UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
FACULDADE DE ODONTOLOGIA

BIBIANA FRANZEN MATTE

ANÁLISE DO PAPEL DO PEPTÍDEO ANTIMICROBIANO NISINA SOBRE A MIGRAÇÃO DE CÉLULAS DE CÂNCER DE BOCA

Porto Alegre
2015

BIBIANA FRANZEN MATTE

ANÁLISE DO PAPEL DO PEPTÍDEO ANTIMICROBIANO NISINA SOBRE A MIGRAÇÃO DE CÉLULAS DE CÂNCER DE BOCA

Trabalho de Conclusão de Curso apresentado ao Curso de Graduação em Odontologia da Faculdade de Odontologia da Universidade Federal do Rio Grande do Sul, como requisito parcial para obtenção do título de Cirurgiã-Dentista.

Orientador: Prof. Dr. Marcelo Lazzaron Lamers

Porto Alegre

2015

CIP - Catalogação na Publicação

Franzen Matte, Bibiana
   Análise do papel do peptídeo antimicrobiano nisina sobre a migração de células de câncer de boca / Bibiana Franzen Matte. -- 2015.
   55 f.

   Orientador: Marcelo Lazzaron Lamers.

   Trabalho de conclusão de curso (Graduação) -- Universidade Federal do Rio Grande do Sul, Faculdade de Odontologia, Curso de Odontologia, Porto Alegre, BR-RS, 2015.

   1. carcinogênese. 2. invasão. 3. metástase. 4. conservante alimentar. 5. antitumoral. I. Lazzaron Lamers, Marcelo, orient. II. Título.

Elaborada pelo Sistema de Geração Automática de Ficha Catalográfica da UFRGS com os dados fornecidos pelo(a) autor(a).

Dedico este trabalho aos meus pais, Ceci Franzen Matte e Carlos Guilherme Matte, e ao meu irmão, Nicholas Franzen Matte, pelo apoio incondicional em todos os momentos deste processo. Desejo ser uma profissional da Odontologia íntegra como vocês me demonstram ser em suas áreas.

À minha família, pelo apoio a cada escolha, pela torcida a cada desafio e pela alegria a cada conquista.

Aos meus amigos e colegas de Faculdade, em especial ao Alexander Pompermayer Jardine, por estar ao meu lado desde o primeiro dia e que continues assim por muito mais.

AGRADECIMENTOS

Ao Prof. Marcelo Lazzaron Lamers, orientador deste trabalho. Obrigada por todos estes anos de orientação, dedicação e confiança no meu trabalho como aluna de iniciação científica. Tu és o principal responsável pelo meu apreço em pesquisa e pelo desenvolvimento de uma visão crítica de tudo que nos rodeia. Serei sempre grata.

À Profª. Anna Cristina Medeiros Fossati, por transmitir todo o seu conhecimento e participar ativamente durante a minha formação.

À Profª. Yvonne Kapila e ao pesquisador Pachiyappan Kamaranjan, que me receberam e me ensinaram com muito carinho e paciência no ano em que passei com eles na Universidade de Michigan

Aos meus colegas de laboratório, em especial aos alunos Paloma Campos e Leonardo Diel, ao mestre Carlos, aos doutorandos Alessandro Menna Alves e Grasieli Ramos e a pós-doutorando Lisiane Bernardi, pelo companheirismo em todos os trabalhos e horas no laboratório e também fora dele. Vocês fizeram cada dia ser mais especial.

RESUMO

MATTE, Bibiana Franzen. **Análise do peptídeo antimicrobiano nisina sobre a migração de células de câncer de boca**. 2015. 53 f. Trabalho de Conclusão de Curso (Graduação em Odontologia) – Faculdade de Odontologia, Universidade Federal do Rio Grande do Sul, Porto Alegre, 2015.

O câncer de boca é a sexta neoplasia maligna mais comum e ainda são necessários esforços para melhorar o seu protocolo terapêutico. Os processos de invasão e metástase estão relacionados com a recorrência do tumor e a resistência terapêutica. Estes dois processos estão relacionados com o perfil migratório de células malignas. Com o objetivo de buscar por novas opções de tratamento, o uso de peptídeos antimicrobianos demonstrou promissor potencial antitumoral. A nisina é um peptídeo antimicrobiano produzido pela *Lactococcus lactis* e que é utilizada como conservante alimentar. Além disso, este peptídeo apresenta potencial em reduzir a carcinogênese de células de câncer de cabeça e pescoço por modular a proliferação celular e induzir a apoptose. Neste estudo, foi avaliado os efeitos da nisina no processo de migração de células de câncer de boca. Após o tratamento com a nisina, a proliferação celular de células de câncer de boca reduziu de uma maneira dose-dependente (redução de 40% da proliferação em 800μg/ml). As células tratadas com altas doses de nisina (50μg/ml) em placas com fundo coberto por agarose não conseguiram estabelecer esferóides. A velocidade de migração reduziu 60% após o tratamento (24h, 25μg/ml) com nisina e também ocorreu impacto na trajetória espacial das células migratórias, indicando que a nisina modula a migração celular antes de ter efeito sobre a proliferação de células de câncer de boca. Apesar dos avanços, ainda são necessários estudos para compreender os mecanismos de ação da nisina no processo migratório. Contudo, como a nisina é segura para o consumo humana, ela apresenta um grande potencial como uma nova terapia para o câncer.

Palavras-chave: Carcinogênese. Invasão. Metástase. Conservante alimentar. Antitumoral.

ABSTRACT

MATTE, Bibiana Franzen. **Analysis of the antimicrobial peptide nisin on the migration of oral squamous cell carcinoma.** 2015. 53 p. Final Paper. (Graduation in Dentistry) – Faculdade de Odontologia, Universidade Federal do Rio Grande do Sul, Porto Alegre, 2015.

Oral cancer is the sixth most common cancer and there is still need to improve its therapeutics. Invasion and metastasis are related with cancer recurrence and drug-resistance therapy. These two processes are related to cancer cells migratory behavior. In order to research new therapeutics approaches, the use of antimicrobial peptides has shown several antitumor potential. Nisin is an antimicrobial peptide produced by *Lactococcus lactis* and it is used as a food preservative. It also has potential to attenuate head and neck squamous cell carcinoma tumorigenesis by modulating cell proliferation and inducing apoptosis. In the present study, we evaluated the effects of nisin in the migration process of oral cancer cells. After treatment with nisin, oral cancer cell proliferation decreased in a dose-dependent manner (40% decreased proliferation with 800μg/ml). Spheroid formation was impaired when cells were treated in agarose-coated plates with high concentrations of nisin (50μg/ml). Migration speed was 60% reduced after treatment (24h, 25μg/ml) with nisin and there was also an impact on spatial trajectory of cell migration, indicating that nisin starts to modulate cell migration prior to the effect in cell proliferation. Besides advances, it is still necessary more studies regarding the mechanisms of action of nisin in the migration process. However, as nisin is safe for human consumption, it emerges as a great potential for cancer therapy.

Keywords: Tumorgenesis. Invasion. Metastasis. Food preservative. Antitumor.

SUMÁRIO

1 INTRODUÇÃO

1.1 ASPECTOS GERAIS DAS NEOPLASIAS MALIGNAS

Classicamente, neoplasia maligna, também chamada de câncer ou tumor maligno, é uma patologia marcada pela proliferação desordenada de uma população celular geneticamente alterada, as quais apresentam capacidade de invasão dos tecidos vizinhos e migração para diferentes locais do organismo (metástase) (RODRIGUES; CAMARGO, 2010). O relatório da Organização Mundial da Saúde (OMS) de 2014 apontou que são diagnosticados 14 milhões de novos casos de câncer por ano e que até o ano de 2035 este número deve aumentar para 24 milhões de diagnósticos/ano devido ao aumento da expectativa de vida das populações. A OMS ainda indica que é necessário o investimento para diagnóstico precoce além de combater os fatores de risco como o fumo, o etilismo e a obesidade. Os tipos de tumores malignos mais comuns são o de pulmão e próstata nos homens, e o de mama nas mulheres.

Em relação ao carcinoma espinocelular oral, a última estimativa mundial apontou que ocorreriam cerca de 300 mil novos casos e 145 mil óbitos para o ano de 2012. Desses, cerca de 80% ocorreram em países em desenvolvimento. No Brasil, foram estimados 11.280 novos casos de câncer bucal em homens – sendo o quinto tipo de câncer mais comum – e 4.010 em mulheres para o ano de 2014 (INSTITUTO NACIONAL DE CÂNCER JOSÉ ALENCAR GOMES DA SILVA, 2015).

O processo de tumorigênese inicia-se a partir de um grupo de células que, expostas a fatores mutagênicos como cigarro (químico), radiação ultra-violeta (físico) ou vírus (biológicos), sofreram mutações genéticas, principalmente em genes relacionados ao controle da proliferação e da apoptose celular, resultando em crescimento descontrolado. Conforme ocorre a progressão do tumor, as células neoplásicas também alteram seu metabolismo, passando a apresentar um comportamento alterado em relação aos demais tecidos do organismo (TEICHER; LINEHAN; HELMAN, 2012; ZHANG, G. et al., 2013; ZHANG, Y. et al., 2013). Recentemente, observou-se que as células tumorais são capazes de remodelar o seu território originando um microambiente tumoral, o qual é composto por uma matriz extracelular remodelada e por células tumorais, inflamatórias, endoteliais e fibroblastos, as

quais estão em constante comunicação através da liberação de diversas citocinas (BECKER et al., 2013; GOMES et al., 2013; HANAHAN; WEINBERG, 2011; JAIN, 2013; TSANTOULIS et al., 2007). De uma maneira geral, são características das células neoplásicas: 1- manutenção da sinalização para proliferação, 2- escape dos supressores de crescimento, 3- fuga da destruição pelo sistema imune, 4-imortalidade replicativa, 5- promoção de inflamação crônica, 6- indução de angiogênese e linfangiogênese, 7- mutação e instabilidade genômica, 8- resistência à morte celular, 9- desregulação do sistema energético, e 10- ativação da invasão tecidual e metástase (HANAHAN; WEINBERG, 2011).

O foco terapêutico da maioria destas neoplasias malignas é o tratamento cirúrgico e/ou radioterapia e/ou quimioterapia. Tratamentos cirúrgicos são, normalmente, bastante invasivos em que ocorre grande perda de tecido o que, muitas vezes, tem como consequência diminuição da qualidade de vida do paciente. Já a radioterapia e a quimioterapia são procedimentos que possuem muitos efeitos adversos ao paciente, uma vez que não conseguem diferenciar o tecido maligno do tecido sadio (HOSKIN; RAMAMOORTHY, 2008). Nas últimas décadas, muitas pesquisas foram realizadas para aprimorar o tratamento dos tumores malignos, sendo observados alguns avanços terapêuticos. Entretanto, devido à complexidade dos fatores que envolvem a doença, são necessárias novas estratégias terapêuticas que abordem uma grande gama de fatores relacionados ao comportamento tumoral, com o intuito de aumentar a sobrevida desses pacientes, além de melhorar a sua qualidade de vida.

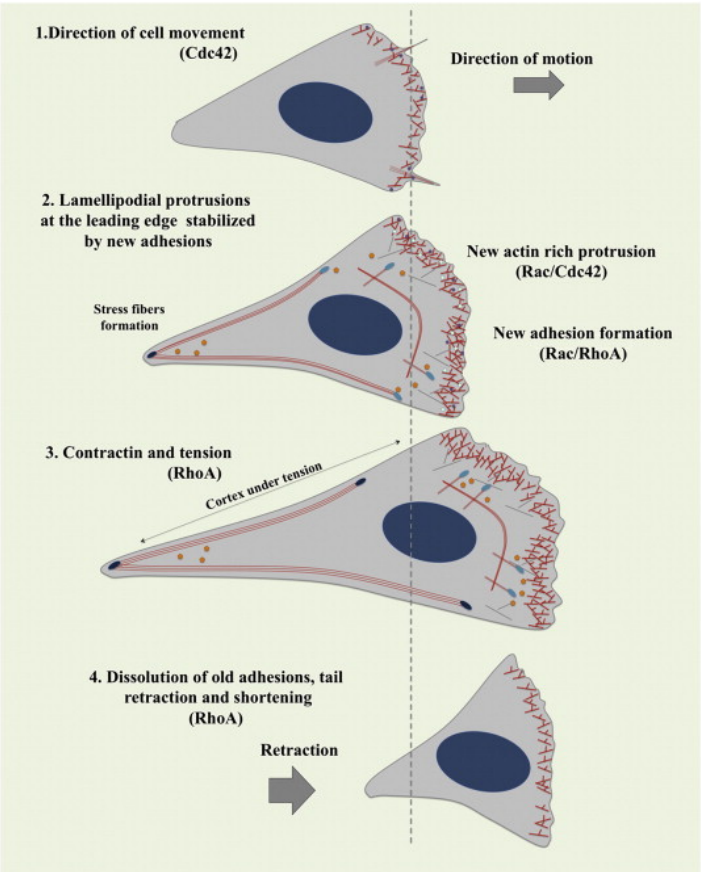
1.2 MECANISMOS DE INVASÃO TUMORAL

A invasão tecidual e o desenvolvimento de potencial metastático de tumores malignos são as maiores causas de insucessos clínicos em termos de terapia e prognóstico. As invasões celulares, que podem ser coletivas ou individuais, são caracterizadas por alterações moleculares importantes, tais como modificação da adesão entre as células e da adesão à matriz extracelular, facilitando a migração e o processo de invasão (ETIENNE-MANNEVILLE, 2008; FRIEDL, 2004; GUARINO, 2007; PAINTER; ARMSTRONG; SHERRATT, 2010; SAHAI, 2005).

A migração celular, no entanto, não é um fenômeno presente apenas em processos patológicos, mas também em eventos fisiológicos fundamentais como na embriogênese e na

cicatrização. O movimento migratório inicia com a formação de uma projeção de membrana através da polimerização da actina e conseguinte formação de adesões focais entre a célula e a matriz extracelular. A partir destas adesões, ocorre a ancoragem das proteínas de citoesqueleto actina e miosina que ao entrarem em contato, permitem a hidrolização do ATP, transformando a energia química em movimento e realizando a contração do corpo celular em direção determinada pela projeção de membrana. Seguido da contração celular, ocorre a liberação das adesões focais na porção posterior da célula para finalizar o movimento (figura 1). Todos os momentos são orquestrados por diversas proteínas para que este importante processo possa ocorrer. A estrutura da miosina é dividida em duas partes principais: a cabeça e a cauda. A cabeça é formada por duas cadeias pesadas de miosina e cada cadeia pesada apresenta um par de cadeia leve que atua junto dos filamentos de actina. (CHOI et al., 2008; GARDEL et al., 2010; LAUFFENBURGER; HORWITZ, 1996; PAROS; HORWITZ; SCHWARTZ, 2010; SANDQUIST et al., 2006; WEBB; HORWITZ, 2003).

Figura 1 – Ilustração das etapas envolvidas durante a migração celular.



Fonte: HANNA; EL-SIBAI, 2013.

O íon cálcio (Ca^{+2}) é um dos principais sinalizadores para as diversas etapas da migração celular (LEE et al., 1999; RIDLEY et al., 2003; WEI et al., 2009). A presença deste íon é necessária para que ocorra a formação das adesões focais na porção anterior da célula, assim como o Ca^{+2} também realiza a fosforilação da cadeia leve de miosina (MLC) pela sua ligação com a cinase da cadeia leve de miosina (MLCK), a qual é Ca^{+2}-dependente. A partir da fosforilação da MLC, a miosina II contrai para realizar a contração do corpo celular e a retração da porção posterior da célula. Para que ocorra retração do corpo celular, as adesões com a matriz extracelular são liberadas através da ação da m-calpain que é uma protease dependente de cálcio (BETAPUDI et al., 2010; FRANCO et al., 2004; TSAI; MEYER, 2012; TSAI et al., 2014). Portanto, a homeostase de Ca^{+2} é fundamental para que ocorra todas as etapas da migração celular (PREVARSKAYA; SKRYMA; SHUBA, 2011).

Em processos fisiológicos, as células migram em um sentido específico para que se desenvolva a função requerida como, por exemplo, em um processo de cicatrização para que ocorra o fechamento das duas margens da ferida. Contudo, em um processo patológico tumoral, as células migratórias não são coordenadas, estão randomicamente orientadas e se dividem em grupos de confusa organização ou se isolam, levando à alteração da estrutura tecidual. No tecido epitelial, por exemplo, ocorre a ruptura da lâmina basal e invasão do tumor para o tecido adjacente. Dentro deste processo invasivo, ocorre a transição de uma migração coletiva para uma migração individual que são etapas essenciais de um processo conhecido como transição epitélio-mesênquima (EMT) e serve como um bom indicador de progressão do tumor. Assim sendo, a invasão tumoral facilita a emergência das metástases, espalhando as células neoplásicas para outras partes do corpo e contribuindo para a formação de novos tumores (ETIENNE-MANNEVILLE, 2008; FRIEDL, 2004; GUARINO, 2007; SAHAI, 2005)

Visto à baixa melhora na sobrevida dos pacientes tratados de maneira convencional (cirúrgica) nos últimos anos e a complexidade de eventos que ocorrem em carcinomas, torna-se necessário buscar estratégias complementares que atuem em diferentes momentos da progressão tumoral, não apenas restrita à atividade proliferativa, mas que também englobe a capacidade invasiva das células. Uma classe potencial de elementos com estas características são os peptídeos antimicrobianos.

1.3 PEPTÍDEOS ANTIMICROBIANOS

Peptídeos antimicrobianos (PAM) são peptídeos produzidos pelas diferentes espécies do reino animal e vegetal que são, em sua maioria, peptídeos catiônicos separados espacialmente por regiões hidrofóbicas. Atuam no sistema imune inato e na defesa dos organismos contra infecções por bactérias, fungos, vírus e protozoários (ZASLOFF, 2002).

Devido à crescente resistência de bactérias aos antibióticos, o estudo dos PAMs começou a ser aprofundado como alternativa terapêutica. Estes peptídeos possuem amplo espectro de ação contra bactérias gram-positivas e gram-negativas, assim como baixa incidência de resistência terapêutica (SEO et al., 2012) O seu mecanismo de ação nas bactérias ocorre pela formação de poros na membrana citoplasmática destes patógenos. Existem diferentes teorias propostas para explicar este mecanismo de lise (LEUSCHNER; HANSEL, 2004). No entanto, é demonstrado que a função destes peptídeos depende de suas características bioquímicas como a sequência de aminoácidos, a estrutura tridimensional, anfipaticidade e a hidrofobicidade (HOSKIN; RAMAMOORTHY, 2008).

Recentemente, estes peptídeos também tem demonstrado potencial uso como agentes antitumorais (LEUSCHNER; HANSEL, 2004). Não são todos os PAMs que possuem capacidade de atingir células malignas, porém, aqueles que conseguem, podem ser divididos em duas categorias: (1) PAMs que possuem potencial contra bactérias e células malignas, mas não contra células mamíferas não-tumorais; (2) PAMs que apresentam potencial citotóxico contra bactérias, células malignas e células mamíferas não-tumorais (HOSKIN; RAMAMOORTHY, 2008).

Similar à ação dos PAMs em bactérias, estes também irão formar poros na membrana plasmática de células malignas, levando-as a entrarem em apoptose. A lesão na membrana plasmática específica em células tumorais ocorre devido a maior carga negativa expressada por moléculas aniônicas nas membranas destas células enquanto que membranas plasmáticas de células saudáveis apresentam carga neutra. De acordo com Hoskin e Ramamoorthy (2008), bactérias e células neoplásicas apresentam membranas com carga negativa o que pode contribuir para compreendermos a similar efetividade destes peptídeos como agentes antimicrobianos e antitumorais (DOBRZYNSKA; SKRZYDLEWSKA; FIGASZEWSKI, 2013; SZACHOWICZ-PETELSKA et al., 2013; UTSUGI et al., 1991).

A literatura científica nos apresenta uma grande quantidade de PAMs que já demonstraram sucesso *in vitro* e *in vivo* como potenciais terapêuticos em diferentes tipos de tumores sólidos – como câncer de mama, próstata, boca e bexiga – assim como nos tumores

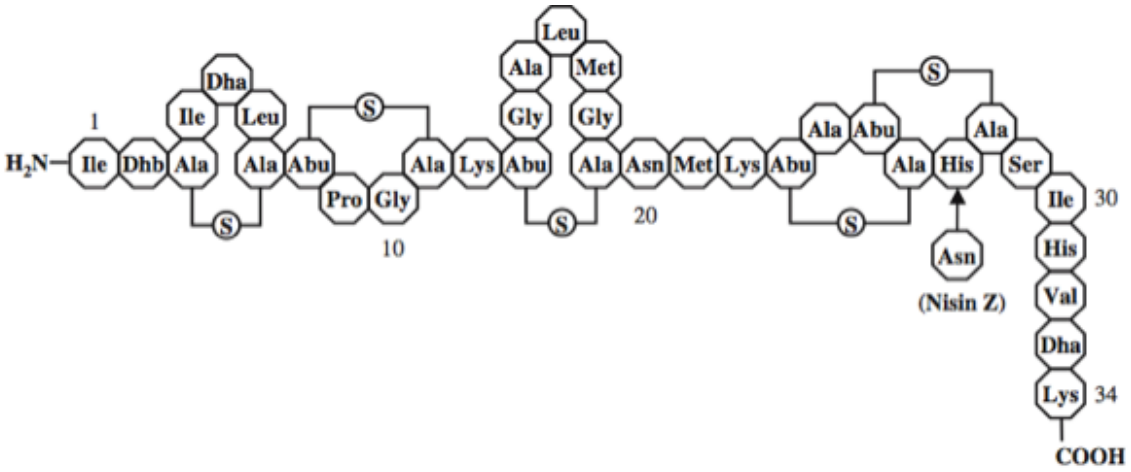
hematológicos – leucemias e linfomas (GASPAR; VEIGA; CASTANHO, 2013). Estes peptídeos foram extraídos de diversas fontes da natureza como: pele de anfíbios, secreções bacterianas e leite de vaca (PUSHPANATHAN; GUNASEKARAN; RAJENDHRAN, 2013). Existe um banco de dados em que podem ser pesquisados os antimicrobianos com potencial anticancerígeno (WANG, 2015). Além dos efeitos dos PAMs em induzir a apoptose de células neoplásicas, também são relatados efeitos de reduzir a migração celular (HSIAO et al., 2013; WANG et al., 2012) e apresentar potencial anti-angiogênico e anti-linfangiogênico (LEE et al., 2014; SZACHOWICZ-PETELSKA et al., 2013). Neste contexto, a nisina aparece como um potencial candidato no uso terapêutico de PAMs no tratamento de tumores.

1.4 NISINA

Nisina é um pequeno PAM com características catiônicas e hidrófobas que possui trinta e quatro aminoácidos (figura 2). É produzido pela estirpe bacteriana *Lactoccus lactis* que pertence a Classe I de bacteriocinas denominadas lantibióticos (CHEIGH; PYUN, 2005). Durante a fase de crescimento exponencial, ocorre a maior produção deste peptídeo e, ao entrar na fase de crescimento estacionário, já não há mais produção de nisina.

Nisina apresenta atividade antimicrobiana contra bactérias gram-positivas e gram-negativas através da realização de poros nas membranas destas bactérias (BREUKINK; DE KRUIJFF, 1999). Existem seis tipos de variantes naturais de nisina, diferenciados nominalmente por A, Z Q, F, U e U2 e diferem quanto a posição de aminoácidos (FIELD et al., 2008).

Figura 2 - Representação esquemática da estrutura química do peptídeo nisina formada por 34 aminoácidos. Os variantes naturais dependem da modificação de um peptídeo na posição 27.



Fonte: CHEIGH; PYUN, 2005.

A despeito dos muitos estudos realizados com os PAMs, poucos são permitidos para uso na indústria alimentícia ou como agentes antibióticos. Nisina é considerada segura para uso em humanos e obteve sua aprovação pela World Health Organization (WHO) em 1969 e pela Food and Drug Administration (FDA) em 1988. Atualmente, a nisina é utilizada como preservativo alimentar em diversos alimentos como leites, queijo, vegetais enlatados e alimentos infantis em diversos países. No Brasil, por exemplo, é permitido adicionar ao queijo a quantidade máxima de 12,5 mg/kg (AGENTES..., 2010).

Além da função estabelecida deste peptídeo como conservante alimentar, diferentes autores descreveram suas outras aplicações possíveis. Já foi demonstrado que a nisina pode ser utilizada em alguns tipos de infecções como na mastite (FERNANDEZ et al., 2008), em dermatite atópica (VALENTA; BERNKOP-SCHNURCH; RIGLER, 1996) e em infecções do trato respiratório (DE KWAADSTENIET; DOESCHATE; DICKS, 2009) por apresentar efeito antimicrobiano contra infecções por *Staphylococcus aureus*. Outro estudo demonstrou interesse em desenvolver as qualidades de solubilidade e estabilidade do peptídeo através de engenharia proteica (ROLLEMA et al., 1995). Também foi verificada a possibilidade de desenvolvimento de comprimidos contendo nisina (UGURLU et al., 2007). Portanto, percebe-se que existe um grande interesse pela pesquisa de novas aplicações para este PAM, devido a sua alta eficácia e baixa citotoxicidade.

Recentemente, a nisina começou a ser testada quanto ao seu potencial de inibir o crescimento de células malignas uma vez que outros antimicrobianos demonstram ter este

potencial. Joo e colaboradores (2012) evidenciaram que nisina induziu apoptose e diminuiu o crescimento de células neoplásicas originárias de carcinoma espinocelular de cabeça e pescoço *in vitro* e *in vivo*. A ação da nisina nestas células ocorre através de aumento intracelular de cálcio, indução de bloqueio do ciclo celular e da ativação de um regulador de transporte de cátions – CHAC1. Este efeito ocorreu em células malignas sem, no entanto, ocorrer em queratinócitos primários. Portanto, nisina demonstrou possuir potencial terapêutico antitumoral (JOO et al., 2012). Este mesmo grupo de pesquisadores, relatou que a utilização de uma formulação de nisina mais pura apresentou melhores efeitos em induzir apoptose e diminuir o crescimento de células de câncer de cabeça e pescoço *in vitro* e *in vivo*, sendo que o variante natural Z apresentou maiores reduções de proliferação celular do que a nisina A. Além disso, foi demonstrado que a nisina induz a apoptose de células endoteliais o que levou a redução da densidade vascular nos tumores que tiveram contato com a droga (KAMARAJAN et al., 2015). Outro grupo de pesquisadores, avaliou o efeito combinado da nisina com a doxorrubicina, que é um medicamento antineoplásico utilizado contra diversos tipos de tumores, em tumores de pele induzidos por 7,12-dimetilbenzantraceno. Após um mês de tratamento, foi observado que o efeito adjunto das duas drogas resultou em menor crescimento dos tumores quando comparado com os grupos tratados com os medicamentos isolados e o grupo sem tratamento (PREET et al., 2015). Estes dados indicam que nisina pode ser considerada como uma droga em potencial para tratamentos de câncer.

Visto que: 1- os efeitos da nisina estão relacionados à incorporação de Ca^{+2}; 2- seus efeitos sobre a proliferação celular e apoptose estão estabelecidos com doses elevadas da substância; e 3- o comportamento migratório ser finamente regulado por elementos reguladores como o Ca^{+2}, é possível que concentrações menores nisina influencie o comportamento migratório de células tumorais, com um potencial papel no combate à invasão e metástase tumoral.

2 ARTIGO CIENTÍFICO

O desenvolvimento do trabalho está apresentado na forma de artigo científico de periódico em inglês, o qual será submetido para publicação no periódico *PlosOne*.

Analysis of the antimicrobial peptide nisin on the migration of oral squamous cell carcinoma

Running Title: Analysis of the antimicrobial peptide Nisin on the migration of oral squamous cell carcinoma

Bibiana Franzen Matte*, Paloma Santos de Campos*, Leonardo Diel*, Yvonne Kapila[+], Alessandro Menna Alves*, Marcelo Lazzaron Lamers*[#]

* Federal University of Rio Grande do Sul, Brazil; School of Dentistry;

[+] University of Michigan, United States of America; School of Dentistry;

[#] Federal University of Rio Grande do Sul, Brazil; Morphological Sciences Department, Health Basic Sciences Institute

Corresponding Author:

Marcelo Lazzaron Lamers

E-mail: marcelo.lamers@ufrgs.br

Address: Department of Morphological Sciences, Institute of Basic Health Science, Federal University of Rio Grande do Sul

Rua Sarmento Leite, 500, subsolo, Porto Alegre, RS, Brazil, CEP 90.050-170

Phone: +55 51 33085011

Abstract

Oral cancer is the sixth most incident cancer and there is still need to improve its therapeutics. Invasion and metastasis are related with cancer recurrence and drug-resistance therapy. These two processes are related to cancer cells migratory behavior. In order to research new therapeutics approaches, the use of antimicrobial peptides has shown several antitumor potential. Nisin is an antimicrobial peptide produced by *Lactococcus lactis* and it is used as a food preservative. It also has potential to attenuate head and neck squamous cell carcinoma tumorigenesis by modulating cell proliferation and inducing apoptosis. In the present study, we evaluated the effects of nisin in the migration process of oral cancer cells. After treatment with nisin, oral cancer cell proliferation decreased in a dose-dependent manner (40% decreased proliferation with 800μg/ml). Spheroid formation was impaired when cells were treated in agarose-coated plates with high concentrations of nisin (50μg/ml). Migration speed was 60% reduced after treatment (24h, 25μg/ml) with nisin and there was also an impact on spatial trajectory of cell migration, indicating that nisin starts to modulate cell migration prior to the effect in cell proliferation. Besides advances, it is still necessary more studies regarding the mechanisms of action of nisin in the migration process. However, as nisin is safe for human consumption, it emerges as a great potential for cancer therapy.

Keywords: tumorgenesis, invasion, metastasis, food preservative, antitumor

Introduction

Cancer is one of the leading causes of death worldwide and Oral Squamous Cell Carcinoma (OSCC) is the most incident malignant neoplasm in the oral cavity. Patients diagnosed with OSCC are usually treated with surgery, radiotherapy and chemotherapy. Because of the limited anatomic area and the invasive behavior of the tumor, surgery procedures are often destructive and, frequently, the complete removal of the tumor mass is not viable[1]. Few alternatives of therapy for these individuals are available and, besides advances in research, the low (~50%) 5-year survival rate for patients with OSCC has not improved in the past decades[2,3]. There is, therefore, urgency for research into new therapeutic approaches for patients with OSCC, specially regarding the invasive and metastatic behavior of the tumor.

Epithelial-derived tumor cells usually undergo epithelial to mesenchymal transition (EMT), which is characterized by molecular changes such as loss of epithelial cell-cell adhesion markers and modulation of migration-related signaling pathways that lead to a more invasive and metastatic behavior[4-7]. The process of cell migration, in summary, initiates with a cell protrusion at the leading edge, formation of adhesions with the extracellular matrix (ECM), contraction of the cell body and detachment of adhesions at the cell rear. These steps rely in the coordinated activation/assembly of several proteins that are orchestrated by different signals, such as regulatory proteins and ions[8,9]. Cell migration is observed in several physiological situations, but a deregulation in this process might lead to different pathological conditions, as observed during cancer metastasis. The development of drugs that modulate cell migration only on tumor cells represents a potential target for cancer therapy[4-7] in order to improve patient survival rate and quality of life.

It has been demonstrated that different antimicrobial peptides have cytotoxic effects against cancer cells, but not to normal cells[10-12]. For instance, nisin is produced by fermentation of the gram-positive bacterium Lactococcus lactis[13] and is approved for use as a food preservative for preventing bacterial growth since 1969 by the World Health Organization and since 1988 by the Food and Drugs Administration. Since nisin is safe for human consumption, it emerges as an interesting drug option for future anti-cancer treatment. It was demonstrated that nisin effectively reduces and prevents tumorigenic properties *in vitro* and *in vivo* in OSCC[14], reduces angiogenesis *in vitro* and *in vivo*[15] and it has additive effect when combined with doxorubicin in DMBA-induced skin carcinogenesis[16]. It is already proved that high doses of nisin decrease cancer cell proliferation, but there is a need to analyze possible effects of nisin on others cancer cells abilities such as invasion and metastasis.

To address the possible role of nisin during cell migration, we analyzed the effects of low levels of nisin on cancer cell proliferation, migration and spheroid formation. Herein, we observed that low doses of nisin did not change cell proliferation or spheroid formation of an OSCC cell line, but was able to reduce migration velocity and impaired migration directionality. Our data suggests that nisin arises as a possible future drug for restraining oral cancer invasion and metastasis.

Methods

Nisin

Nisin Z is one of the natural variants of nisin produced by fermentation of *Lactococcus lactis*. An ultrapure form (95% content) was obtained from Handary (Brussels,

Belgium). Nisin was reconstituted in water to use in all experiments.

Cell culture

It was used a highly invasive OSCC cell line (SCC25 – CRL-1628) obtained from American Type Culture Collection, ATCC, Manassas, VA. Cells were maintained in Dulbecco's modified Eagle's medium F12 (DMEM F12) containing 10% fetal bovine serum, 1% penicillin and streptomycin, hydrocortisone, glutamine and HEPES.

Cell proliferation

Cells were plated in a 96-well plate (2 x 10^3 cells/well) and then treated in the next day with increasing doses of nisin (12.5, 25, 50 and 800 μg/ml) for 24 hours. To determine the effect of nisin on cell proliferation, the CyQUANT® NF Cell Proliferation Assay Kit was used according to manufacturer's instructions (Invitrogen).

Spheroid assay

Three-dimension culture model resembles avascular tumor and reestablish morphological, functional and mass transport properties of the corresponding tissue *in vivo*. We tested the effect of increasing doses of nisin on cancer spheroid according to this procol[18]. Cells were plated in a non-adherent agarose-coated 96-well plate (3x10^4 cells/well) with or without an immediate nisin treatment (12.5, 25, 50 and 800 μg/ml). After 24 hours, pictures were taken from spheroids with charge coupled device camera (Bioptika CMOS 5.0) attached to an inverted microscope (INV3, Bel Photonics, Piracicaba, Brazil) using IScapture 3.6.7 Software. At this time point, we did a late nisin treatment (24-hour after cells were plated) and then analyzed them after 24 hours again.

Migration assay

Cells were plated in a 6-well plate ($1x10^5$ cells/well) and then treated with nisin (12.5 and 25 μg/ml) for 24 hours. After the treatment, cells were detached with trypsin-0.25% EDTA, washed and plated on fibronectin-coated glass-bottomed dishes (2 μg/ml) in DMEM F12 supplemented without drug for 3 h in incubator (37°C, 5% CO2). For analysis of the migration properties, phase microscopy time-lapse images were captured for a period of 20 h at 10 min intervals (migration speed and spatial trajectory (ST)) with a charge coupled device camera (Axiocam mrn, Zeiss, Göttingen, Germany) attached to an inverted microscope (Axio Observer Z1, Zeiss, Göttingen, Germany) using AxioVision Software (Zeiss, Göttingen, Germany). The values for the assessment of migration speed and ST were obtained using Image J (*National Institute of Health*, MA, USA) software, and the data were processed as previously described[17]. For ST analysis, a polar plot graph was constructed, which represents the spatial trajectory developed by each migratory cell, where the X and Y coordinates of each cell trajectory were normalized to start at a virtual (X = 0 and Y = 0) position.

Statistical analysis

Statistical analysis was performed with the Statistical Package for the Social Sciences version 17.0 (SPSS Inc, Chicago, IL, USA). Data were presented as number, percentage, mean and standard deviation. To compare means between groups was applied t-test and analysis of variance (ANOVA) followed by Tukey's post-test. The level of statistical significance was 5% ($p < 0.05$).

Results

Nisin reduces cell proliferation dose-dependently in oral squamous cell carcninoma

To evaluate the effect of nisin in cell proliferation, we incubated an oral squamous cell

carcinoma lineage (SCC25) with different concentrations of nisin (12.5, 25, 50 and 800 μg/mL) for 24 hours. It was observed that there was a decrease in cell proliferation only at high dosages of nisin (Fig. 1; 40% decrease with 800 μg/ml; $p<0,05$). This demonstrates that there is a dose-dependent relationship between Nisin effect and OSCC proliferation *in vitro*, and low doses show low impact in the proliferation rate.

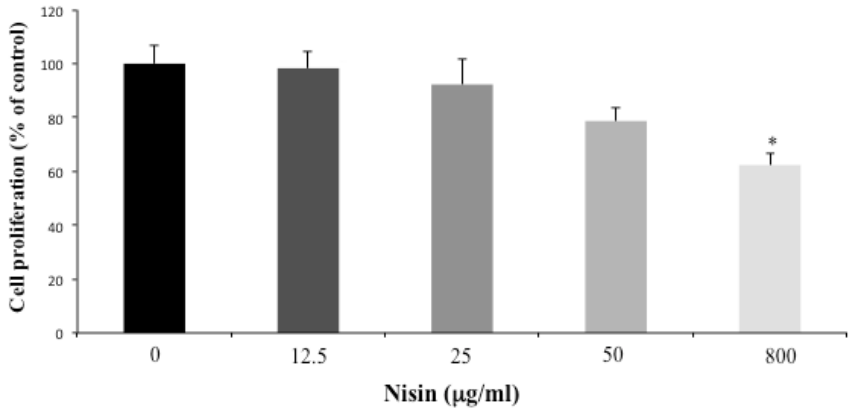


Figure 1. Nisin modulates OSCC proliferation in a dose-dependent manner. An OSCC lineage (SCC-25) was treated with increasing doses of Nisin for 24 hours and then tested with CyQUANT® NF Cell Proliferation Assay Kit (n=3; $p<0.05$; ANOVA test).

Nisin impairs spheroid formation

A 3D cell culture model can mimic drug response more similar to a tumor *in vivo* when compared to a monolayer cell culture *in vitro*[18]. We incubated an OSCC cell line (SCC-25) in agarose-coated plates and treated with increasing doses of nisin (12.5, 25, 50 and 800μg/mL) in two time points. We tested an immediate treatment when nisin was added to each well at the time of plating and a late treatment when nisin was added 24 hours after plating the cells and the spheroid was already formed. In the immediate treatment, high-dose treatment (50 and 800μg/mL) impaired spheroid formation (Fig. 2). However, when the

spheroid was already established, there was no visual impact of 24-hour nisin treatment even in high doses, most probably because cell tightly interactions in a spheroid culture is different then a monolayer culture which can alternate how drugs influence cancer cells.

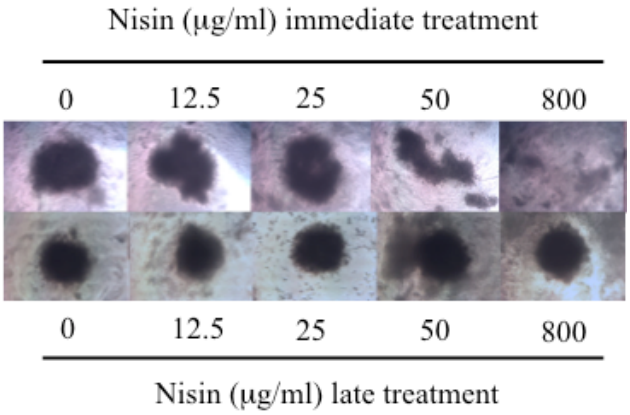


Figure 2. Nisin impairs OSCC spheroid formation. OSCC cells were incubated in agarose-coated plates and treated with increasing doses of nisin (12.5, 25, 50 and 800μg/mL) in two time points: immediate treatment (cells were plated with nisin) and late treatment (nisin was added 24 hours after cells were plated). High doses of nisin impairs spheroid formation in the immediate treatment. However, when the spheroid was already established, there was no visual impact on late treatment (n=3).

Nisin reduces cell migration speed and impairs directionality

Since metastasis is an important process for cancer progression and it relies in an increase on cell migration, we analyze the potential effect of nisin in cell migration. We incubated the highly invasive OSCC cell line (SCC-25) for 24 hour with two different concentrations of nisin (12.5 and 25 μg/ml), since higher doses impaired cell viability during the time-lapse assay. After this incubation, cells were tripsinised, plated in migration promoting conditions and imaged for 20h (time interval of 10min). From the time-lapse movies, individual migratory cells were tracked and we measured migration speed and directionality. After treatment with 12.5μg/ml of nisin, there was no significant change in migration speed when compared to control cells. However, the highly invasive OSCC cells

pre-treated with 25μg/ml of nisin showed a 60% decrease in migration speed when compared to control (Fig. 3A; n=3; $p<0.05$; ANOVA test). The qualitative analysis of the spatial trajectory of each migratory cell also indicates that control cells showed a high directionality, while cells treated with 25μg/ml of nisin resulted with the majority of cells that migrated in a circle trajectory and did not explore the field (Fig. 3B). Taken together, these results suggest that low doses of nisin, besides no changes on proliferation, are able to reduce migration speed and impair migratory capacity of tumor cells.

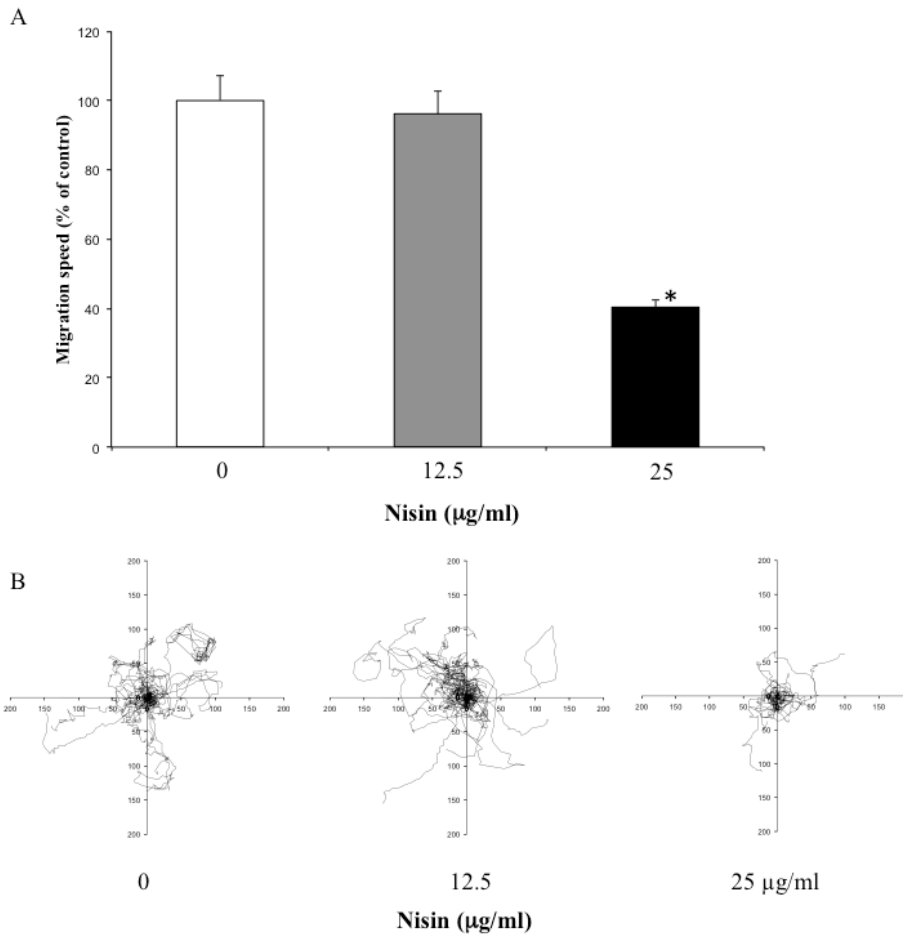


Figure 3. Nisin impairs cell migration speed and directionality. OSCC cells were treated 24 hours with nisin, plated on fibronectin 2μg/ml and imaged using time-lapse analysis. (A) Nisin reduces cell migration speed in a dose-dependent manner (60% with 25μg/ml; n=3; $p\leq0,001$). (B) Each line in the plot represents one cell spatial trajectory. In control and in treatment with 12.5μg/ml of nisin, cells reached longer distances when compared to treatment with 25μg/ml.

Discussion

Most of the cancer therapies target the proliferation process of tumor cells and, as a consequence, they are not able to achieve slow-dividing cells that might be responsible for cancer metastasis and recurrence. Tumors present remarkable characteristics as activating invasion and metastasis, inducing angiogenesis and tumor-promoting inflammation known as cancer hallmarks[19]. Since cancer cells act in a various number of pathways, tumor therapeutics should target those different capabilities in order to increase patient survival rate. As presented in this study, nisin can target migrating cells and, therefore, might interfere in the invasion and metastasis process of oral cancer.

The commonly used chemotherapeutic agents are not able to differentiate cancer cells from normal cells and tissues. As a consequence, cancer treatment has various side effects and there is still a high incidence of treatment resistance and failure. Antimicrobial peptides selectively act in tumor cells like cecropin B[20] and maganin[21]. Nisin has been used as a food preservative for over forty years and studies showed that nisin is selective for head and neck cancer cells and it did not present side effect in an *in vivo* study[14]. There is also evidence that a high content nisin (95% ultrapure) induced the greatest level of apoptosis in cancer cells compared to a low content and that the natural variant nisin Z (95%) showed increased cell proliferation reduction compared to nisin A (95%)[15]. In head and neck squamous cancer cell lines, the use of nisin doses as 100 and 200μg/ml showed a 40% reduction on cell proliferation[15]. In our study, we also used a high content of nisin Z (95%). However, it was necessary high doses of nisin (800μg/ml) to achieve the same percentage of reduction in cell proliferation in oral cancer cells. This result might be from the different cell lineages used. As demonstrated, nisin can differentiate tumor cells from normal cells and it is capable of

reducing cell proliferation that are great characteristics for novel tumor drugs.

Our study also showed that nisin impairs sphere formation, in agreement with a previous study[15]. Interestingly, Huang et al. tested the natural antimicrobial peptide Cecropin B (CB1) on lung cancer spheroids. CB1 did not interfere in the assembly of tumor spheroids, but it disrupted the spheroids once they were established[22]. In order to survive in a 3D culture condition, cells must adhere tightly to each other and this might reflect the absence of effect of nisin in a spheroid culture. Taken together our data, we showed that only high doses of nisin reduce cell proliferation and impair spheroid formation of an OSCC cell line.

Cell migration is a key step for cancer metastasis and several studies try to identify drugs that show a selective modulation of migration properties[2]. In this study, we demonstrated that low levels of nisin reduces cell migration speed and impairs spatial trajectory in OSCC cell line. Similarly, another antimicrobial peptide, Ixonsin-B amide, also reduces migration of breast cancer cells in lower doses prior to the dose needed to affect cell viability[23]. It was reported that Ixonsin-B amide reduced migration in a wound healing assay that represents a mass-migration type of movement. In our study, we analyzed with a time-lapse assay in migrating conditions to promote individual cell movements, which is more similar to a cancer invasion phenotype. Since low doses of nisin diminish cancer cell migration, it is important to continue studying how we can apply its properties on cancers-patients therapeutics.

Nisin acts by increasing calcium influx and activating CHAC1 which is a cation transporter and, therefore, it might deregulates calcium homeostasis during cell migration[14]. Migrating cells requires a transient gradient of calcium, because several steps are regulated by calcium as cleavage of focal adhesions proteins and cell body contraction[24-26]. Focal adhesion turnover are characterized by formation and disassembly of cell adhesion with the

extracellular matrix. This process depends on Ca^{+2} and it provides cell stability to continue the migration process[25]. As shown in this study, OSCC cells treated with nisin presented a circle trajectory behavior, which could indicate that the cell could not assembly proteins with the ECM and continue the migratory process. Cytoskeleton proteins, as actin and myosin, are responsible for cell contraction during the migration process. Myosin is formed by two heavy chains and by four myosin light chains (MLC). This latter part is regulated by myosin light chain kinase (MLCK) that is directly controlled by Ca^{+2} [27-29]. Since nisin increases calcium influx, we hypothesize that migration process is impaired by nisin for its modulation on MLCK. Further studies are needed to elucidate this mechanism.

In summary, nisin reduces cell migration speed and impairs migration directionality, prior to a decrease in cell proliferation and spheroid formation. As nisin is safe for human consumption and studies are demonstrating its anti-tumoral properties, it emerges as a future anti-cancer therapeutic approach.

References

1. Argiris A, Karamouzies MV, Rabens D, Ferris RL. Head and neck cancer. Lancet. 2008; 371: 1695-709

2. Bsoul SA, Huber MA, Terezhalmy GT. Squamous cell carcinoma of the oral tissues: a comprehensive review for oral healthcare providers. J Contemp Dent Pract. 2005;6:1- 16.

3. Siegel R, Naishadham D, Jemal A. Cancer statistics, 2012. CA Cancer J Clin. 2012;1:10–29

4. Sahai E. Mechanisms of cancer cell invasion. Curr Opin Genet Dev 2005; Feb 15(1): 87-96.

5. Guarino M. Epithelial-mesenchymal transition and tumour invasion. Int J Biochem Cell Biol 2007; 39(12): 2153-60.

6. Etienne-Manneville S. Polarity proteins in migration and invasion. Oncogene 2008; Nov 24 27(55): 6970-80.

7. Painter KJ, Armstrong NJ, Sherratt JA. The impact of adhesion on cellular invasion processes in cancer and development. J Theor Biol 2010; Jun 7 264(3): 1057-67.

8. Lauffenburger DA, Horwitz AF. Cell migration: a physically integrated molecular process. Cell 1996; Feb 9 84(3): 359-69.

9. Webb DJ, Horwitz AF. New dimensions in cell migration. Nat Cell Biol 2003; Aug 5(8): 690-2.

10. Mader JS, Hoskin DW. Cationic antimicrobial peptides as novel cytotoxic agents for cancer treatment. Exp Opin Investing Drugs 2006;15:933–946

11. Cruciani RA, Barker JL, Zasloff M, Chen H-C, Colamonici O. Antibiotic magainins exert cytolytic activity against transformed cell lines through channel formation. Proc Natl Acad Sci 1991: 88:3792–3796.

12. Hoskin, DW, Ramamoorthy, A. Studies on anticancer activities of antimicrobial peptides. Biochim Biophys Acta 2008: Feb 1778(2): 357-375.

13. Cheigh CI, Pyun YR. Nisin biosynthesis and its properties. Biotechnology letters 2005; Nov 27(21): 1641-8.

14. Joo EN, Ritchie K, Kamarajan P, Miao D, Kapila YL. Nisin, an apoptogenic bacteriocin and food preservative, attenuates HNSCC tumorigenesis via CHAC1. Cancer Medicine. 2012; 1(3): 295-305.

15. Kamarajan P, Hayami T, Matte B, Liu Y, Danciu T, Ramamoorthy A, Worden F, Kapila S, Kapila Y. Nisin ZP, a bacteriocin and food preservative, inhibits head and neck cancer tumorigenesis and prolongs survival. PloS One 2015; Jul 1;10 (7).

16. Preet S, Bharati S, Panjeta A, Tewari R, Rishi P. Effetct of nisin and doxorubicin on DMBA-induced skin carcinogenesis – a possible adjunct therapy. Tumor biology 2015; May: 1-8.

17. Lamers ML, Almeida ME, Vicente-Manzanares M, Horwitz AF, Santos MF. High glucose-mediated oxidative stress impairs cell migration. PLoS One. 2011;6(8): e22865. doi: 10.1371/journal.pone.0022865. pmid:21826213

18. Friedrich J, Seidel C, Ebner R, Kunz-Schughart LA. Spheroid-based drug screen: considerations and practical approach. Nature Protocols 2009; 4 (3): 309-324.

19. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell 2011; Mar 144 (5): 646-674.

20. Srisailam S, Kumar TKS, Arunkumar AI, Leung KW, Yu C, Chen HM. Crumpled structure of the custom hydrophobic lytic peptide cecropin B3. Eur J Biochem 2001; 268: 4278–4284

21. Takeshima K, Chikushi A, Lee K-K, Yonehara S, Matsuzaki K. Translocation of analogues of the antimicrobial peptides magainin and buforin across human cell membranes. J Biol Chem 2003;278:1310–1315

22. Huang C, Huang H, Forrest MD, Pan Y, Wu W, Chen H. Inhibition effect of a custom peptide on lung tumors. Plos One 2014; Oct 9 (10): 1-13

23. Hsiao Y, Wang K, Tsai S, Chao W, Lung FT. Anticancer activities of an antimicrobial peptide derivative of Ixonsin-B amide. Bioorg Med Chem Lett 2013; Aug 23: 5744-5747.

24. Brundage, R. A., Fogarty, K. E., Tuft, R. A. & Fay, F. S. Calcium gradients underlying polarization and chemotaxis of eosinophils. Science 1991; 254, 703–706.

25. Ridley, A. J. et al. Cell migration: integrating signals from front to back. Science 2003; 302, 1704–1709

26. Prevarskaya, N, Skryma, R, Shuba, Y. Calcium in tumour metastasis: new roles for

knows actors. Nature Reviews Cancer 2011;11: 609-618.

27. Betapudi V, Raj V, Beach JR, Egelhoff T. Novel regulation and dynamics of myosin II acitivation during epidermal wound responses. Exp Cell Res 2010; Feb 316: 980-991.

28. Tsai FC, Meyer T. Ca^{2+} pulses control local cycles of lamellipodia retraction and adhesion along the front of migrating cells. Curr Biol 2012; May 22(9): 837-842.

29. Tsai FC et al. A polarized Ca^{2+}, diacyglycerol and STIM1 signalling regulates directed cell migration. Nat Cell Biol 2014; Jan 16: 133-144

3 CONCLUSÃO

De acordo com trabalho apresentado, o peptídeo antimicrobiano nisina possui capacidade de reduzir a velocidade de migração celular e alterar a direcionalidade de células de câncer de boca em baixas doses quando ainda não se observa efeitos importantes na proliferação celular e na formação de esferóides. A nisina reduz a proliferação celular e impede a formação de esferóides quando as células estão expostas a altas doses. Pelos seus efeitos em diferentes características de células neoplásicas, a droga apresenta potencial como futuro agente terapêutico em pacientes com câncer de boca.

REFERÊNCIAS

AGENTES antimicrobianos químicos e naturais. **Food Ingredients Brasil**, [S.1], n. 15, p. 36-42, 2010. Disponível em: <http://www.revista-fi.com/materias/155.pdf>. Acesso em: 29 out. 2015.

BECKER, J. C. et al. Immune-suppressive properties of the tumor microenvironment. **Cancer Immunol. Immunother.**, Berlim, v. 62, no. 7, p. 1137-1148, July 2013.

BETAPUDI, V. et al. Novel regulation and dynamics of myosin II acitivation during epidermal wound responses. **Exp. Cell Res.**, Orlando, v. 316, p. 980-991, Feb. 2010.

BREUKINK, E.; DE KRUIJFF, B. The lantibiotic nisin, a special case or not? **Biochim. Biophys. Acta**, Amsterdam, v. 1462, no. 1-2, p. 223-234, Dec. 1999.

CHEIGH, C. I.; PYUN, Y. R. Nisin biosynthesis and its properties. **Biotechnol. Lett.**, Dordrecht, v. 27, no. 21, p. 1641-1648, Nov. 2005.

CHOI, C. K. et al. Actin and alpha-actinin orchestrate the assembly and maturation of nascent adhesions in a myosin II motor-independent manner. **Nat. Cell Biol.**, London, v. 10, no. 9, p. 1039-1050, Sept. 2008.

DE KWAADSTENIET, M.; DOESCHATE, K. T.; DICKS, L. M. Nisin F in the treatment of respiratory tract infections caused by Staphylococcus aureus. **Lett. App. Microbiol.**, Oxford, v. 48, no. 1, p. 65-70, Jan. 2009.

DOBRZYNSKA, I.; SKRZYDLEWSKA, E.; FIGASZEWSKI, Z. A. Changes in electric properties of human breast cancer cells. **J. Membr. Biol.**, New York, v. 246, no. 2, p. 161-166, Feb. 2013.

ETIENNE-MANNEVILLE, S. Polarity proteins in migration and invasion. **Oncogene**, Basingstoke, v. 27, no. 55, p. 6970-6980, Nov. 2008.

FERNANDEZ, L. et al. The bacteriocin nisin, an effective agent for the treatment of staphylococcal mastitis during lactation. **J. Hum. Lact.,** Thousand Oaks, v. 24, no. 3, p. 311-316, Aug. 2008.

FIELD, D. et al. The generation of nisin variants with enhanced activity against specific Gram-positive pathogens. **Mol. Microbiol.**, Oxford, v. 69, p. 218-230, July 2008.

FRANCO, J. S. et al. Calpain-mediated proteolysis of tail regulated adhesion dynamics. **Nat. Cell Biol.**, London, v. 6, p. 977-983, Sept. 2004.

FRIEDL, P. Prespecification and plasticity: shifting mechanisms of cell migration. **Curr. Opin. Cell Biol.**, London, v. 16, no. 1, p. 14-23, Feb. 2004.

GARDEL, M. L. et al. Mechanical integration of actin and adhesion dynamics in cell migration. **Annu. Rev. Cell Dev. Biol.**, Palo Alto, v. 26, p. 315-333, May 2010.

GASPAR, D.; VEIGA, A. S.; CASTANHO, M. A. From antimicrobial to anticancer peptides: A review. **Front. Microbiol.**, Lausanne, v. 4, p. 1-16, Oct. 2013.

GOMES, F. G. et al. Tumor angiogenesis and lymphangiogenesis: tumor/endothelial crosstalk and cellular/microenvironmental signaling mechanisms. **Life Sci.**, Amsterdam, v. 92, no. 2, p. 101-107, Feb. 2013.

GUARINO, M. Epithelial-mesenchymal transition and tumour invasion. **Int. J. Biochem. Cell Biol.**, Amsterdam, v. 38, no. 16, p. 2153-2160, July 2007.

HANAHAN, D.; WEINBERG, R. A. Hallmarks of cancer: the next generation. **Cell**, Cambridge, v.144, no. 5, p. 646-74, Mar. 2011.

HANNA, S.; EL-SIBAI, M. Signaling networks of Rho GTPases in cell motility. **Cell Signal.**, Oxford, v. 25, no. 10, p. 1955-1961, Oct. 2013.

HOSKIN, D. W.; RAMAMOORTHY, A. Studies on anticancer activities of antimicrobial peptides. **Biochim. Biophys. Acta**, Amsterdam, v. 1778, no. 2, p. 357-375, Feb. 2008.

HSIAO, Y. et al. Anticancer activities of an antimicrobial peptide derivative of Ixosin-B amide. **Bioorg. Med. Chem. Lett.**, Oxford, v. 23, p. 5744-5747, Aug. 2013.

INSTITUTO NACIONAL DE CÂNCER JOSÉ ALENCAR GOMES DA SILVA. **Síntese de resultados e comentários**, 2015. Disponível em: <http://www.inca.gov.br/estimativa/2014/sintese-de-resultados-comentarios.asp >. Acesso em: 29 out. 2015.

JAIN, R. K. Normalizing tumor microenvironment to treat cancer: bench to bedside to biomarkers. **J. Clin. Oncol.**, Alexandria, v. 31, no. 17, p. 2205-2218, June 2013.

JOO, N. E. et al. Nisin, an apoptogenic bacteriocin and food preservative, attenuates HNSCC tumorigenesis via CHAC1. **Cancer Med.**, Malden, v. 1, no. 3, p. 295-305, Dec. 2012.

KAMARAJAN, P. et al. Nisin ZP, a bacteriocin and food preservative, inhibits head and neck cancer tumorigenesis and prolongs survival. **PloS One**, San Francisco, v. 10, no. 7, p. 1-20, July 2015.

LAUFFENBURGER, D. A.; HORWITZ, A. F. Cell migration: a physically integrated molecular process. **Cell**, Cambridge, v. 84, no. 3, p. 359-369, Feb. 1996.

LEE, E. et al. Inhibition of breast cancer growth and metastasis by a biomimetic peptide. **Sci. Rep.**, London, v. 4, p. 1-13, Nov. 2014.

LEE, J. et al. Regulation of cell movement is mediated by stretch-activated calcium channels. **Nature**, London, v. 400, p. 382-386, May 1999.

LEUSCHNER, C.; HANSEL, W. Membrane disrupting lytic peptides for cancer treatments. **Curr. Pharm. Des.**, Schiphol, v. 10, no. 19, p. 2299-2310, July 2004.

MADER, J. S. et al. Bovine lactoferricin inhibits basic fibroblast growth factor- and vascular endothelial growth factor165-induced angiogenesis by competing for heparin-like binding sites on endothelial cells. **Am. J. Pathol.**, Philadelphia, v. 169, no. 5, p. 1753-1766, Nov. 2006.

PAINTER, K. J.; ARMSTRONG, N. J.; SHERRATT, J. A. The impact of adhesion on cellular invasion processes in cancer and development. **J. Theor. Biol.**, Amsterdam, v. 264, no. 3, p. 1057-1067, June 2010.

PARONS, J. T.; HORWITZ, A. R.; SCHWARTZ, M. A. Cell adhesion: integrating cytoskeletal dynamics and cellular tension. **Nat. Rev. Mol. Cell Biol.**, London, v. 11, no. 9, p. 633-643, Sept. 2010.

PREET, S. et al. Effetct of nisin and doxorubicin on DMBA-induced skin carcinogenesis – a possible adjunct therapy. **Tumour Biol.**, Dordrecht, p. 1-8, May 2015.

PREVARSKAYA, N.; SKRYMA, R.; SCHUBA, Y. Calcium in tumour metastasis: new roles for known actors. **Nat. Rev. Cancer**, London, v. 11, p. 609-618, Aug. 2011.

PUSHPANATHAN, M.; GUNASEKARAN, P.; RAJENDHRAN, J. Antimicrobial peptides: versatile biological properties. **Int. J. Pept.**, New York, v. 2013, p. 1-15, June 2013.

RIDLEY, A. J. et al. Cell Migration: Integrating signals from front to back. **Science**, New York, v. 5651, no. 302, p. 1704-1709, Dec. 2003.

RODRIGUES, M. A. M.; CAMARGO, J. L. V. Carcinogênese. In: FRANCO, M. et al. **Patologia:** processos gerais**.** São Paulo: Atheneu, 2010. Cap. 15, p. 256-272.

ROLLEMA, H. S. et al. Improvement of solubility and stability of the antimicrobial peptide nisin by protein engineering. **Appl. Environmental Microbiol.**, Washington, v. 61, no. 8, p. 2873-2878, Aug. 1995.

SAHAI, E. Mechanisms of cancer cell invasion. **Curr. Opin. Genet. Dev.**, London, v. 15, no. 1, p. 87-96, Feb. 2005.

SANDQUIST, J. C. et al. Rho kinase differentially regulates phosphorylation of nonmuscle myosin II isoforms A and B during cell rounding and migration. **J. Biol. Chem.**, Baltimore, v. 281, no. 47, p. 35873-35883, Nov. 2006.

SEO, M. D. et al. Antimicrobial peptides for therapeutic applications: a review. **Molecules**, Basel, v. 17, no. 10, p. 12276-12286, Oct. 2012.

SZACHOWICZ-PETELSKA, B. et al. Phospholipid composition and electric change in healthy and cancerous parts of human kidneys. **J. Mem. Biol.**, [S.1], v. 246, no. 5, p. 421-425, May 2013.

TEICHER, B. A.; LINEHAN, W. M.; HELMAN, L. J. Targeting cancer metabolism. **Clin. Cancer Res.,** Denville, v. 18, no. 20, p. 5537-5545, Oct. 2012.

TSAI, F. C. et al. A polarized Ca^{2+}, diacyglycerol and STIM1 signalling regulates directed cell migration. **Nat. Cell Biol.**, London, v. 16, p. 133-144, Jan. 2014.

TSAI, F. C.; MEYER, T. Ca^{2+} pulses control local cycles of lamellipodia retraction and adhesion along the front of migrating cells. **Curr. Biol.**, Cambridge, v. 22, no. 9, p. 837-842, May 2012.

TSANTOULIS, P. K. et al. Advances in the biology of oral cancer. **Oral Oncol.**, Oxford, v. 43, no. 6, p. 523-534. July 2007.

UGURLU, T. et al. Colonic delivery of compression coated nisin tablets using pectin/HPMC polymer mixture. **Eur. J. Pharm. Biopharm.**, Amsterdam, v. 67, no. 1, p. 202-210, Aug. 2007.

UTSUGI, T. et al. Elevated expression of phosphatidylserine in the outer leaflet of human tumor cells and recognition by activated human blood monocytes. **Cancer Res.**, Denville, v. 51, no.11, p. 3062-3066, June 1991.

VALENTA, C.; BERNKOP-SCHNURCH, A.; RIGLER, H. P. The antistaphylococcal effect of nisin in a suitable vehicle: a potential therapy for atopic dermatitis in man. **J. Pharm. Pharmacol.**, West Sussex, v. 48, no. 9, p. 988-991, Sept. 1996.

WANG, G. **The antimicrobial peptide database**. Disponível em: <http://aps.unmc.edu/AP/database/query_input.php>. Acesso em: 29 out. 2015.

WANG, K. et al. Effect of human beta-defensin-3 on head and neck cancer cell migration using micro-fabricated cell islands. **Head Neck Oncol.**, London, v. 4, no. 41, p. 1-8, June 2012.

WEBB, D. J. HORWITZ, A. F. New dimensions in cell migration. **Nat. Cell Biol.**, London, v. 5, no. 8, p. 690-692, Aug. 2003.

WEI, C. et al. Calcium flickers steer cell migration. **Nature**, London, v. 457, p. 901-905, Feb. 2009.

ZASLOFF, M. Antimicrobial peptides of multicellular organisms. **Nature**, London, v. 415, p. 389-391, Jan. 2002.

ZHANG, G. et al. Unraveling the mystery of cancer metabolism in the genesis of tumor-initiating cells and development of cancer. **Biochim. Biophys. Acta**, Amsterdam, v. 1836, no. 1, p. 49-59. Aug. 2013.

ZHANG, Y; YANG, J. M. Altered energy metabolism in cancer: a unique opportunity for therapeutic intervention. **Cancer Biol. Ther.**, Philadelphia, v. 14, no. 2, p. 81-89, Feb. 2013.

ANEXO A – ARTIGO CIENTÍFICO

A parte inicial deste trabalho foi desenvolvido em parceria com a Profª. Yvonne Kapila da Universidade de Michigan. Este trabalho foi publicado na revista PlosOne e está em anexo neste trabalho.

PLOS ONE

RESEARCH ARTICLE

Nisin ZP, a Bacteriocin and Food Preservative, Inhibits Head and Neck Cancer Tumorigenesis and Prolongs Survival

Pachiyappan Kamarajan[1], Takayuki Hayami[2], Bibiana Matte[1], Yang Liu[1], Theodora Danciu[1], Ayyalusamy Ramamoorthy[3], Francis Worden[4], Sunil Kapila[2], Yvonne Kapila[1]*

1 Department of Periodontics and Oral Medicine, School of Dentistry, University of Michigan, Ann Arbor, Michigan, United States of America, **2** Department of Orthodontics and Pediatric Dentistry, School of Dentistry, University of Michigan, Ann Arbor, Michigan, United States of America, **3** Department of Chemistry and Biophysics, College of Literature, Science, and the Arts, University of Michigan, Ann Arbor, Michigan, United States of America, **4** Department of Internal Medicine, School of Medicine, University of Michigan, Ann Arbor, Michigan, United States of America

* ykapila@umich.edu

OPEN ACCESS

Citation: Kamarajan P, Hayami T, Matte B, Liu Y, Danciu T, Ramamoorthy A, et al. (2015) Nisin ZP, a Bacteriocin and Food Preservative, Inhibits Head and Neck Cancer Tumorigenesis and Prolongs Survival. PLoS ONE 10(7): e0131008. doi:10.1371/journal.pone.0131008

Editor: Hari K. Koul, Louisiana State University Health Sciences Center, UNITED STATES

Received: February 10, 2015

Accepted: May 26, 2015

Published: July 1, 2015

Data Availability Statement: All relevant data are available within the paper and its Supporting Information file.

Funding: This study was supported by the University of Michigan MCUBED Funding #U036798 to YK, FPW, and AR. BM was supported by the Brazilian CAPES and CNPq sponsored, Science Without Borders Program. YL was supported by the Scholars Program from Peking University School and Hospital of Stomatology.

Abstract

The use of small antimicrobial peptides or bacteriocins, like nisin, to treat cancer is a new approach that holds great promise. Nisin exemplifies this new approach because it has been used safely in humans for many years as a food preservative, and recent laboratory studies support its anti-tumor potential in head and neck cancer. Previously, we showed that nisin (2.5%, low content) has antitumor potential in head and neck squamous cell carcinoma (HNSCC) *in vitro* and *in vivo*. The current studies explored a naturally occurring variant of nisin (nisin ZP; 95%, high content) for its antitumor effects *in vitro* and *in vivo*. Nisin ZP induced the greatest level of apoptosis in HNSCC cells compared to low content nisin. HNSCC cells treated with increasing concentrations of nisin ZP exhibited increasing levels of apoptosis and decreasing levels of cell proliferation, clonogenic capacity, and sphere formation. Nisin ZP induced apoptosis through a calpain-dependent pathway in HNSCC cells but not in human oral keratinocytes. Nisin ZP also induced apoptosis dose-dependently in human umbilical vein endothelial cells (HUVEC) with concomitant decreases in vascular sprout formation *in vitro* and reduced intratumoral microvessel density *in vivo*. Nisin ZP reduced tumorigenesis *in vivo* and long-term treatment with nisin ZP extended survival. In addition, nisin treated mice exhibited normal organ histology with no evidence of inflammation, fibrosis or necrosis. In summary, nisin ZP exhibits greater antitumor effects than low content nisin, and thus has the potential to serve as a novel therapeutic for HNSCC.

Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth leading cause of death worldwide. Patients diagnosed with HNSCC are treated with surgery, radiotherapy and

Competing Interests: The authors have declared that no competing interests exist.

chemotherapy. Given its anatomical location, HNSCC surgical resection is often destructive and, frequently, complete removal of the tumor mass is not a viable option, often rendering these patients with incurable disease following treatments with chemoradiotherapy [1]. There are limited chemotherapeutic options for patients once their disease is no longer amenable to cure, and the low 5-year survival rates for patients with metastatic HNSCC have not improved in decades [2,3]. These facts emphasize the urgency of improving treatment options for such patients.

A few reports have suggested that antimicrobial peptides or bacteriocins have cytotoxic effects against cancer cells [4–8]. Given this interesting premise, we explored the cytotoxic and antitumor properties of the antimicrobial peptide, nisin, and found that it blocks HNSCC tumorigenesis [9]. Nisin is a 34-amino acid polycyclic antibacterial peptide that is produced by fermentation of the gram-positive bacterium *Lactococcus lactis*. Many antibacterial agents are effective against similar bacterial species; however, nisin has broad-spectrum effects, as it also inhibits gram-negative bacteria [10]. Nisin is not toxic to animals and is safe for human consumption [11]. Nisin was approved for human use as a food preservative by the world health organization (WHO) in 1969 and by the food and drug administration (FDA) in 1988, and it has been given a generally-regarded-as-safe (GRAS) designation by the FDA [12]. It is estimated that 0.94 to 2.24 mg of nisin are consumed per person per day in the US [12]. While bacteriocins, like nisin, have been used for decades in preventing bacterial growth in foods, they have only recently been tested for prevention of growth or induction of apoptosis of cancer cells. Apoptosis or programmed cell death is a natural process that eliminates unwanted or older cells. However, cancer cells are resistant to apoptosis. Thus, there is great effort to understand the mechanisms that regulate apoptosis in these cells so that novel agents can be developed to induce apoptosis of cancer cells. Nisin may serve in this capacity and development of nisin as a cancer therapeutic can be readily pursued following dosing determinations.

Recently, we reported that nisin, may serve as a novel potential therapeutic for treating HNSCC [9]. Nisin mediates these effects by inducing preferential apoptosis, cell cycle arrest, and reducing cell proliferation in HNSCC cells, compared to primary keratinocytes. Nisin also reduces HNSCC tumorigenesis in vivo. Mechanistically, nisin exerts these effects on HNSCC, in part, through cation transport regulator homolog 1 (CHAC1), a proapoptotic cation transport regulator and through a concomitant CHAC1-independent influx of extracellular calcium [9,13]. These findings support the use of nisin as a potentially novel therapeutic for HNSCC, and since nisin is safe for human consumption as a food preservative, its translation into a clinical setting may be facilitated.

Nisin acts by altering the integrity of the cellular membrane and forming short-lived pores, thereby changing the membrane potential [14]. Nisin becomes immersed in the cell membrane through the cationic portions of the amino acids extending to one side of the molecule. These cationic portions interact with the negatively charged phospholipid heads, while the hydrophobic portion of nisin interacts with the membrane core [15]. Nisin's involvement with the membrane, therefore, mediates phospholipid reorganization and allows an influx of ions [14,16]. Since HNSCC cells and primary keratinocytes differ in their lipid membrane composition and function and response to calcium fluxes, nisin's ability to alter the transmembrane potential and membrane composition of cells may lead to differential effects on these cells [17–22]. Indeed, our data support this premise as the basis for the nisin-mediated differential apoptotic cell death and reduced proliferation of HNSCC cells compared to primary keratinocytes [9].

Given nisin's role as a safe bacteriocin for food preservation, and the knowledge that other bacteriocins possess proapoptotic properties against cancer cells, our findings on the effects of nisin on HNSCC tumorigenesis help provide a basis for nisin as a potential novel therapeutic for HNSCC. Thus, our focus in the current study was to extend this baseline knowledge.

Previously, we used a 2.5% nisin (low content) formulation. In this study, our aim was to test the efficacy of a 95% nisin (high content) on HNSCC cell apoptosis and proliferation in vitro and on tumorigenesis in vivo. Specifically, we focused on its translational potential by examining a highly pure, food grade form of nisin for its in vivo and long term effects. To this end, we tested two naturally occurring, high nisin content variants, nisin ZP and nisin AP. Considering nisin's safety for human consumption and given its in vitro and in vivo efficacy in HNSCC, nisin could be developed as a novel cancer therapeutic for HNSCC.

Materials and Methods

Institutional Review Board Approval

This study has been approved by and was conducted in accordance with the regulations set for by the institutional review board and the committee on the use and care of animals at the University of Michigan.

Cell culture

Four human HNSCC cell lines were used for these studies. HNSCC cell line authentication and origin was provided by their sources and published extensively. The human HNSCC cell lines, UM-SCC-17B (supraglottis/soft tissue-neck) and UM-SCC-14A (floor of mouth) were provided by Dr. Thomas Carey (Professor, University of Michigan, MI) [23]. The oral SCC cell line HSC-3 (tongue) was provided by Dr. Randall Kramer (Professor, University of California, San Francisco, CA) [24]. The oral SCC cell line, OSCC-3 (tongue) was provided by Dr. Mark Lingen (Professor, University of Chicago). HNSCC cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 1% penicillin, and 1% streptomycin. Normal-human umbilical vein endothelial cells (HUVECs), and accompanying cell culture media and supplements (EGM-2 Bullet Kits) were obtained from Lonza (Allendale, NJ). Human recombinant VEGF165 was obtained from Sigma-Aldrich (St. Louis, MO) and Matrigel Matrix was obtained from BD Biosciences (San Jose, CA). HUVECs were cultured in EGM-2 basal media at 37°C in 5% CO_2.

Nisin

Nisin A and nisin Z are two naturally occurring variants of nisin that are produced by fermentation of *Lactococcus* lactis and they differ by a single amino acid residue at position 27; histidine in nisin A and asparagine in nisin Z [25]. High content forms of these two variants, nisin ZP and nisin AP (95% content/ultrapure; % weight/weight; hydrous potency $\geq$38,000 IU/mg) were purchased from Handary (Brussels, Belgium) and low content nisin A (2.5% content in balance sodium chloride and denatured milk solids; potency $\geq$1,000,000 per IU/g) was purchased from Sigma-Aldrich (N5764). Nisin ZP and AP and low content nisin were reconstituted in water and used for all experiments.

Cell Proliferation and Colony Formation Assays

To determine the effect of nisin on cell proliferation, the CyQUANT NF Cell Proliferation Assay Kit was used according to manufacturer's instructions (Invitrogen/Life Technologies, Grand Island, NY). For colony formation assays, 2000 cells were seeded in a 6-well plate. Following overnight incubation, the cells were treated with different concentrations of nisin ZP for 48 hours. After treatment, fresh media was added every 2 days for a period of 10 days. Colonies were then stained with 0.5% crystal violet and colonies that contained >50 cells were counted. Experiments were repeated in triplicate.

Orasphere Assay

Sphere assays were used to assess anchorage-independent growth, a property thought to contribute to metastatic potential. HNSCC oraspheres (UM-SCC-17B) were prepared as previously reported [26–29]. In brief, cells that survive anchorage withdrawal form multicellular aggregates or oraspheres. Oraspheres were developed by maintaining cells under suspension conditions on poly (2-hydroxyethyl methacrylate) (poly-HEMA) coated plates (7.5 mg/mL in 95% ethanol, Sigma-Aldrich) for 36 hours. An orasphere is defined as an aggregate of cells that is at least 50 μm in diameter. The total area occupied by oraspheres in each well was quantified for each treatment condition using NIS-Elements BR4.13.04 imaging software. Experiments were performed in triplicate. Nisin was added to each well at the time of cell plating.

Apoptosis Assays

To determine the effects of three different forms of nisin on HNSCC cell apoptosis three different assays were performed. Apoptosis was assessed in nisin treated cells using a direct staining method, a flow cytometry-based assay, and a western blotting approach to evaluate known apoptotic signaling proteins.

Apoptosis: Staining and Microscopy

Ethidium bromide and acridine orange (EB/AO) staining was used to measure apoptosis as previously described [30]. For these assays, cells were plated in 96-well plates at 2×10^4 cells/cm^2, and after 24 hours, treated with various concentration of nisin (0, 100, 200, 400 and 800 μg/mL) for 24 hours. Cells were then stained with an EB/AO stain. EB was obtained from Bio-rad (Berkeley, CA) and AO was obtained from Acros Organics (Geel, Belgium). The EB/AO dye reagent was comprised of 100μg/ml of ethidium bromide and 100μg/ml of acridine orange in PBS. The EB/AO dye was added to each well, removed, and then images of stained cells were captured using a microscope equipped with a digital imaging system (Eclipse 50i Nikon, Melville, NY). Cells were counted and, based on their color, were classified as either vital, apoptotic or necrotic. AO permeates all cells and makes the nuclei appear green. EB is only taken up by cells when cytoplasmic membrane integrity is lost, and it stains the nuclei so they appear red. Thus, early apoptotic cells appear green with bright green dots in the nuclei due to chromatin condensation and nuclear fragmentation. Late apoptotic cells appear orange (combination of green and red colors) with more significant chromatin condensation and nuclear fragmentation. Necrotic cells appear orange but their nuclear morphology resembles that of viable cells, thus there is an absence of chromatin condensation. The percentage of cells in each category was determined by counting a minimum of 100 cells. Experiments were performed in triplicate.

Apoptosis: Flow cytometry

The percentage of apoptotic cells induced by nisin treatment was determined by flow cytometry. Briefly, cells were detached by incubation with enzyme-free dissociation buffer (Invitrogen), pelleted by centrifugation, and stained with Annexin V (BD Pharmingen) for analysis by flow cytometry (FACSDiVa Cell sorter, Becton Dickinson).

Apoptosis: Western Blotting

To evaluate the effects of nisin ZP on the expression of pro-apoptotic proteins in HNSCC cells, we performed Western blot analyses of UM-SCC-17B cells that were treated with nisin ZP for 18 hours. A calpain inhibitor was also examined in this context for its ability to reverse the

apoptotic effects induced by nisin ZP; namely blocking PARP cleavage. The calpain inhibitor was purchased from Sigma-Aldrich (A6185, St. Louis, MO), reconstituted in water, and used at a concentration of 500 nM. Following various treatments, cells were collected, washed once with phosphate-buffered saline and lysed for 30 minutes while on ice in radioimmunoprecipitatation assay (RIPA) buffer (R0278, Sigma-Aldrich) that contained a 1% protease inhibitor cocktail (P8340, Sigma-Aldrich). Lysates were adjusted for protein concentration with the BCA protein assay kit (Bio-Rad, Berkeley, CA). Protein lysates were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to Immobilon-P membranes (Millipore, Billerica, MA). Western blot analyses were performed using an anti-poly (ADP-ribose) polymerase-1 (PARP, SC-7150, Santa Cruz Biotechnology, Santa Cruz, CA) primary antibody, a caspase-3 primary antibody (SC-7148, Santa Cruz Biotechnology), a calpain primary antibody (MAB3083, Millipore) followed by a horseradish peroxidase-conjugated anti-rabbit antibody (SC-2004, Santa Cruz Biotechnology) or anti-mouse antibody (A9044, Sigma-Aldrich). Blots were then developed with the ECL-plus detection system (Pierce). To evaluate the samples for equal protein loading, membranes were stripped and reprobed with an anti-β-actin antibody (SC-1615, Santa Cruz Biotechnology).

In Vitro Angiogenesis Assay

To determine the effect of nisin ZP on angiogenesis, we performed in vitro sprout assays [31]. For sprout assays, HUVECs were trypsinized and suspended in endothelial cell growth media (EGM-2) containing 50 ng/ml of recombinant vascular endothelial growth factor (VEGF) and various concentrations of nisin ZP (0, 100, 400 and 800 μg/mL). Cells were seeded at 1×10^4 cells/cm^2 in Matrigel coated 96-well plates and incubated overnight. Images of the sprouts were captured using the EVOS XL Core Imaging System (Life Technologies, Grand Island, NY), then total sprout length was measured using Image J (National Institutes of Health). Assays were performed in triplicate.

Immunohistochemical Analyses

To evaluate nisin's effects on angiogenesis in vivo, immunohistochemical analyses were used to evaluate CD31 expression, an endothelial cell adhesion molecule, in mouse tumor tissue sections. Briefly, after antigen retrieval by microwave pretreatment (citrate buffer, 10 mM, pH 6.0), slides were incubated with a CD31 primary antibody (DIA-310, Dianova, Hamburg, Germany) overnight at 4°C. After diaminobenzidine chromogen (DAB) reaction, slides were counterstained with routine hematoxylin. Immunohistochemical staining for CD31 was graded and scored by a pathologist in a blinded manner.

Oral cancer mouse model

To examine the in vivo antitumor effects of nisin ZP, an oral cancer floor-of-mouth mouse model was used. UM-SCC-17B cells were injected submucosally into the floor of the mouth in mice as previously described [9, 28, 29, 32]. All protocols for the in vivo studies were approved by the Committee on the Use and Care of Animals at the University of Michigan. Specifically, cells were grown to 70% confluence, suspended in DMEM, mixed with an equal volume of growth factor-reduced Matrigel basement membrane matrix (BD Biosciences, San Jose, CA) at a final concentration of 1.25 x 10^4/0.05 mL. Six-week-old athymic nude mice (NCr-nu/nu strain, NCI, Frederick, MD) were anesthetized by intraperitoneal injection with 100 mg/kg ketamine and 10 mg/kg xylazine. A total volume of 0.05 mL of SCC cell/Matrigel suspension was injected submucosally into the floor of the mouth. Three weeks after tumor cell injections and upon confirmation that tumors were established, animals were equally distributed into six

groups: a control group that was given water (equal volume/control) by oral gavage for 3 weeks and five different treatment groups that were given nisin treatment by oral gavage for 3 weeks. Following tumor cell injections, mice were monitored on alternate days. The treatment groups consisted of mice that received nisin ZP at two different concentrations (400 and 800 mg/kg per day) and mice that received nisin AP at two different concentrations (400 and 800 mg/kg per day). There was also another group of mice that was given nisin ZP (800 mg/kg per day) over an extended period of time (months). Following completion of nisin administration, mice were euthanized by CO_2 overdose and cervical dislocation, then tumors were harvested, rinsed in phosphate-buffered saline (PBS), imaged, and fixed overnight in 10% buffered formalin. A digital caliper was used to determine the tumor volume using the formula a x a x $b/2$, where a is the smaller dimension. In general, mice were euthanized if mice exhibited physiologic signs of stress from inability to eat or drink, weight loss, or when tumor volumes reach a range of 300–500mm^3, and thus mice were generally euthanized at approximately 3 weeks for the short-term protocol. Mice were euthanized by a CO_2 overdose.

Statistical Analysis

In general, values were expressed as means ± SD. Intergroup differences were analyzed by the analysis of variance (ANOVA) and Tukey-Kramer HSD test. For the in vivo studies, independent t-tests with unequal variance were used.

Results

Nisin ZP and nisin AP induce significant HNSCC cell apoptosis dose-dependently and beyond that in low content nisin

Treatment with both nisin ZP (95%) and nisin AP (95%) induced significant increases in apoptosis in HNSCC cells (UM-SCC-17B and HSC-3) compared to treatment with low content nisin (2.5%) (Fig 1A and 1B). Apoptosis was measured using ethidium bromide and acridine orange (EB/AO) staining and microscopy. The effects of nisin ZP and AP on apoptosis were dose-dependent and increased as the concentrations of nisin ZP and AP increased from 200 to 400 μg/ml. Although the differences in apoptosis between nisin ZP and nisin AP were not significant, nisin ZP exhibited better solubility, and thus most experiments were performed with nisin ZP.

Furthermore, the effects of nisin ZP on HNSCC cell apoptosis were significant throughout a broad range of doses from 100, 200, 400 and 800 μg/mL (Fig 1C–1E) and in several HNSCC cell lines (UM-SCC-17B and HSC-3). Coordinately, cells treated with increasing doses of nisin ZP exhibited significantly decreasing numbers of vital cells and significantly increasing numbers of apoptotic cells, whereas the number of necrotic cells stayed relatively low and constant. In contrast, primary keratinocytes did not exhibit enhanced levels of apoptosis like that seen in HNSCC cell lines (Fig 1F).

Nisin ZP induces apoptosis via calpain, caspase 8 and PARP cleavage

To further examine the mechanism of nisin-mediated apoptosis, we employed additional apoptotic assays and surveyed known apoptotic signaling proteins. Four different HNSCC cell lines were examined for their nisin responsiveness. Nisin significantly increased apoptosis in all four HNSCC cells lines as assessed by annexin V staining and flow cytometry (Fig 2A).

Given our previous findings that nisin mediates calcium-dependent apoptosis, we explored the potential involvement of calpain, a known calcium dependent mediator of apoptosis [9]. Indeed, nisin treated HNSCC cells exhibited decreased expression levels of the calpain 1 small

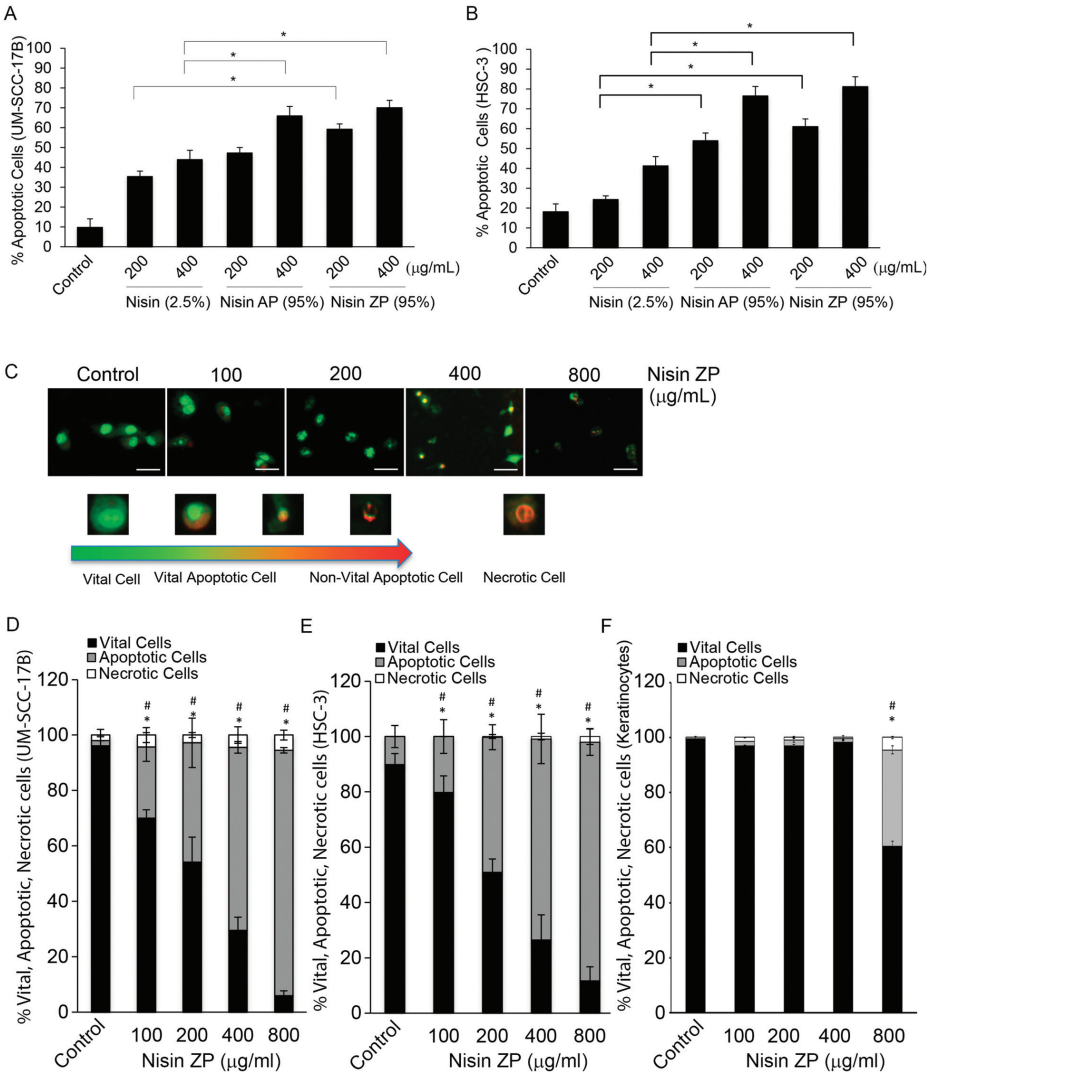


Fig 1. Nisin ZP and nisin AP induce significant HNSCC cell apoptosis dose-dependently and beyond that in low content nisin. *A and B*, Graphs showing changes in percentage of apoptotic HNSCC cells (HSC-3 and UM-SCC-17B) treated with control media or media containing 2.5% nisin, 95% nisin AP, or 95% nisin ZP for 24 h. Cells were then stained using an ethidium bromide and acridine orange (EB/AO) stain and counted. Comparisons between groups relative to controls were analyzed by ANOVA with the level of significance set at *p<0.05. *C*, Microscopic images showing morphologhy of HNSCC cells treated with control media or media containing nisin ZP (100 to 800 μg/mL) for 24 h then stained with EB/AO (Scale bars, 100 μm). *D-F*, Graphs showing changes in percentage of vital, apoptotic, and necrotic cells (UM-SCC-17B, HSC-3 and normal oral keratinocytes) treated with control media or media containing nisin ZP (400 or 800 μg/mL) for 24 h. Comparisons between groups relative to controls were analyzed by ANOVA with the level of significance set at *p<0.05 vital cells; # p<0.05 apoptotic cells.

doi:10.1371/journal.pone.0131008.g001

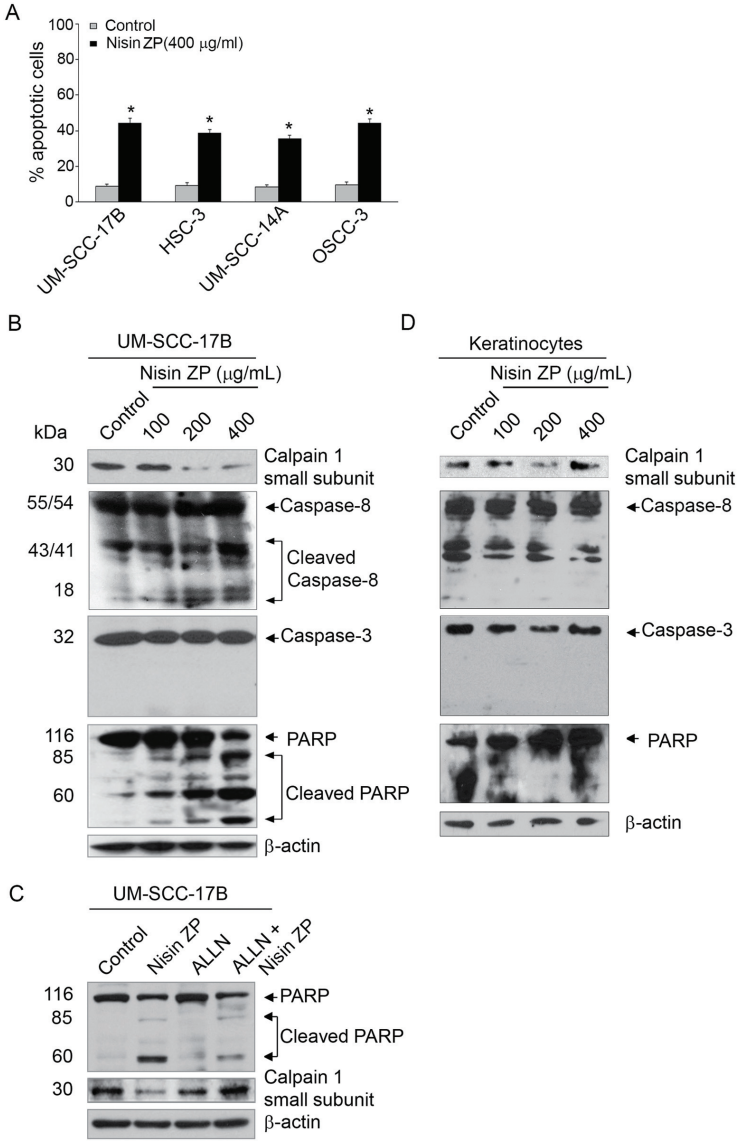


Fig 2. Nisin ZP induces calpain, caspase-8 and PARP activation in HNSCC cells dose-dependently. *A*, Graphs showing changes in percentage of apoptotic cells (UM-SCC-17B, HSC-3, UM-SCC-14A and OSCC-3) treated with control media or media containing nisin ZP (400 μg/mL) for 24 h. Cells were then stained with Annexin V and apoptosis was assessed by flow cytometry. Comparisons between groups relative to controls were analyzed by ANOVA with the level of significance set at *p<0.05. *B*, Immunoblots showing calpain 1,

caspase-8, caspase-3, and PARP protein levels in HNSCC (UM-SCC-17B) and *C*, normal human oral keratinocytes cell lysates after treatment with nisin ZP for 18 h. *D*, Immunoblots showing PARP and calpain 1 activation in HNSCC cell lysates after treatment for 18 h with control media, media containing nisin ZP (400 μg/mL), media containing a calpain inhibitor (ALLN, 500 nM), or media containing both a calpain inhibitor (ALLN, 500 nM) and nisin (400 μg/mL). *D*, An immunoblot for ß-actin shows the loading controls.

doi:10.1371/journal.pone.0131008.g002

subunit, indicative of calpain 1 activation (Fig 2B). The decreasing expression of the calpain 1 small subunit mirrored the increasing doses of nisin ZP from 100 to 400 μg/mL, and thus was dose-dependent.

To further explore the mechanism of apoptosis induced by nisin ZP, caspase-8 and caspase-3 cleavage were examined in this context. Nisin induced caspase-8 activation in a dose-dependent manner (Fig 2B). However, treatment of HNSCC cells with nisin ZP induced caspase-3 independent apoptosis (Fig 2B). Western blotting for caspase-3 in nisin ZP treated cells revealed steady levels of caspase-3 expression and the absence of caspase-3 cleavage regardless of the nisin dose tested (Fig 2B).

Nisin ZP treated cells also exhibited poly (ADP-ribose) polymerase (PARP) cleavage, a hallmark of apoptosis (Fig 2B). PARP cleavage increased dose-dependently as nisin ZP doses increased. To determine whether nisin-mediated PARP cleavage was a calpain-dependent mechanism, a calpain inhibitor (ALLN) was examined in this context (Fig 2C). Treatment of HNSCC cells with both nisin ZP and a calpain inhibitor, effectively diminished PARP cleavage, compared to cells treated with nisin alone. Normal human oral keratinocytes did not exhibit activation of calpain, caspase-8, caspase-3 or PARP in response to treatment with nisin ZP (Fig 2D). Thus, nisin ZP induced apoptosis of HNSCC cells via activation/cleavage of calpain, caspase-8 and PARP, but independent of caspase-3 cleavage.

Nisin ZP and nisin AP significantly reduce HNSCC cell proliferation time- and dose-dependently

The effects of nisin ZP and AP on HNSCC cell proliferation were examined next, and we found that treatment with both nisin ZP and nisin AP significantly reduced HNSCC cell (UM-SCC-17B) proliferation (Fig 3A). The effects of nisin ZP and AP on HNSCC cell proliferation were dose-dependent, since proliferation levels decreased as the concentration of nisin ZP and AP increased from 400 to 800 μg/mL. In addition, the differences in HNSCC cell proliferation induced by nisin ZP versus nisin AP treatment were significant, such that the effects induced by nisin ZP were more pronounced. Thus, given the greater effects of nisin ZP in reducing proliferation and given the improved solubility of nisin ZP over nisin AP, nisin ZP was selected for further study. The effects of nisin ZP on HNSCC cell proliferation were significant throughout a broad range of doses from 100 to 800 μg/ml and in several HNSCC cell lines (Fig 3B). Also, the nisin ZP-mediated reduction in HNSCC cell proliferation was time-dependent, showing significant effects at 6, 12, 24, and 48 h (Fig 3C). The reduced cell proliferation likely reflects in part the cell cycle arrest mediated by nisin [9]; further evidenced by decreased phosphorylation of the cell cycle checkpoint marker, cdc2 (S1 Fig).

Nisin ZP reduces HNSCC cell colony formation

We further explored the effects of nisin ZP on cell proliferation by measuring its long-term effects using colony formation assays. In agreement with the short-term proliferation assays, treatment with nisin ZP inhibited colony formation in HNSCC cells (Fig 3D and 3E). The inhibitory effects on colony formation were dose-dependent, since colony formation diminished significantly as the nisin ZP dose increased from 400 to 800 μg/mL. HNSCC cells treated

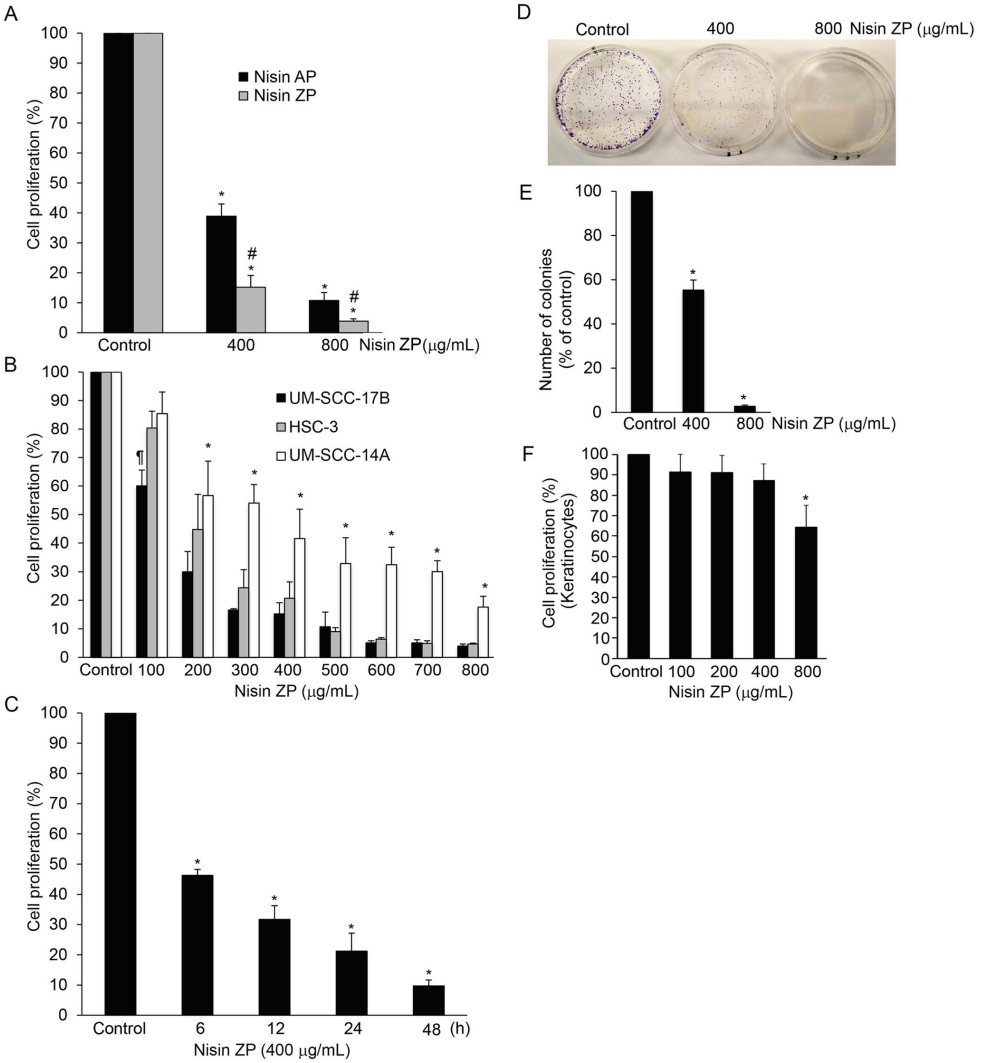


Fig 3. Nisin ZP and nisin AP significantly reduce HNSCC cell proliferation time- and dose-dependently and clonogenic capacity. *A*. Graphs showing changes in cell proliferation in UM-SCC-17B cells treated with control media or media containing nisin AP or ZP (400 or 800 μg/mL) for 48 h. Comparisons between groups relative to controls were analyzed by ANOVA with the level of significance set at *p<0.05. # Comparison between nisin AP and nisin ZP *p≤0.05. *B*. Graphs showing changes in cell proliferation in HNSCC cells (UM-SCC-17B, HSC-3 and UM-SCC-14A) treated with control media or media containing increasing doses of nisin ZP (100 to 800 μg/mL) for 48 h. Comparisons between groups relative to controls were analyzed by ANOVA with the level of significance set at *p<0.05. ¶Comparison between the 100 μg/ml group relative to the control for UM-SCC-17B cells *pμ0.05. *C*. Graphs showing changes in cell proliferation in UM-SCC-17B cells treated with control media or media containing nisin ZP (400 μg/mL) for 6, 12, 24 and 48 h. Comparisons between groups relative to controls were analyzed by ANOVA with the level of significance set at *p<0.05. *D*, The images are of UM-SCC-17B cells treated for 48h with control media or media containing nisin ZP (400 or 800 μg/mL) then cultured for 10 days, stained with crystal violet and imaged to evaluate

clonogenic capacity. *E*, The graph shows the percentage of colonies present relative to controls for assays measuring clonogenic capacity. Comparisons between groups relative to controls were analyzed by ANOVA with the level of significance set at *p<0.05. *F*. Graphs showing changes in cell proliferation in normal human oral keratinocytes treated with control media or media containing nisin ZP (100, 200, 400 or 800 μg/mL) for 48 h. Comparisons between groups relative to controls were analyzed by ANOVA with the level of significance set at *p<0.05.

doi:10.1371/journal.pone.0131008.g003

with these two doses exhibited a 2 and 50-fold decrease in clonogenic capacity. Nisin did not inhibit proliferation in normal human oral keratinocytes like that in HNSCC cells (Fig 3F).

Nisin ZP blocks orasphere formation

We further found that treatment with nisin ZP significantly blocked orasphere formation or anchorage independent growth (Fig 4A and 4B). Orasphere formation or anchorage independent growth is a property that contributes to tumorigenic potential. Nisin ZP's effects on orasphere formation were dose-dependent, such that total orasphere area decreased steadily following treatment with nisin doses from 100, 200, 400 and 800 μg/mL.

Nisin ZP induces endothelial cell apoptosis and inhibits angiogenic sprouting

We previously showed that treatment with low content nisin inhibited tumor growth and resulted in small pale tumors, suggesting that nisin affected the tumor blood supply. We also previously reported that nisin's antitumor effects were mediated by CHAC1, a proapoptotic inducer in endothelial cells [9]. Thus, to determine whether nisin ZP affects angiogenesis, we evaluated the effects of nisin ZP on endothelial cell apoptosis and sprouting. Human umbilical

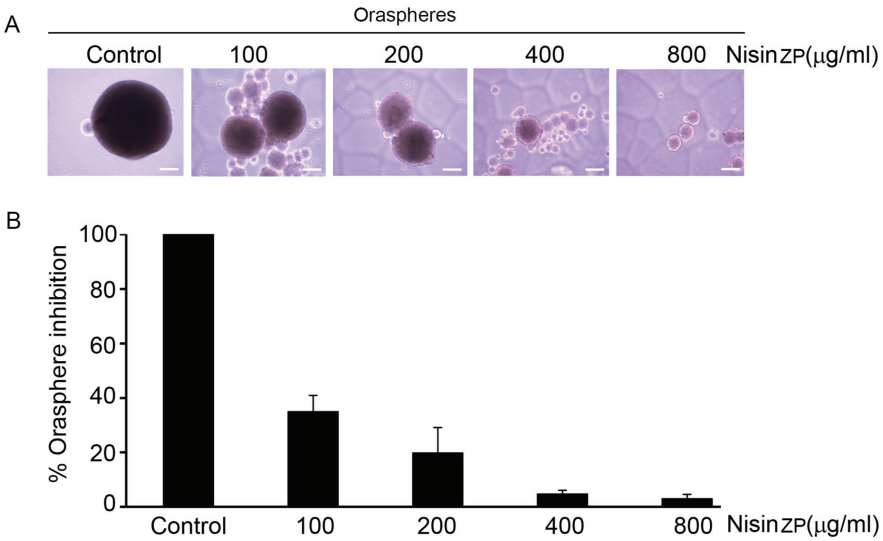


Fig 4. Nisin ZP inhibits orasphere formation in HNSCC cells. *A*, Phase contrast images and *B*, graph showing percentage of orasphere inhibition (total area) in HNSCC cells (UM-SCC-17B) cultured under suspension conditions and treated with control media or media containing nisin ZP (100 to 800 μg/ml) for 36 h. Comparisons between groups relative to controls were analyzed by ANOVA with the level of significance set at *p<0.05.

doi:10.1371/journal.pone.0131008.g004

vein endothelial cells (HUVECs) treated with different concentrations of nisin ZP (100, 200, 400 and 800 μg/mL) exhibited significantly increasing levels of apoptosis when compared to cells treated with media control (Fig 5A and 5B). Consistently, the vital cell fraction decreased coordinately with increasing doses of nisin, whereas the necrotic cell fraction remained small and constant throughout the treatment with different nisin doses. HUVECs treated with different concentrations of nisin ZP (100, 400 and 800 μg/mL) also exhibited significantly decreased sprouting when compared to cells treated with the media control (Fig 5C and 5D). Thus, nisin ZP induces significant endothelial cell apoptosis and inhibits angiogenic sprouting dose-dependently. Nisin may thereby promote further tumor reduction due to its anti-apoptotic and anti-proliferative effects on both tumor cells and endothelial cells.

Nisin AP and ZP reduces HNSCC tumor burden in vivo

To further study the effect of nisin ZP on HNSCC tumorigenesis, we used a well-characterized floor-of-mouth oral cancer xenograft mouse model (Fig 6, Table 1). After 3 weeks of treatment, mice receiving nisin ZP and nisin AP exhibited significantly reduced tumor volumes compared to controls (Fig 6, Table 1). All concentrations of nisin tested significantly reduced mean tumor volumes (13.5, 88.5, 59.2, and 66.75 mm^3 for nisin ZP 800 mg/kg, nisin ZP 400 mg/kg, nisin AP 800 mg/kg, and nisin AP 400 mg/kg, respectively) compared to controls (232.8 mm^3). In general, control treated mice exhibit physiologic signs of stress from inability to eat or drink when tumor volumes reach a range of 300–500mm^3, and thus mice were generally euthanized at approximately 3 weeks for the short-term protocol.

In contrast, mice in the long-term treatment group, which were given 800 mg/kg nisin ZP, survived extended periods of time without compromising eating or drinking. At 9 weeks, tumor volumes for this group of mice were as follows: mouse 1 was 356 mm^3, mouse 2 was 395 mm^3, mouse 3 was 91.9 mm^3, and mouse 4 was 126 mm^3 (Table 1). The survival times for these mice were as follows: mouse 1 survived 9 weeks, mouse 2 survived 9 weeks, mouse 3 survived 12 weeks, mouse 4 survived 16 weeks. Additionally, tumor volume for mouse 3 was 367 mm^3 (12 weeks) and mouse 4 was 2745 mm^3 (16 weeks) at euthanasia. Despite the large tumor volume in mouse 4, eating and drinking were not compromised until the latter time points. For this long treatment group of mice the mean tumor volume was 242 mm^3 at 9 weeks (Table 1). Importantly then, the mean tumor volume for control treated mice at 3 weeks was 232.8 mm^3, whereas the equivalent mean tumor volume in the nisin treated mice (242 mm^3) was reached at 9 weeks. Thus nisin treatment extended the time required for mice to reach comparable tumor volumes. Furthermore, nisin ZP treatment reduced intratumoral microvessel density (Fig 7). There were no histological differences in the liver, lung, and kidney organs of nisin treated mice compared to the control treated mice (Fig 8). Specifically, there was no histological evidence of inflammation, fibrosis or necrosis in these organs following nisin treatment even at the highest tested dose of 800 μg/ml.

Discussion

The current data on nisin ZP extend the baseline information for nisin and support the concept that nisin ZP may be a useful therapeutic for HNSCC, since nisin ZP promotes HNSCC cell apoptosis, suppresses HNSCC cell proliferation, and blocks angiogenic processes, orasphere formation and tumorigenesis in vivo. Translation of nisin ZP from the mouse setting to the human setting requires a dose conversion. Using the standard national cancer institute/national institute of health (NCI/NIH) dosage conversion factor of 12 for mouse to man an 800 mg/kg body weight/day of nisin ZP given to mice would translate to 66.7 mg/kg for humans [33]. FDA has accepted the highest dose tested in the Frazer et al., study (3,330,000

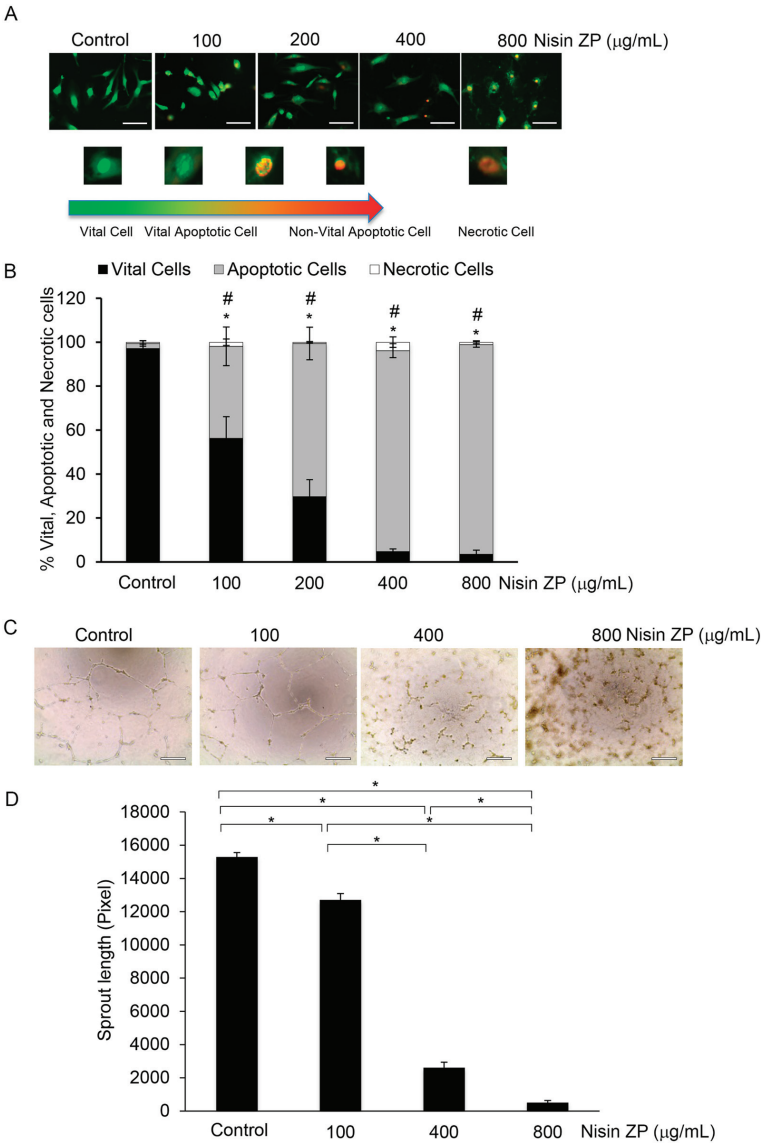


Fig 5. Nisin ZP induces endothelial cell apoptosis and inhibits angiogenic sprouting dose-dependently. *A*, Morphological observation of HUVEC cells treated with control media or media containing nisin ZP (100 to 800 μg/mL) for 24h. Cells were then stained with an ethidium bromide and acridine orange (EB/AO) stain, imaged, and counted (Scale bars, 100 μm). *B*, Graph showing percentage changes in vital, apoptotic, and necrotic HUVEC cells treated with control media or media containing nisin ZP (100 to 800 μg/mL) for 24 h. Comparisons between groups relative to controls were analyzed by ANOVA with the level of significance set at *p<0.05 vital cells; # p<0.05 apoptotic cells. *C*, Microscopic images of sprouting assays for cells (HUVEC) treated with control

media or media containing nisin ZP (100 to 800 μg/mL) for 24h then imaged and counted (Scale bars, 100 μm). *D*, Graphs showing the total sprout length achieved by cells treated as indicated in C. Comparisons between groups relative to controls were analyzed by ANOVA with the level of significance set at *p<0.05.

doi:10.1371/journal.pone.0131008.g005

units of nisin per kg of diet, equivalent to 83.25 mg per kg of diet) as the no-observed-effect-level (NOEL) and affirmed the generally regarded as safe (GRAS) status of nisin derived from *Lactococcus lactis* [12,34]. Typically, nisin is added to foods in the range of 0.25 to 37.5 mg/kg [35]. Also, a manufacturer of a liquid pure nisin A (>2.5% concentration) recommends adding 7.5–100 ml/L in beverages, including fruit juices and alcoholic beverages [36]. Acute toxicity or the LD_{50} in male and female rates was reported to be 9.2 g/Kg and 8.81 g/Kg and 8.81 g/Kg,

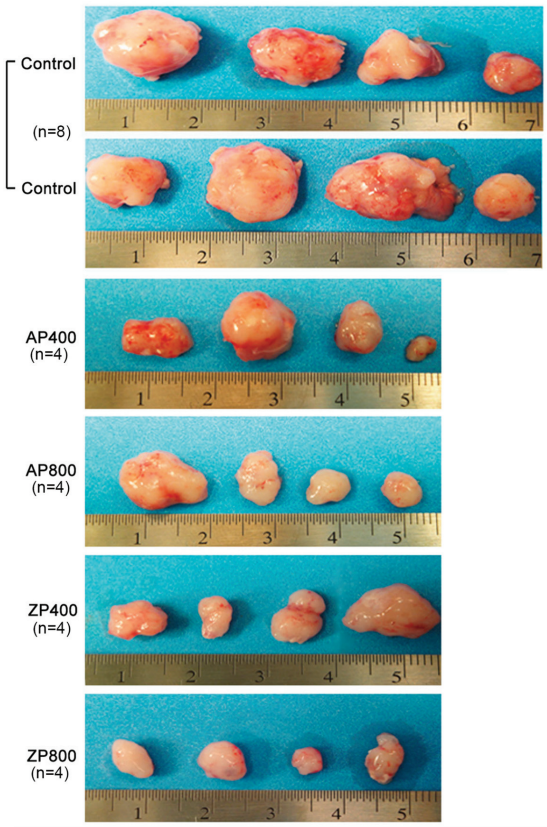


Fig 6. Nisin ZP reduces HNSCC tumor burden in mice. Images show the dissected tumors obtained from mice injected with UM-SCC-17B cells then treated with either water (control) or nisin AP or nisin ZP (400 or 800 mg/kg body weight/day) for 3 weeks.

doi:10.1371/journal.pone.0131008.g006

Table 1. Tumor volumes for mice injected with UM-SCC-17B cells then treated with water (control) or nisin (400 or 800 mg/kg body weight/day), using a short term (3 weeks) or long term treatment approach (9 weeks).

	Short Term Treatment					Long Term Treatment	
Animal number	**Control (mm3)**	**AP400 (mm3)**	**AP800 (mm3)**	**ZP400 (mm3)**	**ZP800 (mm3)**	**Animal Number**	**ZP800 (mm3)**
1	374	57	191	39	12	1	356
2	202	179	24	13	25	2	395
3	75	26	9	68	4	3	91.9
4	50	5	13	234	13	4	126
5	204						
6	584						
7	299						
8	75						
Mean volume	**232.8**	**66.75***	**59.2***	**88.5***	**13.5***		**242***

Statistical analysis: Independent t-test with unequal variances
*p<0.05.

doi:10.1371/journal.pone.0131008.t001

respectively [36]. Thus, a theoretical therapeutic dose for nisin (66.7 mg/kg) in humans would be approximately twice that already present in some foods (37.5/mg/kg). In addition, this dose does not approximate the reported toxic doses in rodents and is within the NOEL designation by the FDA.

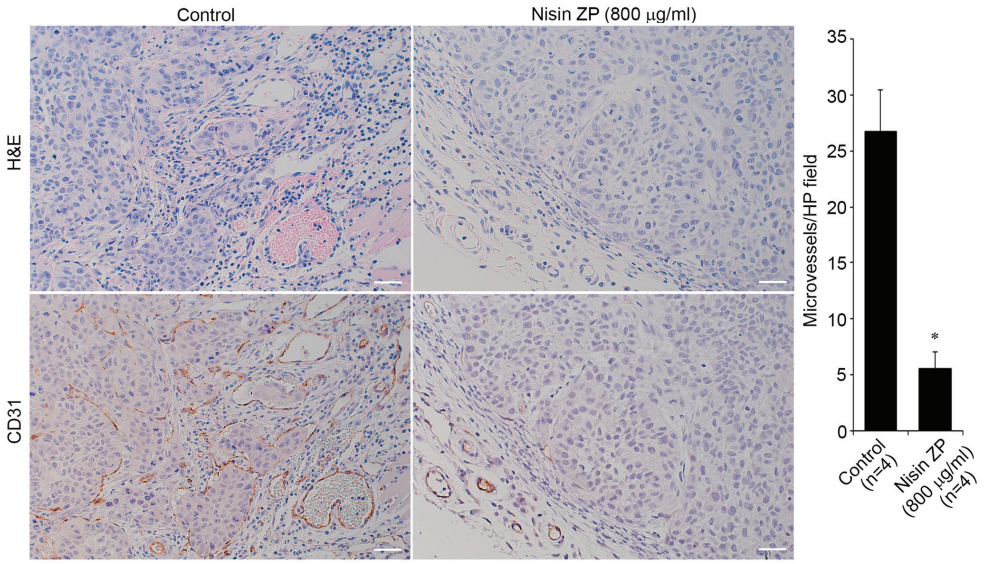


Fig 7. Nisin ZP reduces tumor microvessel density. *A*, Representative images of histological sections stained with H&E (top), and histological sections immunostained for CD31 (bottom) to identify blood vessels. *B*, Graph showing the results of microvessel quantification from five high power fields per tumor (*p<0.05).

doi:10.1371/journal.pone.0131008.g007

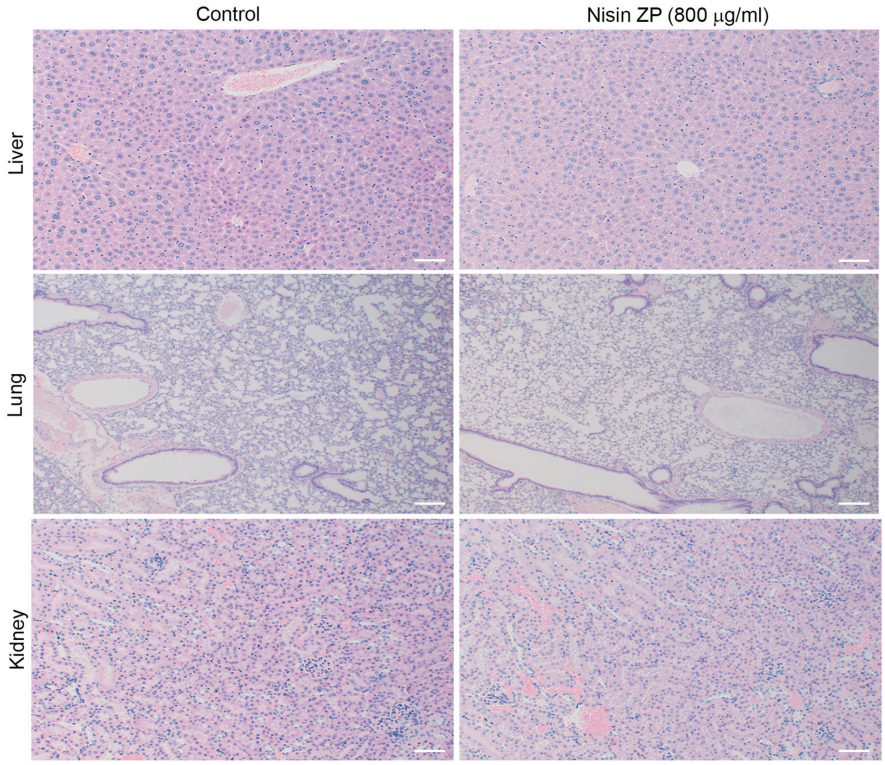


Fig 8. Nisin ZP does not elicit histological signs of toxicity in the liver, lung and kidney of mice. Images of H&E-stained histological sections of liver, lung and kidney from mice injected with UM-SCC-17B cells then treated with either water (control) or nisin ZP (800 mg/kg body weight/day) for 3 weeks.

doi:10.1371/journal.pone.0131008.g008

Bacteriocins, are antimicrobial peptides that have been examined to a limited extent for their antineoplastic potential. Purified bacteriocins, including pyocin, colicin, pediocin, and microcin have shown inhibitory properties against neoplastic cell lines and in xenograft mouse models [37–47]. Nisin has already been used effectively in vivo as a therapeutic for infections. In humans, nisin was an effective alternative to antibiotics for the treatment of staphylococcal mastitis during lactation in women and it was used as a sanitizer against the mastitis pathogens *Staphylococcus* and *Streptococcus* species in lactating cows [48–50]. Our recent work also supports nisin as a potential therapeutic for HNSCC. Thus, given nisin's known safety and use for over 40 years as a food preservative, its antitumor effects in vitro and in vivo, its use in humans as a therapeutic to treat infections, nisin ZP stands poised for development as a cancer therapeutic for HNSCC.

Although, a caspase cascade marked by activation and cleavage of caspase-3 is a hallmark of apoptosis, there are reports of caspase-3 independent apoptosis [51]. For example, p53-mediated cell cycle arrest and apoptosis is mediated through a caspase-3-independent, but caspase-9-dependent pathway in MCF-7 human breast cancer cells and leukemia cells [52,53]. Other

mediators of apoptosis, such as calpain have been implicated in both caspase-3 dependent and independent apoptosis [54–60]. Calpain activation via a $Ca2^{+}$ flux plays an essential role in eliciting an apoptosis-inducing factor (AIF)-mediated caspase-independent apoptosis and necroptosis in HeLa cells [61,62]. Additionally, CD44 ligation induces caspase-independent cell death via a novel calpain/AIF pathway in human erythroleukemia cells and U87MG human gliomas [63,64]. Thus, we explored the role of calpain in this study. Our data support the concept that nisin ZP induces a calpain-dependent mechanism of apoptosis that does not depend on caspase-3 cleavage (Fig 2B). In summary, nisin ZP, via induction of calpain-dependent apoptosis in HNSCC cells, induction of apoptosis in endothelial cells, and inhibition of HNSCC cell proliferation, inhibits tumorigenesis that exhibits reduced intratumoral microvessel density. Nisin ZP's in vivo antitumor effects and known safety as a food preservative, position nisin ZP for future development as a therapeutic for HNSCC.

Supporting Information

S1 Fig. Nisin treatment decreases cdc2 phosphorylation in HNSCC cells. Western blots showing that nisin treatment (80 μg/ml nisin 2.5% purity; 24 h) decreases phosphorylation of the cell cycle checkpoint marker, cdc2, in HNSCC cells (UM-SCC-17B) compared to control/media treatment. A western blot for cdc2 shows that total protein levels for cdc2 do not change with nisin treatment. ß-actin served as a loading control.
(EPS)

Acknowledgments

This study was supported by the University of Michigan MCUBED Funding #U036798 to YK, FPW, and AR. BM was supported by the Brazilian Federal Agency for support and evaluation of graduate education (CAPES) and the Brazilian National Council for scientific and technological development (CNPq), Science Without Borders Program. YL was supported by the Scholars Program from Peking University School and Hospital of Stomatology. We thank Adam Banda for helpful assistance in the laboratory.

Author Contributions

Conceived and designed the experiments: PK AR FW YK TD. Performed the experiments: PK TH BM YL. Analyzed the data: PK TH BM YL AR FW SK YK TD. Contributed reagents/materials/analysis tools: TD. Wrote the paper: PK BM AR FW SK YK.

References

1. Nonzee NJ, Dandade NA, Patel U, Markossian T, Agulnik M, Argiris A, et al. Evaluating the supportive care costs of severe radiochemotherapy-induced mucositis and pharyngitis: results from a Northwestern University Costs of Cancer Program pilot study with head and neck and nonsmall cell lung cancer patients who received care at a county hospital, a Veterans Administration hospital, or a comprehensive cancer care center. Cancer. 2008; 113: 1446–1452. doi: 10.1002/cncr.23714 PMID: 18683883
2. Bsoul SA, Huber MA, Terezhalmy GT. Squamous cell carcinoma of the oral tissues: a comprehensive review for oral healthcare providers. J Contemp Dent Pract. 2005; 6: 1–16.
3. Siegel R, Naishadham D, Jemal A. Cancer statistics, 2012. CA Cancer J Clin. 2012; 62: 10–29. doi: 10.3322/caac.20138 PMID: 22237781
4. Farkas-Himsley H, Zhang YS, Yuan M, Musclow CE. Partially purified bacteriocin kills malignant cells by apoptosis: programmed cell death. Cell Mol Biol (Noisy-le-grand). 1992; 38: 643–651.
5. Farkas-Himsley H, Hill R, Rosen B, Arab S, Lingwood CA. The bacterial colicin active against tumor cells in vitro and in vivo is verotoxin 1. Proc Natl Acad Sci U S A. 1995; 92: 6996–7000. PMID: 7624357
6. Koczulla AR, Bals R. Antimicrobial peptides: current status and therapeutic potential. Drugs. 2003; 63: 389–406. PMID: 12558461

7. Sand SL, Haug TM, Nissen-Meyer J, Sand O. The bacterial peptide pheromone plantaricin A permeabilizes cancerous, but not normal, rat pituitary cells and differentiates between the outer and inner membrane leaflet. J Membr Biol. 2007; 216: 61–71. PMID: 17639368
8. Hoskin DW, Ramamoorthy A. Studies on anticancer activities of antimicrobial peptides. Biochim Biophys Acta. 2008; 1778: 357–375. PMID: 18078805
9. Joo NE, Ritchie K, Kamarajan P, Miao D, Kapila YL. Nisin, an apoptogenic bacteriocin and food preservative, attenuates HNSCC tumorigenesis via CHAC1. Cancer Med. 2012; 1: 295–305. doi: 10.1002/cam4.35 PMID: 23342279
10. Brotz H, Sahl HG. New insights into the mechanism of action of lantibiotics–diverse biological effects by binding to the same molecular target. J Antimicrob Chemother. 2000; 46: 1–6.
11. Cleveland J, Montville TJ, Nes IF, Chikindas ML. Bacteriocins: safe, natural antimicrobials for food preservation. Int J Food Microbiol. 2001; 71: 1–20. PMID: 11764886
12. http://www.fda.gov/ucm/groups/fdagovpublic/2fdago/foodsgen/documents/document/ucm266587.pdf5
13. Mungrue IN, Pagnon J, Kohannim O, Gargalovic PS, Lusis AJ. CHAC1/MGC4504 is a novel proapoptotic component of the unfolded protein response, downstream of the ATF4-ATF3-CHOP cascade. J Immunol. 2009; 182: 466–476. PMID: 19109178
14. Moll GN, Clark J, Chan WC, Bycroft BW, Roberts GC, Konings WN, et al. Role of transmembrane pH gradient and membrane binding in nisin pore formation. J Bacteriol. 1997; 179: 135–140. PMID: 8981990
15. Wiedemann I, Breukink E, van Kraaij C, Kuipers OP, Bierbaum G, de Kruijff B, et al. Specific binding of nisin to the peptidoglycan precursor lipid II combines pore formation and inhibition of cell wall biosynthesis for potent antibiotic activity. J Biol Chem. 2001; 276: 1772–1779. PMID: 11038353
16. Giffard CJ, Ladha S, Mackie AR, Clark DC, Sanders D. Interaction of nisin with planar lipid bilayers monitored by fluorescence recovery after photobleaching. J Membr Biol. 1996; 151: 293–300. PMID: 8661516
17. Eckert RL. Structure, function, and differentiation of the keratinocyte. Physiol Rev. 1989; 69: 1316–1346. PMID: 2678169
18. Gasparoni A, Fonzi L, Schneider GB, Wertz PW, Johnson GK, Squier CA. Comparison of differentiation markers between normal and two squamous cell carcinoma cell lines in culture. Arch Oral Biol. 2004; 49: 653–664. PMID: 15196983
19. Ponec M, Havekes L, Kempenaar J, Lavrijsen S, Vermeer BJ. Defective low-density lipoprotein metabolism in cultured, normal, transformed, and malignant keratinocytes. J Invest Dermatol. 1984; 83: 436–440. PMID: 6209343
20. Ponec M, Kempenaar J, Boonstra J. Regulation of lipid synthesis in relation to keratinocyte differentiation capacity. Biochim Biophys Acta. 1987; 921: 512–521. PMID: 2444262
21. Tertoolen LG, Kempenaar J, Boonstra J, de Laat SW, Ponec M. Lateral mobility of plasma membrane lipids in normal and transformed keratinocytes. Biochem Biophys Res Commun. 1988; 152: 491–496. PMID: 2452631
22. Tripathi P, Kamarajan P, Somashekar BS, MacKinnon N, Chinnaiyan AM, Kapila YL, et al. Delineating metabolic signatures of head and neck squamous cell carcinoma: phospholipase A2, a potential therapeutic target. Int J Biochem Cell Biol. 2012; 44: 1852–1861. doi: 10.1016/j.biocel.2012.06.025 PMID: 22743333
23. Brenner JC, Graham MP, Kumar B, Saunders LM, Kupfer R, Lyons RH, et al. Genotyping of 73 UM-SCC head and neck squamous cell carcinoma cell lines. Head Neck. 2010; 32: 417–426. doi: 10.1002/hed.21198 PMID: 19760794
24. Matsumoto K, Horikoshi M, Rikimaru K, Enomoto S. A study of an in vitro model for invasion of oral squamous cell carcinoma. J Oral Pathol Med. 1989; 18: 498–501. PMID: 2607470
25. Mulders JW, Boerrigter IJ, Rollema HS, Siezen RJ, de Vos WM. Identification and characterization of the lantibiotic nisin Z, a natural nisin variant. Eur J Biochem. 1991; 201: 581–584. PMID: 1935953
26. Kantak SS, Kramer RH. E-cadherin regulates anchorage-independent growth and survival in oral squamous cell carcinoma cells. J Biol Chem. 1998; 273: 16953–16961. PMID: 9642258
27. Zhang Y, Lu H, Dazin P, Kapila Y. Squamous cell carcinoma cell aggregates escape suspension-induced, p53-mediated anoikis: fibronectin and integrin alphav mediate survival signals through focal adhesion kinase. J Biol Chem. 2004; 279: 48342–48349. PMID: 15331608
28. Kamarajan P, Alhazzazi TY, Danciu T, D'Silva N J, Verdin E, Kapila YL. Receptor-interacting protein (RIP) and Sirtuin-3 (SIRT3) are on opposite sides of anoikis and tumorigenesis. Cancer. 2012; 118: 5800–5810. doi: 10.1002/cncr.27655 PMID: 22674009

29. Kamarajan P, Shin JM, Qian X, Matte B, Zhu JY, Kapila YL. ADAM17-mediated CD44 cleavage promotes orasphere formation or stemness and tumorigenesis in HNSCC. Cancer Med. 2013; 2: 793–802. doi: 10.1002/cam4.147 PMID: 24403253

30. Ribble D, Goldstein NB, Norris DA, Shellman YG. A simple technique for quantifying apoptosis in 96-well plates. BMC Biotechnol. 2005; 5: 12. PMID: 15885144

31. Ponce ML. Tube formation: an in vitro matrigel angiogenesis assay. Methods Mol Biol. 2009; 467: 183–188. doi: 10.1007/978-1-59745-241-0_10 PMID: 19301671

32. Alhazzazi TY, Kamarajan P, Joo N, Huang JY, Verdin E, D'Silva NJ, et al. Sirtuin-3 (SIRT3), a novel potential therapeutic target for oral cancer. Cancer. 2011; 117: 1670–1678. doi: 10.1002/cncr.25676 PMID: 21472714

33. (https://ncifrederick.cancer.gov/Lasp/Acuc/Frederick/Media/Documents/ACUC42.pdf).

34. Frazer AC, Sharrant M., and Hickman J. R.. 1962. The biological effects of food additives. I. Nisin. J Sci Food Agric, 13:32–42.

35. Delves-Broughton J. 2005. Nisin as a food preservative. Food Australia-.

36. (http://www.handary.com/download/product/WhiteNisinAMSDS100152.pdf).

37. Cornut G, Fortin C, Soulieres D. Antineoplastic properties of bacteriocins: revisiting potential active agents. Am J Clin Oncol. 2008; 31: 399–404. doi: 10.1097/COC.0b013e31815e456d PMID: 18846002

38. Lagos R, Tello M, Mercado G, Garcia V, Monasterio O. Antibacterial and antitumorigenic properties of microcin E492, a pore-forming bacteriocin. Curr Pharm Biotechnol. 2009; 10: 74–85. PMID: 19149591

39. Saito H, Watanabe T. Effect of a bacteriocin produced by Mycobacterium smegmatis on growth of cultured tumor and normal cells. Cancer Res. 1979; 39: 5114–5117. PMID: 498138

40. Yates KR, Welsh J, Udegbunam NO, Greenman J, Maraveyas A, Madden LA. Duramycin exhibits antiproliferative properties and induces apoptosis in tumour cells. Blood Coagul Fibrinolysis. 2012; 23: 396–401. doi: 10.1097/MBC.0b013e3283538875 PMID: 22543977

41. Shaikh F, Abhinand P, Ragunath P. Identification & Characterization of lactobacillus salavarius bacteriocins and its relevance in cancer therapeutics. Bioinformation. 2012; 8: 589–594. doi: 10.6026/97320630008589 PMID: 22829737

42. Kaur G, Singh TP, Malik RK. Antibacterial efficacy of Nisin, Pediocin 34 and Enterocin FH99 against Listeria monocytogenes and cross resistance of its bacteriocin resistant variants to common food preservatives. Braz J Microbiol. 2013; 44: 63–71. doi: 10.1590/S1517-83822013005000025 PMID: 24159285

43. You T, Hu W, Ge X, Shen J, Qin X. Application of a novel inhibitor of human CD59 for the enhancement of complement-dependent cytolysis on cancer cells. Cell Mol Immunol. 2011; 8: 157–163. doi: 10.1038/cmi.2010.35 PMID: 21258360

44. Hu W, Ge X, You T, Xu T, Zhang J, Wu G, et al. Human CD59 inhibitor sensitizes rituximab-resistant lymphoma cells to complement-mediated cytolysis. Cancer Res. 2011; 71: 2298–2307. doi: 10.1158/0008-5472.CAN-10-3016 PMID: 21252115

45. Smith A, Blois J, Yuan H, Aikawa E, Ellson C, Figueiredo JL, et al. The antiproliferative cytostatic effects of a self-activating viridin prodrug. Mol Cancer Ther. 2009; 8: 1666–1675. doi: 10.1158/1535-7163.MCT-08-1012 PMID: 19509266

46. Tsugu H, Onishi H, Fukushima T, Lee S. Anti-tumor activity of de novo designed small globular protein (SGP) in vivo. Anticancer Res. 2006; 26: 4043–4046. PMID: 17195455

47. Ihle NT, Williams R, Chow S, Chew W, Berggren MI, Paine-Murrieta G, et al. Molecular pharmacology and antitumor activity of PX-866, a novel inhibitor of phosphoinositide-3-kinase signaling. Mol Cancer Ther. 2004; 3: 763–772. PMID: 15252137

48. Cao LT, Wu JQ, Xie F, Hu SH, Mo Y. Efficacy of nisin in treatment of clinical mastitis in lactating dairy cows. J Dairy Sci. 2007; 90: 3980–3985. PMID: 17639009

49. Wu J, Hu S, Cao L. Therapeutic effect of nisin Z on subclinical mastitis in lactating cows. Antimicrob Agents Chemother. 2007; 51: 3131–3135. PMID: 17606675

50. Fernandez L, Delgado S, Herrero H, Maldonado A, Rodriguez JM. The bacteriocin nisin, an effective agent for the treatment of staphylococcal mastitis during lactation. J Hum Lact. 2008; 24: 311–316. doi: 10.1177/0890334408317435 PMID: 18689718

51. Bao JJ, Le XF, Wang RY, Yuan J, Wang L, Atkinson EN, et al. Reexpression of the tumor suppressor gene ARHI induces apoptosis in ovarian and breast cancer cells through a caspase-independent calpain-dependent pathway. Cancer Res. 2002; 62: 7264–7272. PMID: 12499268

52. Cui Q, Yu JH, Wu JN, Tashiro S, Onodera S, Minami M, et al. P53-mediated cell cycle arrest and apoptosis through a caspase-3- independent, but caspase-9-dependent pathway in oridonin-treated MCF-7 human breast cancer cells. Acta Pharmacol Sin. 2007; 28: 1057–1066. PMID: 17588343

53. Bai X, Kinney WH, Su WL, Bai A, Ovrutsky AR, Honda JR, et al. Caspase-3-independent apoptotic pathways contribute to interleukin-32 gamma-mediated control of Mycobacterium tuberculosis infection in THP-1 cells. Bmc Microbiology. 2015; 15.

54. Yoo JO, Lim YC, Kim YM, Ha KS. Transglutaminase 2 promotes both caspase-dependent and caspase-independent apoptotic cell death via the calpain/Bax protein signaling pathway. J Biol Chem. 2012; 287: 14377–14388. doi: 10.1074/jbc.M111.326074 PMID: 22418443

55. Kang H, Han BS, Kim SJ, Oh YJ. Mechanisms to prevent caspase activation in rotenone-induced dopaminergic neurodegeneration: role of ATP depletion and procaspase-9 degradation. Apoptosis. 2012.

56. Selimovic D, Ahmad M, El-Khattouti A, Hannig M, Haikel Y, Hassan M. Apoptosis-related protein-2 triggers melanoma cell death by a mechanism including both endoplasmic reticulum stress and mitochondrial dysregulation. Carcinogenesis. 2011; 32: 1268–1278. doi: 10.1093/carcin/bgr112 PMID: 21693538

57. Karmakar S, Choudhury SR, Banik NL, Ray SK. Activation of Multiple Molecular Mechanisms for Increasing Apoptosis in Human Glioblastoma T98G Xenograft. J Cancer Sci Ther. 2010; 2: 107–113. PMID: 21666767

58. Diwakarla S, Mercer LD, Kardashsyan L, Chu PW, Shin YS, Lau CL, et al. GABAergic striatal neurons exhibit caspase-independent, mitochondrially mediated programmed cell death. J Neurochem. 2009; 109 Suppl 1: 198–206. doi: 10.1111/j.1471-4159.2009.05937.x PMID: 19393028

59. Zhang Y, Bhavnani BR. Glutamate-induced apoptosis in neuronal cells is mediated via caspase-dependent and independent mechanisms involving calpain and caspase-3 proteases as well as apoptosis inducing factor (AIF) and this process is inhibited by equine estrogens. BMC Neurosci. 2006; 7: 49. PMID: 16776830

60. Pozo-Guisado E, Merino JM, Mulero-Navarro S, Lorenzo-Benayas MJ, Centeno F, Alvarez-Barrientos A, et al. Resveratrol-induced apoptosis in MCF-7 human breast cancer cells involves a caspase-independent mechanism with downregulation of Bcl-2 and NF-kappaB. Int J Cancer. 2005; 115: 74–84. PMID: 15688415

61. Lu JR, Lu WW, Lai JZ, Tsai FL, Wu SH, Lin CW, et al. Calcium flux and calpain-mediated activation of the apoptosis-inducing factor contribute to enterovirus 71-induced apoptosis. J Gen Virol. 2013; 94: 1477–1485. doi: 10.1099/vir.0.047753-0 PMID: 23515028

62. Delavallee L, Cabon L, Galan-Malo P, Lorenzo HK, Susin SA. AIF-mediated caspase-independent necroptosis: a new chance for targeted therapeutics. IUBMB Life. 2011; 63: 221–232. doi: 10.1002/iub.432 PMID: 21438113

63. Artus C, Maquarre E, Moubarak RS, Delettre C, Jasmin C, Susin SA, et al. CD44 ligation induces caspase-independent cell death via a novel calpain/AIF pathway in human erythroleukemia cells. Oncogene. 2006; 25: 5741–5751. PMID: 16636662

64. Jeong JC, Shin WY, Kim TH, Kwon CH, Kim JH, Kim YK, et al. Silibinin induces apoptosis via calpain-dependent AIF nuclear translocation in U87MG human glioma cell death. J Exp Clin Cancer Res. 2011; 30: 44. doi: 10.1186/1756-9966-30-44 PMID: 21501525