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# Cytotoxic activity and cell specificity of a novel LHRH peptide drug conjugate, *D*-Cys6-LHRH vedotin, against ovarian cancer cell lines.

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ORCID ID: 0000-0002-1567-3983**Abstract**

Ovarian cancer is the most deadly female gynaecological malignancy in developed countries and new treatments are urgently needed. The luteinising hormone releasing hormone (LHRH) peptide drug conjugate Zoptarelin doxorubicin is one such potential new drug modality that entered clinical trials for treating LHRH receptor-positive gynaecological cancers. However, development stopped after disappointing phase 3 results in 2017. We believe the lack of efficacy was due to linker instability and payload potency. In this work, we replaced its linker-toxin with vedotin (MC-VC-PABC-MMAE), yielding the novel peptide drug conjugate *D*-Cys6-LHRH vedotin. A GI50 and cell specificity comparison against cancerous and non-cancerous ovarian cell lines showed significantly superior bioactivity and selectivity over Zoptarelin doxorubicin (GI50 4 vs 453 nM) and other chemotherapeutic drugs used for treating ovarian cancers. Our results suggest *D*-Cys6-LHRH vedotin can potentially be used as a treatment for ovarian cancer.

## Key words

Luteinising hormone releasing hormone; gonadotropin-releasing hormone; peptide drug conjugate; ovarian cancer; Zoptarelin doxorubicin

## Introduction

Ovarian cancer is the most deadly gynaecological malignancy in women living in developed countries (Emons et al., 2014). In the United States, approximately 20,000 women were diagnosed in 2022 with an estimated 13,000 dying from the disease (Siegel et al., 2022). First-line treatment involve debulking surgery followed by combination chemotherapy using paclitaxel and a platinum drug (Emons et al., 2014). However, approximately 80% of patients relapse and face a poor prognosis using second-line chemotherapies with a progression free survival of 3 to 4 months and overall survival of approximately 1 year (Emons et al., 2014), flagging the urgent need for new drugs.

A major limitation of chemotherapeutic drugs is that they are non-selective, entering both cancerous and non-cancerous cells alike, resulting in off-target toxicity. Developing drugs that are specific for cancer cells can overcome this drawback. A possible solution is to conjugate a cytotoxic drug to a cancer cell-targeting biological molecule which serves as a vector to seek out cancer cells expressing receptors specific for the biological molecule. Upon receptor binding, the vector is internalised together with the drug. A potential vector is the 9-residue peptide, luteinizing hormone-releasing hormone (LHRH; also known as gonadotropin-releasing hormone; Fig. 1), involved in sex organ development, function, reproduction and possibly cancer cell growth (Imai et al., 1994; Nagy & Schally, 2005). LHRH receptors are deemed good cancer drug targets as they are not expressed by normal non-reproductive tissues (Gründker et al., 2002), while expressed on the cell surfaces of approximately 70–80% ovarian carcinomas (Völker et al., 2002; Nagy & Schally, 2005).Hence, Tulane University scientists designed a peptide drug conjugate by conjugating the cytotoxic drug, doxorubicin, to a *D*-lysine6-modified analog of LHRH *via* an esterase-cleavable hemiglutarate linker (Fig. 1) (Nagy et al., 1996; Nagy et al., 2000). This peptide drug conjugate, AN-152/AEZS-108/**Zoptarelin doxorubicin**/Zoptrex, was shown to enter LHRH receptor-positive endometrial and ovarian cancer cells *in vitro* (Günthert et al., 2004). After showing promising results in a mouse xenograft study (Gründker et al., 2002), Zoptarelin doxorubicin entered clinical trials in 2005 to treat patients with LHRH receptor-positive gynaecological tumours (Nagy & Schally, 2005; Emons et al., 2010; Emons et al., 2014) but its development was terminated in 2017 after failing to outperform doxorubicin in a phase 3 trial (Hoppenz et al., 2020; Wu et al., 2023). It has been suggested that the ester linker was too metabolically unstable, resulting in doxorubicin’s premature release from the peptide carrier before cell entry (Wu et al., 2023).Another reason could also be due to doxorubicin’s relatively poor cytotoxic activity when used as a drug payload. Indeed, Bristol Myers Squibb’s antibody drug conjugate (ADC), BMS-182248/BR96-doxorubicin, which also utilised doxorubicin as a payload, failed to show efficacy against breast and gastric cancer patients in two clinical trials (Tolcher et al., 1999; Ajani et al., 2000). Doxorubicin’s average GI50 against 39 cancer cell lines was reported to be 631 nM (Doronina et al., 2003) and this was postulated to lack the potency required as an effective payload as less than 0.01% of injected ADCs are estimated to enter targeted tumour cells (Beck et al., 2017). Hence, ADCs designed for cancer treatment utilise more potent payloads; the most popular being monomethyl auristatin E (MMAE), first reported by Seattle Genetics in 2003 (Doronina et al., 2003). MMAE is an ultra-potent tubulin binder that prevents tubulin polymerisation, resulting in cell death with an average 3 nM GI50 against 39 cancer cell lines, compared to doxorubicin’s 631 nM (Doronina et al., 2003). When conjugated *via* a para-aminobenzyloxycarbamoyl (PABC) spacer to a cathepsin-cleavable valine-citrulline (VC) dipeptide linked to a maleimidocaproyl (MC) moiety, this clinically-proven linker-toxin is named ‘vedotin’(Fig. 1) and is currently utilised by four FDA-approved ADCs for treating various cancers (Dumontet et al., 2023). Based on its popularity and commercial availability, we conjugated MC-VC-PABC-MMAE to the LHRH peptide analog *D*-Cys6-LHRH to yield peptide drug conjugate ***D*-Cys6-LHRH vedotin** (Fig. 1 and Scheme 1). Its GI50 and cell selectivity against various cancer and non-cancer human cell lines were compared to Zoptarelin doxorubicin and ovarian cancer chemotherapeutic drugs paclitaxel and doxorubicin to gauge its potential for use as a cancer drug candidate.

## Fig. 1



**Fig. 1**. Structures of LHRH, peptide drug conjugates AN-152/AEZS-108/Zoptarelin doxorubicin/Zoptrex and *D*-Cys6-LHRH vedotin. Enzyme-cleavable linkers and cytotoxic payloads are denoted in blue and red respectively. Enzyme-cleavable bonds are highlighted pink. Asterisk represents residue 6.

## Method

Clinical candidate Zoptarelin doxorubicin was synthesised based on a reported method in a patent (US5843903A) and details are recorded in the supplementary file. Peptide drug conjugate *D*-Cys6-LHRH vedotin was synthesised by coupling peptide *D*-Cys6-LHRH (GL Biochem, catalog# 534114) to MC-VC-PABC-MMAE (ABCR GmbH, catalog# AB456059) (Scheme 1; synthetic details recorded in supplementary file). Doxorubicin and paclitaxel were purchased from Merck (catalog# D1515 and T7191 respectively) while MMAE was purchased from MedChemExpress (catalog# HY-15162). LHRH receptor-positive (OVCAR-3), receptor-negative (SK-OV-3) ovarian cancer cell lines and lung fibroblast (MRC-5) were purchased from ATCC. The non-cancerous human ovarian (H-6036) cell line was purchased from Cell Biologics. Cell line culturing method and GI50 assay details can be found in the supplementary file. Experiments were conducted in triplicates and GI50s determined using Prism software (GraphPad).

## Scheme 1



**Scheme 1**. *D*-Cys6-LHRH vedotin synthesis route. Enzyme-cleavable linker MC-VC-PABC and MMAE denoted in blue and red respectively. Cathepsin-cleavable bond highlighted pink.

## Results

GI50s are summarised in Table 1. Our cell panel consisted of LHRH receptor-positive (OVCAR-3) and receptor-negative (SK-OV-3) ovarian cancer cell lines used in Zoptarelin doxorubicin’s preclinical development studies (Gründker et al., 2002; Günthert et al., 2004). Non-cancerous human ovarian (H-6036) and lung fibroblast (MRC-5) cell lines were also included for selectivity comparison. Paclitaxel, a first-line chemotherapeutic drug used for ovarian cancer (Emons et al., 2014), was observed to be highly cytotoxic against all cell lines, including non-cancerous ovarian and human fibroblasts (GI50s 1−5 nM; Table 1). Unsurprisingly, common and serious side-effects experienced by more than 50% patients dosed with this drug included anaemia, neutropenia, peripheral neuropathy, nausea, vomiting, myalgia, arthralgia and alopecia. Similarly, doxorubicin was also shown to be non-specific towards all the cell lines (GI50s 60−311 nM; Table 1), below the reported average 631 nM GI50 in a study involving 39 cancer cell lines (Doronina et al., 2003). It is noteworthy that doxorubicin exhibited moderate GI50s of 311 and 218 nM against OVCAR-3 and SK-OV-3 respectively, supporting earlier *in vitro* data (Patankar et al., 2013; Pouyafar et al., 2019). Tubulin binder MMAE was found to be ultra-toxic against all cell lines (GI50s 0.5−1.1 nM; Table 1), supporting the findings from an earlier report (Doronina et al., 2003). It is therefore unsurprising that MMAE has never been approved as a drug on its own. The LHRH analog and carrier peptide, *D*-Cys6-LHRH, was inactive against all cell lines (GI50s > 50,000 nM; Table 1).

Clinical candidate Zoptarelin doxorubicin exhibited moderate GI50s against OVCAR-3 and SK-OV-3 (GI50s 453 and 358 nM respectively; Table 1), 1.5- to 3-fold less potent compared to doxorubicin. Surprisingly, it was found to be active against the non-cancerous ovarian cell line, H-6036 (GI50 180 nM; Table 1), suggesting the cell line expresses LHRH receptors. Indeed, Gifu University researchers reported low levels of LHRH receptors on the surfaces of non-malignant ovarian cell lines (Imai et al., 1994), suggesting that determining LHRH receptor expression levels is imperative before drug administration to prevent off-target toxicity and adverse events.

Our novel peptide drug conjugate *D*-Cys6-LHRH vedotin exhibited high potency against the LHRH receptor-positive OVCAR-3 cell line, on par with paclitaxel (GI50s 4 nM; Table 1) and 113-fold more potent than clinical candidate Zoptarelin doxorubicin (GI50 4 vs. 453 nM; Table 1). Selectivity-wise, *D*-Cys6-LHRH vedotin showed a GI50 of 52 nM against non-cancerous H-6036 ovarian cells with a 13-fold therapeutic index (4 vs. 52 nM GI50; Table 1). Against non-cancerous MRC-5 fibroblasts, the therapeutic index increased almost 50-fold (4 vs. 191 nM GI50; Table 1), highlighting its potential as an ovarian cancer drug candidate. Interestingly, *D*-Cys6-LHRH vedotin is also active against SK-OV-3 (GI50s 49 nM; Table 1), confirming that this cell line does express LHRH receptors, supporting data from an earlier report (Imai et al., 1994).

## Table 1

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  |  |  | GI50 (nM) |  |  |
|  | ovarian adenocarcinoma | ovarian adenocarcinoma |  | non-cancerous ovarian epithelial cells | non-cancerous lung fibroblast |
| Test compound | OVCAR-3 | SK-OV-3 |  | H-6036 | MRC-5 |
| Paclitaxel | 4 | 5 |  | 1 | 2 |
| Doxorubicin | 311 | 218 |  | 60 | 134 |
| MMAE | 0.5 | 0.8 |  | 0.6 | 1.1 |
| *D*-Cys6-LHRH | > 50,000 | > 50,000 |  | > 50,000 | > 50,000 |
| Zoptarelin doxorubicin | 453 | 358 |  | 180 | 382 |
| *D*-Cys6-LHRH vedotin | 4 | 49 |  | 52 | 191 |

**Table 1**. GI50s (nM) of test compounds against various human cell lines.

In conclusion, we show that it is possible to significantly improve Zoptarelin doxorubicin’s potency and selectivity by simply replacing the linker-toxin moiety with vedotin. As both Zoptarelin doxorubicin and the linker-toxin vedotin have been shown to be chemically stable *in vitro* and *in vivo* (Nagy et al., 2000; Doronina et al., 2003), future experiments should involve a head-to-head *in vivo* efficacy study between *D*-Cys6-LHRH vedotin and Zoptarelin doxorubicin in a mouse ovarian cancer xenograft model.

## Abbreviations

ADC antibody drug conjugate

Cys cysteine

FDA Food and Drug Administration

GI50 half-maximal growth inhibitory concentration

MC maleimidocaproyl

MMAE monomethyl auristatin E

OVCAR-3 ovarian carcinoma cell line 3

PABC para-aminobenzyloxycarbamoyl

SK-OV-3 Sloan Kettering ovarian cancer cell line 3

VC valine-citrulline

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## Conflict of Interest Disclosure

The authors declare no conflicts of interest.

## Data Availability

All supplementary data are found in the supplementary file.

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