

## Regular Article

## Effect of Metabolic Inhibitors on the Hepatic Disposition of 5-Fluorouracil after Application to the Rat Liver Surface

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We evaluated the effects of 5-fluorouracil (5-FU) metabolic inhibitors, gimeracil or uridine, on the hepatic disposition of 5-FU after application to the liver surface in rats, aiming to enhance the availability of 5-FU in the liver. 5-FU solution with or without metabolic inhibitors was applied to the rat liver surface using a cylindrical diffusion cell. The liver, blood and the remaining solution in the diffusion cell were collected at specified times, and assayed for 5-FU content. 5-FU absorption properties were not altered by addition of gimeracil and uridine. The 5-FU concentration in the diffusion cell attachment site of the rat liver (site 1) at 0.1–0.4 M ratios of gimeracil to 5-FU was significantly higher than that of the control. On the contrary, the addition of uridine did not increase the 5-FU concentration at site 1. At a 0.1 M ratio of gimeracil to 5-FU, the maximum 5-FU plasma concentration was the lowest, and the area under the 5-FU concentration–time curve at site 1 was 3.4 times greater than that of the control. We demonstrated that applying 5-FU with gimeracil to the rat liver surface could increase the availability of 5-FU in the liver.

**Key words** liver surface; 5-fluorouracil; disposition; dihydropyrimidine dehydrogenase; gimeracil; uridine

Hepatocellular carcinoma (HCC) is the second most frequent cause of cancer-related death worldwide.<sup>1)</sup> In many cases, chronic hepatitis and hepatic cirrhosis trigger the development of HCC.<sup>2)</sup> Even radical surgery, a potentially curative treatment for HCC, is limited by cancer progression and hepatic functional reserve.<sup>3)</sup> In addition, HCC frequently recurs after radical surgery in the residual liver tissue because of progressive disease.<sup>4)</sup> Although chemotherapy has been applied to treat the advanced or recurrent HCC, most of anticancer drugs cannot exert the desired effect on HCC because of high chemoresistance rate.<sup>5,6)</sup>

Local therapies such as percutaneous ethanol injection,<sup>7,8)</sup> intratumoral injection<sup>9–11)</sup> and intra-arterial infusion<sup>12)</sup> have been performed to enhance the therapeutic effect of anticancer drugs on HCC. However, optimal delivery of anticancer drugs to HCC lesion is difficult through these routes because of distribution in the normal surrounding tissue or rapid efflux into the systemic circulation from the injection site.

Therefore, we have proposed that the liver surface is a new drug administration route, and investigated the absorption and disposition characteristics of several marker compounds after direct application to the rat liver surface.<sup>13–18)</sup> To use this route as a local HCC therapy, we showed that 5-fluorouracil (5-FU), a pyrimidine anticancer drug, was site-selectively and continuously delivered in the liver after application to the liver surface in rats.<sup>19,20)</sup> We have also investigated other organs as an administration route of 5-FU.<sup>21,22)</sup> Although viscous additives to 5-FU solution improved site-selective accumulation of 5-FU,<sup>23)</sup> the increase in 5-FU concentration at the application site was not large enough.

5-FU has been widely used in chemotherapy regimens against a variety of cancers such as liver,<sup>24)</sup> colon,<sup>25)</sup> stomach<sup>26)</sup> and pancreatic cancer.<sup>27)</sup> However, approximately 85% of administered 5-FU is catabolized by dihydropyrimidine dehydrogenase (DPD).<sup>28)</sup> 5-FU catabolism must be sup-

pressed to enhance its anticancer effect.

DPD inhibitors have been used as adjuvants to enhance the anticancer effect of 5-FU. TS-1<sup>®</sup> and UFT<sup>®</sup> are oral agents that combine tegafur with the DPD inhibitors, gimeracil and uracil, respectively. TS-1<sup>®</sup> is combined at a 0.4 molar ratio of gimeracil to tegafur. UFT<sup>®</sup> is combined at a 4 molar ratio of uracil to tegafur. These agents could increase the availability of 5-FU by inhibiting DPD activity, leading to an improved anticancer effect by 5-FU.<sup>29–31)</sup> Uridine was also reported to decrease the catabolism of 5-FU by inhibiting DPD.<sup>32)</sup>

For liver surface application of 5-FU, DPD inhibitors are expected to enhance its concentration in the liver and keep the application site concentration high. In addition, 5-FU in combination with the DPD inhibitor would be useful to develop a pharmaceutical formulation for application of 5-FU to the liver surface to treat HCC.

In the present study, we chose gimeracil and uridine as DPD inhibitors. We examined the absorption and distribution in the liver after 5-FU application to the rat liver surface using various combination ratios of the DPD inhibitor. After determining the optimal combination ratio of the DPD inhibitor, we investigated the effect of the DPD inhibitor on the availability of 5-FU in the liver after application to the rat liver surface.

## MATERIALS AND METHODS

**Chemicals** 5-FU was purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Gimeracil and uridine were obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). All other chemicals were of reagent grade.

**Animal Experiments** All animal experiments in this study conformed to the Guidelines for Animal Experimentation at Nagasaki University (approval number: 1006100863-2, approval date: May 28, 2010). Male Wistar rats (230–270 g) were anaesthetized with pentobarbital sodium (50 mg/kg, in-

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traperitoneally (i.p.)). The left femoral artery was cannulated using polyethylene tubes. A cylindrical diffusion cell (i.d. 9mm, 0.64cm<sup>2</sup>) was attached to the liver surface (left lateral lobe) using Aron Alpha (Daichisankyo Co., Ltd., Tokyo, Japan) after laparotomy. 5-FU (0.2mL of 10mg/mL solution) in an isotonic phosphate buffer (pH 7.4) in the presence or absence of gimeracil (0.05, 0.1, 0.2 and 0.4 molar ratio to 5-FU) or uridine (1.0, 2.0, 3.0 and 4.0 molar ratio to 5-FU) was added into the diffusion cell. The applied dose of 5-FU was set according to our previous study.<sup>23)</sup> A piece of aluminum foil was placed on the top of the diffusion cell to prevent evaporation.

To determine plasma 5-FU concentrations, blood samples from the left femoral artery were collected at selected times (15, 60, 120 and 180min) and centrifuged. The solution in the diffusion cell was withdrawn at the pre-determined times to calculate the remaining amount of 5-FU in the diffusion cell. The liver was excised following perfusion with saline from the portal vein. The excised liver was divided into three sites; the region under the diffusion cell attachment site (site 1); the applied lobe except for site 1 (site 2); and the non-applied lobes (site 3). Each liver sample was weighed and homogenized using two-fold volumes of their weight in isotonic phosphate buffer (pH 7.4).

**Analytical Methods** 5-FU concentrations in the liver homogenate, the plasma and the solution remaining in the diffusion cell were measured using previously reported methods<sup>33,34)</sup> with some modifications. Briefly, 300μL of the liver homogenate, the plasma sample, or the solution remaining in the diffusion cell were mixed with a solution containing 150μL of 20μg/mL 5-bromouracil dissolved in isotonic phosphate buffer (pH 7.4) as an internal standard, with 500μL of 20% anhydrous sodium sulfate, and 100μL of 1M sodium acetate buffer (pH 4.8). The mixtures were shaken for 10min after addition of 4mL of ethyl acetate and centrifuged at 1630×*g* for 10min at 4°C. The organic layers (3mL) were collected and 4mL of fresh ethyl acetate was added to the residue. The mixtures were shaken for 10min and centrifuged at 1630×*g* for 10min at 4°C. The organic

layers (4mL) were collected and 7mL of the mixed organic layers were evaporated. The extracted residues were dissolved in 500μL of distilled water and washed twice with 1mL of hexane. Samples (100μL) were injected onto the HPLC column (Cosmosil-packed column 5C<sub>18</sub>-PAQ, 4.6mm i.d.×150mm, Nacalai Tesque, Kyoto, Japan). A HPLC system with a variable-wavelength UV detector (SPD-10A, Shimadzu Co., Ltd., Kyoto, Japan) was used in the reverse-phase mode. The detector wavelength, flow rate, and column temperature were set at 266nm, 0.5mL/min, and 25°C, respectively. The mobile phase consisted of 10mM sodium acetate buffer (pH 4.7). The absorption ratio of 5-FU after application to the liver surface in rats was calculated from the remaining amount of 5-FU in the diffusion cell.

**5-FU Area under the Curve (AUC) of the Plasma or Liver Concentration–Time Profile** The 5-FU plasma and liver concentration–time profile up to 180 and 360min after application to the rat liver surface was analyzed according to the following Eqs. 1 and 2:

$$AUC_p = \int_0^{180} C_p dt \quad (1)$$

$$AUC = \int_0^{360} C dt \quad (2)$$

where *t* is the time (min), *C<sub>p</sub>* is the plasma concentration of 5-FU (μg/mL) and *AUC<sub>p</sub>*, is the area under the plasma concentration–time curve (μg×min/mL plasma). The *AUC<sub>p</sub>* was calculated using the linear trapezoidal formula. *C* is the liver concentration of 5-FU (μg/g), and *AUC*, the area under the liver concentration–time curve (μg×min/g liver), was also calculated using the linear trapezoidal formula.

**Statistical Analysis** Statistical comparisons were performed using Dunnett's test after an analysis of variance for multiple groups. *p*<0.05 compared with the control group (in the absence of DPD inhibitor) was considered to be significant. All values were expressed as the mean value±standard error (S.E.) of at least independent different three experiments.

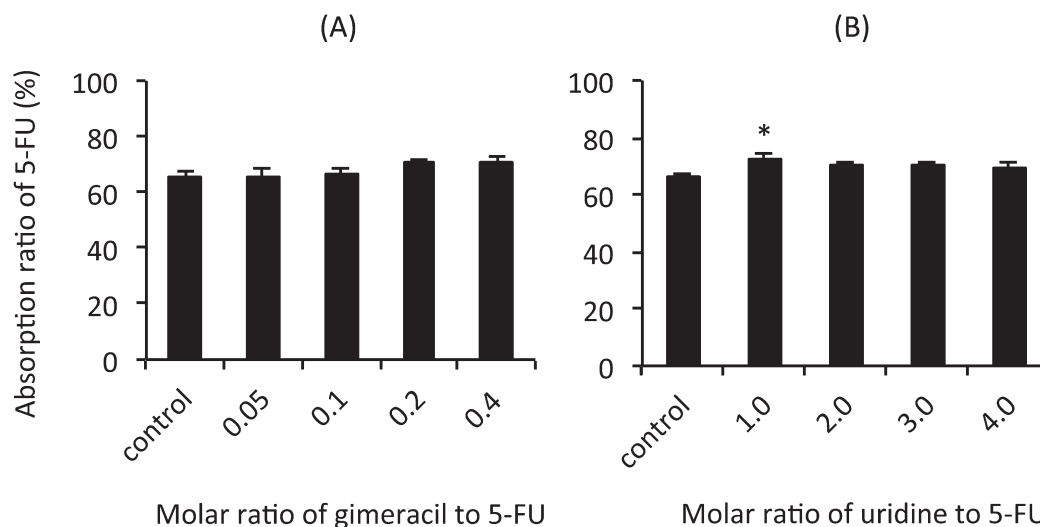


Fig. 1. Absorption Ratio of 5-FU at 180min after Application to the Rat Liver Surface at the Various Molar Ratios of Gimeracil (A) or Uridine (B) to 5-FU

Each bar represents the mean±S.E. of at least three experiments. Significantly different from the result at control: \**p*<0.05.

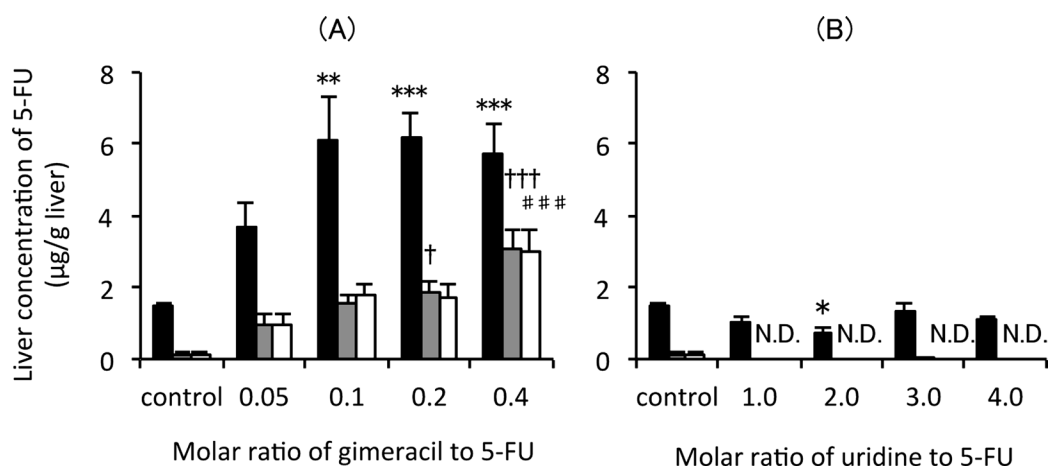


Fig. 2. Liver Concentration of 5-FU at 180min after Application to the Rat Liver Surface at the Various Molar Ratios of Gimeracil (A) or Uridine (B) to 5-FU in Rats

Site 1 (closed bar), site 2 (gray bar) and site 3 (open bar). Each bar represents the mean±S.E. of at least three experiments. Significantly different from the result at site 1 of control: \* $p<0.05$ , \*\* $p<0.01$ , \*\*\* $p<0.001$ , at site 2 of control: † $p<0.05$ , ‡ $p<0.001$ , at site 3 of control: ### $p<0.001$ . N.D.: not detected.

## RESULTS

**Effect of DPD Inhibitors on the Absorption of 5-FU after Application to the Liver Surface in Rats** Figure 1 shows the absorption ratio of 5-FU at 180min after application to the rat liver surface under the various molar ratios of DPD inhibitors to 5-FU. The absorption ratios of 5-FU from the rat liver surface were approximately 70% under each condition.

**Effect of DPD Inhibitors on the Distribution of 5-FU at the Different Hepatic Sites of the Liver after Application to the Liver Surface in Rats** Figure 2 shows the liver concentration of 5-FU at 180min using various molar ratios of DPD inhibitors to 5-FU after application to the liver surface in rats. 5-FU concentrations at site 1 using a 0.1–0.4 molar ratio of gimeracil to 5-FU were significantly increased approximately 4-fold compared with the control (Fig. 2A). The concentration ratios at site 1 using a 0.1 and 0.2 molar ratio of gimeracil to 5-FU and the control were 4.2 in both cases: the highest for each condition. However, combining uridine did not enhance 5-FU concentration at site 1 (Fig. 2B).

**Plasma Concentration Profiles of 5-FU after Application to the Rat Liver Surface in the Presence of Gimeracil** Figure 3 illustrates the plasma concentration profiles of 5-FU after application to the rat liver surface under various molar ratios of gimeracil. The maximum 5-FU plasma concentration was increased based on the molar ratio of gimeracil to 5-FU. However, the plasma concentration profiles of 5-FU were not altered by combining uridine, even in the presence of a 4.0 molar ratio to 5-FU (data not shown).

The  $AUC$  of the plasma concentration profile up to 180min after administration of 5-FU ( $AUC_p$ ) at control, 0.05, 0.1, 0.2 and 0.4 molar ratio of gimeracil to 5-FU was calculated to be 137.5, 216.6, 346.7, 376.5 and 567.7 ( $\mu\text{g}\times\text{min}/\text{mL}$  plasma), respectively. The  $AUC_p$  at a 0.1 molar ratio of gimeracil to 5-FU was the lowest among the 0.1–0.4 molar ratios of gimeracil to 5-FU.

Table 1 summarizes the concentration ratios of 5-FU at each site and plasma under the various molar ratios of gimeracil to 5-FU. These concentration ratios at a 0.1 molar ratio of gimeracil to 5-FU were the highest among the 0.1–0.4 molar ratios. In contrast, these concentration ratios could not be cal-

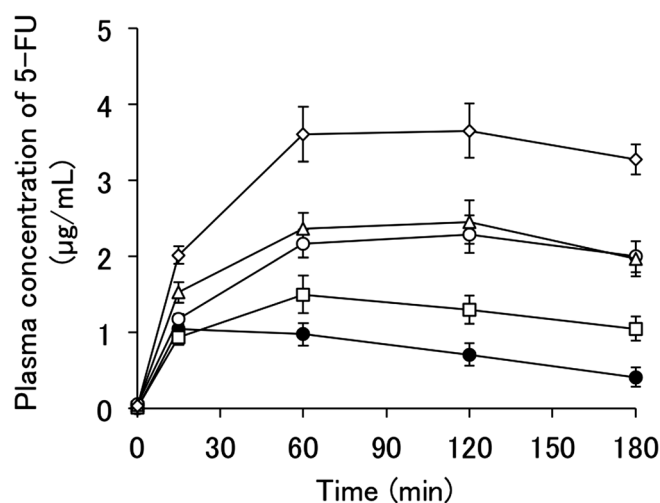


Fig. 3. 5-FU Plasma Concentration Profiles after Application to the Rat Liver Surface at the Various Molar Ratios of Gimeracil to 5-FU

Key: Molar ratio of gimeracil to 5-FU is 0 (control, ●), 0.05 (□), 0.1 (○), 0.2 (△) and 0.4 (◇). Each point represents the mean±S.E. of at least three experiments.

Table 1. Ratio of 5-FU Liver Concentration at 180min at Each Site and in the Plasma after Application to the Rat Liver Surface in the Presence or Absence of Gimeracil

	Molar ratio of gimeracil to 5-FU				
	Control	0.05	0.1	0.2	0.4
Site 1/site 2	13.2	3.8	3.8	3.4	1.9
Site 1/site 3	10.4	3.8	3.5	3.5	1.9
Site 1/plasma	3.5	3.5	3.1	3.1	1.8

culated in the presence of uridine, because the concentrations of 5-FU at sites 2 and 3 were below the limit of detection (data not shown).

The distribution in the rat liver and plasma concentration profiles of 5-FU suggested that the optimal ratio of the DPD inhibitor was a 0.1 molar ratio of gimeracil to 5-FU. We then examined the effect of a 0.1 molar ratio of gimeracil on the absorption and hepatic disposition of 5-FU after application to the rat liver surface.

**5-FU Absorption Profiles from the Rat Liver Surface at a 0.1 Molar Ratio of Gimeracil** Figure 4 shows a semi-log plot of the remaining amount of 5-FU in the diffusion cells after application to the rat liver surface in the presence or absence of gimeracil. The semi-log plots show straight lines for both conditions. The first-order absorption rate constant,  $k_a$ , of 5-FU from the rat liver surface in the absence or presence of a 0.1 molar ratio of gimeracil was calculated to be  $5.7 \times 10^{-3}$  and  $5.0 \times 10^{-3} \text{ min}^{-1}$ , respectively.

**5-FU Liver Concentration Profiles after Application to the Rat Liver Surface at a 0.1 Molar Ratio of Gimeracil** Figure 5 shows the 5-FU liver concentration after application to the rat liver surface in the presence or absence of a 0.1 molar ratio of gimeracil. In the presence of gimeracil, 5-FU was preferentially distributed at site 1 after 15, 60, 180 and 360 min (Fig. 5B), which was similar to the control (Fig. 5A). The 5-FU concentration at site 1 was immediately increased at 15 min in the presence of gimeracil. Even after 360 min, the 5-FU concentration at site 1 remained approximately 2.5 times higher than the control.

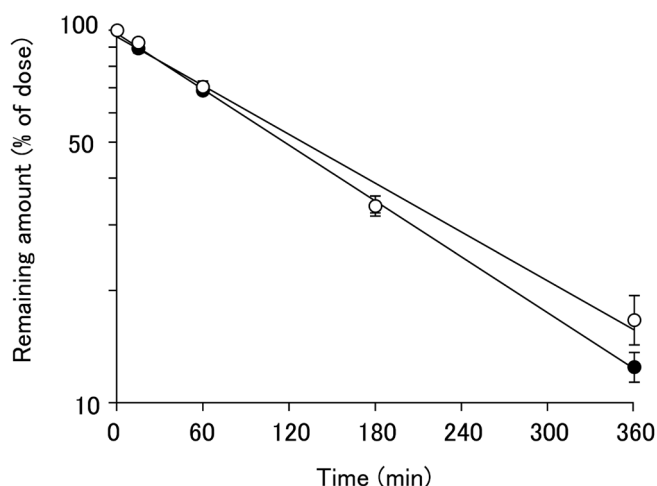


Fig. 4. Semi-log Plot of Remaining Amount of 5-FU in the Diffusion Cell after Application to the Rat Liver Surface in the Presence or Absence of Gimeracil

Key: Control (●), 0.1 molar ratio of gimeracil to 5-FU (○). Each point represents the mean  $\pm$  S.E. of at least three experiments.

The AUC of the 5-FU liver concentration profile was calculated as an index of regional availability. Figure 6 shows the AUC of each site until 360 min after application to the rat liver surface. The AUC of the site 1 5-FU concentration–time profile at a 0.1 molar ratio of gimeracil was 3.4 times higher than that of the control. The AUC ratios of site 1 to sites 2 or 3 in the presence of a 0.1 molar ratio of gimeracil to 5-FU were 4.8 and 4.0, respectively, although the corresponding ratios in the absence of gimeracil were 5.6 and 22.5, respectively.

## DISCUSSION

We propose the liver surface as a new drug application site, and we showed that site-selective and continuous delivery of 5-FU could be accomplished by applying it directly to the rat liver surface.<sup>13–20,23</sup> Because 5-FU is mostly catabolized and inactivated by DPD,<sup>28</sup> we expected that combining DPD inhibitors enable us to increase 5-FU availability at the admin-

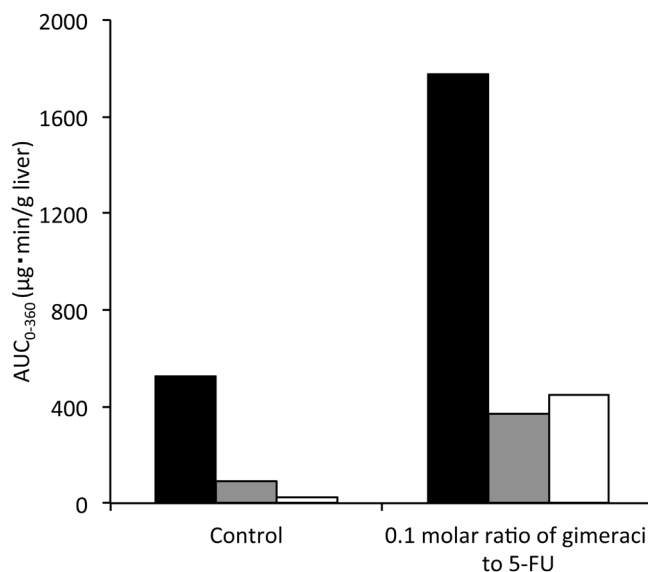


Fig. 6. The 5-FU Area under the Curve up to 360 min at Each Site in the Rat Liver after Application to the Liver Surface at a 0.1 Molar Ratio of Gimeracil or Control

Key: Site 1 (closed bar), site 2 (gray bar), site 3 (open bar).

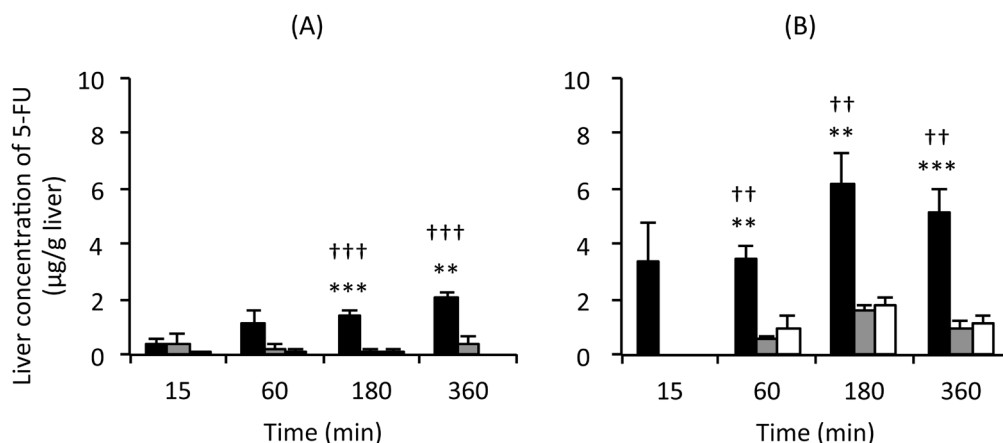


Fig. 5. Liver Concentration of 5-FU after Application to the Rat Liver Surface

(A) Control, (B) 0.1 molar ratio of gimeracil to 5-FU. Key: Site 1 (closed bar), site 2 (gray bar), site 3 (open bar). Each point represents the mean  $\pm$  S.E. of at least three experiments. Significantly different from the result at site 2: \*\* $p < 0.01$ , \*\*\* $p < 0.001$  or site 3: †† $p < 0.01$ , ††† $p < 0.001$  compared with site 1, respectively.

istration site. In this study, we chose gimeracil and uridine as candidates for promising DPD inhibitors that can be applied to the rat liver surface. Uracil was not appropriate as a DPD inhibitor to apply together with 5-FU. That was because of difficulty in preparing the solution containing 5-FU and uracil, due to its poor solubility in water. This study aimed to examine the effect of DPD inhibitors on the hepatic disposition of 5-FU after application to the liver surface in rats, and to investigate the optimal combination ratio of the DPD inhibitor referring to TS-1® and UFT®.

As a first step, we studied the effect of gimeracil and uridine on the absorption properties of 5-FU after application to the rat liver surface using a cylindrical diffusion cell. The absorption ratio of 5-FU from the rat liver surface at 180 min was approximately 70% in the presence or absence of gimeracil or uridine (Fig. 1). This result suggests that gimeracil and uridine can not influence 5-FU absorption from the rat liver surface.

The 5-FU concentration at site 1 at 180 min was significantly increased by the addition of a 0.1–0.4 molar ratio of gimeracil, but the concentration was not altered by the addition of a 1.0–4.0 molar ratio of uridine (Fig. 2). Gimeracil, a stronger inhibitor of DPD than uracil,<sup>35)</sup> was considered to inhibit 5-FU catabolism in the rat liver, resulting in an increase in the 5-FU concentration at site 1 compared with the control. In contrast, uridine was supposed to lower DPD activity after metabolism to uracil by uridine phosphorylase in the liver. DPD-inhibition activity of uracil metabolized from uridine did not seem to be strong enough to increase 5-FU concentration at site 1. The absorption ratio of uridine from the rat liver surface at 180 min at a 4.0 molar ratio of uridine to 5-FU was 27% (data not shown). The low absorption ratio of uridine was considered to be a factor to be unchanged 5-FU concentration at site 1 in the presence of uridine. The concentration of 5-FU at site 1 at a 0.1–0.2 molar ratio of gimeracil to 5-FU was the highest (4.2 times higher) compared with control. These results suggest that addition of gimeracil to 5-FU increase the availability of 5-FU in the liver after application to the liver surface.

It has been reported that the systemic side effects caused by 5-FU were related to the plasma concentration.<sup>36)</sup> To reduce systemic side effects, it is necessary to keep the plasma concentration of 5-FU as low as possible. The maximum plasma concentrations of 5-FU and  $AUC_p$  were increased in the presence of gimeracil as compared with the control (Fig. 3). The exposure of 5-FU in systemic circulation was considered to be increased by inhibiting the 5-FU catabolism in the liver. In contrast, the plasma concentration of 5-FU (Fig. 3) and  $AUC_p$  at a 0.1 molar ratio of gimeracil to 5-FU was the lowest among the conditions. The concentration ratio of site 1 to site 2, site 3 or plasma at a 0.1 molar ratio of gimeracil to 5-FU was the highest among the 0.1–0.4 molar ratios of gimeracil to 5-FU (Table 1). Although these concentration ratios were lower than control, the site-selectivity of 5-FU was sufficiently high at the 0.1 molar ratio of gimeracil to 5-FU. Taking these into considerations, the 0.1 molar ratio of gimeracil to 5-FU could be an appropriate ratio to increase the availability of 5-FU in the liver after application to the liver surface.

We then focused on the effect of a 0.1 molar ratio of gimeracil to 5-FU on the absorbability and hepatic disposition of 5-FU after application to the rat liver surface. In the presence or absence of gimeracil, 5-FU was absorbed from the rat liver

surface according to first-order kinetics (Fig. 4). The 5-FU  $k_a$  value in the presence of gimeracil decreased to 86% of the control. This decrease might be explained by a result of the decrease in the 5-FU concentration gradient between the applied solution and site 1.

The concentration of 5-FU at site 1 was increased immediately, and remained higher than the control until 360 min in the presence of a 0.1 molar ratio of gimeracil to 5-FU (Fig. 5). The absorption ratio of 5-FU and gimeracil from the rat liver surface at 180 min at a 0.1 molar ratio of gimeracil to 5-FU were 66% and 44% (data not shown), respectively. These results suggest that the inhibitory effect of gimeracil on 5-FU catabolism occur immediately, and sustain for at least 360 min. The  $AUC$  of the tumor 5-FU concentration–over-time profile was reported to correlate with inhibition of cancer cell proliferation.<sup>37)</sup> Although the  $AUC$  ratios of site 1 to sites 2 or 3 in the presence of gimeracil were lower than control, the  $AUC$  of the site 1 5-FU concentration profile in the presence of gimeracil was considerably higher than that of the control (Fig. 6). This raises the possibility that the 5-FU anticancer effect would be increased by gimeracil after application to the rat liver surface.

In our previous report, 5-FU was distributed evenly in the liver and eliminated rapidly from the liver after intravenous (i.v.) administration in rats.<sup>19,20)</sup> On the other hand, 5-FU was preferentially distributed at site 1, and its concentration was sustained after application to the rat liver surface.  $AUC_p$  after application to the rat liver surface was lower than that of i.v. administration. Furthermore, the concentration of 5-FU at site 1, site 2 and site 3 at a 0.1 molar ratio of gimeracil to 5-FU were 2.9, 3.3 and 3.6  $\mu\text{g/g}$  liver, respectively, at 180 min after its i.v. administration.  $AUC_p$  (643.3  $\mu\text{g} \times \text{min/mL}$  plasma) after i.v. administration was approximately twice higher than that of application to the rat liver surface. These results suggest that the 5-FU availability in the liver is enhanced with low exposure in the systemic circulation by combining gimeracil to 5-FU after application to the liver surface, compared to i.v. administration.

Although the present study suggests that administering 5-FU with gimeracil directly to the liver surface can be an effective treatment for patients with HCC, clinical use of the cylindrical diffusion cell that was used to administer the 5-FU mixture is not feasible. In this study, we used the cylindrical diffusion cell to limit the 5-FU application area in the presence or absence of gimeracil or uridine, to closely examine their effect on 5-FU absorption and hepatic disposition. Therefore, pharmaceutical formulations such as sheets or hydrogel formulations are required for clinical use. Our results suggested that gimeracil is a promising candidate to add to formulations containing 5-FU for application to the liver surface in clinical for treatment of patients with liver cancer. These results are expected to be beneficial for developing formulations for clinical application of 5-FU to the liver surface.

## CONCLUSION

We found that administration of 5-FU with gimeracil onto the rat liver surface could increase the availability of 5-FU in the liver. We showed that combining gimeracil and 5-FU at a 0.1 molar ratio could be the optimal ratio to apply to the rat liver surface.

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**Conflict of Interest** The authors declare no conflict of interest.

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