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
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 deoxy-
ribonucleotides [5]. Also dNT-1 is very active with these
two deoxyribonucleotides but, in addition, dephosphory-
lates 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 thymi-
dine and deoxyctydine 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 intramitochon-
drial 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 treat-
ment 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 result-
ing 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 sensi-
tivity 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]. Experime-
tnts 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 positively related to low levels of a
clinical studies with patients suffering from leukemia the
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virus[19]. The rationale of the treatment is to use analogs
including dNT-1. A different scenario can be envisaged for
the patient
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 affect-
ing 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 experi-
ments 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 pre-
pared from commercially available nucleosides by chemi-
cal synthesis (AZT, d4T, araC, dFdC, ddC, BVdU, FdU)
[21] or by enzymatic phosphorylation with deoxyctydine
kinase (araG, CdA) or with Drosophila melanogaster deox-
yucleoside 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 (31P and
1H NMR). All were purified by HPLC and were devoid of
inorganic phosphate. 3TCMP was obtained from Dr. Clau-
dia Pasti, Institut Pasteur, Paris. The compounds listed in
Table 3 were originally prepared by Dr. M. Endová, 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 (5')-1-[2'-deoxy-
3',5'-O-(1-phosphono)benzylidene-β-D-threo-pentofuranos-
yl]thymine (DPB-T) and (±)-1-trans-(2-phosphono-
methoxy)cyclopentyl)uracil (PMcP-U) shown in Fig. 1
were synthesized as described [23,24]. 2

3'-dUMP and 14C-IMP for enzyme assays were from
Amer sham Biosciences and Moravek, respectively. DE52
Diethylaminoethyl cellulose was from Whatman and Phen-
yl 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.
obtained from Dr. S. Sugano (Institute of Medical Science, University of Tokyo, Japan), as a template with forward primer 5'-CATACATAGCGCAGCGTGC and reverse primer 5'-CGGTGGATCCTGTGGGCCTGCCCTTCC. The PCR product was ligated between NdeI and BamH1 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°C to an OD600 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/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 (3H-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 at varying inhibitor and substrate concentrations. In all detailed analysis of the kinetics of the reactions was made.

Table 1 shows that the mouse dNT-1 enzyme was active of the analogs also as substrates for the mouse enzyme. 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 cN-II, but no activity with dNT-1 and dNT-2. The adenine nucleotide analog CdAMP showed 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 cN-II, but no activity with dNT-1 and 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 1

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

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.

(2 mM) and are recorded in percent of the dephosphorylation of dUMP (for dNT-1 and dNT-2) or IMP (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...
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 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

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

All experiments were made at pH 7.5.

### Table 3

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

<table>
<thead>
<tr>
<th>Nucleoside phosphonate analogs</th>
<th>Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-(2-Phosphonomethoxyethyl)-5-nitouracil</td>
<td>–</td>
</tr>
<tr>
<td>1-(2-Phosphonomethoxyethyl)-5-bromocytosine</td>
<td>Weak inhibitor of dNT-1</td>
</tr>
<tr>
<td>1-(2-Phosphonomethoxyethyl)-5-nitocytosine</td>
<td>–</td>
</tr>
<tr>
<td>2′,3′-O-Phosphonomethyleneцитidine</td>
<td>–</td>
</tr>
<tr>
<td>2′,3′-O-Phosphonomethylenearuridine</td>
<td>–</td>
</tr>
<tr>
<td>2′,3′-O-Phosphonomethylenethylidine</td>
<td>–</td>
</tr>
<tr>
<td>9-(2-Phosphonomethoxyethyl)xanthine</td>
<td>Weak inhibitor of dNT-2</td>
</tr>
<tr>
<td>2′,3′-O-(1-Phosphono)ethylidenecytidine</td>
<td>–</td>
</tr>
<tr>
<td>2′,3′-O-(1-Phosphono)ethylidenearuridine</td>
<td>–</td>
</tr>
<tr>
<td>2′,3′-O-(1-Phosphono)benzylidenecytidine</td>
<td>–</td>
</tr>
<tr>
<td>2′,3′-O-(1-Phosphono)benzylidenearuridine</td>
<td>–</td>
</tr>
<tr>
<td>2′,3′-O-(1-Phosphono)benzylidenethylidine</td>
<td>–</td>
</tr>
<tr>
<td>1-(2-Deoxy-3,5-O-phosphonomethylene-β-D-threo-pentofuranosyl)thymine</td>
<td>–</td>
</tr>
<tr>
<td>(S)-1-[2′-Deoxy-3′,5′-O-(1-phosphono)benzylidenel-β-D-threo-pentofuranosyl]thymine</td>
<td>Inhibitor of dNT-2 (DPB-T)</td>
</tr>
<tr>
<td>5-Bromo-(S)-HPMPC</td>
<td>–</td>
</tr>
<tr>
<td>(±)-1-cis-(2-Phosphonomethoxycyclohexyl)uracil</td>
<td>–</td>
</tr>
<tr>
<td>1-(3-Dimethylamino-2-phosphonomethoxypropyl)cytosine</td>
<td>–</td>
</tr>
<tr>
<td>1-(3-Amino-2-phosphonomethoxypropyl)cytosine</td>
<td>–</td>
</tr>
<tr>
<td>(±)-1-trans-(2-Phosphonomethoxycyclopropyl)cytosine</td>
<td>Weak inhibitor of dNT-1</td>
</tr>
<tr>
<td>(±)-1-trans-(2-Phosphonomethoxycyclohexyl)cytosine</td>
<td>–</td>
</tr>
<tr>
<td>(±)-1-trans-(2-Phosphonomethoxycyclopentyl)cytosine</td>
<td>–</td>
</tr>
<tr>
<td>(±)-1-trans-(2-Phosphonomethoxycyclohexyl)uracil</td>
<td>Inhibitor of dNT-1 and dNT-2 (PMcP-U)</td>
</tr>
<tr>
<td>(±)-1-trans-(2-Phosphonomethoxycyclopentyl)uracil</td>
<td>–</td>
</tr>
<tr>
<td>(±)-1-trans-(2-Phosphonomethoxycycloheptyl)uracil</td>
<td>–</td>
</tr>
</tbody>
</table>
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...
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

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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.
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. 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.

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