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Synthesis and Metabolism of BTN3A1 Ligands: Studies on Modifications of the Allylic Alcohol

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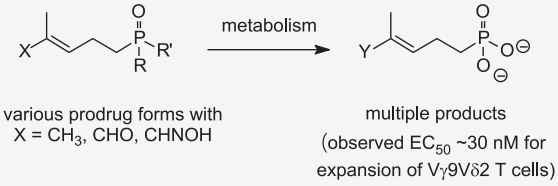
ABSTRACT: (*E*)-4-Hydroxy-3-methyl-but-2-enyl diphosphate (HMBPP) and its phosphonate analogs are potent phosphoantigens. HMBPP contains an (*E*)-allylic alcohol which interacts with the molecular target BTN3A1 giving an antigenic signal to activate Vγ9Vδ2 T cells. As probes of BTN3A1 function, we prepared prodrug derivatives of the HMBPP analog C-HMBP that lack the (*E*)-allylic alcohol or have modified it to an aldehyde or aldoxime and evaluated their biological activity. Removal of the alcohol completely abrogates phosphoantigenicity in these compounds while the aldoxime modification decreases potency relative to the (*E*)-allylic alcohol form. However, homoprenyl derivatives oxidized to an aldehyde stimulate Vγ9Vδ2 T cells at nanomolar concentrations. Selection of phosphonate protecting groups (i.e., prodrug forms) impacts the potency of phosphoantigen aldehydes, with mixed aryl acyloxyalkyl forms exhibiting superior activity relative to aryl amidate forms. The activity correlates with the cellular reduction of the aldehyde to the alcohol form. Thus, the functionality on this ligand framework can be altered concurrently with phosphonate protection to promote cellular transformation to highly potent phosphoantigens.



KEYWORDS: *Butyrophilin, BTN3A1, ligand, phosphoantigen, prodrugs*

The Vγ9Vδ2 T cells are a subset of human T cells that contribute to immunity against infectious diseases and cancer.[1] Unlike most T cells, the Vγ9Vδ2 T cells are activated by small phosphorus-containing molecules known as "phosphoantigens" rather than peptide antigens.[2] Isopentenyl diphosphate (IPP, **1a**, Figure 1) was the first identified natural phosphoantigen for human Vγ9Vδ2 T cells.[3−5] Isomerization of IPP by isopentenyl diphosphate isomerase affords dimethylallyl diphosphate (DMAPP, **2a**), which itself may function as a phosphoantigen.[6,7] These diphosphates are more active relative to the corresponding monophosphates IP (**1b**, 233-fold) and DMAP (**2b**, 3-fold), suggesting that the *β* phosphate increases specific recognition of the phosphoantigens.[8] Additional studies demonstrated that these T cells selectively recognize the methylerythritol 4-phosphate (MEP) pathway intermediate (*E*)-4-hydroxy-3-methyl-but-2-enyl diphosphate (HMBPP, **3**), an immediate precursor to IPP biosynthesis. HMBPP is the most potent natural phosphoantigen, with activity at concentrations 4−6 log units lower than IPP.[9−11] Thus, both the (*E*)-allylic alcohol of HMBPP and the diphosphate group are important determinants of phosphoantigenicity.

Because diphosphates of potential clinical importance have low stability, Boëdec and colleagues designed analogs and stereoisomers of the highly potent HMBPP. As a class, the isosteric analogs of HMBPP containing phosphonates, C-HMBP (**4a**) and C-HMBPP (**4b**), were more stable than

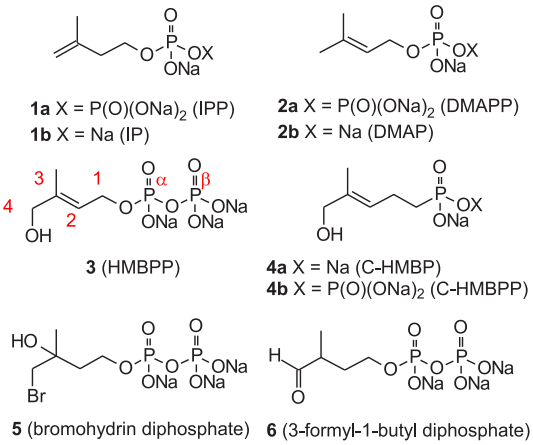


Figure 1. Relevant phosphoantigens. Endogenous phosphoantigens (**1a** and **2a**) and their monophosphate analogs (**1b** and **2b**). The most potent natural ligand for BTN3A1, HMBPP (**3**). Phosphonate analogs C-HMBP (**4a**) and C-HMBPP (**4b**). Bromohydrin diphosphate (**5**) and 3-formyl-1-butyl diphosphate (**6**).

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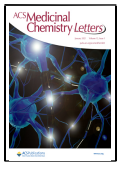


HMBPP, with longer duration of activity.[12] The *E*-olefins were significantly more potent relative to corresponding *Z*-olefin compounds, with a difference in EC_{50} values of greater than 500-fold, revealing a strong preference to *E*-geometry for optimal Vγ9Vδ2 T cell stimulation.[13]

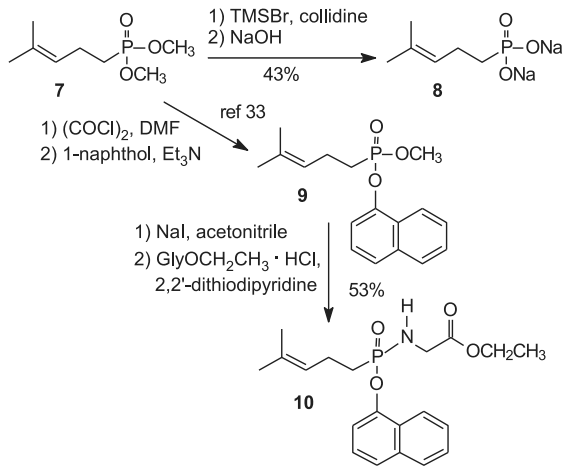
Phosphoantigens are now known to bind to a shallow, basic binding pocket in the intracellular B30.2 domain of BTN3A1.[14−20] BTN3A1 is a single pass transmembrane protein, which associates with other butyrophilins including BTN2A1, BTN3A2, and BTN3A3. Phosphoantigen binding in the intracellular domain causes conformational changes to the extracellular domain which, during interactions between Vγ9Vδ2 T cells and phosphoantigen containing cells, enables the BTN3A1 complex to bind the Vγ9Vδ2 T cell receptor (TCR).[21−24] During HMBPP binding to BTN3A1, the β-phosphate interacts directly with arginine residues (R442, 448, and 499) and the α-phosphate interacts indirectly with tryptophan (W421) and methionine (M424) residues. Importantly, the *E*-allylic alcohol of HMBPP forms a hydrogen bond with the histidine (H381) side chain, an interaction that triggers a conformational transition in H381 and the movement of nearby tryptophan residues that include W421.[16] While not fully understood, this key interaction with H381 in the intracellular domain leads to extracellular changes that allow the Vγ9Vδ2 TCR to detect HMBPP-bound BTN3A1 with greater sensitivity than detection of IPP and DMAPP.[16,19,25]

While IPP and DMAPP lack the (*E*)-allylic alcohol, these endogenous diphosphates do weakly stimulate Vγ9Vδ2 T cells.[8] It is not clear if DMAPP itself functions as a phosphoantigen, or if its activity is due to its cellular conversion to IPP.[26] Nevertheless, some studies have focused on increasing intracellular concentrations of these phosphoantigens to provoke TCR-BTN3A1 interactions.[27] Because C-HMBP phosphonamidates are more active than HMBPP,[28−30] we chose to evaluate whether DMAPP functions as a direct phosphoantigen through preparation of a parallel prodrug analog of C-DMAP. We hypothesized that a C-DMAP phosphonamidate would require lower effective concentrations to stimulate a T cell response comparable to DMAPP. Furthermore, given that the diphosphate **5** is a known phosphoantigen[31] and the aldehyde **6** was once proposed to be a natural phosphoantigen,[32] the requirement for an allylic alcohol in active compounds may be more apparent than real.

To evaluate the impact of removal of the C-HMBP (*E*)-allylic alcohol, synthesis of C-DMAP (**8**) began with the previously reported phosphonate **7** (Scheme 1).[19] Treatment of phosphonate **7** with bromotrimethylsilane and collidine followed by sodium hydroxide afforded the sodium salt **8**. To access the C-DMAP 1-naphthyl phosphonamidate, treatment of phosphonate **7** with oxalyl chloride and a catalytic amount of dimethylformamide followed by addition of 1-naphthol and triethylamine provided the mixed phosphonate ester **9**.[33] The remaining methyl ester in phosphonate **9** then was cleaved selectively by reaction with sodium iodide in acetonitrile at reflux, and the resulting salt then was coupled to glycine ethyl ester hydrochloride in the presence of triphenylphosphine and 2,2′-dithiodipyridine to afford phosphonamidate **10**. Compound **10** represents a monophosphonate analog of DMAPP protected as the phosphonamidate.

Early efforts to determine the organic framework of the more active exogenous phosphoantigen, now thought to be HMBPP, initially assigned the compound as the aldehyde 3-formyl-1-butyl-diphosphate (**6**).[34] Our preparation of C-HMBP phosphonamidates concludes with a selenium dioxide-mediated oxidation, which provides the required *E*-allylic alcohol as a single olefin isomer.[35,36] However, this reaction step can favor an aldehyde product either by increasing reaction temperature or time. In some cases, chromatography of the crude oxidation mixture provided a percentage of recovered starting material in addition to some aldehyde product. In theory, isolation of a mixture of aldehyde and alcohol after minimal purification from the selenium dioxide step would provide material suitable for treatment with manganese dioxide to drive the mixture forward to exclusively aldehyde product. Though aldehydes are generally avoided in drug development due to chemical reactivity, we were interested in evaluation of aldehyde-containing phosphoantigens because that same reactivity can lead to useful biological probes[37] as well as occasionally better drug molecules.[38]

Scheme 1. Synthesis of C-DMAP and Its Phosphonamidate Derivative



While the identity of the mycobacterial phosphoantigen described by Belmant and colleagues remains unclear, the prenyl phosphate stimulated Vγ9Vδ2 T cell proliferation at nanomolar concentrations.[32] Furthermore, their results provided evidence of a terminal aldehyde (C4 in the phosphate which would correspond to C5 in the phosphonate).[34] Bromohydrin diphosphate (BrHPP, **5**), a modified prenyl derivative, also triggered responses of human Vγ9Vδ2 T cells at nanomolar concentrations.[31] Thus, to investigate the impact of other modifications of a known BTN ligand relative to their potential to stimulate an immune response, some C-HMBP aldehyde analogs were prepared for biological evaluation. Considering that recent literature provides strong evidence for the necessity of a ligand to interact with H381 of BTN3A1, we also prepared a phosphonate aldoxime which places a potential hydrogen bond donor off of the C5 position.[16,39,40]

Synthesis of the aldehyde phosphonamidates began with phenyl phosphonamidates **11**−**13** (Scheme 2).[33] Treatment of each of the phosphonamidates **11**, **12**, and **13** with a catalytic amount of selenium dioxide afforded C-HMBP phosphonamidates **14**, **15**, and **16**, in addition to aldehydes **17**, **18**, and **19**, respectively.

The C-HMBP phosphonamidates containing ethyl esters of glycine provide greater potency, on average, relative to methyl and isopropyl compounds.[28] Therefore, the ethyl ester compound **18** was taken to the target aldoxime (**20**).

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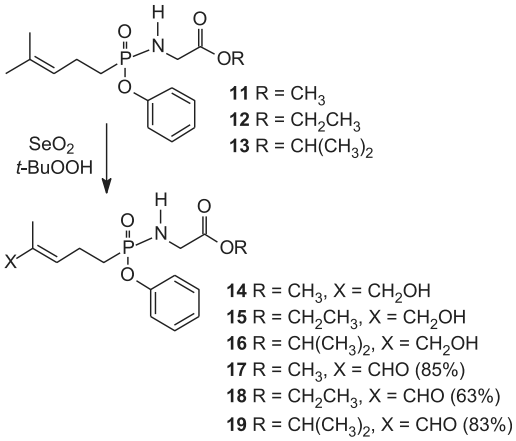
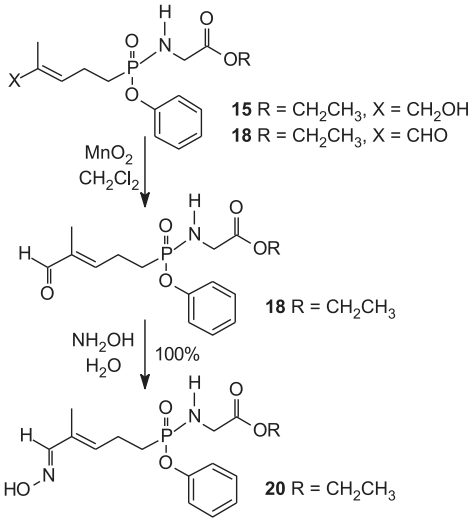
Scheme 2. Synthesis of C-HMBP C5 Aldehydes of Phosphonamidates and Their Previously Reported Alcohols[28]



Treatment of the alcohol/aldehyde mixture (**15**/**18**) with manganese dioxide in dichloromethane provided additional aldehyde for the next synthetic step (Scheme 3). Subsequent reaction with hydroxylamine in water allowed for rapid conversion to a quantitative amount of aldoxime **20**.

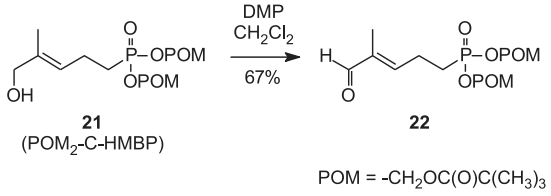
Scheme 3. Synthesis of C-HMBP C5 Modified Aldoxime



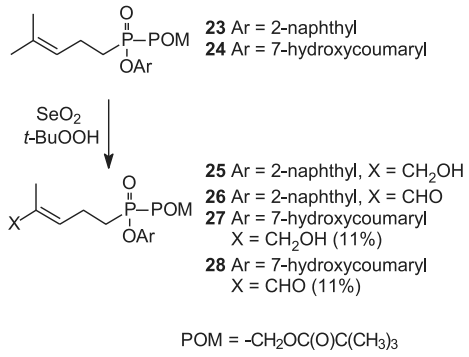
After establishing reaction conditions that modify the (*E*)-allylic alcohol of C-HMBP to an aldehyde, the bis-pivaloyloxymethyl (POM) derivative **22** and some mixed aryl acyloxyalkyl compounds were prepared to allow for comparison of their activities against other known prodrug forms of C-HMBP. Synthesis of compound **22** began with POM_2-C-HMBP (**21**).[19] Treatment of POM_2-C-HMBP with Dess–Martin periodinane (DMP) afforded the target bis-(acyloxyalkyl) ester **22**, in sufficient quantity to allow for bioassay of this aldehyde (Scheme 4).

Mixed aryl acyloxyalkyl forms of C-HMBP have been found to increase activity relative to C-HMBP.[33,41] To compare the new aldehydes across prodrug classes, we began our synthesis of the C5 aldehyde **26** with the 2-naphthyl phosphonate **23** (Scheme 5).[41] The coumaryl phosphonate **24** was prepared by a parallel sequence to that of compound **23** except 2-naphthol was replaced with 7-hydroxycoumarin. Finally, treatment of

Scheme 4. Synthesis of POM_2-C-HMBP Aldehyde by Oxidation with the Dess–Martin Periodinane



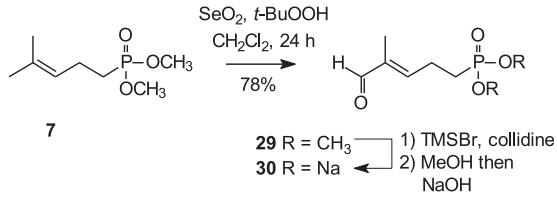
Scheme 5. Synthesis of C-HMBP Aldehydes of Mixed Aryl Acyloxyalkyl Phosphonate Esters



each mixed phosphonate ester, either **23** or **24**, with selenium dioxide resulted in oxidation to afford aldehydes **26** and **28**, respectively. Chromatography of the oxidation mixture also provided the corresponding alcohol counterpart (compounds **25**[33] and **27**) to each of the target aldehydes. Isolation of the aldehydes proceeded in sufficient quantities to allow bioassay without the need for an additional manganese dioxide or DMP oxidation step.

Finally, the last target of interest in this series was the aldehyde salt **30** (Scheme 6), whose activity could be

Scheme 6. Attempted Synthesis of the Aldehyde Salt 30



compared to salts studied previously. Unfortunately, this compound proved to be elusive. The dimethyl ester **7** was chosen as the starting phosphonate because methyl esters should be easiest to cleave.[42] Oxidation of this phosphonate to the corresponding aldehyde **29** could be accomplished in good yield when the SeO_2 oxidation was allowed to proceed for an extended time. However, all attempts to hydrolyze the ester **29** to the corresponding salt **30** under standard conditions went unrewarded. Because this might be due to the instability of the aldehyde itself,[38] further efforts were postponed pending the results of the biological studies on the available compounds.

All of the available compounds were tested for cellular toxicity to K562 cells using a cell viability assay (Figure S1). Only compounds **17**, **22**, and **26** showed cell toxicity, which was weak and only visible at a dose of 100 μM. This indicates that the presence of the aldehyde in these molecules is not inherently toxic to the cells. The newly synthesized compounds

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were next evaluated for their ability to stimulate proliferation of human Vγ9Vδ2 T cells from peripheral blood using a flow cytometry method to quantify the number of cells positive for the Vγ9Vδ2 TCR after stimulation and growth periods. The C-DMAP sodium salt **8** was completely inactive up to 100 μM. Additionally, C-DMAP phosphonamidate **10** was also completely inactive up to 100 μM (Table 1). This is in contrast to its corresponding C-HMBP 1-naphthyl/GlyOEt phosphonamidate which displayed an EC_{50} of 0.36 nM, a greater than 270,000-fold difference.[19,28] These findings support a model in which it is unlikely that DMAPP functions as a direct phosphoantigen but rather acts through cellular conversion to IPP or another endogenous phosphoantigen, though we cannot exclude an unrecognized defect in conversion of the C-DMAP prodrug to the active form.

Because the allylic alcohol of HMBPP plays an important role in the immune response of Vγ9Vδ2 T cells, we became interested in evaluation of a modified C-HMBP analog with a different hydrogen bond donor at this position. Despite the presence of a hydrogen bond donor at the C5 position, the aldoxime **20** was about 40-fold less potent relative to the corresponding aldehyde **18**, with an EC_{50} of 790 nM vs 20 nM, respectively. It is possible that differences in molecular geometry including bond length and position contribute to the observed difference in potency.

Evaluation of the new aldehydes revealed strong stimulants of Vγ9Vδ2 T cell proliferation at nanomolar concentrations. In this assay, the most potent of the aldehyde phosphonamidate set proved to be phenyl phosphonamidate **18** bearing an ethyl ester of glycine, with an EC_{50} of 20 nM. However, as a class, the aldehyde phosphonamidates (**17**−**19**) required an average of 44-fold higher concentrations to stimulate Vγ9Vδ2 T cells proliferation relative to their corresponding C-HMBP phenyl phosphonamidates. Nevertheless, the aldehydes required comparable concentrations to those reported for 3-formyl-1-butyl-diphosphate by Belmant and colleagues.[34] It is possible that the activity reported then was due to HMBPP itself, rather than an aldehyde. However, our findings clearly indicate that the presence of an aldehyde at a comparable position within the butyrophilin ligand affords potent derivatives of the analog of HMBPP (i.e., C-HMBP) which require nanomolar concentrations to elicit an immune response. Furthermore, these bioactivity results bolster our earlier SAR finding with C-HMBP phosphonamidates of glycine, showing increased potency for ethyl ester compounds relative to methyl and isopropyl esters.

The bis(acyloxyalkyl) ester **22** stimulated proliferation of Vγ9Vδ2 T cells at comparable effective concentrations to phosphonamidates **17** and **19**, with EC_{50} values of 47 nM and 49 nM, respectively. However, the activity of compound **22** was more similar to that of POM_2-C-HMBP (**21**), with the aldehyde only 9-fold less potent than its corresponding alcohol.

Aldehydes **26** and **28** of the mixed acyloxy aryloxy class[41] stimulate proliferation of Vγ9Vδ2 T cells at low nanomolar levels, with EC_{50} values of 4.6 nM and 12 nM, respectively. These two mixed aryl acyloxyalkyl esters with an aldehyde stimulated T cell proliferation at concentrations comparable to their corresponding alcohol, only requiring 2-fold higher concentrations, which was not statistically significant by 2-way ANOVA. A similar though less pronounced pattern of activity was observed in ELISA assays for detection of interferon γ (Table S2) produced by Vγ9Vδ2 T cells after exposure to phosphoantigen loaded K562 cells. Therefore, the allylic functionality on this prenyl phosphate framework can be altered concurrently with phosphonate protection to retain high potency.

Table 1. Activity of C5 Modified C-HMBP Compounds for Expansion of Vγ9Vδ2 T Cells from Peripheral Blood Mononuclear Cells[d]

#	Structure	cLog P	EC_{50} [μM] (95% CI)[a]	Fold difference vs. C-HMBP analog
4a[19]	X = CH_2OH R = OH R' = OH	-0.24	4.0	NA[b]
21[19]	X = CH_2OH R = POM R' = POM	3.42	0.0054	NA
27	X = CH_2OH R = POM R' = 7-HC	3.61	0.008 (0.0038 to 0.017)	NA
8	X = CH_3 R = OH R' = OH	1.00	>100	>25
10	X = CH_3 R = Gly-Et R' = 1-Nap	4.45	>100	>270,000
20	X = CHNOH R = Gly-Et R' = Phe	2.23	0.79 (0.11 to 5.8)	>2,100
17	X = CHO R = Gly-Me R' = Phe	2.12	0.047 (0.037 to 0.059)	31
18	X = CHO R = Gly-Et R' = Phe	2.50	0.020 (0.016 to 0.026)	56
19	X = CHO R = Gly-iPr R' = Phe	2.86	0.049 (0.030 to 0.078)	45
22	X = CHO R = POM R' = POM	3.87	0.047 (0.019 to 0.12)	9
28	X = CHO R = POM R' = 7-HC[c]	4.07	0.012 (0.0097 to 0.015)	2
26	X = CHO R = POM R' = 2-Nap	5.20	0.0046 (0.0026 to 0.0080)	2

[a]CI = confidence interval. [b]NA = not applicable (n = 3). [c]7HC = 7-hydroxycoumaryl. [d]Fold difference is relative to **4a** (for compound **8**) or its equivalent prodrug form (for other compounds) as previously determined.[19,28,41].

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Because the aldehydes may be reduced in the cytosol to the corresponding C-HMBP or form glutathione conjugates, we investigated the cellular metabolism of selected compounds. We used basic extraction conditions[43] and ion pairing LCMS[44] to quantify the phosphonate metabolites formed following treatment with selected prodrugs (Supporting Information). As expected, treatment of K562 cells for 1 h with the control prodrug **21** produced the free phosphonic acid payload in its allylic alcohol form, as well as the monoacid intermediate (Figure 2). Compound **21** was not converted to aldehyde

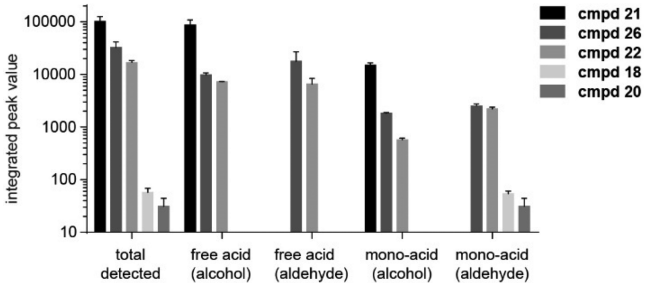

Figure 2. Conversion of selected aldehyde (or aldoxime) prodrugs to their free acid alcohol forms in K562 cells. Treatment for 1 h with the indicated compound results in a two-step biological release of the phosphoantigen payload from the prodrug groups to generate the phosphono monoacid and free-acid forms. Simultaneously, the allylic aldehyde is reduced to the alcohol form (n = 2).

forms. All of the compounds examined in this study (**18**, **20**, **22**, and **26**) were at least partially converted to the monoacid in its aldehyde (or aldoxime) form. Compounds **22** and **26** additionally drove formation of the free phosphonic acid aldehyde. Interestingly, both compounds also formed the free acid alcohol. Measurable reduction of the aldehydes to their alcohol forms was observed in the intact prodrugs, the monoacids, and the free acids. Masses consistent with compound **22** metabolites in $[M+307-H]^-$ form were also found, indicative of glutathione conjugation. Dehydration to the DMAP monoacid was also observed. Taken together, these aldehydes are reduced to the alcohol forms in cells at significant levels, and the amount of alcohol form generated correlates with the potency of the prodrug forms in cellular assays.

In conclusion, these studies report novel modifications to the allylic framework of C-HMBP and their impact on human Vγ9Vδ2 T cell proliferation. Removal of the (*E*)-allylic alcohol completely abrogates activity, while replacement with an aldoxime (i.e., **20**) reduces but does not eliminate activity. In contrast, aldehyde replacements are well tolerated. Six examples including phosphonamidate, bis-acyloxy ester, and mixed acyloxy aryloxy ester forms of C-HMBP are provided, all modified to include an aldehyde and all function as stimulants of Vγ9Vδ2 T cell proliferation at nanomolar concentrations, with two aldehydes (**26** and **28**) having similar activity to alcohol **21**. The latter two compounds were metabolized in cells to produce the corresponding alcohol form. We suspect that the combination of the aldehyde with a rapidly metabolized prodrug form[45] enables cell entry and conversion to the alcohol form, whereas the slower uptake of phosphonamidate forms may allow for extracellular aldehyde metabolism that slows cell entry. In theory, this aldehyde modification could serve as a synthetic handle for further derivation of C-HMBP analogs. One example of such a modification, the quantitative conversion of an aldehyde (**18**) to its corresponding C5 aldoxime (**20**) is reported here.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsmedchemlett.0c00586.

Experimental procedures for synthetic chemistry, bioassay protocols, LC traces, and ^{1}H, ^{13}C, and ^{31}P NMR spectra (PDF)

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$^{\nabla}$N.A.L. and C.M.S. contributed equally to this manuscript. The manuscript was written through contributions of all authors, and all authors have given approval to the final version of the manuscript.

Notes

The authors declare the following competing financial interest(s): A.J.W. and D.F.W. own shares in Terpenoid Therapeutics, Inc. The current work did not involve the

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company. The other authors have no financial conflicts of interest.

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