0026-895X/95/050898-09$3.00/0

MOLECULAR PHARMACOLOGY, 47:898–906 (1995).

Altered Topoisomerase I and II Activities in Suramin-Resistant Lung Fibrosarcoma Cells

SOPHIE LELIÈVRE, YOUSSEF BENCHOKROUN, and ANNETTE KRAGH LARSEN

Department of Structural Biology and Pharmacology, CNRS URA 147, Institut Gustave Roussy, 94805 Villejuif Cedex, France

Received October 6, 1994; Accepted February 17, 1995

SUMMARY

To better understand the molecular basis for the cytotoxic effects of suramin, we have developed suramin-resistant DC-3F/SU 1000 cells by continuous exposure of fibrosarcoma cells to increasing concentrations of suramin. The suramin resistance (~10-fold) is not associated with changes in uptake or intracellular distribution of the drug. The sensitivity to actinomycin D, cytarabine, aphidicolin, hydroxyurea, vincristine, and 5-fluorouracil is unaltered. In contrast, DC-3F/SU 1000 cells are about 2-fold resistant to classical DNA topoisomerase II inhibitors such as doxorubicin, amsacrine, and etoposide, whereas the cells are 1.5-fold more sensitive to the topoisomerase I inhibitor camptothecin. The cross-resistance to topoisomerase II inhibitors occurred earlier than the collateral sensitivity to camptothecin. Amsacrine- and etoposide-induced DNA-protein complex formation is reduced about 2-fold in DC-3F/SU 1000 cells, compared with DC-3F cells, whereas camptothecin-induced DNA-protein complex formation is increased 1.5-fold. Western blot analysis of cellular lysates from the two cell lines shows no significant differences in the level of topoisomerase II, whereas the level of topoisomerase I is increased 2.5-fold in DC-3F/SU 1000 cells. The catalytic activities of topoisomerases I and II in nuclear extracts from DC-3F/SU 1000 cells are both about 2-fold higher than those in extracts from DC-3F cells, whereas amsacrine- and etoposide-induced DNA-protein complex formation is comparable between the two cell lines. Taken together, our results support the involvement of DNA topoisomerases in the cytotoxic activity of suramin. We further believe that the DC-3F/SU 1000 cells may be a useful model for the elucidation of factors that lead to low, clinically relevant, levels of resistance to topoisomerase II inhibitors.

Suramin is a hexasulfonated naphthylurea that has been used in the treatment of sleeping sickness and other parasitic diseases for almost 70 years (1). More recently, suramin has shown antitumor activity toward several metastatic cancers refractory to conventional chemotherapy, such as prostatic carcinoma (for recent reviews, see Refs. 2–4). The use of suramin is limited by a variety of toxic side effects, the most serious of which is a Guillain-Barré neuropathy syndrome (2). Further progress in the use of suramin, such as the selection of clinically useful drug combinations and analog development, is currently hindered by an incomplete understanding of its primary mechanism(s) of action, although multiple potential targets have been proposed. Suramin has been shown to inhibit the binding of various growth factors to their receptors and to dissociate receptor-bound growth factors (Refs. 2–4 and references cited therein). Suramin also interacts with many different nuclear enzymes, such as DNA topoisomerase II and DNA polymerases (5–10). In addition, anti-invasive properties have been attributed to suramin (11, 12).

The study of resistant cell lines is one of the most powerful means to understand the mechanisms of action of drugs. We recently described the development and properties of suramin-resistant DC-3F/SU 1000 cells, which were derived from the DC-3F Chinese hamster fibrosarcoma cell line (13). We now report that these cells exibit altered sensitivity to DNA topoisomerase I and II inhibitors, which is associated with altered topoisomerase I and II activities *in vitro* as well as *in vivo*.

Materials and Methods

Drugs and chemicals. Amsacrine [4′-(9-acridinylamino)-3-methanesulfon-*m*-aniside] and etoposide [4′-demethylepipodophyllotoxin-9-(4,6-*O*-ethylidene-β-D-glucopyranoside)] were kind gifts from Dr. Jerzy Konopa (Technical University of Gdansk, Gdansk, Poland). Suramin (Bayer, Leverkusen, Germany) was donated by Drs. Heine and Hamel (Bayer). Hydroxyurea and aphidicolin were obtained from Sigma (La Verpillière, France). Vincristine sulfate (Oncovin) and doxorubicin (Adriablastine) were purchased from Eli Lilly (Saint-Cloud, France) and from Roger-Bellon (Neuilly-sur-Seine, France), respectively. Actinomycin D (Lyovac) was from Merck, Sharp, and Dohme (Rahway, NJ), and cytarabine (Aracytine) was supplied by Upjohn (Paris, France). 5-Fluorouracil was obtained from Roche (Neuilly-sur-Seine, France). Camptothecin was a generous gift from Dr. Christine Jaxel (Orsay, France). [^{3}H]Suramin (9

S.L. and Y.B. are Fellows of the Association pour la Recherche sur le Cancer (Villejuif, France).

ABBREVIATIONS: SDS, sodium dodecyl sulfate; PBS, phosphate-buffered saline; BSA, bovine serum albumin; SV40, simian virus 40.

Ci/mmol) was purchased from Moravek Biochemicals (Brea, CA), [^{14}C]amsacrine (>99% pure; 20.8 mCi/mmol) was kindly provided by the Drug Synthesis and Chemistry Branch, National Cancer Institut (Bethesda, MD), and [^{3}H]etoposide (1 Ci/mmol), L-[^{14}C(U)]leucine (316 mCi/mmol), and [6-^{3}H]thymidine (30 Ci/mmol) were obtained from Amersham Corp. (Les Ulis, France). All other chemicals were of reagent grade.

DNA substrates. Supercoiled plasmid pBR322 DNA (>95% form I) was purchased from Boehringer Mannheim. Highly catenated kinetoplast DNA (form I) was purified from *Trypanosoma cruzi* (generously provided by Dr. Guy Riou, Institut Gustave Roussy Villejuif, France) after DNA extraction and sucrose sedimentation (14).

Antibodies. Antiserum against both α and β forms of DNA topoisomerase II was recovered from rabbits after four subcutaneous injections (at 30-day intervals) with 300 μg of purified yeast topoisomerase II, as described (15). Monoclonal antibodies directed toward topoisomerase I and topoisomerase IIα (16) were generously provided by Dr. A. I. Scovassi, Instituto di Genetica Biochimica ed Evoluzionistica del C.N.R. (Pavia, Italy). Monoclonal antibodies directed toward topoisomerase IIβ were a kind gift from Dr. I. Hickson, Imperial Cancer Research Fund, University of Oxford (Oxford, England).

Cells and culture medium. The DC-3F Chinese hamster fibrosarcoma cell line has been described previously, as have the media and growth conditions (10, 17). The DC-3F/SU 1000 suramin-resistant cells were obtained after about 18 months of continuous exposure to increasing concentrations of suramin in the growth medium (13). The resistant cells are able to grow in the presence of 1 mg/ml suramin and the resistant phenotype is stable for at least 10 months.

Cytotoxicity. Cell survival was measured by colony formation in the continuous presence of drug-containing medium, as described previously (13). Each experiment was done at least twice, with each point determined in triplicate.

Intracellular localization of suramin. The intracellular location of suramin in DC-3F and DC-3F/SU 1000 cells was determined by electron microscopy after 24-hr exposure to [^{3}H]suramin (50 μM). For quantitative analysis, a total of 40 cells/cell line were analyzed as described previously (10).

Drug accumulation assays. Drug uptake in DC-3F and DC-3F/SU 1000 cells was determined as described (13). Briefly, exponentially growing cells were exposed to radiolabeled amsacrine (200 nM) and etoposide (680 nM). At the indicated times, the cells were washed twice with ice-cold 0.154 M NaCl and detached with trypsin. Cell suspensions were harvested and counted in 15 ml of scintillation fluid. The experiments were done at least twice, with each point determined in triplicate.

DNA-protein complex formation in intact cells. To measure the capacity of DNA topoisomerase II to form cleavable complexes, the KCl-SDS precipitation assay of DNA-protein complexes was performed as described by Zwelling *et al.* (18). Cells were incubated in the presence of [^{14}C]leucine (0.2 μCi/ml) and [^{3}H]thymidine (0.6 μCi/ml) for 18 hr to label the DNA and proteins. The cells were exposed to various drug concentrations for 1 hr at 37°. To stop the reaction, a solution of 1.25% SDS, 5 mM EDTA, pH 8.0, and 0.8 mg/ml denatured salmon testis DNA (final concentrations) was added. The cell lysate was passed through a 22-gauge needle 10 times, heated to 65° for 15 min, and then precipitated with 100 mM KCl on ice. The precipitate was washed three times and dissolved in water, and the radioactivity was determined with a Packard liquid scintillation counter. To measure the capacity of DNA topoisomerase I to form cleavable complexes, the same process was used, except that the first incubation was performed at 37° and the cell lysates were precipitated with 250 mM KCl. Data are expressed as the ratio of ^{3}H-labeled DNA to ^{14}C-labeled protein. The protein serves as an internal measure of the exact number of cells used for any given experimental condition.

In another series of experiments, nuclei were isolated as described previously (19) and drug-induced DNA-protein complex formation was determined as described for whole cells. All experiments, whether with whole cells or with isolated nuclei, were done at least four times, with each point determined in triplicate.

Western blotting. To prepare cell lysates, 2×10^7 exponentially growing cells were scraped into ice-cold PBS, centrifuged for 4 min at $400 \times g$, and then washed twice with 20 ml of cold PBS and once with 20 ml of buffer C (1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 50 μg/ml leupeptin, and 10 μg/ml trypsin inhibitor in PBS). Pellets were resuspended at 2.5×10^7 cells/ml of lysis buffer, heated for 5 min at 68°, and passed five times through a 21-gauge needle. Protein concentrations were measured using the bicinchoninic acid assay (Pierce), according to the procedure recommended by the supplier. The different suspensions were adjusted to the same protein concentration with lysis buffer, and 20–100 μg of protein (1 volume of sample with 0.5 volume of 3× loading buffer consisting of 375 mM Tris·HCl, 6% SDS, 30% glycerol, 4% 2-mercaptoethanol, and 2% bromphenol blue) were loaded in each well. Proteins were separated on 7.5% polyacrylamide-SDS gels and then transferred to nitrocellulose filters in 0.192 M glycine, 0.025 M Tris, 20% methanol, at 4° for 5 hr. Nitrocellulose membranes were saturated overnight at 4° in PBS, pH 7.4, containing 3% BSA. The strips were then incubated in PBS/0.1% BSA containing either a purified polyclonal antibody that recognizes both topoisomerase II isoforms (1/500 dilution, 4 hr at room temperature), a monoclonal antibody directed toward the topoisomerase IIα isoform (1/50 dilution, 3 hr at room temperature), a monoclonal antibody directed toward the topoisomerase IIβ isoform (1/3 dilution, overnight at 4°), or a monoclonal antibody directed toward topoisomerase I (1/100 dilution, 2 hr at room temperature). After three 15-min washes in PBS/0.1% Tween 20, the strips were incubated for 2 hr at room temperature in PBS, with 0.1% BSA and 0.1% Tween 20, containing a 1/500 dilution of a peroxidase-conjugated goat anti-mouse IgG or goat anti-rabbit IgG (Sigma). After four washes in PBS/Tween 20 as described above, antibody binding was detected by enhanced chemiluminescence (Amersham Life Sciences, Amersham, UK). Quantitative evaluations were performed by densitometric scanning of the strips using a Joyce-Loebl Chromoscan 3 densitometer. All experiments were done with at least two different cellular lysates.

Nuclear extracts. All steps were done at 4°. To obtain nuclear extracts, about 7×10^7 exponentially growing cells were scraped into 0.154 M NaCl and centrifuged ($500 \times g$ for 4 min). Pellets were washed with 20 ml of buffer A (5 mM KH_2PO_4, pH 7, 0.1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 10 μg/ml trypsin inhibitor, 10 mM 2-mercaptoethanol, 2 mM $MgCl_2$). Cells were resuspended at 10^7 cells/ml in buffer A including 125 nM okadaic acid and were rotated gently for 1 hr. The swollen cells were disrupted by 80 strokes of a Dounce type A homogenizer, and the nuclei were pelleted by centrifugation at $500 \times g$ for 10 min. The pelleted nuclei were resuspended in 3 packed cell volumes of nuclei extraction buffer (buffer A with 0.35 M NaCl, 25 μg/ml aprotinin, and 125 nM okadaic acid) and incubated for 30 min, with gentle stirring. Nuclear extracts were centrifuged at 12,000 $\times g$ for 15 min, and supernatants were adjusted to the same protein concentration by dilution with nuclei extraction buffer. Nuclear extracts were immediately frozen with 20% glycerol (final concentration) and stored at −20°. The nuclear extracts were used for topoisomerase assays within 4 days. Both decatenation and relaxation assays were done at least three times with different nuclear extracts.

Decatenation assay. The reaction mixture contained 50 mM Tris·HCl, pH 8, 10 mM $MgCl_2$, 150 mM KCl, 5 mM EDTA, 5 mM dithiothreitol, 1 mM ATP, and 200 ng of kinetoplast DNA. The reaction was initiated by the addition of nuclear extract and was allowed to proceed at 30° for the indicated times. Reactions were stopped by addition of 1% SDS, 0.5% bromphenol blue, and 30% glycerol. The samples were electrophoresed in 1.2% agarose gels at 5 V/cm for 6 hr, in Tris/borate/EDTA buffer, pH 8.3. Liberated minicircles were quantified by densitometric scanning of photographic

negatives of the ethidium bromide-stained agarose gels with a Joyce-Loebl Chromoscan 3 densitometer.

Relaxation assay. Reaction conditions were as described above, except that no ATP was used and kinetoplast DNA was replaced by 150 ng of pBR322 DNA. Electrophoresis was in 1% agarose gels at 2 V/cm for 18 hr. DNA bands were quantitated by scanning photographic negatives of the ethidium bromide-stained agarose gels with a Joyce-Loebl Chromoscan 3 densitometer. Levels of DNA relaxation were determined by monitoring the loss of the supercoiled band. Under the conditions used, the intensity of bands in the negative was directly proportional to the amount of DNA present.

DNA-protein complex formation with nuclear extracts. SV40 DNA was 5′-end labeled at the *Acc*I site as described previously (20). Briefly, SV40 DNA was linearized with *Acc*I and then the DNA 5′ termini were dephosphorylated with calf alkaline phosphatase and labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. The DNA was purified by phenol/chloroform extraction and ethanol precipitation after each step and at the end of the labeling procedure. The precipitation of end-labeled DNA by nuclear extracts was quantified by the KCl-SDS precipitation method, as described previously (18). Results are expressed as the amount (counts/minute) precipitated in the presence of drug minus that precipitated in the absence of drugs, using a fixed concentration of nuclear extract proteins. All experiments were done with at least three different nuclear extracts, with each point determined in duplicate.

Topoisomerase II-induced cleavage of end-labeled SV40 DNA. The linear, 5′-labeled, SV40 DNA described in the previous section was subjected to a second enzyme digestion with *Eco*RI. This procedure generates uniquely 5′-end-labeled DNA fragments, which can be used to map unequivocally the DNA cleavage sites generated by topoisomerase II. End-labeled DNA (50,000 cpm) was reacted for 15 min at 30° with nuclear extracts from DC-3F and DC-3F/SU 1000 cells, in the absence or presence of amsacrine and etoposide, in a reaction mixture containing 50 mM Tris·HCl, pH 8, 10 mM $MgCl_2$, 150 mM KCl, 5 mM EDTA, 5 mM dithiothreitol, and 1 mM ATP. Reactions were stopped by the addition of a solution of 1% SDS, 20 mM EDTA, and 0.5 mg/ml proteinase K (final concentrations) and were incubated for an additional 1 hr at 42°. Samples were loaded into 1.2% agarose gels in Tris/borate/EDTA buffer containing 0.1% SDS, to remove DNA-bound drug molecules that otherwise would retard the electrophoretic migration of DNA fragments. Agarose gels were run at 2 V/cm overnight, dried, and autoradiographed with Amersham Hyperfilm MP.

Results

Suramin resistance and drug uptake. Suramin-resistant DC-3F/SU 1000 cells derived from the DC-3F Chinese hamster lung fibrosarcoma cell line are about 10-fold resistant to suramin, as determined by colony formation in the continuous presence of the drug (Table 1). The resistance cannot be explained by decreased drug uptake, because drug accumulation is the same for suramin-sensitive and -resistant cells (data not shown). To determine whether the resistance might be linked to altered intracellular distribution of the drug, sensitive and resistant cells were treated with 50 μM [^{3}H]suramin for 24 hr, followed by autoradiography of cellular sections. A quantitative determination of the cellular distribution of suramin shows that the density of silver grains is 2–3-fold higher over the nucleus than over the cytoplasmic area, which confirms our previous findings (10). No significant difference was found between the cellular distribution of grains in sensitive cells and that in resistant cells; the nucleus to cytoplasm ratio is 2.53 ± 0.03 for DC-3F cells and 2.84 ± 0.03 for DC-3F/SU 1000 cells.

TABLE 1

Cross-resistance of suramin-resistant DC-3F Chinese hamster fibrosarcoma cells

Drugs	ED_{50}[a]		Degree of resistance[b]
	DC-3F	DC-3F/SU 1000	
	nM		
Suramin	28×10^3	290×10^3	10.4
Vincristine	2.8	2.9	1
Actinomycin D	2.1	2.3	1
Cytarabine	34	33	1
Aphidicolin	310	310	1
Hydroxyurea	127×10^3	127×10^3	1
5-Fluorouracil	2.4	2.4	1
Amsacrine	6.2	10.8	1.8
Etoposide	48	95	2
Doxorubicin	2.8	4.6	1.7
Camptothecin	46	30	0.65

[a] The cytotoxic effects were determined by colony formation in the continued presence of drug. Each value is the result of at least two individual experiments, each done in triplicate. The standard error was <10% of the mean in all cases.

[b] Ratio of the ED_{50} for the resistant cells to the ED_{50} for the sensitive parental cells.

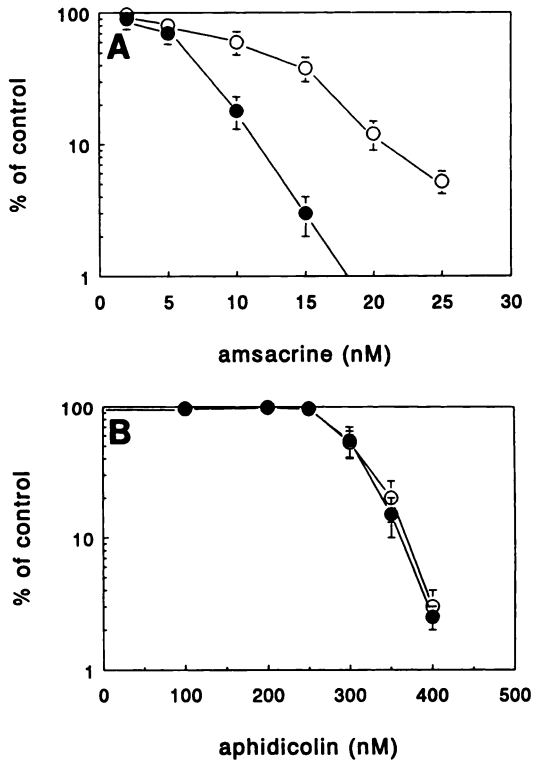

Fig. 1. Cytotoxic effects of amsacrine (A) or aphidicolin (B) on DC-3F (●) and DC-3F/SU 1000 (○) cells, as determined by colony formation in the continued presence of drug. Each value is the average of two individual experiments, each done in triplicate. *Bars*, standard deviations.

Cross-resistance and drug uptake. The cross-resistance of DC-3F/SU 1000 cells to various anticancer drugs was determined. No altered sensitivity was observed with respect to different antineoplastic agents known to interact with nuclear targets such as DNA polymerase, RNA polymerase, and various enzymes involved in DNA metabolism (Table 1). However, a 2-fold resistance was observed toward the DNA topoisomerase II inhibitors amsacrine, etoposide, and doxo-

rubicin. This is further illustrated in Fig. 1, which compares the cytotoxic effects of amsacrine and aphidicolin on suramin-sensitive and -resistant cells. Interestingly, DC-3F/SU 1000 cells are about 1.5-fold more sensitive to the topoisomerase I inhibitor camptothecin (Table 1).

The cross-resistance to topoisomerase II inhibitors could be due to reduced drug uptake or to altered topoisomerase II activity. Fig. 2 shows that the accumulation of etoposide and amsacrine is comparable for sensitive and resistant cells.

Formation of covalent DNA-protein complexes in intact cells. The KCl-SDS precipitation assay was used to determine the covalent interaction between DNA and topoisomerase II. Fig. 3, A and B, shows that the DNA-protein complex formation induced by amsacrine or etoposide is reduced 2-fold in resistant cells, compared with the sensitive parental cell line. The same results were obtained with isolated nuclei from the two cell lines (data not shown). The KCl-SDS precipitation assay was also used to determine the covalent interaction between DNA and topoisomerase I. Fig. 4C shows that the DNA-protein complex formation induced by camptothecin is increased 1.5-fold in DC-3F/SU 1000 cells, compared with DC-3F cells.

Development of cross-resistance. DC-3F/SU 150 cells, which represent an early stage in the development of suramin resistance (13), were used to determine the chronological order in which the altered sensitivity to topoisomerase I and II inhibitors occurred. Fig. 5 shows that these cells already exhibit cross-resistance to etoposide (about 1.3-fold),

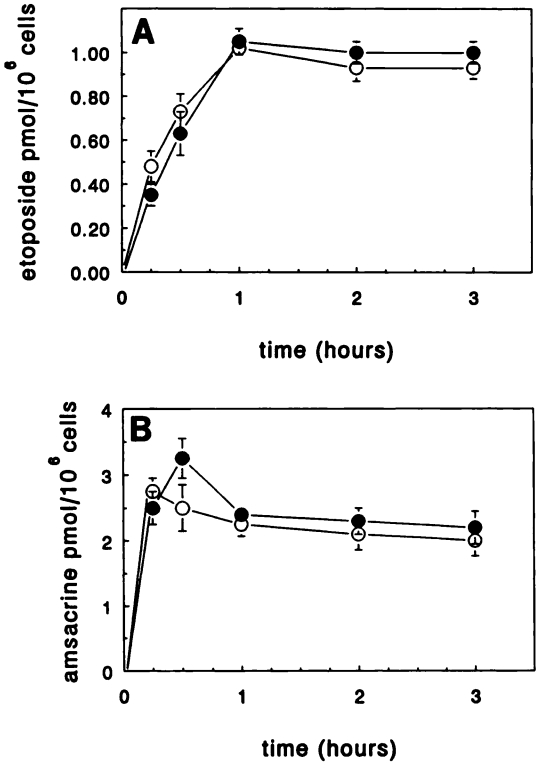

Fig. 2. Accumulation of etoposide (A) or amsacrine (B) as a function of time. DC-3F (●) and DC-3F/SU 1000 (○) cells were incubated in the presence of [^{3}H]etoposide (680 nM) or [^{14}C]amsacrine (200 nM) for the indicated times, and the amount of intracellular drug was determined as described in Materials and Methods. The amount of drug is expressed as pmol/10^6 cells. Values are means of two individual experiments, each done in triplicate. *Bars*, standard deviations.

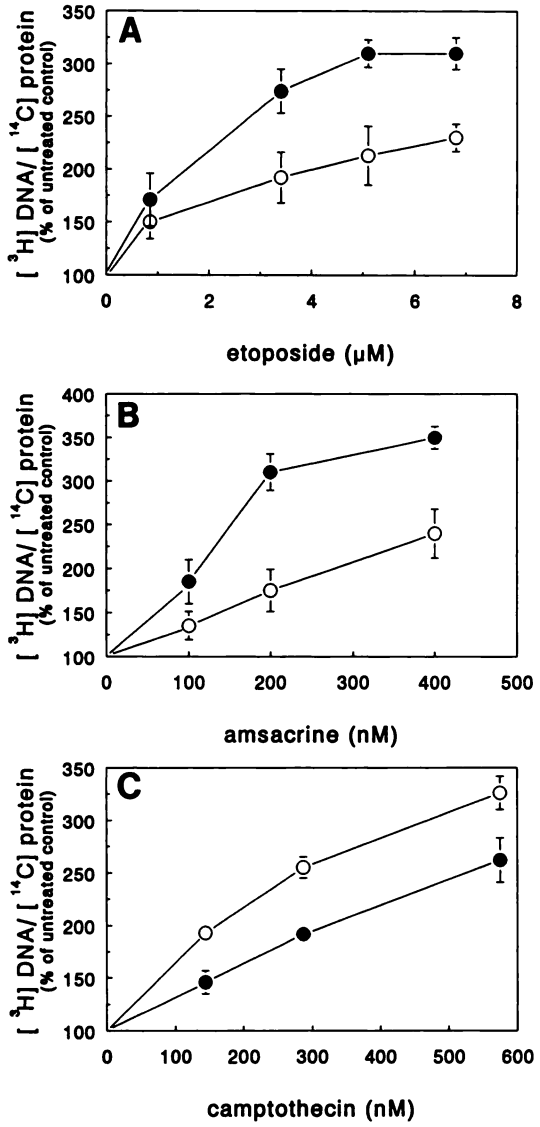

Fig. 3. Drug-stimulated DNA-protein complex formation in DC-3F (●) and DC-3F/SU 1000 (○) cells. DNA and protein were radiolabeled as described in Materials and Methods, and the cells were treated with the indicated concentrations of etoposide (A), amsacrine (B), or camptothecin (C) for 1 hr at 37°. Data are expressed as the amount of radiolabeled DNA precipitated with the cellular protein. Data shown are means of four independent experiments. *Bars*, standard deviations.

whereas the sensitivity to camptothecin is the same as that of the parental DC-3F cells. Therefore, the collateral sensitivity to topoisomerase I inhibitors seems to occur later than the acquisition of cross-resistance to topoisomerase II inhibitors.

Cellular levels of DNA topoisomerases I and II. The levels of DNA topoisomerase II present in DC-3F and DC-3F/SU 1000 cells were quantitated by Western blot analysis of cellular lysates. The blots were probed either with polyclonal anti-topoisomerase II antibodies that recognize both topoisomerase II isoforms (Fig. 5A) or with monoclonal antibodies specific for either the topoisomerase IIβ (Fig. 5B) or topoisomerase IIα (Fig. 5C) isoform. Densitometric analysis of the Western blots shows no significant differences in the levels of topoisomerase IIα and -β between the two cell lines. The levels of DNA topoisomerase I present in the two cell lines were also determined. Fig. 5D shows that the levels

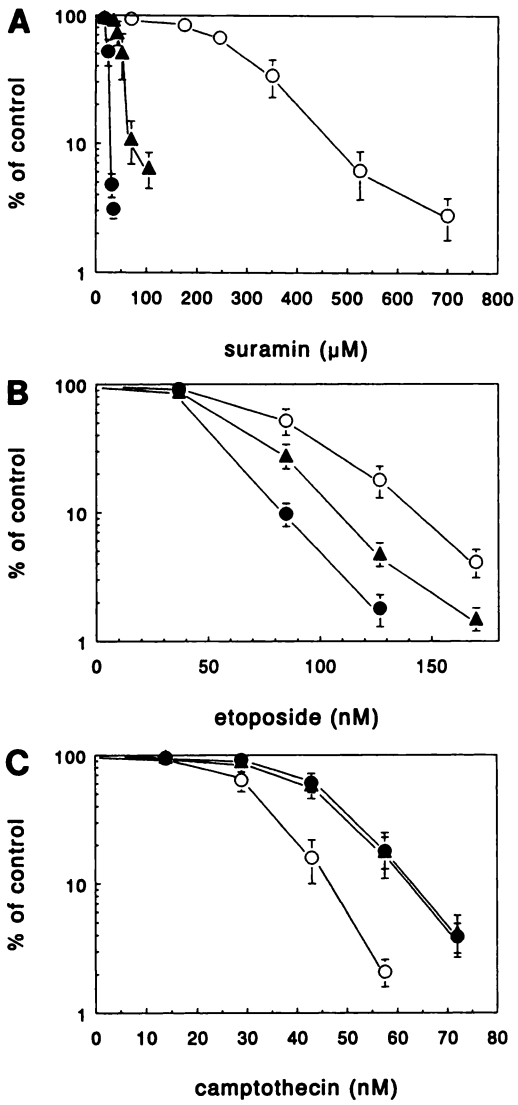

Fig. 4. Cytotoxic effects of suramin (A), etoposide (B), or camptothecin (C) on DC-3F (●), DC-3F/SU 150 (▲), or DC-3F/SU 1000 (○) cells, as determined by colony formation in the continued presence of drug. Each value is the average of two individual experiments, each done in triplicate. *Bars*, standard deviation.

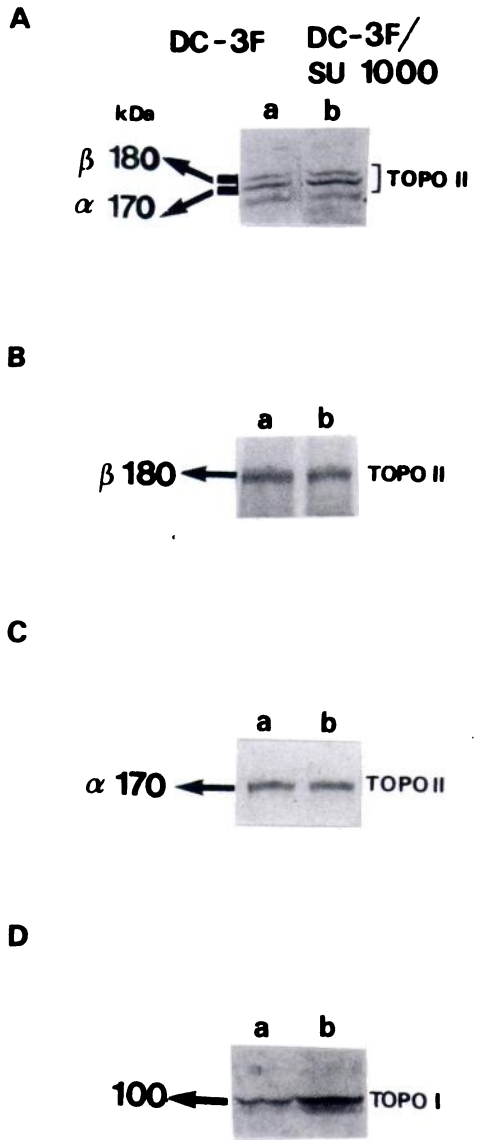

Fig. 5. Western blot analysis of cellular lysates from DC-3F (*lanes a*) and DC-3F/SU 1000 (*lanes b*) cells. Whole-cell lysates were subjected to SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, and probed with antibodies directed toward both topoisomerase II (*TOPO II*) isoforms (A), topoisomerase IIβ isoform (B), topoisomerase IIα isoform (C), or topoisomerase I (*TOPO I*) (D). *Numbers on the left*, size markers (in kDa).

of immunoreactive DNA topoisomerase I are increased about 2.5-fold in DC-3F/SU 1000 cells, compared with the parental DC-3F cells.

Topoisomerase activities in nuclear extracts. Nuclear extracts from sensitive and resistant cells were prepared and the catalytic activity of topoisomerase II was determined by decatenation of kinetoplast DNA. Fig. 6 shows that the catalytic activity is about 2-fold higher in 0.35 M NaCl nuclear extracts from DC-3F/SU 1000 cells than in similar extracts from DC-3F cells. This is not a result of differential extraction of topoisomerase II, because Western blot analysis shows that similar amounts of both topoisomerase IIα and -β are present in nuclear extracts from the two cell lines (data not shown).

The catalytic activity of topoisomerase I in nuclear extracts from sensitive and resistant cells was determined by relaxation of supercoiled DNA in the absence of ATP. Fig. 7 shows that the catalytic activity is about 2-fold higher in nuclear extracts from DC-3F/SU 1000 cells than in similar extracts from DC-3F cells.

Drug-induced DNA-protein complex formation with nuclear extracts. A comparison of the level of drug-stimulated DNA-protein complexes induced by nuclear extracts from the two cell lines revealed no clear differences, in contrast to the findings with whole cells. Fig. 8 shows the distribution of double-stranded DNA cleavage of the SV40 genome stimulated by amsacrine in the presence of nuclear extracts from sensitive (Fig. 8, *lane 2*) or resistant (Fig. 8, *lane 3*) cells. The results indicate that the distribution of topoisomerase II cleavage sites on the SV40 DNA is similar for nuclear extracts from the two cell lines.

Discussion

We have previously shown that suramin penetrates to the nucleus of DC-3F Chinese hamster fibrosarcoma cells, where

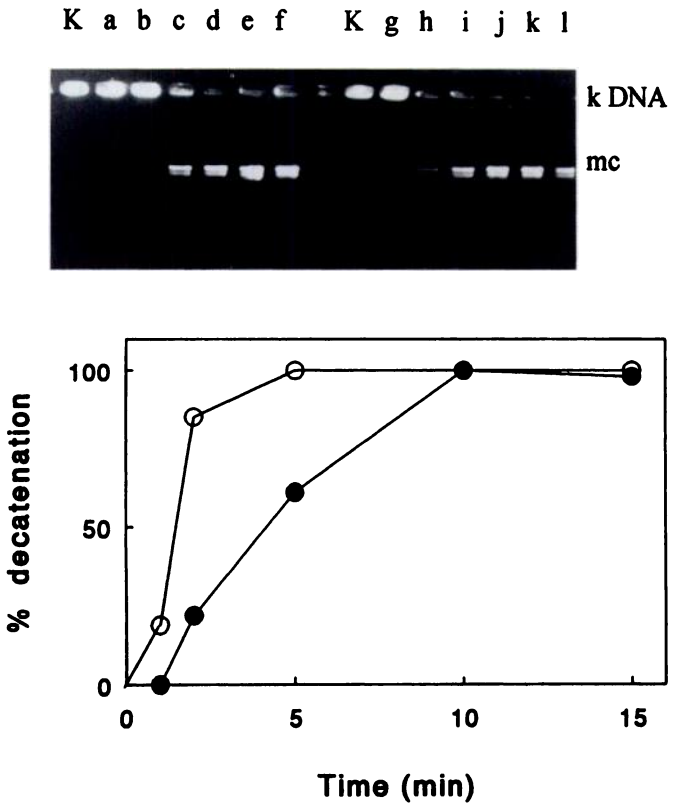

Fig. 6. Catalytic activity of topoisomerase II in nuclear extracts from DC-3F (●) and DC-3F/SU 1000 (○) cells. Kinetoplast DNA (*lane K*) was incubated for 0, 1, 2, 5, 10, or 15 min with nuclear extracts from DC-3F (*lanes a-f*, respectively) or DC-3F/SU 1000 (*lanes g-l*, respectively) cells. *kDNA*, kinetoplast DNA; *mc*, decatenated minicircles. Data shown are typical of four independent experiments with three different nuclear extracts.

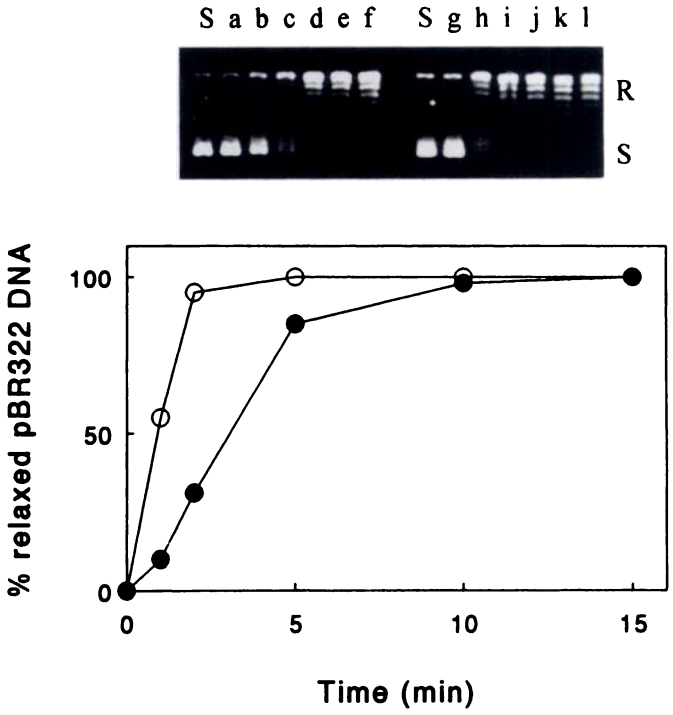

Fig. 7. Catalytic activity of topoisomerase I in nuclear extracts from DC-3F (circf) and DC-3F/SU 1000 (○) cells. Supercoiled pBR322 DNA (*lanes S*) was incubated for 0, 1, 2, 5, 10, or 15 min with nuclear extracts from DC-3F (*lanes a-f*, respectively) or DC-3F/SU 1000 (*lanes g-l*, respectively) cells. *S*, supercoiled DNA; *R*, relaxed DNA. Data shown are typical of four independent experiments with three different nuclear extracts.

it interacts with DNA topoisomerase II. We have also demonstrated that cells resistant to 9-hydroxyellipticine, which have an altered topoisomerase II activity, are cross-resistant to suramin (10). More recently, it has been shown that suramin also interacts with DNA topoisomerase I and II in human prostate cancer cells (21). These results suggest that DNA topoisomerases are targets of suramin action and that this action might play a role in the cytotoxic activity of the drug.

To further understand the molecular basis for the cytotoxic effects of suramin, we have developed suramin-resistant DC-3F/SU 1000 cells by exposing sensitive DC-3F cells to increasing drug concentrations. The resistant cells grow in the presence of 1 mg/ml suramin and are about 10-fold resistant to the drug, compared with the sensitive parental cells (13).

We now show that the suramin-resistant cells are cross-resistant to classical topoisomerase II inhibitors such as amsacrine, etoposide, and doxorubicin. The ability of these drugs to stabilize a covalent reaction intermediate (the cleavable complex) between DNA and topoisomerase II is generally believed to be the first step in the reaction cascade that eventually results in cell death. The drug-induced DNA-protein complex formation was determined by the KCl-SDS precipitation assay in DC-3F and DC-3F/SU 1000 cells. Exposure to amsacrine or etoposide leads to a dose-dependent increase in the formation of DNA-protein complexes in both cell lines. However, at any given dose, the total amount of complexes is reduced by about 40% in the suramin-resistant cells, compared with the parental cells. Similar results are found with isolated nuclei exposed to amsacrine or etoposide, which excludes the possibility that the effect could be due to differential drug uptake. Furthermore, the difference in the formation of DNA-protein complexes is not a result of quantitative differences in the amount of topoisomerase II present in the two cell lines, because Western blot analysis of whole-cell lysates shows no significant differences in the levels of the two topoisomerase II isoforms between sensitive and resistant cells. In contrast, Western blot analysis shows a clear increase in the amount of topoisomerase I in the DC-3F/SU 1000 cells, which is accompanied by an increase in the formation of camptothecin-stabilized DNA-protein complexes. This is likely the reason for the collateral sensitivity of the DC-3F/SU 1000 cells to camptothecin.

To determine the chronological order in which the altered sensitivities to topoisomerase I and II inhibitors occurred, a cell line that represents an early stage in the development of suramin resistance was studied. The results show that the collateral sensitivity to camptothecin seems to occur later than the acquisition of cross-resistance to topoisomerase II inhibitors. This suggests that topoisomerase II may be more sensitive than topoisomerase I to suramin exposure *in vivo*. Alternatively, the expression of topoisomerase I could have been up-regulated to compensate for the altered topoisomerase II activity.

Suramin is a potent inhibitor of several nuclear enzymes, such as DNA polymerase α and RNA polymerases, *in vitro* (5–9). If these enzymes are also inhibited by suramin in living cells and this plays a role in the cytotoxicity, we would

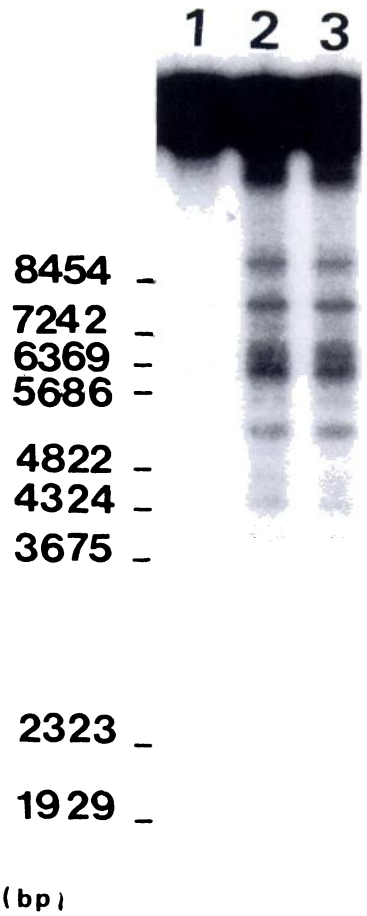

Fig. 8. Topoisomerase II-stabilized cleavage of SV40 DNA. Linear SV40 DNA (*lane 1*) was incubated with 5 μM amsacrine in the presence of nuclear extracts from DC-3F (*lane 2*) or DC-3F/SU 1000 (*lane 3*) cells, followed by treatment with SDS and proteinase K. *Numbers on the left*, genomic positions of cleavage sites.

expect the DC-3F/SU 1000 cells to show an altered sensitivity to drugs such as aphidicolin and cytarabine (which act on DNA polymerase α) and actinomycin D (which acts on RNA polymerases). However, our results show that the sensitivity to these drugs is the same for sensitive and resistant cells. Therefore, even a potent effect of suramin on purified enzymes *in vitro* may not reflect the *in vivo* situation or prove the pharmacological importance of these targets.

To further analyze the changes in topoisomerase activities that had occurred in the DC-3F/SU 1000 cells, 0.35 M NaCl nuclear extracts were prepared from sensitive and resistant cells and their properties were compared. The catalytic activity of topoisomerase I in nuclear extracts from DC-3F/SU 1000 cells is about 2-fold higher than that in similar extracts from DC-3F cells, as determined by the relaxation of supercoiled DNA in the absence of ATP. This difference in catalytic activity is consistent with the increased amount of topoisomerase I in DC-3F/SU 1000 cells. Nuclear extracts from suramin-resistant cells also show about 2-fold higher catalytic activity of topoisomerase II, as measured by decatenation of kinetoplast DNA, compared with extracts from the sensitive parental cells. This cannot be attributed to differential extraction of cellular topoisomerase II, because Western blot analysis shows that similar amounts of topoisomerase IIα and -β are present in nuclear extracts from sensitive and resistant cells.

To evaluate the effects of amsacrine and etoposide on topoisomerase II in the nuclear extracts, drug-stabilized DNA-protein complex formation was determined by the KCl-SDS precipitation method. In contrast to the results observed for living cells, no clear differences in the levels of drug-stabilized DNA-protein complexes were apparent between the two cell lines.

Therefore, the alterations in topoisomerase II activities in DC-3F/SU 1000 cells include 1) about 2-fold reduction in the formation of drug-stabilized DNA-protein complexes *in vivo*, 2) no quantitative changes in the amount of cellular topoisomerase II, 3) increased (~2-fold) catalytic activity *in vitro*, and 4) no difference in the formation of drug-stabilized DNA-protein complexes *in vitro*. These properties are, to the best of our knowledge, different from those reported before for other cell lines resistant to topoisomerase II inhibitors. The DC-3F/SU 1000 cells may therefore be a useful model for the elucidation of factors that can lead to low levels of resistance to topoisomerase inhibitors.

The interpretation that seems to fit best with our data is that the topoisomerase II in the suramin-resistant cells could have undergone some post-translational modifications, such as increased phosphorylation. It has previously been shown that phosphorylation of topoisomerase II by casein kinase II or protein kinase C *in vitro* leads to 2–3-fold increased catalytic activity (22–24). In contrast, phosphorylation has little effect on the level of drug-stabilized cleavable complexes (25). These changes are comparable to those we observed for the DC-3F/SU 1000 cells *in vitro*. The *in vivo* effects of the phosphorylation of topoisomerase II are not known, but it is thought that phosphorylation could also play a role in the nuclear localization of the enzyme. If the nuclear localization of topoisomerase II is indeed altered in the resistant cells, then this might explain the *in vitro/in vivo* discrepancy in drug-induced protein-DNA complex formation.

Our results raise some fundamental questions regarding the mechanism of action of topoisomerase II inhibitors in general. Although all topoisomerase II inhibitors, by definition, are able to inhibit the catalytic activity of topoisomerase II *in vitro*, it is generally agreed that their biological effects might fall into two broad categories. The first group includes the classical topoisomerase II inhibitors such as amsacrine, etoposide, and doxorubicin, which stabilize the cleavable complex. In this case it is probably not the inhibition of the catalytic activity as such that is the lethal event but, rather, the cleavable complex itself somehow triggers cell death. This concept is supported by the finding that drug-induced cleavable complex formation is reduced in a number of cell lines resistant to these drugs (for a recent review, see Ref. 26). The reduced complex formation is usually associated with a decrease in the amount and/or catalytic activity of the enzyme.

The second group of topoisomerase II inhibitors is a heterogenous collection of compounds that inhibit the catalytic cycle at steps different from the cleavable complex and includes fostriecin (27), merbarone (28), bis(2,6-dioxopiperazine) derivatives (29, 30), aclacinomycin (31), novobiocin (32), and suramin (33). The cytotoxic effects of these "new" topoisomerase II inhibitors are presumably a direct

result of inhibition of the catalytic activity of DNA topoisomerase II, which is an essential enzyme (33, 34). Therefore, one would expect cells resistant to this group of topoisomerase II inhibitors to have an increased amount and/or catalytic activity of topoisomerase II. This is consistent with our present results, because the suramin-resistant cells exibit an increased catalytic activity of topoisomerase II. What was initially surprising is that cells resistant to either category of topoisomerase II inhibitors show cross-resistance to compounds from the other category, rather than a collateral sensitivity, which was expected based on the changes in topoisomerase II activity and/or amounts present in cell lines resistant to the two categories of inhibitors. The level of cross-resistance to inhibitors from the other group is often quite modest, compared with the level of resistance to compounds from the same group. For example, cells resistant to 9-hydroxyellipticine are 140-fold resistant to amsacrine but only 7-fold resistant to suramin under comparable conditions (10). Similarily, suramin-resistant cells are 10-fold resistant to suramin but barely 2-fold resistant to etoposide and amsacrine (this report). A slight cross-resistance to new topoisomerase inhibitors has also been reported for teniposide-resistant CEM cells (35). Taken together, these results suggest that the two classes of topoisomerase II inhibitors may have some, but not all, properties in common.

It is possible that the two categories of topoisomerase II inhibitors act on the same pool of nuclear topoisomerase II. Time-lapse, three-dimensional, wide-field microscopy of living *Drosophila melanogaster* embryos injected with rhodamine-labeled topoisomerase II has shown at least three different pools of topoisomerase II associated with the chromosomes, as well as a pool of topoisomerase II localized in the nucleolus (36). It is currently not known what controls the different intranuclear localizations or whether the different pools are equally accessible to topoisomerase II inhibitors. However, it is likely that the intranuclear localization is, at least in part, determined by protein-protein interactions, because topoisomerase II has been shown to form molecular complexes with casein kinase II (15) as well as with chromosome scaffold protein 2 (37). Such complex formation might result in a molecular environment that renders the topoisomerase II less accessible to its inhibitors. Interestingly, it has been shown that suramin can interfere with the formation of complexes between topoisomerase II and casein kinase II (15). It is therefore possible that long term suramin exposure may have resulted in the selection of cells in which such protein-protein interactions are altered.

Taken together, our results support the involvement of DNA topoisomerases in the cytotoxic activity of suramin. We further believe that the DC-3F/SU 1000 cells may be a useful model for the elucidation of factors that lead to low, clinically relevant, levels of resistance to topoisomerase II inhibitors.

Acknowledgments

We gratefully acknowledge the expert technical assistance of Jeannine Couprie and Marie-Christine Bouger. We thank Annie Viron and Marie-Thérèse Maunoury for assistance with electron microscopy and statistical analysis, respectively. We also thank Dr. Mary Danks for kindly providing us with a detailed protocol for the KCl-SDS precipitation experiments. We are grateful to Dr. Andrzej Skladanowski for critical review of the manuscript.

References

1. Hawking, F. Suramin: with special reference to onchocerciasis. *Adv. Pharmacol. Chemother.* **15**:289–322 (1978).
2. La Rocca, R. V., C. A. Stein, and C. E. Myers. Suramin: prototype of a new generation of antitumor compounds. *Cancer Cells* **2**:106–115 (1990).
3. Larsen, A. K. Suramin: an anticancer drug with unique biological effects. *Cancer Chemother. Pharmacol.* **32**:96–98 (1993).
4. Stein, C. A. Suramin: a novel antineoplastic agent with multiple potential mechanisms of action. *Cancer Res.* **53**:2239–2248 (1993).
5. Waring, M. J. The effects of antimicrobial agents on ribonucleic acid polymerase. *Mol. Pharmacol.* **1**:1–13 (1965).
6. De Clercq, E. Suramin: a potent inhibitor of the reverse transcriptase of RNA tumor viruses. *Cancer Lett.* **8**:9–22 (1979).
7. Spigelman, Z., A. Dowers, S. Kennedy, D. Disorbo, M. O'Brien, R. Barr, and R. McCaffrey. Antiproliferation effects of suramin on lymphoid cells. *Cancer Res.* **47**:4694–4698 (1987).
8. Ono, K., H. Nakane, and M. Fukushima. Differential inhibition of various deoxyribonucleic and ribonucleic acid polymerases by suramin. *Eur. J. Biochem.* **172**:349–353 (1988).
9. Jindal, H. K., C. W. Anderson, R. G. Davis, and J. K. Vishwanatha. Suramin affects DNA synthesis in HeLa cells by inhibition of DNA polymerases. *Cancer Res.* **50**:7754–7757 (1990).
10. Bojanowski, K., S. Lelièvre, J. Markovits, J. Couprie, A. Jacquemin-Sablon, and A. K. Larsen. Suramin is an inhibitor of DNA topoisomerase II *in vitro* and in Chinese hamster fibrosarcoma cells. *Proc. Natl. Acad. Sci. USA* **89**:3025–3029 (1992).
11. Nakajima, M., A. De Chavigny, C. E. Johnson, J. I. Hamada, C. A. Stein, and G. L. Nicolson. Suramin: a potent inhibitor of melanoma heparanase and invasion. *J. Biol. Chem.* **266**:9661–9666 (1991).
12. Pienta, K. J., W. B. Isaac, D. Vindivich, and C. S. Coffey. The effects of basic fibroblast growth factor and suramin on cell motility and growth of rat prostate cancer cells. *J. Urol.* **145**:199–202 (1991).
13. Lelièvre, S., and A. K. Larsen. Development and characterization of suramin-resistant Chinese hamster fibrosarcoma cells: drug-dependent formation of multicellular spheroids and a greatly enhanced metastatic potential. *Cancer Res.* **54**:3993–3997 (1994).
14. Riou, G. F., and W. E. Gutteridge. Comparative study of kinetoplast DNA in culture, blood and intracellular forms of *Trypanosoma cruzi*. *Biochimie (Paris)* **60**:365–379 (1978).
15. Bojanowski, K., O. Filhol, C. Cochet, E. M. Chambaz, and A. K. Larsen. DNA topoisomerase II and casein kinase II associate in a molecular complex that is catalytically active. *J. Biol. Chem.* **268**:22920–22926 (1993).
16. Negri, C., R. Chiesa, A. Cerino, M. Bestagno, C. Sala, N. Zini, N. M. Maraldi, and G. C. B. Astaldi Ricotti. Monoclonal antibodies to human DNA topoisomerase I and the two isoforms of DNA topoisomerase II: 170- and 180-kDa isozymes. *Exp. Cell Res.* **200**:452–459 (1992).
17. Salles, B., J.-Y. Charcosset, and A. Jacquemin-Sablon. Isolation and properties of Chinese hamster lung cells resistant to ellipticine derivatives. *Cancer Treat. Rep.* **66**:327–338 (1982).
18. Zwelling, L. A., M. Hinds, D. Chan, J. Mayes, K. L. Sie, E. Parker, L. Silberman, A. Radcliffe, M. Beran, and M. Blick. Characterization of an amsacrine-resistant line of human leukemia cells. *J. Biol. Chem.* **264**: 16411–16420 (1989).
19. Pommier, Y., R. E. Schwartz, L. A. Zwelling, D. Kerrigan, M. R. Mattern, J.-Y. Charcosset, A. Jacquemin-Sablon, and K. W. Kohn. Reduced formation of protein-associated DNA strand breaks in Chinese hamster cells resistant to topoisomerase II inhibitors. *Cancer Res.* **46**:611–615 (1986).
20. Capranico, G., F. Zunino, K. W. Kohn, and Y. Pommier. Sequence-selective topoisomerase II inhibition by anthracycline derivatives in SV-40 DNA: relationship with DNA binding affinity and cytotoxicity. *Biochemistry* **29**:562–569 (1990).
21. Yamazaki, H., A. Dilworth, C. E. Myers, and B. K. Sinha. Suramin inhibits DNA damage in human prostate cancer cells treated with topoisomerase inhibitors *in vitro*. *Prostate* **23**:25–36 (1993).
22. Ackerman, P., C. V. C. Glover, and N. Osheroff. Phosphorylation of DNA topoisomerase II by casein kinase II: modulation of eukaryotic topoisomerase II activity *in vitro*. *Proc. Natl. Acad. Sci. USA* **82**:3164–3168 (1985).
23. Sahyounn, N., M. Wolf, J. Besterman, T.-S. Hsieh, M. Sander, H. Le Vine, K.-J. Chang, and P. Cuatrecasas. Protein kinase C phosphorylates topoisomerase II: topoisomerase II activation and its possible role in phorbol ester-induced differentiation of HL-60 cells. *Proc. Natl. Acad. Sci. USA* **83**:1603–1607 (1986).
24. Rottman, M., H. C. Schröder, M. Gramzow, K. Renneisen, B. Kurelec, A. Dorn, U. Friese, and W. E. G. Müller. Specific phosphorylation of proteins in pore complex-laminae from the sponge *Geodia cydonium* by the homologous aggregation factor and phorbol ester: role of protein kinase C in the phosphorylation of topoisomerase II. *EMBO J.* **6**:3939–3944 (1987).
25. DeVore, R. F., A. H. Corbett, and N. Osheroff. Phosphorylation of topoisomerase II by casein kinase II and protein kinase C: effects of enzyme-mediated DNA cleavage/religation and sensitivity to the antineoplastic

drugs etoposide and 4′-(9-acridinylamino)methane-sulfon-*m*-aniside. *Cancer Res.* **52:**2156–2161 (1992).
26. Cummings, J., and J. F. Smyth. DNA topoisomerase I and II as targets for rational design of new anticancer drugs. *Ann. Oncol.* **4:**533–543 (1993).
27. Boritzki, T. J., T. S. Wolfard, J. A. Besserer, R. C. Jackson, and D. W. Fay. Inhibition of type II topoisomerase by fostriecin. *Biochem. Pharmacol.* **37:**4063–4068 (1988).
28. Drake, F. H., G. A. Hofmann, S.-M. Mong, J. O. Bartus, R. P. Hertzberg, R. K. Johnson, M. R. Mattern, and C. K. Mirabelli. *In vitro* and intracellular inhibition of topoisomerase II by the antitumor agent merbarone. *Cancer Res.* **49:**2578–2583 (1989).
29. Tanabe, K., Y. Ikegami, R. Ishida, and T. Andoh. Inhibition of topoisomerase II by antitumor agents bis(2,6-dioxopiperazine) derivatives. *Cancer Res.* **51:**4903–4908 (1991).
30. Ishida, R., T. Miki, T. Narita, R. Yui, M. Sato, K. R. Utsumi, K. Tanabe, and T. Andoh. Inhibition of intracellular topoisomerase II by antitumor bis(2,6-dioxopiperazine) derivatives: mode of cell growth inhibition distinct from that of cleavable complex-forming type inhibitors. *Cancer Res.* **51:**4909–4916 (1991).
31. Jensen, P. B., P. S. Jensen, E. J. F. Demant, E. Friche, B. S. Sørensen, M. Sehested, K. Wasserman, L. Vindeløv, O. Westergaard, and H. H. Hansen. Antagonistic effect of aclarubicin on daunorubicin-induced cytotoxicity in human small cell lung cancer cells: relationship to DNA integrity and topoisomerase II. *Cancer Res.* **51:**5093–5099 (1991).
32. Rappa, G., A. Lorico, and A. C. Sartorelli. Development and characterization of a WEHI-3B D^+ monomyelocytic leukemia cell line resistant to novobiocin and cross-resistant to other topoisomerase II-targeted drugs. *Cancer Res.* **52:**2782–2790 (1992).
33. Wang, J. C. DNA topoisomerases. *Annu. Rev. Biochem.* **54:**665–697 (1985).
34. Di Nardo, S., K. Voelkel, and R. Sternglanz. DNA topoisomerase II mutant of *Saccharomyces cerevisiae*: topoisomerase II is required for segregation of daughter molecules at the termination of DNA replication. *Proc. Natl. Acad. Sci. USA* **81:**2616–2620 (1984).
35. Chen, M., and W. T. Beck. Teniposide-resistant CEM cells, which express mutant DNA topoisomerase II α, when treated with non-complex-stabilizing inhibitors of the enzyme, display no cross-resistance and reveal aberrant functions of the mutant enzyme. *Cancer Res.* **53:**5946–5953 (1993).
36. Swedlow, J. R., J. M. Sedat, and D. A. Agard. Multiple chromosomal populations of topoisomerase II detected *in vivo* by time-lapse, three-dimensional wide-field microscopy. *Cell* **73:**97–108 (1993).
37. Ma, X., N. Saitoh, and P. J. Curtis. Purification and characterization of a nuclear DNA-binding factor complex containing topoisomerase II and chromosome scaffold protein 2. *J. Biol. Chem.* **268:**6182–6188 (1993).

Send reprint requests to: Annette Kragh Larsen, CNRS URA 147, Institut Gustave Roussy PR II, Villejuif 94805 Cedex, France.