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Fusion Proteins in the Fight against Colorectal Cancer: Assessing the Cytotoxic Potential of Recombinant Nisin-Arginine Deiminase Fusion Protein

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ABSTRACT

Background: A promising strategy in cancer therapy involves the production of fusion proteins, which entail the fusion of two distinct proteins. This study aimed to produce and assess the cytotoxic effects of the Nisin-arginine deiminase (ADI) fusion protein on the SW480 cell lines, a common model for studying colorectal cancer (CRC).
Materials & Methods: The designed Nisin-ADI gene fragment sequence was sent to Biomatik Company for synthesis in pET-28a vectors between SacI and HindIII restriction enzyme sites. *Escherichia coli* (*E. coli*) DH5α and BL21 were utilized for cloning and protein expression, respectively. The recombinant fusion protein expression was induced by Isopropyl ß-D-1-thiogalactopyranoside (IPTG) and purified using Ni2+-nitrilotriacetic acid [1] resin affinity chromatography. Sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting were conducted to analyze the purified protein. The cytotoxic effect of the purified recombinant fusion protein on SW480 and NIH3T3 cells, as a control, was evaluated using 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay.
Findings: The results of this study showed that the fusion protein had a significant impact on the SW480 cell lines. The Half-maximal inhibitory concentration (IC50) of the fusion protein was 30 µg/mL, indicating that it effectively inhibited the growth of cancer cells. However, the fusion protein did not significantly affect the control group.
Conclusion: This study provides helpful insights into the potential application of recombinant Nisin-ADI fusion proteins as a potential treatment option for colorectal cancer. The potential for selective targeting of cancer cells is promising as normal cells are unaffected by this fusion protein.

Keywords: Nisin, Arginine deiminase, Fusion protein, Colorectal cancer, Cytotoxicity

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Introduction

Colorectal cancer (CRC) is the third most prevalent cancer among both men and women, with approximately 1.93 million new cases and 935,000 deaths in 2020 [2]. It is estimated that in 2023, around 52,550 people will die from colorectal cancer in the United States [3]. Early detection and treatment of colorectal cancer can increase survival chances. While various treatment methods, including surgery, chemotherapy, radiation therapy, and hormone therapy, are employed to combat cancer, conventional anticancer treatments often fall short of complete remission.

Moreover, it has been observed that radiation or chemotherapy frequently results in significant side effects, and drug resistance is commonly observed [4,5]. Consequently, exploring new anticancer approaches and substances with minimal side effects has become critical. In recent years, researchers have directed their attention toward utilizing live or degraded bacteria, their products, or compounds derived from bacteria for treating certain malignancies. Within this context, certain bacterial proteins and peptides have demonstrated antimicrobial and cytotoxic effects on cancer cells, positioning them as promising bioactive compounds and potential anticancer agents [6,7].

In this regard, various studies have reported that Nisin and arginine deaminase (ADI) proteins separately have potential anticancer properties [8-10].

Therefore, combining these two proteins to create a novel anticancer compound for evaluating its effects on cancer cells becomes a compelling pursuit.

Recombinant protein fusion represents an approach that has been proposed for targeting tumors using bacterial proteins and peptides. These fusions hold the potential to serve as novel and potentially effective agents against various cancers. Such treatment modalities offer the advantage of a higher therapeutic index and lower toxicity compared to conventional treatments. Researchers can generate a more potent compound that can effectively target and combat cancer cells by fusing two proteins.

This increased potency allows for using smaller doses, thereby minimizing the side effects of conventional treatments. Nisin, a polycyclic antimicrobial cationic peptide composed of 34 amino acids and produced by *Lactococcus lactis* subspecies *lactis*, has demonstrated selective cytotoxic effects on certain cancer cell lines and has been shown to induce apoptosis. Nisin's mechanism of action involves binding to the cell membrane, leading to membrane permeabilization, the release of intracellular contents, and apoptotic cell death [11,12].

On the other hand, clinical investigations have extensively examined the mechanism of cancer cell death resulting from arginine deprivation using ADI-degrading enzymes. ADI, an enzyme derived from bacteria, can convert arginine into citrulline and ammonia. Some tumors, such as melanoma and hepatocellular cancer, depend on the amino acid arginine (auxotrophic arginine). Thus, enzymatic degradation of extracellular arginine presents a potential therapeutic approach for various arginine auxotrophic tumors [13]. ADI has been studied as a potential antitumor drug for treating arginine-auxotrophic tumors, such as hepatocellular carcinomas (HCCs), melanomas, and colorectal cancer [13-15].

Objectives: Given the importance of the recombinant fusion protein in triggering toxicity in cancer cells, the objective of this study was to design and produce a recombinant Nisin-arginine deaminase fusion protein and evaluate its toxicity on the SW480 colorectal cancer cell lines in comparison to the NIH3T3 control cells.

Materials and Methods

Bacterial strains and materials: *Escherichia coli* (*E. coli*) DH5α for cloning, *E. coli* BL21 (DE3) for protein expression, and SW480 cell-lines, as well as NIH3T3 (mouse embryo fibroblast cells), were purchased from Pasteur Institute of Iran, Tehran, Iran. The Ni-NTA purification resin was purchased from QiagenTM, South Korea. TAKARA, Japan, provided the SacI and HindIII restriction enzymes. Antibiotics and Anti-poly-histidine antibody-HRP were bought from Sigma-Aldrich, Germany. Isopropyl-β-D-1-thiogalactopyranoside (IPTG) was purchased from Merck, Germany. All chemicals were purchased from reliable suppliers and used as recommended by the manufacturer. A Plasmid DNA extraction kit was obtained from Bio Basic, Canada.

Bioinformatics evaluations: Bioinformatics evaluations were performed to determine the characteristics of the fusion protein. The sequences of Nisin and ADI were retrieved from the NCBI website, and a flexible fusion protein was created by linking the two proteins with the (Gly-Gly-Gly-Ser) x2 sequence. Online software such as Raptor X and Protparam, as well as ChimeraX software [16], were employed to optimize the codons and determine the structure and characteristics of the fusion protein. The resulting gene fragment sequence was synthesized in pET-28a vectors between SacI and HindIII restriction enzyme sites by Biomatik Company (Canada).

Cloning, Expression, and purification of the recombinant protein: Competent *E. coli* DH5α cells were used to transform the recombinant pET28a-*Nisin-adi* plasmid through heat shock. After overnight culturing in Luria Bertani (LB) broth medium containing 100*ng/µl* ampicillin, bacterial cells were harvested by centrifugation, and plasmids were extracted by the plasmid extraction kit (Bioneer, Korea). Plasmid DNA concentration was measured by NanoDrop (Thermo Scientific, USA). The purified plasmids were then transformed into *E. coli* BL21 expression hosts. Transformed cells were then plated onto LB-ampicillin plates and grown overnight at 37 °C. Plasmid DNA was extracted from the transformed colonies and checked for the presence of the insert by double digestion with restriction enzymes. The positive clones were then isolated and stored for further use. IPTG then induced expression of the recombinant protein at 0.5 M final concentration for 4 hours at 37 °C. Ni2+-NTA resin (Invitrogen, USA) affinity chromatography purified the recombinant fusion protein. SDS-PAGE and Western blotting with an anti-his-tag antibody analyzed the purified protein. The Bradford protein assay was used to measure protein concentration, with bovine serum albumin serving as the standard**.**

Cell Lines and Culture: SW480 cells, a human cell line derived from a primary colorectal adenocarcinoma, as well as NIH3T3 cells, were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37°C and 5% CO2.

Antiprolifrative effects evaluation: To evaluate the antiproliferative effects of purified recombinant Nisin-ADI, SW480 cells were seeded at a density of 10,000 cells per mL in 96-well microplates and incubated at 37°C for 24 hours to allow for cell adhesion. Subsequently, the culture medium was replaced with 100 µL of fresh medium containing varying concentrations of Nisin-ADI (10, 20, 30, 40, 50, 75, and 100 µg/mL). The cells were then further incubated for 24 hours at 37°C. Following each incubation period, the cells were gently washed with phosphate-buffered saline (PBS) to remove any residual Nisin-ADI.

To assess cell viability, 5 mg of MTT [3-(4,5 dimethylethiazol-2-yl) 2,5 diphenyl

tetrazolium bromide] (Sigma, Germany) was added to each well, and the microplates were incubated for an additional 2 hours. Subsequently, 100 µL of dimethyl sulfoxide (DMSO) was added to dissolve the blue formazan crystals formed by viable cells. The absorbance was measured at 570 nm using an ELISA plate reader. All concentrations were tested in triplicate, and the optical density values obtained were averaged.

To determine the half-maximal inhibitory concentration (IC50) of Nisin-ADI, dose-response curves were generated using the optical density values. These IC50 values represent the concentration of Nisin-ADI required to inhibit cell proliferation by 50%. Additionally, NIH3T3 cells were subjected to the same experimental procedures as a control to compare the cytotoxic effects of Nisin-ADI on different cell lines.

The obtained IC50 values were compared between SW480 cells and NIH3T3 cells to assess the selectivity and potential toxicity of Nisin-ADI towards cancerous versus non-cancerous cells. These experiments were conducted in triplicate to ensure the reliability of the results.

Statistical analysis: The study results were statistically analyzed using SPSS V. 18 to compare the two data sets and determine if there were any significant differences using Student's t-test statistics. P values less than 0.05 were considered statistically significant.

Findings

Bioinformatic analysis: The fusion protein structure prediction suggested that the linker would effectively separate the two proteins, allowing them to carry out their functions (Figure 1) independently. Biochemical characterization of the fusion protein provided additional insights, including its molecular weight of 54417 Daltons and an isoelectric point of 6.32. Moreover, the fusion protein exhibited an aliphatic index of 84/11. The estimated half-life was 30 hours in mammalian reticulocytes in vitro, over 20 hours in yeast in vivo, and 10 hours in *E. coli* in vivo.

Production and confirmation of the recombinant fusion protein: The optimal expression conditions were determined to be an IPTG concentration of 0.5 mM, a temperature of 37°C, and an incubation time of 4 hours post-induction. To assess protein expression, both IPTG-induced and non-induced samples (serving as the negative control) were subjected to analysis using SDS-PAGE. Following staining with Coomassie Brilliant Blue 250-R, a distinct band corresponding to the recombinant Nisin-ADI fusion protein was observed. The molecular weight of the recombinant fusion protein was determined to be 54.5 kDa. The presence of the induced band at approximately 54.5 kDa aligned well with the anticipated molecular weight. Given the presence of a poly-histidine sequence at the C-terminus of the protein, a nickel affinity chromatography column was employed for protein purification. Confirmation of the protein production and purification process involved conducting electrophoresis and observing the presence of a distinct and pure band corresponding to the desired protein size. This unambiguous detection of a single band provides strong evidence for the successful production and purification of the protein. Western blot analysis was employed to assess the generated fusion protein's antigenicity. The purified protein was subjected to electrophoresis using SDS-PAGE gel and subsequently transferred onto a nitrocellulose membrane. Notably, a distinct brown strip corresponding to the location of the recombinant protein was observed on the nitrocellulose membrane. This observation confirms the specific reaction between the fusion protein and the

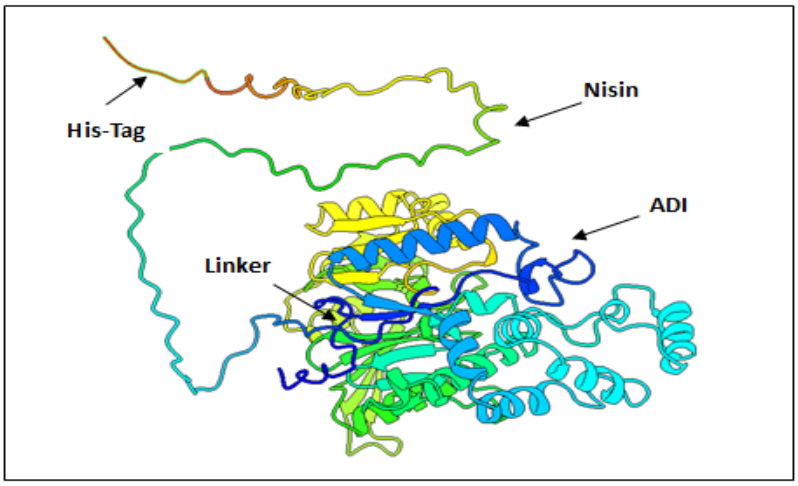

Figure 1) ChimeraX software predicted 3D structure of recombinant Nisin-ADI based on amino acid sequence

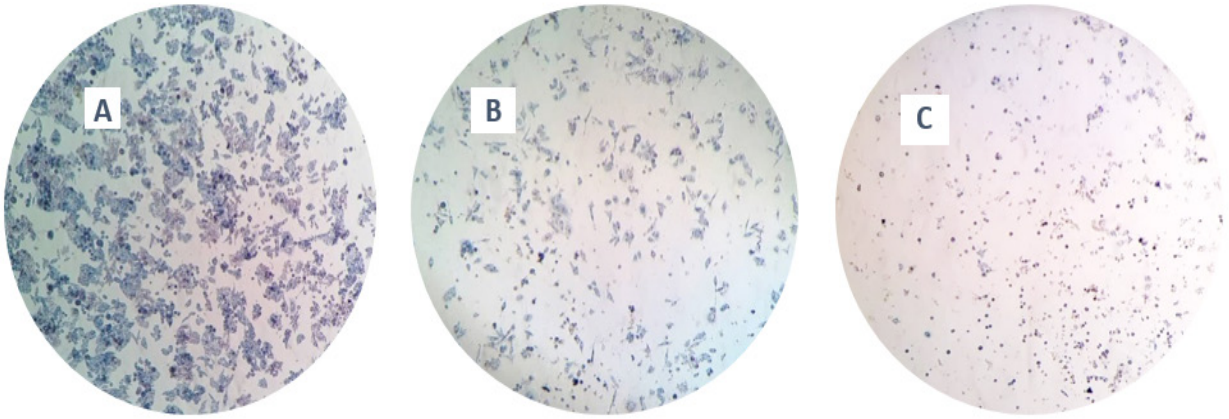

Figure 2) Light microscopy view depicting cytotoxic effects of Nisin-ADI recombinant fusion protein on SW480 tumor cells. A: untreated, B and C, treated with 25 and 50 µg/mL of Nisin-ADI, respectively (×40), results indicated dose-dependent cytotoxicity of evaluated recombinant after 24-hour incubation.

antibodies used in the experiment.

Antiproliferative effects of the recombinant fusion protein: The present study investigated the cytotoxic effects of the Nisin-ADI recombinant fusion protein on SW480 tumor cells. The IC50 value, indicative of the concentration required to inhibit cell growth by 50%, was approximately 30 µg/mL. Results demonstrated a signifig cant reduction in cell proliferation across all tested concentrations of the Nisin-ADI recombinant fusion protein (Figure 2). Notably, the highest level of cell lethality was observed at a concentration of 100 µg/mL after 24 hours of incubation. In contrast, control NIH3T3 cells exhibited no notable decrease in viability when exposed to varying fusion protein concentrations for 24 hours (Figure 3). These findings highlight the selective cytotoxicity of the recombinant fusion protein towards cancer cells, as it induced significant cytotoxic effects compared to the normal control cells (P<0.0035).

Discussion

CRC is the 3rd most commonly diagnosed cancer in the world. It is estimated to cause approximately 5.7 million deaths yearly [17]. CRC is accounting for 10.2% of all cancer cases. It is the second most common cancer

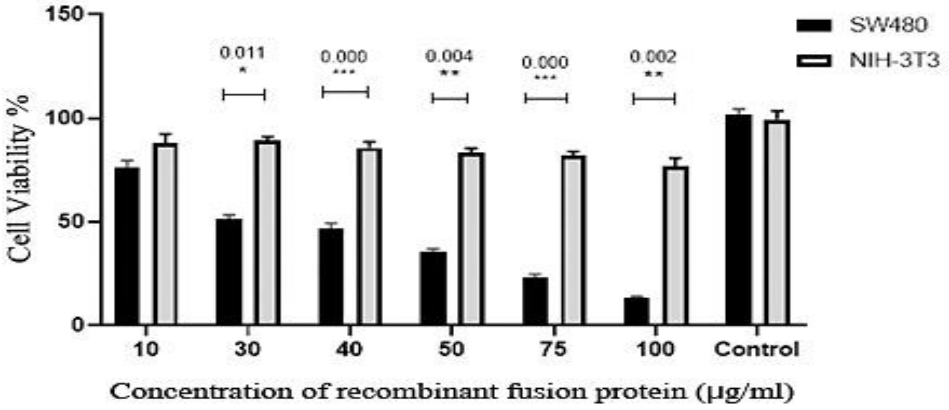

Figure 3) Cell viability percentages based on MTT results, (t-test analysis: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$).

in women (after breast cancer) and the third most common in men (after lung and prostate cancer). Although colorectal cancer can be a serious and life-threatening disease, early detection and advances in treatment have greatly improved survival rates [18]. Relative survival rates for rectal cancer patients were 68% overall after five years. Localized cancer has a 90% survival rate when diagnosed early [19]. The failure of conventional anticancer therapies and their side effects are major causes of recurrence and poor prognosis in colorectal cancer patients [20]. Therefore, researchers need to search for appropriate and efficient novel therapeutic strategies.

Bacterial-derived compounds are effective in combating cancer. It has been shown to reduce the side effects of other treatments and may even improve their effectiveness. This approach may be a promising option for cancer treatment [21]. Over the years, many studies have been conducted to synthesize and produce bacterial proteins and peptides as potential anticancer drugs and bioactive compounds [22,23]. These studies have focused on the ability of bacteria to produce proteins and peptides with specific therapeutic properties. Bacterial recombinant fusion protein technology has revolutionized the field of biotechnology and medicine. This technology involves the combination of two or more different proteins to create a single protein with enhanced features or functions [24,25]. Given the notable role of recombinant fusion proteins in eliciting toxicity in cancer cells, the aim of this investigation was to develop and synthesize a recombinant fusion protein combining Nisin and arginine deaminase. Subsequently, we sought to assess its cytotoxic effects on the SW480 colorectal cancer cell line, juxtaposed against the NIH3T3 control cells. Nisin and ADI can be used to produce recombinant fusion proteins [9,26]. The antimicrobial properties of Nisin are due to its ability to bind to the bacterial cell membrane and create pores that disrupt membrane integrity; this leads to intracellular contents' leakage, ultimately resulting in bacterial cell death [27]. Nisin exerts anticancer effects by inducing apoptosis and inhibiting cell growth. Nisin has also been shown to reduce tumor growth and metastasis in animal models and is non-toxic to healthy cells. They can interact with components of the cancer cell membrane, leading to direct cell death. It also induces the expression of pro-apoptotic proteins,

further contributing to its anticancer effects [28]. Zhang et al. investigated Nisin effects on human colon cancer cells and discovered that doses of 20-80 µg/mL effectively suppressed cell growth. The study further elucidated the mechanisms involved, highlighting the role of the Akt pathway, Bcl-2 family, and mitochondrial pathway in Nisin-induced apoptosis [29]. In a separate study by Zhu et al. (2016), Nisin demonstrated similar anticancer properties in human pancreatic cancer cells. The researchers observed that 20-40 µg/ml doses induced apoptosis and inhibited proliferation. These findings suggest that Nisin could be a potential therapeutic agent against pancreatic cancer. Additionally, Lu et al. (2018) explored the effects of Nisin on hepatocellular carcinoma cells. They found that Nisin suppressed the growth of liver cancer cells at 10-40 µg/ml doses by inducing apoptosis and causing cell cycle arrest. On the other hand, ADI is a protein that catalyzes the hydrolysis of arginine to citrulline and ammonia. This enzyme is found in various microorganisms, including bacteria and fungi. ADI has a strong interest in cancer research due to its capability to reduce arginine, a crucial amino acid necessary for the survival of numerous cancer cells. Studies have shown that cancer cells lacking the enzyme argininosuccinate synthetase (ASS) cannot synthesize arginine and are, therefore, dependent on exogenous sources of this amino acid. By depleting arginine levels using ADI, cancer cells lacking this enzyme are vulnerable to cell death [30]. Clinical trials have been conducted to test the efficacy of ADI in treating various types of cancer, including hepatocellular carcinoma, melanoma, and sarcoma. The results from these trials have been promising, with some patients demonstrating partial or complete responses to treatment. A clinical evaluation of the safety and efficacy of pegylated rADI (ADI-PEG 20) in patients with advanced hepatocellular carcinoma. The trial used a dose of 36 mg/m2 ADI-PEG 20, and the results showed an overall survival benefit in the treatment group compared to the control group [31]. Another preclinical study published in 2021 reported that rADI alone or combined with paclitaxel exhibited potent antitumor activity against triple-negative breast cancer. The researchers used a dose of 100 U/kg of rADI in their experiments [32]. These data make Nisin and ADI proteins attractive options for cancer treatment; this is especially true because both proteins are non-toxic to healthy cells, rendering them much safer than traditional chemotherapeutic treatments. The results of the current study showed the potential of the Nisin-ADI fusion protein to induce cell death in the SW-480 cancer cell line. The findings of this study suggest that the Nisin-ADI recombinant fusion protein may have the potential as an additional treatment for colorectal cancer. The IC50 value obtained, which indicates the protein concentration required to inhibit the growth of half of the cells, was about 30µg/mL.

The study also found that the protein significantly reduced cell proliferation at all concentrations tested. These results are promising and suggest that the Nisin-ADI recombinant fusion protein may effectively combat tumor cell growth. It is important to note that the control NIH3T3 cells did not show any decrease in viability, indicating that the protein is selective in its effects on tumor cells.

It is worth mentioning that this study did not delve into the mechanisms underlying the cytotoxic effects of the Nisin-ADI fusion protein. Further investigations should focus on elucidating the molecular pathways involved and identifying the specific targets of this fusion protein in colorectal cancer cells. However, overall, the results of the current study are a step forward in the

exploration of potential treatments for cancer.

Conclusion

In conclusion, while more research is needed to fully understand the potential benefits and limitations of recombinant Nisin-ADI fusion protein as an additional treatment option for colorectal cancer, its development and initial cytotoxic effects on SW480 cell lines represent an important step forward in the fight against colorectal cancer. Its ability to target cancer cells offers a new approach to treatment that could improve outcomes for patients with this devastating disease.

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Ethical permission: The study protocol and ethical issue were approved by the Ethics Committee of Alborz University of Medical Sciences (IR.ABZUMS.REC.1398.198).

Authors' contributions: Study design; MD, BH, Laboratory experiments; BH, NB, SM, and MG, Data analysis; MD, and FS, Writing of the manuscript; BH, MD, and HG. Assumes overall responsibility for the accuracy and integrity of the manuscript; MD, BH, and FS.

Conflicts of interests : None declared by Authors.

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Consent to participate: This section does not apply to this article because it was completely a laboratory study and no patient or participant was included in it.

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