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Interference by Doxorubicin with DNA Unwinding in MCF-7 Breast Tumor Cells

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SUMMARY

The capacity of doxorubicin to inhibit topoisomerase II in the MCF-7 breast tumor cell line is supported by the induction of protein-associated single-strand breaks in DNA, as well as by interference with the decatenation activity of nuclear extracts. Doxorubicin also produces non-protein-associated DNA strand breaks (at a supraclinical concentration of 5 μM), which may indicate damage mediated via the generation of free radicals. However, no strand breaks are detected in DNA of MCF-7 cells at the IC_{50} for doxorubicin (approximately 0.1 μM). At doxorubicin concentrations of 0.05, 0.1, and 0.5 μM, at which growth is inhibited by approximately 15, 50, and 75%, respectively, doxorubicin interferes with radiation-induced unwinding of DNA; doxorubicin also produces a concentration-dependent inhibition of DNA synthesis that corresponds closely to growth inhibition. These studies suggest that DNA strand breaks fail to fully account for the antiproliferative activity of doxorubicin in the MCF-7 breast tumor cell line. Compromised DNA synthesis associated with interference with DNA unwinding may contribute to growth inhibition in MCF-7 cells exposed to doxorubicin.

Doxorubicin (Adriamycin) is one of the primary chemotherapeutic agents utilized for the treatment of breast cancer (1). However, the mechanism of action of this antineoplastic drug in breast tumors as well as in other neoplastic diseases appears to be complex and unclear. In addition to its capacity to intercalate into DNA and to inhibit macromolecular biosynthesis (2), doxorubicin has been reported to interact directly with the cell membrane (3) and to inhibit the enzyme helicase, which unwinds DNA for replication (4, 5). One of the primary targets for doxorubicin is thought to be the enzyme topoisomerase II (6), where inhibition of DNA religation results in the induction of protein-associated strand breaks in DNA (7). In the particular case of the MCF-7 breast tumor cell line, doxorubicin has also been reported to produce free radicals (8) and to promote lipid peroxidation (9).

Despite the fact that doxorubicin could potentially induce DNA damage in the MCF-7 breast tumor cell line, via either the generation of free radicals or the inhibition of topoisomerase II, there is virtually no information on the capacity of doxorubicin to induce DNA strand breaks in these cells.[1] It should be noted that the role of DNA damage in doxorubicin toxicity is somewhat equivocal. In P388 murine leukemic cells (12) and in a human myeloma cell line (13), a fairly strong correlation has been noted between doxorubicin toxicity and the induction of (primarily double-strand) breaks in DNA. In contrast, in some tumor cell lines, doxorubicin has been shown to produce very low levels of damage (14, 15); at their IC_{50} values no damage has been detected for doxorubicin and daunorubicin in a number of studies in different tumor cell lines (16–19).

The goal of the present studies was to assess the capacity of doxorubicin to induce DNA damage in the MCF-7 breast tumor cell line and to determine the nature of this damage and its potential relationship with growth inhibition. The results presented in this report indicate that bulk damage to DNA fails

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[1] Fisher and Patterson (10) have recently reported detection of single-strand breaks at 0.1 μM doxorubicin in MCF-7 cells, using the alkaline elution technique. However, the IC_{50} value in those experiments was approximately 19-fold higher than that reported previously for MCF-7 cells (11). In addition, those investigators failed to determine whether doxorubicin-induced damage to DNA was protein associated, indicative of inhibition of topoisomerase II. Consequently, it is difficult to assess the direct relevance of those studies.

ABBREVIATIONS: *m*-AMSA, 4′-(9-acridinylamino)methanesulfon-*m*-anisidide; DMSO, dimethylsulfoxide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; VP-16, etoposide (4′-demethylepipodophyllotoxin-9-(4,6-*O*-ethylidene-β-D-glucopyranoside)); VM-26, teniposide (4′-demethylepipodophyllotoxin-4-(4,6-*O*-thenylidene-(β-D-glucopyranoside)); kDNA, kinetoplast DNA.

to correspond to the antiproliferative activity of doxorubicin. In addition, we report a newfound capacity of doxorubicin to interfere with the unwinding of DNA and we propose that this effect may contribute to the inhibition of DNA synthesis and of growth in the MCF-7 breast tumor cell line.

Experimental Procedures

Materials. Dulbecco's modified Eagle medium (catalogue number 56-439-110) was obtained from Hazelton Research Products (Denver, PA), L-glutamine, penicillin/streptomycin (10,000 units/ml penicillin and 10 mg/ml streptomycin), and fetal bovine serum were obtained from Whittaker Bioproducts (Walkersville, MD), and defined bovine calf serum was obtained from Hyclone Laboratories (Logan, UT). Trypsin-EDTA (10×) (0.5% trypsin, 5.3 mM EDTA) was obtained from GIBCO Laboratories (Grand Island, NY). *m*-AMSA was generously provided by the Drug Synthesis and Chemistry Branch of the National Cancer Institute (Bethesda, MD). *m*-AMSA was dissolved in DMSO (Aldrich Biochemicals, Milwaukee, WI) and maintained as a frozen stock solution for a maximum period of 2–3 weeks. Drug was diluted in incubation medium on the day of the experiment. The radiolabeled compounds, [^{3}H]thymidine (75Ci/mmol) and [^{3}H]uridine (27.3Ci/mmol) were obtained from ICN Radiochemicals (Irvine, CA); [α-^{32}P]dATP (80Ci/mmol) was obtained from Dupont-NEN Research Products (Boston, MA). T4 phage topoisomerase II was a generous gift from Dr. Kenneth N. Kreuzer, Duke University Medical Center. Restriction enzymes were obtained from New England Biolabs (Beverly, MA). DMSO, bisbenzimide trihydrochloride (Hoechst 33258), proteinase K, MTT, thymidine, and trichloroacetic acid were obtained from Sigma Chemical Co. (St. Louis, MO). Tetrapropylammonium hydroxide was obtained from Kodak Chemicals (Rochester, NY). Agarose was obtained from GIBCO BRL (Gaithersburg, MD). All other chemicals were reagent or molecular biology grade, as appropriate.

Cell line. The MCF-7 breast tumor cell line was kindly provided by the laboratory of Dr. Kenneth Cowan at the National Cancer Institute (Bethesda, MD). Cells were maintained at 30°, under 5% CO_2, in Dulbecco's modified Eagle medium supplemented with 0.292 mg/ml glutamine, penicillin/streptomycin (0.5 ml/100 ml of medium), 5% fetal bovine serum, and 5% defined bovine serum.

Growth inhibition assay. The capacity of doxorubicin to interfere with growth of the MCF-7 breast tumor cell line was determined using the MTT dye assay (20), as described in detail previously (19). Briefly, cells subcultured at a density of 1×10^4 cells/ml in 96-well microplates (Costar, Cambridge, MA) were incubated for 2 hr at 37° with varying concentrations of doxorubicin. Drug-containing medium was aspirated, and cells were washed with incubation medium and permitted to grow for an additional 72 hr before determination of viable cell number.

Alkaline unwinding assay for single-strand breaks. Single-strand breaks in DNA were monitored using the alkaline unwinding procedure (21), as described in detail previously (22). Briefly, cells in 75-cm^2 T flasks (Costar) were incubated with drug for 2 hr at 37°, drug-containing medium was aspirated, cells were washed with phosphate-buffered saline on ice, released from flasks by incubation for 5 min at 37° with 0.05 mg/ml trypsin/0.02 mg/ml EDTA, collected in ice-cold phosphate-buffered saline, pH 7.4, and centrifuged at 4°, and cell number was determined before analysis for strand breakage. The assay is based on the differential binding and fluorescence of the indicator bisbenzimide trihydrochloride (Hoechst 33258) to single-strand and double-strand DNA after a fixed period of alkaline denaturation. After drug treatment (group B), the double-strand DNA remaining, F, was determined using the relationship $F = (B - C)/(A - C)$, where A, B, and C are the mean fluorescence levels in groups A (no unwinding), B (timed unwinding), and C (total unwinding), respectively. F values (21) were converted to rad equivalence based on standardization of DNA damage, using a ^{137}Cs irradiator to produce graded amounts of strand breakage. This assay was internally controlled by monitoring the relative binding of the indicator bisbenzimide trihydrochloride (Hoechst 33258) to single-strand and double-strand DNA. In experiments using concentrations of doxorubicin that may interfere with dye binding to double-strand DNA, a correction was applied, as indicated by Kanter and Schwartz (21). To assess interference with radiation-induced unwinding after a 2-hr drug exposure, cells were washed with phosphate-buffered saline on ice, released from flasks by incubation for 5 min at 37° with 0.05 mg/ml trypsin/0.02 mg/ml EDTA, collected in ice-cold phosphate-buffered saline, pH 7.4, and centrifuged at 4°, and cell number was determined. The cells were kept on ice and exposed to 1500 rad equivalents of ionizing radiation from a ^{137}Cs irradiator before analysis of radiation-induced unwinding as described above.

Alkaline and neutral elution assays for DNA damage. Strand breaks in DNA were further analyzed using either alkaline elution for single-strand breaks (23) or the neutral elution assay procedure for double-strand breaks (23), as described in detail previously (19). Cells were labeled for 24 hr with 0.1 μCi/nmol [^{3}H]thymidine, followed by washing and incubation for an additional 24 hr in thymidine-free medium before incubation with drug and processing as described for the alkaline unwinding assay. Approximately 5×10^5 cells were lysed either on polyvinyl chloride filters (Gelman Corp., Ann Arbor, MI) for alkaline elution or on polycarbonate filters (Nucleopore Corp., Cambridge, MA) with 2% SDS/0.02 M EDTA. Proteinase K (0.5 mg/ml) was used to eliminate DNA-protein cross-linking and to distinguish between direct and protein-associated strand breaks in the alkaline elution assay. DNA was eluted using tetrapropylammonium hydroxide at either pH 12.1 (for alkaline elution) or pH 9.6–10 (for neutral elution), at a flow rate of 0.8 ml/hr; fractions were collected over a 17-hr period and analyzed by scintillation counting. Damage was normalized based on rad equivalence determined by exposure of MCF-7 cells to various doses of ionizing radiation, using a ^{137}Cs source.

Preparation of MCF-7 nuclear extracts with 0.35 M NaCl. Nuclear extracts of MCF-7 cells were prepared according to the procedure of Sullivan *et al.* (24), as modified by Danks *et al.* (25). Approximately 1×10^8 exponentially growing cells were released from flasks by incubation for 5 min at 37° with 0.05 mg/ml trypsin/0.02 mg/ml EDTA, collected in ice-cold phosphate-buffered saline, pH 7.4, and centrifuged at 4°. Cells and reagents were kept on ice to prevent protein degradation. Cells were permeabilized by suspension in 1.75 ml of hypotonic buffer (buffer G of Ref. 24) (5 mM K_2HPO_4, 2 mM $MgCl_2$, 4 mM dithiothreitol, 0.1 mM disodium EDTA, 1 mM PMSF, pH 7.9) until approximately 90% of the cell population stained with trypan blue. After centrifugation for 5 min at 400 × *g*, the pellet was resuspended in buffer G with 0.25 M sucrose, at a concentration of 5×10^8 cells/8 ml. Each 8-ml suspension was layered over 3 ml of buffer G with 0.3 M sucrose and centrifuged for 20 min at 2000 × *g*. Buffer J (5 mM KH_2PO_4, 4 mM dithiothreitol, 1 mM disodium EDTA, 1 mM PMSF, pH 7.0) was added in a volume equal to one half of the pellet, and the suspension was placed on ice for 15 min. Nuclei were extracted with buffer L (40 mM Tris, 0.7 M NaCl, 1 mM PMSF, 4 mM dithiothreitol, 20% glycerol, pH 7.5), vortexed, and placed on ice for 30 min. The mixture was centrifuged at 100,000 × *g* for 1 hr, and the pellet was resuspended with 30% glycerol and stored at −20°.

Catalytic activity of topoisomerase II. The catalytic activity of topoisomerase II was measured by decatenation of kDNA as described by Sahai and Kaplan (26) and modified by Sullivan *et al.* (27). kDNA from the mitochondria of *Crithidia fasiculata* was labeled with [^{3}H]thymidine and isolated as described by Marini *et al* (28). Each 40-μl assay solution contained 50 mM Tris, pH 7.5, 85 mM KCl, 10 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.5 mM Na_2EDTA, 30 μg/ml bovine serum albumin, 1 mM ATP, 1 μg of [^{3}H]kDNA, and 1.4 μg of nuclear extract, plus doxorubicin (0.5–5 μM), *m*-AMSA (2.5–37.5 μM), or VP-16 (25–150 μM). After incubation at 30° for 30 min, reactions were stopped by addition of 10 μl of 2.5% SDS, 0.025 M Na_2EDTA, pH 8.0, in 50% glycerol. Tubes were then centrifuged for 15 min at 8000 × *g* at 20°. Duplicate 10-μl supernatant samples were then counted in 3.5 ml of Ecolite(+) (ICN Biochemicals, Irvine, CA), in a liquid scintillation counter. Decatenation was quantitated relative to controls assayed in

the absence of drugs, after subtraction of counts found in DMSO or water controls in the absence of nuclear extract.

DNA cleavage assays. Plasmid pBR322 was linearized with *Eco*RI. The 3′ termini were extended using the Klenow fragment of *Escherichia coli* DNA polymerase I (Boehringer Mannheim Corp., Indianapolis, IN) in the presence of [α-^{32}P]dATP and dTTP. One end of the plasmid was removed with *Hind*III, leaving a 4336-base pair fragment labeled at the *Eco*RI cleavage site. Substrate for the assay consisted of 0.20 ng of the ^{32}P-labeled plasmid combined with 0.3 μg of similarly digested unlabeled pBR322 DNA. MCF-7 nuclear extract was added to reaction mixtures (25 μl) containing DNA, 0.5 mM ATP, 0.5 mM dithiothreitol, 40 mM Tris·HCl, pH 7.8, 10 mM $MgCl_2$, 60 mM KCl, 0.5 mM Na_2EDTA, 30 μg/ml bovine serum albumin, and the appropriate concentration of drug. Samples were incubated for 30 min at 37° and the reaction was terminated by the addition of 5 μl of 20% SDS. To remove covalently attached topoisomerase, the reaction mixtures were treated with proteinase K at a final concentration of 100 μg/ml and incubated at 37° for 1 hr. Five microliters of gel loading buffer (20%, w/v, Ficoll, 0.25%, w/v, bromphenol blue, 0.25%, w/v, xylene cyanol) were added and the DNA was analyzed by electrophoresis through a 0.8% agarose gel in TBE running buffer (0.045 M Tris-borate, 1.0 mM EDTA) for 15 hr at 1 V/cm. The results of the cleavage reactions were visualized by autoradiography.

Macromolecular biosynthesis. DNA and RNA biosynthesis was measured by monitoring the incorporation of radiolabeled thymidine (DNA) and uridine (RNA) into acid-precipitable material, as described in detail previously (29). MCF-7 cells, cultured in 24-well plates (Costar), were incubated with drug for 2 hr at 37°. After removal of the drug, the cells were incubated in 2 ml of Hanks' balanced salt solution containing ^{3}H-labeled precursor. Thymidine incorporation was assessed over a 40-min period, whereas uridine incorporation was assessed over a 120-min period. Cells were removed from the wells by trypsinization, precipitated with 10% trichloroacetic acid, and filtered through Millipore AP filters (Millipore Corporation, Bedford, MA) containing 1 mM concentrations of the appropriate precursor. The filters were washed and the associated radioactivity was determined after addition of Beckman Readi-Solv scintillation mixture (Beckman Instruments, Fullerton, CA). The slopes of the lines describing the incorporation of the radiolabeled precursor were then used to compare the rates of macromolecular biosynthesis.

Analysis of data. Data analysis was performed using Student's *t* test. Drug effects that resulted in *P* values of 0.05 or below were considered statistically significant.

Results

The IC_{50} value for growth inhibition in MCF-7 breast tumor cells exposed for 2 hr to the anthracycline antibiotic doxorubicin was approximately 0.1 μM (Fig. 1), which is similar to that reported by Kramer *et al.* (11). To evaluate the relationship between inhibition of tumor cell growth by doxorubicin and induction of DNA damage in MCF-7 cells, single-strand breaks in DNA were assessed using the alkaline elution assay. Fig. 1 indicates that strand breaks were detected at concentrations of 0.5, 1, and 5 μM doxorubicin; however, no breaks were detected at 0.1 μM, a concentration at which growth is inhibited by 48.9%.

DNA single-strand breaks induced by doxorubicin could be either a consequence of the inhibition of topoisomerase II (6), which would result in protein-associated strand breaks (7), or a result of the generation of free radicals (8, 30), which should produce direct or non-protein-associated strand breaks. Fig. 2 presents the results of a representative alkaline elution study designed to define the nature of these strand breaks. It should be noted that this and subsequent figures presenting alkaline and neutral elution data are plotted on a linear (rather than

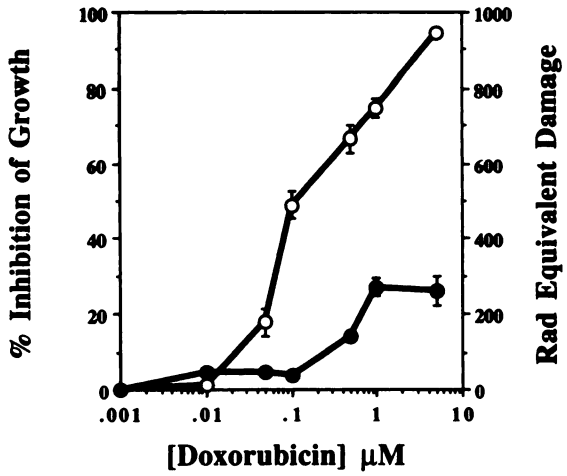

Fig. 1. Relationship between single-strand breaks induced by doxorubicin as detected using alkaline elution and growth inhibition determined using the MTT dye assay. MCF-7 cells were incubated with various concentrations of doxorubicin for 2 hr at 37°, drug was removed, and cells were processed for elution as described in Experimental Procedures. Elutions were performed in the presence of proteinase K. Data for growth inhibition (○) represent means ± standard errors for 10 experiments. Data for single-strand breaks (●) represent means ± standard errors for three experiments.

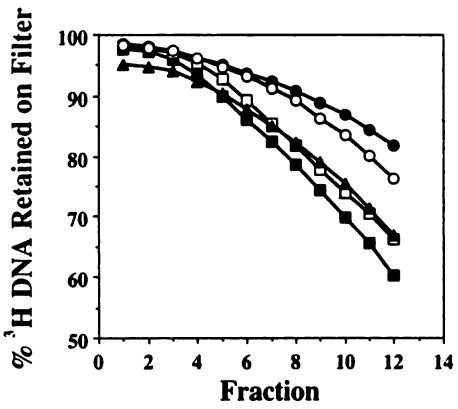

Fig. 2. Discrimination between protein-associated and non-protein-associated DNA strand breaks using alkaline elution. Studies were performed as described for Fig. 1, except that elutions were performed using protein-retentive filters in the absence and presence of proteinase K. The data are representative of three independent experiments. ●, Control, no drug; ○, 1 μM doxorubicin, without proteinase K; ■, 1 μM doxorubicin, with proteinase K; ▲, 5 μM doxorubicin, without proteinase K; □, 5 μM doxorubicin, with proteinase K.

logarithmic) scale and with an abbreviated ordinate, to clarify the extent of drug effects on DNA integrity. The breaks in DNA induced by 1 μM doxorubicin were primarily protein associated, because only a small degree of damage was observed in the absence of proteinase K digestion of the DNA-protein complex; consequently, the damage induced by 1 μM doxorubicin is likely to be mediated primarily by interference with topoisomerase II. In contrast, Fig. 2 indicates that, at a doxorubicin concentration of 5 μM, the protein-associated damage was abrogated in favor of the production of damage that was direct or non-protein associated and that is therefore likely to be free radical mediated (8) (the lines describing elution in the absence and presence of proteinase K in cells treated with 5 μM doxorubicin are essentially superimposable, and damage at

5 μM doxorubicin does not appear to exceed that observed at 1 μM doxorubicin, as in Fig. 1).

The apparent abrogation of topoisomerase II-mediated single-strand breaks in favor of direct or non-protein-associated breaks at 5 μM doxorubicin could be explained by the fact that, at higher concentrations of drug, intercalation into DNA and distortion of the helix may prevent binding and DNA cleavage mediated by topoisomerase II (6). The induction of strand breaks that are non-protein associated (and, presumably, free radical mediated) at elevated doxorubicin concentrations is consistent with earlier work in L1210 cells (31). Although various laboratories have reported evidence of free radical-mediated toxicity in MCF-7 breast tumor cells (8, 9, 30), the non-protein-associated damage in our studies was noted at supraclinical concentrations of drug; consequently, these breaks would not be thought to contribute to clinically relevant drug toxicity.

Because the toxic or antiproliferative effects of doxorubicin have been more closely associated with double-strand breaks, compared with single-strand breaks, in DNA (12), studies were designed to assess the capacity of doxorubicin to produce double-strand breaks in the DNA of MCF-7 breast tumor cells. Fig. 3 indicates that elution at pH 9.6 of DNA from MCF-7 breast tumor cells treated with doxorubicin at a concentration of 1 μM failed to demonstrate enhancement of elution rates over control conditions, indicating the absence of double-strand breaks at this concentration. At 5 μM doxorubicin, however, there was a slight increase in elution rates over control, indicating double-strand breaks. *m*-AMSA, included as a positive control, demonstrated unequivocal double-strand DNA cleavage.

To evaluate the capacity of doxorubicin to induce double-strand cleavage of DNA mediated by topoisomerase II *in vitro*, cleavage was assessed using a linearized ^{32}P-labeled pBR322 DNA substrate incubated with a 0.35 M NaCl nuclear extract from the MCF-7 breast tumor cell line in the presence of doxorubicin or *m*-AMSA. Fig. 4 demonstrates that, whereas *m*-AMSA (at concentrations where growth of MCF-7 cells is inhibited by 20–70%) (32) produced double-strand cleavage (Fig. 4, *lanes 6-8*), concentrations of doxorubicin that produce a similar degree of growth inhibition failed to do so (Fig. 4, *lanes 9-12*). A similar absence of double-strand DNA cleavage

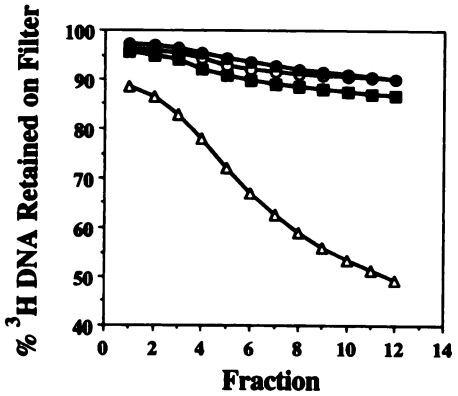

Fig. 3. Detection of double-strand breaks in MCF-7 cells exposed to doxorubicin, using neutral elution. Cells were exposed to doxorubicin as described for Fig. 1. Elutions were performed using non-protein-retentive filters in the presence of proteinase K at pH 9.6. The data are representative of three independent experiments. ●, Control, no drug; ○, 1 μM doxorubicin; ■, 5 μM doxorubicin; △, 10 μM *m*-AMSA.

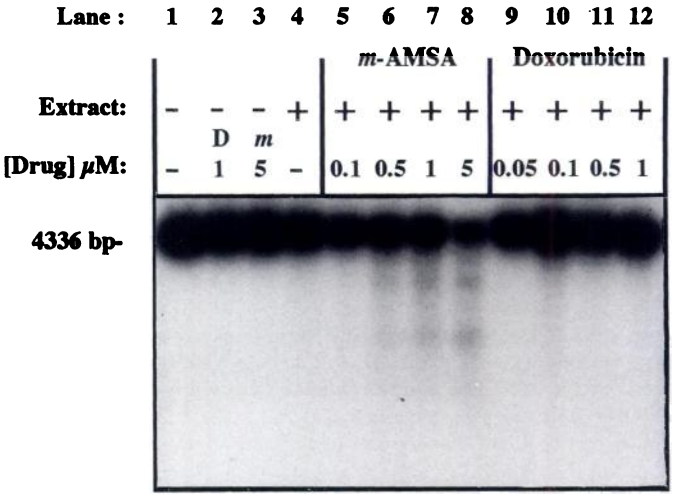

Fig. 4. DNA cleavage activity of MCF-7 topoisomerase II in the presence of *m*-AMSA and doxorubicin. A mixture of end-labeled fragments of pBR322 (20 ng), as described in Experimental Procedures, and similarly processed unlabeled pBR322 DNA (0.3 μg) was incubated with MCF-7 nuclear extract in the presence of increasing concentrations of *m*-AMSA and doxorubicin. Nuclear extract-free reactions (−) were performed in the presence of 1 μM doxorubicin (*D*) or 5 μM *m*-AMSA (*m*), to detect drug-induced DNA cleavage. The DNA was resolved by agarose gel electrophoresis and visualized by autoradiography. The autoradiograph shown here is representative of six independent experiments.

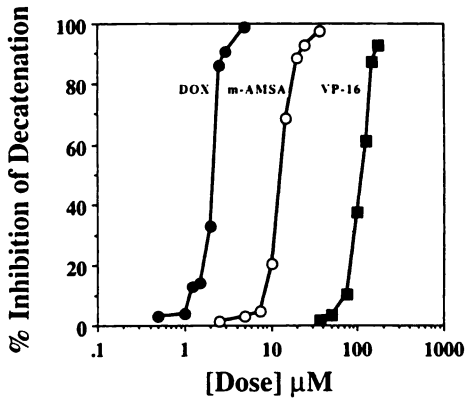

Fig. 5. Effect of various topoisomerase II inhibitors on decatenation of DNA using nuclear extracts from MCF-7 cells. Nuclear extracts (1.4 μg) were combined with [^{3}H]kDNA (1 μg) in the presence and absence of various concentrations of doxorubicin (*DOX*), *m*-AMSA, and VP-16. After a 30-min incubation at 30°, reactions were stopped by addition of 2.5% SDS and decatenated DNA was quantitated after centrifugal separation of precipitated catenated DNA, as described in Experimental Procedures. Results shown are representative of three independent experiments performed on separate days.

in the presence of doxorubicin was observed using T4 phage topoisomerase II (data not shown), consistent with the report of Huff and Kreuzer (33).

Although doxorubicin fails to stimulate double-strand DNA cleavage, it nevertheless interferes with the catalytic activity of topoisomerase II from the MCF-7 breast tumor cell line. Fig. 5 presents the results of studies designed to assess the capacity of various topoisomerase II inhibitors to inhibit the strand-passing activity of the enzyme (nuclear extract), using a decatenation assay. In the experiments presented in Fig. 5, doxorubicin, as well as *m*-AMSA and VP-16, was capable of interfering with the decatenation activity of the nuclear extract, indicating inhibition of the strand-passing activity of topoisom-

erase II or other reactions associated with double-strand DNA cleavage (34, 35).

Studies were also performed utilizing an additional assay, alkaline unwinding, to detect strand breaks in DNA induced by doxorubicin. Fig. 6 indicates that this assay failed to detect breaks by doxorubicin at concentrations up through 1 μM (in contrast to the detection of breaks at doxorubicin concentrations of 0.5 and 1 μM using the alkaline elution procedure, as indicated in Fig. 1) but did detect breaks induced by 5 μM doxorubicin, presumably mediated through free radical production. The alkaline unwinding assay also detected breaks produced by doxorubicin in C6 neuroblastoma cells,[2] and by *m*-AMSA and VM-26 in MCF-7 cells (32, 36). Because the alkaline unwinding assay is capable of detecting 50–100 rad equivalents of DNA damage (21, 22), the absence of detectable breaks in MCF-7 cells incubated with doxorubicin is not a consequence of limited assay sensitivity.

The trivial possibility that the alkaline unwinding assay fails to detect DNA strand breaks that are exclusively single-strand in MCF-7 cells was eliminated by demonstrating the induction of single-strand breaks by the topoisomerase I inhibitor camptothecin (37) (data not shown).

The absence of detectable DNA strand breaks using the alkaline unwinding assay suggests that, whereas doxorubicin does produce DNA breaks in MCF-7 cells, DNA unwinding in alkali is somehow compromised, obviating the differential binding of double-strand and single-strand DNA with the indicator Hoechst dye used in this assay (21). To examine this hypothesis, MCF-7 cells treated with doxorubicin were exposed to ionizing radiation and DNA unwinding was assessed by monitoring the increase in single-strand DNA. Fig. 7 demonstrates that doxorubicin produced a concentration-dependent reduction in the amount of detectable damage (i.e., the amount of single-strand DNA detected), presumably by interfering with the unwinding process. Interference with unwinding was highly variable at 1 μM doxorubicin (data not shown), apparently because this concentration of doxorubicin occasionally produced a small amount of (presumably free radical-mediated) DNA damage, obscuring the effect on unwinding and increasing the total amount of damage observed. The capacity of doxorubicin to interfere with DNA unwinding was observed over unwinding times ranging between 10 min and 2 hr (data not shown). In contrast, *m*-AMSA increased the level of DNA strand breakage over that induced by ionizing radiation alone (data not shown). Another intercalating agent, ethidium bromide, which does not itself induce any DNA breaks, did not interfere with DNA unwinding (Fig. 7).

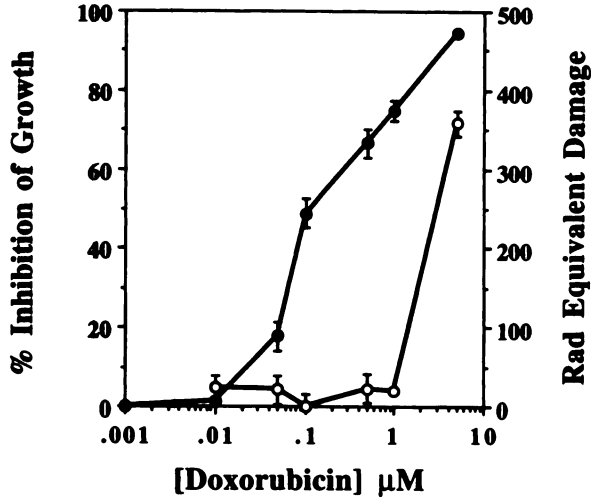

Fig. 6. Relationship between single-strand breaks induced by doxorubicin as detected using alkaline unwinding and growth inhibition determined using the MTT dye assay. Data for growth inhibition (●) represent means ± standard errors for 10 experiments. Data for single-strand breaks (○) represent means ± standard errors for six experiments.

[2] J. Robert, personal communication.

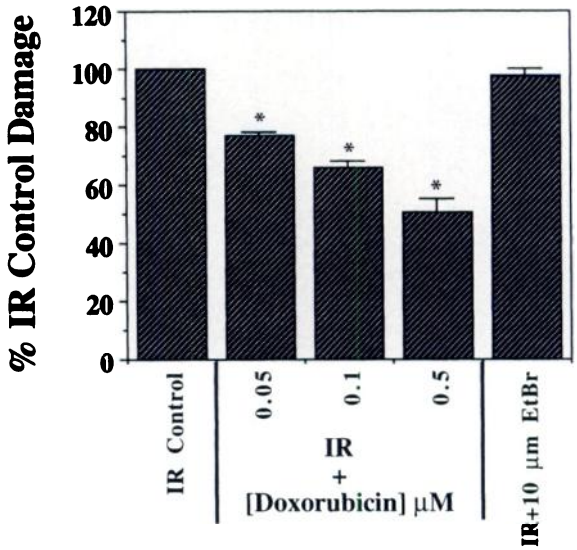

Fig. 7. Capacity of doxorubicin to interfere with alkaline unwinding induced by radiation. MCF-7 breast tumor cells incubated with various concentrations of doxorubicin [or 10 μM ethidium bromide (*EtBr*)] were exposed to ionizing radiation (*IR*) and strand breaks were determined using alkaline unwinding, as described in Experimental Procedures. Data represent means ± standard errors for four experiments. *, Significant at $p < 0.05$.

Although the reduction in DNA damage by doxorubicin is assumed to result from interference with unwinding, the same result would be observed if doxorubicin protected the cell from radiation. To eliminate this possibility, radiation-induced damage was assessed in cells exposed to 0.1 and 0.5 μM doxorubicin, using the alkaline elution assay. However, doxorubicin failed to reduce the damage induced by ionizing radiation (data not shown).

The absence of double-strand breaks in the neutral elution procedure at a doxorubicin concentration as high as 1 μM and the failure of doxorubicin to stimulate double-strand cleavage *in vitro* suggest the possibility that only single-strand breaks are produced because the enzyme-DNA complex is trapped subsequent to cleavage of the first strand in the sequential cleavage reaction (38). In such a case, unwinding of the DNA for replication might be retarded, resulting in the inhibition of DNA synthesis. Studies were conducted to assess the effects of doxorubicin on DNA and RNA biosynthesis in MCF-7 cells treated with doxorubicin. Fig. 8 demonstrates that inhibition of DNA biosynthesis corresponded closely to inhibition of MCF-7 cell growth with doses up to and including 1 μM (r^2 = 0.954). Fig. 8 also shows that inhibition of RNA biosynthesis failed to correspond to growth inhibition, particularly at concentrations of doxorubicin that resulted in the inhibition of unwinding (see Fig. 7).

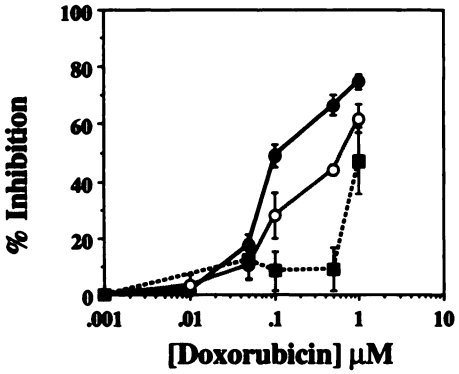

Fig. 8. Relationship between growth inhibition as determined using the MTT dye assay and inhibition of DNA and RNA biosynthesis as determined by incorporation of labeled precursor. Data for growth inhibition (●) were taken from Fig. 1. Data for inhibition of DNA biosynthesis (○) represent means ± standard errors for five separate experiments. Data for inhibition of RNA biosynthesis (■) represent means ± standard errors for three separate experiments.

Discussion

Studies in P388 leukemia cells (12) and in 8226/S myeloma cells (13) have demonstrated a strong correlation between double-strand breaks in DNA and the toxicity of doxorubicin. However, the absence of detectable single- and double-strand breaks in the DNA of MCF-7 breast tumor cells at the IC_{50} suggests that DNA strand breaks mediated by inhibition of topoisomerase II may not be fully responsible for growth inhibition by doxorubicin in this cell line. The absence of detectable DNA damage at the IC_{50} for doxorubicin has also been observed in KBM-3 myeloid leukemia cells (17) and in GLC_4 lung carcinoma cells (18), whereas a dissociation between strand breaks in DNA and the antiproliferative activity of doxorubicin has been reported by our own laboratory for rat hepatoma cells (19) and by other investigators for P388 leukemia cells (39) and mouse fibrosarcoma 935.1 cells (40). In this context, Zwelling *et al.* (15) recently reported minimal DNA damage at a concentration of doxorubicin at which >99.9% of HL-60 cells were killed, whereas Chen *et al.* (41) failed to detect doxorubicin-induced stimulation of DNA-protein cross-link formation using MCF-7 breast tumor cells.

The failure of doxorubicin to produce detectable double-strand cleavage of DNA at clinically relevant concentrations may be reconciled with its capacity to inhibit decatenation (i.e., to interfere with catalytic activity) (42) by virtue of the fact that the decatenation reaction could be blocked at various steps, including strand passage (34, 35). Consequently, a stabilized DNA-topoisomerase II complex, even one in which only single-strand breaks are produced, should also block DNA decatenation.

The exclusive detection of single-strand breaks (by alkaline elution) in MCF-7 breast tumor cells at doxorubicin concentrations associated with the inhibition of topoisomerase II could simply be a consequence of low levels of double-strand breaks (i.e., below the detection limits of the neutral elution assay). Another possibility is that, because the DNA strand cleavage reaction mediated by topoisomerase II is thought to occur sequentially rather than in a concerted fashion (38), doxorubicin might permit topoisomerase II- mediated cleavage of a single strand of the DNA duplex but the subsequent cleavage of the complementary strand and the religation of the cleaved strand would be inhibited. In this regard, Lee and Hsieh (43) have recently reported the induction of a cleavable complex involving only one of the topoisomerase II subunits in the presence of VM-26.

In contrast to the apparent dissociation between strand breaks in DNA and growth inhibition, growth-inhibitory concentrations of doxorubicin are capable of interfering with alkaline-induced unwinding of DNA in MCF-7 cells. One possible mechanism for interference with DNA unwinding is simple intercalation of the doxorubicin into DNA (2). However, it is unlikely that doxorubicin could maintain its association with the DNA backbone under the alkaline conditions of the unwinding assay purely by physical and hydrogen-bonding interactions. Furthermore, another DNA intercalator, ethidium bromide, failed to interfere with DNA unwinding. Another possibility is that compaction of the chromatin by doxorubicin alters the rate of DNA unwinding (44). Again, this is unlikely to be the case with the low concentrations of doxorubicin used in these studies, because a high drug/base ratio is required for chromatin condensation (44).

It is possible that the interference with DNA unwinding in alkali could be a consequence of a cellular enzyme that is normally associated with DNA function (such as topoisomerase II, a polymerase, or a helicase) being "frozen" or locked onto the DNA in a conformation that is stable in alkali, thereby preventing dissociation of the two strands. The inhibition of DNA synthesis by doxorubicin would be consistent with inhibition of unwinding of DNA in the intact cell via interference with the function of DNA-replicative enzymes.

The minimal inhibition of RNA biosynthesis at concentrations of doxorubicin that interfere with unwinding suggests that DNA unwinding may be slowed only at a limited number of sites where the putative DNA-associated enzyme is bound. It is possible that other enzymes, such as topoisomerase I, would still be functional, so that local unwinding for RNA biosynthesis is likely to continue. This would be consistent with the fact that topoisomerase I has been linked with actively transcribed regions on chromatin (45). The abrupt increase in inhibition of RNA biosynthesis at doxorubicin concentrations above 0.5 μM may be related to the freezing of additional enzyme-DNA complexes, which might displace topoisomerase I from DNA binding sites.

The studies presented in this report, which are focused on the initial doxorubicin-induced lesions that may mediate growth arrest, suggest that growth inhibition in MCF-7 breast tumor cells may be related to an effect of doxorubicin at the level of DNA unwinding, rather than to the induction of strand breaks in DNA. It should be noted that apoptosis or programmed cell death has recently been associated with growth arrest induced by doxorubicin in HeLa cells (46). In addition, doxorubicin has been shown to induce apoptosis in thymocytes (47), intestinal epithelium (48), and various tumor cell models, including P388 leukemia cells (49), lung tumor cells (50), and M1 myeloid leukemic cells (51). In contrast, Zwelling *et al.* (52) have reported that doxorubicin fails to produce endonucleolytic cleavage in HL-60 human leukemic cells and Oberhammer *et al.* (53) have demonstrated that the MCF-7 breast tumor cell line appears to be refractory to apoptosis. Preliminary studies in our laboratory, which are consistent with this latter finding, indicate that doxorubicin fails to produce oligosomal DNA fragmentation or apoptotic cell death in the MCF-7 breast tumor cell line.

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