DNA Damage and Cytotoxicity in L1210 Cells by Ellipticine and a Structural Analogue, N-2-(Diethylaminoethyl)-9-hydroxyelliptici
tinum Chloride

Zora Djuric, Carleen K. Everett, and Frederick A. Valeriote
Division of Hematology and Oncology, Department of Internal Medicine, Wayne State University, Detroit, Michigan 48201

ABSTRACT

N-2-(Diethylaminoethyl)-9-hydroxyelliptici
tinum chloride (DHE) is a
structural analogue of ellipticine that is currently a leading compound for clinical trials. We have investigated the mechanism of DNA damage by this compound in murine L1210 leukemia cells using the method of alkaline elution. Although DHE was about 100-fold more cytotoxic than ellipticine, this increased cytotoxicity was not accompanied by greater amounts of DNA strand breakage or protein-DNA cross-linking. The single strand breaks caused by both compounds were protein associated and could be accounted for by the presence of double strand breaks. DNA damage by the compounds therefore was consistent with topoisomerase II inhibition. Unlike DHE, 80% of the DNA damage elicited by ellipticine was repaired within 1 h after removal of drug. For DHE, 20-h incubations in drug-free media were required to obtain 70% repair of single strand DNA breaks. These data indicated that although both ellipticine and DHE may inhibit topoisomerase II, the type of DNA damage which resulted in topoisomerase II inhibition by DHE was much more persistent than the DNA damage elicited by ellipticine.

INTRODUCTION

Ellipticine and its analogues were first identified as highly cytotoxic antitumor compounds in 1967 (1). Subsequent preclinical trials did not demonstrate therapeutic efficacy with these compounds, possibly due to their limited solubility at physiological pH and to their toxicity (2). More recently, newer analogues of ellipticine have been developed to overcome these limitations. One of these analogues, DHE (SR95156B, Delti
tinum), is currently a leading compound for clinical trials (3, 4) and has already completed phase I trials (5). DHE contains a diethy
aminoethyl side chain and a 9-hydroxy substituent (Fig. 1). It is water soluble and displays antitumor efficacy in preclinical studies (3, 4).

Ellipticine and many of its analogues are known to inhibit topoisomerase II, and this property has been linked to anti
tumor activity (reviewed in Ref. 6). However, there is evidence that analogues of ellipticine may interact with DNA differently than the parent compound. Important structural features of active analogues appear to be the substituted quaternary nitrogen and the 9-hydroxy group. Although the parent drug ellipticine as well as its analogues can intercalate into DNA (6), a hydrophobic side chain may stabilize the intercalated complex (7). The 9-hydroxylated analogues also may be able to bind to DNA by hydrogen bonding interactions (8) and by covalent bond formation (9, 10). Metabolism of NMHE (Celtipinium) has been shown to result in formation of a quinone-imine which can bind covalently to DNA (9).

In this study, DNA damage by ellipticine and DHE has been examined in murine L1210 leukemia cells and related to their relative cytotoxic potencies. As determined by the technique of alkaline elution, both compounds caused DNA damage that was consistent with topoisomerase II inhibition; however, the DNA damage caused by DHE was much more persistent and this may contribute to its increased cytotoxicity towards L1210 cells.

MATERIALS AND METHODS

Materials. All chemicals and biochemicals were purchased from Sigma Chemical Co. (St. Louis, MO) except tetrapropylammonium hydroxide which was from RSA Corp. (Ardsel, NY). Polycarbonate filters were obtained from Nucleopore Corp. (Pleasanton, CA), and polynyl chloride filters were obtained from Omega Specialty Instrument Corp. (Chelmsford, MA). Ellipticine was obtained from the Drug Synthesis and Chemistry Branch, National Cancer Institute (Bethesda, MD). DHE was kindly supplied by Sanofi Research (Montpellier, France). NMHE was a gift from Dr. S. Archer (Rensselaear Polytechnic Institute, Troy, NY).

Cell Culture and Cytotoxicity. Murine L1210 cells were obtained from the National Cancer Institute. The cells were grown in suspension culture using RPMI 1640 (Gibco, Grand Island, NY) supplemented with 10 ml/100 ml calf serum, 100 units/ml penicillin, 100 ¡g/ml streptomycin, and 2 ¡g/ml L-glutamine. Cells utilized for the experiments were in the exponential growth phase and all incubations were conducted at 37°C in a 5.5% CO2 atmosphere.

Cells were diluted to a density of 1 ¥ 10^6/ml prior to treatment with compounds. The compounds were dissolved in 20% ethanol/80% 1 mM HCl at concentrations of 0.1 to 1 mM. The solvent alone did not cause any detectable cytotoxicity to L1210 cells. After incubation with the compounds, cells were washed by centrifugation and subjected to alkaline elution analyses. The viability of the cells after treatment with the compounds was verified by trypan blue exclusion. For repair studies, the cells were resuspended in fresh media and incubated at 37°C for various periods of time prior to alkaline elution.

Clonogenic survival assays were utilized to determine cytotoxicity using the method of Chu and Fisher (11). Aliquots of the cells were plated in triplicate on soft agar plates with the same media used for the suspension cell culture. After 2 weeks, colonies were counted and survival of treated cells was calculated relative to that of untreated control cells.

Alkaline Elution. Single strand DNA breaks were determined by the method of alkaline elution as described previously (12). Briefly, 5 ¥ 10^6 cells were placed on 47-mm filters; lysed with 45 mM tetrasodium EDTA-2 M sodium chloride, 2 mg/ml N-lauroylsarcosine, and 500 ¡g/ml protease (pH 10.0); washed with 25 mM tetrasodium EDTA-150 mM sodium chloride, pHi 10.3; and eluted at pH 12.1 with 20 mM EDTA (free acid)-100 mM tetrapropylammonium hydroxide. The DNA content of eluted fractions was determined fluorometrically using Hoechst 33258 dye.

Double strand DNA breaks were determined using 2-µm polycarbonate filters (13). For the assay of double strand breaks, the pH of the lysis, wash, and eluting buffers was 7.2 to prevent detection of alkali
cible sites as double strand breaks (14). The lysis buffer contained 100 mM glycine, 20 mM disodium EDTA, 50 mM Tris (pH 7.2), 0.1% N-
lauroylsarcosine (w/v), and 0.5 mg/ml protease K. The wash buffer was 20 mM disodium EDTA, 50 mM Tris (pH 7.2), and 50 mM glycine. The elution buffer was 20 mM disodium EDTA, 50 mM Tris (pH 7.2), and 50 mM glycine.
50 mM glycine, and 0.8% (v/v) tetrapropylammonium hydroxide.

The single and double strand break frequencies were calculated relative to the elution characteristics of DNA from untreated cells that were irradiated with either 5 or 100 Gy X-rays, respectively, in ice-cold 120 mM sodium chloride, 2.7 mM potassium chloride, and 10 mM potassium phosphate, pH 7.4, with a Siemens (Iselin, NJ) Stabilipan X-ray generator operated at 250 kV and 15 mA with filtration by 1-mm copper. The fraction of DNA retained on the filter after 21 ml of elution was determined and used to calculate DNA damage levels (15). Protein-DNA crosslinks were determined after irradiation of treated and untreated cells with 30 Gy X-rays at 0°C. This irradiation produces short strands of DNA which elute quickly from the filter unless they are linked to protein (16). The alkaline elution procedure was similar to that used for determination of single strand breaks except that 2-μM polyvinylchloride filters were used and no protease was included in the lysis solution. After elution, the filters in the EDTA wash solution were incubated at 37°C with 500 ng/ml proteinase K for 2 h. This step was added prior to the standard 30 min incubation at 65°C and 15 min vortexing in order to liberate all the DNA from the filter for fluorometric analysis. The frequency of protein-DNA cross-links was calculated using the “bound to one terminus” model of Ross et al. (17) as

\[ P_D = \frac{1}{(1 - r)} - \frac{1}{1 - r^2} \times P_R \]

where \( P_D \) is the frequency of drug-induced protein-DNA cross-links, \( P_R \) is the frequency of X-ray induced single strand breaks, and \( r \) and \( r^2 \) are the fractions of DNA eluted with 21 ml in the presence and absence of drug, respectively.

RESULTS

Cytotoxicity. Clonogenic assays were used to determine survival of L1210 cells after 2-h drug exposures. DHE exhibited a steep survival curve relative to ellipticine (Fig. 2A, B). Over 100-fold higher concentrations of ellipticine were required to achieve a similar level of cytotoxicity reached with DHE. NMHE exhibited intermediate cytotoxicity requiring at least 10-fold higher concentrations than DHE for similar levels of survival (Fig. 2C).

DNA Damage. Ellipticine and DHE each elicited single and double strand DNA breaks as well as protein-DNA cross-links in L1210 cells (Table 1). Using concentrations of each compound that resulted in roughly equal frequencies of single strand breakage, ellipticine was much less cytotoxic than DHE (Table 1). An increase in ellipticine concentration to 25 μM produced a 6-fold increase in single strand breakage to 1076 ± 73 rad equivalents (mean ± SD, n = 4); however, the surviving fraction was only decreased 2-fold to 0.24 (Fig. 2B). Similarly, NMHE elicited more single strand DNA breakage than DHE without an accompanying increase in cytotoxicity. Using 2 μM NMHE and 1 μM DHE, which elicited 354 ± 83 and 215 ± 63 rad equivalents of single strand DNA breaks, respectively, the survival of L1210 cells with NMHE was about 10-fold greater (Fig. 2).

The dose-dependent induction of single strand breaks was investigated since some topoisomerase II inhibitors have been shown to exhibit decreased cleavable complex formation at higher drug concentrations (18, 19). This was not the case with DHE which exhibited dose-dependent increases in single strand break formation (Fig. 2A). Although ellipticine did not stay in solution above 25 μM, experiments were conducted with 100 μM ellipticine which decreased the surviving fraction of cells 15-fold to 0.017. At this higher dose, DNA damage levels were 60% lower than those observed at 25 μM (Fig. 2B). NMHE caused a dose-dependent increase in single strand breakage up to 50 μM (Fig. 2C), and the biphasic nature of the survival curve was reflected in the DNA damage curve.

The association of protein with the single strand DNA breaks elicited by DHE was investigated for comparison to published results for ellipticine and NMHE (20). The frequency of protein-DNA cross-linking was within a factor of 2 to the frequency
of single strand breakage indicating that all of the single strand DNA breaks were protein associated (Table 1). Topoisomerase II inhibition also is characterized by double strand DNA breakage (20, 21). The ratios of single to double strand breaks for ellipticine and DHE (Table 1) do differ but are consistent with values that would be obtained for agents which induce exclusively double strand breaks (20).

DNA Repair. Repair of DNA damage following exposure to ellipticine was examined. As shown in Table 2, 80–100% repair of single strand breaks, protein-DNA cross-links, and double strand DNA breaks occurred within 1 h of drug removal following initial incubation with 5 μM ellipticine for two h. Repair of single strand breaks also was rapid using 25 μM ellipticine (80 ± 8% repair, n = 3). After an initial 1-h incubation with 5 μM ellipticine to duplicate published experiments (20), 70% repair of single strand breaks was observed 2 h after removal of drug (105 ± 5 decreased to 30 ± 5 rad equivalents, mean ± mean variance, n = 2). Repair of NMHE-induced single strand breaks was not quite as rapid with 63% repair in 1 h (Table 2).

Repair of DNA damage elicited by DHE was not detected using incubation times similar to those used for ellipticine and NMHE. After treatment of L1210 cells with 1 μM DHE for 2 h followed by incubation in fresh media for 1 h, very little or no repair of DNA damage was detected (Table 2). Allowing a longer repair period (2 h), similar results were obtained after an initial 120- or 30-min incubation with 1 μM DHE, which elicited initial levels of single strand breakage of 285 and 65 rad equivalents, respectively (Fig. 3). With 250 nM DHE, which elicited single strand break frequencies similar to those in 30-min incubations, DNA repair again was not detected (data not shown). This is unlike other topoisomerase II inhibitors where low levels of DNA damage can be repaired readily (19).

Single strand DNA breaks elicited by DHE were still detected 20 h after removal of the compound (Fig. 4). Repair did occur, but it was still less than in cells treated with ellipticine. The values for repair were 68 ± 7% repair for DHE and 96 ± 3% repair for ellipticine-induced single strand breaks.

**DISCUSSION**

DHE possesses structural characteristics which may contribute to its increased antitumor efficacy relative to ellipticine (Fig. 1). In particular, the presence of a quaternary nitrogen is suggested to result in a wider spectrum of activity towards tumor cell lines (6). In addition, analogous to other 9-substituted ellipticine analogues (6–9, 18), it may interact with DNA differently than ellipticine. In this study, we have investigated DNA damage and cytotoxicity by DHE in murine L1210 cells *in vitro* and compared it to that of ellipticine.

The greatly increased cytotoxicity of DHE to L1210 cells relative to ellipticine was not accompanied by greater amounts of DNA damage (Table 1: Fig. 2). This also has been observed with other topoisomerase II inhibitors where the amount of single strand DNA breaks caused by structural analogues does not always reflect cytotoxic potency (reviewed in Refs. 6 and 19). One explanation for this apparent discrepancy may be the complexity of the topoisomerase II interactions with the DNA-drug complex. Many topoisomerase II inhibitors exhibit bell-shaped curves with respect to DNA cleavage; at higher drug concentrations cleavage is suppressed. This has been suggested to be the result of two modes of drug interaction with DNA, namely simple intercalation at low concentrations to stimulate

![Graph](http://example.com/graph.png)

**Fig. 3.** Lack of repair of single strand DNA breaks elicited by DHE in L1210 cells. After incubation of cells with 1 μM DHE for 30 (Δ) or 120 (□) min, cells were washed free of drug and incubated in fresh media for 2 h. △, DNA elution curves for cells with no repair period; △, ○, curves for cells allowed the additional 2-h incubation. Untreated control cells also are indicated with (Φ) and without (Ο) 5 Gy irradiation.

![Graph](http://example.com/graph2.png)

**Fig. 4.** Repair of single strand breaks elicited by either 1 μM DHE (Φ) or 5 μM ellipticine (Δ). Exposure to each compound was for 2 h followed by a 20-h repair period in fresh media. △, Φ, elution curves prior to repair; △, ○, elution curves for cells after 20 h repair. Experiments shown are representative for 3 separate experiments (see "Results").
cleavage and external binding at higher concentrations to suppress DNA cleavage by topoisomerase II (18, 19).

DHE or NMHE did not exhibit decreases in DNA damage at higher concentrations while this effect was seen with ellipticine (Fig. 2). This may be a function of the amount of drug reaching the nucleus. In incubations with purified topoisomerase II or isolated nuclei, decreased DNA cleavage at higher concentrations of DHE has been observed while in intact N417 cells DNA damage increased with dose (22). In cells, cytosolic concentrations of DHE has been observed while in intact N417 cleavage and external binding at higher concentrations to suppress DNA cleavage by topoisomerase II (18, 19).

Topoisomerase II inhibition by ellipticine may result from trapping of the enzyme in a complex with the DNA (21). If topoisomerase interacts with DNA largely by intercalation, as suggested (8), complex formation would be expected to be readily reversible (6). Analogues of ellipticine, however, may be able to interact with DNA via other mechanisms, including covalent binding, hydrogen bonding, stabilized intercalation, and site-specific binding (6–9, 18, 25). Thus, the type of DNA interaction which caused topoisomerase II inhibition by inhibition may be different than that for ellipticine.

In summary, alkaline elution studies indicated that DHE and ellipticine caused topoisomerase II inhibition in L1210 cells. The increased cytotoxicity of DHE to L1210 cells relative to ellipticine was not accompanied by increased amounts of DNA damage. Rather, it appeared that the persistence of DNA damage was related to cytotoxicity and this persistence of damage may be a result of the nature of the interaction(s) with DNA.

ACKNOWLEDGMENTS

We thank Kerry S. Palmer and Julie Dieckman for assistance with the L1210 cell culture and Dr. Wilfried DeNeve for writing the computer program for calculation of DNA damage levels.

REFERENCES

14. Flick, M. B., Warters, R. L., Yamai, S. L., and Kirsch, R. E. Measurement of radiation-induced DNA damage using gel electrophoresis or neutral elution shows an increased frequency of DNA strand breaks after exposure to pH
DNA DAMAGE BY ELLIPTICINE ANALOGUES


DNA Damage and Cytotoxicity in L1210 Cells by Ellipticine and a Structural Analogue, \( N \)-2-(Diethylaminoethyl)-9-hydroxyellipticinium Chloride

Zora Djuric, Carleen K. Everett and Frederick A. Valeriote