MALIGNANT GLIOMA

Experimental studies
with an estrogen-linked cytostatic

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University of Umeå
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ABSTRACT

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Malignant gliomas are the most common primary brain tumors in adults. Patients with these highly malignant tumors have an extremely poor prognosis. The situation with a highly proliferative tumor in a non-proliferating tissue should favor cytostatic treatment but so far the role of conventional chemotherapy has been adjunctive. The concentrations of three sex steroids, estradiol, progesterone and testosterone, were analyzed by radioimmunoassay after celite chromatography in brain tumor samples. Some malignant gliomas had high tissue concentrations of estradiol. Low progesterone levels may suggest steroid consumption. Estramustine (EM), a conjugate of estradiol-17ß and nor-nitrogen mustard had a dose-dependent antiproliferative effect on several human malignant glioma cell lines. At equimolar concentrations the inhibitory effects of the EM complex were clearly more pronounced than those of estradiol and nor-nitrogen mustard given alone or in combination. A specific binding protein (EMBP) is important for the cytotoxic action of EM. Using a mouse monoclonal antibody and an indirect antibodyperoxidase technique, EMBP was demonstrated in human glioma cells. Significant amounts of EMBP were also detected in human brain tumor tissue by radioimmunoassay. The mean concentrations (ng/mg protein) in 16 astrocytomas (2.6) and 7 meningiomas (5.1) were higher (p<0.001) than in 18 samples of normal brain (0.5). The presence of the specific binding protein may suggest a selective binding and effect of EM in human brain tumor tissue. Human glioma cells displayed significant uptake, retention and metabolism of estramustine phosphate (EMP). After incubation with ^3H-EMP a progressive uptake of radioactivity was recorded during 24 hours. Metabolism of parent EMP into estramustine and estromustine, which is a well known part of the metabolic pathway in man, was also demonstrated. A dose-dependent increase in DNA strand breaks was recorded at EMP-concentrations ranging 10-40 µg/ml. The uptake of ^86Rb, used as a tracer for potassium to study ion transport and membrane permeability, was reduced after incubation with EMP. Scanning electron microscopy gave further evidence for membrane damage. According to flow cytometric analyses exponentially growing glioma cells were accumulated in the G2/M stage and the fraction of G1/G0 was reduced. EM seems to attack malignant cells in a multifocal fashion on several vital functions including the microtubule, the nucleus, and the cell membrane. The intact EM complex may be important for effects related to microtubule function which add to the cytotoxic potential of its constituents. These experimental findings justify further investigations on the role of sex hormones in brain tumor growth and development and of hormone-linked cytostatics in clinical treatment.

Key words: malignant glioma, meningioma, glioma cells, sex steroids, estramustine, nor-nitrogen mustard, estramustine-binding protein, DNA damage, cell membrane
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Malignant gliomas are the most common primary brain tumors in adults. Patients with these highly malignant tumors have an extremely poor prognosis. The situation with a highly proliferative tumor in a non-proliferating tissue should favor cytostatic treatment but so far the role of conventional chemotherapy has been adjunctive. The concentrations of three sex steroids, estradiol, progesterone and testosterone, were analyzed by radioimmunoassay after celite chromatography in brain tumor samples. Some malignant gliomas had high tissue concentrations of estradiol. Low progesterone levels may suggest steroid consumption. Estramustine (EM), a conjugate of estradiol-17β and nor-nitrogen mustard had a dose-dependent antiproliferative effect on several human malignant glioma cell lines. At equimolar concentrations the inhibitory effects of the EM complex were clearly more pronounced than those of estradiol and nor-nitrogen mustard given alone or in combination. A specific binding protein (EMBP) is important for the cytotoxic action of EM. Using a mouse monoclonal antibody and an indirect antibodyperoxidase technique, EMBP was demonstrated in human glioma cells. Significant amounts of EMBP were also detected in human brain tumor tissue by radioimmunoassay. The mean concentrations (ng/mg protein) in 16 astrocytomas (2.6) and 7 meningiomas (5.1) were higher (p<0.001) than in 18 samples of normal brain (0.5). The presence of the specific binding protein may suggest a selective binding and effect of EM in human brain tumor tissue. Human glioma cells displayed significant uptake, retention and metabolism of estramustine phosphate (EMP). After incubation with ³H-EMP a progressive uptake of radioactivity was recorded during 24 hours. Metabolism of parent EMP into estramustine and estromustine, which is a well known part of the metabolic pathway in man, was also demonstrated. A dose-dependent increase in DNA strand breaks was recorded at EMP-concentrations ranging 10-40 µg/ml. The uptake of ⁸⁶Rb, used as a tracer for potassium to study ion transport and membrane permeability, was reduced after incubation with EMP. Scanning electron microscopy gave further evidence for membrane damage. According to flow cytometric analyses exponentially growing glioma cells were accumulated in the G₂/M stage and the fraction of G₁/G₀ was reduced. EM seems to attack malignant cells in a multifocal fashion on several vital functions including the microtubule, the nucleus, and the cell membrane. The intact EM complex may be important for effects related to microtubule function which add to the cytotoxic potential of its constituents. These experimental findings justify further investigations on the role of sex hormones in brain tumor growth and development and of hormone-linked cytostatics in clinical treatment.

Key words: malignant glioma, meningioma, glioma cells, sex steroids, estramustine, nor-nitrogen mustard, estramustine-binding protein, DNA damage, cell membrane
This thesis is based on the following papers, which will be referred to in the text by their Roman numerals


VI von Schoultz E, Grankvist K, Gustavsson H, Henriksson R. Estramustine cytotoxicity on malignant glioma cells involves damage on DNA and cell membrane. Submitted.
INTRODUCTION

Malignant gliomas are the most common primary brain tumors in adults. They do not disseminate outside the central nervous system and therefore might be considered as well suited for treatment with surgery and/or radiotherapy. However clinical experience and numerous studies show that few other diseases are so constant in terms of vital prognosis. Despite intensive therapeutic efforts patients with these highly malignant tumors remain to have an extremely poor prognosis. The need for a better understanding of tumor biology and growth, and for new therapeutic strategies is obvious.

Incidence and survival

The annual incidence of primary intracranial tumors varies between 5-12/100,000 according to different epidemiological studies (Brewis et al 1966, Fogelholm et al 1984, Joensen 1989, Percy et al 1972). In Sweden during 1985 a total of 1,221 new cases were reported to the Cancer Registry. This corresponds to 3.2% of all malignant tumors (National Board of Health and Welfare, 1989).

Approximately 50% of primary intracranial tumors are of neuroglial origin and a majority of these represents the highly malignant astrocytomas grade 3 and 4. The group of gliomas also includes astrocytoma grade 1 and 2, oligodendroglioma, mixed oligoastrocytoma, ependymoma, and medulloblastoma (Kernohan et al 1949, Kernohan & Sayre 1952, Rubinstein 1972, Russel & Rubinstein 1989). In recent epidemiological population-based studies the incidence of malignant gliomas was 4.7 in southern Finland (Kallio 1988) and 4.1 in northern Sweden, Fig 1 (von Schoultz et al, to be published). Increasing incidence rates for different gliomas have been reported from the Norwegian Cancer Registry (Hellseth & Mark 1989). The age
distribution of malignant gliomas in northern Sweden is shown in Fig 2 (von Schoultz et al, to be published).

![Fig 1](image1.png)

Fig 1. During 1977-1985 the incidence of gliomas in northern Sweden varied between 2.6-6.7 per 100,000 inhabitants. (Data from, von Schoultz E, Jonsson H, Hartman M, Henriksson R: Malignant gliomas in Northern Sweden. A retrospective study. To be published).

![Fig 2](image2.png)


The etiology of brain tumors is unknown. An increased occurrence within certain occupational groups, like male
dentists, agricultural research workers, public prosecutors, female physicians, welders and metal cutters, glass, porcelain or ceramic workers, and women in the wool industry has been reported (McLaughlin et al 1987). Exposure to chemical agents in petrochemical and vinyl chloride industries (Austin & Schnatter 1983, Beamont & Breslow 1981, Byrén et al 1976) has been suggested as a risk factor as well as radiation (Robertson 1978) and toxoplasma infection (Schuman et al 1967). Case reports indicate the possibility of hereditary factors (Armstrong & Hanson 1969, Thuve 1984, Tupchong et al 1985) and in animal experiments viral induction of malignant gliomas has been demonstrated (Yung et al 1976).

In previous studies of patients with astrocytoma grade 3-4 the overall 2-years survival rate ranged 10-20% and there were very few 5-years survivors (Hatlevoll et al 1985, Leibel & Sheline 1987, Safdari & Ciampi 1986, Salzman 1980, Walker et al 1979). Survival curves for 301 patients from northern Sweden are shown in Fig 3 (von Schoultz et al, to be published). Factors like age,

Aspects on irradiation and chemotherapy

The limited radiation tolerance of the CNS and the relative radio-resistance of glial tumors constitutes a major therapeutic problem. Patients survival is only marginally influenced by intensive efforts with different irradiation doses, fractionation regimens, additional radiosensitizers, stereotactic, and interstitial techniques. Some improvement may be achieved by interstitial high dose irradiation therapy (Brada 1989, Leibel et al 1989).

Further developments in this field are to be expected.

Malignant gliomas are unique in that the majority of tumor cells reside in a non-proliferating pool. In no other organ does there exist such a pronounced kinetic difference between tumor tissue and the surrounding normal brain which is capable of only minimal proliferative activity. Therefore if cell cycle specific antitumor agents could be effectively delivered a selective therapeutic
response might be achieved. However drugs with these properties are still lacking. During the last years several trials of cytostatic agents, in different combinations, schedules and routes of administration have been performed. The chloroethyl nitrosureas have been frequently used and currently carmustine (BCNU) is considered the most effective agent (Hatlevoll et al 1985, Kornblith & Walker 1988, Safdari & Ciampi 1986). Attachment of steroid hormone carrier molecules to alkylating drugs may enhance antitumor efficacy and reduce bone marrow toxicity (Eisenbrand et al 1989). At present the role of chemotherapy for malignant gliomas is adjunctive. The situation with a highly proliferative tumor in a non-proliferating tissue should favor cytostatic treatment. Consequently, studies on penetration, effect mechanisms and metabolism of antitumor agents within the CNS are needed.

Sex steroids in human brain and tumor tissue


Apart from binding to intracellular receptors steroids also interact with monoamine turnover and metabolism (Everitt et al 1974) and alter membrane permeability within the brain (Dufy et al 1979). Estradiol and progesterone have opposite effects on brain excitability (Bäckström et al 1984). Catechol estrogens are the major metabolites of estradiol within the CNS with both estrogenic and catecholamine-like properties. These compounds have been suggested to be involved in a wide range of physiological processes and in estrogen carcinogenesis.
(MacLusky et al 1984, Purdy et al 1983). Conflicting results have been presented on the presence of specific sex steroid receptors in various brain tumors (Courriere et al 1985, Glick et al 1983). Most authors agree that significant amounts of progesterone receptors are present in meningiomas (Brentani et al 1984, Martinez et al 1984). Antiestrogens like tamoxifen and antiprogestins like RU 38486 have been shown to modulate growth of meningioma cells (Olson et al 1987). In malignant gliomas the amounts of estrogen and progesterone receptors seem to be very low or absent (Brentani et al 1984, Martinez et al 1984).

There are several reports on steroid receptors and steroid binding but virtually no data on the concentrations of the different sex steroids in human brain tumor tissue. Moreover there are only few reports on sex steroid distribution in normal human brain (Bixo 1987, Hammond et al 1983, Lanthier & Patwardhan 1986). Circulating sex steroids transported to the brain are apparently concentrated in the CNS (Pardridge et al 1980). Therefore it was considered of interest to determine the concentrations of estradiol, progesterone, and testosterone in different brain tumor tissue specimens.

**Estramustine - an estrogen-linked cytostatic**

Estramustine phosphate (EMP), a cytotoxic nor-nitrogen mustard derivate of estradiol-17ß-phosphate, is generally accepted in the treatment of advanced prostatic carcinoma (Jönsson et al 1977, Madajewics et al 1980). The molecule is illustrated in Fig 4. Estradiol and nor-nitrogen mustard are complexed via a carbamic ester bond at 3-position of the steroid. Water solubility is achieved by
esterification with phosphoric acid at 17-position. In vivo the drug is rapidly dephosphorylated to estramustine (EM) and further oxidized to estromustine (EoM) in the gastrointestinal tract and in target cells (Andersson et al 1981, Gunnarsson & Plym-Forshell 1984). The exact mechanism of action of estramustine on malignant tissue is incompletely understood. Estramustine has been shown to induce mitotic arrest in human prostatic cancer cell lines by interaction with microtubular function and the mitotic spindle (Hartley-Asp 1984, Kanje et al 1985, Stearns & Tew 1985, Wallin et al 1985). The cytotoxic properties of estramustine have been claimed to be exerted specifically by the estramustine complex and unrelated to its alkylating and steroid constituents (Björk et al 1985, Gunnarsson & Plym-Forshell 1984, Tew & Hartley-Asp 1984). The antiproliferative effects on the human prostatic cancer cell line DU-145 were stronger for estramustine than for nor-nitrogen mustard alone while estradiol had no effect at all (Hartley-Asp & Gunnarsson 1982). In the present study the antiproliferative effects of estramustine and its steroid and alkylating constituents upon malignant glioma cells were investigated.

**Estramustine-binding protein**

The presence of a specific binding protein, estramustine-binding protein (EMBP), seems to be important for the
effect. EMBP may facilitate cellular uptake and retention of estramustine in malignant target tissue (Björk et al 1985, Forsgren et al 1979b). EMBP has been described as a major androgen-dependent secretory protein in the rat ventral prostate (Pousette et al 1981), and characterized as an acidic glycopolypeptide with a molecular weight of 54,000 (Björk et al 1982). It is distinct from the estrogen receptor, has a low affinity for estrogens but binds estramustine with a high affinity, dissociation constant (K_d) $2 \times 10^{-8}$ M (Björk et al 1985, Forsgren et al 1979b). A human analogue to this protein has been demonstrated in normal, benign hyperplastic and malignant prostatic tissue (Björk et al 1982) and suggested to act as an accumulator of estramustine and estromustine during clinical therapy (Björk et al 1985).

A polyclonal rabbit antiserum and a mouse monoclonal antibody (Mab EMBP-1) have been raised against purified rat EMBP and found to display cross-reactivity with human tissues (Björk et al 1982, Forsgren et al 1979a). Significant interspecies immunoreactivities have also been demonstrated together with specific staining with the Mab EMBP-1 in normal human prostatic epithelium, prostatic carcinoma and lung cancer (Bergh et al 1988, Nilsson et al 1988). A radioimmunoassay based on the polyclonal rabbit antiserum has previously been used to measure EMBP concentrations in various organs of the rat and also allows quantitative estimates in human tissue cytosol (Björk et al 1982, Forsgren et al 1979a, Forsgren et al 1981, Högberg et al 1979). These antibodies were used in the present study for detection of EMBP in cultured malignant glioma cells and brain tumor tissue samples.
The aims of the present study were

to investigate the antiproliferative effects of an estrogen-linked cytostatic on cultured human malignant glioma cells.

to determine tissue concentrations of estradiol, progesterone, and testosterone in brain tumor specimens.

to compare the antiproliferative effects of the estramustine complex and its constituents on cultured human malignant glioma cells.

to investigate the presence of estramustine-binding protein in cultured human malignant glioma cells and brain tumor tissue.

to study the uptake and metabolism of estramustine phosphate in cultured human malignant glioma cells.

to elucidate the mechanism of action for estramustine cytotoxicity.
MATERIALS AND METHODS

Cell cultures (II, III, V, VI)

The four human glioma cell lines U-87 MG, U-105 MG, U-118 MG, and U-251 MG were used (Westermark et al 1973). The cells were grown as monolayer cultures in Eagle's MEM supplemented with 10% fetal calf serum, penicillin, streptomycin, and amphotericin B. They were incubated at +37°C in humidified atmosphere containing 5-6 % CO₂. Synchronization was achieved by seeding the cells in microtiter wells (Becton & Dickinson labware, Oxnard, CA) in 0.1 ml of Eagle's medium at a concentration of $10^4$ cells/well. The next day the medium was changed to MEM without serum and incubated for 4-6 additional days. The synchrony (>85% cells in $G_0/G_1$) was controlled by flow cytofluorometry.

Cell proliferation (II, III, V, VI)

Cells were seeded in 24-well tissue culture dishes (Costar, Cambridge, MA, USA), at $3.8 \times 10^4$ - $1.2 \times 10^5$ cells/well depending on cell line. Medium was changed three times a week. Cells were harvested by incubation with 0.2 ml EDTA (0.5 mM) for five minutes followed by trypsin 0.1%. Cells were counted in a Linson 431 counter or a coulter multisizer and cell proliferation was calculated. Reversibility experiments as regards the anti-proliferative effect of different agents were performed on all cell lines. On the third day of culture, a group of cells were reincubated with fresh medium devoid of the tested substances.

Flow cytometric analysis (II, III)

For cell cycle analysis the cells were stained according to Vindelöv et al (1983) by applying the staining solution directly on the monolayer after removal of the me-
Fluorescence was analyzed in a Model 4800 A flow cytofluorometer (Bio/Physics Systems Inc., Mahopac, NY, USA) and the DNA curves were obtained in a TN 1705 pulse height analyzer (Tracor Northern Inc., Middleton, WI, USA). In some experiments a FACS II flow cytometer (Becton & Dickinson, Sunnyvale, CA) was used. Forward scatter and red fluorescence were detected, the latter in the FL 2 channel with linear amplification. For each sample 2 x 10^4 cells were analyzed.

**Tissues and tissue sample preparations (I, IV)**

Brain and breast tumor specimens were collected during surgery. Normal human brain without any macroscopic signs of autolysis, was obtained at autopsy performed within four hours. Benign hyperplastic human prostatic tissue was taken by transurethral resection. In all cases a portion of the tissue was submitted for histopathological analysis. Venous blood samples for reference were drawn from patients attending the Department of Oncology. Serum was centrifuged free of formed elements. All tissues and serum samples were immediately frozen and stored at -70°C until assayed in duplicate. For preparation of cytosol, the tissue samples of human brain, brain tumor, and prostate were homogenized in buffer (water solution, pH 7.4; 8.9 g Na_2HPO_4, 3.72 g EDTA-Na_2, 2 g bovine serum albumin, 4.68 g NaCl). A mechanical homogenizer (Ultra Turrax, Model TP 18/10) was used during four 5-second intervals at 75% of maximal speed and with 20-second cooling intervals (on ice). The homogenate was then centrifuged for 20 minutes at 2000 x g and +4°C. The supernatant was collected by a Pasteur pipette.

**Celite chromatography (I)**

Steroids were extracted from tissue samples with 95% ethanol (AB Svensk Sprit, Sundsvall, Sweden) for 7 days.
as described by Bixo et al (1984). The recovery of steroids was 100 %.

Sex steroids in human brain tumor tissue were separated with celite column chromatography (Brenner et al 1973, Bäckström et al 1986). Glass columns (inner diameter 5 mm) were tightly packed with celite (Mansville, Denver, USA), preheated at +600°C for 12 hours and saturated with propylene glycol, to a height of 50 mm. Nitrogen was used to percolate all solvents through the columns. Purified $^3$H-steroids were added to each extract for recovery measurements before the evaporation started. The samples (3.3% of the extract) were dissolved in isooctane saturated with propylene glycol. Isooctane was used as the mobile phase. Progesterone was eluted with 6 ml of isooctane. Other columns were packed with ethylene glycol saturated celite but otherwise the procedures were the same. The samples (40-70% of the extract) were dissolved in 1 ml of isooctane saturated with ethylene glycol before chromatography. Isooctane (4 ml) and 3.5 ml of isooctane:ethylacetate (95:5) were percolated through the columns and taken to progesterone assay. Thereafter testosterone was eluted with 3.5 ml of isooctane:ethylacetate (85:15) and estradiol with another 3.5 ml of isooctane:ethylacetate (50:50). The samples were evaporated under nitrogen and dissolved in ethanol. The solutions were divided for recovery measurements and for radioimmunoassay.

**Hormone and estrogen receptor assays (I)**

The concentrations of estradiol, progesterone, and testosterone in tissue extracts were measured by radioimmunoassay (Bäckström & Södergård 1979). The between assay coefficient of variation was 10 % for estradiol, 6.6 % for progesterone, and 5 % for testosterone. Estrogen receptor values were analyzed by isoelectric focusing
(Fernö et al 1986) and also by an estrogen receptor-enzyme immunoassay kit obtained from Abbot Laboratories, Diagnostic Division, North Chicago, IL, USA. Protein determinations were performed according to Lowry et al (1951) with bovine serum albumin as a standard.

Immunological detection of EMBP (II, IV)

Glioma cells were washed in PBS three times and spun down at 500 x g for ten minutes. The pellets were snap frozen at -70°C and freeze-sectioned at the time of use. The presence of EMBP was assessed by the indirect antibody-peroxidase technique (Bergh et al 1985a, Sternberger 1979). Endogenous peroxidase activity was blocked by addition of H₂O₂ in methanol. The primary mouse monoclonal antibody (Mab EMBP-1) raised against purified rat EMBP, with demonstrated cross reactivity to human EMBP (Bergh et al 1988) was added diluted 1/10 to the sections for 1/2 – 1 hour. After sequential washings in PBS the rabbit anti-mouse avidin-biotin peroxidase-antiperoxidase complexes (Vectastain, Burlingame, CA, USA) were added. The staining reaction was developed in DMSO/ethylcarbozole, followed by counterstaining with haemathoxylin and mounting in glycerol-gelatin. Two small cell lung cancer cell lines U-1285 and U-1906 (Bergh et al 1985b) were used as negative and positive controls respectively. Immunohistochemical staining results were semiquantitatively analyzed.

Polyclonal rabbit antiserum and purified rat EMBP (Pharmacia-LEO Therapeutics AB, Helsingborg, Sweden) were used for radioimmunoassay as described by Forsgren et al (1979a). Radiiodination was performed by using the chloramine-T technique (Hunter & Greenwood 1962) and the specific radioactivity ranged 50-100 µCi/µg protein. The antiserum was diluted 1:5000. A standard curve ranging 5-1000 ng/ml of pure EMBP was established. 100 µl of test
samples, tracer and antiserum were incubated at room temperature for two hours. Sheep anti-rabbit-gammaglobulin-coated particles (DASP, Organon N.V., Oss, Holland) were added for separation of free and bound antigen and tubes were slowly rotated overnight. Samples were centrifuged at 1000 x g for three minutes, washed three times in saline and counted in a liquid scintillation counter.

**Uptake and metabolism of estramustine phosphate (V)**

$^3$H-estramustine phosphate (2,4,6,7-$^3$H)(40 Ci/mmol) was synthesized at Pharmacia-LEO Therapeutics AB, Helsingborg, Sweden. The purity was at least 98% as determined by high performance liquid chromatography (HPLC). $^3$H-EMP was dissolved in 95% ethanol. The final concentration of the solvent in the incubation mixture did not exceed 0.2%. The concentration of $^3$H-EMP in the incubation medium was 20 µg/ml.

The uptake of $^3$H-EMP and its further conversion to $^3$H-EM and $^3$H-EoM in U-251 MG and U-105 MG cells, was investigated during incubation of the cells for 1, 4, 8, 24, and 48 hours.

Radioactivity measurements on the cell pellets, obtained after centrifugation at 1500 x g for 8 min, were performed in a Packard Sample Oxidizer and counted for 10 min in a liquid scintillation counter. Corrections for quenching were made by the external standard ratio method. Metabolites were quantified by high performance liquid chromatography (Kruse et al 1984). The column was a Radial-PAK C18 Cartridge. The mobile phase consisted of methanol/acetate buffer (80/20). The flow rate was 0.80 ml/min and the volume of each fraction was 160 µl. The three references used were estramustine phosphate, estramustine, and estromustine. The localization of the reference compounds was identified by their UV-peaks.
DNA Strand Break Assay (VI)

DNA strand breaks were measured with the DNA precipitation assay (Olive 1988, Olive et al 1988), modified by the use of double radiolabelling according to the principles outlined by Rydberg (1980) and Sandström & Johansson (1987), for detection of DNA strand breaks with improved accuracy. Briefly, cells, either labelled with $^3$H-thymidine or $^{14}$C-thymidine, were incubated with estramustine phosphate or used as untreated control cells. At the end of the drug-treatment period the cells were immediately detached by trypsinization. Drug-treated, $^3$H-labelled cells were then mixed with $^{14}$C-labelled control cells and vice versa. The relative amount of precipitated DNA per sample was determined and the difference of strand breaks between EMP-treated and control cells was calculated with a computer program that corrected the number of counts for spillover between the $^3$H- and $^{14}$C-channels of the liquid scintillation counter (LKB 1217, Bromma, Sweden). The results were expressed as the mean of the differences of the percentage of precipitated DNA in two differently labelled samples. Thus, any damage caused by the labelling itself will cancel out (Rydberg 1980).

$^{86}$Rb-accumulation (VI)

The glioma cells were incubated for 120 min at $+37^\circ$C with or without EMP. They were then washed twice with Eagle's medium and the incubation continued for 120 min in the presence of 28 μmol/l $^{86}$RbCl (Sundström et al 1988). The cells were briefly rinsed, trypsinized, and radioactivity was determined in a liquid scintillation counter (LKB 1217, Bromma, Sweden). The number of counts of treated and untreated cells was compared.

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Scanning Electron Microscopy (VI)

Glioma cells for scanning electron microscopy were cultured as described above. The cells were fixed in 3% glutaraldehyde in 0.1 M phosphate buffer, dehydrated in alcohol, critical point dryfreezed, mounted on stubs, and sputter-coated with gold. A JEOL T330 scanning electron microscope was used.

Hormones and chemicals

Estramustine phosphate [estradiol-3-N-bis(2-chloroethyl)carbamate-17-dihydrogen phosphate], estramustine, estradiol-17ß, and nor-nitrogen mustard were donated by Pharmacia-LEO Therapeutics AB, Helsingborg, Sweden. Progesterone and testosterone were purchased from Sigma Chemical Company, St Louis, MO, USA. Medroxiprogesterone was obtained from Løvens Kemiske Fabrik, Ballerup, Denmark and danazol from Sterling Winthrop, New York, USA. Micronized estramustine and estradiol was dissolved in dimethyl sulfoxide (DMSO). Estramustine phosphate and nor-nitrogen mustard was dissolved in water. These four substances were diluted in medium to concentrations 1 - 40 µg/ml. Progesterone, medroxiprogesterone, testosterone, and danazol were dissolved in DMSO and diluted in medium to $10^{-5} - 10^{-11}$ M. In all solutions the final concentration of the solvent did not exceed 0.1%. Eagle's minimal essential medium (Eagle's MEM) was from GIBCO Ltd, Paisley, Scotland, UK. Fetal calf serum was obtained from Biochrom KG, Berlin, West Germany. $^{86}$RbCl, methyl-14C-thymidine, and methyl-3H-thymidine was from Amersham International, Buckinghamshire, UK. All other chemicals were of analytical grade.
RESULTS AND COMMENTS

Sex steroid concentrations in brain tumor tissue (I)

The tissue concentrations of estradiol in 15 brain tumor specimens ranged 0.04 - 3.32 pg/mg protein and there were no age and sex differences. Five of ten astrocytomas had estradiol concentrations exceeding the lowest value in a reference group of 8 breast cancer samples. No apparent correlation was found between steroid concentrations and estrogen receptor density. High estradiol values were recorded in one 55 year old man (3.32 pg/mg) and a 56 year old woman (1.0 pg/mg) both with the diagnosis of highly malignant astrocytoma. These concentrations were ten to thirty times as high as previously reported in normal female brain (Bixo 1987, Lanthier & Patwardhan 1986).

Testosterone levels were somewhat higher in brain tumors as compared with breast cancers and also in good agreement with previous concentrations found in normal post mortem brain (Bixo 1987, Hammond et al 1983, Lanthier & Patwardhan 1986).

The progesterone concentrations in brain tumors were low. The median value was 0.36 pg/mg whereas previously, concentrations about ten times higher, ranging 5-10 pg/mg were found in "normal" brain (Bixo 1987). The low progesterone value in brain tumor tissue and the very low values in breast cancer (5 out of 8 samples below the limit of detection) might indicate increased steroid consumption during the malignant process.

Specific progesterone receptors are present in breast cancer, in meningiomas and possibly also in certain malignant gliomas (Corriere et al 1985, Glick et al 1983, Martinez et al 1984).
Progesterone reduces brain excitability (Bäckström et al 1984) and antiprogestins are found to inhibit meningioma growth in vivo (Olson et al 1987). The possible association between sex steroid metabolism and breast cancer is frequently discussed whereas the evidence for a connection between hormones and brain tumors are most uncertain. In some highly malignant gliomas there were surprisingly high estradiol concentrations and low amounts of progesterone. Studies on the rat indicate that estramustine-binding protein is sex-steroid dependent (Pousette et al 1981). High concentrations of estradiol in brain tumor tissue might be related to the presence of this protein which displays a low affinity binding for estradiol and estrone (Forsgren et al 1979b).

The current data may indicate that a modulation of steroid metabolism can be of value in the treatment of brain tumors as has been shown for mammary and prostatic carcinomas. In fact, 25-hydroxycholesterol, a potent inhibitor of sterol synthesis, caused a selective decrease of the viability and the sterol content of proliferating rat glioma cells (Maltese et al 1981).

**Dose-dependent antiproliferative effects of estramustine on cultured glioma cells (II,III,V,VI)**

Malignant glioma cells (U-105 MG, U-118 MG, U-251 MG) were grown with different estramustine phosphate and estramustine concentrations. Both substances caused a dose-dependent inhibition of growth of all cell lines tested in the concentration range 1-40 µg/ml. All cell lines were maximally inhibited by a concentration of 20 µg/ml during 6 days incubation. Furthermore a clear inhibitory effect was demonstrated and a reduced cell count was measured already after 24 hours in cell lines U-105 MG and U-251 MG (V). In all investigated cell lines the inhibitory effect was reversed by washing and reincubation in fresh medium.
The antiproliferative effect of the estramustine complex was clearly more pronounced than for the constituents estradiol and nor-nitrogen mustard, whether given alone or in combination Fig 5 (III). Following six days of incubation with estramustine 20 µg /ml the decrease in cell number was 80% for U-251 MG cells. The corresponding figure after 20 µg /ml of nor-nitrogen mustard was 24%. Only a slight antiproliferative effect was recorded for estradiol alone and glioma cells were unaffected by testosterone, medroxiprogesterone, progesterone, and danazol (III)

Micronized estramustine, estradiol, and other steroids were dissolved in dimethyl sulfoxide and diluted in medium. In all solutions the final concentration of the solvent did not exceed 0.1% and should not influence cell proliferation. Fig 6 illustrates a control experiment where U-251 MG cells were grown with increasing concentrations of dimethyl sulfoxide. Even at a concentration of 1% cell proliferation was apparently unaffected.

The finding that estramustine phosphate and estramustine exert antiproliferative effects upon malignant glioma cells is in agreement with previous studies on other
tumor cell lines. The concentrations used here 5-20 μg/ml have similar inhibitory effects on various prostatic tumor and lung cancer cell lines (Hartley-Asp 1984, Westlin et al, unpublished data). The estramustine complex had a stronger inhibitory effect than nor-nitrogen mustard on the human prostatic cancer cell line DU-145 and estradiol alone had no effect (Hartley-Asp & Gunnarsson 1982). In clinical therapy estramustine displays estrogenic properties and estrogen-related side-effects (Daehlin et al 1986). However, according to the present data the estradiol moiety of the compound should account for very little of the cytotoxic action.

Fig 6. A control experiment where U-251 MG cells were grown with increasing concentrations of dimethyl sulfoxide. Even at a concentration of 1% cell proliferation was unaffected. *=untreated cells; o=0.01% DMSO; ●=0.05% DMSO; □ =0.1% DMSO; △ =0.5% DMSO; ■ =1% DMSO.

Estramustine-binding protein in cultured glioma cells and tumor tissue (II,IV)

All studied malignant glioma cell lines (U-87 MG, U-105 MG, U-118 MG, U-251 MG) contained high amounts of estramustine-binding protein as judged from immunohistochemical analyses using the mouse monoclonal antibody Mab EMBP-1. The intensity in positive staining localized to the cytoplasm and the proportion of positive cells (>90%) were the same in all investigated cell lines (II). The staining pattern was similar to that found in different

Significant amounts of estramustine-binding protein were also detected by radioimmunoassay in 16 malignant glioma and 7 meningioma tissue samples. Values in tumorous tissue were markedly higher than in normal brain. There was a tendency to higher values in women and in meningioma samples which however did not reach statistical significance. The highest individual values 9.3 and 9.1 ng/mg were recorded in two women with meningioma. There were no significant age-differences with respect to the concentrations of estramustine-binding protein.

Estramustine-binding protein has been suggested to play an essential role in the mechanism of action of estramustine in the treatment of prostatic carcinoma as regards tissue selectivity and cellular transportation (Björk et al 1985, Högberg et al 1979). The radioimmunoassay used here was based on a polyclonal rabbit antiserum and has previously been employed to measure estramustine-binding protein in various rat and human tissues. The absolute values in human tissues remain uncertain because cross reactivity is difficult to assess in the absence of completely purified human estramustine-binding protein (Björk et al 1982, Forsgren et al 1979a, Forsgren et al 1981, Högberg et al 1979).

Previously estramustine-binding protein has been detected in the pituitary gland and cerebral cortex of male and female rats (Forsgren et al 1979a). According to the immunohistochemical analyses, EMBP in human glioma cells was located to the cytoplasm which is in agreement with earlier findings in other tumor cells (Björk et al 1982). Experiments on prostatic cancer indicate an association between binding protein expression and proliferative activity (Nilsson et al 1988). While the biological role of this glycoprotein within the central nervous system
remains to be elucidated its presence in glioma cells and tissue may have a clinical interest in the management of malignant gliomas. The observation that meningiomas may contain relatively high amounts of estramustine-binding protein (IV) may give new implications as regards the biology and management of this disease.

Uptake and metabolism of estramustine phosphate in glioma cells (V)

During incubation of glioma cell lines U-105 MG and U-251 MG with $^3$H-estramustine phosphate (20 µg/ml) a progressive increase of radioactive uptake until the first 24 hours was recorded. After 48 hours values were stable in U-251 MG cells and even somewhat lower in U-105 MG cells (Fig 7). Already after one hour of incubation significant amounts of $^3$H-estramustine were found in both cell lines. The concentration of this main metabolite of estramustine

![Graph](image_url)

Fig 7. Uptake of $^3$H-EMP and two major metabolites by U-105 MG cells during incubation for 1, 4, 8, 24, and 48 hours (V).
phosphate steadily increased to reach a plateau level at 24 and 48 hours. Only very low amounts of $^3$H-estramustine phosphate were observed. In both cell lines increasing amounts of $^3$H-estromustine were also measured. The relative amount of this secondary metabolite was higher in U-105 MG cells.

The rapid dephosphorylation and metabolism of estramustine phosphate in vivo (Andersson et al 1981) was apparent also in this study of cultured human malignant glioma cells. During 48 hours of incubation only minute amounts of $^3$H-estramustine phosphate were detected in cell pellets, probably due to dephosphorylation in the cells or possibly in the medium. The progressive increase in radioactivity during the first 24 hours is in agreement with previous results. Maximum cellular uptake of $^3$H-estramustine in the human prostatic cancer cell line 1013 L was achieved after 15-20 hours (Kruse & Hartley-Asp 1988 and 1989).

The cellular uptake and efflux of alkylating agents like chlorambucil, melphalan, and busulphan are described as very rapid events (Begleiter & Goldenberg 1983, Harrap & Hill 1970). Estramustine seems different in this respect. The estramustine complex is retained within the cell for a long time (Kruse et al 1984, Kruse & Hartley-Asp 1989). Cellular uptake and retention of estramustine probably reflects the presence of its specific binding protein. Evidently the gradually increasing uptake of estramustine recorded in the present study could not be due to increased cell number in the pellets since a distinct inhibitory effect was observed shortly following delivery of estramustine phosphate and after 24 and 48 hours there was a clear decline in cell number.

In conclusion, a specific uptake and retention of the two main metabolites of estramustine phosphate was demonstrated in glioma cells. The results indicate that the two
investigated cell lines are capable of oxidizing estramustine to estromustine. Both estramustine and estromustine are cytotoxic to prostatic cancer cells (Björk et al 1985) and this oxidation process is a well known part of the metabolic pathway for estramustine phosphate in man (Gunnarsson et al 1984, Gunnarsson & Plym-Forshell 1984). These findings may have clinical implications in the management of malignant gliomas.

Mechanisms for estramustine cytotoxicity (II,III,V,VI)

Alkylating agents are widely used in clinical oncology. In the present study the nor-nitrogen mustard constituent of estramustine was found to exert an inhibitory influence on glioma cell proliferation (III). However at all concentrations the effect of the intact estramustine complex was more pronounced. These findings support the concept that estramustine cytotoxicity is mediated via separate mechanisms.

Earlier studies on prostatic cancer cell lines have suggested microtubules as the main target for the cytotoxic effects of estramustine (Kanje et al 1985, Stearns & Tew 1985, Wallin et al 1985). The influence on microtubule-dependent events was further supported by studies on monocyte phagocytosis (Bjermer et al 1988). Only the microtubule-dependent engulfment was inhibited, whereas the attachment phase was unaffected.

To examine whether a block existed in a certain cell cycle phase, glioma cells were studied by cytofluorometry. Treatment with estramustine phosphate and estramustine caused a large increase in the number of cells in $G_2/M$ compared to controls (II,III). Cells treated with nor-nitrogen mustard displayed a very perturbed DNA profile, with a complete disappearance of cells in the $G_1$ phase and a broad peak over the area where $S$, $G_2$, and $M$
cells were present in the control population. Estramustine did not seem to block cells in resting $G_0$ stage. These findings clearly demonstrate mitotic arrest as a part of the cytotoxicity induced by the estramustine complex.

On the other hand the present data suggest that estramustine cytotoxicity also involves interaction with DNA and cell membrane components (VI). Incubation of U-105 MG and U-251 MG cells with estramustine phosphate caused a dose-dependent increase in DNA strand breaks. In U-251 MG cells an estramustine concentration of 40 $\mu$g/ml caused a reduction in precipitated DNA of 15-20%.

The uptake of $^{86}$Rb by glioma cells was dose-dependently reduced after incubation with estramustine phosphate. $^{86}$Rb has been used as a tracer for potassium in previous studies on ion transport and membrane permeability (Sundström et al 1988). The mean decline in $^{86}$Rb accumulation by U-251 MG cells as compared to controls was 12, 20 and 32% at EMP concentrations of 10, 20 and 40 $\mu$g/ml respectively. Scanning electron microscopy revealed further evidence of cell membrane damage. After incubation with 20 $\mu$g/ml estramustine phosphate, cells were spherical, probably because of a block in metaphase. After EMP treatment cells displayed numerous stub-like projections or blebs, especially over the nuclear area, while the surface of control cells was smooth (Fig 8). Bleb formation and holes have been correlated to loss of cell viability (Noseda et al 1989) and may be related to lipid peroxidation via free oxygen radicals (Norhona-Dutra et al 1988). The present data add further complexity to the interpretation of estramustine action. Membrane damage might be related to previous observations that estramustine, like substances such as diamide and t-butylhydroperoxide, is capable of generation of free oxygen radicals (Grankvist et al 1988). The effects of estramustine phosphate on DNA and cell membrane were also
demonstrated in transformed fibroblasts (Henriksson et al 1990).

Cross-linking and abnormal base-pairing that interferes with DNA replication function are well known effects of alkylating agents like melphalan, cyclophosphamide, chlorambucil and nor-nitrogen mustard. While previously careful experimental analyses have provided solid evidence that interaction with the microtubular function is one important aspect of the effect mechanism for estramustine (Hartley-Asp 1984, Tew et al 1983, Wallin et al 1985) the present results indicate DNA strand breaks and cell-membrane damage as part of the cytotoxic effects of this drug. DNA strand breaks produced by EMP could possibly be concealed by cross-links and further studies are indicated in this respect.

Cellular uptake and action of estramustine is in many ways different from alkylating agents. The estramustine complex is retained in the cell for a long time (Kruse et al 1984, Kruse & Hartley-Asp 1989, von Schoultz et al 1989). The observation that only the estramustine complex inhibits monocyte phagocytosis (Bjermer et al 1988) also supports the concept that this combination between a sex steroid and an alkylating agent has properties similar to those of vinca-alkaloids and other antimitotic agents.

In conclusion, it seems that estramustine may attack malignant cells in a multifocal fashion on several vital functions including the nucleus and the cell membrane. The intact estramustine complex may be important for effects related to microtubule function which add to the cytotoxic potential of its constituents.
Fig 8. Scanning electron micrographs of malignant glioma cells U-251 MG.

a) untreated cell x 3,500
b) after incubation with EMP 20 µg/ml for 24 hours x 3,500.
CONCLUSIONS

* Estramustine - an estrogen-linked cytostatic - exerts a dose-dependent antiproliferative effect on cultured human malignant glioma cells.

* Some malignant gliomas display high tissue concentrations of estradiol. Low progesterone levels in brain tumor tissue may suggest steroid consumption. Further studies are indicated to clarify the role of sex steroids in brain tumor growth and development.

* The antiproliferative effect of the intact estramustine complex is more pronounced than for its constituents nor-nitrogen mustard and estradiol.

* The specific estramustine-binding protein is present in cultured human malignant glioma cells and brain tumor tissue.

* Certain cultured human malignant glioma cells display significant uptake, retention, and metabolism of estramustine phosphate.

* Estramustine seems to attack malignant cells in a multifocal fashion on several vital functions including the microtubule, the nucleus, and the cell membrane.
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