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Design and synthesis of novel distamycin-modified nucleoside analogues as HIV-1 reverse transcriptase inhibitors

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ABSTRACT

Design and synthesis of nucleoside analogues have persistently attracted extensive interest because of their potential application in the field of antiviral therapy, and its study also receives additional impetus for improvement in the ProTide technology. Previous studies have made great strides in the design and discovery of monophosphorylated nucleoside analogues as potential kinase-independent antiretrovirals. In this work, a series of nucleoside phosphoramidates modified by distamycin analogues was synthesized and evaluated as nucleoside reverse transcriptase inhibitors (NRTIs) in HIV-1-infected MT-4 and CEM cells, including variations in nucleoside, alkyl moiety, and the structure of distamycin analogues. These compounds exhibited modest potency with the EC_{50} value in the range of 1.3- to 6.5-fold lower than their corresponding parent drugs in MT-4 cells, which may be attributed to increasing intracellular availability due to the existence of distamycin analogue with favorable hydrophilic–lipophilic equilibrium. Meanwhile, the length of distamycin analogue was considered and assessed as an important factor that could affect antiviral activity and cytotoxicity. Enzymatic and metabolic stability studies have been performed in order to better understand the antiviral behavior of these compounds. The present work revealed the compounds to have a favorable and selective anti-HIV-1 activity in MT-4 and CEM cells, and helped to develop strategies for design and synthesis of effective monophosphorylated nucleoside analogues, which may be applied to antiretroviral research as NRTIs.

1. Introduction

Nucleoside analogues continue to dominate antiviral therapy and also make a significant contribution to the chemotherapy of cancer, HBV (Hepatitis B Virus), and particularly HIV (Human Immunodeficiency Virus) (Hostetler, 2009; Cihlar and Ray, 2010). Without exception, nucleoside analogues with such activity require phosphorylation *in vivo* to their active nucleotide forms. However, it appears that the first phosphorylation to the 5′-monophosphate is the rate-limiting step, since in many cases the ddN (2′,3′-dideoxynucleoside) derivatives have a poor affinity for nucleoside kinases (Balzarini et al., 1987; Johnson et al., 1988). Negatively charged nucleoside monophosphates cannot be used as therapeutic agents because they are unable to cross cell membranes efficiently, and they are readily dephosphorylated on cell surfaces and in extracellular fluids by non-specific enzymes (Ahmadibeni et al., 2011). ProTide, as a pronucleotide technology proposed by McGuigan et al. (2005, 2006) and Mehellou et al. (2009), successfully circumvents the first step of kinase-mediated activation of nucleosides and thus confers significant antiviral activity on inactive parent nucleosides. During the past two decades, various modifications on aryl/alkyl, ester, and amino acid regions of the ProTide and how these changes affect antiviral activity and metabolic stability were described in many reports to achieve this goal (McGuigan et al., 1996; Congiatu et al., 2005; Derudas et al., 2009; Meneghesso et al., 2012). However, research focusing on how to increase hydrophilic–lipophilic equilibrium of nucleoside monophosphorylated analogues with a consequent increase of intracellular availability could be another key point, and not frequent (Perrone et al., 2007).

Distamycin and its analogues containing three 4-amimo-1-methyl-pyrrole-2-carboxylate residues have been proved to possess natural antiviral and antibiotic activity (Grehn et al., 1983, 1986; Pang, 2004). Also, multiple hydrogen-bond donors interacted with the solvent as well as the existence of C-terminal amidine potentially increase polyamide solubility in water and alter cell permeability (Bremer et al., 2000). Conceivably, it will be a novel and challenging design that distamycin as an important masking group was introduced into monophosphorylated nucleoside to complete ProTide approach, and then the intrinsic molecular

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character of distamycin could endow parent nucleoside with favorable intracellular availability with a consequent further increase of antiviral activity. Fortunately, recent efforts in our laboratory have been inspired by the natural distamycin composed of *N*-methylpyrrole carboxamides, and focused on modification of small molecules with the introduction of distamycin analogues to successfully interfere with gene expression (Li et al., 2008, 2010, 2012). Thus, it could provide substantial experiences in reasonably constructing the distamycin nucleoside conjugates as potential anti-HIV prodrugs.

We here presented the synthesis and biological activity of a series of distamycin-modified nucleoside analogues, including variations in nucleoside, alkyl moiety, length of distamycin analogues, and nitrile/amidine terminus (Fig. 1). All these combinations allowed us to extensively study what extent this difference might reflect the relative efficiency of phosphorylation, which might contribute to the progress of antiviral activity, hydrophilic–lipophilic balance and metabolic stability. This paper described our initial attempts in this regard.

2. Materials and methods

2.1. Experimental chemistry

Unless otherwise noted, all materials were obtained from commercial suppliers and dried and purified by standard procedures. ^{1}H NMR and ^{13}C NMR spectra were measured on a Bruker AV600 spectrometer, and chemical shifts in ppm are reported relative to internal Me_4Si ($CDCl_3$ and DMSO-d_6). MS (ESI) mass spectral data were recorded on a Finnigan LCQDECA mass spectrometer. HRMS data were measured using Bruker APEX IV Fourier Transform Ion Cyclotron Resonance Mass spectrometer. Purities of all target compounds ($\geqslant$98%) were determined by an Agilent 1200 HPLC system with a Agilent Zorbax SB-Aq C-18 column (4.6 × 150 mm), UV detector at 295 nm, mobile CH_3CN/H_2O (0.6%trifluoroacetic acid) = 32–67% and flow rate of 1 mL/min. Polyamides with different lengths were prepared according to our previous work (Li et al., 2010). General procedure and data for the synthesis of target compounds were shown in Supporting Information.

2.2. By-products in Pinner reaction

In order to improve the product yield, it should be pointed out that, effective inhibition of by-products in Pinner reaction must be not neglected. Pinner salt, an important reactive intermediate, firstly generated and then converted to amidine with the introduction of dry HCl(*g*) and NH_3(*g*) in proper order in absolute ethanol. Three main side reactions in the process could occur under different conditions, as shown in Scheme 2. Excess ethanol can cause alcoholysis of Pinner salt to successively get compounds 4 and 5 (orthoester). In the presence of HCl(*g*) and another equivalent amount of ethanol, the orthoester could be further converted into ethyl ester 6 which also directly obtained through Pinner salt hydrolysis. Temperature is also one of the main parameters that affected the reaction. At higher than 0 °C, heat decomposition easily occurred in the mixture and resulted in undesired product amide 3. In addition, strictly controlling gas velocity in low flow was found to be preferable, since excess gas turned the reaction mixture to be complex and more than two products detected by TLC.

2.3. In vitro assays of anti-HIV activity and cytotoxicity

MT-4 cells, a human T4-positive cell line were infected with HIV-1 at the multiplicity of infection (MOI) of 0.1, and HIV-infected MT4 cells were incubated for 1.5 h at 37 °C in a 5% CO_2 incubator. Subsequently, cells were cultured in 96-well microtiter plates in the presence of various concentrations of compounds and aliquots of culture supernatants were removed from the wells on the fifth day after infection for p24 antigen assays. The applied p24 enzyme immunoassay (EIA) was the unmodified kinetic which utilizes a murine mAb to HIV core protein coated onto microwell strips to which the antigen present in the test culture supernatant samples binds. Percent viral inhibition was calculated by comparing the p24 values from untreated infected cells. EC_{50} (representing the concentration of compounds reducing HIV-1 replication by 50%) was determined from the concentration–response curve using the median effect method.

CEM/0 or CEM/TK-cells were suspended at 250,000 cells/mL cell culture medium and infected with approximately 100 $CCID_{50}$ (1 $CCID_{50}$ is the 50% cell culture infective dose) of HIV-1. Then, 100 μL of the infected cell suspension was added to the wells of a 96-well microtiter plate containing 100 μL of an appropriate dilution of the test compounds. After 4 days, giant cell formation was recorded microscopically in the HIV-infected CEM cell cultures.

Cytotoxicity of the conjugate was evaluated in parallel with antiviral activity using the 3-(4,5-dimethylthiazol-1-yl)-2,5-diphenyltetrazolium bromide (MTT) method. It was based on the viability of mock-infected cells, as monitored by the MTT method. CC_{50} (representing the concentration of compounds reducing cell viabil-

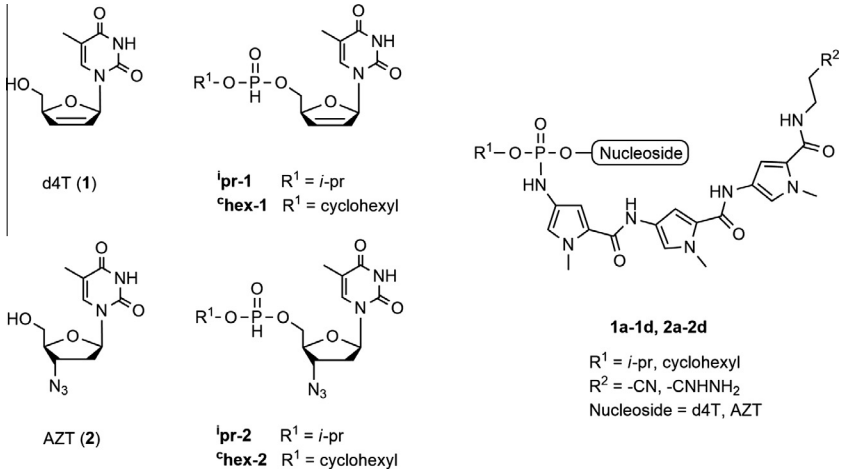
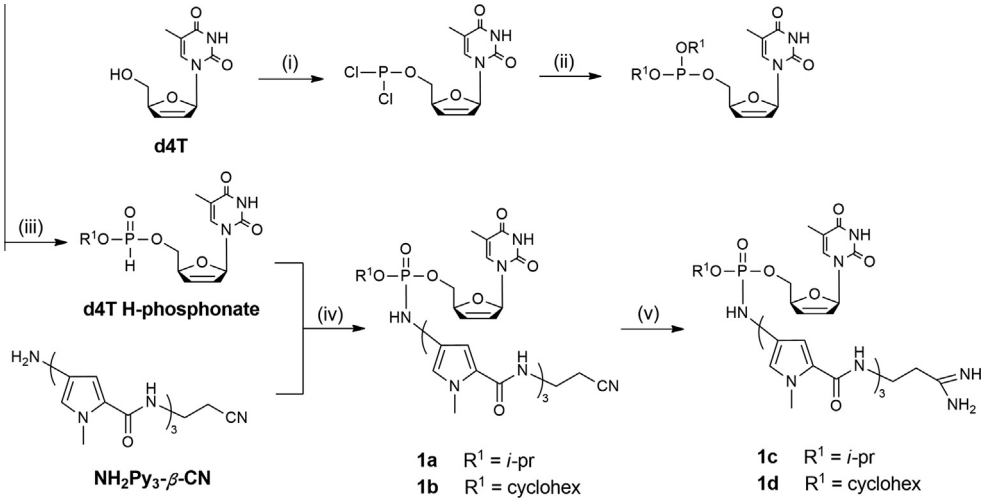
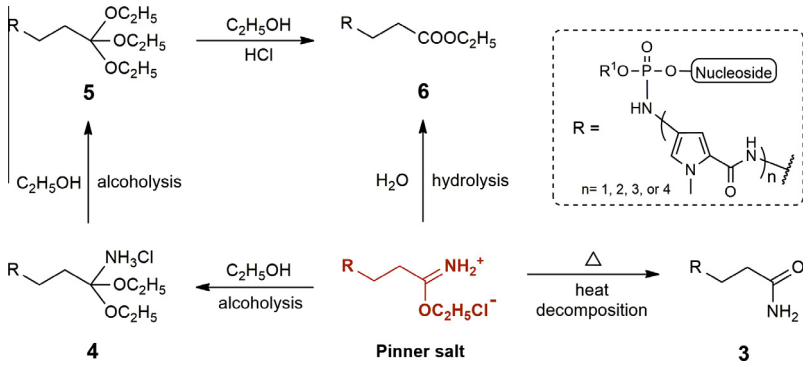


Fig. 1. Structural features of nucleosides and distamycin-modified nucleoside analogues **1a**–**1d** and **2a**–**2d**.



Scheme 1. Reagents and conditions: (i) PCl_3, CH_2Cl_2; (ii) R^1-OH; (iii) Et_3N; (iv) Et_3N/CCl_4, THF/DMF; (v) HCl/EtOH, NH_3(q), EtOH.



Scheme 2. The side reactions in Pinner reaction: hydrolysis, alcoholysis, and heat decomposition.

ity by 50%) was determined from the concentration–response curve using the median effect method.

2.4. Solubility and permeability

Solubility was measured at pH 7.4 by using an HPLC–UV method. Test compounds were initially dissolved in DMSO at 10 mg mL^{-1}. 10 μL of this stock solution was spiked into pH 7.4 phosphate buffer (1.0 mL) with the final DMSO concentration being 1%. The mixture was stirred for 4 h at room temperature and then concentrated at 3000 rpm for 10 min. The saturated supernatants were transferred to other vials for analysis by HPLC–UV. Each sample was performed in triplicate. For quantification, analysis was carried out on Waters 2695 system equipped with a Diode Array Detector. A SB C18 250 × 4.5 mm column was used with an isocratic elution at a flow rate of 1.0 mL min^{-1}. The elution condition was acetonitrile (B) in water (A, with 0.6% trifluoroacetic acid) at 40%. Aqueous concentration was determined by comparison of the peak area of the saturated solution with a standard curve plotted peak area versus known concentrations.

The octanol–water partition coefficient was determined by the traditional shake-flask technique. To 2 mL of a buffer solution (pH 7.4) containing 100 μM of each compound, 2 mL of *n*-octanol, previously saturated with buffer solution, were added. The mixture was shaken for 40 min, centrifuged and the aqueous and organic phases were separated. Samples (10 μL) were injected directly into HPLC (Waters 2695), and test conditions were the same as solubility assay. The partition coefficient (log *P*) was calculated according to the following equation:

$$\log P = \log \frac{[\text{drug}]_o}{[\text{drug}]_{aq}}$$

where $[drug]_o$ is the drug concentration in the organic phase and $[drug]_{aq}$ is the drug concentration in the aqueous phase. In addition, Clog *P* values were calculated with a software package the Molecular Operating Environment (MOE).

2.5. In vitro metabolic stability

Pooled human liver microsomes (HLM) were purchased from Sigma–Aldrich. An incubation mixture with a final volume of 0.1 mL consisted of human liver microsomes in 50 mM KH_2PO_4–K_2HPO_4 phosphate buffer (pH 7.4) and 0.5 μM test compound. The concentration of human liver microsomes was 0.4 mg mL^{-1}. Reactions were started by adding 80 μL of NADPH (final concentration of 1.0 mM). The mixture was incubated at 37 °C for 0 and 30 min. The reaction was terminated by the addition of acetonitrile equivalent to the volume of the reaction mixture. All incubations were made in triplicate. Analysis was carried out on Waters 2695 equipped with a Diode Array Detector. A SB C18 250 × 4.5 mm column was used with a gradient elution at a flow rate of 1.0 mL min^{-1}. The initial elution condition was acetonitrile (B) in water (A, with 0.6% trifluoroacetic acid) at 30%. The concentration of B increased linearly to 90% over 30 min. The mobile phase was

then returned to the initial condition and re-equilibrated for 3 min. The column effluent was analyzed using a Bruker ESQUIRE-LCTM ion trap spectrometer equipped with a gas nebulizer probe. The ion source was operated at 300 °C with the capillary voltage at 11 V. Nitrogen was used as drying gas at a flow rate of 4 L/min.

For metabolic stability determinations, chromatograms were analyzed for parent compound disappearance from the reaction mixtures. The parent compound peak area in the 0 min incubation sample was considered to be the 100% value and parent compound levels were expressed as percent (%) parent remaining.

2.6. Enzymatic assays

5 mg of the appropriate nucleoside analogue was first dissolved in 200 μL of DMSO-d_6 and then 400 μL of Tris buffer solution (pH = 7.4) was added. A ^{31}P NMR was conducted at this stage to see the peaks of the compound in DMSO-d_6 and Tris buffer, so that it acted as a reference (t = 0 h).To this mixture was added 0.3 mg of carboxypeptidase A (purchased from Sigma–Aldrich, >50 units mg^{-1}, EC number 3.4.17.1) that had already been dissolved in 200 μL of Tris buffer. ^{31}P NMR of the reaction mixture was then carried out every 1 h for 24 h at 25 °C.

3. Results and discussion

3.1. Synthesis

Depicted in Scheme 1 is our synthetic route developed to allow for efficient introduction of distamycin moiety on the monophosphorylated d4T through Atherton-Todd reaction under mild conditions. The synthesis of d4T monophosphate started with treatment of d4T with phosphorus trichloride in CH_2Cl_2, to give nucleotide dichloro-phosphate in excellent yield, which was treated in the mixed solution of the corresponding alcoholysis reagent and triethylamine to provide the key intermediate. Another intermediate **NH_2Py_3-β-CN** containing three monomers was synthesized according to our previous similar work (Li et al., 2010). Subsequently, the active P-H from monophosphorylated d4T and NH_2 from distamycin backbone were linked through Atherton-Todd reaction in the presence of tetrachloromethane as catalyst to obtain distamycin-modified d4T with nitrile-terminal, **1a** and **1b**. Direct Pinner reaction of these nitrile-terminal products successfully achieved conversion from nitrile to amidine group, and afforded **1c** and **1d** with moderate yields. Following the same procedure, treatment of nucleoside AZT gave the corresponding compounds **2a**–**2d**.

3.2. Anti-HIV activity and cytotoxicity

All compounds were evaluated for anti-HIV-1 activity and cytotoxicity in human lymphocyte MT-4 and CEM cells, and tests were also conducted in thymidine kinase-deficient CEM cells (Table 1). Both d4T (**1**) and its monophosphorylated derivatives **^{i}pr-1** and **^{c}hex-1** as positive controls exhibited proper potencies and toxicities against HIV-1, with EC_{50} values of 0.27–4.3 μM and CC_{50} values of 74–245 μM in MT-4 and CEM/0 cells, which were consistent with the data previously reported (Pokrovsky et al., 2001). The isopropyl distamycin phosphoramidate (**1b**) with a EC_{50} value of 0.08 μM showed 3.3- and 3.1-fold inhibitory against HIV-1 in MT-4 cell than its parent compounds **1** and **^{i}pr-1** respectively, accompanied with a increasing cytotoxicity (CC_{50} = 98 μM, vs >245 μM for **^{i}pr-1**) due to the presence of distamycin moiety. So, a similar selectivity index (SI) of 1225 was obtained compared to that of **^{i}pr-1** (1448), but significantly higher than that of **1** (274). For the cyclohexyl ester derivatives, it is worth pointing out that the parent compound **^{c}hex-1** having the bulky cyclohexyl group as ester moiety did not show ideal activity (EC_{50} = 1.1 μM, in MT-4 cell), however, **1d** via modification of distamycin showed a 6.5-fold increase in anti-HIV potency, being active at 0.17 μM. As expected, favorable permeability of distamycin derivative could weaken steric-hindrance influence and contribute to intracellular availability, resulting in the increasing of antiviral activity. Additionally, to better demonstrate function of distamycin moiety, the anti-HIV-1 activity of nitrile-terminal **1a** and **1c** were evaluated and results displayed ~2.0-fold lower activity than their corresponding amidine derivatives, suggesting that positively charged amidine terminus may influence the realization of intact membrane permeability. This correlated with their CC_{50} values of 137 μM for **1a** and 113 μM for **1c** recovered from high cytotoxicity.

Striking differences were found for the antiviral activity of tested compounds in HIV-1-infected CEM/TK- cells. Compound **1** proved feebly active against HIV-1 replication in CEM/TK- cells at a concentration as high as ~60 μM. In contrast, the analogues **1a**–**d** showed markedly inhibitory to HIV-1 in CEM/TK- cells. Their

Table 1
Anti-HIV-1 activity and cytotoxicity of distamycin-modified nucleoside conjugates.

Compds.	Nucleoside	R^1	R^2	EC_{50}[a] (μM)			CC_{50}[b] (μM)		SI[c] (MT-4)
				MT-4	CEM/0	CEM/TK-	MT-4	CEM/0	
1	d4T	H	–	0.27	0.28	57.8	74	184	274
^{i}pr-1	d4T	*i*-pr	–	0.25	0.33	5.7	>287	>200	>1148
^{c}hex-1	d4T	Cyclohex	–	1.1	4.3	34.9	>245	>200	>223
1a	d4T	*i*-pr	-CN	0.16	1.12	0.88	137	188	856
1b$_2$	d4T	*i*-pr	-CNHNH$_2$	0.08	0.65	1.07	98	133	1225
1c	d4T	Cyclohex	-CN	0.40	1.39	1.31	113	192	282
1d	d4T	Cyclohex	-CNHNH$_2$	0.17	0.43	0.77	76	120	447
2	AZT	H	–	0.022	0.031	>100	46	>200	2091
^{i}pr-2	AZT	*i*-pr	–	0.026	0.043	3.25	>182	>200	>7000
^{c}hex-2	AZT	Cyclohex	–	0.005	0.032	2.13	>161	>200	>32,200
2a	AZT	*i*-pr	—CN	0.013	0.065	1.15	82	177	6308
2b	AZT	*i*-pr	—CNHNH$_2$	0.007	0.078	1.32	53	89	7571
2c	AZT	Cyclohex	—CN	0.004	0.011	2.19	73	121	18,250
2d	AZT	Cyclohex	—CNHNH$_2$	0.002	0.011	0.97	42	156	23,500
NO_2Py_3-β-CN	–	–	—CN	>100	>100	ND[d]	124	145	ND
NO_2Py_3-β-CNHNH$_2$	–	–	—CNHNH$_2$	>100	>100	ND	55	112	ND

[a] Representing the concentration of compounds reducing HIV-1 replication by 50%.
[b] Representing the concentration of compounds reducing cell viability by 50%.
[c] Selectivity index, SI = CC_{50}/EC_{50}.
[d] Not detection.

EC_{50} values varied between 0.77 and 1.31 μM. Thus, these analogues were at least 44- to 75-fold more effective than **1** in inhibiting HIV-1 replication in CEM/TK- cells. This indicated that a successful bypass of the first phosphorylation step can turn d4T from an inactive compound to a moderately active anti-HIV-1 agent.

As for AZT, antiviral activity and cytotoxicity of three control compounds against HIV-1 were tested, presenting EC_{50} values of 0.022, 0.026 and 0.005 μM for **2**, **^{i}pr-2** and **^{c}hex-2**, respectively. Compared to d4T assay, the similar trend in antiviral potency of **2a**–**2d** was observed via the modification of distamycin analogues, namely slightly increasing activity and cytotoxicity. Although cyclohexyl phosphoramidate **2d** exhibited more favorable inhibitory activity against HIV-1 (EC_{50} = 0.002 μM, in MT-4 cell) compared to **^{c}hex-2**, the considerable cytotoxicity with CC_{50} value of 42 μM drove the selectivity index (23500) down remarkably. In addition, AZT was >3200-fold less active in CEM/TK- than CEM/0 cells, while **2a** and **2b** were only moderately (~17-fold) less inhibitory to HIV-1 replication in CEM/TK- cells than CEM/0 cells, thus confirming the ability of phosphoramidates to deliver AZT monophosphate into cells. As to the influence of distamycin terminus, no striking distinction in activity data was observed between nitrile- and amidine-terminal compounds.

As promoties controls, distamycin analogues **NO_2Py_3-β-CN** and **NO_2Py_3-β-$CNHNH_2$** were devoid of anti-HIV-1 activity at 100 μM in MT-4 and CEM cells, whereas they were cytostatic at CC_{50} of 145 and 112 μM, respectively.

3.3. Structural effect of distamycin on antiviral activity

Carboxamide in each unit contributes available hydrogen-bond donor to interact with solvent, which could provide the substantial basis for increasing aqueous solubility and intracellular availability with a consequent increase of antiviral activity. The problem that influence of amount of *N*-methylpyrrole carboxamide on potential biological activity can be naturally put forward. Here, polyamides containing different amount of *N*-methylpyrrole unites as distamycin analogues were constructed and linked to alkyl monophosphorylated nucleoside, and antiviral data were shown in Table 2. Both **$1b_2$** and **$2d_2$** (n = 2) presented sharply decreased inhibitory potency with EC_{50} values of 0.20 μM and 0.018 μM in MT-4 cells respectively compared to those with intact distamycin, while monocarboxamides **$1b_1$** and **$2d_1$** (n = 1) merely showed initial antiviral activity and cytotoxicity as with their parent compounds. It could be concluded that an integrated distamycin system played a vital role in achieving improvement of biological activity. However, two tested compounds containing four carboxamides displayed unusual results far below the expected, with EC_{50} values of 1.63 μM for **$1b_4$** and 0.07 μM for **$2b_4$**, respectively. The suitable size of masking molecules as an important factor should be considered, since bulky backbone may inhibit the original penetrating ability of distamycin analogues and thus reduce intracellular availability.

3.4. Permeability and aqueous solubility

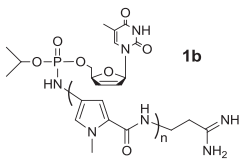
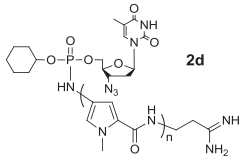
Modification at phosphonate of nucleotide with distamycin analogues expected to improve intracellular availability of the drugs. It can increase the lipophilicity that may lead to potential improvement of intracellular transportation of the compounds. Accordingly, the log *P* values obtained in the present study (Table 3) indicated the modification by distamycin analogues facilitated lipophilic property compared to their parent nucleosides. For instance, compound **1c** (log *P* 1.01; EC_{50} 0.40 μM) exhibited larger log *P* and a 3-fold increase in anti-HIV-1 activity in comparison to parent compound **^{c}hex-1** (log *P* 0.72; EC_{50} 1.1 μM). Compound **2a** (log *P* −1.09; EC_{50} 0.013 μM) showed a 2-fold enhancement in anti-HIV-1 activity compared to **^{i}pr-2** (log *P* −1.12; EC_{50} 0.026 μM). Interestingly, change at distamycin terminal with amidine instead of nitrile group enhanced hydrophilic property, and also further improved anti-HIV activity. For example, amidine-terminal compounds **1d** (log *P* 0.67; EC_{50} 0.17 μM) and **2b** (log *P* −1.58; EC_{50} 0.007 μM) presented lower log *P* and increased anti-HIV-1 activity than their nitrile-terminal compounds **1c** and **2a**. From these data, it can be concluded that antiviral activity of the phosphoramidate prodrugs was strongly dependent on proper hydrophilic–lipophilic equilibrium by promoieties, rather than enhancement of single factor.

Aqueous solubility of the tested compounds was measured (Table 3) and observed that all the distamycin-modified prodrugs also presented favorable equilibrium between oil and water phases. The log *S* ranging from −1 to −5 provided a potential possibility as drugs (Jorgensen and Duffy, 2000). By the introduction of distamycin moieties, compounds **1a**–**d** and **2a**–**d** (log *S* from −2.73 to −3.67) showed more lipophilic than their parent compounds, while **^{i}pr-** compounds exhibited a markedly increased log *S* compared to **^{c}hex-** ones, suggesting increased aqueous solubility. These results were consistent with the permeability assay (log *P*) above.

3.5. Metabolic stability

We next turned our attention to metabolic stability of these nucleoside analogues, which was evaluated by a human liver microsomes incubation assay (Table 3). Compounds **1a**–**d** were

Table 2
Anti-HIV-1 activity and cytotoxicity of **1b** and **2d** with different amount of *N*-methylpyrrole carboxamide.

Compds.	n	EC_{50} (μM)			CC_{50} (μM)		SI (MT-4)
		MT-4	CEM/0	CEM/TK-	MT-4	CEM/0	
 **1b**	1	0.22	1.87	2.13	223	>200	1013
	2	0.20	1.89	2.01	179	>200	895
	3	0.08	0.65	0.17	98	133	1225
	4	1.63	5.33	ND[a]	182	>200	112
 **2d**	1	0.021	0.152	0.133	193	>200	9190
	2	0.018	0.201	0.122	166	>200	9222
	3	0.002	0.011	0.009	42	156	23,500
	4	0.07	ND	ND	221	>200	3157

[a] Not detection.

Table 3
Aqueous solubility, permeability and metabolic stability of nucleoside analogues.

Compds.	log *P*		log *S*		Stability[b]
	Expt.	Calc.[a]	Expt.	Calc.[a]	% remaining
^{i}pr-1	−0.21	−0.17	−2.31	−2.29	52
^{c}hex-1	0.72	1.01	−3.30	−3.11	45
1a	−0.09	−0.06	−2.85	−3.01	72
1b	−0.24	−0.33	−3.05	−3.12	83
1b$_1$	−0.83	−0.88	−2.01	−2.20	44
1b$_2$	−0.55	−0.60	−2.37	−2.66	68
1b$_4$	−0.07	−0.06	−3.07	−3.42	85
1c	1.01	1.12	−3.49	−3.83	67
1d	0.67	0.85	−3.67	−3.93	77
^{i}pr-2	−1.12	−1.23	−1.86	−2.15	48
^{c}hex-2	−0.50	−0.33	−2.85	−3.31	31
2a	−1.09	−1.12	−2.73	−2.88	65
2b	−1.58	−1.39	−2.95	−2.98	71
2c	−0.15	−0.06	−3.52	−3.69	54
2d	−0.30	−0.21	−3.58	−3.80	69
2d$_1$	−0.67	−0.76	−2.85	−2.87	34
2d$_2$	−0.55	−0.49	−3.17	−3.33	63
2d$_4$	−0.09	−0.06	−3.82	−4.10	75

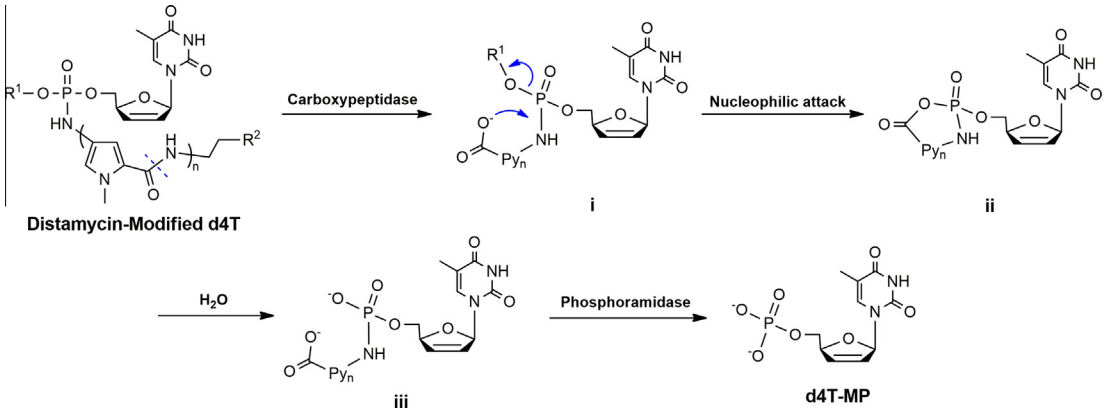
[a] Calculated from a software package the Molecular Operating Environment (MOE).
[b] Human liver microsome incubation assay, data from at least two experiments in parallel.

found to be metabolically stable with 67–83% of their parent compounds. Further, replacing the nitrile group of (**1a** or **1c**) with amidine (**1b** or **1d**) obtained good metabolic stability, but with poor permeability (lower log *P*). A similar result was found in the distamycin-modified AZT series. Attempts to modulate the length of distamycin moieties through *N*-methylpyrrole unit diminished the metabolic stability to some extent (**1b$_{1-4}$** and **2d$_{1-4}$**). Compound **1b$_1$** showed lower metabolic stability and higher log *P* compared to the other three kinds. These results indicated that increasing lipophilicity could be correlated with decreasing stability, and might be also due to increased steric hindrance through distamycin moiety so that the phosphamide would be less available to metabolic enzymes.

3.6. Enzymatic studies

The mechanism of activation of the ProTides involved a first enzymatic activation step mediated by carboxypeptidase-type enzyme(s), which hydrolyzed the amide of the polyamide moiety (Scheme 3). To probe the activation of the nucleosides ProTides to the monophosphorylated form inside cells, we performed an enzymatic study using carboxypeptidase A following the conversion by ^{31}P NMR. On the basis of the favorable activity against HIV-1 and metabolic stability in human liver microsomes, compounds **1b** and **2d** were considered for the evaluation. The assay was carried out by dissolving the compounds in DMSO-d_6 and Tris buffer (pH = 7.4), incubating with the enzyme and recording a blank for each sample before the addition of the enzyme.

In the case of compound **1b** (Fig. 2), the experiment showed a fast hydrolysis of the starting material (δ_p = 9.98 and 9.81, two diastereoisomers) to the intermediate type **i** (Scheme 3) (δ_p = 6.25). This intermediate was then processed to a compound of type **iii**



Scheme 3. Proposed metabolic activation route for d4T phosphoramidates.

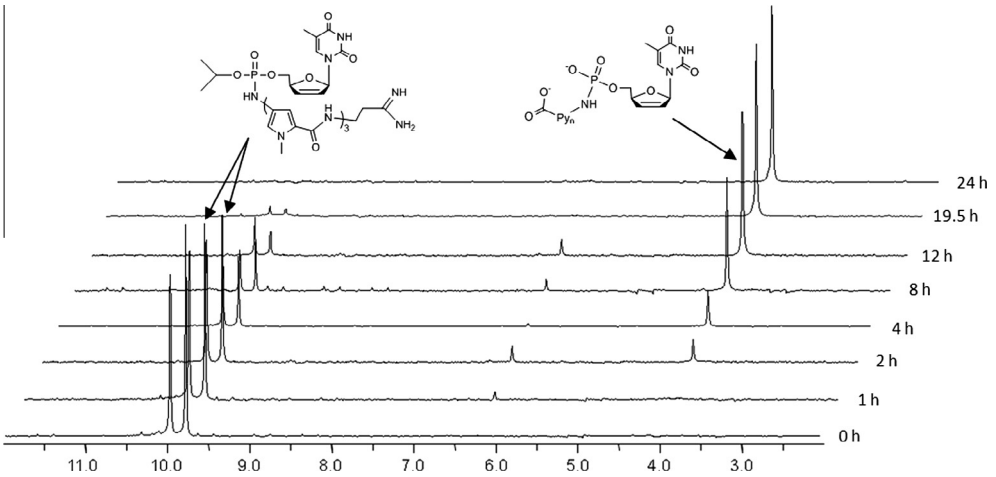


Fig. 2. Carboxypeptidase-mediated cleavage of compound **1b**, monitored by ^{31}P NMR.

(δ_P = 4.01) through the putative intermediate **ii**, which was not detected by ^{31}P NMR. Interestingly, compared with previous work (Derudas et al., 2009; Mehellou et al., 2009), the chemical shift of P changed in the opposite trend due to different electronic effects of substitute groups. In the case of compound **2d**, the experiment showed a similar result in Fig. S1 (see Supporting Information). These results supported the need of activation of nucleoside ProTide in order to deliver the nucleoside monophosphate metabolite. The enzymatic assay correlated well with the in vitro anti-HIV data.

4. Conclusion

In summary, we herein reported the synthesis and antiviral activity of a series of nucleoside monophosphates modified by alkyl moiety and distamycin analogues. Detailed effects on inhibitory activity and cytotoxicity against HIV-1 in MT-4 and CEM cells were evaluated for variations in nucleoside, alkyl ester as well as structure of distamycin analogues. Owing to the introduction of distamycin skeleton, most of tested compounds exhibited favorable antiviral activities with the EC_{50} value in the range of 1.3- to 6.5-fold lower than for their corresponding controls. The increased inhibitory activity against HIV-1 could be attributed to the proper hydrophilic–lipophilic equilibrium of distamycin promoieties. That was not to say that distamycin analogues with any length have the ability to improve antiviral activity of parent compounds via modification, on the contrary, the bulky skeleton may affect cell penetration and reduce biological activity. In addition, enzymatic studies described probable metabolic activation route for delivering nucleoside monophosphate metabolite, and also provided sufficient evidence of the potential of these new compounds as NRTIs. The existence of distamycin analogues inevitably increased cytotoxicity to various degrees, but the current results were still encouraged to design new anti-HIV compounds based on distamycin-modified nucleoside analogues, which will open up interesting and novel perspectives to optimize existing nucleoside drugs and/or to develop new modalities for the treatment of HIV infections.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.antiviral.2013.12.002.

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