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BIOCHEMICAL LESIONS IN DNA ASSOCIATED WITH THE ANTIPROLIFERATIVE EFFECTS OF MITOXANTRONE IN THE HEPATOMA CELL

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Abstract—The H-35 rat hepatoma cell was markedly more sensitive to the anthracenedione mitoxantrone (IC_{50}, 0.05 μM) than to the anthracycline antibiotics daunorubicin (IC_{50}, 0.5 μM) and Adriamycin® (IC_{50}, 2.5 μM). In the rat hepatoma cell, mitoxantrone inhibited DNA and protein syntheses, with minimal effects on RNA synthesis. In contrast to daunorubicin, mitoxantrone induced both DNA strand breaks and DNA–protein cross links. The capacity of mitoxantrone to induce more extensive DNA cleavage than anthracycline antibiotics such as daunorubicin may be related to the sustained cellular retention of mitoxantrone (62% of accumulated drug) as compared to that for daunorubicin (32% of accumulated drug). Protein-associated DNA cleavage is likely to be one of the primary lesions contributing to the antiproliferative activity of mitoxantrone in the hepatoma cell, although marked growth inhibition was observed without corresponding alterations in DNA integrity.

The anthracenedione mitoxantrone (1,4-dihydroxy-5,8-bis[[2-[(2-hydroxethyl)-amino]ethyl]amino]-9,10-anthracenedione dihydrochloride), a synthetic analog of the anthracycline antibiotics, has demonstrated antitumor activity in experimental rodent tumor systems [1–3] as well as in human tumor cell lines [4, 5]. Mitoxantrone has shown clinical effectiveness in the treatment of breast cancer, lymphoma and acute leukemia [6] and recently has shown clinical promise in the treatment of hepatocellular carcinoma [7].

The biochemical lesions which may mediate tumor cell kill by mitoxantrone have proven difficult to define. Mitoxantrone binds to DNA, at least in part by intercalation [8], but via electrostatic interactions as well [9]. Alterations in the melting temperature of DNA induced by mitoxantrone do not appear to correlate with inhibition of cell growth [2], suggesting that intercalation into DNA is not sufficient to account for the antiproliferative activity of mitoxantrone. Mitoxantrone has been shown to induce compaction of isolated chromatin [10, 11] as well as sister chromatid exchange [12], and to inhibit macromolecular biosynthesis in a number of tumor cell lines [2, 5, 13, 14].

Like a variety of other intercalative and non-intercalative antineoplastic drugs, mitoxantrone stimulates DNA strand cleavage mediated by topoisomerase II [15]. In the L1210 leukemia cell, Bowden *et al.* have reported that mitoxantrone induces both protein-associated and non-protein-associated DNA strand breaks [16] while exclusively protein-associated single-strand breaks in DNA have been reported by others in the L1210 murine leukemia line and in Chinese hamster ovary cells [17, 18]. While a lack of direct correspondence between DNA damage and drug toxicity in L1210 leukemia cells in the studies by Bowden *et al.* [16] suggests that these lesions in DNA may not fully explain the antiproliferative action of mitoxantrone, studies using human leukemic cells suggest a correspondence between the induction of DNA strand breaks and cell death in responsive tumor cells [19].

These and other investigators have generally focused on one or two biochemical parameters of mitoxantrone interaction with the cell such as inhibition of DNA synthesis [20], inhibition of protein synthesis [21], induction of sister chromatid exchange [12], binding of mitoxantrone to DNA [11], or production of DNA strand breaks [16, 18, 19, 22]. Recently, Duthie and Grant [23] explored the possibility that free radical mechanisms may mediate mitoxantrone toxicity in the Hep G2 human hepatoma cell line. In view of the recent report that mitoxantrone may have potential utility in the treatment of hepatocellular carcinoma [7], the present studies were designed to assess the capacity of mitoxantrone to induce a *variety* of biochemical alterations in the hepatoma cell in order to attempt to define the lesion(s) which may mediate the antiproliferative activity of this antineoplastic agent.

MATERIALS AND METHODS

Chemicals. Mitoxantrone hydrochloride and [^{14}C]mitoxantrone (60 μCi/μmol) were provided by Lederle Laboratories (Pearl River, NY). Stock solutions of drug were prepared periodically in deionized water and stored at −20°. Concentrations of the stock solutions were determined spectrophotometrically using a molar extinction coefficient of

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19.2×10^3 (Merck Index, 1983). MTT* for the tetrazolium dye assays was obtained from the Sigma Chemical Co. (St Louis, MO). [*methyl*-^{3}H]Thymidine (72 Ci/mmol), [5-^{3}H]uridine (24 Ci/mmol), and [4,5-^{3}H]leucine (56 Ci/mmol) were obtained from ICN Radiochemicals, Division of ICN Biochemicals, Inc. (Irvine, CA). Tetrapropyl ammonium hydroxide used in the alkaline elution assay was obtained from the Eastman Kodak Co. (Rochester, NY). Deferoxamine mesylate was obtained from CIBA (Summit, NJ). All other chemicals were reagent grade.

Culture conditions. Reuber H-35 rat hepatoma cells (also known as the H-4-II-E tumor line), obtained from the American Type Culture Collection (Rockville, MD), have a doubling time of approximately 18 hr. For all studies described in this manuscript, cells were subcultured at densities that ensured logarithmic growth during the assay procecure. Cells were maintained in monolayer culture in "complete medium" consisting of Dulbecco's minimal essential medium (Hazelton Research Products, Denver, PA) supplemented with glutamine (29.2 mg/100 mL), penicillin/streptomycin (0.5 mg/100 mL), 5% fetal calf serum (Life Technologies, Grand Island, NY), and 5% defined bovine calf serum (Hyclone Laboratories, Logan, UT) at 37°, under 5% CO_2.

Assessment of drug effects on cell proliferation. The effects of mitoxantrone, Adriamycin® and daunorubicin on proliferation of the rat hepatoma were assessed by the MTT tetrazolium dye assay [24]. Briefly, hepatoma cells were plated at a density of 3×10^3 cells/mL in flat-bottomed 96-well plates (Costar, Cambridge, MA). After 96 hr, cells were incubated with drugs at various concentrations for 2 hr, washed twice with complete medium, and maintained in complete medium for an additional 72 hr. Medium was decanted, and cells were washed with phosphate-buffered saline prior to incubation with 100 μL of MTT solution (2 mg/mL of MTT in phosphate-buffered saline made fresh and filtered on the day of the assay) at 37° under CO_2 for 3 hr. Unreacted MTT solution was removed by decanting the plate. The blue formazan product was eluted from cells by addition of 100 μL of DMSO, and absorbance was monitored using a model EL310 EIA autoreader (BIOTEK Instruments, Burlington, VT).

To assess the capacity of free radical scavengers and deferoxamine to interfere with drug-induced inhibition of growth, H-35 cells were incubated for 1 hr prior to the addition of 0.1 μM mitoxantrone with one of the following: 3000 units/mL catalase, 1.0 M DMSO, 100 mM mannitol, 300 mM methanol or 10 mM deferoxamine in 100 μL of complete medium. Incubation with scavengers (at one-half the initial concentrations) and mitoxantrone was continued for an additional 2 hr prior to removal of drugs. Cells were maintained in culture medium for an additional 72 hr before assessing cell number utilizing the MTT assay, as described above.

* Abbreviations: MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-dephenyltetrazolium bromide; IC_{50}, the concentration of drug which inhibits cell proliferation by 50%; DMSO, dimethyl sulfoxide; and SDS, sodium dodecyl sulfate.

Cell cycle analysis. Hepatoma cells in log growth were treated with propidium iodide staining solution consisting of 3.8 mM sodium citrate, 0.1% Triton X-100, RNAse B (700 units/mL), and 0.05 mg propidium iodide/mL and refrigerated for 30 min [25]. Cells were dislodged using a rubber policeman. The stained nuclei were analyzed for DNA–propidium fluorescence using a Coulter Telectronics TPS-1 at a laser setting of 36 mW and an excitation wavelength of 488 nm; resulting DNA distributions were analyzed for the proportion of cells in the G_1-S and G_2-M phases of the cell cycle as described by Collins *et al.* [26].

Macromolecular biosynthesis. DNA, RNA, and protein syntheses were assessed by monitoring cellular incorporation of ^{3}H-labeled thymidine, uridine and leucine, respectively, into acid-precipitable material after a 2-hr exposure to drug as well as after an additional 2-hr incubation in drug-free medium. The incubation conditions were identical for this and all other assays, i.e. a 2-hr exposure to drug. Details of this procedure have been presented previously [27]. For measurement of thymidine, uridine and leucine incorporation (DNA, RNA and protein synthesis respectively), each well of a 24-well plate containing adherent H-35 cells was incubated with 2 mL of [^{3}H]thymidine, [^{3}H]uridine or [^{3}H]leucine at specific activities of 13.2 μCi/nmol (approximately 2.2×10^6 cpm/mL) in Hanks' balanced salts solution. Incorporation of thymidine and leucine was assessed over 40 min while uridine incorporation was monitored over 120 min to allow uridine equilibration with a larger intracellular pool. After removal of cells from wells by trypsinization and precipitation with trichloroacetic acid (final concentration, 5%), samples were filtered through Millipore AP filters equilibrated with 10% trichloroacetic acid containing a 1 mM concentration of the unlabeled precursor. Precipitates on the filters were washed three times with the same solutions; filters were dried by vacuum, and associated radioactivity was determined after addition of Beckman Readi-Solv scintillation mixture. Rates of biosynthesis were determined from the slope of the line describing incorporation of radiolabeled precursors into acid-precipitable material.

Induction of DNA single-strand breaks. H-35 rat hepatoma cells were plated in 75 cm^2 canted-neck culture flasks (Costar) at a density of 1×10^4 cells/mL at least 72 hr prior to initiation of the study. Cells were incubated for 24 hr in 12–15 mL of complete medium containing 0.1 μCi/nmol of [^{3}H]thymidine followed by a 24-hr incubation in [^{3}H]thymidine-free medium to permit short fragments of labeled DNA to be incorporated into complete strands.

Cells were treated for 2 hr with various concentrations of mitoxantrone in complete medium. After two washes with phosphate-buffered saline, cells were detached from flasks by incubation with 5 mL of trypsin (0.05 mg) + EDTA (0.02 mg/mL saline) at 37° under 5% CO_2 for 5 min. Cells in the trypsin solution were diluted 1:10 with cold phosphate-buffered saline or complete medium to prevent proteolysis and to limit repair of DNA strand breaks. Cell suspensions were pelleted at 500 *g* in a refrigerated centrifuge and resuspended in cold

phosphate-buffered saline on ice. The alkaline elution procedure utilized is a modification [27] of that described by Kohn *et al.* [28]. Approximately 2.5×10^5 cells were lysed on protein-retentive 0.8-micron polyvinyl chloride filters (Gelman Sciences, Ann Arbor, MI) with 0.2% *N*-lauryl sarcosine/2 M NaCl/0.04 M EDTA (pH 9.7) at room temperature for 30 min. Proteinase K (0.5 mg/mL in lysis solution) was added where appropriate to allow expression of protein-associated DNA damage. Proteinase K treatment digests the topoisomerase II associated with the DNA, permitting the cleaved DNA to elute from the filter. DNA was eluted using tetrapropyl ammonium hydroxide (pH 12.1) at a flow rate of 25–40 μL/min. Fractions were collected over a 17-hr period and analyzed by liquid scintillation counting; [^{3}H]DNA associated with the filter and in cell lysates was quantitated as well.

Double-strand breaks in DNA were monitored by neutral elution [28]. Cells lysed with 2% sodium dodecyl sulfate, 0.02 M EDTA (pH 10) on polycarbonate filters (Nucleopore) in the presence of proteinase K were eluted using tetrapropyl ammonium hydroxide at pH 9.6.

Alkaline unwinding assay for DNA strand cleavage. The alkaline unwinding assay, for determination of DNA strand cleavage, was performed as described by Kanter and Schwartz [29], where DNA cleavage is monitored based on the differential binding of Hoechst dye to single-stranded and double-stranded DNA. H-35 rat hepatoma cells cultured in 75 cm^2 flasks were incubated with various concentrations of mitoxantrone for 2 hr. Where indicated, cells were pretreated with 1 M DMSO for 1 hr prior to mitoxantrone. After removal of drug and detachment of cells using trypsin/EDTA, cells were maintained on ice until initiation of the assay. Cells were resuspended to a density of 1×10^6 cells/mL with phosphate-buffered saline at room temperature. The unwinding interval, in alkali, was 10 min. Fluorescence of the Hoechst dye–DNA complex was determined using a Kratos fluorescence spectrophotometer (excitation wavelength, 350 nm; emission wavelength, 450 nm). DNA cleavage was standardized to rad equivalents using a calibration curve generated by exposing untreated cells to varied doses of radiation. This assay is based on the rate of denaturation of DNA in alkali as a function of the number of drug or radiation-induced nicks and does not discriminate between single-strand and double-strand breaks in DNA or between protein- and non-protein-associated DNA cleavage.

Induction of DNA–protein cross links. DNA–protein cross-link induction was monitored by the procedure of Trask *et al.* [30] in which protein-linked DNA is trapped in an SDS/K$^+$ precipitate. Methodology followed that described by Rowe *et al.* [31]. Hepatoma cells in logarithmic growth were labeled with [*methyl*-^{3}H]thymidine at a final specific activity of 1 μCi/nmol (for induction of DNA–protein cross links, approximately 7×10^5 cpm/mL was used) for approximately 24 hr. Cells were washed with complete medium and detached from plates using trypsin/EDTA, followed by two washes with complete medium. Cells were resuspended to a density of 2×10^5 cells/mL and treated with mitoxantrone for 2 hr; the medium containing mitoxantrone was removed after centrifugation, and cells were lysed by addition of 1 mL of a prewarmed (65°) solution containing 1.25% SDS, 5 mM EDTA (pH 8) and salmon sperm DNA (0.4 mg/mL). Precipitation occurred after addition of KCl to a final concentration of 65 mM. Cells were sonicated with a sonic dismembrator (Fisher, model 300) for 5 sec at a setting of 35 using a 4 mm microtip, cooled on ice for 5 min, and pelleted by centrifugation at 1500 *g* at 4° for 10 min. The pellet was resuspended in a wash solution composed of 10 mM Tris–HCl (pH 8), 100 mM KCl and 1 mM EDTA containing salmon sperm DNA (0.1 mg/mL), and heated at 65° for 10 min. The wash procedure was repeated prior to solubilization of the pellet at 65° in 300 μL H_2O and addition of 5 mL of scintillation fluid. A baseline level of protein-linked DNA is commonly observed when using this assay.

Assays for membrane damage. The integrity of hepatoma cell membranes immediately after a 2-hr incubation with drug was assessed using trypan blue dye exclusion and the MTT tetrazolium dye assay [24].

Accumulation and retention of mitoxantrone. Accumulation and retention of mitoxantrone in the H-35 hepatoma cells were monitored after 2 hr of incubation with [^{14}C]mitoxantrone in complete medium. Incubation was terminated by placing culture dishes on ice and aspirating the medium, after which cells were washed twice with cold phosphate-buffered saline. Cells were gently scraped into cold phosphate-buffered saline using plastic policemen. Cell protein content was determined by the method of Bradford [32]. After centrifugation at 1500 *g* at 4°, the cell pellet was digested with 250 μL of 1 M KOH at 80° for 1 hr and neutralized using 1 M HCl; [^{14}C]mitoxantrone in the hepatoma cell was quantitated by liquid scintillation counting. For drug retention studies, cells were washed once with complete medium and incubated for an additional 2 hr in drug-free medium following the 2-hr exposure to mitoxantrone.

RESULTS

Influence of mitoxantrone on proliferation of the hepatoma cell. Mitoxantrone, an anthracenedione, is a more potent antiproliferative agent in the hepatoma cell than the anthracyclines, Adriamycin® and daunorubicin. The IC_{50} value for inhibition of cell proliferation by mitoxantrone (utilizing a 2-hr drug exposure followed by 72 hr in drug-free medium) was approximately 0.05 μM, similar to that reported in Chinese hamster ovary and human breast tumor cell lines [3, 13, 18]. The H-35 rat hepatoma cell was markedly less sensitive to daunorubicin and Adriamycin®, with IC_{50} values of 0.5 and 2.5 μM respectively. This differential drug sensitivity is consistent with the general observation of enhanced antiproliferative activity of mitoxantrone compared to the anthracycline antibiotics [1, 3, 4]. Mitoxantrone produced a G_2-M block in cell cycle progression, as previously demonstrated in other tumor cell lines [33, 34]. While the control cells showed a G_1-S to G_2-M distribution ratio of 68:32, hepatoma cells

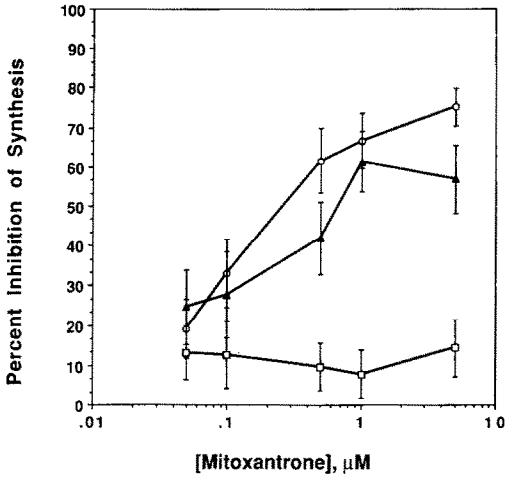

Fig. 1. Inhibition of macromolecular biosynthesis by mitoxantrone. Hepatoma cells were incubated for 2 hr with mitoxantrone at concentrations ranging from 0.05 to 5.0 μM. Drug was removed, and the rates of DNA (○), RNA (□), and protein (▲) syntheses were determined by measuring the incorporation of radiolabeled precursors into acid-precipitable material as described in Materials and Methods. Data are the means ± SE of 6–10 individual experiments. In control cells, thymidine incorporation averaged 1.14 ± 0.024 pmol/hr; leucine incorporation averaged 1.58 ± 0.38 pmol/hr; and uridine incorporation averaged 0.029 ± 0.005 pmol/hr.

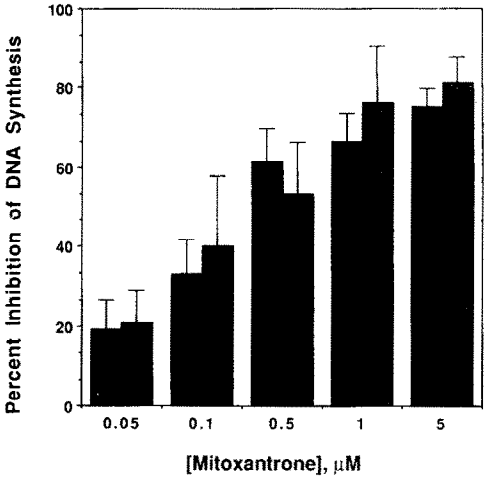

Fig. 2. Lack of recovery of DNA synthesis following a 2-hr incubation with mitoxantrone. H-35 rat hepatoma cells were incubated with mitoxantrone for 2 hr; drug was removed and cells were washed with medium prior to an additional 2-hr incubation in drug-free medium. DNA synthesis was monitored as described in Materials and Methods. At each drug concentration, the dark bar represents the percentage inhibition of DNA synthesis after a 2-hr incubation with mitoxantrone; the striped bar represents the percentage inhibition of DNA synthesis after a 2-hr recovery period in drug-free medium. Data are the means ± SE of 3–10 experiments. Using Students' *t*-test, there were no significant differences between inhibition of DNA synthesis before or after the 2-hr recovery period ($P < 0.9$ at 0.05, 0.1 and 0.5 μM; $P < 0.5$ at 1 and 5 μM). Incorporation of thymidine averaged 1.14 ± 0.024 pmol/hr in control cells and 0.86 ± 0.55 pmol/hr after 2 hr in fresh medium.

treated for 2 hr with 0.1 μM mitoxantrone showed a G_1-S to G_2-M distribution ratio of 33:67.

Alterations in macromolecular biosynthesis induced by mitoxantrone. Figure 1 shows the influence of a 2-hr exposure to mitoxantrone on DNA, RNA and protein syntheses in the H-35 rat hepatoma cell. The IC_{50} value for inhibition of DNA synthesis was 0.25 μM, and, for inhibition of protein synthesis, approximately 0.75 μM; drug effects on RNA synthesis were negligible.

In contrast to a partial reversal of the inhibition of DNA synthesis after termination of incubation with daunorubicin [27], inhibition of DNA synthesis induced by mitoxantrone in the H-35 rat hepatoma was *not* reversed after a 2-hr recovery period in drug-free medium (Fig. 2). This may be related, in part, to the retention of approximately 62% of accumulated mitoxantrone in the hepatoma cell after this recovery period.

Influence of mitoxantrone on DNA integrity. Figure 3 presents the relationship between the extracellular mitoxantrone concentration and the inhibition of cell growth (plotted as percent residual growth) as well as the induction of DNA cleavage; the latter is plotted as percent intact (double-stranded) DNA determined by the alkaline unwinding assay. While the trends of these parameters appear to be generally parallel, it is apparent that inhibition of tumor cell growth failed to demonstrate an absolute correspondence with induction of DNA strand breaks. There was no perceptible double-stranded DNA cleavage (as measured by neutral elution) at mitoxantrone concentrations below 0.5 μM (data not shown).

The DNA cleavage induced by mitoxantrone in the hepatoma cell appeared to be "protein-associated", i.e. DNA damage in the alkaline elution assay was not expressed in the absence of proteinase-K digestion (data not shown). These findings are indicative of drug interference with topoisomerase II and suggest stabilization of a topoisomerase II–DNA "cleavable complex" [31, 35].

Mitoxantrone was also observed to produce DNA–protein cross links, (Fig. 4), consistent with the induction of "protein-associated" DNA strand breaks mediated by drug interference with topoisomerase II [31, 35]. Although the SDS/K^+ coprecipitation assay may lack the sensitivity to reflect small changes in DNA–protein cross-linking activity which may be occurring between 0.01 and 0.1 μM mitoxantrone, cross-link production does appear to accompany the induction of DNA damage up through a mitoxantrone concentration of 1 μM. At 5 μM mitoxantrone, there was an unexpected decline in the extent of DNA–protein cross linking (discussed below).

Influence of free radical scavengers on the antiproliferative activity of mitoxantrone. The free radical scavengers catalase, DMSO, mannitol, and methanol, and the iron chelator, deferoxamine, failed to protect the H-35 rat hepatoma cell from growth inhibition produced by 0.1 μM mitoxantrone

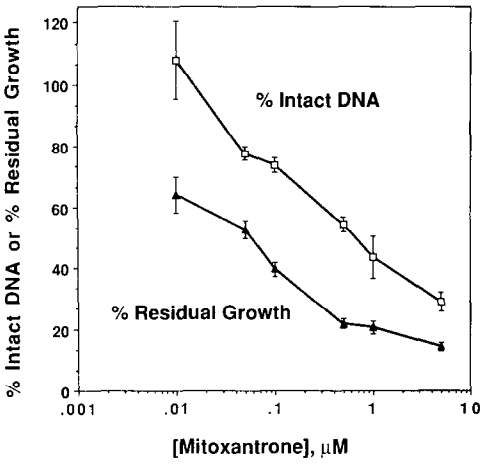

Fig. 3. Relationship between the extracellular mitoxantrone concentration and inhibition of cell growth or induction of DNA damage. Rat hepatoma cells in logarithmic growth in culture were incubated for 2 hr with mitoxantrone at various concentrations between 0.01 and 5.0 μM. Drug was decanted, and cells were washed with medium and permitted to proliferate for 72 hr. Inhibition of growth was determined using the MTT tetrazolium dye assay and is plotted as percent of residual growth. Data are the means ± SE of 8–45 individual experiments. DNA damage was assessed using the alkaline unwinding assay and is plotted as the percentage of intact (double-stranded) DNA. Data are the means ± SE of between 5 and 13 individual experiments. Dye absorbance for control cells averaged 0.442 ± 0.026 units. The average percent intact DNA in control (untreated) cells was 86.6 ± 2.8% in 13 individual experiments.

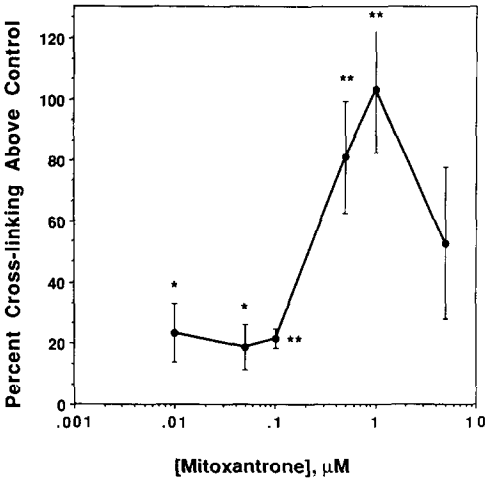

Fig. 4. Induction of DNA–protein cross links in the H-35 rat hepatoma. Cells detached from culture flasks were incubated for 2 hr with concentrations of mitoxantrone ranging between 0.01 and 5.0 μM. After removal of drug, cells were lysed with SDS and cell protein was precipitated with KCl as described in Materials and Methods. Data are the means ± SE of 3–11 experiments. The extent of DNA–protein cross linking was analyzed by comparison with controls [(*)$P < 0.05$; (**)$P < 0.001$]. DNA–protein cross linking at 5 μM did not differ significantly from control ($P < 0.10$). The baseline level of protein-linked DNA was 2.2 ± 0.5 pmol.

(Table 1). In fact, DMSO was found to significantly enhance the growth inhibitory effect of mitoxantrone at concentrations of 0.05 and 0.1 μM ($P < 0.01$ when compared to inhibition in the absence of DMSO), with no apparent enhancement at higher drug concentrations (Table 2).

Influence of DMSO on induction of DNA cleavage by mitoxantrone. The enhanced antiproliferative capacity of 0.05 and 0.1 μM mitoxantrone in the presence of DMSO was not a function of enhanced drug accumulation. At 0.05 μM mitoxantrone, the hepatoma cell accumulated 0.24 ± 0.05 pmol/μg cell protein (control) and 0.12 ± 0.04 pmol/μg (DMSO); at 0.1 μM mitoxantrone, these values were 0.62 ± 0.18 (control) and 0.39 ± 0.09 (DMSO).* Furthermore, treatment of hepatoma cells with DMSO did not alter the percent of accumulated drug retained after incubation of cells in drug-free medium (approximately 62%, data not shown). Despite the fact that DMSO did not enhance cellular accumulation or retention of mitoxantrone, DMSO pretreatment enhanced the capacity of mitoxantrone to produce DNA damage in the hepatoma cell at 0.05, 0.1 and 0.5 μM (Table 2). At 0.5 μM mitoxantrone, enhanced DNA damage was not accompanied by increased antiproliferative activity. DMSO alone produced an additional 80–90 rad equivalents of DNA damage. However, this additional baseline DNA damage does not account for the potentiation of drug-induced DNA cleavage in the presence of mitoxantrone.

Effects of mitoxantrone on membrane integrity and mitochondrial function. The MTT tetrazolium dye assay (which monitors mitochondrial function by

Table 1. Effect of free radical scavengers on inhibition of hepatoma cell growth by mitoxantrone

Pretreatment	% Reduction in cell number	
None	55.6 ± 5.8	
Catalase, 3000 units/mL	40.7 ± 13.8	($P < 0.40$)
DMSO, 1 M	70.2 ± 2.5*	($P < 0.02$)
Mannitol, 100 mM	53.7 ± 3.3	($P < 0.90$)
Methanol, 300 mM	62.9 ± 6.4	($P < 0.40$)
Deferoxamine, 10 mM	70.9 ± 14.0†	($P < 0.90$)

H-35 cells were preincubated for 1 hr with the indicated agents before exposure to 0.1 μM mitoxantrone. Incubation continued for an additional 2 hr before drugs were removed. Cells were permitted to proliferate for 72 hr prior to assessment of cell number using the MTT assay. Dye absorbance for control cells averaged 0.442 ± 0.026 units. Values for percent reduction in cell number relative to control are means ± SE for 3–11 experiments. Numbers in parentheses are P values derived from Students' *t*-test.

* Statistically significant.

† In this series of experiments, the percent inhibition of proliferation induced by mitoxantrone alone was 61.3 ± 12.0.

* The actual mitoxantrone concentrations for these drug uptake and retention studies, after recalculation, were 0.065 μM (0.05 μM desired concentration) and 0.325 μM (0.1 μM desired concentration). Statistical analysis indicated that cellular drug levels were not significantly different in the absence and presence of DMSO ($P < 0.02$).

Table 2. Potentiation of DNA damage in the hepatoma cell by DMSO

[Mitoxantrone], μM	DNA damage in rad equivalents	
	−DMSO	+DMSO
0	118 ± 5	203 ± 15
0.05	220 ± 7 (44.4 ± 4.9%)	461 ± 35 (64.3 ± 2.9%*)
0.1	298 ± 10 (55.8 ± 2.4%)	1064 ± 80 (70.2 ± 2.5%*)
0.5	1202 ± 32 (76.2 ± 1.5%)	4138 ± 213 (79.5 ± 2.6%)
1.0	3643 ± 239 (79.8 ± 1.6%)	ND† (83.2 ± 2.6%)
5.0	6863 ± 181 (85.8 ± 1.0%)	ND (90.0 ± 1.3%)

H-35 cells were preincubated for 1 hr with 1 M DMSO before addition of the indicated concentrations of mitoxantrone. Incubation continued for an additional 2 hr before DNA damage was assessed by the alkaline unwinding procedure. Data, expressed as rad equivalents of DNA damage, are the means ± SE of 5–13 experiments. P values for differences in DNA damage in the absence and presence of DMSO were less than 0.001 at all drug concentrations, as well as in the absence of mitoxantrone. Values in parentheses represent percent inhibition of hepatoma cell growth in the absence and presence of DMSO.

* Inhibition of growth was significantly higher ($P < 0.01$) in the presence of DMSO.

† Not determined.

assessing succinate dehydrogenase activity) was utilized to determine the effect of mitoxantrone on cell viability (and mitochondrial function) immediately following a 2-hr incubation with drug—as in the assays for macromolecular biosynthesis, protection by free radical scavengers, induction of DNA strand breaks and DNA–protein crosslinks. There was little apparent cellular injury at mitoxantrone concentrations below 5 μM; at 1 μM mitoxantrone, a 13.4 ± 3.9% reduction in dye absorbance was observed; at 5 μM mitoxantrone, the highest concentration of drug used, a 32.6 ± 11.9% reduction in dye absorbance was observed as compared to control cells. Studies assessing trypan blue exclusion (i.e. membrane integrity) indicated that cell viability in mitoxantrone-treated cells did not differ significantly from controls over the concentration range of 0.05 to 5 μM. For controls, the percentage of nonviable cells was 9.9 ± 2.6%; at 5 μM mitoxantrone, the percentage of cells which failed to exclude trypan blue was 8.9 ± 6.2%.

DISCUSSION

In the H-35 rat hepatoma, mitoxantrone induces protein-associated DNA strand breaks. DNA cleavage induced by mitoxantrone has been shown to be associated with inhibition of the enzyme topoisomerase II [15], which is involved in DNA replication [36]. Studies in Chinese hamster ovary cells and L1210 leukemia [17, 18] have also indicated that mitoxantrone induces exclusively protein-associated DNA damage (consistent with drug effects at the level of topoisomerase II). However, Bowden *et al.* [16] reported induction of both protein-associated and non-protein-associated damage in the L1210 leukemia.

Induction of DNA strand breaks was accompanied by production of DNA–protein cross links. At a concentration of 5 μM mitoxantrone, DNA–protein cross linking failed to correspond with induction of DNA damage; this observation suggests that DNA damage induced by mitoxantrone in tumor cells may differ from that initiated by classical inhibitors of topoisomerase II [15, 37], and that other biochemical lesions may mediate the antiproliferative effects of this antineoplastic drug. Alternatively, since 5 μM mitoxantrone represents a supra-maximal concentration of drug, reduced cell viability (and the possible release of lysomal enzymes) may account for the reduction of DNA–protein cross linking. Hsiang and Liu [38] have demonstrated previously a decline in DNA–protein cross linking at supra-maximal concentrations of VM-26 and 4′-(9-acridinylamino)-methanesulfon-*m*-anisidide (m-AMSA) in the L1210 leukemia cell.

As reported by Cohen *et al.* in L1210 cells [17], mitoxantrone induced a low level of DNA damage in the rat hepatoma. The induction of protein-associated DNA damage failed to correspond directly with the antiproliferative activity of mitoxantrone. In particular, between 0 and 0.01 μM mitoxantrone, inhibition of cell growth was not accompanied by induction of DNA strand breaks. It is important to note that Bowden *et al.* [16] also failed to observe a direct relationship between protein–associated strand breaks and mitoxantrone toxicity but did observe that mitoxantrone does not induce DNA breaks in isolated nuclei. Furthermore, Epstein and Smith [22] reported that estrogen could enhance mitoxantrone-induced DNA damage in breast cancer cells without a corresponding increase in drug toxicity.

DMSO enhanced both antiproliferative activity and DNA damage induced by low concentrations of mitoxantrone (0.05 and 0.1 μM) without a concomitant increase in cellular levels or cellular retention of drug. In contrast, at higher drug concentrations, increased DNA damage appeared to be dissociable from antiproliferative activity, again raising the question of the relationship between this lesion and drug action. These findings of increased

DNA cleavage in the presence of DMSO are similar to those reported by Pommier *et al.* [39] for Adriamycin®, 5-iminodaunorubicin and m-AMSA in L1210 leukemia cells. Pommier *et al.* suggested that alterations in DNA unwinding and topology induced by DMSO could modify the susceptibility of DNA to enzyme-mediated cleavage. However, there investigators did not observe a corresponding enhancement of cytotoxicity with DNA cleavage.

Although mitoxantrone inhibited DNA synthesis in the hepatoma cell, growth was approximately 5-fold more sensitive to inhibition by mitoxantrone than was DNA synthesis (IC_{50} values of 0.05 and 0.25 μM respectively). This dissociation between the inhibition of DNA synthesis and antiproliferative activity has been reported previously [2]. Consequently, the similarity in the concentration-dependent profiles for inhibition of growth and DNA synthesis may indicate that the inhibition of DNA synthesis is a *consequence* rather than a *cause* of the observed effects of mitoxantrone on cell proliferation. Furthermore, the fact that inhibition of DNA synthesis was sustained for at least 2 hr after drug was removed from the incubation medium is consistent with reports that non-reversible inhibition of DNA synthesis tends to be a function of DNA damage [40].

Inhibition of protein synthesis by mitoxantrone has been associated with nucleolar lesions and condensation of nucleic acids [21]. However, as is the case with DNA synthesis, the dissimilarity in IC_{50} values for inhibition of protein synthesis and inhibition of cell proliferation suggests that these two functions are not causally related.

The apparent capacity of mitoxantrone to induce "protein-associated" DNA strand breaks in the hepatoma cell is in marked contrast to the limited induction of DNA cleavage by daunorubicin [27]. It is possible that the extensive retention of mitoxantrone in this tumor cell (62% of drug as compared with 32% for daunorubicin) [27] accounts for enhanced DNA cleavage as well as the lack of reversibility of DNA synthesis. The difference in drug retention may be due to dissimilar rates of transport and degree of binding to DNA. In addition, topoisomerase II in the hepatoma cell may be differentially sensitive to the anthracycline antibiotics and the anthracenediones. This possibility is currently under investigation in our laboratory.

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