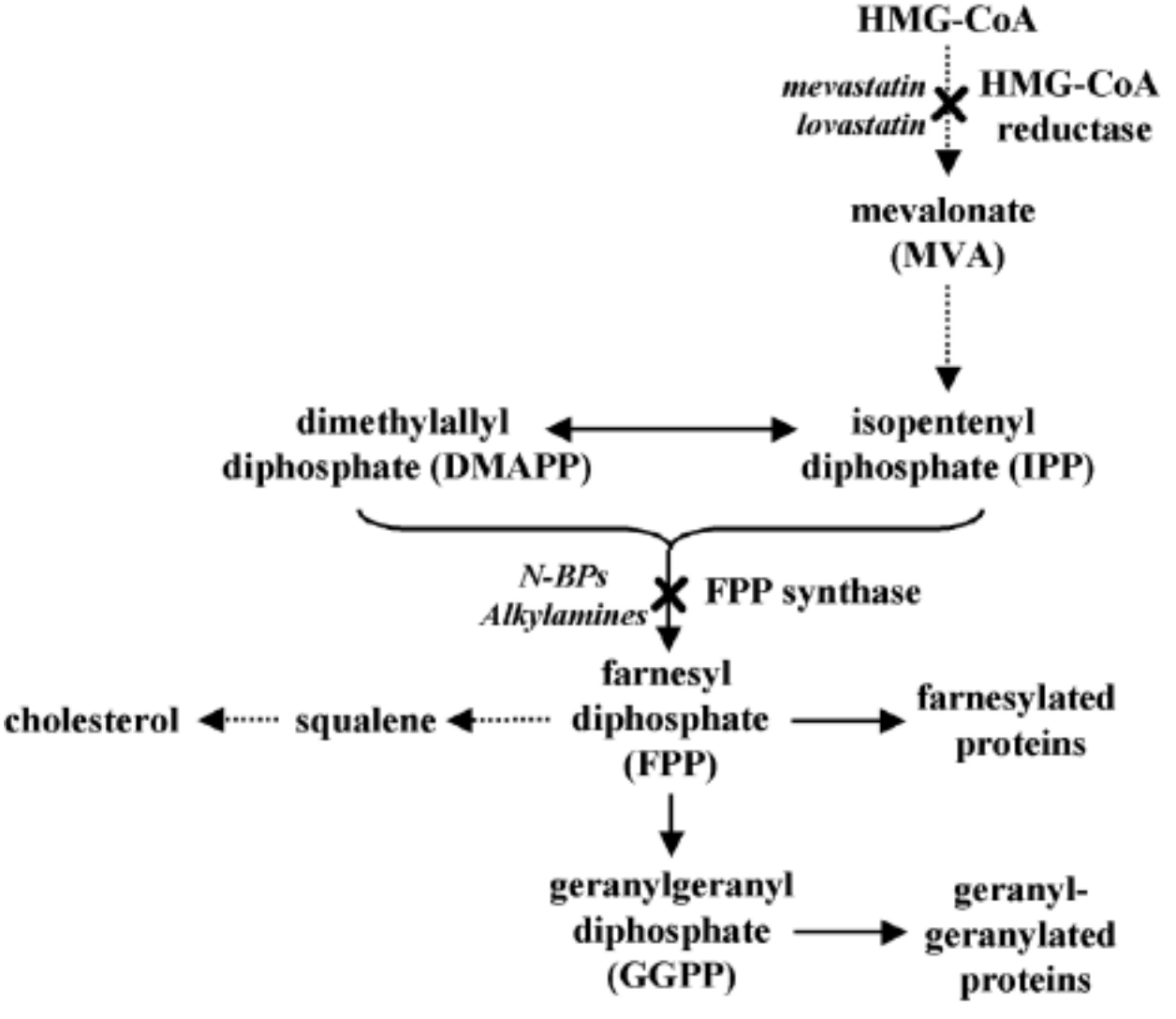

Figure 2. Schematic representation of the mevalonate biosynthetic pathway. Inhibition of FPP synthase by N-BPs or alkylamines prevents the synthesis of FPP and GGPP required for protein prenylation and causing accumulation of upstream isoprenoid intermediates such as IPP/DMAPP.

activating Vγ9Vδ2 T-cells, the alkylamines are far less potent inhibitors of FPP synthase than the N-BPs, which are nanomolar inhibitors of FPP synthase,[10] whilst the alkylamines inhibit only at millimolar concentrations.[9]

If the mechanism of action of the alkylamines is mediated via inhibition of FPP synthase and the intracellular accumulation of IPP, it should be possible to block the stimulatory effect of the alkylamines on Vγ9Vδ2 T-cells by simultaneously treating cells with a statin to inhibit the rate-limiting enzyme in the mevalonate pathway, HMG-CoA reductase, upstream of FPP synthase. Co-administration of 1 μM mevastatin to IBA or SBA-treated PBMCs completely prevented alkylamine-induced IFNγ release and Vγ9Vδ2 T-cell proliferation. This inhibitory effect of mevastatin was largely overcome by replenishing cells with 100 mM mevalonate, suggesting that mevastatin is preventing the stimulatory effect of the alkylamines by inhibiting HMG-CoA reductase and preventing the intracellular accumulation of IPP. To ensure the inhibitory effect of mevastatin on SBA-induced Vγ9Vδ2 T-cell activation and proliferation was not due to a non-specific effect such as cytotoxicity, 1μM IPP was added to the mevastatin-treated PBMCs and induced marked proliferation, suggesting that the inhibitory effect of mevastatin was not due to cytotoxicity. To investigate whether the inhibitory effect of statins was due to the disruption of a co-stimulatory pathway, such as through binding of the statin to the L-site of β2-integrin LFA-1 and thus disrupting the binding of LFA-1 to ICAM-1,[11] we used lovastatin and a closely-related analogue, desoxolovastatin. Both of these compounds have similar affinities for binding LFA-1, but only lovastatin can inhibit HMG-CoA reductase.[11] Only lovastatin was found to prevent SBA-induced IFNγ release and Vγ9Vδ2 T-cell proliferation, whilst desoxolovastatin had no inhibitory effect, lending further support to the notion that statins prevent alkylamine-induced Vγ9Vδ2 T-cell activation and proliferation by inhibiting HMG-CoA reductase and thus preventing the accumulation of IPP following FPP synthase inhibition.

Together with our previous studies with N-BPs,[7] and that of others,[3] we suggest that only the pyrophosphomonoesters are true Vγ9Vδ2-TCR antigens, whilst N-BPs and alkylamines act through an identical mechanism involving the intracellular inhibition of FPP synthase and the accumulation of IPP.

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