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Selective displacement of nuclear proteins by antitumor drugs having affinity for nucleic acids

(mitoxantrone/ametantrone/doxorubicin/actinomycin D/intercalating agents)

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ABSTRACT **The nuclear chromatin binding sites of the antitumor drugs mitoxantrone, ametantrone, doxorubicin, mithramycin, and actinomycin D and the intercalating ligand ethidium were studied by polyacrylamide gel electrophoresis of the proteins released from rat liver nuclei in the presence and absence of these drugs in buffer of low ionic strength (10 mM NaCl). At 25–50 μM free ligand concentration, each drug produced a specific and reproducible pattern of extractable proteins of different molecular weight by (*i*) releasing new proteins, (*ii*) altering the quantity of particular extracted proteins, and/or (*iii*) selectively entrapping other proteins in the nuclei. Ethidium, up to 100 μM, did not affect release of proteins from the nuclei. These results indicate that each ligand either has different binding site(s) in chromatin or modulates chromatin structure in a specific way by changing the affinity of different sets of proteins for their respective binding sites, resulting in their selective extraction or entrapment. The lack of effect of ethidium indicates that intercalation of the ligand to DNA, *per se*, does not alter the release of nuclear proteins. If patterns of nuclear proteins selectively released or retained by antitumor drugs are found to correlate with biological activity, this type of analysis may be helpful in new drug design and screening.**

Many antitumor drugs interact with nucleic acids, and intercalation (1) is the most common type of these interactions (2). It is unclear, however, whether cytotoxic or cytostatic effects of these drugs are a consequence of the intercalation alone or whether other mechanisms are involved. Generally, for a variety of drugs, no correlation is apparent between their antitumor potency and intercalative affinity, and many potent intercalators lack antitumor properties (e.g., 3, 4). It is suspected that chromatin proteins, especially topoisomerases, may play a role in interactions related to antitumor activity (5, 6). In the present study we attempted to characterize changes in nuclear chromatin induced by several anticancer drugs known to interact with nucleic acids as well as by such agents as ethidium, which intercalates into DNA but has no chemotherapeutic value.

Our strategy was to expose isolated cell nuclei to relatively low concentrations of various drugs and to observe whether or not such treatment can release a specific protein. It was postulated that if the ligand binds to the nucleic acid at the site occupied by the protein, it will replace this protein, which upon dissociation may be released from the nucleus. Likewise, any allosteric change in the nucleic acid induced by the drug may alter its binding affinity for some protein(s), which then could dissociate and be released from the nucleus. Thus, analysis of proteins extracted from nuclei by the drugs could provide insight into their binding sites that might be helpful in understanding mechanisms underlying their antitumor activity. Since some of the studied agents are either fluorochromes or analogs of fluorochromes, their binding sites in chromatin and possible modulation of these sites by nuclear proteins are of interest also in cytochemistry.

MATERIALS AND METHODS

Heterozygote nude male rats (SKI:N:NIH-*rnu*/+) of 200–250 g were killed by cervical dislocation during ether anesthesia. Livers were removed and washed with ice-cold buffer A (10 mM Tris/10 mM NaCl/3 mM $MgCl_2$/0.2 mM phenylmethylsulfonyl fluoride, pH 7.4), and the nuclei were isolated by a modification of the method of Prentice and Gurley (7). In brief, the tissue was minced and homogenized in 5 volumes of buffer A. After filtration through four layers of gauze, intact and broken cells were sedimented at 600 × *g* for 10 min. The pellet was resuspended in 5 volumes of buffer A, the nonionic detergent Nonidet P-40 (Sigma) was added to a final concentration of 1.1% (vol/vol), and the suspension was homogenized in a glass homogenizer. During homogenization, purity of the nuclei was checked under a phase-contrast microscope and the procedure was continued until clean nuclei lacking cytoplasmic "tags" were obtained. Nuclei were then sedimented at 600 × *g* for 5 min, resuspended in 3 volumes of buffer B (10 mM Tris/10 mM NaCl/3 mM $MgCl_2$/1 mM $CaCl_2$/0.2 mM phenylmethylsulfonyl fluoride, pH 7.4), layered over a cushion of 0.6 M sucrose in buffer B, sedimented at 1000 × *g* for 10 min, washed with 5 volumes of buffer B, and suspended in buffer B at a concentration of 10^9 nuclei per milliliter. All treatments were done at 0–4°C.

Stock solutions of the studied drugs were made freshly in water at 1 or 2 mg/ml concentration. The following compounds were studied: mitoxantrone (Novantrone) and ametantrone (American Cyanamid, Pearl River, NY), doxorubicin (Adriamycin; Adria Laboratories), mithramycin and actinomycin D (Sigma), and ethidium bromide (Polysciences). These stock solutions were added slowly to nuclear suspensions in buffer B to give the final free ligand concentrations indicated in *Results*. Free drug concentrations were established experimentally in parallel samples by titration of the isolated nuclei and, after their sedimentation, measuring light absorption by the supernatant at the absorption maximum of the drug with an IBM 9140 UV–visible digital spectrophotometer. Nuclei were exposed to drugs for 30 min at 0–4°C, with gentle shaking, and then sedimented (5 min, 600 × *g*). The supernatants were centrifuged (15 min, 4000 × *g*) and treated with 20% (wt/vol) trichloroacetic acid overnight at 0°C. The precipitated proteins were collected by centrifugation (15 min, 3000 × *g*), washed once with a mixture of

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acetone and 0.1 M HCl (7:1, vol/vol) and thrice with acetone, and dried.

The proteins, dissolved either in 0.2 mM phenylmethylsulfonyl fluoride or directly in the electrophoretic buffer, were subjected to one-dimensional gel electrophoresis in 0.1% SDS/10% acrylamide/0.1 M phosphate buffer (pH 7.0) as described by Weber and Osborn (8). Samples (40 μg of protein) were applied to rod gels (0.6 × 8.0 cm) and electrophoresis was carried out at 8 mA per gel for 5 hr. Gels were stained with Coomassie brilliant blue R250 and scanned by using the gel-scanning attachment for the Zeiss PM 6 digital spectrophotometer. Digital data from the measurements were transferred to, and processed by, a Hewlett–Packard 9826 computer and profiles were drawn by a Hewlett–Packard 7225A digital plotter. Special computer programs were developed for data collection and analysis. Densitometric plots were normalized to the maximal peak and with respect to the baseline. For rapid qualitative evaluation the proteins (1–5 μg) were also separated in a discontinuous microslab gel system (Mini Protean II, Bio-Rad) according to Laemmli (9); these gels were stained according to the protocol of the Sigma silver stain kit.

The experiments (protein extractions and electrophoresis) were repeated at least four times and the observed changes in the patterns of proteins eluted by particular ligands were highly reproducible.

RESULTS

Exposure of nuclei to buffer alone results in the release of numerous proteins. This spontaneous release of loosely bound proteins, observed also by others (10, 11), represents the background or "noise" of the present experiments. Changes in this pattern induced by the studied ligands are attributed to the specific effects of these ligands on chromatin.

Three types of changes in protein pattern were observed: (*i*) appearance of new bands in gels, representing release of specific proteins by the ligand; (*ii*) change in density of particular bands, reflecting quantitative changes in release of the proteins; and (*iii*) loss of the bands, compared to the control, indicating entrapment of proteins that otherwise were loosely bound to chromatin but became stabilized in the nuclei in the presence of the ligand.

Figs. 1–3 illustrate the ligand-induced changes in the pattern of proteins extracted from the nuclei. Different sets of proteins were released or retained by each of the studied drugs. Thus, mitoxantrone eluted a 160-kDa protein (Fig. 1, scan B) that was essentially absent from control extracts as well as from extracts induced by ethidium, mithramycin or actinomycin D. Mitoxantrone also increased the relative proportion of proteins of 70, 50, and 27 kDa.

Similarly to mitoxantrone, ametantrone released protein(s) of 160 kDa and increased the relative amount of proteins of 70 and 27 kDa. It also altered the extractability of smaller proteins, retaining a 14-kDa protein and releasing a protein of 22 kDa (Fig. 1, scan C).

Doxorubicin released two or three high molecular mass fractions (180–210 kDa), as well as proteins of 160, 33, and 22 kDa. This drug retained proteins of 43–45 kDa that otherwise were extracted with the buffer (Fig. 2, scan B).

Mithramycin had relatively little effect on protein extractions. In its presence a minor fraction of 150 kDa and proteins of 17–24 kDa were eluted (Fig. 2, scan C).

Ethidium bromide, in concentration up to 100 μM, had no effect on the pattern of proteins released from the nuclei (Fig. 3, scan B). Actinomycin D, on the other hand, suppressed release of most of the nuclear proteins. Least affected were proteins of 70–80 kDa, so when the densitometric scans were normalized to maximum, the peak representing these proteins became predominant (Fig. 3, scan C). A quantitative estimate of proteins released by actinomycin D (data not shown) indicated that, depending on the extraction time and

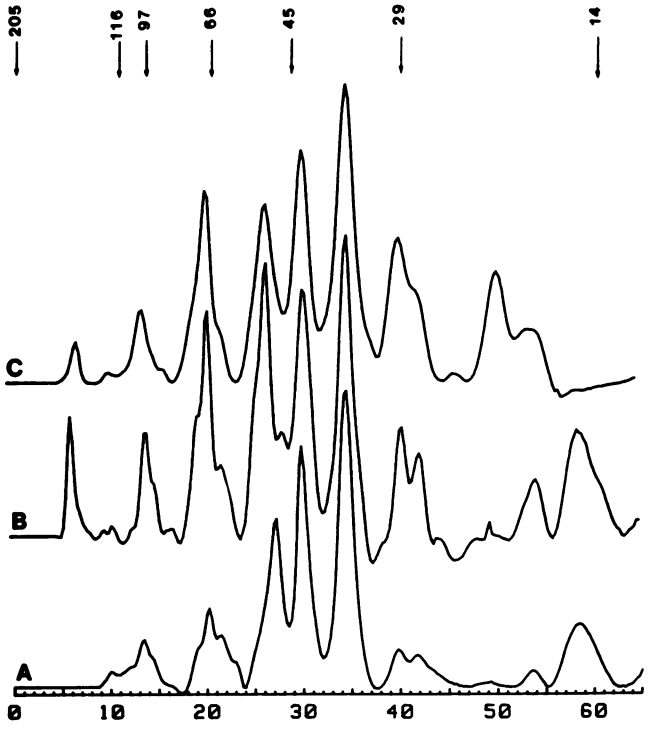

FIG. 1. Densitometric tracing of the gels of proteins released from rat liver nuclei as a result of exposure to buffer alone (scan A), 50 μM mitoxantrone (scan B), or 50 μM ametantrone (scan C). Positions of size markers (kDa) are marked by arrows. The horizontal scale represents length of gels (in millimeters) from the origin, marked as zero.

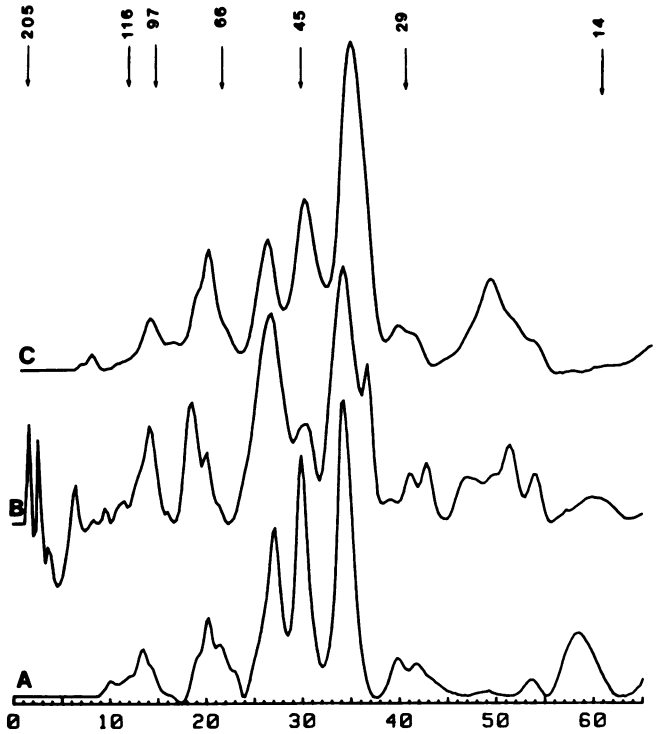

FIG. 2. Densitometric tracing of the gels of proteins released from rat liver nuclei in buffer alone (scan A) or with 50 μM doxorubicin (scan B) or 50 μM mithramycin (scan C).

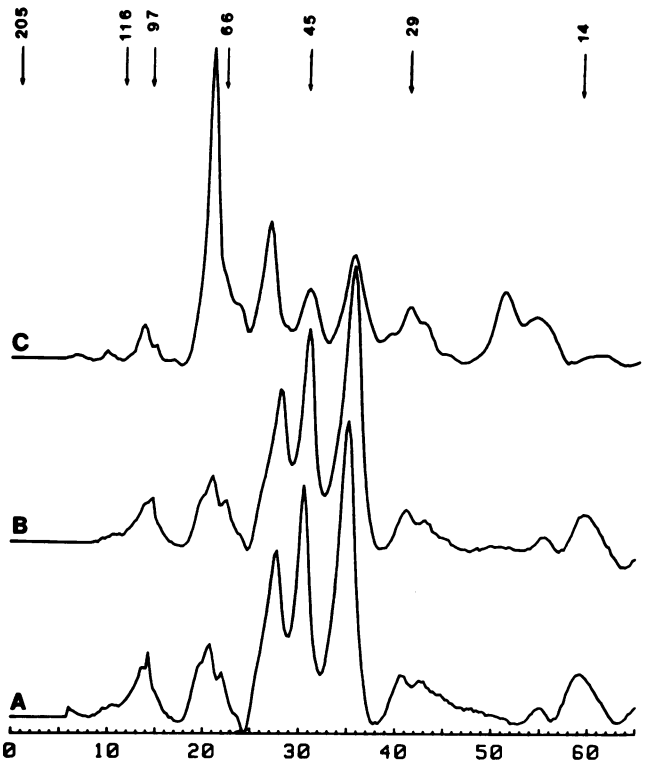

FIG. 3. Densitometric tracing of the gels of proteins released from rat liver nuclei in buffer alone (scan A) or with 100 μM ethidium (scan B) or 50 μM actinomycin D (scan C). Ethidium at 50 μM also had little effect on protein release (data not shown).

the drug concentration, actinomycin D prevented release of nuclear proteins by up to 40%.

The most conspicuous changes in patterns of extraction of nuclear proteins by the studied drugs are summarized in Table 1.

Table 1. Release or retention of nuclear proteins as a result of exposure of rat liver nuclei to various compounds

Compound*	Effect on protein extraction
Mitoxantrone (25 μM)	Releases additional protein fraction of 160 kDa; increases relative quantity of proteins of 70, 50, and 25–30 kDa
Ametantrone (50 μM)	Releases additional fraction of 160 kDa but in lesser quantity than mitoxantrone; increases extraction of 70-kDa protein
Doxorubicin (25 μM)	Releases three or four fractions of 180–210, 160, and 33 kDa; decreases quantity of proteins of 43–45 kDa
Mithramycin (50 μM)	Extracts minor fraction of 150 kDa and suppresses extraction of 43- to 45-kDa proteins
Actinomycin D (50 μM)	Decreases release of most proteins (up to 40%): least affected are proteins of 70–80 kDa
Ethidium (25–100 μM)	No significant effect

Because conditions of electrophoresis were chosen to obtain optimal resolution of proteins of >25 kDa, only such proteins are described. However, changes were observed in extraction of proteins similar in apparent size to histones or high-mobility-group proteins (see Figs. 1–3).

*Concentration of free ligand is given in parentheses.

DISCUSSION

The mechanism of interaction and possible intracellular targets of antitumor drugs are often inferred from studies of the affinity of these drugs for free nucleic acids in solution. Yet the secondary structure and environment of nucleic acids in the cell can significantly modulate these interactions. For instance, the nucleosomal structure and DNA–histone interactions in chromatin decrease the accessibility of DNA to various intercalating ligands (12). Also, the affinity of binding of these ligands to nucleic acids is altered in the cell (13, 14). The assumption that all DNA-intercalating drugs act via intercalative mechanisms appears obvious but may be overly simplistic, inasmuch as no correlation between intercalative binding affinity and antitumor or cytotoxic effects is apparent for a variety of drugs (e.g., 3, 4, 14). Because the mechanisms of drug interactions that are responsible for antitumor activity are not fully understood, new drugs tend to be designed empirically, and no *in vitro* tests to predict their potency exist. Studies characterizing the binding sites of antitumor drugs in the cell are thus of importance because they can provide clues needed for future drug design and may offer a test of drug efficacy.

The present study represents an attempt to characterize the binding sites by analyzing nuclear proteins released by the studied drugs. Of these drugs, mitoxantrone, ametantrone, doxorubicin, actinomycin D, and ethidium are intercalators with strong affinity for DNA, whereas mithramycin is believed to bind to DNA externally (15). Binding of actinomycin D involves also interactions of pentapeptide moieties of this ligand with the narrow groove of the DNA helix (16, 17). Mitoxantrone, ametantrone, and possibly doxorubicin are able to denature and/or condense nucleic acids, and this reaction is highly specific with respect to nucleic acid primary and secondary structure (4, 18). In contrast, ethidium (4) and, because of its bulky structure most likely actinomycin D also, cannot condense nucleic acids at these low concentrations. Of the ligands used in this study, ethidium has the lowest antitumor activity. The isolated nuclei were titrated with the drugs to the point of saturation of the binding sites, evidenced by the presence of the unbound ligand at 25 or 50 mM concentration.

Each drug produced a specific and reproducible pattern of changes in the extractable proteins. There was no apparent relationship between the pattern and the assumed mode of binding of the particular ligand to nucleic acids. For instance, no common feature in the pattern was apparent that would be characteristic of all intercalators or all drugs that condense nuclei acids. Even mitoxantrone and ametantrone, which are very close analogs but differ in their cytotoxic and antitumor potency (4) produced different patterns of extracted proteins. The lack of any effect of ethidium indicates that intercalation *per se* does not alter nuclear chromatin to the extent that there is a change in extractability of the proteins. However, at higher concentrations of ethidium and at higher ionic strength, histones and high-mobility-group proteins can be extracted from nuclei (11, 19–22). It appears therefore that each ligand has somewhat different binding site(s) in nuclear chromatin, since each changes the affinity of different sets of proteins for their respective binding sites in nuclei as reflected either by their selective extraction or entrapment.

The present data do not allow an estimate of whether the "fingerprint" of released nuclear proteins characteristic of a particular drug is in any way related to its antitumor or cytotoxic activity. However, when this approach is applied to a large panel of active drugs and their inactive analogs and to drugs presumed to have different modes of binding (e.g., intercalating vs. externally binding, involving topoisomerase II, etc.), it might reveal associations between the released and/or retained proteins and the biological or antitumor

activity of the drug. If such an association were to be found, the pattern of released proteins would be predictive of drug activity, and this method might be used to screen newly synthesized drugs. By providing an insight into the structure of the binding sites, this approach might be helpful in drug design as well. Further studies are needed to better characterize the proteins specifically released or retained by the drugs, especially their role in chromatin.

Preliminary data indicate that nuclei of other cell types, including tumor cells as well as drug-sensitive and multidrug-resistant cells, exhibit both drug and cell type specificity in quality and quantity of proteins released or retained and that multidrug resistance affects the patterns of their release. This approach therefore may also be of use in characterizing drug sensitivity of the target cells.

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