

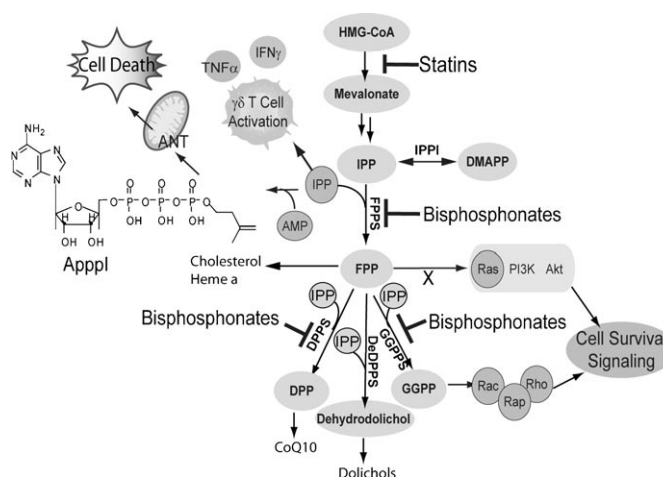
## Lipophilic Pyridinium Bisphosphonates: Potent $\gamma\delta$ T Cell Stimulators\*\*

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Bisphosphonates such as risedronate and ibandronate are widely used to treat a variety of bone resorption diseases, preventing protein prenylation and disrupting osteoclast function.<sup>[1]</sup> Bisphosphonates also activate human  $\gamma\delta$  T cells (expressing the V $\gamma$ 2V $\delta$ 2 T cell receptor), and these activated  $\gamma\delta$  T cells kill tumor cells.<sup>[2,3]</sup> There has thus been interest in using bisphosphonates in cancer immunotherapy, with promising results against B-cell malignancies<sup>[4]</sup> and hormone refractory prostate cancer.<sup>[5]</sup> In a very recent clinical trial, it was shown that zoledronate offered a significant anticancer benefit when added to hormone therapy, reducing the risk of cancer returning by 36%.<sup>[6]</sup> The bisphosphonates used in these trials are, however, extremely polar and are rapidly removed from circulation by binding to bone. We reasoned that it might be possible to develop more lipophilic bisphosphonates<sup>[7]</sup> as  $\gamma\delta$  T cell stimulators that would have

improved cell uptake properties as well as decreased bone binding affinity.<sup>[8]</sup> Herein, we report that novel lipophilic pyridinium bisphosphonates are approximately 250 times more effective in  $\gamma\delta$  T cell activation than any other bisphosphonate drugs.

Current nitrogen-containing bisphosphonates are thought to act primarily by blocking farnesyl diphosphate (FPP) formation in the isoprene biosynthesis pathway (Figure 1),



**Figure 1.** Schematic illustration of several pathways involved in bisphosphonate activity in  $\gamma\delta$  T cells and tumor cells. AMP=adenosine monophosphate, ANT=mitochondrial adenine nucleotide translocase, DMAPP=dimethylallyl pyrophosphate, IFN- $\gamma$ =interferon-gamma. Ras, Rac, Rho, and Rap are small GTPases involved in cell signaling, PI3K is a phosphoinositide-3-kinase involved in intracellular signal transduction, and Akt proteins are involved in cellular survival pathways.

where they act as low-nanomolar FPP synthase (FPPS) inhibitors. Their stimulatory effects are thought to originate in the accumulation of isopentenyl diphosphate (IPP), a known “phosphoantigen” for  $\gamma\delta$  T cells,<sup>[9]</sup> and their effects are blocked by statins.<sup>[10,11]</sup> There are, however, four other targets in this pathway whose inhibition would also increase IPP levels: isopentenyl diphosphate/dimethylallyl diphosphate isomerase (IPPI), geranylgeranyl diphosphate synthase (GGPPS), decaprenyl diphosphate synthase (DPPS), and dehydrodolichyl diphosphate synthase (DeDPPS). Since four of these five enzymes produce long-chain isoprenoids, we reasoned that they might be potently inhibited by more hydrophobic bisphosphonates, which would also confer

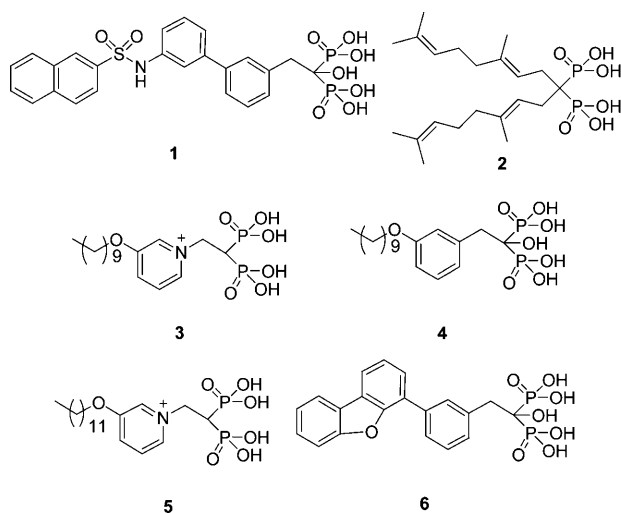
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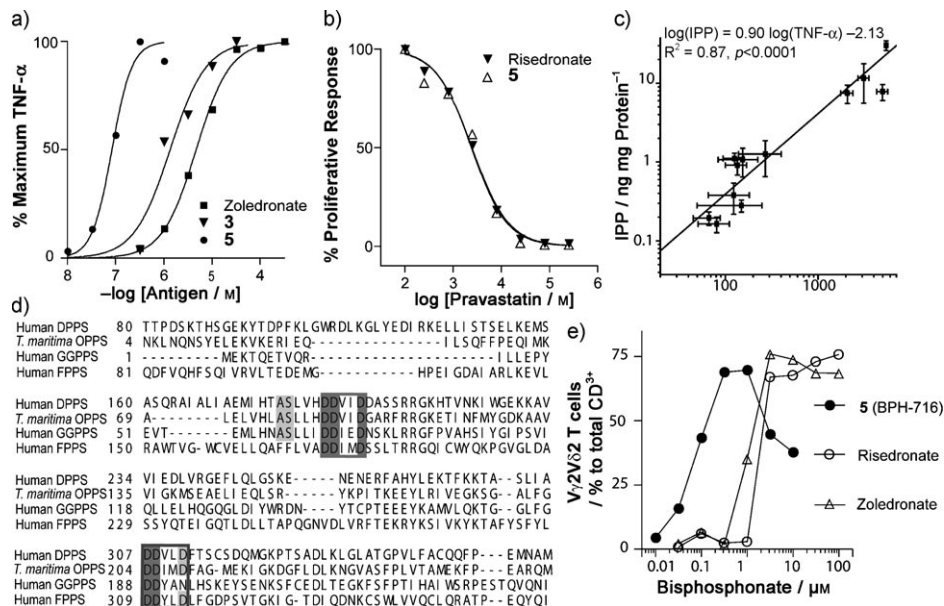
enhanced cell-based activity. To test this idea, we determined the activity of the six lipophilic bisphosphonates **1–6**<sup>[12]</sup> in  $\gamma\delta$  T cell activation. Several of these compounds have been shown to have potent activity in tumor cell killing,<sup>[12]</sup> but do they also activate  $\gamma\delta$  T cells?



We first tested two specific inhibitors (**1**, **2**<sup>[13]</sup>) of GGPPS, which have  $IC_{50}$  (enzyme) values of 2.7, 1.0  $\mu$ M. Neither had major effects on  $\gamma\delta$  T cell activation (tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) release) or proliferation. In a second experiment, we found that long *n*-alkyl-containing bisphosphonates (**3**, **4**) have  $IC_{50}$  values of 280, 590 nM against GGPPS. The pyridinium species (**3**) was a potent (800 nM)  $\gamma\delta$  T cell activator (Figure 2a), while the analogue (**4**) lacking the positive charge feature had much less activity. A longer ( $C_{12}$ ) alkyl chain analogue (**5**) of **3** had even greater activity, with an effective dose ( $ED_{50}$ ) of 70 nM (Figure 2a) in  $\gamma\delta$  T cell activation. Only **3** and **5** were potent FPPS inhibitors (**3**  $IC_{50}$  = 100 nM, **4**  $IC_{50}$  = 548  $\mu$ M, **5**  $IC_{50}$  = 3.8  $\mu$ M). The requirement of a positive charge feature for  $\gamma\delta$  T cell activation is of interest and is reminiscent of the requirement of a positive charge feature (imidazolium, ammonium, guanidinium, sulfonium) in bisphosphonates for FPPS inhibition.<sup>[14,15]</sup> This feature is not required for inhibition of *cis*-prenyl transferases, such as undecaprenyl diphosphate synthase (UPPS), which has 37% identity and 55% similarity to human DeDPPS (and a BLAST *e*-value of  $2 \times 10^{-31}$  between the

two sequences), and since potent UPPS inhibitors (e.g. **6**) have no activity in  $\gamma\delta$  T cell activation, we conclude that  $\gamma\delta$  T cell activation by **3** and **5** is unlikely to be due to inhibition of DeDPPS. Action in the isoprene biosynthesis pathway is clear, as two statins (pravastatin and mevastatin) block  $\gamma\delta$  T cell activation by **3** with the same  $IC_{50}$  values as found for their blocking of  $\gamma\delta$  T cell activation by risenedronate (Figure 2b and Supporting Information, Figure S1). That is, the target is in the isoprenoid pathway downstream of HMG-CoA reductase (HMG-CoA = 3-hydroxy-3-methylglutaryl-coenzyme A). We find no activity of **3** or **5** against IPPI. However, in addition to FPPS, both **3** and **5** inhibit expressed human DPPS (Supporting Information, Figure S2) with  $IC_{50}$  values of 585 (**3**) and 620 nM (**5**).

These results indicate that **3** and **5** can inhibit both FPPS and DPPS, which is expected to result in accumulation of the phosphoantigen IPP. In fact, TNF- $\alpha$  release is directly proportional to IPP levels in the target cells, as shown in Figure 2c (and Supporting Information, Table S1) with  $R^2 = 0.87$  ( $p < 0.0001$ ). Interestingly, the bisphosphonate zoledronate also inhibits DPPS ( $IC_{50} = 5.5 \mu$ M), but the long alkyl pyridinium compounds are more potent. In retrospect, the ability of the cationic bisphosphonates to inhibit FPPS as well as DPPS should not be unexpected, as both enzymes contain the two highly conserved “DDXXD” repeats found in most *trans*-prenyl synthases (including, for example, hexaprenyl diphosphate synthase and octaprenyl diphosphate synthase).<sup>[16]</sup> This conservation is illustrated graphically in the partial sequence alignment between human FPPS and human DPPS (the catalytic subunit 1) in Figure 2d. In FPPS, there



**Figure 2.** V $\gamma$ 2V $\delta$ 2 T cell stimulation by lipophilic bisphosphonates. a)  $\gamma\delta$  T cell stimulation by bisphosphonates evaluated by TNF- $\alpha$  secretion in the presence of CP.EBV (Epstein–Barr virus) B cells. b) Inhibition of bisphosphonate-induced  $\gamma\delta$  T cell proliferative responses by the HMG-CoA reductase inhibitor pravastatin. The  $IC_{50}$  values are both 2.4  $\mu$ M. Mevastatin results are in the Supporting Information, Figure S1. c) Correlation between IPP levels in CP.EBV cells treated with different concentrations of **1**, **3**, **4**, **5**, or zoledronate (determined according to Ref. [20]) and TNF- $\alpha$  release by  $\gamma\delta$  T cells (determined according to Ref. [21]). d) Partial sequence alignment between human FPPS and DPPS. e) Response of blood V $\gamma$ 2V $\delta$ 2 T cells to risedronate, zoledronate, and **5** presented by monocytes.

are two Phe residues that block chain elongation (or the binding of long-chain bisphosphonates), but these residues are Ala, Ser in DPPS, permitting stronger binding of **3** and **5**. And as expected, lipophilic bisphosphonates such as **1** and **4** that are poor FPPS and DPPS inhibitors (FPPS: **1** 126  $\mu\text{M}$ , **4** 0.5  $\mu\text{M}$ ; DPPS: **1** 45  $\mu\text{M}$ , **4** 24  $\mu\text{M}$ ) have essentially no activity in TNF- $\alpha$  release. We thus conclude that these lipophilic bisphosphonates can target both FPPS and DPPS, resulting in elevated IPP levels (and hence, potent  $\gamma\delta$  T cell activation), owing to their more hydrophobic nature.

Intravenous bisphosphonate stimulation of V $\gamma$ 2V $\delta$ 2 T cells in patients for cancer immunotherapy is thought to involve a similar accumulation of IPP in monocytes.<sup>[17–19]</sup> To investigate the effects of the lipophilic bisphosphonates on monocytes, we therefore tested the ability of monocytes in PBMC (peripheral blood mononuclear cell) to stimulate V $\gamma$ 2V $\delta$ 2 T cells in vitro by determining V $\gamma$ 2V $\delta$ 2 T cell expansion. Pulsing of **5** into monocytes present in PBMC stimulated a major expansion of the V $\gamma$ 2V $\delta$ 2 T cell subset with a 12.5-fold lower EC<sub>50</sub> than the most potent nonlipophilic bisphosphonate, zoledronate (the EC<sub>50</sub> was 80 nM for **5** vs. 1.0  $\mu\text{M}$  for zoledronate, Figure 2e). Thus, **5** also strongly stimulates V $\gamma$ 2V $\delta$ 2 T cells ex vivo, when monocytes are used as presenting cells.

Overall, these results are of broad general interest as they show that lipophilic pyridinium bisphosphonates are far more active in  $\gamma\delta$  T cell activation than are the drugs used in several clinical trials.<sup>[4,5]</sup> And since such compounds bind only weakly to bone<sup>[12]</sup> and inhibit GGPPS, they also have direct activity against tumor cell growth and invasiveness,<sup>[12]</sup> opening up the possibility of new and improved routes to combined cancer chemotherapy and immunotherapy using lipophilic bisphosphonates.

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