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Differential Requirement of DNA Replication for the Cytotoxicity of DNA Topoisomerase I and II Inhibitors in Chinese Hamster DC3F Cells

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ABSTRACT

The cytotoxicity of topoisomerase inhibitors is thought to result from the induction of enzyme-mediated DNA breaks. The fact that these breaks reverse rapidly in cells programmed to die, led us to investigate further the cytotoxic mechanisms of topoisomerase I (camptothecin) and topoisomerase II inhibitors (VP-16 and amsacrine) in Chinese Hamster lung fibroblasts (DC3F). Exposures (30 min) to camptothecin produced limited cell killing with approximately 20% of the cells naturally resistant. This resistance was overcome by increasing the drug exposure time. Inhibition of DNA synthesis by 5-min pretreatments with aphidicolin or hydroxyurea abolished the cytotoxicity of camptothecin without changing the level of camptothecin-induced DNA breaks. A good correlation was found between the degree of DNA synthesis inhibition by aphidicolin and the reduction of camptothecin cytotoxicity. In similar experiments performed with topoisomerase II inhibitors, aphidicolin prevented only partially against VP-16- and amsacrine-induced cytotoxicities, yet had no effect upon drug-induced DNA breaks. These results indicate that the production of topoisomerase-mediated DNA breaks by antitumor drugs is not sufficient for cell killing. Instead, an interference of moving DNA replication forks with drug-stabilized topoisomerase-DNA complexes is critical for cell death. The cytotoxicity of camptothecin seemed to be completely related to this process, while that of topoisomerase II inhibitors seemed to involve additional mechanisms in DC3F cells.

INTRODUCTION

DNA topoisomerase II inhibitors are among the most active anticancer agents (Adriamycin, VP-16,[2] VM-26, *m*-AMSA, ellipticines). Although much has been learned in the recent years about the molecular effects of topoisomerase II inhibitors (1–6), the exact mechanism of the cytotoxicity of the drugs remains unknown. Topoisomerase II inhibitors induce enzyme-linked strand breaks (1–6), which can be detected in cells as protein-linked DNA strand breaks (5–11). Nevertheless, the drug-induced DNA breaks, whose frequency is correlated with lethality, reverse in cells which are programmed to die (5–11). The breaks could be sites of DNA recombination (9, 11), but they could also form steric blocks for DNA processing enzymes, such as DNA replication complexes.

Camptothecin has been identified recently as a specific topoisomerase I inhibitor (12–14). As for topoisomerase II inhibitors, the cytotoxicity of camptothecin is correlated with its ability to induce enzyme-mediated DNA breaks (15–18). Topoisomerase I levels are nearly constant during cell cycle, while those of topoisomerase II are high in S phase and proliferating cells (19, 20). In addition, the strong activity of camptothecin in animal solid tumor models (21), has stimulated the recent development of water-soluble camptothecin derivatives as anticancer agents (22, 23).

The present study was undertaken in order to analyze the mechanism of the cytotoxicity of drug-induced topoisomerase-DNA complexes. Since such complexes could form steric blocks for DNA processing enzymes, we tested whether stopping DNA replication fork by brief treatments with the DNA polymerase inhibitor, aphidicolin (24), or with hydroxyurea would affect the cytotoxicity of inhibitors of topoisomerase I (camptothecin) or topoisomerase II (VP-16 and amsacrine). Drug-induced DNA strand breaks were measured by alkaline elution in order to monitor the relationship between topoisomerase-mediated DNA breaks and cytotoxicity. The present study indicates that, particularly in the case of camptothecin, the cytotoxicity of drug stabilized topoisomerase-DNA complexes may result from their interference with moving DNA replication forks.

MATERIALS AND METHODS

Drugs and Materials. Camptothecin (NSC 94600), *m*-AMSA (NSC 249992), and VP-16 (NSC 122819) were obtained from the Drug Synthesis and Chemistry Branch, National Cancer Institute. Aphidicolin and hydroxyurea were purchased from Sigma Chemical Co., St. Louis, MO. Hydroxyurea and VP-16 (10 mM) were dissolved immediately before use in culture medium and dimethyl sulfoxide, respectively. VP-16 was further diluted in H_2O at 0.1 mM. Aphidicolin, camptothecin, and amsacrine stock solutions were made and kept frozen in dimethyl sulfoxide at 10 mM. They were thawed and diluted in H_2O as needed. [^{14}C]Thymidine and [^{3}H]thymidine were purchased from New England Nuclear (Boston, MA). Tissue culture medium and fetal calf serum were purchased from ABI (Columbia, MD) and GIBCO (Grand Island, NY), respectively.

Cell Culture and Drug Treatments. Chinese Hamster lung fibroblasts DC3F cells were a gift from Dr. A. Jacquemin-Sablon (Institut Gustave Roussy, Villejuif, France). Cells were grown in Eagle's minimum essential medium supplemented with 10% fetal calf serum, 0.1 mM nonessential amino acids, 2 mM glutamine, 1 mM sodium pyruvate, 20 mM HEPES, and penicillin/streptomycin, at 37°C in the presence of 5% CO_2. DC3F cells had a doubling time of 12–18 h (8).

All experiments were performed with log phase DC3F cells which had been seeded 12–15 h before at approximately 3×10^5 cells per 25-cm^2 flask in 5 ml of medium. Camptothecin, VP-16, or amsacrine treatments were 30 min, unless otherwise indicated (Fig. 1*B*). Treatments with DNA synthesis inhibitors (aphidicolin & hydroxyurea) were started 5 min before camptothecin, VP-16, or amsacrine treatments, continued throughout the ensuing 30 min, and were ended at the same time as the camptothecin, VP-16, or amsacrine treatments (35 min total treatment time). Dimethyl sulfoxide was added to control cell cultures at the concentration present in the treatment flasks. The dimethyl sulfoxide concentration never exceeded 1% and did not affect cell survival.

Clonogenic Assays. At the end of drug treatments, cells were washed three times with 10 ml warm medium, trypsinized, suspended in warm medium, and counted. 10^2, 10^3, and 10^4 cells were seeded in triplicate into 25-cm^2 flasks in 5 ml of medium for each treatment condition. Cell cultures were incubated for 5–7 days, after which they were washed twice with 10 ml phosphate buffered saline. Colonies were fixed with 95% methanol (15 min), stained with methylene blue (20 min), and counted. The plating efficiency of drug-treated cells was divided by the plating efficiency of untreated cells, to yield a "survival fraction." Results were expressed as the (−)log of the survival fraction (8). The plating efficiency for untreated cells was 70–90%.

DNA Synthesis Inhibition. Cellular DNA was prelabeled by incubating the cell cultures with 0.005–0.01 μCi/ml [^{14}C]thymidine for approximately 18 h. The rate of DNA synthesis was measured by 5-min

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[2] The abbreviations used are: VP-16, etoposide; VP-26, teniposide; *m*-AMSA, amsacrine; HBSS, Hank's balanced salt solution.

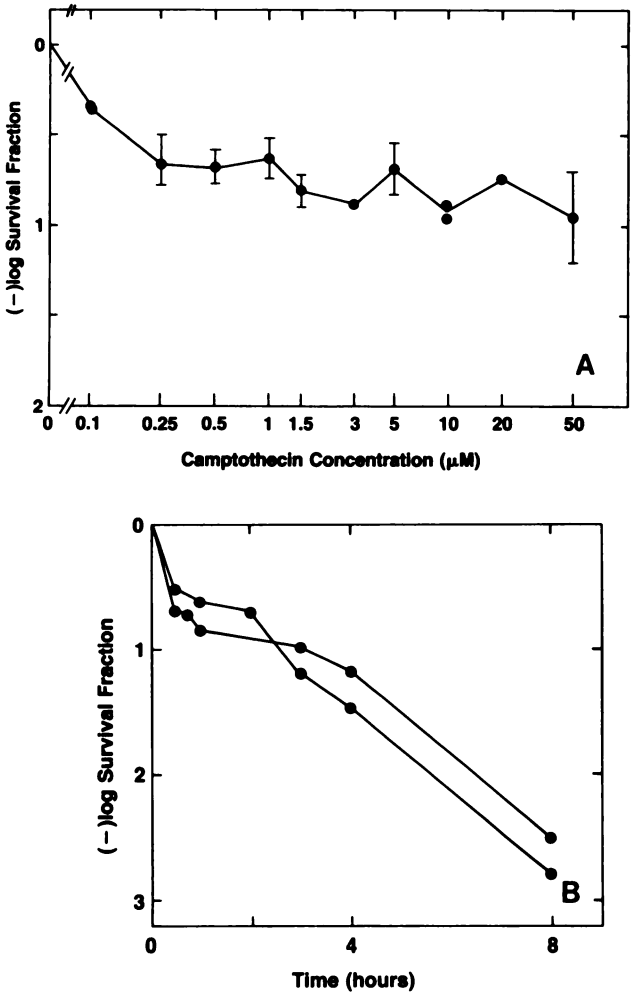

Fig. 1. Cytotoxicity of camptothecin in chinese hamster DC3F cells. *A*, concentration-dependence of 30-min treatments at 37°C. Error bars denote standard deviation of at least three independent experiments. *B*, time-dependence of camptothecin-induced cytotoxicity in DC3F cells. Cells were treated with 0.25 μM camptothecin for the indicated time periods. Two independent experiments are shown.

pulses with 1 μCi/ml [^{3}H]thymidine. Thymidine incorporation was determined as follows: cells were washed twice with ice-cold HBSS, and then scraped on ice into 3 ml HBSS. One-ml aliquots of this suspension were added to 100 μl 100% trichloroacetic acid in duplicate, vortexed, and centrifuged for 6 min in an Eppendorf centrifuge, all at 0–4°C. The precipitates were dissolved with 1 ml 0.4 M NaOH, and counted by liquid scintillation spectrometry. DNA synthesis inhibition was determined as the ratio of [^{3}H]/[^{14}C] in the treated samples over the ratio of [^{3}H]/[^{14}C] in the untreated samples.

Measurement of DNA Single-strand Breaks by Alkaline Elution. The alkaline elution methodology has been described in previous publications (5–11, 14). Briefly, [^{14}C]thymidine-labeled cells (0.02 μCi/ml for 18 h prior to drug treatments) were treated with camptothecin, amsacrine, or VP-16 for 30 min at 37°C. Cell cultures were then dispersed by scraping into 10 ml ice-cold HBSS. In the case of camptothecin the same drug concentration as for drug treatment was used in all the dilution tubes in order to avoid the reversal of the DNA breaks at 0°C (14). Cell suspensions were then loaded onto polycarbonate filters (2-μm pore diameter, Nucleopore Corp., Pleasanton, CA). When the HBSS containing the ^{14}C-labeled-treated cells had dripped through, approximately 0.5 ml ice-cold [^{3}H]thymidine labeled L1210 (internal standard) cells, which had been irradiated with 2000 rads, were loaded. Lysis was immediately performed with sodium dodecyl sulfate and proteinase K (sodium dodecyl sulfate-ProK lysis solution). Elution was with tetrapropylammonium hydroxyl:EDTA, 0.1% sodium dodecyl sulfate, pH 12.1. Fractions were collected every 5 min for 30 min (10, 14). DNA single-strand breaks were expressed in rad-equivalents.

RESULTS

Cytotoxicity of Camptothecin in DC3F Cells. Thirty-min treatments with camptothecin produced limited cytotoxicity with less than one log cell killing (Fig. 1*A*). Drug concentration dependence was only seen at low concentrations (0.1 and 0.25 μM). Increasing camptothecin concentrations above 0.25 μM did not increase cell killing significantly, even when the camptothecin concentration was 200-fold higher (Fig. 1*A*). Therefore 20–30% of the DC3F cells were naturally resistant to 30-min exposures to camptothecin.

Camptothecin-resistance was overcome by increasing the time of drug exposure. An almost linear relationship was found between the cytotoxicity of 0.25 μM camptothecin and time of exposure (Fig. 1*B*). Approximately 3 logs of cell killing were observed after 8-h exposures. These results are in agreement with the previous observation that camptothecin is most cytotoxic in S-phase cells (25), since increasing the time of exposure to camptothecin would increase the fraction of S-phase cells exposed to the drug.

Protective Effect of DNA Synthesis Inhibition against Camptothecin-induced Cytotoxicity. First, it was determined that 35-min exposures of DC3F cells to 10 μM aphidicolin inhibited thymidine incorporation by more than 90% (Table 1) within the first 5 min of drug exposure. DNA synthesis inhibition reversed within 2 h after aphidicolin removal (Table 1). Under these conditions, aphidicolin produced minimal cell killing (survival fraction above 0.8) (Figs. 2 and 4, *top*).

The protocol subsequently chosen to study the effects of

Table 1 *Persistent inhibition of DNA synthesis by topoisomerase inhibitors in Chinese hamster DC3F cells; comparison with aphidicolin and hydroxyurea*

Drug treatments were for 30 min at 37°C. DNA synthesis (expressed as a fraction of control) was determined by measuring trichloroacetic acid precipitable [^{3}H]thymidine after a 5-min pulse at the end of drug treatments. Each value is the mean of two independent determinations.

	Time following drug removal (h)				
	0	0.5	1	2	6
Camptothecin (0.25 μM)	0.54	0.50	0.37	0.44	0.61
VP-16 (5 μM)	0.68	0.44	0.31	0.36	ND[a]
Aphidicolin (10 μM)	0.07	0.20	0.40	0.78	
Hydroxyurea (100 μM)	0.23	0.95			

[a] ND, not determined.

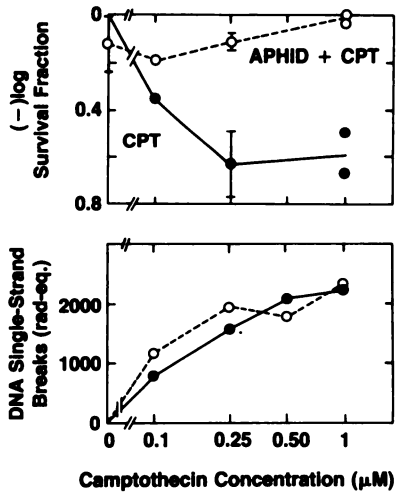

Fig. 2. Effects of aphidicolin on camptothecin-induced cytotoxicity (*top*) and DNA single-strand breaks (*bottom*) in DC3F cells. Cells were either treated with camptothecin alone (——, ●) or camptothecin and aphidicolin (– – – –, ○). Aphidicolin treatments (10 μM) were started 5 min before the addition of camptothecin and continued throughout the 30-min camptothecin treatments. DNA breaks were assayed by alkaline elution immediately at the end of drug treatments; a typical experiment is shown in the lower panel. Error bars denote standard deviations of at least three independent cloning experiments.

DNA synthesis inhibition upon the cytotoxicity and the DNA breaks induced by topoisomerase inhibitors was the following: DC3F cells were treated with 10 μM aphidicolin; 5 min later, camptothecin, VP-16, or amsacrine were added while aphidicolin was kept in the culture medium; finally, drugs were removed from the cell culture simultaneously after 30 min of coincubation. Under these conditions, DNA synthesis was inhibited during the entire time that drug-induced DNA breaks were present, since the breaks induced by camptothecin, amsacrine, and VP-16 form within a few min after drug addition and reverse within 30 min after drug removal (5, 7, 9, 10, 13, 14).

Ten micromolar aphidicolin protected from camptothecin cytotoxicity (Fig. 2, *top*). The protection was complete at camptothecin concentrations lower than 5 μM. It was only partial at higher concentrations.

Camptothecin-induced DNA single-strand breaks were studied in parallel cultures. Fig. 2 (*bottom*) shows the result of a typical experiment. Aphidicolin pretreatment did not affect camptothecin-induced DNA break frequency. Therefore, a dissociation between camptothecin-induced cytotoxicity and DNA breaks was produced by aphidicolin.

In order to confirm that the effect of aphidicolin was directly due to DNA synthesis inhibition, various aphidicolin concentrations producing different degrees DNA synthesis inhibition were used. Fig. 3 shows that the greater the inhibition of DNA synthesis, the better the protection, and that an almost linear relationship existed between the rate of DNA synthesis and camptothecin-induced cytotoxicity (Fig. 3). Additional experiments with hydroxyurea showed also significant protection from camptothecin-induced cytotoxicity (data not shown). Thus, DNA synthesis inhibition during the time of exposure to camptothecin blocked camptothecin cytotoxicity without affecting the formation of DNA breaks.

Effect of Aphidicolin against VP-16- and Amsacrine-induced Cytotoxicity. The same type of experiments were performed with the topoisomerase II inhibitors, VP-16, and amsacrine. As observed in the case of camptothecin, aphidicolin did not affect VP-16-induced DNA break frequency (Fig. 4, *bottom*). However, in contrast to the case of camptothecin, aphidicolin protected only partially against VP-16-induced cytotoxicity (Fig. 4, *top*). Similar results were obtained with amsacrine (data not shown). Increasing the aphidicolin concentration did not prevent the cytotoxicity of topoisomerase II inhibitors further but resulted in a significant cytotoxicity of aphidicolin. These results indicate differences between the cytotoxic mechanism(s) of topoisomerase II and I inhibitors.

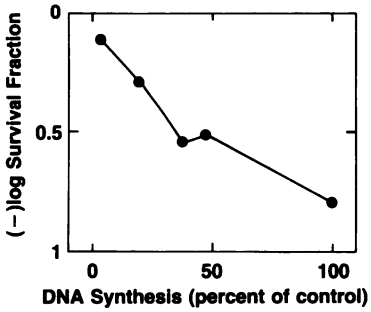

Fig. 3. Relationship between camptothecin-induced cytotoxicity and DNA synthesis in DC3F cells. DNA synthesis was inhibited with different aphidicolin concentrations (0.075–10 μM). Aphidicolin was added 5 min before camptothecin (0.25 μM) and continued throughout the 30-min camptothecin treatments. Duplicate treatments were performed; one set was used for clonogenic assays, and the other for [^{3}H]thymidine pulses (1 μCi/ml for 5 min) in order to monitor DNA synthesis. Each point represents the mean of three determinations for survival and two determinations for DNA synthesis.

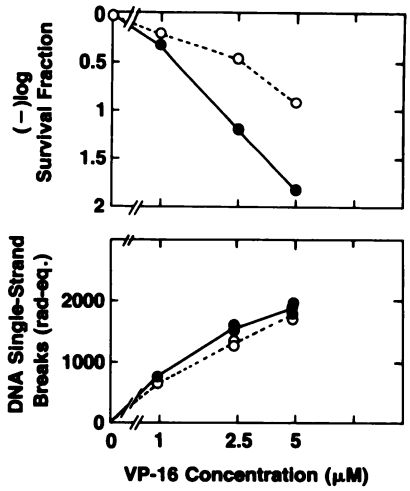

Fig. 4. Effects of aphidicolin on VP-16-induced cytotoxicity (*top*) and DNA single-strand breaks (*bottom*) in DC3F cells. Cells were either treated with VP-16 alone (——, ●) or with VP-16 and aphidicolin (– – – –, ○). Aphidicolin treatments (10 μM) were started 5 min before the addition of VP-16 and continued throughout the 30 min VP-16 treatments. DNA breaks were assayed by alkaline elution. A typical experiment is shown.

Inhibition of DNA Synthesis by Topoisomerase Inhibitors. The requirement of active DNA synthesis for the cytotoxicity of camptothecin suggested that drug-stabilized topoisomerase I-DNA complexes could form lethal blocks for DNA replication forks. The effects of topoisomerase inhibitors upon DNA replication were then tested in comparison with aphidicolin and hydroxyurea (Table 1). Both camptothecin and VP-16 inhibited DNA synthesis. However, this inhibition was never complete even at high drug concentrations.[3] In addition, by contrast to aphidicolin and hydroxyurea, DNA synthesis inhibition was not reversible upon drug removal. This is in contrast with the drug-induced DNA strand breaks induced by topoisomerase inhibitors which reverse within 30 min after drug removal (5, 7, 9, 10, 13, 14). These results show that topoisomerase inhibitors produce a prolonged inhibition of DNA synthesis and suggest that drug-stabilized topoisomerase-DNA complexes interfere with DNA replication complexes.

DISCUSSION

Topoisomerase I inhibition by camptothecin appears to result from the induction of stable topoisomerase I-DNA complexes (12), which can be detected as DNA single-strand breaks by alkaline elution (13, 14). Three types of studies strongly suggest that topoisomerase I-mediated DNA strand breaks are responsible for the cytotoxicity of camptothecin. First, topoisomerase I mutant yeast cells are camptothecin resistant (16, 17). Secondly, human lymphoblastic leukemia cells made resistant to camptothecin fail to produce DNA breaks upon exposure to the drug and have a camptothecin-resistant topoisomerase I (18). Finally, a good correlation is found between the antitumor activity of camptothecin analogues and their inhibitory activity against purified topoisomerase I (15). However, the rapid reversibility of the DNA single-strand breaks after drug removal (13, 14), suggests that the breaks induce irreversible and lethal cellular alterations. The present results showing that aphidicolin protected against the cytotoxicity of camptothecin and yet did not affect camptothecin-induced DNA cleavage indicate that ongoing DNA replication is necessary for camptothecin-induced cytotoxicity. This result is in agreement with those of Horwitz and Horwitz (25) showing that the cytotoxicity of

[3] J. M. Covey and Y. Pommier, unpublished results.

camptothecin is maximum in S-phase cells, while the DNA break frequencies are comparable in S- and G_1-phase cells. Therefore the cytotoxicity of camptothecin-induced topoisomerase I-DNA complexes appears to require active DNA replication.

Topoisomerase I seems to be in close proximity of the DNA replication apparatus (26). As in the case of transcription, it could act as a swivel and relieve the torsional tension that accumulates near a moving replication fork (1, 27). However, analysis of yeast topoisomerase mutants indicates that topoisomerase II can substitute for topoisomerase I during DNA replication since topoisomerase I mutants replicate and are viable (28, 29). Therefore, it seems unlikely that an accumulation of torsional tension within replicating DNA could explain the cytotoxicity of camptothecin. A more attractive possibility is that moving DNA replication forks collide with covalent topoisomerase I-DNA complexes immobilized by camptothecin. Such a collision could lead to the dissociation of replication complexes. This would explain the nonreversible inhibition of DNA synthesis by camptothecin (Table 1).

DNA topoisomerase II is also likely to be in close proximity of the DNA replication apparatus (1). However, the protective effect of aphidicolin against VP-16- and amsacrine-induced cytotoxicity was only partial. These results are in agreement with those of Chow *et al.* (30) and Charcosset *et al.* (31), showing that aphidicolin and hydroxyurea protected partially against the cytotoxicity of topoisomerase II inhibitors, while cycloheximide gave a more complete protection. This partial protection suggests that drug-induced topoisomerase II-mediated DNA breaks could be lethal in the absence of ongoing DNA synthesis. DNA recombinations and mutations at the sites of drug-induced topoisomerase II-DNA complexes could be responsible for this effect (9, 11).

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