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AMANDA ALMEIDA LEITE

CEFALINA REDUZ CÉLULAS TRONCO TUMORAIS DO CARCINOMA ESPINOCELULAR ORAL E SENSIBILIZA AS CÉLULAS TUMORAIS À CISPLATINA

CEPHAELINE DISRUPTS ORAL SQUAMOUS CELL CARCINOMA CANCER STEM CELLS AND SENSITIZES TUMOR CELLS TO CISPLATIN

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Tese apresentada à Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas como parte dos requisitos exigidos para a obtenção do título de Doutora em Estomatopatologia, na Área de Patologia.

Thesis presented to the Piracicaba Dental School of the University of Campinas in partial fulfillment of the requirements for the degree of Doctor in Oral Medicine and Oral Pathology, in Pathology area.

Orientador: Prof. Dr. Pablo Agustin Vargas

Coorientador: Prof. Dr. Luiz Paulo Kowalski

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RESUMO

O carcinoma espinocelular (CEC) oral é uma neoplasia maligna bem reconhecida, responsável por mais de 90% das malignidades orais. Representa um dos sextos tipos de câncer mais comuns em todo o mundo e ainda é responsável por altas taxas de morbidade e mortalidade em pacientes com câncer de cabeça e pescoço. Nas últimas décadas, com o uso de terapias multimodais, houve avanços cruciais no manejo terapêutico do CEC oral. A cirurgia tem sido classicamente utilizada como modalidade de tratamento primário ou em combinação com a radiação e/ou quimioterapia, em diversos contextos clínicos. No entanto, apesar dos esforços, um desafio significativo para os pacientes com CEC oral continua sendo o desenvolvimento de resistência tumoral durante o tratamento, e isso é fundamental para determinar sua sobrevida. Estudos anteriores mostraram que a presença de células-tronco tumorais (CTTs) está relacionada à recorrência do tumor e é responsável por baixas taxas de sobrevida global em pacientes com CEC oral. Nesse contexto, novas estratégias terapêuticas têm surgido com base no melhor entendimento das características moleculares da doença, como a inibição da via de sinalização do NFκB e dos mecanismos envolvidos na compactação da cromatina e na acetilação das histonas. Neste sentido, a Cefalina, um medicamento derivado da *Cephaelis ipecacuanha*, mostrou interferência em vias de proliferação e migração tumoral em carcinomas mucoepidermoides, através da acetilação de histonas e regulação de CTTs. Portanto, no presente estudo, foram avaliadas as propriedades anticancerígenas da cefalina em linhagens celulares de CEC oral, inclusive por meio da regulação de CTTs. As linhagens SCC4, SCC9 e SCC25 foram utilizadas como modelos de CEC in vitro. A viabilidade celular foi determinada pelo ensaio de MTT, e o conteúdo de CTTs foi avaliado pela formação do ensaio de esferas tumorais. A cisplatina (CDDP) e a cefalina foram administradas separadamente nos valores de IC_{50} correspondentes no primeiro dia de cultura e a formação das esferas foi observada diariamente durante cinco dias. O estado de acetilação de histonas (H3K9ac) e a sinalização de NFκB (p65) foram determinados por coloração de imunofluorescência. Todas as análises estatísticas e gráficos foram realizados utilizando o GraphPad Prism 8.0 (GraphPad Software). Nós descobrimos que a administração de uma única dose de cefalina interrompeu a formação de esferas tumorais, enquanto o número de esferas não foi afetado pelo tratamento único com CDDP. Além disso, a cefalina foi capaz de inibir a via NFκB e causar aumento da acetilação de histona H3 em comparação com os grupos controle, sensibilizando as células de CEC oral à CDDP. Um MTT combinado (cefalina + CDDP) mostrou que a cefalina foi eficiente em reduzir

a quantidade de CDDP suficiente para atingir o IC_{50} para as linhagens SCC4 e SCC9. Esses achados sugerem que a cefalina é um potencial agente terapêutico para o CEC oral, interrompendo a capacidade das células cancerígenas de gerarem esferas tumorais. Portanto, a combinação de cefalina e CDDP pode ser um tratamento promissor para o câncer oral. Estudos in vivo são necessários para validar esses achados.

Palavras-chave: Carcinoma espinocelular oral. Câncer oral. Quimioterapia. Célula-tronco tumoral. Resistência tumoral.

ABSTRACT

Oral squamous cell carcinoma (OSCC) is a well-recognized malignant neoplasm that is responsible for more than 90% of oral malignancies. It is one of the sixth most common cancers worldwide and is still responsible for high morbidity and mortality rates in head and neck cancer patients. In recent decades, there have been crucial advances in the therapeutic management of OSCC, with the use of multimodality therapies. Surgery has classically been used as a primary treatment modality or in combination with radiation and/or chemotherapy, in several clinical settings. However, despite efforts, a significant challenge for OSCC patients remains developing tumor resistance during treatment, which is critical in determining their survival. Previous studies showed that the presence of cancer stem cells (CSCs) is related to tumor recurrence and is responsible for poor overall survival rates in patients with OSCC. In this setting, new therapeutic strategies have emerged based on a better understanding of the molecular features, such as the inhibition of the NFκB signaling pathway and mechanisms involved in chromatin compaction and histone acetylation. Recently, Cephaeline, a drug derived from *Cephaelis ipecacuanha*, showed interference in tumor proliferation and migration pathways in mucoepidermoid carcinomas, through histone acetylation and regulation of CSCs. Therefore, in the present study, we evaluated the anticancer properties of cephaeline in OSCC cell lines, including through CSC regulation. SCC4, SCC9, and SCC25 were used as in vitro OSCC models. Cell viability was determined by MTT assay, and CSC content was evaluated by tumorsphere formation. Cisplatin (CDDP) and cephaeline were administered separately in the IC_{50} correspondent values on the first day of culture and the sphere formation was observed daily for five days. The histone acetylation (H3K9ac) status and NFκB signaling were determined by immunofluorescence staining. All statistical analyses and graphics were performed using GraphPad Prism 8.0 (GraphPad Software). We found that a single dose administration of cephaeline disrupted tumorspheres formation, while the number of tumorspheres was not affected by CDDP treatment. Moreover, cephaeline was able to inhibit the NFκB pathway and caused increased acetylation of histone H3 compared with the control groups, sensitizing OSCC cells to CDDP. A combined MTT (cephaeline + CDDP) showed that cephaeline was efficient in reducing the amount of cisplatin enough to reach the IC_{50} for SCC4 and SCC9. These findings suggest that cephaeline is a potential therapeutic agent for OSCC by disrupting the ability of cancer cells to generate tumorspheres. Therefore, the combination of

cephaeline and CDDP might be a promising treatment for oral cancer. In vivo studies are needed to validate these findings.

Keywords: Oral squamous cell carcinoma. Oral cancer. Chemotherapy. Cancer stem cell. Tumor resistance.

SUMÁRIO

1 INTRODUÇÃO

O câncer é um dos principais problemas de saúde pública no mundo. Segundo estimativas da Organização Mundial da Saúde (OMS), em 2019, o câncer foi a primeira ou segunda causa de morte, antes dos 70 anos, na maioria dos países. No ano de 2020, estima-se que houve 19,3 milhões de novos casos de câncer e quase 10 milhões de mortes por câncer em todo o mundo (Sung et al., 2021). A incidência e a mortalidade por câncer vêm crescendo, em parte pelo crescimento e envelhecimento da população, como também pela mudança na distribuição e na prevalência dos fatores de risco, especialmente aqueles associados ao desenvolvimento socioeconômico, devido a incorporação de hábitos e atitudes relacionados à urbanização (sedentarismo, alimentação inadequada, dentre outros) (Bray et al., 2018).

O carcinoma espinocelular de cabeça e pescoço (CECP) está entre as neoplasias malignas mais comuns em todo o mundo, afetando mais de 800.000 pessoas por ano, e causando mais de 400.000 mortes (Johnson et al., 2020; Metzger et al., 2021). No Brasil, é estimado para o triênio 2023-2025, o número de 15.100 novos casos de câncer de cavidade oral por ano; estima-se que para esse triênio, o carcinoma espinocelular (CEC) continue sendo o quinto mais incidente em homens, com 10.900 novos casos/ano apenas em boca. Para as mulheres, as taxas são menores, porém igualmente preocupantes, mostrando 4.200 novos casos/ano (INCA, 2023). Além das mortes diretamente causadas por CECP, sobreviventes deste câncer têm a segunda maior taxa de suicídio (63,4 casos por 100.000 indivíduos) em comparação com sobreviventes de outros tipos de câncer (23,6 casos por 100.000 indivíduos). Sofrimento psicológico e qualidade de vida comprometida pós tratamento são provavelmente os principais fatores envolvidos (Johnson et al., 2020).

A carcinogênese oral é um processo de várias etapas de dano genético acumulado que leva à desregulação celular com interrupção nas sinalizações celulares, reparo do DNA e ciclo celular, que são fundamentais para a homeostase (Bettendorf et al., 2004). Portanto, o processo cancerígeno requer que vários eventos moleculares aconteçam para a transformação de uma célula normal em uma célula cancerosa (Simple et al., 2015). Neste processo, ocorre o acúmulo de mutações genéticas e epigenéticas que levam a superexpressão de oncogenes e/ou o silenciamento de genes supressores tumorais, desenvolvendo células com crescimento e proliferação autônomos, capazes de escapar da morte celular programada (Scully and Bagan, 2009; Simple et al., 2015).

Tratamentos empregados no manejo do CECP

As principais modalidades de terapia curativa para CECP são ressecção cirúrgica, radioterapia e terapia sistêmica (Hartner, 2018) e são baseadas em um contexto individual de cada paciente, levando em consideração localização anatômica, estágio da doença e o contexto funcional (Scully and Bagan, 2009). A cirurgia ou radioterapia pode ser utilizada como modalidade única; no entanto, o tratamento com a modalidade combinada pode oferecer maiores chances de cura (Johnson et al., 2020; Montero and Patel, 2015). Características patológicas indicativas de risco aumentado de recorrência incluem extensão extranodal, margens cirúrgicas exíguas ou comprometidas, ou invasão perineural; quando estas estão presentes, a administração de altas doses de quimioterapia com cisplatina concomitantemente com a radiação, aumenta a sobrevida livre de doença dos grupos de maior risco (Johnson et al., 2020).

No entanto, altas doses de cisplatina, o principal regime quimioterápico utilizado, estão associadas a um risco significativo no desenvolvimento de toxicidades agudas e tardias, como insuficiência renal, perda auditiva, zumbido, náuseas, vômitos e neuropatia (Hartner, 2018). Além disso, o uso da terapia combinada (isto é, a associação de radioterapia e quimioterapia) é conhecida por aumentar as toxicidades tardias da radiação, incluindo disfagia crônica e aspiração, e pode aumentar o risco de mortalidade não relacionada ao câncer em sobreviventes (Johnson et al., 2020).

Associado a isso, é interessante observar que apesar dos esforços conjuntos para a melhoria dos tratamentos atuais, o prognóstico dos CECs avançados continua desfavorável (Bertrand et al., 2014). A taxa de sobrevida global é em torno de 50% e tem se mantido relativamente inalterada nas últimas décadas (Bertrand et al., 2014; Gharat et al., 2016; Scully and Bagan, 2009). Além disso, os tratamentos agressivos continuam reproduzindo altas taxas de recorrência, bem como incapacidades graves para sobreviventes (Facompre et al., 2012). Esse conjunto de fatores ocorre principalmente devido a atrasos durante o processo diagnóstico que impactam o início do tratamento, e ao desenvolvimento de resistência tumoral ao longo da terapia (Metzger et al., 2021).

O conceito de células-tronco tumorais (CTT) e as vias de sinalização associadas a resistência tumoral

Conceitualmente semelhantes às células-tronco normais, as CTTs foram originalmente concebidas como um subconjunto minoritário de células malignas com capacidade de

autorrenovação ilimitada e diferenciação hierárquica (Facompre et al., 2012). O modelo hierárquico postula que a propagação contínua do câncer requer um subconjunto de células tumorais que se dividam assimetricamente, sendo responsáveis por sustentar a maior parte de um tumor, que é composto também por células de proliferação rápida e diferenciação terminal. Por conta disso, atribui-se as CTTs a capacidade de repopulação tumoral após a terapia (Gupta et al., 2011; Simple et al., 2015).

Dessa forma, estudos recentes relacionam a recorrência tumoral e a metástase linfonodal à sobrevivência de CTTs, que se apresentam mais resistentes à radiação e tratamentos quimioterápicos do que a maior parte das células tumorais (Bertrand et al., 2014; Gupta et al., 2011; Lim et al., 2011). Numerosos fatores foram propostos para explicar a resistência das CTTs as terapias, como propensão a quiescência, resistência a apoptose, reparo aprimorado do DNA, regulação positiva de mecanismos de controle do ciclo celular e eliminação de radicais livres (Facompre et al., 2012). Além disso, várias vias moleculares oncogênicas podem ser especificamente aumentadas em CTTs (Simple et al., 2015). Portanto, as tentativas de modular farmacologicamente esse grupo específico representam estratégias terapêuticas promissoras no tratamento do câncer (Facompre et al., 2012; Lim et al., 2011).

Para ilustrar o conceito de resistência tumoral associada a quimioterapia pela presença das CTTs, Guimarães et al. (2016) mostraram, em tumores de glândula salivar, que as modificações nas histonas desempenham um papel importante. De fato, sabe-se que as CTTs exibem cromatina compactada (hipoacetilada), o que não permite a transcrição gênica e o acesso ao DNA, tornando-as menos sensíveis as terapias convencionais (Bertrand et al., 2014; Vivian Petersen Wagner et al., 2018). Dessa forma, eles mostraram que a associação de SAHA, um inibidor de histona desacetilase (iHDAC), a cisplatina, foi capaz de sensibilizar as células tumorais a cisplatina e auxiliar na disrrupção das CTTs (Guimarães et al., 2016).

Para encontrar tratamentos novos e mais eficazes, é de suma importância identificar as assinaturas moleculares e vias de sinalização associadas à resistência tumoral (Wagner et al., 2018). Neste sentido, nosso grupo vem estudando o papel da via do fator de transcrição nuclear kappa B (NFkB) em carcinomas mucoepidermoides (CME) e CECP. A ativação da via do NFkB resulta na translocação deste fator para o núcleo da célula, o que faz com que ele interaja com regiões promotoras de diversos genes e module a transcrição (Almeida et al., 2014; Wagner et al., 2016). Evidências mostram que o NFkB participa dos processos de angiogênese, invasão e metástase (Dai et al., 2009), bem como na resistência ao tratamento radioterápico (Wagner et al., 2016) e quimioterápico (Almeida et al., 2014) em tumores sólidos de cabeça e pescoço.

Dessa forma, novas descobertas farmacológicas que interajam com as CTTs através da acetilação de histonas e da inibição seletiva da via no NFkB, podem representar terapias adjuvantes importantes, sensibilizando o tumor as outras terapias chave (Almeida et al., 2014; Guimarães et al., 2016).

O uso de novas drogas no manejo das CTTs em tumores de cabeça e pescoço

Entre as drogas recentemente testadas em tumores malignos de glândula salivar, está a Emetina. A Emetina é um alcaloide natural derivado da *Psychotria ipecacuanha*, e foi largamente utilizada para o tratamento de amebíase e para induzir o vômito (Lambert, 1918; Sun et al., 2015). Após comprovação de que a Emetina exercia ação apoptótica em outros tumores, como câncer de ovário (Sun et al., 2015) e de pâncreas (Han et al., 2014), nosso grupo testou sua aplicação em CMEs. Foi visto que através da inibição seletiva da fosforilação do IkBα, uma proteína membro de uma família de proteínas celulares que inibem o fator de transcrição NFκB (Jacobs and Harrison, 1998; Verma et al., 1995), houve uma abrupta redução do número de células tumorais viáveis em combinação com a radiação, assim como a redução de CTTs (Wagner et al., 2016).

Recentemente, foi visto que a Cefalina, um análogo desmetilado da Emetina, possuía mecanismo de ação semelhante, e seu uso poderia ser mais bem tolerado pelos pacientes (Yang et al., 2018). A Cefalina se origina a partir da *Cephaelis ipecacuanha,* e é muito próxima da Emetina, diferenciando-se pela presença de um grupo hidroxila. Diversos autores reportaram seu potencial uso para o tratamento de vários tipos de câncer, como carcinoma renal de células claras, câncer colorretal e carcinoma papilar de tireoide (Gulfidan et al., 2022; Mastrogamvraki and Zaravinos, 2020; Xing et al., 2020). Dessa forma Silva et al. (2021) testaram a administração da Cefalina em CMEs. Resultados promissores mostraram interferência em vias de proliferação e migração tumoral, através da acetilação de histonas e regulação de CTTs.

Dessa forma, o objetivo deste trabalho foi avaliar, pela primeira vez, as propriedades anticancerígenas da Cefalina em linhagens celulares de carcinoma espinocelular oral, inclusive por meio da regulação de células-tronco tumorais.

2 ARTIGO

Cephaeline disrupts oral squamous cell carcinoma cancer stem cells and sensitizes tumor cells to cisplatin

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Abstract

Background: Cancer stem cells (CSCs) are commonly related to tumor recurrence being responsible for poor overall survival rates in oral squamous cell carcinoma (OSCC). In the present study, we evaluated the anticancer properties of cephaeline in OSCC cell lines, including OSCC-CSC regulation.

Material and methods: SCC4, SCC9, and SCC25 were used as in vitro OSCC models to assess the cephaeline effects. Cell viability was determined by MTT assay, and CSCs content was evaluated by tumorsphere functional assay. The histone acetylation (H3K9ac) status and NFκB signaling were determined by immunofluorescence staining.

Results: A single dose administration of cephaeline disrupted tumorspheres formation, while the number of tumorspheres was not affected by cisplatin treatment. Also, we found that cephaeline inhibited the NFκB pathway and increased acetylation levels of H3 histone. Finally, cephaeline was able to sensitize OSCC cells to cisplatin.

Conclusion: These findings suggest that cephaeline is a potential therapeutic agent for OSCC by disrupting tumorspheres. In addition, the combination of cephaeline and cisplatin might be a promising treatment for oral cancer. In vivo studies are needed to validate these findings.

Keywords: Oral squamous cell carcinoma. Oral cancer. Chemotherapy. Cancer stem cell. Tumor resistance.

1 Introduction

OSCC is a well-recognized malignant neoplasm that is responsible for more than 90% of oral malignancies[1]. It is one of the sixth most common cancers worldwide and is still responsible for high morbidity and mortality rates in patients with head and neck cancer[2,3]. OSCC treatment is usually complex and involves, in most cases, multimodality strategies[4,5]. Chemotherapy is often reserved for recurrent and metastatic tumors and cisplatin, as a single-agent or in combination therapy, is the standard treatment of choice for advanced disease[5,6]. However, despite efforts, a significant challenge for OSCC patients remains developing tumor resistance, which is critical in determining their survival[6,7].

Tumor resistance to conventional therapies is associated with the presence of cancer stem cells (CSCs)[6,8]. They constitute a subset of malignant cells responsible for the heterogeneity shown by tumors[9]. Therefore, CSCs show additional hallmarks of normal stem cells including resistance to DNA damage and apoptosis[10,11]. Consequently, they are less susceptible to conventional therapies (radiotherapy and chemotherapy), showing different radio and chemosensitivity patterns, and remain viable after treatment, playing a crucial role in tumor recurrence and metastasis[12].

New therapeutic strategies have emerged based on a better understanding of the molecular features of neoplastic cells and their biological behavior. Almeida et al.[6] showed that the activation of the NFκB signaling pathway plays an important role in tumor resistance through mechanisms involved in chromatin compaction and histone deacetylation. Subsequently, other studies demonstrated that the use of Emetine, an NFκB inhibitor, showed promising results to treat salivary gland mucoepidermoid carcinomas (MEC) by CSC regulation[13].

Cephaeline, an alkaloid from *Cephaelis ipecacuanha*, is very close to Emetine, differing by the presence of a hydroxyl group. They regulate the G-quadruplex-mediated alternative splicing, which may affect cellular functions and could represent a possible target cancer therapy[14,15]. Similar to emetine, recently, cephaeline has been presented as a repurposed drug for treating several cancer types such as kidney renal clear cell carcinoma[16], colorectal cancer[17], and papillary thyroid cancer[18]. In addition, Silva et al.[14] proved that the administration of cephaeline in MEC was able to induce histone H3 acetylation and disrupted MEC CSCs.

Unfortunately, only half of the patients with advanced OSCC achieve cures following conventional therapies because of the development of tumor resistance[19]. Therefore, a better

understanding of molecular targets to overcome post-treatment tumor recurrence has been studied for the development of more effective therapeutic strategies. In the present study, we explored for the first time the response of OSCC cells to the administration of cephaeline. We showed the potential role of cephaeline in disrupting OSCC CSCs by the inhibition of the NFκB signaling pathway along with the increase in histone H3 acetylation levels. Also, we demonstrated that cephaeline it's able to sensitize OSCC adherent cells to cisplatin.

2 Material and Methods

2.1 Cell culture

SCC9 and SCC25 cell lines were purchased from American Type Culture Collection (Shanghai, China). The SCC4 cell line was purchased from Bioresource Collection and Research Center (BCRC; Hsinchu, Taiwan, ROC). The SCC4, SCC9, and SCC25 cells were derived from the tongue of male patients aged 55, 25, and 70 years old, respectively. Cell lines were cultured in DMEM/F12 (Hyclone Laboratories Inc.), supplemented with 10% fetal bovine serum (FBS, Thermo Scientific), 1% antibiotics (Invitrogen), and 400-ng/ml hydrocortisone (Sigma-Aldrich), at 37°C in a humidified atmosphere with 5% CO2. When cells reached 70% confluence, they were removed with 0.05% trypsin–EDTA (Invitrogen) and were replated in DMEM/F12 medium to avoid cellular stress and activation of cellular differentiation.

2.2 Cell viability (IC_{50} determination)

Cell viability was determined using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Thermo Scientific). The cells were first treated with cephaeline (Cayman Chemical), and afterward by a combination of cephaeline and cisplatin. Initially, the cells (5×10^4 per well) were seeded in 96-well plates with 500µl of DMEM-F12 medium containing 10% fetal bovine serum per well in a concentration ranging from 1 to 250 nanomolar, for 48h, to identify the optimal concentration of cephaeline capable of inhibiting 50% of cellular proliferation (IC_{50}). Similarly, for combined MTT, the cells were first treated with cephaeline at a concentration ranging from 1 to 250 nanomolar, for 24h, and subsequently with cisplatin for 48h, to identify the impact of cephaeline administration in the IC_{50} cisplatin, which was previously established by our group. In brief, 5 × 104 cells were plated for each group (cephaeline, and cephaeline + cisplatin) and MTT assays were performed at 37°C for 4h.

Formazan precipitated was diluted in ethanol and assessed by absorbance (iMarkTM Microplate Absorbance Reader, BioRad) at 595 nm.

2.3 Tumorspheres formation

We analyzed the role of cisplatin and cephaeline in the maintenance of CSC through the Tumorspheres assay. OSCC cell lines were seeded on ultra-low attachment 6 well plates and cultivated for 5 days in triplicates (D5) (n=3). Cisplatin and cephaeline were administered separately in the IC_{50} correspondent values on the first day (D0) of culture and the tumorsphere formation was observed daily. Images were obtained using a Nikon Eclipse Ti-S microscope. Tumorspheres were counted using Image J software (National Institute of Health).

2.4 Clonogenic Assay

5 x 102 cells/ml were seeded in 6 well plates and cultivated for 7 days (n=3). IC_{50} cephaeline was administered simultaneously with cell seeding and colony formation was observed daily. The cells were stained with 0.1% crystal violet on the seventh day. Colonies that presented >50 cells were counted as surviving colonies using Image J software (National Institute of Health, Bethesda, Maryland, USA). Images were obtained using an Uvitec transilluminator (UVITEC Cambridge).

2.5 Immunofluorescence

OSCC cells were seeded in 6-well plates (5×10^4 cells) with DMEM/F12 supplemented as previously described and treated with Cephaeline (IC_{50}) for 48 h (n=3). Afterward, cells were fixed with formaldehyde 4% for 15 minutes at room temperature. Blockage and cellular permeabilization were performed with 3% (w/v) bovine serum albumin (BSA) and 0.5% (v/v) Triton X-100 in PBS 1X for 1 h. The anti-H3K9ac and anti-p65/NFkB antibodies (Cell Signaling Technology) were diluted in (0.5% [v/v] Triton X-100 in PBS 1X and 1% [w/v] BSA) and incubated overnight. Subsequently, cells were washed and incubated with Alexa 488 and 555 secondary antibodies (Cell Signaling Technology), respectively, and followed by DNA staining using Hoechst 33342 (Cell Signaling Technology). Five fields of each slide were photographed and quantified. Images were taken using a Nikon Eclipse Ti-S microscope and evaluated using Image J software (National Institute of Health).

2.6 Statistical analyses

All statistical analyses and graphics were performed using GraphPad Prism 8.0 (GraphPad Software). One-way analysis of variance (ANOVA) followed by multiple

comparison tests and Student's test. All samples were normalized to 100% following nonlinear regression to fit the data to the nM and μM (inhibitor) vs. response (variable slope) curve. Asterisks denote statistical significance (*p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001; ns: p > 0.05; #: same statistical value).

Results

CDDP fails to reduce the CSC population

Cisplatin is the first-line chemotherapeutic agent for the treatment of various solid tumors, including OSCC, and works very well for rapidly proliferating cancer cells in a bulk tumor but fails to eliminate CSCs[20]. Here, we demonstrated that cisplatin doesn't have impact on OSCC CSCs. Cisplatin IC_{50} was previously established by our group for SCC4, SCC9, and SCC25, and the values were 3.178 μM, 3.891 μM, and 3.493 μM, respectively. Our first step was to verify if the Cisplatin IC_{50} would also have an impact on the CSC population. We noted that it is not significant for any of the cell lines used (Figure 1).

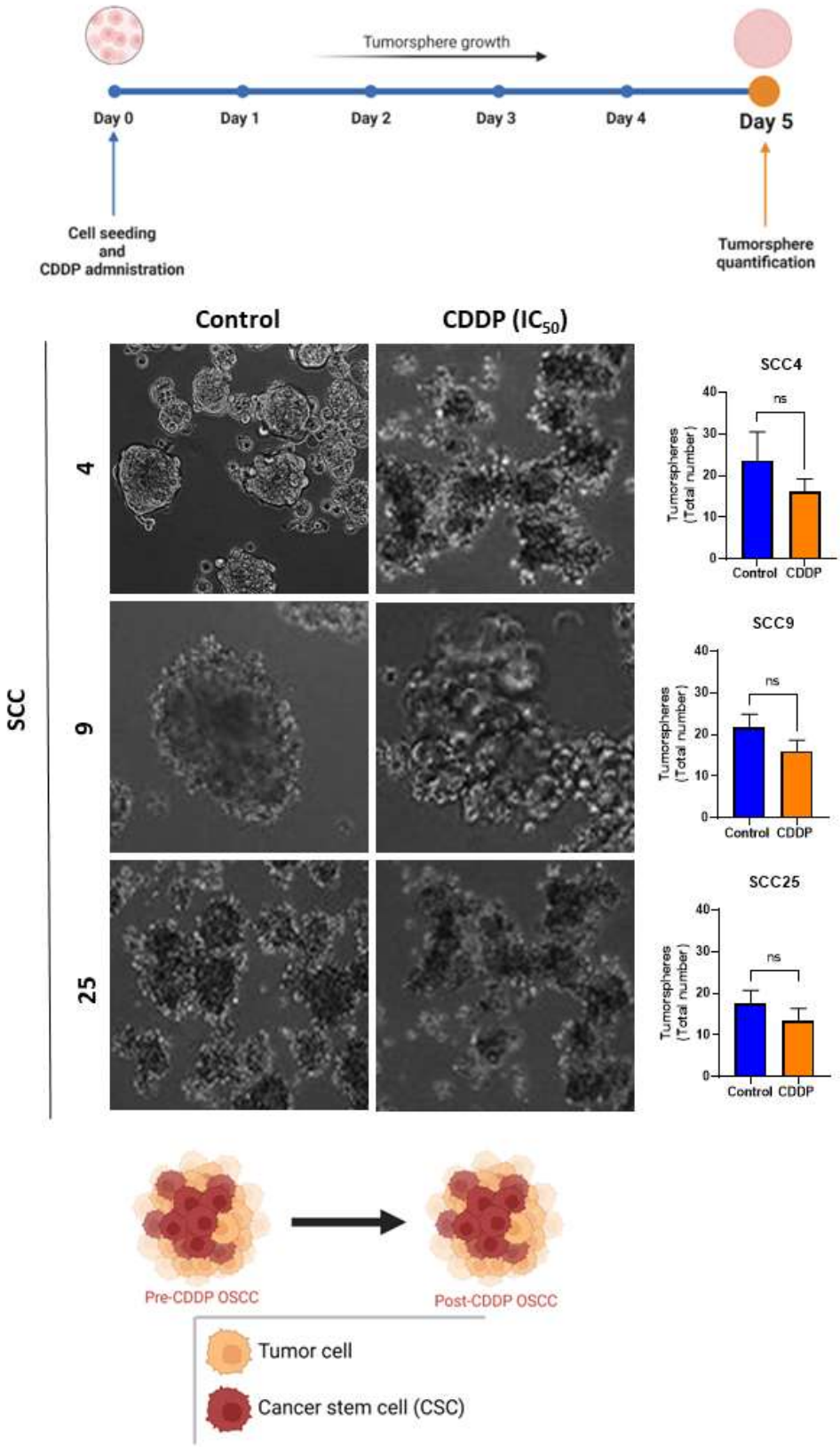


Figure 1. The treatment with CDDP fails to impact the number of tumorspheres in all OSCC cell lines. Original magnification 40X.

Cephaeline strongly reduced OSCC cell viability

Previous studies showed that cephaeline could represent a promising therapeutic strategy to manage several cancer types[14,16–18]. Additionally, it is reported that patients tolerate cephaeline better than emetine[14,21]. Here we investigated the effects of cephaeline on OSCC cell viability using the MTT assay. We discovered that the cells were so sensitive to the drug that we operated with a nanomolar scale, and it was responsible for the impact of 50% on the OSCC cell viability after 48h. The IC_{50} values for SCC4, SCC9, and SCC25 were 10.15 nM, 40 nM, and 19.25 nM, respectively (Figure 2A).

A single dose of Cephaeline efficiently inhibits tumorspheres and colony formation

Other studies have demonstrated that cells growing as a spheroid type in suspension culture conditions exhibits stem cell properties in many normal and malignant tissues[19,22]. Therefore, we used the tumorspheres assay to determine if the cephaeline treatment (IC_{50}) can affect the population of CSC in OSCC cell lines. Interestingly, in SCC4 and SCC25 cell lines, the administrated concentration of cephaeline completely inhibited the formation of tumorspheres (SCC4 ****p<0,0001; SCC25 **p<0,0023). For the SCC9, cephaeline showed a significant reduction in tumorspheres. (SCC9 *p<0,0192) (Figure 2B).

We also investigated cephaeline's ability to prevent the establishment of colonies by the clonogenic assay. A single dose of cephaeline (IC_{50}) was capable of totally inhibiting colonies formation in SCC9 (**p<0,0033). SCC4 and SCC25 showed significantly lower formation of the colonies (SCC4 **p<0,0014; SCC25 ***p<0,0005) (Figure 2C).

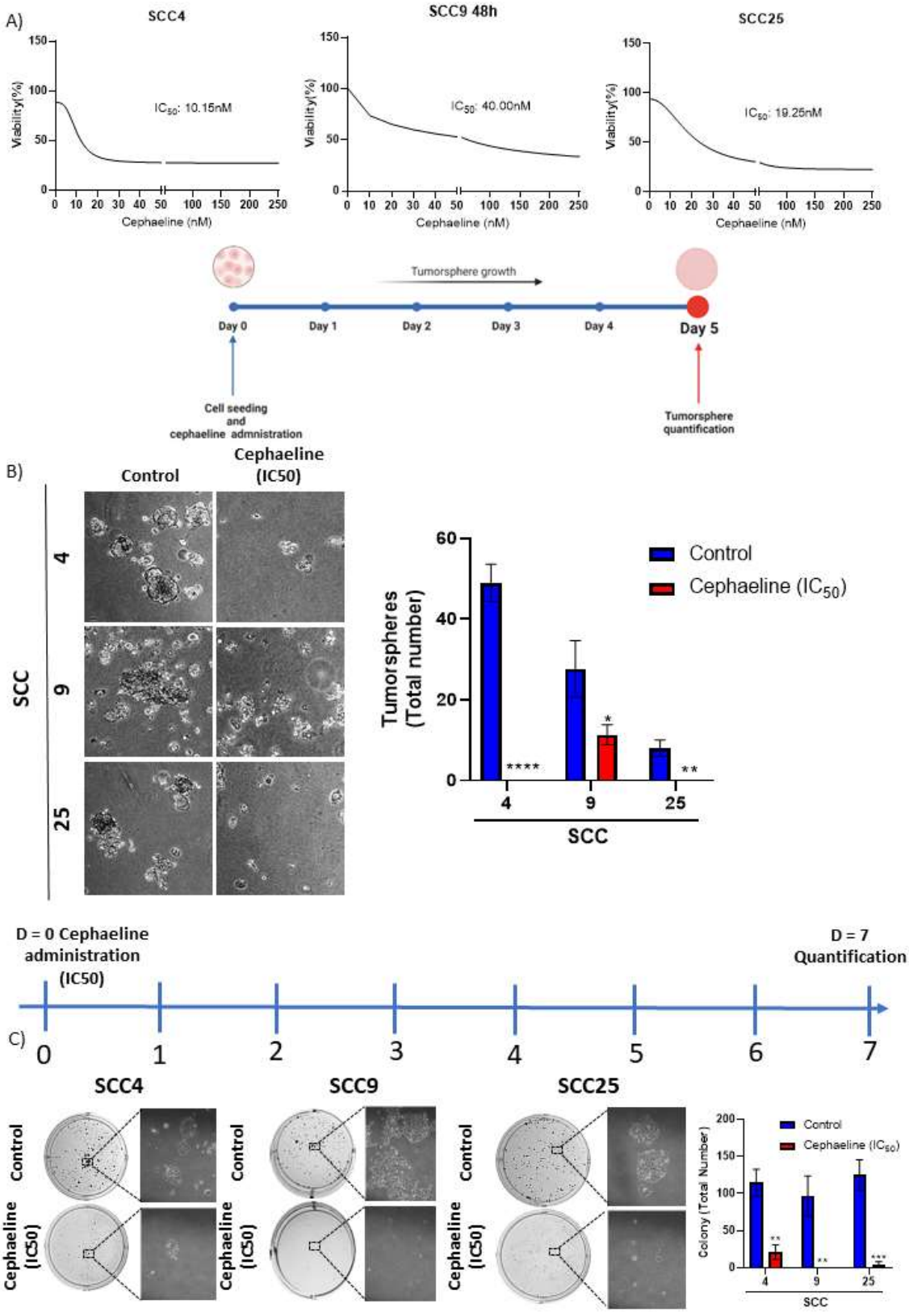


Figure 2. Cephaeline inhibits OSCC cell viability and disrupts the ability of cancer cells to generate colonies and tumorspheres. A) Determination of the IC_{50} of cephaeline in OSCC cells (SCC4, SCC9, and SCC25). B) A single dose of cephaeline completely inhibited the

formation spheres in SCC4 and SCC25 (SCC4 ****p<0,0001; SCC25 **p<0,0023). In SCC9, was observed a significant reduction. (SCC9 *p<0,0192). C) A single dose of cephaeline was capable of totally inhibiting de colonies formation in SCC9 (**p<0,0033). SCC4 and SCC25 showed significantly lower formation of the colonies (SCC4 **p<0,0014; SCC25 ***p<0,0005)

Cephaeline increased histone acetylation levels and reduced NFkB signaling

Normally, cancer cells have strong nuclear NFκB activity, which increases cell survival by inhibiting apoptotic pathways[23]. In addition, was proved that cephaeline causes high histone acetylation in MEC and could represent a promising therapeutic approach for salivary gland tumors[14]. Because of this, we performed an immunofluorescence assay against H3ak9 and p65 (NFκB effector) and tested them in OSCC cell lines. We observed a significant increase in histone acetylation of tumor cells upon cephaeline administration (IC_{50}) for SSC4, SCC9, and SCC25 (***p<0,0002; **p<0,0034; ****p<0,0001, respectively) (Figure 3A).

Following, we assessed cephaeline's ability to inhibit the nuclear NFκB pathway in OSCC tumor cells. As expected, all cell lines showed a transition of p65 expression in nuclear to a cytoplasmic pattern, revealing a lower significant nuclear expression of NFκB in cephaeline-treated groups. (SCC4 *p<0,0199; SCC9 ****p<0,0001; SCC25 ***p<0,0002) (Figure 3B).

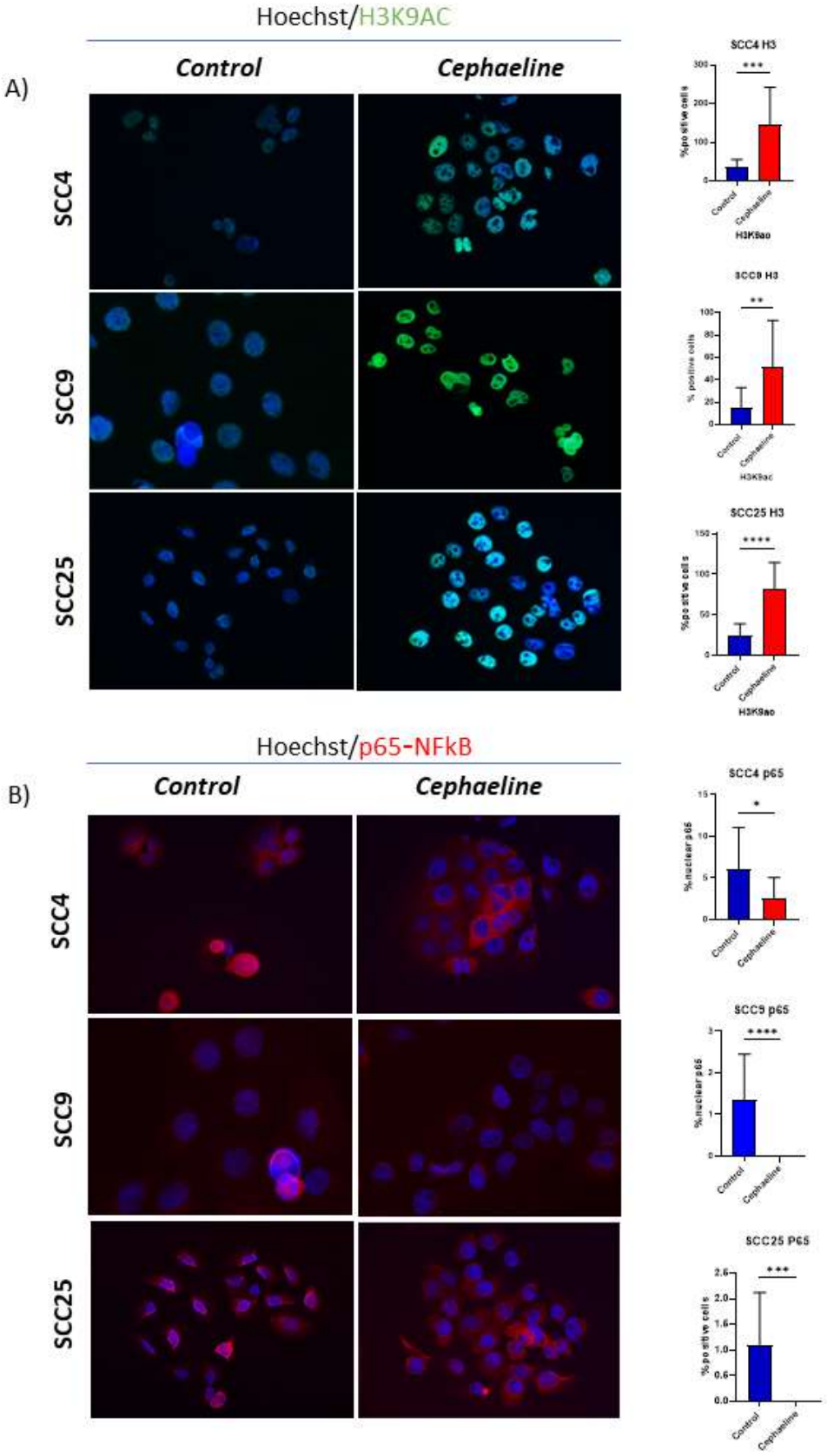
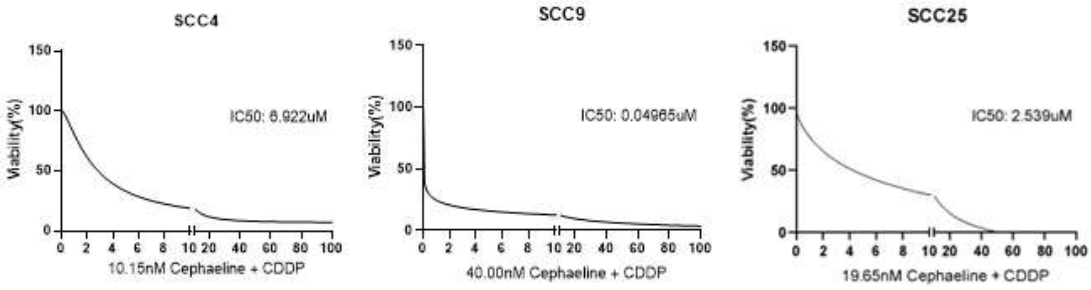


Figure 3. Cephaeline increases H3 levels in OSCC cell lines and reduced nuclear NFkB signaling. A) Immunofluorescence staining of H3K9ac showing increased histone acetylation

in SCC4, SCC9, and SCC25 upon administration of cephaeline for 24h. B) Immunofluorescence staining anti-p65/NFkB antibody showing a transition of the NFκB nuclear expression in control groups to cytoplasmic expression upon administration of cephaeline. Original magnification 40X.

Cephaeline makes OSCC cells more sensitive to Cisplatin

Previous studies showed that tumoral resistance to adjuvant therapies (chemotherapy and ionizing radiation) was linked with interference in NFkB signaling[6,23]. Given the promising results with immunofluorescence against p65/NFκB, we performed a combined MTT with cisplatin and cephaeline. The cells were first treated with cephaeline, for 24 h, and finally, with cisplatin for 48 h. We observed that cephaeline was efficient in reducing the amount of cisplatin enough to reach the IC_{50} for SCC4 and SCC9. However, inexplicably, SCC25 showed an increase in IC_{50} values of required cisplatin (Figure 4).



	CDDP	CDPP + CEPHAELINE
SCC4	3.178uM	2.539uM (⇩ 639uM)
SCC9	3.891uM	0.04965uM (⇩ 3.890,9)
SCC25	3.493uM	6.922uM (⇧ 3.429uM)

Figure 4. Combined MTT with Cephaeline (24h) and CDDP (48h). Cephaeline was efficient in reducing the amount of CDDP enough to reach the IC_{50} for SCC4 and SCC9. SCC25 showed an increase in IC_{50} values of the required CDDP.

Discussion

The current understanding of cancer biology suggests that tumor recurrence is primarily attributed to the resistance of cancer cells to chemo-radiotherapy[23]. Resistance to CDDP remains a challenge in the chemotherapy of several cancers including OSCC[20,24]. In this setting, the CSCs play a critical role in intrinsic therapy resistance, with quiescence, evasion of apoptosis, self-renewal, and tumor-initiating capabilities[11,19]. In the current study, we analyzed the role of CDDP in the maintenance of tumor stem cells through the formation of tumorspheres. Our results corroborate these findings, showing that CDDP fails to eliminate the CSC population in OSCC, which represents a possible scenario of tumor resistance and could be a targeted therapy. (Outras populações elulares?)

Cephaeline is a compound used as an emetic agent and induces vomiting by acting on the stomach lining, therefore, it is mostly recommended as an emergency treatment for accidental poisoning[18]. However, in previous studies, cephaeline was proposed as a candidate drug for the treatment of several cancer types, including MECs[14]. In their study, Silva et al.[14] observed that cephaeline completely inhibited the formation of tumorspheres for two MEC cell lines. Interestingly, we also demonstrated the total inhibition of tumorspheres formation by cephaeline in SCC4 and SCC25, and showed a significant reduction in spheres formation in SCC9.

Indeed, SCC9 is the least sensitive cell line to cephaeline, requiring four times more concentration to reach IC_{50}. Although all cell lines share a common anatomical site and histological features of the primary tumor, interestingly SCC9 is derived from a 25-year-old patient. One of the hypotheses is that OSCC in young patients may represent a more aggressive biological behavior[25]. However, the clinical stage and the mode of invasion seem the most significant prognostic factors[26]. Questions remain; however, this highlights the need for precision medicine and individualized therapies for each patient.

Based on a better understanding of molecular pathways that are involved in the resistant profile, we tested the ability of cephaeline to modulate the chromatin status. We know that histone deacetylation induces chromatin condensation and silencing of tumor suppressor genes which is often observed in quiescent stem cells. Therefore, hypoacetylation likely plays a fundamental role in tumor resistance to chemotherapy[27] because is also associated with poor DNA accessibility to drugs[13]. Additionally, increased chromatin condensation was associated with poor prognosis in cases of OSCC by Webber et al.[28], suggesting that H3K9ac might be

considered a prognostic marker in OSCC. We showed that cephaeline administration increased histones acetylation in all OSCC cell lines. We believe that this mechanism was activated by cephaeline and could be responsible for the better OSCC response to CDDP.

There are several reports describing that NFκB contributes to chemotherapy resistance in malignant salivary gland tumors and OSCC by inhibiting chemotherapy-induced apoptosis[6,13,23]. Therefore, we speculate that inhibition of the NFκB pathway is also responsible for sensitizing OSCC cells to chemotherapy. Thus, we checked the ability of cephaeline to inhibit the NFκB signaling pathway in OSCC. We proved that cephaeline was able to reduce the nuclear expression of NFκB in all OSCC cell lines for the first time. Similar results were shown by Wagner et al.[13] using Emetine associated with Vorinostat, an HDAC inhibitor, in MECs[13,23]. Here, cephaeline was used as a single agent.

The ultimate goal of oncology research is the development of anticancer therapeutic regimes with little toxicity to normal cells[29,30]. In our study, we demonstrated that the OSCC cells are very sensitive to cephaeline, with IC_{50} values on a nanomolar scale. Additionally, we performed a combined MTT with CDDP and cephaeline and we found that cephaeline was also efficient in sensitizing OSCC cells to CDDP, reducing the amount of CDDP enough to reach the IC_{50} in SCC4 and SCC9. This result is especially important for patients that fail their initial chemotherapy cycles due to high toxicity[8]. However, unexpectedly, we observed an increase in the IC_{50} values of the required CDDP in SCC25. This can be explained by the fact that each tumor/patient is unique and presents different features in molecular and physiologic parameters. We also worked with ultra-low doses and used a single administration of the drug. Furthermore, we used only “in vitro” assays to demonstrate the anticancer properties of cephaeline, which represents a limitation. In vivo studies are needed to validate these findings.

Conclusion

In conclusion, we found that cephaeline has anti-cancer properties in OSCC cell lines by disrupting the ability of cancer cells to generate colonies and tumorspheres. Furthermore, we demonstrated for the first time in OSCC that the inhibition of NFκB by cephaeline, in association with increased histone acetylation, impacts the sensibility of the OSCC cells to cisplatin. Cephaeline should be tested “in vivo” as a possible treatment for oral cancer. Furthermore, the use of cephaeline in combination with other chemotherapeutic agents for other cancer cells remains an interesting topic for further studies.

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3 CONCLUSÃO

Concluímos que a cefalina apresenta propriedades anticancerígenas em linhagens de células de CEC oral, interrompendo a capacidade das células cancerígenas de gerarem esferas tumorais. Além disso, demonstramos pela primeira vez em CEC oral, que a inibição da via de NFκB pela cefalina em associação com o aumento da acetilação de histonas, pode interferir na sensibilidade das células de CEC à cisplatina. A cefalina deve ser testada em estudos “in vivo” como um possível tratamento para o câncer bucal. Além disso, o uso de cefalina em combinação com outros agentes quimioterápicos e para outras células cancerígenas continua sendo um tópico interessante para estudos posteriores.

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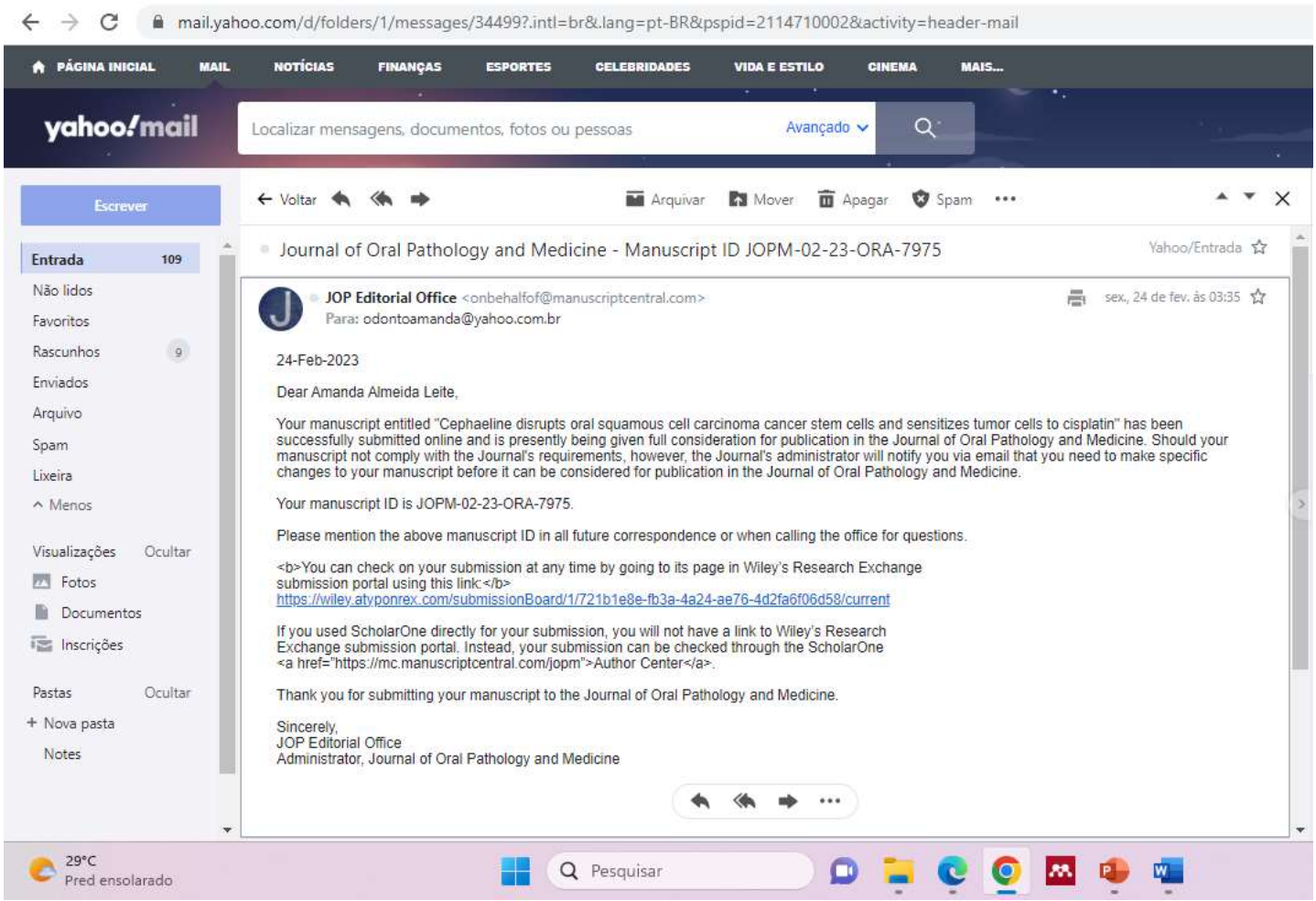
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