Effect of 5-Fluorouracil Treatment on SN-38 Absorption from Intestine in Rats

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5-Fluorouracil (5-FU)-based chemotherapies with irinotecan have been applied for the treatment of cancers, and a common dose-limiting toxicity is neutropenia and diarrhea. In this study, we investigated the effect of 5-FU treatment on expression levels of drug transporters for SN-38 transportation and SN-38 absorption from the intestine following 5-FU treatment. Expression levels of several drug transporters and nuclear receptors in rats after 5-FU treatment were evaluated. SN-38 absorption from the intestine was evaluated by SN-38 concentration levels in serum following SN-38 injection into the intestine of 5-FU treated rats. The levels of renal multidrug resistance protein 2 (Mrp2) on day 4 after treatment (400 mg/kg) showed significant upregulation, 359.2±33.2% (mean±S.E.) of control. Mrp2 levels in the intestine were downregulated to 26.2±8.4% of control. 5-FU treatment (400 mg/kg) also significantly downregurated expression levels of P-glycoprotein (P-gp) and breast cancer resistance protein (Bcrp) to 41.2±14.7%, 15.7±4.3% of control, respectively. To evaluate SN-38 absorption from the intestine, SN-38 was loaded in to the intestine on day 4 after 5-FU treatment. Pretreatment with 5-FU significantly increased SN-38 concentration in the blood 30, 60 and 90 min after SN-38 administration. The area under the curve for SN-38 in the 5-FU group was significantly higher than in vehicle groups. 5-FU treatment decreased expression levels of P-glycoprotein and Bcrp in intestine. The present study suggests that combination chemotherapy of 5-FU with irinotecan (CPT-11) may elevate SN-38 absorption from intestine.

Key words 5-fluorouracil; SN-38; irinotecan; absorption; neutropenia; diarrhea

The pyrimidine analog 5-fluorouracil (5-FU) is an essential drug for metastatic carcinomas of gastrointestinal, breast, ovary, head and neck tumors.[1—4] 5-FU-based combination chemotherapies have been applied for the treatment of tumors[2,5,6]; for example, the FOLFIRI (5-FU, leucovorin and irinotecan) regimen has been a standard chemotherapy for colorectal cancer.[7] Randomized trials have demonstrated improvements in progression-free and overall survival when irinotecan (CPT-11) has been added to fluorouracil and leucovorin in the initial treatment of patients with metastatic colorectal cancer.[6] The most commonly observed toxicities associated with irinotecan are neutropenia, diarrhea, myelosuppression, and alopecia.[7—9]

Irinotecan is a derivative of the natural alkaloid camptothecin which is converted to an active metabolite, SN-38. SN-38 is metabolized predominantly in the liver, where it is inactivated by glucuronidation and excreted through the biliary system. It has been reported that carboxylate forms of SN38-Glu and SN-38 are transported by multidrug resistance protein 2 (Mrp2), suggesting that variability in the uptake of SN-38 and SN38-Glu may imply that interindividual variability in biliary excretion of the metabolites contributes to interpatient variability in CPT-11 toxicity.[10]

The family of ATP binding cassette (ABC) transporters, for example, p-glycoprotein (P-gp), Mrp2 and breast cancer resistance protein (Bcrp), has important roles in the detoxification and excretion of xenobiotics.[11,12] An Mrp isoform, Mrp2, has functions in the terminal excretion of cytotoxic and carcinogenic substances, and plays an important role in detoxification and chemoprevention.[11—14] Mrp2 is important clinically as it modulates the pharmacokinetics of many drugs, and its expression and activity are also altered by certain drugs and disease states.[14] Alterations in Mrp2 expression and/or function could have a variety of clinically important effects. Altered Mrp2 function can change the clearance of many clinically important drugs.[13]

There is accumulated evidence that medical drugs, toxins and health care supplements affect the expression levels of Mrp2[14—16]; however, the effect of 5-FU on the expression of Mrp2 is not clear. Here we report the effect of 5-FU treatment on the expression of Mrp2 in rats. We also investigated the effect of 5-FU treatment on mRNA expression of nuclear receptors: constitutive androstane receptor (CAR), pregnane X receptor (PXR), farnesoid X receptor (FXR), and retinoid X receptor (RXR). The phosphorylation levels of c-jun in the kidney and liver were evaluated. It is known that the promoter region of Mrp2 contains an AP1 recognition site, which is bound with the heterodimer of c-jun and fos.[17—19]

MATERIALS AND METHODS

Reagents A mouse monoclonal antibody specific for anti-multidrug resistance-related protein cMOAT/MRP2 (M_2III-6), anti-glyceraldehyde-3-phosphate dehydrogenase (6C5), anti-β actin (AC-74), anti-P-glycoprotein (C219), and anti-breast cancer resistance protein (bxp-21) was purchased from SANBIO (AM Uden, the Netherlands), Sigma-Aldrich (St. Louis, U.S.A.), Ambion (Austin, U.S.A.), Calbiochem

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(San Diego, U.S.A.) and Kamiya Biomedical (Seattle, U.S.A.), respectively. A rabbit polyclonal antibody (3502-100) specific for anti-phosphorylated c-jun was purchased from BioVision (Mountain View, U.S.A.). Anti-rabbit and anti-mouse immunoglobulin G (IgG) antibodies conjugated with horseradish peroxidase (HRP) as a second antibody were purchased from Nakalai Tesque (Kyoto, Japan). The ECL Western Blotting detection system and Hyperfilm ECL were purchased from Amersham Pharmacia Biotech. Protease inhibitor cocktail tablets (Complete®, ethylenediaminetetraacetic acid (EDTA) free) were purchased from Roche Diagnostics GmbH (Buckinghamshire, U.K.). TaKaRa reverse transcriptase-polymerase chain reaction (RT-PCR) kit AMV Ver. Three and RNA*later*™ were purchased from TaKaRa Biomedicals (Otsu, Japan). TRIzol® Reagent was purchased from Invitrogen (Carlsbad, CA, U.S.A.). Alanine aminotransferase (ALT), aspartate aminotransferase (AST) and creatinine measure kits were purchased from Kainos (Tokyo, Japan). Dulbecco's modified Eagle's medium (DMEM) was purchased from Nissui Seiyaku (Tokyo, Japan). The birilubin measurement kit and all other reagents were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Animals and Treatment Male Wistar rats (body weight; 216±2 g, mean±S.E.; Kyudo, Kumamoto, Japan) were housed under standard conditions (21±2 °C, ventilated rooms, 12 h light/dark cycle). The animals were fed rat chow and allowed free access to water over the experimental period. 5-FU was added to distilled water and then the suspension was titrated with 10 N NaOH until 5-FU dissolved. Rats were treated intraperitoneally (i.p.) with the 5-FU solution (40 mg/ml) at doses of 50, 100, 200 and 400 mg/kg. Vehicle group was treated with saline. Co-treatment with *N*-acetyl-L-cystein was administered per oral at a dose of 1 g/kg (Demeule *et al.*, 2004). *N*-acetyl-L-cystein (nAC) solution was neutralized with sodium hydroxide. At each experimental time point, rats were anesthetized with diethyl ether, and whole blood was collected from the heart. The rats were killed by exsanguinations after blood collection. Target tissues were promptly removed and stored at −30 °C until Western blot analysis. For RT-PCR analysis, the tissues were stored in RNA stabilization solution, RNA*later*™ at 4 °C overnight, and the tissues were stored at −30 °C until RT-PCR analysis. Serum prepared from each rat was stored at 4 °C until analysis. Hemolysate samples were excluded from analysis samples.

Male Wister rats (250—300 g) were treated with 5-FU at a dose of 400 mg/kg, and on day 4, SN-38 was injected into the intestine at a dose of 10 mg/kg. SN-38 was suspended in Polysorbate 80 and Macrogol 400 solution, and then the SN-38 suspension was diluted with saline (SN-38, 10 mg; Polysorbate 80, 0.1 ml; Macrogol 400, 0.9 ml; saline, 9 ml). An indwelling catheter was placed in the subclavian artery under pentobarbital anesthesia before SN-38 treatment. The rats were anesthetized with pentobarbital during blood collection. Animal experiments were performed in accordance with the criteria of Kagoshima University for the use and care of experimental animals (approval No. H19-11-28).

Histological Examination Male Wister rats (250—300 g) were treated with 5-FU at a dose of 400 mg/kg intraperitoneally. Rats were anesthetized with diethyl ether, and the target tissues were removed on day 4. The intestine, liver and kidney were fixed with 10% formaldehyde neutral buffer solution. Five micrometer paraffin sections were cut on a microtome and stained with hematoxylin and eosin for conventional histological examination.

Protein Sample Preparation Protein samples for Western blotting detection, crude plasma membranes, were prepared from rat kidney, liver and intestine. Tissues obtained from individual animals in each group were cryopreserved and homogenized in buffer containing 250 mM sucrose and 5 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES)-tris(hydroxymethyl)-aminomethane (Tris, pH 7.4) with an ultrasonic homogenizer. Whole cell homogenates were centrifuged at 3000×***g*** for 10 min. The supernatants were then centrifuged at 13500×***g*** for 30 min, and pellets containing the crude plasma membranes were resuspended in 10 mM Tris–HCl, pH 7.4, including 250 mM sucrose. For phosphorylated c-jun (p-c-jun) and glyceraldehyde-3-phosphate dehydrogenase (gapdh) detection, the tissues were also homogenized in a buffer containing 20 mM Tris, 5 mM EDTA and 10% sucrose (pH 7.4) with an ultrasonic homogenizer. Protease inhibitor tablets were dissolved in the above-mentioned homogenizing buffer at appropriate concentrations.

Electrophoresis and Western Blotting Protein samples were each mixed with an equal volume of sodium dodecyl sulfate (SDS) sample buffer containing 125 mM Tris–HCl (pH 6.8), 4% SDS, 20% glycerol and 0.005% bromophenol blue. For Mrp2 P-gp and Bcrp detection, electrophoresis on SDS polyacrylamide gels (SDS-PAGE) was performed without sample heat treatment.[16] Protein samples for Mrp2 detection (Liver: 20 μg whole cell homogenate; kidney and intestine: 20 μg crude plasma membranes) were separated by 8% SDS-PAGE. Protein samples for P-gp and Bcrp detection (intestine, 20 μg of whole cell homogenate) were separated by 8% SDS-PAGE. Protein samples for phosphorylated c-jun (p-c-jun), gapdh and β-actin detection (p-c-jun: 25 μg whole cell homogenate; gapdh and β-actin: 10 μg whole cell homogenate) were separated by 12% SDS-PAGE. After SDS-PAGE separation, proteins were transferred to a nitrocellulose membrane. After transfer, the membranes were incubated with 3% skim milk in buffer A (0.35 M NaCl, 10 mM Tris–HCl, pH 8.0, 0.05% Tween 20) for 1 h at room temperature for blocking. The membranes were then incubated with monoclonal antibody either M_2III-6 (400-fold dilution), 6C5 (100000-fold dilution), C219 (1000-fold dilution), bxp-21 (500-fold dilution) or AC-74 (10000-fold dilution) and with polyclonal antibody anti-phosphorylated c-jun (250-fold dilution) in the same blocking solutions. After washing three times with buffer A, the membranes were further incubated with 1000-fold diluted secondary antibody conjugated with HRP. Finally, membranes were rinsed once for 15 min and four more times for 5 min with buffer A. The ECL Western blotting detection system was used for chemiluminescence detection, except for phosphorylated c-jun detection. For phosphorylated c-jun detection, the ECL Advance Western blotting detection system was used.

RNA Isolation and RT-PCR Total RNA was isolated from tissues using TRIzol® Reagent according to the manufacturer's instructions. Single-stranded cDNA was synthesized by reverse-transcriptase (RT) using TaKaRa RNA PCR kit (AMV) ver. 3.0. RT was performed for 10 min at 30 °C

and for 30 min at 42 °C, and the samples were subsequently heated for 5 min at 95 °C to terminate the RT reaction. The subsequent PCR was performed using a PC-801 (ASTEC, Fukuoka, Japan) machine. Briefly, the synthesized cDNA was first incubated at 95 °C for 5 min to denature the primers and cDNA, and the subsequent PCR was performed as in the previous report.[16] For every PCR reaction, gapdh as an internal control and sterile water as a negative control were used.[16] Ten microliters of PCR product was loaded on to a 2% agarose gel and stained with ethidium bromide to visualize the amount of the amplified gene.

Data Analysis Protein concentration was determined by the DC protein assay (Bio-Rad). Optical densities (ODs) were measured by the public domain program Image J (available on the Internet at http://rsb.info.nih.gov/ij/). Individual images were scanned as TIFF images with a GT-9700F color scanner (Seiko Epson). Overall differences among treatments were evaluated by one-way analysis of variance (ANOVA). Statistical significant was performed by William's test to compare with the control group. Statistics analyses of two paired samples were performed by the two-tailed Student's *t*-test. The area under the blood concentration–time curve ($AUC_{0—120}$) was calculated by the liner a trapezoidal approximation method.

SN-38 Assay The total plasma concentration of SN-38 was determined by high-performance liquid chromatography (HPLC) with fluorescence detection using a previously reported modified method.[20] In brief, 20 μl plasma samples were deproteined by treatment with 20 μl methanol. The samples were mixed and spun at 10000 ***g*** for 5 min, and 30 μl supernatant was mixed with 30 μl mobile phase solution. The mixture was centrifuged at 10000 ***g*** for 5 min. Ten microliters of the clear supernatant was injected in to the HPLC System, an LC-10AS HPLC pump, RF-550 spectrofluorometric detector, and C-RSA recorder (Shimadzu Corp., Kyoto, Japan). Each sample was measured 4 times and the average value of the raw data was adopted. The standard curve of the SN-38 concentration was determined with SN-38 standard solution at concentrations of 0.002, 0.02, 0.2 and 1 ng/ml. Column temperature was maintained at 40 °C in a CTO-6A column oven (Shimadzu Corp., Kyoto, Japan) using a reverse-phase column (Mightysil RP-18 GP250-4.6, 250×4.6 mm, i.d. 5 μm; Kanto Chemical Co., Ltd., Japan) by isocratic elution with 50 mmol/l potassium dihydrogen phosphate, 25% acetonitrile with 0.1% *N*,*N*,*N*′,*N*′-tetramethylethylenediamine at 3.5 pH. The mobile phase was delivered at 1 ml/min and the column effluent was monitored at excitation and emission wavelengths of 380 and 532 nm, respectively.

Cell Culture and RT-PCR The human embryonic kidney cell line HEK-293T and the human hepatoma cell line HEP-G2 were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM glutamine and 100 units/ml penicillin at 37 °C in a 5% CO_2 humidified atmosphere. Cell cultures were maintained at exponential growth by replacing the media every 2—3 d. Culture medium was replaced with fresh medium the day before the experiment. HEK-293T and HEP-G2 cells were incubated for 72 h at concentrations of 5 μmol/ml and 500 μmol/ml, respectively. The concentrations of 5-FU for incubation were IC_{50} of the cells, which were determined with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.[21]

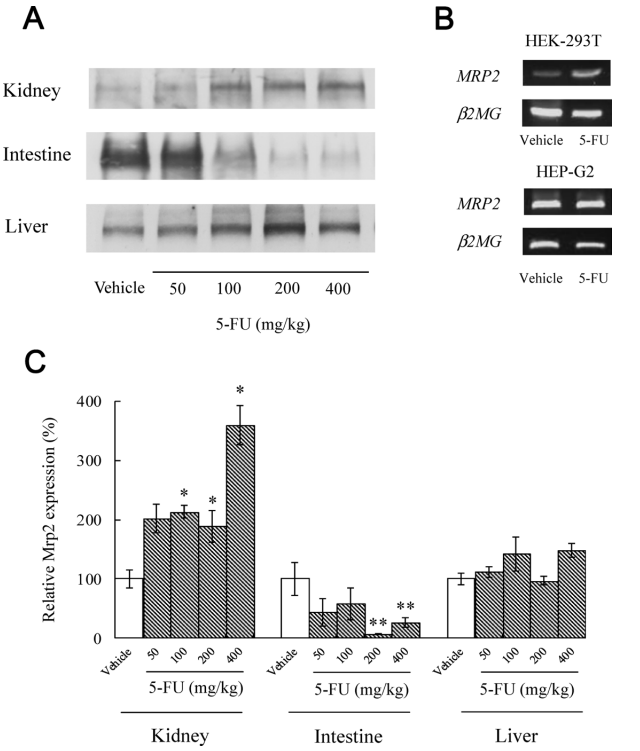

Fig. 1. Effect of 5-FU Treatment on Expression Levels of Mrp2

(A) Rats were intraperitoneally single injected with saline (vehicle) as a control, or 5-FU at a dose of 50, 100, 200 and 400 mg/kg. Tissues were obtained on day 4 after treatment. Mrp2 was detected as 190 kDa proteins by Western blotting. (B) Effect of 5-FU treatment on *MRP2* mRNA expression in HEK293T and HEP-G2 cells. The number of cycles for amplification was determined to be halfway through the exponentially amplifying phase. As an internal standard, β2-myoglobin was used. (C) Relative expression levels of Mrp2 in the kidney, intestine and liver after single injection of 5-FU. Rats were killed on day 4 after 5-FU treatment. Values are the mean±standard error (S.E.) as percentage of normalized ODs, compared with vehicles, $n=6$. Asterisk indicates significance from vehicle group (* $p<0.05$, ** $p<0.01$). Vehicle group was treated with saline (10 ml/kg, i.p.) only.

The reaction was repeated for 22 to 34 cycles, depending on the samples, for 30 s with denaturing at 94 °C, 30 s of annealing at 55 °C and 90 s of primer extension at 72 °C. This was determined to be halfway through the exponentially amplifying phase. The annealing temperature and PCR cycling number depended on the target gene being amplified. For example, 55 °C annealing temperature and 26 cycles for β2-microglobulin (β2MG); 60 °C and 34 cycles for MRP2 of HEK-293T and HEP-G2 cells. The sequences of forward and reverse primers and amplified target genes information are as follows: β2MG forward 5′-CCAGCAGAGAATGGAAAGTC-3′, reverse 5′-CATGTCTCGATCCCACTTAAC-3′; MRP2 forward 5′-ACAGAGGCTGGTGGCAACC-3′, reverse 5′-ACCATTACCTTGTCACTGTCCATGA-3′.[22]

RESULTS

Effect of 5-FU Treatment on Mrp2 Expression Typical immunoblot analysis is shown in Fig. 1A. The Mrp2 protein was detected as a 190-kDa band. Expression levels of *MRP2* mRNA with 5-FU treatment were evaluated in human cancer cells, HEK-293T and HEP-G2. semi-quantitative RT-PCR was performed with specific primers *MRP2* and *β2-microglobulin* (*β2MG*) as housekeeping genes.[22] 5-FU treatment induced *MRP2* mRNA in HEK-293T cells, but not in HEP-G2 cells (Fig. 1B). Mrp2 expression levels were de-

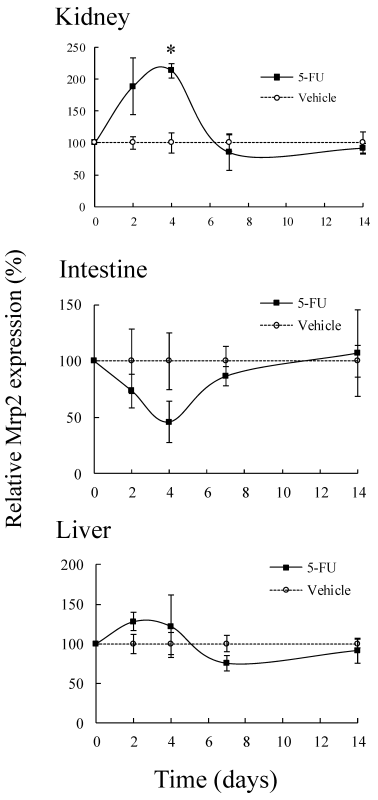

Fig. 2. Temporal Changes in Mrp2 Expression Following 5-FU Treatment

Rats were intraperitoneally single injected with 5-FU (100 mg/kg). Values are the mean±standard error (S.E.) as percentage of normalized ODs, compared with vehicles, $n=6$. Asterisk indicates significance from vehicle group ($*p<0.05$).

scribed as a percentage of the corresponding vehicle- treated group. The Mrp2 expression level in the kidney was increased (Fig. 1C). The expression levels of Mrp2 in the kidney after 5-FU treatment showed significant up-regulation 201.7±24.5% (50 mg/kg), 213.6±11.1% (100 mg/kg), 189.5±26.8% (200 mg/kg), 359.2±33.2% (400 mg/kg, mean±S.E.) of the control. On the other hand, the Mrp2 expression level in the intestine was decreased 43.9±23.2% (50 mg/kg), 57.9±26.2% (100 mg/kg), 7.1±0.7% (200 mg/kg), 26.2±8.4% (400 mg/kg, mean±S.E.) of the control in a dose-dependent manner (Fig. 1C). The alteration of Mrp2 expression levels in the kidney and intestine peaked 4 d after a single injection of 5-FU at a dose of 100 mg/kg (Fig. 2). No significant changes on the expression level of Mrp2 in the liver were observed in the same condition (Fig. 1C). Alteration of Mrp2 expression levels peaked on day 4 after single 5-FU treatment (100 mg/kg), and the expression levels recovered on day 7 after 5-FU treatment (Fig. 2).

Effects of 5-FU Treatment on the Parameters of Blood and Body Weight Blood parameters were measured to evaluate 5-FU toxicity. The serum concentrations of each aspartate aminotransferase (AST), alanine aminotransferase (ALT), total protein (TP) and creatinine (Cr) were measured. Blood parameters (RBC, WBC, AST, ALT, TP and Cr) on day 4 after single 5-FU treatment (50, 100, 200, 400 mg/kg) did not show significant change compared with the vehicle group (Table 1). By day 4 after 5-FU treatment, the 5-FU treatments had significantly decreased body weight. 5-FU administration produced anorexia and diarrhea, so the rats lost body weight, which transiently decreased after a single 5-FU treatment (100 mg/kg), and recovered on day 7 and 14 after 5-FU treatment. Blood parameters (RBC, WBC, AST, ALT,

Table 1. The Biochemical Parameters of Serum and Body Weight on Day 4 after 5-FU (Single Injection, i.p.) Treatment in Rats

	Vehicle	50 mg/kg	100 mg/kg	200 mg/kg	400 mg/kg
Body weight (g)	243.2±2.1	219.2±4.2**	226.0±6.5*	200.5±5.7**	194.3±5.6**
RBC ($\times10^4/\mu$l)	1194.5±104.6	1557.7±64.5	1253.2±122.2	1581.9±77.2	1319.7±104.3
WBC ($\times10^4/\mu$l)	1287.9±67.2	1403.5±41.2	1291.1±57.5	1216.8±38.4	1166.9±36.0
TP (g/dl)	7.45±0.20	7.46±0.59	7.40±0.33	6.83±0.40	6.12±0.30
ALT (IU/l)	103.6±8.0	91.7±9.7	97.1±10.0	79.4±6.7	83.9±5.5
AST (IU/l)	43.2±2.1	37.7±6.0	43.1±1.9	26.6±3.7	23.6±2.0
Cr (mg/dl)	0.34±0.03	0.40±0.03	0.44±0.05	0.46±0.02	0.43±0.03

AST: aspartate aminotransferase, ALT: alanine aminotransferase. Vehicle group was single injected saline (1 ml/kg, i.p.). Data represent mean±S.E.M. ($n=4$—6). Asterisk indicates significance from vehicle group ($**p<0.01$; $*p<0.05$). Overall differences among treatments were evaluated by one-way analysis of variance (ANOVA), with differences between groups evaluated using William's test.

Table 2. The Biochemical Parameters of Serum and Body Weight on Day 2, 4, 7 and 14 after 5-FU (100 mg/kg, Single Injection, i.p. 5-FU) Treatment in Rats

	Day 2		Day 4		Day 7		Day 14	
	Vehicle	5-FU	Vehicle	5-FU	Vehicle	5-FU	Vehicle	5-FU
Body weight (g)	210.8±17.2	212.0±10.8	241.8±5.1	226.0±18.2	259.0±6.4	241.3±6.9	323.3±11.9	314±4.5
RBC ($\times10^4/\mu$l)	1390.5±458.8	682.3±90.9	966.5±302.1	941.3±315.6	1076.0±535.5	1443.5±126.2	1252.3±329.9	1464.5±327.0
WBC ($\times10^4/\mu$l)	1253.0±353.6	1692.3±209.6	1411.0±139.7	1410.5±224.0	1336.8±153.3	1472.0±74.0	1192.5±53.1	1172.0±114.1
TP (g/dl)	5.68±0.69	6.44±0.08	7.45±0.40	8.30±1.06	6.16±0.84	8.10±0.99	7.91±1.47	8.15±0.58
ALT (IU/l)	86.9±28.4	81.4±13.8	103.6±16.1	97.1±20.0	81.0±11.6	77.9±11.8	113.7±20.1	103.7±19.5
AST (IU/l)	14.4±6.6	17.8±5.9	43.2±4.1	43.1±3.8	24.9±0.7	33.0±6.6	30.7±7.9	30.5±3.6
Cr (mg/dl)	0.54±0.01	0.47±0.10	0.34±0.06	0.44±0.11	0.46±0.04	0.45±0.07	0.42±0.06	0.45±0.02

RBC: Red blood cells, WBC: White blood cells, TP: Total protein of serum, ALT: alanine aminotransferase, AST: aspartate aminotransferase, Cr: creatinine. Rats were injected 5-FU intraperitoneally. Date represent means±S.D. ($n=4$—6). Differences between individual treatments were evaluated using the Student's paired t-test.

TP and Cr) after single 5-FU treatment at a dose of 100 mg/kg did not show significant change compared with the vehicle group (Table 2). Four consecutive days of 5-FU treatment (50 mg/kg, i.p.) significantly decreased body weight, WBC, TP, ALT, direct bilirubin and total bilirubin, but not RBC, AST and creatinine levels (Table 3).

Effects of 5-FU Treatment on mRNA Expression of Mrp2 and Nuclear Receptors Expression levels of *Mrp2*, *CAR*, *PXR*, *RXRα* and *FXR* mRNA were investigated to reveal the mechanism of Mrp2 expression with 5-FU treatment. Semi-quantitative RT-PCR was performed with specific primers for *gapdh*, *Mrp2*, *CAR*, *PXR*, *RXRα* and *FXR* to analyze the expression levels of each gene in the liver, intestine and kidney (Table 2). The expression level of *Mrp2* in the kidney was significantly increased to 1.66±0.24-fold of control. Pre-treatment with antioxidant *N*-acetyl-L-cysteine (nAC) did not affect the up-regulation of *Mrp2* in the kidney (Table 4). It was reported that reactive oxygen species (ROS) mediate the up-regulation of Mrp2.[23] The expression levels of *Mrp2* in the intestine and liver and the nuclear receptors in all examined tissues showed no significant difference (Table 4).

Effects of Consecutive Treatment with 5-FU on the Mrp2 Expression and Phosphorylation of c-Jun Four consecutive days of 5-FU treatment (50 mg/kg, i.p.) up-regulated expression levels of Mrp2 in the kidney to 312.2±15.7% (mean±S.E.) of control. On the other hand, 5-FU treatment down-regulated expression levels of Mrp2 in the intestine to 38.8±9.6% (mean±S.E.) of control (Fig. 3A). To investigate how 5-FU treatment increased expression levels of renal Mrp2, we evaluated the phosphorylation levels of c-jun. Rats were injected for 4 consecutive days with saline (vehicle) as a control, or 5-FU (50 mg/kg, i.p.). Rats were killed the day after 5-FU treatment. Phosphorylated c-jun (p-c-jun) was detected as 33 kDa protein (Fig. 3B). Renal p-c-jun was detected following consecutive 5-FU treatment, but not after vehicle treatment. 5-FU treatment did not alter the phosphorylation levels of hepatic p-c-jun (vehicle, 100.0±32.0%; 5-FU, 111.2±21.3%, mean±S.E. optical densities of Mrp2/gapdh). On the other hand, p-c-jun could not

Table 3. Biochemical Parameters of Serum and Body Weight after Constitutive 5-FU (50 mg/kg, 4 d, i.p.) Treatment in Rats

	Vehicle	50 mg, 4 d
Body weight (g)	224.0±2.6	159.1±4.1**
RBC ($\times 10^4/\mu$l)	1341.0±104.8	1656.6±55.7*
WBC ($\times 10^4/\mu$l)	1353.3±31.4	1149.6±49.8**
TP (g/dl)	8.45±0.62	6.37±0.29*
ALT (IU/l)	100.6±6.5	81.7±4.1*
AST (IU/l)	23.1±1.8	27.8±2.1
Total bilirubin (mg/dl)	0.55±0.13	0.18±0.05*
Direct reacting bilirubin (mg/dl)	0.50±0.10	0.17±0.04*
Creatinine (mg/dl)	0.35±0.04	0.37±0.02

AST: aspartate aminotransferase, ALT: alanine aminotransferase, RBC: red blood cells, WBC: white blood cells. Vehicle group was single injected with saline (1 ml/kg, i.p.). Data are the mean±S.E. (n=4—6). Asterisk indicates significance from vehicle group (** $p<0.01$; * $p<0.05$). Difference between individual treatments was evaluated using Student's t-test.

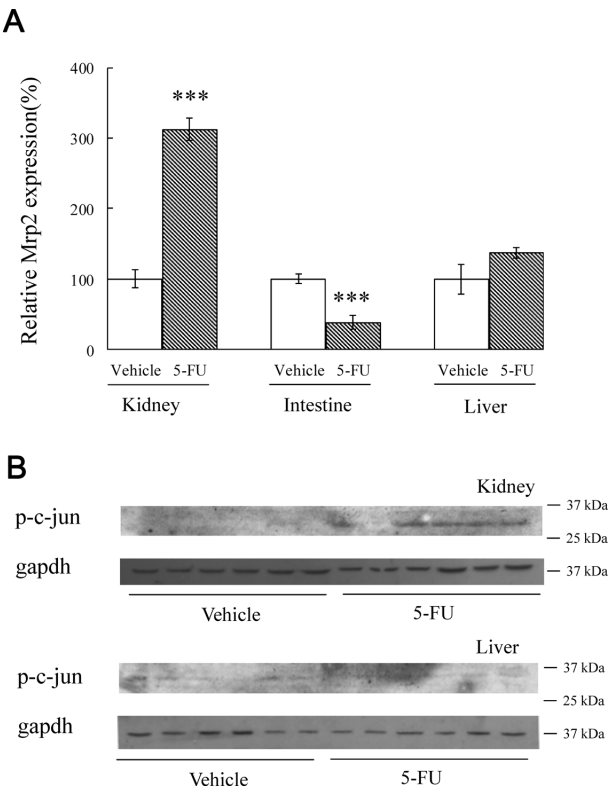

Fig. 3. Effect of 4 Consecutive Days of 5-FU Treatment on Expression Levels of Mrp2 and c-Jun Phosphorylation

Rats were intraperitoneally single injected with 5-FU (50 mg/kg) for 4 consecutive days. (A) Values are the mean±standard error (S.E.) as percentage of normalized ODs, compared with vehicles, n=6. Asterisk indicates significance from vehicle group (*** $p<0.001$). (B) Western blotting analysis for phosphorylation levels of c-Jun after 4 consecutive days of 5-FU treatment. Protein samples for phosphorylated c-jun (p-c-jun) and gapdh detection (p-c-jun; 25 μg whole cell homogenate, gapdh; 10 μg whole cell homogenate) were separated by 12% SDS-PAGE. The polyclonal antibody detects only activated c-Jun when phosphorylated at ser73. Renal p-c-jun was detected following consecutive 5-FU treatment, but not after vehicle treatment. 5-FU treatment showed no change in phosphorylation levels of hepatic p-c-jun (vehicle, 100.0±32.0%; 5-FU, 111.2±21.3%, mean±S.E. optical densities of Mrp2/gapdh).

Table 4. Relative Ratio of Mrp2 and Nuclear Receptors mRNA Expression after 5-FU Treatment

	Kidney			Intestine			Liver		
	Vehicle	5-FU	nAC+5-FU	Vehicle	5-FU	nAC+5-FU	Vehicle	5-FU	nAC+5-FU
Mrp2	1.00±0.10	1.66±0.24*	1.81±0.29*	1.00±0.42	0.82±0.18	1.03±0.47	1.00±0.12	0.62±0.10	0.57±0.41
CAR	1.00±0.10	1.28±0.16	1.06±0.15	1.00±0.35	0.86±0.30	0.94±0.33	1.00±0.12	0.70±0.15	0.56±0.31
PXR	1.00±0.15	1.29±0.15	1.34±0.22	1.00±0.21	0.52±0.19	1.10±0.29	1.00±0.15	0.61±0.11	0.64±0.32
RXRα	1.00±0.08	0.91±0.15	0.88±0.20	1.00±0.39	0.58±0.14	1.01±0.39	1.00±0.19	1.16±0.13	2.12±1.03
FXR	1.00±0.08	0.92±0.11	1.11±0.13	1.00±0.24	0.55±0.27	1.33±0.58	1.00±0.11	0.86±0.10	0.68±0.19

Rats were treated with 5-FU at a dose of 400 mg/kg (i.p., single injection) and were killed 16 h after 5-FU injection. Renal *Mrp2* mRNA expression level was significantly up-regulated by 5-FU treatment. Expression levels of *mRNA* were determined by semiquantitative reverse transcriptase-polymerase chain reaction. Data are the mean±S.E. (n=5—6). Asterisk indicates significance from vehicle group (* $p<0.05$). Overall differences among treatments were evaluated by one-way analysis of variance (ANOVA), with differences between groups evaluated using William's test.

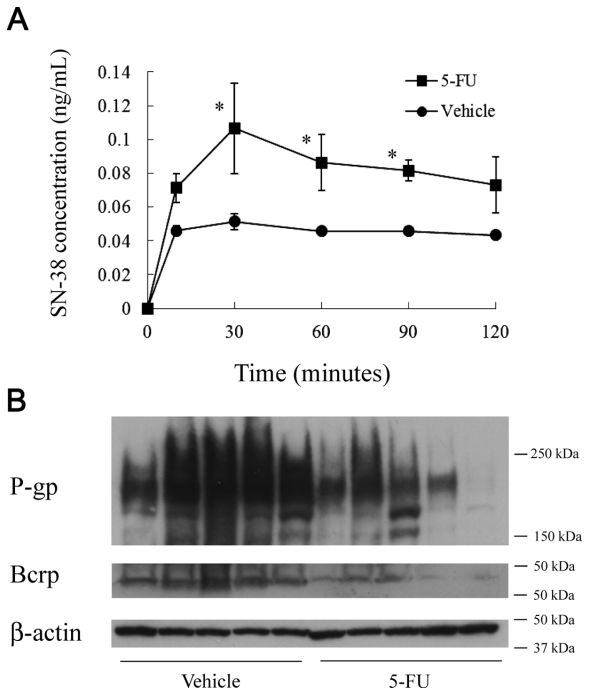

Fig. 4. Effects of Pre-5-FU Treatment on SN-38 Absorption from Intestine and Expression Levels of P-Glycoprotein and Bcrp in the Intestine

(A) SN-38 was injected into the intestine on day 4 after 5-FU treatment at a dose of 400 mg/kg. SN-38 concentration in serum was determined by HPLC analysis. Values are the mean±S.E. (ng·min/ml, $n=5$). Asterisk indicates significance from vehicle group ($*p<0.05$). (B) Western blotting analysis for P-gp, Bcrp and β-actin expressions in the intestine of 5-FU treated rats. Rats were killed after the experiment for SN-38 absorption from the intestine. The detailed procedure for Western blot analysis is described in Materials and Methods.

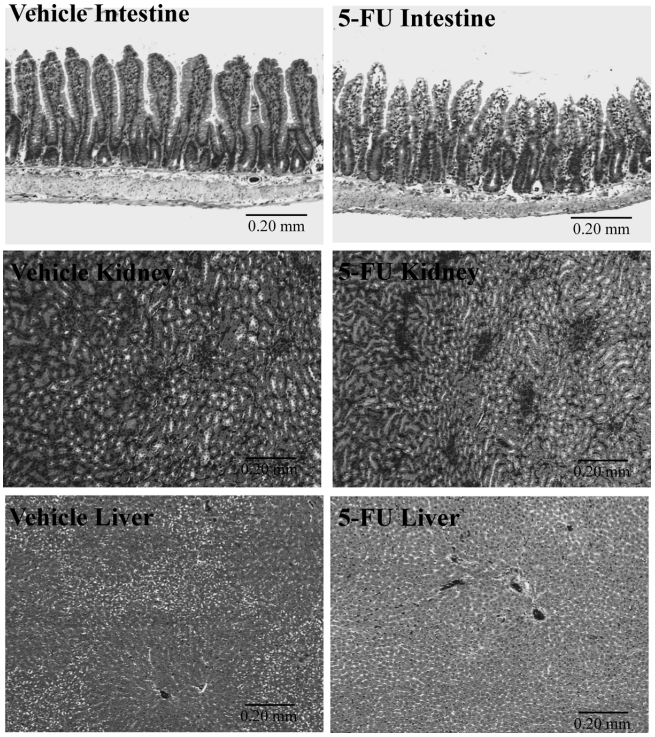

Fig. 5. Microscope Images of the Tissues after the 5-FU Treatment

Rats were intraperitoneally single injected with saline (vehicle) as a control, or 5-FU at a dose of 400 mg/kg. Tissues were obtained on day 4 after the treatment. Five micrometer paraffin sections were cut on a microtome and stained with hematoxylin and eosin for conventional histological examination.

be detected in the intestine.

Effects of 5-FU Treatment on SN-38 Absorption from Intestine and Expression Levels of P-Glycoprotein and Bcrp in the Intestine To evaluate the effect of 5-FU combined treatment with CPT-11 on neutropenia and diarrhea symptoms, the effect of 5-FU or alteration of SN-38 absorption from the intestine was determined. SN-38 was loaded in to the intestine on day 4 after 5-FU treatment at a dose of 400 mg/kg. Blood was collected from indwelling catheter placed in the subclavian artery under pentobarbital anesthesia 10, 30, 60, 90 and 120 min after SN-38 administration. SN-38 concentration in serum was determined by HPLC analysis. SN-38 was readily absorbed from the intestine; SN-38 concentration in the serum was 0.046±0.003, 0.051±0.005, 0.046±0.001, 0.046±0.002 and 0.043±0.001 in vehicle-treated rats, 0.071±0.009, 0.107±0.027, 0.086±0.017, 0.082±0.006 and 0.073±0.016 (ng/ml, means±S.E.) in 5-FU-treated rats 10, 30, 60, 90 and 120 min after SN-38 administration, respectively. Pre-treatment with 5-FU significantly increased SN-38 concentration in the blood 30, 60 and 90 min after SN-38 administration into the intestine. The area under the curve (*AUC*) for SN-38 in the 5-FU group (9.86±1.46 ng·min/ml, means±S.E.) was significantly higher than in the vehicle group (5.37±0.23 ng·min/ml, means±S.E.). Expression levels of P-gp and Bcrp, which transport SN-38, were evaluated after the SN-38 administration experiment. 5-FU treatment decreased the expression levels of P-gp and Bcrp in the intestine to 41.2±14.7 and 15.7±4.3% (mean±S.E.) of control, respectively (Fig. 4B). Histological examination showed that 5-FU treatment injured intestinal mucosal epithelia, but did not injure kidney and liver (Fig. 5).

DISCUSSION

5-FU is an essential drug for anti-cancer chemotherapy. The authors investigated the effect of 5-FU treatment on the expression levels of transporters in rats. 5-FU treatment at a dose of 200 and 400 mg/kg altered the expression levels of Mrp2 in the kidney and intestine (Figs. 1A, C). It has been demonstrated that there is a similarity in the dosage per unit of surface area of anti-cancer drugs for certain laboratory animals and man.[24] The dosage of 5-FU at a dose of 400 mg/kg corresponds to 2222 mg/m^2 in rats.[24] The FOLFIRI regimen is a standard chemotherapy for colorectal cancer, and the regimen involves 5-FU treatment at a dose of 2400 mg/m^2, CPT-11 at a dose of 180 mg/m^2 and leucovorin at a dose of 400 mg/m^2.[25] It was reported that combines bolus with infusional 5-FU/leucovorin regimen was more effective profile than the 5-FU/leucovorin bolus regimen. The activity of 5-FU also has been enhanced by the addition of the biochemical modulator leucovorin.[26] The rats were treated intraperitoneally bolus injection without leucovorin in this study. Therefore, it was assumed that the 5-FU dosage in this study lower than the dose of clinical chemotherapy.

5-FU treatment elevated the expression levels of Mrp2 in the kidney, whereas, it reduced its expression in the intestine (Figs. 1A, C). 5-FU treatment *in vitro* also elevated the expression levels of *MRP2* mRNA in HEK 293T cells, but not HEP-G2 cells (Fig. 1B). The nadir of intestinal Mrp2 expression occurred on day 4 after 5-FU treatment. The expression

level of renal Mrp2 also peaked on day 4. The expression levels of Mrp2 had recovered to normal by one week after treatment (Fig. 2). The biochemical parameters in the serum were also recovered to normal by one week after treatment (Table 2). We evaluated the effect of 5-FU treatment on the expression levels of the nuclear receptor and the phosphorylation of c-Jun to investigate how 5-FU treatment elevated the expression levels Mrp2 in the kidney. It has been reported that the promoter region of the Mrp2 gene has an AP1 binding site which is stimulated by c-jun.[17—19] 5-FU treatment increased *Mrp2* mRNA expression, but did not affect nuclear receptors in the kidney, intestine and liver (Table 4). This result suggests that the phosphorylation of c-jun might be an important basis for Mrp2 expression induced by 5-FU in the kidney. Zhang *et al.* reported that *MRP2* mRNA transcripts in liver are differed from kidney and small intestines. The 5′-untranslated region of MRP2 in regulating its translation and provide a potential mechanism to explain the observed post-transcriptional regulation of MRP2.[27]

It has been reported that 5-FU induces reactive oxygen species (ROS), and ROS induces MRP2 expression.[23,28] It is known that *N*-acety-L-cystein (nAC) is an antioxidant.[28] Treatment with nAC inhibited the up-regulation of Mrp2 expression followed by cisplatin treatment which induces ROS.[29] In this study, nAC treatment did not inhibit the up-regulation of Mrp2 expression. It was therefore assumed that ROS did not affect the expression level of Mrp2 followed by 5-FU treatment.

Recent study revealed a certain mechanism for the regulation of *Mrp2* gene expression. Such as *CAR*, *PXR*, *RXR*α and *FXR* play as a transcription factor of *Mrp2* gene expression. *Mrp2* is activated through binding of a heterodimers of those nuclear receptors.[30—33] The present study showed that a tendency of downregulation of *PXR*, *RXR*α and *FXR* mRNA expression, there were no significant differences in the expression levels of the nuclear receptors between the 5-FU treated group and vehicle group.

Single 5-FU treatment did not show significant differences in body weight, hematological toxicity, total protein, ALT, AST and creatinine in the serum (Tables 1 and 2). Consecutive 5-FU treatment showed hematological toxicity and decreased body weight, total protein, ALT and bilirubin in the serum (Table 3).

Consecutive 5-FU treatment elevated expression levels of Mrp2 in the kidney, while 5-FU reduced its expression in the intestine (Fig. 3A). Wendling *et al.* reported that 5-FU stimulates c-jun phosphorylation of dermal fibroblast cells.[34] Consecutive 5-FU treatment in this study increased the phosphorylation of c-jun in the kidney, but not in the liver (Fig. 3B), suggesting that up-regulated Mrp2 expression in the kidney may be the result of increased c-jun phosphorylation.

It has been reported that cholestasis induced downregulation of Mrp2 expression.[35] In this study, 5-FU treatment decreased serum bilirubin levels. Cholestasis induced by 5-FU treatment decreased intestinal Mrp2 expression levels *in vitro*. 5-FU treatment did not show significant effect on Mrp2 expression levels in rat liver and HEP-G2 cells. It is not clear the effect of 5-FU on signaling mechanism of hepatic Mrp2 expression.

Severe diarrhea induced by CPT-11 treatment remains an unresolved problem and causes dose-limiting toxicity. Additionally, CPT-11 causes Common Terminology Criteria of Adverse Events grade 3—4 diarrhea in at least 40% of patients, leading to premature interruption of chemotherapy.[36] Here, we evaluated the effect of concurrent treatment with 5-FU on the absorption of SN-38, the active metabolite form of CPT-11, from the intestine. 5-FU treatment increased SN-38 concentration and *AUC* in the serum followed by SN-38 injection into the intestine. Efflux transporters in intestine, liver, kidney, brain, testes, and placenta can efflux xenobiotics out of cells and serve as barriers against the entrance of xenobiotics into cells, whereas many xenobiotics enter the biological system *via* uptake transporters. As the barrier against xenobiotics, the intestine has high expression of efflux transporters on the apical membrane of enterocytes, including P-gp, Mrp2 and Bcrp.[37] It was reported that Mrp2, P-gp and Bcrp transport SN-38.[38,39] Downregulation of Mrp2, P-gp and Bcrp caused by concurrent treatment with 5-FU may injure barriers against the entrance of SN-38 into the intestine.

Consequently, concurrent treatment with 5-FU and CPT-11 can result in severe neutropenia and diarrhea. Korenaga *et al.* reported that a 5-FU derivative drug, S-1 injured intestinal mucosa and result in impaired gut barrier function measurable as an increase in intestinal permeability to lactulose and mannitol in rats.[40] The present study showed that 5-FU treatment also injured intestinal mucosa (Fig. 5). Impaired barrier function of intestine may effect on absorption of SN-38 from intestine.

It was demonstrated that docetaxel is a good substrate for MRP2 *in vitro*. van Waterschoot *et al.* reported that Mrp2$^{-/-}$ mice was not significantly different from wild-type, after both oral and intravenous administration. The docetaxel exposure was significantly higher in Mdr1a/b/Mrp2$^{-/-}$ mice in comparison to wild-type mice.[41]

Lopinavir was reported to be a substrate for ABCB1 and ABCC2 *in vitro*. van Waterschoot reported a marked increase (10.6-fold) in systemic exposure (area under the plasma concentration–time curve, *AUC*) in Abcb1a/b/Abcc2$^{-/-}$ mice when compared with wildtype mice. No significant increase in systemic exposure could be determined for Abcc2$^{-/-}$ mice.[42] The pharmacokinetics profiles of the Mrp2/P-gp substrates in gene knockout mice model suggest that P-gp have an important role in absorption of Mrp2/P-gp substrates in the intestine. The reports suggest that downregulation of intestinal P-gp induced by 5-FU treatment may have an important role of an Mrp2/P-gp substrate, SN-38 absorption in the intestine.

Yoshisue *et al.* reported that an oral 5-FU-derivative drug, and 5-chloro-2,4-dihydroxypyridine treatment significantly decreased the activities of both glutathione *S*-transferase and uridine diphosphate glucuronyltransferase (UGT) in the small-intestinal mucosa of rats at day 4 to 48 and 54%, respectively, as compared with those in the vehicle-treated rats. UGT facilitates the excretion of SN-38. Decline of UGT activity caused by 5-FU treatment may affect SN-38 absorption in the intestine.[43]

This study suggests that 5-FU treatment induces renal Mrp2 expression and reduces intestinal Mrp2, P-gp and Bcrp expression. Intestinal toxicity followed by concurrent treatment with 5-FU may promote SN-38 absorption from the intestine.

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