Differential Uptake of Estramustine Phosphate Metabolites and Its Correlation with the Levels of Estramustine Binding Protein in Prostate Tumor Tissue

Peter H. Walz, Per Björk, Per-Olov Gunnarsson, Karin Edman, and Beryl Hartley-Asp

Department of Urology, Kreiskrankenhaus Lüdenscheid, D-58515 Lüdenscheid, Germany, [P. H. W.], and Pharmacia and Upjohn Oncology, S-22007 Lund, Sweden [P. B., P-O. G., K. E., B. H.-A.]

ABSTRACT

Estracyt (EMP) has been used for the treatment of hormone refractory prostate cancer for many years. Recently, new data from combination studies have given rise to new interest in this old drug. Explanations for the synergy found in the clinic are many, but one major factor may be the previous indication that the drug accumulates in the prostate tumor. We have, therefore, examined the level of the four metabolites, estromustine (EoM), estramustine (EaM), estrone, and estradiol in the serum and tumor of 14 patients with T2 and T3 prostate cancer receiving a single i.v. dose of 600 mg of EMP, about 12 h before radical prostatectomy. Because it has been suggested that the uptake into the prostate tumor is due to binding to the estramustine binding protein (EMBP), we have in addition measured the level of EMBP in the prostate tumor tissue. The main serum and tissue metabolite in all patients was EaM followed by EoM, estrone, and estradiol. The levels for EoM ranged from 63.8-162.8 ng/ml in the serum and from 64.8-1209 ng/ml in the prostate tumor, resulting in a mean ratio for serum to tumor of 1:5. The levels for EaM ranged from 8.3-51.4 ng/ml in the serum and 73.9-563.4 ng/ml in the tumor, giving a mean ratio for serum to tumor of 1:13. The levels of EMBP were higher in T2 tumors than in T2 tumors, 54.1 and 40.7 ng/g tissue, respectively. A significant correlation was found between the levels of EaM (r = 0.60) and the levels of EMBP in the tumor. These data demonstrate that 12 h after a single i.v. dose of 600 mg of EMP the levels of the cytotoxic metabolites EoM and EaM are substantially higher in the tumor than in the serum of the same patient and that a correlation exists between the levels of EaM in the tumor and the levels of EMBP.

INTRODUCTION

EMP2 has been used for many years in the treatment of advanced prostate cancer. Recently, combination treatments with vinblastine or etoposide have shown great promise and, in addition, neoadjuvant treatment with EMP has been reported to cause a reduction in the number of positive margins in T2B patients (1-6). This has caused a renewed interest in an old drug. EMP is a prodrug that is rapidly dephosphorylated on oral administration to the two cytotoxic metabolites EMBP and EoM (7). EoM has been shown to be the major circulating metabolite in prostate cancer patients and, thus, the proposed metabolic scheme is seen in Fig. 1. EoM induces dose- and time-dependent metaphase arrest and breakdown of interphase microtubules in several different human tumor cell lines in vitro (8-10), and causes dose-dependent metaphase arrest in hormone-independent human prostate tumor cells (DU 145) implanted in nude mice (11). The effect of EoM on microtubules has been shown to be through binding to both tubulin and the microtubule-associated proteins (12, 13). Similar effects have been found for EaM. In addition, EaM induced a time-dependent increase in apoptosis in BT4C rat glioma cells implanted in the brain in contrast to a lack of effect in normal rat brain tissue (14). Apoptosis was also found in glioma tissue from patients who had received treatment with EMP (14).

Norlén et al. (15) demonstrated that at steady state, after treatment with oral EMP, a preferential retention of EaM occurred in the prostate tumor; on average, EaM was six times higher in the tumor tissue than in the serum. They suggested that this could possibly be due to specific binding of EaM to a protein present in prostate tumor tissue. This protein has many names (prostatein, 224-protein, and EMBP) and is a secretory protein, the function of which is still unknown. It is a M, 46,000 protein, which in the rat ventral prostate and normal human prostate is under androgenic control (16-19). In man, a protein with similar characteristics has been found in normal tissue, benign prostatic hyperplasia, and carcinoma of the prostate, and in addition, in other tumor tissues such as pancreas, breast, lung, and glioma (20-24). In rats, after a single i.v. dose of tritium-labeled EMP, the concentration of radioactivity 24 h later in the ventral prostate was 20 times higher than in the serum (25).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 To whom requests for reprints should be addressed, at Department of Urology, Kreiskrankenhaus Lüdenscheid, Paulmannshöfer Str. 14, D-58515 Lüdenscheid, Germany. Phone: 49-2351-46-36-80; Fax: 49-2351-46-36-82.

2 The abbreviations used are: EMP, estracyt; EaM, estramustine; EoM, estromustine; EMBP, EaM binding protein; E2, estradiol; E1, estrone; LOQ, limit of quantitation; PSA, prostate-specific antigen.

3 Unpublished data.
Similar results have been found in the BT4C rat glioma model, where a 16:1 tumor to serum ratio for EaM was found (20).

In the present study, our aim was to determine whether a correlation exists between the level of EMBP and the levels of EaM and EoM in tumor tissue. Therefore, we have carried out the present investigation on tumor tissue from radical prostatectomies after a single i.v. administration of EMB. In addition, to broaden our knowledge regarding the preferential uptake/retention of these metabolites and E1 and E2 in the tumor, we have also determined the level of these four metabolites in the serum of these patients.

MATERIALS AND METHODS

Study Design

Fourteen patients, 52–74 years of age, with prostate cancer T2–T3 and negative bone scans, participated in the study. All patients were informed about the trial and consented to participate. All patients received a single i.v. injection of 600 mg of EMB, 12–15.5 h before radical retropubic prostatectomy was performed. This time point was chosen to allow for full metabolism of EMB and distribution of the metabolites to occur.

Serum samples were taken at the start of the operation and at the same time as the tissue samples were taken from the prostate tumor (i.e., approximately 90 min apart). All tissue and serum samples were immediately immersed in liquid nitrogen and stored at −70°C until analyzed.

Analysis of Metabolites

Serum. Serum was assayed for EaM, EoM, E2, and E1 using gas chromatography. Briefly, before gas chromatography, the serum samples were purified by C18 solid phase and solvent extraction procedures and derivatized with bis(trimethylsilyl)-trifluoroacetamide. The reagent solution of each sample was divided in two and evaporated. One of the residues was dissolved in xylene, and EaM and EoM were quantified by gas chromatography (HP 5890) with nitrogen-phosphorus detection. The other residue was dissolved in toluene, and E2 and E1 were quantified by gas chromatography with selected ion monitoring (Varian 3400-Finnigan Mat INCONS 500).

The LOQ was 13 ng/ml EaM, 16 ng/ml EoM, 1 ng/ml E2, and 3 ng/ml E1. The precision (CV) of the methods was as follows: for EaM, 7% at 45 ng/ml; for EoM, 7% at 260 ng/ml; for E2, 10% at 5 ng/ml; and for E1, 9% at 22 ng/ml.

Tissues. Tissue was assayed for EaM, EoM, E2, and E1 using gas chromatography according to a method by Andersson et al. (26), modified for methanol extracts and adjusted to the capillary column instrumentation used for serum analyses.

Tissue samples were homogenized with 15 volumes of methanol. The extracts were evaporated, and the residues were dissolved in 2 ml of water, extracted with hexane, and purified on an aluminum oxide column. The eluates were evaporated and derivatized with bis(trimethylsilyl)-trifluoroacetamide. The reagent solutions were further analyzed as described above for serum samples. The LOQs were 5 ng/sample EaM, 7 ng/sample EoM, 1 ng/sample E2, and 4 ng/sample E1. A sample consisted typically of 10–30 ml of extract. The precision of the methods was as follows: for EaM, 18% at 45 ng/sample; for EoM, 11% at 45 ng/sample; for E2, 39% at 6 ng/sample; and for E1, 54% at 6 ng/sample.

Radioimmunochemical Determination of EMBP

The prostatic tissue specimens were homogenized in ice-cold 50 mM Tris-HCl (pH 7.4) containing 0.25 sucrose, 10 mM KCl, 1.5 MgCl2, and 1% (w/v) NP40 using an Ultra-Turrax homogenizer and clarified by centrifugation for 1 h at 105,000 × g. Proteins were precipitated by adding 1 volume of extract to 9 volumes of freeze-cold acetone and centrifugation for 20,000 × g × 10 min. The acetone was removed by decantation, and the precipitate was washed once with 10 volumes of freeze-cold diethylether and centrifuged as above. After decantation, the remaining ether was removed by evaporation under a stream of nitrogen gas, and the dry residue was dis-
solved in 50 mM Tris-HCl (pH 7.4), 10 mM KCl, and 1 mM EDTA and stored at −30°C until analyzed for EMBP.

The RIA used for determination of the EMBP was as described previously (21), with some modifications. Briefly, radiiodinated EMBP (specific activity, 50–100 μCi/μg) was used as tracer and unlabeled EMBP as standard in concentrations from 0.04–330 ng/ml. Each incubate consisted of 0.1 ml each of the radioligand (7 ng/ml), standard, and, protein G-Sepharose 4-B purified rabbit anti-EMBP (1 μg/ml), and was allowed to react for 2 h. Each vial was rotated overnight with 1 ml of a Gamma Bind Plus suspension (0.2 ml slurry/100 ml; Pharmacia Biotech, Upssala, Sweden), and the antigen-antibody complex was recovered by repeated centrifugations (1,500 × g × 5 min) and aspirations. The antibody-bound radioactivity was determined by the counting of the sediment in a Packard COBRA gamma counter (Packard Instruments Co., Downers Grove, IL). Standards and samples were analyzed in duplicates, the latter at least as three serial dilutions to allow the test of congruence with the standard curve when the antigen was present in enough quantities. Values falling within 10% and 90% of tracer bound in the absence of unlabeled competitor were used for calculation of EMBP concentrations.

RESULTS

In six patients, the tumor stage was pT<sub>2</sub>N<sub>0</sub>; in four patients, pT<sub>3</sub>N<sub>0</sub>; in one patient, pT<sub>3</sub>N<sub>1</sub>; and in three patients, pT<sub>3</sub>N<sub>2</sub>. The tumor was highly differentiated in four patients, poorly differentiated in seven patients, and cribriform or anaplastic in three patients. The PSA values ranged from 0.1–75.3 ng/ml, the highest value being found in one patient with a T<sub>3</sub>N<sub>0</sub> tumor (Tables 1 and 2).

The concentrations of EoM, EaM, E<sub>1</sub>, and E<sub>2</sub> in the serum and prostate tumor tissue of the individual patients are given in Table 3. The major serum metabolite, despite the wide variation of 63.8–162.8 ng/ml, was in all patients’ serum after a single i.v. dose of EMP. The serum concentrations of EaM ranged from 8.3 (LOQ) to 51.4 ng/ml. Although the serum concentrations of estrogens were substantially lower, a similar pattern emerges [i.e., E<sub>1</sub> is the major estrogen ranging from 3.4–14.1 ng/ml, with E<sub>2</sub> from 0.4 (LOQ) to 2.3 ng/ml].

The levels of EoM and EaM in the prostate tumor tissue were higher than those found in the serum of the same patient except for one patient (patient 10), where the concentration of EoM was very similar in both tumor and serum. The concentrations in the prostate tumor tissue ranged from 64.8–1209 ng/g for EoM and from 73.9–563.4 ng/g for EaM (Table 3).

The prostate tumor tissue levels of the two estrogens E<sub>1</sub> and E<sub>2</sub> were also higher than those found in the serum of the same patients with the tumor tissue levels ranging from 5.5–58.7 ng/g for E<sub>1</sub> and 4.5–252 ng/g for E<sub>2</sub> (Table 3). The tumor to serum ratios for EoM and EaM in the individual patients are found in Table 4. The mean values show that the concentration of EoM is 4.79 times higher and EaM 13.07 times higher in the serum tissue than in the serum (Table 4).

The levels of EMBP in the prostate tissue from individual patients are found in Table 4 and vary from 6.73–93.1 ng/g tissue. When the tumors are grouped according to stage, the mean value for T<sub>2</sub> tumors is 40.69, and for T<sub>3</sub> tumors 54.07. A significant correlation was found between the level of EMBP in the tumor and the concentration of EaM in the tumor sample [r = 0.64; P = 0.0297 (least square linear regression, two-tailed)]; the correlation was not statistically significant for EoM (r = 0.29; P = 0.323; Fig. 2).

DISCUSSION

The present study demonstrates that a correlation exists between the level of EMBP in prostate tumor tissue and the level of the two major metabolites EaM and EoM after a single i.v. administration of 600 mg of EMP to 14 patients before radical prostatectomy.

In a previous study, it was demonstrated that in 6 patients who had received oral EMP for several years, the level of EaM was on average 6.3 times higher in the prostate tumor than in the serum of these patients; however, no differences were found for EoM (15). In that study, as demonstrated in other studies (27), a depot of the very lipophilic EoM would have built up in fat during the continuous oral dosing. The binding to fat is unspecific as can be seen from the similarity between the quotas for plasma to fat for EaM and EoM; however, as EaM is the major metabolite, much more of this is released to the serum. Therefore, the lack of accumulation of EoM could possibly be due to EaM being continuously available from the fat depot for unlimited binding to serum proteins, whereas the tumor binding sites were saturable at the concentration range found in the tumor. This problem is overcome in the present study as a single i.v. dose was given. The serum to tumor ratios were 1:4.79 (EoM)
Table 3  Serum (ng/ml) and prostatic tissue (ng/g) concentrations of EoM, EaM, E,1, and E2 in prostate cancer patients (n = 14) who received a single dose of EMP >12 h before surgery and blood sampling

<table>
<thead>
<tr>
<th>Patient</th>
<th>Plasma*</th>
<th>EoM (ng/g prostate tissue)</th>
<th>EaM (ng/g prostate tissue)</th>
<th>E (ng/g prostate tissue)</th>
<th>E2 (ng/g prostate tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>90.8</td>
<td>20.0</td>
<td>5.1</td>
<td>(0.6)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>78.7</td>
<td>19.5</td>
<td>7.8</td>
<td>1.1</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>39.6</td>
<td>(8.3)</td>
<td>6.9</td>
<td>(0.7)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>162.8</td>
<td>51.4</td>
<td>14.1</td>
<td>2.3</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>63.8</td>
<td>11.4</td>
<td>(4.7)</td>
<td>(0.4)</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>82.7</td>
<td>25.7</td>
<td>10.1</td>
<td>1.5</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>66.0</td>
<td>20.9</td>
<td>12.3</td>
<td>1.9</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>85.8</td>
<td>17.1</td>
<td>5.7</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>102.7</td>
<td>18.2</td>
<td>11.8</td>
<td>1.3</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>69.2</td>
<td>15.2</td>
<td>8.7</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>94.4</td>
<td>17.7</td>
<td>5.6</td>
<td>1.9</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>147.7</td>
<td>23.8</td>
<td>11.1</td>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>145.1</td>
<td>30.8</td>
<td>3.4</td>
<td>0.9</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>126.9</td>
<td>28.8</td>
<td>7.4</td>
<td>1.3</td>
<td></td>
</tr>
</tbody>
</table>

* Parentheses, one or two replicates at or below the LOQ.

and 1:13.07 (EaM), demonstrating the retention of both metabolites in the prostate tumor tissues.

The retention of metabolites is similar to data from experimental glioma models. In the rat BT4C orthotopic implanted brain tumor model, the serum to tumor ratio was 1:15 for EaM and 1:5 for EoM (14). Similar data have also been generated from patients with gliomas who received 280 mg of oral EaM phosphate before surgery. In 16 patients, a ratio of 16:1 between tumor tissue and serum for EaM was found 2 h after drug delivery (20).

The present data demonstrate a correlation between the level of EMBP and those of EaM and EoM in samples of prostate tumor tissue from patients receiving EMP. This sub-

**Fig. 2** The correlation between the levels of EoM, EaM, and the EMBP found in the prostate tumor tissue of patients.
stantiates the previous hypothesis that it is this protein that is responsible for the selective uptake and retention of the two cytotoxic metabolites in the prostate tumor (15). This opens the possibility of using the presence or absence of EMBP as an indicator for treatment with EAM phosphate. The biological role of this protein in the normal prostate is still unknown, but in tumor tissue the highest levels of EMBP are found in hormone-refractory prostate cancer after androgen ablation (22). It has also been suggested that the tissue EMBP/dihydrotestosterone ratio might be useful as a marker for predicting disease progression (28). In the present study, this association with tumor progression is also indicated as higher levels of EMBP were found in T2 tumors than in T1 tumors.

Recently, it has been demonstrated that EAM in vitro inhibits the efflux of other cytostatic drugs, possibly via an interaction with the P-glycoprotein (29–31). The present data demonstrate that concentrations necessary for achieving this potentiating effect are present in the tumor tissue. This lends credence to the hypothesis that this could be part of the mechanism behind the clinical results demonstrating synergy between EMP and various other cytotoxic agents, such as vinblastine, etoposide, and paclitaxel in the treatment of advanced prostate cancer (1–4).

The tumor levels of the estrogens were also higher in the tumor than the serum, which is probably a result of the metabolism of EoM and EaM in the target organ. It has recently been demonstrated that 6 weeks of oral EMP neoadjuvant therapy not only caused a decrease in the number of positive margins in patients with T2B disease, but that 6 weeks of EMP therapy produced a higher cytological regression grade than 3 months of flutamide treatment (5, 6). Thus, it is not unlikely that in these hormone-dependent tumors it is both the high levels of estrogens and the cytotoxic metabolites found specifically in the tumor that participate in the antitumor effect.

The present study confirms and extends previous studies demonstrating that the levels of EaM and EoM are significantly higher in tumor tissue than in the serum of the same patient 12–15 h after a single dose of EMP administered i.v. In addition, this study demonstrates a correlation between the levels of the EMBP and the levels of EoM and EaM in the prostate tumor tissue, indicating that the EMBP is involved in the selective uptake and retention of EaM and EoM in prostate tumors.

ACKNOWLEDGMENTS
We thank Kertin Heander and Anne Lindell for help with the bioanalysis.

REFERENCES
10. Wang, M., Tew, K. D., and Stearns, M. E. Immunofluorescent studies of the anti-microtubule effects of the anti-cancer drug estramus- 


Differential uptake of estramustine phosphate metabolites and its correlation with the levels of estramustine binding protein in prostate tumor tissue.

P H Walz, P Björk, P O Gunnarsson, et al.