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Combination of Irinotecan (CPT11) and 5-Fluorouracil with an Analysis of Cellular Determinants of Drug Activity

*Valérie Pavillard, Patricia Formento, Philippe Rostagno, Jean–Louis Formento, Jean–Louis Fischel, Mireille Francoual, Marie–Christine Etienne and Gérard Milano**

ONCOPHARMACOLOGY UNIT, CENTRE ANTOINE LACASSAGNE, 06189 NICE CEDEX 2, FRANCE

ABSTRACT. We evaluated the combination SN38 (7-ethyl-10-hydroxycamptothecin) –5fluorouracil (5FU) ± folinic acid (FA) on six human colon cancer cell lines expressing spontaneous sensitivity to both drugs. Tumoral parameters potentially related to drug sensitivity were investigated: topoisomerase I (topo I) cleavable complexes formed with SN38, thymidylate synthase (TS) activity, folylpolyglutamate synthetase activity and dihydropyrimidine dehydrogenase activity. Drugs (SN38 and/or 5FU ± FA) were applied for 72 hr, either sequentially or together. The concentration ratio between SN38 and 5FU was 100. Cytotoxicity (MTT [3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide] test), DNA flow cytometry and isobologram analysis (Chou and Talalay) were performed. Based on 5FU IC_{50} values and isobologram analyses, the most cytotoxic schedule was SN38 followed by 5FU - FA, with high synergistic effects. Flow cytometry indicated that SN38 induced a more or less marked S-G2 block in all cell lines. Sensitivity to SN38, 5FU ± FA, or combinations were not linked to the potential above-cited tumoral parameters. Interestingly, an inverse correlation was demonstrated between TS activity and topo I cleavable complexes ($r^2 = 0.78$, $P = 0.019$). These data emphasize the critical importance of the irinotecan-5FU schedule and strongly support this association for the treatment of potentially 5FU-sensitive tumors. BIOCHEM PHARMACOL **56**;10: 1315–1322, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. 5fluorouracil; irinotecan; SN38; tumoral cell lines; drug combination; colorectal cancer

Colorectal cancers represent one of the most frequent malignant diseases in Western countries, with the highest rates being found in North America and Northern Europe [1]. Almost 50% of patients with colorectal cancer will develop a metastatic disease treated by chemotherapy. For more than 4 decades, 5FU has been the only drug offering acceptable efficacy in the chemotherapeutic treatment of metastatic colorectal cancer; the response rate of 5FU has been shown to be significantly improved by combination with folinic acid [2].

Camptothecins represent a new family of cytotoxic agents. Irinotecan (CPT11)† is a camptothecin derivative which appears to be active in colorectal cancer; interestingly, patients refractory to 5FU may present new tumoral responses following treatment by CPT-11 [3]. Because of the antitumor activity of 5FU and CPT11 in colorectal cancer and due to specific differences in respective targets of drug action and in side-effects, it was logical to try to combine these drugs. Phase I studies associating 5FU and CPT11 have recently been published [4–7]. These protocols were based on preclinical investigations combining the two drugs. From this experimental background, synergetic effects have been reported but there were conflicting results concerning an optimal sequence involving these two drugs. Some investigators pointed out a synergism when CPT-11 was applied before 5FU [8, 9], whereas others recommended the reverse sequence to achieve synergistic effects [10]. These apparent discrepancies may be explained by differences in experimental conditions but also by the fact that a very limited number of cell lines was used, ranging from a single cell line [10] to 4 different cell lines [9]. Peculiar biochemical characteristics of a given cell line may strongly influence the final conclusion and clinical extrapolations. The aim of the present study was to test the cytotoxic effects of the SN38–5FU combination on a panel of 6 different human cancer cell lines of colonic origin expressing a spontaneous sensitivity to both drugs. SN38 was used because CPT11 is inactive per se and needs to be activated in the organism into SN38, the active drug [3]. In addition, relevant cellular determinants of drug action were determined for each cell line and compared to the final cytotoxic efficacy of the drug combination. On the basis of the data in the literature and our own experience in this area, the

* Corresponding author: Dr. Gérard Milano, Oncopharmacology Unit, Centre Antoine Lacassagne, 33, Avenue de Valombrose, 06189 Nice cedex 2, France. Tel. 33 4 92 03 15 53; FAX 33 4 93 81 71 31

† *Abbreviations:* CPT11, 7-ethyl-10-(-4 piperidino)-1-piperidino carbonyloxycamptothecin, irinotecan; DMEM, Dulbecco's modified Eagle's medium; DPD, dihydropyrimidine dehydrogenase; FA, folinic acid; FBS, fetal bovine serum; FPGS, folylpolyglutamate synthetase; 5FU, 5-fluorouracil; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5diphenyltetrazolium bromide; SN38, 7-ethyl-10-hydroxycamptothecin; TS, thymidylate synthase.

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cellular determinants considered for 5FU were TS [11], FPGS [12] and DPD [13]. To predict camptothecin efficacy, and in accordance with Goldwasser *et al.* [14], the cellular capacity to form cleavable complexes between drug and DNA was taken into consideration. Finally, flow cytometry analysis was included to describe the impact generated by drug exposure on cycle phase profile.

MATERIALS AND METHODS

Chemicals

SN38 was kindly provided by Rhône Poulenc Rorer. ^{3}HdUMP labelled at position 5 (16 Ci/mmol) was from Moravek Biochemicals. 14C-glutamic acid tetralabelled (264 Ci/mol) was obtained from Amersham. CH_2FH_4 was prepared from FH4 supplied by Fluka Biochemika in accordance with the procedure provided by Dr Priest (University of South Carolina). Purified TS (3.7 units/mg protein) from *lactobacillus casei* was also from Dr Priest. DMEM (folinic acid-free) and glutamine were from Gibco and FBS was from Dutscher. Penicillin and streptomycin were from Merieux. All other chemicals including aminopterin, MTT and *dl* 5 methyltetrahydrofolate were obtained from Sigma Chemical Co. Cytosolic proteins were measured according to the Bradford assay using the Bio–Rad protein assay kit from Bio–Rad Laboratories with human purified albumin as standard.

CELL LINES

Culture Conditions

Six human cancer cell lines of colonic origin were investigated: COLO 1 (ATCC ref CCL 222), COLO 2 (ATCC ref CCL 227), COLO 4 (ATCC ref CCL 230), CAL 14 (obtained in our institute), CAL 124 (obtained in our institute), and WIDR (obtained from the EORTC). All of them expressed spontaneous sensitivity to anticancer agents (the cells had never been previously exposed to 5FU or SN38). Cell doubling times were between 1.1 and 3.2 days (median 2.2). Cells were grown in a humidified incubator (Sanyo) at 37° with an atmosphere containing 8% CO_2. Cells were routinely cultured in a regular DMEM medium supplemented with 10% FBS (concentration of active folates in the FBS was 10 nM accounting for 1 nM in the culture medium), 2 mM glutamine, 50,000 U/L of penicillin and 80 μM of streptomycin. In order to remain as close as possible to the physiological folate concentration in humans, cells were grown in a folate-controlled medium for ten days before the start of experiments (i.e. folate-free DMEM medium supplemented with 40 nM of *dl* 5 methyltetrahydrofolate plus 0.1 mM *l* ascorbic acid for folate stabilisation). During this period, the folate-controlled medium was renewed after 5–6 days. For cytotoxicity experiments, cells were grown in 96-well microtitration plates (0.32 cm2/well) with the folate-controlled medium. In addition, cells were cultured for 5 days in 175 cm^2 plates with the folate-controlled medium for measurement of cellular parameters (basal conditions): TS, DPD, and FPGS. After 5 days of growth, cells were harvested, washed three times in PBS at 4° and cell pellets containing approximately 50×10^6 cells were stored in liquid nitrogen. In a preliminary experiment, different cell concentrations were tested and the cell concentration of the inoculate giving the longest exponential growth was retained. In all cases, confluence was not reached after 5 days (70–80% of confluence). All investigations (cytotoxicity and biochemical determinations, including measurement of biochemical parameters) were performed in three independent experiments.

Evaluation of Cytotoxicity

Cells were plated in 96-well microtitration plates in order to obtain exponential growth for the whole duration of the experiment (initial cell density 2000–7000 cells/well depending on the cell line). Twenty-four hours later, cells were exposed to drugs: 5FU, 5FU + folinic acid (FA, 1 μM), SN38, or drug combinations. The duration of cell exposure to individual drugs was 72 hr. For drug combinations (5FU → SN38, 5FU-FA → SN38, SN38 → 5FU, SN38 → 5FU-FA), the duration of cell exposure was 72 hr for each drug, thus giving a total duration of 144 hr. For concomitant exposures to both drugs, the duration was 72 hr. 5FU concentrations ranged between 0.001 μM and 10,000 μM (11 concentrations) and SN38 concentrations ranged between 0.00001 μM and 0.1 μM (11 concentrations). Experimental conditions were tested in sextuplicate. Growth inhibition was assessed by the MTT Test 112 hr after the end of drug exposure [15]. Results were expressed as the percentage cell survival and IC_{50} was determined. Concentration ratios between drugs were chosen following examination of the respective dose-response curves for 5FU and SN38. According to this, the ratio 5FU concentration/SN38 concentration was 10^2.

FPGS Assay

Basal FPGS activity was measured on cells grown in the folate-controlled medium, at 70–80% of confluence. Cell pellets were homogenised (50×10^6 cells/mL) in buffer B: 10 mM Tris-HCl buffer pH 7.5 containing 1.5 mM EDTA, 0.5 mM dithiothreitol (DTT) and 10 mM sodium molybdate supplemented with 0.2 mg/mL trypsin soybean inhibitor, freeze-thawed three times and sonicated on an ice-bed four times (10 sec each time) at 10-sec intervals. Cytosols were obtained after centrifugation of the homogenates for 30 min at 105,000 *g* (4°).

FPGS activity was measured according to a method derived from that of Montero and Llorente [16] based on the incorporation of an additional ^{14}C-glutamic acid residue into the glutamate chain of aminopterin. Each cytosol was assayed in duplicate. The assay consisted in incubating 100 μL of cytosol with ^{14}C- glutamic acid (isotopic dilution, 250 μM final concentration) and aminopterin (250

μM final concentration) in a total volume of 250 μL (final concentrations of the buffer pH 8.9 were 100 mM Tris HCl, 20 mM MgCl2, 20 mM KCL, 10 mM ATP and 10 mM β mercaptoethanol). After 2-hr incubation at 37°, the reaction was stopped by the addition of 50 μL of 40% trichloroacetic acid (the reaction was linear according to duration of incubation from 1 to 4 hr). Tubes were then centrifuged for 10 min at 3,000 *g*. The supernatant (80 μL injected) was analysed for the presence of ^{14}C-aminopterin diglutamates by HPLC using an RP18 5 μm Lichrospher 100 column (250 × 4 mm ID) from Merck. Mobile phase A contained 100 mM ammonium acetate/acetonitrile (99/1, pH 5.5) and phase B, 100 mM ammonium acetate/acetonitrile (90/10, pH 5.5). The elution (flow rate 1.5 mL/min) was as follows: 0–15 min, 100% phase A; 15–40 min, 100% phase B; 40–60 min, 100% phase A for equilibration. Typical retention time was 2 min for ^{14}C-glutamic acid and 21 min for ^{14}C-aminopterin diglutamate. Results were expressed as pmol/min/mg prot. The limit of sensitivity was 0.4 pmol/min/mg prot. The intra- and inter-assay reproducibility, determined on cell aliquots obtained from a pool of cell pellets, gave coefficients of variation of 7.2% and 9.4%, respectively.

TS Assay

TS activity was measured according to the tritium-release assay described by Spears and Gustavsson [17]. The assay consisted in incubating 25 μL of cytosol with ^{3}H-dUMP (1 μM final concentration) and CH_2FH_4 (0.62 mM final concentration) in a total volume of 55 μL. After 0, 10, 20, and 30 min of incubation at 37°, the reaction was stopped on ice. The excess of ^{3}HdUMP was removed by adding 300 μL of activated charcoal (15%) containing 4% trichloroacetic acid (5-min centrifugation at 14,000 *g*, room temperature). The $^{3}H_2O$ formed during the incubation was then counted in an aliquot of the above supernatant. Results were expressed as fmoles of $^{3}H_2O$ formed per min per mg of protein, based on the linear regression obtained from the incubation times. The sensitivity limit was 10 fmol/min/mg prot. Inter-assay reproducibility was evaluated through repeated analysis of single-use aliquots of a pooled cytosol: N = 5, mean = 1,110 fmol/min/mg prot, SD = 78.59 fmol/min/mg prot, CV = 7.08%.

DPD Assay

The cells were grown in the folate-controlled medium (75 cm^2 plates) and harvested on reaching 70–80% of confluence. DPD activity was measured according to the method described by Harris *et al.* [18] and under experimental conditions previously described by us [13]. DPD activity determination consisted in the quantification of ^{14}C-dihydro 5-fluorouracil, ^{14}C-fluoro-β-alanine, and ^{14}C-α-fluoroureidopropionic acid, using a previously reported HPLC method [19]. DPD activity was calculated by taking into account the sum of dihydrofluorouracil, fluoro-β-alanine and α-fluoroureidopropionic acid peaks. DPD activity was expressed as pmol of ^{14}C-FU catabolized per min and per mg of protein. Each sample was assayed in duplicate and DPD activity was measured in three independent experiments. The sensitivity limit was 1 pmol/min/mg protein. The inter-assay reproducibility (pooled cell suspension) gave a coefficient of variation of 12% (N = 8).

Quantification of Cleavable Complexes

Cleavable complexes were measured using a Western slot-blot (TopoGEN). Briefly, 10^7 cells grown in medium without folates and fetal calf serum (FCS) were incubated for 30 min in the presence of SN38 at an optimal concentration of 10^{-4} M. A control (without drug) was done in parallel. The medium was then completely removed and cells were lysed by the addition of 1 mL of lysis buffer (1% sarkosyl in Tris-HCl 10 mM, pH: 7.5, EDTA 1 mM). The lysates were laid on top of the gradients and centrifuged at 110,000 *g* for 16 hr at 25°. Fractionation of the gradient was done by collecting 20 fractions of 200 μL from the top of the tube. Fifty μL of each fraction were used to measure optical densities at 260 and 280 nm to localise cleavable complexes. Detection of free and bound topoisomerase I was carried out by Western blotting of fractions on a slot blot device (Schleicher and Schuell Slot Blot): 75 μL of each fraction were diluted in 75 μL of sodium phosphate buffer 25 mM pH: 6.5 and slot blotted on a nitrocellulose membrane. After incubation for 2 hr in blocking solution, the solution was hybridised overnight at 4° with the anti-topoisomerase I antibody provided with the kit. The membrane was then washed and incubated for 2 hr in the presence of 8 μCI of ^{125}I protein A (Amersham). The bands corresponding to cleavable complexes were cut and counted in a γ radioactivity counter.

DNA Flow Cytometry

The cells were cultured in a 16 cm^2 flask (150,000 cells/flask) in 3 mL of medium supplemented with 10% heat-inactivated FCS. After approximately 48 hr at 37°, drugs were added for 72 hr. Next, the cells were washed in PBS, harvested by trypsinisation and centrifuged. The pellet was resuspended in the culture medium to stop the action of trypsin, rinsed with PBS and centrifuged. Then, the cells were exposed to trypsin for 10 min, a mixture of anti-trypsin and RNase for 10 min and finally, 130 mg/mL of propidium iodide prepared in citrate buffer [20]. Specimens were filtered through a 35 mm nylon mesh and analysed on a FACScan (Becton–Dickinson). Dye excitation was 488 nm. Fluorescent emission was measured through a 610 long-pass filter. For each sample, 25,000 cells were analysed. Cell distribution in cell cycle phases was determined on a DNA histogram according to Baisch's method [21]. Histograms were analysed using a specific program included in the FACScan software program.

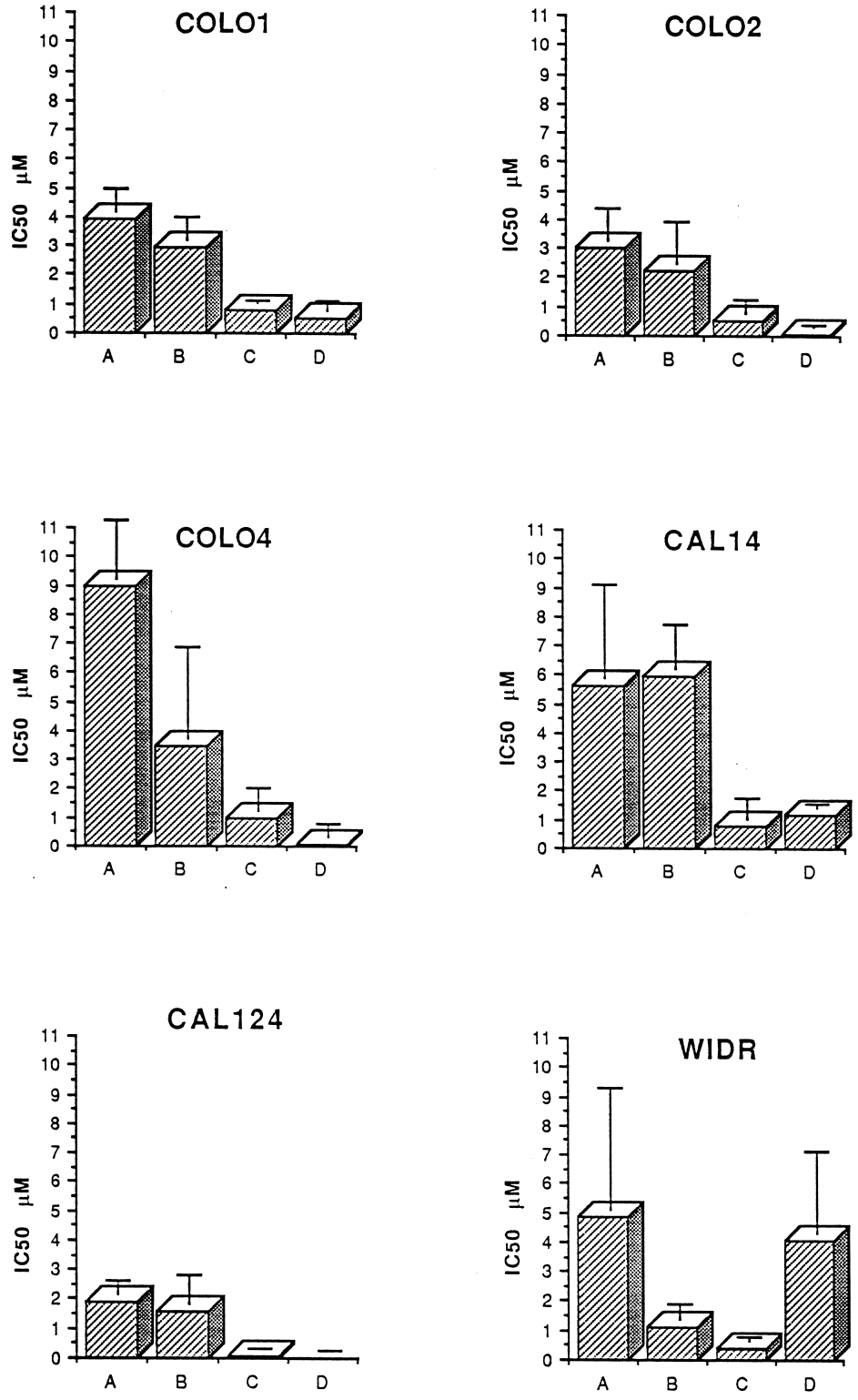

FIG. 1. Final cytotoxicity (drug IC_{50} µM for 5FU and 10–2 µM for SN38) resulting from the drug combinations. A = 5FU-FA alone, B = 5FU-FA before SN38, C = drugs together, D = SN38 before 5FU-FA. Means ± standard deviations (two independent experiments). The IC_{50} values (nM) of SN38 alone (72-hr exposure) for COLO 1, COLO 2, COLO 4, CAL 14, CAL 124, and WIDR were: 7.7, 19.5, 15.0, 11.0, 2.6, and 4.0, respectively.

TABLE 1. Combination indexes and 5FU-SN38 combinations

	Combinations of 5FU and SN38			Combinations of 5FU-FA and SN38		
Cell lines	5FU and SN38	5FU before SN38	5FU after SN38	5FUFA and SN38	5FUFA before SN38	5FUFA after SN38
COLO1	1.29 ± 0.56	0.62 ± 0.27	0.31 ± 0.04	1.44 ± 0.05	0.85 ± 0.40	0.74 ± 0.44
COLO2	0.41 ± 0.16	0.40 ± 0.15	0.64 ± 0.19	0.82 ± 0.26	0.67 ± 0.23	0.34 ± 0.09
COLO4	0.63 ± 0.05	0.68 ± 0.18	0.35 ± 0.09	0.70 ± 0.18	0.32 ± 0.28	0.22 ± 0.1
CAL14	1.36 ± 0.29	0.56 ± 0.17	0.63 ± 0.168	1.23 ± 1.00	1.37 ± 0.53	1.08 ± 0.02
CAL124	0.18 ± 0.16	0.52 ± 0.27	0.21 ± 0.07	0.19 ± 0.16	0.99 ± 0.71	0.05 ± 0.04
WIDR	2.80 ± 0.80	0.64 ± 0.24	2.03 ± 0.35	1.78 ± 1.45	0.64 ± 0.37	4.29 ± 3.1

Means ± standard deviations (two independent experiments).
Friedman rank test, the lowest values when 5FU-FA is exposed after SN38: $P = 0.004$.
For the combination of 5FU alone and SN38, the Friedman rank test gives $P = 0.12$, NS.

Analysis of Data

For *in vitro* investigations, curve fittings were done on Graph Pad Software (ISI). For each parameter studied, the mean values from 2 separate experiments were calculated with standard deviation. Drug combinations were analysed using the method developed by Chou and Talalay [22]: a combination index smaller than 1 or higher than 1 indicates synergism or antagonism, respectively.

RESULTS

Figure 1 shows the results of the drug combinations testing the following sequences: 5FU-FA before or after SN38, and the drugs together. The different sequences were compared at equivalent final cytotoxic effect (drug IC_{50} values, μM for 5FU and 10^{-2} μM for SN38). It follows that, globally, the sequence generating the lowest IC_{50} values and thus proving the most cytotoxic was that which combined 5FU-AF after SN38 ($P = 0.0038$, Friedman rank test). Similar results were observed when associating 5FU alone and SN38 ($P = 0.0012$). It must be stressed that the opposite sequence with 5FU-FA before SN38 was less cytotoxic overall than the combination with the two drugs exposed simultaneously (Fig. 1, $P < 0.001$, Mann–Whitney test).

Table 1 illustrates the results of the isobologram analysis by giving the values of the combination indexes relative to the different sequences tested. Whatever the sequence considered, there was a majority of synergistic effects (combination indexes lower than 1); this was not only true for the combination testing 5FU-FA and SN38 but also for FU alone combined with SN38. The values of the combination indexes were not significantly different when comparing the situations in which 5FU is modulated or not by FA. For the 5FU-FA –SN38 combinations, the sequence significantly influenced the value of the combination index, with the sequence SN38 followed by 5FU-FA generating the lowest combination indexes and thus the highest synergistic effects ($P = 0.004$, Table 1). In contrast, for the FU alone –SN38 combination, the combination index values were not significantly influenced by the sequence tested ($P = 0.12$, Table 1).

Table 2 gives the distribution profile of the cellular levels of the potential tumoral determinants of drug action for all cell lines tested. DPD was quantifiable (above the limit of detection) for COLO1 and WIDR only. The cell-to-cell capacity to form cleavable complexes between topoisomerase I, DNA and SN38 varied threefold between cell lines. Cell lines were compared at equivalent final cytotoxic effect (50% cell survival) for the best combination sequence (5FU-FA after SN38): drug IC_{50} values (μM for 5FU and 10^{-2} μM for SN38) varied 100-fold between 0.04 for CAL 124 (relatively the cell line most sensitive to the effects of the combination) and 4.05 (relatively the most resistant cell line). None of the tested tumoral determinants was significantly related to the indicator of cytotoxic efficacy (respective IC_{50} values). Interestingly, a significant inverse correlation was observed between TS activity and

TABLE 2. Tumoral determinants of drug activity and sensitivity to drugs in combination

Cell lines	IC_{50} values for the best combination sequence (SN38 → 5FU–FA) (5FU, μM; SN38, μM × 10^{-2})	TS (pmol/min/mg)	FPGS (pmol/min/mg)	Cleavable complexes (Cpm/μg DNA)
COLO1	0.55 ± 0.29	2.40 ± 0.01	2.13 ± 0.18	554 ± 103
COLO2	0.06 ± 0.01	7.50 ± 0.40	3.24 ± 0.87	178 ± 74
COLO4	0.05 ± 0.02	2.90 ± 1.20	1.90 ± 0.48	411 ± 13
CAL14	1.21 ± 0.14	7.00 ± 1.30	3.64 ± 0.01	299 ± 91
CAL124	0.04 ± 0.02	8.10 ± 0.85	2.46 ± 0.13	177 ± 17
WIDR	4.05 ± 0.30	5.70 ± 1.70	3.72 ± 0.50	179 ± 52

Means ± standard deviations (two independent experiments).

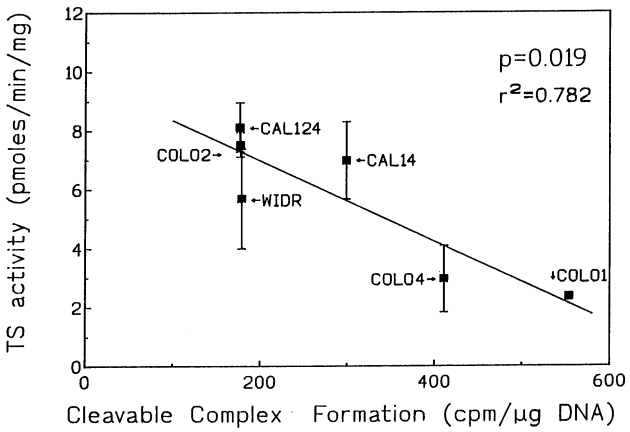

FIG. 2. Regression line between TS activity and cleavable complex formation: six independent cell lines.

the cellular capacity to form cleavable complexes (r^2 = 0.78, P = 0.019); the cell lines with a high level of cleavable complexes had a low TS activity and vice versa (Fig. 2).

Table 3 illustrates, for the whole panel of cell lines investigated, the flow cytometry profile resulting from 72-hr exposure to SN38, which is the first part of the optimal sequence SN38 followed by 5FU-FA. SN38 was applied at variable concentrations ranging from 0.005 µM up to 0.5 µM. Following SN38 exposure, there were profond changes in the distribution of cycle phases. Most cell lines shifted toward a state where the S-G2 phase was predominant. It was noteworthy that for CAL14 there was no obvious effect of SN38 on cycle phases as compared to the controls without drug. It is interesting to note that CAL14 was among the cell lines which were less sensitive to the optimal combination SN38 followed by 5FU-FA (Table 2, IC_{50} at 1.21 µM); in addition, the value of the combination index for CAL14 when this sequence was applied was close to 1 (Table 1), indicating a simple additivity of the respective cytotoxic effects of SN38 and 5FU-FA.

DISCUSSION

The main objective of the present study was to re-evaluate the effects of the association of 5FU and SN38 on a large panel of tumoral cell lines by taking into account the order of association between drugs. From the present results, it appears that the optimal sequence leading to the highest cytotoxic effects is that which combines SN38 before 5FU-FA exposure. This was true for 4/6 investigated cell lines whereas, in the other two (CAL14 and WIDR), the concomitant exposure provided the best cytotoxic action (Fig. 1). This finding highlights the need, in such studies, to cover several cell lines, since drawing conclusions from the investigation of a single cell line may lead to generalisations that are not representative of the phenomenon as a whole. The present data are in agreement with Mans *et al.* [8] and Grivicich *et al.* [9] but differ from the conclusions given by Guichard *et al.* [10], who identified an opposite sequence (5FU-CPT11) for achieving optimal cytotosic effect when combining these two drugs. Besides the importance of the order of association assessed in terms of comparative cytotoxic effects, the interaction between drugs was investigated by isobologram analysis. It was shown that most combination index values were located below 1, thus indicating a majority of synergetic effects whatever the sequence considered. The superiority of the sequence of SN38 followed by 5FU-FA was then confirmed to be the one which generated the highest synergy between drugs overall. In order to account for the preceding observation, flow cytometry analyses were undertaken after exposure of the cell lines to SN38 for 72 hr. In the majority of cases, there was cell recruitment in the S-G2 phase in the presence of SN38 (Table 3). It is known that 5FU acts preferentially on cells which are in the S phase [23], and thus the impact of SN38 on the cell cycle may explain, at least in part, the synergy between these two drugs. In addition, it was found that for the CAL14 cell line, SN 38 had no effect on cell cycle profile. For this cell line, the combination index values resulting from the association of SN38 followed by FU-FA indicated simple additivity (value close to 1). It must be emphasised that the marked effect of camptothecins on cycle phases had previously been observed by Goldwasser *et al.* [24] and Nakatsu *et al.* [25]. These latter authors showed that after 24-hr exposure to SN38, there was a change within the cell cycle, whereby the proportion of cells in the S phase predominated over the G2 phase. Thus, it is possible that 24-hr pre-exposure to SN38 before 5FU-FA would induce greater final cytotox-

TABLE 3. Flow cytometry profile following exposure to SN38

	Control without drug			0.005 µM SN38			0.1 µM SN38			0.5 µM SN38		
Cell lines	G1%	S%	G2%	G1%	S%	G2%	G1%	S%	G2%	G1%	S%	G2%
COLO1	46	43	12	46	40	14	3	26	71	9	45	46
COLO2	76	18	6	33	24	43	1	11	88	0	44	56
COLO4	81	12	7	30	56	14	15	81	4	44	51	5
CAL14	69	22	9	69	22	9	61	15	24	61	14	25
CAL124	53	35	12	46	26	28	19	31	50	20	59	21
WIDR	72	20	8	70	21	9	4	27	69	9	86	6

Cells were treated with different SN38 concentrations (0 for controls) for 72 hr and analysed by flow cytometry as indicated in the Materials and Methods secion.
The data show measurements from an individual experiment that was conducted at least twice with similar results.

icity than the 72-hr period which was adopted in the present study. Other authors have used longer exposure to both drugs (96 hr) and found antagonistic effects in this case [26]. It is thus clear that not only the schedule but also the duration of exposure to these drugs in combination is critical for the obtention of optimal cytotoxic effects. In addition, other explanations complementary to those related to the impact of SN38 on cellular cycle may be advocated in order to account for the origin of the synergy between drugs. For instance, Guichard *et al.* [27] have elegantly demonstrated a CPT11-mediated decrease in thymidylate synthase which is compatible with optimal efficacy of 5FU-FA following an exposure to the camptothecin derivative. Another molecular explanation could be found in the *in vitro* study by Pourquier *et al.* [28], who observed that the presence of uracil in DNA induced new topoisomerase I cleavage sites.

The results obtained with tumoral TS for predicting therapeutic outcome after 5FU-based treatment in colorectal cancer [11, 29, 30] strengthen the idea, as postulated by Leichman *et al.* [30], that the identification of a molecular indicator of response for a cancer could avoid the expense and toxicity of ineffective therapy and allow a more efficient search for new therapies in patients unable to benefit from standard treatment. The present study has also aimed to identify such molecular determinants for the combination CPT11 (SN38)-5FUFA. The molecular indicators were selected herein according to the cellular determinants for each drug considered. In a study similar to the present one, Goldwasser and colleagues [14], exploring 7 colorectal cell lines of the NCI anticancer screen, analysed the link between different topoisomerase I-related parameters and camptothecin activity. They came to the conclusion that cleavable complexes were the best topoisomerase I phenotypic parameter to predict cell sensitivity to camptothecin in the panel of colon cancer cell lines explored. On this basis, it was decided in the present study to include the determination of cleavable complex formation as a putative parameter for predicting cellular responsiveness to the SN38–5FUFA tandem. Concerning 5FU responsiveness, as pointed out above, several experimental and clinical data have shown the determinant role of tumoral TS [11]. In addition, in the light of our recent experience, we added DPD and FPGS because these parameters have demonstrated a link with the efficacy of 5FU-based treatment [12, 13]. In the present study, it was not possible to demonstrate a significant relationship between the respective cellular levels of the potential tumoral determinants of the tested drugs and IC_{50} values resulting from the optimal combination between SN38 and 5FU-FA. This lack of statistical significance may be due to the limited number of cell lines tested in the present study. The heaviness of the present investigations prevented us from extending the number of cell lines studied. If confirmed on a larger number of cell lines, this result would mean that, at the clinical level, the use of these tumoral parameters can be of limited value in predicting the efficacy of the SN38–5FU FA combination. The possibility of examining these different parameters led us to make observations on a significant inverse correlation between cleavable complex formation and TS activity (Fig. 2). To our knowledge, this relationship had never previously been reported. This observation may have pharmacological significance and clinical consequences. Tumoral cells with a low TS have a relatively limited capacity to repair DNA lesions because of low production of dTMP. The observed correlation indicates that the cell lines with this low TS activity and limited DNA repair also have a high capacity to form cleavable complexes; this is an optimal condition both for 5FU and SN38 action and suggests that if such a correlation is confirmed at the clinical level, it would be advisable to give patients the CPT11–5FUFA combination as first-line treatment.

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