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# CELL DEATH INDUCED BY TOPOISOMERASE INHIBITORS

## ROLE OF CALCIUM IN MAMMALIAN CELLS

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**Abstract**—Although the stabilization of topoisomerase II cleavable complexes by etoposide (VP-16) has been recognized to be important for cell killing, the lethal events following the formation of cleavable complexes remain to be elucidated. In an attempt to characterize the biochemical requirements for VP-16-induced cytotoxicity, we examined the effects of calcium depletion in Chinese hamster DC3F cells. Four-hour preincubation in calcium-free medium or in complete medium containing 5 mM [ethylenebis(oxyethylenenitrilo)]tetraacetic acid (EGTA) protected against the cytotoxicity of VP-16. Under these same conditions, the VP-16-induced DNA single-strand break frequency in calcium-depleted cells remained similar to that of control cells. Cell-cycle analysis and thymidine pulse incorporation indicated that calcium depletion did not alter DNA synthesis and cell cycle distribution. Drug-induced cytotoxicity was restored progressively within 4–8 hr after calcium-depleted cells were refed with calcium-containing medium. Calcium depletion also protected against the cytotoxicity of camptothecin, hyperthermia and, to a lesser extent, nitrogen mustard and gamma radiation in DC3F cells. Similar results were obtained in human colon carcinoma HT-29 cells. Our results suggest that topoisomerase II-mediated DNA breaks are only potentially lethal and that calcium-dependent cellular processes are required for the cytotoxicity of topoisomerase inhibitors.

Mammalian DNA topoisomerase II is the target of a wide group of drugs currently used in cancer chemotherapy. Anthracyclines, ellipticines, mitoxantrone, amsacrine and etoposide (VP-16) inhibit topoisomerase II by inducing the formation of "cleavable complexes" [1]. These are covalent enzyme–DNA complexes associated with DNA single- and double-strand breaks. They can be detected as protein-linked DNA strand breaks in drug-treated cells [2]. Camptothecin also induces protein-linked DNA single-strand breaks in mammalian cells [3, 4]. However, camptothecin-induced DNA breaks result from topoisomerase I inhibition [5, 6].

VP-16-induced DNA breaks, like those produced by most other topoisomerase II inhibitors and camptothecin, have been recognized to be important for cell killing although they are rapidly reversible after drug removal [7]. This observation strongly suggests that the formation of cleavable complexes is only potentially lethal and that the cytotoxicity of drug-induced topoisomerase-mediated DNA breaks results from further irreversible cellular lesions.

Programmed cell death (apoptosis) has been implicated in the cytotoxicity of a variety of agents including glucocorticoids, tumor necrosis factor, and cytotoxic lymphocytes [8–13]. Furthermore, treatment of target cells with calcium chelators, such as [ethylenebis(oxyethylenenitrilo)]tetraacetic acid (EGTA), or with a calcium ionophore, has been shown to protect against hormone- and cytotoxic T cell-induced cytotoxicity. Recently, topoisomerase inhibitors have also been reported to induce internucleosomal DNA degradation, suggesting that they may also induce apoptosis [14].

In the present study, we examined the effects of calcium depletion on VP-16-induced cytotoxicity in Chinese hamster lung fibroblasts (DC3F cells) and in human colon carcinoma (HT-29) cells. We found that cytotoxicity was greatly diminished in cells that had been incubated in calcium-depleted medium for 2–4 hr. The reduced cytotoxicity was not due to an effect on topoisomerase II-mediated DNA strand breaks since VP-16-induced DNA breaks were similar in normal and calcium-depleted cells. Calcium was also found to be required for the cytotoxicity of camptothecin and hyperthermia and to a lesser extent for nitrogen mustard and gamma radiation.

### MATERIALS AND METHODS

*Chemicals.* Etoposide (VP-16) was provided by Bristol Laboratories (Syracuse, NY). Fresh solutions (10 mM) were prepared in dimethyl sulfoxide immediately prior to each experiment. Camptothecin and nitrogen mustard were obtained from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute (Bethesda, MD). Camptothecin and nitrogen mustard were dissolved at 10 mM in dimethyl sulfoxide and 0.1 N HCl, respectively, and kept as stock solutions at −80°. Aphidicolin, cordycepin and EGTA were

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purchased from the Sigma Chemical Co. (St. Louis, MO). Radiolabeled nucleotides were purchased from New England Nuclear (Boston, MA). All other chemicals were of reagent grade and purchased from either Sigma or other local sources.

*Cell culture and calcium-depleting treatments.* DC3F Chinese hamster lung fibroblasts, 023 cells (a subclone of the Chinese hamster lung fibroblast cell line CCL 39), subclone 2.2 (023 cells transfected with the human HSP27 gene) [15], and human colon carcinoma HT-29 cell lines were grown in Eagle's Minimum Essential Medium (MEM) (ABI, Columbia, MD) supplemented with 10% heat-inactivated fetal bovine serum (GIBCO, Grand Island, NY), 2 mM glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids (MEM), 100 units penicillin/mL and 100 μg streptomycin/mL (ABI), at 37° in the presence of 5% $CO_2$. Calcium-free medium was made identical to the Eagle's Minimum Essential Medium from an Earle's balanced salt solution (ABI) without calcium and was supplemented with 1 mM EGTA to chelate traces of calcium contained in fetal bovine serum [16]. HL-60 cells were grown in suspension in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, 100 units penicillin/mL and 100 μg streptomycin/mL. Fifteen hours before all experiments, exponentially growing cells were plated onto 25 $cm^2$ flasks ($3 \times 10^5$ cells/flask).

*Gamma irradiation and heat-shock treatments.* Single cell suspensions in Eagle's Minimum Essential Medium with or without EGTA were irradiated with a $^{137}Cs$ source at a dose rate of approximately 6 Gy/min at room temperature prior to cell survival assays. For heat-shock treatments, culture flasks of exponentially growing cells were immersed in a 45° water bath. Immediately after treatment, cells were trypsinized and plated in fresh medium at 37°.

*Colony formation assay.* Cell cultures were washed twice with 10 mL of Hanks' balanced salt solution (37°) and trypsinized; $10^2$, $10^3$ and $10^4$ cells were plated in triplicate in 25 $cm^2$ flasks with 5 mL of fresh medium. Colonies were grown for 5–7 days for DC3F, 023, and 2.2 cells (doubling times of 12–14 hr), and for 12–14 days for HT-29 cells (doubling times of 40–44 hr). Culture flasks were then washed with ice-cold phosphate-buffered saline (PBS), fixed with methanol (95%), and stained with methylene blue (0.05%) [17]. Results are expressed as survival fractions which were calculated by dividing the number of colonies in the treated flasks by the number of colonies in control flasks. Plating efficiencies of control cells were 70–90% for DC3F cells, 40–60% for HT-29 cells, and 30–50% for 023 and 2.2 cells.

*Measurements of DNA single-strand breaks by alkaline elution.* Briefly, cellular DNA was labeled with [$^{14}C$]thymidine (0.02 μCi/mL) for 15 hr. Label was chased for at least 4 hr in isotope-free medium prior to drug treatment. Following treatment, cells were scraped in their culture medium and mixed in 10 mL of ice-cold Hanks' balanced salt solution with internal standard, an aliquot of $^3H$-labeled L1210 cells subjected to a fixed dose of gamma radiation (20 Gy) as described previously [18]. DNA single-strand breaks were then assayed by alkaline elution under deproteinizing conditions [19]. DNA single-strand break frequencies were expressed in rad-equivalents.

*Measurement of thymidine and uridine incorporation.* DC3F cells were prelabeled with [$^{14}C$]thymidine (0.005 μCi/mL) for 24 hr and then chased for an additional 15 hr in isotope-free medium. Cells were then incubated in complete medium with or without EGTA. Rates of nucleotide incorporation were measured by 10-min pulse experiments with [$^3H$]thymidine (1 μCi/mL) or [$^3H$]uridine (10 μCi/mL). Nucleotide incorporation was stopped by removing the isotope-containing medium and by adding 10 mL of ice-cold PBS. Cell cultures were then quickly scraped on ice, pelleted by centrifugation (500 *g*, 5 min, 4°) and washed twice with ice-cold PBS. Acid-insoluble nucleotides were precipitated with 10% trichloroacetic acid. The precipitates were dissolved in 0.4 N NaOH, and radioactivity was monitored by scintillation spectrometry. Results are expressed as the ratio of [$^3H$]/[$^{14}C$] for treated cells over the ratio of [$^3H$]/[$^{14}C$] for untreated cells [20]. Additional controls were performed by adding to the medium either 10 μM aphidicolin (DNA synthesis inhibitor [20]) or 5 μM cordycepin (RNA synthesis inhibitor [21]) for 30 min before pulses.

*Cell cycle analysis.* Exponentially growing cells in complete medium with or without 5 mM EGTA were scraped, harvested by centrifugation, washed twice with ice-cold PBS, and incubated on ice for 1 hr in 70% ethanol. Following fixation, cells were washed twice with ice-cold PBS and incubated at 25° for 1 hr in PBS containing 500 units/mL of RNase A. Cells were again washed twice with PBS, resuspended, and kept at 4° in 250 μL PBS prior to analysis. Cell-cycle analysis was performed using a fluorescence-activated cell analyzer (Becton Dickinson), and data were interpreted using the Cellfit model program.

*Analysis of DNA fragmentation by agarose gel electrophoresis.* At the end of the incubation period following drug treatment, cells were scraped into their culture medium, harvested by centrifugation, and washed twice with ice-cold PBS. The DNA was extracted by a salting-out procedure. Briefly, cells were incubated for 16 hr at 48° in 0.5 M Tris (pH 9.0) containing 20 mM EDTA, 10 mM NaCl, 1% sodium dodecyl sulfate (SDS) and 0.5 mg/mL proteinase K. Following incubation, the salt concentration (NaCl) was raised to 1 M, and tubes were shaken vigorously. Samples were centrifuged (30 min, 500 *g*). Supernatants were collected, 2 vol. of ethanol (95%) was added, and DNA was precipitated. DNA electrophoresis was performed for 14 hr at 2 V/cm in 1.2% agarose gel in Tris-borate buffer (pH 8.0). DNA was visualized after electrophoresis by ethidium bromide staining.

## RESULTS

*Effects of calcium depletion on VP-16-induced cytotoxicity.* To examine the calcium dependence of the cytotoxic effect of VP-16, DC3F cells were

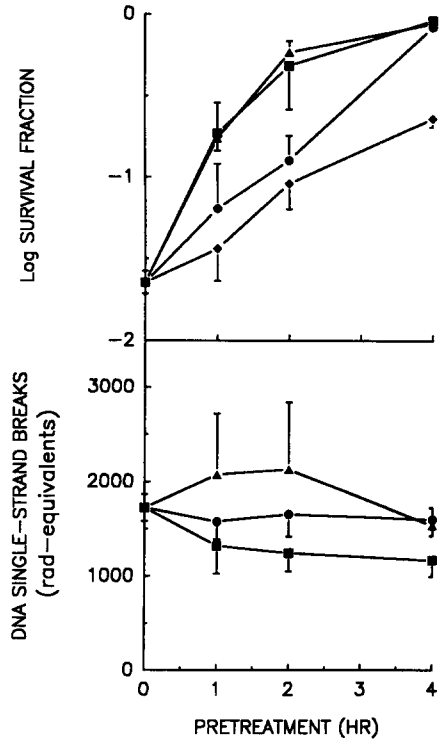


Fig. 1. Time-dependent effect of calcium depletion on VP-16-induced cytotoxicity (upper panel) and DNA single-strand breaks (lower panel). DC3F cells were preincubated either in complete medium containing EGTA at 2 mM (◆), 5 mM (●) or 10 mM (■) or in calcium-free medium (▲). At the indicated times, 5 μM VP-16 was added to the cultures for 30 additional min. Cytotoxicity was measured by colony formation assays and DNA single-strand breaks by alkaline elution. Points and vertical bars represent means and standard deviations of four independent experiments.

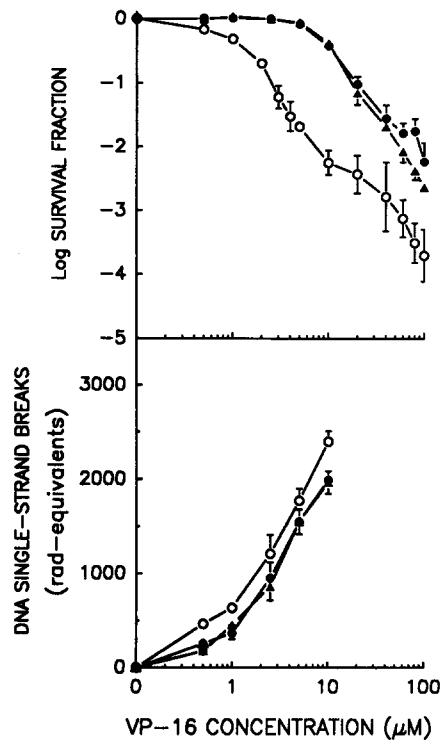


Fig. 2. Effect of calcium depletion on VP-16-induced cytotoxicity (upper panel) and DNA single-strand breaks (lower panel). DC3F cells were preincubated in either complete medium containing no EGTA (○), 5 mM EGTA (●) or in calcium-free medium (▲) for 4 hr. The indicated VP-16 concentrations were then added to the cultures for 30 additional min. Cytotoxicity was measured by colony formation assays and single-strand breaks by alkaline elution. Points and vertical bars represent means and standard deviations of at least three independent experiments. Points without a vertical bar indicate that standard deviations were within symbol size.

preincubated either with various concentrations of EGTA or with calcium-free medium. At various times after calcium depletion, cells were treated with VP-16 for 30 min and cytotoxicity was measured by colony formation assays. As shown in Fig. 1 (upper panel), survival of VP-16-treated cells increased with time of EGTA exposure and concentration. Similar protection was produced by calcium-free medium and 10 mM EGTA in complete medium. It should be noted that EGTA concentrations equal to or above 5 mM produced detachment of a large fraction of the cells after 2 hr of incubation. In this case, detached cells were collected by centrifugation and were plated along with the attached cells in the colony formation assays. EGTA preincubation produced no detectable loss of cell number or viability since the plating efficiency of cells treated with EGTA was similar to that of untreated cells.

Next, the extent of protection produced by calcium depletion was tested over a range of VP-16 concentrations (Fig. 2, upper panel). Maximum protection was evident at VP-16 concentrations lower than 10 μM. Above 10 μM VP-16, an exposure that produced approximately 2 logs of cell kill in the presence of calcium, the VP-16-induced cytotoxicity curves in the absence and presence of calcium were parallel. Thus, over a broad dosage of VP-16, calcium depletion conferred protection which ranged from 1 to 2 logs of cell killing.

*Effects of calcium depletion on DNA single-strand breaks induced by VP-16.* We next examined the effect of calcium depletion on VP-16-induced DNA single-strand breaks. As illustrated in Figs. 1 and 2 (lower panels), various preincubation times in the presence of 5 mM EGTA or in calcium-free medium prior to VP-16 treatment did not alter significantly the VP-16-induced DNA single-strand break frequency. DNA double-strand breaks were also measured under these conditions and were found to be similar in control and in calcium-depleted cells. Therefore, calcium depletion produced by 5 mM EGTA or calcium-free medium had no significant effect on VP-16-induced DNA single- and double-strand breaks.

In contrast, treatment of DC3F cells with 10 mM EGTA for 4 hr reduced VP-16-induced DNA single-strand break frequency by 40% (Fig. 1, lower panel).

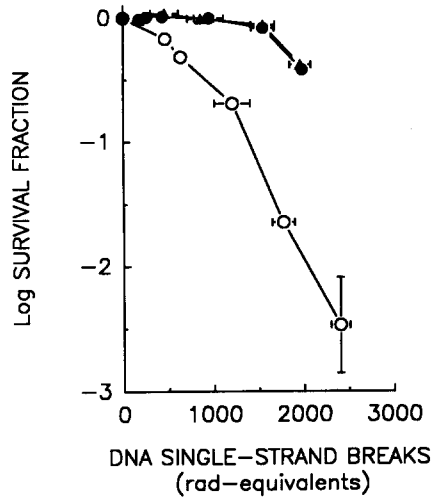


Fig. 3. Relationship between VP-16-induced cytotoxicity and DNA single-strand breaks in DC3F cells treated in complete medium containing no EGTA (○), 5 mM EGTA (●) or in calcium-free medium (▲). EGTA pretreatments were for 4 hr prior to the addition of VP-16 (0.5 to 10 μM) for 30 min. Survival fraction and DNA single-strand breaks were then measured at a given VP-16 concentration as described in Materials and Methods. Points and bars represent the means and standard deviations of three independent experiments. Points without a vertical bar indicate that standard deviations were within symbol size.

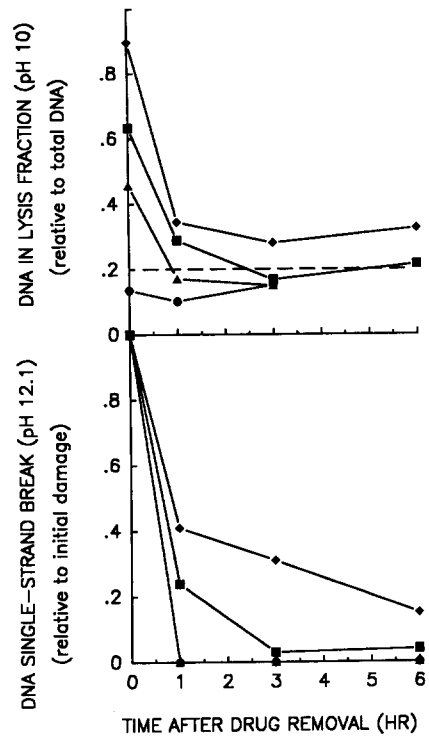


Fig. 4. Reversal of VP-16-induced DNA damage in DC3F cells. DC3F cells were treated with 1 μM (●), 5 μM (▲), 10 μM (■) and 50 μM (◆) VP-16 for 30 min. At the indicated times after drug removal, the fraction of DNA in the lysis solution (upper panel) and DNA single-strand breaks (lower panel) were measured by alkaline elution. The dashed line in the upper panel represents the fraction of DNA in the lysis solution of control cells.

However, this reduction was probably due to reduced intracellular accumulation of VP-16 as verified by experiments checking the effect of EGTA concentrations on drug uptake (data not shown).

The dissociation between VP-16-induced single-strand breaks and cytotoxicity produced by calcium depletion was observed at various VP-16 concentrations (0.5 to 10 μM) (Fig. 2, lower panel). In Fig. 3, survival fractions were plotted as a function of DNA single-strand breaks in order to show more clearly the dissociation between VP-16-induced DNA single-strand breaks and cytotoxicity. Clearly, VP-16-induced DNA strand breaks appear to be only potentially lethal since their occurrence was insufficient for cell killing when cells were preincubated with EGTA or placed in calcium-free medium.

*Reversal of DNA lesions induced by VP-16.* The reversal of VP-16-induced DNA damage was measured by alkaline elution as the fraction of DNA in pH 10 lysis solution (Fig. 4, upper panel) and as DNA single-strand breaks (Fig. 4, lower panel) at different times after drug removal. At 1 and 5 μM VP-16, reversal was complete within 1 hr. At these VP-16 concentrations calcium depletion conferred complete protection. In contrast, at 50 μM VP-16, a concentration where calcium depletion did not confer complete protection, the DNA damage reversal was incomplete even 6 hr after drug removal. Therefore, it is possible that the partial protection conferred by calcium depletion at high VP-16 concentrations (Fig. 2) is related to the persistence of VP-16-induced DNA damage under these conditions.

*Restoration of VP-16-induced cytotoxicity after replating DC3F cells in calcium-containing medium.* The reversibility of the protective effect of calcium depletion was investigated by first incubating cells either in complete medium containing 5 or 10 mM EGTA, or in calcium-free medium for 4 hr, and then replating these cells in complete medium before testing the cytotoxicity of VP-16 at various times (5 μM for 30 min). VP-16-induced cytotoxicity was restored progressively and was complete by 6–8 hr (Fig. 5). This restoration occurred either by adding back complete medium or by adding only calcium chloride (3 mM) to the calcium-free medium (Fig. 5, inset). These results strongly suggest that calcium is the divalent cation involved in the protective effect of EGTA against VP-16-induced cytotoxicity.

*Effect of calcium depletion on the cytotoxicity of camptothecin, nitrogen mustard, gamma radiation, and heat-shock in DC3F cells.* To investigate whether the protective effect of calcium depletion was restricted to the topoisomerase II inhibitor VP-16, we performed similar experiments using the topoisomerase I inhibitor, camptothecin, the alkylating agent nitrogen mustard, gamma irradiation, and hyperthermia. As summarized in Table 1, cell treatment with 5 mM EGTA for 4 hr induced a nearly complete protection against the cytotoxicity

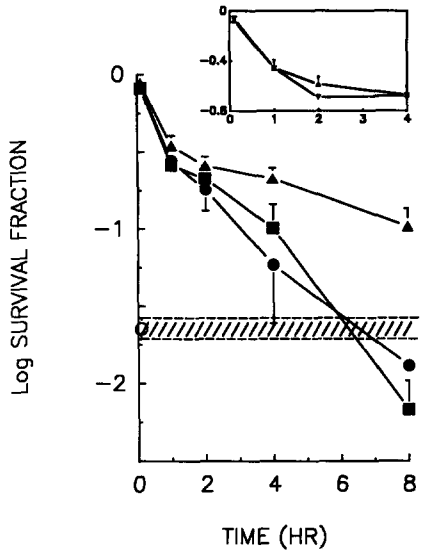


Fig. 5. Restoration of VP-16-induced cytotoxicity after transfer of DC3F cells into calcium-containing medium. DC3F cells were first incubated for 4 hr either in medium containing 5 mM EGTA (●) or 10 mM EGTA (■), or in calcium-free medium (▲) and then incubated in complete medium for various periods of time prior to VP-16 treatment (5 μM for 30 min). Cytotoxicity was measured by colony formation assays. Points and vertical bars represent the mean and standard deviations of three independent experiments. Points without a vertical bar indicate that standard deviations were within symbol size. The hatched area represents the cytotoxicity (mean ± standard deviation) of VP-16 in cells treated in complete medium without calcium depletion. Inset: same except that cells that had been preincubated for 4 hr in calcium-free medium were then incubated either in complete medium (▲) or in calcium-depleted medium to which 3 mM $CaCl_2$ had been added at time 0 (▼).

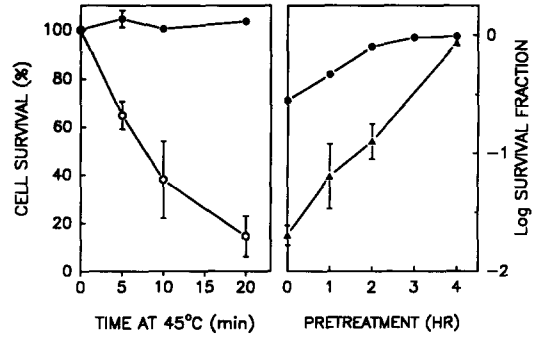


Fig. 6. Effect of calcium depletion on heat-shock-induced cytotoxicity in DC3F cells. Left panel: cells were pretreated without (○) or with 5 mM EGTA (●) in complete medium for 4 hr before heat-shock treatments. Right panel: time-dependent induction of thermotolerance by 5 mM EGTA pretreatment in DC3F cells (●). The time-dependent effect of EGTA pretreatment upon 5 μM VP-16-induced cytotoxicity (▲) is also shown as a comparison. Survival was measured by colony formation assays. Points and bars represent means and standard deviations of at least three independent experiments. Points without a vertical bar indicate that standard deviations were within symbol size.

Table 1. Effect of EGTA on the cytotoxicity of camptothecin, nitrogen mustard, and gamma radiation in DC3F cells

| | Percent cell survival | |
|---|---|---|
| | No EGTA | + 5 mM EGTA |
| Camptothecin | | |
| 0.1 μM | 62.3 ± 11.1 | 99.8 ± 3.8 |
| 1.0 μM | 26.3 ± 7.0 | 88.7 ± 11.0 |
| Nitrogen mustard | | |
| 0.1 μM | 66.2 ± 9.2 | 100.1 ± 7.2 |
| 1.0 μM | 34.2 ± 7.1 | 74.4 ± 23.1 |
| 10.0 μM | 0.8 ± 0.5 | 1.7 ± 1.6 |
| Gamma radiation | | |
| 1 Gy | 70.3 ± 7.1 | 97.6 ± 6.4 |
| 2 Gy | 59.9 ± 4.8 | 67.8 ± 6.2 |
| 3 Gy | 30.5 ± 2.2 | 38.0 ± 2.1 |
| 4 Gy | 18.5 ± 1.6 | 31.5 ± 5.5 |
| 5 Gy | 13.2 ± 2.6 | 23.0 ± 6.4 |

Cells were pretreated with 5 mM EGTA for 4 hr before gamma irradiation or 30-min treatments with camptothecin or nitrogen mustard. Cytotoxicity, expressed as percent cell survival, was assessed by colony formation assays. Values are means ± SEM, N = 6.

of camptothecin, whereas the protection was modest against nitrogen mustard and gamma irradiation.

EGTA pretreatment also induced a strong protection against various exposures to hyperthermia (45°) (Fig. 6, left panel) [22, 23]. Furthermore calcium depletion-induced thermotolerance was time-dependent, and the time course for this effect was comparable to that for protection from VP-16 cytotoxicity (Fig. 6, right panel).

*Effects of calcium depletion on cell proliferation, cell cycle, and thymidine and uridine incorporation.* Because the cytotoxicity of topoisomerase II inhibitors, such as VP-16, has been shown to be reduced in quiescent cells and in cycling cells outside S-phase [24–26], additional experiments were performed to test the effects of calcium depletion upon cell growth, replication and transcription. Preincubation of DC3F cells in complete medium containing 5 mM EGTA for 4 hr did not alter cell cycle distribution or cell proliferation (Fig. 7). DNA synthesis as measured by thymidine incorporation was not changed significantly by EGTA (Table 2). In contrast, uridine incorporation increased markedly 2 hr after addition of 5 mM EGTA (Table 2).

*DNA fragmentation following VP-16 treatment.* Because EGTA has been shown to inhibit apoptosis-associated DNA fragmentation [27, 28], and previous studies have shown that a variety of cytotoxic drugs cause apoptosis-like DNA fragmentation [13, 14], we looked for nucleosome-like DNA fragmentation in VP-16-treated DC3F cells. In the absence of EGTA, no DNA fragmentation could be detected in DC3F cells by agarose gel electrophoresis even 24 hr following VP-16 treatment. However, in HL-60 cells, which have been described as exhibiting an apoptosis-like process [14], DNA fragmentation could be visualized as early as 3 hr following drug treatment (data not shown). This fragmentation was

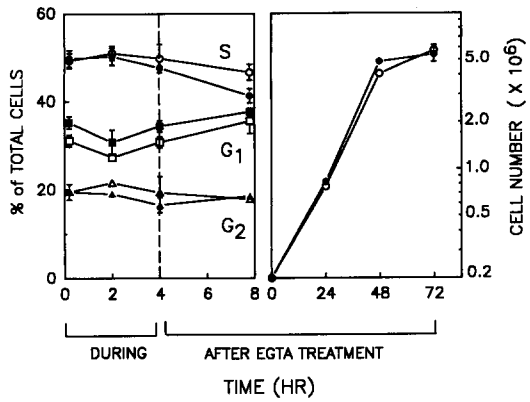


Fig. 7. Effect of calcium depletion on DC3F cell cycle distribution and cell proliferation. Left panel: cells were treated with 5 mM EGTA (closed symbols) at time 0 (untreated cells: open symbols). EGTA was removed and cells fed back in complete medium after 4 hr (dashed vertical line). At the indicated times, cells were collected and cell-cycle analysis was performed. Results are expressed as the percentage of total cells at a specific cell phase, and points and bars represent the mean and standard deviations of four experiments. Right panel: growth curves of DC3F cells treated without or with 5 mM EGTA. DC3F cells were preincubated 4 hr either in complete medium without (○) or with 5 mM EGTA (●). Cells were then replated into several tissue culture flasks (25 $cm^2$) in complete medium. At the indicated times, cells were trypsinized and counted. Each point is the mean of two independent experiments (less than 10% difference between experiments).

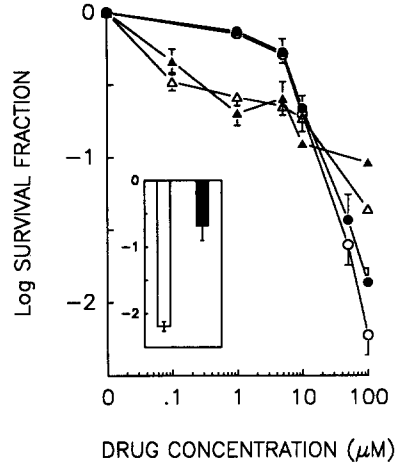


Fig. 8. Cell survival of 023 cells (open symbols) and HSP27-transfected 2.2 cells (closed symbols) treated either with VP-16 (○, ●) or camptothecin (△, ▲). Cytotoxicity was measured by colony formation assays after drug treatment (30 min). Inset: cell survival of 023 cells (open bar) and HSP27-transfected 2.2 cells (closed bar) following hyperthermia treatment (44°, 3 hr). Points and vertical bars represent the means and standard deviations of three experiments.

Table 2. Effect of EGTA on thymidine and uridine incorporation in DC3F cells

| Time with EGTA (hr) | Thymidine incorporation | Uridine incorporation |
|---|---|---|
| 0 | 1.08 ± 0.18 | 1.01 ± 0.01 |
| 1 | 1.19 ± 0.35 | 1.89 ± 0.80 |
| 2 | 0.88 ± 0.11 | 2.93 ± 0.64 |
| 4 | 0.93 ± 0.18 | 1.60 ± 0.10 |

Cells were incubated in complete medium with or without 5 mM EGTA for the indicated times. Rates of nucleotide incorporation were measured by a 10-min pulse experiment. Results are expressed as the ratio of incorporation in EGTA-treated cells over untreated cells; values are means ± SEM, N = 8.

Table 3. Effect of EGTA on the cytotoxicity of VP-16 and camptothecin in HT-29 cells

| | Log survival fraction No EGTA | + 5 mM EGTA |
|---|---|---|
| VP-16 (150 μM) | −1.10 ± 0.32 | −0.24 ± 0.07 |
| Camptothecin (1 μM) | −0.66 ± 0.25 | −0.16 ± 0.09 |

Cells were pretreated with 5 mM EGTA for 4 hr before 30-min treatments with VP-16 or camptothecin. Cytotoxicity was assessed by colony formation assays and is expressed as log survival fraction. Values are means ± SEM, N = 6.

not inhibited by the presence of EGTA. Therefore, we found no evidence that DC3F cells undergo apoptosis following VP-16 treatment and no evidence that calcium depletion may act on a VP-16-induced apoptosis pathway.

*Effect of HSP27 gene expression on VP-16- and camptothecin-induced cytotoxicity.* Similar EGTA treatments have been found to induce phosphorylation of one of the heat-shock proteins, HSP27 [29]. The role of HSP27 in thermotolerance has been further implied because of the finding that cells transfected with the human HSP27 gene are resistant to heat-shock [15]. We examined whether these HSP27-transfected cells were cross-resistant to topoisomerase inhibitors. As illustrated in Fig. 8, HSP27-transfected cells exhibited only modest resistance to VP-16 and camptothecin.

*Effects of calcium depletion on the VP-16-induced cytotoxicity in human colon carcinoma HT-29 cells.* The protective effect of EGTA was not restricted to Chinese hamster DC3F cells since similar results were obtained in human colon carcinoma HT-29 cells treated with the topoisomerase I and II inhibitors, camptothecin and VP-16 (Table 3).

## DISCUSSION

The present report demonstrates that incubation of Chinese hamster DC3F and human colon

carcinoma HT-29 cells in calcium-free or EGTA-containing medium for 2–4 hr blocked the cytotoxicity of VP-16, camptothecin and heat-shock. Under these conditions, the ability of VP-16 to form cleavable complexes with topoisomerase II was not altered. Reduction of drug-induced cytotoxicity without alteration of topoisomerase II-induced DNA breaks has also been observed in cells pretreated with dinitrophenol [30], cycloheximide [31, 32], cordycepin [21] or aphidicolin [20], and in some cell lines that are resistant to topoisomerase II inhibitors [33]. These present findings, with EGTA and calcium depletion, provide further evidence that topoisomerase II-linked DNA breaks ("cleavable complexes") may be necessary but are not sufficient for VP-16-induced cytotoxicity and that other factors may be important as well.

The identification of these other factors will be important in order to understand the cellular determinants of drug action. In the case of topoisomerase I inhibition by camptothecin, active DNA replication complexes appear critical since aphidicolin and hydroxyurea protect completely against camptothecin-induced cytotoxicity [20, 34]. In the case of topoisomerase II inhibitors, however, neither DNA synthesis inhibition by aphidicolin nor RNA synthesis inhibition by cordycepin confer complete cytoprotection against VP-16 [21], indicating that the lethality of topoisomerase II "cleavable complexes" requires cellular factors other than replication or transcription complexes.

Prior to this study, the two most potent cytoprotective agents reported as antagonists to topoisomerase II inhibitors were cycloheximide and dinitrophenol [30, 32, 35]. Neither of these affect "cleavable complex" formation. Dinitrophenol acts primarily by depleting the intracellular pool of ATP and cycloheximide by blocking protein synthesis. The cytoprotective effect of dinitrophenol is rapid and transient, while that of cycloheximide is progressive and persistent, requiring approximately 4 hr for completion and being slowly reversible after removal [32]. Regarding kinetics, the effect of cycloheximide is similar to the protection by calcium depletion in our study. Taken together, these observations indicate that the cytotoxicity of topoisomerase II inhibitors requires calcium, ATP and certain proteins other than DNA topoisomerase II.

Previous observations have indicated that thermotolerance may induce resistance to topoisomerase II inhibitors [36] and that treatment of cells with topoisomerase II inhibitors induces heat-shock proteins [37]. However, no clear relationship between the two processes has emerged. The observation that EGTA pretreatment induced thermotolerance in DC3F cells (Fig. 6) is in agreement with the results of Landry and coworkers [22, 23]. These authors found that EGTA, as well as cycloheximide, induces thermotolerance and that neither of these agents induces new heat-shock protein synthesis, but rather, they stimulate increased phosphorylation of one of the heat-shock proteins, HSP27 [29]. Furthermore, isolation of heat-shock resistant cell lines [38] and transfection experiments of rodent cells with the human HSP27 gene [15] indicated that elevated levels of HSP27 were sufficient to give protection from thermal killing. Our present results showing that HSP27-transfected cells were not cross-resistant to VP-16 or camptothecin suggest that HSP27 is not directly involved in the mechanisms of cellular resistance to topoisomerase inhibitors.

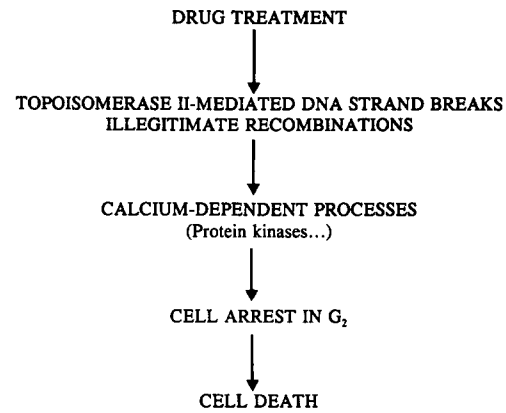


Fig. 9. Schematic representation of cell killing mechanisms by topoisomerase II inhibitors.

Although a programmed cell death mechanism resembling apoptosis has been described in HL-60 cells treated with VP-16 [14], it is unlikely that such a process occurs as an early event following VP-16-induced DNA breaks in DC3F cells since we found no evidence of "nucleosome-like" DNA degradation under conditions of 99% lethality. Thus, at this time, the nature of the calcium-dependent biochemical pathway(s) involved in cell death remains hypothetical.

Since EGTA had to be present for at least 2 hr before VP-16 treatment to exert its blocking action, it is likely that a slow exchangeable calcium pool, rather than extracellular calcium, is involved in the inhibitory process. Furthermore, previous observations indicate that EGTA treatment of Chinese hamster ovary HA-1 cells under calcium-free conditions leads to removal of approximately 95% of cell-associated calcium [39]. Calcium plays an important role as a cofactor of kinases, phospholipases, proteases and nucleases, and is also a second messenger in the signal transduction pathways. Because of the calcium-, ATP-, and protein synthesis dependence of VP-16-induced cytotoxicity, and because of the $G_2$ arrest of cells dying from exposure to topoisomerase inhibitors and its possible relationship to alterations of cyclin phosphorylation [40], it is tempting to speculate that protein kinases may be involved in the cytotoxicity of topoisomerase II-mediated DNA breaks (Fig. 9).

In conclusion, the present study demonstrates that formation of topoisomerase-DNA "cleavable complexes" can be dissociated from cell killing under calcium-depleting conditions. Thus, topoisomerase II-mediated DNA breaks may be necessary but are not sufficient for cell killing by VP-16. Our results also suggest that calcium-dependent cellular processes, possibly related to protein kinase activity,

are important in controlling the cytotoxicity of VP-16, camptothecin and heat-shock.

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