Evaluation of Lipophilins as Determinants of Tumor Cell Response to Estramustine


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Running title: Lipophilin expression and tumor cell response to EM

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ABBREVIATIONS: EMP, estramustine phosphate; EGFP, enhanced green fluorescent protein; RT-PCR, reverse transcription-polymerase chain reaction; HRPC, hormone refractory prostate cancer; EMBP, estramustine binding protein; EM, estramustine; RACE, rapid amplification of cDNA ends; DMSO, dimethylsulfoxide; PBS, phosphate buffered saline, TBS, Tris buffered saline.

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Abstract

Estramustine administered orally as estramustine phosphate (EMP) remains a major tool in hormone refractory prostate cancer chemotherapy. The presence of estramustine binding protein, prostatin, in prostate tissue may be a determinant of response to treatment. Lipophilins are secretory proteins with homology to prostatin. RT-PCR was performed to estimate expression patterns of lipophilins A, B and C in human biopsies and cell lines resistant to estramustine. Although lipophilin A was not expressed in prostate tissue, both lipophilins B and C were expressed in normal and tumor prostate without significant differences. For lipophilin C, a somatic mutation (T to C transition at positions 409 and 412) was found in human tumor samples and absent in normal prostate tissue. No consistent response to EMP was observed in EGFP-tagged lipophilin C transfected PC3 cells compared to parental controls. Among these EGFP-lipophilin C clones, no direct correlation between response to EMP treatment (IC$_{50}$ values) and EGFP expression was observed ($p = 0.73$). Lipophilin C mRNA levels did not vary significantly between wild type and estramustine-resistant cells in prostate (DU145 and PC3) and ovarian (SKOV3) cancer cell lines. Overall, these results suggest that lipophilins are not specific determinants of estramustine efficacy.
Introduction

Estramustine phosphate [estradiol 3-(bis 2-chloroethyl carbamate) 17-dihydrogen phosphate], a clinically administered anticancer drug, is converted to estramustine, its active cytotoxic form, by dephosphorylation. Estramustine is a nor-nitrogen mustard conjugated to estradiol through a carbamate ester bond. This linkage is not susceptible to cleavage (Punzi et al., 1992) and the parent molecule has antitumor activity through the binding and destabilizing of microtubules (Speicher et al., 1994) with resultant antimitotic activity (Stearns et al., 1985). Its clinical utility has been extended through combination protocols with other antimicrotubule drugs (Speicher et al., 1992) to the treatment of hormone refractory prostate cancer (HRPC), with a significant number of positive clinical trial results in the last few years (for example see Hudes et al., 1992). In addition, because of the improved understanding of its pharmacology, the drug has proven its utility in the management of breast cancer (Hamilton and Muggia, 2001), glioblastoma (Rosenthal et al., 2000) and non-Hodgkins lymphoma (Borghaei et al., 2004). Even though estramustine has an established clinical niche, investigators continue to expand its clinical utility and synthesize novel analogues (Nicholson et al., 2002).

In the rat, estramustine can be concentrated intracellularly through binding to the estramustine binding protein, a factor variously referred to as EMBP, steroid binding protein, prostatin or prostatein (Forsgren et al., 1979). A similar process may occur in humans via structurally similar protein subunits, called lipophilins. Lipophilins A, B and C have been described as plausible human counterparts of the C1, C2 and C3 subunits of prostatin (Zhao et al., 1999). One component, lipophilin A, was homologous to the C1 and C2 subunits of rat prostatin, while the other, lipophilin C, was homologous to the C3 subunit and to human mammoglobin, a protein expressed in some breast carcinomas (Becker et al., 1998). These
peptides are within the secretoglobin family, a group of proteins expressed in numerous secretory glands, including mammary, sweat, salivary and pituitary (Watson and Fleming, 1994; Becker et al., 1998; Mukherjee et al., 1999; Sjodin et al., 2003; Sjodin et al., 2005). Secretoglobins are also prevalent in human tears, (heterodimeric lipocalin) and ovarian cancers (lipophilin C) (Lehrer et al., 1998; Glasgow et al., 2002; Adib et al., 2004). Assuming that the human lipophilins may be functional counterparts of prostatin, their properties may include a capacity to bind estramustine. In the present report, we sought to determine the expression pattern for lipophilins in normal and tumor tissue from prostate and to ascertain if expression of lipophilin C impacted response to estramustine.

**Methods**

**Cell culture.** DU145 wild type and estramustine-resistant cells (DU145 EM) were maintained in Dulbecco’s minimal essential media supplemented with 10% fetal calf serum (Mediatech Inc., Herndon, VA), 2 mM L-glutamine, 0.1 mM non-essential amino acids and 100 units/ml penicillin-streptomycin (Invitrogen, Carlsbad, CA). The media used to maintain estramustine-resistant DU145 cells contained 15 µM estramustine. PC3 wild type and estramustine resistant cells (PC3 EM) were maintained in F-12K Nutrient Mixture (Kaighn’s modification, Mediatech) supplemented with 10% fetal calf serum and 100 units/ml penicillin-streptomycin. The media for the estramustine-resistant cells contained 10 µM estramustine. SKOV3 wild type and estramustine-resistant cells (SKOV EM) were maintained in Alpha Modification of Eagle’s Medium (Mediatech) supplemented with 10% fetal calf serum, 2mM L-glutamine and 100 units/ml penicillin-streptomycin. SKOV EM cells were maintained in media containing 15 µM estramustine.
Analysis of lipophilin expression in prostate tumors. Six matched normal and tumor prostate tissue samples were obtained from the Fox Chase Cancer Center Tumor Bank Facility (Philadelphia, PA) and prepared and analyzed by personnel in accordance with the Fox Chase Cancer Center Institutional Review Board. Total RNA from these samples and total RNA from prostate cancer cell lines was isolated using the RNeasy Mini Kit (QIAGEN Inc. Valencia, CA). Two micrograms of total RNA was DNase I treated (Invitrogen), 1 µg of which was used for cDNA synthesis and the remaining RNA served as a negative control for RT-PCR. First strand cDNA synthesis was performed using Super Script II RNase H− Reverse Transcriptase (Invitrogen). cDNA was used as a template for PCR amplification (Advantage cDNA Polymerase Mix, BD Biosciences Clontech, Mountain View, CA) of products 349, 151, and 138 bp in size, respectively for lipophilins A, B and C via using sense and antisense primer pairs listed in Table 1. Also, sequence information for full-length lipophilin C cDNA, was obtained for the six normal and tumor samples. Since lipophilin A expression was not observed any samples, subsequent analysis focused on lipophilins B and C. RT-PCR was performed in triplicate, and bands were quantified by densitometry (ChemiDoc XRS and Quantity One software, BioRad). Relative expression levels of lipophilins versus loading control (actin or 18S ribosomal RNA), was determined for each sample. Lipophilin C RT-PCR data for tumor cell lines was confirmed by real-time PCR (MyiQ with SYBR Green I detection, BioRad).

Cloning of lipophilin B and C. The 5′- and 3′- ends of lipophilin B and C cDNA were generated using a rapid amplification of cDNA ends (RACE)-PCR strategy. Full-length cDNA’s were constructed using a sense primer beginning at the first nucleotide of the 5′-end of lipophilin C and Pst I restriction sites were present in both the 5′- and 3′-end overlapping fragments. Both fragments were cut with Pst I, ligated, cloned into pCR-XL-Topo vector and sequenced. For
lipophilin B, overlap extension was performed for 3′- and 5′-end fragments, and used as a template for PCR with the T7 universal primer (New England BioLabs, Beverly, MA). Both lipophilin B and C PCR-products cloned into pCR-XL-Topo vector (Invitrogen) for sequencing. Sequence information for full-length lipophilin C cDNA, prepared as described above, was obtained from six normal and tumor samples.

For lipophilin B, primer mutagenesis was used to introduce a Kozak sequence, remove the stop codon and create Nhe I and Bam HI restriction sites. Using these restriction sites, lipophilin B cDNA sequence was cloned in-frame with a C-terminal myc-His-tag into pcDNA3.1/myc-His(-)C vector (Invitrogen), further referred to as “His-LB.” For lipophilin C, primer mutagenesis was used to remove the start codon was mutared and an Xho I was generated. The PCR-product was cloned into pCR-XL-Topo vector (Invitrogen) and subcloned into the pEGFP-C3 vector (BD Biosciences Clontech) using the created Xho I site and Eco RI site from the pCR-XL-Topo vector. The lipophilin C cDNA sequence was in frame with an N-terminal EGFP-tag (EGFP-LC).

**Transfection.** DU145 and PC3 cells were transfected with the plasmid constructs EGFP-LC and His-LB, respectively, or vector alone controls, using 15 µl of FuGENE 6 Transfection Reagent (Roche Applied Science, Indianapolis, IN). At 24 h post-transfection, the medium was replaced with serum-free medium and at 60 h, medium was collected from transfected and vector alone transfected cells, centrifuged at 500 × g for 10 minutes and concentrated using Amicon Centriplus YM-10 for EGFP-LC, or YM-3 for His-LB (Millipore, Billerica, MA).

**Western blotting.** Cells were harvested with a cell scraper and homogenized using a syringe fitted with a 21-gauge needle. Cells were lysed in PBS containing 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 10 µg/ml PMSF, 30 µl/ml aprotinin
Cell lysate was then incubated for 45 min on ice and centrifuged at 10,000 × g for 10 minutes at 4°C. The supernatant was collected and protein concentrations were measured using the Bradford method (BioRad). Proteins were separated on a 15% SDS-polyacrylamide gel and transferred to nitrocellulose membranes using an electrophoretic transfer apparatus (BioRad). Membranes probed with anti-His antibody were blocked with 6% bovine serum albumin [BSA (Sigma)] and 0.5% Tween-20 (BioRad). Membranes probed with anti-EGFP were blocked in 5% non-fat dry milk/PBS/0.5% Tween-20 in Tris buffered saline (TBS). Monoclonal anti-penta-His (Qiagen) and polyclonal anti-EGFP (Clontech) antibodies were diluted 1:1000 in blocking buffer. All antibodies were incubated at 25 °C for 1 h or at 4 °C for 16 h. Membranes were probed with horseradish peroxidase-linked secondary antibodies and detected using an enhanced chemiluminescence system (Amersham) with on Kodak film.

**Cell survival assay.** Estramustine phosphate (EMP) in its disodium salt form (Emcyt®, Pharmacia), was dissolved in dimethylsulfoxide (DMSO) and sterilized using a 0.22 µm DMSO-safe syringe filter (Pall Life Sciences, East Hills, NY). Cell viability following EMP exposure was determined in both parental PC3 cells and PC3 transfectants expressing EGFP-LC, referred to as clones A4, A6, C2 and D2. Cells cultured in RPMI 1640/10%FBS (Invitrogen) were seeded at 7000 cells/well a 96-well format and treated with EMP or DMSO-vehicle control for 3 d. A colorimetric, MTT-based assay was used to quantify cell viability. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) was added to the cells for final concentration of 0.5 mg/ml. After a 4 h incubation at 37°C/5% CO₂, cells were permeabilized in 10% sodium dodecyl sulfate/0.1M HCl and incubated again for 16 h. Absorbance was measured on a Benchmark Microplate reader (BioRad). Cell viability, defined as the difference in absorbance at 550 and
690 nm, was determined for cell lines at various concentrations of EMP. IC\textsubscript{50} values were determined using KaleidaGraph\textsuperscript{®} software (Synergy Software).

**Flow cytometry.** PC3 cells and pEGFP-lipophilin C expressing clones were seeded on 10 cm plates, grown to ~80-90% confluence, harvested, counted using a hemocytometer, pelleted by centrifugation at 4°C. After removing media, the cells were washed twice in cold PBS and fixed in 2% paraformaldehyde/PBS pH 7.4 for 1 h at 4°C. Cells were resuspended in PBS at 10\textsuperscript{6} cells/ml for analysis on the FACSCalibur\textsuperscript{TM} analytical flow cytometer (BD Biosciences). EGFP positive cells were gated from a minimum of 10\textsuperscript{4} cells per run. Data are reported as the average percentage of cells gated within the EGFP-positive population.

**Statistics.** Three independent experiments were conducted for analyses of data from RT-PCR, western blotting, cell survival assays and flow cytometry. For cell survival assays (n = 21 for each experiment), and RT-PCR densitometry (n = 3 for each experiment), a student's t test was used to compare mean values. Differences were considered to be significant if \(P\)-values were less than 0.05. Linear regression analysis of mean EGFP expression versus mean IC\textsubscript{50} values for EMP treatment was performed using QuickFit software (Micro-Active Australia Pty Ltd).
Results

Lipophilins B and C are not differentially expressed in human prostate tumors. The expression profiles of specific isoforms of human lipophilin were determined in both clinical prostate samples and in various human cancer cell lines. Using primers for RT-PCR analysis (Table 1) expression of lipophilins A, B and C in human clinical biopsies and cell lines from prostate were analyzed. Lipophilin A levels in all biopsy and cell line samples proved to be below the level of detection (data not shown), while lipophilins B and C (Fig. 1A) were expressed at detectable levels in both normal and tumor tissues. In the examples shown from four individuals (Fig 1A), expression levels were variable, with some paired samples showing apparent differences in expression. However, quantitative analyses of replicates ($n = 15$) showed no statistical difference in expression levels between normal and tumor samples of all 6 patients ($p > 0.05$).

Lipophilin C polymorphism in prostate tumors. Comparative sequence data for cDNA’s from normal and malignant human prostate samples were analyzed to search for the presence of possible somatic mutations. No changes in lipophilin A or B were found ($n = 6$); however, for lipophilin C, a T to C transition was found in tumors at positions 409 and 412 (Fig. 1B). Samples from both normal and tumor tissues were shown to have an additional guanine residue at position 413 when compared to the GenBank sequence. This change was outside of the open reading frame.

Expression of lipophilins in cancer cell lines is unrelated to an estramustine-resistance phenotype. Lipophilin B was detected at equivalent low levels in both wild type and estramustine-resistant DU145 and PC3 cells. Similar low levels were found in SKOV3 ovarian carcinoma cells and a line made resistant to estramustine and there was no enhanced expression
of lipophilin B in the resistant cell line (data not shown). Concurrent with the biopsy data, 
lipophilin C was also expressed at low levels in prostate carcinoma cells (PC3 and DU145) and 
their EM-resistant counterparts (Fig. 2). Similarly, SKOV3 ovarian carcinoma cells and 
corresponding EM-resistant clones (SKOVEM3, SKOVEM10, and SKOVEM15, each adapted 
to grow in 3, 10 and 15 µM EM, respectively) had no differences in lipophilin C expression (Fig. 
2). Relative expression patterns for lipophilin C were confirmed by real time PCR using 18S 
rRNA as a loading control (data not shown). Overall, drug resistance to EM did not seem to 
require modulation of lipophilin C expression by tumor cells, nor did it seem to be a cause and/or 
effect of malignant transformation.

**Epitope-tagged lipophilins are not secreted when transiently expressed in prostate 
cancer cells.** In order to study these secretory proteins, tagged constructs were generated to 
assess whether lipophilins were secreted into the extracellular medium in overexpressing cell 
lines. The pcDNA3.1/myc-His(-)-lipophilin B construct was transfected into PC3 cells and after 
60 h, the medium was collected for detection of any secreted protein, and cells were harvested 
and lysed for analysis of intracellular protein. The myc- His-tag was detected in the cell lysates 
but not in the medium (Fig. 3A). Similarly, DU145 cells were transfected with the pEGFP-C3 
lipophilin C plasmid and the fusion protein was found in the lysate, but not in the medium (Fig. 
3B). However, EGFP alone was detected both in the cell lysates and in the medium of the cells 
transfected with the empty pEGFP-C3 vector (Fig. 3B). These results demonstrated over-
expression of lipophilin B or C in PC3 or DU145 cells, respectively, and suggested that neither 
isoform was secreted in this cellular context.

**Stable expression of a lipophilin C-EGFP fusion protein did not consistently impact 
PC3 cell response to EMP treatment.** Subsequent treatment of the transfected cells with
estramustine phosphate (EMP) showed that increased expression of the EGFP-lipophilin C fusion protein had no impact on cell survival (Fig. 4). IC$_{50}$ values for PC3 parental cells (142 µM) lay within the range of that determined for EGFP-lipophilin C stable clones (from 102 to 191 µM). Relative EGFP expression was determined for each line and was also shown to vary (Fig.4). Linear regression analysis showed that EGFP expression did not correlate with EMP IC$_{50}$ values ($r = 0.21, p = 0.73$).
Discussion

The aim of this study was to investigate what role, if any, lipophilins might play in determining the response of prostate cancer to the anti-microtubule drug estramustine. The clinical formulation, estramustine phosphate, is routinely used in the treatment of hormone refractory prostate cancer. Earlier reports have suggested that the efficacy of the drug in rats may be affected by the presence of prostatin (Forsgren et al., 1979). When homology between prostatin and lipophilin family members was described (Lehrer et al., 1998), there seemed to be value in comparing lipophilin expression patterns and drug response in biopsies and cell lines.

In the present study, two T to C transitions were found in the lipophilin C sequence of human prostate tumor biopsies that were not observed in matched normal tissues. These changes, outside of the open reading frame, did not appear to effect expression levels of lipophilin C, as normal and tumor paired samples did not differ significantly. In prostate cancer cell lines, DU145 and PC3, clones with acquired EM resistance expressed similar levels of lipophilins B and C versus parental controls. In addition, the over-expression of lipophilin C in PC3 cells did not alter the growth-inhibitory effects of estramustine.

Our earlier work showed that acquired resistance to estramustine was accompanied by amplification of the q34 region of chromosome 9, a region containing the ATP binding cassette transporter ABCA2 (Laing et al., 1998), a transporter that is causally linked with sequestration of drug into the endosome/lysosome compartment (Vulevic et al., 2001). The chromosomal localizations of lipophilin genes are: 15q12-q13 (lipophilin A), 10q23 (lipophilin B), and 11q12-q13.1 (lipophilin C) (Lehrer et al., 2000). At least in this estramustine selected resistant cell line, there is no concordance between resistance and those chromosomal regions coding for
lipophilins. This fact would be consistent with the lack of any positive correlation described by the present data.

There are a number of explanations for the low expression levels of lipophilin B and C in the cell culture lines (DU145, PC3 and SKOV3). These cell lines are known to grow in a hormone-independent manner. DU145 and PC3 cells are androgen-receptor negative (Chlenski et al., 2001) and do not bind testosterone. In SKOV3, androgen and progesterone receptors are dramatically down-regulated and estrogen receptor α is mutated (32-bp deletion in exon 1), defining SKOV3 as estrogen receptor-positive, but estrogen-insensitive (Lau et al., 1999). The expression of C3 and C1 subunits of rat prostatin is regulated by androgen (Zhang et al., 1988). These findings, together with in vitro androgen receptor binding to DNA sequences from intron 1 of the prostatin C3 subunit gene suggest that its expression is a function of the quantitative expression levels of the androgen receptor (Claessens et al., 1989). If prostatins and lipophilins are functionally synonymous, expression of the latter may also be androgen-regulated and dependent on the androgen receptor. The lack of androgen receptors in DU145, PC3 and SKOV3 cell lines could provide a rational explanation for the observed low levels or absence of lipophilin subunit expression. In an obverse fashion, the prevalent expression of lipophilins B and C in the human biopsies implies an association with hormone responsiveness.

Because lipophilins have been functionally linked with secretion, there was some concern that this function, if it were to exist in prostate, might complicate the premise of intracellular estramustine concentration. Generally, DU145 and PC3 cells express granular secretions. These granules have properties similar to the human seminal prostasomes, a granular type of secretory product in the human prostate gland cells (Nilsson et al., 1999), illustrating that PC3 and DU145 cells are capable of secretion. However, our data showed that tagged lipophilins were not
secreted in the transfected prostate carcinoma cells. This implies that both the B and C subunits may be necessary to facilitate secretion of these proteins.

Stable transfection of lipophilin C in PC3 cells did not impact the cytotoxic profile of estramustine. This result is consistent with the observation that lipophilin C expression patterns do not correlate with the acquired resistance phenotype. While extrapolation of results gathered from cell lines is not fully representative of a complex prostate tumor microenvironment, the lack of correlation between lipophilin expression and estramustine response supports the conclusion that lipophilins may not be critical determinants of response to estramustine.

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References


Footnotes

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Authors J.M.T. and Z.L contributed equally to the work.

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Figure Legends

**Fig. 1.** Levels of lipophilin B expression in biopsies and prostate cancer cell lines do not correlate to a transformed state or drug resistance phenotype. (A) Representative RT-PCR products of lipophilin B (LB) and lipophilin C (LC) are shown, both normalized to actin, for matched pairs of normal (N) and tumor (T) prostate tissues from 4 patients. (B) Sequence alignment of lipophilin C cDNA from prostate tumor tissue illustrating the T to C transitions at nucleotides 409 and 412, and the insertion of a G nucleotide at position 413 compared to the GenBank sequence. The open reading frame is underlined.

**Fig. 2.** Relative lipophilin C expression is similar between tumor cell lines and their EM-resistant counterparts. Lipophilin C mRNA levels measured by RT-PCR were normalized using products of 18S rRNA as loading controls. PC3, DU145, and SKOV3 cells all express levels of lipophilin C that do not differ significantly ($p > 0.30$) from corresponding estramustine-resistant cell lines (PC3EM, DU145EM, and SKOVEM3, 10 and 15, respectively).

**Fig. 3.** Prostate cancer cells retain epitope-tagged lipophilins B and C intracellularly. (A) Immunoblot detection of His-tagged lipophilin B (His-LB) in the medium and cell lysates for non-transfected control PC3 cells (lane 1) and His-LB transfected PC3 cells (lane 2). Molecular weight marker (M) confirmed the predicted size of the recombinant protein (15kD). (B) Detection of the EGFP epitope for DU145 cells transfected with EGFP-LC (lane 1), vector alone (EGFP-VA, lane 2) and non-transfected cells (lane 3). For both cell lines, lipophilin expression was detected in the cell lysate, with no indication of secretion into the medium.
Fig. 4. Stable EGFP-lipophilin C expression does not correlate to EMP sensitivity in PC3 cells. IC₅₀ values for EMP treatment were calculated by the average of three independent experiments for EGFP-LC clones A4, A6, C2 and D2 and PC3 parental controls. Cell viability was quantified by colorimetric MTT assay following 3 d of EMP exposure. EGFP expression is shown by the average percentage of cells gated within the EGFP-positive population. Flow cytometric analysis was also conducted in triplicate. Linear regression analysis (Quick Fit) showed no correlation between EGFP expression and EMP IC₅₀ values (r = 0.21, p = 0.73).
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Figure 1

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LB

LC

actin

B

GenBank

Normal

Tumor

AATATGAAGAGTAATTAACCTTACCCA......CATCTGTGGAT - TGCTAG...
AATATGAAGAGTAATTAACCTTACCCA......CATCTGTGGATGTTGCTAG...
AATATGAAGAGTAATTAACCTTACCCA......CATCTGTGGATGAGTGCTAG...

409  412
Figure 2

![Bar chart showing relative LC expression across different cell lines.](chart.png)
Figure 3