The Synthesis and in Vitro and in Vivo Stability of 5-Fluorouracil Prodrugs Which Possess Serum Albumin Binding Potency

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For a new drug delivery system of 5-fluorouracil, we prepared prodrugs possessing certain desired properties. The prodrugs, 1-(N-4-chlorophenyl-N-methylcarbamoyl)-5-fluorouracil and 1-(N-2,4-dichlorophenyl-N-methylcarbamoyl)-5-fluorouracil, contain high serum albumin binding potency and a comparably long half life in the bloodstream in vivo to Tegafur. These two prodrugs are expected to be retained in the bloodstream as a polymeric complex with albumin and to circulate in the body for a long time, like a polymeric prodrug.

Keywords 5-fluorouracil; prodrug; protein binding; stability; in vivo; in vitro

Although the precise mechanisms of 5-fluorouracil (5FU) are not fully understood, 1, 2 5FU is widely used in cancer chemotherapy because of its high anti-tumor activity. 3 Since it is eliminated quickly from the body according to its metabolism in the liver, a high dose of 5FU is necessary for clinical treatment, which may result in undesirable side effects. To solve this problem, several types of the drug delivery systems (DDS) of 5FU have been considered. Most research to date has involved the usage of synthetic or natural polymers as the drug carrier, where 5FU is grafted onto the polymer with or without spacer groups. 4, 5 In these systems, however, numerous problems have been caused by the introduction of high molecular weight compounds into the body: antigen toxicity, trapping by the reticuloendothelial system, accumulation in the liver and spleen, restriction of the method of administration, and so on. 6, 7 In order to solve these problems posed by a macromolecular drug carrier, and to establish the most available DDS for 5FU, we have been developing a new system in which a complex of 5FU-prodrugs and serum albumin is formed in the body after the administration of a low molecular weight 5FU derivative into the blood. The complex is then expected to circulate in the body like a polymeric prodrug, and finally 5FU is released from the complex at an appropriate rate by the cleavage of the prodrug. By now, we have shown basic information about the derivation of 5FU for providing high binding affinity to serum albumin, as well as other pharmaco-kinetical data. 8 From the data, it was strongly suggested that our new system had a high possibility for clinical use. To attain information on the release of 5FU from a prodrug by hydrolysis, we tested the in vitro and in vivo decomposition phenomena of two kinds of 5FU prodrugs, 9 in which a phenyl or a substituted phenyl group was introduced to the N-1 or N-3 position in 5FU via a spacer group. It was clear that the hydrolysis or decomposition phenomenon was very different in vitro vs. in vivo, and that the N-1 position in 5FU should be blocked to avoid rapid metabolism in vivo. Taking this information into account, we were able to prepare, in the present study, 5FU prodrugs with desired properties for our new DDS. They have high serum albumin binding potency and a slow rate of prodrug decomposition (hydrolysis) in vivo.

Materials and Methods
Preparation of 1-(4-Chlorobenzoylcarbamoyl)-5-fluorouracil (5FU-1pCZ) To 40 ml of 1,4-dioxane solution containing phosphene (0.040 mol) was added 4-chlorobenzyl alcohol (0.890 g, 6.0 mmol) dissolved in diethyl ether (30 ml). The reaction solution was stirred at −10 °C for 2 h and at 0 °C overnight. After removing the solvent and the remaining excess phosphene with dried nitrogen, the residue was dissolved in dried dimethylformamide (DMF, 20 ml). Then 5FU sodium salt (0.608 g, 4.0 mmol) was added, and the reaction mixture was stirred for 2 h at room temperature (r.t.). After removing the solvent in vacuo, the residual crystal was purified by recrystallization from chloroform to get 5FU-1pCZ with a yield of 22.2% (0.265 g), mp 170.5°C (lit. 100°C). NMR (DMSO-d6): δ 5.43 (s, 2H), 7.30–7.81 (m, 4H), 8.30 (d, 1H).

Preparation of 1-Benzoyloxymethyl-5-fluorouracil (5FU-1BOM) To an aqueous solution of formaldehyde (35%) was added 5FU (0.65 g, 5.0 mmol), and the solution was stirred at 60 °C for 1 h. Benzoic acid (0.733 g, 6.0 mmol) dissolved in 17 ml of water/1,4-dioxane mixed solvent (10/7, v/v) was added at r.t. and the pH of the solution was adjusted to 4 to 5 with 0.1 M NaOH. To the solution 1-ethyl-3-(dimethylaminopropyl)carbodiimide hydrochloride (WSCD-HCl, 1.342 g, 7.0 mmol) was added, and the solution was stirred at r.t. overnight. After removing the solvent in vacuo, the residue was extracted with diethyl ether, washed with 10% NaHCO3 and water, then dried with Na2SO4. The solvent was removed, and to it was added a small amount of diethyl ether which produced a fine crystal of 5FU-1BOM. Yield 0.080 g (6.1%), mp 159.0°C (lit. 183°C). NMR (acetone-d6): δ 5.97 (s, 2H), 7.52–7.70 (m, 3H), 8.03–8.06 (m, 2H), 8.10 (d, 1H).

Preparation of 1-(4-Chlorobenzoyloxymethyl)-5-fluorouracil (5FU-1pCBOM) To an aqueous solution of formaldehyde (35%) was added 5FU (0.65 g, 5.0 mmol), and the solution was stirred at 60 °C for 45 min. Next was added 4-chlorobenzonic acid (1.18 g, 7.5 mmol), then the pH of the solution was adjusted to 4 to 5 with 0.1 M NaOH at 0 °C. To the solution, WSCD-HCl (1.92 g, 10 mmol) was added, and the solution was stirred at r.t. overnight. Extraction was performed with chloroform. The residue obtained from the organic phase by evaporation was applied on silica gel (Kieselgel 60, 35 × 150 mm) eluted with chloroform. The eluted fraction was washed with water to get 5FU-1pCBOM. Yield 0.080 g (5.4%), mp 188.1°C (lit. 198°C). NMR (DMSO-d6): δ 5.89 (s, 2H), 7.65–8.05 (m, 4H), 8.25 (d, 1H).

Preparation of 1-(4-Chlorophenylsulfonyl)-5-fluorouracil (5FU-1pCPS) To the suspension of 5FU (0.260 g, 2.0 mmol) and triethylamine (0.556 ml, 4 mmol) in 1,4-dioxane (4 ml) was titrated 4-chlorobenzensulfonyl chloride (1.27 ml, 6.0 mmol) dissolved in 1,4-dioxane (3.5 ml). The reaction mixture was then stirred for 2 h at room temperature. After filtration, the filtrate was evaporated to dryness. The residue was applied on a silica gel column (Kieselgel 60, 35 × 200 mm) eluted with benzene/ethanol (9/1, v/v). The crystal obtained by the evaporation of the fractions was recrystallized from ethanol to get 5FU-1pCPS. Yield 0.116 g (19.0%), mp 234.5°C (lit. 12)

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Preparation of 1-(4-Chlorophenyl)-N-methylcarbamoyl-5-fluorouracil (5FU-1pCPM)

To the solution of N-methyl-4-chloroaniline (1.42 g, 10 mmol) in anhydrous toluene (30 ml) was added activated charcoal (0.15 g). Trichloromethyl chlorofomate (TCF, 2 ml) was then added at 0°C and the solution was stirred at r.t. for 2 h and refluxed for 2 h. Next nitrogen gas was bubbled at r.t. to remove excess phosgene in the solution. After filtration, the solvent was evaporated. The residual solid was dissolved in 10 ml of DMF, to which was added 5FU sodium salt (1.52 g, 10 mmol) suspended in DMF (70 ml). The reaction mixture was stirred at r.t. for 46 h. After removing the insoluble material, the solvent was evaporated in vacuo. The residual crystal was washed with diethyl ether to get 5FU-1pCPM. Yield: 2.20 g (73.8%), mp 235.0°C. NMR (DMSO-d6): δ: 3.36 (s, 3H), 7.24-7.62 (m, 4H), 8.30 (d, 1H). Found: C, 48.8; H, 3.14; N, 14.05. Calcd. for C12H10O2CNF, C, 48.42; H, 3.05; N, 14.12.

Preparation of 1-(2,4-Dichlorophenyl)-N-methylcarbamoyl-5-fluorouracil (5FU-1DCPM)

To the solution of N-methyl-2,4-dichloroaniline (0.705 g, 4.0 mmol), which was prepared from 2,4-dichloroaniline and dimethyl sulfoxide, in anhydrous toluene (10 ml), was added activated charcoal (0.01 g). At 0°C, TCF (1 ml) was titrated and the solution was stirred until no more phosgene was observed. Chloroform was then added to the solution at r.t. to remove excess phosgene in the solution. After filtration, the solvent was evaporated. The residual solid was dissolved in 4 ml of DMF, to which was added 5FU sodium salt (0.608 g, 4.0 mmol) suspended in DMF (26 ml). The reaction mixture was stirred at r.t. for 47 h. The solvent was removed in vacuo. The residual crystal was recrystallized from acetone to obtain 5FU-1DCPM. Yield: 0.555 g (41.8%), mp 232.4°C. NMR (DMSO-d6): δ: 3.33 (s, 3H), 7.56 (s, 2H), 7.78 (s, 1H), 8.27 (d, 1H). Found: C, 43.45; H, 2.40; N, 12.67. Calcd. for C12H9O2CNF, C, 43.40; H, 2.43; N, 12.65.

Measurement of the Rate of Hydrolysis in the Buffer

The test compound was dissolved in an isotonic phosphate buffer (pH 7.4). The solution was incubated at 37°C, then the aliquot was taken at an appropriate time. The concentration of the test compound was determined by HPLC (Shimazu LC-6A system; column, Cosmosil SC18-AR, 4.6 × 150 mm, NaCl equilibrated phase (pH 5.0) methanol (1/1 or 3/2, v/v); detection at 254 nm with UV-810, Tosoh).

Measurement of the Rate of Hydrolysis in the Plasma

The isotonic phosphate solution of the test compound was mixed with rat plasma (1/1, v/v) and incubated at 37°C. An aliquot was taken from the mixed solution at an appropriate time, which was added 2 volumes of methanol or 0.1 M zinc sulfate. After vortexing, centrifugation (1560 × g) was performed for 5 min. The concentration of the test compound in the supernatant was determined by HPLC as described above.

Measurement of Partition Coefficient

The test compound was dissolved in an isotonic phosphate buffer (pH 7.4) whose concentration was determined by HPLC as described above. To 0.5 ml of the solution, n-octanol (0.5 ml) saturated with phosphate buffer was added. The mixed solution was vortexed at the highest speed for 5 min, then centrifugation (12100 × g) was performed. The upper phase (n-octanol) was removed and the concentration of the test compound in the lower phase (water phase) was determined by HPLC under the conditions described previously.

Protein Binding

To the solution of 5FU-derivative in isotonic phosphate buffer (pH 7.4, 0.2 m) was added human serum albumin (HSA, fraction V, Sigma), resulting in a final concentration of 4.5%. The solution was incubated at 37°C for 20 min. Then the solution was applied on an ultrafiltration membrane tube (Centron, Amicon Co.) and ultrafiltration was performed at 15860 × g for 10 min. The solution remaining on the filter was replaced to the tube, and double the volume of methanol (or 0.1 M zinc sulfate) was added. After vortexing, the supernatant was obtained by centrifugation (15860 × g) for 10 min. The concentration of test compound in the supernatant was determined by HPLC. Also, the concentration of the compound in the filtrate was determined by HPLC. The content of the compound adsorbed to the filter was correlated with the similar experiment without HSA.

Measurement of the Half-Life of the Compound in Vitro

The biological half-life of 5FU derivatives was measured using rats (body weight 200-250 g) under an anesthetized condition. The femoral artery of each rat was cannulated with polyethylene tubing for collection of the blood samples. Then, the test compound dissolved in isotonic phosphate buffer (pH 7.4) was administered to the femoral vein (1.5 mmole/kg), and the blood was taken at appropriate intervals from the femoral artery. After centrifugation for 5 min, the supernatant was mixed with double the volume of methanol or 0.1 M zinc sulfate. The mixed solution was vortexed and centrifuged at 15860 × g for 5 min to remove any protein. The concentration of 5FU or 5FU-derivatives in the supernatant was determined by HPLC as described above.

Results and Discussion

Rate of Hydrolysis in Vitro

Table I summarizes the in vitro data of half-life for the 5FU derivatives prepared in an isotonic phosphate buffer and in 50% rat plasma at 37°C. The half-life of 5FU-1pCZ was very short compared with that of the other 5FU prodrugs in buffer, as well as in the plasma. It was found that the half-life of all 5FU prodrugs in plasma was shorter than that in the buffer, suggesting the contribution of enzymes in the plasma. It was indicated that the effect of the chloride atom attached to a phenyl group of 5FU-prodrug was not simple. In the previous study, the half-life of 3-(4-chlorobenzoyl)-5-fluorouracil was found to be longer than that of 3-benzoyl-5-fluorouracil. And it was suggested that an increase in hydrophobicity affected the rate of hydrolysis of the derivatives. In the case of 5FU-1pCZ, however, the half-life involving hydrolysis in the buffer was shorter than that of 5FU-1Z (1-benzoxycarbonyl-5-fluorouracil, 2.05 h⁻¹). Similar phenomena were found in comparing 5FU-1BOM and 5FU-1pCBOM. In these cases, the effect of a chloride atom may be explained by the electron-withdrawing effect of the chloride atom introduced to the 4-position of a phenyl group. That is, the local electron density on the oxy carbonyl group may be decreased according to the electron-withdrawing effect of the chloride, so that the rate of hydrolysis may be increased. The low yield in the preparation of these compounds may be due to fast hydrolysis. By contrast, in comparing 5FU-1pCPMC and 5FU-1DCPMC, the prolonged half-life may be attributed to an additional chloride atom in the phenyl group. In this case, the electron-withdrawing effect of a chloride atom may also contribute to the shortage of the electron on the hydrolysable part in the compound; however, the electron may be supplied from a 5-fluorouracil group through the resonance structure of the compound. Consequently, the slower in vitro hydrolysis reaction may occur due to the increased local hydrophobicity of the compound.

Partition Coefficients and Protein Binding Potency

Shown in Table II are the partition coefficients and binding potency to HSA of the 5FU derivatives prepared. As shown in the in vitro hydrolysis experiment, where 5FU-1pCZ was quickly hydrolyzed in HSA solution, it was impossible to measure the protein binding percent of that compound. Other 5FU prodrugs prepared here showed relatively higher protein binding potency. In the case of

<table>
<thead>
<tr>
<th>Compound</th>
<th>In buffer (h)</th>
<th>In plasma (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5FU-1pCZ</td>
<td>&lt;1</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>5FU-1BOM</td>
<td>&gt;400</td>
<td>5.3</td>
</tr>
<tr>
<td>5FU-1pCBOM</td>
<td>24</td>
<td>3.2</td>
</tr>
<tr>
<td>5FU-1pCPS</td>
<td>&gt;400</td>
<td>35.9</td>
</tr>
<tr>
<td>5FU-1pCPMC</td>
<td>&gt;400</td>
<td>12.1</td>
</tr>
<tr>
<td>5FU-1DCPMC</td>
<td>&gt;400</td>
<td>43.1</td>
</tr>
</tbody>
</table>

a) In isotonic phosphate buffer at 37°C. b) In 50% rat plasma at 37°C.
TABLE II. Partition Coefficients, Protein Binding and the Half-Life in Vivo of 5FU Prodrugs

<table>
<thead>
<tr>
<th>Compound</th>
<th>Partition coefficient</th>
<th>Protein binding (bound %)</th>
<th>Half life (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tegafur&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.331</td>
<td>22.9&lt;sup&gt;0&lt;/sup&gt;</td>
<td>5.4</td>
</tr>
<tr>
<td>5FU-1pCZ</td>
<td>19.1</td>
<td>n.d.</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>5FU-1BOM</td>
<td>9.42</td>
<td>87.4</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>5FU-1pCBOM</td>
<td>49.7</td>
<td>95.7</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>5FU-1pCPS</td>
<td>2.18</td>
<td>97.5</td>
<td>0.15</td>
</tr>
<tr>
<td>5FU-1pCPMC</td>
<td>1.73</td>
<td>75.7</td>
<td>5.4</td>
</tr>
<tr>
<td>5FU-1DCPMC</td>
<td>6.96</td>
<td>82.5</td>
<td>5.7</td>
</tr>
</tbody>
</table>

<sup>a</sup> n-Octanol/isotonic phosphate buffer. <sup>b</sup> Binding to HSA. <sup>c</sup> Half-life in rat after i.v. administration. <sup>d</sup> 1-(1-Tetrafuranyl)-5-fluorouracil; from reference 8. <sup>e</sup> Examed with rat plasma.

5FU-1pCPS, the protein binding was over 95%, even though its partition coefficient was 2.18. This suggests that the additional binding site in the albumin, which may be due to the sulfonyl group, attributes to the binding phenomenon of this compound.

The *Half-Life of the 5FU-Prodrugs in Vitro* In Table II, the in vivo data of the rate of disappearance of the compounds were also included. Intact 5FU, which may be delivered from 5FU-prodrug, was detected in rat plasma after intravenous administration in all cases. However, three 5FU derivatives (5FU-1pCZ, 5FU-1BOM, 5FU-1pCBOM) were not detected after 10 min of administration. This fast metabolism may be concerned with the fast in vitro hydrolysis measured in 50% rat plasma solution. The half-life of 5FU-1pCPS was found to be short (0.15 h) compared with that of 5FU-1pCPMC and 5FU-1DCPMC (5.4 h and 5.7 h, respectively). This fact indicates that the in vivo half-life of 5FU derivative, which can bind to the albumin molecule with a high affinity, may be attributed not only to the higher binding potency, but also to the steric hindrance (methyl group attached to a nitrogen atom in the carbamoyl group) from the attack of enzymes in the bloodstream and in a metabolic organ, such as the liver.

Additional proof regarding this is the fact that the half-life of comparable compound to 5FU-1pCPMC, 1-phenylcarbamoyl-5-fluorouracil, where the N-methyl group and chloride atom were absent, was only 0.07 min in a buffer solution.<sup>13</sup>

In conclusion, 5FU-1DCPMC or 5FU-1pCPMC may have a high possibility as a clinical treatment agent for cancer chemotherapy because they have a longer half-life in vivo and relatively higher protein binding potency. Both 5FU derivatives are expected to be retained in the blood as a polymeric complex with albumin, and to circulate in the body for a long time. A practical test for estimating the anti-tumor activity of these compounds using animals is currently under examination.

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**References**