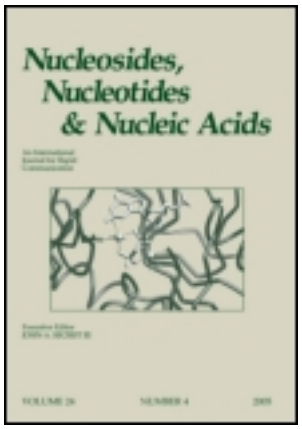

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Synthesis of Novel Nucleoside Analogue Phosphorothioamidate Prodrugs and in vitro Anticancer Evaluation Against RKO Human Colon Carcinoma Cells

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SYNTHESIS OF NOVEL NUCLEOSIDE ANALOGUE PHOSPHOROTHIOAMIDATE PRODRUGS AND IN VITRO ANTICANCER EVALUATION AGAINST RKO HUMAN COLON CARCINOMA CELLS

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□ *Novel phosphorothioamidates of pyrimidine nucleoside analogues have been prepared and evaluated in vitro against RKO human colon cancer cell by the MTT cytotoxicity assay. The parent nucleoside analogues were inactive in this assay, while the phosphorothioamidate prodrugs were active at low uM levels in some cases. The O-isopropyl phosphorothioamidate of 2′,3′-O-isopropylideneuridine containing the L-phenylalanine ethyl ester* ***6f*** *was the most active at 148 uM, a 10-fold enhancement in anticancer activity compared with the parent nucleoside* ***2*** *with no increase in cytotoxicity.*

Keywords Phosphorothioamidate; prodrug; nucleoside; anticancer activity; colon cancer

INTRODUCTION

Many nucleoside derivatives and analogues have been synthesized as important agents for anticancer and antiviral therapy. Preliminary metabolic studies showed that the nucleosides are phosphorylated to triphosphates and inhibit RNA synthesis. Phosphorylation to nucleoside monophosphates by the host cell kinase may be the crucial step in their activation.[1] In many cases, however, nucleoside analogues are poor substrates for the kinases and unable to cross the cell membrane. In order to overcome these problems, their phosphate and phosphoramidate derivatives have been widely used as

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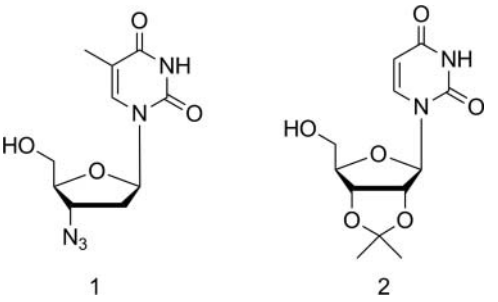


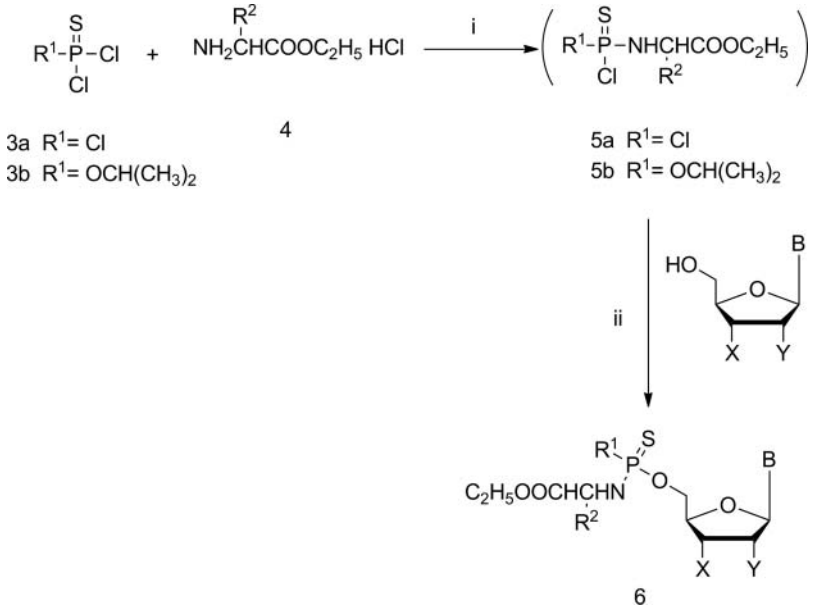
FIGURE 1 Structures of AZT (**1**) and 2′,3′-*O*-isopropylidene uridine (**2**).

prodrug moieties to enhance biological activity.[2–7] Among all reported prodrugs approaches, the class of aryl phosphoramidate nucleoside prodrugs (ProTide) developed by McGuigan has been proven to enhance, in vitro, the activity of parent nucleosides by increasing the formation rate of NuTP by improved intracellular transport and/or bypassing the rate-limiting first phosphorylation step.[8–17] In the past, our group reported the synthesis of several series of nucleoside 5′-phosphorothioamidate prodrugs.[18] Except for herbicidal activity,[19] few investigations on the medicinal activity of phosphorothioamidate derivatives have been reported. Previously, Venkatachalam reported a comparative study of aryl phosphoramidate and aryl thiophosphoramidate derivatives of d4T.[20] By determining the rate of hydrolysis in vitro, they believed that aryl phosphoramidate derivatives of d4T were more active than the corresponding aryl phosphorothioamidate derivatives. However, Bell reported in 2006 that phosphorothioamidates had antimalarial activity and might be a target for new antimalarial drugs.[21] Recently, further studies on antimalarial activity revealed that phosphorothioamidates were more active than their oxo congeners.[22] These findings prompted us to undertake further research on phosphorothioamidate derivatives. Herein, the ProTide strategy has been applied to parent nucleosides (see Figure 1). We report the synthesis of eight novel pyrimidine nucleoside analogue phosphorothioamidate prodrugs **6** (see Table 1) bearing amino acid ester moieties with the aim to improve and/or broaden their biological activity. Both protected 2′,3′-*O*-isopropylidene-uridine **2** and AZT **1**, as nucleoside analogs having a defect in the 2′,3′ positions of ribose ring, have antiviral or anticancer activity by inhibiting the RNA replication in these cells. The anticancer activity of compounds **6** was evaluated in RKO human colon cancer cells.

RESULTS AND DISCUSSION

Chemistry

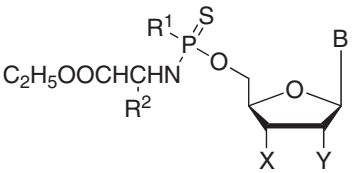
Using the previously described method[18] (see Scheme 1), we synthesized the glycine, L-alanine, L-phenylalanine, and L-valine phosphorothioamidates **6** of parent nucleosides **1** and **2**, each bearing an ethyl ester.



SCHEME 1 General synthetic pathway for the synthesis of phosphorothioamidate prodrugs **6**. Reagents and conditions: (i) Et_3N, THF, −5°C, 2 h; (ii) Et_3N, rt, 24 h.

In brief, thiophosphoryl chloride **3a** was used as starting material, which was condensed with 2-propanol to yield *O*-isopropyl phosphorodichloridothioate **3b**. Two compounds (**3a** and **3b**) were further condensed with L-amino acid ethyl ester hydrochlorides **4** to obtain the dichloridate and monochloridate **5**, respectively. Treatment of chloridates **5** in dry tetrahydrofuran (THF) and triethylamine with **1** or **2** furnished the desired alkyl phosphorothioamidate derivatives **6**. All the phosphorothioamidate prodrugs **6** displayed two

TABLE 1 Structures and yields of phosphorothioamidates **6** synthesized in this work



Compound	R^1	R^2	Amino acid ethyl ester	Nucleoside (B/X/Y)	Yield (%)
6a	OH	Me	L-alaninyl	2′,3′-*O*-isopropylidene uridine	71
6b	OH	i-Pr	L-valinyl	2′,3′-*O*-isopropylidene uridine	74
6c	$OCH(CH_3)_2$	H	Glycinyl	2′,3′-*O*-isopropylidene uridine	62
6d	$OCH(CH_3)_2$	Me	L-alaninyl	2′,3′-*O*-isopropylidene uridine	65
6e	$OCH(CH_3)_2$	i-Pr	L-valinyl	2′,3′-*O*-isopropylidene uridine	58
6f	$OCH(CH_3)_2$	Bn	L-phenylalaninyl	2′,3′-*O*-isopropylidene uridine	53
6g	$OCH(CH_3)_2$	H	Glycinyl	Zidovudine (AZT)	62
6h	$OCH(CH_3)_2$	i-Pr	L-valinyl	Zidovudine (AZT)	66

closely spaced signals in phosphorus-31 nuclear magnetic resonance (^{31}P NMR) because of the presence of diastereoisomers at the phosphorus stereocenter. Unfortunately, the diastereoisomeric mixtures could not be separated by silica gel column chromatography. Their structures have been demonstrated by mass spectrometry (MS), infrared (IR), and a series of one- and 2-dimensional NMR analyses.

Because of the stereochemistry at the phosphorus center, the final compounds were isolated as mixtures of two diastereoisomers. The confirmation of the presence of two diastereoisomers was shown by ^{31}P NMR (two peaks) and ^{1}H and ^{13}C NMR (splitting of many of the nucleoside signals). For example, a pair of peaks at ^{31}P NMR 71.96 and 71.63 ppm was derived from the diastereoisomers **6g**. The ^{1}H NMR signal of five CH_3 groups appeared at 1.98–1.30 ppm as three groups of signals, which could confirm the structure of **6g**. The ^{13}C NMR spectrum of **6g** displayed 17 carbons. The DEPT-135 (135° distortionless enhancement by polarization transfer) indicated the presence of four methylenes (δ_C 65.5, 61.7, 43.4, 37.6), five methines (δ_C 135.3, 85.0, 82.5, 73.0, 60.6), one quaternary carbon (δ_C 111.4), three carbonyl carbon (δ_C 170.6, 163.7, 150.3), and four methyl groups (δ_C 23.7, 23.4, 14.1, 12.5). These carbon signals and the ^{1}H NMR data also supported the fact that **6g** was composed of an isopropoxy group, a nucleoside moiety, and an amino acid moiety, and was further confirmed by the ^{1}H–^{1}H COSY and ^{1}H–^{13}C HMQC experiments.

The IR spectra of compounds **6** were recorded in KBr and are given in the Experimental section. Characteristic frequencies of the azide group in the spectrogram of the compound **6g** were observed at 2111 cm^{-1}. Absorptions at 1692 cm^{-1} were due to the presence of the carbonyl group. Electrospray ionization (ESI) mass spectra showed that the quasimolecular ion peaks of compound **6g** were observed in both the positive mode and the negative mode. The high-resolution mass spectrometry (HRMS) gave a quasimolecular ion at m/z 491.1485 $[M+H]^+$ (calculated 491.1478 for $C_{17}H_{27}N_6O_7PS$).

Anticancer Activity

The anticancer activity of phosphorothioamidate prodrugs **6** against human colon carcinoma cells (RKO cells) was compared with that of the parent nucleosides **1–2**. Meanwhile, cytotoxicity was also evaluated in normal human embryonic kidney cells (293T cells). The activity values expressed as IC_{50} values are shown in Table 2. Phosphorothioamidate prodrugs **6** showed only very modest activity through more potent activity against the cover cell line than the parent nucleosides **1** and **2**, with IC_{50} values >1000 μM. This result could suggest that the phosphorothioamidate approach allows the efficient conversion to its corresponding monothiophosphate in RKO cells, whereas the parent nucleosides were poor substrates for the nucleoside

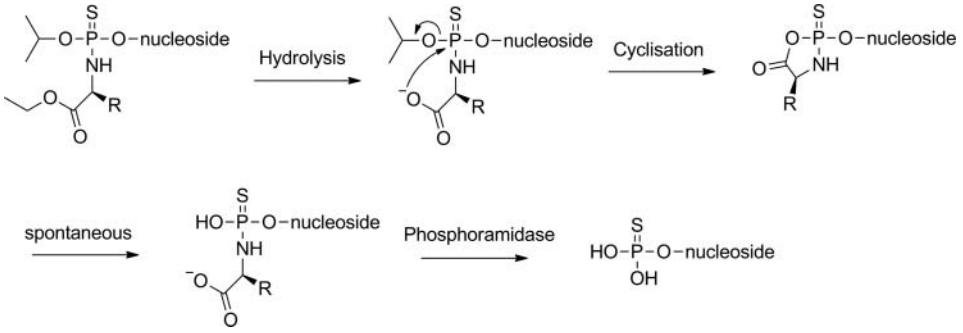
TABLE 2 In vitro cytotoxicity of parent nucleosides **1** and **2** and phosphorothioamidate prodrugs **6** against RKO human colon cancer cells and 293T human embryonic kidney cells

Compound	IC_{50} (uM)	
	RKO	293T
6a	>1000	>1000
6b	>1000	>1000
6c	724	178
6d	276	132
6e	561	677
6f	148	>1000
6g	187	578
6h	160.00	147
1 (AZT)	>1000	53
2	>1000	203

Note: The IC_{50} values were determined from eight different concentrations of compounds and were the means of three separate experiments.

kinases in these cells. We consider that the mechanisms of phosphorothioamidate prodrugs in vitro are likely similar to phosphoramidate prodrugs (see Scheme 2), though the modest activity makes it difficult to draw firm conclusions.

Structure–activity relationships could be observed with regard to the nature of the alkoxy moiety and the amino acid moiety in terms of eventual anticancer activity of the prodrug derivatives. Although prodrugs **6a** and **6b** did not show appreciable activity in RKO cells, prodrugs containing isopropoxy groups (**6c–h**) were more potent than those bearing hydroxyl groups (**6a–b**), with IC_{50} values in the 724–148 μM range. Instead of hydroxyl, prodrugs containing isopropoxy groups (**6c–h**) were more active. According to the metabolism of nucleoside aryl phosphoramidates,[13] alkyl phosphorothioamidates also need four steps to eventually release the nucleoside analogue monothiophosphate (see Scheme 2). Cyclization as the second step involves the oxygen of the carboxylate attacking the phosphorus



SCHEME 2 Postulated activation pathway of phosphorothioamidate prodrugs.

atom and displacing the isopropyl ester to form a five-membered intermediate, which eventually is converted to a monothiophosphate. In this second step, the leaving group ability of the isopropoxy group is expected to be greater than that of the hydroxy group. As a result, prodrugs **6a–b** containing hydroxyl groups should slow down conversion to monothiophosphate. With regard to the amino acid modifications, we found that the introduction of bulky amino acid side chains significantly enhanced potency in the Et-ester derivatives. The L-phenylalanine derivative **6f** and L-valine derivative **6h** showed a bit more anticancer activity than other amino acid derivatives in the uridine and AZT series, respectively. For example, the inhibitory activity of **6f** was fourfold higher than glycine derivative **6c**. We utilized the 293T cells to test the cytotoxicity of the compounds described herein. The results of the cytotoxicity in vitro evaluations showed that all phosphorothioamidate prodrugs **6** were less toxic than the corresponding parent nucleosides **1–2**. For example, prodrugs **6a**, **6b**, and **6f** exhibited an IC_{50} of >1000 μM. Among all investigated compounds, prodrug **6f** showed the most pronounced inhibitory activity against the RKO human tumor cell line and demonstrated some selectivity relative to its effect on the growth of human normal 293T cells.

CONCLUSIONS

A series of phosphorothioamidate prodrugs of pyrimidine nucleoside analogues were prepared and evaluated as inhibitors of RKO tumor cells in vitro. The phosphorothioamidate approach provided novel compounds with modest potency in the anticancer assay when compared with the inactive parent compounds. All phosphorothioamidates tested were only moderately cytotoxic ($IC_{50} > 100$ μM). The most active compound prepared in the series was the *O*-isopropyl L-phenylalanine ethyl ester phosphorothioamidate **6f** with an IC_{50} of 148 μM in the anticancer assay and a selectivity index of >6. This report suggested that phosphorothioamidate prodrugs may bypass the rate-limiting initial phosphorylation of a ribonucleoside analogue and thus confer anticancer activity on an inactive parent nucleoside.

EXPERIMENTAL

Chemistry (General Procedures)

All experiments involving water-sensitive compounds were conducted under scrupulously dry conditions. Triethylamine was dried by refluxing over calcium hydride. THF was distilled from sodium. Nucleosides were dried by storage at elevated temperature over P_2O_5 in vacuo. Proton, carbon, and phosphorus nuclear magnetic resonance (^{1}H, ^{13}C, ^{31}P NMR) spectra were recorded on 400 or 600 MHz Bruker Avance DPX spectrometers.

All ^{13}C and ^{31}P spectra were recorded proton decoupled. All NMR spectra were recorded in $CDCl_3$ or D_2O at room temperature (20°C). Chemical shifts for ^{1}H and ^{13}C spectra are quoted in ppm downfield using tetramethylsilane (TMS). Coupling constants are referred to as *J* values. Chemical shifts for ^{31}P spectra are quoted in ppm relative to an external phosphoric acid standard. Many proton and carbon NMR signals were split due to the presence of (phosphate) diastereoisomers in the samples. ESI mass spectra were acquired using a Bruker ESQUIRELC^{TM} ESI ion trap spectrometer. HR-ESI mass spectra were obtained with an Agilent Technologies ESI-TOF spectrometer. FTIR spectra were recorded at room temperature in the region of 4000–400 cm^{-1} with a PerkinElmer spectrum 65 FTIR spectrometer using KBr pellets.

Preparation of O-isopropyl Phosphorodichloridothioate 3b[23]

2-Propanol (50 mmol) was added dropwise to a stirred solution of **3a** (100 mmol) at room temperature over 10 minutes. Following the addition, pyridine (50 mmol) was added dropwise to the reaction mixture. The reaction mixture was stirred at room temperature for 2 hours. The crude product was purified by reduced pressure distillation to give the title compound as an oil in 84% yield.

Standard Procedure: Preparation of Amino Acid Ethyl Ester Hydrochloride 4

L-amino acid (5.0 mol equiv) in 50 ml dry ethanol was stirred at room temperature; the mixture was saturated by HCl gas and stirred overnight. The solvent was removed under reduced pressure to give the crude product as amino acid ethyl ester hydrochlorides. The product was washed with dry ethanol, concentrated to remove HCl gas, and recrystallized from EtOH-Et_2O to give the white title compound.

Standard Procedure A: Preparation of Phosphorothioamidate Derivatives 6a–b of Nucleosides

Triethylamine (0.5 g, 5.0 mmol) was added dropwise to a stirred solution of **3a** (0.42 g, 2.5 mmol) and amino acid ethyl ester hydrochloride **4** (2.5 mmol) in dry THF (15.0 ml) under nitrogen. The reaction flask was externally cooled by ice bath below 0°C. After completion of the addition of the base, the reaction mixture was allowed to warm to room temperature and stirred for additional 2 hours. On completion of the reaction (as determined by ^{31}P NMR), a solution of **2** (0.71 g, 2.5 mmol) and dry THF (10 ml) was added dropwise to the above reaction mixture over a period of 20 minutes. Additional triethylamine (0.25 g, 2.5 mmol) was then added and the reaction mixture was stirred overnight, filtered, and concentrated in vacuo. After hydrolysis in NH_4OH, the residue was purified by column chromatography

on silica (200–300 mesh) with elution by propanol-NH_4OH-H_2O (30:1:1) to give a white solid **6a–b**.

Standard Procedure B: Preparation of Phosphorothioamidate Derivatives 6c–h of Nucleosides

Triethylamine (0.5 g, 5.0 mmol) was added dropwise with vigorous stirring to a solution of amino acid ethyl ester hydrochloride **4** (2.5 mmol) and **3b** (0.5 g, 2.5 mmol) in dry THF (15 ml) at −5°C over a period of 15 minutes under nitrogen. The reaction mixture was slowly warmed to room temperature with stirring over 2 hours. Upon complete reaction of **3b** (as determined by ^{31}P NMR), a solution of nucleoside **1** or **2** (2.5 mmol) dissolved in dry pyridine (10 ml) and triethylamine (0.25 g, 2.5 mmol) was added with vigorous stirring. The solvent was removed under vacuum after 24 hours. The residue was dissolved in chloroform (20 ml) and washed with 1 M hydrochloric acid solution (2 × 15 ml), saturated sodium bicarbonate solution (2 × 10 ml), and then water (3 × 15 ml). The organic phase was dried ($MgSO_4$) and evaporated under vacuum, and the residue was purified by column chromatography on silica (200–300 mesh) with elution with 5% methanol in chloroform to give a white solid **6c–h**.

2′,3′-O-isopropylidene-uridine-5′-O-[hydroxy(ethyloxy-L-alaninyl)]thiophosphate (Diastereoisomers, 6a). The compound was prepared according to Standard Procedure A by using uridine **2**, L-alanine ethyl ester hydrochloride, and **3a**. Product **6a** was obtained as a white solid (0.85 g, 71%). ^{31}P NMR (162 MHz, D_2O): δ 57.19, 56.77; ^1H NMR (400 MHz, D_2O): δ 7.90, 7.88 (1H, dd, J = 8.0 Hz, H-6), 5.90 (2H, m, H-1′, 5), 4.98 (2H, m, H-2′, 3′), 4.58 (1H, s, H-4′), 4.20–4.11 (2H, m, H-5′), 4.06 (2H, m, OCH_2CH_3), 3.91–3.82 (1H, m, NHCH), 1.60 (3H, s, CH_3), 1.41 (3H, s, CH_3), 1.31 (3H, d, J = 7.2 Hz, $CHCH_3$), 1.25 (3H, t, J = 7.6 Hz, OCH_2CH_3); ^{13}C NMR (100 MHz, D_2O): δ 177.20 (COOEt), 166.46 (C-4), 151.50 (C-2), 142.98, 142.88 (C-6), 114.60, 114.57 (>CMe_2), 102.00, 101.91(C-5), 93.23, 92.92 (C-1′), 85.61, 85.42 (d, $J_{C\text{-}P}$ = 10.0 Hz, C-4′), 84.76, 84.62 (C-2′), 81.13, 81.01 (C-3′), 64.80 (C-5′), 62.38 (OCH_2CH_3), 50.94, 50.53, (d, $J_{C\text{-}P}$ = 8.0 Hz, Ala-C-α), 26.21 (CH_3), 24.49 (CH_3), 20.17, 19.99 (d, $J_{C\text{-}P}$ = 3.0 Hz, Ala-C-β), 13.50, 13.47 ($COOCH_2CH_3$); IR (KBr, cm^{-1}) 3459, 1677, 1401, 1277, 1220, 1116, 978, 938, 905; ESI-MS (pos.): m/z 480 $(M+H)^+$; ESI-MS (neg.): m/z 478 $(M-H)^-$; HRMS-ESI (m/z) calcd for $C_{17}H_{26}N_3O_9PS$ $[M+H]^+$ 480.1206, found 480.1211.

2′,3′-O-isopropylidene-uridine-5′-O-[hydroxy(ethyloxy-L-valinyl)]thiophosphate (Diastereoisomers, 6b). The compound was prepared according to Standard Procedure A by using uridine **2**, L-valine ethyl ester hydrochloride, and **3a**. Product **6b** was obtained as a white solid (0.94 g, 74%). ^{31}P NMR (162 MHz, D_2O): δ 58.22, 57.30; ^1H NMR (400 MHz, D_2O): δ 7.88, 7.86 (1H, dd, J = 8.0 Hz, H-6), 5.94–5.89 (2H, m, H-1′, 5), 4.97 (2H, m, H-2′, 3′), 4.60 (1H, s, H-4′), 4.22–4.16 (2H, m, OCH_2CH_3), 4.08–4.04 (2H,

m, H-5′), 3.58 (1H, dm, NHCH), 1.94 (1H, m, CH(CH_3)$_2$), 1.61 (3H, s, CH_3), 1.42 (3H, s, CH_3), 1.28 (3H, t, J = 7.2 Hz, OCH_2CH_3), 0.90 (6H, m, CH(CH_3)$_2$); ^{13}C NMR (100 MHz, D_2O): δ 176.40 (COOEt), 166.46 (C-4), 151.43 (C-2), 142.82, 142.78 (C-6), 114.48 (>CMe_2), 101.88, 101.79 (C-5), 93.32, 93.15 (C-1′), 85.70, 85.49 (d, $J_{C\text{-}P}$ = 10.0 Hz, C-4′), 84.81, 84.77 (C-2′), 81.08, 81.00 (C-3′), 64.78, 64.73 (C-5′), 62.11 (OCH_2CH_3), 60.81, 60.67 (Val-C-α), 31.99 (Val-C-β), 26.13, 26.10 (CH_3), 24.36 (CH_3), 18.34, 17.65 (Val-CH(CH_3)$_2$), 13.51 ($COOCH_2CH_3$); IR (KBr, cm^{-1}) 3459, 1644, 1384, 1271, 1214, 1116, 962; ESI-MS (pos.): m/z 508 $(M+H)^+$; ESI-MS (neg.): m/z 506 $(M-H)^-$; HRMS-ESI (m/z) calcd for $C_{19}H_{30}N_3O_9PS$ $[M+H]^+$ 508.1519, found 508.1524.

2′,3′-O-isopropylidene-uridine-5′-O-[isopropyl(ethyloxy-glycinyl)]thiophosphate (Diastereoisomers, 6c). The compound was prepared according to Standard Procedure B by using uridine **2**, glycine ethyl ester hydrochloride, and **3b**. Product **6c** was obtained as a white solid (0.79 g, 62%). ^{31}P NMR (162 MHz, $CDCl_3$): δ 71.69, 71.19; ^{1}H NMR (400 MHz, $CDCl_3$): δ 9.10 (1H, br, H-3), 7.50, 7.45 (1H, dd, J = 8.0 Hz, H-6), 5.85 (1H, dd, J = 2.4, 22.4 Hz, H-1′), 5.74 (1H, dd, J = 3.2, 8.0 Hz, H-5), 4.87 (2H, m, H-2′, 3′), 4.74 (1H, m, OCH(CH_3)$_2$), 4.39 (1H, m, H-4′), 4.23–4.19 (4H, m, H-5′, OCH_2CH_3), 3.76–3.72 (3H, m, P-NH, $NHCH_2$), 1.58 (3H, s, CH_3), 1.36–1.26 (12H, m, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ 170.69, 170.61 (COOEt), 163.13 (C-4), 150.05 (C-2), 141.65, 141.40 (C-6), 114.47 (>CMe_2), 102.54, 102.49 (C-5), 93.54, 93.02 (C-1′), 85.31, 84.97 (d, $J_{C\text{-}P}$ = 10.0 Hz, C-4′), 84.57 (C-2′), 80.74, 80.68 (C-3′), 72.73 (OCH(CH_3)$_2$), 66.13, 65.83 (d, $J_{C\text{-}P}$ = 5.0 Hz, C-5′), 61.64, 61.62 (OCH_2CH_3), 43.40, 43.37 (Gly-C-α), 27.18, 27.15 (CH_3), 25.33, 25.31 (CH_3), 23.65, 23.60 (d, $J_{C\text{-}P}$ = 3.0 Hz, OCH(CH_3)$_2$), 14.14 ($COOCH_2CH_3$); IR (KBr, cm^{-1}) 3452, 2988, 1637, 1457, 1384, 1271, 1214, 1108, 987; ESI-MS (pos.): m/z 508 $(M+H)^+$; ESI-MS (neg.): m/z 506 $(M-H)^-$; HRMS-ESI (m/z) calcd for $C_{19}H_{30}N_3O_9PS$ $[M+H]^+$ 508.1519, found 508.1524.

2′,3′-O-isopropylidene-uridine-5′-O-[isopropyl(ethyloxy-L-alaninyl)]thiophosphate (Diastereoisomers, 6d). The compound was prepared according to Standard Procedure B by using uridine **2**, L-alanine ethyl ester hydrochloride, and **3b**. Product **6d** was obtained as a white solid (0.85 g, 65%). ^{31}P NMR (162 MHz, $CDCl_3$): δ 70.66, 70.10; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.23 (1H, br, H-3), 7.51, 7.47 (1H, dd, J = 8.0 Hz, H-6), 5.89–5.75 (2H, m, H-1′, 5), 4.86 (2H, m, H-2′, 3′), 4.73 (1H, m, OCH(CH_3)$_2$), 4.39 (1H, s, H-4′), 4.20 (4H, m, H-5′, OCH_2CH_3), 4.05 (1H, m, P-NH), 3.70 (1H, m, NHCH), 1.60 (3H, s, CH_3), 1.44–1.27 (15H, m, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ 173.57 (COOEt), 163.23 (C-4), 149.83 (C-2), 141.52, 141.39 (C-6), 114.52 (>CMe_2), 102.44 (C-5), 93.38, 92.97 (C-1′), 84.47, 84.24 (d, $J_{C\text{-}P}$ = 10.0 Hz, C-4′), 84.55 (C-2′), 80.74, 80.64 (C-3′), 72.75 (OCH(CH_3)$_2$), 65.54 (C-5′), 61.58, 61.53 (OCH_2CH_3), 46.79 (Ala-C-α), 27.16 (CH_3), 25.33

(CH_3), 23.61, 23.59 (d, $J_{C\text{-}P}$ = 3.0 Hz, OCH($\underline{C}H_3)_2$), 21.08 (Ala-C-β), 14.13 (COOCH$_2\underline{C}H_3$); IR (KBr, cm^{-1}) 3459, 2985, 1692, 1459, 1384, 1273, 1216, 1108, 990; ESI-MS (pos.): m/z 522 $(M+H)^+$; ESI-MS (neg.): m/z 520 $(M-H)^-$; HRMS-ESI (m/z) calcd for $C_{20}H_{32}N_3O_9PS$ $[M+H]^+$ 522.1676, found 522.1670.

2′,3′-O-isopropylidene-uridine-5′-O-[isopropyl(ethyloxy-L-valinyl)]thiophosphate (Diastereoisomers, 6e). The compound was prepared according to Standard Procedure B by using uridine **2**, L-valine ethyl ester hydrochloride, and **3b**. Product **6e** was obtained as a white solid (0.80 g, 58%). ^{31}P NMR (162 MHz, $CDCl_3$): δ 72.36, 72.16; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.83 (1H, br, H-3), 7.52, 7.46 (1H, dd, J = 8.0 Hz, H-6), 5.90–5.76 (2H, m, H-1′, 5), 4.86–4.82 (2H, m, H-2′, 3′), 4.75 (1H, m, OC$\underline{H}$($CH_3)_2$), 4.38 (1H, s, H-4′), 4.23–4.15 (4H, m, H-5′, OC$\underline{H}_2$CH$_3$), 3.87 (1H, m, P-NH), 3.69 (1H, m, NHC$\underline{H}$), 2.07 (1H, m, CHC$\underline{H}$($CH_3)_2$), 1.59 (3H, s, C$\underline{H}_3$), 1.37 (3H, s, C$\underline{H}_3$), 1.32–1.27 (9H, m, COOCH$_2\underline{C}H_3$, OCH($\underline{C}H_3)_2$), 0.93 (6H, dd, J = 6.8, 26.0 Hz, CHCH($\underline{C}H_3)_2$); ^{13}C NMR (100 MHz, $CDCl_3$): δ 172.97, 172.94 (COOEt), 162.95, 162.91 (C-4), 150.00 (C-2), 141.43, 141.21 (C-6), 114.55, 114.51 (>$\underline{C}$Me$_2$), 102.66, 102.55 (C-5), 93.30, 92.66 (C-1′), 85.12 (C-2′), 84.74, 84.58 (d, $J_{C\text{-}P}$ = 10.0 Hz, C-4′), 80.62 (C-3′), 72.86, 72.78 (d, $J_{C\text{-}P}$ = 6.0 Hz, O$\underline{C}$H($CH_3)_2$), 66.07, 65.87 (d, $J_{C\text{-}P}$ = 4.5 Hz, C-5′), 61.28 (O$\underline{C}$H$_2$CH$_3$), 60.46, 60.34 (Val-C-α), 32.19 (Val-C-β), 27.20, 27.16 (CH_3), 25.35, 25.30 (CH_3), 23.62, 23.49 (d, $J_{C\text{-}P}$ = 4.0 Hz, OCH($\underline{C}H_3)_2$), 18.97, 18.84, 17.73, 17.63 (Val-CH($\underline{C}H_3)_2$), 14.25(COOCH$_2\underline{C}H_3$); IR (KBr, cm^{-1}) 3450, 2979, 1693, 1460, 1384, 1271, 1215, 1107, 994; ESI-MS (pos.): m/z 550 $(M+H)^+$; ESI-MS (neg.): m/z 548 $(M-H)^-$; HRMS-ESI (m/z) calcd for $C_{22}H_{36}N_3O_9PS$ $[M+H]^+$ 550.1989, found 550.1981.

2′,3′-O-isopropylidene-uridine-5′-O-[isopropyl(ethyloxy-L-phenylalaninyl)]thiophosphate (Diastereoisomers, 6f). The compound was prepared according to Standard Procedure B by using uridine **2**, L-phenylalanine ethyl ester hydrochloride, and **3b**. Product **6f** was obtained as a white solid (0.79 g, 53%). ^{31}P NMR (162 MHz, $CDCl_3$): δ 70.72, 70.63; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.75 (1H, br, H-3), 7.47, 7.41 (1H, dd, J = 8.0 Hz, H-6), 7.31–7.17 (5H, m, PhH), 5.80–5.61 (2H, m, H-1′, 5), 4.71–4.67 (2H, m, H-2′, 3′), 4.68–4.51 (1H, m, OC$\underline{H}$($CH_3)_2$), 4.30 (2H, m, H-5′), 4.15 (3H, m, H-4′, OC$\underline{H}_2$CH$_3$), 3.92–3.85 (1H, m, P-N$\underline{H}$), 3.68 (1H, m, NHC$\underline{H}$), 3.02 (2H, m, PhC$\underline{H}_2$), 1.58 (3H, s, C$\underline{H}_3$), 1.36 (3H, s, C$\underline{H}_3$), 1.30–1.19 (9H, m, COOCH$_2\underline{C}H_3$, OCH($\underline{C}H_3)_2$); ^{13}C NMR (100 MHz, $CDCl_3$): δ 172.72, 172.56 (d, $J_{C\text{-}P}$ = 9.0 Hz, $\underline{C}$OOEt), 163.05 (C-4), 150.05, 149.98 (C-2), 141.44, 141.22 (C-6), 136.05, 135.92 (PhC), 129.53, 129.49 (PhC-meta), 128.60, 128.55 (PhC-ortho), 127.12(PhC-para), 114.51, 114.45 (>$\underline{C}$Me$_2$), 102.59, 102.55 (C-5), 93.21, 92.72 (C-1′), 85.71, 85.61 (d, $J_{C\text{-}P}$ = 10.0 Hz, C-4′), 85.33 (C-2′), 80.59 (C-3′), 72.84, 72.77 (d, $J_{C\text{-}P}$ = 4.5 Hz, O$\underline{C}$H($CH_3)_2$), 65.91, 65.68 (C-5′), 56.43, 56.24 (O$\underline{C}$H$_2$CH$_3$), 61.54, 61.50 (Phe-C-α), 40.58, 40.45 (d, $J_{C\text{-}P}$ = 6.0 Hz, Phe-C-β), 27.20, 27.18 (CH_3), 25.35 (CH_3), 23.60, 23.49 (d, $J_{C\text{-}P}$ = 4.0 Hz, OCH($\underline{C}H_3)_2$), 14.17 (COOCH$_2\underline{C}H_3$); IR (KBr, cm^{-1})

3467, 2983, 1691, 1456, 1384, 1273, 1216, 1107, 990; ESI-MS (pos.): m/z 599 $(M+H)^+$; ESI-MS (neg.): m/z 597 $(M-H)^-$; HRMS-ESI (m/z) calcd for $C_{26}H_{36}N_3O_9PS$ $[M+H]^+$ 598.1989, found 598.1985.

Zidovudine-5′-O-[isopropyl(ethyloxy-glycinyl)]thiophosphate (Diastereoisomers, 6g). The compound was prepared according to Standard Procedure B by using **1**, glycine ethyl ester hydrochloride, and **3b**. Product **6g** was obtained as a white solid (0.76 g, 62%). ^{31}P NMR (243 MHz, $CDCl_3$): δ 71.96, 71.63; ^{1}H NMR (600 MHz, $CDCl_3$): δ 8.96 (1H, s, H-3), 7.47, 7.42 (1H, 2s, H-6), 6.25 (1H, d, $J = 30$ Hz, H-1′), 4.78 (1H, m, $OCH(CH_3)_2$), 4.38 (1H, s, H-3′), 4.24 (4H, m, H-5′, OCH_2CH_3), 4.08 (1H, s, H-4′), 3.76–3.69 (3H, m, P-NH, $NHCH_2$), 2.45 (1H, s, H-2′), 2.31 (1H, s, H-2′), 1.98 (3H, d, $J = 4.8$ Hz, 5-CH_3), 1.35–1.30 (9H, m, $OCH(CH_3)_2$, $COOCH_2CH_3$); ^{13}C NMR (150 MHz, $CDCl_3$): δ 170.66, 170.60 (d, $J_{C\text{-}P} = 9.0$ Hz, COOEt), 163.68 (C-4), 150.25 (C-2), 135.33, 135.28 (C-6), 111.47, 111.36 (C-5), 85.01, 84.74 (C-1′), 82.52, 82.46 (C-4′), 73.02, 72.99 ($OCH(CH_3)_2$), 65.52, 65.25 (d, $J_{C\text{-}P} = 6.0$ Hz, C-5′), 61.76, 61.74 (OCH_2CH_3), 60.67, 60.62 (C-3′), 43.45, 43.35 (Gly-C-α), 37.55 (C-2′), 23.65, 23.44 (d, $J_{C\text{-}P} = 4.5$ Hz, $OCH(CH_3)_2$), 14.14 ($COOCH_2CH_3$), 12.54 (5-CH_3); IR (KBr, cm^{-1}) 3467, 2981, 2111, 1692, 1469, 1374, 1274, 984; ESI-MS (pos.): m/z 491 $(M+H)^+$; ESI-MS (neg.): m/z 489 $(M-H)^-$; HRMS-ESI (m/z) calcd for $C_{17}H_{27}N_6O_7PS$ $[M+H]^+$ 491.1478, found 491.1485.

Zidovudine-5′-O-[isopropyl(ethyloxy-L-valinyl)]thiophosphate (Diastereoisomers, 6h). The compound was prepared according to Standard Procedure B by using **1**, L-valine ethyl ester hydrochloride, and **3b**. Product **6h** was obtained as a white solid (0.88 g, 66%). ^{31}P NMR (243 MHz, $CDCl_3$): δ 72.94, 72.28; ^{1}H NMR (600 MHz, $CDCl_3$): δ 8.75 (1H, s, H-3), 7.48, 7.40 (1H, 2s, H-6), 6.26, 6.20 (1H, 2s, H-1′), 4.77 (1H, m, $OCH(CH_3)_2$), 4.37 (1H, s, H-3′), 4.22–4.16 (4H, m, H-5′, OCH_2CH_3), 4.08 (1H, s, H-4′), 3.90 (1H, s, P-NH), 3.66 (1H, t, $J = 9.0$ Hz, NHCH), 2.45 (1H, m, H-2′), 2.27 (1H, m, H-2′), 2.09 (1H, m, $CHCH(CH_3)_2$), 2.00 (3H, s, 5-CH_3), 1.34–1.27 (9H, m, $COOCH_2CH_3$), 0.99, 0.90 (6H, d, $J = 6.6$ Hz $CHCH(CH_3)_2$); ^{13}C NMR(150 MHz, $CDCl_3$): δ 172.86 (COOEt), 163.53 (C-4), 150.10 (C-2), 135.09 (C-6), 111.40 (C-5), 84.93, 84.72 (C-1′), 82.45, 82.32 (d, $J_{C\text{-}P} = 10.0$ Hz, C-4′), 73.05, 73.00 ($OCH(CH_3)_2$), 65.40, 65.35 (C-5′), 61.31 (OCH_2CH_3), 60.76 (C-3′), 60.64, 60.42 (Val-C-α), 37.48 (C-2′), 32.20, 32.04 (Val-C-β), 23.62, 23.39 (d, $J_{C\text{-}P} = 5.0$ Hz, $OCH(CH_3)_2$), 18.88, 17.63 (Val-$CH(CH_3)_2$), 14.21 ($COOCH_2CH_3$), 12.48 (5-CH_3); IR (KBr, cm^{-1}) 3454, 2975, 2111, 1692, 1469, 1385, 1273, 996; ESI-MS (pos.): m/z 533 $(M+H)^+$; ESI-MS (neg.): m/z 531 $(M-H)^-$; HRMS-ESI (m/z) calcd for $C_{20}H_{33}N_6O_7PS$ $[M+H]^+$ 533.1948, found 533.1955.

MTT Cytotoxicity Assay

Exponentially growing RKO cell or 293T cell were plated at a density of 8000 or 15,000 cells, respectively, per well into 96-well plates. They were incubated for 24 hours before the addition of drugs. Stock solutions of the

drugs (**1**, **2**, **6**) were initially in dimethyl sulfoxide (DMSO) and further diluted with fresh complete medium.

Cytotoxic activity of phosphorothioamidate prodrugs **6** was determined in RKO and 293T cells using standard tetrazolium MTT assay[24]; 20 μl each of the drug dilutions were added per well and cells were exposed to drugs at a final concentration range of 0–1000 μM. After incubation with the compounds for 48 hours at 37°C in a humidified atmosphere containing 5% CO_2, 20 μl of the medium containing MTT (5 mg/ml) were added to each well. After 4 hours of exposure, the medium was removed and 180 μl of DMSO were added to each well. The metabolized MTT product dissolved in DMSO was quantified by reading the absorbance at 490 nm on a micro plate reader. IC_{50} value, 50% inhibition of cell growth, was calculated from the equation of the logarithmic line determined by fitting the best line (origin 7.0) to the curve formed from the data. All experiments were done at least 3 times, with 6 wells for each of the concentrations of the drugs.

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