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Original Article

Oral squamous cell carcinoma cancer stem cells have different drug sensitive to pharmacological NFκB and histone deacetylation inhibition

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Abstract: Despite many progresses in the development of new systemic therapies for oral squamous cell carcinoma (OSCC), the five-year survival rate of OSCC is low. The traditional chemotherapies approach (cisplatin - CDDP) shows some limitations like drug toxicity, limited efficacy, and drug resistance. Promising studies suggested OSCC cancer stem cells (CSC) presented resistance to CDDP. We have previously studied many targets, and we extensively showed the efficacy of the NFκB signaling and the role of histones acetylation, on different malignant tumors, including adenoid cystic carcinoma and mucoepidermoid carcinoma, but until then the effects of the NFkB inhibitor and histone deacetylase (HDAC) inhibitor on the biology of OSCC were not evaluated. Here we assessed the pharmacological inhibitor of NFκB emetine and HDAC inhibitor SAHA on the behavior of CSC derived from OSCC. Our data suggested that CDDP administration resulted in reduced viability of bulk OSCC cells and increased CSC. A single and isolated shot of emetine and SAHA were able to disrupt CSC by inhibiting the NFκB pathway and increasing the histone acetylation levels, respectively. Further, the combined administration of emetine and SAHA presented the same CSC disruption as seen in emetine alone.

Keywords: Head and neck tumors, oral squamous cell carcinoma, chemotherapy, target therapy, cancer stem cell

Introduction

Oral squamous cell carcinoma (OSCC) is the most frequent type of oral cancer, ranked as the eighth most prevalent cancer worldwide in men [1, 2]. OSCC treatment depends on tumor stage and pathological diagnosis, but usually, it is based on multimodal therapy, involving surgery, radio, and/or chemotherapy [3, 4]. Basically, chemotherapy is administered as a single agent or combined with radiotherapy in advanced OSCC as postoperative adjuvant therapy or as a palliative option [3]. Well-established systemic therapies are recommended for advanced or disseminated OSCC, including cisplatin (CDDP), taxanes, cetuximab, pembrolizumab, and nivolumab, being CDDP the first-line drug [5-7]. However, despite many therapeutic systems available, the five-year survival rate of OSCC is approximately 50%. This could be explained by the traditional therapeutic approach shows limitations, such as high drug toxicity, limited therapeutic effect, and drug resistance [8].

Our previous results suggest that tumor drug resistance to conventional therapies could be associated with the presence of cancer stem cells (CSCs) [9-12]. CSCs are a subpopulation cellular with a high ability for multiplication, which can avoid apoptosis and offer resistance to DNA damage-induced therapies [10, 13, 14]. In addition, emergent studies showed that OSCC CSC presented resistance to CDDP [15]. Therefore, new approaches like immunotherapy and targeted therapies aimed at CSC disruption are urgently needed. Our group has extensively demonstrated several targets, including the NFκB signaling and the role of histones acetylation, as two promisor goals to treat CSCs [16-19].

The NFκB pathway is activated in CSCs from several malignant tumors, like leukemia, glioblastoma, prostate, ovary, breast, pancreatic, and colon cancer [20]. Histone acetylation mediated by pharmacological agents caused an important decrease in CSC [21]. In this study, we assessed the role of CDDP on the behavior of CSCs derived from three OSCC cell lines. Also, we are the first to evaluate the properties of emetine, a well-known emetic drug able to interfere with the NFκB signaling, and SAHA, a pharmacological agent for histone deacetylation inhibition on OSCC. Here, we present the data on the effectiveness of low doses of emetine and SAHA in managing OSCC CSCs. Moreover, we found that the administration of a low concentration of emetine is extremely effective in depleting OSCC enriched CSC population, suggesting a novel therapeutic strategy for OSCC.

Material and methods

Cell culture

SCC4, SCC9, and SCC25 cell lines (American Type Culture Collection - ATCC, Manassas, VA, USA) are derived from tongue squamous cell carcinoma. Cell lines were cultured in DMEM/F-12 (Cytiva - Hyclone Laboratories Inc., Logan, UT) supplemented with 10% of fetal bovine serum (FBS, Thermo Scientific), 400 ng/mL hydrocortisone (Sigma-Aldrich) and 1% antibiotic/antimycotic solution (Invitrogen) at 37°C in a humidified atmosphere with 5% CO_2. Tumor cells were maintained under 70% of confluency to avoid cellular stress and activation of cellular differentiation. Cisplatin (Cayman Chemical Company Ann Arbor, MI, USA), emetine (Cayman Chemical Company Ann Arbor, MI, USA), and SAHA (Cayman Chemical Company Ann Arbor, MI, USA) was administrated at appropriated concentrations, as described below.

IC_{50} determination

CDDP IC_{50} was determined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliμM bromide) assay (Thermo Scientific), in order to identify the optimal concentration capable of inhibiting 50% of cellular viability [22]. OSCC cells were cultured at a concentration ranging from one to 100 μM, for 48 h (n=8). In brief, 5×10^4 cells were seeded into 96-well plates, and after 24 h of cell adhesion, the CDDP was administrated, and then MTT assay was performed at 37°C for 4 h. Formazan precipitated was diluted in ethanol and assessed by absorbance (iMarkTM Microplate Absorbance Reader, BioRad) at 595 nm.

Cancer stem cells

CSC population was determined by tumorspheres assay. The tumorsphere assay is a strong approach to characterize CSCs by enriching them in vitro. 8×10^3 cells was seeded (n=8) on 96-well plates capable to generate a single tumorsphere per well and designed with a rounded shape and featuring low cell adhesion characteristics (Nunclon™ Sphera™ 96-Well, Nunclon Sphera-Treated, U-Shaped-Bottom Microplate), and cultured for 12, 24, and 48 hours. A single dose of CDDP (IC_{50}), emetine (ranging from 0.01 μM to 0.5 μM), and SAHA (ranging from 0.1 μM to 5 μM) were administered along with cellular seeding. Tumorspheres were observed daily. Images were obtained using a Nikon Eclipse Ti-S microscope from all tumorspheres. Tumorspheres were analyzed and quantified the total area, using Image J software (National Institute of Health).

Flow cytometry of cancer stem cells

After establishment of the emetine and SAHA concentrations able to disrupt the tumorspheres behavior, the enzymatic activity of aldehyde dehydrogenase (ALDH) activity (StemCell Technologies, Durham, NC, USA) was investigated to evaluate the CSC derived from

OSCC cells. OSCC cells received Emetine (0.5 μM) and SAHA (5 μM) (n=4), as previously established based on tumorspheres behavior. After 24 h, cells were washed, suspended, and incubated with Aldefluor kit (activated Aldefluor substrate (BODIPY amino acetate) or a negative control (dimethylamino benzaldehyde-DEAB, a specific ALDH inhibitor) for 40 min at 37°C, following the manufacturer's instructions. All samples were analyzed using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA).

Immunofluorescence

Our goal here was to investigate if a lower drug concentration established in the tumorspheres assay was capable of inhibits NFκB and increase H3 histone levels in OSCC. OSCC cells were seeded in 6-well plates (5×10^4 cells) and treated with 0.5 μM of emetine, and 5 μM of SAHA in triplicates for 24 h. After, cells were fixed with formaldehyde 4% for 15 min 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 1× for 1 h. Cells treated with emetine and SAHA receipted p65 antibody (NFκB effector) (Cell Signaling Technology) and anti-H3K9ac antibody (Cell Signaling Technology), respectively. Both antibodies were diluted in (0.5% [v/v] Triton X-100 in PBS 1× and 1% [w/v] BSA) and incubated overnight. Subsequently, cells were washed and incubated with Alexa 555 and 488 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 Nikon Eclipse Ti-S microscope and evaluated using Image J software (National Institute of Health).

Colony assay

5×10^2 cells were seeded into 6-well plate. Established concentration CDDP, emetine, and SAHA were administrated at the same time as cell seeding and allowed to grow for additional ten days in order to form colonies. The drugs were re-administrated along with the media changing, performed every 2 or 3 days. The development of colonies was observed daily. After colony formation, cells were stained with 0.1% crystal violet. Colonies that presented >50 cells were counted as surviving colonies. Images were obtained using an Uvitec transilluminator (UVITEC Cambridge). Colonies were counted using Image J software (National Institute of Health, Bethesda, Maryland, USA).

Statistical analyses

All statistical analyses were performed using GraphPad Prism 8.0 (GraphPad Software). One-way analysis of variance (ANOVA) followed by multiple comparison test and Student's t-test. Asterisks denote statistical significance (*P<0.05; **P<0.01; ***P<0.001; ****P< 0.0001; ns: P>0.05; #: same statistical value). All samples were normalized to 100% following nonlinear regression to fit the data to the μM (inhibitor) vs. response (variable slope) curve. The graphics are created using GraphPad Prism 8.0 (GraphPad Software).

Results

CDDP impacted bulk tumor cells and increase oral squamous carcinoma cancer stem cells

CDDP is the main systemic therapy used in several cancers, including OSCC [23]. Here, we demonstrated that CDDP was very efficient to reduce the viability of bulk tumors while playing an antagonistic effect in CSC. Our first step was carefully established the optimal concentration able to impact 50% of the OSCC cell viability, and as expected, the lower doses of CDDP administration resulted in an important reduction of bulk viable OSCC cells, where the IC_{50} values were 3.178 μM, 3.891 μM, and 3.493 μM for SCC4, SCC9, and SCC25, respectively (**Figure 1A**). Following, we investigated if the IC_{50} CDDP would have the same effect on CSC. Our group have been widely demonstrated the use of tumorspheres to detect CSC. This approach is well established technique to assess the enriching CSCs *in vitro*. Additionally, our group has demonstrated the value of this technique for developing therapies specifically targeting CSCs. Recently, was demonstrated that CDDP cause an increasing in CSC, and this could be explained by the high expression levels of CD44, CD133, ALDHA1, and others [24, 25]. Further, was recently demonstrated a low accumulation of DNA damage in cisplatin-resistant cell lines, suggesting the mechanism of CSCs maintenance [26]. Interestingly, we observed in the current study, different sizes of tumorspheres in all cell lines studied, caused

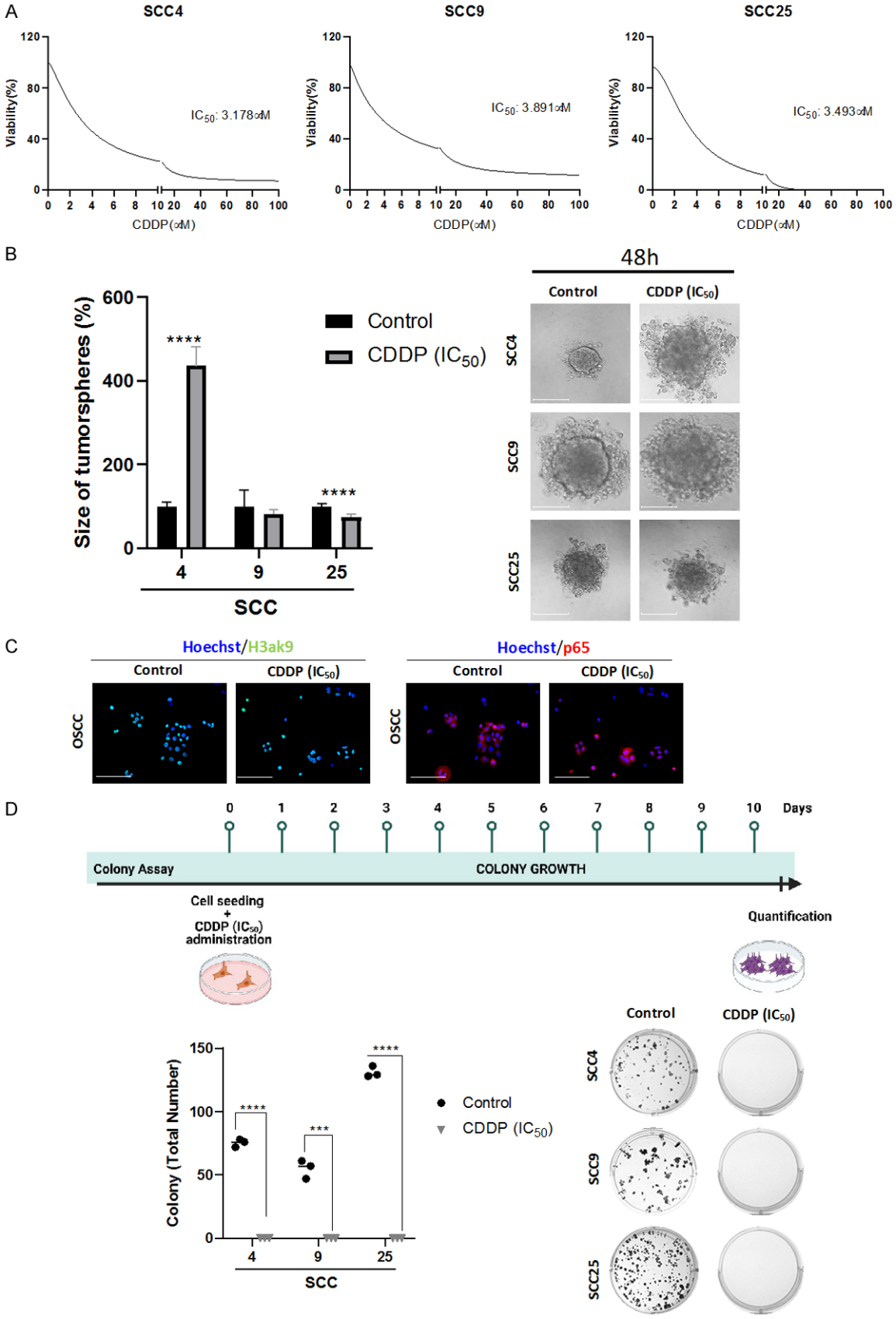

A
SCC4
SCC9
SCC25
Viability(%)
CDDP(∝M)
IC50: 3.178∝M
IC50: 3.891∝M
IC50: 3.493∝M
B
48h
Control
CDDP (IC50)
Size of tumorspheres (%)
****
SCC
SCC4
SCC9
SCC25
C
Hoechst/H3ak9
Hoechst/p65
OSCC
D
Days
Colony Assay
COLONY GROWTH
Cell seeding
+
CDDP (IC50)
administration
Quantification
Colony (Total Number)
***

Figure 1. CDDP impacted bulk tumor and increase oral squamous carcinoma cancer stem cells. A. Determination of the IC_{50} of CDDP in OSCC cells (SCC4, SCC9, and SCC25). B. The treatment with CDDP increased CSC SCC4 (****P<0.001). In SCC9 statistically significant was not found. Original magnification 40×. C. Immunofluorescence staining of p65 and H3K9ac in oral squamous cell carcinoma, illustrated with the SCC4 cell line, after the administration of CDDP (IC_{50}) for 48 h. D. Schedule of clonogenic assay. Colonies individually produced by SCC4, SCC9, and SCC25. Note the completely inhibition of colonies after the IC_{50} CDDP administration (SCC4 and SCC25 ****P<0.0001; SCC9 ***P<0.001).

by the administration of CDDP. SCC4 showed an increase in four folds (****P<0.001), while a slight decrease was seen in SCC25 (****P<0.001). SCC9 does not presented significance statistical after the treatment with CDDP (IC_{50}) (**Figure 1B**).

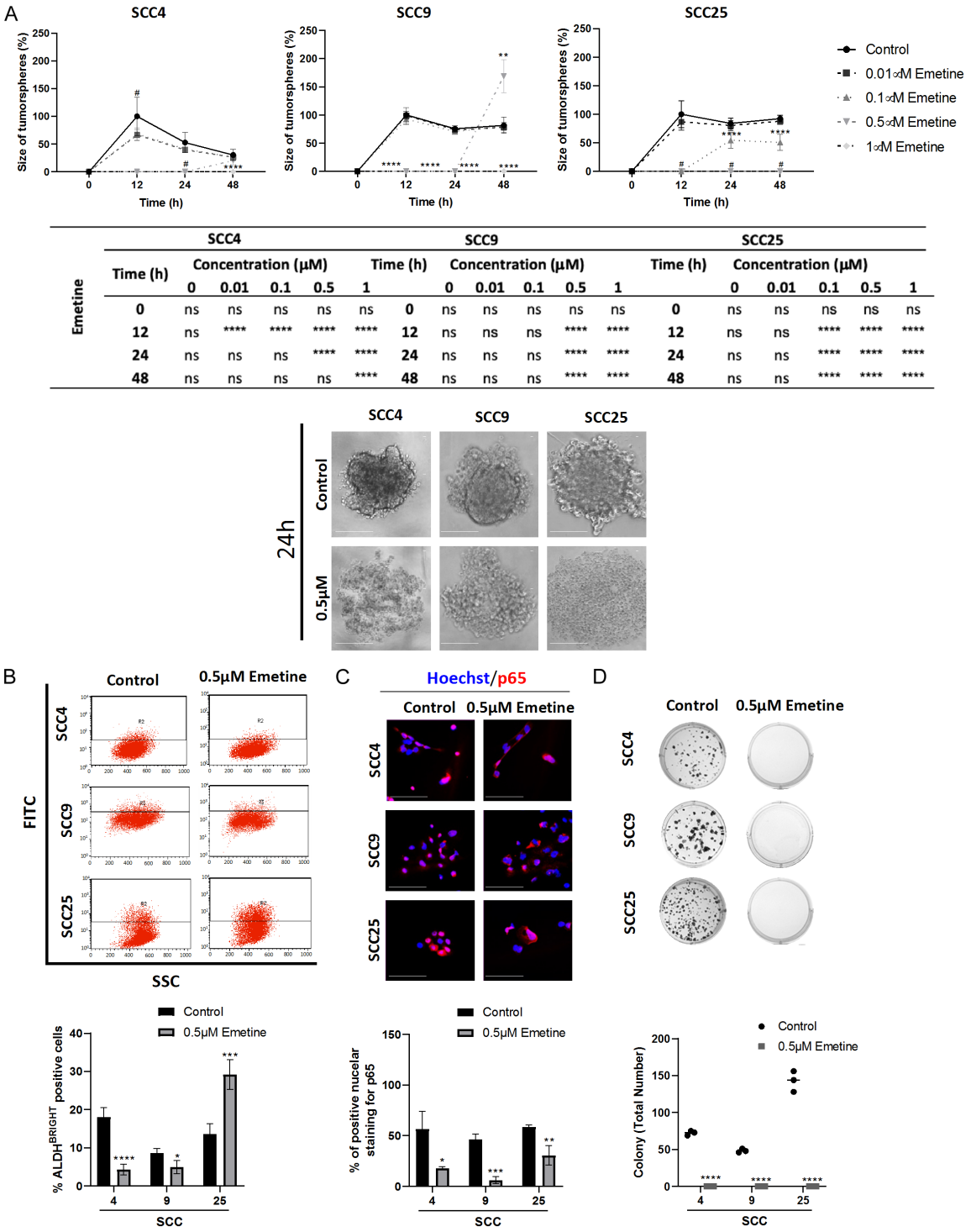
Our previous results demonstrated that the inhibition of NFκB signaling along with the pharmacological inhibition of HDAC represents a promising approach to deter CSC maintenance [17, 21]. Here, we performed an immunofluorescence assay against p65 (NFκB effector), and H3ak9 histone. Interestingly, no difference was seen in the p65 and H3ak9 expression levels after the CDDP (IC_{50}) treatment, illustrated by the SCC4 expression profile (**Figure 1C**). We also evaluated the effects of CDDP on OSCC colony formation, an assay where the cells proliferate faster and do not exhibit drug resistance profiles. Our previous results demonstrated that despite to the CDDP IC_{50} does not kill all the cells in mucoepidermoid carcinoma (MEC) cell lines, this concentration was seeming enough to completely disrupt the ability to form colonies in MEC [17]. Here, we observed that an IC_{50} dose of CDDP was effective in totally depleting colony formation in SCC4 (****P<0.001), SCC9 (***P<0.001), and SCC25 (****P<0.001) cells (**Figure 1D**).

Emetine is extremely competent in disrupting oral squamous carcinoma cancer stem cells by NFκB inactivation

Emetine is a well-known emetic medication, historically used as an antineoplastic agent [27]. Currently, its favorable effects in the *in vitro* treatment of several tumors, including salivary gland cancer, and its ability to interfere with CSC maintenance [17, 21, 28], have been encouraged your testing in other malignancies. First, we decided to explore the effect of emetine as a single agent for OSCC treatment. OSCC cell lines were treated with a range of concentrations of emetine varying from 0.01 μM to 1 μM and cultivated in conditions to form tumorspheres (**Figure 2A**). A time-course assay varying from 0 to 48 h, was performed to evaluate the effects of emetine on tumorspheres formation and resulted in growth inhibition of OSCC tumorspheres SCC4, SCC9, and SCC25. All concentrations evaluated led to tumorspheres reduction in size in comparison with the control group. 0.5 μM concentration was the best dose to always interfere in the tumorspheres architecture, being able to completely disrupt the tumorspheres architecture after 24 h.

Given the promising results, we did run our experiments using the 0.5 μM dose of emetine in 24 h. The high tumorigenic ability of CSC has been extensible assessed by several markers [29]. Our previous studies suggested that ALDH activity along with the tumorspheres assay, compromises a very promisor approach to identify CSC in several carcinomas from head and neck sites, like mucoepidermoid carcinoma, adenoid cystic carcinoma, and head and neck squamous cell carcinoma [10, 16, 30]. Here we showed that emetine administration significantly reduced the number of ALDH+ OSCC tumor cells within 24 hours of treatment in SCC4 (****P<0.001), and SCC9 (*P<0.05). Unexpectedly, we observed an increased number of ALDH+ cells in SCC25 (***P<0.001) (**Figure 2B**).

To further explore the emetine effects on OSCC cells, we assessed whether this emetine concentration could affect nuclear levels of NFκB. Our previous results demonstrated that the inhibition of NFκB signaling represents a promising approach to deter CSC maintenance [21]. Here, we performed an immunofluorescence assay against p65 (NFκB effector). All SCC cells showed a basal level of nuclear expression of NFκB (control group). Also, we observed a significant change in the expression profile of this pathway after the 0.5 μM emetine administration. A transition of p65 expression from a nuclear to a cytoplasmic pattern was seen here. All SCC cell lines showed a significant



Emetine	SCC4					SCC9						SCC25					
Time (h)	Concentration (μM)					Time (h)	Concentration (μM)					Time (h)	Concentration (μM)				
	0	0.01	0.1	0.5	1		0	0.01	0.1	0.5	1		0	0.01	0.1	0.5	1
0	ns	ns	ns	ns	ns	0	ns	ns	ns	ns	ns	0	ns	ns	ns	ns	ns
12	ns	****	****	****	****	12	ns	ns	ns	****	****	12	ns	ns	****	****	****
24	ns	ns	ns	****	****	24	ns	ns	ns	****	****	24	ns	ns	****	****	****
48	ns	ns	ns	ns	****	48	ns	ns	ns	****	****	48	ns	ns	****	****	****

Figure 2. Emetine is extremely competent in disrupting oral squamous carcinoma cancer stem cells by NFκB inactivation. A. A time-course assay varying from 0 to 48 h with the OSCC cells was treated with a range of concentrations of emetine varying from 0.01 μM to 1 μM. Tumorspheres are individually produced by SCC4, SCC9, and SCC25, after 24 hrs of 0.5 μM emetine administration. Comparison among all the treatment times and emetine concentration for SCC4, SCC9, and SCC25. ns: no statistical, t: time. B. Cells exposed to 0.5 μM of emetine for 24 h were collected and processed for ALDH activity using fluorescence-activated cell sorting (FACS) analysis. Here, we presented SSC4, SCC9, and SCC25 representative samples of ALDH+, and the percentage of these positive cells. Note that SCC4 and SCC9 OSCC cells presented a significant reduction in the number of ALDH+ cells (SCC4 ***P<0.001; SCC9 *P<0.05), while an increase in ALDH+ cells was observed in SCC25. C. Immunofluorescence staining of p65 in SCC4, SCC9, and SCC25 cells upon administration of 0.5 μM emetine for 24 h. Note that a single dose of emetine was able to reduce the NFκB levels in all cell lines studied (SCC4 *P<0.05; SCC9 ***P<0.001; SCC25 **P<0.01). D. Colonies individually produced by SCC4, SCC9, and SCC25. Note the completely inhibition of colonies after the 0.5 μM emetine administration (****P<0.0001).

decrease in the nuclear expression of NFκB after administration of emetine (SCC4 *P< 0.05, SCC9 ***P<0.001, and SCC25 **P< 0.01) (**Figure 2C**).

We also assessed the emetine on OSCC colony formation. We observed that 0.5 μM of emetine was effective in completely depleting colony formation in SCC4, SCC9, and SCC25 (****P<0.001) cells (**Figure 2D**).

SAHA is able to reduce oral squamous carcinoma cancer stem cells and increase the histone acetylation levels

SAHA is a histone deacetylase inhibitor, approved by FDA, and evaluated in some clinical trials, including a phase II trial that had demonstrated acceptable efficacy in other head and neck carcinoma (HNSCC), like adenoid cystic carcinoma [31]. We previously showed that pharmacological acetylation of histones by SAHA represents a promising therapeutic strategy to manage mucoepidermoid carcinoma and sensitize HNSCC to CDDP [16, 17, 21]. First, we explore the effect of SAHA as a single agent for OSCC treatment. OSCC cell lines were treated with a range of concentrations of SAHA varying from 0.1 μM to 10 μM and cultivated in conditions to form tumorspheres (**Figure 3A**). Like the one performed with emetine, a time-course assay varying in the same times (0, 12, 24, and 48 h) was executed, and the SAHA administration resulted in progressive growth inhibition of OSCC tumorspheres SCC4, SCC9, and SCC25. 5 μM of SAHA was the best concentration to always interfere in the tumorspheres architecture, being able to significantly reduce the size of tumorspheres in 24 h, in all OSCC cells studied.

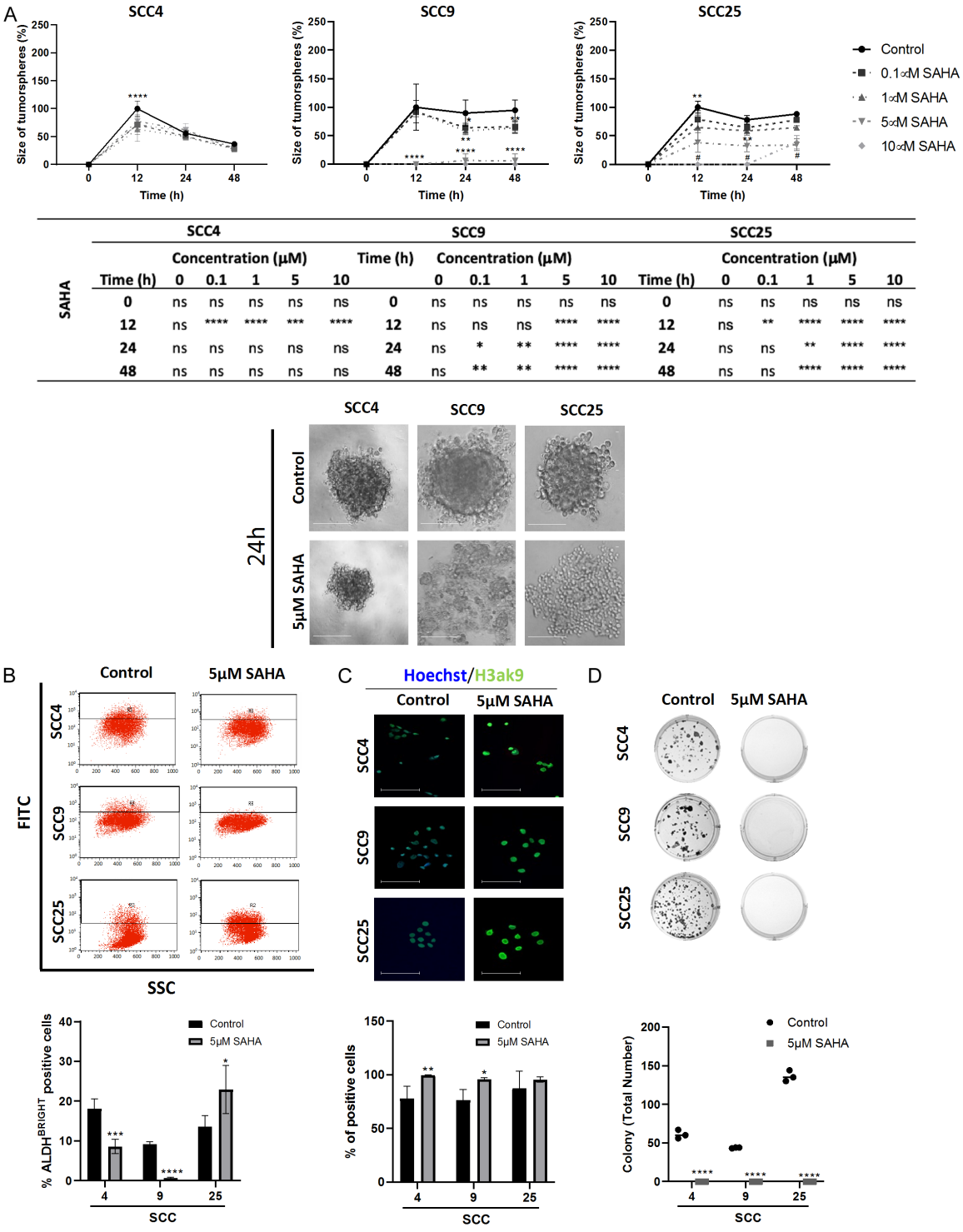
Amid the objectives of the present study, we also decided to explore the effects of CSC identified by the enzymatic activity of ALDH under the SAHA treatment at the previously concentration established. Like to be seen after the treatment with emetine, we found that SCC4 and SCC9 ALDH+ were more sensitive to emetine than SCC25. We demonstrated that SCC4 and SCC9 treated with 5 μM SAHA for 24 h presented a significant reduction in the number of ALDH+ cells (SCC4 ***P<0.001; SCC9 1 μM ****P<0.0001), while SCC25 presented a significant increase (*P<0.05) (**Figure 3B**).

In addition, our previous results showed that H3K9ac may be considered a prognostic marker for OSCC [19], and also the histone modifications could represent a promising therapeutic approach to salivary gland tumors [30]. Here, we performed an immunofluorescence assay against H3ak9, and we observed an increase in histone acetylation of tumor cells upon administration of SAHA, for SCC4 (**P<0.01) and SCC9 (*P<0.05) (**Figure 3C**). We also assessed the SAHA on OSCC colony formation. We observed that 5 μM of SAHA was effective in completely depleting colony formation in SCC4, SCC9, and SCC25 (****P<0.001) cells (**Figure 3D**).

A combination of Emetine and SAHA showed the same effect than emetine alone in disrupting oral squamous carcinoma cancer stem cells

It is fundamental to consider more than one hit to impact CSC, once they present resistance to several therapies. Here, we decided to evaluate if the combination between emetine and SAHA efficiently impact the tumorspheres and we observed that 0.5 μM of emetine associated with 5 μM of SAHA was also effective in completely depleting tumorspheres in all OSCC cells (SCC4, SCC9, and SCC25 ****P<0.001) (**Figure 4A**). Further, likely in the separated therapies, we also assessed the emetine and SAHA combined on OSCC colony formation and observed again the completely depleting colony formation in SCC4, SCC9, and SCC25 (****P<0.001) cells (**Figure 4B**).

Following, we compare the capacity of CDDP, emetine, SAHA, and the combination of emetine + SAHA on tumorspheres maintenance. We found that emetine is the best treatment to completely disrupt the tumorspheres, reducing up to 435 times the size of the SCC4 tumorspheres when compared to the increase caused by CDDP, and in 81 times for SCC9, and 74 times for SCC25. Further, SAHA is able to reduce seven, thirteen, and two times for SCC4, SCC9, and SCC25, respectively. Interestingly, the reduction observed with emetine alone was the same seen in a combination of emetine and SAHA (**Figure 5A**).



SAHA	SCC4						SCC9						SCC25					
Concentration (μM)						Time (h)						Time (h)						
Time (h)	0	0.1	1	5	10		0	0.1	1	5	10		0	0.1	1	5	10	
0	ns	ns	ns	ns	ns	0	ns	ns	ns	ns	ns	0	ns	ns	ns	ns	ns	0
12	ns	****	****	***	****	12	ns	ns	ns	****	****	12	ns	**	****	****	****	12
24	ns	ns	ns	ns	ns	24	ns	*	**	****	****	24	ns	ns	**	****	****	24
48	ns	ns	ns	ns	ns	48	ns	**	**	****	****	48	ns	ns	****	****	****	48

Figure 3. SAHA is able to reduce oral squamous carcinoma cancer stem cells and increase the histone acetylation levels. A. A time-course assay varying from 0 to 48 h with the OSCC cells was treated with a range of concentrations of SAHA varying from 0.1 μM to 10 μM. Tumorspheres are individually produced by SCC4, SCC9, and SCC25, after 24 hrs of 5 μM SAHA administration. Comparison among all the treatment times and SAHA concentration for SCC4, SCC9, and SCC25. ns: no statistical, t: time. B. Cells exposed to 5 μM of SAHA for 24 h were collected and processed for ALDH activity using fluorescence-activated cell sorting (FACS) analysis. Here, we presented SSC4, SCC9, and SCC25 representative samples of ALDH+, and the percentage of these positive cells. Note that SCC4 and SCC9 OSCC cells presented a significant reduction in the number of ALDH+ cells (SCC4 ***P<0.001; SCC9 *P<0.05), while an increase in ALDH+ cells was observed in SCC25. C. Immunofluorescence staining of H3ak9 in SCC4, SCC9, and SCC25 cells upon administration of 5 μM SAHA for 24 h. Note that a single dose of SAHA was able to increase the H3ak9 levels in SCC4 (**P<0.01) and SCC9 (*P<0.05). D. Colonies individually produced by SCC4, SCC9, and SCC25. Note the completely inhibition of colonies after the 5 μM SAHA administration (****P<0.0001).

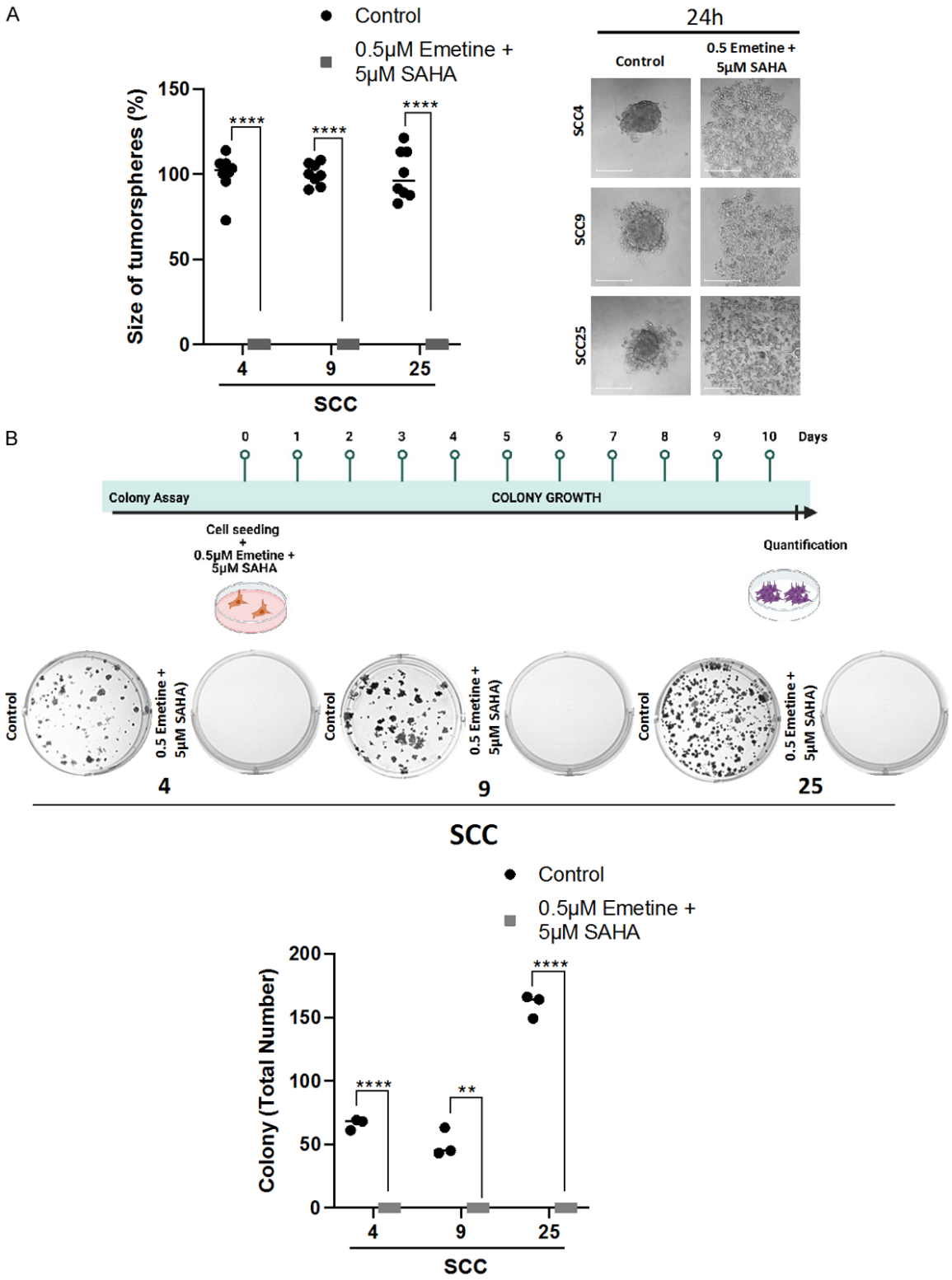
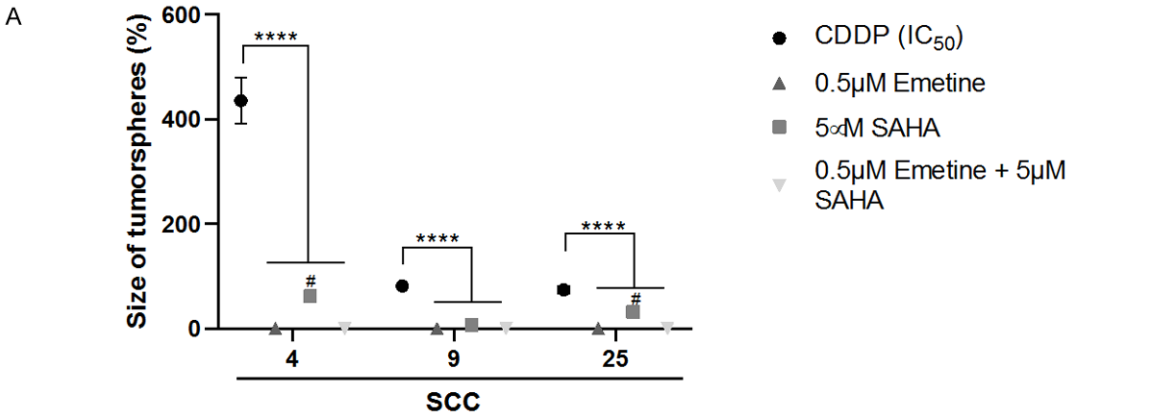


Figure 4. Combined emetine and SAHA are extremely competent in disrupting oral squamous carcinoma cancer stem cells. A. Cells exposed to 0.5 μM emetine + 5 μM SAHA for 24 h were cultivated in conditions to form tumorspheres. Note the complete tumorsphere disruption in SCC4, SCC9, and SCC25 (****P<0.001). Original magnification 40×. B. Schedule of the clonogenic assay. Colonies are individually produced by SCC4, SCC9, and SCC25. Note the completely inhibition of colonies after the emetine + SAHA administration (SCC4 and SCC25 ****P<0.0001; SCC9 **P<0.001).



Tumorspheres	CDDP (IC_{50}) (%)	0.5μM Emetine (%)	5μM SAHA (%)	0.5μM Emetine + 5μM SAHA (%)
SCC4	435.8	0 (▼435)	62 (▼7)	0 (▼435)
SCC9	81.7	0 (▼81)	6.3 (▼13)	0 (▼81)
SCC25	74.4	0 (▼74)	32.2 (▼2)	0 (▼74)

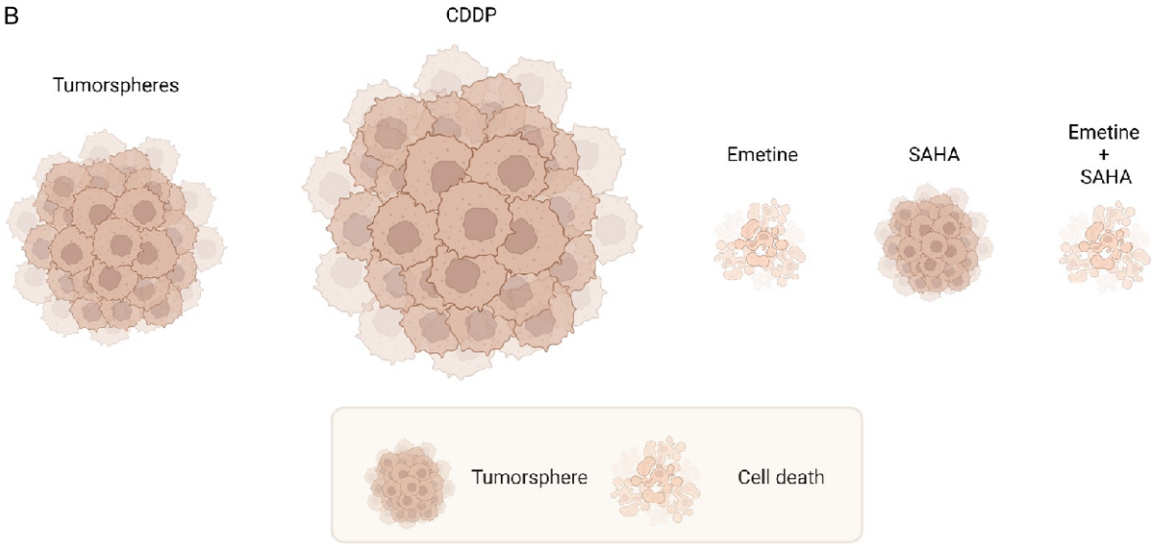


Figure 5. Combined emetine and SAHA were more efficient than CDDP. A. Quantification of tumorspheres after the administration of the different therapies. Note that the emetine + SAHA was responsible for a strong reduction in all cells cell lines when compare with the IC_{50} CDDP. B. Emetine + SAHA is extremely competent in disrupting oral squamous carcinoma cancer stem cells.

Discussion

Treatment for advanced OSCC involves multimodal modalities, however, the tumor response fails, and this is currently associated with CSCs [32, 33]. Usually, new drugs targeting cancer are recognized in adherent human tumor cell lines, where the cells do not show drug resistance profiles as seen in CSC properties [34]. One option to improve success rates in cancer drug development would be to use physiologically relevant in vitro cell models, such as the three-dimensional (3D) sphere model. Our previous studies have demonstrated that tumorspheres are a good approach to identify the stemness potential [10]. Here we visualized, quantified, and investigated the accumulation and distribution of CDDP, emetine, and SAHA in different CSC OSCC populations. Moreover, we presented the data showing that the association between emetine and SAHA can extremely interrupt CSC in OSCC, however, emetine showed superior anti-cancer properties in CSC OSCC cell lines than SAHA.

CDDP has been amply used to treat advanced HNSCC, but although it presents some effectiveness on "*in vitro*" bulk tumors, the tumor cells often could develop resistance to this agent [23]. Our results showed that CDDP can cause an acceptable effect on adherent cells while supporting the increase in CSC. The fact of CSC was associated with tumor resistance, recurrences, and metastasis is already established in the literature, and represents a real problem in terms of prognosis [26]. In this sense, it is important keeping in mind that the CDDP could act by slowing cell cycle and intrinsic resistance to treatment. Therefore, several molecular targets to escape tumor resistance have been investigated, but the works are mainly *in vitro* studies, with a few clinical studies performed.

Previously, our results demonstrated the important role of the NFκB pathway in the control of mucoepidermoid carcinoma CSCs [21, 28]. The NFkB pathway is activated by a pro-inflammatory stimulus, which triggers the activation of the IKK complex, followed by the phosphorylation of IkB-α. These events allow NFκB to translocate to the nucleus where it becomes active, acting as a transcription factor [35]. NFkB plays in the initiation and progression of many cancers, including HNSCC and OSCC [20]. In the present study, we demonstrated that low doses of emetine extremely disrupted CSC by the inhibition of NFkB. The interference of the NFκB signaling pathway results in reduced tumor cell survival and reduced tumor growth [36]. Here, we used emetine to target the NFkB pathway. Emetine is an emetic drug approved by the FDA to treat amoebiasis, which also presented antineoplastic properties by inhibition of IKK-β [37].

Nonetheless, one-hit therapies to treat advanced tumors frequently increase selective tumor cells [38]. Keeping this in mind, we also assessed the low doses of SAHA, which is also able to interfere in the OSCC CSC maintenance, but with less efficiency than emetine alone. But how the chromatin status is a very relevant feature of CSCs, we assessed the association of emetine and SAHA combined on CSCs. The combination of the interference in the NFκB pathway along with the pharmacological acetylation of histones was recently established by our group as a promisor therapy to control CSC [21]. However, here we have presented the data proving that the proposed combined therapy showed the same effect as emetine alone, by the complete breaking of tumorspheres. Moreover, this was the first time that we explored the combination of these two important goals in OSCC. Here, we have shown that any of the antineoplastic agents that we proposed, even if administered separately, have superior properties to those of CDDP for disrupting the CSCs.

Finally, some aspects need to be taken into consideration. At the beginning of the 70s-decade, Phase I and II clinical trials with emetine were performed, but due to some adverse effects, its use was discontinued. However, in the last years, various studies showed again the anticancer activity of emetine in a variety of human carcinoma cell lines [39]. Here, our results were obtained after a single administration of these therapies, and in ultra-low doses to avoid toxicity. Further, the increasing histone acetylation seems to increase the sensitivity of CSC to other chemotherapeutic agents, and the use of two or more hits therapy looks like an optimal method to overcome this issue.

Together, we conclude that emetine can have superior anti-cancer properties than SAHA, in CSC OSCC cell lines, with effective inhibition of tumorspheres formation. However, the use of SAHA could rise the sensitivity to CSC, by the changed in the histone acetylation profile. We also suggested the use of one therapy aimed at CSC along with the use of one aimed at bulk tumor, such as CDDP, to achieve effective success. Further studies are needed to elucidate the mechanism of action of combined therapy and to explore its potential efficacy as a sensitized therapy (**Figure 5B**).

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Disclosure of conflict of interest

None.

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