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Cytotoxicity of Estramustine, a Steroid-Nitrogen Mustard Derivative, through Non-DNA Targets

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SUMMARY

Estramustine is cytotoxic in HeLa and Walker 256 carcinoma cells (with or without acquired resistance to nitrogen mustards) at concentrations equivalent to other alkylating agents. Even at lethal estramustine levels, no damage to DNA occurs. Instead, a disproportionately high amount of intact estramustine binds hydrophobically to the structural proteins of the nucleus, the nuclear matrix. In HeLa cells, estradiol receptors are absent, and estradiol per se is not toxic. Thus, estramustine has a mechanism of action distinct from that of steroids and alkylating agents and may induce cytotoxicity through interactions with the proteins of the nuclear matrix.

INTRODUCTION

Estramustine phosphate [a derivative of estramustine (1); Fig. 1] is an effective chemotherapeutic agent in the treatment of advanced prostatic cancer (2, 3). Prostatic carcinoma cells in culture have been shown to be sensitive to the cytotoxic effects of estramustine (4), although the mechanism by which cytotoxicity is induced is not understood. Other steroid-alkylating agent conjugates, such as prednimustine, have been shown to hydrolyze with a short half-life to the constituent prednisolone and chlorambucil (5). Because of this, cellular toxicity has been found to be a composite of equimolar concentrations of the steroid and alkylating agent given as separate drugs (5). If the cytotoxic potential of estramustine is mediated through its decomposition to estradiol and nornitrogen mustard, it should be possible to detect the biological consequences of both alkylating damage and steroid response. The present study reports the stability of estramustine as the parent compound and demonstrates that in an estradiol receptor-negative cell line, no alkylation occurred at drug concentrations in excess of ID_{100} concentrations. Preliminary indications of the antimitotic activity (6) of estramustine would be consistent with the substantial levels of drug binding to the nuclear matrix, since many of the macromolecules of the matrix are critical to normal spindle formation and subsequent mitotic activity (7, 8).

MATERIALS AND METHODS

Cell cultures. WS[3] cells were maintained in static suspension in Dulbecco's minimal essential medium with glucose (4.5 g/liter) supplemented with 10% fetal bovine serum (MA Bioproducts, Walkerville, Md.) under 5% CO_2. WR cells have been characterized previously as possessing greater than a 20-fold resistance to a broad range of nitrogen mustards (9). HeLa S3 cells were maintained in Spinner flasks in minimal essential medium (Eagle's) (MA Bioproducts, Walkerville, Md.) containing 10% fetal bovine serum.

Colony-forming assays. Cell survival of WR, WS (the parent Walker line), and HeLa was determined by a 0.3% soft agar colony-forming assay as previously described (9). The drug incubations were carried out for 24 hr, with unincorporated drug diluted out of the culture after the incubation period.

Nuclear extraction. Nuclei were isolated by a Titon method described previously (10).

Nuclear drug uptake. Drug uptake was measured by incorporation of radiolabel into the isolated nuclei. Nuclei were solubilized with NCS (New England Nuclear Corporation, Boston, Mass.), neutralized with glacial acetic acid, and counted in a Mark III scintillation counter (Searle Analytic, Des Plaines, Ill.) with ACS (Amersham Searle, Des Plaines, Ill.) as a flour.

Nuclear matrix isolation. The nuclear matrix was isolated as described by Berezney and Coffey (11). Cytoplasmically free nuclei were digested wtih DNase I, followed by washes with 0.2 mM $MgCl_2$ (three washes), 2 M NaCl (three washes), 0.1% Triton, and exhaustive RNase A and DNase I digestion.

Estrogen receptor assay. Estrogen receptors were quantitated by using a dextran-coated charcoal assay (12). Estrogen incubation con-

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[3] The abbreviations used are: WS, Walker 256 carcinoma cells; WR, Walker 256 carcinoma cells resistant to nitrogen mustards; HPLC, high-pressure liquid chromatography; ID_{50}, drug concentration causing 50% growth inhibition.

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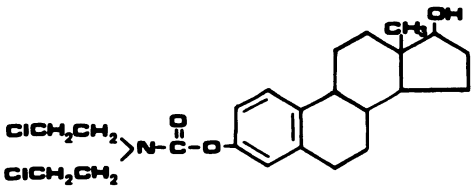

FIG. 1. *Chemical structure of estramustine*

Estradiol is connected to nor-nitrogen mustard through a carbamate ester linkage. The metabolite estromustine has an oxygen atom in position 17β instead of a hydroxyl group.

centrations ranged from 5×10^{-9} M to 3×10^{-10} M. Cytoplasmic receptors were determined from cell-free lysates adjusted to a protein concentration of 2 mg/ml and incubated with varying estrogen concentrations for 16 hr. Dextran-coated charcoal was added to the incubation medium after this period to remove any unbound estrogen. After centrifugation, 1 ml of the supernatant was removed and counted. Nuclear receptors were determined in the same fashion, but with a prior protamine sulfate treatment. Binding status was determined via Scatchard plot analysis.

To determine possible binding of estramustine with estradiol receptors, cell samples pretreated with radiolabeled estramustine were "chased" with concentrations of cold estradiol equivalent to 50, 100, and 1000 times the initial drug concentration. Residual estramustine binding was estimated by scintillation counting.

Alkaline elution assay for DNA damage. By using alkaline elution methodology described previously (13), the presence of DNA damage following estramustine treatment was monitored in log-phase HeLa cells preincubated for 24 hr with [2-^{14}C]dThd (0.02 μCi/ml) (2×10^5 cells per treatment). Drug was administered at a concentration of 1, 2.5, or 5 μg/ml for 2, 12, or 24 hr at 37°. Irradiation with 300 R (^{137}Cs source) at 132 R/min was carried out at 0°. Cells were mixed with 3×10^5 L1210 mouse leukemia cells that had been prelabeled for 24 hr with [6-^{3}H]dThd. The mixture of cells was layered onto polyvinyl chloride filters (2-μm pore, Millipore Corporation, Bedford, Mass.) and lysed with 2% sodium lauryl sulfate and 0.02 M Na_2EDTA (pH 10.0) in the presence of proteinase K. The retained DNA was eluted by pumping tetrapropylammonium hydroxide/0.02 M EDTA (pH 12.1) through the filter at a pump rate of 0.035 ml/min. Three-hour fractions were collected. Samples were counted in a liquid scintillation counter, and the elution patterns of the ^{14}C-labeled HeLa cells and the ^{3}H-labeled L1210 internal standards were computed.

HPLC. Nuclear matrices from log-phase HeLa cells were extracted and solubilized in 3 M guanidine hydrochloride/0.15 M NaCl/0.01 M Na_2HPO_4 (pH 7.0), which was also used as the mobile phase during HPLC separation. Separation was achieved on a Waters liquid chromatographic system (Milford, Mass.), using a 60-cm TSK 3000 SW column (Kratos, Westwood, N. J.). The flow rate was 1 ml/min; chart

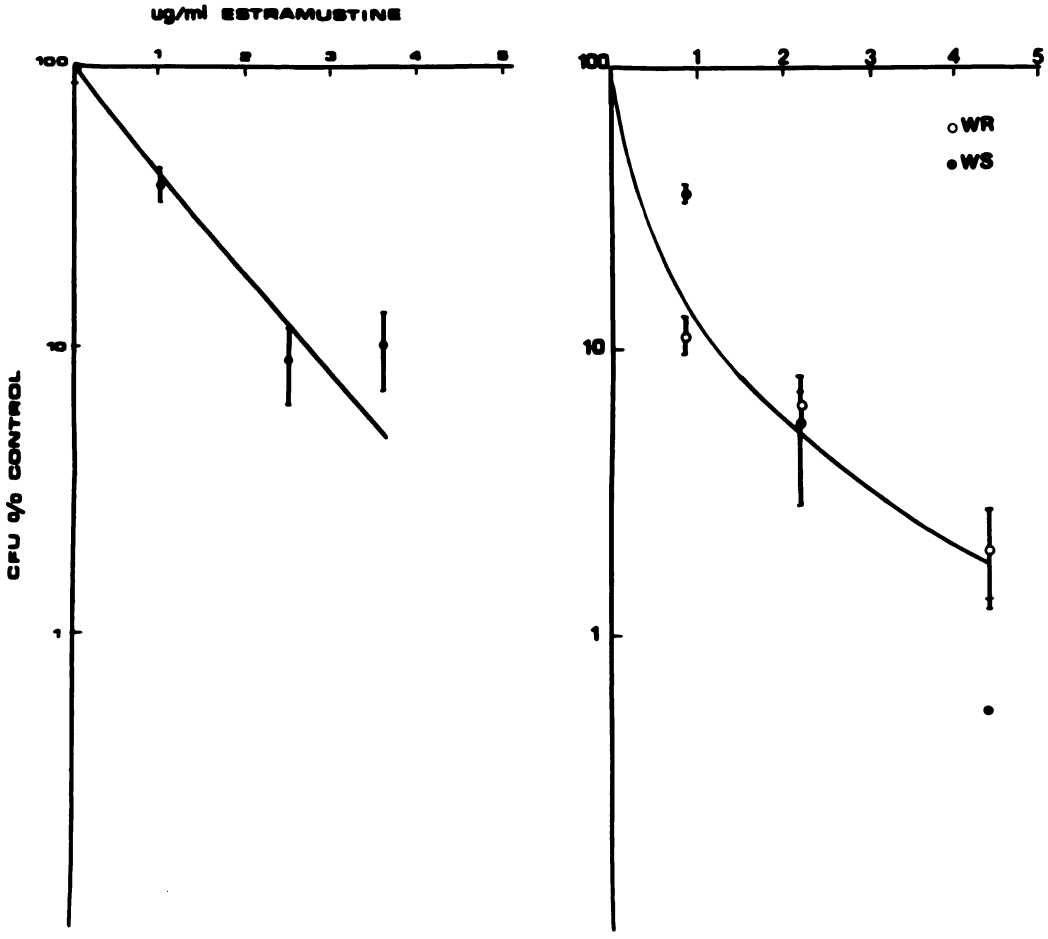

FIG. 2. *Cytotoxicity of estramustine in HeLa cells (left) or Walker 256 carcinoma cells (right) sensitive (WS) or resistant (WR) to nitrogen mustards*

Soft agar colony-forming assays were carried out as described under Materials and Methods (plating efficiencies: HeLa 30%; Walker 70%). Each point represents the mean ± standard deviation for four experiments. The ordinate is a log plot of colony-forming units (*CFU*) expressed as a percentage of vehicle (ethanol)-treated control cultures.

speed, 0.5 cm/min; sensitivity, 0.05 AUFs; absorption, simultaneous A_{280}, A_{260}; 0.7-ml fractions were collected and counted using ACS as a fluor in a Mark III scintillation counter.

Drug treatments. Estramustine and [2,4,6,7-^{3}H]estramustine (102 Ci/mmole) were obtained from AB Leo Research Laboratories (Helsingborg, Sweden). Initial incubations with estramustine were carried out for 24 hr; subsequent experimentation tested 4-, 6-, and 16-hr incubations. For the nuclear incorporation and matrix experiments, 100 μCi of [^{3}H]estramustine were used for 24 hr. [2,3,6,7-^{3}H]Estradiol (104 Ci/mmole) was obtained from Amersham. [2-^{14}C]Thymidine (50 mCi/mmole) and [5-^{3}H]uridine (25 Ci/mmole) were obtained from New England Nuclear Corporation. Cells for HPLC separation were prelabeled with [2-^{14}C]thymidine for 10 min and for 1 hr with [5-^{3}H]uridine.

RESULTS AND DISCUSSION

Figure 2 shows that both HeLa and Walker mammary carcinoma cells were killed by low concentrations of estramustine over a 24-hr period. Cell killing was similar in the HeLa and Walker cell lines (ID_{90} values approximately 2.5 μg/ml for HeLa; 1.5 μg/ml for WS). In addition, no resistance was expressed by the WR cells [resistant to bifunctional nitrogen mustards (9)]. The survival curves for WR and WS were overlapping (Fig. 2, *right panel*). Since acquired resistance to mustards in WR cells has been shown not to be a function of impaired uptake of drug (9), estramustine was not inducing cytotoxicity by classical nitrogen mustard pharmacological activity.

Nuclear incorporation of estramustine (estimated by using radiolabeled drug) in HeLa cells was found to be 1.34% of the available drug in the culture medium. This value was similar to that found for other alkylating agents (10). To estimate the degree of damage to DNA, alkaline elution assays were performed on estramustine-treated HeLa cells (Fig. 3). Methodology has been described elsewhere (13), and previous data have demonstrated extensive DNA strand breakage and cross-linkage following single-agent treatment with nitrogen mustard and other alkylating agents (14). Concentrations of estramustine producing ID_{50} or greater were chosen. Single-strand breaks were assayed by elution of drug-treated cells that were not irradiated. At all drug concentrations and time points, the treated cells were similar to non-drug-treated control cells, showing that the drug was not producing appreciable DNA breaks. The initial rapid elution on the elution profile could be attributed to a degree of DNA degradation occurring as a result of the presence of dead cells. Assays for DNA-DNA cross-links were achieved by treating cells with 300 R prior to elution. Drug cultures showed elution profiles similar to those receiving no estramustine, indicating that DNA interstrand cross-links were not produced by estramustine. Variations of the alkaline elution assay by (a) omitting proteinase K; (b) increasing the irradiation to 3000 R; (c) using drug incubation periods of 4, 6, and 16 hr; and (d) using an estramustine concentration of 10 μg/ml did not alter the elution profiles from those of non-drug-treated cells (data not shown). Thus, estramustine did not induce strand scission or DNA-DNA or DNA-protein cross-links at drug concentrations which are cytotoxic. The potential for estramustine to form chemically reactive electrophiles under *in vitro* conditions was estimated by measuring the alkylation of 4-(*p*-nitrobenzyl)-pyridine by a range of drug concentrations up to 100 μM. This method has been used to detect the presence of alkylating species (15). Data for this assay were negative for all drug concentrations and for incubation periods of up to 24 hr at 37°, suggesting that estramustine did not produce chemically reactive carbonium ions under *in vitro* conditions.

Isolation of the nuclear matrix from HeLa cells using standard procedures (11) revealed that approximately 0.4% of the available estramustine (one-third of the drug localized in the nucleus) was bound to this structural

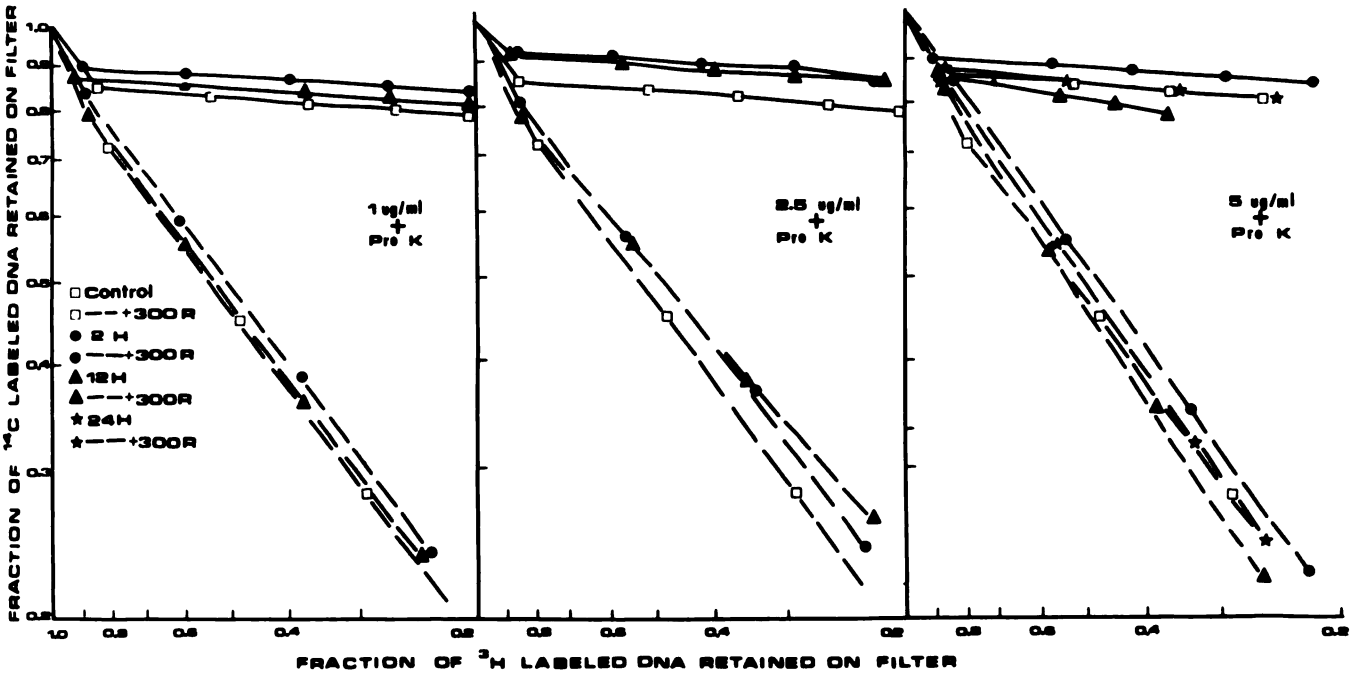

FIG. 3. *Assays for DNA damage following estramustine treatment*
See Materials and Methods for complete experimental details.

component. Since the matrix constitutes approximately 5% of the total nuclear proteins with residual amounts of DNA, RNA, phospholipids, and carbohydrates (11), estramustine is preferentially bound to these components. This preferential interaction is similar to that found for nitrosoureas, where approximately one-third of the nuclear-bound drug (alkylation or carbamoylation) is associated with the matrix (16). Using standard estradiol receptor analysis procedures (12), neither cytoplasmic nor nuclear receptors were detected in these HeLa cells. Exposure to 50 μM estradiol for 24 hr was not cytotoxic as judged by the CFU assay. In addition, bound estramustine could not be removed from the matrix by "chases" with cold estradiol. These factors suggest that drug binding was independent of estradiol receptor sites in HeLa cells. Further characterization of the matrix components was achieved by HPLC (Fig. 4). Conditions were used which favor the separation of the nucleic acid species from the protein components of the matrix. The *cross-hatched* area (14- to 24-min elution) shows the fractions which contained [^{14}C]thymidine and [^{3}H]uridine (i.e., residual low molecular weight DNA and RNA). These fractions are coincident with maximal absorbance at A_{260}. Protein and minor amounts of phospholipids [using standard thin-layer chromatographic procedures, these phospholipids were identified as phosphatidylserine and phosphatidylethanolamine (data not shown)] were found throughout the elution, but were the sole components of Fractions 8–13 min. This was consistent with the presence of the residual inner nuclear membrane in the matrix preparation. Treatment of HeLa cells with [^{3}H]estramustine for 24 hr prior to matrix isolation showed that drug binding localized in Fractions 8–13 min (histogram, Fig. 4), i.e., those fractions with only proteins and phospholipids, no nucleic acids. The lack of drug binding to nucleic acids was consistent with the absence of DNA damage in the alkaline elution assays. When the same matrix preparation was extracted with ethyl acetate, >98% of the drug was removed from the matrix, indicating a noncovalent binding between the drug and the matrix macromolecules. The bound drug was present either as the parent estramustine or as an oxidized metabolite, estromustine (see legend to Fig. 1), which is found to be the major plasma metabolite in man (17). The presence of estromustine was surprising, since previous studies failed to demonstrate this metabolite in an *in vitro* system (4). The relative absence of other metabolities suggests that no cleavage of the carbamate-ester bond had occurred, preventing the formation of estradiol or a mustard alkylating moiety. Since the drug binding to the matrix was resistant to sodium chloride and low molarity guanidine hydrochloride and dissociated by ethyl acetate, the drug interaction with the protein/phospholipid component of the matrix is likely to be hydrophobic in nature.

Thus, estramustine appears to act neither as a steroid nor as an alkylating agent in producing its cytotoxic effect in tumor cells. Approximately two-thirds of the nuclear estramustine is associated with macromolecules which are not part of the matrix. Primarily, these are chromatin components, although the outer nuclear membrane is also removed during matrix isolation. In HeLa cells, the nuclear matrix constitutes approximately 5% of

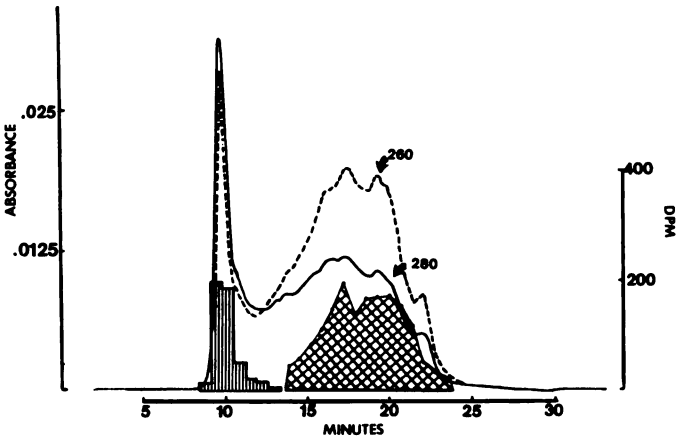

FIG. 4. *HPLC separation of nuclear matrix fractions from estramustine-treated HeLa cells*

Using cells which have been prelabeled with [2-^{14}C]thymidine and [5-^{3}H]uridine, the DNA and RNA content of the matrix was found to be localized within Fractions 14–23 min (*cross-hatched* area). Estramustine binding (*shaded* histogram bars) occurred in Fractions 8–13 min. The abscissa indicates the time of elution in minutes. The left ordinate shows absorbance at either 280 nm or 260 nm in arbitrary units. The right ordinate indicates disintegrations per minute of [^{3}H] estramustine or the mean disintegrations per minute of thymidine and uridine incorporation.

the total nuclear proteins, with residual quantities of phospholipids and nucleic acids (11, 16, 18). The remaining macromolecules are associated with chromatin components. The fact that approximately 40% of the nuclear-localized drug is bound to the 5% of nuclear macromolecules which constitute the matrix demonstrates a high degree of tropism. These interactions are presumably the result of stearic domains of the matrix permitting hydrophobic drug binding. The nuclear matrix is integral to many nuclear functional processes (18) and is conserved through mitosis as the chromosomal scaffold and mitotic apparatus (7, 8). During mitosis, spindle formation and chromatid attachment at the kinetochore is dependent upon the ordered interaction of microtubules and many microtubule-associated proteins. The hydrophobic binding of estramustine to the matrix may be of direct cytotoxic consequence in HeLa cells and other cultured cells through infererence with the ordered assembly of a functional mitotic apparatus. This possibility is further supported by its antimitotic action in human prostatic carcinoma cells, where metaphase arrest was found at cytotoxic concentrations (6). A similar effect of estramustine has been found in HeLa cells. A 24-hr treatment with 0.5 μg/ml produced a significant increase in the mitotic index from 0.9% to 6.0% [estimated by standard labeling techniques (19)]. This was accompanied by the appearance of typical stathmokinetic mitotic figures, and confirmed that estramustine was interfering with spindle formation. Structural comparison of estramustine with other known mitotic inhibitors, such as methyl(5-[2-thienylcarbonyl]-1*H*-benzimidazol-2-yl)carbamate (20), would suggest that the planarity of the substituted perhydrocyclopentanophenanthrene steroid nucleus and the nature of mustard linkage contribute stearic properties that are responsible for the drug's activity. In addition, the estramustine binding protein of the human prostate

(21) has been shown to contain high levels of acidic amino acid residues. The nuclear matrix polypeptides are also acidic in nature (11, 18). It is possible that these acidic properties contribute to the preferential estramustine binding to the matrix and may determine the cellular disposition and binding of the remainder of the drug. The possibility that cytoskeletal elements (e.g., actin, tubulin) are targets for estramustine is currently under investigation. It should be noted that actin has been found to constitute approximately 12% of the nuclear matrix of bovine lymphocytes (22). Presently, it is not clear how cytoskeletal elements interact with the nuclear matrix prior to the spindle formation at mitosis. The observed tropism of estramustine with the nuclear matrix may interfere with such interactions and may be responsible for the observed antimitotic effects.

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