Potential Antiglioma Activity of 9-Hydroxy-2-N-methylellipticine as Determined by Pharmacological and Human Tumor Clonogenic Cell Studies

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ABSTRACT

The antiglioma activity of elliptinium (HME) was investigated in a human glioma clonogenic cell assay. Early passage cells of three human glioma cell lines (SF126, SF375, and SF407) were exposed to HME at the clinically achievable dose of 3 μM for 3 h. At this HME concentration, clonogenic cell survival was reduced by more than 3 logs in SF126 and SF375, and by 0.8 logs in SF407. A study of the kinetics of cell kill showed that whereas at moderate (±1.5 μM) HME doses cell kill increased with treatment time up to a maximum at approximately 3 h, cytotoxicity was more dose than time dependent at higher doses. Flash treatment of SF375 cells with 3 μM HME resulted in more than 2 logs clonogenic cell kill. Using high-pressure liquid chromatography, we investigated the in vitro decay kinetics of HME under our in vitro drug treatment conditions and observed a very rapid, protein nondenpendent 40% drop in HME concentration which was dose dependent and was probably due to HME adsorption on the surface of tissue culture plasticware. Subsequent decay of the drug was very slow, with a decay rate constant of 0.022/h and a half-life of 298 h. In order to determine whether HME crosses the blood-brain barrier, we measured the rat brain capillary permeability coefficient, P, of [3H]HME and [14C]HME. The mean P value of 2.2 x 10^-6 cm/s ± 16% (SD) suggests that HME crosses the blood-brain barrier (t1/2 = 46 min) consistent with its molecular size and octanol-water partition coefficient.

INTRODUCTION

HME, a 9-hydroxy-2-N-methyl derivative of ellipticine, was first described by LePecq et al. (1) and was shown in preclinical studies to have significant antitumor activity against the murine L1210 leukemia and other animal tumors with little accompanying bone marrow toxicity (1, 2). Furthermore, the acute hemolytic and cardiovascular toxicities observed previously with other ellipticine derivatives (3) occurred to a much lesser degree. These observations consequently led to Phase I (4–6) and Phase II (7, 8) clinical trials of HME. In these trials, the drug showed activity against a number of solid human tumors especially of the breast and its associated skin and bone lesions (7–9). Because of its lipophilicity and molecular size, we considered HME to be potentially useful in the treatment of central nervous system cancers. In this study, therefore, we (a) measured HME decay kinetics in a human glioma clonogenic cell culture system in order to optimize in vitro drug exposure conditions and to relate it to clinically achievable drug exposures, (b) examined the effects of HME on the in vitro clonogenic cell growth of 3 human gliomas, and (c) measured rat brain capillary permeability coefficients to determine whether HME crosses the blood-brain barrier. In this report, we will show that HME crosses the blood-brain barrier and that at clinically achievable doses is markedly cytotoxic to human glioma cells in vitro.

MATERIALS AND METHODS

Tumor Cell Lines. Three human glioma cell lines, SF126, SF375, and SF407, were derived from primary surgical biopsy specimens obtained from patients who underwent craniotomies for tumors at our institution. Cells were maintained by serial passage of confluent monolayer cultures, and were screened for and shown to be free of mycoplasma contamination before being used in these studies. The passage numbers of the cell lines were SF126, 11, SF375, 4, and SF407, 2.

Using an indirect immunofluorescence technique, each cell line was characterized by reactivity to an antisum against glial fibrillary acidic protein (10) and/or to a monoclonal antibody (GE2) that recognizes a glial tumor-associated antigen (11). For this, the cells were cultured for 5 days in an 8-chamber tissue culture grade microscope slide (Miles Scientific, Naperville, IL) in a humidified 5% CO2-air atmosphere at 37°C. After removing the culture supernatant, the monolayers were washed twice with cold (4°C) PBS, pH 7.2, fixed in cold methanol, and rinsed once more in cold PBS. The cells were then treated with a rabbit anti-GFAP antisum (kindly provided by Dr. Larry Eng, Stanford University, Palo Alto, CA) or with a mouse anti-human monoclonal antibody, GE2 (kindly provided by Dr. Nicholas de Tribulet, Lausanne, Switzerland). After incubation in a humidified chamber at 37°C for 30 min, the cells were gently rinsed twice with cold PBS and incubated with rhodamine-conjugated mouse anti-rabbit (for GFAP) or a goat anti-mouse antibody (for GE2) for another 30 min at 37°C in a humid chamber. The cell layers were then rinsed twice with cold PBS, mounted in aqueomount, and using phase contrast, examined under UV microscopy (Olympus Vanox fluorescence photomicroscope; Olympus, Tokyo, Japan). GFAP positive cells were counted using an QIP2 cytometer.

Drugs. HME, [3H]HME, and [14C]HME were all obtained in the acetate form from S. A. Labaz-Sanoff, N. V. Bordeaux, France or Brussels, Belgium. The chemical and radiopurity of the radiolabeled HME were determined by the company to be 99% based on analysis by HPLC using a μBondapak reverse-phase column, 70% methanol:30% 0.01 M ammonium acetate solvent system, and detection at 313 nm. Both radiolabeled compounds were used in the brain permeability studies within 2 months of receipt.

In Vitro Decay Kinetics. The kinetics of in vitro drug decay was studied with cultures of SF126 cells. A set of 4 flasks containing 10⁶ SF126 cells, 10⁶ heavily X-irradiated (40 Gy) 9L rat gliosarcoma cells, and MEM supplemented with Earle’s balanced salts and 20% FCS (10 ml total volume) were incubated for 24 h in a humidified atmosphere at 37°C in 5% CO₂. Stock freshly prepared HME solution was then added to each flask to yield a final concentration of 10 μM. One ml of the culture supernatant from each flask was aliquoted at the following time points:

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C is the terminal brain tissue (dpm per ml), and PW is the brain plasma activity curve (dpm per min per ml), Cpi is the terminal plasma (dpm per ml), and 0.28 is a correction factor that accounts for capillary geometry. The measurement of K, has been described previously (per sec), CD is the intercapillary distance (cm), BV is the brain blood volume (ml per g), and P was calculated using the equation

\[ P = \frac{0.28 \text{ (ICD) } K_i}{\sqrt{BV}} + 0.1 \]

where \( K_i \) is the capillary permeability times the surface area coefficient (per sec), ICD is the intercapillary distance (cm), BV is the brain blood volume (ml per g), and 0.28 is a correction factor that accounts for capillary geometry. The measurement of \( K_i \) has been described previously (14)

\[ K_i = \frac{0.93C}{AUC} \left( 1 - PW \frac{C_p}{C} \right) \]

where AUC is the area under the [3H]- and [14C]HME plasma disappearance curve (dpm per min per ml), \( C_p \) is the terminal plasma (dpm per ml), C is the terminal brain tissue (dpm per ml), and PW is the brain plasma water (ml per g).

RESULTS

In Vitro Pharmacokinetics. The decay kinetics of HME, under the culture conditions of the clonogenic assay (MEM, Earle’s balanced salt solution, 20% FCS, 37°C, and 5% CO2) as monitored by HPLC was biexponential with a very rapid 40% drop in HME concentration followed by a subsequent slow decay with a decay rate constant of 0.022/h and a half-life of 298 h (Chart 1). Because the HME decay kinetics were similar in medium with and without 20% serum and with and without cells, the rapid decay phase was most probably due to HME adsorption to the surface of the plastic tissue culture flasks rather than to protein binding or cellular uptake. Consequently, doses used to plot Charts 1, 2, and 3 were based on actual measured HME levels.

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Treatment Duration (hrs)

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<th>8.4</th>
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<td>10^-3</td>
<td>10^-4</td>
<td>10^-5</td>
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</tr>
<tr>
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<td>10^-2</td>
<td>10^-3</td>
<td>10^-4</td>
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<td>10^-6</td>
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Charts. Kinetics of 1.5 μM HME cytotoxicity on human gliomas SF375 and SF407. At each time point, the cells were rinsed twice and incubated for an additional 3 weeks as described in the text. The surviving fraction of clonogenic cells at each time point was determined relative to untreated controls at the same time point. Bars, SD.

IN VITRO CYTOTOXICITY. Chart 2 summarizes the in vitro sensitivities of all 3 human tumors. After a 3-h treatment with 1.5 μM HME, clonogenic cell kills of 1.48 (SF375), 1.18 (SF126), and 0.71 (SF407) logs, respectively, were obtained. A 3-h, 3 μM HME exposure resulted in more than a 3-log clonogenic cell kill in both SF375 and SF126 and 0.8 log kill in the SF407.

Chart 3 illustrates the kinetics of in vitro cytotoxicity of 1.5 μM HME on SF375 and SF407 cells. Maximum clonogenic cell kill was achieved after an approximately 3-h drug exposure. Longer incubation times, even up to 24 h, resulted in little additional cell kill. In contrast, exposure of SF126 cells to a lower dose (0.6 μM) HME resulted in a time-dependent cell kill over 24 h (Chart 4).

The results of flask treatment of SF126 cells with increased doses of HME (drug exposure of only a few s) are shown in Chart 5. There was a dose-dependent clonogenic cell kill of up to 2.4 logs at 3 μM.

Brain Capillary Permeability. Using [3H]- and [14C]HME, rat brain capillary permeability coefficients were determined at 6, 10, and 20 min. Because the 10- and 20-min values for both [3H]- and [14C]HME were similar, the data were pooled. The permeability coefficient obtained from 20 brain samples in 5 rats was found to be 2.2 × 10^{-6} cm/s ± 16% (SD), and the K_i value was 0.015/min (t_1/2 = 46 min).

To verify the permeability value, we measured the octanol-phosphate buffer (pH 7.5) partition coefficients (P0) for [3H]-HME, [14C]HME, and unlabeled HME using the technique of Hansch and Leo (15). The P0 for [3H]HME was 2.6, whereas that for [14C]HME was 0.86. The mean P0 value for unlabeled HME at 0.03 and 0.15 μM was found to be 0.34 ± 0.09.

DISCUSSION

Progress in the chemotherapy of human brain tumors is largely dependent upon the identification of new chemotherapeutic agents which are: (a) active against brain tumor cells; (b) are able to permeate the blood-brain barrier; (c) have a high therapeutic index; and (d) are not cross-resistant with drugs currently used in the treatment of these cancers. The encouraging antitumor activity of HME in advanced breast cancer (9) and the relatively low myelosuppression encountered during Phase I clinical trials coupled with a molecular size and structure suggestive of ability to cross the blood-brain barrier led us to investigate the efficacy of the drug as a potential agent for antiglioma chemotherapy. Concurrently, a limited Phase I clinical trial was carried out at our institution.

The results of 2 separate experiments showed HME to be very stable under our in vitro culture conditions. The rapid initial drop in concentration we observed was probably attributable to adsorption on the surface of the plastic tissue culture treatment flasks and less probably to protein binding or cellular uptake. It

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was dose but not time dependent and was the same in the presence and absence of cells and for medium both with and without 20% FCS supplementation. This observation does not suggest that there may not be a significant rapid uptake of HME by the cells. Our results of cytotoxicity after flash treatment of glioma cells with HME as discussed in other sections of this paper do in fact suggest that there might be a significant rapid cellular HME uptake. However, because of the small number of cells per treatment flask relative to the total surface area of the flask and the volume of medium overlying the cells, any such cellular uptake should not result in a drop in HME concentration as large as was observed. In computing the in vitro drug exposures (AUC), we therefore monitored the HME concentrations by HPLC and adjusted the HME treatment doses to account for the concentration drop. This way, the actual in vitro AUCs to which the cells were exposed could be related to those obtained in clinical pharmacokinetic studies.

In vitro cytotoxicity studies indicate that 2 of the 3 selected malignant glioma cell lines showed highly significant cell kill (≥3 logs) when cells were exposed to 3 μM HME for 3 h. Flash exposure of cells to 3 μM HME resulted in more than a 2-log kill of clonogenic cells. The kinetics study showed that maximum cell kill at 1.5 μM of 1.18 (SF126) and 1.48 (SF375) was achieved after approximately a 3-h treatment. These HME doses at which such high levels of glioma cell kill could be achieved in vitro are well within the range (3 to 12 μM) of plasma levels reported to be achievable in patients after a total i.v. injection of 140 to 160 mg HME over a 1-h period (6). It is interesting to relate the in vitro cytotoxicity levels achieved with HME to the 0.22-log cell kill at clinically achievable doses that discriminate between in vitro sensitivity and resistance to chloroethylnitrosoureas for human gliomas (12). In a Phase I clinical trial performed at our institution, plasma HME levels of 0.74 to 2.45 μM and a plasma AUC average of 3.3 μM·h were obtained for i.v. doses of HME, 2 mg/kg, over 120 to 208 min. Even at in vitro HME exposures within this low plasma AUC range, high levels of clonogenic cell kill of SF375 and SF126 was obtained (Charts 2 and 3). These results indicate that at high HME doses (≥1.5 μM), cytotoxicity appears to be more a dose- than time-related phenomenon. Li and Cowie (16) have observed a similar dose-dependent irreversible inhibition of L1210 cell growth upon contact with ellipticine (the parent compound of HME) for 15 to 20 min. Paolletti et al. (17) have also reported a dose-dependent significant shift in DNA sedimentation rate in sucrose gradients after only a few s exposure of L1210 cells to HME.

Although the actual mechanism of HME cytotoxicities is still not fully understood, our results and those mentioned above suggest that a major cytotoxic lesion is induced by HME very rapidly. One such possible lesion is the peroxidase-H2O2-catalyzed generation of free radicals via an iminoquinone (18, 19). The lipophilicity and the ability of HME to cross the blood-brain barrier were demonstrated by measurement of P0 values and of the rat brain capillary permeability coefficient using established techniques (13, 14). Because of possible differences in radiopurity of [14C]- and [3H]HME thus resulting in differences in P0 values measured with them, the mean P0 value of 0.34 for unlabeled HME at 2 concentrations (0.03 and 0.15 μM) was used.

The permeability coefficient of HME was found to be 2.2 × 10^{-6} cm/s which with a P0 of 0.34 and a molecular weight of 336 for HME is close to the coefficient of 4.8 × 10^{-6} cm/s computed using a linear regression model determined in previous studies of brain capillary permeability (13). Thus, HME permeates the rat blood-brain barrier with a t1/2 of approximately 46 min and at a rate similar to that determined for another anticancer drug, didronomodulcitol (13).

The findings in this report of significant in vitro antitumor activity at clinically relevant doses as well as the blood-brain barrier permeation by HME suggest that the agent or an analogue of HME may be potentially useful in the chemotherapy of patients harboring malignant gliomas. Its relative lack of myelosuppression would further suggest that, provided there is no cross-resistance with the chloroethylnitrosourea, HME might be useful in combination therapy with the chloroethylnitrosoureas or for the treatment of patients with tumors refractory to chloroethylnitrosourea therapy.

A major clinical limitation of HME is its nonhematological toxicities (4, 5, 7–9) which have been shown in Phases I and II clinical trials to include cumulative renal toxicity (especially in patients with preexisting renal dysfunction, immunologically mediated hemolysis, acute phlebitis at the injection site, gastrointestinal disorders and, in some (older) patients, acute cardiovascular toxicity (notably, hypotension and bradycardia). Although as noted previously the exact antitumor mechanism of HME is not yet known, some of these clinically observed toxicities have been associated with the presence of specific chemical groups at certain positions on the HME molecule (20) suggesting that appropriate chemical modification of the parent compound might help improve the therapeutic index of the drug. An in vitro cytotoxicity study with a clonogenic tumor cell assay, as described in this report, might prove useful in comparatively evaluating the potential antitumor activity of future HME analogues.

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REFERENCES


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