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Pivaloyloxymethyl-modified isoprenoid bisphosphonates display enhanced inhibition of cellular geranylgeranylation

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Abstract—Nitrogenous bisphosphonate inhibitors of farnesyl disphosphate synthase have been used clinically for treatment of bone disease. Because many of their effects may be mediated by depletion of geranylgeranyl diphosphate, our group has sought compounds that do this more directly through inhibition of geranylgeranyl diphosphate synthase and we have discovered a number of isoprenoid-containing bisphosphonates that selectively inhibit this enzyme. These compounds have a high negative charge at physiological pH which is necessary for inhibition of the enzyme but may limit their ability to enter cells. Therefore, chemical modifications that mask this charge may enhance their cellular potency. We now have synthesized novel pivaloyloxymethyl-modified isoprenoid bisphosphonates and investigated their ability to inhibit protein geranylgeranylation within cells. We have found that addition of pivaloyloxymethyl moieties to isoprenoid bisphosphonates increases their potency towards cellular geranylgeranylation even though this modification decreases their in vitro inhibition of geranylgeranyl diphosphate synthase. Pivaloyloxymethyl modifications more effectively increase the cellular activity of the more polar isoprenoid bisphosphonates. These results reveal structural relationships between in vitro and cellular activity which may serve as the basis for future development of more potent and/or drug-like inhibitors of geranylgeranyl diphosphate synthase.

1. Introduction

The nitrogenous bisphosphonates (NBPs, Fig. 1) such as pamidronate (**1**), alendronate (**2**), zoledrodrate (**3**) and risedronate (**4**), are structural analogs of diphosphates that are used clinically to treat disorders associated with low bone density including osteoporosis and metastatic bone disease.[1,2] The clinical NBPs are useful in this regard because their negative charge at physiological pH together with the C-1 hydroxyl group leads to very high affinity for bone matrices.[3] In laboratory settings, these drugs also have been shown to have direct growth inhibitory effects on malignant cells,[4,5] to treat animal models of parasitic infections effectively,[6,7] and to activate γδ T-cells.[8] Their clinical use in this regard is more problematic because of poor oral bioavailability,[9–11] rapid elimination, and low distribution to parts of the body other than the bone.[12]

The clinical NBPs deplete cells of farnesyl diphosphate (FPP) and the larger isoprenoid diphosphates that are required for post-translational protein isoprenylation, a modification necessary for activation of many small GTPases.[13] While the NBPs inhibit farnesyl diphosphate synthase (FDPS)[14–18] and thus directly impact farnesyl diphosphate (FPP) synthesis, their cellular effects may be largely a result of downstream geranylgeranyl diphosphate (GGPP) depletion.[19–23] Based on this rationale, we developed a series of bisphosphonates that inhi-

Abbreviations: IPP, isopentenyl diphosphate; FPP, farnesyl diphosphate; GGPP, geranylgeranyl diphosphate; GGDPS, geranylgeranyl diphosphate synthase; FDPS, farnesyl diphosphate synthase; NBP, nitrogenous bisphosphonate.

Keywords: Bisphosphonate; Geranylgeranyl diphosphate; Geranylgeranyl pyrophosphate; Geranylgeranyl diphosphate synthase; Farnesyl diphosphate; Ras; Rap1a; Prenylation; Geranylgeranylation; Prodrug; Pivaloyloxymethyl; POM.

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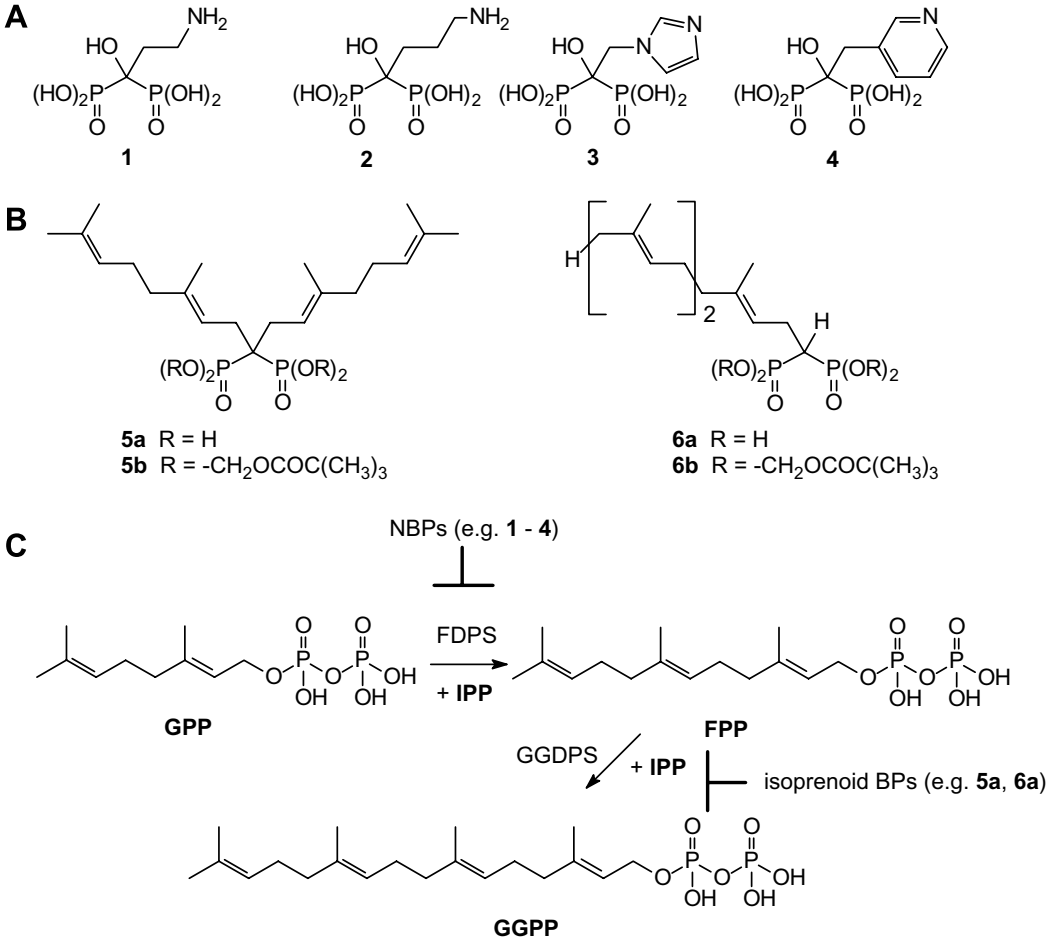


Figure 1. (A) Chemical structures of relevant clinical nitrogenous bisphosphonates pamidronate (**1**), alendronate (**2**), zoledronate (**3**), and risedronate (**4**); (B) chemical structures of isoprenoid bisphosphonates digeranyl bisphosphonate (**5a**), POM-modified digeranyl bisphosphonate (**5b**), farnesyl bisphosphonate (**6a**), and POM-modified farnesyl bisphosphonate (**6b**); (C) inhibition of farnesyl diphosphate synthase by nitrogenous bisphosphonates prevents the biosynthesis of both FPP and GGPP, while inhibition of geranylgeranyl diphosphate synthase by isoprenoid bisphosphonates such as **5a** and **6a** prevents biosynthesis of GGPP and not FPP.

bit geranylgeranyl diphosphate synthase (GGDPS) and directly diminish cellular GGPP levels[24–27] without inhibiting FDPS.

Our library of isoprenoid bisphosphonates can be divided into two groups, dialkyl bisphosphonates that bear two isoprene or isoprenoid substituents and monoalkyl compounds that contain one isoprene group (Fig. 2). We have shown that both types of compounds, including digeranyl bisphosphonate (**5a**) and (*2E, 6E*)- farnesyl bisphosphonate (**6a**), can potently inhibit GGDPS in vitro.[26,27] All of the isoprenoid bisphosphonates are more lipophilic than the clinical NBPs **1**–**4** (which have negative values of *C*log *P*) and thus may exhibit cellular efficacy at significantly lower concentrations. However, like the NBPs, some of the more polar isoprenoid bisphosphonate GGDPS inhibitors exhibit low cellular potency. Therefore, we decided to design and synthesize derivatives of these compounds that temporarily mask their negative charge (i.e. prodrugs). Ideally, these derivatives would enhance the ability of the compounds to enter cells and then be cleaved to yield the active parent compound in effective intracellular concentration.

A prodrug approach has been considered in the case of the NBPs and it was thought that this strategy would be useful to decrease undesirable side effects, increase bioavailability, and/or change the distribution of the drugs to more beneficial locales.[28–30] One report included several pivaloyloxymethyl (POM)-modified compounds in a study of more than 60 bisphosphonates, and found the POM compounds to be the most active in several cell lines.[31] A prodrug approach also has been tried successfully with the use of peptide-modified derivatives of phosphonates[32] and bisphosphonates[33] that increase oral absorption. The oral bioavailability of a POM-modified bisphosphonate inhibitor of squalene synthase was dramatically increased relative to its parent salt.[34] The POM derivative of etidronate has been shown to effectively release the parent drug in liver homogenate following cleavage by unknown esterases.[35] One POM-modified acyclic nucleoside phosphonate had a measured half-life of 14 h in buffer but this fell to just minutes in homogenates of different canine tissues.[36] Therefore, we chose to prepare POM-protected isoprenoid bisphosphonates and study their effectiveness as cellular GGDPS inhibitors.

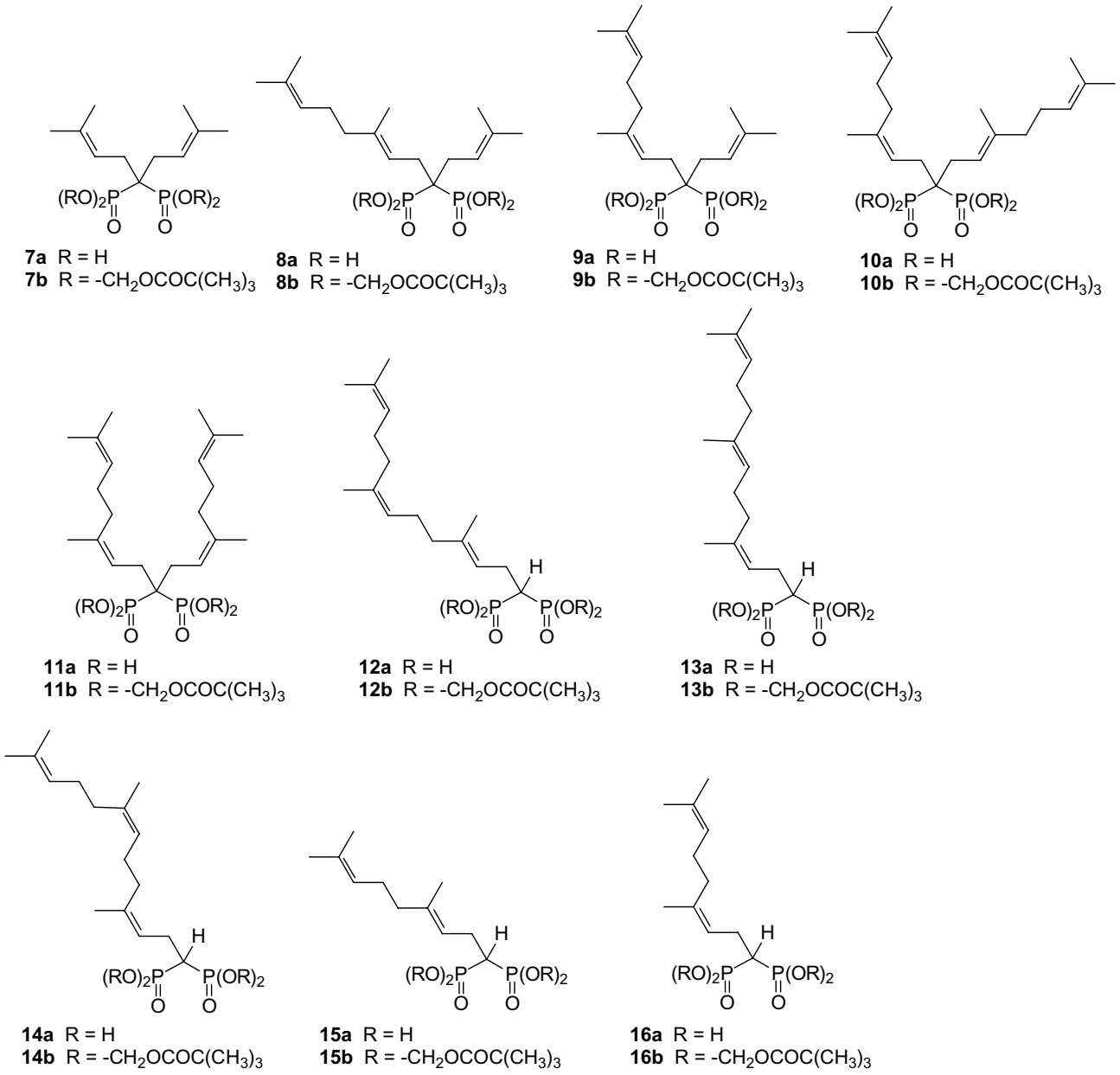
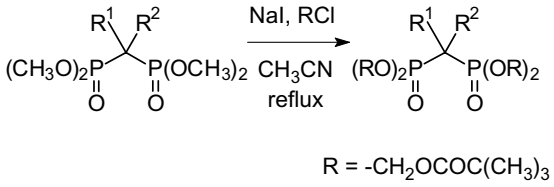


Figure 2. Chemical structures of isoprenoid bisphosphonates examined for biological activity. Compounds were tested both as isoprenoid bisphosphonic acids (**a**) and POM-modified isoprenoid bisphosphonates (**b**).

2. Results and discussion

Synthesis of the POM-modified isoprenoid bisphosphonate isomers **5b**–**14b** began with the respective tetramethyl bisphosphonate esters. The methyl esters already have been described[27,37] or were prepared through a reaction sequence parallel to that used to prepare the corresponding tetraethyl esters.[24,38] Reaction with commercial chloromethyl pivalate (POMCl) and sodium iodide under reflux (Scheme 1) afforded the desired POM-modified isoprenoid bisphosphonates **5b**–**14b** (Table 1) in marginal to modest yields.



Scheme 1. General method for production of POM-modified isoprenoid bisphosphonates.

Addition of the POM moiety to these compounds results in significantly increased hydrophobicity as reflected by changes in the calculated octanol:water partition coefficients (*C*log *P*) of as much as seven orders of magnitude (Table 2). The hydrophobicity of the isoprenoid bisphosphonate acids and their respective POM analogs is consistently much greater than those of the clinical NBPs. While addition of the POM moiety increases

Table 1. Isolated yields for the synthesis of bisphosphonate POM esters according to Scheme 1

Product	Side chains		Yield (%)
	R^1	R^2	
Dialkyl			
5b	Geranyl	Geranyl	18
7b	Prenyl	Prenyl	47
8b	Geranyl	Prenyl	26
9b	Neryl	Prenyl	49
10b	Neryl	Geranyl	42
11b	Neryl	Neryl	39
Monoalkyl			
6b	Farnesyl	H	40
12b	(2*E*,6*Z*)-Farnesyl	H	14
13b	(2*Z*,6*E*)-Farnesyl	H	20
14b	(2*Z*,6*Z*)-Farnesyl	H	16
15b	Geranyl	H	53
16b	Neryl	H	29

the hydrophobicity of all the isoprenoid bisphosphonates, the magnitude of the increase is much larger for the short-chain compounds, which as free acids exhibit higher charge to mass ratios than the longer-chain compounds.

Though it has been shown previously that POM moieties are cleaved from phosphonates in vivo, it was possible that this is not the case for these specific compounds. Observation of a cellular effect would indicate hydrolysis as long as the POM compounds do not inhibit GGDPS in vitro. To determine if the POM-modified compounds are capable of direct GGDPS inhibition, the POM-modified versions (**5b**, **6b**, **8b**) of three of the most potent inhibitors (**5a**, **6a**, and **8a**) of GGDPS were tested for in vitro inhibition of this enzyme. While the bisphosphonic acids **5a** and **6a** have IC_{50}'s for GGDPS in vitro at concentrations below 1 μM,[27,37] the POM-modified isoprenoid bisphosphonates show no activity at 10 μM levels against GGDPS (Fig. 3a). This indi-

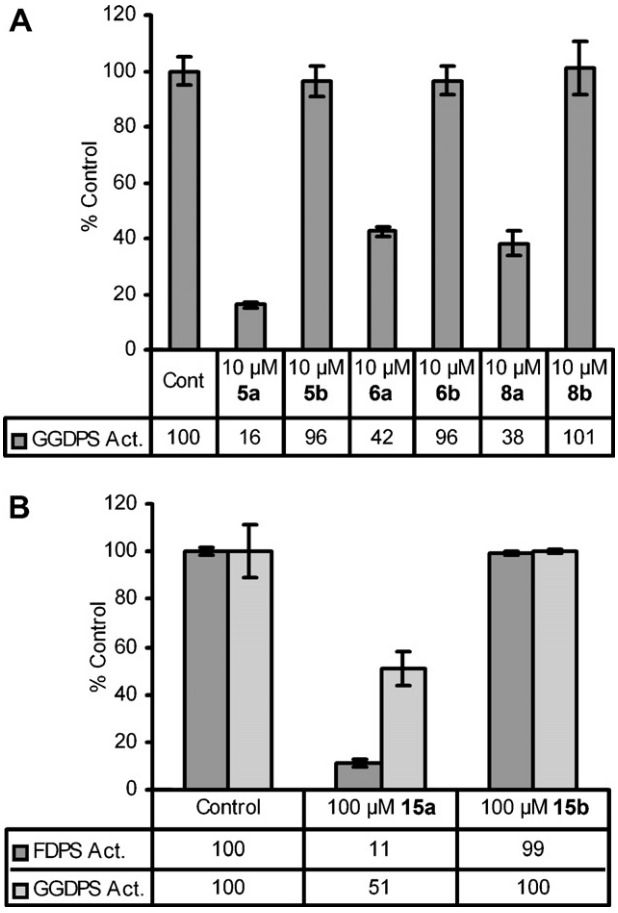


Figure 3. Inhibition of FDPS and GGDPS by selected bisphosphonates. In vitro enzyme assays were performed as described in Section 4 ($n = 3$). (A) GGDPS activity as a percentage of control in the presence or absence of 10 μM of the indicated bisphosphonic acids (**5a**, **6a**, **8a**) or 10 μM of the respective POM-modified bisphosphonates (**5b, 6b, 8b**). (B) Compound **15** (100 μM) inhibits both FDPS and GGDPS as the free acid, but the POM-modified compound does not inhibit either enzyme. All values for bisphosphonic acids differ significantly from control and respective POM-modified bisphosphonates ($p < 0.05$).

Table 2. Hydrophobic modifications increase the toxicity of bisphosphonic acids

	Side chains		Calculated *C*log*P*		K562 toxicity (GI_{50} [μM])	
	R^1	R^2	Acid (a)	POM (b)	Acid (a)	POM (b)
Dialkyl						
5	Geranyl	Geranyl	5.4	11	>100	58
7	Prenyl	Prenyl	1.7	9	>100	7.7
8	Geranyl	Prenyl	3.5	9.9	>100	5.2
9	Neryl	Prenyl	3.5	9.9	>100	21
10	Geranyl	Neryl	5.4	11	>100	>100
11	Neryl	Neryl	5.4	11	>100	>100
Monoalkyl						
6	(2*E*,6*E*)-Farnesyl	H	3.4	9.7	90	7.9
12	(2*E*,6*Z*)-Farnesyl	H	3.4	9.7	nd	2.7
13	(2*Z*,6*E*)-Farnesyl	H	3.4	9.7	nd	2.0
14	(2*Z*,6*Z*)-Farnesyl	H	3.4	9.7	nd	2.9
15	Geranyl	H	1.6	8.9	>100	8.1
16	Neryl	H	1.6	8.9	>100	7.0

Calculated *C*log*P* values for isoprenoid bisphosphonic acids and POM-modified isoprenoid bisphosphonates were determined as described in Section 4. Toxicities of isoprenoid bisphosphonic acids versus POM compounds were determined from quadruplicate dose–response curves of ^3H thymidine incorporation assays at 24 h (nd, not determined).

cates, whether through masking the negative charges or increasing steric hindrance, that the addition of POM moieties to isoprenoid bisphosphonates prevents them from inhibiting GGDPS. This would be expected based on crystal structures of GGDPS which show tight ionic interactions between the enzyme, magnesium ions, and the isoprenoid diphosphate.[39] The inability of the POM-modified isoprenoid bisphosphonates to inhibit GGDPS in vitro means it is highly unlikely that they would inhibit cellular GGDPS without cleavage of the POM groups. Therefore, demonstration of activity in cellular assays for GGDPS inhibition would indicate that these compounds serve as prodrugs.

It should be noted that some of the parent isoprenoid bisphosphonates may inhibit multiple enzymes involved with isoprenoid biosynthesis. For example, farnesyl bisphosphonate (**5a**) has been shown to inhibit squalene synthase, GGDPS, and farnesyltransferase.[27,40,41] We had previously shown that digeranyl bisphosphonate does not inhibit FDPS,[26] but we had not tested the other compounds in our library due to insufficient quantities of purified enzyme. In light of that, all of our compounds of geranyl length or shorter (**5a**, **7a**–**11a**, **15a**, **16a**) now have been tested for their ability to inhibit FDPS, and of those, only geranyl bisphosphonate (**15a**) displayed an IC_{50} below 100 μM. This establishes for the first time that geranyl bisphosphonate inhibits both FDPS (IC_{50} = 20 μM) and GGDPS (Fig. 3b). Although geranyl bisphosphonate is not a potent inhibitor of either enzyme, it may serve as a starting point for further design of dual inhibitors of FDPS and GGDPS. However, it is likely that the addition of a second isoprene chain both enhances the ability of a bisphosphonate to inhibit GGDPS and also decreases its ability to inhibit FDPS.

The POM-modified compounds were assessed for cytotoxicity by ^{3}H thymidine incorporation in K562 chronic myelogenous leukemia cells (Table 2). All of the previously described isoprenoid bisphosphonic acids, as well as the clinical NBPs, generally exhibit low cytotoxicity levels at 24 h with GI_{50} values routinely above 100 μM in K562 cells. The POM-modified isoprenoid bisphosphonates are significantly more cytotoxic with 24 h GI_{50} values in the sub 10 μM range. Because the POM group itself is not significantly toxic in this range, this finding indicates that addition of the POM moiety enhances cellular internalization of the isoprenoid bisphosphonates. The toxicity was most enhanced for short-chain dialkyl bisphosphonates **7b**–**9b** and for all mono alkyl bisphosphonates tested (**6b**, **12b**–**16b**). The toxicity was enhanced less for the compounds which contained the greatest number of carbon atoms (i.e. the lowest charge to mass ratio in the bisphosphonic acids). This suggests a threshold for internalization with these compounds in that the negative charge on the larger isoprenoid bisphosphonates does not dramatically diminish their ability to enter cells.

Finally the compounds were tested for their ability to inhibit protein geranylgeranylation in K562 cells (Fig. 4). As a model for protein geranylgeranylation, we studied

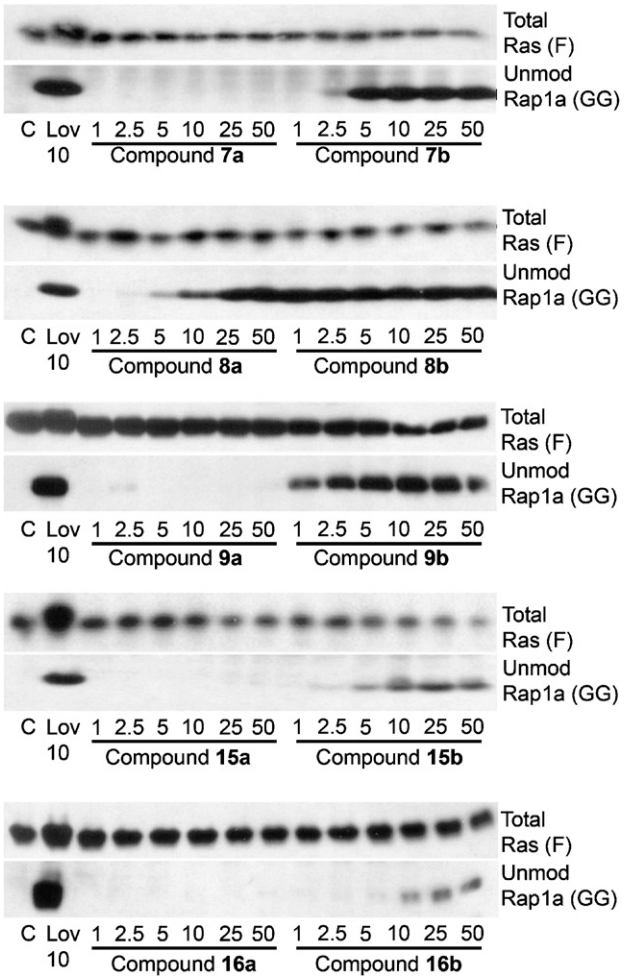


Figure 4. Western blot analysis of K562 cells treated with indicated pairs of free acids and POM-modified isoprenoid bisphosphonates. Inhibition of protein geranylgeranylation is depicted by the appearance of an unmodified Rap1a band.

the accumulation of non-geranylgeranylated Rap1a by Western blot analysis as described previously.[24] Because the Rap1a antibody detects only the unmodified protein, inhibition of Rap1a geranylgeranylation can be determined by the appearance of a band on the Western blot analysis. In contrast, the Ras antibody detects both a lower modified Ras band and an upper unmodified Ras band, and therefore inhibition of Ras farnesylation is detected by the appearance of the upper band. The ability of some isoprenoid bisphosphonates to inhibit cellular geranylgeranylation is significantly enhanced by addition of the POM moiety. This supports the assumption that once inside the cells, the POM moiety is cleaved to release the active parent compound. The most striking examples of this effect are displayed by compounds **7**–**9**,**15**, and **16**. In particular, bisphosphonic acids **7a** and **9a** both display greater than 50 μM IC_{50} values for inhibition of both FDPS and GGDPS in vitro and no inhibition of protein geranylgeranylation at 50 μM concentrations. However, the corresponding POM-modified compounds **7b** and **9b** were able to inhibit cellular geranylgeranylation at concentrations well below 10 μM, and they did so without any effect on pro-

Table 3. Inhibition of geranylgeranylation by isoprenoid bisphosphonic acids and POM-modified isoprenoid bisphosphonates

	Side chains		GGDPS IC_{50} (μM)	Inhibition of Rap1a modification in intact K562 cells (μM)	
	R^1	R^2	Acid (a)	Acid (a)	POM (b)
Dialkyl					
5	Geranyl	Geranyl	0.2	25	10
7	Prenyl	Prenyl	>100	>50	5
8	Geranyl	Prenyl	3	25	1
9	Neryl	Prenyl	60	50	5
10	Geranyl	Neryl	7	25	25
11	Neryl	Neryl	6	25	10
Monoalkyl					
6	Farnesyl	H	0.1	10	10
12	(2*E*,6*Z*)-Farnesyl	H	0.6	50	10
13	(2*Z*,6*E*)-Farnesyl	H	40	>50	>50
14	(2*Z*,6*Z*)-Farnesyl	H	70	>50	>50
15	Geranyl	H	10	>50	10
16	Neryl	H	100	>50	>50

GGDPS IC_{50} values for free acids were determined previously.[27] To determine Rap1a inhibition, cells were incubated with 1, 2.5, 5, 10, 25, and 50 μM concentrations of each compound. The experimental concentration required for cellular inhibition of Rap1a processing equivalent to 10 μM of lovastatin was determined by quantitative Western blot analysis.

tein farnesylation. This suggests that the cellular membrane may act as a barrier to both entry and exit of the isoprenoid bisphosphonic acid. Notably, compound **15a**, which is a weak dual inhibitor of FDPS and GGDPS in vitro, potently and preferentially inhibits cellular geranylgeranylation when POM-modified (**15b**).

As a group, the POM-modified compounds generally exhibit enhanced cellular potency for inhibition of geranylgeranylation (Table 3). Parallel to the trend in toxicity, addition of the POM moiety confers the largest increase in ability to inhibit cellular geranylgeranylation upon the compounds with the highest charge to mass ratios, specifically those with side chains of 10 carbon atoms or less and 16 or fewer total carbon atoms. Previously studied isoprenoid bisphosphonates that inhibit geranylgeranylation as free acids also significantly inhibit cellular geranylgeranylation when POM-modified (e.g. **5**, **6**, and **8**). However, addition of the POM moiety does not produce effects that are as dramatic because these compounds are more hydrophobic in themselves.

3. Conclusions

It is clear that GGDPS is emerging as a novel cellular target with possible applications in the treatment of bone diseases. Based on the recent revelations of inhibitory diphosphate-binding sites[39] and multiple potential inhibitor[42] binding sites on GGDPS, it is perhaps not surprising that dialkyl isoprenoid bisphosphonates such as digeranyl bisphosphonate **5a** potently inhibit this enzyme. However, in order for these types of compounds to be useful in targeting locations distinct from the bone, it most likely would be necessary to employ a prodrug strategy. Because it has been shown that NBPs as free acids enter the cell through endocytosis[43,44] and treatment with high concentrations is necessary to see biological effects, a prodrug strategy may also be a useful way to increase their cellular activity. Results presented herein clearly demonstrate the ability of bisphosphonate prodrug modifications to enhance the cellular potency of isoprenoid bisphosphonates.

4. Experimental

4.1. Chemical synthesis

4.1.1. General procedure for synthesis of pivaloyloxymethyl bisphosphonate esters. To bisphosphonate starting material (1 equiv) dissolved in acetonitrile was added POMCl (5 equiv) and sodium iodide (4 equiv) at room temperature under an inert atmosphere of argon. The reaction mixture was allowed to reflux overnight before addition of water and ether. The quenched material was extracted with ether before a sodium thiosulfate wash of the organic layers. The combined organic extracts were then dried ($MgSO_4$) and concentrated under vacuo. The resulting yellow residue was purified via flash chromatography (silica gel, 25% ether in hexanes) to afford the desired POM bisphosphonate ester.

4.1.1.1. Tetrapivaloyloxymethyl (6*E*,11*E*)-2,6,12,16-tetramethylheptadeca-2,6,11,15-tetraene-9,9-diyldiphosphonate (5b). Yield: 99 mg, 18%; ^{1}H NMR δ 5.64–5.76 (m, 8H), 5.31(t, J = 7.1 Hz, 2H), 5.05–5.10 (m, 2H), 2.57(td, J_{HP} = 16.7 Hz, J = 7.2, 4H), 1.94–2.08 (m, 8H), 1.67 (s, 6H), 1.61 (s, 6H), 1.59 (s, 6H), 1.23 (s, 36H); ^{13}C NMR δ 176.9 (4C), 139.0 (2C), 131.6 (2C), 124.4 (2C), 117.9 (t, J_{CP} = 7.5 Hz, 2C), 82.4 (t, J_{CP} = 2.9 Hz, 4C), 46.6 (t, J_{CP} = 130.7H), 40.3 (2C), 38.8 (4C), 28.6–28.7 (m, 2C), 27.1 (12C), 26.8 (2C), 25.9 (2C), 17.9 (2C), 16.4 (2C); ^{31}P NMR δ 25.0. Anal. Calcd for $C_{45}H_{78}O_{14}P_2$: C, 59.72; H, 8.69. Found: C, 59.72; H, 8.71.

4.1.1.2. Tetrapivaloyloxymethyl (3*E*,7*E*)-4,8,12-trimethyltrideca-3,7,11-triene-1,1-diyldiphosphonate (6b). Yield: 1.07 g, 40%; ^{1}H NMR δ 5.65–5.78 (m, 8H), 5.19–5.28 (m, 1H), 5.06–5.14 (m, 2H), 2.42–2.73 (m, 3H), 1.93–2.11 (m, 8H), 1.68 (s, 3H), 1.56–1.65 (m, 9H), 1.23 (s, 36H); ^{13}C NMR δ 176.95 (2C), 176.92 (2C), 138.5, 135.4, 131.4, 124.5, 124.1, 120.4 (t, J_{CP} = 7.5 Hz), 82.1–82.4 (m, 4C), 39.93, 39.9, 38.9 (4C), 38.8 (t, J_{CP} = 132.9 Hz), 30.5, 26.9 (12C), 26.8, 25.9, 23.7 (t, J_{CP} = 5.2 Hz), 17.9, 16.3, 16.2; ^{31}P NMR δ 22.7. Anal. Calcd for $C_{40}H_{70}O_{14}P_2$: C, 57.40; H, 8.43. Found: C, 57.67; H, 8.70.

4.1.1.3. Tetrapivaloyloxymethyl 2,8-dimethylnona-2,7-diene-5,5-diyldiphosphonate (7b). Yield: 709 mg, 47%; ^{1}H NMR δ 5.62–5.77 (m, 8H), 5.27 (t, J = 7.5 Hz, 2H), 2.61 (td, J_{HP} = 17.9 Hz, J = 7.5, 4H), 1.72 (s, 6H), 1.61 (s, 6H), 1.23 (s, 36H); ^{13}C NMR δ 176.9 (4C), 135.4 (2C), 117.9 (t, J_{CP} = 8.25 Hz, 2C), 82.3 (4C), 46.7 (t, J_{CP} = 132 Hz, 1C), 38.9 (4C), 28.8 (t, J_{CP} = 7.5 Hz, 2C), 27.1 (12C), 26.3 (2C), 18.1 (2C); ^{31}P NMR δ 25.6; HRMS (ESI, *m*/*z*) calcd for $(M+H)^+$ $C_{35}H_{63}O_{14}P_2$: 769.3693. Found: 769.3693.

4.1.1.4. Tetrapivaloyloxymethyl (*E*)-2,8,12-trimethyltrideca-2,7,11-triene-5,5-diyldiphosphonate (8b). Yield: 6 mg, 26%; ^{1}H NMR δ 5.57–5.79 (m, 8H), 5.21–5.36 (m, 2H), 5.02–5.14 (m, 1H), 2.53–2.72 (m, 4H), 1.95–2.13 (m, 4H), 1.45–1.75 (m, 15H), 1.25 (s, 36H); ^{13}C NMR δ 176.9 (4C), 139.0, 135.3, 131.6, 124.3 (2C), 117.7–118.2 (m,1C), 82.3 (4C), 46.6 (t, J_{CP} = 130.5 Hz), 40.2, 38.9 (4C), 30.5, 28.7, 27.0 (12C), 26.7, 26.2, 25.8, 18.1, 17.8, 16.4; ^{31}P NMR δ 26.0; HRMS (ESI, *m*/*z*) calcd for $(M+Na)^+$ $C_{40}H_{70}O_{14}P_2$: 859.4139. Found: 859.4134.

4.1.1.5. Tetrapivaloyloxymethyl (*Z*)-2,8,12-trimethyltrideca-2,7,11-triene-5,5-diyldiphosphonate (9b). Yield: 148 mg, 49%; ^{1}H NMR δ 5.78–5.62 (m, 8H), 5.36–5.24 (m, 2H), 5.14–5.05 (m, 1H), 2.70–2.52 (m, 4H), 2.06–2.00 (m, 4H), 1.72 (s, 6H), 1.67 (s, 3H), 1.60 (s, 6H), 1.23 (s, 36H); ^{13}C NMR δ 176.8 (2C), 176.6 (2C), 138.9, 135.3, 131.7, 124.2, 118.5 (t, J_{CP} = 8.0 Hz), 117.9 (t, J_{CP} = 8.1 Hz), 82.3 (t, J_{CP} = 2.7 Hz, 4C), 46.5 (t, J_{CP} = 130.1 Hz), 38.8 (4C), 32.0, 28.9 (t, J_{CP} = 3.9 Hz), 28.4 (t, J_{CP} = 5.2 Hz), 27.0 (12C), 26.4, 26.2, 25.8, 23.9, 18.1, 17.7. ^{31}P NMR δ 25.6; HRMS (ESI, *m*/*z*) calcd for $(M+Na)^+$ $C_{40}H_{70}O_{14}P_2$: 859.4139. Found: 859.4144.

4.1.1.6. Tetrapivaloyloxymethyl (6*Z*,11*E*)-2,6,12,16-tetramethylheptadeca-2,6,11,15-tetraene-9,9-diyldiphosphonate (10b). Yield: 218 mg, 42%; ^{1}H NMR δ 5.79–5.60 (m, 8H), 5.36–5.26 (m, 2H), 5.14–5.04 (m, 2H), 2.62 (td, J_{HP} = 16.5 Hz, J = 6.9 Hz, 4H), 2.12–1.90 (m, 8H), 1.72 (s, 3H), 1.67 (s, 6H), 1.59 (s, 9H), 1.23 (s, 36H); ^{13}C NMR δ 176.8 (2C), 176.7 (2C), 139.0, 138.9, 131.8, 131.5, 124.3, 124.2, 118.5 (t, J_{CP} = 6.5 Hz), 117.8 (t, J_{CP} = 7.6 Hz), 82.4–82.0 (m, 4C), 46.5 (t, J_{CP} = 130.4 Hz), 40.2, 38.8 (4C), 32.0, 28.8 (t, J_{CP} = 3.6 Hz), 28.4 (t, J_{CP} = 4.9 Hz), 27.0 (12C), 26.8, 26.4, 25.8 (2C), 23.9, 17.8, 17.7, 16.3; ^{31}P NMR δ 25.7; HRMS (ESI, *m*/*z*) calcd for $(M+Na)^+$ $C_{45}H_{78}O_{14}P_2$: 927.4764. Found: 927.4762.

4.1.1.7. Tetrapivaloyloxymethyl (6*Z*,11*Z*)-2,6,12,16-tetramethylheptadeca-2,6,11,15-tetraene-9,9-diyldiphosphonate (11b). Yield: 608 mg, 39%; ^{1}H NMR δ 5.77–5.62 (m, 8H), 5.32 (t, J = 6.6 Hz, 2H), 5.14–5.05 (m, 2H), 2.62 (td, J_{HP} = 17.1 Hz, J = 1.2, 4H), 2.16–1.96 (m, 8H), 1.72 (s, 3H), 1.71 (s, 3H), 1.67 (s, 6H), 1.60 (s, 3H), 1.59 (s, 3H), 1.23 (s, 36H); ^{13}C NMR δ 176.8 (4C), 139.0 (2C), 131.7 (2C), 124.2 (2C), 118.4 (t, J_{CP} = 7.1 Hz, 2C) 82.3 (t, J_{CP} = 2.7 Hz, 4C), 46.4 (t, J_{CP} = 130.8 Hz), 38.8 (4C), 32.0 (2C), 28.5 (t, J_{CP} = 4.5 Hz, 2C), 27.0 (12C), 26.5 (2C), 25.4 (2C), 23.9 (2C), 17.7 (2C); ^{31}P NMR δ 25.5; HRMS (ESI, *m*/*z*) calcd for $(M+K)^+$ $C_{45}H_{78}O_{14}P_2$: 943.4504. Found: 943.4506.

4.1.1.8. Tetrapivaloyloxymethyl (3*E*,7*Z*)-4,8,12-trimethyltrideca-3,7,11-triene-1,1-diyldiphosphonate (12b). Yield: 117 mg, 14%; ^{1}H NMR δ 5.80–5.66 (m, 8H), 5.28–5.18 (m, 1H), 5.16–5.03 (m, 2H), 2.74–2.42 (m, 3H), 2.20–1.80 (m, 8H), 1.69 (s, 3H), 1.61 (s, 9H), 1.23 (s, 36H); ^{13}C NMR δ 176.9 (2C), 176.8 (2C), 138.4, 135.3, 131.4, 125.7, 124.2, 120.3 (t, J_{CP} = 3.9 Hz), 82.5–81.9 (m, 4C), 39.9, 38.9 (4C), 38.7 (t, J_{CP} = 132.9 Hz), 32.1, 27.0 (12C), 26.7, 26.4, 25.8, 23.6 (t, J_{CP} = 5.0 Hz), 23.4, 17.8, 16.2; ^{31}P NMR δ 23.0; HRMS (ESI, *m*/*z*) calcd for $(M+Na)^+$ $C_{40}H_{70}O_{14}P_2$: 859.4139. Found: 859.4137.

4.1.1.9. Tetrapivaloyloxymethyl (3*Z*,7*E*)-4,8,12-trimethyltrideca-3,7,11-triene-1,1-diyldiphosphonate (13b). Yield: 124 mg, 20%; ^{1}H NMR δ 5.82–5.62 (m, 8H), 5.22 (t, J = 6.3 Hz, 1H), 5.17–5.06 (m, 2H), 2.75–2.40 (m, 3H), 2.16–1.92 (m, 8H), 1.70 (s, 3H). 1.68 (s, 3H), 1.60 (s, 6H), 1.24 (s, 36H); ^{13}C NMR δ 176.9 (2C), 176.8 (2C), 138.4, 135.6, 131.4, 124.5, 123.9, 121.1 (t, J_{CP} = 6.8 Hz), 82.4–81.9 (m, 4C), 39.9, 38.8 (4C), 38.7 (t, J_{CP} = 130.8 Hz), 31.9, 27.0 (12C), 26.8, 26.4, 25.8, 23.7, 23.4 (t, J_{CP} = 5.0 Hz), 17.8, 16.1; ^{31}P NMR δ 23.0; HRMS (ESI, *m*/*z*) calcd for $(M+Na)^+$ $C_{40}H_{70}O_{14}P_2$: 859.4139. Found: 859.4144.

4.1.1.10. Tetrapivaloyloxymethyl (3*Z*,7*Z*)-4,8,12-trimethyltrideca-3,7,11-triene-1,1-diyldiphosphonate (14b). Yield: 78 mg, 16%; ^{1}H NMR ($CDCl_3$) δ 5.75–5.65 (m, 8H), 5.22 (t, J = 4.2 Hz, 1H), 5.14–5.06 (m, 2H), 2.71–2.44 (m, 3H), 2.07–1.93 (m, 8H), 1.69 (s, 3H), 1.68 (s, 6H), 1.61 (s, 3H), 1.23 (s, 36H); ^{13}C NMR δ 176.9 (2C), 176.8 (2C), 138.3, 135.7, 131.7, 124.9, 124.5, 121.2 (t, J_{CP} = 6.8 Hz), 82.4–82.0 (m, 4C), 38.9 (4C), 38.9 (t, J_{CP} = 131.7 Hz), 32.2, 32.1, 26.9 (12C), 26.8, 29.0, 26.2, 25.9, 23.7–23.4 (m, 3C), 17.8; ^{31}P NMR δ 22.7; HRMS (ESI, *m*/*z*) calcd for $(M+Na)^+$ $C_{40}H_{70}O_{14}P_2$: 859.4139. Found: 859.4144.

4.1.1.11. Tetrapivaloyloxymethyl (*E*)-4,8-dimethylnona-3,7-diene-1,1-diyldiphosphonate (15b). Yield: 1.11 g, 53%; ^{1}H NMR δ 5.66–5.76 (m, 8H), 5.23 (t, J = 7.5 Hz, 1H), 5.04–5.22 (m, 1H), 2.42–2.73 (m, 3H), 1.93–2.11 (m, 4H), 1.67 (s, 3H), 1.61 (s, 3H), 1.59 (s, 3H), 1.23 (s, 36H); ^{13}C NMR δ 177.0 (2C), 176.9 (2C),

138.4, 131.7, 124.2, 120.5 (t, J_{CP} = 7.5 Hz), 82.1–82.4 (m, 4C), 39.9, 38.9 (4C), 38.8 (t, J_{CP} = 142.0 Hz), 27.3, 27.0 (12C), 26.8, 25.9, 17.9, 16.3; ^{31}P NMR δ 22.7. Anal. Calcd for $C_{35}H_{62}O_{14}P_2$: C, 54.68; H, 8.13. Found: C, 54.98; H, 8.30.

4.1.1.12. Tetrapivaloyloxymethyl (*Z*)-4,8-dimethylnona-3,7-diene-1,1-diyldiphosphonate (16b). Yield: 300 mg, 29%; ^{1}H NMR δ 5.76–5.65 (m, 8H), 5.23 (t, J = 7.2 Hz, 1H), 5.14–5.05 (m, 1H), 2.73–2.41 (m, 3H), 2.08–1.98 (m, 4H), 1.69 (d, J = 1.2 Hz, 3H), 1.68 (s, 3H), 1.60 (s, 3H), 1.23 (s, 36H); ^{13}C NMR δ 176.9 (2C), 176.8 (2C), 138.3, 131.9, 124.1, 121.2 (t, J_{CP} = 8.2 Hz), 82.4–82.0 (m, 4C), 38.9 (4C), 38.9 (t, J_{CP} = 132.0 Hz), 32.0, 27.0 (12C), 26.4, 25.8, 23.5, 23.4 (t, J_{CP} = 4.7 Hz), 17.8; ^{31}P NMR ($CDCl_3$) δ 22.6; HRMS (ESI, *m/z*) calcd for $(M+Na)^+$ $C_{35}H_{62}O_{14}P_2$: 791.3513. Found: 791.3516.

4.2. Biological methods

4.2.1. Enzyme assays. GGDPS assays were performed as described previously[26] but with slight modifications. GST-tagged recombinant human FDPS and GGDPS were expressed in BL21 gold bacteria from a GeneCopoeia plasmid (Germantown, MD) by induction with IPTG. Proteins were purified by affinity chromatography or by incubation with glutathione agarose followed by centrifugation and washed three times with PBS. GGDPS reaction mixtures contained 20 μM FPP, 40 μM ^{14}C IPP, buffer (50 mM Imidazole, pH 7.5, 0.5 mM $MgCl_2$, 0.5 mM $ZnCl_2$) and inhibitor in a final volume of 20 μL. FPP synthase assays were performed by the method of Dunford.[45] Each reaction mixture contained 20 μM GPP, 40 μM ^{14}C IPP, and FDPS in 20 μL of reaction buffer (50 mM Tris, pH 7.7, 10 mM NaF, 2 mM $MgCl_2$, and 1 mg/mL BSA). Following a 10-min pre-incubation with indicated bisphosphonates, reactions were initiated by simultaneous addition of isoprenoid substrates. Reactions proceeded for 1 h at 37 °C then longer isoprenoids were extracted with 1 ml saturated butanol and washed three times with 300 μL saturated water. The amount of ^{14}C incorporation into GGPP was detected by liquid scintillation-counting.

4.2.2. Cell culture and incubations. K562 leukemia cells were obtained from ATCC (Manasas, VA) and cultured according to ATCC protocol. Asynchronous suspension cultures (5×10^6 cells/5 ml) in fresh media were incubated for 24 h in the presence or absence of the indicated concentrations of bisphosphonates.

4.2.3. Western blot analysis. Protein concentrations were determined using BCA method (Pierce). Proteins were resolved by electrophoresis on a 12% gel and transferred to a PDVF membrane. Primary and secondary antibodies were added sequentially for 45 min and proteins were visualized using an ECL chemiluminescence detection kit (Amersham). Anti-pan-Ras was obtained from Inter-Biotechnology (Tokyo, Japan). Rap1a (sc-1482) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). HRP-conjugated anti-mouse secondary was obtained from Amersham and HRP-conjugated anti-goat was obtained from Santa Cruz Biotech. Upon visualization proteins were quantified using ImageJ software.

4.2.4. Determination of *C*log *P* values. Theoretical *C*log *P* values were determined using Molinspiration Property Calculation Service (www.molinspiration.com).

4.2.5. Statistical analysis. Unpaired two-tailed *t*-tests were used to calculate statistical significance. Comparisons were done relative to the control or between pairs of free acids and POM-modified compounds. All columns in bar graphs represent the mean of the indicated number of replicates. An α level of 0.05 was set as the level of significance.

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