Anti-Cancer Drugs 2001, **12,** pp. 741–751

Preclinical report

Combination of oxaliplatin and irinotecan on human colon cancer cell lines: activity *in vitro* and *in vivo*

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The *in vitro* and *in vivo* combination of oxaliplatin and irinotecan was investigated in a panel of four human colon cancer cell lines and their counterpart xenografts. *In vitro* and *in vivo* experiments demonstrated a synergistic or additive interaction in three cell lines (HCT-116, HCT-8 and HT-29) and an antagonism in SW-620 cells. Since there were clearly opposite interactions depending on the cell line, we further investigated cellular determinants possibly involved in the interaction between the two drugs in HCT-8 and SW-620 cells. Irinotecan slowed down the early platinum–DNA adducts repair (1 h after oxaliplatin exposure) in the presence of irinotecan only in HCT-8 cells (p=0.03, n=3). Moreover, a decrease of the expression of two proteins of the nucleotide excision repair (NER) system, ERCC1 and XPA, was observed. None of these effects was seen in SW-620 cells. Irinotecan induced apoptosis with an increase of poly(ADP-ribose) polymerase (PARP) cleavage in SW-620 cells (60 versus 7% basal level). Pretreatment of these cells with oxaliplatin abolished the increase in PARP cleavage induced by irinotecan (29%). In HCT-8 cells, a very little PARP cleavage was observed whatever the drug treatment. The persistence of platinum–DNA adducts in the presence of irinotecan could be due to a direct impact of irinotecan on NER gene expression or to an indirect effect on topoisomerase I activity. Complementary studies are required to determine if the cellular parameters identified in this study could be translated at the clinical level to predict clinical response after combined treatment with oxaliplatin and irinotecan in humans. [© 2001 Lippincott Williams & Wilkins.]

Key words: Apoptosis, ERCC1, human colon cancer cell lines, irinotecan, nucleotide excision repair, oxaliplatin, poly(ADP-ribose) polymerase, synergism, XPA.

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Introduction

Of the many thousands of platinum compounds synthesized over the last 30 years, a few dozen have reached pre-clinical and early clinical development. Of these, until recently, only cisplatin and carboplatin were available. Additionally, the diaminocyclohexane (DACH) family was shown to be non-cross-resistant with cisplatin. The current definition of the anticancer agent group proposed recently by the NCI from analysis of its *in vitro* cytotoxic screening program identified the DACH family as a completely new group of anticancer agents with a mechanism of action different from that of conventional platinum agents. Among the DACH compounds, oxaliplatin demonstrated a great potential against human colon cancer cell lines while cisplatin was barely cytotoxic.[1] Clinical development of this drug in colorectal cancer has resulted in its marketing approval in several countries.[2] Moreover, pre-clinical data and clinical trials demonstrated a great synergy between 5-fluorouracil (5-FU) and oxaliplatin in colorectal cancer.[3] Irinotecan, a topoisomerase (Topo) I inhibitor, was also introduced a few years ago in second-line therapy of metastatic colon and rectal cancer.[4,5] Several Topo I inhibitors were combined with platinum derivatives, mostly cisplatin, *in vitro* and *in vivo*. Synergy was observed when combining irinotecan and cisplatin,[6] or topotecan and cisplatin[7] or 9-aminocamptothecin and cisplatin.[8] Few studies investigated the combination of oxaliplatin and Topo I inhibitors.[9] Bissery *et al*. found an additive effect of the combination of oxaliplatin and irinotecan in a human sarcoma xenograft model when both drugs were administered simultaneously.[10] More recently, Zeghari-Squalli *et al*. demonstrated a sequence dependency when combining oxaliplatin and SN-38,

the active metabolite of irinotecan, in a human colon cancer cell line HT-29.[11] They showed that synergy was due, in part, to the delayed reversal of oxaliplatin DNA interstrand crosslinks by SN-38. However, no information was given concerning downstream events like the repair process of this damage or apoptosis. Repair of platinum adducts has been shown to be carried out by the nucleotide excision system (NER).[12] Two proteins seem critical in this process: XPA and ERCC1. The level of expression of these two proteins has been associated with cisplatin resistance both in ovarian cell lines and in ovarian cancer tissues.[13,14] Moreover, Topo I inhibitors induced apoptosis in tumor cells. Poly(ADP-ribose) polymerase (PARP) is a nuclear protein showing a typical cleavage pattern after cell exposure to genotoxic drugs. Topo I inhibitors were shown to induce PARP cleavage—this cleavage being correlated with apoptosis.[15]

Here we describe the *in vitro* combination of oxaliplatin and irinotecan in a panel of four human colon cancer cell lines. We extended our *in vitro* results to *in vivo* experiments to assess both the toxicity and efficacy of oxaliplatin and irinotecan combination. We specifically investigated cell determinants possibly involved in the interaction between oxaliplatin and irinotecan: platinum–DNA adducts repair, NER gene expression and PARP cleavage.

Material and methods

Drugs

Rhône Poulenc Rorer (Neuilly sur Seine, France) provided irinotecan. Oxaliplatin was obtained from Sanofi (Gentilly France). For animal studies, irinotecan was diluted in 0.9% sodium chloride solution and oxaliplatin was diluted in 5% glucose solution.

Antibodies

Rabbit anti-PARP and mouse anti-actin antibodies were from Roche (Meylan, France). Horseradish peroxidase conjugated anti-immunoglobulins were from Amersham (Les Ulis, France).

Cell culture

HT-29, HCT-8, SW-620 and HCT-116 were obtained from ATCC (Rockville, MD). Cells were grown as monolayers in RPMI 1640 medium supplemented with 5% fetal calf serum and 1% L-glutamine at 37°C in a humidified atmosphere containing 5% CO_2. Cells were split once a week using Trypsin/EDTA (0.25%/0.02%). Doubling times were 24 h for HCT-8 and HCT-116, and 28 h for HT-29 and SW-620.

Cytotoxicity assay

Drug concentrations that inhibit 50% of cell growth (IC_{50}) were determined using the sulforhodamine B technique.[16] Cells were plated on day 1 in 96-well plates. The cell density was 1500 cells/well for HCT-8 and HCT-116, 2200 cells/well for HT-29, and 4500 cells/well for SW-620 in a volume of 150 μl/well. In each plate, one column contained control cells and nine columns had increasing concentrations of drugs. For each column, six wells were used. Cells were treated on day 2 with oxaliplatin for 1 h (final concentration range 0.5–250 μM) or irinotecan for 24 h (range 0.05–25 μM) in a volume of 50 μl. The drug concentrations were reduced in the combination with oxaliplatin concentrations ranging from 0.25 to 250 μM for oxaliplatin and from 25 nM to 25 μM for irinotecan. The oxaliplatin:irinotecan ratio was 1:5 for HT-29 and SW-620, and 1:10 for HCT-116 and HCT-8. This ratio corresponds to the IC_{50} ratio of each cell line. After drug exposure, the medium was replaced with 200 μl of fresh drug-free medium and cells were grown for 72 h after the end of drug exposure. The cells were then precipitated with 50 μl of ice-cold 50% trichloracetic acid for 60 min at 4°C, rinsed 6 times with water and air-dried. Fixed cells were stained with 50 μl of 0.4% sulforhodamine B solution in 0.1% acetic acid, rinsed with 0.1% acetic acid solution and air-dried. Sulforhodamine B was re-dissolved with 150 μl of 10 mM Tris buffer, pH 10.5. Optical densities were measured at 540 nm with a Multiskan multisoft apparatus (Labsystems, Les Ulis, France). Growth inhibition curves were plotted as percentage of control cells and IC_{50} values were determined by interpolated graph analysis. For the combination, cells were exposed to oxaliplatin for 1 h, rinsed once with saline solution and then exposed to irinotecan for 24 h.

The cytotoxicity of combinations was compared to the cytotoxicity of each drug alone in every experiment and each experiment was performed 3 times.

Median-effect analysis

Median-effect analysis was used to determine interactions between oxaliplatin and irinotecan. Dose–response interactions (antagonism, additivity or synergism) were expressed as a non-exclusive case. Combination indexes were calculated according to Chou and Talalay.[17,18] $CI < 0.8$ indicates synergism, $CI > 1.2$ indicates antagonism and CI=0.8–1.2 indicates additivity of the drugs.

Animals

Female Swiss athymic mice, 4–5 weeks old, purchased form Iffa Credo (St Germain sur l'Abresle, France), were housed in filter-capped cages kept in a sterile facility and maintained in accordance with the usual standards. After a 2-week quarantine, they were used for chemotherapeutic studies.

Tumor models

Xenografts of the four cell lines were obtained after s.c. injection of 10^7 cells. Drug activity was evaluated only against advanced-stage tumors. Seven days after cell inoculation, mice bearing 100–250 mm^3 s.c. tumors were pooled and randomly assigned to different groups of 10 mice. Two perpendicular diameters of the tumors were measured 3 times weekly with a caliper square by the same investigator. Each tumor volume was calculated according to the following equation: V (mm)=d^2 (mm^2) $\times D$ (mm)/2, where d and D are the smallest and the largest perpendicular tumor diameters, respectively. Animal body weights were recorded 3 times a week and mortality was checked daily. The experiments lasted until tumor volumes reached 5 times the initial volume.

Treatment

Drugs were administered i.v. via the caudal vein in a volume of 10 μl/g of body weight. Mice received oxaliplatin as a single i.v. injection on day 1 at a dose of 10 mg/kg. They received i.v. irinotecan from day 1 to 5 at a dose of 20 mg/kg/day. The control group was treated from day 1 to 5 with i.v. injections of saline.

Evaluation of toxicity and antitumor activity

The maximum body weight loss (MBWL) and the number of toxic deaths monitored toxicity. Antitumor activity was evaluated by the time to reach 5 times the initial volume (*Td*) and by specific growth delay (*SGD*) calculated according the following equation:

$$SGD\ (\%) = \frac{Td(treated) - Td(controls)}{Td(controls)} \times 100$$

Quantification of platinum–DNA adducts by atomic absorption spectrometry (AAS)

Cells were treated with 40 μM oxaliplatin for 1 h. At the end of the incubation, medium was replaced by either drug-free medium or by medium containing irinotecan for 24 h (the oxaliplatin:irinotecan ratio was maintained as in the cytotoxicity study). Cells were then rinsed twice with phosphate-buffered saline and frozen at -20°C until analysis. DNA was extracted by phenol:chloroform and redissolved in water. DNA concentration was determined by spectrometric absorption at 260 nm. DNA was then digested by 1000 UI of nuclease S1 for 24 h at 37°C. Platinum concentration was evaluated on a Perkin-Elmer atomic absorption spectrometer AAnalyst 600. Calibration curves were established for each determination using platinum solution containing 5.25 to 262 pg/μl platinum and validated by controls. The limit of quantification was 2.5 pg/μl platinum. Results were expressed as pg Pt/μg DNA.

Determination of XPA and ERCC1 expression by reverse transcription-polymerase chain reaction (RT-PCR)

Cells were treated with 40 μM oxaliplatin for 1 h. At the end of the incubation, medium was either replaced by drug-free medium or by medium containing irinotecan for 6 h (the oxaliplatin:irinotecan ratio was maintained as in the cytotoxicity study). Cells were then rinsed twice with PBS and frozen until RNA extraction. Total RNA was extracted using Trizol solution (Life Technologies, Cergy-Pontoise, France). Then 2 μg of RNA were reversed transcribed using Ready-to-go You prime first-strand kit (Amersham Pharmacia, Piscataway, NJ). The following primers were used: XPA primers (XPA S: 5′-GCATGGCTAATG-TAAAAGCA-3′; XPA AS: 5′-TCCTGTGGACTTCCTTT-GC-3′), ERCC1 primers (ERCC1 S: 5′-GAGCTGGCTAA-GATGTGTAT-3′; ERCC1 AS: 5′-AGGCCAGATCTTCTC-TTGAT-3′) and β-actin primers (BA S: 5′-ATCTGGCAC-CACACCTTCTACAATGAGCTGCG-3′ and BA AS: 5′-CGTCATACTCCTGCTTGCTGATCCACATCTGC-3′).

The 100-μl reaction mixture contained 10 mM Tris, pH 9, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 0.2 mg/ml bovine serum albumin, 50 μM of each dNTP, 0.5 U Taq polymerase and either 20 pmol of XPA primers, 30 pmol of ERCC1 primers or 5 pmol of β-actin primers. Amplification conditions were: denaturation 1 min at 94°C, hybridization 1 min at 60°C and extension 1.5 min at 72°C, for 35 cycles. Reaction products were electrophoretically separated in 2% agarose gel in TBE buffer and visualized after ethidium bromide staining. PCR products were quantitated using Image Quant software.

Results were expressed as ratio of intensity of the bands from ERCC1 or XPA and β-actin.

PARP determination by Western blotting

Cells were treated with 40 μM oxaliplatin for 1 h. At the end of the incubation, medium was replaced by either drug-free medium or by medium containing irinotecan for 24 h (the oxaliplatin:irinotecan ratio was maintained as in the cytotoxicity study). At the end of incubation, cells were placed in drug-free medium for 72 h. Cells were then sonicated in lysis buffer (62.5 mM Tris, 4 M urea, 100 mM DTT, 2% SDS, 10% glycerol and 0.04% Bromophenol blue) and centrifuged at 13 000 *g* for 10 min. Samples containing 20 μg protein were separated by PAGE consisting in 4% (w/v) acrylamide stacking gel and 7.5% (w/v) separating gel. The running gel buffer was 25 mM Tris, 250 mM glycine and 0.1% SDS. Proteins were electrotransferred on Hybond P membranes (Amersham Pharmacia). Membranes were then blocked with 5% non-fat dry milk solution in TBS–Tween for 1 h at room temperature and then incubated overnight at 4°C with primary antibody (1/10 000 dilution for anti-PARP and 1/25 000 dilution for anti-α-actin). After washing, the membranes were incubated with horseradish peroxidase-conjugated anti-immunoglobulin (1/5000) in TBS–Tween for 90 min at room temperature.

Bands were visualized with enhanced chemiluminescence reagent (ECL+; Amersham Pharmacia Biotech) on Hyperfilm MP (Amersham Pharmacia Biotech). Intensity of the bands was quantified using Image Quant (Moldyn; Amersham Pharmacia Biotech). Results were normalized with α-actin intensity.

Statistical analysis

Statistical tests were performed after verification of the normal distribution of the population for the different parameters studied. The data are presented as average ± standard deviation. Comparisons were carried out after controlling for the homogeneity of the variances. The variance analysis was performed on equilibrated groups (same number of mice per group) and included the calculation of the interaction between oxaliplatin and irinotecan, and the statistical significance of this interaction. The level of significance for all tests was 5%.

Results

Cytotoxicity of oxaliplatin and irinotecan on colon cell lines

The growth inhibition experiments were performed using 1-h exposure with oxaliplatin or 24-h exposure with irinotecan in HCT-8, HCT-116, SW-620 and HT-29 cells. The results are summarized in Table 1. For oxaliplatin, the IC_{50}s ranged from 12 μM for SW-620 to 45.9 μM for HCT-8. HCT-116 cells were the most sensitive to irinotecan (2.2 μM); HT-29 being the least sensitive (7.8 μM).

Median-effect analysis of oxaliplatin and irinotecan combination *in vitro*

Combination of oxaliplatin and irinotecan was investigated by exposing the cells to oxaliplatin for 1 h, followed by a 24-h exposure to irinotecan and analyzed by median-effect analysis, developed by Chou and Talalay.[17,18] Figure 1 represents the median-effect analysis for the four cell lines, HCT-8, HCT-116, SW-620 and HT-29. The combination indexes producing 50% growth inhibition (CI_{50}) were 0.83 ± 0.13, 0.66 ± 0.25, 1.42 ± 0.5 and 0.77 ± 0.14 for HCT-8, HCT-116, SW-620 and HT-29, respectively. Thus, a significant synergistic effect was observed in one cell line (HCT-116), an additive effect in two others (HCT-8 and HT-29), and an antagonist effect in SW-620 cells.

Toxicity of the combination of oxaliplatin and irinotecan in mice

The optimal doses of irinotecan and oxaliplatin were chosen according to previous studies.[10–19] While irinotecan and oxaliplatin induced low or no toxicity (5 and 1.5% of body weight loss, respectively), the combination of 10 mg/kg oxaliplatin on day 1 and 20 mg/kg irinotecan (day 1–5) induced a maximum body weight loss of 20 ± 7%. Thus, there was greater than additive toxicity when combining oxaliplatin and irinotecan. However, this toxicity was tolerable, since only four toxic deaths out of 70 treated mice were observed.

Antitumor activity of oxaliplatin and irinotecan *in vivo*

The antitumor activity of oxaliplatin, irinotecan and the combination of both drugs was expressed by the

Table 1. Growth inhibition of irinotecan and oxaliplatin in a panel of four cell lines: HCT-8, HCT-116, SW-620 and HT-29

	HCT-8	HCT-116	SW-620	HT-29
Oxaliplatin (μM)	46 ± 4	38 ± 7	12 ± 4	24 ± 6
Irinotecan (μM)	6.4 ± 2	2.2 ± 0.5	3.5 ± 0.5	7.8 ± 0.9

Cells were exposed to oxaliplatin (0.5–250 μM) for 1 h or irinotecan (0.05–25 μM) for 24 h. Results are expressed as the concentration that inhibits 50% of growth in comparison with controls (IC_{50}). Values are mean ± SD of three independent experiments.

time necessary to reach 5 times the initial tumor volume (*Td*) and specific growth delay (*SGD*). Table 2 summarizes the *Td* obtained for oxaliplatin, irinotecan and the combination of both drugs on the four xenografts studied, and Figure 2 illustrates the *SGD*s obtained with the different treatments and xenografts. Oxaliplatin was rather inefficient against this panel. Irinotecan showed a significant antitumor effect in all the four cell lines with *SGD*s ranging from 15.9 to 130.2%. The combination of oxaliplatin and irinotecan showed a significant synergism on HCT-8 xenografts, an additive effect on HT-29 and HCT-116 xenografts, and less than an additive effect on SW-620 xenografts.

By combining *in vitro* and *in vivo* data, a clear antagonism between oxaliplatin and irinotecan was observed in the SW-620 cell line, whereas an additive or synergistic interaction was noted in HCT-8, HCT-116 and HT-29 cells (Table 3). Consequently, molecular approaches have been explored in two cell lines: SW-620 and HCT-8.

Quantification of platinum–DNA adducts by AAS

We determined the amount of platinum–DNA adducts in the presence or absence of irinotecan. We studied two timepoints: 1 and 24 h after the end of oxaliplatin exposure. Figure 3 shows that at the end of the oxaliplatin treatment, the amounts of platinum–DNA adducts were about the same in SW-620 and HCT-8 cells. SW-620 cells showed the same decrease in platinum–DNA adducts when exposed to 1 h irinotecan or free medium (p=0.25, n=3). Conversely, in HCT-8 cells, the presence of irinotecan slowed down the repair of platinum–DNA adducts compared to free medium (p=0.03, n=3). Twenty-four hours after oxaliplatin, the presence of irinotecan did not produce any significant difference in platinum–DNA adducts level in SW-620 and HCT-8 cells.

Table 2. *In vivo* antitumor activity of oxaliplatin, irinotecan and the combination of both drugs in the four cell lines grown as xenografts: HCT-8, HCT-116, SW-620 and HT-29

	HCT-8	HCT-116	SW-620	HT-29
Controls	15.7±3.3	12±1.3	17.7±3.9	12.6±3.4
Oxaliplatin	15.5±3.6	11.7±2.6	16.6±2.0	14.4±2
Irinotecan	18.2±4.8	27.6±2.3	39.2±4.8	21.4±2.7
Oxaliplatin+ irinotecan	23.8±4.3	29.2±3.4	40.2±6.6	26.4±2.2

Mice received either a single i.v injection of 10 mg/kg of oxaliplatin on day 1, i.v injection of 20 mg/kg of irinotecan from day 1 to 5 or i.v injection of 10 mg/kg of oxaliplatin on day 1 and 20 mg/kg of irinotecan from day 1 to 5. The antitumor effect is expressed as the time to reach 5-fold the initial tumor volume in days (*Td*). Each group contained at least eight animals.

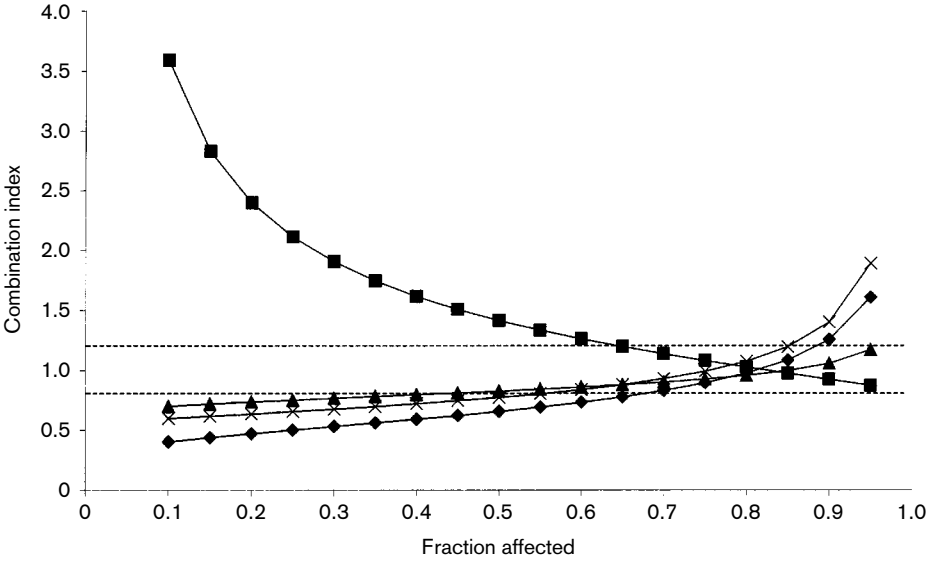

Figure 1. Median-effect analysis of the combination of oxaliplatin and irinotecan on the four cell lines: HCT-8 (▲), HCT-116 (◆), SW-620 (■) and HT-29 (X). Combination indexes are presented as a function of the fraction of cells affected by the cytotoxic effect. CI>1.2 indicates antagonism, CI=0.8–1.2 indicates additivity and CI<0.8 indicates additivity. Values are means of three independent experiments.

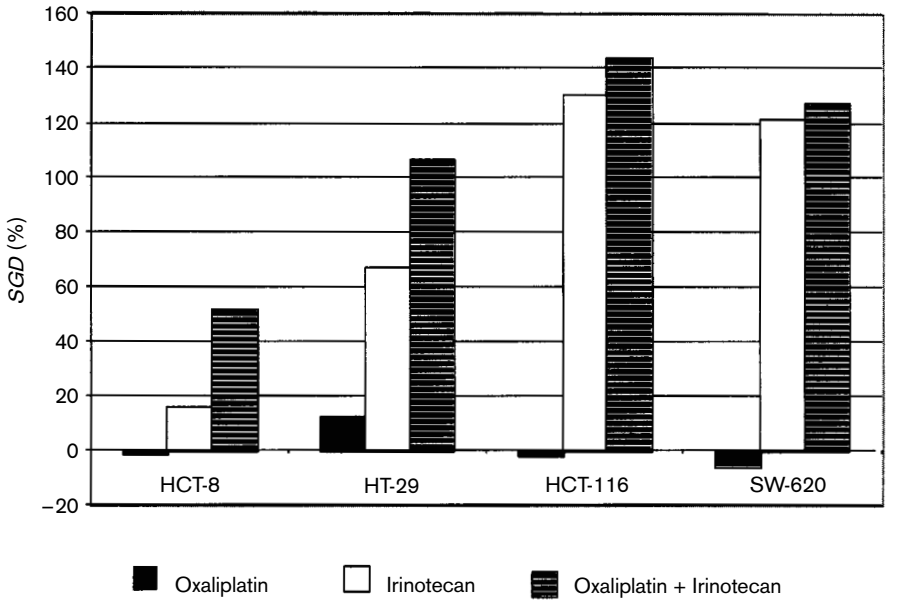

Figure 2. Efficacy of oxaliplatin (10 mg/kg day 1), irinotecan (20 mg/kg/day, for 5 days) and the combination of the two drugs against HCT-8, HT-29, HCT-116 and SW-620 xenografts. Treatment started when tumor volumes were 100–250 mm^3. Results are mean of 10 mice and expressed as specific growth delay (%) (*SGD*).

Table 3. Type of interaction (synergistic, additive or antagonistic) observed *in vitro* and *in vivo* on the panel of four cell lines: HCT-8, HCT-116, SW-620 and HT-29

	HCT-8	HCT-116	SW-620	HT-29
In vitro interaction	additivity	synergism	antagonism	additivity
In vivo interaction	synergism	additivity	antagonism	additivity

XPA and ERCC1 expression after oxaliplatin and irinotecan exposure *in vitro*

We investigated the impact of oxaliplatin ± irinotecan on XPA and ERCC1 expression 6 h after the end of oxaliplatin exposure using a RT-PCR technique. Results are presented Figure 4(a and b). The expression of ERCC1 mRNA increased after exposure of HCT-8 cells to oxaliplatin, while no change was seen in SW-620 cells. In HCT-8 cells, the combination of oxaliplatin and irinotecan decreased both ERCC1 and XPA mRNA expression compared to oxaliplatin alone (70 and 78% decrease, respectively). In SW-620 cells, conversely, modification of neither XPA nor ERCC1 expression was observed after oxaliplatin+irinotecan exposure. It seemed that the down-regulation of both XPA and ERCC1 was related to the synergistic effect of the oxaliplatin and irinotecan combination in HCT-8 cells.

PARP cleavage after oxaliplatin and irinotecan exposure *in vitro*

Figure 5 presents PARP cleavage determined by Western blot analysis after exposure of HCT-8 and SW-620 cells to oxaliplatin, irinotecan and the combination of these two drugs. The basal level of PARP cleavage was rather low in both HCT-8 and SW-620 cell lines (7 and 15%, respectively). After oxaliplatin exposure, PARP cleavage was not modified in HCT-8, but increased significantly in SW-620 cells (3.3-fold). Irinotecan induced a moderate increase of PARP cleavage in HCT-8 (1.3-fold increase), but had a major impact in SW-620 cells: PARP cleavage increased from 7 to 60%. The combination of oxaliplatin and irinotecan did not produce any further increase in PARP cleavage in HCT-8 cells, while it totally abolished the increase induced by irinotecan in SW-620 cells. Thus, the blockade of PARP cleavage in SW-620 cells by oxaliplatin could be a factor in the antagonism between oxaliplatin and irinotecan.

Discussion

This study evaluated the combination of oxaliplatin and irinotecan in a panel of four human colon cell lines, both *in vitro* and *in vivo*. A previous *in vitro* study reported a synergy when combining oxaliplatin and SN-38, the active metabolite of irinotecan, on

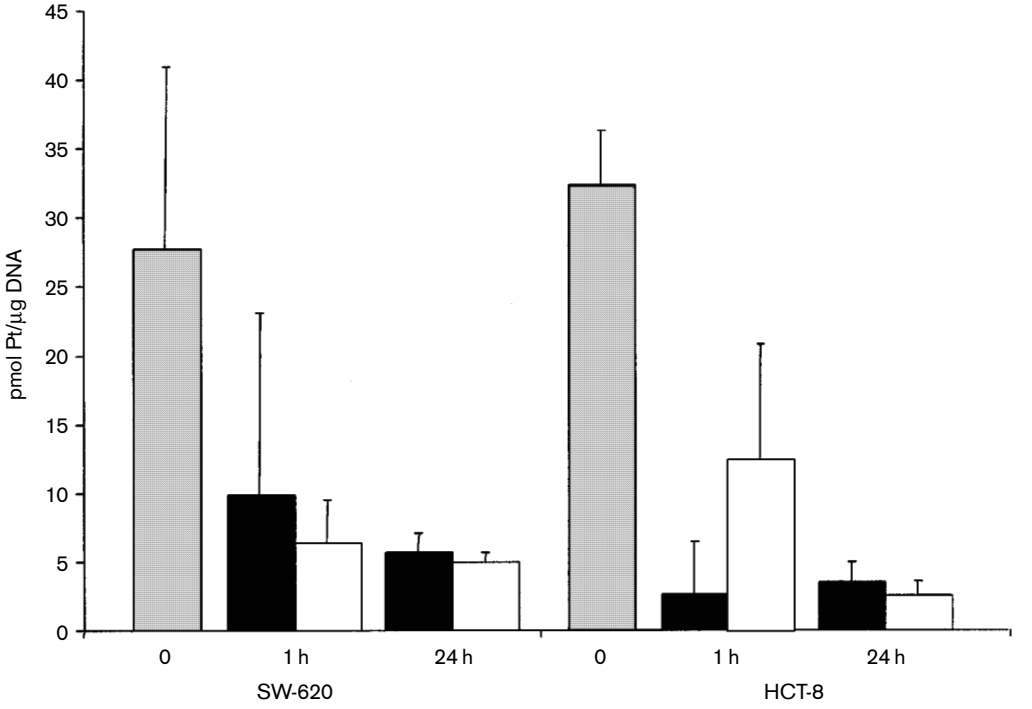

Figure 3. Quantification of platinum–DNA adducts after oxaliplatin ± irinotecan in HCT-8 and SW-620 cells. Cells were treated with oxaliplatin for 1 h (▨) followed by drug-free (■) or irinotecan-containing medium (□) for 1 and 24 h. DNA was extracted using phenol:chloroform. Platinum content was measured by AAS. Results are expressed as pg Pt/μg DNA.

the ovarian cancer cell line IGROV-1.[9] *In vivo*, Bissery *et al.* showed an additive effect when combining oxaliplatin and irinotecan in sarcoma xenografts.[10] The aim of our work was to extend the investigation to several cell lines in order to show if synergism could always be achieved and, if it was not the case, to identify the cellular determinants predicting a positive interaction between the two drugs. We therefore conducted both *in vitro* and *in vivo* experiments combining oxaliplatin and irinotecan on a panel of four different colon cell lines and their xenograft counterparts. We used a 1-h *in vitro* exposure to oxaliplatin and a 24-h exposure to irinotecan. We previously demonstrated that in HT-29 cells at the concentration of 10 μM, irinotecan was converted over 24 h in cytotoxic concentrations of SN-38.[19] The choice of a prolonged exposure was also based on the mechanism of cytotoxicity of the camptothecin analogs (collision between a cleavable complex stabilized by the inhibitor and a replication fork)[20] and the results from preclinical studies from Houghton *et al.*[21] Finally, Kawato *et al.* showed *in vitro* that irinotecan can exert its antitumor activity after transformation to SN-38 via a carboxylesterase, but also has its own inhibitory effects on DNA and RNA synthesis at concentrations corresponding to the concentrations we used (about 10 μM).[22] Our *in vitro* study demonstrated that synergism or additivity between oxaliplatin and irinotecan was observed in three out of four cell lines in our panel: synergism in HCT-8, HCT-116 and HT-29, and a clear antagonism in SW-620 cells. To complete this evaluation, we performed *in vivo* studies using the same panel of four cell lines. The combination of oxaliplatin and irinotecan *in vivo* was associated with a greater toxicity, as compared to both drugs used as single agents. We previously reported such a synergistic toxicity with the 5-FU–irinotecan combination.[23] Surprisingly, we were unable to reproduce *in vivo* the data obtained *in vitro* for the cytotoxicity of oxaliplatin. In tumor xenografts, we did not observe any modification in the time to reach five initial volumes compared to controls. Our data are in agreement with those reported by Fink *et al.*[24] on HCT-116 xenografts using a higher oxaliplatin dose level: 12 mg/kg. The *in vivo* antitumor activity of irinotecan was in good agreement with the *in vitro* data: all xenografts responding to irinotecan with *Td*s significantly greater than the controls. These results were consistent with those reported by Houghton *et al.* demonstrating complete or partial regression in five of eight human colon carcinoma cell lines using a 2-week administration schedule.[21]

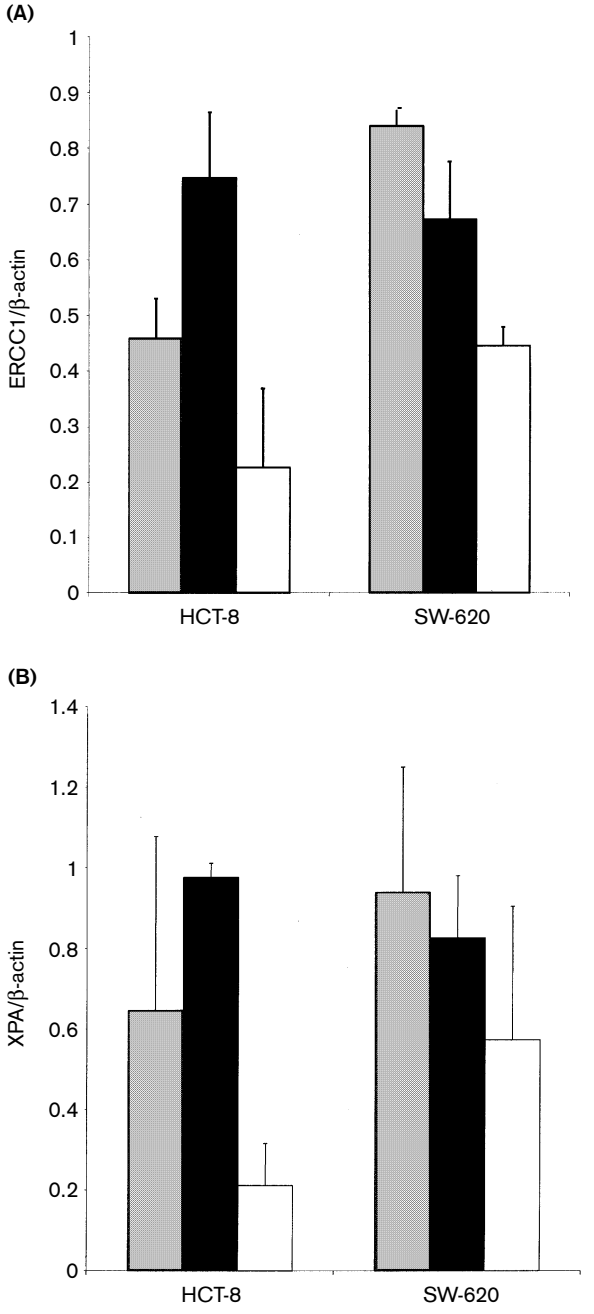

Figure 4. ERCC1 (A) and XPA (B) expression determined by RT-PCR (β-actin amplification is the internal control) on HCT-8 and SW-620 cells. Cells were treated with 40 μM oxaliplatin 1 h $\pm$ irinotecan for 6 h. Total RNA (2 μg) was reversed transcribed and amplified using specific primers. After electrophoresis on agarose gel and staining with ethidium bromide, band intensity was determined and results are expressed as the ratio of either XPA or ERCC1 band and β-actin band. Results are means of two independent experiments. (▨) Controls, (■) oxaliplatin and (□) oxaliplatin + irinotecan.

We observed a synergistic or additive antitumor effect of oxaliplatin and irinotecan combination in HT-29, HCT-116 and HCT-8, and an antagonism in SW-620 xenografts as we found *in vitro* (Table 3). Since the synergy of the combination of oxaliplatin and irinotecan was clearly absent in SW-620 cells and present in HCT-8 cells, it was interesting to identify cellular determinants involved in the interaction between the two drugs in these cell lines.

Zeghari-Squalli *et al.*[11] demonstrated that the supra-additivity observed *in vitro* when combining oxaliplatin and SN-38 was associated with reciprocal interactions: a pre-exposure of cells to oxaliplatin enhanced the cytotoxic effect of SN-38 with a prolonged DNA elongation inhibition and a greater block in S phase. On the other hand, SN-38 slowed down the reversion of oxaliplatin-induced interstrand cross-links. The same explanation has been proposed by Masumoto *et al.*[25] in a squamous cell carcinoma cell line HST-1 with the combination of cisplatin and SN-38, and by Goldwasser *et al.*[8] with cisplatin and 9-aminocamptothecin. We evaluated the formation and the repair of platinum–DNA adducts induced by oxaliplatin and showed that irinotecan slowed down the early repair (1 h after oxaliplatin exposure) of platinum–DNA adducts in HCT-8 cells, while it had no impact on SW-620 cells. Thus, platinum–DNA adduct repair seemed to be the first step in the interaction between oxaliplatin and irinotecan.

Reardon *et al.* demonstrated that oxaliplatin–DNA adducts were repaired by NER repair just like the other platinum compounds, cisplatin and JM216.[26] Reed *et al.* demonstrated that the increase of ERCC1, a NER gene, was a prerequisite for the expression of the other NER genes in response to cisplatin-induced damages.[27] ERCC1 and XPA gene expression were higher in patients with ovarian carcinoma not responding to cisplatin than responders.[13] We determined ERCC1 and XPA mRNA expression after oxaliplatin $\pm$ irinotecan exposure of HCT-8 and SW-620 cells. ERCC1 and XPA expression increased in HCT-8 cells after oxaliplatin exposure, which was consistent with the low sensitivity of these cells to oxaliplatin. A down-regulation of both XPA and ERCC1 expression was observed in HCT-8 cells when combining oxaliplatin and irinotecan, while no impact on either of these genes was seen in SW-620 cells under the same conditions. Zeghari-Squalli *et al.* hypothesized that the presence of oxaliplatin-induced DNA damage could transiently increase Topo I activity, thus resulting in more Topo I-mediated DNA damage and increased cytotoxicity.[11] Our data indicated that irinotecan might have also an impact on ERCC1 and XPA gene expression, directly or indirectly through the persistence of platinum–DNA adducts.

The apoptosis induced by Topo I inhibitors visualized by the cleavage of the PARP was reported

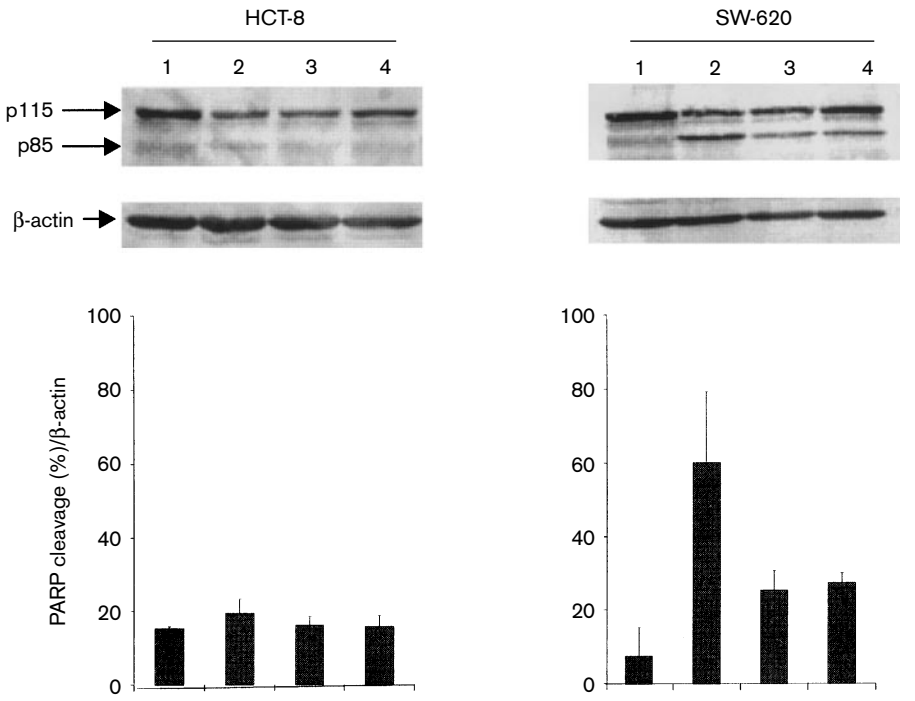

Figure 5. PARP cleavage determined by Western blot analysis on HCT-8 and SW-620 cells. Cells were treated with 40 μM oxaliplatin for 1 h $\pm$ irinotecan for 24 h. PARP cleavage was evaluated 72 h after the end of the exposure. Cells were lysed and analyzed by SDS–PAGE, and immunoblotted with anti-PARP and anti-actin antibodies. Lane 1, control cells; lane 2, irinotecan-treated cells; lane 3, oxaliplatin-treated cells; lane 4, oxaliplatin+irinotecan-treated cells. The arrows show the position of both 115 and 85 kDa PARP bands, and the 42 kDa α-actin band. Bands intensity was quantified using ImageQuant software. Results are means of two independent experiments.

by Whitacre *et al.* as a predictive marker of Topo I inhibitor antitumor effect.[15] We determined the apoptosis mediated by oxaliplatin and irinotecan by Western blotting of the cleaved PARP. Oxaliplatin did not produce any significant PARP cleavage in either HCT-8 or SW-620 cells. Irinotecan alone induced PARP cleavage in SW-620 cells (60% in treated cells compared to 7% in control cells), but the pretreatment of these cells with oxaliplatin abolished the ability of irinotecan to induce PARP cleavage. We hypothesized that in SW-620 cells, the most sensitive to oxaliplatin, the DNA damage caused by oxaliplatin induced downstream events which did not cause PARP cleavage. This process prevented irinotecan from further exerting its cytotoxic effect through apoptosis.

Topo I is involved in DNA repair of many different cytotoxic drugs and is suspected to be present on DNA during this process.[20] It has also been demonstrated that cisplatin can induce p53 *in vitro*. Gobert *et al.* demonstrated that p53 and Topo I form a molecular complex *in vitro*, which stimulates the catalytic activity of Topo I.[28] In MCF-7 cells treated with mitomycin C, p53 is translocated from the cytoplasm to the nucleus, leading to an increase in Topo I activity. In p53 wild-type cells (HCT-8), p53 can interact with the Topo I after oxaliplatin-induced DNA damage and increase the cytotoxicity in the presence of Topo I inhibitors. In cells with mutated p53 (SW-620 cells), oxaliplatin-induced cytotoxicity does not involve p53 and prevents the interaction with Topo I. This could decrease the impact of Topo I inhibitors in this situation.

Further investigations are needed to extend these findings to clinical samples. However, the amount of biological material is a concern. A sufficient amount of tumour sample would be necessary to investigate both NER gene expression and PARP cleavage. The RT-PCR method is particularly suited for clinical studies on small biological samples due to its high sensitivity. As for the quantification of PARP cleavage, Whitacre *et al.*[15] have already demonstrated the feasibility of the method on clinical samples.

Conclusion

At the clinical level, different phase I clinical trials have been performed using weekly, biweekly or every 3

weeks administration of this drug combination.[29] Myelosuppression was the dose-limiting toxicity and, as in animals, the doses of both drugs recommended for phase II clinical trials were lower than those used in single-drug chemotherapy. Preliminary encouraging response data in phase I trials and an overall response rate of 42% has been documented in advanced colorectal cancer refractory to fluoropyrimidines. Knowledge of the predictive parameters of the response could increase the response rate by refining the selection of patients that could benefit from this treatment.

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(Received 8 May 2001; revised form accepted 19 July 2001)