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

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Z. Naturforsch. **52 c,** 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-nitrouracil, 5-fluorouracil, 6-aminouracil, 4,6-dihydroxy-5-nitropyrimidine 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 [E C 2.4.2.4.] and uridine [E C 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, Niedzwicki *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, Niedzwicki *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.

Abbreviations: dThdPase, thymidine phosphorylase; UrdPase, uridine phosphorylase; DFUR, 5-deoxy-5-fluorouridine; FUR, 5-fluorouridine; FUdR, 5-fluoro-2'deoxyuridine; 5-FU, 5-fluorouracil; 5-BrU, 5-bromouracil; 5-NiU, 5-nitrouracil; U, uracil; 6-AU, 6-aminouracil; 6-BTU, 6-benzyl-2-thiouracil; 4,6-DHNiP, 4,6-dihydroxy-5-nitropyrimidine; dThd, thymidine; T, thymine, U, uracil, AMT, allyloxymethylthymine; AMU, allyloxymethyluracil; AMFU, allyloxymethyl-5-fluorouracil; AMC, allyloxymethylcytosine;Th 5, 1[(1',3-dihydroxy)-2,2'-(propoxymethyl)] thymine.

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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, were obtained by Ozierov (Ozierov *et al.*,1991), Th5 by Dramiński (Rutkowski and Dramiński, 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 placentas with our own modifications. The

0939–5075/97/0900–0670 $ 06.00

tissues were homogenized in 4 volumes of ice cold buffer A [(1 mM EDTA, 0.02% 2-mercaptoethanol, 2 mM phenylmethanesulfonyl fluoride (PMSF), 20 mM sodium phosphate buffer, pH 7.5)] and centrifuged at 50000 x *g* for 1h.

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 M 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 (Iltzch *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).

Compound	Uterine leiomyomas			Uteri		
	dThdPase activity [U/mg of protein]	% of inhibition	K_i [μM]	dThdPase activity [U/mg of protein]	% of inhibition	K_i [μM]
Control	9.30 ± 0.60			0.650 ± 0.035		
U	7.07 ± 0.55	24.0 (p= 0.05)	158.0	0.640 ± 0.055		
6-AU	5.94 ± 0.36	36.1 (p= 0.005)	88.0	0.448 ± 0.030	31.0 (p=0.01)	130.0 comp.inh.
5-FU	4.35 ± 0.40	53.2 (p= 0.001)	43.0	0.363 ± 0.025	44.0 (p=0.002)	72.8 comp.inh.
5-NiU	0.86 ± 0.05	90.8 (p= 0.001)	5.1	0.200 ± 0.015	69.0 (p=0.001)	25.3 comp.inh.
5-BrU	0.19 ± 0.02	98.0 (p= 0.001)	1.1	0.062 ± 0.005	90.5 (p=0.001)	6.0 comp.inh
4,6-DHNiP	2.76 ± 0.21	70.3 (p= 0.001)	21.0	0.510 ± 0.050	22.0 (NS)	211.0 comp.
6-BTU	4.00 ± 0.25	57.2 (p=.0.001)	75.5	0.370 ± 0.024	43.0 (p=0.001)	137.0 m.inh.
AMT	5.20 ± 0.46	44.0 (p= 0.002)	127.0	0.360 ± 0.026	44.6 (p=0.001)	128.6 uncomp.inh.
AMU	9.31 ± 0.68			0.652 ± 0.072		
AMFU	8.15 ± 0.59	12.4 (p=0.02)		0.598 ± 0.063	8.0 (p=0.02)	
Th 5	8.41 ± 0.51	9.6 (p=0.05)		0.624 ± 0.056	4.0 (p=0.01)	
AMC	9.32 ± 0.75			0.660 ± 0.065		

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.
dThdPase from human uterine leiomyomas and uteri was purified 40-fold and 10-fold, respectively.
U, uracil; 6-AU, 6-aminouracil; 5-FU, 5-fluorouracil; 5-NiU, 5-nitrouracil; 5-BrU, 5-bromouracil; 4,6-DHNiP, 4,6-dihydroxy-5nitropyrimidine; 6-BTU, 6-benzyl-2-thiouracil; AMT, allyloxymethylthymine; AMU, allyloxymethyluracil; AMFU, allyloxymethyl-5-fluorouracil; Th 5, 1[(1',3-dihydroxy)-2,2'(propoxymethyl)]thymine; AMC, allyloxymethylcytosine.

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 μM and 1.06 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 μM next to 6-amino-5-bromouracil and 6-aminothymine with a K_i of 6 and 11 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 4,6DHNiP 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 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).

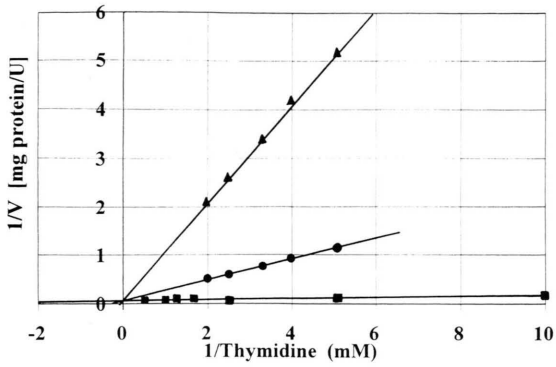

Fig. 1. Lineweaver-Burk plot of dThdPase activity in human uterine leiomyoma (■-■-■), in presence 0.1 mM 5-BrU (▲-▲-▲) and in presence 0.1 mM 5-NiU (●-●-●). 1 unit of enzyme activity (U) is defined as the quantity that catalyzes the formation of 1.0 μmol of free thymine per hour [μM T/h]. *V* is expressed as specific activity of dTdPase [U/mg protein]. 40-fold purified dThdPase (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 poins to K_{mi}=19.08 mM), in presence of 0.1 mM 5-NiU crosses *x*-axis at value -0.243 (which points to K_{mi}=4.12 mM).
Regression equations of the enzyme activity lines are:
y=0.0359 + 1.0343 x for r=0.999 in presence 5-BrU,
y=0.0597 + 0.2182 x for r=0.998 in presence 5-NiU,
y=0.0544 + 0.0103 x for r=0.993 without inhibitor,
Course of line indicates a competitive type of inhibition.

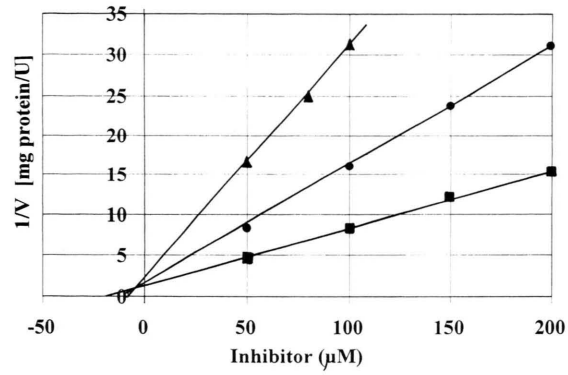

Fig. 2. Dixon plot for the effect of 5-BrU concentration on dThdPase activity in human uterus.
1 unit of enzyme activity (U) is defined as the quantity that catalyzes the formation of 1.0 μmol of free thymine per hour [μM T/h]. *V* is expressed as specific activity of dTdPase [U/mg protein]. 10-fold purified dThdPase (spec. activity 1.44 U/mg protein) were used in experiments. Substrate concentrations: (▲-▲-▲) 0.1 mM dThd; (●-●-●) 0.2 mM dThd; (■-■-■) 0.4 mM dThd. Reactions were carried out as described in Materials and Methods. Each point of the line – the mean ± S. D. for four experiments.
Course of line indicates a competitive type of inhibition with K_i = 6.0 μM.

Iltzch and Klein (1993) have reported that *T.gondi* UrdPase is similar to both mammalian UrdPase and dThdPase 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 dThdPase 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 dThdPase 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 dThdPase (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 dThdPase 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-aminothymine is a potent inhibitor for dThdPase activity (Langen *et al.,* 1967; Niedzwicki *et al.,* 1981). 6-AU, on the other hand, inhibited less the activity of the dThdPase 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 μM for uterine leiomyomas and K_i=130 μM for adjacent uterine tissue) (Table I).

The observed stronger inhibitory influence of these compounds on the activity of dThdPase from the studied leiomyomas may result from a better DEAE-Sepharose purification, greater sensitivity of dThdPase to temperature, shorter half-life, or greater specific activity of the enzyme in compari-

son with the one isolated from the uteri themselves (data not published).

Drabikowska *et al.*, (1987) investigated the acyclonucleoside analogues consisting of 5- and 5,6- substituted uracils as the potential inhibitors of UrdPase *E.coli*, but none of the compounds was a substrate or inhibitor of *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 acyclonocleosides (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 μM and 127.0 μM, respectively (Table I). This compound inhibits also the synthesis of dTMP and dGMP *in vivo* and dTMP, *in vitro* (Modrzejewska *et al.*, 1996). Other acyclonucleosides have been synthesized with antiviral and antitumor activties (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).

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