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Characterization of a Subline of P388 Leukemia Resistant to Amsacrine: Evidence of Altered Topoisomerase II Function

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SUMMARY

Sensitive (P388/S) and amsacrine-resistant (P388/amsacrine) sublines of P388 leukemia were cloned *in vitro* and tested for differential chemosensitivity against a panel of drugs. P388/amsacrine, resistant both *in vivo* and *in vitro* to amsacrine, was cross-resistant to other putative topoisomerase II inhibitors including teniposide, etoposide, bisantrene, and doxorubicin. P388/amsacrine, was however, as sensitive as cloned P388/S to camptothecin, an inhibitor of topoisomerase I. The pattern of cross-resistance suggested that an alteration in topoisomerase II may be involved in the resistance of P388/amsacrine to these drugs. No differences in the uptake of amsacrine were detected between the two sublines. Cross-resistance to vinblastine was evident in P388/amsacrine; however resistance to vinblastine was associated with alterations in uptake or efflux of the drug. The number of protein-concealed single-strand breaks induced in whole cells by amsacrine, teniposide, bisantrene, and camptothecin was measured. Diminished numbers of strand breaks in the resistant subline were consistent with decreases in DNA-protein crosslinks. In the absence of drug treatment, resistant cells sustained approximately one-half as many single-strand breaks and DNA-protein crosslinks as the sensitive cells during preparation of nuclei. As measured by the P4 phage DNA unknotting assay, 0.35 M NaCl nuclear extracts from P388/S contained approximately 2.3-fold more topoisomerase II catalytic activity than did extracts from P388/amsacrine. The amount of protein that immunoreacted with a specific antibody to calf thymus topoisomerase II was also decreased in the resistant cells. These data suggest that alterations in topoisomerase II which lead to differential drug sensitivities are partially responsible for the resistance of P388/amsacrine to a specific group of drugs.

DNA topoisomerases I and II are critical nuclear enzymes which regulate three-dimensional DNA structure (1, 2). Several classes of cytotoxic agents, including DNA intercalators (e.g., amsacrine and doxorubicin) and epipodophyllotoxins, induce topoisomerase II-mediated cleavage of DNA *in vitro* (3–8) and protein-concealed DNA strand breaks in cultured mammalian cells (3–5, 8). Camptothecin, a potent cytotoxic plant alkaloid which is active against a number of experimental murine neoplasms (9), induces topoisomerase I-mediated single-strand cleavage of DNA *in vitro* (10, 11) and protein-concealed single-strand breaks in cells (12). These drugs appear to stabilize topoisomerase-DNA covalent complexes, which can be trapped as "cleavable complexes" upon denaturation and protease treatment (3, 6, 13, 14).

Amsacrine has been shown to have antitumor activity in experimental animal tumor models and in humans with acute myelogenous leukemia (15, 16). Evidence linking an interaction with topoisomerase II and the cytotoxicity and antitumor activity of amsacrine has been reported (8, 17). In addition, it has been shown that amsacrine binds to DNA (17), produces DNA single-strand breaks (18), interacts with copper, generating free radical-induced DNA strand breaks (19), and reacts with sulfhydryl groups on small molecules (e.g., glutathione) and proteins (20, 21). To define more clearly the biochemical pharmacology of amsacrine and to provide a cellular system to evaluate analogues of this compound, an amsacrine-resistant subline of P388 lymphocytic leukemia (P388/amsacrine) was developed by serial passage of tumor cells in mice treated with amsacrine (22). Complete resistance to the antitumor effects of amsacrine developed after 20 transplant generations of drug exposure. Resistance to amsacrine was found to be stable in the absence of drug exposure and did not appear to be due to alterations in the cellular uptake or efflux of amsacrine (23). The unique pattern of cross-resistance of P388/amsacrine may provide a tool to help elucidate the mechanism(s) of resistance. The cells are partially or completely resistant to a number of

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ABBREVIATIONS: P388/amsacrine, amsacrine-resistant subline of P388 leukemia; P388/S, amsacrine-sensitive (wild-type) subline of P388 leukemia; A20, clonally derived *in vitro* subline from P388/amsacrine; Wt20, clonally derived *in vitro* subline from P388/S; PBS, phosphate-buffered saline; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; EGTA, ethylene glycol bis (β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid.

structurally unrelated drugs, including etoposide (VP-16), teniposide (VM-26), mitoxantrone, and doxorubicin (22). Because each of these agents, like amsacrine, produces protein-concealed DNA strand breaks in cells (3, 4, 8) and induces topoisomerase II-mediated strand cleavage of DNA *in vitro* (3–8), it is possible that alterations in topoisomerase II are responsible for resistance in P388/amsacrine. To test this hypothesis, we have undertaken a detailed *in vitro* pharmacological and biochemical comparative evaluation of the P388/S and P388/amsacrine tumor lines. We report here the *in vitro* cloning of cell lines from their respective *in vivo* tumors and evaluation of: 1) chemosensitivity of the cloned lines *in vitro* and *in vivo*, 2) the ability of putative topoisomerase II inhibitors to produce protein-concealed DNA strand breaks in whole cells and nuclei isolated from the resistant and sensitive clones, and 3) topoisomerase II activity in nuclear extracts from the two cloned cell lines. The data suggest that an alteration in drug sensitivity at the level of topoisomerase II is in part responsible for the chemorefractory phenotype of P388/amsacrine.

Materials and Methods

Drugs and chemical reagents. Amsacrine, teniposide, etoposide, mitoxantrone, camptothecin, ellipticine, and bisantrene were kindly provided by the Drug Synthesis and Design and Natural Products Branches of the National Cancer Institute, Bethesda, MD. Vinblastine, mitomycin C, dactinomycin, cisplatin, and doxorubicin were purchased from Sigma Chemical Co., St. Louis, MO. Other reagents were purchased from Sigma or Thomas Scientific, Swedesboro, NJ.

***In vitro* cloning and maintenance of P388 tumor cell lines.** P388/S and P388/amsacrine tumors were maintained by serial intraperitoneal passage in syngeneic DBA/2 mice from cryopreserved stocks, as described previously (22). P388/S and P388/amsacrine cells were inoculated intraperitoneally (10^6 cells) into DBA/2 mice. On day 7, the ascites tumors were removed from the mice. Cells were washed twice by centrifugation in PBS and then suspended in RPMI 1630 medium supplemented with 20% fetal calf serum, 100 μg/ml penicillin, 100 μg/ml streptomycin, 10 μM 2-mercaptoethanol, and 0.6% noble agar in a total volume of 5 ml in round bottom polypropylene tubes. Following 6–7 days of incubation at 37° in a 5% CO_2-humidified incubator, microscopic colonies were visible. Under these conditions, the cloning efficiency of both tumors was 0.5–1%. Individual colonies were then removed from the soft agar and grown as suspension cultures in the medium described above, minus agar. Cultures were determined to be free of *Mycoplasma* contamination (24). Individual cultures were stored in liquid nitrogen. All *in vitro* experiments were carried out with cultures of the clones which had undergone ≤30 passages.

Soft agar clonogenic assay. Cultures were treated with various concentrations of the drugs for 1 hr at 37°. Treated cells were then diluted (1:200) into the *in vitro* cloning medium and incubated for 7 days at 37° to permit development of macroscopic colonies, which were stained with tetrazolium salts (0.1%) for 1–2 days and then enumerated with a Biotran III automatic totalizer (New Brunswick Scientific Co.). Cloning efficiency for each of the cloned tumor cell lines in this assay was 2–3%. The mean ± standard deviation of triplicate samples was determined for each drug concentration. The data were expressed by plotting the log of the surviving fraction (cloning efficiency in drug-treated samples/cloning efficiency in controls) versus drug concentration.

Cellular accumulation assay. [9-^{14}C]Amsacrine (10^2 Ci/mol, Stanford Research Inst., Menlo Park, CA) was incubated with 2×10^6 A20 and Wt20 cells in 0.5 ml of RPMI 1630 medium (containing supplements as described above). Cells were incubated at 37° with increasing concentrations of amsacrine for 30 min or with 20 μM amsacrine for various times from 0 to 60 min. Cells were transferred to glass microfiber filters in a sampling manifold (Millipore Corp., Bedford, MA) and were washed five times with 5 ml of cold PBS containing 50 μM nonradiolabeled amsacrine. Filters were then dried and placed into scintillation vials with 5 ml of Econofluor (New England Nuclear, Boston, MA), and cell-associated radioactivity was measured in a Beckman LS 9800 counter (Beckman Instruments, Palo Alto, CA). [G-^{3}H]Vinblastine sulfate (9.8 Ci/mM; Amersham Corp., Arlington Heights, IL) was incubated at a concentration of 20 nM with 3×10^6 cells in 0.5 ml of RPMI 1630 medium (with supplements) for time intervals up to 90 min at 37°. At each time of measurement, 5 ml of cold PBS were added to the cells, which were collected by centrifugation at 1000 rpm for 5 min at 4°. Cell pellets were washed once with 5 ml of cold PBS, resuspended in 0.5 ml of PBS, and transferred to vials containing 10 ml of HPB scintillation fluid (Beckman Instruments) and 5 ml of H_2O for determination of cell-associated radioactivity as described above.

Determination of protein-concealed DNA strand breaks. DNA filter elution assays were performed as described by Kohn *et al.* (25). Drug-treated and untreated control cells were mixed with equal numbers of ^{3}H-labeled cells that had been irradiated with 300 rads in a Gammacell 40 cesium source (Atomic Energy of Canada, Ltd.) (dose rate 134 rads/min). Cells were deposited onto polycarbonate membrane filters (2-μm pore diameter; Nucleopore Corp., Pleasanton, CA) and lysed with a solution containing 0.1 M glycine, 0.025 M disodium EDTA, 2% SDS, pH 10, with or without 0.5 mg/ml proteinase K. Elution was carried out with tetrapropylammonium hydroxide/EDTA/0.1% SDS, pH 12.1, at a flow rate of 0.04 ml/min. Fractions were collected every 3 hours for a total of 18 hr. Single-strand break frequency was calculated and results were expressed as "rad equivalents" of DNA strand breakage.

In experiments in which isolated nuclei were evaluated for protein-concealed DNA strand breaks and DNA-protein crosslinks, labeled cells were washed and incubated in radiolabel-free medium for 1 hr. Nuclei were prepared by a modification of the method of Filipski and Kohn (26). Cells were incubated in nucleus buffer [150 mM NaCl/1 mM KH_2PO_4/5 mM $MgCl_2$/1 mM EDTA/0.2 mM dithiothreitol/10% (v/v) glycerol/0.1 mM phenylmethylsulfonyl fluoride, pH 6.4], containing 0.15% Triton X-100 for 10 min at 0–4° and then centrifuged and washed with Triton-free nucleus buffer. Nuclei were incubated with various concentrations of drug in Triton-free nucleus buffer for 1 hr and then assayed for protein-concealed DNA strand breaks.

DNA-protein crosslinking. DNA-protein crosslinking was estimated by a gravity drip method adapted by Bartus *et al.* (27) from Minford *et al.* (28). [^{3}H]Thymidine-labeled cells or nuclei (isolated as described above) were deposited onto polyvinyl chloride filters (Millipore Corp.) mounted in Swinnex holders (25) and lysed by passing through the filter 5 ml of 0.2% Sarkosyl, 40 mM EDTA, 2 M NaCl, pH 10. The lysed material was washed with 2 ml of 20 mM Tris, 10 mM EDTA, pH 7.9. Lysis and wash fractions were collected into 20-ml scintillation vials. Flow through the filters was interrupted by clamping off the tubing attached to the filter units positioned beneath the funnels, and 2 ml of Fe(II)$(NH_4)_2(SO_4)_2$-EDTA solution were added to each funnel to randomly degrade the DNA (29). Solutions were prepared 1–2 hr before use as follows. Stock solutions of 200 mM dithiothreitol and 5 mM Fe(II)$(NH_4)_2(SO_4)_2$ were prepared, the latter stirred at room temperature for 30 min. To 20 mM Tris-10 mM EDTA, pH 7.9, were added Fe(II)$(NH_4)_2(SO_4)_2$ stock to 5 μM and dithiothreitol stock to 1 mM. The Fe(II)-EDTA mixture was added to the funnels and kept in contact with the lysed cells at room temperature for 30 min, after which it was allowed to drip from the funnels by the removal of the clamps. Excess Fe(II) was removed by washing with 20 mM Tris, 10 mM EDTA, pH 7.9. Fe(II) and wash solutions were collected into 20-ml scintillation vials. DNA fragments not attached to protein were removed by alkaline elution with four 5-ml additions of tetrapropylammonium hydroxide (pH 12.1), each one collected into a 20-ml scintillation vial. Radioactivity remaining on the filters and that in each eluted fraction were determined as described previously (28), and the fraction of the total radioactivity remaining on the filter was

calculated. This value was considered to be directly proportional to the extent of DNA-protein crosslinking (25), and values obtained from various cell preparations could be compared to ascertain relative DNA-protein crosslinking.

Preparation of nuclear extracts. Nuclei prepared from 10^8 cells were resuspended in 1 ml of nucleus buffer at 4°. Five M NaCl was slowly added to a final concentration of 0.35 M, and nuclei were extracted for 30 min by gentle rotation at 4° and then centrifuged at 1,800 rpm for 20 min at 4°. Supernatants were collected into Eppendorf tubes and centrifuged again at 12,000 × *g* for 10 min at 4° to remove any nuclei or insoluble material. Extracts were assayed for protein concentration (30) or P4 unknotting activity (31), electrophoresed on SDS-polyacrylamide gels, or assayed for topoisomerase II content by immunoblotting within 24 hr of nucleus extraction.

Topoisomerase II assay. Topoisomerase II catalytic activity in nuclear extracts was assayed using the P4 unknotting assay described previously (31).

Protein gel electrophoresis and immunoblotting. Polyacrylamide gels (7.5%) were run at 25 mamp for 2 hr in a circulating water bath at 4°. Proteins were electrotransferred to nitrocellulose (0.2 μM pore size) for 16 hr at 60 V at 4° using a Bio-Rad Trans-Blot cell as described (32). Blots were incubated sequentially with a polyclonal antibody to calf thymus topoisomerase II (kindly provided by Dr. Leroy Liu, Johns Hopkins University) and ^{125}I-protein A, and then dried. Antibody-reactive proteins were visualized by autoradiography as described by Halligan *et al.* (33).

Results

Selection and propogation of cloned cell lines. The development, *in vivo* growth characteristics, and chemosensitivity of P388/amsacrine have been reported (22). To obtain clonally derived cell lines for *in vitro* characterization, P388/S and P388/amsacrine tumors were cloned in soft agar (see Materials and Methods). Individual clones were selected randomly and established as suspension cultures. Two clones of each tumor type were evaluated further. All four clones had doubling times of 10 ± 1 hr and grew to approximately the same maximum cell density (2.3 ± 0.4 × 10^6/ml).

***In vitro* chemosensitivity of clones from P388/S and P388/amsacrine.** Each clone was evaluated for sensitivity to amsacrine in the *in vitro* soft agar clonogenic assay (Fig. 1). The dose response curves obtained for the two P388/S clones (Wt19 and Wt20) were identical: 0.13 μg/ml amsacrine for 1 hr decreased clonogenic capacity by 1 log (IC_{90}). Although the shapes of the dose response curves were the same for the sensitive and resistant clones, the curves for both P388/amsacrine clones (A19 and A20) were shifted to the right of those for Wt19 and 20 with IC_{90} values of approximately 2.5 μg/ml (Fig. 1). Thus, the clones selected from the amsacrine-resistant tumor displayed approximately 20-fold resistance to amsacrine *in vitro*. Wt20 and A20 were selected for further studies. Both of these clones were maintained (in the absence of amsacrine) as suspension cultures. The clones were routinely passed up to 40 times, during which time the A20 cells maintained a stable level of resistance to amsacrine.

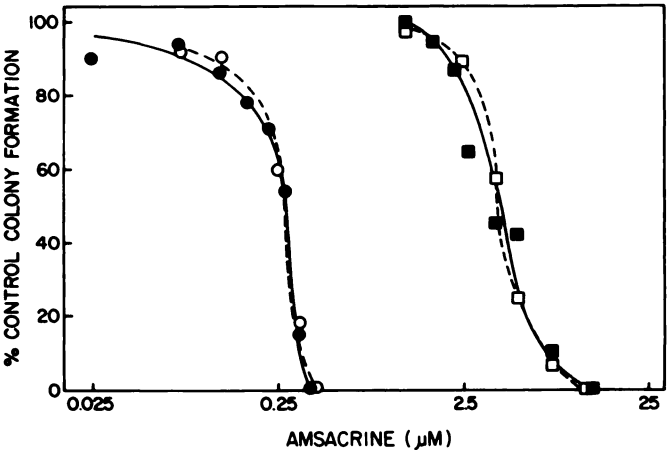

Fig. 1. Response of cloned cell cultures from P388/S [Wt19 (○) Wt20 (●)] and P388/amsacrine [A19 (□), A20 (■)] to a 1-hr treatment with amsacrine as determined in the soft agar colony formation assay. Values are the result of three separate determinations in which standard deviations were within 10% for each value.

A20 and Wt20 were evaluated for their *in vitro* sensitivity to a variety of structural and mechanistic types of antitumor agents (Table 1). A20 cells were resistant to a number of agents in whose mechanism of action topoisomerase II has been implicated (teniposide, ellipticine, doxorubicin, bisantrene, and dactinomycin). Of all drugs tested, A20 cells were most resistant (200-fold) to bisantrene. No cross-resistance to cisplatin or camptothecin and only weak resistance to mitomycin C were detected. In addition, A20 cells were resistant to vinblastine, an agent reported to bind to tubulin (34), by a factor of 10. When evaluated *in vivo* as an intraperitoneal tumor in mice and compared to the P388/S tumor, the A20 clone maintained resistance to amsacrine, ellipticine, doxorubicin, bisantrene, dactinomycin, and vinblastine (data not shown).

Cellular accumulation of amsacrine and vinblastine by A20 and Wt20 clones. Kessel *et al.* (23) reported that P388/S and P388/amsacrine cells accumulate radiolabeled amsacrine and daunorubicin to similar extents, suggesting that resistance is not the result of impaired cellular drug accumulation. To determine whether this phenotypic characteristic of the P388/amsacrine tumor was retained in the A20 clone, we measured uptake and accumulation of labeled amsacrine in A20 and Wt20 cells (Fig. 2). Following 30 min of incubation, the cellular accumulation of radiolabel was similar in both cell types over a range of amsacrine concentrations (Fig. 2A). The time course of uptake was similar in A20 and Wt20 cells exposed to 20 μM amsacrine, with rapid uptake and attainment of steady state within 20 min (Fig. 2B). In contrast, studies using [^{3}H] vinblastine showed that the rates of accumulation and steady

TABLE 1

***In vitro* sensitivity of cloned amsacrine-sensitive and -resistant P388 tumor lines[a]**

Drug	Wt20 IC_{90}[b]	A20 IC_{90}	Resistance ratio[c]
	μM	μM	
Amsacrine	0.33	6.3	19
Teniposide	0.15	1.5	10
Bisantrene	0.21	43	200
Ellipticine	0.41	2	5
Doxorubicin	0.07	0.7	10
Dactinomycin	0.015	0.12	8
Cisplatin	1.7	1.7	1
Camptothecin	2.3	2.9	1.3
Mitomycin C	1.3	3.2	2.5
Vinblastine	0.04	0.4	10

[a] As determined in a soft agar clonogenic assay following a 1-hr exposure to the drug.

[b] Concentration required to inhibit colony formation by 90% relative to control. Values are the result of three separate determinations in which standard deviations were within 10% for each value.

[c] A20 (IC_{90}) divided by Wt20 (IC_{90}).

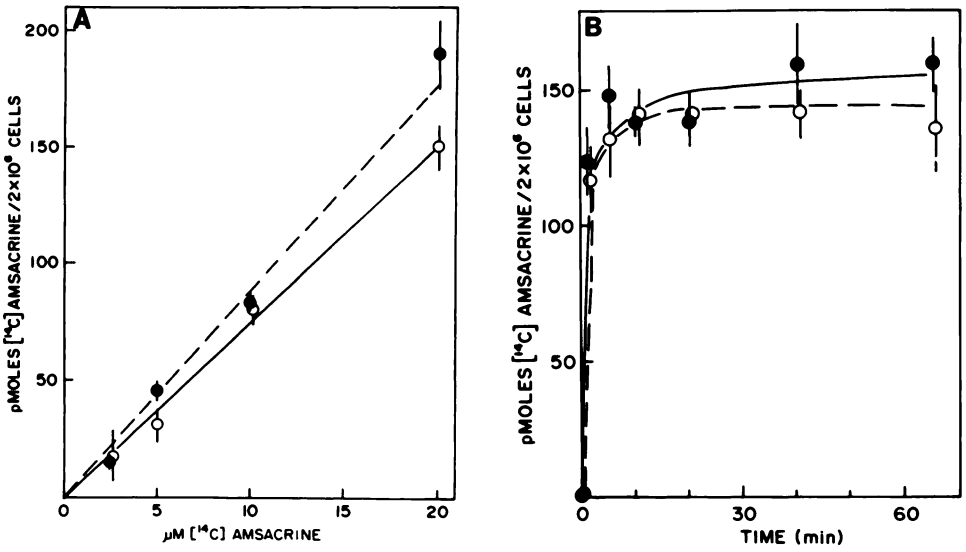

Fig. 2. Cellular accumulation of [^{14}C]amsacrine by Wt20 (●) and A20 (○) cells. Cells were incubated with [^{14}C]amsacrine at various concentrations for 30 min (A) or with 20 μM [^{14}C]amsacrine for increasing time intervals (B). Cell-associated radioactivity was determined as described in Materials and Methods. Points represent the average of two determinations from separate experiments, and the *bars* represent their range.

state concentrations achieved in A20 and Wt20 cells were different. After 30 min of incubation, the P388/amsacrine cells accumulated approximately 75% less [^{3}H]vinblastine than did the P388/S cells (Fig. 3).

Formation of protein-concealed DNA strand breaks in sensitive and resistant cells. Trapping of DNA topoisomerase II by amsacrine and other putative topoisomerase II inhibitors can be detected in intact cells by DNA-filter elution measurements of protein-concealed DNA strand breaks. Amsacrine, teniposide, and bisantrene generated far more single-strand breaks in Wt20 than in A20 cells (Fig. 4, A, B, and D). Camptothecin produced dose-dependent increases in single-strand breaks in both the Wt20 and A20 cells (Fig. 4C). At the concentrations of camptothecin tested, there were no differences in the total numbers of single-strand breaks produced in the two cell lines. Vinblastine at concentrations up to 100 μM produced no single-strand breaks in either cell type (data not shown).

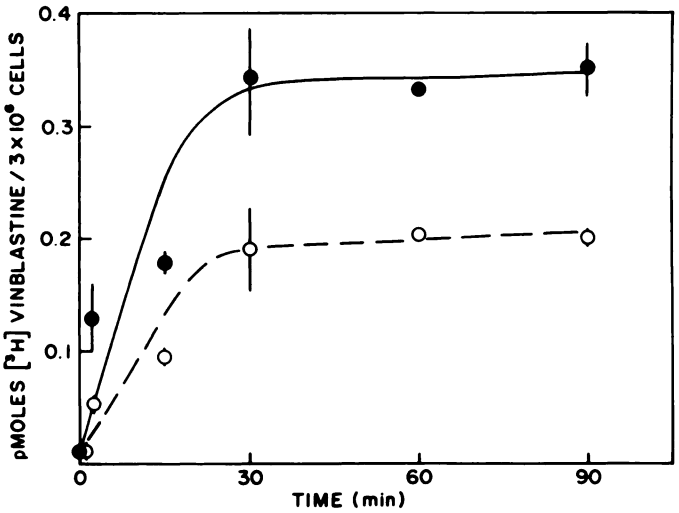

Fig. 3. Cellular accumulation of [^{3}H]vinblastine by Wt20 (●) and A20 (○) cells. Cells were incubated with 20 μM [^{3}H]vinblastine for increasing time intervals of 90 min. Cell-associated radioactivity was determined as described in Materials and Methods. Points represent the average of two determinations from separate experiments, and the *bars* represent their range.

DNA strand breakage induced by drugs in isolated nuclei was assayed to determine if there were cytoplasmic effects that could have affected the yields of single-strand breaks (Fig. 5). The differences in the numbers of single-strand breaks produced in A20 and Wt20 cells by amsacrine or teniposide were observed in isolated nuclei, as well as in whole cells. Incubation of nuclei isolated from either Wt20 or A20 cells with bisantrene did not result in DNA single-strand breaks. This result is consistent with the results of Bowden *et al.* (35), who demonstrated that bisantrene and a structurally related anthracene, mitoxantrone, both produced protein-concealed DNA strand breaks in whole L1210 leukemia cells but not in isolated nuclei.

Production of DNA strand breaks and DNA-protein crosslinks during the isolation of nuclei from A20 or Wt20 cells. The Triton X-100 method of nucleus preparation is a relatively mild technique, yet it produces DNA that elutes more rapidly from filters than DNA from corresponding lysed cells (26). The amount of this intrinsic DNA strand breakage depends upon cell type. Although DNA from either Wt20 or A20 untreated cells sustained the same negligible amount of strand breakage, DNA from Wt20 isolated nuclei consistently eluted more rapidly than that from A20 nuclei (Fig. 6). Because: 1) this difference in fragility of chromosomal DNA was revealed by detergent-based nucleus isolation, and 2) detergent treatment unmasks protein-associated DNA strand breakage that is mediated by topoisomerases (26), intact cells and isolated nuclei were assayed for DNA-protein covalent links after 60 min at 37° in the absence of any drug. The assay was based on that developed by Minford *et al.* (28). Proteinase-sensitive retention of randomly fragmented DNA from lysed cells on protein-adsorbing filters (polyvinyl chloride) was taken as a quantitative measure of relative DNA-protein linking (28). The filters retained <5% of the fragmented DNA from lysed A20 or Wt20 whole cells (data not shown), but 10–20 or 30–40% of the fragmented DNA of lysed nuclei isolated from A20 or Wt20 cells, respectively (Fig. 7). Proteinase K reduced the retention of DNA to background amounts, confirming that DNA fragments were retained by protein binding (Fig. 7). Thus, nuclei isolated from amsacrine-resistant cells by detergent treatment were deficient in both strand breaks and DNA-protein crosslinks assayed in the absence of drug.

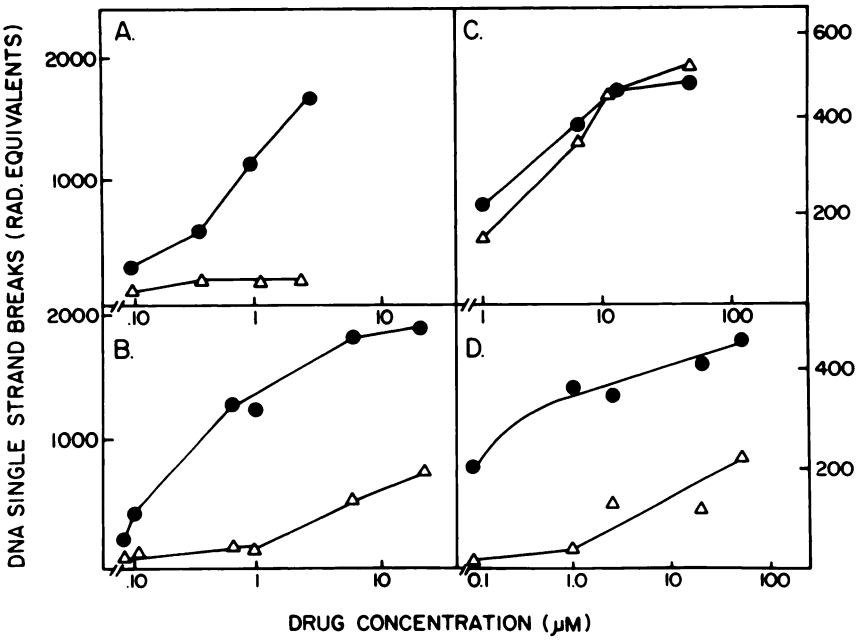

Fig. 4. Concentration dependence of protein-concealed DNA single-strand breakage in Wt20 (●) or A20 (△) cells incubated with amsacrine (A), VM-26 (B), camptothecin (C), and bisantrene (D). Cell lysis occurred in the presence of 0.5 mg/ml proteinase K. Data were calculated from alkaline elution kinetics as described by Kohn *et al.* (25). Data are averages from two or more independent experiments. The average error of the alkaline elution assay is 10–15%.

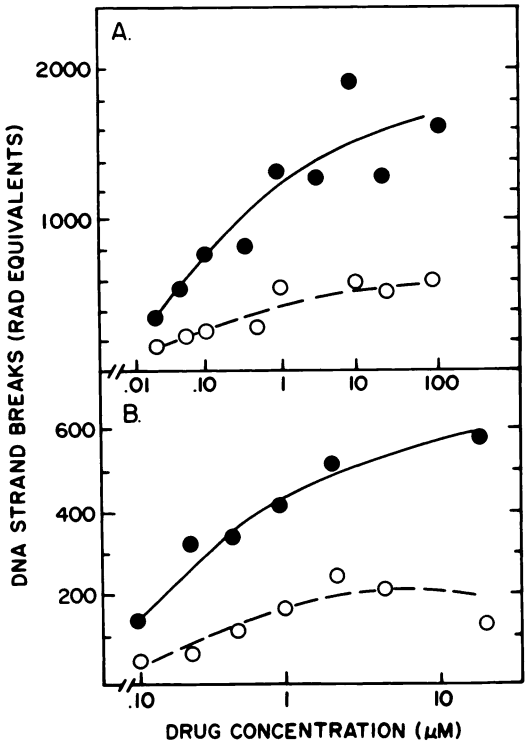

Fig. 5. Concentration dependence of protein-concealed DNA single-strand breakage in isolated nuclei from Wt20 (●) or A20 (○) incubated with amsacrine (A) or VM-26 (B). Nuclei were lysed in the presence of 0.5 mg/ml proteinase K. Data are averages from two or more independent experiments; the average error of the alkaline elution assay is 10–15%.

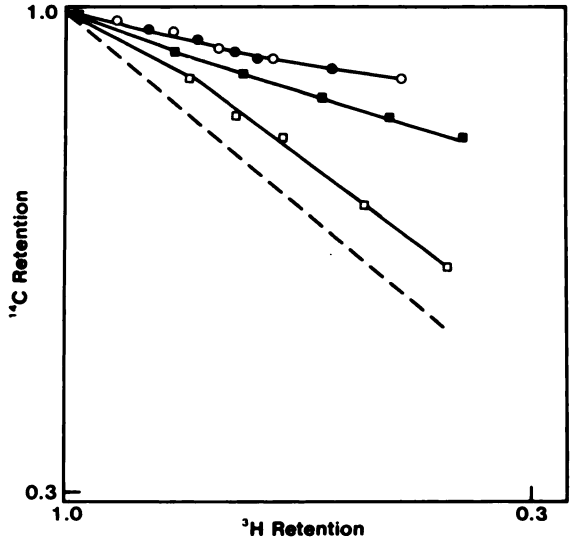

Fig. 6. Alkaline elution rates of DNA from untreated Wt20 (○, □) or A20 (●, ■), cells (○, ●) or isolated nuclei (□, ■). – – –, the average elution rate of DNA from cells irradiated with 300 rads.

Topoisomerase II activity in 0.35 M NaCl extracts from A20 and Wt20 nuclei. Differences in the sensitivity of A20 and Wt20 to the protein-concealed DNA strand breakage that were: a) induced by topoisomerase II inhibitors, and b) observed in isolated nuclei without drug exposure could be due to quantitative and/or qualitative changes in their topoisomerase II. To test whether there were quantitative differences in this enzyme in A20 and Wt20 cells, topoisomerase II catalytic activity was assayed in 0.35 M NaCl nuclear extracts of each line by measuring the ability of the extracts to unknot P4 phage DNA. Nuclear extracts from 10^6 cells of A20 and Wt20, containing approximately equal amounts of protein, were serially diluted to determine the maximum dilution at which one unit of P4 unknotting activity could be measured (Fig. 8). In this assay, P4 knotted DNA migrates as a smear representing a heterogeneous population of DNA molecules containing various numbers of topological knots (31). Upon the removal of the knots via the catalytic strand-passing activity of topoisomerase II, the circular form of P4 DNA migrates as a single band directly above the trailing end of the smear representing knotted DNA. One unit of enzyme is defined as the amount of enzyme required to completely unknot 0.4 μg of P4 DNA. As shown in Fig. 8, the extract from A20 cells contained one unit

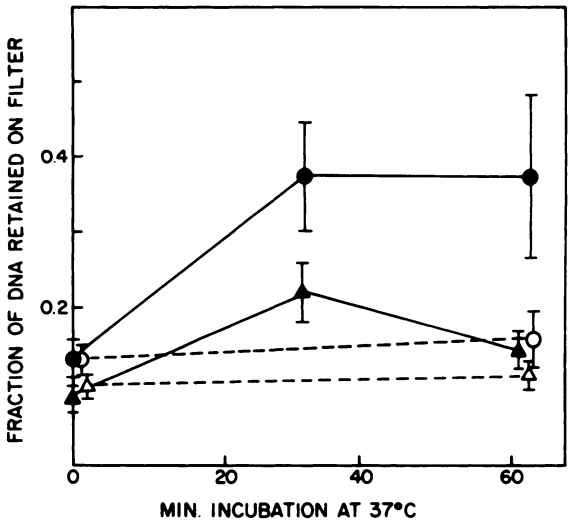

Fig. 7. DNA-protein crosslinks in nuclei isolated from Wt20 (●) and A20 (▲) cells. Radiolabeled nuclei were isolated by the Triton method and incubated at 37° for increasing time intervals in nucleus buffer and then lysed with (○, △) or without (●, ▲) 0.5 mg/ml proteinase K. The nuclei were eluted from polyvinyl chloride filters and the fraction of the total [^{3}H]DNA retained on the filters was measured. Points represent three determinations and the *bars* indicate standard errors.

of enzyme at a dilution of 1:400, whereas the Wt20 extract contained one unit of enzyme at 1:800. Thus, when the extracts were normalized for equivalent protein concentration, those of Wt20 nuclei contained approximately 2.3-fold more topoisomerase II activity than did those of A20 nuclei. This result was obtained in three independent experiments in which topoisomerase II activity in nuclear extracts of Wt20 was 1.7-, 1.9-, and 3.2-fold that in A20 extracts. The amount of topoisomerase II in each cell type was determined using a polyclonal antibody to topoisomerase II isolated from calf thymus (33). As indicated by immunoblot analysis, the number of topoisomerase II molecules in Wt20 and A20 cells differed as well.

In lysates of both Wt20 and A20 cells, the antibody reacted primarily with two proteins of molecular weights 173,000 and 164,000 (Fig. 9). These proteins were observed in whole cell lysates and in nuclear extracts, in both of which the ratio of the 164-kDa protein to the 173-kDa protein was greater in Wt20 than in A20 cells. The ratio of the total of 164 kDa + 173 kDa protein from A20:Wt20 was approximately 3:1 as determined by densitometric analysis of immunoblots for both whole cells and nuclear extracts, in good agreement with the ratios of P4 unknotting activity (see above). Immunoblot analysis of Wt20 or A20 nuclear pellets, after extraction, demonstrated that extraction had removed 90–95% of both 164- and 173-kDa topoisomerase II antibody-reactive proteins (data not shown).

Discussion

These studies were performed to establish an *in vitro* system to assess the role of topoisomerase II in multiple drug resistance. Consistent with such a role, the cell line that we have cloned from the P388/amsacrine tumor is resistant to amsacrine-induced cell killing and production of protein-concealed DNA strand breaks. Moreover, the congruity of biochemical and biological data obtained with other, structurally diverse compounds known to interact with topoisomerase II strongly suggests that a change in drug sensitivity at the level of this target enzyme is involved in the resistance of A20 cells to a variety of antineoplastic agents.

Recently, a number of reports have implicated topoisomerase II in the resistance of various cell lines to intercalators and/or epipodophyllotoxins. For example, 20-fold resistance to epipodophyllotoxins was obtained by mutagenesis and single-step selection in Chinese hamster ovary cells (36). This cell line was subsequently found to be resistant to a variety of other antineoplastic agents (37). Glisson *et al.* (38) have shown that the multidrug resistance phenotype of these cells may be the result of a qualitative change in topoisomerase II that alters the interaction of drugs with the enzyme or enzyme-DNA complex. Pommier *et al.* (39) investigated the mechanism of resistance of a Chinese hamster lung cell line selected for resistance to 9-hydroxyellipticine; the cells are also resistant to amsacrine, daunorubicin, vincristine, and methotrexate (40). Although their resistance to some of the drugs could in part be due to reduced uptake, as is common in pleiotropic resistance, the cells were not deficient in the uptake of 9-hydroxyellipticine,

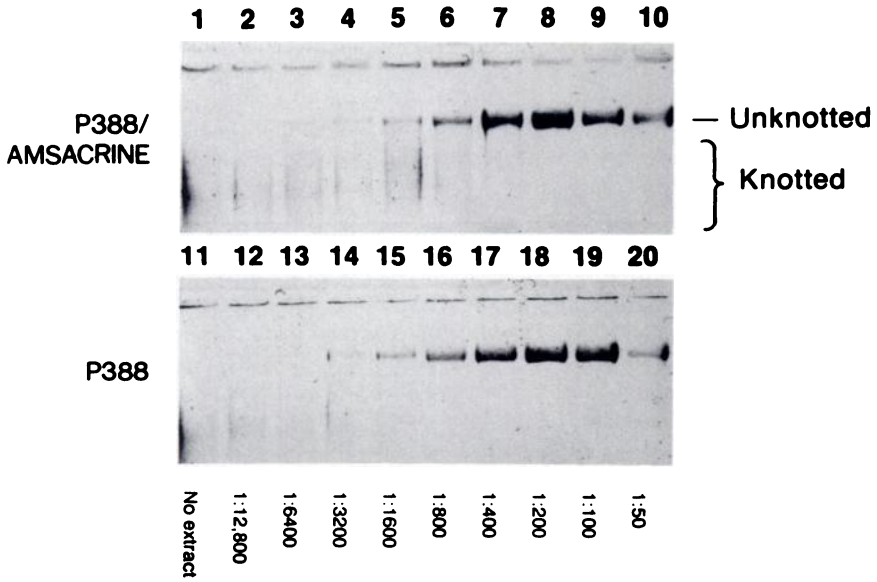

Fig. 8. DNA strand passing activity of 0.35 M NaCl extracts of nuclei from A20 (lanes *2–10*) and Wt20 (lanes *12–20*). Nuclear extracts from 10^6 cells at dilutions of 1:12,800 (lanes *2* and *12*), 1:6,400 (lanes *3* and *13*), 1:3,200 (lanes *4* and *14*), 1:1600 (*5* and *15*), 1:800 (*6* and *16*), 1:400 (*7* and *17*), 1:200 (*8* and *18*), 1:100 (*9* and *19*), and 1:50 (*10* and *20*) were incubated with 0.4 μg of P4 DNA and analyzed on 0.7% agarose gels as described previously (31). Lanes *1* and *11* contain P4 DNA incubated in the absence of nuclear extract.

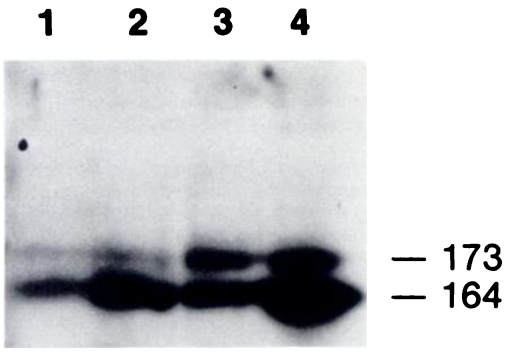

Fig. 9. Immunoblotting of SDS-polyacrylamide gel of A20 (lanes *1* and *3*) and Wt20 (lanes *2* and *4*) whole cell lysates (lanes *1* and *2*), 0.35 M NaCl extracts of isolated nuclei (lanes *3* and *4*). Lanes *1* and *2* contained the amount of material normalized to 10^6 cells. Lanes *3* and *4* contained the amount of material normalized to 10^6 nuclei. Equivalent amounts of protein were loaded into lanes *3* and *4*. The samples were run into a 7.5% polyacrylamide gel, transferred to nitrocellulose, and assayed for their reactivity to rabbit anti-topoisomerase II antiserum. *Right*, molecular weights (kDa) as determined by molecular weight markers run in the gel.

amsacrine, or etoposide but, rather, in protein-concealed DNA strand breakage induced by these drugs (40). Recent studies suggest that a line of P388 leukemia cells is resistant to doxorubicin by a mechanism that is independent of the presence of an energy-driven active extrusion pump (41). The resistant cells are characterized by a marked reduction in anthracycline-induced, protein-concealed DNA strand breakage which cannot be accounted for by reduced intracellular drug accumulation. A similar pattern of cross-resistance to putative topoisomerase II inhibitors has also been identified in an amsacrine-resistant subline of HL-60 human leukemia (42).

The A20 clone of P388 leukemia described here has a number of pharmacologic and biochemical characteristics in common with those of the intercalator or epipodophyllotoxin-resistant lines described above. The observation that amsacrine and teniposide produce far fewer protein-concealed DNA strand breaks in A20 whole cells or isolated nuclei than in Wt20 cells or nuclei suggests that resistance is linked specifically to a decrease in topoisomerase II-mediated damage in DNA. Consistent with this specificity is the finding that camptothecin, an inhibitor of topoisomerase I (12), is equally toxic to both A20 and Wt20 cells and induces the same amount of protein-concealed DNA strand breakage in both. Given that resistance of A20 to amsacrine does not appear to be due to altered transport of the drug, a plausible cause of resistance is a modification of either topoisomerase II itself or of some other factor which participates in its regulation, expression, or function.

A20 cells appear to be deficient in two kinds of lesions that are associated with the action of topoisomerase II on DNA: DNA-protein crosslinks detected in the absence of drug (Fig. 7) and DNA strand breaks induced by a topoisomerase-acting drug (Fig. 4). Although these results do not prove that topoisomerase II generated the strand breaks or DNA-protein crosslinks in cells or nuclei, they demonstrate a difference between A20 and Wt20 cells in the response of intact chromatin to treatment with detergent or topoisomerase II-active drugs. Both treatments are known to produce the intermediate or "cleavable" complex of the topoisomerase II reaction (14). A20 cells contain fewer topoisomerase II molecules and lower topoisomerase enzymatic activity per cell than do Wt20 cells (Figs. 8 and 9), consistent with fewer topoisomerase-mediated DNA lesions. It has been reported that levels of topoisomerase II activity are directly correlated with sensitivity to drugs such as etoposide and doxorubicin (43, 44). In addition, preliminary studies have shown that amsacrine- or teniposide-induced DNA double strand cleavage of purified plasmid DNA mediated by Wt20 nuclear extracts is 5 to 10 times greater than that mediated by A20 extracts.[1] We are currently determining whether this difference is due to changes in the target enzyme or is the result of some other factor which modulates the interaction of the drugs with topoisomerase II differentially in A20 and Wt20 cells. The latter would be similar to the 9-hydroxyellipticine-resistant subline of Chinese hamster cells, whose resistance to topoisomerase II inhibitors is due not to the absence of drug-sensitive topoisomerase II but, rather, to the presence of a 55-kDa modulating factor that may be related to topoisomerase I (39). As shown in Fig. 9 and described in Results, two major proteins in P388 cells react with a polyclonal topoisomerase II antibody and differ in relative amounts in A20 and Wt20 cells, there being more of the 164-kDa protein, relative to the 173-kDa protein, in Wt20 cells than in A20 cells. Recent data from our laboratory have demonstrated that, upon isolation from P388 cells, both proteins possess toposiomerase II activity, and have suggested that the 164-kDa protein is not a proteolytic fragment of the 173-kDa protein. In addition, the catalytic activity (P4 DNA unknotting) of the 164-kDa protein is approximately 5- to 10-fold more sensitive than that of the 173-kDa protein to the inhibitory effects of amsacrine.[2]

A20 cells were more resistant to bisantrene than to amsacrine, to which resistance was generated *in vivo* (Table 1). Bisantrene produces protein-concealed DNA strand breaks in cells (Fig. 4; Ref. 35) but not in isolated nuclei (34)[3] and inhibits the catalytic activity of purified topoisomerase II (7). Cross-resistance of A20 cells to bisantrene is consistent with its anti-topoisomerase II activity and protein-associated strand break production. However, we cannot account for the approximately 10-fold greater resistance of A20 cells to bisantrene than to amsacrine on the basis of protein-associated, single-strand breakage. At the IC_{90} (drug concentrations giving 10% survival), amsacrine produced 5–7 times and bisantrene 10–15 times as many strand breaks in Wt20 as in A20 cells. This result is not unexpected, as cytotoxicity may not be proportional to the numbers of protein-concealed single-strand breaks (35, 45). As bisantrene does not produce protein-free DNA strand breaks in L1210 cells (35), free-radical mechanisms are unlikely. Enhanced resistance to bisantrene may be due to effects other than the interaction of the drug with topoisomerase II-DNA. The fact that no breaks are produced by bisantrene in isolated nuclei could mean that cellular activation of the drug (in which A20 cells could be defective) is required for its topoisomerase-DNA interaction, or that perturbations of chromatin structure by nucleus isolation render the chemical microenvironment unfavorable for drug-DNA-topoisomerase interactions. Our data do not permit us to distinguish among several possible explanations, including increased drug efflux (see below), for enhanced resistance of A20 cells to bisantrene.

[1] S. R. Per, and H. F. Bartus, unpublished observations.
[2] F. H. Drake, J. P. Zimmerman, F. L. McCabe, H. F. Bartus, S. R. Per, D. M. Sullivan, W. E. Ross, M. R. Mattern, R. K. Johnson, S. T. Crooke, and C. K. Mirabelli, submitted for publication.
[3] S. R. Per, unpublished results.

The *in vitro* and *in vivo* cross-resistance pattern of A20 cloned cells was not entirely consistent with that reported for the P388/amsacrine tumor from which the clone was derived. While the cloned cell line, like P388/amsacrine, is resistant to agents known to produce topoisomerase II-mediated DNA damage, it is also cross-resistant to a number of agents to which the original P388/amsacrine tumor was sensitive. These agents include dactinomycin, for which there is evidence of interaction with topoisomerase II *in vitro* (7). There are no data indicating that vinblastine or mitomycin C interacts with topoisomerase II. Although the mechanism of action of vinblastine and related *Vinca* alkaloids has not been clearly elucidated, the agents appear to produce cytotoxic effects by interaction with tubulin and microtubules (34). Although we have not investigated the possibility that alterations in tubulin levels and/or *Vinca*-binding sites on tubulin in A20 cells could contribute to their apparent 20-fold resistance to vinblastine, the data suggest that resistance may be in part related to decreased uptake and/or increased efflux of the drug (Fig. 3). Tsuruo *et al.* (46) have described a *Vinca*-resistant P388 tumor line, the resistance of which was clearly linked to an enhanced rate of drug efflux. Recently, a number of multidrug-resistant cell lines have been isolated which are resistant to *Vinca* alkaloids, doxorubicin, actinomycin D, and other agents (47, 48). Decreased drug accumulation as a result of increased drug efflux has been demonstrated in such multidrug-resistant cell lines (49). These multidrug-resistant cells contain and express an amplified gene, mdr1, which is transcribed into a 4.5-kilobase mRNA (50–53) and translated into a 170-kDa membrane glycoprotein (54, 55). This protein appears to bind to the drugs and transport them out of the cell. Therefore, the resistance to vinblastine as well as some degree of the total level of resistance to doxorubicin, actinomycin D, and bisantrene may be mediated in part by an overexpression of the 170-kDa membrane glycoprotein by the A20 cells. It is unclear whether the additional resistance of the A20 clone to vinblastine relative to the P388/amsacrine tumor characterized originally is the result of subsequent passage of the tumor *in vivo*. We have been unable to reestablish the comparative *in vivo* chemosensitivity profile of the uncloned P388/amsacrine subline, due to changes in its growth kinetics. Alternatively, the potential for changes in the phenotype of cloned cells derived from heterogeneous polyclonal tumors has been demonstrated (56). In order to investigate this as a potential explanation for the vinblastine resistance in A20 cells, we are currently evaluating the *in vivo* chemosensitivity of A20 and Wt20.

The data presented herein, directly linking resistance to amsacrine, other intercalators, and epipodophyllotoxins to a decrease in drug-induced, protein-concealed DNA strand breaks and showing changes in the amounts of topoisomerase II in resistant cells, provide further support for topoisomerase II as a major target of these drugs. The cloned, amsacrine-resistant A20 cells will provide a model to evaluate both *in vitro* and *in vivo* the biochemical mechanisms which underlie topoisomerase II-mediated resistance in tumors.

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References

1. Vosberg, H.-P. DNA topoisomerases: enzymes that control DNA conformation. *Curr. Top. Microbiol. Immunol.* **114**:91–102 (1985).
2. Wang, J. C. DNA topoisomerases. *Annu. Rev. Biochem.* **54**:665–697 (1985).
3. Pommier, Y., M. R. Mattern, R. E. Schwartz, and L. A. Zwelling. Absence of swivelling at the sites of intercalator-induced protein-associated DNA strand breaks in mammalian cell nucleoids. *Biochemistry* **23**:2922–2927 (1984).
4. Ross, W. E., D. L. Glaubiger, and K. W. Kohn. Protein-associated DNA breaks in cells treated with adriamycin and ellipticine. *Biochim. Biophys. Acta* **519**:23–30 (1978).
5. Ross, W. E., D. L. Glaubiger, and K. W. Kohn. Quantitative and qualitative aspects of intercalator-induced DNA strand breaks. *Biochim. Biophys. Acta* **562**:41–50 (1979).
6. Ross, W., T. Rowe, B. Glisson, J. Yalowich, and L. Liu. Role of topoisomerase II in mediating epipodophyllotoxin-induced DNA cleavage. *Cancer Res.* **44**:5857–5860 (1984).
7. Tewey, K. M., T. C. Rowe, L. Yang, B. D. Halligan, and L. F. Liu. Adriamycin-induced DNA damage mediated by mammalian DNA topoisomerase II. *Science (Wash. D. C.)* **226**: 466–468 (1984).
8. Zwelling, L. A., S. Michaels, L. C. Erickson, R. S. Ungerleider, M. Nichols, and K. W. Kohn. Protein-associated deoxyribonucleic acid strand breaks in L1210 cells treated with the deoxyribonucleic acid intercalating agents 4′(acridinylamino)methanesulfon-*m*-anisidide and adriamycin. *Biochemistry* **20**:6553–6563 (1981).
9. Gallo, R. C., J. Whang-Peng, and R. H. Adamson. Studies on the antitumor activity, mechanism of action, and the cell cycle effects of camptothecin. *J. Natl. Cancer Inst.* **46**:789–795 (1971).
10. Snapka, R. M. Topoisomerase inhibitors can selectively interfere with different stages of Simian virus 40 DNA replication. *Mol. Cell. Biol.* **6**:4221–4227 (1986).
11. Hsiang, Y., R. Hertzberg, S. Hecht, and L. F. Liu. Camptothecin induces protein-linked DNA breaks via mammalian DNA topoisomerase I. *J. Biol. Chem.* **260**:14873–14878 (1985).
12. Mattern, M. R., S.-M. Mong, H. F. Bartus, C. K. Mirabelli, S. T. Crooke, and R. K. Johnson. Relationship between the intracellular effects of camptothecin and the inhibition of DNA Topoisomerase I in cultured L1210 cells. *Cancer Res.* **47**:1793–1798 (1987).
13. Chen, G. L., L. Yang, T. C. Rowe, B. D. Halligan, K. M. Tewey, and L. F. Liu. Nonintercalative antitumor drugs interfere with the breakage-reunion reaction of mammalian DNA topoisomerase II. *J. Biol. Chem.* **259**:13560–13566 (1984).
14. Liu, L. F., T. C. Rowe, L. Yang, K. M. Tewey, and G. L. Chen. Cleavage of DNA by mammalian DNA topoisomerase II. *J. Biol. Chem.* **258**:15365–15370 (1983).
15. Cain, B. F. and G. J. Atwell. The experimental antitumor properties of three congeners of the acridylmethanesulphonanilide (AMSA) series. *Eur. J. Cancer* **10**:539–545 (1974).
16. Waring, M. J. DNA binding characteristics of acridylmethanesulphonanilide drugs: comparison with antitumor properties. *Eur. J. Cancer* **12**:995–1001 (1976).
17. Nelson, E. M., K. M. Tewey, and L. F. Liu. Mechanism of antitumor drug action: poisoning of mammalian DNA topoisomerase II by 4′-(9-acridinylamino)methanesulfon-*m*-anisidide. *Proc. Natl. Acad. Sci. USA* **81**:1361–1365 (1984).
18. Burr-Furlong, N., J. Sato, T. Brown, F. Chavez, and R. B. Hurlbert. Induction of limited DNA damage by the antitumor agent Cain's acridine. *Cancer Res.* **38**:1329–1335 (1978).
19. Wong, A., C.-H. Huang, and S. T. Crooke. Mechanism of deoxyribonucleic acid breakage induced by 4′-(9-acridinylamino)methanesulfon-*m*-anisidide and copper: role for cuprous ion and oxygen free radicals. *Biochemistry* **23**:2946–2952 (1984).
20. Wong, A., C.-H. Huang, S.-M. Hwang, A. W. Prestayko, and S. T. Crooke. Formation of thiol adducts of 4′-(9-acridinylamino)methanesulphon-*m*-anisidide and their binding to deoxyribonucleic acid. *Biochem. Pharmacol.* **35**:1655–1662 (1986).
21. Wong, A., and S. T. Crooke. Studies on the fluorescence labeling of human red blood cell membrane ghosts with 4′-(9-acridinylamino)methanesulfon-*m*-anisidide. *Biochem. Pharmacol.* **34**:3265–3273 (1985).
22. Johnson, R. K., and W. S. Howard. Development and cross-resistance characteristics of a subline of P388 leukemia resistant to 4′-(9-acridinylamino)methanesulphon-*m*-anisidide. *Eur. J. Cancer Clin. Oncol.* **18**:479–484 (1982).
23. Kessel, D., C. Wheeler, T.-H. Chou, W. S. Howard, and R. K. Johnson. Studies on a mode of resistance to *m*-AMSA. *Biochem. Pharmacol.* **31**:3008–3010 (1982).
24. Chu, M. Y., and G. A. Fisher. The incorporation of ^{3}H-cytosine arabinoside and its effect on murine leukemic cells (L5178Y). *Biochem. Pharmacol.* **17**:753–767 (1968).
25. Kohn, K. W., R. A. G. Ewig, L. C. Erickson, and L. A. Zwelling. Measurements of strand breaks and cross-links by alkaline elution, in *DNA Repair: A*

Laboratory Manual of Research Techniques (E. C. Friedberg and P. C. Hanawalt, eds.). Marcel Dekker, New York, 379–401, (1981).
26. Filipski, J., and K. W. Kohn. Ellipticine-induced protein-associated DNA breaks in isolated L1210 nuclei. *Biochim. Biophys. Acta* **698**:280–286 (1982).
27. Bartus, H. F., M. R. Mattern, S.-M. Mong, C. K. Mirabelli, J. O. Bartus, G. A. Hofmann, and R. K. Johnson. Evaluation of a modified protein filter binding assay for the rapid detection of potential topoisomerase inhibitors. *Proc. Am. Assoc. Cancer Res.* **27**:71, abstr. 1538 (1986).
28. Minford, J., Y. Pommier, J. Filipski, K. W. Kohn, D. Kerrigan, M. Mattern, S. Michaels, R. Schwartz, and L. A. Zwelling. Isolation of intercalator dependent protein-linked DNA strand cleavage activity from cell nuclei and identification as topoisomerase II. *Biochemistry* **25**:9–16 (1986).
29. Hertzberg, R. P., and P. B. Dervan. Cleavage of DNA with methidiumpropyl-EDTA-iron (II): reaction conditions and product analyses. *Biochemistry* **23**:3934–3945 (1984).
30. Bradford, M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**:248–254 (1976).
31. Liu, L. F., and J. L. Davis. Novel topologically knotted DNA from bacteriophage P4 capsids: studies with DNA topoisomerases. *Nucleic Acids Res.* **9**:3979–3989 (1981).
32. Towbin, H., T. Staehelin, and J. Gordon. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA 76:* 4350–4354 (1979).
33. Halligan, B. D., K. A. Edwards, and L. F. Liu. Purification and characterization of a type II DNA topoisomerase from bovine calf thymus. *J. Biol. Chem.* **260**:2475–2482 (1985).
34. Hamel, E. Antimitotic drugs and tubulin-nucleotide interactions, in *Developmental Cancer Chemotherapy* (R. I. Glazer, ed.). CRC Press, Boca Raton, FL, 131–164 (1984).
35. Bowden, G. T., R. Roberts, D. S. Alberts, Y.-M. Peng, and D. Garcia. Comparative molecular pharmacology in leukemic L1210 cells of the anthracene anticancer drugs mitoxantrone and bisantrene. *Cancer Res.* **45**:4915–4920 (1985).
36. Gupta, R. Genetic, biochemical and cross-resistance studies with mutants of Chinese ovary cells resistant to the anticancer drugs VM-26 and VP16-213. *Cancer Res.* **43**:1568–1574 (1983).
37. Glisson, B., R. Gupta, P. Hodges, and W. Ross. Cross-resistance to intercalating agents in an epipodophyllotoxin-resistant Chinese hamster ovary cell line: evidence for a common intracellular target. *Cancer Res.* **46**:1939–1942 (1986).
38. Glisson, B., R. Gupta, S. Smallwood-Kentro, and W. Ross. Characterization of acquired epipodophyllotoxin resistance in a Chinese hamster ovary cell line: loss of drug-stimulated DNA cleavage activity. *Cancer Res.* **46**:1934–1938 (1986).
39. Pommier, Y., D. Kerrigan, R. E. Schwartz, J. A. Swack, and A. McCurdy. Altered DNA topoisomerase II activity in Chinese hamster cells resistant to topoisomerase II inhibitors. *Cancer Res.* **46**:3075–3081 (1986).
40. 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**:611–616 (1986).
41. Capranico, G., T. Dasdia, and F. Zunino. Comparison of doxorubicin-induced DNA damage in doxorubicin-sensitive and -resistant P388 murine leukemia cells. *Int. J. Cancer* **37**:227–231 (1986).
42. Odaimi, M., B. S. Andersson, K. B. McCredie, and M. Beran. Drug sensitivity and cross-resistance of the 4′-(9-acridinylamino)methanesulfon-*m*-anisidide-resistant subline of HL-60 human leukemia. *Cancer Res.* **46**:3330–3333 (1986).
43. Sullivan, D. M., B. S. Glisson, P. K. Hodges, S. Smallwood-Kentro, and W. E. Ross. Proliferation dependence of topoisomerase II mediated drug action. *Biochemistry* **25**:2248–2256 (1986).
44. Silber, R., L. F. Liu, Y. H. Hsiang, S. Kirschenbaum, B. Bank, and M. Potmesil. DNA topoisomerase II was not detected in lymphocytes from patients with B-cell chronic lymphocytic leukemia. *Proc. Am. Assoc. Cancer Res.* **27**:248 (1986).
45. Pommier, Y., L. A. Zwelling, M. R. Mattern, L. C. Erickson, D. Kerrigan, R. Schwartz, and K. W. Kohn. Effects of dimethyl sulfoxide and thiourea upon intercalator-induced DNA single-strand breaks in mouse leukemia (L1210) cells. *Cancer Res.* **43**:5718–5724 (1983).
46. Tsuruo, T., H. Iida, S. Tsukagoshi, and Y. Sakurai. Overcoming of vincristine resistance in P388 leukemia *in vivo* and *in vitro* through enhanced cytotoxicity of vincristine and vinblastine by verapamil. *Cancer Res.* **41**:1967–1972 (1981).
47. Beck, W. T., T. J. Mueller, and L. R. Tanzer. Altered surface membrane glycoproteins in *Vinca* alkaloid-resistant human leukemia lymphoblasts. *Cancer Res.* **39**:2070–2076 (1974).
48. Biedler, J. C., and R. H. F. Peterson. Chemotherapeutic agents, in *Molecular Actions and Targets for Cancer* (A. C. Sartorelli, J. S. Lazo, and J. R. Bertino, eds.). Academic Press, Inc., New York, 453–482 (1981).
49. Ling, V. Multidrug-resistant mutants, in *Molecular Cell Genetics* (M. M. Gottesman, ed.). John Wiley and Sons, Inc., New York, 773–787 (1985).
50. Gros, P., J. Croop, I. Roninson, A. Varshavsky, and D. E. Housman. Isolation and characterization of DNA sequences amplified in multidrug-resistant hamster cells. *Proc. Natl. Acad. Sci. USA* **83**:337–341 (1986).
51. Gudkov, A. V., J. S. Massino, O. B. Chernova, and B. P. Kopnin. Gene amplification in Djungarian hamster cell lines possessing decreased plasma membrane permeability for colchicine and some other drugs. *Chromosoma (Berl.)* **92**:16–24 (1985).
52. Fojo, A., J. Whang-Peng, M. M. Gottesman, and I. Pastan. Amplification of DNA sequences in human multidrug-resistant KB carcinoma cells. *Proc. Natl. Acad. Sci. USA* **82**:7661–7665 (1985).
53. Shen, D. W., A. Fojo, J. E. Chin, I. B. Roninson, N. Richert, I. Pastan, and M. M. Gottesman. Increased expression of the mdr1 gene in various human multidrug-resistant cell lines can precede the gene amplification event. *Science (Wash. D. C.)* **232**:643–645 (1986).
54. Riordan, J. R., K. Denchars, N. Kartner, M. Alon, J. Trent, and V. Ling. Amplification of P-glycoprotein genes in multidrug resistant mammalian cell lines. *Nature (Lond.)* **316**:817–819 (1985).
55. Shen, D. W., C. Cardarelli, J. Hwang, M. Cornwell, N. Richert, S. Ishii, I. Pastan, and M. M. Gottesman. Multidrug-resistant human KB carcinoma cells independently selected for high-level resistance to colchicine, adriamycin or vinblastine show changes in expression of specific proteins. *J. Biol. Chem.* **261**:7762–7770 (1986).
56. Poste, G. H., and R. Greig. On the genesis and regulation of cellular heterogeneity in malignant tumors. *Invasion Metastasis* **2**:137–176 (1982).

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