Cyclosaplin analog peptides design and analysis of their cytotoxicity effects on breast cancer cell line (MDA-MB-231) and human leukemia cell line (K562)

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Research Article

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Abstract

Objective: It was the aim of this study to develop Cyclosaplin analogs and assess the anticancer effects of those analog peptides on MDA-Mb-231 as well as K562 cell lines. The analogs of Cyclosaplin peptide (Cyclosaplin-2A and Cyclosaplin-7G) were designed and then investigated by online web server predictor AntiCP. The analog peptides were applied to MDA-MB-231 and K562 cells in various concentrations and for various periods of time. The anticancer potential was confirmed by the MTT assay. Hemolytic activity also was assessed. In order to investigate the apoptotic effects of peptides on cancer cells, various tests such as morphological examination, Giemsa test, and DNA fragmentation were performed. Lactate dehydrogenase leakage also was examined to confirm the effects of analogs.

Results: Our experimental and computational data show that the analog peptides have anticancer potential. The MTT assay and morphological study confirmed the anticancer effects. Other tests also confirmed the anticancer effect of the analog peptides. According to hemolytic assays, none of the analog peptides possess any hemolytic activity against human erythrocytes, indicating the compounds are not toxic for normal cells. Analog peptides found in this study were shown to have anticancer potential on two human cancer cell lines.

Introduction

One of the leading causes of death worldwide has long been cancer, which is regarded as a severe health problem [1–3]. Cancers have been identified so far, including the most common ones such as Bladder, Breast, Colon, Rectal, Leukemia, Lung, Lymphoma, Prostate, and so forth [3–5].

A number of factors contribute to the formation of carcinogens, including lifestyle, chemical exposure, heredity, genetics, immune system disorders, and environmental circumstances [6, 7].

Every treatment method is limited in its application to specific organs, stages of cancer progression, and medical conditions of the patient [8–10]. One of the common procedures by delivering a cytotoxic substance to the cancer cells is Chemotherapy [8, 11]. The major problem with conventional chemotherapy is the inability to separate cancer cells from normal cells to deliver the cytotoxic agent to them and kill not only cancer cells but also normal cells [2, 9, 10]. Other disadvantages of these methods include high costs, long treatment cycles, drug resistance, altered biodistribution, and difficulty eliminating chemicals [10, 12–14]. Current cancer treatment methods and their complications have led scientists to search for a more effective medicine and approach to defeat cancer [15, 16].

The discovery of anticancer peptides (ACPs), a type of short peptide generally has led to the emergence of a novel alternative treatment for cancer [13, 15, 17]. Biologically-based natural substances are more target-specific and have fewer side effects than conventional cancer therapies [13, 15]. A cationic quality of ACP, due to the negative charge on cancer cell membranes, makes them unique for binding the cancer cells compared to others. Amphipathic and hydrophobic properties of anticancer peptides are vital for Specific penetration into cancer cell membranes [4, 18–21]. In this study, we focused on designing an

anticancer peptides with a high level of anticancer potential to change and improve some of its features to achieve the novel anticancer peptides. As a result, Cyclosaplin, a cyclic peptide with eight amino acids (RLGDGCTR) which Abheepsa Mishraa et al. (2014) purified from somatic seedlings of Santalum album L. was used as a framework peptide [22].

Materials And Methods

Bioinformatic prediction

In this study, the in silico model, AntiCP server (webs.iiitd.edu.in/raghava/anticp/submission.php) [23] was used to predict and design ACPs before synthesis and study on certain cancer cells. Several physiochemical features including charge, hydrophobicity, hydrophilicity, amphipathicity and molecular weight of parent peptide were considered for designing the analogs.

In order to understand the properties of the new peptide sequence, it was submitted. Then, the server displayed the result as 'ACP' or 'non-ACP' along with the prediction score and physiochemical properties.

Peptide preparation

Cyclosaplin-2A and Cyclosaplin-7G peptides (2A and 7G) were synthesized by TAG Copenhagen company (Denmark, UK). HPLC (250 × 4.6 mm C18 column) was used to purify over 95% of the synthesized peptides. The peptides were eluted in acetonitrile/water mixed with 0.1% trifluoroacetic acid from 5–95% at a flow rate of 1 ml/min. The peptides were further characterized by mass spectrometry.

Cell cultures

MDA-MB-231, human breast cancer cells, and K562, human leukemia cells were obtained from the Pastor Institute in February 2018 (Iran, Tehran). Cells were cultured according to the cell culture protocol [24]. Both cells were seeded in 96-well microtiter plates at a density of 5×10^4 cells/ well.

MTT assay

Cell viability count and cytotoxicity of ACPs were performed using MTT assay [25]. Briefly, cells were exposed to the five various concentrations (10, 15, 50, 100, 150 µg/ml) of 2A and 7G in each plate, followed by incubation for various periods. Plates were read at 570 nm using a ELISA analyzer (ELISYS UNO). The results were expressed as IC_{50}, representing the concentration at which cell viability was reduced by 50%.

Determination of cells’ morphology

5×10^4 cells/well MDA-MB-231 and K562 cells were cultured and incubated for 24 h. Then, both cells were treated with the IC_{50} concentration of 2A and 7G for 48 h. After incubation, treated and control MDA-MB-231 and K562 cells were collected to observe morphological changes of cells related to the apoptotic effects under inverted microscopy.

Giemsa staining

Giemsa staining was also performed cytologically to assess the apoptotic morphology to evaluate the apoptosis of cell death in MDA-MB-231 and K562 cells. Treated cells and Control cells were collected and washed according to the Giemsa Staining method [24]. The morphological changes of apoptosis cells were observed by light microscopy.

Hemolytic Assay

The hemolytic examination was aimed to assess whether 2A and 7G caused oxidative damages to the erythrocyte membrane or not. Erythrocytes suspension is prepared according to the protocol [26]. The first well served as a negative control containing only solvent and the last served as a positive control containing 20 µL of 0.1% Triton X-100 in 0.85% saline. Finally, the absorbance of the supernatant was noted spectrophotometrically at 560 nm. The average value was calculated from triplicate assays.

Lactate Dehydrogenase (LDH) leakage assay

The growth inhibitory effects of 2A and 7G were further investigated through LDH into the culture medium upon damage to the plasma membrane. Treated cells with IC_{50} concentration of each peptide and without peptides (negative control) were incubated for 48 hours according to the protocol [27]. The absorption of each well at 490 nm was then measured by a ELISA analyzer.

DNA laddering assay

Untreated and treated both cells with 2A and 7G were extracted using a DNA extraction kit (QIAwave DNA Blood & Tissue Kit) according to the manufacturer's protocol. Cells extracted DNA along with 100 bp DNA ladder was loaded on to 1.5% agarose gel. The gel was visualized under the Gel Doc system to determine DNA fragmentation.

Statistical Analysis

Each test in the in vitro anticancer assay was carried out in triplicate and the results were expressed as mean ± SEM. Hemolytic test analysis was carried out using one-way ANOVA.

Results

Synthesis of designed peptides

The development of novel ACPs effects are extremely time-consuming and often expensive, so computational methods are absolutely essential before applying them clinically. As a result, due to the importance of ACPs, we have improved the quality of identification in the study using a strong AntiCP predictor (https://webs.iiitd.edu.in/raghava/anticp/). Based on amino acid composition and binary profiles, AntiCP develops support vector machine models (SVMs) to predict ACPs.

In this study, as part of the process of designing the analogs of Cyclosaplin, much attention was paid to physicochemical properties of the peptides such as hydrophobicity, hydrophilicity, amphipathicity, charge, etc. As a result, in one analog of the peptide, the amino acid lysine was substituted with an alanine at position 2 (RAGDGCTR) to increase hydrophobicity, whereas, in another analog, the amino acid tyrosine was substituted with the amino acid glycine at position 7 (RLGDGCGR). A linear structure was also introduced for analog peptides, rather than a cyclic format.

All the physicochemical properties of the Cyclosaplin and its analogs were evaluated (Table 1.). Despite the changes made to the parent peptide, it was found that the major physicochemical properties of the parent peptide and its analogs are very similar based on the results of the AntiCP predictor server.

Table 1
Characteristics of both analog peptides as compared to the parent peptide

Peptide	**Parent**	**2A**	**7G**
Sequenc	RLGDGCTR	RAGDGCTR	RLGDGCGR
No of residues	8	8	8
Format	Cyclo	Linear	Linear
AntiCP predictor	Anticp	Anticp	Anticp
Hydrophobicity	-0.44	-0.48	-0.40
Hydrophilicity	0.73	0.89	0.78
Amphipathicity	0.61	0.61	0.61
Charge	+ 1	+ 1	+ 1
Mr(Da)	877.09	835.00	833.04

RP-HPLC was used to purify the synthetic peptides, and MALDI-MS was used to confirm their molecular weights. Hydrophobic-hydrophilic balances of amphipathic peptides have also been quantitatively determined via RP-HPLC frequently. For amphipathic specifications, according to the TAGC Company's suggestion, a ratio of 1 to 1 of water and acetonitrile is an appropriate solution for these peptides.

MTT assay

The MTT assay was performed on both cells to measure the cytotoxicity of 2A and 7G. The MTT assay indicated that the cell viability decreased with increasing peptide concentration for both peptides (Fig. S1.).

In order to assess the anticancer activity of the analog peptides, we calculated the IC_{50} values of two cancer cell lines after 48 hours of incubation. On MDA-MB-231 cells with a concentration of 70 µg/ml of 2A and 90 µg/ml of 7G, and with a concentration of 10 µg / ml of 2A and 15 µg / ml of 7G on K562 cells

in 48 hours, less than 50% of the cells were viable (Fig. S2.). Other tests were conducted on these concentrations.

Determination of cells' morphology

An inverted microscope showed many distinct morphological changes in both 2A and 7G treated cells compared to untreated. Apoptotic cell signaling manifests itself in terms of membrane wrinkling, cytoplasm condensation, cell degradation (change from a spindle or round shape to a wrinkled shape), and slow growth and progression of cells in the vicinity of the peptide drug. The morphological changes demonstrated that concentration and time are likely to affect cell morphology (Fig. S4. and Fig. S5.)

Giemsa staining

As seen with Giemsa staining, in MDA-MB-231 and K562 cells treated with IC_{50} concentration of 2A and 7G, nuclei with chromatin condensation, cell size depletion, disruption of membrane integrity, and formation of apoptotic bodies were detected by a light microscope (Fig. S6. And Fig. S7.).

Hemolytic Assay

An RBC hemolysis test was used to determine if analog peptides were capable of damaging membranes. 2A and 7G, Triton X-100 (positive control) and solvent (negative control) demonstrated differential anti-hemolytic activity. An analysis of the hemolytic activity of analog peptides at their highest IC_{50} concentrations illustrated neither 2A nor 7G ($P > 0.005$) exhibited potent anti-hemolytic action compared to the solvent. In contrast, Triton X-100 as a strong hemolytic substance illustrated most hemolytic activity ($P < 0.006$) (Table S1).

Lactate Dehydrogenase (LDH) leakage assay

Due to the fact that damaged cells are fragmented completely after prolonged incubation with drug substances, LDH has been shown to act as a reliable biomarker of patient response to treatment in cancer patients. In this study, the percentage of LDH leakage from the treated cells increased as a function of dose compared to the control cells in 48 hours. The amount of LDH produced by MDA-MB-231 cells exposed to 2A at IC_{50} concentration was 76.6%, and 7G was 74.5%. While, for K562 cells exposing of 2A at IC_{50} concentration was 59% and 7G was 56% (Fig. S3.).

DNA laddering assay

Further investigation into the apoptotic pathway was carried out by determining the DNA fragmentation assay. The results of the study reveal that MDA-MB-231 and K562 cells samples treated with 2A or 7G showed significant inter nucleosomal fragmentation. In Fig. 1, it can be seen that the DNA ladder was created following exposure to 2A and 7G at IC_{50} concentration.

Discussion

Two analog peptides were designed and investigated in this study. Although the parent peptide has undergone some changes, the analogs were found to also possess anticancer properties despite being short in length. The amino acid content of a peptide influences its bioactivity. Therefore, we examined how new amino acids alter the function of analogs and how they impact cancer cells. Unlike the parent peptide, analogs were synthesized linearly and not in a cyclic form. The first step after synthesis was the dissolution of analogous peptides in water and organic solvents (acetonitrile), which showed that they were amphipathic like their parent. The MTT result to determine the IC_{50} value was clearly different between analogs and their parent, which was probably due to the linear structure of analogs and the amino acid substitution. The possibility that the analogs would be toxic to normal cells was ruled out by assessing their efficacy on normal cells through hemolytic assays. In this study, Giemsa staining confirmed appoptisis changes, while scanning electron microscopy verified this for parent peptides.

As a whole, MDA-MB-231 cells reacted more favorably with the parent peptide than its analogs. In addition, of the two analogs, 2A exhibited a greater cytotoxicity effect than 7G on both cancer cells. Further enhancement of analogs anticancer activity may be achieved by modifying peptide properties such as their hydrophobicity, structure or net charge.

Conclusions

Analog peptides, in particular 7A, showed anticancer properties against MDA-MB-231 and K562 cells. A series of tests confirmed the effect of peptides on cancer cells. Additionally, the hemolytic test confirmed these peptides' ineffectiveness against normal cells.

Limitations

Additional tests required to detect apoptosis and preapoptosis are unable to be performed due to a lack of equipment.

Abbreviations

ACP, Anticancer peptide; 2A, Cyclosaplin-2A; 7G, Cyclosaplin-7G

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

Data is available from corresponding authors upon request.

Competing interests

The authors declare that they have no competing interests.

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Authors' Contributions

Parisa Kadkhodaei Elyaderani: Writing - Original Draft, Conceptualization, Methodology, Validation. Elham Abbasi: Formal analysis. Ali Mohammad Asgharian: Supervision.

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Informed consent

All authors have read and approved the contents and manuscript.

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Figures

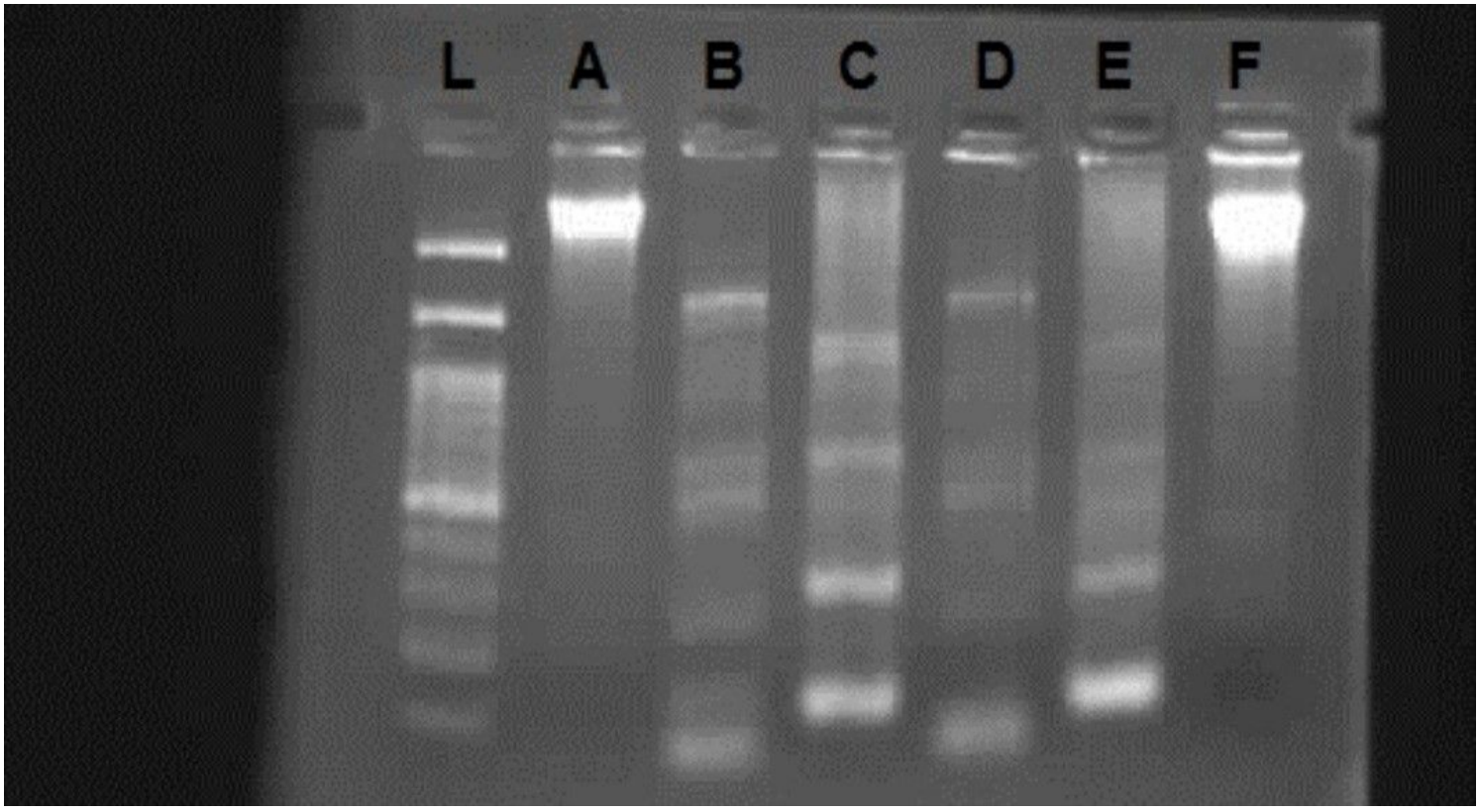

Figure 1

DNA laddering assay. L) 100 bp ladder. A) MDA-MB-231 cells DNA (untreated cells). B) Treated MDA-MB-231 cells by IC_{50} concentration of Cyclosaplin-2A. C) Treated MDA-MB-231 cells by IC_{50} concentration of Cyclosaplin-7G. D) Treated K562 cells by IC_{50} concentration of Cyclosaplin-2A. E) Treated MDA-MB-231 cells by IC_{50} concentration of Cyclosaplin-7G. F) K562 cells DNA (untreated cells)

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