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Original Paper

Sequence-dependent Growth Inhibition and DNA Damage Formation by the Irinotecan–5-Fluorouracil Combination in Human Colon Carcinoma Cell Lines

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We evaluated irinotecan (CPT-11) together with 5-fluorouracil (5-FU) for improved cell growth inhibition with respect to that by either agent alone in the human colon carcinoma cell lines SW620, HT-29 and SNU-C4. Cells were exposed for 24 h to each drug, as well as to various combinations and sequences of low, fixed doses of one drug with higher varying doses of the other, cultured for two more days in drug-free medium and then assessed for growth response with the sulphorhodamine B assay. Multiple drug effect analysis was used to evaluate the data, which were then related to the amount of DNA damage occurring in the cells which was determined by a fluorescence-enhancement assay for DNA unwinding. Cellular responses were also related to thymidylate synthase topoisomerase I and carboxyl esterase activities, which were assessed by a ligand-binding and a ^{3}H-release assay; a DNA decatenation assay; and a spectrophotometric method, respectively. IC_{50} values for 5-FU alone in the SW620, HT29 and SNU-C4 cells were 15.3 ± 0.8, 8.2 ± 1.3 and 2.2 ± 0.7 μM, respectively, and for CPT-11 2.0 ± 0.9, 2.5 ± 0.5 and 3.8 ± 0.3 μM, respectively. The differential responses to 5-FU alone were possibly determined by differences in substrate affinity and conversion rate of thymidylate synthase (K_m of approximately 7.5, 5.0 and 2.5 μM and V_0 of approximately 800, 200 and 2400 μM/h, respectively). The comparable cellular responses to CPT-11 alone might be accounted for by the counterbalancing effects of differences in topoisomerase I (1, 1, and 1.5 arbitrary units, respectively) and carboxyl esterase activities (5055 ± 1789, 4080 ± 752, 1713 ± 522 mU/mg, respectively). IC_{20} CPT-11 prior to 5-FU was additive to synergistic in SW620, HT-29 and SNU-C4 cells (CIs of 0.7 ± 0.1). By contrast, pretreatment with IC_{20} 5-FU antagonised the CPT-11-mediated growth inhibition (CIs of 1.9 ± 0.4, 1.7 ± 1.1, 2.5 ± 0.9, respectively). Simultaneous drug treatment did not produce more cell growth inhibition than either drug alone in the SW620 and the HT-29 cells, but was additive or antagonistic in the SNU-C4 cells (CIs of 1.1 ± 0.3 and 2.2 ± 1.4), depending on the ratio of the drugs. Increased DNA damage in the SW620 and HT-29 cells was only seen when IC_{20} CPT-11 preceded IC_{50} 5-FU, resulting in approximately 40 and 25%, respectively, more lesions than for IC_{50} 5-FU alone. In the SNU-C4 cells, not only such a treatment, but also simultaneous drug treatment produced (30 to 60%) more DNA damage than either drug alone. Our results show clear sequence-dependent antiproliferative effects and DNA damage formation by CPT-11 and 5-FU at combinations of low, fixed doses with higher, varying doses in cultured human colon carcinoma cells, and may be of relevance to the design of improved chemotherapeutic regimens in this disease.

Key words: colon carcinoma cell lines, irinotecan (CPT-11), 5-fluorouracil (5-FU), combinations, cell growth inhibition, DNA damage, drug interactions, thymidylate synthase, topoisomerase I, carboxyl esterase

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INTRODUCTION

CARCINOMAS OF the colorectal region represent 5–10% of malignancies in man, but are among the three leading causes of cancer mortality [1]. This is for an important part due to the limited responsiveness of advanced disease to existing forms of treatment including chemotherapy [2].

The most commonly used protocols incorporate the fluorinated pyrimidine antimetabolite 5-fluorouracil (5-FU), that produces as a single agent objective clinical responses in 10–15% of patients which unfortunately last less than one year [2, 3]. Addition of leucovorin (5-formyl-tetrahydrofolate) can increase response rates approximately 2-fold [4, 5], which is due to prolonged inhibition of thymidylate synthase and thus DNA synthesis, resulting from stabilisation of a ternary complex formed among the 5-FU metabolite 5-fluorodeoxyuridine monophosphate (FdUMP), thymidylate synthase, and 5,10-methylene-tetrahydrofolate [3–6]. This finding provided the rationale for the current use of 5-FU plus leucovorin as first-line therapy in advanced colorectal carcinoma [2, 3, 7].

Recently, several effective alternative treatment options became available for patients with colorectal cancer who relapse after a 5-FU-based chemotherapy. Among these are the use of novel active agents such as the thymidylate synthase inhibitor Tomudex [8], the platinum analogue oxaliplatin [9], and the topoisomerase I-inhibiting agent irinotecan (7-ethyl-10-[4-(1-piperidino)-1-piperidino] carbonyloxy-camptothecin; CPT-11). The latter drug is a novel, semisynthetic, water-soluble derivative of camptothecin, a lead compound isolated from the bark and wood of the Chinese tree *Camptotheca acuminata* [10]. CPT-11 is converted into the much more potent topoisomerase I inhibitor SN-38 by carboxyl esterases that are abundantly present in the liver [10], but can also be found in other tissues and cells [11].

In phase II clinical studies, CPT-11 demonstrated significant activity against various malignancies including advanced colorectal carcinoma. In this disease, response rates of 15–32 and 17–25% were noted in chemotherapy-naïve and pretreated, progressing patients, respectively [12–16]. These observations prompted the evaluation of combinations of CPT-11 and 5-FU (plus leucovorin) in patients with advanced colorectal carcinoma. The use of both agents together [17–20] or sequentially [21–24] has yielded encouraging results when compared with other strategies [7].

Data from preclinical studies provided additional justification to conduct these clinical trials, showing improved antitumour effects with both drugs at certain combinations over those with either agent alone. For instance, 5-FU concurrently with either CPT-11 or SN-38 was additive in HCT-8 and/or HT-29 human colon cancer cell lines [25, 26], MOLT-3 human T cell leukaemia cells [27], as well as Suit-2 human pancreatic carcinoma cells and primarily cultured cells obtained at surgery [28]. More importantly, giving SN-38 before 5-FU plus leucovorin led to synergism in six human colon carcinoma cell lines [29]. However, synergism was also found with CPT-11 or SN-38 subsequent to 5-FU in HT-29 cells [25, 30], while in another study antagonism was reported with 5-FU given 1 h before SN-38 in HCT-8 and HT-29 cells [26].

Thus, the best way to use the CPT-11–5-FU combination has yet to be established. Furthermore, when used in the clinic, such a protocol should avoid the occurrence of severe side-effects such as grade 3–4 gastrointestinal and bone marrow toxicity, potential risks of both these drugs alone when given at full doses [2, 10]. Therefore, in this study we examined the *in vitro* antiproliferative effects of, and the DNA damage induced by CPT-11 and 5-FU in various combinations and sequences using low, fixed doses of one drug with higher, varying doses of the other. To roughly simulate tumour heterogeneity, we used the differentially drug-sensitive SW620, HT-29, and SNU-C4 human colon carcinoma cell lines [31], which we further characterised in terms of thymidylate synthase, topoisomerase I and carboxyl esterase activities. We used CPT-11 rather than SN-38, in order to achieve a better correlation with current ongoing clinical trials, in which combinations of the former agent and 5-FU are employed [16–24].

MATERIALS AND METHODS

Drugs and chemicals

5-FU was from Hoffman-La Roche Inc. (Nutley, New Jersey, U.S.A.) and Yakult Honsha Co. Ltd (Tokyo, Japan) kindly provided CPT-11. Stock solutions of 1 mM of each drug were prepared in Hank's Balanced Salt Solution (Interlab, São Paulo, SP, Brazil), which were diluted with cell culture medium to the desired concentrations for each experiment. All other chemicals used were from our laboratory stock and were of the highest grade available.

Cell culture and cell line maintenance

The HT-29 human colon carcinoma cell line was from the American Type Culture Collection (Rockville, Maryland, U.S.A.). R. Camalier from the National Cancer Institute (Bethesda, Maryland, U.S.A.) provided the SW620 human colon carcinoma cell line. The SNU-C4 cell line was provided by one of the authors (G.J.P.). The cells were maintained in RPMI 1640 medium (Interlab, Sao Paulo, SP, Brazil) containing 10% (v/v) fetal calf serum (Cultilab, Campinas, SP, Brazil) and 2% (w/v) L-glutamine (Sigma Chemical Co., St Louis, Missouri, U.S.A.), at a temperature of 37°C and in a humidified atmosphere of 5% CO_2 in air. Only exponentially growing cell cultures having a viability >95%, as confirmed by trypan blue exclusion, were used for experiments. Evaluation of cell densities before, as well as at various periods of time after inoculation (see below), indicated proliferation rates of approximately 22, 23 and 26 h for SW620, HT-29, and SNU-C4 cell lines, respectively.

Cell growth inhibition studies

Cells were collected by trypsinisation, separated into single-cell suspensions in culture medium containing 50 μg/ml of gentamicin, and inoculated into 96-well microplates at densities of 5×10^3 cells per 100 μl per well. After culturing for 24 h, triplicate cultures were treated for 24 h with either 5-FU or CPT-11 alone, or with the drugs together at various combinations and sequences in final volumes of 200 μl per well. Following removal of the drug-containing medium, the cells were cultured for two additional days in drug-free medium.

Cellular responses were assessed by means of the sulphorhodamine B (SRB) assay [32], involving *in situ* fixation with trichloroacetic acid, staining with SRB, and solubilisation of cell-bound SRB with Trizma base (all from Sigma Chemical Co.). The latter was colorimetrically assessed with a Model 750 Automatic Microplate Reader (Cambridge Technology, Inc., Watertown, Massachusetts, U.S.A.).

Absorbances were read at a wavelength of 515 nm and semi-logarithmically plotted against drug concentrations.

From the resulting dose–response curves IC_{50} values were derived, i.e. drug concentrations leading to 50% inhibition of cell growth when compared with untreated controls. Data were corrected for background absorption, which was determined from wells containing medium alone, or drug-containing medium alone, but no cells.

Muliple drug effect analysis

Interactions between CPT-11 and 5-FU were assessed using a computer programme for multiple drug effect analysis [33]; Elsevier-Biosoft; Cambridge, U.K.). The programme enables calculation of combination indices (CIs) which, when smaller than 1, equal to 1, or greater than 1, indicate synergism, additivity or antagonism, respectively, between two drugs.

CIs were calculated by the formula:

$$CI = (D)_1/(D_x)_1 + (D)_2/(D_x)_2$$

where $(D_x)_1$, and $(D_x)_2$ are the concentrations of CPT-11 alone or 5-FU alone, giving x% growth inhibition, and $(D)_1$ and $(D)_2$ the drug concentrations in combination inhibiting cell growth also x%. $(D_x)_1$ and $(D_x)_2$ were calculated by the median-effect equation of Chou and Talahay [33]:

$$D_x = D_m[FA/(1 - FA)]^{1/m}$$

where D_m is the median-effect dose, FA is the fraction affected, and m the slope of the median-effect plot. Data were evaluated by taking the means of the CIs at FAs of 0.50, 0.75, 0.90 and 0.95.

Determination of thymidylate synthase activity

Thymidylate synthase activity was evaluated with two previously described assays, namely a ligand-binding assay to determine the number of available binding sites for FdUMP [34], and a ^{3}H-release assay to determine thymidylate synthase catalytic activity, i.e. the rate of conversion of deoxyuridine monophosphate (dUMP) into deoxythymidine monophosphate (dTMP) [35]. Enzyme suspensions were made from 5×10^7 untreated cells by sonication on ice in 0.2 M Tris–HCl pH 7.4, 20 mM β-mercaptoethanol, 100 mM NaF, and 15 mM cytidine monophosphate. Protein contents of the samples was measured by the method of Lowry [36] and by using the BioRad assay.

For the ligand-binding assay, 50 µl enzyme suspension was incubated for 1 h at 37°C with 50 µl of 6.5 mM 5,10-methylene-tetrahydrofolate, 135 µl of 0.2 M Tris–HCl pH 7.4, and 10 µl of 0.57 µM [6-^{3}H]-FdUMP as substrate. Reactions were stopped by the addition of 500 µl of 10% (w/v) neutral charcoal and 250 µl was used for radioactivity counting. Data were expressed as fmoles FdUMP bound per mg protein.

For the ^{3}H-release assay, [5-^{3}H]-dUMP was used as a substrate at the non-saturating and saturating concentrations of 1 and 10 µM, respectively. Thus, 25 µl of enzyme suspension (at different dilutions) was incubated for 30 min at 37°C with 5 µl of 6.5 mM 5,10-methylene tetrahydrofolate, 10 µl of 0.2 M Tris–HCl pH 7.4 buffer and 10 µl of [5-^{3}H]-dUMP. The potential inhibition of thymidylate synthase was also measured by substituting the Tris–HCl buffer for 10 µl of 0.05 µM FdUMP. Reactions were terminated by the addition of 50 µl of ice-cold 35% (w/v) trichloroacetic acid and 250 µl of 10% (w/v) neutral charcoal and 150 µl supernatant was used for liquid scintillation counting. Data were expressed as pmoles dUMP converted per h per mg protein.

From the results of both assays, substrate affinity (K_m), velocity of substrate saturation (V_{max}), initial substrate conversion rate ($V_0 = V_{max}/K_m$), enzymic properties (catalytic activity/FdUMP binding), as well as relative substrate-mediated inhibition of thymidylate synthase (K_i) were derived.

Determination of topoisomerase I activity

Topoisomerase I activity was assessed in nuclear extracts prepared from 5×10^6 cells as described previously [37]. Thus, cells were suspended in ice-cold 1 mM KH_2PO_4 pH 6.4, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM ethyleneglycol bis(β-aminoethylether)-N,N,N′N′, tetraacetic acid, 0.2 mM dithiothreitol (DTT), 1 mM phenylmethylsulphonyl fluoride (PMSF) and 0.3% (v/v) Triton X-100, and nuclei were pelleted by low-speed centrifugation at 150×*g*, 10 min at 4°C. The nuclear fractions were vigorously mixed at 0°C with 10 mM Tris–HCl pH 7.5, 25 mM KCl, 5 mM $MgCl_2$, 1 mM DTT and 1 mM PMSF, followed by extraction at 4°C with 50 mM Tris–HCl pH 7.5, 1 mM ethylenediamminetetraacetic acid, 1 M NaCl, 1 mM DTT and 1 mM PMSF. The nuclear extracts were centrifuged for 20 min at 16 000×*g* and at 4°C. Aliquots of the supernatants were then used to assess topoisomerase I activity or protein content.

Topoisomerase I catalytic activity was evaluated by relaxation of supercoiled plasmid DNA as previously described [37], using a topoisomerase I assay Kit from TopoGen (Columbus, Ohio, U.S.A.). Relaxation was for 30 min at 37°C and carried out by incubating 5 µl nuclear extract (at different dilutions) in a final volume of 20 µl containing 0.2 µg pHOT1 plasmid DNA in 50 mM Tris–HCl pH 8, 120 mM KCl, 10 mM $MgCl_2$, 0.5 mM ATP, 0.5 mM DTT and 30 µg/ml of bovine serum albumin. Reactions were terminated by the addition on ice of 5% (w/v) sarkosyl, 0.125% (w/v) bromophenol blue and 25% (v/v) glycerol. Excess enzyme was degraded at 37°C for 60 min with 50 µg/ml of proteinase K.

Relaxed DNA was separated from supercoiled DNA by electrophoresis at 20 V for 4 h in a 1% (w/v) agarose gel containing 0.5 µg/ml of ethidium bromide, which was immediately photographed. Negatives were used for densitometry, and topoisomerase I activities were expressed relative to each other.

Determination of cellular carboxyl esterase activity

Cellular carboxyl esterase activity was determined in microsomal fractions as previously described [38]. Thus, samples of 5×10^8 untreated cells were suspended in 3 ml of 0.25 M sucrose, homogenised by sonication on ice in 3 ml of 0.25 M sucrose, and centrifuged at 4°C for 30 min at 16 000×*g*. The supernatants were collected and centrifuged at 4°C for 60 min at 105 000×*g* to sediment the microsomal fractions, which were resuspended in 1 ml of 0.1 M Tris–HCl pH 8.5 and disrupted by sonication on ice. Digitonin was added to a final concentration of 15 mg/ml, and the carboxyl esterase activity was solubilised by stirring for 60 min on ice. These suspensions were centrifuged for 2 h at 105 000×*g*, and aliquots of the supernatants were used to determine either carboxyl esterase activity or protein content.

The former was carried out by mixing 12.5 μg microsomal extract with 100 mM Tris–HCl pH 8.2 and 1 mM *para*-nitrophenylacetate in a final volume of 1 ml, and measuring the amount of *para*-nitrophenol formed over 2 min at a wavelength of 405 nm. Data were expressed as mU carboxyl esterase per mg protein.

DNA damage assay

The DNA damage in the cells after the treatments mentioned above was assessed using a fluorescence-enhancement assay for DNA unwinding (FADU) as previously described [39, 40]. The assay is based on: (a) the time-dependent unwinding of duplex DNA under alkaline conditions to yield single-stranded DNA; (b) the acceleration of this process after the introduction of DNA damage by, for instance, cytotoxic agents; and (c) the differential molar fluorescence of duplex DNA versus single-stranded DNA upon complexation with the fluorescent chromosome stain bisbenzamide.

Thus, cultures of 5×10^6 cells were treated for 24 h with 5-FU or CPT-11 alone, or with the drugs together at various combinations and sequences. Following removal of the drug-containing medium, the cultures were either immediately assessed for DNA damage (on day 1), or grown for two more days in drug-free medium prior to the assessment of the DNA damage (on day 3).

To this end, adhering cells were washed, collected by centrifugation, and resuspended in phosphate-buffered saline (PBS) at densities of 10^6 cells per ml. Each suspension was divided into three aliquots to measure the fluorescence of denatured DNA (B: blank) and intact DNA (T: total), in order to correct for the values of the actual drug effect in the third part (P). The latter reflects the percentage of intact, double-stranded DNA remaining in the surviving cells, and was calculated by the formula:

$$\text{percentage double-stranded DNA} = (P-B)/(T-B)\times 100\%.$$

Fluorescence was measured at excitation and emission wavelengths of 353 nm and 451 nm, respectively, and data were corrected for background values determined from samples without cells.

Statistics

Data presented are means ± standard deviations (S.D.s) of at least three experiments performed in triplicate, and P values <0.05 were taken to indicate statistical significance (Student's t-test for paired samples).

RESULTS

Chemosensitivity of SW620, HT-29 and SNU-C4 cells to 5-FU, CPT-11 and combinations

The antiproliferative effects of 5-FU and CPT-11 in the SW620, HT-29, and SNU-C4 human colon carcinoma cell lines were assessed after 24 h drug exposure, followed by 2 days culturing in drug-free medium.

Comparison of IC_{50} values (Table 1) showed that the SW620 and the HT-29 cells were relatively resistant to 5-FU (IC_{50} values of 15.3 μM ± 0.8 and 8.2 μM ± 1.3, respectively), while the SNU-C4 cells were relatively sensitive to this drug (IC_{50} value of 2.2 μM ± 0.7). The three cell lines displayed smaller differences in their sensitivity to CPT-11, the IC_{50} values being 2.0 ± 0.9, 2.5 ± 0.5 and 3.8 ± 0.3, respectively.

We subsequently examined the effects of weakly cytotoxic, fixed concentrations of one agent on the cell growth inhibition exerted by the other, using various combinations and sequences. Thus, cells were incubated for 24 h with either 5-FU or CPT-11 together with CPT-11 or 5-FU, respectively, at approximately 20% growth-inhibitory concentrations (IC_{20}). Alternatively, cells were exposed for 22 h to serial dilutions of either 5-FU or CPT-11, after 2 h pretreatment with CPT-11 or 5-FU, respectively, at IC_{20} concentrations.

As shown in Table 1 and Figure 1, pretreatment with CPT-11 improved the growth-inhibitory effects of 5-FU approximately 2-, 4- and 2-fold in SW620, HT-29 and SNU-C4 cells, respectively. Multiple drug effect analysis (Table 2) revealed CIs of 0.7 ± 0.1 in all three cell lines, suggesting that prior IC_{20} CPT-11 acted additively to synergistically with 5-FU. On the other hand, pretreatment with 5-FU at IC_{20} concentrations led to a decrease of approximately 2-fold, 2-fold and 4-fold in CPT-11-mediated growth inhibition in SW620, HT-29 and SNU-C4 cells, respectively (Table 1, Figure 2). CIs were 1.9 ± 0.4, 1.7 ± 1.1 and 2.5 ± 0.9, respectively, suggesting antagonistic interactions between the drugs with this sequence.

None of the simultaneous treatments significantly altered the cell growth inhibition by each drug separately in the SW620 and HT-29 cells (Table 1; Figures 1 and 2). However, in contrast, in the SNU-C4 cells, simultaneous CPT-11 IC_{20} antagonised the antiproliferative effect of 5-FU approximately 2-fold (CI of 2.2 ± 1.4; Tables 1 and 2; Figure 1), while simultaneous 5-FU IC_{20} added to that of CPT-11

Table 1. *IC_{50} values (μM; means ± S.D., n≥3) in human colon carcinoma cell lines upon treatment for 24 h with 5-FU or CPT-11 alone, or at the indicated combinations and sequences*

	Cell lines		
	SW620	HT-29	SNU-C4
IC_{50}-5FU upon:			
5-FU alone, 24 h	15.3 ± 0.8	8.2 ± 1.3	2.2 ± 0.7
CPT-11 IC_{20} + 5-FU, 24 h simultaneously	13.9 ± 5.0	10.9 ± 1.2	5.4 ± 0.9*
CPT-11 IC_{20} 2 h, then 5-FU 22 h	9.3 ± 0.6*	1.5 ± 0.5	1.1 ± 0.2*
IC_{50} CPT-11 upon:			
CPT-11 alone, 24 h	2.0 ± 0.9	2.5 ± 0.5	3.8 ± 0.3
5-FU IC_{20} + CPT-11, 24 h simultaneously	2.1 ± 0.9	2.2 ± 0.4	2.2 ± 0.2†
5-FU IC_{20} 2 h, then CPT-11 22 h	5.3 ± 0.3†	5.5 ± 0.7†	18.2 ± 0.6†

Cellular responses were assessed after culturing for two additional days in drug-free medium using the sulphorhodamine B assay. *Significantly different from 5-FU alone ($P<0.05$, Student's t-test). †Significantly different from CPT-11 alone ($P<0.05$, Student's t-test). S.D., standard deviation.

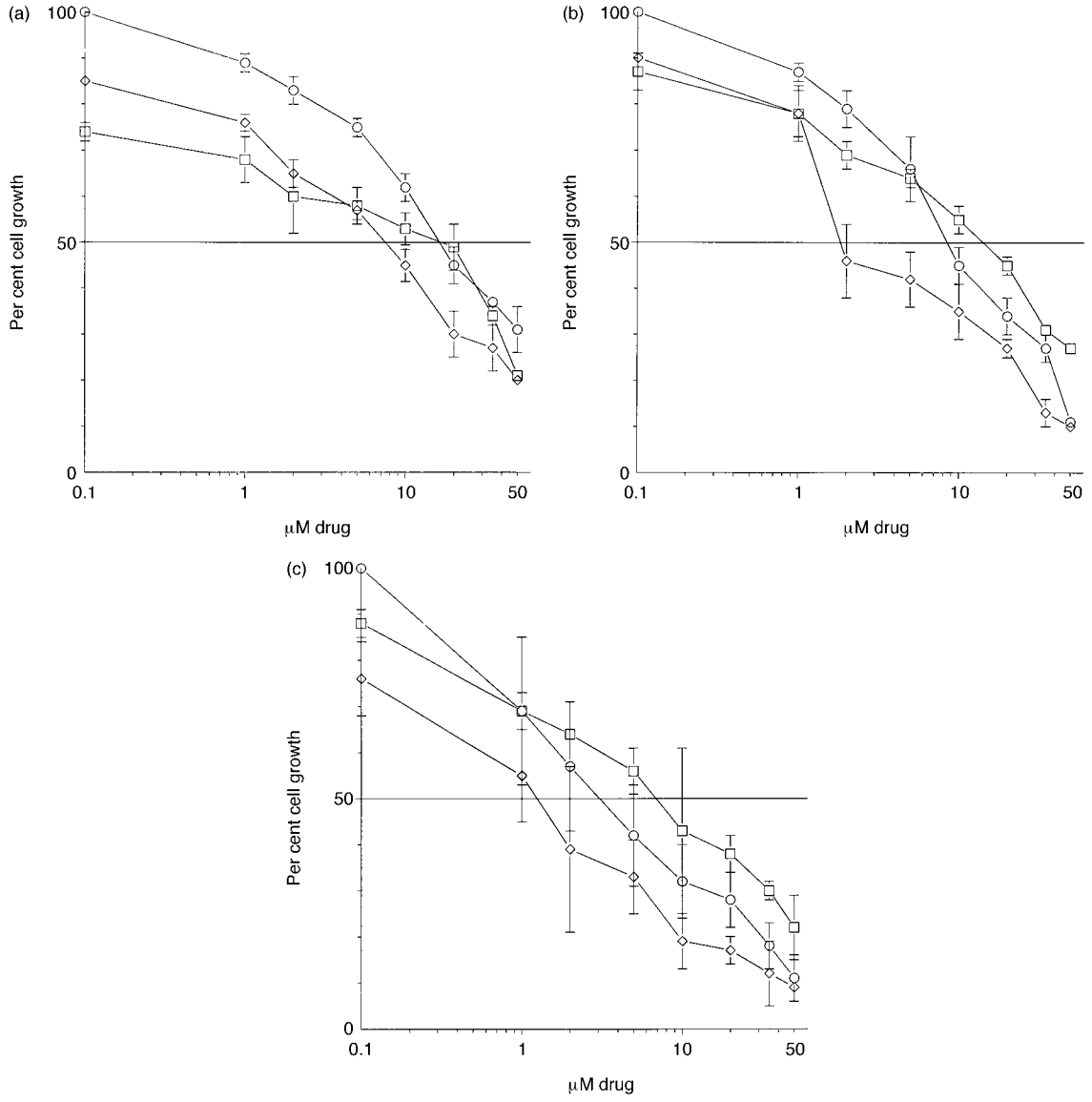

Figure 1. Inhibition of proliferation of (a) SW620; (b) HT-29; and (c) SNU-C4 human colon carcinoma cell lines by 24 h 5-FU alone (○); 24 h IC_{20} CPT-11 together with 5-FU (□); and 2 h IC_{20} CPT-11 followed by 22 h 5-FU (◇). Data points are means ± S.D.s (vertical bars; $n \geq 3$). IC_{50} values are given in Table 1.

Table 2. Combination indices (CIs; means ± S.D., n ≥ 3) from multiple drug effect analysis of combinations of CPT-11 and 5-FU in human colon carcinoma cell lines

	Cell lines		
Combination	SW620	HT-29	SNU-C4
CPT-11 IC_{20} + 5-FU, 24 h simultaneously	1.0 ± 0.2	1.3 ± 0.1	2.2 ± 1.4
CPT-11 IC_{20} 2 h, then 5-FU 22 h	0.7 ± 0.1	0.7 ± 0.1	0.7 ± 0.1
5-FU IC_{20} + CPT-11, 24 h simultaneously	1.0 ± 0.2	2.7 ± 0.8	1.1 ± 0.3
5-FU IC_{20} 2 h, then CPT-11 22 h	1.9 ± 0.4	1.7 ± 1.1	2.5 ± 0.9

The analyses were carried out after 2 additional days in drug-free medium. From each experiment, means of CIs were calculated at fractions affected of 0.50, 0.75, 0.90, and 0.95, and these values were used to calculate the data presented. CI < 1, = 1, or > 1 indicate synergism, additivity and antagonism, respectively.

with approximately the same factor (CI 1.1 ± 0.3; Tables 1 and 2; Figure 2).

Thus, pretreatment with low-dose CPT-11 potentiated 5-FU-mediated cell growth inhibition, while prior low-dose 5-FU antagonised that by CPT-11 in all three cell lines. Depending on the ratio of the drugs, simultaneous 5-FU–CPT-11 treatment led to potentiation or antagonism in SNU-C4 cells alone.

Target enzyme activities in SW620, HT-29 and SNU-C4 cells

To examine whether the differences among the three cell lines in sensitivity to 5-FU could be due to differences in thymidylate synthase activity, various aspects of thymidylate synthase kinetics were determined. The number of available FdUMP binding sites, and catalytic activity at non-saturating (1 μM) or saturating dUMP levels (10 μM) did not differ significantly between the SW620 and SNU-C4 cells, but were approximately 6-fold lower in the HT-29 cells (Table

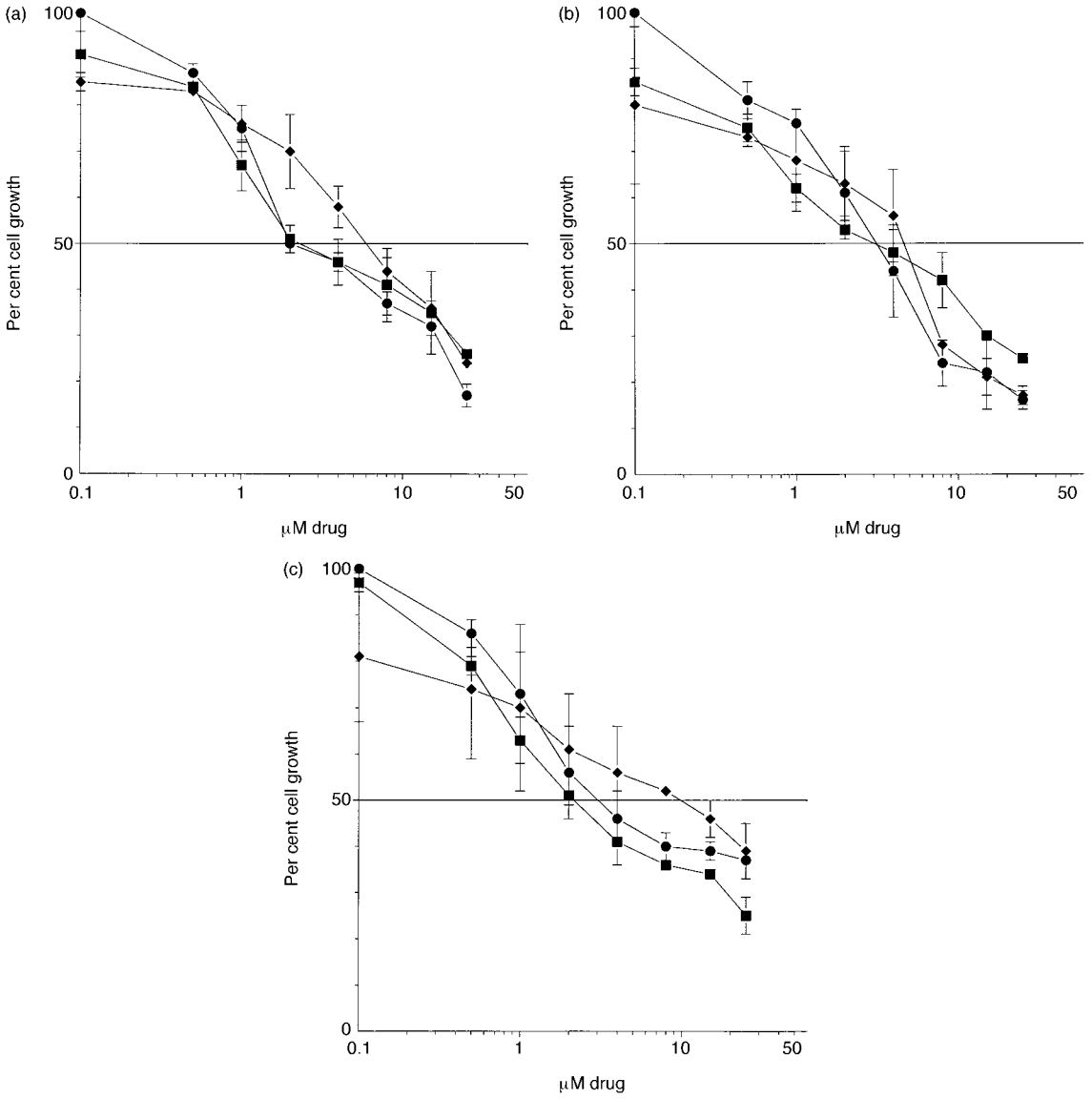

Figure 2. Inhibition of proliferation of (a) SW620; (b) HT-29 and (c) SNU-C4 human colon carcinoma cell lines by 24-h CPT-11 alone (●); 24-h IC_{20} 5-FU together with CPT-11 (■); and 2-h IC_{20} 5-FU followed by 22-h CPT-11 (◆). Data points are means ± S.D.s (vertical bars; $n \geq 3$). IC_{50} values are given in Table 1.

3). The same held true for V_{max}, which was approximately 6000, 6000 and 1000 pmoles/h/mg in SW620, SNU-C4, and HT-29 cells, respectively. Thymidylate synthase catalytic activity in the three cell lines also did not show significant differences in enzymic properties, and relative inhibition by FdUMP was similar (K_i values for FdUMP of approximately 1.5 nM in the three cell lines). K_m values for dUMP were approximately 2.5, 5.0, and 7.5 μM in SNU-C4, HT-29, and SW620 cells, respectively. The efficiency V_0 of the enzyme is calculated as the ratio between V_{max} and K_m and was approximately 12 and 4 times higher in the SNU-C4 and SW620 cells, respectively, than in the HT-29 cells. Considering the chemosensitivity ranking of the cell lines (SNU-C4 > HT-29 > SW620; Table 1), these data suggest that their response to 5-FU was mainly determined by the V_{max}/K_m ratio rather than by available FdUMP binding sites and potential catalytic activity of thymidylate synthase.

To examine whether topoisomerase I activity levels could account for the responses of the cell lines to CPT-11, cataly-

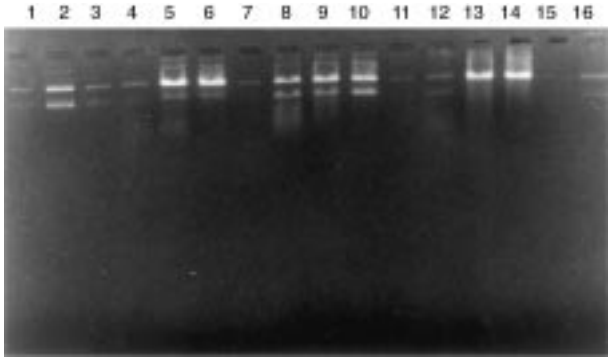

Figure 3. Decatenation of pHOT1 plasmid DNA by dilutions of topoisomerase I-containing nuclear extracts from SW620 (lanes 3–6), HT-29 (lanes 7–10), and SNU-C4 (lanes 11–14) human colon carcinoma cell lines. Details of the assay are in Materials and Methods. Markers for relaxed and supercoiled DNA are given in lanes 1 and 2, and lane 16 depicts the separation of plasmid DNA by purified topoisomerase I.

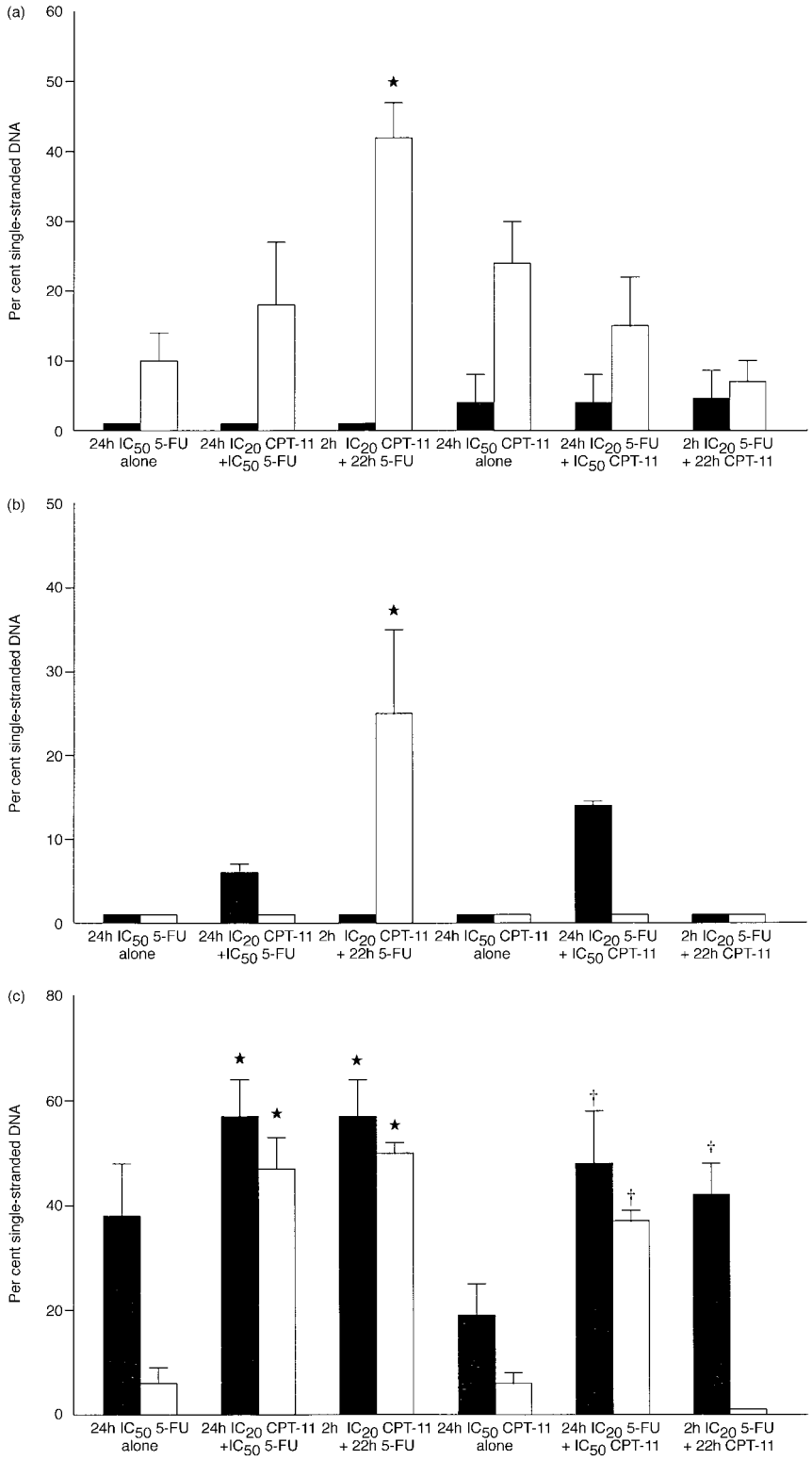

Figure 4. DNA damage in (a) SW620; (b) HT-29 and (c) SNU-C4 human colon carcinoma cell lines upon treatment for 24 h with 5-FU of CPT-11 alone or at the indicated combinations and sequences, either immediately after drug treatment (day 1: dark bars), or after culturing for two additional days in drug-free medium (day 3: light bars). DNA damage was evaluated as % single-stranded DNA (means ± S.D., $n \geq 3$) using a fluorescence-enhancement assay for DNA unwinding, and was expressed relative to that found with untreated controls. Typical background DNA damage in untreated cells was, in agreement with literature data [23, 24] approximately 30%, probably introduced during processing of the samples (*significantly different from 5-FU alone, $P < 0.05$, Student's *t*-test. ‡Significantly different from CPT-11 alone, $P < 0.05$, Student's *t*-test).

Table 3. Target enzyme activity levels in untreated human colon carcinoma cell lines

	Cell lines		
	SW620	HT-29	SNU-C4
FdUMP binding (fmoles/mg)	1170 ± 645	206 ± 61	1374 ± 237
TS catalytic activity (pmoles/h/mg)			
At 1 μM dUMP	764 ± 320	187 ± 120	1775 ± 816
At 10 μM dUMP	3428 ± 2286	700 ± 281	4718 ± 1433
Topoisomerase I activity (arbitrary units)	1	1	1.5
Carboxyl esterase (mU/mg protein)	5055 ± 1789	4080 ± 752	1713 ± 522

Data are means ± S.D.s with $n \geq 3$.

tic activities were assessed in nuclear extracts, and estimated from band intensities by density scanning of agarose gels. We also assessed the possible involvement of activating capacity in cellular responses to CPT-11, by determining carboxyl esterase activity in microsomal extracts.

Topoisomerase I catalytic activity in the SNU-C4 cells was approximately 1.5-fold greater than that in the HT-29 and SW620 cells (Figure 3; Table 3). In contrast, carboxyl esterase activity in the SNU-C4 cells was approximately 2.5 times lower than that in the other two cell lines (approximately 2000 mU/mg protein compared with approximately 4000–5000 mU/mg protein; Table 3). These differences between the enzyme activities might counterbalance each other's effects on cellular responses to CPT-11, tentatively explaining the comparable IC_{50} values in the three cell lines.

Sequence-dependent production of DNA damage

DNA damage in the cells was examined after treatment for 24 h with 5-FU or CPT-11 at IC_{50} concentrations alone; for 24 h with 5-FU or CPT-11 at IC_{50} concentrations together with CPT-11 or 5-FU, respectively, at IC_{20} concentrations; and for 2 h with CPT-11 or 5-FU at IC_{20} concentrations followed by 22 h with 5-FU or CPT-11, respectively, at IC_{50} concentrations. DNA damage was evaluated in adhering cells on days 1 and 3, in order to obtain indications about the lesions introduced immediately after drug treatment, as well as about those remaining behind after a 2-day recovery period in drug-free medium. Under all experimental conditions and throughout the entire incubation period, no more than 10% of floating cells were seen. Figure 4 summarises the data from these studies.

With none of the treatments lesions were detected on day 1 in the SW620 and HT-29 cells. In the SNU-C4 cells, all 5-FU-containing treatments caused significant DNA damage. Furthermore, when compared with IC_{50} 5-FU alone, addition of IC_{20} CPT-11, either sequentially or concomitantly, led to approximately 20% more lesions in this cell line. Also, IC_{50} CPT-11 alone did not cause detectable DNA damage in the SNU-C4 cells, but in combination with IC_{20} 5-FU approximately 40–50% damage was introduced, regardless of the schedule.

On day 3, significant DNA damage in the SW620 and HT-29 cells (30–40%) was only observed when IC_{20} CPT-11 had preceded IC_{50} 5-FU. In the SNU-C4 cells, the damage introduced by both the combination of IC_{50} 5-FU with IC_{20} CPT-11 and that of IC_{20} 5-FU simultaneously with IC_{50} CPT-11 was retained, while that generated by IC_{50} 5-FU alone and that by IC_{20} 5-FU prior to IC_{50} CPT-11 was no longer detectable.

DISCUSSION

Using the SW620, HT-29, and SNU-C4 human colon carcinoma cell lines we showed in this study, that prior treatment with CPT-11 at hardly growth-inhibiting concentrations (IC_{20}) potentiated 5-FU-mediated growth inhibition (2- to 4-fold), but that 5-FU at these concentrations before CPT-11 led to antagonistic interactions (2- to 4-fold). Simultaneous drug treatment did not affect the antiproliferative effects of the drugs alone in the SW620 and HT-29 cell lines, but in the SNU-C4 cell line, simultaneous low-dose 5-FU was additive to CPT-11-induced growth inhibition, while simultaneous low-dose CPT-11 antagonised that by 5-FU (approximately 2-fold).

Although the three cell lines differed significantly (up to 8-fold) in their sensitivity to 5-FU they displayed much smaller differences (approximately 2-fold) in their response to CPT-11. These findings are in agreement with the absence of cross-resistance between both drugs, as well as with the demonstrated efficacy of CPT-11 in 5-FU-resistant colon carcinoma [12–16].

Assessment of thymidylate synthase kinetics suggested that differences in both substrate affinity (K_m) and conversion rate (V_0) were the main determinants for the different response of the cell lines to 5-FU. This is supported by our recent studies (data not shown), where cells were treated for 24 h with 5-FdUMP and then left for 2 days in drug-free medium. These studies gave a sensitivity ranking of approximately 3:2:1 in the SW620, HT-29 and SNU-C4 cells, respectively (IC_{50} values of 14.5 ± 2.0, 9.5 ± 3.5 and 5.5 ± 0.4 μM, respectively). Interestingly, this 3:2:1 ranking is comparable with values obtained for the K_m in the 3 cell lines (2.5, 5.0 and 7.5 μM in SNU-C4, HT-29 and SW620 cells, respectively). However, it should be taken into account that several other factors such as transmembrane transport and metabolising pathways [3, 41] were probably also involved in 5-FU sensitivity [3, 41].

In the case of CPT-11, the comparable responses of the cell lines might be explained by the counterbalancing effects of differences in topoisomerase I and carboxyl esterase activities. Additional support for this suggestion is provided by their similar response to 24 h treatment with SN-38 followed by 2 days in drug-free medium (IC_{50} values of 4.8 ± 1.2, 6.0 ± 1.3, and 5.1 ± 1.4 nM in SW620, HT-29 cells, and SNU-C4 cells, respectively; data not shown).

The different effects of the various drug treatments led us to hypothesise further that they would cause different types of cellular damage. Although capable of inducing multiple types of lesions, 5-FU has been suggested to generate mainly DNA-directed lesions at exposure periods of 24 h or less [41].

Such damage could be introduced upon blockage of DNA synthesis and DNA repair through thymidylate synthase inhibition by FduMP [42], as well as through interference with chain elongation and DNA stability after incorporation of 5-fluoro-2′-deoxyuridine-5′-triphosphate (FdUTP) [43].

None the less, 5-FU alone at IC_{50} concentrations caused significant DNA damage (approximately 40%) in the SNU-C4 cells alone which was removed when the cells were recultured for 2 days in drug-free medium. This might be due to excision repair of the apyrimidinic sites created upon cleavage of the glycosyl bonds of FdUMP residues in the DNA [41]. This phenomenon is likely to be operative at short drug exposures followed by a recovery period of approximately two doubling times. The absence of detectable DNA damage in the SW620 and HT-29 cells might be due to the lesser susceptibility of these cell lines to FdUMP-induced, DNA-directed damage when compared with the SNU-C4 cell line. The growth-inhibiting effects of 5-FU in the three cell lines observed on day 3 despite the absence of apparent DNA strand-breakage could be mediated by other lesions, e.g non-excised fluorouracil- or uracil-containing sites [44, 45], and/or RNA-directed damage generated upon incorporation of 5-fluorouridine-5′-triphosphate (FUTP) into various RNA species [41].

In contrast, although initially not causing detectable DNA lesions, IC_{50} 5-FU preceded by IC_{20} CPT-11 led 2 days later to approximately 40 and 25% damage in the SW620 and HT-29 cells, respectively (Figure 4a and b). In the SNU-C4 cells, this combination caused approximately 60% DNA damage that was almost retained until day 3. Apparently, prior low-dose CPT-11 enhanced the 5-FU-inducible DNA damage, accounting for the greater cell growth inhibition over that by 5-FU alone. These observations might be explained by interference of prior CPT-11 with unscheduled DNA synthesis, resulting in unsuccessful attempts to excise and repair fluorouracil- or uracil-containing DNA [46, 47]. A comparable mechanism has been suggested to underlie the synergistic cell killing by ionising radiation and topotecan in Chinese hamster ovary cells and mouse P388 leukaemia cells [48], as well as the potentiation of cisplatin cytotoxicity by preceding SN-38 in HST-1 human squamous carcinoma cells [49].

If prior CPT-11-mediated perturbation of the DNA synthesis machinery is necessary to modulate 5-FU-induced cell growth inhibition, simultaneous IC_{20} CPT-11 can be expected to have a less profound impact on this phenomenon. This is, because unlike with CPT-11 prior to 5-FU, under these conditions the cells would have maintained their ability to resist the 5-FU-induced damage. Particularly in the more 5-FU-resistant SW620 and HT-29 cells, the effects of CPT-11 separately would be relatively small, which may not significantly modify the cell growth inhibition and the DNA damage by 5-FU alone. Our data (Table 1 and Figure 4) indicate that this was indeed the case.

Simultaneous CPT-11 at IC_{20} concentrations might affect 5-FU-mediated cell growth inhibition even less in the approximately 2-fold less CPT-11-sensitive SNU-C4 cells. Indeed, we even observed a reduction in the anti-proliferative effects of 5-FU with this treatment (Table 1). Surprisingly however, this combination also introduced 60% persistent DNA damage. This unexpected finding may be tentatively explained by assuming that these lesions represented non-lethal damage, for instance non-lethal apyrimidinic sites brought to light as strand breaks after alkali treatment during the FADU. Although this assumption needs to be verified in further studies, the possible production of such non-lethal damage has been described before for the semi-quinone free radical of the topoisomerase II-inhibiting agent etoposide [50].

Although implicated in its mechanism of action [10], CPT-11 alone at IC_{50} concentrations did not cause detectable DNA strand breaks in any of the cell lines (Figure 4). However, at these concentrations, insufficient SN-38 could have been produced to cause appreciable DNA strand breakage. Indeed, from cellular pharmacokinetic studies with HT-29 cells treated with CPT-11 [13, 25] it can be inferred that under our experimental conditions at the most 0.5 nM SN-38 and only approximately 10 rad equivalents of DNA strand breaks were formed, too low to be detectable with the FADU [39]. However, such low numbers of strand breaks may still affect cell viability, as suggested before for camptothecin [51], and as indicated by the cell growth inhibition we observed on day 3.

IC_{50} CPT-11 preceded by IC_{20} 5-FU also did not affect DNA integrity in the SW620 and HT-29 cells (Figures 4a and b, respectively), but caused significant DNA damage in the SNU-C4 cells which was, however, eliminated 2 days later (Figure 4c). These lesions were probably largely attributable to 5-FU which caused in this cell line acute DNA damage that was repaired on day 3. Notwithstanding, IC_{20} 5-FU before CPT-11 led to 2-fold (SW620 and HT-29 cells) or 4-fold (SNU-C4 cells) less cell growth inhibition than CPT-11 alone (Table 1). These apparent antagonising effects could be due to FdUMP- and/or FdUTP-mediated inhibition of DNA synthesis [3, 42, 43], disabling the anti-proliferative effects of CPT-11, the cytotoxicity of which is known to require ongoing DNA replication [10]. The greater antagonism in the SNU-C4 cell line when compared with the SW620 and HT-29 cell lines might be due to its greater 5-FU sensitivity, possibly allowing for more efficient 5-FU-mediated S phase blockage and thus precluding CPT-11 cytotoxicity.

5-FU at IC_{20} concentrations given concurrently with CPT-11 presents a different situation. The effects of CPT-11 probably prevailed over those of 5-FU in the relatively 5-FU-resistant and rather CPT-11 sensitive SW620 and HT-29 cells (Table 1). Accordingly, this treatment in these two cell lines did not result in an increase in the percentage of single stranded DNA (Figure 4).

Taken together, our results support the use of CPT-11 alone in 5-FU-resistant colon carcinoma, and the use of CPT-11 before 5-FU because of the sequence-dependent antiproliferative effects of the combination [25–30]. Furthermore, prior treatment with CPT-11 at a weakly cytotoxic, fixed concentration can sensitise 5-FU resistant colon cancer cells to 5-FU. Moreover, in 5-FU-sensitive tumour cells low-dose 5-FU may also add to CPT-11 cytotoxicity (Table 1). These phenomena seem to be mediated by the sequence-dependent introduction of DNA lesions with different effects on cell viability.

To understand better the molecular basis of the observed interactions between the drugs, future studies should focus on the effects of low doses of CPT-11 or 5-FU on cellular determinants relevant to drug cytotoxicity. Assessing, for instance, the incorporation of ^{3}H-labelled thymidine in the presence of hydroxyurea, our initial efforts in this direction (data not shown) suggest that the treatments may interfere

with unscheduled DNA synthesis. This provides support for the above-mentioned suggestion that the potentiation of 5-FU by preceding CPT-11 could be due to interference with the excision repair pathway. Similarly, we are currently examining the impact of the drugs on thymidylate synthase and topoisomerase I activity, longevity of CPT-11-stabilised cleavable complexes, cell cycle phase distribution and plasma membrane integrity.

The results from the present study emphasise the importance of proper scheduling of CPT-11 and 5-FU in the design of treatment schedules for patients with advanced colorectal cancer. Both untreated and treated patients often are insensitive to systemic (5-FU-based) initial and follow-up therapies. Together with available pharmacokinetic information about the drugs, our findings can help to rationally design effective as well as weakly toxic protocols with the CPT-11–5-FU combination.

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