ChemPubSoc Europe

DOI: 10.1002/cmdc.201600465

CHEMMEDCHEM Full Papers

Targeting Cancer Cells with a Bisphosphonate Prodrug

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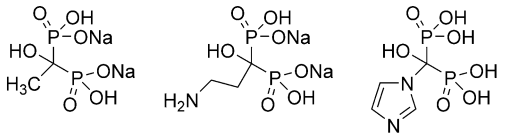
Nitrogen-containing bisphosphonates have antitumor activity in certain breast cancer and myeloma patients. However, these drugs have limited oral absorption, tumor cell entry and activity, and cause bone side effects. The potencies of phosphorylated antiviral drugs have been increased by administering them as prodrugs, in which the negative charges on the phosphate moieties are masked to make them lipophilic. We synthesized heterocyclic bisphosphonate (BP) prodrugs in which the phosphonate moieties are derivatized with pivaloyloxymethyl (pivoxil) groups and that lack the hydroxy "bone hook" on the geminal carbon. When the lipophilic BP prodrugs enter tumor cells, they are converted into their active forms by intracellular esterases. The most active BP prodrug, tetrakispivaloyloxymethyl 2-(thiazole-2-ylamino)ethylidene-1,1-bisphosphonate (**7**), was found to potently inhibit the in vitro growth of a variety of tumor cell lines, especially hematopoietic cells, at nanomolar concentrations. Consistent with this fact, compound **7** inhibited the prenylation of the RAP1A small GTPase signaling protein at concentrations as low as 1–10 nM. In preclinical studies, **7** slowed the growth of human bladder cancer cells in an immunodeficient mouse model. Thus, **7** is significantly more active than zoledronic acid, the most active FDA-approved BP, and a potential anticancer therapeutic.

Introduction

Geminal bisphosphonates (BPs) are synthetic compounds containing P-C-P groups that are metabolically stable analogues of diphosphate.[1] BPs were first synthesized more than 100 years ago and have a number of industrial uses.[2] Because BPs bind to bone hydroxyapatite[3] and inhibit bone resorption,[4] a variety of BPs have been developed as therapeutics for the treatment of bone-related diseases, such as Paget's disease, osteoporosis, and hypercalcemia of malignancy.[5]

First-generation BPs are simple non-nitrogen-containing compounds, such as etidronate and clodronate, that can be metabolically incorporated into a β-γ-methylene analogue of ATP. The non-hydrolyzable ATP analogue inhibits ATP-dependent intracellular enzymes like mitochondrial ADP/ATP translocase, leading to cell death.[6] Second-generation BPs, such as pamidronate and alendronate, contain alkylamine moieties and function to inhibit farnesyl diphosphate synthase (FDPS) in the mevalonate pathway, blocking the synthesis of downstream farnesyl and geranylgeranyl diphosphate metabolites. Transfer of the aliphatic chains of the diphosphates is required for the activation of small guanosine triphosphate (GTP)-binding proteins, such as Ras, Rho, Rac, Rab, and Rap, because the aliphatic chains function to anchor the proteins to the cell membrane for signaling. These GTP-binding signaling proteins are essential for the proliferation, survival, and migration of cells. Release of BPs from bone and uptake into osteoclasts causes osteoclast apoptosis and decreases bone resorption.[7–9] Third-generation BPs, including risedronate and zoledronic acid (Zol) that have nitrogen-containing heterocyclic moieties, exhibit more potent antiresorptive activity than second-generation BPs through stronger inhibition of FDPS.[10]



The addition of Zol to standard treatments for breast cancer and multiple myeloma improved disease-free survival and overall survival, respectively.[11–15] These improvements appeared to be independent of preventing bone-related events, suggesting that BPs have antitumor activity besides preventing bone metastases. Our goal was to develop BPs specifically de-

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Supporting information and the ORCID identification number(s) for the author(s) of this article can be found under http://dx.doi.org/10.1002/cmdc.201600465.

signed as cancer therapeutics for improved activity. However, heterocyclic BPs, such as Zol and minodronate, fit well into the active site of FDPS[16] making it difficult to design BPs with higher activity.

An alternative approach to increase BP activity is to improve BP cell entry. Cellular uptake of BPs through fluid-phase endocytosis is enhanced by Ca^{2+}, suggesting that the negatively charged hydrophilic P-C-P structure limits BP entry into cellular vesicles.[17] Oldfield and co-workers have reported several approaches to improving cellular uptake through the addition of a 9–12-carbon acyl chain to Zol and other BPs[18,19] or by masking the negatively charged P-C-P structure with pivoxil esters.[20] For Zol, addition of a 9–12-carbon acyl chain decreases FDPS inhibition by two- to three-fold, while increasing activity in cellular assays by up to 22-fold.[19] Similarly, the pivoxil derivatives of mono- and dialkyl-BPs that inhibit FDPS or geranylgeranyl diphosphate synthase (GGDPS) were consistently more potent than their acid forms at inhibiting the prenylation of RAP1A in K562 cells and K562 proliferation.[21] In this study, we have increased the cellular uptake and activity of nitrogen-containing BPs by masking the phosphonate charges with pivoxil groups resulting in novel BP prodrugs. These prodrugs have greatly increased bioactivity against hematopoietic tumor cells.

Results and Discussion

We synthesized BP prodrugs where the phosphonate charges are masked by pivoxil groups, and evaluated the effect of pivoxil addition on their biological activity in vitro and in vivo. We first synthesized seven BP prodrugs and their corresponding active acid forms (Figure 1). Five of the pairs (**2**–**6**/**9**–**13**) were based on NE11809 (compound **8**)[22,23] by varying the position of the methyl group on the heterocyclic ring, by the addition of a halogen group, or by addition of a nitrogen atom to the heterocyclic ring. A final compound substituted a thiazole for the pyridine group. The synthesis of BP pivoxil derivatives is detailed in Supporting Information, Figure S1. All of the BP prodrugs inhibited the growth of U937, a human histiocytic lymphoma cell line, more efficiently than their acid forms (Table 1 and Supporting Information, Figure S2). For example, compound **7** was found to be 100-fold more potent than its acid form, **14** (IC_{50} against U937 was 260 nM for **7**, versus 26000 nM for **14**). Compound **1** inhibited U937 cells 185-fold more efficiently than its acid form, **8**. However, the effect of pivoxil derivatization of BPs was variable, ranging from 5- to 1618-fold (mean increase in potency: 291 ± 588-fold). Similar increases in activity of BP pivoxil derivatives compared with their acid forms were noted with EJ-1 bladder carcinoma cells (Table 2 and Supporting Information, Figure S3). Again, the effect was variable depending on the BP, ranging from 1.2- to 422-fold (mean increase: 123 ± 163-fold). For EJ-1, compound **7**

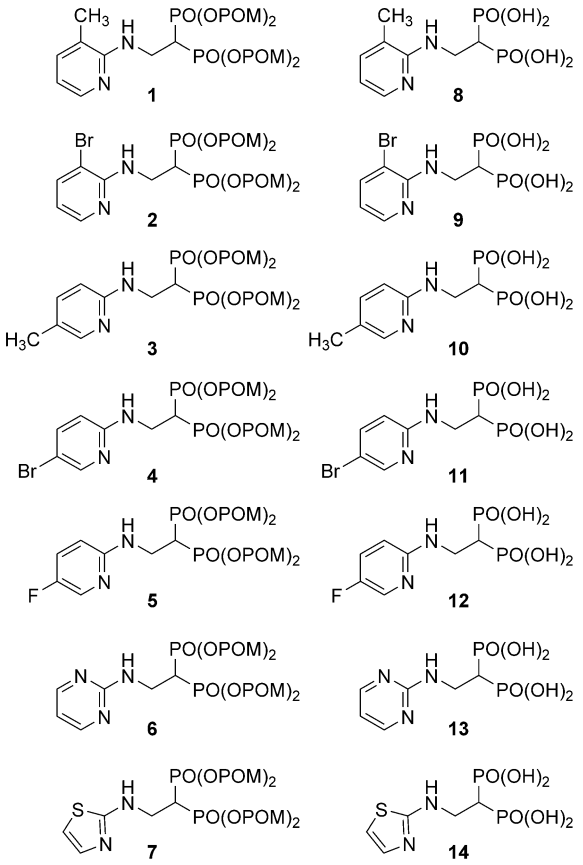


Figure 1. Structures of pivoxil esters and their acid forms. Details of synthesis are summarized in the Experimental Section.

Table 1. Comparison of pivoxil esters (PE) with their acid forms (H) in growth inhibition of the U937 histiocytic lymphoma cell line.[a]

PE/H	IC_{50} [μM] PE	H	Ratio IC_{50}(PE)/IC_{50}(H)
1:8	5.3 ± 0.4	980 ± 80	1:185
2:9	0.68 ± 0.05	1100 ± 200	1:1618
3:10	5.6 ± 0.7	29 ± 1	1:5
4:11	3.6 ± 0.3	200 ± 3	1:56
5:12	5.3 ± 0.4	68 ± 1	1:13
6:13	2.4 ± 0.0	150 ± 6	1:63
7:14	0.26 ± 0.01	26 ± 1	1:100

[a] Experimental details are described in Supporting Information, Figure S2.

Table 2. Comparison of pivoxil esters (PE) with their acid forms (H) in growth inhibition of the EJ-1 bladder carcinoma cell line.[a]

PE/H	IC_{50} [μM] PE	H	Ratio IC_{50}(PE)/IC_{50}(H)
1:8	11 ± 0	13 ± 2	1:1.2
2:9	2.3 ± 0.1	970 ± 30	1:422
3:10	5.4 ± 0.6	7.9 ± 0.6	1:1.5
4:11	7.8 ± 0.2	290 ± 10	1:37
5:12	5.7 ± 1.3	22 ± 2	1:3.9
6:13	0.09 ± 0.01	23 ± 2	1:256
7:14	0.026 ± 0.014	3.7 ± 0.2	1:142

[a] Experimental details are described in Supporting Information, Figure S3.

Table 3. Comparison of **7** (pivoxil ester, PE) with **14** (acid form, H) in growth inhibition of solid tumor cell lines.[a]

Cell line	Origin	IC_{50} [μM]		Ratio
		7 (PE)	**14** (H)	IC_{50}(**7**)/IC_{50}(**14**)
786-0	Renal cell carcinoma	0.88 ± 0.15	25 ± 1	1:28
MKN1	Gastric carcinoma	1.2 ± 0.8	100 ± 60	1:83
OST	Osteosarcoma	0.81 ± 0.20	66 ± 5	1:81
PC-3	Prostate carcinoma	0.22 ± 0.06	100 ± 20	1:455
PK1	Pancreatic cancer	0.34 ± 0.06	110 ± 20	1:324
LK-2	Squamous NSCLC[b]	0.80 ± 0.33	35 ± 1	1:44
G-361	Melanoma	0.12 ± 0.02	20 ± 4	1:167
TFK-1	Cholangiocarcinoma	0.10 ± 0.05	30 ± 1	1:300
MRK-nu-1	Mammary carcinoma	0.74 ± 0.03	210 ± 10	1:284
EJ-1	Bladder carcinoma	0.026 ± 0.014	3.7 ± 0.2	1:142

[a] Experimental details are described in Supporting Information, Figure S4. [b] Non-small-cell lung carcinoma.

inhibited 142-fold more than **14**. Given the high activity of **7**, growth inhibition by **7** and **14** were determined for cell lines derived from solid tumors. Again, **7** was 28- to 455-fold more potent than its acid form, **14** (Table 3 and Supporting Information, Figure S4). Similarly, growth inhibition of hematopoietic cell lines was enhanced by pivoxil protection, with **7** being 100- to 3714-fold more active than **14** (Table 4 and Supporting Information, Figure S5).

Based on these findings, we synthesized 28 additional BP pivoxil and other esters (compounds **15**–**42**, Figure 2) and assessed their ability to inhibit the growth of the U937 and EJ-1 cell lines (Table 5). However, again, **7** was the most potent inhibitor of U937 and EJ-1 cell growth. Therefore, we focused further studies on **7**. To determine its potential clinical usefulness, **7** was tested against a variety of tumor cell lines in comparison with Zol, the most potent FDA-approved BP (Figure 3 and Supporting Information, Figure S6 and Table S1). Compound **7** was found to be, on average, 27-fold more active than Zol for 52 solid tumor cell lines. Several tumor cell lines such as the bladder cancer cell line EJ-1 and the gastric tumor cell lines GCIY and MKN45 were highly sensitive to **7**. Only two solid tumor cell lines were more sensitive to Zol than **7**. Compound **7** was found to be even more active on average against hematopoietic cell lines. Hematopoietic cell lines averaged 796-fold (±1442-fold) more sensitive to growth inhibition with **7** than Zol. For example, HL60 (promyelocytic leukemia) was 5679-fold and PEER (T-cell lymphoma) was 391-fold more sensitive to **7** than Zol. The IC_{50} value for **7** averaged 515 nM for B-cell malignancies, 116 nM for T-cell malignancies, and 107 nM for myeloid malignancies versus 85 100, 55 000, and 85 557 nM, respectively, for Zol (Figure 3 and Supporting Information, Figure S6 and Table S1). Thus, while solid tumors were 27-fold more susceptible to **7** than Zol, lymphoma and myeloid hematopoietic cell lines were markedly more susceptible to **7** than to Zol.

To determine its mechanism of action, the effect of **7** on RAP1A prenylation in tumor cells was assessed. Compound **7** strongly inhibited RAP1A geranylgeranylation in six tumor cell lines, with inhibition detected at concentrations as low as 1–10 nM (Figure 4). As expected based on its inhibition of FDPS, Zol also inhibited prenylation, but required concentrations of at least 10 000–100 000 nM. For example, whereas unprenylated RAP1A was detected in EJ-1 with **7** at 1 nM, Zol required a concentration

Table 5. Inhibition of U937 and EJ-1 cell proliferation by additional pivoxil esters.[a]

Compd	IC_{50} [μM]		Compd	IC_{50} [μM]	
	U937	EJ-1		U937	EJ-1
7	0.26 ± 0.01	0.026 ± 0.014	**30**	6.6 ± 0.3	10 ± 1
39	0.65 ± 0.11	0.059 ± 0.006	**19**	6.7 ± 0.3	9.5 ± 0.5
35	1.1 ± 0.3	3.5 ± 0.5	**16**	6.8 ± 0.4	15 ± 1
31	2.2 ± 0.1	1.7 ± 0.2	**20**	6.9 ± 0.6	16 ± 1
34	2.5 ± 0.2	1.1 ± 0.4	**26**	7.0 ± 0.3	8.7 ± 0.4
17	2.6 ± 0.1	4.3 ± 0.5	**15**	7.1 ± 0.5	16 ± 0
24	3.0 ± 0.2	6.3 ± 0.5	**28**	7.1 ± 0.3	6.0 ± 0.4
18	3.3 ± 0.2	4.8 ± 0.5	**36**	7.9 ± 0.2	15 ± 1
22	3.4 ± 0.1	3.7 ± 0.4	**37**	7.9 ± 0.6	16 ± 2
23	3.5 ± 0.6	4.5 ± 0.4	**27**	8.9 ± 1.0	13 ± 1
33	5.0 ± 0.5	4.2 ± 0.4	**38**	11 ± 2	8.5 ± 0.3
21	6.1 ± 0.5	9.0 ± 0.6	**42**	17 ± 1	16 ± 1
25	6.2 ± 0.5	8.8 ± 0.7	**41**	50 ± 3	56 ± 2
32	6.3 ± 0.2	12 ± 2	**40**	70 ± 1	79 ± 7
29	6.6 ± 0.1	12 ± 3			

[a] Experimental details are described in the Supporting Information.

Table 4. Comparison of **7** (pivoxil ester, PE) with **14** (acid form, H) in growth inhibition of lymphoma and leukemia hematopoietic cell lines.[a]

Cell line	Origin	IC_{50} [μM]		Ratio
		7 (PE)	**14** (H)	IC_{50}(**7**)/IC_{50}(**14**)
MOLT-3	T-cell acute lymphoblastic leukemia	0.13 ± 0.09	180 ± 20	1:1385
MOLT-4	T-cell acute lymphoblastic leukemia	0.16 ± 0.12	120 ± 10	1:750
PEER	T-cell acute lymphocytic leukemia	0.023 ± 0.009	82 ± 6	1:3565
C1R	B-cell lymphoma	0.078 ± 0.018	79 ± 6	1:1013
SCC-3	Non-Hodgkin's lymphoma	0.18 ± 0.08	66 ± 3	1:367
Daudi	Burkitt's lymphoma	0.43 ± 0.52	76 ± 5	1:177
Raji	Burkitt's lymphoma	0.27 ± 0.01	120 ± 10	1:444
Ramos-RA1	Burkitt's lymphoma	0.11 ± 0.07	75 ± 5	1:682
THP-1	Acute monocytic leukemia	0.035 ± 0.061	130 ± 10	1:3714
U937	Histiocytic lymphoma	0.26 ± 0.01	26 ± 1	1:100
K562	Erythroleukemia	0.029 ± 0.006	16 ± 2	1:552

[a] Experimental details are described in Supporting Information, Figure S5.

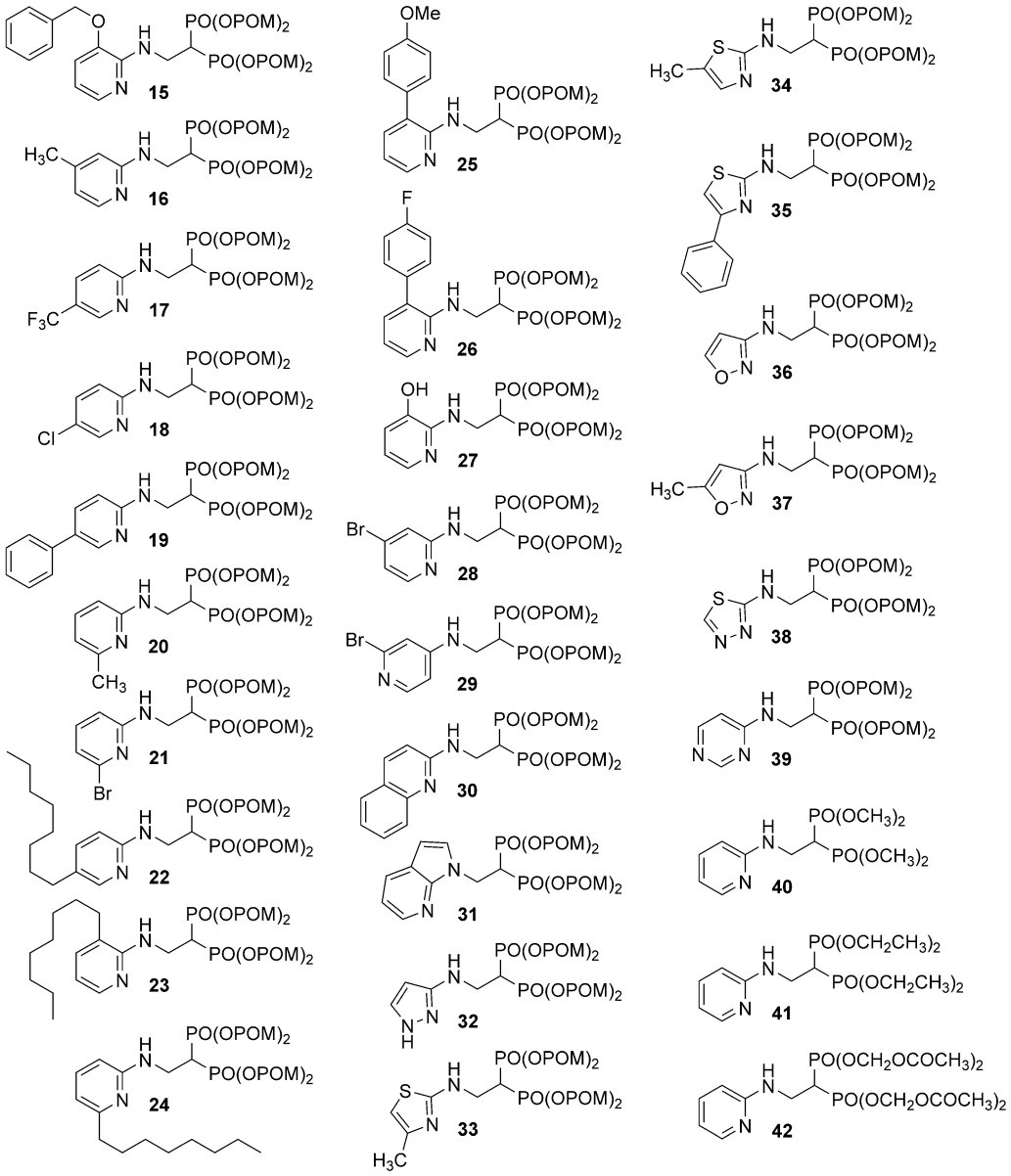


Figure 2. Structures of pivoxil esters. Details of synthesis are summarized in the Experimental Section and Supporting Information (chemistry and NMR spectra).

of ~10000 nM. The differences between **7** and Zol are similar to those noted with growth inhibition, albeit somewhat greater in magnitude. Thus, the activity of **7** is consistent with inhibition of isoprenoid biosynthesis. Given that most strong inhibitors of GGDPS contain mono- or diacyl chains,[24–26] the activity of **7** is likely due to inhibition of FDPS, although dual FDPS and GGDPS inhibition remains possible,[27] as does direct or indirect inhibition of signaling kinases in addition to the inhibition of FDPS.[28,29]

To assess the activity of **7** against tumors in vivo, the effect of **7** on the growth of EJ-1 bladder carcinoma cells was assessed in immunodeficient NOG mice. Luciferase-expressing EJ-1 bladder carcinoma cells were intraperitoneally inoculated into NOG mice and the mice were then treated with compound **7** twice a week. Compound **7** significantly inhibited the growth of EJ-1 from weeks 4 through 7, such that at seven weeks, EJ-1 tumor growth was decreased by 78% in mice treated with compound **7** relative to control mice (Figure 5). Thus, compound **7** has direct therapeutic benefits in the treatment of human cancer in this preclinical mouse model.

The development of phosphate and phosphonate prodrugs has been an important advance in anticancer and antiviral therapy.[31–33] Esterification of phosphate- and phosphonate-containing drugs can increase their gastrointestinal absorption, survival in the systemic circulation, and cell penetration. A number of different FDA-approved drugs contain the pivoxil group used here or the related disoproxil group with acceptable levels of toxicity. Adefovir dipivoxil and tenofovir disoproxil fumarate have been used to treat hepatitis B virus and HIV while cefditoren pivoxil is a broad-spectrum cephalosporin an-

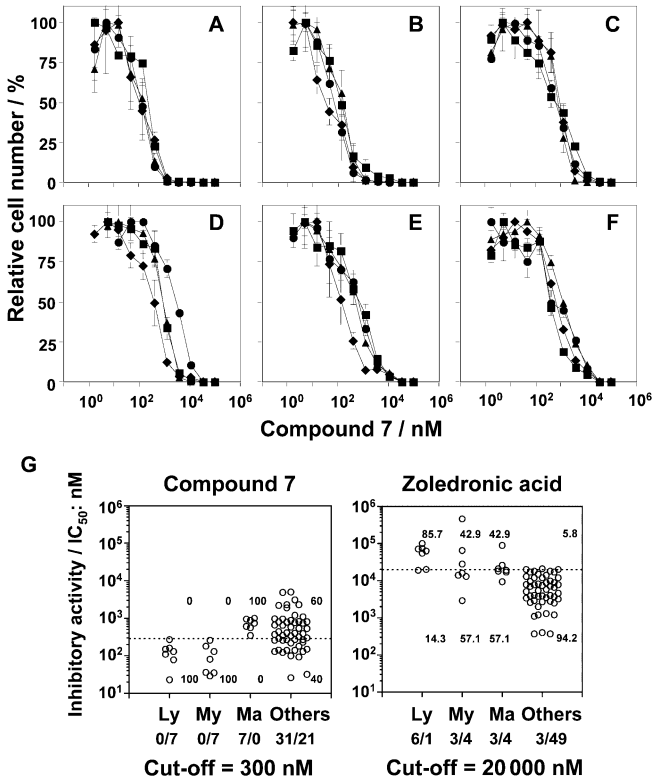


Figure 3. Tumor cell growth inhibition by compound **7** in comparison to Zol. A)–F) Dose-dependent inhibition of tumor cell growth by compound **7**. Tumor cells were grown, harvested and resuspended at 1×10^4 cells mL^{-1} in complete RPMI 1640 medium. A total of 0.05 mL of the cell suspension was added to flat-bottomed 96-well plates, followed by 0.05 mL of three-fold serial dilutions of compound **7**. After incubation at 37 °C with 5% CO_2 for four days, 0.1 mL of CellTiter-Glo reagent (Promega, Madison, WI, USA) was added and the luminescence resulting from ATP was measured using an ARVO luminometer (PerkinElmer, Foster City, CA, USA). All experiments were performed in triplicate: A) lymphomas: ● MOLT-3, ▲ J.RT3-T3.5, ■ Raji, ◆ Ramos (RA 1); B) myeloid leukemias: ● HL60, ▲ SCC-3, ■ P31/FUJ, ◆ NOMO-1; C) mammary carcinomas: ● YMB-1-E, ▲ HMC-1-8, ■ MCF-7, ◆ MDA-MB-231; D) renal cell carcinomas: ● VMRC-RCW, ▲ UOK121, ■ Caki-1, ◆ A-704; E) pancreatic carcinomas: ● KP4-1, ▲ KP4-2, ■ KP4-3, ◆ MiaPaCa2; and F) other tumor cells: ● 24TKB, ▲ MKN1, ■ Colo320, ◆ TAKAO. G) Comparison of compound **7** (left panel) with Zol (right panel) concentrations required for half-maximal growth inhibition of various tumor cell lines. Ly = lymphomas, My = myeloid leukemia, Ma = mammary carcinomas, and Others = other tumor cells. Cut-off values were arbitrarily set at 300 nM for **7** and 20 000 nM for Zol. Details of Zol growth inhibition are given in Supporting Information, Figure S6.

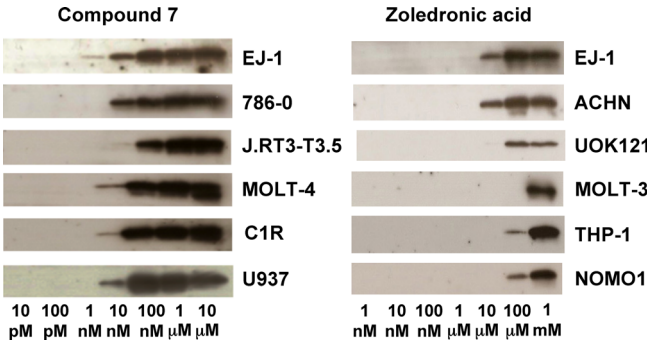


Figure 4. Inhibition of geranylgeranylation of RAP1A in tumor cells by compound **7**. Tumor cells were cultured with **7** or Zol for 16 h, lysed, and the proteins were separated by SDS-PAGE, transferred onto PVDF membranes and probed with anti-unprenylated RAP1A antibody. Data for inhibition of geranylgeranylation of RAP1A by Zol are from Idrees et al.[30] and are included for comparison. Note that the maximum concentration used for **7** was 100-fold less than that used for Zol.

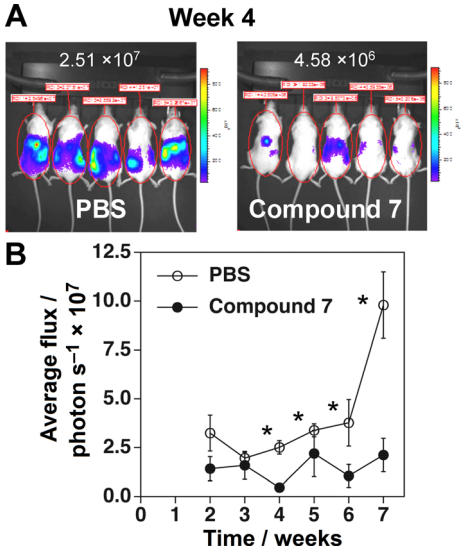


Figure 5. Compound **7** inhibits EJ-1 bladder carcinoma growth in immunodeficient NOG mice. A) Whole-body bioluminescence imaging of tumor cells. Immunodeficient NOG mice were intraperitoneally (i.p.) inoculated with 1×10^6 EJ-1 cells transfected with pGL4.10[luc2] on day 0 and treated with 2 μg/0.1 mL compound **7** (97.1 μg kg^{-1}) or 0.1 mL PBS i.p. on days 3 and 6. On day 7, 0.1 mL of 15 mg mL^{-1} VivoGlo™ luciferin (Xenogen, Alameda, CA, USA) was injected i.p., and the mice were placed in the specimen chamber and photon emissions transmitted from the mice were then measured. This treatment regimen was repeated seven times, and the images from day 28 are shown. Luciferase units are photons per second per mouse. B) Inhibition of tumor growth by compound **7** in vivo. The growth of EJ-1 was monitored for weeks 2–7 as described in (A), and the average photon flux per second for four to five mice is plotted ± SEM. *$p < 0.05$ by Mann–Whitney U test.

tibiotic. Besides pivoxil acyloxyalkyl esters, it may be possible to further improve targeting of hematopoietic tumors using aryloxy phosphoramidate esters.[33–35] The tenofovir alafenamide aryloxy phosphoramidate prodrug has increased levels in plasma and lymphatic tissue and is 1000-fold more active against HIV than tenofovir[36] and is more active in patients, with fewer side effects than its disoproxil derivative.[37,38] Unlike nucleoside prodrugs, nitrogen-containing BP prodrugs would be given intermittently and their predicted reduced deposition into bone would minimize jaw osteonecrosis and atypical fractures.

The mechanism for the high activity of **7** for hematopoietic tumors may reflect increased uptake by **7** coupled with increased dependence of hematopoietic tumors on prenylated Ras or other GTPases for their growth. Whereas hematopoietic cancer cells were more resistant to Zol than non-hematopoietic cancer cells (mean EC_{50} value of 77 800 nM versus 9600 nM), they were more sensitive to **7** (mean EC_{50}: 240 nM versus 770 nM). This suggests that BP uptake by hematopoietic tumors is lower than with other tumors; therefore, the in-

creased uptake of the **7** prodrug results in higher antiproliferative activity. To further improve the biological activity of nitrogen-containing BPs, it is necessary to examine and compare their cell penetration, lipophilicity, and metabolic stability in detail.

Conclusions

In conclusion, masking the negatively charged P-C-P structure of BPs with pivoxil esters greatly increases their capacity to inhibit tumor cell growth. The most active BP pivoxil ester, compound **7**, was found to be particularly effective at inhibiting the growth of hematopoietic cells, with IC_{50} values generally between 20 and 200 nM, whereas the IC_{50} values for ZoI were up to 5679-fold higher, being generally greater than 20000 nM. Besides the direct effect of **7** on tumor growth, **7** also expands cytotoxic Vγ2Vδ2 T cells in vitro and can be used in combination with adoptively transferred Vγ2Vδ2 T cells in vivo to enhance tumor control in the NOG mouse model (Tanaka et al., unpublished results). Moreover, we speculate that **7** may exhibit less bone deposition due to its lack of free phosphonate moieties as well as the absence of the hydroxy group (bone hook) on the germinal carbon of the P-C-P structure. Although further research is required, BP prodrugs could increase the effectiveness of BP treatment for both hematopoietic and non-hematopoietic solid tumors.

Experimental Section

General

Thin-layer chromatography (TLC) was performed on pre-coated plates (0.25 mm, silica gel plate 60 F_{245}, Merck Millipore, MA, USA). Column chromatography was conducted using silica gel (Kanto Chemical Co. Inc., Chuo-ku, Tokyo, Japan). All reactions were conducted under an atmosphere of air unless otherwise noted. Unless otherwise stated, reagents were purchased from commercial sources and used without further purification. ^{1}H and ^{13}C NMR spectra were obtained in $CDCl_3$ solution on a JNM-AL-400 spectrometer (^{1}H NMR at 400 MHz, ^{13}C NMR at 100 MHz) and a JNM-ECA-500 spectrometer (^{1}H NMR at 500 MHz, ^{13}C NMR at 125 MHz) (JEOL Ltd., Akishima, Tokyo, Japan). ^{1}H NMR chemical shifts were referenced to tetramethylsilane (TMS) (0.00 ppm) and ^{13}C NMR chemical shifts to $CDCl_3$ (77.0 ppm). Chemical shift values are expressed in parts per million (ppm). The following abbreviations were used for peak multiplicities: s, singlet; d, doublet; t, triplet; q, quartet; quin, quintet; sept, septet; m, multiplet; and br, broad. IR spectra were recorded on an FT/IR-4100 (JASCO Corp., Hachioji, Tokyo, Japan). Mass spectra and high-resolution mass spectra were recorded on JMS-HX/HX 110A (JEOL Ltd.). NMR spectral information and physical data are provided in the Supporting Information (chemistry and NMR spectral information).

General procedure for the synthesis of 1,1-bisphosphonic acid pivoxil esters (Scheme 1)

Tetramethyl vinylidene-1,1-bisphosphonate (S1): Following the protocol of Degenhardt and Burdsall,[39] diethylamine (6.3 mL, 60 mmol) was added to a solution of paraformaldehyde (9.0 g, 300 mmol) in methanol (230 mL) at room temperature and stirred at 65 °C for 30 min. A solution of tetramethyl methylenediphosphonate (14 g, 60 mmol) in methanol (10 mL) was added, and the reaction mixture was held at reflux for 1.5 h. The resulting mixture was concentrated in vacuo to give a crude product, which was used for the next reaction without further purification. The crude product was dissolved in toluene (200 mL) and treated with *p*-toluenesulfonic acid monohydrate (114 mg, 0.6 mmol). The reaction mixture was held at reflux using a Dean–Stark trap for 16 h and then diluted with $CHCl_3$. The resulting mixture was washed with brine, dried over $MgSO_4$, filtered and concentrated in vacuo to give a crude product that was purified by silica gel column chromatography (MeOH/$CHCl_3$ = 10%) to yield 8.9 g (60%) of the title compound as a colorless oil.

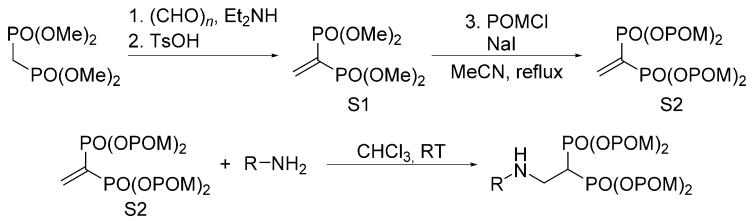
Tetrakispivaloyloxymethyl vinylidene-1,1-bisphosphonate (S2): NaI (10.2 g, 68 mmol) and chloromethyl pivalate (POMCl, 12.4 mL, 85 mmol) were added to a solution of tetramethyl vinylidene-1,1-bisphosphonate (4.2 g, 17 mmol) in MeCN (85 mL) and then held at reflux for 14 h. After addition of H_2O, the resulting mixture was extracted with EtOAc, and the organic layer was washed with brine, dried over Na_2SO_4, filtered and concentrated in vacuo to give a crude product that was purified by silica gel column chromatography (EtOAc/hexane = 50%) to yield 3.8 g (35%) of the title compound as a pale-yellow oil.

Synthesis of 1,1-bisphosphonic acid tetrakispivoxil esters

Tetrakispivaloyloxymethyl 2-(thiazole-2-ylamino)ethylidene-1,1-bisphosphonate (7): 2-Aminothiazole (20 mg, 0.2 mmol) was added to a solution of tetrakispivaloyloxymethyl vinylidene-1,1-bisphosphonate (64 mg, 0.1 mmol) in $CHCl_3$ (0.4 mL) and stirred at room temperature for 1 h. The resulting mixture was concentrated in vacuo to give a crude product, which was purified by silica gel column chromatography (EtOAc/$CHCl_3$ = 50%) to yield 69 mg (92%) of the title compound as colorless solid. The following compounds were prepared using the same synthetic procedure: **1**–**6** and **15**–**26**.

Tetrakispivaloyloxymethyl 2-(3-hydroxypyridine-2-ylamino) ethylidene-1,1-bisphosphonate (27): To a solution of tetrakis-pivaloyloxymethyl 2-(3-benzyloxypyridine-2-ylamino)ethylidene-1,1-bisphosphonate (**15**, 253 mg, 0.30 mmol) in EtOAc (15 mL) was added 10% Pd/C (62 mg, 0.058 mmol) and stirred under H_2 (1 atm) at room temperature for 1.5 h. The resulting mixture was filtered and concentrated in vacuo to give a crude product, which was purified by silica gel column chromatography (EtOAc) to afford 196 mg (87%) of the title compound. The following compounds were prepared using the same synthetic procedure: **28**–**39**.

Tetramethyl 2-(pyridine-2-ylamino)ethylidene-1,1-bisphosphonate (40): 2-Aminopyridine (47 mg, 0.50 mmol) was added to a solution of tetramethyl vinylidene-1,1-bisphosphonate (122 mg,



Scheme 1. General procedure for the synthesis of 1,1-bisphosphonic acid pivoxil esters.

0.50 mmol) in THF (1 mL) and stirred at room temperature for 34 h. The resulting mixture was concentrated in vacuo to give a crude product that was purified by silica gel column chromatography (MeOH/EtOAc = 5–100%) to yield 171 mg (99%) of the title compound.

Tetraethyl 2-(pyridine-2-ylamino)ethylidene-1,1-bisphosphonate (41): 2-Aminopyridine (376 mg, 4.0 mmol) was added to a solution of tetraethyl vinylidene-1,1-bisphosphonate (600 mg, 2.0 mmol) in $CHCl_3$ (8 mL) and stirred at room temperature for 3 h. The resulting mixture was concentrated in vacuo to give a crude product, which was purified by silica gel column chromatography (MeOH/$CHCl_3$ = 10%) to yield 772 mg (98%) of the title compound. Compound **42** was prepared by using the same synthetic procedure.

General procedure for the synthesis of 1,1-bisphosphonic acids (Scheme 2)

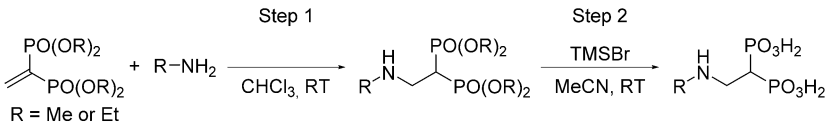
2-(5-Methylpyridine-2-ylamino)ethylidene 1,1-bisphosphonic acid (10). 2-Amino-5-methylpyridine (108 mg, 1.0 mmol) was added to a solution of tetraethyl vinylidene-1,1-bisphosphonate (150 mg, 0.50 mmol) in $CHCl_3$ (2 mL) and stirred at room temperature for 1 h. The resulting mixture was concentrated in vacuo to give a crude product that was purified by silica gel column chromatography (MeOH/$CHCl_3$ = 10%) to yield 212 mg (99%) of tetraethyl 2-aminoethylidene-1,1-bisphosphonate. Tetraethyl 2-aminoethylidene-1,1-bisphosphonate (102 mg, 0.25 mmol) was dissolved in MeCN (2 mL) and treated with bromotrimethylsilane (TMSBr, 0.20 mL, 1.5 mmol). The resulting mixture was stirred at room temperature for 4 h. The mixture was concentrated in vacuo to give a crude product that was purified by recrystallization from acetone/H_2O to yield 55 mg (74%) of the title compound. The following compounds were prepared using the same synthetic procedure: **8**–**9** and **11**–**14**.

Biological assays

In vitro tumor cell growth inhibition: Tumor cells were grown, harvested, and resuspended at 1×10^4 cells mL^{-1} in complete RPMI 1640 medium. A total of 0.05 mL of the cell suspension was added to flat-bottomed 96-well plates, followed by 0.05 mL of three-fold serial dilutions of POM esters. After incubation at 37 °C with 5% CO_2 for 4 days, 0.1 mL of CellTiter-Glo reagent (Promega, Madison, WI, USA) was added, and the luminescence resulting from ATP was measured using an ARVO luminometer (PerkinElmer, Foster City, CA, USA). All experiments were performed in triplicate. The concentrations required for 50% tumor cell growth inhibition (IC_{50} values) are shown. The sources for the cell lines are detailed in Idrees et al.[30]

Inhibition of geranylgeranylation of RAP1A in tumor cells: Tumor cells were resuspended in 90 mL of complete RPMI 1640 medium supplemented with 10% fetal bovine serum (Sigma, St. Louis, MO, USA), 10^{-5} M 2-mercaptoethanol (Invitrogen Corp., Carlsbad, CA, USA), 100 IU mL^{-1} penicillin (Meiji Seika Kaisha, Ltd., Chuo-Ku, Tokyo, Japan), and 100 μg mL^{-1} streptomycin (Meiji Seika Kaisha) and grown overnight at 37 °C with 5% CO_2 in 225 cm^2 flasks. Compound **7** was then added to the flasks to the concentrations indicated above. After incubation for 16 h, the cells were harvested and resuspended in 100 μL lysis solution containing 1% NP-40 (Wako Pure Chemical Industries Ltd., Chuo-ku, Osaka, Japan), 0.1% sodium dodecyl sulfate (SDS; Tokyo Chemistry Industry Co., Ltd., Chuo-Ku, Tokyo, Japan), and 0.5% sodium deoxycholate (Wako) in microcentrifuge tubes. After centrifugation at 15000 rpm for 10 min, the supernatants were transferred to new tubes and SDS-urea buffer containing 6.7 M urea (Wako), 5% SDS (Tokyo Chemistry Industry), 100 mM Tris·HCl buffer, pH 7.4 (Wako), 0.25% bromophenol blue (Wako), and 50 mM dithiothreitol (Wako) were added to give a protein concentration of 5 mg mL^{-1}. The samples were loaded on 15% polyacrylamide slab gels (Daiichi Pure Chemicals Co., Ltd., Chuo-ku, Tokyo, Japan) at 50 μg lane^{-1}, and electrophoresed at 120 mA h^{-1}. The proteins were then transferred onto Polyscreen (R) PVDF Transfer Membranes (PerkinElmer Inc., Waltham, MA, USA) treated with goat anti-unprenylated RAP1A antisera (× 500, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), and horseradish peroxidase-conjugated anti-goat IgG antisera (×5000, KPL Inc., Gaithersburg, MD, USA), followed by SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL, USA). Although not shown, controls using goat anti-RAP1A and anti-GAPDH antisera (Santa Cruz Biotechnology) were included in this study. Chemiluminescence was detected on Amersham Hyperfilm™ MP (GE Healthcare Ltd., Little Chalfont, Buckinghamshire, UK) using a Fuji Medical Film Processor FP M100 (Fuji Film Co., Ltd., Ashigara, Kanagawa, Japan).

*Inhibition of EJ-1 bladder tumor cell growth by **7** in NOG immunodeficient mice*: EJ-1 tumor cells (1×10^6) stably transfected with pGL4.10[luc2] (Promega, Madison, WI, USA) were intraperitoneally (i.p.) inoculated into immunodeficient NOG mice (obtained from the Central Institute for Experimental Animals, Kawasaki, Kanagawa, Japan). Mice were treated i.p. with 2 μg compound **7** in 0.1 mL PBS (97.1 μg kg^{-1} body weight for mice weighing 20.6 g), on days 3 and 6. On day 7, 0.1 mL of 15 mg mL^{-1} VivoGlo™ luciferin (Xenogen, Alameda, CA, USA) was i.p. injected, and the mice placed in a specimen chamber mounted with a CCD camera cooled to −120 °C (In Vivo Imaging System, Waltham, MA, USA). Then, the photon emission transmitted from the mice was measured. The greyscale photographic images and bioluminescence color images were superimposed. This treatment regimen was repeated for seven weeks, and the images for week 4 are shown. Arbitrary luciferase units were expressed as average flux (photons per second per mouse), and the photon intensity values per mouse were plotted with time. The growth of EJ-1 was monitored for weeks 2–7, and the average photon flux per second for four to five mice is plotted ± SEM. Statistical significance was assess by the non-parametric Mann–Whitney *U* test. The animal studies were conducted in accordance with the relevant laws and institutional guidelines, and were approved by the local IACUC committee.



Scheme 2. General procedure for the synthesis of 1,1-bisphosphonic acids.

Acknowledgements

The authors thank Ms. Chiyomi Inoue for technical assistance. This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Culture, Sports, and Technology of Japan (MEXT), by "Special Coordination Funds for Promoting Science and Technology" from MEXT and Astellas Pharma Inc. through the "Formation of Center for Innovation by Fusion of Advanced Technologies" program, by "Platform for Drug Discovery, Informatics, and Structural Life Science" from MEXT, by a Grant-in Aid for Translational Research from Kyoto University and Japan Agency for Medical Research and Development (AMED) (to Y.T.), and by the US Department of Veterans Affairs (Veterans Health Administration, Office of Research and Development, Biomedical Laboratory Research and Development) Grant 1I01BX000972-01A1, US National Cancer Institute Grants CA097274 (University of Iowa/Mayo Clinic Lymphoma Specialized Program of Research Excellence) and P30A086862 (Core Support), and the University of Iowa Carver College of Medicine Carver Medical Research Initiative Grant (to C.T.M.). C.T.M. is the Kelting Family Scholar in Rheumatology.

Keywords: anticancer agents · bisphosphonates · farnesyl diphosphate synthase · lipophilicity · prodrugs

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Received: September 12, 2016
Revised: October 10, 2016
Published online on ■■ ■■, 0000

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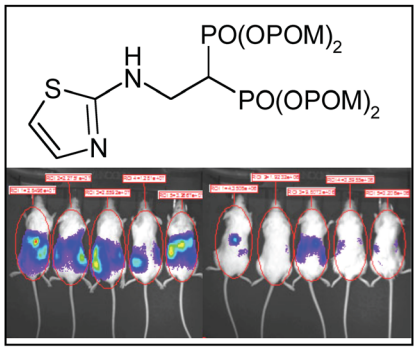

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Targeting Cancer Cells with a Bisphosphonate Prodrug

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