Topoisomerase I-related Parameters and Camptothecin Activity in the Colon Carcinoma Cell Lines from the National Cancer Institute Anticancer Screen¹

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ABSTRACT

Camptothecin (CPT) derivatives are a new family of anticancer agents which are selective inhibitors of DNA topoisomerase I (topi) and have entered clinical trials with promising results. The cellular determinants for CPT activity were studied in the seven cell lines of the National Cancer Institute anticancer screen: colo205, SW620, HCT116-HT29, HCC2998-HCT15, and KM12. The differential sensitivity range was approximately 17-fold between KM12 and colo205 cells. CPT uptake varied only by less than a factor of three among the cell lines. Topi mRNA, measured by Northern blotting analysis, and topi protein levels, measured by Western blotting, varied by 2-fold or less among the cell lines and were correlated neither with the CPT cytotoxicity nor the levels of cleavable complexes measured by alkaline elution in the various cell lines. An overall log-linear correlation was observed between CPT-induced topi- cleavable complexes and growth inhibition, indicating the importance of cleavable complex formation rather than topi levels for cell killing in this panel of cell lines. Also, some cell lines displayed marked growth inhibition differences with minimal differences in cleavable complexes and S-phase fraction, suggesting that parameters downstream from the cleavable complexes are also critical for CPT cytotoxicity.

INTRODUCTION

During the last decade, cancer chemotherapy lacked original molecules to improve the prognosis of solid tumors and metastatic cancers. Recently, two new families of cytotoxic agents have been discovered, taxol and CPT³ derivatives. CPT derivatives have a unique mechanism of action, and their original spectrum of activity, both in xenografts (1) and in the first clinical trials, have born new hopes for therapeutic improvements (2, 3). Phase I/II clinical trials of CPT derivatives are in progress, and promising activity has been observed in various malignancies and especially in colorectal carcinomas (4–8). Irinotecan (CPT-11) already appears as the most active CPT derivative. CPT is isolated by Monroe E. Wall, Mansukh C. Wani (Research Triangle Institute, Research Triangle Park, NC). Stock solutions of CPT were prepared in DMSO at 10 mm. [methyl-³H]thymidine, 2-[¹⁴C]thymidine (specific activity, 20 and 0.05 Ci/mmol, respectively), and [α-³²P]ATP (specific activity, 6000 Ci/mmol) were purchased from NEN Research Products (Boston, MA).

CPT was isolated by Monroe E. Wall, Mansukh C. Wani, and co-workers 30 years ago as the active alkaloid of extracts from the Chinese tree Camptotheca acuminata (10). Almost 10 years later, CPT was found to inhibit selectively a new target, eukaryotic DNA topi (11). Topi is ubiquitous and is required for Drosophila development (12). Topi relaxes DNA supercoiling by making transient single-strand breaks (13, 14, 15). These breaks are coupled with the transient formation of a covalent DNA-enzyme intermediate termed as cleavable complex. CPT specifically and reversibly stabilizes cleavable complexes by inhibiting their religation (11, 16–18). The mechanism of CPT cytotoxicity is thought to be the consequence of a collision between moving replication forks and CPT-stabilized cleavable complexes (19–21).

A better understanding of the cellular determinants for CPT activity is needed for optimal clinical use and to address the controversy between dose escalation of short treatments versus low doses during long exposure for optimal clinical responses. Identification of predictive parameters for cell sensitivity also would make it eventually possible to develop drug sensitivity assays for the clinic. One way to answer these questions is to compare topi-related parameters in cell lines which naturally exhibit differences in CPT sensitivity. Using the seven colon cell lines of the NCI anticancer cell screen as a tool (miniclinic), we studied the topi-related parameters: topi mRNA and protein levels, cell doubling-time, and cell cycle distribution that are already used in ongoing clinical studies and measurement of cleavable complex formation. We find that topi levels are not predictive of drug cytotoxicity but rather an overall log-linear relationship between CPT-induced cleavable complexes and cytotoxicity. These observations suggest that measurement of topi-induced DNA damage rather than topi levels should be monitored during clinical use of CPT and derivatives. A higher benefit of extended CPT exposure rather than dose escalation is also suggested by the present results.

MATERIALS AND METHODS

Drugs and Chemicals. CPT and [³H]CPT (labeled at position 9; specific activity, 17 Ci/mmol) were a kind gift from Drs. Monroe E. Wall and Mansukh C. Wani (Research Triangle Institute, Research Triangle Park, NC). Stock solutions of CPT were prepared in DMSO at 10 mm. [methyl-³H]thymidine, 2-[¹⁴C]thymidine (specific activity, 20 and 0.05 Ci/mmol, respectively), and [α-³²P]ATP (specific activity, 6000 Ci/mmol) were purchased from NEN Research Products (Boston, MA).

Cell Culture and Radiolabeling. All cell lines, colo205, SW620, HCT116, HT29, HCC2998, HCT15 and KM12, were provided by Dr. Dominick Scudiero (NCI, Frederick, MD) and were grown in monolayer cultures in RPMI 1640 supplemented with 5% heat-inactivated FCS and 2 mm glutamine. No antibiotic was added to the medium. The cells were trypsinized and passed 2–3 times a week.

Growth Inhibition Assays. Approximately 10⁵ cells were seeded in T25 flasks, and cell counts were checked from control flasks every 24 h. When the cells were in exponential growth phase and reached 4 x 10⁵ cells/flask (usually after 48 h), treatments were performed with various CPT concentrations for 1 or 24 h at 37°C. Drug treatments were ended by rinsing cells twice in preheated PBS; then they were diluted in 5 ml of preheated medium. Every 24 h, for each concentration of CPT, one separate T25 flask was taken, and the cells were trypsinized and counted for 4 days using a Coulter Counter (Coulter Electronics Inc., Hialeah, FL). Growth inhibition was calculated as: N3-Co/C3-Co, where N3 is the number of cells in the CPT-treated sample at day 3, C3 the number of control cells at day 3, and C0 the cell number at day 0, before drug treatment. When the amount of cells was less than the initial number seeded, the growth inhibition appeared as more than 100%, reflecting the cytotoxic effect.

Cell Cycle Determination. Cells were harvested, washed in ice-cold PBS (pH 7.4), fixed in 70% ethanol, washed twice in ice-cold PBS, and treated with RNase (30 min at 37°C; 500 units/ml; Sigma Chemical Co., St. Louis, MO); cellular DNA was stained with 500 μl of 50 μg/ml propidium iodide. Cells

¹ The abbreviations used are: CPT, camptothecin; topi, topoisomerase I; NCI, National Cancer Institute; DPC, DNA protein cross-links; GI₅₀, concentration of CPT that inhibits 90% growth.

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were stored at 4°C prior to analysis. Cell cycle determinations were performed using a Becton Dickinson fluorescence-activated cell analyzer, and data were interpreted using the SFIT model program provided by the manufacturer. Results represent the mean of triplicate determinations in which a minimum of 15,000 cells were assayed for each determination.

**Northern Blot Analysis of DNA Topi.** Immobiloblots were performed as described previously (22, 23). The whole cell lysates from each cell line were subjected to gradient 4–12% SDS-PAGE, and the proteins were transferred to a nitrocellulose filter (Immobilon-P; Millipore Corporation, Bedford, MA). The filters were incubated overnight at 4°C in PBS containing 5% (w/v) milk and mouse monoclonal topi antibodies (C21), kindly provided by Dr. Yung-chi Cheng (Yale University, New Haven, CT; Ref. 24). Membranes were then incubated for 1 h in PBS with sheep peroxidase-conjugated secondary anti-mouse IgM antibody (Amersham, IL). Development was performed according to the manufacturer specifications using Amersham films.

**Immunoblot Analysis of DNA Topi.** Immunoblots were performed as described previously (25). mRNA was isolated using oligo-d(T) column (oligotex-DT; Qiagen, Chatsworth, CA). Two µg mRNA were subjected to electrophoresis in a 1% agarose gel containing 0.66 M formaldehyde and then transferred to a nylon membrane (Duralose-UV; Stratagene, La Jolla, CA). The filter was hybridized with each 32P-labeled cDNA probe in 50% formamide at 43°C for 24 h. Human topi cDNA (T18) used as a probe was kindly provided by Dr. William C. Earnshaw (Johns Hopkins University School of Medicine, Baltimore, MD; Ref. 25). Human G3PDH cDNA probe was purchased from Clontec (Palo Alto, CA) and used as internal standard. The filter was then washed twice in 2X SSC-0.1% SDS and then twice in 0.5X SSC-0.1% SDS at 65°C [1X SSC = 0.15 M NaCl-0.015 M sodium citrate (pH 7.0)]. Membranes were scanned using Phosphorimage (Molecular Dynamics, Sunnyvale, CA), and quantification was performed using IMAGE QUANT Ver.3.22. The expression level of topi was calculated as the ratio between pixels of the topi band and those of the corresponding G3PDH band.

**CPT Uptake.** This technique was similar to that used previously by Pomnier et al. (27). Briefly, 107 cells were incubated in 10 ml medium for 1 h in presence of 1 µM mixture of unlabeled and labeled CPT. At the end of the treatment, cells were scraped, centrifuged, and resuspended in 1 ml of CPT-containing medium. Nine hundred µl of the cell suspension was layered above 0.5 ml of silicone oil (Versitube F50; General Electric Co., Waterford, NY) in a microcentrifuge tube and centrifuged for 5 min at 12,000 X g. The bottom of the tube was cutoff and placed in a liquid counting scintillation vial. The cell pellet was solubilized by the addition of 2.5 ml of 0.4 M NaOH and incubated overnight at 37°C before counting.

**Determination of Drug-stabilized Cleavable Complexes Frequency.** Nondeproteinizing, DNA-denaturing alkaline elution was used to determine the amount of DPC as described previously (28, 29). Briefly, colon cells were labeled with 0.02 µCi/ml of [14C]thymidine for 1 to 2 doubling times at 37°C. All cells were then chased in nonradioactive medium for at least 4 h before drug treatment. After a 1-h CPT treatment, the cells were irradiated on ice with 30 Gy prior to elution. Cells were gently layered onto polyvinylchloride-acryllic copolymer filters (Metricel DM-800; Gelman Sciences, Ann Arbor, MI) and lysed using a solution containing 0.2% sodium sarkosyl-2 M NaCl-0.04 M EDTA (pH 10.0) in the absence of proteinase K. CPT concentrations were maintained constant until cell lysis to avoid topi-cleavable complex reversal that is known to take place even at 4°C after drug dialysis (30). DNA was eluted with tetrapropylammonium hydroxide-EDTA (pH 12.2) without SDS. Fractions were collected at 3-h intervals for 15 h. DPC frequencies were calculated according to the bound-to-one terminus model and expressed in rad-equivalents (28, 29).

**RESULTS**

**Differential Sensitivity of the Colon Cell Lines of the NCI Anticancer Drug Screen Cell Screen and Time-dependence of Camptothecin-induced Growth Inhibition.** Fig. 1 shows growth inhibition assays after 1 and 24 h of exposure to CPT. The GI50 was 14- to 40-fold higher after 1-h exposure compared to 24-h exposure. The ranking of the cell lines was the same in both assays, and after a 1-h exposure to CPT, the KM12 cells were 17 times more resistant than the colo205 cells (Table 1). Three cell lines, colo205, HCT116 and SW620, were very sensitive, with GI50 (24 h) less than 0.03 µM and GI50 (1 h) less than 0.6 µM CPT. Two cell lines, HT29 and HCC2998, were intermediate, with GI50 (24 h) between 0.045 to 0.065 µM and GI50 (1 h) between 0.7 and 0.9 µM CPT. Two cell lines were more resistant, HCT15 and KM12, with GI50 (24 h) higher than 0.1 µM CPT and GI50 (1 h) around 3 µM CPT.

**Topi mRNA and Protein Expression and Cell Cycle Kinetics**

**Do Not Predict Cell Sensitivity to CPT.** As shown in Table 1, the range of S-phase fraction was 38–52% within these cells. High S-phase fraction (more than 45%) was observed both in sensitive cells (SW620) or in the most resistant cells (KM12). Low S-phase fraction could be associated with high sensitivity (colo205) or resistant phenotype (HCT15). Doubling times were not correlated with CPT sensitivity either (Table 1).

**Topi detection by Western blot analysis showed the expected M, 100,000 size in all cell lines with minor proteolysis products (Fig. 2). Little variation was seen among the seven cell lines.**
expression did not correlate with sensitivity since the HCT116-sensitive cells and the resistant KM12 had the same protein expression (Table 1). Therefore, top1 expression does not predict cell sensitivity in this panel of cell lines. Even when taking into account both top1 expression and S-phase fraction, cell sensitivity could not be predicted since KM12 had both the highest top1 expression and highest S-phase fraction, while SW620 had the same S-phase fraction but a lower top1 expression.

Top1 mRNA levels were measured by Northern and dot-blot analysis and normalized to the HT29 cell line top1 mRNA level (Fig. 2; Table 1). The predicted 4.1-kilobase size was observed for top1 mRNA in all cell lines (data not shown). In agreement with the top1 protein levels, top1 mRNA levels exhibited little (approximately 30%) variation among the cell lines, in contrast with the large range of sensitivity (Fig. 1; Table 1). The relative top1 mRNA levels were not correlated with CPT sensitivity; when compared to the HT29 cells, the most sensitive cells could have higher levels (colo205) or lower levels (HCT116 and SW620) of top1 mRNA. In contrast, the most resistant cell line, KM12, had the same level of top1 mRNA than the HT29 cells and only 15% less than the most sensitive colo205 cell line.

Top1 mRNA and Protein Expression and CPT Uptake Are Not Correlated with Top1 Cleavable Complexes. Top1 activity was assayed by measuring the amount of drug-sensitive (chromatin-associated, enzyme associated with cleavable complexes (or DPC) in cells by alkaline elution. Fig. 3 shows the dose-response curves of CPT-stabilized cleavable complexes in the seven cell lines over a wide range of CPT concentration. Generally, the more sensitive cells had high frequencies of DPC, while the more resistant cells had low frequencies. The range in DPC frequency within the seven cell lines was best detected at 10 μM CPT and reached 2.5-fold (788 to 1900 rad-eq). The colo205 cells had a particular behavior, with a highest frequency of DPC between 0.1 and 1 μM and a plateau above 0.5 μM CPT.

While top1 mRNA and protein expressions varied only within a factor of 2 in the seven cell lines, the amount of cleavable complexes could vary by 5.3-fold at 0.5 μM CPT (Fig. 3). Thus, the relationship between top1 mRNA/protein expression and the amount of cleavable complexes was very different from one cell line to another. The top1 mRNA level was well correlated with cleavable complexes in the HCC2998 and HCT15 cells (low expression, low amount of cleavable complexes). On the other hand, SW620 had low top1 mRNA and protein levels with high amounts of cleavable complexes. For three cell lines, the relationship depended on the concentration of camptothecin used; for colo205, top1 mRNA and cleavable complexes at low concentrations of CPT were relatively high, while cleavable complexes tended to plateau above 0.5 μM CPT. HT29 and KM12 had similar top1 mRNA expression and comparable cleavable complexes up to 1 μM CPT, while at high concentrations of CPT, HT29 produced 1.6-fold more cleavable complexes than KM12.

Top1 protein levels were consistent with top1 mRNA levels (Table 1; Fig. 2). However, the KM12 cell line which exhibited a low level of cleavable complexes (Fig. 3) and the lowest sensitivity to CPT (Table 1; Fig. 1) had the highest top1 protein level (Fig. 2). Taken together, these results demonstrate no relationship between top1 mRNA or protein expression and top1-induced cleavable complexes in cells.

CPT uptake was determined using [3H]CPT. Table 1 shows the results from at least two independent experiments. CPT uptake varied only within a factor of 2 in the colon cell lines. The sensitive HCT116
cells had a 2-fold higher CPT uptake. This was associated with high cleavable complexes and might participate to their high sensitivity. On the other hand, SW620, another sensitive cell line, had the lowest CPT uptake. The other cell lines did not differ significantly one from the other. Therefore, no simple relationship could be drawn either between CPT uptake and cleavable complexes or between CPT uptake and CPT sensitivity.

Log-linear Relationship between Frequency of CPT-induced Cleavable Complexes and Cytotoxicity. Fig. 4 represents the amount of cleavable complexes as a function of growth inhibition over a wide range of CPT concentrations. Taken all the cell lines together, in spite of their various sensitivity to CPT, the best representation was log-linear. When the concentration of drug increased, the amount of stabilized cleavable complexes was enhanced and so did the growth inhibition. More than 100% growth inhibition was found at the highest drug concentrations for the most sensitive cell lines, as the number of cells counted 72 h after drug treatment was less than the number of seeded cells.

The log-linearity of this relationship indicates that identical increases in cleavable complexes have different consequences on toxicity, depending on the range of cleavable complexes. The same increase in DNA damage is associated with a higher increase in toxicity in the lower range than in the higher range of cleavable complexes. Moreover, low amounts of cleavable complexes could be enough to induce strong growth inhibition.

However, close examination of the data shows that additional factors are probably involved. For instance, approximately 100 rad-equivalent cleavable complexes (corresponding to approximately one cleavable complex/10^6 nucleotides; Refs. 28 and 29) induced 40% decrease in DNA damage as compared to untreated cells. SW620 and COLO205, very sensitive cell lines, had similar CPT uptake. However, the amount of CPT-stabilized cleavable complexes was found to be different. SW620 had the lowest CPT uptake. This was associated with high production of cleavable complexes and might participate to their high sensitivity. On the other hand, COLO205 and SW620, another sensitive cell line, had the lowest CPT uptake. The other cell lines did not differ significantly one from the other. Therefore, no simple relationship could be drawn either between CPT uptake and cleavable complexes or between CPT uptake and CPT sensitivity.

In this study, using the colon cell lines from the NCI Anticancer Drug screen, we have found a log-linear relationship between CPT-induced DNA cleavable complexes and growth inhibition. Top1 mRNA and protein expression and S-phase fraction were not predictive for sensitivity to CPT. Moreover, we have confirmed the time-dependency of CPT-induced toxicity and that optimum toxicity can be achieved with low CPT concentration for a long treatment period.

The time-dependence of the camptothecin-induced growth inhibition appears in the experiments comparing the toxicity of 1 versus 24 h exposure to CPT. Twenty-four h exposure required about 14 to 40 times lower concentrations of CPT to achieve similar cytotoxicity. These data are in agreement with the model of cytotoxicity of CPT proposed earlier (19–21) that only the cleavable complexes that are reached by moving replication forks lead to DNA damage. This model provides arguments to administer CPT derivatives by continuous infusion rather than bolus administration (2, 3, 31). However, 1 h exposure to CPT inhibited the growth of a larger cell fraction than expected from the fraction of cells in S-phase. We had already noticed this apparent discrepancy in studies with CPT and its more potent derivative, 10,11-methylenedioxy camptothecin in HT29 cells and suggested that S-phase fraction, as defined by flow cytometry, probably underestimates the fraction of cells synthesizing DNA (32). S-phase fraction also was not correlated with CPT cytotoxicity in cell lines with similar top1 levels. This is in agreement with studies on xenografts models in which CPT derivatives have shown high efficiency despite the low S-phase fraction usually observed in these models (3). Hence, CPT may also be toxic outside of S-phase, through collisions between top1-cleavable complexes and DNA repair processes which physiologically appear at any phase of the cell cycle. This possibility would be consistent with the top1 down-regulation observed after ionizing radiation-induced DNA damage and its correlation with better survival (33).

Top1 expression has been found to be decreased in CPT-resistant cell lines selected after exposure to the drug (24, 34, 35). However, selection might introduce a bias, since to select a highly resistant cell line, one top1 allele is expected to be silent or lost while the other is mutated (51); therefore, for experimental reasons, top1 expression is expected to be decreased. Here, we used a panel of cell lines which exhibit natural differences in sensitivity to CPT. This situation should be clinically more relevant. According to the replication fork collision model (21), it is expected that the greater the S-phase fraction and the higher the top1 expression, the more likely CPT will be toxic. However, we found that top1 mRNA and protein expressions are not predictive for cell sensitivity; low top1 expression could be associated with high sensitivity to CPT (colon205 cells), and high expression could be observed with resistance (KM12 cells). Our findings in the colon cell lines is in opposition with previous observations, which suggested a good predictive value of top1 expression for cellular or tumor sensitivity (36, 37). Differences between cell type is not surprising, taking into account the importance of individual downstream signaling and repair pathways (21). Both top1 expression and S-phase fraction are probably too much upstream from the lethal events to have any predictive value in many cases. The ability of the cells to repair the CPT-induced DNA damage and their sensitivity to undergo apoptosis are also critical parameters for CPT sensitivity.

Top1 mRNA and protein expression were not found to predict the amount of CPT-induced cleavable complexes, the latter being the best predictors of CPT cytotoxicity. Differential CPT uptake might explain, in part, higher detection of cleavable complexes in HCT116 cells. However, no correlation between CPT uptake and the amount of CPT-stabilized cleavable complexes was found since SW620 and
KM12 cells had same cleavable complexes from 0.01 to 5 μM CPT, while KM12 had higher CPT uptake. Since the discrepancy between top1 expression and top1-cleavable complexes could not be explained by differential CPT transport, this observation underlies the importance of posttranslational modifications for top1 regulation. Dephosphorylation abolishes catalytic activity and CPT sensitivity (38, 39, 40, 41), while phosphorylation by protein kinase C increases the formation of top1-cleavable complexes (41). Poly(ADP-ribosylation) inhibits top1 catalytic activity (42–45), and 3-aminobenzamide, a poly(ADP ribose) inhibitor, reduces the amount of CPT-induced cleavable complexes (46). The complexity of top1 regulation is also exemplified by the importance of chromatin structure that limits and regulates the association of top1 with its DNA target. Cleavable complexes may be prevented to form and/or change their chromatin distribution depending upon nucleosome structure (47), concentrations of polyamines (48), and DNA methylation (49). Therefore, it is important to bear in mind, for clinical trials of CPT and its derivatives, that top1 expression, although convenient for measurement, may not be well correlated with drug efficacy.

In contrast to top1 expression, the amount of functional top1-cleavable complexes, measured in cells by alkaline elution, was a relatively good parameter to predict sensitivity to CPT; the higher was the amount of cleavable complexes stabilized by the drug, the greater was the cell sensitivity. This parameter might be interesting to consider to monitor drug sensitivity of individual patients. However, alkaline elution is a long method that works best when cellular DNA is labeled (28) and, therefore, cannot be transferred to the clinic. Others methods to measure drug-induced DNA damages are warranted. Several authors have suggested to use PCR techniques and have shown that, in very precise experimental conditions, the reduction of DNA amplification is proportional to the amount of DNA damage (50).

In conclusion, we are using the cell lines of the NCI anticancer screen as a tool (miniclinic) to study the cellular determinants for sensitivity to CPT and its derivatives. In vivo cleavable complexes were the best top1 phenotypic parameter to predict cell sensitivity to CPT in this panel of colon cell lines. The log-linear relationship between CPT-induced DNA damage and survival and the differences in CPT toxicity observed after 1 and 24 h exposure suggest that increases in drug exposure are probably more cytotoxic and more clinically relevant than dose escalation.

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