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The Ends of Lecirelin on the Destruction of Ovarian Cancer Cells

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ABSTRACT:

As a gynecological cancer, ovarian cancer has the highest lethality among females on the globe. Evidence shows that the GnRH agonists and antagonists dramatically inhibit the growth of most cancer cell lines expressing the GnRH receptor. The current research focuses on the effectiveness of three novel GnRH analogs showing anticancer activities on the cell lines of ovarian cancer. The present investigation synthesized Lecirelin in the solid phase and changed the peptide chain's C-terminal. Two ovarian cancer cell lines were examined for the impacts of the purified peptides. The hydrazide derivative with an approximate concentration of 30 µg/mL (lC_{50}~30) can kill more than half of the ovarian cancer cells, while the Lecirline needs twice more active ingredient to kill this amount of the cells (lC_{50}~47, p = 0.001). Lecirelin and its hydrazide derivative have anticancer activity against ovarian cancer cells, and this GnRH analog may be considered a promising candidate for further studies.

Keywords: Lecirelin; Ovarian cancer; Gonadotropin-releasing hormone (GnRH); Peptide; Hydrazide; MTT.

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1. Introduction

A main community health problem is cancer throughout the world. As reported by the World Health Organization, approx. one in six mortalities is caused by cancer [1]. The occurrence of most deaths (70%) in low- and middle-income countries has caused the utmost harm to the mentioned countries [2]. The limited accessibility of remedial drugs, particularly chemotherapy medicines, is a major reason for the heightened death rate. However, the limitations of this method include cancer cells' acquired or innate multidrug resistance, absence of selectiveness, fast removal of medicines from the circulatory system, and toxic aftereffects caused by heightened dosages needed for treatment effectiveness. Recently, studies on cancer have turned to a method with higher selectivity and target orientation, utilizing therapies that show superior effectiveness with lower aftereffects [3].

Ovarian cancer is known to cause the utmost lethality as a gynecological cancer in females globally, being the eighth most prevalent malign tumor illness in females [4, 5]. Due to the lack of early detection methods (owing to nonspecific symptoms), the diagnosis in 70% of cases with this cancer occurs in progressive phases. The disease is detected in merely 20% of patients at phase I [6].

In hormone therapy, hormones or hormone-blocking medicines are used to counteract cancers, which is a systemically treating modality mostly utilized in the treatment of stromal tumors in ovaries [7].

The wide use of gonadotropin-releasing hormone (GnRH) in clinical medicine has started since its discovery and synthesis in 1971 [8-11]. Studies show that ovarian cancer cells express the GnRH receptor (GnRH-R), which regulates proliferation and metastasis in these cells. Available reports indicate the significant inhibition of the growth of most cancer cell lines expressing the GnRH receptor by GnRH agonists and antagonists [5].

After identifying its amino acid sequence, numerous GnRH agonist and antagonist analogs were designed and subjected to clinical trials. The primary reason for this is its short half-life, which is between 2 to 4 minutes due to degradation by peptidase enzymes. By altering the sequence through deletion, addition, or substitution of amino acids at various positions, there is an aim to increase the half-life and efficacy of GnRH [12].

GnRH analogs could serve as suitable alternatives for the treatment of various cancers, including ovarian cancer. GnRH-R and FSH, LH, and estradiol receptors are expressed in most cancer tissues of ovaries [13,14].

After administrating GnRH analogs, the profound hypoestrogenism condition occurs by blocking the hypothalamic-pituitary-ovarian axis. The abundant sex steroid receptors in fibroid tissue support tumor growth. Therefore, the deprived estrogen condition caused by GnRH analogs in women with fibroids has therapeutic benefits, which leads to the reduction of tumor volume and improvement of symptoms [15]. In investigations that evaluated several GnRH analogs, a reduced volume of about 4% was observed after six months of treatment, and improvements in this symptom were reported by 97% of females who complained of atypical hemorrhage following 6 months [16].

At present, the clinical use of GnRH analogs is not common for ovarian cancer treatment. Some clinical trials assessing the usage of GnRH analogs demonstrated moderate effectiveness [17].

Lecirelin (a GnRH analog) and Cetrorelix (an antagonist) have been examined in patients who have platinum-resistant ovarian cancer. Whereas partially improved patients were 9% and 18%, the disease stabilized in 26% and 35%, respectively [18,19].

GnRH analogs may serve as a valued preventive factor counteracting sterility resulting from chemotherapy by inhibiting fast cellular turnover in developing follicles containing various kinds of cells targeted accidentally throughout anticancer therapies [20].

To synthesize peptidomimetic structures, researchers have tried considerably to overwhelm the undesirable features and remedial shortcomings of peptides. Söderhäll et al. claimed the peptide's improved p-stacking interactions with hydrophobic groups when they were introduced into the GnRH sequence, thereby increasing its efficacy [21].

So far, many non-peptide compounds with hydrazide or hydrazide-hydrazone substitution have been studied as anticancer drugs, and the presence of these groups in many compounds has increasingly affected the compound's properties [22-24].

Hydrazides are a group of compounds with an aza- (-NH-NH2) functional group, which are attached to the carbonyl group. The current experiment dealt with the design, synthesis, and analysis of three novel GnRH analogs for their anticancer action compared to Lecirelin. The structure of the novel derivatives resembles Lecirelin with different C-Terminals to enhance the hydrophobic properties of the peptide with no changes in the active site of the peptide.

A stronger interplay is expected between the novel analog and the GnRH receptor, resulting in enhanced peptide action, as reported previously about the molecular mode of action of ligand-receptor interplay with the GnRH receptor [25,26].

2. Materials & Methods

2.1 Chemicals

Resin, coupling reagents, and protected amino acids for peptide synthesis were purchased from GL-Biochem, a Chinese company. Solvents and other chemical reagents were obtained from Merck, Germany. All cell lines (normal fibroblast cells (Hu02), A2780, and A2780-cp) were purchased from the Pasteur Institute of Iran.

The following chemicals and the medium components were purchased from Sigma: L-glutamine, fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), Dimethylsulfoxide (DMSO), penicillin-streptomycin antibiotic (Pen-Strep), 3-(4, 5 dimethylthiazol-2-yl)-2,5- diphenyl-tetrazolium bromide (MTT).

2.2 Peptide synthesis

Peptide sequences were synthesized using the solid-phase peptide synthesis (SPPS) strategy and following the Fmoc protocol on a 2-chlorotrityl chloride resin substrate. At each coupling stage, the amino acid addition was checked with the Kaiser test. After completing the synthesis with the side-chain protections and the amino-terminal still protected, the sequence was cleaved from the resin using a 1% solution of trifluoroacetic acid.

A total of 0.5 mmol of the fully protected nonapeptide with a free carboxylic end was dissolved along with 0.9 mmol of the coupling agent TBTU and 1.1 mmol of the corresponding ammonium salt in a flask containing 1.5 mL of 1-methyl-2-pyrrolidone solvent to amidate the sequence's end. After the mixture was stirred and all components had dissolved, three mmol of N-methylmorpholine base was added to the solution. Thin-layer chromatography (TLC) monitored the reaction's progress using a 1:2:10 ethyl acetate:methanol: water system. After five hours, water was added dropwise to the flask contents. The formed precipitate was separated by filtration and transferred to a vacuum oven after being washed again with water.

For the synthesis of the hydrazide derivative, after dissolving one mmol of the fully protected nine-sequence

(nonapeptide) with a free carboxylic end in methanol (3 mL), three mmol of thionyl chloride was added at -10 degrees Celsius, and the reaction mixture was stirred at room temperature for one day. After the reaction was complete at this stage and the carboxylic end was converted to a methyl ester, the reaction solvent was evaporated under vacuum, and the reaction residue precipitated in a mixture of methanol and diethyl ether solvents, then separated by filtration. In the subsequent step, a flask containing water (5 mL), 1.2 mmol of sodium carbonate, and one mmol of the amino acid methyl ester was stirred for 15 minutes. Following this period and after dissolution, one mmol of di-tert-butyl dicarbonate in 3 mL of acetone was introduced to the reaction mixture and stirred at room temperature for one day. Subsequently, the acetone was evaporated under vacuum, and the reaction residue was washed with ethyl acetate (20 mL) and petroleum ether (20 mL). After that, 50 mL of ethyl acetate was added to the reaction mixture, and the pH was adjusted to 4 using citric acid, followed by stirring the mixture for 20 minutes. After this period, the organic phase was extracted and washed with saturated brine until the pH was adjusted to 5. Following the dehydration of the resulting organic phase with magnesium sulfate and the evaporation of the solvent under vacuum, the product precipitated in the reaction vessel. In the next step, after dissolving the resulting Fmoc-Peptide(protect)-OMe (1 mmol) in methanol (5 mL), 3.5 mmol of hydrazide hydrate was added, and the reaction mixture was stirred at room temperature for one day. After the reaction was complete at this stage and the corresponding Fmoc-Peptide (protect)-NHNH2 synthesis, the reaction solvent was evaporated under vacuum, and the reaction residue was purified by washing with methanol.

Each millimole of the synthesized sequences with side-chain protecting groups was added to a flask containing 2 mL of a 25% piperidine solution in ethyl acetate to remove the Fmoc protection from the amino terminus of the sequence. The progress of the reaction was monitored by thin-layer chromatography (TLC) using a 1:2:20 ethyl acetate: methanol: water system. After 2 hours, the reaction was complete. The flask contents were transferred to a separatory funnel and extracted with water (4×10 mL). The aqueous phase containing the desired product was then freeze-dried and obtained as a powder.

The K reagent was utilized to remove side-chain protections. Specifically, the solid powder obtained from the previous step was introduced into a flask containing the K reagent mixture (20 mL trifluoroacetic acid, 2 mL water, and 0.3 mL triisopropyl silane). The mixture was stirred for 2.5 hours, after which the solvent was evaporated under vacuum until the solution's volume was reduced to 2 mL. Diethyl ether was gradually added to the residual contents. The resulting precipitate was then filtered off and dried.

2.3 RP-HPLC

The crude Lecirelin and the derivatives were purified using a KNAUER Advanced scientific instrument HPLC system (H. Knauer, Bad Homburg, Germany) with a preparative C18 column (100 Å, 10 µm, 250 mm × 20 mm). Linear gradient elution (0 min 5% B; 5 min 5% B; 100 min 100% B; 120 min 100% B) with eluent A (0.1% TFA in water) and eluent B (0.1% TFA in ACN/H_2O (70:30, v/v)) were used at a flow rate of 10 mL/min. Peak detection was done at 218 nm. The pureness of the compounds was proved with an Agilent 1200 HPLC system. A water C18 column (100 Å, 5 µm, 150 mm × 4.6 mm) was used as a stationary phase. A linear gradient elution (0 min 5% B; 5 min 5% B; 52 min 100%; 80 min 100%) was utilized at a 1 mL/min flow rate with the eluents above. Peak detection was performed at 218 nm.

2.4 Mass Spectrometry

The reaction product was identified using mass spectral data. An Agilent Triple Quadrupole LC/MS 6410 equipped with a Diode array detector, bin pump, and gas nebulizer, series 1200, and a mass spectrometer featuring electrospray ionization with a 3.5 kV voltage source and a 50 PSI gas nebulizer pressure was used.

2.5 Investigating the impact of Lecirelin and other synthesized derivatives on ovarian cancer cells

The MTT assay was utilized to assess the cytotoxic effects of synthetic peptides on cancer cells and ensure their non-toxicity towards healthy and normal cells. This assay briefly involves taking the targeted cancer cells from the nitrogen storage and culturing them in cell culture flasks with RPMI 1640 medium, supplemented with 1% penicillin-streptomycin (penstrep) and 10% fetal bovine serum. After the cells have proliferated, they are passaged, counted, and seeded at a density of 5000 cells per well in a 96-well cell culture plate. The plate is then incubated overnight in a carbon dioxide incubator (5%) at 37 degrees Celsius to fully allow the cells to adhere to the bottom of the microplate. A specific amount of the compounds (20-60 µg/mL) being tested (peptides) is dissolved in PBS buffer. The pH of each sample is measured and then adjusted to between 7.5 and 7.6 with sterile 1N sodium hydroxide solution. In the subsequent step, the buffer was used to adjust the volume of all samples to 1.5 mL. After preparing the cells, the desired peptide compound was added to each plate well in varying concentrations and then incubated (notably, each concentration was replicated across three wells to ensure a more precise outcome through averaging). The treatment with each sample

concentration was repeated three times at 24-hour intervals.
Twenty-four hours following the final treatment [11], the cells underwent microscopic and visual examination for cell death, followed by the execution of the MTT assay.
For the final step, the culture medium on the microplate was first removed, and the wells were washed with PBS buffer. Then, 100 microliters of RPMI 1640 medium without serum were added to each well of the microplate, followed by the addition of 10 microliters of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), a substance necessary for staining living cells. This setup was then incubated for 2 hours at 37 degrees Celsius. Afterward, the supernatant was removed, and the microplate was washed twice with PBS buffer. Then, 100 microliters of dimethyl sulfoxide (DMSO) solution were added to each well and shaken for 5 minutes to ensure the solution's color became uniform. Subsequently, the absorbance was measured at a wavelength of 570 nanometers using an ELISA reader [12].
The viability of the cells is determined by averaging the absorbance values obtained for each sample at every concentration. Higher absorbance indicates greater cell viability, while lower absorbance signifies increased cell death and a greater effect of the compound on the cells.

2.6 Statistical analysis

In the present study, all the experiments were performed in triplicate. Differences between the mean cell viability rate in each concentration of different compounds were analyzed using a one-way analysis of variance (ANOVA) and the Tukey-HSD test. Also, a one-way ANOVA test was performed to determine the differences in the mean cell viability rate in each compound between different concentrations. For the calculation of 50 lethal concentrations of each compound, Probit regression analysis. Moreover, data analysis was done using the SPSS statistical package (version 27.0) (SPSS Inc., Chicago, IL, USA). For all analyses, (p<0.05) was considered statistically significant.

3. Results & Discussion

3.1 Identification of the structure of Lecirelin derivatives

After purifying the compounds, mass spectrometry was employed to identify and confirm their structures.
The mass spectrum of the compound reveals that, for Lecirelin, the principal peak for $[M+H]^+$ occurs at m/z=1210.7117, while the peak for $[M+2H]^{2+}/2$ appears as the base peak (the tallest peak in the spectrum) at m/z=605.3711.

In pertains to the mass spectrum of the **M1** derivative, the main peak at m/z = 1196.5000 corresponds to $[M+H]^+$, and the base peak appears at m/z = 598.9000, related to $[M+2H]^{2+}/2$. Furthermore, the mass spectrum of the **M2** derivative shows two peaks at m/z = 1181.6000 and m/z = 673.0000, corresponding to $[M+H]^+$ and $[M+2H]^{2+}/2$, respectively. In the mass spectrum of the **M3** derivative is presented, where the principal peak with a mass-to-charge ratio of 1249.6000 relates to $[M+H]^+$, and another peak at m/z = 625.5000 is associated with the $[M+2H]^{2+}/2$ base peak. All spectra confirm the synthesized structures (Figure 1).

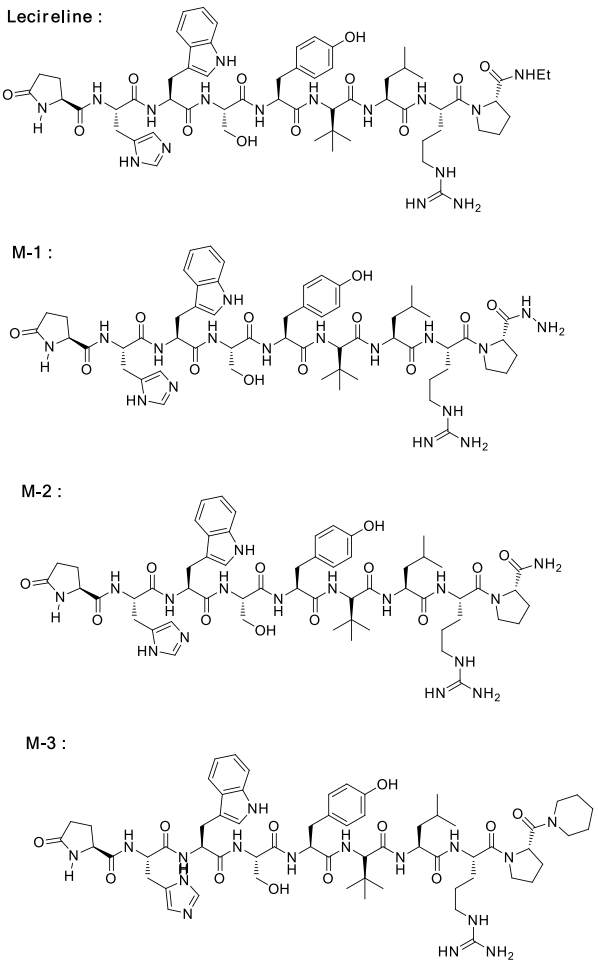

Figure 1. Sequence and structure of Lecirelin and its

3.2 MTT Assay Results

This research project aimed to design and synthesize new derivatives of Lecirelin to enhance its efficacy. To achieve this, after conducting studies on this class of compounds, changes were made in the end substituent, alterations in the stereochemistry of the amino acids used

in the structure, and modifications in the steric hindrance of the structure, leading to the design of three new derivatives. These structures were synthesized and purified; the accuracy of their synthesis was examined by mass spectrometry. After conducting control tests, their efficacy in ovulation (article under review) and their impact on two ovarian cancer cell lines (A2780 and A2780 CP) and one healthy fibroblast cell line (Hu02) were evaluated.

Here, the assay was conducted on the A2780 ovarian cancer cell line (sensitive to cisplatin), the A2780 CP (cisplatin-resistant), and the fibroblast cell line (HU02) as a healthy cell model. A2780 is an ovarian cancer cell line derived from an untreated patient's endometrioid adenocarcinoma tumor.The cell viability assay results indicate that the Lecirelin sample and its derivatives had no effect on healthy fibroblast cells.

3.2.1 Cell Line A2780

In concentrations 50 and 60 µg/mL, no significant difference was seen in terms of cell survival percentage between concentrations of 50 and 60 µg/mL in all four compounds. However, the cell survival percentage at 60 µg/ml was lower than at 50 µg/mL. As mentioned in the article, the survival rate of cancer cells was below 20% (16.55% and 13.32%) using 50 and 60 µg/mL of compound **M1**, respectively.

Among the different compounds at a concentration of 50 µg/ml, the **M1** compound had a significant difference with all three other compounds ($p<0.05$), and the two compounds, **M2** and **M3,** had no significant difference in cell viability. Except for compound **M1**, compound L showed the lowest cell viability among others.

The **M1** compound had a significant difference with all three groups ($p<0.05$), and the **M2** and **L** compounds did not have significant differences with each other using a concentration of 60 µg/ml. The M3 group had the highest survival rate in this dose.

3.2.2 Cell line A2780 cp

In concentrations of 50 and 60 µg/mL, there was a significant difference in cell viability in all four compounds between concentrations of 50 and 60 µg/mL ($p<0.001$). Moreover, at the 60 µg/mL concentration, the cell survival percentage was lower than the 50 µg/mL concentration.

Among the different compounds at a concentration of 50 µg/ml, only the M1 group showed a significant difference from the other groups ($p<0.001$), and there was no difference between the other groups in terms of cell viability.

In concentration 60 µg/mL, the **M1** compound had a significant difference with all three groups ($p<0.01$), and the **M2** and L compounds did not have significant

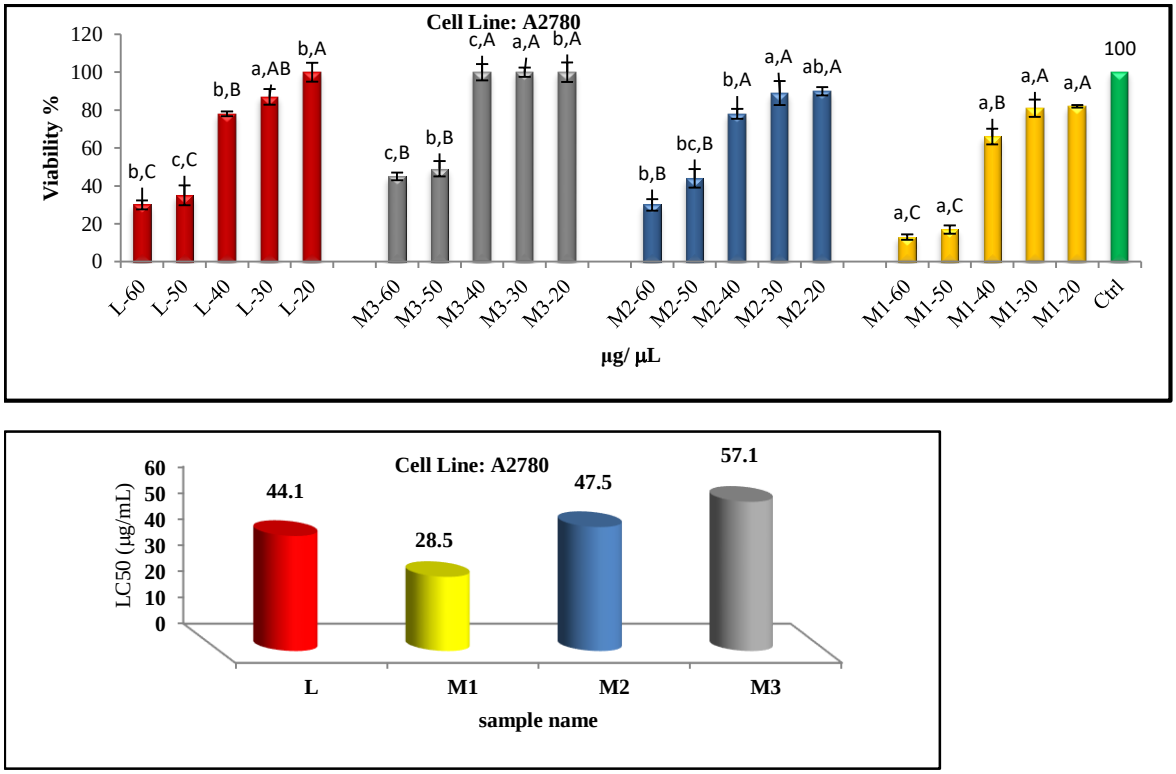

Figure 2. (A) Effects of treatment with different concentrations of the Lecirelin (**L**-Red column) and its derivatives (**M1**-Yellow column, **M2**-Blue column, and **M3**-Gray column) on the A2780 cell line. Individual columns represent the cell number percentage compared to the control (Green column). (B) Determination of the IC50 value on the A2780 cell line.

differences with each other. The **M3** group had the highest survival rate in this dose (Figures 2 and 3).

The decapeptide structure of the GnRH hormone was explored by the research team of Dr. Schally in 1971 [10]. Afterward, it was found that this peptide was involved in reproductive function control [11]. More investigations unveiled that GnRH receptors were present in melanomas [27], brain [28], lung [29], pancreas [30], and gynecological cancers [31]. The selectively expressing GnRH receptors ideally target novel treatment modalities with minimum aftereffects. Research has shown that GnRH analogs can manage advanced reproductive system tumors that are not treatable with chemotherapy [32,33]. Additionally, tumors outside the reproductive system, e.g., melanomas that possess GnRH receptors, can be managed using these compounds [5].

According to earlier studies, the development of ovarian cancer may be stimulated or inhibited by steroid hormones secreted from the hypothalamic-pituitary-ovarian axis. Research indicates the expression of hormonal receptors in the cancer cells of ovaries, thereby mediating the growth-stimulating or inhibitive impacts of hormones on these cells. Some clinically conducted trials have investigated hormonal therapy agents. Medium effectiveness and limited aftereffects were documented in most of the trials focusing on patients with recurring or resistant ovarian cancer [34].

The goserelin remedial usage in benign gynecological disorders (dysfunctional uterine bleeding, endometriosis, fertility disorders, and uterine leiomyomata) has been surveyed in an extensive review by Perry and Brogden [35, 36]. A study by Zhang et al. demonstrated that goserelin enhanced cellular apoptosis in epithelial ovarian cancer (EOC) across three cell lines: A2780, SKOV3, and SKOV3-ip.

Additionally, goserelin exhibits anticancer activity in prostate and breast cancer treatment, as well as benign gynecological conditions [37].

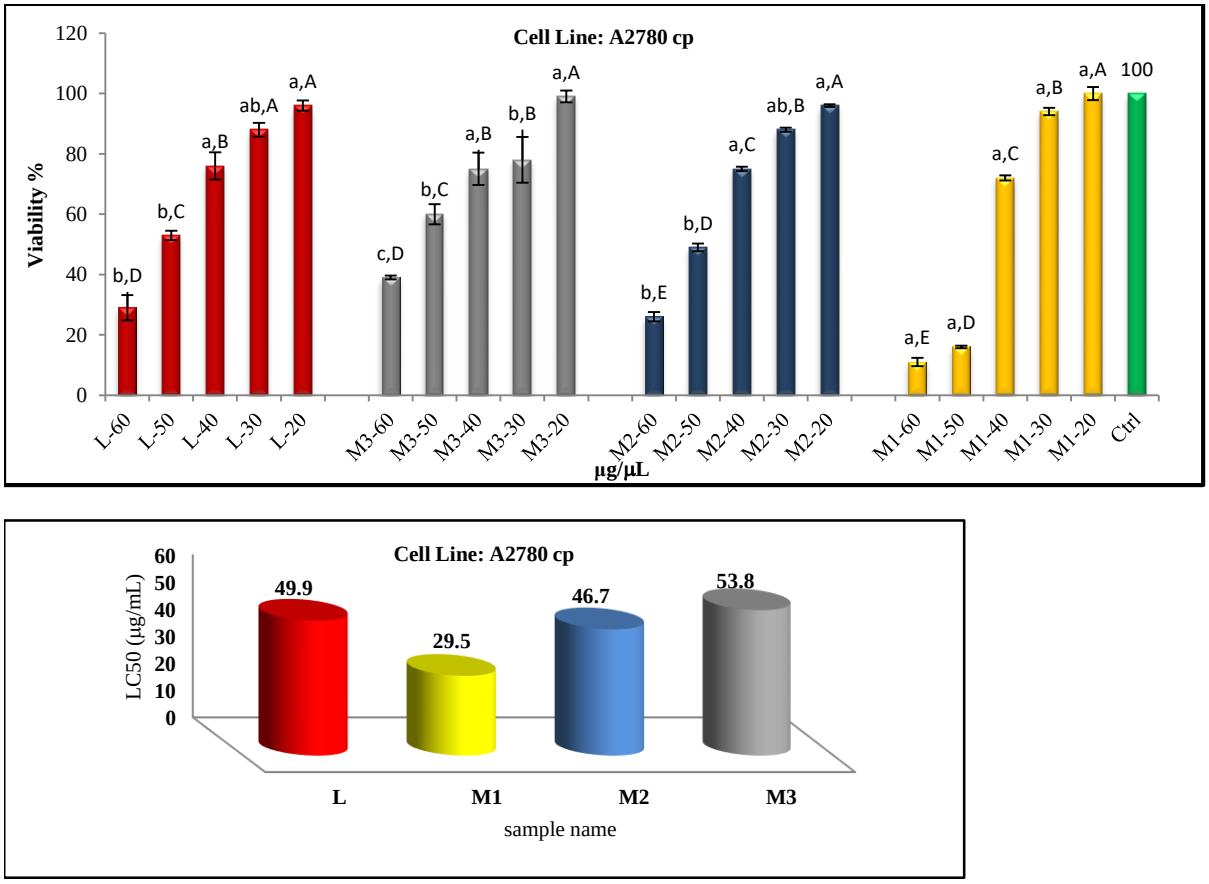

Figure 3. (A) Effects of treatment with different concentrations of the Lecirelin (**L**-Red column) and its derivatives (**M1**-Yellow column, **M2**-Blue column and **M3**-Gray column) on the A2780 cp cell line. Individual columns represent the cell number percentage compared to the control (Green column). (B) Determination of IC50 value on A2780 cp cell line. *Values are means±SD of three replicates. [a-d] Within each concentration column marked with different letters (lowercase) are significantly different between compounds (ANOVA, Tukey's HSD test, $p<0.05$). [A-E] Within each compound column marked with different letters (uppercase) are significantly different between concentrations (ANOVA, Tukey's HSD test, $p<0.05$).

Leuprolide is a GnRH peptide analog that is usable as an alleviative therapy for prostate cancer and other certain disorders [38].

M1 derivative with hydrazide end with lC_{50}= 28.5, 29.5 µg/mL can kill half of A2780 and A2780 cp cancer cells, respectively (Figures 2 and 3). Meanwhile, Lecirelin can destroy half of the cells at 44.1 and 49.9 µg/mL concentrations, which require about twice more active ingredients. Furthermore, two other derivatives with amino and cyclohexyl ends need to use much higher amounts of the sample to destroy half of the cancer cells. The lower the amount of the active ingredient used, the less likely the dose will be prescribed for patients, resulting in fewer aftereffects.

An exact comparison cannot be made since the effect of Lecirelin and its analogs has not so far been investigated on ovarian cancer cells.

Nevertheless, these results demonstrate that this compound and its hydrazide derivative are effective in this category of cancer cells; hence, these compounds can be further studied in the future.

The results demonstrate a dose-dependent pattern in which the treatment of cells by increasing the Lecirelin concentration leads to a higher percentage of cancer cell eradication.

4. Conclusion

Since treatment of the ovarian cancer cells with lower concentration (<50 µg/µL) of the new derivatives of Lecirelin does not affect cell viability, we conclude the GnRH receptors on these cells have low affinity, and they will activated using high concentrations of GnRH agonists.

In addition, by altering the acidic tail sequence of lecithin from ethylamide to hydrazide, the survival rate of cancer cells at a concentration of 60 micrograms per well decreased from over 30% to 14%. This effect is likely due to the increased polarity resulting from the modification at the sequence's end, aligning with findings from Soderhall's studies. Specifically, incorporating such functional groups in the new analog's sequence enhances its protease stability, thereby boosting its biological activity [21]. Given that no studies have yet examined the effects of lecithin on ovarian cell lines, lecithin, and its amide derivative can be investigated as candidate drugs for the treatment of ovarian cancer.

All research in this area requires extensive studies and the determination of impactful parameters such as toxicity, dosage, and mode of application. These results assist in refining future research in this field, leading to the design of newer compounds with higher efficacy in fertility and combating the progression and proliferation of ovarian cancer cells.

Acknowledgments

None.

Conflict of interest

There is no conflict of interest.

Ethics

None.

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