Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical Pharmacology 68 (2004) 711–718
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Enantioselectivity of ribonucleotide reductase: a first study using stereoisomers of pyrimidine 2′-azido-2′-deoxynucleosides

Béatrice Roy[a], Annalisa Verri[b], Andrea Lossani[b], Silvio Spadari[b], Federico Focher[b], Anne-Marie Aubertin[c], Gilles Gosselin[a], Christophe Mathé[a], Christian Périgaud[a,*]

[a]*UMR 5625 CNRS-UM II, Université Montpellier II, case courrier 008, place E. Bataillon, 34095 Montpellier Cedex 5, France*
[b]*Istituto di Genetica Molecolare IGM-CNR, CNR, Via Abbiategrasso 207, I-27100 Pavia, Italy*
[c]*Unité 544 I.N.S.E.R.M.-Université L. Pasteur, Institut de Virologie de la Faculté de Médecine, 3, rue Koeberlé, 67000 Strasbourg, France*

Received 31 October 2003; accepted 6 May 2004

Abstract

In this paper, the enantioselectivity of ribonucleotide reductase (RNR, EC 1.17.4.1), a pivotal enzyme involved in DNA biosynthesis, was studied using the β-D and β-L stereoisomers of 2′-azido-2′-deoxynucleosides of uracil and cytosine. The corresponding 5′-diphosphate derivatives in the D-configuration have been extensively studied as mechanism-based inhibitors of the enzyme. The original L-enantiomers were synthesized and evaluated in vitro. In cell culture experiments, only the cytosine derivative with a D-configuration was found cytostatic and able to deplete dNTP pools in response to RNR inhibition. In the case of the uracil enantiomeric pair, this result correlates with an inefficient intracellular monophosphorylation as demonstrated in testing their substrate properties against human uridine-cytidine kinase 1. Regarding cytosine analogues, human deoxycytidine kinase was found to be able to phosphorylate both enantiomers with comparable efficiency but only the D-stereoisomer was active in human cell culture. The interaction of the β-D and β-L stereoisomers of 2′-azido-2′-deoxyuridine 5′-diphosphate with purified *Escherichia coli* RNR was also examined. Inactivation of the enzyme was only observed in the presence of the D-stereoisomer, demonstrating that RNR exhibits enantiospecificity with respect to the natural configuration of the sugar moiety, as far as 2′-azido-2′-deoxynucleotides are concerned.

Keywords: Ribonucleotide reductase; Enantioselectivity; L-Nucleosides; 2′-Azidonucleosides; Deoxycytidine kinase; Uridine-cytidine kinase

1. Introduction

The therapeutic efficacy of nucleoside analogues depends on their ability to mimic natural counterparts, thus interacting with viral and/or cellular enzymes and inhibiting important processes in the metabolism of nucleic acids and their components. This requires the metabolization of nucleosides to their corresponding phosphorylated forms by kinases and/or other activating enzymes virally encoded or naturally occurring in cells. In the last years, L-nucleoside analogues have drawn considerable attention as antiviral or antitumoral agents [1–3]. This result has hastened studies about the enantioselectivity of enzymes involved in nucleotide biosynthesis and possible relevance for the development of novel drugs with the inverted optical configuration [4,5]. Work on this topic focused on nucleoside kinases catalyzing the first, often rate-limiting, phosphorylation step of nucleoside analogues [6]. For example, deoxycytidine kinase (dCK, EC 2.7.1.74), which is involved in the activation of various antiviral and antitumoral nucleosides, is characterized by a considerably low selectivity both for the substrate and for the phosphate

Abbreviations: ATP, adenosine 5′-triphosphate; CMP, cytidine 5′-monophosphate; CDP, cytidine 5′-diphosphate; dCK, 2′-deoxycytidine kinase; D-Cyd, β-D-cytidine; D-dCyd, β-D-2′-deoxycytidine; dNTPs, 2′-deoxyribonucleoside 5′-triphosphates; DTT, dithiothreitol; HIV, human immunodeficiency virus; N_3-D-dCyd, 2′-azido-2′-deoxy-β-D-cytidine; N_3-L-dCyd, 2′-azido-2′-deoxy-β-L-cytidine; N_3-D-dCydDP, 2′-azido-2′-deoxy-β-D-cytidine 5′-diphosphate; N_3-D-dUrd, 2′-azido-2′-deoxy-β-D-uridine; N_3-L-dUrd, 2′-azido-2′-deoxy-β-L-uridine; N_3-D-dUrdDP, 2′-azido-2′-deoxy-β-D-uridine 5′-diphosphate; N_3-L-dUrdDP, 2′-azido-2′-deoxy-β-L-uridine 5′-diphosphate; RNR, ribonucleotide reductase; D-Urd, β-D-uridine; UCK1, uridine-cytidine kinase 1; UMP, uridine 5′-monophosphate

* Corresponding author. Tel.:+33 467144776; fax: +33 467042029.
E-mail address: perigaud@univ-montp2.fr (C. Périgaud).

0006-2952/$ – see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2004.05.002

donor. This enzyme phosphorylates not only 2′-deoxycytidine, but also 2′-deoxyadenosine, 2′-deoxyguanosine and several pyrimidine and purine deoxyribonucleosides modified both on the base and on the sugar ring [4,7,8]. Similarly to herpes virus thymidine kinases [4,9–12], dCK lacks enantioselectivity, being able to phosphorylate both enantiomers of dCyd [7] and some L-nucleoside analogues [4,7,8]. Moreover, dCK can also use L-ATP as phosphate donor [13]. These results prompted us to evaluate the enantioselectivity of other cellular enzymes. In this respect, ribonucleotide reductase (RNR, EC 1.17.4.1) plays a central role in DNA biosynthesis and repair. This enzyme catalyzes the reduction of all four ribonucleotides into 2′-deoxyribonucleotides [14,15] and constitutes a target for antiproliferative agents as illustrated by the anticancer drugs hydroxyurea [16] and gemcitabine [17]. Furthermore, the use of inhibitors of RNR in association to anti-HIV nucleoside analogues has been suggested as an additional strategy in the treatment of AIDS [18].

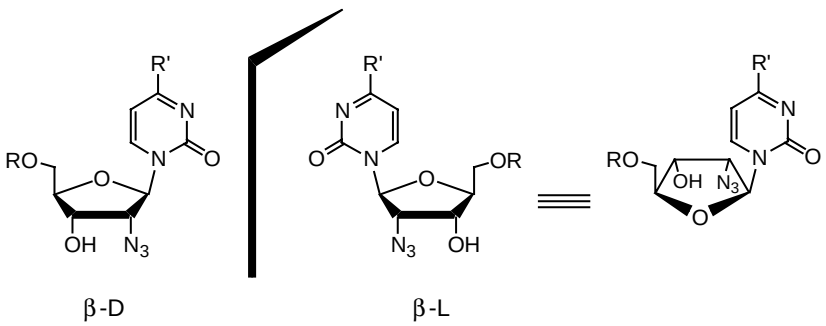
Herein, we report the first study about the enantioselectivity of RNR using, as biochemical tools, enantiomeric pairs of 2′-azido-2′-deoxynucleoside derivatives of uracil and cytosine (Fig. 1). Indeed, the D-nucleoside 5′-diphosphates have been shown to be potent inhibitors of RNR and the in vitro biological activity as well as cellular metabolism of the parent nucleosides have been investigated [19–21]. In this study, the hitherto unknown L-nucleosides have been synthesized. Their effects on tumor cell growth and dNTP pools were evaluated in comparison to their antipodes. The substrate properties of the four tested nucleosides were also determined by using recombinant human dCK and uridine-cytidine kinase 1 (UCK1, EC 2.7.1.48). Finally, the interaction of 2′-azido-2′-deoxy-β-L-uridine 5′-diphosphate with purified *E. coli* RNR, the prototype for human RNR, was studied.

2. Materials and methods

2.1. Chemicals

Bacterial media components were from Difco. Restriction and modification enzymes were from Promega, Sigma or Roche Molecular Biochemicals. Cytidine was from Roche Molecular Biochemicals. [^{3}H]-deoxycytidine 25 Ci/mmol and [^{3}H]-uridine 25 Ci/mmol were from ICN. [^{3}H]CDP (specific activity: 28 Ci/mmol) was from Amersham France SA. Tris(tetra-*n*-butylammonium) hydrogen pyrophosphate was purchased from Sigma.

N_3-D-dUrd and N_3-D-dCyd were synthesized following established protocols [22]. N_3-L-dUrd and N_3-L-dCyd were stereospecifically synthesized by multistep reaction sequences from 2,2′-anhydro-L-uridine [23] which was converted into the corresponding 2′-azido-2′-deoxy derivatives following the same protocol developed for the synthesis of N_3-D-dUrd and N_3-D-dCyd. Their structures and purities were ascertained. The physico-chemical properties of the 2′-azido-2′-deoxy L-nucleosides were identical (except for the $[\alpha]_D^{20}$ value) to those corresponding to the D-nucleosides [24,25]. The synthesis of the nucleotides N_3-D-dUrdDP and N_3-L-dUrdDP involved the 5′-tosylation of the corresponding nucleosides, followed by displacement of the leaving group by pyrophosphate [26,27]. Nucleotides were purified on DEAE-Sephadex A-25 (elution: linear gradient of TEAB pH 7.6 from 10 to 300 mM) followed by chromatography on RP18 (elution: water to methanol 50%). The triethylammonium counter ions were exchanged to sodium by passing the nucleotide solution through a DOWEX-AG 50WX2-400 column (Fluka). Yields were 12–13%. N_3-D-dUrdDP: $[\alpha]_D^{20}$ +3 (*c* 1.0, H_2O); N_3-L-dUrdDP: $[\alpha]_D^{20}$ −3 (*c* 1.0, H_2O); ^{1}H NMR (D_2O, 400 MHz) δ 7.86 (d, 1H, $J = 8.1$, H_6), 5.89 (d, 1H,



R = H,	R' = OH :	N_3-D-dUrd	N_3-L-dUrd
	R' = NH_2 :	N_3-D-dCyd	N_3-L-dCyd
R = $P_2O_6^{3-}$, R' = OH :		N_3-D-dUrdDP	N_3-L-dUrdDP

Fig. 1. Structures of the studied pyrimidine β-L and β-D-2′-azido-2′-deoxynucleosides and their 5′-diphosphate derivatives.

$J = 5.1$, $H_{1'}$), 5.85 (d, 1H, $J = 8.1$, H_5), 4.53 (pt, 1H, $J = 5.1$, $H_{3'}$), 4.30 (pt, 1H, $J = 5.3$, $H_{2'}$), 4.22–4.05 (m, 3H, $H_{4'}$, $H_{5'}$, $H_{5''}$); ^{13}C NMR (D_2O, 400 MHz) δ 166.2, 151.5, 141.3, 102.6, 86.9, 83.5, 69.8, 65.4, 64.2; ^{31}P NMR (D_2O, 400 MHz) δ −9.7 (d, $J_{P-P} = 20.6$), −11.2 (d, $J_{P-P} = 20.6$); MS FAB$^+$ *m/z* 474 $(M + H)^+$, 452 $(M - Na + 2H)^+$, 430 $(M - 2Na + 3H)^+$; FAB$^-$ *m/z* 472 $(M - H)^-$, 450 $(M - Na)^-$; UV (H_2O) λ_{max} 260 nm (ε 9200); HRMS ($C_9H_{12}O_{11}N_5P_2Na_2$), calcd 473.9804, found 473.9817 for N_3-D-dUrdDP, found 473.9856 for N_3-L-dUrdDP.

2.2. Cell lines and MTT cell viability assay

The human MT-4 and T_4-lymphoblastoid CEM/SS cell lines, obtained from NIH AIDS Research and Reference Reagent Program, were maintained at 37 °C in RPMI 1640–GlutamaxI medium (Invitrogen) supplemented with 25 mM HEPES (pH 7.4), 100 IU/mL penicillin, 100 μg/mL streptomycin, and 10% heat-inactivated fetal calf serum.

The growth of CEM/SS and MT-4 cells was determined after 5 days of incubation in absence or presence of different concentrations of the tested compounds, using the MTT method [28]. Results were expressed in CC_{50}, the concentration required to reduce cell viability by 50%.

2.3. Construction of recombinant bacterial expression vector for human dCK and UCK1

One microgram of total RNA extracted from HeLa cells using the Rneasy Mini kit (Qiagen) was retrotranscribed by using RT–PCR method.

In order to clone human dCK, total cDNA was then amplified using the following specific primers: 5′-GGAATGGCTAGCCCGCCCAAGAGAAGCTGCCCG-3′ (primer 1, sense) and 5′-AGCGAATTCACAAAGTACTCAAAAACTCTTTGACC-3′ (primer 2, antisense), presenting the *Nhe*I and *Eco*RI restriction sites, respectively. The amplified region (793 bp long), containing the complete dCK coding sequence, was inserted into pTrcHisA (Invitrogen) (pHis-dCK). His-tagged dCK sequence encodes for the complete 271 amino acids enzyme containing a 6-His tag at its NH_2-terminus and the substitution of the third amino acid of the cDNA from threonine to serine.

To clone human UCK1 total cDNA was amplified by using the following specific primers: 5′-GAGATGGCTAGCGCGGGAGGCGAAGACTGCG-3′ (primer 1, sense) and 5′-TGCGCCGAGAGGAATTCGTGGGTGTGGG-3′ (primer 2, antisense), presenting the *Nhe*I and *Eco*RI restriction sites, respectively. The amplified region (935 bp long), containing the complete UCK1 [29] coding sequence, was inserted in pTrcHisA (pHis-UCK1). His-tagged UCK1 sequence encodes for the complete 277 amino acids enzyme with a 6-His tag at its NH_2-terminus. The sequence of the recombinant UCK1 shows a single nucleotide difference (G_{284} in place of A_{284}), when compared to the published cDNA sequence [29], which leads to an amino acid substitution, namely Gly_{95} in place of Asp_{95}.

2.4. Expression and purification of recombinant human dCK and UCK1 from bacterial cells

Expression and purification of the human dCK and UCK1 were carried out as described by the manufacturer of the Ni-NTA Superflow resin (Qiagen). Briefly, two fresh overnight saturated cultures of *E. coli* (DH5α strain) transformed with pHis-dCK or pHis-UCK1, respectively, were diluted 1:100 in 1 l of 2XYT broth [30] containing ampicillin (60 μg/mL) and incubated at 37 °C with shaking. At 0.6 OD_{600}, isopropylthio-β-D-galactoside (IPTG, Sigma) was added to a final concentration of 1 mM, and the cultures were incubated for further 4 h at 37 °C. The bacterial cell pellets were independently resuspended in four volumes of lysis buffer (50 mM sodium phosphate (pH 8.0), 300 mM NaCl, 10 mM imidazole, 1 mM PMSF and 1 mg/mL lysozyme) and incubated on ice for 30 min. Cells were then sonicated on ice, and the lysates were centrifuged at 10,000 × *g* for 30 min at 4 °C. Supernatants were then independently loaded on a Ni-NTA Superflow column (1 mL) at a flow rate of 0.25 mL/min. The column was first washed with lysis buffer and then with 50 mM sodium phosphate buffer (pH 8.0), containing 300 mM NaCl and 20 mM imidazole. The protein was then step-eluted with 250 mM imidazole in 50 mM sodium phosphate buffer (pH 8.0), 300 mM NaCl and 1 mg/mL BSA. Fractions were collected for enzymatic activity analysis. The enzymes, collected from peak fractions, was dialyzed, against 50 mM Tris–HCl (pH 7.5), containing 20% glycerol and 1 mM DTT, and then frozen in liquid nitrogen until used.

2.5. dCK and UCK1 assays

dCK was assayed with a radiochemical method which measures the formation of [^{3}H]dCMP from [^{3}H]dCyd as previously described [7]. UCK1 was assayed with a radiochemical method which measures the formation of [^{3}H]UMP from [^{3}H]Urd. The enzyme was incubated at 37 °C in 25 μL of a mixture containing 50 mM Tris–HCl (pH 7.4), 10 mM $MgCl_2$, 10 mM ATP, 1 mM DTT and 1 mM [^{3}H]Urd (5 cpm/pmol). Then, UCK1 assay follows the procedure described for dCK [7]. When nucleoside analogues were tested as possible substrates of human kinases, 100 μM of each compound was incubated at 37 °C for 30 min in a mixture (25 μL) containing 30 mM HEPES K^+ (pH 7.5), 6 mM $MgCl_2$, 6 mM ATP, 0.5 mM DTT and an amount of enzyme of dCK or UCK1 which phosphorylates approximately 0.5–1.5 nmol of the natural substrate, dCyd and Urd, respectively. Samples were then heated at 100 °C for 5 min and centrifuged for 15 min at 8000 × *g* in an Eppendorf bench fuge. Supernatants were then subjected to HPLC analysis.

2.6. HPLC separation of nucleosides and nucleotides

The reverse phase chromatography method employing the HPLC system (Shimadzu) was used in order to separate nucleosides from nucleotides. An Alltima C18-Nuc 100A 5 U column (4.6 mm × 25 cm, Alltech) was used at room temperature in the following conditions: injection volume, 20 μL; UV detection at 260 nm; linear gradient (40 min): buffer A (20 mM KH_2PO_4, pH 7.5) to buffer B (20 mM KH_2PO_4, pH 5.2, 60% methanol); flow rate: 0.5 mL/min.

2.7. Treatment of CEM/SS and determination of dNTPs

CEM/SS cells (0.8×10^6 cells/mL) were distributed in microtest plates (2 mL/well), afterwards 105 μL of drugs (final concentration, 200 μM) were added. After 6 h at 37 °C, cells were collected, counted, monitored for viability by trypan blue staining, and then washed with ice-cold PBS. After centrifugation at 500 × *g* for 10 min, cell pellets were extracted with 60% cold methanol, heated for 2 min at 95 °C and centrifuged at 17,000 × *g* for 30 min. The supernatants were taken to dryness by lyophylization, and then resuspended in 50 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$. Quantification of dNTPs was performed using an enzymatic assay based on the catalytic elongation of radiolabeled oligonucleotide primers annealed to complementary templates [31].

2.8. Ribonucleotide reductase assay

E. coli ribonucleotide reductase proteins R1 and R2 were purified from overproducing strains as previously reported [32,33]. In brief, 2 μM of RNR was incubated for 2 h at 30 °C in the presence of 0.3–40 μM N_3-D-dUrdDP or N_3-L-dUrdDP. Afterwards, the mixture was diluted 10-fold by adding the substrate solution and incubated for 10 min at 30 °C to measure the residual RNR activity. We prepared the protein solution (70 μL) containing 3.3 μM R1, 3.3 μM R2, 17 mM DTT, 17 mM $MgCl_2$, 22 μM *E. coli* thioredoxin (IMCO Corporation Ltd AB) and 2.5 mM ATP in 58 mM HEPES buffer (pH 7.6). We also prepared the substrate solution (945 μL) containing 0.56 mM CDP, 21 μCi of [^{3}H]CDP, 10 mM DTT, 10 mM $MgCl_2$, 13 μM *E. coli* thioredoxin and 1.5 mM ATP in 35 mM HEPES buffer (pH 7.6). The protein and the substrate solutions were prepared just before use and kept at 4 °C. The protein solution (3 μL) was distributed in eppendorf tubes. To each tube was added 2 μL of an aqueous solution of N_3-L-dUrdDP or N_3-D-dUrdDP; the final concentration ranged between 0.3 and 40 μM. In the control experiment, 2 μL of water was added to 3 μL of the protein solution. The samples were incubated for 2 h at 30 °C and then briefly centrifuged to recover the whole volume. The enzymatic reaction was started by adding 45 μL of the substrate solution. After 10 min at 30 °C, the reaction was stopped by heating at 95 °C for 2 min. Nucleotides were converted to their corresponding nucleosides by treatment for 2 h at 37 °C with 5 μL of *Crotalus adamanteus* venom (200 μg/mL). At the end of hydrolysis, samples were heated at 90 °C for 2 min and diluted by adding 200 μL of water. Precipitates formed after heating were pelleted by centrifugation for 20 min at 17,000 × *g*. Supernatants were removed and analyzed by HPLC on a 5 μM Zorbax ODS C18 column connected to a Berthold LB 506 C-1 flow-through radioactivity monitor. An isocratic elution was performed at 1 mL/min with a 10 mM sodium acetate buffer (pH 4.8), 4% methanol, supplemented with 1% of the ion-pairing reagent pentanesulfonic acid (Waters, Low UV PIC-B5).

3. Results

3.1. Inhibition of cell growth by stereoisomers of 2′-azido-2′-deoxynucleoside derivatives of uracil and cytosine

The enantiomeric pairs of 2′-azido-2′-deoxynucleoside derivatives of uracil (N_3-D-dUrd and N_3-L-dUrd) and of cytosine (N_3-D-dCyd and N_3-L-dCyd) were comparatively evaluated for their inhibitory effects on cell growth in two human T_4-lymphoblastoid cells. In accordance with the literature [34,35], N_3-D-dCyd showed a significant toxicity with CC_{50} values at 7.2 ± 3.3 μM and 4.2 ± 1.3 μM in CEM/SS and MT-4, respectively (data not shown). The three other compounds failed to show any significant inhibitory activity at concentrations up to 100 μM.

3.2. Effects of pyrimidine 2′-azido-2′-deoxynucleosides on intracellular dNTPs

The effects of the studied nucleoside analogues on cellular RNR were evaluated indirectly by measuring their impact on dNTP pools in the CEM/SS cell line. Using HU as positive control, the concentrations of the four natural dNTPs were determined after cell incubation during 6 h with pyrimidine 2′-azido-2′-deoxynucleosides at 200 μM (Table 1). A comparable decrease in the dATP and in the dCTP pools was observed for HU and N_3-D-dCyd. This result is in accordance with the literature data showing that N_3-D-dCyd is able to be intracellularly converted into its corresponding diphosphate form (N_3-D-dCydDP), a potent inhibitor of RNR [19]. In contrast, N_3-L-dCyd, N_3-D-dUrd and N_3-L-dUrd had no impact on the four dNTP pools. It is interesting to note that N_3-D-dUrd is completely inactive on RNR in cell cultures whereas N_3-D-dUrdDP is a powerful inactivator of RNR, with efficiency comparable to N_3-D-dCydDP [19]. This discrepancy can be explained by the inefficient monophosphorylation of N_3-D-dUrd inside cells

Table 1
Effect of 2′-azidopyrimidines (200 μM) on the intracellular dNTP pools in CEM/SS after 6 h of incubation at 37 °C

Treatment	Picomoles of dNTP per 10^6 viable cells			
	dATP	dGTP	dCTP	dTTP
No drug control	26.1 ± 1.7	15.8 ± 1.5	12.0 ± 1.6	27.6 ± 1.1
HU	16.8 ± 1.6*	11.7 ± 1.2**	7.7 ± 0.5*	23.7 ± 3.2
N_3-D-dUrd	25.0 ± 0.8	15.5 ± 0.9	12.3 ± 0.6	27.4 ± 1.2
N_3-L-dUrd	28.3 ± 1.3	16.6 ± 0.9	12.6 ± 0.5	30.3 ± 1.9
N_3-D-dCyd	16.5 ± 3.2*	12.2 ± 1.4**	5.1 ± 1.2*	25.4 ± 3.6
N_3-L-dCyd	27.8 ± 2.5	16.8 ± 1.6	11.8 ± 0.9	27.2 ± 2.2

HU (200 μM) was tested as a reference. Data represent the mean ± S.D. of at least three independent experiments. *$P < 0.01$ or **$P < 0.05$ vs. the control value in Student's *t*-test.

Table 4
Percent of phosphorylation of pyrimidine nucleosides and pyrimidine nucleoside analogues by human dCK and UCK1

Compound	dCK	UCK1
D-dCyd	100	nd[a]
D-Cyd	37	100
N_3-D-dCyd	74	0
N_3-L-dCyd	78	0
D-Urd	0	100
N_3-D-dUrd	0	0
N_3-L-dUrd	0	0

Hundred percent of phosphorylation corresponds to 1.35 nmol of monophosphate produced in 30 min at 37 °C. Each compound was present at 100 μM (2.5 nmol/25 μl) in the assay.
[a] nd: not determined.

[21]. Consequently, we investigated the monophosphorylation of the four nucleoside analogues using human purified kinases, namely dCK and UCK1.

3.3. Cloning, expression and characterization of human dCK and UCK1

Human dCK and UCK1 were purified from *E. coli* crude extract in a single chromatographic step by using a Ni-NTA Superflow column. In the assay conditions reported in Section 2, recombinant dCK and UCK1 possess a specific activity of 1500 and 6700 U/mg, respectively (one unit is defined as the amount of enzyme catalyzing the formation of one nanomole of monophosphate in 1 h at 37 °C). Tables 2 and 3 report the kinetic parameters of the enzymes. Interestingly, recombinant UCK1 phosphorylates D-Urd following a biphasic kinetic (negative cooperativity) with K_m values of 0.225 and 4.14 mM, respectively at low (≤0.5 mM) or at high (≥0.5 mM) uridine concentration (Table 2). These data indicate that UCK1, at high substrate concentration, has a 20-fold reduced affinity for the substrate but a 7-fold higher V_{max}. The catalytic efficiency of the enzyme, calculated in both conditions as k_{cat}/K_m, indicates that UCK1 has a two- to three-fold higher catalytic efficiency at low (more physiological) substrate concentration.

3.4. Phosphorylation of pyrimidine 2′-azido-2′-deoxynucleosides

In assay conditions allowing the phosphorylation of 1.35 nmol of dCyd to dCMP in 30 min at 37 °C, human dCK was found able to phosphorylate approximately 1 nmol of both D- and L-N_3-dCyd (Table 4). Tested as substrates for human dCK, D- and L-N_3-dCyd showed K_m values with three and two orders of magnitude higher than those found for the natural substrate D-dCyd (0.13 μM), with values at 133 and 12.5 μM, respectively (Table 3). However, comparison of k_{cat}/K_m ratios for D-dCyd and its analogues demonstrates that the enzyme has catalytic efficiencies for the D- and L-enantiomers of N_3-dCyd which are, respectively, only 60- and 12-fold times lower than that of D-dCyd. Finally, tested as inhibitors of D-dCyd (0.2 μM) phosphorylation, D- and L-N_3-dCyd showed IC_{50} values at 220 μM and 45 μM, respectively (data not shown). In marked contrast to dCK, UCK1 was found highly specific for the natural substrates D-Urd and D-Cyd, being unable to phosphorylate their L-enantiomers (data not shown). Here

Table 2
Kinetic properties of recombinant human UCK1 with ATP as phosphate donor

Substrate	K_m (mM)	V_{max} (pmol min^{-1} μg^{-1})	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} M^{-1})
D-Urd ≤ 0.5 mM	0.225	267	0.14	6.22×10^2
D-Urd ≤ 0.5 mM	4.14	1700	0.9	2.17×10^2

k_{cat} was calculated by using a molecular mass of 31 kDa.

Table 3
Kinetic properties of recombinant human dCK with ATP as phosphate donor

Substrate	K_m (M)	V_{max} (pmol min^{-1} μg^{-1})	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} M^{-1})
D-dCyd	1.30×10^{-7}	8.9	4.5×10^{-3}	3.46×10^4
N_3-D-dCyd	1.33×10^{-4}	141.0	7.12×10^{-2}	5.35×10^2
N_3-L-dCyd	1.25×10^{-5}	72.0	3.60×10^{-2}	2.88×10^3

k_{cat} was calculated by using a molecular mass of 30 kDa.

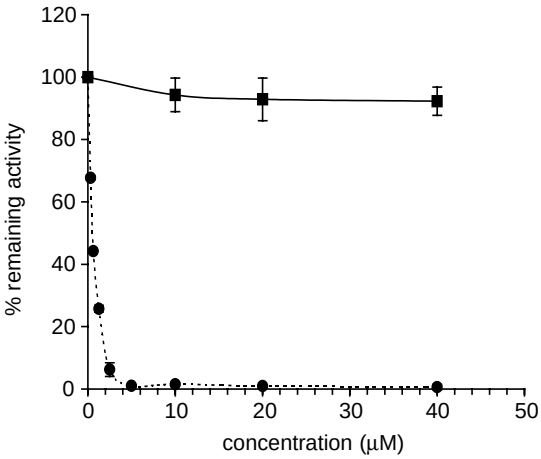


Fig. 2. Enantioselective inactivation of RNR by N_3-D-dUrdDP (●) is not observed with N_3-L-dUrdDP (■). RNR (2 μM) was preincubated for 2 h at 30 °C with each of the two 2′-azidonucleotides at the indicated concentrations. Afterwards, the mixture was diluted 10-fold by adding the radiolabeled substrate [^{3}H]CDP and incubated for 10 min at 30 °C to measure the residual RNR activity.

we found that it is also incapable of activating to monophosphate either Urd analogues or Cyd analogues (Table 4).

3.5. Influence of the natural configuration of the sugar moiety on RNR inhibition

In the literature, inactivation of RNR by N_3-D-dUrdDP and N_3-D-dCydDP has been studied using the *E. coli* enzyme, which is the prototype for the mammalian RNR [19,36–39]. To study the enantioselectivity of RNR, we compared the reactivity of N_3-D-dUrdDP and N_3-L-dUrdDP towards the enzyme. For inactivation experiments, a stoichiometric R1/R2 mixture was first incubated for 2 h at 30 °C with the nucleotide analogues in the presence of the allosteric effector ATP. The remaining catalytic activity was measured after an extended 10 min incubation in the presence of the radiolabeled substrate [^{3}H]CDP. Fig. 2 shows a dose-dependent decrease of RNR residual activity in the presence of N_3-D-dUrdDP. Approximately one equivalent of inhibitor resulted in the complete inactivation of the enzyme. This result is in agreement with literature [36]. By contrast, the L-enantiomer was found strictly inactive, even when a 20-fold excess of nucleotide over RNR was used.

4. Discussion

The aim of this study was to assess for the first time the enantioselectivity of RNR and also to find possible new inhibitors of the enzyme. For this purpose, we synthesized enantiomeric pairs of 2′-azido-2′-deoxynucleoside derivatives of uracil and cytosine. These structures were chosen on the basis of the potent inhibitory properties of N_3-D-dCydDP and of N_3-D-dUrdDP towards RNR.

We found that N_3-L-dUrd had no activity in human cell culture and was not phosphorylated by human dCK and UCK1. On the other hand, N_3-L-dCyd had no effect on dNTP pools of the human cell line CEM-SS in spite of being phosphorylated by human dCK. Since UMP/CMP kinase has a relaxed enantioselectivity for the substrate site [40], this result strongly suggests that N_3-L-dCydDP is inactive towards human RNR. We also carried out experiments using the pure recombinant *E. coli* enzyme, the prototype for mammalian RNR, in the presence of N_3-D-dUrdDP and N_3-L-dUrdDP. A stoichiometric inactivation of RNR was observed in the presence of N_3-D-dUrdDP, whereas the L-enantiomer was strictly inactive, even at high concentrations. Thus, using pyrimidine 2′-azido-2′-deoxynucleotides as models, we found that RNR exhibits enantiospecificity for the D-configuration of the sugar moiety.

Class I ribonucleotide reductases found in eukaryotes and *E. coli* are composed of two homodimeric subunits named R1 and R2. The large R1 contains the active site that binds all four physiological substrates (CDP, UDP, GDP, or ADP), in addition to two different allosteric sites that bind nucleoside triphosphate effector molecules (ATP, dATP, dTTP, dGTP). Subunit R1 also contains five redox active cysteines essential for catalysis. Subunit R2 harbors a stable free tyrosyl radical near an essential iron center. Binding of a substrate triggers electron and proton transfers from R2 to R1, leading to abstraction of H3′ from C3′ of the sugar ring [14,15]. Crystallographic studies have shown that the 3′-oxygen of the natural substrate is hydrogen bonded to the side chain of Glu441, whereas the 2′-oxygen atom is hydrogen bonded to the side chains of Asn437 and Cys225 [15]. Moreover, Cys439 is in van der Waals contact with the 3′-carbon atom in a suitable position for removing the 3′-hydrogen [15].

The substrate binding site of RNR can accommodate several nucleoside analogues modified on the sugar ring [41]. Experiments with isotopically labeled derivatives of N_3-D-dUrdDP have provided much insight into the mechanism of *E. coli* RNR inhibition. Inactivation is accompanied by the destruction of the catalytically essential tyrosyl radical, cleavage of the 3′-carbon-hydrogen bond, and production of 1 equivalent each of uracil, inorganic pyrophosphate, and nitrogen [37]. The R1 subunit is also covalently modified and partially inactivated by reaction with N_3-D-dUrdDP [37]. Our results indicate that 2′-azido-β-L-2′-deoxyuridine 5′-diphosphate is inactive against *E. coli* RNR. We assume that the 3′-carbon atom of 2′-azido-β-L-2′-deoxyuridine 5′-diphosphate may be too far from Cys 439 to remove the 3′-hydrogen atom, an essential step in the activity of 2′-azido-β-D-2′-deoxyuridine 5′-diphosphate. Moreover, the presence of an azido group in the 2′-position of 2′-azido-β-L-2′-deoxyuridine 5′-diphosphate may be detrimental for the binding of this nucleoside analogue to the active site of RNR. In continuation to this work, the interaction of the enantiomers of

the natural substrates, and also those of the natural allosteric effectors, with *E. coli* and mammalian ribonucleotide reductases should be studied.

Acknowledgments

This work was supported by EC Grant QLK3-CT-2001-00506. Financial support to B.R. by CNRS is gratefully acknowledged. We thank Michel Lepoivre and Hans Eklund for insightful discussions.

References

[1] Sommadossi JP, Antiviral β-L-nucleosides specific for hepatitis B virus infection. In: Chu CK, editor. Recent advances in nucleosides: chemistry and chemotherapy. Amsterdam: Elsevier; 2002. p. 417–32.
[2] Gumina G, Song GY, Chu CK. L-Nucleosides as chemotherapeutic agents. FEMS Microbiol Lett 2001;202:9–15.
[3] Zemlicka J. Enantioselectivity of the antiviral effects of nucleoside analogues. Pharmacol Ther 2000;85:251–66.
[4] Spadari S, Maga G, Verri A, Focher F. Molecular basis for the antiviral and anticancer activities of unnatural L-β-nucleosides. Exp Opin Invest Drugs 1998;7:1285–300.
[5] Focher F, Spadari S, Maga G. Antivirals at the mirror: the lack of stereospecificity of some viral and human enzymes offers novel opportunities in antiviral drug development. Curr Drug Targets Infect Disord 2003;3:41–53.
[6] Eriksson S, Munch-Petersen B, Johansson K, Eklund H. Structure and function of cellular deoxyribonucleoside kinases. Cell Mol Life Sci 2002;59:1327–46.
[7] Verri A, Focher F, Priori G, Gosselin G, Imbach JL, Capobianco M, et al. Lack of enantiospecificity of human 2′-deoxycytidine kinase: relevance for the activation of β-L-deoxycytidine analogs as antineoplastic and antiviral agents. Mol Pharmacol 1997;51:132–8.
[8] Maury G. The enantioselectivity of enzymes involved in current antiviral therapy using nucleoside analogues: a new strategy? Antivir Chem Chemother 2000;11:165–89.
[9] Spadari S, Maga G, Focher F, Ciarrocchi G, Manservigi R, Arcamone F, et al. L-Thymidine is phosphorylated by herpes simplex virus type 1 thymidine kinase and inhibits viral growth. J Med Chem 1992;35:4214–20.
[10] Spadari S, Maga G, Verri A, Bendiscioli A, Tondelli L, Capobianco M, et al. Lack of stereospecificity of some cellular and viral enzymes involved in the synthesis of deoxyribonucleotides and DNA: molecular basis for the antiviral activity of unnatural L-β-nucleosides. Biochimie 1995;77:861–7.
[11] Spadari S, Ciarrocchi G, Focher F, Verri A, Maga G, Arcamone F, et al. 5-Iodo-2′-deoxy-L-uridine and (*E*)-5-(2-bromovinyl)-2′-deoxy-L-uridine: selective phosphorylation by herpes simplex virus type 1 thymidine kinase, antiherpetic activity, and cytotoxicity studies. Mol Pharmacol 1995;47:1231–8.
[12] Maga G, Verri A, Bonizzi L, Ponti W, Poli G, Garbesi A, et al. Lack of stereospecificity of suid pseudorabies virus thymidine kinase. Biochem J 1993;294:381–5.
[13] Verri A, Montecucco A, Gosselin G, Boudou V, Imbach JL, Spadari S, et al. L-ATP is recognized by some cellular and viral enzymes: does chance drive enzymic enantioselectivity? Biochem J 1999;337:585–90.
[14] Jordan A, Reichard P. Ribonucleotide reductases. Annu Rev Biochem 1998;67:71–98.
[15] Eklund H, Uhlin U, Farnegardh M, Logan DT, Nordlund P. Structure and function of the radical enzyme ribonucleotide reductase. Prog Biophys Mol Biol 2001;77:177–268.
[16] Stevens MR. Hydroxyurea: an overview. J Biol Regul Homeost Agents 1999;13:172–5.
[17] Storniolo AM, Enas NH, Brown CA, Voi M, Rothenberg ML, Schilsky R. An investigational new drug treatment program for patients with gemcitabine: results for over 3000 patients with pancreatic carcinoma. Cancer 1999;85:1261–8.
[18] Garcia F, Plana M, Arnedo M, Ortiz GM, Miro JM, Lopalco L, et al. A cytostatic drug improves control of HIV-1 replication during structured treatment interruptions: a randomized study. AIDS 2003;17:43–51.
[19] Thelander L, Larsson B, Hobbs J, Eckstein F. Active site of ribonucleoside diphosphate reductase from *Escherichia coli*: inactivation of the enzyme by 2′-substituted ribonucleoside diphosphates. J Biol Chem 1976;251:1398–405.
[20] Akerblom L, Reichard P. Azidocytidine is a specific inhibitor of deoxyribonucleotide synthesis in 3T6 cells. J Biol Chem 1985;260:9197–202.
[21] Kang SH, Cho MJ. Biological activity and phosphorylation of 2′-azido-2′-deoxyuridine and 2′-azido-2′-deoxycytidine. Nucleosides Nucleotides 1998;17:1077–88.
[22] Mc Gee DPC, Vargeese C, Zhai Y, Kirschenheuter GP, Settle A, Siedem CR, et al. Efficient synthesis of 2′-amino-2′-deoxypyrimidine 5′-triphosphates. Nucleosides Nucleotides 1995;14:1329–39.
[23] Holý A. Nucleic acid components and their analogues. CLIII. Preparation of 2′-deoxy-L-ribonucleosides of the pyrimidine series. Collect Czech Chem Comm 1972;37:4073–87.
[24] Verheyden JPH, Wagner D, Moffat JG. Synthesis of some pyrimidine 2′-amino-2′-deoxynucleosides. J Org Chem 1971;36:250–4.
[25] Hobbs J, Sternbach H, Sprinzl M, Eckstein F. Polynucleotides containing 2′-amino-2′-deoxyribose and 2′-azido-2′-deoxyribose. Biochemistry 1973;12:5138–45.
[26] Davisson VJ, Davis DR, Dixit VM, Poulter CD. Synthesis of nucleotide 5′-diphosphates from 5′-O-tosyl nucleosides. J Org Chem 1987;52:1794–801.
[27] Wnuck SF, Chowdhury SM, Garcia PI, Robins MJ. Stereodefined synthesis of O3′-labeled uracil nucleosides. 3′-[^{17}O]-2′-azido-2′-deoxyuridine 5′-diphosphate as a probe for the mechanism of inactivation of ribonucleotide reductases. J Org Chem 2002;67:1816–9.
[28] Moog C, Wick A, Le Ber P, Kirn A, Aubertin AM. Bicyclic imidazo derivatives, a new class of highly selective inhibitors for the human immunodeficiency virus type 1. Antivir Res 1994;24:275–88.
[29] Van Rompay AR, Norda A, Linden K, Johansson M, Karlsson A. Phosphorylation of uridine and cytidine nucleoside analogs by two human uridine-cytidine kinases. Mol Pharmacol 2001;59:1181–6.
[30] Sambrook J, Fritsch EF, Maniatis T. Molecular cloning: a laboratory manual. New York: Cold Spring Harbor Laboratory; 1989.
[31] Roy B, Beuneu C, Roux P, Buc H, Lemaire G, Lepoivre M. Simultaneous determination of pyrimidine or purine deoxyribonucleoside triphosphates using a polymerase assay. Anal Biochem 1999;269:403–9.
[32] Sjöberg BM, Hahne S, Karlsson A, Jörnvall H, Göransson M, Uhlin BE. Overproduction and purification of the B2 subunit of ribonucleotide reductase from *Escherichia coli*. J Biol Chem 1986;261:5658–62.
[33] Larsson B, Karlsson A, Sahlin M, Sjöberg BM. Radical formation in the dimeric small subunit of ribonucleotide reductase requires only one tyrosine 122. J Biol Chem 1988;263:17780–4.
[34] Kang SH, Sinhababu AK, Cory JG, Mitchell BS, Thakker DR, Cho MJ. Cellular delivery of nucleoside diphosphates: a prodrug approach. Pharm Res 1997;14:706–12.
[35] Kang SH, Sinhababu AK, Cho MJ. Synthesis and biological activity of bis(pivaloyloxymethyl)ester of 2′-azido-2′-deoxyuridine 5′-monophosphate. Nucleosides Nucleotides 1998;17:1089–98.

[36] Salowe SP, Ator MA, Stubbe J. Products of the inactivation of ribonucleoside diphosphate reductase from *Escherichia coli* with 2′-azido-2′-deoxyuridine 5′-diphosphate. Biochemistry 1987;26:3408–16.

[37] Salowe S, Bollinger JM, Ator M, Stubbe J, Mc Cracken J, Peisach J, et al. Alternative model for mechanism-based inhibition of *Escherichia coli* ribonucleotide reductase by 2′-azido-2′-deoxyuridine 5′-diphosphate. Biochemistry 1993;32:12749–60.

[38] Van der Donk WA, Stubbe J, Gerfen GJ, Bellew BF, Griffin RG. EPR investigations of the inactivation of *E. coli* ribonucleotide reductase with 2′-azido-2′-deoxyuridine 5′-diphosphate: Evidence for the involvement of the thiyl radical of C225-R1. J Am Chem Soc 1995;117:8908–16.

[39] Sjöberg BM, Gräslund A, Eckstein F. A substrate radical intermediate in the reaction between ribonucleotide reductase from *Escherichia coli* and 2′-azido-2′-deoxynucleoside diphosphates. J Biol Chem 1983;258:8060–7.

[40] Pasti C, Gallois-Montbrun S, Munier-Lehmann H, Veron M, Gilles A-M, Deville-Bonne D. Reaction of human UMP-CMP kinase with natural and analog substrates. Eur J Biochem 2003;270:1784–90.

[41] Robins MJ. Mechanism-based inhibition of ribonucleotide reductases: new mechanistic considerations and promising biological applications. Nucleosides Nucleotides 1999;18:779–93.