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Cephaeline is an inductor of histone H3 acetylation and inhibitor of mucoepidermoid carcinoma cancer stem cells

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Abstract

Aim: To evaluate the potential use of Cephaeline as a therapeutic strategy to manage mucoepidermoid carcinomas (MEC) of the salivary glands.

Material and Methods: UM-HMC-1, UM-HMC-2, and UM-HMC-3A MEC cell lines were used to establish the effects of Cephaeline over tumor viability determined by MTT assay. *In vitro* wound healing scratch assays were performed to address cellular migration while immunofluorescence staining for histone H3 lysine 9 (H3k9ac) was used to identify the acetylation status of tumor cells upon Cephaeline administration. The presence of cancer stem cells was evaluated by the identification of ALDH enzymatic activity by flow cytometry and through functional assays using in vitro tumorsphere formation.

Results: A single administration of Cephaeline resulted in reduced viability of MEC cells along with the halt on tumor growth and cellular migration potential. Administration of Cephaeline resulted in chromatin histone acetylation as judged by the increased levels of H3K9ac and disruption of tumorspheres formation. Interestingly, ALDH levels were increased in UM-HMC-1 and UM-HMC-3A cell lines, while UM-HMC-2 showed a reduced enzymatic activity.

Conclusion: Cephaeline has shown anti-cancer properties in all MEC cell lines tested by regulating tumor cells' viability, migration, proliferation, and disrupting the ability of cancer cells to generate tumorspheres.

KEYWORDS
cancer stem cells, chemotherapy, epigenetic, head and neck tumors, salivary gland cancer, target therapy

1 | INTRODUCTION

Mucoepidermoid carcinoma (MEC) is the most common salivary gland malignancy presenting low survival rates for high-grade tumors and advanced stages of the disease.[1,2] The most common treatment for MEC is surgical excision, associated or not to postoperative radiotherapy, however, there is a lack of effective therapies for tumors where surgery is not indicated or in tumor presenting distant metastasis, thereby requiring systemic therapy.[3] Radiotherapy may be recommended as an adjuvant therapy to surgery, while chemotherapy is mainly indicated as a palliative treatment as it is associated with low response rates and poor survival.[4]

Epigenetic events have been shown to play a role in MEC behavior. Deregulation of the epigenetic machinery controlling histone acetylation directly affects the initiation and progression of tumors. The acetylation of histones and consequent decondensation of the tumor chromatin is a critical mechanism that guarantees gene transcription of DNA repair genes leading to the repair of the genome.[5,6] Several studies consider histones as a critical mechanism involved in the control of cancer stem cells (CSCs). Furthermore, emerging studies suggest that tumor resistance to conventional therapies is associated with the presence of CSCs.[7,8]

CSCs constitute a subpopulation of cancer cells capable of self-renewal and presenting multipotency. Like stem cells, CSCs can evade apoptosis while presenting resistance to DNA damage-induced therapies due to the abnormal expression of proteins involved in drug transport.[9] Recently, we have shown that administration of cisplatin induces the accumulation of MEC CSCs *in vitro*,[8] and that ionizing radiation is ineffective against CSCs from a metastatic MEC cell line.[10] Furthermore, we have shown that by interfering with the NFkB signaling along with the pharmacological acetylation of histones represents a promising therapeutic strategy to manage MEC tumor growth and proliferation.[10]

In this study, we propose to assess the anti-cancer properties of Cephaeline, a desmethyl analog of Emetine. Zhang et al.[11] indicated that Cephaeline modulates quadruplex G-dependent alternative splicing, which are structures that regulate essential cellular functions, and could be a promising target therapy. We have previously explored the effects of low doses of Emetine, a well-known emetic medication that have shown promising results in the treatment of MEC and its CSCs.[10,12] Cephaeline is an alkaloid isolated from the Cephaelis ipecacuanha and closely related to Emetine, differing by the presence of a methoxy group in Emetine and a hydroxyl group in Cephaeline. Furthermore, Cephaeline is better tolerated by patients than Emetine.[13]

Here, we present the data on the effectiveness of Cephaeline in managing MEC cells viability, growth, and migration. We also present the impact of Cephaeline on the population of ALDH positive cells along its effects on chromatin compactation and in the ability of tumor cells to generate tumorspheres. Our findings support that Cephaeline interferes with the regulation of multiple oncogenic processes, including cellular viability, growth and migration, and CSC maintenance. Furthermore, our group was the first to investigate the anti-cancer properties of Cephaeline in MEC.

2 | MATERIAL AND METHODS

2.1 | Human mucoepidermoid carcinoma cells

UM-HMC-1, UM-HMC-2, and UM-HMC-3A MEC cell lines were originally established at the University of Michigan School of Dentistry and described by Warner et al.[14] UM-HMC-1 is derived from a minor salivary gland, UM-HMC-2 is derived from a parotid gland, and UM-HMC-3A is derived from a local tumor recurrence in a minor salivary gland. Cell lines were cultured in DMEM/High Glucose (Hyclone Laboratories Inc.) supplemented with 10% fetal bovine serum (FBS, Thermo Scientific), 1% antibiotics (invitrogen), 1% L-glutamine (Invitrogen), 20 ng/ml epidermal growth factor (PeproTech), 400-ng/ml hydrocortisone (Sigma-Aldrich), and 5-μg/ml insulin (Sigma-Aldrich), 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.

2.2 | Viability and cellular growth

Cell viability was determined using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Thermo Scientific). In order to identify the optimal concentration of Cephaeline (Cayman Chemical) capable of inhibiting 50% of cellular proliferation (IC_{50}), cells were cultured at a concentration ranging from 0.01 to 30 μM, for 48 h ($n = 8$). In brief, 5×10^4 cells were plated into 96-well plates and MTT assay was performed at 37°C for 4 h. Formazan precipitated was diluted in ethanol and assessed by absorbance (iMark™ Microplate Absorbance Reader, BioRad) at 595 nm. Cellular growth was assessed using trypan blue. Here, 10^5 cells were seeded in 24-well plates with DMEM/high glucose supplemented as previously described and received a single Cephaeline dose at the corresponding IC_{50} concentration. The effects of Cephaeline administration on cellular growth were evaluated at the time points 24, 48, and 72 h in triplicates.

2.3 | Scratch migration assay

Cells were seeded in high density (5×10^4 cells) in 24-well plates with DMEM/high glucose supplemented. After 24 h, cells presenting 100% confluency were scraped using a sterile 200-µl pipette tip to create cell-free (wound) areas. After three washes with PBS, cells were treated with Cephaeline at the corresponding IC_{50} and the wound area was evaluated at 0, 6, 12, 24, and 48 h time points. All scratch assays were performed in triplicate. Images were obtained using a Nikon Eclipse Ti-S microscope. Cell migration was calculated using Image J software (National Institute of Health) and expressed as the percentage of wound closure.

2.4 | Immunofluorescence

MEC cells were seeded in 6-well plates (5×10^4 cells) with DMEM/high glucose supplemented as previously described and treated with Cephaeline (IC_{50}) in triplicates for 24 and 48 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 1X for 1 h. Anti-H3K9ac antibody (Cell Signaling Technology) was 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 secondary antibody (Cell Signaling Technology) following by DNA staining using Hoechst 33342 (Cell Signaling Technology). Ten 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).

2.5 | Flow cytometry of cancer stem cells

CSCs from MEC cell lines were identified by Aldehyde dehydrogenase (ALDH) enzymatic activity by flow cytometry using Aldefluor kit (StemCell Technologies) following the manufacturer's instructions. In brief, MEC cells received Cephaeline at the corresponding IC_{50} concentration ($n = 4$). After 24 h, cells were washed, suspended, and incubated with an Aldefluor kit for 40 min at 37°C. All samples were analyzed using a FACSCalibur flow cytometer (BD Biosciences).

2.6 | Tumor sphere formation

MEC cells were seeded at a concentration of 3×10^3 cells on ultra-low attachment plates and cultured for 5 days. Cephaeline was administered along with cellular seeding. Sphere formation was observed daily. Images were obtained using a Nikon Eclipse Ti-S microscope. Tumor spheres were counted using Image J software (National Institute of Health).

2.7 | Statistical analysis

All statistical analyses were performed using GraphPad Prism 8.0 (GraphPad Software). One-way and two-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: $p > 0.05$). All samples were normalized to 100% following nonlinear regression to fit the data to the µM (inhibitor) vs. response (variable slope) curve.

3 | RESULTS

3.1 | Cephaeline inhibits cellular viability, growth, and migration in MEC cell lines

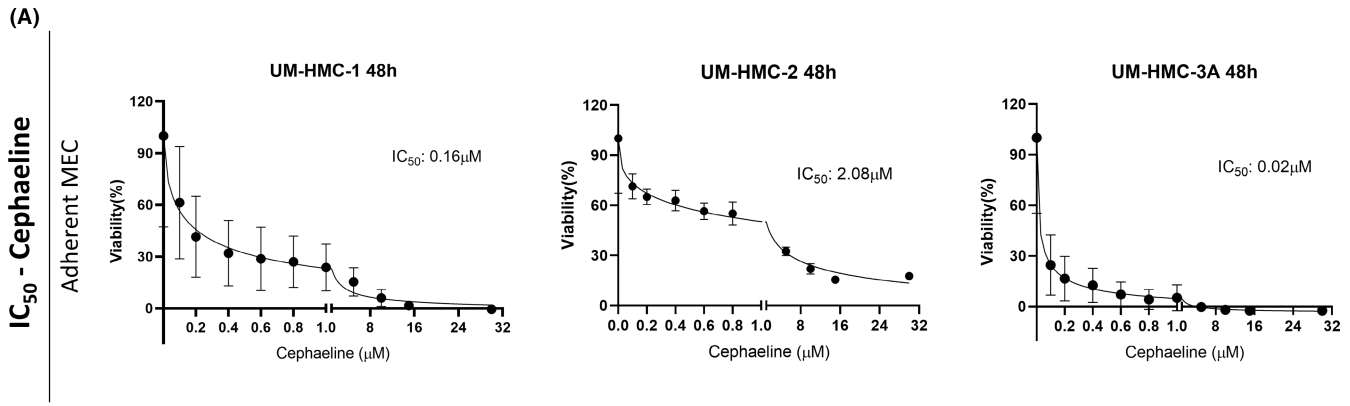
Initially, we examined the effects of Cephaeline in cellular viability, growth, and migration of MEC cell lines. First, we verified the cell viability by MTT assay and determined the inhibition concentration of 50% (IC_{50}) of the cells. The IC_{50} values of Cephaeline were 0.16, 2.08, and 0.02 µM for UM-HMC-1, UM-HMC-2, and UM-HMC-3A, respectively (Figure 1A).

Next, MEC cell lines were treated with the appropriated IC_{50} concentration of Cephaeline and assessed for cell growth (Figure 1B). A time-course assay to evaluate the effects of Cephaeline on tumor cell growth resulted in growth inhibition of MEC cell lines UM-HMC-1 ($^{***}p < 0.001$) and UM-HMC-2 ($^*p < 0.05$). Interestingly, although we observed growth inhibition of UM-HMC-3A upon Cephaeline administration, it did not achieve statistical significance. Furthermore, we observed a progressive difference in tumor growth between treated and control groups, with the largest difference observed at 72 h after a single administration of Cephaeline in UM-HMC-1 and UM-HMC-2 cell lines.

To further verify the effects of Cephaeline on the aggressive behavior of tumor cells, we explored the impact of the drug on the migration properties of MEC cells using a scratch assay. Our results showed that Cephaeline significantly reduced cell migration compared with control cells in all MEC cell lines. Cephaeline was able to significantly inhibit tumor migration at 48 and 60 h time points ($^{****}p < 0.0001$) in UM-HMC-1 (Figure 2A). In comparison, UM-HMC-2 and UM-HMC-3A cell lines presented reduced migration as early as 24 h after Cephaeline administration (UM-HMC-2 $^{****}p < 0.0001$ and UM-HMC-3A $^*p < 0.05$) (Figure 2B,C).

3.2 | Cephaeline progressively increases histone acetylation

Following this, we decided to evaluate if Cephaeline impacts histone acetylation in MEC cell lines. Previous reports have demonstrated that the acetylation of histones can induce differentiation of tumor cells and inhibit proliferation and invasion.[15] Furthermore, our group showed that histone modifications could represent a



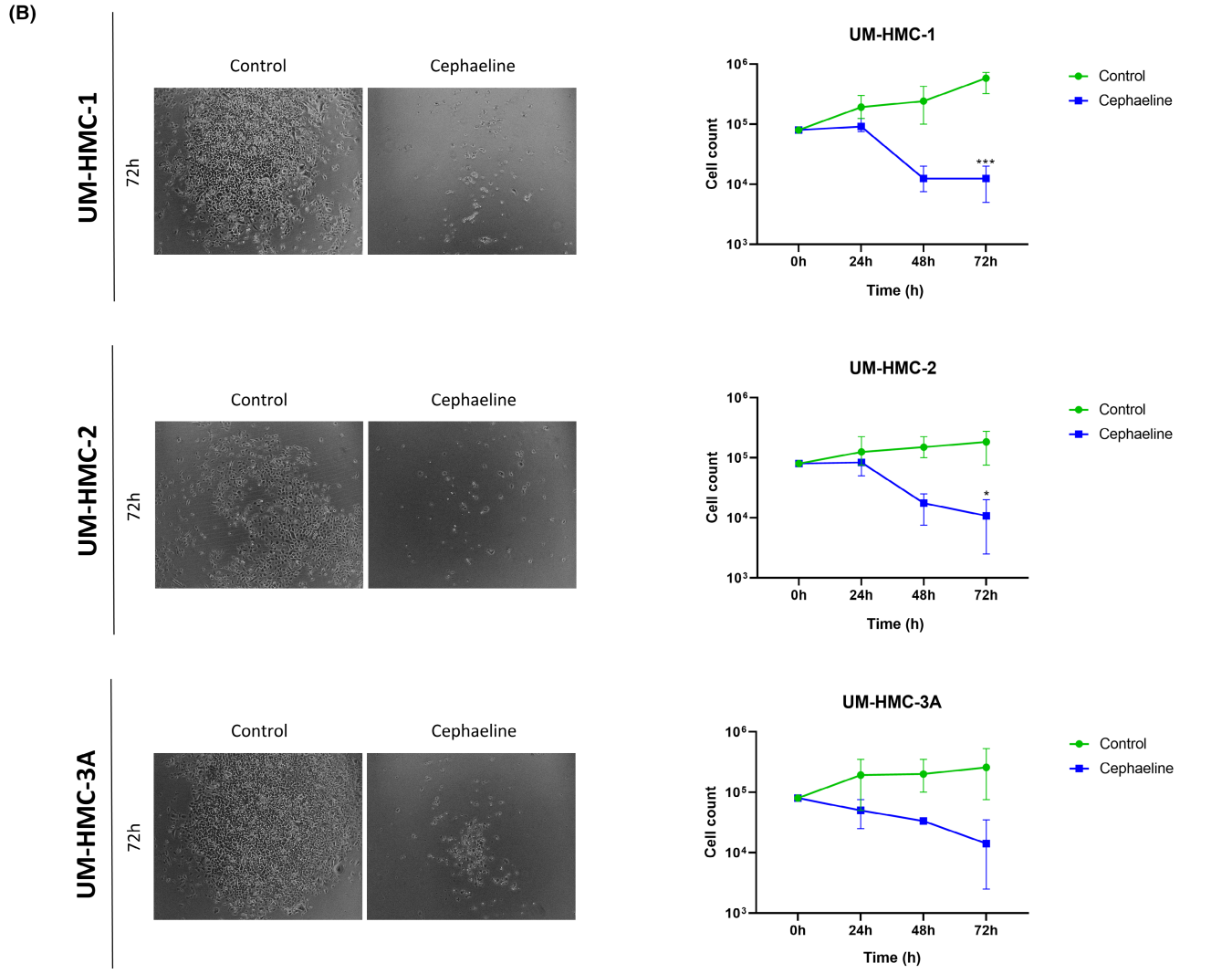


FIGURE 1 Cephaline inhibits viability and growth cellular in MEC tumor cell lines. (A) Determination of the IC_{50} of Cephaline in MEC cells (UM-HMC-1, UM-HMC-2, and UM-HMC-3A). (B) The treatment with Cephaline inhibits UM-HMC-1 (***$p > 0.001$) and UM-HMC-2 (*$p > 0.05$) cell proliferation, with a stronger effect after 72 h. In UM-HMC-3A, statistically significant was not found. Original magnification 40X

promising therapeutic strategy to manage MEC.[12,16] Using immunofluorescence assay, we observed a significant change in histone acetylation of tumor cells upon administration of Cephaline. All MEC cell lines underwent significant acetylation of histone H3 at lys9 as early as 24 h after administration of Cephaline (UM-HMC-1, ****$p < 0.0001$, UM-HMC-2 ****$p < 0.0001$; and UM-HMC-3A **$p < 0.01$) (Figure 3A–C). The acetylation levels of histone H3 lys9 were further maintained for up to 48 h after a single dose of Cephaline (UM-HMC-1 ****$p < 0.0001$; UM-HMC-2 ****$p < 0.0001$; and UM-HMC-3A *$p < 0.01$) (Figure 3A–C).

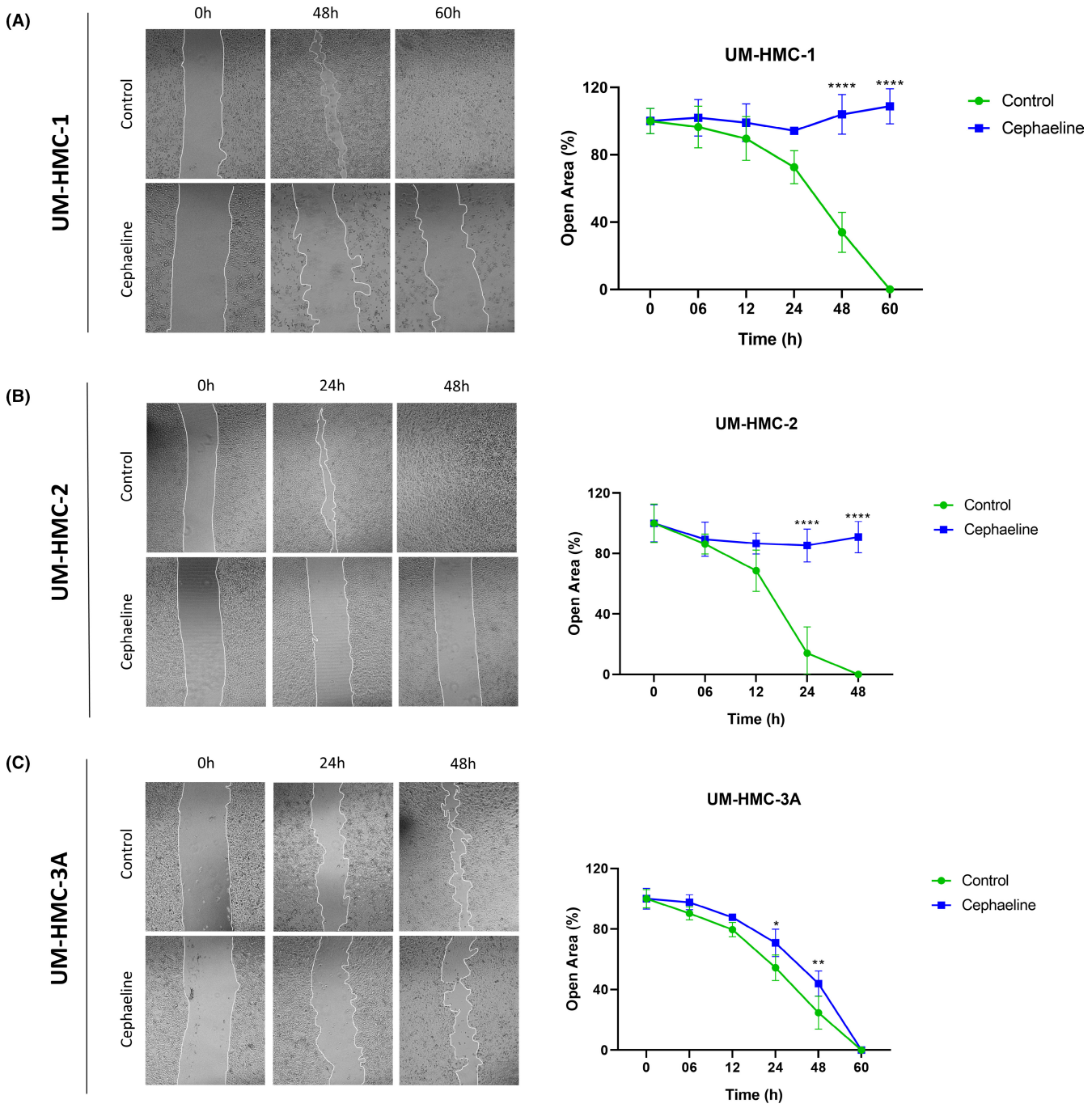


FIGURE 2 Cephaeline inhibits migration cellular in MEC tumor cell lines. The treatment with Cephaeline significantly reduced cell migration in comparison with control cells with stronger effect after 48 and 60 h in (A) UM-HMC-1 (****$p < 0.0001$) and for 24 and 48 h in (B) UM-HMC-2 (****$p < 0.0001$) and (C) UM-HMC-3A (**$p < 0.01$). Original magnification 40X

3.3 | Distinct effects of Cephaeline over the population of CSCs

CSCs constitute a subpopulation of cancer cells recently described in MEC.[10,12,17] Here, we investigated whether the administration of Cephaeline would impact the population of CSC of MEC through the identification of the enzymatic activity of ALDH. MEC stem cells have been identified by high expression levels of ALDH by Adams et al.[17] and Wagner et al.[12]. Here, we show that Cephaeline administration significantly reduced the number of ALDH + MEC tumor cells within 24 h of treatment in UM-HMC-2 (*$p < 0.05$). Unexpectedly, we observed an increased number of ALDH + cells in UM-HMC-1 (*$p < 0.05$) and UM-HMC-3A (*$p < 0.05$) (Figure 4A). To further explore the effects of Cephaeline on MEC CSCs we decided to run a functional assay by culturing tumorspheres in the presence of Cephaeline. Previous studies have shown that MEC cell lines can

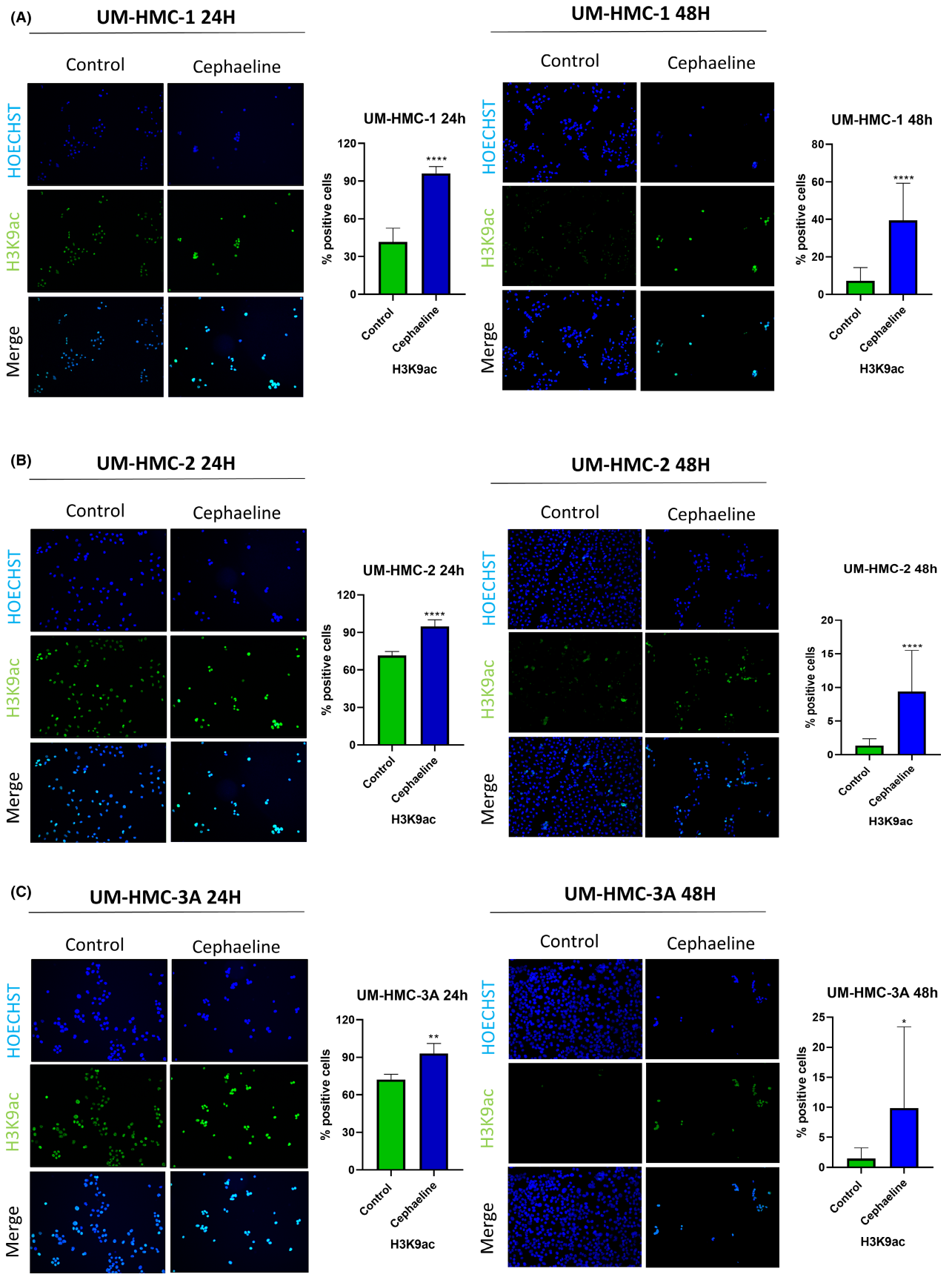


FIGURE 3 Cephaeline increases H3 levels in MEC cell lines. Immunofluorescence staining of H3K9ac in (A) UM-HMC-1, (B) UM-HMC-2, and (C) UM-HMC-3A cells upon administration of Cephaeline for 24 and 48 h. Note that Cephaeline increases histone acetylation in all MEC cell lines in both times. Original magnification 40X

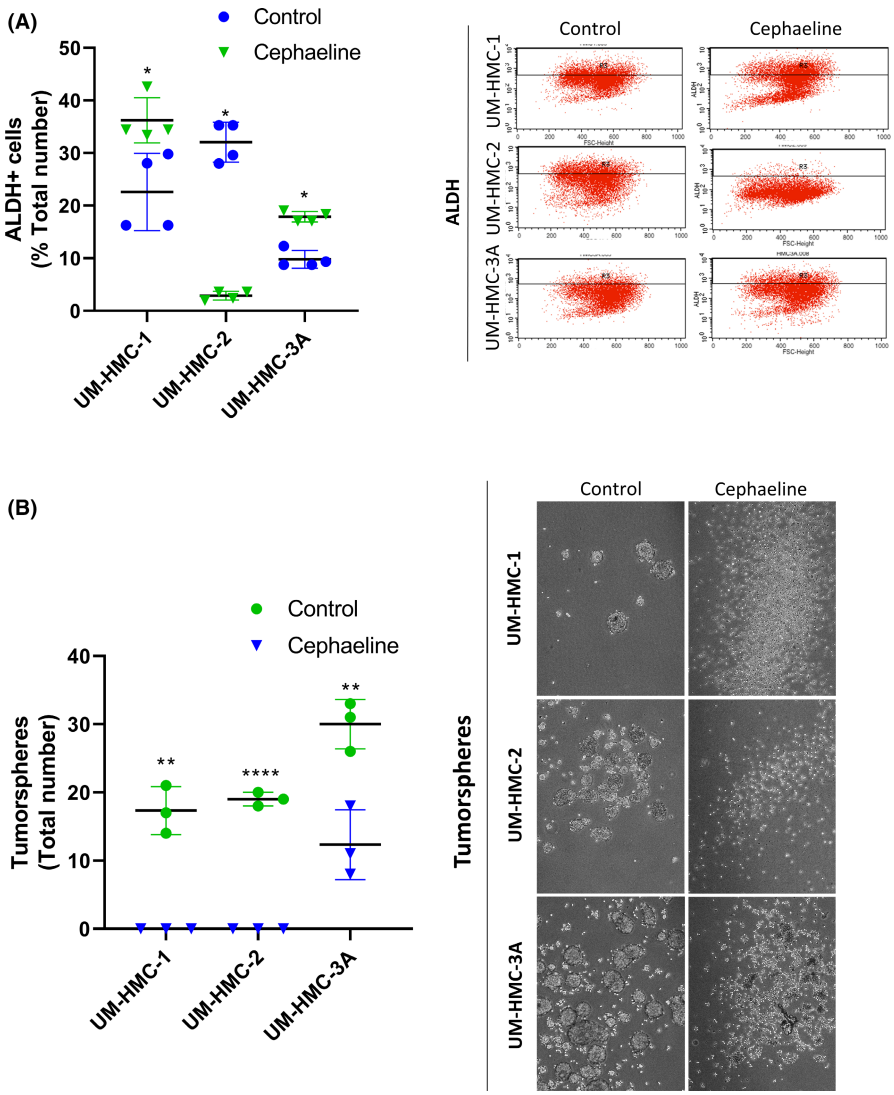


FIGURE 4 CSC in MEC cell lines. (A) Cells exposed to Cephaeline for 24 h were collected and processed for ALDH activity using fluorescence-activated cell sorting (FACS) analysis. Here, we presented UM-HMC-1, UM-HMC-2, and UM-HMC-3A representative samples of ALDH+, and the percentage of these positive cells. (B) Tumorspheres individually produced by UM-HMC-1, UM-HMC-2, and UM-HMC-3A. Original magnification 40X

generate tumorspheres when cultured under ultra-low adhesion conditions.[8,12] Here, we observed that Cephaeline completely inhibited the formation of tumorspheres in UM-HMC-1 (**$p < 0.01$) and UM-HMC-2 (****$p < 0.0001$) tumor cells. Interestingly, UM-HMC-3A was able to generate tumorspheres in the presence of Cephaeline, although at significantly lower levels than the control group (**$p < 0.01$) (Figure 4B).

4 | DISCUSSION

Management of MEC follows similar protocols in the use for other malignancies from the salivary glands. Unfortunately, therapeutic strategies to manage disseminated diseases and metastatic tumors are lacking, and new strategies are needed.[3,18] Although several chemotherapy protocols have been studied in the management of MEC, a generally low level of response is observed.[19–21] Therefore, in this pioneering study, we investigate the effects of Cephaeline on MEC cell line behavior. Furthermore, we evaluated the impact of Cephaeline on histone acetylation, a key epigenetic machinery associated with the maintenance of CSCs. Our results suggested that Cephaeline is a promising drug to manage MEC by inhibiting viability and cell growth, and migration, along with the ability to reduce the number and size of tumorspheres.

Our first step was to carefully identify the IC_{50} for each MEC cell line as a standard method to test new anti-cancer therapies *in vitro*.[22] Each cell line presented a different IC_{50} value. UM-HMC-2 required 13 times more concentration of Cephaeline than UM-HMC-1, while a considerably lower IC_{50} was found for UM-HMC-3A. This exciting finding can be related to the fact that these cell lines are derived from MEC tumors presenting different clinical characteristics. Although all cell lines share a common clinical stage (IV) and histological grade (intermediate), the anatomical site of the primary tumor and histological characteristics such as the presence of angio/perineural invasion can be pointed out. UM-HMC-2 is derived from the parotid gland and demonstrates higher resistance to Cephaeline and presented perineural invasion. UM-HMC-1, however, was isolated from a minor salivary gland tumor and demonstrated higher sensitivity to Cephaeline and no signs of perineural invasion.[14] Surprisingly, UM-HMC-3A, the most

sensitive cell line to Cephaeline, is derived from a more aggressive tumor isolated from a local recurrence of the left hard palate and presenting perineural and angiolymphatic invasion. These results reinforce the importance of personalized medicine. Each tumor/patient has unique features in molecular and physiologic parameters, susceptible to environmental and behavior changes, which results in the necessity of personalized interventions.[23]

After establishing the IC_{50}, we investigated the impact of a single administration of Cephaeline on MEC growth. We had previously shown that Emetine inhibited MEC cell line survival[12] and, therefore, expected to see a similar effect of Cephaeline. Indeed, we observed that Cephaeline significantly reduced cell growth in UM-HMC-1 and UM-HMC-2 MEC cell lines. Our results corroborate with[24] findings that showed reduced cellular growth of chronic lymphocytic leukemia after administration of Cephaeline. To further explore the impact of Cephaeline on MEC tumors, we performed a scratch assay to analyze cell migration. Identifying new therapies capable of hindering tumorigenic properties as enhanced motility is key to preventing disease relapse in distant organs.[25] Herein, we found that Cephaeline had a significant impact on cell migration assessed by scratch assay. Our study is the first to demonstrate that the administration of Cephaeline interferes in malignant cell migration.

Histone deacetylation is an epigenetic event linked to tumor progression, leading to chromatin compaction and silencing of tumor suppressor genes.[15,26–28] We had previously shown the critical role of histone modifications on CSC maintenance in MEC tumors[12] (Markman et al., 2019). Herein, we showed that Cephaeline administration increased histones acetylation in all MEC cell lines. This mechanism could be responsible for the MEC response to Cephaeline, since the histone acetylation in tumor cells induces cell differentiation, inhibiting proliferation and invasion.[15]

Salivary gland tumors are highly heterogeneous, presenting CSCs dispersed throughout the tumor mass. CSCs constitute a subpopulation of tumor cells with an inherent self-renewing characteristic and greater potential to generate metastasis. The presence of CSCs is also associated with chemoresistance phenotypes and consequently increased recurrence rates after therapy.[8] Here, we found that Cephaeline hampers the ability of MEC to form tumorspheres. Our group has demonstrated that tumorspheres grown under low-attachment conditions express stem cell markers at significantly higher levels compared with cells cultured in monolayer.[29] Along tumorspheres, the presence of ALDH+ cells is also used to identify the CSC fraction of cells within MEC tumors.[12] In a previous study, we found a reduction in the ALDH + population in three MEC cell lines after treatment with Emetine (analog of Cephaeline).[12] Thus, our hypothesis was that Cephaeline would exert a similar effect. Surprisingly, we found that Cephaeline impacts in different forms the ALDH + population in MEC cell lines examined. We observed a significant decrease in ALDH + cells in UM-HMC-2, while a substantial increase was seen in UM-HMC-1 and UM-HMC-3A. The dose of Cephaeline used for UM-HMC-2 might have played a role in this result since this cell line was more resistant to the drug, and a higher dose of Cephaeline was needed to achieve IC_{50}. Yet, the results from

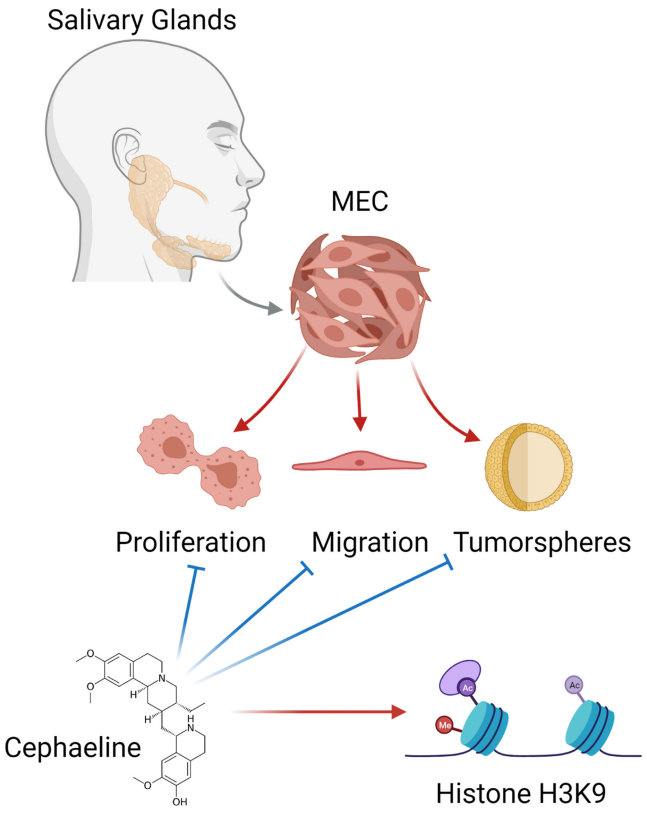


FIGURE 5 Role of Cephaeline in MEC cell lines. Cephaeline has anti-cancer properties in MEC cell lines through the regulation of proliferation, migration, and ability to reduce the number and size of tumorspheres, along with significantly increased histone acetylation

UM-HMC-1 and UM-HMC-3A cell lines were unexpected. The use of IC_{50} doses has shown positive results in all the assays performed in this manuscript suggesting its efficacy. Also, Emetine decreased the ALDH + cell population in both UM-HMC-1 and UM-HMC-3A, as previously demonstrated by us.[12] These findings suggest that despite having a similar chemical structure, Cephaeline and Emetine might act in distinct ways, reverberating in different responses concerning CSC maintenance. The mixed antitumor activities expressed by Cephaeline in UM-HMC-3A performed by downregulation and upregulation in several processes of tumor survival might be questioned due to the low dose of Cephaeline used for UM-HMC-3A; however, this cell line was more sensitive to this drug, verified through cell viability assay and IC_{50} determination. Furthermore, we demonstrated Cephaeline anticancer properties only in "in vitro" assays, this represents a restriction.

While this result might represent a limitation of Cephaeline therapy in MEC, some aspects need to be taken into consideration. These results were obtained after a single administration of the drug; thus, continuous treatment might have a different impact on tumor cells. Also, we tested Cephaeline as a single agent. Cancer cells, and more specifically CSC, are endowed with intrinsic resistance mechanisms to evade chemotherapy. The use of combined therapy represents a promising approach to overcome this issue. As we have demonstrated

here, Cephaeline acts by increasing histone acetylation and therefore, might serve as an epi-drug. Compacted chromatin leads to poor DNA accessibility to drugs,[12] and changes in chromatin configuration by other epi-drugs, such as Vorinostat, increase the sensitivity of CSC to other chemotherapeutic agents, such as Cisplatin.[8,30] Further studies are needed to elucidate the mechanism of action of Cephaeline and to explore its potential efficacy as a combined therapy.

Together, we conclude that Cephaeline can have anti-cancer properties in MEC cell lines by regulating multiple processes, including cell growth and migration. Our results also suggest that Cephaeline acts by modulating histone H3 acetylation, inducing chromatin relaxation along with effective inhibition of tumorspheres formation (Figure 5).

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CONFLICT OF INTEREST

None declared.

AUTHOR CONTRIBUTION

LCS and GBB contributed to conception, design, data acquisition, and interpretation, drafted and critically revised the manuscript. VPW, MDM, GCJ, CHS, RMC, and PAV contributed to data acquisition and interpretation, drafted and critically revised the manuscript. GZR, MAL, ARSS, LPK, and JEN contributed to the draft and critically revised the manuscript. All authors gave their final approval and agreed to be accountable for all aspects of the work.

ETHICS STATEMENT

The study did not require approval from the ethics committee.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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