Inhibition of 5'-Deoxy-5-fluorouridine Phosphorolysis by Acyclothyminde in Tumor Cell Homogenates

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The inhibitory effect of acyclothyminde(AcT, 5-methyl-1/(2'-hydroxyethoxymethyl) uracil), a potent pyrimidine nucleoside phosphorylase (PyNPase) inhibitor, on 5'-deoxy-5-fluorouridine (5'-DFUR) phosphorolysis in human and mouse tumor cell homogenates was measured. Competitive inhibition was observed in MKN-74 and Lewis lung carcinoma (LLC), whereas non-competitive inhibition was observed in HeLa. The strength of the inhibitory effect by AcT showed the following pattern: HeLa < human normal intestine < mouse normal intestine < Colon 26 < LLC < MKN-74 < DLD-1. From the kinetic parameter obtained, we simulated the inhibitory effect of AcT on 5'-DFUR phosphorolysis in tumor cells and the intestine. These data indicated that AcT was more sensitive in normal mouse intestine than in Colon 26 and LLC, and that orally administered AcT can reduce the intestinal toxicity of 5'-DFUR without reducing the antitumor effect in the mouse. The present finding may have an important implication for attempts to introduce AcT, a potent PyNPase inhibitor, into the clinic.

Key words pyrimidine nucleoside phosphorylase; 5'-deoxy-5-fluorouridine; acyclothyminde; inhibitor

5'-Deoxy-5-fluorouridine (5'-DFUR) is a prodrug of 5-fluorouracil (5-FU),1,2 and is used orally in Japan in the treatment of such human solid tumors as those in the breast, colon, uterine cervix and stomach.3,4 The phosphorolytic conversion of 5'-DFUR to 5-FU by pyrimidine nucleoside phosphorylase (PyNPase) is required for its activity.1,2 There are two distinct PyNPase: one is thymidine phosphorylase (EC2.4.2.4) in human and rabbit, which catalyzes the phosphorolysis of thymidine and is reported to be specific for 2'-deoxyribonucleosides;5,6 the other is uridine phosphorylase (EC2.4.2.3) in mouse and rat, which is relatively nonspecific, as it also cleaves uridine and thymidine.7,8 In both species, since PyNPase activity is greater in tumors than in normal tissues, 5'-DFUR was effectively converted to 5-FU in the target tumors.1,2,9-11 However, its activity in the intestinal tract is much greater than in other normal tissue, indicating that some part of the orally administered 5'-DFUR is converted to 5-FU in the intestinal tract before reaching the target tumor in mouse.12-14 This undesirable regeneration of 5-FU can cause gastrointestinal toxicity, namely diarrhea, which was the dose-limiting factor in clinical trials.3,4 Inhibition of intestinal PyNPase activity and reduction of the conversion of 5'-DFUR to 5-FU in the intestinal tract may alleviate the intestinal toxicity of 5'-DFUR administered orally.13 We reported that acyclothyminde [AcT, 5-methyl-1/(2'-hydroxyethoxymethyl) uracil] showed the highest inhibitory effect on the phosphorolytic conversion of 5'-DFUR to 5-FU in intestinal tissue homogenates among pyrimidine acyclonucleosides,16,17 a series of PyNPase inhibitors,18 and the oral co-administration of 5'-DFUR with AcT alleviated the intestinal toxicity without reducing the antitumor activity to mice bearing Lewis lung carcinoma19 and Colon 26.20 However, species differences probably exist in the inhibitory effect of AcT, so it cannot be assumed that these findings would hold true in humans; the effect of AcT on 5'-DFUR phosphorolysis in human tumor cells has still not been elucidated. In the present study, we examined this inhibitory effect on 5'-DFUR phosphorolysis in various human tumor cell homogenates from colon carcinomas DLD-1, stomach carcinomas MKN74, and uterine cervix carcinomas HeLa, and in mouse tumor cell homogenates from Lewis lung carcinoma (LLC) and Colon 26.

MATERIALS AND METHODS

Chemicals 5'-DFUR was generously provided by Nippon Roche Co. (Kamakura, Japan), and 5-FU was generously provided by Kyowa Hakko Co. (Tokyo, Japan). AcT was prepared from corresponding thymine and 2-(chloromethoxy) ethyl benzoate according to the general method reported by Kelley et al.20 6-Benzyl-2-thiouracil was purchased from Sigma Chemical Co. (St. Louis, MO.). All other chemicals were of reagent grade.

Cell Extracts Lewis lung carcinoma cells were maintained by sc transfer every 2 weeks into C57BL/6 mice. Tissue was homogenized in approximately 10 volumes of ice-cold 0.15 M isotonic phosphate buffer (pH 6.0) for 3 min in a Teflon homogenizer. The homogenate was centrifuged (600 × g, 20 min) at 4°C to remove the nuclei, and the supernatant was used for the enzyme assay. HeLa, DLD-1, MKN-74, and Colon 26 cells were maintained at 37°C in RPMI1640 with 10% heat-inactivated fetal bovine serum and 10 μg/ml kanamycin in a plastic tissue culture flask (75 cm2) in a humified incubator under 5% CO2 and 95% air. Exponentially growing cells were harvested from the monolayer culture by trypsinization. A suspension of the tumor cells was then centrifuged at 500 × g for 10 min, the cell pellet was washed twice with 10 volumes of appropriate ice-cold 0.15 M isotonic phosphate buffer solution, and then resuspended in 3 volumes of the same

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solution. Cell suspensions were sonicated on ice and centrifuged at 5000×g for 90 min. The supernatant was collected and stored at −70 °C until enzyme assay.

**Enzyme Assays** The enzyme assays were carried out at 37 °C using 1 ml of the cell homogenate (500 µg protein/ml) diluted with the isotonic phosphate buffer (pH 6.8). The mixture was preincubated at 37 °C for 5 min. The experiments were initiated by adding both 5'-DFUR and the inhibitor to 1 ml of the preincubated homogenate in a 3 ml glass tube. The phosphorolysis rates of 5'-DFUR (velocity, \( V \)) were calculated from the amount of 5-FU converted from 5'-DFUR. Kinetic constants (\( K_m \) and inhibition constants (\( K_i \)) were obtained from double-reciprocal plots of velocity versus 5'-DFUR concentration using the standard Lineweaver-Burk method. All of the latter plots calculated by least-squares fitting of the experimental data were linear.

**Sample Analysis** The proteins and macromolecules in the 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. All samples were analyzed by HPLC as described previously. Protein concentrations in the preparations were determined by the method of Lowry et al.

**Data Analysis** The \( V_{\text{max}} \) and \( K_m \) for 5'-DFUR phosphorolysis and the \( K_i \) of the inhibitors were analyzed using the following equations for enzyme kinetics.

**Competitive inhibition:**

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

**Non-competitive inhibition:**

\[
V = \frac{V_{\text{max}} \times S}{S + K_m}
\quad (2)
\]

where \( V \) is the rate of phosphorolysis, and \( S \) and \( I \) are the concentrations of 5'-DFUR and its inhibitors at midpoint. Under competitive inhibition, the \( V_{\text{max}} \) is unchanged but the apparent \( K_m \), which equals \( K_m(1 + I/K_i) \), increases with inhibitor concentration. Under non-competitive inhibition, the \( K_m \) is unchanged but \( V_{\text{max}} \), which equals \( V_{\text{max}}/(1 + I/K_i) \), decreases with inhibitor concentration. The percent inhibition of 5'-DFUR phosphorolysis without an inhibitor was considered 0%, and the reduction in phosphorolysis rate was expressed as percent inhibition.

\[
\text{percent inhibition} = \left(1 - \frac{V_{\text{with AcyT}}}{V_{\text{without AcyT}}}\right) \times 100
\quad (3)
\]

**RESULTS AND DISCUSSION**

We previously reported that AcyT showed the highest inhibitory effect among pyrimidine acyclonucleosides on the phosphorolytic conversion of 5'-DFUR by PyNPase deprived of intestinal homogenates. In the present study, AcyT was tested for its inhibitory effect on the conversion in human and mouse tumor cell homogenates. Table 1 shows \( K_m \), \( K_i \) and \( V_{\text{max}} \) values for the phosphorolysis in these homogenates. Similar \( K_m \)s, 1.29 mm and 1.05 mm, were observed in DLD-1 and HeLa, respectively. AcyT showed a higher inhibitory effect in DLD-1 and MKN-74 than in HeLa; the inhibitory effect in HeLa was even weaker than that in normal human intestine. Similar \( K_m \)s, 1.34 × 10⁻⁷ mm and 1.07 × 10⁻² mm, were observed in LLC and Colon 26, respectively. The strength of the inhibitory effect (\( 1/K_i \)) showed the following pattern: HeLa < human normal intestine < mouse normal intestine < LLC < Colon 26 < MKN-74 < DLD-1.

Figure 1 shows a Lineweaver-Burk plot for the inhibitory effect of AcyT in cell homogenates. Competitive inhibition was observed in MKN-74, DLD-1, Colon 26 and LLC, but non-competitive inhibition was observed in HeLa. Using another PyNPase inhibitor, 6-benzyl-2-thiouracil, however, competitive inhibition was observed in HeLa (Fig. 2). There are two distinct PyNPase, one is thymidine phosphorylase, and the other is uridine phosphorylase, though the former predominates in both HeLa and normal human intestine. In normal human intestinal homogenates, competitive inhibition was observed, el Kouni et al. suggested the existence of tissue-specific isozymes of PyNPase in humans. In this study, AcyT showed three different inhibitory effects: high-affinity (\( K_i < 2 \times 10^{-2} \) mm) competitive inhibition in MKN-74, DLD-1, mouse intestine, LLC and Colon 26, low-affinity (\( K_i > 0.1 \) mm) competitive inhibition in human intestine, and low-affinity non-competitive inhibition in HeLa. These results indicate that substrate specificity varies between PyNPases from different human cells and/or tissues, and suggests the existence of isozymes.

![Table 1. Kinetics Parameters for the Phosphorolysis of 5'-DFUR by PyNPase in Human and Mouse Carcinoma Cell Homogenates](image-url)

<table>
<thead>
<tr>
<th>Homogenates</th>
<th>( K_m ) (mm)</th>
<th>( K_i ) (mm)</th>
<th>( V_{\text{max}} ) (nmol/min/µg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td></td>
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<tr>
<td>Intestine¹⁸</td>
<td>0.57±0.04</td>
<td>0.36±0.03</td>
<td>5.32±0.44</td>
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<tr>
<td>MKN-74</td>
<td>0.40±0.08</td>
<td>7.47×10⁻² ±0.75×10⁻³</td>
<td>1.70×10⁻³ ±0.17×10⁻³</td>
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<tr>
<td>DLD-1</td>
<td>1.29±0.10</td>
<td>3.10×10⁻² ±0.17×10⁻³</td>
<td>3.07×10⁻³ ±0.24×10⁻³</td>
</tr>
<tr>
<td>HeLa</td>
<td>1.05±0.03</td>
<td>1.55±0.18</td>
<td>1.18×10⁻² ±0.14×10⁻³</td>
</tr>
<tr>
<td>Mouse</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intestine¹⁹</td>
<td>1.03±0.09</td>
<td>1.76×10⁻² ±0.10×10⁻²</td>
<td>8.56±0.75</td>
</tr>
<tr>
<td>LLC</td>
<td>2.07±0.09</td>
<td>1.34×10⁻² ±0.05×10⁻²</td>
<td>3.36×10⁻² ±0.15×10⁻²</td>
</tr>
<tr>
<td>Colon 26</td>
<td>1.76±0.14</td>
<td>1.07×10⁻² ±0.33×10⁻²</td>
<td>3.70×10⁻² ±0.29×10⁻²</td>
</tr>
</tbody>
</table>

Values represent mean ± S.D. of three experiments.
If AcyT inhibits PyNPase in target tumor cells, the antitumor activity of 5'-DFUR might be reduced. We showed that orally administered 5'-DFUR at a dose of 1.0 mmol/kg was effective for LLC in vivo, though it caused intestinal toxicity; orally co-administered AcyT reduced the intestinal toxicity. Ninomiya et al. measured the concentration of 5'-DFUR in the small intestine and tumor after the oral administration of 5'-DFUR at a dose of 1.0 mmol/kg to mice implanted with LLC or Colon 26. The concentration of 5'-DFUR was in the range of 3000 to 12000 nmol/g tissue in the small intestine, and was less than 10 nmol/g tissue in tumor tissues. From these data, the concentration of 5'-DFUR, 1 to 10 mm and 10 μM, was estimated in the intestine and target tumor, respectively, and we simulated the inhibition of 5'-DFUR phosphorylation by AcyT in the tumors and the intestine (Fig. 3). These data points were calculated using Eq. 3 the values from Table 1, and ref. 18. The simulated profile for the inhibition of PyNPase in Colon 26 was similar to that of LLC: the inhibitory effect increased with an increase in the molar ratio of AcyT. The simulated profile for the mouse intestine was different from that obtained with LLC and Colon 26; AcyT at the ratio (AcyT/5'-DFUR) of 0.1 showed a high inhibitory effect.
Fig. 3. Simulated Profiles for the Inhibition of 5′-DFUR Phosphorolysis by AcyT

These data points were calculated using equation (3). For details, see "Materials and Methods." (1) Simulated profile for the inhibition of 5′-DFUR (10 μM) phosphorolysis in Colon 26 and LLC. (2) Simulated profile for the inhibition of 5′-DFUR (1 mM and 10 mM) phosphorolysis in mouse intestine. Kinetic parameter values (Km, K, Vmax) in Table 1.

(>75%). Since AcyT was more sensitive in the intestine than in the tumor, it was expected to be able to prevent 5′-DFUR phosphorolysis only in the intestinal tract, but not in the tumors. We previously reported that orally co-administered AcyT with 5′-DFUR could reduce its intestinal toxicity without reducing its antitumor effect in mice bearing LLC, (13) and this simulation was consistent with the results of the in vivo investigation.

In conclusion, the present study demonstrates that species differences exist in the inhibitory effect of AcyT, and also indicates that substrate specificity varies between PyNPase from different human cells and/or tissues. The present finding may have an important implication for efforts to introduce AcyT, a potent PyNPase inhibitor, into the clinic.

REFERENCES