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Camptothecin Induction of a Time- and Concentration-Dependent Decrease of Topoisomerase I and Its Implication in Camptothecin Activity

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SUMMARY

Camptothecin (CPT) has been shown to induce protein-linked DNA breaks (PLDB), which are stabilized intermediates of topoisomerase I (TOP1) activity. Due to the reversible nature of PLDB and the need for replication fork movement for CPT toxicity, both the time of CPT exposure and TOP1 levels are determinants of CPT toxicity. Therefore, the effects of CPT exposure on TOP1 over time were examined in an established human cell line, KB. Using an *in vivo* KCl-SDS co-precipitation assay, it was determined that 1 hr of CPT exposure resulted in a concentration-dependent increase in PLDB that reached a maximum at 5 μM CPT. However, prolonged incubations with CPT revealed a concentration- and time-dependent decrease in CPT-induced PLDB formation. The most rapid loss of PLDB was within 6 hr. Neither aphidicolin nor cycloheximide cotreatments altered the PLDB decrease induced by CPT. Immunoblot analysis revealed a reduction in TOP1 protein upon CPT exposure, whereas RNA analysis revealed no changes, which suggested a post-transcriptional mechanism of TOP1 down-regulation. The CPT-induced reduction was specific for TOP1, because actin and tubulin levels were unaltered by CPT exposure. Finally, clonogenic assays revealed a small but statistically significant decrease in CPT toxicity throughout the CPT exposure period. Because PLDB formation based on TOP1 levels is an important step in the toxicity of CPT, the CPT-induced TOP1 reduction could be a transient mechanism of resistance for cells to avoid toxic levels of PLDB.

The plant alkaloid CPT and its more water-soluble analogs are promising compounds for the treatment of cancer (1, 2). Exposure to CPT has been shown to inhibit DNA and RNA synthesis and to induce reversible fragmentation of DNA (3–7). In cells, the action of CPT was found to be mediated through the action of the nuclear DNA-unwinding enzyme TOP1 (8). TOP1 activity has been associated with several DNA topological events, such as replication, transcription, and recombination (for review, see Refs. 9–11). CPT has been shown not to bind DNA or TOP1 alone, but when CPT was added to a TOP1 and DNA mixture nicks were introduced into the DNA (8, 12). CPT stabilizes the reaction intermediate that has TOP1 covalently bound to the 3′ terminus of the DNA phosphate backbone (8, 13, 14). It is the stabilization of the single-stranded DNA reaction intermediate, termed the cleavable complex, that results in higher steady state levels of TOP1-DNA adducts, called PLDB.

The PLDB produced by CPT were shown to be rapidly reversible upon CPT removal (8, 13); within 15 min PLDB levels had almost completely reverted to background levels. Using synchronized cell lines, it was shown that cells in S phase were at least 100-fold more sensitive to the killing effects of CPT (15); however, the PLDB levels induced by CPT were equal in all phases of the cycle (16), and TOP1 levels were shown to remain constant throughout the cell cycle (17). Also, APH, a DNA polymerase inhibitor, was shown to protect against CPT toxicity while not affecting PLDB levels (18). Therefore, whereas PLDB formation was necessary for CPT toxicity, additional events were needed. From these observations, a replication fork collision model for CPT toxicity was proposed (14, 19, 20), in which a moving replication fork collides with the reversible PLDB to produce a dsDNA break, which transforms the rapidly reversible PLDB into a more permanent dsDNA break. Studies showed the production of dsDNA breaks near sites of active replication, the production of the dsDNA breaks required replication fork movement, and the dsDNA breaks were not rapidly reversible but persisted within the cell (21).

Two important determinants of CPT toxicity were the levels of TOP1 and the duration of CPT exposure. The greater the amount of TOP1, the greater the number of potentially lethal lesions (PLDB) that would be produced. In certain cell

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ABBREVIATIONS: CPT, camptothecin; TOP1, topoisomerase I; PLDB, protein-linked DNA break(s); APH, aphidicolin; dsDNA, double-stranded DNA; CHX, cycloheximide; SDS, sodium dodecyl sulfate; kb, kilobase(s); TBST, Tris-buffered saline/Tween.

lines CPT toxicity correlated with levels of TOP1, although exceptions were numerous (18, 22–24). Also, cell lines selected for resistance to CPT have shown decreased TOP1 levels, compared with CPT-sensitive counterparts (25–27). Regarding the time of CPT exposure, because CPT toxicity has been shown to be S phase specific (5, 15) only a portion of a given cell population would be susceptible to the cell killing effects of CPT over a period of time (18). Because the production of PLDB by CPT was shown to be rapidly reversible upon CPT removal and other S phase-specific factors were necessary for toxicity, the reversible nature of the PLDB produced by CPT further emphasizes the importance of time in CPT toxicity.

Because both time and CPT levels are considered to be determinants of CPT toxicity, we wanted to characterize the cytotoxic actions of CPT over the duration of CPT exposure in a human cell line. The current models of CPT activity assume that TOP1 levels remain constant throughout the exposure to CPT, because TOP1 levels and CPT-induced PLDB have been shown to remain constant throughout the cell cycle (16, 17). If the levels of TOP1 change over the period of exposure to CPT, then the potential toxicity of CPT would also change over the period of CPT administration. Understanding the efficacy of CPT over time is crucial for designing effective dosage strategies with CPT either as a single agent or in combination with other drugs.

Experimental Procedures

Materials. APH, CHX, and CPT stock solutions were dissolved in dimethylsulfoxide and stored at −20°. Dimethylsulfoxide concentrations were consistently <0.1% and did not alter results. All other chemicals were of reagent grade or better.

Cell culture. The established human nasopharyngeal cell line KB was cultured in RPMI 1640 medium, supplemented with 5% fetal bovine serum and 1% kanamycin, in a humidified 5% CO_2 incubator at 37°. The doubling time for KB cells was 19 hr. Growth inhibition assays (constant exposure for three doublings) determined the IC_{50} value for KB cells to be 9.8 nM CPT.

Quantification of PLDB. For quantification of steady state PLDB levels, a modified *in vivo* KCl-SDS co-precipitation assay was developed (28). The DNA of mid-logarithmic phase cultures was labeled with [^{14}C]thymidine (0.1 μCi/ml) for 24–48 hr. Cells were harvested, counted, and plated onto six-well plates (1–2 × 10^5 cells/well) in [^{14}C]thymidine-free medium for 16 hr. After drug exposure, medium was aspirated and 1 ml of prewarmed (37°) lysis buffer (1.25% SDS, 5 mM EDTA, 0.4 mg/ml sheared salmon sperm DNA) was immediately added to each well. After it was transferred to a tube, the viscous lysate was sheared by being passed through a 22-gauge needle five times. After a 10-min incubation at 65°, 0.25 ml of 325 mM KCl was added, and the tubes were vortex-mixed and placed on ice for 10 min. The precipitates were pelleted (2000 × *g* for 10 min at 4°), the supernatants were aspirated, and 1 ml of a wash solution (10 mM Tris, pH 7.5, 100 mM KCl, 1 mM EDTA, 0.1 mg/ml sheared salmon sperm DNA) that had been prewarmed to 65° was added to the pellets, followed by vortex-mixing. The tubes were incubated at 65° for 10 min, with occasional vortex-mixing. The tubes were again placed on ice for 10 min, followed by pelleting of the white precipitate as before. A second round of washing and pelleting was performed as described above. The resulting pellets were resuspended in 1 ml of distilled water and transferred to scintillation vials. Ten milliliters of scintillation cocktail were added to the vials, and the total radioactivity (counts/minute) was determined by scintillation counting.

PLDB time course assays. The PLDB time course assay involved a preincubation period with a designated drug or drug combination, followed by a 30-min pulse with a high concentration (5 μM) of CPT to quantify the maximal amounts of PLDB capable of being produced by CPT at that time. After the 30-min CPT pulse, cultures were processed for PLDB quantification by the *in vivo* KCl-SDS co-precipitation assay indicated above.

Immunoblot analysis of TOP1. For immunoblotting experiments, cells were harvested, washed in phosphate-buffered saline, and counted. Cells were centrifuged, resuspended in 1× Laemmli loading buffer (63 mM Tris, pH 6.8, 2% SDS, 5% 2-mercaptoethanol, 10% glycerol), and boiled for 10 min. The resulting whole-cell lysates were loaded onto 7.5% SDS-polyacrylamide gels and run at 4-mA constant voltage for 16 hr, in 1× SDS-polyacrylamide gel electrophoresis running buffer. The resulting gels were sandwiched between two pieces of nitrocellulose paper in transfer buffer (50 mM NaCl, 10 mM Tris·HCl, pH 7.5, 2 mM EDTA, 0.5 mM 2-mercaptoethanol) for approximately 30 hr, to yield two Western blots. For TOP1 immunoblotting, filters were blocked with 5% dried milk in phosphate-buffered saline for 20 min. A 1/1000 dilution of an anti-TOP1 monoclonal antibody developed in our laboratory (29) was added to this solution, and the primary hybridization was conducted at 4° for 16 hr, with shaking. After three successive washings in 1× TBST (10 mM Tris·HCl, pH 7.5, 150 mM NaCl, 0.15% Tween 20), an anti-mouse IgM-peroxidase conjugate (1/1000 dilution) in 1× TBST was added for 1 hr at room temperature, with shaking. After three washes in 1× TBST, chemiluminescence was used to detect the peroxidase conjugate with exposure to X-ray film. Band intensities were quantified by densitometric scans of the resulting X-ray films.

TOP1 RNA analysis. Total RNA was isolated according to an acid guandinium isolation procedure (30). Size fractionation on agarose-formaldehyde gels and transfer to nylon membranes were performed according to established procedures (31). A TOP1 cDNA fragment (29) corresponding to the 3′-end 1.8 kb of the TOP1 cDNA was labeled with ^{32}P by using a random primer kit and was used to probe RNA blots.

Clonogenic time course assay. The clonogenic time course assay consisted of a 14–16-hr preincubation period with the indicated drug(s), followed by removal of drug and a wash in drug-free medium. After removal of the wash medium, cultures were incubated in drug-free medium for 30 min, after which a 4-hr exposure period was initiated by the addition of increasing concentrations of CPT. After the 4-hr CPT exposure period, cultures were washed once with drug-free medium, and then drug-free medium was added for an eight-generation incubation to allow for colony formation. After culturing, cells were fixed and stained with a 0.5% methylene blue solution in 50% ethanol. Visible colonies were counted to give cloning efficiencies for each treatment.

Results

PLDB levels. After a 1-hr exposure to increasing concentrations of CPT, steady state levels of PLDB increased in a concentration-dependent manner, followed by a plateau of PLDB levels (Fig. 1A). To confirm that the precipitated DNA was protein linked, cell lysates were digested with proteinase K before KCl precipitation, which abolished precipitated [^{14}C]thymidine counts (data not shown). Upon removal of CPT, PLDB levels have been shown to quickly reverse to background levels. To confirm the reversibility of PLDB (8, 13) after 1 hr of CPT incubation, CPT-free medium was added to cultures, followed by KCl-SDS co-precipitation at designated times. As shown in Fig. 1B, steady state PLDB levels were rapidly reduced upon CPT removal in the KB line. After 5 min of drug-free incubation, approximately 70% of PLDB were reversed. By 60 min of CPT-free exposure

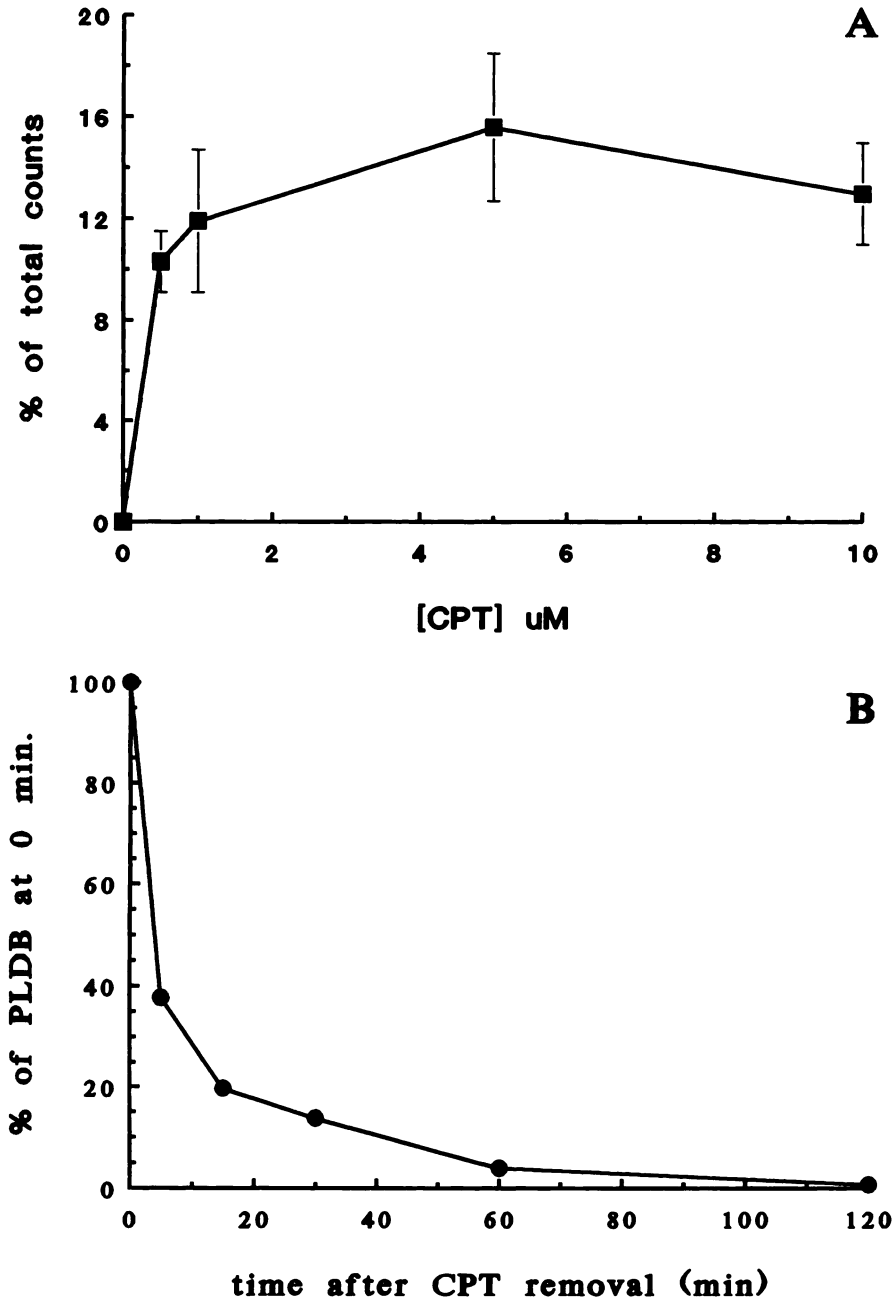

Fig. 1. *In vivo* KCl-SDS co-precipitation assay. A, Steady state levels of PLDB in KB cells after a 1-hr incubation with CPT at the designated concentration. Data represent means ± standard deviations from at least three experiments. B, Reversibility of PLDB. After incubation of cells with 5 μM CPT for 1 hr, CPT was removed and replaced by drug-free medium. Reactions were stopped for the KCl-SDS co-precipitation assay, to quantitate steady state PLDB levels. Data represent the mean values of triplicate samples, with standard deviations of <10%.

PLDB levels were <10% of the values measured during CPT exposure.

Changes in PLDB upon CPT exposure. To determine whether PLDB levels remained constant throughout the CPT exposure, the following KCl-SDS time course assay was performed. Cells were preincubated with several different CPT concentrations for different periods of time, after which the cells were given a pulse of 5 μM CPT for 30 min, which, according to Fig. 1A, induced the maximal levels of PLDB possible after the preincubation period. If the PLDB levels remained constant over time, then a graph of PLDB levels versus time would result in a plateau of PLDB levels, equal to the plateau found in Fig. 1A.

In the experiments reported in Fig. 2, KB cells were preincubated with 7 nM to 5 μM CPT for various periods of time. After a 30-min pulse with 5 μM CPT, the graphs of PLDB levels versus time did not show plateaus but displayed dramatic decreases in PLDB levels over time. CPT induced a time- and concentration-dependent decrease in the maximal levels of PLDB induced by a pulse of 5 μM CPT. The most dramatic loss of concentration-dependent PLDB formation came within the first 6 hr of preincubation. The concentration-dependent decrease in PLDB leveled off from 18 hr to 24 hr.

Effects of APH coincubation on PLDB decreases. The effects of halting replication fork movement on the CPT-induced PLDB decreases were examined by APH coincubation. APH preincubation alone did not alter the PLDB levels produced by the 5 μM CPT pulses over time, nor did it alter the 1 μM CPT-induced decreases in PLDB over time (Fig. 3), because preincubation with CPT plus APH or CPT alone produced identical results. Therefore, replication fork move-

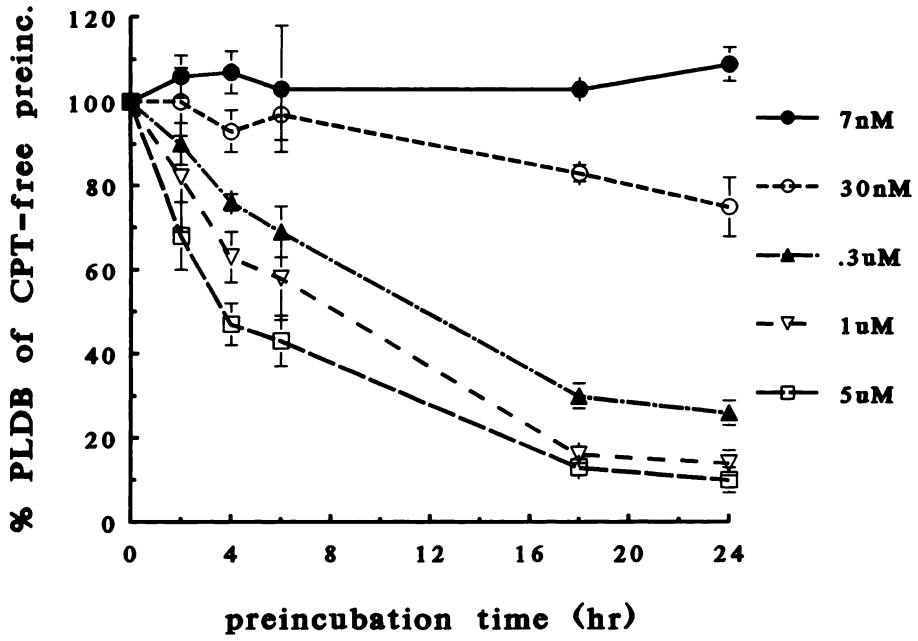

Fig. 2. PLDB time course assay. KB cells were processed through a PLDB time course assay as described in Experimental Procedures. The CPT concentrations shown (*numbers to the right*) represent concentrations of CPT during the preincubation period. Data represent mean ± standard deviation values from triplicate samples.

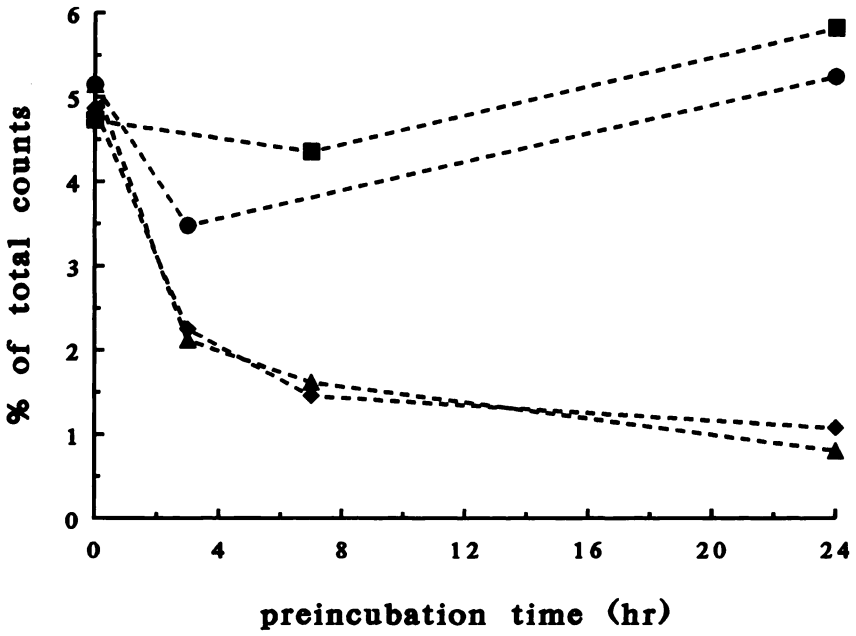

Fig. 3. Effects of APH coincubation on CPT-induced PLDB decreases. The KCl-SDS PLDB time course assay was repeated with the designated preincubation conditions. ●, Control, drug-free preincubation; ▲, 1 μM CPT; ■, 7.5 μM APH; ◆, 1 μM CPT plus 7.5 μM APH. After preincubation periods, cells were pulsed with 5 μM CPT for 30 min to determine the maximal PLDB induced by CPT at that time. Data represent mean values of triplicate samples, with standard deviations of <10%.

ment was not necessary for the observed CPT-induced decreases in PLDB over time. Additionally, as expected, the APH coincubation resulted in the elimination of CPT toxicity, as determined by clonogenic assays (data not shown). Therefore, based on the fact that PLDB decreases were observed with the elimination of toxicity, the decreases were not due simply to the killing of cells over time.

Effects of CHX on PLDB decreases. It was possible that the PLDB decrease was due to the induction of a proteolytic enzyme that degraded the TOP1 within the PLDB. If the induction of this enzyme required new protein synthesis, then coincubation with CHX, a protein synthesis inhibitor, would abolish the observed PLDB decreases. To test this, KCl-SDS time course assays were performed with CHX coincubation. CHX at 1 μM was shown to inhibit protein synthesis by 95% (data not shown). As shown in the KCl-SDS time course assays in Fig. 4, CHX did not produce PLDB alone, nor did CHX alter any changes in PLDB levels induced by sublethal (7 nM) or lethal (1 μM) concentrations of CPT. Therefore, new protein synthesis was not necessary for the CPT-induced decreases in PLDB over time.

Immunoblot analysis of TOP1 decreases. To confirm that the PLDB decreases reflected decreases in TOP1 protein, immunoblots of TOP1 were examined at the same time points as those used to show decreases in PLDB upon CPT exposure. In the KB line, cells were preincubated with 1 μM CPT and APH, to prevent CPT toxicity. At the indicated time points, the drug medium was removed, the cultures were washed with drug-free medium, and the cultures were incubated for 1 hr in drug-free medium. The final 1 hr of incubation in drug-free medium allowed for the total reversal of all PLDB, which would deplete the TOP1 band on immunoblots

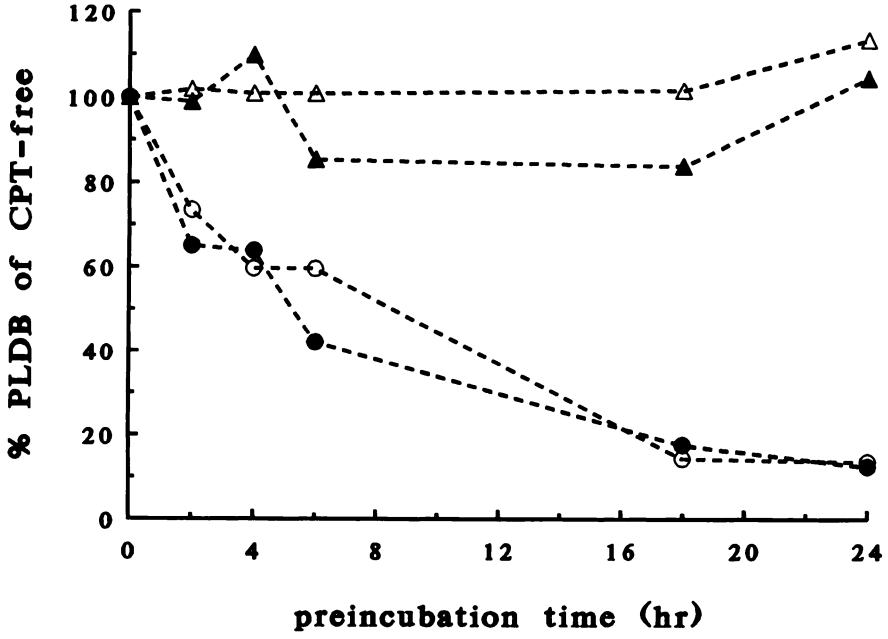

Fig. 4. Effects of CHX on CPT-induced decreases in PLDB. Two different CPT concentrations, 7 nM (*triangles*) and 1 μM (*circles*), were used in the preincubation step of the PLDB time course assay. CHX (4.5 μM) was either included in the preincubation step (*filled symbols*) or not included in the preincubation step (*open symbols*). Data represent the mean value of triplicate samples, with standard deviations of <10%.

(Fig. 1B). The resulting TOP1 immunoblots displayed dramatic decreases in TOP1 protein from 1 to 19 hr of CPT exposure (Fig. 5A). Densitometric scans of the resulting 100-kDa bands in KB cells revealed that the bands at 4, 8, and 19 hr had 54%, 25%, and 16%, respectively, of the TOP1 protein in the 0-hr sample. Additionally, APH incubation alone did not alter TOP1 levels after a 19-hr exposure. To determine whether the decreases in TOP1 upon CPT exposure were the result of a general proteolytic event or the specific degradation of TOP1, the Western blots described above were also probed with actin- and tubulin-specific antibodies. The resulting actin (Fig. 5B) and tubulin (Fig. 5C) immunoblots revealed no changes in either of these proteins throughout the period of exposure to CPT plus APH. If the CPT-induced decreases in TOP1 were due to the activation of a proteolytic enzyme, then proteolysis was apparently specific for TOP1.

TOP1 RNA analysis. Finally, total RNA was isolated from cultures treated with CPT plus APH for up to 19 hr and was probed for TOP1 RNA content (Fig. 6). No changes in TOP1 RNA levels were found throughout the CPT plus APH exposure, from 2 hr to 19 hr.

Assessment of CPT toxicity over time. If TOP1 levels are not constant over time and if CPT toxicity is determined by TOP1 levels, then CPT toxicity should not remain constant with time. To examine the ability of CPT-induced decreases in TOP1 to lower CPT toxicity, the rate of CPT toxicity during CPT exposure was investigated. Because sublethal CPT concentrations (<9.8 nM) did not cause decreases in PLDB, much higher (and more toxic) CPT concentrations were necessary to induce TOP1 decreases. Therefore, it was necessary to use highly toxic CPT concentrations to induce TOP1 decreases without killing the cells. This was possible due to the unique features of coincubation of APH with CPT. It was known that APH coincubation 1) prevents CPT toxicity, 2) does not prevent PLDB formation, and 3) does not alter CPT-induced decreases in PLDB in KB cells. Therefore, with preincubation of cells with CPT and APH for 14 hr, the CPT-induced decreases occur without CPT toxicity. After the 14-hr preincubation, CPT and APH were washed out, fol-

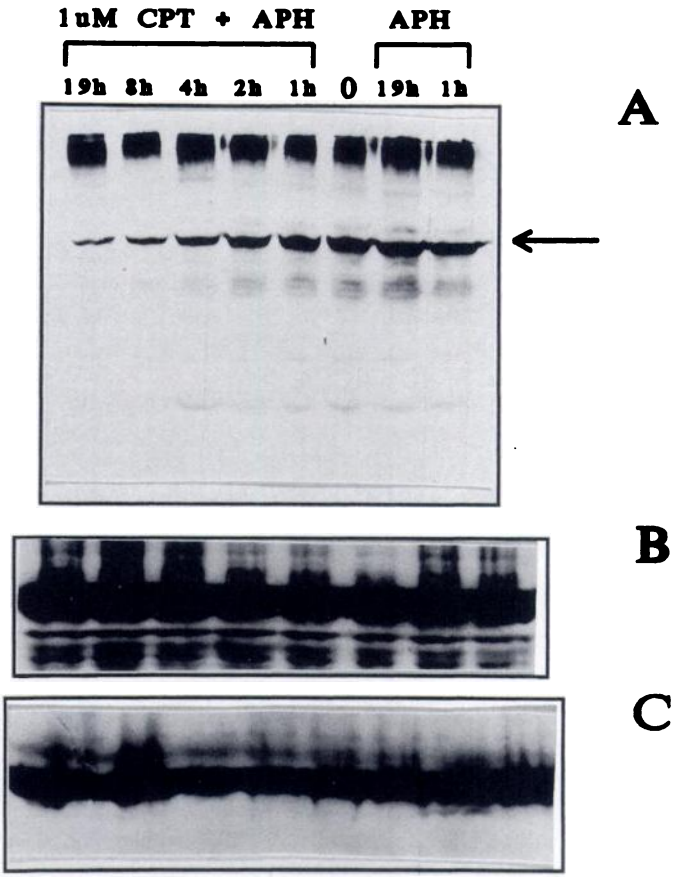

Fig. 5. Immunoblot analysis of CPT-induced decreases in TOP1 protein levels. KB cells were preincubated with either APH (7.5 μM) or APH plus 1 μM CPT for the number of hours indicated (*numbers at the top of each lane*). *Lane 0*, untreated control KB protein levels. After preincubations, cells were incubated in CPT-free medium for 1 hr and harvested. Cells (5×10^5) were boiled in 1× Laemmli sample buffer and fractionated on 7.5% SDS-polyacrylamide gels. Western blots were incubated with either a TOP1-specific monoclonal antibody (A), an actin-specific polyclonal antibody (B), or a tubulin-specific polyclonal antibody (C). The resulting bands were visualized by chemiluminescence. *Arrow*, intact 100-kDa TOP1 band.

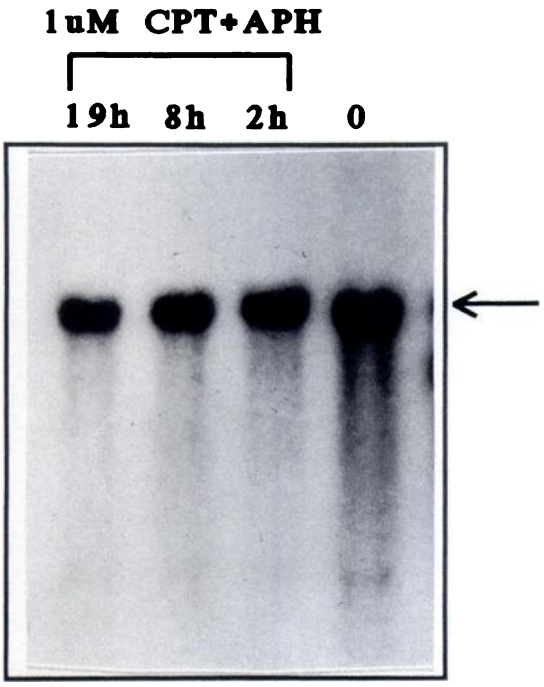

Fig. 6. Quantitation of TOP1 RNA levels during CPT incubation. KB cells were treated with 1 μM CPT plus 7.5 μM APH for the indicated times. After incubations, RNA was isolated from cultures, sized fractionated on agarose-formaldehyde gels, and transferred to nylon membranes. The membranes were probed with a ^{32}P-labeled probe corresponding to the 3′-end 1.8 kb of the TOP1 cDNA. The resulting 4.2-kb TOP1 transcripts were visualized by autoradiography. *Arrow*, 4.2-kb TOP1 RNA.

lowed by a 30-min drug-free incubation and a 4-hr period of exposure to increasing concentrations of CPT. After the 4-hr exposure period, cells were washed free of CPT and drug-free medium was added for quantification of colony-forming ability.

An additional consequence of the APH preincubation step that could influence the fraction of cells killed is the resulting cell cycle perturbation (32). Because APH halts all replication fork movement, cells that were in S phase or entered S phase during APH exposure were stopped. The resulting cell cycle distribution after a 14-hr preincubation showed an accumulation of cells in late G_1 phase, unchanged S phase populations, and decreased G_2/M populations, as shown in Table 1. The cycle distribution after exposure to 1 μM CPT for 14 hr was different from the cycle distribution after APH exposure; the CPT exposure was characterized by an accumulation of mid-S phase cells. Coincubation with CPT and APH produced identical cycle distributions, compared with APH alone, which were characterized by an accumulation of cells on the G_1/S border. The actual DNA histograms were identical for the APH and CPT plus APH preincubations (data not shown). Therefore, the only difference between cultures preincubated with APH alone and those preincubated with CPT plus APH was the CPT-induced decrease in TOP1 content.

TABLE 1

Cell cycle analysis of KB cells after the preincubation period of the clonogenic time course assay

The preincubation period lasted 14 hr and included either no drugs (None), CPT (1 μM), APH (7.5 μM), or CPT and APH. At the end of the preincubation period, cells were harvested and fixed, and DNA was stained with propidium iodide. Cell cycle distribution analyses based on DNA content were performed with a FACS IV flow cytometer, and accepted mathematical models were used to determine population percentages in each phase.

Treatment	Distribution		
	G_1	S	G_2/M
		%	
None	53	22	25
CPT	6	86	8
APH	68	21	11
CPT + APH	68	20	12

The resulting survival curves are shown in Fig. 7. The first observation was that the maximal cell kill after APH preincubation correlated with the percentage of cells in or entering S phase during the 4-hr incubation. This elevated fraction of cell killing (compared with untreated controls) was an expected side effect of the APH incubation. Comparison of KB cells pretreated with APH and with CPT plus APH revealed a low but significant decrease in CPT sensitivity (Fig. 7), especially at 6× (p = 0.004) and 9× (p = 0.002) CPT concentrations. Therefore, in the KB line, CPT-induced decreases in TOP1 were able to diminish CPT toxicity.

Discussion

TOP1, the intracellular target of CPT, did not remain constant throughout the CPT exposure period. CPT induced a time- and concentration-dependent decrease in TOP1, TOP1-associated PLDB, and, to a small extent, CPT toxicity. In both PLDB formation and immunoblotting assays, the most rapid phase of decrease was found from 2 to 8 hr, with a plateau of TOP1 and PLDB levels from 16 to 24 hr. Regarding the concentration dependence of CPT, exposure to 0.3 μM CPT resulted in significant loss of TOP1 in the KB line. Neither replication fork movement nor protein synthesis was necessary for PLDB decreases. Also, the TOP1 decreases were specific for TOP1, because analysis of actin and tubulin levels revealed no changes throughout the period of exposure to CPT. Finally, analysis of TOP1 RNA revealed no decreases in TOP1 RNA levels throughout the period of exposure to CPT, which indicated a post-transcriptional level of TOP1 regulation.

The concept of transient protection was introduced (33) as a potential modulator of drug resistance. Transient protection describes an induced state of resistance, conferred by immediate factors, that does not involve genetic changes and is not permanent. From our observations, it appears that CPT exposure can induce this state of transient protection to a small extent in KB cells. The IC_{50} value for CPT (9.8 nM) in KB cells did not produce PLDB above background levels. Therefore, it appears that KB cells need only very low levels of PLDB for toxicity. Because the CPT-induced decreases did not result in complete PLDB elimination, there probably were still enough PLDB present to kill these cells. Additionally, treatment of KB cells with sublethal CPT concentrations (7 nM) (Fig. 2) did not diminish PLDB levels over time; therefore, no transient protection by sublethal CPT concentrations would be expected. We are currently characterizing CPT-resistant KB sublines that require much higher PLDB levels for toxicity. When CPT toxicity over time was determined in these resistant sublines, dramatic decreases in CPT toxicity were observed.

Previous studies showed depletion of the TOP1 band in

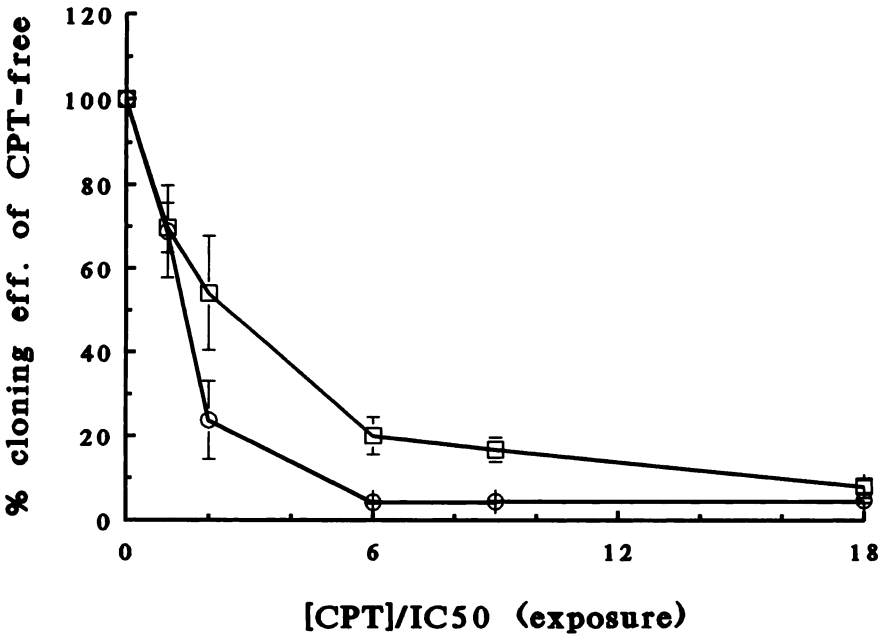

Fig. 7. Clonogenic time course assay. KB cells were preincubated for 14 hr with either 7.5 μM APH (○) or APH and 1 μM CPT (□), followed by 30 min of drug-free incubation. Preincubated cultures were then exposed to CPT concentrations (expressed as multiples of the IC_{50} value, which was determined by growth inhibition assays; see Experimental Procedures) for 4 hr, followed by assessment of toxicity by cloning efficiency. CPT-free samples had identical preincubation conditions but did not receive CPT during the 4-hr exposure period. Data represent mean ± standard deviation. Student *t* test *p* values (three experiments) were as follows: 1×, $p = 0.907$; 2×, $p = 0.034$; 6×, $p = 0.004$; 9×, $p = 0.002$; 18×, $p = 0.024$.

immunoblots produced after exposure to CPT (13, 34). The resulting PLDB, which consisted of TOP1, CPT, and DNA, were covalently complexed by the addition of the sample buffer, which contained SDS. The attachment of TOP1 to high molecular weight DNA prevented its migration into the SDS-polyacrylamide gel, which resulted in depletion of TOP1 in immunoblots. This depletion of TOP1 by CPT was used as evidence to show that the target of CPT was TOP1. Therefore, one possible interpretation of the decreases in TOP1 that occurred upon CPT exposure was that the PLDB depleted TOP1 from the immunoblots. However, several factors rule out the possibility of TOP1 band depletion by CPT. First, before all cells were harvested for SDS-polyacrylamide gel electrophoresis, they were incubated in CPT-free medium for 1 hr. The PLDB reversibility data showed that at 60 min after CPT removal 95% of the PLDB were reversed (Fig. 1B). Another point involves the gradual loss of the TOP1 band on the immunoblots. CPT has been shown to produce PLDB as quickly as 5 min after addition, but the TOP1 band from cultures incubated for 1 hr was nearly equal to that of untreated cultures. If the depletion of TOP1 in the immunoblots were due to band depletion by PLDB formation, then the band at 1 hr and those at later time points should have been depleted equally and not gradually, as shown. Finally, because the KCl-SDS assay measured TOP1 complexed to DNA via PLDB and the immunoblots of whole-cell lysates measured all TOP1 that was not involved in PLDB, the sum of the values from the two assays at the same time point during CPT exposure should give a complete accounting of the TOP1 in the cells. Because the decreases in TOP1 protein levels and the decreases in PLDB levels coincided, the possibility of band depletion seemed improbable.

The mechanism responsible for the down-regulation of TOP1 by CPT is under investigation. As stated above, a post-transcriptional event was suggested by the fact that no changes in TOP1 mRNA levels were seen during CPT exposure. If a proteolytic enzyme was responsible, then it appeared to be specific for the TOP1 protein, because actin and tubulin levels were not altered by CPT. Also, because CHX coincubation did not prevent the down-regulation of PLDB, the induction of a proteolytic enzyme did not require new protein synthesis. Investigations into the half-life of TOP1 in response to CPT are underway. Two recent studies have characterized a similar proteolytic loss of topoisomerase II protein and PLDB upon exposure to topoisomerase II poisons (35, 36). It will be interesting to determine whether the proteolytic enzyme responsible for topoisomerase II loss in response to topoisomerase II poisons and the enzyme responsible for TOP1 loss in response to CPT are the same.

If the CPT-induced decreases in TOP1 and CPT toxicity prove to be a common response, then significant clinical implications would result. If a cell population was able to survive the initial high PLDB levels generated by CPT, then the resulting diminished PLDB levels present later might not prove to be toxic. The ability of cells to survive the initial hours of CPT exposure is plausible, especially due to the S phase-specific nature of CPT killing. If a cell population did not enter the CPT-vulnerable S phase until several hours after CPT exposure, then the PLDB levels would be greatly reduced, compared with initial levels, and it could be possible for these cells to traverse the S phase unharmed by the lower PLDB levels present.

Questions to address involve the ability of the more water-soluble CPT derivatives (topotecan and irinotecan), which are currently being evaluated in clinical trials, to induce the same responses as found in this study with CPT. If the CPT derivatives produce time- and concentration-dependent decreases in TOP1, are they all of similar rates and magnitudes? In terms of the time dependence, dosing schedules would be greatly affected by these decreases. If toxicity permits, these results would favor a high dose of CPT for a short period, compared with perfusions of CPT over longer time periods, because even low levels of circulating CPT would initiate decreases in TOP1 levels and CPT sensitivity. Additionally, the dosage schedule must allow time for TOP1 levels to revert back to levels present before CPT exposure. Investigations into the rates of reversal of the CPT-induced decreases in TOP1 are necessary to further exploit this phe-

nomenon, because the possibility exists that different cell types would reverse TOP1 levels at different rates.

These studies provided insight into the use of TOP1 levels for predicting the sensitivity of tumor cells to CPT. Care must be taken with respect to the time when the sample is taken for TOP1 analysis, because even a 4-hr exposure to CPT could dramatically decrease apparent TOP1 levels. More permanent effects of CPT exposure, such as the induction of dsDNA breaks, would be more consistent indicators of CPT activity. In addition to the effects that CPT-induced decreases in TOP1 would have on dosage schedules for CPT, additional consequences could be found in combination therapy with CPT. Because CPT toxicity decreases over time, the question of when CPT is used in a combination strategy becomes significant. The use of CPT simultaneously with other compounds versus the sequential administration of CPT and other agents would result in different toxicity profiles.

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