Colorectal cancer (CRC) is one of the leading causes of cancer-related morbidity and mortality in Western and well-developed countries. Several environmental factors, including lifestyle, food, and body mass, play important roles in the induction and progression of CRC. Generally, surgery is the first-line treatment for patients with early-stage CRC (stage I or II), and it has been reported to afford a positive prognosis. However, surgery is less effective in patients with more advanced CRC (stage III); furthermore, patients with involvement of the lymph nodes are at a 50% risk of relapse following resection. Therefore, adjuvant chemotherapy is often prescribed for patients with lymph node involvement in order to reduce the risk of recurrence.

5-Fluorouracil (5-FU), an analogue of the natural pyrimidine uracil (Ura), is an anti-cancer agent that is widely used in the management of patients with cancers of the gastrointestinal tract, breast, head, and neck. At present, 5-FU remains the single, most effective chemotherapeutic agent for the treatment of CRC. Some studies have shown a relationship between systemic plasma levels of 5-FU and treatment efficacy. Increased objective responses have been demonstrated when higher 5-FU area under the curve (AUC) values are maintained. However, the optimal method of using 5-FU remains debatable. Gamelin et al. conducted a randomized, phase III multicentre clinical trial involving 208 patients with metastatic CRC and reported wide pharmacokinetic (PK) variability and a large distribution of the optimal dose of 5-FU to achieve target 5-FU plasma levels. To achieve the prescribed target concentration levels, dose increase was required in 68% of the patients. The study investigators concluded that individual 5-FU dose adjustment on the basis of PK monitoring, not body surface area, is needed for an improved objective response rate, a higher survival rate, and fewer grade III/IV toxicities. In the field of oncology, researchers are focusing on the identification of predictive markers to improve the efficacy of 5-FU and decrease the likelihood of severe toxicity, which remain a challenge.

Dihydropyrimidine dehydrogenase (DPD) is the rate-limiting step in the catabolism of 5-FU, and approximately 90% of administered 5-FU is metabolized by DPD in the liver. The PK of 5-FU can be affected by changes in DPD activity in the liver, suggesting that the level of liver DPD activity is critical in determining 5-FU efficacy and toxicity. Thus, measuring the level of liver DPD before 5-FU treatment to enable the adjustment of an individual’s dose is a possible strategy to improve efficacy and reduce toxicity. However, clinically, it is not feasible to detect DPD activity levels directly in the liver before 5-FU treatment. Given that 5-FU and Ura are metabolized by the same pathway with DPD as the key, rate-limiting enzyme, the measurement of Ura and its product, dihydouracil (UH2), in plasma may provide an estimate of 5-FU degradation as well as DPD levels, and thereby 5-FU clearance prior to treatment. Moreover, considering the relationship between the systemic plasma levels of 5-FU and treatment efficacy, the UH2/Ura plasma ratio, measured prior to 5-FU treatment, may be an indirect, reliable, and convenient biomarker to assess not only DPD activity level and 5-FU clearance but also 5-FU treatment efficacy and tumor growth. Predicting the efficacy of 5-FU could be particularly helpful for determining individual 5-FU doses and would also be cost-effective, because extensive and expensive medical care is often required to manage metastatic carcinoma induced by insufficient anti-cancer agent treatment. To investigate this hypothesis, we determined the variations in the ratio of UH2/Ura and the PK parameters of 5-FU at different time points during the day in rats with 1,2-dimethylhydrazine-induced CRC. Using 5-FU for seven days. This study focused on the relationship between the ratio of UH2/Ura in plasma and tumor volume (TV) after 5-FU treatment, we conducted pharmacodynamic (PD) studies in CRC rats by using 5-FU for seven days. This study focused on the relationship between the ratio of UH2/Ura in plasma and 5-FU treatment efficacy, which has not been previously described. The measurement of the UH2/Ura ratio in plasma before 5-FU treatment would be beneficial, enabling an individual’s 5-FU dose to be adjusted and the clinical efficacy of 5-FU treatment to be improved while preventing toxicity.
MATERIALS AND METHODS

Materials 5-FU, Ura, dihydrouracil (UH2), bovine serum albumin (BSA), methylene blue, the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH), and ethylenediamine tetraacetic acid (EDTA) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). 5-Bromouracil (5-BU), which was used as an internal standard in the liquid chromatography/tandem mass spectrometry (LC-MS) analysis as described below, was obtained from Sigma-Aldrich Co. (Steinheim, Germany). DMH and aminoethylisothiouronium bromide were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Potassium phosphate, magnesium chloride, benzamidene, and sucrose were supplied by Nacalai Tesque Inc. (Kyoto, Japan). We obtained 2-mercaptoethanol from Kanto Chemical Co., Inc. (Tokyo, Japan). All rats were housed in a temperature- and humidity-controlled facility with a 12-h light/dark cycle for at least 5 d after arrival from Hamamatsu, Japan). All rats were housed in a temperature-controlled facility with a 12-h light/dark cycle for at least 5 d. The concentration of UH2 and Ura. The concentration of UH2 and Ura in plasma and the circadian variation of the UH2/Ura ratio in plasma and the activity of DPD in the liver of CRC rats were determined in the experiments one week after the last administration of DMH in order to eliminate the effect of DMH.

Animal Preparation All experimental protocols involving animals were performed in accordance with the Kyoto Pharmaceutical University Guidelines for Animal Experimentation. Male Wistar Hannover rats 10 weeks of age (weighing 315±17 g) were purchased from Nippon SLC Co., Ltd. (SLC, Hamamatsu, Japan). All rats were housed in a temperature-controlled facility with a 12-h light/dark cycle for at least 5 d before use. The animals were fed standard rodent chow. Free access to food and water was permitted prior to experimentation. DMH solutions were prepared immediately before use as described by Li and Li.16) DMH was dissolved in saline at a concentration of 10% w/v. CRC rats were subcutaneously administered DMH solution in the groin region at a dosage of 40 mg/kg, once a week for 10 weeks. CRC rats were used in the experiments one week after the last administration of DMH in order to eliminate the effect of DMH.

Plasma UH2/Ura Ratio and DPD Activity Assay The circadian variation of the UH2/Ura ratio in plasma and the activity of DPD in the liver of CRC rats were determined using the methods described by Jang et al. and Tateishi et al., respectively, with minor modifications.15,17) Briefly, CRC rats were anesthetized by intraperitoneal (i.p.) injection of 50 mg/kg sodium pentobarbital and euthanized by cervical dislocation. After cardiac puncture, blood was collected into heparinized centrifuge tubes and centrifuged at 2500 × g for 10 min at 25°C to separate the plasma for determining the concentration of UH2 and Ura. The concentration of UH2 and Ura in plasma was measured using LC-MS/MS as described below. After perfusion with phosphate-buffered saline (PBS) using an infusion pump, the liver was harvested, blotted using filter paper, and weighed for cytosol preparation. Cytosol was prepared using a previously described method.19) The livers were minced and homogenized in 35 mmol/L potassium phosphate (pH 7.4), 2.5 mmol/L magnesium chloride, 10 mmol/L 2-mercaptoethanol, 0.25 mol/L sucrose, 1.0 mmol/L benzamidene, 1.0 mmol/L aminoethylisothiouronium bromide, and 5.0 mmol/L EDTA (using three-fold volumes of each sample weight) using a glass homogenizer. The resulting homogenate was centrifuged at 9000×g for 20 min at 4°C. Subsequently, the supernatant was centrifuged at 10000×g for 60 min at 4°C. The supernatant was removed and used as the cytosolic fraction in the subsequent assay. The protein concentration of the cytosolic fraction was determined using the method of Lowry et al.20) Cytosolic incubation was carried out in a final volume of 1.0 mL with 35 mmol/L potassium phosphate (pH 7.4), 2.5 mmol/L magnesium chloride, 10 mmol/L 2-mercaptoethanol, 200 µmol/L NADPH, and 500 µg of cytosolic protein. Each sample was pre-incubated at 37°C for 20 min. The reaction was initiated by the addition of 5-FU solution at a final concentration of 40 µmol/L. After 20 min, the reaction was stopped by the addition of 500 µL of ice-cold methanol. DPD activity was determined by measuring the recovery of 5-FU.

5-FU PK Analysis in CRC Rats Prior to the 5-FU PK studies, CRC rats were fasted overnight with free access to water. After being anesthetized with 50 mg/kg (i.p.) sodium pentobarbital, rats were placed supine on a heating pad under a surgical lamp to maintain their body temperature during the experiment. All CRC rats were randomly allocated to 12 groups based on administration time and PD studies (Fig. 1). Each group was administered 20 mg/kg 5-FU solution (10 mg/mL in saline) intravenously (i.v.) at different times of the day (7, 13, or 19 h). The 5-FU solutions were administered to rats at 7 h (Groups 1, 4, 7, 10), 13 h (Groups 2, 5, 8, 11), or 19 h (Groups 3, 6, 9, 12) for one day (Groups 1–3), three days (Groups 4–6) or seven days (Groups 7–12). PK studies were conducted on Day 1 (Groups 1–3), Day 3 (Groups 4–6), or Day 7 (Groups 7–9). To evaluate the pre-therapeutic ratio of UH2/Ura plasma levels as a predictor of the PK parameters of 5-FU and tumor growth, 0.50-mL aliquots of blood samples from the external left jugular vein were collected into heparinized centrifuge tubes at a predetermined time before 5-FU administration. After the administration of 5-FU to the external right jugular vein, 0.25-mL aliquots of blood samples from the external left jugular vein were collected into heparinized centrifuge tubes at a predetermined time. The blood samples were centrifuged at 9000×g for 10 min to collect plasma. These samples were stored at −80°C until 5-FU analysis using LC-MS as described below.

5-FU PD Analysis in CRC Rats Aberrant crypt foci (ACF), which have been used for the early detection of factors that influence colorectal carcinogenesis in rats and can be induced by DMH, and TV were identified and measured using a previously reported method with some modifications.19,20) After the PK studies, PD experiments were performed on Days 3 (Groups 4–6) or Day 7 (Groups 7–9). In Groups 10–12, PD studies were conducted on Day 10 (Groups 10–12; Fig. 1). Moreover, saline, which was the solvent used in the 5-FU solutions, was administered to control CRC rats at 13 h for 7 d, and PD studies were performed on Days 3, 7, and 10. CRC rats were anesthetized using 50 mg/kg (i.p.) sodium pentobarbital. After cardiac puncture, buffered formalin phosphate (10%) was immediately injected into the colon by intubation into the anus using a 30-mL Terumo syringe® (Terumo Co., Tokyo, Japan) to ensure that the colon was distended and longitudinally. It was then immersed in 10% buffered formalin phosphate for 24 h. The fixed colon segments were stained in 0.2% methylene blue solution dissolved in distilled water for 10 min. ACF were examined at 100 magnification using a Nikon XF-PH-21 photomicroscope equipped with phase optics (Nikon Co., Tokyo, Japan). Aberrant crypts were distinguished...
from the surrounding normal crypts by their increased size, significantly increased distance from the laminae to the basal surface of the cells, and the easily discernible pericryptal zone. Crypt multiplicity was determined by the number of crypts in each focus, and was categorized as containing 1, 2, 3, 4, or more aberrant crypts/focus. Moreover, the dimensions of the tumors were also measured using a SKM-Z100A-PC microscope (Saitoh Kougaku Co., Yokohama, Japan). TV was determined using the following formula: 

\[ TV = \frac{(L \times W)}{2} \]

with the W and L variables representing the width and length of the tumors, respectively. 21) ACF and the tumor dimensions were recorded using a SKM-Z100A-PC microscope (Saitoh Kougaku Co.).

**PK Analysis** Standard PK parameters obtained from the plasma concentration–time curve of 5-FU were calculated using non-compartmental PK analysis with WinNonlin® Version 6.1 software (Pharsight Co., Mountain View, CA, U.S.A.). The terminal slope (\( \lambda_z \)) was determined by the linear regression of at least three data points from the terminal portion of the plasma concentration–time curve. The area under the plasma concentration–time curve from 0 to the last measured time point (\( AUC_{0-\text{last}} \)) was calculated using the linear trapezoidal rule up to the last measured plasma concentration (\( C_{p(\text{last})} \)). The area under the plasma concentration–time curve from 0 to infinity (\( AUC_{0-\infty} \)) was extrapolated to infinity using the correction term \( C_{p(\text{last})}/\lambda_z \). The elimination half-life (\( t_{1/2} \)) was calculated using the formula \( t_{1/2}=\ln 2/\lambda_z \). The area under the first moment curve from 0 to infinity (\( AUMC_{0-\infty} \)) was also calculated using the linear trapezoidal rule and the addition of the concentration term after the last measured point (\( t_{(\text{last})} \)) to infinity, namely, \( t_{(\text{last})}C_{p(\text{last})}/\lambda_z + C_{p(\text{last})}/\lambda_z^2 \). The mean residence time (MRT) was calculated using the formula \( \frac{AUMC}{AUC_{0-\infty}} \). Total plasma clearance (CL) was calculated using the formula \( D/AUC_{0-\infty} \), with D being the administered dose of 5-FU. The steady-state volume of distribution (\( V_{dss} \)) was calculated by multiplying CL by MRT.

**LC-MS/MS Assay for UH2 and Ura** The assays for UH2 and Ura in plasma were carried out according to a previously reported method22) with some modifications. The LC-MS/MS system consisted of an API 3200 triple quadrupole mass spectrometer equipped with a turbo ion spray sample inlet as an interface for electrospray ionization (ESI), an Analyst Workstation (Applied Biosystems, CA, U.S.A.), an LC-10AD micropump (Shimadzu Corp., Kyoto, Japan), and an AS8020 automatic sample injector (Tosoh, Tokyo, Japan). The mobile phase of acetonitrile–0.1% formic acid (20:80, v/v) was degassed and pumped through a Quicksorb ODS column (2.1 mm×150 mm, 5 \( \mu \)m; Chemco Scientific Co., Ltd., Osaka, Japan) at a flow rate of 0.2 mL/min and with the column temperature maintained at 25°C. The ionization was carried out via the turbo ion spray inlet in the positive ion mode. The flow rates of the nebulizer gas, curtain gas, and collision gas were set at 8.0, 2.0, and 4.0 l/min, respectively. The ion spray voltage and temperature were set at 5500 V and 500°C, respectively. The declustering potential, the entrance potential, the collision energy, and the collision cell exit potential were set at 36.0, 12.0, 27.0, and 4.0 V for UH2, and 46.0, 10.5, 21.0, and 4.0 V for Ura, respectively. Multiple reaction-monitoring analysis was performed with transition m/z 115.1→73.0 for UH2 and m/z 113.1→55.0 for Ura. Standard samples were prepared by adding aliquots of UH2 and Ura stock solutions to 3% BSA solution. Standard and unknown plasma
samples (200 μL) were added to 150 μL of 2% ZnSO₄ in 50% methanol and vortexed vigorously for 15 s. Next, 5.0 mL iso-propanol–ethyl acetate (15:85, v/v) was added, vortexed for 30 s, and centrifuged for 5 min at 12000×g. The organic layer was transferred to a glass tube and evaporated to dryness at 60°C under a stream of nitrogen. Prior to injection, 150 μL of the 15% methanol solution was added to the resulting residue and vortexed for 30 s. A 100-μL aliquot was injected into the LC-MS/MS system. The lower limits of detection and the limits of quantification for UH2 and Ura were less than 10 ng/mL from 200 μL of each matrix.

**LC-MS Assay for 5-FU** The assays for the analysis of 5-FU in plasma as well as the other samples were performed using LC-MS. The LC-MS system (Shimadzu Corp.) consisted of an SIL-10A system controller, an LC-10ADvp pump, an SPD-10A UV detector, an SIL-10ADvp automatic injector, a CTO-10A column oven, and an LC-MS-QP8000a mass spectrometer equipped with a CLASS-8000 workstation. Mass spectrometry was performed using the negative ion-atmospheric pressure chemical ionization (APCI) mode. The internal standard used was 5-BU. LC-MS analysis was performed using a Quicksorb ODS column (2.1 mm×150 mm, 5 μm; Chemco Scientific Co., Ltd.) and maintained at 60°C for all separations. The mobile phase consisted of 5.0 mmol/L ammonium formate–acetonitrile (10:90, v/v) at a flow rate of 0.2 mL/min. The m/z values observed for 5-FU and 5-BU were 129.0 and 189.0, respectively. Standard samples were prepared by adding aliquots of 5-FU stock solutions to a drug-free matrix. Standard and unknown samples were added to 100 μL of the internal standard (5-BU, 250 μg/mL in 50% methanol) and 150 μL of 2% ZnSO₄ in 50% methanol, and vortexed vigorously for 15 s. Diethyl ether (1.0 mL) was then added, vortexed for 30 s, and centrifuged for 5 min at 12000×g. The aqueous phase was frozen in a cold bath at −10°C. Next, the organic layer was transferred to HPLC sample vials and evaporated to dryness at 60°C. Prior to injection, 100 μL of the mobile phase was added to the resulting residue and vortexed for 30 s. A 60-μL aliquot was injected into the LC-MS system. The lower limits of detection and the limits of quantification for 5-FU were less than 0.05 μg/mL from 100 μL of each matrix.

**Statistical Analysis** All values are expressed as mean ± standard error (S.E.). Correlations between the ratio of UH2/Ura and DPD activity levels in the liver, 5-FU plasma CL, 5-FU plasma AUC₀⁻∞, or TV on Day 10 were assessed by simple linear regression analysis. Data were analysed using one-way analysis of variance (one-way ANOVA). The differences between the means were considered statistically significant when p<0.05.

**RESULTS**

**ACF and Tumors in the Colons of CRC Rats** Figure 2 shows a topographic view of the colons of control and CRC rats. All rats treated with DMH developed abnormal and hyperplastic crypts in the colon, whereas no abnormal or hyperplastic crypts were found in the colons of control rats.

**Circadian Variation of the Plasma UH2/Ura Ratio and Activity Levels of DPD in the Liver** Circadian variation was observed in the plasma UH2/Ura ratio and in liver DPD activity levels (Fig. 3). DPD activity levels in the liver were found to be highest at 13 h (1.77±0.12 nmol/min/mg protein) and lowest at 19 h (0.26±0.05 nmol/min/mg protein), with a difference that was approximately six-fold (Fig. 3A). The ratio of UH2/Ura in plasma was highest at 13 h (0.43±0.02) and lowest at 19 h (0.24±0.01), with a difference that was approximately two-fold (Fig. 3B). By plotting the plasma UH2/Ura ratio against liver DPD activity levels, a linear relationship was inferred, with r²=0.934 (Fig. 3C).

**5-FU PK Analysis in CRC Rats at Different Times of
Figure 4 shows the mean plasma 5-FU concentration–time curves after CRC rats were administered i.v. bolus injections at 7, 13, or 19 h for 1, 3, or 7 d. Table 1 summarizes the PK parameters of 5-FU observed for each group and the plasma ratio of UH2/Ura that was determined before the PK experiments. After i.v. bolus injection, circadian variation was observed in the PK parameters, and particularly in $t_{1/2}$, $CL$, and $AUC_{0–∞}$, on Day 1. After repeated 5-FU administration, no circadian variation was found in the PK parameters or in the plasma ratio of UH2/Ura, whereas decreases were observed in the plasma ratio of UH2/Ura and $CL$ on Day 7, and the $AUC_{0–∞}$ on Day 7 was increased compared with Day 1. Figure 5 illustrates the correlation between the plasma ratio of UH2/Ura that was determined before the PK experiments and the PK parameters. Linear correlations with the plasma ratio of UH2/Ura were demonstrated for $CL$ ($r^2=0.905$) and $AUC_{0–∞}$ ($r^2=0.843$).

**5-FU PD Analysis in CRC Rats at Different Times of Day and the Correlation between UH2/Ura and Tumor Volume** Figure 6 shows the time-courses of the number of ACF after CRC rats were administered either vehicle or 20 mg/kg 5-FU at 7, 13, or 19 h for seven days. After the first administration of 5-FU, the total number of ACF (ACF-1, ACF-2, ACF-3, and ACF-4$^+$) was decreased in CRC rats treated with 5-FU, whereas the number of ACF in control CRC rats was increased. The total number of ACF in the rats that were administered 5-FU at 13 h (high liver DPD activity-level rats) was slightly higher compared with the number observed in rats that received 5-FU at 7 or 19 h (low or intermediate DPD activity-level rats).

Figure 7A illustrates the tumor growth curves observed in CRC rats administered (i.v.) either vehicle or 20 mg/kg 5-FU at 7, 13, or 19 h for 7 d. After the first administration of 5-FU, TV was decreased in CRC rats treated with 5-FU, whereas TV was found to be increased in control CRC rats. Interestingly, significant differences in TV were observed between the groups on Day 10; TV was the highest ($1.56±0.40$ mm$^3$) in the rats that were administered 5-FU at 13 h (high liver DPD activity-level rats) and lowest ($0.57±0.21$ mm$^3$) in the rats that received 5-FU at 19 h (low DPD activity-level rats), with a difference of approximately three-fold (Fig. 7B). Furthermore, by plotting the TV on Day 10 against the ratio of UH2/Ura in plasma that was measured prior to 5-FU treatment, a linear relationship was inferred, with $r^2=0.988$ (Fig. 8).

**DISCUSSION**

The standard approach for determining 5-FU dosage has been to use body surface area, which is generally recommend-
ed according to the maximum dose tolerated that had been established in early phase clinical trials. Dosing based on body surface area is associated with a considerable variability in plasma 5-FU levels of approximately 100-fold, and this inter- and intra-patient PK variability is a major contributor to treatment failure. Therefore, screening individuals for sensitive biomarkers that are indicative of the efficacy of 5-FU before treatment would enable the selection of an optimal therapeutic scheme for each patient on a rational basis.

**DMH** is a widely used and highly specific carcinogen

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**Fig. 4.** Mean Plasma Concentration Profiles of 5-FU after i.v. Administration of 20mg/kg 5-FU to CRC Rats at Different Times of the Day for 1, 3 or 7 d

(A) Mean plasma concentration profiles of 5-FU after i.v. administration of 20mg/kg 5-FU at 7, 13, or 19h on Day 1. (B) Mean plasma concentration profiles of 5-FU after i.v. administration of 20mg/kg 5-FU at 7, 13, or 19h for 3 d on Day 3. (C) Mean plasma concentration profiles of 5-FU after i.v. administration of 20mg/kg 5-FU at 7, 13, or 19h for 7 d on Day 7. ○ 7h; △ 13h; □ 19h. Results are presented as the mean±S.e. of 4 rats.

**Fig. 5.** Linear Correlations with the Plasma UH2/Ura Ratio for CL (L/h/kg) (A) and AUC$_{0–\infty}$ (µg·h/mL) (B) after i.v. 20mg/kg 5-FU Administration

The plasma ratio of UH2/Ura was determined before the PK experiments. CL and AUC$_{0–\infty}$ obtained from the plasma concentration–time curve of 5-FU in Fig. 4 were calculated using non-compartmental PK analysis using a computer program, WinNonlin® Version 6.1 (Pharsight Co., Mountain View, CA, U.S.A.), n=36.

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**Table 1.** Biomarker That Was Determined before the PK Experiments and PK Parameters after i.v. Administration of 5-FU (20mg/kg) to CRC Rats at 7, 13, or 19h

<table>
<thead>
<tr>
<th>Day 1</th>
<th>Day 3</th>
<th>Day 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>7 h (Group 1)</td>
<td>13 h (Group 2)</td>
<td>19 h (Group 3)</td>
</tr>
<tr>
<td>13 h (Group 4)</td>
<td>19 h (Group 5)</td>
<td>19 h (Group 6)</td>
</tr>
<tr>
<td>7 h (Group 7)</td>
<td>13 h (Group 8)</td>
<td>19 h (Group 9)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Day 1</th>
<th>Day 3</th>
<th>Day 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>UH2/Ura</td>
<td>0.30±0.01</td>
<td>0.39±0.03**</td>
<td>0.25±0.01*††</td>
</tr>
<tr>
<td>PK parameters</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$t_{1/2}$ (h)</td>
<td>0.25±0.02</td>
<td>0.37±0.03*</td>
<td>0.56±0.04**††</td>
</tr>
<tr>
<td>CL (L/h/kg)</td>
<td>1.51±0.11</td>
<td>1.81±0.09*</td>
<td>1.42±0.09††</td>
</tr>
<tr>
<td>$V_d_{ss}$ (L/kg)</td>
<td>0.39±0.02</td>
<td>0.52±0.07</td>
<td>0.67±0.03**</td>
</tr>
<tr>
<td>AUC$_{0–\infty}$ (µg·h/mL)</td>
<td>13.5±1.1</td>
<td>11.2±0.6*</td>
<td>14.2±0.9††</td>
</tr>
</tbody>
</table>

**p<0.01, *p<0.05 statistically significant difference against rats treated at 7h on the same day. ††p<0.01, †p<0.05 statistically significant difference against rats treated at 13h on the same day. **p<0.01, *p<0.05 statistically significant difference against rats treated at the same time on Day 1. ††p<0.01, †p<0.05 statistically significant difference against rats treated at the same time on Day 3. Each value represents the mean±S.E. of 4 rats.**
for the induction of CRC in rats, which is a model that is known to closely parallel the disease in humans in terms of disease presentation and gross and microscopic pathology. 28) In fact, the DMH-induced CRC model is a well-established, well-received, and widely used model of experimental colon carcinogenesis; Rahman et al. investigated the distribution of colon-specific sodium alginate microspheres containing 5-FU in the gastrointestinal tract, and performed PK studies and measured TV in CRC rats. 29) Furthermore, DMH-induced CRC rats have been used to score intermediate biomarkers of colon carcinogenesis using ACF. 19,20) In this study, we injected rats subcutaneously with DMH and observed tumor growth and ACF in the colons of CRC rats (Fig. 2). Our observations confirmed the usefulness of the subcutaneous injection of DMH for inducing CRC in rats for the purposes of investigating a correlation between the ratio of UH2/Ura in plasma and PK parameters and TV after 5-FU treatment.

The plasma ratio of UH2/Ura and DPD activity levels in the liver were shown to display a significant circadian variation and a correlation was obtained between the two measures (Fig. 3) that was found to be similar to the correlation published in a previous report. 15) Previous studies have firmly established the existence of a circadian rhythm and a correlation between DPD activity in the rat liver and the ratio of UH2/Ura in rat plasma, although the underlying mechanisms are still unknown. 13,30) Jiang et al. reported that the levels of DPD in rat liver are highest at around noon and lowest at around midnight 15); the peak levels of DPD in rats occurred during rest time and during the troughs in active times. Our study confirmed that this circadian rhythm exists, and that the ratio of UH2/Ura in plasma is correlated with DPD activity levels in the liver. Boisdron-Celle et al. reported that measuring the UH2/Ura ratio appeared to be the single best method for determining DPD deficiency and activity level for clinical use. 31) These observations indicate that the UH2/Ura ratio reflects the circadian variation of DPD activity levels in the liver and that this ratio could serve as an indirect, reliable, and convenient biomarker for liver DPD activity levels in CRC rats. Moreover, in this study, the PK parameters of 5-FU were also shown to exhibit a circadian variation; the i.v. 5-FU bolus injection administered to CRC rats at different times of day yielded significant circadian variations in CL and AUC on Day 1, although no circadian variation was observed in the PK parameters after the repeated administration of 5-FU (Table 1). By plotting the CL and AUC against the ratio of UH2/Ura in plasma measured before the PK experiments, a linear relationship was inferred; a positive correlation was found between CL and the UH2/Ura ratio in plasma, whereas a negative correlation was observed between AUC and the ratio of UH2/Ura in plasma (Fig. 5). Previous reports have shown that a circadian rhythm exists in the concentrations of 5-FU after its infusion in clinics, 32) and the inverse relationship was observed between the circadian profile of 5-FU plasma concentration and DPD activity levels in these patients. 33) DPD is the rate-limiting step in the catabolism of 5-FU 13) and approximately 90% of the 5-FU administered is metabolized by DPD in the liver. 14) On the basis of these findings, the metabolism of 5-FU varies with the circadian variation of DPD activity
levels in the liver, and the plasma ratio of UH2/Ura is a useful predictor of CL and AUC$_{0-\infty}$ after the administration of 5-FU to CRC rats. The absence of circadian variation observed in PK parameters after repeated 5-FU administration in CRC rats raises the possibility that DPD activity may be inhibited by 5-FU administration, although we could not elucidate this from our study. Determining the ratio of UH2/Ura in plasma could be recommended before 5-FU-based treatment to identify colorectal cancers patients who are at risk because of altered 5-FU disposition.

After the first administration of 5-FU, the total number of ACF and TV were found to be decreased in CRC rats treated with 5-FU, whereas these values were increased in control CRC rats (Figs. 6, 7A). Interestingly, significant differences in TV between the groups were observed on Day 10; TV on Day 10 was the highest in rats with high DPD activity levels (1.56±0.40 mm$^3$) and lowest in rats with low DPD activity levels (0.57±0.21 mm$^3$), with a difference of approximately three-fold (Fig. 7B). Moreover, the total number of ACF on Day 3 in the rats with high liver DPD activity levels was slightly increased. The mechanism underlying the cytotoxicity of 5-FU has been ascribed to several factors, including the misincorporation of fluoronucleotides into RNA and DNA, and the inhibition of nucleotide synthetic enzyme thymidylate synthase; the mechanism of action depends on the concentration of 5-FU.$^{34}$ Furthermore, with respect to the sensitivity of tumor cells, circadian variation in DNA synthesis and cellular proliferation in the gastrointestinal mucosa have been documented in both animals and humans; DNA synthesis occurs rapidly during times of activity when the lowest DPD levels are observed.$^{35,36}$ These observations indicate that the fluctuations in 5-FU concentration induced by the variation in DPD activity levels and tumor cell sensitivity may affect the degree of TV decrease and the number of ACF; a time-modified administration of 5-FU, according to DPD activity levels during the day, could maximize the efficacy of 5-FU treatment. In addition, by plotting the TV on Day 10 against the ratio of UH2/Ura in plasma measured before treatment with 5-FU, a linear relationship was inferred, with $r^2 = 0.988$ (Fig. 8). These results suggest that the ratio of UH2/Ura in plasma, measured prior to 5-FU treatment, may be an indirect, reliable, and convenient biomarker to assess not only liver DPD activity levels and PK parameters of 5-FU, but also 5-FU treatment efficacy. Predicting the efficacy of 5-FU by using the plasma ratio of UH2/Ura would be particularly helpful for determining individual 5-FU doses and would be cost-effective for anti-cancer agent treatment.

Caution should be exercised in the extrapolation of DPD circadian variation from rats to humans. Rats are more active in the dark and less active in the light, whereas humans are the opposite; the peak DPD levels in both rats and humans were shown to occur during rest time and in the troughs during times of activity.$^{15,36}$ Therefore, when extrapolating DPD variation from rats to humans, differences in the life cycle of the two species must always be taken into consideration.

The findings of the present study suggest that the ratio of UH2/Ura in plasma, measured prior to 5-FU treatment, may serve as an indirect biomarker for assessment of DPD activity levels in the liver, 5-FU PK parameters, and 5-FU treatment efficacy. Several studies have investigated the correlation between the ratio of UH2/Ura and DPD activity levels or 5-FU treatment toxicity and have reported that the systemic...
measurement of DPD activity levels using the UH2/Ura ratio in plasma could optimize 5-FU-based chemotherapy and minimize life-threatening toxicity.\textsuperscript{15,37-40} Given the relationship between the systemic plasma levels of 5-FU and treatment efficacy and toxicity, these observations suggest that pre-therapeutic assessment of the UH2/Ura ratio in plasma would be useful for predicting 5-FU PK, thereby enabling the adjustment of an individual's 5-FU dose and improving the clinical efficacy of 5-FU treatment while preventing toxicity. However, at least two limitations of this study were identified. First, the DMH-induced CRC rat model does not represent the complexity of the human disease and does not replace studies carried out with patient material. However, it is a valuable tool for developing and evaluating a variety of novel cancer chemopreventive agents or emerging therapeutic strategies that are difficult to address in humans. Another limitation is that it is difficult to determine tumor growth curves in the same CRC rat because the measurement of colon TV requires a laparotomy. Therefore, to evaluate the net correlation between the ratio of UH2/Ura and 5-FU treatment efficacy, further investigation is warranted under clinically relevant conditions.

CONCLUSION

In conclusion, the present data have several potentially important implications. First, the ratio of UH2/Ura may be an indirect, reliable, and convenient biomarker in CRC rats. The metabolism of 5-FU varies with the circadian variation of DPD activity levels in the liver, and the ratio of UH2/Ura in plasma is a useful predictor of CL of DPD activity levels in the liver, and the ratio of UH2/Ura in plasma would be useful for predicting 5-FU PK, thereby enabling the adjustment of an individual's 5-FU dose and improving the clinical efficacy of 5-FU treatment while preventing toxicity. However, at least two limitations of this study were identified. First, the DMH-induced CRC rat model does not represent the complexity of the human disease and does not replace studies carried out with patient material. However, it is a valuable tool for developing and evaluating a variety of novel cancer chemopreventive agents or emerging therapeutic strategies that are difficult to address in humans. Another limitation is that it is difficult to determine tumor growth curves in the same CRC rat because the measurement of colon TV requires a laparotomy. Therefore, to evaluate the net correlation between the ratio of UH2/Ura and 5-FU treatment efficacy, further investigation is warranted under clinically relevant conditions.

Acknowledgements  This study was supported by a Grant-in-Aid for Scientific Research (C) (No. 24590223) from MEXT (Ministry of Education, Culture, Sports, Science and Technology) of Japan.

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June 2013
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