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Synergistic cytotoxicity of cisplatin and topotecan or SN-38 in a panel of eight solid-tumor cell lines in vitro

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Abstract The cytotoxicity of cisplatin alone and in combination with topotecan (TPT) or SN-38, two novel topoisomerase I (topo I) inhibitors, was determined in a panel of eight well-characterized human solid-tumor cell lines. Interactions between cisplatin and these topo I inhibitors were investigated using three different administration schedules: (1) simultaneous incubation (C + T and C + S), (2) cisplatin followed by TPT or SN-38 (C → T and C → S), and (3) TPT or SN-38 followed by cisplatin (T → C and S → C). Median-effect analysis revealed synergistic cytotoxicity in seven of the eight cell lines used. In addition, a significant schedule-dependent synergistic cytotoxicity was found in three of the cell lines used, with C → T (or C → S) being the most active schedule. The formation and repair of total cisplatin-DNA adducts in the IGROV-1 ovarian cancer cell line and its cisplatin-resistant subline $IGROV_{CDDP}$ was not significantly affected by TPT on simultaneous incubation. In contrast, the number of cisplatin-DNA interstrand cross-links detected in the IGROV-1 and $IGROV_{CDDP}$ lines at certain time points was significantly lower after coincubation of the cells with TPT. Assessment of the cell-cycle distribution revealed an accumulation of cells in the G_2/M phase after exposure to cisplatin. After exposure to TPT a different pattern was observed that was cell-type-specific and dependent upon the TPT concentration. Although up to 4-fold differences in topo I activity were observed in this panel of cell lines, these differences did not appear to be related to the synergy observed between cisplatin and TPT or SN-38. The observed synergy may at least partly be explained by the increased retention of cisplatin-DNA interstrand cross-links in the presence of topo I inhibitors.

Key words Cisplatin · Topoisomerase I · Topotecan · SN-38 · Synergy · In vitro · Schedule

Abbreviations *ER* Estrogen receptor · F_a fraction affected · F_u fluorescence yield from untreated cells · F_t fluorescence yield from treated cells · $IC_{50}(5\ day)$ IC_{50} after 5 days of continuous exposure · $IC_{50}(3\ day)$ IC_{50} after 3 days of exposure · *ISC* interstrand cross-links · *SRB* sulforhodamine B · *topo I* topoisomerase I · *TPT* topotecan

Introduction

Cisplatin is one of the most widely used anticancer drugs in the clinical setting and shows good responses in the treatment of testicular cancer, non-small-cell (NSCLC) and small-cell lung cancer (SCLC), ovarian cancer, and head and neck cancer [24, 41]. The antitumor activity of cisplatin is supposed to be correlated with its interaction with DNA. Cisplatin is capable of forming potentially lethal intra- and interstrand DNA cross-links [14, 33]. The clinical use of cisplatin is frequently limited by intrinsic or acquired resistance. Among other reasons, this resistance to cisplatin has been ascribed to increased repair of cisplatin-DNA adducts [11, 32, 37]. The importance of DNA repair in cisplatin toxicity is also evident in a testicular cancer cell line that has a decreased repair rate of cisplatin-DNA adducts as compared with a bladder-carcinoma cell line [3]. This deficiency in DNA repair has been shown to contribute to the remarkable sensitivity of this testicular cell line to cisplatin [16].

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Because of this apparent relationship between the sensitivity of tumor cells to cisplatin and DNA repair, inhibition of this repair may be clinically important in cisplatin chemotherapy.

A promising class of agents in this respect are the topoisomerase I (topo I) inhibitors. Topoisomerases are involved in many important processes in the cell, e.g., in topologic changing of the cellular DNA structure and in DNA replication and transcription [15, 42, 43]. Topo I inhibitors such as topotecan (TPT) and SN-38 (the active metabolite of irinotecan) show a broad spectrum of antitumor activity in pre-clinical studies with a unique mechanism of action, targeting topo I. Both TPT and irinotecan are now in clinical phase I, II, and III trials and yield good responses in many tumor types as single agents or in combination chemotherapy [35, 36, 38]. Due to their nonoverlapping toxicity and different mechanisms of action, the combination of topo I inhibitors with cisplatin is an attractive subject for further clinical study. There are many reports about the function of topo II inhibitors in cisplatin-DNA adduct repair [1, 12, 13]. However, the role of topo I in the formation and repair of these cisplatin-DNA adducts has not yet been fully identified and, consequently, there is a need for data on this subject. Recently, studies about the interaction between cisplatin and topo I inhibitors such as TPT, irinotecan, or SN-38 have revealed a synergistic effect between topo I inhibitors and cisplatin [7, 21, 28].

In the present study the synergy between cisplatin and TPT or SN-38 was investigated using a panel of eight human solid-tumor cell lines covering a broad tumor spectrum and a broad range of sensitivities to cisplatin and topo I inhibitors. In addition, the schedule dependency of this combination was investigated by administration of cisplatin and TPT or SN-38 using three different schedules. Total and interstrand adduct kinetics in the presence and absence of TPT or SN-38 were monitored in an attempt to explain the synergy between these drugs. Furthermore, topo I catalytic activity as well as cell-cycle distributions were determined to investigate the possible relationship between these parameters, which by themselves are important for the activity of TPT and SN-38, and synergy between cisplatin and these topo I inhibitors.

Materials and methods

Chemicals and drugs

TPT was a generous gift from Smith Kline Beecham Pharmaceuticals (King of Prussia, Pa., USA). SN-38 was generously supplied by Rhône-Poulenc Rorer (Alfortville, France). Cisplatin was obtained from Lederle (Wolfratshausen, Germany). Plasmid pBR322 DNA, dithiothreitol (DTT), bovine serum albumin (BSA), bromophenol blue, sulforhodamine B (SRB), ethidium bromide, and DNase I [E.C. 3.1.21.1] were supplied by Sigma (St. Louis, Mo., USA). Proteinase K was purchased from Merck (Darmstadt, Germany). RPMI 1640 and Dulbecco's modified Eagle's medium (DMEM) were obtained from Gibco BRL (Life Technologies B.V, Breda, The Netherlands). Bovine calf serum (BCS) was obtained from Hyclone (Logan, Utah, USA). Ethylene glycol tetraacetic acid (EGTA) was purchased from Serva (Heidelberg, Germany). Propidium iodide was supplied by Calbiochem (La Jolla Calif., USA).

Cell lines and culture conditions

The human WiDr colon cancer, the H226 undifferentiated lung cancer, the A498 renal-cell cancer, the M-19 melanoma, the IGROV-1 ovarian adenocarcinoma and its 8-fold cisplatin-resistant subline $IGROV_{CDDP}$ as well as the estrogen receptor (ER)-positive MCF7 breast-cancer cell lines were cultured in RPMI 1640 supplemented with 10% BCS, 10 m*M* $NaHCO_3$, 2 m*M* glutamine, 110 IU penicillin/ml, 100 µg streptomycin/ml, 45 µg gentamycin/ml, and 10 µg insulin/ml. Cells were kept in continuous logarithmic growth at 37 °C in a humidified atmosphere of 5% CO_2 in air. The ER-negative EVSAT breast-cancer cell line was cultured in DMEM supplemented with 5% BCS and other supplements as outlined above. The resistance of the $IGROV_{CDDP}$ cell line to cisplatin can partly be explained by reduced accumulation of cisplatin [20, 27].

Cytotoxicity assay

Exponentially growing cells were trypsinized and plated (2,000 cells/200 µl per well, except for the WiDr cell line: 1,600 cells/200 µl per well) in 96-well microplates (Costar Corporation, Cambridge, Mass., USA). Subsequently, the cells were allowed to attach for 48 h at 37 °C in an atmosphere containing 5% CO_2. Following this attachment period, 100 µl drug solution (diluted with RPMI 1640 or DMEM) was added to the wells at day 2. Next, the cells were incubated for 5 days at 37 °C in an atmosphere of 5% CO_2 in air. Depending on the administration schedule, the second drug was added at day 4. In this case the drug dilution was prepared separately and the appropriate dilution was added to the respective wells in a small volume of 10 µl without washing away of the first drug. Subsequently, cells were incubated for another 3 days. Individuals survival curves for cisplatin and TPT or SN-38 following 5 or 3 days of exposure (i.e., exposure to drug at day 2 or 4, respectively) were also generated. Treatment with the single agent (control) was exactly the same as that used in the scheduled combinations, i.e., IC_{50} values were determined using a 5-day exposure period (100 µl drug, serially diluted) as well as using a 3-day period of exposure. In the latter case, cells were treated as described for the combination experiment, i.e., at day 2, 100 µl blank medium was serially diluted and at day 4 the drug was added to each individual well in a small volume of 10 µl. Cytotoxicity was evaluated using the SRB method [31]. In brief, at day 7, cells were fixed with 10% TCA and stained with SRB (0.4% in 1% acetic acid) for 1 h. After washing of the unbound SRB, bound SRB was dissolved in 10 m*M* TRIS buffer and the absorption at 540 nm was determined using a Bio-Rad 450 microplate reader. Each agent was tested in quadruplicate in at least three independent experiments.

Drug combination schedules and data analysis

In the combination study the following combination schedules were applied: (1) cisplatin and TPT or SN-38 were applied simultaneously for 5 days, starting from day 2 (C + T or C + S); (2) cells were incubated with cisplatin for 5 days, starting from day 2, combined with incubation with TPT or SN-38 from day 4 through day 7 (C → T or C → S); and (3) cells were incubated with TPT or SN-38 for 5 days, starting from day 2, combined with incubation with cisplatin from day 4 through day 7 (T → C or S → C). In the latter two schedules the second drug was added in a 10-µl volume.

In drug combination experiments, TPT or SN-38 was combined with cisplatin at a constant molar ratio, depending on the IC_{50} values determined for cisplatin and TPT or SN-38 following the respective incubation period (see Table 1, Fig. 1). Drug interactions were evaluated using the median-effect analysis as described

by Chou et al. [6, 7, 25]. The combination index for each fractional effect was calculated using the following equation:

$$\text{Combination index} = d_1/D_1 + d_2/D_2,$$

where D_1 and D_2 are the doses of drug 1 and drug 2 that by themselves produce a given fractional effect (such as the IC_{50}) and d_1 and d_2 are the doses that produce the same fractional effect in combination. Combination indices are generally interpreted as zero interaction or additive cytotoxicity when CI = 1, as a synergistic effect when CI < 1, and as an antagonistic effect when CI > 1.

Quantitation and kinetics of total cisplatin-DNA adducts and cisplatin-DNA interstrand cross-links

DNA was isolated as previously described [30], with minor modifications [26], and was quantitated spectrophotometrically. The total amount of platinum bound to DNA was determined with atomic absorption spectrophotometry (AAS) [26, 34] using a Perkin-Elmer 3030B atomic absorption spectrophotometer equipped with an HGA 600 flameless system and an AS 60 autosampler (Uberlingen, Germany). Prior to this measurement the isolated DNA was DNAse I-digested. Subsequently, the sample was injected using the 4-times multiple injection feature of the AAS apparatus. Adduct levels are expressed as picograms of platinum per microgram of DNA (pg Pt/μg DNA).

The relative amount of cisplatin-DNA interstrand cross-links was determined by an ethidium bromide assay [1, 8] using a Perkin-Elmer LS-3B fluorescence spectrophotometer. Ethidium bromide fluorescence was measured before and after denaturation of the DNA using an excitation wavelength of 525 nm and an emission wavelength of 580 nm. The relative amount of interstrand cross-links was calculated using the following formula [8]:

$$\%ISC = [(F_t - F_u)/(1 - F_u)] \times 100\%,$$

where $\%ISC$ is the percentage of interstrand DNA cross-links in cisplatin-treated cells, F_t is the fluorescence in cisplatin-treated cells, and F_u is the fluorescence in untreated cells (blanks). F_t and F_u were calculated as the ratio of ethidium bromide fluorescence detected after and prior to denaturation of the DNA, respectively.

For monitoring of the kinetics of total cisplatin-DNA adducts and ISC, IGROV-1 or $IGROV_{CDDP}$ cells [60–75% confluent in T75 flasks (Greiner, Frickenhausen, Germany)] were exposed for 2 h to 10 μg cisplatin/ml alone or to 10 μg cisplatin/ml combined with 10 ng TPT/ml. After 2 h of incubation, cells were washed twice with phosphate-buffered saline (PBS), drug-free medium was added, and culturing was continued. In the case of combined cisplatin/TPT treatment, 10 ng TPT/ml was again added to the medium immediately after this washing procedure. Directly and at 4, 8, 24, and 48 h after the end of exposure to cisplatin, flasks were withdrawn and cells were harvested by trypsinization and washed with PBS. Subsequently, cells were resuspended in 3 ml TE buffer (10 m*M* TRIS-HCl, 2 m*M* EDTA, and 0.4 *M* NaCl, pH 7.3) and stored at −20 °C until DNA isolation. DNA was isolated, and the adducts were quantitated as described above.

Flow cytometry analysis

Cell-cycle distribution was monitored following exposure of the cells to IC_{50}(5 day) doses of cisplatin or TPT. Furthermore, the effect of simultaneous exposure to a combination of cisplatin and TPT [at 50% of their IC_{50}(5 day) does each] was monitored. Cells were exposed to cisplatin and/or TPT continuously, and flasks were withdrawn at different times following the start of incubation. To obtain single-cell suspensions, cells were trypsinized and washed twice with RPMI culture medium without phenol red. Cells were stained with propidium iodide (100 mg/l) and the DNA content was analyzed according to the method of Krishan [23] on a FACScan flow cytometer (Becton Dickinson, Etten-Leur, The Netherlands), with excitation being set at 488 nm. The following parameters were measured: forward light scatter (FLS), perpendicular light scatter (PLS), and fluorescence of the DNA-PI complex (563–607 nm). Cell debris was excluded from analysis by an appropriate FLS threshold setting.

Topo I catalytic activity

Crude nuclear extracts were prepared as described elsewhere [44]. After isolation the nuclear extracts were diluted 1:1 with 99% glycerol and stored in small aliquots at −80 °C for a maximal period of 1 month. The protein concentrations were determined according to the Bradford method using the Bio-Rad assay [5]. In separate experiments, IGROV-1 cells were incubated with 10 or 40 μg cisplatin/ml for 48 h prior to the preparation of nuclear extracts.

Topo I catalytic activity was assayed by monitoring of the relaxation of 250 ng supercoiled pBR322 DNA [44] using serial dilutions of nuclear extracts. The reaction mixture was incubated at 37 °C for 30 min. After stopping of the reaction, samples were analyzed by electrophoresis on a 1% agarose gel. After staining with ethidium bromide, bands were visualized on a UV light table and photographed with Polaroid (667) positive/negative films. Activity is expressed as units per milligram of protein, in which 1 unit is the amount of protein capable of completely relaxing 250 ng DNA in 30 min at 37 °C.

Statistical analysis

Statistical evaluation was performed using Student's *t*-test, and $P < 0.05$ was applied as the significance level.

Results

Characterization of the cell lines

The doubling times and sensitivities of the cells to cisplatin, TPT, and SN-38 are summarized in Table 1. The WiDr cell line was the fastest growing, whereas H226

Table 1 Doubling time (*DT*) and IC_{50} of cisplatin, TPT, and SN-38 in a panel of 8 human tumor cell lines as determined using the SRB assay

Cell line	DT (h)	IC_{50}(5 day) (ng/ml)			IC_{50}(3 day) (ng/ml)		
		Cisplatin	TPT	SN-38	Cisplatin	TPT	SN-38
IGROV-1	28	145 ± 17	6.5 ± 1.9	1.5 ± 0.2	1,984 ± 326	541 ± 337	143.5 ± 86
$IGROV_{CDDP}$	26	1,285 ± 285	23.0 ± 3.4	4.6 ± 0.5	7,449 ± 1,269	831 ± 163	400 ± 157.5
MCF7	24	676 ± 205	5.4 ± 0.6	0.9 ± 0.1	7,525 ± 1,648	422 ± 51	184.8 ± 45.2
EVSAT	25	687 ± 104	10.3 ± 2.1	1.0 ± 0.1	16,505 ± 219	281 ± 141	49.3 ± 17.7
M-19	24	1,210 ± 492	19.6 ± 10	3.2 ± 1.7	4,598 ± 1,068	552 ± 353	270.9 ± 28.9
H226	43	1,275 ± 518	35.1 ± 31	7.3 ± 3.8	16,319 ± 822	466 ± 66	195.8 ± 65.9
A498	33	925 ± 357	21.9 ± 4.7	6.2 ± 0.8	12,355 ± 345	172 ± 32	110.8 ± 51.5
WiDr	18	609 ± 95	12.6 ± 2.9	4.1 ± 2.3	12,770 ± 125	229 ± 63	106.9 ± 9.3

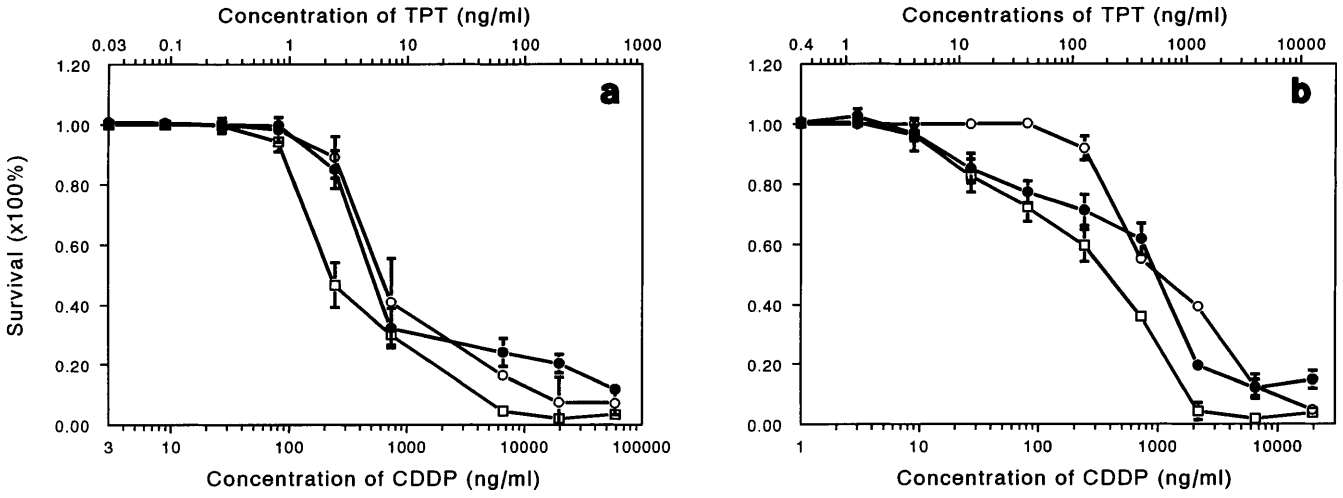

Fig. 1a,b Cell-proliferation curves generated for the MCF7 cell line. **a** Cells were exposed continuously to cisplatin (○), to TPT (●), or to a combination of cisplatin and TPT (□) for 5 days. A fixed cisplatin:TPT ratio of 100:1 [approximately equals the IC_{50}(5 day) ratio of the individual drugs in the MCF7 cell line; see Table 1] was used in the combination experiment. **b** Cells were exposed continuously to cisplatin for 5 days (○), to TPT for 3 day. (starting at day 4; ●), or to a combination of cisplatin (5 days exposure) and TPT (3 days exposure, from day 4; □). The fixed ratio of cisplatin/TPT in this scheduled combination experiment was 2.7:1 [approximately equals the IC_{50}(5 day)/IC_{50}(3 day) ratio of the individual drugs in the MCF7 cell line; see Table 1]. Data represent mean values ± SD for at least 3 independent experiment

was the slowest growing cell line, their doubling times being 18 and 43 h, respectively. The doubling times of the other cell lines ranged between 24 and 33 h. The IGROV-1 cell line was most sensitive to cisplatin, whereas MCF7 was most sensitive to TPT and SN-38 over a 5-day incubation period.

Cytotoxicity of combined exposure to cisplatin and TPT or SN-38

The results of combination experiments using cisplatin and TPT are shown in Figs. 1 and 2. In all cell lines tested except the A498 cell line a markedly synergistic interaction between cisplatin and TPT was observed. When SN-38 was used, analogous results were obtained (summarized in Table 2). A comparison of the different combination schedules showed that the (C → T) and (C → S) treatments resulted in the most pronounced synergy. In the IGROV-1, $IGROV_{CDDP}$, and MCF7 cell lines this schedule yielded significantly higher synergy ($P < 0.05$) than did the other combination schedules. Also in the other cell lines except for H226 the C → T schedule tended to the most active. However, in these cases the difference observed between the (C → T) schedule and the other schedules did not reach statistical significance. In contrast, in the H226 cell line the most pronounced synergy was observed in the (T → C) schedule (CI at $F_a = 0.5:0.46 \pm 0.07$).

Effect of TPT on the kinetics of total cisplatin-DNA adducts and cisplatin-DNA interstrand cross-links

The kinetics of total cisplatin-DNA adducts and of ISC were evaluated in the IGROV-1 and cisplatin-resistant $IGROV_{CDDP}$ cell lines. TPT did not affect the formation and removal of total cisplatin-DNA-adducts as measured by AAS (data not shown). However, in both the IGROV-1 and the $IGROV_{CDDP}$ cell lines the number of cisplatin-DNA ISCs was increased in the presence of TPT (Fig. 3). At nearly all time points the number of ISCs detected in the presence of TPT were higher than those found in the absence of TPT. In the IGROV-1 cell line the number of ISCs detected at 8–48 h following exposure to cisplatin was 10–29% higher in the presence of TPT (Fig. 3a), whereas this difference was 30–39% in the resistant $IGROV_{CDDP}$ cell line (Fig. 3b). In any individual case, DNA-adduct levels detected at time points 4, 8, 24, and 48 h in the presence of cisplatin and TPT were higher than those observed with cisplatin alone. Using the paired *t*-test, we demonstrated that the numbers of ISCs were significantly higher when TPT was present after 48 h $P = 0.008$ and $P = 0.02$ for IGROV and $IGROV_{CDDP}$, respectively).

Cell-cycle distribution

The cell-cycle distribution observed following exposure to cisplatin, TPT, or a combination of the two is shown

►

Fig. 2 Combination index (CI) plotted as a function of the F_a in the indicated cell lines using three different schedules as described in Materials and methods (○) C → T, i.e., exposure to cisplatin for 5 days combined with exposure to TPT for 3 days (starting 2 days later); (□) C + T, i.e., simultaneous exposure to cisplatin and TPT for 5 days; and (●) T → C, i.e., exposure to TPT for 5 days combined with exposure to cisplatin for 3 days (starting 2 days later). Cisplatin and TPT were combined according to their IC_{50} ratios, which were determined both for a 5-day and a 3-day exposure period. Data represent mean values ± SD for at least 3 independent experiments

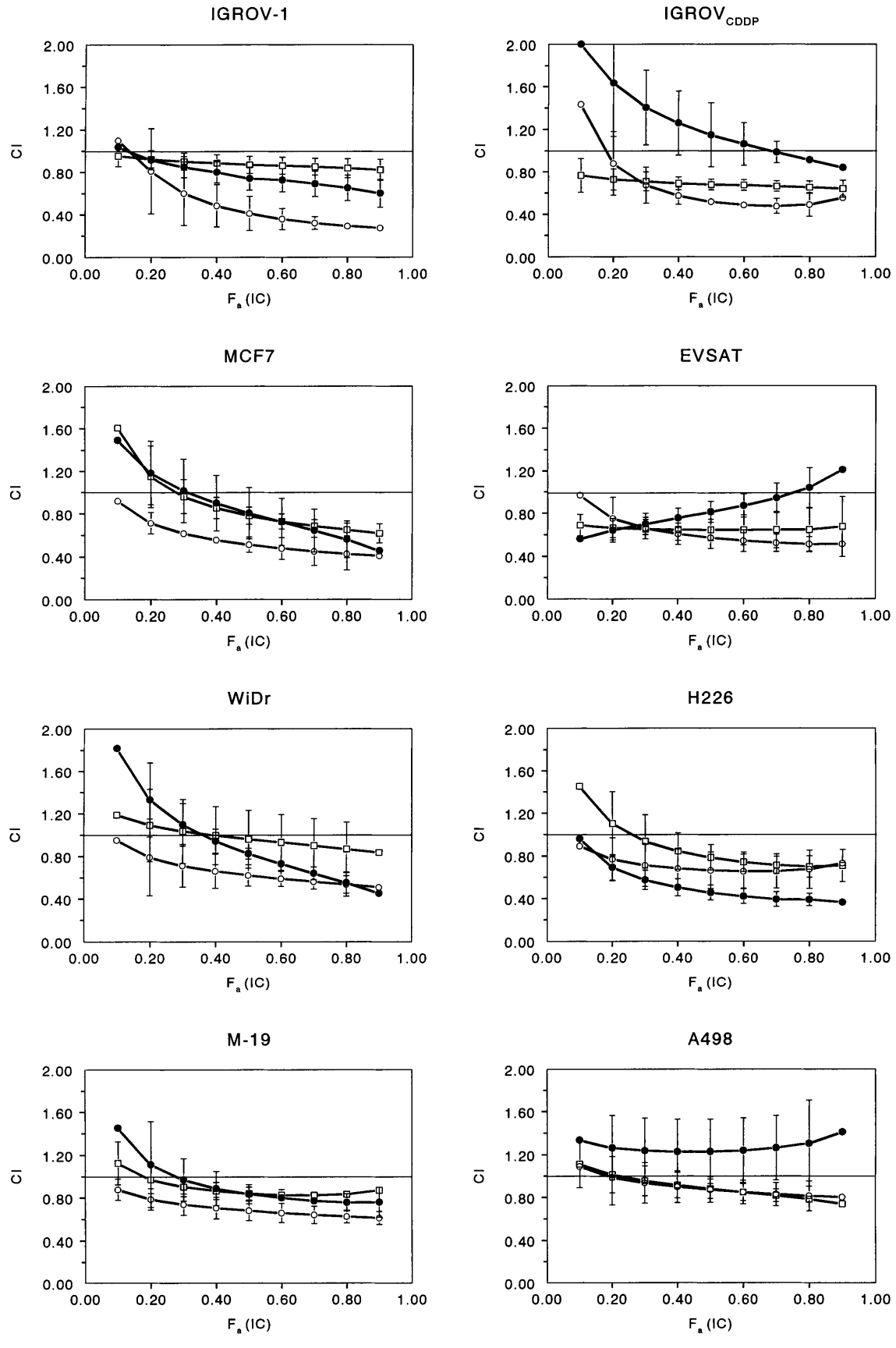
IGROV-1
IGROV$_{CDDP}$
MCF7
EVSAT
WiDr
H226
M-19
A498
CI
F_a (IC)
0.00
0.40
0.80
1.20
1.60
2.00
0.20
0.60
1.00

Table 2 Combination index (*CI*) determined at an Fa value of 0.5 for cisplatin, TPT, and SN-38 in a panel of 8 human solid-tumor cell lines. Cells were incubated with cisplatin and TPT or SN-38 for 5 days ($C + T$ and $C + S$) or to cisplatin for 5 days, followed by TPT or SN-38 for 3 days, starting 2 days later. Drugs were added as described in Materials and methods

Cell lines	CI (F_a = 0.5)			
	C + T	C + S	C → T	C → S
IGROV-1	0.83 ± 0.08	0.77 ± 0.15	0.41 ± 0.16	0.51 ± 0.12
$IGROV_{CDDP}$	0.68 ± 0.05	1.03 ± 0.12	0.52 ± 0.02	0.60 ± 0.07
MCF7	0.78 ± 0.08	0.81 ± 0.07	0.51 ± 0.07	0.51 ± 0.11
EVSAT	0.64 ± 0.12	0.77 ± 0.10	0.57 ± 0.10	0.54 ± 0.12
M-19	0.84 ± 0.06	0.85 ± 0.18	0.68 ± 0.09	0.67 ± 0.11
H226	0.78 ± 0.12	0.73 ± 0.09	0.66 ± 0.17	0.67 ± 0.09
A498	0.88 ± 0.09	0.89 ± 0.08	0.87 ± 0.12	0.91 ± 0.19
WiDr	0.96 ± 0.27	0.71 ± 0.11	0.62 ± 0.10	0.46 ± 0.04

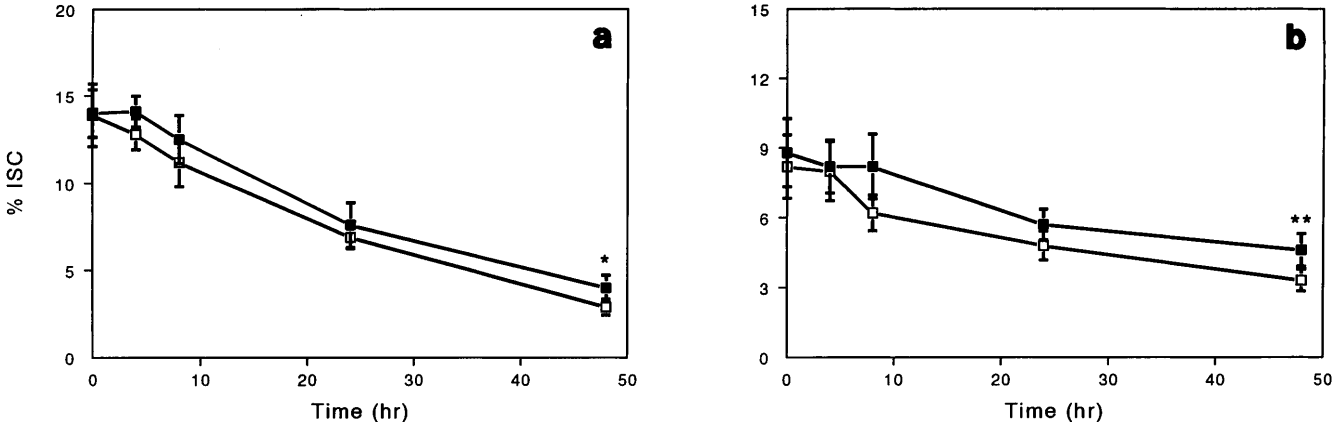

Fig. 3a,b Kinetics of interstrand cross-links (*ISC*) after a 2-h period of incubation of **a** IGROV-1 and **b** $IGROV_{CDDP}$ with cisplatin at 10 μg/ml in the presence (■) or absence (□) of TPT at 10 ng/ml for 48 h. Following exposure to cisplatin, cells were washed and culturing was continued in the presence or absence of TPT. Directly and at 4, 8, 24, and 48 h after the end of cisplatin exposure, cells were harvested. Subsequently, DNA was isolated using high-level salt extraction [30]. Relative numbers of interstrand cross-links were determined using the ethidium bromide fluorescence assay as described in Materials and methods. Data represent mean values ± SD for at least 4 independent experiments. $*P = 0.008$, $**P = 0.02$

in Fig. 4. Continuous exposure of cells to IC_{50}(5 day) doses of cisplatin for 24 or 48 h resulted in an increase in the fraction of cells in the G_2/M phase with a corresponding decrease in the fraction of cells in G_1. Furthermore, in the H226 cell line a significant increase in the number of cells in the S phase was observed. Interestingly, continuous exposure of cells to TPT (IC_{50} dose) also resulted in accumulation in the G_2/M phase for most of the cell lines tested. Only H226 cells were arrested in the S phase. Following treatment of H226 and A498 cell lines with TPT, almost no cell was observed in the G_1 phase. Combination of cisplatin and TPT [both at 50% of their IC_{50}(5 day) dose] resulted in a combined effect of the drugs alone, i.e., most of the cell lines accumulated in the G_2/M phase.

To mimic the most active schedule in the IGROV-1 line, i.e., C → T (see Fig. 2), IGROV-1 cells were exposed to an IC_{50}(5 day) dose of cisplatin for 5 days and to an IC_{50}(3 day) dose of TPT for 3 days (starting at day 4), the drugs being applied alone and in combination (each drug at 50% of its respective IC_{50} dose). Cells were monitored for up to 5 days after the start of incubation (Table 3). Untreated IGROV-1 cells displayed no major change in cell-cycle distribution during this period. Following administration of cisplatin, cells accumulated in the G_2/M phase. Interestingly, exposure of the cells to high concentrations of TPT [IC_{50} at shorter duration (3 days)] resulted in increased arrest of the cells in the G_1 and S phases as compared with the 5-day period of incubation with lower amounts of TPT. Also in the case of the C → T exposure, at time-points 72, 96, and 120 h, 32%, 11.8%, and 19.3% more of the cells, respectively were in the G_1 phase as compared with the C + T schedule. Furthermore, the number of cells in the S phase increased by 18.3%, 36.9%, and 38.5% at these respective time points, with a concomitantly lower number of cells being observed in G_2/M.

Topo I catalytic activity

Topo I catalytic activity was evaluated as described in Materials and methods. For most cell lines, equal catalytic activities were observed (Fig. 5, Table 4). However, the MCF7 line displayed a 2-fold higher level of activity, whereas the A498 cell line showed a level of activity 2 times lower than did the other cell lines. Incubation of IGROV-1 or $IGROV_{CDDP}$ cell with cisplatin (10 or 40 μg/ml) for 48 h prior to nucleus extraction did not have a significant effect on topo I catalytic activity (data not shown).

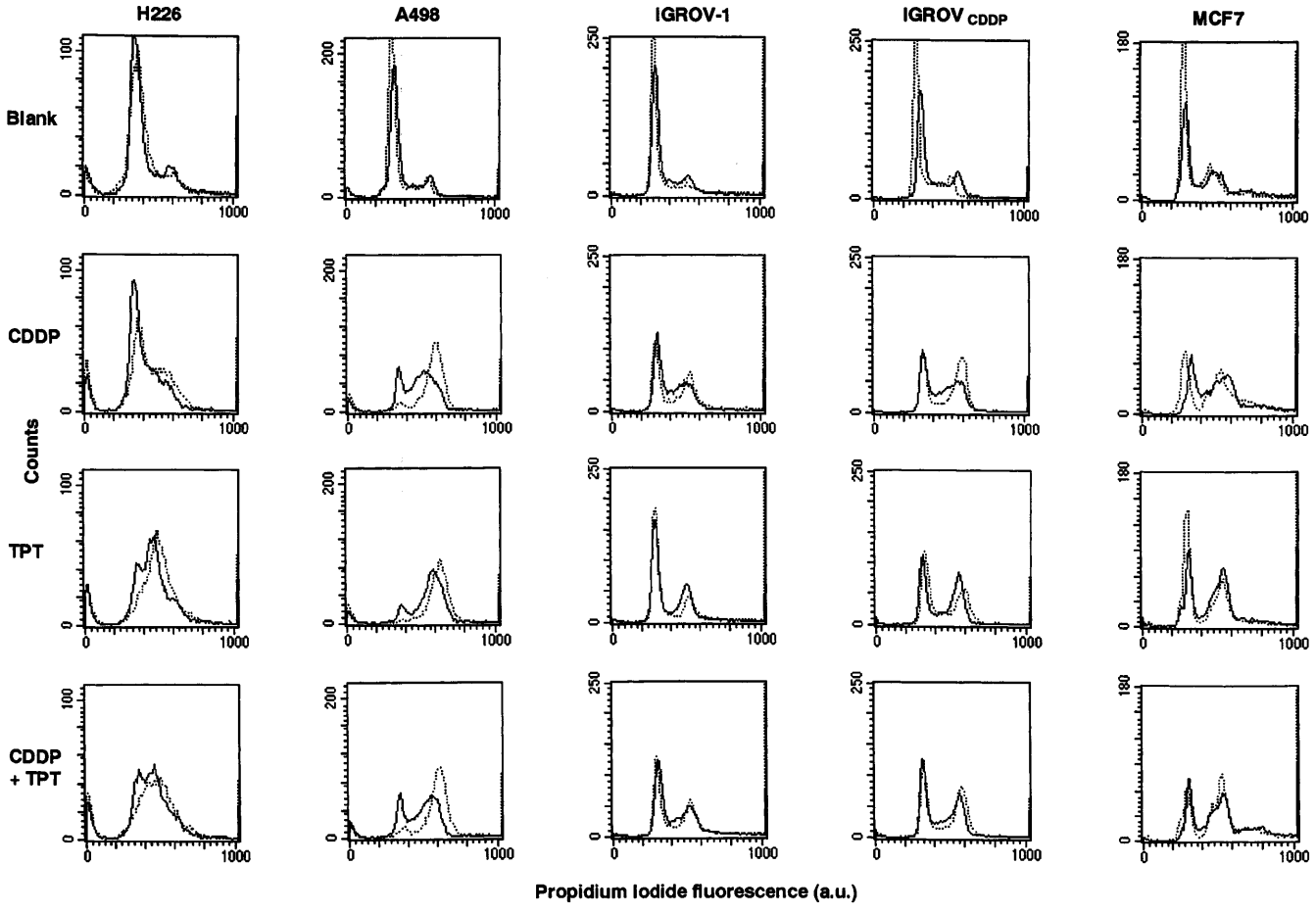

Fig. 4 DNA cell-cycle analysis following exposure of H226, A498, IGROV-1, $IGROV_{CDDP}$, and MCF7 cells in exponential growth to IC_{50}(5 day) concentrations of TPT or cisplatin for 48 h or to cisplatin and TPT simultaneously (in this case, both at 50% of their IC_{50} doses). After 24 (—) and 48 (---) h of exposure, cells were fixed and stained with propidium iodide

Discussion

In this study the interaction between cisplatin and topo I inhibitors was investigated using a panel of eight human solid-tumor cell lines. Two different topo I inhibitors, TPT and SN-38, were used, and the drugs were applied using three different schedules. In analogy to previously published reports [7, 28, 29], synergistic effects were observed in most of the cell lines tested. In addition to this, the observed cytotoxicity appeared to be schedule-dependent, with the C → T and C → S schedule yielding the highest levels of synergy in most of the cell lines. This type of schedule dependency has not been reported by other investigators. In the report by Masumoto et al. [29], all combined schedules of cisplatin and SN-38 showed synergy, but the maximal effect was obtained upon simultaneous administration. The difference between these results and ours may be attributable to the different cell type that was used. A second possible explanation may be the different incubation periods used for the drugs. Most scheduling experiments have been performed using a relatively short period of exposure (1–2 h) to TPT or SN-38 followed by removal of the drug. As TPT and SN-38 have been shown to be preclinically and clinically more active upon prolonged administration [17], we reasoned it to be more relevant to use longer incubation periods for TPT and SN-38. This different approach may have resulted in different synergistic effects.

The synergistic cytotoxicity of cisplatin and TPT upon simultaneous administration may at least partly be caused by the increased retention of DNA ISCs. Also for SN-38, higher levels of ISCs have been observed following incubation in the presence of cisplatin [28]. In our study, no effect of TPT on total cisplatin-DNA adduct repair was observed on the basis of the total numbers of cisplatin-DNA lesions determined by AAS. Importantly, although ISCs represent only a small portion of the total amount of DNA adducts, they may be critical for cell survival [19, 45].

As reported by other investigators [4, 39, 40], upon treatment with cisplatin, all cell lines accumulated in the G_2/M phase. The effect of TPT on cell-cycle distribution appeared to be cell-type-dependent. Although arrest in the S phase has been reported for camptothecin [18], in our panel, at IC_{50} doses of TPT, only H226 cells accumulated in that cell-cycle phase (Fig. 4), whereas the

Table 3 Cell-cycle distribution observed in the IGROV-1 cell line upon exposure to IC_{50} doses of cisplatin or TPT for 5 days or of TPT for 3 days and using the C → T and the C + T schedule as described in Materials and methods

Hour	Blank			Cisplatin			TPT (5 days)			TPT (3 days)			C + T			C → T		
	G_1	S	G_2	G_1	S	G_2	G_1	S	G_2	G_1	S	G_2	G_1	S	G_2	G_1	S	G_2
0	62.7	13.3	21.0															
24	67.8	11.3	17.9	47.4	17.9	31.7	56.0	9.3	31.8				47.8	14.2	33.7	55.3	14.9	26.6
48	75.0	7.0	12.7	43.4	9.3	40.1	62.6	5.9	25.3				46.5	8.2	37.7	60.3	7.7	25.2
72	76.2	7.6	12.2	41.9	9.0	43.9	54.6	7.0	31.9	70.5	11.0	13.1	45.0	9.3	40.1	59.4	11.0	23.8
96	77.5	7.6	11.9	37.6	6.8	48.5	46.7	7.8	38.0	70.2	10.8	14.6	43.3	8.4	42.9	48.4	11.5	31.0
120	76.3	8.1	13.8	41.1	10.5	43.9	51.6	7.3	35.4	69.1	14.9	14.9	46.1	6.5	40.8	55.0	9.0	31.2

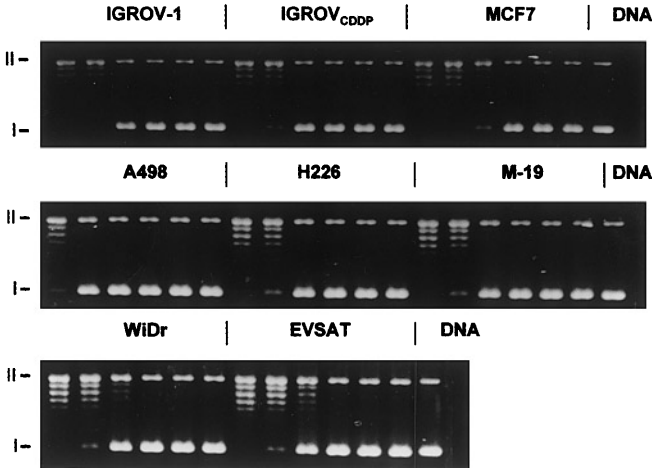

Fig. 5 Topo I catalytic activity detected in tumor cell lines. Topo I catalytic activity was assayed by monitoring of the relaxation of 250 ng supercoiled pBR322 DNA [44] using serial dilutions of nuclear extracts. For all cell lines, 375, 188, 94, 47, 23, and 12 ng nuclear extract was added (*from left to right*). After 30 min of incubation at 37 °C, samples were analyzed by electrophoresis on a 1% agarose gel. At the *outer right lanes* the pBR322 DNA marker was added (*I* Supercoiled DNA, *II* relaxed DNA)

Table 4 Topo I catalytic activity detected in tumor cell lines. Activity was monitored as described in Materials and methods. Experiments were performed two times using independently prepared nuclear extracts. Activity is expressed as units/mg protein, in which 1 unit is the amount of protein capable of completely relaxing 250 ng DNA in 30 min at 37 °C. For all cell lines, catalytic activity was equal in the two different nuclear extracts. Catalytic activity in the IGROV-1 cell line was set to 1

Cell lines	Units/mg protein (relative topo I activity)
IGROV-1	5,200 (1)
IGROV$_{CDDP}$	5,200 (1)
MCF7	10,400 (2)
EVSAT	5,200 (1)
M-19	5,200 (1)
H226	5,200 (1)
A498	2,600 (0.5)
WiDr	5,200 (1)

other cell lines tested accumulated in the G_1 and/or G_2/M phase. No apparent difference was noted in the effects of TPT on cell-cycle distribution between TPT-sensitive cell lines, i.e., IGROV-1 and MCF7, and the less sensitive cell lines H226 and IGROV$_{CDDP}$. Apparently, the effect on cell-cycle distribution by TPT is cell-type-dependent. Probably other topo I-related parameters, e.g., formation of cleavable complexes, play a role in this matter. At IC_{50} (5 day) doses the A498 cell line displayed a very marked accumulation in the G_2/M phase following treatment both with cisplatin and with TPT. Interestingly, the use of higher concentrations of TPT for shorter duration (3 days) revealed an increased number of cells arrested in the G_1 and S phases (Table 3), which is in line with the results of other studies [9, 10]. Hence, the schedule and incubation concentration of cisplatin and TPT seem to influence the cell-cycle distribution. In the C → T schedule significantly more cells arrested in the G_1 and S phases as compared with the C + T schedule, with a concomitant reduction occurring in the number of cells in G_2/M ($P = 0.03$). Whether the changed cell-cycle distribution, which appears to be mainly caused by the higher concentration of TPT in the C → T schedule (Table 3), contributes to the degree of synergy remains to be elucidated.

It is well known that topo I levels may affect cellular sensitivity to TPT and SN-38 [2, 22]. The MCF7 cell line, which proved to be the most sensitive cell line in our panel upon exposure to TPT or SN-38 for 5 days, also displayed the highest degree of topo I catalytic activity. Under these conditions the A498 cell line, showing a lower level of catalytic activity, was much less sensitive. However, upon incubation with TPT or SN-38 for 3 days the relative sensitivities of the tumor cells changed. The effect of topo I catalytic activity on the synergy observed between cisplatin and TPT or SN-38 is unclear. The degree of synergy seen in the A498 cell line was lower than that observed in the other cell lines, all having a higher amount of catalytic activity. Furthermore, the MCF7 cell line showed a relatively high degree of synergy in the C → T schedule. However, other cell lines, i.e., IGROV-1 and IGROV$_{CDDP}$, showed the same degree of synergy as did MCF7 while having a 2-fold lower level of catalytic activity. Therefore, the cellular topo I activity does not appear to be crucial for synergy between cisplatin and topo I inhibitors. Possibly, differences in repair between the cell lines may attenuate the effect of topo I levels. Repair of cisplatin adducts in these cell lines is currently under investigation.

In conclusion, the combination of cisplatin with TPT or SN-38 yielded a synergistic cytotoxicity in most human solid-tumor cell lines in our panel. Furthermore,

this synergy appeared to be schedule-dependent, with the C → T schedule being most active. Analyses of cell-cycle distribution did not reveal an explanation for the synergy or the schedule dependency of this phenomenon. Also, topo I catalytic activity did not appear to be related to the observed synergistic effects. However, the increased retention of cisplatin-DNA ISCs may contribute to the synergy between cisplatin and topo I inhibitors when the latter are applied simultaneously. Additional studies to assess the effect of topo I inhibitors on repair of the major cisplatin-DNA adducts, i.e., the GG- and AG-intrastrand cross-links, are currently being performed.

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