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Report

Inhibition of Intestinal Pyrimidine Nucleoside Phosphorylases

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The activity of 5'-deoxy-5-fluorouridine (dFUR) depends on its activation to 5-fluorouracil (FU) by pyrimidine nucleoside phosphorylases. These enzymes are found in tumors and normal tissues, with the highest activity in the small intestines. The present study examined the inhibition of dFUR phosphorolysis in intestinal tissues. dFUR metabolism in intestinal homogenates was inhibited by uracil (U), uridine (UR), and thymidine (TdR), which are the normal substrates for the phosphorylases. Conversely dFUR reduced the metabolism of these inhibitors. A good agreement was found between the observed data and the computer-fitted data using the equations for competitive inhibition between dFUR and the inhibitors. In the absence of inhibitors, the V_{max} of dFUR phosphorolysis was 47.1 ± 4.9 μM/min and the apparent K_m was 910 ± 167 μM. The V_{max} was unaltered by the inhibitors, while the K_m was increased with increasing inhibitor concentrations. The maximal inhibition of dFUR metabolism by UR and TdR was about 80%. The K_i's were 372 μM for U, 87.2 μM for UR, and 112 μM for TdR and are orders of magnitude higher than their reported endogenous serum concentrations. The rate of dFUR phosphorolysis to FU in the intact intestinal epithelial crypt cells, indicated by the ratio of FU to dFUR in the intracellular fluid, was reduced by UR in a concentration-dependent fashion. These data indicate that the naturally occurring pyrimidines inhibit competitively the dFUR metabolism by the intestinal phosphorylases, that this inhibition occurs at concentrations much higher than the circulating endogenous levels, and that phosphorolysis is the major route of dFUR metabolism.

KEY WORDS: inhibition; intestinal phosphorylases; 5-fluorouracil; 5'-deoxy-5'-fluorouridine; uridine; thymidine; uracil.

INTRODUCTION

5'-Deoxy-5-fluorouridine (dFUR)[4] is a metabolic prodrug of 5-fluorouracil (FU) (1,2). The activation of dFUR to FU by phosphorylases is required for its activity (3). Both compounds are used in the palliative treatment of solid tumors (2). FU is active by the intravenous route but has little or no activity by the oral route, while dFUR is active by both routes (3–8). However, the intestinal toxicity of dFUR in patients by the oral route is greater than by the intravenous route (8).

Phosphorylases catalyze the reversible conversion from (pyrimidine base plus ribose-1-phosphate) to (nucleoside plus inorganic phosphate) (9). These enzymes are present in tumors and in various normal tissues. The small intestine, liver, kidney, and lung have the highest activity, while the bone marrow contains little or no activity (10–13). We showed that the conversion of dFUR by rat intestinal homogenates is 80% faster than in liver homogenates and estimated that the small intestine metabolizes about 10% of the dFUR presented to the tissue during a single passage (13). This represents a significant amount of FU in terms of activity, because FU is 100 times more potent than dFUR on a molar basis (14), and may explain the high intestinal toxicity of dFUR. The inhibition of intestinal phosphorylases and hence the reduction of the activation of dFUR may spare the intestinal tissue from drug toxicity. This study examined the inhibition of dFUR phosphorolysis in intestinal tissue homogenates and intestinal cells by uridine (UR), uracil (U), and thymidine (TdR), which are the nontoxic and normal substrates for the pyrimidine nucleoside phosphorylases (9).

METHODS

Chemicals. All chemicals including FU (MW 130.1), U (MW 112.1), UR (MW 244.2), and TdR (MW 242.2) were obtained from Sigma Co. (St. Louis, Mo.) and Mallinckrodt Co. (Paris, Ky.), dFUR (lot 0305001, MW 246.2) was from Hoffman LaRoche Inc. (Nutley, N.J.), [6-^{3}H]dFUR (sp act, 450 μCi/μmol) was from Moravek Biochemical (City of Industry, Calif.), and tissue culture supplies were from the Grand Island Biological Co. (Grand Island, N.Y.).

Inhibition of dFUR Phosphorolysis in Intestinal Homogenates. Three- to six-month-old female Fischer rats (Charles River Breeding Laboratories, Kingston, N.J.) were used in groups of two to five animals per experiment. The

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[4] Abbreviations used dFUR, 5'-deoxy-5-fluorouridine; FU, 5-fluorouracil; TdR, thymidine; U, uracil; UR, uridine; HPLC, high-pressure liquid chromatography.

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13,000g supernatant fraction of intestinal homogenates was obtained as described (13). Our previous study showed that the dFUR metabolism by intestinal homogenates is linear at an enzyme concentration range of 5 to 25% and that the dFUR metabolism was fastest during the first 10 min (13). Preliminary experiments in this study confirmed the linear phosphorolysis rate during the first 15 min. Drugs were incubated with 12.5% (w/v) intestinal homogenate, 1.5% KCl, 10 mM phosphate, and 2.5 mM $MgSO_4$ (pH 7.4), at 37°C under 95% O_2 and 5% CO_2. The dFUR concentration ranged from 60 to 1200 μM, and inhibitor concentrations from 0 to 1200 μM. The initial metabolic rates of dFUR and inhibitors were measured by their disappearance over 3 to 9 min.

Inhibition of dFUR Phosphorolysis in Intestinal Crypt Cells. Epithelial cells were dissociated from everted small intestinal segments using 1.5 mM EDTA (14). The villus cells which dissociated during the first 15 min were discarded, and the crypt cells which dissociated during the subsequent 60 min were collected. The cell viability determined by trypan blue exclusion was >70%. Drugs ([6-^{3}H]dFUR with or without UR) were incubated with 5 million cells/ml of RPMI-1640 culture medium containing 20 mM phosphate, 20 mM glucose, 12 mM $MgCl_2$, and 10% fetal bovine serum, at 37°C under 95% O_2 and 5% CO_2. The cell pellet (about 50 µl in volume) obtained after centrifugation was washed three times with 2 ml of ice-cold saline. The intracellular contents were extracted as described below. The concentrations of dFUR and UR in the extracellular culture medium and in the intracellular extracts were measured.

Sample Analysis. The proteins and macromolecules in washed cell pellets and incubation mixtures were precipitated with acetonitrile. Ten micrograms of 5-bromouracil was added as the internal standard. The supernatant fraction was analyzed by HPLC using a published reversed-phase HPLC assay (15). Elution volumes were 6.0, 6.9, 7.5, 8.9, 10.0, 19.0, and 27.2 ml for U, FU, thymine, UR, 5-bromouracil, TdR, and dFUR, respectively. The lower detection limits were 4 μM for dFUR and TdR, 120 μM for UR, and 240 μM for FU, U, and thymine. In the experiments using radiolabeled dFUR, the HPLC eluting fractions corresponding to dFUR and its metabolite FU were collected and the radioactivity was determined by liquid scintillation counting.

Data Analysis. The V_{max} and K_m for dFUR phosphorolysis and the K_i of the inhibitors were analyzed using the following equations for enzyme kinetics with competitive inhibition (16).

$$V = \frac{V_{max} \times S}{S + K_m(1 + I/K_i)} \quad (1)$$

and the linearized form,

$$\frac{S}{V} = \frac{S}{V_{max}} + \frac{K_m}{V_{max}}\left(1 + \frac{I}{K_i}\right) \quad (2)$$

where V is the rate of phosphorolysis, and S and I are the concentrations of dFUR and inhibitors at midpoint. Under competitive inhibition, the V_{max} is unchanged but the apparent K_m which equals K_m $(1 + I/K_i)$ increases with inhibitor concentration. The S/V versus S plot, for a single inhibitor at several concentrations, gives a set of parallel lines with each line corresponding to an inhibitor concentration. The computer fitting of experimental data to these equations was done using a SAS program.

RESULTS

The nonenzymatic aqueous hydrolysis of dFUR, determined in a preliminary experiment, had an apparent first-order rate constant of 0.005 hr^{-1} at 37°C and pH 7.4. The nonenzymatic degradation at a concentration of 1200 μM is calculated to be 0.1 μM/min, which is negligible compared to the enzymatic rate of >8 μM/min. Upon incubation with intestinal homogenates, the disappearance of UR, TdR, and dFUR was accompanied by the appearance of U, thymine, and FU, indicating that these nucleosides were metabolized by the phosphorylases (9). The extensive inhibition between these compounds further indicates that phosphorolysis is the major route of dFUR metabolism (see below). Because the products of nucleoside phosphorolysis, i.e., pyrimidine bases, are further metabolized by dihydrouracil dehydrogenase (17), which is a different enzyme also present in tissue homogenates, quantitation of the product formation would underestimate the phosphorolysis rate. Hence the rate of enzymatic phosphorolysis was measured by the substrate disappearance rate.

Figure 1 shows the Michaelis–Menten plot of dFUR phosphorolysis in the intestinal homogenates and the effect of increasing TdR concentrations. The linearized S/V versus S plot for the dFUR phosphorolysis rate in the presence of different TdR concentrations (Fig. 2) shows that the slopes of the lines are similar, indicating that the V_{max} remained unchanged while the apparent K_m increased with increasing TdR concentrations. Similar plots were obtained for UR and U (not shown). For all three inhibitors, the computer-fitted values by Eqs. (1) and (2) were within 7.7 ± 5.9% of the observed values. The predictability of the experimental data by Eqs. (1) and (2) for competitive inhibition, the unchanged

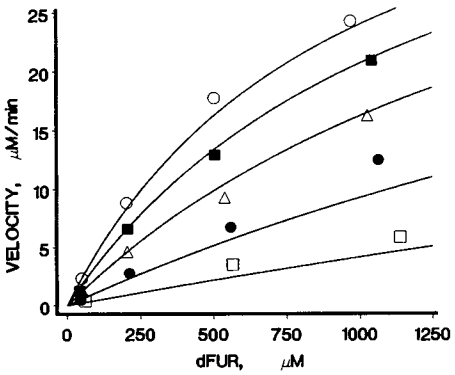

Fig. 1. Michaelis–Menten plot for the inhibition of dFUR phosphorolysis by TdR. The rate (V) of dFUR phosphorolysis in an intestinal enzyme solution was determined at the indicated midpoint dFUR concentrations in the presence of thymidine at various midpoint concentrations: 0 μM (control; open circles), 34 μM (filled squares), 98 μM (open triangles), 342 μM (filled circles), and 1044 μM (open squares). The lines linking the data points were computer fitted using Eq. (1).

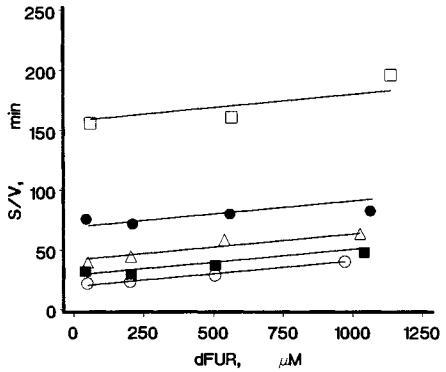

Fig. 2. Linearized S/V versus V plot for the inhibition of dFUR phosphorolysis by TdR. The symbols are the same as in the legend to Fig. 1. The lines linking the data points were computer fitted using Eq. (2).

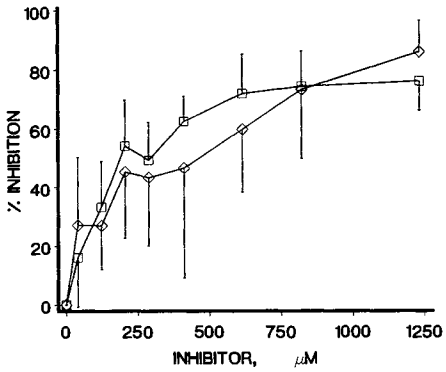

Fig. 3. Inhibition of dFUR phosphorolysis by UR and TdR. dFUR (200 μ*M*) was incubated with intestinal homogenates with or without the inhibitors UR (diamonds) and TdR (squares) at various initial concentrations. The rate of dFUR phosphorolysis without inhibitors was taken as 100%, and the reduction in phosphorolysis rate was expressed as the percentage inhibition. Data represent the mean of six experiments.

V_{max} and the increased apparent K_m indicate a competitive inhibition between these compounds (16). The data in Fig. 1 were fitted to Eq. (1) by least-squares nonlinear regression and solved for the V_{max} and K_m of dFUR phosphorolysis and the K_i of the inhibitors. The data in Fig. 2 were fitted to Eq. (2) by least-squares linear regression, under the constraint of a common slope for all inhibitor concentrations, and solved for the K_m and V_{max} of dFUR phosphorolysis. The K_m's and V_{max}'s obtained from these equations are comparable (Table 1). Figure 3 shows the results of a series of experiments (N = 6) indicating the progressive reduction of the disappearance rate of dFUR (initial concentration of 200 μ*M*) by increasing TdR and UR concentrations. The maximal inhibition was 80% by 600 μ*M* UR or TdR. A further increase in UR and TdR concentration to 1200 μ*M* did not enhance the inhibition. These data indicate that UR and TdR inhibited the dFUR degradation and that dFUR metabolism is primarily (>80%) by the pyrimidine nucleoside phosphorylases.

Upon incubation with intestinal homogenate, the concentrations of the inhibitors declined monoexponentially (r > 0.98) during the incubation period, but the apparent half-lives increased with increasing concentrations. The half-lives were 3 min at 400 μ*M* and 9.7 min at 1200 μ*M* for UR, 27 min at 400 μ*M* and 33 min at 1200 μ*M* for TdR, and 16 min at 800 μ*M* and 22 min at 2400 μ*M* for U. In comparison, the half-life of dFUR (without inhibitors) was 15 min at an initial concentration of 60 μ*M*, 19.5 min at 600 μ*M*, and 26.6 min at 1200 μ*M*. The concentration-dependent degradation half-lives of dFUR and the inhibitors suggests a nonlinear and saturable metabolism in the intestines. The half-lives of UR, TdR, and U at an initial concentration of 400 μ*M* (800 μ*M* for U) were increased by 20, 17, and 34% when the dFUR concentration in the incubation mixture was increased from 60 to 1200 μ*M*. The increase in degradation half-lives of the inhibitors by dFUR further supports a competitive inhibition between these compounds. The possible interactions between U and dFUR include a competition for the phosphorylases and a competition between the dFUR metabolite FU and U for the dihydrouracil dehydrogenase (17) also present in tissue homogenates.

Additional experiments examined the interaction between dFUR and UR in intact isolated intestinal epithelial crypt cells, which are the target cell for anticancer drug toxicity (2). In the presence of 5 million cells/ml, the apparent half-life of dFUR in the extracellular culture medium was 67 min at a starting concentration of 60 μ*M* and increased to

Table I. Inhibition of dFUR Phosphorolysis[a]

	U	UR	TdR
Nonlinear regression, Eq. (1)			
V_{max} of dFUR (μ*M*/min)	47.5 ± 2.4	46.7 ± 3.7	45.9 ± 5.3
K_m of dFUR (μ*M*)	906 ± 114	780 ± 162	918 ± 20
K_i (μ*M*)	372 ± 37	87.2 ± 16.8	124 ± 17
Linear regression, Eq. (2)			
V_{max} of dFUR (μ*M*/min)	50.0 ± 4.1	50.0 ± 5.7	41.4 ± 4.8
K_m of dFUR (μ*M*)	1089 ± 162	881 ± 215	764 ± 195

[a] The phosphorolysis of dFUR in the 13,000*g* supernatant of rat intestinal homogenate (12.5%) and its inhibition by competing substrates were determined at 37°C. The data were computer fitted to Eq. (1) by least-squares nonlinear regression and to Eq. (2) by least-squares linear regression, using SAS programs. Best-fit values ± asymptotic standard errors are presented.

123 min at 600 μ*M*. UR was metabolized faster, with apparent half-lives of <5, 6.1, 9.4, and 17.0 min at starting concentrations of 60, 160, 300, and 600 μ*M*. The concentration-dependent metabolism of UR and dFUR by intact intestinal crypt cells and the more rapid degradation of UR compared to dFUR are consistent with the results obtained with intestinal homogenates. Results of three experiments showed that the intracellular conversion of [6-^{3}H]dFUR to [6-^{3}H]FU, measured by the ratio of [6-^{3}H]FU to [6-^{3}H]dFUR in the intracellular extract after a 60-min incubation, was decreased to 87.5, 52.1, 44.3, 42.7, and 15.0% by 50, 100, 150, 600, and 6000 μ*M* UR. These results indicate a concentration-dependent inhibition of dFUR conversion in intact crypt cells to FU by UR.

DISCUSSION

This study established the competitive inhibition of dFUR metabolism in rat intestinal homogenates and intact epithelial crypt cells by the normal substrates of pyrimidine nucleoside phosphorylases, UR, TdR, and U. The K_i's of these compounds (100–400 μ*M*) were defined in this study and are orders of magnitude higher than their serum concentrations in humans [2–8 μ*M* for UR, 0.04–1 μ*M* for TdR, and undetectable for U (<0.5 μ*M*); from Refs. 18–22], indicating that the circulating levels of U, TdR, and UR are not sufficient to inhibit dFUR activation.

The cytotoxicity of dFUR requires its activation by phosphorylases (3,10–12). An inhibition of these enzymes in a tissue may therefore spare the tissue from drug toxicity. TdR and UR are nontoxic (18,19,23) and are good candidates for this purpose. A localized inhibition in the intestines is necessary to achieve a selective protection of the intestines without compromising the antitumor activity of dFUR. Based on the rapid clearance of UR and its metabolite U in humans (19), the nearly complete extraction of UR by perfused rat livers after a single passage (24–26), the low oral bioavailability of TdR in humans (18), and the rapid intestinal metabolism of UR and TdR shown in the present study, it can be speculated that these compounds if given orally will be eliminated by presystemic metabolism and will not enter the systemic circulation in appreciable quantities. Hence, they may provide selective protection of the intestines from dFUR toxicity. Data from this study suggest several factors which can complicate a selective rescue. First, the concentration-dependent degradation half-lives of these compounds suggest saturable first-pass metabolism, which may result in increased systemic availability of the inhibitors and loss of the selective protection. Second, upon their absorption UR and TdR are metabolized to their bases, U and T. As shown in this study, these bases may inhibit the conversion of dFUR to FU. Third, by competing for the pyrimidine enzymes, U and T may inhibit the activation of FU to cytotoxic nucleotides and/or its degradation (17), which will reduce and/or potentiate the toxicity of FU. Hence the protection of the intestines from the dFUR toxicity depends on the fine tuning of the dose of the inhibitors. Further studies to determine the pharmacokinetic interaction between dFUR and UR and the effect of UR on the therapeutic selectivity of dFUR are ongoing in our laboratory.

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