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Design, Synthesis, and Anticancer Activity of Phosphonic Acid Diphosphate Derivative of Adenine-Containing Butenolide and Its Water-soluble Derivatives of Paclitaxel with High Antitumor Activity

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Abstract—Synthesis of adenine derivative of triphosphono-γ-(*Z*)-ethylidene-2,3-dimethoxybutenolide **4** was accomplished by treatment of phosphonate **3** with 5-phosphoribosyl 1-pyrophosphate in the presence of 5-phosphoribosyl 1-pyrophosphate synthetase. It was found that triphosphonate **4** functions as an irreversible stoichiometric inactivator of the *Escherichia coli* ribonucleoside diphosphate reductase (RDPR). Triphosphonate **4** exhibited potent inhibitory activity against murine leukemias (L1210 and P388), breast carcinoma (MCF7), and human T-lymphoblasts (Molt4/C8 and CEM/0) cell lines. Paclitaxel ester derivatives of adenine-containing triphosphono-γ-(*Z*)-ethylidene-2,3-dimethoxybutenolide **8**–**10** were also synthesized. Like triphosphonate **4**, compound **8** exhibited inhibitory property toward RDPR. It also induced microtubule assembly similar to paclitaxel (**5**). The structure of the chlorodiester linker in **8** was found to account for this dual property. After treatment of MCF7 cells with compounds **4**, **5**, and **8**, fluorescence microscope examination demonstrated the presence of nucleus shrinkage or segmentation. Bifunctional prodrug **8** exhibited higher lipophilicity than **4** and higher water-solubility than **5**. Pro-dual-drug **8** exhibited more pronounced anticancer activity relative to that of the triphosphonate **4** and paclitaxel (**5**). In contrast, compound **9**, resulting from the linkage of triphosphonate **4** and paclitaxel (**5**) through a diester unit, was only found to function as a highly water-soluble prodrug for paclitaxel (**5**). It induced microtubule assembly in vitro, but did not show inhibitory property toward RDPR. On the other hand, compound **10**, an aggregate of triphosphonate **4** and paclitaxel (**5**), neither functioned as an inhibitor of RDPR nor exhibited microtubule assembly stimulating activity in vitro.

Introduction

Mechanism-based inhibition of enzymes crucial to metabolic pathways involved in cell division is an attractive concept for the rational design of chemotherapeutic agents.[1] Such an enzyme in the nucleic acid manifold is ribonucleoside diphosphate reductase (RDPR) (EC 1.17.4.1).[2] RDPR catalyzes the reduction of four purine and pyrimidine ribonucleotides by direct substitution of the 2′-OH group of the ribonucleotides with hydrogen.[3,4] The physiological reducing species are the mercapto groups of thioredoxin, which are regenerated from the oxidized form by NADPH-dependent thioredoxin reductase.[5,6] Inhibition of reductases by certain ribonucleotide analogues obstructs the route for the replication of the genetic materials necessary for

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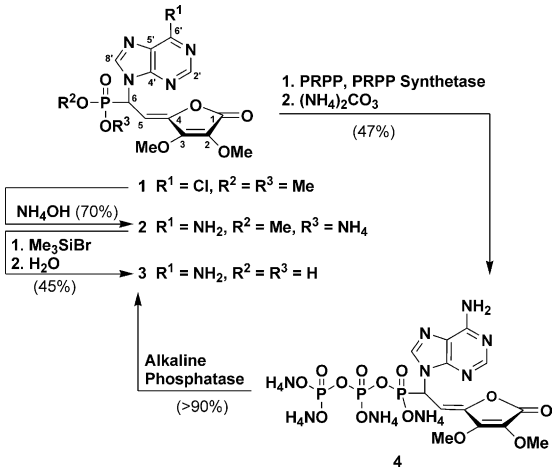
cancer cell division.[7] However, a well-known problem in anticancer therapy through inhibition of RDPR is the bioavailability of this type of phosphorylated derivatives. Nucleoside-5′-diphosphate or triphosphate analogues do not easily penetrate cellular membranes due to their low lipophilicity.[8]

On the other hand, paclitaxel (Taxol®, **5**) is a highly lipophilic anticancer agent for a variety of tumors (i.e., L1210 and P388 leukemias, MX-1 mammary tumor, B16 melanoma, and CX-1 colon tumor xenografts),[9] due to its unique ability to inhibit cell division as well as other interphase processes by stabilizing microtubules.[9] In the presence of paclitaxel, microtubules resist depolymerization, thus interfering with the G2 and M phases of the cell cycle.[9d] A major problem encountered in the use of paclitaxel for cancer chemotherapy is one of low water-solubility, which makes its formulation for injection difficult.[10] To date, a great deal of research has been devoted towards the modification of paclitaxel in order to create a more water-soluble and, therefore, more easily formulated and delivered drug.

Prodrug strategy may provide a utilitarian solution to the biodistribution of potential drugs having either low lipophilicity or low water-solubility. This strategy consists of transient modification of the physiochemical properties of a given compound through chemical derivatization. Such temporary chemical modification is usually designed to alter bioavailability while the inherent pharmacological properties of the parent drug remain intact.[10] Prodrugs are designed, once the barrier to delivery has been circumvented, to be converted into the active drugs, in vivo, either by an enzymatic mechanism[10] or simple hydrolysis[8,11] initiated under physiological pH conditions.

Herein we report the synthesis of adenine-containing triphosphono-γ-(*Z*)-ethylidene-2,3-dimethoxybutenolide **4**, which was found to be an alternate substrate for RDPR and exhibited notable anticancer activity. Polar molecule **4** possesses high water-solubility, but very low lipophilicity. Consequently, the intracellular delivery of triphosphonate **4** from a highly lipophilic prodrug (i.e., **8**) is expected to enhance its intracellular inhibitory property toward RDPR, which may result in profound activity against malignant tumor cell lines.



Scheme 1. Preparation of adenine-containing triphosphono-γ-(*Z*)-ethylidene-2,3-dimethoxybutenolide **4**.

Attempts to increase lipophilicity of RDPR inhibitors are heretofore undescribed. The induction of high water-solubility to paclitaxel ester derivative **8** by its triphosphonate moiety, encouraged us to use compound **4** in particular as RDPR inhibitor. As such, two compounds, triphosphonate **4** and paclitaxel (**5**), with different modes of action are linked to each other. The adducts **8**, **9**, and **10** overcome the physiochemical disadvantages of both parent compounds. Depending on the spacer, both parent compounds, only one compound, or none of them are released under physiological conditions.

Results

Chemical and biological reactions in the synthesis of adenine-containing triphosphono-γ-(*Z*)-ethylidene-2,3-dimethoxybutenolide 4 (Scheme 1)

6-Chloropurine-containing dimethylphosphono-γ-(*Z*)-ethylidene-2,3-dimethoxybutenolide **1**[12] was reacted with NH_4OH in refluxing CH_3CN to produce the corresponding adenine monoammonium phosphonate salt **2** as a mixture of two diastereoisomers in 70% yield. Sequential treatment of **2** with Me_3SiBr/DMF and H_2O gave phosphonic acid **3** in 45% yield over two steps. Adenine-containing phosphono-γ-(*Z*)-ethylidene-2,3-dimethoxybutenolide **3** and adenosine 5′-monophosphate (AMP), as a reference compound, were individually incubated with PRPP and PRPP synthetase during which time, the reaction proceeded linearly. The assays were terminated after 6 h by the addition of MeOH and the crude products purified on a DEAE Sephadex column with ammonium carbonate to afford diphosphate **4** (triphosphate equivalent, 47% yield) and ATP (>95% yield), respectively.[13]

The structure of **4** was confirmed by its cleavage with alkaline phosphatase first to a diphosphate analogue then to monophosphate **3** (>90% yield). On the other hand, compound **4** was found not to be a substrate for snake venom or spleen phosphodiesterase. It remained intact even after 24 h incubation at physiological conditions.

Kinetic parameters for PRPP synthetase with phosphonate 3

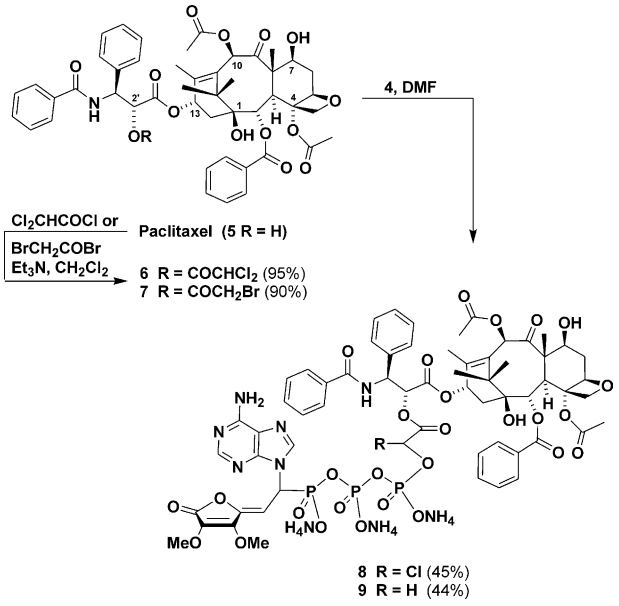
Using an established procedure,[13] the kinetic parameters for PRPP synthetase were determined. The substrate affinity of phosphonate **3** ($K_m = 6.5$ mM) for the enzyme was found to be 27-times less than that of AMP ($K_m = 0.24$ mM). The V_{max} for conversion of monophosphonate **3** ($V_{max} = 0.12$ μmol/unit/h) to triphosphonate **4** is 120 times lower than that for the conversion of AMP ($V_{max} = 14.5$ μmol/unit/h) to ATP. Inhibitory effect of phosphonate **3** ($K_i = 46.5$ mM) toward phosphorylation of AMP was also evaluated.[13]

Synthesis of paclitaxel ester derivatives of adenine-containing triphosphono-γ-(*Z*)-ethylidene-2,3-dimethoxybutenolide 8 and 9 (Scheme 2)

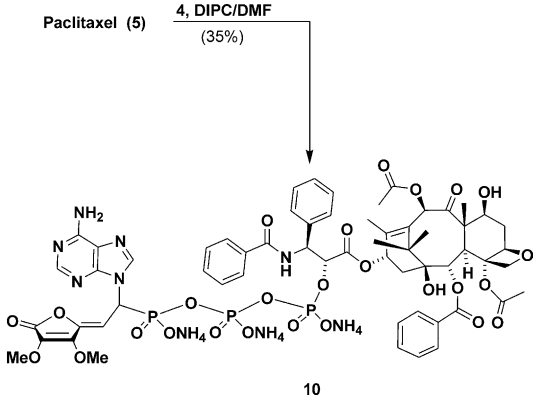
Acylation of paclitaxel (**5**) with dichloroacetyl chloride in the presence of Et_3N in CH_2Cl_2 at −15 °C gave 2′-dichloroacetyl taxol (**6**) (95% yield). Alkylation of triphosphonate **4** with compound **6** (DMF, 25 °C) then afforded prodrug **8** (45% yield). Likewise, treatment of paclitaxel (**5**) with bromoacetyl bromide in the presence of Et_3N in CH_2Cl_2 at −15 °C gave 2′-bromoacetyl taxol (**7**) (90% yield), which was subsequently alkylated with triphosphonate **4** to give prodrug **9** (44% yield).

Synthesis of adenine-containing paclitaxel 2′-triphosphono-γ-(*Z*)-ethylidene-2,3-dimethoxybutenolide 10 (Scheme 3)

Condensation of triphosphonate **4** with paclitaxel (**5**) in DMF using diisopropylcarbodiimide (DIPC) as condensing agent gave phosphate ester **10** in 35% yield.



Scheme 2. Synthesis of paclitaxel ester derivatives of adenine-containing triphosphono-γ-(*Z*)-ethylidene-2,3-dimethoxybutenolide **8** and **9**.



Scheme 3. Synthesis of adenine-containing paclitaxel 2′-triphosphono-γ-(*Z*)-ethylidene-2,3-dimethoxybutenolide **10**.

Lipophilicity and solubility tests

Lipophilicity and water-solubility were determined by distribution between 1-octanol and water according to the methods reported by Baker et al.[14] (Table 1). Paclitaxel derivatives of adenine-containing triphosphonobutenolide **8**, **9**, and **10** were observed to exhibit higher water-solubility than that exhibited by paclitaxel (**5**). Prodrugs **8** and **9**, as well as compound **10**, also exhibited higher lipophilicity as compared to adenine-containing triphosphonobutenolide **4**.

In general, the solubility of impure polar molecules (i.e., **4**) in organic solvents (i.e., 1-octanol) is higher than that of their pure forms. Accordingly, we observed 27 times increment in the lipophilicity of **4** when an equimolar mixture of **4**+**5** was tested (see Table 1). However, this combination did not improve the water-solubility of paclitaxel (**5**), as expected.

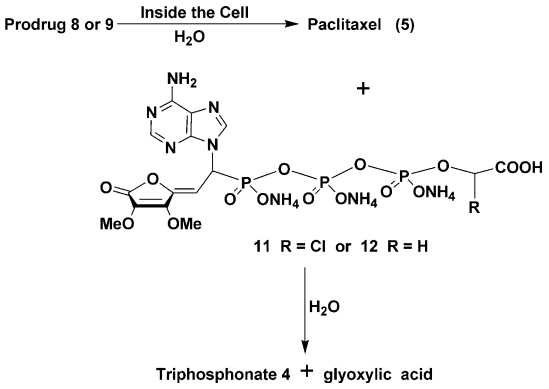
Hydrolysis of paclitaxel ester derivatives 8–10 in deuterated water, phosphate buffer solution, and human plasma (Scheme 4)

Paclitaxel-containing triphosphonate **8** was completely hydrolysed to paclitaxel (**5**), triphosphonate **4**, and glyoxylic acid in a mixture of DMSO-d_6 and distilled D_2O (pD 5.8, 1:1 mL/mL), a mixture of DMSO-d_6 and phosphate buffer solution (pH 7.2, 1:1 mL/mL), and in human plasma at 37 °C after 17.0, 2.0, and 0.40 h, respectively. Analysis of the hydrolysis reaction in DMSO-d_6/D_2O after 7.0 h by ^1H NMR showed the presence of triphosphonate ester **11**, suggesting that it is the intermediate in the formation of triphosphonate **4**. On the other hand, hydrolysis of paclitaxel-containing triphosphonate **9** in DMSO-d_6/D_2O (43 h), DMSO-d_6/phosphate buffer solution (4.5 h), and in human plasma (0.65 h) produced paclitaxel (**5**) and phosphoester derivative **12**. In this case, triphosphonate **4** or glyoxylic acid was not detected. However, paclitaxel-containing butenolide **10** was found to be intact in water or in buffer solution at 37 °C even after 55 h; yet in human plasma, it was hydrolyzed completely to paclitaxel (**5**) and triphosphonate **4** within 15 h.

Table 1. Solubility and lipophilicity of triphosphonate **4**, paclitaxel (**5**), an equimolar mixture of **4**+**5**, prodrugs **8** and **9**, as well as the triphosphonate ester **10**

Compd	Solubility in water (μM)	Solubility in 1-octanol (μM)	log P (1-octanol/water)[a]
Triphosphonate **4**	43×10^6	48.9	−5.9
Paclitaxel (**5**)	58.6	59.0×10^6	6.0
4+**5**[b]	412×10^5	1327	−4.5
4+**5**[c]	56.7	58.2×10^6	6.0
8	29×10^3	56×10^3	0.3
9	26×10^3	58×10^3	0.3
10	14×10^3	69×10^3	0.7

[a]Partition coefficients were calculated as $P=[\text{substrate}]_{\text{1-octanol}}/[\text{substrate}]_{H_2O}$.
[b]Solubility in water and lipophilicity of **4** in an equimolar mixture of **4**+**5**.
[c]Solubility in water and lipophilicity of **5** in an equimolar mixture of **4**+**5**.



Scheme 4. Hydrolysis of the prodrugs **8** and **9** to their respective parent drugs.

Inhibition of *Escherichia coli* RDPR

Using methodology previously described by Stubbe et al.,[15] compounds **4**, **8**, **9**, **10**, and **12** were evaluated for inhibitory property against *E. coli* RDPR (EC 1.17.4.1). The remaining enzyme activity was assayed as described by Steeper and Steuart.[16] The *E. coli* RDPR (1.35 μM) was significantly inactivated upon incubation with triphosphonate **4** or prodrug **8** by concentration-dependent process. While inactivation of the enzyme achieved immediately by **4**, its inactivation by paclitaxel-containing triphosphonate **8** occurred after ca. 2 h. This is consistent with the rate of hydrolysis of prodrug **8** to parent drug **4** in the reaction media. The maximal inactivation at 10, 25, 50, and 100 μM concentrations used was 100% of the original enzyme activity. On the other hand, at concentrations as low as 0.1 and 1.0 μM of **4**, instantaneous inactivation ($t_{1/2}$ <10 s) of 10–15% and 69–78% of the enzyme was observed, respectively. No change in the remaining enzyme activity was detected over 10 s to 2 h. Such rapid inactivation did not allow the determination of time-dependent inactivatory property of triphosphonate **4** or its transport form **8** towards RDPR. Chromatography of the inactivated enzyme on a Sephadex G-50 column, resulted in no recovery of the RDPR activity. In addition, dithiothreitol (DTT) did not protect RDPR from inactivation by **4** or **8**. Thus, the inactivation may be due to an irreversible stoichiometric complexation of **4** with the active site of the enzyme.[17] On the other hand, compounds **9**, **10**, and **12** were found not to be inhibitors of RDPR.

Effect of paclitaxel ester derivatives of adenine-containing triphospho-γ-(*Z*)-ethylidene-2,3-dimethoxybutenolide 8–10 on microtubule assembly in vitro

The ability of paclitaxel (**5**), prodrugs **8** and **9**, as well as 2′-paclitaxel phosphoester **10** to induce microtubule assembly in vitro at a concentration of 7.0 μM were examined according to an established procedure.[18] The percent of tubulin which had polymerized was determined to be 70% for paclitaxel (**5**), 64% for **8**, and 34% for **9**. 2′-Paclitaxel phosphoester **10** failed to exhibit microtubule assembly stimulating activity in vitro. HPLC analysis, after 3.5 h, demonstrated that 2′-paclitaxel esters **8** and **9** were degraded, respectively, to 97 and 42% paclitaxel (**5**), while the paclitaxel phosphoester derivative **10** remained intact. The conversions of **8** and **9** to paclitaxel (**5**) may account for their activity on microtubule assembly.

Anticancer activity in vitro

Inhibition of the proliferation of murine leukemias (L1210 and P388), breast carcinoma (MCF7), human T-lymphocytes (Molt4/C8 and CEM/0), human embryonic lung cell (HEL), and normal fibroblast (Hef522) by 50% (IC_{50})[19] in the presence of compounds **2**–**10**, **12**, glyoxylic acid, 9-(β-D–arabinofuranosyl)cytosine (ara-C), equimolar mixtures of **4**+**6**, **5**+**11**, and **4**+**5**+glyoxylic acid were carried out in vitro. Furthermore, compounds **4**, **5**, and **8**–**10** were tested for inhibition of the proliferation of B16 melanoma cells.[18] A mixture of DMSO/D_2O (8:2 v/v) was used to solve the compounds and their mixtures. The results are listed in Table 2.

Effect of adenine-containing triphosphonobutenolide 4, paclitaxel (5), and bifunctional prodrug 8 on microtubule bundle formation in MCF7 cells

The effects of compounds **4**, **5**, and **8** at their IC_{50} (Table 2) on microtubule cycloskeletone were examined by immunofluorescence microscopy using tubulin antibodies (see Experimental). Triphosphonate **4** at 0.34 μM did not exhibit differential microtubule assembly as compared with the untreated MCF7 cells after 6.5 h. Under the same conditions, however, both paclitaxel (**5**) (2.71×10^{-3} μM) and prodrug **8** (0.20×10^{-3} μM) showed similar ability to induce the formation of microtubule bundles as well as different distribution of microtubules in apoptotic MCF7 cells (Fig. 1).

Apoptotic morphology in MCF7 cells caused by adenine-containing triphosphonobutenolide 4, paclitaxel (5), or bifunctional prodrug 8

MCF7 cells were treated with higher concentrations than IC_{50} of compounds **4** ($4\times IC_{50}$), **5** ($2\times IC_{50}$), and **8** ($2\times IC_{50}$). Chromatin staining of the cells revealed nucleus shrinkage or fragmentation after 8.0 h (Fig. 2). This morphological change is associated with cell death occurring by a process called apoptosis.

Discussion

An unusual potential pathway for the intracellular conversion of monophosphonate **3** to its biologically active anabolite **4** is demonstrated. The rate of phosphorylation of **3** to **4** by PRPP synthetase is, however, 120-times less than that of the AMP. Thus, phosphonate **3** did not exhibit high activity against malignant tumor cell lines. Triphosphonate **4** showed significant inhibitory property towards RDPR. Consequently, it exhibited notable anticancer activity (see Table 2).

Because lower dosage of two drugs in combination has shown better efficacy than individual drug applied alone,[20a] the combined modality may be beneficial in

Table 2. Inhibitory effects, IC_{50} (μM), of butenolides **2** and **3**, triphosphonate **4**, paclitaxel (**5**), 2′-dichloroacetyl taxol (**6**), 2′-bromoacetyl taxol (**7**), prodrugs **8** and **9**, equimolar mixtures of **4**+**6**, **5**+**11**, and **4**+**5**+glyoxylic acid as well as the triphosphonate esters **10** and **12**, glyoxylic acid, and ara-C in DMSO/D_2O (8:2 v/v) on the growth of malignant tumor cell lines, human embryonic lung cell (HEL), and normal fibroblast (Hef522)[a]

Compd	L1210	P388	MCF7	Molt4/C8	CEM/0	B16 melanoma	HEL	Hef522
ara-C	0.17±0.00	0.14±0.01	1.03±0.02	0.65±0.00	0.78±0.10	—	1.25±0.13	1.98±0.05
2	85.49±2.71	49.87±1.98	>120	92.65±3.07	>120	—	74.18±2.45	80.46±2.96
3	11.42±1.15	7.10±0.87	13.90±1.01	9.50±1.92	14.60±1.12	—	45.76±1.11	62.17±1.87
4	0.62±0.01	0.58±0.02	0.34±0.01	0.75±0.04	0.81±0.13	15.60±1.01	35.87±0.98	53.14±1.26
Paclitaxel (**5**)	4.50 ± 10^{-3}	5.64 ± 10^{-3}	2.71 ± 10^{-3}	6.71 ± 10^{-3}	7.03 ± 10^{-3}	21.50 ± 10^{-3}	0.73±0.06	1.89±0.09
6	4.36 ± 10^{-3}	5.29 ± 10^{-3}	2.34 ± 10^{-3}	6.80 ± 10^{-3}	6.31 ± 10^{-3}	—	0.58±0.04	2.05±0.07
7	5.47 ± 10^{-3}	6.83 ± 10^{-3}	3.95 ± 10^{-3}	7.21 ± 10^{-3}	5.96 ± 10^{-3}	—	0.47±0.01	1.72±0.08
Prodrug (**8**)	0.54 ± 10^{-3}	0.62 ± 10^{-3}	0.20 ± 10^{-3}	0.49 ± 10^{-3}	0.81 ± 10^{-3}	3.20 ± 10^{-3}	0.86±0.02	2.45±0.03
4+**6**	2.90 ± 10^{-3}	2.31 ± 10^{-3}	1.77 ± 10^{-3}	4.65 ± 10^{-3}	4.06 ± 10^{-3}	—	—	—
5+**11**	2.01 ± 10^{-3}	1.98 ± 10^{-3}	1.02 ± 10^{-3}	3.98 ± 10^{-3}	3.67 ± 10^{-3}	—	—	—
4+**5**+glyoxylic acid	2.34 ± 10^{-3}	2.03 ± 10^{-3}	1.56 ± 10^{-3}	4.17 ± 10^{-3}	3.99 ± 10^{-3}	—	—	—
Prodrug (**9**)	3.89 ± 10^{-3}	4.16 ± 10^{-3}	3.01 ± 10^{-3}	5.92 ± 10^{-3}	6.70 ± 10^{-3}	17.00 ± 10^{-3}	0.94±0.00	1.21±0.06
10	5.70±0.11	4.28±0.07	7.99±0.86	5.83±0.09	6.51±0.14	1.20±0.04	113±3.67	120±2.35
12	45.63±1.38	20.32±2.06	68.74±1.70	>120	68.75±2.55	—	83.49±2.14	97.54±3.14
Glyoxylic acid	69.86±1.94	77.67±3.41	80.09±2.14	74.25±2.81	97.84±3.09	—	120±3.75	>120
DMSO/D_2O (8:2 v/v)	>120	>120	>120	>120	>120	>120	>120	>120

[a]The IC_{50} values were estimated from dose–response curves compiled from three independent experiments and represent the compound concentration (μM) required to inhibit cell proliferation by 50%. The mean (±SD) for **5**–**9**, **4**+**6**, **5**+**11**, and **4**+**5**+glyoxylic acid was $<0.04\times10^{-3}$ in all cases, except in B16 melanoma where (±SD) was $<0.87\times10^{-3}$.

the treatment of patients who cannot tolerate high dosages of the drugs. One approach to combination therapy involves the linkage of two anticancer agents targeting at different pathways associated with the cell division. As such, the cancer cells are expected to become more susceptible to a dual drug, which can be judged by its lower IC_{50} relative to the parent drugs (compare IC_{50} values of **4**, **5**, and **8** in Table 2).

The ability of a drug to penetrate the cell membrane and exhibit biological activity can be correlated to its lipophilicity.[8,20b] Triphosphonate **4** possessed high water-solubility, but very low lipophilicity (Table 1). Consequently, it was conjugated with paclitaxel (**5**), a highly lipophilic antitumor agent, via a chlorodiester unit. In comparison with triphosphonate **4**, the lipophilicity of the resulting bifunctional prodrug **8** was about 1000 times. Furthermore, prodrug **8** was also found to exhibit 500 times more water solubility than paclitaxel (**5**). Thus, in comparison with adenine-containing triphosphonobutenolide **4** and paclitaxel (**5**), paclitaxel 2′-triphosphonate ester **8** possessed superior bioavailability and greater anticancer activity.

Like impure organic compounds, a mixture of polar molecule **4** with paclitaxel (**5**) showed more lipophilicity for adjuvant compound **4** in comparison with individual pure triphosphonate **4** (see Table 1). As such, an increment in the cell penetration of **4** is expected. Triphosphonate **4** and its derivative **11** are alternate substrates for RDPR, which is crucial to a metabolic pathway involved in the cell division. On the other hand, paclitaxel (**5**) and its derivative **6** inhibit cancer cell division by stabilizing microtubules. Thus, relative to anticancer activity of individual compounds, a synergistic effect on the activity of their mixtures, capable of attacking different cellular targets, was predicted. Indeed, when the key experiments for a pro-dual-drug concept were carried out, it was found that equimolar mixtures of **4**+**6**, **5**+**11**, and **4**+**5**+glyoxylic acid exhibited about two times more anticancer activity than paclitaxel (**5**), but less activity relative to that of prodrug **8** (see Table 2).

2′-Paclitaxel esters with electron-withdrawing substituents in the α-position show remarkable rate enhancements of hydrolysis.[10] Similarly, from a hydrolytic perspective, the ease of hydrolysis of novel prodrug **8** to paclitaxel (**5**) and phosphoester intermediate **11** is due to the presence of electron-withdrawing substituents (i.e., Cl and OPO_2O–) at the α-position of its 2′-ester unit. Likewise, the terminal phosphate in **11** is intrinsically more labile due to the chloro-substitution in α-position—note that phosphate hydrolysis may occur either by water attack at the phosphorus center, accelerated by an inductive or hyperconjugative α-chloro effect, or by water attack at the C–Cl bond, followed by rapid cleavage of the hemiacetal to afford triphosphonobutenolide **4** and glyoxylic acid. As such, prodrug **8** and triphosphonate **4** exhibited similar inhibitory property toward RDPR.

The concept of pro-dual drug was further corroborated by studying microtubule bundle formation and apoptosis in MCF7 cells in the presence of prodrug **8** as well as parent drugs **4** and **5**. It is reported that introduction of an acyl moiety at the 2′-position of paclitaxel resulted in the loss of ability to promote microtubule assembly, but not cytotoxicity.[18] The ability of bifunctional prodrug **8** to induce microtubule assembly in vitro or inside MCF7 cells (see Fig. 1) is due to its hydrolysis to paclitaxel (**5**). As shown in Fig. 2, chromatin staining of MCF7 cells incubated with **4** (1.36 μM), **5** (5.42×10^{-3} μM), or **8**

(0.40×10^{-3} μM) revealed different numbers of apoptotic nuclei. Indeed, the ability of compounds **4**, **5**, and **8** to induce apoptosis correlates well with their anticancer activity (Table 2).

To prove the rationale behind the design of our new bifunctional prodrug **8**, we synthesized 2′-paclitaxel ester derivative **9** in which a chlorine atom is replaced with a hydrogen atom. Ester **9**, under physiological conditions, was hydrolyzed to paclitaxel (**5**) and stable phosphoester **12**. Prodrug **9** exhibited similar anticancer activity in vitro when compared to paclitaxel (**5**); yet it did not exhibit inhibitory property against RDPR. Unlike phosphoester intermediate **11**, phosphoester **12** (see Scheme 4) was not hydrolyzed to triphosphonate **4** under physiological conditions. This result confirms the importance of chlorine atom at the α-position of the ester linkage in dual-action anticancer prodrug **8**. It should be noted that since the linking moiety employed is an ester, substantial rate enhancement due to non-specific esterases may also occur in vivo and contribute to the effective use of the prodrugs.

Unlike triphosphonate **4**, its phosphoester derivative **12** did not inhibit RDPR; yet exhibited weak anticancer activity (see Table 2). This may be due to the slow rate of hydrolysis of **12** to **4** by the action of phosphoesterases inside the malignant tumor cell lines. On the basis of this observation, and also to realize the importance of the linker in the biological activity of prodrugs **8** and **9**, we synthesized paclitaxel 2′-triphosphono-γ-(*Z*)-ethylidene-2,3-dimethoxybutenolide **10**, which was found to be about 250 times more soluble in water than paclitaxel (**5**). We hypothesized that, in the presence of phosphodiesterases, paclitaxel (**5**) and triphosphonate **4** may be liberated inside the tumor cells as potential drugs. Derivative **10**, however, was found to be stable toward hydrolysis in either buffer solution (pH 7.2) or in water (pH 5.8). Consequently, it did not show activity on microtubule assembly. The activity of compound **10** against the examined malignant tumor cell lines in vitro was found to be about 1000 times less than that of paclitaxel (**5**), and at least 10 times less than that of the triphosphonate **4** (Table 2). Accordingly, the controlled release of the drugs based on the enzymatic cleavage of

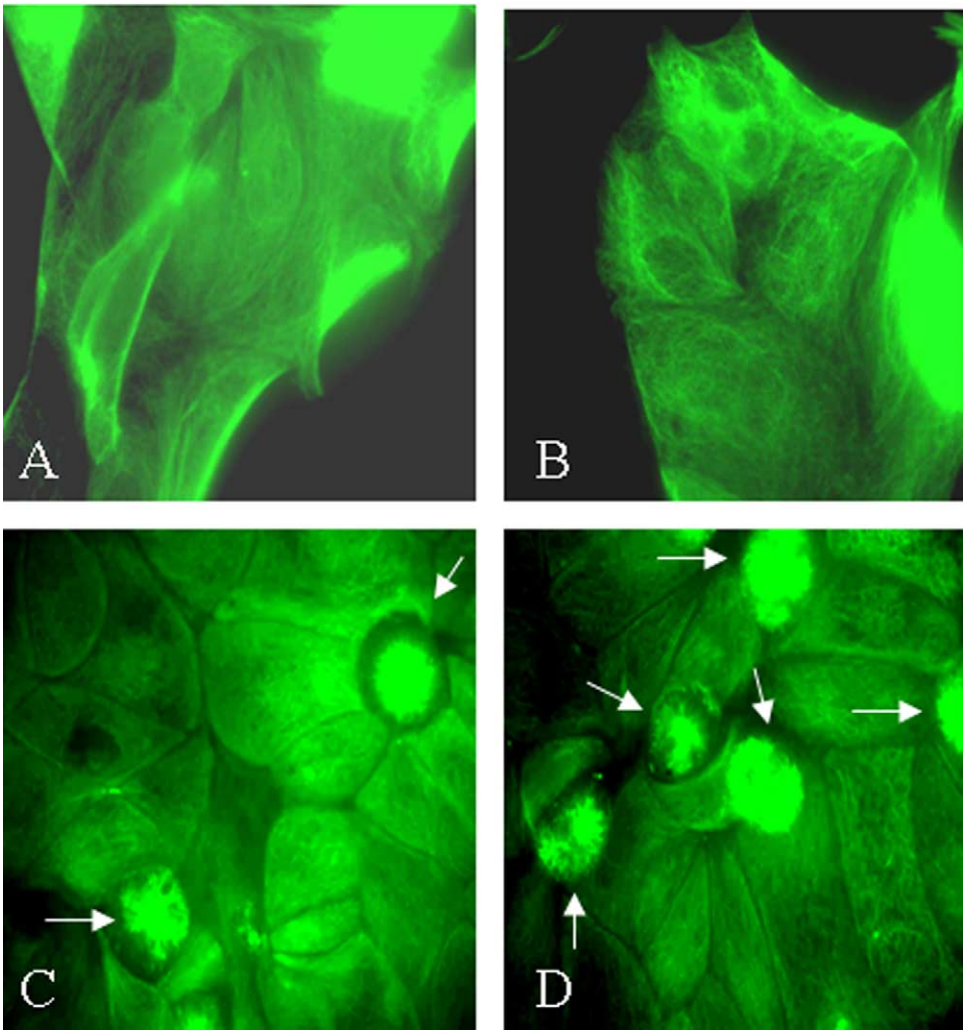


Figure 1. Immunofluorescence staining of microtubules in control and drug-treated MCF7 cells. Microtubules were labeled with a mouse monoclonal anti-tubulin antibody and detected with an FITC-conjugated secondary anti-mouse IgG: (A) untreated cells; (B) cells treated with 2.94 μM adenine-containing triphosphonobutenolide **4**; (C) treatment with 2.71×10^{-3} μM of paclitaxel (**5**); (D) treatment with 0.98×10^{-3} μM of bifunctional prodrug **8**. Microtubule distributions of apoptotic cells are indicated by white arrows.

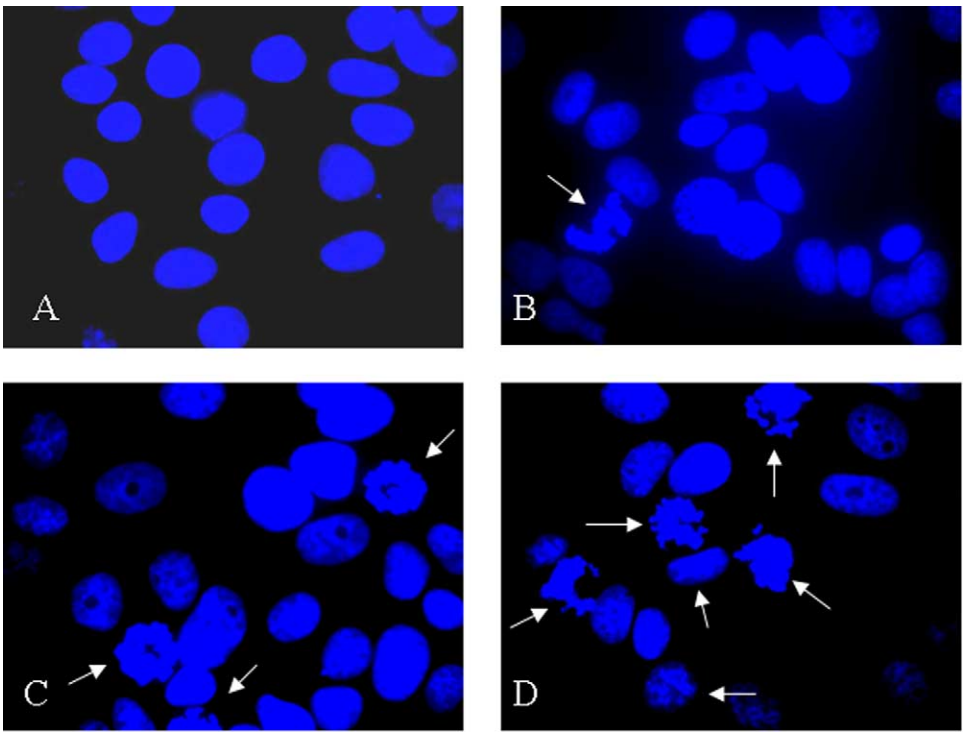


Figure 2. Drug-induced apoptosis in MCF7 cells: (A) untreated cells; (B) cells treated with 11.76 μM adenine-containing triphosphonobutenolide **4**; (C) treatment with 5.42×10^{-3} μM of paclitaxel (**5**); (D) treatment with 1.96×10^{-3} μM of bifunctional prodrug **8**. Apoptotic nuclei are indicated by white arrows.

paclitaxel 2′-triphosphonate **10** was seemed to not be an effective approach.

Finally, chemical stability of paclitaxel derivatives is critical to their formulation since partial degradation of conjugates can lead to precipitation of the poorly water soluble paclitaxel. Compounds **8**–**10** in water (3.0 mg/mL) remain clear and precipitate free for >15 h. They also appear to show reasonable solid-state stability on storage at 25 °C.

Conclusions

A novel adenine-containing triphosphono-γ-(*Z*)-ethylidene-2,3-dimethoxybutenolide **4** was synthesized by chemical and biological strategies. Compound **4** is a significant inhibitor for RDPR and exhibited notable anticancer activitiy against murine leukemias (L1210 and P388), breast carcinoma (MCF7), and human T-lymphoblasts (Molt4/C8 and CEM/0) cell lines. Triphosphonate **4** was found not to be significantly active against proliferation of B16 melanoma cells. On the other hand, monophosphonate **3** exhibited weak activity against malignant tumor cell lines. This lack of significant activity is proposed to be a consequence of its slow rate of phosphorylation by PRPP synthetase in cellular system. Paclitaxel ester derivatives of adenine-containing triphosphono-γ-(*Z*)-ethylidene-2,3-dimethoxybutenolide **8** and **9** as well as adenine-containing paclitaxel 2′-triphosphono-γ-(*Z*)-ethylidene-2,3-dimethoxybutenolide **10** were also synthesized. Paclitaxel derivatives **8**–**10** were observed to show much higher water solubility than that exhibited by paclitaxel (**5**). They also showed much higher lipophilicity as compared to adenine-containing triphosphonobutenolide **4**. By virtue of its linker, bifunctional prodrug **8** was easily hydrolyzed to paclitaxel (**5**) and triphosphonate **4** under physiological conditions. Therefore, the biological activity of **8** stems from both the adenine-containing triphosphonobutenolide (RDPR inhibitor) and paclitaxel (microtubule assembly activator) moieties. Under identical conditions, the hydrolysis of prodrug **9** to paclitaxel (**5**) and triphosphonate derivative **12** was found to be much slower, with biological activity only comparable to that of paclitaxel (**5**). Paclitaxel 2′-triphosphonate **10**, however, was found to not act as a significant prodrug.

Experimental

General

For anhydrous reactions, glassware was dried overnight in an oven at 120 °C and cooled in a desiccator over anhydrous $CaSO_4$ or silica gel. Reagents including *E. coli* alkaline phosphatase, NADPH, ATP, and Hoechst 33342 were purchased from Sigma (St. Louis, MO, USA) or Fluka (Switzerland). Antibodies were obtained from Boehringer Mannheim (Indianapolis, IN, USA). Solvents, including dry ether and tetrahydrofuran (THF), were obtained by distillation from the sodium ketyl of benzophenone under nitrogen. Other solvents, including chloroform, dichloromethane, ethyl acetate, and hexanes were distilled over CaH_2 under nitrogen. Absolute methanol and ethanol were purchased from Merck (Germany) and used as received. *E. coli* RDPR was isolated as described.[15b]

Melting points were obtained with a Büchi 510 melting point apparatus. Infrared (IR) spectra were recorded on a Beckman IR-8 spectrophotometer. The wavenumbers reported are referenced to the 1601 cm^{-1} absorption of polystyrene. NMR spectra were obtained on a Varian XL-300 (300 MHz) Spectrometer. DMSO-d_6, D_2O, or $CDCl_3$ was used as solvent; Me_4Si (δ 0.00 ppm) was used as an internal standard. All NMR chemical shifts are reported as δ values in parts per million (ppm) and coupling constants (*J*) are given in hertz (Hz). The splitting pattern abbreviations are as follows: s, singlet; d, doublet; t, triplet; q, quartet; br, broad; m, unresolved multiplet due to the field strength of the instrument; and dd, doublet of doublets. For ^{31}P NMR, acetone-d_6 or D_2O was used as solvent; $PO(Me)_3$ (δ 1.59 ppm) was used as an internal standard. UV–vis spectroscopy was carried out using an HP8452A diode array spectrophotometer. Microanalyses were performed on a Perkin-Elmer 240-B microanalyzer.

Purification refers to gravity column chromatography on a DEAE Sephadex or Merck Silica Gel 60 (particle size 230–400 mesh). Analytical TLC was performed on precoated plates purchased from Merck (Silica Gel 60 F_{254}). Compounds were visualized by use of UV light.

(*Z*)-4-[2-Ammonium methylphosphono-2-(adenine-9-yl)-ethylidene]-2,3-dimethoxy-$\Delta^{\alpha,\beta}$-butenolide (distereoisomeric mixture, 2). To a solution of **1** (4.12 g, 9.60 mmol) in CH_3CN (50 mL) was added concentrated NH_4OH solution (100 mL). The solution was refluxed for 6 h. The solvents were evaporated, and the residue was crystallized from EtOH to give **2** (2.69 g, 6.72 mmol) in 70% yield. R_f = 0.14 (EtOAc/MeOH = 4:1); mp 211–214 °C; IR (KBr): ν = 3210–3153 (NH_2), 3065 ($C_{8'}H$), 3054 ($C_{2'}H$), 2945 (C_5H), 1780 (C=O), 1695 (C=C), 1221 (P=O) cm^{-1}; UV (EtOH): λ_{max} (lgε) = 212 (4.60), 260 nm (4.25); ^1H NMR (300 MHz, DMSO-d_6/D_2O): δ = 3.71 (d, *J* = 11.5 Hz, 1.5H, $P(OCH_3)$), 3.76 (d, *J* = 11.5 Hz, 1.5H, $P(OCH_3)$), 3.80 (br s, 3H, C_2OCH_3), 4.24 (br s, 3H, C_3OCH_3), 6.14 (dd, *J* = 16.0, 31.8 Hz, 0.5H, PCH), 6.23 (dd, *J* = 16.2, 32.2 Hz, 0.5H, PCH), 6.96–7.23 (m, 1H, =CH), 7.65, 7.83, 8.32 8.41 (4 s, 2H, $C_{2'}H + C_{8'}H$); ^{13}C NMR (300 MHz, DMSO-d_6): δ = 51.87, 52.01 ($POCH_3$), 59.98, 60.01 (2-OCH_3), 60.20, 60.46 (3-OCH_3), 82.16, 83.14 (C_6), 98.79, 99.87 (C_5), 126.13, 127.51 (C_4), 132.98, 133.04 ($C_{5'}$), 141.11, 142.20 (C_2), 144.62, 145.31 ($C_{2'}$), 147.98, 148.45 ($C_{4'}$), 149.79, 150.12 (C_3), 155.86, 156.41 ($C_{6'}$), 156.95, 157.02 ($C_{8'}$), 167.89, 167.96 (C=O);^{31}P NMR (300 MHz, acetone-d_6): δ = 16.37, 16.45; MS (DCl, 200 eV): *m*/*z* (%): 415 (95) [M + 1, phosphorus cluster]$^+$; elemental analysis calcd (%) for $C_{14}H_{19}N_6O_7P$ (414.3480): C, 40.58; H, 4.62; N, 20.28; P, 7.48; found: C, 40.47; H, 4.58; N, 20.30; P, 7.41.

(*Z*)-4-[2-Dihydrogenphosphono-2-(adenine-9-yl)ethylidene]-2,3-dimethoxy-$\Delta^{\alpha,\beta}$-butenolide (3). To a solution of **2** (0.41 g, 1.0 mmol) in DMF (10 mL) was added Me_3SiBr (1.09 g, 7.02 mmol). After the solution was stirred at 25 °C for 7 h, a mixture of MeOH and H_2O [6:3 (v/v), 18 mL] was added, and the solvents were evaporated. The crude residue was purified by use of column chromatography (resine XAD-4, H_2O) to afford **3** (0.17 g, 0.45 mmol) in 45% yield. R_f = 0.09 (EtOAc/MeOH = 4:1); mp 264 °C (dec.); IR (KBr): ν = 3100–3450 (OH, NH_2), 3112 ($C_{8'}H$), 3098 ($C_{2'}H$), 3021 (C_5H), 1776 (C=O), 1689 (C=C), 1215 (P=O) cm^{-1}; UV (EtOH): λ_{max} (lgε) = 210 (4.57), 259 nm (4.19); ^1H NMR (300 MHz, DMSO-d_6/D_2O): δ = 3.78 (s, 3H, C_2OCH_3), 4.19 (s, 3H, C_3OCH_3), 6.32 (dd, *J* = 16.4, 32.5 Hz, 1H, PCH), 7.19 (br d, *J* = 16.9 Hz, 1H, =CH), 7.78, 8.12 (2 s, 2H, $C_{2'}H + C_{8'}H$); ^{13}C NMR (300 MHz, DMSO-d_6): δ = 60.20 (2-OCH_3),60.29 (3-OCH_3), 84.08 (C_6), 91.65 (C_5), 124.00 (C_4), 135.10 ($C_{5'}$), 137.41 (C_2), 143.15 ($C_{2'}$), 149.65 ($C_{4'}$), 153.08 (C_3), 157.21 ($C_{6'}$), 157.43 ($C_{8'}$), 166.27 (C=O);^{31}P NMR (300 MHz, acetone-d_6): δ = 14.67; MS (70 eV): *m*/*z* (%): 383 (70) [M, phosphorus cluster]$^+$; elemental analysis calcd (%) for $C_{13}H_{14}N_5O_7P$ (383.2576): C, 40.74; H, 3.68; N, 18.27; P, 8.09; found: C, 40.68; H, 3.55; N, 18.29; P 8.13.

5-Phosphoribosyl 1-pyrophosphate synthetase in the preparation of (*Z*)-4-[2-triphosphono-2-(adenine-9-yl)-ethylidene]-2,3-dimethoxy-$\Delta^{\alpha,\beta}$-butenolide (4) and ATP. Substrate affinities of phosphonate **3** and AMP for PRPP synthetase as well as the inhibitory effect of **3** against the enzyme were evaluated according to an established procedure.[13] The resulting triphosphonate **4** (47% yield) and ATP (>95% yield) were, individually, purified by using a DEAE Sephadex A-25 column (1.5×15 cm) that had been equilibrated in ammonium carbonate (20 mM). A linear gradient of ammonium carbonate (400 mL) was used to elute **4**. The purity of **4** was determined to be 99.6% by analytical HPLC (anion-exchange Partisphere column eluted with a gradient of 8.0 mM to 1.0 M ammonium phosphate, pH 5.47) monitored at 260 nm: mp >250 °C (dec.); UV (EtOH): λ_{max} (lgε) = 214 (4.38), 260 nm (4.27); ^1H NMR (300 MHz, D_2O): δ = 3.60 (s, 3H, C_2OCH_3), 4.25 (s, 3H, C_3OCH_3), 6.82 (dd, *J* = 17.2, 33.1 Hz, 1H, PCH), 7.19 (dd, *J* = 17.3, 22.3 Hz, 1H, =CH), 7.99, 8.30 (2 s, 2H, $C_{2'}H + C_{8'}H$);^{31}P NMR (300 MHz, D_2O): δ = 17.86 (d, *J* = 28.5 Hz, α-P), −22.48 (dd, *J* = 19.6, 28.5 Hz, β-P), −7.97 (d, *J* = 19.6 Hz, γ-P).

Treatment of triphosphonate 4 with *E. coli* alkaline phosphatase. Triphosphonate **4** (24 mM) was treated with alkaline phosphatase (60 IU/mL) and 2-amino-2-methylpropanol (65 mM, pH 9.8) at 37 °C. Over 12 h period sequential conversion of triphosphonate **4** to diphosphonate and then to monophosphonate **3** was observed. After 15 h, chromatography was performed on thin-layer PEI-cellulose in 0.8 M LiCl/0.8 M formic acid 1:1 (v/v) (R_f = 0.09 for triphosphonate **4**, 0.26 for diphosphonate analogue, and 0.64 for monophosphonate **3**) to afford **3** in >90% yield.

2′-Dichloroacetylpaclitaxel (6). To a solution of paclitaxel (**5**) (0.146 g, 0.170 mmol) and Et_3N (0.018 g, 0.18 mmol) in dry CH_2Cl_2 (8.0 mL) at −15 °C was added dropwise dichloroacetyl chloride (0.027 g, 0.18 mmol). The reaction mixture was stirred for 2.0 h. The solution was then partitioned between CH_2Cl_2 (30 mL) and water (40 mL). The CH_2Cl_2 solution was washed with water (2×50 mL); then it was dried over $MgSO_4$ and

filtered. Evaporation under reduced pressure and purification of the residue by use of column chromatography (CH_2Cl_2) afforded **6** (0.15 g, 0.16 mmol) in 95% yield. R_f = 0.29 (hexanes/EtOAc = 3:1); mp 171–173 °C (dec.); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.07 (s, 3H, H_3C_{17}), 1.24 (s, 3H, H_3C_{16}), 1.67 (s, 3H, H_3C_{18}), 1.84 (s, 3H, H_3C_{19}), 2.23 (s, 3H, $OCOCH_3$), 2.52–2.79 (m, 2H, H_2C_6), 2.36 (s, 3H, $OCOCH_3$), 2.31–2.34 (m, 2H, H_2C_{14}), 3.71 (d, J = 6.8 Hz, 1H, HC_3), 4.19 (d, J = 8.3 Hz, 1H, HC_{20}), 4.28 (d, J = 8.3 Hz, 1H, HC_{20}), 4.36 (dd, J = 31.3, 8.3 Hz, 1H, HC_7), 4.50–4.86 (br, 2H, 2×OH), 4.94 (br d, 1H, HC_5), 5.65 (d, J = 7.0 Hz, 1H, HC_2), 5.77 (d, J = 2.4 Hz, 1H, $HC_{2'}$), 5.95 (dd, J = 8.8, 2.4 Hz, 1H, $HC_{3'}$), 6.21–6.28 (m, 3H, $HCCl_2 + HC_{10} + HC_{13}$), 6.87 (d, J = 8.8, 1H, NH), 7.23–7.90 (m, 15H, 3×C_6H_5); MS (FAB): m/z (%): 965 (84%) [M + 1, chlorine cluster]$^+$; elemental analysis calcd (%) for $C_{49}H_{51}NO_{15}Cl_2$ (964.8399): C, 61.00; H, 5.33; N, 1.45; Cl, 7.35; found: C, 60.97; H, 5.41; N, 1.50; Cl, 7.34.

2′-Bromoacetylpaclitaxel (7). Compound **7** (0.149 g, 0.153 mmol) was prepared in 90% yield from **5** (0.146 g, 0.170 mmol) and bromoacetyl bromide (0.036 g, 0.18 mmol) in the presence of Et_3N (0.018 g, 0.18 mmol) in dry CH_2Cl_2 (8.0 mL) by the method used for the synthesis of **6**. R_f = 0.32 (hexanes/EtOAc = 3:1); mp 163–166 °C (dec.); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.08 (s, 3H, H_3C_{17}), 1.26 (s, 3H, H_3C_{16}), 1.69 (s, 3H, H_3C_{18}), 1.83 (s, 3H, H_3C_{19}), 2.21 (s, 3H, $OCOCH_3$), 2.53–2.80 (m, 2H, H_2C_6), 2.38 (s, 3H, $OCOCH_3$), 2.30–2.33 (m, 2H, H_2C_{14}), 3.70 (d, J = 6.8 Hz, 1H, HC_3), 4.17 (d, J = 8.5 Hz, 1H, HC_{20}), 4.27 (d, J = 8.5 Hz, 1H, HC_{20}), 4.36 (dd, J = 30.8, 8.2 Hz, 1H, HC_7), 4.50–5.01 (br, 2H, 2′ OH), 4.88 (br d, 1H, HC_5), 5.59 (d, J = 2.5 Hz, 1H, $HC_{2'}$), 5.60 (s, 2H, CH_2Br), 5.67 (d, J = 7.1 Hz, 1H, HC_2), 5.97 (dd, J = 8.8, 2.5 Hz, 1H, $HC_{3'}$), 6.26–6.29 (m, 2H, $HC_{10} + HC_{13}$), 6.85 (d, J = 8.8, 1H, NH), 7.21–7.86 (m, 15H, 3′ C_6H_5); MS (FAB): m/z (%): 975 (79) [M + 1, bromine cluster]$^+$; elemental analysis calcd (%) for $C_{49}H_{52}NO_{15}Br$ (974.8458): C, 60.37; H, 5.38; N, 1.44; Br, 8.20; found: C, 60.21; H, 5.49; N, 1.37; Br, 8.18.

(*Z*)-4-[2-(Paclitaxel-2′-*O*-carbo-α-chloromethyl)triphosphono-2-(adenine-9-yl)ethylidene]-2,3-dimethoxy-Δ$^{\alpha,\beta}$-butenolide (8). To a solution of 6 (0.322 g, 0.333 mmol)) in DMF (15.0 mL) at 25 °C was added ammonium triphosphonate **4** (0.204 g, 0.333 mmol). The mixture was stirred for 16.0 h. The solvent was then evaporated under reduced pressure and the residue was crystallized from MeOH to give **8** (0.23 g, 0.15 mmol) in 45% yield. R_f = 0.21 (EtOAc); mp > 250 °C (dec.); ^{1}H NMR (300 MHz, $CDCl_3/D_2O$): δ = 1.10 (br s, 3H, H_3C_{17}), 1.26 (br s, 3H, H_3C_{16}), 1.82 (br s, 3H, H_3C_{18}), 1.80 (br s, 3H, H_3C_{19}), 2.22 (s, 3H, $OCOCH_3$), 2.50–2.83 (m, 2H, H_2C_6), 2.41 (br s, 3H, $OCOCH_3$), 2.28–2.41 (m, 2H, H_2C_{14}), 3.76 (br d, 1H, HC_3), 3.76 (s, 3H, C_2OCH_3), 4.20 (s, 3H, C_3OCH_3), 4.23 (br d, 2H, H_2C_{20}), 4.45 (m, 1H, HC_7), 5.07 (br d, 1H, HC_5), 5.72 (br d, 1H, HC_2), 5.81 (br d, 1H, $HC_{2'}$), 5.99 (br d, 1H, $HC_{3'}$), 6.11–6.53 (m, 4H, $HCCl + HC_{10} + HC_{13+}PCH$), 7.20–7.94 (m, 16H, =CH + 3′ C_6H_5), 7.69, 8.08 (2 br s, 2H, $C_{2'}H + C_{8'}H$); elemental analysis calcd (%) for $C_{62}H_{75}N_9O_{28}ClP_3$ (1,522.7820): C, 48.90; H, 4.96; N, 8.27; Cl, 2.33; P, 6.11; found: C, 48.86; H, 4.81; N, 8.32; Cl, 2.30; P, 6.07.

(*Z*)-4-[2-(Paclitaxel-2′-*O*-carbomethyl)triphosphono-2-(adenine-9-yl)ethylidene]-2,3-dimethoxy-Δ$^{\alpha,\beta}$-butenolide (9). Compound **9** (0.43 g, 0.29 mmol) was prepared in 44% yield from **7** (0.392 g, 0.666 mmol) and ammonium triphosphonate **4** (0.408 g, 0.666 mmol) in DMF (18.0 mL) by the method used for the synthesis of **8**. R_f = 0.24 (EtOAc); mp > 250 °C (dec.); ^{1}H NMR (300 MHz, $CDCl_3/D_2O$): δ = 1.09 (br s, 3H, H_3C_{17}), 1.22 (br s, 3H, H_3C_{16}), 1.80 (br s, 3H, H_3C_{18}), 1.84 (br s, 3H, H_3C_{19}), 2.25 (s, 3H, $OCOCH_3$), 2.48–2.76 (m, 2H, H_2C_6), 2.44 (br s, 3H, $OCOCH_3$), 2.30–2.52 (m, 2H, H_2C_{14}), 3.81 (br d, 1H, HC_3), 3.80 (s, 3H, C_2OCH_3), 4.26 (s, 3H, C_3OCH_3), 4.33 (br d, 2H, H_2C_{20}), 4.50 (m, 1H, HC_7), 5.10 (br d, 1H, HC_5), 5.69 (br d, 1H, HC_2), 5.79 (br s, 2H, OCH_2CO), 5.84 (br d, 1H, $HC_{2'}$), 6.02 (br d, 1H, $HC_{3'}$), 6.10–6.52 (m, 3H, $HC_{10} + HC_{13+}PCH$), 7.28–8.13 (m, 16H, =CH + 3×C_6H_5), 7.70, 8.12 (2 br s, 2H, $C_{2'}H + C_{8'}H$); elemental analysis calcd (%) for $C_{62}H_{76}N_9O_{28}P_3$ (1,488.2900): C, 50.04; H, 5.15; N, 8.47; P, 6.25; found: C, 50.18; H, 5.27; N, 8.52; P, 6.39.

(*Z*)-4-[2-(Paclitaxel-2′-*O*-triphosphono)-2-(adenine-9-yl)-ethylidene]-2,3-dimethoxy-Δ$^{\alpha,\beta}$-butenolide (10). Ammonium triphosphonate **4** (0.30 g, 0.49 mmol) was dissolved in acetic acid (3.0 mL). After 5 min, the solvent was evaporated and the residue was dissolved in DMF (7.0 mL). To this solution were added paclitaxel (**5**) (0.42 g, 0.49 mmol), DIPC (0.064 g, 108 μL, 0.51 mmol) and DMAP (0.10 g, 0.78 mmol). The resulting solution was stirred at 25 °C for 24 h. The reaction mixture was diluted with water (20 mL) and extracted with EtOAc (40 mL). The EtOAc solution was washed with water (3′ 50 mL); then it was dried over $MgSO_4$ and filtered. Evaporation under reduced pressure and purification of the residue by use of silica gel column chromatography (EtOAc) afforded the corresponding triacid of **10** (MS (FAB): m/z (%):1,380 (65) [M + 1, phosphorus cluster]$^+$), which was suspended in ammonium carbonate solution (20 mL, 150 mM) and stirred for 1 h. Filtration afforded triammonium salt **10** (0.24 g, 0.17 mmol) in 35% yield. R_f = 0.31 (EtOAc); mp 224–227 °C (dec.); ^{1}H NMR (300 MHz, $CDCl_3/D_2O$): δ = 1.12 (s, 3H, H_3C_{17}), 1.19 (br s, 3H, H_3C_{16}), 1.67 (br s, 3H, H_3C_{18}), 1.81 (s, 3H, H_3C_{19}), 2.17 (br s, 3H, $OCOCH_3$), 2.50–2.80 (m, 2H, H_2C_6), 2.33 (s, 3H, $OCOCH_3$), 2.29–2.48 (m, 2H, H_2C_{14}), 3.81 (br d, 1H, HC_3), 3.78 (s, 3H, C_2OCH_3), 4.21 (s, 3H, C_3OCH_3), 4.21 (br d, 2H, H_2C_{20}), 4.39 (m, 1H, HC_7), 4.75 (br d, 1H, HC_5), 5.58 (br t, 1H, $HC_{2'}$), 5.67 (br d, 1H, HC_2), 5.93 (br d, 1H, $HC_{3'}$), 6.18–6.54 (m, 3H, $HC_{10} + HC_{13+}PCH$), 7.18–8.09 (m, 16H, =CH + 3×C_6H_5), 7.79, 8.36 (2 s, 2H, $C_{2'}H + C_{8'}H$); MS (FAB): m/z (%): 1,431 (48) [M + 1, phosphorus cluster]$^+$; elemental analysis calcd (%) for $C_{60}H_{74}N_9O_{26}P_3$ (1,430.2499): C, 50.39; H, 5.22; N, 8.81; P, 6.50; found: C, 50.27; H, 5.10; N, 8.97; P, 6.69.

Lipophilicity and solubility tests. An individual solution of **4**, **5**, an equimolar mixture of **4** + **5**, and **8**–**10** (30.0

mg) in 1-octanol (15.0 mL) was shaken with distilled water (pH 5.8, 15.0 mL) for 2.0 h. The layers were separated, and their concentrations were determined by an UV spectrophotometer at 227 (for **5**, **4**+**5**, and **8**–**10**) or 260 (for **4**, **4**+**5**, and **8**–**10**) nm.[14,21] The solubility and lipophilicity data obtained for compounds **8**–**10**, possessing a joint skeleton of **4** and **5**, at UV absorbance of paclitaxel (**5**) (227 nm) were found to be consistent (limit error ±13%) with those obtained for **8**–**10** at UV absorbance of adenine-containing triphosphonobutenolide **4** (260 nm). Reported results are the average of both determinations for **8**–**10** in duplicate (Table 1).

Hydrolysis of prodrugs 8, 9, and 2′-paclitaxel phosphoester 10 in a mixture of DMSO-d_6 and D_2O (1:1 mL/mL, pD 5.8) or a mixture of DMSO-d_6 and phosphate buffer (1:1 mL/mL, pD 7.2)-(^{1}H NMR study). Compounds **8**, **9**, and **10** (0.10 mmol) were individually dissolved in a mixture of DMSO-d_6 (2.0 mL) and D_2O (2 mL, pD 5.8) or a mixture of DMSO-d_6 (2.0 mL) and deuterated phosphate buffer (2.0 mL, pD 7.2). The ^{1}H NMR spectra at 25 °C were taken immediately. The mixtures were then incubated at 37 °C for various periods of time. The spectra of **8** completely changed to that of the eliminated compounds **4**, **5**, and glyoxylic acid after 17.0 h (in DMSO-d_6/D_2O) and 2.0 h (in DMSO-d_6/deuterated phosphate buffer), respectively. On the other hand, after 7.0 h in DMSO-d_6/D_2O, prodrug **8** was hydrolyzed to paclitaxel (**5**) and intermediate **11**. Each mixture was extracted with $CDCl_3$ (2×3.0 mL) to remove paclitaxel (**5**), which was found to be identical with an authentic sample. Each aqueous solution was frozen and lypholized to afford chloro-intermediate **11** or a mixture of triphosphonate 4 and glyoxylic acid as evidenced by ^{1}H NMR analysis. For **11**: mp 130–132 °C (dec.); ^{1}H NMR (300 MHz, DMSO-d_6/D_2O): δ = 3.63 (s, 3H, C_2OCH_3), 4.24 (s, 3H, C_3OCH_3), 6.01 (br s, 1H, OCHCl), 6.78–6.99 (m, 1H, PCH), 7.10–7.31 (m, 1H, =CH), 7.85, 8.26 (2 br s, 2H, $C_{2'}H + C_{8'}H$). Following the same method, compound 9 was found to be converted to 5 and 12 after 43 h (in DMSO-d_6/D_2O) and 4.5 h (in DMSO-d_6/deuterated phosphate buffer), respectively, while 10 was stable even after 55 h. For 12: mp > 250 °C (dec.); UV (EtOH): λ_{max} (lgε) = 216 (4.24), 260 nm (4.19); ^{1}H NMR (300 MHz, D_2O): δ = 3.65 (s, 3H, C_2OCH_3), 4.30 (s, 3H, C_3OCH_3), 5.52 (s, 2H, OCH_2CO), 6.85 (dd, J = 18.0, 32.3 Hz, 1H, PCH), 7.15–7.28 (m, 1H, =CH), 7.86, 8.24 (2 br s, 2H, $C_{2'}H + C_{8'}H$).

Hydrolysis of paclitaxel triphosphonate esters 8–10 in the presence of human plasma. Paclitaxel triphosphonate esters **8**–**10** were separately dissolved in $CHCl_3$ at a concentration of 15 mg/mL. From each stock solution 40 μL was transferred, individually, to 1.5-mL tubes and the solvent in each tube was evaporated by bubbling nitrogen gas into the respective solution. A few drops of human plasma then was added to each tube, and the mixtures were incubated at 37 °C for various periods of time. In all cases, 2-propanol (300 μL) was added at the proper intervals to precipitate the proteins, and the resulting solution was vortexed and centrifuged for 4.0 min. Supernatant solution (250 μL) of each tube was transferred into another vials, diluted with distilled water (300 μL), and then extracted with $CHCl_3$ (300 μL) to remove paclitaxel (**5**), which was found to be identical with an authentic sample. The aqueous solutions were, respectively, analyzed by HPLC, employing an anion-exchange Partisphere column, using a gradient of 8.0 mM to 1.0 M ammonium phosphate as mobile phase. We observed that compound **8** was hydrolyzed to **4**, **5**, and glyoxylic acid after 0.40 h. Compound **9** was hydrolyzed to **5** and **12** after 0.65 h, and compound **10** was completely converted to **4** and **5** in human plasma media after 15 h.

Evaluation of the effectiveness of triphosphonate 4, paclitaxel triphosphonate esters 8–10, and triphosphonate ester 12 against RDPR. All compounds **4**, **8**–**10**, and **12** were evaluated for inhibitory property against *E. coli* RDPR (EC 1.17.4.1),[15] and the remaining enzyme activity was determined.[16] The following is a representative procedure: *E. coli* RDPR was isolated as described,[15b] and used by the method of Baker et al.[15a] Briefly, HEPES (50.0 mM, pH 7.60), $MgSO_4$ (15.0 mM), EDTA (1.0 mM), ATP (1.60 mM), NADPH (0.50 mM), thioredoxin (12.0 μM), thioredoxin reductase (0.79 μM), R1 subunit (12.8 μM), and R2 subunit (12.8 μM) along with variable concentrations of the inhibitor **4** were incubated at 37 °C for different times (20 s–20 min). During the reactions, the respective solutions scanned repeatedly from 190 to 620 nm. The remaining enzyme activity for the conversion of CDP to dCDP was determined by the method of Steeper and Steuart.[16] Control experiments were performed with no inhibitor in the incubation mixture.

Microtubule assembly experiment. The ability of paclitaxel (**5**), prodrugs **8** and **9**, as well as 2′-paclitaxel phosphoester **10** to induce microtubule assembly in vitro were examined.[18] The following is a representative procedure: To PEM buffer solution (0.10 M Pipes, pH 7.1, 1.0 mM EGTA, and 0.8 mM $MgSO_4$) was added tubulin (12.0 μM), bifunctional prodrug **8** (7.0 μM), and GTP (7.0 μM). The increase in turbidity (microtubule polymerization) was monitored at 30 °C by the increase in apparent absorbance at 350 nm using a temperature-controlled spectrophotometer. To determine the amount of assembled tubulin, the samples were centrifuged (200,000*g* for 4.0 min) at 37 °C. The pellet was dissolved in cold PEM buffer and allowed to depolymerize on ice for 25 min. The protein concentration in the supernatant after centrifugation at 4 °C was determined by the method of Bradford.[22]

Anticancer test procedure in vitro. Murine leukemias (L1210 and P388), breast carcinoma (MCF7), human T-lymphoblasts (Molt4/C8 and CEM/0), human embryonic lung cell (HEL), and normal fibroblasts (Hef522) were cultured in DMEM supplemented with 10% FBS, 2.0 mM glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin in a humidified atmosphere with 5% CO_2 at 37 °C.[19] Under this condition, the generation time for L1210, P388, MCF7, Molt4/C8, CEM/0, HEL, and Hef522 cells was about 13, 12, 17, 18, 21, 45, and 48 h, respectively. Compounds **2**–**10**, **12**, glyoxylic acid,

ara-C, and equimolar mixtures of **4**+**6**, **5**+**11**, and **4**+**5**+glyoxylic acid in DMSO/D_2O (8:2 v/v), at various concentrations, were added to the cells (200 cells/mL) in their exponential phase of growth. The cell numbers of the control cultures, as well as that of the cultures supplemented with the test compounds, were determined after 24, 48, and 72 h of growth. The IC_{50} values were estimated from dose–response curves compiled from three independent experiments and represent the compound concentration (μM) required to inhibit proliferation of the respective malignant tumor cell lines by 50% after 72 h incubation (Table 2).

B16 melanoma cell proliferation. Inhibition of proliferation of B16 melanoma cells by compounds **4**, **5**, and **8**–**10** were studied according to the reported procedure by Mathew et al.[18] Results are illustrated in Table 2.

Observation of microtubule assembly in MCF7 by immunofluorescence microscopy. Cells were grown on coverslips and remained untreated or were incubated with adenine-containing triphosphonobutenolide **4** (0.34 μM), paclitaxel (**5**) ($2.71\times10-^{3}$ μM), or bifunctional prodrug **8** (0.20×10^{-3} μM).[23] After 6.5 h, coverslips were air-dried and fixed with freshly prepared 3.7% paraformaldehyde in phosphate-buffered saline for 10.0 min. Residual aldehyde was quenched with 0.10 M glycine in PBS for 5.0 min. Cells were permeabilized for 2.0 min at room temperature with 0.5% Triton X-100 in PBS and blocked for 14.0 h at 4.0 °C with normal goat serum in PBS containing 0.2% bovine serum albumin and 50.0 mM NH_4Cl (1:1000 μL/μL). Microtubule staining was carried out by primary anti-E7 mouse antibody in PBS containing 0.2% BSA (1:20 μL/μL) for 45.0 min. Then, it was treated with goat secondary anti-mouse FITC Fab fragment in PBS containing 0.2% BSA (1:100 μL/μL) for 20.0 min. The coverslips were mounted on glass slides by elvanol containing 0.2% paraphenylene diamine and viewed under a Zeiss fluorescence microscope.

Observation of apoptotic morphology in MCF7 cells by a fluorescence microscope. Cells were grown on coverslips and remained untreated or were incubated with adenine-containing triphosphonobutenolide **4** (1.36 μM), paclitaxel (**5**) (5.42×10^{-3} μM), or bifunctional prodrug **8** (0.4×10^{-3} μM) for 8.0 h. The coverslips were prepared in the same manner as described above. Nuclei were labeled with Hoechst 33342 in PBS (1.0 μg mL^{-1}) for 15.0 min in the dark at room temperature.[24] The coverslips were mounted on glass slides and viewed under a Zeiss fluorescence microscope.

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