Proliferation-dependent Topoisomerase II Content as a Determinant of Antineoplastic Drug Action in Human, Mouse, and Chinese Hamster Ovary Cells

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ABSTRACT

We have shown previously that quiescent Chinese hamster ovary (CHO) cells are less sensitive than log phase CHO cells to the cytotoxic and DNA cleavage effects of etoposide, a drug which appears to act via DNA topoisomerase II. This loss of sensitivity was associated with a decrease in topoisomerase enzyme activity in nuclear extracts of the quiescent cells. We have now extended our observations by examining the basis for the reduction in enzyme activity during quiescence. DNA topoisomerase II content, as assayed by immunoblotting with a polyclonal rabbit anti-topoisomerase II antiserum, was virtually absent in nuclear extracts of quiescent CHO cells in contrast to logarithmically growing cells. This suggests that the previously demonstrated loss of enzyme activity in CHO cells is a function of reduction in content rather than posttranslational modifications of the enzyme. Quiescent human lymphoblastic CCRF cells also exhibited reduced topoisomerase II content compared to actively proliferating cultures, but the difference was less than that observed in CHO cells. In contrast, log and plateau phase cultures of mouse leukemia L1210 cells exhibited similar topoisomerase II content. Reduction in enzyme content correlated with the ability of these cell lines to accumulate during quiescence with a G0-G1 content of DNA. Sensitivity to the DNA cleavage effects of etoposide in dividing and nondividing cells correlated well with enzyme content. As has been observed with CHO cells, both CCRF and L1210 cells in plateau phase were more resistant to the cytotoxic effects of etoposide than those actively dividing. The result with L1210 cells was surprising, however, in light of the equivalent DNA damage observed under the two growth conditions. Our data indicate that topoisomerase II enzyme content is proliferation dependent in some but not all cells and suggest that while enzyme content may be important in drug resistance in some cell types, other factors may decrease the sensitivity of the cell to cleavable complex formation as well.

INTRODUCTION

The role of DNA topoisomerase II (EC 5.99.1.3) in intracellular genetic processes and its enzymology have recently been reviewed in detail (1, 2). Briefly, this enzyme reversibly cuts double stranded DNA and becomes covalently linked to the 5'-termini at the break site via phosphotyrosyl bonds. This intermediate DNA-enzyme “cleavable complex” is isolable upon treatment with a protein denaturant and presumably represents the reaction intermediate of the breaking-rejoining mechanism. In vivo topoisomerase II is involved in the separation of daughter chromosomes at the conclusion of DNA synthesis (3, 4), has been shown to be part of the multienzyme “reputase complex” (5), and, more recently, has been shown to be associated with the nuclear matrix in chromosome scaffolds (6, 7). It is thus thought to serve both a structural and a functional role for chromatin.

A novel enzyme role that has been demonstrated for topoisomerase II is its mediation of the effects of several of the intercalating and nonintercalating antitumor drugs (8). The former includes such clinically important compounds as Adriamycin, m-AMSA, and mitoxantrone, while the latter is principally represented by the epipodophyllotoxins etoposide and teniposide. By a mechanism which is still not clear, these drugs stabilize the cleavable complex formed between topoisomerase II and DNA, resulting in increased DNA scission and concomitant inhibition of the rejoicing reaction (9). This is experimentally expressed as the formation of protein-associated DNA breaks when assayed by the alkaline elution technique (10).

Both topoisomerase II activity and drug sensitivity have been shown to vary as a function of cell proliferation (11–14), although this may not be uniform for all cell lines. For instance, this laboratory has shown previously that Chinese hamster ovary cells become resistant to the cytotoxic and DNA cleaving effects of etoposide and m-AMSA during quiescence and that this correlates well with topoisomerase enzyme activity in nuclear extracts from these cells (14). Since cleavable complex formation requires the presence of topoisomerase II and appears to correlate well with drug cytotoxicity, it is important to determine the range of effects cell-proliferative status may exert on these parameters. Whether the changes observed by us and others are due to alterations in enzyme content or result from posttranslational modifications of the enzyme has not been clear. The point is especially relevant in light of recent evidence that topoisomerase II can undergo phosphorylation (15) or poly(ADP)ribosylation (16) with very different effects on enzyme activity. Furthermore, the generality of our observations in CHO cells is unclear, since preliminary data suggest that certain malignant cells may differ in their sensitivity to etoposide-induced cleavable complex formation under the two growth conditions. In this paper, we have extended our previous work by determining the basis for the previously observed reduction in enzyme activity during quiescence, and we demonstrate for the first time that cells may differ in their regulation of topoisomerase II content as a function of proliferation. In addition, we have examined in greater detail the relationship of proliferation to sensitivity to the DNA cleavage and cytotoxic effects of etoposide. Our results indicate that although drug-induced DNA cleavage may occur during quiescence in some cells, the cytotoxic effect of these cleavable complexes is, nonetheless, attenuated, suggesting that other factors are involved in the lethal event.

MATERIALS AND METHODS

Mouse leukemia L1210 cells and human lymphoblastic CCRF cells were grown in suspension cultures in RPMI 1630 medium with 20% fetal calf serum and α-minimal essential medium with 10% fetal calf serum, respectively. α-Minimal essential medium, with 5% fetal calf serum, was used to grow wild type CHO cells in suspension cultures.

Received 9/22/86; revised 2/26/87, 4/30/87; accepted 5/7/87.

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1This work was supported by USPHS Grants CA-24586 and CA-40884.

2To whom requests for reprints should be addressed.

1The abbreviations used are: m-AMSA, 4′-(9-acridinylamino)methanesulfonamide; etoposide, 4′-demethylepipodophyllotoxin 4(4,6-O-ethylidene-β-D-glucopyranoside); PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol; CHO, Chinese hamster ovary.
All media were obtained from Gibco Laboratories, Grand Island, NY. CCRF and CHO cells were grown in the presence of 5% CO₂, and all three cell lines were incubated at 37°C with penicillin (100 IU/ml) and streptomycin (100 μg/ml) added to the culture media. Cell numbers were determined by a Coulter Counter and viability was determined by trypan dye exclusion. All cell lines were cultured from initial seeding through plateau growth without replenishment of the media. For all three cell lines, the term “plateau phase” implies that (a) there has been no net increase in cell number over the previous 24 h period, (b) DNA synthesis, as measured by thymidine incorporation, is less than 10% of that exhibited by logarithmically growing cells, and (c) cell viability is equivalent to that of logarithmically growing cells.

[¹⁴C]Thymidine (57 mCi/mmoll was obtained from ICN (Irvine, CA). [³H]Thymidine (20 Ci/mmol) was from New England Nuclear (Boston, MA). Safety-Solve was obtained from Research Products International Corporation (Mt. Prospect, IL). Moravek Biochemicals, Inc. (Brea, CA) supplied [¹⁴C]Hepetidine (200 mCi/mmoll. Etoposide was obtained from Bristol-Myers Co. (St. Louis, MO). Bethesda Research Laboratories (Gaithersburg, MD) supplied PMSF, DT, and glycerol. Reagents for sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot electrophoresis were obtained from Bio-Rad Laboratories (Richmond, CA).

Drug Treatment. Plateau phase L1210 or CCRF cells were radiola
deled during log phase growth with [¹⁴C]thymidine. At different stages of cell growth, the medium was replaced with fresh medium at 37°C for 1 h, and experimental cell flasks were then treated with various concentrations of etoposide for 60 min at 37°C. Etoposide was incubated in dimethyl sulfoxide, and control cells were treated with dimethyl sulfoxide alone. After incubation with the drug, the cells were washed two to three times with cold phosphate-buffered saline (via centrifugation in a Beckman TJ-6R centrifuge at 2000 rpm for 10 min), resus
pended in cold media, and kept on ice to preclude repair of DNA strand breaks. Etoposide-induced DNA single strand break frequency was then determined by alkaline elution.

Alkaline Elution. Both DNA high single strand break frequency and low single strand break frequency were determined by the alkaline elution technique (17, 18). [³H]Thymidine-labeled L1210 or CCRF cells were used as an internal standard.

Cytotoxicity Assays. Drug-induced cytotoxicity was assessed for both log and plateau phase cells by colony-forming assays in soft agar. CCRF and L1210 suspension cultures were grown to log (2-4 x 10⁸ cells/ml) and plateau (1.5-2 x 10⁸ cells/ml) densities in the presence of 5% CO₂. One x 10⁶ total cells were pelleted by centrifugation at 2500 x g for 10 min. The pellets were resuspended in 30 ml of Buffer A, centrifuged, and washed twice more with 30 ml of Buffer A. The cells were then swollen in 10 ml of Buffer B and allowed to sit on ice for 20 min. A detergent (1 ml of 10% Nonidet P-40) was added to the mixture and the mixture was gently triturated and finally left on ice for 15 min. The cells were then Dounce homogenized with 20 strokes of a tight pestle and the nuclei were collected at 2500 x g for 10 min. The pellet was resuspended in 2 ml of Buffer C and layered over 0.6 ml of Buffer D. The nuclei were then sedimented in a swinging bucket rotor at 6000 x g for 10 min, and the nuclear pellet was resuspended in 0.5 to 1 ml of Buffer E and left on ice for 15 min. An equal volume (0.5 to 1 ml) of Buffer F was added to the above solution, and this was left at 4°C for 30 min. Finally, 10% glycerol (w/v) was added to the above solution, and this was ultracentrifuged at 100,000 x g for 60 min. The supernatant from the last centrifugation contains topoisomerase II activity. This supernatant was stored at -20°C and used for the Western blot analysis.

Topoisomerase II was isolated from the nuclei of CCRF and L1210 cells which had been grown to log (2-4 x 10⁸ cells/ml) and plateau (1.5-2.5 x 10⁸ cells/ml) densities. One x 10⁶ cells from suspension cultures were pelleted by centrifugation at 1500 x g for 10 min. The pellet was resuspended in 30 ml of Buffer A, centrifuged, and washed twice more with 30 ml of Buffer A. The cells were then swollen in 10 ml of Buffer B and allowed to sit for 30 min on ice. After Dounce homogenization with 10 strokes of a tight pestle, the nuclei were collected by centrifugation at 2500 x g for 15 min. The nuclear pellet was resuspended in 2 ml of Buffer C and carefully layered over 0.6 ml of Buffer D. After centrifugation at 2000 x g for 10 min, the nuclear pellet was resuspended in 0.5 to 1 ml of Buffer J, and this solution was placed at 4°C for 15 min. Nuclear topoisomerase II was extracted by adding an equal volume (0.5 to 1 ml) of Buffer K and allowing the solution to sit on ice for 30 min. Glycerol (10%, w/v) was added to the above solution, it was ultracentrifuged, and the supernatant was stored as above. Protein concentrations of the nuclear extracts were deter
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Western Blot Analysis of Nuclear Extracts Containing Topo

RESULTS

Western Blot Analysis of Nuclear Extracts Containing Topo

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actively dividing. This observation has important implications for sensitivity to drugs such as etoposide which act via topoisomerase II. In order to determine whether this reduction in enzyme activity is a function of posttranslational modification of the enzyme or a loss of enzyme content and to determine the extent to which malignant cells differ, we have assayed topoisomerase II content by immunoblotting. DNA topoisomerase II was extracted with 1 M NaCl from the nuclei of CHO, CCRF, and L1210 cells, and equal amounts of nuclear protein were subjected to Western blot electrophoresis as described in "Materials and Methods." The results of these experiments are shown in Fig. 1. In contrast to log phase CHO cells, no topoisomerase II was detected in 50 μg of plateau phase CHO nuclear extract. Thus, etoposide-induced DNA damage and cytotoxicity appear to correlate with nuclear topoisomerase II levels in wild type CHO cells. Western blot electrophoresis of equivalent amounts of protein from topoisomerase II containing nuclear extracts of log and plateau phase CCRF cells demonstrates that there is approximately 3- to 4-fold more topoisomerase II by visual inspection in log phase cells than plateau phase cells. Western blot analysis of L1210 nuclear extracts indicates that there is no discernable difference in immunoblotted topoisomerase II between log and plateau cells.

Cell Cycle Distribution as a Function of Proliferation. The differences in topoisomerase II content observed in plateau phase from the three cell lines suggested that although all three were in plateau phase, other differences in phenotype may exist. It has long been recognized that under conditions of growth arrest, different cell lines exhibit varying degrees of ability to enter a G₀-like state. In order to examine this issue in our cells, DNA histograms, generated by flow cytometry, were obtained for all three cell lines as a function of proliferation (Table 1). Plateau phase CHO cells were found to have a relatively high G₁ fraction when compared with their log counterpart. Compared to log CCRF cells, those in plateau also have a higher fraction of cells in the G₁ compartment, although the difference is less than for CHO cells. In contrast, L1210 cells were observed to maintain the same percentage of cells in G₁, S, and G₂-M in confluent cultures as compared to those in log phase growth.

Etoposide-induced DNA Damage in Log and Plateau Phase L1210 and CCRF Cells. In order to characterize topoisomerase-mediated DNA scission in situ in malignant cells, the amount of DNA damage following etoposide exposure was quantified by alkaline elution as described in "Materials and Methods.” As seen in Figs. 2 and 3, sensitivity to etoposide-induced DNA cleavage is affected by proliferative status in CCRF cells. Cells at log and plateau densities were exposed to 1–50 μM etoposide [low sensitivity elution (Fig. 2)] or 0.1–1 μM etoposide [high sensitivity elution (Fig. 3)]. The low sensitivity alkaline elution experiments demonstrated that plateau CCRF cells remain relatively sensitive to etoposide-induced DNA damage. However, the maximum DNA damage observed in log phase CCRF cells is approximately 1000 rad equivalents greater than plateau cells. The difference in strand break frequency in log and plateau CCRF cells at low drug concentrations is emphasized in the high sensitivity alkaline elution. Decreased cell viability did not account for the differences in DNA damage observed, because both log and plateau phase CCRF cells maintained a viability ≥95%.

Mouse leukemia L1210 cells were also examined for etoposide sensitivity. Log and plateau phase L1210 cells were treated with 1–50 μM etoposide [low sensitivity elution (Fig. 4)] or 0.1–1 μM etoposide [high sensitivity elution (Fig. 5)]. These experiments demonstrated, in contrast to CHO and CCRF cells, that plateau phase L1210 cells maintain the same drug sensitivity as compared with their log counterpart when assayed by either a high or low sensitivity alkaline elution. This correlates well

![Fig. 1. Western blot electrophoresis of nuclear extracts from log and plateau phase L1210, CCRF, and CHO cells. All three cell lines were grown in suspension cultures to log and confluent numbers, and topoisomerase II was extracted with 1 M NaCl from the nuclei of these cells. Equivalent amounts of nuclear protein were loaded on the gel; in this experiment, each lane represents 50 μg of protein. These experiments demonstrated that log (Lane 1) and plateau (Lane 2) phase L1210 cells have approximately equal concentrations of nuclear topoisomerase II, that log phase CCRF cells (Lane 3) have more nuclear topoisomerase II than confluent CCRF cells (Lane 4), and that plateau phase CHO cells (Lane 6) have undetectable amounts of nuclear topoisomerase II in comparison to log phase CHO cells (Lane 5).](image)

![Table 1 Cell cycle distribution by flow cytometry as a function of proliferation](image)

<table>
<thead>
<tr>
<th>% in G₁</th>
<th>% in S</th>
<th>% in G₂-M</th>
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<tbody>
<tr>
<td>Log</td>
<td>Plateau</td>
<td>Log</td>
</tr>
<tr>
<td>L1210 cells</td>
<td>36.5</td>
<td>38.9</td>
</tr>
<tr>
<td>CCRF cells</td>
<td>46.3</td>
<td>65.8</td>
</tr>
<tr>
<td>CHO cells</td>
<td>51.0</td>
<td>81.2</td>
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![Fig. 2. Low sensitivity alkaline elution of human lymphoblastic CCRF cells treated with etoposide (VP-16). Suspension cultures of log and plateau phase CCRF cells were exposed to etoposide for 60 min, and the amount of DNA damage was quantified as DNA single strand breaks. DNA scission is expressed as the equivalent radiation dose which would result in that degree of strand breakage. Points, means; Bars, SD.](image)
Fig. 3. High sensitivity alkaline elution of human lymphoblastic CCRF cells treated with etoposide (VP-16). Log and plateau phase CCRF cells were treated with lower doses of etoposide for 60 min, and the amount of DNA damage was quantified by an alkaline elution procedure that detects 50–1000 rad equivalents. Plateau phase cells are clearly less sensitive to etoposide than log phase cells.

Fig. 4. Low sensitivity alkaline elution of mouse leukemia L1210 cells treated with etoposide (VP-16). Log and plateau phase L1210 cells were exposed to low concentrations of etoposide, and the amount of DNA damage was quantified by the high sensitivity alkaline elution procedure. These curves demonstrate that even at low etoposide concentrations, there is a minimal difference in drug-induced DNA damage between log and plateau phase L1210 cells.

Fig. 5. High sensitivity alkaline elution of mouse leukemia mouse L1210 cells treated with etoposide (VP-16). L1210 cells, at different stages of proliferation, were exposed to low concentrations of etoposide, and the amount of DNA damage was quantified by the high sensitivity alkaline elution procedure. These curves demonstrate that even at low etoposide concentrations, there is a minimal difference in drug-induced DNA damage between log and plateau phase L1210 cells.

with Western blot analysis of enzyme content. The cell viabilities for both log and plateau phase L1210 cells were >95%.

Uptake of [3H]Etoposide by Log and Plateau Cells. To determine if the differences in etoposide-induced DNA scission in log and plateau cells were a function of drug uptake, appropriate cells were exposed to [3H]etoposide as described under “Materials and Methods.” Cells were exposed to two to four different drug concentrations (10–50 μM etoposide) in a minimum of two separate experiments for each log and plateau density. Within a 10% error, there were no differences between log and plateau phase cell drug uptake for CCRF cells or L1210 cells (data not shown).

Etoposide-induced Cytotoxicity of CCRF and L1210 Cells. Our next objective was to correlate etoposide-induced cytotoxicity in CCRF and L1210 cells with DNA damage and enzyme content and to compare this to our previously published data with CHO cells (14). In that work, we demonstrated a near total absence of cytotoxicity in plateau phase cells following exposure to etoposide. In our current work, cytotoxicity assays of drug-treated log and plateau phase CCRF cells (Fig. 6) show that a correlation exists between strand break frequency and survival. Plateau phase CCRF cells, which are moderately sensitive to etoposide-induced DNA damage, were found to be moderately sensitive to the cytotoxic effects of this drug.

An unexpected observation was encountered in the cytotoxicity assays of etoposide-treated L1210 cells. Log and plateau density L1210 cells have nearly identical drug-induced DNA strand break frequency. However, their sensitivity to etoposide cytotoxicity is markedly different (Fig. 7). At 20 μM etoposide, there is approximately a 2-log difference in survival. Thus,
etoposide-induced strand break frequency does not appear to correlate well with cytotoxicity in L1210 cells.

DISCUSSION

In this extension of our previous work, we have compared in detail three cell lines which differ in their degree of growth regulation. We have been able to show that topoisomerase II activity, as measured by drug-induced cleavage, and enzyme content, as measured by immunoblotting, are not uniformly diminished in all cell lines during quiescence. Furthermore, although we find that drug-induced DNA breakage correlates well with enzyme content, other factors are apparently important in determining cytotoxicity. Our data have clinical implications, since they suggest that regulation of topoisomerase content may differ as a function of the malignant phenotype.

The proliferation dependence of topoisomerase activity has been demonstrated previously by a number of investigators. Duguet et al. found a 10-fold increase in topoisomerase II activity in nuclear extracts from regenerating rat liver following partial hepatectomy (11) and an 18-fold stimulation in topoisomerase II in nuclear extracts from concanavalin A-stimulated guinea pig lymphocytes (13). Stimulation of human fibroblasts and Swiss/3T3 mouse fibroblasts by epidermal growth factor results in increased topoisomerase II activity as well (12). Our laboratory has also shown that quiescent Chinese hamster ovary cells exhibit diminished topoisomerase II activity by decatenation assays and by sensitivity to etoposide and m-AMSA (14).

None of these aforementioned studies, however, assayed topoisomerase content. The importance of this fact is underscored by the observation that phosphorylation of topoisomerase II can stimulate enzyme activity (15) while poly(ADP)ribosylation inhibits it (16). Earnshaw et al. (6) reported that in MSB-1 cells stained with an anti-topoisomerase II antibody and analyzed by flow cytometry, the only cells which failed to stain with antibody were found to have a G1 content. They did not, however, examine proliferation per se. Our results strongly suggest that loss of enzyme content is the major reason for the observed decreased activity seen in quiescent cells. While we cannot exclude the possibility that posttranslational modifications of the enzyme occur as well, in general, enzyme content correlates closely with activity in our hands.

In addition to the issue of posttranslational modifications of the enzyme, we believe that assaying topoisomerase II content is important because of the number of factors which can interfere with assays of enzyme activity in these crude extracts. For example, both sodium chloride and potassium chloride can decrease enzyme activity in vitro at concentrations greater than 150 mM, and ATP is similarly inhibitory at concentrations greater than 1.5 to 2 mM (20, 21). Furthermore, a number of proteins other than topoisomerase II are present in nuclear extracts, and some of these have been shown previously to affect enzyme activity (22, 23). Decatenation and catenation assays are particularly subject to influence by extraneous elements in the reaction mixture because they are bimolecular. That only a 4-fold difference in decatenation of kinetoplast DNA (enzyme...
activity) was observed in log versus plateau phase CHO cells (14) while a much greater difference in immunoblotted topoisomerase II (enzyme content) was found is most reasonably explained by attenuation of activity by the salt and other factors present in the crude nuclear extract. Our results, therefore, emphasize the importance of measuring enzyme content. We have used nuclear extracts to measure enzyme content in log and plateau phase cells. One obvious concern regarding such studies is the possibility that extractable topoisomerase II may not adequately reflect cellular content. We believe that this is not the case for two reasons: (a) immunoblotting of the residual nuclei following salt extraction resulted in the detection of no enzyme; (b) immunoblotting of sonicated whole cells resulted in the same pattern as that observed with nuclear extracts (data not shown).

Our results represent the first demonstration that topoisomerase II content may not be regulated the same in all cell types. In the limited number of cell lines we have examined, it would appear that topoisomerase content correlates well with the ability of the cell to accumulate with a G0-G1 DNA content during quiescence. In this regard, it may behave similarly to other enzymes which are found in diminished concentrations when cells enter quiescence (24). Since there is a great deal of variability in growth regulation among tumor cells and in particular their ability to arrest in G0 under conditions of high density or serum deprivation (25), we anticipate that there will likewise be considerable heterogeneity among tumors in their topoisomerase content. Further work will be required to determine if factors other than cell cycle control are involved in regulating the amount of enzyme in the cell.

There is substantial evidence that cleavable complex formation induced by intercalating agents and epipodophyllotoxins generally correlates well with drug cytotoxicity. Structure-activity relationships of closely related epipodophyllotoxins (26) and aminocaridines (27) both demonstrate a close correlation between the ability of the agents to induce cleavable complex formation and their cytotoxicity. Interference with the cleavable complex formation induced by etoposide strongly inhibited the cytotoxicity of the agent as well (28). Finally, several cell lines which are highly resistant to epipodophyllotoxins and intercalating agents also exhibit decreased cleavable complex formation in response to the drugs and altered topoisomerase II activity in vitro (although the enzyme itself may not necessarily be altered) (19, 29, 30). In general, our data agree with prevailing concepts regarding cytotoxicity and cleavable complex formation. CHO and CCRF cells exhibit fewer strand breaks and less cytotoxicity in plateau phase. Our experiments with L1210 cells, however, are somewhat divergent. Since sensitivity to strand breakage is equal in log and plateau phase, it is surprising that plateau phase cells are more resistant to drug cytotoxicity than log phase L1210 cells. From a practical standpoint, the data suggest that topoisomerase content (and, therefore, strand breakage) may not uniformly predict tumor sensitivity to intercalating agents and epipodophyllotoxins in the nonproliferative fraction. From a theoretical viewpoint, however, the data reiterate the concept that events beyond the cleavable complex formation may be important in the expression of lethality. Brief exposure to etoposide or several different classes of intercalating agents can result in considerable cell killing in spite of the fact that the cleavable complexes which result from this drug exposure are transient, being virtually totally resealed within 30 to 120 min following drug removal (17, 31, 32). It is thus likely that the cleavable complex formation sets into motion a series of events which culminates in cell death. We suggest that elucidation of the mechanism of L1210 cell resistance during plateau phase may shed light on the response of the cell to cleavable complex formation.

ACKNOWLEDGMENTS

The authors wish to acknowledge the excellent assistance of Alexandre Q Fair in the preparation of this manuscript.

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PROLIFERATION DEPENDENCE OF TOPOISOMERASE II


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