Inhibition of 5'-Deoxy-5-fluorouridine Phosphorolysis by Acyacyclidinenucleosides in Intestinal Tissue Homogenates

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This study examined the inhibitory effect of acyacyclidinenucleosides on 5'-deoxy-5-fluorouridine (5'-DFUR) phosphorolysis in intestinal tissue derived from rabbit, rat, mouse, and human. 5'-Bromoacyclouridine, 5-fluroacyclouridine, acyclouridine, and 5-nitroacyclouridine showed little or only moderate effect, but acylothymidin [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 (acyclouridine), 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 F_max was unaltered by increased inhibitor concentration. The maximal inhibitory effect of acyclouridine on 5'-DFUR phosphorolysis in rat homogenates was over 90%. The K/K_m was 0.63 in human, 2.14 in rabbit, 1.09 x 10^-2 in rat, and 1.71 x 10^-2 in mouse. These data show that acyclouridine is a competitive inhibitor of 5'-DFUR phosphorolysis, and that it can inhibit only uridine phosphorylase but also thymidine phosphorylase.

Keywords 5'-deoxy-5-fluorouridine; 5-fluorouracil; acyclouridine; thymidine phosphorylase; uridine phosphorylase; pyrimidine nucleoside phosphorylase

5'-Deoxy-5-fluorouridine (5'-DFUR) is a prodrug of 5-fluorouracil (5-FU), and is used orally in the treatment of human malignancies. The phosphorolytic activation of 5'-DFUR to 5-FU by pyrimidine nucleoside phosphorylase (PyNase) is required for its activity. There are two distinct PyNase. 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. 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. PyNase activity is greater in tumors than in normal tissues. Since PyNase 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 leukopenia and thrombocytopenia when compared with 5-FU.

PyNase 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. This undesirable regeneration of 5-FU can cause gastrointestinal toxicity, such as diarrhea, nausea and vomiting, abdominal pain and anorexia. Diarrhea, which occurred most frequently (26.3%), was the dose-limiting factor in clinical trials.

The inhibition of intestinal PyNase and hence the reduction of the activation of 5'-DFUR may spare the intestinal tissue from drug delivery. Among nucleoside analogues, acyclorimidinenucleosides show a strong inhibitory effect on nucleoside phosphorylase. Phosphorolytic degradation of 5-fluoro-2'-deoxyuridine to 5-FU is strongly inhibited by various acyclorimidinenucleosides, resulting in potentiation of its activity against L1210 in vivo. Low toxicity of several analogues has also been noted. Because of these properties, oral co-administration of acyclorimidinenucleoside and 5'-DFUR was studied in our laboratory. We have reported the pharmacokinetic interaction between 5'-DFUR and acyclorimidine[5-methyl-(2'-hydroxyethoxymethyl) uracil]. After the oral co-administration of 5'-DFUR with acyclorimidine (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 acyclorimidine reduced the intestinal toxicity without reducing the antitumor activity to mice bearing Lewis lung carcinoma.

In the present study, we examined the inhibitory effects of acyclorimidinenucleosides 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.). Ancyldinenucleosides were prepared from corresponding 5-substituted pyrimidines and 2-chloromethoxy) ethyl benzoate according to the general method reported by Kelley et al. Acetonitrile and n-hexane were HPLC grade and purchased from Wako Pure Chemicals Co. (Tokyo, Japan). All other chemicals

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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 phosphorylation 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 phosphorylation 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 (Km) of the 5'-DFUR phosphorylase were determined with four levels of 5'-DFUR (in the range 0.25–2.0 mM). The Km values were obtained from double-reciprocal plots of velocity versus 5'-DFUR concentration using the standard Lineweaver–Burk method. 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 (KI) 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.

**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 (SPD-6A, Shimadzu), and a chromatographic 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. (1951).

**RESULTS AND DISCUSSION**

**Selection of an Inhibitor** Five 5-substituted acyclopurimidinenucleosides including acyclothymidine (AcT) were evaluated for their ability to inhibit 5'-DFUR phosphorylase at a concentration of 2.0 mM in human intestinal homogenates, thymidine phosphorylase dominant enzyme system. Table I summarizes the percent
TABLE I. Inhibition of 5'-DFUR Phosphorolysis in Human Intestinal Tissue Homogenate by Acyclopirimidinenucleosides

<table>
<thead>
<tr>
<th>Acyclopirimidinenucleoside</th>
<th>R</th>
<th>Percent inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acyclothymidine</td>
<td>CH₃</td>
<td>48.8 ± 2.0</td>
</tr>
<tr>
<td>Acyclouridine</td>
<td>H</td>
<td>19.8 ± 1.6</td>
</tr>
<tr>
<td>5-Fluorooacyclouridine</td>
<td>F</td>
<td>6.2 ± 0.4</td>
</tr>
<tr>
<td>5-Bromoacyclouridine</td>
<td>Br</td>
<td>2.2 ± 0.2</td>
</tr>
<tr>
<td>5-Nitroacyclouridine</td>
<td>NO₂</td>
<td>27.7 ± 1.2</td>
</tr>
</tbody>
</table>

Values represent means ± S.E. of three experiments. a) 5'-DFUR (2.0 mM) was incubated in human intestinal tissue homogenates with or without the acyclopirimidinenucleoside (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 = \( \frac{1 - \text{conversion to 5-FU with inhibitor}}{\text{conversion to 5-FU without inhibitor}} \) × 100

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

<table>
<thead>
<tr>
<th>Species</th>
<th>Human</th>
<th>Rabbit</th>
<th>Rat</th>
<th>Mouse</th>
</tr>
</thead>
<tbody>
<tr>
<td>( K_m ) (mM)</td>
<td>0.57 ± 0.04</td>
<td>0.44 ± 0.03</td>
<td>0.89 ± 0.06</td>
<td>1.03 ± 0.07</td>
</tr>
<tr>
<td>( V_{max} ) (µmol/min)</td>
<td>2.66 ± 0.20</td>
<td>9.09 ± 0.74</td>
<td>9.26 ± 0.62</td>
<td>4.96 ± 0.34</td>
</tr>
<tr>
<td>( K_i ) (mM)</td>
<td>0.36 ± 0.03</td>
<td>0.94 ± 0.09</td>
<td>0.97 × 10⁻²</td>
<td>1.76 ± 10⁻²</td>
</tr>
<tr>
<td>( K_i/K_m )</td>
<td>0.63 ± 0.05</td>
<td>2.14 ± 0.20</td>
<td>1.09 × 10⁻²</td>
<td>1.71 × 10⁻²</td>
</tr>
</tbody>
</table>

Values represent means ± S.E. of three experiments. a) The phosphorolysis of 5'-DFUR in the 600 × 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 acyclopirimidinenucleosides: 48.8%. 5-Bromoacyclouridine and 5-fluoroacyclouridine showed little effect; these two acyclopirimidinenucleosides were reported as weak inhibitors of FUdR phosphorolytic degradation. Aycouridine and 5-nitroacyclouridine showed moderate effect, 19.8% and 27.7%, respectively, though both had been reported as strong uridine phosphorolase inhibitors. AcyT has been a strong inhibitor of FUdR phosphorolytic degradation in intestinal homogenates prepared from mouse and rat, uridine phosphorolase dominant enzyme system. This data indicate that AcyT can inhibit not only uridine phosphorolase in rat and mouse but also thymidine phosphorolase 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.

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 acyclopirimidinenucleosides. 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 (\( t_{1/2} > 1000 \text{ min} \)), and has no antitumor, 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...
phosphorylase than to thymidine phosphorylase, because this inhibitory effect was higher in the rat and mouse; $K_i/K_m$ was $1.09 \times 10^{-2}$ and $1.71 \times 10^{-2}$ 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 possible 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.

REFERENCES