In vitro synergistic antitumor activity of a combination of 5-fluorouracil and irinotecan in human colon cancer

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Abstract. The combination of irinotecan and a fluoro-pyrimidine is widely accepted as a treatment for advanced colorectal cancer. However, evaluable data on the feasibility of these combinations has not been presented, and an optimal sequence for administration has not been experimentally and clinically determined. The sequential effect of a combination of 5-FU and CPT-11 in the human colon cancer cell line LoVo was evaluated by WST-8 colorimetric assay. The cytotoxicy and cell cycle distributions of each drug were analyzed by apoptosis assay and flow cytometry. Further, the potential mechanisms of the sequence-dependent effects were investigated by a microarray technique, and confirmed by Western blot analysis. The cytotoxicity of 5-FU (10, 100, 1000 μM) followed by CPT-11 (1 μM) was significantly greater than that of CPT-11 (1 μM) followed by 5-FU (10, 100, 1000 μM) (p<0.05). In cell cycle distribution, 5-FU exposure for 24 h increased the S phase fraction in a dose-dependent manner; though there was no significant difference in cell cycle distribution in 24 h CPT-11 (0.01-1 μM) exposure. Microarray analysis revealed that expressions of some apoptosis related genes such as Bcl-2 changed, and were correlated with sequence-dependent cytotoxicity of the 5-FU→CPT-11 sequence. Western blot analysis confirmed that the Bcl-2/Bax ratio was lower after 5-FU→CPT-11 sequence than before. The sequence-dependent cytotoxic effect may depend on the sensitizing effect of 5-FU pre-treatment on CPT-11 cytotoxicity. 5-FU followed by CPT-11 administration may be an optimal sequence for IFL treatment of advanced colon cancer.

Introduction

Since randomized trials showed an increase in the response rate and a modest increase in survival when irinotecan (CPT-11)

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was added to 5-fluorouracil (5-FU) and leucovorin (LV) (1,2), the combination of these agents (IFL) has become a widely accepted treatment for patients with advanced colorectal cancer. However, no evaluable data on the feasibility of these combinations has been presented. In the United States, IFL consisting of bolus 5-FU and LV in combination with 90 min infused CPT-11, as reported by Saltz *et al*, has been widely used as a standard treatment for metastatic colorectal cancer (CRC) (1). By contrast, infused 5-FU, instead of bolus 5-FU injection, has been used in the European IFL regimen (2). However, toxicity and treatment related deaths of IFL are reported to be higher than that of the 5-FU/LV combination (3,4). Although modifying dose, duration, and administration for IFL has been attempted to decrease toxicity and the death rate, an optimal administration sequence for these agents remains uncertain (5-8). Experimental studies to determine an optimal sequence of drug administration were also inconclusive, and most reports have favored a sequence in which CPT-11 was given before 5-FU (9-12). Other reports have shown that the reverse sequence was equally as effective (13,14).

In Japan, our pharmacokinetic modulating chemotherapy (PMC), which has a low toxicity rate, has also proven highly effective in treating colorectal cancer (15-17). PMC consists of a continuous i.v. infusion of 5-FU over 24 h for 1 day a week at 600 mg/m²/day, and an oral dose of uracil-tegafur (UFT), a 5-FU derivative, at 400 mg/day for 5-7 days per week, repeated every week. A recent *in vitro* study at our institute showed that the PMC regimen targets at least two different phases of the cell cycle (18). These two pathways depend on the integrity of the schedule oriented cell cycle check points; G2-M arrest and mitotic catastrophe at lower 5-FU, and G1-S arrest and apoptosis at higher 5-FU. This may be the reason why PMC is one of the more effective regimens against colo-rectal cancer among the various 5-FU-based chemotherapies (19). Theoretically, we consider the combination of CPT-11, LV and PMC to be the most aggressive front-line treatment for advanced colorectal cancer since the activity of CPT-11 is very schedule-dependent and S-phase-specific (20). From a sequential viewpoint, the dual antitumor effect of 5-FU by prior PMC treatment may enhance the subsequent effect of CPT-11. Indeed, we instigated dose intensification of CPT-11 for unresectable colorectal cancer, and found a marked response and a manageable level of toxicity in this modification of PMC.

The present study aimed to clinically evaluate the role of the administration sequence of the combination of 5-FU with CPT-11 in human colon cancer. For this we used a fixed dose of CPT-11 (at a clinically relevant drug concentration) with varying doses of 5-FU (PMC therapeutic range). We focused on p53-dependent apoptosis and used the colon cancer cell line LoVo (wild-type p53), since in a previous study we found dual antitumor effect of 5-FU regardless of mutations in p53 gene (18). Additionally, we investigated genes associated with sequence-dependent cytotoxicity using a DNA microarray to clarify the biochemical mechanism of the synergistic interaction.

Cell lines and culture conditions. The human colon cancer cell line LoVo was obtained from the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University (Miyagi, Japan). LoVo cells were grown in monolayer culture in RPMI-1640 (Sigma-Aldrich, Inc., St. Louis, MO. USA) supplemented with fetal bovine serum [FBS, 10% (v/v), Gibco BRL, Tokyo, Japan], glutamine (2 mM), penicillin (100000 units/l), streptomycin (100 mg/l), and gentamycin (40 mg/l) at 37°C in a 5% CO_2 environment. For routine passage, cultures were spilt 1:10 when they reached 90% confluence, generally every 3 days. For all experiments, cells at the fifth to ninth passage were used. All experiments were performed with exponentially growing cells.

Anticancer agents. 5-fluorouracil was obtained from Sigma Aldrich and reconstructed in distilled water. Irinoteacan was purchased from Toronto Research Chemicals Inc (North York, ON, Canada). These two drugs were dissolved in appropriate concentrations with distilled water and stored at -20°C until used in the experiments.

Concept of the experimental protocol. Although the IC_{50} value (a drug concentration responsible for 50% growth inhibition) of each drug was generally used for the combination study, as much as possible we used the concentration of each drug as determined from clinical use. 5-FU concentrations were referenced to our previous reports and to drug information obtained from Kyowa Hakko Kogyo (Tokyo, Japan). Assays of 5-FU plasma concentrations in 23 patients receiving PMC showed serum concentrations of 5-FU ranged from 88 to 1323 ng/ml (0.1-10 μM). Drug information on 5-FU from Kyowa Hakko Kogyo shows that plasma concentration of 5-FU reaches 15.3 μg/ml (100 μM) after bolus injection of 5-FU 500 mg/body, and also reaches 0.6 μ/ml (5 μM) during continuous infusion of 5-FU 60 mg/kg/48 h.

CPT-11 concentrations were also referenced to plasma concentrations obtained from clinical use, as found in information on irinotecan hydrochloride (CPT-11) from Daiichi Pharmaceutical Co., Ltd (Tokyo, Japan), showing that plasma concentrations of CPT-11 reach 0.7-7.1 μg/ml (1-10 μM) after drip infusion of CPT-11 50-350 mg/m².

Drug concentration, exposure time and administration schedule. We adopted clinically relevant concentrations of 5-FU and CPT-11. Although we should consider the doubling time of LoVo cells and subsequently decide exposure time; for experimental simplicity, we used just the 249-h exposure time for each drug. Final concentrations used ranged from 0.1 to 1000 μM for 5-FU and from 0.01 to 100 μM for CPT-11.

For the cytotoxicity of each drug, LoVo cells in an exponential growth phase were treated for 24 h with various concentrations of 5-FU or CPT-11. After discarding media containing each drug and replacing it with fresh media, cytotoxicity was evaluated using WST-8 colorimetric assay. For the combination study, LoVo cells were exposed to the first drug (5-FU or CPT-11) for 24 h. After discarding media containing the first drug and replacing it with fresh media, the second drug was administered and incubated for 24 h. Cytotoxic evaluation was similarly performed. Drug-exposure schedules were determined as follows: i) no treatment; ii) 5-FU alone (0.1, 1, 10, 100, and 1000 μM for 24 h); iii) CPT-11 alone (0.01, 0.1, 1, 10, and 100 μM for 24 h); iv) 5-FU (0.1, 1, 10, 100, and 1000 μM for 24 h) followed by CPT-11 (1 or 10 μM for 24 h); v) CPT-11 (1 or 10 μM for 24 h) followed by 5-FU (0.1, 1, 10, 100, and 1000 μM for 24 h). Experiments were done in triplicate for each time point, and averages (means) with standard deviations (S.D.) were calculated.

Growth inhibition assay. The cytotoxicity was evaluated by WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulfophenyl)-2H-tetrazolium, monosodium salt] colorimetric assay. SW480 cells (5000 cells/well) were seeded into 96-well cell plates (Becton Dickinson Labware, NJ, USA) in 100 μl of culture medium for 24 h prior to drug exposure. After 24-h preincubation, cells were treated with various concentrations of 5-FU or CPT-11 for various durations for the different protocols.

After drug exposure for indicated concentrations and hours, the medium was discarded and replaced with 90 μl of fresh medium followed by adding 10 μl WST-8 reagent solution (Cell Counting Kit, Dojindo Laboratories, Japan) and incubated for 4 h at 37°C in the incubator.

Cell viability was determined according to colorimetric comparison by reading optical density (OD) values from a microplate reader (SoftMax, Molecular Devices Corporation, CA) at an absorption wavelength of 450 nm. Cytotoxicity was evaluated by a Cell Counting Kit according to manufacturer's instruction.

Apoptosis assay. The Chemicon ssDNA Apoptosis ELISA Kit (Chemicon International, Inc., CA, USA) that detects denatured DNA with monoclonal antibody (mAb) to single-strand DNA (ssDNA) was used according to the manufacturer's instructions. In brief, LoVo cells (5x10³ per well) were treated with formamide to denature DNA in apoptotic cells and then stained with a mixture of mAb to ssDNA and peroxidase-labeled secondary antibody. Color development was quantified using a Microplate Reader at 405 nm. Comparison of the absorbance of the treated sample with both negative and positive controls determined the extent of ssDNA in apoptotic cells.

Flow cytometric analysis. Cell cycle distribution was determined by DNA content analysis after propidium iodide staining. Cells were treated with various concentrations of each drug for 24 h. They were then harvested, fixed in 70% ethanol, incubated with 2 mg/ml RNase, and stained in

50 μg/ml of propidium-iodide solution. The DNA content of approximately 1×10^5 stained cells was analyzed using a FACScan flow cytometer. The fraction of the cells in G0-G1, S, and G2-M phases were analyzed by DNA program software.

Total RNA extraction. Concentrations of 5-FU at 10 or 100 μM for 24 h and CPT-11 at 1 or 10 μM for 24 h were selected for total RNA extraction because combinations using these concentrations showed significant differences in sequence-dependent cytotoxicities. LoVo cells (5000) were seeded on plastic tissue culture flasks (75 cm²) in 15 ml of culture medium. After 24- or 48-h preincubation, cells were treated with 5-FU at 10 or 100 μM for 24 h followed by CPT-11 at 1 or 10 μM for 24 h (A), and by the reverse sequence (B). Tumor cells were washed with phosphate-buffered saline (PBS) after drug treatment and harvested with trypsin. After that, total RNA was extracted using an RNeasy Midi kit (Qiagen Inc., Chatsworth, CA) according to the manufacturer's instructions.

Microarray analysis. Microarray analyses were performed as previously (21). Concentrations of each total RNA were measured and verified for quality for analysis. A) 5-FU at 10 μM for 24 h followed by CPT-11 at 1 μM for 24 h and the reverse sequence, B) CPT-11 at 1 μM for 24 h followed by 5-FU at 10 μM for 24 h, were selected for microarray analysis. Finally, fluorescent signals were detected on a confocal laser scanner (HB GeneArray Scanner, Affymetrix) and analyzed with the DenasisArray (Hitachi software engineering) and Excel (Microsoft. Redmond, WA). Genes with average intensity ratio that showed a >2-fold or 0.5-fold change were selected. Consequently, selected genes were further analyzed for statistical comparison.

Protein extraction and Western blot analysis. LoVo cells were exposed to combinations of first 5-FU and then CPT-11 for 24 h each in various administration sequences. After drug treatment, cells were homogenized in lysis buffer (Tris-buffered saline, pH 7.5, containing 1% Triton X-100) for 5 min on ice. After centrifugation at 15000 rpm for 15 min at 4˚C, the supernatants were collected and frozen at -20˚C until analysis. The protein concentration was measured by BCA protein assay (Pierce, Rockford, IL). Lysates containing 10 μg total protein were mixed with an equal volume of 2X Laemmli loading buffer containing 2-mercaptoethanol and heated at 100˚C for 5 min. The samples were electrophoretically separated in 12.5% gradient polyacrylamide gels containing 0.1% SDS at 25 mA for 2 h followed by semi-dry transfer to an Immun-Blot PVDF membrane (Bio-Rad Laboratories, Hercules, CA) at 12 V for 2 h. The membranes were blocked with 5% skim milk in Tris-buffered saline, pH 7.5, containing 0.1% Tween 20 (TBS-T) for 1 h at room temperature, and then incubated with a primary antibody diluted in TBS-T/5% skim milk for 1 h at room temperature. The primary antibodies used were: a mouse monoclonal anti-Bcl-2 antibody (1:250; Santa Cruz Biotechnology, Santa Cruz, CA), and a mouse monoclonal anti-Bax antibody (1:500; Dako Corp., Carpinteria, CA). After 3 washes in TBS-T, the blots were incubated with alkaline phosphatase-conjugated goat anti-mouse IgG (Promega

Corp., Madison, WI, USA) diluted 1:1000 in TBS-T/5% skim milk for 1 h at room temperature. Following treatment with an enhanced chemiluminescence detection solution, the blots were exposed to X-ray film for autoradiographic visualization of the bands. The film was scanned and the relative quantities of the protein bands were analyzed by densitometry using CS Analyzer version 2.0 (ATTO Corporation, Japan).

Statistical analysis. Statistical analysis of patient data including various clinicopathological characteristics was performed using analyzing software (Stat View, version 5; Abacus Concepts, Inc., Berkeley, CA). Results are expressed as mean ± SD (standard deviation). Unpaired Student's t-test and Mann-Whitney U test were used for comparison among unpaired groups. Spearman rank correlations test was done for statistical correlations. P values <0.05 were considered statistically significant.

Results

Growth inhibition of LoVo cells by 5-FU and CPT-11. The cytotoxic effects of 5-FU and CPT-11 in LoVo cells were assessed after 24 h drug exposure, followed by WST-8 colorimetric assay. The concentrations of each drug used in this study are based on our previous reports and plasma levels in clinical use. 5-FU and CPT-11 treatment inhibited LoVo cell growth in a dose-dependent manner (Fig. 1). After 24-h exposure, approximately 40-60% of the growth inhibitory effect was observed after 5-FU treatment ranging from 10 to 1000 μM concentrations. For CPT-11 24-h exposure, 10 μM and 100 μM CPT-11 induced approximately 40% and 80% growth inhibition, respectively.

These result indicate that LoVo cells were inhibited at higher levels of 5-FU in PMC (5-FU-based chemotherapy) therapeutic range; 10 μM, and at the clinically relevant concentration of CPT-11; 1-10 μM.

Cytotoxic effects of 5-FU and CPT-11 sequence combination. Clinically, 5-FU in the IFL regimen is used as a bolus injection (Saltz regimen) or by continuous infusion (FOLFILI regimen). In contrast, CPT-11 is usually used as a 90-min infusion. Concentrations of 5-FU have more variations than those of CPT-11 because of the various administration methods. Taking these findings together, CPT-11 was used at a fixed concentration of 1 μM (or 10 μM), and 5-FU was used at serial concentrations of 0.1-1000 μM for sequential study.

The cytotoxicity of 5-FU (10, 100, 1000 μM) followed by CPT-11 (1 μM) was significantly greater than that of CPT-11 (1 μM) followed by 5-FU (10, 100, 1000 μM) (respectively, p<0.01). (Fig. 2) Although 24-h exposure of CPT-11 (1 μM) alone induced 10% growth inhibition in LoVo cells, the growth inhibitions of 5-FU (10, 100, and 1000 μM for 24 h)→CPT (1 μM for 24 h) were 40%, 60%, and 80%, respectively. The cytotoxicity of 5-FU→CPT is also greater than serial concentrations of 5-FU alone.

This shows that pretreatment with 5-FU increases the cytotoxic effect of the subsequently administered CPT-11. The cytotoxicity of serial 5-FU→CPT-11 (1 μM) sequence is almost similar to that of serial 5-FU→CPT-11 (10 μM), while the other conditions are the same.
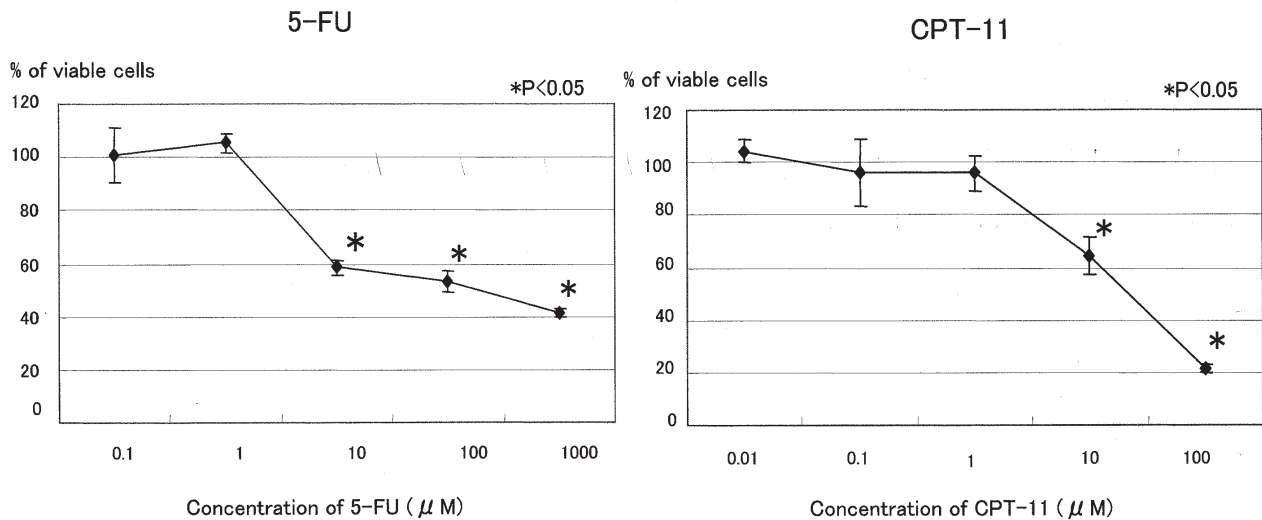
Figure 1. Growth inhibition of LoVo cells by 5-FU and CPT-11. LoVo cells exposed to indicated concentrations of 5-FU or CPT-11 for 24 h. Data represent three separate modified MTT experiments, each done in quadruplicate.
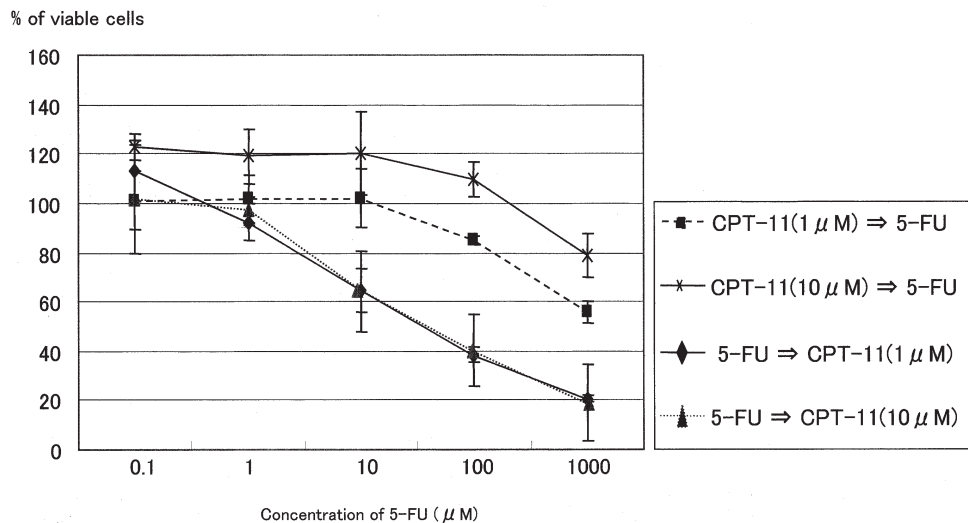

Figure 2. Cytotoxic effects of the combination sequence of 5-FU and CPT-11. 5×10^5 tumor cells were cultured in 96-well microplates. After 24 h incubation, cells were exposed to the first drug (5-FU or CPT-11) for 24 h. Thereafter the medium containing the first drug was changed and then tumor cells were treated with the second drug (CPT-11 or 5-FU) for 24 h. Modified MTT assays were performed as described as Materials and methods.

This phenomenon prompted us to consider further study in respect to apoptosis and cell cycle distribution.

Apoptosis in LoVo cells for 5-FU and CPT-11 treatment. As shown in Fig. 3, apoptosis in LoVo cells was slightly induced at the point of 10, 100, and 1000 μM 5-FU treatment for 24 h. In contrast, apoptosis is significantly induced in just 100 μM CPT-11 treatment for 24 h compared with other concentrations of CPT-11 treatment. Although there were discrepancies in cell death between the cytotoxicity evaluated by WST-8 colorimetric assay and apoptosis evaluated by ssDNA detection kit, this sequential effect may be synergistic, rather than additive for both 5-FU and CPT-11 antitumor effects.

Cell cycle distribution in LoVo for 5-FU and CPT-11 treatment. 5-FU exposure for 24 h increased the S phase fraction in a dose-dependent manner compared with controls. There were no significant differences in cell cycle distribution among the various concentrations (except for 10 and 100 μM) of CPT-11 for 24 h exposure (Fig. 4). Taken the above findings together, sequence-dependent cytotoxicity may depend on the increase in S phase fraction due to pretreatment 5-FU because CPT-11 antitumor activity is cell cycle specific, acting more effectively against cancer cells accumulated in S phase.

Identification of genes correlated with sequence-dependent cytotoxicity of 5-FU and CPT-11. To identify this sequence-dependent cytotoxic mechanism, we carried out cDNA-based microarray analysis after treatment of LoVo human colon cancer cells with: a) 5-FU (10 μM) followed by CPT-11 (1 μM) and b) CPT-11 (1 μM) followed by 5-FU (10 μM). The exposure time for each drug was 24 h. RNA derived from cells treated with (a) or (b) was reverse transcribed, labeled
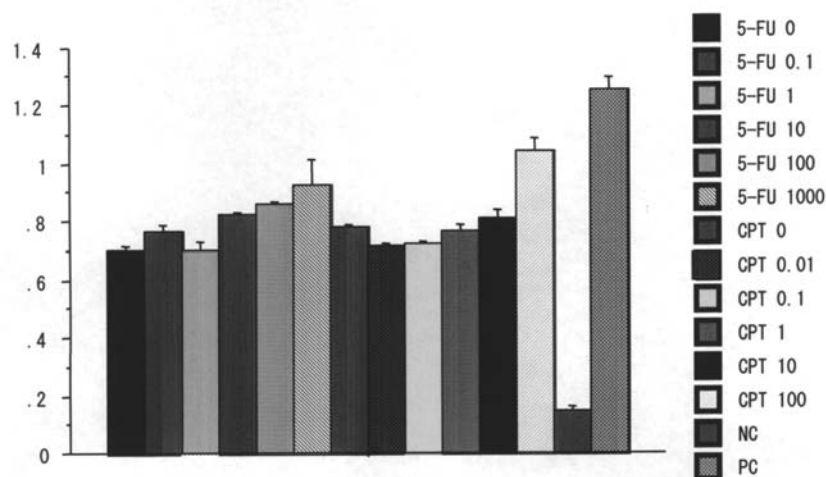
Figure 3. Apoptosis in LoVo cells from 5-FU and CPT-11 treatment. LoVo cells were induced to apoptosis at a significantly higher rate by 5-FU (1000 μM) and CPT-11 (1 μM, 10 μM) exposure for 24 h than any other drug exposure pattern.
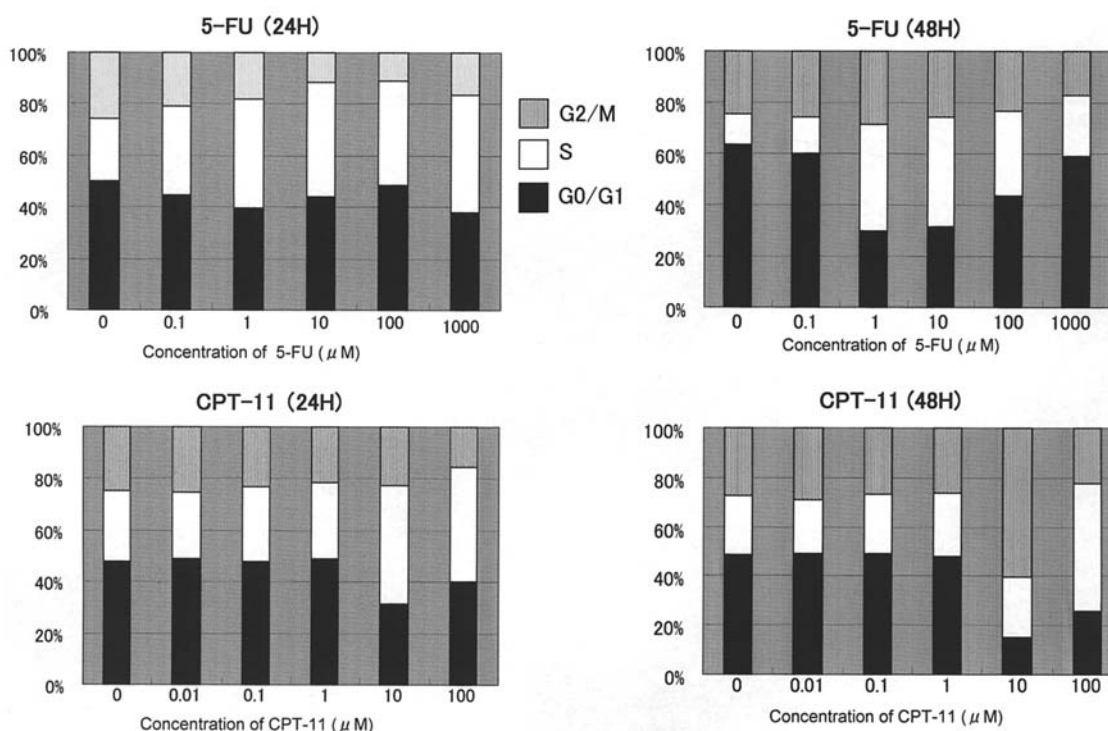

Figure 4. Cell cycle distribution in LoVo from 5-FU and CPT-11 treatment. There are not significant differences in cell cycle distribution among the various concentrations (except for 10 and 100 μM) of CPT-11 for 24 h exposure.

and hybridized to a 20000 gene cDNA microarray. Bound cDNA was detected using Cy3 (a) and Cy5 (b) reporter dyes. The expression profiles in the: a) 5-FU followed by CPT-11 and b) CPT-11 followed by 5-FU were compared and expressed as a Cy3: Cy5 ratio. We found that 22 genes were up-regulated >2-fold and 14 genes were down-regulated <2-fold (Tables I and II).

Protein level of Bcl-2/Bax ratio by Western blot analysis. To verify the microarray analysis, we carried out Western blot analysis for Bcl-2/Bax expression ratio, and found that the

5-FU→CPT-11 sequence caused the regulation of apoptosis by decreasing the Bcl-2/Bax expression ratio (Fig. 5).

Discussion

A widely accepted treatment regimen for colorectal cancer has not yet been established from identification of the basic mechanism, but rather by feedback from clinical trials. Various mechanisms have been proposed for combined fluorouracil and CPT-11 chemotherapy, but none alone can explain all of the interactions by clarifying a basic mechanism.

Table I. Genes down-regulated by the 5-FU→CPT-11 sequence.

Accession no.	Gene	Function
NM_014736	KIAA0101 gene product	
NM_054012	Argininosuccinate synthetase	
NM_003135	Ensembl genscan prediction AceGene oligo matches these RefSeq numbers	
M13994	B-cell CLL/lymphoma 2	Apoptosis
XM_027630	Hypothetical protein xp_027630; loc89959	
NM_001562	Interleukin 18 (interferon-gamma-inducing factor)	Tumor supressor gene
NM_015874	Recombining binding protein suppressor of hairless (Drosophila)	Apoptosis
NM_052837	Secretory carrier membrane protein 3	
NM_004069	Adaptor-related protein complex 2, sigma 1 subunit	
NM_014409	TAF5-like RNA polymerase II, p300/CBP-associated factor (PCAF)-associated factor, 65 kDa	Transcription
NM_004616	Transmembrane 4 superfamily member 3	Oncogene
NM_002639	Serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 5	Oncogene
NM_000893	Kininogen	
NM_022802	C-terminal binding protein 2	Transcription

Table II. Genes up-regulated by the 5-FU→CPT-11 sequence.

Accession no.	Gene	Function
D38112	Atpase subunit 6	
AF026246	herv-e integrase; pol	
NM_032036	tlh29 protein precursor; tlh29	
AF346626	pr-domain containing protein 17	Tumor suppressor gene
XM_051270	Hypothetical protein xp_051270; loc93417	
NM_003489	Receptor interacting protein 140; nrip1	Transcription
BC002439	tat-interacting protein (30 kDa)	
XM_018351	Hypothetical protein xp_018351; loc94852	
XM_006730	Hypothetical protein xp_006730; itga7	
BC012137	Similar to syntaxin 5a	
XM_037969	Hypothetical protein dkfzp434n1415; dkfzp434n1415	
NM_005083	u2 small nuclear ribonucleoprotein auxiliary factor, small subunit-related protein 1; u2af1rs1	
XM_009179	xaa-pro dipeptidase; loc126241	
NM_000264	Patched (drosophila) homolog; ptch	Tumor suppressor gene
NM_018457	dkfzp564j157 protein; dkfzp564j157	
L39610	tcrdv3j1	
NM_023068	Sialoadhesin; sn	Immunology
AF044333	pleiotropic regulator 1; plrg1	
XM_017039	Hypothetical protein xp_017039; loc95731	
NM_020368	Disrupter of silencing 10; sas10	
NM_000944	Protein phosphatase 3 (formerly 2b), catalytic subunit, alpha isoform (calcineurin a alpha); ppp3ca	Apoptosis
NM_022773	Hypothetical protein flj12681; flj12681	

A: 5-FU   (10uM, 24 h)
B: 5-FU   (10uM, 24 h) → CPT-11 (1uM, 12 h)
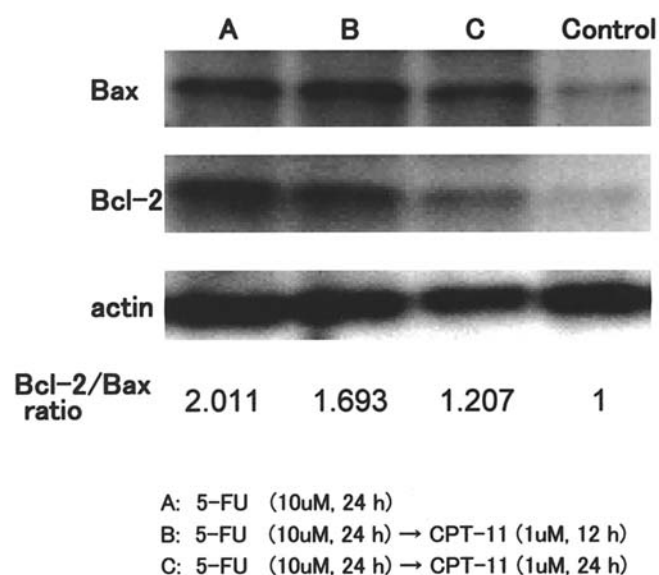C: 5-FU   (10uM, 24 h) → CPT-11 (1uM, 24 h)

Figure 5. Protein level of the Bcl-2/Bax ratio by Western blot analysis. 5-FU→CPT-11 sequence causes regulation of apoptosis by decreasing the Bcl-2/Bax expression ratio.

To better understand and improve chemotherapy, it is important that efforts are made to elucidate the basic mechanism, as we showed previously (18,22). In these earlier studies, we obtained an important clue regarding the mechanism of synergistic interaction in IFL combinations. Namely, colon cancer cells exposed to 5-FU are targeted through two different pathways, depending on the integrity of the schedule oriented cell cycle check points; G2-M arrest and mitotic catastrophe at a lower dose (10 ng/ml in HCT116; wild-type p53), and G1-S arrest and apoptosis at a higher dose (100 ng/ml in HCT116). On the basis of the results, the development of a PMC regimen in combination with CPT-11 that targets different checkpoints may heighten efficacy and broaden the selectivity of 5-FU. At the time that IFL combinations were being developed, much less was known about toxicities and the schedule dependence of CPT-11, than those of 5-FU. In a conventional IFL regimen, Saltz et al reported that 5-FU did not affect CPT-11 cytotoxicity in a CPT-11→5-FU sequence and that SN38 AUC (area under concentration) in CPT-11→5-FU was significantly lower than that in 5-FU→CPT-11, suggesting that CPT-11→5-FU sequence has less toxicity than 5-FU→CPT-11, but with an equal antitumor effect (23). Falcon et al also demonstrated that SN38 AUC in CPT-11→5-FU is 40% lower than that of 5-FU→CPT-11; indicating that CPT-11→5-FU is associated with less toxicity (24). They both recommended CPT-11→5-FU in IFL rather than the 5-FU→CPT-11 sequence because it is less toxic. In our experimental study, the sequential administration of 5-FU→CPT-11 in LoVo colon cancer cells was the more cytotoxic sequence than the reverse one. The cytotoxicity of this sequence was 4-8 times greater than that of CPT-11 alone. Our results showing the superiority of the 5-FU→CPT-11 sequence are in agreement with the report by Guichard et al (13,14). They explained this sequential effect in part from the fact that intracellular CPT-11 concentration was higher in 5-FU followed by CPT-11 exposure, than in CPT-11 alone;

suggesting the 5-FU→CPT-11 sequence enhanced subsequent CPT-11 cytotoxicity. In contrast, Pavillard et al reported that cell accumulation in the S phase fraction by prior CPT-11 treatment enhanced subsequent 5-FU, acting preferentially on the S phase, indicating the most cytotoxic effect of CPT-11→5-FU (10). These discrepancies may be caused by the differences in cell lines, culture conditions, drug-exposure schedules, cytotoxicity evaluation methods, and the timing of evaluation. In this study, apoptosis of each drug for 24 h exposure was not significantly different among the various concentrations except for the 100 μM CPT-11 treatment. Cell cycle analysis showed that 5-FU exposure for 24 h increased the S phase fraction in a dose-dependent manner. A possible explanation of sequence-dependent cytotoxicity is that by increasing the S phase fraction from the pretreatment with 5-FU, the S phase acting drug, CPT-11, may act more effectively against cancer cells. To clarify this potential mechanism, we investigated genes associated with sequence-dependent cytotoxicity using a DNA microarray. This study revealed that 22 up-regulated and 14 down-regulated genes correlated with the sequence dependent cytotoxicity of the 5-FU→CPT-11 sequence. Of these genes, some have already been shown to be associated with apoptosis. For example, genes showing up-regulated expression included protein phosphatase 3. Protein phosphatase 3 (PPP3, formerly PP2B, Calcineurin), a serine/theronine phosphatase that is tightly regulated by Ca^{2+}/calmodulin and plays critical roles in many calcium-mediated signal transduction pathways (25,26). Several studies have shown that PPP3 stimulates NF-κB by enhancing inactivation of its inhibitory molecule NF-κB alpha (27,28). NF-κB is also essential in p53-mediated cell death and induction p53 causes an activation of NF-κB that correlates with the ability of p53 to induce apoptosis (29). On the other hand, expression of Bcl-2, an anti-apoptotic gene, was down-regulated by 5-FU→CPT-11 sequence. To verify the microarray analysis, we next carried out Western blot analysis for the Bcl-2/Bax expression ratio, and found that the 5-FU→ CPT-11 sequence caused up-regulation of apoptosis by decreasing the Bcl-2/Bax expression ratio. These results suggested that 5-FU pretreatment like PMC, may play an important role in sensitizing CPT-11 cytotoxicity and so resulting in apoptosis, with p53 dependence.

In conclusion, the cytotoxic effects of the combination of 5-FU and CPT-11 are schedule-dependent in human colon cancer cells. The sequence of 5-FU→CPT-11 could be of importance with resultant apoptosis in the treatment of colorectal cancer. Research into an appropriate administration sequence should help us to establish a modifying IFL regimen with less toxicity. To better understand and improve chemotherapy, we need to investigate more deeply the combination of PMC and CPT-11, in both experimental and clinical settings.

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