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Cellular Interactions of 5-Fluorouracil and the Camptothecin Analogue CPT-11 (Irinotecan) in a Human Colorectal Carcinoma Cell Line

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ABSTRACT. CPT-11 (irinotecan) is a DNA topoisomerase I inhibitor active against metastatic colorectal carcinoma. We investigated, in a human colon carcinoma cell line, HT-29, the effects of CPT-11 and 5-fluorouracil (5FU) combinations. A strong synergism between CPT-11 and 5FU was observed after sequential exposure and only additivity or antagonism after simultaneous exposure. When cells were first exposed to 5FU, the product of cellular CPT-11 concentrations versus time (CxT) was 6895 ± 1020 pmol · hr/10^6 cells, while it was 3875 ± 121 pmol · hr/10^6 cells with CPT-11 alone ($p < 0.01$). The same phenomenon was observed with SN-38: 148.2 ± 49.5 versus 83.4 ± 23.6 pmol · hr/10^6 cells ($p < 0.05$). Consequently, the formation of protein-DNA complexes was 1.4 times greater with 5FU pretreatment than with CPT-11 alone ($p = 0.03$). Moreover, the incorporation of 5FU derivatives into DNA was multiplied by a factor of 1.5 24 hr after CPT-11 exposure. When cells were first incubated with CPT-11, the decrease in thymidylate synthase (TS) activity was identical to that obtained after 5FU exposure (1.09 to 0.023 pmol/min/mg protein), but this decrease persisted for 24 hr (0.014 pmol/min/mg protein) ($p = 0.035$). At the same time, a 1.8-fold increase in the incorporation of 5FU derivatives into DNA and a 2-fold increase in DNA-protein complex formation were evidenced. With the two sequential associations, we observed a persistent S-phase arrest, as compared with CPT-11 alone. These results suggest that CPT-11 and 5FU combinations are of clinical interest, and mechanisms of interaction between the two drugs seem to be multifactorial. BIOCHEM PHARMACOL **55**;5:667–676, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. 5-fluorouracil; CPT-11; thymidylate synthase; topoisomerase I; synergism, DNA-protein complexes

Topoisomerase I (topo I)§ is a nuclear enzyme, discovered by Wang in 1971, that is essential for the maintenance of DNA topology [1]. Topo I acts by nicking a single DNA strand, passing the intact strand through the nick and then resealing the nick, resulting in a decrease in the linking number by one. Thus, it unwinds supercoiled DNA and appears to function mainly during DNA transcription and DNA replication. Few drugs are known to interact with topo I. Camptothecin (CPT), a natural product derived from the Chinese tree *Camptotheca acuminata*, was shown in the late 1970s to have antitumor activity [2]. However, the relative water insolubility of CPT and its important toxicity in clinical trials (hemorrhagic cystitis) have led to the development of hydrophilic derivatives [3–5]. New CPT analogues (including topotecan, CPT-11 (irinotecan) and 9-aminocamptothecin) have improved the overall therapeutic efficacy [5–9]. At present, there is substantial clinical interest in CPT-11, a water-soluble derivative that has been shown to have activity mainly in metastatic colorectal carcinoma [10, 11]. This drug must be metabolized into SN-38 (7-ethyl-(10-hydroxy camptothecin) to be active. The enzyme responsible for such a conversion is a carboxyesterase present in many tissues, mainly in liver [12].

Camptothecin and its analogues reversibly stabilize DNA-topo I cleavable complexes [13]. Precisely how this stabilization leads to cell death is not well understood. Because CPT is S-phase specific, it is hypothesized that DNA replication forks collide with DNA-topo I complexes, resulting in several changes including fork arrest, topologically (or strain-) induced DNA double strand breaks, G_2 cell cycle arrest, and cell death [14–16]. This mechanistic model and complementary *in vitro* data suggest that topo I inhibitors would be most clinically efficacious if present in tumors continuously over a long period of time, similar to

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§ *Abbreviations:* 5FU, 5-fluorouracil; CPT, camptothecin; CPT-11, irinotecan or 7-ethyl-10-[4-(1-piperidino)-1-piperidino]-carbonyloxy-camptothecin mTHF, methylene tetrahydrofolate; [^{3}H]dUMP, tritiated deoxyuridine monophosphate; DTT, dithiothreitol; TS, thymidylate synthase; FdUMP, fluorine deoxyuridine monophosphate; FdUTP, fluorine deoxyuridine triphosphate; Topo I, topoisomerase I; FCS, fetal calf serum; TCA, trichloroacetic acid; TDI, time-schedule dependency index; CxT, product of cellular concentrations versus time; SN-38, 7-ethyl-(O-hydroxy camptothecin.

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5-fluorouracil (5FU). 5FU, an analogue of uracil base, is the most active single agent in colorectal carcinoma. Its mechanism of action includes an inhibition of thymidylate synthase (TS) activity; moreover, 5FU is incorporated into DNA and RNA synthesis [17, 18]. The difference in the mechanism of action and the resistance of these two drugs provides a rationale for testing them in combination. The combined effects of topo I inhibitors and 5FU have been studied by our group [19] and others [20–24]. Overall, synergistic cytotoxicities were reported mainly when cells were sequentially exposed to the drugs, whatever the order of exposure, whereas only an additive or antagonistic cytotoxicity was shown when cells were simultaneously exposed to both drugs.

Here, we report the different mechanisms by which CPT-11 and 5FU interact synergistically in HT-29 cells. Pretreatment of cells with 5FU induced an increase in cellular CPT-11 and SN-38 uptake and a concomitant increase in DNA-protein complexes. With the other sequence, we demonstrated a persistent inhibition of TS. The two different sequential schedules led to a similar cell cycle effect with a persistent S-phase arrest.

MATERIALS AND METHODS

Drugs and Chemicals

CPT-11 and SN-38 were provided by Rhône Poulenc Rorer Laboratories (Vitry sur Seine, France). 5FU was purchased from Roche Laboratories. RPMI 1640 and fetal calf serum (FCS) were purchased from SEROMED. [6-^{14}C] 5FU (specific activity: 56 mCi/mmol) was purchased from Moravek. [Methyl-^{3}H]thymidine (20 Ci/mmol) was from Amersham. Sulforhodamine B, trichloroacetic acid, and camptothecin were purchased from Sigma Chemicals. Phenol was from Bioprobe Systems.

Cell Cultures

The HT-29 colon cancer cell line was from American Type Culture Collection. Cells were grown as monolayers in RPMI 1640 medium supplemented with 5% FCS at 37° in a humidified atmosphere containing 5% CO_2. Cells were trypsinized once a week with trypsin/EDTA (0.25/0.02%) and medium was changed once a week. Doubling time of the cell line was 22 ± 5 hr. Cells were determined to be free of mycoplasms by PCR assay.

Cytotoxicity Assays

The cytotoxic studies were performed using the sulforhodamine B technique [25], representing the percentage of growth inhibition induced by increasing drug concentrations. Cytotoxicity was evaluated by the IC_{50} value. To optimize the search for an interaction between 5FU and CPT-11, a previous experiment was performed in which the dose-growth inhibition curves of CPT-11 and 5FU after 1- or 24-hr time exposure were compared according to Matsushima *et al.* [26]. The time-schedule dependency index (TDI) was calculated by dividing the IC_{50} at 1-hr exposure by that at a 24-hr exposure. The method used was identical to that described hereafter, except that cell exposure to 5FU or CPT-11 alone was 1 hr or 24 hr. On Day 1, 2500 cells/well in a volume of 150 μL were plated in 96-well plates. In each plate, one column contained cells not exposed to drugs, while nine columns contained cells exposed to increasing concentrations of drugs. For each drug or drug combination, six wells were used. On Day 2 or on Days 2 and 3, 5FU or CPT-11 were added in a volume of 50 μL resulting in a series of final concentrations ranging from 0.4 μM to 200 μM for 5FU and from 0.8 μM to 400 μM for CPT-11. For combination studies, the concentrations of both drugs were tenfold reduced with a 5FU: CPT-11 ratio of 1:2 (molar ratio of individual IC_{50} values) and 3 different schedules were used: 5FU for 24 hr and then CPT-11 for 24 hr; CPT-11 for 24 hr and then 5FU for 24 hr; 5FU and CPT-11 simultaneously for 24 hr.

After drug exposure, the medium in control and drug-containing wells was removed and replaced by 200 μL of fresh drug-free medium, and the cells were cultured for three doubling times after the end of drug exposure. Then, cells were precipitated with 50 μL ice-cold 50% TCA and fixed for 60 min at 4°, rinsed sixfold with water and air dried. Fixed cells were colored with 50 μL of 0.4% of sulforhodamine B/0.1% of acetic acid solution, rinsed with 0.1% acetic acid solution and air dried. Sulforhodamine was redissolved in 150 μL/well of 10 mM Tris buffer pH 10 and 495 nm OD was measured in a Labsystems Multiscan Multisoft apparatus. The cytotoxicity of combinations was compared to the cytotoxicity of each drug alone in every experiment and each experiment was performed three times. For the different studies described hereafter, cells were exposed to the IC_{50} values for both drugs.

Intracellular CPT-11 and SN-38 Accumulation

Drug accumulation, net uptake and net efflux of CPT-11 and SN-38 were performed by the HPLC technique [27, 28]. The method was validated in our laboratory. For this study, 10^7 cells were plated in 10-cm^2 Petri dishes. The following day, they were exposed to CPT-11 or 5FU alone or in combination for 24 hr. After different times of incubation (5, 15, 30 min and 1, 3, 6, and 24 hr), cells were harvested using trypsin, counted, centrifuged, resuspended in 200 μL of PBS solution and stored at −20°C until analysis. Analysis was performed using a standard curve composed of 10^7 cells, spiked with different concentrations of CPT-11 (50 to 5,000 ng), SN-38 (0.5 to 50 ng) and 0.01 N HCl in a final volume of 300 μL. The experiment included three levels of controls, treated as samples. Samples were lysed by sonication and 100 μL of 0.01 N HCl was added. Fifty microliters of internal standard (camptothecin solution 40 μg/mL) and 350 μL of acetonitrile/methanol (50/50) solution were added in all samples and standards.

After vortexing and centrifugation, the supernatant was analyzed.

The HPLC analysis was performed using a Nucleosil C18 5-μm column (300 mm × 3.9 mm), with a fluorescent detection (excitation at 355 nm and emission at 515 nm). The mobile phase consisted of 0.1M potassium phosphate/3 mM sodium heptane sulfonate, pH 4)/Acetonitrile (66/34) at a flow rate of 1 mL/min. Retention times were 4.83 ± 0.02 min, 6.29 ± 0.06 min, and 7.68 ± 0.1 min for CPT-11, SN-38, and CPT, respectively. The detection limit was 50 ng/sample for CPT-11 and 0.5 ng/sample for SN-38. The experiment was validated when there was less than 10% deviation of the control values. Intracellular amounts of CPT-11 and SN-38 were corrected by cell number and expressed as picomoles per 10^6 cells. The product of the cellular concentrations of CPT-11 or SN-38 versus time (CxT) were calculated by the trapezoidal method. Results are the means of three independent experiments.

Thymidylate Synthase Activity (EC 2.1.1.45)

Cells were exposed to CPT-11 and 5FU combinations or to CPT-11 and FU alone. After incubation, cells were harvested with trypsin, counted, centrifuged, and pellet was resuspended in 50 mM Tris HCl buffer pH 7.3 containing 2 mM DTT at a density of 4 × 10^6 cells/mL. Cells were sonicated and ultracentrifuged at 20,000 × *g* for 30 min at 4°. Cytosolic protein concentration was determined with the Bradford method [29]. TS activity was determined according to Beck *et al.* [30]. Briefly, samples containing [^{3}H]dUMP (110 nM final concentration), mTHF (0.62 mM final concentration), and 25 μL cytosol in a total volume of 60 μL were incubated for 15 min at 37°. The excess of [^{3}H]dUMP was removed by adding 300 μL of activated charcoal solution in 4% TCA at 4°. After centrifugation for 5 min at 10,000 × *g*, 150 μL of supernatant was counted in a tri-carb Packard scintillation counter. Linearity with proteins (in the range of 0.1–0.4 mg/mL) and with incubation time (between 5 and 60 min) were checked. Results are expressed as pmoles of ^3H_2O formed per minute per milligram of protein. Each experiment was performed in duplicate and results are the means of four independent experiments.

KCl-SDS Precipitation Assay for Protein-DNA Complexes

The *in vitro* formation of topoisomerase I-DNA complexes was quantitated using the SDS precipitation assay described previously [31]. Logarithmically growing cells (2.5 × 10^5) were labeled by adding [methyl-^{3}H] thymidine (Specific activity, 20 Ci/mmol) into the medium to a final concentration of 0.25 μCi/mL. After an overnight incubation, the medium was replaced by medium containing CPT-11 and 5FU alone or in combination for 24 hr. Cells were then rinsed with cold PBS and lysed by 1 mL of a prewarmed (65°) lysis solution (1.25% SDS, 5 mM EDTA (pH 8.0) salmon sperm DNA (0.4 mg/mL)). The lysate was transferred to a 1.5-mL Eppendorf tube containing 250 μL of a 325 mM KCl solution. After vortexing, the sample was cooled on ice for 10 min and centrifuged for 10 min at 4°. The pellet was resuspended in 1 mL of prewarmed wash solution (10 mM Tris-HCl (pH 8), 100 mM KCl, 1 mM EDTA, salmon sperm DNA (0.1 mg/mL)) and placed at 65° for 10 min. The suspension was cooled on ice for 10 min and centrifuged for 10 min at 4°. After a second wash and centrifugation, the pellet was resuspended in 200 μL H_2O (65°) and combined with 5 mL of scintillation liquid (Packard). The radioactivity was determined in a Wallac 1409 liquid scintillator counter. Each experiment done in triplicate was repeated three times.

Incorporation of 5FU into DNA

Cells growing in log phase were harvested and plated into 10-cm^2 Petri dishes at a density of 7.5 × 10^6 cells/Petri. The next day, the cells were exposed for 24 hr under the same conditions as for accumulation, except that the 5FU solution contained 890 nM of [^{14}C]5FU. After treatment, cells were rinsed twice with PBS and stored at −20° until analysis. Cells were lysed with 3 mL of extraction buffer (0.3 M NaCl, 5 mM EDTA, 0.5% SDS) containing 50 μg/mL of proteinase K and incubated overnight at 37°. Lysate was mixed with 1 volume of Tris-saturated phenol (pH 8) and centrifuged for 10 min at 2,000 × *g*. The upper aqueous phase was removed and mixed with 1 volume of phenol/chloroform. DNA contained in the aqueous phase was precipitated with 0.2 volume of 10 M ammonium acetate and 2 volumes of absolute ethanol. After two washes with 70% ethanol, DNA was air-dried and then redissolved in 10 mM Tris-HCl, 1 mM EDTA, pH 8. DNA concentration was evaluated by spectrometry. DNA was denatured at 65° and radioactivity was measured in a Tri-carb Packard liquid scintillation counter. Results were expressed as picomole 5FU per microgram DNA. Results presented are the means ± SD of four independent experiments.

Cell Cycle Determination

Exponentially growing cells (10^7) were exposed to CPT-11 and 5FU for 24 hr alone or in combination. After incubation, cells were harvested with trypsin, counted, and aliquots of 10^6 cells were stored at −20°C until analysis. For analysis, cells were gently mixed with 220 μL buffer A (0.3% trypsin in 3.4 mM trisodic citrate, 0.1% NP-40, 1.5 mM spermine tetrahydrochloride and 0.5 mM Tris base (pH 7.6)), and left at room temperature for 10 min. Then, 180 μL of buffer B (5% trypsin inhibitor, 1% RNAse A in 3.4 mM trisodic citrate, 0.1% NP-40, 1.5 mM spermine tetrahydrochloride and 0.5 mM Tris base (pH 7.6)) was added. After 10-min at room temperature, 180 μL of buffer C (4.16% propidium iodide in 3.4 mM trisodic citrate,

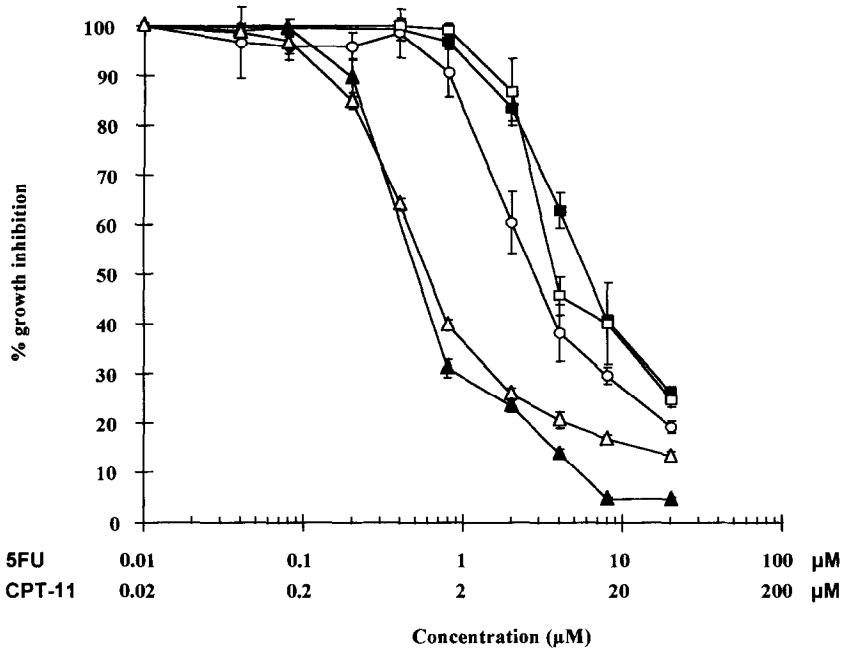

FIG. 1. Concentration-effect curves after CPT-11 (□), 5FU (■), CPT-11 then 5FU (△), CPT-11 and 5FU (○), and 5FU then CPT-11 (▲) on HT-29 cell line. Cells were treated for 24 hr with each drug. Cytotoxicity was evaluated by the SRB technique. Values are means ± SD of three independent experiments.

0.1% NP-40, 4.8 mM spermine tetrahydrochloride and 0.5 mM Tris base (pH 7.6)) was added. After 15 min at 4° in the dark, analysis was performed using a Becton Dickinson cell analyzer and data were interpretated using LYSIS II software. Three independent experiments were performed.

RESULTS

Analysis of Cytotoxic Effects of Two-Drug Combination

When cells were exposed to 5FU or CPT-11 alone for 1 hr, the IC_{50} values were 112 ± 32 µM and 550 ± 85 µM, respectively. When the time of exposure was 24 hr, the IC_{50} values were 8.3 ± 4.8 µM and 13.8 ± 10 µM for 5FU and CPT-11, respectively. The mean respective high TDI of 5FU (11.2) and CPT-11 (55.3) led us to use a 24-hr exposure for drug combination. Fig. 1 depicts the concentration-effect curves of CPT-11, 5FU and combinations against HT-29 cells. When cells were simultaneously exposed to both drugs, to obtain 50% of growth inhibition, the relative contributions of each drug were 0.42 and 0.6 for 5FU and CPT-11, respectively. When 5FU exposure preceded CPT-11 exposure, the relative IC_{50} values were 0.098 and 0.132 for 5FU and CPT-11, respectively. Finally, when cells were exposed to CPT-11 before 5FU, the relative IC_{50} values decreased to 0.093 for 5FU and 0.117 for CPT-11. Thus, as described previously, [19], when cells were sequentially exposed to both CPT-11 and 5FU, a strong and significant synergism was observed. When cells were simultaneously exposed to CPT-11 and 5FU, an additive or antagonistic effect was noted. These results provided the basis for the study of the mechanism of action of CPT-11 and 5FU. The first steps of mechanism of action of CPT-11 and 5FU were investigated, i.e., intracellular accumulation of CPT-11 and its active metabolite SN-38, and thymidylate synthase activity.

Intracellular Accumulation of CPT-11 and Intracellular Formation of SN-38

When cells were first exposed to 5FU, the product of cellular CPT-11 concentrations versus time (CxT) was 6895 ± 1020 pmol · hr/10^6 cells, while it was 3875 ± 121 pmol · hr/10^6 cells with CPT-11 alone ($p < 0.01$) (Fig. 2A). The same phenomenon was observed with SN-38: 148.2 ± 49.5 pmol · hr/10^6 cells versus 83.4 ± 23.6 pmol · hr/10^6 cells ($p < 0.05$). The pretreatment by 5FU did not influence the CPT-11 metabolism as shown by a constant ratio of CPT-11/SN-38 CxT.

When cells were exposed simultaneously to both drugs, the uptake of CPT-11 or SN-38 was close to that obtained after CPT-11 exposure alone (3298 ± 869 pmol · hr/10^6 cells and 72.9 ± 40.6 pmol · hr/10^6 cells for CPT-11 and SN-38, respectively) (Fig. 2B). Finally, when cells were exposed to CPT-11 before 5FU, no modification of CPT-11 and SN-38 efflux was shown.

Thymidylate Synthase Activity

Table 1 summarizes TS activity in HT-29 cells according to the different treatment schedules. When cells were exposed to CPT-11 alone, no modification of TS activity was observed for 48 hr. When cells were exposed to 5FU for 24 hr, an important decrease in TS activity was observed (from 0.77 to 0.024 pmol/min/mg protein). Twenty-four hours later, TS activity increased again (0.042 pmol/min/mg protein). When cells were first exposed to 5FU or simultaneously exposed to both drugs, the profile of TS inhibition was identical with that observed with 5FU alone. By contrast, when cells were preincubated for 24 hr with CPT-11, the decrease in TS activity was identical to that obtained after 5FU exposure (1.09 to 0.023 pmol/min/mg

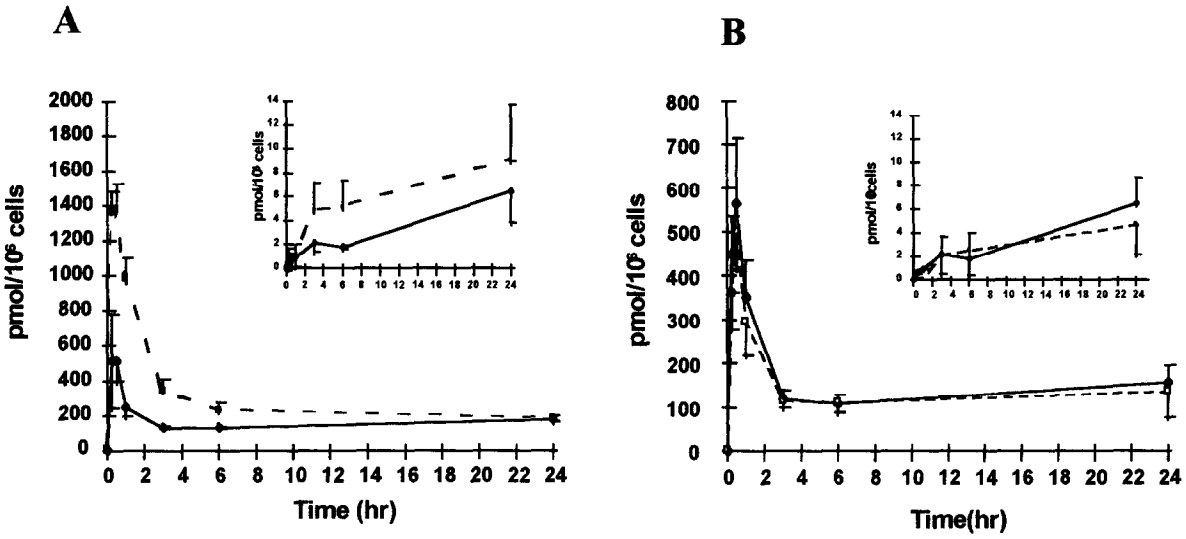

FIG. 2. A, Cellular concentration versus time curves of CPT-11 and SN-38 (insert) after treatment of cells with CPT-11 alone (—◆) or cells treated sequentially with 5FU before CPT-11 exposure (—■). B, Cellular concentration versus time curves of CPT-11 and SN-38 (insert) after treatment of cells with CPT-11 alone (—◆) or cells treated with 5FU and CPT-11 simultaneously (—□).

protein), but this decrease persisted for 24 hr (0.014 pmol/min/mg protein), and a statistically significant difference was noted 24 hr after the end of treatment (p = 0.035). The differences between simultaneous and sequential exposure to 5FU and CPT-11 in terms of cytotoxicity could be explained by the differences described below: modification of CPT-11 and SN-38 uptake and persistence of TS inhibition.

These pharmacologic events may have some consequences, and this led us to compare DNA-topo I complexes, 5FU incorporation into DNA synthesis, and cell cycle distribution in the sequential schedules.

DNA Topoisomerase I Complexes

Figure 3 summarizes the formation of protein-DNA complexes in HT-29 cells after the two sequential schedules. When cells were exposed to CPT-11 alone, a two-fold increase in protein-DNA complexes was observed after 24 hr exposure in comparison with untreated cells. During the 48 hr after the end of exposure, protein-DNA complexes decreased progressively to reach the initial level. When cells were first exposed to 5FU, the formation of protein-DNA complexes was 1.4 times greater at the end of CPT-11 exposure than that obtained after CPT-11 alone (p = 0.03). However, 24 hr after the end of CPT-11, the persistence of protein-DNA complexes was similar (Fig. 3A). When cells were first incubated with CPT-11, protein-DNA complexes were two-fold greater than with CPT-11 alone 48 hours after CPT-11 exposure (Fig. 3B) (p = 0.0005).

Incorporation of 5FU into DNA

Figure 4 shows the incorporation of [^{14}C]5FU derivatives into DNA in cells exposed to 5FU alone and 5FU combined with CPT-11, whatever the order. When cells were exposed to 5FU alone, radioactivity accumulated into DNA during 5FU exposure and then decreased. When cells were exposed to 5FU before CPT-11, the incorporation of

TABLE 1. Thymidylate synthase activity in HT-29 cells according to the different sequences of exposure to 5FU and CPT-11

	Thymidylate synthase (pmol/min/mg protein)				
Time (hr)	0	24	48	72	96
Controls	2.8 ± 1.6				
5FU alone		0.052 ± 0.047	0.048 ± 0.05	0.075 ± 0.037	0.135 ± 0061
CPT-11 alone		2.91 ± 0.96	1.43 ± 0.59	1.85 ± 0.28	
CPT-11 before 5FU		2.91 ± 0.96	0.057 ± 0.075	0.021 ± 0.0068*	0.033 ± 0.02*
5FU before CPT-11		0.052 ± 0.047	0.053 ± 0.038	0.046 ± 0.023	
5FU + CPT-11 simultaneously		0.040 ± 0.009	0.053 ± 0.031	0.062 ± 0.048	

* Significantly different from controls treated with 5FU alone at $p < 0.05$.

Cells were treated with IC_{50} of 5FU and CPT-11 for 24 hr as described in "Materials and Methods." Each value represents the mean ± SD of three independent experiments.

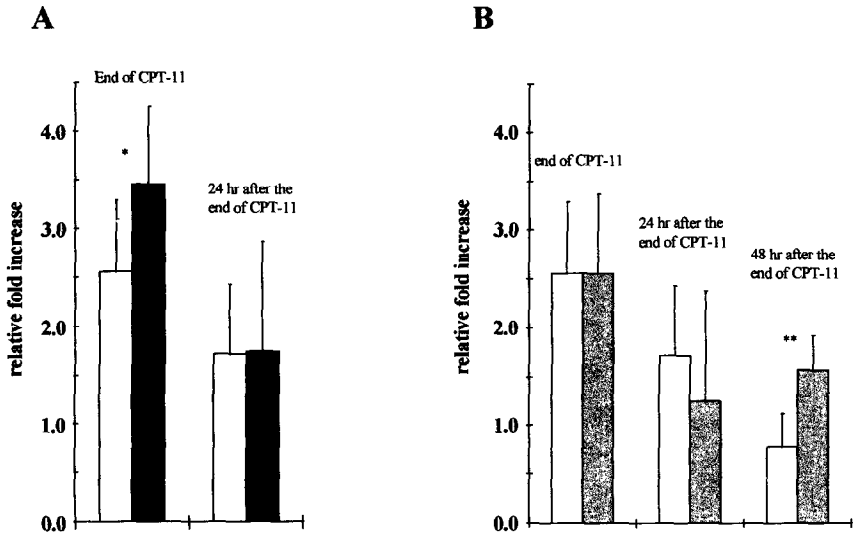

FIG. 3. Relative fold increase in SDS/KCl precipitable protein-DNA complexes in comparison with untreated HT-29 cells. A, Cells were exposed to CPT-11 alone for 24 hr (□) or to CPT-11 for 24 hr before treatment with 5FU for 24 hr (▨). B, Cells were exposed to CPT-11 alone for 24 hr (□) or to 5FU for 24 hr and then to CPT-11 for 24 hr (■). Data are expressed as relative fold increase from controls of three independent experiments. *And **: statistically different from controls treated with CPT-11 alone at $p < 0.05$ and 0.01, respectively.

radioactivity into DNA was not significantly different from that observed with 5FU alone (2.83 ± 0.71 pmol/μg DNA vs. 1.92 ± 0.57 pmol/μg DNA for the combination and 5FU alone, respectively). However, this incorporation increased in cells treated with the 5FU and CPT-11 combination at 72 hr, which corresponds to 24 hr after the end of CPT-11 treatment (2.71 ± 0.4 pmol/μg DNA for the combination and

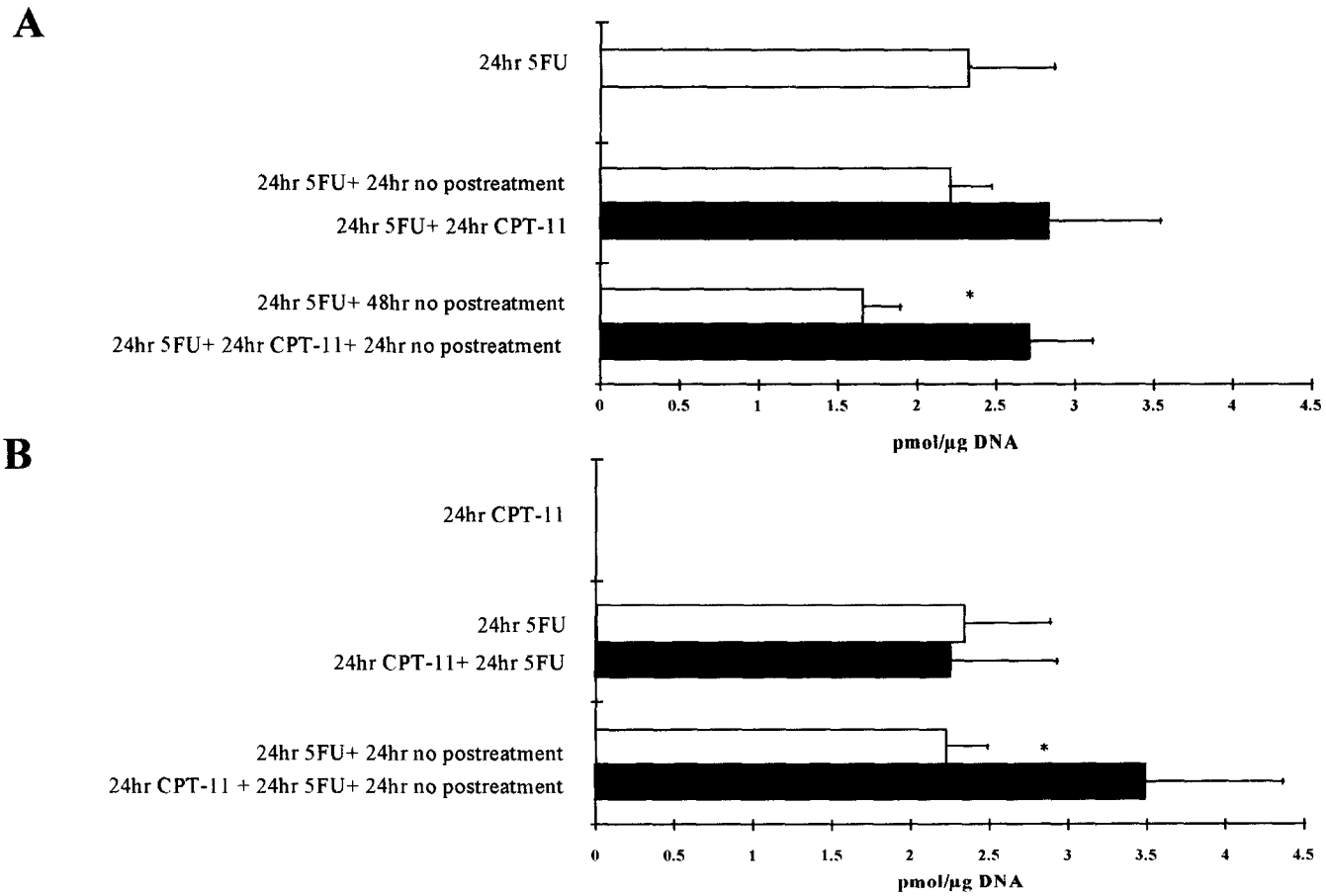

FIG. 4. Incorporation of [^{14}C]5FU into DNA derivatives as a function of time and of different combinations. A, Cells were exposed to 5FU alone for 24 hr (□) or to CPT-11 for 24 hr and then to 5FU for 24 hr (▨). B, Cells were exposed to 5FU alone for 24 hr (□) or to 5FU for 24 hr and then to CPT-11 for 24 hr (■). Data are the means ± SD of four independent experiments and are expressed as picomoles 5FU incorporated per microgram DNA. *, Statistically different from controls treated with 5FU alone at $p < 0.05$.

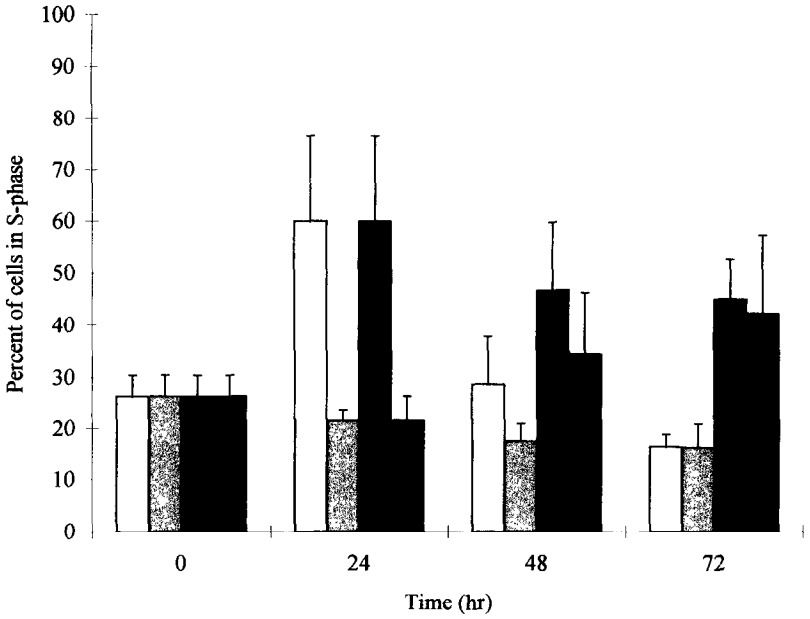

FIG. 5. Cell cycle analysis of HT-29 cells treated with CPT-11 alone (□), with 5FU alone (▨), with CPT-11 then with 5FU (■), and with 5FU then with CPT-11 (▩). Results are expressed as percentage of cells in S-phase at the indicated times. Data are means ± SD of three independent experiments.

1.65 ± 0.23 pmol/μg DNA for 5FU alone; $p = 0.0166$) (Fig. 4A).

When cells were exposed to CPT-11 before 5FU, the incorporation into DNA at the end of treatment with 5FU was close to that obtained with 5FU alone until 48 hr (2.24 ± 0.67 pmol/μg DNA for the combination and 2.86 ± 0.67 pmol/μg DNA for 5FU alone). However, 24 hr later, radioactivity incorporation into DNA continued to increase in cells treated with the combination and at 72 hr, there was a statistically significant difference (3.47 ± 0.87 pmol/μg DNA) in comparison with 5FU alone (1.92 ± 0.57 pmol/μg DNA) ($p = 0.0249$) (Fig. 4B).

When cells were first exposed to 5FU, the increase in protein-DNA complexes occurred at the end of CPT-11 treatment, and the disappearance of these complexes was related to an increase in 5FU derivatives into DNA. When cells were first exposed to CPT-11, the increase in DNA-protein complexes was concomitant to the increase in 5FU incorporation into DNA.

Cell Cycle Analysis

The percentage of cells in S-phase after CPT-11, 5FU and combinations is shown in Figure 5. 5FU treatment did not modify the percentage of S-phase cells, while CPT-11 induced an accumulation of cells in S-phase (60%). This arrest disappeared progressively within 48 hr after the end of the treatment. In the two sequential schedules, the same arrest was observed but persisted for 72 hr at which time 50% of cells were always blocked in S-phase, a percentage significantly higher than with CPT-11 alone ($p = 0.025$ and 0.002 for 5FU than CPT-11 and CPT-11 then 5FU, respectively).

DISCUSSION

The combination of 5FU and a topo I inihibitor such as CPT-11 seems to be a very interesting strategy for cancer chemotherapy. Significant clinical response rates have been reported with CPT-11 in metastatic colorectal cancer [10, 11, 32] and clinical trials are ongoing combining these two drugs [33, 34].

In the present study, we have attempted to elucidate the molecular mechanisms responsible for the synergistic effect noted *in vitro* with such a combination. To study the combination of drugs, a 24-hr exposure was chosen because 5FU and CPT-11 are time schedule-dependent drugs as suggested by the TDI defined by Matsushima *et al.* [26]. A synergistic cytotoxicity was only observed when cells were sequentially exposed to both drugs, while a single additive or antagonistic effect was noted with simultaneous exposure [19].

Our strategy was to study the first step of the mechanism of action of each drug: the uptake of CPT-11 and the conversion into its active metabolite SN-38, and for 5FU, the TS inhibition. 5FU demonstrated a duality of its mechanism of action: brief exposure with 5FU induced an RNA-directed toxicity, while TS inhibition and DNA-directed toxicity occurred with prolonged exposure [18]. Furthermore, Pizzorno *et al.* demonstrated that in the HT-29 cell line, the high rate of FdUMP formation, the increase in dUMP pool size, and TS inhibition were consistent with a DNA-directed toxicity [35]. As we used 24-hr exposure of 5FU, we focused our study on TS inhibition and 5FU incorporation into DNA.

In the sequence in which 5FU exposure preceded CPT-11 incubation, the first step was the modification of CPT-11 accumulation due to 5FU pretreatment. It was remarkable that the product of cellular CPT-11 concentra-

tions versus time was twofold greater with 5FU pretreatment. Such a modification in cellular CPT-11 or SN-38 uptake was not observed when cells were simultaneously exposed to 5FU and CPT-11 and could partially explain why a simultaneous exposure to both drugs did not induce a synergistic cytotoxicity. 5FU was reported to induce modification in cell surface due to changes in the glycosylation of glycoproteins [36, 37]. This could modify the permeability of cells to CPT-11. The increase in CPT-11 cellular uptake resulted in an equivalent increase in SN-38 cellular concentrations, demonstrating that 5FU pretreatment had no influence on the activity of carboxylesterase, which is the enzyme responsible for the transformation of CPT-11 into its active metabolite SN-38. It was shown [12] that CPT-11 itself possessed a poor antiproliferative effect, and that its metabolite SN-38 played a major role in the cytotoxic effect of CPT-11. The increase in SN-38 CxT when cells were treated with 5FU prior to CPT-11 was correlated with an increase in protein-DNA breaks measured by SDS/KCl precipitation. In HT-29 cells, SN-38 is the most potent Topo I inhibitor to form protein DNA breaks [38]. This potency was not due to an induction of more cleavage sites but to a greater stability of the cleavage sites formed, one correlated with induced cytotoxicity [39]. Finally, the disappearance of these DNA-protein complexes was associated with an increase in 5FU incorporation into DNA. There has been much speculation concerning the role of topoisomerases in DNA repair. Topo I has been localized in areas of the genome undergoing high rates of transcription [40]. These areas have also been shown to repair DNA damage at a much faster rate than other, nontranscriptionally active areas [41]. Topo I therefore may be directly or indirectly involved in DNA repair processes [42, 43]. In X-irradiation-CPT association, it was demonstrated that when CPT exposure followed X-irradiation, the observed synergy was due to a recruitment of the Topo I for DNA repair [44]. In the 5FU-CPT-11 combination, a similar mechanism could be involved, since Topo I was implicated in the repair of abasic sites created after 5FU exposure [45]. The cytotoxicity is reinforced by the fact that the repair was carried out with fraudulous incorporation of FdUTP into DNA.

TS inhibition by 5FU is due to the formation of a ternary complex including TS, FdUMP, and methylene tetrahydrofolate [17]. In the present study, we demonstrated that pretreatment of cells with CPT-11 before 5FU exposure induced a TS inhibition that persisted for a long time. When cells were exposed to 5FU alone, TS activity decreased rapidly by 24 hr and then increased after the end of drug exposure. The same phenomenon was observed when cells were simultaneously exposed to both drugs, partially explaining why a single additivity or an antagonism was observed in this case. When cells were exposed to CPT-11 prior to 5FU, TS activity continued to decrease after the end of 5FU treatment (Table 1). It is well established that a maintenance of TS inhibition leads to a superior cytotoxic activity [46]. The critical role of thymidylate synthase inhibition in the cytotoxic interaction between CPT-11 and 5FU was confirmed by the fact that synergy was also observed between raltitrexed, a specific TS inhibitor, and SN-38 [20].

When CPT-11 exposure preceded 5FU exposure, the increase in protein-DNA complexes occurred 24 hr after the end of 5FU exposure. It was concomitant to the increase in 5FU derivative incorporation into DNA. As above, a parallel could be made between the association of ionizing radiation and Topo I inhibitors. A synergy was observed when ionizing radiation followed camptothecin exposure in DC3F cells. It was hypothesized that CPT induced "sublethal" DNA damage that was converted into lethal DNA damages with the addition of radiation-induced damage [47]. A similar phenomenon could be suggested with 5FU and CPT-11, the cytotoxicity resulting in cumulative DNA damage through drug-stabilized cleavable complexes formed during CPT-11 and fraudulous incorporation of 5FU derivatives into DNA.

The combination of CPT-11 and 5FU is synergistic *in vitro*. This synergism is schedule-dependent. Modifications in the CPT-11 uptake on the one hand and the persistence of TS inhibition on the other have the same consequences: an increase in protein-DNA complexes and in 5FU incorporation into DNA. The modifications of these two factors induced the same cell cycle impact (persistent S-phase blockage [15, 48]) and finally, an equivalent degree of synergy. How can this information be used to design an optimal schedule for combining 5FU and CPT-11, especially in tumor types such as metatastatic colorectal cancer? Recent preliminary preclinical and clinical data suggest that such an association is feasible without overlapping clinical toxicities [33, 34].

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References

1. Wang JC, DNA topoisomerases. *Annu Rev Biochem* **54:** 665–697, 1985.
2. Wall ME, Wani MC, Cook CE, Plumer KH, McPhail AT and Sim GA, Plant antitumor agents. I. The isolation and structure of camptothecin, a novel alkaloidal leukemia and tumor inhibitor from *Canptotheca acuminata*. *J Am Chem Soc* **88:** 3888–3890, 1966.
3. Gottlieb JA, Guarino AM and Call JB, Preliminary pharmacological and clinical evaluation of camtothecin sodium (NSC-100880). *Cancer Chemother Rep* **54:** 461–470, 1970.
4. Moertel CG, Schutt AJ and Reitemeier RJ, Phase II study of camptothecin (NSC-100880) in the treatment of advanced gastrointestinal cancer. *Cancer Chemother Rep* **56:** 95–101, 1972.
5. Muggia FM, Creaven PJ and Hansen H, Phase I clinical trial of weekly and daily treatment with camptothecin (NSC-100880); Correlation with preclinical studies. *Cancer Chemother Rep* **56:** 515–521, 1972.
6. Armand JP, Ducreux M, Mahjoubi M, Abigerges D, Bugat R, Chabot G, Herait P, de Forni M and Rougier P, CPT-11

(irinotecan) in the treatment of colorectal cancer. *Eur J Cancer* **31A:** 1283–1287, 1995.
7. Burris HA, Rothenberg ML, Kuhn JG and Von Hoff DD, Clinical trials with the topoisomerase I inhibitors. *Semin Oncol* **19:** 663–669, 1992.
8. Giovanella BC, Stehlin JS, Wall ME, Wani MC, Nicholas AW, Liu LF, Silber R and Potmesil M, DNA topoisomerase I—targeted chemotherapy of human colon cancer in xenografts. *Science* **246:** 1046–1048, 1989.
9. Kingsbury WD, Boehm JC, Jakas DR, Holden KG, Hecht SM, Gallagher G, Caranfa MJ, McCabe FL, Faucette LF, Johnson RK et al., Synthesis of water-soluble (aminoalkyl)camptothecin analogues: inhibition of topoisomerase I and antitumor activity. *J Med Chem* **34:** 98–107, 1991.
10. Rothenberg M, Eckardt JR, Kuhn JG, Burris HAR, Nelson J, Hilsenbeck SG, Rodriguez GI, Thurman AM, Smith LS, Eckhardt SG, Weiss GR, Elfring GL, Rinaldi DA, Schaaf LJ and Von-Hoff DD, Phase II trial of irinotecan in patients with progressive or rapidly recurrent colorectal cancer. *J Clin Oncol* **14:** 1128–1135, 1996.
11. Rougier P, Bugat R, Douillard JY, Culine S, Suc E, Brunet P, Becouarn Y, Ychou M, Marty M, Extra JM, Bonneterre J, Adenis A, Seitz JF, Ganem G, Namer M, Conroy T, Negrier S, Merrouche Y, Burki F, Mousseau M, Herait P and Mahjoubi M, Phase II study of irinotecan in the treatment of advanced colorectal cancer in chemotherapy-naive patients and patients treated with fluorouracil-based chemotherapy. *J Clin Oncol* **15:** 251–260, 1997.
12. Kawato Y, Aonuma M, Hirota Y, Kuga H and Sato K, Intracellular roles of SN-38, a metabolite of the camptothecin derivative CPT-11, in the antitumor effect of CPT-11. *Cancer Res* **51:** 4187–4191, 1991.
13. Hertzberg RP, Caranfa MJ and Hecht S, On the mechanism of topoisomerase I inhibition by camptothecin: evidence for binding to an enzyme-DNA complex. *Biochemistry* **28:** 4629–4638, 1989.
14. Avemann K, Knippers R and Koller T, Camptothecin, a specific inhibitor of type I DNA topoisomerase I, induces DNA breakage at replication forks. *Mol Cell Biol* **8:** 3026–3034, 1988.
15. Hsiang YE, Lihou MG and Liu LF, Arrest of replication forks by drug-stabilized topoisomerase I-DNA cleavable complexes as a mechanism of cell killing by camptothecin. *Cancer Res* **49:** 5077–5082, 1989.
16. Zhang H, D'Arpa P and Liu LF, A model for tumour cell killing by topoisomerase poisons. *Cancer Cells* **2:** 23–27, 1990.
17. Diasio RB and Harris BE, Clinical pharmacology of 5-fluorouracil. *Clin Pharmacokinet* **16:** 215–237, 1989.
18. Sobrero AF, Aschele C and Bertino JR, Fluorouracil in colorectal cancer—a tale of two drugs: implications for biochemical modulation. *J Clin Oncol* **15:** 368–381, 1997.
19. Guichard S, Cussac D, Hennebelle I, Bugat R and Canal P, Sequence-dependent activity of the irinotecan—5FU combination in human colon cancer model HT-29 *in vitro* and *in vivo*. *Int J Cancer*, in press.
20. Aschele C, Baldo C, Guglielmi A, Sobrero A and Bertino JR, Sequence-dependent synergism between tomudex and irinotecan in human colon cancer cells *in vitro*. 9th NCI EORTC Symposium on new drugs in cancer therapy, Abstract 302, 88, 1996.
21. Erlichman C, Svingen PA, Mullany SA and Kaufmann SH, Synergistic interaction of SN-38 and 5FU/leucovorin. *Proc Am Assoc Cancer Res* **37:** 294, 1996.
22. Kano Y, Suzuki K, Akutsu M, Suda K, Inoue Y, Yoshida M, Sakamoto S and Miura Y, Effects of CPT-11 in combination with other anti-cancer agents in culture. *Int J Cancer* **50:** 604–610, 1992.
23. Kaufmann SH, Peereboom D, Buckwalter CA, Svingen PA, Grochow LB, Donehowet RC and Rowinsky EK, Cytotoxic effects of topotecan combined with various anticancer agents in human cancer cell lines. *J Natl Cancer Inst* **88:** 734–741, 1996.
24. Mans DRA, Brondani da Rocha A, Vargas Schwartzbold C and Schwartsmann G, Assessment of the efficacy of the irinotecan—5 fluorouracil combination in a panel of human colon carcinoma cell lines. *Proc Am Assoc Cancer Res* **37:** 291, 1996.
25. Skehan P, Storeng R, Scudiero D, Monks A, McMahon J, Vistica D, Warren JT, Bokesch H, Kenney S and Boyd MR, New colorimetric cytotoxicity assay for anticancer-drug screening. *J Natl Cancer Inst* **82:** 1107–1112, 1990.
26. Matsushima Y, Kanzawa F, Hoshi A, Shimizu E, Nomori H, Sasaki Y and Saijo N, Time-schedule dependency of the inhibiting activity of various anticancer drugs in the clonogenic assay. *Cancer Chemother Pharmacol* **14:** 104–107, 1985.
27. Barilero I, Gandia D, Armand JP, Mathieu Boue A, Re M, Gouyette A and Chabot GG, Simultaneous determination of the camptothecin analogue CPT-11 and its active metabolite SN-38 by high-performance liquid chromatography: application to plasma pharmacokinetic studies in cancer patients. *J Chromatogr* **575:** 275–280, 1992.
28. Rivory LP and Robert J, Reversed-phase high-performance liquid chromatographic method for the simultaneous quantitation of the carboxylate and lactone forms of the camptothecin derivative irinotecan, CPT-11, and its metabolite SN-38 in plasma. *J Chromatogr B Biomed Appl* **661:** 133–141, 1994.
29. Bradford MM, A rapid and sensitive method for quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72:** 248–254, 1976.
30. Beck A, Etienne MC, Chéradame S, Fischel JL, Formento P, Renée N and Milano G, A role for dihydropyrimidine dehydrogenase and thymidylate synthase in tumor sensitivity to fluorouracil. *Eur J Cancer* **30A:** 1517–1522, 1994.
31. Wang Z and Sinha BK, Interleukin-1a-induced modulation of topoisomerase I activity and DNA damage: implications in the mechanisms of synergy with camptothecins *in vitro* and *in vivo*. *Mol Pharmacol* **49:** 269–275, 1996.
32. Conti JA, Kemeny NE, Saltz LB, Huang Y, Tong WP, Chou TC, Sun M, Pulliam S and Gonzalez C, Irinotecan is an active agent in untreated patients with metastatic colorectal cancer. *J Clin Oncol* **14:** 709–715, 1996.
33. Saltz LB, Kanowitz J, Kemeny NE, Schaaf L, Spriggs D, Staton BA, Berkery R, Steger C, Eng M, Dietz A, Locker P and Kelsen DP, Phase I clinical and pharmacokinetic study of irinotecan, fluorouracil, and leucovorin in patients with advanced solid tumors. *J Clin Oncol* **14:** 2959–2967, 1996.
34. Saltz L, Shimada Y and Khayat D, CPT-11 (irinotecan) and 5-fluorouracil: a promising combination for therapy of colorectal cancer. *Eur J Cancer* **32A(suppl.3):** S24–S31, 1996.
35. Pizzorno G, Sun Z and Handschumacher RE, Aberrant cell cycle inhibition pattern in human colon carcinoma cell lines after exposure to 5-fluorouracil. *Biochem Pharmacol* **49:** 553–557, 1995.
36. Peters GJ, Pinedo HM, Ferwerda W, de Graaf TW and van Dijk W, Do antimetabolites interfere with the glycosylation of cellular glycoconjugates? *Eur J Cancer* **26:** 516–523, 1990.
37. Kessel D, Cell surface alterations associated with exposure of leukemia L1210 cells to fluorouracil. *Cancer Res* **40:** 322–324, 1980.
38. Tanizawa A, Fujimori A, Fujimori Y and Pommier Y, Comparison of topoisomerase I inhibition, DNA damage, and cytotoxicity of camptothecin derivatives presently in clinical trials. *J Natl Cancer Inst* **86:** 836–842, 1994.
39. Tanizawa A, Kohn KW, Kohlhagen G, Leteurtre F and Pommier Y, Differential stabilization of eukaryotic DNA topoisomerase I cleavable complexes by camptothecin derivatives. *Biochemistry* **34:** 7200–7206, 1995.

40. Fleischmann G, Pflugfelder G, Steiner EK, Javaherian K, Howard GC and Elgin SCR, *Drosophila* DNA topo-isomerase I is associated with transcriptionally active regions of the genome. *Proc Natl Acad Sci USA* **81:** 6958–6962, 1984.
41. Bohr VA, Phillips DH and Hanawalt PC, Heterogeneous DNA damage and repair in the mammalian genome. *Cancer Res* **47:** 6426–6436, 1987.
42. Downes CS and Johnson RT, DNA topoisomerases and DNA repair. *BioEssays* **8:** 179–184, 1988.
43. Stevnsner T and Bohr VA, Studies on the role of topoisomerases in general, gene- and strand-specific DNA repair. *Carcinogenesis* **14:** 1841–1850, 1993.
44. Boothman DA, Wang M, Schea RA, Burrows HL, Strickfaden S and Owens JK, Posttreatment exposure to camptothecin enhances the lethal effects of x-rays on radioresistant human malignant melanoma cells. *Int J Radiat Oncol Biol Phys* **24:** 939–948, 1992.
45. Pourquier P, Ueng LM, Kohlhagen G, Mazumber A, Gupta M, Kohn KW and Pommier Y, Effects of Uracil incorporation, DNA mismatches, and abasic sites on cleavage and religation activities of mammalian topoisomerase I. *J Biol Chem* **272:** 7792–7796, 1997.
46. van Laar JAM, ven der Wilt CL, Rustum YM, Noordhuis P, Smid K, Pinedo HM and Peters GJ, Therapeutic efficacy of fluoropyrimidines depends on the duration of thymidylate synthase inhibition in the murine colon 26-B carcinoma tumor model. *Clin Cancer Res* **2:** 1327–1333, 1996.
47. Chen AY, Okunieff P, Pommier Y and Mitchell JB, Mammalian DNA topoisomerase I mediates the enhancement of radiation cytotoxicity by camptothecin derivatives. *Cancer Res* **57:** 1529–1536, 1997.
48. Tsao YP, D'Arpa P and Liu LF, The involvement of active DNA synthesis in camptothecin-induced G2 arrest: altered regulation of p34cdc2/cyclin B. *Cancer Res* **52:** 1823–1829, 1992.