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Disruption of oncogenic pathways in mucoepidermoid carcinoma: CREB inhibitor 666.15 as a potential therapeutic agent

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ABSTRACT

Objectives: Mucoepidermoid carcinoma (MEC) is the most common malignant salivary gland tumour with around 50 % of cases carrying the CRTC1-MAML2 translocation. The CREB pathway has been associated with the transforming activity of this translocation. The aim of this study was to determine the effects of CREB inhibition on MEC cell behaviour *in vitro*. **Material and Methods:** Two translocation-positive (UM-HMC-2 and H292) and one translocation-negative (H253) MEC cell lines were treated with 666.15, a CREB inhibitor. Drug IC50 doses were determined for each cell line. Clonogenic and spheroid assays were used to assess survival, including percentage of cancer stem cells, and transwell and scratch assays evaluated invasive and migratory capacities, respectively. Immunofluorescence staining was used to determine E-cadherin expression. **Results:** CREB inhibition significantly reduced the number of surviving colonies and spheroids and delayed cell invasion in all cell lines, but this was more significant in the fusion positive, UM-HMC-2 cells. The expression of E-cadherin was significantly higher in treated UM-HMC-2 and H292 cells. **Conclusion:** CREB inhibition with 666.15 impaired key MEC oncogenic behaviours associated with metastasis and drug resistance, including cell invasion and survival.

Introduction

Salivary gland tumours (SGTs) are a group of lesions with heterogenous microscopic features and biological behaviours that comprise around 5% of neoplasms in the head and neck region. Mucoepidermoid carcinoma (MEC) is the most prevalent malignant SGT in both adults and children [1–4] but can occur as a primary tumour in sites other than salivary glands, such as the lungs, representing the most common malignant SGT in this location [5]. The 5-year survival rate of MEC varies from 57 % to 92 % [6,7], however, it is important to highlight that 5-year survival rates of high-grade tumours are significantly lower; 32 % and 26 % in the presence of nodal or distant metastasis, respectively [8]. Moreover, some cases diagnosed at an earlier clinical stage as low- or intermediate-grade can exhibit unexpected aggressive behaviour. There are currently no effective systemic therapies available for MECs with surgery remaining the treatment of choice despite the debilitating side effects which can lead to increased morbidity and poor quality of life for the patients [9]. Tumour resistance to conventional therapies has been associated with the presence of a subpopulation of cancer cells capable of self-renewal and multi-lineage differentiation, known as cancer stem cells (CSCs) [10].

A growing number of studies have shown the presence of a recurrent chromosomal translocation t(11;19)(q21;p13) in MEC, resulting in a fusion transcript comprised of exon 1 of the CRTC1 gene at 19p13 and exons 2 to 5 of the MAML2 gene. The CRTC1 gene (CREB Regulator Transcriptional Coactivator), also known as MECT1, encodes a 75KDa protein which controls the expression of specific cAMP-response element binding protein (CREB)-activated genes. The MAML2 gene encodes a 140KDa protein, a member of the master mind-like family, which acts as a co-activator for Notch receptor transcriptional activation and signalling [11,12]. The reported frequency of translocation-positive (TP) MEC patients ranges from 34 % to 81 % with the presence of the translocation being associated with improved survival rates [13,14]. A systematic review published by our group [15] highlighted controversy

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surrounding the real prognostic value of the translocation, as 3 out 10 publications included in the review found no correlation with survival rates [16–18].

Wu and colleagues [19] have shown that the transforming activity of the CRTC1-MAML2 fusion results from the unique activity generated by both the CREB-binding domain and the MAML2 TAD. CREB targets are up-regulated by the translocation at RNA and protein levels [19]. Pharmacological inhibition of CREB, therefore, could reduce the oncogenic activity of MEC cells; this has not yet been tested. 666.15 has previously been identified as a potent inhibitor of CREB with efficacious anti-cancer activity both *in vitro* and *in vivo* [20]. Additionally, a study performed by Li and colleagues [21] found that pharmacological inhibition of CREB was well-tolerated *in vivo*, indicating that such an inhibitor could provide a promising cancer therapeutic. The aim of this study was to investigate the effects of CREB inhibition, through the 666.15 compound, on MEC cell behaviour *in vitro* by evaluating cell invasion, migration, survival and the percentage of remaining CSCs.

Material and methods

Cell lines and treatment

Three MEC cell lines: UM-HMC-2 (intermediate-grade, parotid gland, TP), H253 (ATCC® HTB-41 undifferentiated high-grade, submandibular gland, TN) and H292 (ATCC® CRL-1848™, primary pulmonary, TP) were studied. UM-HMC-2, kindly provided by Dr. Jaques Eduardo Nör, was established at the University of Michigan [22] and was cultured in DMEM-High glucose (Hyclone Laboratories Inc., Logan, UT, USA), supplemented with 10 % Foetal Bovine Serum (FBS, Thermo Scientific, Waltham, MA, USA), 1 % antibiotics (Invitrogen, Carlsbad, CA, USA), 1 % L-glutamine (Invitrogen, Carlsbad, CA, USA), 20 ng/mL epidermal growth factor (PeproTech, Rocky Hill, NJ, USA), 400 ng/mL hydrocortisone (Sigma-Aldrich, St. Louis, MO, USA) and 5 μg/mL insulin (Sigma-Aldrich). H253 and H292 were acquired from the American Type Culture Collection (ATCC, Manassas, VA, USA) and were maintained according to the ATCC prescribed guidelines.

The cells were grown at 37 °C with 5 % CO_2 in a standard bench-top CO_2 incubator, monitored daily using a phase contrast microscope, and cultured to a maximum of 70 % confluence before passage, to avoid stress. All experiments were carried out prior to cells reaching a maximum of 10 passages. Cells were treated with the CREB inhibitor, 666.15 (Sigma-Aldrich) after the IC50 of the drug had been established for each cell line using a crystal violet viability assay in monolayer adhered cells [23]. For UM-HMC-2, H253 and H292 cells, the IC50 doses were 0.136 μM, 0.042 μM and 0.289 μM, respectively. All cell lines were routinely tested to confirm the absence of mycoplasma contamination.

Clonogenic assay

Cells were seeded at 5×10^2 cells/well in 6-well culture plates and after overnight incubation were treated with a single dose of the drug, using the dose previously established. Cells were allowed to grow for an additional 7 days before colonies were fixed with Methanol and Acetic Acid (7:1) and stained with 0.1 % crystal violet. The results were assessed using a conventional optical microscope and only colonies that presented > 50 cells were considered [24]. Experiments were performed in triplicate.

Spheroid assay

Before cell seeding, a grid was drawn on the back of the culture plate to enable orientation during spheroid counting. A total of 9×10^2 cells/well were seeded into 6-well Corning® Costar® Ultra-Low attachment plates (Merck KGaA, Darmstadt, Germany). On day 5, cells were treated with the CREB inhibitor, 666.15. The media was not changed, as the cells were grown in suspension and the spheroid count was performed on day 7 using a phase contrast microscopy. Experiments were performed in triplicate.

Scratch assay

MEC cells were seeded at 5×10^5 in 12-well culture dishes maintained at 37 °C and grown to confluence in normal growth media. Two hours before scratching, the cells were treated with 2 μg/mL Mitomycin C (Sigma-Aldrich) with the optimal dose of Mitomycin C having been previously calculated to ensure minimal loss of viability, with maximum inhibition of cell division. After removing the media containing Mitomycin C and washing the cells with PBS, a wound was created by scratching the cells with a 200 μL pipette tip. After 24 h, the three cell lines were treated with the CREB inhibitor, 666.15, as previously detailed. Cells were allowed to migrate into the wound area until the control group had achieved complete wound closure. Forty-eight hours after creating the wound, cell migration of H292 cells was not observed, either in the control or treated groups, and thus this cell line was excluded from this part of our study; only results from UM-HMC-2 and H253 were analysed. Photographs were taken at 0, 4, 8, 12 h for UM-HMC-2 cells and 0, 8, 16, 44, 48 h for H253 cells, using a digital camera attached to a phase contrast microscope. Experiments were performed in quadruplicate, and the same two areas of each well were analysed at each time point. The wound area was measured using the MRI Wound Healing Tool plugin (https://dev.mri.cnrs.fr/projects/imagejmacros/wiki/Wound_Healing_Tool) in ImageJ (National Institutes of Health, Bethesda, MD, USA), and a relative wound closure (%) was determined by normalising the values to the wound area at T0.

Transwell invasion assay

Cell invasion through an extracellular matrix (ECM) substitute was assessed using 8 μm, 24-well Millicell Cell Culture Inserts (Millipore, Billerica, MA, USA), coated with Matrigel (Sigma-Aldrich). MEC cells were seeded at $1x10^4$ into the upper chamber of the insert, using normal cell culture media and were allowed to adhere. After 24 h, the upper chamber media was replaced with 2 % FBS media, normal media (10 % FBS) remained in the bottom chamber. The cell density and time of invasion was optimised for each cell line and the CREB inhibitor was administered following the protocol outlined for the scratch assay. Membranes were washed with PBS and the non-invading cells removed from the upper surface of the membrane by “scrubbing” with a cotton swab. The membranes were then fixed with methanol, stained with haematoxylin and eosin (H&E), and mounted on glass slides. The experiments were performed in triplicate and three representative images per membrane were captured using a digital camera attached to a conventional optical microscope. The number of invading cells was determined using the cell counter plugin from ImageJ. Slides were blinded before photograph capture to prevent bias. The “Invasion Index” was further calculated, and represents the ratio of the percent invasion of the treated membranes versus the percent invasion of the control membranes.

Immunofluorescence

3×10^5 cells MEC cells were seeded into 6-well plates and treated with the CREB inhibitor, 666.15 as previously described. After 24 h, cells were fixed with methanol at −20 °C for 6 min, washed three times with PBS and blocked with 3 % (w/v) bovine serum albumin (BSA) and 0.5 % (v/v) Triton X-100 in PBS for 1 h. Cells were incubated overnight at 4 °C with E-Cadherin primary antibody (Cell Signaling Technology) diluted in 0.5 % (v/v) Triton X-100 and 1 % (w/v) BSA in PBS. Subsequently, cells were washed three times and incubated with Cy3 (red) fluorophore secondary antibody (Cell Signaling Technology) followed by DNA staining using Hoechst 33342 (Cell Signaling Technology). Three fields of each slide were photographed using an Olympus BX63 fluorescence

microscope. Experiments were performed in triplicate.

Statistical analysis

GraphPad Prism (GraphPad Software, San Diego, CA, USA) was used for statistical analysis. Data from clonogenic, spheroid,and transwell migration assays were compared using student *t*-test and the scratch assay using two-way ANOVA followed by Tukey's multiple comparison test. Asterisks denote statistical significance (*$p < 0.05$; ** $p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$, and NS > 0.05).

Results

We determined the effects of the CREB inhibitor 666.15 on important cell functions, associated with resistance, such as the number of surviving colonies and spheroids, and with tumour metastasis, including cell migration and invasion. The detailed results of each assay are described below.

Effect of CREB inhibition on MEC cell survival and CSC number

The clonogenic [25] and spheroid [26] assays were used to evaluate the impact of CREB inhibition on the survival of CSCs. Disparities in the number, size and morphology of colonies and tumour spheroids varied between the cell lines. The colony forming ability and number of tumour spheroids was higher in H292 cells, followed by H253 and UM-HMC-2 cells, however, UM-HMC-2 cells formed denser colonies but UM-HMC-2 tumour spheroids had a looser architecture (Figs. 1 and 2). CREB inhibition significantly reduced the number of surviving colonies and tumour spheroids compared to untreated cells in all MEC cell lines, particularly for UM-HMC-2 where surviving isolated cells were unable to form colonies following drug treatment.

Effect of CREB inhibition on MEC cell migration and invasion

Cell migration was assessed using a scratch assay and to guarantee that only cell migration, and not cell proliferation, was responsible for wound closure the cells were treated with a low dose of a cell cycle arrest antagonist (Mitomycin C). The time for the wounds to close following treatment with the CREB inhibitor was significantly delayed in both the UM-HMC-2 and H253 cell lines (Fig. 3), however, the differences were more significant for UM-HMC-2, supporting the important role of CREB activation during cell migration related to translocation positive MEC cells.

The effect of 666.15 on cell invasion was evaluated through the use of transwell membranes coated with Matrigel, which mimic the ECM components needed during tumour progression [27]. Optimal time to invasion was determined for each cell line and H253 cells invaded faster than either UM-HMC-2 or H292 cells. This baseline difference in invasive capacity might be due to distinctive proteinases produced by the different cells. Treatment with 666.15 significantly delayed cell invasion in all cell lines evaluated **(**Fig. 4**).**

Immunofluorescence

Previous reports have demonstrated that increased E-cadherin expression can induce differentiation of tumour cells and inhibit proliferation and invasion [28]. Immunofluorescence demonstrated significantly higher expression of E-cadherin in the UM-HMC-2 and H292 cells following 24 h treatment with the CREB inhibitor, however, no significant difference was observed in H253 cells (Fig. 5).

Discussion

The gold standard treatment for MEC is surgical excision, but this therapeutic approach can lead to significant morbidity impacting the patients' quality of life. Unresectable tumours, or cases of disseminated disease, lack effective systemic therapy. The available chemotherapy drugs for this carcinoma are outdated and are mainly indicated as palliative management, with low response rates and consequently poor survival rates [29]. Other carcinomas, including those arising in the breast [30] and prostate [31], are effectively treated with selective targeted inhibition of signalling pathways involved in tumour progression based on their individual molecular signatures. The same rationale needs to be followed for salivary gland cancers.

The CREB pathway is involved in the activity of the main molecular signature of MEC, the CRTC1/MAML2 translocation [19] and thus our study was focused on the role of CREB pharmacological inhibition on

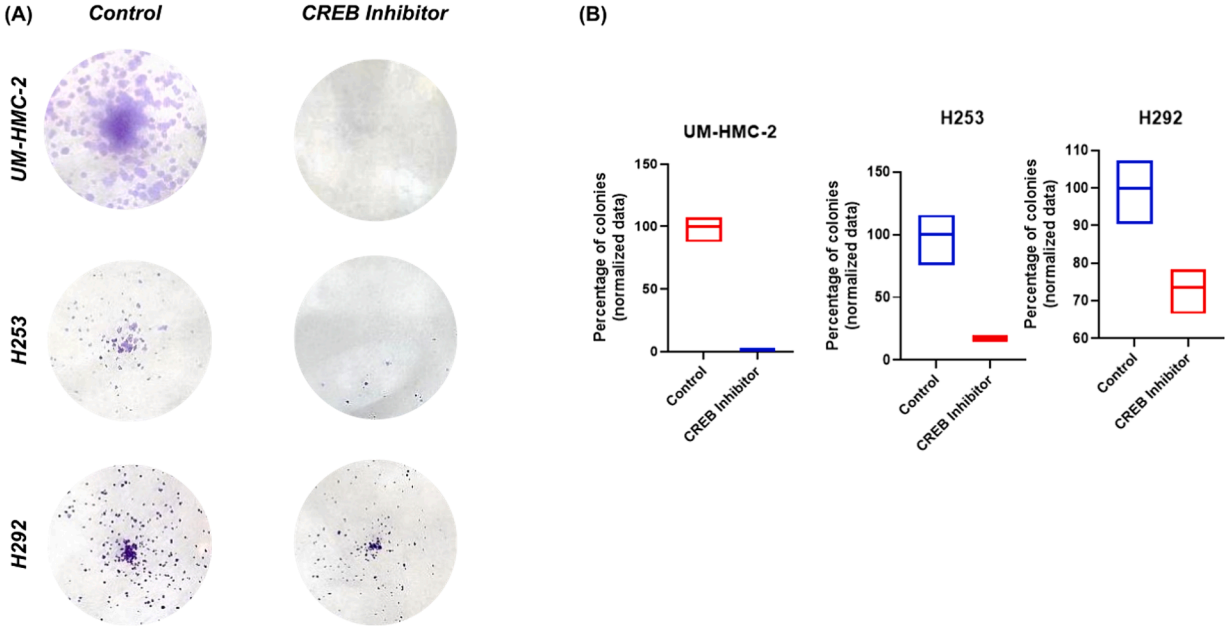

Fig. 1. (A) Comparison of stained colonies in control and CREB inhibitor 666.15 groups. The drug reduced the size and number of colonies. **(B)** Quantitative analysis revealed a significant reduction in the number of surviving colonies (more than 50 cells) in all MEC cell lines (* $p < 0.05$; ** $p < 0.01$; ****$p < 0.0001$).

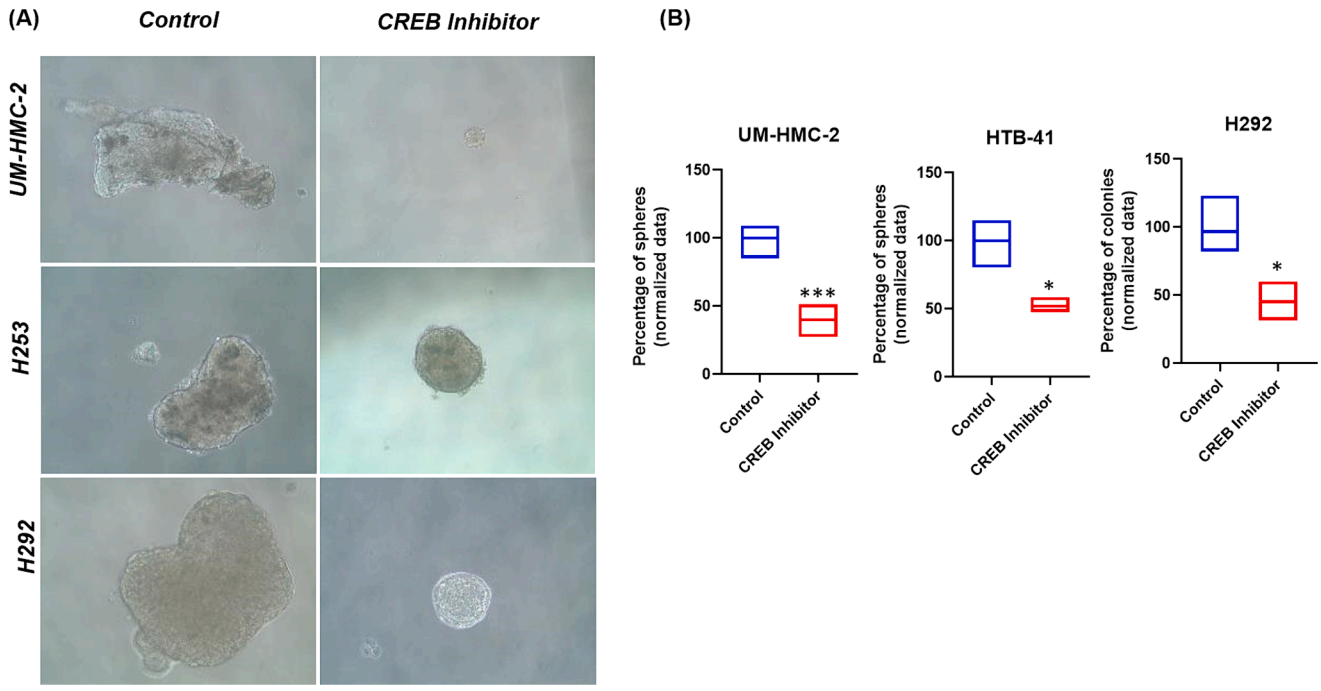

Fig. 2. (**A**) Representative images of tumour spheroid under phase contrast microscopy. Note that cells under CREB inhibitor 666.15 treatment formed spheres with lower size. (**B**) Quantitative analysis revealed a significant reduction in the number of surviving spheres in all MEC cell lines (*** < 0.001; * $p < 0.05$).

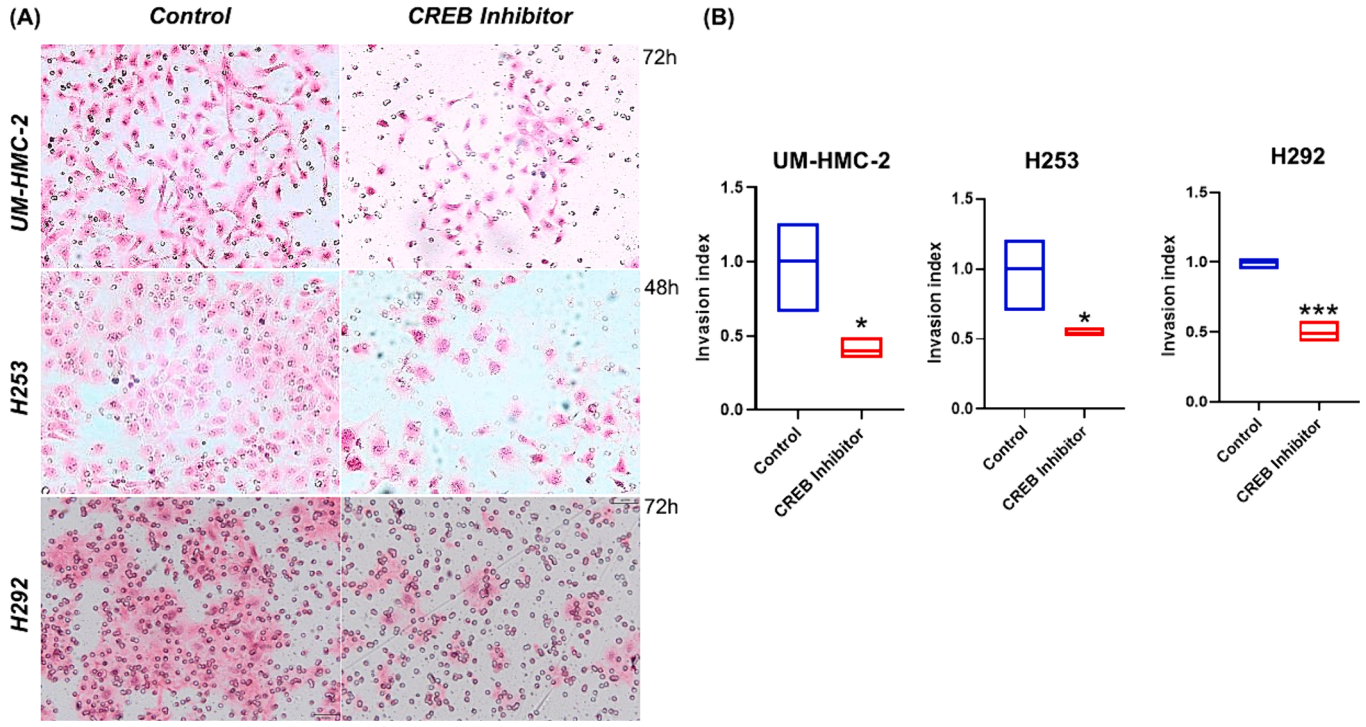

Fig. 3. **(A)** CREB inhibition delays cell migration in UM-HMC-2 cell line. The treatment with CREB inhibitor 666.15 significantly reduced cell migration in comparison with control cells with stronger effect after 8 h (*$p > 0.05$; ****$p > 0.0001$). **(B)** CREB inhibition delays cell migration in H253 cell line. The treatment with CREB inhibitor 666.15 significantly reduced cell migration in comparison with control cells in 8 h, 16 h, and 44 h time points (****$p > 0.0001$; NS – not significant).

key oncogenic events related to disease relapse and metastasis. To the best of our knowledge this is the first study to investigate the effects of CREB inhibition in a salivary gland carcinoma. Our results suggest a promising effect of the CREB inhibitor 666.15 in impairing MEC cell migration, invasion, and survival *in vitro*.

Identified initially by Montminy and Bilezikjian in 1987 [32], CREB, a 43 kDa protein, is a member of the basic leucine zipper (bZIP) transcription factors. Currently, it is recognized that CREB regulates more than 4,000 genes and its activation is involved in several cellular mechanisms, such as inflammation, immune response, and cell cycle progression [33]. Reduced CREB activation is largely associated with the onset of neurological diseases, including schizophrenia and Alzheimer's

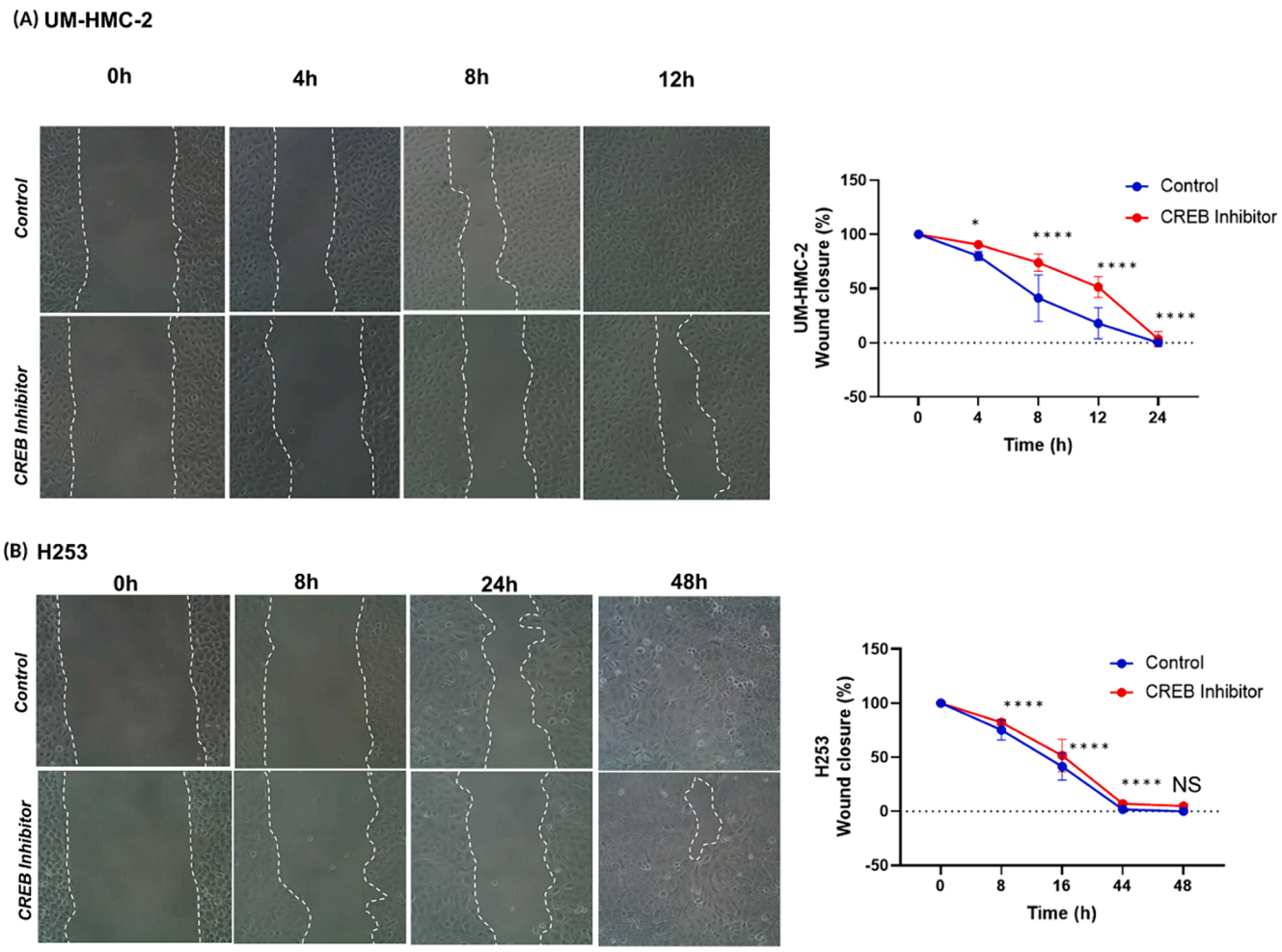

Fig. 4. **(A)** Representative images of invading cells followed by drug administration. **(B)** Quantitative analysis revealed that CREB inhibitor 666.15 treatment were effective in disrupting cell invasive capacities in all cell lines. (*$p < 0.05$; ***$p < 0.001$).

disease [34,35], while the hyperactivation of CREB is often associated with neoplastic diseases, including acute lymphoblastic leukaemia [36], acute myeloid leukaemia [37], melanoma [38], renal [39], prostate [40], oesophageal [41], pancreatic [42] and breast carcinomas [43], and brain tumours [44]. In these situations, CREB is associated with aberrant signal transduction caused by the deregulated expression of downstream genes that control the hallmarks of cancer including proliferation, apoptosis, angiogenesis, metastasis, immune surveillance, and metabolism, and the generation of tumour stem cells, which lead to the initiation and progression of tumours [45].

Taking into consideration CREB involvement in neoplastic transformation, its inhibition is increasingly becoming a potential therapeutic strategy for cancer treatment. Several CREB modulators have been developed and investigated as chemical modulators of cancer development. There are two main pharmacological strategies: "CREB inhibitors" and "CREB-related pathway inhibitors" [46]. The synthesis of 3-(3-aminopropoxy)-*N*-[2-[[3-[[(4-chloro-2-hydroxyphenyl)amino] carbonyl]-2-naphthalenyl]-oxy]ethyl]-2-naphthalenecarboxamide hydrochloride inhibitor (CREB inhibitor 666.15) was first described by Xie et al. [20] as a potent and selective inhibitor of CREB-mediated gene transcription without harming normal cells. This compound is a highly efficient CREB-CBP inhibitor that weakly affects NFκB activity by blocking the CBP-NFκb interaction. Although CREB is postulated as a general transcriptional activator, *in vivo* experimental murine studies of 666.15 recorded no effects on kidney or heart function, and it was, therefore, well tolerated in the mouse model [21].

To guarantee safety, low doses are necessary. Initial investigation with 666.15 suggested an IC50 dose of 0.081 ± 0.04 μM [21], however, our results found slightly higher IC50 doses for 2 cell lines, but these remained in the μM range (0.136 μM, 0.042 μM and 0.289 μM for UM-HMC-2, H253, and H292, respectively). These different concentrations can be related to the fact that the cell lines are derived from MEC with different clinicopathological features. Although UM-HMC-2 and H292 both harbour the CRTC1-MAML2 fusion, UM-HMC-2 has an intermediate histological grade and demonstrated perineural invasion in the patient and H292 was derived from a cervical lymph node metastasis of a pulmonary MEC; these cell lines represent MEC with the worst biological behaviour and consequently required a higher IC50 dose to affect cell proliferation. Indeed, these results endorse the current concept of personalized cancer therapy because each patient has unique features in their biological and molecular profile.

It is well known that the aberrant activation of CRTCs in tumours is related to oncogenic activities, such as migration, invasion, and metastasis. This is also strengthened by the fact that mutations in CRTCs have been shown to be key drivers in the development and progression of tumorigenesis [46,47]. This fusion protein is localized in the nucleus and has no known enzymatic activity making it traditionally difficult to target. Significant efforts have been directed into identifying critical signalling pathways downstream of the CRTC1-MAML2 fusion in order to identify therapeutic approaches. We included two TP cell lines, HMC-2 (TP, intermediate grade MEC) and H292 (TP, primary lung MEC), and even though they need a higher IC50 dose to inhibit cell growth, they presented more significant results for all assays, suggesting that CREB inhibitors would be more effective in patients with this molecular

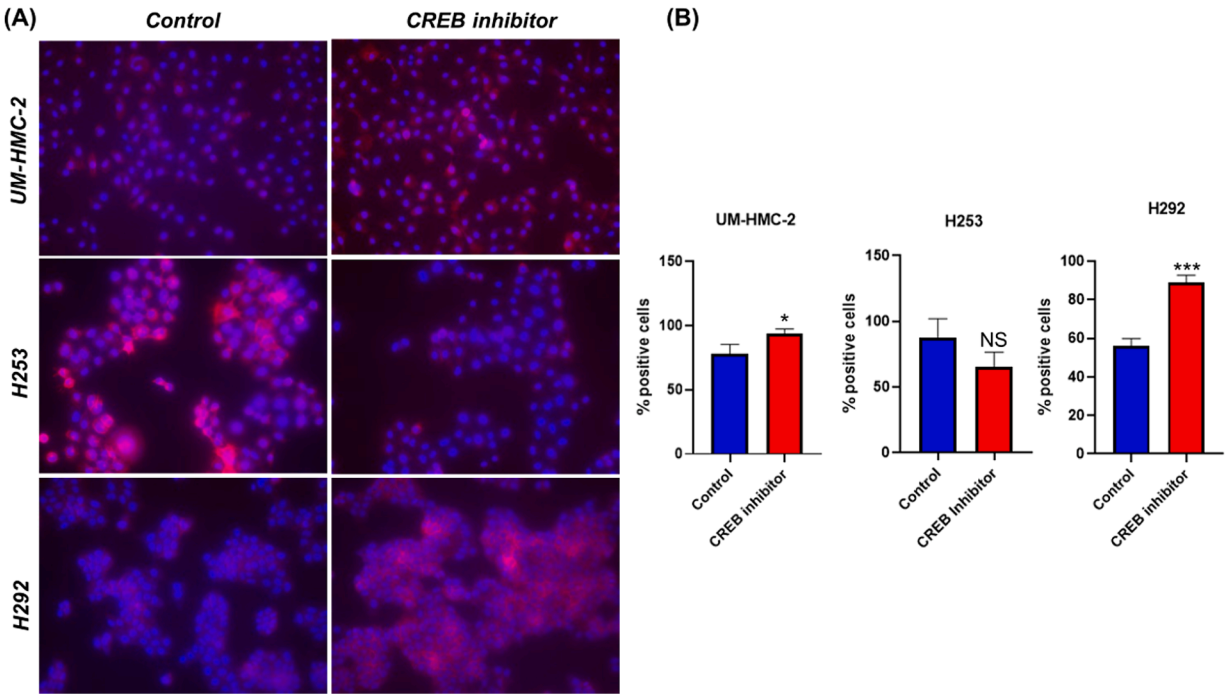

Fig. 5. Immunofluorescence reaction for the E-cadherin protein showing a higher cytoplasmic expression in UM-HMC2 and H292 cells, and lower cytoplasmic intensity in H253 cells after 24 h CREB inhibitor 666.15 administration (*$p < 0.05$; ***$p < 0.001$; NS – not significant).

signature. Interestingly, the TN cell line also showed a positive response, which could indicate other alternative routes for CREB activation but additional studies are needed to investigate this further.

MEC cell survival, through a clonogenic assay was shown to be reduced following CREB inhibition, which is in agreement with previous studies that have shown that in human MEC cells, the knockdown of the CRTC1-MAML2 translocation reduced cell growth and survival [19,48]. CREB knockdown has also been shown to impair the growth of B-cell precursor acute lymphoblastic leukaemia cells [36]. The clonogenic assay results are further supported by our spheroid analysis, suggesting a significant impact of 666.15 on CSCs. New drugs must be effective in eliminating this highly resilient cell population to achieve disease control or cure [10,26]. Fujishita et al. [49] identified that knockout of CREB in colorectal cancer cells reduced their spheroid-forming and metastasis-initiating ability. We know that CSC survival is based on complex interactions from many intrinsic and extrinsic factors and thus additional studies are needed to fully determine if CREB represents a vital pathway for MEC CSC survival.

Therapies capable of hampering other malignant properties such as motility and invasion are important in preventing tumour spread and metastatic disease [50]. The motility of cells is mediated by epithelial-mesenchymal transition (EMT), a dynamic process in which epithelial cells acquire properties of mesenchymal cells such as enhanced cell motility and invasiveness. E-cadherin, a calcium-dependent cell adhesion protein, is one of the most studied genes associated with EMT and is known to be regulated by a number of EMT transcriptional factors. Downregulation of E-cadherin has been shown to increase cancer cell proliferation, invasiveness, and metastasis [28]. Wang et al. [51] demonstrated that silencing CREB suppressed EMT by modulating EMT-related proteins and proteolytic enzymes, such as matrix metalloproteinase (MMP)-2 and MMP-9 in renal cell carcinoma cells. As mentioned previously, the CREB inhibitor 666.15 significantly reduced the migration and invasion capacities of MEC cells in our study but in addition we have also demonstrated that 666.15 led to the upregulation of E-cadherin on TP MEC cells (UM-HMC-2 and H292). Taken together, these data indicate that CREB inhibition in MEC may impair cell functions associated with the metastatic process.

Conclusions

We conclude that treatment of MEC cell lines with the CREB inhibitor, 666.15, results in anticancer properties through the reduction of survival and invasion and by delaying the migration of MEC cells *in vitro*, particularly for MEC cells harbouring the CRTC1-MAML2 translocation. This data supports the concept that pharmacologically targeting CREB may represent a promising strategy for patients diagnosed with MEC, especially for those with TP positive tumours.

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CRediT authorship contribution statement

Maria Eduarda Pérez-de-Oliveira: Data curation, Formal analysis, Investigation, Methodology, Writing – original draft. **Vivian Petersen Wagner:** Conceptualization, Data curation, Writing – review & editing. **Colin D. Bingle:** Data curation, Writing – review & editing. **Pablo Agustin Vargas:** Conceptualization, Writing – review & editing. **Lynne Bingle:** Conceptualization, Data curation, Project administration, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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