Effects of irinotecan/5-fluorouracil combination on thymidylate synthase and cell cycle in human colon carcinoma cell line

ABSTRACT

Thymidylate synthase (TS) is responsible for the de novo synthesis of thymidylate, which is required for DNA synthesis and repair TS and is an important target for 5-fluorouracil (5-FU). In this study we investigated whether the greater cytotoxicity of irinotecan (CPT-11) followed by 5-FU in HT-29 and SNU-C4 cell lines was related to TS-mRNA expression or activity. Thus, cells were exposed for 24 h to each drug, alone or in combination, and assessed for colony formation, TS catalytic activity, TS-mRNA expression and cell cycle distribution. Pre-treatment with CPT-11 at IC_{20} increased the 5-FU cytotoxicity in HT-29 and SNU-C4 cells. TS catalytic activity and TS-mRNA expression suggested that the differences in sensitivity to 5-FU among the cell lines were not correlated to TS profile. When we exposed cells to CPT-11 at IC_{20} and subsequently to 5-FU at IC_{50}, the impact on TS activity and mRNA expression were the same as observed with 5-FU alone. CPT-11 induced G2/M arrest, while 5-FU arrested cells in S-phase in both cell lines. When cells were treated with CPT-11 followed by 5-FU we observed an increased in 5-FU-inducible S-phase arrest in both cell lines. Our findings suggested that the greater cytotoxicity of CPT-11/5-FU combination in HT-29 and SNU-C4 cell lines are not related to interference with thymidylate synthase.

KEY WORDS: *Thymidylate Synthase, Colon Carcinoma Cell Lines, 5-Fluorouracil, Irinotecan, Cell Cycle.*

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INTRODUCTION

The combination of irinotecan (CPT-11) with 5-Fluorouracil (5-FU) has been extensively investigated in view of its therapeutic efficacy against advanced colorectal carcinoma. *In vitro* and *in vivo* evidence suggests schedule-dependent cytotoxic interactions for the combination of thymidylate synthase (TS) inhibitors and CPT-11 or its active metabolite SN-38 (1,2,3,4). Several studies with different schedules of CPT-11/5-FU are still under investigation to identify a more practical and tolerable treatment option (5). The enhanced antitumor activity of this combination over that of each agent alone, might be related to the synergistic antiproliferative effects seen in cultured human colon carcinoma cells treated with CPT-11 followed by 5-FU (4,5,6). However, the exact mechanism of interaction of these drugs still remains unclear. It has been suggested that the optimal response of this combination is dependent not only on the sequence of administration, but also on the duration of exposure to the drugs (3,4).

Even though it is proposed that inhibition of TS is the main mechanism of 5-FU cytotoxicity, abnormal RNA and protein processing after incorporation of fluorouridine-5'-triphosphate (FUTP) into various RNA species, and premature chain termination and/or DNA strand breakage upon incorporation of fluorodeoxyuridine-5'-triphosphate (FdUTP) into DNA, may also contribute to varying degrees to 5-FU cytotoxicity (7). CPT-11 is a semi-synthetic derivative of the natural alkaloid camptothecin (8). CPT-11 acts by introducing single-strand DNA breaks upon the stabilization of DNA-topoisomerase I complexes (9).

Thymidylate synthase is a critical target for cancer treatment and represent the major target for the widely used anticancer agent 5-fluorouracil (5-FU) and for new TS inhibiting folate antagonists, such as Raltitrexed and ALIMTA (10,11). TS catalyzes the methylation of dUMP to dTMP for which 5,10-methylene-tetrahydrofolate (CH_2-THF) is the methyl donor (12,13). The activity levels and amount of this en-

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zyme fluctuates with the cell cycle progression with peak activity and maximum content occurring during the S phase (14). Although the cell-cycle-dependency of TS gene expression (15), some evidences demonstrated that the proliferation is an important factor for changes in TS levels (16). The TS gene is located on chromosome 18q, for which a high percentage of alterations have been reported in human colon tumors (17). Among others mechanisms of 5-FU resistance, the most frequent are TS gene amplification increased TS mRNA, insufficient TS inhibition by FdUMP, elevated levels of TS after treatment (18).

To elucidate the role of TS, we investigated whether the interaction of CPT-11/5-FU could be related to TS activity and mRNA expression in human colon carcinoma cell lines.

MATERIALS AND METHODS

Drugs and Chemicals

5-FU was obtained from Hoffman-La Roche Inc (Nutley; NJ, USA), and CPT-11 (CAMPTOSAR®) from Pharmacia & Upjohn Company (Kalamazoo, Michigan, USA). The [5-^{3}H]dUMP (specific activity 19 Ci/mmol) was obtained from Amersham International (Buckinghamshire, United Kingdom).

Cell Culture Maintenance

The HT-29 human colon carcinoma cell line was obtained from American Type Culture Collection (Rockville; MD, USA). The SNU-C4 human colon carcinoma cell line was made available by one of the authors (Dr. G.J. Peters). The cells were maintained in RPMI 1640 medium containing 10% (v/v) fetal calf serum at a temperature of 37 ºC in a humidified atmosphere of 5% CO_2 in air.

Drug Treatments

The cytotoxic effects and interference with thymidylate synthase were examined by exposing cells: (1) during 24 h to the IC_{50} of either 5-FU or CPT-11; and (2) during 2 h with the IC_{20} of CPT-11 followed by 22 h to the IC_{50} of 5-FU. Control cells were cultured during 24 h without drug treatment.

Colony Formation Studies

To investigate the cytotoxic effect of drug treatments we used a clonogenic assay as described previously (19). Briefly, HT-29 and SNU-C4 cells were seeded into 6-well plates (300 cells/well) after treatment and incubated during 7 days. Then, the cells were fixed with 70% ethanol and counterstained with 0.5% crystal violet. Colonies of 50 cells or more were scored as originating from a single clonogenic cell. The survival fraction (SF) was then calculated as:

$$SF = \frac{\text{Number of colonies in treated cells} \times 100}{\text{Number of colonies in untreated control}}$$

Thymidylate Synthase Activity

Thymidylate synthase activity was evaluated with a [^{3}H]-release assay to determine thymidylate synthase catalytic activity, *i.e.* the rate of conversion of deoxyuridine monophosphate (dUMP) into deoxythymidine monophosphate (dTMP) (20). Enzyme suspensions were made from 5 x 10^7 untreated cells by sonication on ice in 0.2 M Tris-HCl pH 7.4, 20 mM β-mercaptoethanol, 100 mM NaF, and 15 mM cytidine monophosphate. Protein contents of the samples were measured spectrophotometrically at 590 nm as described by Bradford (21) using the Bio-Rad Protein Assay Dye Reagent with bovine serum albumin as a standard. [5-^{3}H]-dUMP was used as a substrate at a concentration of 10 µM. Thus, 25 µL enzyme suspensions were incubated for 60 min at 37 ºC with 5 µl of 6.5 mM 5,10-methylenetetrahydrofolate, 10 µL of Tris-HCl buffer, and 10 µL of [5-^{3}H]-dUMP. Reactions were terminated by the addition of 50 µl of ice-cold 35% (w/v) trichloroacetic acid and 250 µL of 10% (w/v) neutral charcoal, and 150 µL supernatant was used for liquid scintillation counting. Data were expressed as pmoles dUMP converted per h per mg protein.

RNA Extraction and Synthesis of cDNA

Total RNA was extracted from 1 x 10^6 cells by Chomczynski & Sacchi (22) method, and quantified spectrophotometrically. First strand cDNA synthesis was performed in a total volume of 10 µL containing 5 µg total RNA, 50 pmol of TS antisense primer and 50 pmol ß-actin antisense primer, 33 units ribonuclease inhibitor, 10 mM dNTP mix, 0.1 M DTT, 250 mM Tris-HCl, and 200 units Mu-MLV reverse transcriptase (Gibco, Rockville, MD). The reaction was incubated at 37°C for 60 min, and then stopped by heat inactivation. The cDNA was stored in aliquots at –20°C.

Thymidylate Synthase mRNA Expression

mRNA levels for TS were estimated by semiquantitative methods as previously described (23,24). Briefly, simultaneous amplifications of TS and ß-actin as an internal standard were performed. The primers used were: the TS sense primer (5'-AGATCCAACACATCCTCCGCT-3'), the TS antisense primer (5'-CAGAACACACGTTTGGTTGTCA-3'), the ß-actin sense primer (5'-CGGGAAATCGTGCGTGACAT-3') and the ß-actin antisense primer (5'-GGAGTTGAAGGTAGTTTCGTG-3'), all of which were designed according to previous study (23). Generally, 10 µl cDNA were amplified in a total volume of 50 µL containing, 50 mM $MgCl_2$, 8.5 mM dNTPs, 1.25 unit of Taq DNA Polymerase (Promega) and specific sense primers (50 pmol of each TS primer and 50 pmol ß-actin) using a 9600 thermal cycler (Perkin Elmer, CA). Initial heating at 94°C for 3 min was followed by 40 PCR cycles (95°C 1 min, 55°C 1 min and 30 seconds, 72°C 1 min). Amplifica-

tion was terminated after a final extension step at 72°C of 5 min. The linear range of amplification was established for each cDNA. Amplified DNA fragments were separated on an ethidium bromide stained 2.0 % agarose gel. Gel pictures were scanned, and the density of each band was quantitated using image analysis software. Relative amounts of mRNA for TS were calculated as TS/ß-actin ratios.

Flow Cytometry Analysis

After treatment, cells were harvested by trypsinization and washed in ice-cold PBS. Samples of floating and adherent cells (5×10^5) were then fixed in 70% ethanol at 4ºC overnight. The 600 x g centrifuged cell pellet was washed in 1 mL ice-cold PBS, and resuspended in 0.5 mL PBS. To a 0.5 mL cell sample, 0.5 mL RNAse A was added, followed by 1 mL propidium iodide (25). Samples were incubated, in the dark, at room temperature for 30 min, and kept at 4ºC in the dark until measured. Cells (15,000) were assessed using the FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA, USA). The DNA content was analyzed using a ModFit 2.0 software (Becton Dickinson, San Jose, CA, USA).

Statistics

Data presented are mean ± SD or mean ± SEM of at least three experiments. One-way ANOVA followed by *Tukey's* test were used to indicate statistical significance. The differences were considered significant when $P < 0.05$. For correlation analysis *Pearson's* coefficient were used.

RESULTS

Cytotoxic Effects of Drugs Combination

The cytotoxic effects of 5-FU and CPT-11 in the HT-29, and SNU-C4 human colon carcinoma cell lines were examined after 24 h drug treatments by colony formation assay. A concentration dependent cytotoxicity was observed with all treatments (Figure 1). When HT-29 cells were exposed to 5-FU alone or CPT-11 alone for 24 h the dose necessary to inhibit 50% (IC_{50} values) the formation of colonies was 8.5 µM and 2.5 µM, respectively. The inhibition of 50% of colony formation in SNU-C4 cells occurred when the cells were exposed to 2.5 µM and 4.0 µM of 5-FU and CPT-11, respectively.

When the cells were exposed to CPT-11 for 2 h at IC_{20} (1.5 and 1.0 µM, for HT-29 and SNU-C4 cell lines, respectively; Table 1) followed by 5-FU for 24 h at IC_{50}, the relative values of Survival Fraction (SF) decreased about 2-fold in HT-29 cells, and 3-fold in SNU-C4 cells when compared to 5-FU alone ($p < 0.05$; Figure 1). When we compared this combination treatment (CPT-11 followed by 5-FU) with CPT-11 at IC_{50}, we noticed a decreased in SF of 2-fold in both cell lines ($p < 0.05$; Figure 1). Thus, as described previously (1-4), when cells were treated with sequential CPT-11 followed by 5-FU a greater effect was observed, when compared to both agents alone.

Thymidylate Synthase Activity

We evaluated the TS activity in order to determine if the observed differences in the sensitivity of the HT-29 and SNU-C4 cell lines to the combination treatment were related to changes in TS activity. The basal level of TS was 1.4-fold higher in SNU-C4 cells when compared to the HT-29 cell line ($p < 0.05$; Figure 2). When HT-29 and SNU-C4 cells were exposed to 5-FU alone, we observed a decreased of TS activity of 3.7-fold and 2.6-fold, respectively, when compared to untreated controls ($p < 0.05$; Figure 2). When we exposed the cells to CPT-11 at IC_{50} no significant alteration on TS activity was observed in both cell lines (Figure 2).

The CPT-11 followed by 5-FU treatment showed a similar pattern than that with the 5-FU at IC_{50} in both cell lines ($p < 0.05$; Figure 2). However, when we compared this treatment with CPT-11 at IC_{50}, we observed a decreased of 3-fold in both cells lines ($p < 0.05$; Figure 2).

Thymidylate Synthase mRNA Expression

Taking into account that TS catalytic activity could be regulated by its mRNA (24), we determined whether the TS-mRNA levels were involved in the different responses to the combination treatments. The basal TS-

Table 1 – Effect of treatment with 5-FU at IC_{50}; 2 h IC_{20} CPT-11 followed by 22 h 5-FU at IC_{50}; and 24 h CPT-11 at IC_{50} on cell cycle distribution of HT-29 and SNU-C4 human colon carcinoma cell lines. Data was expressed as the mean ± SD of three different experiments. *significantly different from untreated control.

	HT-29			SNU-C4		
	G0/G1	S	G2/M	G0/G1	S	G2/M
Untreated Control	56.6 ± 3.8	25.6 ± 0.5	16.9 ± 2.0	61.2 ± 1.3	16.6 ± 3.8	18.9 ± 4.3
5-FU at IC50	43.7 ± 0.4	39.8 ± 1.0*	12.1 ± 0.2	44.4 ± 2.3	45.5 ± 1.0*	14.9 ± 1.3
CPT-11 > 5-FU	38.0 ± 3.4	52.5 ± 0.3*	10.7 ± 1.0	43.7 ± 0.3	45.8 ± 2.3*	15.6 ± 2.3
CPT-11 at IC50	37.5 ± 1.5	25.7 ± 0.7	36.2 ± 4.7*	42.1 ± 7.1	16.3 ± 0.7	40.8 ± 7.4*

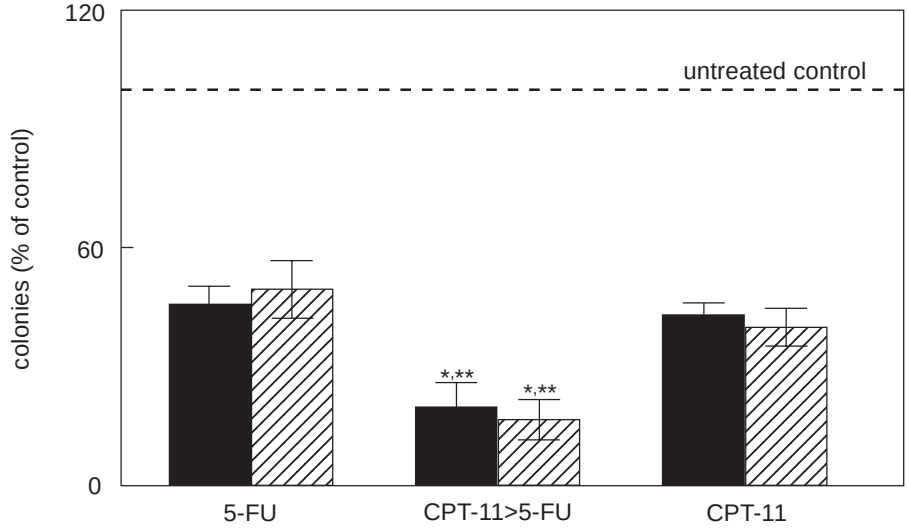


Figure 1 – Effect of treatment with 5-FU alone; 2 h IC_{20} CPT-11 followed by 22 h 5-FU and 24 h CPT-11 alone, in HT-29 (■) and SNU-C4 (▨) human colon carcinoma cells survival fraction. Following treatment, cells were incubated during 10 days. Colonies containing ≥ 50 cells were scored. Data was expressed as the percentage of the survival fractions of cells versus the different treatments. Data was plotted as the mean ± SD of three different experiments. *significantly different from 5-FU at IC_{50};**significantly different from CPT-11 at IC_{50}.

mRNA levels demonstrated that SNU-C4 cells had 1.3-fold more TS-mRNA expression than the HT-29 cell line ($p < 0.05$; Figure 3).

The treatment with CPT-11 at IC_{20} followed by 5-FU IC_{50} decreased the TS-mRNA expression in 14% in HT-29 cells, and in 24% in SNU-C4 cell line, when compared to untreated controls (Figure 3). When compared to 5-FU at IC_{50}, this treatment (CPT-11 at IC_{20} followed by 5-FU at IC_{50}) increased TS-mRNA expression in about 30% in both cell lines (Figure 3; $p < 0.05$). Treatment with CPT-11 at IC_{50} showed the same TS-mRNA profile of the untreated controls, in both cell lines (Figure 3).

TS activity and TS-mRNA expression were compared within the same samples to determine if the levels of TS-mRNA correlated to TS catalytic activity. A linear relation was noted between TS catalytic activity and TS-mRNA expression in SNU-C4 cells ($r^2 = 0.6038$), while HT-29 cell line did not show correlation ($r^2 = 0.2503$).

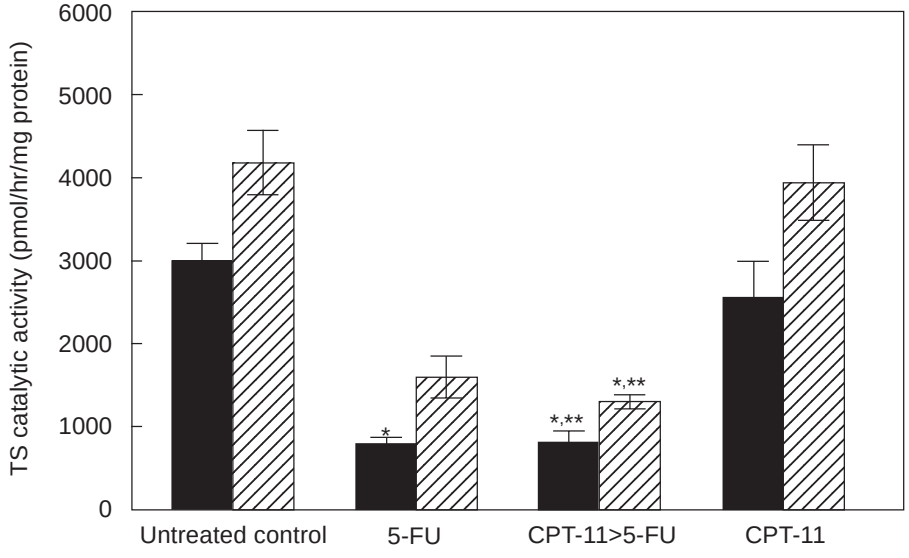


Figure 2 – Effect of treatment with 5-FU at IC_{50}; 2 h IC_{20} CPT-11 followed by 22 h 5-FU at IC_{50} and 24 h CPT-11 at IC_{50}, in HT-29 (■) and SNU-C4 (▨) human colon carcinoma cells on TS catalytic activity. TS catalytic activity in untreated and treated cells were expressed as pmol/hr/mg protein (mean ± SEM) of three different experiments. *significantly different from untreated control;**significantly different from CPT-11 at IC_{50}.

Interference on Cell Cycle Distribution

To further investigate if the interaction of CPT-11 with 5-FU, cell cycle regulation was evaluated using a flow cytometry. After the 24 h treatment with either drugs alone or combined, we observed a similar pattern of cell cycle distribution in both cell lines. In both untreated cell lines, 50-60 % of the cells were in G1 phase, 15-25 % in S phase and about 17 % in G2/M phase (Table 1).

The flow cytometry analysis demonstrated that HT-29 cells were arrested in S-phase after exposure to 5-FU alone or CPT-11 at IC_{20} followed by 5-FU at IC_{50}. The most pronounced effect on cell cycle was observed when we treated cells with CPT-11 at IC_{20} followed by 5-FU at IC_{50}. This treatment caused an increased of approximately 50% of cells in S-phase ($p < 0.05$; Table 1). In SNU-C4 cells the treatments containing 5-FU increased in 2.8-fold the number of cells in S-phase ($p < 0.05$; Table 1). In both cell lines, the treatment with CPT-11 alone increase in 2-fold the number of cells in G2/M phase (Table 1).

DISCUSSION

The combination of 5-FU with the topoisomerase I inhibitor CPT-11 is an important strategy in the treatment of advanced colon carcinoma. Previous studies have demonstrated that the sy-

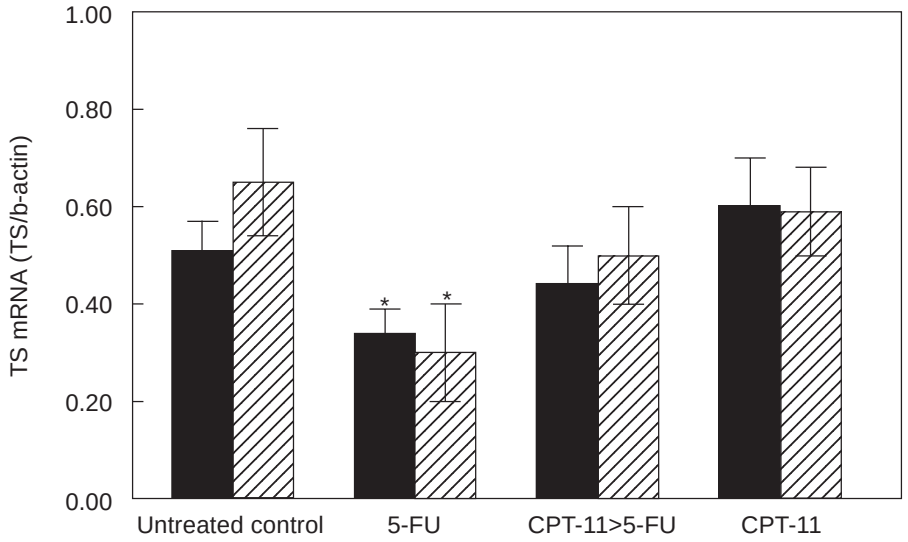


Figure 3 – Effect of treatment with 5-FU at IC_{50}; 2 h IC_{20} CPT-11 followed by 22 h 5-FU at IC_{50} and 24 h CPT-11 at IC_{50}, in HT-29 (■) and SNU-C4 (▨) human colon carcinoma cells on TS-mRNA. Data were expressed as TS/ß-actin ratio (mean ± SD) of three different experiments. *significantly different from untreated control.

nergy between TS and topoisomerase I inhibitors is greatest when the topoisomerase I inhibitors are administered prior to the TS inhibitors (1,2,3,4). However, the mechanisms involved in this synergism, have not been elucidated. In the present study, we investigated the effects of the combination with CPT-11/5-FU on cell cycle, TS activity and TS-mRNA expression on the HT-29 and SNU-C4 cell lines. We demonstrated, in previously study (1), that prior treatment with CPT-11, at a low cytotoxic concentration (IC_{20}), potentiated 5-FU-mediated cytotoxicity. Although the two cell lines (HT-29 and SNU-C4) differed significantly (up to 8-fold) in their sensitivity to 5-FU, they showed less difference (approximately 2-fold) in their response to CPT-11. These findings are in agreement with the absence of cross-resistance between both drugs, as well as with the demonstrated efficacy of CPT-11 in 5-FU-resistant colon carcinoma (26,27,28).

The 5-FU treatment significantly decreased the TS-mRNA expression and TS catalytic activity in both cell lines. This is in agreement with Peters *et al* (29-31) who reported a decrease in TS activity in cell lines (30), and TS-mRNA in patients (31) after 23 h of 5-FU treatment, and with Xu *el al.* (32) that demonstrated a depletion of free TS after 24 h treatment in cell lines. In line with previous studies (30,33), it was found that 5-FU induced a concentration dependent TS inhibition.

When HT-29 and SNU-C4 cells were treated with CPT-11 at IC_{20} followed by 5-FU we observed a similar TS activity inhibition as with 5-FU alone. These effects most probably are due only to 5-FU at IC_{50} concentration, and not associated with CPT-11 at IC_{20} interference on neither TS activity nor TS-mRNA expression. Our results corroborate a previous study (33), which suggested that SN-38 (a CPT-11 metabolite) does not interfere with TS expression. Indeed, CPT-11 at IC_{20} did not induce significant modification on TS activity or TS-mRNA expression. The same held true for CPT-11 at IC_{50}.

The pattern of cell cycle distribution observed in this study demonstrated that in both cell lines CPT-11, at IC_{50} concentration, mainly induced G2/M arrest, while 5-FU preferentially induced arrest in S-phase. Previous studies (34,35), have found that 5-FU induced S-phase arrest. This arrest should be induced by inhibition of DNA synthesis in the middle of replication due to dTTP depletion (36). In this study, the S-phase arrest was more pronounced in the SNU-C4 cell line compared to HT-29 cells. It is possible that this effect was due to a more specific inhibition of thymidylate synthase by 5-FU or its metabolite FdUMP in SNU-C4 cells. When cells were treated with CPT-11 followed by 5-FU we noticed an enhance in 5-FU-inducible S-phase arrest in both cell lines. This could explain the greater cell growth inhibition observed with this combination.

In summary, our data indicated that the synergistic response observed with the CPT-11 followed by 5-fU combination could not be attributed simply to an enhanced effect of CPT-11 on the expression or activity of thymidylate synthase. Yet additional investigations are required, our results suggested that alterations on cell cycle phase distribution play an important role in the effects observed with this combination treatment.

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