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Original Article

Repurposing NFκB and HDAC inhibitors to individually target cancer stem cells and non-cancer stem cells from mucoepidermoid carcinomas

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Abstract: Drug resistance remains a major obstacle in the treatment of mucoepidermoid carcinomas (MEC) leading to tumor recurrence, disease progression, and metastasis. Emerging evidence suggests that drug resistance is mediated by the presence of a highly adaptative subpopulation of cancer cells known as cancer stem cells (CSC). We have previously reported that solid tumors use NFkB signaling as a chemotherapy-resistant mechanism. We have also shown that interfering with the epigenome of solid tumors is an effective strategy to control the population of CSC. Here, we sought to investigate the effects of the NFkB inhibitor emetine and the HDAC inhibitor SAHA on the biology of MEC CSC and assessed whether this combination therapy would favor the standard of care therapy comprised of the administration of Cisplatin (CDDP). Our findings suggested that the administration of low concentrations of emetine and SAHA is more effective in disrupting CSC in MEC, while the administration of emetine in combination with CDDP constitutes an effective therapy to target non-CSC MEC tumor cells.

Keywords: Head and neck tumors, salivary gland cancer, chemotherapy, target therapy, NFκB, epigenetic, cancer stem cells

Introduction

Mucoepidermoid carcinoma (MEC) is the most common malignancy from the salivary glands presenting a 5-year disease-free survival of 76% [1-4]. Typically, patients with low- and intermediate-grade MEC have a favorable prognosis, while high-grade tumors are associated with a poor prognosis [5-7]. The majority of MEC are detected at early stages and are usually associated with a good prognosis, being surgical resection the standard treatment. Nonetheless, metastatic and unresectable MEC are typically managed with radiotherapy and platinum-based chemotherapy [8-11]. Unfortunately, malignant salivary gland tumors (SGT) are found to present a modest response to platinum-based therapies [12, 13]. Much of the resistance observed in SGT can be related to the presence of cancer stem cells (CSC), similar to other tumors like breast cancer [14-17]. CSC constitutes a subpopulation of cancer cells capable of self-renewal while retaining multipotency properties. Like stem cells, CSC can evade apoptosis while presenting resistance to DNA damage-induced therapies through different mechanisms, including the abnormal expression of proteins involved in drug transport

[18, 19]. Our group has shown that the administration of Cisplatin (CDDP) induces the accumulation of MEC CSC *in vitro* [15].

In the search for novel therapeutic strategies, we have identified several targets, including the NFκB signaling and the epigenetic machinery of histones, as two potential targets involved in tumor behavior. Our initial studies involved the successful delivery of emetine to disrupt NFκB signaling and SAHA as a histone deacetylase inhibitor capable of interfering with the epigenome of solid tumors [20-23]. We have also shown that MEC tumors underwent significant acetylation of histone H3 upon administration of Cephaeline, an analog to emetine [24]. Remarkably, DNA decondensation mediated by pharmacological-induced acetylation of histones resulted in profound impairment of the subpopulation of CSC.

Here, we investigated the effects of emetine and SAHA in CSC derived from three MEC cell lines and assessed whether this combination was able to sensitize MEC tumor cells to CDDP. Moreover, we found that administration of low concentration of emetine and SAHA is effective in depleting MEC CSC, suggesting that our proposed combination therapy may constitute a novel therapeutic strategy to disrupt CSC in MEC. Furthermore, we found that administration of emetine in combination with CDDP is more effective in targeting non-CSC MEC tumor cells.

Methods

Cell lines and proliferation assay

UM-HMC1, UM-HMC2, and UM-HMC3A MEC cell lines were cultured as we previously described [25]. Cells were maintained under 70% of confluence to avoid cellular stress and activation of cellular differentiation. Cells received CDDP (Cayman Chemical Company Ann Arbor, MI, USA), emetine (Cayman Chemical Company Ann Arbor, MI, USA), and SAHA (Cayman Chemical Company Ann Arbor, MI, USA). Cell proliferation was determined using MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) assay (Thermo Scientific, Waltham, MA, USA). MEC cells (5×10^4) were seeded in 96-well to establish the concentration of emetine and SAHA. Cells receiving emetine were cultured with 22 ηM and 440 ηM of the inhibitor for 24 h (n=8). MEC receiving SAHA were cultured at 1 μM, 5 μM, and 10 μM of the HDAC inhibitor. Briefly, an MTT assay was performed at 37°C for 4 h, and the Formazan precipitated was diluted in ethanol and assessed by absorbance (iMarkTM Microplate Absorbance Reader, BioRad) at 595 nm.

Immunofluorescence

MEC cells were seeded in 6-well plates (5×10^4 cells) and 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. Cells treated with emetine were incubated overnight with NFκB p65 Antibody (Cell Signaling Technology, Danvers, MA, USA), and cells treated with SAHA were incubated overnight with Anti-H3K9ac antibody (Cell Signaling Technology, Danvers, MA, USA); both antibodies were diluted in (0.5% (v/v) Triton X-100 in PBS 1X and 1% (w/v) BSA). Cells were washed and incubated with Alexa 555 and Alexa 488 secondary antibodies (Cell Signaling Technology, Danvers, MA, USA), followed by DNA staining using Hoechst 33342 (Cell Signaling Technology, Danvers, MA, USA). Images were taken using a Nikon Eclipse Ti-S microscope and evaluated using Image J software (National Institute of Health, Bethesda, Maryland, USA).

Flow cytometry

CSC derived from MEC cell lines were identified by flow cytometry through the enzymatic activity of aldehyde dehydrogenase (ALDH) activity (StemCell Technologies, Durham, NC, USA), following the manufacturer's instructions. In brief, MEC cells were treated with 1 μM, 5 μM, and 10 μM of SAHA (Histone acetylation) (n=4) for 24 h. MEC cells were washed, suspended and incubated with activated Aldefluor substrate (BODIPY amino acetate) or a negative control (dimethylamino benzaldehyde-DEAB, a specific ALDH inhibitor) for 45 min at 37°C. All samples were analyzed using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA).

Tumorspheres assay

MEC cells were seeded at a concentration of 3×10^3 cells on ultra-low attachment plates

(Corning® Costar® Ultra-Low attachment multi-well plates, CLS3471 Sigma) and cultured for five days. All cells received administration of CDDP, SAHA, emetine, or a combination of drugs. Tumorsphere formation was observed daily. Images were obtained using a Nikon Eclipse Ti-S microscope (Nikon). Tumorspheres were counted using Image J software (National Institute of Health, Bethesda, Maryland, USA).

Colony assay

MEC cells (3×10^2) were plated into 6-well. Established concentration drugs were administrated at the same time as cell seeding and allowed to grow for additional seven days in order to form colonies. 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).

IC_{50} determination

We used MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) assay (Thermo Scientific, Waltham, MA, USA) to identify the optimal concentration of the drugs capable of inhibiting 50% of cellular proliferation (IC_{50}). In brief, 5×10^4 cells were plated into 96-well plates. Further, cells were sensitized with emetine, SAHA, and emetine/SAHA for 24 h. The range of CDDP from 1 μM to 100 μM was incubated for more than 24 h. MTT assay was performed as we previously described [24].

Statistical analysis

All statistical analyses were performed using GraphPad Prism 8.0 (GraphPad Software, San Diego, CA, USA). 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.

Results

CDDP reduces the viability of MEC cells but has a limited effect on tumorspheres

The use of CDDP-based therapies provides a modest control over advanced SGT [26]. Most recently, it has been shown that administration of CDDP alone does not induce significant cytotoxicity to CSC, although it induces apoptosis of non-CSC MEC cells [27]. Here, we performed the viability assay by MTT and determined the inhibitory concentration of 50% of the cells (IC_{50}). MEC cells present different IC_{50} values for CDDP ranging from 16.28 μM, 6.35 μM, and 18.10 μM for UM-HMC-1, UM-HMC-2, and UM-HMC-3A, respectively (**Figure 1A**). Next, we decided to explore the impact of CDDP on tumorspheres. We decided to use this approached aiming to assess the MEC stemness ability. Therefore, MEC cells were cultured under ultra-low adhesion conditions and treated with the appropriated IC_{50} (**Figure 1B**). We observed that CDDP alone reduces the number of tumorspheres significantly in UM-HMC1 ($^{***}P<0.001$). However, we observed no statistical difference in the number of tumorspheres upon administration of CDDP to UM-HMC2 and UM-HMC3A cell lines (ns $P>0.05$). To further explore the effects of CDDP on MEC cells, we cultured MEC cells in low-density culture to allow colony formations. Here, we used the appropriated CDDP IC_{50} on day 0 and evaluated colony formation by day 7 (**Figure 1C**). As expected, we showed that CDDP caused a total inhibition of colony-forming in UM-HMC1 ($^{****}P<0.001$), UM-HMC2 ($^{****}P<0.0001$), and UM-HMC3A ($^{***}P<0.001$), once the CDDP administration represent a great target to adherent cells. However, in this model, the cells proliferate faster and do not exhibit drug resistance profiles, thus neglecting the CSC properties.

A single dose of emetine reduces tumor proliferation and NFκB activity of MEC cells

Our group has previously demonstrated that NFκB inhibition is a viable target to control MEC growth by reducing the viability of MEC non-CSC and partially disrupting CSC [21, 22]. Also, we demonstrated that emetine administration can disrupt the NFκB activity, by the reduction of phosphorylated IκB-α and down-

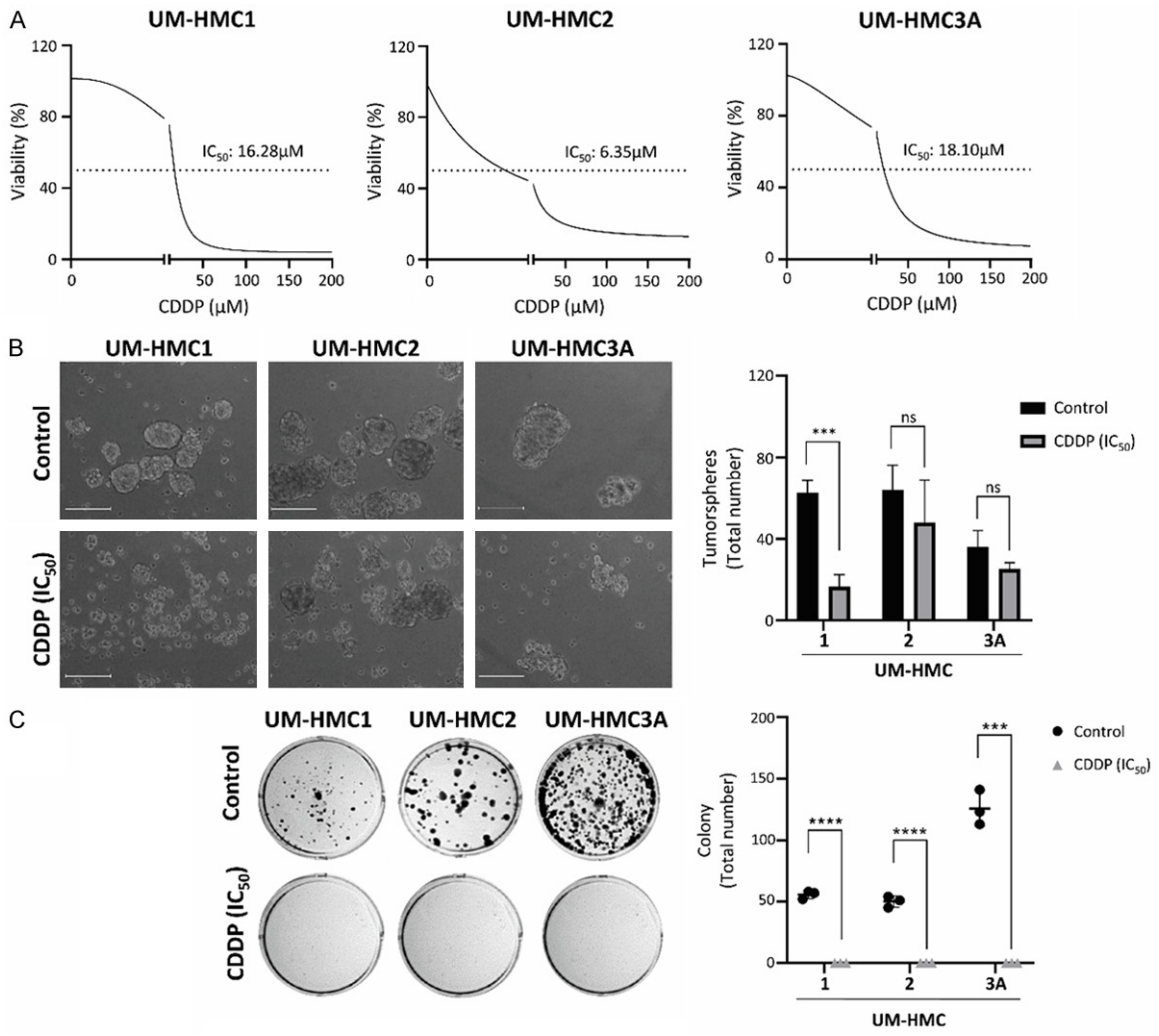

Figure 1. CDDP inhibits the viability and differently affects tumorspheres and colony formation in MEC cell lines. A. Determination of the IC_{50} of CDDP (ranging from 1 μM to 200 μM) in MEC cell lines (UM-HMC-1, UM-HMC-2, and UM-HMC-3A) by MTT assay. B. The treatment with CDDP reduces UM-HMC1 (IC_{50}: 16.28 μM) tumorspheres (***P<0.001) and did not impact UM-HMC2 (IC_{50}: 6.35 μM) and UM-HMC3A (IC_{50}: 18.10 μM) tumorspheres. Note the difference in tumorspheres shape after the administration of CDDP. C. The colony formation after seven days was totally inhibited by CDDP (IC_{50}) in UM-HMC1 (****P<0.001), UM-HMC2 (****P<0.0001), and UM-HMC3A (***P<0.001). Scale bar 125 μM.

regulation of IKK-β [21]. Based on previous studies and skills, we decided to use a lower (22 ηM) and higher (440 ηM) concentration of emetine for 24 h. We observed that a lower dose of emetine (22 ηM) was sufficient to disrupt cellular proliferation of UM-HM3A (*P< 0.05) cell line, while a higher dose of emetine (440 ηM) efficiently disrupted the proliferation of all MEC cell line (**Figure 2A**).

However, our goal here was to establish as low as possible effective doses of emetine capable of inhibits NFκB levels in MEC cells. For this, we assessed whether emetine could affect nuclear levels of NFκB. Towards this goal, we performed an immunofluorescence assay against p65 (NFκB effector). All MEC cell lines presented nuclear expression of NFκB at the base level (control) (**Figure 2B**, immunofluorescence panel). Administration of emetine efficiently reduced the nuclear levels of NFκB at both concentrations presenting overall similar inhibitory values for UM-HMC1 (*P<0.05), UM-HMC2 (***P<0.001), and UM-HMC3A (**P<0.01) (**Figure 2B**). Given the promising results, we decided to follow up our experiments using the lower

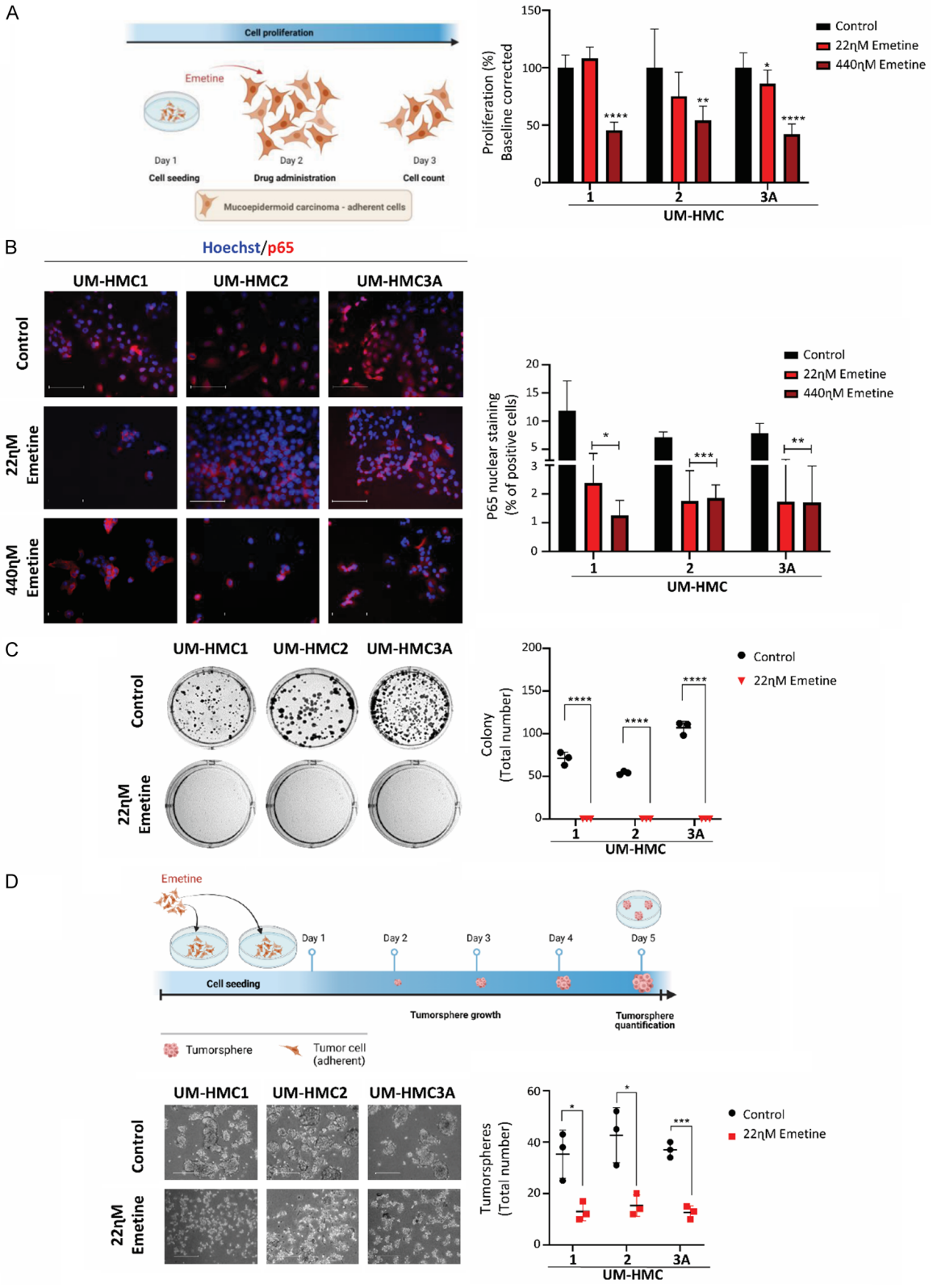

Figure 2. A single dose of emetine reduces proliferation and NFκB activity, inhibits colony formation, and impacts tumorsphere formation. A. The treatment with emetine inhibits the MEC cell line proliferation seen by MTT assay. Note that a 22 ηM dose of emetine only is sufficient to inhibit the UM-HMC3 proliferation after 24 h. Further, a strong effect in proliferation was shown with the 440 ηM dose in all MEC cell lines, also after 24 h. B. All MEC cell lines

presented NFκB nuclear expression (Immunofluorescence, stained with p65). The treatment with 22 ηM and 440 ηM of emetine reduced the NFκB nuclear expression levels, after 24 h of treatment (200× original magnification). C. 22 ηM of emetine was administrated on day 0, and the colony formation assay was evaluated on day 7. Representative images of colonies fixed and stained with 0.1% crystal purple. Note that a single dose of emetine can complete disrupting of colony formation in all MEC cell lines (****P<0.001). D. Schedule of emetine administration used in tumorsphere assay. A single dose of emetine (22 ηM) determined NFκB nuclear expression) was administrated on day 0. Tumorspheres were assessed on day 5. Tumorspheres individually produced by UM-HMC-1, UM-HMC-2, and UM-HMC-3A. Original magnification 200×. Note that a single dose of emetine (22 ηM) is able to reduce UM-HMC1 (*P<0.05), UM-HMC2 (*P<0.05), and UM-HMC3A (***P<0.001) tumorspheres. Scale bar 125 μM.

dose of 22 ηM of emetine. Next, we decided to explore the effects of low doses of emetine on the colony-forming properties of MEC cells. Here, we decide to follow with the lower emetine dose (22 ηM) because although this concentration was efficient just on UM-HMC3a proliferation, they reduced the nuclear levels of NFκB in all MEC cell lines. Administration of emetine was carried over as a single dose on day 0, and the colony formation assay was evaluated on day 7. Here, we demonstrated that a single dose of emetine efficiently disrupted colony formation in all MEC cell lines (****P<0.001) (**Figure 2C**).

To further explore the properties of MEC CSC, we cultured all MEC cells under ultra-low adhesion conditions and evaluated the effects of a single dose of emetine administered on day 0 of tumorsphere formation and maintained for 5 days. Our strategy entails the potential use of emetine as a chemopreventive drug. We observed that emetine was capable of reducing the number of tumorspheres derived from UM-HMC1 (*P<0.05), UM-HMC2 (*P< 0.05), and UM-HMC3A (***P<0.001) cell lines (**Figure 2D**).

A single dose of SAHA reduces MEC proliferation and reduces the population of CSC

Here we selected a concentration of SAHA based on its ability to reduce the number of CSC as judged by the ALDH enzymatic activity. We have previously shown that epigenetic events play an important role in MEC behavior [28]. Our experience along emerging findings indicates that interfering with the levels of histone acetylation constitutes a promising strategy for disrupting CSC [15, 22, 24]. Here, we observed that low doses of SAHA ranging from 1 to 10 μM could efficiently inhibit MEC proliferation (**Figure 3A**). SAHA is an FDA approved class I and II HDAC inhibitor widely used to treat different malignant tumors, by histone acetylation and growth arrest, differentiation, and apoptosis impacted [22]. Amid our findings, we decided to explore the effects of low doses of SAHA on the population of CSC using the Aldefluor assay. We have previously shown that MEC stem cells are endowed with high expression levels of ALDH [22, 24]. Here, we found that UM-HMC2 and UM-HMC3A MEC cells treated with SAHA for 24 h presented a significant reduction in the number of ALDH+ cells (UM-HMC2 ****P<0.0001; UM-HMC3A 1 μM **P<0.01, 5 μM and 10 μM ****P<0.0001). Interestingly, an increase in ALDH+ cells was observed in UM-HMC1 for all concentrations of SAHA (**Figure 3B**). Although ALDH is a well-known marker of cancer stem cells, one previous study performed by our group has shown that ALDH accounts for 7-20% of all cells within a tumorsphere. This observation suggests the existence of different stem cell populations withing the tumorsphere and that ALDH may only identify a subset of these stem cells, that in the case of UM-HMC1 are not affected by SAHA [29].

Next, we used the lowest dose of SAHA (1 μM) on UM-HMC1, UM-HMC2, and UM-HMC3A cells, followed by the immunofluorescence staining for H3K9ac after 24 h of treatment. Administration of SAHA resulted in increased histone acetylation in all MEC cell lines, especially for UM-HMC1 (**Figure 3C**). We further explored the ability of SAHA to prevent the formation of colony foci by adding the drug along with cellular seeding and following for 7 days. A single dose of SAHA was able to completely prevent the formation of colonies of UM-HMC1 and UM-HMC2 cell lines (***P<0.001) and reduce colony formation of UM-HMC3A (***P<0.001) (**Figure 3D**).

Further, we decided to culture all MEC cell lines under ultra-low adhesion conditions to evaluate the effects of SAHA over tumorspheres after 5 days of treatment. We observed that SAHA was also efficient in reducing the number of tumorspheres for all MEC cell lines (UM-

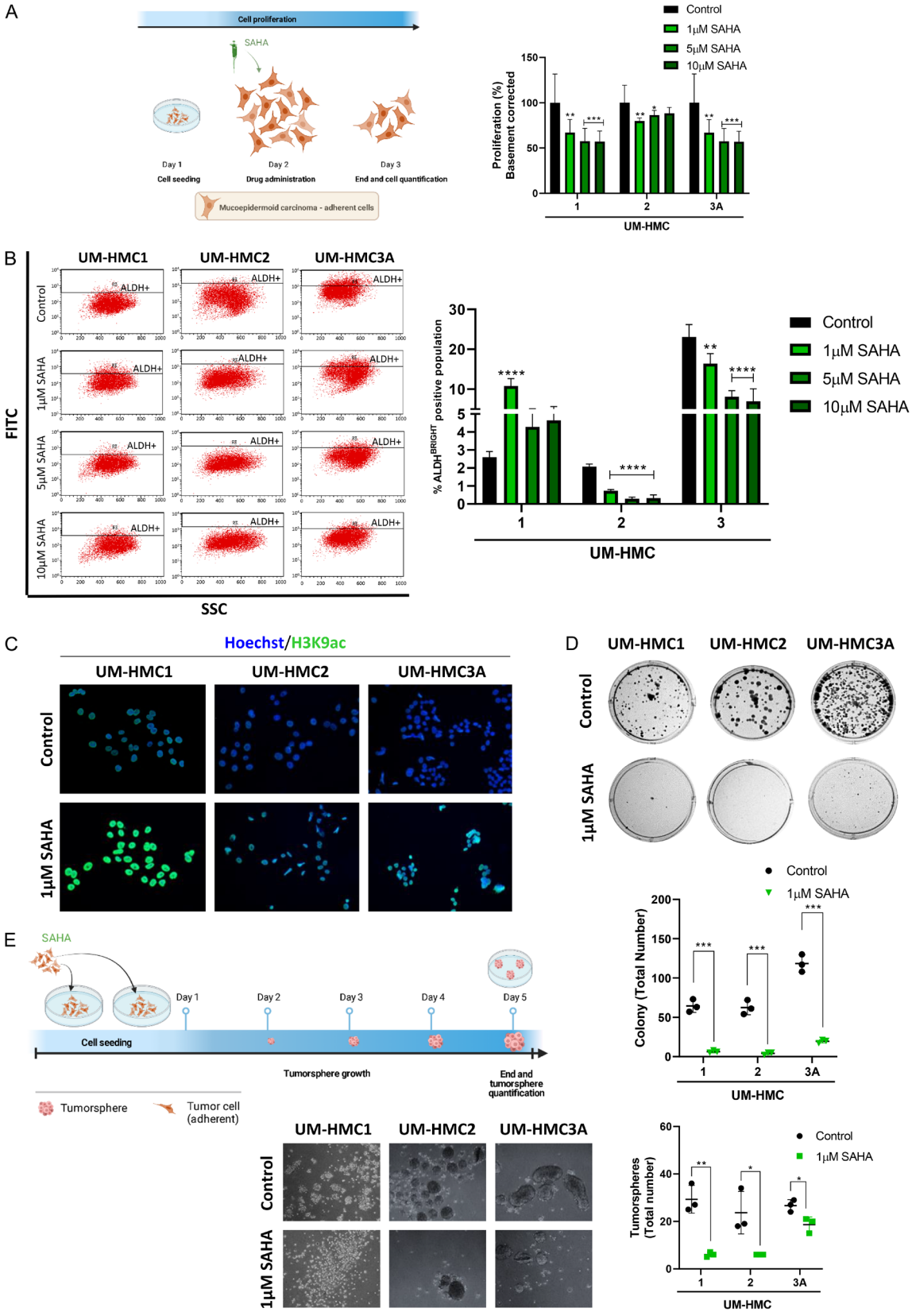

Figure 3. A single dose of SAHA reduces the proliferation, distinctly affects the ALDH+ cells, reduces colony formation, and impacts the tumorsphere formation. A. The treatment with SAHA inhibits the MEC cell line proliferation seen by MTT assay. B. Cells exposed to SAHA (1 μM) for 24 h were collected 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. Note that UM-HMC2 and UM-HMC3A MEC cells presented a significant reduction in the number of ALDH+ cells (UM-HMC2 ****P<0.0001; UM-HMC3A 1 μM **P<0.01, 5 μM and 10 μM ****P<0.0001), while an increase in ALDH+ cells was observed in UM-HMC1 for all concentrations of SAHA. C. Immunofluorescence staining of H3K9ac in UM-HMC-1, UM-HMC-2, and UM-HMC-3A cells after the administration of SAHA (1 μM) for 24 h. Note that SAHA increases histone acetylation in all MEC cell lines (200× original magnification). D. Representative images of colonies fixed and stained with 0.1% crystal purple. The administration of SAHA (1 μM) was done on day 0, and the colony formation assay was evaluated on day 7. Note that a single dose of SAHA (1 μM) can complete disrupting of colony formation in all UM-HMC1 and UM-HMC2 cell lines (***P<0.001) and reduce UM-HMC3A (***P<0.001). E. Schedule of SAHA administration used in tumorsphere assay. A single dose of SAHA (1 μM, determined by ALDH expression) was administrated on day 0. Tumorspheres were assessed on day 5. A single dose of SAHA (1 μM) was administrated and assessed in the same protocol described above. Original magnification 200×. Note that a single dose of SAHA (1 μM) was also able to reduce tumorspheres in all MEC cell lines; however, a minor reduction was observed in UM-HMC3A (*P<0.05). Scale bar 125 μM.

HMC1: **P<0.01; UM-HMC2 and UM-HMC3A: *P<0.05) (**Figure 3E**).

Combined administration of emetine and SAHA efficiently disrupts the formation of CSC

Here we decided to evaluate the effects of SAHA and emetine as a combination therapy to manage MEC tumors. This showed a good approach, once solid tumors are often refractory to monotherapies as compared to the effects of therapeutic regimens using two or more drugs. Also, we previously showed a promisor effect of this drug combination on MEC cells [22], but now, we assessed a ultra-low drug concentrations, in order to minimize side effects and to exploring new goals to chemosensitivity. Firstly, we performed a clonogenic assay, where we administrated the combination therapy of emetine and SAHA on day 0 and analyzed the colony formation for 7 days. We observed a complete inhibition of colonies in all MEC cell lines (****P<0.001) (**Figure 4A**). Next, we treated MEC cells with a combination of emetine and SAHA on day 0, followed by the analysis of the total number of tumorspheres formed after 5 days (**Figure 4B**). We observed a strong inhibition on tumorsphere formation for all MEC cell lines (UM-HMC1 ***P<0.001; UM-HMC2 *P<0.05; and UM-HMC3A **P<0.01) (**Figure 4C**). Following, we compared the tumorsphere inhibitory ability of emetine, SAHA, and the combination of both drugs with the frequently used chemotherapeutic agent, CDDP. We found that SAHA alone is capable of efficiently inhibiting the formation of tumorspheres compared with CDDP alone in UM-HMC2 (**P< 0.01) (**Figure 4D**). Further, we showed that SAHA in combination with emetine achieved the best inhibitory effect on tumorsphere formation in UM-HMC1 and UM-HMC3A (*P<0.05, **P<0.01).

Sensitizing MEC cell lines to CDDP

After our data confirmed that the proposed therapy reduced CSC, we investigated if SAHA and/or emetine were also capable of sensitizing MEC cells to CDDP. We previously demonstrated that SAHA alone could reduce the concentration of CDDP required for IC_{50} levels [15]. Here, MEC cells were sensitized with emetine, SAHA, or emetine/SAHA combination for 24 hours, followed by administration of CDDP (IC_{50}) (**Figure 4E**). We found that emetine was the best optimal to sensitize MEC cells to CDDP, resulting in a reduction of IC_{50} from 16.2 μM to 1.6 μM for UM-HMC1, from 6.3 μM to 3.9 μM for UM-HMC2, and 18.10 μM to 2.6 μM for UM-HMC3A (**Table 1**). SAHA was able to sensitize UM-HMC1 and UM-HMC3A cells to CDDP, where it was observed a reduction of IC_{50} in 1.58 folds for UM-HMC1 and 1.32 folds for UM-HMC3A. Emetine/SAHA combination therapy also reduced CDDP IC_{50} values (3.4 μM, 2.8 μM, and 3.6 μM for UM-HMC1, UM-HMC2, and UM-HMC3A, respectively). Finally, it is important to notify that although all cell lines arise from MEC, each one present different clinical feature. UM-HMC-1 was isolated from a minor salivary gland tumor, UM-HMC-2 is derived from the parotid gland, and UM-HMC-3A is derived from a more aggressive tumor isolated from a local recurrence of the left hard palate [25]. Therefore, despite these MEC cell lines sharing a common clinical stage (IV) and histological grade (intermediate), these different clinicopathological features can explain the individual effects on each one and support the importance of personalized medicine.

Also, our study suggests that emetine is more efficient in sensitizing MEC cells to CDDP and that emetine/SAHA combination therapy is

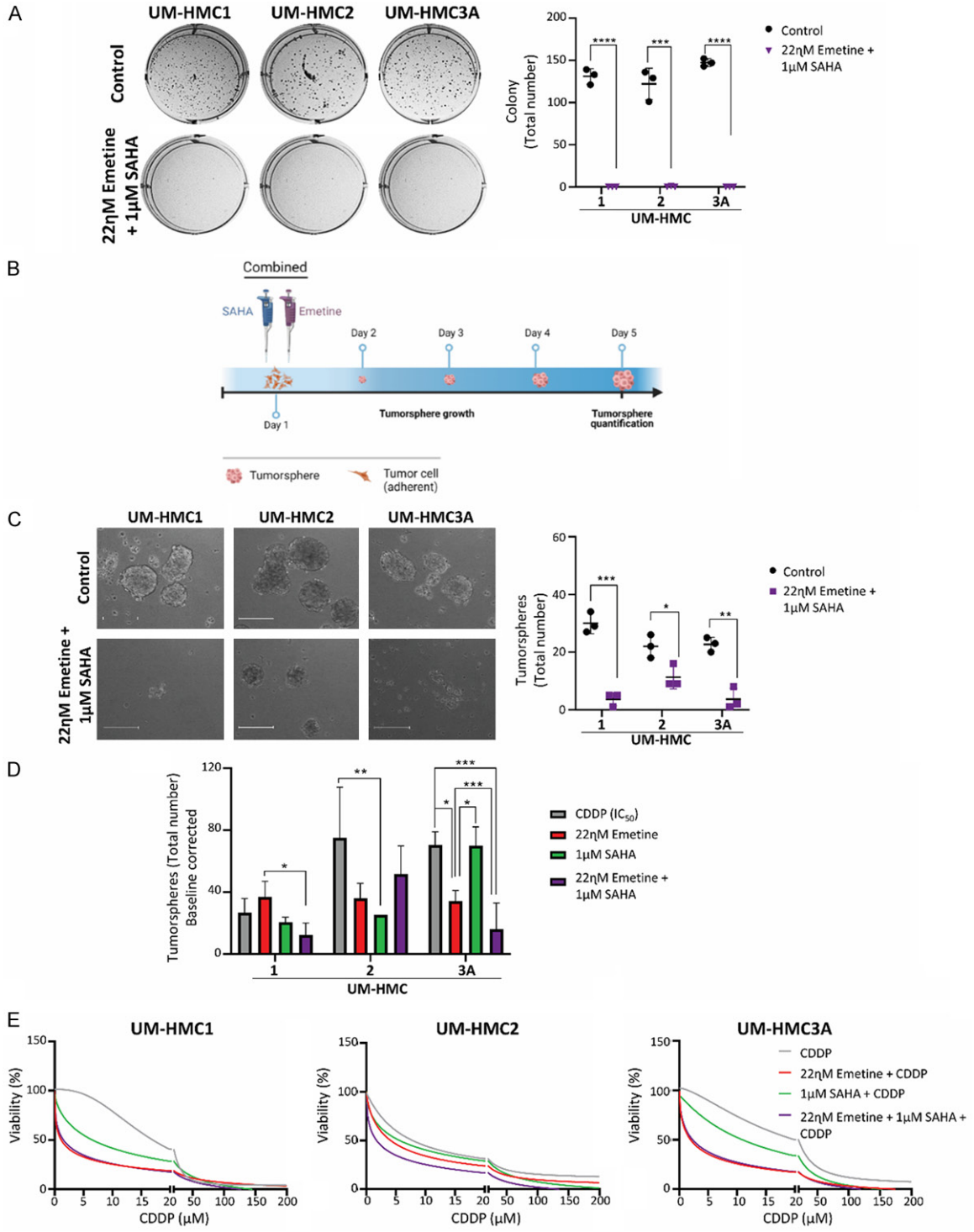

Figure 4. Both emetine and SAHA on MEC cell lines. A. Representative images of colonies fixed and stained with 0.1% crystal purple. The administration of emetine (22 ηM)/SAHA (1 μM) was done on day 0, and the colony formation assay was evaluated on day 7. Note that a single dose of a combinated therapy (emetine (22 ηM) and SAHA (1 μM) can complete disrupting of colony formation in all MEC cell lines (UM-HMC1 and UM-HMC3a ****P<0.001 and UM-HMC2 ***P<0.01). B. Schedule of emetine/SAHA combined administration used in tumorsphere assay (emetine (22 ηM) and SAHA (1 μM). C. A combination of these drugs (emetine (22 ηM) and SAHA (1 μM) was administrated on day 0. Tumorspheres were assessed on day 5. Tumorspheres are individually produced by UM-HMC-1, UM-HMC-2, and UM-HMC-3A. Original magnification 200×. Note that a proposed therapy reduced UM-HMC2 (*P<0.05) and completely inhibited UM-HMC1 (***P<0.001) and UM-HMC3A (**P<0.01) tumorspheres. D. Combining the results of

tumorspheres from all cell lines, we observed that the combined therapy was more efficient in reducing the number of tumorspheres in UM-HMC3A (**P<0.01), and UM-HMC1 (*P<0.05), while administration of SAHA alone was more efficient in UM-HMC2 (**P<0.01). E. IC50 of CDDP before and after sensitization with emetine (22 ηM), SAHA (1 μM), or the combination of emetine and SAHA (22 ηM of emetine + 1 μM of SAHA). Scale bar 125 μM.

Table 1. Comparison of IC_{50} of CDDP before and after the sensitizing therapies

Adherent MEC cell lines	Associated IC_{50} CDDP (μM)	Associated IC_{50} CDDP (μM) to emetine	Associated IC_{50} CDDP (μM) to SAHA	Associated IC_{50} CDDP (μM) to emetine + SAHA
UM-HMC1	16.28	1.69 (↓ 9.65)	10.32 (↓ 1.58)	3.48 (↓ 4.68)
UM-HMC2	6.36	3.91 (↓ 1.63)	8.52 (↑ 1.34)	2.86 (↓ 2.22)
UM-HMC3A	18.10	2.67 (↓ 6.79)	13.67 (↓ 1.32)	3.69 (↓ 4.91)

↓: decreased; ↑: increased.

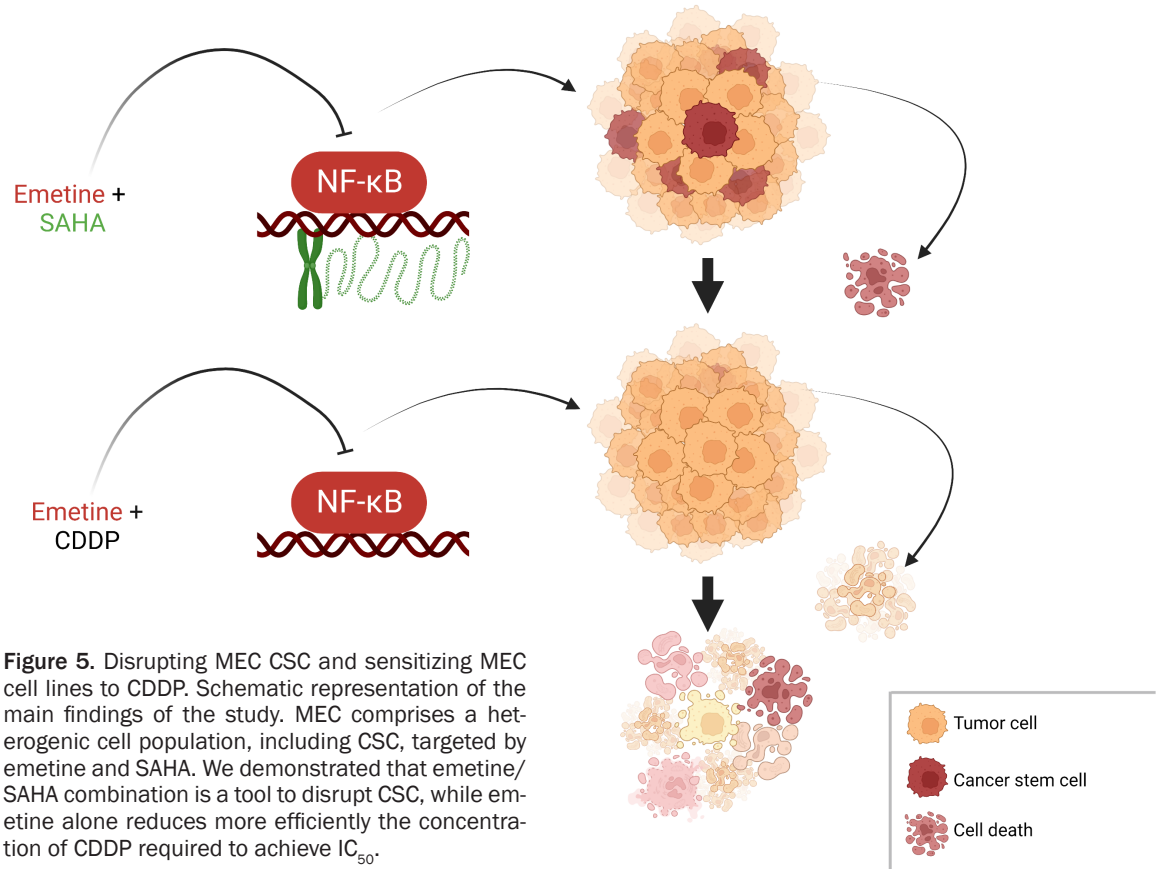

Figure 5. Disrupting MEC CSC and sensitizing MEC cell lines to CDDP. Schematic representation of the main findings of the study. MEC comprises a heterogenic cell population, including CSC, targeted by emetine and SAHA. We demonstrated that emetine/SAHA combination is a tool to disrupt CSC, while emetine alone reduces more efficiently the concentration of CDDP required to achieve IC_{50}.

effective in reducing the number of CSC. Our data also shows that despite the combined administration of emetine/SAHA is not as efficient in CDDP sensitization, it is necessary for a better disruption of CSC (**Figure 5**).

Discussion

Despite considerable investments in cancer research, drug discovery, and development, approval rates for new cancer drugs are ≤5%, half of the other therapeutic areas [30]. Here, we assessed a novel therapeutic strategy by repurposing drugs already approved by FDA. Emetine is an approved drug to treat amoebiasis; however, it also exhibits an antitumor effect [31]. Emetine is a drug purified from the ipecac root shown to inhibit IKK-β [21], a trigger for canonical activation of NFκB and signaling [32]. SAHA is a well-known HDAC inhibitor studied in various malignancies. SAHA has good oral bioavailability and tolerability, as suggested by

clinical data on hematological malignancies [16]. We provided evidence that combination therapy using both drugs can effectively disrupt CSC in MEC tumors.

Surgery is still considered the gold standard treatment for MEC [11]. Chemotherapy is often used in cases of tumor relapse or the presence of metastasis [27]. Further, drug resistance remains a significant obstacle in managing MEC tumors, resulting in tumor recurrence, disease progression, and metastasis. Emerging evidence suggests that tumor resistance may be conferred by the presence of CSC [24]. Our group has focused on the development of novel therapeutic strategies to target MEC CSC as a strategy to mitigate the gain of tumor resistance. In our previous studies, we showed that while CDDP induces the accumulation of MEC CSC in vitro, targeting the NFκB signaling and interfering with the chromatin organization of tumors using HDAC inhibitors constitutes promising strategies to manage MEC [15, 21, 22, 24]. Here, we investigated the effects of emetine and SAHA, either administered isolated or in combination, over the behavior of MEC CSC and as sensitizing agents to CDDP. Our results suggest that the association of emetine and SAHA seems to be the best pharmacological strategy to disrupt MEC CSC. Meanwhile, emetine functions as an effective sensitizer for CDDP targeting MEC cells.

The delivery of chemotherapy to cancer patients is often disrupted by its side effects. This issue is particularly real for platinum-based therapies that illicit several undesired side effects resulting in either a dose reduction or the interruption of treatment [33]. A new class of drugs known as sensitizing agents are being explored to address the issue of drug toxicity. Our group is interested in identifying and validating drugs with sensitizing properties that can support the chemotherapeutic agent's current effectiveness while being administered at lower doses. We are particularly interested in identifying sensitizing agents capable of targeting CSC and non-CSC tumor cells when administered in combination with CDDP. We have successfully identified several of these agents for head and neck solid tumors, including squamous cell carcinomas, adenoid cystic carcinomas, and mucoepidermoid carcinomas [21, 22, 34-37]. However, this is the first time we have explored the combination therapy of two sensitizing agents administered at low doses combined with CDDP. Here we confirmed the effects of emetine on MEC tumors previously published by our group. We further demonstrate that a single administration of low concentrated emetine was sufficient to drop 12 times the required dose of CDDP to achieve IC_{50} values on MEC cells. The perspective repurposes of a well-known and well-tolerated drug-like emetine to sensitize CDDP is an exciting finding with promising clinical translation.

Moreover, we have previously published that pharmacological inhibition of histone deacetylase interferes with the regulation of multiple oncogenic processes, including CSC maintenance [22]. Here, we showed that pharmacological decondensation of the chromatin (histone acetylation) resulted in profound impairment of CSC maintenance. We also demonstrate that by combining SAHA and emetine, we further disrupted the population of CSC in one of our cell lines beyond what was achieved with the administration of SAHA alone.

Together, we suggest the crucial role of NFκB and HDAC inhibitors on MEC and suggest that emetine/SAHA combination, a candidate for drug repurposing, could constitute a novel therapy targeting CSC in MEC and as an efficient strategy to sensitize MEC tumors to CDDP.

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Disclosure of conflict of interest

None.

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