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Unlocking the chromatin of adenoid cystic carcinomas using HDAC inhibitors sensitize cancer stem cells to cisplatin and induces tumor senescence

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Abstract

Adenoid cystic carcinoma (ACC) is an uncommon malignancy of the salivary glands that is characterized by local recurrence and distant metastasis due to its resistance to conventional therapy. Platinum-based therapies have been extensively explored as a treatment for ACC, but they show little effectiveness. Studies have shown that a specific group of tumor cells, harboring characteristics of cancer stem cells (CSCs), are involved in chemoresistance of myeloid leukemias, breast, colorectal and pancreatic carcinomas. Therapeutic strategies that target CSCs improve the survival of patients by decreasing the rates of tumor relapse, and epigenetic drugs, such as histone deacetylase inhibitors (HDACi), have shown promising results in targeting CSCs. In this study, we investigated the effect of the HDACi Suberoylanilide hydroxamic acid (Vorinostat), and cisplatin, alone or in combination, on CSCs and non-CSCs from ACC. We used CSCs as a biological marker for tumor resistance to therapy in patient-derived xenograft (PDX) samples and ACC primary cells. We found that cisplatin reduced tumor viability, but enriched the population of CSCs. Systemic administration of Vorinostat reduced the number of detectable CSCs in vivo and in vitro,

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Conflict of interest statement

The authors declare that they have no conflict of interest.

and a low dose of Vorinostat decreased tumor cell viability. However, the combination of Vorinostat and cisplatin was extremely effective in depleting CSCs and reducing tumor viability in all ACC primary cells by activating cellular senescence. These observations suggest that HDACi and intercalating agents act more efficiently in combination to destroy tumor cells and their stem cells.

Keywords

Adenoid cystic carcinoma; Cancer stem cells; Chemoresistance; Vorinostat; Cisplatin

1. Introduction

Adenoid cystic carcinoma (ACC) are rare and highly aggressive neoplasms of salivary glands and the breast. ACC of the salivary gland represents approximately 22% of all salivary gland malignancies; these tumors are slow-growing and have a high potential for local recurrence (Hitre et al., 2013; Duberge et al., 2012; Hotte et al., 2005). The treatment of choice for ACC is surgical resection followed by postoperative radiotherapy. While the effectiveness of chemotherapy in treating ACC has been extensively studied, outcomes remain poor (de Haan et al., 1992; Licitra et al., 1991; Lagha et al., 2012). Following treatment, 29% to 40% of patients with ACC experience a disease-free survival of 10 years (Fordice et al., 1999). The majority of deaths from salivary ACC are due to local recurrence and distant metastasis and are associated with resistance to conventional therapy. Due to the low incidence of ACC in the general population, the molecular events underlying tumor progression, metastasis, and resistance to treatment are underexplored; as such, this presents a major roadblock for understanding the biology of ACC and for expanding ACC therapy beyond the limited number of effective treatments (Liu et al., 2015a).

Chemotherapy is a standard treatment for patients with advanced disease, including nonresectable, recurrent, or metastatic tumors. Due to chemoresistance, the majority of patients die within three years (Papaspyrou et al., 2011). Indeed, cisplatin and other platinum-based therapies do not present clear benefits to patient’s survival rates (Adelstein et al., 2012). Intrinsic or acquired resistance limits the efficacy of the cisplatin, as evidenced by 30% of patients with primary disease, and N70% of relapsed patients, showing resistance to cisplatin (Murdoch, 2007). Several mechanisms contribute to cisplatin chemoresistance, including alterations in molecular signaling underlying drug influx, drug metabolism, apoptosis, cell survival regulators and DNA repair (Shen et al., 2012; Huang et al., 2014). Recent studies show that cancer stem cells (CSCs) are involved in tumor development, chemoresistance and radioresistance of different tumors including head and neck cancers (Le et al., 2014).

CSCs, a small fraction of the tumor population, are responsible for tumor initiation and growth. CSCs are quiescent, self-renew, and are inherently resistant to chemotherapy and radiotherapy thereby contributing to treatment failure (Dragu et al., 2015; Pozzi et al., 2015; Vidal et al., 2014; Qiu et al., 2015). Conventional chemotherapy targets proliferating cancer cells but fails to target dormant or slow cycling cancer cells. Initially, chemotherapy and

radiotherapy destroy the majority of cancer cells, leading to reduced tumor volume. However, the CSCs that evade treatment eventually undergo cell division resulting in disease relapse (Liu et al., 2015b). Therapeutic strategies that target CSCs have the potential to improve overall survival by decreasing the incidence of tumor relapse. One strategy to reduce CSCs is via global acetylation of histones (Shukla and Meeran, 2014; Wagner et al., 2016; Guimaraes et al., 2016).

Vorinostat (Suberoyanilide hydroxamic acid, SAHA) is an efficient pan-class I and class II Histone Deacetylase inhibitor approved in 2006 for the treatment of lymphoma (Marks, 2007; Park et al., 2016). Recent studies have demonstrated that Vorinostat has significant anti-tumor activity for many solid tumors (Khan and La Thangue, 2012; Kumar et al., 2015). Vorinostat is currently been used as a single agent in phase II clinical trial for adenoid cystic carcinoma but it is showing poor response rate (Chae et al., 2015). However, Vorinostat has been efficiently used in combination with other chemotherapeutic agents in several solid tumors (Park et al., 2016; Gumbarewicz et al., 2016; Pan et al., 2016; Pettke et al., 2016; Yoo et al., 2016; Pili et al., 2017) and as an efficient sensitizer for salivary gland tumor (Guimaraes et al., 2016).

In this study, we investigated the efficacy of combined therapy with Vorinostat (an FDA-approved histone deacetylation inhibitor - HDACi) and cisplatin in reducing CSCs and tumor cells from ACC primary cells. We used the population of CSCs as a biological marker for tumor resistance to therapy. Surprisingly, cisplatin enhanced the accumulation of CSCs in ACC patient-derived xenografts (PDX) and primary cell lines. In contrast, Vorinostat reduced the number of CSCs in PDX samples and from primary tumor cells 8 h after treatment. However, prolonged administration of Vorinostat caused an increase in CSCs in ACC primary cells despite an overall reduction in tumor cell viability. We found that the combination of Vorinostat and cisplatin efficiently depleted CSCs and reduced primary tumor cell viability via cellular senescence. Collectively, our findings suggest that combined therapy using Vorinostat and cisplatin may be a viable strategy to prevent the development of tumor resistance in ACC tumor cells.

2. Materials and methods

2.1. Patient-derived xenograft

PDX was generated by South Texas Accelerated Research Therapeutics (START) in collaboration with the Adenoid Cystic Carcinoma Research Foundation. Briefly, mice received 4 cycles of Vorinostat (100 mg/kg) or 4 cycles of cisplatin (1.5 mg/kg) ($n = 3$). Each cycle corresponded to 5 days of Vorinostat or cisplatin administration and 2 days off treatment. Mice were sacrificed after 2 months, and tumors were collected. Paraffin-embedded tissues were submitted to the Laboratory of Epithelial Biology at the University of Michigan for processing; an immunofluorescence assay was used to detect histone H3 acetylation and ALDH1 expression.

2.2. Immunohistochemistry/immunofluorescence

For immunohistochemical staining, the slides were incubated overnight with anti-Acetyl-Histone H3 (Cell Signaling, Danvers, MA, USA) and then for 60 min at room temperature (RT) with the anti-rabbit secondary antibody (Vector Laboratories, Burlingame, CA, USA). The vector DAB detection system was used following incubation with diaminoben-zidine tetrahydrochloride (DAB, Sigma-Aldrich Corp., St. Louis, MO, USA) and staining with Mayer's hematoxylin. Slides from PDX tissues were incubated overnight with anti-ALDH1 (BD Biosciences, San Jose, CA, USA) and anti-Acetyl-Histone H3 (Cell Signaling, Danvers, MA, USA). Slides were then incubated for 60 min at RT with FITC or TRITC-conjugated secondary antibody and stained with Hoechst 33,342 for visualization of DNA content.

2.3. Primary cells

Adenoid Cystic Carcinoma cells lines UM-HACC1, UM-HACC2A, and UM-HACC-6 were initially described by Warner et al. (2013). Cells were maintained in a 5% CO_2 humidified incubator at 37 °C and cultured in RPMI 1640 (Thermo Scientific, Waltham, MA, USA) supplemented with 10% Fetal Bovine Serum (Thermo Scientific), 1% antibiotic (Invitrogen, Carlsbad, CA, USA), 1% $_L$-glutamine (Invitrogen), 20 ng/ml epidermal growth factor (Sigma–Aldrich, St. Louis, MO, USA), 400 ng/ml hydrocortisone (Sigma–Aldrich), and 5 μg/ml insulin (Sigma–Aldrich). Cells were treated with Vorinostat (SAHA) (Cayman Chemical Company Ann Arbor, MI, USA) and cisplatin (Sigma–Aldrich, St. Louis, MO, USA).

2.4. IC50 determination

Cell proliferation was determined using the CellTiter 96TM Aqueous non-radioactive cell proliferation kit (Promega) according to manufacturer instructions. Approximately 5000 cells were plated into 96-well plates in quintuplicate. Cells were treated with control (vehicle), Vorinostat (0.1, 0.25, 0.5, 1.0, 2.5, 5.0 and 10.0 μM), or cisplatin (2.5,5.0, 7.5, 10.0, 12.5, 15.0, 17.5 and 20.0 μg/ml) for 24 h. Cells were incubated with MTS at 37 °C for 4 h, and the results were assessed by absorbance (Bio-Tek EL-311, Bio-Tek Instruments) at 490 nm.

2.5. Tumorsphere formation assay

The sphere formation assay was performed as previously described (Almeida et al., 2016). Briefly, cells were plated on ultra-low attachment 6-well plates (Corning, New York, USA) and allowed to grow for 5 days. Spheres growing in suspension were collected at day 5 and transferred to a glass slide by centrifugation (4 °C) at 1500 rpm for 10 min using a cytospin system. Spheres were stained with hematoxylin and eosin and mounted in aqueous mounting media (Sigma).

2.6. Immunofluorescence

Cells were placed on glass coverslips in 12-well plates and fixed with absolute methanol at −20 °C for 5 min. Cells were blocked with 0.5% (v/v) Triton X-100 in PBS and 3% (w/v) bovine serum albumin (BSA) and incubated with anti-Acetyl-Histone H3 (Lys9) (Cell Signaling, Danvers, MA, USA), anti-CK7 (BD Biosciences, San Jose, CA, USA), and anti-

CK14 (Cell Signaling, Danvers, MA, USA) as indicated. Cells were then washed three times and incubated with FITC or TRITC-conjugated secondary antibody for 60 min at RT and then stained with Hoechst 33,342 for visualization of DNA content. Images were taken using a QImaging ExiAqua monochrome digital camera attached to a Nikon Eclipse 80i Microscope (Nikon, Melville, NY) and visualized with QCapturePro software.

2.7. Flow cytometry

ACC cancer stem cell-like cells were identified by aldehyde dehydrogenase (ALDH) activity combined with CD44-APC expression (BD Biosciences, clone G44–26) using flow cytometry. The Aldefluor kit (StemCell Technologies, Durham, NC, USA) was used according to the manufacturer's instructions to identify cells with high ALDH enzymatic activity. Cells with or without pretreatment, as indicated in individual experiments, were suspended with activated Aldefluor substrate (BODIPY amino acetate) or negative control (dimethylamino benzaldehyde, a specific ALDH inhibitor) for 45 min at 37 °C. All samples were analyzed using a FACS Canto IV (BD Biosciences) at the University of Michigan Flow Cytometry Core.

2.8. β-Galactosidase assay

Tumor cell lines HACC2A and HACC6 were cultured under normal culture conditions followed by treatment with Vorinostat, cisplatin, or their combination. An SA-β-Gal detection kit (Cell Signaling, Senescence β-Galactosidase Staining Kit #9860) was used to detect cells undergoing senescence. In cells treated with Vorinostat and cisplatin, a blue color was visualized under a microscope (200× total magnification) for 16 h.

2.9. Statistical analysis

Statistical analysis was performed using GraphPad Prism (GraphPad Software, San Diego, CA). Statistical analysis of total cell number was performed using unpaired Student's *t*-test. The sphere adhesion assay was assessed by two-way analysis of variance (ANOVA) followed by the Bonferroni posttest. CSC clonogenic potential was analyzed using one-way ANOVA followed by Tukey's multiple comparison tests. Asterisks denote statistical significance (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; and NS $p > 0.05$).

3. Results

3.1. The presence of cancer stem cells in ACC patient-derived xenograft (PDX) and primary cell culture of ACC

Epigenetic mechanisms control chromatin modifications during development and in response to environmental and hormonal stimuli. Histone acetylation is one of the most frequent epigenetic alterations that affects chromatin stability. Histone charge modifications influence the interaction between DNA and histone core proteins by altering nucleosome contacts and exposing binding sites for transcription (Kimura, 2013; Messier et al., 2016). Epigenetic modifications upregulate various tumorigenic pathways that are associated with poor clinical outcomes in patients (Jones and Baylin, 2002).

We examined the acetylation status of histone H3 in PDX tumors receiving cisplatin or Vorinostat and a possible correlation with levels of Aldehyde dehydrogenases (ALDH), an enzyme highly expressed in stem cells (Moreb, 2008). PDX tumor samples were graciously provided by South Texas Accelerated Research Therapeutics (START) in collaboration with the Adenoid Cystic Carcinoma Research Foundation.

Cisplatin alone did not alter the acetylation of histone H3 (Fig. 1A and B, ns $p > 0.05$). However, compared to vehicle, cisplatin-induced the accumulation of ALDH1 positive cells (Fig. 1C, $^{**}p < 0.01$). As expected, Vorinostat alone caused an abrupt increase in acetylated histone H3 (Fig. 1A and B, $^{***}p < 0.001$) but only marginally reduced $ALDH^+$ cells in PDX (Fig. 1A and C, ns $p > 0.05$). Interestingly, Cisplatin and Vorinostat presented opposite profiles of histone acetylation and $ALDH^+$ cells accumulation in PDX models (Fig. 1B and C, $^{***}p < 0.001$). These findings suggest that cisplatin triggers the accumulation of CSCs in ACC, similar to what we found in mucoepidermoid carcinomas (Guimaraes et al., 2016). Similarly, recent studies have shown that cisplatin induces the accumulation of CSCs in HNSCC xenograft mice and contributes to tumor relapse (Nor et al., 2014; Adams et al., 2013) and the combination of Cisplatin and Vorinostat may be a strategy to ACC treatment.

Low passage primary human ACC cells have been isolated and successfully cultured (Acasigua et al., 2015; Warner et al., 2016). These cells are positive for cytokeratin 7 and 14, confirming they originate from the salivary gland (CK7) and epithelial cells (CK14) (Fig. 1D) (Tsubochi et al., 2000).

A small population of cancer cells with the characteristics of stem cells can survive, proliferate and form sphere-shaped colonies when cultured in anchorage-independent conditions (Almeida et al., 2016; Dontu et al., 2003). We examined whether four primary ACC cells lines retained a subpopulation of CSCs using the sphere-forming assays as a method to enrich CSCs. The four ACC cell lines generated approximately 20 colonies of tumorspheres after 5 days of culture in ultra-low adhesion conditions (Fig. 1E), suggesting they retain their CSCs. Therefore, primary ACC cell cultures can serve as an important tool for identifying strategies that target CSCs.

3.2. Low doses of Vorinostat control the population of CSCs and reduce tumor viability of ACC

HDACi is a drug that induces global chromatin decondensation by acetylating histones. Histone acetylation often leads to cell cycle arrest, enhanced tumor susceptibility to conventional cytotoxic treatment, and selective toxicity in transformed cells (Frank et al., 2010; Duvic and Vu, 2007). Vorinostat targets histone deacetylase classes I and II, inhibiting tumor cell proliferation and mitosis (Kelly et al., 2005; Sakajiri et al., 2005; Marks et al., 2004; Secrist et al., 2003) and inducing cell cycle arrest, differentiation or apoptosis (Richon et al., 2000; Warrener et al., 2003). We and others have shown that Vorinostat is an efficient sensitizing agent to chemotherapy in several types of cancers, including mucoepidermoid carcinomas (Guimaraes et al., 2016), glioblastomas (Barazzuol et al., 2015), and refractory cutaneous T-cell lymphomas (West and Johnstone, 2014). HDACi also depletes CSCs from squamous cell carcinoma and mucoepidermoid carcinoma of the oral cavity (Guimaraes et al., 2016; Giudice et al., 2013). Although the mechanism involved in HDACi-induced

disruption of CSCs is poorly understood, it is clear that histone acetylation induces quiescent CSCs to differentiate into more mature tumor cells (Yoshida et al., 1995; Struhl, 1998; Melcer et al., 2012).

We examined the effects of Vorinostat in ACC tumor cells and their CSCs. We first determined the half-maximal inhibitory concentration (IC50) that affects tumor cells growing in monolayer and tumorspheres (Fig. 2A). Note that the IC50 is one-fold lower for cells in tumorspheres compared to cells in monolayer. Further, HACC1 was the most sensitive to Vorinostat when grown in monolayer and ACC52 was the most sensitive when grown in suspension (spheres) (Fig. 2A).

The primary tumor cells showed differing doubling times. We used HACC2A and HACC6 cells, which had similar doubling times, for the remaining assays. Our strategy was to sensitize ACC to chemotherapy by targeting CSCs using HDACi. To this end, we used low doses of Vorinostat, as determined by the IC50 in tumorspheres, to induce histone acetylation at relevant levels for CSCs. Interestingly, Vorinostat reduced CSCs in HACC2A cells for up to 48 h, as determined by CD44 expression and ALDH enzymatic activity ($ALDH^{bright}$) (Fig. 2B - HACC2A). HACC6 CSCs were initially resistant to Vorinostat, an effect that was overcome after 5 days of treatment (Fig. 2B - HACC6). Unexpectedly, low doses of Vorinostat also significantly reduced the viability of tumor cells cultured in monolayer (Fig. 2C, $^{***}p < 0.001$ after 5 days of treatment).

3.3. Cisplatin as a single agent triggers the accumulation of CSCs in adenoid cystic carcinoma

Conventional anticancer therapeutics, such as cisplatin, rely on proliferating cells to generate DNA adducts and induce cell death (Wozniak and Blasiak, 2002). We first determined the IC50 of cisplatin in adherent tumor cells and in suspension as tumorspheres (Fig. 3A) and then examined the effects of cisplatin on CSCs in HACC2A and HACC6 tumor cells. Cells were treated with cisplatin for five days using the concentrations obtained from the IC50 for adherent tumor cells, and CSCs were identified ($ALDH^{bright}$ $CD44^{+}$) using flow cytometry. Cisplatin-induced accumulation of CSCs in ACC tumor cells as early as 8 h after treatment (Fig. 3B). Previous studies have shown that cisplatin causes the accumulation of CSCs in mucoepidermoid carcinomas from the salivary glands (Guimaraes et al., 2016) and in head and neck squamous carcinomas (Nor et al., 2014). As expected, cisplatin efficiently reduced tumor viability beginning 8 h after administration in HACC2A and 24 h after administration in HACC6 (Fig. 3C, $^{***}p < 0.001$ and $^{**}p < 0.01$). These findings also suggest that ACC may develop resistance to intercalating agents by inducing the accumulation of CSCs.

3.4. Combined administration of Vorinostat and cisplatin disrupts CSCs and sensitizes tumor cells to chemotherapy

We recently showed that head and neck squamous cell carcinomas resistant to chemotherapy are characterized by increased chromatin compaction that is driven by histone deacetylation (Almeida et al., 2014). We also observed that compacted chromatin prevents the influx of DNA damage repair (DDR) molecules to the nucleus, prohibiting the activation of apoptosis. However, HDACi reverses this process and sensitizes tumor cells to chemotherapy (Almeida

et al., 2014). Furthermore, we have observed that tumorspheres cannot retain their organization for >5 h following administration of HDACi, suggesting that maintenance of CSCs is incompatible with chromatin acetylation (Giudice et al., 2013). In this study, we examined the therapeutic efficacy of combined administration of low doses of Vorinostat (tumorsphere-relevant) with cisplatin. We first assessed whether this combination would reduce CSCs in HACC2A and HACC6 cells (Fig. 4A). Vorinostat enhanced the efficacy of cisplatin, resulting in complete depletion of CSCs in HACC2A (***$p < 0.001$) and a 79.73% reduction of CSCs (from a total of 100% of CSC in the untreated samples to a 20.2% of CSC after 5 days of treatment) (Fig. 4A and B, **$p < 0.01$). Combined therapy was more effective than Vorinostat alone in reducing CSCs (Fig. 4B).

We next found that the combination of Vorinostat and cisplatin significantly reduced viability in both cell lines after 5 days of treatment (Fig. 4C, ***$p < 0.001$). Further analysis revealed that combined therapy reduced viability by 73.5% of HACC2A and by 77.5%of HACC6 tumor cells. These findings suggest a superior efficiency of combined Vorinostat and cisplatin therapy compared to single agent treatment (Fig. 4D, ***$p < 0.001$).

Together, these results suggest that targeted disruption of CSCs using HDACi is an effective strategy to sensitize ACC tumor cells.

3.5. Combined administration of Vorinostat and cisplatin in ACC primary cells boosts activation of cellular senescence

Cellular senescence causes irreversible cell cycle arrest in which cells remain metabolically active but with a limited lifespan (Kuilman et al., 2010; Bartkova et al., 2006). Senescence occurs through alterations in the p53/p21WAF1 and p16INK4A/pRB pathways and changes in chromatin organization, resulting in increased cell volume (Deruy et al., 2014). We have observed that exposure of tumor cells to HDACi causes senescence-like morphological changes in cells (Newbold et al., 2016). We examined whether Vorinostat and cisplatin combination therapy drive ACC tumor cells to senescence. Cellular senescence can be detected by γ-H2AX foci formation, the accumulation of senescence-associated β-Galactosidase, and the accumulation of $p16^{ink4}$. Cisplatin alone induced the accumulation of γ-H2AX foci due to its DNA intercalating effects (Fig. 5A, ***$p < 0.001$). Vorinostat alone did not cause significant accumulation of γ-H2AX foci in any of the ACC cells (Fig. 5A, ns $p > 0.05$). Combined therapy also induced the accumulation of γ-H2AX foci in both ACC cell lines (Fig. 5A, ***$p < 0.001$). Using another marker of senescence, we observed a modest increase in SA-β-Gal accumulation in response to cisplatin but not Vorinostat (Fig. 5B-graphics-CDDP). Interestingly, combined therapy led to significant accumulation of SA-β- Gal in both ACC cell lines beyond the levels achieved with cisplatin alone (Fig. 5B, ***$p < 0.001$ for both cell lines). We confirmed our observations using immunohistological detection of $p16^{ink4}$. Similar to SA-β-Gal, $p16^{ink4}$ accumulated in tumor cells in response to combination treatment (Fig. 5C, ***$p < 0.001$). Although not significant, there was also a small accumulation of $p16^{ink4}$ in cells treated with cisplatin alone. Collectively, our results suggest that Vorinostat sensitizes tumor cells to cisplatin through a mechanism that combines a reduction in CSCs with activation of cellular senescence. Dual administration of Vorinostat and cisplatin may constitute a feasible therapeutic strategy to treat ACC patients.

4. Discussion

ACC is one of the most common salivary gland cancers, second only to mucoepidermoid carcinoma. Almost one-quarter of all patients diagnosed with malignant salivary gland tumors have ACC, which originates from the intercalated duct of all major and minor salivary glands in the oral cavity. The histological features of ACC vary from a solid cellular growth to tubular or cribriform patterns that are comprised of epithelial and myoepithelial cells. The incidence of ACC is higher during the 5th and 6th decades of life, and clinical progression is considerably slow. Such indolent growth is reflected in the high survival rates within the first 5 years of diagnosis (70–90%), particularly when compared to other malignancies. However, the long-term prognosis of patients with ACC is poor, with a survival rate of 35–40% after 15 years and 10% after 20 years. Moreover, the therapies that target ACC are not standardized (Chae et al., 2015) and are often ineffective in treating advanced or metastatic disease (Dillon et al., 2016). There have been few advances in understanding the biology of ACC because disease incidence is low, resulting in a small number of samples available for use in research. Furthermore, tumor cell lines are difficult to establish due to the heterogeneous cellular composition of ACC tumors. Recent advances in patient-derived xenografts (PDX) for ACC resulted in the development of primary cultures of ACC tumors derived from PDX mice (Warner et al., 2016).

Cancer cells use epigenetic mechanisms to control tumor growth, invasion, and resistance to therapy. Novel therapeutic approaches targeting epigenetic modifications are considered the new frontier in cancer therapy. In fact, pharmacological modulation of epigenetic alterations is expected to overcome drug resistance that occurs with current treatments (Kumar et al., 2015). Therapies that sensitize solid tumors may be particularly beneficial for diseases known to be resistant to standard treatments. HDACi induces global chromatin acetylation following cellular differentiation (Newbold et al., 2016); however, the molecular mechanisms underlying HDACi efficiency as a sensitizing therapy remain poorly understood. Sensitizing therapies are especially important for tumors in which the first line of treatment is inefficient, such as ACC. Recent evidence has shown that CSCs are a subpopulation of tumor cells characterized by increased resistance to conventional therapies and by a propensity to perpetuate tumorigenesis (Frank et al., 2010). In this study, we proposed sensitizing ACC tumor cells to chemotherapy by inducing the acetylation of tumor histones with HDACi. Recent reports reveal that osteosarcoma becomes enriched with CSCs following cisplatin treatment. Similarly, ovarian cancer (Wiechert et al., 2016), mucoepidermoid carcinomas (Guimaraes et al., 2016), and head and neck squamous carcinoma (Nor et al., 2014) display increased CSCs in response to chemotherapy. Therefore, accumulation of CSCs is a potential mechanism underlying tumor resistance to therapy (Yu et al., 2016). We showed that ACC tumor cells had an unexpected accumulation of CSCs following cisplatin administration (Fig. 3B). Interestingly, cisplatin monotherapy was still effective at reducing non-CSC tumor cells (Fig. 3C). The mechanism of action of cisplatin involves crosslinking DNA to generate high levels of DNA double-strand breaks (Fig. 5A). Interestingly, cisplatin also induced modest activation of cellular senescence (Fig. 5B). Other platinum-based chemotherapies, such as Oxaliplatin, induce cellular senescence, an irreversible process that blocks cell division and results in cell death (Seignez et al., 2014;

Zhao et al., 2004). Indeed, activation of senescence is a known driver of tumor suppression (Sager, 1991; Rodriguez-Brenes et al., 2015; Reddel, 2000).

We found that administration of Vorinostat to PDX mice resulted in a modest reduction in ALDH positive cells, suggesting a potential adverse effect on CSCs (Fig. 1A and C). Our initial hypothesis was that Vorinostat, like the majority of HDACi, would induce differentiation of CSCs. Indeed, Vorinostat-induced differentiation has been reported in primary sarcoma cell lines (Hrzenjak et al., 2006) and human tumor cells derived from the liver (Yamashita et al., 2003), lung (Platta et al., 2007), and breast (Newbold et al., 2016; Munster et al., 2001; Salvador et al., 2013). Also, human pluripotent and embryonic stem cells have high levels of HDAC that result in differentiation (Yang et al., 2014; Etchegaray et al., 2015; Kretsovali et al., 2012). We found that Vorinostat alone caused a reduction in CSCs in ACC cells; however, one of the cell lines showed a rebound in CSCs to pre-treatment levels after 5 days of Vorinostat (Fig. 2B - HACC2A). These findings suggest that each ACC tumor may present a different “window of opportunity” during the administration of Vorinostat. Thereby, a careful analysis on the efficacy of Vorinostat in reduce the population of CSCs should be accessed prior to therapy. Nonetheless, Vorinostat alone reduced non-CSC tumor cells in ACC cells (Fig. 2C). It is important to note that the effects of Vorinostat on non-CSC cells were achieved using very low levels the drug, established as the IC50 dose for tumorspheres (Fig. 2A - tumorspheres). Interestingly, Vorinostat did not activate DNA damage response or cellular senescence (Fig. 5).

Cisplatin and Vorinostat alone produced exciting results, but their combination was extremely efficient in disrupting CSCs and non-CSCs. After 5 days of combined therapy, CSCs were completely depleted in HACC2A cells, and a 3.9-fold reduction in CSCs occurred in HACC6 cells (Fig. 4A and B). Vorinostat and cisplatin also reduced the viability of non-CSC cells in ACC cell lines (Fig. 4C and D), as evidenced by a2.78-fold reduction in viability in HACC2A cells and a 3.45-fold reduction in viability in HACC6 cells within the first 5 days of treatment (Fig. 4D).

The benefits of Vorinostat and cisplatin were evident after an initial rise in CSCs that occurred between 8 and 24 h after treatment (Fig. 4A). The spike in CSCs may be due to activation of cellular senescence, a process that follows increased replicative stress and the accumulation of DNA damage foci. Replicative stress can be activated by several mechanisms including oncogene-induced-senescence and the administration of pharmacological agents capable of perturbing replication (Courtois-Cox et al., 2006; Wells et al., 2013; Dobbelstein and Sorensen, 2015). Cell senescence was initially described in 1961 by Hayflick and Moorhead as an irreversible process triggered by several events, including cytotoxic drugs (Kuilman et al., 2010; Hayflick and Moorhead, 1961; Ewald et al., 2010). Activation of senescence results in the accumulation of DNA double-strand breaks. Within 16 h of cisplatin and Vorinostat treatment, we observed accumulation of γ- H2AX foci, especially in tumor cells receiving combined therapy (Fig. 5A). Cells undergoing senescence began to accumulate endogenous lysosomal β-galactosidase and to express $p16^{ink4}$ after 48 h of treatment (Fig. 5B and C). It is interesting that Vorinostat alone did not result in increased DNA double strand breaks, but it did augment the accumulation of double strand breaks, β-galactosidase and $p16^{ink4}$ expression induced by cisplatin (Fig. 5A).

In summary, the combination of Vorinostat and cisplatin is effective in depleting CSCs from ACC and in reducing tumor viability. As a potential mechanism of action, Vorinostat potentiates the ability of cisplatin to induce DNA double strand breaks and activate cellular senescence. Although the precise mechanism by which Vorinostat sensitizes cells to cisplatin remains unknown, we predict that enhanced chromatin relaxation, resulting in a more effective bond between cisplatin and DNA, leads to increased crosslinking and transcription of senescence- associated genes. In sum, our findings suggest that the combination of Vorinostat and cisplatin may be a promising therapy for targeting ACC tumors.

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Abbreviations:

ACC	adenoid cystic carcinoma
ALDH1	aldehyde dehydrogenase 1
BSA	bovine serum albumin
CD44-APC	cell surface glycoprotein CD44 – allophycocyanin
CK7	cytokeratin 7
CK14	cytokeratin 14
CSC	cancer stem cells
DAB	3,3′-diaminobenzidine
DDR	DNA damage repair
FITC	fluorescein isothiocyanate
HDACi	histone deacetylase inhibitor
HNSCC	head and neck squamous cell carcinoma
MTS	dimethylthiazol sulfate
PDX	patient derivate xenograft
RPMI	Roswell Park Memorial Institute medium
RT	room temperature

SAHA	suberoylanilide hydroxamic acid
TRITC	tetramethylrhodamine
UM-HACC1	University of Michigan - human adenoid cystic carcinoma 1 cell line
UM-HACC2A	University of Michigan - human adenoid cystic carcinoma 2A cell line
UM-HACC6	University of Michigan - Human adenoid cystic carcinoma 6 cell line

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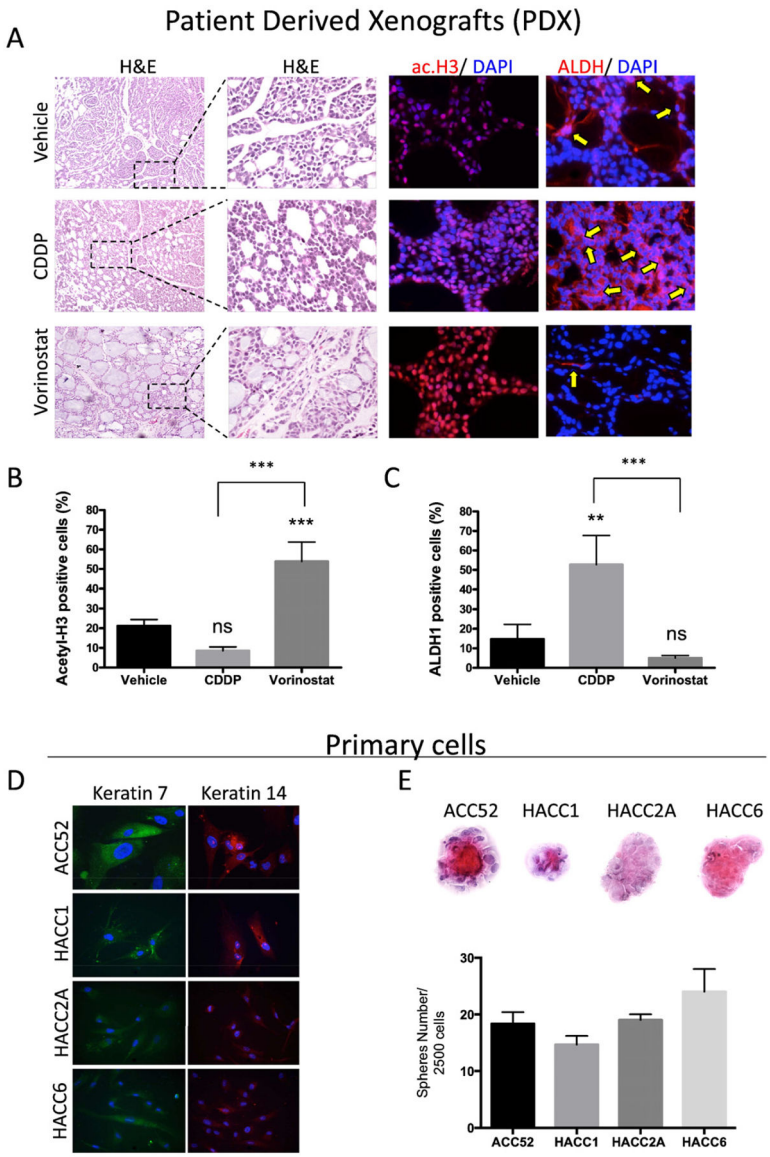

Fig. 1.

Levels of cancer stem cells in patient-derived xenograft (PDX) and primary cells of ACC. A. PDX tissue samples stained with hematoxylin and eosin (left) and identification of histone acetyl-H3 (Lys9) and ALDH (right) by immunofluorescence following administration of vehicle, cisplatin (CDDP) or Vorinostat. B. Quantification of cells positive for histone acetyl-H3 (Lys9). C. Quantification of cells positive for ALDH. Five fields were quantified using ImageJ 1.50i software (National Institutes of Health, USA); NS (non-significant, $p > 0.05$), ** ($p < 0.01$) and *** ($p < 0.001$). D. Immunofluorescence of the primary cellular culture of ACC stained with anti-Keratin 7 and anti-Keratin 14 antibodies. E. Tumorsphere assays to analyze the presence of cancer stem cells in ACC primary cells. Cells were grown in ultra-low adhesion for 5 days, the spheres were stained with hematoxylin and eosin following cytospin, and the total number of spheres were quantified. The assay was performed in triplicate.

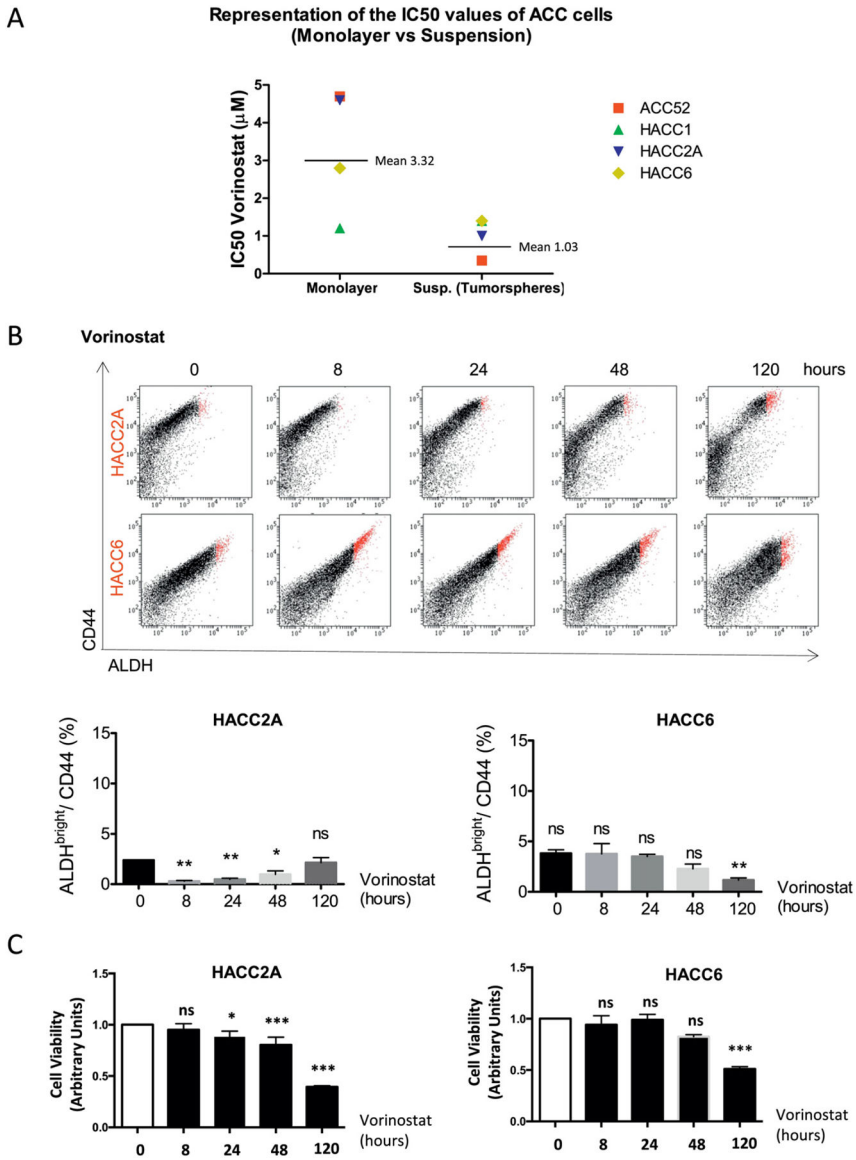

Fig. 2.

Effect of Vorinostat on cancer stem cells. A. Representative IC50 values for ACC primary cells receiving Vorinostat and cultured in monolayer or in suspension (tumorspheres). For adherent cells, the IC:50 was determined using an MTT assay; cells were seeded in 96-well plates in quintuplicate and treated with Vorinostat (0.1, 0.25, 0.5, 1.0, 2.5, 5.0 and 10.0 μM) for 24 h (Mean ± SEM of 3.325 ± 0.8320). For tumorspheres, cells were grown in ultra-low adhesion for 5 days, and Vorinostat (0.1, 0.25, 0.5, 1.0, 2.5, 5.0 and 10.0 μM) was administrated for 24 h (Mean ± SEM of 1.038 ± 0.2478). Following cytospin, cells were stained with hematoxylin and eosin. The assay was performed in triplicate, and the total number of spheres was counted. Overall, tumorspheres require a significant lower dose of Vorinostat compared to same cellls growing under attchment conditions (monolayer) (*$p <$ 0.05). B. Accumulation of cancer stem cells in HACC2A and HACC6 was determined using a flow cytometry assay for ALDH and CD44 following treatment with Vorinostat for

different times. Cells were treated with the IC:50 concentration of Vorinostat identified in tumorspheres (1 μM for HACC2A and 1.4 μM for HACC6). The assay was performed in triplicate, and the percentage of $ALDH^+/CD44^+$ was plotted in the graphs. NS (non-significant, $p > 0.05$), *$p < 0.05$, **$p < 0.01$. C. MTT assay to determine the effect of Vorinostat on cell viability. ACC primary cells were treated with Vorinostat for 0, 8, 24, 48 or 120 h at the tumorsphere IC:50 concentration. Cells were seeded in quintuplicate, and the assay was performed in triplicate. NS (non-significant, $p > 0.05$), *$p < 0.05$, ***$p < 0.001$.

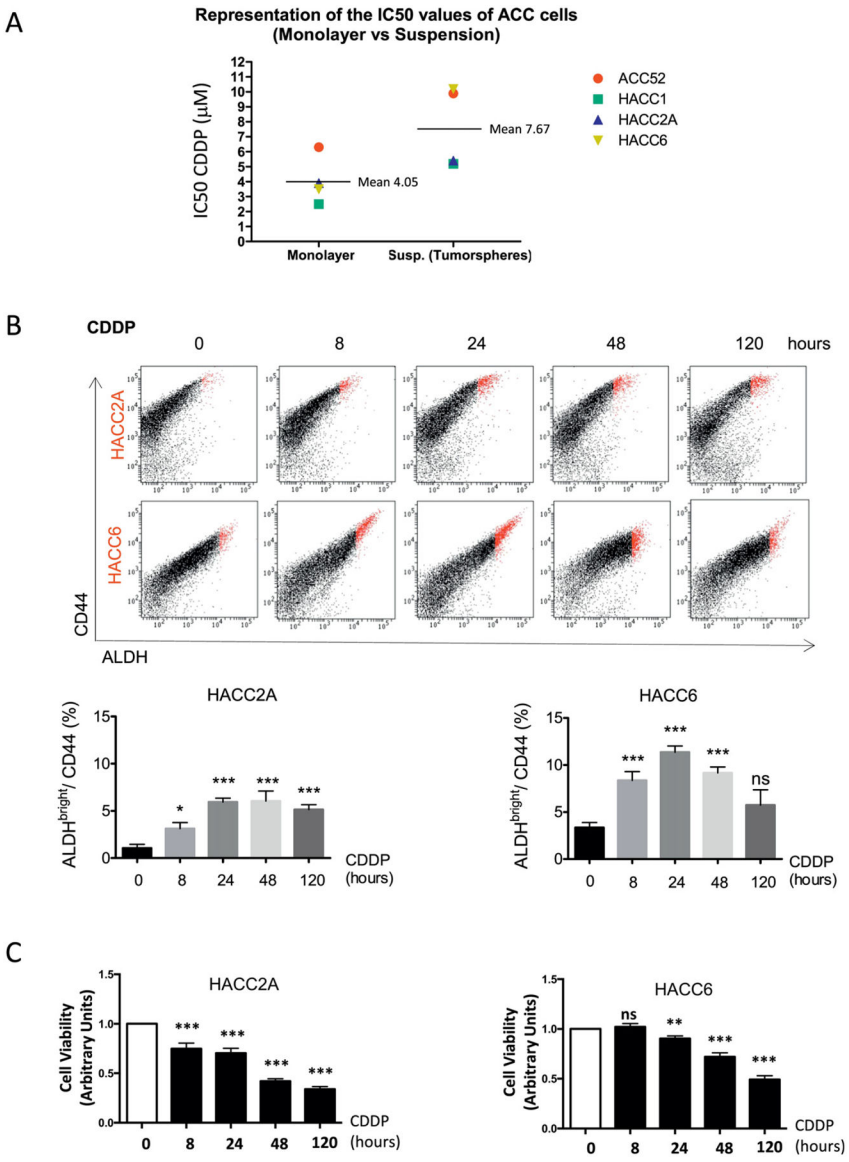

Fig. 3.

Administration of Cisplatin (CDDP) promotes accumulation of cancer stem cells. A. Determination of Cisplatin IC:50 in adherent ACC primary cells and tumorspheres. IC:50 determination for adherent cells after 24 h of cisplatin treatment (2.5, 5.0, 7.5, 10.0, 12.5, 15.0, 17.5 and 20.0 µg/ml) (Mean ± SEM of 4.050 ± 0.8057). MTT was performed in quintuplicate. For tumorspheres, cells were grown in ultra-low adhesion for 5 days, and Cisplatin (2.5, 5.0, 7.5, 10.0, 12.5, 15.0, 17.5 and 20.0 µg/ml) was administrated for 24 h (Mean ± SEM of 7.675 ± 1.373). Following cytospin, cells were stained with hematoxylin and eosin. The assay was performed in triplicate, and the total number of spheres was counted. B. Flow cytometry assays to determine the accumulation of cancer stem cells. Cells positive (%) for ALDH and CD44 were detected in HACC2A and HACC6 after different times of treatment with CDDP at IC:50. NS (non-significant, $p > 0.05$), *$p < 0.05$, ***$p < 0.001$. C. Cell viability was determined using MTT after different times of CDDP

administration. Cells were seeded in quintuplicate, and the assay was performed in triplicate. NS (non-significant, $p > 0.05$), $^{**}p < 0.01$, $^{***}p < 0.001$.

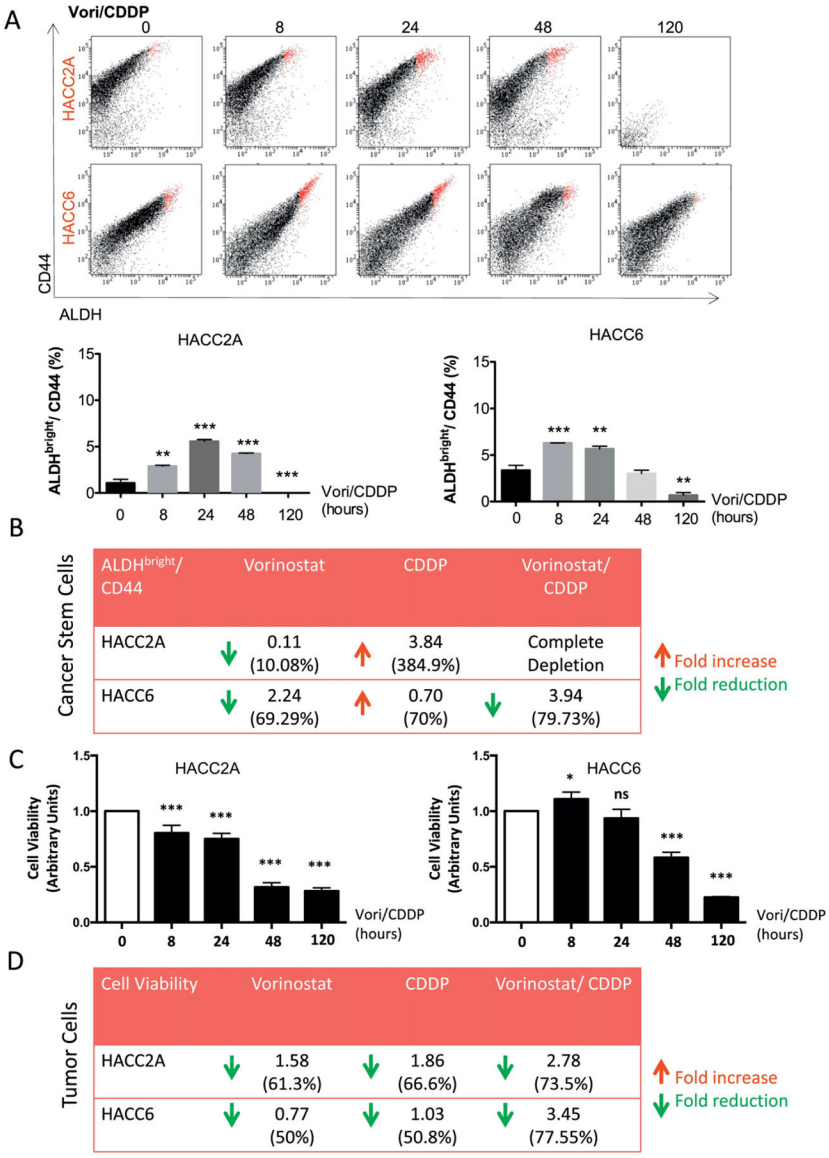

Fig. 4.

Efficacy of combined Vorinostat/Cisplatin (CDDP) treatment to reduce cancer stem cells and tumor cells. A. Accumulation of cancer stem cells was determined by flow cytometry to detect ALDH and CD44 in HACC2A and HACC6 cells after different times of treatment with Vorinostat (1 μM for HACC2A and 1.4 μM for HACC6) and CDDP (3.0 μg/ml for HACC2A and 3.5 μg/ml for HACC6). The assay was performed in triplicate, and the percentage of $ALDH^+/CD44^+$ was plotted in the graphs. NS (non-significant, $p > 0.05$), $^{**}p < 0.01$, $^{***}p < 0.001$. B. Accumulation of cancer stem cells after 120 h of treatment with Vorinostat or CDDP alone or in combination. Fold regulation and percentages were calculated using untreated cells (0 h of treatment) as the control. C. Cell viability was performed using MTT to determine the effect of combined treatment (Vorinostat/CDDP) in ACC cells. Primary cells were treated with Vorinostat/CDDP for 0, 8, 24, 48 or 120 h. Cells were seeded in quintuplicate, and the assay was performed in triplicate. NS (non-significant,

$p > 0.05$), *$p < 0.05$, ***$p < 0.001$. D. Cell viability was determined after 120 h of combined Vorinostat/CDDP treatment. Fold regulation and percentages were calculated using untreated cells (0 h of treatment) as the control.

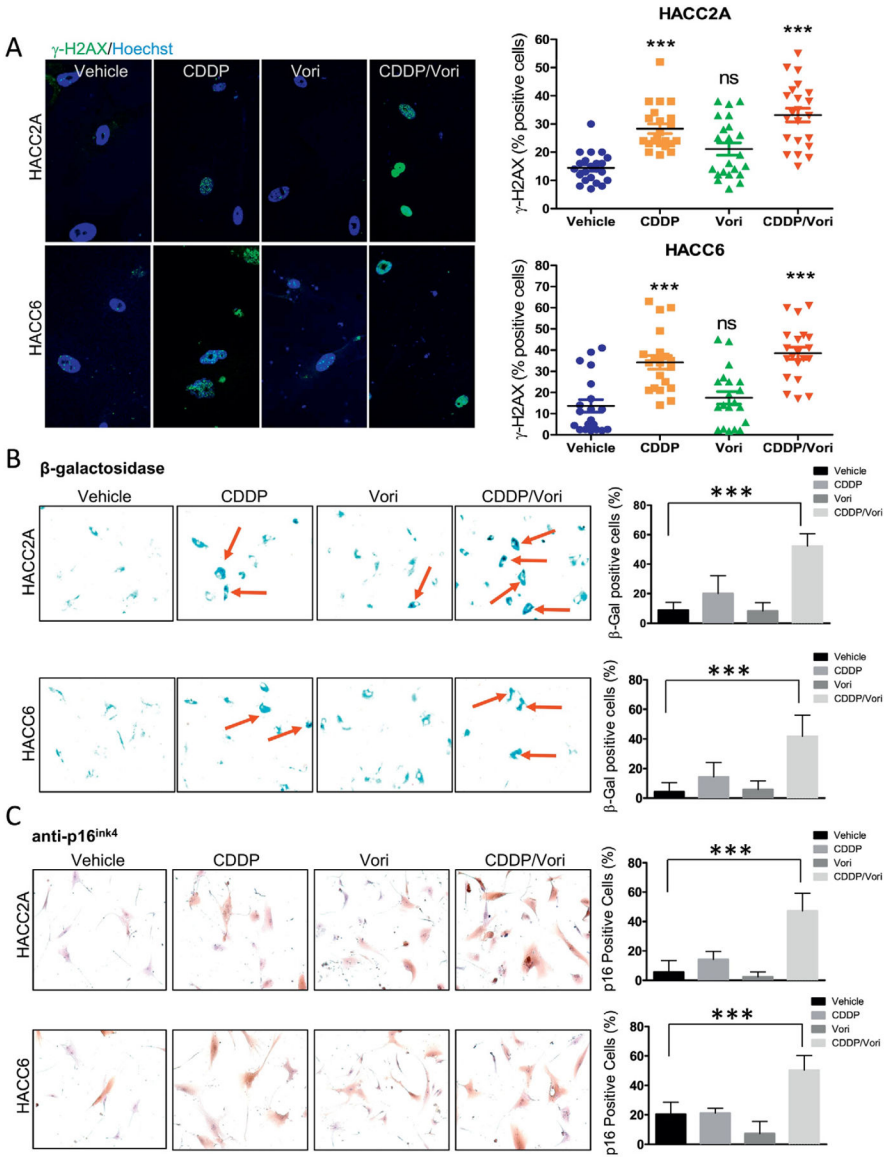

Fig. 5.

Simultaneous administration of Vorinostat and Cisplatin (CDDP) drives ACC primary cells to senescence. A. Immunofluorescence to detect γ-H2A.X staining in ACC cells after administration of Vorinostat, CDDP or both in combination (left). γ-H2A.X positive cells were quantified using ImageJ 1.50i software (National Institutes of Health, USA). Results of quantification of 5 fields are plotted in graphs (right); ns (non-significant, $p > 0.05$) and ***$p < 0.001$. B. Levels of β-galactosidase were detected using the SA-β-Gal detection kit (Cell Signaling, Senescence β-Galactosidase Staining Kit) after administration of Vorinostat, CDDP or their combination (left). Positive cells in five fields representing each treatment were quantified using ImageJ 1.50i. Results are plotted in graphs (right). ***$p < 0.001$. C. Accumulation of $p16^{INK4}$ protein was detected using immunocytochemistry after administration of Vorinostat, CDDP or their combination (left). Positive cells in five fields

representing each treatment were quantified using ImageJ 1.50i (right). Results are plotted in graphs (right). ***$p < 0.001$.