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PHOSPHOROLYSIS OF (*E*)-5-(2-BROMOVINYL)-2′-DEOXYURIDINE (BVDU) AND OTHER 5-SUBSTITUTED-2′-DEOXYURIDINES BY PURIFIED HUMAN THYMIDINE PHOSPHORYLASE AND INTACT BLOOD PLATELETS

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Abstract—Various 5-substituted-2′-deoxyuridines (dUrd), including 5-ethyl-,5-propyl-, 5-trifluoromethyl-, 5-hydroxymethyl-, 5-formyl-, 5-vinyl-, (*E*)-5-(2-chlorovinyl)-, (*E*)-5-(2-bromovinyl)-, 5-fluoro-, 5-chloro-, 5-bromo-, 5-iodo-, 5-cyano-, 5-thiocyano-, 5-nitro- and 5-amino-dUrd, were shown to be effective substrates for the thymidine (dThd) phosphorylase isolated from human blood platelets. Some of dUrd analogs, i.e. the highly potent and selective antiherpes agent (*E*)-5-(2-bromovinyl)-dUrd, were degraded more rapidly than the natural substrates, dUrd and dThd. All dUrd analogs were also readily catabolised by intact human blood platelets. The potent inhibitors of thymidine phosphorylase, 6-amino-thymine and 6-amino-5-bromo-uracil, strongly inhibited the phosphorolysis of (*E*)-5-(2-bromovinyl)-dUrd by both purified enzyme and intact platelets.

Several 5-substituted-2′-deoxyuridines (dUrd) exhibit antitumor or antiviral properties. Thus, thymidine (dThd), 5-fluoro-dUrd and 5-trifluoromethyl-dUrd are used in the clinical therapy of some varieties of cancer [1–5], and 5-iodo-dUrd (IDU) and 5-trifluoromethyl-dUrd (TFT) are widely used as antiviral agents for the topical treatment of herpes simplex keratitis [6, 7]. Recently, some other 5-substituted dUrd derivatives have proven to be more potent and less toxic antiherpes agents than IDU and TFT [8–17]; foremost among these new antiherpes agents is (*E*)-5-(2-bromovinyl)-dUrd, which has been applied successfully in humans in the topical treatment of herpetic keratitis [18, 19] and the systemic (peroral) treatment of disseminated herpes zoster infection [20]. Moreover, some of the new 5-substituted dUrd analogs are potent inhibitors of the growth of tumor cells *in vitro*, particularly murine and human tumor cell lines [21, 22], and could, therefore, be considered as potential antitumor agents.

The dUrd analogs need to be phosphorylated to the 5′-monophosphate stage to exert their antiproliferative effect (by inhibition of thymidylate synthetase [21–23]) or to the 5′-triphosphate stage to exhibit their antiviral effect (by inhibition of the virus-induced DNA-polymerase [24] or incorporation into viral DNA [25, 26]). The degradation of the dUrd analogs by phosphorolytic cleavage between the uracil ring and sugar moiety obviously represents a limitation for their potential therapeutic use as either antitumor or antiviral agents. Therefore, it seemed interesting to determine the susceptibility of the dUrd analogs to degradation by pyrimidine nucleoside phosphorylases.

In mammals, two pyrimidine nucleoside phosphorylases are known to catalyse the phosphorolytic degradation of dThd and dUrd: thymidine phosphorylase (E.C. 2.4.2.4.) and uridine phosphorylase (E.C. 2.4.2.3.), the first one being specific for the deoxyribosyl moiety. 5-Halogeno derivatives of dUrd are also substrates for partially purified pyrimidine nucleoside phosphorylases [27, 28] and Nakayama *et al.* [29] reported the phosphorolysis of some 5-substituted dUrd derivatives by thymidine phosphorylase purified from horse liver. Thymidine phosphorylase seems to play a major role in the degradation of thymidine in man [30, 31]. Since (some of) the 5-subsituted dUrd analogs are aimed for clinical use, we chose the human thymidine phosphorylase as enzyme source to examine the rate of phosphorolysis of the dUrd analogs.

Previously we have shown that human blood platelets contain thymidine phosphorylase as the sole pyrimidine nucleoside phosphorylase and we have isolated and characterized this enzyme [32]. Moreover, intact human blood platelets have also been used to monitor the intracellular degradation of thymidine [33]. In the present study, we have investigated a series of 17 dUrd analogs as substrates for both purified human thymidine phosphorylase and intact human blood platelets. For (*E*)-5-(2-bromovinyl)-dUrd we also investigated the possibility of inhibiting this phosphorolysis by two potent inhibitors of thymidine phosphorylase, 6-amino-thymine and 6-amino-5-bromo-uracil.

MATERIALS AND METHODS

Chemicals

dThd, dUrd, 5-amino-dUrd, 5-trifluoromethyl-dUrd, 5-hydroxymethyl-dUrd, 5-fluoro-dUrd, 5-

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bromo-dUrd, 5-iodo-dUrd were obtained from Sigma Chemical Co and 5-chloro-dUrd from Calbiochem Behring Corp. The source of the other compounds was as follows: 5-nitro-dUrd [12, 34], 5-ethyl-dUrd [8, 35], 5-propyl-dUrd [10], 5-vinyl-dUrd [15], (*E*)-5-(2-bromovinyl)-dUrd [15, 36], (*E*)-5-(2-chlorovinyl)-dUrd [17, 36], 5-formyl-dUrd [16, 37] 5-cyano-dUrd [13], 5-thiocyano-dUrd [9, 38]. 6-Amino-thymine was prepared by alkaline cyclisation of α-methyl-cyanoacetyl urea according to the procedure of Bergman and Johnson [39]; 5-bromo-6-amino-uracil was synthesized by direct halogenation of 6-amino-uracil [40].

Human blood platelet thymidine phosphorylase

Purified thymidine phosphorylase was prepared from washed human blood platelets as previously described [32]. After chromatography on hydroxyapatite, the resulting thymidine phosphorylase was homogeneous upon sodium dodecyl sulfate gel electrophoresis; the purified enzyme catalysed the phosphorolysis of deoxyribonucleosides of thymine and uracil but also the direct pentosyl transfer from these nucleosides to uracil or thymine: these properties were characteristic of a thymidine phosphorylase [32]. It had a sp. act. of 10 U/mg of protein; one unit of enzyme activity was the amount of protein that produced the conversion of 1 μmole of thymidine per min at 37°C and pH 5.7 with 0.1 mM thymidine and 10 mM phosphate as substrates. At this phosphate concn, the K_m for thymidine and the catalytic constant values were respectively 140 μM and 55/sec (unpublished results).

Nucleoside phosphorolysis by purified thymidine phosphorylase

A continuous spectrophotometric assay was used according to the method described by Krenitsky for uridine [41] modified by Nakayama *et al.* [29] for 5-substituted dUrd. The change in absorbance was continuously monitored at the wavelengths where the difference between the deoxynucleoside and its free base was maximal ($\lambda\Delta A_{max}$): dUrd (270 nm), dThd (275 nm), 5-ethyl-dUrd (276 nm), 5-propyl-dUrd (277 nm), 5-hydroxymethyl-dUrd (273 nm), 5-formyl-dUrd (288 nm), 5-trifluoromethyl-dUrd

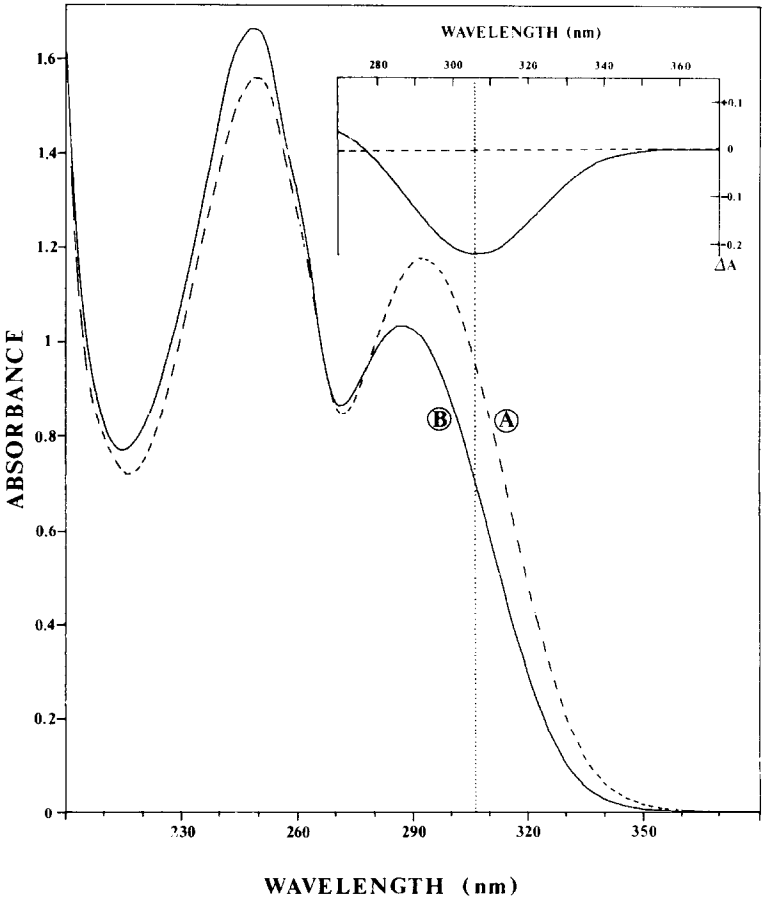

Fig. 1. Spectrum of (*E*)-5-(2-bromovinyl)-dUrd before (A) and after conversion to (*E*)-5-(2-bromovinyl)-U (B) with thymidine phosphorylase. Experimental conditions were those described in Materials and Methods. The difference spectrum (insert) (B) minus (A) shows that the ΔA_{max} is obtained at 306 nm; at this wavelength 0.1 mmole of nucleoside cleaved in base corresponds to an extinction of 0.22; this value is used in kinetic experiments to expressed velocities as nmole of product formed per min per ml of reaction mixture.

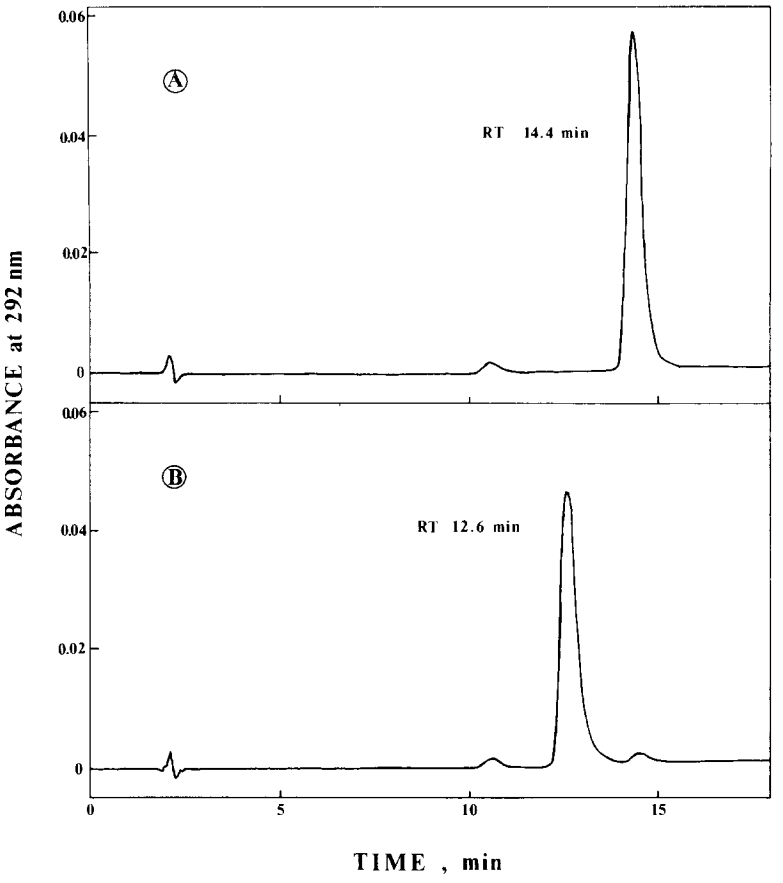

Fig. 2. HPLC analysis of (*E*)-5-(2-bromovinyl)-dUrd degradation by thymidine phosphorylase. Samples are the same than in Fig. 1. (A) Chromatogram of a 0.1 mM nucleoside solution before thymidine phosphorylase action; (B) after enzyme action 98% of the nucleoside was transformed in base. Peaks were identified by elution position and quantitated by peak areas of marker compounds. Chromatographic conditions: column, radial pak c_{18}; linear gradient from 20 to 60% of the high eluent (see Materials and Methods) in 15 min; flow rate, 2 ml/min; 40 μl injection; wavelength, 292 nm.

(270 nm), 5-fluoro-dUrd (278 nm), 5-chloro-dUrd (286 nm), 5-bromo-dUrd (289 nm), 5-iodo-dUrd (300 nm), 5-vinyl-dUrd (301 nm), (*E*)-5-(2-chlorovinyl)-dUrd (304 nm), (*E*)-5-(2-bromovinyl)-dUrd (306 nm), 5-amino-dUrd (304 nm), 5-nitro-dUrd (346 nm), 5-cyano-dUrd (283 nm), 5-thiocyano-dUrd (282 nm). Completion of the reaction was verified by HPLC analysis (see infra). The change in absorbance was continuously monitored at the $\lambda\Delta A_{max}$: the reaction mixtures consisted of 0.1 mM nucleoside (a non saturating concentration) in potassium phosphate buffer pH 5.7 and enzyme at a final concn of 1.25 μg/ml. For example, the spectra of (*E*)-5-(2-bromovinyl)-dUrd and of its resulting base after enzyme action were shown in Fig. 1.

Human blood platelet suspensions

EDTA-treated blood was centrifuged at 150 *g* for 15 min to remove erythrocytes and nucleated cells; the supernatant platelet rich plasma was centrifuged at 2000 *g* for 20 min. The sedimented platelets were suspended in 1 mM EDTA/0.15 M NaCl/0.01 M Tris–HCl (pH 7.4) and resedimented by centrifugation. This operation was repeated twice more. The final suspension contained 6.10^8 platelets/ml of washing buffer.

Nucleoside degradation by intact human blood platelets

The phosphorolysis of the 5-substituted dUrd analogs by intact blood platelets was measured by HPLC (see below). The incubation mixture contained 1 mM EDTA, 0.15 M NaCl, 1 mM sodium phosphate, 0.01 M Tris–HCl (pH 7.4), 0.1 mM 5-substituted dUrd and 3.10^8 platelets/ml. Incubations were performed at 37°; at different times, 200 μl fractions were taken off, rapidly cooled in ice and centrifuged for 10 min at 3000 *g* and 2°. The supernatant (40 μl) was then analysed by HPLC.

Inhibition studies

The inhibition of phosphorolysis of (*E*)-5-(2-bromovinyl)-dUrd by thymidine phosphorylase inhibitors was determined at the initial velocities of the phosphorylase reaction in the presence of various concns of the inhibitors. As reference point served the cleavage of (*E*)-5-(2-bromovinyl)-dUrd in the absence of the inhibitor.

HPLC method

The 5-substituted dUrd analogs were separated from the corresponding 5-substituted uracils by HPLC on a reverse phase Radial Pak C_{18} column (Waters) eluted by a linear gradient according to the method of Hartwick *et al.* [42] with some modifications due to the nature of the 5-substituent. A Waters HPLC system, with a M-450 detector, was used in this study, at a wavelength corresponding to the maximal absorbance of the nucleoside. The mobile phase was a mixture of 0.01 M potassium phosphate (pH 5.5) buffer (low eluent) and methanol–potassium phosphate buffer (80:20) solution (high eluent), at a flow rate of 2 ml/min. The slope of the gradient was chosen according to the nucleoside assayed. The peak areas of the nucleoside were used to follow the reaction. The chromatograms from a 0.1 mM (*E*)-5-(2-bromovinyl)-dUrd solution before and after treatment with thymidine phosphorylase are shown in Fig. 2.

RESULTS

Phosphorolysis by purified thymidine phosphorylase

All the 5-substituted dUrd analogs were effective substrates for human blood platelet phosphorylase (Fig. 3). The velocity of degradation was dependent on the nature of the 5-substituent. In comparison with the pattern of degradation shown for dUrd, the alkyl substituents either increased (formyl and trifluoromethyl) or decreased (hydroxymethyl) the velocity (Fig. 3A). The unsaturated vinyl substituent considerably increased the substrate affinity for thymidine phosphorylase (Fig. 3B), while the corresponding saturated ethyl group did not modify the rate of degradation; this increase in degradation rate was even higher if the vinyl radical was halogenated (Fig. 3B). Introduction of an halogen at the C-5 position of the uracil ring also increased the reaction velocity although F proved less efficient in this regard than either Cl, Br or I (Fig. 3C). Nitrogen-containing substituents had a differential effect: a nitro group did not cause a marked change, while a cyano or thiocyano group increased the velocity and an amino group decreased it (Fig. 3D). Thus, the 5-substituted dUrd analogs were cleaved at different velocities depending on the nature of the 5-substituent. In Table 1, these compounds were classified according to their initial velocities of phosphorolysis. This order corresponds to phosphorolytic degradation at pH 5.7 and it is not excluded that it may be different at another pH.

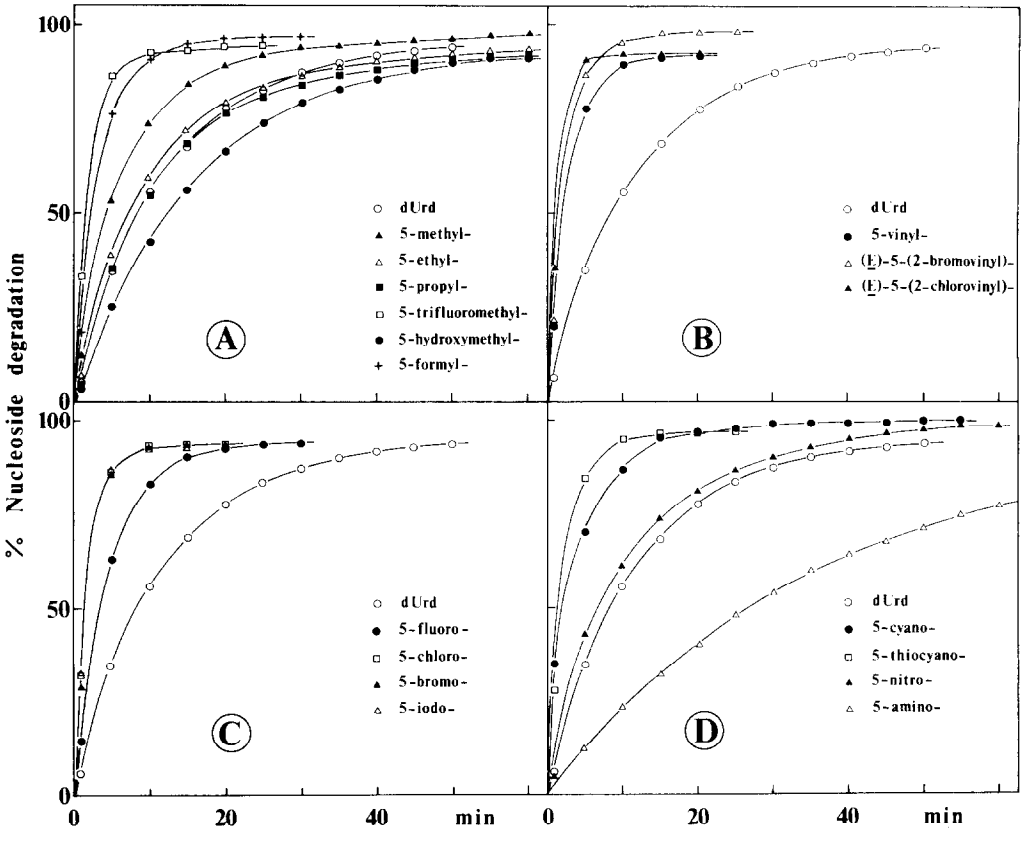

Fig. 3. Kinetics of degradation of 5-substituted 2′-deoxyuridines by human thymidine phosphorylase. A 0.1 mM solution of each nucleoside was incubated at 37°C with thymidine phosphorylase in 10 mM potassium phosphate pH 5.7. The percentage of degradation was determined by continuous monitoring of the absorbance at $\lambda\Delta A_{max}$ (the wavelength corresponding to the maximal difference of absorbance between a nucleoside and its base). 3A: alkyl substituents; 3B: vinyl substituents; 3C: halogen substituents; 3D: nitrogenous substituents.

Table 1. Initial velocities of phosphorolysis of 5-substituted dUrd derivatives by purified thymidine phosphorylase

Compounds	Initial velocity*
1. (*E*)-5-(2-chlorovinyl)-dUrd	40
2. (*E*)-5-(2-bromovinyl)-dUrd	36
3. 5-Iodo-dUrd	35
4. 5-Bromo-dUrd	35
5. 5-Chloro-dUrd	35
6. 5-Thiocyano-dUrd	30
7. 5-Trifluoromethyl-dUrd	30
8. 5-Formyl-dUrd	27
9. 5-Vinyl-dUrd	26
10. 5-Cyano-dUrd	24
11. 5-Fluoro-dUrd	20
12. 5-Methyl-dUrd	13
13. 5-Nitro-dUrd	12.5
14. 5-Ethyl-dUrd	10
15. 5-Propyl-dUrd	9.5
16. dUrd	9.5
17. 5-Hydroxymethyl-dUrd	6
18. 5-Amino-dUrd	2.5

* Velocities are expressed as nmole of base formed per min and per ml of reaction mixture in the conditions described in Materials and Methods.

Phosphorolysis in intact blood platelets

It has been shown previously that dThd penetrates into the blood platelets where it is rapidly catabolized to thymidine which is then secreted by the cell: the total reaction can be monitored by measuring the concns of extracellular thymine and thymidine [32, 33]. This was also the case for (*E*)-5-(2-bromovinyl)-dUrd: when 4.10^8 platelets per ml were incubated for 2 hr with 0.1 mM (*E*)-5-(2-bromovinyl)-dUrd, 95% of the nucleoside present in the medium was degraded. Intracellular nucleoside was not detectable. For the base the intra-and extracellular concns were identical. The platelet vol (about 6 μl) was negligible in comparison to the volume of the extracellular medium (1 ml); consequently, and as shown previously for thymidine, degradation of the nucleosides by the platelets could be monitored by measuring the extracellular concns of the nucleosides. Moreover, the sum of nucleoside and base remained nearly constant and equal to the initial concn of the nucleoside irrespective of the time at which the nucleoside and base concns were determined. Therefore, the degradation of the nucleosides could be followed by analysis of the supernatant fluid of the platelets. As shown in Fig. 4, all 5-substituted dUrd analogs were gradually converted to their respective free bases, when they were incubated with washed human blood platelets. The velocities of degradation were different depending on the nature of the 5-substituent. As could be

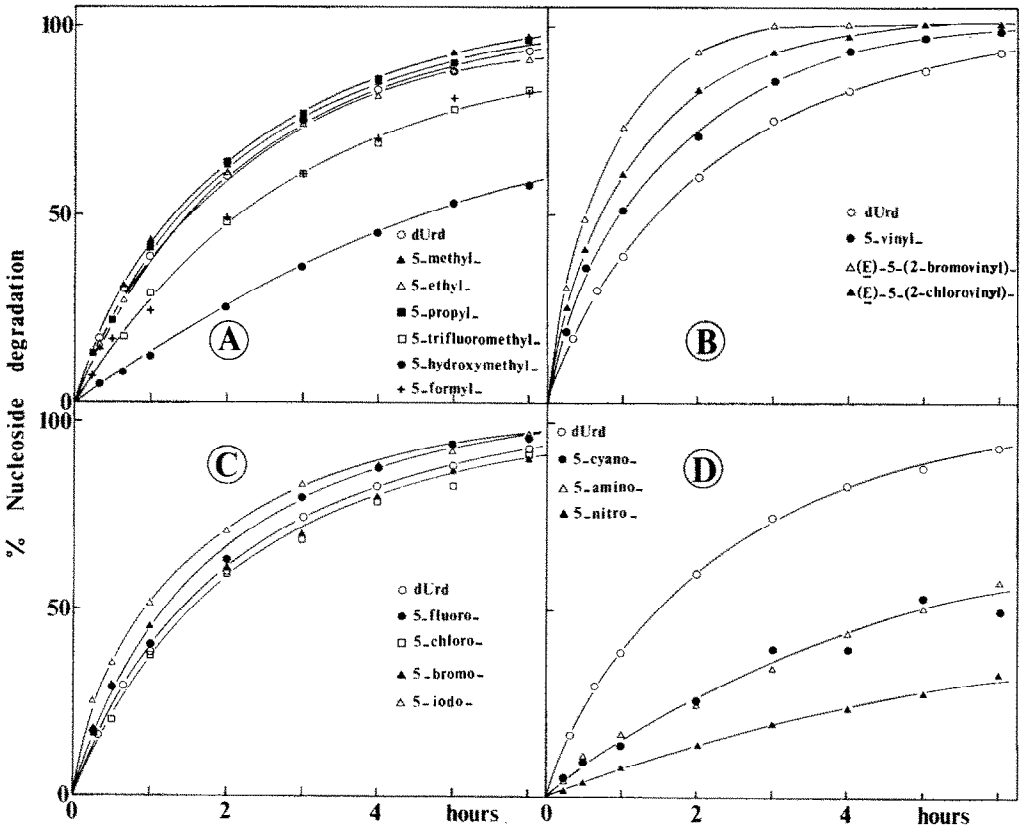

Fig. 4. Kinetics of degradation of 5-substituted 2'-deoxyuridines by intact human blood platelets. A 0.1 mM solution of each nucleoside was incubated at 37°C with a suspension of intact blood platelets (3.10^8 platelets/ml). The percentage of degradation was determined by HPLC. 4A: alkyl substituents; 4B: vinyl substituents; 4C: halogen substituents; 4D: nitrogenous substituents.

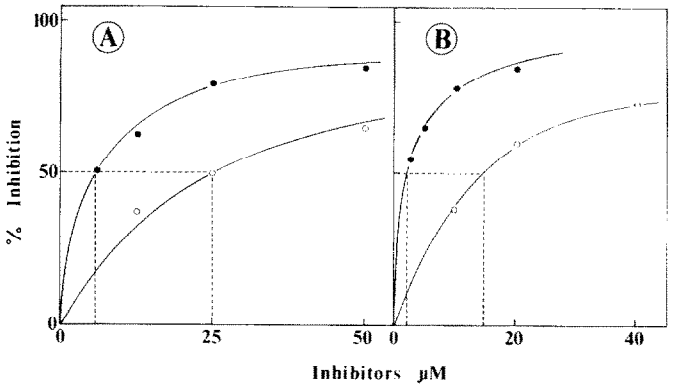

Fig. 5. Inhibition of phosphorolysis of (*E*)-5-(2-bromovinyl)-dUrd by 6-amino-thymine (A) and 6-amino-5-bromo-uracil (B). Intact blood platelets (○—○) or purified thymidine phosphorylase (●—●) were incubated with 0.1 mM (*E*)-5-(2-bromovinyl)-dUrd in the presence of different concns of 6-amino-thymine or 6-amino-5-bromo-uracil. Initial velocities were registered spectrophotometrically at 306 nm for the purified enzyme and by HPLC for the blood platelet suspension. The percentage of inhibition was determined in comparison with the cleavage of (*E*)-5-(2-bromovinyl)-dUrd in the absence of the inhibitors.

inferred from the nucleoside half-life times, the platelets degraded the 5-substituted dUrd analogs in the following order (of decreasing degradation velocity): (*E*)-5-(2-bromovinyl)-dUrd > (*E*)-5-(2-chlorovinyl)-dUrd > 5-iodo-dUrd > 5-vinyl-dUrd > dUrd, 5-methyl-, 5-ethyl-, 5-propyl-, 5-fluoro-, 5-chloro-, 5-bromo-dUrd>5-formyl-, 5-trifluoromethyl-dUrd > 5-amino-, 5-hydroxymethyl-, 5-cyano-dUrd > 5-nitro-dUrd. The 5-thiocyano-dUrd was peculiar in that it was also apt to a spontaneous degradation. After 24 hr, all the nucleosides, except 5-amino- and 5-nitro-dUrd, were entirely transformed to their respective bases.

Inhibition of the phosphorolysis of (E)-*5-(2-bromovinyl)-dUrd*

Several uracils substituted at either C-5 or C-6, or both, by small hydrophobic groups have been described as potent inhibitors of mammalian thymidine phosphorylase [43–45]. We have shown previously that 6-amino-thymine and 6-amino-5-bromo-uracil were potent inhibitors of the isolated and intracellular human blood platelet thymidine phosphorylase [33]. These two substituted uracils were not investigated for their ability to inhibit the degradation of the most promising antiherpetic agent, (*E*)-5-(2-bromovinyl)-dUrd (Fig. 5A and B). The two bases considerably decreases the velocity of the degradation of (*E*)-5-(2-bromovinyl)-dUrd by intact platelets and isolated thymidine phosphorylase. With the purified enzyme the concns (ID_{50}) required for 50% inhibition of phosphorolysis of a 100 μM solution of (*E*)-5-(2-bromovinyl)-dUrd were 2 μM and 6 μM for 6-amino-5-bromo-uracil and 6-amino-thymine, respectively. With intact platelets the ID_{50} were 15 and 25 μM, respectively. From these values, it can be concluded that the inhibitors were more effective on purified thymidine phosphorylase than on intact platelets.

DISCUSSION

Human blood platelets contain only one pyrimidine nucleoside phosphorylase, i.e., thymidine phosphorylase; they represent an attractive model to study the degradation of thymidine and specific inhibitors thereof at the cellular level [32, 33]. We have now investigated the degradation of several thymidine analogs by intact platelets and purified thymidine phosphorylase. This degradation was monitored by HPLC for intact platelets and continuous spectrophotometric recording for the isolated enzyme; hence, colorimetric or isotopic methods were avoided.

All 5-substituted dUrd analogs proved to be efficient substrates for the human thymidine phosphorylase. As noted previously for thymidine [33], intact human blood platelets split the 5-substituted dUrd derivatives to release their respective bases which were then excreted into the extracellular medium. Thus, nucleoside degradation by intact platelets was recorded by measuring the nucleoside concentration in the extracellular medium.

The 5-substituted dUrd analogs were degraded to a lesser extent by intact platelets than by purified thymidine phosphorylase. This differential degradation could be due to the intracellular environment of the enzyme, but also to differences in the uptake of the nucleoside by the platelet or excretion of the base from the platelet; as a consequence the base could accumulate within the platelet and inhibit the reaction. When the ratios of the half-lives of the nucleosides in presence of free enzyme or intact platelets were compared (Table 2), dUrd derivatives (i.e. 5-propyl-,5-amino- and 5-ethyl-dUrd) showed a ratio similar to that of dUrd (about ten), while all other dUrd derivatives showed a markedly higher ratio. For 5-trifluoromethyl-, 5-nitro- and 5-cyano-dUrd the half-life time ratios went up to 100 (Table 2). It would be interesting to find out why such substrate as 5-nitro-dUrd and 5-cyano-dUrd are less rapidly degraded than the 5-alkyl-dUrd analogs by intact platelets, while these nucleosides have similar degradation velocities with the purified enzyme. On the other hand, 5-amino-dUrd which was the least effective of all the dUrd analogs as substrate for the

Table 2. Rates of phosphorolysis of 5-substituted dUrd derivatives by purified thymidine phosphorylase and intact blood platelets

Compounds	HLT_I/HLT_E*
1. dUrd	9
2. 5-Propyl-dUrd	9
3. 5-Amino-dUrd	10
4. 5-Ethyl-durd	11
5. 5-Methyl-dUrd	18
6. 5-Fluoro-dUrd	19
7. (*E*)-5-(2-bromovinyl)-dUrd	20
8. 5-Hydroxymethyl-dUrd	23
9. 5-Vinyl-dUrd	27
10. (*E*)-5-(2-chlorovinyl)-dUrd	32
11. 5-Iodo-dUrd	37
12. 5-Formyl-dUrd	44
13. 5-Bromo-dUrd	58
14. 5-Chloro-dUrd	63
15. 5-Trifluoromethyl-dUrd	106
16. 5-Nitro-dUrd	108
17. 5-Cyano-dUrd	133

* HLT_I and HLT_E correspond to the half-life times of the nucleosides in the presence of intact platelets or purified thymidine phosphorylase, respectively.

purified enzyme, did not experience additional difficulties in its degradation by intact platelets.

Since all compounds, and in particular (*E*)-5-(2-bromovinyl)-dUrd, proved susceptible to cleavage by thymidine phosphorylase and also by intact blood platelets, it is likely that such degradation may limit the efficacy of 5-substituted dUrd analogs as antiviral or antitumor agents in the whole organism. Indeed, dUrd analogs are rapidly degraded *in vivo*, as has been shown directly for thymidine [46–48], 5-fluoro-dUrd [49] and 5-trifluoromethyl-dUrd [4] in man, and for 5-ethyl- and 5-iodo-dUrd in mice [50, 51]. Similarly, (*E*)-5-(2-bromovinyl)-dUrd has a limited half-life in mice [52].

Therefore, measures were considered to prevent or inhibit the degradation of (*E*)-5-(2-bromovinyl)-dUrd, so as to potentiate its efficacy *in vivo* whenever this may seem necessary. Two compounds, 6-amino-thymine and 6-amino-5-bromo-uracil, which were previously shown to inhibit the degradation of thymidine [33], also inhibited the degradation of (*E*)-5-(2-bromovinyl)-dUrd by both isolated thymidine phosphorylase and intact blood platelets. Moreover, these compounds exhibited similar ID_{50} values for thymidine and (*E*)-5-(2-bromovinyl)-dUrd degradation. It would now seem mandatory to investigate whether these inhibitors also suppress the breakdown of (*E*)-5-(2-bromovinyl)-dUrd *in vivo* and increase its potency in experimental animal models.

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