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Synthesis and biological evaluation of diastereoisomerically pure *N,O*-nucleosides

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ABSTRACT

Several *N,O*-nucleosides have been synthesized in good yields by direct 1,3-dipolar cyclization methodology, in the absence of solvent. A remarkable *cis* stereoselectivity (de 98%) was observed by tuning the substituents on the nitrone moiety. A good number of these *N,O*-nucleosides have been evaluated for cytotoxic activity against selected cellular lines. Some of the tested compounds have proven to be potential antiproliferative drugs.

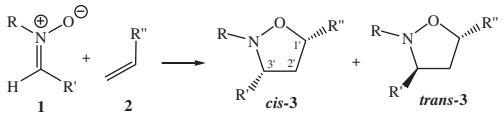
1. Introduction

Many strategies for the preparation of modified nucleosides have been reported in last decades in response to the pressing need of new treatments against virus infections. In a recent approach to these derivatives the ribose unit has been replaced by either carbo- or heterocyclic rings, being *N,O*-containing moieties, that is, isoxazolidine and isoxazoline nucleosides, particularly promising.[1] This was the case of the substituted isoxazolidine pyrinodemin-A which displayed cytotoxicity, of isoxazolidinium salts used in tumor therapy[2] and of isoxazolidines bearing an allylic oxygen active against a number of human cancer cell lines.[3] Furthermore, phosphonated carbocyclic 2′-oxa-3′-aza nucleosides showed powerful inhibiting activity toward the reverse transcriptase of the human retrovirus T-cell leukemia/lymphotropic virus type 1.[4,5]

The synthesis of isoxazolidinyl nucleosides is usually carried out using a classical 1,3-dipolar cycloaddition that represents the most successful protocol for the construction of biologically active derivatives as the already mentioned modified isoxazolidines, amino acids, β-lactams, amino carbohydrates, and alkaloids.[6] In 1,3-dipolar cycloadditions to isoxazolidines, the dipolarophiles are usually alkenes, whereas dipoles are represented by suitable nitrones. The regioselectivity and stereoselectivity of this reaction has been explained by frontier molecular orbital theory. In particular, the cycloaddition of nitrones with mono-substituted electron-rich alkenes involves a dominant $HOMO_{alkene}$–$LUMO_{nitrone}$ interaction, as a consequence of an inverse electron demand (IED).[7,8] Several investigations, including theoretical studies, confirmed that this reaction is characterized by almost exclusive *ortho* regioselectivity i.e. formation of 1′-substituted isoxazolidines **3**-type only, while the *cis–trans* selectivity depends on different factors as the possible interconversion between *E/Z* isomers of **1**[9,10] and the bulk of the substituents present on both nitrone and alkene (Scheme 1).

Our contribution to this field[11,12] has been devoted toward the synthesis of modified nucleosides based on the *N,O*-heterocyclic ring **6**, obtained through microwave irradiated[13–15] direct cyclization of suitable nitrones **4** and unprotected vinylated nucleobases **5** (Scheme 2). The 1,3-dipolar cycloadditions were conveniently carried out in environmentally acceptable conditions as the absence of solvent and the quantitative recover/recycle of unreacted **4**, present in stoichiometric excess.

In this paper we describe the synthesis of a new class of *N,O*-nucleosides obtained in high diastereoisomeric *cis–trans* excess,



Scheme 1. Synthesis of *N,O*-nucleosides via 1,3-dipolar cycloaddition of nitrones and substituted alkenes.

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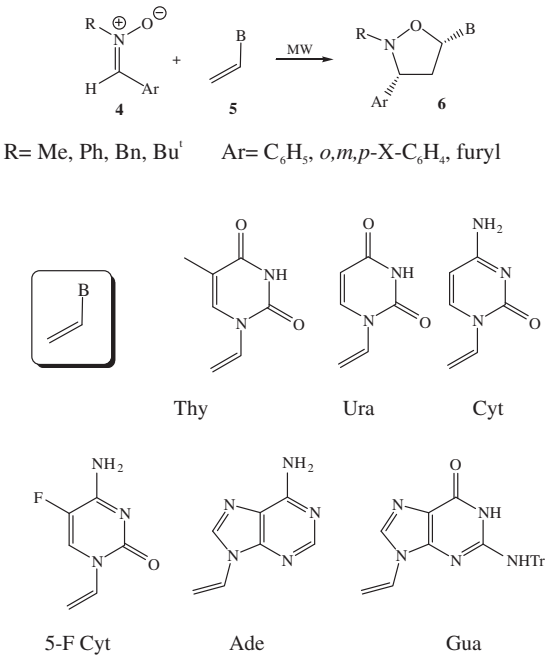


Scheme 2. Direct uncatalyzed synthesis of *N,O*-nucleosides under microwave conditions.

by tuning the substituents on the nitrone moiety. The nitrones used in this investigation, in particular those possessing the *C*-aryl *N*-*tert*-butyl structures, were proved to possess neuroprotective properties.[16–18] The obtained *N,O*-nucleosides have been evaluated for cytotoxic activity against lymphoblastoid cell lines (LCL), in vitro EBV-transformed B lymphocytes, JiJoye cells and Jurkat cells. Some of the tested compounds have proven to be potential antiproliferative drugs.

2. Chemistry

The strategy of the synthetic approach is fast and simple consisting in the direct reaction of the selected nitrone and the unprotected vinyl nucleobase in the absence of solvent and/or catalyst (Scheme 2). As shown in Table 1, the cycloadducts are formed in good yield and with a remarkable *cis–trans* selectivity, in some cases higher than 99:1 (de 98%).[19] The stereoselectivity of the reaction may be predicted taking into account the possible geometries of approach of the two reacting species.[8] The obtainment of the *cis* adduct is explained by invoking either an *exo* approach of the alkene to the (*Z*) nitrone isomer or an *endo* approach to the (*E*) nitrone isomer (Scheme 3). The corresponding opposite parallel may be done in the case of *trans*-cycloadducts.

The ^{1}H NMR analysis, carried out on the different nitrones, confirmed that *N*-*tert*-butyl, aryl derivatives exist almost exclusively as (*Z*)-isomers. On the other hand, and as explained in details in next paragraphs, the *cis–trans* ratio is in favor of the *cis* cycloadduct, thus proving that the approach of the diene–dienophile couple for this cycloaddition is predominantly of (Z)-*exo* type (path **a** of Scheme 3 and entries 1–7 of Table 1). In contrast, with *N*-methyl, *N*-phenyl, and *N*-benzyl nitrones either the formation under microwave conditions of small quantities of (*E*) isomers by possible *E/Z* interconversion, or a significant presence of a second reaction channel for the diene-dienophile approach give account for the formation of minor amounts of *trans*-cycloadducts. In order to discriminate between the two possibilities we have carried out specific ^{1}H NMR and NOESY experiments on *C*-phenyl-*N*-methyl nitrone **4**. Variable-temperature NMR spectra showed the presence of the sole (*Z*)-isomer, even after heating at 80 °C for 24 h[10] or after MW-irradiation of the nitrone for 10 min, thus excluding an *E/Z* isomerisation. Consequently the formation, together with the *cis* product, of minor amounts of *trans*-cycloadduct may be explained by the occurrence of a second reaction pathway for the diene-dienophile approach, that is, the (*Z*)-*endo* path **d**. This latter reaction channel is not active in the case of bulky *N*-substituents due to a disfavored transition state (TS 1) if compared with the approach of smaller *N*-alkyl nitrone derivatives to the dienophile (TS 2), Figure 1.

The proton assignment of the different cycloadducts was confirmed by ^{1}H NMR, COSY, and NOESY spectra, whereas the stereochemical assignment has been performed by NOE experiments carried out on both diastereoisomers of 4′-aza-4′-(*N*-methyl)-3′-phenyl-2′,3′-dideoxycytidine **6n** (Fig. 2). For the major isomer the NOE measurements showed that irradiation of H_A increased the resonances of protons H_D of 1.5% and that of proton H_B of 4%, unambiguously indicating a *cis* relationship of these protons. On the other hand, the irradiation of H_A of the minor isomer showed an increase of ca. 3% on proton H_B and no NOE effect on proton H_D, thus confirming a *trans* relationship of these protons.

The *cis–trans* ratio of the *N,O*-nucleosides has been further confirmed by HPLC analysis. It is interesting to note that for all the mixtures of stereoisomers examined the minor diastereoisomer, possessing *trans* stereochemistry, elutes earlier than the major *cis* one, on a Jupiter 10 μ C-18 column. Although these findings should not lead to a direct diastereoisomer configuration assignment,[20] they may help in the definition of the stereochemical course of the reaction and in the one-step separation/purification of the isomers, especially for biological assays.[21]

3. Biological assays

Most of the *N,O*-nucleoside derivatives prepared according to this procedure were evaluated by in vitro assays for their antiproliferative activity against human lymphoblastoid cell lines (LCL), JiJoye cells, an EBV-positive Burkitt cell line[22] and Jurkat cells, a human T-cell lymphoblast-like cell line.[23,24] Lymphoblastoid cell line were obtained by the in vitro infection of B lymphocytes purified from donor S.C. with supernatants of the EBV producer B 95.8 cell line.[25] Cells were treated with the test compounds at concentrations ranging from 1 to 100 μM, for 72 h. Growth inhibition induced by the tested compounds was assessed by the 3-(4,5-dimethylthiazolyil-2)-2,5-diphenyltetrazolium bromide (MTT) assay. The pertinent results are collected in Table 2. The results are expressed in IC_{50} values of the indicated compounds tested against the three different cell lines.

Most of the newly synthesized compounds posses an antiproliferative activity, being derivatives **6e, 6m**, and **6o** particularly efficient in inhibiting cell proliferation. Figure 3 showed the activity of these *N,O*-nucleosides over a three days period range. The *o*-chloro compound **6m** and the *N,O*-nucleoside **6o** demonstrated the better capacity to inhibit cell proliferation of B- and T-derived cell lines. To note that a previously reported ADF derivative is characterized by low cytotoxicity and induction of high levels apoptosis on lymphoid and monocytois cells,[5a] whereas an *o*-chloro isoxazolidine is converted to the corresponding β-lactam, class of compounds possessing unique biological activity.[26]

4. Conclusions

N,O-nucleosides possessing different nucleobases (Thy, Ura, F-Ura, Ade, Cyt) in position 1′ have been synthesized in good chemical yield and remarkable stereoselectivity by 1,3-dipolar

Table 1
Solvent-free 1,3-dipolar cycloadditions of unprotected vinyl nucleobases with different nitrones under microwave irradiation

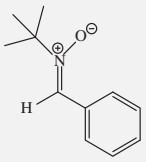
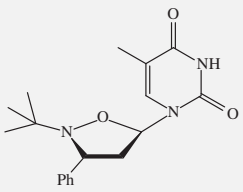
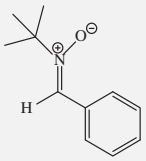
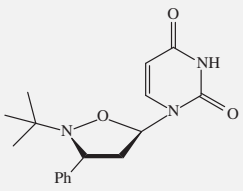
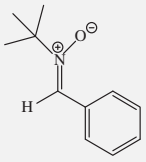
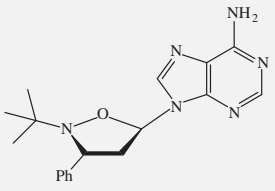
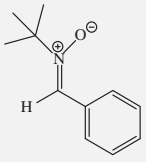
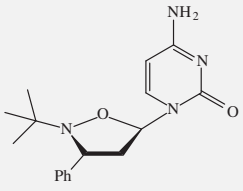
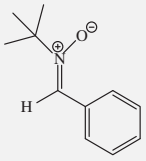
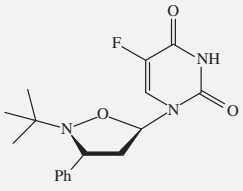
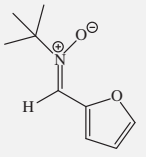
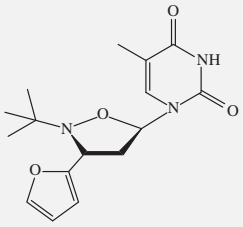
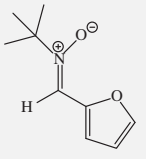
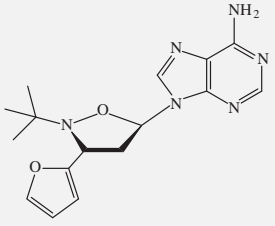
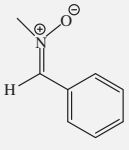
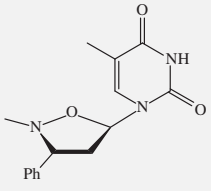
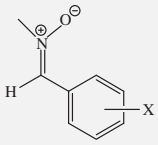
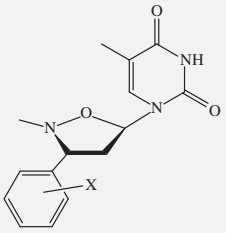
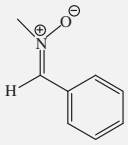
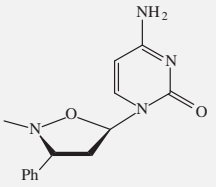
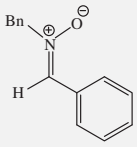
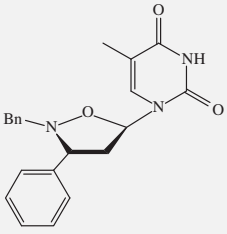
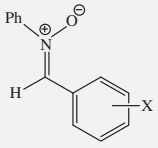
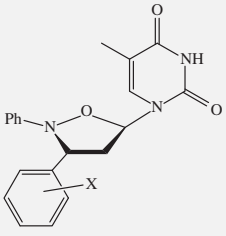
Entry	*N*-Vinyl nucleobase	Nitrone	*t* (min)	Product		de[b] (%)	Yield[c] (%)
1[a]	Thy		10		**6a**	98	95
2[a]	Ura		13		**6b**	98	75
3[a]	Ade		10		**6c**	92	79
4[a]	Cyt		10		**6d**	92	70
5	F-Ura		10		**6e**	98	70
6	Thy		11		**6f**	98	81
7	Ade		35		**6g**	94	78
8	Thy		15		**6h**	60	80

Table 1 (*continued*)

Entry	*N*-Vinyl nucleobase	Nitrone	*t* (min)	Product		de[b] (%)	Yield[c] (%)
9	Thy		20		**6i** X = *o*-OH	60	62
10			22		**6l** X = *p*-OH	62	60
11			20		**6m** X = *o*-Cl	80	80
12	Cyt		25		**6n**	50	50
13	Thy		10		**6o**	60	80
14	Thy		30		**6p** X = *p*-NO_2	60	90

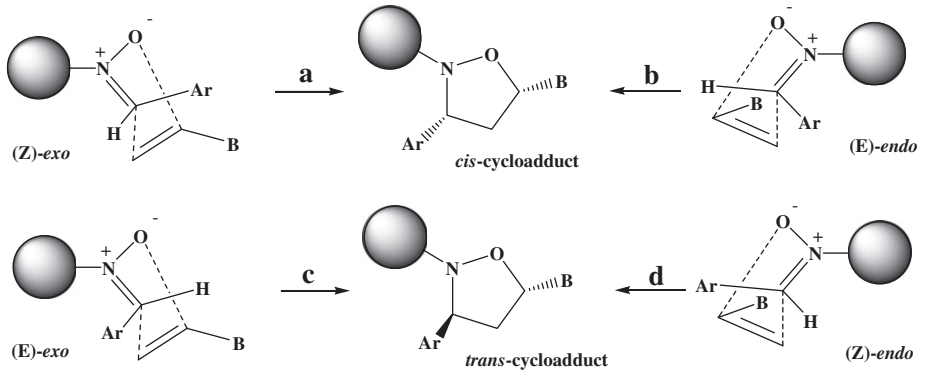
[a] MW power 850 W, other cases 750 W.
[b] de = (*cis*–*trans*) × 100 see Ref. 19.
[c] Isolated yields.

cycloaddition of suitable nitrones and vinyl nucleobases. The diastereoisomeric excess is in many cases of 98% in favor of the *cis* isomer, especially when a bulky alkyl group is present on the nitrone moiety. Regarding to their biological activity some of the tested compounds have proven to be potential antiproliferative drugs. In particular the Thy-phenyl-substituted compound **6o** and the *o*-chloro analog **6m** were able to prevent the proliferative activity at a relatively low concentration.

5. Experimental section

5.1. General

Vinyl nucleobases[27] and nitrones[12,14] were synthesized according to published procedures. ^{1}H NMR spectra were recorded at 300 MHz in $CDCl_3$ using tetramethylsilane (TMS) as internal standard (Bruker ACP 300 MHz). Chemical shifts are given in parts



Scheme 3. Geometries of approach of reacting nitrone and alkene.

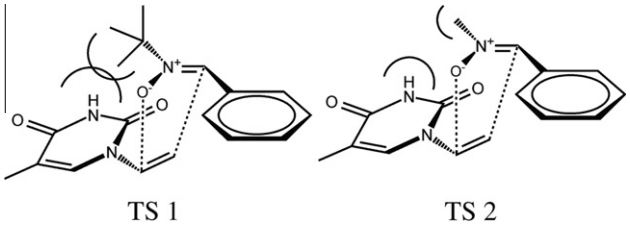


Figure 1. Model transition states for the diene-dienophile approach.

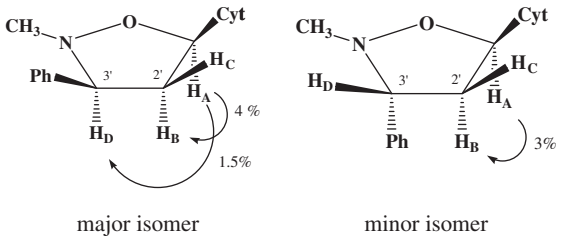


Figure 2. Relevant NOE effects on *cis*-and *trans*-**6n**.

Table 2
In vitro cytotoxic activity of *N,O*-nucleoside derivates

Compound	IC_{50}[a] (μM)	IC_{50}[b] (μM)	IC_{50}[c] (μM)
6h	82.5 ± 4.5	60.6 ± 1.9	76.6 ± 1.4
6a	87.1 ± 6.2	68.8 ± 3.4	72.2 ± 3.2
exo-**6n**	>100	>100	>100
endo-**6n**	>100	>100	>100
exo-**6q**	>100	>100	>100
endo-**6q**	>100	>100	>100
6e	67.5 ± 8.2	63.1 ± 3.4	60.6 ± 6.5
6f	>100	>100	>100
6m	52.6 ± 13.1	28.5 ± 3.5	8.8 ± 4.4
6o	55.2 ± 6.1	36.9 ± 1.8	19.3 ± 2.4

[a] LCL_s, [b]JiJoye and [c]Jurkat cell lines were exposed to concentrations of the indicated compounds, ranging from 1 to 100 μM, or control medium. Values indicate the IC_{50}, detected by MTT test after72 h of incubation. Results are presented as a mean ± SD of four independent experiments performed in triplicates.

per million from TMS and coupling constants in hertz. The COSY and NOESY spectra were recorded at 500 MHz in $CDCl_3$ or Py-d_5 (Bruker Avance 500 MHz). NOE experiments were performed in Py-d_5 on a Varian Mercury 400 MHz. The *cis–trans* product composition was established by ^{1}H NMR spectroscopy and by HPLC analysis. The mass spectrometric experiments were carried out on a Finnigan LCQ Deca, equipped with an electrospray ionization source. Standard experimental conditions are as follows: sample concentration10^{-6} M; elution solvent MeOH; flowrate 8 μL min^{-1}; nebulizing gas 40 units flow rate; spray voltage 4 kV; capillary voltage 14 V; capillary temperature 270 °C. Melting points were obtained on a Kofler apparatus. HPLC analysis were performed on a Hewlett-Packard 1100 Series, UV detector monitored at 254 nm, using a JUPITER 10 μ C-18, (25 cm), 0.1% TFA H_2O/MeOH 9:1, 1.0 mL/min.

5.2. Typical experimental procedure

The selected vinyl nucleobase (0.1 mmol) and the nitrone (0.2 mmol) are co-grinded in a mortar and further mixed in a vortex. The mixture of the two solids is transferred in a 50 mL Pyrex container that is placed within an unmodified household microwave oven, at 750 W or 850 W irradiation power. After the appropriate time the reaction mixture is dissolved in the minimum quantity of $CHCl_3$ and submitted to flash chromatographic separation, using variable mixtures of chloroform and methanol. The nitrone in excess is recovered and may be re-used. The cycloadducts were analyzed by HPLC and ^{1}H NMR to establish the diastereoisomeric *cis–trans* ratio. Yields were calculated on isolated compounds. Further purification by column chromatography was carried out for all the compounds submitted to biological evaluation. The purity of these samples was re-confirmed by HPLC, see Supplementary data for spectra.

5.3. *N,O*-Nucleosides

5.3.1. *cis*-4′-Aza-4′-(*N*-*t*-butyl)-3′-phenyl-2′,3′-dideoxythymidine (6a)

White solid, mp 245–246 °C. ^{1}H NMR ($CDCl_3$): δ 1.08 (9H,s, *t*-Bu), 1.95–2.04 (m, 3H, Thy CH_3), 2.40 (1H, ddd, J = 3.5, 7.8, 14 Hz, H_c), 3.29 (1H, ddd, J = 7.3, 8.4, 14 Hz, H_b), 4.24 (1H, t, J = 8.4 Hz, H_d), 6.05 (1H, dd, J = 3.5, 7.3 Hz, H_a), 7.21–7.50 (5H, m, Ph), 7.90 (1H, m, Thy H_6), 9.52 (1H, s_b, NH). ^{13}C NMR ($CDCl_3$): δ 12.88, 26.15, 49.94, 59.54, 62.75, 83.14, 109.81, 127.07, 127.66, 128.76, 136.26, 141.34, 150.33, 163.91. ESI-MS $[MH]^+$ m/z 330. Anal. Calcd for $C_{18}H_{23}N_3O_3$: C, 65.63; H, 7.04; N, 12.76. Found: C, 65.57; H, 7.00; N, 12.81.

5.3.2. *cis*-4′-Aza-4′-(*N*-*t*-butyl)-3′-phenyl-2′,3′-dideoxyuridine (6b)

White solid, mp 256–257 °C. ^{1}H NMR ($CDCl_3$): δ 1.10 (9H,s, *t*-Bu), 2.39 (1H,ddd, J = 3.2, 8.1, 14 Hz, H_c), 3.33 (1H, ddd, J = 7.3, 8.7, 14 Hz, H_b), 4.21 (1H, t, J = 8.1 Hz, H_d), 5.66 (1H, d, J = 8.1, Ura H_5), 6.30 (1H, dd, J = 3.2, 7.3 Hz, H_a), 7.22–7.52 (5H, m, Ph), 8.18 (1H, d, J = 8.1 Hz, Ura H_6), 9.55 (1H, s_b, NH); ^{13}C NMR ($CDCl_3$): δ 25.22, 47.33, 57.75, 66.10, 85.64, 108.81, 126.81, 127.02, 128.31, 139.43, 143.75, 151.52, 164.01. ESI-MS $[MH]^+$ m/z 316. Anal. Calcd for $C_{17}H_{21}N_3O_3$: C, 64.74; H, 6.71; N, 13.32. Found: C, 64.81; H, 6.66; N, 13.28.

5.3.3. *cis*-4′-Aza-4′-(*N*-*t*-butyl)-3′-phenyl-2′,3′-dideoxyadenosine (6c)

White solid, mp 264–265 °C. ^{1}H NMR ($CDCl_3$): δ 1.12 (9H, s, *t*-Bu), 2.66 (1H, ddd, J = 2.7, 7.9, 13.8 Hz, H_c), 3.43 (1H, ddd, J = 7.6, 8.9, 13.8 Hz, H_b), 4.31 (1H, dd, J = 7.6, 8.9 Hz, H_d), 6.38 (1H, dd, J = 2.7, 7.6 Hz, H_a), 7.30–7.52 (5H, m, Ph), 8.30 (1H, s, Ade H_2), 8.55 (1H, s, Ade H_8), 10.00 (2H, s_b, NH_2); ^{13}C NMR ($CDCl_3$): δ 23.75, 47.32, 59.45, 65.34, 82.56, 127.61, 129.43, 130.28, 140.53, 121.65, 141.23, 148.84, 153.25, 158.11. ESI-MS $[MH]^+$ m/z 339. Anal. Calcd for $C_{18}H_{22}N_6O$: C, 63.89; H, 6.55; N, 24.83. Found: C, 63.83; H, 6.58; N, 24.79.

5.3.4. *cis*-4′-Aza-4′-(*N*-*t*-butyl)-3′-phenyl-2′,3′-dideoxycytidine (6d)

White solid, mp 276–277 °C. ^{1}H NMR ($CDCl_3$): δ 1.08 (9H, s, *t*-Bu), 2.30–2.42 (1H, m, H_c), 3.30–3.44 (1H, m, H_b), 4.21 (1H, t, J = 7.83, H_d), 5.88 (1H, d, J = 7.3, Cyt H_5), 6.04 (1H, dd, J = 3.2, 7.3 Hz, H_a), 7.00–7.80 (6H, m, Ph + Cyt H_6), 10.03 (2H, s_b, NH_2); ^{13}C NMR ($CDCl_3$): δ 27.22, 45.30, 58.77, 68.23, 87.41, 98.04, 126.91, 127.88, 128.74, 140.21, 142.92, 157.28, 168.10. ESI-MS $[MH]^+$ m/z 315. Anal. Calcd for $C_{17}H_{22}N_4O_2$: C, 64.95; H, 7.05; N, 17.82. Found: C, 65.02; H, 7.00; N, 17.78.

5.3.5. *cis*-4′-Aza-4′-(*N*-*t*-butyl)-3′-phenyl-2′,3′-dideoxy-5-fluorouridine (6e)

White solid, mp 252–253 °C. ^{1}H NMR ($CDCl_3$): δ 1.82 (9H, s, *t*-Bu), 2.27–2.44 (1H, m, H_c), 3.28–3.44 (1H, m, H_b), 3.92 (1H, dd,

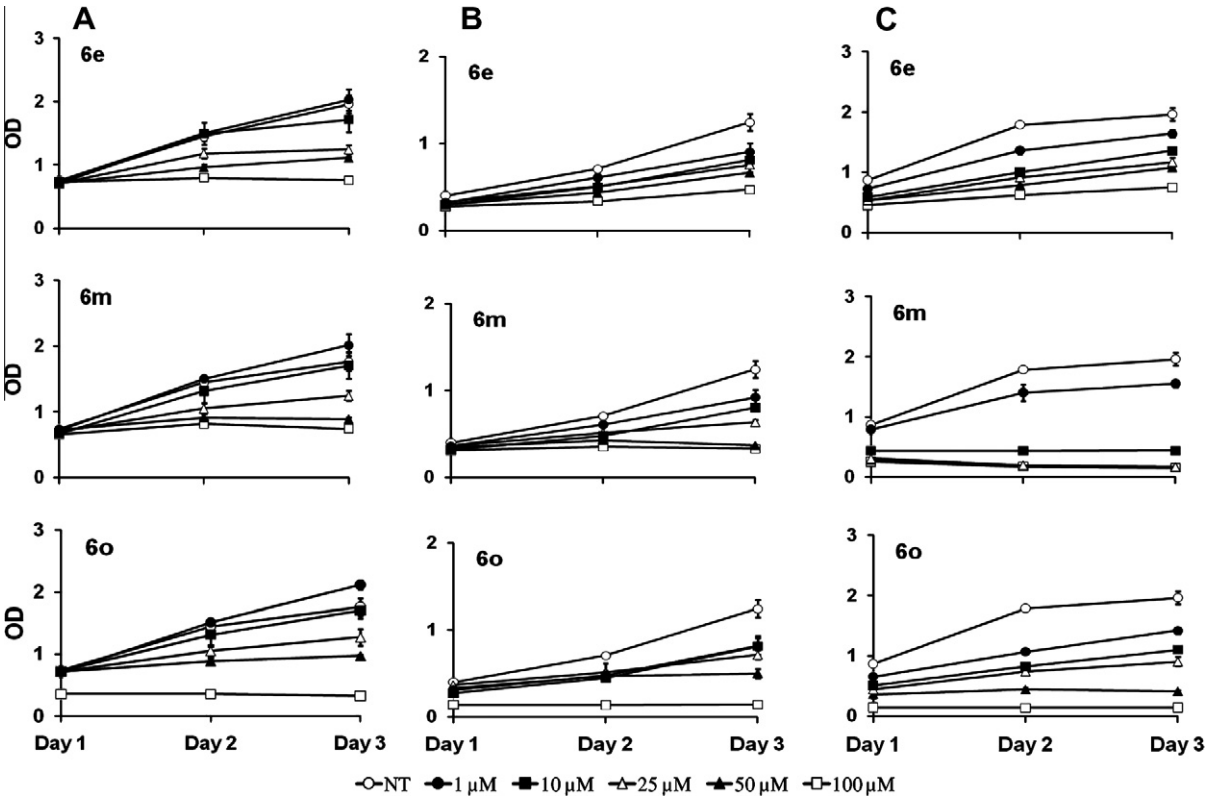


Figure 3. Effect of *N*,*O*-nucleoside **6e**, **6m**, and **6o** derivates, at concentrations ranging from 1 to100 μM, on cell proliferation. LCL_s (A), JiJoye (B), and Jurkat (C) cells were incubated for the indicated time in the presence of compounds **6e**, **6m**, and **6o** at concentrations ranging from 1 to100 μM. Viable cells were measured by MTT test. One representative experiment performed in triplicates ± SD is shown.

J = 7.6, 10.0 Hz H_d), 6.03 (1H, dd, *J* = 3.2, 7.6 Hz, H_a), 7.12–7.60 (6H, m, Ph + Ura H_6), 9.15 (1H, s, NH); ^{13}C NMR ($CDCl_3$): δ 27.83, 44.86, 59.21, 67.34, 83.89, 125.89, 128.10, 128.89, 130.34, 138.21, 142.57, 153.43, 160.05. ESI-MS $[MH]^+$ *m*/*z* 334. Anal. Calcd for $C_{17}H_{20}FN_3O_3$: C, 61.25; H, 6.05; F, 5.70; N, 12.61. Found: C, 61.19; H, 6.08; F, 5.73; N, 12.57.

5.3.6. *cis*-4′-Aza-4′-(*N*-*t*-butyl)-3′-furyl-2′,3′-dideoxythymidine (6f)

White solid, mp 240–241 °C. ^{1}H NMR ($CDCl_3$): δ 1.17 (d, *J* = 1.3 Hz, 3H, Thy CH_3), 1.99 (s, 9H, *t*-Bu), 2.24–2.37 (m, 1H, H_c), 2.74–2.95 (m, 1H, H_b), 3.13 (t, *J* = 7.5, 1H, H_d), 6.07 (dd, *J* = 3.7, 7.2 Hz, 1H, H_a), 7.10–7.32 (m, 3H, Ar), 7.73 (d, *J* = 1.3 Hz, 1H, Thy H_6), 9.32 (s_b, 1H, NH); ^{13}C NMR ($CDCl_3$): δ 13.34, 23.09, 45.87, 54.87, 60.41, 85.64, 103.78, 111.05, 113.31, 136.91, 143.52, 151.64, 154.67, 164.67. ESI-MS $[MH]^+$ *m*/*z* 320. Anal. Calcd for $C_{16}H_{21}N_3O_4$: C, 60.17; H, 6.63; N, 13.16. Found: C, 60.21; H, 6.58; N, 13.20.

5.3.7. *cis*-4′-Aza-4′-(*N*-*t*-butyl)-3′-furyl-2′,3′-dideoxyadenosine (6g)

White solid, mp 253–254 °C. ^{1}H NMR ($CDCl_3$): δ 1.12 (9H, s, *t*-Bu), 2.66 (1H, ddd, *J* = 2.7, 7.9, 13.8 Hz, H_c), 3.43 (1H, ddd, *J* = 7.6, 8.9, 13.8 Hz, H_b), 4.31 (1H, dd, *J* = 7.6, 8.9 Hz, H_d), 6.38 (1H, dd, *J* = 2.7, 7.6 Hz, H_a), 7.30–7.52 (5H, m, Ph), 8.30 (1H, s, Ade H_2), 8.55 (1H, s, Ade H_8), 10.00 (2H, s_b, NH_2); ^{13}C NMR ($CDCl_3$): δ 26.87, 47.11, 51.54, 57.32, 61.09, 83.76, 107.67, 110.65, 122.44, 141.76, 142.62, 152.54, 154.43, 157.22. ESI-MS $[MH]^+$ *m*/*z* 329. Anal. Calcd for $C_{16}H_{20}N_6O_2$: C, 58.52; H, 6.14; N, 25.59. Found: C, 58.47; H, 6.10; N, 25.62.

5.3.8. *cis*-4′-Aza-4′-(*N*-methyl)-3′-*o*-hydroxyphenyl-2′,3′-dideoxythymidine (6i)

Pale yellow solid, mp 173–174 °C. ^{1}H NMR ($CDCl_3$): δ 1.99 (3H, d, *J* = 1.8 Hz, Thy CH_3), 2.62–2.74 (1H, m, H_c), 2.85–2.96 (1H, m, H_b), 3.01 (3H, s, CH_3), 3.18–3.26 (1H, m, H_d), 6.27 (1H, dd, *J* = 5.3, 7.2 Hz, H_a), 7.10–7.38 (4H, m, Ar), 8.34 (1H, d, *J* = 1.8 Hz, Thy H_6), 9.98 (1H, s, NH), 12.35 (1H, s_b, OH); ^{13}C NMR ($CDCl_3$): δ 12.86, 42.78, 45.01, 71.34, 84.67, 111.56, 118.34, 120.54, 126.39, 126.78, 130.21, 136.98, 153.27, 157.76, 165.34. ESI-MS $[MH]^+$ *m*/*z* 304. Anal. Calcd for $C_{15}H_{17}N_3O_4$: C, 59.40; H, 5.65; N, 13.85. Found: C, 59.44; H, 5.68; N, 13.80.

5.3.9. *cis*-4′-Aza-4′-(*N*-methyl)-3′-*p*-hydroxyphenyl-2′,3′-dideoxythymidine (6l)

Pale yellow solid, mp 169–170 °C. ^{1}H NMR ($CDCl_3$): δ 1.89 (3H, d, *J* = 1.3 Hz, Thy CH_3), 2.52–2.68 (1H, m, H_c), 2.77–2.89 (1H, m, H_b), 3.01 (3H, s, CH_3), 3.22–3.36 (1H, m, H_d), 6.35 (1H, dd, *J* = 5.2, 7.7 Hz, H_a), 7.10–7.30 (4H, m, Ar), 8.24 (1H, d, *J* = 1.3 Hz, Thy H_6), 9.80 (1H, s, NH), 12.23 (1H, s_b, OH); ^{13}C NMR ($CDCl_3$): δ 13.21, 44.29, 46.45, 72.65, 87.90, 111.02, 117.32, 130.74, 131.42, 137.32, 151.44, 158.21, 164.89. ESI-MS $[MH]^+$ *m*/*z* 304. Anal. Calcd for $C_{15}H_{17}N_3O_4$: C, 59.40; H, 5.65; N, 13.85. Found: C, 59.47; H, 5.62; N, 13.83.

5.3.10. *cis*-4′-Aza-4′-(*N*-methyl)-3′-*o*-chlorophenyl-2′,3′-dideoxythymidine (6m)

Yellow solid, mp 158–159 °C. ^{1}H NMR ($CDCl_3$): δ 1.99 (3H, d, *J* = 1.2 Hz, Thy CH_3), 2.18 (1H, ddd, *J* = 4.2, 9.8, 13.9 Hz, H_c), 2.74 (3H, s, CH_3), 3.48 (1H, ddd, *J* = 7.8, 7.9, 13.9 Hz, H_b), 4.21 (1H, dd, *J* = 7.8, 9.8 Hz, H_d), 6.28 (1H, dd, *J* = 4.2, 7.9 Hz, H_a), 7.18–7.55

(4H, m, Ar), 7.76 (1H, d, J = 1.2 Hz, Thy H_6), 9.58 (1H, s_b, NH); ^{13}C NMR ($CDCl_3$): δ 12.78, 43.25, 45.94, 68.48, 82.90, 110.64, 127.05, 127.47, 129.05, 129.92, 133.72, 134.94, 135.80, 150.56, 164.13. ESI-MS $[MH]^+$ m/z 322. Anal. Calcd for $C_{15}H_{16}ClN_3O_3$: C, 55.99; H, 5.01; Cl, 11.02; N, 13.06. Found: C, 55.94; H, 4.97; Cl, 11.05; N, 13.09.

5.3.11. *cis*-4′-Aza-4′-(*N*-methyl)-3′-phenyl-2′,3′-dideoxycytidine (6n)

Pale yellow solid, mp 252–253 °C. ^{1}H NMR (Py-d_5): δ 2.44 (1H, ddd, J = 4.3, 10.0, 14.0 Hz, H_c), 2.58 (3H, s, CH_3), 3.41 (1H, ddd, J = 7.6, 7.8, 14.0 Hz, H_b), 3.62 (1H, dd, J = 7.6, 10.0 Hz, H_d), 6.12 (1H, d, J = 7.4 Hz, Cyt H_5), 6.56 (1H, dd, J = 4.3, 7.8 Hz, H_a), 7.20–7.45 (5H, m, Ar), 8.20 (1H, d, J = 7.4 Hz, Cyt H_6), 8.28 (2H, s_b, NH_2); ^{13}C NMR (Py-d_5): δ 41.16, 47.37, 71.77, 82.61, 92.84, 126.47, 126.89, 127.53, 136.14, 139.49, 155.18, 165.71. ESI-MS $[MH]^+$ m/z 273. Anal. Calcd for $C_{14}H_{16}N_4O_2$: C, 61.75; H, 5.92; N, 20.57. Found: C, 61.81; H, 5.89; N, 20.53.

5.3.12. *cis*-4′-Aza-4′-(*N*-phenyl)-3′-*p*-nitrophenyl-2′,3′-dideoxythymidine (6p)

Yellow solid, mp 188–189 °C. ^{1}H NMR ($CDCl_3$): δ 1.97 (3H, d, J = 1.2 Hz, Thy CH_3), 2.62–2.81 (1H, m, H_c), 3.25–3.42 (1H, m, H_b), 3.68–3.80 (1H, m, H_d), 3.85 (2H, s, CH_2-Ph), 6.56 (1H, m, H_a), 7.20–7.70 (9H, m, Ar), 7.78 (1H, d, J = 1.2 Hz, Thy H_6), 10.1 (1H, s_b, NH); ^{13}C NMR ($CDCl_3$): δ 12.88, 47.07, 69.37, 83.25, 111.26, 116.35, 118.16, 124.50, 125.37, 127.92, 129.24, 134.79, 145.97, 147.92, 153.44, 163.73. ESI-MS $[MH]^+$ m/z 395. Anal. Calcd for $C_{20}H_{18}N_4O_5$: C, 60.91; H, 4.60; N, 14.21. Found: C, 60.87; H, 4.63; N, 14.25.

For characterization of compounds **6h** and **6o** see Ref. 12.

5.4. Biological assays

Cells were maintained in RPMI 1640, supplemented with 10% newborn bovine serum, penicillin (100 U/mL) and streptomycin (100 U/mL) and glutamine (2 mM); the pH of the medium was 7.2 and incubation was at 37 °C in a 5% CO_2 atmosphere. Cells were routinely passaged every three days. Compounds were solubilized in DMSO at 20 mM and diluted in medium (RPMI + 10% FCS) before use. The viability of cells was analyzed by colorimetric assay with MTT (thiazollyl blue). MTT assay, based on conversion of the yellow tetrazolium salt MTT to purple formazan crystals by metabolically active cells provides a quantitative determination of viable cells.[28] The cells were seeded in triplicate in 96-well plate at a density of 50×10^3 in 50 μL of RPMI + 10% FCS and treated with the test compounds at concentrations ranging from 1 to 100 μM. Untreated cells were placed in every plate as a negative control. After 1–3 days of culture 200 μL of MTT were added to each well and plates were incubated at 37 °C and 5% CO_2. After 2 h the MTT crystals were solubilized with 100 μL of lysing buffer (50% DMF + 20% SDS, pH 4.7); after 24 h spectrophotometric absorbance of each sample was then measured at 570 nm.[29]

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.08.024.

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