Eur. J. Med. Chem. 35 (2000) 1011–1019

PII: S0223-5234(00)01184-3/SCO

Short communication

Unnatural enantiomers of 5-azacytidine analogues: Syntheses and enzymatic properties

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Received 17 April 2000; revised 21 June 2000; accepted 13 July 2000

Abstract – Although 2′-deoxy-β-D-5-azacytidine (Decitabine) and β-D-5-azacytidine display potent antileukemic properties, their therapeutic use is hampered by their sensitivity to nucleophiles and to deamination catalysed by cytidine deaminase. As shown earlier [Shafiee M., Griffon J.-F., Gosselin G., Cambi A., Vincenzetti S., Vita A., Erikson S., Imbach J.-L., Maury G., Biochem. Pharmacol. 56 (1998) 1237–1242], β-L-enantiomers of cytidine derivatives are resistant to cytidine deaminase. We thus synthesized several 5-azacytosine β-L-nucleoside analogues to evaluate their enzymatic and biological properties. 2′-Deoxy-β-L-5-azacytidine (L-Decitabine), β-L-5-azacytidine, 1-(β-L-*xylo*-furanosyl)5-azacytosine, and 1-(2-deoxy-β-L-*threo*-pentofuranosyl)5-azacytosine were stereospecifically prepared starting from L-ribose and L-xylose. D- and L-enantiomers of 2′-deoxy-β-5-azacytidine were weak substrates of human recombinant deoxycytidine kinase (dCK) compared to β-D-deoxycytidine, whereas both enantiomers of β-5-azacytidine or the L-*xylo*-analogues were not substrates of the enzyme. As expected, none of the presently reported derivatives of β-L-5-azacytidine was a substrate of human recombinant cytidine deaminase (CDA). The prepared compounds were tested for their activity against HIV and HBV and they did not show any significant activity or cytotoxicity. In the case of L-Decitabine, this suggests that the enantioselectivities of concerned enzymes other than dCK and CDA might not be favourable. © 2000 Éditions scientifiques et médicales Elsevier SAS

β-L-5-azacytidine / L-decitabine / synthesis / deoxycytidine kinase / cytidine deaminase

1. Introduction

β-D-5-Azacytidine (β-D-5-azaC), **1**, and 2′-deoxy-β-D-5-azacytidine (β-D-5-azadC), **2**, are important antileukemic agents used in clinical treatment [1, 2]. The latter compound (Decitabine) is a potent antineoplastic compound against human myeloid leukemic cells, more active than β-D-arabinofuranosylcytosine (araC) [3]. It has been shown that both β-D-5-azaC and β-D-5-azadC interfere with the process of DNA methylation and that the antileukemic activity of these compounds originates from their ability to induce the synthesis of hypomethylated DNA in cells [4, 5]. After triphosphorylation and incorporation into cellular DNA, **1** or **2** forms a covalent adduct with the DNA methylating enzyme DNA-(cytosine-5)methyltransferase which is therefore inhibited [6, 7]. This may induce cell differentiation [8] or may activate silent genes, particularly tumor suppressor genes [9, 10].

The use of β-D-5-azaC or β-D-5-azadC suffers however from several drawbacks. The inclusion of an extra nitrogen atom into the cytosine nucleus increases its chemical sensitivity with respect to nucleophiles and accounts for the instability of the compounds in aqueous solution [11]. Decitabine has thus to be administered as a freshly reconstituted solution every 4 to 6 h [12]. Furthermore, several mechanisms of drug resistance operate [10]. A deficiency in deoxy-

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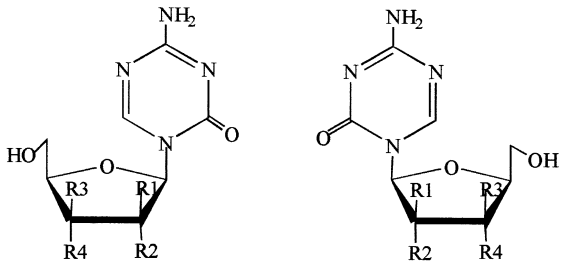


1 (R1 = R3 = H, R2 = R4 = OH) **3** (R1 = R3 = H, R2 = R4 = OH)

2 (R1 = R2 = R3 = H, R4 = OH) **4** (R1 = R2 = R3 = H, R4 = OH)

5 (R1 = R4 = H, R2 = R3 = OH)

6 (R1 = R2 = R4 =H, R3 = OH)

Figure 1. β-D- and β-L-5-azacytidine analogs studied as substrates of human dCK and human CDA.

cytidine kinase (dCK) or the inhibition of this enzyme by an excess of the feedback inhibitor dCTP hampers the phosphorylation of the drug. Another major cause of resistance is induced by the ubiquitous cellular enzyme cytidine deaminase (CDA) since deamination of **1** or **2** results in total loss of activity [10, 13, 14].

We have previously shown that a number of cytidine analogues having the unnatural L stereochemistry are both substrates of human deoxycytidine kinase and resistant to human cytidine deaminase [15]. We therefore stereospecifically prepared the L-enantiomers of **1** and **2** and other analogues (*figure 1*), and we studied their enzymatic properties with respect to dCK and CDA in the hope of getting phosphorylation, a lack of deamination of these compounds and possibly an efficient incorporation into DNA. One β-L-5-azacytidine analogue, namely 2′,3′-dideoxy-β-L-5-azacytidine, was previously prepared and shown to display anti-HBV activity but no cytotoxicity [16].

2. Chemistry

The previous syntheses of 5-azacytidine analogues are based either on the construction of the *s*-triazine ring from a conveniently substituted sugar or on the coupling of the silylated heterocycle and an halogenosugar. Thus, β-D-5-azaC and β-D-5-azadC have been obtained using both methods [17–23]. To avoid the limiting step of nucleophilic deprotection, the benzoylated sugar was reduced with sodium borohydride in HMPA and then reoxidized with bistrimethylsilyltrifluoroacetamide to afford the nucleoside analogues in improved yields [24, 25]. 2′,3′-Dideoxy-β-D-5-azacytidine was prepared from β-D-5-azacytosine using the Barton–McCombie method [26] whereas the L-enantiomer was obtained by coupling the 5′-protected 1-acetoxy-β-L-2,3-dideoxyribose and the silylated heterocycle [16, 27].

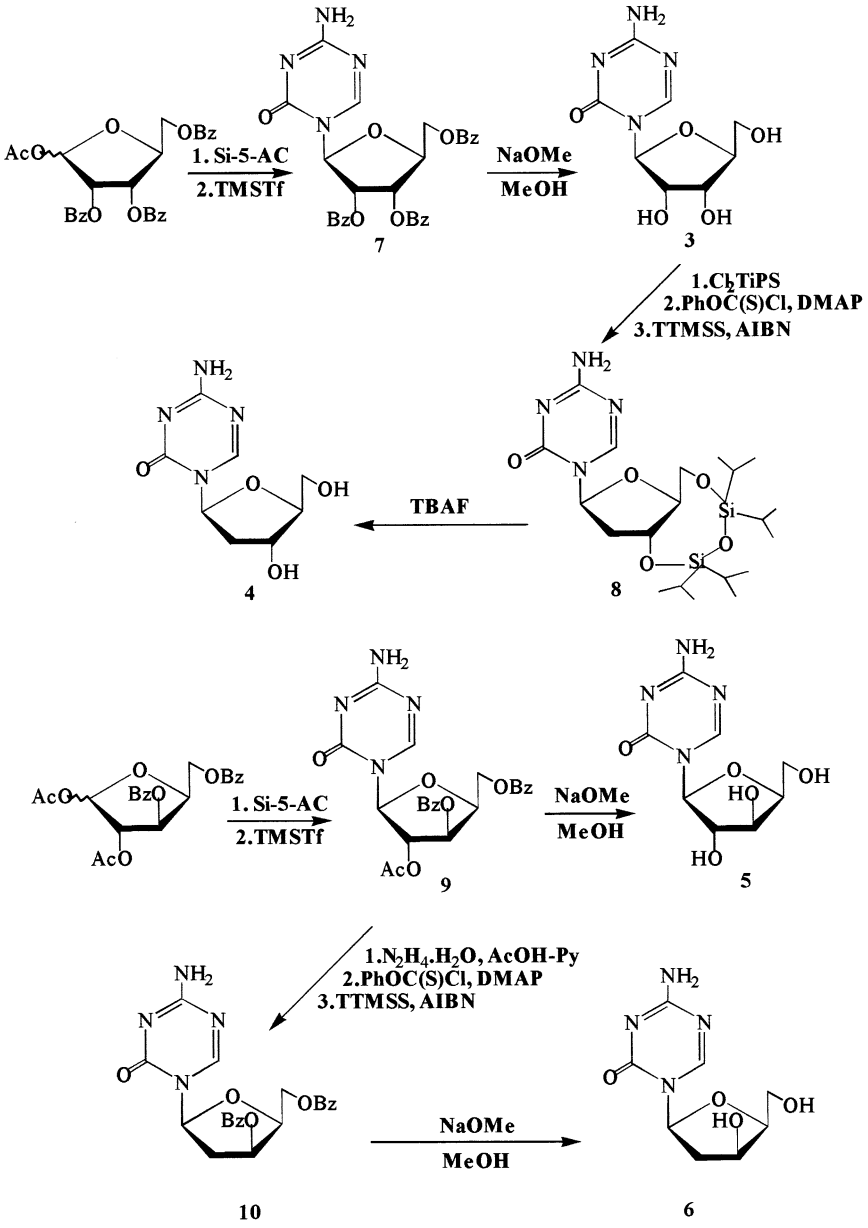
Most previous syntheses of 5-azacytidine analogues yield both α- and β-anomers often difficult to separate. For this reason, we used regio- and stereospecific methods to prepare the L-enantiomers **3–6** starting from L-ribose or L-xylose. 1-*O*-Acetyl-2,3,5-tri-*O*-benzoyl-β-L-ribofuranose [28] was coupled with silylated 5-azacytidine in the presence of trimethylsilyltriflate. According to Baker's rule [29], the 2′-benzoyl group controls the stereochemistry of the substitution and only the β-anomeric derivative **7** was obtained. Deprotection of **7** catalysed by sodium methanolate in methanol afforded β-L-ribofuranosyl-5-azacytosine, **3**, in good yield. The 3′- and 5′-positions of **3** were then protected using dichlorotetraisopropyldisiloxane. A Barton–McCombie elimination [30] of the 2′-hydroxyl group of the 3′,5′-diprotected compound followed by deprotection gave 2′-deoxy-β-L-*erythro*-pentofuranosyl-5-azacytosine (L-Decitabine), **4**, in 42% overall yield from **3** (*figure 2*).

1,2-Di-*O*-acetyl-3,5-di-*O*-benzoyl-L-*xylo*-furanose [31] was similarly condensed with silylated 5-azacytosine giving exclusively the β-anomeric derivative **9**. Deprotection with sodium methanolate afforded β-L-xylofuranosyl-5-azacytosine, **5**, in good yield. Deacetylation of **9** followed by a Barton–McCombie elimination of the 2′-hydroxyl group and debenzoylation yielded 2′-deoxy-β-L-*threo*-pentofuranosyl-5-azacytosine, **6** (*figure 2*).

3. Biological results and discussion

The enzymatic properties of β-L-5-azacytidine derivatives **3–6** were determined with respect to human recombinant deoxycytidine kinase, to human recombinant cytidine deaminase, and compared to the properties of the corresponding β-D-enantiomers when available.

Deoxycytidine kinase is a key enzyme in the transformation of nucleoside analogues to the correspond-



Si-5-AC: 5-Azacytosine silylated with hexamethyldisilazane.
TMSTf: Trimethylsilyl triflate.
Cl_2TiPS: 1,3-Dichloro-1,1,3,3-tetraisopropyldisiloxane.
DMAP: 4-Dimethylaminopyridine.
TTMSS: Tris(trimethylsilyl)silane.
AIBN: α,α'-Azoisobutyronitrile.
TBAF: Tetrabutylammonium fluoride.

Figure 2. Syntheses of the β-L-5-azacytidine analogs **3**–**6**.

ing 5′-triphosphate derivatives, whereas cytidine deaminase often deactivates cytidine derivatives with antiviral properties. Several studies of their action on the D-enantiomers **1** and **2** have been previously reported, and evaluations of the substrate character of β-D-5-azaC with respect to dCK have shown that it is

either low or non-existent. Thus, kinetics in the presence of dCK from calf thymus gave a K_m of 1200 μM and a substrate efficiency of only 0.4 % compared to β-D-dC [32]. Human dCK extracted from leukemic cells has been reported to have no affinity for β-D-5-azaC [33]. In contrast, β-D-5-azaC appears to be a substrate of uridine-cytidine kinase from various sources [34, 35]. Concerning β-D-5-azadC (Decitabine), all existing studies indicate that this compound is an average substrate of human or mammal dCK, with K_m ranging from 29 to 71 μM depending on the origin of the enzyme and on the conditions [36, 37]. More recently, human dCK has been reported to display with Decitabine 20 % of its activity compared to β-D-dC [38].

Most of the preceding kinetic studies of the enzymatic phosphorylation of β-D-5-azaC or -dC used radioactivity to analyse the reaction medium. To take directly into account the chemical decomposition of D- or L-5-azacytidine derivatives due to hydrolytic opening of the *s*-triazine ring [11, 24], we used HPLC to follow the kinetics of phosphorylation of **1–6** in the presence of human dCK. Under our conditions, substrate reversible decomposition was held below 2 % for the duration of the kinetics. With 5mM ATP as phosphate donor, only 2′-deoxy-β-D-5-azacytidine, **2**, and 2′-deoxy-β-L-5-azacytidine, **4**, were phosphorylated (*figure 3*, *table I*). The efficiencies of phosphorylation were similar for the two enantiomers and they were both largely below the efficiency of 2′-deoxy-β-D-cytidine phosphorylation (*table I*). The value of the K_m constant for 2′-deoxy-β-D-5-azacytidine is similar to the constants previously determined [36, 37]. The weak enantioselectivity displayed by human dCK in this case ($E_{L/D} = 0.66$) is consistent with its stereochemical properties regarding cytidine, adenosine or guanosine derivatives [15, 39]. In contrast, no phosphorylation was observed in the case of both enantiomers of β-5-azacytidine, **1** and **3**. Concerning β-D-5-azacytidine, our findings are in agreement with previous results [35] and they further indicate that human dCK has similar properties with both enantiomers of β-5-azaC or of β-5-azadC. The β-L-*xylo*- or 2′-deoxy-*xylo*-derivatives **5** and **6** did not display any substrate properties and, based on the considerably relaxed enantioselectivity of human dCK, it is likely that their antipodes are also mediocre substrates.

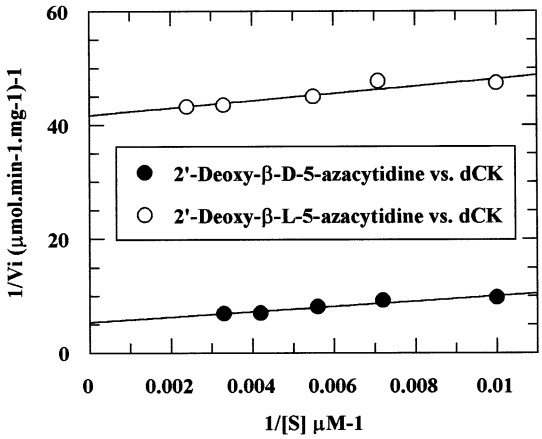


Figure 3. Lineweaver–Burk plots obtained in the phosphorylation of 2′-deoxy-β-D-5-azacytidine and β-L-5-azacytidine catalysed by human dCK.

Numerous studies have evaluated the sensitivity of β-D-5-azacytidine and 2′-deoxy-β-D-5-azacytidine to deamination catalysed by cytidine deaminase. The source of the purified enzyme has a considerable

Table I. Kinetic parameters corresponding to the phosphorylation of 2′-deoxy-D- or L-5-azacytidine and the other prepared analogues catalysed by human deoxycytidine kinase[a].

Compound	K_m (μM)	V_m (μmol/min per mg)	V_m/K_m (relat)
2′-Deoxy-β-D-5-azacytidine, **2**	94 ± 19	0.19 ± 0.02	0.075
2′-Deoxy-β-L-5-azacytidine, **4**	17 ± 4	0.024 ± 0.0006	0.05
2′-Deoxy-β-D-cytidine	9 ± 1.6	0.24 ± 0.01	1
β-D-5-Azacytidine, **1**	[b]	[b]	
β-L-5-Azacytidine, **2**	[b]	[b]	
β-L-Xylofuranosyl-5-azacytidine, **5**	[b]	[b]	
2′-Deoxy-β-L-xylofuranosyl-5-azacytidine, **6**	[b]	[b]	

[a] The reaction medium contained 5 mM ATP, 5 mM $MgCl_2$, the substrate (50–500 μM) and the enzyme (30 μg/mL) at 37 °C.
[b] No formation of monophosphate after incubation of the substrate and dCK (500 μg/mL) for 1 h at 37 °C.

influence on the K_m values of the two compounds [13, 40–42] which may partly result from their instability. Using a low temperature (25 °C) and short kinetic durations allowed us to limit the decomposition of the substrates as observed from HPLC analysis. Only the D-enantiomers **1** and **2** were substrates of human CDA (K_m values 225 and 690 μM, respectively) whereas no deamination occurred for the L-enantiomers **3–6**, under the same conditions and in the presence of increased concentrations of enzyme.

The prepared L-nucleoside analogues **3–6** were tested as inhibitors of HIV replication in CEM-SS and MT-4 cell systems or as inhibitors of HBV replication in HepG2 cells following standard protocols [43]. None of the compounds displayed any significant antiviral or cytotoxic effect. Several causes could account for the lack of activity. Our study only shows that L-Decitabine may be monophosphorylated in cells and is resistant to enzymatic deamination. The substrate properties and enantioselectivities of cellular nucleotide kinases, viral DNA polymerases or other concerned enzymes with respect to the reported compounds have not been investigated and could be unfavourable, even if HIV- or HBV DNA polymerases have been shown to accept triphosphates of some L-nucleoside analogues as substrates or inhibitors [44–47].

4. Experimental protocols

4.1. Chemistry

Melting points were determined in capillary tubes on a Gallenkamp MFB-595-010 M apparatus and are uncorrected. UV spectra were recorded on an Uvikon 810 (Kontron) spectrophotometer. ^{1}H-NMR spectra were obtained with a Brüker AC 250 spectrometer. Chemical shifts (δ) are reported in ppm. Deuterium exchange and decoupling experiments were performed to confirm proton assignments. FAB mass spectra were recorded in the positive-ion or negative-ion mode on a JEOL DX 300 mass spectrometer: the matrix was a mixture (50/50, v/v) of glycerol and thioglycerol (G/T). Specific rotations were measured on a Perkin-Elmer model 241 spectropolarimeter. Elemental analyses were carried out by the Service Central de Microanalyse du CNRS and were within $\pm$ 0.4 % of the calculated values, unless otherwise stated. L-Ribose and L-xylose were purchased from Interchim-France.

4.1.1. 1-(β-L-Ribofuranosyl)5-azacytosine **3**

A suspension of 5-azacytosine (2g, 17.9 mmol) in hexamethyldisilazane (132 mL) and a catalytic amount of ammonium sulfate was refluxed for 18 h under dry argon. After evaporation, the oily residue was dissolved in anhydrous 1,2-dichloroethane (125 mL). To this solution were added 1-*O*-acetyl-2,3,5-tri-*O*-benzoyl-β-L-ribofuranose (6.02 g, 12 mmol) in 1,2-dichloroethane (62 mL) and trimethylsilyltriflate (4.33 mL, 23.8 mmol). The mixture was left standing at room temperature under stirring for 5h, then diluted in chloroform (100 mL) and neutralized with a saturated solution of sodium hydrogenocarbonate. The organic layer was separated, washed twice with 150 mL of water, dried and evaporated. The residue was purified by silica gel column chromatography. 1-(2,3,5-Tri-*O*-benzoyl-β-L-ribofuranosyl)5-azacytosine, **7**, was eluted with a 0–2% gradient of methanol in dichloromethane (4.65 g, 70%). To a stirred suspension of this compound (4.65 g, 8.36 mmol) in anhydrous methanol (1.8 mL), a solution of sodium methanolate in methanol (0.2 M, 4.2 mL, 0.84 mmol) was slowly added under anhydrous conditions. The reaction mixture was stirred for 4h at room temperature then left standing at 4 °C. 1-(β-L-Ribofuranosyl)5-azacytosine crystallized as a white solid which was filtered, washed with water and recrystallized in methanol with a minimum of water (1.55 g, 76%): mp 233–235 °C; UV (ethanol 95) λ_{max} 244 nm, ε 6500; ^{1}H-NMR (DMSO-d_6) δ 8.57 (s, 1H, H-6), 7.52 (bs, 2H, NH_2), 6.63 (d, 1H, H-1′, $J_{1'-2'} = 4.9$ Hz), 5.43 (bs, 1H, OH-2′), 5.17 (s, 1H, OH-5′), 5.06 (bs, 1H, OH-3′), 4.1–3.9 (m, 2H, H-2′ and H-3′), 4.82 (m, 1H, H-4′), 3.7–3.5 (m, 2H, H-5′; H-5″); MS (FAB > 0, G/T): 245 $[M+H]^+$, 113 $[BH_2]^+$, (FAB < 0, G/T): 243 $[M-H]^-$, 111 $[B]^-$; $[\alpha]_D^{20} = -30$ (c 1.02, DMSO) (Lit. for the enantiomer: $[\alpha]_D^{20} = +22.4$ (c 1, H_2O) [18]). Anal. $C_8H_{12}N_4O_5$, 1/5 H_2O (C, H, N).

4.1.2. 1-[3,5-O-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-β-L-ribofuranosyl]5-azacytosine

1,3-Dichloro-1,1,3,3-tetra-isopropyldisiloxane (0.65 mL, 2.26 mmol) was slowly added to a suspension of 1-(β-L-ribofuranosyl)5-azacytosine (0.50g, 2.05 mmol) in anhydrous pyridine (20 mL) under argon. After stirring for 1 h at room temperature, the mixture was diluted with ethyl acetate (60 mL) and a saturated aqueous solution of sodium hydrogenocarbonate (20 mL). The organic layer was washed with water (20 mL), dried and evaporated. The residue was chromatographed on silica gel (eluent: 0 to 4 % gradient of methanol in dichloromethane). The product was obtained as a white

powder (0.84 g, 84 %): mp 160–165 °C; UV (ethanol 95) λ_{max} 242, ε 5500; ^{1}H-NMR (DMSO-d_6) δ 8.31 (s, 1H, H-6), 7.56 (bd, 2H, NH_2), 5.79 (bs, 1H, OH-2′), 5.53 (bs, 1H, H-1′), 4.3–4.1 (m, 2H, H-2′, H-3′), 4.1–3.8 (m, 3H, H-4′, H-5′, H-5″), 1.1–1.0 (m, 28H, iPr); MS (FAB > 0, G/T): 973 $[2M+H]^+$, 487 $[M+H]^+$, 113 $[BH_2]^+$, (FAB < 0, G/T): 971 $[2M-H]^-$, 485 $[M-H]^-$, 111 $[B]^-$; $[\alpha]_D^{20} = +27$ (c 1.02, DMSO).

4.1.3. 1-[2-Deoxy-3,5-O-(1,1,3,3-tetra-isopropyl-disiloxane-1,3-diyl)-β-L-erythro-pentofuranosyl]5-azacytosine ***8***

Phenoxy (thiocarbonyl) chloride (0.13 mL, 1.57 mmol) and 4-dimethylaminopyridine (0.38 g, 3.15 mmol) were added to a solution of 1-(3,5-*O*-TiPS-β-L-ribofuranosyl)5-azacytosine (0.51 g, 1.05 mmol) in anhydrous acetonitrile (32 mL). The suspension was stirred for 4 h at room temperature under argon. After evaporation, the residue was dissolved in dichloromethane (10 mL), washed with water (2 × 10 mL), with hydrochloric acid 0.5 N (10 mL), and finally with water (2 × 10 mL). The residue obtained after solvent evaporation was co-evaporated with anhydrous dioxane then dissolved in this solvent (8.5 mL). Tris(trimethylsilyl)silane (0.4 mL, 2.62 mmol) and α,α′-azoisobutyronitrile (0.05 g, 0.31 mmol) were added to the resulting solution which was then heated to 100 °C for 2 h. After cooling, the evaporation of the solvent gave a residue which was chromatographed on silica gel (eluent: 0 to 2 % gradient of methanol in dichloromethane). The product **8** was obtained as a white solid: mp 150–153 °C; UV (ethanol 95) λ_{max} 242, ε 5700; ^{1}H-NMR (DMSO-d_6) δ 8.12 (s, 1H, H-6), 7.41 (bd, 2H, NH_2), 5.77 (dd, 1H, H-1′, $J_{1'-2'} = 7.5$ Hz, $J_{1'-2''} = 2.5$ Hz), 4.48 (dd, 1H, H-3′, $J_{3'-2'} = 5.8$ Hz, $J_{3'-2''} = 5.7$ Hz), 3.9–3.7 (m, 2H, H-5′, H-5″), 3.6–3.5 (m, 1H, H-4′), 2.4–2.2 (m, 2H, H-2′, H-2″), 1–0.7 (m, 28H, iPr); MS (FAB > 0, G/T): 941 $[2M+H]^+$, 471 $[M+H]^+$, 113 $[BH_2]^+$, (FAB < 0, G/T): 469 $[M-H]^-$, 111 $[B]^-$; $[\alpha]_D^{20} = +81$ (c 1.10, DMSO).

4.1.4. 1-(2-Deoxy-β-L-erythro-pentofuranosyl)5-azacytosine ***4***

A solution of tetrabutylammonium fluoride (1M/THF, 0.91 mL, 0.91 mmol) was slowly added to a solution of 1-(2-deoxy-3,5-*O*-TiPS-β-L-*erythro*-pentofuranosyl)5-azacytosine (0.21 g, 0.46 mmol) in anhydrous tetrahydrofuran (8.2 mL) under stirring. The mixture was left 4h at room temperature under stirring, then it was evaporated to dryness. The oily residue was added to a minimum of methanol. The precipitate of 1-(2-deoxy-β-L-*erythro*-pentofuranosyl)5-azacytosine (86 mg, 84%) was recrystallized in methanol with a minimum of water: mp 192–195 (Litt. for the enantiomer: 191–193 °C [18]); UV (ethanol 95): λ_{max} 243 nm ε 6600 (Lit. for the enantiomer: λ_{max} 244 nm, ε 6900 [20]); ^{1}H-NMR (DMSO-d_6) δ 8.49 (s, 1H, H-6), 7.51 (bs, 2H, NH_2), 6.0 (m, 1H, H-1′), 5.22 (bs, 1H, OH-3′), 5.06 (bs, 1H, OH-5′), 4.21 (bs, 1H, H-3′), 3.8–3.5 (m, 3H, H-4′, H-5′, H-5″), 4.3–2.1 (m, 2H, H-2′, H-2″); MS (FAB > A, G/T) 217 $[M+H]^+$, 113 $[BH_2]^+$, (FAB < 0, G/T): 215 $[M-H]^-$, 107 $[S]^-$; $[\alpha]_D^{20} = -44$ (c 1.02, DMSO). Anal. $C_8H_{12}N_4O_4$, 1/10 H_2O (C, H, N).

4.1.5. 1-(2-O-Acetyl-3,5-di-O-benzoyl-β-L-xylofuranosyl)5-azacytosine ***9***

5-Azacytosine (3.65 g, 32.6 mmole) was suspended in hexamethyldisilazane in the presence of a catalytic amount of ammonium sulfate. The mixture was refluxed for 18 h under dry argon. After evaporation, the residual oil was dissolved in anhydrous 1,2-dichloroethane (240 mL). This solution was added to a solution of 1,2-di-*O*-acetyl-3,5-di-*O*-benzoyl-L-xylofuranose (9.5 g, 21.6 mmol) [31] and trimethylsilyltriflate (7.88 mL, 43.4 mmol) in 100 mL of 1,2-dichloroethane. After 5 h at room temperature under stirring, the reaction mixture was diluted with chloroform (200 mL) and neutralized with an aqueous solution of sodium hydrogenocarbonate. The organic layer was washed with water (2 × 300 mL), dried and evaporated. The crude residue was purified by silica gel column chromatography and the product was eluted with a 0–5% gradient of methanol in dichloromethane. The final product obtained as a foam was crystallized in absolute ethanol as white crystals: mp 116–118 °C; UV (ethanol 95) λ_{max} 228 nm, ε 25800; ^{1}H-NMR (DMSO-d_6) δ 8.45 (s, 1H, H-6), 7.9–7.4 (m, 12H, 2PhCO and NH_2), 5.83 (d, 1H, H-1′, $J_{1'-2'} = 2.5$ Hz), 5.74 (d, 1H, H-3′, $J_{3'-4'} = 4.2$ Hz), 5.54 (t, 1H, H-2′, $J_{1'-2'} = J_{2'-3'} = 2.1$ Hz), 4.8–4.6 (m, 3H, H-4′, H-5′, H-5″); MS (FAB > 0, G/T): 495 $[M+H]^+$, 383 $[S]^+$, 105 $[PhCO]^+$ (FAB < 0, G/T): 493 $[M-H]^-$, 111 $[B]^-$, 121 $[PhCO_2]^-$. $[\alpha]_D^{20} = -95$ (c 0.98, DMSO).

4.1.6. 1-(β-L-xylofuranosyl)5-azacytosine ***5***

To a stirred suspension of 1-(2-*O*-acetyl-3,5-di-*O*-benzoyl-β-L-xylofuranosyl)5-azacytosine (0.3g, 0.61mmol) in dry methanol (1.8 mL), a methanolic solution of sodium methanolate (0.02 M, 0.3 mL, 0.06 mmol) was slowly added at room temperature and under argon. After 4h of stirring, the mixture was left overnight at room temperature, and 1-(β-L-xylofuranosyl)5-azacy-

tosine was filtered and washed with methanol. The solid obtained as a white powder was recrystallized in methanol with a minimum of water (114 mg, 77%): mp 110–111 °C; UV (ethanol 95) λ_{max} 244 nm, ε 4990; ^{1}H-NMR (DMSO-d_6) δ 8.18 (s, 1H, H-6), 7.39 (bs, 2H, NH_2), 5.75 (d, 1H, OH-2′, $J_{2'\text{-OH}} = 3.7$ Hz), 5.50 (s, 1H, H-1′), 5.31 (d, 1H, OH-3′, $J_{3'\text{-OH}} = 2.8$ Hz), 4.74 (t, 1H, OH-5′, $J_{5'\text{-OH}} = J_{5''\text{-OH}} = 5.6$ Hz), 4.11 (m, 1H, H-4′), 3.92 (d, 1H, H-2′, $J_{2'\text{-OH}} = 2.9$ Hz), 3.85 (bs, 1H, H, H-3′), 3.7–3.6 (m, 2H, H-5′, H-5″); MS (FAB > 0, G/T): 245 $[M + H]^+$, (FAB < 0, G/T): 243 $[M - H]^-$: $[\alpha]_D^{20} = +15$ (c 1.02, DMSO). Anal. $C_8H_{12}N_4O_5$ (C, H, N).

4.1.7. 1-(3,5-Di-O-benzoyl-β-L-xylofuranosyl)5-azacytosine

To a solution of 1-(2-*O*-acetyl-3,5-di-*O*-benzoyl-β-L-xylofuranosyl)5-azacytosine (5 g, 10.1 mmol) in a mixture of acetic acid (20 mL) and pyridine (85 mL), hydrazine hydrate 80 % (6.2 mL, 0.10 mol) was added at room temperature under stirring. After 3h, the reaction was stopped by adding 50 mL of acetone and stirring was continued for 45 min. After partial evaporation, the mixture was poured in water (200 mL) and ethyl acetate was added (200 mL). The organic layer was washed with a saturated aqueous solution of sodium hydrogenocarbonate (2 × 200 mL) and with water (2 × 200 mL). After drying and evaporation, the residue was chromatographed on a silica gel column (eluent: 0 to 5 % gradient of methanol in dichloromethane) giving the product as a solid (2.06 g, 45 %) which was recrystallized in ethanol with a minimum of dichloromethane: mp 110–111 °C; UV (ethanol 95) λ_{max} 227 nm, ε 25300; ^{1}H-NMR (DMSO-d_6) δ 8.61 (s, 1H, H-6), 7.9–7.4 (m, 12H, 2PhCO and NH_2), 6.38 (bs, 1H, OH-2′), 5.64 (s, 1H, H-1′), 5.37 (d, 1H, H-3′, $J_{2'\text{-}3'} = 3.5$ Hz), 4.8–4.6 (m, 3H, H-4′, H-5′, H-5″), 4.37 (s, 1H, H-2′); MS (FAB > 0, G/T): 905 $[2M + H]^+$, 341 $[S]^+$, 113 $[BH_2]^+$, 105 $[PhCO]^+$, (FAB < O, G/T): 903 $[2 - H]^-$, 111 $[B]^-$, 121 $[PhCO_2]^-$; $[\alpha]_D^{20} = -123$ (c 1.35, DMSO).

4.1.8. 1-(2-Deoxy-3,5-di-O-benzoyl-β-L-threo-pentofuranosyl)5-azacytosine **10**

To a solution of 1-(3,5-di-*O*-benzoyl-β-L-xylofuranosyl)5-azacytosine (1.27 g, 2.81 mmol) in dry acetonitrile (86.5 mL), phenoxy(thiocarbonyl) chloride (0.58 mL, 4.21 mmol) and 4-dimethylaminopyridine (1.03 g, 8.43 mmol) were added under stirring. The mixture was left under stirring for 2h at room temperature, then it was evaporated under vacuum. The residue was dissolved in dichloromethane (50 mL), and the solution was washed with water (2 × 50 mL), with cold hydrochloric acid (0.5 N, 50 mL) and again with water (2 × 50 mL). Drying and evaporation gave the 2′-*O*-phenyloxycarbonyl intermediate which was coevaporated with anhydrous dioxane then dissolved in the same solvent (22.7 mL). To this solution, tris(trimethylsilyl)silane (1.05 mL, 7.03 mmol) and α,α'-azoisobutyronitrile (0.14 g, 0.84 mmol) were added, and the mixture was heated at 100 °C for 2h. After cooling and evaporation, the residue was chromatographed on silica gel using a 0–2% gradient of methanol in dichloromethane. 1-(2-Deoxy-3,5-di-*O*-benzoyl-β-L-*threo*-pentofuranosyl)5-azacytosine (0.91 g, 75 %) was obtained as a white solid which was recrystallized in absolute ethanol: mp 170–173 °C; UV (ethanol 95) λ_{max} 228 nm, ε 25700; ^{1}H-NMR (DMSO-d_6) δ 8.51 (s, 1H, H-6), 7.9–7.4 (m, 12H, 2PhCO and NH_2), 6.15 (q, 1H, H-1′, $J_{1'\text{-}2'} = 7.4$ Hz, $J_{1'\text{-}2''} = 1.7$ Hz), 5.68 (t, 1H, H-3′, $J_{2'\text{-}3'} = J_{3'\text{-}4'} = 5.2$ Hz), 4.8–4.6 (m, 3H, H-4′, H-5′, H-5″), 3.0–2.9 (m, 1H, H-2′), 2.4 (ld, 1H, H-2″, $J_{2'\text{-}2''} = 15.4$ Hz); MS (FAB > 0, G/T): 873 $[2M + H]^+$, 437 $[M + H]$, 325 $[S]^+$, 113 $[BH_2]^+$, 105 $[PhCO]^+$, (FAB < 0, G/T): 435 $[M - H]^-$, 121 $[PhCO_2]^-$; $[\alpha]_D^{20} = -138$ (c 1.05, DMSO).

4.1.9. 1-(2-Deoxy-β-L-threo-pentofuranosyl)5-azacytosine **6**

A solution of sodium methanolate in methanol (0.2 M, 0.23 mL, 0.05 mmol) was added to a suspension of 1-(2-deoxy-3,5-di-*O*-benzoyl-β-L-*threo*-pentofuranosyl)-5-azacytosine (225 mg, 0.52 mmol) in anhydrous methanol (3.9 mL) under argon. After 2h at room temperature, 1-(2-deoxy-β-L-*threo*-pentofuranosyl)5-azacytosine precipitated as a white solid which was filtered, washed with methanol, and recrystallized in methanol with a minimum of water (92 mg, 77 %): mp 180–182 °C; UV (ethanol 95) λ_{max} 243 nm, ε 5000 ^{1}H-NMR (DMSO-d_6) δ 8.35 (s, 1H, H-6), 7.40 (bs, 2H, NH_2), 5.92 (d, 1H, H-1′, $J_{1'\text{-}2'} = 6.7$ Hz), 5.19 (bs, 1H, OH-5′), 4.75 (bs, 1H, OH-3′), 3.9–3.6 (m, 3H, H-4′, H-5′, H-5″), 2.4 (m, 1H, H-2′), 1.95 (d, 1H, H-2″, $J_{2'\text{-}2''} = 14.4$ Hz); MS (FAB > 0, G/T) 229 $[M + H]^+$, (FAB < 0, G/T) 227 $[M - H]^-$; $[\alpha]_D^{20} = +15$ (c 1.02, DMSO). Anal. $C_8H_{12}N_4O_4$ (C, H, N).

4.2. Enzymatic studies

Recombinant human deoxycytidine kinase has been prepared and purified using human dCK cDNA [48] cloned into the pET19b vector [49]. The preparation

contained a histidine tag sequence and was more than 90% pure. The presence of the histidine tag had no effect on the substrate kinetics of the enzyme [49]. The specific activity of purified dCK was 60 U/mg protein, 1 unit of enzymatic activity being defined as the amount of enzyme catalysing the phosphorylation of 1 nmol of β-D-2′-deoxycytidine/min at 37 °C. Recombinant human cytidine deaminase was prepared and purified as reported [40]. The specific activity of pure CDA was 62 U/mg protein, 1 unit being defined as the amount of enzyme catalysing the deamination of 1 μmole of β-D-cytidine per min at 37 °C. β-D-dC, β-D-5-azaC and β-D-5-azadC were obtained from Sigma.

In kinetic studies with dCK, the reaction medium contained 50 mM Tris–HCl pH 7.6, 5 mM ATP, 5 mM $MgCl_2$, 1 mM dithiothreitol, 15 mM KCl, a concentration of substrate in the range of 50 to 500 μM depending on the experiment, and 30 μg/mL of dCK. The reaction was run at 37 °C until about 10 % of the substrate had been transformed, and experiment length did not exceed 30 min. The compounds were considered as non substrates of the enzyme if there was no phosphorylation after 1 h at 37 °C in the presence of dCK 500 μg/mL. Analysis of the reaction medium was performed by HPLC on a Hypersil ODS 3 μ column using a Waters System Alliance 2690 equipped with a diode array detector Waters 996. 10 min of isocratic elution using eluent A [5 mM Pic A-Waters (ion pairing agent: tetrabutylammonium hydrogen sulfate and phosphoric acid)] followed by a 30-min gradient from eluent A to eluent B (5 mM Pic A in 50% aqueous acetonitrile) allowed the separation of all reactants and products using a flow rate of 1 mL/min. The retention times in min of each substrate and its 5′-monophosphate (when observed) were, respectively: **1** or **3** (2.1), **2** or **4** (3.5, 11.2), **5** (3.1), **6** (4.1). The retention times of ATP and ADP were 24.6 min and 21 min, respectively. Identifications of products resulted from their UV spectra which were determined at several wavelengths to identify the eventual decomposition products. Based on the known absorptions of the decomposition products [11, 24], it was estimated that less than 5 % of the substrate was decomposed at the end of any kinetic study. For each concentration of substrate, the kinetic curves were determined by at least three measurements of substrate transformation in function of time. Using the GraFit program (Erithacus Software, 1992), the initial rates were obtained and used to determine the apparent V_m and K_m parameters and standard deviations according to the Lineweaver–Burk method.

In kinetics of deamination catalysed by CDA, the reaction mixture consisted of Tris–HCl 0.1 M pH 7.5, the substrate (100–400 μM) and the enzyme (4.2 μg/mL) at 25 °C. To hold substrate decomposition below 5%, the kinetic duration was limited to 5 min and it was assumed that the rate measured after that time and under these conditions was not very different from the initial rate. Kinetics were followed by HPLC as in the case of dCK but using instead an isocratic elution with triethylammonium acetate 20 mM pH 6.6 at a flow rate of 1 mL/min. The retention times in min of the substrates were: **1** (3.15), **2** (5.25), **3** (3.10), **4** (5.30), **5** (3.15), and **6** (4.00). The corresponding products of reversible decomposition were eluted at 2.3 min, 6.4 min, 2.25 min, 6.4 min, 2.4 min, and 5.00 min, respectively. Under these conditions, no deamination product of compounds **1** and **2** was detected using UV detection between 220 and 400 nm, and especially at 240 nm near the maximum of absorption of 1-methyl-5-azauracil [50]. For this reason, the progress of reaction was measured solely from the decrease in intensity of the peak due to the reactant. This was possible with good accuracy because of the precision and reproducibility of the automatic injector of the Waters System Alliance 2690. A nucleoside analogue was considered as non substrate if there was no detectable reaction after incubation of the compound in 400 μM concentration in the presence of CDA (42 μg/mL) for 1 h at 25 °C.

Acknowledgements

This work was financed by the Agence Nationale de Recherches sur le SIDA (ANRS, France) and the Centre National de la Recherche Scientifique (CNRS, France). We thank Dr A.-M. Aubertin (Université Louis Pasteur, Strasbourg, France) and Professor J.-P. Sommadossi (University of Alabama at Birmingham, AL, USA) for the results of the biological evaluations.

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