Abstract. Background: The aim of this study was to clarify the molecular mechanisms of the sequence-dependent antitumor activity of SN-38 and 5-fluorouracil (5-FU) against colon cancer cells. Materials and Methods: KM12SM and HCT116 colon cancer cells were exposed to 5-FU and/or SN-38 in various conditions. The nature of interactions was determined by median-effect analysis. Cell cycle, apoptosis, and expression of thymidylate synthase (TS) were analyzed. Results: A strong synergism was observed after initial sequential exposure of SN-38, and the activity was enhanced by a 24 h-interval to the drug-exposure. Antagonism was observed after low-dosage initial sequential exposure of 5-FU. Low-dosage 5-FU caused G2 arrest and high-dosage 5-FU caused G1 arrest. TS protein level significantly decreased after exposure to SN-38. Conclusion: The sequence dependency between SN-38 and 5-FU against colon cancer cells may be related to the dual action on cell cycle regulation by 5-FU and to the down-regulation of TS level by SN-38.

Irinotecan (CPT-11) has been shown to be an active agent in colorectal cancer. Single-agent chemotherapy with irinotecan in metastatic colorectal cancer produced response rates of 15-32% in chemonaive patients and 18-27% in 5-fluorouracil (5-FU) pretreated patients (1, 2). Various attempts have been made to improve the objective response rate and survival time of 5-FU/leucovorin (LV). One of the most successful of which has been its combination with irinotecan, being currently one of the standard therapies for metastatic colorectal cancer (3, 4). It seems to be a reasonable and sound rationale for combining these two active drugs in colorectal cancer because of the following reasons; i) 5-FU and CPT-11 act against different intracellular targets: 5-FU inhibiting thymidylate synthase and CPT-11 targeting the DNA topoisomerase I enzyme; ii) CPT-11 has demonstrated in vivo activity against both chemonaive and 5-FU refractory colorectal cancer patients; iii) topoisomerase I levels are reported to be substantially higher in colorectal cancer than in normal tissue (5); iv) no significant adverse pharmacokinetic interaction has been demonstrated between the two drugs; v) in vitro and in vivo data on combination therapy using 5-FU and CPT-11 or SN-38 (active form of CPT-11) showed some additive or synergistic effect when the two drugs were used sequentially (6-8), in general, synergism was always observed when cells were first exposed to CPT-11 or SN-38, although inconsistent results were obtained with the reverse sequential-exposure schedule (9, 10). However, the molecular mechanisms on synergistic effects of the combination are still unclear.

Recently, oral fluoropyrimidines such as capecitabine and S-1 have been used in combination with CPT-11 instead of infusion or bolus 5-FU (11, 12). The efficacy of oral fluoropyrimidines is reportedly less toxic and with equal anti-tumor activity compared to 5-FU/leucovorin (LV) (13, 14). Moreover, the administration schedule using oral fluoropyrimidines can be conveniently organized with respect to the sequence and the interval in combination with CPT-11. With regard to combination cytotoxicity of CPT-11 and 5-FU, it has been reported that the optimal therapeutic synergy was achieved when CPT-11 was administered 24 h before 5-FU (8). This evidence suggests that the interval between drug administrations may enhance the activity of combination therapy using CPT-11 and oral fluoropyrimidines. In the present study, the molecular mechanisms of sequence-dependency of antitumor activity in relation to cell cycle responses, apoptosis, and regulation of thymidylate synthase (TS), and significance of the interval between the sequential exposure of SN-38 first on cytotoxicity have been clarified.
Materials and Methods

Anticancer drugs. SN-38 was provided by Yakult Honsha Ltd (Tokyo, Japan) and 5-FU was a kind gift from Kyowa Hakko Kogyo (Tokyo, Japan).

Cell culture. The KM12SM colon cancer cell line was a kind gift from Dr. Motowo Nakajima (Johnson & Johnson K.K., Tokyo, Japan). The HCT116 colon cancer cell line was obtained from the ATCC (Rockville, MD, USA). Each cell line was cultured in RPMI-1640 (Invitrogen, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS) at 37˚C/5% CO2 in 75-cm2 culture dishes. The cells were trypsinized once a week with trypsin/EDTA (0.25%/0.02), and the medium was changed twice a week.

Drug-exposure and cell proliferation assay. The amount of drug needed to achieve a 50% inhibition in cell growth (IC50) was determined using the Premix WST-1 cell proliferation assay system (Takara Bio Inc., Otsu, Japan). On day 1, 1.5×104 cells/well in a volume of 150 μL were plated onto 96-well plates. On each plate, one column contained cells not exposed to drugs and 9 columns contained cells exposed to increasing concentrations of drugs. For each drug or drug concentration, 6 wells were used for the cell proliferation assay.

On day 2 (at 24 h after cell seeding), 5-FU and/or SM38 were added in a volume of 50 μL, resulting in a series of final concentrations ranging from 0.2 to 200 μM for 5-FU, and from 0.1 to 100 μM for SN-38, (each series included 0 M as a control), and then the cells were incubated for 24 h. On day 3 (at 24 h of drug exposure), the medium in the control and drug-containing wells was replaced by 200 μL of fresh drug-free medium and the cells were cultured for 48 h. In the case of sequential exposure of 5-FU and SN-38 (sequence A: 5-FU first, sequence B: SN-38 first, sequence C: SN-38 first with 24 h-interval), the medium was replaced by 200 μL of opposite drug-containing medium on day 3 or day 4, and the cells were further cultured for 24 h or for 0 h (Figure 1). The concentrations in the combinations were reduced 10-fold with a 5-FU/SN-38 ratio of 12.5 for KM12SM and of 5:4 for HCT116 according to the molar ratios of the individual IC50. To each well, 20 μL of Premix WST-1 were added and the viable cells were counted using a microplate reader (Model 450 Microplate Reader, Bio-RAD, Hercules, USA), and the inhibition in cell growth was calculated. The IC50 of 5-FU and SN-38 against KM12SM and HCT116 cells were determined.

Median-effect analysis of interaction between 5-FU and SN-38. The most generally accepted mathematical model used to calculate combined drug effects is median-effect analysis (15). The median-effect equation is defined as: 
\[ \frac{fa}{fu} = \frac{Dx}{Dm} \]

Where fa and fu are the fraction of cells affected and unaffected (therefore, fu=1−fa), by a specific dosage, Dx; Dm is the concentration for the median-effect dosage, and m is the coefficient depicting the shape of the dosage-effect curve (m=1, >1 and <1, indicate a hyperbolic, sigmoidal and negative sigmoidal curve, respectively). The plot of the logarithmic form of the above equation yields a straight line where m is the slope and log (Dm) is the intercept. The interaction between the two agents can then be quantified from the following equation:

Combination Index (CI) = \( \frac{(D1/Dx)1 + (D2/Dx)2}{1/Dx} \)

(D1) is the first drug’s dosage in combination with x-percent inhibition, and (D2) is the 2nd drug’s dosage in combination with x-percent inhibition, whereas (Dx)1 and (Dx)2 are the dosages of the individual drug which gives the same inhibition. CI values of <1, 1 and >1 indicates synergy, additive and antagonism, respectively.

Cell cycle analysis by flow cytometry. A total of 2.5×104 tumor cells in a 6-well dish containing 4 mL RPMI-1640 supplemented with 10% FBS were incubated overnight, and then incubated for 24 h with 5-FU or SN-38 at various concentrations followed by further 24 h incubation with medium only. The tumor cells were harvested and fixed in 70% ethanol, and then stained with propidium iodide (0.2 mg/mL). Then, the cellular DNA content was measured using a standard FACScan (Becton Dickinson Immunocytometry Systems, San Jose, USA). Typically, the fluorescent signals from 10,000 cells were collected, and the result displayed as a frequency-distribution histogram (DNA histogram).

Apoptosis detection by annexin V-EGFP staining. A total of 5×105 tumor cells in a 6-well dish containing 4 mL RPMI-1640 supplemented with 10% FBS were incubated overnight. They were then treated with a combination of 5-FU and SN-38 at a concentration each one fifth of IC50 for a total of 72h (indicated in Figure 1), collected and then resuspended in 500 μL of binding buffer. Five μL of an enhanced green fluorescent protein fusion of annexin V (Annexin V-EGFP; Medical & Biological Laboratories, Nagoya, Japan) and 5 μL of propidium iodide were added to the cells, and the cells then incubated at room temperature for 5 min in the dark. The Annexin V-EGFP binding cells were counted by flow cytometry using an FITC signal detector.

Western blot analysis. Cells were cultured in a 25-cm2 dish up to confluence, and were then treated with various dosages of SN-38 for 0 h to 48 h. The cells were washed in PBS and then directly lysed in a sample buffer (0.5 M Tris-HCL, pH 6.8, 10% glycerol, 10% SDS, 6% mercaptoethanol, 0.05% bromophenol blue). Protein (10 μg) was separated on SDS-PAGE gel at 120 V for 1.5 h. After electrophoresis, the proteins were transferred to a PVDF membrane (Bio Rad Lab., USA) at 100 V for 1 h. The membrane was blocked in PBS containing 0.1% Tween-20 (PBS-Tween) with 5% skimmed milk at room temperature for 1 h, subsequently incubated with anti-TS antibodies (Abcam K.K. Tokyo, Japan) at 4˚C overnight. After washing with PBS-Tween, the membrane was
incubated with a horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotecnology, USA). The membrane was washed with PBS-Tween, and the signal was detected using an ECL detection kit (Amersham Pharmacia Biotech, Sweden). A mouse monoclonal antibody against beta-actin (Sigma-Aldrich Fine Chemicals) was used to control for protein loading. The amounts of each protein were quantified as the ratio to beta-actin. Quantification of band densities was performed using the public domain NIH Image software (version 1.60).

Statistical analysis. All data were analyzed using the chi-square test, Fisher’s exact test and Student’s t-test. A p-value less than 0.05 was considered statistically significant.

Results

IC50 of 5-FU and SN-38 against colon cancer cells. The IC50 of 5-FU was 12.1±4.4 μM against KM12SM cells, and 4.5±1.6 μM against HCT116 cells. The IC50 of SN-38 was 5.1±2.2 μM against KM12SM cells, and 3.8±1.8 μM against HCT116 cells.

Combination Index (CI) of 5-FU and SN-38. Figure 2 and Table I show the respective Combination Index (CI) on KM12SM and HCT116 cell growth by the combination of 5-FU and SN-38. On the combined effects of the two drugs judged from the CI value calculated from the 10 to 80% inhibition in cell growth from 3 independent experiments, the CI was 1.35±0.15 in sequence A, and 1.31±0.06 in simultaneous treatment when 20% of cell growth was inhibited (fa=0.2) in KM12SM cells, greater than 1, showing an antagonistic effect of the two drugs. On the other hand, the CI was 0.69±0.11 in sequence B, and 0.68±0.07 in sequence C, when 20% of cell growth was inhibited (fa=0.2) in KM12SM cells, less than 1, showing a synergistic effect. Of interest, the CIs tended to decrease according to the increase in drug concentration, in any combination schedule. When 80% of cell growth was inhibited (fa=0.8) in KM12SM cells, the CI was 0.77±0.14 in sequence A, and 0.84±0.06 in simultaneous treatment, less than 1, showing a synergistic effect of the two drugs. Similar results were found in HCT116 cells.

Effect of 5-FU and SN-38 on cell cycle. Flow cytometry showed that 38.1% of the KM12SM cells were in the G0/G1 phase, 53.8% in S, and 8.0% in the G2/M phase, without

![Figure 2. Median-effect analysis of interaction between 5-FU and SN-38. Human colon carcinoma KM12SM or HCT116 cells were incubated as described in Materials and Methods. Combination Index (CI) values are: CI>1, antagonism; CI=1, additive; CI<1, synergism. Values are means±SD of 3 independent experiments. A; sequence A, B; sequence B, C; sequence C, S; simultaneous exposure.](image-url)
exposure to the drug. After treatment with two fifths of IC\textsubscript{50} of 5-FU or SN-38 for 24 h, and further 24 h incubation with medium only, approximately 24.1\% and 43.0\% of cells were arrested in the G\textsubscript{2} phase, respectively. On the other hand, at IC\textsubscript{50} of 5-FU or SN-38, the ratio of cells in the G\textsubscript{0}/G\textsubscript{1} phase increased to 44.7\% after treatment with 5-FU, and the ratio of cells in the G\textsubscript{2}/M phase increased to 44.1\% after treatment with SN-38 (Figure 3). Similar results were obtained in the HCT116 cells (data not shown).

Detection of Annexin V-EGFP staining cells (apoptotic cells) using flow cytometry. A flow cytometric annexin V-EGFP assay was performed to determine the rate of apoptosis. After the colon cancer cells were treated with a combination of 5-FU and SN-38 at a concentration each of one fifth of IC\textsubscript{50} for a total of 72 h (corresponding fa=0.2 in sequence A or simultaneous combination, fa=0.3-0.4 in sequence B or C), the rate of apoptotic cells were 18.1\% in simultaneous treatment, 24.4\% in sequence A, 33.3\% in sequence B, and 36.1\% in sequence C, in the KM12SM cells. The rate of apoptosis was 15.3\% in simultaneous treatment, 21.5\% in sequence A, 35.2\% in sequence B, and 61.5\% in sequence C, in the HCT116 cells (Figure 4). The ratio increased when the cells were treated with SN-38 first (sequence B or C).

Regulation of TS protein level by SN-38. As shown in Figure 5, Western blot analysis indicated that the TS protein was reduced in a dosage-dependent manner and the down-regulation was maximized up to 48 h after treatment with SN-38 for 24 h in the HCT116 cells. Similar results were found in the KM12SM cells (data not shown).

Discussion

The in vitro experiment clearly demonstrated that the cytotoxic interaction between SN-38 and 5-FU was schedule dependent. Simultaneous exposure of these two agents for 24 h was antagonistic. Sequential exposure to 5-FU for 24 h followed by SN-38 for 24 h had antagonism at low dosage, and synergistic effects at high dosage. On the other hand,
sequential exposure to SN-38 for 24 h followed by 5-FU for 24 h had dramatic synergistic effects. Moreover, the 24 h-interval between SN-38 and 5-FU enhanced the cytotoxicity of the sequential exposure of SN-38 first. With regard to the optimal sequence, most reports have favored a sequence in which CPT-11 or SN-38 were given before 5-FU (6-8), although some other reports have shown that the reverse sequence was equally or more effective (9, 10). In general, synergism was always observed when cells were first exposed to CPT-11 or SN-38.

With regard to the molecular mechanisms for sequence dependent antitumor effect of these two agents, several explanations for the sequence dependency have been reported. Inoue et al. (16) reported that exposure to 5-FU down-regulated both MDR1 (a drug transporter) and bcl-2 (apoptotic pathway) mRNA and simultaneously upregulated carboxylesterase 2 mRNA expression, suggesting enhancement of subsequent CPT-11 cytotoxicity. Firstly, pre-treatment of 5-FU might modify the cellular pharmacology of CPT-11, namely, intracellular CPT-11 concentration is higher in 5-FU followed by CPT-11 exposure than in CPT-11 alone, resulting in synergy when cells were first exposed to 5-FU (17). Another explanation has been made from the integration of the schedule-oriented cell cycle check points. Pavillard et al. (18) reported that cell accumulation in the S phase fraction by initial CPT-11 treatment enhanced subsequent 5-FU cytotoxicity, acting preferentially on the S phase, indicating the most cytotoxic effect of CPT-11 followed by 5-FU exposure. With regard to the cell accumulation by CPT-11 treatment, Goldwasser et al. (19) reported its dependency on the drug concentration and on the cell type. SW620 cells were arrested in G2 at low concentration and in the S phase at high concentration, while KM12 cells did not show any S-phase arrest. The data indicated

**Figure 4. Flow cytometric analysis for Annexin V-EGFP staining of cells.** The cells were treated with various sequences of 5-FU and SN-38 at concentrations each of one fifth of IC_{50} for 24 h. Apoptosis was measured as described in Materials and Methods and represents the total cell death (early and late apoptosis). a; histogram, FL1-H represents Annexin V fluorescence intensity, b; the ratio of apoptotic cells.

**Figure 5. Western blot analysis of TS protein level after treatment with SN-38.** The HCT116 cells were treated with various concentrations of SN-38 for 24 h. TS protein level was determined by Western blot. The quantification of TS protein expression by densitometric scanning is shown in the bottom panel as the fold change from the control. Lanes 1 and 6; control, Lane 2; IC_{50}/100 for 24 h, Lane 3; IC_{50}/10 for 24 h, Lane 4; IC_{50}/5 for 24 h, Lanes 5 and 7; IC_{50} for 24 h, Lane 8; IC_{50} for 24 h and further incubation with drug-free medium for 24 h.
that both the KM12SM cells and the HCT116 cells were consistently arrested in G2 at any concentration of CPT-11. In contrast, both the cell types were arrested in G2 at low 5-FU concentration and in G1 at high 5-FU concentration. The differential accumulation of cells would be one possible explanation for the dosage-dependent cytotoxicity of the 5-FU followed by SN-38 shown in the present study. In other words, since both 5-FU and SN-38 are S phase-specific-killing agents, the cell accumulation in the G2 phase by the first exposure to low dosage 5-FU is thought to cause a relative decrease in the G1/S phase cell population, resulting in antagonism when followed by SN-38. Moreover, the G2 cell arrest by initial exposure to low dosage of 5-FU might be prolonged by subsequent exposure of SN-38, suggesting a low induction of apoptosis (20). Indeed, Annexin V-EGFP staining indicated a low induction of apoptosis in sequence A, and a high induction in sequence B or C which might be augmented through 5-FU activity. With regard to apoptosis induction, Grivicich et al. (21) have reported that an increase in apoptosis could be explained by a disruption in the mitochondria membrane potential which induced caspase activation when CPT-11 was followed by 5-FU.

The synergy of sequential combination can also be explained by the regulation of the activity of one agent at specific target sites (TS, topoisomerase I). It has been shown that SN-38 down-regulated TS protein level in a dosage-dependent manner, resulting in enhanced 5-FU cytotoxicity. Guichard et al. (17) also showed that the decrease in TS activity contributed to synergy in sequential exposure of CPT-11 followed by 5-FU. Similar combination effects were found when CPT-11 or SN-38 were followed by raltitrexed, a specific TS inhibitor (22). Moreover, it has been shown that UCN-01, a protein kinase C/cyclin-dependent kinase inhibitor, suppressed the TS gene expression and enhanced the 5-FU-induced apoptosis in a sequence-dependent manner (23). Of interest, down-regulation of the TS protein was maximized up to 48 h, by 24 h exposure of SN-38, suggesting enhancement in cytotoxicity of the sequential exposure of SN-38 first by the 24 h-interval between SN-38 and 5-FU. These results may provide a rationale for the favorable clinical results of 2-day interval in combination metronomic chemotherapy using CPT-11 followed by oral 5-FU derivative doxifluridine, an intermediate metabolite of capecitabine, for colorectal cancer (24).

It is concluded that the sequence dependency between SN-38 and 5-FU against colon cancer cells might be related to the dual action of cell cycle regulation by 5-FU (G1 and G2) and of the down-regulation of the TS level by SN-38. The optimal sequence of this combination chemotherapy might be the exposure to CPT-11 (SN-38) followed after a 24 h-interval by 5-FU. These findings are worthy of development in combination chemotherapy using CPT-11 and oral fluoropyrimidines such as S-1 and capecitabine for colorectal cancer.

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References


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