Am J Cancer Res 2017;7(12):2577-2586
www.ajcr.us /ISSN:2156-6976/ajcr0064162

*Original Article*

# Combined efficacy and mechanism of trifluridine and SN-38 in a 5-FU-resistant human colorectal cancer cell lines

Kazuaki Matsuoka, Teiji Takechi

*Translational Research Laboratory, Taiho Pharmaceutical Co., Ltd., 224-2 Ebisuno, Hiraishi, Kawauchi-cho 771-0194, Japan*

Received August 21, 2017; Accepted November 1, 2017; Epub December 1, 2017; Published December 15, 2017

**Abstract:** Trifluridine/tipiracil (FTD/TPI or TFTD, also known as TAS-102) with a molar ratio of 1:0.5, is a novel combination of FTD, an antineoplastic thymidine analog, and TPI, an inhibitor of thymidine phosphorylase. It has been approved as a treatment for unresectable advanced or recurrent colorectal cancer. Irinotecan (CPT-11) is an active agent in colorectal cancer. The administration order of drugs is a critical issue in clinical combination therapy. In this study, we evaluated the *in vitro* simultaneous and sequential combination efficacy of FTD and SN-38, an active metabolite of CPT-11, against human colorectal 5-fluorouracil (5-FU) resistant cell line DLD-1/5-FU and the parental cells DLD-1. The sequential exposure to SN-38 for 24 h followed by sequential exposure to FTD for 24 h or vice versa was more effective for cell survival than the simultaneous exposure of both drugs for 24 h. Furthermore, compared with simultaneous exposure, sequential exposure induced DNA damage, G2/M cell cycle arrest with increasing sub-G1 positive cells, and apoptosis in both DLD-1 and DLD-1/5-FU cells. In particular, in DLD-1/5-FU cells, sequential exposure to SN-38 followed by FTD induced apoptosis more than FTD followed by SN-38. Thus, the sequential treatment with SN-38 followed by FTD may be useful for the combination therapy of FTD/TPI and CPT-11 against relapsed colorectal cancer after 5-FU-based chemotherapy.

**Keywords:** Trifluridine, tipiracil, FTD/TPI, TAS-102, 5-FU resistant cells, colorectal cancer

## Introduction

Colorectal cancer (CRC) is the third most commonly diagnosed cancer in the world [1]. The management of patients with metastatic CRC (mCRC) requires the systemic administration of cytotoxic drugs. Patients with mCRC who receive chemotherapy have shown a median overall survival of more than 2 years [1] compared to standard care alone (5 months) [1, 2]. The combination therapies FOLFOX [5-fluorouracil (5-FU)/leucovorin (LV) and oxaliplatin (L-OHP)] and FOLFIRI [5-FU/LV and irinotecan (CPT-11)] have been established as effective cytotoxic regimens for the treatment of mCRC [1]. CPT-11 is a prodrug analog of the alkaloid camptothecin that is converted to the active metabolite SN-38 by carboxylesterases [3]. This metabolite is approximately 1000 times more potent than CPT-11 at inhibiting Topo I *in vitro* and plays an essential role in the mechanism of action of CPT-11 [4].

Trifluridine/tipiracil (FTD/TPI or TFTD, also known as TAS-102) is a combination of FTD and TPI with a molar ratio of 1:0.5. FTD, an antineoplastic thymidine analog [5], is the active antitumor component of FTD/TPI [6, 7] and exerts its antitumor activity through two actions: its monophosphate form inhibits thymidylate synthase [8] and its triphosphate form is incorporated into DNA, thereby inhibiting DNA synthesis. TPI potently inhibits thymidine phosphorylase [9], an enzyme that degrades FTD. Therefore, TPI helps maintain adequate concentrations of orally administered FTD in the plasma [9], thereby potentiating its antitumor activity.

Some clinical studies on FTD/TPI have recently been conducted [10-12]. In the RECOURSE study which is an international, multicenter randomized, double-blind phase III study, FTD/TPI significantly improved overall and progression-free survival compared with the placebo group and had a manageable safety profile in patients

with mCRC refractory to standard chemotherapies [13]. While FTD/TPI is promising for metastatic colorectal cancer, it is warranted to enhance its antitumor efficacy by combination with other agents especially for earlier treatment lines for metastatic colorectal cancer.

The establishment of drug-resistant cancer cell lines may be one of the most useful clinically relevant models to evaluate treatment after standard chemotherapy. Previously, we had established the 5-FU-resistant colorectal cancer cell line DLD-1/5-FU wherein 5-FU is incorporated into the RNA at low levels because of decreased orotate phosphoribosyltransferase activity [14]. In addition, we had investigated the combination therapy of FTD/TPI and CPT-11 in a DLD-1/5-FU-bearing nude mice model [15]; however, the administration sequence of these drugs was not investigated in detail. Because both are DNA-targeting drugs, the order of administration is thought to be essential for the tumor growth-inhibitory effect. In the present study, we investigated whether the growth-inhibitory effect was enhanced when FTD was used simultaneously or sequentially with SN-38, which is an active metabolite of CPT-11 against DLD-1/5-FU cells.

## Materials and methods

### *Chemicals and reagents*

FTD was obtained from Yuki Gosei Kogyo Co., Ltd. (Tokyo, Japan). Dimethyl sulfoxide (DMSO) and 7-Ethyl-10-hydroxycamptothecin (SN-38) were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan) and Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan), respectively.

### *Cell lines*

DLD-1 cells were obtained from the Health Science Research Resources Bank (Tokyo, Japan). DLD-1 cells were repeatedly exposed to stepwise increasing concentrations of 5-FU up to 100 μM for 120 h. The 5-FU-resistant cell line DLD-1/5-FU was established by this process over 8 months after long-term culture in the presence of 5-FU, as previously described [14]. Cells were cultured at 37°C in a humidified atmosphere of 95% air and 5% $CO_2$ in RPMI 1640 media supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 mg/mL streptomycin. These cells were authenticated in 2014 by analysis of short tandem repeats.

### *Clonogenic cell survival assay*

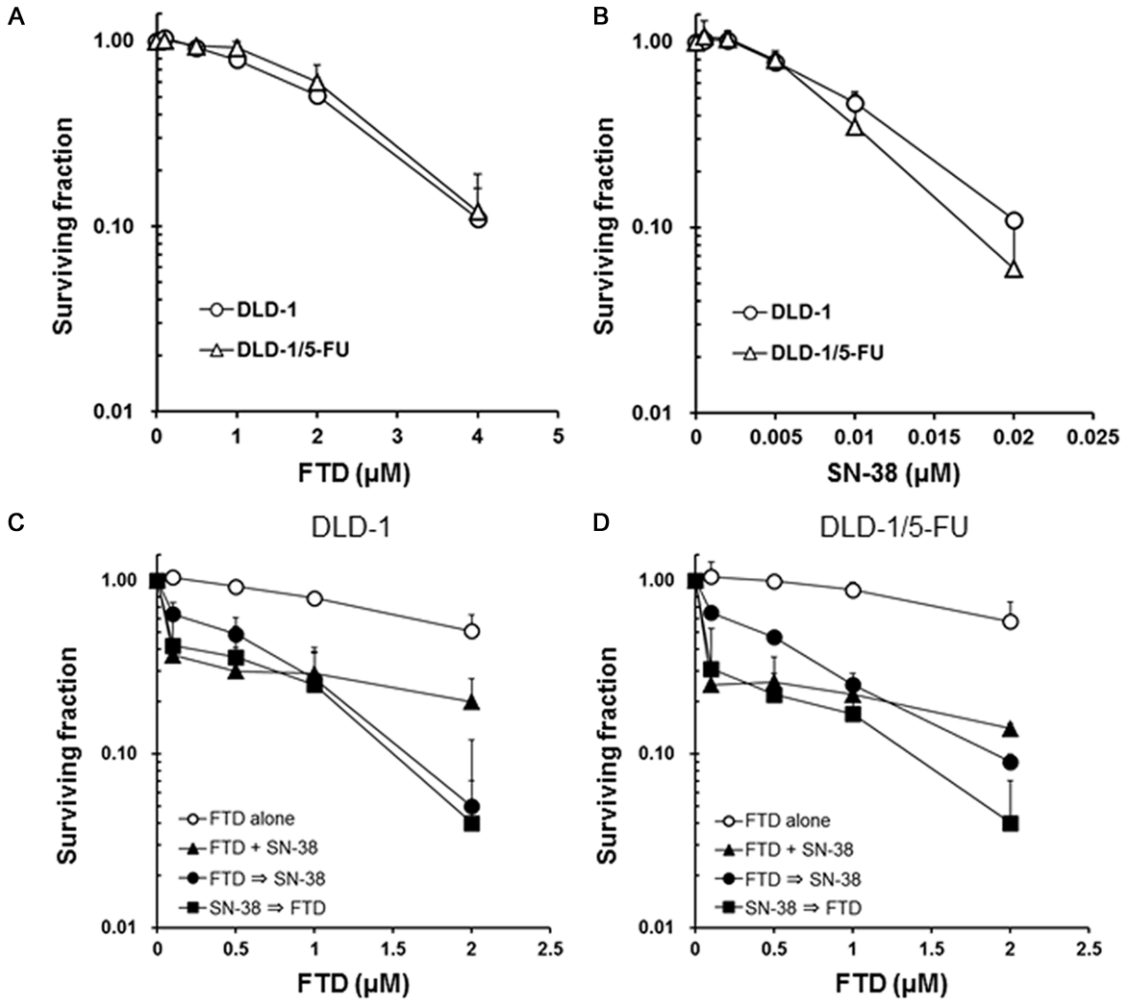
The colorectal cancer cell line DLD-1 and 5-FU-resistant DLD-1/5-FU were plated at concentrations of 100-3000 cells/plate in duplicates in 6-well plates. Sixteen hours after plating, the cells were treated with SN-38 and FTD as follows: (1) exposure to either 0.1-4.0 μM FTD or 0.0005-0.02 μM SN-38 for 24 h, (2) simultaneous exposure to 0.1-2.0 μM FTD and 0.01 μM SN-38 for 24 h followed by no drug exposure for 24 h, (3) sequential exposure to 0.1-2.0 μM FTD for 24 h followed by exposure to 0.01 μM SN-38 for 24 h, or (4) sequential exposure to 0.01 μM SN-38 for 24 h followed by exposure to 0.1-2.0 μM FTD for 24 h. Ten to twelve days after removal of the drug, cells were fixed with 2% glutaraldehyde and stained with 0.05% crystal violet; subsequently, the number of colonies containing at least 50 cells was determined. The plating efficiency (PE) was calculated by dividing the number of colonies by the number of cells plated. The surviving fraction (SF) was determined by dividing the PE of the drug-treated cells by the PE of DMSO (vehicle) or 0.01 μM SN-38 alone.

### *Comet assay*

Alkaline comet assay was performed according to a previous report [7]. After drug treatment, cells were collected, resuspended in ice-cold PBS at $1\times10^5$ cells/mL, mixed with low melt agarose (1:10 ratio), and spread on comet slides (Trevigen, Gaithersburg, MD, USA). The slides were successively submerged in lysis and alkaline (300 mM NaOH, 1 mM EDTA, pH > 13) solutions after the agarose had solidified. The slides were then subjected to electrophoresis for 30 min at 21 V in an alkaline solution (200 mM NaOH, 1 mM EDTA, pH > 13) under dark conditions. Subsequently, the cells were fixed with 70% ethanol and stained with SYBR Gold (Thermo Fisher Scientific, Waltham, MA, USA). The tail moment was determined using CometScore software (TriTek Corp., Sumerduck, VA, USA).

### *Immunoblotting*

Cell pellets were lysed in RIPA buffer (Thermo Fisher Scientific) containing protease inhibitor




E

| Cells | FTD (μM) | FTD alone | FTD + SN-38 | FTD ⇒ SN-38 | SN-38 ⇒ FTD |
|---|---|---|---|---|---|
| DLD-1 | 0.1 | 1.04 ± 0.03 | 0.37 ± 0.05*** | 0.64 ± 0.10* | 0.42 ± 0.18* |
| | 2.0 | 0.51 ± 0.12 | 0.20 ± 0.07* | 0.05 ± 0.02* | 0.04 ± 0.02* |
| DLD-1/5-FU | 0.1 | 1.01 ± 0.05 | 0.25 ± 0.11** | 0.65 ± 0.11* | 0.31 ± 0.22* |
| | 2.0 | 0.60 ± 0.14 | 0.14 ± 0.07* | 0.09 ± 0.04* | 0.04 ± 0.03* |

**Figure 1.** Clonogenic cell survival assay of trifluridine (FTD) and SN-38 in various treatment schedules against DLD-1 and DLD-1/5-FU cells. DLD-1 and 5-FU resistant DLD-1/5-FU cell lines were plated at appropriate concentrations in duplicate in 6-well plates. Cells were exposed to either 0.1-4.0 μM FTD (A) or 0.0005-0.02 μM SN-38 for 24 h (B). DLD-1 (C) and DLD-1/5-FU cells (D) were exposed to FTD alone, in combination with 0.1-2.0 μM FTD and 0.01 μM SN-38 for 24 h, 0.1-2.0 μM FTD for 24 h followed by 0.01 μM SN-38 for 24 h, or 0.01 μM SN-38 for 24 h followed by 0.1-2.0 μM FTD for 24 h. Ten to twelve days after removal of the drug the number of colonies was determined. Data from three independent experiments are represented as the mean ± SD, surviving fraction (SF) in 0.1-2.0 μM FTD followed by 0.01 μM SN-38 or vice versa was calculated by assuming SF as 1.00 when 0.01 μM SN-38 alone was treated for 24 h (E). The mean SF values of simultaneous or sequential combination of 0.1 or 2.0 μM FTD and 0.01 μM SN-38 in DLD-1 and DLD-1/5-FU cells. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ represent significant differences compared with 0.1 or 2.0 μM FTD alone.

cocktail and phosphatase inhibitor cocktail (Nacalai Tesque, Inc., Kyoto, Japan) and incubated for 30 min on ice. The supernatant was cleared by centrifugation at 15,000×*g* for 15

min at 5°C. The protein concentration was determined using the BCA Protein Assay Kit (Thermo Fisher Scientific), and equal amounts of protein (10 μg/lane) were resolved by SDS-PAGE. An ImageQuant LAS 3000 Mini system (GE Healthcare UK Ltd., Buckinghamshire, UK) was used for the detection. The following antibodies were used: anti-poly (ADP-ribose) polymerase (PARP) [9542S; Cell Signaling Technology (CST), Beverly, MA, USA], anti-Cleaved PARP (5625S; CST), anti-Caspase-3 (9662S; CST), anti-Cleaved Caspase-3 (9664S; CST), anti-TopoI (TG2012-4; Topogen, Inc., Columbus, OH, USA), and anti-β-actin (clone AC-74; Sigma-Aldrich, St. Louis, MO, USA).

*Apoptosis detection and cell cycle analysis*

The PE Annexin V Apoptosis Detection Kit I (BD Biosciences, San Jose, CA, USA) was used to detect apoptosis by flow cytometry. After drug treatment, cells were harvested, washed with PBS, and pelleted by centrifugation at 2,500×g for 5 min at 5°C. They were resuspended to $1\times10^5$ cells/100 μL in a binding buffer to which 5 μL of Annexin V and 5 μL of 7-aminoactinomycin D (7-AAD) were added and then incubated in the dark for 15 min at room temperature. Next, 400 μL of binding buffer was added and the cells were immediately processed with a BD Accuri C6 flow cytometer (BD Biosciences). Cells were separated in Annexin-V positive, Annexin-V and 7-AAD double positive, and negative subpopulations using FlowJo software (Tree Star, Inc., Ashland, OR, USA). Apoptosis was calculated as a percentage of the sum of Annexin-V positive and Annexin-V and 7-AAD double positive subpopulations. Cell cycle analysis was conducted as follows: After drug treatment, cells were fixed with 70% ice-cold ethanol to evaluate the cell cycle. Cells were stained with 5 μg/mL propidium iodide supplemented with 10 μg/mL RNase A to determine the DNA content. Samples were then analyzed using the same instrument and software as mentioned above.

*Statistical analysis*

Statistical analyses were performed using Welch's *t*-test or the paired Wilcoxon test with JMP (SAS Institute Inc., Cary, NC, USA). Results were considered statistically significant if $P < 0.05$.

## Results

*Cell killing effects of FTD in combination with SN-38*

The ability of FTD and SN-38 alone to potentiate cell killing was evaluated using a clonogenic cell survival assay. DLD-1 and DLD-1/5-FU cells treated with FTD or SN-38 alone for 24 h resulted in a concentration-dependent decrease in survival (**Figure 1A** and **1B**). Because no significant differences were observed in the SF between the cells, the results for the DLD-1/5-FU cells demonstrated no cross-resistance to either FTD or SN-38.

Next, we evaluated the efficacy when the cells were exposed to the simultaneous combination of the two drugs for 24 h or the sequential combination of two drugs (FTD for 24 h followed by SN-38 for 24 h or vice versa). In this experiment, we used a fixed concentration of 0.01 μM SN-38 from the result of the $IC_{50}$ value from the clonogenic cell survival assay of SN-38 alone. **Figure 1C** and **1D** show the results obtained after simultaneous exposure to FTD and SN-38, the sequential combination of FTD and SN-38, or vice versa in DLD-1 and DLD-1/5-FU cells. After simultaneous exposure, the SF was not altered and even increased with the concentration of FTD from 0.1 to 2.0 μM (closed triangle, **Figure 1C** and **1D**). In contrast, the SF after both sequential combinations decreased in an FTD concentration-dependent manner (closed circle and closed square, **Figure 1C** and **1D**). **Figure 1E** shows the mean SF summary of the DLD-1 and DLD-1/5-FU cells after their sequential exposure to FTD and SN-38. After sequential exposure to 0.1 or 2.0 μM FTD with minimum and maximum concentrations in this experiment followed by 0.01 μM SN-38 or vice versa, the mean SF of both sequential treatments in DLD-1 and DLD-1/5-FU cells were significantly lower ($P < 0.05$) than those after exposure to FTD alone. In addition, although it was not significant, in DLD-1/5-FU cells, sequential exposure to SN-38 followed by FTD tended to have a stronger cell killing effect than sequential exposure to FTD followed by SN-38.

*Induction of DNA damage in combination of FTD with SN-38*

Since FTD and SN-38 are DNA-targeting drugs, we evaluated DNA damage induced by the

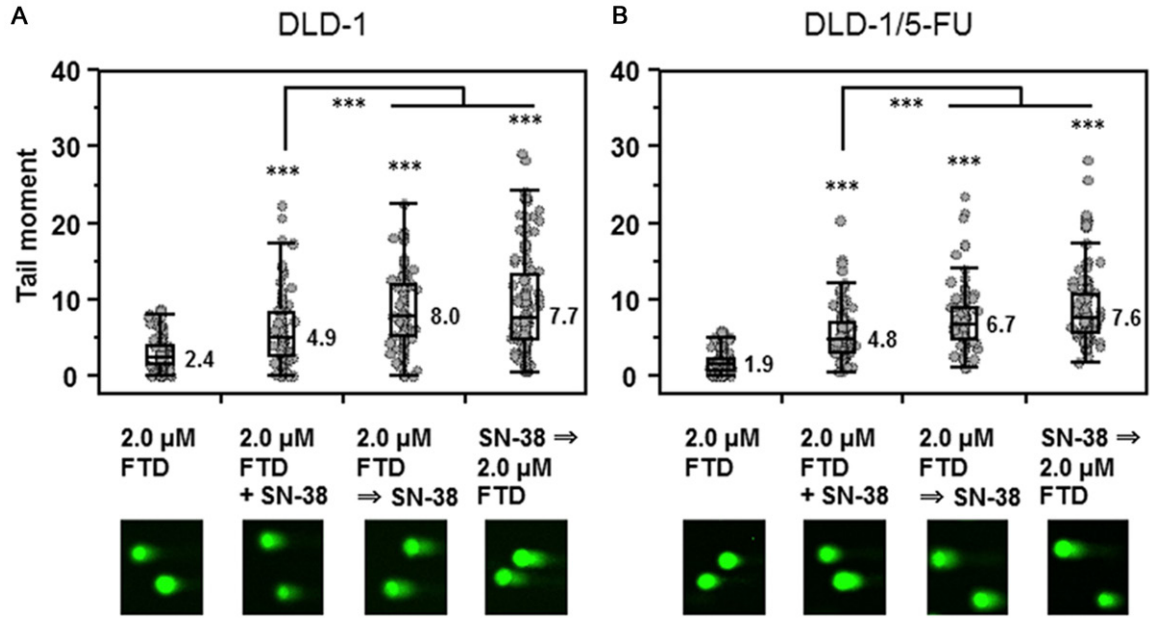



**Figure 2.** DNA damage in DLD-1 and DLD-1/5-FU cells after exposure to trifluridine (FTD) and SN-38. DLD-1 and 5-FU resistant DLD-1/5-FU cell lines were plated in 60 mm-dishes. Cells were treated in combination with 2.0 μM FTD and 0.01 μM SN-38 for 24 h, 2.0 μM FTD for 24 h followed by 0.01 μM SN-38 for 24 h, or 0.01 μM SN-38 for 24 h followed by 2.0 μM FTD for 24 h. DNA damage in DLD-1 (A) and DLD-1/5-FU cells (B) was measured using the alkaline comet assay and the tail moment was determined. The lines are the median and the bars indicate the interquartile range. ***$P < 0.001$.

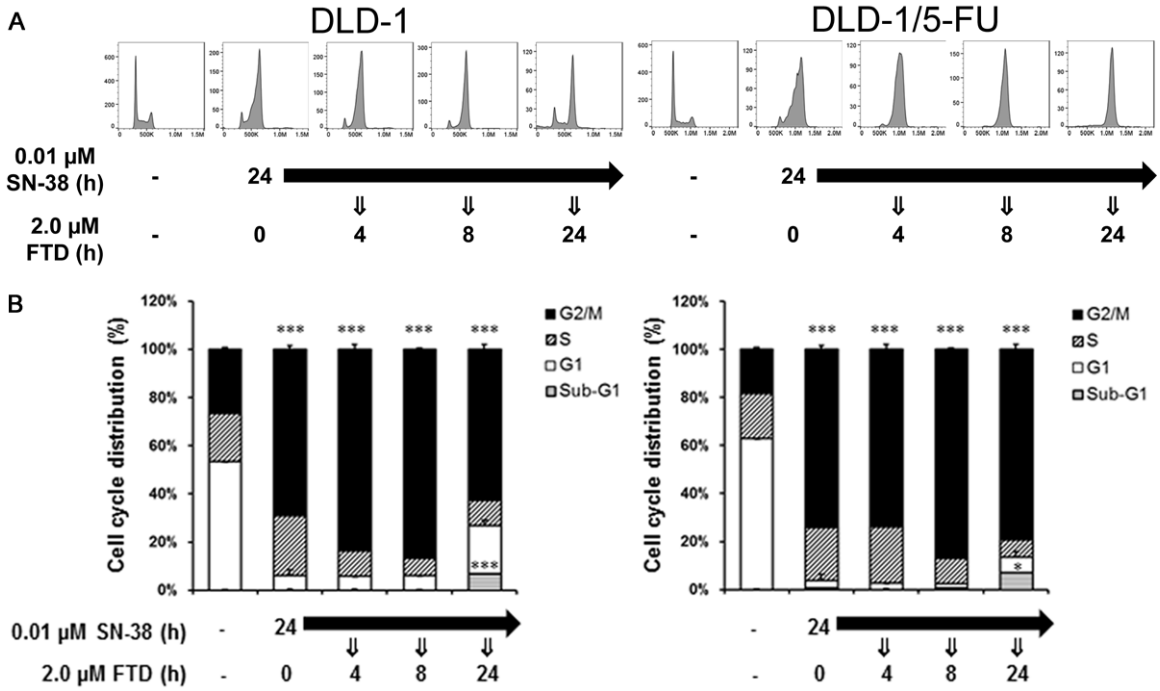



**Figure 3.** Cell cycle distribution of sequential exposure, SN-38 followed by trifluridine (FTD). DLD-1 and 5-FU resistant DLD-1/5-FU cell lines were plated in 60 mm-dishes. Cells were treated with 0.01 μM SN-38 for 24 h followed by 2.0 μM FTD for 24 h. Cells collected at indicated times were stained with propidium iodide and analyzed by flow cytometry. Representative cell cycle histogram (A) and cell cycle distribution (B) data from three experiments are represented as the mean ± SD. *$P < 0.05$, ***$P < 0.001$.

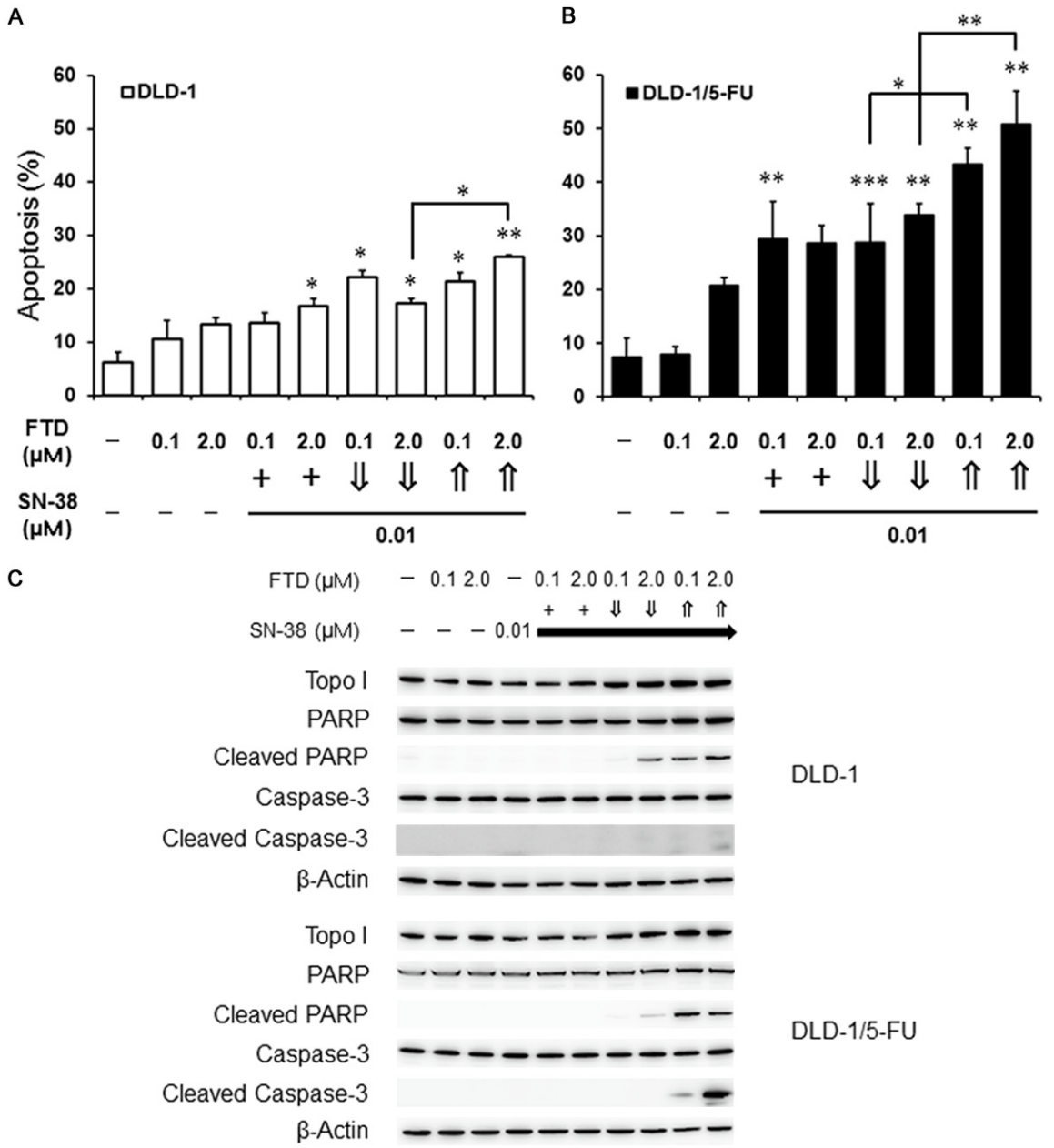



**Figure 4.** Apoptosis in DLD-1 and DLD-1/5-FU cells induced by the simultaneous and sequential combination of trifluridine (FTD) and SN-38. DLD-1 and 5-FU resistant DLD-1/5-FU cell lines were plated in 60 mm-dishes. Cells were treated with FTD (0.1 or 2.0 μM) alone for 24 h; in combination with FTD (0.1 or 2.0 μM) and 0.01 μM SN-38 for 24 h, FTD (0.1 or 2.0 μM) for 24 h followed by 0.01 μM SN-38 for 24 h; or 0.01 μM SN-38 for 24 h followed by FTD (0.1 or 2.0 μM) for 24 h. DLD-1 (A) and DLD-1/5-FU cells (B) were double stained with Annexin V and 7-AAD for flow cytometry analysis. Data from three independent experiments are represented as the mean ± SD. **P* < 0.05, ***P* < 0.01, ****P* < 0.001. Detection of apoptotic markers including cleaved PARP and cleaved caspase-3 by immunoblot in DLD-1 and DLD-1/5-FU cells (C). Equal loading was confirmed with β-actin.

exposure to FTD and SN-38 at the most effective cell killing concentration of clonogenic cell survival assays. DLD-1 and DLD-1/5-FU cells were simultaneously (2.0 μM FTD and 0.01 μM SN-38) or sequentially (2.0 μM FTD followed by 0.01 μM SN-38 or vice versa) exposed to FTD and SN-38, and DNA damage were evaluated using the alkaline comet assay. Interestingly, in both DLD-1 and DLD-1/5-FU cells, the median comet tail moment in sequential exposure to FTD followed by SN-38 or vice versa was significantly higher ($P < 0.001$) than that in simultane-

ous exposure to FTD and SN-38 (**Figure 2A** and **2B**). These results suggest that the simultaneous and sequential combination of FTD and SN-38 effectively induce DNA damage. In addition, no difference in DNA damage was observed between DLD-1 and DLD-1/5-FU cells.

*Cell cycle distribution after exposure to FTD and SN-38*

Increasing DNA damage caused by SN-38 reportedly induces S and G2/M cell cycle arrest [16, 17]. Therefore, we investigated how FTD affects the cell cycle after SN-38 treatment. After 24 h of SN-38 treatment, DLD-1 and DLD-1/5-FU cells accumulated in the S and G2/M phases. After 24 h of the subsequent FTD treatment, the cells in the S phase gradually decreased compared with those treated with SN-38 alone, which finally accumulated in the G2/M phase (62.8% ± 1.5: DLD-1, 79.5% ± 2.2: DLD-1/5-FU) along with sub-G1 accumulation (6.7% ± 0.2: DLD-1, 6.9% ± 1.2: DLD-1/5-FU) (**Figure 3A** and **3B**). The accumulation in the G2/M phase in DLD-1/5-FU cells after SN-38 treatment followed by FTD was persistent compared with that in the DLD-1 cells.

*Induction of apoptosis through the intrinsic apoptotic signaling pathway after exposure to FTD and SN-38*

Cell cycle arrest by anticancer drugs such as 5-FU and paclitaxel reportedly induces apoptosis [18]; therefore, we evaluated whether the combination of FTD and SN-38-associated cytotoxicity is a result of apoptosis. To quantify the number of cells undergoing apoptosis, DLD-1 and DLD-1/5-FU cells were simultaneously (0.1 or 2.0 μM FTD and 0.01 μM SN-38) or sequentially (0.1 or 2.0 μM FTD followed by 0.01 μM SN-38 or vice versa) exposed to FTD and SN-38 and the stages of apoptosis were quantified. In DLD-1 cells (**Figure 4A**), which strongly showed G2/M arrest with 0.01 μM SN-38 followed by 2.0 μM FTD, after sequential exposure (2.0 μM FTD followed by 0.01 μM SN-38 or vice versa), the percentage of apoptotic cells were 17.3 ± 1.6 and 26.1 ± 3.0 compared with that using 2.0 μM FTD alone (13.4 ± 1.2) ($P < 0.05$ and $< 0.01$ vs. FTD alone, respectively). In DLD-1/5-FU cells (**Figure 4B**), which also strongly showed G2/M arrest with 0.01 μM SN-38 followed by 2.0 μM FTD, after sequential exposure (2.0 μM FTD followed by 0.01 μM SN-38 or vice versa), the percentage of apoptotic cells were 33.9 ± 3.0 and 50.8 ± 4.4 compared with that using 2.0 μM FTD alone (20.8 ± 3.2) (both $P < 0.01$ vs. FTD alone). Notably, the highest percentage of apoptosis was observed in DLD-1/5-FU cells sequentially treated with SN-38 followed by FTD, then in cells sequentially treated with FTD followed by SN-38, and the lowest percentage of apoptosis was noted in cells with simultaneous drug exposure (**Figure 4B**).

The cleavages of PARP and caspase-3, which are proapoptotic markers, were substantially induced after sequential exposure to FTD followed by 0.01 μM SN-38 or vice versa in both DLD-1 and DLD-1/5-FU cells (**Figure 4C**). Furthermore, the amount of both cleaved PARP and cleaved caspase-3 increased more in DLD-1/5-FU cells than in DLD-1 cells, suggesting that the induction of apoptosis in DLD-1/5-FU cells was much stronger than in DLD-1 cells. Similar to the G2/M-phase arrest in the cell cycle analysis, the apoptosis in DLD-1/5-FU cells was more strongly induced than that in DLD-1 cells.

Meanwhile, no alteration in the expression of Topo I, which is a target of SN-38, was observed regardless of exposure to FTD alone, simultaneously, or sequentially. This result suggests that FTD does not potentially interact with the Topo I protein.

## Discussion

In the present study, we evaluated the cell growth-inhibitory effects of the simultaneous or sequential combination of FTD with SN-38 in DLD-1 and DLD-1/5-FU colorectal cancer cells. We found that interactions between these drugs were schedule dependent and more effective when treated sequentially with SN-38 followed by FTD than simultaneous or sequential treatment of FTD followed by SN-38. These effects were involved in the induction of DNA damage and apoptosis.

We previously reported that the antitumor activity of FTD/TPI and CPT-11 combination therapy was significantly superior to that of both monotherapies against a DLD-1/5-FU-bearing nude mice model [15]. In the study, FTD/TPI was orally administered twice a day from days 1 to 14 and CPT-11 was adminis-

tered intravenously on days 1 and 8. Because the sequential administration of these drugs was not investigated *in vivo*, we evaluated the sequential treatment of FTD and SN-38 in detail *in vitro*. Our findings suggest that the sequential exposure to FTD followed by SN-38 or vice versa was further effective in both DLD-1 and DLD-1/5-FU cells compared with simultaneous exposure (**Figure 1**). This is the first finding of the sequential combination effects by FTD and SN-38 against 5-FU-resistant cells.

Since both FTD and SN-38 target DNA, we focused on DNA damage, cell cycle distribution, and apoptosis to elucidate a cell death mechanism with the combination of FTD and SN-38. In both DLD-1 and DLD-1/5-FU cells, the combination of FTD and SN-38 induced DNA damage (**Figure 2**). In particular, compared with the simultaneous combination, the sequential exposure to FTD followed by SN-38 or vice versa significantly induced DNA damage (**Figure 2**). It has been reported that SN-38 induces single strand breaks by inhibiting Topo I [4], while FTD causes DNA dysfunction with few DNA double strand break (DSB) [7, 19]. Because the sequential exposure has stronger effects than the simultaneous exposure, one drug may modulate the other. For instance, FTD is thought to enhance Topo I inhibition or to inhibit repair after DNA damage by SN-38.

Previous studies have shown that SN-38 induces S- and G2/M-phase arrest [16, 17], while FTD induces G2-phase arrest with its massive incorporation into DNA [7]. Interestingly, the sustained G2/M arrest in the case of SN-38 followed by FTD treatment was observed in both DLD-1 and DLD-1/5-FU cells (**Figure 3**). In particular, the G2/M arrest in DLD-1/5-FU cells was persistently maintained compared with that in DLD-1 cells. It has been reported that a prolonged G2/M arrest leads to an inhibition of cell growth and proliferation through activation of apoptotic pathways [20-22]. The sequential combination of SN-38 followed by FTD in DLD-1/5-FU cells significantly induced apoptosis (**Figure 4A** and **4B**). This result was reflected by the induction of the proapoptotic markers cleaved PARP and cleaved caspase-3 (**Figure 4C**). The apoptosis induction observed in DLD-1/5-FU cells seems to be attributed to the prolonged G2/M arrest in the case of SN-38 followed by FTD.

Meanwhile, similar to a previous report that FTD alone hardly affected Topo I protein levels [23], they were hardly affected regardless of simultaneous or sequential treatment with FTD and SN-38 (**Figure 4C**). These results demonstrated that FTD did not affect Topo I activity in colon or gastric cancer cells. Collectively, exposure to SN-38 followed by FTD in DLD-1/5-FU cells was more effective in cell killing, cell cycle arrest, and apoptosis induction than that in DLD-1 cells; however, no difference in DNA damage was observed in the treatment order. We speculated that FTD first delayed DNA repair after DNA damage by SN-38.

Our results suggest that the combination of FTD and SN-38 is effective against 5-FU-resistant cells. The sequence dependency between SN-38 and FTD may be related to the S and G2/M arrest with DSB by SN-38 [4, 24], followed by G2 arrest by FTD incorporated into DNA [7], and finally to the effect leading to apoptosis. However, these results are only based on two cell lines, and it is unclear why the sequential treatment of SN-38 followed by FTD is more effective in DLD-1/5-FU cells. Further investigations are needed to elucidate the effect of the administration order of these drugs and their mechanisms. In addition, because we did not investigate the sequential exposure of these drugs on normal cells, further *in vivo* and clinical studies are needed to evaluate the balance between efficacy and toxicity in the future.

In conclusion, our findings suggest that a combination of FTD/TPI and CPT-11 is useful for relapsed colorectal cancer after 5-FU-based treatment. Furthermore, the order of drug administration may affect efficacy in clinical therapies.

## Acknowledgements

The authors would like to thank Enago for English language editing.

## Disclosure of conflict of interest

KM and TT are employees of Taiho Pharmaceutical Co. Ltd (Tokyo, Japan).

**Address correspondence to:** Dr. Kazuaki Matsuoka, Translational Research Laboratory, Taiho Pharmaceutical Co., Ltd., 224-2 Ebisuno, Hiraishi, Kawauchi-cho 771-0194, Japan. E-mail: kazu-matsuoka@taiho.co.jp

## References


[1] Gustavsson B, Carlsson G, Machover D, Petrelli N, Roth A, Schmoll HJ, Tveit KM, Gibson F. A review of the evolution of systemic chemotherapy in the management of colorectal cancer. Clin Colorectal Cancer 2015; 14: 1-10.

[2] Scheithauer W, Rosen H, Kornek GV, Sebesta C, Depisch D. Randomised comparison of combination chemotherapy plus supportive care with supportive care alone in patients with metastatic colorectal cancer. BMJ 1993; 306: 752-755.

[3] Takimoto CH, Wright J, Arbuck SG. Clinical applications of the camptothecins. Biochim Biophys Acta 1998; 1400: 107-119.

[4] Kawato Y, Aonuma M, Hirota Y, Kuga H, Sato K. Intracellular roles of SN-38, a metabolite of the camptothecin derivative CPT-11, in the antitumor effect of CPT-11. Cancer Res 1991; 51: 4187-4191.

[5] Heidelberger C, Parsons DG, Remy DC. Syntheses of 5-trifluoromethyluracil and 5-trifluoromethyl-2'-deoxyuridine. J Med Chem 1964; 7: 1-5.

[6] Tanaka N, Sakamoto K, Okabe H, Fujioka A, Yamamura K, Nakagawa F, Nagase H, Yokogawa T, Oguchi K, Ishida K, Osada A, Kazuno H, Yamada Y, Matsuo K. Repeated oral dosing of TAS-102 confers high trifluridine incorporation into DNA and sustained antitumor activity in mouse models. Oncol Rep 2014; 32: 2319-2326.

[7] Matsuoka K, Iimori M, Niimi S, Tsukihara H, Watanabe S, Kiyonari S, Kiniwa M, Ando K, Tokunaga E, Saeki H, Oki E, Maehara Y, Kitao H. Trifluridine induces p53-dependent sustained G2 phase arrest with its massive misincorporation into DNA and few DNA strand breaks. Mol Cancer Ther 2015; 14: 1004-1013.

[8] Langenbach RJ, Danenberg PV, Heidelberger C. Thymidylate synthetase: mechanism of inhibition by 5-fluoro-2'-deoxyuridylate. Biochem Biophys Res Commun 1972; 48: 1565-1571.

[9] Fukushima M, Suzuki N, Emura T, Yano S, Kazuno H, Tada Y, Yamada Y, Asao T. Structure and activity of specific inhibitors of thymidine phosphorylase to potentiate the function of antitumor 2'-deoxyribonucleosides. Biochem Pharmacol 2000; 59: 1227-1236.

[10] Overman MJ, Kopetz S, Varadhachary G, Fukushima M, Kuwata K, Mita A, Wolff RA, Hoff P, Xiong H, Abbruzzese JL. Phase I clinical study of three times a day oral administration of TAS-102 in patients with solid tumors. Cancer Invest 2008; 26: 794-799.

[11] Overman MJ, Varadhachary G, Kopetz S, Thomas MB, Fukushima M, Kuwata K, Mita A, Wolff RA, Hoff PM, Xiong H, Abbruzzese JL. Phase 1 study of TAS-102 administered once daily on a 5-day-per-week schedule in patients with solid tumors. Invest New Drugs 2008; 26: 445-454.

[12] Doi T, Ohtsu A, Yoshino T, Boku N, Onozawa Y, Fukutomi A, Hironaka S, Koizumi W, Sasaki T. Phase I study of TAS-102 treatment in Japanese patients with advanced solid tumours. Br J Cancer 2012; 107: 429-434.

[13] Mayer RJ, Van Cutsem E, Falcone A, Yoshino T, Garcia-Carbonero R, Mizunuma N, Yamazaki K, Shimada Y, Tabernero J, Komatsu Y, Sobrero A, Boucher E, Peeters M, Tran B, Lenz HJ, Zaniboni A, Hochster H, Cleary JM, Prenen H, Benedetti F, Mizuguchi H, Makris L, Ito M, Ohtsu A. Randomized trial of TAS-102 for refractory metastatic colorectal cancer. N Engl J Med 2015; 372: 1909-1919.

[14] Murakami Y, Kazuno H, Emura T, Tsujimoto H, Suzuki N, Fukushima M. Different mechanisms of acquired resistance to fluorinated pyrimidines in human colorectal cancer cells. Int J Oncol 2000; 17: 277-283.

[15] Nukatsuka M, Nakagawa F, Saito H, Sakata M, Uchida J, Takechi T. Efficacy of combination chemotherapy using a novel oral chemotherapeutic agent, TAS-102, with irinotecan hydrochloride on human colorectal and gastric cancer xenografts. Anticancer Res 2015; 35: 1437-1445.

[16] Maurya DK, Ayuzawa R, Doi C, Troyer D, Tamura M. Topoisomerase I inhibitor SN-38 effectively attenuates growth of human non-small cell lung cancer cell lines in vitro and in vivo. J Environ Pathol Toxicol Oncol 2011; 30: 1-10.

[17] Wallin A, Svanvik J, Holmlund B, Ferreud L, Sun XF. Anticancer effect of SN-38 on colon cancer cell lines with different metastatic potential. Oncol Rep 2008; 19: 1493-1498.

[18] Spina A, Sorvillo L, Chiosi E, Esposito A, Di Maiolo F, Sapio L, Caraglia M, Naviglio S. Synergistic cytotoxic effects of inorganic phosphate and chemotherapeutic drugs on human osteosarcoma cells. Oncol Rep 2013; 29: 1689-1696.

[19] Lenz HJ, Stintzing S, Loupakis F. TAS-102, a novel antitumor agent: a review of the mechanism of action. Cancer Treat Rev 2015; 41: 777-783.

[20] Weaver BA, Cleveland DW. Decoding the links between mitosis, cancer, and chemotherapy: The mitotic checkpoint, adaptation, and cell death. Cancer Cell 2005; 8: 7-12.

[21] Lee SJ, Langhans SA. Anaphase-promoting complex/cyclosome protein Cdc27 is a target for curcumin-induced cell cycle arrest and apoptosis. BMC Cancer 2012; 12: 44.

[22] Orth JD, Loewer A, Lahav G, Mitchison TJ. Prolonged mitotic arrest triggers partial activation of apoptosis, resulting in DNA damage and p53 induction. Mol Biol Cell 2012; 23: 567-576.

[23] Temmink OH, Hoebe EK, Fukushima M, Peters GJ. Irinotecan-induced cytotoxicity to colon cancer cells in vitro is stimulated by pre-incubation with trifluorothymidine. Eur J Cancer 2007; 43: 175-183.

[24] Torigoe S, Ogata Y, Matono K, Shirouzu K. Molecular mechanisms of sequence-dependent antitumor effects of SN-38 and 5-fluorouracil combination therapy against colon cancer cells. Anticancer Res 2009; 29: 2083-2089.