Light, Fluorescent, and Electron Microscopic Analysis of Cultured Breast Tumor Cells (T-47D) Treated with 9,10-Anthracenedicarboxaldehyde Bis[(4,5-dihydro-1H-imidazol-2-yl)hydrazone] Dihydrochloride

Michael T. Tseng¹ and Ahmad R. Safa²

Department of Anatomy and the J. Graham Brown Regional Cancer Center, University of Louisville Health Sciences Center, Louisville, Kentucky 40292

ABSTRACT

The influence of bisantrene on T-47D human breast tumor cells was assessed by colony-forming assay in soft agar and by light, fluorescence, and electron microscopy. Test solutions of bisantrene solubilized in distilled water or dimethyl sulfoxide were added to cultures at final concentrations between 0.01 and 60 μg/ml. Brightly fluorescent particles appeared in a concentration-dependent fashion after cultures were treated with water-soluble bisantrene at concentrations greater than 0.1 μg/ml. Similar fluorescent crystals appeared in culture media when concentrations of the dimethyl sulfoxide-dissolved drug exceeded 10 μg/ml. Clonogenic survival as defined by soft agar assay indicated significant reproductive impairment in cells treated with concentrations greater than 1 μg/ml (p < 0.01). Nuclear and cytoplasmic fluorescence was evident in treated cells. Cells that survived 24-hr drug treatment displayed round nuclei with watery nucleolus when examined under the light microscope. Under the electron microscope, nuclei of these cells revealed fragmentation of the nucleolar complex and a highly electron-lucent nucleolus presumably either an acute drug reaction at the site of injection or a specific bisantrene-induced cellular reaction. Such localized electron-rich structure (1).

INTRODUCTION

Bisantrene, 9,10-anthracenedicarboxaldehyde bis[(4,5-dihydro-1H-imidazol-2-yl)hydrazone] dihydrochloride, is a "second generation" intercalating anticancer compound currently undergoing Phase II trials. It exhibits tumoridical effects similar to Adriamycin but without cardiotoxicity (2). Inhibition of DNA and RNA synthesis is compatible with its planar, tricyclic, electron-rich structure (1).

The main toxic effects in human patients receiving bisantrene have been reversible leukopenia and local cutaneous reactions (19, 21). Cellulitis in the infused extremities of a few patients given bisantrene at doses exceeding 60 mg/sq m has signified presumably either an acute drug reaction at the site of injection or a specific bisantrene-induced cellular reaction. Such localized responses may have resulted from the presence of precipitated drug at the injection sites. Recently, evidence for intravascular deposition of the drug in humans and rabbits and for its possible implication in the induction of phlebitis has been reported (15).

To test such a hypothesis in vitro, cultured mammary tumor cells were exposed to media containing soluble and precipitated bisantrene. The surviving cells were evaluated by clonogenic assay in soft agar and were examined by visible light, fluorescence, and electron microscopy to localize subcellular effects. Results are presented and discussed in this paper.

MATERIALS AND METHODS

Cell Culture and Drug Treatment. T-47D human breast tumor cells were seeded at a density of 5 × 10⁵ cells/well in multiwell culture plates (16.4 mm, Costar plastic) containing Eagle’s minimal essential medium supplemented with 10% fetal calf serum, hormones, and antibiotics (16) and were maintained in a humidified 5% CO₂-95% air atmosphere at 37°C. Two to 3 days later, cultures reached 50 to 70% confluence and were used for this study. Bisantrene (Lederle, Pearl River, N. Y.) was first dissolved in either sterile distilled water or DMSO.³ Appropriate dilutions with supplemented Eagle’s minimal essential medium to final concentrations between 0.01 and 60 μg/ml were made prior to addition to test cultures. In all cases, the DMSO concentration in the drug-containing growth medium was less than 1%. Twenty-four hr later, monolayer cultures were washed 3 times in Ca²⁺- and Mg²⁺-free phosphate-buffered saline and examined with an inverted microscope before removal from culture wells by a trypsin-Versene mixture and counting in a Coulter Counter. The viability of cells in treated cultures and controls was checked by the trypan blue dye exclusion test. Drug uptake by the T-47D cells was assessed by fluorescence microscopy using a 510 M extinction filter.

Clonogenic Assay. The single-cell suspension was adjusted to a final concentration of 5 × 10⁶ cells/ml in the presence of 10⁻³, 10⁻⁴, or 10⁻⁶ M bisantrene. Cells were incubated with or without bisantrene for 1 hr at 37°C. The colony-forming assay used in this study has been described previously (9, 10). In brief, cells to be tested were suspended in 0.3% agar (Difco Laboratories, Detroit, Mich.) in Connaught Medical Research Laboratories Medium 1066 supplemented with 15% horse serum (M. A. Bioproducts, Walkersville, Md.), insulin (0.5 μg/ml), prolactin (1 μg/ml), penicillin (10,000 units/ml), streptomycin (10,000 units/ml), and Fungizone (25 μg/ml) to provide a final cell density of 5 × 10⁶ cells/ml. One ml of this suspension was transferred by pipet into a 35-mm Petri dish containing 1 ml of 0.5% bactoagar (Difco) in enriched McCoy’s Medium 5A. Cultures were set up in triplicate and incubated at 37°C in a humidified 5% CO₂-95% air atmosphere.

Colonies containing 30 or more cells appeared in about 14 to 21 days, at which time, the number of colonies and treated plates was counted using an inverted-phase microscope.

Electron Microscopy. For ultrastructural analysis, cells were collected and pelleted in 1.5-ml conical microcentrifuge tubes, fixed in glutaraldehyde-paraformaldehyde fixative, and postfixed in 1% aqueous osmium tetroxide as described earlier (20). Thin sections were cut on a Porter-Blum MT-2 ultramicrotome and stained with uranyl acetate and lead citrate before being examined in a Philips 300 electron microscope.

RESULTS

Solubility of Bisantrene in Culture Medium. Bisantrene dissolves readily in either sterile water or DMSO. In cultures treated with aqueous solutions of bisantrene at final concentrations

¹ To whom requests for reprints should be addressed.
² Recipient of a postdoctoral fellowship from the Graduate School, University of Louisville.

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between 0.01 and 60 μg/ml, small yellowish rhomboid crystals appeared in a concentration-dependent fashion and were observed even at the lowest concentrations used. Cultures treated with bisantrene dissolved in DMSO, on the other hand, showed no crystals at final concentrations between 0.01 and 10 μg/ml. At higher concentrations, small crystals appeared.

**Clonogenic Survival of T-47D Mammary Tumor Cells after Bisantrene Treatment.** T-47D tumor cells grew as spherical colonies between 10 and 14 days. A concentration-dependent decline in colony-forming capacity was observed after 1-hr incubation with bisantrene (Table 1). At concentrations lower than 0.1 μg/ml, 77% of the bisantrene-treated cells survived. When drug concentration increased from 1 to 10 μg/ml, colony-forming capacity declined from 33.5 to 0.004%, respectively. Above 10 μg of bisantrene per ml, no colonies were formed.

**Morphology of the T-47D Mammary Tumor Cells before and after Bisantrene Treatment.** T-47D mammary tumor cells proliferate in our laboratory with an estimated doubling time of 32 hr. Three days after seeding, patches of polygonal epithelial cells are established (Fig. 1). After 24-hr exposure to bisantrene at concentrations exceeding 1 μg/ml, surviving cells are highly uniform in appearance. The nuclei in these cells are distinct, and the cells seem more rounded than untreated cells (Fig. 2). Except for occasional large inclusion bodies in cultures containing precipitated bisantrene, no differences in morphology of the surviving cells have been observed by light microscopy regardless of the drug concentration used.

**Fluorescent Microscopy Analysis.** Strongly fluorescent crystals appeared in the growth medium when bisantrene dissolved in water was added to achieve final concentrations of 0.1 to 60 μg/ml. After exposure to 0.1 to 10 μg of bisantrene per ml, surviving cells displayed faint nuclear fluorescence and clusters of brightly fluorescent particles in the cytoplasm (Fig. 3). Those treated with the same concentrations of the drug dissolved in DMSO also exhibited faint nuclear fluorescence, but the bright cytoplasmic particles were not present (Fig. 4). Cells exposed to concentrations of bisantrene higher than 10 μg/ml in either water or DMSO exhibited much stronger fluorescence. Unlike the faint fluorescence displayed by cells exposed to lower drug concentrations, the entire nuclear matrix became fluorescent. A few dark spots resembling nucleolar complexes were also present. Cytoplasmic fluorescence was evidenced by scattered, brightly fluorescent particles (Fig. 5).

**Ultrastructure of T-47D Tumor Cells before and after Bisantrene Treatment.** The ultrastructural morphology of T-47D tumor cells prior to drug treatment is illustrated in Fig. 6. A dominant feature of the untreated controls is the presence of large, centrally placed nuclei filled with clumps of heterochromatin, well-dispersed euchromatin, and a large nucleolar complex. Indentation of the nuclear surface is seen frequently. In the cytoplasm, a well-developed Golgi complex, short segments of rough endoplasmic reticulum, occasional electron-opaque granules, and a few elongated mitochondria and clear vacuoles are distributed randomly. The cell surface shows a few short microvilli. No ultrastructural effects were observed in T-47D cells treated with 1% DMSO. Consistent with the light microscopic observations, cells surviving bisantrene treatment principally showed nuclear changes. At low drug concentrations (0.1 to 5 μg/ml), euchromatin predominated in the nuclear matrix. Abnormal nuclei in which the granular and fibrillar components had begun to separate were evident (Fig. 7). The appearance of numerous small, clear vesicles near the cell surface and slight swelling of the mitochondria marked the cytoplasmic response. At higher drug concentrations (10 to 100 μg/ml), groups of small electron-opaque particles resembling fragmented heterochromatin were contrasted starkly against the electron-lucent nucleoplasm (Fig. 8). Several altered nucleoli containing both fibrillar and granular components were seen in the smoothly contoured nuclei (Fig. 9). Cytoplasmic alterations were limited to the formation of membrane-enclosed lysosomal complexes containing a few irregularly shaped, electron-opaque particles and an accumulation of small vesicles near the plasmalemma (Fig. 10).

A feature unique to the majority of cells exposed to bisantrene concentrations exceeding 10 μg/ml is the presence of intracellular membrane-enclosed crystalline inclusions (Fig. 11). Multilayered aggregates with rod-like substructure can be seen within the inclusion bodies, which at times are incompletely enclosed by membrane (Figs. 11 and 12). The internal structure of the inclusions becomes less regular after they are completely enclosed and lysis begins. Small myelin bodies were found frequently within the lysosomes. The nuclear response in these cells appeared qualitatively similar to that in cells treated with drug solutions free of crystals.

**DISCUSSION**

Light, fluorescent, and electron microscopic observations presented here point clearly to the cell nucleus as the principal target of bisantrene. Minimal disturbance of other organelles was evident in cells treated with soluble bisantrene and in those containing bisantrene crystals.

The presence of crystalline inclusions in cells exposed to medium containing bisantrene crystals demonstrates the phagocytic capability of mammary tumor cells. This phagocytic tendency, while not usually thought of as a property of nontransformed mammary cells, has been expressed by cultured Syrian hamster embryonic cells and Chinese hamster ovary cells (4). Costa and Mollienauer (4) have reported that, once internalized, nickel sulfide crystals remain in the cell cytoplasm. Subsequent data from the same laboratory reported lysosomal dissolution of the internalized particles and migration of lead molecules into the nucleus, but nickel salt crystals per se did not appear to enter the cell nucleus in this case (3). In another study, platinum uracil blue, after internalization by HeLa cells, is located in phagosomes but not in the cell nucleus (7). In contrast, migration of lead-induce inclusion bodies into the nucleus of the cultured kidney epithelial cells occurred within 24 hr (12). Since crystalline particles were not formed in the nucleus of T-47D cells, the mechanism for the genotoxicity of bisantrene may be similar to that of nickel compounds in which lysosomal disintegration of bisantrene crystals facilitates nuclear entrance of the active derivative.

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**Table 1**

<table>
<thead>
<tr>
<th>Bisantrene concentration (μg/ml)</th>
<th>No. of colonies</th>
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<tr>
<td>0</td>
<td>126.5 ± 15.8</td>
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<tr>
<td>0.01</td>
<td>119.0 ± 29.2</td>
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<tr>
<td>0.1</td>
<td>97.5 ± 32.7</td>
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<tr>
<td>1.0</td>
<td>45.2 ± 24.0*</td>
</tr>
<tr>
<td>10.0</td>
<td>0.5 ± 1.0*</td>
</tr>
<tr>
<td>60.0</td>
<td>0</td>
</tr>
</tbody>
</table>

* n = 4 for all experimental groups.

0 Indicates significant decline in colony formation at p < 0.01, 2-sided t test.

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* Indicates significant decline in colony formation at p < 0.01, 2-sided t test.

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**References**
A feature common to most DNA intercalators is the ability to cause single-stranded DNA scission and breakage of the associated DNA-protein cross-links. Intercalation alters DNA supercoiling and increases the susceptibility of single-stranded DNA to nuclease attack (16, 17). At various concentrations of the drug dissolved in either DMSO or distilled water, the nucleoli of surviving cells are similar to those found in liver and in cardiac and skeletal muscle of rat after in vivo exposure to Adriamycin (13), or after in vitro treatment of cells with Adriamycin, carminomycin, or marcellomycin (5, 14). Cells treated with high concentrations of bisantrene appear more rounded than untreated cells. This may be caused by interaction of the drug with the plasma membrane. Many studies have demonstrated the interaction of anthracycline drugs with membrane phospholipids (6, 8). The reproductive viability of the bisantrene-treated cells was studied by clonogenic assay in soft agar. In anchorage-dependent cultures, nonviable cells tend to detach from culture plates and are washed away during cell harvesting (11, 18). The remaining cells have been judged to be viable by the dye exclusion test (18). Hence, the morphological features reported here are those of viable T-47D tumor cells after bisantrene treatment. Their proliferative potential, as addressed by clonogenic assay, suggests that most of the stem cells retain the ability to divide after exposure to bisantrene at concentrations of 1 \( \mu g/ml \) or less.

Our data do not bear directly on the possible connection between precipitated bisantrene and the celluities observed in some patients. That there are no lesions specific to cells containing crystalline particles may argue that locally high concentrations of bisantrene do not themselves cause the local complications seen in patients.

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