Anticancer activity of cloned Nisin as an alternative therapy for MCF-7 breast cancer cell line

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ARTICLE INFO

Original paper

Article history:
Received: May 04, 2023
Accepted: June 25, 2023
Published: September 30, 2023

Keywords:
breast cancer, MCF-7, Gibson cloning, Nisin, cytotoxicity

ABSTRACT

Despite advancements in treatment and detection, cancer remains one of the most common causes of death worldwide. Conventional chemotherapeutic drugs used to treat cancer have non-specific toxicity toward normal body cells, which leads to several adverse effects. Second, malignancies are known to develop resistance to chemotherapy over time. As a result, the demand for novel anticancer drugs is growing daily. The most frequent type of cancer among women is breast cancer. Utilizing cloned Nisin as an anticancer was the purpose of this study using Gibson cloning and a cell-free peptide synthesis system, then purification of the target protein. The antiproliferative effect of Nisin against a breast cancer MCF-7 cell line was also determined using an MTT assay, and viability in cell lines was measured using acridine orange and propidium iodide. Our findings demonstrate the successful isolation and cloning of the NisA gene in addition to inducing peptide synthesis system and then purification of a target protein. MTT assay results indicate that Nisin exhibits a high and selective cytotoxicity against the MCF-7 cell line with an IC50 value of 11.68 μg/ml. This data suggest that the NisA gene had in vitro antiproliferative effect against breast cancer cell. However, more research, including a combination of the NisA gene with other anticancer therapy in clinical use. In addition, in vivo studies are required.

Introduction

Cancer is considered one of the most causative agents of mortality worldwide (1). The new cases of cancer in 2020 reached 18.1, and 10 million deaths were reported; it is expected by 2030, the number of deaths will be ~17 million (2); therefore, these figures highlight how urgent it is to develop new potent therapies. The incidence of cancer all over the world is increasing, as over three years, cancer prevalence has risen by 23%, and it has been stated that breast cancer is one of the most prevalent cancers in Kurdistan (3).

One of the most common types of malignant cancer, breast cancer, can be fatal and significantly negatively affects both physical and mental health (4). Furthermore, over the past few decades, Asian nations have seen an increase in breast cancer incidence rates (5). According to estimates, 2.1 million new cases and 630 thousand deaths from breast cancer occurred in women globally in 2018; despite recent improvements in breast cancer therapies, including targeted therapy, the prognosis for patients with advanced-stage and recurring disease is still poor, for instance, just 22% of stage IV breast cancer patients survive for five years (6).

Conventional cancer treatments, such as surgery, radiation, chemotherapy, and immunotherapy, all have certain drawbacks (7). As well as the complex physiology of different types of malignancy makes treatment challenging, including issues with size, place, stage, and metastasis within the tumour (8). Additionally, resistance frequently develops to decrease the initial efficacy of chemotherapy, radiation, and immunotherapy, resulting in poor tumour control and various adverse effects during or after the treatment (9). The disadvantages and challenges of cancer therapy make the patient suffer from the side effect of those therapies (10).

One of the main barriers preventing chemotherapy from being effective in treating breast cancer is multidrug resistance (11). As paclitaxel and doxorubicin, a first-line therapy with substantial anticancer efficacy, are frequently used to treat breast cancer, this can result in resistance (12). Several studies have identified potential causes for paclitaxel resistance, including altered signal transduction, dysregulation of the P glycoprotein (Pgp) drug efflux pump, alterations in tubulin structure, and suppression of the activation of apoptotic pathways (13,14).

There are many alternative therapies for cancerous cells, such as gene therapy, diet therapy, photodynamic therapy, insulin potentiating therapy, HAMLET (human alpha-lactalbumin made lethal to tumour cells), telomerase therapy, hyperthermia therapy, dichloroacetate, non-invasive RF cancer treatment, and bacteriotherapy, that have been proposed to enhance and increase the effectiveness of conventional cancer therapy (15). However, those treatments are not efficient enough to be used as anticancer alone because of poor tumour control, and many side effects that occur during or after the treatment (16).

Consequently, there is a critical need for cancer cell-
specific targeted therapies that can either be employed as a stand-alone cancer treatment or as adjuvants to reduce the therapeutic doses of established anticancer medications (17). As protein therapies expand in popularity, the scientific community has begun looking into bacteriocins as potential new cancer medicines. Earlier reviews discussed how bacteriocins affect mammalian cells (18).

Nisin is one of the main bacteriocins used as an anticancer in many studies (19), which is a model type A lantibiotic that is encoded by four genes found in lantABCT clusters (20). It has a low-affinity membrane penetration as well as the creation of particular pores that are lipid II-dependent and causes membrane permeabilization (21). Because of that Kamarajan and colleagues have concentrated on examining the translational potential of a high-purity version of nisin Z to treat Head and neck squamous cell carcinomas (HNSCCs) (22). Additionally, Kamarajan and colleagues demonstrated that HNSCC cells that are extremely resistant to ionizing radiation and cisplatin could undergo apoptosis when high-purity versions of NisA and Z are combined with cisplatin in vivo (Global Medical Discovery, 2015).

Nisin interacts with the anionic properties of the phospholipid heads in the plasma membrane of cancer cells, as seen in its antimicrobial action. As a result, it causes the membrane to reorganize, creating momentary openings that let calcium ions enter the cytoplasm (19). According to other reports, Nisin enters the plasma membrane, polymerizes, and creates pores that permit calcium inflow and cytoplasmic content outflow (23). Calpain, a calcium-dependent protease that breaks down cellular substrates and plasma membranes, was then triggered by the rise in intracellular calcium concentration. This is one of the main processes that eventually results in apoptosis and the breakdown of the cellular architecture (24).

This study aims to find the anticancer activity of cloned NisA as a natural peptide produced by microorganisms against the MCF-7 breast cancer cell line. Evaluating the cytotoxicity effect by measuring its IC50 compared to positive control paclitaxel and commercially available Nisin.

Materials and Methods

Isolation of Nisin-producing Lactococcus lactis spp. lactis

Homemade cheese samples were collected for this research inside sterile packaging materials, the cheese samples (10 grams) were homogenized in 90 ml of Buffered Sodium Chloride-Peptone Solution at 37°C for 24 hours (25), then cultured on MRS agars (Merck, USA), a loopful of each was spotted for 24 hours at 37 °C. The colony’s morphological characteristics (colour, size, shape, margin, and surface) were examined visually. The isolated strain is next tested for catalase test and Gram staining (26). To produce pure culture, a single colony from a petri dish was selected, added to 5 ml of MRS broth, and cultured overnight. After that 500 ul of the overnight culture was transferred to 500 ul of 30% glycerol and kept at -20 °C as a stock culture.

Molecularly confirmation of species was performed by PCR using two primers (Forward: GCGGGCTGCTAATACTA; Reverse: ATCTACGCACTTACCCGTAC) for 16S rRNA of Lactococcus lactis spp. Lactis uses the optimal conditions of denaturation at 94°C for 1 min, annealing at 55°C for 30 s and extension at 72°C for 2 min, 35 cycles (27).

Identification and purification of NisA gene.

Potential NisA gene was identified by colony PCR amplification using two primers(Forward: ATGAGTTAACAAGATTATCATTTTGC; Reverse: TATTATCTTACGTTAATCTACAATGAC) (this study). A single colony was picked up from the MRS agar plate, which was then mixed while suspended in a PCR Eppendorf tube holding 10 ul of sterile distilled water. Then, using the optimal conditions of denaturation at 94°C for 1 min, annealing at 46°C for 30 s, and extension at 72°C for 2 min, 35 cycles, 1 ul was utilized as the DNA template (28). The PCR products were sorted in a 1.5% agarose gel by electrophoresis (29). DNA bands were stained with a safe dye (Addbio, South Korea) and then were made visible. Following that, the proper gene segment was extracted from the agarose gel using AddPrep Fragment Purification Kit (Addbio/ Korea); following that, it was stored at -20°C for further analysis.

Cloning of NisA gene

Performing the Gibson cloning (30) by designing a set of primers containing the recognition sites of the overlapping sequences of PET-3a plasmid vector (GenScript/China), which includes T7 promoter region to enable bacterial gene expression and 6xHistag for purification purposes, The NisA gene was prepared to be cloned into the plasmid using the F and R primers have the following sequences: AGAAGGAGATATACATATGGAGTACAAAAGATT TTAA for the Forward primer and TAGTTATTGCTCAG CTTA*GTTGTTATGTTGATGATG TTTACTCTACG TGAATACACT for the Reverse primer (bold: Start and Stop Codons, italic: pET-3a overlapping sequences; underlined: Nisin Specific Sequences; * 6xHistag).

The PCR amplification was carried out in a 25 µl mixture in a DNA thermocycler. The conditions consisted of 45 cycles of 94°C for 20 sec, 60°C for 20 sec, and 72 h for 1 min. PCR products were visualized on gel and purified using the AddPrep Fragment Purification Kit (Addbio/ Korea). Then extracted PCR amplicon was sequenced with Illumina Nextera XT in order to sure about the gene direction and find any mutation that may occur during amplification by using IGV 2.3 from Broad Institute is available at (https://software.broadinstitute.org/software/ igv/igv2.3) software for analysis. The target PCR product and vector are mixed with the presence of an enzyme at 50 °C for 15 minutes and transferred to the ice to get the constructed vector that contains the NisA gene according to the kit’s instructions.

NEBuilder HiFi DNA Assembly from (New England Biolabs/ England) was used for expression cloning. Then Gibson Assembly reaction mix was transformed with Heat shock in bacteria (NEB Stable Competent Escherichia coli high Efficiency from NEBuilder HiFi DNA Assembly) (31). The transformed E. coli was grown in 950 ml of prewarmed 37 °C SOC media, and the cells were grown at 37 °C with 220 rpm shaking without the use of antibiotics and then plated on LB agar (Merck, USA) containing 50 mg/ml ampicillin with same environmental condition. The next day, colonies observed on plates were transferred to 2 ml antibiotic-containing liquid LB solution with a sterile pipette tip and incubated at 37 °C overnight with 220 rpm
shaking.

A plasmid extraction kit (Addbio/Korea) was used to extract the plasmid for the screening of cells that took the constructed plasmid containing the NisA gene by using PCR with the same utilized primers.

Expression and purification of NisA

Obtained plasmids with known concentrations should be sequenced before the peptide synthesis step to ensure they contain the correct sequences. This step was performed with Next Generation Sequencing – NGS in the Illumina Miseq platform.

Finally, the "PURExpress in vitro Protein Synthesis Kit" (New England Biolabs/England) is used following the manual instruction to express the NisA gene. Using "NEBExpress Ni Spin Columns" (New England Biolabs/England) to separate expressed NisA from another peptide that is expressed on the constructed plasmid and use the 6-Histag signal that is added just before the stop codon of the target gene during the cloning. Then NisA quantification was evaluated fluorometrically using a "Qubit Protein Assay kit" from Thermo Fisher Scientific in the USA.

The purified protein confirmed using SDS-PAGE, according to (32), Nisin has a molecular weight of 3.4 kDa. After the products were added to the gel's wells, they were electrophoretically separated, linked to an electric current, and stained with Coomassie blue.

Tissue culture

The MCF-7 human breast cancer cell line was purchased from the Iraqi Center for Cancer and Medical Genetic Research at the University of AL-Mustansiriyah. Cells were grown in MEM medium (Santa Cruz Biotechnology, USA), supplemented by 10 % fetal bovine serum (FBS) (Capricon, Germany), 100 ug/mL penicillin, and 100 µg/mL streptomycin (Capricon, Germany), at 37°C in a humidified environment with 95% O2 and 5% CO2 (33).

Cell viability by MTT assay

Using a conventional colorimetric MTT test, drug activity was evaluated based on the ability of live cells to metabolically convert yellow, water-soluble tetrazolium salt into purple, non-soluble formazan product. When the enzymes in living cells called mitochondria succinate dehydrogenases are active, this reaction occurs (34).

In summary, trypsinization (Capricorn, Germany) was used to harvest cells in exponential growth, which was then plated out in 96-well plates at a density of 104 cells per 100 ul/well and given an overnight interval for attachment. After that, the medium was taken out, and fresh medium was introduced along with various concentrations of paclitaxel as the positive control, Nisin cloning, and commercial Nisin 2.5% (Freda, China) as follows (3.1µg/ml, 6.25µg/ml, 12.5µg/ml, 25µg/ml, 50µg/ml, 100µg/ml) for 72 hours. MTT (Bio-World, USA) was added to the incubation media at 37 °C for 4 hours. Once the medium was removed for treatment, 50 ul DMSO (Santacruz Biotechnology, USA) was applied to the cells to dissolve the MTT formazan crystals (35). The amount of colour production was measured at 492 nm with a microtiter plate reader (Gennex Lab, USA). To determine the IC50 values, dose-response curves for each compound alone were drawn. The data were statistically analyzed using an unpaired t-test with Graph Pad Prism 6 (36). The values were presented as the mean ± SD of triplicate measurements (37).

Apoptosis estimation (propidium iodide/Acrifline orange assay)

The viability in cell lines was measured using (AO/PI) (38). For this purpose, 5*10^4 cells/well were seeded in a plate and further treated with IC50 of Nisin and Taxol for 24 hours at a 37 °C incubator. The tested wells received exactly 50µl of the AO/PI stain mixture (at room temperature) for 30 seconds. Then, the stain was removed. The images were taken using a Leica fluorescent microscope (39). The fluorescent intensity was measured by fluorescent microscopy using Image J Software.

Results

Morphological and biochemical properties of Lactococcus lactis spp. Lactis.

Potential colonies were isolated from homemade cheese samples. After excluding the strains with bacilli and cocco-bacilli shapes, pure isolates that shared (gram-positive, cocci, and catalase-negative) characteristics with small, raised, and circular colony morphology were selected (Figure 1).

Molecular identification of Lactococcus lactis spp. Lactis. and NisA gene

According to PCR amplifications, the 16S primer was used as the basis for the PCR process. The results revealed that amplified DNA fragments on agar gel electrophoresis had both copies of the 16S rRNA gene, about 700 bp, and target NisA gene, about 170 bp, which is used to identify family and species of bacteria and isolation of the NisA gene, respectively (Figure 2).

Preparation of plasmid and NisA gene

Amplification of the NisA gene that contains the recognition sites of the overlapping sequences of PET-3a plas-

![Figure 1. Microscopic properties of Lactococcus lactis spp. Lactis (40X magnification microscope).](image)

![Figure 2. A Lactococcus lactis spp. lactis bands on 1.5% agarose gel. Lane 1. 100 bp ladder. Lane 2 and 3 samples. B. The amplified NisA gene. Lane 1) DNA Ladder 100 bp. Lane 2,3) Negative control. Lane 4) NisA gene.](image)
mid vector and 6xHistag was conducted, and the outcome of PCR was pictured on gel and purified, an expected bond of NisA gene after designed with sequence needed for cloning. The PCR product was purified and sequenced, as mentioned before, and then inserted into the “pET-3a plasmid vector”. This backbone contains T7 promoter region to enable bacterial gene expression. As well as constructed plasmid pictured on gel electrophoresis with a specific bond, as shown in (Figure 3).

**Constructed plasmid transformation to E. coli**

Using Competent *Escherichia coli* high Efficiency from NEBuilder HiFi DNA Assembly as host transformation is performed by heat shock. Then after incubation plated on LB agar, the colony was observed as small, white, and clear. After that colony was picked up for PCR reaction to confirm the amplification of recombinant DNA, the presence of the band showed that the target gene had been amplified (Figure 4).

**Induction of gene expression and isolation of peptide**

Constructed plasmid DNA isolated from the colony in order to synthesize the Nisin peptide using cell-free peptide synthesis "PURExpress system. The purity of the lantibiotics, which is about 420 ng/μl was also evident from Qubit Protein Assay. Tricine-SDS gel electrophoresis showed single bands with identical apparent molecular weights of 3,400 kDa (Figure 5).

**In vitro cytotoxicity of Nisin**

The cytotoxicity of Nisin against MCF-7 cells was evaluated using an MTT assay. The viability of MCF-7 cells decreased from 91.8% to 36.4% in a concentration-dependent of cloned Nisin from 3.125 to 100 μg/ml, as well as commercial Nisin exhibited an apoptotic effect on MCF-7 at 50.8% with maximum concentration, compared with paclitaxel the viability of cell line decreased from 77 % to 25.4 % in a concentration from 3.125 to 100 μg/ml (Figure 6). The highest rate of cell toxicity occurred after cells were exposed to the highest concentration of Nisin over 72 hours, according to our findings; additionally, Nisin was able to induce marked apoptosis in the MCF-7 cell line, as opposed to a control group.

The survival of MCF-7 cells was significantly inhibited by NisA with IC50: 11.68 μg/ml. Anti-proliferator effect of produced NisA on the MCF-7 cell line was a little bit different from commercial Nisin with IC50: 5.079 μg/ml. As well as the IC50 of positive control for paclitaxel was 14.62 μg/ml (Figures 7 and 8).

**Morphological analysis**

Changes in cell morphology were evaluated using an inverted microscope. The morphological analysis showed that compared to the control or untreated cells (Figure 9A), treatment of cells with cloned Nisin (concentration at IC50) led to cell lysis (Figure 9B). However, commercial Nisin had close results with cloned nisin (Figure 9C), but in the case of chemotherapy treatment apoptosis was clear with significant cell lysis (Figure 9D).
Apoptosis (AO/PI) double staining
The changes in the cell morphology of MCF-7 after being treated with Nisin, Nisin commercial, and Paclitaxel at IC50 concentrations for 24 hours were studied using AO/PI dual staining method. AO and PI are intercalating, nucleic acid-specific dyes which emit a green and orange fluorescence, respectively, when bound to DNA. AO stains healthy nuclei and cell cytoplasm an emerald green colour, whereas PI is only taken by cells when cytoplasmic membrane integrity is lost and stains the nuclei with the bright orange-red. The apoptotic cells evaluated based on DNA damage from the AO/PI staining, it was confirmed that Nisin, Nisin commercial and paclitaxel-induced apoptosis in MCF-7 cells leads to cell death (Figure 10).

Discussion
Depending on the patient's overall health and the severity of the illness, common cancer treatments include surgery, chemotherapy, and radiation therapy. Each of these therapies has disadvantages (40,41).

However, daily advancements are being made in treating breast cancer, and drug-resistance cases are still prevalent (42). Though, this is presently one of the biggest problems facing cancer studies. For instance, 50% of women with HER2-positive breast cancer exhibit or develop trastuzumab resistance. Additionally, standard chemotherapy is still the first-line treatment for patients with triple-negative breast cancer, and these patients frequently develop drug resistance (43).

A wide range of commonly utilized anticancer medicines has decreased clinical efficacy because of rising mammalian tumour recurrence and significant chemotherapeutic side effects. Therefore, the need to create complementary or synergistic anticancer medications with low adverse effects remains continual (44). Scientists are therefore searching for novel medicines that are more successful in the treatment process and have fewer adverse effects owing to the spread of cancer. Antimicrobial peptides, particularly bacteriocins, are one of the most recent treatments that have been approved in many research (45).

On the other hand, researchers have worked on a combination of therapeutic interventions to give patients a chance to get the most out of their treatment while reducing or eliminating toxic effects, recurrence, and resistance, which gives a normal life for patients (46).

Nisin is the most well-characterized bacteriocin of Lactococcus lactis (LAB origin, having first been isolated in 1951 (47). In various research, the anticancer effects of Nisin in head and neck squamous cell carcinoma (HNSCC) were investigated (22,48). In another investigation, the apoptotic impact of Nisin on colon cancer cells (SW480) was assessed (49). Nisin exhibited a cytotoxic effect on gastrointestinal (AGS and KYSE-30), hepatic (HepG2), and blood (K562) cancer cell lines, according to a different study (50). Additionally, Nisin causes selective apoptosis and reduces oral squamous cell carcinoma (OSCC) cell...
proliferation (51). Since Nisin enhanced HNSCC cell apoptosis, suppressed HNSCC cell proliferation, inhibited angiogenesis, prevented the development of HNSCC or inhibited tumorigenesis in vivo, and increased survival in vivo, the data suggest the function of Nisin as an alternative therapy for HNSCC (22), Assuming that the FDA has approved Nisin’s no-observed-effect level (NOEL) in humans to be 83.25 mg/kg (66.7 mg/kg was utilized in mice as a cancer treatment dosage).

In the current study, we attempted to evaluate the cytotoxicity of Nisin on MCF-7 that cloned with Gibson cloning and synthesis using a cell-free peptide synthesis system as a simple method. So, making a huge amount of Nisin by cloning was one of the primary objectives of this research using Gibson cloning, speed, efficiency, scarless assembly with vector, and versatility. Additionally, assembly does not depend on the presence of restriction sites within a specific sequence to be synthesized or cloned, as well as saves time-consuming. In the current investigation, the NisA genes were cloned, expressed, and verified using SDS-PAGE in laboratory settings.

According to the findings of this study, the isolated cloned Nisin and commercial Nisin may also have therapeutic potential for breast cancer cells comparing the paclitaxel used as a control. The IC50 was 11.68 µg/ml for cloned Nisin, 5.079 µg/ml for commercial Nisin, and paclitaxel 14.62 µg/ml against MCF-7 cell line for 72 hours, which have a great effect on reducing cells viability. This is agreed with the result obtained by Avand and his colleagues (2018) that demonstrate that Nisin has an IC50 value of 5 uM and has high and specific cytotoxicity against the MCF-7 cell line (52). Our finding showed that the effect of Nisin on the MCF-7 cell line was significant compared to the paclitaxel effect on breast cancer; this may be due to Nisin inducing programmed cell death, cell cycle arrest, and reduced cell proliferation (53).

Nisin caused cell death in MCF-7 cells by inducing apoptosis, as determined by the DNA damage found in the apoptotic cells after AO/PI staining. This method is the most effective way for assessing cell viability because it enables dual-fluorescence detection (live and dead), which eliminates the possibility of inaccurately counting populations of membrane-poor cells, such as late apoptotic and necrotic cells, as well as cellular debris. Nisin inhibited the MCF-7 cell line’s growth and triggered apoptosis (18). It is worth mentioning that the FDA has authorized the peptide nisin, which is GRAS (generally recognized as safe) and has the potential to be used in clinical settings and in biomedical disciplines over the previous two decades (54).

The major interest is in the discovery of bacteria-producing Nisin, which potentiates the activity of anticancer cell lines. This study was only limited to the in vitro effect in MCF-7 cells with regard to cytotoxicity as measured by the MTT assay. The current study showed Nisin has increasing cytotoxicity effect on the MCF-7 cell line. One of our upcoming research objectives will be the use of chemotherapy or nanoparticles in combination with cloned Nisin and nisin-paclitaxel conjugation. As well as, for future in vitro studies, it is suggested that more cancer cell lines be included. The mechanistic interaction between Nisin and the chemotherapeutic agents should also be investigated.

Acknowledgments
The authors would like to express special thanks of gratitude to all the staff of Vin Laboratory especially Dr. Ameer Ibrahim Ahmed for their tremendous support and assistance. Additionally, the authors would like to express their gratitude to Dr. Zahra Khairi Ramadhun, Dr. Meqdad S. Ahmed and Dr. Ayaz M. Mamsin for their help during this study.

Interest conflict
The authors declare that they have no conflict of interest.

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