Induction of Alkylator (Melphalan) Resistance in HL60 Cells Is Accompanied by Increased Levels of Topoisomerase II Expression and Function

Q. Q. PU and W. R. BEZWODA

Division of Clinical Hematology and Medical Oncology, Department of Medicine, University of the Witwatersrand Medical School, Parktown, South Africa

This paper is available online at http://www.molpharm.org

ABSTRACT

Human leukemic HL60 cells were selected for resistance to alkylating agents by stepwise exposure to increasing concentrations of L-phenylalanine mustard (melphalan). The resulting resistant cell line (*R*-HL60) was 4-fold resistant (melphalan IC_{50} value, 27.84 ± 4.2 μM) to melphalan compared with parental HL60 cells (melphalan IC_{50} value, 6.9 ± 1.78 μM). Nuclear extracts from *R*-HL60 cells possess a $\sim$4-fold increase in DNA topoisomerase II activity compared with parental HL60 cells. As determined using Western blot analysis, the level of topoisomerase IIα protein expressed in *R*-HL60 cells was approximately 3-fold that of parental HL60 cells. However, there were no differences observed in the level of topoisomerase IIβ protein, in the topoisomerase I activity, or in the level of topoisomerase I protein expression comparing the two cell lines. *R*-HL60 cells were 5-fold more sensitive than parental HL60 cells to the cytotoxic effect of the topoisomerase II inhibitor doxorubicin. The sensitivity to the cytotoxic effects of the topoisomerase I inhibitor camptothecin did not differ in *R*-HL60 and parental HL60 cell lines. Preincubation with doxorubicin significantly increased melphalan-induced interstrand DNA cross-link formation and cytotoxicity in *R*-HL60 cells compared with the parental HL60 cells. The affinity of topoisomerase II for UV-irradiated cross-linked HL60 DNA was increased by $\sim$2.5-fold compared with that of HL60 native DNA. The affinity of topoisomerase II for both UV-irradiated (cross-linked) and native DNA was significantly decreased after doxorubicin pretreatment. Elevated topoisomerase II activity and the increased affinity of topoisomerase II for cross-linked DNA in melphalan-resistant cells seems to contribute to alkylator resistance by changing DNA topology, thereby facilitating DNA repair.

L-Phenylalanine mustard (melphalan) is a rationally designed alkylating agent active against ovarian cancer, myeloma, breast cancer, and rhabdomyosarcoma. Because melphalan is the active agent, requiring no further metabolic activation, the drug has become a model for studying the mechanisms of alkylator resistance. Melphalan resistance can be mediated by a number of mechanisms, including altered drug transport, increased drug detoxification, or increased removal/repair of DNA interstrand cross-links (Redwood and Colvin, 1980; Batist et al., 1989; Bailey et al., 1992), acting either singly or in combination. We have demonstrated previously that in lymphoid cells, a major mechanism for melphalan resistance is an increased rate of removal of DNA-interstrand cross-links (Bezwoda and Pu, 1997). Such increased rates of interstrand-DNA cross-link removal may be caused either by increased activity of DNA repair enzymes or by alteration of DNA topology.

In eukaryotic cells, two major topoisomerases (I and II) catalyze changes in the topological conformation of DNA by the concerted breakage of single or double strands. Topoisomerase II action is important in DNA replication, transcription, recombination, and mitosis (Wang, 1987). Topoisomerase II is localized to AT-rich DNA regions, where it forms a significant part of the mitotic chromosomal scaffold (Earnshaw et al., 1985; Nelson et al., 1986). Two isoenzyme forms of topoisomerase II, α and β, are expressed in mammalian cells. The two forms differ with respect to molecular size (170 kDa versus 180 kDa, respectively), cleavage site, thermal stability, and catalytic capacity (Drake et al., 1987, 1989). The enzyme described first, topoisomerase IIα, is expressed preferentially in proliferating cells (Heck and Earnshaw, 1986; Hsiang et al., 1988) and is cell-cycle regulated (Heck et al., 1987). The topoisomerase IIβ enzyme, on the other hand, seems to be expressed at equivalent levels in proliferating and quiescent cells (Woessner et al., 1990, 1991).

In this study, we investigated topoisomerase I and II expression and activity in melphalan-resistant HL 60 cells (*R*-HL60) and in parental HL60 cells. The results show that elevated levels of topoisomerase II are associated with resistance to melphalan and that such *R*-HL60 cells are more

---

ABBREVIATIONS: melphalan, L-phenylalanine mustard; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; TE, Tris/EDTA; *R*-HL60, melphalan-resistant HL60 cell line.

sensitive than parental HL60 cells to the topoisomerase II inhibitor doxorubicin.

Experimental Procedures

Materials. Melphalan was obtained as >99.5% pure 4-[bis(2 chloroethyl)amino]-L-phenylalanine from Wellcome (Dartford, UK). Melphalan solutions were prepared daily in 70% ethanol containing an equimolar concentration of hydrochloric acid. To minimize the hydrolysis, further dilutions were made in aqueous medium immediately before use. RPMI 1640 was purchased from Highveld Biologicals (Johannesburg, South Africa). Doxorubicin and camptothecin were obtained from Topogen (Columbus, OH).

Cell Lines and Tissue Culture. HL60 cells and resistant HL-60 (*R*-HL60) cells were grown in RPMI 1640 that contained 10% fetal calf serum, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Incubations were performed at 37°C in a humidified atmosphere with 5% CO_2. *R*-HL60 cells were selected by intermittent exposure of surviving cells to increasing concentrations of melphalan. HL60 cells (2×10^5 per 50-ml flask) were treated for 1 h with the IC_{50} concentration of the drug. The cultures were observed daily and allowed to grow until they reached the initial cell density or greater. Melphalan concentrations for subsequent exposure were increased primarily on the basis of recovery time. For short recovery times (1–2 weeks), a 1.0- to 1.5-fold increase in concentration was used. For recovery times of 2 to 4 weeks, the increase was smaller (10–50%). After 6 months of intermittent exposure, a stable subline (*R*-HL60) demonstrating a 4-fold increase in the IC_{50} melphalan concentration was grown out.

Cell Number. Cell numbers were determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, based on active mitochondrial reduction of MTT. The assay used incorporated the modifications of Twentyman and Luscombe (1987). Assays were performed using 5×10^4 cells in 96-well microtiter plates in 200 μl of culture medium. Cells were exposed to melphalan at concentrations ranging from 0 to 40 μM for 96 h and to doxorubicin or camptothecin at concentrations ranging from 0 to 5.0 μM for 96 h. Cells were then washed with fresh medium, followed by addition of 20 μl of MTT (5 mg/ml) in 200 μl of medium and incubated for 4 h. Medium was then aspirated and formazan solubilized in dimethyl sulfoxide. Absorbance was read at 540 nm within 30 min in an enzyme-linked immunosorbent assay plate reader. Each experiment was performed in triplicate at each of the drug concentrations. After treatment with each of the drugs, the surviving fraction of cells compared with control (untreated) cells was calculated for each drug concentration. Cell number was plotted versus drug concentration, and IC_{50} values were calculated from dose- response curves as the concentration of drugs that reduced the number of viable cells to 50% of control.

Determination of Interstrand-DNA Cross-Links. HL60 cells were suspended in RPMI 1640 at 2×10^6 cells/ml. Cells were exposed to various concentrations of melphalan at 37°C. Interstrand-DNA cross-link formation was determined by an ethidium bromide fluorescence assay based on the method of De Jong et al. (1991a). After hypotonic lysis (10 mM/l Tris, pH 7.4, 1 mM EDTA, and 0.2% Triton X100) the contents of each well were precipitated in cryotubes for 24 h at −20°C in 50% isopropanol and 0.5 M NaCl. The precipitates were pelleted by centrifugation, air dried, resuspended in Tris/EDTA (TE) buffer and treated with 1 U/tube of DNase-free RNase at 37°C for 1 h followed by proteinase K (0.5 mg/ml) treatment (25 μl/tube) for another hour. Aliquots of the resulting lysates were denatured by heating at 100°C for 5 min and then rapidly cooled to 23°C. Samples were then added to 3 ml of ethidium bromide (10 μg/ml) in 20 mM K_2HPO_4, and 0.4 mM EDTA buffer, pH 12.0, in tubes wrapped in aluminum foil to prevent light-induced cleavage of DNA by ethidium bromide. Fluorescence was measured both before and after denaturation in 1-cm^2 cuvettes at an excitation wavelength of 525 nm and an emission wavelength of 580 nm in an LS50 variable wavelength

spectrofluorometer (Perkin Elmer, Norwalk, CT). The percentage of interstrand cross-linked DNA was determined by measuring the difference in fluorescence of denatured control cell lysates and denatured control cell samples according to the formula: $C_t\% = (ft - fc/1 - fc) \times 100$, where $C_t\%$ = the percentage of interstrand cross-linked DNA on treated cells, ft = fluorescence intensity after heat denaturation divided by fluorescence intensity before heat denaturation in treated cells, and fc = the same fluorescence measurement in control cells.

Preparation of Nuclear Extracts. A procedure for isolation of nuclear and cytosolic fractions was developed based on the methods of Chow et al. (1985) and Matsumoto et al. (1993). In brief, cells in the exponential phase of growth were treated with detergent buffer (1% Nonidet P-40, 30 mM HEPES, 200 mM sucrose, 40 mM NaCl, 5 mM $MgCl_2$, and 5 mM EGTA, pH 8.0), with constant swirling for 10 to 15 min. The cell suspension was centrifuged at 400g for 10 min and the supernatant was saved as the cytosolic fraction. The sedimented nuclei were then washed three times by resuspending the pellet in buffer A (50 mM HEPES, 10% sucrose, and 10 mM β-mercaptoethanol, pH 7.5) and repelleting. To the final pellet was added an equal volume of buffer B [50 mM HEPES, 10% sucrose, 10 mM β-mercaptoethanol, and 0.7 M NaCl (final NaCl concentration, 0.35 M)]. After extraction for 60 min at 4°C, the mixture was centrifuged at 100,000g for 60 min, and the supernatant was saved as the nuclear extract. The cytosolic and nuclear extracts were dialysed against buffer C (50% glycerol, 50 mM Tris · HCl, 0.5 mM dithiothreitol, 1 mM EDTA, 1 mM EGTA, and 260 mM NaCl, pH 7.5) for 6 h. A combination of six protease inhibitors, including soybean trypsin inhibitor (10 μg/ml), leupeptin (50 μg/ml), pepstatin (1 μg/ml), aprotinin (20 μg/ml), benzamidine (1 mM), and phenylmethylsulfonyl fluoride (1 mM), were prepared just before each experiment and added to each of the above-mentioned buffers just before each experiment. The protein concentrations in the extracts were determined by the method of Bradford (1976).

Topoisomerase I and II Assays. Measurement of the topoisomerase II catalytic activity in cytosol and nuclear extracts was performed using the topoisomerase II decatenating method (Topogen), which is a modification of the method of Marini et al. (1980). Photographic negatives of the ethidium bromide-stained agarose gels were scanned with a REP Scanning Densitometer (Helena Laboratories, Beaumont, TX) and the quantity of liberated minicircles was measured as a percentage of total kinetoplast DNA (kDNA). Results are expressed in arbitrary units and the ratio of activity in *R*-HL60 cells to that in parental HL60 cells was used for comparison.

Topoisomerase I activity was determined using the topoisomerase I assay kit from Topogen, essentially as described by Liu and Miller (1981). The ratio of open circular species (form II) to total DNA loaded, expressed as percentage of total DNA, was quantified using the same densitometer.

Western Blot Analysis. Proteins from the nuclear extract and cytosol fractions of HL60 and *R*-HL60 cell lines were electrophoresed (100 μg of protein/lane) in 7.5% SDS-polyacrylamide gels and transferred to nitrocellulose paper by the method of Harker et al. (1991). The nitrocellulose strips were preincubated in blocking buffer (3% BSA-5% nonfat dry milk in PBS) overnight at 4°C. Transferred, immobilized topoisomerase proteins were detected using purified mouse antihuman DNA topoisomerase IIα p170 (Topogen) and anti-topoisomerase IIβ p180 (PharMingen, San Diego, CA) monoclonal antibody, or rabbit polyclonal, monospecific, antihuman topoisomerase I antibody (Topogen). Blots were incubated with primary antibody for 4 h at 37°C, then washed with a wash buffer (1% BSA in PBS containing 0.2% Tween 20). The bound antibodies were visualized with alkaline phosphatase-linked sheep antimouse or antirabbit IgG, using 5-bromo-4 chloro-3-indolyl phosphate and nitroblue tetrazolium chloride substrates. Each experiment included controls for nonspecific binding using nonimmune rabbit serum in place of specific antibody and a negative control without primary antibody. The relative amounts of topoisomerase IIα, IIβ, and I proteins seen on

Western blots were quantified densitometrically. Specified regions of the film images of the Western blot were digitized by the scanner and the area and image intensities were calculated. The image intensity was calibrated against internal computer standards and values are expressed as arbitrary units relative to the standards. A linear relationship was present between the value of the integration units and the amount of protein extract loaded on the gel in the range (30–250 μg of protein) (data not shown). Protein extracts (100 μg) were used for all comparative experiments.

Preparation of HL60 Native DNA. A procedure for isolation of DNA from HL60 cells was developed based on the method of Gross-Bellard et al. (1972). Briefly, HL60 cells were suspended in 1 volume of digestion buffer (100 mM NaCl, 10 mM Tris-Cl, 25 mM EDTA, 0.5% SDS, and 0.1 mg/ml proteinase K, pH 8.0). Samples were incubated in a shaking incubator in tightly capped tubes for 12 to 18 h at 50°C. An equal volume of phenol/chloroform/isoamyl alcohol was used for extraction, followed by centrifugation for 10 min at 1700*g* (3000 rpm in Sorvall H1000B rotor; Sorvall Instruments, Newton, CT). This organic extraction was repeated twice. The aqueous layer was transferred to a new tube and .5 volumes of 7.5 M ammonium acetate and 2 volumes of 100% ethanol were added. The solution was centrifuged for 2 min at 1700*g*. Organic solvents and salt were removed by two dialyses against 100 volumes of TE buffer for 24 h. Residual RNA was removed by adding 0.1% SDS and 1 μg/ml DNase-free RNase and incubating for 1 h at 37°C. The organic extraction and purification was repeated and the resultant purifed DNA was resuspended in TE buffer at 1 mg/ml.

Preparation of Cross-Linked DNA. HL60 DNA was suspended at 100 μg/ml in TE buffer. Samples in a thin layer were irradiated at room temperature with UV light (360-nm peak) 12 cm below four FL-15BLB fluorescent lamps. The incident fluence rate was 30 W/m^2, measured with a Model J221 Blak-Ray ultraviolet meter (Ultraviolet Products, San Gabriel, CA). The solution was extracted with phenol/chloroform/isoamyl alcohol. After ethanol precipitation, the DNA was resuspended in TE buffer at 1 mg/ml. The formation of cross-links was determined by ethidium bromide fluorescence assay mentioned above. Cross-linked DNA accounted for 80% of total DNA after UV treatment.

Iodination of Topoisomerase II. Topoisomerase II was iodinated by the Iodogen method (Salacinski et al., 1981), purified by contricon 100 (Amicon, Beverly, MA) and formulated in topoisomerase II storage buffer (15 mM sodium phosphate, pH 7.1, 700 mM NaCl, 0.1 mM EDTA, 0.5 mM dithiothreitol, and 50% glycerol) at the concentration of 1 U/μl (1 U will decatenate 0.2 μg of kDNA in 30 min at 37°C). The specific activity of the radiolabeled material is 600 cpm/mU topoisomerase II.

Affinity of Topoisomerase IIα to DNA. HL60 cell DNA (5 μg) or HL60 cell UV-irradiated cross-linked DNA was incubated with various concentrations of ^{125}I-labeled topoisomerase IIα in Topo II assay buffer (50 mM Tris-Cl, 120 mM KCl, 10 mM MgCl$_2$, 0.5 mM dithiothreitol, and 30 μg BSA/ml, pH 8) at 37°C for 1 h. For some experiments, the ^{125}I-labeled topoisomerase II was in the assay buffer with 5 μM doxorubicin preincubation for 30 min. Reaction mixture were collected on DEAE-cellulose filters, washed with 5% trichloroacetic acid, and counted by a Packard (Meriden, CT) 5330 gamma-ray counter. There were duplicate determinations for each point. Nonspecific binding was defined as bound ^{125}I-labeled topoisomerase II that remained in the presence of a 100-fold excess of the unlabeled topoisomerase II, except for the competitive binding. The specific binding was determined by subtracting the nonspecific binding from total binding. Scatchard analysis was employed to determine the maximal binding (B_{max})

Neutral Comet Assay of Double-Strand DNA Breaks. The single-cell comet electrophoresis assay was performed according to the method of Olive et al. (1991). Cells were suspended in PBS at a density of 5×10^5/ml and treated with doxorubicin at a dose of 10 μM at 37°C for 3 h. After the treatment, 1.5 ml of 1% low-gelling-temperature agarose gel (Sigma, St. Louis, MO) at 40°C were added

to a tube containing 0.5 ml of cold cell suspension. The contents were quickly pipetted onto a frosted microscope slide and allowed to gel for about 1 min on a cold surface. Then slides were immersed for 5 h at 50°C in 0.5% SDS and 30 mM EDTA, pH 8.3. After lysis, slides were thoroughly rinsed overnight in large volumes of 90 mM Tris/90 mM boric acid/2 mM EDTA buffer. Slides were placed in horizontal gel electrophoresis chambers containing 1 liter of 90 mM Tris/90 mM boric acid/2 mM EDTA buffer at 0.6 V/cm for 30 min. DNA was stained by immersing slides in 2.5 μg/ml propidium iodide for 30 min.

An Olympus (Tokyo, Japan) epifluorescence microscope attached to a camera and image analysis system was used to quantify the different parameters of the comets. Generally, 100 comets were analyzed per slide. Results were expressed in terms of tail moment, which is defined as the product of the percentage of DNA in the tail multiplied by the tail length.

Statistical Methods. Differences between two cell lines were tested for statistical significance by Student's *t* test.

Results

Cytotoxicity and Interstrand DNA Cross-Link Formation. The effects of melphalan on growth and survival of HL60 cells and *R*-HL60 cells is shown in Fig. 1A. *R*-HL60 cells showed a 4-fold increase in the IC$_{50}$ concentration (27.8 $\pm$ 4.2 μM) of melphalan compared with parental HL60 cells (6.9 $\pm$ 1.8 μM; $p < .01$). The effects of doxorubicin (Fig. 1B), a topoisomerase II inhibitor, and camptothecin (Fig. 1C), a topoisomerase I inhibitor, were also studied. Statistical analysis showed that HL60 cells were significantly more sensitive to melphalan than *R*-HL60 cells ($p < .05$); however, parenteral HL60 cells were significantly more resistant to doxorubicin (doxorubincin IC$_{50}$, 1.3 $\pm$ 0.8 μM) than *R*-HL60 (doxorubincin IC$_{50}$, 0.25 $\pm$ 0.15 μM; $p < .05$). There was no difference in IC$_{50}$ dose for camptothecin when HL60 cells and *R*-HL60 cells were compared (camptothecin IC$_{50}$, 1.02 $\pm$ 0.25 μM versus 0.92 $\pm$ 0.30 μM, respectively). On the other hand, there was a significant ($p < .01$) difference in IC$_{50}$ dose for melphalan between *R*-HL60 (27.84 $\pm$ 4.2 μM) and *R*-HL60 cell with 0.05 μM doxorubicin pretreatment (5.3 $\pm$ 2.21 μM); there was no difference between HL60 (6.9 $\pm$ 1.78 μM) and
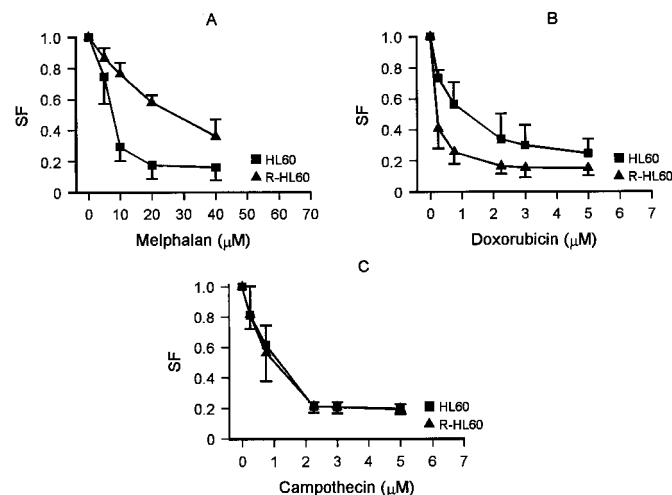

Fig. 1. Viable cell fraction (as a proportion of control) after treatment with melphalan (A), doxorubicin (B), and camptothecin (C) against HL60 (■) and *R*-HL60 cells (▲). Values are the mean $\pm$ S.D. of five independent experiments.

HL60 with 0.05 μM doxorubicin pretreatment (6.1 $\pm$ 1.13 μM).

Interstrand-DNA cross-link (C_t%) formation after a brief (60-min) exposure to various concentrations of melphalan (measured after washing cells and a further drug-free incubation for 4 h) demonstrated a clear dose-response relationship between increasing concentrations of melphalan and C_t% in both cell lines studied. The percentage of interstrand-DNA cross-link formation present at the end of 4 h of drug-free incubation at any given concentration of melphalan was substantially lower in R-HL60 cells compared with that found in parental HL60 cells (Fig. 2).

Kinetics of Interstrand-DNA Cross-Linking. To better define the kinetics of the formation and removal of inter-strand-DNA cross-links, C_t values were determined at 0, 4, and 24 h after a 60-min incubation with 10 μM melphalan. After melphalan exposure for 60 min, the cells were washed and reincubated in drug-free medium for the stated times. Viability and metabolic activity of cells was monitored through this incubation and remained >90% at all test times.

The pattern of interstrand-DNA cross-link formation showed that there was an initial rise, with a peak at 5 h followed by a decline resulting from DNA repair/cross-link removal. Comparison of interstrand-DNA cross-link formation after melphalan exposure of HL60 and R-HL60 cells showed statistically significantly differences at all stated times for all comparisons, except for that between HL60 cells and HL60 cells pretreated with 0.05 μM doxorubicin (Fig. 3).

Exponential functions for the rate of removal of interstrand-DNA cross-links were calculated from the data for each concentration according to the equation: $Y = ae^{-ct}$, where Y = the percentage of interstrand-DNA cross-links remaining at t hour after the time of maximum cross-linking, a = the maximum amount of interstrand-cross-linking as estimated by linear regression analysis, and c = the rate constant for the removal of interstrand-DNA-cross-links. From these exponential functions, the $T_{1/2}$ for removal of interstrand-DNA cross-links was calculated. The $T_{1/2}$ values showed significant differences for each of the groups except between HL60 cell and HL60 cell with 0.05 μM doxorubicin 6 h-pretreatment (Table 1).

Topoisomerase II Activity and Expression in HL60 cells and R-HL60 Cells. The strand-passing activity of to-

poisomerase II was measured by decatenation of kDNA. This reaction, resulting in release of double-stranded minicircles from the catenated kDNA network, is ATP-dependent and specific for topoisomerase II (Fig. 4A). Figure 4A shows the results obtained using 0.35 M NaCl nuclear extracts at various dilutions. Enhanced decatenation activity was observed in R-HL60 cells. In a set of five independent experiments, a mean increase of 4.28-fold in catalytic activity was found in nuclear extracts of R-HL60 (topoisomerase II catalytic activity, 100.7 $\pm$ 39 U) compared with parental HL60 cells (topoisomerase II catalytic activity, 23.5 $\pm$ 10.8 U; $p <$ .01).

The levels of topoisomerase IIα and IIβ proteins in six independently prepared whole-cell extracts were determined by Western blot analysis using monoclonal antitopoisomerase IIα and IIβ antibodies. The identity of the topoisomerase as topoisomerase IIα was confirmed by the molecular size determination, the identical electrophoretic mobility of the purified 170-kDa topoisomerase IIα (Topogen) as a marker, and by use of the monoclonal antitopoisomerase IIβ antibodies. Densitometric analysis of the immunoblots (Fig. 4B) showed that the level of topoisomerase IIα protein was increased approximately 3-fold (3.25 $\pm$ 0.99) in whole-cell extracts of R-HL60 cells compared with parental HL60 cells, but no difference in the level of topoisomerase IIβ protein was observed between the two cell lines.

Affinity of Topoisomerase II for DNA. Figure 5A shows the ability of the unlabeled topoisomerase to compete with ^{125}I-labeled topoisomerase II for binding to DNA. The unlabeled topoisomerase II competed efficiently with ^{125}I-labeled topoisomerase II in a dose-dependent manner. The time-course analysis indicated that the maximal specific binding of ^{125}I-labeled topoisomerase II to DNA was reached after 60 min of incubation at 37°C (Fig. 5B). Scatchard analysis re-
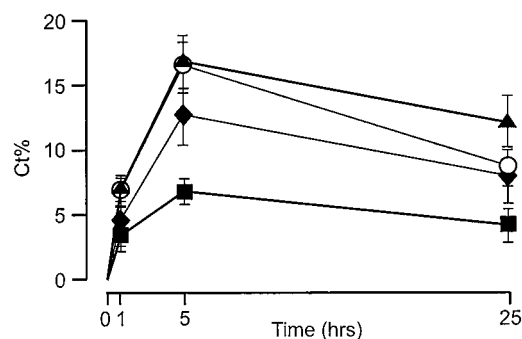
Fig. 3. Interstrand-DNA cross-links (Ct%) after 60-min exposure to 10 μM melphalan (MLN) and a 24-h drug-free incubation in: A, HL60 cells (○); B, HL60 cells with doxorubicin pretreatment (6 h) (▲); C, R-HL60 (■); D, R-HL60 with doxorubicin pretreatment (6 h) (♦). Time 0 represents the start of incubation with melphalan, which was continued for 1 h, followed by washing and then drug-free incubation for another 24 h.
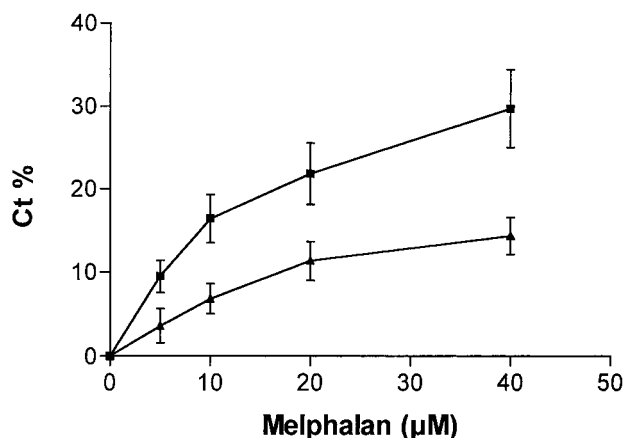
Fig. 2. Percentage of interstrand-DNA cross-link formation (Ct%) after 60-min melphalan exposure followed by 4-h drug-free incubation in HL60 cells (■) and R-HL60 cells (▲). C_t was significantly different ($p <$ .05) when comparing R-HL60 and parental HL60 cells.

TABLE 1

Regression analysis of kinetics of interstrand-DNA cross-link formation and removal

$T_{1/2}$, half-life (hours) of interstrand-DNA cross-links. Significant differences ($p <$ .01) for each comparison except between treated and untreated HL60 cells.

	Regression	$T_{1/2}$
HL60 cells	$Y = 17.32e^{-0.008t}$	86.64
HL60 cells, pretreated[a]	$Y = 17.15e^{-0.007t}$	99.02
R-HL60 cells	$Y = 9.34e^{-0.032t}$	21.66
R-HL60 cells, pretreated[a]	$Y = 13.74e^{-0.016t}$	43.32

[a] 0.05 μM doxorubicin for 6 h.

vealed that the affinity of topoisomerase II for HL60 UV-irradiated, cross-linked HL60 DNA was increased ~2.47-fold compared with that of native HL60 DNA. Doxorubicin decreased topoisomerase II binding to both native DNA and
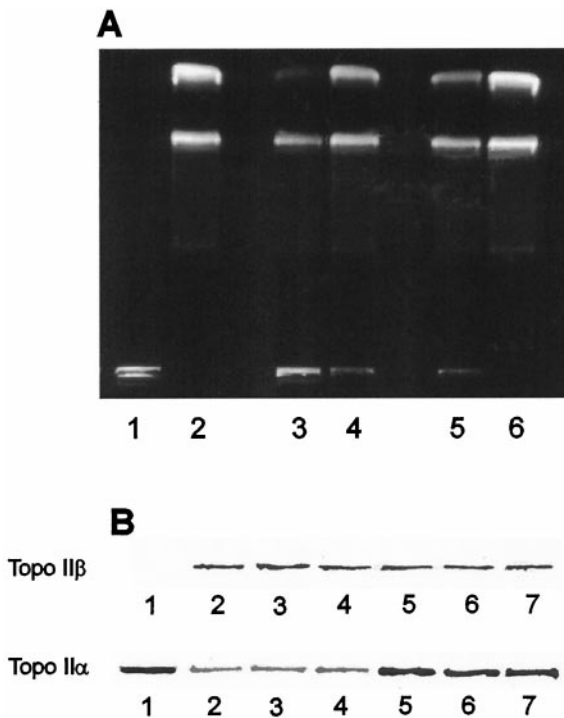

Fig. 4. A, topoisomerase II activity in nuclear extracts from HL60 cells and *R*-HL60 cells. Lane 1, kDNA control; lane 2, decatenated kDNA control; lanes 3 and 5, HL60 nuclear extracts (containing 50 ng and 100 ng of protein/lane); lanes 4 and 6, *R*-HL60 nuclear extracts (containing 50 ng and 100 ng of protein/lane). B, topoisomerase II protein levels, by Western blot analysis of whole-cell lysates from HL60 cells and *R*-HL60 cells. Lane 1, the purified p170 kDa topoisomerase II maker (Topogen); lanes 2 to 4, HL 60 cell extracts; lanes 5 to 7, *R*-HL60 cell extracts. Each sample contained 100 μg of total cellular protein per lane. The blots were stained with a combination of monoclonal antitopoisomerase IIα p170 antibody and monoclonal antitopoisomerase IIβ p180 antibody (Phar-Mingen).
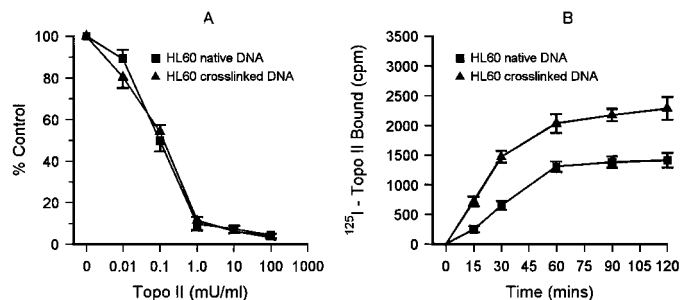

Fig. 5. A, competitive binding of unlabeled topoisomerase II to HL60 native DNA and HL60 UV-irradiated, cross-linked DNA. DNA was incubated with 10 mU/ml ^{125}I-labeled topoisomerase II for 1 h at 37°C in the topoisomerase II assay buffer. Specific binding was determined by subtracting the nonspecific binding (in the presence of 1 U/ml unlabeled topoisomerase II) from total binding. Values shown represent the percentage of specific binding of ^{125}I-labeled topoisomerase II alone. B, time course of binding of ^{125}I-labeled topoisomerase II to HL60 native DNA and HL60 cross-linked DNA. DNA was incubated with 10 mU/ml ^{125}I-labeled topoisomerase II with or without a 100-fold excess of unlabeled topoisomerase II for the indicated times at 37°C. Points represent means ± S.D. These determinations were repeated three time with similar results.

cross-linked DNA by 3.39-fold and 2.94-fold, respectively (Table 2 and Fig. 6, A and B).

Effect of the Topoisomerase II Inhibitor, Doxorubicin, on Interstrand-DNA Cross-Link Formation in HL60 and *R*-HL60 Cells. A 6-h exposure to a minimally cytotoxic concentration (<9% growth inhibition of HL60 and 11% growth inhibition of *R*-HL60 cells after 96 h of exposure) of doxorubicin (0.05 μM) resulted in interstrand-DNA cross-link formation of 0.6% and 1.6% in HL60 and *R*-HL60 cells, respectively. Interstrand-DNA cross-link formation was significantly enhanced in *R*-HL60 cells pre-exposed to 0.05 μM doxorubicin for 6 h followed by a 1-h incubation with melphalan (10 μM) and then assayed at both 4 ($p < .05$) and 24 h ($p < .01$) compared with parenteral HL60 cells (Fig. 7). Pre-exposure to 0.05 μM doxorubicin decreased the IC$_{50}$ value for melphalan concentration for *R*-HL60 cells from 27.84 ± 4.2 to 5.3 ± 2.21 μM.

Effect of Doxorubicin on Double-Strand DNA Breakage in HL60 and *R*-HL60. DNA is able to migrate toward the anode in an electric field when cells are embedded in agarose and lysed. Individual cell DNA was visualized by fluorescence microscope. Each cell has the appearance of a "comet," with brightly fluorescent head and tail, and an intensity that is related to the amount of double-stranded DNA breakage sustained by the cell. The neutral comet assay measures only double-strand breaks (Olive et al., 1991). HL60 cells and *R*-HL60 cells were examined for DNA double-strand breaks induced by doxorubicin. The average tail moment of *R*-HL60 cells increased ~1.7-fold compared with that of HL60 cells (Fig. 8).

Topoisomerase I Activity and Expression in HL60 and *R*-HL60 Cells. The possibility that the increase in topoisomerase II activity might be balanced by decreased topoisomerase I activity was also investigated. Quantification of relaxed DNA forms in six independently prepared extracts of *R*-HL60 and HL60 showed a ratio of 1.27 ± 0.32 ($p > .05$), which indicates similar topoisomerase I activity in both cell lines. The level of topoisomerase I protein was determined in five independent extracts by Western blot analysis. Densitometric analysis showed no significant difference in the level of topoisomerase I protein expressed in the two cell lines. The ratio of topoisomerase I proteins in *R*-HL60 extracts to HL60 cells was calculated to be 1.13 ± 0.16 ($p > .05$).

Discussion

Antineoplastic drugs that alkylate DNA are used extensively in cancer chemotherapy, either alone or in combination

TABLE 2
Affinity of topoisomerase II for native and for UV-irradiated (cross-linked) DNA

	B_{max}	
	mU/ml	
No pretreatment		
HL60 native DNA	51.52 ± 4.68	
		$p < .05$
HL60 UV-irradiated (cross-linked) DNA	127.29 ± 9.84	
Pretreatment with 5 μM doxorubicin		
HL60 native DNA	15.19 ± 2.34	
		$p < .05$
HL60 UV-irradiated (cross-linked) DNA	43.21 ± 4.17	

B_{max}, topoisomerase II concentration at which DNA is maximally occupied by topoisomerase II.

with other classes of chemotherapeutic drugs. One of the principal factors limiting the effectiveness of alkylating agents is cellular resistance to the alkylating effect. There is considerable evidence from a number of in vitro models, including both human and animal tumor cell lines (Frei et al., 1985; Louie et al., 1985; Robson et al., 1985) that the cytotoxicity of these drugs results directly from interstrand DNA cross-link formation (Ross et al., 1978; Hansson et al., 1987; Erickson et al., 1997; Ewig and Kohn, 1997). The alkylator-resistant phenotypes are multifactorial and include decreased uptake, increased glutathione content, and increased DNA repair activity (Bungo et al., 1990; Richon et al., 1990). In addition to these previously defined mechanisms, the results of the current study demonstrate that alkylator resistance is critically determined by the capacity to repair interstrand-DNA cross-links, which in turn is determined by increased topoisomerase IIα activity, resulting from an increase in topoisomerase IIα protein expression.

Melphalan-resistant HL60 cells showed increased topoisomerase IIα activity and protein expression proportional to the degree of melphalan resistance. These findings, taken in conjunction with the studies of the kinetics of DNA cross-link formation in the sensitive and resistant cell lines, suggest that enhanced DNA repair attributable to increased topoisomerase IIα activity with increased binding of topoisomerase IIα to cross-linked DNA in R-HL60 is responsible for the resistance to alkylating agents. These melphalan-resistant (R-HL60) cells were more sensitive to the cytotoxic effects of the topoisomerase II inhibitor doxorubicin, exposure to which resulted in increased double-strand DNA breakage in R-HL60 cells. Inhibition of topoisomerasse II activity by minimally cytotoxic concentrations of doxorubicin was able to significantly increase the amount of interstrand-DNA cross-link formation after melphalan exposure in R-HL60 cells, thus reversing the alkylator-resistant phenotype.

Increased topoisomerase II activity and binding thus seem to be the rate-limiting step for DNA repair. Although we could not exclude, in the present study, that additional DNA repair mechanisms were also increased, the restoration of melphalan sensitivity by topoisomerase II inhibition suggests that this is the most important mechanism. In addition, specificity of the increase of topoisomerase II level and activity was further suggested by the observation that there was no increase in topoisomerase I expression or alteration in sensitivity to the topoisomerase I inhibitor camptothecin in R-HL60 cells compared with parental HL60. These results are consistent with earlier studies demonstrating that increased topoisomerase II expression was associated with resistance to mechlorethamine and cisplatin and that this resistance was associated with increased topoisomerase II binding to cisplatin-damaged DNA (Eder et al., 1995). A possible correlation between topoisomerase II activity and cellular resistance to alkylating agents in the mechlorethamine resistant Raji-HN$_2$ cell line and in a cisplatin-resistant human lung cancer cell line (Tan et al., 1987; De Jong et al., 1991a) has also been described.

Topoisomerase II has been identified as the putative cellular target of many clinically active antineoplastic agents, including the aminoacridines, anthracyclines, and epipodo-
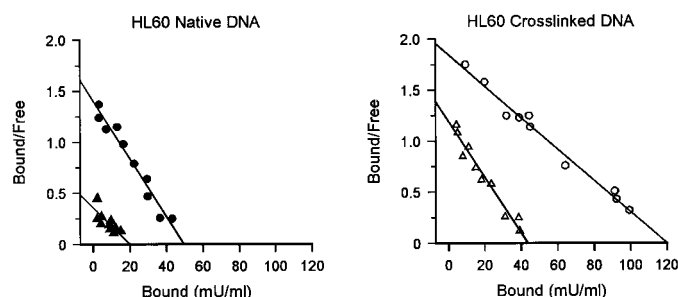

Fig. 6. Scatchard analysis of the binding of ^{125}I-labeled topoisomerase II to HL60 native DNA (A) and HL60 UV-irradiated, cross-linked DNA (B). DNA was incubated with different concentrations of ^{125}I-labeled topoisomerase II (10 mU/ml to 1 U/ml) with (▲, △) or without (●, ○) pretreatment with 5 μM doxorubicin for 30 min at 37°C in the presence or absence of a 100-fold excess of the unlabeled topoisomerase II for 1 h at 37°C. The results in each panel represent data from one of three experiments, each of which gave similar results.
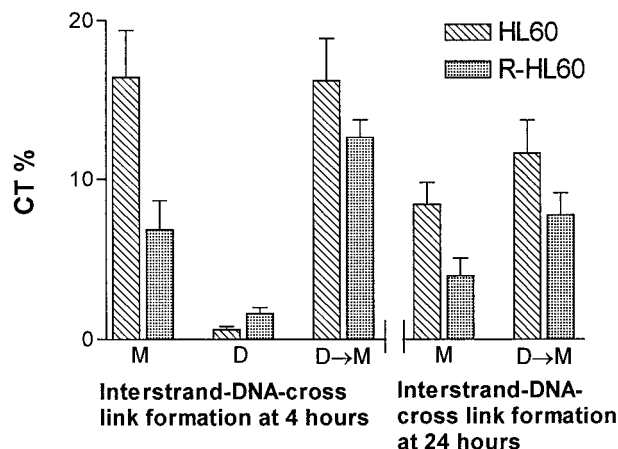

Fig. 7. The effect of doxorubicin (D) pretreatment on melphalan (M)-induced interstrand-DNA cross-link formation. D, 0.05 μM doxorubicin for 6-h; M, 10 μM melphalan for 1 h; D → M, preincubation with 0.05 μM doxorubicin for 6 h and then 10 μM melphalan treatment for 1 h. Cells were then washed and $CT\%$ was measured after 4- and 24-h drug-free incubations. Values are the mean ± S.D. of 10 experiments, each performed in triplicate.
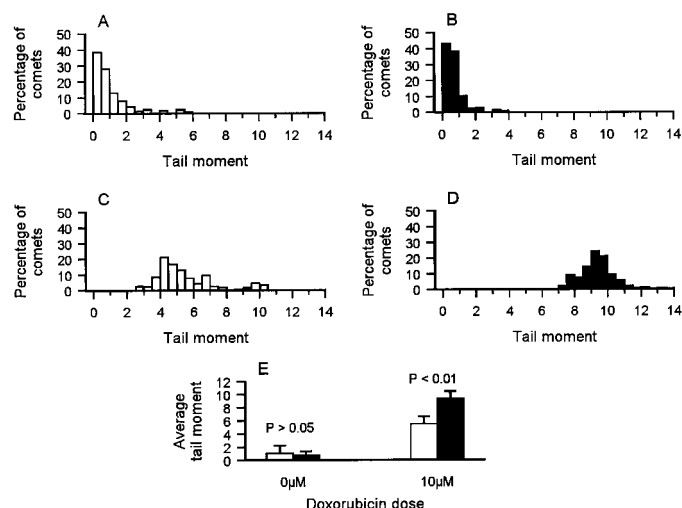

Fig. 8. Doxorubicin-induced double-stranded DNA Breaks of HL60 cells and R-HL60 cells. Double-stranded DNA breaks were measured by the neutral comet assay. Comets were analyzed by calculating the "tail moment" for each cell. In A–D, representative histograms from 100 comets are shown. A, HL60 cells without doxorubicin pretreatment; B, R-H L60 cells without doxorubicin pretreatment; C, HL60 cells pretreated with 10 μM doxorubicin; D, R-HL60 cells pretreated with 10 μM doxorubicin; E, the means ± S.D. (error bars) of tail moments for 100 comets are shown.

phyllotoxin (Glisson and Ross, 1987; Liu, 1989). Sensitivity to these agents in vitro seems to correlate with cellular topoisomerase II levels. Previous studies have shown that topoisomerase II inhibitors can also partially reverse resistance to the alkylating agents (Tan et al., 1987; De Jong et al., 1993), although the mechanism of this effect has not been fully defined.

The results of the current study provide further insights into the mechanisms of alkylator resistance and provide a mechanistic framework for the clinical use of alkylating agents in combination with topoisomerase II inhibitors such as doxorubicin and epipodophyllotoxins. The findings provide a rationale for the clinical use of combination chemotherapy regimens based on alkylating agents and topoisomerase II inhibitors.

References

Bailey HH, Gipp JJ, Ripple M, Wilding G and Mulcahy RT (1992) Increase in gamma-glutamylcysteine synthetase activity and steady-state messenger RNA levels in melphalan-resistant Du-145 human prostate carcinoma cells expressing elevated glutathione levels. *Cancer Res* **52**:5115–5118.

Batist G, Torres-Garcia S, Demuys JM, Greene D, Lehnert S, Rochon M and Panisci L (1989) Enhanced DNA cross-link removal: The apparent mechanism of resistance in a clinically relevant melphalan-resistant human breast cancer cell line. *Mol Pharmacol* **36**:224–230.

Bezwoda WR and Pu QQ (1997) In-vitro assessment of melphalan induced inter-strand-DNA cross-links in lymphocytes provides a rapid method for assessment of alkylator sensitivity in patients with lymphoproliferative disorders. *Int J Oncol* **10**:1061–1068.

Bradford MN (1976) 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.

Bungo M, Fujiwara Y, Kasahara K, Nakagawa K, Ohe Y, Irino S and Saijo N (1990) Decreased accumulation as a mechanism of resistance to cis-diamminedichloroplatinum (II) in human non-small cell lung cancer cell lines: Relation to DNA damage and repair. *Cancer Res* **50**:2549–2553.

Chow KC, Johnson TL and Pearson GD (1985) A novel method for the detection and quantitation of eukaryotic topoisomerase I. *Biotechniques* **3**:290–296.

De Jong S, Timmer-Bosscha H, DeVries E and Mulder NH (1993) Effect of novobiocin on cisplatin cytotoxicity and DNA interstrand crosslink formation in a cisplatin resistant small cell lung cancer cell line. *Int J Cancer* **53**:110–117.

De Jong S, Zijlstra JG, Mulder NH and DeVries EGE (1991a) Lack of cross resistance to fostreicin in a human small cell carcinoma cell line showing topoisomerase II-related drug resistance. *Cancer Chemother Pharmacol* **28**:461–464.

De Jong S, Zijlstra JG, Timmer-Bosscha H, Mulder NH and De Vries EGE (1991b) Detection of DNA cross-link in tumor cells with the ethidium bromide fluorescence assay. *Int J Cancer* **37**:557–561.

Drake FH, Hofmann GA, Bartus HF, Mattern MR, Crooke ST and Mirabelli CK (1989) Biochemical and pharmacological properties of p170 and p180 forms of topoisomerase II. *Biochemistry* **20**:8154–8160.

Drake FH, Zimmerman JP, Mccabe FL, Bartus HF, Per SR, Sullivan DM, Ross WE, Matteern MR, Johnson RK, Crooke ST and Mirabelli CK (1987) Purification of topoisomerase II from amsacrine-resistant P388 leukemia cells: Evidence for two forms of the enzyme. *J Biol Chem* **34**:16739–16747.

Earnshaw WC, Halligan BC, Crooke CA, Heck MMS and Liu LF (1985) Topoisomerase II is a structural component of the mitotic chromosome scaffold. *J Cell Biol* **100**:1706–1715.

Eder JP Jr, Chan VT, Ng SW, Rizvi NA, Zacharoulis S, Teicher BA and Schnipper LE (1995) DNA Topoisomerase IIα Expression is associated with alkylating agent resistance. *Cancer Res* **55**:6109–6116.

Erickson LC, Bradley MO and Kohn KW (1997) Strand breaks in DNA from normal and transformed human cells treated with 1, 3- bis (2-chloroethyl)-1-nitrosourea. *Cancer Res* **37**:3744–3750.

Ewig RAG and Kohn KW (1997) DNA damage and repair in mouse leukemia L1210 cell treated with nitrogen mustard, 1, 3 bis (2-chloroethyl)-1-nitrosourea, and other nitrosoureas. *Cancer Res* **37**:2114–2122.

Frei E, Cucchi C and Rosowsky A (1985) Alkylating agent resistance: In vitro studies of human cell lines. *Proc Natl Acad Sci USA* **82**:2158–2162.

Glisson BS and Ross WE (1987) DNA topoisomerase II: A primer on the enzyme and its unique role as a multidrug target in cancer chemotherapy. *Pharmacol Ther* **32**:89–106.

Gross-Bellard M, Oudet P and Chambon P (1972) Isolation of high-molecular-weight DNA from mammalian cells. *Eur J Biochem* **36**:32–35.

Hansson J, Lewensohn R, Ringborg U and Nilsson B (1987) Formation and removal of DNA crosslinks induced by melphalan and nitrogen mustard in relation to drug-induced cytotoxicity in human melanoma cells. *Cancer Res* **47**:2631–2637.

Harker WG, Slade DL, Drake FH and Parr RL (1991) Mitoxantrone resistance in HL60 leukemia cells: Reduced nuclear topoisomerase II catalytic activity and drug induced DNA cleavage in association with reduced expression of the topoisomerase II beta isoform. *Biochemistry* **30**:9953–9961.

Heck MMS and Earnshaw WC (1986) Topoisomerase II: A specific marker for cell proliferation. *J Cell Biol* **103**:2569–2581.

Heck MMS, Hittelman WN and Earnshaw WC (1987) Differential expression of DNA topoisomerases during the eukaryotic cell cycle. *Proc Natl Acad Sci USA* **85**:1086–1090.

Hsiang Y, Wu H-Y and Liu LF (1988) Proliferation-dependent regulation of DNA topoisomerase II in cultured human cells. *Cancer Res* **48**:3230–3235.

Liu LF (1989) DNA topoisomerase poisons as antitumor drugs. *Ann Rev Biochem* **58**:351–375.

Liu LF and Miller KG (1981) Eukaryotic DNA topoisomerase: Two forms of type I DNA topoisomerases from HeLa cell nuclei. *Proc Natl Acad Sci USA* **78**:3487–3491.

Louie KG, Behrens BC, Kinsella TJ, Hamilton TC, Grotzinger KR, McKoy WM, Winker MA and Ozols RF (1985) Radiation survival parameters of antineoplastic drug sensitive and resistant human ovarian cancer cell lines and their modification by glutathione sulfoximine. *Cancer Res* **45**:2110–2115.

Marini JC, Miller KG and England PT (1980) Decatenation of kinetoplast DNA by topoisomerases. *J Biol Chem* **255**:4976–4979.

Matsumoto Y, Fujiwara T, Honjo Y, Sasaoka N, Tsuchida T and Nagao S (1993) Quantitative analysis of DNA topoisomerase I activity in human and rat glioma: Characterization and mechanism of resistance to antitopoisomerase chemical, camptothecin-II. *J Surg Oncol* **53**:97–103.

Nelson WG, Pienta KJ, Barrack ER and Coffey DS (1986) The role of the nuclear matrix in the organization and function of DNA. *Ann Rev Biophys Chem* **15**:457–475.

Olive PL, Wlodek D and Banath JP (1991) DNA double-strand breaks measured in individual cells subjected to gel electrophoresis. *Cancer Res*. **51**:4671–4676.

Redwood WR and Colvin M (1980) Transport of melphalan by sensitive and resistant L1210 cells. *Cancer Res* **40**:1144–1149.

Richon VW, Schulte N and Eastman A (1990) Multiple mechanisms of resistance to *cis*-diamminedichloroplatinum (II) in human non-small cell lung cancer cell lines: Relation to DNA damage and repair. *Cancer Res* **50**:2549–2553.

Robson CN, Alexander J, Harris AL and Hickson ID (1985) Isolation and characterization of a Chinese hamster ovary cell line resistant to bifunctional nitrogen mustards. *Cancer Res* **45**:5304–5309.

Ross WE, Ewing RAG and Kohn KW (1978) Differences between melphalan and nitrogen mustard in the formation and removal of DNA crosslinks *Cancer Res* **38**:1502–1506.

Salacinski PRP, McLean C, Sykes JEC, Clement-Jones VV and Lowry PJ (1981) Iodination of proteins, glycoproteins, and peptides using a solid-phase oxidizing agent, 1,3,4,6-tetrachloro-3α,6α-diphenyl glycoluril (Iodogen). *Anal Biochem* **117**:136–146.

Tan KB, Mattern MR, Boyce RA and Schein PS (1987) Elevated DNA topoisomerase II activity in a nitrogen mustard resistant human cells. *Prot Natl Acad Sci USA* **84**:7668–7671.

Twentyman PR and Luscombe MA (1987) Study of some variables in a tetrazolium dye (MTT) based assay for cell growth and chemosensitivity. *Br J Cancer* **56**:279–285.

Wang JC (1987) Recent studies of DNA topoisomerase. *Biochim Biophys Acta* **909**:1–9.

Woessner RD, Chung TDY, Hofmann GA, Mattern MR, Mirabelli CK, Drake FH and Johnson PK (1990) Differences between normal and ras-transformed NIH-3T3 cells in expression of the 170kD and 180kD forms of topoisomerase II. *Cancer Res* **50**:2901–2908.

Woessner RD, Mattern MR, Mirabelli CK, Johnson RK and Drake FH (1991) Proliferation-and cell cycle-dependent differences in expression of the 170 kilodalton and 180 kilodalton forms of topoisomerases II in NIH-3T3 cells. *Cell Growth & Differ* **2**:209–214.