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ORIGINAL ARTICLE

Hamid R. Bahadori · Mark R. Green
Carlo V. Catapano

Synergistic interaction between topotecan and microtubule-interfering agents

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Abstract *Purpose*: Topotecan is a topoisomerase I inhibitor with demonstrated anticancer activity in preclinical and clinical studies. The purpose of the present study was to evaluate drug-drug interactions in therapeutic regimens that would combine topotecan with microtubule-interfering agents, such as Taxol and vinblastine. *Methods*: The cytotoxic activities of various drug combinations and schedules of administration were measured in a colon cancer cell line using the MTT assay. Western blot and flow cytometry were performed to determine the effects of Taxol and vinblastine on topoisomerase I and Bcl-x_L protein levels and cell cycle distribution. *Results*: Brief incubation of colon cancer cells with low concentrations of either Taxol or vinblastine increased the efficacy of a subsequent treatment with topotecan. Preincubation of cells with vinblastine or Taxol reduced by 10- to 40-fold the concentration of topotecan necessary to induce a 50% decrease in cell survival. The effects were maximal when the cells were treated for 5 h with microtubule-interfering agents and then incubated for 19 h in drug-free medium before the addition of topotecan. Under these conditions, both Taxol and vinblastine caused an increase in topoisomerase I protein levels, fraction of S phase cells, and extent of Bcl-x_L phosphorylation immediately prior to the addition of topotecan. All these factors may contribute to the increased efficacy of topotecan observed with sequential therapy. *Conclusion*: Combinations of topotecan and microtubule-interfering agents result in synergistic anticancer activity when the drugs are administered sequentially. The promising preclinical data presented here encourage clinical testing of these drug combinations using a sequential schedule of administration.

Keywords Topotecan · Taxol · Vinblastine · Topoisomerase I · Bcl-x_L · Colon cancer · Cell cycle

Abbreviations *CPT:* camptothecin · *IC_{50}:* concentration that inhibits cell survival 50% · *MIA:* microtubule interfering agent · *Topo I:* DNA topoisomerase I · *TPT:* topotecan · *TX:* Taxol · *VBL:* vinblastine · *VCR:* vincristine

Introduction

CPT and its derivatives constitute a relatively new class of anticancer agents with a unique mechanism of action [27]. The intracellular target of these compounds is the nuclear enzyme topo I [42]. This 100,000 kDa monomeric protein modulates the topological structure of DNA and is involved in multiple functions, such as replication, transcription and recombination. The reaction catalyzed by topo I involves the formation of single-strand DNA breaks with the enzyme covalently linked to the 3′ termini of the broken DNA strands [35]. This is followed by passage of the complementary strands through the transient breaks, resealing of the broken DNA and dissociation of the topo I-DNA complexes. CPTs bind and stabilize the intermediate topo I-DNA complexes [14, 25]. Formation of CPT-topo I-DNA ternary complexes is necessary but not sufficient to induce cytotoxicity [10]. Cell killing by CPTs has been shown to be a multistep process, in which DNA replication plays an essential role. Collisions of advancing DNA replication forks with CPT-topo I-DNA ternary complexes result in arrest of DNA replication and conversion of the transient single-strand breaks into permanent double-strand breaks located at the forks [8, 13, 15, 34, 36, 39]. Accumulation of these permanent DNA breaks leads to cell death by apoptosis [10, 17].

H.R. Bahadori · M.R. Green · C.V. Catapano (✉)
Department of Medicine,
Division of Hematology/Oncology and Hollings Cancer Center,
Medical University of South Carolina,
86 Jonathan Lucas Street, Charleston,
South Carolina, 29425, USA
E-mail: catapanc@musc.edu
Tel.: +1-843-7926648
Fax: +1-843-7923200

According to this model, many factors, including topo I level, fraction of S phase cells, and susceptibility to the induction of apoptosis, are important determinants of cellular responses to CPTs [19, 24].

Preclinical and clinical studies have shown that CPTs are active in several tumor types, including colon, ovarian, lung, breast and pancreatic cancers [27, 37]. Two water-soluble CPT derivatives, TPT and irinotecan, have received extensive clinical evaluation and have been approved in the U.S. for treatment of ovarian and colon cancer, respectively [37]. In recent years, much effort has been devoted to developing combinations of topo I inhibitors with other anticancer drugs that have nonoverlapping mechanisms of action [9]. Encouraging results have been obtained with cisplatin, topo II-directed drugs, classic alkylating agents, platinum, and certain antimetabolites in combination with topo I inhibitors [2, 9]. Variable results, however, have been reported for combinations of topo I inhibitors with MIAs. TPT and TX are synergistic in a teratocarcinoma cell line [7] and in primary cultures of tumor cells from patients with cancers of various origins [16]. In contrast, combinations of topo I inhibitors with either TX or VCR are antagonistic in human lung cancer [18] and malignant glioma cell lines [5, 31]. In these studies cells were generally exposed to the two drugs simultaneously and continuously. In experiments with a human malignant glioma cell line, Schmidt et al. [31] noted a moderate additive effect when TPT followed VCR in a sequential schedule of treatment. Also, in the only in vivo preclinical study of a combination of MIAs and topo I inhibitors, sequential administration of VCR and TPT resulted in synergistic activity in 9 of 14 tumor xenografts [38]. The combination was antagonistic only in two tumor xenografts, while no interaction was observed in a VCR-resistant subline. These results suggest that the interaction between topo I inhibitors and MIAs might be cell-type specific and depend on the schedule of administration of the drugs.

In the present study, we evaluated the effects of combining TPT with either TX or VBL on the growth of a colon cancer cell line. We found that pretreatment of the cells with MIAs greatly reduced the concentration of TPT required for inhibition of cell growth. The effects of TX and VBL on the cellular response to TPT were associated with changes in topo I protein level, cell cycle distribution, and phosphorylation of Bcl-x_L following incubation of colon cancer cells with MIAs.

Materials and methods

Cell culture conditions and drug incubation

SW 480 colon cancer cells were obtained from American Type Culture Collection (ATCC, Manassas, Va.) and grown as a monolayer in McCoy's 5A medium supplemented with 10% fetal bovine serum. At the start of each experiment, cells were harvested by trypsinization, counted and diluted to the required cell density. After 24 h, cells were treated with the indicated drugs. TPT and TX were obtained from SmithKline Beecham (King of Prussia, Pa.) and Bristol Myers Squibb (Princeton, N.J.), respectively. VBL was purchased from Sigma (St Louis, Mo.). Stock solutions were prepared by dissolving the drugs in DMSO at a concentration of 10 m*M* and were stored at –20°C. For each experiment, an aliquot of drug solution was thawed, diluted in medium to the desired concentrations and used immediately after preparation.

MTT cytotoxicity assay

Cells were diluted to 2.5×10^4 cells/ml and plated (100 µl/well) into 96-well plates. After 24 h, the medium was removed and substituted with freshly prepared medium containing the appropriate drugs. Unless otherwise indicated, cells were incubated with TX and VBL for 5 h. At the end of this incubation, the medium was removed and cells were washed twice with medium and incubated in the absence of drug for 19 h. Some samples were then left untreated and the remaining samples were treated with various concentrations of TPT for 72 h. The number of viable cells was determined at the end of the 96-h incubation by the MTT assay as described previously [6]. Three replicates were made for each treatment group, and each experiment was repeated at least twice.

Western blot analysis

Cells were plated in T25 flasks at a density of 2×10^5 cells/ml. After 24 h, cells were treated for 5 h with either TX or VBL. At the end of the drug incubation, the medium was removed and substituted with drug-free medium. Cells were then harvested 19 h later. A separate set of cells was treated with TX or VBL continuously for 24 h. Cells were washed twice with PBS and lysed in a buffer containing 10 m*M* Tris-HCl, pH 6.8, 2 m*M* EDTA, 2% SDS and protease inhibitor cocktail (Sigma). Cell lysates were sonicated with two 10-s pulses using a Virsonic sonicator at 6% output power (28.5 W). Following centrifugation at 10,000 *g* for 15 min, supernatants were collected, heated at 95°C for 5 min and either used immediately for Western blotting or stored at –70°C until analysis (usually 2 to 5 days later). Preliminary experiments showed that short-term storage of the samples at –70°C did not affect detection of the proteins under analysis and the results obtained from fresh and frozen samples were identical.

Protein concentrations in cell lysates were determined using the BCA assay (Pierce, Rockford, Ill.). Aliquots of either 40 or 80 µg of protein were mixed with loading buffer and separated on 8% and 12% SDS-polyacrylamide gels. Proteins were transferred to nitrocellulose membranes. The blots were incubated overnight at 4°C in 10 m*M* Tris, pH 7.5, 144 m*M* NaCl, 0.05% Tween 20, and 5% dried milk and then in the same buffer containing primary antibodies. Blots were developed using horseradish peroxidase-conjugated secondary antibodies and an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Piscataway, N.J.). The monoclonal antibody against human Topo I (C-21) was a gift from Dr. Y.-C. Cheng. Antibodies against β-actin (I-19), Bcl-2 (N-19) and Bcl-x_L (S-18) were purchased from Santa Cruz Biotechnology (Santa Cruz, Calif.). The antibody against lamin B (Ab-1) was purchased from Oncogene Research Products (Boston, Mass.). To examine phosphorylation of Bcl-x_L, the phosphatase inhibitors vanadate and sodium fluoride were added to the lysis buffer at concentrations of 1 and 25 m*M*, respectively. Samples were then processed as described above.

Cell cycle distribution

Cells were plated at a density of 2×10^5 cells/ml in six-well plates, incubated at 37°C for 24 h, and then treated with TX or VBL for 5 h. Cell cycle analysis was performed immediately after drug incubation or 24 h and 48 h following drug removal. At each timepoint, cells were harvested by trypsinization, washed twice with PBS and fixed in 70% ethanol. Samples were then stored at 4°C

until analysis. Cells were recovered by centrifugation, stained with propidium iodide and analyzed by flow cytometry as previously described [22].

Data analysis

Cell survival data were analyzed using Student's *t*-test to evaluate the significance of any difference observed between cells treated with the individual or combined drugs. Drug interactions were also analyzed according to the isobole model [3]. Drug concentrations were expressed as fraction of IC_{50} of the individual drugs ($1/IC_{50}$) and then equitoxic doses of the individual and combined drugs were plotted to generate isobolograms. Ratios between the survival observed with the combined drugs and the survival expected for the same drug doses in the case of additive interaction were also calculated. For an additive interaction the effect on cell survival would be equal to the product of the effect of each individual drug [40]. Ratios of observed/expected survival equal to or below 0.8 would indicate synergistic interaction, whereas ratios equal to or above 1.2 would indicate subadditive or antagonistic interaction [16, 40].

Results

Figure 1 shows the effects of TPT and TX, either alone or in combination, on the survival of SW480 cells. When used as single agents, TPT and TX at a concentration of 1 μ*M* decreased the number of viable cells by about 50% and 40%, respectively, compared to untreated control cells. Incubation with concentrations of TPT and TX lower than 1 μ*M* had only minimal effects on cell survival. When cells were incubated with TX for 5 h and then, following a 19-h growth in drug-free medium, treated with TPT, a significant increase in the cytotoxic activity of the combination compared to either drug alone was observed. For example, a 50% reduction in cell survival was seen when cells were pretreated with 25 n*M* TX and then treated with 50 n*M* TPT, although at these concentrations neither TX nor TPT alone affected cell survival.

As shown in Table 1, the concentration of TPT necessary to inhibit cell survival by 50% was decreased approximately 20- and 40-fold when cells were pretreated with 25 and 100 n*M* TX, respectively, compared to cells treated with TPT alone. Thus, preincubation with low, noncytotoxic concentrations of TX sensitized colon cancer cells to the cytotoxic activity of TPT. Analysis of the data with the isobole method confirmed that the combination of TX and TPT with the sequential schedule of treatment was synergistic. As shown in Fig. 1, the plot of the concentrations that reduced cell survival by 50% was concave in shape to the left of the zero isobole curve. When analyzed according to an additive model, the combination of TX and TPT was also found to be synergistic. The ratios between the observed survival and the survival expected for additive interactions were equal to or less than 0.8 for all the doses tested, with most of the values under 0.6.

Further studies were done to determine whether the incubation in drug-free medium following TX treatment was important. We did not observe a synergistic interaction when TPT was added to the cells immediately following the 5-h incubation with TX (data not shown). On the other hand, the incubation time in drug-free medium following TX treatment could be extended from 24 h up to 48 h without losing the positive effect of the pretreatment with TX (Fig. 2). Taken together, these results suggest that a change induced by TX and relevant

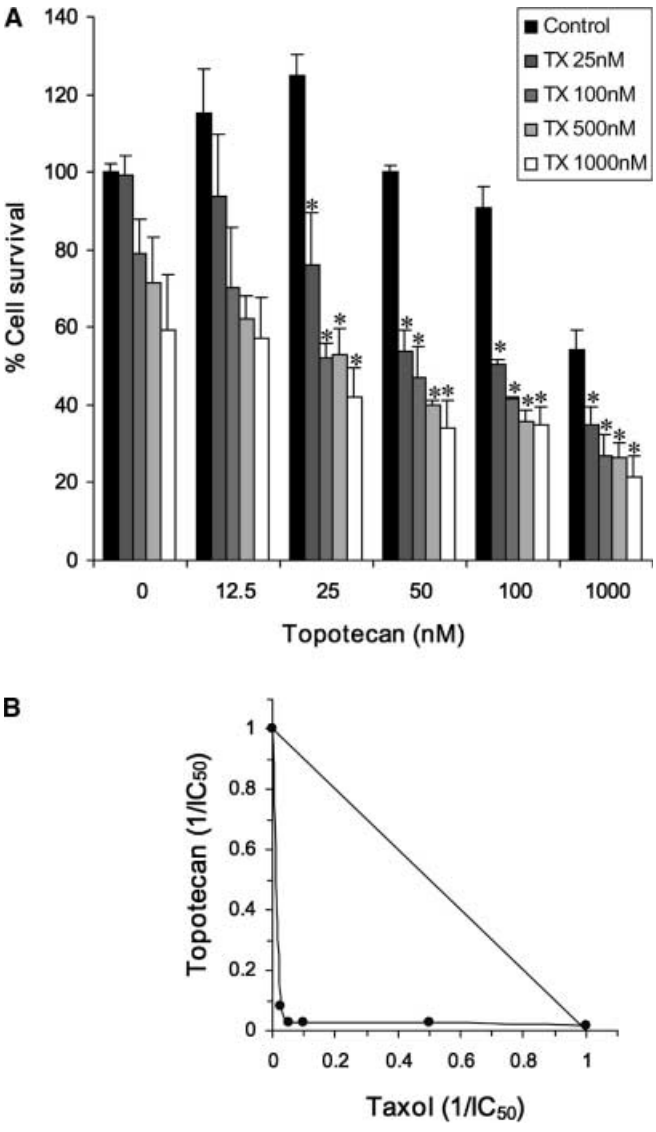

Fig. 1A, B Modulation of the cytotoxic activity of topotecan by Taxol. **A** Cells were incubated with Taxol for 5 h, washed and incubated in drug-free medium for 19 h. Topotecan was added and the cells were incubated for an additional 72 h (total 96 h). Cell viability was determined by the MTT assay as described in Materials and methods. The results are expressed as means ± SD of the percent cell survival compared to untreated control cells. Triplicate samples were analyzed in each experiment, and the experiment was repeated three times with identical results (*$P \leq 0.05$ vs survival of cells treated with either drug alone). **B** Isobologram of equitoxic doses of Taxol and topotecan alone or in combination. Drug doses are expressed as fraction of concentrations of the individual drugs required to inhibit survival by 50%. The diagonal line represents the zero interaction isobole. The concave curve to the left of the zero isobole indicates a synergistic interaction between the two drugs

to the sensitivity of cells to TPT probably occurred during post-TX incubation and persisted for at least 48 h.

We also measured survival of cells that were incubated with TX for 24 h and then treated with TPT immediately after removal of TX, or were exposed simultaneously to TX and TPT for 24 h. Survival under these treatment conditions was generally not different from survival of cells exposed to identical doses of TX alone (data not shown). Simultaneous treatment with TX and TPT resulted in an antagonistic interaction with survival ratios for all the doses tested above 1.2. A moderate additive effect of the combined treatment was seen only at the highest concentration of TPT in cells pretreated with TX for 24 h. The lack of synergy in these experiments further suggested that the gap between TX and TPT treatment was important. These results are also in agreement with previously published data that show that simultaneous incubation with MIAs and topo I inhibitors has no advantage compared to either drug alone [5, 18, 31].

We investigated whether another MIA had effects similar to those of TX on the sensitivity of SW480 cells to TPT. As shown in Fig. 3, incubation with VBL sensitized the cells to subsequent treatment with TPT. The concentration of TPT required to reduce cell survival by 50% was decreased about 10- and 20-fold when cells were pretreated with 25 and 50 n*M* VBL, respectively (Table 1). VBL alone at these concentrations had only

Table 1 Changes in IC_{50} of topotecan in cells pretreated with Taxol or vinblastine

Drug	Topotecan IC_{50} (n*M*)[a]	Fold decrease
No drug	1000	1
Taxol 25 n*M*	50	20
Taxol 100 n*M*	25	40
Vinblastine 25 n*M*	100	10
Vinblastine 50 n*M*	50	20

[a]Concentrations of topotecan that reduced cell survival by 50% were calculated from the data presented in Figs. 1 and 3

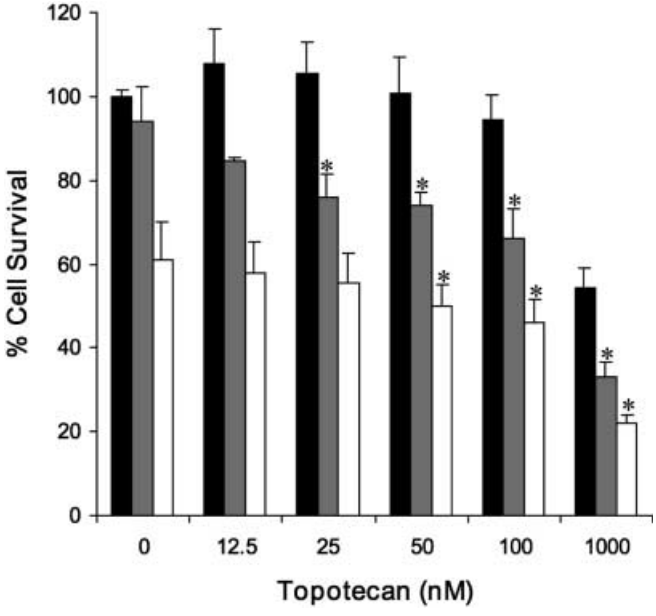

Fig. 2 Effects of Taxol pretreatment and incubation in drug-free medium on the cytotoxic activity of topotecan. Cells were treated with Taxol for 5 h, incubated in drug-free medium for 48 h and then treated with topotecan. Cell survival was determined after 96 h by the MTT assay and the results are presented as described in the legend to Fig. 1. Taxol concentrations were 0 (*black bars*), 100 n*M* (*gray bars*), and 500 n*M* (*white bars*). (* $P \leq 0.05$ vs survival of cells treated with either drug alone)

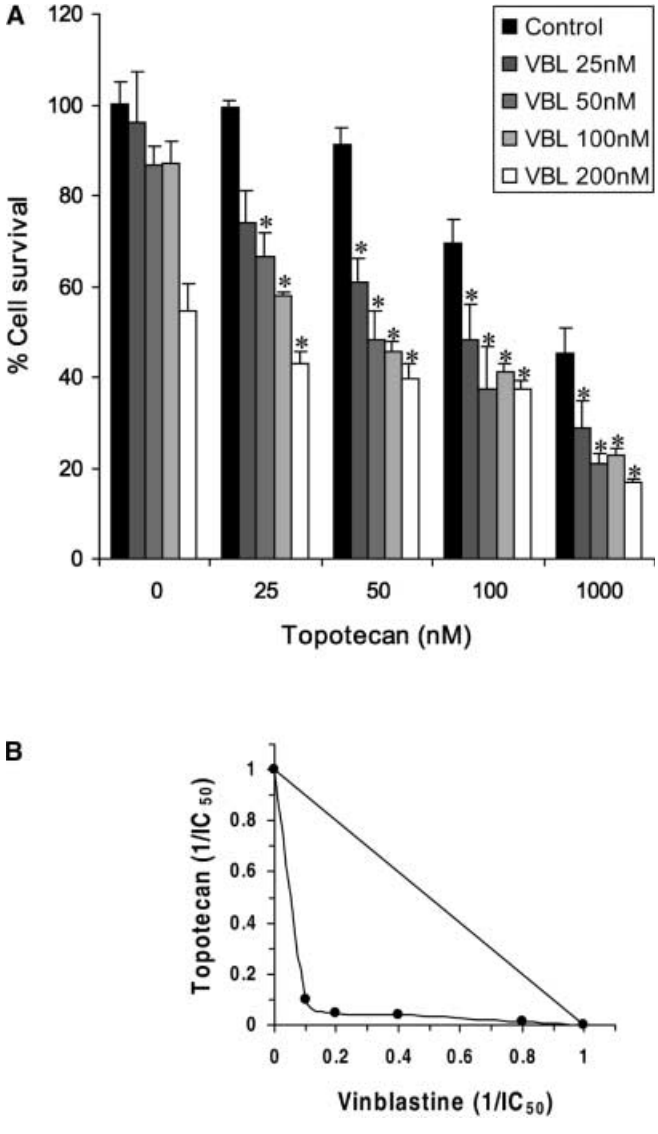

Fig. 3A, B Modulation of the cytotoxic activity of topotecan by vinblastine. **A** Cells were incubated with vinblastine for 5 h, washed and incubated in drug-free medium for 19 h. Cells were then treated with topotecan for 72 h. Cell viability was determined by the MTT assay and results are presented as described in the legend to Fig. 1 (*$P \leq 0.05$ vs survival of cells treated with either drug alone). **B** Isobologram of equitoxic doses of vinblastine and topotecan alone or in combination. The plot with a concave curve to the left of the zero isobole indicates a synergistic interaction between the two drugs

minimal effects on cell survival. A plot of the doses of VBL and TPT that produced 50% inhibition of cell survival gave a concave curve similar to that from the TX and TPT combination, confirming that the interaction was synergistic. In addition, the ratios of observed/expected survival were all equal to or less than 0.8. As in the case of TX, simultaneous treatment with VBL and TPT or treatment with TPT immediately following a 24-h incubation with VBL did not result in a synergistic interaction (data not shown). These results, therefore, indicate that two structurally and mechanistically different MIAs had similar effects on the sensitivity of colon cancer cells to TPT.

Further studies were carried out to identify factor(s) that could mediate MIA-induced cell sensitization to TPT. A major determinant of response to CPTs is the level of topo I [19, 24]. We speculated that TX and VBL might sensitize cells to a subsequent treatment with TPT by increasing the topo I level. In the presence of a higher level of topo I, a greater number of topo I-DNA complexes could be trapped by TPT, thus resulting in increased cytotoxicity. Therefore, we measured topo I protein level by Western blotting in cells either untreated or treated for 5 h with TX or VBL and then incubated in drug-free medium for 19 h. A second set of samples was either left untreated or treated with TX and VBL continuously for 24 h. As shown in Fig. 4, topo I was increased about twofold in cells incubated with TX or VBL for 5 h and then left untreated for 19 h. In contrast, no changes in topo I level were observed in cells treated with TX or VBL continuously for 24 h compared to untreated cells. Topo I protein level was also slightly increased (50–75% higher than in untreated control cells) when cells were harvested 43 h after drug treatment (data not shown). It is worth noting that only full-length topo I and lamin B were detected in the immunoblots from both untreated and drug-treated cells, suggesting that the brief treatment with low concentrations of TX and VBL did not affect the integrity of these proteins. This was consistent with the absence of morphological changes associated with apoptosis in drug-treated cells examined at the time of harvesting for Western blot analysis.

Another parameter that is known to affect the sensitivity of cancer cells to CPTs is the number of cells in the S phase of the cell cycle at the time of drug treatment [19]. Collisions between CPT-topo I-DNA complexes and DNA replication forks in S phase cells are believed to be critical events leading to the conversion of reversible cleavable complexes into lethal damage [25]. Cell cycle effects of MIAs, such as TX and VBL, have been widely described. These agents are known to arrest cells predominantly in the G_2-M phase. Limited information, however, is available on the effects of transient exposure to relatively low concentrations of these agents on cell cycle distribution. The results obtained in SW480 cells are summarized in Table 2. At the end of the 5-h incubation with TX or VBL, cells accumulated, as expected, in G_2-M. By 19 h after drug washout, however, the number of cells in G_2-M was drastically reduced and increased numbers of TX- and VBL-treated cells were found in S phase compared to untreated cells. The number of S phase cells was also slightly higher (5–7%) in TX- and VBL-treated samples compared to control samples at 43 h after completion of drug treatment (data not shown).

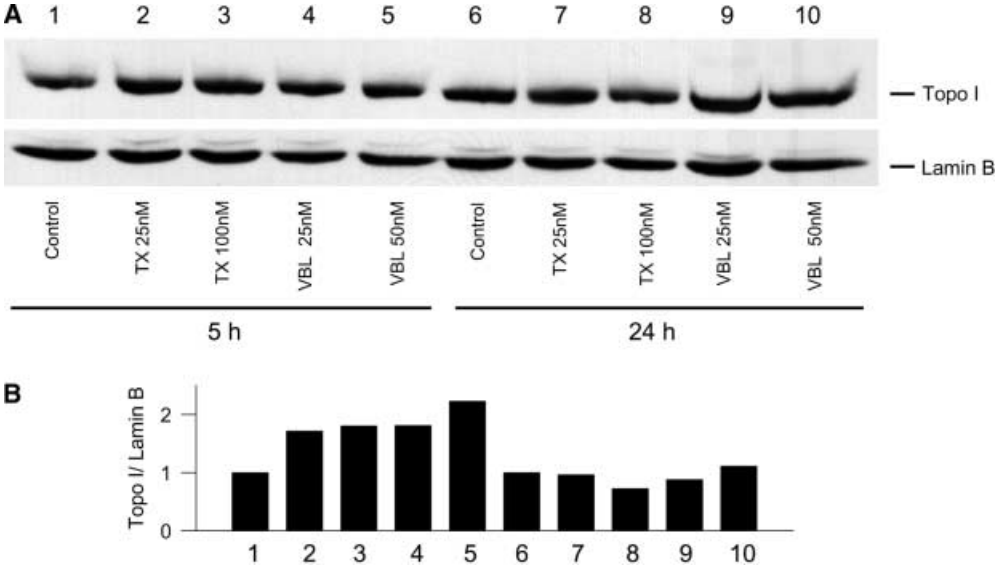

Fig. 4A, B Changes in topo I protein level in cells treated with Taxol and vinblastine. **A** Immunoblot analysis of topo I and lamin B (*lanes 1 to 5* cells were left untreated or treated with the indicated drugs for 5 h and then grown in drug-free medium for 19 h; *lanes 6 to 10* cells were left untreated or treated with TX or VBL for 24 h). Proteins were separated on 8% polyacrylamide gels and transferred to nitrocellulose membranes as described in Materials and methods. The membranes were incubated with monoclonal antibodies against topo I and lamin B. **B** Quantification of topo I levels by densitometric analysis using the Gel Pro Analyzer software (Media Cybernetics, Silver Spring, Md.) and normalized to lamin B levels (*samples 1 to 10* are as indicated in **A**). The experiment was repeated three times with similar results. The SD from the mean of the three experiments was less than 10%

Table 2 Effects of TX and VBL on cell cycle distribution. Cells were incubated with TX and VBL for 5 h and harvested either immediately after drug treatment (5-h time-point) or after a 19-h incubation in drug-free medium (24-h time-point). Cell cycle distribution of fixed and propidium iodide-stained samples was determined by flow cytometry as described in Materials and methods

Drug	Time-point (h)	Drug concentration (n*M*)	G_1	G_2-M	S
TX	5	0 (control)	52.0 ± 0.7	16.6 ± 0.1	31.4 ± 0.8
		100	20.9 ± 0.1	57.1 ± 1.6	22.2 ± 1.6
		500	17.3 ± 0.1	62.1 ± 1.3	20.7 ± 1.2
	24	0 (control)	50.7 ± 0.7	14.7 ± 2.2	34.6 ± 2.8
		100	31.4 ± 1.3	27.3 ± 1.9	41.3 ± 3.3
		500	25.4 ± 0.2	39.8 ± 0.5	34.8 ± 0.2
VBL	5	0 (control)	52.5 ± 0.4	16.4 ± 1.6	31.3 ± 1.8
		25	17.6 ± 1.6	52.8 ± 1.9	29.8 ± 3.5
		100	21.6 ± 2.0	47.3 ± 2.0	31.1 ± 4.0
	24	0 (control)	52.9 ± 1.1	13.3 ± 0.6	33.9 ± 0.5
		25	32.8 ± 1.3	16.0 ± 0.7	51.2 ± 2.0
		100	32.6 ± 1.9	21.2 ± 3.3	46.3 ± 1.4

Apoptosis antagonists, such as Bcl-2, decrease the sensitivity of cancer cells to a variety of anticancer drugs, including CPTs [1, 41]. Bcl-2 is undetectable in SW480 cells (data not shown). However, these cells express high levels of another Bcl family member, Bcl-x_L. Like Bcl-2, Bcl-x_L has antiapoptotic activity and can protect cells from CPT-induced cell death [23, 32]. Moreover, MIAs have been shown to decrease Bcl-x_L expression in prostate cancer cells [21] and induce its phosphorylation in cancer cell lines of diverse origin [26]. It has been proposed that both these events could play a role in MIA-induced apoptotic cell death [21, 26]. We hypothesize that similar changes in Bcl-x_L might occur in SW480 colon cancer cells following TX and VBL treatment and facilitate the induction of apoptosis by a subsequent treatment with TPT. Therefore, we examined whether TX or VBL altered Bcl-x_L protein level or its phosphorylation state in these cells. Cells were either treated with drugs for 5 h and incubated for an additional 19 h in drug-free medium, or treated continuously for 24 h. As shown in Fig. 5, Bcl-x_L was detected in immunoblots of SW480 cell lysates as two major bands, with the slowest migrating form corresponding to phosphorylated Bcl-x_L as described previously [26]. The total amount of Bcl-x_L protein in both groups of drug-treated cells was not significantly different from the level in untreated cells. However, phosphorylation of Bcl-x_L was enhanced in TX- and VBL-treated cells compared to untreated cells, as indicated by the increased intensity of the slow-migrating band. The change in phosphorylation was more pronounced in cells treated with drugs for 24 h than in cells treated for 5 h only, and in VBL-treated cells compared to TX-treated cells.

Discussion

CPT derivatives, such as TPT and irinotecan, have demonstrated activity in preclinical and clinical studies against a variety of cancers and are currently included in an increasing number of chemotherapy regimens [37]. Therefore, it is particularly important to understand how to optimally combine these compounds with other anticancer drugs in order to improve the efficacy of treatment. In the present study, we examined the combination of TPT and MIAs. Our results indicate that pretreatment of SW480 colon cancer cells with either TX or VBL, even at low, noncytotoxic concentrations, increased the sensitivity of the cells to TPT. Combinations of CPTs and MIAs have been tested previously in a limited number of preclinical studies and have been frequently found to be nonsynergistic or even antagonistic [5, 18, 31]. In most studies, however, the drugs were administered simultaneously and continuously. A sequential schedule of treatment was used in two instances only [31, 38]. In agreement with our results, additive or synergistic effects were observed in both these cases. Our studies confirmed that simultaneous treatment with TPT and MIAs did not result in synergistic activity. Furthermore, we observed that, in addition to sequential administration, a period of approximately 24 h, during which cells were incubated in drug-free medium after TX and before TPT treatment, was required in order to obtain maximal synergistic effect.

We investigated the mechanisms underlying the positive interaction between TPT and MIAs. Our hypothesis was that pretreatment with MIAs could sensitize cells to subsequent treatment with TPT by acting at multiple steps along the pathway that leads to TPT-induced cell death. We found that cells treated for 5 h with TX or VBL followed by incubation in drug-free medium for approximately 24 h exhibited higher levels of topo I. The increase in topo I levels could have been due to an effect of MIAs on the stability of topo I protein or RNA. Alternatively, MIAs could affect transcription of the topo I gene. Indeed, TX and other MIAs have been shown to activate transcription factors and induce expression of a variety of genes by a yet-undefined mechanism [4].

We observed also that a greater number of cells were in the S phase of the cell cycle 24 h after the 5-h treatment with TX or VBL compared to untreated control cells. Apparently, cells that were initially arrested in G_2-M progressed through the cell cycle following removal of the drugs and were found in S phase 24 h later. The increase in S phase cells was still present, although attenuated, at 48 h after TX and VBL treatment. A

recent study of the cell cycle effects of short-term TX treatments in ovarian cancer cells reached a similar conclusion. At low concentrations of TX, the G_2-M block was reversible and an increase in cells in S phase was detected after 24–48 h [33]. Independently of the specific mechanisms, it is reasonable to assume that the combination of these two factors could significantly increase the sensitivity of cells to TPT. Cells with a higher topo I content would be expected to be more sensitive to CPTs due to the possibility of forming an increased number of cytotoxic CPT-topo I-DNA complexes [19, 24]. Also, S phase cells would be more sensitive to CPTs because of the increased probability of transforming CPT-topo I-DNA complexes into permanent DNA damage [19].

Less clear is the role of the increased Bcl-x_L phosphorylation that we observed in cells treated with TX and VBL. The level of the apoptosis antagonist Bcl-x_L is an important determinant of response to anticancer drugs, including CPTs [23, 32], and TX has been previously shown to affect Bcl-x_L levels [21]. However, MIAs did not affect Bcl-x_L protein levels in our experiments with colon cancer cells. Phosphorylation has been proposed as an alternative way to decrease antiapoptotic activity of Bcl-x_L and, perhaps, increase sensitivity to anticancer drugs [26]. In our experiments, we saw a modest increase in the levels of phosphorylated Bcl-x_L following a 5-h treatment with TX and VBL. It is possible that this increase could contribute to the enhanced cytotoxic activity of TPT. The amount of phosphorylated Bcl-x_L was even greater in cells treated with MIAs for 24 h continuously than in cells incubated for only 5 h. However, the 24-h incubation with MIAs followed immediately by TPT did not result in increased sensitivity to TPT cytotoxicity. An additive/synergistic interaction was observed only with the highest concentration of TPT (1 μM) using this treatment schedule. It is likely that the increased phosphorylation of Bcl-x_L, like phosphorylation of Bcl-2 [11, 20, 30], coincides with accumulation of cells continuously exposed to MIAs in G_2-M. If this were the case, cells treated continuously with MIAs and arrested in G_2-M would be prevented from progressing through the cell cycle and entering the S phase. Thus, G_2-M arrest and reduced number of S phase cells would counteract the potentially positive effect of Bcl-x_L phosphorylation on the cytotoxic activity of TPT.

The results presented here have direct relevance to the design of new chemotherapy regimens that include both MIAs and CPTs. Results of previous preclinical studies of similar drug combinations suggest that sensitization of cells to topo I inhibitors by MIAs is not a phenomenon unique to the colon cancer cell line used in the present study [7, 16, 31, 38]. Also, the effects of MIAs on cell cycle, topo I expression and bcl-XL phosphorylation that we think are mechanistically associated with this sensitization have been observed by others in various cancer cell lines [4, 12, 26, 33]. An increase in topo I protein level has also been observed in peripheral blood lymphocytes from patients with solid tumors 24 h after a 3-h infusion of TX [28]. It is also important to note that the treatment conditions that were used in our study closely resembled the common schedules of administration of these drugs. TPT is frequently administered intravenously as a continuous infusion over 3 to 7 days or, more recently, orally in a multiday schedule [37]. VBL

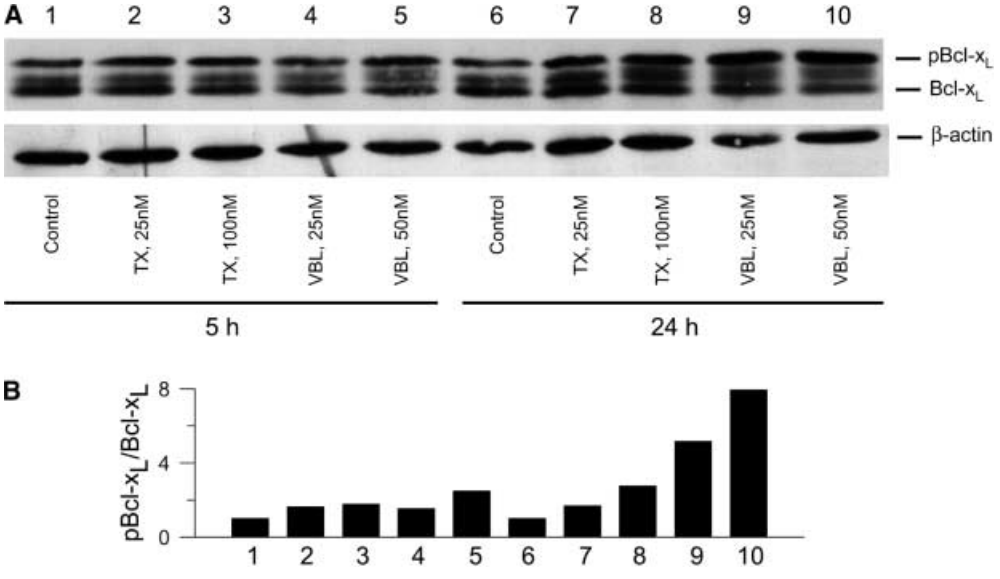

Fig. 5A, B Effects of Taxol and vinblastine on Bcl-x_L protein levels and phosphorylation. **A** Immunoblot analysis of Bcl-x_L and β-actin. Cells were incubated with the indicated drugs as described in the legend to Fig. 4. Cell lysates were prepared in the presence of phosphatase inhibitors. Proteins were separated on a 12% polyacrylamide gel and transferred to a nitrocellulose membrane, which was incubated with antibodies for Bcl-x_L and β-actin. **B** Quantification of the relative amounts of Bcl-x_L and phosphorylated Bcl-x_L (pBcl-x_L). The intensities of the bands corresponding to Bcl-x_L and pBcl-x_L were determined by densitometric analysis using the Gel Pro Analyzer software. The Bcl-x_L/pBcl-x_L ratio in drug-treated cells was normalized to the ratio in untreated control cells (*samples 1 to 10* are as indicated in **A**)

and TX are frequently given intravenously as brief infusions. Therefore, the treatment schedule suggested by our results – i.e. a short infusion of a MIA followed approximately 24 h later by prolonged treatment with TPT or other CPTs – could be easily incorporated into clinical regimens. Indeed, phase I clinical studies with a design similar to that proposed here have been carried out at our institution to test the feasibility of this approach [28, 29]. Additional studies based on these observations are planned.

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