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Degradation of Topoisomerase I Induced by Topoisomerase I Inhibitors Is Dependent on Inhibitor Structure but Independent of Cell Death

QIN FU, SEUNG-WHAN KIM, HONG-XIN CHEN, SUSAN GRILL and YUNG-CHI CHENG

Yale University, School of Medicine, Department of Pharmacology, New Haven, Connecticut

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ABSTRACT

DNA topoisomerase I (top I) is the target of the antitumor drug camptothecin (CPT) and its analogs. CPT induces dose- and time-dependent degradation of top I. Degradation of top I also occurs in a CPT-resistant cell line and, therefore, is not a consequence of cell death. Top I degradation is preceded by the appearance of a high molecular weight ladder of top I immunoreactivity and can be blocked by specific inhibitors of the proteasome. We compared the effects of five top I poisons [CPT, topotecan, 6-*N*-formylamino-12,13-dihydro-1,11-dihydroxy-13-(β-D-glucopyranosyl)-5H-indolo[2,3-a]pyrrolo[3,4-c]carbazole-5,7(6H)-dione (NB506), camptothecin-(*para*)-4β-amino-4′-O-demethyl Epipodophyllotoxin (W1), and camptothecin-(*ortho*)-4β-amino-4′-O-demethyl Epipodophyllotoxin (W2)] on cleavable complex formation and top I degradation. Although all five drugs induced cleavable complex formation, two of the drugs, NB506 and W1 did not induce top I degradation.

Camptothecin (CPT) is isolated from extracts of the *Camptotheca accumminata* tree native to China (Wall and Wani, 1996). A number of CPT analogs, such as 9-aminocamptothecin, topotecan (TPT), and irinotecan, have shown impressive antitumor activity against a variety of tumors in clinical trials (Rothenberg, 1997). DNA topoisomerase I (top I) is the target of CPT. top I is an essential nuclear enzyme which can change the topological state of DNA by breaking one strand of a DNA double helix, allowing free rotation of the cleaved strand, and then rejoining the phosphodiester backbone of DNA (Porter and Champoux, 1989). top I relaxes both positively and negatively supercoiled DNA and plays important roles in a variety of cellular processes involving DNA such as DNA replication, RNA transcription, and DNA repair (Downes and Johnson, 1988; Roca, 1995; Wang, 1996).

The mechanism of top I involves a transient covalent bond between top I and the 3′ end of the cleaved DNA strand. CPT stabilizes this "cleavable complex" which results in the accumulation of top I-DNA adducts known as protein-linked DNA breaks (PLDBs) (Hsiang et al., 1985). PLDB formation is reversible once CPT is removed. However, if a DNA synthesis fork collides with the PLDB, irreversible DNA damage occurs, leading to cell death (Pommier et al., 1994; Liu et al., 1996).

Preventing the collision of DNA synthesis forks with PLDBs, either by inhibiting DNA synthesis or by reducing the amount of top I available to form PLDBs, can protect cells against CPT-mediated cytotoxicity. The DNA synthesis inhibitor aphidicolin (APH) could protect cells from CPT cytotoxicity (Hsiang et al., 1989; Kaufmann, 1998). Several CPT-resistant cells have been selected that have reduced top I activity resulting from mutant top I genes, reduced top I expression, or posttranslational modification of top I such as phosphorylation or poly-ADP ribosylation (Pommier et al., 1996).

Prolonged treatment of cells with CPT also leads to top I down-regulation, resulting in a reduction in PLDBs. D'Arpa and colleagues (Desai et al., 1997) have reported that top I protein is ubiquitinated and then degraded by the proteasome in response to CPT treatment. We confirm these results and report that CPT-induced top I down-regulation occurs in the nucleus of cells from the human epidermoid carcinoma cell line (KB cells) and is independent of cell death. We also examine the effects of several other top I poisons on top I expression and find that 6-*N*-formylamino-12,13-dihydro-1,11-dihydroxy-13-(β-D-

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ABBREVIATIONS: CPT, camptothecin; TPT, topotecan; top I, topoisomerase I; top II, topoisomerase II; NB506, 6-*N*-formylamino-12,13-dihydro-1,11-dihydroxy-13-(β-D-glucopyranosyl)-5H-indolo[2,3-a]pyrrolo[3,4-c]carbazole-5,7(6H)-dione; MG132, carbobenzoxyl-L-leucyl-L-leucyl-L-leucinal; E64, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane; IBU, *i*-BuNH-Eps-Leu-Pro-OH; W1, camptothecin-(*para*)-4β-amino-4′-O-demethyl Epipodophyllotoxin; W2, camptothecin-(*ortho*)-4β-amino-4′-O-demethyl Epipodophyllotoxin; APH, aphidicolin; PLDB Protein-linked DNA break; DCI 3, 4-dichloroisocoumarin; TPCK tosyl-phenylalanine choromethyl ketone; YVAD-CMK Ac-Tyr-Val-Ala-Asp-Chloromethyl ketone; PAGE, polyacrylamide gel electrophoresis; VP-16, 4β-amino-4′-*O*-demethyl epipodophyllotoxin.

glucopyranosyl)-5H-indolo[2,3-a]pyrrolo[3,4-c]carbazole-5,7(6H)-dione (NB506) and a novel topoisomerase inhibitor, camptothecin-(*para*)-4β-amino-4′-O-demethyl Epipodophyllotoxin (W1), do not induce top I down-regulation.

Materials and Methods

Cells

KB cells, derived from an epidermoid carcinoma in the mouth of an adult male Caucasian, were purchased from the American Type Culture Collection (Rockville, MD). The cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2. The growth medium used was RPMI 1640 supplemented with 5% fetal bovine serum, and 100 μg/ml kanamycin. CPT-resistant cells, KBCPT100 (Beidler et al., 1996), were maintained in RPMI 1640 medium supplemented with 100 nM CPT, 10% fetal bovine serum, and 100 μg/ml kanamycin. Before each experiment, KBCPT100 cells were cultured in CPT-free medium for 3 days.

Drugs and Antibodies

Mouse monoclonal antibody C21, an antitop I-specific antibody, was developed in this laboratory (Zhou et al., 1989). TPT was obtained from the National Cancer Institute (Bethesda, MD). NB506 was kindly provided by Dr. T. Yoshinari and Dr. S. Nishimura from the Banyu Tsukuba Research Institute, Tsukuba, Japan. W1 and camptothecin-(*ortho*)-4β-amino-4′-O-demethyl Epipodophyllotoxin (W2) were kindly provided by Dr. K. H. Lee from the University of North Carolina. Lactacystin was kindly provided by Dr. E. J. Cory from Harvard University. *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E64) and *i*-BuNH-Eps-Leu-Pro-OH (IBU) were kindly provided by Dr. Barbara J. Gour from McGill University, Montreal, Canada. APH was purchased from Sigma Chemical Co. (St. Louis, MO). MG132, calpain inhibitor I, 3, 4-dichloroisocoumarin (DCI), and tosyl-phenylalanine chloromethyl ketone (TPCK), were purchased from Boehringer Mannheim Corporation (Indianapolis, IN). Interleukin-1β-converting enzyme inhibitor I, Ac-Tyr-Val-Ala-Asp-chloromethyl ketone (YVAD-CMK), and carbobenzoxyl-L-leucyl-L-leucyl-L-leucinal (MG132) was purchased from Calbiochem-Novabiochem Corporation (La Jolla, CA).

Processing of Cell Lysates

Total Cell Lysates. Cells (5×10^5) were plated in a T-25 flask in 5 ml of growth medium overnight. The next day, a drug was added and the cells were grown for the indicated time. At the end of the incubation, cells were washed twice with PBS, incubated in drug-free medium for 30 min, and then treated with pancreatin to dislodge the cells from the flask. The cells were centrifuged and washed with PBS. The resulting cell pellet was resuspended in 1× Laemmli loading buffer (63 mM Tris, pH 6.8, 2% SDS, 5% 2-mercaptoethanol, 10% glycerol) and boiled for 5 min. The cell lysates were either used immediately or stored at −70°C. All samples were boiled again for another 5 min immediately before SDS-polyacrylamide gel electrophoresis (PAGE) gel analysis. Samples were normalized with an equal cell number per lane.

High Molecular Weight top I Analysis. The method of Desai (Desai et al., 1997) was followed. After drug treatment, cells were quickly harvested by pancreatin treatment and cell pellets were chilled on ice for 5 min. Ice-cold high-salt solution (50 mM Tris, pH 7.4, 0.8 M NaCl, 0.5% Nonidet P-40, 5 mM $MgCl_2$, 5 μg/ml leupeptin, 5 μg/ml pepstatin, 5 μg/ml aprotinin, 5 mM *N*-ethylmaleimide) was added to the cell pellet. The lysate was then added to an equal volume of 10 mM cysteine in water. Finally, one-third volume of 3 × SDS-PAGE sample buffer was added to the lysate. Samples were normalized to equal cell number before loading on the SDS-PAGE gel.

Cell Fractionation. KB cells were treated with 25 μM CPT for 10 min, 30 min, 1 h, 2 h, and 4 h. At each time point, cells were quickly harvested with pancreatin, washed twice with cold PBS, resuspended in buffer N (50 mM Tris, pH 7.4, 0.5% NP40, 5 mM $MgCl_2$, 5 μg/ml leupeptin, 5 μg/ml pepstatin, 5 μg/ml aprotinin), and incubated on ice for 30 min. The cytosolic fraction was obtained by centrifugation for 5 min at 5000 rpm in a microcentrifuge and then collecting the supernatant. Unfractionated cells and the nuclei were extracted with ice-cold high-salt buffer. Samples from an equal number of cells were loaded in each lane for SDS-PAGE analysis.

Western Blotting

Proteins were separated by either a 7% acrylamide gel or a 4 to 12% gradient gel. After electrophoresis, proteins were transferred onto a nitrocellulose blotting membrane, BioTrace NT (Pall Gelman Science, Ann Arbor MI) with a Bio-Rad mini trans-blot electrophoretic transfer cell (Bio-Rad Laboratories, Hercules CA). The resulting filter was blocked with 5% nonfat dried milk in PBST (0.15% Tween 20 in PBS) overnight with shaking at 4°C. The blot was incubated with the primary antibody, either antitop I antibody (C21, 1/1000) or antiactin in a blocking buffer for 2 h at room temperature. The blot was then washed at room temperature three times for 15 min and incubated with a secondary antibody (1/1000) in blocking solution for 1 h at room temperature. The secondary antibody was either antimouse IgM (μ chain specific, peroxidase conjugated, 1/1000; Sigma, St. Louis, MO) or antimouse polyvalent Ig (peroxidase conjugated, 1/1000, Sigma). After another three 15-min washes at room temperature with PBST, enhanced chemiluminescence (DuPont, Wilmington, DE) was used to detect the peroxidase conjugate by exposure to X-ray film.

PLDB Analysis

PLDBs were quantified by a potassium/SDS coprecipitation assay (Beidler and Cheng, 1995). PLDB formation reached a plateau level (defined as 100%) when cells were treated with 10 μM CPT.

Results

Degradation of top I Is CPT Dosage- and Incubation Time-Dependent but Cell Death-Independent. It has been previously reported that top I protein expression is down-regulated in response to CPT treatment in the KB cell line (Beidler and Cheng, 1995) and in other cell lines (Desai et al., 1997). However, due to the cytotoxic effect of CPT on these cells, it is possible that the reduction in top I protein is related to cell death rather than being directly caused by CPT exposure. We compared the effect of CPT on top I expression in CPT-sensitive versus CPT-resistant cell lines in an attempt to uncouple top I down-regulation from CPT-induced apoptosis. The CPT-resistant KBCPT100 cell line was derived from human KB cells by selecting for growth in media supplemented with CPT. KBCPT100 cells are resistant to a variety of top I poisons but not to other classes of drugs, including topoisomerase II (top II) poisons. The mechanism of CPT resistance is unknown because KBCPT100 cells have normal top I expression and activity (Beidler et al., 1996). The LC_{50} value of CPT, defined as the concentration that produced a 50% reduction in colony-forming ability after one generation time exposure, was 12 nm for kB cells and was 3500 nm, a 300-fold higher concentration, for CPT-resistant variant.

To compare top I down-regulation in CPT-sensitive and CPT-resistant KB cells, the two cell lines were treated with CPT at various concentrations for 8 h. The doubling time of KB cells is 19 h and that of KBCPT100 cells is 29 h. The cells were then incubated in drug-free medium for another 30 min to allow for reversal of the cleavable complex. As shown in

Fig. 1A, both cell lines show a similar pattern of top I down-regulation at increasing concentrations of CPT. Similarly, the time course of top I down-regulation in CPT-sensitive and CPT-resistant cells incubated in 1 μM CPT is identical (Fig. 1B). These results indicate that CPT-induced top I down-regulation is not a consequence of cell death. Down-regulation is also independent of newly synthesized protein because the protein synthesis inhibitor cycloheximide did not affect top I protein down-regulation in either cell line (Fig. 1C).

We also studied the reversibility of top I down-regulation. CPT-sensitive and CPT-resistant cells were pretreated with 1 μM CPT for 12 h. Because previous work suggested that APH, a DNA synthesis inhibitor, would prevent CPT cytotoxicity without affecting CPT-induced down-regulation (Beidler and Cheng, 1995), 7.5 μm APH was included with the CPT pretreatment for the CPT-sensitive cells. Then, drugs (CPT and APH) were removed and the cells were incubated in a drug-free medium. Cells were harvested at different time points (0, 4, 8, 24, 48, 72, and 96 h) and top I protein levels were examined by Western blotting with a top I-specific monoclonal antibody. The top I expression level recovered to the pretreatment level after 48 to 72 h (Fig. 2). Recovery of top I is slightly faster in CPT-sensitive cells. This phenomenon may be related to the faster growth rate of the parental cells.

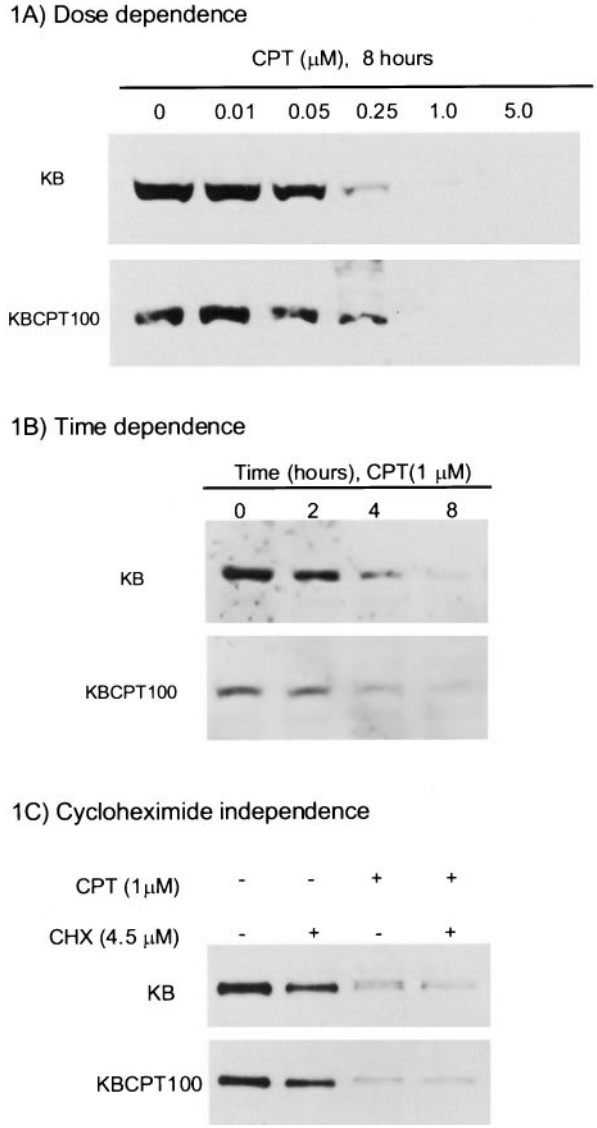

Fig. 1. CPT dose- and incubation time-dependent but protein synthesis-independent of top I down-regulation. 1A, KB and KBCPT100 cells were treated with different concentrations of CPT for 8 h. 1B, KB and KBCPT100 cells were treated with 1 μM CPT for 0, 2, 4, or 8 h. 1C, KB or KBCPT100 cells were incubated with 1 μM CPT and 4.5 μM cycloheximide (CHX) for 8 h. Cells were then washed and allowed to grow in a drug-free medium for another 30 min (enough time for reversal of cleavable complex). Cells were then harvested with pancreatin and the cell pellet was lysed directly in a cell lysis buffer. An equal number of cells were loaded in each lane. Western blots of the cell lysates were probed for top I with C-21 antibody.

Degradation of top I Can Be Blocked by Selected Protease/Proteasome Inhibitors. We next investigated the possibility that proteolysis is responsible for the observed decline in top I protein levels in CPT-treated cells by using specific protease inhibitors. Eight different protease inhibitors were tested for their ability to block CPT-induced down-regulation (Fig. 3A). Two proteasome inhibitors, lactacystin and MG132 (Rock et al., 1994; Fenteany et al., 1995; Fenteany and Schreiber, 1998), blocked top I down-regulation. The inhibition of top I degradation by MG132 was dose-dependent (Fig. 3B). As a control, MG132 or lactacystin alone had no effect on top I expression (Fig. 3A, top gel). This result suggests the involvement of the ubiquitin/proteasome pathway in the degradation of top I and is consistent with a recent report that described ubiquitination of top I upon CPT treatment (Desai et al., 1997). Calpain inhibitor I, a Ca^{2+}-dependent cysteine protease inhibitor, can also block top I degradation. However, two other cysteine protease inhibitors 1-*trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane and IBU (Sreedharan et al., 1996), did not block the top I degradation. This result suggested that the ability of calpain inhibitor I to block top I degradation is not due to inhibition of cysteine proteases in general, but probably due to inhibition of a very specific cysteine protease. The down-regulation of top I was not affected by an interleukin-1β-converting enzyme inhibitor I inhibitor, YVAD-CMK. Two serine protease inhibitors, DCI and TPCK, were evaluated. DCI could only partially block degradation and TPCK had no effect.

Proteins degraded by the proteasome pathway are typi-

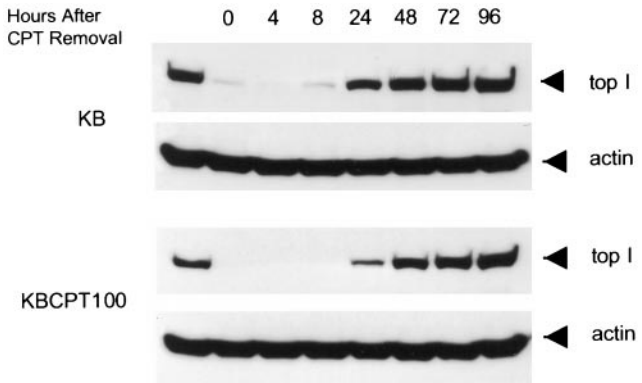

Fig. 2. Reversibility of down-regulated top I after CPT removal. KB cells were pretreated with 1 μM CPT and 7.5 μM APH for 12 h (KBCPT100 cells were treated with 1 μM CPT) and then cells were washed and grown in a drug-free medium for the times indicated. At each time point, 0, 4, 8, 24, 48, 72, and 96 h after drug removal, cells were harvested. Cell lysates were prepared as described in *Materials and Methods*. Equal amounts of protein (50 μg of protein of cell lysate for KB cells and 65 μg of protein of cell lysate for KBCPT100 cells) were loaded for each time point. Western blots were probed with C-21 and anti-Actin monoclonal antibody.

cally ubiquitinated before degradation. Consistent with this prediction, we observed a high molecular weight ladder of top I immunoreactivity when an immunoblot for top I from cells treated with CPT was overexposed (Fig. 4). The high molecular weight top I ladder appeared after 10 min of CPT treatment, before top I down-regulation was apparent, and declined at later time points. Down-regulation of top I is likely to be a specific response to CPT treatment because top II was not down-regulated in CPT-treated cells and we observed no change in the profile of ubiquinated proteins on an immunoblot of total cellular protein probed with an antiubiquitin antibody (data not shown).

Intracellular Localization of top I after CPT Treatment. We investigated the localization of top I during CPT-induced down-regulation in KB cells by subcellular fractionation. KB cells were treated with 25 μM CPT for various times and then fractionated into cytosol and nuclei fractions. Top I was present only in the nuclear fraction at all time points (Fig. 5). The high molecular weight ladder of top I immunoreactivity was also observed only in the nuclear fraction. This result suggests that top I is ubiquitinated and subsequently degraded within the nuclei. Alternatively, degradation could occur rapidly after ubiquitinated top I has been translocated to the cytoplasm.

The Effect of Other top I Poisons on top I Protein Down-Regulation. Although CPT has been extensively characterized in the laboratory, it has limited utility in clinical settings due to its high toxicity (Rothenberg, 1997). Therefore, we wished to determine whether other top I poisons could also induce top I down-regulation. For this study, we chose two well known top I poisons, TPT and NB506 (Fukasawa et al., 1998, Yoshinari et al., 1995), and two newly designed drugs, W1 and W2. The new drugs are conjugates of CPT and the top II inhibitor, 4β-amino-4′-*O*-demethyl epipodophyllotoxin (VP-16). The CPT moiety in both drugs is conjugated through the seven position of the B ring—a modification known to have only minor effects on top I inhibitory activity (Wang et al., 1997). The major difference between W1 and W2 is that the linkage between the CPT moiety and the aromatic ring of the epipodophyllotoxin analog is *para* for W1 and *ortho* for W2 (Bastow et al., 1997). W1 and W2 have similar cytotoxic effects in KB cells with an LC_{50} of 44 nM for W1 and 75 nM for W2 (JY Chang, X Guo, HX Chen, ZL Jiang, HK Wang, KF Bastow, XK Zhu, J Guan, KH Lee, YC Cheng, submitted). The CPT-resistant KBCPT100 cells are partially cross-resistant to both WI and W2, whereas the VP-16-resistant KB/7D cell line does not suggest that the primary mode of cytotoxicity of these conjugates is via top I (JY Chang et al., submitted).

The dose dependence of top I down-regulation was compared for the five drugs (Fig. 6). We observed that 1 μM CPT, TPT, and W2 each induced approximately 50% down-regulation of top I in a 4-h exposure. However, 1 μM W1 and NB506 did not induce down-regulation under these conditions. Identical results were observed for the CPT-resistant KBCPT100 cells (data not shown). To test for ubiquitination of top I, we looked for induction of a high molecular weight ladder of top I immunoreactivity after a 10-min incubation with the five drugs. Again, high molecular weight top I immunoreactive

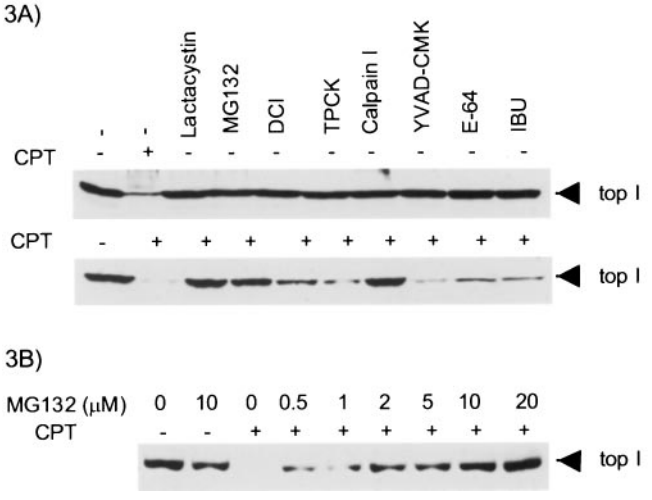

Fig. 3. Prevention of CPT-induced top I down-regulation by selected protease inhibitors. 3A, KBCPT100 cells were incubated with protease inhibitors (100 μM lactacystin, 20 μM MG132, 93 μM DCI, 200 μM TPCK, 50 μM calpain inhibitor I, 10 μM YVAD-CMK, 20 μM E-64, and 20 μM IBU) alone or with 1 μM CPT for 8 h. 3B, KBCPT100 cells were incubated with 1 μM CPT and increasing concentration of MG132 (0, 0.5, 1, 2, 5, 10, and 20 μM) for 8 h. Cells were washed free of drugs and the incubations were continued in drug-free medium for another 30 min. Cells were harvested and cell pellets were lysed directly in lysis buffer. Equal numbers of cells were loaded for each lane. Western blots were probed with C-21 antibody.

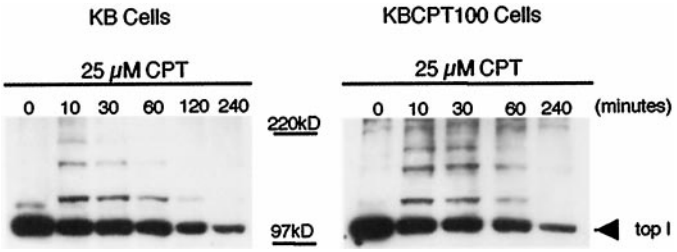

Fig. 4. High molecular weight top I ladder. KB or KBCPT100 cells were treated with 25 μM CPT for 10 min, 30 min, 1 h, 2 h, and 4 h. At each time point, cells were quickly treated with pancreatin and the cell pellet was collected by centrifugation. The cell pellet was extracted with high-salt (0.8 M NaCl) lysis buffer (see *Materials and Methods* for details) and an equal number of cells were loaded in each lane. Western blots were probed with C-21 antibody.

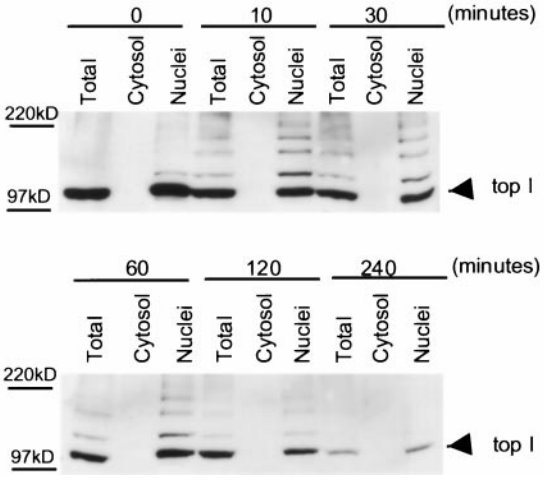

Fig. 5. Subcellular localization of top I. KB cells were treated with 25 μM CPT for 10 min, 30 min, 1 h, 2 h, and 4 h. At each time point, cells were fractionated into cytosol or nuclei fractions (see *Materials and Methods*). The nuclei fraction was extracted with 0.8 M NaCl. Cytosol or nuclear fractions from an equal number of cells were loaded for SDS-PAGE analysis. Blots were probed with C-21 antibody.

bands were induced by CPT, TPT, and W2, but not by W1 or NB506 (Fig. 7). These results are consistent with the model that top I is ubiquitinated before degradation. We did not observe significant down-regulation of top I in Fig. 7 because 10 min of drug treatment is too short a time to observe down-regulation.

The formation of PLDBs is an intermediate step in the cytotoxic mechanism of topoisomerase inhibitors. Therefore, we measured the induction of PLDBs by the five drugs to determine whether there is a correlation between the induction of PLDBs and top I down-regulation. As a cautionary note, we point out that although W1 and W2 act primarily via top I, these CPT/VP-16 conjugates may also induce PLDBs via a top II-mediated mechanism. We observed that all five drugs induced PLDBs in a dosage-dependent manner (Fig. 8). CPT and TPT induced the highest percentage of PLDBs, whereas W2, W1, and NB506 had weaker effects. However, because 10 μM W2, W1, and NB506 induced similar levels of PLDBs (36, 28, and 25% of PLDBs respectively), whereas only W2, but not W1 or NB506 (at 10 μM), induced top I down-regulation, there is no direct correlation between PLDB formation and top I down-regulation.

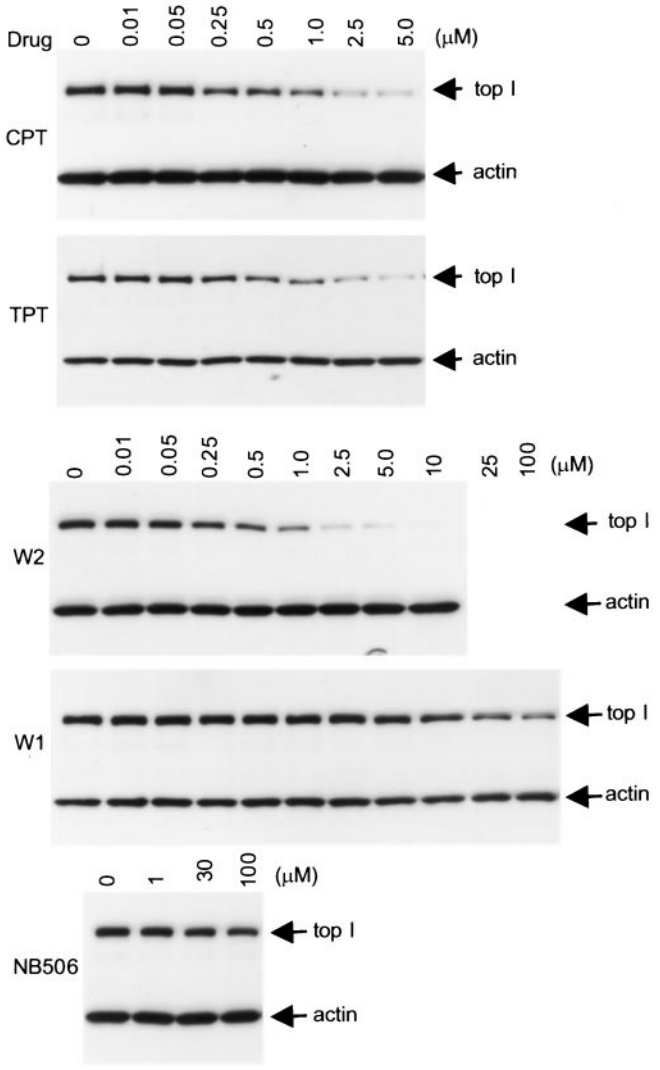

Fig. 6. Different effects of top I poisons on top I protein down-regulation. KB cells were treated with CPT, TPT, W1, W2, or NB506 at the indicated concentrations for 4 h. Then cells were washed and allowed to grow in drug-free medium for another 30 min (enough time for reversal of cleavable complex). Then cells were harvested with pancreatin and the cell pellet was lysed directly with cell lysis buffer. An equal number of cells were loaded for each lane. Western blots of the cell lysate were probed for top I with C-21 and anti-actin monoclonal antibody.

Discussion

Our laboratory has previously shown that CPT can induce top I down-regulation at all stages of the cell cycle (Beidler and Cheng, 1995). Here, using CPT-resistant cells, our results (Fig. 1) demonstrate that the down-regulation of top I is independent of CPT-induced cell death and that top I protein levels recover after CPT removal. These observations have important implications for the design of future clinical trials

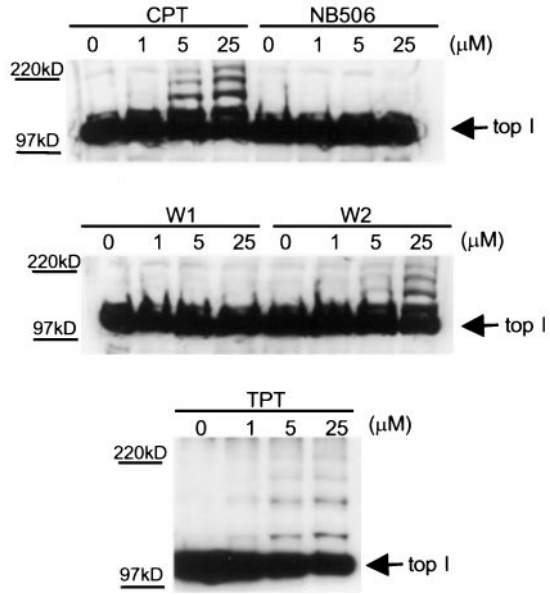

Fig. 7. Different effects of top I poisons on the high molecular weight top I ladder formation. KBCPT100 cells were treated with 0, 1, 5, or 25 μM CPT, TPT, NB506, W1, or W2 for 10 min. Cells were quickly harvested by pancreatin treatment and the cell pellet was collected by centrifugation. The cell pellet was extracted with high-salt (0.8 M NaCl) lysis buffer (see *Materials and Methods*) and an equal number of cells were loaded in each lane. Western blots were probed with C-21 antibody.

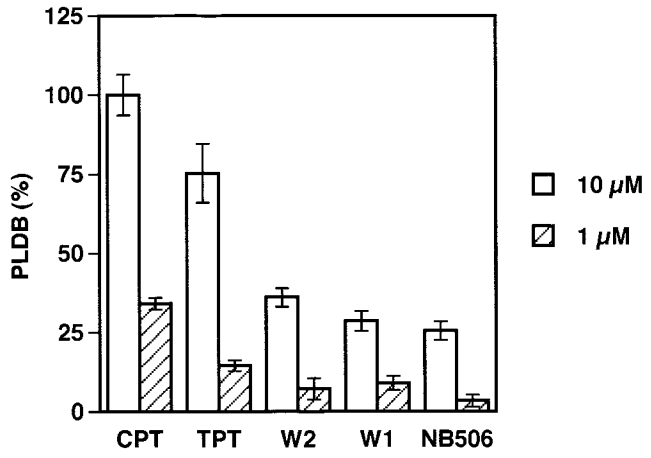

Fig. 8. PLDB formation induced by five topoisomerase poisons. KB cells were treated with 1 μM and 10 μM concentrrations of each drug, and the steady-state levels of PLDBs were measured with an in vivo K-SDS coprecipitation assay (Beidler and Cheng, 1995). The quantity of PLDBs induced by 10 μM CPT was defined as 100%.

of top I poisons. Prolonged treatment with CPT analogs may reduce the amount of top I protein in target tumor cells, thereby reducing the efficacy of the treatment. Our results suggest that expression of top I in target tumors will recover after the top I poison has been metabolized. Therefore, we suggest that high dosages, short exposure times, and multiple cycles of treatment should be considered for future clinical trials of top I poisons. Alternatively, it may be advantageous to use a CPT conjugate like W2 that traps top I-DNA cleavable complexes without inducing top I down-regulation.

Loss of top I activity in response to CPT results from a dose- and concentration-dependent loss of top I protein. We tested a panel of protease inhibitors to determine whether a protease is responsible for top I down-regulation. MG132, a proteasome inhibitor, inhibited CPT-induced top I down-regulation in a dose-dependent manner, suggesting involvement of the proteasome. We confirmed proteasome involvement by inhibiting top I down-regulation with the more specific proteasome inhibitor lactacystin. Short-lived proteins such as cyclins (Krek, 1998), p53 (Whitesell et al., 1997), and IkBa (Ghoda et al., 1997) are marked for degradation by the proteasome by covalent modification with a polyubiquitin chain consisting of multiple 8.6-kDa adducts. In contrast to other proteins known to be degraded by the proteasome, top I has a long half-life in normal cells and is not cell cycle-regulated. Nevertheless, long exposure of Western blots revealed induction of a high molecular weight ladder of top I immunoreactivity 10 min after addition of CPT. These high molecular weight adducts are likely to be ubiquitinated top I as previously reported for CHO cells by Desai et al (1997). The high molecular weight top I-immunoreactive bands were observed before top I degradation induced by TPT and W2 as well as CPT, but were not seen after 10 min of incubation with W1 or NB506. Thus, there is a strong correlation between the induction of high molecular weight top I immunoreactivity and subsequent top I degradation.

It was suggested by others that when cells are treated with TPT, top I is rapidly redistributed (Buckwalter et al., 1996; Danks et al., 1996). Danks et al. (1996) also observed increased cytoplasmic concentrations of top I protein. However, our result (Fig. 5) and that of Buckwalter(1996) do not support such an observation. We detected reduced top I protein in the nuclear fraction upon CPT treatment but did not see increased cytoplasmic top I protein. Proteasomes are found in the nucleus as well as in the cytoplasm of liver cells (Rivett et al., 1992). However, cell type-dependent differences in proteasome localization have also been observed (Palmer et al., 1994). It would be very interesting to see whether this top I redistribution phenomenon, which was observed in tissue cells, could be duplicated in patient samples, or whether different types of cancer cells differ in top I redistribution.

In addition to the proteasome-specific inhibitors lactacystin and MG132, we observed that DCI and calpain inhibitor I also inhibited top I degradation (Fig. 3). The effect of DCI was partial and may represent a low potency inhibitory effect on the proteasome (Nannmark et al., 1996). For calpain inhibitor I, there are several possible inhibitory mechanisms. Calpain inhibitor I may act upstream of the 26S proteasome pathway. Another possibility is that calpain inhibitor I may prevent a post-translational modification of top I that is necessary for subsequent polyubiquitination. The third possibility invokes a novel lactacystin- and MG132-sensitive proteolysis pathway (Glas et al., 1998).

We compared the ability of five drugs to induce the formation of PLDBs and top I degradation. Although PLDBs were induced by all five drugs, NB506 and W1, a conjugate of CPT and VP-16, did not induce top I down-regulation. Therefore, the formation of top I-DNA-cleavable complexes is not sufficient to induce top I degradation. The crystal structure of a top I-DNA complex was recently solved (Redinbo et al., 1998; Stewart et al., 1998). The authors suggested a model based on their structure for the binding of CPT to top I-DNA complexes. Our current hypothesis is that CPT and W1 have different effects on the conformation of the top I-DNA complex that regulate its ability to be recognized by the ubiquitin-conjugating enzymes and, subsequently, to be degraded. W1 may be more useful for long-term chemotherapy due to its failure to induce top I down-regulation.

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Send reprint requests to: Dr. Yung-Chi Cheng, Yale University, School of Medicine, Department of Pharmacology, 333 Cedar St., New Haven, CT 06520. E-mail: cheng.lab@yale.edu