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Chapter 3

The Cytotoxic, Antimicrobial and Anticancer Properties of the Antimicrobial Peptide Nisin Z Alone and in Combination with Conventional Treatments

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.71927

Abstract

Nisin is an antimicrobial peptide commonly used as a food preservative since 1969. This peptide has potent antimicrobial activity against several Gram-positive bacterial strains, including clinically important and resistant pathogens. The combination of nisin with conventional antibiotics has been shown to improve the antimicrobial activity of these antibiotic agents. Apart from the antimicrobial properties of nisin, this AMP also displays promising anticancer potential towards several types of malignancies. The nisin Z variant is able to induce selective cytotoxicity in melanoma cells compared to non-malignant cells. It was shown that nisin Z disrupts the cell membrane integrity of melanoma cells and that cytotoxicity is likely due to the activation of an apoptotic pathway. In addition, when used in combination with the conventional chemotherapeutic agents, nisin Z has the potential to enhance the cytotoxicity of these chemotherapeutic agents against cultured melanoma cells. Nisin Z has great potential for clinical application considering its low cytotoxicity to non-malignant cells and its effectiveness against Gram-positive bacterial strains and certain cancers.

Keywords: melanoma, antimicrobial peptide nisin Z, combination therapy, selective cancer cytotoxicity, chemotherapeutic agents, antibiotic resistance

1. Introduction

Antimicrobial peptides (AMPs) are produced by all known living species and exhibit direct microbial killing activity while also playing an important role in the innate immune system [1]. This diverse group of peptides is found in all living species and may be promising alternatives

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or serves as additives to current antibiotics [2–4]. Many of the more than 2000 known AMPs have been demonstrated to exhibit broad-spectrum antibacterial activity [5], and bacteria are less likely to develop resistance to these peptides compared to conventional antibiotics [6, 7].

The lantibiotic nisin, produced by *Lactococcus lactis*, has promising potential for clinical application with its *Generally Regarded as Safe* (GRAS) status. This AMP was approved by the World Health Organisation (WHO) in 1969 and the US Federal Food and Drug Administration (FDA) in 1988 for the use as a food preservative [8]. Despite being extensively utilised for food preservation for nearly 50 years, there is very little indication of resistant mutants arising in food products treated with this AMP [8, 9].

Nisin is primarily used for its antibacterial activity. However, AMPs, and especially bacteriocins, display selectivity towards cancer cells [10]. Due to the toxicity associated with many conventional chemotherapeutic agents, as well as the development of chemotherapy resistance [11–13], there is a need for the development of novel anti-cancer therapies. Furthermore, to overcome chemotherapy resistance, the efficacy of chemotherapeutic agents can be enhanced by the co-administration of multi-functional agents to achieve synergistic interactions [14, 15]. The ability of nisin to increase the activity of the chemotherapeutic drug doxorubicin was investigated *in vivo* by Preet and co-workers. Nisin, when used in combination with doxorubicin, enhanced the anti-cancer activities of doxorubicin. Apoptosis could be detected upon treatment of mice with induced skin carcinogenesis. However, the exact mechanism by which nisin exerts its anti-cancer activities was not known [16].

2. Antimicrobial properties of the antimicrobial peptide nisin

A report published in 2016 projects that resistance to antibiotics could potentially lead to 10 million deaths per year by 2050 [17]. Moreover, the estimated economic impact of microbial resistance will be massive, costing nearly 100 trillion US dollars while leading to sharp decreases in the gross domestic product. Microbial resistance against conventional antibiotic agents is a serious hazard to the effective treatment of numerous diseases. This upsurge in antibiotic resistance has stimulated research into the development of alternative antimicrobial agents. Antimicrobial peptides are considered promising alternatives to current antibiotics and have the potential to replace certain antibiotics or to be used synergistically in combination with existing antimicrobial agents [2, 18].

2.1. Anti-bacterial effects of Nisin

Nisin was discovered in the same year as penicillin, but was quickly overshadowed by this antibiotic due to penicillin's ease of mass production and low manufacturing costs [19]. Nisin is a 3.5 kDa polycyclic peptide consisting of 34 amino acids and is produced by the non-pathogenic bacteria *Lactococcus lactis* [20]. Two naturally occurring variants of this peptide are nisin A and nisin Z. These two variants are structurally identical with the exception a single amino acid at position 27, where histidine occurs in nisin A while asparagine is found in nisin Z [20]. Both variants display similar antimicrobial activity but nisin Z is more soluble at neutral pH [21, 22].

In Gram-positive bacteria, nisin exhibits a dual mode of action by binding to lipid II on the bacterial membrane resulting in the inhibition of cell wall synthesis and the formation of pores in the bacterial cell membrane [23]. The antimicrobial effects of nisin Z against Gram-negative bacteria are largely inadequate. However, the activity towards Gram-negative bacteria can be improved by using ethylenediaminetetraacetic acid (EDTA) and the non-ionic surfactant Tween®80 [24, 25] (**Figure 1**).

The glycopeptide antibiotic, vancomycin, also binds to lipid II to inhibit cell wall synthesis, albeit at a different amino acid moiety. Vancomycin is one of the last line treatments against several Gram-positive antibiotic-resistant bacteria including methicillin-resistant *Staphylococcus aureus* (MRSA) [26, 27]. Disturbingly, clinical variants of MRSA have been isolated of which the lipid II pentapeptide have mutated to acquire resistant to vancomycin. These strains contain the *vanA*-type gene cluster where the terminal D-Ala has been changed to D-Lactate in the lipid II pentapeptide [28]. Due to its different binding motif, nisin remains active against the *vanA*-type resistant strains [29]. This shows the potential of nisin to bolster the antimicrobial defences against antibiotic-resistant bacterial strains. Nisin has a promising potential for clinical application with its GRAS status and approval by both the FDA and WHO, considering its low cytotoxicity and the fact that it is considered safe for human consumption. Currently, it is employed as a food preservative in nearly 50 countries to guard food against spoilage resulting from pathogens such as *Staphylococcus aureus, Listeria monocytogenes*, and *Clostridium botulinum* [30]. In addition, nisin has also been demonstrated to possess antibacterial activity against several clinically

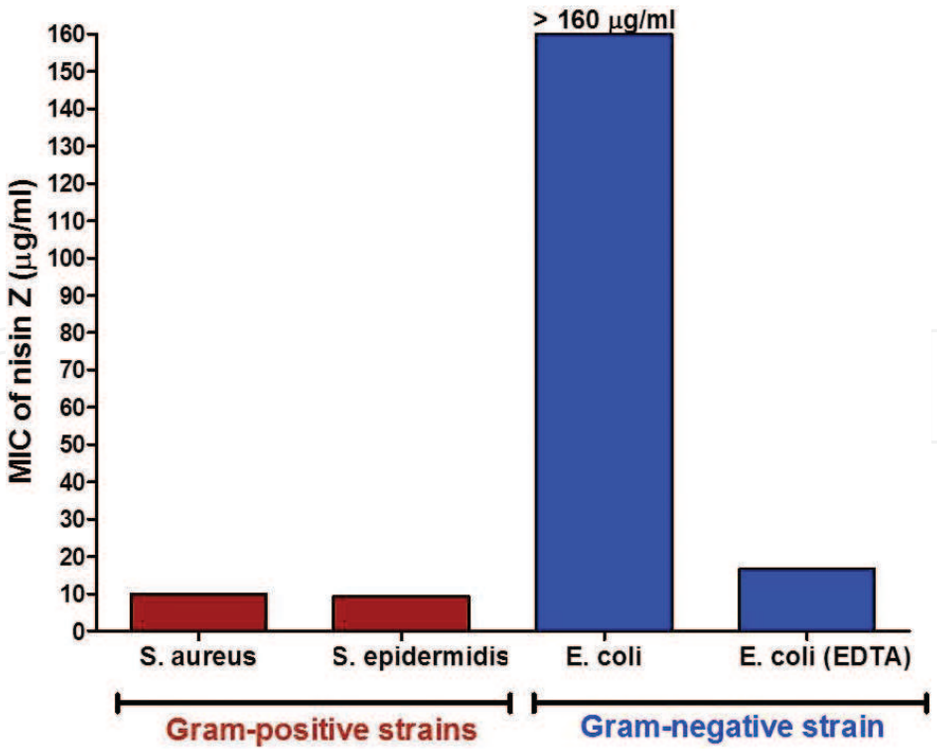

Figure 1. Minimum inhibitory concentrations (MIC) of nisin Z for Gram-positive and Gram-negative bacterial strains. The effect of EDTA (200 μM) on the MIC of *E. coli* is also demonstrated.

relevant pathogens including vancomycin-resistant *Enterococci*, *Streptococcus pneumonia*, and methicillin-resistant *Staphylococcus aureus* [31, 32].

Mastitis-causing *Staphylococcus* strains have a tendency to develop resistance to antibiotics [33, 34]. Nisin has been successfully applied as a sanitizer against mastitis causing *Staphylococcus* and *Streptococcus* species in lactating cows even when these species are antibiotic resistant [35, 36]. Three nisin-based products were developed for the treatment of bovine mastitis, namely Ambicin N® (Applied Microbiology, Inc., New York) and Mast Out® as well as Wipe Out® Dairy wipes (ImmuCel Corporation, Maine, USA) [30]. *In vivo* nisin has also been shown to be an effective and safe alternative to antibiotics in the treatment of staphylococcal mastitis during lactation in pregnant women [37].

Antibacterial agents possessing various modes of action are particularly of interest in the fight against antimicrobial resistance as it is considered to be more challenging for bacteria to develop resistance against multiple mechanisms concurrently. This has proven true in the case of nisin, as there is very little evidence of transmissible and stable resistance occurring after nearly 50 years of treating food products with this AMP [37–39].

2.2. AMPs as antibiotic adjuvant therapy

The discovery and subsequent development of a wide range of antibiotics have revolutionised modern health care. Over the last century, the introduction of antibiotics drastically reduced morbidity and mortality. Today, antibiotics are readily available to the global population, and effective antibiotic agents have been developed against the majority of illness-causing bacteria. Ironically, the success of antibiotics has resulted in these drugs being misused, leading to the accelerated development of antimicrobial resistance amongst many bacterial species. Antibiotic resistance is making the effective treatment of numerous infections no longer achievable and there is a pressing need for alternative therapeutic approaches.

Antibiotic adjuvant therapy (to achieve synergistic interactions, although additive interactions are also favoured) can be considered as a promising strategy to combat antibiotic resistance. Combination of antibiotics and AMPs that possess different modes of action are valuable in the fight against antimicrobial resistance as it is unlikely for bacteria to develop resistance against multiple mechanisms simultaneously. Several studies have demonstrated synergism between nisin and conventional antibiotics. Nisin displayed synergism with the antibiotics, colistin and clarithromycin, against the common Gram-negative bacteria, *Pseudomonas aeruginosa* [40]. Synergistic effects were also observed with streptomycin, penicillin, rifampicin and lincomycin against *P. fluorescens* as well as the antibiotic-resistant variants of this strain [41]. Daptomycin, teicoplanin and ciprofloxacin displayed synergism against MRSA biofilms [4]. In a study by Dosler and Gerceker, nisin-antibiotic combinations were shown to have synergistic interactions against clinical isolates of methicillin-susceptible *S. aureus* (MSSA), MRSA and *Enterococcus faecalis*. A major finding from their study was that a high incidence of synergistic interactions occurred with a nisin-ampicillin combination against MSSA and nisin-daptomycin combination against *E. faecalis* strains [42]. When nisin is combined with

penicillin, chloramphenicol or ciprofloxacin, biofilm formation of *E. faecalis* was significantly reduced [43].

In a previous study, we also evaluated the interaction of the nisin Z variant with conventional antibiotics [24]. Antibiotic-nisin Z combinations (1:1) were evaluated on *Staphylococcus epidermidis* (ATCC 12228) and *Staphylococcus aureus* (ATCC 12600) seeing as nisin is principally effective against Gram-positive bacterial species. Several conventional antibiotics with different mechanisms of action against Gram-positive bacteria were selected and included methicillin; vancomycin; ampicillin; tetracycline; gentamicin and novobiocin. The minimum inhibitory concentration (MIC) was used as a reflection of the bacterial cytotoxicity following exposure to the antibiotic-nisin Z combinations. The MIC was determined using a modified broth microdilution method [44], where the p-iodonitrophenyltetrazolium violet (INT) was replaced with the yellow tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). The interactions between the antibiotics and nisin were determined using the fractional inhibitory concentrations (FIC) [45] and values were interpreted as ΣFIC ≤0.5 synergistic, ΣFIC >0.5–1.0 additive, ΣFIC >1.0 and <4.0 indifferent and ΣFIC ≥4.0 antagonistic.

Bacterial treatment with nisin Z-antibiotic combinations resulted in the identification of three additive and two synergistic combinations. Nisin Z displayed an additive effect (ΣFIC >0.5 to 1.0) when combined with ampicillin and gentamicin in *S. aureus* (**Table 1**).

Bacterial strain	**MIC of nisin Z (μg/ml)**	**MIC of antibiotic (μg/ml)**	**Nisin Z:antibiotic (1:1)**
			ΣFIC
S. epidermidis	Nisin Z (9.17)	Methicillin (1.88)	2.68
		Vancomycin (2.50)	2.55
		Ampicillin (16.67)	**0.71**
		Tetracycline (80.00)	3.65
		Gentamicin (1.04)	2.23
		Novobiocin (1.46)	**0.50**
S. aureus	Nisin Z (10.00)	Methicillin (1.88)	1.06
		Vancomycin (2.50)	2.50
		Ampicillin (1.04)	**0.66**
		Tetracycline (0.47)	1.40
		Gentamicin (6.67)	**0.94**
		Novobiocin (2.29)	**0.17**

Highlighted values represent ΣFIC values which indicate positive interactions between nisin Z and antibiotics where; ≤0.5 synergistic, >0.5–1.0 additive, 1.1–3.9 indifferent and ≥4.0 antagonistic.

Table 1. MIC values and ΣFIC values for antibiotic-nisin Z combinations.

Furthermore, *S. epidermidis* treated with ampicillin-nisin Z combination also showed an additive interaction. Novobiocin-nisin Z combinations showed synergistic interactions when used against *S. epidermidis* and *S. aureus.* Novobiocin, as part of the aminocoumarins antibiotic group, is able to indirectly block DNA replication by effectively inhibiting bacterial DNA gyrase. Novobiocin-nisin Z combination was particularly effective in the treatment of *S. aureus* as a dramatic reduction in the ΣFIC was witnessed. This may be due to the different, but complementary, mechanisms of actions of nisin Z and novobiocin. As the lipid II-nisin Z complex forms pores in the bacterial membrane, hydrophobic novobiocin can pass through the cell membrane to interact with the DNA gyrase of *S. aureus*. This is only speculation and the exact synergistic mechanism of should be examined further. This *in vitro* study shows the potential of nisin Z for the use as an adjuvant with conventional antibiotics. AMP-antibiotic combination therapy may aid in reinforcing the defences against resistant organisms by making it more challenging for a bacterial strain to adapt to multiple antimicrobial mechanisms. Furthermore, novobiocin is used for the treatment of mastitis in lactating cows [46]; and as previously mentioned, some nisin-based products have been developed for the treatment of mastitis. The synergistic interactions between nisin and novobiocin make this combination especially of interest for developing novel formulations for the treatment of mastitis.

3. Cytotoxic effects of nisin on non-malignant mammalian cells

It is clear that nisin is an effective antimicrobial agent which can inhibit the growth of/kill several Gram-positive bacterial species, including food-borne pathogens such as *Staphylococcus aureus*, *Listeria monocytogenes* and *Clostridium botulinum* as well as exhibiting activity against many clinical important pathogens such as vancomycin-resistant *Enterococci* (VRE), *Streptococcus pneumonia* and MRSA [32, 47, 48]. Despite having exceptional antimicrobial activity, many AMPs also exhibit high toxicity to mammalian cells. An example of a cytotoxic AMP is melittin, the main active component of apitoxin (bee venom). Melittin has an excellent antibacterial activity and the antimicrobial mechanism of this AMP is most likely the permeabilisation of cell membranes by pore formation resulting in cell lysis and death [49]. Although melittin has effective broad-spectrum antimicrobial activity, this AMP is extremely toxic to mammalian cells even at very low concentrations.

As mentioned before, nisin has a *Generally Regarded as Safe* (GRAS) status and is considered safe for human consumption. The Accepted Daily Intake (ADI) of nisin was determined by the FDA as 2.94 mg/per day (0.049 mg/kg body weight/day) in 1988, prior to receiving GRAS status [50]. In a study by Joo and co-workers, mice were exposed to a concentration of nisin more than ×1000 (150 mg/kg body weight/day) the recommended ADI over a period of 3 weeks with no signs of cytotoxicity [51]. In another study, mice were treated with doses of 800 mg/kg body weight/day (more than 10 000 times higher than the recommended ADI) ultra-pure nisin Z for 3 weeks without any evidence of toxicity [52]. In both these studies, long-term (>3 weeks) treatment with high concentrations of nisin did not result in any observable toxicity.

http://dx.doi.org/10.5772/intechopen.71927

We also investigated cytotoxicity of nisin Z towards mammalian cells using the MTT assay to measure metabolic activity and the lactate dehydrogenase (LDH) assay to indicate membrane integrity. The non-malignant human immortalised keratinocyte (HaCaT) cells were employed for cytotoxicity testing and cultured under normal conditions [24]. Briefly, HaCat cells were seeded in a 96-well plate and incubated until ~90% confluent. Synthetic melittin was used (≥97% HPLC from Sigma-Aldrich) as a positive AMP control for cytotoxicity. After 24 h of exposure to nisin Z or melittin (2.5–40 μg/ml), the MTT assay was performed as described previously [24]. The ability of NAD(P)H-dependent cellular oxidoreductase enzymes to reduce MTT to formazan is considered a reflection of the number of viable cells present. Cell viability is expressed as a percentage relative to the untreated control, which was set as being 100% viable. For an assay positive control, cells were exposed to 0.01% Triton-X 100 (Sigma-Aldrich, St Louis, MO, USA).

To investigate the effect of the two AMPs on cell membrane integrity, the CytoTox-ONE™ Homogeneous Membrane Integrity Assay (Promega, Madison, WO, USA) was employed. This assay determines the release of lactate dehydrogenase (LDH) into the culture media from cells with impaired cell membranes. HaCat cells were exposed to melittin and nisin Z as described earlier. A lysis solution (Promega) was used as a maximum LDH release positive control. The LDH release assay was performed as described previously [24]. Results are conveyed relative to the untreated control (set to 0% LDH release) and the maximum release sample (set to 100% LDH release).

Cytotoxicity data (**Figure 2**) shows that nisin Z did not negatively affect the cell viability of HaCat cells.

The MTT assay indicates that the ability of NAD(P)H-dependent cellular oxidoreductase enzymes to reduce MTT to formazan was not affected by the exposure to the tested nisin

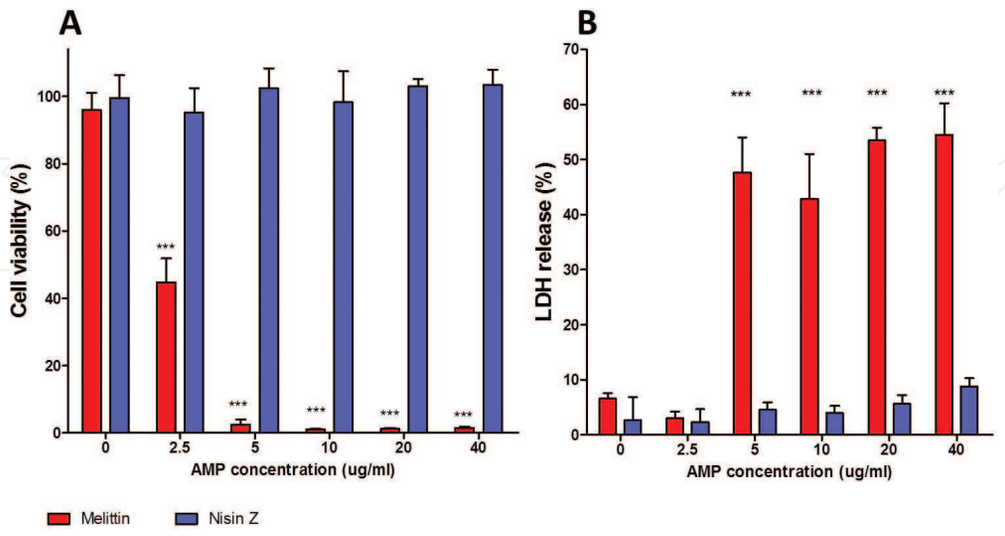

Figure 2. Cytotoxicity assay of HaCat cells exposed to the AMPs melittin and nisin Z. (A) MTT assay and (B) LDH release assay. Vehicle control groups are represented by 0 mg/ml. Values represent mean stdev n = 3. ***$p < 0.001$ compared to the vehicle control group.

Z concentrations. Indicating that nisin Z did not negatively affect the cell viability of HaCat cells. The LDH assay also showed that there was no significant increase in the release of LDH, indicating that nisin Z did not cause any measurable membrane damage. On the other hand, both the MTT and LDH assays showed that relatively low concentrations of melittin led to a considerable increase in cytotoxicity in HaCat cells.

These *in vitro* results eco many of the recent *in vivo* findings, showing that nisin exposure to non-malignant cells has very little to no cytotoxic effects. Even at concentrations of nisin Z that exceeds the MICs for *S. aureus* and *S. epidermidis* (**Figure 1**) no toxicity was observed. Keeping in mind that nisin is an effective antimicrobial agent against several Gram-positive bacterial species, including clinical important and resistant pathogens, this AMP shows promising potential for clinical application.

4. Cytotoxic effect of nisin on malignant cells

Over the last few decades, great strides have been made in cancer treatment and therapies, leading to the steady decline of cancer death rates [53]. Despite these developments, many current cancer therapies are still associated with high cytotoxicity and lack specificity. There is consequently still a need for the development of novel anti-cancer therapies. AMPs, especially bacteriocins, display selectivity towards cancer cells [10]. These AMPs are, therefore, potential alternative candidates to current chemotherapeutic agents. AMPs can also be applied as adjuvants to chemotherapeutic agents to lower the therapeutic doses needed with the intention of quelling the toxicity of these treatments.

Studies have previously investigated the anti-tumour potential of nisin *in vitro* and *in vivo* for head and neck squamous cell carcinoma (HNSCC) [52]. The study by Joo and co-workers indicated that nisin has the ability to selectively induce apoptosis, cell cycle arrest and reduce cell proliferation in HNSCC cells, compared to primary keratinocytes *in vitro* [51]. *In vivo*, nisin treatment reduced the overall tumour burden compared to non-nisin treated groups, in a floor-of-mouth oral cancer xenograft mouse model. Also, to examine the mechanism by which nisin facilitates its anti-proliferative and pro-apoptotic effects on HNSCC cells, the effect of nisin-treatment on the expression of 39,000 genes was examined by using Affymetrix gene arrays. The expression of multiple genes was altered, including those in the apoptotic and cell cycle pathways, membrane physiology, energy and nutrient pathways, ion transport and signal transduction and protein binding pathways. The *CHAC1* gene, a cation transport regulator and apoptosis mediator were dramatically up-regulated. This study was the first to show that the antibacterial food preservative nisin could effectively reduce and prevent tumorigenesis both *in vitro* and *in vivo.*

4.1. Cytotoxic effects of nisin Z on melanoma cells

We also evaluated the potential of nisin Z to induce selective cytotoxicity towards human melanoma cells *in vitro*. Melanoma is the leading cause of skin cancer-related deaths [54, 55].

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Contrary to most types of cancer, the frequency of melanoma has been increasing over the last three decades [54]. In addition to a high mortality rate, Melanoma cells also have a sinister tendency to rapidly develop resistance to mainstream chemotherapeutic agents [12, 13]. *In vitro* cytotoxicity of the nisin Z was determined by employing the MTT assay, LDH assay and flow cytometric apoptosis and necrosis analyses. The non-malignant human keratinocyte (HaCat) cell line was used as a control. The MTT and LDH assays were performed, as described previously [56]. The flow cytometric FITV Annexin V apoptosis assay (BD Pharmingen™, BD Biosciences, San Jose, CA, USA) was employed for the detection of apoptotic cytotoxicity. FITC Annexin emits green fluorescence and its presentation indicates early apoptotic events, while propidium iodide (PI) emanates red fluorescence and is associated with late apoptotic or necrotic cells.

The quantitative colourimetric MTT assay was used to investigate the cytotoxic effect of nisin Z on cultured melanoma cells as well as non-malignant keratinocytes. There is a clear concentration-dependent decline in cell viability observed in melanoma cells exposed to nisin Z (**Figure 3A**).

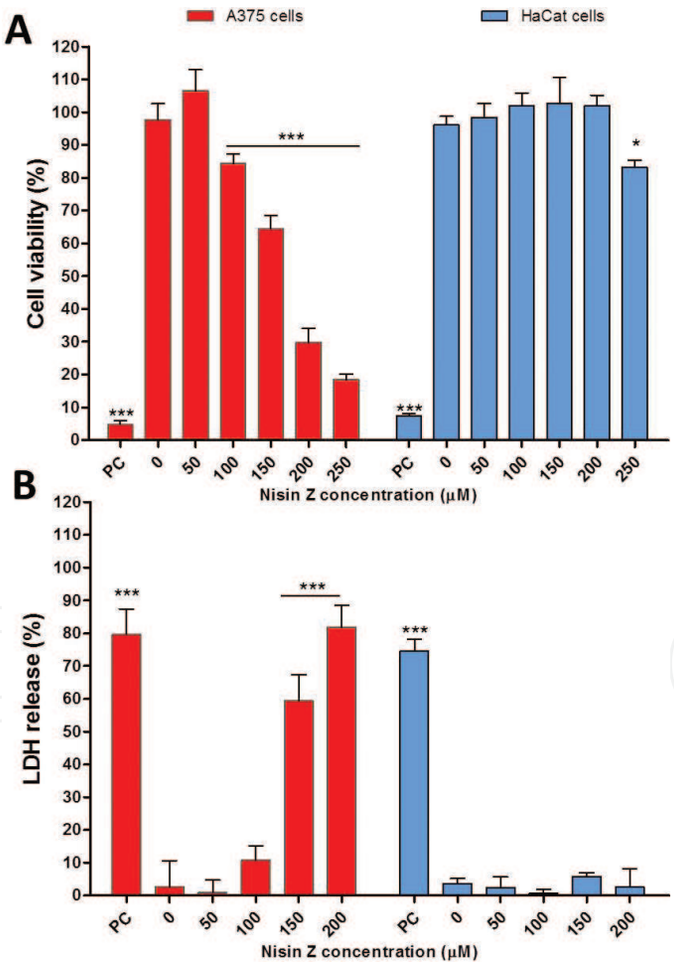

Figure 3. Cytotoxic effects of nisin Z on melanoma (A375) cells. (A) Cell viability was determined using the MTT assay. (B) LDH release from cells following treatment with nisin Z. Keratinocytes (HaCat) were used as a non-malignant control. * $p < 0.05$ and *** $p < 0.001$ compared to the control groups.

A significant increase in cytotoxicity is observed in melanoma cells after exposure to relatively low concentrations of nisin Z. The IC_{50} value of melanoma cells exposed to nisin Z is approximately 180 μM. Conversely, the non-malignant keratinocytes exposed to nisin Z presented with considerably higher cell viability, with an IC_{50} value more than double that of its malignant counterpart. To examine whether the observed cytotoxicity of melanoma cells exposed to nisin Z is the result of membrane damage, the LDH assay was performed. This assay measures the release of lactate dehydrogenase, the cytosolic enzyme, as a result of cellular plasma membrane damage. Results suggest that the exposure of melanoma cells to nisin Z concentrations of 150 μM and higher (**Figure 3B**) lead to in a significant increase in LDH release. No significant LDH release was detected in the non-malignant keratinocytes after nisin Z exposure, indicating very little membrane damage. Both, the basic cytotoxicity assays (MTT and LDH assay) suggest that nisin Z is selectively more toxic towards cultured melanoma cells compared to non-malignant cells.

Flow cytometry was used to investigate whether the cytotoxicity observed in melanoma cells was of apoptotic or necrotic origin. For the non-malignant keratinocyte cells, the flow cytometric analysis indicated that >98% of the cells exposed to 50 μM nisin Z could be considered viable and is comparable to the untreated control (**Figure 4**).

A small increase in cytotoxicity is observed at higher concentration. Melanoma cells exposed to 50 μM nisin Z showed a much larger early apoptosis (>17%) population than their non-malignant counterparts. A significant increase in cytotoxicity is observed in melanoma cells exposed to higher concentrations of nisin Z, resulting in approximately half of the cancer cells undergoing apoptosis/necrosis after being exposed to nisin Z concentrations of 100 μM or higher. These results confirm the basic viability data that nisin Z is more selectively cytotoxic to melanoma cells and give an indication that the cell death observed in these cells is probably due to the activation of an apoptotic pathway.

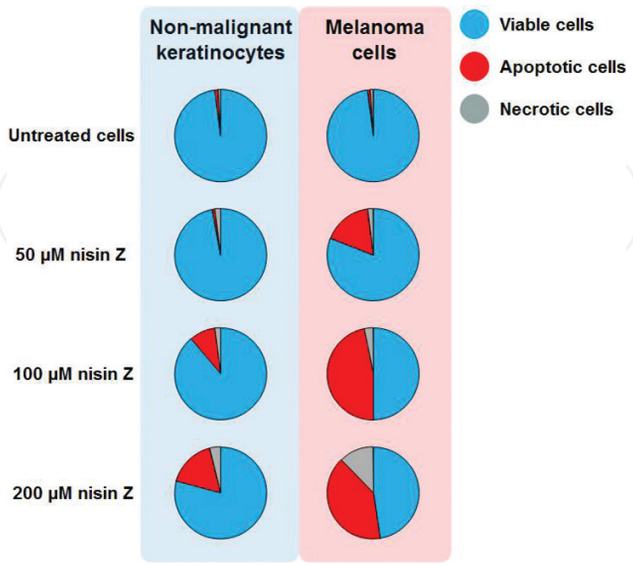

Figure 4. Pie graphs representing the cell population sizes of viable, apoptotic and necrotic non-malignant keratinocyte (HaCat) and melanoma (A375) cells after exposure to 50–200 μM of nisin Z for 24 h.

4.2. The potential of nisin Z to increase the cytotoxicity and selectivity of conventional chemotherapeutic agents

Due to the toxicity associated with some conventional chemotherapeutic agents, as well as the constant threat of malignancies evolving chemotherapy resistance [11–13], there is a necessity for the development of novel anti-cancer therapies. To combat chemotherapy resistance, the efficacy of chemotherapeutic agents can be enhanced by the co-administration of multifunctional agents to achieve synergistic interactions [14, 15].

As stated earlier, there is an abundance of studies which investigated the use of nisin as an adjuvant to conventional antibiotics [4, 40–42, 57]. It has been shown that nisin displays anticancer properties; however, inadequate focus has been given to applying nisin as an adjuvant for chemotherapeutic agents. The ability of nisin to increase the activity of the chemotherapeutic drug, doxorubicin, was investigated *in vivo* by Preet and co-workers [16]. Doxorubicin (Adriamycin) is traditionally employed to treat breast cancer, bladder cancer, lymphoma, and acute lymphocytic leukaemia, to name a few. When combining nisin with doxorubicin, enhanced anti-cancer activities were observed and apoptosis could be detected upon treatment of mice with induced skin carcinogenesis as well as a slight increase in oxidative stress. However, the exact mechanism by which nisin exerts its anti-cancer activities was not determined [16]. It is suggested that AMPs, which display anticancer activity, should be used in combination with conventional chemotherapeutic agents to enhance the effectiveness of these treatments, prevent recurrence of cancer following treatment and possibly reduce instances of chemotherapy resistance [58, 59]. Other studies have also shown that AMPs have the potential to enhance the effectiveness of conventional chemotherapeutic agents. The cytotoxicity of etoposide and cisplatin could be enhanced through the combination with magainin A and magainin G, respectively [60]. More recently, it was shown that the combination of melittin and 5-Fluorouracil enhanced cytotoxic effects against squamous skin cancer cells, while simultaneously reducing the toxicity to normal keratinocytes [61]. There are currently no AMPs that have entered into clinical trials or that are in preclinical development as cancer therapeutics. However, peptide-derived therapies are being recognised for the selectivity and anticancer effectiveness and have been investigated in clinical trials [59]. For example, the peptide asparagine-glycine arginine tumour homing peptide (NGR-hTNF) has completed phase 1 clinical trials and is waiting to enter phase 2 clinical trials to test its effectiveness when used in combination with cisplatin for the treatment of several refractory solid tumours including melanomas [62].

Based on the findings that nisin Z is more selectively cytotoxic to melanoma cells, the cytotoxic effect of the combination of nisin Z with conventional chemotherapeutic agents was investigated in cultured melanoma cells. The effect of combinations of nisin Z with conventional chemotherapeutic agents (5-Fluorouracil, etoposide, hydroxyurea) on A375 (melanoma) and HaCat (non-malignant keratinocyte) cells was determined by the MTT assay. Cells were exposed to different concentrations of the respective chemotherapeutic agents independently and in combination with 150 μM of nisin Z for 24 h. Following exposure, the MTT assay was performed as described earlier. Blank and background measurements were subtracted and cell viability is expressed as a percentage relative to the untreated control, which was set as

100% viable. Possible synergistic interactions were evaluated by comparing the cytotoxicity of combination treatment with mono-treatment on melanoma cells.

The chemotherapeutic agent 5-Fluorouracil can inhibit RNA and DNA synthesis leading to cell death. The combination of nisin Z with 5-Fluorouracil increased the cytotoxicity to melanoma cells over the entire concentration range tested compared to the mono-treatment of 5-Fluorouracil ($p < 0.05$) (**Figure 5A**), with no significant increase in toxicity to non-malignant keratinocytes (**Figure 5B**).

The 5-Fluorouracil treatment is initially cytotoxic at 50 μM ($p < 0.01$ compared to the control), whereas the combination of 5-Fluorouracil and nisin Z only begins to induce toxicity at 200 μM ($p < 0.001$ compared to the control) in the non-malignant keratinocytes. Results indicate that the 5-Fluorouracil-nisin Z combination is more cytotoxic to melanoma cells compared to the mono-treatment. The anti-cancer activity of 5-Fluorouracil may, therefore, be enhanced by combination treatment with nisin Z. Etoposide is a chemotherapeutic agent that is able to induce DNA strand breaks in cancer cells by interfering with type II topoisomerase, ultimately inducing apoptosis. When combining etoposide with nisin Z it was found that the activity towards melanoma cells was enhanced compared to mono-treatment across the entire concentration range ($p < 0.001$) (**Figure 5C**), with no increase in cytotoxicity to non-malignant keratinocytes (**Figure 5D**). In melanoma cells, the combination of nisin Z with etoposide had a higher level of activity at the lowest concentration tested compared to the highest concentration for mono-treatment ($p < 0.001$). The anti-cancer activity of etoposide can, therefore, be significantly enhanced through the combination of nisin Z. Hydroxyurea is able to induce DNA damage and inhibit DNA synthesis. The combination of nisin Z with hydroxyurea was able to increase the cytotoxicity to melanoma cells at concentrations of between 25 and 400 μM compared to the mono-treatment of hydroxyurea ($p < 0.01$) (**Figure 5E**), with no significant increase in toxicity to non-malignant keratinocytes (**Figure 5F**).

To evaluate if possible synergistic interactions occurred between the chemotherapeutic agents and nisin Z, the cytotoxicity of melanoma cells following the mono-treatment of the respective chemotherapeutic agents (50 μM) was compared to that of the mono-treatment of nisin Z (150 μM), followed by that of the combination (50 μM chemotherapeutic agent +150 μM nisin Z). Synergism occurs when the combined effects of the different components are greater than their individual effects. The cell viability of melanoma cells was significantly lower for all combinations compared to mono-treatment with the chemotherapeutic agent alone ($p < 0.05$) (**Figure 6**). However, the only combination that displayed synergism was the combination of nisin Z with etoposide.

The AMP nisin, which is considered safe for human consumption, not only displays antibacterial properties, but also anti-cancer activities. Although the use of nisin as an adjuvant for conventional antibiotics has been investigated extensively, there are few studies investigating nisin as an adjuvant for conventional chemotherapeutic agents. Nisin also exhibits immune-modulatory properties. We have shown that nisin Z induces selective cytotoxicity towards melanoma cells through an apoptotic pathway. These properties make nisin Z an attractive anti-cancer agent to be used alone or in combination with current chemotherapeutic agents to enhance anti-cancer properties of these agents, while also potentially combatting chemotherapy resistance. Here,

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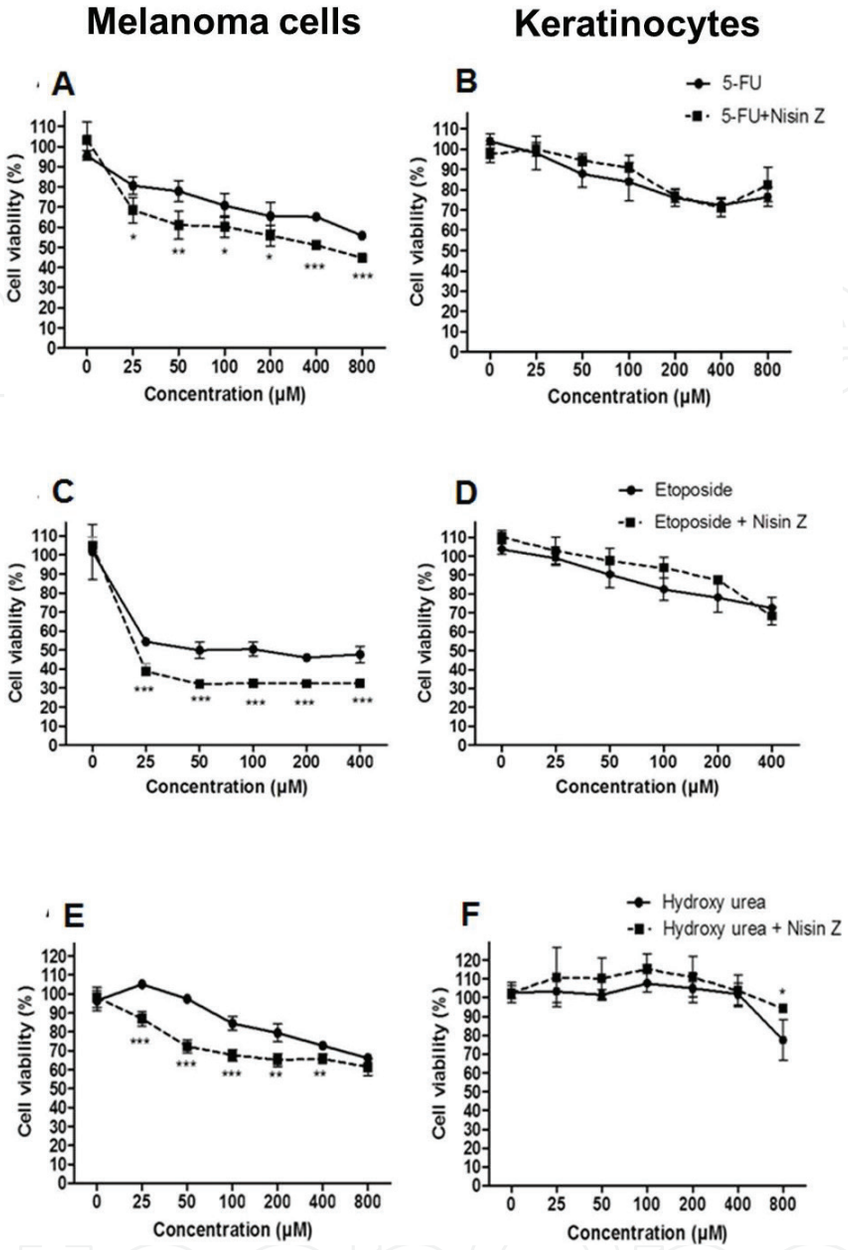

Figure 5. Cytotoxicity of chemotherapeutic agents in combination with nisin Z on melanoma (A375) cells and non-malignant keratinocytes (HaCat) as determined by the MTT assay. (A) Melanoma cells exposed to 5-Fluorouracil (FU) combinations. (B) HaCat exposed to 5-FU combinations. (C) Melanoma cells exposed to etoposide combinations. (D) HaCat exposed to etoposide combinations. (E) Melanoma cells exposed to hydroxyurea combinations. (F) HaCat exposed to hydroxyurea combinations. Vehicle control groups were included and are represented by 0 μM. Results are expressed relative to the untreated controls which were set as being 100% viable. Bars represent the standard deviation, n = 4. *$p < 0.05$, **$p < 0.01$ and *** $p < 0.001$ for combination compared to chemotherapeutic agent alone.

it was shown that combinations of nisin Z with 5-Fluorouracil, hydroxyurea and etoposide was able to enhance the cytotoxicity to melanoma cells, while no significant increase in toxicity toward non-malignant keratinocytes were observed. Especially of interest is the consequence of nisin Z on the effectiveness of etoposide, seeing as etoposide resistance is known in melanoma [63, 64]. The combination of nisin Z with etoposide was able to significantly and selectively

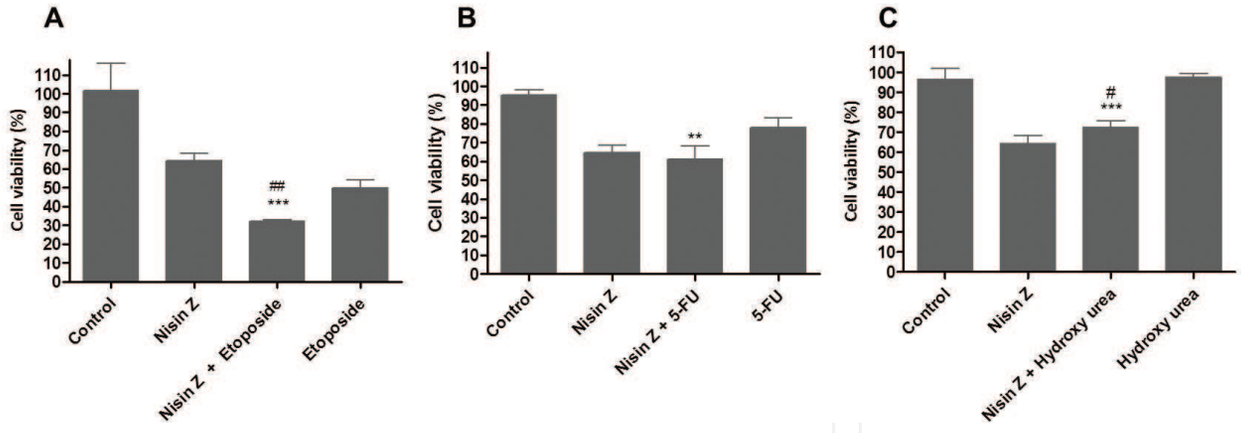

Figure 6. Cytotoxicity results for mono-treatment and combinations of chemotherapeutic agents (50 μM) and nisin Z (150 μM) as determined by the MTT assay. Nisin Z was combined with (A) etoposide, (B) 5-Fluorouracil and (C) hydroxyurea. Bars represent the average and error bars the standard deviation, n = 4. **$p < 0.01$ and ***$p < 0.001$ for combination compared to chemotherapeutic agent alone. #$p < 0.05$ and ##$p < 0.01$ for combination compared to nisin Z alone.

enhance the cytotoxic effect etoposide to melanoma cells. Synergism was also observed when combining nisin Z and etoposide with regards to the cytotoxic effect in melanoma cells. Based on all the properties of nisin Z and its GRAS status it could, therefore, be considered as an adjuvant for conventional chemotherapeutic agents.

5. Conclusion

The majority of AMPs exhibit direct microbial killing activity and occur in all living species as an important part of their innate immune system. Due to the co-evolution of AMPs and bacteria, bacterial species are less likely to develop resistance to these peptides compared to conventional antibiotics. The lantibiotic, nisin, has a promising potential for clinical application as it exhibits very low cytotoxicity to mammalian cells, while displaying potent antimicrobial activity against several common foods borne and clinically important Gram-positive bacteria. The use of nisin against Gram-negative bacteria still remains limited. Nisin can be considered as a promising adjuvant for antibiotics in the treatment of Gram-positive bacteria. Antibiotic-nisin combinations can potentially be used to lower the therapeutic dose of antibiotic treatments, while also enhancing the antimicrobial activity by employing multiple modes of action. With multiple antimicrobial mechanisms concurrently in play, these combinations can hinder the development of antibiotic resistance. We have demonstrated that nisin Z displays synergism when combined with novobiocin against *S. aureus*. This bacterial species is associated with mastitis. Both nisin-based products and novobiocin are used for the treatment of mastitis. The synergistic interactions between nisin and novobiocin make this combination, especially of interest for developing novel formulations for the treatment of mastitis (**Figure 7A**).

Apart from the antimicrobial properties of nisin, this AMP also displays promising anticancer potential towards several types of malignancies. This chapter discussed the anti-cancer potential of nisin Z towards cultured melanoma cells. Results showed that this AMP is more

cytotoxic to melanoma cells compared to non-malignant keratinocytes. It was shown that nisin Z disrupts the cell membrane integrity of melanoma cells, while also inducing apoptosis in the majority of exposed malignant cells (**Figure 7B**). Taking into account these anticancer properties of nisin Z, the cytotoxicity of nisin Z-chemotherapeutic agent combinations to melanoma cells was compared to the mono-treatment with selected conventional chemotherapeutic agents. This study indicated that when used in combination with the conventional chemotherapeutic agents (5-Fluorouracil, hydroxyurea and etoposide), nisin Z has the potential to enhance the cytotoxicity of these conventional chemotherapeutic agents against cultured melanoma cells. Synergism was observed between the nisin Z and etoposide combination. However, this study was only limited to the *in vitro* effect in melanoma cells with regards to cytotoxicity as measured by the MTT assay. For future *in vitro* studies, it is suggested that more cancer cell lines be included. The mechanistic interaction between nisin Z and the chemotherapeutic agents should also be investigated. It is also suggested that *in vivo* studies be conducted similarly to that by Preet and co-workers to assess whether the combination of nisin Z with these conventional chemotherapeutic agents are able to reduce melanoma tumorigenesis *in vivo* [16]. The effective dosages also need to be determined with *in vivo* assays. Nisin Z has great potential for clinical application considering its low cytotoxicity to non-malignant cells and the effectiveness of this AMP against Gram-positive bacterial strains and certain cancers. However, detailed antimicrobial and anticancer mechanistic interaction studies analysis are lacking and many *in vitro* results must still be confirmed within *in vivo* systems.

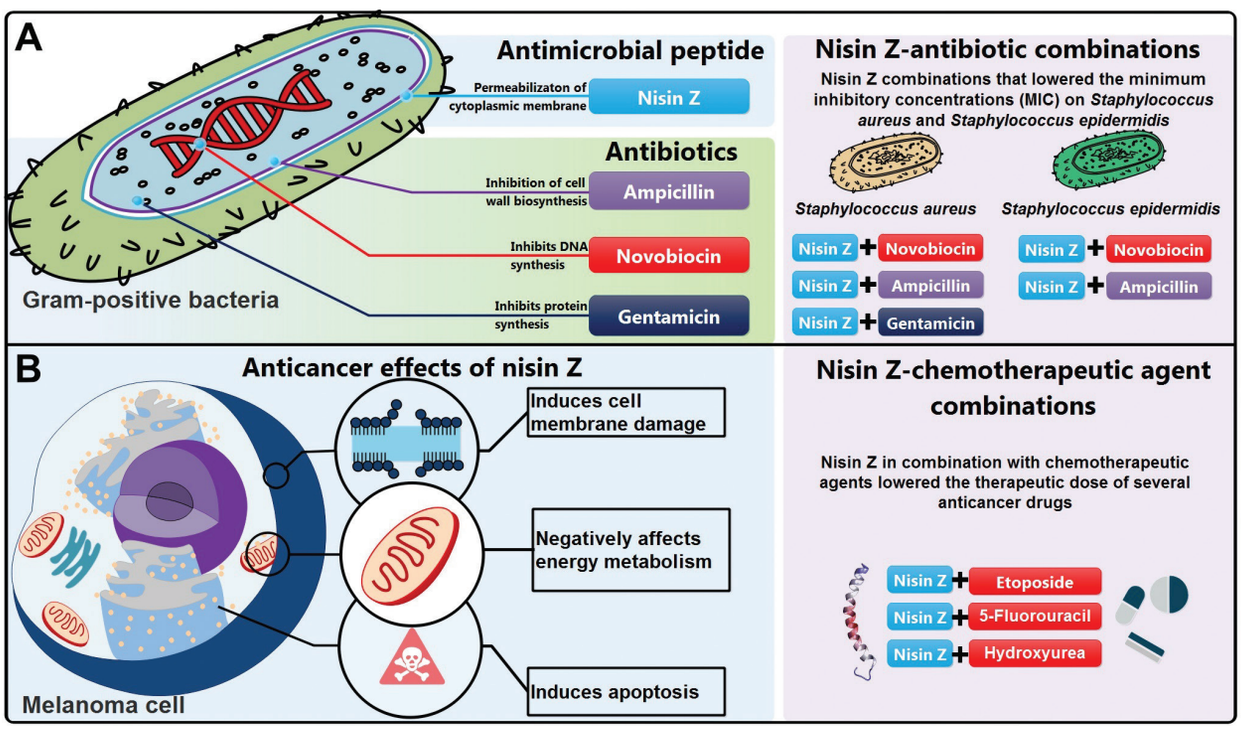

Figure 7. Summary of the antimicrobial and anticancer properties of nisin Z alone and in combination with conventional therapies. (A) The antimicrobial effects and mechanisms of action of nisin Z and selected antibiotics alone and in combination on gram-positive bacteria. (B) The cytotoxic effect of nisin Z on cultured melanoma cells and combinations of this AMP with conventional chemotherapeutic agents.

Acknowledgements

AL is grateful for financial assistance from the National Research Foundation (NRF) of South Africa (Grant Number 94942). Opinions expressed and conclusions arrived at are those of the authors and are not to be attributed to the NRF.

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