Cellular Pharmacology of the Combination of the DNA Topoisomerase I Inhibitor SN-38 and the Diaminocyclohexane Platinum Derivative Oxaliplatin[1]

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ABSTRACT

CPT-11, a DNA topoisomerase I inhibitor, and oxaliplatin, a diaminocyclohexane platinum derivative, are cytotoxic agents that have demonstrated clinical antitumor activity in colorectal cancer. Given the therapeutic potential of their combination, we studied the cellular pharmacology of SN-38, the active metabolite of CPT-11, and oxaliplatin in the human colon cancer HT29 cell line. Growth inhibition was studied after a 1- or 24-h exposure to SN-38 or oxaliplatin, given alone or in combination. The cytotoxicity analysis by the isobolograms method elicited synergy. SN-38 delayed the reversion of oxaliplatin-induced DNA interstrand cross-links (ISCs), measured in cells by alkaline elution. The amount of detectable ISCs 15 h after a 1-h exposure to 10 μM oxaliplatin was 27% of the ISC peak levels and increased to 68% in the presence of 0.1 μM SN-38. The presence of oxaliplatin DNA adducts led to a 3.3-fold increase in the SN-38-induced DNA elongation inhibition, as measured by pulse-labeling alkaline elution. Inhibition of DNA and RNA synthesis was longer after exposure to the combination of oxaliplatin and SN-38 than after exposure to each agent alone. Consistently, flow cytometry analyses revealed that preexposure to oxaliplatin enhanced SN-38-induced S-phase arrest. Filter binding assays indicated that the cells arrested in S-phase at 48 h were undergoing apoptosis. Hence, supra-additive cytotoxicity appears related to major modifications in the cellular response to DNA damage rather than to changes in DNA damage formation. The combination of CPT-11 and oxaliplatin induced a 2-fold higher tumor growth reduction *in vivo* than did oxaliplatin alone at feasible nonlethal doses. This study provides a rationale for the optimal use of CPT-11 and oxaliplatin in combination.

INTRODUCTION

The clinical development of CPT-11 (1, 2) and oxaliplatin (3–5) has led to their availability in advanced colorectal cancer patient treatment. CPT-11 is a camptothecin derivative and acts as the prodrug of a more potent DNA topo I[4] inhibitor, the 7-ethyl-10-hydroxy-camptothecin, also known as SN-38. The topo I enzyme relaxes DNA supercoiling and, thus, resolves topological constraints during DNA replication, transcription, and probably DNA repair and genetic rearrangements (6–8). This nuclear enzyme catalyzes DNA relaxation by making transient DNA single-strand breaks (6, 8, 9). Camptothecin derivatives inhibit the religation of topo I-DNA covalent cleavable complexes (9–11). The cytotoxicity of topo I-mediated DNA damage results from collisions between topo I-cleavable complexes and advancing replication forks, which create DNA double-strand breaks (6–8, 11). CPT-11 has demonstrated antitumor efficacy in a wide range of solid tumor xenografts (12, 13), including colorectal cancers. Oxaliplatin [DACH oxalato-platinum(II); oxaliplatin] is the only representative of the DACH platinum agents that has been successfully developed. The mechanism of action of oxaliplatin is mediated by the formation of DNA adducts. Platinum complexes with DACH carrier are of considerable interest because of their lack of cross-resistance *in vitro* with cisplatin and carboplatin (14–18). Clinical studies have confirmed that oxaliplatin is active in patients with cisplatin-resistant diseases, such as colorectal cancer (3, 5, 19). Because CPT-11 and oxaliplatin have wide spectra of antitumor activity, different mechanisms of action, and a nonoverlapping toxicity profile, the clinical development of their combination is of major interest. The administration of CPT-11 after oxaliplatin during the first Phase I clinical trial combining these two agents (20) was based on the present *in vitro* results.

We explored the cellular pharmacology of the combination of SN-38 and oxaliplatin, both in view of the therapeutic potential of the combination in the clinic and in an attempt to investigate the cellular interactions between camptothecin derivatives and platinum derivatives.

MATERIALS AND METHODS

Drugs and Chemicals. SN-38, CPT-11, and oxaliplatin were provided by Dr. F. Lavelle (Rhône-Poulenc Rorer, Vitry

[4] The abbreviations used are: topo I, topoisomerase I; DACH, *trans*-L-1,2-diaminocyclohexane; MTT, (4,5 dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; CI, combination index; ISC, interstrand cross-link; MTD, maximal tolerated dose.
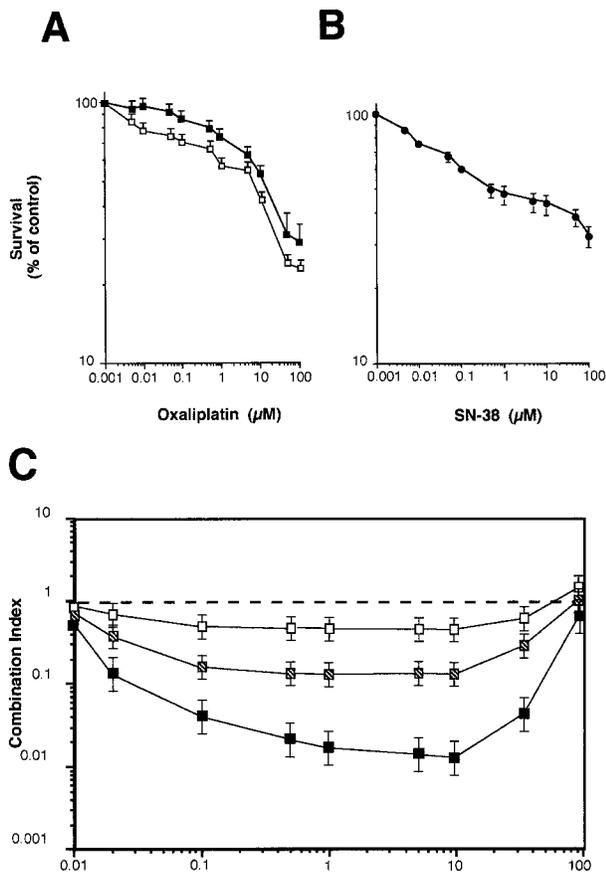
Fig. 1 Cytotoxicity after 24-h exposure to oxaliplatin, SN-38, or the combination of oxaliplatin and SN-38 in the human HT29 colon cancer cell line. Survival of HT29 cells was determined using MTT assay. *A*, oxaliplatin (■) and oxaliplatin plus 0.01 μM SN-38 (□). *Data points*, means of three to six experiments; *bars*, SD. *B*, cytotoxicity of SN-38 alone. *Bars*, SD. *C*, synergy was studied when oxaliplatin and SN-38 were combined, giving SN-38 before (□), with (▨), or after (■) oxaliplatin, using the method described by Chou *et al*. (21, 22). CI > 1, antagonism; CI < 1, synergism; CI = 1, additive effect.

sur Seine, France). Stock solutions of SN-38 were prepared in DMSO at 10 mM. Oxaliplatin was prepared extemporaneously for both *in vitro* and *in vivo* experiments and diluted in 5% dextrose immediately prior to use. CPT-11 was dissolved in 5% dextrose prior to use. [Methyl-^{3}H]thymidine, [^{14}C]thymidine (specific activities, 87 and 0.056 Ci/mmol, respectively), and [^{3}H]uridine (specific activity, 22 Ci/mmol) were purchased from NEN Research Products (Boston, MA).

Cell Culture. HT29 human colon adenocarcinoma cells were provided by Dr Y. Pommier (National Cancer Institute, NIH, Bethesda, MD) and were grown in monolayer cultures in RPMI 1640 supplemented with 5% heat inactivated FCS and 2 mM glutamine. No antibiotics were added to the medium. The cells were trypsinized and passed twice a week.

Cytotoxicity Assays. Cytotoxicity was determined by colony formation assays. Briefly, exponentially growing cells (2×10^5 cells/ml) were plated 24 h before drug treatment to allow them to attach as single cells. Following drug treatment, cells were washed twice with 5 ml of PBS preheated at 37°C and

trypsinized. Colony-forming ability was determined in triplicate by cloning 10^3 cells into 25-cm² flasks. Colonies were stained with methylene blue and counted after 14 days. Cytotoxicity was assessed from at least two different experiments. The cloning efficiency for untreated cells was 75%.

Growth inhibition was measured using the MTT (Sigma Chemical Co., St. Louis, MO) dye assay described previously (21) to evaluate the growth-inhibitory effects of the cytotoxic agents. Cell suspensions containing 2×10^4 viable cells/ml were seeded into 96-well microtiter plates. After a 48-h incubation, the cells were treated for 24 h with various concentrations of SN-38 (5×10^{-3} to 10^2 μM), oxaliplatin (10^{-2} to 10^3 μM). Combination assays were performed using 0.05 μM SN-38 with various concentrations of oxaliplatin. In the sequential schedule, SN-38 was administered 24 h prior to or after oxaliplatin. Seventy-two h following exposure to the drugs, 50 μl of a 125 μM MTT solution were added to each well, and the plates were incubated at 37°C for another 3 h to allow MTT metabolism into formazan crystals. The formazan crystals were finally solubilized adding 50 μl of 25% SDS solution (Sigma) into each well. Absorbance at 540 nm was measured using a Delta Soft ELISA analysis program for a Macintosh computer interfaced with a Bio-Tek microplate reader (model EL-340; BioMetallics, Princeton, NJ). Wells containing only RPMI 1640-fetal bovine serum and MTT were used as controls. Each experiment was performed using eight replicated wells for each drug concentration and carried out independently at least three times. The GI_{50} was defined as the concentration that reduced the absorbance in each test by 50%. The absorbance was calculated as: [(mean absorbance of eight wells containing drug) − (absorbance of eight control wells)]/[(mean absorbance of eight drug-free wells) − (absorbance of eight control wells)] $\times$ 100.

Analysis of Combination Effects. Combination analysis was performed using the method described by Chou *et al*. (21, 22) and a Calcusyn software program for automated analysis (Biosoft, Cambridge, United Kingdom). The influence on the combination of the two drugs was evaluated by comparing the sequential assays with assays involving oxaliplatin or SN-38 exposures alone. The combination effect was evaluated from isoeffect analysis CIs, calculated as follows: CI = $C_{oxaliplatin}/Cx_{oxaliplatin}$ + C_{SN-38}/Cx_{SN-38}, where $Cx_{oxaliplatin}$ and Cx_{SN-38} are the concentrations of oxaliplatin and SN-38 alone, respectively, needed to achieve a given effect (*x*%) and $C_{oxaliplatin}$ and C_{SN-38} are the concentrations of oxaliplatin and SN-38 needed for the same effect (*x*%) when the drugs were combined. These concentrations were calculated for each experiment and for each combination experiment at a fixed concentration ratio. The CIs were calculated under the assumption of a mutually exclusive drug interaction, *i.e.*, that the effect of SN-38 on topo I may influence the effect of oxaliplatin on DNA and *vice versa*. The combination was considered as positive (synergistic) when the combination index was <1 and negative (antagonistic) when it was >1.

DNA Synthesis Inhibition. Cellular DNA of exponentially growing cells was labeled with 0.005 μCi/ml [^{14}C]thymidine (53.6 mCi/mmol) for 48 h at 37°C. The rate of DNA synthesis was measured by 10-min pulses with 1 μCi/ml [methyl-^{3}H] thymidine (80.9 Ci/mmol). Tritiated thymidine incorporation was stopped by washing cell cultures twice in ice-cold HBSS and then scraping in 4 ml of ice-cold HBSS. One ml aliquots were transferred in Eppendorf tubes and precipitated with 100 μl of 100% trichloroacetic acid. Samples were vortexed, mixed, and centri-

Table 1 Growth inhibition and cytotoxicity of SN-38, oxaliplatin, and the combination of SN-38 and oxaliplatin[a]

| | SN-38[b] | L-OHP[c] | L-OHP+SN-38[d] | SN-38 → L-OHP[e] | | L-OHP → SN-38[f] | |
				3 h	24 h	3 h	24 h
$GI_{50\ (1h)}$	1.8	36	0.6	0.76	0.71	0.45	0.79
$IC_{50\ (1h)}$	2.5	42	0.9	1.1	0.95	0.66	1

[a] Data are expressed in μM. L-OHP, oxaliplatin; $GI_{50\ (1h)}$, concentration inhibiting 50% cell growth after a 1-h exposure; $IC_{50\ (1h)}$, concentration inhibiting 50% clonogenic efficacy after 1-h exposure.

[b] Effect of SN-38 alone.

[c] Effect of oxaliplatin alone.

[d] Effect of SN-38 when combined with 10 μM oxaliplatin for 1 hour, using simultaneous administration.

[e] SN-38 given 3 or 24 h prior to oxaliplatin.
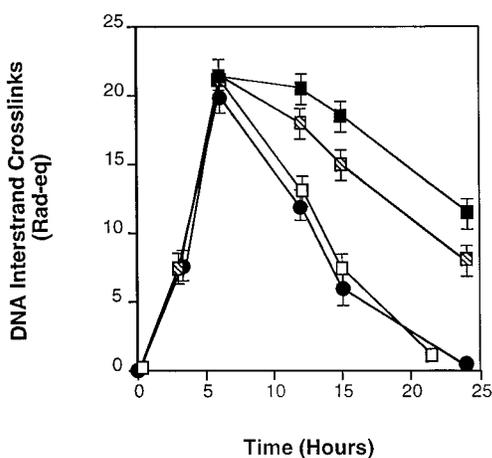
[f] Oxaliplatin given 3 or 24 h prior to SN-38.

Fig. 2 Effect of SN-38 on the kinetics of oxaliplatin-induced DNA ISCs. HT29 cells were exposed to 10 μM oxaliplatin alone (●) or 10 μM oxaliplatin plus 0.1 μM SN-38; SN-38 was given with (▨), 24 h before (□), or 6 h after (■) oxaliplatin.

fuged for 10 min at high speed in microfuge at 4°C. The precipitates were dissolved overnight at 37°C in 0.5 ml of 0.4 M NaOH. Samples were counted by dual-label liquid scintillation counting, and ^{3}H values were normalized using ^{14}C counts. Inhibition of DNA synthesis was calculated as the ratio of ^{3}H/^{14}C ratio in treated samples to ^{3}H/^{14}C in control cells.

RNA Synthesis Inhibition. The assay was performed as described in the paragraph above for the DNA synthesis inhibition assay. Pulses of [^{3}H]uridine at 1 μCi/ml were used to label RNA.

Measurement of DNA ISCs. ISCs were measured using the alkaline elution technique (23). DNA ISCs were assayed after a 300-rad irradiation of the cells. After irradiation, the filter elution assay was performed as described previously (23). The relative ISC frequency was obtained by the formula: ISC = {[(1 − R_0)/(1 − R_1)]$^{1/2}$ − 1} × 300 rad-eq, where 300 represents the standard irradiation dose (3 Gy = 300 rad) used to introduce random DNA single-strand breaks, R_0 represents the DNA retention of control cells treated only with the standard irradiation at a fixed elution end point, and R_1 represents the DNA retention of experimental cells treated with oxaliplatin and the standard dose of radiation at the same fixed elution end point.

Measurement of DNA Elongation. DNA elongation was measured using pulse-labeling alkaline elution, as described

previously (24–26). Exponentially growing HT29 cells were pulse-labeled for 15 min with 1 μCi/ml [methyl-^{3}H] thymidine, washed twice in preheated PBS, and incubated in radioactivity-free medium with 1 μM SN-38 at 37°C. After 1 h, the cells were washed twice in preheated PBS and maintained at 37°C. The aliquots were removed and analyzed by alkaline elution as described above, except that [^{14}C]thymidine-labeled cells were used as internal controls. The extent of DNA elongation, expressed as a percentage of control, was calculated as: [(R_0 − r)/(R_0 − r_x)] × 100, where r_x and r are the fractions of pulse-labeled [methyl-^{3}H] DNA retained on filter for control and SN-38 treated cells, respectively, and R_0 is the fraction of [^{14}C]DNA retained on filter for control cells. r_x, r, and R_0 were all determined at the same time elution point. Several time elution points were used to determine the kinetics of SN-38-induced DNA elongation. Thereafter, the effect of oxaliplatin alone or in combination with SN-38 was determined using 6- and 12-h elution time points.

Cell Cycle Determination. Cells were harvested, washed twice in ice-cold PBS (pH 7.4), fixed in 1 ml of 70% ethanol for 30 min at 4°C, washed twice in ice-cold PBS, treated for 30 min at 37°C with PBS containing 200 units/ml RNase (Sigma), and cellular DNA was stained with 1 ml of 500 μg/ml propidium iodide (Sigma). Cells were stored at 4°C prior to analysis. Cell cycle determinations were performed using a FACScan flow cytometer (Coulter Electronics, Paris, France), and data were interpreted using the multicycle AV model program, provided by the manufacturer. Each experiment was performed in triplicate.

Filter Binding Assay. This filter methodology has been described previously and allows the quantitation of DNA fragmentation (27). Briefly, cells were deposited on a protein-adsorbing filter (Metricel; Gelman Science, Ann Harbor, MI), washed with 3 ml of 0.02 mM Na2EDTA (pH 10), and lysed with 5 ml of LS10 [2 M NaCl, 0.04 M Na2EDTA, and 0.2% sarkosyl (pH 10)], followed by washing with 5 ml of 0.02 mM Na2EDTA (pH 10). Lysis fraction (L), Na2EDTA for washing (E), and filters (F) were collected and counted by liquid scintillation (28). DNA fragmentation was calculated as the percentage of DNA eluting from the filter as: DNA fragmentation = 100 × (L + E)/(L + E + F).

***In Vivo* Antitumor Activity.** The antitumor effect of CPT-11 and oxaliplatin was investigated in the GR-1 mouse mammary tumor model. GR-1 mice were obtained from M. Sluyser (NKI, Amsterdam, The Netherlands). Mice care was in agreement with institution guidelines. Treatments were admin-
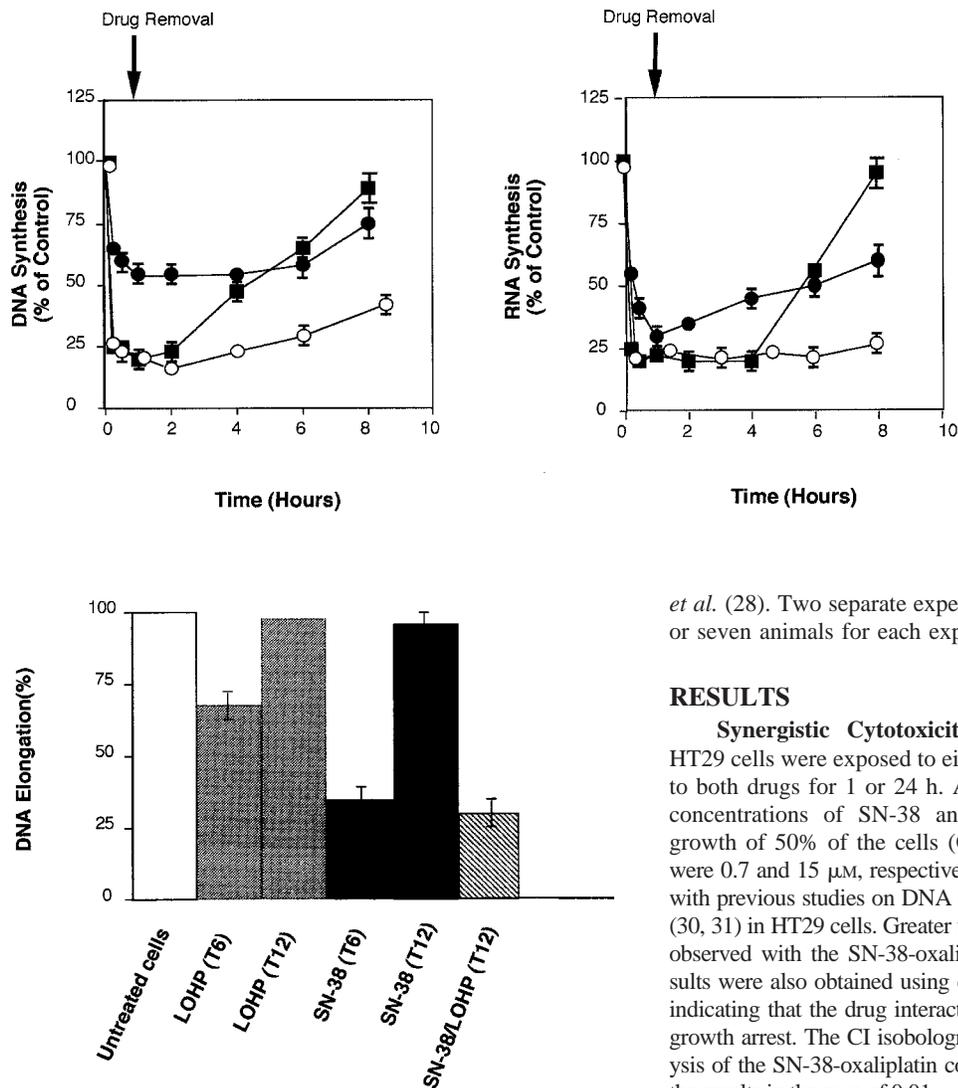
Fig. 3 Kinetics of DNA and RNA synthesis inhibitions. DNA (*left*) and RNA (*right*) synthesis was measured during and after a 1-h exposure to 10 μM oxaliplatin (●), 0.1 μM SN-38 (■), or 10 μM oxaliplatin plus 0.1 μM SN-38 (○). *Bars*, SD.

Fig. 4 Effect of oxaliplatin on SN-38-induced DNA elongation inhibition. Cells were treated for 1 h with either 0.1 μM SN-38, 10 μM oxaliplatin, or 10 μM oxaliplatin plus 0.1 μM SN-38. DNA elongation was measured 6 and 12 h after drug removal, using pulse-labeling alkaline elution.

istered by i.p. injection. The MTDs of oxaliplatin and CPT-11 alone and in combination were determined. The MTD was defined as the dose leading to reversible 25–30% body weight loss, without toxic death. The GR-1 human xenografts (1 × 10^6–2 × 10^6 cells) were transplanted s.c. into the hind limbs. Treatment started by day 13, after the tumor became palpable. Treatments were performed on day 10 after transplantation. The mean tumor volume on day 10 was 100 ± 50 mm^3. The animals were weighed concurrently with tumor measurements. In all cases, the mice were sacrificed before the tumor load or their general condition reduced their mobility. At the time of sacrifice, the tumor was excised, weighed, and, when required, embedded in paraffin and microscopically analyzed. Antitumor activity was defined following the recommendations of Schabel

et al. (28). Two separate experiments were conducted using six or seven animals for each experimental group.

RESULTS

Synergistic Cytotoxicity of SN-38 and Oxaliplatin. HT29 cells were exposed to either SN-38 or oxaliplatin alone or to both drugs for 1 or 24 h. As shown in Fig. 1, *A* and *B*, the concentrations of SN-38 and oxaliplatin that inhibited the growth of 50% of the cells (GI_{50}) when given alone for 24 h were 0.7 and 15 μM, respectively. Both results were in agreement with previous studies on DNA topo I inhibitors (29) or oxaliplatin (30, 31) in HT29 cells. Greater than additive growth inhibition was observed with the SN-38-oxaliplatin combination. Consistent results were also obtained using colony formation assays (Table 1), indicating that the drug interaction led to cell lethality rather than growth arrest. The CI isobologram equation was used in the analysis of the SN-38-oxaliplatin combination data. Fig. 1*C* illustrates the results in the case of 0.01 μM SN-38 combined with oxaliplatin. Similar results were obtained with various SN-38 concentrations in addition to a fixed dose of oxaliplatin, as summarized in Table 1. Our data extend the synergy previously described between DNA topo I inhibitors and cisplatin (23, 32) to the DACH platinum derivative oxaliplatin.

HT29 cells were incubated for 1 h with SN-38 and oxaliplatin, using either sequence, and a time interval ranging from 1 to 24 h. A supra-additive effect was found with both sequences but was maximal when oxaliplatin was administered first (Fig. 1*C*). Moreover, using the administration sequence oxaliplatin followed by SN-38, separated by a short time interval (3 h), there was higher cytotoxicity than either simultaneous administration or sequential administration with a 24-h interval (Table 1). This latter unexpected result led us to explore the molecular and cellular interactions likely to occur within a few hours of drug exposure and to reverse within 24 h.

Kinetics of Oxaliplatin-induced ISCs in HT29 Cells. The effect of SN-38 on the kinetics of oxaliplatin-induced ISCs was measured in cells by alkaline elution (Fig. 2). Very few ISCs were detected in cells when exposed to 10 μM oxaliplatin for 1 h. The exposure to 0.01 to 10 μM SN-38 failed to elicit ISC formation (data not shown). SN-38 coexposure did not affect the
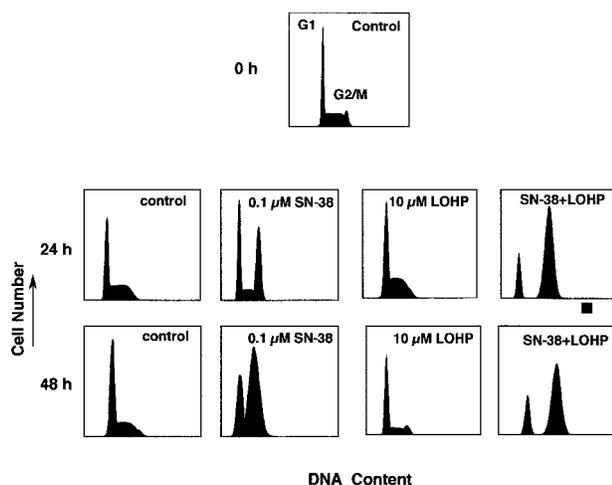
Fig. 5 Cell cycle response after oxaliplatin and SN-38-induced DNA damage. Flow cytometry was measured in HT29 cells 24 and 48 h after a 1-h exposure to 0.1 μM SN-38 or 10 μM oxaliplatin.

Fig. 6 Effect of the sequence of administration on the cell cycle response to oxaliplatin- and SN-38-induced DNA damages. Cell cycle distribution was measured by cytofluorimetry in HT29 cells 48 h after 1-h treatments with 0.1 μM SN-38 or 10 μM oxaliplatin, either alone or in combination. *Left,* untreated cells (*top*), 0.1 μM SN-38 alone (*middle*), and 10 μM oxaliplatin alone (*bottom*). *Right*, SN-38 was given either simultaneously (*top*), 24 h before oxaliplatin (*middle*), or 24 h after oxaliplatin (*bottom*).

formation of oxaliplatin ISCs but was associated with a 2-fold increase in ISCs 15 h after oxaliplatin removal. SN-38 preexposure had less effect on the reversion of oxaliplatin-ISCs than simultaneous exposure, whereas this effect was even more marked if SN-38 was given 6 h after oxaliplatin treatment. These results indicate that SN-38 slows down the reversion of ISCs. This effect is time dependent, with maximal inhibition of ISCs reversion obtained when SN-38 is administered close to the peak of oxaliplatin-induced ISCs.

Kinetics of DNA and RNA Synthesis Inhibition following SN-38- and Oxaliplatin-induced DNA Damage. The effect of the time interval between oxaliplatin and SN-38 administration on both cytotoxicity and ISCs led to a study of the kinetics of DNA and RNA synthesis inhibition. DNA and RNA synthesis was measured during and after a 1-h exposure to oxaliplatin, SN-38, and a combination of both (Fig. 3). Oxaliplatin inhibited DNA synthesis less profoundly but for a longer period of time than SN-38. SN-38 inhibited DNA and RNA synthesis for 4–6 h, with both DNA and RNA synthesis recovering fully 8 h after SN-38 removal. The addition of SN-38 to oxaliplatin increased the duration and extent of DNA and RNA synthesis inhibition (Fig. 3).

Effect of SN-38 on DNA Elongation in Presence and in Absence of Oxaliplatin. To study whether the inhibition of DNA synthesis in replicons in the vicinity of oxaliplatin-induced DNA adducts might be related to the observed increase in cytotoxicity, the kinetics of DNA elongation were studied using the pulse-labeled alkaline elution technique (Fig. 4). DNA elongation inhibition after a 1-h exposure to 0.1 μM SN-38 lasted for 6–8 h. DNA elongation was still inhibited when SN-38 was administered 0 to 6 h prior to oxaliplatin ISC formation (during or within 6 h after exposure to oxaliplatin), indicating that it could contribute to the longer half-life and toxicity of oxaliplatin ISCs. On the other hand, SN-38 treatment resulted in a longer DNA elongation inhibition, in the presence of oxaliplatin ISCs, with profound DNA elongation inhibition 12 h after SN-38 removal, suggesting irreversible DNA breakage.
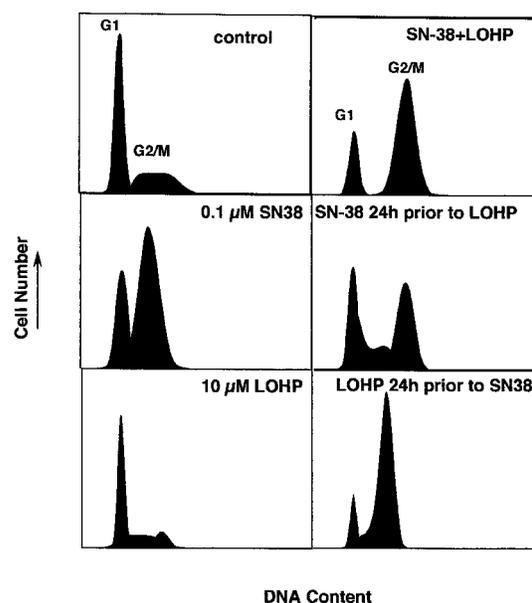
Cell Cycle Response and Apoptosis following SN-38- and Oxaliplatin-induced DNA Damage. The effects of SN-38- and oxaliplatin-induced DNA damages on cell cycle distribution and apoptosis were studied in HT29 cells. The cells were treated for 1 h, and cell cycle distribution was analyzed 24 and 48 h after drug removal, using cytofluorimetry techniques. Simultaneous 1-h exposure to 10 μM oxaliplatin and 0.1 μM SN-38 led to G_2 arrest and, to a lesser extent, G_1 arrest. Although the exposure to oxaliplatin alone led to G_2 arrest (Fig. 5), the cells treated with oxaliplatin prior to SN-38 did not arrest in G_2 but accumulated in S phase (Fig. 6). This S-phase block was consistent with the previous observation of prolonged SN-38-induced DNA elongation inhibition after oxaliplatin DNA adduct formation (Fig. 4). Because S-phase arrest is a common hallmark of the death induced by DNA topo I inhibitors (24, 26), apoptotic DNA fragmentation was measured in cells treated with the combination of SN-38 and oxaliplatin. A high rate of DNA fragmentation was detectable in cells at 48 h, confirming that S-phase arrested cells were undergoing apoptosis (Fig. 7). These results suggested that preexposure to oxaliplatin enhanced SN-38 cytotoxicity.

***In Vivo* Antitumor Activity of CPT-11 and Oxaliplatin.** Hormone-independent GR-1 mouse mammary tumors were used to evaluate the antitumor effects of CPT-11, oxaliplatin, and their combination *in vivo*. The aim of this *in vivo* study was to evaluate whether the concentrations of SN-38 and oxaliplatin required *in vitro* to observe molecular and cellular interactions leading to supra-additive cytotoxicity might be achievable *in vivo*. The MTD of oxaliplatin was 20 mg/kg. Oxaliplatin and CPT-11 given alone at doses of 10 and 30 mg/kg, respectively,
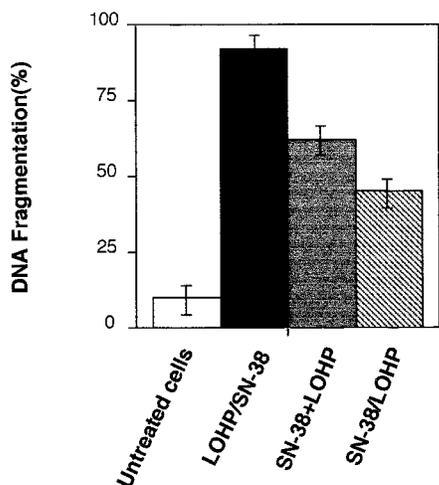
Fig. 7 Apoptotic DNA fragmentation. DNA fragmentation was quantitated using a filter binding assay 48 h after treatment with 0.1 μM SN-38 plus 10 μM oxaliplatin, using sequential (/) or simultaneous (+) administration.

showed no adverse effects and demonstrated antitumor activity against GR-1 mouse mammary tumors. The MTD of CPT-11 was 30 mg/kg when combined with 10 mg/kg oxaliplatin. Oxaliplatin and CPT-11 could be given simultaneously at two separate injection sites without a significant increase in toxicity. The addition of 10 mg/kg oxaliplatin to 30 mg/kg CPT-11 enhanced the antitumor activity, the combination being about twice as active as single drug treatments (Fig. 8). These results indicated that active doses of CPT-11 and oxaliplatin could be achievable in combination and suggested antitumor activity *in vivo* in GR-1 mouse mammary tumors.

DISCUSSION

We assessed the *in vitro* synergy between SN-38 and oxaliplatin. The cytotoxic supra-additivity observed was associated with evidence of reciprocal interactions: preexposure to oxaliplatin enhanced the toxic effects of SN-38, with more prolonged DNA elongation inhibition and more pronounced S-phase block, whereas slower reversion of oxaliplatin-induced ISCs was seen in the presence of SN-38, which might be explained by the effect of SN-38 on RNA and/or DNA synthesis.

Low levels of resistance to oxaliplatin are associated with the increased expression and activity of γ-glutamyl transpeptidase and glutathione levels (33). Because SN-38 did not affect the peak level of ISCs, the increased cytotoxicity could not be explained by changes in oxaliplatin accumulation or in detoxification enzymes.

Topo I inhibitors have been found to potentiate the cytotoxicity of other DNA-damaging agents, such as ionizing radiation, and alkylating agents (34). However, the mechanism of the synergy remained poorly understood. Synergism has also been found with cisplatin, associated with slower reversion of cisplatin-induced ISCs (23, 32). Cisplatin interacts synergistically with DNA synthesis inhibitors, whatever the mechanism of action leading to DNA synthesis interruption. Aracytin (35), 5-fluorouracil (36), camptothecin derivatives (23, 32), and, more recently, gemcitabine (37) have been shown to have supra-additive *in vitro* cytotoxicity when combined with cisplatin. Our
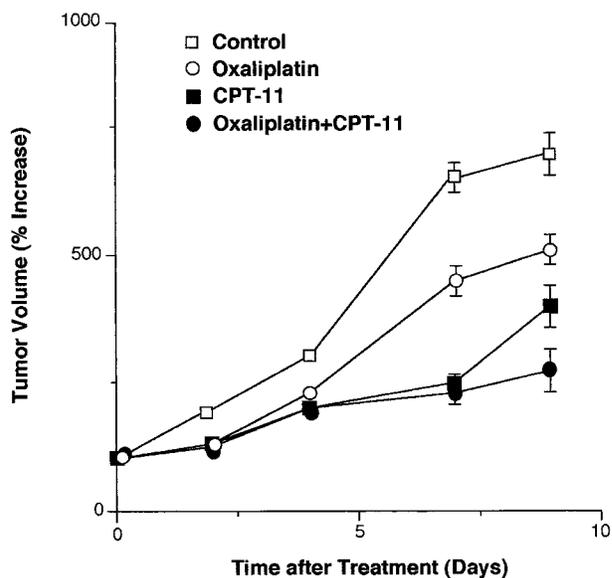

Fig. 8 Antitumor activity of CPT-11 oxaliplatin in combination with oxaliplatin in GR-1 mouse mammary tumors. Mice bearing s.c. tumors were treated i.p. with: no treatment (□), 30 mg/kg CPT-11 (■), 10 mg/kg oxaliplatin (○), or the combination of 30 mg/kg CPT-11 and 10 mg/kg oxaliplatin (●). *Data points*, means of two independent experiments; *bars*, SD.

results indicate that DNA and RNA synthesis inhibition following topo I-mediated DNA damage may also slow down the reversion of oxaliplatin-induced ISCs. Maximal cytotoxicity is observed with simultaneous DNA and RNA synthesis inhibition and the onset of oxaliplatin DNA ISCs formation.

The cytotoxicity of platinum compounds is believed to be a consequence of the formation of platinum-DNA adducts. However, DACH platinum DNA adduct structures differ from those of cisplatin and carboplatin, given the nature of the non-leaving ligand, with the DACH carrier ligand modifying the N—Pt—N bond angle (14). Loss of DNA mismatch repair may increase the tolerance to cisplatin-induced DNA adducts. The failure of the mismatch repair proteins to recognize the oxaliplatin DNA adducts may account for the antitumor activity of oxaliplatin in mismatch repair-deficient cells (16). Topo I may act as a specific mismatch site-nicking enzyme (38) and can be trapped on DNA depending on the location of a given DNA damage relative to the topo I cleavage site (38). An interaction between the oxaliplatin ISCs repair enzyme machinery and topo I is a possibility to consider for the increased duration of oxaliplatin ISCs in the presence of a topo I inhibitor.

Oxaliplatin-induced DNA damage sensitized the cells to the toxic effects of SN-38. Both the duration of SN-38 induced DNA elongation and the percent of cells arrested in S-phase increased when the cells were exposed to oxaliplatin prior to SN-38. The increase in topo I activity after DNA damage observed *in vitro* (38) might be translated *in vivo* by a transient increase in topo I activity and/or topo I-cleavable complex formation, resulting in more topo I-mediated DNA damage and increased cytotoxicity. Unfortunately, it was not possible to detect topo I-cleavable complexes after oxaliplatin exposure because oxaliplatin also induced high levels of DNA-protein

crosslinks (data not shown). S-phase block is not observed after oxaliplatin treatment but is a characteristic feature of topo I inhibitor cytotoxicity (24, 26). Thus, the enhanced S-phase block only observed with the sequence oxaliplatin followed by SN-38 suggests that SN-38 cytotoxicity is higher in presence of oxaliplatin-induced DNA damage. This effect might be the result of DNA breakage in the vicinity of oxaliplatin ISCs, making *de novo* DNA elongation impossible.

Direct correlations between quantitative DNA damage and cytotoxicity are seldom elicited, underlying the role of the cellular response to DNA damage for cytotoxicity (16, 26, 27, 29). We found that oxaliplatin may increase the cytotoxicity of topo I-mediated DNA damage and that SN-38 may interfere with the repair of oxaliplatin DNA ISCs.

Combinations of CPT-11 with any DNA damaging agent are expected to be supra-additive, and thus its association with oxaliplatin is of particular interest in colorectal cancer patients. Several clinical trials have explored the combination of cisplatin and CPT-11 (39–41). Interaction between cisplatin and CPT-11 was suggested by the marked decrease in the MTD of each compound given in combination (39, 40). A Phase I clinical trial combining oxaliplatin and CPT-11, given once every 3 weeks, has already been completed (20). A similar pharmacodynamic potentiation was observed in the absence of detectable pharmacokinetic interaction. Thus, the interaction between cisplatin or oxaliplatin and CPT-11 is likely to occur at the cellular level. Our findings reinforce this hypothesis showing that SN-38 and oxaliplatin reciprocally affect the cellular response to DNA damage. These *in vitro* findings may contribute to a better knowledge of the combination of topo I inhibitors and platinum derivatives cellular pharmacology, contributing to a rationale for the optimal development of the combination of CPT-11 and oxaliplatin.

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