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# Influence of Mitoxantrone on Nucleic Acid Synthesis on the T-47D Breast Tumor Cell Line

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Mitoxantrone exerts growth inhibitory effects, suppresses [$^3$H]-thymidine as well as [$^3$H]-uridine incorporation, and induces ultrastructural alterations in T-47D human breast tumor cells. At low concentration ($10^{-9}$M) the drug induced little effect on cell proliferation; cell growth kinetics were inhibited at a concentration of $10^{-5}$M. [$^3$H]-thymidine and [$^3$H]-uridine incorporation declined rapidly at the concentrations tested ($10^{-9}$, $10^{-7}$, and $10^{-5}$ M), revealing a potent effect on metabolic activity of the cultured cells. The sharpest decline in DNA and RNA synthesis occurred within the first 2 hr of drug treatment. Serial ultrastructural examinations indicated definitive alterations in chromatin structure, disintegration of nucleolar components as early as 2 hr after drug treatment, and complete segregation of nucleolar components following 8-hr exposure to concentrations of the drug between $10^{-5}$ and $10^{-7}$ M. A distinct increase in the density of mitochrondrial matrix was evident. The in vitro data presented in this report demonstrate the growth inhibitory and antimetabolic effects of mitoxantrone on human breast tumor cells and suggest that the drug may be a promising antitumor agent.

**Key words: mitoxantrone, nucleic acid synthesis, breast tumor cell line, uridine, thymidine**

Mitoxantrone(1,4-dihydroxy-5,8-bis{{{2-2[(2-hydroxyethyl)amino]ethyl}-amino}}-9,10-anthracenedione dihydrochloride) (NSC 301739) is a new synthetic anthraquinone compound currently being evaluated for antitumor activity in phase I-II trials in humans [1, 2]. It has been shown that mitoxantrone exerts pronounced antitumor activity both in vivo and in vitro [3-6]. The drug binds to both DNA and RNA in nuclear chromatin, with a preference for the nucleolus, and to cytoplasmic RNA [4]. It has been suggested that the drug intercalates into double-stranded nucleic acid [4, 5, 7] which in turn may impair DNA transcription and RNA processing [3, 4]. Mitoxantrone blocks cell division at the G2 phase, inhibits colony formation in soft agar, stops RNA and DNA synthesis, and induces alterations in the chromatin structure [3, 4]. It has been reported that mitoxantrone is less cardiotoxic than adriamycin [8].

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In this study, we have investigated the sequential influence of mitoxantrone on DNA and RNA synthesis, cell proliferation, and ultrastructure in T-47D human breast tumor cells. Its effects on nucleic acid synthesis and cell ultrastructure reveal function-structure alterations induced by the drug.

## MATERIALS AND METHODS

### Cell Culture Conditions

Exponentially growing T-47D human breast tumor cells (80th passage) were maintained by plating $1 \times 10^5$ cells per well in multiwell plates (16.4-mm diameter, Costar Plastic [Cambridge, MA]) containing Eagle minimum essential medium (EMEM) supplemented with 10% fetal calf serum (MA Bioproducts, Walkersville, MD) 1 μg/ml cortisone, 5 μg/ml insulin (Sigma Chemical Corp, St Louis, MO), 1 μg/ml prolactin (National Institute of Arthritis, Metabolism, and Digestive Diseases, Bethesda, MD), 10,000 units/ml penicillin, 10,000 units/ml streptomycin, and 25 μg/ml fungizone. Cells were grown for 2–3 days before treatment with the drug in a humidified 5% $CO_2$-95% air atmosphere.

### Drug Treatment

A stock solution of mitoxantrone (American Cyanamid Company, Lederle Laboratories Division, Pearl River, NY) was prepared in sterile buffered 0.9% NaCl solution and subsequent dilutions for incubation with cells were made with supplemented EMEM.

To evaluate the effects of mitoxantrone on DNA and RNA synthesis and cell ultrastructure, exponentially growing monolayer cultures were treated with the drug at $10^{-5}$M, $10^{-7}$M, and $10^{-9}$M for 2 hr. For serial analysis of these drug-induced effects, cultured cells were exposed to $10^{-7}$ M mitoxantrone for 1, 2, 4, 8, and 24 hr. After washing the individual wells three times in $Ca^{++}$-$Mg^{++}$-free phosphate-buffered saline, the attached cells were trypsinized in a trypsin-Versene mixture (MA Bioproducts, Walkersville, MD) and cells were counted using a Coulter counter. The number of viable cells, as defined by the trypan blue dye exclusion test, was determined by hemocytometer count for each culture and the relative number of viable cells/ml was plotted as a percentage of control values.

### Nucleic Acid Synthesis In Vivo

After drug treatment cells were washed twice with EMEM to remove the drug and incubated with 2 μCi/ml [$^3$H]-thymidine (6.7 Ci/mmole, New England Nuclear, Boston, MA) or 3 μCi/ml [$^3$H]-uridine (26.4 Ci/mmole, New England Nuclear, Boston, MA). At designated intervals (ie, 2, 4, 6, 8, and 24 hr) the cultures were washed with EMEM, trypsinized, and collected by centrifugation at 600g for 2 min. The cells were lysed in 0.2 μl of a 0.05% solution of Zaponin (10 gm/liter saponin and 8.3 gm/liter sodium chloride, Curtin Matheson Scientific, Inc., Houston, TX) in EMEM. The lysate was transferred to 2GF/C Whatman filter papers (Fisher Scientific, Cincinnati, OH), washed three times with cold 10% trichloroacetic acid (Sigma Chemical Corp., St Louis, MO) and twice with 95% ethanol. The filters were then dried, placed into vials containing 3 ml of Aquasol-2 (New England Nuclear), and the radioactivity was determined in a Beckman Model LS7500 liquid scintillation counter. The background counts were subtracted from all values. For each data point, $5 \times 10^5$ cells/ml were used and all experiments were repeated three times.

### Transmission Electron Microscopy

For ultrastructural analysis, cells were collected and pelleted in 1.5-ml conical microcentrifuge tubes, then fixed in 1% glutaraldehyde-3% paraformaldehyde for 1 hr at 4°C. After fixing, the cells were washed overnight in 1% cacodylate buffer and postfixed in 1% osmium tetroxide for 2 hr, dehydrated in ascending concentrations of ethanol, and embedded in Araldite 502. Cells were then sectioned, stained with uranyl acetate and lead citrate, and examined on a Philips 300 electron microscope.

## RESULTS

### Inhibition of Cell Growth in Monolayer Culture Following Treatment With Mitoxantrone

The ability of mitoxantrone to inhibit cell growth over a 24-hr period was determined at concentrations of $10^{-5}$ M, $10^{-7}$ M, and $10^{-9}$ M and the results are shown in Figure 1. Population doubling time at a seeding density of $5 \times 10^4$ cells/well in untreated cultures was approximately 24 hr. The number of cells at the initiation of experiments was $1 \times 10^5$ cells/well. After 24-hr treatment with $10^{-5}$ M mitoxantrone, the number of cells remained unchanged, while doubling of the cell population was observed in untreated samples. At $10^{-9}$ M, mitoxantrone exerted no significant effect on cell growth. While growth inhibitions at $10^{-9}$ M and $10^{-7}$ M were 10 and 25%, respectively, no growth was achieved at a drug concentration of $10^{-5}$ M.

### Influence of Mitoxantrone on Nucleic Acid Synthesis

**DNA.** The effect of mitoxantrone on DNA synthesis in T-47D human breast tumor cell line is presented in Figures 2 and 3. A sharp decrease in the rate of

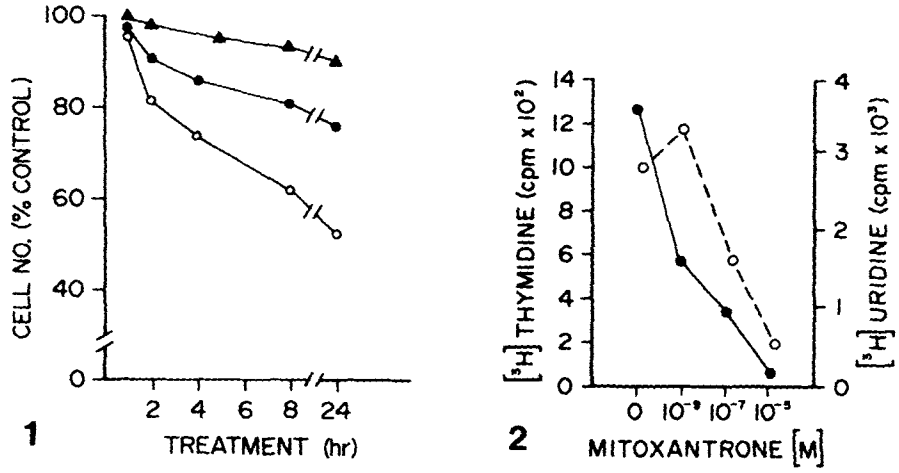


Fig. 1. Dose-response curve of T-47D human breast tumor cells treated with $10^{-9}$ M (▲), $10^{-7}$ M (●), and $10^{-5}$ M (○) mitoxantrone. Each point represents the average response of triplicate plates of cells. Standard deviation from the mean within each point is less than 10%.

Fig. 2. The effect of different concentrations of mitoxantrone on DNA synthesis (●) and RNA synthesis (○) after 2-hr exposure to the drug.

incorporation of [$^3$H]-thymidine following 2-hr treatment with $10^{-5}$–$10^{-9}$ M mitoxantrone and incubation of cells with 2 $\mu$Ci [$^3$H]-thymidine for 45 min was observed (Fig. 2). The rate of thymidine incorporation at $10^{-5}$ M was 3.5% and at $10^{-9}$ M about 40% of control values. The time-dependent effects of various concentrations of mitoxantrone on the incorporation of [$^3$H]-thymidine into acid-precipitable material by the cultured cells are presented in Figure 3. The results demonstrate a clear dose- and time-dependent inhbition of DNA synthesis during a 24-hr treatment period. The most conspicuous effects of the drug, regardless of the concentration, were observed in the first 2 hr of exposure, after which the rate of incorporation decreased gradually and leveled off beyond 4 hr (Fig. 3). The data also indicated that 24 hr of treatment with $10^{-5}$ M mitoxantrone completely abolishes DNA synthesis.

**RNA.** To determine whether mitoxantrone affected RNA synthesis, T-47D cells were treated for 2 hr with different concentrations of the drug. The rates of incorporation of [$^3$H]-uridine following 2-hr exposure to $10^{-5}$ M, $10^{-7}$ M, or $10^{-9}$ M mitoxantrone and 45-min incubation with [$^3$H]-uridine are seen in Figure 2. At $10^{-9}$M there was a slight increase in incorporation of [$^3$H]-uridine. However, as the drug concentration increased, there was a linear decrease in incorporation.

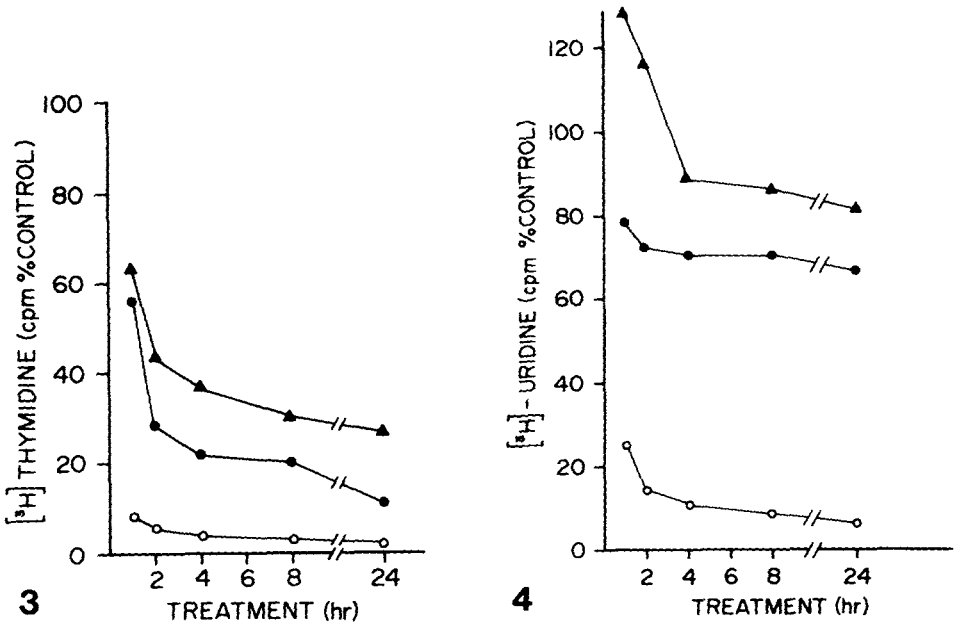


Fig. 3. Suppression of [$^3$H]-thymidine incorporation in the cultured cells treated with $10^{-9}$ M (▲), $10^{-7}$ M (●), and $10^{-5}$ M (○) mitoxantrone during sequential treatment for 24 hr. Before incubation of cells with [$^3$H]-thymidine for 45 min, cells were washed twice with EMEM (1 ml each). Standard deviation from the mean for each point is less than 10%. For each data point $5 \times 10^5$ cells were incubated for 45 min with the labelled thymidine.

Fig. 4. Inhibition of RNA synthesis in the cultured cells treated with $10^{-9}$ M (▲), $10^{-7}$ M (●), and $10^{-5}$ M (○) mitoxantrone during serial treatment for 24 hr. For each data point $5 \times 10^5$ cells were incubated for 45 min with [$^3$H]-uridine.

Measurements on the cultured cells over 24 hr revealed a rapid decrease in incorporation of [$^3$H]-uridine during the first 4 hr of treatment with $10^{-5}$ M and $10^{-7}$ M concentrations of the drug, followed by nearly constant low levels during the subsequent 18 hr of exposure. At $10^{-9}$ M an increase in incorporation during the first 2 hr of treatment was observed (Fig. 4). This slight elevation was, however, transient since at longer intervals [$^3$H]-uridine incorporation declined.

## Ultrastructure of T-47D Breast Tumor Cells Before and After Mitoxantrone Treatment

Untreated cells possess a large spherical to ovoid nucleus, one or two distinct nucleoli containing spherical electron-dense particles (granular component), well-defined fibrillar centers (nucleolar organizers), and fibrillar components (Fig. 5). In addition to numerous mitochondria, there are several vacuoles, Golgi complexes, segments of rough-walled endoplasmic reticulum (RER), a few bundles of tonofilaments, small vesicles, and lipid inclusions.

Two hours after the addition of $10^{-5}$ M mitoxantrone early disintegration and separation of granular and fibrillar components of the nucleoli were evident and the nucleolar periphery appeared to merge with the surrounding nucleoplasm (Fig. 6). Other nuclear changes included clumping of chromatin and the appearance of hetero-

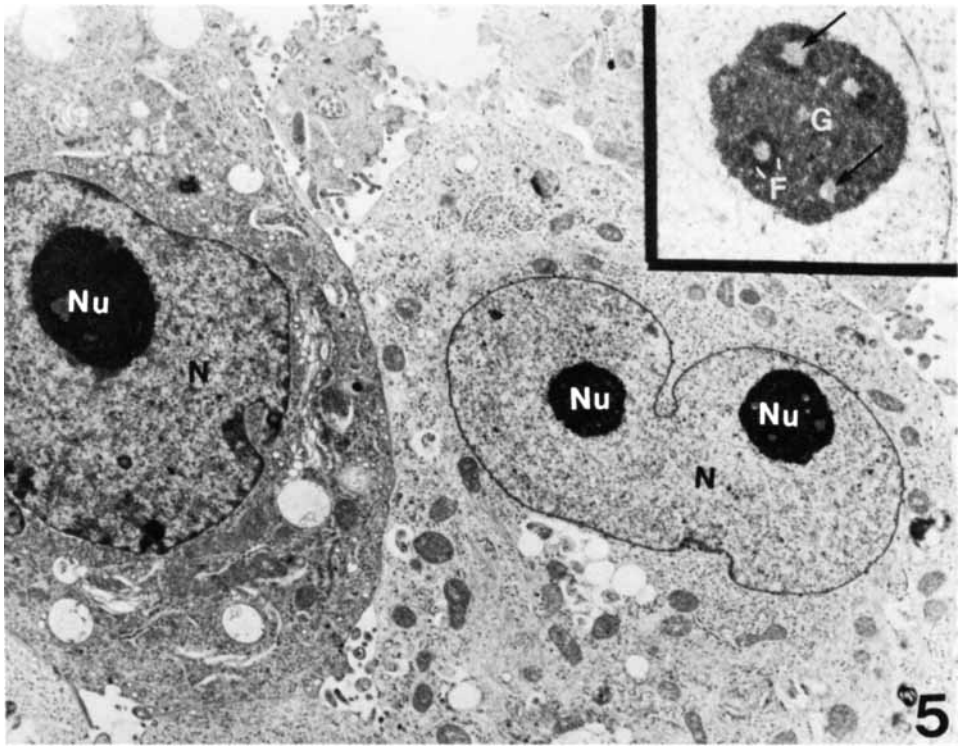


Fig. 5. A cluster of T-47D human breast tumor cells from a nontreatment control. Note the presence of large nuclei (N) with dispersed euchromatin and nucleoli (Nu) containing nucleolar organizers (arrows) and granular (G) and fibrillar (F) components (insert). ×4,800, ×12,500.

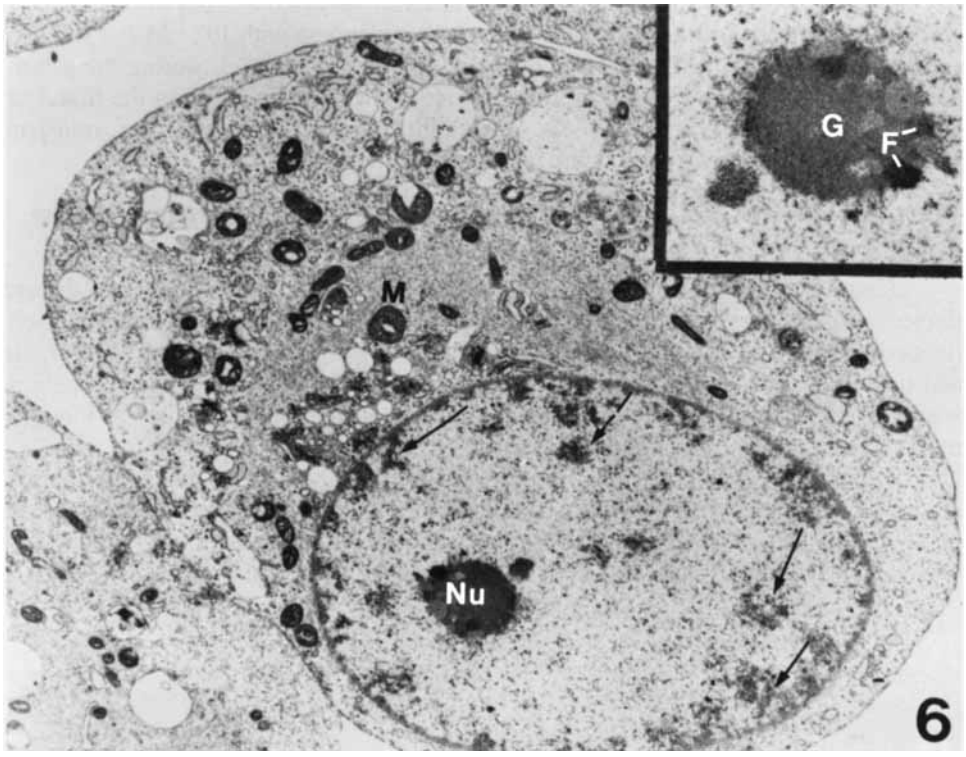


Fig. 6. A T-47D human breast tumor cell treated with $10^{-5}$ M mitoxantrone for 2 hr. Note clumping of chromatin, appearance of heterochromatin in the interior and periphery of the nuclei (arrows), and disintegration of granular (G) and fibrillar (F) components of the nucleoli (insert). The increase in density of mitochrondria (M) is also evident. ×6,900, ×11,500.

chromatin aggregates. In the cytoplasm, an increase in the density of mitochrondrial matrix, disorganization of mitochondrial cristae, and dilation of rough-walled endoplasmic reticulum were evident. After 6 hr, the most conspicuous drug-induced effects were in the nucleolus. Disintegration and segregation of the fibrillar and granular components of the nucleolus were seen (Fig. 7). Central and peripheral heterochromatin were clearly distinguished from the interchromatin material and a slight dilatation of nuclear envelopes was visible. Small blebs on the plasma membrane were occasionally seen (Fig. 7).

After 24-hr exposure of cells to $10^{-5}$ M mitoxantrone, nucleoli were small, compact, and highly electron-dense. Segregation of nucleolar components and the absence of nucleolar organizers were apparent (Fig. 8). In addition, separation of the perinucleolar chromatin from the nucleolar surface created an electron-lucent halo around the nucleolus (Fig. 8). The mitochondrial matrix remained dense and disorganization of cristae persisted. Serial examination of cells treated with $10^{-7}$ M mitoxantrone for 24 hr showed dispersion of euchromatin and distinct separation of nucleolar components. At $10^{-9}$ M mitoxantrone only minor disruption of the fibrillar and granular components of the nucleolus was evident after similar treatment. The

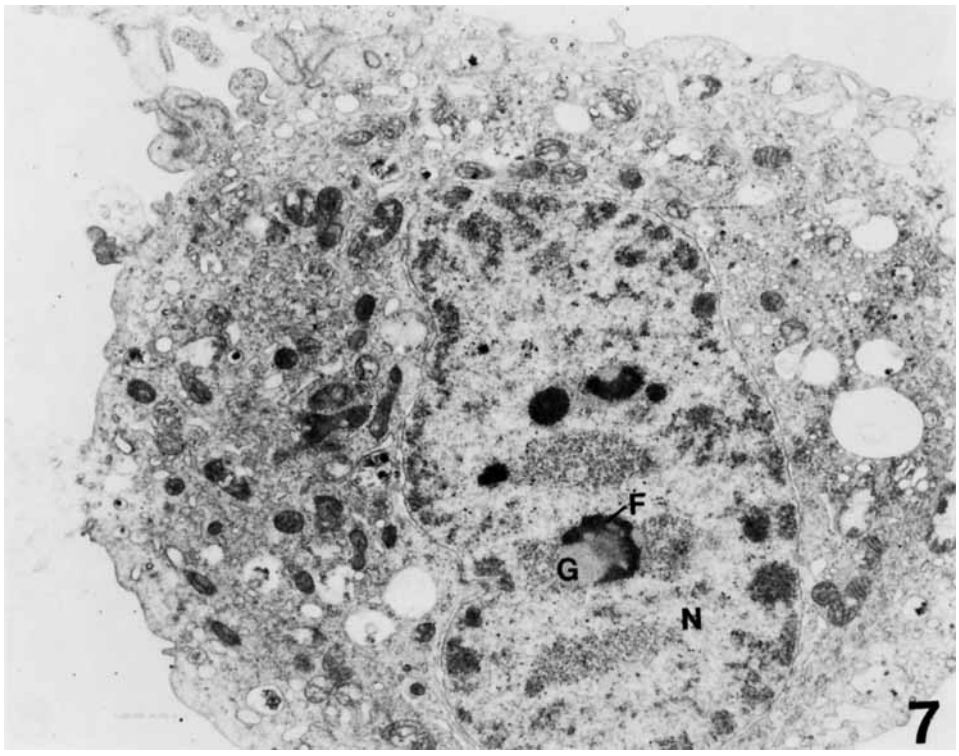


Fig. 7. Appearance of T-47D human breast tumor cell following 8-hr treatment with $10^{-5}$ M mitoxantrone. Note the segregation of fibrillar (F) and granular components (G) of the nucleoli and presence of prominent heterochromatin clumps in the periphery of the nuclei (N). Appearance of small blebs on the plasma membrane as also notable. ×7,800.

only cytoplasmic change seen at either drug concentrations was a slight increase in electron density of the mitochondria.

## DISCUSSION

In this study the growth-inhibitory and antimetabolic effects of mitoxantrone on cell growth, nucleic acid synthesis, and the ultrastructure of T-47D breast tumor cells in monolayer culture were determined. About 10% growth inhibition was achieved after 24 hr of exposure to $10^{-9}$ M mitoxantrone. However, exposure to concentrations in excess of $10^{-7}$ M for 24 hr increased growth-inhibitory rates. At $10^{-5}$ M (5.26 $\mu$g/ml) mitoxantrone exposure total inhibition of cell growth was observed.

The data presented here indicate that at the concentrations used, the drug suppresses cell proliferation and DNA synthesis. At the lowest concentration ($10^{-9}$ M), 24-hr exposure to the drug reduced DNA synthesis to about 25% that of controls. This effect was progressively accentuated at $10^{-7}$ M and $10^{-5}$ M, with reduction to 12% and 0.5%, respectively. This is presumably a result of intercalation of the drug into the DNA molecule [4, 5, 7]. An interesting finding was that most of the effect of

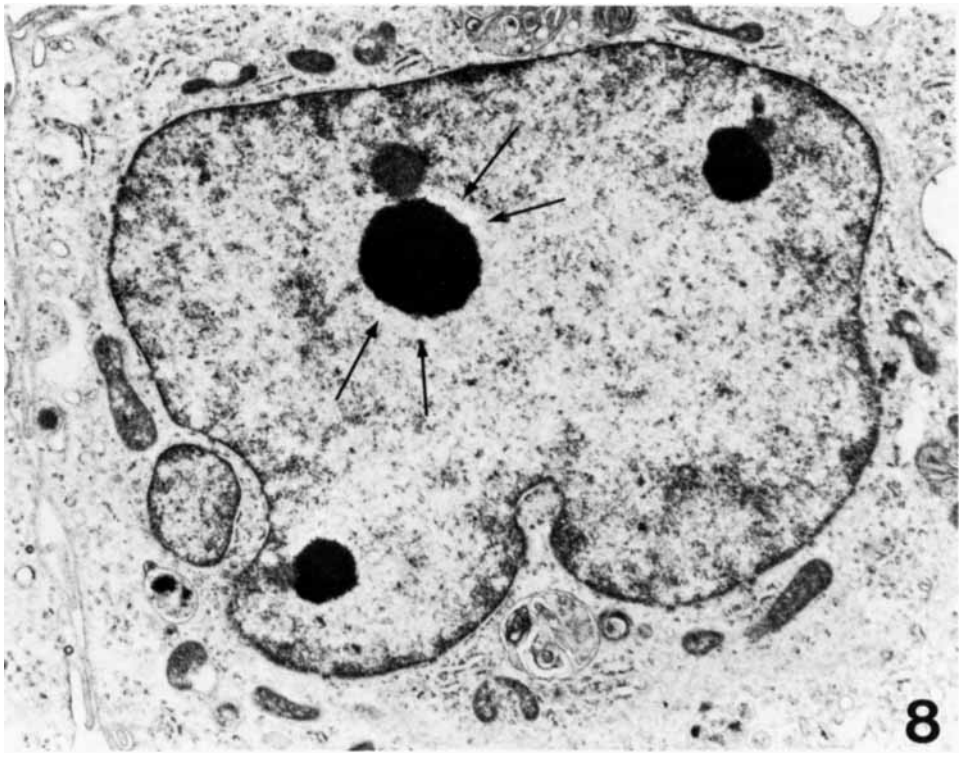


Fig. 8. Distinct segregation of nucleolar components and appearance of compact and dense nucleoli in cells treated for 24 hr with $10^{-5}$ M mitoxantrone. Presence of an electron-lucent halo around the surface of nucleoli and perinucleolar chromatin is evident (arrows). Mitochrondrial matrix remains dense. ×14,700.

the drug on DNA synthesis occurred with 2 hr of treatment. It has been shown that in addition to inhibiting DNA and RNA synthesis, most DNA-intercalating agents induce single-strand cleavage in DNA and associated DNA-protein cross links. In addition, alteration of DNA supercoiling and increased susceptibility of single-stranded DNA to nuclease attack are usually associated with intercalation [9, 10].

The mechanism by which mitoxantrone arrests cells at G2 phase of the cell cycle [3] and inhibits the cell proliferation is not known but may resemble that observed for adriamycin [11–13] in which low concentrations of the drug inhibit the cell division-dependent RNA synthesis required for the synthesis of division-dependent protein [11, 12] and high concentrations induce chromosomal aberrations including chromosome stickiness, chromatid exchange, breaks, and gaps [11]. Such results indicate that there is a good correlation between damage to the genome and the inhibition of cell cycle progression after adriamycin treatment. A similar mechanism of action and also the possibility of interference of the drug with the mitotic spindle has been proposed for a mitoxantrone analog, 9,10-anthracenedione-1,4-bis-{{2{(2-hydroxyethyl)amino}-ethyl}-diacetate (ANT) [14, 15].

Consistent with the inhibition of DNA synthesis, concentrations of the drug from $10^{-5}$ M to $10^{-7}$ M suppressed RNA synthesis. However, following treatment with $10^{-9}$ M mitoxantrone for 2 hr, incorporation of labelled uridine was slightly increased although not statistically significant. This result is consistent with a previous report [3] in which a slight enhancement of uridine incorporation was observed following exposure of Friend leukemia cells to 0.001 $\mu$g/ml of dehydroxyanthraquinone (DHAQ), an analog of mitoxantrone. The exact mechanism for this effect is not known. A rapid reduction in uridine incorporation during the first 2 hr of exposure to $10^{-5}$–$10^{-7}$ M mitoxantrone indicates the potent inhibitory effect of the drug on transcription of DNA, processing of RNA and RNA polymerases, and perhaps translation of the genetic information to protein synthesis. While a decrease in the rate of RNA synthesis has been observed here and reported elsewhere [3, 5, 7], it is interesting to note that a marked increase in cellular RNA has been observed in different mammalian cells treated with analogs of mitoxantrone, DHAQ [3], or ANT [14]. A similar increase in RNA has been reported when progression of the cell cycle in stimulated lymphocytes or in Chinese hamster ovary cells was prevented by hydroxyurea and 5-fluorodeoxyuridine [16, 17]. The increase in cellular RNA in the presence of such chemical compounds may be the result of continued RNA synthesis in the absence of cell proliferation (accumulation of cells in the G2 phase of the cell cycle) [see 3, 14].

The ultrastuctural observations in this study show prominent nuclear alterations and, specifically, a series of nucleolar changes that with higher doses and longer treatment duration lead to complete separation of the nucleolar components. The clumping of heterochromatin in nuclei after exposure to $10^{-5}$–$10^{-7}$ M mitoxantrone resemble those of Friend leukemia cells treated with the mitoxantrone analog ANT [15] and the recently reported effect of adriamycin on cultured mammary tumor cells [18]. The alterations of nucleolar structure presented here resemble those reported in studies of liver, cardiac, and skeletal muscle of the rat after in vivo treatment with adriamycin or carminomycin [19, 20] and correlate well with the ultrastructural changes seen after exposure of cultured Novikoff hepatoma cells to adriamycin, carminomycin, or marcelomycin [21]. While the mechanism for the segregation of nucleolar components in mitoxantrone-treated cells is unclear, it might resemble those which have been described for anthracyclines and actinomycin D [21–25]. Binding of such drugs to the DNA which contains the rRNA cistrons (rDNA) and nucleolar deoxyribonucleoproteins (rDNP) might contribute to the segregation of fibrillar and granular components of the nucleoli [21, 24, 25]. The condensation of the rDNP and its asymmetrical localization has been demonstrated by Ebstein [23] using [$^3$H]-actinomycin D autoradiography. Recher et al [22], using a human cell line derived from a metastatic carcinoma of the cervix, have also proposed that the primary effects of actinomycin D are condensation and retraction of the nucleolar DNP.

The data presented in this report demonstrate a preferential effect of mitoxantrone on chromatin structure, and specifically on the nucleolus. This correlates well with the biochemical data of Kapuscinski et al [4], who reported the accumulation of a mitoxantrone analog (DHAQ) in nucleoli and a high affinity of the drug for DNA. Such information combined with evidence for increased RNA accumulation, intercalation of the drug into DNA and double-stranded (HnRNA or rRNA) regions of RNA

[3], plus the suppression of RNA synthesis reported here strongly imply inhibitory effects of mitoxantrone on transcription of DNA and processing of RNA.

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