The Activity of Thymidine Phosphorylase Obtained from Human Uterine Leiomyomas and Studied in the Presence of Pyrimidine Derivatives

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Z. Naturforsch. 52c, 670–675 (1997); received March 10/May 9, 1997

Thymidine Phosphorylase, Pyrimidine Derivatives, Leiomyoma, Uterus

Partially purified samples of thymidine phosphorylase were obtained from four preparations of human uterine leiomyomas and uteri using the method of Yoshimura et al. (1990), Biochim. Biophys. Acta 1034, 107–113. Among the studied twelve pyrimidine derivatives, 5-bromouracil, 5-nitouracil, 5-fluorouracil, 6-aminouracil, 4,6-dihydroxy-5-nitopyrimidine are competitive inhibitors, while allyloxymethylthymine is an uncompetitive inhibitor of thymidine phosphorylase activity. 6-benzyl-2-thiouracil inhibits the activity of the enzyme in a mixed way. The most potent inhibitor of the thymidine phosphorylase activity is 5-bromouracil and uracil the weakest one. Stronger inhibition of these compounds on the activity of thymidine phosphorylase was found in uterine leiomyomas than in uteri.

Introduction

Two pyrimidine nucleoside phosphorylases are present in the cytosol of mammalian cells, i.e. thymidine [EC 2.4.2.4.] and uridine [EC 2.4.2.3.] (Krenitsky et al., 1964). These two enzymes play an important role in nucleoside degradation, as well as in the process of recovery (salvage) of pyrimidine (Granharov et al., 1991). Inhibitors of these enzymes were applied in chemotherapy by decreasing the in vivo degradation of pyrimidine nucleosides analogues exhibiting antitumor activity (Mukherjee et al., 1963, Niedzwiecki et al., 1981). A number of inhibitors of uridine phosphorylase (UrdPase) activity are known which are, among others, 5-substituted uracil derivatives (Baker et al., 1970, Woodman et al., 1980, Niedzwiecki et al., 1983). Little is known, however, of thymidine phosphorylase (dThdPase) inhibitors, and therefore, we have decided to study uracil derivatives as inhibitors of dThdPase isolated from human uteri and uterine leiomyomas.

Materials and Methods

The investigated material were four leiomyomas and uteri operated in women aged 30–45 years. Histopathological results revealed the endometrium to be in the proliferative stage. Directly after surgery the tissues were deep-frozen (−70 °C) and then taken for analysis.

Chemicals

5-FU, 6-AU, 4,6-DHNiP, 6-BTU, dThd, T, U were obtained from Sigma Chemical Company St.Louis, MO, USA; 5-BrU, 5-NiU from Fluka AG Loba Chemie (Wien). AMU, AMT, AMFU, AMC, 5-NiU were obtained by Ozierov (Ozierov et al., 1991), Th5 by Draminski (Rutkowski and Draminski, 1991). DEAE-Sepharose was obtained from Pharmacia Fine Chemicals. All other chemicals were of reagent or analytical grade and were used without further purification.

Enzyme preparation

dThdPase was partially purified according to the method described by Yoshimura et al. (1990) for human placenta with our own modifications. The
tissues were homogenized in 4 volumes of ice cold buffer A [(1 mM EDTA, 0.02% 2-mercaptoethanol, 2 mM phenylmethanesulfonfyl fluoride (PMSF), 20 mM sodium phosphate buffer, pH 7.5)] and centrifuged at 50000 x g for 1 h.

The supernatant was mixed overnight with 40% saturation of ammonium sulfate at 4 °C, then the precipitate was dissolved in buffer A, dialyzed against this buffer and next dialyzed against buffer B (1 mM EDTA, 0.02% 2-mercaptoethanol, 10 mM tris(hydroxymethyl)aminomethane-maleate, pH 6.5). The dialysed solution was applied to a DEAE-Sepharose (Pharmacia) column and eluted with a linear gradient of NaCl (50–250 mM). Then the active fractions were pooled and stored at −70 °C, or were taken for analysis. dThdPase from human uterine leiomyomas and uteri was purified 40-fold and 10-fold, respectively.

**Enzyme assays**

dThdPase activity was assayed by the spectrophotometric method described by Yoshimura et al. (1990) in our own modification, using the transformation of thymine from thymidine in the presence of arsenate. The incubation mixture of 0.5 ml final volume contained 0.1 mM Tris-arsenate buffer (pH 6.5), 10 mM dThd and crude or partially purified enzyme. After 1 h incubation at 37 °C, the reaction was stopped by adding 0.5 ml 1 N NaOH and the thymine formed was measured with absorbance at 300 nm. The inhibition studies were performed in the same conditions, but the incubation mixture of 0.5 ml final volume contained an appropriate concentration of inhibitor and substrate. The protein content was determined according to the method described by Bradford (1976).

One unit of activity of dThdPase was defined as the amount of the enzyme which was required to form 1 µM of free base per 1 h. Specific activity was defined as the number of the enzyme activity units per milligram of protein. $K_m$ values were calculated from eight substrate concentrations using equations described by Michal (1974). $K_i$ values were calculated according to the same method after the kind of inhibition had been determined using Dixon plot (Michal G. 1974).

**Results and Discussion**

The 5-FU derivatives, i.e. 5-FUR, 5-FUdR and 5-DFUR are converted to 5-FU by pyrimidine nucleoside phosphorylases. One of them is dThdPase specific for pyrimidine 2'deoxy-nucleosides, mainly dThd which may be responsible for the degradation of FUdR to 5-FU, primarily in human tumors. The other – UrdPase specific mainly for Urd is responsible for degradation of FUR, and also FUdR in murine tumors (Razzel et al., 1967; Kono et al., 1983; Miwa et al., 1986). Degradation of the drugs by phosphorylases is the limiting factor of their antitumor activity and, therefore, it appeared pertinent to find inhibitors of these enzymes. In fact, the application of FUR together with 2,2'-anhydro-5-ethyluridine (ANEUR), which is a potent inhibitor of UrdPase, markedly enhanced the antitumor activity of FUR and FUdR (Veres et al., 1985 and 1987). ANEUR is not inhibitory for dThdPase and, hence, it should not interfere with the conversion of DFUR to 5-FU by dThdPase in human tumors (Kono et al., 1983). Strong inhibitors of dThdPase, however, are 6-anilino- and 6-(1-naphthylmethylamino-) derivatives of uracil, which were investigated in E.coli (Baker et al., 1970). On the other hand, they did not inhibit or inhibited insignificantly (less than 10%) the effect of dThdPase on FUdR in five different mammalian preparations (Woodman et al., 1980). There are, therefore, differences between chemical and steric properties of dThdPases from eukaryotes and prokaryotes. The investigations on the enzyme have been mainly carried out with animal material. A detailed study of kinetics for dThdPase isolated from mouse liver revealed to be consistent with Michaelis-Menten kinetics (Itzch et al., 1985). The importance of using human phosphorylases to evaluate potential inhibitors of these enzymes for clinical use should be emphasised. Daker et al. (1990) reported that dThdPase from intact blood cells follows the Michaelis-Menten kinetics with $K_m = 0.1–0.19$ mM. In our research dThdPase isolated from uteri and uterine leiomyomas, and partially purified (fractionation to saturation with ammonium sulfate, chromatography on DEAE-Sepharose) also revealed the hyperbolic kinetics with $K_m = 0.2$ mM for uterine leiomyomas and 0.25 mM for the uteri. This enzyme was subject to the effect of compounds deriving from uracil. They were 5-BrU, 5-NiU, 5-FU, 6-BTU, 4,6-DHNiP, 6-AU, U, AMU, AMFU, AMC, T 5 and AMT. These results revealed a few inhibitors of this enzyme (Table I).
Table I. Effect of pyrimidine derivatives on partially purified dThdPase activity from human uterine leiomyomas and uteri (see Materials and Methods). The compounds (0.1 mM) were tested using dThd as substrate (0.2 mM).

<table>
<thead>
<tr>
<th>Compound</th>
<th>dThdPase activity [U/mg of protein]</th>
<th>% of inhibition</th>
<th>K_i [µM]</th>
<th>dThdPase activity [U/mg of protein]</th>
<th>% of inhibition</th>
<th>K_i [µM]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>9.30 ± 0.60</td>
<td>24.0 (p=0.05)</td>
<td>158.0</td>
<td>0.650 ± 0.035</td>
<td></td>
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<tr>
<td>U</td>
<td>7.07 ± 0.55</td>
<td>6.0 (p=0.001)</td>
<td>31.3</td>
<td>0.640 ± 0.055</td>
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<tr>
<td>6-AU</td>
<td>5.94 ± 0.36</td>
<td>36.1 (p=0.005)</td>
<td>88.0</td>
<td>0.448 ± 0.030</td>
<td></td>
<td></td>
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<tr>
<td>5-FU</td>
<td>4.35 ± 0.40</td>
<td>53.2 (p=0.001)</td>
<td>43.0</td>
<td>0.363 ± 0.025</td>
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<td></td>
</tr>
<tr>
<td>5-NiU</td>
<td>0.86 ± 0.05</td>
<td>90.8 (p=0.001)</td>
<td>5.1</td>
<td>0.200 ± 0.015</td>
<td></td>
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<tr>
<td>5-BrU</td>
<td>0.19 ± 0.02</td>
<td>98.0 (p=0.001)</td>
<td>1.1</td>
<td>0.062 ± 0.005</td>
<td></td>
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<tr>
<td>4,6-DHNIP</td>
<td>2.76 ± 0.21</td>
<td>70.3 (p=0.001)</td>
<td>21.0</td>
<td>0.510 ± 0.050</td>
<td></td>
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</tr>
<tr>
<td>6-BTU</td>
<td>4.00 ± 0.25</td>
<td>57.2 (p=0.001)</td>
<td>75.5</td>
<td>0.370 ± 0.024</td>
<td></td>
<td></td>
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<tr>
<td>AMT</td>
<td>5.20 ± 0.46</td>
<td>44.0 (p=0.002)</td>
<td>127.0</td>
<td>0.360 ± 0.026</td>
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<tr>
<td>AMU</td>
<td>9.31 ± 0.68</td>
<td></td>
<td></td>
<td>0.652 ± 0.072</td>
<td></td>
<td></td>
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<tr>
<td>AMFU</td>
<td>8.15 ± 0.59</td>
<td>12.4 (p=0.02)</td>
<td></td>
<td>0.598 ± 0.063</td>
<td></td>
<td></td>
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<tr>
<td>Th 5</td>
<td>8.41 ± 0.51</td>
<td>9.6 (p=0.05)</td>
<td></td>
<td>0.624 ± 0.056</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AMUc</td>
<td>9.32 ± 0.75</td>
<td></td>
<td></td>
<td>0.660 ± 0.065</td>
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</table>

1 unit of enzyme activity (U) is defined as the quantity that catalyzes the formation of 1.0 µmol of free thymine per hour. Specific activity of dThdPase is expressed as U/mg of protein. Each value: the mean ± SD for four experiments, NS-mean non significant (i.e. p>0.05), p-values were calculated using Student’s t-test, comp., uncomp. or m.inh. indicates competitive, uncompetitive or mixed inhibition.

Desgranges has also reported, that the activity of dThdPase from blood platelets is inhibited competitively by various C-5 or C-6 substitutions of uracil (Desgranges et al., 1982).

In our study 5-BrU appeared to be the most potent and competitive inhibitor for dThdPase isolated from both uteri and from uterine leiomyomas with $K_i = 6.1 \text{ µM}$ and 1.06 $\text{ µM}$, respectively (Table I, Figs 1, 2). This result has not revealed a significant difference in sensitivity of dThdPase isolated from a benign tumor (uterine leiomyoma) and from adjacent tissue (uterus) to 5-BrU, but pointed to a different sensitivity of dThdPase isolated from these human tissues in comparison with this enzyme isolated from other mammalian tissues. The inhibition studies with mouse liver dThdPase did not reveal an inhibition at the maximum 5-BrU concentration of 2 mM (Granharov et al., 1991). 5-BrU, however, studied in intact platelets was one of the most active inhibitors with $K_i$ of 28 $\text{ µM}$ next to 6-amino-5-bromouracil and 6-aminothymine with a $K_i$ of 6 and 11 $\text{ µM}$, respectively (Desgranges et al., 1982).

5-NiU, on the other hand, appeared to be an equally effective inhibitor for dThdPase as 5-BrU in tissues studied by us (Table I, Fig. 1) and in tissues from other sources. At the concentration of 0.1 mM 5-NiU inhibited dThdPase from uterine leiomyomas and uteri in 90.8% and 69%, respectively. In mouse liver 5-NiU at the concentration of 0.4 mM inhibited dThdPase by 50% (Granharov et al., 1991). The results of the inhibition study for 5-NiU in HeLa cells, mouse liver and human leukocytes appeared to be similar in case of both dThd in our study and FUdR as substrates for this enzyme (Woodman et al., 1980).

The next position, in inhibitory potency is taken by 4,6-DHNiP which inhibits dThdPase activity more effectively in leiomyomas – 70% inhibition, at the inhibitor concentration of 0.1 mM (Table I), than in mouse liver – less than 10% inhibition at the maximal inhibitor concentration of 2 mM (Granharov et al., 1991).

A less inhibitory potency is exhibited by 6-BTU, as showed in Table I (mixed inhibition, $K_i = 75.5 \text{ µM}$ for the leiomyomas). Interesting to note, that neither of the single-substituted compounds, i.e. 2-thiouracil or 6-benzyluracil binds significantly to mammalian dThdPase, whereas the combination of these substitutions results in a 5-fold enhancement in binding relative to uracil (Niedzwicki et al., 1983).
1 unit of enzyme activity (U) is defined as the quantity that catalyzes the formation of 1.0 μmol of free thymine per hour [μM T/h]. V is expressed as specific activity of dTdpase [U/mg protein]. 40-fold purified dTdpase (spec. activity 18.6 U/mg protein) were used in experiments. Each point of the line – the mean ± S. D. for four experiments. Line of the enzyme activity without inhibitor, crosses x-axis at value -5 (which points to \( K_m = 0.2 \) mM), in presence of 0.1 mM 5-BrU crosses x-axis at value -0.0524 (which points to \( K_{i1} = 19.08 \) mM), in presence of 0.1 mM 5-NiU crosses x-axis at value -0.243 (which points to \( K_{i2} = 4.12 \) mM).

Regression equations of the enzyme activity lines are:

\[ y = 0.0359 + 1.0343 \times \text{for } r=0.999 \text{ in presence 5-BrU}, \]
\[ y = 0.0597 + 0.2182 \times \text{for } r=0.998 \text{ in presence 5-NiU}, \]
\[ y = 0.0544 + 0.0103 \times \text{for } r=0.993 \text{ without inhibitor}, \]

Course of line indicates a competitive type of inhibition.

Iltzch and Klein (1993) have reported that T. gondi UrdPase is similar to both mammalian UrdPase and dTdpase with respect to binding of uracil analogues substituted at the 5-position with electron-withdrawing groups. Substitutions at these types of groups for the C-5 hydrogen binding to all these enzymes, although the increase in binding to mammalian UrdPase and dTdpase is much greater than it is for T. gondi UrdPase. For example, a bromo- group at the 5-position (5-BrU) increases binding to mammalian dTdpase and UrdPase 28- and 21- fold, respectively, but increases binding to T. gondi UrdPase only about 2-fold. These enzymes are also similar with respect to substitutions at the 6-position (Iltzch et al., 1993). Substitution at C-5 position with electron-withdrawing groups enhances binding significantly: bromo- 28-fold, chloro- 26-fold, iodo- 17-fold, nitro- 13-fold, fluoro- 2-fold for mammalian dTdpase (Niedzwicki et al., 1983). The studied compounds may be arranged in the following sequence, depending on the potency of the inhibitory effect: 5-BrU >5-NiU >5-FU >U. The last mentioned compound at the concentration of 0.1 mM exhibited only a 24% inhibition for dTdpase activity from uterine leiomyomas and no inhibition for uteri. The inhibition of these compounds is competitive (Dixon plot, Fig. 2).

Literature data showed that 6-aminothyminne is a potent inhibitor for dTdpase activity (Langen et al., 1967; Niedzwicki et al., 1981). 6-AU, on the other hand, inhibited less the activity of the dTdpase cleaving FUdR to various degrees depending on the source of the enzyme (Woodman et al., 1980). In comparison with previously mentioned inhibitors in our study 6-AU appeared to be less effective \( (K_i = 88 \mu M \text{ for uterine leiomyomas and } K_i = 130 \mu M \text{ for adjacent uterine tissue}) \) (Table I).

The observed stronger inhibitory influence of these compounds on the activity of dTdpase from the studied leiomyomas may result from a better DEAE-Sepharose purification, greater sensitivity of dTdpase to temperature, shorter half-life, or greater specific activity of the enzyme in compari-
Drabikowska et al., (1987) investigated the acyclonucleoside analogues consisting of 5- and 5,6- substituted uracils as the potential inhibitors of UrdPase \textit{E. coli}, but none of the compounds was a substrate or inhibitor of \textit{E. coli} dThdPase (Drabikowska et al., 1987). Also other authors have reported on acyclonucleosides inhibiting UrdPase activity which did not affect dThdPase activity (Niedzwiecki et al., 1981; Naguib et al., 1993; Goudgaon et al., 1993). However, one from among the allyloxymethyl pyrimidine acyclonucleosides (AMC, AMFU, AMU, Th 5 and AMT) studied by us, i.e. allyloxymethylthymine (AMT) appeared to be, using Dixon plot, an uncompetitive inhibitor of dThdPase. The enzyme from uteri and uterine leiomyomas was inhibited by AMT by 44% with a $K_i$ of 128.6 $\mu$m and 127.0 $\mu$m, respectively (Table I). This compound inhibits also the synthesis of dTMP and dGMP \textit{in vivo} and dTMP, \textit{in vitro} (Modrzejewska et al., 1996). Other acyclonucleosides have been synthesized with antiviral and antitumor activities (Trinh et al., 1994; Lazrek et al., 1995; Sommadossi et al., 1995) and therefore, further, complex investigations of their influence on metabolism in man are necessary.

This easily available material (about 30% of women have uterine leiomyomas, usually removed surgically) may appear valuable for the study of dThdPase inhibitors owing to the fact, that as it has recently been reported, dThdPase is considered to be a platelet-derived endothelial cell growth factor (PD-ECGF) (Usuki et al., 1992; Usuki et al., 1994; Sumizawa et al., 1993; Moghaddam et al., 1992) directly engaged in cancerous cells proliferation (Takahashi et al., 1995; Toi et al., 1995). A correlation between dThdPase expression in tumors with malignancy on the other hand identifies this enzyme as a target for antitumor strategies by specific inhibitors of dThdPase (Moghaddam et al., 1995).


