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ORIGINAL PAPER

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Estramustine phosphate enhances the effects of hyperthermia and induces the small heat shock protein HSP27 in the human prostate carcinoma cell line PC-3

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Abstract The antimicrotubule drug estramustine phosphate (EMP) has been shown to sensitize prostate carcinoma cells to radiation via synchronization at the G2/M phase of the cell cycle. This synchronization may also render cells more sensitive to hyperthermia, providing a rationale for multimodal treatment approaches. We have investigated the effects of EMP and hyperthermia, as well as the regulation of heat shock proteins (HSP) in the PC-3 prostatic carcinoma cell line. Cells were incubated with four doses of EMP for 48 h followed by a 1-h hyperthermia treatment ranging from 41°C to 44°C. Cell cycle distribution at the end of the EMP incubation was investigated by flow cytometry. Cytotoxicity was assessed by colony formation assays. HSP accumulation was investigated by Western immunoblotting. Doses of 1, 5, 10 and 15 μM EMP synchronized 27, 28, 46, and 68% of PC-3 cells at G2/M. With 5, 10 and 15 μM, a sensitizing effect of EMP was assessed at hyperthermic temperatures of 42, 43 and 44°C. EMP did not alter the expression of HSP72, but substantially induced the synthesis of HSP27 in PC-3 cells. Our data show that EMP sensitizes PC-3 cells to hyperthermia induced cytotoxicity. This observation supports the rationale for multimodal treatment approaches in locally advanced prostate cancer.

Keywords Prostate cancer · Estramustine phosphate · Hyperthermia · Heat shock proteins

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Introduction

The clinical management of locally advanced prostate cancer often involves androgen deprivation, radiation, and chemotherapy. Among the few antineoplastic agents that have been clinically accepted in the treatment of advanced prostate cancer, estramustine phosphate (EMP) is of significant value based on its specific mechanisms of action and the relatively low occurrence of side effects. EMP is an antimicrotubule agent consisting of 17β-estradiol linked to nitrogen mustard. The intracellular active compound, estramustine, causes the mitotic arrest of cells by direct binding to tubulin leading to microtubule depolymerization, stathmokinesis, and cell death [6, 12]. Studies describing a limited effectiveness of an EMP monotherapy [15, 23] have initiated experimental and clinical efforts to combine EMP with other antimicrotubule agents such as vinblastine, taxanes, or etoposide, and other treatment modalities such as radiation [3, 11, 13, 17, 18, 25, 30].

Recent studies in prostate cancer cell lines and in human glioma cells have shown that EMP acts as a radiation sensitizer [9, 28, 34]. The in vitro data were confirmed in the Dunning rat prostate carcinoma model and in nude mice transplanted with the DU145 human prostate carcinoma [10, 33]. In a phase II human trial Khil et al. demonstrated that the combination of EMP, vinblastine and radiation leads to a significant clinical and biochemical (as measured by levels of prostate-specific antigen) tumor control in locally advanced prostate cancer (60% free of biochemical relapse after 60 months in the group of patients with pretreatment PSA levels between 21 and 50 ng/ml) [17]. A 73% disease-free survival after 3 years was observed in another, recently published study combining neoadjuvant estramustine and etoposide with concurrent estramustine and radiotherapy [3].

The observation of a sensitizing effect of EMP to radiation is based on the fact that EMP synchronizes cells at the G2/M phase of the cell cycle when they are known to be most vulnerable to radiation-induced

damage. In addition to the S phase of the cell cycle, hyperthermia has also been shown to kill cells preferentially at the G2/M phase by directly affecting the microtubular system [8, 31, 32].

Recent studies in prostate cancer have shown that hyperthermia is a powerful tool in combination with radiation. However, these studies have also pointed out the need for further investigation of effective temperatures, combination approaches, treatment schedules and molecular mechanisms associated with hyperthermia [1, 2, 7, 26]. In this study, we investigated whether EMP sensitizes cells to hyperthermia induced cellular damage in vitro in the hormone insensitive human prostate carcinoma cell line PC-3. We also analyzed the regulation of the 27- and 72-kDa heat shock proteins (HSP27 and HSP72) that are synthesized and accumulated during cellular stress in order to protect cells against a variety of subsequent stress stimuli, including hyperthermia and certain antineoplastic agents [5, 21, 27].

Materials and methods

Cell line and culture conditions

The human prostate carcinoma cell line PC-3 was used in this study [16]. PC-3 cells were grown in RPMI 1640 containing 10% heat inactivated FBS with 10 mM HEPES (pH 7.0), 2 mM L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin and were maintained in a humidified atmosphere containing 5% CO_2/air at 37°C.

Estramustine phosphate and hyperthermia treatment

The EMP was a generous gift from Pharmacia (Helsingborg, Sweden). It was dissolved in sterile water to a 1-mM stock solution, sterile filtered, and stored at room temperature for no more than 8 h. Prior to each experiment, appropriate dilutions were prepared in RPMI 1640 containing 10% FBS.

Cells were grown to 70% confluence in 25 cm^2 tissue culture flasks. The cells were rinsed with HBSS and then exposed to various concentrations of EMP (1, 5, 10, or 15 μM) for 48 h at 37°C.

At the end of this incubation period, cells receiving hyperthermia were placed into a precision water bath set at temperatures ranging from 41.0°C to 44.0°C (±0.1°C) for 1 h in a humidified atmosphere containing 5% CO_2/air. Cells not exposed to hyperthermia were kept at 37°C for 1 h. After treatment, cells were rinsed twice with HBSS and then either used for cell cycle analysis, Western blot or the colony formation assay.

Western blot analysis of HSP27 and HSP72

Cells were collected, lysed by four freeze-thaw cycles, and total cellular protein was determined using the Bradford method. An amount of 20 μg of total protein (for HSP72 immunoblotting) or 40 μg of total protein (for HSP27 immunoblotting) for each sample were separated on a 12.5% SDS PAGE gel and transferred to nitrocellulose. The membranes were blocked by soaking in 10% FCS, 10% bovine serum albumin (BSA) in TBS (TBS; 10 mM Tris, 150 mM NaCl, pH 8.0) for 3 h at room temperature. The blots were rinsed once in TBS with 0.5% Tween-20, three times in TBS, then probed for 1 h with monoclonal alkaline phosphate-linked antibodies specific for either the HSP27 or HSP 72 (SPA-800 or SPA-810, StressGen Biotechnologies, Victoria, B.C., Canada). The blot was incubated with an alkaline phosphatase linked secondary antibody, re-rinsed, and then incubated in 0.45 mM 5-bromo-4-chloro-3-indolyl phosphate (BCIP, Sigma, St. Louis, Mo., USA) and 0.27 mM nitro blue tetrazolium (NBT, Fisher Scientific, Fair Lawn, N.J., USA), in AP buffer (100 mM Tris, 100 mM NaCl, and 5 mM $MgCl_2$, pH 9.5) until the bands developed.

Cell cycle analysis

Cell cycle analysis was performed using the propidium iodide (PI) staining procedure. Briefly, after treatment the cells were scraped and single cell suspensions of 10^6 cells in 200 μl PBS were prepared. Then 2 ml of 70% ethanol were added and the tubes were incubated for 30 min at 4°C. After incubation, cells were spun down for 5 min at 250 *g*, resuspended in 800 μl PBS, 100 μl RNase A (1 mg/ml, Sigma) and 100 μl PI (400 mg/ml, Sigma) were added, and the tubes were incubated for 30 min at 37°C. After resuspension, cells were analyzed using a Becton Dickinson flow cytometer set at 488 nm. Cell cycle data were calculated using the ModFit program (Verity Software, Topsham, Mass., USA).

Colony formation assay

The ability of cells to form colonies was assessed by the method of La Rocca et al. [19]. Briefly, after treatment with hyperthermia or EMP, cells were trypsinized, counted with a hemocytometer, plated in triplicate in 25 cm^2 culture flasks, and cultured in a humidified 5% CO_2 atmosphere incubator. After 12 days of incubation, the cells were stained with 1% (w/v) methylene blue for 10 min and colonies containing at least 50 cells were scored. The surviving fraction was determined as a percentage of the number of colonies per plated cells in each treated group versus the number of colonies per plated cells in the control group for each experiment. A minimum of three culture flasks were used per concentration and all clonogenic experiments were repeated at least twice.

Statistical analysis

The nonparametric data were statistically analyzed using the Mann-Whitney U-test with $P < 0.05$ indicating significance. The software program GraphPad 3.0L (PRISM, San Diego, Calif., USA) was used for statistical calculations.

Results

Effects of EMP on cell cycle distribution

EMP caused a dose-dependent alteration in the cell cycle distribution of PC-3 cells. As shown in Table 1, only minor changes in the cell cycle of treated cells compared to untreated controls were observed at 1 and 5 μM EMP. Doses of 10 and 15 μM EMP caused a significant synchronization with an arrest of 46% and 68% of PC-3 cells in the G2/M phase.

Effects of hyperthermia and EMP on cytotoxicity

The PC-3 cells' ability to form colonies was used to assess the cytotoxic effects of hyperthermia and EMP. As shown in Fig. 1, these experiments demonstrate a dose-dependent cytotoxic effect following hyperthermia. Compared to the control group, a surviving fraction of 84% ± 11%, 55% ± 9%, 22% ± 6.3%, and 4% ± 1.6% was calculated for the 1-h exposure at hyperthermic

Table 1. Flow cytometry results of cell cycle analysis were obtained using the propidium iodide staining procedure. A total of 10,000 cells were analyzed for each concentration of estramustine phosphate (*EMP*). The data could be reproduced in three independent experiments. Doses of 1, 5, 10 and 15 μM EMP synchronized 27, 28, 46, and 68% of PC-3 cells at the G2/M phase of the cell cycle

μM EMP	%G1	%S	%G2-M
Control	47.4 ± 3.3	30.3 ± 1.0	22.1 ± 2.4
1	47.1 ± 4.9	26.6 ± 5.3	27.0 ± 2.0
5	42.6 ± 4.0	29.0 ± 1.4	28.3 ± 3.0
10	30.3 ± 2.6	22.6 ± 1.9	46.0 ± 4.0
15	12.2 ± 0.1	16.5 ± 1.0	68.0 ± 0.8

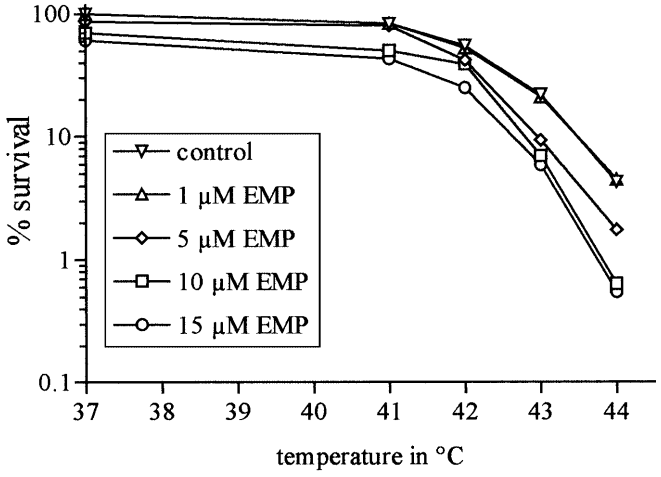

Fig. 1. The effect of the combination of hyperthermia and estramustine phosphate (*EMP*) on cellular survival as measured by clonogenic survival. Each data point represents the mean of at least two independent experiments. Hyperthermia and EMP alone caused dose-dependent cytotoxic effects on PC-3 cells. When hyperthermia of 41°C for 1 h was combined with EMP we observed no differences at doses of 1 and 5 μM EMP, but a significantly enhanced cytotoxicity at 10 and 15 μM EMP. This sensitizing effect was also observed with 5, 10, and 15 μM EMP at temperatures of 42, 43 and 44°C

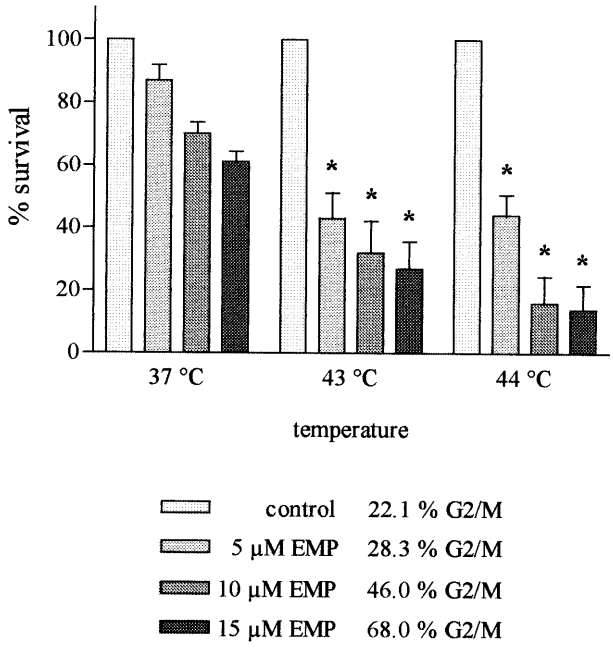

Fig. 2. The sensitizing effect of 5, 10, and 15 μM estramustine phosphate (*EMP*) on survival of cells exposed to hyperthermic temperatures of 43 and 44°C. Data are presented as the percent of the surviving fraction at each EMP concentration compared to the surviving fraction of cells without EMP pretreatment at the same temperature (set as 100%). Each percentage is determined from at least two independent experiments. Mean values and standard deviations are shown. * indicates significance ($P < 0.05$) compared to the same EMP concentration at 37°C

temperatures of 41, 42, 43, and 44°C, respectively. When cells were exposed to 1 μM EMP for 48 h, we observed no cytotoxic effects. At doses of 5, 10, and 15 μM EMP, survival was 87% ± 12%, 70% ± 9%, and 61% ± 8%, respectively.

At 41°C we observed no significant differences between hyperthermia and the combination of hyperthermia and 1 or 5 μM EMP. However, 41°C combined with either 10 or 15 μM EMP caused a significant enhancement of the cytotoxic effects.

At 42, 43, and 44°C there was no difference when hyperthermia was combined with 1 μM EMP compared to hyperthermic exposure alone. At these temperatures, cytotoxic effects were significantly elevated in a dose-dependent relation at 5, 10, and 15 μM EMP when compared to either hyperthermia alone or EMP treatment alone. In Fig. 2 the significant sensitizing effect of 5, 10, and 15 μM EMP at temperatures of 43°C and 44°C has been demonstrated by comparing the percent of surviving cells at each EMP concentration to cells not pre-treated with EMP.

Effect of EMP on HSP27 and HSP72 accumulation in PC-3 cells

Untreated PC-3 cells constitutively expressed both HSP27 and HSP72 at low levels. HSP72 accumulation did not change after a 48-h incubation in EMP (Fig. 3). HSP27 accumulation exhibited a dose-dependent accumulation following EMP treatment (Fig. 3).

Discussion

Our studies reveal that concentrations of EMP, which synchronize PC-3 cells at the G2/M phase of the cell cycle, sensitize these cells to hyperthermia-induced cytotoxicity. EMP also induces the accumulation of HSP27 but not of HSP72.

EMP, which is dephosphorylated in the cytoplasm to estramustine causes depolymerization of microtubules by binding directly to tubulin. This leads to spindle disassembly which finally causes the arrest of cells in the G2/M phase of the cell cycle [6]. Based on this specific mode of action, studies have shown a sensitizing effect of EMP when combined with radiation in

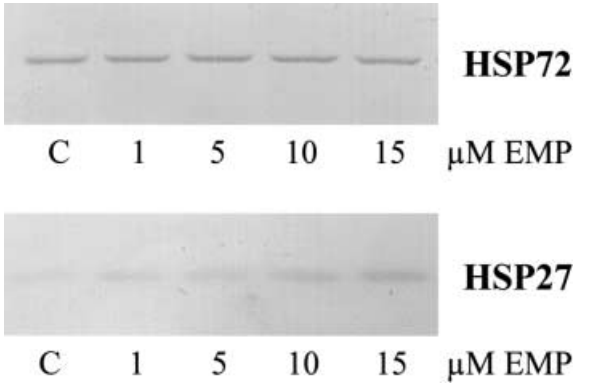

Fig. 3. The expression of HSP72 and HSP27 was analyzed by Western immunoblot following a 48-h estramustine phosphate (*EMP*) treatment. PC-3 cells constitutively expressed both proteins at low levels. Whereas no differences in HSP72 expression were observed, we did note a dose-dependent induction of HSP27

human glioblastoma cells and also in prostate cancer models in vivo and in vitro [9, 28, 33, 34]. In glioblastoma cells, Yoshida et al. reported no effects on cell cycle distribution and cytotoxicity at a dose of 1 μM EMP for 24 h. At a dose of 10 μM EMP, the G2/M fraction was significantly enhanced and cytotoxic effects were potentiated by a factor of 8.5 [34]. Similar results have been observed in prostate cancer cells both in vivo and in vitro. In the studies of Eklöv et al. on DU-145 human prostate cancer cells, 5 μg/ml (approx. 8.5 μM) EMP caused a 23% sensitization (expressed as radiation dose-modifying factor and measured by clonogenic survival) [9]. In a similar study, cells lines which have high levels of EMP binding protein (DU-145, breast carcinoma, MCF-7, and malignant glioma U-251) were compared to cell lines without EMP binding protein such as the colon carcinoma cell line HT-29 and the cervical carcinoma cell line HeLa-S_3 [28]. In that study, 5 μM EMP for 48 h caused 31.7% of the DU-145 to arrest in the G2/M phase compared to 23.7% of controls. We obtained similar results in this study using PC-3 cells. A 48-h incubation with 1, 5, 10, or 15 μM EMP resulted in a significant number of cells arrested at the G2/M phase compared with the control (beginning with 27.0% at G2/M at 1 μM EMP compared to 22.1% in controls; Table 1). In a study by Pienta and Lehr, 1 μM EMP had no effect but, in contrast to our findings, 10 μM EMP resulted in 95% cytotoxicity. No information about cell cycle distribution was given in that study [25].

Taken together, these in vitro studies show a positive correlation between the number of cells arrested in the G2/M phase of the cell cycle and the degree of sensitization to radiation. Similar results have been seen when this therapeutic rationale was applied to animal models of prostate carcinoma. Widmark et al. reported a potentiation of the combined treatment of EMP and radiation compared to radiation alone with a significant tumor growth delay in Copenhagen rats carrying the Dunning R3327 prostatic carcinoma [33].

Cells are known to be most sensitive to radiation-induced damage during the G2/M phase of the cell cycle. Hyperthermic treatment is effective in both, mainly in the late S phase but also in the G2/M phase of the cell cycle [31, 32]. The observation of a complementary mode of action of radiation and hyperthermia provides the rationale for the combination of these two modalities. The establishment of multimodal strategies may further enhance the therapeutic efficacy of locally advanced disease. There is no information in the literature regarding the combination of EMP and hyperthermia. However, in a study by Lloyd et al. the effects of hyperthermia and treatment with an androgen (5-α-dihydrotestosterone) and antiandrogen (hydroxyflutamide) on cellular growth and survival of the prostate carcinoma cell lines LNCaP and DU 145 was investigated. Previous studies demonstrate that the combination of hyperthermia and hydroxyflutamide significantly enhance cytotoxicity and inhibit the cell's ability to develop thermotolerance [22]. In addition, Dermietzel and Streffer studied the effects of hyperthermia on the microtubular system in melanoma cells. In their study, hyperthermia, similar to the known effect of EMP, caused profound damage to the microtubular system associated with changes in the phenotypical appearance of the cells [8].

In line with the observations made by combining EMP and radiation, we found a dose-dependent sensitization of EMP and hyperthermia which can be positively correlated to the fraction of cells arrested in the G2/M phase, as shown for 5, 10, and 15 μM EMP in Fig. 2. Our results suggest that the level of sensitization to hyperthermia may be lower than to radiation. Although the effect of hyperthermia is known to be most pronounced in the late S phase of the cell cycle, our data provide evidence that the synchronization of cells in the G2/M phase results in a profound cytotoxicity. Thus, the EMP pretreatment might be most effective when combining both hyperthermia and radiation therapy. The analysis of trimodal treatments with various combinations of EMP, radiation, and hyperthermia will be the aim of further in vitro studies.

The cellular effects of hyperthermia are associated with the synthesis and accumulation of HSPs. These range in molecular weight from 8 to over 110 kDa. Members of the 70-kDa family have been shown to be the most thermosensitive. Although the precise function of these proteins is not yet fully understood, they are essential for cellular survival under hyperthermic stress and are associated with tolerance to subsequent, otherwise lethal thermal events. Both HSP72 and HSP27 have been shown to be involved in thermotolerance and resistance to certain chemotherapeutic agents [5, 21, 27]. HSP27, which is homologous to α-crystalline, has an important function in the binding cytoskeletal proteins [5]. Given the effects of EMP on the microtubular system as well as its estrogenic function, we evaluated the influence of EMP exposure on HSP27 and HSP72. As expected, HSP72 exhibited weak basal expression which was not altered by EMP treatment. In contrast, HSP27

accumulation occurred in a dose-dependent manner after EMP treatment. HSP27 is one of the proteins synthesized in certain mammalian cells following treatment with steroid hormones, including estrogens. Expression of HSP27 has been shown in a variety of estrogen-responsive human tissues and also in breast cancer and in endometrial carcinomas [4, 5, 29]. In these carcinomas the expression of HSP27 has been correlated with prognosis, and for endometrial carcinoma, with the degree of tumor differentiation and the presence of estrogen and progesterone receptors [5]. There is strong evidence that the regulation of HSP27 in PC-3 cells is also under the control of estrogens such as EMP. Lau et al. have recently shown that PC-3 cells express both the estrogen receptor-α and the estrogen receptor-β and that estrogens as well as antiestrogens act as growth inhibitors [20].

Although the precise function of HSP27 in carcinoma cells is only partially understood, this protein might be one of the important mediators of resistance to antineoplastic agents. Resistance for doxorubicin, colchicine, and vincristine has been shown in cell lines transfected with the structural gene for human HSP27 [14]. Antineoplastic agents such as cisplatin can also induce HSP27 synthesis [24]. Thus, the induction of certain HSPs, which can confer a protective effect, needs to be considered in the establishment of effective treatment schedules in combination therapies of chemotherapeutic agents with radiation and/or hyperthermia.

Our results clearly indicate a sensitizing, dose-dependent effect of EMP on hyperthermically induced cytotoxicity in PC-3 prostatic carcinoma cells. The data provide a rationale for trimodal treatment approaches combining EMP, hyperthermia, and radiation for the management of advanced prostatic carcinoma. The analysis of heat shock protein regulation will be of importance for determining optimal doses and treatment schedules to investigate the development of cellular resistance.

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