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THE HUMAN BLOOD PLATELET: A CELLULAR MODEL TO STUDY THE DEGRADATION OF THYMIDINE AND ITS INHIBITION

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Abstract—Intact platelets catabolize extracellular thymidine into thymine. Studies of the concentration dependent degradation of thymidine by intact platelets indicate a Michaelis mechanism with an apparent K_m of about 0.12 mM and a V_{max} of 2.5 nmoles/min for 3×10^8 platelets. This degradation process is inhibited by various nucleosides, pyrimidine bases and C-5 or C-6 substituted uracils. Cytidine, deoxycytidine, adenosine and deoxyadenosine seem to inhibit thymidine degradation by reducing the intracellular transport of thymidine. Uridine inhibits both the thymidine transport and the activity of the phosphorolytic enzyme, thymidine phosphorylase (EC 2.4.2.4). Some substituted uracils are specific inhibitors of thymidine phosphorylase activity. 6-Amino-5-bromouracil, the most active of them, either with acellular extracts or purified thymidine phosphorylase, is also the best inhibitor of thymidine degradation in intact human platelets. Platelets constitute a new model to study the efficiency of specific inhibitors on thymidine catabolism in an 'human intact cell' which contains only one pyrimidine nucleoside phosphorylase, the thymidine phosphorylase.

During the last years, a great interest has been focused on the clinical use of thymidine in humans. First, this nucleoside has been used to modulate the toxic activity of some anti-cancer agents such as methotrexate or 5-fluorodeoxyuridine (for review, see [1]). More recently, it has been shown that, *in vivo*, thymidine plays an important role in the regulation of the proliferation of some cellular types, mainly neoplastic cells [2, 3]. However, a rapid catabolism restrains the therapeutic effectiveness of thymidine [4–6]. Thymidine phosphorylase seems to be the main agent for the degradation of thymidine in man [7, 8] and an increase of the chemotherapeutic efficacity of this nucleoside may be mediated by the inhibition of this enzyme.

In prokaryotes, the inhibition of thymidine phosphorylase has been extensively studied. These works have allowed the definition of two kinds of inhibitors: natural nucleosides [9–11] and synthetic products, mostly uracils bearing bulky groups at C-6 [12, 13]. However, most of these last products are inefficient on the mammalian thymidine phosphorylase, because of important differences between the active sites of bacterial and mammalian enzymes [14–17]. That implies the need to undertake similar investigations on mammalian enzymes to study the inhibition of thymidine degradation in mammals. The inhibition of mammalian thymidine phosphorylase by natural nucleosides has not been studied; however, some uracils substituted at either C-5 or C-6, or both, by small hydrophobic groups have been described as potent inhibitors of this enzyme [15–17]. These studies had been performed with purified or partially purified enzyme, and not with intact cells or *in vivo*. Recently we have shown that intact human platelets can rapidly convert extracellular thymidine in thymine [18]. The catabolism of thymidine has been elucidated in whole platelets; thymidine yields deoxyribose-1-phosphate that remains in the cells and thymine which is excreted in the extracellular medium. In human platelets, this reaction is catalysed by thymidine phosphorylase which has been isolated and characterized; we have checked that these cells do not contain uridine phosphorylase [18].

Human platelets are anucleated cells produced by the cytoplasmic division of megakaryocytes; consequently, they exhibit a low level of DNA synthesis. Nevertheless, the catabolism of thymidine into thymine is the main pathway of utilization of this nucleoside (Fig. 1). Therefore, platelets can be used as a suitable cellular model to study the degradation of thymidine and its inhibition.

MATERIALS AND METHODS

Chemicals. Thymidine, thymine, uracil, uridine, deoxyuridine, 6-aminouracil, 5-nitrouracil, and other natural bases or nucleosides were obtained from Sigma Chemical Co. (St. Louis, MO). 6-Aminothymine was prepared by alkaline cyclization of α-methyl-cyanoacetyl urea according to the procedure of Bergman and Johnson [19]; 5-bromo-6-amino-uracil was synthesized by direct halogenation of 6-aminouracil [20]. [Me-^{3}H]Thymidine (1 Ci/mmole) was obtained from CEA (France).

Human platelets. Platelets were isolated by differential centrifugations from EDTA-anticoagulated human blood, resuspended in 1 mM EDTA/0.15 M NaCl/0.01 M Tris–HCl (pH 7.4) and washed three times in this buffer. The final suspension contained

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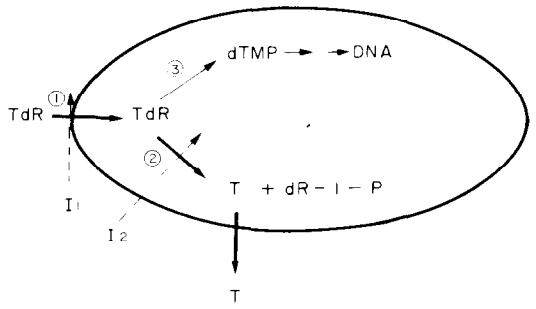


Fig. 1. Scheme of thymidine metabolism in a human blood platelet. Extracellular thymidine (TdR) penetrates into the cell where it is rapidly catabolized in thymine (T) which is excreted out of the platelet; the other product deoxyribose-1-phosphate (dR-1-P) remains in the cell. The other metabolic pathway, i.e. the salvage pathway, leading to DNA synthesis (3) is negligible in comparison with the catabolic one. The thymidine degradation can be limited by inhibitors (I_1) of thymidine transport (1) or by inhibitors (I_2) of thymidine phosphorylase activity (2).

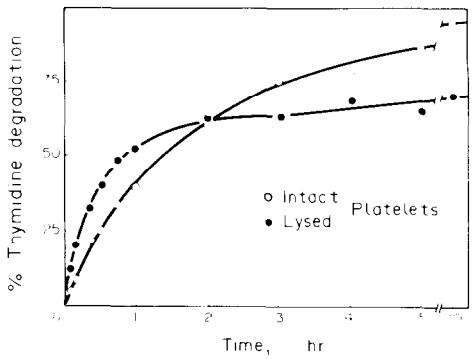


Fig. 2. Time course of thymidine degradation by intact or lysed human platelets. A platelet suspension (3×10^8 cells/ml) was incubated at 37° with 0.1 mM thymidine in presence of 1 mM sodium phosphate. Aliquots were taken at the time indicated and thymidine degradation was measured by thin layer chromatography. The same experiment was carried out with an acellular extract of the same platelet suspension.

approximately 1.2×10^9 platelets/ml. Platelet lysates were prepared by addition of 0.5% (v/v) Triton X-100 to this suspension.

Thymidine phosphorylase purification. Purified thymidine phosphorylase was prepared from washed human platelets as previously described [18].

Degradation of thymidine. The phosphorolysis of thymidine by purified thymidine phosphorylase, by intact or lysed platelets, was measured with a radio-isotopic technique. In 100 μl, the incubation medium contained 1 mM EDTA, 0.15 M NaCl, 1 mM sodium phosphate, 0.01 M Tris–HCl (pH 7.4), 2 μCi of labelled thymidine, and unlabelled thymidine to have a final concentration of 0.1 mM. The inhibitor's concentration is specified in the text; the enzyme source was purified thymidine phosphorylase (0.03 μg of proteins) or 3×10^8 intact or lysed platelets. The reaction was started by the addition of the enzyme source. Incubations were performed at 37° for different times. The reaction was stopped with 100 μl of 20% trichloracetic acid and the precipitate was removed by centrifugation.

Labelled thymine and thymidine were separated on Silica Gel 60 F 254 plates (Merck, Darmstadt, F.R.G.) eluted by the upper phase of a mixture of ethyl acetate/water/formic acid (60:35:5) [21]. Thymine and thymidine spots were located under ultraviolet light at 254 nm, scraped off and counted in a liquid scintillation spectrometer.

RESULTS

Experimental model. When incubated with thymidine, washed human platelets catabolize almost entirely this nucleoside in thymine. Lysed platelet extracts also catalyse the phosphorolysis of thymidine with a higher initial velocity but, in our experimental conditions, the reaction is not complete and reached a plateau when 70% of the thymidine had been catabolized (Fig. 2). The initial velocities of thymidine degradation, both by intact platelets or acellular extracts, were proportional to the platelet number (Fig. 3). The rate of thymidine degradation was linear with time during approximately 8 min for intact platelets and 4 min for acellular extracts with platelet concentration less than 5×10^8/ml. All other experiments described in this paper have been done for these incubation times.

Inhibition by nucleosides. The effects of some nucleosides on thymidine degradation by intact or lysed platelets are shown in Fig. 4. Most nucleosides at concentrations ranging from 1 to 8 mM are suitable inhibitors of thymidine degradation by intact platelets. On the contrary, only deoxyuridine, which is also a substrate of the phosphorolytic reaction, and uridine have an inhibitory effect on the lysed platelets; other nucleosides show little or no inhibition at all. These results show that most nucleosides assayed act probably as inhibitors of thymidine uptake by intact platelets, but, except deoxyuridine and uridine, do not act on thymidine phosphorylase itself. We have verified this hypothesis with purified platelet enzyme; only uridine exerted an inhibitory action with an ID_{50} of 1 mM, comparable to the ID_{50} obtained with intact or lysed platelets.

Inhibition by substituted uracils. Friedkin and Rob-

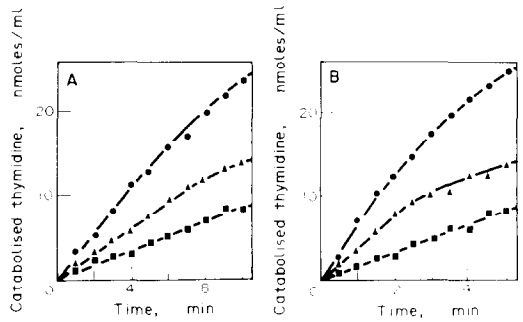


Fig. 3. Influence of platelet number on the thymidine degradation by intact platelets (A) or cell-free extracts (B). (■) 0.25, (▲) 0.5, (●) 1×10^9 cells/ml. Other experimental conditions are the same as in Fig. 2. It is clearly shown that the rate of degradation is twice as great in B as in A.

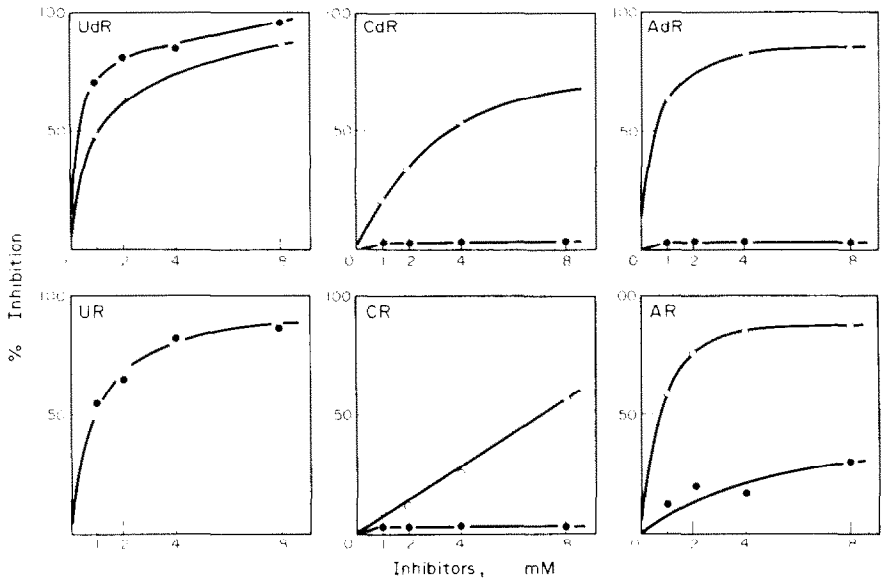
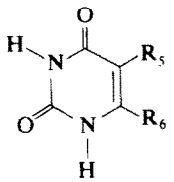


Fig. 4. Effects of some nucleosides on thymidine degradation by human platelets. Intact (○) or lysed (●) platelets (3×10^8 cells/ml) were incubated with 0.1 mM thymidine in the presence of different concentrations of various nucleosides: deoxyuridine (UdR), uridine (UR), deoxycytidine (CdR), deoxyadenosine (AdR), adenosine (AR), and cytidine (CR). Initial velocity of thymidine degradation is studied and the percentage of inhibition induced by each inhibitor is determined in comparison with thymidine cleavage in the corresponding non-inhibited sample.

erts have reported that free pyrimidine bases released in the phosphorolytic reaction, produce a relatively weak inhibition of mammalian thymidine phosphorylase [22]. Thymine and uracil, as well as some 5- and 6-substituted uracils known to be potent inhibitors of mammalian thymidine phosphorylase [15–17], have been assayed for their ability to inhibit the degradation of thymidine by intact or lysed platelets (Table 1). It can be seen that some inhibition was enhanced by the introduction of different groups in position 5 or 6 of the pyrimidine ring. With intact platelets, the ID_{50} values decrease in the following order: uracil, 5-fluorouracil, thymine, 6-aminouracil, 5-nitrouracil, 5-bromouracil, 6-aminothymine and 6-amino-5-bromouracil. The most powerful inhibitor, 6-amino-5-bromouracil, was 250-fold more effective than uracil and 90-fold more than thymine. The kinetics of the inhibition of thymidine phosphorolysis by 6-amino-5-bromouracil in the intact platelet system as shown in Fig. 5 demonstrate that the inhibition is competitive in the range of the concentrations used. Thymine, 5-bromouracil and 6-aminothymine, show an identical pattern. The K_m for thymidine phosphorolysis by intact platelets was in the range of 0.1–0.14 mM. The inhibitory constants (K_i) for 6-amino-5-bromouracil, 6-aminothymine, 5-bromouracil and thymine were 6, 11, 28 and 270 μM, respectively. With acellular

Table 1. Inhibition of thymidine phosphorolysis by 5- and 6-substituted uracils



R_5	R_6	Inhibitors	Catabolic source: Intact platelets	Catabolic source: Acellular extracts or purified platelet TdR-Pase
			(ID_{50}* μM)	
—H	—H	Uracil	2000	1300
—NO_2	—H	5-Nitrouracil	70	17
—F	—H	5-Fluorouracil	825	475
—Br	—H	5-Bromouracil	45	31
—H	—NH_2	6-Aminouracil	375	275
—Br	—NH_2	6-Amino-5-bromouracil	8	5
—CH_3	—H	Thymine	725	280
—CH_3	—NH_2	6-Aminothymine	22	13

* ID_{50} is the concentration of the inhibitor required for 50% inhibition of phosphorolysis of 100 μM thymidine.

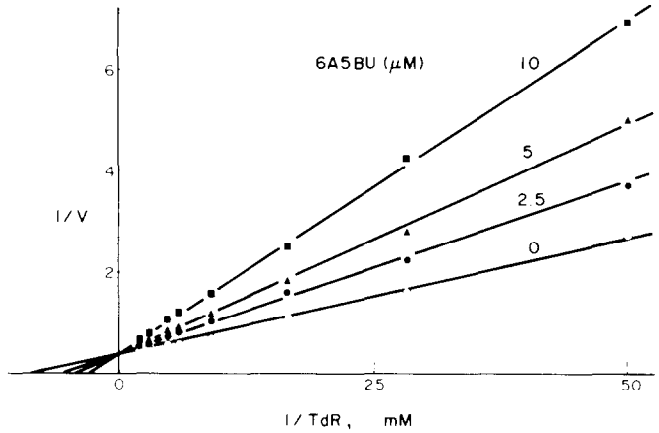


Fig. 5. Lineweaver–Burk plot for thymidine degradation inhibition by 6-amino-5-bromouracil in intact platelets. Thymidine degradation by intact platelets (3×10^8 cells/ml) was determined with various concentrations of thymidine with or without 6-amino-5-bromouracil (6A5BU) at different concentrations. Velocity (V) is expressed as nmoles of TdR degraded by 3×10^8 platelets/min. From the curves, the following constants are calculated: $K_m = 0.120$ mM and $K_i = 6\,\mu$M. The V_m is approximately 2.5 nmoles/3×10^8 platelets/min.

extracts, these inhibitory constants were 2, 8, 15 and 72 μM, respectively.

From Table 1, it can also be concluded that the different compounds assayed are more effective on lysed than on intact platelets. The ID_{50} obtained with acellular extracts are in the order of those obtained with purified platelet thymidine phosphorylase.

Inhibition and transport. Most nucleosides, such as cytidine, deoxycytidine, adenosine and deoxyadenosine, act only on intact platelets, probably by inhibiting the transmembrane transport of thymidine. Other products such as uridine or substituted uracils act either on intact platelets or with acellular extracts. If these last molecules are specific inhibitors of the thymidine phosphorylase activity, they could also inhibit the uptake of thymidine. We have studied the degradation and the intra- and extracellular distribution of radiolabelled thymidine after incubation with platelets in the presence of some of these inhibitors. Table 2 summarizes one of these experiments. It can be seen that the amount of total radioactivity of the extracellular medium remains nearly constant in the four samples. However, as expected, the percentage of degraded thymidine is less important with all the inhibitors. On the contrary, important variations can be noticed in the radioactivity linked to the TCA-soluble intracellular material. When platelets have been incubated with uridine or deoxyadenosine, the intracellular radioactivity is less important than that found in control or in 6-amino-5-bromouracil samples. This result indicates that 6-amino-5-bromouracil does not interfere with the uptake of thymidine; on the contrary, uridine acts in two ways, by inhibiting thymidine transport (Table 2) and thymidine phosphorylase activity (Fig. 4). It should be noticed that the intracellular free thymidine is greater with uridine and 6-amino-5-bromouracil than with deoxyadenosine because the intracellular degradation of thymidine is partially inhibited by uridine and 6-amino-5-bromouracil but not by deoxyadenosine; the non-degraded thymidine is very faint in the absence of inhibitor.

DISCUSSION

As pointed out in the introduction, thymidine can

Table 2. Uptake and degradation of thymidine by intact platelets

	Extracellular medium		Intracellular TCA-soluble material			
Inhibitor	Total R.A. 10^{-6} cpm	% Degradated thymidine	Total R.A. 10^{-3} cpm	TdR	T	dTxP
0	24.8	52.7	128	4	104	20
6A5BU	24.4	13.2	128	33	72	23
UR	24.1	5.1	72	33	20	19
AdR	24.7	10.4	59	16	24	19

Intact human platelets (3.4×10^8/ml) were incubated with 10 μCi tritiated thymidine without inhibitor (0) or with 0.064 mM 6-amino-5-bromouracil (6A5BU), 10 mM uridine (UR) or deoxyadenosine (AdR). After 30 min at 37° the cells were sedimented by centrifugation, lysed and the macromolecules were precipitated by cold trichloracetic acid. The supernatant—intracellular TCA-soluble material—and the extracellular medium were analysed for their radioactivity. Thymidylates (dTxP) were separated from thymidine (TdR) and thymine (T) by thin-layer chromatography as described in Materials and Methods.

play several important roles *in vivo*; the inhibition of its degradation could be a way to increase its therapeutic effects. However, to be active, thymidine has to penetrate into the cells. Consequently, the inhibitors of thymidine degradation employed *in vivo* must be inhibitors of the enzyme activity responsible for degradation but not for thymidine transport. Some nucleosides are known to inhibit cellular transport of thymidine in mammalian cells [23–25] but their influence on thymidine degradation has not been studied. On the other hand, several substituted uracils have been described to inhibit the enzyme thymidine phosphorylase [15, 17] but their roles on intact cells or on thymidine transport have not been determined.

In a previous paper [18], we have reported that human platelets catabolize exogenous thymidine by a phosphorolytic reaction due to the thymidine phosphorylase. We took advantage of this property to study the inhibition of thymidine degradation on an 'intact human cell': the blood platelet. This model permits us to define three kinds of inhibitors of thymidine degradation according to their behaviour with intact cells. The first group is constituted by nucleosides, as cytidine, deoxycytidine, adenosine, and deoxyadenosine, that inhibit thymidine degradation only by inhibiting the intracellular transport of thymidine. In the second group we find some compounds such as uridine which can affect both thymidine transport and thymidine phosphorylase activity. Finally, substituted uracils of the third group block only the phosphorolytic enzyme.

The results obtained on human purified thymidine phosphorylase or crude acellular extracts are in agreement with those previously reported with mammalian thymidine phosphorylase from other sources [15, 17]. The introduction of an amino group in position 6 of the pyrimidine ring increases the inhibitory effect and this is more marked if a halogen, especially bromine, is introduced in position 5. This pattern is identical with intact human platelets although the modified uracils are less effective than in the acellular system. One of the causes of this decrease could be a limited uptake of these inhibitors into the platelets and we are actually studying some compounds that could penetrate more easily into these cells.

Furthermore, 5-halogenodeoxyuridines, particularly 5-fluorodeoxyuridine, are also substrates for thymidine phosphorylase [7, 26, 27]. It has been shown that 5-fluorodeoxyuridine is a potent carcinostatic agent *in vivo* [28] but that its effectiveness is decreased because it is rapidly degraded [28, 29]. The inhibition of nucleoside phosphorylases was a possibility to inhibit the degradation of FUdR [17, 30]. Platelet thymidine phosphorylase was able to cleave 5-halogenodeoxyuridines [18] just as did intact human platelets. The inhibition of 5-fluorodeoxyuridine degradation could also be studied in this intact human cell containing only one pyrimidine nucleoside phosphorylase. Consequently, intact human platelets represent a useful tool for the definition of products able to inhibit thymidine and 5-fluorodeoxyuridine degradation at the cellular level, for the study of their inhibition mechanism and for their selection before they are tested *in vivo*.

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