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Simona Romanelli · Paola Perego · Graziella Pratesi
Nives Carenini · Monica Tortoreto · Franco Zunino

In vitro and in vivo interaction between cisplatin and topotecan in ovarian carcinoma systems

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Abstract Topotecan, a camptothecin analogue, is a specific inhibitor of topoisomerase I approved for use in the treatment of patients with refractory ovarian carcinoma. The drug's mechanism of action suggests a potential efficacy of drug combinations incorporating DNA-damaging agents. In an attempt better to define a rational basis for drug combination we examined the effect of topotecan on the cytotoxicity and antitumor activity of cisplatin in an ovarian carcinoma system growing in vitro and in vivo as a tumor xenograft. The in vitro cell system included a cisplatin-sensitive cell line, IGROV-1, and a cisplatin-resistant subline, IGROV-1/Pt0.5, which is characterized by p53 mutation and loss of normal function of the wild-type gene of the parental cell line. This cell system was chosen since the cell sensitivity to DNA-damaging agents appears to be dependent on p53 gene status. Cytotoxicity was assessed by the growth inhibition assay using different schedules: (a) a 1-h period of cisplatin exposure followed by a 24-h topotecan treatment and (b) a 1-h period of simultaneous exposure to cisplatin and topotecan. In the case of the sequential schedule, an additive interaction was observed in IGROV-1 and IGROV-1/Pt0.5 cells. When the simultaneous schedule was used, a synergistic interaction, more evident for the cisplatin-sensitive cells, was found. On the basis of these observations at a cellular level, the effect of concomitant administration of the two drugs (i.e., the most favorable schedule) was studied in the IGROV-1 tumor xenograft, which is moderately responsive to cisplatin and topotecan. Suboptimal doses of each drug (with a low dose of topotecan, 5.1 mg/kg) achieved an antitumor effect comparable with or superior to that of the optimal dose of a single treatment (tumor weight inhibition, 60%), thus indicating a pharmacological advantage of the combination over the single treatment. However, an increase in the topotecan dose (7.1 mg/kg) was associated with an evident increase in the toxicity of the combination, thereby suggesting that the drug interaction was not tumor-specific. Although the molecular basis of the drug interaction is not clear, it is likely that inhibition of topoisomerase I affects the ability of cells to repair cisplatin adducts. Such findings may have pharmacological implications since they suggest the potential clinical interest of topoisomerase I inhibitors in combination with cisplatin.

Key words Cisplatin · Topotecan · Ovarian cancer

Introduction

Platinum compounds are recognized as the most effective agents in the treatment of ovarian carcinoma [16]. Unfortunately, the development of resistance during treatment constitutes an obstacle to the cure of even initially responsive tumors [1]. The basis of resistance to platinum drugs has not been conclusively identified, but several mechanisms have been proposed, including reduced drug accumulation [8], drug sequestration through intracellular thiols [12, 14], decreased DNA platination, increased repair [7], and tolerance to DNA damage [15]. Recently, decreased susceptibility to cisplatin-induced apoptosis has been associated with the cisplatin-resistance phenotype [18]. The frequent development of resistance to platinum compounds in ovarian carcinoma has stimulated the clinical evaluation of new drug combinations in an attempt to improve the efficacy of the pharmacological treatment of advanced disease.

The topoisomerase I inhibitor topotecan [22] is one of the most promising drugs available for evaluation of new protocols in terms of a unique mechanism of action

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S. Romanelli · P. Perego (✉) · G. Pratesi · N. Carenini · M. Tortoreto · F. Zunino
Istituto Nazionale per lo Studio e la Cura dei Tumori, Via Venezian 1, I-20133 Milan, Italy Tel.: + 39-2-2390237; Fax: + 39-2-2390764

and novelty of the therapeutic approach. Elucidation of drug interaction at a cellular level may provide a rational basis for optimization of clinical use in combination with conventional effective drugs (e.g., cisplatin). In particular, topoisomerase I inhibitors have been proposed as promising agents to be combined with DNA-damaging agents, since topoisomerase I might have a role in DNA repair [3]. A number of cell studies have suggested a variable interaction of topotecan with DNA-damaging agents, including cisplatin. The nature of the interaction is apparently dependent on the cell type examined, the drug used in combination, and the schedule of drug treatment. However, all these studies were limited to in vitro cell systems.

Since the efficacy of cisplatin in ovarian carcinoma could be related to p53 gene status [19], the aim of our study was to examine the interaction of cisplatin and topotecan in a sensitive ovarian carcinoma cell line with wild-type p53 and a subline selected for resistance to cisplatin characterized by p53 mutation [18]. To define better the pharmacological relevance of the synergistic interaction we extended the study of drug combinations to an in vivo model, using the IGROV-1 cell line growing in nude mice. The results support the pharmacological interest of the combination of cisplatin and topotecan.

Materials and methods

In vitro studies

The IGROV-1 cell line originated from the ovarian adenocarcinoma of an untreated patient [2]. Its cisplatin-resistant variant IGROV-1/Pt0.5, selected by continuous exposure to increasing concentrations of cisplatin, was highly resistant to cisplatin [18]. Cells were maintained as monolayer cultures in RPMI 1640 supplemented with 10% fetal calf serum and 1% glutamine. The resistant subline exhibited karyotypic features similar to those of the parental cell line but displayed a somewhat reduced proliferation rate.

Cell survival was evaluated by the growth inhibition test as previously described elsewhere [18]. In brief, cells in the logarithmic phase of growth were harvested and plated into 6-well plates. IGROV-1 and IGROV-1/Pt0.5 cells were exposed to drugs according to different schedules and sequences: (a) a 1-h period of cisplatin exposure followed by a 24-h period of topotecan exposure and (b) a 1-h period of simultaneous exposure to cisplatin and topotecan. Cells were harvested and counted using a Coulter Counter (PBI Electronics, Luton, UK) at 72 h following exposure to cisplatin.

Cisplatin (Platinex), obtained from Bristol-Myers Squibb (Rome, Italy), was diluted in saline. Topotecan [10-hydroxy-9-dimethylaminomethyl-(S)-camptothecin], supplied by Smith Kline Beecham (King of Prussia, Pa.), was dissolved in water and diluted in culture medium.

In vivo studies

Female athymic nude Swiss mice (8–10 weeks old) used in the study were purchased from Charles River Laboratories (Calco, Italy) and maintained in standard conditions according to European Community Directive 86/609/CEE. The IGROV-1 tumor xenograft was maintained as a line by successive transplants involving the s.c. injection of tumor fragments into both flanks of mice. Tumor weight (TW) was calculated biweekly by measurements of diameters with a Vernier caliper according to the formula $d^2 \times D/2$, where d and D are the shortest and longest diameters, respectively.

Chemotherapeutic treatment was started when the mean tumor weight was about 50 mg. Cisplatin was dissolved in saline and topotecan, in distilled water, and both were delivered i.v. on a schedule of q7d × 3, 10 ml/kg body weight. When given in combination, the drugs were delivered as topotecan immediately after cisplatin. At 10 days after the last treatment the percentage ratio between the TW (T) of treated mice and the TW of controls (C) was calculated (T/C%).

For assessment of drug toxicity, animals were weighed biweekly and mean body-weight percentage variations were calculated as $Dbw\% = [100 - (bw_7/bw_1)] \times 100$, where bw_7 represents the mean body weight at 7 days after the last drug treatment and bw_1, that at the day of the first treatment. No control animal died during the experiment; therefore, all deaths in treated mice were considered as toxicity-related mortality.

Analysis of drug interaction

The effect of the combination of cisplatin and topotecan was evaluated according to the method of Kern et al. [11]. In brief, in cell line studies the expected cell survival (S_{exp}, defined as the product of the survival observed with drug A alone and the survival observed with drug B alone) and the observed cell survival (S_{obs}) for the combination of A and B were used to construct an index (R): $R = S_{exp}/S_{obs}$. An R index of 1 (additive effect) or lower indicated the absence of synergism. Synergism was defined as any value of R greater than unity. In our experience a synergistic interaction may be of pharmacological interest when R values are around 2.0 [17]. For in vivo studies, R was calculated from expected and observed T/C% values.

Northern-blot analysis

Total RNA was purified from exponentially growing cells by cell lysis with guanidine isothiocyanate and centrifugation in a cesium chloride gradient [6]. An aliquot of 20 μg of total RNA was electrophoresed in a formaldehyde-containing 1% agarose gel and transferred onto a nylon membrane. A 0.7-kb human topoisomerase I cDNA fragment was purified from the plasmid pGEM-4-DI, kindly provided by Dr. L. Liu (Piscataway, N.J.). A β-actin probe was used as a control for loading. DNA probes were ^{32}P-labeled with a random primer kit (Amersham, Little Chalfont, UK). Prehybridization was carried out for at least 4 h at 42 °C in 50% formamide, 5 × SSC (3 *M* sodium chloride, 0.3 *M* sodium citrate, pH 7), 5× Denhart's solution, 0.2% SDS (sodium dodecyl sulfate), and 50 μg of denaturated salmon sperm. Hybridization was performed for 18 h at 42 °C in the same buffer containing 10% dextran sulfate and ^{32}P-labeled DNA probes. Final washes of the filters were performed at 50 °C for 20 min in 0.5 × SSC and 0.1% SDS.

Results

In vitro studies

The cisplatin-resistant subline IGROV-1/Pt0.5 exhibited a factor of resistance to the selecting agent of about 10. The sensitivity of IGROV-1 and IGROV-1/Pt0.5 cell lines to topotecan as evaluated after 1 or 24 h of exposure is shown in Table 1. A slight degree of cross-resistance to topotecan was observed, with the degree of resistance being similar after a 1-h and a 24-h period of exposure. Since Northern-blot analysis showed similar

Table 1 Sensitivity of IGROV-1 and IGROV-1/Pt0.5 cells to topotecan

Cell line	IC_{50}^{a} (μg/ml) 1 h	RI[b]	IC_{50}^{a} (μg/ml) 24 h	RI[b]
IGROV-1	0.22 ± 0.1		0.012 ± 0.004	
IGROV-1/Pt0.5	0.54 ± 0.24	2.4	0.029 ± 0.012	2.4

[a] The cytotoxic effect of topotecan was measured by the growth inhibition assay. IC_{50} is defined as the drug concentration leading to a 50% inhibition of cell growth. Data represent mean values ± SD for 1–6 independent experiments

[b] RI, resistance index: ratio of the IC_{50} of the resistant cell line to the IC_{50} of the sensitive cell line

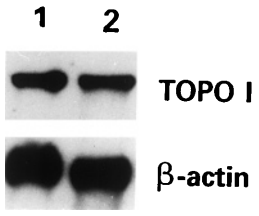

Fig. 1 Northern-blot analysis of topoisomerase I expression in IGROV-1 (*lane 1*) and IGROV-1/Pt0.5 (*lane 2*). An aliquot of 20 μg of total RNA was fractionated in a 1% agarose formaldehyde-containing gel, transferred to a nylon filter, and hybridized with the topoisomerase I probe

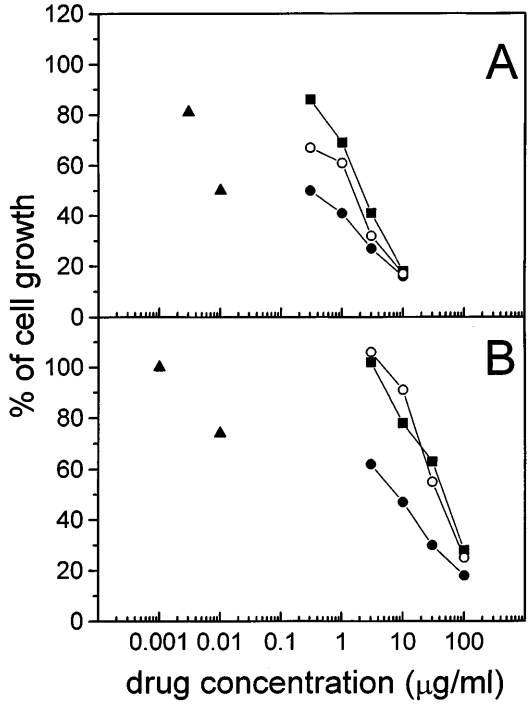

Fig. 2A,B Effect of cisplatin applied alone and in combination with topotecan on **A** IGROV-1 and **B** IGROV-1/Pt0.5 cells after a 1-h period of exposure to cisplatin followed by a 24-h period of topotecan exposure. **A** Cisplatin alone (■); cisplatin + topotecan, 0.01 μg/ml (●); cisplatin + topotecan, 0.003 μg/ml (○); topotecan alone (▲). **B** Cisplatin alone (■); cisplatin + topotecan, 0.01 μg/ml (●); cisplatin + topotecan, 0.001 μg/ml (○); topotecan alone (▲). SD never exceeded 10% ($n = 3$)

mRNA levels of topoisomerase I gene expression in the cisplatin-sensitive and resistant cells (Fig. 1), the reduced sensitivity to topotecan of the cisplatin-resistant subline could not be ascribed to a reduced expression of the target enzyme.

With the aim of evaluating the interaction between cisplatin and topotecan in these ovarian carcinoma cell systems, we combined the two drugs according to two schedules: (a) a sequential schedule – a 1-h period of cisplatin treatment followed by a 24-h period of topotecan treatment and (b) a simultaneous schedule – a 1-h period of exposure to both cisplatin and topotecan. These schedules were selected as the most favorable, since topoisomerase I is involved in the cell response to DNA damage [3]. In all the experiments, increasing concentrations of cisplatin were combined with two topotecan concentrations.

In the sequential treatment, increasing concentrations of cisplatin followed by the IC_{20} or IC_{50} (0.003 and 0.01 μg/ml, respectively) of topotecan caused a greater cell kill than that observed for cisplatin alone in IGROV-1 cells (Fig. 2A). R index values of around 1 were reached (Fig. 3A), suggesting that the interaction was additive. Similar results were obtained in the IGROV-1/Pt0.5 cells (Fig. 2B, 3B). When a 1-h period of simultaneous exposure was used (Fig. 4), we observed that the combination of increasing concentrations of cisplatin with topotecan at 0.01 (corresponding to a nontoxic dose in both cell systems) or 0.1 μg/ml (corresponding to the IC_{20} and IC_{30} in IGROV-1/Pt0.5 and IGROV-1 cells, respectively) generally resulted in an increase in the cytotoxic effect of cisplatin alone. R index values were higher than 1 for the drug combination in the IGROV-1 cells only when topotecan was combined with the two highest cisplatin concentrations (Fig. 5A). In IGROV-1/Pt0.5 cells the trend was similar, with values being > 1 for most cisplatin concentrations combined with topotecan, but the synergistic interaction was less marked than that observed in the parental cells (Fig. 5).

In vivo studies

To investigate the therapeutic advantage of the potentiation of cisplatin cytotoxicity by topotecan we studied the effect of cisplatin in combination with topotecan using the most favorable schedule (immediately after cisplatin) in the sensitive IGROV-1 tumor xenografted

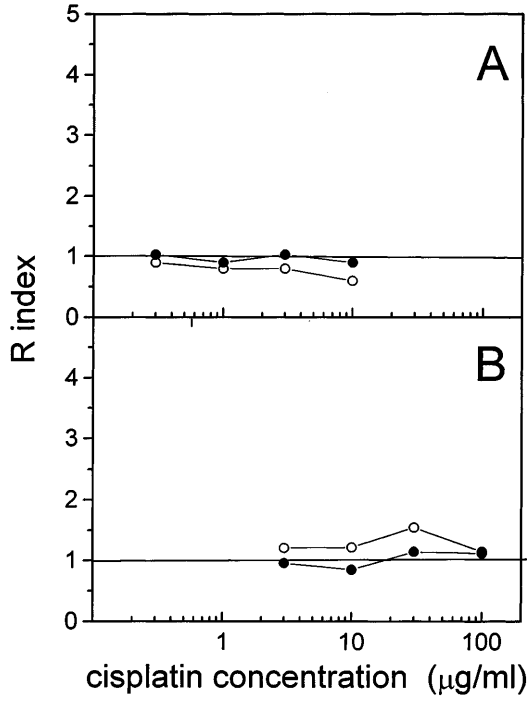

Fig. 3A,B Evaluation of the interaction between cisplatin and topotecan. Sequential treatment: 1-h cisplatin treatment followed by 24-h treatment with topotecan. R index values are plotted against cisplatin concentrations. **A** IGROV-1: cisplatin + topotecan, 0.01 μg/ml (●); cisplatin + topotecan, 0.003 μg/ml (○). **B** IGROV-1/Pt0.5: cisplatin + topotecan, 0.01 μg/ml (○); cisplatin + topotecan, 0.001 μg/ml (●)

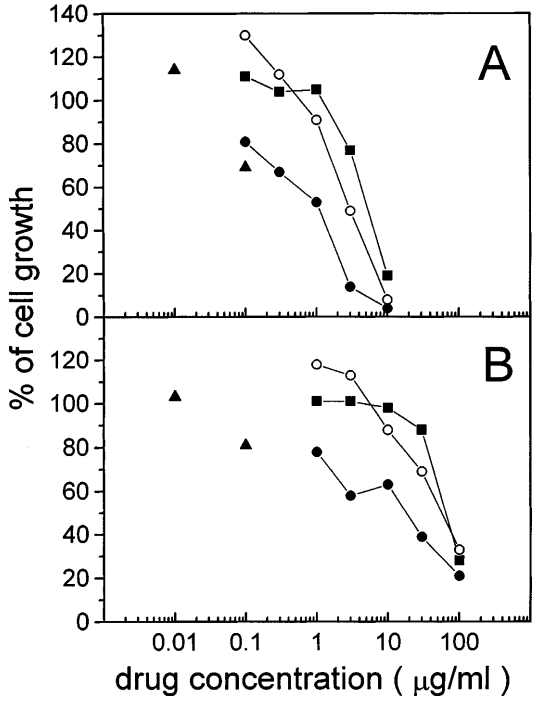

Fig. 4A,B Effect of cisplatin applied alone and in combination with topotecan on **A** IGROV-1 and **B** IGROV-1/Pt0.5 cells after a simultaneous 1-h period of exposure. **A** Cisplatin alone (■); cisplatin + topotecan, 0.1 μg/ml (●); cisplatin + topotecan, 0.01 μg/ml (○); topotecan alone (▲). **B** Cisplatin alone (■); cisplatin + topotecan, 0.1 μg/ml (●); cisplatin + topotecan, 0.01 μg/ml (○); topotecan alone (▲). SD never exceeded 10% ($n = 3$)

s.c. into nude mice (Table 2). The IGROV-1 tumor was moderately responsive to either drug alone, since at the respective optimal doses [6 mg/kg cisplatin and 10 mg/kg topotecan given every 7 days for a total of three times (q7d × 3)], tumor growth was around 40% for either drug with no toxic death. When suboptimal doses of each drug were combined (using a topotecan dose level of 5.1 mg/kg) the combination achieved a tumor growth inhibition comparable with those achieved by the single-agent therapy (i.e., about 40% T/C). In addition, a higher degree of antitumor efficacy (24% T/C) was achieved by a tolerated combination of suboptimal doses of each drug (4.2 mg/kg cisplatin plus 5.1 mg/kg topotecan). The combination of 3 mg/kg cisplatin plus 7.1 mg/kg topotecan achieved a 26% T/C value and an R index of 1.77, but it was toxic (2/5 mice died due to toxicity and the mean weight loss was 17% in this group). Therefore, it seems that although some level of synergism between the two drugs was maintained even in the in vivo system, the therapeutic advantage is limited since the therapeutic index of the drug combination worsened as a consequence of an increase in lethal toxicity.

Discussion

A number of in vitro observations have suggested a pharmacological relevance of the topotecan and cisplatin combination [5, 10]. Phase I studies evaluating sequences of topotecan and cisplatin have recently been reported [21]. Since the drug schedule may be an important determinant of the activity and toxicity of combined cisplatin and topotecan, in the present study we explored two different schedules to simulate sequences that could reflect drug exposure in the clinical setting (i.e., simultaneous treatment with a bolus injection of the two drugs or sequential treatment with a bolus injection of cisplatin followed by a slow infusion of topotecan). On the basis of the rationale relating topoisomerase I to the DNA-repair process, the latter schedule was expected to be very promising. However, in our cell systems, potentiation of the cytotoxic effects of cisplatin was obtained only by simultaneous exposure to the drugs.

The relevance of an appropriate schedule to achieve optimal efficacy of the combination of topotecan with other DNA-damaging agents has been emphasized in a previous study with Chinese hamster V79 cells [5]. However, in that study a 1-h period of concurrent exposure to cisplatin and topotecan was less effective than a 1-h period of exposure to cisplatin at the beginning of a prolonged infusion (24 h) of topotecan. In contrast, our results are consistent with the previously reported in vitro synergy between cisplatin and 9-aminocamptothecin using the same schedule and a sensitive ovarian

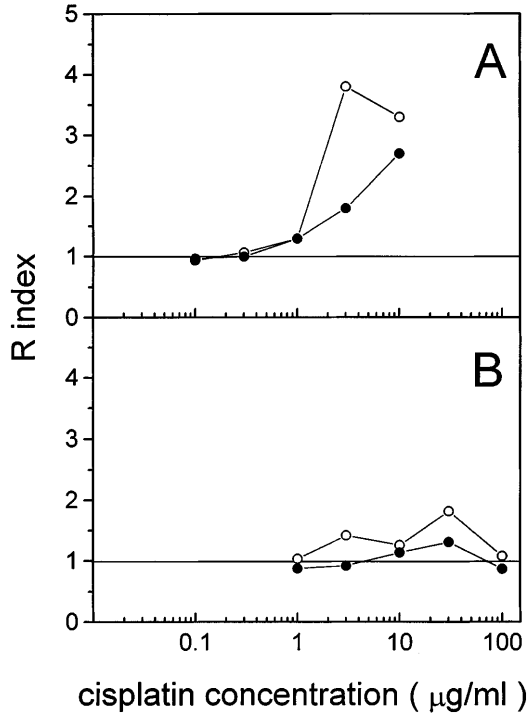

Fig. 5A,B Evaluation of the interaction between cisplatin and topotecan. Simultaneous 1-h period of exposure. **A** IGROV-1: cisplatin + topotecan, 0.1 μg/ml (○); cisplatin + topotecan, 0.01 μg/ml (●). **B** IGROV-1/Pt0.5: cisplatin + topotecan, 0.1 μg/ml (○); cisplatin + topotecan, 0.01 μg/ml (●)

carcinoma cell line (IGROV-1) [9]. A plausible explanation for the variable efficacy of different schedules in different systems would be that the interaction between topotecan and DNA-damaging agents is dependent on the cell type and its biochemical background. This interpretation is also consistent with a variable radiosensitization by topotecan in human cell lines [13].

The results presented herein suggest that the potentiation of cisplatin cytotoxicity by topotecan is dependent not only on the cell type but also on the mechanism of drug resistance. We have previously reported that the IGROV-1/Pt0.5 subline, selected following continuous exposure to cisplatin, is characterized by loss of wild-type p53 function as a consequence of mutation [18]. It is likely that such mutation is one of the relevant alterations underlying the development of cisplatin resistance and cross-resistance to topotecan, since resistance is also associated with reduced susceptibility to apoptosis induction by either agent [4]. Thus, it may be that the nature of the interaction (i.e., additive or synergistic) reflects the cell's ability to respond to drug-induced DNA damage. Such an interpretation is consistent with the observation that the synergistic effect was more marked and more evident at highly cytotoxic concentrations in the cell system more responsive to the DNA-damaging agent. Thus, it is likely that the cisplatin/topotecan combination is more favorable when other mechanisms of resistance are involved. Indeed, the observed cross-resistance between cisplatin and topotecan is related to the specific mechanism of cisplatin resistance [4].

To understand better the pharmacological relevance of drug interaction we studied the concurrent combination of cisplatin and topotecan in the treatment of the parental IGROV-1 tumor growing in nude mice. A marginal potentiation of the drug's antitumor effect was observed in the combination using suboptimal dose levels of each drug. An ineffective dose of cisplatin (3 mg/kg) enhanced the antitumor activity of topotecan. However, the drug interaction was also associated with an increase in toxicity. Although the best antitumor

Table 2 Antitumor activity of cisplatin and topotecan given as single agents and in combination on human IGROV-1 tumor xenografts

Drug	Dose[a] (mg/kg)	TW[b] (mg)	Observed[c] T/C%	Expected[d] T/C%	Expected/observed R	Number of toxic deaths/ Total number of mice	BW%[c]
Control	–	678 ± 341				0/5	+13
Cisplatin	3.0	640 ± 290	94			0/4	+2
	4.2	338 ± 218	50			0/5	−4
	6.0	295 ± 125	43			0/5	−9
Topotecan	5.1	372 ± 112	55			0/4	+2
	7.1	330 ± 146	49			0/5	+12
	10	272 ± 127	40			0/5	+4
Cisplatin + topotecan	3 + 5.1	271 ± 108	40	52	1.3	0/5	+4
	3 + 7.1	176 ± 59	26	46	1.77	2/5	−4
	4.2 + 5.1	159 ± 59	24*	27.5	1.14	1/5	−4
	4.2 + 7.1	210 ± 74	31	24.5	0.79	2/5	+3

* $P < 0.05$ vs the group treated with topotecan at 10 mg/kg; Student's *t*-test
[a] Drugs were delivered i.v. at days 7, 14, and 21 after the tumor inoculum. In the groups treated with the drug combination, cisplatin was followed immediately topotecan
[b] Tumor weight at 10 days after the last drug treatment. Mean values ± SD are reported
[c] Observed T/C%: TW in treated mice TW in control mice ×100
[d] Expected T/C%: product of the observed T/C% of each drug alone/100
[e] Body weight change % at 10 days after the last drug treatment and relative to the body weight at the 1st day of treatment

effects were achieved with the drug combination (26% tumor growth as compared with around 40% caused by single treatment with either drug at its maximum tolerated dose), the therapeutic advantage in terms of the therapeutic index appears questionable. However, since the increase in toxicity was probably related to myelotoxicity, a major toxic effect of topotecan [20], it is noteworthy that the side effect could be controlled by adequate hematological support, such as the use of colony-stimulating factors in clinical therapy; thus, the combination may be of pharmacological interest for future clinical approaches in the treatment of ovarian carcinoma.

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