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Effect of Cellular ATP Depletion on Topoisomerase II Poisons. Abrogation of Cleavable-Complex Formation by Etoposide But Not by Amsacrine

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ABSTRACT

Topoisomerase (topo) II poisons have been categorized into ATP-independent and -dependent drugs based on in vitro studies. We investigated drug-induced topoII-DNA complexes in intact cells almost completely depleted of ATP. Virtually no DNA single-strand breaks (SSBs), as measured by alkaline elution, were detected in energy-depleted cells treated with the topoII poisons etoposide, teniposide, daunorubicin, doxorubicin, mitoxantrone, or clerocidin. This inhibition was reversible; subsequent incubation with glucose restored the level of DNA SSBs. The effect of ATP depletion was specific for topoII, because topoI-mediated cleavable complexes induced by camptothecin were unaffected by ATP depletion. Furthermore, etoposide-induced DNA-protein complexes and DNA double-strand breaks, as measured by filter elution techniques, and topoIIα and -β trapping, as measured by a band depletion assay, were completely inhibited by energy depletion. Differences in drug transport could not explain the effect of ATP depletion. The topoII poison amsacrine (*m*-AMSA) was unique with respect to ATP dependence. In ATP-depleted cells, *m*-AMSA-induced DNA SSBs, DNA double-strand breaks, DNA-protein complexes, topoIIα and -β trapping were only modestly reduced. The accumulation of *m*-AMSA was reduced in ATP-depleted cells, which indicates that drug transport could contribute to the modest decrease in *m*-AMSA-induced cleavable complexes. In conclusion, drug-induced topoII-DNA complexes were completely antagonized in ATP-depleted cells, except in the case of *m*-AMSA. One possible interpretation is that *m*-AMSA mainly produces prestrand passage DNA lesions, whereas the other topoII poisons tested exclusively stabilize poststrand passage DNA lesions in intact cells.

Topoisomerase (topo) II catalyzes the passing of an intact DNA duplex through a transient break in another DNA duplex. Several antitumor drugs, including etoposide (VP-16) (Yang et al., 1985), amsacrine (*m*-AMSA; 4′-(9-acridinylamino)methanesulfon-*m*-anisidide) (Nelson et al., 1984), and doxorubicin (Tewey et al., 1984), target topoII. The cytotoxic effect of these drugs, designated topoII poisons, is not caused by classical catalytic inhibition of the enzyme. On the contrary, cell killing is associated with the inhibition of the religation step in the catalytic process whereby the topoII-DNA complexes, designated cleavable complexes, are stabilized. The catalytic process of topoII is ATP dependent. The binding of ATP to topoII is sufficient to support DNA strand passage, whereas ATP hydrolysis is required for enzyme turnover (Osheroff et al., 1983). In vitro, ATP is not a requirement for topoII-mediated DNA cleavage, although ATP stimulates cleavage 2- to 3-fold (Tewey et al., 1984; Osheroff, 1986). Drug-mediated DNA cleavage can occur both before and after strand passage (Robinson and Osheroff, 1991). Liu and coworkers (Chen and Liu, 1994; Frydman et al., 1997; Li and Liu, 1998) proposed the categorization of topoII poisons into ATP-independent and -dependent drugs based on their in vitro studies. However, our knowledge of the role of ATP on the action of topoII-targeting drugs in intact cells is limited. In isolated nuclei, VP-16-induced DNA single-strand breaks (SSBs) are stimulated by the presence of extranuclear ATP (Glisson et al., 1984; Woynarowski et al., 1988). Furthermore, the presence of either sodium azide or 2,4-dinitrophenol (DNP), which reduce ATP pools to a third, abrogate VP-16 cytotoxicity without changing the level of cleavable complexes (Kupfer et al., 1987). This article reports on investigations of the ability of various topoII poisons to induce DNA lesions in almost completely ATP-depleted whole cells in an attempt to differentiate whether they act at different steps in the catalytic cycle.

Materials and Methods

Cell Lines. The human small-cell lung cancer cell line OC-NYH (also designated GLC-2) (de Leij et al., 1985) and the murine leukemia cell line L1210 were used. Cell cultures were performed in RPMI

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ABBREVIATIONS:topo, topoisomerase; SSBs, single-strand breaks; DPCs, DNA protein complexes; DSBs, double-strand-breaks; FCS, fetal calf serum; DMSO, dimethyl sulfoxide; *m*-AMSA, amsacrine; VP-16, etoposide; DNP, 2,4-dinitro-phenol.

1640 medium supplemented with 10% fetal calf serum (FCS) plus penicillin and streptomycin. Depletion of cellular ATP was done by incubating cells in PBS with 5% FCS in the presence of 10 mM sodium azide and 10 mM 2-deoxyglucose or in the presence of 1 mM DNP and 10 mM 2-deoxyglucose (all from Sigma Chemical Co., St. Louis, MO). Non-ATP-depleted cells were incubated in PBS with 5% FCS enriched with 10 mM glucose. The modulators were added 10 min before treatment with drug.

Drugs. Drugs used were kept in aliquots at −20°C and thawed just before use. Camptothecin (CPT; Sigma) and clerocidin (a generous gift from Dr. Poul Rasmussen, Leo Pharmaceuticals, Ballerup, Denmark) were dissolved in dimethyl sulfoxide. Doxorubicin (Pharmacia & Upjohn; Copenhagen, Denmark) and daunorubicin (Rhone-Poulenc Rorer; Birkerod, Denmark) were dissolved in sterile water. *m*-AMSA (Parke-Davis; Frederiksberg, Denmark) delivered in *N*,*N*-dimethylacetamide solution was further diluted in acid lactose. Etoposide, teniposide (both from Bristol-Myers Squibb; Lyngby, Denmark), and mitoxantrone (Lederle; Glostrup, Denmark) were in solution for infusion.

Antibodies. Mouse monoclonal antibody to topoI was generously provided by Dr. Y-C Cheng (Yale University, New Haven, CT; Chang et al., 1992). Rabbit polyclonal antibody against the carboxyl terminus (residues 1513–30) of topoIIα was obtained from CRB Diagnostics (Cheshire, UK). Rabbit polyclonal antibody against topoIIβ was purchased from BioTrend (Cologne, Germany).

Measurement of DNA SSBs. DNA damage was quantified by the alkaline elution filter method as described by Kohn et al. (1981). [^{3}H]Thymidine-labeled L1210 cells used as internal standard were exposed to 100 mM H_2O_2 for 60 min on ice, corresponding to an irradiation dose of 300 rad (Szmigiero and Studzian, 1988). [^{14}C]Thymidine-labeled NYH cells were treated with drug for 1 h at 37°C. Inhibitors of oxidative phosphorylation or glycolysis were added 10 min before drug treatment. Standard and experimental cells were layered on the filter (Nucleopore filter, 2.0 μm pore size) immediately before lysis with 5 ml of lysis solution (2% SDS, 0.1 glycine, and 0.025 M disodium EDTA, pH 10.0). After completion of lysis, 1.5 ml of lysis solution supplemented with 0.5 mg/ml proteinase K (Sigma) was added on the filter. DNA was eluted with tetrapropyl-ammoniumhydroxide-EDTA supplemented with 0.1% SDS at pH 12.1. Fractions were collected at 20-min intervals for 2 h, with an elution rate of 0.125 ml/min. DNA SSB frequencies were expressed in rad-equivalents and calculated as described by Kohn et al. (1981).

Measurement of DNA-Protein Complexes (DPCs). DPCs were measured using 0.8 μm Metricel DM-800 filters (Gelman Sciences, Ann Arbor, MI). No internal standard cells were used. Before drug treatment, cells were incubated on ice with 5 mM H_2O_2 corresponding to a dose of 3000 rad (Szmigiero and Studzian, 1988). Cells were incubated with drug in PBS with 5% FCS in the presence of either 10 mM glucose or 10 mM 2-deoxyglucose and 10 mM sodium azide for 1 h at 37°C. When layered on the filter, cells were lysed with 5 ml of sarcosyl-EDTA lysis solution (2.0 M NaCl, 0.2% sodium lauryl sarcosine, and 0.04 M disodium EDTA, pH 10.0). Elution was performed as above, except SDS and proteinase K were not included. Decreased elution rate is associated with the formation of DPCs as the protein moiety adheres to the filter. Percentage retention of [^{3}H]thymidine-labeled DNA was plotted against elution time.

Measurement of DNA Double-Strand Breaks (DSBs). DSBs were measured by neutral elution (pH 9.6). No internal standard cells were used. Cells were treated with drug in PBS with 5% FCS in

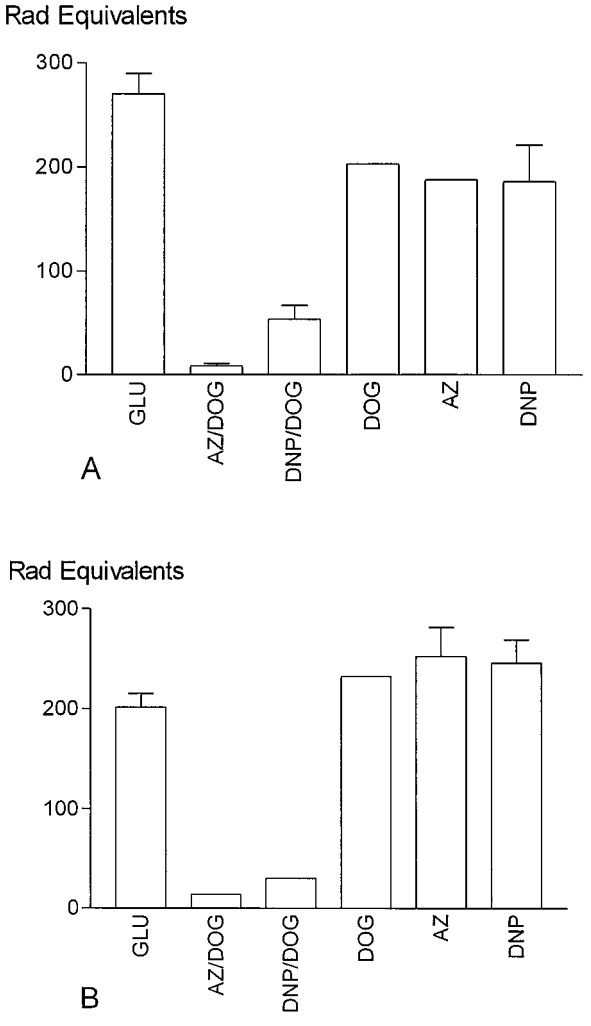

Fig. 1. Etoposide-induced DNA SSBs in NYH (A) and L1210 (B) cells. Cells incubated in PBS with 5% FCS were treated with 3 μM VP-16 at 37°C for 60 (NYH) or 30 min (L1210). Glucose (10 mM; GLU), 10 mM sodium azide, and 10 mM 2-deoxyglucose (AZ/DOG), 1 mM DNP and 10 mM 2-deoxyglucose (DNP/DOG), 10 mM 2-deoxyglucose (DOG), 10 mM sodium azide (AZ), or 1 mM DNP were added 10 min before drug treatment. DNA SSBs were measured by alkaline elution and expressed as rad-equivalents. Error bars indicate S.D.s of two to eight independent experiments.

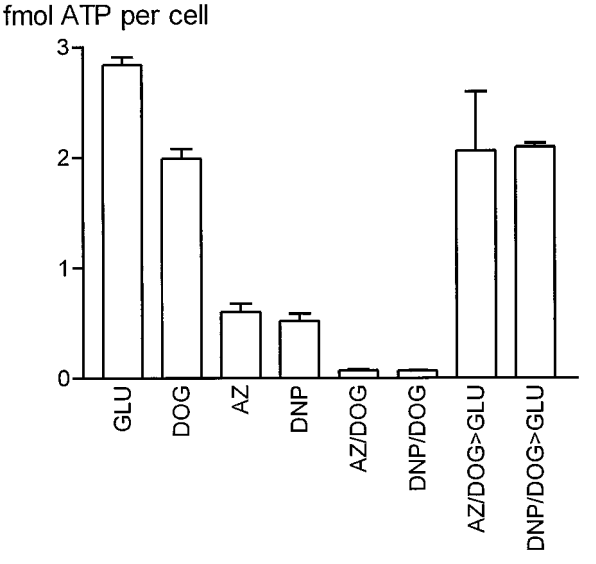

Fig. 2. Measurement of intracellular ATP in NYH cells using the luciferin-luciferase assay. ATP was measured after a 15-min incubation in PBS with 5% FCS supplemented with 10 mM glucose (GLU), 10 mM 2-deoxyglucose (DOG), 10 mM sodium azide (AZ), 1 mM DNP, 10 mM sodium azide and 10 mM 2-deoxyglucose (AZ/DOG), or 1 mM DNP and 10 mM 2-deoxyglucose (DNP/DOG). Additionally, cells preincubated in AZ/DOG or DNP/DOG for 15 min were washed and transferred to glucose enriched PBS for 5 min (AZ/DOG > GLU; DNP/DOG > GLU) before measuring ATP. Error bars indicate S.D.s of two to six independent experiments.

the presence of either 10 mM glucose or 10 mM 2-deoxyglucose and 10 mM sodium azide for 1 h at 37°C. Neutral elution was performed as described above for the alkaline elution measuring DNA SSBs, except elution was done at pH 9.6. Percentage retention of [^{3}H]thymidine-labeled DNA was plotted against elution time.

Drug Accumulation. Cells (5×10^6) were incubated with DNase I (Sigma) at 0.025% for 30 min to dissolve nuclei from dead cells (Versantvoort et al., 1992). Thereafter, cells were incubated with increasing concentrations of [^{3}H]VP-16 (Moravek Biochemicals Inc., Brea, CA) in PBS (57.0 mM NaCl, 5.0 mM KCl, 1.3 mM $MgSO_4$, 51.0 mM Na_2HPO_4, 9.0 mM NaN_2PO_4, pH 7.45) to which 5% FCS was added. Cells were either preincubated with 10 mM glucose or 10 mM sodium azide and 10 mM 2-deoxyglucose. After 60 min at 37°C, the cells were spun down at 150*g* for 5 min and washed twice with ice-cold PBS. Cell pellets were solubilized in 0.8 ml of 0.5 N KOH at 70°C for 1 h and analyzed by liquid scintillation counting. Measurements of *m*-AMSA accumulation were done as above, except 2×10^6 cells were used and the drug was extracted by incubating the pellets in 0.3 N HCl with 50% ethanol for 30 min. The pellets were then spun down and the supernatants were collected. The *m*-AMSA concentration was measured by a spectrophotometer at 435 nm (Skovsgaard, 1978).

Band Depletion Assay. Topo-DNA complexes were measured by a band-depletion assay as described previously by Liu and coworkers (Hsiang and Liu, 1988; Desai et al., 1997). Cells (1×10^6) were preincubated in PBS with 5% FCS supplemented with 10 mM glucose or 10 mM 2-deoxyglucose and 10 mM sodium azide for 10 min followed by drug treatment for 60 min. Cells were pelleted and the pellets were vortexed vigorously before lysis with 400 μl of SDS containing lysis solution (50 mM Tris/HCl, pH 6.8, 15% sucrose, 12 mM EDTA, 3% SDS, 10% β-mercaptoethanol, and 0.1% bromphenol blue). After 5 min on a boiling-water bath, lysates were passed through a 27-gauge syringe 10 times. To avoid air bubbles, lysates were briefly spun down before they were loaded on a 7.5% SDS-polyacrylamide gel. After blotting, membranes were blocked in 10% nonfat milk in PBS buffer with 0.05% Tween 20 and probed overnight with antibodies against either topoI (1:5,000), topoIIα (1:5,000), or topoIIβ (1:10,000). Horseradish peroxidase-linked sheep anti-mouse or anti-rabbit antibodies (Amersham, Buckinghamshire, UK) were used as secondary antibodies. Membranes were incubated in a mixture of luminol and peroxide (Pierce, Rockford, IL) for 5 min with a subsequent exposure to a film. All steps were performed at room temperature. A molecular weight standard was included in each blot. Blots were scanned using Scion Image software from Scion Corporation (Frederick, MD).

ATP Measurements. Intracellular ATP was measured by the luciferin-luciferase method using the kit FL-ASC from Sigma according to the manufacturer's instructions. Sodium azide, DNP, or 2-deoxyglucose were added either alone or in combination to cell suspensions of 0.7×10^6 cells/ml PBS supplemented with 5% FCS. At the indicated time points (Fig. 2), ATP was released by adding 150 μl of cell suspension to 300 μl of ATP-releasing agent. Equal volumes of cell lysate and assay mix solution containing luciferase and luciferin

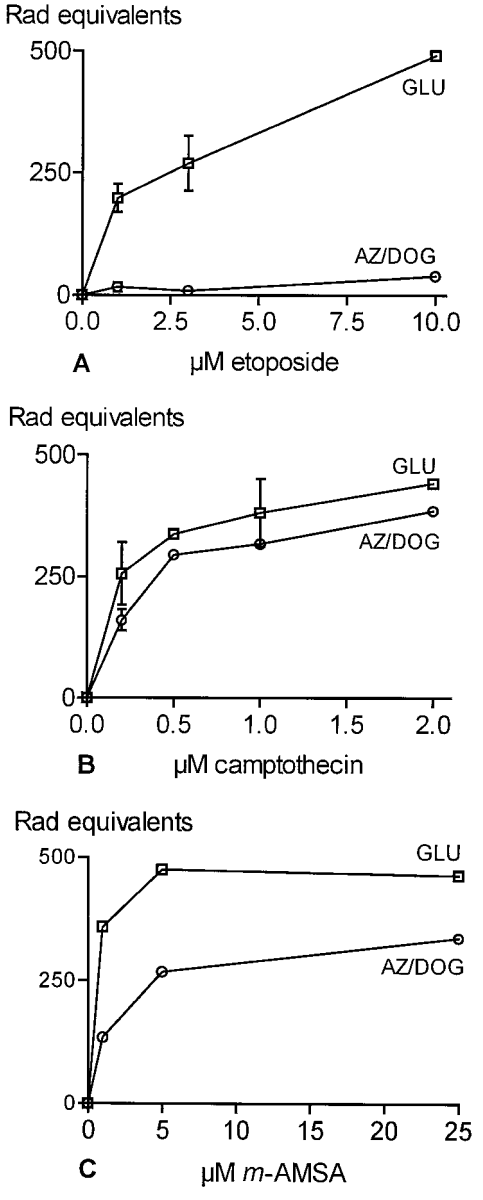

Fig. 3. Dose-response curve of VP-16- (A), CPT- (B), and *m*-AMSA- (C) induced DNA SSBs in NYH cells incubated in the presence of 10 mM glucose (GLU, □) or 10 mM sodium azide and 10 mM 2-deoxyglucose (AZ/DOG, ○). Experiments were performed as indicated in Fig. 1. Error bars indicate S.D.s of two to eight independent experiments.

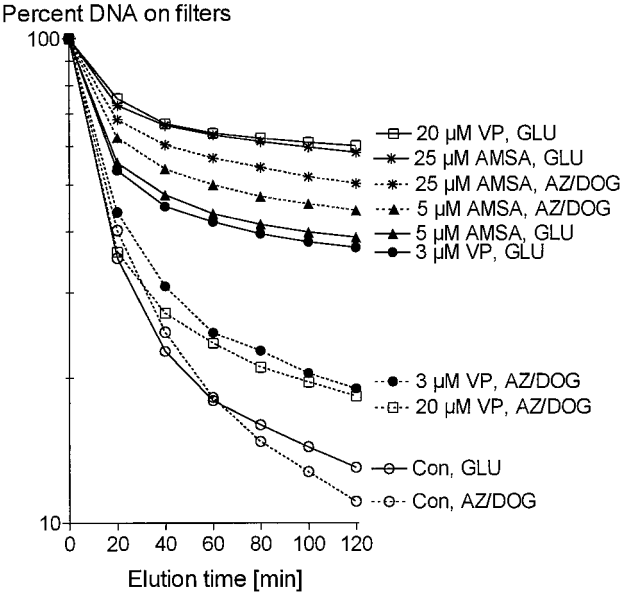

Fig. 4. Measurement of VP-16- and *m*-AMSA-induced DPCs. Cells were treated with drug as indicated in Fig. 1. Before drug treatment, cellular DNA was fragmented by incubating cells on ice with 5 mM H_2O_2. Drug-mediated DPCs adhere to the filters, resulting in decreased elution rates. Unbroken lines, treatment in the presence of 10 mM glucose (GLU); dotted lines, treatment in the presence of 10 mM sodium azide and 10 mM 2-deoxyglucose (AZ/DOG). ○, no drug (Con); ●, 3 μM VP-16; □, 20 μM VP-16; ▲, 5 μM *m*-AMSA; ∗, 25 μM *m*-AMSA. Percentage of labeled DNA remaining on the filters is plotted logarithmically on the *y*-axis.

were rapidly mixed in a glass scintillation vial and immediately counted in a Packard scintillation counter (Packard, Meriden, CT) set to measure single photons.

Results

Effects of ATP Depletion on Etoposide-Mediated DNA SSBs. Depletion of cellular ATP by uncouplers of oxidative phosphorylation such as sodium azide and DNP increases survival in teniposide- or VP-16-treated L1210 cells without reduction in cleavable complex formation (Kupfer et al., 1987; Lock et al., 1996). In accordance with these findings, we found that both sodium azide and DNP had minor influence on the level of VP-16-induced DNA SSBs in NYH (Fig. 1A) and in L1210 cells (Fig. 1B). However, ATP depletion is not complete using sodium azide or DNP alone. Similar to other reports (Kupfer et al., 1987), we found that sodium azide or DNP reduced the level of ATP to approximately 20% of the level in cells incubated in glucose enriched PBS (Fig. 2). Glycolysis is one possible source for residual ATP when oxidative phosphorylation is blocked. To obtain complete ATP depletion, we coincubated cells with 2-deoxyglucose, a competitive inhibitor of glycolysis and inhibitor of oxidative phosphorylation. Using this approach, we were able to further reduce the cellular ATP concentration to 2% of base levels, which approached the detection limit of the luciferase assay (Fig. 2). This nearly complete ATP depletion almost completely blocked formation of VP-16-induced DNA SSBs. As seen in Fig. 1, DNA SSBs dropped to a level that was almost undetectable in both cell lines using the alkaline elution technique. In contrast, 2-deoxyglucose alone did not prevent VP-16-induced DNA SSBs (Fig. 1) in accordance with the modest effect of 2-deoxyglucose on intracellular ATP levels (Fig. 2). Furthermore, we measured DNA SSBs induced by various doses of VP-16 to determine whether the effects of ATP depletion could be surpassed by increasing drug dose. Using doses of VP-16 in the range of 1 to 10 μM, the level of DNA SSBs in ATP-depleted NYH cells remained near the detection limit of the alkaline elution assay, which indicates that the presence of ATP is an absolute requirement for the generation of DNA SSBs (Fig. 3A). To further substantiate that the effect of ATP depletion is specific for the formation of topoII-mediated cleavable complexes, we used the topoI-targeting drug CPT as a negative control. As seen in Fig. 3B, CPT-induced DNA SSBs in ATP-depleted NYH cells are comparable with those of cells treated in the presence of 10 mM glucose over a wide range of CPT doses.

Effects of ATP Depletion on Etoposide-Mediated DPCs. A hallmark of topoisomerase-mediated cleavable complexes is the covalent binding of the enzyme to the 5′ end of the broken DNA strands. To ensure that the DNA SSBs measured under deproteinizing conditions were actually protein linked, we measured DPCs by performing alkaline elution under nondeproteinizing conditions. In contrast to alkaline elution measuring DNA SSBs, DPCs are detected as a reduction in elution rates, because the protein moiety bound to H_2O_2-fragmented DNA will adhere to the filters (compare control cells with 20 μM VP-16-treated cells in the presence of glucose in Fig. 4). In accordance with the DNA SSB measurements, we found that ATP depletion using sodium azide and 2-deoxyglucose almost completely prevented the induction of DPCs by 3 and 20 μM VP-16, as shown in Fig. 4.

Effects of ATP Depletion on Etoposide-Mediated TopoII-DNA Adducts. Using a band-depletion assay, we measured intracellular trapping of topoII to DNA. Nontrapped topoII is readily detected by Western blotting. In contrast, the presence of topoII-DNA adducts results in the depletion of immunoreactive topoII because migration into the polyacrylamide gel is hindered by the attached DNA (Hsiang and Liu, 1988; Desai et al., 1997). Confirming the data obtained using elution techniques, 25 μM VP-16 was unable to trap either topoIIα or -β in ATP-depleted cells as opposed to cells

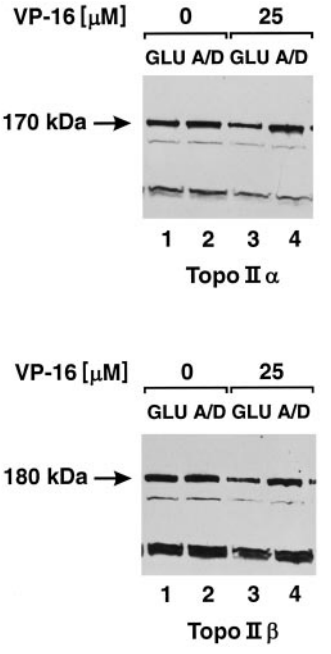

Fig. 5. Band depletion assay. Cells were treated with 25 μM VP-16 (lanes 3 and 4) or without drug (lanes 1 and 2) in the presence of 10 mM glucose (GLU) or 10 mM sodium azide and 10 mM 2-deoxyglucose (A/D). Free topoIIα (top) and topoIIβ (bottom) were detected in SDS-lysed cells by Western blotting. Formation of DNA-topoII complexes results in depletion of immunoreative bands. The intensities of 170- and 180-kDa bands in VP-16 treated cells relative to those in control cells defined as 100% were as follows: lane 3 relative to lane 1: 75% (topoIIα) and 60% (topoIIβ); lane 4 relative to lane 2: 110% (topoIIα) and 98% (topoIIβ). Similar results were found in four independent experiments. Positions of the 170- and 180 kDa bands are indicated at the left side.

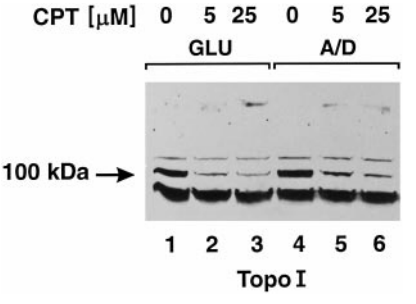

Fig. 6. Band depletion assay. Cells were treated with 5 and 25 μM CPT (lanes 2, 3, 5 and 6) or without drug (lanes 1 and 3) in the presence of 10 mM glucose (GLU) or 10 mM sodium azide and 10 mM 2-deoxyglucose (A/D). Free topoI was detected in SDS-lysed cells by Western blotting. Formation of DNA-topoI complexes results in depletion of immunoreative bands. The intensities of 100-kDa bands in CPT-treated cells relative to those in control cells defined as 100% were as follows: lanes 2 and 3 relative to lane 1: 30 and 21%, respectively; lanes 5 and 6 relative to lane 4: 44 and 26%, respectively. Similar results were found in four independent experiments. Position of the 100-kDa band is indicated at the left side.

with normal ATP pools, as shown in Fig. 5. Even at 100 μM VP-16, energy-depleted cells remained refractory to induction of topoII-DNA complexes (not shown). In contrast, 5 and 25 μM CPT induced topoI-mediated trapping equally effectively in control and ATP-depleted cells, as seen in Fig. 6.

Effect of ATP Depletion on Cellular Uptake of Etoposide. Because whole cells were used in these experiments, one might speculate that a reduced intracellular drug accumulation could be responsible for the observed effects of ATP depletion. To rule out this possibility, we measured the accumulation of radiolabeled VP-16. Under conditions similar to those of the alkaline elution assay, ATP depletion did not decrease cellular uptake of VP-16. In fact, drug accumulation was slightly increased in ATP-depleted cells, which excludes drug transport as a contributor to the effects of ATP depletion (Fig. 7A).

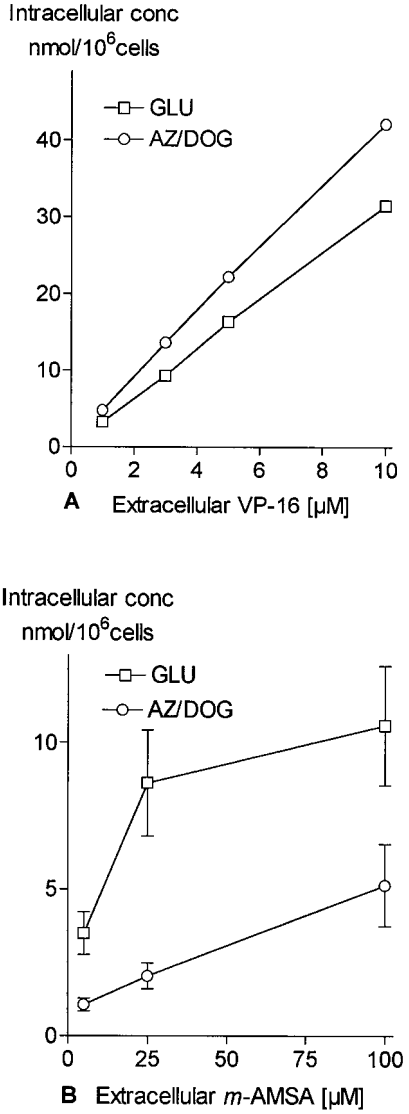

Fig. 7. Accumulation of [^{3}H]VP-16 (A) and *m*-AMSA (B) in the presence of 10 mM glucose (GLU, □) or 10 mM sodium azide and 10 mM 2-deoxyglucose (AZ/DOG, ○) in NYH cells. Cells were incubated for 1 h in PBS with 5% FCS at varying extracellular concentrations of VP-16 and *m*-AMSA indicated on the *x*-axis. Intracellular drug concentrations are indicated in nanomoles per 10^6 cells. Intracellular drug concentrations were measured in a liquid scintillation counter (VP-16) or by using a spectrophotometer (*m*-AMSA).

Reversibility of Effects of ATP Depletion. If ATP depletion is the crucial event preventing the formation of VP-16-mediated DNA SSBs, one would expect that restoration of ATP pools would abolish the prevention of DNA SSBs. To investigate whether this is the case, cells were treated for 30 min with VP-16 in the presence of sodium azide and 2-deoxyglucose and then, after a wash, cells were transferred to PBS enriched with glucose. We found that this strategy did indeed restore ATP levels. The level of ATP reached approximately 75% of control 5 min after cells were transferred to PBS with glucose (Fig. 2). As shown in Fig. 8, inhibition of VP-16 induced DNA SSBs by ATP depletion is reversible, because subsequent incubation with glucose completely restored the level of DNA SSBs.

***m*-AMSA and Other TopoII Poisons.** We investigated the influence of ATP depletion on other poisons of topoII, including teniposide, the intercalating agents doxorubicin, daunorubicin, mitoxantrone, and *m*-AMSA, as well as clerocidin, an inducer of heat- and salt-stable DNA breaks. All compounds showed a pattern similar to that of VP-16 (not shown), except for *m*-AMSA. Interestingly, *m*-AMSA was partly capable of inducing DNA SSBs independent of the presence of ATP. Depending on the drug concentrations used, the level of DNA SSBs in ATP-depleted L1210 (not shown) and NYH (Fig. 3C) cells ranged from 40 to 75% of the level in cells with normal ATP pools. The level of DNA SSBs were unaffected by the presence of sodium azide or DNP alone (not shown). We verified that DNA lesions induced by *m*-AMSA in ATP-depleted cells were indeed protein bound by performing alkaline elution under nondeproteinizing conditions. As shown in Fig. 4, approximately the same level of *m*-AMSA-induced DPCs were detected in cells with normal and depleted ATP pools. Furthermore, we measured DNA DSBs by neutral elution. The levels of *m*-AMSA-induced DNA DSBs in the absence of ATP were only slightly reduced compared with those in cells treated in the presence of glucose. In contrast, VP-16-induced DSBs were completely antagonized by ATP depletion (Fig. 9). In addition, *m*-AMSA trapped a substantial part of the free fraction of topoIIα and -β to DNA in ATP-depleted cells using the band-depletion assay. Densitometric scanning showed that *m*-AMSA depleted approximately 20 to 40% of the free fraction of topoIIα and -β in ATP-deprived cells compared with approximately 35 to 65% trapped in cells with normal ATP levels (Fig. 10). These data are in marked contrast to the complete inability of VP-16 to trap topoII in energy-depleted cells (Fig. 5). Cellular accumulation of *m*-AMSA was reduced by 50 to 80% in ATP-depleted cells compared with cells with normal ATP levels (Fig. 7B), which excludes the possibility that increased drug uptake contributed to *m*-AMSA-induced DNA lesions in the absence of ATP.

Discussion

Several studies have reported that the cytotoxicity of topoII poisons are dramatically antagonized by inhibitors of oxidative phosphorylation, such as DNP and sodium azide, without reducing the number of intracellular cleavable complexes (Kupfer et al., 1987; Shibuya et al., 1991; Lock et al., 1996). However, intracellular ATP pools were only reduced by two thirds (Kupfer et al., 1987). The present study confirms that reduction of ATP levels to 20% by DNP or sodium

azide had no influence on the yield of drug-induced DNA lesions. Interestingly, the protection against cytotoxicity by uncouplers of oxidative phosphorylation is not limited to topoII poisons. Kaufmann et al. (1989) showed that DNP prevented VP-16 and the topoI poison CPT from inducing apoptosis and cytotoxicity as assessed by clonogenic assay. These and subsequent observations (Thakkar and Potten, 1993; Lock et al., 1996; Haga et al., 1998) indicate that processes occurring downstream from cleavable complex formation, such as inhibition of energy-requiring steps in the apoptotic pathway, might be responsible for the protective effect of DNP. Furthermore, inhibition of RNA and DNA synthesis have also been proposed as crucial events (Shibuya et al., 1991; Lock et al., 1996). Interestingly, DNP was able to rescue cells in culture medium from *m*-AMSA-induced cytotoxicity, although ATP levels were unaffected (Shibuya et al., 1991), which indicates that the antagonizing effect of DNP also can operate independently of ATP levels. Because ATP levels are reduced only in part by inhibitors of oxidative phosphorylation, it is not possible to conclude based on previous studies that induction of drug-mediated cleavable complexes in intact cells can occur in the absence of ATP. To determine whether ATP is a requirement for drug-mediated topoII-DNA complexes in intact cells, we used 2-deoxyglucose in combination with DNP or sodium azide to render cells nearly devoid of ATP, corresponding to 2% of ATP levels in control cells. We found that the addition of 2-deoxyglucose almost completely prevented the formation of DNA SSBs using several known topoII poisons. Similarly, Fry (1990) found a 4-fold reduction in VP-16-induced DNA SSBs in L1210 cells incubated in the presence of DNP and 2-deoxyglucose. We excluded the possibility that altered drug transport could account for the effects of sodium azide/2-deoxyglucose. Furthermore, the effects of ATP depletion seem to be operating at the level of cleavable-complex formation, because protein-bound DNA breaks and both topoIIα and -β

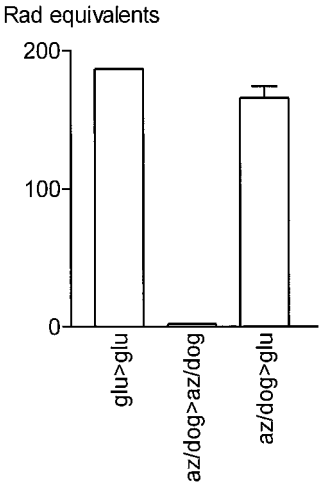

Fig. 8. Inhibition of VP-16-induced DNA SSBs by ATP depletion is reversible. Cells were treated with 3 μM VP-16 for 30 min in PBS followed by a wash and a subsequent rechallenge with VP-16 for an additional 30 min. Modulation of ATP levels in the two 30-min incubation periods were as follows: addition of 10 mM glucose in both periods (glu>glu); addition of 10 mM sodium azide and 10 mM 2-deoxyglucose in both periods (az/dog>az/dog); and addition of 10 mM sodium azide and 10 mM 2-deoxyglucose in the first treatment period followed by transfer to glucose-enriched PBS (az/dog>glu). DNA SSBs were measured by alkaline elution. Error bars indicate S.D.s of two experiments.

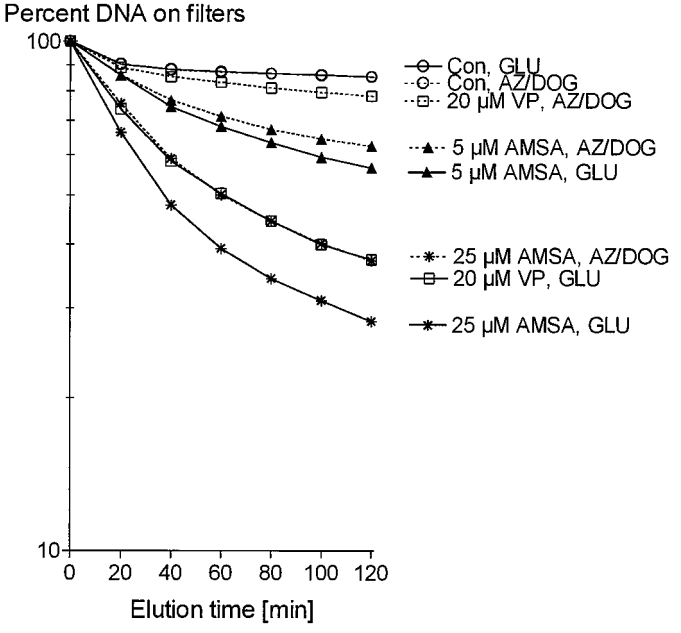

Fig. 9. Measurement of VP-16- and *m*-AMSA-induced DNA DSBs by use of neutral elution (pH 9.6). Cells were treated with drug as indicated in Fig. 1. Unbroken lines, treatment in the presence of 10 mM glucose (GLU); dotted lines, treatment in the presence of 10 mM sodium azide and 10 mM 2-deoxyglucose (AZ/DOG). ○, no drug; □, 20 μM VP-16; ▲, 5 μM *m*-AMSA; ∗, 25 μM *m*-AMSA. Percentage of labeled DNA remaining on the filters is plotted logarithmically on the *y*-axis.

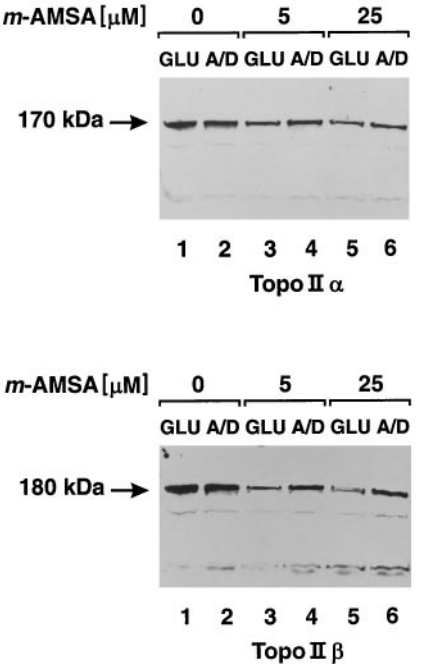

Fig. 10. Band depletion assay. Cells were treated with 5 and 25 μM *m*-AMSA (lanes 3, 4, 5, and 6) or without drug (lanes 1 and 2) in the presence of 10 mM glucose (GLU) or 10 mM sodium azide and 10 mM 2-deoxyglucose (A/D). Free topoIIα (top) and topoIIβ (bottom) were detected in SDS-lysed cells by Western blotting. Formation of DNA-topoII complexes results in depletion of immunoreative bands. The intensities of 170- and 180-kDa bands in *m*-AMSA-treated cells relative to those in control cells defined as 100% were as follows: lanes 3 and 5 relative to lane 1: 64 and 51% (topoIIα), 44 and 37% (topoIIβ), respectively; lanes 4 and 6 relative to lane 2: 76 and 58% (topoIIα), 65 and 60% (topoIIβ), respectively. Similar results were found in two independent experiments. The positions of the 170- and 180-kDa bands are indicated at the left side.

trapping were abrogated by sodium azide and 2-deoxyglucose. The critical role of ATP depletion was underscored by the finding that VP-16-mediated DNA SSBs reappeared as intracellular ATP levels were restored by transferring cells to glucose-enriched PBS. In addition, these experiments show that topoII is not irreversibly inactivated by ATP depletion.

Because the topoI poison CPT efficiently induced DNA SSBs and DNA-topoI adducts, regardless of cellular ATP status, it is tempting to hypothesize that the differential effect of topoI and topoII poisons is caused by the requirement of ATP for the catalytic cycle of topoII. However, *m*-AMSA behaved quite differently than all other topoII poisons tested. In ATP-depleted cells, *m*-AMSA-induced DNA SSBs, DPCs, and DNA DSBs were reduced only modestly. Furthermore, *m*-AMSA trapped an appreciable level of DNA-topoIIα and -β complexes, as opposed to the complete inefficiency of VP-16 to trap topoII in the absence of cellular ATP. Thus, it seems that a substantial part of *m*-AMSA-induced DNA lesions and trapping of topoIIα and -β occur in an ATP-independent manner. Transport studies excluded the possibility that ATP depletion resulted in increased *m*-AMSA accumulation, which would circumvent the antagonistic effect of ATP depletion. Rather, it seems that the decreased accumulation of *m*-AMSA might partly contribute to the modest reduction in DNA lesions in ATP-depleted cells. Thus, in terms of creating DNA lesions, *m*-AMSA seems at least as effective in ATP-depleted cells as in cells with normal ATP levels at a given intracellular concentration.

Apparently, *m*-AMSA does not rely on an catalytically active enzyme to induce DNA lesions, as opposed to VP-16. In vitro, ATP is not a requirement for DNA cleavage using purified DNA (Tewey et al., 1984). The binding of ATP to topoII induces DNA strand passage, whereas ATP hydrolysis is required for enzyme turnover (Osheroff et al., 1983). Thus, it seems that either strand passage or the enzyme's ability to undergo repeated enzymatic cycles is essential for the formation of VP-16-induced DNA breaks, as opposed to *m*-AMSA. The present model cannot differentiate between these two possibilities. However, in vitro data indicate that the two compounds differ with respect to ATP dependence. In contrast to VP-16, *m*-AMSA is a potent inhibitor of topo-mediated ATP hydrolysis (Robinson et al., 1993) and strand passage (Corbett et al., 1993), which implies that in the presence of *m*-AMSA, poststrand passage cleavage is less likely to occur. This notion is consistent with observations showing that VP-16 has a more pronounced ability to stabilize the poststrand passage cleavage complexes than *m*-AMSA (Robinson and Osheroff, 1991). Liu and coworkers have categorized topoII poisons into ATP-independent and -dependent drugs on the basis of in vitro studies. They proposed that ATP-dependent drugs interact preferentially with the salt-stable, closed-gate conformation of topoII, whereas ATP-independent drugs may interact preferentially with the open-gate conformation (Chen and Liu, 1994). They found, as we did, that VP-16-, teniposide-, and doxorubicin-induced breaks are stimulated by ATP (Chen and Liu, 1994; Li and Liu, 1998), whereas menadione, β-lapachone (Frydman et al., 1997), and amonafide induce topoII-mediated cleavage independently of ATP. Our preliminary data show that menadione in vivo displays an even more pronounced ATP independence than *m*-AMSA. Furthermore, in support of this view, we have shown previously that cellular exposure to VP-16 prevents topoII from being extracted from nuclei with high salt extraction (Sehested and Jensen, 1996), whereas *m*-AMSA virtually has no effect on high salt extraction of nuclear topoIIα and -β (not shown). In conclusion, the lack of cellular ATP is associated with the complete inability of VP-16 to stabilize intracellular topoII cleavable complexes, as opposed to *m*-AMSA, which retains its ability to poison topoII in vivo. These in vivo data could reflect in vitro observations indicating that VP-16, among other topoII poisons, interacts with the closed-gate conformation of the enzyme, whereas *m*-AMSA may interact primarily with the open-clamp conformation of topoII, resulting in prestrand passage cleavage. The use of sodium azide and 2-deoxyglucose to deplete ATP seems a feasible method to discriminate between ATP-independent and -dependent topoII poisons and may thus further our insight in the molecular action of topoII poisons.

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