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Down-regulation of topoisomerase II by camptothecin does not prevent additive activity of the topoisomerase II inhibitor etoposide *in vitro*

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Topoisomerases (Topo) I and II are cellular enzymes that catalyze the relaxation of topologically strained DNA and that are involved in a number of DNA-related processes. Their complete inhibition by Topo I and II inhibitors gives promise for improvements in the treatment of malignant diseases. However, preclinical studies showed down-regulation of Topo II protein expression by Topo I inhibitors, which may preclude the useful application of combined topoisomerase inhibition in the clinic. We investigated the efficacy of the combination of etoposide (ETP) and camptothecin (CPT) in human gastric and lung cancer cell lines with different sensitivity towards ETP. The cytotoxicity of different drugs was assessed by the sulforhodamine B assay. Drug interactions were evaluated by isobologram analysis. The polymerase chain reaction and flow cytometry were employed for examination of the *mdr1* (multidrug resistance type 1) phenotype. As reported by others, incubation of the P glycoprotein (P-gp)-negative tumor cell lines with the Topo I inhibitor CPT resulted in a significant down-regulation of Topo II protein expression. This was obviously due to changes in the cell cycle distribution of the cells induced by the treatment, with a marked increase of cells in G_2/M phase and a consecutive decrease of S phase cells. Despite these biochemical changes, isobologram analysis showed additive cytotoxic activity of CPT and ETP in all the cell lines, independent of whether the drug incubation was performed simultaneously or sequentially. These data indicate that down-regulation of Topo II protein by CPT does not prevent additive activity of CPT and ETP *in vitro*, and therefore combined Topo I and II inhibition may be useful for investigation in clinical trials.

Key words: Camptothecin, etoposide, *in vitro* studies, isobologram analysis, topoisomerase inhibitors.

Introduction

DNA topoisomerases (Topo) are nuclear enzymes that catalyze the relaxation of topologically strained DNA by transient single-strand (Topo I) and double-strand (Topo II) breakage. Moreover, these catalytic proteins are involved in various DNA processes including replication, transcription, recombination and repair.[1–3] Two forms of DNA topoisomerases are defined: Topo I and Topo II, with its isoenzymes α and β.[2] Beside other differences between the two forms of topoisomerases it is of importance that Topo I is expressed continously during the cell cycle, whereas Topo II expression increases during the S phase of the cell cycle and is almost absent in quiescent cells.[4]

The Topo II inhibitor etoposide (ETP), an epipodophyllotoxin, has shown broad antitumor activity, and therefore plays an essential role in the chemotherapy of solid tumors and malignant systemic diseases. The Topo I interactive drug camptothecin (CPT) and its derivatives belong to the most promising new anticancer drugs. Originally extracted from the plant *Camptotheca accuminata*, the semisynthetic derivatives irinotecan (CPT-11) and topotecan showed significant antitumor activity in a number of solid tumors, including gastric and lung cancer.[5–7]

Topo I or II inhibitor-related cytotoxicity appears to be correlated with the induction of drug-stabilized DNA-topoisomerase cleavable complexes,[1,8] which has been associated with the regulation of Topo I/II gene and protein expression[9,10] or alterations of Topo I/II structure and function.[11–13] Since tumor cells which proved to be resistant towards Topo II inhibitors often showed increased sensitivity to Topo I inhibitors[8,14,15] and because of the different toxicity profile of topoisomerase inhibitors, the combined application of Topo I and II inhibitors might be clinically useful.

However, experimental data showing alterations in cell cycle distributions of tumor cells induced by Topo I inhibitors (CPT), which might lead to decreased activity of especially Topo II inhibitors (e.g. ETP),[16,17] have questioned the applicability of the combined use of Topo I and II inhibitors.

The aim of the present study was to evaluate whether down-regulation of Topo II protein induced by treatment with CPT would negatively affect the cytotoxic activity of Topo II inhibitors.

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Materials and methods

Drugs and chemicals

Cell culture media were obtained from Gibco/Life Technology (Eggenstein, Germany). Doxorubicin and cisplatin were provided by Pharmacia (Erlangen, Germany) and Bristol-Myers Squibb (München, Germany), respectively. AmpliTaq polymerase was from Perkin-Elmer Cetus (Norwalk, CT). All other chemicals were supplied by Sigma (Deisenhofen, Germany).

Cell lines

The NCI large cell lung cancer cell lines H460 and H661 were obtained from the ATCC (Rockville, MD). The gastric adenocarcinoma cell line HM51 was established by our group and has been described previously.[18]

Cytotoxicity assay

The cytotoxicity of different drugs was assessed by the sulforhodamine B assay.[19] The assay was performed in 96-well microtiter plates (Falcon, Heidelberg, Germany) seeding the tumor cells at a density of 500–1000 cells/well. After 24 or 48 h, 100 μl of medium containing different concentrations of the cytotoxic drug was added for 2 h. Total incubation time of the cells was 120 h. All experiments were done in triplicate. The drug concentration that inhibited cell growth by 50% (IC_{50}) was obtained from semilogarithmic dose–response plots.

The standard isobologram methodology (50% isodose) was used to determine the interaction of ETP and CPT. The schedule-dependent interactions of these drugs were classified as synergistic (upward concavitiy), additive (linear) or antagonistic (downward concavity) as described by Berenbaum.[20] We defined three different treatment schedules: simultaneous incubation (2 h ETP+2 h CPT), sequential incubation 1 (2 h ETP followed by 2 h CPT 24 h later) and sequential incubation 2 (2 h CPT followed by 2 h ETP 24 h later).

Western blot analysis of Topo II

Cells were lysed in 50 mM Tris-HCl buffer, pH 8.0, containing 100 mM NaCl, 0.5% SDS, 0.5% sodium deoxycholate, 0.5% Nonidet P-40, 10 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride and 0.4 IU/ml aprotonin. The protein content was determined by the BioRad DC protein assay (BioRad, Hercules, CA). Samples of 40 μg protein of whole cell lysates were dissolved on a 7.5% SDS–polyacrylamide gel and electroblotted to nitrocellulose (BioRad). For Topo II immunoblotting, membranes were blocked with 5% (w/v) dried non-fat milk in phosphate-buffered saline. Topo II-specific binding of the rabbit polyclonal antibody against Topo II (TopoGen, Columbus, OH) was visualized with the enhanced chemiluminescence detection system (Amersham, Arlington Heights, IL) using an anti-rabbit horseradish peroxidase-labeled goat monoclonal antibody (Amersham).

Polymerase chain reaction (PCR) for P-glycoprotein (P-gp) expression

PCR was carried out with 2 U of AmpliTaq polymerase and 35 pmol of the primers (sense primer: 5′-GGACGATAACGTTACTACCC-3′; antisense primer: 5′-AGTCCTCGTCTTCAAACTTG-3′ corresponding to nucleotides of the MDR1 cDNA) in a final volume of 25 μl. Amplification was performed by 30 cycles according to the method of Noonan.[21] Each cycle of PCR included 45 s of denaturation at 95°C, 45 s of primer annealing at 60°C and 2 min of extension/synthesis at 72°C. Samples were separated on 1.4% agarose gels and stained with ethidium bromide.

Flow cytometry

Detection of P-gp was performed by incubation of the cells with the UIC-2 antibody (Dianova, Hamburg, Germany), followed by a FITC-conjugated goat anti-mouse IgG (Becton Dickinson, Heidelberg, Germany). A mouse IgG2a served as the negative control. After appropriate gating, cellular fluorescence was determined by an Epics XL flow cytometer (Coulter, Krefeld, Germany).

Cytometry was also used to quantitate the cell cycle distribution of the cell lines at different times after drug incubation. DNA was stained with propidium iodide and DNA content of the cells was measured at 635 nm using the multicycle software of Phoenix Flow Systems (San Diego, CA).

Results

In vitro cytotoxicity

The cytotoxicity of ETP, CPT, doxorubicin (DOX), vincristine (VCR) and paclitaxel (TAX) was determined

in the different cell lines using the sulforhodamine B assay (Table 1). Twenty-four hours after cell seeding each drug was given for 2 h, resembling short-term infusions of these drugs in the clinic.[22]

The sensitivity of the cell lines towards the Topo II interactive drug ETP differed with IC_{50} values ranging from 1.0 to 10.0 μM, whereas it was comparable for the Topo I drug CPT. This difference was not related to the different MDR1 phenotype of the cell lines, since all three were P-gp-negative by flow cytometry and did not show overexpression of the MDR1 gene by PCR reaction. In accordance with these results, the three cells lines showed similar sensitivity towards different drugs, which are substrates for P-gp-mediated efflux, such as DOX, VCR and TAX (Table 1).

Cell cycle analysis

Expression of Topo I and II has been shown to be related to the cell's state during the cell cycle.[2,4] Thus, the treatment-related changes in the cell cycle distribution were examined. Exemplary for the three cell lines, the data obtained from the H460 cells are shown in Figure 1. Exposure to CPT resulted in a G_2/M block after 24 h, increasing the proportion of cells in G_2/M from 25 to more than 60%. In parallel, the proportion of cells in S phase dropped from about 40 to 30%. This influence on the cell cycle distribution was independent of whether CPT was given alone or in combination with ETP. However, exposure of cells to ETP alone resulted in a slight increase of cells in the G_1 phase (Figure 1).

Western blot analysis of Topo II protein expression

The effect of CPT on Topo II protein expression was investigated and the results of a representative Topo II

Table 1. Cytotoxicity of ETP, CPT, DOX, VCR and TAX in human gastric and lung cancer cell lines (mean IC_{50} values in μM)

	IC_{50} for cell line		
	H460	H661	HM51
ETP	1.0	10	7.0
CPT	0.05	0.15	0.1
DOX	0.14	1.1	0.5
VCR	0.5	0.2	0.3
TAX	0.15	0.05	0.45

The standard deviation was below 15%, results are expressed as the mean values of three independent experiments.

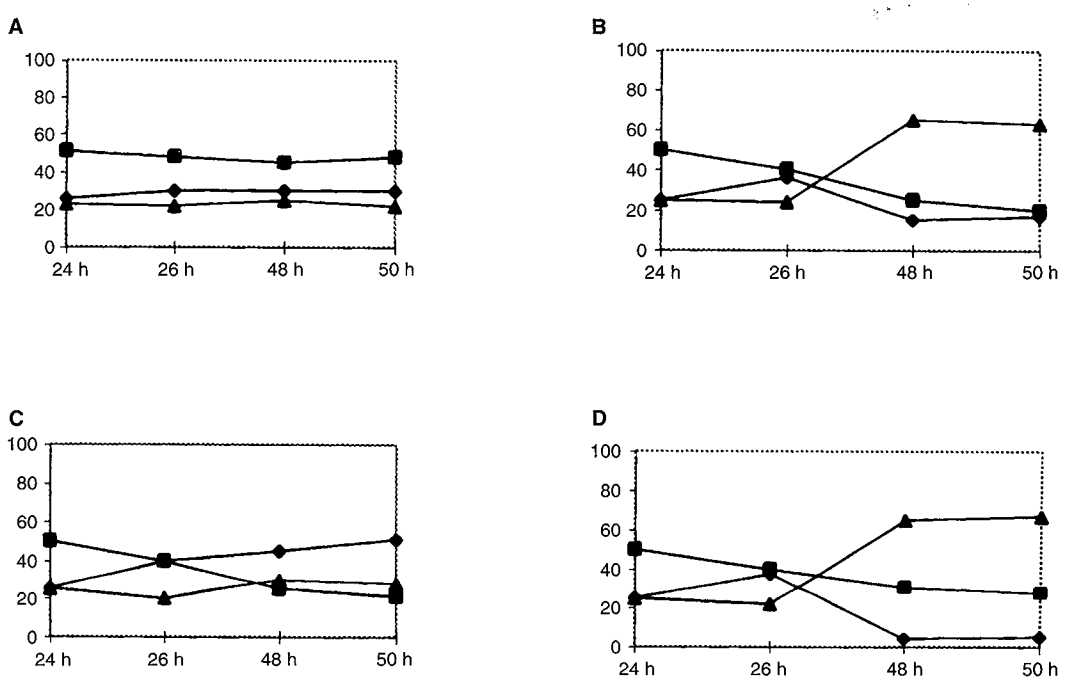

Figure 1. Cell cycle distribution in the cell line H460 between 24 and 50 h after cell seeding. (A) Cells growing in drug-free medium. (B) Incubation with the IC_{50} of CPT beginning 24 h after seeding for 2 h. (C) Incubation with the IC_{50} of ETP beginning 24 h after seeding for 2 h. (D) Incubation with the IC_{50} of CPT followed by ETP, beginning 24 h after seeding for 2 h, each. Diamonds, $G_{0/1}$ phase; squares, S phase; triangles, G_2/M.

immunoblot are shown in Figure 2. While untreated H460 cells showed the intact M_r 180 000 Topo II protein band, exposure to CPT induced a down-regulation of Topo II protein. This down-regulation was significant at the dose of 2 μM CPT, resembling the IC_{50} in this cell line, and it proved to be concentration dependent.

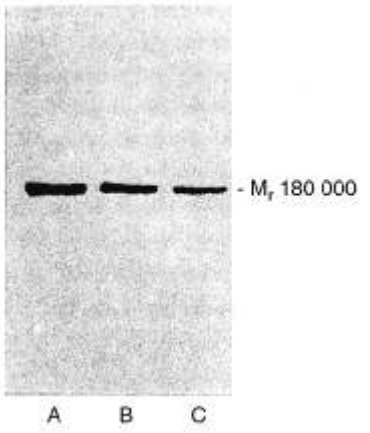

Figure 2. Western blots of DNA Topo II in whole cell lysates of H460 cells. Equal amounts of protein (40 μg) from each of the preparations were separated on a 7.5% SDS–PAGE gel, transferred to nitrocellulose paper and reacted with Topo II antiserum. (A) No preincuabtion. (B) Preincubation with 2 μM (IC_{50}) of CPT. (C) Preincubation with 20 μM (10-fold IC_{50}) of CPT.

In vitro interaction of ETP and CPT

Our data showed that exposure of cells to CPT indeed resulted in a marked depletion of cells in S phase after 24 h, which was accompanied by a significant down-regulation of Topo II protein. To evaluate whether these changes would affect the activity of Topo II inhibitors, we performed drug interaction studies using different schedules. As outlined in Figure 3, the simultaneous application of CPT and ETP produced additive cytotoxic effects in all three cell lines. The

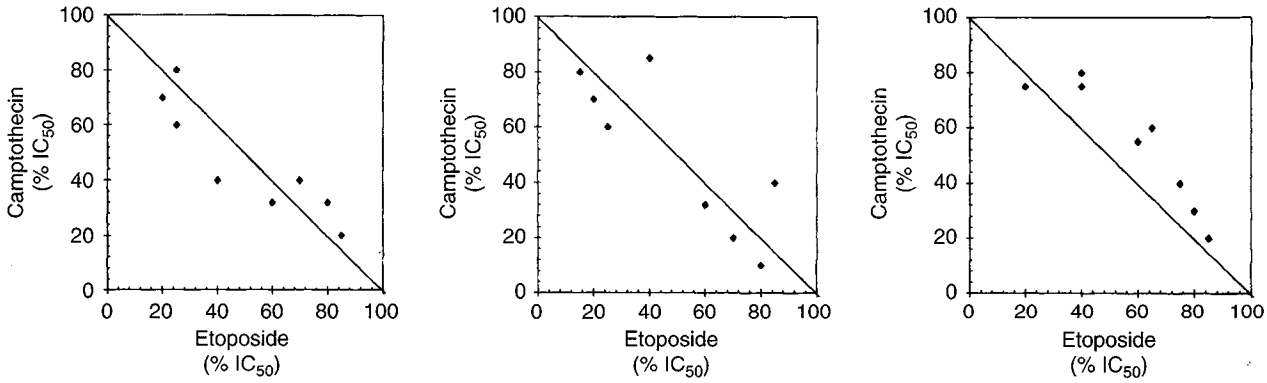

Figure 3. Isobologram analysis after simultaneous incubation with ETP and CPT in the cell lines HM51 (left), H661 (middle) and H460 (right).

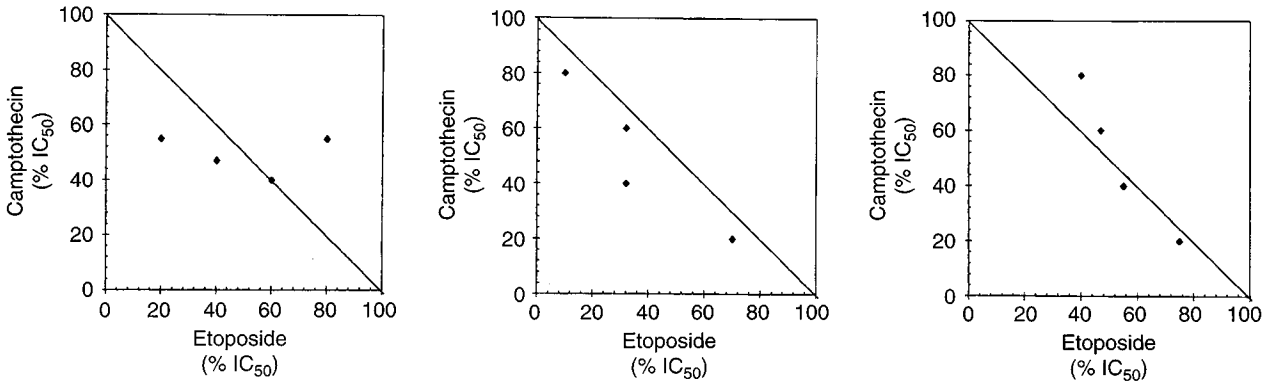

Figure 4. Isobologram analysis after sequential incubation with CPT, followed 24 h later by ETP in the cell lines HM51 (left), H661 (middle) and H460 (right).

same pattern of drug interaction was seen when a sequential application was used and ETP was given 24 h after CPT (Figure 4); at a time where the cells showed down-regulation of Topo II. Thus, the activity of ETP appears to be unaffected, though Topo II levels are decreased after CPT treatment.

Discussion

Topo I and II share a common role in resolving topological constraints in DNA in association with other cellular functions, such as transcription, translation and chromatid separation.[1–3] According to the importance of these enzymes with regard to cell function, topoisomerase inhibitors revealed broad antitumor activity in preclinical and clinical studies.[2,6–8]

There is evidence that Topo I and II inhibitors commonly do not show cross-resistance *in vitro*, which is particularly important, because these enzymes are in part able to substitute their cellular function.[7] Additionally, different Topo inhibitors essentially do not show overlapping toxicity. All these facts suggest that the combination of Topo inhibitors may be a step forward in the treatment of cancer.

However, preclinical data have shown that incubation of tumor cells with Topo I inhibitors (and in part also with Topo II inhibitors) causes a down-regulation of Topo II protein expression.[16,17] Since reduced Topo II expression proved to be an important mechanism of tumor resistance towards Topo II inhibitors, the combination of Topo inhibitors could result in antagonistic activity of the drugs. Indeed, this antagonism has been reported by preclinical investigations *in vitro* and *in vivo*.[23,24]

In this regard, we built up an *in vitro* model to investigate the hypothesis of down-regulation of Topo II protein expression by Topo I inhibitors and to study its influence on different combination schedules of Topo inhibitors. To rule out effects of membrane protein-mediated drug efflux, we used three cell lines which were MDR1/P-gp-negative. In these cell lines we investigated the effect of the Topo I inhibitor CPT and the Topo II inhibitor ETP—alone and in combination—on the cell cycle distribution. As reported by other groups,[16,17] exposure to CPT altered the cell cycle distribution by increasing the number of cells in G_2/M phase and concomitantly decreasing S phase cells after 24 h (Figure 1). In contrast to published data however, incubation with ETP did not significantly change the cell cylce distribution compared to controls in the cell lines studied here. This difference may be explained by the lower dose of ETP (IC_{50}) used in our studies. In parallel with the G_2/M block of the tumor cells after exposure to CPT, down-regulation of Topo II protein was shown by immunoblotting (Figure 2). This down-regulation proved to be dose dependent, but was already significant at the IC_{50} of each cell line. Since reduced intracellular levels of topoisomerases have shown to be one of the most important factors for cellular resistance,[1,8,9] combinations of Topo inhibitors could theoretically prove to be antagonistic and therefore should be avoided in the clinic. However, isobologram analysis of the combined *in vitro* cytotoxicity of ETP and CPT revealed additive activity of both drugs in all the three cell lines, independent of whether simultaneous or sequential drug incubation was performed, indicating that negative interactions did not take place. These *in vitro* results are in accordance with preliminary clinical data, where the combination of CPT-11 and ETP showed promising results in a phase I study in patients with refractory solid tumors.[25]

These data clearly show that cells appear to be still sensitive to Topo II inhibitors even after CPT induced down-regulation of Topo II protein. The biochemical reasons for these findings are currently unclear. One possible explanation might be that cells which have already been exposed to a Topo I inhibitor are unable to utilize Topo I for the substitution of Topo II function when exposed to ETP, a fact that may compensate the reduced Topo II protein levels of the cells. Thus, it seems worthwhile to investigate combined Topo inhibition in clinical trials.

Conclusion

Taken together, these data comfirm investigations of others indicating that the exposure of tumor cells to Topo I inhibitors results in changes of the cell cylce distribution and down-regulation of topo II protein. Despite these effects, treatment with CPT does not negatively affect the cytotoxic activity of the topo II interactive drug ETP. Therefore, combined Topo I and II inhibition may be useful for investigation in clinical trials.

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References

1. Beck WT, Danks MK, Wolverton JS. Resistance of mammalian tumor cells to inhibitors of DNA topoisomerase II. *Adv Pharmacol* 1994; **29B:** 145–69.
2. Chen AY and Liu LF. DNA topoisomerases: essential enzymes and lethal targets. *Annu Rev Pharmacol Toxicol* 1994; **34:** 191–218.
3. Wang JC. DNA topoisomerases. *Annu Rev Biochem* 1995; **54:** 665–72.
4. Heck MM, Mittelman WN, Earnshaw WC. Differential expression of DNA topoisomerase I and II during the eukaryotic cell cycle. *Proc Natl Acad Sci USA* 1988; **85:** 1086–90.
5. Kambe M, Wakui A, Nakao I, *et al.* A late phase II study of irinotecan in patients with advanced gastric cancer. *Proc Am Soc Clin Oncol* 1993; **12:** 198.
6. Masuda N, Fukuoka M, Kusunoki Y, et al. CPT-11: a new derivative of camptothecin for the treatment of refractory or relapsed small-cell lung cancer. *J Clin Oncol* 1992; **10:** 1225–9.
7. Bonomi P. Non-small cell lung cancer chemotherapy. In: Pass HI, Mitchell JB, Johnson DH, Turisi AT, eds. *Lung cancer: principles and practice.* Philadelphia: Lippincott-Raven 1996; 811–23.
8. Beck WT. Unknotting the complexities of multidrug resistance: the involvement of DNA topoisomerases in drug action and resistance. *J Natl Cancer Inst* 1989; **81:** 1683–5.
9. Friche E, Danks MK, Schmidt CA, Beck WT. Decreased DNA topoisomerase II in daunorubicin-resistant Ehrlich ascites tumor cells. *Cancer Res* 1991; **51:** 4213–8.
10. Eng WK, McCabe FL, Tan KB, *et al.* Development of a stable camptothecin-resistant subline of P388 leukemia with reduced topoisomerase I content. *Mol Pharmacol* 1990; **38:** 471–80.
11. Danks MK, Schmidt CA, Cirtain MC, *et al.* Altered catalytic activity of and DNA cleavage by DNA topoisomerase II from human leukemic cells selected for resistance to VM-26. *Biochemistry* 1988; **27:** 8861–9.
12. Kapoor R, Slade DL, Fujimori A, Pommier Y, Harker W. Altered topoisomerase I expression in two subclones of human CEM leukemia selected for resistance to camptothecin. *Oncol Res* 1995; **7:** 83–95.
13. Hsiung Y, Jannatipour M, Rose A, *et al.* Functional expression of human topoisomerase II alpha in yeast: mutations at amino acids 450 or 803 of topoisomerase II alpha result in enzymes that can confer resistance to anti-topoisomerase II agents. *Cancer Res* 1996; **56:** 91–9.
14. Tan KB, Mattern MR, Eng WK. Nonproductive rearrangement of DNA topoisomerase I and II genes: correlation with resistance to topoisomerase inhibitors. *J Natl Cancer Inst* 1989; **81:** 1732–6.
15. Sugimoto Y, Tsukahara S, Oh-hara T, *et al.* Decreased expression of DNA topoisomerase I in camptothecin-resistant tumor cell lines as determined by a monoclonal antibody. *Cancer Res* 1990; **50:** 6925–30.
16. Beidler DR, Chang JY, Zhou B, Cheng Y. Camptothecin resistance involving steps subsequent to the formation of protein-linked DNA breaks in human camptothecin-resistant KB cell lines. *Cancer Res* 1996; **56:** 345–53.
17. Tsao YP, D'Arpa P, Liu LF. The involvement of active DNA synthesis in camptothecin-induced G_2 arrest: altered regulation of $P34^{cdc2}$/cyclin B. *Cancer Res* 1992; **52:** 1823–9.
18. Harstrick A, Bokemeyer C, Scharnofske M, *et al.* Preclinical activity of a new platinum analogue, lobaplatin, in cisplatin-sensitive and -resistant human testicular, ovarian, and gastric carcinoma cell lines. *Cancer Chemother Pharmacol* 1993; **33:** 43–7.
19. Skehan P, Storeng R, Scudiero D. New colorimetric cytotoxicity assay for anticancer drug screening. *J Natl Cancer Inst* 1990; **82:** 1107–12.
20. Berenbaum MC. What is synergy? *Pharmacol Rev* 1989; **41:** 93–140.
21. Noonan KE, Beck C, Holzmayer TA, *et al.* Quantitative analysis of MDR1 (multidrug resistance) gene expression in human tumors by polymerase chain reaction. *Proc Natl Acad Sci USA* 1990; **87:** 7160–4.
22. Chabner BA, Collins JM. Plant alkaloids. In: *Cancer chemotherapy: principles and practice* Philadelphia: Lippincott 1992; 253–69.
23. Kaufmann SH. Antagonsim between camptothecin and topoisomerase II-directed chemotherapeutic agents in a human leukemia cell line. *Cancer Res* 1991; **51:** 1129–36.
24. Houghton JA, Cheshire PJ, Hallman JD, *et al.* Evaluation of irinotecan in combination with 5-fluorouracil or etoposide in xenograft models of colon adenocarcinoma and rhabdomyosarcoma. *Clin Cancer Res* 1996; **2:** 107–18.
25. Karato A, Sasaki Y, Shinkai T, *et al.* Phase I study of CPT-11 and etoposide in patients with refractory solid tumors. *J Clin Oncol* 1993; **11:** 2030–5.

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