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Original article

Synthesis, structure–activity relationship and antiviral activity of 3′-*N*, *N*-dimethylamino-2′,3′-dideoxythymidine and its prodrugs☆

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ABSTRACT

A probable NRTI molecule, viz. 3′-*N*,*N*-dimethylamino-2′,3′-dideoxythymidine (**4**) and its 5′-*O*-carboxyl ester prodrugs – 5′-(*N*-α-BOC-L-phenylalanyl)-3′-*N*,*N*-dimethylamino-2′,3′-dideoxythymidine (**5**), 5′-L-phenylalanyl-3′-*N*,*N*-dimethylamino-2′,3′-dideoxythymidine (**6**) and 5′-decanoyl-3′-*N*,*N*-dimethylamino-2′,3′-dideoxythymidine (**7**) have been synthesized and screened against HIV, HSV-1 and 2, parainfluenza-3, vesicular stomatitis and several other viruses. The compound **6** showed good antiviral activity with EC_{50} value 0.03 μM (SI = 8) against VSV in Hela and HEL cell lines. However, the lead compound **4** and its derivatives **5**, **6** and **7** showed no remarkable activity against HIV-1 and other viruses. Molecular docking studies with HIV-1 RT using DS 2.5 and pymol softwares have shown marked differences in the interaction patterns between the lead compound **4** and AZT.

1. Introduction

HIV-1 reverse transcriptase (HIV-1 RT), a primary target for developing anti-HIV drugs, is the enzyme responsible for generating linear dsDNA from ssRNA – the genetic material of HIV-1. This dsDNA acts as a provirus and leads to viral expression through integration in the host genome, transcription and translation processes.

There are several drugs that inhibit HIV-1 RT and they are classified as nucleos(t)ide RT inhibitors (N(t)RTIs) and non-nucleoside RT inhibitors (NNRTIs). The NRTIs have emerged as important therapeutic agents for the development of anti-HIV drugs [1–4]. The NRTIs after intracellular phosphorylation to their active triphosphate form by cellular kinases [5], act as competitive inhibitors with respect to the dNTP substrates. These molecules when incorporated by HIV-1 RT in the growing template/primer, cause chain termination [6,7] since they lack 3′-OH group, which is necessary for chain elongation and thus, they inhibit HIV-1 RT [8,9]. Despite their tremendous utility, nucleoside analogues may cause serious cellular toxicity by interacting with human DNA polymerase γ [10], present in mitochondria, which results in mitochondrial dysfunctions. Thus NRTIs, show many side effects [11–13]. In addition to this, four more significant obstacles currently limiting the clinical application of nucleoside analogues are oral absorption, cellular uptake, short half-life and viral resistance [14]. So, there is a need for new nucleoside mimics and their prodrugs to overcome the limitations. We have already reported several nucleosidic [15,16] and non-nucleosidic molecules [17] with significant antiviral activities.

The NRTIs, by virtue of acting as chain terminators of (−) strand DNA synthesis during reverse transcription, have been developed as potential drugs against HIV/AIDS. In order to explore such new inhibitors, we report here the synthesis of 3′-*N*,*N*-dimethylamino-2′,3′-dideoxythymidine (DMAT) and its 5′-*O*-ester prodrugs with amino and fatty acids. The lead molecule, DMAT, was designed keeping AZT, an approved NRTI drug against HIV/AIDS, in mind, where azido group has been replaced by dimethylamino function, Fig. 1. Thus, this molecule being a dideoxynucleoside is expected to act as NRTI against HIV-1 RT enzyme. Similarly, the prodrugs, having biodegradable ester linkages, are supposed to enhance the cellular uptake due to their high lipophilic nature.

All these molecules have been evaluated for their activity against various viruses, like HIV-1, vesicular stomatitis, HSV-1 & 2, reovirus, parainfluenza virus and many others.

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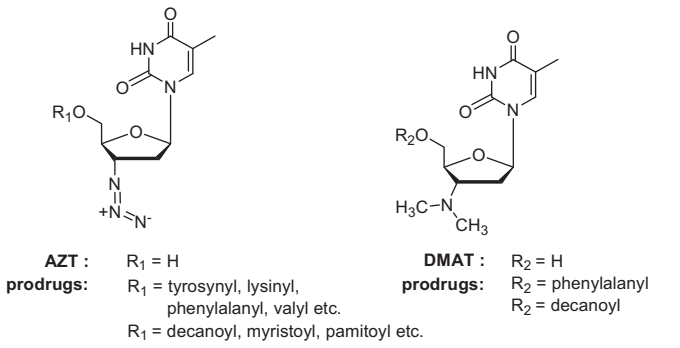


Fig. 1. Structures of AZT and 3′-*N*,*N*-dimethylamino-2′,3′-dideoxythymidine and their prodrugs.

2. Chemistry

A new thymidine analogue, viz. 3′-*N*,*N*-dimethylamino-2′,3′-dideoxythymidine has been synthesized from thymidine as shown in Scheme 1. 5′-*O*-(4,4′-Dimethoxytrityl)-3′-mesylthymidine, obtained after 5′-OH selective protection with DMTCl followed by mesylation with MsCl, when reacted with potassium phthalimide witnessed the introduction of nucleophile (phthalimide ion) into the sugar moiety [18]. During conversion of **1**–**2**, the elimination of C3′-mesyloxy group was effected through intramolecular displacement by attack of carbonyl group of thymidine at C2, which resulted in formation of 2,3′-anhydronucleoside intermediate. This result was well in accordance with the literature data. It is suggested that under this condition, phthalimide ion is introduced into the 'down' (ribo) configuration [19]. The 3′-phthalimide derivative **2** was treated with methanolic methylamine, which through methanolysis and aminolysis resulted in formation of compound **3** [20]. Reaction of **3** with formic acid and aqueous formaldehyde (40%) yielded **4**. In this reaction, formaldehyde used was the source of methyl groups, while formic acid supplied the hydrogens involved in the reduction. This treatment also removed the DMT group from compound **3**. As expected, the UV absorption spectra of all intermediates were similar to that of thymidine, while dissimilar R_f values showed that all these changes took place in the sugar moiety and not in the aglycon part [19].

3. Results and discussion

3.1. Antiviral activity

All compounds **4**–**7** have been screened for their antiviral potential against HIV and several other viruses and the results are shown in Table 1. The lead compound DMAT and its prodrugs were found inactive against HIV-1 ROD and HIV-1 IIIB strains and showed toxicity. Compounds **4** and **5** have showed similar values of CC_{50} and EC_{50} against all viruses studied and thus no activity was observed, while 5′-L-phenylalanyl derivative **6**, having free amino function was potentially active at an EC_{50} of 0.03 μM against VSV in HeLa and HEL cell cultures. The EC_{50} value of compound **6** has been found to be eightfold lower than its CC_{50} value (SI = 8). Besides this, it has also showed moderate activity at an EC_{50} of around 0.05 μM against HSV-1 & 2, coxsackie B4, and RSV in HeLa cell culture. At the same EC_{50} value, it was found active against VV, TK HSV, in HEL cell lines; PIV-3, RV-1, SV and PTV in Vero cell culture and ECV (FIPV) and FHV in CRFK cell culture. The 5′-decanoyl derivative **7** has shown some activity against FCV (FIPV) and FHV in CRFK cell culture.

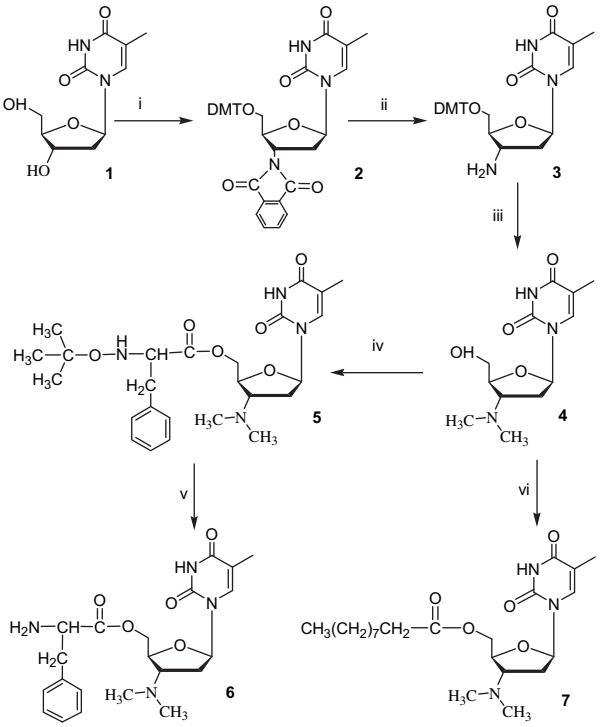
All compounds were also tested against influenza A H1N1/H3N2 subtype and Influenza B in MDCK cell lines. However, none of these compounds was able to inhibit cytopathic effects of Influenza A or B virus at subtoxic concentrations or the highest concentration tested.

It was observed that compounds **6** and **7** only, showed antiviral properties against VSV (EC_{50} 0.03) and FCV (EC_{50} 0.05), respectively. However, the antiviral potency of the prodrug **6** was still significantly lower than that of established antiviral drugs – (S)-DHPA, BVDU and ribavirine against VSV as was evident from its CC_{50} and EC_{50} values shown in Table 1.

Since nucleosides exhibit only modest bioavailability [21], we prepared the prodrug molecules using amino and fatty acids to improve their bioavailability [22,23] and chemical stability. This was done to ensure enhanced cellular uptake and sustained release of drug molecule without compromising its properties [24–27].

The lead compound **4** and its prodrug **5** did not show any antiviral activity, whereas the prodrug **7** showed activity, albeit, too little against FCV and FHV. However, the prodrug **6** showed good activity against VSV with SI value equal to 8. The enhanced reactivity of free amino acid ester prodrug may be attributed to its electronic activation by the positively charged amino terminus at physiological pH, which derives support from the fact that L-amino acid esters enhance nucleoside absorption via human peptide transporters (hPEPT1) of active transport system [28,29]. Furthermore, since bone marrow progenitor cells lack this active transport system for amino acid, prodrug **6** is expected to show reduced toxicity at bone marrow level. Therefore, compound **6** has potential to be developed as an effective antiviral agent against VSV.

The lead compound **4**, being a 2′,3′-dideoxynucleoside, was expected to act as chain terminator and thus, interfere with viral cell metabolism and its prodrugs were designed and synthesized to enhance its cellular uptake. However, this molecule showed almost no anti-HIV activity and rather proved cytotoxic. This might be



i. DMTCl, MsCl, potassium phthalimide, DMAP, TEA, Pyr; ii. methylamine in methanol, HCl, 105°C; iii. HCOOH/ HCHO (40%), 70°C; iv. BOC-phenylalanine, DCC, DMAP, pyr, r.t.; v. TFA/ DCM; Et_3N/DCM, r.t.; vi. $C_9H_{19}COCl$, DMAP, pyr, r.t.

Scheme 1. Synthesis of DMAT (**4**) and its prodrugs (**5**–**7**).

Table 1
Antiviral and cytotoxic effects of compounds **4**–**7**.

Compound	Virus	a	b	c	d	e	f	g	h	i	j	k	l	m	n	o	p	q	r	s	t
	Cell	HeLa	HeLa	HeLa	HEL	HEL	HEL	HEL	HEL	Vero	Vero	Vero	Vero	Vero	CRFK	CRFK	MDCK	MDCK	MDCK	MTT	MTT
4	EC_{50}	>0.37	>0.37	>0.37	>0.37	>0.37	>0.37	>0.37	>0.37	>0.37	>0.37	>0.37	>0.37	>0.37	>0.37	>0.37	>0.37	>0.37	>0.37	>0.46	>0.46
	CC_{50}	>0.37	>0.37	>0.37	>0.37	>0.37	>0.37	>0.37	>0.37	>0.37	>0.37	>0.37	>0.37	>0.37	>0.37	>0.37	–	–	–	–	–
5	EC_{50}	>0.20	>0.20	>0.20	>0.20	>0.20	>0.20	>0.20	>0.20	>0.20	>0.20	>0.20	>0.20	>0.20	>0.20	>0.20	>0.20	>0.20	>0.20	>0.16	>0.16
	CC_{50}	>0.20	>0.20	>0.20	>0.20	>0.20	>0.20	>0.20	>0.20	>0.20	>0.20	>0.20	>0.20	>0.20	>0.20	>0.20	–	–	–	–	–
6	EC_{50}	0.03	>0.05	>0.05	>0.05	>0.05	>0.05	0.03	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.24	>0.24	>0.24	>0.18	>0.18
	CC_{50}	0.24	0.24	0.24	0.24	0.24	0.24	0.24	0.24	>0.24	>0.24	>0.24	>0.24	>0.24	>0.24	>0.24	–	–	–	–	–
7	EC_{50}	>0.23	>0.23	>0.23	>0.23	>0.23	>0.23	>0.23	>0.23	>0.23	>0.23	>0.23	>0.23	>0.23	>0.05	>0.05	>0.23	>0.23	>0.23	>0.15	>0.15
	CC_{50}	>0.23	>0.23	>0.23	>0.23	>0.23	>0.23	>0.23	>0.23	>0.23	>0.23	>0.23	>0.23	>0.23	>0.23	>0.23	–	–	–	–	–
(S)-DHPA	EC_{50}	146	>250	146	–	–	–	–	–	>250	>250	>250	>250	>250	–	–	–	–	–	–	–
	CC_{50}	>250	>250	>250	–	–	–	–	–	>250	>250	>250	>250	>250	–	–	–	–	–	–	–
Ribavirin	EC_{50}	10	30	30	10	50	85	>250	125	95	250	111	>250	50	–	–	7	9	9	–	–
	CC_{50}	>250	>250	>250	>250	>250	>250	>250	>250	>250	>250	>250	>250	>250	–	–	>100	>100	>100	–	–
Brivudine	EC_{50}	–	–	–	0.08	50	2	>250	50	–	–	–	–	–	–	–	–	–	–	–	–
	CC_{50}	–	–	–	>250	>250	>250	>250	>250	–	–	–	–	–	–	–	–	–	–	–	–
Ganciclovir	EC_{50}	–	–	–	0.03	0.03	>100	>100	0.03	–	–	–	–	–	>100	2.6	–	–	–	–	–
	CC_{50}	–	–	–	>100	>100	>100	>100	>100	–	–	–	–	–	>100	>100	–	–	–	–	–
Oseltamivir-carboxylate	EC_{50}	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0.07	1.7	4	–	–
	CC_{50}	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	>100	>100	>100	–	–
Amantidin	EC_{50}	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	45	4		–	–
	CC_{50}	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	>100	>100	>100	–	–
Rimantidin	EC_{50}	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	20	0.8		–	–
	CC_{50}	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	>100	>100	>100	–	–

a = Vesicular stomatitis virus; b = coxsackie virus B4; c = respiratory syncytial virus; d = herpes simplex virus-1(KOS); e = herpes simplex virus-2 (G); f = vaccinia virus; g = vesicular stomatitis virus; h = herpes simplex virus-1 TK-KOS ACV; i = para-influenza-3 virus; j = reovirus-1; k = sindbis virus; l = coxsakie virus B4; m = punta toro virus; n = feline corona virus (FIPV); o = feline herpes virus; p = influenza A H1N1 subtype; q = influenza A H3N2 subtype; r = influenza B; s = HIV-1 ROD; t = HIV IIIB.
EC_{50} = compound concentration (in μM) required to reduce virus yield by 50%, CC_{50} = compound concentration (in μM) required to reduce cell viability by 50%.

Table 2
Drug characteristics of thymidine, AZT and DMAT.

Molecules	M.W.	Mol. vol.	No. of H-acceptors	No. of H-donors	TPSA	log *P*
Thymidine	242.231	207.189	7	3	104.557	−1.433
AZT	267.245	224.063	9	2	134.084	−0.099
DMAT	269.301	245.078	7	2	87.567	−0.771

expected because of introduction of dimethylamino group at 3′-C of ribose moiety, which did not show any interaction with HIV RT during molecular docking experiments. The lead molecule **4** was designed because of its similarity with AZT, but it completely failed in fulfilling the objectives. It was surprising to see that replacement of azido group by dimethylamino group turned the molecule highly toxic.

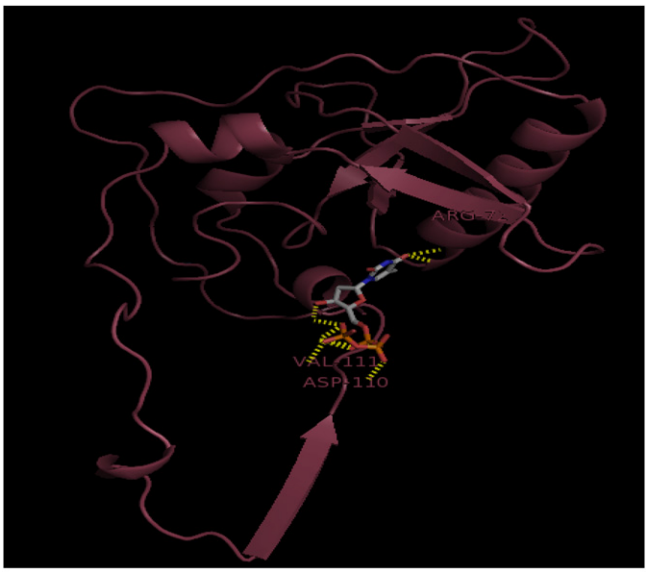
3.2. Molecular docking

The fact that the lead molecule DMAT (and its prodrugs) being a dideoxynucleoside and having structural features similar to AZT, did not show any activity against HIV and rather proved toxic, prompted us to study its interaction with HIV-1 RT.[1] We performed elaborate molecular computational studies using the software Discovery Studio (DS) 2.5. This molecule showed some striking differences with AZT – the approved anti-HIV drug as shown in Table 2. The physiochemical studies suggested that both the molecules – AZT and DMAT, followed Lipinski's rule of five but they have marked differences in their molecular volume, total polar surface area (TPSA) and log *P* values. Similarly, the docking experiment also showed quite different patterns of their interaction with HIV-1 RT (Figs. 2–4). Although DMAT formed one more H-bond with HIV-1 RT than AZT, the crucial interactions as shown by AZT were missing in the case of DMAT. The azido function on AZT formed three H-bonds – one with Arg72 and two with Asp113 (the amino acid constituting the dNTP-binding site), whereas the dimethylamino function on DMAT formed no H-bonds at all. Similarly, the H-bond formed by phosphate moiety of AZT (and TTP as well) with Val111 was missing in the case of DMAT. However, the aglycan part in both the molecules formed two H-bonds with Arg72 through oxo group and "*O*" atom in the sugar ring. The stability of DMATTP–RT complex was also less than that of AZTTP–RT or TTP–RT complexes as is evident from their minimization energies. A comparative account of interaction of TTP, AZTTP and DMATTP and stability of their respective complexes with HIV-1 RT has been shown in Table 3. Thus, the physiochemical and docking studies suggested the possible reasons for inactivity of the lead molecule and its prodrugs.

4. Conclusion

In conclusion, we have synthesized 3′-*N*,*N*-dimethylamino-2′,3′-dideoxythymidine **4** and its 5′-*O*-carboxyl ester prodrug derivatives **5**–**7** and evaluated their antiviral activity against HIV-1 (III_B and ROD strains), HSV-1/2, vesicular stomatitis, parainfluenza-3 virus and many others. It was observed that compounds **6** and **7** only, showed moderate antiviral properties against VSV (EC_{50} 0.03) and FCV (EC_{50} 0.05), respectively. Further mechanistic studies on molecules **6** and **7** are in progress against vesicular stomatitis, feline corona and feline herpes viruses.

The inference drawn from the present studies that for a nucleoside analogue to be an effective NRTI, being a dideoxy derivative is not sufficient enough criterion and rather it must possess the characteristic features too for requisite interactions at the dNTP-binding site of HIV-1 RT enzyme shall definitely help in designing new NRTI molecules.



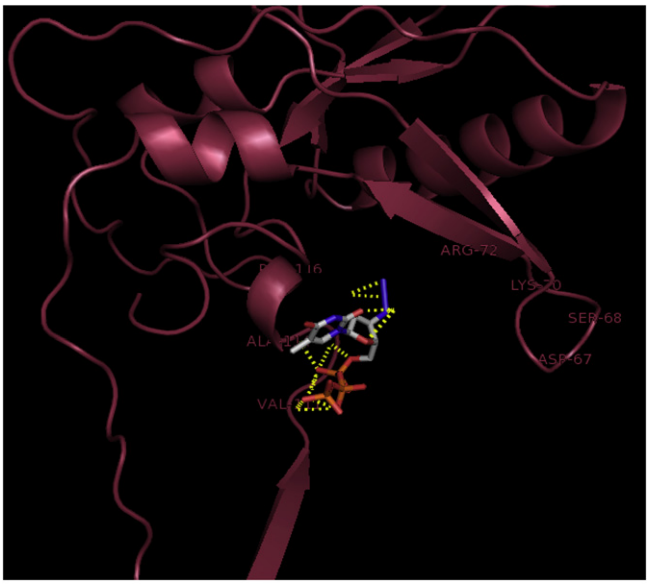
Minimization energy: (-) 61.20318 kcal/mol, Number of H-bonds formed-12
{Phosphate (9) – Val 110, Val 111; C=O of base (2) - Arg 72; 3' OH (1) – Asp 113}

Fig. 2. Interaction of TTP with HIV-1 RT at dNTP-binding site.

5. Experimental

5.1. Chemistry

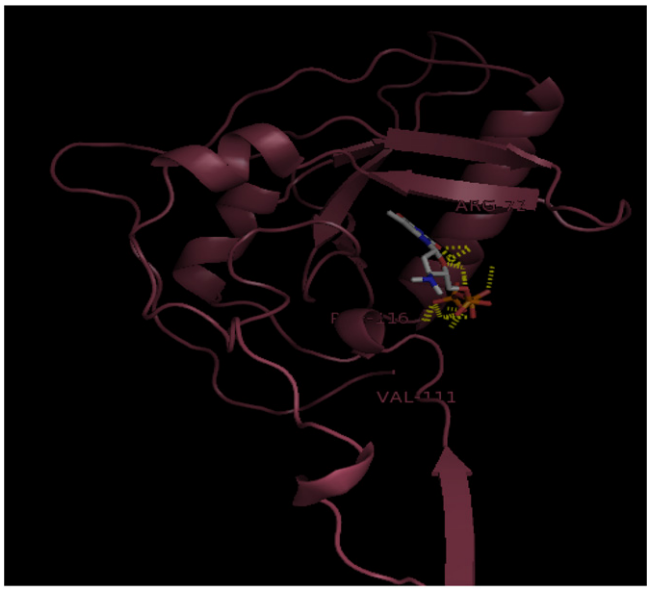
Chemicals were obtained from E. Merck India Ltd, India and Sigma–Aldrich Chemical Company, USA. Melting points were determined on electrothermal apparatus and are uncorrected.



Minimization energy: (-) 63.93809kcal/mol, Number of H-bonds-13
{Phosphate (8) - Val 111, Asp 113; C=O of base (1) - Arg 72; Sugar 'O' (1) – Arg 72; $N=N^{+}=N^{-}$ (3) - Arg 72, Asp 113}

Fig. 3. Interaction of AZTTP with HIV-1 RT at dNTP-binding site.

[1] This facility was not available in the beginning.



Minimization energy: (-) 53.81129kcal/mol, Number of H-bonds-14
{Phosphate (10) – Glu 44, Lys 46, Asp 113; C=O of base (2) - Arg 72;
Sugar ring 'O' (2) – Arg 72}

Fig. 4. Interaction of DMATTP with HIV-1 RT at dNTP-binding site.

Silica gel for TLC and column chromatography (60–120 mesh) was obtained from E. Merck India Ltd. UV measurements were carried out on Hitachi 220S spectrophotometer. HPLC analyses were performed using $250 \times 4.6\ mm^2$ C18 column and solvent system MeOH:H_2O (6:4) at a flow rate of 1 ml/min on 6 AD Binary Gradient Shimadzu HPLC system. ^1H NMR spectra were recorded on DRX 300 MHz instrument using DMSO-d_6 as solvent and TMS as an internal standard. ^{13}C NMR spectra were recorded in $CDCl_3$ on a Varian XL-300 spectrometer operating at 75 MHz. Mass spectra were obtained using a Thermofinnigan TRACE-DSQ Electrospray Ionization (ESI) mass spectrometer. Elemental analyses were carried out on a Perkin–Elmer 240-C analyzer. All solvents were dried and distilled prior to use.

*5.1.1. 5′-O-(4,4′-Dimethoxytrityl)-3′-phthalimidothymidine (**2**)*

Thymidine (2.415 g, 10 mmol) was suspended in dry pyridine (~100 ml) in a 250 mL round bottomed flask and added DMAP (61 mg, 0.05 equivalent), TEA (1.9 ml, 1.4 equivalent) and DMTCl (4.1 g, 1.2 equivalent) and stirred the reaction mixture for 2.5 h. TLC at this point showed complete disappearance of thymidine. The contents were cooled to 0 °C and methanesulfonyl chloride (1.23 ml, 10.89 mmol) was added dropwise. Stirring was continued further for 2 h and the reaction mixture was allowed to warm up to 25 °C. After stirring the reaction mixture for an additional 30 min, the contents were poured into crushed ice/water and the viscous mass so obtained was extracted in chloroform (50 ml) and washed with water (2×10 ml). The organic phase was dried over anhydrous sodium sulfate, filtered and evaporated on a rotary evaporator to obtain the crude product. Silica gel column purification with a linear gradient up to 10% ethyl acetate in hexane, afforded the pure compound, 5′-*O*-(4,4′-dimethoxytrityl)-3′-mesyl-2′,3′-dideoxythymidine, which was refluxed for 12 h with potassium phthalimide (9.0 g) in dimethylformamide (450 ml). The solvent was removed under reduced pressure and the residue extracted with ethyl acetate. The ethyl acetate layer was finally washed with water (3×10 ml), dried over anhydrous Na_2SO_4 and concentrated to get the title compound in crude form (4.72 g, 70%).

*5.1.2. 5′-O-(4,4′-Dimethoxytrityl)-3′-amino-2′,3′-dideoxythymidine (**3**)*

The compound **2** (4.34 g) was stirred with methylamine (16 ml) in methanol at 105 °C for 20 h. The reaction mixture was evaporated to an oily residue, washed with water, dissolved in ethanol (100 ml) and treated with hydrochloric acid to neutralize the residual methylamine. The acidic solution was concentrated under reduced pressure, which resulted in crystallization. The crystals were separated and the residual water and acids were removed by co-evaporation with benzene under reduced pressure from the mother liquor, which was further subjected to crystallization. The crystalline residue was triturated with ethanol and ether. The title compound was obtained as colorless crystals (3.04 g, 87%).

*5.1.3. 3′-N,N-Dimethylamino-2′,3′-dideoxythymidine (**4**)*

5′-*O*-(4,4′-Dimethoxytrityl)-3′-amino-2′,3′-dideoxythymidine (3.04 g, 5 mmol) dissolved in a mixture of 98–100% formic acid (6 ml) and 40% aqueous formaldehyde (6 ml) was refluxed at 70 °C for 20 min to give light orangish syrup. The reaction mixture was concentrated under reduced pressure and partitioned between water and ethyl acetate. Compound **4** (1.15 g, 77%) obtained in water fraction was crystallized with aqueous ethanol: mp 180–180.5 °C; R_f: 0.17 (DCM:MeOH 9.5:0.5); UV(EtOH) λ_{max} 265 nm; C18 HPLC (265 nm) $t_R = 1.47$ min; ^1H NMR (DMSO); δ 1.95 (s, 3H, 5-CH_3); 2.15 (m, 2H, H-2′); 2.27 (s, 6H–N $(CH_3)_2$) ; 2.97 (m,1H, H-3′); 3.64 (m, 2H, H-5′); 4.08 (m,1H, H4′); 5.87 (m, 1H, H-1′); 7.54 (s, 1H, H-6);10.02 (s, 1H, NH, Thy); ^{13}C NMR ($CDCl_3$): δ 15.7, 31.5, 39.8, 55.7, 63.9, 77.2, 78.6, 109.3, 135.8, 152.6, 164.5; ESI-MS *m/z* 269 (M^+). Anal. calcd for $C_{12}H_{19}N_3O_4$: C, 53.50; H, 7.06; N, 15.60; found: C, 53.10; H, 6.92; N, 15.50.

*5.1.4. 5′-(N-α-BOC-L-phenylalanyl)-3′-N,N-dimethylamino-2′,3′-dideoxythymidine (**5**)*

Dicyclohexylcarbodiimide (515 mg, 2.5 mmol) was added to a stirred solution consisting of 3′-*N*,*N*-dimethylamino-2′,3′-dideoxythymidine (269 mg, 1 mmol), DMAP (183.25 mg, 1.5 mmol,) and *N*-α-t-boc-L-phenylalanine (397.95 mg, 1.5 mmol) in pyridine (10 ml) under anhydrous condition. The progress of reaction was monitored by TLC. After the reaction was complete (70–72 h), the separated DCU was filtered off. The filtrate was evaporated to dryness under reduced pressure and the title compound was purified by column chromatography over silica gel using a mixture of ethyl acetate/hexane (8.5:1.5) as eluent. Yield: (269 mg, 62%); mp 97 °C; R_f : 0.46 (DCM:MeOH 9.5:0.5); UV (EtOAc) λ_{max} 265 nm. C18 HPLC (265 nm) $t_R = 2.24$ min; ^1H NMR (DMSO-d_6); δ 1.23 (s, 9H, t-

Table 3
Interaction of Thymidine, AZT and DMAT triphosphates with HIV-1 RT.

Molecule	Total number of H-bonds	Bonds formed with PO_4 unit	Bonds formed with C=O on base	Bonds formed with sugar moiety	Bonds formed with 3′ functional group	Minimization energy (kcal/mol)
TTP	11	8 Val110, 111	1 Arg72	1 Arg72	1 (OH) Asp113	−61.20318
AZTTP	13	8 Val111,	1 Arg72	1 Arg72	3 (N_3) Arg72, Asp113	−63.93809
DMATTP	14	10 Asp113, Glu44, Lys46	1 Arg72	1 Arg72	0 (N–$(CH_3)_2$)	−53.81129

boc); 1.95 (s, 3H, 5-CH_3); 2.13 (m, 2H, H-2′); 2.26 (s, 6H, −N $(CH_3)_2$); 2.95 (m,1H, H-3′); 3.18 (d, 2H, CH_2−amino acid); 3.86 (t, 1H, CH−amino acid); 4.20 (m, 2H, H-5′); 4.58 (m,1H, H-4′); 5.85 (m, 1H, H-1′); 7.26–7.08 (m, 5H, Ar); 7.55 (s, 1H, H-6); 10.05 (s, 1H, NH, Thy); ^{13}C NMR ($CDCl_3$): δ 15.8, 31.6, 33.3, 39.2, 39.7, 55.8, 60.6, 67.2, 74.6, 78.5, 109, 127.8, 128.1, 125.4, 134.8, 140.9, 127.7, 152.2,172; ESI-MS m/z (M^+) 488. Anal. calcd for $C_{25}H_{36}N_4O_6$: C, 61.46; H, 7.43; N, 11.47; found: C, 61.15; H, 7.30; N, 11.38.

5.1.5. 5′-L-Phenylalanyl-3′-N,N-dimethylamino-2′,3′-dideoxythymidine (**6**)

Compound **5** (150 mg, 0.30 mmol) was added slowly to a mixture of 30% TFA and methylene chloride (10 ml). The solution was stirred under anhydrous condition for 30 min. The solvent was removed under vacuum and the residue treated with triethylamine in methylene chloride to afford the title molecule. The product was purified by silica gel column chromatography using ethyl acetate/methanol (4:1) as eluent. Yield: (80 mg, 63%) mp 219–220 °C; R_f : 0.29 (DCM:MeOH 9.5:0.5) UV (EtOAc) λ_{max} 265 nm. ^{1}H NMR (DMSO-d_6): δ 1.94 (s, 3H, 5-CH_3); 2.15 (m, 2H, H-2′); 2.26 (s, 6H, −N $(CH_3)_2$); 2.95 (m,1H, H-3′); 3.17 (d, 2H, CH_2−amino acid); 3.85 (t, 1H, CH−amino acid); 4.22 (m, 2H, H-5′); 4.56 (m,1H, H-4′); 4.65 (s, 2H, $-NH_2$); 5.87 (m, 1H, H-1′); 7.22–7.18 (m, 5H, Ar); 7.54 (s, 1H, H-6);10.03 (s, 1H, NH, Thy); ^{13}C NMR ($CDCl_3$): δ 15.2, 31.5, 39.4, 39.7, 55.2, 60.1, 67.2, 74.1, 78.9, 109.2, 125.7, 127.0, 128.7, 134.1, 140.9, 152.3,172.8, ESI-MS m/z (M^+) 416. Anal. Calcd for C_{42} H_{41} N_7 O_{12}: C, 60.35; H, 4.91; N, 11.73 found: C, 60.14; H, 4.70; N, 11.61.

5.1.6. 5′-Decanoyl-3′-N,N-dimethylamino-2′,3′-dideoxythymidine (**7**)

Decanoyl chloride (0.20 ml, 1 mmol) was added dropwise to an ice-cold stirred solution consisting of the lead compound **4** (269 mg, 1 mmol), DMAP (0.32 g, 2.6 mmol) in pyridine (25 ml) and the reaction mixture stirred overnight at room temperature under anhydrous condition. Reaction mixture was concentrated under reduced pressure and partitioned between ethyl acetate and water. The organic fraction was concentrated to a residue and the product was purified by column chromatography over silica gel using ethyl acetate/hexane (9.5:0.5) as eluent. Yield: (200 mg, 59%) mp 160 °C; R_f : 0.84 (DCM:MeOH 9.5:0.5); UV (EtOAc) λ_{max} 265 nm, C18 HPLC (265 nm) $t_R = 2.84$ min; ^{1}H NMR (DMSO-d_6) δ 0.97 (t, 3H); 1.29 (m, 10H); 1.33 (q, 2H); 1.68 (m, 2H,); 1.93 (s, 3H, 5-CH_3); 2.13 (m, 2H, H-2′); 2.25 (t, 2H,); 2.27 (s, 6H, −N $(CH_3)_2$); 2.97(m,1H, H-3′); 4.21 (m, 2H, H-5′); 4.57 (m,1H, H-4′); 5.85 (m, 1H, H-1′); 7.57 (s, 1H, H-6); 10.04 (s, 1H, NH, Thy); ^{13}C NMR ($CDCl_3$): δ 14.0, 15.8, 23.8, 25.4, 29.7, 30.6, 31.5, 33.6, 39.4, 67.2, 74.9, 109.73, 134.8, 152.5, 164.4, 172.0; ESI-MS m/z 423 (M^+) Anal. calcd for $C_{22}H_{37}N_3O_5$: C, 62.33; H, 8.73; N, 9.91; found: C, 62.19; H, 8.51; N, 9.52.

5.2. Biological evaluation

5.2.1. Antiviral assays

Antiviral assays were based on inhibition of virus-induced cytopathogenicity in various cell cultures following the established procedures [29–32]. The assays were performed against several viruses, viz. HIV, herpes simplex virus type 1 (strain KOS), herpes simplex virus type 2 (strain G), cytomegalovirus (CMV), sindbis virus (SV), parainfluenza virus type-3 (PIV-3) and reovirus type-3, vesicular stomatitis virus (VSV), coxsackie virus (Coxs V) and respiratory syncytial virus (RSV) using CRFK, HEL, HeLa, Vero and CD-4 cell lines.

5.2.2. Virus cytopathogenecity

Cell cultures were prepared in microtiter trays and inoculated with 100 CCID50 (1 CCID50 corresponding to the virus stock dilution that proved infective for 50% of cell cultures). After 1 h virus adsorption to the cells at 37 °C, the residual virus was replaced by cell culture medium (Eagle minimum essential medium supplemented with 3% fetal calf serum) and various concentrations of the test compounds. Virus cytopathogenicity was recorded as it reached completion in the untreated virus-infected cell cultures, i.e., at 1–2 days for vesicular stomatitis virus; 2 days for coxsackie; 2–3 days for vaccinia, herpes simplex type 1 and 2 and sindbis; 4 days for respiratory syncytial virus and 6–7 days for reo and parainfluenza viruses [33,34]. The antiviral activity of compounds is expressed as EC_{50} (the concentration (μM) required to inhibit virus-induced cytopathogenicity by 50%).

5.2.3. Cytotoxicity assays

Cytotoxicity of all compounds was assessed on the basis of two parameters: (i) alteration of normal cell morphology, and (ii) inhibition of macromolecule (DNA, RNA and protein) synthesis. Cytotoxicity (CC_{50}) of compounds was examined by trypan blue exclusion test. To evaluate cytotoxicity, uninfected confluent cell cultures treated with various concentrations of test compounds were incubated in parallel with virus-infected cell cultures prepared in plastic trays containing 24 wells (16 mm diameter; Falcon plastics). After 2 days of incubation at 37 °C in a CO_2 incubator, when the cell cultures were confluent, culture medium was removed from each well and 1 ml of maintenance medium containing serial concentrations of the test compounds was added. For cell control, 1 ml of maintenance medium without compound was added. All cultures were incubated at 37 °C, and after 2 and 7 days of incubation, compounds were withdrawn and the viability of the cells was determined by the trypan blue exclusion method.

5.2.4. Anti-HIV assay

Antiviral screening against HIV-1 (IIIB and ROD strains) was monitored by the efficiency of test compounds to inhibit syncytia formation after HIV infection of MT-4 cells following the MTT method [34–36]. The activity of compounds against HIV-1 was monitored by inhibition of HIV-1-induced cytopathogenecity in MT-4 cells. Briefly, MT-4 cells (3×104 cells per well in 96 well plate) were cultured in microdilution trays in presence of various concentrations of test compounds added immediately after infection with 50% cell culture infective doses of HIV-1. After 5 days of incubation at 37 °C, the number of viable cells was determined by the MTT (3′-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide) method.

5.2.5. Molecular modeling study

Molecular docking of compound **4** and its prodrugs **5**, **6** and **7** into the dNTP-binding site of HIV-1 RT was carried out using DS 2.5 software and PDB code 3E01 of HIV-1 RT. CDOCKER was used and flexible docking was done. Stimulated annealing was done to get the energy-minimized structures of the molecules. The energy-minimized forms were then docked into HIV-1 RT. Visualization was done using PyMol software. All molecular modeling studies were performed on an Intel Pentium 2.99 GHz processor, 1.99 GB RAM with Windows XP professional version 2002 operating system.

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