J. Pharm. Pharmacol. 1986, 38: 426–431
Received October 2, 1985

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Phosphorolysis of 5′-deoxy-5-fluorouridine in human plasma, serum and blood platelets

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During the course of the study of 5′-deoxy-5-fluorouridine (5′dFUrd) plasma protein binding using ^{19}F NMR spectroscopy, phosphorolytic cleavage of 5′dFUrd into 5-fluorouracil (5FU) was observed. This transformation was due to the enzymatic content of residual blood cells present in plasma, since the percentage conversion of 5′dFUrd into 5FU was lower as the number of residual blood cells fell, and an 'acellular' plasma or a serum effected a negligible phosphorolyis of 5′dFUrd. As platelets were the contaminants of the plasma samples studied, and a concentrate of human platelets demonstrated a high phosphorolytic activity towards 5′dFUrd, it was concluded that these blood cells were responsible for the 5′dFUrd cleavage. Thymidine phosphorylase, being the only pyrimidine nucleoside phosphorylase in human platelets, is suggested by the present results to catalyse 5′dFUrd phosphorolysis and is therefore not as specific for 2′-deoxyribonucleosides as has been reported.

5′-Deoxy-5-fluorouridine (5′dFUrd) is a new antineoplastic fluoropyrimidine active against several animal and human tumours. 5′dFUrd antitumour activity is related to its metabolic conversion into 5-fluorouracil (5FU); 5′dFUrd is thus a 5FU prodrug (Bollag & Hartmann 1980; Ishitsuka et al 1980). In previous papers, we have reported the analysis of 5′dFUrd metabolite pools in human biofluids using a new method: fluorine-19 NMR (^{19}F NMR) (Malet-Martino et al 1983, 1984). In this study, we have observed two signals for 5′dFUrd in plasma samples of patients treated with 5′dFUrd, one corresponding to unbound 5′dFUrd and the other to 5′dFUrd bound to plasma proteins (Fig. 1). We therefore decided to undertake a systematic study of the 5′dFUrd plasma protein binding using ^{19}F NMR. Unfortunately, during the course of these experiments, we came up against the problem of plasmatic 5′dFUrd cleavage into 5FU (Fig. 1). Even if human plasma protein binding of 5FU has been reported to be low (≃10%, Garrett et al 1977), the presence of 5FU makes a serious study of 5′dFUrd protein binding difficult because of the competition between these two fluorinated drugs for binding sites on plasma proteins.

The work presented in this article was designed to determine the causes of the 5′dFUrd plasmatic conversion into 5FU and thereby define the experimental conditions for the study of fluoropyrimidines binding which would prevent this cleavage.

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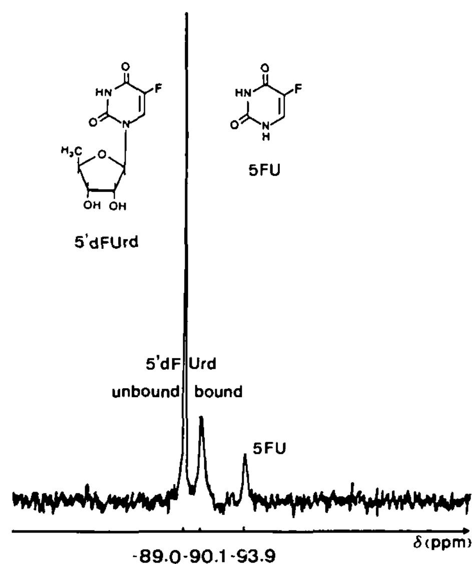

FIG. 1. ^{19}F NMR spectrum of a plasma sample (lot A) spiked with 5′dFUrd ($6{\cdot}7 \times 10^{-4}$ M). 5FU comes from 5′dFUrd cleavage during the time of NMR recording. Chemical shifts, expressed in ppm, are related to CF_3COOH (5% aqueous solution) as an external standard. Negative numbers indicate upfield direction from the reference. Number of scans 7 × 6000; recording time 22 h 45 min.

MATERIALS AND METHODS

Materials

5′dFUrd was a gift from Laboratoires Hoffman-La Roche (Basle, Switzerland). 5-Bromouracil (5BrU), 5FU and human serum albumin were from Sigma.

Preparation of plasma, serum, blood cells and platelets
Plasmas Lots A–J were obtained from Centre de Transfusion Sanguine of Toulouse. They were from blood, freshly collected on acid-citrate dextrose (ACD) (lots C–F, H) or citrate-phosphate dextrose (lots A, B, G, I, J) anticoagulant. Samples of lots F-J were counted using both a Malassez cell and a Coulter counter.

The 'acellular' plasma sample K was prepared by centrifugation at 4500*g* for 15 min of blood freshly collected on ACD anticoagulant. The plasma was carefully discarded and counted using both a Malassez cell and a Coulter counter.

The serum sample L was prepared from blood freshly collected in dry Vacutainer tubes. The tubes were kept at 37 °C for 30 min, centrifuged at 2000*g* for 15 min and the serum was then carefully discarded.

The blood cell pellet, obtained by centrifugation at 4500*g* for 15 min of blood freshly collected on ACD anticoagulant, was washed three times with 0·9% NaCl solution and resuspended in an equal volume of this medium.

Freshly collected blood on ACD anticoagulant was centrifuged at 200*g* for 10 min to remove erythrocytes and nucleated cells; the supernatant platelet rich plasma was then centrifuged at 2000*g* for 15 min. The sedimented platelets were washed three times with 0·9% NaCl solution and then resuspended in an equal volume of this medium.

The various samples studied after freeze-thawing were examined after three cycles of freeze-thawing at −20 °C.

Determination of inorganic phosphorus in plasma and serum samples
The phosphate ion level of the plasma and serum samples was measured by dosing the inorganic phosphorus using a P-Kit Biomérieux. The principle of the assay is a colorimetric determination of plasma and serum phosphorus using ammonium heptamolybdate which forms a phosphomolybdate complex in the presence of the reducing agent (ferrous sulphate).

Procedure for NMR assay
^{19}F NMR spectra were recorded at 250 MHz on a Cameca 250 FT spectrometer interfaced with a 16K memory Nicolet 80 computer. Spectra were run without proton decoupling and with no frequency field lock in 5 mm diameter tubes. The resonance positions were measured from the H_2O proton signal which is always positioned arbitrarily for any sample at the same frequency and referenced to an external standard CF_3COOH (5% aqueous solution) resonance peak. The instrumental settings were established as follows: probe temperature 25 °C; sweep width 33 333 Hz; pulse width 1·6 μs; recycling time 1·95 s; computer resolution 4·1 Hz/point; receiving filter out; line broadening caused by exponential multiplication 4–20 Hz depending on the signal-to-noise ratio of the spectrum. The spectra were usually acquired in blocks of 6000 scans.

The sample to be analysed (700 μl) was placed in an NMR tube. An aqueous solution of 5′dFUrd and relaxation reagent ($Cr(acac)_3$, $2 \cdot 7 \times 10^{-3}$ M) was then added (it was verified that this concentration of $Cr(acac)_3$ did not modify the spectra). The volume of the added solution never exceeded 10% of the biological medium volume. Estimates of the percent composition of resonances in the spectra were obtained by integration of areas of the expanded resonances (40 Hz cm^{-1}) and were reproducible within 5–10% from same samples depending on the signal-to-noise ratio of the peaks.

Procedure for HPLC assay
Extraction. Plasma (500 μl) was spiked with an aqueous solution of 5′dFUrd ($7 \cdot 74 \times 10^{-3}$ M) and internal standard, 5BrU ($4 \cdot 62 \times 10^{-4}$ M), to obtain concentrations of $7 \cdot 04 \times 10^{-4}$ M for 5′dFUrd and $1 \cdot 15 \times 10^{-5}$ M for 5BrU, and were incubated for 24 h at 25 °C. Tris buffer (500 μl, pH = 6) and 7 ml of ethylacetate were then added. The mixture was vortex-mixed for 1 min, then centrifuged at 3000*g* for 20 min. The upper organic layer was removed and evaporated to dryness at 45 °C. The residue was redissolved in 200 μl water and this solution was injected into the chromatograph. Under these conditions of extraction, the percentage recovery determined with plasma samples spiked with 5FU (final concentration $1 \cdot 31 \times 10^{-5}$ M) was approximately 70%.

Chromatographic conditions
The chromatographic analyses were performed using a Waters Associates model 510 apparatus equipped with a variable wavelength UV detector. 5′dFUrd, 5FU and 5BrU were determined at 280 nm. The column was a reverse phase type C_{18} Nova-Pak (5 μm), 15 cm long and having a 3·9 mm internal diameter (Waters Associates). The mobile phase was 5 mM tetrabutylammonium phosphate and 1·5 mM potassium phosphate buffer (pH = 7) at a flow rate of 1 ml min^{-1}. The retention times for 5FU, 5BrU

and 5′dFUrd were 4·15, 5·40 and 7·40 min, respectively. The calibration curve (ratios of the 5FU to 5BrU peak areas versus concentrations of 5FU) was obtained after analysis of plasma samples to which increasing concentrations of 5FU (3×10^{-6}–5×10^{-5} M) were added together with a constant concentration of 5BrU ($1·12 \times 10^{-5}$ M). The reproducibility was ±15% by analysing a plasma pool containing $1·13 \times 10^{-5}$ M of 5FU and $1·15 \times 10^{-5}$ M of 5BrU.

RESULTS AND DISCUSSION

The ^{19}F NMR study of 5′dFUrd binding to plasma proteins was first carried out with frozen-thawed plasma. We noticed a 5′dFUrd conversion into 5FU as a function of the lot of frozen-thawed plasma used (Table 1). It was not a chemical cleavage since 5′dFUrd aqueous solution never led to 5FU at physiological pH nor even at any pH between 0·5 and 12. This cleavage was due to a protein since a urea 8 M–dithioethanol 0·2 M denatured plasma did not cleave 5′dFUrd (Table 1). It was not, however, a conversion due to a non-specific protein since a solution of human serum albumin (HSA) did not induce 5′dFUrd cleavage (Table 1). 5′dFUrd conversion was thus enzymatic; it required the presence of phosphate ions in the medium. We let a plasma sample from lot B (inorganic phosphorus concentration $4·52 \times 10^{-3}$ M) cleave 5′dFUrd for 44 h; the percentage of 5′dFUrd phosphorolysis reached a plateau (≃24%) at about 25 h. If phosphate ions at two different concentrations ($1·4 \times 10^{-3}$ and $4·8 \times 10^{-3}$ M) were added to two plasma samples from lot B at t = 29 h (i.e. during the plateau), an increase in the phosphorolysis percentage was noticed; this percentage reached 32–33% after about 43 h for both phosphate ion concentrations (Fig. 2).

Table 1. 5′dFUrd phosphorolysis in human plasma, blood cells and platelets determined using ^{19}F NMR.

	Inorganic phosphorus[a] (M)	5′dFUrd concn (M)	Time of NMR recording (h)	% phosphorolysis[b]
Frozen-thawed plasma A	$4·23 \times 10^{-3}$	$1·2 \times 10^{-3}$	22	7
B	$4·52 \times 10^{-3}$	$1·2 \times 10^{-3}$	22	17
C	$1·23 \times 10^{-3}$	$1·2 \times 10^{-3}$	22	2
A	$4·23 \times 10^{-3}$	$6·1 \times 10^{-4}$	19	12
B	$4·52 \times 10^{-3}$	$6·1 \times 10^{-4}$	19	20
D	$1·19 \times 10^{-3}$	$6·1 \times 10^{-4}$	19	15
E	$1·32 \times 10^{-3}$	$6·1 \times 10^{-4}$	19	13
Urea-dithioethanol denatured plasma		$1·0 \times 10^{-3}$	16	0
HSA aqueous solution (6×10^{-4} M)		$1·0 \times 10^{-3}$	19	0
Frozen-thawed blood cells		$1·1 \times 10^{-3}$	24	37
Frozen-thawed platelets		$7·0 \times 10^{-4}$	24	52

[a] The range of expected values of inorganic phosphorus in plasma and serum of adults is $0·8 \times 10^{-3}$–$1·61 \times 10^{-3}$ M. The abnormally high values of some samples are due to the fact that blood was collected on an anticoagulant containing phosphate ions (citrate-phosphate dextrose anticoagulant); they are however in agreement with those expected for a plasma resulting from a blood collected on this anticoagulant.

[b] The percentage of phosphorolysis does not correspond to the percentage of 5FU present at time t (end of the recording) but corresponds to the percentage of 5FU determined after t h of recording, i.e. to the sum of 5FU estimated *each 1·95 s* during the recording time. The accuracy of phosphorolysis percentage

$$\frac{\text{area of 5FU signal}}{\text{areas of (5FU + 5′dFUrd) signals}}$$

is within 5%.

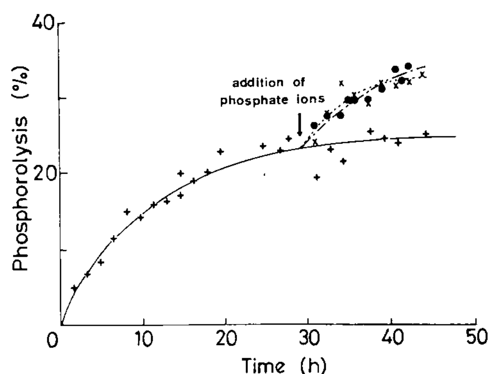

FIG. 2. 5′dFUrd phosphorolysis in plasma sample (lot B). At 29 h, addition of phosphate ions ($1·4 \times 10^{-3}$ M (×) and $4·8 \times 10^{-3}$ M (●)). 5′dFUrd concentration: $1·11 \times 10^{-3}$ M. The variation of the 5′dFUrd concentration due to the addition of phosphate ions is negligible. The time of each NMR recording block being 3·25 h, the percentage of 5′dFUrd phosphorolysis is considered to be representative of the state of 5′dFUrd conversion 1·625 h after the beginning of each NMR recording block.

In animal and human tumours, the antitumour activity of 5′dFUrd is manifested after its cleavage into 5FU by pyrimidine nucleoside phosphorylases. It is known that these enzymes are phosphate-dependent (Krenitsky 1968). We therefore concluded that 5′dFUrd phosphorolytic cleavage observed in plasma was catalysed by pyrimidine nucleoside phosphorylases. There are two distinct pyrimidine nucleoside phosphorylases: (a) thymidine phosphorylase (EC 2.4.2.4; thymidine : orthophosphate deoxyribosyl transferase) which catalyses the phosphorolysis of thymidine and is reported to be specific for 2′-deoxyribonucleosides; it is not inhibited by 1-(2′-deoxy-β-D-glucopyranosyl)-thymine (GPT) (Friedkin & Roberts 1954; Langen & Etzold 1963; Zimmerman 1964), and (b) uridine phosphorylase (EC 2.4.2.3; uridine : orthophosphate ribosyl transferase) (Pontis et al 1961; Bose & Yamada 1977). Recently, Woodman et al (1980) distinguished

between uridine phosphorylase activity that primarily cleaves uridine but not thymidine and is inhibited by GPT, and uridine-deoxyuridine phosphorylase that cleaves uridine, 2'-deoxyuridine and thymidine and is inhibited by GPT.

There is controversy concerning the presence of pyrimidine nucleoside phosphorylases in human plasma or serum. Birnie et al (1963) have found that human serum is totally lacking in nucleoside phosphorylase activity. A low thymidine phosphorylase activity has been detected in human serum (Heldin et al 1977; Gan et al 1981). Pauly (1977a, b) reported the identification of thymidine phosphorylase in human plasma. For Woodman (1979), thymidine phosphorylase does not occur in plasma and the activity found by Pauly is an artifact due to the method of plasma preparation which disrupts the granulocytes during the process of ultrafiltration.

The frozen-thawed plasma samples used (lots A-E) were from Centre de Transfusion Sanguine (CTS) and could contain some blood cells, which we thought could be responsible for the phosphorolysis of 5'dFUrd; a frozen-thawed cell pellet obtained after blood centrifugation does cleave 5'dFUrd (37% of 5'dFUrd was cleaved after 24 h of NMR recording) (Table 1). We then examined the ^{19}F NMR behaviour of plasma samples from CTS and of serum samples which had been previously counted. As phosphate ions are involved in the phosphorolysis reaction, the inorganic phosphorus level (representative of the phosphate ions) was determined for the various samples studied (Table 2). F, G, I and J plasma samples had a variable number of residual blood cells (mostly platelets); they led to variable phosphorolysis rates ranging between 3 and 6%. The H plasma had a low platelet count and produced a very weak rate of phosphorolysis which was not accurately measurable with ^{19}F NMR (<3%). The rates of phosphorolysis did not vary according to the phosphate ion content of the samples since samples with different inorganic phosphorus levels led to similar phosphorolysis levels (compare A/E for 5'dFUrd concentration $6{\cdot}1 \times 10^{-4}$ M in Table 1 and F/G in Table 2), while samples with very similar inorganic phosphorus levels produced different phosphorolysis levels (compare A/B in Table 1 and F/K in Table 2). It therefore seems that the variable cleavage rate found in various plasma samples is the result of the random presence of residual blood cells. To prove this, we attempted to prepare an 'acellular' plasma; the resulting plasma sample still contained 1700 platelets mm^{-3}. Under these conditions, the ^{19}F NMR method could not detect any phosphorolysis after 24 h of NMR recording whether the studied sample was fresh (not frozen) or frozen-thawed and even after 60 h for the frozen-thawed

Table 2. 5'dFUrd phosphorolysis in human plasma and serum determined using ^{19}F NMR and HPLC.

	Blood cells count (mm^{-3})					^{19}F NMR		HPLC	
	Platelets	Leucocytes	Erythrocytes	Inorganic phosphorus[a] (M)	5'dFUrd concn (M)	Time of NMR recording (h)	% phosphorolysis[b]	Incubation time (h)	% phosphorolysis
Frozen-thawed plasma F	66000	0	0	$1{\cdot}03 \times 10^{-3}$	7×10^{-4}	24	6		
Frozen-thawed plasma G	75000	100	0	$3{\cdot}55 \times 10^{-3}$	7×10^{-4}	24	5		
Fresh plasma G						24	4·5		
Frozen-thawed plasma H	12500	50	0	$1{\cdot}29 \times 10^{-3}$	7×10^{-4}	24	[c]	24	1·8
Frozen-thawed plasma I	36000	0	0	$4{\cdot}52 \times 10^{-3}$	7×10^{-4}	30	4		
Frozen-thawed plasma J	44500	150	2000	$4{\cdot}00 \times 10^{-3}$	7×10^{-4}	24	3		
'Acellular' frozen-thawed plasma K	1700	0	0	$1{\cdot}00 \times 10^{-3}$	7×10^{-4}	24	0	24	1·0
						60	0		
'Acellular' fresh plasma K						24	0	24	0·6
Frozen-thawed serum L	120	0	0	$1{\cdot}19 \times 10^{-3}$	7×10^{-4}	24	0	24	0·8
Fresh serum L						24	0	24	0·5

[a] The range of expected values of inorganic phosphorus in plasma and serum of adults is $0{\cdot}8 \times 10^{-3}$–$1{\cdot}61 \times 10^{-3}$ M. The abnormally high values of some samples are due to the fact that blood was collected on an anticoagulant containing phosphate ions (citrate-phosphate dextrose anticoagulant); they are however in agreement with those expected for a plasma resulting from a blood collected on this anticoagulant.

[b] The percentage of phosphorolysis does not correspond to the percentage of 5FU present at time t (end of the recording) but corresponds to the percentage of 5FU determined after t h of recording, i.e. to the sum of 5FU estimated *each 1·95 s* during the recording time. The accuracy of phosphorolysis percentage ([area of 5FU signal]/[areas of (5FU +5'dFUrd) signals]) is within 10%

[c] 5FU is detected but is too low to be determined accurately.

sample (Table 2). The same result was obtained for fresh or frozen-thawed serum samples which are more easily prepared without cells, the latter being in the clot (Table 2).

As NMR is not a very sensitive method, we have also studied the phosphorolysis rate of H and K plasmas and L serum, using HPLC which is more sensitive. With this method, we noticed that the phosphorolysis rate of plasma H was about 2% and those of plasma K and serum L were even lower (≤1%) (Table 2). Woodman (1979) obtained with thymidine a phosphorolysis rate comparable to those observed with K and L samples; he considered it as corresponding to a negligible level of thymidine phosphorylase activity. We may therefore assume the phosphorolysis of 5′dFUrd to be negligible in the 'acellular' samples (plasma K and serum L).

The two methods used in our study show that the nucleoside phosphorylase activity found in plasmas A-J is a consequence of plasma contamination by blood cells since (i) the percentage of 5′dFUrd conversion into 5FU is lower as the number of residual blood cells falls, (ii) an 'acellular' plasma demonstrates a negligible nucleoside phosphorylase activity. To study plasma protein binding of 5′dFUrd and probably of other fluoropyrimidines (work in progress), it is therefore necessary to use a plasma or a serum carefully prepared to avoid cell damage or contamination.

Concerning the blood cells, the literature reports the following data. Thymidine phosphorylase is the single pyrimidine nucleoside phosphorylase in human platelets (Desgranges et al 1981). Gallo & Perry (1969) and Desgranges et al (1981) have reported that human leucocytes contain both uridine and thymidine phosphorylases, but Woodman et al (1980) contest the uridine phosphorylase activity. As extracts of erythrocytes do not catalyse the splitting of pyrimidine nucleosides, neither thymidine phosphorylase, nor uridine phosphorylase are present in human red blood cells (Friedkin & Kalckar 1961; Gan et al 1981).

As plasmas F-J were contaminated with platelets, we examined the phosphorolytic activity on 5′dFUrd of a human platelet concentrate, entirely free of erythrocytes and containing 0·07% of leucocytes: after freezing, the platelets cleaved 52% of 5′dFUrd during 24 h of NMR recording (Table 1). These blood cells are therefore responsible for the observed cleavage of 5′dFUrd into 5FU in plasma.

Thymidine phosphorylase is not released from platelets (Desgranges et al 1981), in contrast to leucocytes; Marsh & Perry (1964) found a thymidine phosphorylase activity in the medium after intact leucocytes were incubated in phosphate buffer. It is therefore conceivable that the extremely low level of thymidine phosphorylase activity found in plasma K and serum L could be due to the release of traces of this enzyme from intact leucocytes into plasma (Woodman 1979). However, our results show that this residual activity may also be due to the fact that it is very difficult to obtain a plasma or serum sample completely free of platelets. Residual platelets may well be responsible for the very weak nucleoside phosphorylase activity found in plasma K and serum L.

Thymidine phosphorylase is the only pyrimidine nucleoside phosphorylase in human platelets (Desgranges et al 1981). Our results show that this enzyme catalyses 5′dFUrd phosphorolysis. This may at first seem surprising as thymidine phosphorylase has been described as being highly 2′-deoxyribonucleosides specific. Recently, however, Kono et al (1984) found that the activation of 5′dFUrd in human tumours was catalysed by thymidine phosphorylase. Our results agree with these findings and suggest that the substrate specifity of the human thymidine phosphorylase is broader than has been reported (Zimmerman 1964; Desgranges et al 1981).

Acknowledgements
The authors would like to thank Drs Calot and Mariniere, Centre de Transfusion Sanguine de Toulouse, for their assistance, and Hoffman-La Roche Laboratories for the generous gift of 5′dFUrd.

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