mTOR Inhibition Ablates Cisplatin-induced Stemness in Mucoepidermoid Carcinoma

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Materials and Methods

Cell lines

We used human salivary gland MEC lines (UM-HMC-1, UM-HMC-3A, UM-HMC-3B), passages 85-90, generated and characterized in our laboratory for these studies (Warner et al. 2013). The salivary gland medium consisting of DMEM (Gibco; Waltham, MA, USA), 10% FBS (Gibco), 1% L-Glutamine (Gibco), 1% Antibiotic-Antimycotic (Gibco), 400 ng/mL hydrocortisone (Sigma Aldrich, St. Louis, MO, USA), 20 ng/mL epidermal growth factor (Sigma), and 5 µg/mL insulin (Sigma) was used to culture MEC cells, as described (Warner et al. 2013). Cell line authenticity was verified by short tandem repeat (STR) profiling performed in an independent commercial laboratory (Appendix Fig. 1).

Sulforhodamine-B (SRB) Assay

We used the SRB assay to evaluate the effect of mTOR pathway inhibitors on cell viability, as described (Skehan et al 1990). Briefly, the SRB reagent binds stoichiometrically to proteins under mild acidic conditions and then can be extracted using basic conditions. The amount of bound dye can be used as a proxy for cell density (i.e. compounded effect of treatment on cell proliferation and cell survival). Here, we seeded $1x10^3$ MEC cells/well in 96-well plates. The following day, cells were exposed to serial dilution concentrations of Buparlisib (Selleckchem; Houston, TX, USA), temsirolimus (Selleckchem), AZD8055 (Selleckchem), or PF4708671 (Selleckchem) with or without cisplatin (Teva Pharmaceuticals; Parsippany, NJ, USA) for 24 to 72 hours in quadruplicate wells per condition. Cells were fixed and stained for the SRB assay, as shown (Nör et al. 2014). Here, and throughout this manuscript, studies were independently repeated at least 3 times to ensure reproducibility of the data.

Salisphere Assay

Non-adherent spheroids (named salispheres) were generated with salivary MEC cell lines, as we shown (Adams et al. 2015; Krishnamurthy and Nör 2013). Cells were counted, seeded in triplicate wells (2 x 10^3 cells/well) of 6-well ultra-low attachment plates (Corning; Corning, NY, USA), and cultured with the salisphere culture medium consisting of DMEM-F12 (Invitrogen; Carlsbad, CA, USA), 1% Glutamax (Gibco), 1% Antibiotic-Antimycotic (Gibco), 1% N2 Supplement (Gibco), 20 ng/mL epidermal growth factor (Sigma), 20 ng/mL basic fibroblast growth factor (Sigma), 10 µg/mL insulin (Sigma), and 1 µM dexamethasone (Sigma). After 24 hours, we treated cells with serial dilution concentrations of temsirolimus and/or cisplatin or Bmi-1 inhibitor (PTC-209; Tocris Bioscience, Bristol, UK) for 8 days. Then, we measured the size and the number of salispheres, *i.e.* floating spheroids of ≥50 cells.

Flow cytometry

MEC cancer stem-like cells (CSCs) are characterized by high ALDH activity and high CD44 expression (Adams et al. 2015). Single cell suspensions were generated either from trypsinization of UM-HMC cell lines or digestion of tumor tissues, as shown (Adams et al. 2015). Cells were immersed with Aldefluor® substrate (StemCell Technologies; Vancouver, BC, Canada) or ALDH inhibitor diethylaminobenzaldehyde (DEAB) for 40 minutes at 37ºC. Then, we washed and immersed cells with anti-human CD44 antibody (APC; BD Pharmingen; Franklin Lakes, NJ, USA) for 30 minutes at 4ºC. We analyzed 100,000 DAPI-negative cells to determine ALDH activity and CD44 expression using flow cytometry. Data were analyzed from triplicate wells per condition.

MEC Patient-Derived Xenograft (PDX) model

To generate MEC PDX (UM-HMC-PDX-18), we subcutaneously transplanted fragments of the UM-HMC-18 tumor (*in vivo* passage 5) in the dorsal region of severe combined immunodeficient (SCID, CB-17; Charles River; Wilmington, MA, USA) male mice. Tumor volume was measured using the formula: volume (mm^3) = L x $W^2/2$ (L, length; W, width). When mean tumor volume

reached 460 mm^3, we randomly allocated mice into 6 groups, as follows: 1) PBS daily via intraperitoneal (i.p.) injection (placebo control, n=6); 2) 5 mg/kg temsirolimus (Sigma Aldrich) daily via i.p. injection (n=6); 3) 5 mg/kg cisplatin (Sigma Aldrich) weekly via i.p. injection (n=6); 4) 5 mg/kg temsirolimus daily + 5 mg/kg cisplatin weekly (both drugs started at the same time, n=8); 5) 5 mg/kg temsirolimus daily + 5 mg/kg cisplatin weekly (temsirolimus started 1 week before cisplatin, n=8); 6) 5 mg/kg cisplatin weekly + 5 mg/kg temsirolimus daily (cisplatin started 1 week before temsirolimus, n=6). After 10 days, we euthanized mice, retrieved PDX tumors and divided into 3 pieces (*i.e.* one for flow cytometry and two smaller pieces for western blots and histology). All *in vivo* experiments followed the ARRIVE (Animal Research: Reporting In Vivo Experiments) guidelines, and an animal protocol was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of University of Michigan (approval number: PRO00007199).

Western Blot Analysis

We plated UM-HMC-1, UM-HMC-3A, or UM-HMC-3B cell lines, serum starved overnight, and treated cells with 0-10 μM temsirolimus, 0-1 μM cisplatin, or 0.1 μM temsirolimus together with 0.1 μM cisplatin for 24 hours. We used Nonidet P-40 (NP-40; Sigma) buffer to prepare protein lysates from cell lines or UM-HMC-PDX-18 tissues and loaded protein lysates onto 9% SDS-PAGE gels. Membranes were immersed overnight at 4ºC with one of the following primary antibodies: mouse anti-human phospho-mTOR Ser2448 (1:1000; Cell Signaling, Danvers, MA, USA), mouse anti-human mTOR (1:2000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse anti-human Raptor (1:500; Santa Cruz), mouse anti-human Rictor (1:500; Santa Cruz), rabbit anti-human phospho-S6K1 Thr421/Ser424 (1:2000; Cell Signaling), rabbit anti-human S6K1 (1:2000; Cell Signaling), rabbit anti-human phospho-AKT Ser473 (1:2000; Cell Signaling), rabbit anti-human AKT (1:50,000; Cell Signaling), rabbit anti-human Bmi-1 (1:10,000; Cell Signaling), or mouse anti-human GAPDH (1:40,000,000; Chemicon, Billerica, MA, USA). Membranes were exposed to affinity-purified secondary antibodies (1:2000) conjugated with

HRP (Jackson Laboratory). Immunoreactive proteins were visualized by SuperSignal West Pico chemiluminescent substrate (Thermo Fisher Scientific, Waltham, MA, USA).

Histological analyzes

We deparaffinized and rehydrated by immersing formalin-fixed, paraffin-embedded tissue sections in xylene and descending concentrations of ethanol. For immunohistochemical and immunofluorescence staining, we performed antigen retrieval with Trypsin (Sigma Aldrich) at 37°C for 1 hour or with citrate buffer (Thermo Fisher Scientific) in a pressure cooker following manufacturer's instructions. Then, tissues were immersed with 0.1% triton X-100 (Fisher Scientific, Fair Lawn, NJ, USA) for 20-30 minutes, followed by 3% hydrogen peroxide (Fisher Scientific) for 20-30 minutes and Background Sniper (Biocare Medical, Parcheco, CA, USA) for 20-30 minutes at room temperature. Tissues were exposed to anti-human Pan-cytokeratin (1:200; Santa Cruz) or anti-F4/80 (macrophage) antibody (1:100; Abcam) overnight at 4°C. After incubation with primary antibody, tissues were treated with MACHI3 (Biocare Medical) for 20 minutes at room temperature, MACHI 3 HRP polymer (Biocare Medical) for 20 minutes at room temperature, and then with DAB for 3 minutes. Hematoxylin (Vector Laboratories, Burlingame, CA, USA) was used to counterstain for 20-30 seconds. For immunofluorescence staining, tissues were immersed with anti-human ALDH1 (1:100; Abcam, Cambridge, England) and anti-human CD44 (1:400; Cell Signaling) overnight at room temperature. After incubation with primary antibodies, tissue sections were exposed to Alexafluor 488 (Anti-Rabbit; Invitrogen, San Diego, CA, USA) and Alexafluor 594 (Anti-Mouse; Invitrogen) for 1 hour at room temperature. Then, tissues sections were covered by cover slip using Vectashield with DAPI (Vector Laboratories, Burlingame, CA, USA). The number of ALDH-positive and CD44-positive cells was counted in randomly selected fields (5 per tumor). In selected experiments, specimens were also stained for Periodic Acid Schiff (PAS) (Sigma) following manufacturer's instructions. All images were taken with Nikon Eclipse E800 Fluorescence Microscope.

Statistical analysis

We analyzed the impact of treatment on tumor volume, number and size of salispheres, cell viability, and CSC fraction using one-way ANOVA followed by appropriate post hoc test (Tukey test). Statistical significance was determined at $P<0.05$ throughout this manuscript.

References

Krishnamurthy S, Nör JE. 2013. Orosphere assay: a method for propagation of head and neck cancer stem cells. Head Neck. 35(7):1015–1021.

Skehan P, Storeng R, Scudiero D, Monks A, McMahon J, Vistica D, Warren JT, Bokesch H, Kenney S, Boyd MR. 1990. New colorimetric cytotoxicity assay for anticancer-drug screening. J Natl Cancer Inst. 82(13):1107–1112.

Warner KA, Adams A, Bernardi L, Nor C, Finkel KA, Zhang Z, McLean SA, Helman J, Wolf GT, Divi V, et al. 2013. Characterization of tumorigenic cell lines from the recurrence and lymph node metastasis of a human salivary mucoepidermoid carcinoma. Oral Oncol. 49(11):1059–1066.

A

Genetic locus	Cell line (UM-HMC-1, p# 2)	Cell line (UM-HMC-1, p# 88)
D3S1358	15, 16	15, 16
D7S820	11, 12	11, 12
vWA	16	16
FGA	18.2, 20	18.2, 20
D8S1179	15	15
D21S11	30, 31	30, 31
D18S51	12, 17	12, 17
D5S818	10, 13	10, 13
D13S317	12, 13	12, 13
D16S539	11	11
TH01	7, 9	7, 9
TPOX	6, 9	6, 9
CSF1PO	7, 11	7, 11
AMEL	X, Y	X, Y
Penta D	6, 13	6, 13
Penta E	9, 10	9, 10

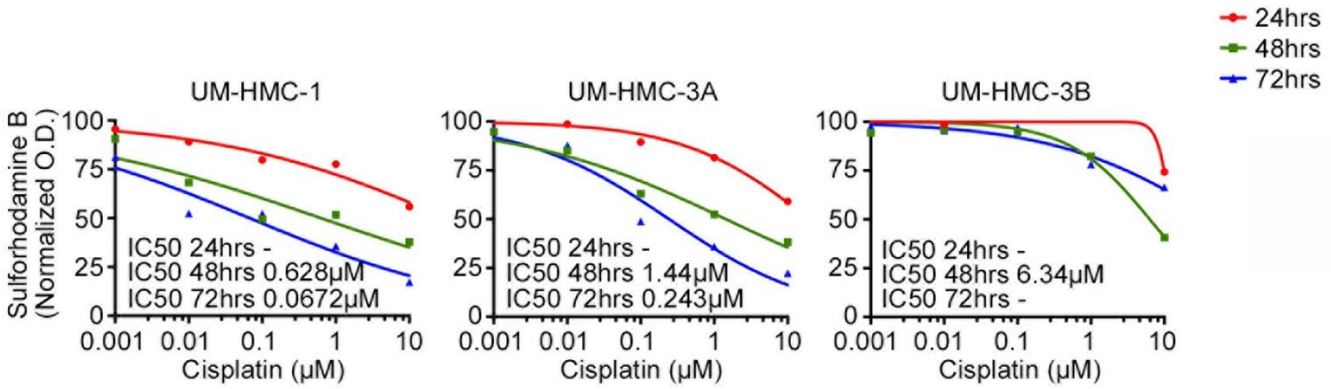
B

Genetic locus	Tumor specimen (recurrence)	Cell line (UM-HMC-3A, p# 89)
D3S1358	14	14
D7S820	8, 9	8, 9
vWA	15	15
FGA	10, 21	10, 21
D8S1179	13	13
D21S11	29, 32.2	29, 32.2
D18S51	12, 16	12, 16
D5S818	11, 12	11, 12
D13S317	8, 11	8, 11
D16S539	12, 13	12, 13
TH01	8	8, 9
TPOX	9, 12	9, 12
CSF1PO	12	12
AMEL	X	X
Penta D	10, 14	10, 14
Penta E	9, 17	9, 17

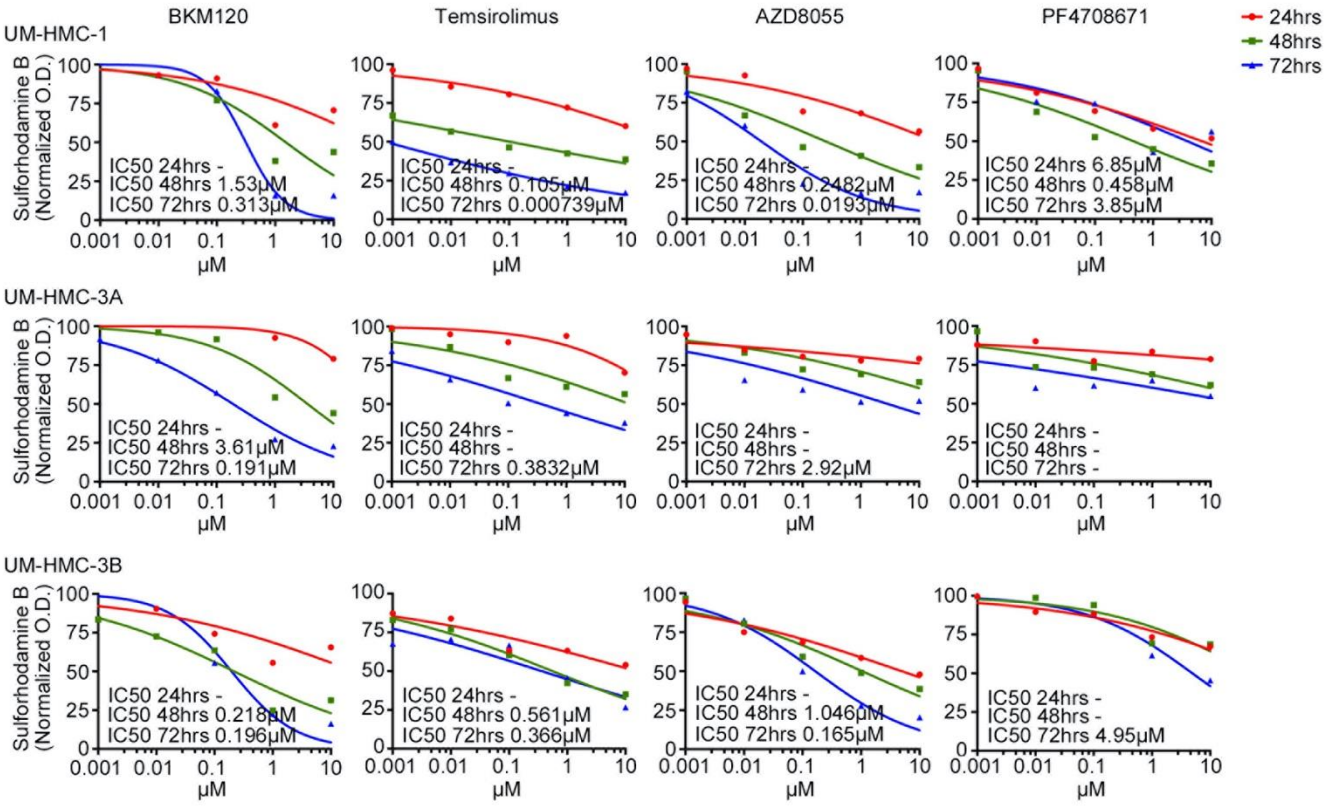
C

Genetic locus	Tumor specimen (metastasis)	Cell line (UM-HMC-3B, p# 90)
D3A1358	14	14
D7S820	8, 9	8, 9
vWA	15	15
FGA	10, 21	10, 21
D8S1179	13	13
D21S11	29, 32.2	29, 32.2
D18S51	12, 16	12, 16
D5S818	11, 12	11, 12
D13S317	11	8, 11
D16S539	12, 13	12, 13
TH01	8, 9	8, 9
TPOX	9, 12	9, 12
CSF1PO	12	12
AMEL	X	X
Penta D	10, 14	10, 14
Penta E	9, 17	9, 17

Appendix Fig. 1. Genotypic match of the UM-HMC cell lines used in this study. **A,** Table depicting the short tandem repeat (STR) profile of UM-HMC-1 (passage 1) against UM-HMC-1 cells used here (passage 89). **B,** Table depicting the STR profile of the human MEC specimen and the UM-HMC-3A cell line (passage 89). **C**, Table depicting the STR profile of the human MEC specimen and the UM-HMC-3B cell line used here (passage 90).



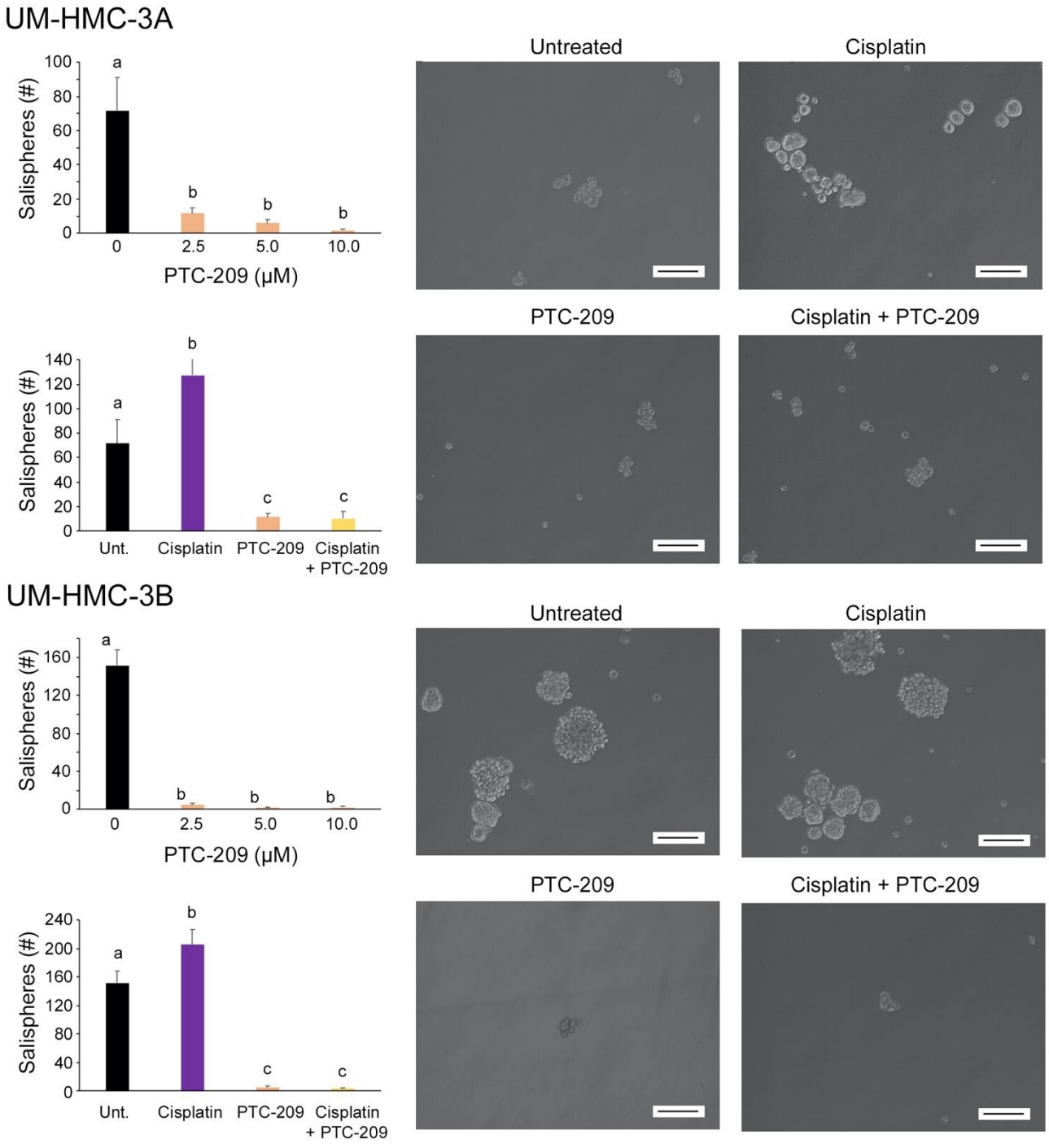
Appendix Fig. 2. Effect of cisplatin on bulk MEC cell viability. The cytotoxicity of cisplatin after 24, 48, and 72 hours was evaluated by the SRB assay in UM-HMC-1, UM-HMC-3A, and UM-HMC-3B cell lines. Data were normalized against vehicle control, and is derived from triplicate wells per condition.



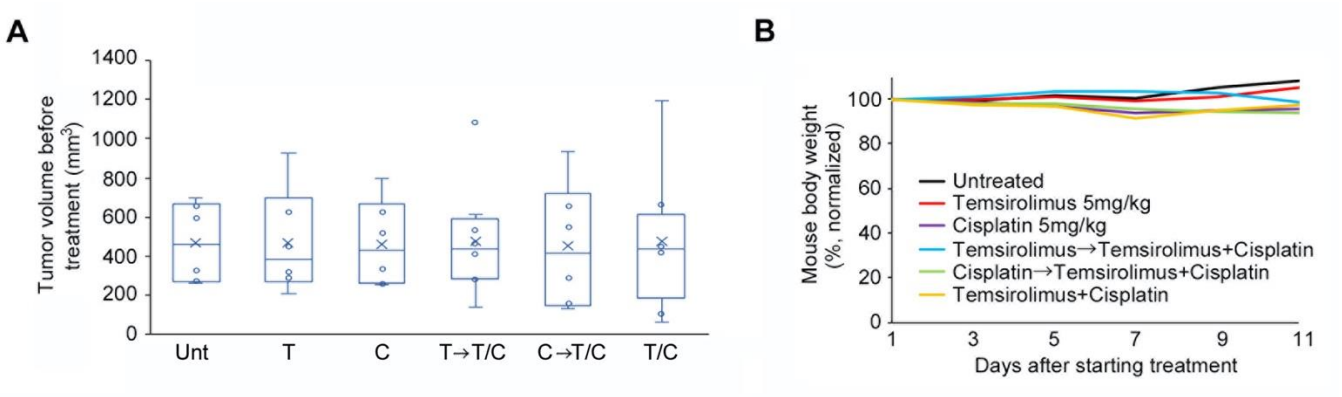
Appendix Fig. 3. Effect of mTOR inhibitors on bulk MEC cell viability. The cytotoxicity of mTOR pathway inhibitors (BKM120, temsirolimus, AZD8055, PF4708671) after 24, 48, or 72 hours was evaluated by the SRB assay in UM-HMC-1, UM-HMC-3A, and UM-HMC-3B cell lines. Data were normalized against vehicle control, and is derived from triplicate wells per condition.

Cell line						
UM-HMC-1	Temsirolimus (μM)	0.0001	0.001	0.01	0.1	1
	Cisplatin (μM)	1	1	1	1	1
	24 hrs CI	11.9774	0.70806	0.45914	0.63627	0.27484
	48 hrs CI	28.5475	0.99039	0.62213	0.44288	0.43365
	72 hrs CI	10.5819	0.60585	0.28996	0.24968	0.42646
UM-HMC-3A	Temsirolimus (μM)	0.0001	0.001	0.01	0.1	1
	Cisplatin (μM)	1	1	1	1	1
	24 hrs CI	18.4491	1.36374	0.41465	0.3882	0.25291
	48 hrs CI	436.529	13.8867	1.03811	0.6681	0.7469
	72 hrs CI	12.1631	2.98133	1.89234	0.87426	0.67665

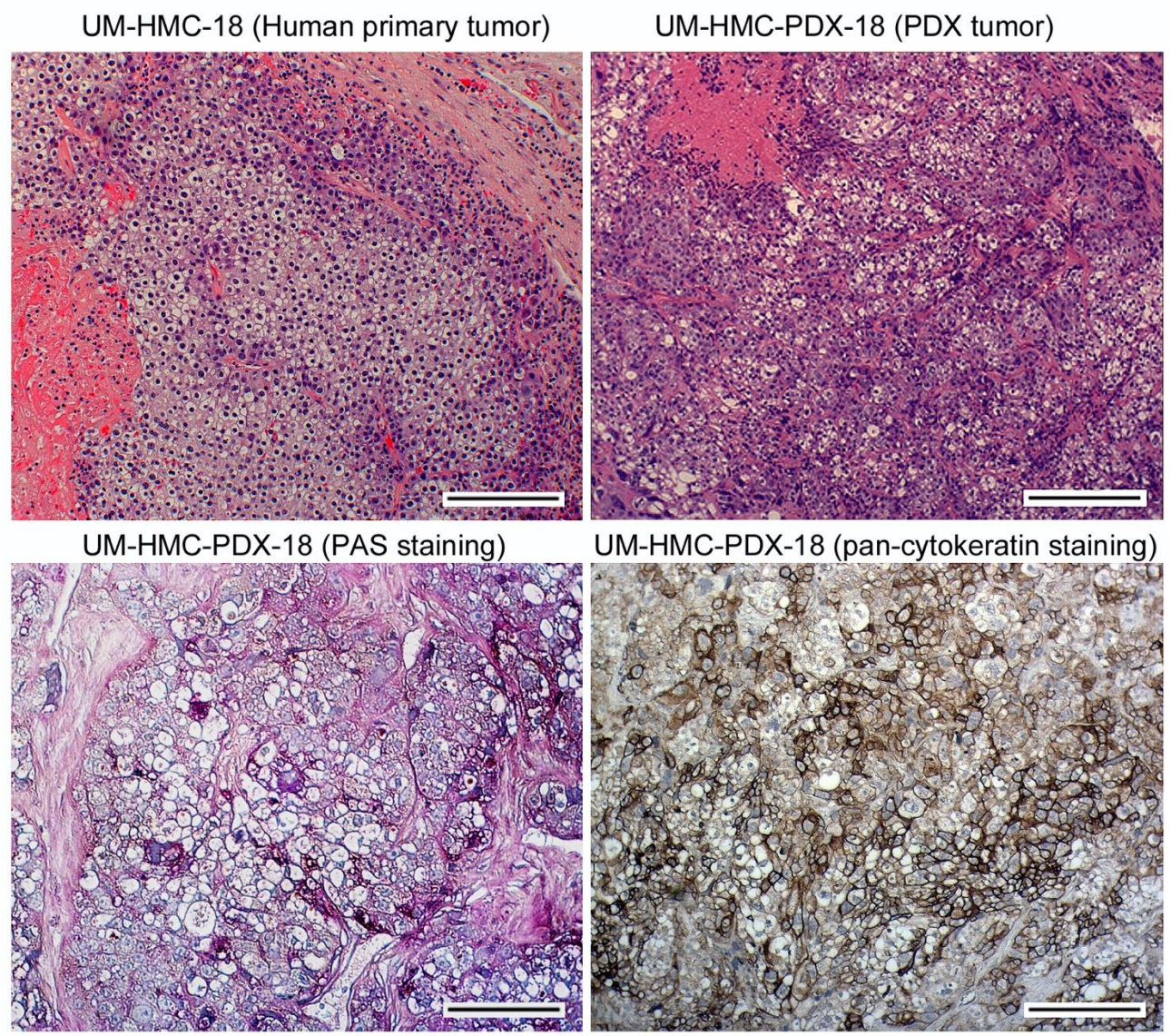
Appendix Fig. 4. Combination index (CI)-isobologram method that allows quantitative determination of drug interactions, where CI<1, =1, and >1 indicate synergism, additive effect, and antagonism (respectively). This method was used to quantify the impact of combination 0.0001-1 μM temsirolimus and 1 μM cisplatin on MEC cell viability (UM-HMC-1, UM-HMC-3A).



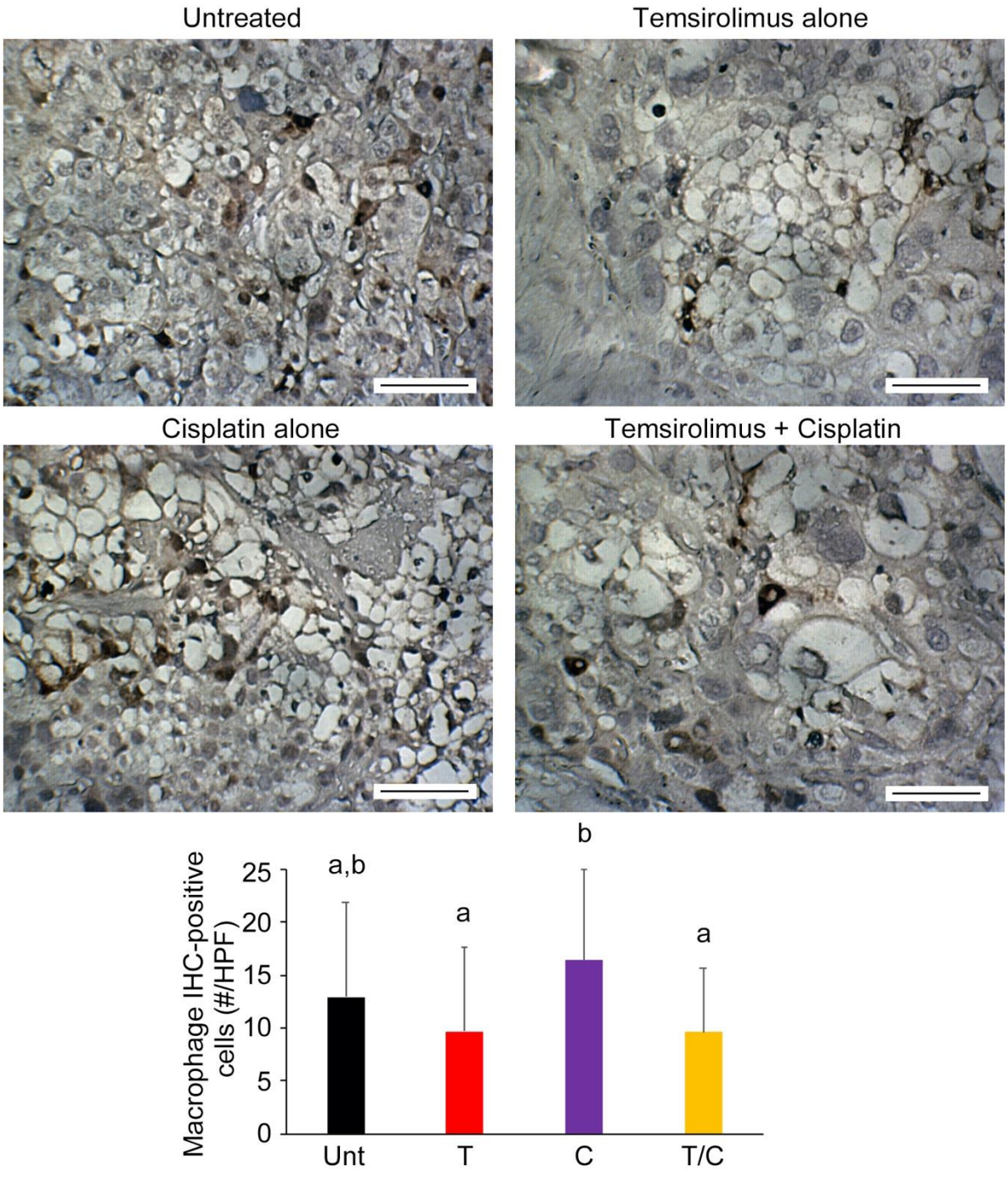
Appendix Fig. 5. Effect of Bmi-1 inhibitor (PTC-209) and/or cisplatin on salispheres. Bar graphs illustrate the number of salispheres per well generated from UM-HMC-3A and UM-HMC-3B cells treated with 0-10 μM PTC-209 (top graphs) or 2.5 μM PTC-209 and/or 0.1 μM cisplatin (bottom graphs). Representative photographs of salispheres after treatment with cisplatin (0.1 μM) and/or PTC-209 (2.5 μM). Scale bars represent 100 μm (x100). Different lowercase letters in graphs represent statistical significance at $P<0.05$.



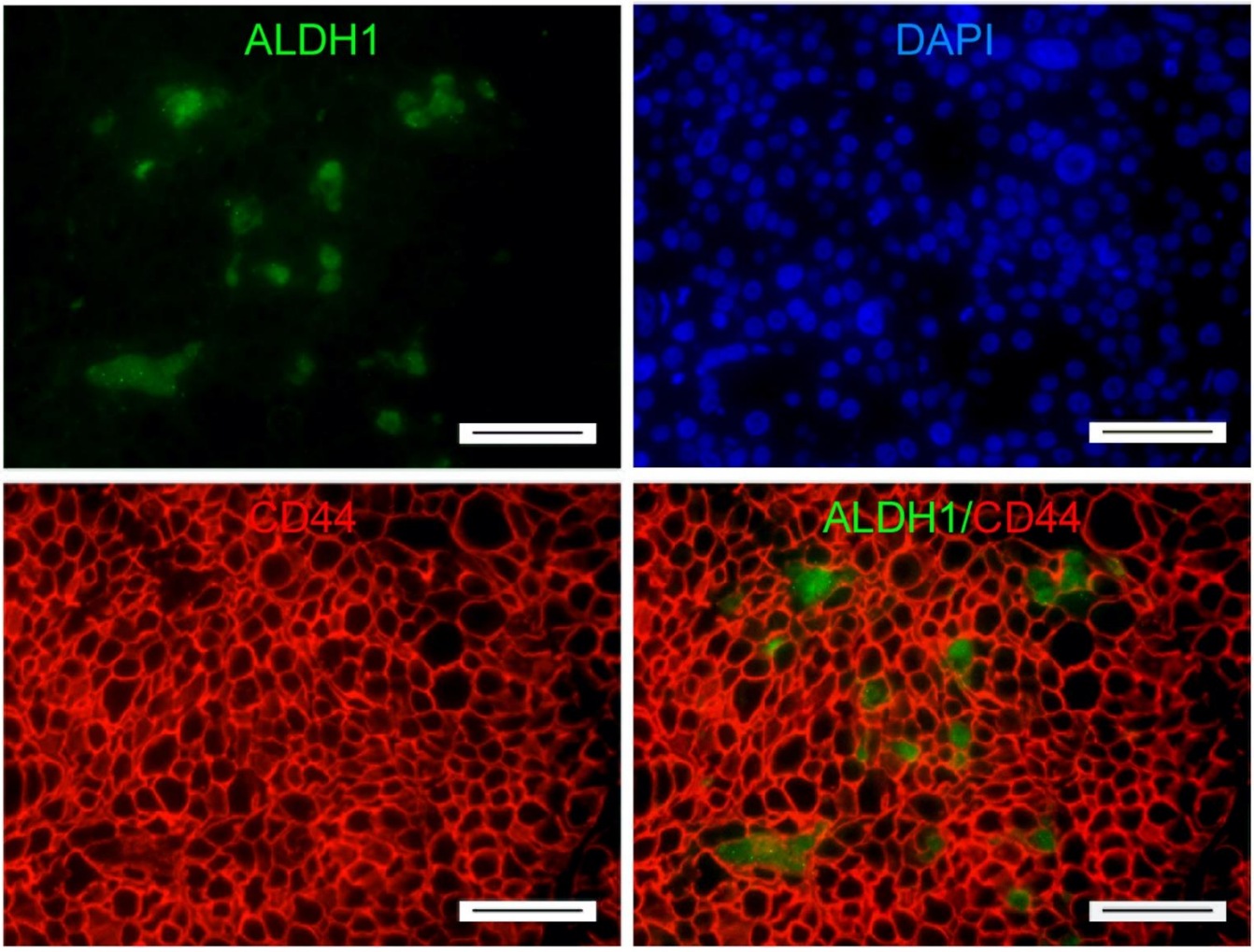
Appendix Fig. 6. Effect of temsirolimus and/or cisplatin on tumor growth in MEC PDX model. **A**, Tumor volume before start of treatment: Untreated (Unt.), temsirolimus (T), cisplatin (C), temsirolimus first combination therapy (T → T/C), cisplatin first combination therapy (C → T/C), and temsirolimus and cisplatin at same time (T/C). The average tumor volumes in each treatment group were between 451.2 to 476.6 mm^3. **B**, Graph depicting mouse body weight during treatment. Data were normalized against body weigh before starting treatment. UM-HMC-PDX-18 tumors treated with PBS (Untreated, black), temsirolimus (red), cisplatin (purple), temsirolimus first combination therapy (light blue), cisplatin first combination therapy (green), or temsirolimus and cisplatin at same time (yellow).



Appendix Fig. 7. UM-HMC-PDX-18 tumors retain the histological features of the primary human tumor. Representative histological sections (HE) of the human primary tumor (UM-HMC-18) from which the UM-HMC-PDX-18 xenograft tumors were generated. The PDX tumors were also stained with PAS (lower left) and pan-cytokeratin (lower right). Scale bars represent 100 µm (x100).



Appendix Fig. 8. Representative photomicrographs of UM-HMC-PDX-18 tumor tissue sections upon immunohistochemical staining for macrophages (anti-F4/80 antibody). Images were taken at 400x magnification. Scale bars represent 50 μm. Bar graph illustrating the number of positive cells for macrophage per high power field (HPF: 400X magnification) in tissues treated with temsirolimus (red), cisplatin (purple), or temsirolimus and cisplatin at same time (yellow). Different lowercase letters in graphs represent statistical significance at $P<0.05$.



Appendix Fig. 9. Representative photomicrographs of immunofluorescence staining of UM-HMC-PDX-18 tumor tissue sections stained for ALDH1 (green), CD44 (red), and DAPI (blue). Images were taken at 400x magnification. Scale bars represent 50 μm.