

Nisin Augments Doxorubicin Permeabilization and Ameliorates Signaling Cascade during Skin Carcinogenesis

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Abstract

Background: Multidrug resistance exhibited by cancerous cells has proved to be a big hurdle in the development of an effective anti-cancer therapy. We previously demonstrated nisin-doxorubicin adjunct therapy to exhibit strong additive anti-cancer effect against DMBA-induced murine skin carcinogenesis but the mechanism remained unexplored.

Methods: The *in vivo* tumoricidal activity of the combination was validated in terms of animal bioassay observations while *ex vivo* anti-cancer effect was monitored by employing HaCat cell lines.

Results: The combination was found to be additive *in vivo* as evidenced by larger decreases in mean tumor burden and tumor volumes. The IC₅₀ values of nisin and DOX alone were evaluated to be 16 µg/ml and 4 µg/ml respectively while the sub-inhibitory concentration of DOX was reduced to 2 µg/ml when nisin was used as an adjunct. Q values calculated using MTT assays indicated the combination to be synergetic than additive. The mechanism was found to involve enhanced membrane permeabilization as well as reduced expression of NF-Kb, TNF-α, TNF-β, IL-1 and IL-6.

Conclusion: As combined effect of nisin and DOX even at halved concentrations was significantly higher than either drug alone, these observations might help in lowering the chemotherapeutic doses of DOX thereby decreasing its associated side effects.

Keywords: Nisin; Doxorubicin; Synergy; Cytokines; Nuclear factor-Kb; Skin cancer

Introduction

Severe toxicity and low efficacy of existing anti-cancer drugs against multi drug resistant cancer cells still precludes the successful development of a novel class of agents for successful clinical use [1,2]. It necessitates the exploitation of alternative anti-cancer strategies with minimal side effects which are unaffected by common mechanisms of chemo-resistance.

In this context the development of various cationic antimicrobial peptides (AMPs) as anticancer peptides (ACPs) is currently being considered to be a promising and interesting alternative [3,4]. These AMPs and/or ACPs are effector molecules of the innate immune system and possess direct antibacterial, anticancer as well as immunomodulatory properties. Owing to their cationic charge, they also tend to be very selective towards the cancerous cells thereby sparing the normal cells of the body [5]. Numerous AMPs like gomesin, temporin, human neutrophil peptide (HNP)-1 and dermaseptin etc. [6] have been reported to exert strong anticancer effect against various types of cancers such as breast, prostate, ovarian and skin cancers [7]. Considering the immense amount of AMP sequences deciphered till date [8] such peptide based drugs can prove out to be vital novel anti-cancer pharmaceuticals with minimal side effects.

A yet another emerging approach to tackle multi drug resistance and associated side effects of conventional single agent chemotherapy is the use of multi-component anti-cancer therapeutics [9]. Currently; nearly all successful chemotherapy regimens are combinations of multiple agents given simultaneously thereby achieving better therapeutic efficacy and minimal side effects [10]. In this context, various cationic peptides have also been reported to enhance the efficacy of conventional chemotherapeutics against several cancer cell lines [11]. Amongst AMPs, nisin in particular makes a good anti-cancer drug candidate as it is the only bacteriocin approved as “Generally Regarded as Safe” (GRAS) compound for use as food preservative in over 50 countries

[12]. We have also recently demonstrated an additive *in vivo* anti-cancer effect of nisin in conjunction with a conventional chemotherapeutic; doxorubicin (DOX) against murine skin carcinogenesis [13]. The present study further delineates the underlying mechanism of nisin-DOX additive anticancer action *in vivo* (in a murine model) and *in vitro* (using HaCaT cell lines) against skin carcinogenesis.

Materials and Methods

Material

All the chemicals used in the present study were of analytical grade and were obtained from standard companies. ELISA plates, tissue culture plates (96 /8 well), culture dishes, Serological plates, Roux culture bottles, 0.2µm sterile syringe filters (non-pyrogenic). DMBA (7,12-Dimethylbenzylanthracene), TPA (12-O-tetradecanoylphorbol-13-acetate), RPMI 1640 (Rose Well Park Memorial Institute Lab no. 1640) (Hi-media, India), doxorubicin nisin and 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide were procured from Sigma Aldrich. N-phenyl-1-naphthylamine (NPN), bovine serum albumin (BSA), 5,5-dithiobis-2-nitrobenzoic acid (DTNB), thiobarbituric acid (TBA), ethanol, formaldehyde, hydrogen peroxide (H₂O₂), benzene, paraffin wax, xylene, trichloroacetic acid (TCA), sodium chloride (NaCl), EDTA, Folin's Reagent, sodium carbonate, copper sulphate,

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Received November 14, 2015; **Accepted** November 25, 2015; **Published** December 19, 2015

Citation: Preet S, Pandey SK, Saini N, Koul A, Rishi P (2015) Nisin Augments Doxorubicin Permeabilization and Ameliorates Signaling Cascade during Skin Carcinogenesis. Transl Med 6: 161. doi:10.4172/2161-1025.1000161

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potassium tartarate and other chemicals used were obtained from reputed Indian manufacturers (Sisco Research Laboratory, Central Drug House, SD fine chemicals).

Animals: Female BALB/c mice, 6-8 weeks old (18-30g), bred in Central Animal House of Panjab University, Chandigarh (India) were used in the present study. All the animals received standard pellet diet (M/S Ashirwad Industries Pvt. Ltd., Punjab, India) and water *ad libitum*. The experimental protocols were approved by the Institutional Animal Ethics Committee of Panjab University, Chandigarh (India).

Methods

Experimental design and tumor induction: The mice in the test group were challenged with three subcutaneous consecutive doses of dimethylbenzanthracene (DMBA) (1 mg/0.2 ml in olive oil) at an interval of seven days [13] following this all animals were kept under normal conditions in polypropylene cages. Fifteen weeks post-DMBA challenges, the animals in the test group were further segregated into five subgroups. The therapy with nisin and DOX was started post 15 weeks of DMBA injection, where nisin was injected sub-cutaneously (s.c) at alternate days and doxorubicin was administered intravenously (i.v) twice a week at an interval of three days for two months duration.

Group A: Untreated tumor group.

Group B: Received DOX 10 mg/kg b.w. (body weight)

Group C: Received nisin 50 mg/Kg b.w.

Group D: Received nisin + DOX 25 mg/Kg+5mg /Kg b.w.

Group E: Received nisin + DOX 50 mg/Kg+10mg /Kg b.w.

Tumor analysis: During the tumor induction study and treatment period the animals were carefully observed for the presence of lesions/papillomas/ tumors on a weekly basis and the tumor diameters were measured using Vernier calliper (s). Post-treatment period, animals were sacrificed and tumors were exercised for further analysis. Total numbers of tumors were also counted at the end of the treatment period. Tumor volume and tumor burden were calculated using the following formula [14]:

Mean Tumor Volume: $4/3\pi r^3$ (r= radius of tumor)

Mean Tumor Burden: mean tumor volume \times mean number of tumors

Percentage Tumor Inhibition: (tumor volume of untreated tumor - tumor volume of treated tumor)/tumor volume of untreated tumor $\times 100\%$

Cell culture and maintenance: The human keratinocyte cell line, HaCaT [15] was a kind gift from a friend from Institute of Microbial Technology (IMTECH), Chandigarh, India and was maintained in RPMI 1640 (Sigma Aldrich chemicals, St Louis, MO, USA), supplemented with 10% (vol/vol) heat inactivated (56°C, 30 minutes) fetal bovine serum (FBS; Himedia), 100 IU/ml penicillin, and 100 IU/ml streptomycin at 37°C in humidified air with 5% CO₂ in the departmental cell culture facility.

Cell Viability assay: The cell viability was analyzed by MTT (3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide) assay [16]. HaCaT cells (9×10^3) were plated in triplicate in a 24-well cell culture plates (containing RPMI-1640) at 37°C in a humidified CO₂ (5%) incubator overnight. Complete medium was replaced after 24 h with 100 μ l of fresh medium (RPMI-1640) containing various concentrations of drugs. After a further 24 h, cells were incubated

with MTT at 37°C for 4 h. Thereafter DMSO was added to dissolve the formazan crystals and the absorbance at 492 nm was measured with a microplate reader. Jin's formula [17] was used to further quantify the synergistic effect of the combination treatment of nisin and DOX. The formula is: $Q = Ea + b / (Ea + Eb - Ea \times Eb)$, where Q is the combination index; Ea+b represents the cell proliferative inhibition rate of the combined drug; Ea and Eb are signs of the cell proliferative inhibition rate of each drug. After calculation: $Q < 0.85$, $Q > 1.15$ and $0.85 < Q < 1.15$ indicate antagonism, synergy, and additive effect, respectively.

Permeabilization Assay: The fluorescent dye NPN assay was used to determine outer membrane permeability of the peptides [18]. Briefly, HaCat cells (9×10^3) were treated with various sub-inhibitory concentrations with Nisin, Doxorubicin and combination of both (nisin+DOX). NPN was added to 2 ml of cell suspension in a quartz cuvette to give a final concentration of 10 μ M, and the background fluorescence was measured (excitation wavelength=350 nm, emission wavelength=420 nm). After addition of the drugs, the increase in fluorescence was measured using an F-4500 fluorescence spectrophotometer (Hitachi, Japan). Polymyxin B was used as a positive Control because of its strong outer membrane permeability properties. Relative Florescence units (fluorescence value of cell suspension with the test substance and NPN subtracted with the corresponding value of the cell suspensions and NPN without the test substance) were measured by spectra analysis ranging from 350- 550 nm.

Cytokine measurements by ELISA: Level of cytokines were measured using commercially available kits in the serum obtained from mice (Mouse Cytokine 20-Plex, Novex[®] Life Technologies) in all the *in vivo* treatment groups as well as in cell supernatants of HaCat cells (BD Bioscience) incubated with various peptide-drug combinations. Briefly, Micro-ELISA plates were coated with either/or the cell supernatant and serum sample and incubated at 37°C for 1 hour. Following this, each sample was tested in duplicate for the levels of TNF- α , TNF- β , IL-1, IL-6, and NF kappa B by ELISA according to the manufacturers' instructions. Optical densities were measured using an ELISA reader at 450 nm. Cytokine concentrations were calculated using standard curves that were performed for each ELISA plate.

Statistical Analysis: Quantitated Data is represented as Mean \pm SD of three independent values. The data were analysed by Student's test for the tumor statistics experiment and by one way ANOVA followed by post hoc test for the other experiments.

Results

Tumor induction studies

Subcutaneous DMBA administration led to the formation of fully blown tumors after fifteen weeks which was accompanied by skin hardening, drying and formations of lesions at different stages (Figure 1D). In DOX-alone treated mice, the percentage tumor inhibition was observed to be 21%, 24.39% and 24% after two, six and eight weeks of chemotherapy respectively. Interestingly, an augmentation in this anti-cancer activity was observed when nisin was co-administered along with DOX as the percentage tumor inhibition was increased to 37.31%, 36.61% and 42.5% ($*p \leq 0.05$) (Figure 1C) after two, six and eight weeks of adjunct chemotherapy. The recorded mean tumor volume in untreated tumor induced group after fifteen weeks of DMBA administration (sat the start of the therapy) was 293 ± 6.02 mm³ which further increased to 650 ± 36.05 mm³ after eight weeks. On the other hand, tumor volumes were found to be reduced to 322 mm³ ($^{\$}p \leq 0.05$) (Figure 1A) in groups treated with nisin + DOX after eight weeks of therapy as compared to DOX-alone treated group the volume was

decreased to $524 \pm 25.25 \text{ mm}^3$ only. A similar additive response of the combination could be observed in decreases in respective tumor burdens in all the groups (Figure 1B).

Cell viability assay

HaCat cells were treated with various concentration(s) (0-64 $\mu\text{g/ml}$) of nisin and DOX alone and in combination with each other and the percentage cell viability was found to be decreased in a concentration dependent manner (Figure 2A). The inhibitory concentration (s) IC_{50} values of DOX and nisin against were evaluated to be 4 $\mu\text{g/ml}$ and 16 $\mu\text{g/ml}$ respectively. Interestingly, when the cells were incubated in the presence of various combined concentrations of these two agents, the IC_{50} value of DOX was found to be halved to 2 $\mu\text{g/ml}$ in presence of as low as 2 $\mu\text{g/ml}$ of nisin. Based on IC_{50} value of each drug alone, the effect of the combination on cell growth was also evaluated using MTT assays (REF). According to Jin's Formula¹⁶, the Q values at 2 $\mu\text{g/ml}$ as well as at 4 $\mu\text{g/ml}$ of both the agents together were 1.15 thereby revealing a significant anti-cancer synergy between nisin and doxorubicin (Figure 2B).

Membrane permeabilization studies

A dose dependent response in terms of relative fluorescence units (RFUs) could be observed when nisin was added to HaCat cells in the presence of NPN thereby indicating that nisin has the potential to permeabilize the cell membrane of these cancerous cells. Interestingly, incubation of the cells with NPN in presence of both nisin and DOX resulted in a marked increase (approx. tenfold) in RFUs as compared to RFU values observed when DOX alone was added to the cells (in presence of NPN) (Figure 3). Figure 3 Inset shows the mean relative fluorescence values at various concentrations of test substances and a light micrograph of HaCat cells.

ELISA

An increase in levels of NF κ B, TNF- α and (TNF- β) in tumor-induced groups as well as in untreated HaCaT cell lines was observed by ELISA estimations. On the other hand, nisin-DOX combinatorial therapy resulted in significantly ($p \leq 0.001$) reduced levels of all these cytokines in serum as well as in cell supernatants (Figure 4A-4C). An almost similar trend in decrease in levels of tumorigenic cytokines IL-1 and IL-6 could be observed upon treatment with the drugs alone as well as in conjunction with each other as compared to untreated groups

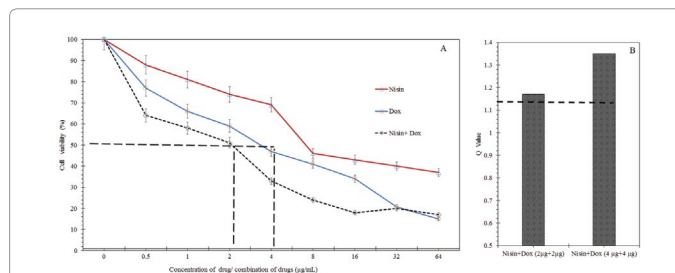


Figure 2: A) Percentage cell viability of HaCat cells after incubation with nisin (4 $\mu\text{g/ml}$) and DOX(4 $\mu\text{g/ml}$) alone and nisin(4 $\mu\text{g/ml}$) + DOX(4 $\mu\text{g/ml}$) for 24 hours. Results are expressed as percentage of the control \pm SD of three independent experiments. **B)** combination index (Q) of nisin + DOX (2+2 $\mu\text{g/ml}$) and nisin + DOX (4+4 $\mu\text{g/ml}$), where $Q < 0.85$, $Q > 1.15$ and $0.85 < Q < 1.15$ indicate antagonism, synergy, and additive effect respectively.

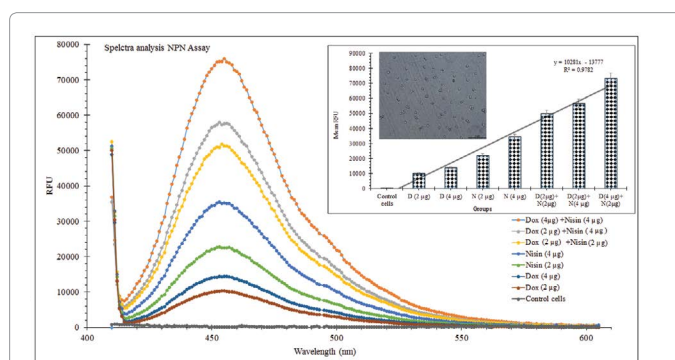


Figure 3: Uptake of NPN in the presence of various subinhibitory concentrations of nisin and DOX alone and in combination as monitored at an excitation wavelength of 350 nm and an emission wavelength of 420 nm. Values are represented as means \pm S.D. of three independent values. Inset shows the mean relative fluorescence values at various concentrations of test substances along with a light micrograph of HaCat cells.

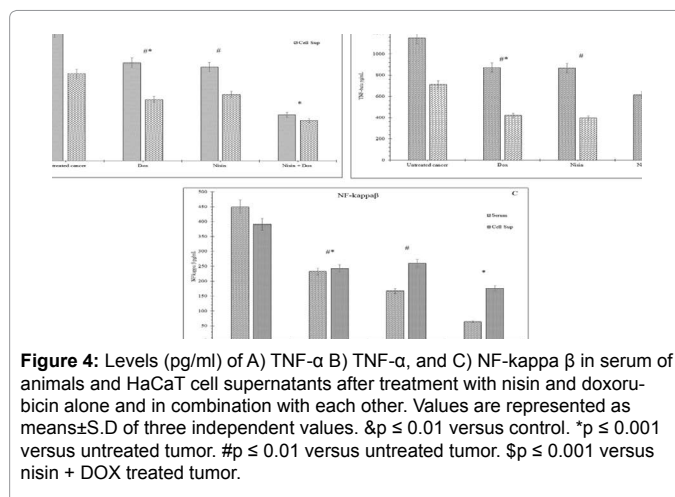


Figure 4: Levels (pg/ml) of A) TNF- α B) TNF- β , and C) NF- κ beta in serum of animals and HaCaT cell supernatants after treatment with nisin and doxorubicin alone and in combination with each other. Values are represented as means \pm S.D. of three independent values. $\&p \leq 0.01$ versus control. $*p \leq 0.001$ versus untreated tumor. $\#p \leq 0.01$ versus untreated tumor. $\$p \leq 0.001$ versus nisin + DOX treated tumor.

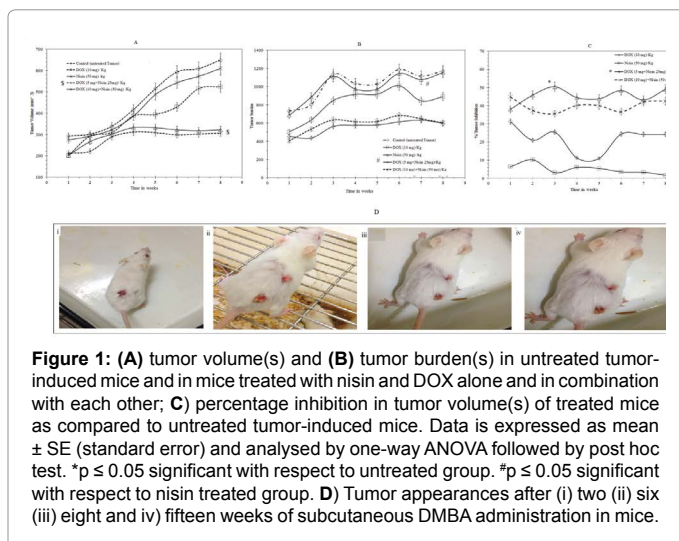
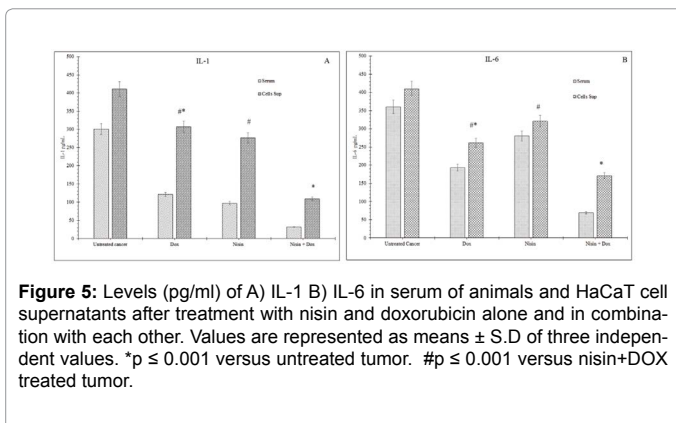


Figure 1: (A) tumor volume(s) and **(B)** tumor burden(s) in untreated tumor-induced mice and in mice treated with nisin and DOX alone and in combination with each other; **(C)** percentage inhibition in tumor volume(s) of treated mice as compared to untreated tumor-induced mice. Data is expressed as mean \pm SE (standard error) and analysed by one-way ANOVA followed by post hoc test. $*p \leq 0.05$ significant with respect to untreated group. $\#p \leq 0.05$ significant with respect to nisin treated group. **(D)** Tumor appearances after (i) two (ii) six (iii) eight and (iv) fifteen weeks of subcutaneous DMBA administration in mice.

(Figure 5A and 5B). Remarkably, approx. two fold decreases in the levels of IL-1 and IL-6 could be observed when both the agents were used together indicating the additive immunomodulatory role of the combination.

Discussion

In addition to the use of multi-component therapeutics (employing different anticancer compounds with different intracellular targets),



development of antimicrobial peptides (AMPs) as anti-cancer peptides (ACPs) is yet another emerging promising strategy for development of novel anti-cancer agents. In this context, we recently demonstrated that four weeks of nisin co-administration significantly enhances the *in vivo* chemotherapeutic effect of doxorubicin against DMBA-induced skin tumors in mice and also indicated that the adjunct therapy needs to be monitored over a longer period in order to completely understand the underlying mechanism(s) responsible for this observed additive effect [13].

The present study initially aimed at validating the *in vivo* additive anti-cancer effect of nisin-DOX combination (also at lower doses than used alone) over a regimen of two months post-fifteen weeks of established skin carcinogenesis. The *in vivo* additive effect displayed by the combination in terms of larger decreases in tumor volumes and burdens was in concordance with our previous report. An interesting finding was that nisin could also reduce the *in vivo* chemotherapeutic dose of DOX as the additive anti-cancer effect was still retained when the agents were used together in combination at half the doses than used alone. This can be attributed to an increased uptake of DOX by tumor cells in presence of nisin leading to more intracellular accumulation of DOX thereby causing an enhanced anti-cancer effect.

In order to further explore the possible mutual interactions occurring between nisin and DOX, their sub inhibitory concentration (s) alone and in combination against HaCaT cells were determined. The IC_{50} value of DOX was found to be halved in the presence of as low as 2 $\mu\text{g/ml}$ of nisin and the Q values [16] also revealed a significant anti-cancer synergy between nisin and DOX (Figure 2B). Interestingly, these results revealed that this particular combination, earlier perceived to be additive by us is in-fact synergetic in its anticancer action. These findings also affirmed that nisin as an adjunct has the potential to lower the minimum chemotherapeutic dose of DOX required to achieve significant anti-cancer effects thereby decreasing the associated side effects of high dose single agent chemotherapy.

To investigate the possible mechanism by which this combination might exerts an increased anti-cancer effect, nisin mediated permeabilization of NPN in HaCat cell lines was carried out by calculating relative fluorescence units in the presence of various sub inhibitory concentrations of DOX and nisin alone as well as in combination. NPN fluoresces weakly in an aqueous environment but strongly in the hydrophobic interior of cell membranes. Upon destabilization of the cellular membrane, the dye enters the damaged membrane, where it emits stronger fluorescence [19]. Approximately ten fold increases in RFU observed with the combination clearly indicated that nisin being a pore forming bacteriocin allows increased

uptake of doxorubicin by the cancer cells thereby leading to an enhanced anti-cancer effect demonstrated by the combination in our previous experiments [13,20]. Our observations are strongly supported by a previous report [11] wherein certain membrane active amphipathic peptides have been revealed to produce pores or channels and bind quickly to the surface of negatively charged HeLa cells via a strong electrostatic interaction. We hereby hypothesise that nisin being cationic in nature might be selectively toxic to the negatively charged HaCat cells thereby sparing the zwitterionic eukaryotic cells based on the fact that outer membrane of cancerous cells is negatively charged due to the presence of certain glycoproteins [21]. In addition to this, preferential facilitation of the uptake of DOX by tumor cells in presence of nisin might also help in improving the therapeutic index of this very important chemotherapeutic agent as doxorubicin is known to exert cardiotoxicity at higher doses which limits the maximum effective dose of this agent that can be used to cure cancers.

Many anticancer drugs have been shown to act through controlling or antagonizing the effect of nuclear factor- κ B [22,23] which acts as a major regulator of tumor phenotype and also controls the expression of pro-inflammatory molecules and cytokines like TNF-alpha, TNF-beta, IL-1 and IL-6 etc. In concordance with various earlier reports wherein increased levels of these cytokines have been found to be associated with a wide spectrum of cancers [24-26], our results also revealed that along with changes in NF- κ B, the levels of TNF- α , TNF- β , IL-1, IL-6 were modulated in response to DMBA and in untreated HaCaT cells. However, nisin-DOX adjunct therapy down regulated this increased expression of NF κ B and TNF- α , with a concomitant decrease in the levels of tumorigenic interleukins, IL-1 and IL-6 thereby indicating a strong immunomodulatory effect of the combination. Previously also, inhibition of TNF- α by various cancer preventive agents like green tea polyphenols, tamoxifen, and aspirin has been reported to be associated with recession of various cancers [27]. Moreover, the immuno-modulatory efficacy of nisin [28] as well as various other peptide-drug combinations [29] has been well documented. The present study reports that down regulation of these crucial players of carcinogenesis and inflammation is a prominent route by which nisin-DOX combination might exert a synergetic anti-cancer effect. Since apoptotic cell death is a non inflammatory process, decreased levels of proinflammatory cytokines observed in the present study might be correlated to the apoptogenic activity revealed by the combination in our previous report.

The results of the present study indicate that nisin by selectively targeting the cancer cells might increase the selectivity as well as intracellular concentration of DOX thereby leading to an enhanced synergetic anticancer effect. An enhanced cancer- cell specific DNA intercalation by DOX and consequently augmented immunomodulation by nisin makes this combination a functionally dual option for developing strategies to handling the chemotherapy induced side effects and development of drug resistance in cancer cells.

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