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Advances in Brief

Estramustine Sensitizes Human Glioblastoma Cells to Irradiation[1]

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Abstract

Estramustine is an estradiol-based antimicrotubule agent that accumulates in malignant glioma cells, resulting in a concentration-dependent inhibition of proliferation. This agent has been shown to synchronize human glioma cells at G_2-M consistent with its known effects on the mitotic spindle and potentially could be used as a radiation enhancer. We determined the effects of estramustine on the cell cycle of glioblastoma cells by flow cytometry. These findings were compared with clonogenic survival in cells pretreated with varying concentrations of estramustine prior to irradiation. These experiments indicated that 24 h treatment with 1 μM estramustine had no effect on the percentage of G_2-M cells and did not enhance the cytotoxic effects of radiation while 10 μM estramustine increased the G_2-M fraction by 100% associated with a potentiation factor as high as 8.5 and a relative radiation sensitivity at 70% cytotoxicity of 5.2 compared with 15.4 for control cells. Estramustine can be administered p.o. on a daily schedule with minimal systemic toxicity. These data suggest that estramustine may be an effective radiation enhancer for glioblastoma.

Introduction

With conventional therapy the median survival for patients with malignant gliomas of the brain is around 1 year and the majority of patients die as a result of recurrence of the tumor within a few cm of the original lesion (1). Radiation therapy has been shown to be an important treatment modality for malignant gliomas and recent clinical studies have shown that increasing the dose of focal irradiation can increase survival in selected patients (2, 3). However, the risk of immediate and delayed toxicity limits the amount of irradiation that can be used safely to treat brain tumors in a majority of patients. The identification of a radiation enhancer may improve the local tumor control and increase the effectiveness of current radiation management. An optimal radiation enhancer should accumulate within malignant gliomas and be administered throughout the course of radiation treatment with minimal systemic toxicity. Estramustine, a conjugate of estradiol and nitrogen mustard, has been shown to meet many of these criteria. This agent accumulates within tissues containing estramustine binding protein, a binding site expressed in malignant gliomas, and has potent *in vitro* antiproliferative effects on human glioblastoma (4–8). Drug uptake in glioma cells has been shown to depolymerize microtubules resulting mitotic arrest associated with an accumulation of cells at G_2-M (8, 9). Pretreatment with estramustine enhances the effects of radiation on human prostatic carcinoma cells *in vitro* (10). On the basis of these observations we sought to determine whether estramustine also could be used as a radiation sensitizer in human glioblastoma. The human glioblastoma cell line HS683 was preincubated with varying concentrations of estramustine prior to treatment with ionizing radiation to examine drug effects on radiation sensitivity. Cell survival by clonogenic assay and the effects on distribution of cells in the cell cycle were determined by flow cytometry.

Materials and Methods

A human glioblastoma cell line, HS683 (American Tissue Collection, Rockville, MD), was used for these experiments (4, 8). Cell cultures were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (both from Sigma Chemical Co., St. Louis, MO), penicillin and streptomycin in a humidified tissue culture chamber at 37°C in 5% CO_2. At the time of experimentation cells were lifted from subconfluent cultures with 0.25% trypsin in 5 mM EDTA, counted on a hemocytometer, and plated at the appropriate density according to the experimental protocol below.

Pure estramustine was supplied by the manufacturer, Kabi-Pharmacia AB, Helsingborg, Sweden. The drug was dissolved in DMSO[3] for a stock solution of 10^{-2} M and stored at −20°C. This solution was diluted in medium to obtain the required concentrations for study. In each experiment control specimens were used and treated with Dulbecco's modified Eagle's medium in 0.1% DMSO to reflect the amount of vehicle in the drug-treated samples. All studies were performed in triplicate.

For flow cytometry cells were plated at a density of 3×10^6 cells in a 150-cm flask and allowed to attach overnight. These cells were exposed to 0.1% DMSO or 1 or 10 μM estramustine for 4, 8, 12, 16, or 24 h after which the cells were harvested with trypsin and collected by centrifugation. The resulting cell pellet was resuspended by vortexing and washed three times in phosphate buffered saline. After the final wash the cell suspension was further diluted with ice cold ethanol (95%) added in 2-ml volumes for a total suspension volume of 8 ml. For each sample cells were resuspended in 1 mg/ml RNase (Sigma) for 30 min at 37°C and then stained with 0.05 mg/ml propidium iodide (Sigma) for 1 h on ice. Flow cytometric analysis was performed with a FACS IV flow cytometer (Becton-Dickinson, San Jose, CA). The cells were excited at 488 nm and the emission was collected above 590 nm. A minimum of 2×10^4 cells were analyzed for each sample. Cell cycle analysis was performed according to the mathematical model of Jett (11).

Clonogenic survival was determined according to the method of Tishler *et al.* (12). Cells were plated (10^3/well) in 6-well plates and allowed to attach for 48 h. A minimum of 6 wells were used for each drug concentration and plates were treated with 0.1% DMSO or 1, 5, or 10 μM estramustine in 2-ml volumes for 24 h. Cells from each experiment were acutely irradiated with a ^{137}Cs source (J. L. Shepherd, San Fernando, CA) at 3.4 Gy/min from 0.6 to 4.8 min (2, 4, 8, 16 Gy) while exposed to drug. Following radiation the experimental medium was removed by aspiration and the cells were cultured in drug free media for 7 to 10 days. Wells were washed with phosphate buffered saline and stained with cresyl violet (0.25%) in methanol and colonies greater than 50 cells were counted with a ×8 lens.

Clonogenic capacity was calculated by a modification of the method of Wheeler *et al.* (13). Relative clonogenicity (RC) was determined as the number of cells plated normalized by the number of cells treated at the same concentration of estramustine without radiation. The effects of estramustine on radiation sensitivity were further expressed by the PF and RRS. The RRS was determined as the dose of radiation required to achieve a given level of cytotoxicity at each concentration of estramustine and without estramustine.

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[3] The abbreviations used are: DMSO, dimethyl sulfoxide; RRS, relative radiation sensitivity; PF, potentiation factor.

$$RC = \frac{\text{No. of colonies from treated cells/no. of cells seeded}}{\text{No. of colonies from untreated cells/no. of cells seeded}}$$

$$PF = \frac{RS \text{ from radiation alone}}{RS \text{ from radiation} + EM \atop RS \text{ from } EM \text{ alone}}$$

Results and Discussion

The percentage of HS683 cells in G_2-M varied from 19 to 26% within a 24-h study period. Treatment of HS683 with 1 μM estramustine for up to 24 h did not increase G_2-M cells. In contrast, there was a time dependent increase in the percentage of G_2-M cells with 10 μM estramustine (Table 1). At this concentration the proportion of G_2-M cells appeared to reach a maximum by 16 h and was sustained at 24 h. Accumulation in the G_2-M phase was associated with a decrease in the fraction of G1 cells.

The effects of estramustine and radiation alone on HS683 and the effects of pretreatment with estramustine before irradiation are demonstrated in Fig. 1*A* where data have been normalized to no irradiation and no estramustine. The 1 μM concentration of estramustine had a minimal effect on the slope of the survival curve compared with control. However, a progressive decrease in clonogenic capacity with increasing doses of irradiation was noted at 5 and 10 μM concentrations of estramustine. Further demonstration of the interaction between estramustine and irradiation are shown in Fig. 1*B* where survival curves are presented relative to no radiation at the same concentration of estramustine. There is a significant change in the slope of the 5 and 10 μM curves relative to control and the 1 μM curves. The dose-response data shown in Fig. 1*B* were analyzed using a least squares fit to the multitarget, single-hit model. The D_0s of these dose-response curves decreased from 13.5 Gy in the absence of drug to 10.3, 5.7, and 4.4 Gy at 1, 5, and 10 μM estramustine, respectively. Neither the values of the extrapolation number, n (0.89, 0.93, 0.85, and 0.95) nor the values of the surviving fraction at 2 Gy calculated from the fitted curves, SF_2 (0.79, 0.76, 0.60, 0.60) differed significantly at 0, 1, 5, and 10 μM estramustine, respectively. The significant decrease in the D_0 and constant value of n are in accord with the changes which would be expected if estramustine acted as a dose modifying radiosensitizer. The statistically insignificant decrease in SF_2 is likewise compatible with the small change expected from the dose modifying radiosensitization at this low radiation dose. The apparent radioresistance of the cultures may reflect the experimental protocol used in these studies, rather than an unusual intrinsic radioresistance of the HS683 glioblastoma cell line. A different protocol would be needed to define the radiation dose-response curves for single HS683 cells and to allow rigorous comparisons with single cell survival curves for other cell lines (14).

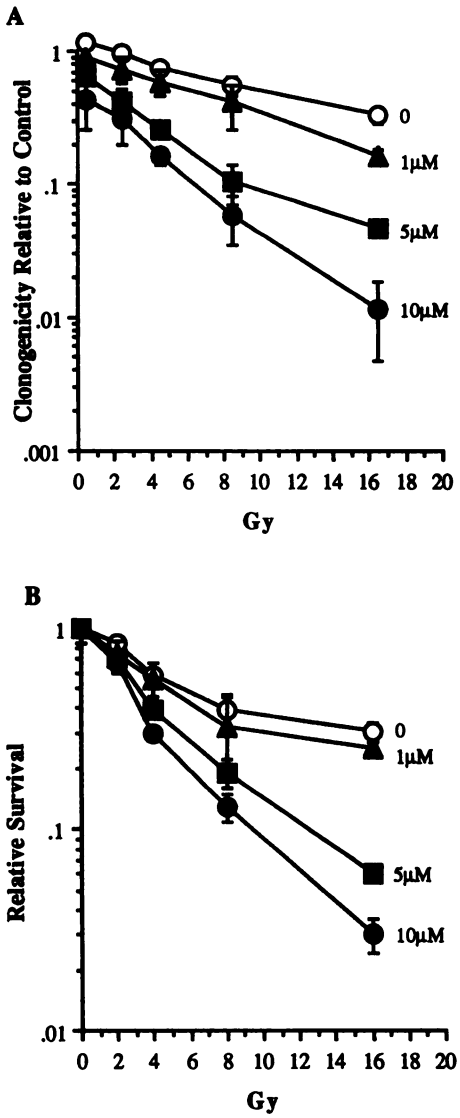

Fig. 1. Radiation survival curves for HS683 cells following 24 h treatment with 0.1% DMSO (0) or with 1, 5, or 10 μM estramustine. In *A* clonogenicity is expressed as a percentage of control values for no irradiation and no estramustine. Changes related to estramustine alone are demonstrated by the decrease in clonogenicity at 0 radiation. In *B* survival curves have been normalized to no radiation at each concentration of estramustine. The radiation enhancing effect of estramustine is shown by changes in the shape of curves at 5 and 10 μM. *Bars*, SE.

Table 1 *Flow cytometry results on H5683 cells treated with 10 μM estramustine for 4–24 h*

	% G_1	% S	% G_2-M
4 h			
C[a]	54	24	22
EM	46	26	28
8 h			
C	55	21	24
EM	31	33	36
12 h			
C	47	34	19
EM	18	43	39
16 h			
C	49	30	21
EM	17	35	49
24 h			
C	45	29	26
EM	7	35	58

[a] C, control (0.1% DMSO); EM, estramustine.

The radiosensitizing effects of pretreatment of HS683 with estramustine is further illustrated in Figs. 2 and 3. The 1 μM concentration had a minimal effect on the PF, and the RRS varied from control values only at 70% cytotoxicity. However, pretreatment with 5 and 10 μM estramustine resulted in a concentration dependent enhancement of the effects of irradiation. For example, at 16 Gy the PF for 10 μM is 8.5 and for 5 μM is 4.6. Fig. 3 demonstrates the dose of irradiation required to achieve a given level of cytotoxicity with or without pretreatment. There is a dramatic increase in cytotoxicity of irradiation for the 5 and 10 μM pretreated cells. For example, a 70% cytotoxicity was achieved with 15.3 Gy while the same level of toxicity was noted at 5.2 Gy in cells pretreated with 10 μM estramustine.

Eklov *et al.* (10) have shown that at concentrations ranging from 5 to 20 μg/ml estramustine enhanced radiation sensitivity by 23 to 30% in human prostatic carcinoma. Our data suggest that estramustine also

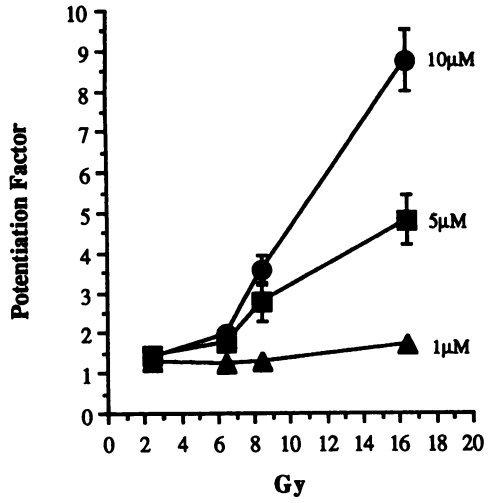

Fig. 2. Potentiation factor following pretreatment of HS683 cells for 24 h with 1, 5, or 10 μM estramustine. *Bars*, SE.

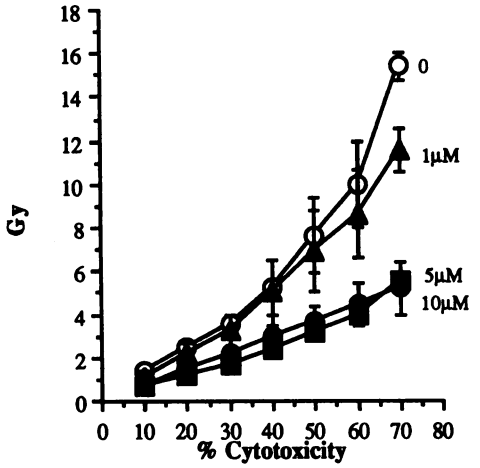

Fig. 3. Radiation dose required to achieve a given level of cytotoxicity (relative radiation sensitivity) following pretreatment for 24 h with 0.1% DMSO (0) or 1, 5, or 10 μM estramustine. *Bars*, SE.

is a potent radiation sensitizer in human glioblastoma and that, as anticipated, this effect as a radiation sensitizer correlates with synchronizing HS683 in G_2-M. Pretreatment with 1 μM estramustine did not significantly increase the percentage of HS683 cells in G_2-M and had virtually no radiation sensitizing effect, whereas the 10 μM concentration of estramustine increased the percentage of G_2-M cells by up to 100% and increased the cytotoxic effects of irradiation.

Estramustine has antimicrotubule activity on several human malignancies (15, 16) and it is an attractive agent for use in the treatment of malignant gliomas for several reasons. Its potent *in vitro* effects against human glioblastoma and accumulation in glial tumors may be determined by the expression of estramustine binding protein (17, 18) within these tumors (9). Its noncovalent binding with tubulin and/or microtubule associated proteins results in depolymerization of microtubules, inhibition of the mitotic spindle and subsequent arrest of cells at G_2-M potentially making these synchronized cells more susceptible to irradiation. Furthermore, estramustine can be administered p.o. an a daily schedule with minimal systemic toxicity and modest immunosuppression or alkylating activity (19, 20). These findings suggest that estramustine may be an effective agent for use during radiation therapy for malignant cerebral gliomas and warrants further laboratory and clinical investigation.

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