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Water Soluble Prodrugs of the Antitumor Agent 3-[(3-Amino-4-methoxy)phenyl]-2-(3,4,5-trimethoxyphenyl)cyclopent-2-ene-1-one

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Abstract—Fourteen prodrugs of the antitumor agent 3-[(3-amino-4-methoxy)phenyl]-2-(3,4,5-trimethoxyphenyl)cyclopent-2-ene-1-one (**1**) were prepared to improve its water solubility and potency. These prodrugs include α-amino acid (**1a**–**1h**), aliphatic amino acid (**1i**–**1l**), phosphoramidate (**1m**), and phosphate (**1n**) derivatives. All of the prodrugs showed improved water solubility. A number of the amino acid prodrugs (**1a**, **1b**, **1d**–**1f**, **1h**, **1j**, and **1k**) exhibited more potent antitumor activity compared to the parent compound (**1**). The phosphate prodrug **1n** also offered a potent antitumor activity, but the phosphoramidate **1m** did not show any antitumor activity in vivo. None of the prodrugs exhibited significant toxicities in mice. These results indicate that the design and preparation of the amino acid prodrugs (**1a**, **1b**, **1d**–**1f**, **1h**, **1j**, and **1k**) and phosphate prodrug (**1n**) are beneficial for enhancing the antitumor activity of **1**. The similar approaches may be used to improve water solubility and bioactivity of other poorly soluble aromatic amines.

Introduction

Previously, we have reported an antitumor agent 3-[(3-amino-4-methoxy)phenyl]-2-(3,4,5-trimethoxyphenyl)cyclopent-2-ene-1-one (**1**, Fig. 1), a novel analogue of combretastatin A-4 (CA-4, Fig. 1).[1] Compound **1** exhibited very potent cytotoxicity against various tumor cell lines with IC_{50} values at low nanomolar range (18.2–22.2 nM). However, only modest antitumor activity was observed in vivo when **1** was administered ip to BDF1 mice bearing Lewis lung carcinoma (3LL) cells at 40 mg/kg/day (the tumor mass inhibition rate of compound **1** was 59% compared to 71% produced by etoposide, a clinical anticancer drug which was used as a positive control). The unexpected low antitumor activity was thought to be due to its low bioavailability, which was caused, at least in part, by its poor aqueous solubility. It has been shown that compound **1** and its hydrochloride salt had water solubilities of 0.8 and 2.1 mg/mL, respectively, at 25 °C. A maximum dose of 40 mg/kg/day of **1** in the form of hydrochloride salt was possible with the use of media such as Tween 80 or Cremophor®.[1] Noteworthy however, at this dose the compound showed no sign of toxicity in the treated mice. Thus, the improvement of the water solubility is a strategy to increase administration dosage and to improve therapeutic values of this antitumor agent.

Introduction of an ionizable pro-moiety into the structure of compounds is one common approach to improve the water solubility. For example, amino acid prodrugs have frequently been investigated for alcohol drugs.[2–6] Amide prodrugs in general have been used limitedly due to their relative stability in vivo. Activated amide prodrugs such as amides of α-amino acids, however, have favorable water solubility and generate a parent drug(s) in vivo.[2,3] The bioconversion of those amide prodrugs is due to the strong electron-withdrawing effects of the α-protonated amino group, which activates the amide linkage towards hydrolysis, and partly (or predominantly) due to intramolecular catalysis or assistance by the neighboring amino group, the so-called folding effects.[2] This observation led us to design and synthesize the amino acid prodrugs **1a**–**h** (Fig. 1,

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Scheme 1). Compound **1h** in particular can be viewed either as a water soluble prodrug of **1** or a hybrid of compound **1** and melphalan, a drug currently used in cancer chemotherapy.

The folding effect toward the bioconversion of certain aliphatic amino acid prodrugs has also been observed.[2] Based on this observation, three aliphatic amino acid prodrugs (**1i–k**) with varying chains were designed and synthesized (Fig. 1, Scheme 2). The prodrug **1l** was synthesized to examine the importance of both the folding effects in the bioconversion process of **1i–k**, and the electron-withdrawing effects of the α-protonated amino group in **1a–h**. By incorporation of the phenyl group between the amide moiety and the amino group compound **1l** has no α-amino group, thus the electron-withdrawing effects should be absent. In addition, the intramolecular catalytic reaction of the amino group is no longer possible for sterical reasons. Compound **1l** is expected to have a similar water solubility with that of **1i–k** since the amino group is not directly attached to the phenyl ring but through a methylene group.

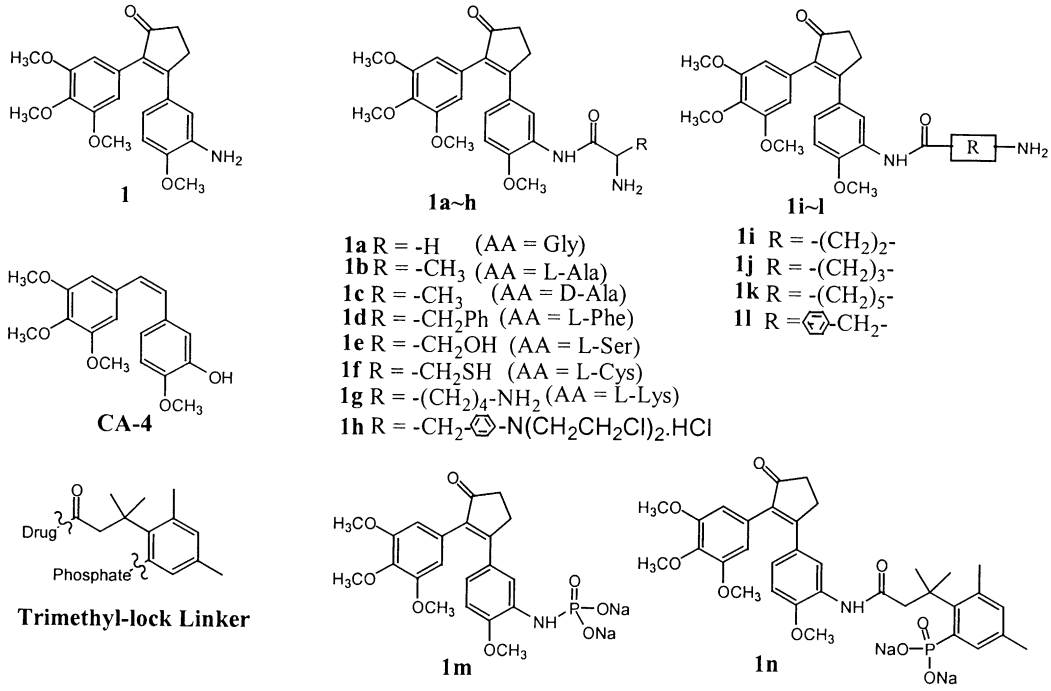
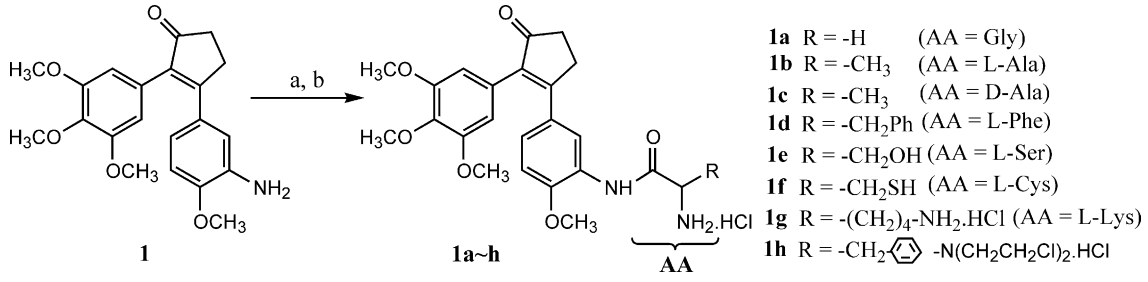
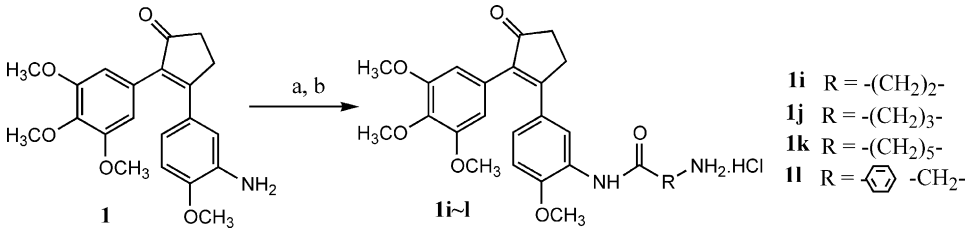


Figure 1. Structures of 3-[(3-amino-4-methoxy)phenyl]-2-(3,4,5-trimethoxyphenyl)cyclopent-2-ene-1-one (**1**), combretastatin A-4 (CA-4), and designed prodrugs.



Scheme 1. Reagents and conditions: (a) protected amino acid (*N*-Boc-gly for **1a**, *N*-Boc-L-Ala for **1b**, *N*-Boc-D-Ala for **1c**, *N*-Boc-L-Phe for **1d**, *N*-Fmoc-*O*-*tert*-Bu-L-Ser for **1e**, *N*-Boc-*S*-trityl-L-Cys for **1f**, diBoc-L-Lys for **1g**, and *N*-Boc-Melphalan for **1h**), DCC, HOBt·H_2O, DMF; (b) 4 N HCl-dioxane, rt, 3 h (for **1a–d** and **1g–h**); piperidine/CH_2Cl_2 then 4 N HCl-dioxane, rfx, 3 h (for **1e**); or 3 N HCl-aq. AcOH, 90 °C, 1.5 h (for **1f**).



Scheme 2. Reagents and conditions: (a) HOOC-R-NHBoc, DCC, HOBt·H_2O, DMF (b) 4 N HCl-dioxane, rt, 3 h.

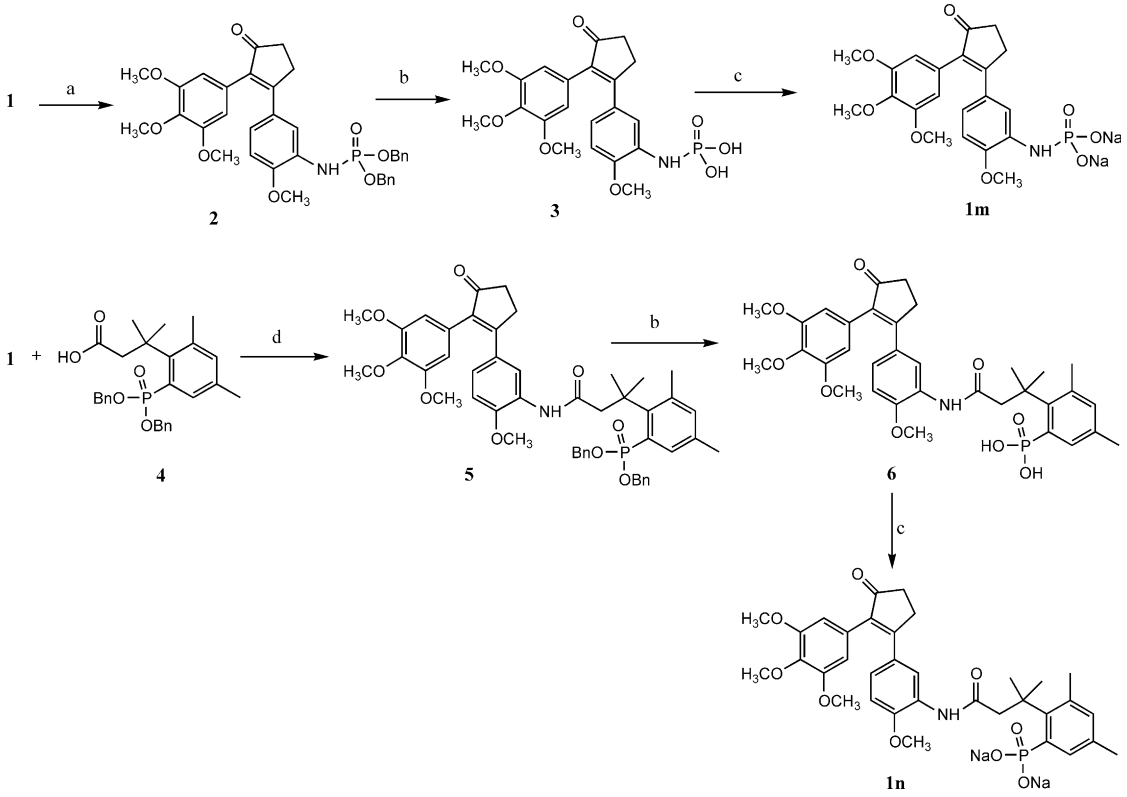
The use of phosphates as an ionizable pro-moiety is another approach to improve the water solubility. We initially designed the phosphoramidate **1m** as a prodrug of **1**. However, phosphoramidates and phosphate prodrugs of steric alcohols often suffer from poor bioconversion in vivo.[2,3] Prodrugs with various linked phosphates have been designed to overcome such hurdles.[7–15] Different masked lactone linkers have been developed for this purpose.[10–15] Among these, a trimethyl-lock linker (Fig. 1) has been demonstrated to have favorable pharmacokinetic properties.[15] We have therefore chosen this linker and synthesized a phosphate prodrug **1n**.

In this paper, the synthesis and results from the biological evaluation of these prodrugs (**1a–n**) are presented and discussed.

Chemistry

The syntheses of amino acid or short-chain aliphatic amino acid amide hydrochlorides of **1** are shown in Schemes 1 and 2. The synthetic sequences involved coupling of **1** with protected amino acids followed by deprotection step. Commercially available Boc-amino acids (*N*-*tert*-butyloxycarbonylamino acids) were used including *N*-Boc-Gly, *N*-Boc-L-Ala, *N*-Boc-D-Ala, *N*-Boc-L-Phe, *N*-diBoc-L-Lys, *N*-Boc-β-Ala. For cysteine and serine, *N*-Boc-*S*-trityl-L-Cys and *N*-Fmoc-*O*-*tert*-Bu-L-Ser were used. Short-chain aliphatic amino acids [γ-aminobutyric acid, 6-aminohexanoic acid (caproic acid)] and 4-aminomethyl benzoic acid were protected with Boc by reacting with Boc anhydride [$(Boc)_2O$] in aq NaOH. The coupling reaction of **1** with protected amino acids was carried out in the presence of DCC/$HOBt{\cdot}H_2O$ as coupling reagents. Removal of the Boc protecting group was performed by stirring in 4 N HCl-dioxane to yield the hydrochloride form of **1**-amino acid amides (**1a–d**, **1g**, **1h**, and **1i–l**) directly. For the synthesis of **1e** the deprotection step was carried out in piperidine to remove the Fmoc group followed by refluxing in 4 N HCl-dioxane to deprotect the hydroxy group and to yield the hydrochloride salt of **1e** directly. In the case of 1f the coupling product of 1 and *N*-Boc-*S*-trityl-L-Cys was refluxed in HCl/aq AcOH to remove both the Boc and trityl protecting groups to afford the hydrochloride salt of **1f**.

The synthesis of the phosphate prodrugs **1m** and **1n** is shown in Scheme 3. The phosphoramidate **1m** was obtained via three steps. The first step was involved the phosphorylation of **1** by tetrabenzyl pyrophosphate to give a 1-dibenzylphosphoramidate intermediate (**2**). The intermediate **2** was deprotected with trimethylsilyl iodide (generated in situ) at room temperature to generate a debenzylated **1**-dihydroxyphosphoramidate (**3**) which was reacted with sodium methoxide to give the disodium phosphoramidate **1m** in 45% overall yield. Reaction of 1 with 3-[2′-(dibenzylphosphono)oxy-4′,6′-dimethylphenyl)-3,3-dimethylpropionic acid (**4**)[15] in the presence of DCC/$HOBt{\cdot}H_2O$ as coupling reagents gave



Scheme 3. Reagents and conditions: (a) $P_2O_7Bn_4$, TEA, MeCN, rfx; (b) TMS-Cl, NaI, acetonitrile, rt; (c) THF, MeONa, rt; (d) 3-[2′-(dibenzylphosphono)oxy-4′,6′-dimethylphenyl)-3,3-dimethylpropionic acid (**4**),[15] DCC, $HOBt{\cdot}H_2O$, CH_2Cl_2.

5. The intermediate 5 was debenzylated by trimethylsilyl iodide to furnish 6, which was reacted with sodium methoxide, as described for the case of 1m, to yield 1n in 22% overall yield.

All final products were obtained in sufficient purity after purification through a silica gel column eluting with 7.5–10% MeOH in CH_2Cl_2 and/or recrystallization from appropriate solvents. ^1H NMR, IR, HR-MS and elemental analysis were used to confirm the structures of compounds. IR spectra revealed the peaks at 1640–1660 cm^{-1} range due to the presence of the newly added amide carbonyl group in addition to the ketone signals appeared at 1690–1720 cm^{-1} range.

Biological Results and Discussion

Cytotoxicity of the synthesized prodrugs was determined in two tumor cell lines including B16 (murine melanoma) and HCT116 (human colon tumor). The results are summarized in Table 1.

It has been mentioned earlier that amides in general are too stable to be effective as prodrugs. However, most of the amino acid prodrugs of 1 showed potent cytotoxicities in both tumor cell lines. Thus, the prodrugs 1a–k would be cytotoxic per se or would likely be conversed to the parent compound. The electron-withdrawing effects of the α-protonated amine groups present in 1a–h or folding effects of the chained aliphatic amines in 1i–k might facilitate the bioconversion of these prodrugs. In attempt to gain some insights into the importance of these effects, compound 1l was synthesized and evaluated. In this compound the amino group is not attached at the α-position but is linked to an amide group through a bulky phenyl moiety and thus the electron-withdrawing effects of the α-protonated amine group is absent and the folding effects should be blocked. Compound 1l was found to be rather inactive (IC_{50} values of >2000 nM) compared to the parent 1 (18.2–22.2 nM). These results suggest that the prodrugs 1a–k were unlikely cytotoxic per se but were conversed to the parent 1 under cell culture conditions. Thus, the electron-withdrawing effects of the α-protonated amine groups and the folding effects of the chained aliphatic amines towards the cleavage of the amide linkages seemed to be important for the bioconversion of these prodrugs. Exceptionally however, the D-amino acid prodrug 1c was found to be less active than its corresponding L-amino acid prodrug 1b. It has been shown previously that prodrugs with D-amino acid(s) are much more stable to enzymatic hydrolysis than that with L-amino acid(s).[16] This could be the reason for the low activity observed with 1c. A similar result has also been observed with other D-amino acid prodrugs.[17]

The decreased activity of the phophoramidate 1m indicates that this prodrug might not cleave under cell culture conditions or the rate of hydrolysis was too low for

Table 1. Cytotoxicity and antitumor activity of prodrugs 1a–j

Compd	Cytotoxicity[a] (IC_{50}[b] nM)		Antitumor activity		
	B16	HCT116	Dose[c] (mg/kg/day)	Body weight changes (%)	IR[d] (%)
1a (Gly)	149	160	76	+5.6	72.2
1b (L-Ala)	157	197	75	+7.3	66.7
1c (D-Ala)	682	1512	75	+11.4	28.4
1d (L-Phe)	132	125	87	+7.5	65.7
1e (L-Ser)	67	91	78	+8.6	77.4
1f (L-Cys)	75	77	81	+7.5	78.2
1g (L-Lys)	77	65	84	+11.2	59.7
1h (Melphalan)	32	47	109[e]	+9.2	76.3
1i (β-Ala)	136	107	75	+10.1	17.0
1j (4-AA)	67	82	77	+10.0	76.2
1k (6-AA)	143	151	82	+6.2	73.6
1l (Scheme 22)	>2000	>2000	nt[f]		
1m (Scheme 23)	>2000	>2000	84	+14.8	—[g]
1n (Scheme 23)	84	89	116	+11.4	74.5
1.HCl	22.2	17.2	40	+10.5	59.0
Adriamycin	62	40	Nt		
Etoposide[h]	nt		36	0	78.6
Melphalan[i]	nt		50	−6.5	39.7
Vehicle			0.2 mL/mice/day	+19.7	0

[a]Cancer cell lines: B16, murine melanoma; HCT116, colon cancer.
[b]A sample's concentration which produces a 50% reduction in cell growth. The values shown were the averages from a triplicate experiment.
[c]Dose equivalent to 60 mg/kg/day of 1. HCl.
[d]Inhibiton rate (%) = $(1-T/C)\times100$, calculated on day 15; T = mean tumor volume of the sample-treated group and C = mean tumor volume of the negative control group; mice were implanted sc with 3LL cells (10^7 cells/0.2 mL PBS/mice), and the drugs were administered ip in 0.2 mL vehicle (5% DMSO + 20% cremophor/saline).
[e]Equivalent to 50 mg/kg/day of melphalan.
[f]Not tested.
[g]Inactive (IR < 10%).
[h]Etoposide, used as a positive control, injected on the days 1, 5, and 9 at 36 mg/kg/day.
[i]Melphalan was given at 50 mg/kg/day with the same schedule as prodrugs.

the prodrug to observe any activity. The highly hydrophilic nature of the prodrug might effectively prevent the compound from crossing the lipophilic layer of the cellular membrane, rendering it noncytotoxic. In contrast, the phosphate **1n** exhibited potent cytotoxicity against both tumor cell lines, suggesting that the prodrug was bioconversed to the parent compound **1**.

To examine whether the prodrugs are conversed to the parent compound **1** in vivo, the antitumor activities were determined in BDF1 mice bearing 3LL cells. The phosphoramidate **1m** was also evaluated as a representative of the in vitro inactive compounds.

We have previously reported that compound **1** did not manifest significant toxicity in mice at 40 mg/kg/day.[1] Therefore, increase of dosage is possible given that the pharmacokinetic profile of the compound is improved. All of the synthesized prodrugs offer substantial increased water solubility (Table 2), which permit the dosing up to 100 mg/kg/day. However, preliminary experiments with **1a** indicated that this prodrug showed signs of toxicity in mice at doses greater than 76 mg/kg/day, equivalent to 60 mg/kg/day of **1**. As a result, the evaluation of this prodrug series was performed at doses of compounds that are equivalent to 60 mg/kg/day of **1**, hypothesizing that 100% bioconversion is achieved in vivo (Table 1). Etoposide, a drug currently used in cancer chemotherapy was administered to one group at 36 mg/kg/day on day 1, 5, 9 and used as a positive control. The prodrugs were reconstituted in 0.2 mL vehicle (5% DMSO + 20% Cremophor®/saline) and dosed from day 1 and every other day until day 11 (totals six injections). The results summarized in Table 1 demonstrate that most of the amino acid prodrugs showed more potent antitumor activity than the parent compound **1** in BDF1 mice bearing 3LL cells. Careful analyses of the data indicated that the antitumor activity, in general, seemed to correlate to the cytotoxicity. The glycine prodrug **1a** showed a higher inhibition rate (IR) value (72.2%) than the L-alanine prodrug of **1** (**1b**, IR value of 66.7%), suggesting that the presence of the α-methyl group might somewhat lower the rate of the amide linkage hydrolysis. The D-alanine prodrug **1c**, which was much less active in vitro compared to other L-amino acid prodrugs, was found to have a marginal antitumor activity (IR value, 28.4%). Compounds **1e** and **1f**, the serine and cystein prodrugs of **1**, exhibited more potent activities than that of **1a** (IR values of 77.4 and 78.2%, respectively, vs 72.2% of **1a**) despite the presence of the bulkier α-methylenehydroxy and α-methylenethiol groups. Thus, the presence of the α-methylenehydroxy and α-methylenethiol moieties were favorable for the bioactivity of this prodrug, probably due to the possible roles of the hydroxy and thiol groups in the cleavage of the prodrug to the parent compound. The melphalan prodrug **1h** also offered a good regression of tumor mass with an IR value recorded at 76.3%, higher than that (59%) of **1**. When melphalan was administered to one group of mice at 50 mg/kg/day, a dose equivalent to its content in **1h** given at 109 mg/kg/day, it showed only moderate activity (IR value of 39.7%). Thus, the hybrid **1h** showed a good synergistic activity. Moreover, the toxicity of melphalan was notable, as manifested by the body weight loss of the melphalan-treated mice (Table 1), while **1h** exhibited no toxicity at the given dose. These results indicate the benefits of the design of hydrid prodrugs in this way.

Among three amino acid prodrugs containing aliphatic chains, for example, **1i–k**, the most potent activity was observed with **1j**. **1k** was found to be slightly less potent than **1j**, while **1i** showed only marginal activity. These results suggest that the folding effects of the terminal amino group might play an important role towards the amide hydrolysis of these prodrugs, as the formation of the more stable five-membered ring intermediate under the amino-mediated intramolecular cyclative cleavage of **1j** was more favorable, compared to the constrained four- or less stable seven-membered ring intermediates in the cases of **1i** and **1k**, respectively (Fig. 2). Hence, **1j** was likely converted to the parent drug **1** at a faster rate than **1i** and **1k**. The inactivity of the phosphoramidate **1m** which was also inactive in vitro, suggests that this type of prodrugs is stable in vivo, and thus the proposed cleavage of this prodrug catalyzed by phosphoramidases as shown in Figure 3 unlikely occurred fast enough for the prodrug to be effective. Meanwhile, compound 1n exhibited good antitumor activity with an IR value of 74.5%. Previously, Nicolaou et al.[15] have shown that trimethyl-lock linked phosphate prodrugs of amines which have structural similarity with prodrug **1n**, were effectively cleaved in the presence of alkaline

Table 2. Water solubility of the prodrugs **1a–n**

Prodrug	Solubility (mg/mL)	Prodrug	Solubility (mg/mL)
1a (Gly)	>10[a]	**1h** (Melphalan)	4.67
1b (L-Ala)	6.65	**1i** (β-Ala)	7.12
1c (D-Ala)	6.43	**1j** (4-AA)	6.81
1d (L-Phe)	5.94	**1k** (6-AA)	5.27
1e (L-Ser)	6.75	**1l** (Scheme 2)	5.12
1f (L-Cys)	4.68	**1m** (Scheme 3)	>10[a]
1g (L-Lys)	>10[a]	**1n** (Scheme 3)	>10[a]

[a]Due to limited amounts of the prodrugs, no further effort was made to measure the maximum solubility of these compounds.

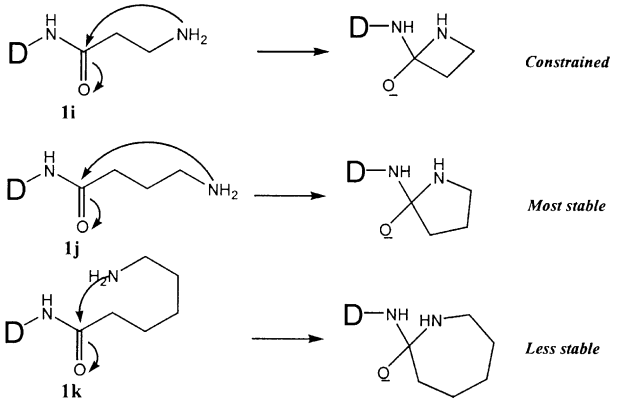


Figure 2. Possible pathways for generation of **1** from prodrugs **1i–k** (D, a parent drug).

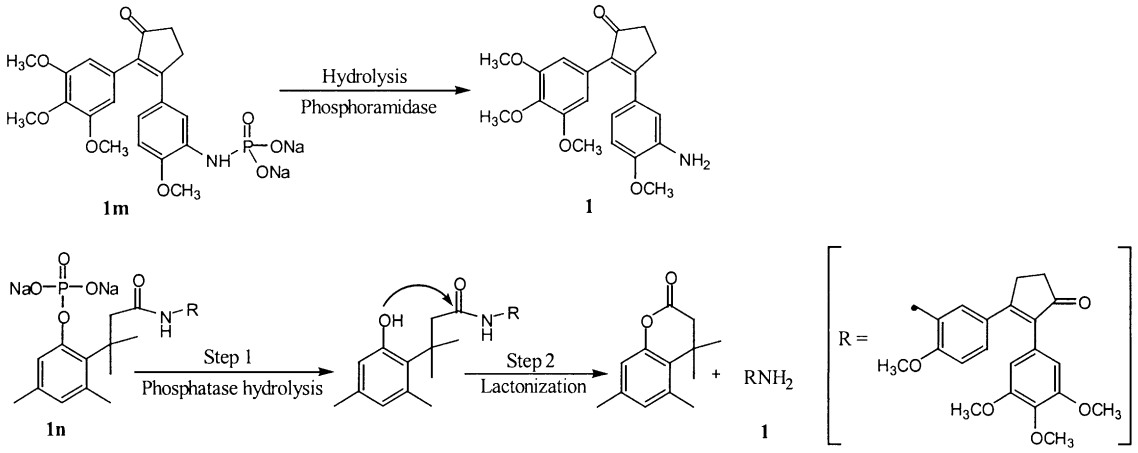


Figure 3. Possible pathways for generation of **1** from prodrugs **1l** and **1m**.

phosphatases (AP), regardless of the nature of amine parent drugs. Alkaline phosphates are widely distributed in a variety of tissues such as liver, kidney tubules, and intestinal epithelium. Though we have not performed experiments to confirm the cleavage mechanism for the prodrug, the results indicate that the in vivo cleavage of **1n** likely occurred as depicted in Figure 3.

In summary, we have reported in this paper the synthesis and biological evaluation of a series of amino acid prodrugs and phosphate prodrugs of the antitumor agent 3-[(3-amino-4-methoxy)phenyl]-2-(3,4,5-trimethoxyphenyl)cyclopent-2-ene-1-one (**1**). Most of the amino acid prodrugs exhibited more potent antitumor activity compared to the parent compound. The phosphate prodrug **1n** showed a potent antitumor activity. None of the prodrugs showed significant toxicity as manifested by no body weight loss of prodrugs-treated mice. These results indicate that the design and preparation of the amino acid prodrugs and phosphate prodrug like **1n** are beneficial for enhancing the antitumor activity of **1**. Similar strategies are possible for other aromatic amine drugs, which are often poorly soluble in aqueous systems.

Experimental

Common chemicals and solvents were purchased from commercial sources. Solvents were distilled before use. Protected amino acids were purchased from either Sigma Aldrich Co. Ltd. (USA) or Fluka Co. Ltd. (USA). All ^{1}H NMR spectra were recorded using a Varian-Gemini 300 (300 MHz) spectrometer with tetramethylsilane as the internal standard. Chemical shifts are reported in ppm (δ) and coupling constants are reported in Hz. Infrared spectra were measured using KBr plates on a Perkin-Elmer 1600 series FTIR. Melting points were determined using an electrothermal IA6304 open capillary melting point apparatus and are uncorrected. High-resolution mass spectra (HRMS) were measured on JEOL JMS-HX110/HX110 instrument. Syntheses of compounds and biological procedures are described as follow. For convenient follow-up, the compounds are described in the same order they appeared in Schemes 1–3.

1-Gly. HCl (1a). All glasswares were flame dried, and the reaction was carried out under nitrogen atmosphere. HOBt·H_2O (207 mg, 1.53 mmol) and DCC (271 mg, 1.2 mmol) were added to a solution of **1** (369 mg, 1 mmol) in anhydrous DMF (5 mL) at 0 °C. The reaction mixture was kept under continuous stirring for 15 min. *N*-Boc-Gly (209 mg, 1.2 mmol) in the solid form was then added at once, and the reaction mixture was stirred for additional 12 h while it was gradually allowed to reach ambient temperature. The solvent was then removed under reduced pressure. The crude **1**-*N*-Boc-Gly was purified on a silica gel column using 33% ethyl acetate in hexane. **1**-*N*-Boc-Gly was directly dissolved in 4 N HCl-dioxane (10 mL) and stirred at room temperature for 3 h. After removal of solvent, the residue was purified by silica gel column chromatography eluting with 7.5% MeOH in CH_2Cl_2 to furnish 355 mg of **1a**. Yield: 76%; mp 145 °C; IR (KBr) 3441 (NH), 1690 (CO ketone), 1664 (CO amide) cm^{-1}; ^{1}H NMR (DMSO-d_6, 300 MHz) δ 2.58–2.66 (2H, m), 2.75–2.87 (2H, m), 3.74 (6H, s), 3.80 (2H, br), 3.81 (3H, s), 3.89 (3H, s), 6.54 (2H, s), 6.67 (1H, d, J = 8.12 Hz), 6.71 (1H, dd, J = 8.12, 2.00 Hz), 6.78 (1H, d, J = 2.00 Hz). Anal. calcd for $C_{23}H_{27}ClN_2O_6$: C, 59.67; H, 5.88; N, 6.05. Found C, 59.84; H, 6.01; N, 6.26.

1-L-Ala. HCl (1b). This compound was synthesized according to the same procedure described for **1a** using *N*-Boc-L-Ala instead of *N*-Boc-Gly. Yield: 68%; mp 178 °C; IR (KBr) 3405 (NH), 1715 (CO ketone), 1676

(CO amide) cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz) δ 1.58 (3H, d, $J = 7.20$ Hz), 2.66–2.82 (2H, m), 2.87–3.01 (2H, m), 3.78 (6H, s), 3.81 (3H, s), 3.87 (3H, s), 4.53 (1H, q, $J = 7.20$ Hz), 6.51 (2H, s), 6.70 (1H, d, $J = 9.50$ Hz), 6.76 (1H, dd, $J = 9.50$, 2.10 Hz), 6.85 (1H, d, $J = 2.00$ Hz). Anal. calcd for $C_{24}H_{29}ClN_2O_6$: C, 60.44; H, 6.13; N, 5.87. Found C, 60.02; H, 6.18; N, 6.11.

1-D-Ala. HCl (1c). This compound was synthesized by the same procedure described for **1a** using *N*-Boc-D-Ala instead of *N*-Boc-Gly. Yield: 69%; mp 139 °C; IR (KBr) 3376 (NH), 1703 (CO ketone), 1655 (CO amide) cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz) δ 1.62 (3H, d, $J = 7.08$ Hz), 2.64–2.80 (2H, m), 2.89–3.03 (2H, m), 3.81 (3H, s), 3.84 (6H, s), 3.88 (3H, s), 4.33 (1H, q, $J = 7.09$ Hz), 6.57 (2H, s), 6.71 (1H, dd, $J = 7.78$, 1.98 Hz), 6.74 (1H, d, $J = 7.78$ Hz), 6.82 (1H, d, $J = 1.99$ Hz). Anal. calcd for $C_{24}H_{29}ClN_2O_6$: C, 60.44; H, 6.13; N, 5.87. Found C, 60.24; H, 6.28; N, 6.09.

1-L-Phe. HCl (1d). This compound was synthesized by the same procedure described for **1a** using *N*-Boc-L-Phe instead of *N*-Boc-Gly. Yield: 78%; mp 173 °C; IR (KBr) 3445 (NH), 1693 (CO ketone), 1667 (CO amide) cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz) δ 2.61–2.75 (2H, m), 2.87–3.00 (3H, m), 3.25–3.51 (2H, m), 3.82 (3H, s), 3.87 (6H, s), 3.91 (3H, s), 4.63 (1H, m), 6.49 (2H, s), 6.66 (1H, d, $J = 8.01$ Hz), 6.71 (1H, dd, $J = 8.01$, 2.05 Hz), 6.78 (1H, d, $J = 2.05$ Hz), 7.01–7.35 (5H, m). Anal. calcd for $C_{30}H_{33}ClN_2O_6$: C, 65.15; H, 6.01; N, 5.07. Found C, 64.99; H, 6.13; N, 5.26.

1-L-Ser. HCl (1e). 1-*N*-Fmoc-*O*-*tert*-Bu-L-Ser was synthesized by the same procedure described for **1a** using *N*-Fmoc-*O*-trityl-L-Ser instead of *N*-Boc-Gly. The Fmoc group was removed by stirring 1-*N*-Fmoc-*O*-*tert*-Bu-L-Ser in piperidine/CH_2Cl_2 for 3 h. Removal of the *O*-*tert*-Bu group was achieved by refluxing 1-*O*-*tert*-Bu-L-Ser in 4 N HCl-dioxane for 3 h. 1-L-Ser. HCl (**1e**) was purified as described for **1a**. Yield: 52%; mp 157 °C; IR (KBr) 3510 (OH), 3454 (NH), 1700 (CO ketone), 1668 (CO amide) cm^{-1}; ^1H NMR (DMSO-d_6, 90 MHz) δ 2.61–2.69 (2H, m), 2.77–2.93 (2H, m), 3.73 (6H, s), 3.81 (3H, s), 3.89 (3H, s), 3.90–3.98 (2H, m), 4.12–4.21 (1H, m), 6.66 (2H, s), 6.78 (1H, d, $J = 2.10$ Hz), 6.81–6.99 (2H, m). Anal. calcd for $C_{24}H_{29}ClN_2O_7$: C, 58.48; H, 5.93; N, 5.68. Found C, 58.27; H, 6.11; N, 5.61.

1-L-Cys. HCl (1f). 1-*N*-Boc-*S*-trityl-L-Cys was synthesized by the same procedure described for **1a** using *N*-Boc-S-trityl-L-Cys instead of *N*-Boc-Gly. Concomitant deprotection of *N*-Boc and S-trityl groups was achieved by refluxing 1-*N*-Boc-*S*-trityl-L-Cys in 3 N HCl-AcOH for 3 h. Purification method of 1-L-Cys. HCl (**1f**) was the same as described for **1a**. Yield: 47%; mp 138 °C; IR (KBr) 3450 (NH), 1697 (CO ketone), 1671 (CO amide) cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz) δ 2.63–2.75 (2H, m), 2.79–3.02 (2H, m), 3.74 (6H, s), 3.82 (3H, s), 3.88 (3H, s), 3.94–4.01 (2H, m), 4.10–4.18 (1H, m), 6.55 (2H, s), 6.70 (1H, d, $J = 8.00$ Hz), 6.74 (1H, dd, $J = 8.00$, 2.01 Hz), 6.84 (1H, d, $J = 2.01$ Hz). Anal. calcd for $C_{24}H_{29}ClN_2O_6S$: C, 56.63H, 5.74; N, 5.50. Found C, 56.71; H, 5.82; N, 5.49.

1-L-Lys. 2HCl (1g). This compound was synthesized by the same procedure described for **1a** using N_α, N_ε-diBoc-L-Lys instead of *N*-Boc-Gly. Yield: 68%; mp 123 °C; IR (KBr) 3460 (NH), 1691 (CO ketone), 1665 (CO amide) cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz) δ 1.35–1.40 (2H, m), 1.67–1.82 (2H, m), 1.88–1.95 (2H, m), 2.58–2.71 (2H, m), 2.88–3.02 (4H, m), 3.76 (6H, s), 3.81 (3H, s), 3.89 (3H, s), 4.09–4.13 (1H, m), 6.67 (2H, s), 6.69 (1H, d, $J = 8.02$ Hz), 6.75 (1H, d, $J = 2.11$ Hz), 6.89 (1H, dd, $J = 8.02$, 2.11 Hz). Anal. calcd for $C_{27}H_{37}Cl_2N_3O_6$: C, 56.84; H, 6.54; N, 7.37. Found C, 57.02; H, 6.55; N, 7.18.

1-L-*p*-[bis(2-chloroethyl)]amino-Phe. 2HCl (1h). This compound was synthesized by the same procedure as described for **1a** using *N*-Boc-L-p-[bis(2-chloroethyl)]amino-Phe (*N*-Boc-Melphalan) instead of *N*-Boc-Gly. Yield: 48%; mp 172 °C; ^1H NMR (DMSO-d_6, 300 MHz) δ 2.55–2.69 (2H, m), 2.78–2.96 (2H, m), 3.60–6.68 (8H, m), 3.74 (6H, s), 3.81 (3H, s), 3.87 (3H, s), 5.06 (1H, m), 6.08 (1H, br), 6.54 (2H, s), 6.66 (1H, d, $J = 8.22$ Hz), 6.72 (1H, dd, $J = 8.22$, 2.07 Hz), 6.88 (1H, d, $J = 2.07$ Hz), 8.25 (1H, s). Anal. calcd for $C_{34}H_{41}Cl_4N_3O_6$: C, 55.94; H, 5.66; N, 5.76. Found C, 56.05; H, 5.49; N, 5.48.

1-β-Ala. HCl (1i). This compound was synthesized by the same procedure as described for **1a** using *N*-Boc-β-Ala instead of *N*-Boc-Gly. Yield: 88%; ^1H NMR (DMSO-d_6, 300 MHz) δ 2.59–2.68 (2H, m), 2.77–3.02 (4H, m), 3.74 (6H, s), 3.86 (3H, s), 3.89 (3H, s), 3.99 (2H, t, $J = 6.91$ Hz), 6.51 (2H, s), 6.65 (1H, d, $J = 8.17$ Hz), 6.69 (1H, dd, $J = 8.17$, 2.11 Hz), 6.75 (1H, d, $J = 2.11$ Hz). Anal. calcd for $C_{24}H_{29}ClN_2O_6$: C, 60.44; H, 6.13; N, 5.87. Found C, 60.18; H, 6.21; N, 5.98.

1-4-Aminobutyric amide. HCl (1j). This compound was synthesized by the same procedure described for **1a** using 4-(*N*-Boc-amino)butyric acid instead of *N*-Boc-Gly. Yield: 80%; ^1H NMR (DMSO-d_6, 300 MHz) δ 1.51–2.23 (2H, m), 2.62–2.76 (2H, m), 2.78–3.05 (4H, m), 3.73 (6H, s), 3.87 (3H, s), 3.89 (3H, s), 4.12 (2H, t, $J = 7.08$ Hz), 6.55 (2H, s), 6.67–6.69 (2H, m), 6.78 (1H, d, $J = 2.15$ Hz). Anal. calcd for $C_{25}H_{31}ClN_2O_6$: C, 61.16; H, 6.36; N, 7.22. Found C, 60.98; H, 6.32; N, 7.18.

4-(*N*-Boc-Amino)butyric acid was obtained by the following procedure: 4-aminobutyric acid (1.03 g, 10 mmol) was dissolved in 20 mL dioxane, 10 mL water, and 10 mL NaOH solution (1 N aq) at 0 °C. To this solution was added 2.4 g $(Boc)_2O$ (11 mmol). The resulting mixture was stirred for 30 min and concentrated in vacuo to 15 mL. The pH of the mixture was adjusted to 3–4 using HCl (1 N aq) and extracted with ethyl acetate twice (20 mL each). The organic layers were combined, dried over sodium sulfate and solvent was removed at reduced pressure using evaporator. The residue was dried and used directly without any further purification.

1-6-Aminohexanoic amide. HCl (1k). This compound was synthesized by the same procedure as described for

1a using 6-(*N*-Boc-amino)hexanoic acid (*N*-Boc-caproic acid) instead of *N*-Boc-Gly. Yield: 78%; ^{1}H NMR (DMSO-d_6, 300 MHz) δ 1.42–2.47 (6H, m), 2.64–2.77 (2H, m), 2.80–3.00 (2H, m), 3.05–3.14 (2H, m), 3.75 (6H, s), 3.87 (3H, s), 3.89 (3H, s), 4.17 (2H, t, J = 7.41 Hz), 6.52 (2H, s), 6.65 (1H, dd, J = 7.88, 2.31 Hz), 6.72–6.83 (2H, m). Anal. calcd for $C_{27}H_{35}ClN_2O_6$: C, 62.48; H, 6.80; N, 5.40. Found C, 62.29; H, 6.87; N, 5.78.

6-(*N*-Boc-Amino)hexanoic acid (*N*-Boc-caproic acid) was obtained by the same procedure as described for 4-(*N*-Boc-amino)butyric acid.

1-4-Aminomethylbenzoic amide. HCl (1l). This compound was synthesized by the same procedure as described for **1a** using 4-(*N*-Boc-amino)methyl benzoic acid instead of *N*-Boc-Gly. Yield: 64%; ^{1}H NMR (DMSO-d_6, 300 MHz) δ 2.60–2.73 (2H, m), 2.85–2.94 (2H, m), 3.82 (3H, s), 3.87 (6H, s), 3.91 (3H, s), 6.49 (2H, s), 6.67 (1H, d, J = 8.11 Hz), 6.73 (1H, dd, J = 8.12, 2.13 Hz), 6.79 (1H, d, J = 2.05 Hz), 7.03–7.25 (2H, m), 7.66–7.80 (2H, m). Anal. calcd for $C_{29}H_{31}ClN_2O_6$: C, 64.62; H, 5.80; N, 5.20. Found C, 64.55; H, 5.87; N, 5.31.

4-(*N*-Boc-Amino)methyl benzoic acid was obtained by the same procedure described for 4-(*N*-Boc-amino)-butyric acid.

Disodium salt of 1-phosphoramidate (1m)

1-Dibenzyloxyphosphoramidate (2). Compound **1** (1.05 g, 2.84 mmol) was added to 30 mL of dry MeCN followed by addition of TEA (0.4 mL, 3.10 mmol). The mixture was heated to reflux for approximately 5 min. Solid tetrabenzyl pyrophosphate (1.68 g, 3.12 mmol) was added to the warm reaction mixture. The reaction mixture was kept under continuous stirring at reflux for 1 h and then allowed to cool to room temperature. To the reaction milieu was added petroleum ether (30 mL), and the insoluble precipitate was removed by filtration. After removal of all solvents under reduced pressure, a residue was eluted from a silica gel column using gradient ethyl acetate in hexane as eluent to give 1.43 g of **2**. Yield: 77%; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 2.65–2.76 (2H, m), 2.79–3.00 (2H, m), 3.88 (6H, s), 3.91 (3H, s), 3.95 (3H, s), 5.28 (4H, overlap), 6.49 (2H, s), 6.75–6.88 (3H, m), 7.00–7.33 (10H, m). Anal. calcd for $C_{35}H_{36}NO_8P$: C, 66.76; H, 5.76; N, 2.22. Found C, 66.51; H, 5.85; N, 2.29.

1-Dihydroxyphosphoramidate (3). A mixture of **2** (1.31 g, 2 mmol) and sodium iodide (1.2 g, 8 mmol) in anhydrous acetonitrile (15 mL) was stirred under nitrogen and trimethylsilyl chloride (1 g, 8 mmol) was added dropwise. The solution was kept under vigorous stirring for 3 h and quenched with water. The acidified reaction mixture was extracted with ethyl acetate three times. The organic layers were combined and the solvent was removed under reduced pressure rotary evaporation. The final solid product was obtained (884 mg) by trituration in petroleum ether. Yield: 88%; ^{1}H NMR (CD_3OD, 300 MHz) δ 2.68–2.77 (2H, m), 2.83–3.05 (2H, m), 3.86 (6H, s), 3.93 (6H, overlap), 6.51 (2H, s), 6.77–6.82 (2H, m) 6.89 (1H, m), 9.82 (2H, br, overlap). Anal. calcd for $C_{21}H_{24}NO_8P$: C, 56.13; H, 5.38; N, 3.12. Found C, 56.38; H, 5.28; N, 3.17.

Disodium salt of 3 (1m). Sodium methoxide (104 mg, 2.0 mmol) was added to a solution of 3 (820 mg, 1.83 mmol) in MeOH (5 mL). The resulting mixture was stirred for 12 h at room temperature and concentrated. The residue was recrystallized from cold acetone/water. Yield: 75%; ^{1}H NMR (CD_3OD, 300 MHz) δ 2.63–2.74 (2H, m), 2.85–3.01 (2H, m), 3.87 (6H, s), 3.91 (3H, s), 3.94 (3H, s), 6.50 (2H, s), 6.75 (1H, d, J = 9.00 Hz), 6.80 (1H, dd, J = 8.89, 1.95 Hz), 6.85 (1H, d, J = 1.95 Hz). HRMS m/z 494.1207 $(M+H)^+$. calcd for $C_{21}H_{22}NNa_2O_8P$: 493.0911.

Disodium salt of 1-{3-[2′-(dihydroxyphosphono)oxy-4′,6″-dimethylphenyl]-3,3-dimethylpropionic} amide (1n)

1-{3-[2′-(Dibenzyloxyphosphono)oxy-4′,6′-dimethylphenyl]-3,3-dimethylpropionic} amide (5). DCC (112.5 mg, 0.50 mmol) and HOBt·H_2O (84 mg, 0.63 mmol) were added to a solution of 3-[2′-(dibenzylphosphono)oxy-4′,6′-dimethylphenyl)-3,3-dimethylpropionic acid (**4**)[15] (200 mg, 0.415 mmol) in anhydrous CH_2Cl_2 (50 mL) at 0 °C. The reaction mixture was kept under continuous stirring for 15 min. Compound **1** (139 mg, 0.377 mmol) was then added at once, and the reaction mixture was stirred for additional 24 h while it was gradually allowed to reach ambient temperature. The solvent was removed under reduced pressure rotary evaporation, and the desired product was eluted from a silica gel column. Yield: 52%; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.67 (6H, s), 2.11 (3H, s), 2.45 (3H, s), 2.68 (2H, s), 2.71–2.82 (2H, m), 2.89–3.04 (2H, m), 3.78 (3H, s), 3.85 (6H, s), 3.91 (3H, s), 5.12–5.18 (4H, m), 6.49 (2H, s), 6.73 (1H, s), 6.79–7.05 (4H, m), 7.36–7.45 (10H, m). Anal. calcd for $C_{48}H_{52}NO_9P$: C, 70.49; H, 6.41; N, 1.71. Found C, 70.68; H, 6.59; N, 1.66.

1-{3-[2′-(Dihydroxyphosphono)oxy-4′,6′-dimethylphenyl]-3,3-dimethylpropionic} amide (6). This compound was prepared from **5** as described for **3**. Yield: 71%; ^{1}H NMR (CD_3OD, 300 MHz) δ 1.62 (6H, s), 2.14 (3H, s), 2.40 (3H, s), 2.74 (2H, s), 2.75–2.87 (2H, m), 2.91–3.08 (2H, m), 3.81 (3H, s), 3.87 (6H, s), 3.94 (3H, s), 6.51 (2H, s), 6.62 (1H, s), 6.81 (1H, d, J = 7.80 Hz), 6.89 (1H, dd, J = 7.80, 2.21 Hz), 7.05 (1H, s), 7.07 (1H, d, J = 2.21 Hz). 9.64 (2H, br, overlap). Anal. calcd for $C_{34}H_{40}NO_9P$: C, 64.04; H, 6.32; N, 2.20. Found C, 64.21; H, 6.31; N, 2.28.

Disodium salt of 1-{3-[2′-(dihydroxyphosphono)oxy-4′,6′-dimethylphenyl]-3,3-dimethylpropionic} amide (1n). This compound was prepared from **6** as described for **1m**. Yield: 61%; ^{1}H NMR (CD_3OD, 300 MHz) δ 1.65 (6H, s), 2.12 (3H, s), 2.43 (3H, s), 2.73 (2H, s), 2.75–2.84 (2H, m), 2.92–3.00 (2H, m), 3.82 (3H, s), 3.85 (6H, s), 3.91 (3H, s), 6.50 (2H, s), 6.63 (1H, s), 6.79 (1H, d, J = 8.10 Hz), 6.87 (1H, dd, J = 8.10, 2.25 Hz), 6.93 (1H, d, J = 2.25 Hz), 7.02 (1H, s). HR-MS m/z 682.3811 $(M+H)^+$. calcd for $C_{34}H_{38}NNa_2O_9P$: 681.2147.

Cytotoxicity assays

Tumor cells were maintained in plastic dishes in RPMI-1640 medium supplemented with 10% fetal bovine serum. On day 0, 180 μL of a tumor cell suspension (3×10^4 cells/mL in culture medium) were seeded in each well of 96-well plates. The plates were incubated in a 5% CO_2 incubator at 37 °C for 24 h and then samples in 20 μL culture medium were added at various concentrations. The plates were incubated for another 48 h. Cytotoxicity was measured by SRB's method as described in literature[18] with slight modifications.[19,20] The values shown for these compounds are averages of three determinations.

Water solubility

The solubilities of prodrugs were measured by adding excess quantities of the prodrugs to a predetermined volume of distilled water. The resulting mixtures were stirred at 25 °C for 24 h and the solutions were filtered. Appropriately diluted filtrates were assayed by HPLC. No chemical degradation was observed.

Antitumor experiments

Antitumor experiments were carried out as described in our previous reports.[19,20] Briefly, 3LL cells were inoculated sc into BDF1 mice on the day 0 (1×10^7 cells/mouse/0.2 mL PBS). Prodrugs were dissolved in a medium comprising of 5% DMSO and 20% Cremophor®. Each compound was dosed with six injections from day 1 and every other day. Body weights of mice were tracked every day and tumor sizes were measured with calipers from day 9. Tumor volumes were calculated by the following equation: tumor volume (mm^3) = [length (mm) × width $(mm)^2$]/2. The inhibition rate was evaluated as $(1-T/C)\times100\%$ (where T is the mean tumor volume of the treated group, C is the mean tumor volume of the control group). Each group consisted of 10 mice. Etoposide was administered at day 1, 5, and 9 at 36 mg/kg/day. No death was recorded within the experimented period.

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