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ORIGINAL ARTICLE

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Potentiation of etoposide-induced apoptosis by staurosporine in human tumor cells is associated with events downstream of DNA-protein complex formation

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Abstract Protein kinase inhibitors have demonstrated potential for use in the therapy of human cancers, in particular leukemia. Staurosporine, a protein kinase inhibitor with broad specificity, enhances the cytotoxic effects of various antitumor agents with different modes of action. The topoisomerase II inhibitor, etoposide, has shown clinical activity against a wide range of tumor types. *Purpose*: The purpose of this study was to assess the effects of staurosporine on etoposide-induced cell death processes in a human tumor of epithelial origin. *Methods*: Modulation of etoposide-induced apoptosis by staurosporine in HeLa cells was assessed by cell morphology, extraction of low molecular weight DNA, quantitation of DNA-protein complexes, and measurements of rates of DNA synthesis. The effects on cellular genes implicated in apoptosis were determined by Northern and Western blotting, along with assays of cyclin-dependent kinase activities. *Results*: Staurosporine exhibited a two- to three-fold potentiation of apoptosis caused by etoposide in HeLa cells when applied concurrently, or immediately following etoposide removal, but did not alter the quantity of DNA-protein complexes produced by etoposide. Etoposide-induced apoptosis, and its potentiation by staurosporine, were associated with reduced c-*myc* expression, and a moderate increase in $p21^{WAF1/CIP1}$ mRNA and protein levels. Inhibitors of cyclic AMP-dependent protein kinase and protein kinase C, which exhibit greater specificity than staurosporine, were without effect on apoptosis caused by etoposide, whereas use of the tyrosine phosphatase inhibitor, vanadate, resulted in its abrogation. The potentiation of etoposide-induced apoptosis by staurosporine was associated with a significant increase in cyclin A-dependent kinase activity. In addition, etoposide caused substantial inhibition of DNA synthesis. *Conclusion*: These results indicate that staurosporine potentiates apoptosis through events which occur downstream of DNA damage, and implicate unscheduled activation of cyclin A-dependent kinase during inhibition of DNA synthesis as a possible cause.

Key words Apoptosis · Staurosporine · Etoposide · Tyrosine kinase · Topoisomerase II

Abbreviations *etoposide* 4′-demethyl-epipodophyllotoxin-9-(4,6-*O*-ethylidene-β-D-glucopyranoside) · *ara-C* 1-[β-D-arabinofuranosyl] cytosine · *cdk* cyclin-dependent kinase · *DMSO* dimethylsulfoxide · *H-89* *N*-[2-((*p*-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide · HCl · *P-gp* P-glycoprotein · *PKA* cyclic AMP-dependent protein kinase · *PKC* protein kinase C · *PMA* phorbol-12-myristate 13-acetate · *SDS* sodium dodecylsulfate · *ssDNA* salmon sperm DNA · *ST* staurosporine · *vanadate* sodium orthovanadate

Introduction

The epipodophyllotoxin, etoposide, belongs to one of many classes of antitumor agents which exert cytotoxicity by stabilizing covalent "cleavable" — complexes between DNA and the α and β isozymes of topoisomerase II (reviewed in reference 8). The transfer of etoposide-treated mammalian tumor cells into drug-free medium results in rapid drug efflux (half-life 3 min) and resealing of DNA lesions (half-life 20–60 min)

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[1, 35]. Ensuing cell cycle events include inhibition of DNA synthesis and transient S phase delay followed by G2 arrest [19, 21, 25, 31], the latter being associated with delayed activation of the cdk, $p34^{cdc2}$ [30, 31]. Alterations in gene expression provoked by exposure to topoisomerase II poisons have been reported to involve repression of c-*myc* [19, 57] and induction of c-*jun* protooncogenes [45]. Also, in cells which express wildtype p53 tumor suppressor protein, topoisomerase II inhibitors are potent inducers of the DNA damage-responsive pathway which leads to increased transcriptional activity of p53, induction of the $p21^{WAF1/CIP1}$ cdk inhibitor, and G1 arrest or apoptosis [14, 27, 37, 54].

The topoisomerase II-interactive agents appear to induce two morphologically distinct modes of death in human tumor cells, apoptosis and mitotic catastrophe [34]. Apoptosis is characterized by the rapid entry of cells into a programmed cell death cascade involving cell shrinkage, chromatin condensation around the nuclear periphery, plasma membrane blebbing, controlled DNA degradation, and fragmentation of cells into membrane-bound "apoptotic bodies" (reviewed in references 23 and 28). Mitotic catastrophe (also termed genomic or reproductive death, micronucleation or multinucleation) is reminiscent of the effects of gross chromosomal damage imposed on cycling cells by a variety of DNA-damaging agents [7, 44, 55], and is preceded by G2 arrest [32–34]. Both mechanisms of death have been observed in a single population of human tumor cells exposed to etoposide [34].

A surge of interest in apoptosis was generated following the demonstration that it could be regulated by c-Myc and p53, and inhibited by the product of the *bcl*-2 oncogene (reviewed in reference 53). A major component of the DNA damage-responsive apoptotic pathway is mediated by wildtype p53 [37], although there is clear evidence that apoptosis induced by topoisomerase II inhibitors also occurs through p53-independent pathways [50]. In addition, induction of $p21^{WAF1/CIP1}$ by etoposide is not restricted to cells which express wildtype p53 [24]. Certainly, Bcl-2 inhibits both p53-dependent and -independent modes of cell death induced by etoposide [22, 26, 50]. While deregulated expression of c-*myc* induces apoptosis under a variety of cellular stresses [15, 36], observing its behavior in nontransfected tumor cells would suggest that c-Myc does not play a direct role in apoptosis induced by the epipodophyllotoxins [19, 36, 57]. Finally, unscheduled activation of cdks appears to play a role in apoptosis induced by multiple stimuli, including etoposide [39, 48, 49], although this remains controversial [43].

Modulators of protein kinases are under consideration for use in combination with cytotoxic drug therapy for the treatment of human cancers, in particular leukemia. ST is a nonspecific inhibitor of PKC which appears to have greater cytotoxicity against leukemia cells than normal human bone marrow progenitor cells when used in combination with the topoisomerase II inhibitor daunorubicin [29]. The rationale behind the use of PKC inhibitors centers around the ability of PKC to phosphorylate, and thereby activate, the product of the multidrug resistance (*mdr*1) gene, P-gp [6, 17]. Interestingly, PKC also phosphorylates topoisomerase IIα, resulting in *decreased* drug-induced DNA cleavage in vitro [12], and exposure of human tumor cells to the PKC activator PMA attenuates etoposide-induced DNA cleavage [59]. These studies suggest an alternative pathway by which PKC inhibitors may enhance the cytotoxicity of topoisomerase II inhibitors.

ST also inhibits PKA, cdks and tyrosine kinases [18, 41, 52], and enhances apoptosis induced by agents which cause S phase arrest in human tumor cells through non-PKC/P-gp mechanisms [20, 39]. Therefore, additional studies on the mechanism by which ST potentiates apoptosis induced by topoisomerase II inhibitors in human tumors of epithelial origin are warranted. Our results demonstrated that ST potentiates etoposide-induced apoptosis in HeLa cells in concert with activation of cyclin A-dependent kinases, repression of c-*myc* and upregulation of $p21^{WAF1/CIP1}$. In addition, we demonstrated that vanadate causes a concentration-dependent inhibition of etoposide-induced apoptosis, suggesting that tyrosine dephosphorylation may play a critical role in apoptosis and its potentiation in this human tumor cell line.

Materials and methods

Reagents

Etoposide, Giemsa stain, penicillin, PMA, sodium pyruvate, ssDNA, streptomycin, Triton X-100, vanadate, and Wright stain were purchased from Sigma Chemical Company (St. Louis, Mo.). H-89 and calphostin C were purchased from Calbiochem (La Jolla, Calif.). Kamiya Biomedical Company (Thousand Oaks, Calif.) supplied ST, protein A-Sepharose was purchased from Pharmacia (Piscataway, N.J.), and Millipore (Burlington, Mass.) supplied polyvinylidene difluoride membrane. Magna NT nylon membrane and the Promega nick translation kit were purchased through Fisher Scientific (Cincinnati, Ohio), while the Amersham Corporation (Arlington Heights, Ill.) supplied horseradish peroxidase-conjugated antibodies and reagents for enhanced chemiluminescence. Dulbecco's modified Eagle's medium, minimum essential medium, recombinant human insulin, fetal bovine serum, and molecular biology grade agarose were purchased from GIBCO BRL (Life Technologies, Frederick, Md.). Calf serum was purchased from Hyclone Laboratories (Logan, Utah), and all radioactively labeled compounds were supplied by DuPont NEN (Boston, Mass.). All other chemicals were purchased from Sigma or Fisher.

Preparation and characterization of COOH-terminal $p34^{cdc2}$ peptide polyclonal antisera have been described previously [34]. Polyclonal antisera raised against human cyclin A and COOH-terminal $p33^{cdk2}$ peptide were a generous gift from Drs. G. Draetta and M. Pagano (Mitotix, Cambridge, Mass.). Monoclonal antibodies against human c-Myc (Ab-1, clone 9E10), p53 (Ab-2, clone PAb1801) and $p21^{WAF1}$ (Ab-1, clone EA10) were purchased from Oncogene Science (Uniondale, N.Y.), while an antiphosphotyrosine

monoclonal antibody (clone 4G10) was supplied by Upstate Biotechnology (Lake Placid, N.Y.). Human wildtype p53 cDNA in pCMV-Neo [2] and human WAF1 cDNA in the pZL vector [13] were generously supplied by Dr. B. Vogelstein (Johns Hopkins Oncology Center, Baltimore, Md.), while human c-*myc* cDNA in pLNCX was kindly provided by Drs. N. Quintrell and J.M. Bishop (University of California, San Francisco, Calif.).

Cell culture and drug treatments

HeLa cells, derived from a squamous cell carcinoma of the cervix (clone S3 from American Type Culture Collection, Rockville, Md.) were maintained as a monolayer in Dulbecco's modified Eagle's medium supplemented with 10% calf serum, penicillin (100 units/ml), and streptomycin (100 μg/ml) at 37 °C in a humidified atmosphere containing 5% CO_2. The human breast cancer cell line, MCF-7 (also from ATCC), was grown as a monolayer in minimum essential medium containing nonessential amino acids and Earle's balanced salt solution, supplemented with sodium pyruvate (1 m*M*), recombinant human insulin (10 μg/ml), 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μg/ml) at 37 °C in a humidified atmosphere containing 5% CO_2.

Stocks in DMSO were prepared of etoposide (100 m*M*), ST (200 μ*M*), calphostin C (200 μ*M*), H-89 (4 m*M*), and PMA (10 μg/ml). These were stored at −20 °C in the dark. Vanadate was dissolved in distilled water at 100 m*M* and stored at −20 °C. During experiments control cultures received equivalent solvent treatment, which did not exceed 0.4% DMSO.

Apoptotic methodology

Morphologically, the proportion of apoptotic cells was estimated by light microscopy of Wright/Giemsa-stained cytospins, as detailed elsewhere [34]. For each data point 1000 cells were counted per experiment. Biochemically, apoptotic low molecular weight DNA fragments were extracted from 2×10^6 control or drug-treated cells by a Triton X-100 lysis method described in detail by Kamesaki et al. [26].

Assay of DNA-protein complexes and rates of DNA synthesis

A potassium-SDS precipitation assay was used to measure etoposide-induced DNA-protein complexes without any contribution from nonprotein-associated apoptotic DNA fragmentation. The method employed has been described in detail elsewhere [40]. Briefly, exponentially dividing cells were labeled with [^{14}C]leucine (0.2 μCi/ml) and [methyl-^{3}H]thymidine (0.6 μCi/ml) for 24 h. Following exposure to etoposide, ST or solvent control for 1 h at 37 °C, the reaction was stopped with 0.5 ml 2.5% SDS, 10 m*M* EDTA containing 0.8 mg/ml ssDNA. The lysate was passed 20 times through a 23-gauge needle, and heated to 65 °C for 5 min. Proteins were precipitated by the addition of KCl to 0.1 *M* and a 15-min incubation on ice. Following centrifugation (10 000*g* for 10 min at 4 °C), the pellets were washed three times with 10 m*M* Tris.Cl (pH 8), 100 m*M* KCl, 1 m*M* EDTA containing 0.1 mg/ml ssDNA, each wash included sequential 5-min incubations at 65 °C and on ice. The final pellet was dissolved in distilled water (65 °C for 15 min), centrifuged (10 000*g* for 15 s), and the radioactivity in the supernatant determined. DNA-protein complex formation for each treatment was expressed as the ratio of ^{3}H-labeled DNA to ^{14}C-labeled protein.

In a control experiment to test the sensitivity of the technique, etoposide (50 μ*M*, 1 h) caused over fivefold more DNA-protein complexes in wildtype Chinese hamster ovary cells than in the sevenfold etoposide-resistant SMR_5 cell line (data not shown) [56].

Rates of DNA synthesis were estimated by the incorporation of [methyl-^{3}H]-thymidine (35 Ci/mmol, 1 μCi/ml) into trichloroacetic acid-precipitable material over a 1-h labeling period. Results were expressed as a percentage of solvent-treated controls.

Cyclin-dependent kinase assays, Western and Northern blotting

Assays of immunoprecipitated cyclin A-dependent, cyclin B1-dependent, p34^{cdc2} and p33^{cdk2} kinase activities, and Western blot analysis of proteins, were carried out as described previously [31, 32, 34] except that the lysis buffer used was calcium- and magnesium-free phosphate-buffered saline containing 1% NP-40, 10 m*M* sodium fluoride, 5 m*M* sodium pyrophosphate and 1 m*M* vanadate plus the protease inhibitors aprotinin, leupeptin, pepstatin A, soybean trypsin inhibitor, *N*-tosyl-L-phenylalanine chloromethyl ketone (each at 10 μg/ml) and 1 m*M* phenylmethylsulfonyl fluoride. Protein concentration in lysates was estimated by the BCA assay (Pierce Chemical Company, Rockford, Ill.), using bovine serum albumin as standard. Equal amounts of protein (100 μg) were loaded into each lane for Western blots, or used in immunoprecipitation reactions for kinase activity assays. Cdk activity was estimated by phosphorylation of histone H1 following immunoprecipitation with polyclonal antisera [30].

RNA was extracted using the method of Chomczynski and Sacchi [9]. RNA (10 μg) from each sample was denatured with glyoxal and DMSO, and separated by electrophoresis through a 1% agarose gel. The RNA was vacuum transferred to a nylon membrane, and equal loading/transfer was verified by staining with methylene blue [46]. The c-*myc*, p53 and p21^{WAF1} transcripts were detected by nick-translated cDNA inserts using standard techniques [46]. The post-hybridization washes were 1 × SSPE/0.1% SDS (20 min, room temperature) and 0.2 × SSPE/0.1% SDS (30 min, 68 °C), following which the membrane was exposed to Fuji RX film at −70 °C.

Statistics

Unless stated otherwise, numerical data presented are the mean of at least two, and normally four, experiments. Standard errors (SE), unless shown, were less than 10%. Student's *t*-test was used to evaluate the significance of differences: only *P*-values < 0.05 were considered significant. Western and Northern blots are representative of at least three experiments.

Results

The effects of staurosporine on etoposide-induced apoptosis

HeLa cells which were exposed to 50 μ*M* etoposide for 4 or 8 h exhibited a gradual increase in the proportion of morphologically apoptotic cells (Fig. 1A). The inclusion of 20 or 50 n*M* ST during drug treatment increased apoptosis by between two- and threefold at both time-points (Fig. 1A). This potentiation was observed consistently throughout all experiments. Fig. 1B shows a single representative experiment in which 20 or 50 n*M* ST caused between a 2.5- and 4-fold increase in apoptosis at two etoposide concentrations during an 8-h exposure. ST alone at concentrations of 20 or 50 n*M* had no effect on apoptosis induction during the

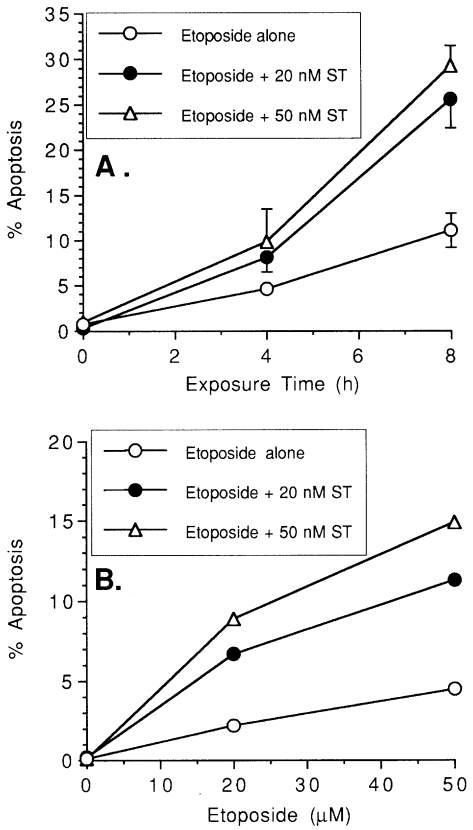


Fig. 1A, B Potentiation of etoposide-induced apoptosis in HeLa cells by cotreatment with ST. The proportion of cells undergoing apoptosis was determined morphologically by counting 1000 Wright/Giemsa-stained cells per data point per experiment. **A** Cells were exposed to etoposide alone (*open circles*) or etoposide plus 20 n*M* (*closed circles*) or 50 n*M* (*open triangles*) ST for 4 or 8 h. Values are the means ± SE of four experiments. **B** Cells were exposed to 0, 20 or 50 μ*M* etoposide in the absence (*open circles*) or presence of 20 n*M* (*closed circles*) or 50 n*M* (*open triangles*) ST for 8 h. Values are from a single experiment

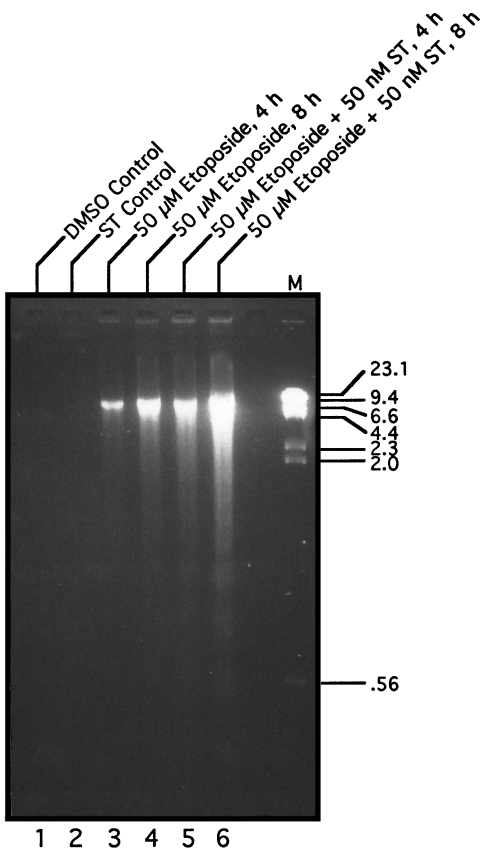


Fig. 2 ST increases the quantity of low molecular weight DNA extracted from etoposide-treated HeLa cells. Control cultures were exposed to DMSO vehicle (*lane 1*) or 50 n*M* ST (*lane 2*) for 8 h. Cells were exposed to 50 μ*M* etoposide for 4 (*lanes 3 and 5*) or 8 h (*lanes 4 and 6*) in the absence (*lanes 3 and 4*) or presence (*lanes 5 and 6*) of 50 n*M* ST. The migration distance and size (kb pairs) of DNA standards (Lambda/HindIII) are shown to the right (*M*)

experiments described here. However, we did detect a slight increase in apoptosis induced by 100 n*M* ST alone over an 8-h exposure and, consequently, 20 or 50 n*M* ST concentrations were used in subsequent studies.

The morphological data presented in Fig. 1 were confirmed biochemically by the ability of ST to increase the amount of low molecular weight apoptotic DNA fragments extracted from etoposide-treated cells (Fig. 2). Solvent- or ST-treated controls yielded no, or very little, DNA (Fig. 2, lanes 1 and 2) whereas increasing amounts were extracted from cells exposed to etoposide for 4 or 8 h (lanes 3 and 4). Simultaneous ST/etoposide treatment significantly augmented the quantity of low molecular weight DNA extracted at both time-points (lanes 5 and 6). These data were also verified using the terminal deoxynucleotidyl transferase assay which measures nonprotein-associated DNA breaks (data not shown).

ST at nontoxic concentrations is able to enhance etoposide-induced apoptosis in HeLa cells by between two- and threefold. Subsequent experiments were designed to assess the various mechanisms which have been proposed for synergy between chemotherapeutic drugs and ST, with respect to etoposide/ST combinations in their effect on apoptosis in a human tumor cell line.

Etoposide-induced DNA-protein complexes

Critical to form a hypothesis regarding the mechanism by which ST enhances apoptosis is the quantity of DNA-protein complexes induced by etoposide. Inhibition of PKC by ST may decrease phosphorylation of PKC targets, which include topoisomerase IIα [12] and P-gp [6, 17], and thereby affect the ability of etoposide to either reach, or interact with, its target. In order to estimate the number of DNA lesions caused by etoposide or etoposide/ST combinations without the

contribution of apoptotic DNA degradation, it was necessary to quantify covalent DNA-protein interactions. The SDS-KCl precipitation assay was used to measure etoposide-induced DNA-protein complexes in HeLa cells. In cells exposed to etoposide alone, or in combination with ST, a concentration-dependent increase in DNA-protein complexes was observed (Fig. 3). No significant differences were detected in DNA-protein complexes when ST was included in the etoposide treatment.

The DNA-protein complex data do not support the hypothesis that the modulating effects of ST are due to an increase in the quantity of drug-induced DNA lesions brought about by dephosphorylation of topoisomerase II or P-gp. Instead, the data suggest that ST modifies secondary cellular responses to DNA lesions. This hypothesis is supported by the results of experiments shown in Fig. 4, in which cells were exposed to ST only after removal of etoposide. Under these conditions, ST would be expected to have minimal effect on the formation of etoposide-induced DNA lesions. Nevertheless, ST was still able to enhance apoptosis 2.5-fold within 6 h of etoposide removal, and continued to potentiate over an extended exposure (Fig. 4).

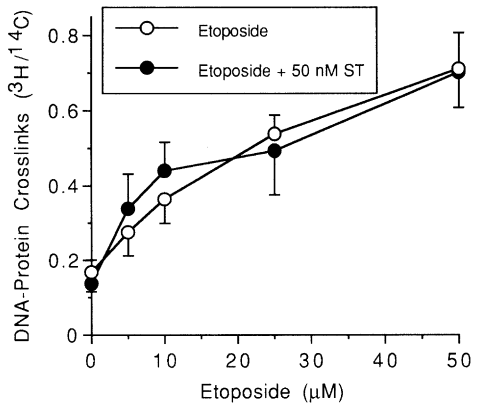


Fig. 3 ST has no effect on the quantity of etoposide-induced DNA-protein complexes. HeLa cells were exposed to various etoposide concentrations for 1 h in the absence (*open circles*) or presence (*closed circles*) of 50 n*M* ST, then analyzed for DNA-protein complex formation. Values are the means ± SE of four experiments

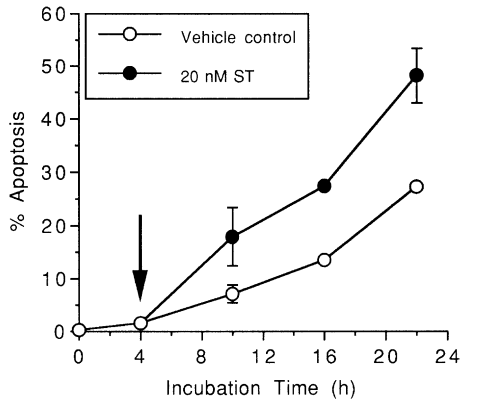


Fig. 4 ST enhances apoptosis when applied following removal of etoposide. HeLa cells were exposed to etoposide (25 μ*M*, 4 h), washed twice in warm PBS (*arrow*), and incubated in etoposide-free medium containing solvent control (*open circles*) or 20 n*M* ST (*closed circles*). At the appropriate times cells were harvested and the proportion of morphologically apoptotic cells estimated. Values are the means ± SE of two experiments

Behavior of cell cycle regulatory proteins in cells exposed to etoposide and ST

The role of cdk activation in apoptosis induced by a variety of stimuli remains controversial [43, 48, 49]. However, ST-induced entry of S phase-arrested HeLa cells into apoptosis is associated with unscheduled activation of cyclin A-dependent kinases ($p34^{cdc2}$ and $p33^{cdk2}$) [39]. In addition, the c-Myc oncoprotein has been implicated in apoptosis induction under conditions of cell cycle arrest in cells of differing lineage [15, 36], but is downregulated upon exposure of human tumor cell lines to epipodophyllotoxins [19, 57]. The cdk inhibitor, $p21^{WAF1/CIP1}$, is induced by p53-dependent and p53-independent pathways [14, 24, 38]. While the $p21^{WAF1/CIP1}$ response to ionizing radiation seems to be strictly p53-dependent [38], its induction by etoposide appears not to be exclusively mediated by p53 [24].

For these reasons we studied the behavior of several proteins implicated in apoptosis and cell cycle control, during etoposide/ST exposures. No significant differences were detected in intracellular concentrations of cyclin A, $p34^{cdc2}$ and $p33^{cdk2}$ (Fig. 5). However, exposure to etoposide for 8 h caused a decrease in both mRNA and protein c-Myc levels (compare lanes 1 and 5 in Figs. 5 and 6). ST, while having no effect on c-Myc in cells which were not exposed to etoposide (Figs. 5 and 6, lanes 2 and 3), did cause a concentration-dependent attenuation of c-*myc* gene expression in etoposide-treated cells at 8 h (compare lane 5 with lanes 7 and 9 in Figs. 5 and 6). The apparent increase in c-Myc mRNA levels following 4-h etoposide/ST exposures was not reflected by significant changes in c-Myc protein levels (compare lanes 4, 6 and 8 in Figs. 5 and 6). Downregulation of expression was not detected for the other genes examined (Figs. 5–7). Therefore, progression of etoposide-treated cells into apoptosis, and its augmentation by ST, is associated with specific downregulation of c-*myc* gene expression.

No consistent differences were detected in p53 transcript or protein levels in HeLa cells exposed to etoposide with or without ST cotreatment (Figs. 5 and 6). However, increased levels of $p21^{WAF1/CIP1}$ transcript were detected in cells exposed to etoposide for 4 or 8 h (Fig. 6, lanes 4 and 5), and these levels were substantially augmented by simultaneous ST exposure at both

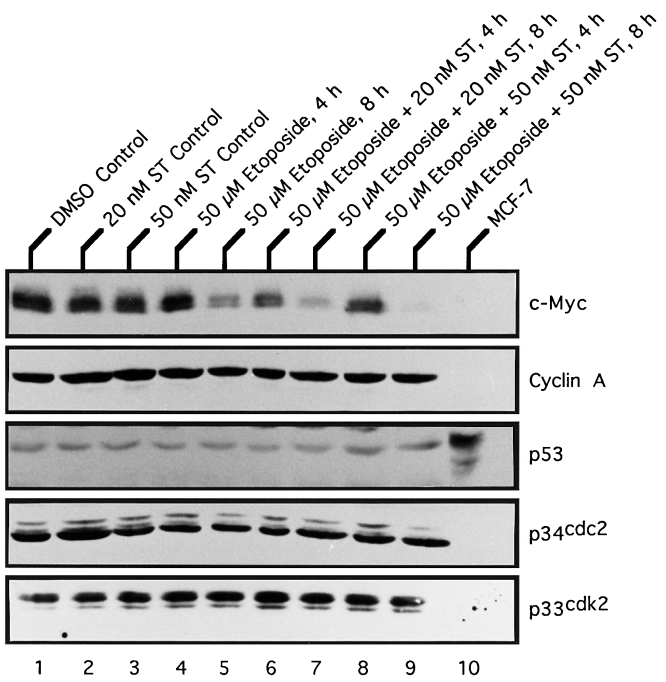


Fig. 5 Behavior of cell cycle regulatory proteins in etoposide-treated cells. HeLa cells (*lanes 1–9*) were exposed to DMSO (*lane 1*), 20 n*M* ST (*lane* 2) or 50 n*M* ST (*lane 3*) for 8 h as controls. Parallel cultures received 50 μ*M* etoposide alone for 4 h (*lane* 4) and 8 h (*lane 5*), or etoposide plus 20 n*M* ST for 4 h (*lane 6*) and 8 h (*lane 7*), or etoposide plus 50 n*M* ST for 4 h (*lane 8*) and 8 h (*lane 9*). Protein extract (100 μg) from each treatment was analyzed by Western blot. Shown are the 62–64 kDa c-Myc, 60 kDa cyclin A, p53, $p34^{cdc2}$, and $p33^{cdk2}$ proteins. The p53 blot includes a control of MCF-7 cells exposed to etoposide (50 μ*M*, 1 h) (*lane 10*), because of the low levels of p53 protein detected in HeLa cells. The figure is representative of at least three separate experiments

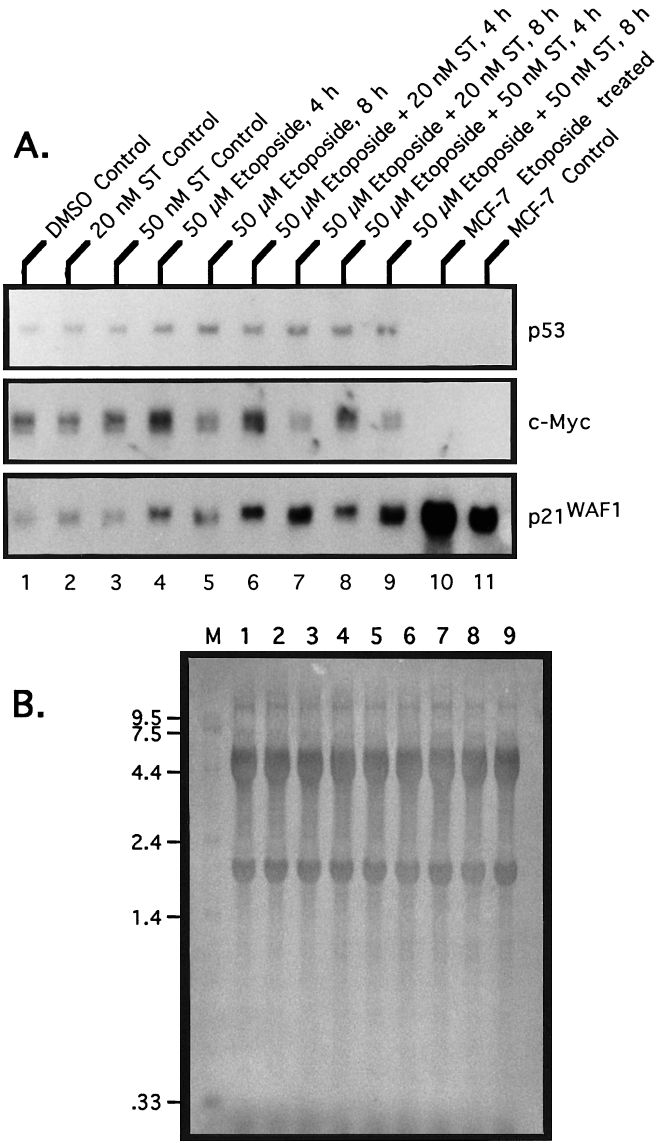


Fig. 6A, B Levels of p53, c-*myc*, and $p21^{WAF1/CIP1}$ transcripts in HeLa (*lanes 1–9*) and MCF-7 (*lanes 10 and 11*) cells exposed to etoposide. The drug treatments were exactly as described for Fig. 5, except that MCF-7 controls were included only in the $p21^{WAF1/CIP1}$ blot, and consisted of solvent control (*lane 11*) or cells exposed to etoposide (50 μ*M*, 1 h) and harvested 2 h following removal of drug (*lane 10*). **A** Representative blots of at least three experiments show the 2.8 kb p53, 2.3 kb c-*myc*, and 2.1 kb $p21^{WAF1/CIP1}$ transcripts. **B** methylene blue-stained membrane following RNA transfer to verify equal loading. The migration distances and size (kb) of RNA standards are shown to the left of the membrane (*lane M*)

time-points (Fig. 6, lanes 6–9). The basal levels of $p21^{WAF1/CIP1}$ transcript, and its increase upon etoposide exposure, were substantially diminished in HeLa cells compared to MCF-7 cells (Fig. 6, lanes 10 and 11) which express wildtype p53 (4). For this reason it was difficult to obtain quality Western blots of $p21^{WAF1/CIP1}$ protein expression in HeLa cells. However, increased levels of $p21^{WAF1/CIP1}$ protein were detected upon etoposide/ST cotreatment (Fig. 7, lanes 5 and 6). Again, the response was significantly less than in MCF-7 cells exposed to etoposide for only 1 h (Fig. 7, lanes 7–10).

The use of additional modulators to study etoposide-induced apoptosis

ST is a potent PKC inhibitor, but also exhibits inhibitory activity against PKA, cdks and tyrosine kinases [18, 41, 52]. Also, PKA inhibitors have been shown to enhance radiation-induced apoptosis in a Burkitt's lymphoma cell line [16], while the ability of ST to potentiate apoptosis caused by ara-C in HL-60 cells does not appear to reflect inhibition of PKC [20]. Consequently, we tested other modulators or agents with greater specificity than ST for their effects on etoposide-induced apoptosis in HeLa cells. Cotreatment with the tyrosine phosphatase inhibitor vanadate [51] caused a concentration-dependent inhibition of apoptosis, in contrast to the potentiation observed with ST (Fig. 8). A concentration of 80 μ*M* vanadate caused

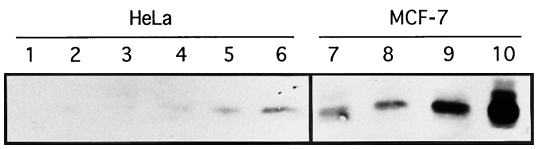


Fig. 7 $p21^{WAF1/CIP1}$ protein levels in HeLa (*lanes 1–6*) or MCF-7 (*lanes 7–10*) cells exposed to etoposide. Protein extract (100 μg) from each treatment was analyzed by Western blot. HeLa cells were exposed to solvent (*lane 1*) or 50 n*M* ST (*lane 2*) controls for 8 h, etoposide (50 μ*M*) for 4 h (*lane 3*) and 8 h (*lane 4*), or etoposide plus 50 n*M* ST for 4 h (*lane 5*) and 8 h (*lane* 6). MCF-7 cells were exposed to solvent control (*lane* 7) or etoposide (50 μ*M*, 1 h; *lanes 8–10*) and harvested at 0 h (*lane 8*), 1 h (*lane 9*) or 2 h (*lane 10*) following removal of drug

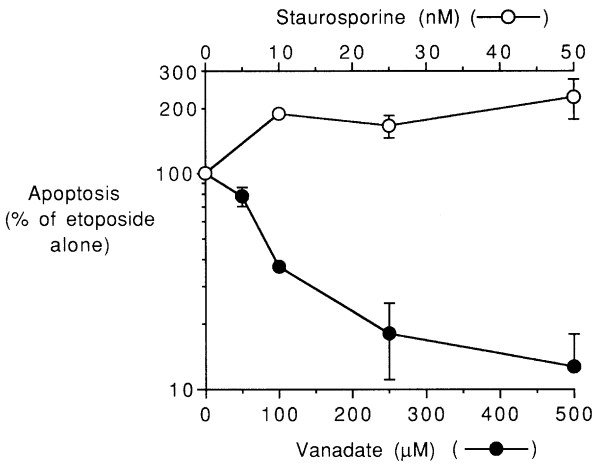


Fig. 8 Contrasting effects of ST and vanadate on etoposide-induced apoptosis in HeLa cells. Following exposure to etoposide (50 μ*M*, 10 h) in the presence of increasing concentrations of ST (*open circles*) or vanadate (*closed circles*), cells were harvested and the proportion of morphologically apoptotic cells determined by counting 1000 cells per data point per experiment. Values are the means ± SE of two separate experiments. Etoposide treatment alone caused a mean of 18.5% apoptosis

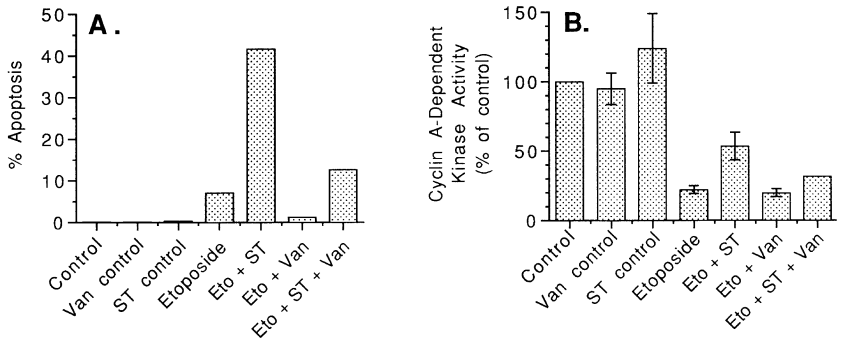
50% inhibition of the apoptotic effects of etoposide. However, while we did not directly measure PKA or PKC activities, we were unable to demonstrate any effects on etoposide-induced apoptosis using the PKC activator PMA, the PKC inhibitor calphostin C and the PKA inhibitor H-89 at concentrations which have been shown to exert physiological effects in other tissue culture model systems (data not shown) [16, 20]. These results indicate that the tyrosine phosphorylation status of a critical component of the apoptotic machinery controls etoposide-induced apoptosis in HeLa cells.

The effects of ST or vanadate on apoptosis, cdk activities, and DNA synthesis in etoposide-treated cells

Premature activation of cyclin A-dependent kinase activity is associated with apoptosis induction in S phase-arrested HeLa cells [39], while tyrosine dephosphorylation is required for activation of cdks [42]. In addition, topoisomerase II poisons exert transient S phase arrest [19, 21, 25, 34]. For these reasons, and based on the results of the experiments described above, cdk activities were studied in relation to apoptosis and DNA synthesis inhibition in HeLa cells exposed to etoposide/ST/vanadate combinations.

Figure 9A demonstrates that ST potentiated, and vanadate inhibited, apoptosis when applied simultaneously with etoposide, in agreement with data shown in Figs. 1 and 8. Figure 9A also shows that ST and vanadate antagonized each other's effects. Estimates of cdk activities in cell lysates revealed that etoposide treatment alone caused between 68 and 85% repression of cyclin A-dependent, cyclin B1-dependent, $p34^{cdc2}$ and $p33^{cdk2}$ kinases (Fig. 9B, and data not shown), in agreement with previous studies [31, 32, 49]. ST partially prevented inhibition of cyclin A-dependent kinase in etoposide-treated cells to a level of activity which was 2.4-fold higher than etoposide alone (Fig. 9B), a statistically significant difference ($P = 0.039$). The effects of etoposide/ST/vanadate combinations on cyclin A-dependent kinase activities bore a striking resemblance to their effects on apoptosis (compare Fig. 9A with Fig. 9B). ST also demonstrated similar effects on $p34^{cdc2}$ and $p33^{cdk2}$, but not cyclin B1-dependent, kinase activities (data not shown). However, unlike cyclin A-dependent kinase activity, the differences between etoposide alone and etoposide plus ST did not

Fig. 9A, B The effects of etoposide, ST and vanadate treatment on apoptosis and cyclin A-dependent kinase activity in HeLa cells. Cells were exposed for 12 h to solvent control (control), 250 μ*M* vanadate (*Van control*), 50 n*M* ST (*ST control*), 50 μ*M* etoposide (*Etoposide*), or etoposide plus ST/vanadate (*Eto + ST*, *Eto + Van*, *Eto + ST + Van*). **A** The proportion of morphologically apoptotic cells. **B** Cyclin A-dependent kinase activity, expressed as a percentage of solvent-treated controls. Values are the means of two experiments. SEs, unless shown, were less than 10%



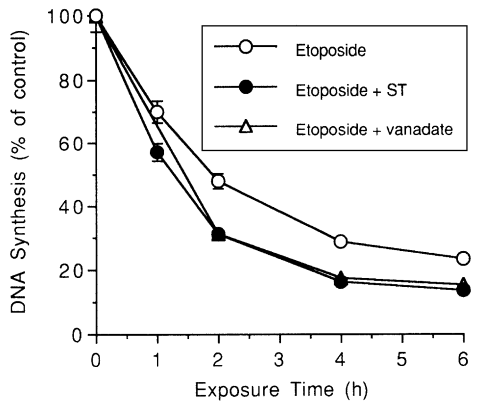


Fig. 10 The effects of etoposide ± ST or vanadate on rates of DNA synthesis. Cells were exposed to etoposide (50 μ*M*, *open circles*) ± 50 n*M* ST (*closed circles*) or 250 μ*M* vanadate (*open triangles*), and DNA synthesis measured during the final hour of treatment. DNA synthesis is expressed as a percentage of solvent-treated controls. Values are the means ± SE of two experiments

reach statistical significance, and it was not possible to identify which cyclin A kinase subunit was activated.

Finally, the effects of etoposide, ST and vanadate on rates of DNA synthesis were studied. Fig. 10 shows that etoposide caused a time-dependent inhibition of DNA synthesis, which was mildly exacerbated by simultaneous ST or vanadate exposure.

Discussion

This study showed the following in HeLa cells: (1) ST at sublethal concentrations potentiated etoposide-induced apoptosis, as measured by the two criteria of cell morphology and DNA degradation; (2) the mechanism of apoptotic enhancement by ST involved events unrelated to the quantity of etoposide-induced DNA lesions; and (3) simultaneous inhibition of DNA synthesis and activation of cyclin A-dependent kinase may have accounted for the ability of ST to potentiate etoposide-induced apoptosis.

Since ST is a potent, but non-specific, inhibitor of PKC [41, 52], it was necessary to determine whether ST was acting to increase etoposide-induced DNA lesions caused by the drug via inhibition of PKC. This has been suggested to result in increased intracellular drug accumulation via inhibition of P-gp [29] (unlikely since HeLa cells do not overexpress P-gp), or via decreased topoisomerase IIα phosphorylation by PKC [12, 59]. Our results, which show that DNA-protein complex formation was unaffected by ST, and that ST potentiated etoposide-induced apoptosis even when applied following removal of etoposide, exclude both of these possibilities. Instead, our results resemble those which show that ST increases apoptosis in human tumor cells exposed to nontopoisomerase II-interactive S phase-arresting agents, neither of which are substrates of P-gp [20, 39].

In agreement with the studies of Grant et al. [20], our results argue against the involvement of PKC in the mechanism of potentiation of apoptosis in HeLa cells exposed to etoposide. Moreover, as alluded by Grant et al. in their studies with ara-C and ST [20], we present additional evidence, by the use of vanadate to inhibit etoposide-induced apoptosis, that ST may impose its apoptotic-enhancing effects by inhibiting a tyrosine kinase. This hypothesis is further strengthened by observations that specific inhibitors of PKC and PKA did not enhance etoposide- or ara-C-induced apoptosis in human tumor cells (this study; [16, 20]).

What, then, are the identities of the tyrosine kinase and its substrate(s)? It is tempting to speculate on the involvement of cdks, whose activity is intimately associated with tyrosine phosphorylation status (reviewed in reference 42). Unscheduled activation of cyclin A-dependent kinases ($p34^{cdc2}$ and $p33^{cdk2}$) is associated with induction of apoptosis by ST in S phase-arrested HeLa cells [39], while premature activation of cyclin B1-dependent kinase ($p34^{cdc2}$) has been observed in HL60 cells exposed to a variety of DNA-damaging agents, including etoposide [49]. Also, $p34^{cdc2}$ has been shown to be rapidly tyrosine phosphorylated by $p56/p53^{lyn}$ kinase in HL60 cells exposed to ara-C [58]. In our studies, the observations that etoposide alone severely represses the activities of cyclin A-dependent, cyclin B1-dependent, $p34^{cdc2}$ and $p33^{cdk2}$ kinases makes their precise contribution to apoptosis difficult to interpret. However, ST did cause a significant activation of cyclin A-dependent kinase which was abrogated by vanadate (Fig. 9B), findings which correlated with their respective effects on etoposide-induced apoptosis. The effects of ST and vanadate on $p34^{cdc2}$ and $p33^{cdk2}$ kinase activities were similar to those on cyclin A-dependent kinase activity, in contrast to cyclin B1-dependent kinase activity. However, the differences in $p34^{cdc2}$ and $p33^{cdk2}$ kinase activities may not have reached statistical significance owing to either cyclin A sharing both catalytic partners [39, 42] or the severe inhibitory effects of etoposide alone. Additional studies are required utilizing synchronous cells to identify the kinase subunit implicated in cyclin A-mediated apoptosis.

In light of the observations reported above, we propose that apoptosis caused by etoposide in HeLa cells is a complex, multifactorial process involving cleavable complex formation, cell cycle arrest and inhibition of DNA synthesis, of which downregulation of c-*myc* may merely be a sequela. We also propose that additional stresses may be superimposed on this process to result in enhancement of the apoptotic cascade. These additional stresses may include unscheduled cyclin A-dependent kinase activation or induction of $p21^{WAF1/CIP1}$. However, induction of $p21^{WAF1/CIP1}$ would be expected

to inhibit cdk activity [14]. This apparent anomaly leads us to hypothesize that the combined activation of cyclin A-dependent kinase by ST and increased expression of $p21^{WAF1/CIP1}$ during the S phase arrest imposed by etoposide results in increased levels of apoptosis. The exacerbation by ST of etoposide-induced DNA synthesis inhibition alone cannot explain its effects on apoptosis, owing to the disparate effects of vanadate on these processes (Figs. 8 and 10). Again, this hypothesis is consistent with the effects of ST on apoptosis induced by other DNA synthesis inhibitors [20, 39], without the added complexity of their causing apoptosis via cleavable complex formation.

At this point we are unable to conclude whether $p21^{WAF1/CIP1}$ induction is dependent upon p53, because the wildtype status of p53 in HeLa cells is compromised by the human papillomavirus-18 E6 protein [47]. However, we observed no significant changes in p53 protein expression throughout these experiments, which suggests that the $p21^{WAF1/CIP1}$ increase may be more closely associated with etoposide-induced cell cycle arrest rather than mediated through a p53 response to DNA lesions [24]. Other workers have demonstrated that downregulation of c-*myc* is unlikely to be the cause of chemotherapeutic drug-induced apoptosis [19, 36, 57]. While our studies do not allow us to conclude to the converse, the degree and specificity of c-*myc* attenuation does correlate with the extent of apoptosis induced by etoposide with or without ST cotreatment.

Regardless of the mechanism by which ST enhances etoposide-induced cell death, this study demonstrates that ST itself, or agents which exert similar effects, may have a broader range of therapeutic potential than originally expected. For example, tumor necrosis factor potentiates apoptosis in S phase-arrested HeLa cells [39], and enhances the cytotoxicity of topoisomerase II inhibitors [3]. Etoposide has activity against a variety of human cancers, with myelosuppression being the principal dose-limiting toxicity [11]. Simultaneous ST exposure increases the cytotoxicity of the topoisomerase II inhibitor, daunorubicin, against human leukemic cells but not normal bone marrow progenitors [29]. However, prior exposure of human leukemia cells to ST either compromises, or has no effect on, subsequent exposure to topoisomerase II poisons [5, 60]. Therefore, providing they are administered in the appropriate schedule to maximize their modulating effects, protein kinase inhibitors may demonstrate therapeutic specificity against human solid tumors in combination with topoisomerase II inhibitors. An interesting property of ST is the acute sensitivity of normal human cells to a G1 block, in contrast to the insensitivity of transformed cells [10]. This may lead to therapeutic exploitation considering the preferential S and G2 phase cytotoxicity exhibited by the majority of antitumor agents in clinical use.

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