Targeted destruction of prostate cancer cells and xenografts by lytic peptide-βLH conjugates

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SUMMARY
In a series of experiments conducted \textit{in vitro}, we have established the concept that conjugates of the lytic peptides Hecate or Phor14 with a fragment of the beta chain of hCG (designated βLH) selectively destroy both androgen sensitive and insensitive human prostate cancer cells. Extraction of steroids from the culture medium by charcoal reduced the ability of the conjugates to kill LNCaP, BRF41T, and PC-3 cells. Addition of hormones known to up-regulate LH receptors (estradiol, testosterone or FSH) to the culture medium restored the ability of the conjugates to kill these cell lines. The toxicity of the conjugates (EC\textsubscript{50}s) to these cell lines was closely correlated to their LH binding capacities (nmol/t10 cells). In a series of \textit{in vivo} experiments we have shown that both the Hecate and Phor14-βLH conjugates are remarkably effective in causing tumor cell necrosis and cessation of tumor growth in nude athymic mice. Treatment with Hecate-βLH (12 mg/kg body weight) resulted in a reduction of tumor burden (mg tumor/g body weight) from 60 to 14 (P<0.0001); treatment with Phor14-βLH (12 mg/kg body weight) reduced tumor burden to 27 mg (P<0.0001). Treatment with a high dose of Phor14-βLH (24 mg/kg body weight) reduced the tumor burden from 60 to 12 mg/g (P<0.0001). Pretreatment of animals receiving a low dose of Phor14-βLH (12 mg/kg) with either estradiol or follicle stimulating hormone (FSH) resulted in reduction of tumor burden from 60 to 11 mg/g. Administration of a second 3-week treatment after a one month recovery period caused complete regression of more than 75 percent of the tumors. No changes in body weight or histological abnormalities were found in any of the organs examined, except the testes.

INTRODUCTION
Presently available treatments for advanced, hormone resistant prostate cancer are only marginally effective and newer agents are needed to selectively kill the cancer cells [4]. We developed a novel approach for the treatment of prostate cancer. We hypothesized that an amphipathic membrane disrupting peptide linked to a protein hormone, like luteinizing hormone (LH), for which receptors are expressed in both androgen dependent and independent prostate cancer cells, could serve as an effective drug. Intensive studies on the lytic peptide conjugate Hecate-βLH \textit{in vitro} clearly established the concept that lytic peptides linked to a fragment (amino acids 81-95) of the beta chain of LH (fig. 1) specifically target and destroy prostate cancer cells that express LH receptors [6]. The ability of the lytic peptide-βLH conjugate to destroy Chinese Hamster Ovary (CHO) cells was significantly (p<0.02) greater in LH receptor transfected cells than in wild type cells, which contain no LH receptors. A further increase (p<0.02) in the ability of the lytic peptide-βLH to kill CHO cells was seen when ZnCl\textsubscript{2} was added to cells transfected with the rat LH receptor fused to a methylthionine promoter (fig. 2). The βLH fragment was shown to compete with the lytic peptide-βLH for binding sites on the LH receptor in LNCaP prostate cancer cells (fig. 3).

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**Phor14-βLH:** Lys Phe Ala Lys Phe Ala Lys Phe Ala 5 10
Lys Phe Ala Lys // Ser Tyr Ala Val Ala Leu Ser Cys Gln Cys 15 20
Ala Leu Cys Arg Arg 25

**Hecate-βLH:** Phe Ala Leu Ala Leu Lys Ala Leu Lys Ala Leu 5 10
Leu Lys Lys Leu Lys Ala Leu Lys Ala Leu // Ser Tyr 15 20 25
Ala Val Ala Leu Ser Cys Gln Cys Ala Leu Cys Arg Arg 30 35

Fig. 1. Sequences of the conjugates of the amphipathic alpha-helical cationic peptides, hecate (1-23) or Phor14 (1-14), with the hCG fragment (81-95). Cysteine-bridge formation was prevented during synthesis by protection with ACM (acetylamidomethyl)-groups.

![Graph 1](image1)

**Fig. 2.** Cytotoxicity of Hecate-βLH to wild type CHO cells. CHO cells transfected with the LH receptor gene (rCHO) and transfected CHO cells treated with ZnCl₂ (rCHO activ.) to increase the number of LH receptors. Cytotoxicity was measured at concentrations of 0 - 100 μM of Hecate-βLH by trypan blue staining and lactate dehydrogenase (LDH) release. The values are the means of two independent experiments. *p<0.05 for CHO vs rCHO and rCHO activated vs rCHO. (from Leuschner et al., 6)

![Graph 2](image2)

**Fig. 3.** Cytotoxicity of Hecate-βLH in the presence and absence of the βLH fragment. Cytotoxicity was measured in LNCaP cells to Hecate-βLH (10 and 25 μM) in the presence of 0 and 50 μM βLH (81-95) fragment. Cell viability was determined by trypan blue staining of the cells. The values express the means of two experiments. *p<0.01. (from Leuschner et al., 6)
The toxicities of the lytic peptide-βLH conjugate differed among prostate cancer cell lines and their differences were related to their LH binding capacities (table 1). Removal of steroids from the culture media reduced the capacity of the lytic peptide-βLH conjugate to kill prostate cancer cells and addition of estradiol (E2), testosterone, FSH or EGF, compounds known to up-regulate LH receptors in some cell lines, to the steroid free media restored the toxicity of the conjugate in all but one cell line (figs. 4, 5) [6]. Treatment of male nude mice with Hecate-βLH (12 mg/kg body weight) injected via the tail vein on days 15, 22 and 28 after tumor inoculation significantly reduced tumor burden, compared to control mice (fig. 6).

**Table 1. LH Receptors in prostate cancer cell lines and Chinese Hamster Ovary (CHO) cells in complete and charcoal treated (cc) media**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Binding affinity Kd [nM]</th>
<th>Binding Capacity [fmol/10^6 cells]</th>
</tr>
</thead>
<tbody>
<tr>
<td>BRF 41 T (Complete media)</td>
<td>10±3.5</td>
<td>106±9</td>
</tr>
<tr>
<td>BRF 41 T (CC media)</td>
<td>14.8±5.8</td>
<td>65±10*</td>
</tr>
<tr>
<td>Du145</td>
<td>3.3±1.5</td>
<td>87±11</td>
</tr>
<tr>
<td>LNCaP (Complete media)</td>
<td>5.3±1.2</td>
<td>48±5</td>
</tr>
<tr>
<td>LNCaP (CC media)</td>
<td>1.11±0.44</td>
<td>16±1**</td>
</tr>
<tr>
<td>rCHO</td>
<td>2±0.7</td>
<td>49±5†</td>
</tr>
<tr>
<td>rCHO activated</td>
<td>4.4±0.9</td>
<td>176±11</td>
</tr>
</tbody>
</table>

*p<0.001 vs. complete media  
** p<0.0001 vs. complete media  
† p<0.0001 vs. activated media

**Fig. 4. Effect of addition of 17β-estradiol (E2) or dihydrotestosterone (DHT) in the presence or absence of tamoxifen (T) on the sensitivity of BRF 41 T (A) and LNCaP cells (B) to Hecate-βLH (12.5 and 25 μM, respectively) in steroid free media. Cell viability was measured by trypan blue staining. *p<0.002 compared to the charcoal treated samples, #p<0.001, compared to E2 and DHT treated samples. The values are means of two independent experiments. (from Leuschner et al., 6)**
Fig. 5. Cytotoxicity of Hecate-βLH to LNCaP (25 μM), BRF 41 T and DU145 (12.5 μM) cells in the presence of epidermal growth factor (EGF) or follicle stimulating hormone (FSH). Cell viability was measured by LDH release. *p<0.002 vs. charcoal treated media. The values are the means of two different experiments. (from Leuschner et al., 6)

Fig. 6. Effect of Hecate-βLH on PC-3 xenografts in nude mice. A/ Tumor growth during treatment of PC-3 xenografts with Hecate-βLH. PC-3 prostate cancer cells were implanted into male nude mice (6 weeks of age). Treatments with Hecate-βLH (12 mg/kg body weight) were performed via tail vein injection on days 15, 22 and 28 post tumor inoculation. B/ Tumor burden in Hecate-βLH treated and saline control mice bearing PC-3 xenografts. Tumor weight was determined 36 days post inoculation for each group. n=12 per group; *p<0.0001 compared to saline control tumor bearing mice. (from Leuschner et al., 6)
Collectively, these experiments clearly established the concept that prostate cancer cells can be targeted through their LH receptors and that the toxicity of the targeting drug can be increased by prior treatments in vitro with substances that up-regulate LH receptors.

In the present study we have extended these results to another less toxic lytic peptide conjugate (Phor14-βLH) and have tested the concept that "up-regulation" of LH receptors increases the sensitivity of human prostate cancer xenografts in nude mice to the lytic peptide conjugates. We have also compared the abilities of Hecate-βLH and Phor14-βLH to destroy human xenografts in vivo and examined the potential of these tumors to resume growth after cessation of treatment.

Phor14 is a 14 amino-acid amphipathic synthetic peptide, which is less toxic to both bacterial and mammalian cells than Hecate. We conjugated Phor14 to a 15-amino acid fragment of the β-chain of hCG (fig. 1), hereafter referred to as βLH. This 29 amino acid conjugate (Phor14-βLH) is expected to have low antigenicity because of its relatively small size, yet it retains the ability to bind LH receptors [9]. Membrane disrupting peptides exhibit strong toxicity to mammalian cells. As a mechanism of cell lysis, it has been suggested that pore/ion channels are formed by disorganizing lipids and plasma membrane proteins, causing the release of phospholipids from the plasma membranes [3, 7, 8].

MATERIALS AND METHODS

Experimental outlines

In the first experiment, the effect of Phor14-βLH conjugate on androgen dependent and independent prostate cancer cell lines was investigated. In the second experiment, the effect of steroids on the toxicity of the conjugate to LNCaP and PC-3 cells was studied by reconstituting the charcoal treated media with estradiol or testosterone, alone or in combination with the estrogen receptor antagonist tamoxifen. The effect of FSH pretreatment on the toxicity of the conjugate to PC-3 and LNCaP cells was investigated in experiment 3. Lactate dehydrogenase (LDH) and trypan blue staining were used as measures of cell viability. In the fourth experiment the effects of administration of Phor14-βLH at two concentrations and in the presence or absence of estradiol or FSH pre-treatments on PC-3 xenografts in athymic nude mice was studied. In the fifth and final experiment, the ability of PC-3 xenografts in nude mice to recover from Phor14-βLH treatments, and the effects of pre-treatments after a 4-week recovery period were studied.

Cell cultures

The immortalized human primary prostate cancer cell line, BRF 41 T, was obtained from the Biological Research Faculty and Facility, (Jamasville, MD) and cultured in BRFF-HPC 1 medium containing 100 IU/ml penicillin and 100 μg/ml streptomycin. "BRFF-HPC-1" was also obtained from Biological Research Faculty and Facility, (Jamasville, MD). The human prostate cancer cell line LNCaP (derived from lymph node metastasis), DU145 (derived from brain metastasis), and PC-3 (derived from bone metastasis), were obtained from the American Type Culture Collection (Rockville, MD). LNCaP cells were grown in RPMI 1640 medium containing 1.5 g/l sodium bicarbonate, 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin. 4.5 g/l glucose, 10 mM HEPES, 1 mM sodium pyruvate and 10% fetal bovine serum. PC-3 cells were cultured in F12-K medium with 2 mM L-glutamine, 1.5 g/l sodium bicarbonate, 100 IU/ml penicillin and 100 μg/ml streptomycin. DU145 cells were grown in Eagle's minimum essential medium (MEM) with 2 mM L-glutamine and Earle's BSS, 1.5 g/l sodium bicarbonate, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 100 IU/ml penicillin and 100 μg/ml streptomycin and 10% fetal bovine serum. Fetal bovine serum was purchased from Hyclone (Logan, UT), RPMI 1640, MEM and Hams F 12 media were obtained from Life Technologies, (Gaithersburg, MD). The cultures were maintained at sub-confluency at 37°C in a humidified atmosphere of 5% CO₂:95% air. Steroid free media were obtained by charcoal/dextran treatment.

Drugs

Phor14-βLH and Hecate-βLH were synthesized and purified in the Protein Facility, Department of Chemistry, Louisiana State University, Baton Rouge, LA. Figure 1 shows the sequences of Phor14-βLH and Hecate-βLH.
Cytotoxicity studies
Cells were grown to 70% confluency and kept in complete or charcoal treated media for 48h. Treatment with Phor14-βLH (0 - 300 μM) was initiated by adding various concentrations of the drug dissolved in saline to the supernatant. The cultures were incubated for 4h at 37°C in a humidified atmosphere. Reconstitution experiments were performed by growing cells to 70% confluency, changing the supernatant to the charcoal treated media for 48h and then adding 5 nM 17β-estradiol with and without 100 nM tamoxifen, 10 nM testosterone with and without tamoxifen, or FSH (10 or 30 ng/ml) to charcoal treated media for an additional 48h. Treatment of the cells with Phor14-βLH was performed as described above, using 50 μM for LNCaP and PC-3 cells. Cell viability was determined by trypan blue exclusion and LDH release.

Determination of cell viability
Treated and untreated cells were incubated with trypan blue for 10 minutes, after which 300 cells per well were examined for dye exclusion. Lactate dehydrogenase activity released from the cells was determined from samples drawn from the supernatants directly after treatment and analyzed within 3h using the colorimetric LDH assay kit according to the manufacturer's instructions (Sigma, St. Louis, MO). LH receptor binding capacities were determined by radio-receptor assay as previously described [6].

Tumor establishment in nude mice
Athymic BalB/c male nude mice (4 weeks of age) purchased from Harlan Sprague Dawley, IN, were housed in autoclaved cages fitted with a high efficiency filter-top and with autoclaved bedding. The animals were fed irradiated Purina chow (5350) and autoclaved water. The room was kept at 25°C with a 12 hour light-dark cycle. At 6 weeks of age the animals (25-30 g body weight) were subcutaneously implanted with 1 x 10⁶ PC-3 cells suspended in 0.1 ml of PBS and 0.3 ml of phenol red free Matrigel (Collaborative Biomedical Products, Beckton Dickinson Labware, Bedford, MA) using a 27 gauge needle [15]. Control mice received PBS and Matrigel. Body weights were determined weekly, and tumor size was monitored beginning 14 days after tumor inoculation by measuring with a microcaliper. In experiment 4, animals with tumor volumes of 100-150 mm³ were allotted to the following treatment groups: a/ saline control, b/ Phor14-βLH 12 mg/kg, c/ pre-treatment 3 days with 0.15 mg/d E₂, then Phor14-βLH, 12 mg/kg [15], d/ pre-treatment 3 days with 3 μg/d FSH s.c. prior to Phor14-βLH, 12 mg/kg and e/ Phor 14-βLH, 24 mg/kg. The conjugates were administered via tail vein injection. The control animals received saline injections. There were 12 animals per group. In preliminary studies, it was determined that the maximum non-toxic dose is 24 mg/kg for Phor 14-βLH and 12 mg/kg for Hecate-βLH. The animals were treated once a week for 3 weeks, euthanized 7 days after the last treatment and a complete necropsy was performed. Body weights, tumor weights and organ weights were recorded. The tissues were fixed in 10% neutral PBS buffered formaldehyde, embedded in paraffin, sectioned and stained with hematoxylin-eosin. The tumors were studied histologically and all other tissue was stored for later detailed histological studies. In experiment 5, three groups of PC-3 tumor-bearing animals were treated as follows a/ saline control; three injections at days 15, 22, and 28, and sacrificed at 48 days, b/ Phor14-βLH (24 mg/kg) at days 15, 22, and 28, and again at days, 63, 70, and 77, and c/ FSH pretreated (3 μg/day for 3 days) plus Phor14-βLH (12 mg/kg) administered as in group b. The control group consisted of 6 mice; the Phor14-βLH and the FSH plus Phor14-βLH groups consisted of 12 and 10 mice, respectively. No treatments were given between days 29 and 63. Control animals were autopsied after tumors reached 300 mm³. This study was approved by the institutional animal care and use committee and animal care was in compliance with the principles of laboratory animal care of the NIH, USA.

Statistical analysis
Statistical analyses were conducted using Student's t test and ANOVA. Differences were considered significant at p < 0.05.
RESULTS
Toxicity of Phor14-βLH to prostate cancer cells
Figure 7 illustrates the ability of Phor14-βLH at concentrations between 0 and 300 μM to kill various prostate cancer cells cultured in complete media (experiment 1). Cell viability was determined by trypan blue exclusion and LDH release. The toxicity (EC₅₀) of Phor14-βLH was concentration dependent in all cell lines and differed among cell lines (p<0.01 for LNCaP compared to BRF 41 T, PC-3 and DU145, and p<0.05 for BRF 41 T compared to PC-3 and DU145; table 2). A decreasing sensitivity among the cell lines was observed; BRF 41 T (EC₅₀ 29.3 ± 1.5 μM), PC-3 (EC₅₀ 34.8 ± 2.5 μM), DU145 (EC₅₀ 36.3 ± 1.8 μM) and LNCaP cells (EC₅₀ 46.9 ± 2.6 μM).

*Fig. 7. Cytotoxicity of Phor14-βLH to prostate cancer cells (BRF 41 T, PC-3, LNCaP and DU145). Cell viability was determined by trypan blue staining and lactate dehydrogenase release of cells treated with Phor14-βLH at concentrations of 0 - 300 μM in complete media. The values represent the means of two experiments.*
Table 2. Sensitivities of prostate cancer cells to Phor14 alone and Phor14-βLH and the relationship of sensitivity of cell lines to LH binding capacity

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Phor14 Alone (EC₅₀)</th>
<th>Phor14-βLH–Complete Medium (EC₅₀)</th>
<th>LH-Receptor Binding Capacity [fmol/10⁶ cells]</th>
</tr>
</thead>
<tbody>
<tr>
<td>LNCaP</td>
<td>26.3±3.5 μM²</td>
<td>46.9±2.6 μM</td>
<td>48.16±5* /16.1°±1⁰C</td>
</tr>
<tr>
<td>PC-3</td>
<td>25.6±3.9 μM⁴</td>
<td>34.8±2.5 μM</td>
<td>n.d.</td>
</tr>
<tr>
<td>DU145</td>
<td>28.8±2.6 μM⁴</td>
<td>36.3±1.8 μM</td>
<td>87.64±11*</td>
</tr>
<tr>
<td>BRF41 T</td>
<td>29.5±0.8 μM</td>
<td>29.3±1.5 μM⁸</td>
<td>106±9°/65.5°±10⁰C</td>
</tr>
</tbody>
</table>

The LH receptor capacity is given for complete* and CC (charcoal treated) medium n.d. = not determined, EC₅₀ according to Hill Equation.
A/ p<0.05 vs. LNCaP; B/ p<0.05 vs. PC-3, DU145; C/ p<0.0001 vs. complete media; D/ p<0.001 vs. complete media; E/ p<0.05 vs. conjugate; n = 8.

PC-3 and LNCaP cell lines responded to steroid removal with a 50% reduction in sensitivity to the lytic peptide conjugate at a concentration of 50 μM (p<0.02) (figs. 8A, B). The relationship between cytoxicity and LH receptor expression is shown in table 2. LH receptor binding capacity was significantly reduced (p < 0.001) by steroid removal from the media. LNCaP cells with the highest EC₅₀ had the lowest LH binding capacity, while BRF 41T cells with the lowest EC₅₀ had the highest receptor binding capacity.

Fig. 8. Effect of addition of 17β-estradiol (E₂) or testosterone (Testo) in the presence or absence of tamoxifen (Tam) on the sensitivity of LNCaP (A) and PC-3 cells (B) to Phor14-βLH (50 μM) in steroid free media. Cell viability was measured by trypan blue staining. *p< 0.0002 compared to the charcoal treated samples, #p< 0.001, compared to E₂ or Testo treated samples. The values are means of two independent experiments.
Reconstitution of charcoal treated media with steroids and FSH

The results of experiment 1, designed to determine which steroids were responsible for the decreased sensitivity of the Phor14-βLH seen after charcoal treatment are also shown in fig. 8. The addition of 17β-estradiol (5 nM) to the charcoal treated medium completely restored the sensitivity of LNCaP and PC-3 (figs. 8A, B) to the Phor14-βLH conjugate (p<0.001). In LNCaP cells, an androgen dependent prostate cancer cell line, testosterone fully restored the sensitivity (p<0.001), but this effect was absent in the androgen independent prostate cancer cell line PC-3 (p>0.5). To determine a possible mechanism involved in this effect, the estrogen receptor antagonist tamoxifen was added to the medium. Addition of tamoxifen abolished the ability of both estrogen and testosterone to restore the sensitivity of LNCaP cells to Phor14-βLH after charcoal treatment (p<0.001) (fig. 8A). Tamoxifen alone was without effect on the sensitivity of charcoal treated cells to Phor 14-βLH.

In experiment 3, we tested whether FSH had any effects on the sensitivity of LNCaP and PC-3 prostate cancer cells to Phor14-βLH. FSH caused a concentration dependent increase (p<0.01) in the sensitivity of LNCaP cells to the conjugate; PC-3 cells responded with maximal sensitivity at the lower concentration (fig. 9).

Fig. 9. Cytotoxicity of Phor14-βLH to A/ LNCaP (50 μM) and B/ PC-3 (50 μM) cells in the presence of follicle stimulating hormone (FSH). Cell viability was measured by trypan blue staining of cells. *p<0.005. The values are the means of two different experiments.
In vivo studies
As may be seen from fig 10A and B the first injection of Phor14-βLH, at both the 12 and 24 mg/kg levels, immediately stopped growth of the PC-3 tumors. Pretreatment of tumor-bearing mice with E₂ or FSH prior to the administration of 12 mg/kg Phor14-βLH also arrested tumor growth immediately. The tumor burden was decreased (fig. 11) from 60.2 mg/g body weight in tumor bearing

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**Fig. 10.** Effect of Phor14-βLH on PC-3 xenografts in nude mice. A/ Tumor growth during treatment of PC-3 xenografts with Phor14-βLH. Treatments with Phor14-βLH (12 mg/kg body weight and 24 mg/kg body weight) were given via tail vein injection on days 15, 22. and 29 post tumor inoculation. B/ Tumor growth during treatment with Phor14-βLH (12 mg/kg body weight) and Phor14-βLH (12 mg/kg body weight) after pretreatment with E₂ (0.150 mg/d) or FSH (3 μg/d) sc. for 3 days prior to the Phor14-βLH treatment. *p < 0.0001 significantly different compared to saline control tumor bearing mice; n = 12/group

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**Fig. 11.** Tumor burden in Phor14-βLH treated and saline control mice bearing PC-3 xenografts. Tumor weight was determined at 36 days post inoculation for each group. * p < 0.0001 significantly different from saline control tumor bearing mice, ** p < 0.0001 significantly different from the Phor14-βLH 12 mg/kg group; n = 12/group.
control animals to 27.2 mg/g body weight in treated animals receiving the lower concentration of the conjugate (12 mg/kg) (p<0.0001), and from 60.2 mg/g body weight to 11.8 mg/g body weight in animals treated with 24 mg/kg Phor14-βLH (p<0.0001), suggesting a concentration dependent effect on the tumors. Pretreatment of animals with E₂ or FSH prior to the administration of the lower dose of conjugate (Phor14-βLH 12 mg/kg) resulted in tumor burdens of only 11.9 and 11.1 mg/g body weight, respectively (p<0.0001), as compared to 27.2 mg/g body weight in animals treated with 12 mg/kg Phor14-βLH. Body weights were not affected by the treatment (data not shown) nor were organ weights of prostate, testes, seminal vesicles and liver. Pre-treatment of xenograft bearing mice with E₂ or FSH alone did not influence the tumor burden (data not shown).

Histologically, the tumors of treated animals were found to consist mainly of necrotic cells and fluid. The untreated tumors consisted of sheets of cells with large vesicular hyperchromatic nuclei and prominent nucleoli. Many mitotic figures were seen. In most instances the tumor margin was rich in blood vessels which penetrated the rim of the neoplastic tissue. Necrosis was most severe in tumors of mice pretreated with FSH and E₂. Tumors of animals receiving the lower dose (12 mg/kg Phor14-βLH) and pretreated with FSH had more necrotic cells than tumors from animals pretreated with E₂. Tumors of mice treated with 12 mg/kg Phor14-βLH showed less necrosis than E₂ and FSH pretreated animals. The tumors from animals treated with 24 mg/kg Phor14-βLH had the most necrotic cells. All tumors of the treated mice were pale and poorly vascularized, although some intact vessels were present within the tissue.

No histological abnormalities were noted in the other tissues examined (liver, spleen, heart, kidney, adrenal, pancreas, lung and pituitary), except the testes. Although testes weights were unaffected, the interstitial cells were shrunken and pyknotic. Primary and secondary spermatocytes and spermatids were virtually absent from the tubules, and few spermatozoa were seen in the epididymides.

In the fifth experiment, the tumor bearing mice were treated with Phor14-βLH or FSH plus Phor14-βLH for three weeks, as in the previous experiment, and then allowed a one month recovery period before being retreated with the same doses of Phor14-βLH and FSH plus Phor14-βLH. The results are shown in fig. 12 and table 3. It is evident that both treatments effectively destroyed...
Table 3. Tumors in nude mice after treatment once a week for three weeks, followed by a one-month recovery period and a second three-week treatment period.

<table>
<thead>
<tr>
<th>Tumor Conditions</th>
<th>Saline Controls</th>
<th>Phor14-βLH 24 mg/kg (%)</th>
<th>FSH Pretreated Phor14-βLH (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of animals</td>
<td>6</td>
<td>12</td>
<td>10</td>
</tr>
<tr>
<td>Animals with no visible tumors</td>
<td>0</td>
<td>7(58)</td>
<td>5(50)</td>
</tr>
<tr>
<td>Animals with liquid filled cysts</td>
<td>0</td>
<td>2(17)</td>
<td>3(30)</td>
</tr>
<tr>
<td>Animals with solid tumors</td>
<td>6(100)</td>
<td>3(25)</td>
<td>2(20)</td>
</tr>
<tr>
<td>Tumor burden (mg/kg)</td>
<td>100±13</td>
<td>23±6</td>
<td>21±10</td>
</tr>
</tbody>
</table>

¹ cysts contained no detectable tumor tissue; ² only animals with solid tumors are included.

tumors during the 12-week experimental period. More than 75 percent of the treated animals had no tumor or had a fluid filled cyst at the tumor site at the time of autopsy. The tumors in the saline treated animals continued to grow rapidly; all animals became cachectic and were sacrificed by day 48. Tumor growth was not reinitiated during the 28 day interruption of treatments.

**DISCUSSION**

Together with our previous findings outlined above, these experiments clearly establish the concept that prostate cancer cells can be targeted through their LH receptors and that the toxicity of the targeting drug can be increased *in vivo* by prior treatments with hormones (FSH and E₂) known to up-regulate LH receptors. The data also demonstrate that the effect of Phor14-βLH to PC-3 xenografts is concentration dependent. The βLH conjugate of Phor14 is less toxic to prostate cancer cells than Phor14 alone, which is in agreement with our results with Hecate and its βLH conjugate. Moreover, the same order of sensitivity among the cells lines was observed with BRF41 T>PC-3>DU145>LNCaP and cells with higher sensitivity were shown to have more LH binding capacity than cells with lower sensitivity. These results support the concept that the sensitivity of prostate cancer cells to the conjugate is dependent on LH receptor expression [6].

LH receptor expression has been intensively studied in ovarian granulosa cells, where it is regulated by FSH and E₂ [1, 5, 11, 13]. Our previous data on LNCaP, BRF41 T and PC-3 cells showed a reduced sensitivity to Hecate-βLH upon steroid removal [6]. The present data are in agreement with our previous findings with the more toxic lytic peptide conjugate. Physiological concentrations of E₂ restored the sensitivity of LNCaP and PC-3 cell lines, suggesting a direct effect of E₂ on the expression of LH receptors in prostate cancer cells. Estrogen receptors have been detected in LNCaP and PC-3 cells [2]. Testosterone was effective in restoring the sensitivity in the androgen dependent LNCaP cell line. In contrast, testosterone failed to restore the sensitivity in the androgen independent PC-3 cells, which do not express androgen receptors [14], suggesting that androgens might be involved in the expression of LH receptors in androgen sensitive prostate cancer cells. In these cells, testosterone could be converted to estradiol through aromatase, which has been detected in LNCaP cells [10].

Tamoxifen, an estrogen receptor antagonist [12], attenuated the effect of estradiol and testosterone, suggesting involvement of genomic estrogen receptor function in the regulation of LH receptors in prostate cancer cells. PC-3 cells express estrogen [2] and FSH receptors [14]. Pretreatment of tumor bearing animals with estradiol and FSH significantly increased the sensitivity to the lytic peptide conjugate, confirming the results obtained *in vitro*. The remarkable ability of both Phor14-βLH and Hecate-βLH to selectively destroy prostate cancer cells of human origin *in vivo*, suggests that it may be possible to develop an effective treatment for some prostate cancers based on LH receptor manipulation and targeting. The long-term studies in which the tumor bearing nude mice were treated for 3 weeks with Phor14-βLH and FSH, left untreated for 4 weeks,
and then treated for an additional 3 weeks are of particular interest. Tumor volume measurements (fig. 12) indicate that the tumors did not grow during the period when no treatments were given. When autopsied after the second 3-week treatment period more than 75 percent of all treated animals had no tumor or had a fluid filled cyst containing no viable tumor cells.

Surprisingly, histological studies revealed no damage in treated animals to tissues other than the testes, that have been reported to contain LH receptors. Blood vessels in tumors removed after 3 weeks of treatment appeared to be intact, despite the extensive necrosis of the tumor cells. Weights of prostates, testes, seminal vesicles, and livers were not affected by 3-week treatments. The reasons for the lack of effects of treatments in organs reported to contain LH receptors is unknown. Perhaps, the numbers of receptors in these organs are so small that insufficient lytic peptide is bound to the cell to cause damage. Alternatively, receptors in these organs that are not primary LH target organs may not bind the 15-amino acid segment of the hCG beta chain used in these conjugates.

We conclude that the lytic peptide conjugates, Phor14-βLH and Hecate-βLH, are capable of killing androgen dependent and androgen independent prostate cancer cells in vitro and in vivo, their toxicity is directly dependent on the LH receptor expression of the cells. The LH receptors in prostate cancer cells can be up-regulated in vitro by estradiol, testosterone, and FSH. This novel approach will extend our knowledge about prostate cancer cell growth and development, receptor regulation and the treatment of androgen independent prostate cancers and may provide new treatments for androgen independent prostate cancer.

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REFERENCES