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A NOVEL DIMERIC FLUOROPYRIMIDINE MOLECULE BEHAVES AS A REMOTE PRECURSOR OF 5-FLUORO-2′-DEOXYURIDINE IN HUMAN ERYTHROCYTES

ANNA GASPARINI, MARCO GIOVINE, GIANLUCA DAMONTE, MICHELA TONETTI, TERESA GRANDI,* MAURO MAZZEI,* ALESSANDRO BALBI,* LUIGI SILVESTRO,† UMBERTO BENATTI and ANTONIO DE FLORA‡

Institute of Biological Chemistry, University of Genova, V.le Benedetto XV, 1, 16132 Genova; *Institute of Pharmaceutical Sciences, University of Genova, V.le Benedetto XV, 3, 16132, Genova; and †Respharma Pharmacological Research, Via Belfiore 57, 10125 Torino, Italy

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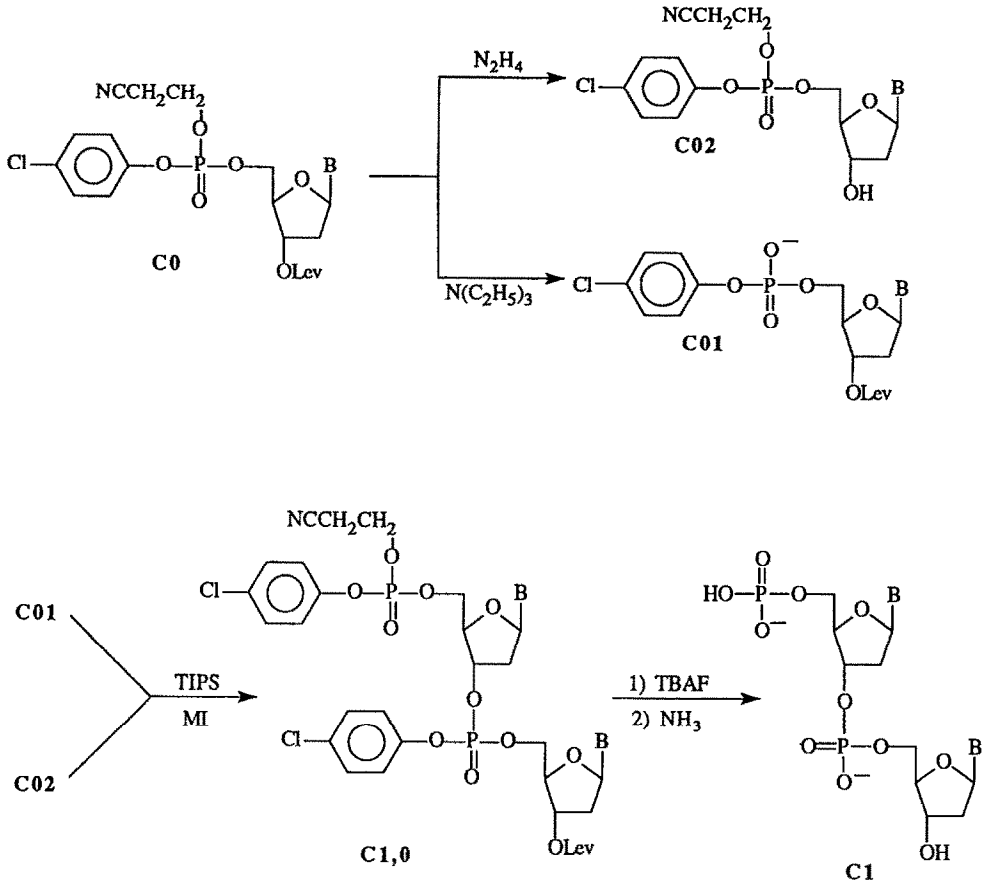
Abstract—A new dimeric fluoropyrimidine molecule (5-fluoro-2′-deoxyuridilyl-(5′→3′)-5-fluoro-2′-deoxy-5′-uridylic acid, Compound 1) was chemically synthesized from two separately deblocked 5-fluoro-2′-deoxyuridine mononucleotide moieties. Other structurally related nucleotides, 5-fluoro-2′-deoxyuridine-5′-diphosphate (FdUDP), 5-fluoro-2′-deoxyuridine-5′-triphosphate (FdUTP) and 5-fluoro-2′-deoxyuridine-3′,5′-bisphosphate were also synthesized. The structures of all synthesized molecules were verified by mass spectrometric analyses and were consistent with expected molecular mass values. The metabolic patterns of conversion of Compound 1 were investigated both in human erythrocyte lysates and in intact erythrocytes previously loaded with this molecule according to a highly conservative encapsulation procedure. In hemolysates, Compound 1 was transformed to 5-fluoro-2′-deoxyuridine (FUdR) and to 5-fluorouracil (FU) through the intermediate formation of 5-fluoro-2′-deoxyuridine-5′-monophosphate (FdUMP). In intact red cells, Compound 1 still generated FUdR (and to a lesser extent FU), that was then released outside. The conversion pathway involves a phosphodiesterase-catalysed hydrolysis of Compound 1 into two FdUMP molecules, followed by further dephosphorylation to FUdR and by partial conversion to FU. Unlike hemolysates, Compound 1-loaded intact erythrocytes featured transient formation of FdUDP and FdUTP, both metabolites representing storage compounds for the final and sustained production of FUdR and FU. Therefore, human erythrocytes can behave as bioreactors ensuring the time-controlled production and delivery of the two powerful antitumor drugs FUdR and FU from encapsulated Compound 1. This new molecule and other compounds as well (e.g. FdUDP and FdUTP) can be viewed as useful pre-prodrugs, exploitable for intraerythrocytic bioconversion reactions.

Key words: erythrocytes; 5′-fluoro-2′-deoxyuridine; fluoropyridimidine prodrugs; pre-prodrugs; 5-fluorouracil; cellular bioreactors

Increasing attention is devoted to new systems of controlled *in vivo* delivery of biologically active molecules, the primary aim being to overcome some disadvantages of conventional schedules of administration [1]. Undesired effects are most critical for chemicals that display cytotoxic activities, as often observed with chemotherapeutic agents, especially antiviral and antitumor drugs. In many cases, distinctive patterns of drug release (e.g. continuous vs pulsatile) elicit quite different toxicities and pharmacological responses [2]. The rationale for pursuing innovative procedures of delivery is based upon the strict correlation between pharmacological activity and pharmacokinetic parameters such as area under the curve (AUC), peak plasma levels and half-life [3].

A promising strategy towards improvement of therapies with highly toxic molecules is the exploitation of autologous cells as bioreactors competent to the time- and site-controlled production and release of these drugs. Such a strategy relies on the proper *in vitro* engineering of autologous cells (either by genetic modifications or by loading with suitable chemicals), thereby allowing them to display the new desired function; i.e. release of the toxic pharmacological agent following their re-administration to the same patient. An example of this trend is the use of TIL (tumor infiltrating lymphocytes) preliminarily transfected with the tumor necrosis factor gene [4]. Another cell-based system of drug delivery is represented by human and animal erythrocytes [5–12]. These cells have been proposed as ideal carriers for many chemicals because of their biocompatibility, the ease and reproducibility of the procedures for their loading with several drugs and the possibility of targeting them to specific organs and tissues [13–16]. An additional and major advantage is the metabolic potential of erythrocytes to convert non-diffusable encapsulated pro-drugs to their membrane-diffusable active forms, by virtue of endogenous enzymes catalysing such conversions [17, 18]. Obviously, the specificity and kinetic properties of intraerythrocytic enzymes involved in bioconversion reactions should

‡ Corresponding author. Tel. (39)-10-3538151; FAX (39)-10-354415.



Scheme 1. Synthesis of Compound 1. B = 5-fluorouracil; Lev = levulinic group.

dictate the choice of molecules that can be encapsulated in red cells to achieve the production of a specific drug.

We have previously described the conversion of the encapsulated prodrug FdUMP§ to its pharmacologically active form FUdR in human and mouse erythrocytes [17, 19]. FUdR is an antineoplastic drug showing selective cytotoxicity toward liver metastases from colorectal carcinomas [20]. Although such prodrug to drug conversion was unequivocally demonstrated to occur in the FdUMP-loaded erythrocytes, its rate was found to be too high to be compatible with an efficient anticancer therapy. Procedures designed to slow down the intraerythrocytic production and subsequent release of FUdR, including alkalinization of red cells or co-encapsulation of ATP and GTP (thereby inhibiting this conversion), proved unsuitable for this purpose [17, 19]. Accordingly, an alternative approach was developed in order to down-regulate FUdR release: the reconstruction within erythrocytes of a longer sequence of reactions still terminating with the FdUMP to FUdR conversion. This study involved the chemical synthesis of a properly tailored pre-prodrug represented by a new fluoropyrimidine dimer, that was demonstrated to generate FdUMP within erythrocytes. The kinetic and metabolic features of this model of drug production and release seem to fit the requirements of chemotherapy with FUdR.

§ Abbreviations: Compound 1 (C 1), 5-fluoro-2'-deoxyuridilyl-(5'→3')-5-fluoro-2'-deoxy-5'-uridylic acid; FdUMP, 5-fluoro-2'-deoxyuridine-5'-monophosphate; FdUDP, 5-fluoro-2'-deoxyuridine-5'-diphosphate; FdUTP, 5-fluoro-2'-deoxyuridine-5'-triphosphate; FUdR, 5-fluoro-2'-deoxyuridine; FU, 5-fluorouracil; TIPS, 2,4,6,-tri-isopropylbenzenesulfonyl chloride; MI, 1-methylimidazole; TBAF, tetrabutylammonium fluoride; TBA, ter-butyl-ammonium hydroxide; Htc, hematocrit; PCA, perchloric acid.

MATERIALS AND METHODS

Chemicals. FUdR, FdUMP (sodium salt) and FU were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). All other reagents were purchased from the Aldrich Chemical Co. (Gillingham, U.K.) and were of the highest purity available.

Synthesis of Compound 1. This compound was synthesized according to the general method described by Mazzei *et al.* [21], starting from the completely protected monomer β-cyanoethyl-*p*-chlorophenyl - 5' - (5 - fluoro - 2' - deoxyuridine

phosphate)-3'-levulinate (C 0), (see Scheme 1). Details on synthesis of the protected monomer (C 0) and on its treatment in order to obtain separate removal of the levulinic group from the 3' position (C 02) and of the β-cyanoethyl group from the 5' position (C 01), respectively, have been reported elsewhere [22]. Briefly, the following steps were used to this purpose: (1) protection of the 5'-OH of 5-fluoro-2'-deoxyuridine by dimethoxytrityl chloride (DMT); (2) protection of the 3'-OH by levulinic anhydride; (3) removal of DMT at 5'; (4) introduction in 5' of the phosphate group by *p*-chlorophenyldichlorophosphate; (5) introduction of the β-cyanoethyl group by reaction with 3-hydroxypropionitrile to obtain C 02. C 01 and C 02 were obtained by treatment of C 0 with triethylamine or hydrazine hydrate, respectively (see Scheme 1).

The mixture of C 01 (0.025 mmol) and C 02 (0.025 mmol) was then heated at 40° for 30 min in the presence of TIPS (0.1 mmol) and MI (60 μL), using 1 mL anhydrous pyridine as solvent. After cooling to 25°, the solution was evaporated three times with 2 mL toluene and the residue dissolved in 2 mL chloroform and purified by silica gel chromatography (linear gradient from 100% $CHCl_3$ to 90% $CHCl_3$/10% ethanol). The collected fractions were evaporated under reduced pressure and precipitated by petroleum ether. Final deprotection from C 1,0 to Compound 1 was achieved by treatment with 1 mL of 0.33 M TBAF in aqueous pyridine for 24 hr at 25°. After evaporation the residue was treated with 100 mL of 60% NH_3 for 48 hr at 25°. The fully deprotected dimer Compound 1 was purified by HPLC (anionic and reverse-phase columns). Anion chromatography was performed as follows: linear gradient from 30% acetonitrile/70% H_2O to 30% acetonitrile/70% 1 M KH_2PO_4 (pH 7.0) in 60 min at a flow rate of 5 mL/min (retention time of C 1: 9 min). After evaporation under reduced pressure, the residue was dissolved in 2 mL water and injected into a reverse-phase column. The chromatographic conditions were: linear gradient from 0.05 M $LiClO_4$ to 0.05 M $LiClO_4$ in 20% acetonitrile in 30 min at a flow rate of 5 mL/min (retention time of C 1: 6 min). The eluate from the reverse-phase column was evaporated under reduced pressure. The residue was resuspended in 0.3 mL water and precipitated as lithium salt by adding 3 mL of 2% $LiClO_4$ in acetone.

The fully protected dimer (C 1,0, see Scheme 1) was obtained at a 70% yield. The final deblocked dimer (C 1) was obtained at a 50% yield. The absorption maximum of C 1 was 269 nm in water. The procedure used for synthesis of C 1 enabled us to obtain a dinucleotide carrying the phosphate group in 5' since a protected phosphate is already present in that position in the starting material C 0. This is the main difference in respect to other procedures of synthesis in solution that are based on the phosphotriester approach [23]. Moreover, the proposed method makes it possible to obtain relatively large amounts (i.e. 100 mg) of dinucleotide C 1 at quite low cost. Since it is possible to remove the β-cyanoethyl and levulinic groups selectively from C 1,0 using triethylamine in anhydrous medium for the first, and hydrazine hydrate in neutral medium for the second, the present procedure facilitates the introduction of suitable active groups in 5' or 3', thus allowing the synthesis of a wide variety of C 1 analogs to be tested for bioconversion reactions.

Synthesis of 5-fluoro-2'-deoxyuridine phosphorylated derivatives. FdUDP was synthesized according to the general procedure described by Feldhaus *et al.* [24] for the synthesis of adenosine 5'-tetraphosphate.

FdUTP was synthesized as follows: FdUMP (0.1 mmol) was treated with diphenylphosphochloridrate to obtain the activated compound. Sodium pyrophosphate (0.025 mmol in 1.25 mL H_2O) was converted to the pyridinium salt by using a Dowex 50W-X8 (pyridinium form) and eluted with 50% aqueous methanol and vacuum-dried. Tri-*n*-octylamine (0.75 mmol) and methanol (1.25 mL) were added to the residue and stirred for 30 min at 25°. Methanol was removed under vacuum and the resulting oil dried with 1.25 mL *N*,*N*-dimethylformamide by repeated (three) evaporations. The residue was dissolved in 0.5 mL anhydrous pyridine and 0.1 mL hexamethylphosphotriamide and then added to the activated FdUMP. The solvent was removed by evaporation. After 0.1 mL pyridine was added, the mixture was stirred for 24 hr at 25° and vacuum-dried. Water (2 mL) was added to the residue, the pH adjusted to 8.0 with 1 M KOH and the solution extracted twice with ether.

5-fluoro-2'-deoxyuridine-3'5'-bisphosphate was prepared using the cyanoethylphosphate method described by Tener [25], with the following modifications: FUdR (0.15 mmol) was dissolved in 300 mL cyanoethylphosphate stock solution [25] and dried twice with anhydrous pyridine. Then, 190 mg *N*,*N*-dicyclohexylcarbodiimide and 3.2 mL anhydrous pyridine were added to the residue and the mixture stirred for 2 days. Water (0.46 mL) was added and the mixture stirred for 30 min. Upon further addition of 0.46 mL of water, the mixture was stirred for 30 min and then evaporated. The residue, resuspended in 1.6 mL water, was filtered to remove the precipitate containing the dicyclohexylurea, which was washed again with 1.6 mL water. The filtrate and the wash were combined, then 3.2 mL 1 N NaOH were added and the mixture heated under reflux for 40 min in order to remove the cyanoethyl group. The solution was then cooled to 25° and poured onto a Dowex 50W-X8 (H^+) column to remove Na^+ ions. The pH was then adjusted to 7.5 with $Ba(OH)_2$ to precipitate inorganic phosphate. Finally, the supernatant, reduced to a small volume (5 mL), was supplemented with 10 mL ice-cold ethanol to precipitate the barium salt of the nucleotide.

Mass spectrometry analysis. Mass spectra were acquired using a triple quadrupole tandem mass spectrometer PE-Sciex API III equipped with an ionspray atmospheric pressure ionization source or a single quadrupole HP Engine 5989-A equipped with an electrospray ion source. The sample flow was introduced using a micro-pump syringe (Harvard Apparatus). Mass spectra were acquired in negative mode scanning over a range that included expected molecular masses of the phosphorylated molecules

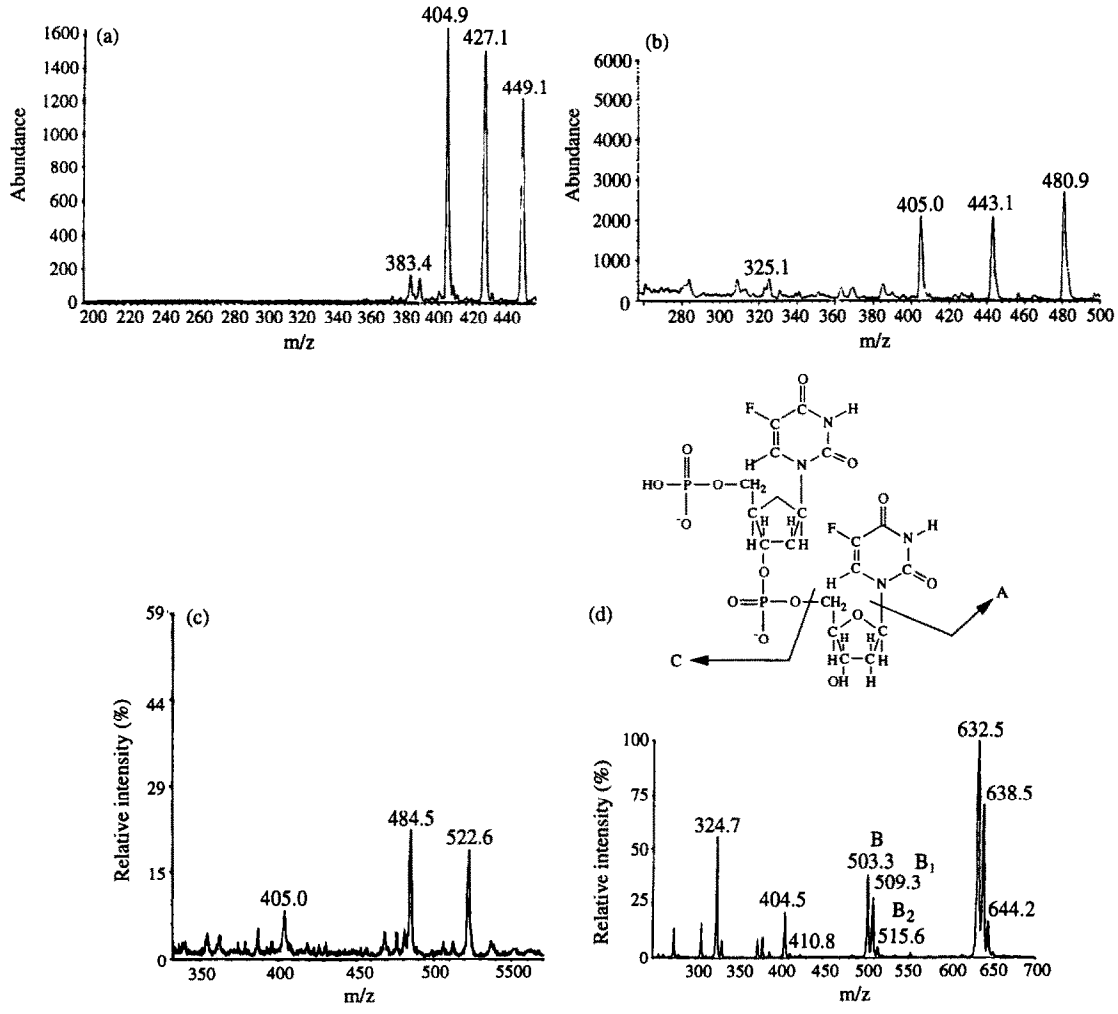


Fig. 1. M.s. characterization of Compound 1 and related fluoropyrimidine derivatives. (a) Spectrum of 5-fluoro-2′-deoxyuridine-3′,5′-bisphosphate: $(m - H)^- = 404.9$, $(m + Na^+ - 2H)^- = 427.1$, $(m + 2Na^+ - 3H)^- = 449.1$. (b) Spectrum of FdUDP: $(m - H)^- = 405.0$, $(m + K^+ - 2H)^- = 443.1$, $(m + 2K^+ - 3H)^- = 480.9$. (c) Spectrum of FdUTP: $(m - H)^- = 484.5$, $(m + K^+ - H)^- = 522.6$. (d) Spectrum of Compound 1: $(m - H)^- = 632.5$, $(m + Li^+ - 2H)^- = 638.5$, $(m + 2Li^+ - 3H)^- = 644.2$. Fragments of the molecule were identified as A, B, B1, B2 and C, where C = 404.5, B = $(m - A - H)^-$, B1 = $(B + Li^+ - 2H)^-$ and B2 = $(B + 2Li^+ - 3H)^-$.

(FdUDP, FUdR-3′,5′ bisphosphate, FdUTP and Compound 1) and of related compounds, as well as their possible adducts with Na^+, K^+ and Li^+.

Incubation in hemolysates. Washed and packed erythrocytes were hemolysed by freezing and thawing three times. Compound 1 was then added at a final concentration of 1.5 mM and incubated at 37° in the hemolysates under sterile conditions. At various times, samples were withdrawn and kept at −20° until analysis.

Dialysis encapsulation. Dialysis encapsulation was achieved under sterile conditions by the hypotonic dialysis and isotonic resealing method [7, 12, 17]. A two-step procedure was used, consisting of a first dialysis of the washed and packed erythrocytes (80% Htc) against 70 vol. of hemolysing buffer (5 mM Na_2HPO_4 supplemented with 4 mM $MgCl_2$, pH 7.2, 26 mOsm) for 35 min at 4° under gentle rotation. This step was followed by addition of 10–15 mM Compound 1 inside the dialysis bag and further dialysis for 15 min under the same conditions. Erythrocytes were then resealed by dialysing them for 40 min at 4° against phosphate-saline buffer pH 7.4 supplemented with 10 mM glucose and adenosine, 310 mOsm [26]. The loaded erythrocytes were then extensively washed with ice-cold NaCl and incubated at 37° in autologous plasma at a 10% Htc. At various times, 1 mL aliquots of the incubation mixture were withdrawn. Samples were immediately frozen at −20° after the separation of erythrocytes from plasma and kept frozen until analysis.

Extraction and analysis of nucleotides. HPLC analyses were carried out on neutralized perchloric acid extracts [27] of aliquots from the incubated

erythrocyte suspensions (both red cells and plasma) or hemolysates. The samples were injected into an HP 1090 instrument (Hewlett-Packard, Palo Alto, CA, U.S.A.) equipped with an HP ODS Hypersil 4.6 × 60 mm 3 μm particle size column. The solvent program was a linear gradient starting at 100% of buffer A (0.1 M KH_2PO_4 with 5 mM TBA, pH 4.9) and increasing up to 100% buffer B (0.1 M KH_2PO_4 with 5 mM TBA, 40% methanol, pH 4.9) in 35 min. The flow rate was 0.4 mL/min and the eluted compounds were monitored with a spectrophotometric detector set at 270 nm. Retention times for the various compounds were (in min): FU, 2.74; FUdR, 6.07; FdUMP, 9.09; AMP, 11.76; FdUDP, 13.60; FUdR-3′,5′-bisphosphate, 14.10; ADP, 17.76; FdUTP, 18.82; ATP, 21.00 and C 1, 21.30.

RESULTS

Figure 1 shows the m.s. spectra obtained by direct flow injection of the synthesized compounds in negative ionization mode. In any spectrum the molecular ions and the corresponding K^+, Na^+ and Li^+ adducts could be identified. In the spectrum of Compound 1 other relevant ions, identified as molecular fragments A, B, B1, B2, C (Fig. 1d), were also detected.

The metabolic patterns of conversion of Compound 1 were investigated both in erythrocyte lysates and in intact erythrocytes following encapsulation of this compound. As shown in Fig. 2, hemolysates were highly efficient in removing Compound 1, which fell to nearly 30% after 60 min and was almost undetectable at 3 hr. At shortest incubation times (within 60 min), FUdR and FdUMP proved to be the major products of this conversion, with a small amount of FU being formed (Fig. 2). From 60 min onwards, a divergent pattern was observed for FdUMP (declining rapidly until 3 hr and showing a slower rate of decrease later on) and for FUdR (linearly increasing up to 3 hr and then reaching a plateau). After Compound 1 was exhausted, i.e. from 3 to 24 hr, a moderate formation of FU was observed, which corresponded exactly to the sum of the decays of FdUMP and FUdR. This quantitative correlation identifies the pool of these two compounds as the source of FU.

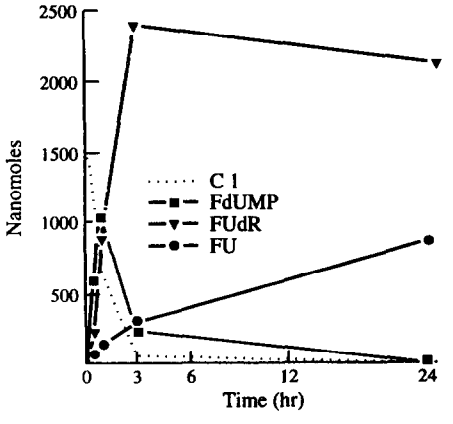


Fig. 2. Metabolic patterns of conversion of 1.5 mM Compound 1 in hemolysed erythrocytes. Incubations were performed at 37°, as reported in Materials and Methods. At the times indicated, samples were withdrawn and their PCA extracts analysed by HPLC. Results of a single experiment are shown for the sake of clarity. Variability in five different experiments never exceeded 16%.

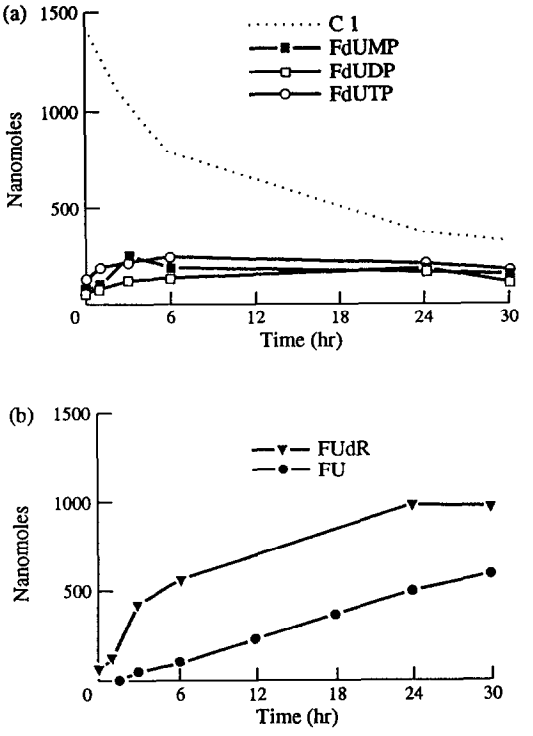


Fig. 3. Metabolic patterns of conversion of Compound 1 encapsulated within intact erythrocytes. The loaded cells (1.4 mM Compound 1) were incubated at 37° in plasma at 10% Htc under sterile conditions: (a) levels of phosphorylated derivatives in the erythrocyte fraction; (b) release of the membrane-diffusable molecules FUdR and FU in the plasma fraction. Results of a single experiment are shown. Variability in four different experiments never exceeded 12%.

No other metabolites of Compound 1 were found throughout incubation with the hemolysates. The patterns illustrated in Fig. 2 and obtained with a 1.5 mM concentration of Compound 1 were seen constantly within a range of starting concentrations from 1.5 to 3.4 mM Compound 1 (not shown). These patterns are consistent with the immediate and coordinated metabolic flow of Compound 1 to FdUMP as intermediate compound and eventually to FUdR and FU.

Encapsulation of Compound 1 in erythrocytes led to a final intracellular concentration of 1.5–3 mM. Analysis of major metabolic properties of Compound 1-loaded erythrocytes (including rates of glycolytic and hexose monophosphate shunt pathways, intracellular levels of ATP, ADP, AMP and GSH, and osmotic fragility curves) revealed no perturbation

whatsoever as related to encapsulation of Compound 1 and its metabolism (not shown). The metabolic pathways of conversion of Compound 1 within intact human erythrocytes were investigated in PCA extracts of Compound 1-loaded red cells obtained at various times of incubation in autologous plasma (see Materials and Methods). Both intraerythrocytic metabolites and those released into the supernatant plasma were analysed. Figure 3a shows that the decrease in intraerythrocytic Compound 1 following its encapsulation was much lower than in the corresponding hemolysates (Fig. 2): thus, at 30 hr of incubation (performed under sterile conditions), approximately 30% of the original amount of Compound 1 was still present in the red cells. Figure 3a also shows that three major by-products of Compound 1 were detectable in the erythrocyte extracts. Of these, one was immediately identified as FdUMP, while the other two proved to be the corresponding diphosphate and triphosphate forms, respectively. This conclusion was reached by comparing the UV spectra and HPLC retention times of the two compounds with those of authentic FdUDP and FdUTP, synthesized as described in Materials and Methods and identified by m.s. (Fig. 1b and c, respectively). As shown in Fig. 3a, the three phosphorylated metabolites of Compound 1 were already present at zero time, indicating that onset of conversion had taken place immediately after internalization, i.e. during the resealing step.

Inspection of Fig. 3a clearly indicates a lack of quantitative correlation between the disappearance of Compound 1 and the formation of its three phosphorylated metabolites. This reflects the immediate release of FUdR from the Compound 1-loaded erythrocytes, as actually observed to occur in the corresponding supernatant (Fig.3b). The only other metabolite released from erythrocytes was FU. However, the output patterns of the two compounds were sharply different. FUdR was produced intracellularly and released from the red cells very rapidly over the first 3 hr and much more slowly from 3 to 30 hr of incubation. Conversely, FU showed a delayed formation as compared to FUdR and afterwards increased in a parallel fashion to FUdR, until reaching relatively high extra-erythrocytic levels at 30 hr (Fig. 3b).

The finding of FdUDP and FdUTP in the Compound 1-loaded erythrocytes suggested the idea to investigate whether these nucleotides too can behave as precursors of the active fluoropyrimidine drugs FUdR and FU. Figure 4 shows that this was the case with FdUDP-loaded red cells. Indeed, a progressive, although non-linear, decrease of intraerythrocytic FdUDP was apparent which was paralleled by the concomitant release of FUdR and FU from the red cells. The system also showed a continuous decrease in intracellular FdUTP and FdUMP down to their almost complete disappearance (Fig. 4). Similar results were obtained after encapsulation of FdUTP (data not shown).

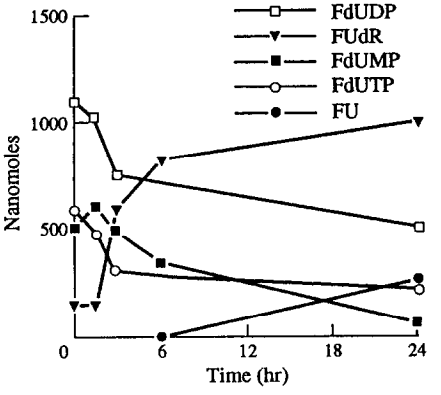


Fig. 4. Metabolism of erythrocyte-encapsulated FdUDP (2.2 mM final concentration). Both FUdR and FU released in the plasma fraction and the phosphorylated derivatives formed inside the red cells are shown in the figure. Results of a representative experiment are shown for the sake of clarity. The range of variability in three different experiments never exceeded 15%.

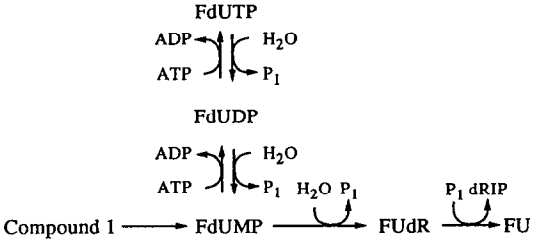


Fig. 5. Proposed pathways of metabolic conversion of fluoropyrimidines derived from Compound 1 in human erythrocytes. Each molecule of Compound 1 yields two molecules of FdUMP.

DISCUSSION

The procedure of chemical synthesis of Compound 1 allowed us to investigate properly its putative role of precursor of pharmacologically active fluoropyrimidine molecules in biological systems (hemolysates and intact erythrocytes). Indeed, the metabolic patterns of conversion of Compound 1 taking place in human erythrocyte lysates and also in intact red cells seem to suggest a major two-step pathway. The first reaction is a direct phosphodiesterase-catalysed hydrolysis of the dinucleotide structure to two FdUMP molecules (Figure 5). Evidence for specificity of cleavage of the 3′,5′-phosphodiester bond comes from the failure to observe any trace of 5-fluoro-2′-deoxyuridine-3′,5′-bisphosphate, in spite of a search for this hypothetical derivative by means of synthesis of the authentic compound (see Materials and Methods).

In hemolysates, there is clear evidence that FdUMP, once formed from Compound 1, has no other fate than hydrolysis to yield FUdR—a reaction catalysed by the deoxyribonucleotide—specific isoenzyme of erythrocyte pyrimidine 5′-nucleotidase identified by Paglia *et al.* [28]. On the other hand, the data obtained with the Compound 1-loaded

erythrocytes demonstrate that within the intact red cells, dephosphorylation of FdUMP to FUdR is balanced by phosphorylation of FdUMP to both FdUDP and FdUTP (Fig. 3). Another major difference between hemolysates and intact erythrocytes in metabolizing Compound 1 is a quantitative one, since the disappearance of Compound 1 is clearly unrestrained in the hemolysates as compared to the patterns observed within red cells (see Figs 2 and 3a for comparison). Disruption of the erythrocyte structure could also account for the qualitatively different patterns of metabolism, allowing the hydrolytic activity of the pyrimidine 5′-nucleotidase to prevail over the re-phosphorylating activities involved in the formation of FdUDP and FdUTP that takes place intracellularly. These two enzyme activities cannot yet be identified safely, although the FdUMP to FdUDP conversion may result from a side activity of adenylate kinase and the subsequent phosphorylation to yield FdUTP is probably catalysed by the erythrocyte nucleoside diphosphate kinase described by Mourad and Parks [29]. In a previous study performed by encapsulating FdUMP as a direct precursor of FUdR in human erythrocytes, no detectable formation of FdUDP or FdUTP had been observed [17]. The apparent discrepancy with the present findings seems to be related to a different steady state concentration of FdUMP in the two cases. Thus, high levels of this deoxyribonucleotide in the red cell, as obtained by its direct encapsulation, would favor the low affinity pyrimidine 5′-nucleotidase over the kinase enzymes responsible for the production of FdUDP and FdUTP. Progressive formation of FdUMP from Compound 1, taking place in the erythrocyte through the phosphodiesterase reaction, might shift the balance between the two alternative pathways of FdUMP, thereby allowing its phosphorylation as well. This view is supported by the high K_m value of the pyrimidine 5′-nucleotidase toward FdUMP, i.e. 7.7 ± 1.2 mM [17]. The normal metabolic competence of the Compound 1-loaded erythrocytes during incubation, witnessed by almost undetectable hemolysis and persistence of the starting ATP/ADP/AMP ratio, seems to be a prerequisite for further phosphorylation of FdUMP.

Both FdUDP and FdUTP appear to be important sources of the two metabolites released (FUdR and FU), from 3 hr onwards. Indeed, up to 3 hr incubation of the Compound 1-loaded erythrocytes, the kinetic patterns of FUdR output (that of FU being undetectable) suggest the direct two-step pathway resulting from a combination of phosphodiesterase and pyrimidine 5′-nucleotidase reactions (Fig. 3a and b). Afterwards, FdUDP and FdUTP seem to represent "storage" metabolites of FUdR, accounting for the sustained intraerythrocytic production of FUdR. Experiments in which FdUDP was directly encapsulated in red cells and found to be converted to extracellularly released FUdR demonstrate this to be the case (Fig. 4). Accordingly, FdUDP (and FdUTP as well) can be considered as alternative pre-prodrugs suitable for encapsulation in erythrocytes.

The formation of FU observed in both hemolysates and loaded erythrocytes may be linked to the experimental *in vitro* system used in the present study. Specifically, the FUdR released extracellularly during the first hours of incubation can diffuse back into the erythrocyte by means of the membrane nucleobase transporter [30] and then be metabolized to FU through the pyrimidine nucleoside phosphorylase reaction. Therefore, this step should not occur *in vivo*, since the steep concentration gradient between the red cell and the surrounding plasma would result in the irreversible extrusion of FUdR from the circulating erythrocytes.

The introduction of an additional, phosphodiesterase-catalysed step upstream to the final bioconversion reaction resulting in the production of FUdR therefore has two major and distinctive consequences as compared to simple FdUMP metabolism [17]: (a) formation of FU in addition to FUdR, and (b) partial channeling of FdUMP to di- and tri-phosphorylated derivatives that represent transient fluoropyrimidine reservoirs for further formation of FUdR (and of FU) through sequential dephosphorylation steps (Fig. 5). While the former feature may only be apparent and related to the "closed" system of erythrocytes in their incubation mixture, the latter event appears to be substantial in determining a major property of the bioreactor, i.e. sustained FUdR output.

A biochemical approach to therapy like the one pursued in this study seems to be promising. It is based on programmed patterns of drug biodisposition occurring in specific types of cells according to peculiar complements of bioconverting enzymes. These patterns are reminiscent of other pathways for the intracellular processing of bioactive molecules such as the synthesis of pro-enzymes or of pro-hormones followed by selective hydrolytic steps to generate the corresponding active enzymes or hormones, respectively.

The present findings represent the basis for developing an animal model of pre-prodrug-loaded erythrocytes behaving as "active" delivery systems of FUdR. In this regard, an additional requirement is a specific hepatic targeting of FUdR-releasing erythrocytes. In fact, current locoregional chemotherapy in humans by means of peristaltic pumps implanted in the hepatic artery is not free of toxicity and technical complications. In mice and dogs, red cells loaded with antineoplastic drugs can be properly treated to be selectively targeted to the liver [15, 31]. This represents a further indication that the development of suitable prodrugs or, as in the present study, or pre-prodrugs to be encapsulated in erythrocytes, is increasingly required.

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