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Hecate-FSHβ33-53C/S lytic peptide conjugate selectively kills targeted follicle stimulating hormone receptor (FSHR)-positive cancer cells

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ABSTRACT

Background: The follicle stimulating hormone (FSH) receptor (FSHR), is expressed primarily in the gonads, also found in ovarian and prostate cancers, and in tumor vessel endothelial cells. We investigated the potential of a targeted cytotoxic approach using Hecate-FSHβ, a conjugate derived from a lytic peptide Hecate, an analog of bee venom melittin, and the β subunit of FSH, to selectively eliminate FSHR-positive cancer cells.
Methods: Hecate-FSHβ-mediated cytotoxicity was tested in human granulosa tumor cell line KGN, human embryonic kidney HEK-293 cell line stably transfected with human FSHR cDNA (HEK293-FSHR) and mock-transfected HEK-293 cells as FSHR-negative control cells. Tested variant Hecate-FSHβ33–53C/S with cysteine residues replaced by serine, was evaluated for its cytotoxicity towards FSHR-positive cells.
Results: Hecate-FSHβ33–53C/S demonstrated the highest specific cytotoxicity towards FSHR-positive cells (KGN and HEK293-FSHR vs. control). In competition studies, cotreatment with recombinant human FSH (rhFSH) reduced the cytotoxic effect of the conjugate on these cells, highlighting FSHR specificity. In xenograft models of HEK293-FSHR, Hecate-FSHβ33–53C/S alone or in combination with a gonadotropin releasing hormone (GnRH) antagonist (Cetrorelix, CTX) significantly inhibited tumor growth. No synergistic effect was observed with co-administered Hecate-FSHβ33–53C/S and CTX. Hecate-FSHβ33–53C/S induced necrosis in tumor cells, whereas CTX triggered apoptosis. Hecate-FSHβ33–53C/S did not produce any side effects. CTX treatment caused increased spleen size and inhibited spermatogenesis, leading to reduced testis weight, which aligns with expected gonadal effects.
Conclusions: Hecate-FSHβ33–53C/S is highly effective in selectively targeting and killing FSHR-expressing cancer cells, with minimal side effects, suggesting its potential as a therapeutic option for cancers expressing FSH receptors.

1. Introduction

Follicle-stimulating hormone (FSH) and its receptor (FSHR) are involved mainly in reproduction [1,2]. In females, FSH promotes the maturation of ovarian follicles and regulates the synthesis of estrogens, whereas in males, FSH regulates spermatogenesis and activates the

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function of testicular seminiferous tubule cells [3]. Under pathological conditions, FSHR was found in granulosa cell tumors and ovarian epithelial cancers [4], as well as in endometrial cancer [5] and neuroendocrine tumors in the appendix [6]. FSHR is localized in different primary tumor vessel endothelial cells (such as prostate, breast, colon, pancreas) [7] as well as in the vasculature of lung, breast, prostate, colon, kidney, and leiomyosarcoma cancer metastases [8]. These findings suggest that FSHR is a potential cellular marker for different tumors and could be used for targeted cancer therapy [9].

Naturally occurring membrane-disrupting lytic peptides serve as defense molecules in bacteria, insects, plants and invertebrates [10]. They share common characteristics such as total positive charge, linearity, and amphipathic and alpha-helical structures in a hydrophobic environment [10]. An example of a lytic peptide displaying antiviral and anticancer properties is melittin, a venom component of the honey bee (*Apis mellifera*) [11,12]. Hecate, an analog of melittin, is a 23 amino acid peptide (FALALKALKKALKKLKKALKKAL) [13] with a high content of positively charged lysine and nonpolar leucine and alanine [14] that exhibits high anticancer activity. Although the Hecate backbone had highly cytotoxic effects on different cancer cell lines *in vitro,* a definitive effect *in vivo* is still needed [15,16]. Hecate has also been used successfully as an antiviral [17–20], antimicrobial [21], for FSHR expressing tumors using adaptive T cells transfer [22] or in bispecific antibody-based therapeutics [23].

It was hypothesized that treatment with Hecate could be enhanced *in vivo* by its conjugation with a specific ligand for the receptor localized on the cancer cell membrane. This fusion with a 15-amino acid chain of human chorionic gonadotropin (hCG) β-subunit (residues 81–95) (Hecate-βhCG) significantly increased the specificity of Hecate-mediated cytotoxicity in cancer cells expressing the LH/hCG receptor (LHCGR) both *in vitro* and *in vivo*. Hecate-βhCG conjugates have been shown to specifically destroy ovarian [24], prostate [15,25], Leydig and granulosa [26], breast [16] and adrenocortical [27] tumor cells. Moreover, the Hecate-βhCG conjugate was able to inhibit the growth of prostate [28] and breast [16] metastatic cancer cells. Owing to high selectivity toward primary and metastatic cancer cells, rapid metabolism and a lack of immunoreactivity, no side effects were observed after Hecate-βhCG conjugate treatment. Mice treated with Hecate-βhCG conjugates revealed only reversible changes in gonads, namely, smaller tubule diameters and shrunken interstitial cells in males [29], whereas no changes in ovaries or the uterus could be observed in females [30].

In this study, we designed and tested 12 different conjugates of Hecate fused with fragments of the FSH β-subunit to target FSHR-expressing cancer cells. The Hecate-FSHβ conjugate that displayed the highest specific cytotoxicity *in vitro* was selected for further *in vivo* xenograft treatment studies. To reduce endogenous FSH competition for receptor binding, we additionally cotreated xenografted mice with the GnRH antagonist cetrorelix (CTX) alone with the Hecate-FSHβ conjugate.

2. Material and methods

2.1. Test compounds

Lytic peptide Hecate backbone (also was available from earlier synthesis) [26] and Hecate-FSHβ conjugates were synthesized as one chain and additional conjugation process was not involved (similar process as before, [26,31]), with a TETRAS peptide synthesizer at Peptides and Elephants GmbH (Potsdam, Germany). The purities of the compounds were confirmed via HPLC analysis. The synthesized peptides were lyophilized and stored at −80 °C. (Supplementary Table 1).

2.2. Cell lines and culture conditions

The human embryonic kidney-293 cell line (HEK-293) (ATCC: CRL-1573), human ovarian cancer cell lines SKOV-3 (ATCC: HTB-77) and OVCAR-3 (HTB-161) were obtained from American Type Culture Collection (ATCC; Manassas, VA, USA). The human granulosa cell tumor cell line KGN was kindly donated by Dr. T. Yanase (Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan). The HEK-293-FSHR cell line was generated via stable transfection of HEK-293 cells with the FLAG-hFSHR/pcDNA3.1 expression plasmid with Lipofectamine® LTX with Plus™ Reagent transfection reagent (Thermo Fisher Scientific Inc., Renfrewshire, UK) in Opti-MEM® I Reduced Serum Media (Thermo Fisher Scientific). To determine the functionality of FSHR, cAMP production was measured after treatment with recombinant human FSH (rhFSH). The cells were incubated at 37 °C in a humidified atmosphere in the presence of 5 % CO_2. A cell suspension was obtained with digestion solution (0.25 % trypsin and 0.02 % EDTA).

2.3. Cytotoxicity test

Hecate-FSHβ conjugate-mediated cytotoxicity in HEK-FSHR, HEK293, SKOV-3, OVCAR-3 and KGN cells was tested via the CytoTox 96® Assay (Promega, USA). The CytoTox 96® Assay quantitatively measures lactate dehydrogenase (LDH), a stable cytosolic enzyme that is released into the medium upon membrane perforation. To assess LDH release, the cells were seeded onto 96-well plates (15,000 cells per/well) and incubated overnight in 100 µl of complete medium. After 18 h, the medium was replaced with stimulation medium [DMEM/F12 supplemented with 0.5 % (v/v) fetal calf serum (Thermo Fisher Scientific), penicillin (100 IU/ml) and streptomycin (100 IU/ml) (Sigma—Aldrich)], and the cells were treated for 1.5 h with Hecate backbone or Hecate-FSHβ conjugates in the concentration range of 0.5–5 µM. For the competitive binding study, the cells exposed to Hecate-FSHβ conjugates were pretreated with 100 IU/L rhFSH for 30 min and 1.5 h, respectively.

2.4. Tumor xenografts

All mouse studies were approved by the local ethics committee of the Medical University of Bialystok, Poland. Male athymic nude mice (Crl: NU(NCr)-Foxn1^{nu}, 8–10 weeks old, 20–23 g) purchased from Charles River Laboratories International, Inc. (Sulzfeld, Germany) were housed two to three per cage and provided with sterilized pellet chow and water. The animals were maintained in a pathogen-free mouse colony at the Center of Experimental Medicine (Bialystok, Poland).

The HEK293-FSHR cells were harvested with trypsin when near confluence, centrifuged (1100 rpm, RT, 5 min) and resuspended in sterile non-supplemented DMEM/F12 medium mixed 1:1 with Matrigel® matrix (Corning, Corning, USA). The obtained cell suspension (1×10^6 cells/0.2 ml) was injected subcutaneously into the interscapular area. When the xenograft volume reached 150–200 mm^3 (5–9 days after implantation), the xenograft-bearing mice were randomly divided into six treatment groups: 1) 0.9 % NaCl, control (CTR, n = 12), 2) Hecated backbone 12 mg/kg/72 h (n = 10), and 3) Hecated-FSHβ 12 mg/kg/72 h (n = 11). The in vivo treatment injections were In this study, we tested whether the Hecate-FSHβ conjugate can selectively target and destroy HEK293-FSHR cells.

The mice were injected on day 7, 14 and 21 and all the mice were sacrificed on day 26, 5 days after the last injection. Total blood was collected via heart puncture. Body weight, selected organ and xenograft weights/volumes and sizes were recorded at the time of necropsy. Xenografts and testes were fixed in 4 % PFA or snap frozen in liquid nitrogen for future histological or gene expression analysis, respectively.

2.5. Total RNA isolation, reverse transcription and quantitative PCR

Total RNA from cell lines, xenografts and mouse tissues was isolated via the TRIzol reagent (Life Technologies, Thermo Fisher Scientific) according to the standard protocol. The total RNA concentration was quantified via a NanoDrop 1000 spectrophotometer (NanoDrop Technologies, Wilmington, Delaware, USA) and quantified via gel

electrophoresis. After DNase-I treatment (Sigma—Aldrich), 1000 ng of total RNA was transcribed in a thermal cycler under the following conditions: 25 °C for 10 min, 48 °C for 60 min (additional step for highly structured RNA), 85 °C for 5 min, and hold at 4 °C; the SensiFAST™ cDNA Synthesis Kit (Bioline, London, UK) was used. Gene expression analysis (qPCR) was performed via the DyNAmo HS SYBR Green qPCR Kit (Thermo Fisher Scientific) and standard primers: *FSHR* F: GGAATGCCATTGAACTGAGG, R: TTGGGAAGGTTGGAGAACAC, 133 bp; *PPIA* F: ACTTCGAGCAAGAGATGGCCA, R: GACTCCATGCCCAGGAAGGA, 142 bp. A CFX96 Touch™ real-time PCR detection system (Bio-Rad, Hercules, CA) was used. Primer sequences for the GNRHR (116 bp) were F: CAGAGCCCTTTGCCCATAATA, R: TGGTTACTGACTCCTCCAAATG and PPIA (144 bp); F: GCCAAGACTGAGTGGTTGGATG; R: GAGTTGTCCACAGTCAGCAATGG.

2.6. cAMP production

Extracellular cAMP production in HEK293-FSHR, HEK-293 and KGN cells was determined according to the modified radioimmunoassay method using iodinated succinyl-cAMP [32]. In brief, cells were seeded onto 24-well plates (80,000 cells/well) and grown overnight in culture medium. Before stimulation, the cells were starved for 12 h in serum-free medium. cAMP decay was prevented by adding 3-isobutyl-1-methylxanthine (IBMX; Sigma) to the stimulation medium. The cells were stimulated without or with 10, 100 or 1000 IU/L of rhFSH or 10 μM forskolin (FRK), which was used as a positive control, and the medium for cAMP determination was collected after a 1 h incubation at 37 °C in a 5 % CO_2 humidified atmosphere.

2.7. Immunocytochemistry and immunohistochemistry

HEK293-FSHR cells were grown on Millicell EZ Slide 8-well glass (Merck Millipore, Darmstadt, Germany) overnight in culture medium, fixed with 4 % paraformaldehyde in PBS (15 min, room temperature) and washed with PBS (3 × 5 min). The cells were washed in PBS (3 × 5 min) and incubated for 30 min with blocking solution (3 % BSA in PBS with 0.05 % Tween 20; PBST) at RT. Thereafter, the cells were incubated in a humidified chamber for 1 h at RT with the following antibodies: anti-hFSHR (FSHR323; 5μg/ml; kindly donated by Dr. Ghinea) and FLAG sequence (F7425, 1:300, Sigma—Aldrich) diluted in blocking solution. The washed cells were incubated with goat anti-rabbit, goat anti-mouse or donkey anti-goat IgG conjugated with Alexa Fluor 488 or Alexa Fluor 594 (all diluted 1:250, Thermo Fisher Scientific) for 45 min in the dark (RT). The cell nuclei were visualized via DAPI staining (1 μg/ml in PBS). The slides were mounted in mounting medium (101098–042, Vector Laboratories, Burlingame, CA).

Formalin-fixed paraffin Section (5 μm) of HEK293-FSHR xenografts were deparaffinized, hydrated and boiled in 10 mM citric acid (pH 6.0) for 15 min for antigen retrieval. Endogenous peroxidase activity was reduced by incubation with 3 % H_2O_2 for 15 min at RT, and the sections were blocked with bovine serum albumin (3 % BSA) for 1 h at RT. Afterwards, the sections were incubated with primary anti-Ki67 (Dako) and anti-cleaved PARP1 (Cell Signaling) antibodies in blocking solution overnight at 4 °C. The slides were washed 3x for 5 min in PBST, and a DAKO EnVision+ HRP-conjugated system (Dako, Glostrup, Denmark) was used as the secondary antibody. The signal was visualized via 3'3-diaminobenzidine tetrahydrochloride (DAB, Dako). The sections were counterstained with Meyer's hematoxylin for 30 sec, washed, dehydrated and mounted with Pertex (Histolab Products AB, Gothenburg, Sweden).

2.8. RNAScope

In situ hybridization of formalin-fixed paraffin-embedded (FFPE) blocks of xenografts was performed with an RNAscope FFPE 2.0 HD Detection Kit Brown [33] (Advanced Cell Diagnostics (ACD), Hayward, California, USA, CAT# 310033) as previously described [34]. In brief, 5 μm FFPE cell sections were pretreated under standard conditions and incubated with the following prewarmed probes: a mouse *Fshr* probe (Cat No. 400461), positive control probes for low-abundance transcripts Mm-*Polr-2a*, #312471) and a negative control probe (*DapB*, ACD-310043) for 2 h at 40 °C in a HybEZ(TM) Oven (ACD). The slides were washed twice in 1X wash buffer for 2 min. Subsequent hybridization amplifiers were applied for 30 min (AMP 1, 3, 5) or 15 min (AMP 2, 4, 6) and incubated at 40 °C in a HybEZ(TM) oven (AMPs) with 2 min double washes between washes. To visualize the signal, an equal volume mixture of brown-A and brown-B was added on top of the sections and incubated at RT for 10 min. After double washing with ddH_2O and counterstaining for 2 min, fresh 50 % Gill's Hematoxylin (Vector Laboratories, Burlingame, CA, USA) slides were washed with ddH_2O and dipped in 0.02 % ammonia water for 20 s. Dehydration was performed with fresh ethanol (70 % for 2 min, twice with 100 % for 2 min) and xylene for 5 min. Slides were mounted with Pertex (Histolab Products AB, Gothenburg, Sweden).

2.9. FSH concentration measurement

The plasma levels of FSH were evaluated via an immunofluorometric assay via the Delfia® Enhancer system (PerkinElmer, Turku, Finland) according to the protocol described earlier by van Casteren et al. [35]. The rat FSH standard (gift from Dr. Albert Parlow from The National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, Maryland) was diluted in Diluent II buffer (DELFIA® Diluent II, PerkinElmer) to concentrations ranging from 50 ng/ml to 0.02 ng/ml. Enhanced fluorescence signals (DELFIA® Enhancement Solution, PerkinElmer) were measured with Victor^2 (Perkin Elmer). The assay sensitivity was 0.1μ g/L, with an intra-assay CV of 4.3 % and an interassay CV of 10.4 % at 4.8 μg/L. The intra- and interassay coefficients of variation for these assays were less than 10 %.

2.10. Statistical analysis

Numerical data are presented as the mean ± SEM. To analyze statistical significance, one-way ANOVA with Dunnett's multiple comparison post hoc test with a 95 % confidence interval was used (GraphPad PRISM v. 5., GraphPad Software Inc., San Diego, CA). The results were considered statistically significant at the $P < 0.05$ level.

3. Results

3.1. Hecate-FSHβ conjugates

We analyzed twelve variants of lytic Hecate-FSHβ conjugates (Suppl Fig 1.). Variants were generated via the conjugation of a lytic backbone (FALALKALKKALKKLKKALALKKAL) with a native or modified fragment of the FSHβ subunit (FSHβ33–53 YTRDLVYKDPARPKIQKTCTF and FSHβ81–95 QCHCGKCDSDSTDCT) or their combination (FSHβ33–53 +81–95 YTRDLVYKDPARPKIQKTCTFQCHCGKCDSDSTDCT). Additionally, cysteine residues in the FSHβ33–55 and 85–91 fragments were replaced by serines (C/S) or alanines (C/A) or stabilized by acetamidomethyl groups (Acm) to prevent disulfide linkage formation [26, 31], resulting in conjugate structure variation or oxidation, respectively (Suppl Fig 1.).

3.2. Hecate-FSHβ conjugates specifically destroy FSHR-positive cells in vitro

Hecate-FSHβ-mediated cytotoxicity was analyzed in three *in vitro* models: the endogenously expressed FSHR human granulosa cell tumor line KGN [36], the human FSHR stably transfected human embryonic kidney HEK293 cell line (HEK293-FSHR) and the FSHR-negative HEK293 control cell line. In contrast to HEK293-FSHR cells, KGN cells

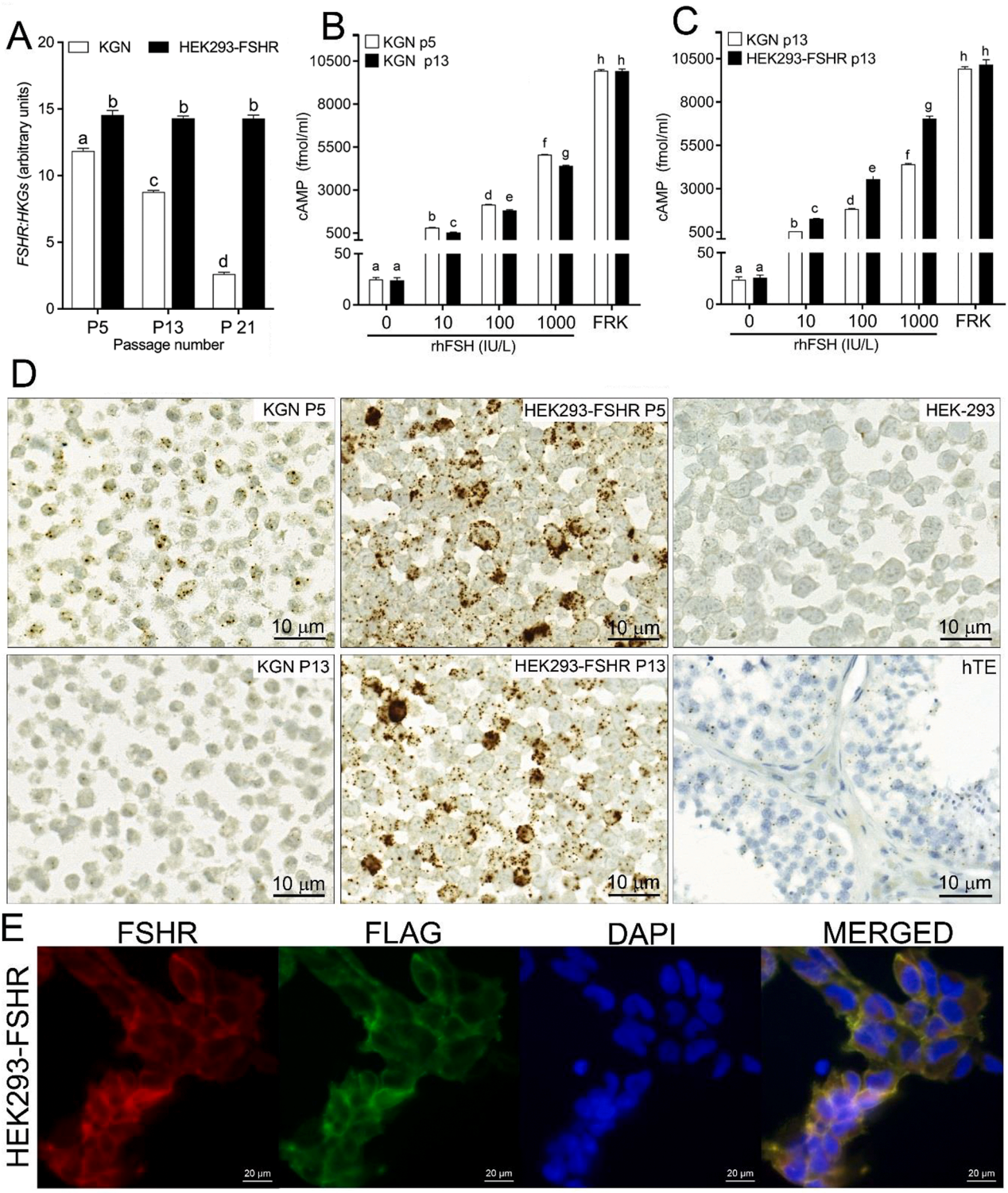

Fig. 1. Functional characterization of KGN and HEK293-FSHR cells. (A) qPCR relative expression of *FSHR* over 21 passages (p5, p13, p21) in KGN and HEK293-FSHR cells. Each bar represents the mean ± SEM of relative gene expression run in triplicate. (B-C) rhFSH-stimulated cAMP production in KGN and HEK293-FSHR cells. Each bar represents the mean ± SEM of three independent experiments (n = 8/experiment). Different letters above the bars indicate that the difference between them is statistically significant ($P \leq 0.05$). (D) RNAScope *in situ* hybridization of *FSHR* transcripts in KGN and HEK293-FSHR cells. HEK-293 and human testis (TE) cells were used as negative and positive controls for FSHR expression, respectively. (E) Immunofluorescence colocalization of human FSHR and FLAG in HEK293-FSHR cells. KGN, Human granulosa tumor cells; HEK293-FSHR, human embryonic kidney HEK-293 cell line stably transfected with human FSHR cDNA; Hecate-FSHβ33-53C/S, tested variant Hecate lytic peptide conjugated with modified fragment of the FSHβ subunit (FSHβ33–53) and cysteine residues replaced by serine (C/S), TE, human testis, FLAG.

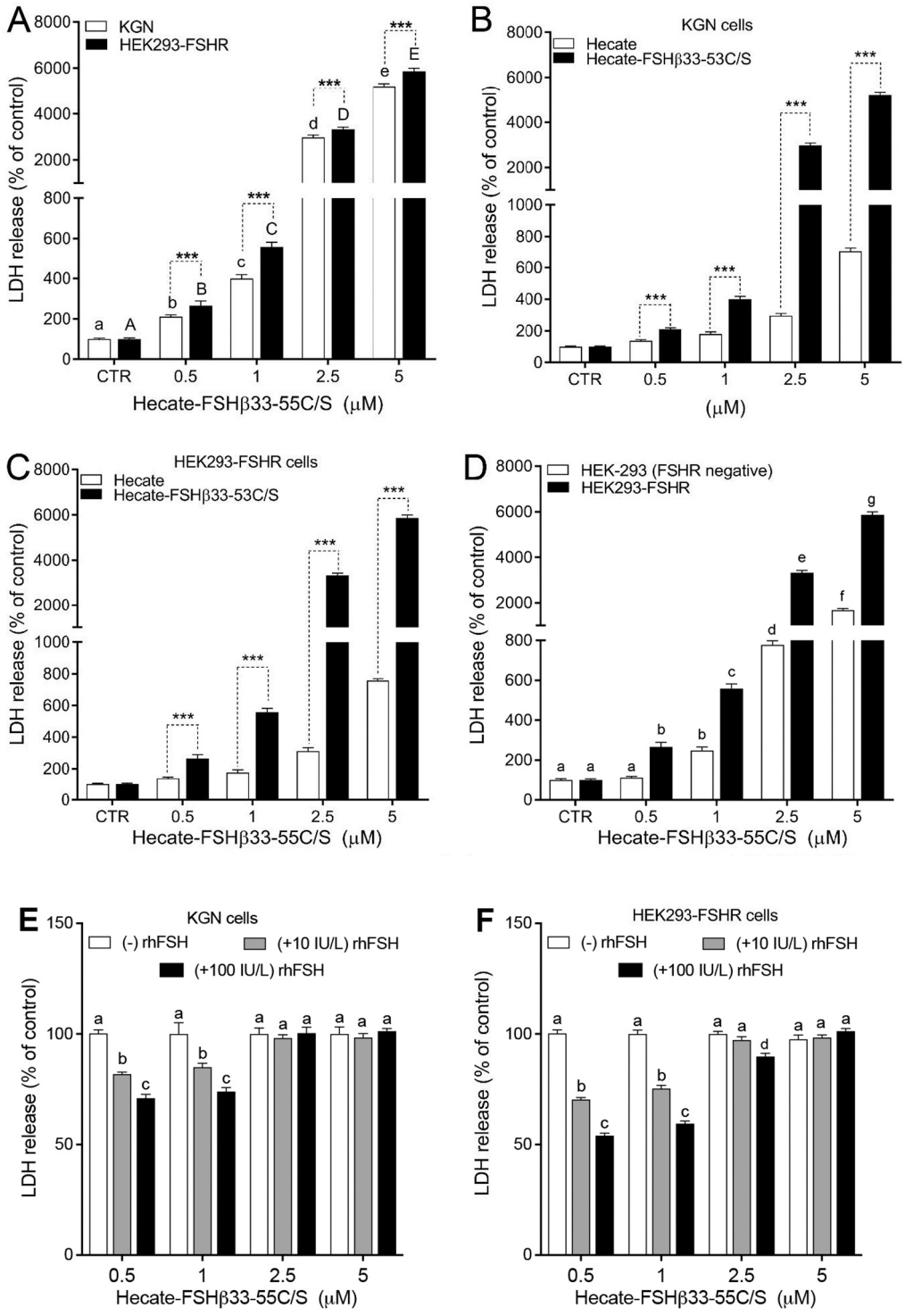

Fig. 2. Characterization of the cytotoxicity and specificity of the Hecate-FSHβ33–53C/S conjugate *in vitro*, as determined by the release of lactose dehydrogenase (LDH) into the culture supernatant (A-D). (A) Dose-dependent Hecate-FSHβ33–53C/S–induced cytotoxicity in KGN and HEK293-FSHR cells. (B-C) Comparison of Hecate and Hecate-FSHβ33–53C/S cytotoxicity in KGN and HEK293-FSHR cells. (D) Comparison of the sensitivity of HEK-293 and HEK293-FSHR cells to FSHβ33–53C/S cytotoxicity. The values are presented as the means ± SEMs of three independent experiments (n = 8/experiment) in three different passages of the cell line. Bars with different superscript letters differ significantly from each other ($P \leq 0.05$). Asterisks indicate additionally significant differences (*$P \leq 0.05$; ***$P \leq 0.001$) between the indicated groups. Effects of rhFSH (10 IU/L and 100 IU/L) pretreatment and cotreatment on Hecate-FSHβ33–53C/S-mediated cytotoxicity in KGN and HEK293-FSHR cells (E, F). The values are presented as the means ± SEMs of three independent experiments (n = 8/experiment) in three different passages of the cell line. Different letters above the bars indicate that the difference between them is statistically significant ($P \leq 0.01$). KGN, Human granulosa tumor cells; HEK293-FSHR, human embryonic kidney HEK-293 cell line stably transfected with human FSHR cDNA; Hecate-FSHβ33–53C/S, tested variant Hecate lytic peptide conjugated with modified fragment of the FSHβ subunit (FSHβ33–53) and cysteine residues replaced by serine (C/S); rhFSH, recombinant human follicle stimulating hormone.

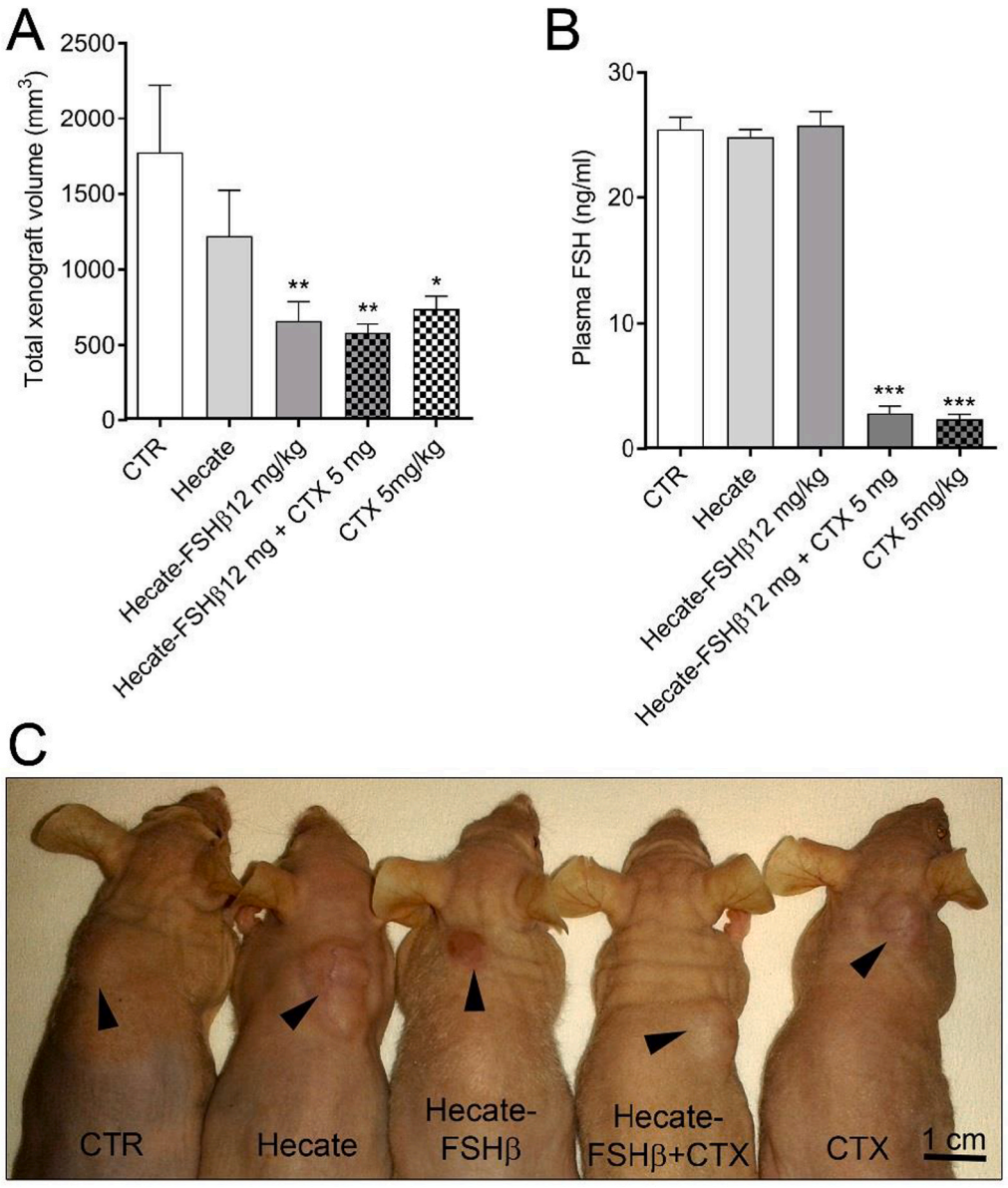

Fig. 3. Effects of Hecate-FSHβ33–53C/S and CTX treatments on HEK293-FSHR xenograft growth in nude mice. (A) Total xenograft volume at necropsy. (B) Plasma FSH levels. (C) Representative images of HEK293-FHSR xenografts that developed subcutaneously in the interscapular area of nude mice. The values are the means ± SEMs (n = 10–12). Asterisks/hashtags indicate significant differences between the CTR group and the treatment or indicated groups (*P ≤ 0.05; ***P ≤ 0.001). KGN, Human granulosa tumor cells; HEK293-FSHR, human embryonic kidney HEK-293 cell line stably transfected with human FSHR cDNA; Hecate-FSHβ33–53C/S, tested variant Hecate lytic peptide conjugated with modified fragment of the FSHβ subunit (FSHβ33–53) and cysteine residues replaced by serine (C/S).

presented a passage-dependent (P5, P13, P21) decrease in *FSHR* expression (Fig. 1 A), followed by reduced rFSH-stimulated cAMP production (Fig. 1 B, C). FSHR protein expression in the cells can be seen in Fig. 1D. The stable membrane localization of FSHR, which colocalized with a FLAG reporter, was confirmed in HEK293-FSHR cells (Fig. 1E).

Compared with the other tested conjugates, the screening of conjugate-mediated cytotoxicity revealed that Hecate-FSHβ33–55C/S was the most efficient at the two lowest doses (0.5 and 1 μM) on HEK293-FSHR cells (Fig. S1). To determine the mechanism of CTX action, we analyzed additionally the GnRH receptor mRNA expression (GNRHR) in HEK-293 and HEK293-FSHR cells and GNRHR were expressed in both cell lines (Fig. S2).

Further characterization revealed a dose-dependent effect of Hecate-FSHβ33–55C/S on both KGN and HEK293-FSHR cells, with stronger effects on the latter (Fig. 2 A). To analyze the selectivity of Hecate-FSHβ for FSHR-positive cells, we compared the cytotoxicity between the Hecate-FSHβ conjugate and Hecate backbone in KGN and HEK293-FSHR cells and the cytotoxicity of Hecate-FSHβ between HEK293 and HEK293-FSHR cells. Compared with the Hecate backbone, the Hecate-FSHβ33–55C/S conjugate had a significantly greater dose-dependent cytotoxic effect on FSHR-positive cells (Fig. 2 B, C) and more efficiently targeted (from 2.4- to 4.2-fold) HEK293-FSHR cells than did the Hecate backbone (Fig. 2 D). We further used 2 additional human ovarian cancer cell lines without FSHR-expression (OVCAR-3 and SKOV-3) [22], along with FSHR-expressing HEK293-FSHR and KGN cells and determined the release of lactose dehydrogenase (LDH) into the culture supernatant (Fig Suppl S3). This result additionally showed that Hecate-FSHβ33–55 C/S conjugate had a significantly greater dose-dependent cytotoxic effect on FSHR-positive cells (Fig Suppl S3).

Hecate-FSHβ33–55 C/S conjugate-mediated cytotoxicity at doses ranging from 0.5 to 2.5 μM decreased after pre- and co-stimulation with 10 or 100 IU/L rhFSH (Fig. 2 E, F), indicating competition between

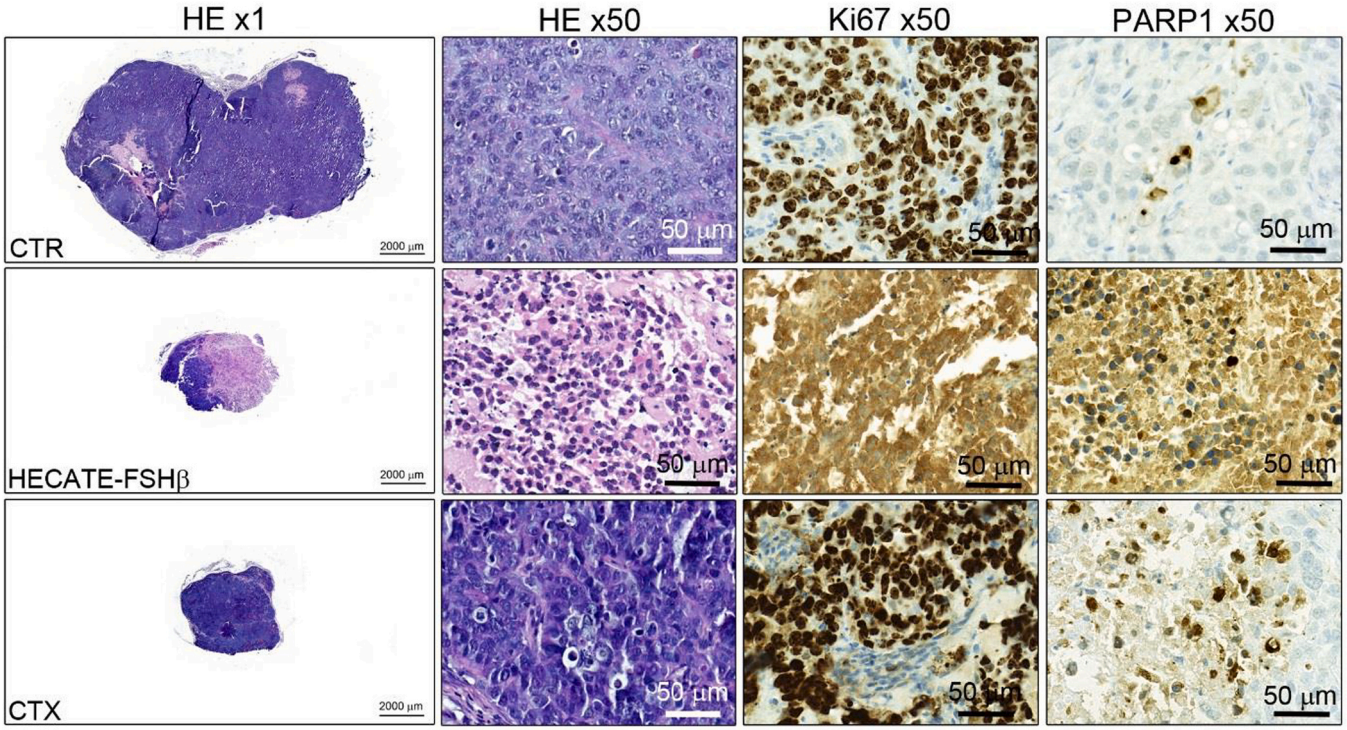

Fig. 4. Histology and immunohistochemical staining for nuclear Ki-67 proliferation markers and cytoplasmic PARP-1 apoptosis markers in the xenografts of control, Hecate-FSHb33–53C/S-treated and CTX-treated mice.

Hecate-FSHβ and endogenous FSH for binding to FSHR *in vivo*.

3.3. *Hecate-FSHβ conjugates inhibit xenograft growth*

Owing to the unstable expression of FSHR in KGN cells (gradual decrease in the mRNA expression of *FSHR* and reduced FSH-stimulated cAMP production over passages), the efficacy of Hecate-FSHβ33–55 C/S conjugate treatment *in vivo* was tested in HEK293-FSHR cells xenografted into nude mice. Compared with the control and Hecate backbone, Hecate-FSHβ33–55 C/S alone or in combination with CTX inhibited the growth of the HEK293-FSHR xenografts (Fig. 3 A, C). Moreover, CTX treatment alone also significantly inhibited xenograft growth (Fig. 3 A, C) and, as expected, significantly reduced FSH levels (Fig. 3 B). The inhibition of tumor xenograft growth over the course of the experiment could be followed from the measurements on day 5, 8, 11, 14, 17, 20 23 and terminal 26 (Fig Suppl S4). Histopathological analysis of xenograft tissues revealed apparent necrotic changes in the xenografts of Hecate-FSHβ33–55 C/S-treated mice (Fig. 4). CTX treatment-induced apoptosis manifested histologically as shrunken cells with condensed cytoplasm and pyknotic and fragmented nuclei (arrowheads, Fig. 4), as confirmed by cytoplasmic staining of the apoptosis marker PARP1 (Fig. 4). In contrast to those in the control and CTX groups, the proliferation marker KI67 in Hecate-FSHβ33–55 C/S-treated xenografts was not localized, most likely because of the selected destruction of the FSHR-containing intact cells (Fig. 4).

Treatment with Hecate or Hecate-FSHβ33–55C/S caused no changes in body, spleen or testis weight (Fig. 5 A-C). CTX alone or in combination with Hecate-FSHβ33–55C/S significantly increased the weight of the spleen and reduced the weight of the testes (Fig. 5B, C). Histology of the testes of CTX-treated mice inhibited spermatogenesis but not the abundance of Leydig or Sertoli cells (Fig. 5D).

4. Discussion

The FSHβ subunit contains two specific fragments, 33–53 and 81–96, which were confirmed to be potent antagonists of FSHR activation [37,38]. In our study, original or modified (Cys replaced by Ser and Ala or covalently linked to Acm groups) FSHβ fragments were conjugated with the lytic peptide Hecate to selectively target and destroy FSHR-expressing cancer cells. It has been reported that Cys residues in peptides may undergo rapid oxidation and are prone to form disulfide linkages [39]. An earlier study on modified synthetic analogs of hFSHβ revealed that replacing Cys with Ser did not affect receptor binding affinity but was deleterious to its agonistic activity [40,41]. Compared with other modifications, the increased cytotoxicity of the Hecate-FSHβ33–55 C/S conjugate is most likely due to its increased stability after the replacement of Cys51 with Ser. In previous studies, greater cytotoxicity was also observed in the Phor21-βCG(Ala) conjugate with Cys in the βCG81–96 sequence replaced by Ala [16,28,42] or in the Hecate-βCG conjugate, with cysteines covalently linked to Acm groups [25–27]. In these studies, the Phor21-βCG(Ala) conjugate significantly reduced primary tumor weight in nude mice xenografted with human prostate and breast cancer cells or metastatic cells from the bone marrow and lymph nodes [16,28,42]. It is important to mention here that although melittin and its transposed analogue D-melittin has been shown to be immunogenic [43], relatively small and rapidly metabolized Hecate and Phor 21 peptides are not immunogenic [30].

In this study, the specificity and cytotoxicity of synthesized Hecate-FSHβ conjugates were tested in KGN and versatile *in vitro* model HEK293-FSHR cells, as well as in 3 cell lines non-expressing FSHR as controls (HEK-293, OVCAR-3 and SKOV3). The KGN cell line was established from a patient with invasive ovarian granulosa cell carcinoma [36]. Originally, KGN cells expressed functional FSHR, and its stimulation resulted in increased aromatase activity [36]. However, owing to the gradual loss of FSHR expression in cultured KGN cells observed in our study, we generated and used a stable FSHR-expressing HEK293-FSHR cell line for xenograft studies. HEK-293 cells are from a human embryonic kidney cell line transfected with adenovirus type 5 DNA and can be classified as a tumorigenic cell line [44]. HEK-293 cells display negatively charged membranes, unlimited division and stable

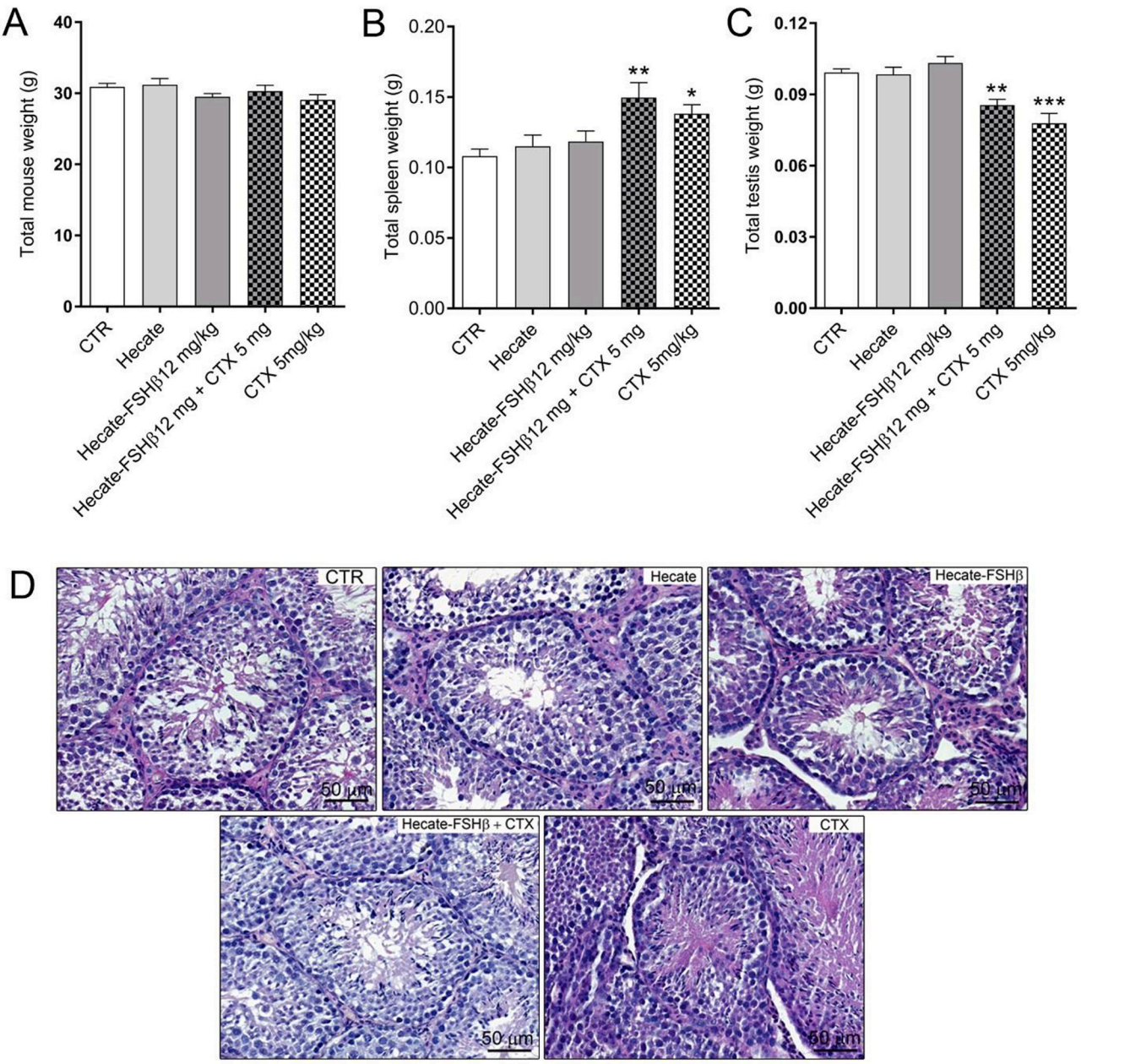

Fig. 5. Effects of Hecate-FSHβ33–53C/S conjugate and CTX treatments on the total body, spleen and testis weights of nude mice xenografted with HEK293-FSHR cells (A-C). Each bar represents the mean value of the total weight measured at necropsy (n = 8–12). Asterisks indicate significant differences (*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.01) between the control and treated groups. Histology of a representative mouse testis control that was not treated or treated with the Hecate-FSHβ33–53C/S conjugate and CTX or their combination (D).

growth in nondemanding culture conditions *in vitro* or when xenografted in nude mice [44]. An advantage of the HEK293-FSHR mode was the high and stable expression of FSHR in these cells, and FSHR-negative HEK-293 cells served as controls for the assessment of the selectivity of the tested compounds *in vitro* and *in vivo*.

The cytotoxic effect of the lytic peptide Hecate has been successfully tested in combination with βCG (Hecate-βCG) to target LHCGR-positive breast [45,46], prostate [25], ovarian [26] and adrenal [27] cancer cells. Very recently, a fragment of the FSHβ subunit conjugated to the lytic peptide Phor18 was shown to inhibit the growth of PC-3 prostate cancer cell line xenografts by targeting FSHR-expressing cancer cells or endothelial cells of the tumor vessels [47]. A conjugate of the FSHβ33–53 chain and the cationic peptide $G(IIKK)_3I\text{-}NH_2$ ($FSHR_{33–53}$-IIKK) has been shown to exert strong cytotoxic effects on different FSHR-positive cell lines [48]. However, in the last two studies [47,48], *FSHR* mRNA expression or the functionality of FSHR were tested in the treated cell lines, which raises concerns about the specificity of their action through FSHR [47,48]. Recently, utility of targeting FHSR in ovarian cancer by highly immune potent bispecific tools focused on FSHR and CD3 was reported [23]. Namely, mAbs targeting the external domain of FSHR (D2AP11, a potent FSHR surface–targeted mAb) to develop a bispecific T cell engager, which induced in vitro specific and potent killing of different genetic and immune escape ovarian cancer FSHR-expressing cells, as well as attenuated tumor burden in in vivo ovarian cancer-challenged mouse models [23]. Another interesting approach was to utilize FSHR as a target in the redirected T-therapy cell for ovarian cancer [22]. In this later study, human T-cells transduced to express anti-FSHR immunoreceptors were specifically immunoreactive against FSHR-expressing human and mouse ovarian cancer cell lines in a MHC-non-restricted manner, which

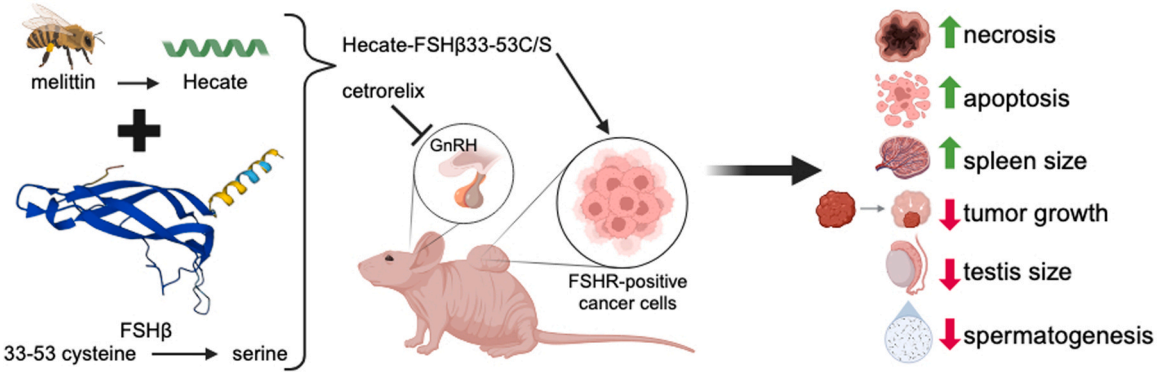

Fig. 6. Graphic Abstract. Schematic overview of the Hecate-FSHβ33–53C/S conjugate or cetrorelix (CTX) selectively targeting and killing the FSHR-positive cancer cells. (Figure created with BioRender.com).

mediated effective lysis of only FHSR-expressing tumor cells in vitro [22]. The outgrowth of human ovarian cancer xenografts in immunodeficient mice was also significantly inhibited by this adoptive transfer of FSHR-redirected T-cells in vivo [22].

The inhibition of xenograft growth by the Hecate-FSHβ33–55 C/S and CTX treatments observed in our study involved two different mechanisms. In contrast to chemical cytotoxic compounds, such as enzyme inhibitors or antagonists, lytic peptides perforate the cell membrane, inducing cell swelling and bursting and resulting in rapid cell death [25,26]. Therefore, Hecate-FSHβ33–55 C/S, like other lytic peptide-based conjugates, induced rapid necrosis [25,26]. Conversely, CTX treatment could directly act on HEK293-FSHR cells that express GnRHR. Our findings are supported by other studies showing that CTX has direct antiproliferative and proapoptotic effects on a number of human cancers, including prostate, colorectal, endometrial, lung, and ovarian tumors [49]. A schematic overview of the Hecate-FSHβ33–53C/S conjugate or CTX selectively targeting and killing the FSHR-positive cancer cells are shown in Fig. 6.

The significant reduction in Hecate-FSHβ33–55 C/S-mediated cytotoxicity caused by dose dependent rhFSH cotreatment in KGN or HEK293-FSHR cells indicated competition between the conjugate and FSH for the FSHR binding site. However, a xenograft study revealed only a nonsignificant reduction in xenograft growth after treatment with both Hecate-FSHβ33–55 C/S and CTX compared with each compound alone. This could be explained by either too low intraspecific FSH levels and/or high Hecate-FSHβ33–55 C/S concentrations or by the treatment being too short. In the future, pharmacokinetics of Hecate-FSHβ33–55 C/S in vivo needs to be established, as well as further characterization of the half-life and stability of the compound in vivo, which will be helpful to analyze its feasibility.

In conclusion, the Hecate-FSHβ33–55 C/S conjugate had a strong specific cytotoxic effect on cancer cells expressing FSHR. High selectivity with low or no systemic toxicity suggests considerable therapeutic potential in treating cancers that express FSHR. Moreover, the use of a GnRH antagonist seems to be justified not only as a cotreatment with the Hecate-FSHβ33–55 C/S conjugate to eliminate the competition between FSH and the conjugate for binding to FSHR in cancer cells but also for its direct action on cancer cells expressing the GnRH receptor.

CRediT authorship contribution statement

Li Xiangdong: Validation, Investigation. **Rivero-Muller Adolfo:** Investigation, Formal analysis. **Kreuzer Oliver J:** Formal analysis. **Doroszko MIlena:** Investigation, Formal analysis. **Stelmaszewska Joanna:** Investigation, Formal analysis. **Pulawska-Moon Kamila:** Methodology, Investigation, Formal analysis. **Ponikwicka-Tyszko Donata:** Methodology, Investigation, Formal analysis. **Chrusciel Marcin:** Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Huhtaniemi Ilpo:** Writing – review & editing, Validation, Investigation. **Rahman Nafis:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Investigation, Funding acquisition, Data curation, Conceptualization. **Wolczynski Slawomir:** Validation, Supervision, Investigation. **Ziecik Adam J:** Validation, Investigation.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2025.118022.

Data Availability

Research data supporting this publication are available from the Corresponding Author upon reasonable request.

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