Inhibition of 5′-Deoxy-5-fluorouridine Phosphorolysis by Acyclopyrimidinenucleosides in Intestinal Tissue Homogenates

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This study examined the inhibitory effect of acyclopyrimidinenucleosides on 5′-deoxy-5-fluorouridine (5′-DFUR) phosphorolysis in intestinal tissue derived from rabbit, rat, mouse, and human. 5-Bromoacyclouridine, 5-fluoroacyclouridine, acyclouridine, and 5-nitroacyclouridine showed little or only moderate effect, but acyclothymidine [5-methyl-1-(2′-hydroxyethoxymethyl)uracil] showed strong inhibitory effect on 5′-DFUR phosphorolysis in intestinal tissue homogenates derived from human. In the absence of inhibitor (acyclothymidine), the V_{max} of 5′-DFUR phosphorolysis was 2.66 μmol/min and the K_m was 0.57 mM in human intestinal homogenates. The V_{max} was unaltered by increased inhibitor concentration. The maximal inhibitory effect of acyclothymidine on 5′-DFUR phosphorolysis in rat homogenates was over 90%. The K_i/K_m was 0.63 in human, 2.14 in rabbit, 1.09×10^{-2} in rat, and 1.71×10^{-2} in mouse. These data show that acyclothymidine is a competitive inhibitor of 5′-DFUR phosphorolysis, and that it can inhibit not only uridine phosphorylase but also thymidine phosphorylase.

Keywords 5′-deoxy-5-fluorouridine; 5-fluorouracil; acyclothymidine; thymidine phosphorylase; uridine phosphorylase; pyrimidine nucleoside phosphorylase

5′-Deoxy-5-fluorouridine (5′-DFUR) is a prodrug of 5-fluorouracil (5-FU),[1,2] and is used orally in the treatment of human malignancies. The phosphorolytic activation of 5′-DFUR to 5-FU by pyrimidine nucleoside phosphorylase (PyNPase) is required for its activity. There are two distinct PyNPase.[3–6] One is thymidine phosphorylase (EC 2.4.2.4) in human and rabbit, which catalyzes the phosphorolysis of thymidine and is reported to be specific for 2′-deoxyribonucleosides.[6–8] The other is uridine phosphorylase (EC 2.4.2.3) in mouse and rat, which acts primarily on uridine, though a broad substrate specificity has been reported.[9,10] These enzymes are present in tumors and in various normal tissues. Since PyNPase activity is greater in tumors than in normal tissues, 5′-DFUR is effectively converted to 5-FU in tumors. The higher therapeutic index of 5′-DFUR over that of 5-FU can therefore be at least partially explained by its minimal activation in normal tissues like bone marrow, which results in relatively low myelotoxicity such as leukocytopenia and thrombocytopenia when compared with 5-FU.[2,11–14]

PyNPase activity in the intestinal tract, however, is much greater than in other normal tissues: orally administered 5′-DFUR molecules can be converted to 5-FU in the intestinal tract before they reach the target tissue or tumor.[13] This undesirable regeneration of 5-FU can cause gastrointestinal toxicity, such as diarrhea, nausea and vomiting, abdominal pain and anorexia.[14] Diarrhea, which occurred most frequently (26.3%), was the dose-limiting factor in clinical trials.[15]

The inhibition of intestinal PyNPase and hence the reduction of the activation of 5′-DFUR may spare the intestinal tissue from drug delivery. Among nucleoside analogues, acyclopyrimidinenucleosides show a strong inhibitory effect on nucleoside phosphorylase.[16–18] Phosphorolytic degradation of 5-fluoro-2′-deoxyuridine to 5-FU is strongly inhibited by various acyclopyrimidinenucleosides, resulting in potentiation of its activity against L1210 *in vivo*.[19] Low toxicity of those analogues has also been noted.[18] Because of these properties, oral co-administration of acyclopyrimidinenucleoside and 5′-DFUR was studied in our laboratory. We have reported the pharmacokinetic interaction between 5′-DFUR and acyclothymidine[5-methyl-(2′-hydroxyethoxymethyl) uracil].[20] After the oral co-administration of 5′-DFUR with acyclothymidine (at an equimolar concentration), the AUC (area under the plasma concentration–time curve) values for 5′-DFUR and 5-FU increased. A study in our laboratory also showed that the oral co-administration of 5′-DFUR with acyclothymidine reduced the intestinal toxicity without reducing the antitumor activity to mice bearing Lewis lung carcinoma.[21]

In the present study, we examined the inhibitory effects of acyclopyrimidinenucleosides on 5′-DFUR phosphorolysis in intestinal tissue homogenates derived from rabbit, rat, mouse and human to investigate their inhibitory effects and to select the most promising modulator.

MATERIALS AND METHODS

Chemicals 5′-DFUR was generously provided by Nippon Roche Co. (Kamakura, Japan), 5-FU was generously provided by Kyowa Hakko Co. (Tokyo, Japan), and 5-Chlorouracil was purchased from Sigma Chemical Co. (St. Louis Mo.). Acyclopyrimidinenucleosides were prepared from corresponding 5-substituted pyrimidines and 2-(chloromethoxy) ethyl benzoate according to the general method reported by Kelley *et al.*[22] Acetonitrile and *n*-hexane were HPLC grade and purchased from Wako Pure Chemicals Co. (Tokyo, Japan). All other chemicals

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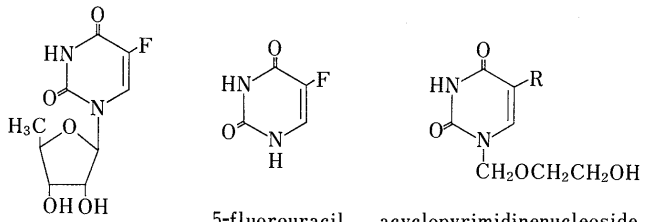

Fig. 1. Chemical Structures of the Acyclopyrimidinenucleosides

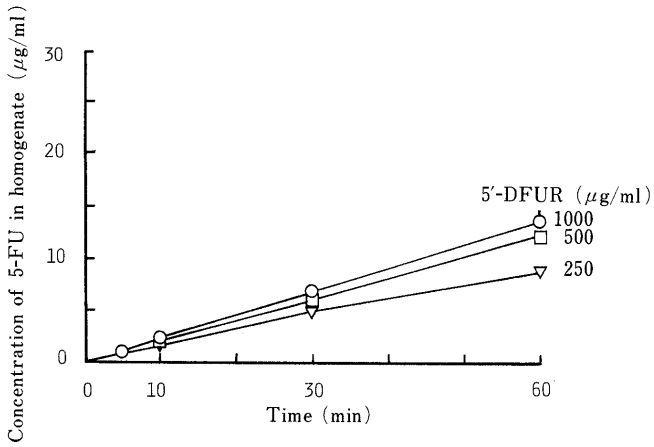

Fig. 2. The Conversion Behavior of 5′-DFUR to 5-FU in Human Intestinal Tissue Homogenates

Values represent means S.E. of three experiments.

were of reagent grade. Structures of the above nucleoside analogues are shown in Fig. 1.

Tissue Extracts Human intestinal tissues obtained in surgical operations were stored at −70 °C. Male New Zealand white rabbits 2 to 3 months old and weighing about 2 to 3.5 kg, male Donryu rats 4 to 5 weeks old and weighing about 190—210 g, and male C57BL/6 mice 5 weeks old and weighing about 20—25 g were sacrificed to obtain small intestines. Five grams of the fresh tissue was homogenized in approximately 10 volumes of ice-cooled 0.15 M isotonic phosphate buffer (pH 6.8) for 3—5 min in a Teflon homogenizer. The homogenates were centrifuged (600 × *g*, 20 min) at 4 °C to remove the nuclei, and 1 ml of the homogenates was transferred to small glass tubes (3 ml) and stored at −80 °C until enzyme assay.

Inhibition of 5′-DFUR Phosphorolysis by Acyclopyrimidinenucleosides in Intestinal Homogenates The enzyme assays were carried out at 37 °C using 1 ml of the homogenate (500 μg·protein/ml) diluted with the isotonic phosphate buffer (pH 6.8). The mixture was preincubated at 37 °C for 5 min. 5′-DFUR was prepared in homogenate to give concentrations of 0.25, 0.5, 1.0, and 2.0 mM. The experiments were initiated by adding 5′-DFUR and the inhibitor to 1 ml of the preincubated homogenate in a 3 ml glass tube. The phosphorolysis of 5′-DFUR to 5-FU in the homogenates incubated at 37 °C was followed by periodic sampling of the reaction mixture and HPLC analysis. The initial phosphorolysis rates were measured at 0—30 min. Linear reaction kinetics were maintained for the period of the measurement (Fig. 2). The phosphorolytic degradation rates (velocity, *V*) were calculated from the amount of 5-FU converted from 5′-DFUR. Kinetic constants (K_m) of the 5′-DFUR phosphorolysis were determined with four levels of 5′-DFUR (in the range 0.25—2.0 mM). The K_m values were obtained from double-reciprocal plots of velocity *versus* 5′-DFUR concentration using the standard Lineweaver–Burk method.[23] Studies of inhibition kinetics were made with four levels of 5′-DFUR (0.25, 0.5, 1.0, and 2.0 mM) for each of three inhibitor levels and for a control mixture lacking inhibitor. Inhibition constants (K_i) were obtained from replots of inhibitor concentrations *versus* slopes of double-reciprocal plots of velocity *versus* 5′-DFUR concentration. All of the latter plots were linear, and calculated by least-squares fitting of experimental data.

200 μl of homogenate
- add 400 μl acetonitrile (containing 1.0 μg/ml 5-Chlorouracil)
- mix for 2 min
- centrifuge at 1000 × *g* for 3 min

400 μl of supernatant
- add 100 μl 0.5 M sodium phosphate, monobasic
- add 3.0 ml ethyl acetate
- shake for 10 min
- centrifuge at 1500 × *g* for 5 min

organic layer
- evaporate *in vacuo*

residue
- dissolve in 300 μl mobile phase[a]

sample solution for HPLC assay

Fig. 3. Pretreatment Method of Homogenate Samples for HPLC Assay

a) ethyl acetate: *n*-hexane: formic acid: water = 50 : 50 : 0.5 : 0.2.

Sample Analysis The proteins and macromolecules in incubation mixtures were precipitated with acetonitrile, containing 1.0 μg/ml of 5-chlorouracil as the internal standard. The supernatant fraction was analyzed by HPLC. The method of pretreatment of the homogenate samples for HPLC assay is shown in Fig. 3. The samples were injected into a HPLC column equipped with an automatic injector (SIL-9A, Shimadzu, Japan), a variable wavelength spectrophotometer (SPD6A, Shimadzu), and a chromatograph terminal (CR-3A, Shimadzu). All analyses were performed on a Lichrosorb Si-100 column (4 × 300 mm, Merck Co., Darmstadt, Germany). Column temperature was maintained at 30 °C, elution was carried out at 2.0 ml/min, and absorbance was monitored at 268 nm. Protein concentrations in the preparations were determined by the method of Lowry *et al.*[24]

RESULTS AND DISCUSSION

Selection of an Inhibitor Five 5-substituted acyclopyrimidinenucleosides including acyclothymidine (AcyT) were evaluated for their ability to inhibit 5′-DFUR phosphorolysis at a concentration of 2.0 mM in human intestinal homogenates, thymidine phosphorylase dominant enzyme system. Table I summarizes the percent

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TABLE I. Inhibition of 5'-DFUR Phosphorolysis in Human Intestinal Tissue Homogenate by Acyclopyrimidinenucleosides[a]

Acyclopyrimidinenucleoside	R[b]	Percent inhibition[c]
Acyclothymidine	CH_3	48.8±2.0
Acyclouridine	H	19.8±1.6
5-Fluoroacyclouridine	F	6.2±0.4
5-Bromoacyclouridine	Br	2.2±0.2
5-Nitroacyclouridine	NO_2	27.7±1.2

Values represent means±S.E. of three experiments. *a*) 5'-DFUR (2 mM) was incubated in human intestinal tissue homogenates with or without the acyclopyrimidinenucleoside (2 mM) for 30 min. The percent inhibition of 5'-DFUR phosphorolysis without inhibitor was taken as 0%, and the reduction in phosphorolysis rate was expressed as percent of inhibition. *b*) R in Fig. 1.

c) percent of inhibition $= \left(1 - \frac{\text{conversion to 5-FU with inhibitor}}{\text{conversion to 5-FU without inhibitor}}\right) \times 100$

TABLE II. Kinetic Parameters for the Phosphorolysis of 5'-DFUR by PyNPase in the Intestinal Tissue Homogenates[a]

	Human	Rabbit	Rat	Mouse
K_m (mM)	0.57 ±0.04	0.44 ±0.03	0.89 ±0.06	1.03 ±0.07
V_{max} (μmol/min)	2.66 ±0.20	9.09 ±0.74	9.26 ±0.62	4.96 ±0.34
K_i (mM)	0.36 ±0.03	0.94 ±0.09	0.97×10^{-2} ±0.11×10^{-2}	1.76 ± 10^{-2} ±0.17×10^{-2}
K_i/K_m	0.63 ±0.05	2.14 ±0.20	1.09×10^{-2} ±0.12×10^{-2}	1.71×10^{-2} ±0.16×10^{-2}

Values represents means±S.E. of three experiments. *a*) The phosphorolysis of 5'-DFUR in the $600\times g$ supernatant of the intestinal tissue homogenate (500 μg·protein/ml) was determined at 37 °C. 5'-DFUR (0.25—2.0 mM) was incubated with intestinal tissue homogenate with or without AcyT (0.02—2.0 mM).

inhibition calculated from the amount of 5-FU converted from 5'-DFUR in 30 min. AcyT showed the highest inhibitory effect among the acyclopyrimidinenucleosides: 48.8%. 5-Bromoacyclouridine and 5-fluoro-acyclouridine showed little effect; these two acyclopyrimidinenucleosides were reported as weak inhibitors of FUdR phosphorolytic degradation.[19] Acyclouridine and 5-nitroacyclouridine showed moderate effect, 19.8% and 27.7%, respectively, though both had been reported as strong uridine phosphorylase inhibitors.[16,19] AcyT has been a strong inhibitor of FUdR phosphorolytic degradation in intestinal homogenates prepared from mouse and rat, uridine phosphorylase dominant enzyme system.[19] These data indicate that AcyT can inhibit not only uridine phosphorylase in rat and mouse but also thymidine phosphorylase in human and rabbit.

5'-DFUR Phosphorolysis Activity and Inhibitory Effect by Acyclothymidine in the Intestinal Tissue Homogenates

Table II shows the kinetic parameters for the phosphorolysis of 5'-DFUR in the intestinal tissue homogenates. The phosphorolysis activity in rat and rabbit was higher than that in human and mouse. The V_{max} for phosphorolysis of 5'-DFUR by PyNPase in the intestinal homogenates derived from rat and rabbit were very close, 9.26 and 9.09 μmol/min per 500 μg protein, respectively. However, the affinity (K_m) of PyNPase for 5'-DFUR differed: 0.89 mM in rat and 0.44 mM in rabbit. The V_{max} in mouse was 4.96 μmol/min per 500 g protein, and K_m was 1.03 mM. The V_{max} in human was 2.66 μmol/min per 500 μg protein, while K_m in human was 0.57 mM.

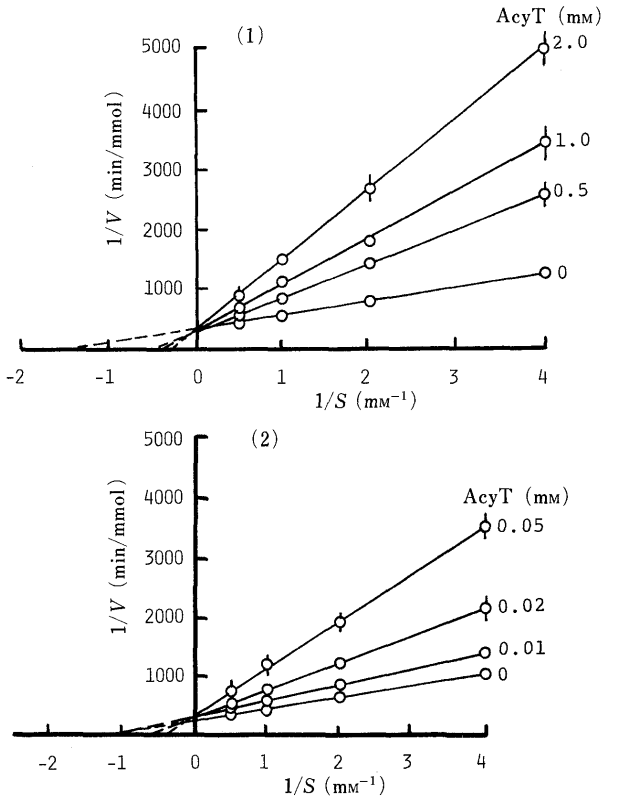

Fig. 4. The Lineweaver–Burk Plot for Inhibition of PyNPase by AcyT
(1) human; (2) mouse. Values represent means±S.E. of three experiments.

Figure 4 shows a typical Lineweaver–Burk plot for the inhibitory effect of AcyT in the human and mouse intestinal homogenates. Competitive inhibition was observed in all homogenates. Higher inhibition of 5'-DFUR phosphorolysis was noted in the intestinal homogenates derived from mouse and rat than from human or rabbit. Since the K_m values depended on the homogenates, K_i/K_m was used for the relative evaluation among the species. K_i/K_m in intestinal homogenates derived from human, rabbit, rat, and mouse was 0.63, 2.14, 1.09×10^{-2}, and 1.71×10^{-2}, respectively. The inhibitory effect of AcyT was higher in intestinal homogenates derived from mouse and rat than from human or rabbit.

If the conversion of 5'-DFUR to 5-FU in the intestinal tract is inhibited, intestinal toxicity can be reduced after the oral administration of 5'-DFUR with inhibitor. Selective protection of the intestines without compromising the antitumor activity of 5'-DFUR is required for a promising modulator. AcyT showed the highest inhibitory effect of the acyclopyrimidinenucleosides. A desired modulator is required to have biological and chemical stability and low toxicity, as well as inhibitory effect. AcyT, which shows the strongest inhibitory effect, was stable in homogenate studies ($t_{1/2}>1000$ min),[18] and has no anticancer, antiviral, or antimicrobial activities; these data make it appear a desirable modulator. We evaluated the competitive inhibitory effect of 5'-DFUR by AcyT and found it differed among the homogenates derived from the four species. This may suggest that the inhibitory effect of phosphorylation by AcyT is more sensitive to uridine

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phosphorylase than to thymidine phosphorylase, because this inhibitory effect was higher in the rat and mouse; K_i/K_m was 1.09×10^{-2} and 1.71×10^{-2} of the uridine phosphorylase dominant enzyme system than in the human and rabbit; K_i/K_m was 0.63 and 2.14 of the thymidine phosphorylase dominant enzyme system. Despite the existence of considerable differences in the inhibitory effect of AcyT among species, which makes it necessary to avoid simple extrapolation from animal experiments to human, the effect intestinal homogenate could still indicate the possibile clinical use of AcyT.

Further studies on the effect of AcyT on the therapeutic selectivity of 5'-DFUR as well as its active metabolite 5-FU are ongoing in our laboratory.

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