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Cytosolic and mitochondrial deoxyribonucleotidases: activity with substrate analogs, inhibitors and implications for therapy

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Abstract

Nucleoside analogs act as prodrugs that must be converted to 5′-phosphates by intracellular kinases to become active in the treatment of viral and oncological diseases. Activation may be reversed by dephosphorylation if the 5′-phosphates are substrates for 5′-nucleotidases. Dephosphorylation by cytosolic enzymes decreases the efficacy of the analogs, whereas dephosphorylation by mitochondrial enzymes may decrease mitochondrial toxicity. Both effects may influence the outcome of therapy. We investigated the dephosphorylation of the 5′-phosphates of commonly used nucleoside analogs by two cytosolic (cN-II and dNT-1) and one mitochondrial (dNT-2) nucleotidase. Most uracil/thymine nucleotide analogs were dephosphorylated by all three human enzymes but cytosine-containing nucleotide analogs were inactive. Only cN-II showed some activity with the monophosphates of the two purine analogs 2-chloro-2′-deoxyadenosine and 9-β-D-arabinosylguanine. We conclude that overproduction of any of the three 5′-nucleotidases cannot explain development of resistance against cytosine analogs but that overproduction of cN-II could lead to resistance against purine analogs. Of the tested analogs, only (*E*)-5-(2-bromovinyl)-2′-deoxyuridine was preferentially dephosphorylated by mitochondrial dNT-2. We propose that in future developments of analogs this aspect be considered in order to reduce mitochondrial toxicity. We tested inhibition of dNT-1 and dNT-2 by a large variety of synthetic metabolically stable nucleoside phosphonate analogs and found one (PMcP-U) that inhibited dNT-1 and dNT-2 competitively and a second (DPB-T) that inhibited dNT-2 by mixed inhibition. Both inhibitors are useful for specific 5′-nucleotidase assays and structural studies and may open up possibilities for therapy.

Keywords: Nucleoside analogs; 5′-Nucleotidases; Prodrug activation; Mitochondrial toxicity; Resistance; Enzyme inhibitors

1. Introduction

5′-Nucleotidases are ubiquitous enzymes that dephosphorylate nucleoside monophosphates producing nucleosides and inorganic phosphate. The enzymes show varying specificities for the sugar and base moieties. The cDNAs of seven enzymes have been cloned [1–7]. We are particularly interested in two related 5′-nucleotidases that prefer deoxyribonucleotides as substrates, one located in the cytosol (=dNT-1) [4] and the other in mitochondria (=dNT-2) [5]. Both in humans and in the mouse the genes for dNT-1 and dNT-2 map to the same chromosome and have identical intron/exon organization. The amino acid sequences of all four proteins are approximately 50% identical, disregarding

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Abbreviations: cN-II, high K_M 5′-nucleotidase; dNT-1, cytosolic 5′(3′)-deoxyribonucleotidase; dNT-2, mitochondrial 5′(3′)-deoxyribonucleotidase; DPB-T, (*S*)-1-[2′-deoxy-3′,5′-*O*-(1-phosphono)benzylidene-β-D-*threo*-pentofuranosyl]thymine; HIV, human immunodeficiency virus; PMcP-U, (±)-1-*trans*-(2-phosphonomethoxycyclopentyl)uracil; HPLC, high pressure liquid chromatography; araC, 1-β-D-arabinofuranosylcytosine; araG, 9-β-D-arabinosylguanine; araT, 1-β-D-arabinosylthymine; AZT, 3′-azidothymidine; BVdU, (*E*)-5-(2-bromovinyl)-2′-deoxyuridine; CdA, 2-chloro-2′-deoxyadenosine; ddC, 2′,3′-dideoxycytidine; dFdC, 2′,2′-difluoro-2′-deoxycytidine; d4T, 2′,3′-didehydro-2′,3′-dideoxythymidine; FdU, 5-fluoro-2′-deoxyuridine; 3TC, 2′-deoxy-3′-thiacytidine; monophosphates are abbreviated by addition of MP to the abbreviation of nucleoside.

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the leader sequences for the two mitochondrial enzymes [8]. The structures of human dNT-2 [9] and dNT-1,[1] including the active site of the enzymes, were recently determined and a detailed reaction mechanism was proposed for their catalytic function.

We proposed that both dNT-1 and dNT-2 function in the homeostasis of deoxynucleoside triphosphate pools required for DNA synthesis [10]. dNT-2 has a narrow specificity for dUMP and dTMP among natural deoxyribonucleotides [5]. Also dNT-1 is very active with these two deoxyribonucleotides but, in addition, dephosphorylates other deoxyribonucleotides, notably dGMP and dIMP [4]. We found that in cultured cells the catabolic activity of mouse dNT-1 counteracted the anabolic activity of thymidine and deoxycytidine kinases, thereby preventing the accumulation of pyrimidine deoxyribonucleotides in the cytosol and providing evidence for a regulatory role of the enzyme [11]. dNT-2 probably has a similar intramitochondrial function with respect to thymidine and deoxyuridine nucleotides. In humans intramitochondrial accumulation of dTTP leads to a severe genetic disease [12], attesting to the importance of a strict control of this pool.

5′-Nucleotidases contribute to the outcome of the treatment of cancer and viral diseases with nucleoside analogs. To interfere with DNA synthesis the analogs must first be phosphorylated and activated by cytosolic kinases. The efficiency of this process may be compromised if the resulting 5′-monophosphate is a good substrate for a cytosolic nucleotidase. Within a group of related nucleosides, an analog whose phosphorylated form is a poor substrate for dNT-1 would be more effective than a related analog that is rapidly dephosphorylated. A further concern is that sensitivity of cells to therapy by a nucleoside analog and the development of resistance against treatment might be related to the activity of cytosolic 5′-nucleotidases [13]. Experiments from different laboratories have demonstrated that cells kept in culture in the presence of increasing concentrations of an analog and developing resistance against the analog may increase their content of a soluble 5′-nucleotidase [14–16]. In clinical studies with patients suffering from leukemia the success of treatment was posi-tively related to low levels of a cytosolic nucleotidase (high K_M 5′-nucleotidase = cN-II) in the patient's cells [17]. Also another cytosolic 5′-nucleotidase (=cN-I) may play a role as cell lines overproducing cN-I showed increased resistance towards CdA, dFdC and ddC [18]. It was suggested that nucleotidase inhibitors might increase the efficacy of some analogs [13].

Such considerations apply to cytosolic 5′-nucleotidases, including dNT-1. A different scenario can be envisaged for the mitochondrial dNT-2. Mitochondrial toxicity is a major problem during treatment with some nucleoside analogs, in particular during long-term treatment of HIV and hepatitis B virus [19]. The rationale of the treatment is to use analogs that selectively interfere with viral replication without affecting cellular nuclear DNA polymerases. In several cases the mitochondrial DNA polymerase is, however, affected and the ensuing deficit in mitochondrial DNA replication gives severe side-effects [20]. dNT-2 operates inside mitochondria and can there lower the concentration of the phosphorylated analogs provided that they are substrates for the enzyme. The monophosphate of an ideal nucleoside analog used in therapy thus should be a poor substrate for dNT-1 and other cytosolic nucleotidases but a good substrate for dNT-2.

Here we investigate the ability of the 5′-monophosphates of various nucleoside analogs used in cancer and virus therapy to act as substrates for dNT-2 and two cytosolic nucleotidases, dNT-1 and cN-II. We also describe experiments with two nucleoside phosphonoalkyl derivatives that are inhibitors of 5′-nucleotidases.

2. Materials and methods

2.1. Materials

The 5′-phosphates of the nucleoside analogs were prepared from commercially available nucleosides by chemical synthesis (AZT, d4T, araC, dFdC, ddC, BVdU, FdU) [21] or by enzymatic phosphorylation with deoxycytidine kinase (araG, CdA) or with *Drosophila melanogaster* deoxynucleoside kinase (araT). The sources of the kinases and their use for the phosphorylation of the nucleoside analogs were described earlier [22]. The purity of all nucleoside monophosphates was >99% as based on NMR data (^{31}P and ^{1}H NMR). All were purified by HPLC and were devoid of inorganic phosphate. 3TCMP was obtained from Dr. Claudia Pasti, Institut Pasteur, Paris. The compounds listed in Table 3 were originally prepared by Dr. M. Endovà, Dr. R. Liboska and Dr. A. Holy in Prague, Chech Republic. Mouse dNT-1 [4] and human dNT-2 [5] were homogeneous enzymes prepared earlier. The inhibitors (*S*)-1-[2′-deoxy-3′,5′-*O*-(1-phosphono)benzylidene-β-D-*threo*-pentofuranosyl]thymine (DPB-T) and (±)-1-*trans*-(2-phosphonomethoxycyclopentyl)uracil (PMcP-U) shown in Fig. 1 were synthesized as described [23,24].[2]

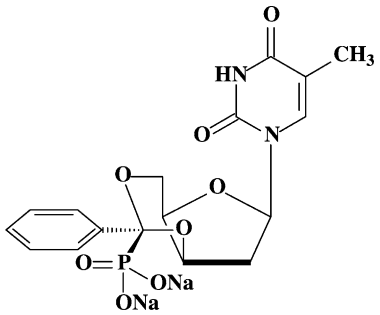
^{3}H-dUMP and ^{14}C-IMP for enzyme assays were from Amersham Biosciences and Moravek, respectively. DE52 Diethylaminoethyl cellulose was from Whatman and Phenyl Sepharose from Amersham Biosciences.

2.2. Expression and purification of recombinant human dNT-1

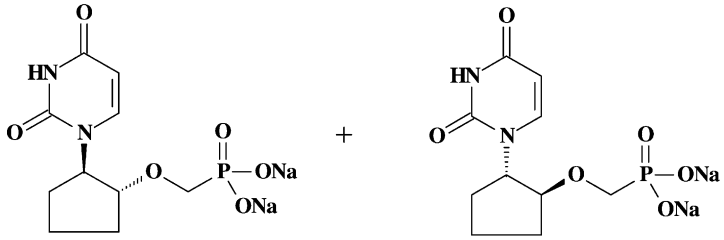
The coding sequence of human dNT-1 (accession no. AF154829) was amplified by PCR using clone KAT02154

[1] Rinaldo-Mathis *et al.*, unpublished data.

[2] In the originally given structures (Rampazzo *et al.*, 2002b) DPB-T contained erroneously a bromomethyl in place of the benzyl group and was named BPE-T, PMcP-U contained erroneously a hexyl instead of a pentyl group and was named PMcH-U.



(*S*)-1-[2'-Deoxy-3',5'-*O*-(1- phosphono)benzylidene-β-D-*threo*-pentofuranosyl]thymine (sodium salt) (DPB-T)



1:1 enantiomeric mixture

(±)-1-trans-(2-phosphonomethoxycyclopentyl)uracil (sodium salt) (PMcP-U)

Fig. 1. Structural formulas of DPB-T and PMcP-U.

obtained from Dr. S. Sugano (Institute of Medical Science, University of Tokyo, Japan), as a template with forward primer 5′-CATACATATGGCGCGGAGCGTGCGCG and reverse primer 5′-CGGTGGATCCTGTGGGCCTGCCCT-TCC. The PCR product was ligated between *Nde*I and *Bam*H1 sites of the pET20b vector (Novagen). The construct was transformed into *Escherichia coli* BL21(DE3)-*plysS*. The bacterial culture (9 L) was grown at 37° to an OD_{600} of 0.6 and induced for 3 hr with 0.4 mM isopropyl-β-D-thiogalactoside. After centrifugation the washed bacterial pellet was lysed by repeated freezing and thawing in 50 mM Tris–HCl/2 mM DTT/1 mg/mL lysozyme. Streptomycin sulfate at the final concentration of 1% (w/v) and a mixture of protease inhibitors for bacterial cell extracts (Sigma) were added and the suspension was centrifuged at 100,000 *g* for 30 min. The clear supernatant solution was then fractionated between 35 and 55% saturation with ammonium sulfate, the precipitate was dissolved in buffer A (20 mM Tris–HCl pH 7.5/20% (v/v) glycerol/2 mM DTT/1 mM EDTA) and dialyzed extensively against buffer A. After centrifugation the supernatant solution contained in a total volume of 35 mL 357 mg of protein and 3200 units of dNT-1 activity. Part of the solution (163 mg protein) was adsorbed to a 65 mL column of DE52 and eluted with a linear gradient of 0–150 mM NaCl (160 + 160 mL) in buffer A. The enzyme (760 units of dNT-1 activity) eluted as a sharp peak around 75 mM NaCl with a specific activity of 85 units/mg protein in the peak fraction. This material served for the crystallization of dNT-1 and subsequent structure determination. The combined side fractions (8 mg protein in 16 mL) were concentrated by filtration to 4.5 mL, solid ammonium sulfate was added to a final molarity of 1.5 M and the solution was adsorbed to a 6 mL column of Phenyl Sepharose CL4B (Amersham Biosciences). The column was eluted with a linear gradient (15 + 15 mL) of ammonium sulfate (1.5–0 M) with enzyme activity appearing at the very end of the gradient in a broad peak (2 mg of protein, specific activity 140 units/mg protein). All fractions gave a single band at 26 kDa on denaturing SDS 12% (w/v) polyacrylamide electrophoresis.

2.3. Expression and purification of recombinant cN-II

Plasmid pNT-3 [25] was transformed into *Escherichia coli* BL21 (DE3)pLysS. Expression of cN-II, preparation of the bacterial extract and fractionation with ammonium sulfate was made as described above for dNT-1. Extracts from non-transformed bacteria showed no demonstrable 5′-nucleotidase activity. The dialyzed supernatant was applied directly to a column of Affi-Gel Blue (Bio-Rad) and cN-II was purified by stepwise elution with NaCl as described [26]. Immediately after elution from the column the enzyme had a specific activity of 64 units/mg protein but the highly dilute solution rapidly lost activity. We therefore added bovine serum albumin to a final concentration of 1 mg/mL to the chromatographic fractions emerging from the column. Enzyme activity was then completely stable. Aliquots of frozen solutions were used

for our experiments. On denaturing SDS–polyacrylamide gel electrophoresis a concentrated solution of the enzyme (without added bovine serum albumin) showed a strong band at 65 kDa, expected from the monomer molecular mass of cN-II, together with several very faint additional bands. We estimate that the final preparation of cN-II was approximately 50% pure.

2.4. Enzyme assays

dNT-1, dNT-2 and cN-II activities were determined by the earlier described specific assays with labeled dUMP or IMP [27]. One unit of enzyme activity corresponds to the dephosphorylation of 1 μmol substrate per min under these conditions. Specific activity is units per mg protein. Protein was determined by the method of Bradford with crystalline bovine serum albumin as standard.

Preliminary tests for inhibition of dNT-1 and dNT-2 by a large variety of nucleotide analogs were made at 1 mM concentration of both the analog and substrate (^{3}H-dUMP) at pH 5.5 (pH optimum). For DPB-T and PMcP-U a further detailed analysis of the kinetics of the reactions was made at varying inhibitor and substrate concentrations. In all kinetic experiments care was taken not to dephosphorylate more than 10–15% of the substrate. The dephosphorylation of other 5′-phosphates, including 5′-phosphates of nucleoside analogs (all at 2 mM) was determined from the formation of inorganic phosphate [28]. In that case, cN-II activity was determined at pH 6.5, dNT-1 and dNT-2 were determined at pH 7.5. Some positive cases were analyzed to determine K_M and V_{max} values. V_{max} values were obtained from the extrapolated maximal specific activity of the Lineweaver–Burk plots. All experiments were made in duplicates at different concentrations of the enzymes and repeated at least once. Activities of the four enzymes with the different analogs are expressed as percentage of their specific activity with the standard substrates (dUMP for dNT-1 and dNT-2, IMP for cN-II). We used a single preparation of each enzyme for all experiments, with excellent reproducibility of the 100% values for the specific activities. At the indicated pH-values and at 2 mM substrate concentrations the specific activities were 40 units/mg of protein for human dNT-1, 90 units/mg for mouse dNT-1, 160 units/mg for dNT-2 and 54 units/mg for cN-II.

3. Results

3.1. Specificity of human dNT-1, dNT-2 and cN-II for substrate analogs

The results in Table 1 demonstrate the ability of the three enzymes to dephosphorylate the 5′-phosphates of various nucleoside analogs used in viral and cancer therapy. Activities were measured at a single substrate concentration (2 mM) and are recorded in percent of the dephosphorylation of dUMP (for dNT-1 and dNT-2) or IMP (for cN-II).

Table 1
Dephosphorylation of 5′-phosphates of natural nucleosides and nucleoside analogs by human dNT-1, dNT-2 and cN-II and mouse dNT-1

Substrate	Relative dephosphorylation			
	Human dNT-1	Human dNT-2	Human cN-II	Mouse dNT-1
dUMP	100	100	23	100
IMP	nd	nd	100	nd
FdUMP	217	82	11	108
BVdUMP	8.5	28	1.5	19
dTMP	98	48*	16	100
AZTMP	22	2	6	103
d4TMP	4	9	0.6	3
araTMP	0.1	1	0.2	nd
dCMP	0.5	0*	nd	16
ddCMP	1	0.1	1	18
dFdCMP	2	0.1	0.6	20
araCMP	0.1	0.1	0.2	0.2
3TCMP	1.5	0.2	0.2	1.2
dAMP	7	1*	7.5	nd
CdAMP	0.5	0.1	4.5	nd
dGMP	73	2*	72	nd
araGMP	1	0.3	3.5	nd

All assays were made at 2 mM substrate concentration at pH 7.5 with dNT-1 and dNT-2, at pH 6.5 with cN-II. The activities are given as percentage of the specific activity of the enzymes with dUMP (for dNT-1 and dNT-2) or IMP (for cN-II). 100 percent correspond to 40 units/mg of protein for human dNT-1, 90 units/mg for mouse dNT-1, 160 units/mg for dNT-2 and 54 units/mg for cN-II. Asterisk (*) from [5]; nd: not determined.

Among the nucleotides-containing uracil or thymine as the base, all except araTMP were dephosphorylated. For both human dNT-1 and dNT-2 dTMP was the next best natural substrate after dUMP, and FdUMP was an even better substrate for dNT-1. BVdUMP was also a good substrate for dNT-2 but showed less activity with the other two enzymes. The anti-HIV analog AZTMP was a better substrate for dNT-1 than for the other two human enzymes while d4TMP, also employed extensively in HIV treatment, was rather poorly dephosphorylated by all enzymes.

No cytosine nucleotide analog was dephosphorylated by any of the three human enzymes, with the possible exceptions for the low activity of dNT-1 for dFdCMP and 3TCMP. We also tested two purine analogs used in cancer therapy, araGMP and CdAMP. araGMP showed some activity with the cytosolic enzymes but was inactive with dNT-2. The adenine nucleotide analog CdAMP showed some activity with cN-II, but no activity with dNT-1 and dNT-2.

In earlier experiments mouse dNT-1 had the ability to dephosphorylate dCMP, whereas human dNT-1 was now found to be inactive, indicating a difference in the substrate specificity for the two enzymes. We therefore tested some of the analogs also as substrates for the mouse enzyme. Table 1 shows that the mouse dNT-1 enzyme was active with ddCMP and dFdCMP in contrast to human dNT-1, but not with araCMP or 3TCMP. Remarkably, mouse dNT-1

Table 2
K_M and V_{max} values for the dephosphorylation of selected nucleotide analogs by human dNT-1 and dNT-2 and mouse dNT-1

Substrate	Human dNT-1			Mouse dNT-1			Human dNT-2		
	K_M (mM)	V_{max} (units/mg)	V_{max}/K_M	K_M (mM)	V_{max} (units/mg)	V_{max}/K_M	K_M (mM)	V_{max} (units/mg)	V_{max}/K_M
dUMP	1.5	49	33	0.8	110	138	0.1	110	1100
dTMP	1.5	64	43	1.4	156	111	0.2	74	370
AZTMP	2.4	21	9	3.6	238	66	2.5	8.4	3.4
d4TMP	>5	–	–	>5	–	–	0.3	14	47
FdUMP	1.9	160	84	2.9	200	69	0.1	140	1400
BVdUMP	1.9	6	3	2.8	38	14	0.7	56	80

All experiments were made at pH 7.5.

dephosphorylated AZTMP much more efficiently than the human enzyme. Also FdUMP and BVdUMP were good substrates.

To further study the differences between mouse dNT-1, human dNT-1 and human dNT-2 we determined the K_M and V_{max} values for some uracil/thymine nucleotide analogs (Table 2). To approach conditions in living cells the experiments were done at pH 7.5 and not at the acid pH-optima of the enzymes. We carried out at least two experiments with each analog but their limited supply did not allow extensive kinetic experiments at high substrate concentrations. The K_M values of d4TMP for the dNT-1 enzymes could not be determined with any degree of accuracy. Nevertheless it is evident that generally the K_M values with dNT-2 were 3- to 30-fold lower than with the two dNT-1 enzymes, the exception being AZTMP. With this nucleotide the three enzymes showed very similar K_M values of approximately 2.5 mM, but the values for V_{max} were strikingly higher for mouse dNT-1 resulting in a considerably higher V_{max}/K_M ratio.

3.2. Inhibitors of deoxyribonucleotidases

A series of nucleoside phosphonate analogs (Table 3) were tested for their effect on the dephosphorylation of dUMP by dNT-1 or dNT-2. At equimolar concentration with the substrate most of them were completely inactive, three showed very weak activity (63–72% remaining activity), and two (DPB-T and PMcP-U, Fig. 1) were strong inhibitors of dNT-2 (Table 3). PMcP-U also inhibited dNT-1 whereas DPB-T was almost specific for dNT-2. We studied the two strong inhibitors in more detail in kinetic experiments.

Table 3
Inhibitory effect of nucleoside phosphonate analogs against dNT-1 and dNT-2

Nucleoside phosphonate analogs	Inhibition
1-(2-Phosphonomethoxyethyl)-5-nitrouracil	–
1-(2-Phosphonomethoxyethyl)-5-bromocytosine	Weak inhibitor of dNT-1
1-(2-Phosphonomethoxyethyl)-5-nitrocytosine	–
2′,3′-*O*-Phosphonomethylenecytidine	–
2′,3′-*O*-Phosphonomethyleneuridine	–
2′,3′-*O*-Phosphonomethylenethymidine	–
9-(2-Phosphonomethoxyethyl)xanthine	Weak inhibitor of dNT-2
2′,3′-*O*-(1-Phosphono)ethylidenecytidine	–
2′,3′-*O*-(1-Phosphono)ethylideneuridine	–
2′,3′-*O*-(1-Phosphono)ethylidenethymidine	–
2′,3′-*O*-(1-Phosphono)benzylidenecytidine	–
2′,3′-O-(1-Phosphono)benzylideneuridine	–
2′,3′-*O*-(1-Phosphono)benzylidenethymidine	–
1-(2-Deoxy-3,5-*O*-phosphonomethylene-β-D-*threo*-pentofuranosyl)thymine	–
(*S*)-1-[2′-Deoxy-3′,5′-*O*-(1-phosphono)benzylidene-β-D-*threo*-pentofuranosyl]thymine	Inhibitor of dNT-2 (DPB-T)
5-Bromo-(*S*)-HPMPC	–
(±)-1-*cis*-(2-Phosphonomethoxycyclohexyl)uracil	–
1-(3-Dimethylamino-2-phosphonomethoxypropyl)cytosine	–
1-(3-Amino-2-phosphonomethoxypropyl)cytosine	–
(±)-1-*trans*-(2-Phosphonomethoxycyclopentyl)cytosine	Weak inhibitor of dNT-1
(±)-1-*trans*-(2-Phosphonomethoxycyclohexyl)cytosine	–
(±)-1-*trans*-(2-Phosphonomethoxycycloheptyl)cytosine	–
(±)-1-*trans*-(2-Phosphonomethoxycyclopentyl)uracil	Inhibitor of dNT-1 and dNT-2 (PMcP-U)
(±)-1-*trans*-(2-Phosphonomethoxycyclohexyl)uracil	–
(±)-1-*trans*-(2-Phosphonomethoxycycloheptyl)uracil	–

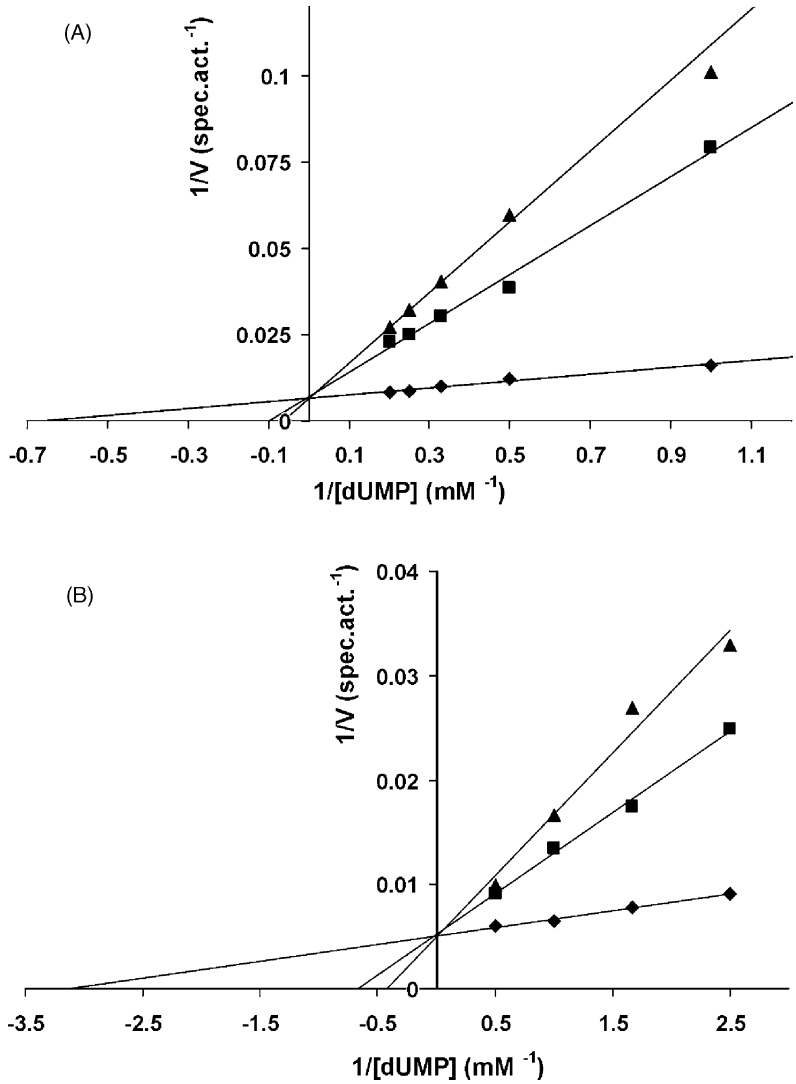


Fig. 2. Lineweaver–Burk plots for the inhibition of human dNT-1 (A) and dNT-2 (B) by PMcP-U. No inhibitor (◆); 0.15 mM PMcP-U (■); 0.25 mM PMcP-U (▲).

To determine the type of inhibition we made a series of experiments at the pH optima of the enzymes with results shown in Figs. 2 and 3. In the Lineweaver–Burk plots of Fig. 2, PMcP-U shows clear competition with dUMP for both dNT-1 (Fig. 2A) and dNT-2 (Fig. 2B). From the curves we calculate a K_i of 26 μM (K_M for dUMP = 1.5 mM) for the inhibition of dNT-1 and a K_i of 40 μM (K_M for dUMP = 0.3 mM) for dNT-2. Inhibition of dNT-2 by DPB-T was less straightforward. The primary Lineweaver–Burk plot of Fig. 3A shows a series of curves in which the inhibition by various concentrations of the inhibitor was determined at increasing substrate concentrations. The inhibited curves do not intersect at the ordinate or at the abscissa with the curve obtained in the absence of inhibitor. The same result was found in two additional experiments. The inhibition therefore appears to be of a mixed type. In the secondary plot of Fig. 3B we assume a mixed linear inhibition and can then calculate a K_i value of 70 μM for the inhibitor (K_M for dUMP = 0.5 mM).

4. Discussion

The results of Table 1 are of interest in connection with the therapeutic use of nucleoside analogs. AZT, d4T, ddC and 3TC are all used during multidrug treatment of HIV infection [19]. Fluorouracil, dFdC and araC are used to treat both leukemias and some solid tumors. HIV infections require intensive long-time chemotherapy and under those circumstances several of the analogs give rise to toxic effects that can be ascribed to their interference with mitochondrial DNA replication [19]. Because of mitochondrial toxicity treatment with these nucleoside analogs of HIV-infected patients without clinical symptoms of AIDS is sometimes not instituted. In the design of new active analogs one of the important principles should be to minimize their effect on mitochondrial DNA synthesis. One avenue in this direction is to design nucleoside analogs whose triphosphates show a low affinity for mitochondrial DNA polymerase. We now suggest

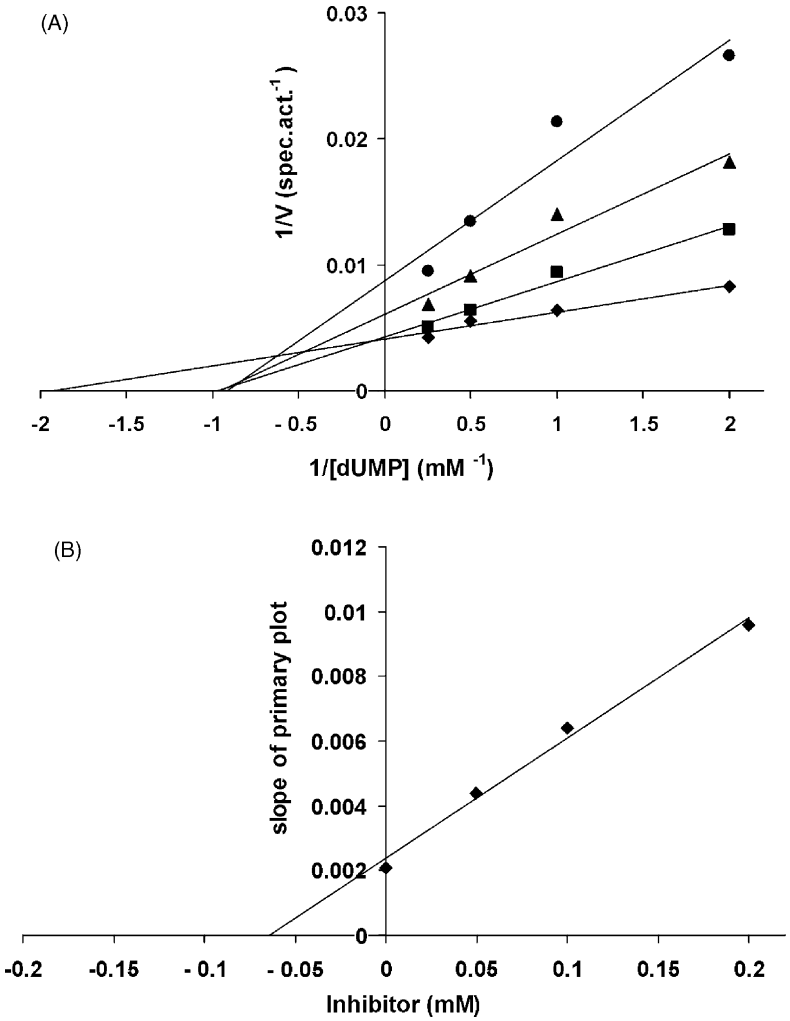


Fig. 3. A Lineweaver–Burk plot for the inhibition of dNT-2 by various concentrations of DPB-T. No inhibitor (◆); 0.05 mM (■); 0.1 mM (▲); 0.2 mM (●). Panel B shows a secondary plot of the data of panel A assuming a mixed linear inhibition with the slopes of the inhibitor curves on the ordinate and the inhibitor concentration on the abscissa.

a second avenue based on the fact that mitochondrial and cytosolic deoxyribonucleotide pools form separate compartments: to design nucleoside analogs whose monophosphates are good substrates for dNT-2 but not dNT-1. Such an approach is aided by the recent elucidation of the X-ray structures for the two deoxyribonucleotidases ([9] and Footnote 1).

Tables 1 and 2 show that none of the four phosphorylated nucleoside analogs employed in HIV treatment are good substrates for dNT-2. The best results in this respect were obtained with d4TMP. AZTMP, the most commonly used analog, was poorly dephosphorylated by this enzyme. The activity with human dNT-1 was better. During treatment with azidothymidine the intramitochondrial pool of azidothymidine phosphates may therefore actually be less modified by a 5′-nucleotidase than the cytosolic pool. Prolonged treatment with this analog is indeed known to result in mitochondrial toxicity [19]. The cytosine analogs show no activity with either deoxyribonucleotidase and are consequently not deactivated in mitochondria.

When human and mouse dNT-1 were compared for the dephosphorylation of AZTMP (Table 2) the latter enzyme had a 7-fold higher activity. The two enzymes also differed greatly in their ability to dephosphorylate various cytosine-containing analogs, in particular for ddCMP and dFdCMP (Table 1). These results may explain the greater toxicity of ddC for human cells than mouse cells [29]. Taken together our results stress the importance of using human systems when testing the metabolism and toxicity of nucleoside analogs.

An involvement of a soluble 5′-nucleotidase in disease was originally suggested as an explanation for the specific toxicity of deoxyadenosine for T lymphoblasts in children suffering from adenosine deaminase deficiency [30,31]. Accumulation of dATP occurs preferentially in these cells. dATP induces apoptosis [32] and causes a severe immune deficiency. Compared to other lymphoblasts, T lymphoblasts contain little 5′-nucleotidase [30]. Later work by Carson's group demonstrated that deoxyadenosine-resistant T lymphoblasts in culture contained an increased

activity of the cytosolic cN-II [14]. Moreover, low activity of cN-II and high activity of deoxycytidine kinase in lymphocytes from leukemic patients favored a positive response to treatment with CdA, a non-hydrolyzable analog of deoxyadenosine [13]. Deoxycytidine kinase catalyzes the phosphorylation of CdA. More recently, two CdA-resistant HL60 cell lines were shown to contain an increased cN-II activity but no change in deoxycytidine kinase [16]. These cells were not cross-resistant to cytosine nucleoside analogs. In a different study with HL60 cells resistance to CldA developed by loss of deoxycytidine kinase without impairment of cN-II and these cells were also resistant to the cytosine nucleoside analogs [33]. These studies suggest that resistance to deoxyadenosine and CldA can arise from a decrease in deoxycytidine kinase activity and/or an increase in cN-II. In the former but not in the latter case the cells become cross-resistant to cytosine nucleoside analogs. At variance with this conclusion appears to be that a 10-fold overexpression of cN-II in HEK-293 fibroblasts [34] did not lead to an increased resistance to deoxyadenosine. This kind of experiment should, however, be repeated in a lymphoblastoid cell line.

The substrate specificity of 5′-nucleotidases shown in Table 1 demonstrates that both cN-II and dNT-1 can dephosphorylate dAMP and that cN-II is the only enzyme dephosphorylating CdAMP even though the relative activity for the two nucleotides is low compared to the activity with the prime substrate IMP. Nevertheless an increase in cN-II activity may well decrease the size of the adenine deoxynucleotide pools and lead to an increased resistance. Neither enzyme shows a reasonable activity with cytosine nucleotides. In particular, araCMP was completely inactive. This agrees with findings showing that CdA-resistant cells overproducing cN-II were not cross-resistant to cytosine-containing nucleoside analogs [16]. The recent report [17] that low expression of the mRNA for cN-II increases the success of treatment of leukemic patients with araC needs further corroboration.

The two inhibitors DPB-T and PMcP-U were earlier used by us successfully to distinguish between the activity of dNT-1 and dNT-2 [27] and to demonstrate the specific presence of dNT-2 inside mitochondria [10]. X-ray crystallographic studies revealed that both inhibitors bind to the active site of dNT-2 and can be used to identify amino acid residues involved in their binding.[3] Whereas PMcP-U occupies the whole active site, DPB-T only occupies the region that binds the pyrimidine ring. This may be related to our kinetic experiments that demonstrate a pure competitive inhibition of the enzymes by PMcP-U. It is clear that this inhibitor is mutually exclusive with dUMP and does not act as alternative substrate of the enzymes because of the stable unmetabolizable C–P bond in its structure. The mixed-type inhibition of the dNT-2 by DPB-T suggests that this inhibitor may change the dissociation constant for dUMP and thus affect the affinity of the enzyme for the natural substrate. Also this inhibitor cannot act as an alternative substrate. PMcP-U and DPB-T are two synthetically pure inhibitors of 5′-nucleotidases, useful for in depth studies of the role and properties of these enzymes.

[3] Rinaldo-Mathis and Nordlund, unpublished data.

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