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ORIGINAL ARTICLE

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Effect of adding the topoisomerase I poison 7-ethyl-10-hydroxycamptothecin (SN-38) to 5-fluorouracil and folinic acid in HCT-8 cells: elevated dTTP pools and enhanced cytotoxicity

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Abstract *Purpose*: To determine the effect of combined treatment with 7-ethyl-10-hydroxycamptothecin (SN-38, the active metabolite of irinotecan) and 5-fluorouracil/folinic acid (5FU/FA) in vitro using HCT-8 human intestinal adenocarcinoma cells. *Methods*: Cell survival was examined using colony forming assays. Cell cycle distribution before and after treatment was assessed by flow microfluorimetry. Levels of thymidylate synthase (TS) and topoisomerase I (topo I) in untreated and treated cells were determined by immunoblotting. Changes in deoxynucleotide pools were examined by high-performance liquid chromatography. *Results*: Clonogenic assays revealed that colony formation was decreased by 50% after a 24-h exposure to 8 ± 2 n*M* SN-38 or 12 ± 3 μ*M* 5FU, the latter being assayed in the presence of 2 μ*M* FA. When treatment with 5FU/FA was followed by SN-38, the cytotoxicity was similar to that observed with 5FU/FA alone. In contrast, when HCT-8 cells were exposed to both agents simultaneously or to SN-38 followed by 5FU/FA, the cytotoxicity was greater than that of SN-38 or 5FU/FA treatment alone. Investigation of the mechanistic basis for this sequence dependence revealed that SN-38 treatment was associated with a dose- and time-dependent decrease in conversion of [5-^{3}H]-2′-deoxyuridine to [^{3}H]-H_2O and thymidylate in intact cells. Immunoblotting failed to reveal any decrease in TS protein that could account for the decreased activity. High-performance liquid chromatography revealed that SN-38 treatment was associated with increased levels of the deoxynucleotide dTTP and decreased levels of dUTP. Flow microfluorimetry revealed that a 24-h treatment with 10 n*M* SN-38 resulted in accumulation of HCT-8 cells in late S and G_2 phases of the cell cycle, with a further increase in the G_2 fraction during the 24 h after SN-38 removal. *Conclusions*: These observations are consistent with a model in which SN-38 sequentially induces diminished DNA synthesis, elevated dTTP pools, inhibition of dUMP synthesis and enhanced toxicity of 5FU/FA. Accordingly, sequencing of irinotecan and 5FU/FA might be important in combining these agents into an effective treatment for colorectal cancer.

Key words Irinotecan · Deoxynucleotide pools · Thymidylate synthase · Fluoropyrimidines · Colon cancer

Abbreviations *CI* combination index · *dCMP* deoxycytidine monophosphate · *dGTP* deoxyguanosine triphosphate · *dNTP* deoxyribonucleotide triphosphate · *dTTP* deoxythymidine triphosphate · *dUMP* deoxyuridine monophosphate · *dUTP* deoxyuridine triphosphate · *FA* folinic acid · *5FU* 5-fluorouracil · IC_{50}, dose that inhibits the measured effect by 50% · LD_{50}, dose that inhibits colony formation by 50% · MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide · PBS calcium-, magnesium-free Dulbecco's phosphate-buffered saline · *SDS* sodium dodecylsulfate · *SN-38* 7-ethyl-10-hydroxycamptothecin · *TK* thymidine kinase · *topo* topoisomerase · *TS* thymidylate synthase

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Introduction

5FU is currently the single most active agent available for the treatment of colorectal cancer. When used in

conjunction with FA (leucovorin), 5FU has a response rate of 23% for metastatic colon cancer [1, 25, 27]. Until recently, there was no effective treatment for patients with metastatic colorectal cancer that had progressed following 5FU-based therapy.

Several recent phase I and phase II studies have indicated that the topo I-directed agent irinotecan has a response rate of 20–30% in patients with metastatic colon cancer (reviewed in references 2 and 8), including patients who have progressed or recurred after fluoropyrimidine therapy [34]. These observations raise the question of whether 5FU and irinotecan can be productively combined for the treatment of colorectal cancer.

Based on the mechanism of action of these agents, there is reason to believe that they might be antagonistic. 5FU is converted to 5-fluoro-2′-deoxyuridine monophosphate, which inhibits TS, decreases dTTP pools, inhibits DNA synthesis and causes a G_1/S cell cycle arrest (reviewed in reference 13). SN-38, the active metabolite of irinotecan, stabilizes covalent complexes between topo I and nuclear DNA. When moving replication complexes encounter the drug-stabilized topo I–DNA complexes, DNA double-strand breaks result (reviewed in references 6, 11, 31, 39, 40). In response to these DNA double-strand breaks, cells accumulate in the G_2 phase of the cell cycle [41, 43]. In other words, 5FU might be expected to inhibit the DNA synthesis that is required for the cytotoxicity of SN-38; and SN-38 might be expected to cause cells to accumulate in G_2 rather than S phase, where they would be more sensitive to 5FU.

A previous study has suggested that addition of 5FU to irinotecan results in additive cytotoxicity in Molt3 leukemia cells when survival is assessed using an MTT assay after 3 days of continuous exposure to one or both agents [20]. The MTT assay, however, has subsequently been criticized as an unreliable index of cell survival in certain human leukemia cell lines [16, 29]. In view of the widespread clinical interest in combining 5FU and irinotecan [14, 30, 36–38, 45], the effect of combining SN-38 and 5FU was reevaluated in the present study. For these experiments, clonogenic assays were utilized to evaluate the toxicity of these treatments in HCT-8 human intestinal adenocarcinoma cells [42]. To better approximate the regimen that is utilized clinically, FA was added to 5FU. We report a sequence-dependent synergy between SN-38 and 5FU/FA and provide a potential mechanistic explanation for this observation. A preliminary account of this work has been presented [9].

Materials and methods

Reagents

SN-38 was a kind gift from Upjohn/Pharmacia (Kalamazoo, Mich.). 5FU and FA were purchased from Sigma (St. Louis, Mo.). [5-^{3}H]-2′-Deoxyuridine (24.9 Ci/mmol) was obtained from Moravek (Brea, Calif.). Monoclonal antibodies to topo I [5], TS [19] and histone H1 [23] were kindly provided by Drs. Y-C. Cheng (Yale University School of Medicine, New Haven, Ct.), P. G. Johnston (NCI Navy, Bethesda, Md.), and J. Sorace (Veterans' Affairs Medical Center, Baltimore, Md.). Chicken antilamin B1 antiserum was raised as described previously [21].

Clonogenic assays

HCT-8 cells obtained from American Type Culture Collection (Rockville, Md.) were routinely passaged in RPMI-1640 medium containing 5% heat-inactivated fetal bovine serum, 100 U/ml penicillin G, 100 μg/ml streptomycin, and 2 m*M* glutamine (medium A). For clonogenic assays, trypsinized HCT-8 cells were resuspended in medium A containing 5% dialyzed fetal bovine serum in place of nondialyzed serum (medium B), plated in 35-mm tissue culture plates (500 cells/plate) and incubated for 14–16 h to allow cells to adhere. SN-38 and/or 5FU (prepared as 1000-fold concentrated stocks in dimethylsulfoxide and H_2O, respectively) were added to the indicated concentrations. FA was present throughout the 5FU exposure at 2 μM, a concentration that was shown in preliminary experiments to be sufficient for maximal sensitization of this cell line to 5FU. After a 24-h incubation, plates were washed twice with serum-free RPMI-1640 medium, refed with medium B, and incubated for 10 days. The resulting colonies were stained with Coomassie brilliant blue so that visible colonies could be counted. Control (diluent-treated) plates typically contained 150–200 colonies.

To examine the effect of sequencing the treatments, cells were plated in medium B as described above, incubated with one drug for 24 h as indicated, washed twice, incubated with the second drug in medium B for 24 h, washed and incubated in drug-free medium B for the duration of the 10 day incubation period. In conjunction with these experiments, exposure to the single agents took place at the same time as exposure to the drugs in sequence.

Analysis of combined drug effects

Combined drug effects were analyzed by the median effect method [7] as previously described [24]. In brief, cells were treated with serial dilutions of each drug individually and with both drugs simultaneously or sequentially at a fixed ratio of doses that typically corresponded to 1/2, 5/8, 3/4, 7/8, 1 and $1_{1/2}$ times the individual IC_{50} values. The fractional survival (f) was calculated by dividing the number of colonies in drug-treated plates by the number of colonies in control plates. Log[(1/f)−1] was plotted against log (drug dose). From the resulting graphs, the x intercept (log IC_{50}) and slope m (a measure of sigmoidicity) were calculated for each drug and for the combination by the method of least squares. These parameters were then used [7] to calculate doses of the individual drugs and the combination required to produce varying levels of cytotoxicity (f = 0.95, 0.90, 0.85,…,0.05) according to the equation:

$$\mathrm{Dose}_f = \mathrm{Dose}_{IC_{50}}[(1-f)/f]^{1/m} \quad (1)$$

Because the two drugs were administered at a fixed ratio, the dose of the combination required to produce fractional survival f could be divided into the component doses $(D)_1$ and $(D)_2$ of drugs 1 and 2, respectively. For each level of cytotoxicity (f = 0.95, 0.90, 0.85, 0.05) the combination index (CI)[7] was then calculated according to the equation:

$$\mathrm{CI} = (D)_1/(D_f)_1 + (D)_2/(D_f)_2 + \alpha(D)_1(D)_2/(D_f)_1(Df)_2 \quad (2)$$

where $(D)_1$ and $(D)_2$ are the concentrations of the combination required to produce survival f, $(D_f)_1$ and $(D_f)_2$ are the concentrations of the individual drugs required to produce f, and $\alpha = 1$ or 0 depending on whether the drugs are assumed to be mutually nonexclusive or mutually exclusive, respectively [7]. In this method, synergy is indicated by CI < 1, additivity by CI = 1, and antagonism by CI > 1 [7].

Unless otherwise indicated, experiments were repeated until three replicates yielded correlation coefficients $R > 0.9$ for all three

median effect lines [7]. Results of multiple experiments are summarized by indicating the mean ± standard deviation of the CI at the indicated level of colony inhibition. Because SN-38 and 5FU/FA act on different intracellular targets, they were assumed throughout to be mutually nonexclusive. CI values calculated under the assumption that these agents are mutually exclusive are also presented in the figures.

Whole cell TS assay

Subconfluent HCT-8 cells growing in six-well plates were incubated with increasing concentrations of SN-38 in 2 ml RPMI-1640/10% dialyzed fetal calf serum containing 125 U/ml penicillin G, and 1.25 μg/ml streptomycin. Whole cell TS activity was assayed as previously described [32, 44]. In brief, 3 μCi[5-^{3}H]-2′-deoxyuridine was added to each well. At 10, 20, 30, 40, 50 and 60 min of incubation, 100-μl aliquots of medium were removed from each well and diluted with 200 μl of ice cold 2% (w/v) trichloroacetic acid containing 10% (w/v) activated charcoal. The resulting sample was vortexed, incubated on ice for 15 min and centrifuged at 12 000 g for 10 min at 4 °C. The radioactivity in a 100-μl aliquot of supernatant (representing [^{3}H]-labeled H_2O produced by the TS reaction) was quantitated by scintillation counting. The amount of product released was plotted as a function of incubation time, and the slope (indicating product released per unit time) was determined by the method of least squares. The rate of [^{3}H]-labeled H_2O release from drug-treated cells was compared to that from diluent-treated cells.

Determination of dNTP levels

dNTP pools were examined by HPLC as described by Houghton et al. [17]. In brief, cells treated for 24 h with 0–30 n*M* SN-38 were washed in ice-cold PBS containing 0.1% (w/v) glucose and lysed in 220 μl 0.5 *M* perchloric acid at 4 °C. After centrifugation at 12 000 *g* for 30 s at 4 °C, supernatants were neutralized with an equal volume of freon/tri-*N*-octylamine followed by 15 s of vortexing. In order to oxidize the ribonucleotides, 300 μl of the aqueous upper phase was treated with 30 μl 0.2 *M* sodium periodate followed, within a few minutes, by 18.75 μl 4 *M* methylamine. After a 30-min incubation at 37 °C, 3 μl 1 *M* rhamnose was added to destroy any remaining periodate. HPLC was then performed on a Beckman Model 366 System Gold chromatograph equipped with a Model 168 diode array detector. dNTPs were separated by ion pair reverse-phase HPLC on an Alltech 7-μm nucleotide/nucleoside column (250 × 4.6 mm) under the conditions described by Houghton et al [17]. Elution was monitored at 254 nm. Authentic dATP, dGTP, dUTP and dTTP subjected to treatment with perchloric acid, freon/tri-*N*-octylamine and sodium periodate were utilized as standards to permit quantitation of dNTP pools. Retention times for dATP, dGTP, dUTP and dTTP were 7.6, 11.8, 16.1, and 26.5 min, respectively.

Flow cytometry

Cells were incubated for 24 h with the indicated concentration (typically two times the LD_{50}) of SN-38 or 5FU/FA. Cells were then washed and incubated in drug-free medium for 0, 8, 16, or 24 h. At the completion of the incubation, cells were released by trypsinization, sedimented at 200 *g* for 10 min, washed twice with ice-cold PBS, and fixed by dropwise addition of ethanol to a final concentration of 50% (v/v). After incubation for at least 1 h at 4 °C, samples were washed twice with PBS, resuspended in 0.1% (w/v) sodium citrate containing 1 mg/ml RNase A and incubated at 37 °C for 15 min. Propidium iodide was then added to a final concentration of 50 μg/ml. After a 15-min incubation at 21 °C in the dark, samples were subjected to flow microfluorimetry on a Becton-Dickinson FACScan (San Jose, Calif.) using an excitation wavelength of 488 nm and an emission wavelength of 585 nm. Histograms were analyzed using ModFit software (Verity Software House, Topsham, Me.).

Immunoblotting

To examine the effect of drug treatment on topo I and TS polypeptide content, cells were incubated with individual agents as indicated elsewhere in the text, washed in serum-free ice-cold RPMI-1640, solubilized in buffer consisting of 250 m*M* Tris-HCl (pH 8.5 at 21 °C) containing 6 *M* guanidine hydrochloride, 10 m*M* EDTA, 1% (v/v) β-mercaptoethanol and 1 m*M* α-phenylmethylsulfonyl fluoride (freshly added from a 100 m*M* stock in anhydrous isopropanol), and prepared for electrophoresis as previously described [22]. Samples containing 50 μg of protein were solubilized in SDS sample buffer consisting of 4 *M* urea, 2% (w/v) SDS, 62.5 m*M* Tris-HCl (pH 6.8) and 1 m*M* EDTA, heated to 65 °C for 20 min, separated on SDS-polyacrylamide gels containing a linear 5–15% acrylamide gradient, and transferred to nitrocellulose. Immunoblotting was performed [22] using antibodies to topo I [5] or TS [19]. To confirm that lanes were loaded equally, blots were reprobed with antiserum that recognizes lamin B_1, a major structural polypeptide of the nucleus [21], and with monoclonal antibody to histone H1, a chromatin protein that is present in equal amounts in all diploid nuclei.

Results

Effect of simultaneous exposure to SN-38 and 5FU/FA

When HCT-8 human adenocarcinoma cells were incubated for 24 h with increasing concentrations of 5FU in the presence of 2 μ*M* FA and subsequently assayed for colony formation, an LD_{50} of 12 ± 3 μ*M* ($n = 6$) was observed (solid line, Fig. 1A). The LD_{50} of SN-38 was 8 ± 2 n*M* ($n = 9$) on the same schedule (solid line, Fig. 1B). When cells were exposed to both treatments simultaneously, more cells were killed than when cells were exposed to each treatment alone (dashed lines, Fig. 1A,B).

The median effect method [7] was used to compare the observed cytotoxicity of the combination with the cytotoxicity that would be expected if the effects of the two agents were strictly additive. Of the various methods for assessing drug interactions that have been proposed (reviewed in references 3, 7 and 12), the median effect method was chosen because it requires data sets that are within the limits of experimental feasibility (50–100 tissue culture plates) in colony-forming assays. In applying this method, log $[(1/f)-1]$ versus log(drug dose) was plotted for each treatment (Fig. 1C). From the resulting lines, the x intercept and slope were determined and then used to calculate the CI, a parameter that indicates whether the doses of the two agents required to produce a given degree of cytotoxicity are greater than ($CI > 1$), equal to ($CI = 1$), or less than ($CI < 1$) the doses that would be required if the effects of the two drugs were strictly additive. For the combination of SN-38 and 5FU/FA, the CI calculated under the assumption that the treatments were mutually nonexclusive was 1.5 ± 0.1 (mean ± SD, $n = 3$) at the IC_{50} of the combination and >1 over much of the entire range of cytotoxicity (Fig. 1D, solid line), suggesting that the effects of these two agents were less than additive.

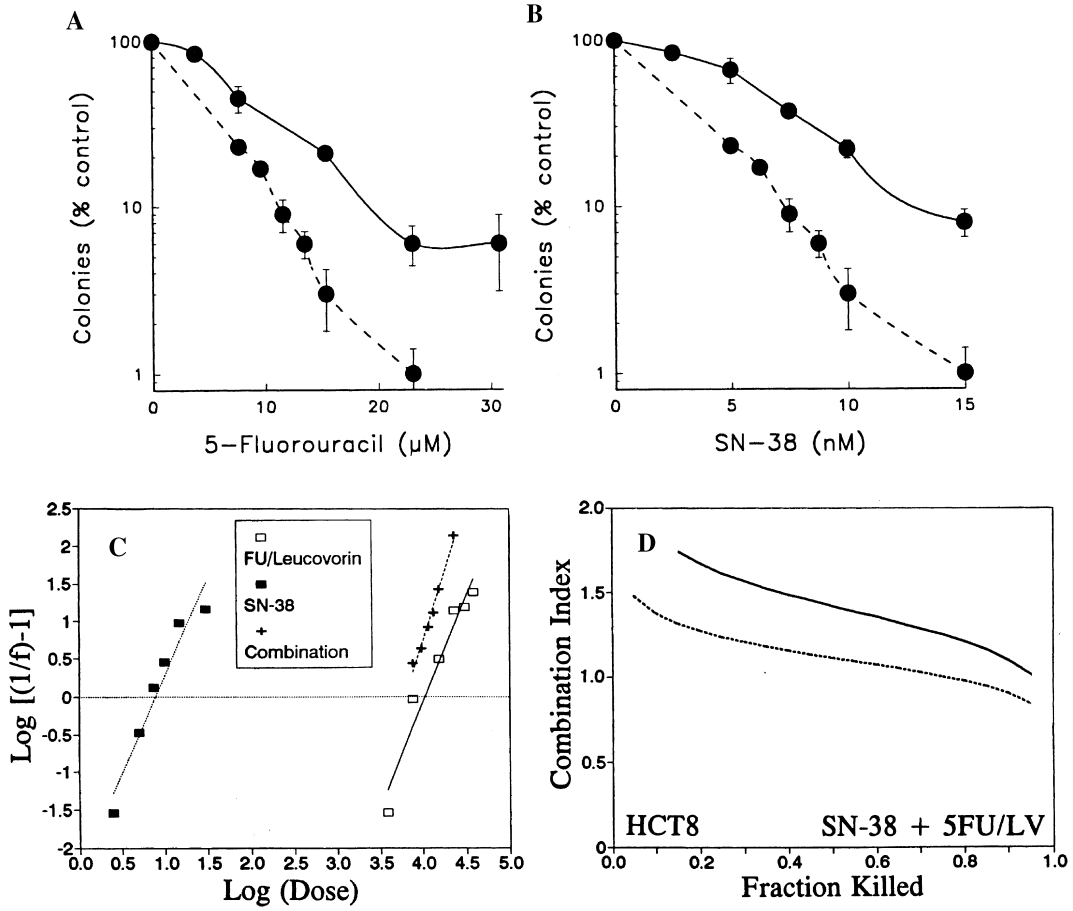

Fig. 1A–D Cytotoxicity of 5FU/FA and SN-38 individually and simultaneously in HCT-8 cells. **A, B** Cells were incubated for 24 h with 5FU in the present of 2 μ*M* FA (**A**, *solid line*), SN-38 alone (**B**, *solid line*), or a fixed 1540: 1 molar ratio of 5FU and SN-38 in the presence of 2 μ*M* FA (**A** and **B**, *dotted lines*). Colony formation was plotted relative to diluent-treated cells. **C** Median effect plot of data in **A** and **B**. **D** Plot of CI vs cytotoxicity calculated from data in **C** under the assumptions that agents are mutually nonexclusive (*solid line*) or mutually exclusive (*dotted line*). Error bars in **A** and **B**, mean ± 1 standard deviation of triplicate plates. Results are representative of three separate experiments

Effect of sequential exposure to SN-38 and 5FU/FA

In subsequent experiments, the effect of sequential exposure to the two treatments was examined. In performing these experiments, it was noted that each of the single treatments (SN-38 or 5FU/FA) was less effective when the agent was administered on the second day after plating as opposed to the first day (cf. solid lines in Fig. 2B vs Fig. 1B or Fig 2D vs Fig 1A). This effect, which has previously been reported with other agents [4], appears to reflect the fact that more cells must be killed to inhibit colony formation to the same extent after the cells have doubled. Accordingly, to provide a basis for comparison, each experiment included treatments with the single agents at exactly the same time that the cells were treated with each agent in sequence. Results of this analysis are depicted in Fig. 2.

When HCT-8 cells were exposed to 5FU/FA followed by SN-38 (Fig. 2A–C), the combination was not much more effective than 5FU/FA alone (Fig. 2A). Consistent with this observation, analysis by the median effect method revealed that the combined effects were less than additive, with a CI of 1.3 ± 0.2 and 1.4 ± 0.3 ($n = 3$) at the LD_{50} and LD_{90}, respectively, of the combination (Fig. 2C). In contrast, when 5FU/FA followed SN-38, the combination was again more effective than either treatment alone (Fig. 2D, E). At the IC_{50} concentration of 5FU/FA, for example, it was observed that pretreatment with SN-38 at approximately its IC_{50} diminished colony formation to 14 ± 8% ($n = 4$) of control (untreated) values.

Effect of SN-38 on TS activity, TS polypeptide levels, and dNTP pools

To assess the mechanistic basis of this sequence-dependent interaction, we examined the possibility that SN-38 might alter the cellular activity of TS and/or perturb the cell cycle distribution in such a way that cells were more likely to traverse S phase during the 24 h after SN-38 removal. To address the first of these possibilities, HCT-8 cells were incubated with increasing concentrations of SN-38 for 24 h, then assayed for TS activity using a

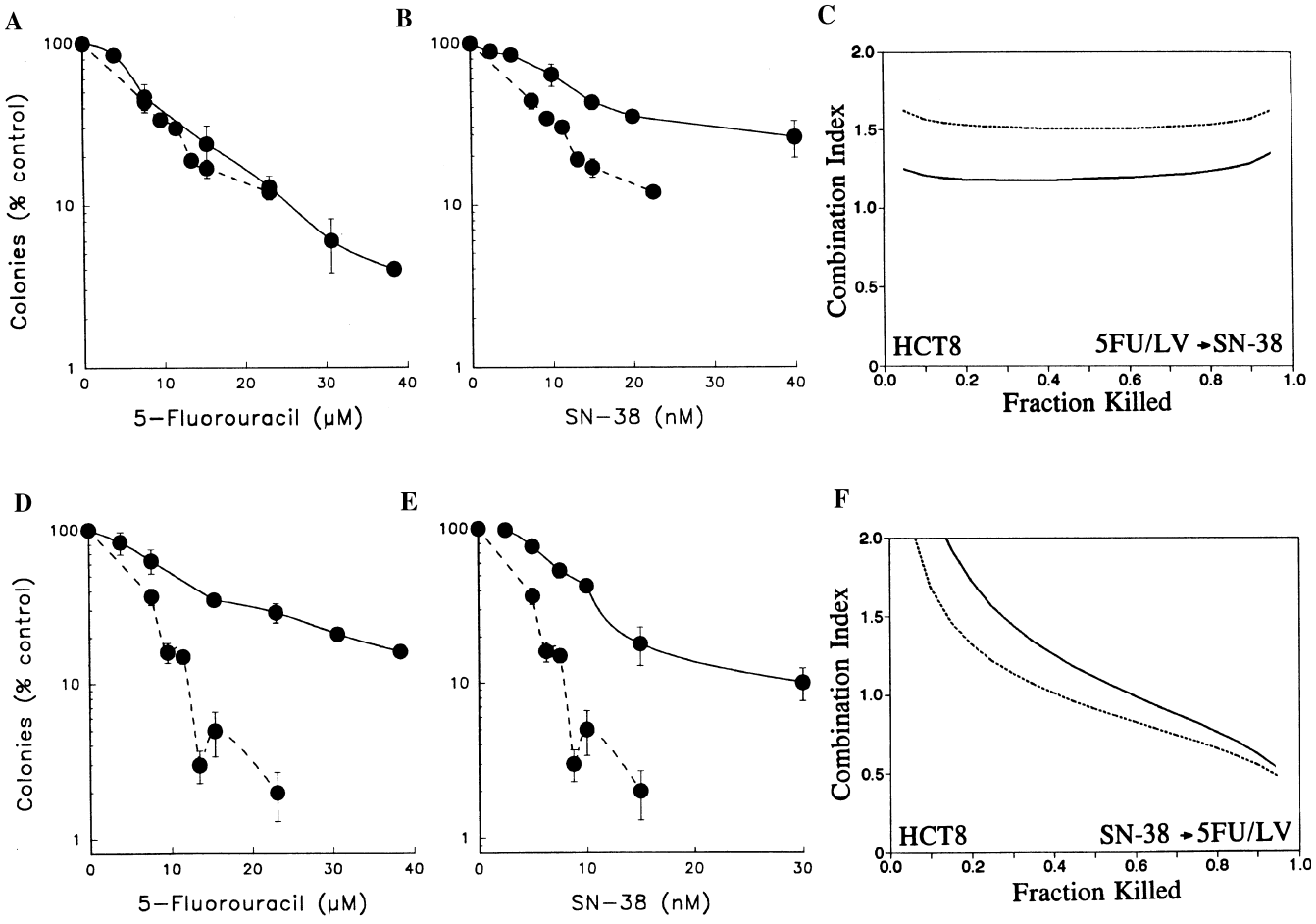

Fig. 2A–F Cytotoxicity of sequential SN-38 and 5FU/FA in HCT-8 cells. **A–C** cells were treated with 5FU/FA (24 h) followed by SN-38 (24 h) at a 1020:1 molar ratio or with the individual agents at the corresponding times. **A, B** Colony formation after treatment with the combination (*dotted lines*) or the individual agents (*solid lines*) is plotted relative to diluent-treated samples. **C** The CI calculated from the same data under the assumption that agents are mutually nonexclusive (*solid line*) or mutually exclusive (*dotted line*) is plotted relative to cytotoxicity. **D–F** Cells were treated with SN-38 (24 h) followed by 5FU/FA (24 h) at a 1:1540 molar ratio or with the individual agents at the corresponding times. Data are plotted as described for **A–C**. Results are representative of three separate experiments each

previously described whole cell assay [32, 44]. SN-38 treatment for 24 h resulted in a dose-dependent decrease in conversion of [5-^{3}H]-2′-deoxyuridine to [^{3}H]-H_2O (Fig. 3A).

Because the decrease in [^{3}H]-H_2O generation in the whole cell assay might reflect either a decrease in TS polypeptide content or a change in endogenous nucleotide pools (or both), these parameters were assayed in further experiments. To examine potential changes in TS polypeptide content, cells treated with SN-38 for 24 h were incubated in drug-free medium for 0–24 h, and subjected to immunoblotting with a monoclonal anti-TS antibody. Results of this experiment revealed that TS protein was similar to control levels in cells harvested at the end of a 24-h SN-38 treatment (Fig. 3B, lanes 5–8) or during the ensuing 24 h, the time when 5FU/FA would be present if cells were treated with the two agents in sequence (Fig. 3B, lanes 9–12).

To search for SN-38-associated changes in deoxynucleotide pools, perchloric acid lysates of cells treated with SN-38 for 0–24 h were neutralized, treated with sodium periodate to oxidize ribonucleotides, and then subjected to reverse-phase HPLC. Results of these assays are summarized in Fig. 3F. In brief, a 24-h treatment with 10 n*M* SN-38 was associated with a 1.5- to 2.5-fold increase in dTTP pools and a > 50% decrease in dUTP pools.

Effect of SN-38 and 5FU/FA on cycle cell distribution

To evaluate the potential role of cell cycle perturbations in the sequence-dependent synergy depicted in Fig. 2, HCT-8 cells were treated with SN-38 or with 5FU/FA for 24 h, then washed and harvested at periodic intervals over the next 24 h. Flow microfluorimetry revealed that 24 ± 5% and 10 ± 4% (mean ± SD, $n = 5$) of the cells were in S and G_2 phases of the cell cycle, respectively, after diluent treatment (Fig. 4A). After a 24-h exposure to 30 μ*M* 5FU and 2 μ*M* FA, there was little discernible effect on cell cycle distribution (Fig. 4B). In contrast, after a 24-h exposure to 10 n*M*

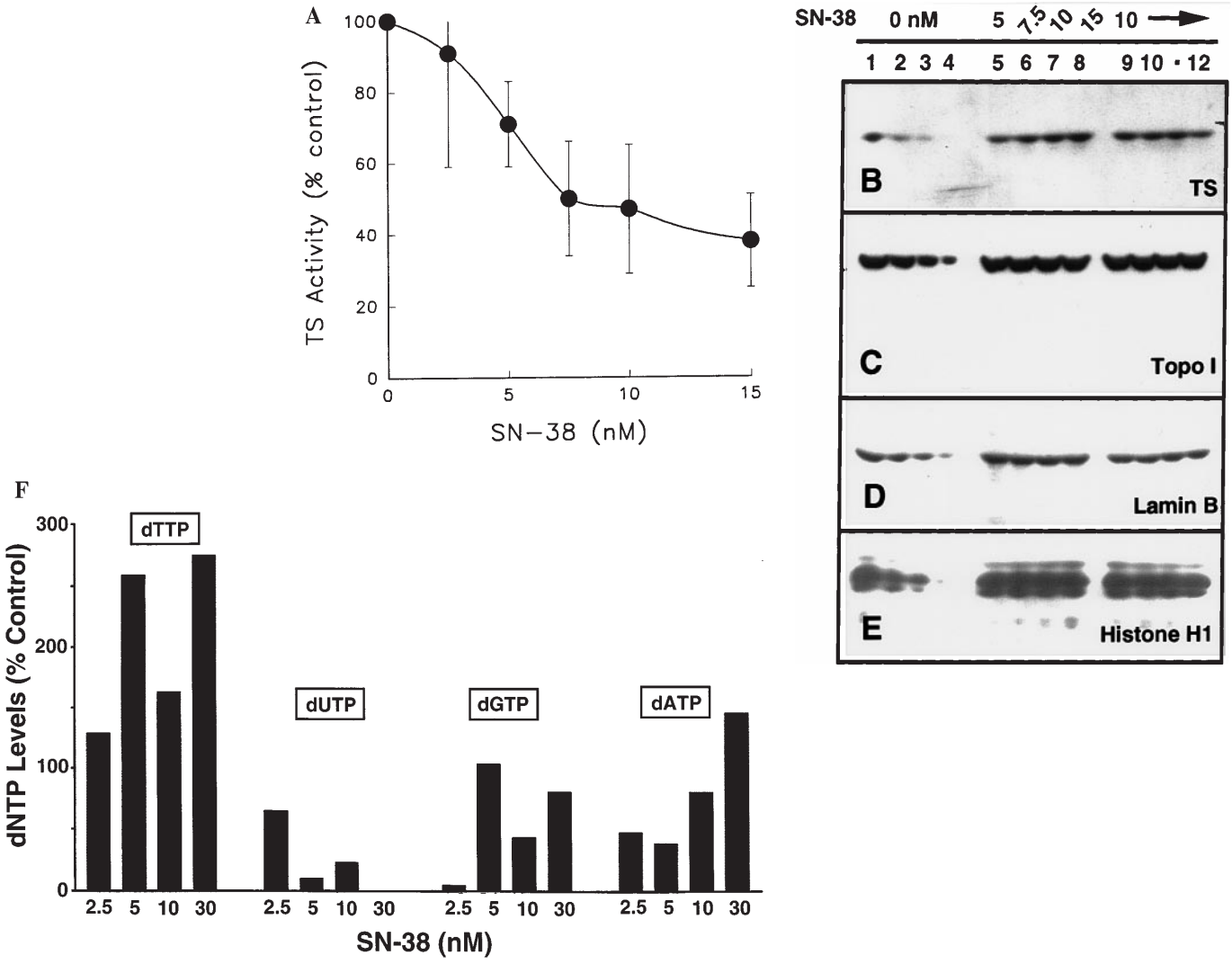

Fig. 3A–F Effect of SN-38 on TS activity, TS polypeptide content and dNTP pools. **A** Cells were incubated for 24 h with the indicated concentration of SN-38. Whole cell TS activity was then determined as described in Methods. **B–E** Cells were treated with the indicated concentration of SN-38 for 24 h and immediately lysed (*lanes 1–8*) or washed and incubated in drug-free medium B for 4, 8, 16 or 24 h (*lanes 9–12*, respectively) prior to lysis. After samples were prepared for SDS-polyacrylamide gel electrophoresis, lanes were loaded with 50 μg of protein (*lanes 1* and *5–12*) or with 25, 12.5 and 5 μg of protein (*lanes 2–4*, respectively). After polypeptides were transferred to PVDF membrane, immunoblotting was performed using probes for TS (**B**), topo I (**C**), lamin B_1 (**D**) or histone H1 (**E**). **F** Effect of SN-38 on dNTP pools. After HCT-8 cells were incubated with the indicated concentration of SN-38 for 24 h, perchloric acid extracts were prepared for HPLC as indicated in Methods. dNTP levels in control cells expressed as pmol/10^6 cells (mean ± standard deviation, $n = 3–6$) were: dATP, 40 ± 6; dGTP, 25 ± 7; dUTP, 7.7 ± 4.7; and dTTP, 19 ± 5

SN-38, 79 ± 5% of the cells contained greater than diploid (S or G_2/M phase) DNA content (Fig. 4C), indicating that the cells had been delayed in their progression through these phases of the cell cycle. Further experiments indicated that this cell cycle perturbation occurred in a dose-dependent fashion. During the 24 h after removal of SN-38 (the time when 5FU/FA would be present in the sequential experiments), the percentage of S phase cells decreased as cells progressively moved through the later part of S phase and accumulated in G_2 (Fig. 4D).

Discussion

Despite the widespread clinical interest in treating colorectal cancer with irinotecan and 5FU [10, 14, 30, 36–38, 45], there is relatively little information about the optimal method of combining these two drugs. In the present study, multiple complementary techniques were utilized to assess the effect of combining 5FU/FA and SN-38, the active metabolite of irinotecan. These experiments led to several potentially important observations.

First, a sequence-dependent interaction of SN-38 and 5FU/FA was observed. This interaction was less than additive when cells were treated with the two drugs simultaneously or with 5FU/FA followed by SN-38. In contrast, the interaction was synergistic when SN-38 was

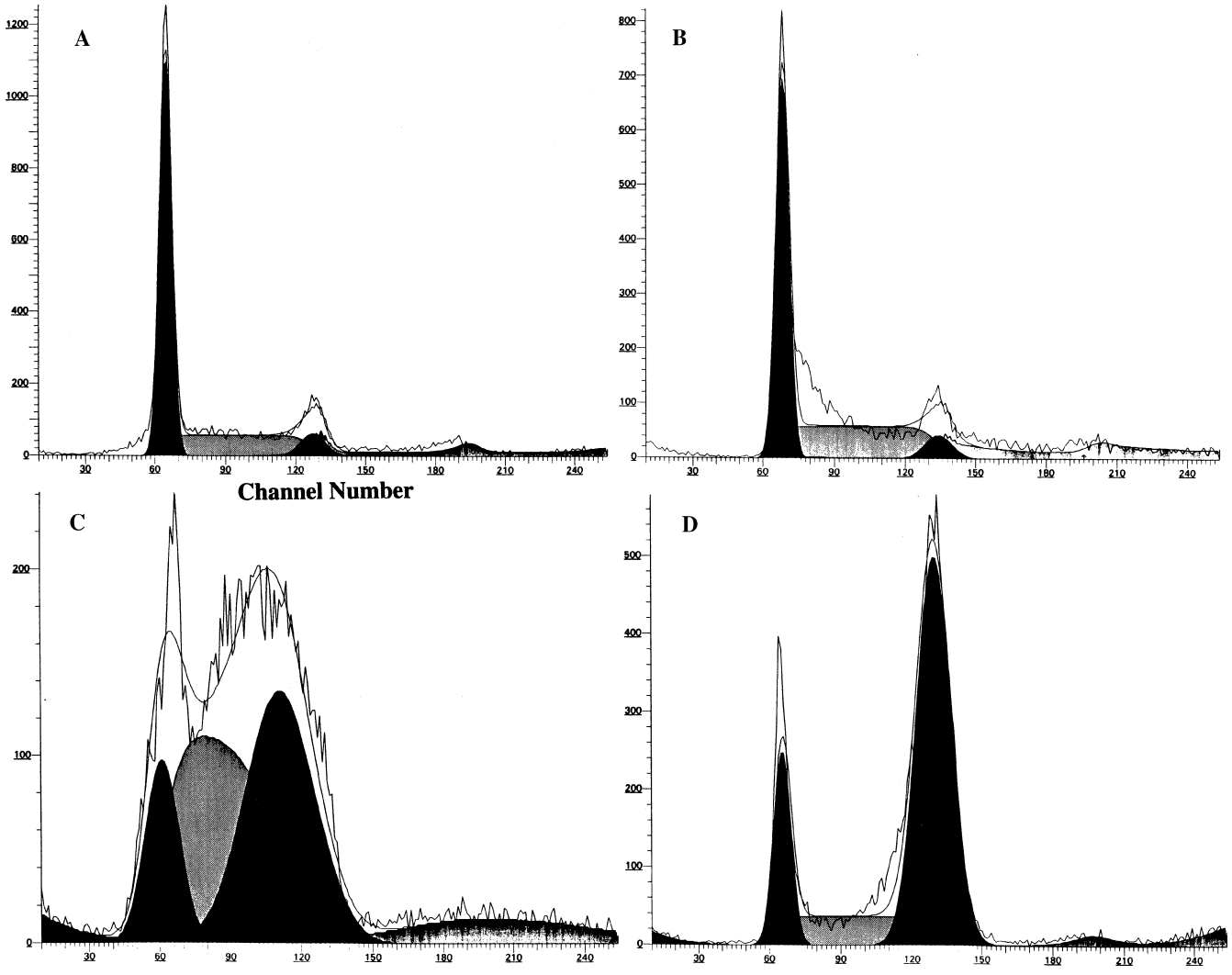

Fig. 4A–D Effect of 5FU/FA and SN-38 on cell cycle distribution. After incubation for 24 h with diluent (**A**), 30 μ*M* 5FU in the presence of 2 μ*M* FA (**B**), or 10 n*M* SN-38 (**C**), cells were fixed, stained with propidium iodide, and subjected to flow microfluorimetry. Alternatively, SN-38-treated cells were washed and plated in drug-free medium for 24 h (D)

followed by 5FU/FA. Other studies recently published in abstract form have likewise revealed that the latter sequence is synergistic [15, 26, 46]. These results raise the possibility that scheduling of 5FU and irinotecan might be important in determining the toxicity in normal tissues and/or tumor cells in the clinical setting.

Second, SN-38 treatment was observed to result in accumulation of cells in S phase (Fig. 4C). This observation is clearly different from previous reports that camptothecin causes a G_2 arrest [41, 43]. Previous experiments, however, were performed by treating cells for 1 h with very high concentrations of camptothecin followed by prolonged incubation under drug-free conditions. In contrast, the present studies were performed by exposing cells to therapeutically achievable nanomolar concentrations of SN-38 for a period of time that corresponds to a few serum half-lives in vivo [35]. The resulting S phase accumulation, which has also been reported after treatment of HeLa cells with low concentrations of camptothecin [28], presumably reflects a slowing of DNA replication.

This S phase slowing was accompanied by a decrease in the release of [^{3}H]-H_2O from [5-^{3}H]-2′-deoxyuridine in the intact cells (Fig. 3A). This decrease in activity occurred in the face of constant TS protein levels (Fig. 3B), prompting us to hypothesize that deoxynucleotide pools had been altered by the SN-38 treatment. Consistent with this hypothesis, increased dTTP pools were observed in cells treated with SN-38 for 24 h (Fig. 3F).

This elevation of dTTP pools provides the basis for a model (Fig. 5) that accounts for the observed synergy of the SN-38 → 5FU/FA sequence as well as the decreased generation of [^{3}H]-H_2O from [5-^{3}H]-2′-deoxyuridine observed in the whole cell assay. In brief, the increase in dTTP pools after SN-38 exposure would be expected to inhibit dCMP deaminase and TK [18, 33]. The inhibition of dCMP deaminase would cause depletion of dUMP, the endogenous nucleotide that competes with

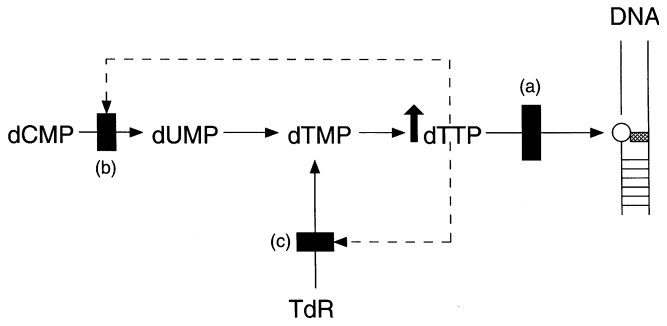

Fig. 5 **A** model for the effect of SN-38 on thymidylate metabolism. Inhibition of DNA synthesis leads to elevated dTTP pools (*a*). These elevated dTTP pools inhibit dCMP deaminase (*b*), leading to decreased dUMP (and enhanced toxicity of 5FU/FA). In addition, the elevated dTTP pools inhibit TK (*c*), leading to decreased conversion of [^{3}H]-2′-deoxyuridine to [^{3}H]-dUMP and ultimately to [^{3}H]-H_20 in the whole cell TS assay. Further support for this model can be found in reference 18

5-fluoro-2′-deoxyuridine monophosphate for binding to TS. This dUMP depletion would be expected to enhance 5FU-associated inhibition of TS, explaining the enhanced cytotoxicity of 5FU/FA after SN-38 treatment (Fig. 2). In addition, the inhibition of dCMP deaminase and depletion of dUMP would account for the decreased dUTP pools (Fig. 3F). On the other hand, the dTTP-associated inhibition of TK, the enzyme required to phosphorylate [5-^{3}H]-2′-deoxyuridine in order for TS to act upon this substrate, would explain the decreased production of [^{3}H]-H_2O from [5-^{3}H]-2′-deoxyuridine in intact cells (Fig. 3A) despite the presence of unaltered levels of TS protein (Fig. 3B). Thus, all of the observed effects can be explained by the elevated dTTP levels.

As noted above, the accumulation of cells in S phase during the 24-h SN-38 treatment (Fig. 4C) presumably reflects inhibition of DNA synthesis caused by the SN-38-stabilized topo I-DNA complexes or the DNA double-strand breaks that accumulate as replication forks interact with these complexes. Although the mechanism by which SN-38 causes elevation of dTTP was not directly investigated in the present study, it is likely that the inhibition of DNA synthesis and the consequent lack of dTTP consumption cause the dTTP pools to increase until a new steady state is achieved when dCMP deaminase and TK activity are sufficiently inhibited. Further studies are required to determine whether similar effects occur in other cell lines and with other agents that cause a slowing in S phase.

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References

1. Advanced Colorectal Cancer Meta-Analysis Project (1992) Modulation of fluorouracil by leucovorin in patients with advanced colorectal cancer: evidence in terms of response rate. J Clin Oncol 10: 896
2. Armand JP, Ducreux M, Mahjoubi M, Abigerges D, Bugat R, Chabot G, Herait P, de Forni M, Rougier P (1995) CPT-11 (Irinotecan) in the treatment of colorectal cancer. Eur J Cancer 31A: 1283
3. Berenbaum MC (1989) What is synergy? Pharmacol Rev 41: 93
4. Bible K, Kaufmann SH (1997) Cytotoxic synergy between flavopiridol (NSC 649890, L86-8275) and various antineoplastic agents: importance of sequence of administration. Cancer Res 57: 3375
5. Chang J-Y, Dethlefsen LA, Barley LR, Zhou BS, Cheng Y-C (1992) Characterization of camptothecin-resistant Chinese hamster lung cells. Biochem Pharmacol 43: 2443
6. Chen AY, Liu LF (1994) DNA Topoisomerases: essential enzymes and lethal targets. Annu Rev Pharmacol Toxicol 34: 191
7. Chou T-C, Talalay P (1984) Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. Adv Enzyme Regul 22: 27
8. Conti JA, Kemeny NE, Saltz LB, Huang Y, Tong WP, Chou T-C, Sun M, Pulliam S, Gonzalez C (1996) Irinotecan is an active agent in untreated patients with metastatic colorectal cancer. J Clin Oncol 14: 709
9. Erlichman C, Svingen PA, Mullany SA, Kaufmann SH (1996) Synergistic interaction of SN-38 and 5FU/leucovorin. Proc Am Assoc Cancer Res 37: 294
10. Fonseca R, Goldberg RM, Erlichman C, Kaufmann SH, Sloan JA, Miller LL (1997) Phase I study of CPT-11/5FU/leucovorin. Proc Am Assoc Cancer Res 38: 76
11. Froelich-Ammon SJ, Osheroff N (1995) Topoisomerase poisons: harnessing the dark side of enzyme mechanism. J Biol Chem 270: 21429
12. Greco WR, Bravo G, Parsons JC (1995) The search for synergy: a critical review from a response surface perspective. Pharmacol Rev 47: 331
13. Grem JL (1996) 5-Fluoropyrimidines: In: Chabner BA, Longo DL (eds) Cancer Chemotherapy and Biotherapy. Lippincott-Raven Publishers, Philadelphia, Second, p 149
14. Grossin F, Barbault H, Benhammouda A, Rixe O, Antoine E, Auclerc G, Weil M, Nizri D, Farabos C, Mignard D, Mahjoubi M, Khayat D, Bastian G (1996) A phase I pharmacokinetics study of concomitant CPT-11 (C) and 5FU (F) combination. Proc Am Assoc Cancer Res 37: 168
15. Guichard MJ, Caliaro G, Houin R, Bugat P, Canal P (1996) Sequential exposure to CPT-11 and 5FU is synergistic in human colon carcinoma HT-29 cell line. Proc Am Assoc Cancer Res 37: 292
16. Hanauske A-R (1993) In vitro assays for antitumour activity: more pitfalls to come? Eur J Cancer 29A: 1502
17. Houghton JA, Tillman DM, Harwood FG (1995) Ratio of 2′-deoxyadenosine-5′-triphosphate/thymidine-5′-triphosphate influences the commitment of human colon carcinoma cells to thymineless death. Clin Cancer Res 1: 723
18. Jackson RC (1978) The regulation of thymidylate biosynthesis in Novikoff hepatoma cells and the effects of amethopterin, 5-fluorodeoxyuridine, and 3-deazauridine. J Biol Chem 253: 7440
19. Johnston PG, Liang C-M, Henry S, Chabner BA, Allegra CJ (1991) Production and characterization of monoclonal antibodies that localize human thymidylate synthase in the cytoplasm of human cells and tissue. Cancer Res 51: 6668
20. Kano Y, Suzuki K, Akutau M, Suda K, Inoue Y, Yoshida M, Sakamoto S, Miura Y (1992) Effects of CPT-11 in combination with other anti-cancer agents in culture. Int J Cancer 50: 604
21. Kaufmann SH (1989) Additional members of the rat liver lamin polypeptide family: structural and immunological characterization. J Biol Chem 264: 13946
22. Kaufmann SH, McLaughlin SJ, Kastan M, Liu LF, Karp JE, Burke PJ (1991) Topoisomerase II levels during granulocytic maturation in vitro and in vivo. Cancer Res 51: 3534
23. Kaufmann SH, Karp JE, Jones RJ, Miller CB, Schneider E, Zwelling LA, Cowan K, Wendel K, Burke PJ (1994)

Topoisomerase II levels and drug sensitivity in adult acute myelogenous leukemia. Blood 83: 517
24. Kaufmann SH, Peereboom D, Buckwalter CA, Svingen PA, Donehower RC, Rowinsky EK (1996) Cytotoxic effects of topotecan combined with various anticancer agents in human cancer cell lines. J Natl Cancer Inst 88: 734
25. Leichman CG, Fleming TR, Muggia FM, Tangen CM (1995) Phase II study of fluorouracil and its modulation in advanced colorectal cancer: a Southwest Oncology Group Study. J Clin Oncol 13: 1303
26. Mans DR, Brondani da Rocha A, Vargas Schwartzbold C, Schwartsmann G (1996) Assessment of the efficacy of the irinotecan (CPT-11)/5-fluorouracil (5-FU) combination in a panel of human colon carcinoma cell lines. Proc Am Assoc Cancer Res 37: 291
27. Moertel CG (1994) Chemotherapy for colorectal cancer. N Engl Med 330: 1136
28. Nelson WG, Kastan MB (1994) DNA strand breaks: the DNA template alterations that trigger p53-dependent DNA damage response pathways. Mol Cell Biol 14: 1815
29. Pagliacci MC, Spinozzi F, Migliorati G, Fumi G, Smacchia M, Grignani F, Riccardi C, Nicoletti I (1993) Genistein inhibits tumour cell growth in vitro but enhances mitochondrial reduction of tetrazolium salts: a further pitfall in the use of the MTT assay for evaluating cell growth and survival. Eur J Cancer 29 A: 1573
30. Parnes HL, Tait N, Conley B, Van Echo D (1995) A phase I study of CPT-11, weekly bolus 5-FU and leucovorin in patients with metastataic cancer. Oncol Rep 2: 1131
31. Potmesil M (1994) Camptothecins: from bench research to hospital wards. Cancer Res 54: 1431
32. Pressacco J, Hedley DW, Erlichman C (1994) ICI D1694 and idoxuridine: a synergistic antitumor combination. Cancer Res 54: 3772
33. Rode W, Scanlon KJ, Moroson BA, Bertino JR (1980) Regulation of thymidylate synthetase in mouse leukemia cells (L1210). J Biol Chem 255: 1305
34. Rothenberg ML, Eckardt JR, Kuhn JG, B, HA Nelson J, Hilsenbeck SG, Rodriguez GI, Thurman AM, Smith LS, Eckhardt SG, Weiss GR, Elfring GL, Rinaldi DA, Schaaf LJ, Von Hoff DD (1996) Phase II trial of irinotecan in patients with progressive or rapidly recurrent colorectal cancer. J Clin Oncol 14: 1128
35. Rowinsky EK, Grochow LB, Ettinger DS, Sartorius SE, Lubejko BG, Chen TL, Rock MK, Donehower RC (1994) Phase I and pharmacological study of the novel topoisomerase I inhibitor 7-ethyl-10-[4-(1-piperidino)-1-piperidino] carbonyloxycamptothecin (CPT-11) administered as a ninety-minute infusion every 3 weeks. Cancer Res 54: 427
36. Saltz LB, Kanowitz J, Kemeny NE, Schaaf L, Spriggs D, Staton BA, Berkery R, Steger C, Eng M, Dietz A, Locker P, Kelsen DP (1996) Phase I clinical and pharmacokinetic study of irinotecan, fluorouracil, and leucovorin in patients with advanced solid tumors. J Clin Oncol 14: 2959
37. Sasaki Y, Ohtsu A, Shimada Y, Ono K, Saijo N (1994) Simultaneous administration of CPT-11 and fluorouracil: alteration of the pharmacokinetics of CPT-11 and SN-38 in patients with advanced colorectal cancer. J Natl Cancer Inst 86: 1096
38. Shimada Y, Sasaki Y, Sugano K, Shirao K, Kondo H, Yokota T, Saito D, Tamura T, Ohe Y, Shinkai T (1993) Combination phase I study of CPT-11 (irinotecan) combined with continuous infusion of 5-fluorouracil (5FU) in metastatic colorectal cancer. Proc Am Soc Clin Oncol 12: 196
39. Slichenmyer WJ, Rowinsky EK, Donehower RC, Kaufmann SH (1993) The current status of camptothecin analogues as antitumor agents. J Natl Cancer Inst 85: 271
40. Snapka RM, Permana PA (1993) SV40 DNA replication intermediates: analysis of drugs which target mammalian DNA replication. Bioessays 15: 121
41. Tobey R (1975) Different drugs arrest cells at a number of distinct stages in G_2. Nature 254: 245
42. Tompkins WAF, Watrach AM, Schmale JD, Harris JA (1974) Cultural and antigenic properties of newly established cell strains derived from adenocarcinomas of the human colon and rectum. J Natl Cancer Inst 52: 1101
43. Tsao YP, D'Arpa P, Liu L (1992) The involvement of active DNA synthesis in camptothecin-induced G2 arrest: altered regulation of P34cdc2/cyclin B1. Cancer Res 52: 1823
44. Yalowich JC, Kalman TI (1985) Rapid determination of thymidylate synthase activity and its inhibition in intact L1210 leukemia cells in vitro. Biochem Pharmacol 34: 2319
45. Yamao T, Shimada Y, Shirao K, Kondo H, Matsumura Y, Sugano K, Saito D, Ohtsu A, Boku N, Yoshida S, Sasaki Y, Ono K (1996) Phase I study of CPT-11 combined with sequential 5FU in metastatic colorectal cancer (CRC). Proc Am Soc Clin Oncol 15: 481
46. Zeghari-Squalli N, Misset JL, Goldwasser F (1997) Mechanism of the in vitro interaction between SN38 and 5-FU (abstract). Proc Am Assoc Cancer Res 38: 19