Comparative Molecular Pharmacology in Leukemic L1210 Cells of the Anthracene Anticancer Drugs Mitoxantrone and Bisantrene

George T. Bowden, Robin Roberts, David S. Alberts, Yei-Mei Peng, and Dava Garcia

ABSTRACT

1,4-Dihydroxy-5,8-bis[2-[(2-hydroxyethyl)aminoethyl]amino]-9,10-anthracenedione (mitoxantrone) and 9,10-anthracenedicarboxaldehyde bis[(4,5-dihydro-1H-imidazol-2-yl)hydrazone] dihydrochloride (bisantrene) were evaluated for their abilities to cause cytotoxicity and interact with cellular DNA using leukemic L1210 cells. On a molar basis mitoxantrone has been found to be 7-fold more toxic than bisantrene. Using a nucleoid sedimentation technique, bisantrene caused changes in DNA supercoiling which were characteristic of an intercalating drug, but mitoxantrone did not induce these changes. Both drugs were found to interact with cellular DNA with tight but noncovalent binding. Both drugs also induced nonprotein-associated double- and single-strand DNA breaks, but with mitoxantrone only one of the DNA single-strand breaks were protein associated, whereas with bisantrene all the DNA single-strand breaks were protein associated. The cytotoxicity produced by bisantrene at a given frequency of protein-associated DNA strand breaks was low. However, with mitoxantrone at an equivalent DNA strand break frequency, the cytotoxicity was high. Treatment of isolated L1210 nuclei with either drug did not result in DNA single-strand breaks. It can be concluded that bisantrene binds DNA in whole cells by an intercalative, electrostatic interaction and induces non-protein-associated DNA strand breaks.

INTRODUCTION

Bisantrene and mitoxantrone (Chart 1) are two new anthracene drugs which have shown significant activity against a wide variety of animal tumor models (1–5). Both agents have proven activity in patients with breast cancer (6–8); however, mitoxantrone but not bisantrene appears effective in the treatment of acute leukemia and lymphoma in Phase II clinical trials (9–12). Although mitoxantrone and bisantrene were the first totally synthetic DNA binders to reach clinical trial and show promise of usefulness, the exact mechanism of action is unknown for either of these drugs. Knowledge of their mechanism of action could aid in further development of the anthracene group of compounds.

The initial results of animal tumor studies as well as toxicological and clinical investigations using bisantrene and mitoxantrone compared to doxorubicin suggested that these anthracenes compounds were similar to the anthracycline antibiotics. However, the anthracyclines and these anthracene drugs are quite different in chemical characteristics, structure-activity relationships, and dose responsiveness in terms of cytotoxicity (13). On these bases, one would predict that their respective mechanisms of action are likely different.

Mitoxantrone has been shown to be distinctly different from doxorubicin or bisantrene in its cytotoxicity to P388 leukemia. Mitoxantrone was shown to be 20-fold more potent than doxorubicin and 100-fold more potent than bisantrene in a cytotoxicity assay. These results suggest that there may be significant differences in the mechanism of cytotoxicity of mitoxantrone as compared to bisantrene or doxorubicin.

We have observed similar differences between bisantrene and mitoxantrone in terms of cytotoxicity using mouse L1210 leukemia cells in culture. The planar structures of both drugs suggest that they may act as DNA intercalating agents and therefore may induce protein-associated DNA strand scissions. Using L1210 leukemia cells, we have explored potential differences between the two drugs in terms of their interactions with cellular DNA.

MATERIALS AND METHODS

Cell Cultures and Labeling Conditions. The L1210 cells used in these experiments were supplied by R. A. G. Ewig, National Cancer Institute, Bethesda, MD. L1210 cells were grown in suspension culture as described previously (14). Cultures were routinely tested for Mycoplasma and found to be contamination free. Uniform labeling of the DNA was obtained as described previously (14).

Drug Treatment. Unlabeled and 14C-radiolabeled bisantrene as well as unlabeled and 1H- or 14C-radiolabeled mitoxantrone were obtained from the Medical Research Division, American Cyanamid Co., Pearl River, NY. Stock solutions of the drugs were made up, and the cells were treated as described previously (14). Drug treatments were terminated by placing the cells at 4°C and washing the cells twice with cold Hank’s balanced salt solution. Cells were counted, and aliquots of the appropriate cell number were used for the assays listed below.

Cell Survival. Cells treated for 1 h with various concentrations of bisantrene or mitoxantrone as described above were assayed for colony growth in soft agar by the method of MacPherson and Montagnier (15). Colonies were counted after 7 days of incubation at 37°C. Cloning efficiency of control cells was 85 to 96%.

Nucleoid Sedimentation on Neutral Sucrose Gradients. The sedimentation properties of nucleoids following drug treatment were assayed by a modified procedure (14) from Mattern and Painter (16).

Alkaline Elution Assay. The alkaline elution technique for the detection of protein-associated DNA single-strand breaks was carried out by a modified procedure (14) described by Ross et al. (17, 18). Data were analyzed to calculate DNA single-strand break frequencies using equations developed by Ross et al. (18). In this laboratory, it was found that the rate of elution of DNA from the filter was dependent on DNA single-strand size. When L1210 cells were X-irradiated on ice and then analyzed...
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![Chart 1. Structures of substituted anthraquinones.](image_url)

Table 1. Structures of substituted anthraquinones.

<table>
<thead>
<tr>
<th>Anthraquinone</th>
<th>Structure</th>
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<tbody>
<tr>
<td>Mitoxantrone</td>
<td><img src="image_url" alt="Structure" /></td>
</tr>
<tr>
<td>Bisantrene</td>
<td><img src="image_url" alt="Structure" /></td>
</tr>
</tbody>
</table>

RESULTS

Cell Survival. Assessment of clonogenic survival of L1210 cells after a 1-h treatment with either bisantrene or mitoxantrone was made by studying anchorage-independent growth in soft agar (Chart 2). On a molar basis, mitoxantrone was found to be 7-fold more toxic than bisantrene ($D_0$ bisantrene, 18 ¿¿M; $D_0$ mitoxantrone, 2.5 ¿¿M). There was no evidence for a shoulder in the survival curves for either drug.

Changes in DNA Supercoiling. Neutral sucrose gradients were used to determine if mitoxantrone in vivo induced changes in DNA supercoiling which were indicative of DNA intercalation. In a previous publication (14), it was shown that bisantrene induced a biphasic change in the sedimentation coefficient of the nucleoids as a function of drug concentration. This biphasic change is typical for the classical intercalating drug, ethidium bromide, and indicated that bisantrene acts as an intercalating drug. When L1210 cells were treated in suspension culture for 1 h at 37°C with mitoxantrone and the resulting nucleoids were sedimented through sucrose gradients, no statistically significant change in the relative sedimentation distance resulted (Chart 3). As a positive control, a similar experiment with bisantrene was carried out, and a typical biphasic response in the relative sedimentation was observed (Chart 3). There, however, remained the possibility that mitoxantrone could be inducing changes in DNA supercoiling due to DNA intercalation which were obscured by non-protein-associated DNA strand breaks. (we will show evidence for these DNA SSB in Table 1). To eliminate the non-protein-associated DNA SSB, we isolated nuclei from L1210 cells, incubated the nuclei with various concentrations of mitoxantrone at ice temperature, and then sedimented nucleoids through sucrose gradients as described previously. Incubation of the nuclei at ice temperature prevents DNA SSB but would allow intercalation if it occurred. We found no significant change in the sedimentation of treated nucleoids compared to control nucleoids (data not shown). A positive control experiment was carried out in which isolated nuclei were treated with bisantrene and by DNA alkaline elution (including proteinase K treatment), the fraction of DNA remaining on the filter at a fixed elution interval showed a first-order relationship to X-ray dose. Plots of the log of retention versus time of elution were linear with X-irradiation. Alkaline sucrose sedimentation studies have indicated that 1,084 breaks/10^9 nucleotides are produced per rad of X-rays.

Isolation and Treatment of L1210 Nuclei. A modification of a procedure described by Wozniak and Ross (20) and originally developed by Filipski and Kohn (21) was used to isolate L1210 nuclei. Briefly, whole cells were washed in cold Buffer A (1 mM KH₂PO₄·0.5 mM MgCl₂-15 mM NaCl-2 mM ethyleneglycol bis(β-aminoethyl ether)-N,N',N''-tetraacetic acid-4 mM disodium EDTA-0.3 mM spermine-1 mM spermidine) at pH 6.4. The cells were resuspended in 1 ml of the above buffer and were lysed with 9 ml of Buffer B (1 mM KH₂PO₄·0.5 mM MgCl₂-15 mM NaCl-1 mM ethyleneglycol bis(β-aminoethyl ether)-N,N',N''-tetraacetic acid plus 0.3% Triton X-100 (Eastman Kodak Co., Rochester, NY). Immediately, 40 ml more of the original buffer were added, and the nuclei were sedimented by centrifugation at 900 x g for 15 min. The presence of intact nuclei was confirmed by phase microscopy. The intact nuclei were resuspended in 2 ml of the original buffer. The nuclei were then treated with drug for 1 h at 37°C, and the alkaline elution assay as described above was performed to detect protein-associated DNA single-strand breaks.

CsCl Gradient Centrifugation of Cellular DNA. Whole L1210 cells were treated with radiolabeled bisantrene or mitoxantrone for 1 h at 37°C. The harvested cells were pelleted, and nuclei were isolated as described above. Chromatin was isolated by sonicating the nuclei, and the chromatin was centrifuged on a CsCl gradient (density = 1.7 g/ml). The gradients were centrifuged at 33,000 rpm in a Beckman 50 Ti rotor for 48 h. The gradients were fractionated from the bottom, the absorbance at 260 nm was read, and the radioactivity in each fraction was determined by scintillation counting. Evidence for covalent binding of the drug to DNA was sought by attempting extraction of the radioactivity with normal butyl alcohol.

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Charts. Effects of mitoxantrone and bisantrene on nudeoid sedimentation. L1210 cells were treated for 1 h with various concentrations of mitoxantrone or bisantrene, and nucleoids were sedimented in neutral sucrose gradients as described in "Materials and Methods." To obtain the relative distance sedimented, the peak fraction number for each sample containing cells which were treated with mitoxantrone (O) or bisantrene (●) divided by the total number of fractions was divided by the same parameter calculated for the untreated control sample in the same centrifugation run. This quantity (i.e., relative distance sedimented) is normalized from one run to another. Points, mean; bars, SE.

at ice temperature, and nucleoids were sedimented through sucrose gradients. We found a typical biphasic response in the relative sedimentation of these nucleoids (data not shown). Therefore, we have observed both in vivo at 37°C and with isolated nuclei at ice temperature that bisantrene induced changes in DNA supercoiling typical of classical intercalating drugs, while mitoxantrone did not.

CsCl Banding of Drug-bound Cellular DNA. To determine the nature of any potential drug binding to cellular DNA, L1210 cells were treated for 1 h with either 14C-radiolabeled mitoxantrone or bisantrene. Nuclear DNA was isolated from treated cells and banded on neutral CsCl gradients. Both absorbance at 260 nm and 14C radioactivity banded at a buoyant density of 1.7 g/ml (Chart 4) for each drug. The data for bisantrene were obtained but are not shown. To determine whether the drug binding was covalent, the peak fractions were pooled and extracted with normal butyl alcohol. Greater than 95% of the 14C radioactivity and presumably the drug were extractable from the DNA using normal butyl alcohol (data not shown). These data indicate that both bisantrene and mitoxantrone bind tightly but noncovalently to cellular DNA.

DNA Strand Break Induction. The formation of DNA single-strand breaks in L1210 cells was examined following exposure to various concentrations of both bisantrene and mitoxantrone for 1 h. DNA single-strand breaks were quantitated using the alkaline elution assay with or without proteinase K treatment of the cell lysates on the filters. As has been shown previously, no detectable strand breaks were found with bisantrene without proteinase K treatment. However, DNA single-strand breaks were detected without proteinase K when the cells were treated with mitoxantrone (Table 1). Approximately 66% of the strand breaks were protein associated with mitoxantrone, whereas the majority of the strand breaks induced by bisantrene were protein associated (14). Mitoxantrone was also shown to induce DNA single-strand breaks in a dose-dependent manner (data not shown). A comparison of toxicity and frequency of drug-induced DNA single-strand breaks for mitoxantrone and bisantrene at equal molar concentrations (Table 2) indicates that treatment with the drugs resulted in the same level of DNA single-strand breaks but completely different levels of reduction in cell survival. Mitoxantrone was 20-fold more toxic than bisantrene at the same dose level and at the same level of DNA strand breaks. The relationship between DNA single- and double-strand breaks was also determined for both drugs utilizing a newly developed neutral elution assay to measure the frequencies of the double-strand breaks.

Table 1

<table>
<thead>
<tr>
<th>Drug</th>
<th>µM</th>
<th>Survival (%)</th>
<th>No. of DNA SSB/10^6 nucleotides</th>
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<tr>
<td>Bisantrene</td>
<td>10</td>
<td>49.9</td>
<td>0.331</td>
</tr>
<tr>
<td>Mitoxantrone</td>
<td>10</td>
<td>2.4</td>
<td>0.311</td>
</tr>
</tbody>
</table>

Table 2

Comparison of cytotoxicity and frequency of DNA single-strand breaks between mitoxantrone and bisantrene at equal molar concentrations

A comparison was made between the cytotoxicity and frequency of drug-induced DNA single-strand breaks for mitoxantrone and bisantrene at equal molar concentrations.

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Due to the nature of the neutral elution assay, it was not possible to determine the absolute DNA double-strand break frequency. Instead, this frequency was determined in ionizing radiation rad equivalents. With this information, it was possible to determine the absolute ratio of DNA single-strand breaks to DNA double-strand breaks by comparing the rad equivalents for the two types of DNA strand breaks and knowing the estimated ratio of DNA single-strand to double-strand breaks produced by ionizing radiation as determined by a gradient technique. The reported values for the ratio of X-ray-induced single- to double-strand breaks using alkaline and neutral sucrose gradients have been in the range of 10 to 40. DNA single-strand break frequencies determined using alkaline elution assay include DNA singlestrand breaks arising from DNA double-strand breaks as well as true single-strand breaks. The relationship between measured and true break frequencies is given in Table 3 for bisantrene and mitoxantrone. For bisantrene these ratios ranged from 0.3 to 7.2, and for mitoxantrone the values ranged from 1.0 to 10.

**Treatment of Isolated L1210 Nuclei with Bisantrene and Mitoxantrone.** To investigate further the potential difference in the mechanisms involved in the production of DNA single-strand breaks induced by bisantrene and mitoxantrone, the production of single-strand breaks in treated, isolated nuclei was studied. Isolated nuclei were treated at 37°C for 1 h, and the nuclei were lysed and treated on the filters with proteinase K. The results of these experiments (Chart 5) indicated that both bisantrene and mitoxantrone require the presence of cytoplasm to produce DNA strand breaks. Published evidence indicates that intercalating agents at high concentrations can actually inhibit strand breakage by topoisomerase II, and thus it was possible that failure to observe strand breaks in the isolated nuclei was related to the nature of the dose-response curve and not a requirement for cytoplasm. Therefore, we treated isolated nuclei with lower concentrations (0.1 and 1 μM) of bisantrene and mitoxantrone. Again we observed no significant DNA SSB even at these low concentrations (data not shown). A control experiment was carried out to show that the nuclei isolation procedure did not interfere with the detection of DNA strand breaks. Intact cells were treated with bisantrene or mitoxantrone, and nuclei were isolated from these cells. DNA single-strand breaks were detected in these nuclei (Chart 5). Therefore, the nuclei isolation procedure did not interfere with the detection of DNA strand breaks.

**DISCUSSION**

Bisantrene and mitoxantrone are two new anthrane derivatives which are undergoing clinical trial for treatment of human cancers. Both drugs have proven activity in patients with metastatic breast cancer (6–8), but only mitoxantrone has also shown significant activity against acute lymphocytic and nonlymphocytic leukemia, malignant lymphomas, and hepatomas (9–12). Although the mechanism(s) of action of these drugs is not understood, a number of investigators have clearly established that the drugs inhibit RNA and DNA synthesis and intercalate DNA in *in vitro* reactions (23–25). The purpose of our investigations was to study *in vivo* the nature of the interactions of these two drugs with cellular DNA.

Bisantrene does behave as a classical intercalating drug *in vivo* (14), since we have shown that bisantrene causes changes in DNA supercoiling typical of DNA intercalating agents and protein-associated DNA breaks which are detectable by neither alkaline elution nor nucleoid sedimentation in the absence of proteinase K. These protein-associated DNA breaks could therefore correspond to topoisomerase II-DNA complexes trapped by the intercalator (26, 27). In contrast, we have shown that mitoxantrone, which on a molar basis is more toxic than bisantrene, induces DNA SSB which are non-protein associated. We have also shown that mitoxantrone does not act as a classical intercalating drug.
calcating drug in changing DNA supercoiling, but instead may cause in vivo DNA compaction due to electrostatic interaction with the anionic exterior of the DNA helix. Therefore, we have shown that there are major differences in the way bisantrene and mitoxantrone interact with cellular DNA.

The question arises as to why DNA strand breakage does not affect nucleoid sedimentation when cells are treated at 37°C with bisantrene or mitoxantrone. Since DNA strand breaks are protein associated with bisantrene, it is possible that covalent linkage of integral proteins to DNA in the nucleoid would prevent DNA strand breaks from affecting supercoiling. Since the majority of the DNA strand breaks induced by mitoxantrone are protein associated, the non-protein-associated DNA strand breaks which could affect supercoiling may not be at a level detectable by the nucleoid sedimentation technique.

Mitoxantrone is structurally related to a series of substituted anthraquinones and has features known to be essential for DNA intercalation (planar, electron-rich chromophore). The presence of side chains bearing basic amino groups which could bind electrostatically to the phosphate groups of DNA may enhance intercalation. Indeed, mitoxantrone and related drugs have been shown to intercalate purified DNA in the test tube (23, 28). Most of these drugs show strong DNA binding as evidenced by $\Delta Tm$ values equal to or greater than those produced by the anthraquinone antibiotics. However, the ability of these drugs to increase the melting temperature of DNA and to inhibit nucleic acid synthesis in vitro does not always correlate with in vivo antitumor activity (23). This raises the possibility of a mechanism of action other than or in addition to DNA intercalation.

Data presented in this paper suggest that mitoxantrone when added to intact cells does not act as a classical intercalating drug as measured by the nucleoid sedimentation technique. However, we have reported in this paper the rather strong binding of mitoxantrone to cellular DNA using CsCl gradient sedimentation of DNA extracted from intact cells treated with mitoxantrone. This tight binding could be associated with the electrostatic interaction of mitoxantrone with the anionic exterior of the DNA. Scatchard plots of mitoxantrone binding to DNA by equilibrium dialysis analysis indicated two binding sites. The strong binding site may be associated with intercalation into DNA base pairs, and the weaker binding site may possibly be associated with secondary binding of the side chains by electrostatic interaction with the anionic exterior of the DNA helix. Therefore, there remains the possibility that even though mitoxantrone contains the requisite planar chromophore, the presence of two extended alkyl residues in positions 1 and 4 may preclude the smooth incorporation of all parts of the molecule. As pointed out by Durr et al. (23), this is supported by the observed greater intercalative binding of the less hindered ethidium molecule. These observations have led some investigators to speculate that DNA binding includes both partial intercalation and external binding.

It has also been shown that mitoxantrone is capable of inducing condensation and compaction of isolated chromatin. This has been attributed to extensive supercoiling of the DNA (29). Factors in addition to intercalation may play a role, since ethidium bromide is inactive in this system. These include ionic interactions of inter- and intrastrand cross-links in addition to DNA intercalation. Lown et al. (30), using flow microfluorimetry measurements on relaxed PM2 DNA reacted with mitoxantrone, have shown the formation of lace-like networks of DNA linked together in a compacted fashion. This phenomenon has been attributed to inter-DNA linking by the charged side arms of mitoxantrone.

The production of protein-associated DNA single- and double-strand breaks has been associated with the action of DNA intercalating drugs. We have previously shown (14) that bisantrene induces both protein-associated DNA single-strand breaks as well as DNA-protein cross-links in a ratio of one to one. Bisantrene-induced DNA strand breaks appear to be protein associated. In contrast, we have found that mitoxantrone induces both protein-associated and non-protein-associated DNA strand breaks. Of interest is the fact that mitoxantrone contains a hydroquinone structure which could potentially generate free radicals during NADPH and O2 reaction with reductase enzymes, and DNA strand breaks can be generated by this mechanism (31).

Besides the non-protein-associated DNA strand breaks, mitoxantrone induces protein-associated strand breaks which could have resulted from the partial insertion of the mitoxantrone molecule into the DNA. There is also in vitro cell-free experimental evidence that drugs with either weak or no intercalating activity have the ability to induce the formation of a topoisomerase II-DNA complex (26). This same complex could be formed in a cell treated with these drugs.

Experimental results reported by Zwelling et al. (32) and Ross and Bradley (33) have indicated that there are wide differences between ratios of SSB to DSB produced by different intercalating agents in the order acridinyl anisidine > Adriamycin > ellipticine. It has been suggested that ellipticine produces DNA DSB almost exclusively (17). Our results obtained with both bisantrene and mitoxantrone indicate ratios of SSB to DSB very similar to those found for Adriamycin and certainly less than those found for acridinyl anisidine. It has been speculated that, if the DNA strand breaks were induced enzymatically, the intercalators may differ widely in their relative stimulation of topoisomerases producing single-strand versus double-strand breaks.

Besides identifying the types of DNA damage induced by bisantrene and mitoxantrone in treated whole cells, we have determined the action of the drugs on isolated nuclei. We found that neither drug induced DNA single-strand breaks (protein or non-protein associated) in isolated nuclei. Thus, it would appear that the cytoplasm of intact cells is necessary for drug action. However, we cannot exclude the possibility that the failure to see DNA strand breakage in isolated nuclei could be a function of the buffer system used for these experiments. Recently Wozniak and Ross (20) showed that the podophyllotoxin VP-16 [4'-demethylepipodophyllotoxin-9-(4,6-O-ethylidene-β-D-glucopyranoside) (etoposide)] does induce both DNA SSBs and double-strand breaks in isolated nuclei, indicating that for VP-16 cytoplasmic components are not required for drug action.

As other investigators have reported, we have not found a positive correlation between the frequency of protein-associated DNA strand breaks and the level of cytotoxicity induced by bisantrene and mitoxantrone. Pommier et al. (27) have reported in studies of the modification of intercalator-induced protein-associated DNA strand breaks by thiourea and dimethyl sulfoxide that alterations in intercalator-induced DNA scission were not accompanied by corresponding alterations in cytotoxicity. Our data taken together with Pommier’s data would suggest a clear dissociation of intercalator-induced strand break production and
lethality.

In conclusion, data have been presented which are consistent with the idea that the two anthracene drugs, mitoxantrone and bisantrene, differ from each other in the way they interact with DNA in the intact cell. Though not directly addressed in this paper, this difference could indicate that mitoxantrone and bisantrene have different mechanisms of cytotoxic action. The differences in mechanisms of action could explain differences in the spectrum of activity of the drugs in preclinical and clinical trials.

REFERENCES

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