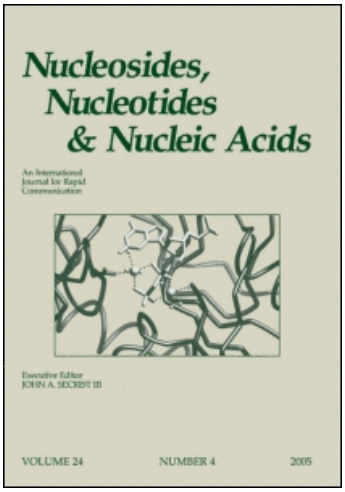


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# Synthesis, In Vitro Anticancer Evaluation, and Interference with Cell Cycle Progression of N-Phosphoamino Acid Esters of Zidovudine and Stavudine

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## ABSTRACT

A series of N-diisopropylphosphoryl (DIPP) L-amino acid ester prodrugs of zidovudine (AZT) (**3a–3e**) and stavudine (d4T) (**4a–4e**) has been prepared. The activity of these compounds against MCF-7 cells (human pleural effusion breast adenocarcinoma cell line) and K562 cells (human chronic myeloid leukemia (CML) cell line) was evaluated. In difference from that of AZT amino acid phosphoramidates, the alophatic amino acid esters of AZT were found to be more cytotoxic than the aromatic analogues toward MCF-7 cell. Two DIPP-L-amino acid esters of d4T **4b** ($CC_{50}$ = 83 μM) and **4c** ($CC_{50}$ = 182 μM) were found to be more cytotoxic than the parent drug toward K562 cells. MCF-7 and K562 cell cycle disturbance was investigated showing detectable blockade in the S phase when exposed to biologically active AZT, **3a**, **3b**, **3c**, **4b** and **4c**, indicating that they inhibit cell growth by blocking cell cycle progression. Together with previous reports, present findings suggest that anti-breast cancer activity of AZT may be due to hamper DNA synthesis.

*Key Words:* N-phosphoamino acid ester; AZT; d4T; K562; MCF-7.

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## INTRODUCTION

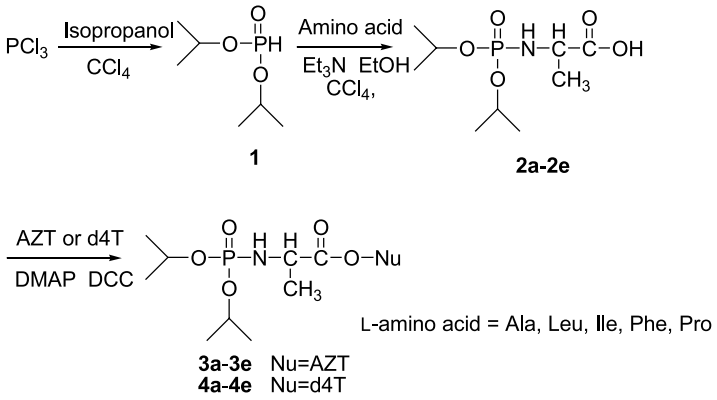
Nucleosides and nucleotides have demonstrated widespread utility as antiviral and anticancer therapeutics.[1] Two of them, 2′,3′-dideoxy-3′-azidothymidine (AZT) and 2′,3′-dideoxy-2′, 3′-didehydrothymidinene (d4T), have antiretroviral activity against human immunodeficiency virus (HIV).[2,3] These drugs are phosphorylated to an active triphosphate form after entry into the cells, followed by its incorporation into DNA by host cell polymerases and then act as DNA chain terminator and/or competitor by blocking the incorporation of the respective deoxynucleoside 5′-triphosphate.[4] Although originally designed as an antitumor agent, AZT has demonstrated far more utility as an antiviral agent and shown only marginal antineoplastic activity compared to standard anticancer chemotherapeutics.[5] Recently, AZT has shown strong inhibition of growth of Sp2/0 cells in vitro[6] and the evidence of the anti-breast cancer activity in vitro and in vivo.[7] Nevertheless, preclinical evaluation of d4T and its derivatives toward mammary tumor animal models has not been reported.

Unfortunately AZT and d4T-based therapies suffer from several drawbacks such as absolute dependence on host cell kinase-mediated activation leading to low activity, short terminal half-life, relatively small volume of distribution, clinical toxicities that include bone myelosuppression, myopathy and hepatic abnormalities.[8,9] In order to overcome the drawbacks, a lot of work has been reported aimed at developing 5′-O-ester prodrugs of AZT and d4T.[10,11] However, the inability or insolubility of lipophilic prodrugs of AZT within the gastrointestinal tract, or slow in vivo release of AZT, limits their oral bioavailability.[12] L-Amino acid-AZT chimeras can serve as alternate prodrugs, which are relatively stable to plasma esterases with longer plasma half-life, and yet are hydrolyzed by the cellular enzymes of the target cells, to enhance AZT absorption via amino acid transport system.[12–15] Moreover, bone marrow progenitor cells may lack the active transport system for amino acids,[16] which may lead to reduced toxicity. L-Amino acid ester prodrugs were less cytotoxic than AZT, suggesting that the slow release of AZT from prodrugs may be related to the observed reduction in toxicity. Some N-phosphoryl amino acids have favorable biological activities and medicinal application in therapy of neurasthenia, dermatosis, parkinsonism, calcium phosphate fame and so on.[17] Previous studies reported from our laboratory have demonstrated that the N-diisopropylphosphoryl (DIPP) amino acids called of mini activating enzyme are important biological small molecules by chemical modification, such as cleavage of DNA,[18] ester exchange and N→O phosphoryl migration,[19] self-activated peptide-formation[20] and interesterification with nucleosides to form nucleotides and oligonucleotides.[21] Therefore, we now design DIPP-L-amino acid esters of AZT and d4T in an attempt to enhance the anticancer activity, membrane penetrating, half-life, stability of AZT and d4T, reduce toxicity of AZT and d4T, and investigate the utility of these compounds as anti-breast cancer and anti-leukemia agents in vitro.

## RESULTS AND DISCUSSION

### Chemical

In order to introduce DIPP-L-amino acid moiety at the 5′-O-position of AZT and d4T, we began by synthesizing the DIPP-L-amino acids according to the method we




***Scheme 1.*** Synthesis of DIPP-L-amino acid esters of AZT and d4T.



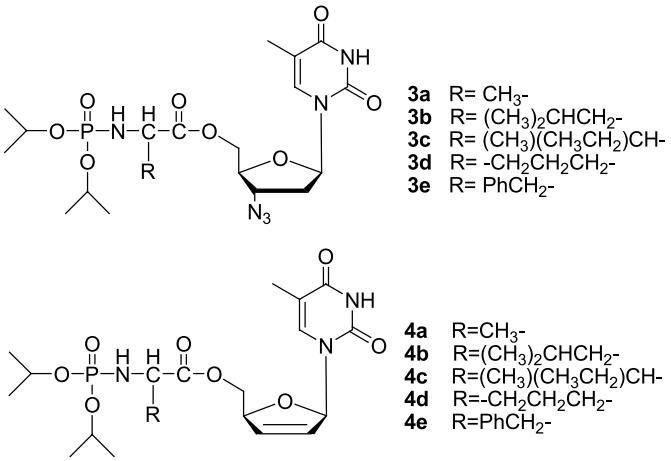
have previously reported.[22] $PCl_3$ and isopropanol were subjected to Arbozov reaction followed by treatment with saturated aqueous sodium hydrogencarbonate solution to obtain **1** (diisopropyl-H-phosphonate, DIPPH). Reaction of **1** with nonpolar L-amino acids, L-alanine, L-leucine, L-isoleucine, L-phenylalanine, and L-proline under Atherton-Todd reaction conditions gave DIPP-L-amino acids **2a–2e** in yields ranging from 83 to 95% (Scheme 1).

During recent years, carbodiimides, and especially N, N′-dicyclohexylcarbodiimide (DCC), have been widely used as condensing agents in ester synthesis.[23] The esterification reaction in the case of AZT was found to be dependent upon the requirement for 4-N, N-dimethylaminopyridine (DMAP) as a catalyst.[13] The 5′-O-esterification of AZT and d4T by N-phosphoryl amino acids **2** was carried out using DCC and DMAP as the condensing agent. Compound **3a–3e** and **4a–4e** were isolated in 72 to 80% yield as white powder solids after purification by silica gel column chromatography (dichloromethane/methanol 20:1).

There is no protecting and deprotecting procedure in the synthesis of N-phosphoryl amino acid esters of AZT and d4T that is unlike the synthesis of amino acid esters of AZT.[13] Thus, these esters of AZT and d4T were prepared in a more facile way in high yield (Scheme 1).

Before biological evaluation, the stability of selected esters **3b** and **4c** in culture medium (10% heat-inactivated fetal bovine serum (FBS)) and in PBS (phosphate buffered saline, pH 7.4) was determined by incubating each compound at a concentration of 100 μM over the course of 30 h at 37°C. There is no measurable decomposition for **3b** and less than 1% decomposition for **4c** over 30 h. Consequently, as was observed for amino acid phosphoramidates of 5-fluoro-2′-deoxyuridine (FUdR)[24] and AZT,[25] the intact esters of AZT and d4T over the 30 h period necessary for completion of an anticancer assay.

In compare to amino acid esters of acyclovir[26] with half-lives in PBS (pH 7.4) at 37°C ranging from 4.2 h to 16.6 h and some amino acid esters of AZT[13] of which approximately 75% resulted in hydrolysis for up to 4 h under these conditions, the N-phosphoryl protected amino acid esters of AZT and d4T were found to be highly stable in culture medium (10% FBS) or PBS (pH 7.4) at 37°C.




***Scheme 2.*** DIPP-L-amino acid esters of AZT and d4T.



## Biological

DIPP-L-amino acid ester of AZT (3a–3e) and DIPP-L-amino acid ester of d4T (4a–4e) were evaluated for their in vitro cytotoxicity toward the MCF-7 cells and K562 cells. The $CC_{50}$ value of esters of AZT and d4T against MCF-7 and K562 cells are summarized in Table 1. Their cytotoxicity was compared to that of parent drugs, AZT and d4T. The effects of varying the α-substituents, the carboxyl end substitutions, on the anticancer activity of the esters toward MCF-7 and K562 were compared.

The amino acid α-substituent of DIPP-L-amino acid ester of AZT plays an important role in cytotoxicity toward MCF-7 cells. Similar with the structure activity relationship for a series of AZT amino acid phosphoramidates,[25] increasing the size of the α-substituent from methyl (**3a**) to isobutyl (**3b**, **3c**) diminished the cytotoxicity, and aliphatic L-amino acid esters **3b** (L-leucine) and **3c** (L-isoleucine) with the same long size of the α-substituent showed similar cycotoxicity, suggesting that steric interactions may play an important role. However, compounds **3d** (L-phenylalanine) and **3e** (L-proline) with large aromatic and cycloalkyl α-substituent are lack of cytotoxicity compared to the other N-phosphoryl amino acid esters of AZT in Table 1, whereas the amino acid phosphoramidates of AZT[25] with large aromatic α-substituents (phenylalanine and tryptophan residues) show remarkable cytotoxicity toward MCF-7 cells. Thus, the hydrophobic character of the α-substituent of the L-amino acid moiety contributes to the cytotoxicity of DIPP-L-amino acid esters of AZT. In Table 1, the lack of activity of d4T and improved activity for **4b** and **4c** toward K562 cells show that the introduction of DIPP-L-leucine and DIPP-L-isoleucine moiety to d4T remarkably enhance the anti-CML activity of d4T.

To probe the anticancer mechanism for these esters, we investigated their perturbation of MCF-7 and K562 cell cycle progression and compared to that of their parent drugs. Exposing MCF-7 cells to AZT, **3a**, 3**b** and **3c** induced cell cycle perturbations in essentially similar manner. Cell cycle analysis showed that AZT, **3a**, 3**b** and **3c** produced a dose-dependant accumulation of cells in the S phase with a

***Table 1.*** In vitro activity of DIPP-L-amino acid esters of AZT and d4T against MCF-7 and K562 cell lines.

| R | Compound | $CC_{50}$ (μM)[a] MCF-7 | $CC_{50}$ (μM)[a] K562[b] |
|---|---|---|---|
| $CH_3-$ | **3a** | 10 | >200 |
| $(CH_3)_2CHCH_2-$ | **3b** | 34 | >200 |
| $(CH_3)(CH_3CH_2)CH-$ | **3c** | 51 | >200 |
| $PhCH_2-$ | **3d** | >200 | >200 |
| $-CH_2CH_2CH_2-$ | **3e** | >200 | >200 |
| $CH_3-$ | **4a** | >200 | >200 |
| $(CH_3)_2CHCH_2-$ | **4b** | >200 | 83 |
| $(CH_3)(CH_3CH_2)CH-$ | **4c** | >200 | 182 |
| $PhCH_2-$ | **4d** | >200 | >200 |
| $-CH_2CH_2CH_2-$ | **4e** | >200 | >200 |
| | d4T | – | >200 |
| | AZT | 1.9 | >200 |

[a]$CC_{50}$ is the concentration required to inhibit cell proliferation by 50% compared to control.
[b]Cisplatin, in the same system showed cytotoxicity ($CC_{50}$ = 1.2 μM) toward K562 cells.



corresponding depletion in the amount of cells in the $G_1$ and $G_2$/M phase, and a detectable blockade in the S phase (Fig. 1). Cell cycle analysis of K562 exposed to **4b** and **4c** at various concentration for 24 h also revealed a dose-dependent increase in percentage of S compartment, and decrease in the percentage of cells in the $G_1$ and $G_2$/M compartment, and produced a detectable blockade in the S phase (Fig. 2). The activity of cell cycle perturbation of these compounds is associated with their activity against MCF-7 and K562 cells, suggesting that blocking cell cycle progression was at least one of the mechanisms by which AZT and its three esters affected MCF-7 cell growth and two d4T esters affected K562 cell growth.

The different percentage of AZT, **3a**, **3b**, and **3c** treated cells in debris compartment (cells undergoing cell death or apoptosis) correlate with their cytotoxicity toward MCF-7 cells. The lack of cytotoxicity of **4b** and **4c** toward K562 cells indicates that their anti-CML activity in 24 h is due to inhibition of cell proliferation but not killing cells.

The inhibition of cell cycle progression observed on AZT may well be explained by the reported main intracellular activity of AZT. Although a correlation between the levels of AZT incorporation into genomic DNA and cytotoxicity has been obtained for cultured colon cancer (HCT-8) cells[27] and AZT-MP but not AZT-TP is responsible for the toxicity toward CEM cells associated with AZT,[28,29] the study on the mechanism for the antiproliferative activity of AZT toward MCF-7 cells only showed that the increased amount of AZT-MP and AZT-TP maybe responsible for the enhanced sensitivity of MCF-7 cells compared to CEM cells,[7] and whether the enhanced cytotoxicity of AZT phosphoramidates is related to enhanced levels of AZT-MP, AZT-TP, or a unique bioactivity associated with the phosphoramidates remains undetermined.[25] It was previously reported that AZT and dideoxycytidine (ddC) blockaded the PBMC and CEM cell cycle progression in the S phase and the effect on cell growth by AZT and ddC may represent a generalized pattern involving a variety of cells of

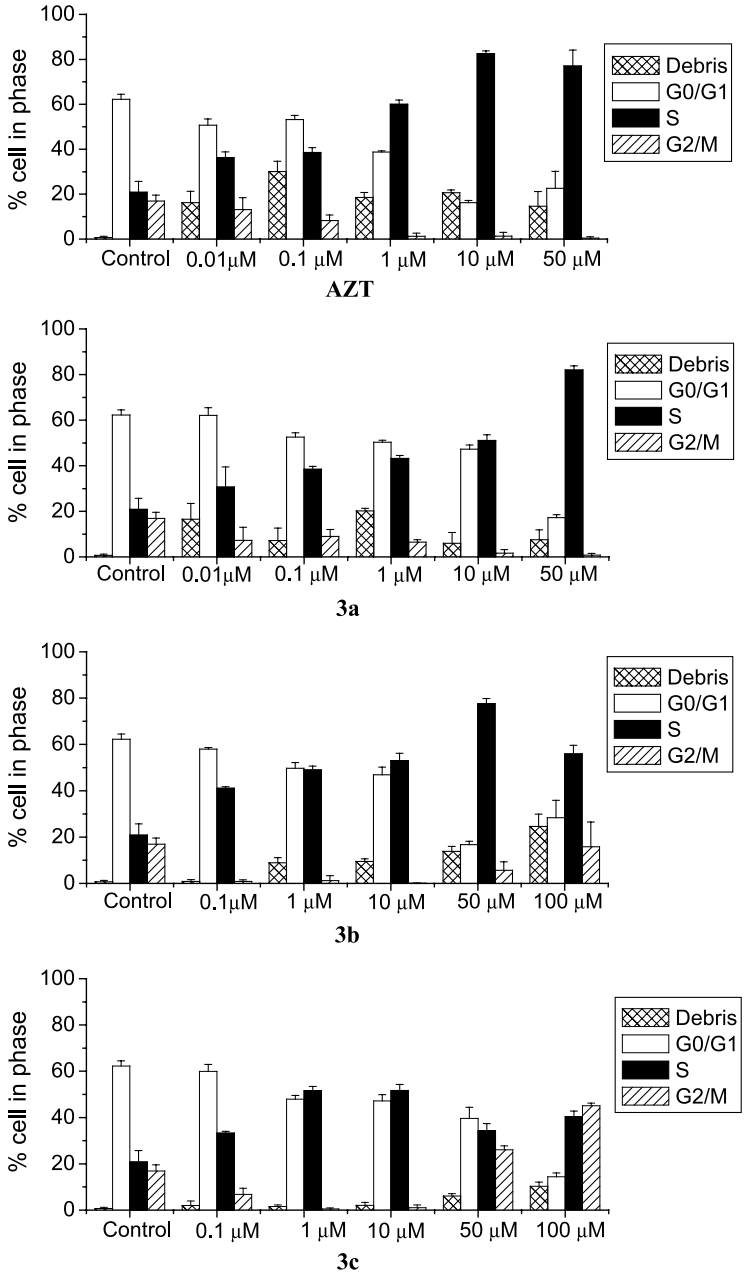



***Figure 1.*** Cell cycle perturbation induced by various concentration of AZT, **3a**, **3b** and **3c** on MCF-7 cells over time. Drugs were added at the beginning of the cultures and cell cycle phases (Debris, $G_0/G_1$, S and $G_2/M$) assessed by flow cytometry after 48 h.

hematopoietic orgin.[4] These drugs act as substrates so that the corresponding nucleoside monophosphate moiety is incorporated into replicating DNA leading to DNA chain termination.[30] Other anticancer nucleosides Fludarabine and Cladribine applied in treatment of CLL (chronic lymphocytic leukaemia) and Ara-C applied in AML (acute myelogeous leukaemia) incorporated into DNA after activation and terminated the DNA chain elongation, killing cells or inducing apoptosis in those cells in the S phase of the cell cycle.[31–33] Since an impairment of DNA synthesis including regulation of nucleotide metabolism[34] and DNA incorporation[35] upon dideoxynucleoside challenge has been reported in several cell models, the mechanism for anti-breast cancer activity of AZT may be due to detriment of DNA synthesis. But whether AZT acts as an anti-metabolite or DNA chain terminator or both in MCF-7 cells remains to be determined.

In summary, DIPP-L-amino acid esters of AZT and d4T were disclosed as novel prodrugs. We have demonstrated that the hydrophobic character of amino acid α-substituents of DIPP-L-amino acid esters of AZT plays an important role in in vitro anti-breast cancer activity, preferably a small aliphatic L-amino acid side chain. The large aromatic and cycloalkyl α-substituents significantly reduce the activity of the DIPP-L-amino acid esters of AZT. Among a series of DIPP-L-amino acid esters of d4T, compounds **4b** and **4c** can act as potential growth inhibitors of the human chronic myeloid leukemia cell line, K562. Cell cycle analysis has showed that AZT, **3a**, **3b**, **3c**, **4b** and **4c** blockade the cell cycle in the S phase in similar manner, indicating that the anticancer activity of these compounds is due to retarding cell cycle progression. The

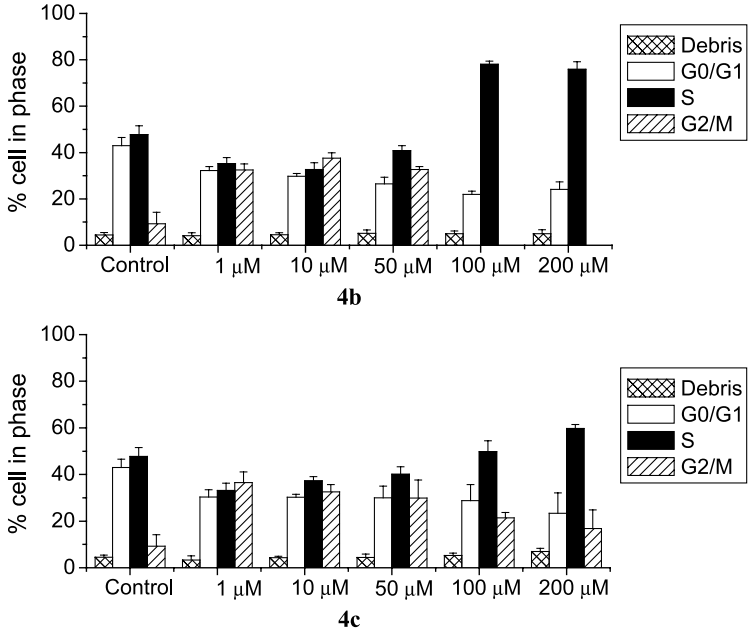



***Figure 2.*** Cell cycle perturbation induced by various concentration of **4b** and **4c** on K562 cells over time. Drugs were added at the beginning of the cultures and cell cycle phases (Debris, $G_0/G_1$, S and $G_2/M$) assessed by flow cytometry after 24 h.

cytotoxicity of AZT toward MCF-7 may own to the impairment of DNA synthesis. Further study for intracellular uptake and metabolism of these prodrugs will be needed to determine the anticancer mechanism.

## EXPERIMENTAL SECTION

### Chemical Procedures

$^{1}H$- and $C^{13}$-NMR spectra were recorded on a Varian INOVA 500 and JEOL AL 300 spectrometers. $^{31}P$ NMR spectra were recorded on a Bruker AC 200 with 85% $H_3PO_4$ as an external standard. ESI-MS spectra were obtained on a ESQUIRE-LC mass spectrometer. Analytical TLC was performed on GF254 silica gel plates. Column chromatography was performed with 200–300 mesh silica gel. Anhydrous tetrahydrofuran, ethyl acetate and triethylamine were refluxed and distilled from sodium. Anhydrous ethanol was prepared by using magnesium turnings.



**General procedure for the synthesis of 2′,3′-dideoxy-3′-azidothymidine-5′-(2-(N-(O,O′-diisopropoxy)-phosphorylamino) acid) esters (3a–3e) and 2′,3′-dideoxy-2′, 3′-didehydrothymidinene-5′-(2-(N-(O,O′-diisopropoxy)-phosphorylamino) acid) esters (4a–4e).** To a solution of the nucleoside (AZT or d4T) (1 equiv.), DMAP (1.5 equiv.) and N-(O,O′-diisopropoxy)-phosphorylamino acid (**2a–2e**)[22] (1.1 equiv.) in anhydrous ethyl acetate were added DCC (1.2 equiv.) in batches at 0°C. The reaction mixture was stirred overnight at room temperature, then the side product N, N′-Dicyclohexyl carbamide (DCU) was filtered. After evaporation of the solvent, the residue was dissolved in methylene chloride and purified by column chromatography ($CH_2Cl_2$:$CH_3OH$ = 20:1). The fractions containing the desired product were pooled together, and the solvent was evaporated in vacuo, yield of the isolated products as white powder solids. $^{1}H$ NMR, $^{13}C$ NMR, $^{31}P$NMR and ESI-MS data are indicated below each compound.

**2′,3′-dideoxy-3′-azidothymidine-5′-(2-(N-(O,O′-diisopropoxy)-phosphorylamino)-proprionic acid) ester (3a).** Yield of **3a** (80%); $^{31}P$ NMR ($CDCl_3$, 81 MHz, δ ppm): 5.55; $^{1}H$ NMR ($CDCl_3$, 500 MHz, δ ppm); 9.92 (br, 1H, H-3), 7.11 (d, 1H, H-6, $J$ = 2.0 Hz), 5.96 (dd, 1H, H-1′, $J$ = 6.5 Hz, 6.0 Hz), 4.55 (m, 2H, $-OCH(CH_3)_2$), 4.33 (d, 2H, H-5′, $J$ = 4.0 Hz), 4.29 (m,1H, H-3′), 3.99 (m, 1H, H-4′), 3.92 (m,1H, H-α), 3.63 (dd, 1H, $J$ = 10.0 Hz, –PO–NH–), 2.44 (m, 2H, H-2′), 1.88 (S, 3H, 5-$CH_3$),1.37 (d, 3H, $J$ = 7.5 Hz, H-β), 1.23–1.26 (m, 12H, $-OCH(CH_3)_2$); $^{13}C$ NMR (125 MHz, $CDCl_3$, δ ppm): 173.48 (C=O), 163.96 (C-4), 150.23 (C-2), 135.86 (C-6), 111.23(C-5), 86.13 (C-1′), 81.44 (C-4′), 71.20 ($-O-CH-(CH_3)_2$), 63.71 (C-5′), 60.26 (C-3′), 50.10 (C-α), 37.04 (C-2′), 23.63 (d, $^{2}J$ = 5.5 Hz, $-O-CH-(CH_3)_2$), 20.71 (d, $^{3}J$ = 3.6 Hz, $CH_3$-β), 12.44 ($CH_3$-5); ESI-MS (+): m/z = 503 $[M + H]^+$.

**2′,3′-dideoxy-3′-azidothymidine-5′-(2-(N-(O,O′-diisopropoxy)-phosphorylamino)-4-methyl-petanoic acid) ester (3b).** Yield of **3b** (87%); $^{31}P$ NMR ($CDCl_3$,81 MHz, δ ppm): 5.70; $^{1}H$ NMR ($CDCl_3$, 500 NHz, δ ppm): 9.65 (br, 1H, H-3), 7.19 (d, 1H, H-6, $J$ = 1.0Hz), 6.07 (dd, 1H, H-1′, $J$ = 6.5 , 6.5 Hz), 4.60 (m, 2H, $-OCH(CH_3)_2$),

4.41 (dd, 1H, H-5′, $J$ = 5.0 Hz, 12.5 Hz), 4.31 (dd, 1H, H-5′, $J$ = 3.0 Hz, 12.0Hz), 4.29 (m, 2H, H-3′), 4.07 (m, 1H, H-4′), 3.87 (M, 1H, H-α), 3.44 (dd, 1H, –PO–NH–, $J$ = 10.0 Hz, 10.0Hz), 2.45 (m, 2H, H-2′), 1.94 (s, 3H, 5-$CH_3$), 1.79 (m, 1H, H-γ), 1.53 (m,2H, H-β), 1.28–1.32 (m, 12H,–OCH($CH_3$)$_2$), 0.94 (d, 6H, $J$ = 6.5 Hz, $CH_3$-δ); $^{13}$C NMR (125 MHz, $CDCl_3$, δ ppm): 173.59 (C=O), 164.03 (C-4), 150.23 (C-2), 135.46 (C-6), 111.16 (C-5), 85.45 (C-1′), 81.29(C-4′), 71.18 (–O–$CH$–($CH_3$)$_2$), 63.76 (C-5′), 60.45 (C-3′), 50.03 (C-α), 43.57 (D, $^3J$ = 7.2 Hz, C-β), 36.98 (C-2′), 24.23 (C-γ), 23.62 (–O–CH–($CH_3$)$_2$), 22.55, 21.79 ($CH_3$-δ), 12.47 ($CH_3$-5). ESI-MS (+): m/z = 545 $[M + H]^+$.

**2′,3′-dideoxy-3′-azidothymidine-5′-(2-(N-(O,O′-diisopropoxy)-phosphoryl-amino)-3-methyl-petanoic acid) ester (3c).** Yield of **3c** (90%); $^{31}$P NMR ($CDCl_3$, 81 MHz, δ ppm): 6.16; $^1$H NMR ($CDCl_3$, 500 MHz, δ ppm): 9.38 (br, 1H, H-3), 7.19 (d, 1H, $J$ = 1.0 Hz, H-6), 6.05 (dd, 1H, H-1′, $J$ = 6.5 Hz, 6.5 Hz), 4.60 (m, 2H, –O$CH$($CH_3$)$_2$), 4.41 (dd, 1H, H-5′, $J$ = 5.0 Hz, 12.0 Hz), 4.31 (dd, 1H, H-5′, $J$ = 3.5 Hz, 12.0 Hz), 4.28 (m, 2H, H-3′), 4.06 (dd, 1H, $J$ = 9.5 Hz, 5.5 Hz, H-4′), 3.76 (m, 1H, H-α), 3.38 (dd, 1H, –PO–NH–, $J$ = 10.0 Hz, 10.0 Hz), 2.45 (m, 2H, H-2′), 1.94 (s, 3H, 5-$CH_3$), 1.76 (m, 1H, H-β), 1.48 (m, 1H, H-γ), 1.27–1.32 (m, 12 H, –OCH($CH_3$)$_2$), 1.18 (m, 1H, H-γ), 0.90–0.95 (m , 6H, 2$CH_3$); $^{13}$C NMR (125 MHz, $CDCl_3$, δ ppm) : 172.57 (C=O), 163.98 (C-4), 150.24 (C-2), 135.39 (C-6), 111.16 (C-5), 85.44 (C-1′), 81.22 (C-4′), 71.11 (d, $^2J$ = 5.5 MHz, –O–$CH$–($CH_3$)$_2$), 63.57 (C-5′), 60.24 (C-3′), 58.89 (C-α), 38.93 (d, $^3J$ = 7.2 Hz, C-β), 36.95 (C-2′), 24.69 (C-γ), 23.58 (–O–CH–($CH_3$)$_2$), 15.20, 11.30 ($CH_3$), 12.40($CH_3$-5); ESI-MS (+): $m/z$ = 545 $[M + H]^+$.

**2′,3′-dideoxy-3′-azidothymidine-5′-(N-(O,O′-diisopropoxy)-phosphorylamino-pyrrolidine-2-carboxylic acid) ester (3d).** Yield of **3d** (89%); $^{31}$P NMR ($CDCl_3$, 81 MHz, δ ppm): 4.26; $^1$H NMR ($CDCl_3$, 500 MHz, δ ppm): 9.39 (br, 1H, H-3), 7.29 (D, 1H, H-6, $J$ = 2.0 Hz), 6.14 (dd, 1H, H-1′, $J$ = 6.5 Hz, 6.0 Hz), 4.71 (m, 1H –O$CH$($CH_3$)$_2$), 4.55 (m, 1H, –O$CH$($CH_3$)$_2$), 4.43 (dd, 1H, H-5′, $J$ = 5.0 Hz, 12.0 Hz), 4.30 (dd, 1H, H-5′, $J$ = 4.0 Hz, 12.0 Hz), 4.24–4.33 (m, 2H, H-3′, H-α), 4.10 (dd, 1H, $J$ = 5.0 Hz, 4.0 Hz, H-4′), 3.25 (m, 2H, H-δ), 2.45 (m, 1H, H-2′), 2.33 (m, 1H, H-2′), 2.21 (m, 1H,H-β), 1.96 (s, 3H, 5-$CH_3$), 1.92 (m, 3H,, H-β, H-γ), 1.28–1.34 (m, 12H, –OCH($CH_3$)$_2$); $^{13}$C NMR (125 MHz, $CDCl_3$, δ ppm) : 173.86 (C=O), 163.74 (C-4), 150.16 (C-2), 135.26 (C-6), 111.38 (C-5), 85.22 (C-1′), 81.56 (C-4′), 70.80 (–O–CH–($CH_3$)$_2$), 63.61 (C-5′), 60.56 (C-3′), 60.22 (d, $^2J$ = 5.5 Hz, C-α), 46.88(C-δ), 37.23 (C-2′), 31.35 (d, $^3J$ = 9.0 Hz, C-β), 25.38 (d, $^3J$ = 9.0 Hz, C-γ), 23.84, 23.65 *(–O–CH–($CH_3$)$_2$), 12.46 (CH-5); ESI-MS(+): m/z = 529 $[M + H]^+$.

**2′,3′-dideoxy-3′-azidothymidine-5′-(2-(N-(O,O′-diisopropoxy)-phosphoryl-amino)-3-phenylpropanoic acid) ester (3e).** Yield of **3e** (86%); $^{31}$P NMR ($CDCl_3$, 81 MHz, δ ppm): 5.37; $^1$H NMR ($CDCl_3$, 500 MHz, δ ppm): 9.76 (br, 1H, H-3), 7.23–7.30 (m, 3H, Ph-H), 7.18 (d, 2H, $J$ = 6.5 Hz, Ph-H), 7.11 (s, 1H, H-6 ), 6.01 (dd, 1H, H-1′, $J$ = 7.5 Hz, 6.0 Hz), 4.54 (m, 2H, –O$CH$($CH_3$)$_2$), 4.30 (dd, 1H, H-5′, $J$ = 3.5 Hz, 12.5 Hz), 4.22 (dd, 1H, H-5′, $J$ = 3.5 Hz, 12.5 Hz), 4.12 (m, 1H, H-α, 3.90 (m, 1H, H-4′), 3.67 (m, 1H, H-3′), 3.55 (dd, 1H, –PO–NH–, $J$ = 10.0 Hz, 10.0 Hz), 3.11 (dd, 1H,. H-β, $J$ = 6.0 Hz, 13.5 Hz), 2.97 (dd, 1H, H-β, $J$ = 7.5 Hz, 13.5 Hz),

2.28–2.33 (m, 1H, H-2′), 2.12–2.18 (m,1H, H-2′), 1.98 (s, 3H, 5-$CH_3$), 1.22–1.30 (m, 12H, –OCH($CH_3$)$_2$); $^{13}$C NMR (125 MHz, $CDCl_3$, δ ppm): 172.55 (C=O), 163.79 (C-4), 150.16 (C-2), 136.00, 135.36 (C-6), 129.30, 128.67, 127.22 (PhC), 111.30 (C-5), 85.35 (C-1′), 81.20 (C-4′), 71.35 (–O–*CH*–($CH_3$)$_2$), 63.58 (C-5′), 59.63 C-3′, 56.17 (C-α), 41.03 (d, $^3J$ = 5.5 Hz, C-β), 37.18 (C-2′), 23.69 (–O–CH–(*$CH_3$*)$_2$), 12.68 ($CH_3$-5); ESI-MS (+): m/z = 579 [M + H]$^+$.

**2′,3′-dideoxy-2′,3′-didehydrothymidinene-5′-(2-(N-(O,O′-diisopropoxy)-phosphorylamino)-proprionic acid) ester (4a).** Yield of **4a** (87%); $^{31}$P NMR ($CDCl_3$,81 MHz, δ ppm): 5.59; $^1$H NMR ($CDCl_3$, 500 MHz, δ ppm): 10.08 (br, 1H, H-3), 7.17 (d, 1H, H-6, $J$ = 1.0 Hz), 7.01 (m, 1H, H-1′), 6.30 (d, 1H, $J$ = 6.0 Hz,H-3′), 5.94 (d, 1H, $J$ = 5.0 Hz, H-2′), 5.05 (br, 1H, H-4′), 4.58 (m, 2H, –O*CH*($CH_3$)$_2$), 4.43 (dd, 1H, H-5′, $J$ = 12.0, 5.0 Hz), 4.27 (dd, 1H, H-5′, $J$ = 12.0, 3.5 Hz), 3.92 (m, 1H, H-α), 3.65 (dd, 1H, $J$ = 10.0 Hz, 10.0 Hz, –PO–NH–), 1.94 (s, 3H, 5-$CH_3$), 1.40 (d, 3H, $J$ = 7.5 Hz, H-β), 1.25–1.32 (m, 12H, –OCH(*$CH_3$*)$_2$); $^{13}$C NMR (125 MHz, $CDCl_3$, δ ppm): 173.70 (d, $^3J$ = 6.0 Hz, C=O), 163.90 (C-4), 150.85 (C-2), 134.87 (C-6), 132.65 (C-3′), 127.50 (C-2′), 111.17 (C-5), 89.73 (C-1′), 83.69 (C-4′), 71.00 (d, $^2J$ = 5.7 Hz, –O–*CH*–($CH_3$)$_2$), 65.42 (C-5′), 49.81 (C-α), 23.58 (–O–CH–(*$CH_3$*)$_2$), 20.85 (d?$^3J$ = 5.2 Hz, $CH_3$-β), 12.43 ($CH_3$-5); ESI-MS(+): m/z = 460 [M + H]$^+$.

**2′,3′-dideoxy-2′,3′-didehydrothymidinene-5′-(2-(N-(O,O′-diisopropoxy)-phosphorylamino)-4-methyl-petanoic acid) ester (4b).** Yield of **4b** (95%); $^{31}$P NMR ($CDCl_3$, 81 MHz, δ ppm): 5.85; $^1$H NMR ($CDCl_3$, 500 MHz, δ ppm): 9.59 (br, 1H, H-3), 7.15 (d, 1H, H-6, $J$ = 1.0 Hz), 7.00 (m, 1H, H-1′), 6.30 (d, 1H, $J$ = 5.5 Hz, H-3′), 5.94 (m, 1H, H-2′), 5.05 (br, 1H, H-4′), 4.58 (m, 2H, –O*CH*($CH_3$)$_2$), 4.37 (dd, 1H, H-5′, $J$ = 12.0, 5.5 Hz), 4.27 (dd, 1H, H-5′, $J$ = 12.0, 4.5 Hz), 3.82 (m, 1H, H-α), 3.29 (dd, 1H, $J$ = 10.0 Hz, 10.0 Hz, –PO–NH–), 1.94 (s, 3H, 5-$CH_3$), 1.76 (m, 1H, H-γ), 1.53 (m, 2H, H-β), 1.25–1.32 (m, 12 H, –OCH(*$CH_3$*)$_2$), 0.91–0.93 (m, 6H, $J$ = 6.5 Hz, $CH_3$-δ); $^{13}$C NMR (125 MHz, $CDCl_3$, δ ppm): 173.99 (C=O), 163.80 (C-4), 150.78 (C-2), 134.89 (C-6), 132.85 (C-3′), 127.47 (C-2′), 111.38 (C-5), 89.98 (C-1′), 83.72 (C-4′), 71.17 (d, $^2J$ = 6.6 Hz, –O–*CH*–($CH_3$)$_2$), 65.56 (C-5′), 52.93 (C-α), 43.80 (d, $^3J$ = 6.7 Hz, C-β), 24.27 (C-γ), 23.68(–O–CH–(*$CH_3$*)$_2$), 22.62, 22.00 ($CH_3$-δ), 12.63 ($CH_3$-5); ESI-MS (+): *m/z* = 502 [M + H]$^+$.

**2′,3′-dideoxy-2′,3′-didehydrothymidinene-5′-(2-(N-(O,O′-diisopropoxy)-phosphorylamino)-3-methyl-petanoic acid) ester (4c).** Yield of **4c** (84%); $^{31}$P NMR ($CDCl_3$, 81 MHz, δ ppm): 6.29; $^1$H NMR ($CDCl_3$, 500 MHz, δ ppm): 9.49 (br, 1H, H-3), 7.16 (d, 1H, $J$ = 1.0 Hz, H-6), 6.99 (m, 1H, H-1′), 6.29 (dd, 1H, $J$ = 4.5 Hz, 1.5 Hz, H-3′), 5.94 (d, 1H, J = 5.0 Hz, H-2′), 5.05 (br, 1H, H-4′), 4.58 (m, 2H, –O*CH*($CH_3$)$_2$), 4.43 (dd, 1H, H-5′, $J$ = 5.0 Hz, 12.0 Hz), 4.24 (dd, 1H, H-5′, $J$ = 4.0 Hz, 12.0 Hz), 3.71 (m, 1H, H-α), 3.32 (dd, 1H, –PO–NH–, $J$ = 10.0 Hz, 10.0 Hz), 1.95 (s, 3H, 5-$CH_3$), 1.76 (m, 1H, H-β), 1.44 (m, 1H, H-γ), 1.25–1.32 (m, 12 H, –OCH(*$CH_3$*)$_2$), 1.15 (m, 1H, H-γ), 0.87–0.94 (m , 6H, 2$CH_3$); ESI-MS (+): *m/z* = 502 [M + H]$^+$.

**2′,3′-dideoxy-2′,3′-didehydrothymidinene-5′-(N-(O,O′-diisopropoxy)-phosphorylamino-pyrrolidine-2-carboxylic acid) ester (4d).** Yield of **4d** (92%); $^{31}$P NMR ($CDCl_3$,81 MHz, δ ppm): 4.30; $^1$H NMR ($CDCl_3$, 500 MHz, δ ppm): 8.98 (br, 1H,

H-3), 7.22 (d, 1H, H-6, $J$ = 1.0 Hz), 6.79 (m, 1H, H-1′), 6.33 (m, 1H, H-3′), 5.91 (d, 1H, H-2′), 5.04 (br, 1H, H-4′), 4.73 (m, 1H, $-OCH(CH_3)_2$), 4.52 (m, 1H, $-OCH(CH_3)_2$), 4.44 (dd, 1H, H-5′, $J$ = 12.0, 5.0 Hz), 4.28 (m, 1H, H-α), 4.24 (dd, 1H, H-5′, $J$ = 12.0, 4.0 Hz) 3.25 (m, 2H, H-δ), 2.16 (m, 1H, H-β), 1.94 (s, 3H, 5-$CH_3$), 1.91 (m, 3H, H-β, H-γ), 1.27–1.34 (m, 12H, $-OCH(CH_3)_2$); $^{13}$C NMR (125 MHz, $CDCl_3$, δ ppm): 174.00 (C=O), 163.65 (C-4), 150.62 (C-2), 135.18 (C-6), 133.17 (C-3′), 127.19 (C-2′), 111.18 (C-5), 90.06 (C-1′), 84.22 (C-4′), 71.70 (d, $-O-CH-(CH_3)_2$), 65.03 (C-5′), 60.08 (d, $^2J$ = 7.1 Hz, C-α), 46.86 (C-δ), 31.39 (d, $^3J$ = 8.1 Hz, C-β), 25.30 (d, $^3J$ = 8.1 Hz, C-γ), 23.67, 23.62*($-O-CH-(CH_3)_2$), 12.49 ($CH_3$-5); ESI-MS (+): m/z = 486 $[M + H]^+$.

**2′,3′-dideoxy-2′,3′-didehydrothymidinene-5′-(2-(N-(O,O′-diisopropoxy)-phosphorylamino)-3-phenylpropanoic acidacid) ester (4e).** Yield of **4e** (87%); $^{31}$P NMR ($CDCl_3$, 81 MHz, δ ppm): 5.48; $^1$H NMR ($CDCl_3$, 500 MHz, δ ppm): 9.10 (br, 1H, H-3), 7.23–7.28 (m, 3H, Ph-H), 7.16 (m, 2H, Ph-H), 7.05 (d, 1H, H-6, $J$ = 1.0 Hz), 6.97 (m, 1H, H-1′), 6.05 (d, 1H, $J$ = 6.0 Hz, H-3′), 5.80 (m, 1H, H-2′), 4.93 (br, 1H, H-4′), 4.52 (m, 2H, -$OCH(CH_3)_2$), 4.30 (dd, 1H, H-5′, $J$ = 12.0, 5.0 Hz), 4.16 (dd, 1H, H-5′, $J$ = 12.0, 4.0 Hz), 4.08 (m, 1H, H-α), 3.30 (m, 1H, –PO–NH–), 3.01 (m, 2H, H-β), 1.963 (s, 3H, 5-$CH_3$), 1.20–1.30 (m, 12H, $-OCH(CH_3)_2$); $^{13}$C NMR (125 MHz, $CDCl_3$, δ ppm): 172.62 (C=O), 163.56 (C-4), 150.66 (C-2), 135.02 (C-6), 132.92 (C-3′), 127.20 (C-2′), 135.74, 128.57, 127.20 (PhC), 111.36 (C-5), 89.85 (C-1′), 83.50 (C-4′), 71.29 ($-O-CH-(CH_3)_2$), 65.52 (C-5′), 55.78 (C-α), 40.80 (C-β), 23.68 ($-O-CH-(CH_3)_2$), 12.67 ($CH_3$-5); ESI-MS(+): m/z = 536 $[M + H]^+$.

**Determination of decomposition rates in culture medium and PBS.** The aqueous stability of selected **3b** and **4c** was assessed in culture medium (with 10% fetal bovine serum) and PBS (pH 7.4) at 37°C in 100 μM in triplicate. Decomposition of each compound was detected by HPLC at 4, 30, 60, 120 h. For culture medium solution, cold chromatographical grade methanol in equal volume to the solution was added to precipitate proteins, and d4T or AZT in PBS was added as an internal standard. Samples were then centrifuged (13200g, 10 min, 4°C), and a 20 μL aliquot from the methanolic solution was subjected to HPLC analysis. Analysis of the remaining ester was performed on a 4.6 × 250 mm 5 μm Kromasil reverse-phase C18 column. A standard curve (20–300 μM) based on peak area was constructed for the compounds with d4T or AZT used as the internal standard. The compounds were eluted by using a gradient of 85% 50 mM ammonium acetate and 15% acetonitrile running at 1 mL/min. The concentration of the remaining ester was determined from the standard curve. Decomposition rates were determined by linear plots of the remaining concentration of the ester relative to time.

## Biological Procedures

All compounds were evaluated in vitro cytotoxicity toward the human pleural effusion breast adinocarcinoma cell line MCF-7 and human chronic myeloid leukemia cell line K562. The concentration causing 50% cell growth inhibition ($CC_{50}$) of each compound was determined using MTT (3-[(4,5-Dimethylthiazol-2-yl)]-2,5-diphenyltetrazolium bromide) assay, dose-response curves for each cell line were measured

with five different drug concentration, and the $CC_{50}$ compared with the control was calculated.

### MTT Assay

MCF-7 cells and K562 cells were cultured in DMEM and RPMI 1640 medium respectively supplemented with 10% fetal bovine serum (FBS). MCF-7 cells and K562 cells were plated in 96-well sterile plates at a density of $1 \times 10^5$ cells /mL in 90 μL of medium with 10 μL of samples in 1 × PBS (phosphate buffered saline) to achieve different final concentrations (0.001, 0.01, 0.1, 1, 10, and 100 μM for MCF-7 and 1, 10, 50, 100, 200 μM for K562), and were incubated at 37°C in a humidified 5% $CO_2$ atmosphere for 48h and 24 h respectively, pulsed with 10 μL of MTT (5mg/mL) and incubated for an additional 4 h under the above-mentioned conditions. 100 μL DMSO was added to all wells and mixed thoroughly. The cell viability was evaluated by measurement of the absorbance at 490nm, using a Spectra MAX Plus model Microelisa Reader. Inhibition Rate (IR%) was calculated as follows: IR% = (mean control absorbance-mean experimental absorbance)/mean control absorbance × 100%.

### Cell Cycle Analysis

After 48 h and 24 h respectively incubation with different concentrations of compounds and without compounds, control and treated MCF-7 and K562 cells were harvested by centrifugation and washed by PBS twice. Fixation was performed in fixing solution (70% ethanol) overnight, then the 200μl RNAse of 1mg/mL was added and the cells were cultured at 37°C for 2 h. After centrifugation and stained by 500μl propidium iodide (PI) of 0.05 mg/mL (with 1 mg/mL Sodium citrate and 2 mg/mL Triton X-100) for 30 min, the cells were immediately assayed by FACSCalibur Flow Cytometer (BETON DICHINSON) with 488 nm excitation. DNA analysis was performed by acquiring up to 15,000 events using the ModFIT software. Data are mean of three experiments performed in triplicate.



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