The effects of a low therapeutic dose of βCG fragment-lytic peptide conjugates on ovarian function and gonadotropin secretion in ewes: A randomized controlled trial

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SUMMARY

The main objective of this experiment was to determine and compare the effects of two lytic peptide conjugates, Phor21-βCG(ala) and βCG(ala)-Phor21, at a low therapeutic dose (0.2 mg/kg body weight i.v.), on periovulatory ovarian and endocrine activity, and ensuing luteal function in an ovine experimental model. We hypothesized that the dense expression of LH/hCG receptors on the preovulatory follicle would present an appropriate target for the drugs and disrupt normal ovarian dynamics in sheep. Serum levels of reproductive hormones and ultrasonographic images were used for the assessment of periovulatory events following drug administration in 14
Rideau Arcott ewes; seven animals served as controls. Ovulations were synchronized with intravaginal progestogen-releasing sponges (medroxy-progesterone acetate, 60 mg) that were left in place for 12 days and a single i.m. injection of 750 IU of equine chorionic gonadotropin (eCG) given at sponge withdrawal. Both drugs were administered by i.v. injection 36 h post sponge removal/eCG injection, during the period of increasing LH responsiveness of potential ovulatory follicles and around the expected onset of the preovulatory surge of gonadotropins. No difference (p>0.05) was detected in the number of luteal structures per ewe in control versus treated animals during early luteogenesis. After drug administration, peak FSH concentrations were higher (p<0.05) in Phor21-ßCG(ala)-treated compared to control ewes and circulating estradiol concentrations were lower (p<0.05) in ßCG(ala)-Phor21-treated animals. Mean serum progesterone concentrations were lower (p<0.05) in ßCG(ala)-Phor21-treated than control ewes during the luteal phase post-treatment. There were no differences (p>0.05) in the percentage of ewes that lambed or lamb characteristics between the three groups at lambing 9 months post-treatment. In summary, neither Phor21-ßCG(ala) nor ßCG(ala)-Phor21 demonstrated adverse effects on the ovulatory process but the treatment with ßCG(ala)-Phor21 significantly depressed follicular and luteal steroidogenesis. With a lack of evidence for disruptive effects on endocrine function and fertility, these observations support the use of Phor21-ßCG(ala) as a cancer pharmaceutical. Reproductive Biology 2010 10 3: 195–213.

Key words: conjugated lytic peptides, reproductive tissue cancer, gonadotropins, chorionic gonadotropin, LH/CG receptor, sheep, ultrasonography

INTRODUCTION

Cancer continues to be a major health concern globally. Reproductive cancers are of particular concern, as breast and prostate cancers fall behind only lung cancer as the leading number of new cases annually [8]. Worldwide, cases of ovarian cancer also come close to 190,000 per annum. Despite the significant investment of time and resources into cancer research, there
has been almost no change in the survival rates of metastatic cancer patients in the last 30 years [19]. A number of drawbacks plague current treatment modalities such as chemotherapy. The toxic effect it has on all proliferating cells severely compromises patient immunity, causes dramatic hair loss, nausea, cachexia, and limits the tolerable therapeutic dose, which ultimately prolongs the treatment. To overcome these adverse effects, the focus in cancer treatment has shifted to receptor-targeted therapy, and more recently, the novel use of hormone molecules (or their receptor-targeting fragments) conjugated with lytic peptides.

The most extensive and promising research has been in targeting cancers of the reproductive tract (i.e., prostate, ovaries and endometrium) and mammary glands. The distinct requisite for targeting the reproductive tissue cancers is that they naturally express the lutropin/human chorionic gonadotropin (LH/hCG) receptor, whereas expression of this receptor in other somatic tissues is relatively small [27]. Preliminary results in mice have shown promise for the two lytic-peptide conjugates in particular, hecate-beta chorionic gonadotropin (hecate-ßCG) and Phor21-beta chorionic gonadotropin (Phor21-ßCG), both in vitro [14, 18, 19, 23, 30] and in vivo [14, 19, 23]. In all in vivo studies using a small rodent model, a significant decrease in tumour load was achieved after only three applications given at weekly intervals. More impressively, a significant effect has consistently been reported after only the first dose [14, 23].

While the primary focus of in vivo studies in rodents has been to decrease tumour burden, the impact of ßCG-lytic peptide conjugates on the surrounding receptor-positive tissue morphology has been addressed only in brief. In a study using hecate-ßCG and OVCAR-3 (ovarian cancer cells) xenografts in nude mice, Gawronska et al. [14] conducted histological examinations, which provided first evidence of abnormal ovarian morphology post-treatment. Approximately 80% of the treated mice exhibited severe degeneration of primary, secondary and tertiary ovarian follicles, and absent or poorly organized corpora lutea (CL). However, the primordial follicles were unaffected and subsequent development of these follicles resulted in the restoration of ovulatory cycles in about 12 weeks [19, 22, 24]. These findings present interesting supposition for a transient disruption of LH-dependent ovarian
processes that merits further investigation. It would be of value to assess what effects the conjugated lytic peptides have on the kinetics of the reproductive system and what damage, if any, they induce in unintended ovarian structural targets in a large domestic animal model.

The basic structure of the lytic peptide/βCG fragment conjugates consists of an alpha helical, amphipathic, synthetic membrane-disrupting peptide, such as hecate (23 amino acid residues) or Phor21 (21 amino acids), linked to a 15-amino-acid (81–95) fragment of the beta subunit of hCG. The fragment of the β unit of hCG allows for targeted delivery of the lytic peptide to the tissues expressing the shared LH/hCG receptor by binding to the receptor with high affinity [22, 23]. The proposed mechanism for cell death involves the disruption of plasma membrane lipids and proteins, creating plasma membrane pores that allow unregulated ionic flow leading to rapid cellular necrosis [22]. The entire process is completed in only minutes after the lytic peptide interacts with the membrane [22].

Through progressive analysis, Hansel et al. [18] have established Phor21-βCG(ala) as a likely candidate for further trials. The efficacy with which this compound inhibits cancer growth and reduces tumour burden at relatively low doses, while causing little damage to receptor-negative cells, has made it a most appealing candidate drug. Phor21-βCG(ala) differs structurally from Phor21-βCG in that three cysteine residues have been replaced by alanine. To date, an assessment of Phor21-βCG(ala) effects on normally developing ovarian structures in a non-rodent species has not been reported. Thus, Phor21-βCG(ala) and its enantiomer, βCG(ala)-Phor21 have been selected for the present study in ewes.

Towards the end of early stages of folliculogenesis (i.e., recruitment of follicles from the primordial/primary reserve pool), ovarian follicles in most mammalian species become responsive to gonadotropins, namely follicle-stimulating hormone (FSH) and luteinizing hormone (LH; [6, 12, 13]). The initial growth response of the follicle seems to be heavily dependent on FSH; however, this shifts to LH-dependence in the terminal stages of antral follicle maturation, coinciding with an increase in circulating estradiol-17β [3]. Notably, it is this shift to LH dependency that appears critical for the selection of follicles within mammalian species that display
follicular dominance [16]. An increase in LH/hCG receptors allows the follicle to use LH for its continued growth and maximize the response to the preovulatory LH surge. The LH surge initiates ovulation of the oocyte and luteinization of the remaining granulosa cells into the corpus luteum (CL). Since the ability of estrogen to increase receptor expression is strongly exhibited during the follicular phase of the ovulatory cycle, augmented cytotoxic effects on growing follicles can be anticipated during the terminal phase of antral follicular growth [4]. This dense expression of LH receptors in preovulatory follicles forms the basis of our proposed follicle ablation by Phor21 conjugated to the βCG fragment.

A typical interovulatory interval in the ewe lasts ~17 days, and the estrous cycle comprises on average 3 or 4 well-defined waves of antral follicular growth [2, 3, 11]. As proof of principle, sheep provide a good model for the human female reproductive processes because of their similar body size and hormonal cues governing the ovarian activity during the ovulatory cycle [7, 25]. Hence, the main objective of this report was to describe the effects of two βCG-lytic peptide conjugates (Phor21-βCG(ala) and its enantiomer, βCG(ala)-Phor21) on terminal antral follicle development and endocrine function, ovulation, and luteogenesis in ewes. We hypothesized that the preovulatory antral follicles would be actively targeted by conjugated lytic peptides as the hormonal profile at the end of the follicular phase (i.e., high follicular estrogenicity triggering an increase in gonadotropin secretion) creates an optimal environment for the binding of the βCG fragment. The increase in LH receptor expression should make these follicles highly susceptible to the cytotoxic actions of the conjugates. As a result, ultrasonographic imaging and hormone profile should detect the failure of ostensibly ovulatory-sized follicles to ovulate and/or complete luteinization post-ovulation.

MATERIALS AND METHODS

All experimental procedures were carried out in accordance with the Canadian Council on Animal Care guidelines and had been approved by the local
Animal Care Committee. Clinically healthy and sexually mature Rideau Arcott ewes (n=21) of comparable age (mean±SEM: 3.2±0.3 years; range: 3 to 7 years) and weight (mean±SEM: 83.9±1.6 kg; range: 69 to 91 kg) were obtained from the research herd at the Ponsonby Research Station near Guelph (latitude: 43°33’ N). Animals were given access to dry sheltered pens separate from rams and provided with a maintenance diet of alfalfa hay, with water and iodized salt licks available ad libitum. Ewes were then randomly assigned to one of the 3 groups: 1/ Phor21-ßCG(ala) (0.2 mg/kg i.v.); 2/ ßCG(ala)-Phor21 (0.2 mg/kg i.v.); or 3/ saline only controls. The doses of the conjugated lytic peptides used in this experiment were the minimal effective therapeutic doses determined in previous mouse model studies [19].

Estrus was synchronized in all ewes (May-June) with intravaginal progestogen-releasing sponges (Veramix®; medroxyprogesterone acetate, MAP; 60 mg) that were left in place for 12 days. Sponge removal (day=0) was coupled with an i.m. injection of 750 IU of equine chorionic gonadotropin (eCG; Pregnecol™ 6000; Bioniche Animal Health, Belleville, ON, Canada) in accordance with a previously established protocol for the induction of ovulation in sheep [26]. The preovulatory peak of LH surge occurs approximately 46 h after injection in eCG-treated anestrous ewes primed with MAP-releasing vaginal sponges [26]. Thus, the lytic peptide conjugates in this study were administered by i.v. injection 36 h post sponge removal/eCG injection, during the period of high estrogen production/increasing LH responsiveness of potential ovulatory follicles and around the expected onset of the preovulatory discharge of gonadotropic hormones (fig. 2).

At the beginning of the breeding season (September) following the treatment with the conjugated lytic peptides, estrus was synchronized in all ewes by a 12-day treatment with EAZI-BREED™CIDR® Sheep and Goat Devices (0.3 g progesterone in inert silicone elastomer; Pharmacia & Upjohn, Rydalmere, NSW, Australia). The ewes were bred by 4 Rideau Arcott rams introduced 48 h after CIDR withdrawal. The number, sex and birth weight of lambs were recorded at lambing induced with a single i.m. dose of dexamethasone (20 mg; Unidex®, Univet Pharmaceuticals, Milton, ON, Canada) on day 140 of gestation.
Figure 1. Two of the four cavitated corpora lutea (CL; A and B) and a follicular cyst (C) detected ultrasonographically in a Phor21-ßCG(ala)-treated ewe; two follicular cysts (D and E) and a CL (F) detected in a ßCG(ala)-Phor21-treated ewe. Arrowheads delineate the borders of the CL, a scale bar in the panel D corresponds to 10 mm.
Figure. 2. Serum FSH (A), LH (B) and estradiol-17β (C) concentrations determined by frequent blood sampling conducted every 2 h during the 24-60-h period after sponge removal in eCG-primed ewes. Ewes were treated with Phor21-ßCG(ala), ßCG(ala)-Phor21 (both at the dose of 0.2 mg/kg i.v.) or saline only (controls) at 36 h post-sponge removal (dashed line). Statistical comparisons were only performed on hormone concentrations obtained during the 36-60-h period (i.e., after drug injections).
Frequent blood samples were collected via an indwelling jugular catheter at 2-h intervals, from 24 h to 60 h after MAP sponge removal, to detect the preovulatory LH/FSH peaks and time at which they occurred, and to determine serum concentrations of estradiol-17β (E$_2$-17β) post-treatment. Subsequently, blood samples were drawn from all ewes via jugular venipuncture into vacutainers (Becton Dickinson Vacutainer Systems; Franklin Lakes, NJ, USA) twice daily, between 3 and 6 days after sponge withdrawal, and then once daily until the end of the luteal phase (the 13-day period), for measurements of circulating concentrations of progesterone (P$_4$). All samples were allowed to clot at room temperature for 18–24 h, and serum was harvested by centrifugation at 1500×g for 10 min and stored at −20°C until assayed. Hormone analyses were conducted externally by facilities at the University of Saskatchewan (Saskatoon, SK, Canada) using validated radioimmunoassay procedures. Analyses included the quantification of LH [28], FSH [29], E$_2$-17β [21], and P$_4$ concentrations [10]. The sensitivities of assays were as follows: LH and FSH assays: 0.1 ng/ml; E$_2$-17β: 1.0 pg/ml; and P$_4$: 0.03 ng/ml. Concentrations of LH and FSH are given in terms of the standards NIAMDDoLH-24 and NIDDK.oFSH.RPl, respectively; both generously provided by Dr. A.F. Parlow (National Pituitary Agency, NIH, Bethesda, MD, USA). All samples for LH and FSH were analyzed in a single assay. The intra-assay coefficients of variation (CVs) for reference sera with mean LH concentrations of 0.44 or 2.33 ng/ml were 10.3 and 4.6%, respectively. The intra-assay CVs for reference sera with mean FSH concentrations of 0.52 or 2.90 ng/ml were 4.8 and 3.0% respectively. The intra- and interassay CVs for ovine reference sera analyzed for E$_2$-17β (means: 10.2 or 25.6 pg/ml) were 9.0 and 12.0% or 6.9 and 8.3%, respectively, and for P$_4$ (means: 1.40 or 14.23 ng/ml) they were 5.8 and 9.6% or 5.5 and 8.6%, respectively.

Ewes of all groups underwent transrectal ovarian ultrasonography 3 days after treatment to confirm the presence of CL with the use of high-resolution B-mode ultrasound equipment (Aloka SSD-3500SX; Aloka Co., Tokyo, Japan) connected to a 7.5-MHz bi-plane transducer [13, 22]. Ovaries were displayed on the viewing screen of an ultrasound scanner at 1.5X to 2X image magnification using a linear-array component of the bi-plane
βCG-lytic peptide conjugates

One animal from the group receiving βCG(ala)-Phor21 was dropped from the study after exhibiting signs of mastitis and vaginitis, which led to discomfort during scanning. These are not attributed to our interventions and partial data from this animal were subsequently excluded from analyses. Luteal structures were identified in all remaining 20 animals and appeared as solid or cavitated CL (fig. 1). Total numbers of luteal structures per ewe did not differ between animals treated with Phor21-βCG(ala) and βCG(ala)-Phor21 (2.0±0.2 and 2.5±0.4, respectively), nor from controls (2.4±0.4). Two
ewes, one from each drug treatment group, presented with ovarian follicular cysts. A Phor21-ßCG(ala)-treated ewe, in addition to four CL detected ultrasonographically on the left ovary, had a 14-mm follicular cyst present on the right ovary. A ßCG(ala)-Phor21-treated ewe with a single ovulation and CL on the right ovary, had two follicular cysts (11 and 14 mm in diameter, respectively) on the contralateral ovary. No such abnormalities were detected in control animals.

Although numerically different, the overall pattern of peri-ovulatory LH/FSH and E$_2$-17β secretion was not altered significantly by administration of either drug (p>0.05), as shown in fig. 2A-C. The mean timing of the LH/FSH peak, LH peak concentration and AUC for serial FSH/LH concentrations did not differ (p>0.05) between control, ßCG(ala)-Phor21 and Phor21-ßCG(ala)-treated groups (tab. 1). However, the mean FSH peak concentration was greater (p<0.05) in Phor21-ßCG(ala)-treated ewes compared with controls. Mean AUC for serum E$_2$-17β concentrations was lower in ßCG(ala)-Phor21-treated ewes than in untreated controls and the ewes that had received Phor21-ßCG(ala) (tab. 1). All but one Phor21-ßCG(ala)-treated ewe exhibited a decline in circulating P$_4$ concentrations by the end of the sampling period; elevated, mid-luteal concentrations of luteal progesterone were recorded in that animal until day 19 after progestin sponge removal/eCG treatment. Mean serum P$_4$ concentrations were lower (p<0.05) in ßCG(ala)-Phor21-treated ewes than control animals from days 6 to 15, and they were lower in ßCG(ala)-Phor21-treated than in Phor21-ßCG(ala)-treated animals on days 8–11, 13 and 14 after sponge removal (fig. 3).

There were no differences (p>0.05) in the percentage of ewes that lambed ($\chi^2$-test; 86% (6/7), 86% (6/7) and 83% (5/6); for controls, Phor21-ßCG(ala)-treated and ßCG(ala)-Phor21-treated ewes, respectively) and mean numbers of lambs per ewe (live births: 2.1±0.5, 2.5±0.6 and 1.9±0.5, respectively), and no differences (p>0.05) between the groups for lamb birth weights (4.1±0.1 kg, 4.0±0.2 kg and 4.1±0.2 kg, respectively) or the percentage of male lambs born per ewe (54%, 48% and 43%, respectively) during the breeding season after the treatment.
Figure. 3. Systemic progesterone ($P_4$) concentrations from day 3 to day 19 after medroxyprogesterone acetate (MAP) sponge withdrawal and eCG treatment of ewes that received Phor21-$\beta$CG(ala), $\beta$CG(ala)-Phor21 or saline. Both drugs at the dose of 0.2 mg/kg i.v. or saline only (controls) were administered 36 h after MAP sponge removal. Daily $P_4$ concentrations were significantly lower in $\beta$CG(ala)-Phor21-treated ewes than control ewes from days 6 to 15, and they were significantly lower in $\beta$CG(ala)-Phor21-treated than in Phor21-$\beta$CG(ala)-treated animals on days 8-11, 13 and 14 after MAP sponge removal/eCG injection (day 0).

DISCUSSION

Previous studies exploring the use of lytic peptides conjugated to the fragments of gonadotropin receptor ligands as cancer therapeutics have consistently demonstrated a potent and targeted killing effect on various cancer cell lines [19]. The selective cell death of receptor-positive cancer cells has been confirmed in several studies using ovarian epithelial tumour xenographs in mice - an effect shown to have increased with the number of LH/
Table 1. Pituitary and ovarian endocrine responses to drug treatments

<table>
<thead>
<tr>
<th>Hormone Group/Variable</th>
<th>n</th>
<th>Time to peak (h)**</th>
<th>Peak concentration</th>
<th>AUC for serial hormone concentrations***</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>LH</strong></td>
<td></td>
<td></td>
<td>ng/ml</td>
<td></td>
</tr>
<tr>
<td>Control*</td>
<td>7</td>
<td>45.7±3.0</td>
<td>26.5±4.8</td>
<td>33.1±18.5</td>
</tr>
<tr>
<td>Phor21-ßCG(ala)</td>
<td>7</td>
<td>44.6±2.1</td>
<td>44.1±11.1</td>
<td>30.1±18.1</td>
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<tr>
<td>ßCG(ala)-Phor21</td>
<td>6</td>
<td>45.0±2.4</td>
<td>42.1±13.3</td>
<td>38.7±17.1</td>
</tr>
<tr>
<td><strong>FSH</strong></td>
<td></td>
<td></td>
<td>ng/ml</td>
<td></td>
</tr>
<tr>
<td>Control*</td>
<td>7</td>
<td>46.6±2.8</td>
<td>3.2±0.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.3±0.3</td>
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<tr>
<td>Phor21-ßCG(ala)</td>
<td>7</td>
<td>45.4±2.5</td>
<td>5.1±0.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.2±0.5</td>
</tr>
<tr>
<td>ßCG(ala)-Phor21</td>
<td>6</td>
<td>46.0±2.4</td>
<td>4.6±0.8&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>1.9±0.4</td>
</tr>
<tr>
<td><strong>Estradiol-17β</strong></td>
<td></td>
<td></td>
<td>pg/ml</td>
<td></td>
</tr>
<tr>
<td>Control*</td>
<td>7</td>
<td>42.6±2.4</td>
<td>14.4±1.1</td>
<td>33.0±2.0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
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<td>41.4±1.5</td>
<td>14.4±0.6</td>
<td>34.3±2.2&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>ßCG(ala)-Phor21</td>
<td>6</td>
<td>43.7±3.9</td>
<td>13.6±2.1</td>
<td>28.5±3.3&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

*control: saline-treated ewes; **time from medroxyprogesterone acetate (MAP) sponge removal/eCG injection to maximum hormone concentration; ***AUC: area under the curve for hormone concentrations between 36 h and 60 h after MAP sponge removal/eCG injection; abwithin columns, means for each hormone denoted by different letters are different (p<0.05)
hCG receptors present on the cell surface [14, 22]. Thus, our hypothesis was that the dense expression of LH/hCG receptors on ovarian antral follicles during the periovulatory period in ewes would present a similar target for these agents. In this study, however, we failed to demonstrate a powerful disruptive effect of either conjugated lytic peptide, at a low therapeutic dose, on the ovulatory response. Instead, there was a moderate stimulatory effect on the serum concentrations of gonadotropic hormones, more pronouncedly FSH, after administration of Phor21-ßCG(ala) and suppression of ovarian steroidogenesis after the treatment with ßCG(ala)-Phor21.

Elevated peaks of FSH concentrations could be observed during the 24-h period after Phor21-ßCG(ala) treatment (tab. 1). The timing of the LH and FSH peaks as well as the amplitude of the preovulatory LH surge did not appear to be altered by the present drug treatments (tab. 1). All these values, however, were in keeping with previous reports for timing of the preovulatory gonadotropin surge in ewes that had been primed with medroxyprogesterone acetate and received eCG at the time of MAP sponge withdrawal [1, 5, 26]. The treatment with ßCG(ala)-Phor21, but not that with Phor21-ßCG(ala), had a suppressive effect on follicular estradiol and luteal progesterone production in the ewes of the present study. A clear explanation for these responses is uncertain, but several possibilities exist.

The concentration of the drugs used in the present study was based on the minimum effective doses determined in a small rodent model [19] in order to minimize the risk of potential adverse effects to experimental animals. At these therapeutic levels, there was no apparent effect of either drug on the ovulatory response with the moderate deviations in the FSH profiles only following Phor21-ßCG(ala) injections. More pronounced effects, characterized by a significantly diminished estradiol production and residual suppression of progesterone biosynthesis, appeared to be exerted by ßCG(ala)-Phor21. It appears that at the present dosage (0.2 mg/kg), the Phor21-ßCG(ala) merely acted as an LH-like substrate rather than the lytic agent initiating the destruction of large antral follicles and developing luteal structures, thus contributing to a slightly greater concentration of this gonadotropin available for binding the existing LH/hCG receptors and/or competing with endogenous LH for binding with its receptors. Once
bound, the normal response of the preovulatory follicular theca cells to LH is the secretion of androgens, which are then aromatized to estrogens by the granulosa cells. Estrogen release in the late follicular phase has a stimulatory effect on pituitary expression of LH and FSH, increasing their secretion into the circulatory system and producing the elevated surge in gonadotropins during the preovulatory period. It remains uncertain why the predominant effects of Phor21-ßCG(ala) observed in this study were on the FSH secretion.

A second explanation is that Phor21-ßCG(ala) functioned much as it should and left all healthy ovarian cells unharmed despite their dense LH/hCG receptor expression. The principle caveat for the use of the conjugated lytic peptides in the treatment of cancers is the negative membrane charge that cancer cells have in comparison to normal healthy cells. The expression of LH/hCG membrane-bound receptors should promote the binding of Phor21-ßCG(ala) and ßCG(ala)-Phor21 to the cells of preovulatory antral follicles and forming luteal glands. The gonadotropin fragment of the drug conjugate would act as a ligand to facilitate binding to the ovarian structures, but thereafter fail to cause follicle ablation because they lack the negative surface charge largely responsible for lytic peptide binding. Evidence exists to support this as a likely supposition. In a study by Leuschner and Hansel [22], the Chinese Hamster Ovary (CHO) cells transfected with LH/hCG receptors were less susceptible to the killing effects of the conjugated lytic peptides tested than MDA-MB-435S breast cancer cells, despite being receptor positive. Thus, in addition to their dependence on receptor capacities and binding affinity, the lytic effects of Phor21-ßCG(ala) and ßCG(ala)-Phor21 are also, if not predominantly, mediated by the cell surface membrane charge. Therefore, it may also be inferred from the present study that the difference exists between the two enantiomers in their ability to “anchor” the cell disrupting peptide onto the surface of gonadotropin responsive follicular cells.

When testing the effects of a drug designated for human use in animal models, the question of inter-species variation in the presence and functioning of the ligand/receptor systems can compromise the applicability of the findings. Binding affinity of the conjugated lytic peptides has been examined more thoroughly in previous in vitro studies using human cancer
xenographs [14, 22]. Structurally, the ovine LH receptor (GeneBank accession #: L36329 & L36115) shares a greater than 90% homology to human LH receptor. Thus, it is unlikely that a failure of the drug to bind the receptors based on structure variation was a factor in the present study. However, the binding affinity of the βCG-lytic peptide conjugates for normal (non-cancerous) receptor-positive tissue has not been fully characterized in vivo. Inter-species variation in the half-life (T½) of the conjugates is another factor to consider. Estimated T½ of Phor21-βCG in rats was 2.25 h [20]. If a similar clearance rate was present in ewes, the drugs injected may have essentially been eliminated in the 8-h period between injection and the peak of LH release. Unfortunately, there are no data on half-life of the drugs in ewes.

An intriguing observation in two animals, one receiving Phor21-βCG(ala) and the other βCG(ala)-Phor21, was the development of ovarian cysts. Also, the presence of persistent CL secreting large amounts of progesterone after the expected onset of luteolysis was noted in one Phor21-βCG(ala)-treated animal. Corpora lutea with a prolonged lifespan are sporadically seen in normally cycling ewes but their etiology remains uncertain [3]. The formation of ovarian cysts is commonly associated with a decrease in LH levels or availability within the ovarian environment at the outset of the ovarian cycle, either just before ovulation (follicular cyst or unovulated persistent follicles) or shortly after follicle rupture (luteinized cyst; [4, 9, 15, 17, 20]). As such, the formation of ovarian follicular cysts in the two ewes of the present study could be considered a plausible side effect of the drug that competes for the LH/hCG receptor and interferes with the functioning of endogenous LH. In considering the two affected animals, retrospective inspection of individual hormonal profiles failed to confirm the altered levels of serum FSH/LH or estradiol during the preovulatory discharge of gonadotropins. Hence, it was most likely a decrease in the ability of the follicles to respond to LH due to competition for receptor binding (a Phor21-βCG(ala)-treated ewe) and/or the “damage” to the LH/hCG receptors themselves (a βCG(ala)-Phor21-treated ewe). The former would suggest that the lytic peptide conjugates, and especially Phor21-βCG(ala), were actually less potent than endogenous LH although they might bind to ovarian targets with similar affinity.
In summary, our present observations may be interpreted to suggest that the administration of low therapeutic doses of the two conjugated lytic peptides tested in this study does not appear to affect the normal ovarian cycle and fertility. Contrary to our expectations, neither Phor21-βCG(ala) nor βCG(ala)-Phor21 demonstrated adverse effects on the ovulation rate. We demonstrated that there were no carry-over effects of the treatment on lamb productivity in sheep. Analyses of estrogen and progesterone concentrations revealed that a single injection of βCG(ala)-Phor21 depressed ovarian steroidogenesis. It is attractive to suggest that this may have important bearing on the use of βCG(ala)-Phor21 in contraceptive and luteolytic roles when higher concentrations of the drug are used. At present, with a lack of evidence for strong adverse effects of Phor21-βCG(ala) on terminal follicle development and ovulation, our results support the use of this particular conjugate as a cancer pharmaceutical and provide further support towards its safety in reproductive-aged patients.

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