Targeting tumor-associated MUC1 overcomes anoikis-resistance in pancreatic cancer

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The third leading cause of cancer-related deaths in the United States is pancreatic cancer, more than 95% of which is pancreatic ductal adenocarcinoma (PDA). The incidence rate of PDA nearly matches its mortality rate and the best treatment till date is surgical resection for which only 25% are eligible. Tumor recurrence and metastasis are the main causes of cancer-related mortality. MUC1 is a transmembrane glycoprotein expressed on most epithelial cells. It is overexpressed and aberrantly glycosylated in cancer and is known as tumor-associated MUC1 (tMUC1). More than 80% of PDAs express tMUC1. A monoclonal antibody called TAB004 has been developed specifically against human tMUC1 extracellular domain. We report that treatment with TAB004 significantly reduced the colony forming potential of multiple PDA cell lines while sparing normal pancreatic epithelial cell line. Binding of TAB004 to tMUC1 compromised desmosomal integrity, induced ER stress and anoikis in PDA cells. The mechanisms underlying TAB004′s antitumor effects were found to be reduced activation of the EGFR-PI3K signaling pathway, and degradation of tMUC1, thereby reducing expression of its transcriptional targets, c-Src and c-Myc. This reduction in oncogenic signaling triggered anoikis as indicated by reduced expression of antiapoptotic proteins, PTBH2 and BCL2. TAB004 treatment slowed the growth of PDA xenograft compared to IgG control and enhanced survival of mice when combined with 5-FU. Since TAB004 significantly reduced colony forming potential and triggered anoikis in the PDA cells, we suggest that it could be used as a potential prophylactic agent to curb tumor relapse after surgery, prevent metastasis and help increase the efficacy of chemotherapeutic agents.

Abbreviation: 5-FU, 5-Fluoro Uracil; APAF1, Apoptotic Peptidase Activating Factor 1 ASB16-AS16 Antisense RNA 1; ASNS, Arpaparig Synhaste ATP4-Activating Transcription Factor 4; BCA, bicinchoninic acid assay; BCL2, B-cell lymphoma 2; BIP, Binding immunoglobulin protein (GRP 78); Br1, Brd-2 inhibitor of transcription 1; CCB1, Collagen And Calcium Binding EGF Domains 1 CDH23-Cadherin Related 23; CIAF1, cytokine-induced inhibitor of apoptosis; CNT2, Contactin 2; Go-IP, Co-Inmunoprecipitation; COXIV, Cytochrome C Oxidase Subunit 4I1; CT, Cytosplasmic Tail Cyclohydrolase; DAVID, Database for Annotation, Visualization and Integrated Discovery; DEGs, Differentially Expressed Genes; DGCA, Differential Gene Correlation Analysis; E2F1, E2F Transcription Factor 1; ECD, Extracellular domain; ECM, Extracellular matrix; EF2, Elongation Factor 2; EGFR, Epidermal Growth Factor Receptor; EGLN3, Egl nine homolog 3; ER, Endoplasmic Reticulum; FACS, Fluorescence Associated Cell Sorting; FAK, Focal Adhesion Kinase; FGF, Fibroblast Growth Factor; FITC, Fluorescein isothiocyanate; GDC, Genomic Data Commons; GO, Gene Ontology; GRP75, Glucose Regulated Protein 75; HI-FBS, heat-inactivated fetal bovine serum; HPDE, Human Pancreatic Ductal Epithelial Cell; IgG, Immunoglobulin; IPA, Ingenuity Pathway Analysis; KEGG, Kyoto Encyclopedia of Genes and Genomes Limma-linear models for microarray data; MTHFD2, Methylene tetrahydrofolate Dehydrogenase (NADP + Dependent) 2; Methylenetetrahydrofolate MTHT3-4,5-Dimethylthiazole-2-y)-2,5-Diphenyltetrazolium Bromide; MUC1, Mucin1; MUC1, N-Mucin 1 N-terminal domain; MYH9, Myosin H9; NF-kB, Nuclear factor kappa-light-chain-enhancer of activated B cells; O.D., Optical Density; PBS, Phosphate Buffered Saline; PDA, Pancreatic Ductal Adenocarcinoma; PDGF, Platelet Derived Growth Factor; PHGDH, phosphoglycerate dehydrogenase; PI3K, phosphoinositol 3-kinase; PIK3CB, Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit beta isoform; PHKB, Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha isoform; PKB, Phosphatidylinositol-3-kinase regulatory subunit alpha; POI, Point of Interest; PSAT1, Phosphoserine Aminotransferase 1; PSM, Peptide Spectrum Matches; PTBH2, Peptidyl-tRNA hydrolase 2; RMA, Robust Multi-Array Average; RPM, Rotations per Minute; S.D., Standard Deviation; S.E.M., Standard Error of Mean; SCAMP5, Secretory carrier-associated membrane protein 5; SDS, Sodium Dodecil Sulphate; SLC6A9, solute carrier family 6; SLC7A5, solute carrier family 7 A5; SRC, Steroid Receptor Coactivator; STAT3, Signal transducer and activator of transcription 3; TCGA, The Cancer Genome Atlas; TMEM127, Transmembrane Protein 127; tMUC1, tumor-associated MUC1; TRIB2, Tribbles homolog 2; UBES2, ubiquitin-conjugating enzyme; VEGF, Vascular Endothelial Growth Factor; WB, Western Blot; WRAP73, WD repeat containing protein 73; WT, wild type; ZC3H4, Zinc Finger CCCH-Type Containing 4

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Introduction

Currently, cancer is the second leading cause of death worldwide and the main cause is not the primary tumor, but metastasis and recurrence. Cancer cells metastasize after successful detachment from surrounding cells or the extracellular matrix (ECM), migrating to distal locations, followed by reattachment, and proliferation in the new site.1 Cancer cells employ various mechanisms to resist anoikis, thus enhancing their invasiveness and potential to metastasize. These include ligand-dependent or independent oncogenic signals that induce pro-survival signaling, leading to increased stemness, drug resistance, proliferation, and invasion.2 Anoikis is an apoptotic event, which is activated to clear detached cells3 via induction of both the intrinsic and extrinsic apoptotic pathways. Pro-apoptotic events are activated in the mitochondria to trigger the activity of effector caspases for elimination of detached cells and restoration of tissue homeostasis. For detached cells that have undergone epithelial-to-mesenchymal transition, it is crucial to resist anoikis to colonize a new organ. Anoikis resistance is an emerging hallmark of metastatic malignancies since it provides cells with an advantage of anchorage-independent survival during tumor dissemination.3 Different factors have been reported to aid in anoikis-resistance in cancer cells including but not limited to changes in cell adhesion molecules, enhanced production of reactive oxygen species and growth factors such as Epidermal growth factor (EGF), Fibroblast growth factor (FGF), and Vascular endothelial growth factor (VEGF), activation of oncogenic signaling pathways, upregulation of stemness factors, hypoxia and autophagy.2 Especially in pancreatic ductal adenocarcinoma (PDA), the anoikis resistance mechanisms are mostly driven by changes in glucose metabolism, STAT3 upregulation, BCL2 activation and activation of c-Src in a FAK independent manner.2 The PI3K-Akt pathway has been reported to drive anoikis resistance in anchorage-independent cells.2 Mucin 1 or MUC1 is a heterodimeric transmembrane glycoprotein with a hyperglycosylated extracellular N-terminal domain that extends out of the cell surface. MUC1 is expressed on the apical surface of glandular or luminal epithelial cells of most all normal tissues. The MUC1 single polypeptide chain is cleaved by auto-proteolysis at a sea-urchin sperm protein enterokinase and agrin domain to generate 2 peptide fragments and heterodimeric MUC1. The β subunit or MUC1-C contains a C-terminal cytoplasmic domain (MUC1-CT), a hydrophobic transmembrane domain and a short extracellular domain (ECD) with 58 amino acids that is noncovalently attached to the N-terminal ECD (MUC1-N) or α subunit.34 MUC1-ECD engages in extracellular signaling through sensing of external stimuli, followed by reprogramming of downstream gene expression profiles of cells.55 In tumor cells, MUC1 loses its apical localization, is overexpressed, hypo- and aberrantly glycosylated, which exposes its core peptide and makes it accessible to immunotherapy.10 Aberrantly glycosylated MUC1 (designated tMUC1) shows increased intracellular uptake by clathrin-mediated endocytosis and decrease in degradation. Thus, hypoglycosylation may foster MUC1-induced oncogenic signaling by decreasing its cell surface levels and increasing intracellular accumulation.4 The receptor-like functions of MUC1 provide adhesive/antiadhesive functions to the cells. The MUC1-CT subunit is 72 amino acid residues-long and acts as a highly busy docking site for numerous signaling molecules.11 It directly interacts with several transcription factors, and physically occupies multiple promoter regions.4 Although, MUC1 lacks a DNA binding domain, it acts as a transcriptional co-activator or adaptor protein and its presence in transcriptional complexes significantly enhances recruitment of other co-factors and promoters of oncogenic transcription factors.12 Transcriptional regulation and other oncogenic functions are significantly modulated by post-translational modifications of MUC1-CT. For example, the MUC1-CT has 7 tyrosine residues that are highly conserved across all mammalian species. Phosphorylation of these tyrosine residues increases the binding affinity of multiple kinases to MUC1-CT, thus enabling activation of downstream oncogenic signaling.5 Overexpression of MUC1 has been long been associated with high metastatic potential and poor prognosis in cancer patients.12 MUC1 O-glycosylation has been recently reported to promote resistance to anoikis, and removing the glycosyl residues opened up interaction of cell death receptors to their ligands, leading to apoptosis.13 Both MUC1-ECD and MUC1-CT were found to contribute to anoikis-resistance in epithelial cancer cells.13-15 Several antibodies have been developed against MUC1 for therapeutic use and a few of them are in clinical trials. A thorough comparison of these antibodies has been made previously.10 TAB004 is a mouse monoclonal IgG1 antibody that was developed by immunizing Balb/c mice with lysates from MUC1-expressing tumors that spontaneously developed in a transgenic tMUC1 bearing mouse.12 The epitope of TAB004 is present within the tandem repeat sequence STAPPYHVN (AA950-958) of hypo-glycosylated tMUC1.17-19 This area is accessible for antigenic binding in MUC1 but is blocked by large, glycosylated branches in normal MUC1. TAB004 has unique complementarity determining regions in both the heavy and the light chains and binds the antigen with high affinity (3ng/ml, 20PM), and does not bind unrelated antigens. TAB004 can differentiate between the normal and MUC1 based on its aberrant glycosylation. The specificity of TAB004 has been confirmed in an immunohistochemistry study with over 430 tissue samples from normal, benign, and malignant breast tissue and by demonstrating tumor targeting in transgenic animal models that have normal MUC1 in their epithelia. Several studies showing TAB004’s tumor specificity, and its targeted imaging and therapeutic potential have been published.20-28 However, the mechanism of action of TAB004 in intracellular signaling has not yet been elucidated. The rationale for this study is to understand the intracellular oncogenic signaling changes post binding of TAB004 to surface tMUC1 in human PDA cells. Our long-term goal is to optimize therapeutic strategies to prevent metastasis and recurrence in epithelial cancers. There is significant lack of understanding of the mechanism(s) of action of tumor-specific antibodies with regards to how it may alter intracellular oncogenic signaling within a cell. Thorough analysis of the intracellular signaling of an antibody after binding to its target may clarify some of the causes of resistance development and its clinical potential. In this study, we show that targeting tMUC1-ECD with TAB004 antibody significantly alters intracellular oncogenic signaling and slows tumor growth by overcoming anoikis-resistance.

Materials and Methods

Data analysis from TCGA

RNA sequencing data

RNA-seq data were downloaded from the GDC (Genomics Data Commons Portal) for all available cancer types, with a majority from TCGA
(The Cancer Genome Atlas) program. A total of 15,112 samples were included for the analysis, 13,509 tumor and 1603 normal samples. Tumor types chosen included primary and metastatic solid tumor tissue samples (n = 13,509) and normal samples available in GDC (n = 1603) were also downloaded for the analysis. Data was downloaded using the gdc-client tool. Samples were considered to have low MUC1 expression if the expression value is less than 100, otherwise the sample was considered to have high MUC1 expression.

Plot for MUC1 expression in normal and tumor samples was generated from the DGCA (1.0.2) package in R (3.6.3).

Gene correlation analysis

Gene correlation analysis was run using the R package: DGCA (Differential Gene Correlation Analysis). DGCA calculates gene-gene correlations between different groups and the correlation significance values. Genes with the lowest expression levels were filtered out by the dispersion measure, with the filter dispersion percentile at 0.3. This dataset was the input to run differential correlation analysis across tumor and normal samples, identifying gene correlations with the MUC1 gene. P value adjustment was done with the Benjamini & Hochberg correction method.

Survival analysis

Survival analyses for select genes and overall survival were computed using the Kaplan-Meier estimate and plots were made using ggplot2 (3.3.5) package in R (3.6.3). Tumor (12,042) samples from 48 cancer projects were included for this analysis. Overall survival time was used for this analysis. The survival (3.2-13) package in R (3.6.3) calculated the survival curve estimated, which was plotted using ggplot2 (3.3.5) package in R.

KEGG pathway analysis

Pathway analysis was performed for the following genes: BCL2, EGF, EGRF, MYC, PIK3CB, PIK3CD, PIK3R1, PTHR2, and SRC. These genes were input to the Database for Annotation, Visualization, and Integrated Discovery (DAVID 2021) to identify associated pathways. DAVID contains a large knowledge base of functional annotations including GO terms, KEGG pathways, etc. Functional annotation results were filtered to only include those with a Benjamini and Hochberg adjusted P value of <0.05. KEGG pathways were selected for further analysis and visualization. The bar plot figure of KEGG pathways was plotted using ggplot2 (3.3.6) in R (4.1.0).

Cell culture

Human pancreatic ductal epithelial cell line HPDE, PDA cell lines Capan2, CFPAC, HPAFII and MiaPaCa2, hepatocellular carcinoma cell lines SNU449 and SNU475, ovarian cancer cell lines SKOV3 and CAOV3, and breast cancer cell lines HCC1937 and HCC70 were obtained from ATCC (Manassas, VA). Cell lines were maintained in Dulbecco’s Modified Eagle Medium (DMEM; Gibco) supplemented with 10% heat-inactivated fetal bovine serum (HI-FBS; Gibco or Hyclone), 3.4 mM l-glutamine, 90 units (U) per ml penicillin, 90 μg/ml streptomycin, and 1% nonessential amino acids (Cellgro) and cultured at 37°C and 5% CO₂.

Flow cytometry for MUC1 expression

PDA cells were harvested and washed with PBS, followed by incubation with IgG-conjugated to FITC or TAB004-conjugated to FITC in flow buffer (5% FBS in PBS) for 30 minutes in dark on ice. Then the cells were washed and suspended in flow buffer and analyzed by FACS Diva. The data was analyzed using Flow Jo software.

MUC1 mutant generation and transfection

MUC1 Y0, Y1F through Y7F were created using the Quick-Change mutagenesis kit (Stratagene, La Jolla, CA). Briefly, primers based on the MUC1 sequence were designed containing single-base alterations resulting in mutation of the tyrosine residues (Y) in MUC1-CT to phenylalanine (F) as shown schematically (Fig 6, A). Successful mutations were confirmed with DNA sequencing. MUC1-CT mutants and MUC1 WT were cloned into the pLNCX.1 vector consisting of the neomycin resistance gene for retroviral infection. Cells were transfected with Lipofectamine 3000 (Thermo Fisher) according to the manufacturer’s protocol and maintained in complete DMEM containing Geneticin (G418; Invitrogen, Carlsbad, CA). Note: Cells designated as MiaPaca2.Neo were transfected with only the empty vector with neomycin resistance gene. Cells designated as MiaPaca2.MUC1 represent cells expressing full length MUC1 that consists of the ECD, the transmembrane domain and wild type cytoplasmic tail domain. Cells designated as MiaPaca2.Y0 represent cells expressing full length MUC1 that consists of the ECD, the transmembrane domain and mutant cytoplasmic tail domain in which all the 7 tyrosines are replaced by phenylalanine. Similarly, cells designated as MiaPaca2.Y1F through Y7F represent cells expressing full length MUC1 with the tyrosine at that position replaced by phenylalanine, respectively. Every passage of MiaPaca2 transfected cells was maintained in a final concentration of 150 μg/ml of the antibiotic G418 (50 mg/ml) (Thermo Fisher) to ensure positive selection.

Cell survival assay by MTT

Cells were plated at a seeding density of 1 × 10² cells per well in a 96-well plate and grown overnight. Cells were left untreated or treated with Gemcitabine, Paclitaxel, 5-FU, TAB004 alone or IgG isotype control antibody for 24, 48, and 72 hours. Next, MTT (Biotium, Hayward, Calif) solution was added (20 μL/well) to cells and incubated for an additional 3–4 hours. In the final step, media was removed, formazan was dissolved in dimethyl sulfoxide (200 μL/well), and the absorbance read at 560 nm using a colorimetric plate reader. The O.D. value of treated group were calculated as a percentage of the O.D. values of the IgG treated group and plotted as a bar graph in GraphPad Prism. A P value of <0.05 was considered significant.

Colony forming assay

About 500–1000 cancer cells were plated in a 6-well tissue culture plate and allowed to adhere overnight. Next day, the cells were treated with increasing concentrations of TAB004 (5, 10, 15, and 20 μg/ml) for 7–14 days (depending on the doubling time of each cell line). The highest concentration of 20 μg/ml of IgG was used as the isotype control. After 7–14 days, the media was removed, colonies were washed with PBS and fixed with 3:1 solution of Methanol: Acetic acid for 5 minutes, followed by staining with 0.5% (w/v) of Crystal Violet in Methanol for 15 minutes. Then the colonies were washed under running tap water, images were taken, and colonies were counted manually. Colonies consisting of >25 cells were considered. The number of colonies in TAB004 treated wells were calculated as a percentage of colonies in the IgG treated wells and plotted as a kill curve in GraphPad Prism. A P value of <0.05 was considered significant.

Cell detachment assay

About 1000 CFPAC cells were plated in 24-well plates and allowed to adhere overnight. Next day, cells were treated with 20 μg/ml of IgG or TAB004 for 5 hours and images were taken with a microscope at 20x.
Invasion assay

Cells were serum starved for 18 hours before plating for the invasion assay. About 50,000 PDA cells were plated over transwell inserts (Sarstedt) precoated with diluted Matrigel (1:1) in serum free media, with 10 and 20 μg/ml of IgG and TAB004. The cells were allowed to invade through the Matrigel coating for 48 hours toward the serum-containing medium in the bottom chamber. After 48 hours, all the wells were swabbed with a cotton swab, followed by staining of all inserts with 5% crystal violet. The excess stain was washed off and the inserts were allowed to dry overnight. The membrane was cut and dipped in 10% acetic acid for 10 minutes to elute the dye, which was read by a colorimetric plate reader at 560 nm. Percent invasion was calculated as (O.D. of TAB004 treated sample (x μg/ml) / O. D. of IgG treated sample (x μg/ml)) × 100.

Microarray analysis

CFPAC and MiaPaca2 cells were grown overnight in complete DMEM with heat-inactivated FBS. Next day, the cells were treated with 10 μg/ml of IgG or TAB004 for 24 hours and then RNA was extracted from the cells using the Qiagen RNA Mini kit according to the manufacturer’s protocol. The Clarion S transcriptomics was performed by Thermo Fisher Scientific.

Differential gene expression analysis

Differential gene expression analysis was performed on the MiaPaca2 and CFPAC cell line microarray data using the limma (3.42.2) package in R (3.6.3). Three separate differential gene expression analyses were performed: MiaPaca2 data, CFPAC data, and then combined analysis of both MiaPaca2 and CFPAC data. Normalization was done using RMA. Limma identified differentially expressed genes (DEGs) between the IgG control and TAB004 antibody groups. Genes with an adjusted P value of < 0.05 were considered statistically significant. The top 30 DEGs between the 2 groups in both CFPAC and MiaPaca2 cells were presented using a heatmap.

Pathway analysis

The top 30 genes (by unadjusted P value < 0.05 between IgG and TAB004 treatment) were input into Ingenuity Pathway Analysis (IPA) to identify top associated pathways in both MiaPaca2 and CFPAC datasets.

Lysotracker deep red staining for autophagosome formation

About 1000−2000 MiaPaca2 and CFPAC cells were plated in a 24-well plate overnight, next day they were treated with either PBS or 100 μM of Chloroquine phosphate (autophagy inhibitor) for 2 hours followed by treatment with either 20 μg/ml IgG or TAB004 for 5 hours. Lysotracker Deep Red (75nM) (Thermo Fisher Scientific) was added to the cells and incubated at 37°C, 5% CO2 for 30−60 minutes according to manufacturer’s protocol. Fresh medium was added to the cells and photographs were taken with a fluorescence microscope at 20x after applying a filter for Texas Red. Percentage of area positive for red fluorescence from 5 independent fields in the IgG-treated, TAB004-treated and chloroquine phosphate + TAB004-treated cells were calculated using Image J software and plotted as a bar graph in GraphPad Prism. A P value of < 0.05 was considered as significant.

JC1 staining for mitochondrial membrane potential damage

About 1000−2000 cells were plated in a 24-well plate overnight, next day they were treated with IgG or TAB004 for 48 hours. After 48 hours, cells were incubated for 30 minutes with JC-1 dye (Cayman Chemicals) according to the manufacturer’s protocol. Images were taken using a fluorescence microscope after applying a filter for FITC. Percentage of area positive for green fluorescence from 3 randomly selected fields in the IgG-treated and TAB004-treated wells were calculated using Image J software and plotted as a bar graph in GraphPad Prism. A P value of < 0.05 was considered as significant.

Western blot

CFPAC cells were serum-starved for 48−72 hours and treated with 10 μg/ml of IgG or TAB004 for 10, 20, and 30 minutes for analysis of phosphorylation. For detection of other proteins, CFPAC cells were treated with 10 μg/ml and 20 μg/ml of IgG or TAB004 for 48 hours. Cell lysates were prepared using complete lysis buffer (Lysis buffer and 1X Halt Protease and Phosphatase Inhibitor), and 25−60 μg of protein was subjected to denaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot. The polyvinylidene fluoride membrane was blocked for 30 minutes with commercial blocking buffer (Thermo Fisher Scientific) and probed with anti-MUC1 antibody CT2 (1:500 from 0.5 mg/ml stock), TAB004 antibody for MUC1-N (1:5000 from 8.41 mg/ml stock), Phospho-EGFR (Cell Signaling Technology), EGFR, Lamin A/C (Santa Cruz Biotechnology), phospho-PISK, PISK, c-Src, STAT-3, c-Myc, Apaf1, Cleaved Caspase 3, full-length Caspase 3, Cleaved Caspase 9, full-length Caspase 9, COX-IV, and β-actin (1:5000) (Cell Signaling Technology) and Ubiquitin (ABclonal) antibodies. All the antibodies were used at a dilution recommended by the manufacturer, unless mentioned otherwise. Appropriate secondary antibodies conjugated to horseradish peroxidase were used at a dilution of 1:5000, and protein detected using the chemiluminescence kit (Thermo Dura).

Densitometric analyses

The bands on Western blot were quantified using image analysis software Image J from the National Institutes of Health (Bethesda, MD). The target protein bands were normalized with their corresponding β-actin bands on the same membrane and plotted as a ratio in GraphPad Prism 9.0.

Co-immunoprecipitation and mass spectrometry

CFPAC cells were serum-starved for 24 hours and treated with 10 μg/ml of IgG or TAB004 for 20 minutes. After that, the media was removed, cells were washed with PBS and lysate was collected with complete lysis buffer (Lysis buffer with 1X Halt Protease and Phosphatase inhibitor), with the help of a cell scraper. The lysate was vortexed briefly and then sonicated and kept on ice for 10 minutes, followed by centrifugation at 14,000 RPM for 15 minutes at 4°C. The supernatant was collected in a fresh tube, BCA assay was performed by Pierce BCA Assay kit (Thermo Fisher Scientific) to estimate protein concentration as per the manufacturer’s protocol.

For mass spectrometry, 2 μg of lysate was used to pull down tMUC1 with 100 μg of TAB004 antibody using the Pierce Co-IP kit. All the steps were performed according to the manufacturer’s protocol. Protein concentration and purity of the eluate were measured using Nanodrop (Thermo Fisher Scientific), and at least 0.5 μg of protein was used for analysis. Mass spectrometry was performed by Poochon Scientific LLC. At first, double digestion of the MUC1 protein was performed with both Trypsin and Chymotrypsin, followed by mass spectrometry of the digested peptide fragments. A summary of the proteins identified were analyzed in the IgG treated vs the TAB004 treated samples, and the relative abundance of binding to MUC1 (PSM) was analyzed. A ratio of the PSM in the TAB treated and IgG treated samples was generated for each binding partner and the ones with a fold change of ≥2 with a Pvalue of < 0.05 were plotted as a bar graph in GraphPad Prism.

For co-immunoprecipitation, 1 mg of lysate was used to pull down MUC1 with 100 μg of MUC1-CT2 antibody or an anti-Armenian Hamster IgG control antibody using the Pierce Co-IP kit (Cat. No. 26149). All the steps were performed according to the manufacturer’s protocol. For WB, the eluate was mixed with 5X SDS sample loading buffer supplied
by the manufacturer and run on an SDS gel and protocol for WB was followed.

Xenograft studies

Ten 6–8-weeks-old athymic nude, Foxn1

mice (strain number 002019; 5 female and 5 male) were purchased from The Jackson Laboratories and housed at UNC Charlotte’s vivarium. These mice were injected subcutaneously (n = 10) with 1 \times 10^{5} CFPAC cells (50 μl) with Matrigel (50 μl) (total = 100 μl) in a 1:1 ratio, mixed with 500 μg/ml solution of IgG or TAB004 prepared in sterile PBS into the left flank. Once the tumors reached a palpable size (~3 x 3 mm, ~5 days post-tumor inoculation), tumor measurements were taken twice a week. 500 μg/ml of IgG or TAB004 in sterile PBS (50 μl) was injected once a week intratumorally. Mice were monitored three times a week for general health and tumor volumes were measured by Vernier Calipers every 5 days over 40 days until endpoint and once euthanized, tumor wet weight was taken, and tissues collected in formalin (Fig 7, A–E). This study and all procedures were performed after approval from the Institutional Animal Care and Use Committee of UNC Charlotte under IACUC protocol number 21-008.

For the combination treatment, 6–8-weeks-old athymic nude mice (n = 12) were injected with 5 \times 10^{5} CFPAC cells subcutaneously (50 μl) with Matrigel (50 μl) (total = 100 μl) in a 1:1 ratio, into the left flank. Mice were randomized into 4 groups and the treatments were (1) PBS control, (2) TAB004 (500 μg/ml), (3) 5-FU (20 mg/kg), and (4) TAB004 (500 μg/ml) + 5-FU (20 mg/kg). For groups 2 and 4, on the day of injection, CFPAC cells were mixed with 500 μg/ml solution of TAB004 prepared in sterile PBS and then injected into the mice. 5-FU injection was started only after the tumors reached a palpable size. Treatments were injected once a week for 60 days. Measurements were taken with Vernier Calipers every 5 days and the survival of the mice were monitored (Fig 7, F–I). Two-way repeated measures ANOVA was used to determine difference between groups over the period of treatment using GraphPad Prism 9.0.

Immunohistochemistry

Paraffin-embedded sections were incubated at 60°C for 30 minutes in a humidity chamber. Then slides were washed in 3 changes of Xylene for 3 minutes each, followed by hydration in 100%, 90%, and 70% ethanol for 2 minutes each. For nonenzymatic antigen retrieval, sections were heated to 85°C in Dako antigen retrieval solution for 90 minutes and cooled for 20 minutes; all subsequent steps occurred at room temperature. To quench endogenous peroxidase, slides were rinsed and incubated in 2% H₂O₂ in methanol for 10 minutes. Sections were then washed, blocked in 50% FBS in PBS for 45 minutes, and incubated overnight with primary antibodies. Sections were incubated for 1 hour with secondary antibody, developed with a dianobenzidine (DAB) substrate (Vector Inc., Burlingame, CA), counterstained with hematoxylin, washed, dehydrated in 70%, 90%, and 100% ethanol for 2 minutes each, followed by 3 changes of Xylene for 3 minutes each and mounted with Permount. Primary antibodies used were Armenian hamster anti-MUC1-CT CT2 antibody (1:50), TAB004 antibody (1:100) and cleaved Caspase 3 antibody (1:150). Secondary antibodies used were mouse anti-Armenian hamster HRP conjugated antibody (1:100, Jackson Labs) and anti-mouse HRP conjugated antibody (Cell Signaling Technology) (1:100). Mouse IgG was used as negative control. Immunopositivity was assessed using light microscopy and images taken at 100× magnification. Images were quantified using ImageJ software and plotted in GraphPad Prism 9.0.

Statistical analysis

Statistical analysis was performed with GraphPad Prism software 9.0 (La Jolla, CA). Student’s t-test or unpaired Mann–Whitney test was used to determine differences between means of 2 groups. One-way ANOVA or Kruskal–Wallis test was used to compare between 3 or more groups and multiple comparison tests were performed when necessary. A P value of <0.05 denoted by an asterisk (*) was considered statistically significant.

Results

MUC1 is overexpressed in majority of epithelial cancers, correlates with poor overall survival and anoiiks-resistance genes

We first confirmed MUC1 overexpression in majority of epithelial cancers as analyzed from the TCGA database (Fig 1, A). Compared to normal samples (n = 1603), the expression of MUC1 was significantly higher in tumor samples across multiple tumor types (n = 13,509) (Fig 1, A). Overexpression of MUC1 was found to be significantly correlated (P = 0.017) with poorer overall survival in epithelial tumors (n = 12,042) across 48 different tumor types (Fig 1, B). The TCGA dataset was analyzed for significantly DEGs in normal vs tumor samples in human PDA (data not shown). The list of DEGs was curated for finding a subset of 9 genes that are associated with anoikis resistance, as determined from the literature, and from those that were significantly upregulated in PDA (Fig 1, C). These 9 genes belong to the family of growth factor and its receptors (EGF, EGFR), GPCRs (PI3K and PI3KR), nonreceptor tyrosine kinases (c-Src), transcription factors (c-Myc) or regulators of apoptosis (PTHR2 and BCL2). These genes were found to be significantly associated with stemness, drug resistance, regulation of actin cytoskeleton, focal adhesion, autophagy, and apoptosis by KEGG pathway analysis (Fig 1, D). Survival data showed that overexpression of these 9 genes significantly correlated with poor overall survival (Fig 1, E). The list from Fig 1, C was further filtered to find correlation with MUC1 expression levels in normal vs tumor samples. Seven out of 9 genes showed a trend of positive correlation with MUC1 expression levels in normal vs tumor samples in PDA (Fig 1, F). PTHR2 and SRC were filtered out due to high variability. The types of epithelial cancers analyzed and patient characteristic table are in the Supplementary Tables 1 and 2.

Treatment with TAB004 reduces survival, colony forming potential, and invasion in PDA cells

A panel of PDA cell lines were analyzed for their MUC1 expression using TAB004-FTTC, that binds to tumor-associated MUC1 (tMUC1) (Fig 2, A) and using another antibody directed to MUC1-CT (Supplementary Fig 1, A). CFPAC and Capan 2 cells expressed high levels of tMUC1 on their surface, HPAF II expressed medium levels and MiaPaca2 expressed low levels of tMUC1. TAB004 treatment significantly reduced the colony forming ability of PDA cells in a dose dependent manner (Fig 2, B). Representative images of the colony forming plates for Capan 2 and CFPAC are also shown in Fig 2, B. The percentage of tMUC1 positive cells and IC₅₀ values for each cell line are shown in the table (Fig 2, C). In general, PDA cells expressing higher tMUC1 showed higher IC₅₀. To confirm that cell survival was impacted, we used the MTT assay on CFPAC, MiaPaca2, and normal HPDE cells and show that post TAB004 treatment, the PDA cells have 50% survival while the normal cell line remains unaffected (Fig 2, D). HPDE normal cells did not form colonies and therefore was not included in the colony forming assay. Furthermore, TAB004 treatment significantly reduced the percent of PDA cells that invaded the matrix in a trans-well invasion assay (Fig 2, E). When tested in combination with other chemotherapy drugs, TAB004 enhanced the antitumor efficacy of 5-FU and Gemcitabine but not paclitaxel as measured by significant reduction in survival of CFPAC cells in the combination group of TAB004 and drugs (Supplementary Fig 1, B). TAB004 significantly reduced the colony forming potential of other epithelial cancer cells including breast, ovarian and hepatocellular carcinoma expressing varying levels of MUC1 (Supplementary Fig 1, C).
Fig 1. MUC1 is overexpressed and correlates with anoikis-resistance and poor overall survival. (A) MUC1 gene expression values in normal and tumor samples from the pan-cancer analysis. A total of 15,112 samples were included, 13,509 tumor and 1603 normal samples. Plot was generated from the Differential Gene Correlation Analysis (DGCA) (1.0.2) package in R (3.6.3). (B) Kaplan-Meier survival analysis with MUC1 gene expression from pan-cancer analysis. (C) Heatmap showing scaled expression values for genes PIK3R1, PIK3CD, PIK3CB, EGFR, EGF, BCL2, MYC, PTRH2, and SRC in samples obtained from the TCGA pancreatic cancer (PAAD) project. (D) Pathway analysis was performed for the following genes: BCL2, EGF, EGFR, MYC, PIK3CB, PIK3CD, PIK3R1, PTRH2, and SRC, and the list of KEGG pathways that these genes were significantly associated with are shown as a bar plot. (E) Kaplan-Meier survival analyses with expression of the 9 genes from pan-cancer analysis. (F) Heatmap showing gene correlation values with MUC1 expression, calculated from DGCA, for PIK3R1, PIK3CD, PIK3CB, EGFR, EGF, MYC, and BCL2 genes in normal vs. tumor samples (PAAD project) (The SRC and PTRH2 genes were filtered out during preprocessing filtering steps).
Treatment with TAB004 reduces activation of EGFR-PI3K pathway as measured by phosphorylation of EGFR and PI3K

To evaluate the effect of TAB004 treatment on downstream oncogenic signaling, we assessed the EGFR-PI3K pathway post treatment with TAB004. CFPAC cells were serum-starved overnight and treated with EGF with or without pretreatment with IgG or TAB004. EGF induced phosphorylation of EGFR and PI3K in CFPAC cells within 10 minutes (Fig 3, A and B). When the cells were pretreated with TAB004 for 20 minutes, EGFR and PI3K phosphorylation was significantly reduced (Fig 3, A and B).

Gene expression changes induced by treatment with TAB004 in PDAC cells

To further understand the underlying mechanism of TAB004’s anti-tumor effects, we performed transcriptomics on 2 PDAC cell lines CFPAC (tMUC1-high) and MiaPaca2 (tMUC1-low) post 24-hours treatment with either IgG or TAB004. The DEGs in CFPAC were primarily metabolism regulating genes or cell-cycle and apoptosis regulating genes (Fig 4, A). The DEGs were involved in cell cycle regulation and apoptosis (E2F1, WRAP73, ZC3H4, GIAPIN1, TMEM127, TRIB2), protein degradation (ASB16-AS1 and UBE2S), cell-cell adhesion, ECM remodeling and migration (CDH23, CNTN2, CCBE1), endoplasmic reticulum stress and hypoxia (SCAMP5, EGLN3) (Fig 4, B). UBE2S is an E3 Ubiquitin ligase.

In MiaPaca2, most changes were observed in the amino acid transport and metabolism (SLC6A9, PSAT1, MTHFD2, PHGDH), ER stress and autophagy (ATF4 and autophagy (ATF4 and SLC7A5) (Fig 4, A and B). ER stress and nutrient deprivation usually lead to autophagy in cancer cells as an early stress response. To confirm increased lysosomal activity and induction of autophagy, we treated MiaPaca2 and CFPAC cells with IgG or TAB004 with or without pretreatment with autophagy inhibitor chloroquine phosphate and stained the cells with Lysotracker Deep Red. A significant increase in number of cells undergoing autophagy (as determined by increased lysosomal activity) was observed 5 hours post TAB004 treatment as compared with IgG treatment, which was reversed on blocking autophagy (Fig 4, C).

TAB004 leads to degradation of MUC1, disrupts MUC1-CT signaling, and induces apoptosis

Assuming that TAB004 treatment induces nutrient deprivation and autophagy (based on Fig 4), we evaluated if the treatment with TAB004 led to increased degradation of tMUC1 protein itself in CFPAC cells. After 48 hours of TAB004 treatment, there was significant reduction in cytoplasmic MUC1 levels, although no significant change in nuclear MUC1 was observed (Fig 5, A). We hypothesize that due to degradation of MUC1, the binding site for many desmosomal and cell adhesion proteins and intracellular kinases and protooncogenes may be reduced, thus leading to “anoikis”. TAB004 treatment significantly reduced protein levels of EGFR, c-Src, STAT3, c-Myc, and MUC1 (using both the MUC1-N and MUC1-CT antibodies) (Fig 5, B). These binding partners also happen to be transcriptional targets of MUC1-CT.31-34 Thus, the essential drivers of survival, stemness and anoikis resistance may have decreased binding to MUC1-CT, therefore, reducing subsequent downstream signaling.

Since the EGFR-PI3K activation was reduced post treatment with TAB004 (Fig 3) and because this pathway is known to confer anoikis resistance in cancer cells,35 we determined the levels of their
downstream effectors PTRH2 and BCL2 after 48 hours of TAB004 treatment (Fig 5, C). Treatment with TAB004 showed reduction in expression levels of PTRH2 and BCL2 (Fig 5, C) as well as showed increased expression of Apaf1 and cleavage of Caspases 9 and 3 (Fig 5, D). Data show that treatment with TAB004 led to increased cleavage of Caspase 8 (Fig 5, E). Cleavage of Caspase 8 into the p18 subunit is a hallmark of activation of the extrinsic apoptotic pathway. Release of cytochrome C is a hallmark of intrinsic or mitochondrial apoptosis activation. Thus, we isolated mitochondria in TAB004-treated CFPAC cells by subcellular fractionation and found that cytochrome C subunit 4 (COX IV) was released only in the cytosolic fraction of the TAB004 treated cells (Fig 5, F). To check if there is indeed mitochondrial membrane damage, we stained TAB004-treated MiaPaca2.Neo (expressing empty vector) cells with JC-1 dye and found that TAB004 induced mitochondrial membrane damage.

Fig 2. TAB004 reduces tumorigenic properties of PDA cells. (A) Expression of tMUC1 in PDA cells HPDE, Capan2, CFPAC, HPAFII and MiaPaca2 (left to right). (B) (Left) Graph showing percent of colonies formed by the 4 PDA cell lines with increasing concentrations of TAB004 (5, 10, and 20 μg/ml) after 7 days. Percentage of colonies were calculated as (number of colonies in TAB004 treated wells/number of colonies in IgG treated wells) X 100) (Right). Representative images showing the number of colonies in 20 μg/ml IgG and TAB004 treated Capan2 (top) and CFPAC cells (bottom). (C) Table showing the MUC1 expression levels and IC50 of TAB004 on the 4 PDA cells. (D) Graphs showing the percentage of survival in CFPAC, MiaPaca2, and HPDE (left to right) cells after 48 hours of treatment with 80 μg/ml of TAB004 by MTT assay. (E) Invasion was determined by standard transwell assay and results are presented as percentage of cells invading the Matrigel after treatment with 50 μg/ml of IgG and TAB004 for 48 hours. Percent invasion was calculated as (O.D. of TAB004 treated sample with conc. x/ O.D of IgG treated sample with conc. x) X 100). All the experiments were performed in 3 independent replicates and the data are shown as + SEM, P < 0.05 *, P < 0.01 **, P < 0.001 *** , P < 0.0001 ****

Fig 3. TAB004 blocks EGFR-PI3K pathway. (A) Western blot showing levels of P-EGFR, EGFR, P-PI3K, and PI3K before and after treatment with 10ng/ml of EGF for 10 minutes and 10 μg/ml of IgG or TAB004 for 20 minutes. β-actin was used as endogenous loading control. (B) Densitometric analyses showing the ratios of expression levels of the phosphorylated proteins and their total counterparts after normalization with β-actin levels. All the experiments were performed in 3 independent replicates with similar results and one representative image is shown.
in PDA cells (Fig 5, G). The quantification of the number of apoptotic cells is shown as percentage of area positive for JC1 green fluorescence in Fig 5, G insert. Therefore, TAB004 induced activation of both the extrinsic and intrinsic apoptotic pathways that generally occurs on activation of anoikis.

**Differential response of MUC1 cytoplasmic tail mutants to treatment with TAB004**

Various nonreceptor and receptor tyrosine kinases phosphorylate MUC1-CT at specific tyrosine residues which enables MUC1-CT to function as a co-transcription factor to regulate gene expression (schematic shown in Fig 6, Aii). First, we show that binding of TAB004 to tMUC1-N leads to phosphorylation of its tMUC1-CT at tyrosine 1229 (Y6) within 10 minutes and this phosphorylation increases at 20 minutes and declines at 30 minutes (Fig 6, Aii). Since c-Src is known to phosphorylate MUC1-CT at Tyr1229,33 we hypothesized that the kinase responsible for CT phosphorylation is c-Src, especially since there was also c-Src phosphorylation at 10 minutes post TAB004 treatment and it declined by 20 minutes (Supplementary Fig 2, A). Furthermore, treatment with SRC-inhibitor PP2 reversed phosphorylation of MUC1 and c-Src (Supplementary Fig 2, B). Therefore, to assess if binding of TAB004 to tMUC1 blocks signaling through the tyrosines in MUC1-CT and confers TAB004’s anti-colony forming effects (shown in Fig 2), we generated point mutations in MUC1-CT to replace the tyrosines (Y) with phenylalanine (F) (Fig 6, Aiii) and transfected MiaPaca2 cells with the mutant constructs. The western blot showing the expression of MUC1 in the transfected MiaPaca2 cells is presented in Supplementary Fig 1, D. We observed differential sensitivity to TAB004 mediated inhibition of colony forming potential in the mutants. We report that Y0, Y6F, and Y7F were the least sensitive to TAB004 with the highest IC50 dose (Fig 6, B). Representative Images of the colony forming results are shown in Fig 6, C. At 10 μg/ml,
TAB004 treatment did not significantly reduce the colony forming potential of YO, Y6F, and Y7F (Fig 6, D). JC-1 staining of TAB004 treated cells showed highest mitochondrial membrane damage in MiaPaca2Neo cells (cells that have been transfected with an empty vector and deemed as the true control for the other mutants), followed by MiaPaca2.MUC1 and almost no mitochondrial membrane damage in MiaPaca2.Neo cells after 48 hours (Fig 6, E). Therefore, MUC1-CT signaling via its tyrosines plays a significant role in the TAB004 mediated inhibition of colony formation and mitochondrial membrane damage.

**Treatment with TAB004 significantly reduces tumor growth in vivo, induces apoptosis, and shows reduction in MUC1 expression**

To verify if TAB004 has any significant therapeutic efficacy alone, we treated CFPAC xenograft bearing nude mice with either IgG control or TAB004 once a week for 6 weeks. TAB004 slowed down tumor growth significantly compared to IgG (Fig 7, A). At endpoint, the tumor burden in TAB004 treated samples were significantly less than the IgG treated samples (Fig 7, B), and it did not have any adverse effect on the body weight of the animals (Fig 7, C). TAB004 treated tumor tissues showed an increased level of cleaved Caspase 3 compared to the IgG treated tissues (Fig 7, D). These data confirmed the intracellular activation of apoptotic signaling induced by TAB004 treatment in vivo. In addition, MUC1-N and MUC1-CT were both significantly reduced in TAB004-treated tissues (Fig 7, E). To assess if TAB004 enhances the antitumor efficacy of 5-FU in vivo, we treated CFPAC xenograft bearing nude mice with PBS control, TAB004, 5-FU, and TAB004 + 5-FU. As expected, TAB004 alone was sufficient to significantly slow down tumor growth, and when combined with 5-FU, TAB004 enhanced its antitumor efficacy and prolonged the survival of mice (Fig 7, F and H). Mice in the combination (TAB004 + 5FU) group survived longer than the rest of the groups. Although there was no significant difference in tumor growth rate between the treatment groups themselves, TAB004 + 5-FU was the most effective treatment compared to PBS control group showing significant reduction in tumor growth rate, tumor volume, and enhanced survival without any adverse effects on the animals’ body weights (Fig 7, F–I).

**TAB004 disrupts the desmosomal assembly and colony forming factors by degrading their association with MUC1**

To prove that apoptosis was induced by disruption of cell-cell adhesion, we performed mass spectrometry analysis of CFPAC cells treated with TAB004 for 20 minutes and found decreased binding of cell-attachment molecules Desmplakin, Junction Plakoglobin (γ-catenin), Desmoglein, Keratin, Desmocollin, and Galectin-7 with MUC1 (Fig 8, A), indicating that TAB004 triggers detachment of cells from the matrix. TAB004 also inhibited phosphorylation and activation of Desmplakin at Serine 2209, inhibited N-Acetylation of Junction Plakoglobin and induced methylation loss and N-acetylation gain of Keratins 14 and 16 with TAB004 for 20 minutes and found decreased binding of cell-attachment molecules Desmplakin, Junction Plakoglobin (γ-catenin), Desmoglein, Keratin, Desmocollin, and Galectin-7 with MUC1 (Fig 8, A), indicating that TAB004 triggers detachment of cells from the matrix. TAB004 also inhibited phosphorylation and activation of Desmplakin at Serine 2209, inhibited N-Acetylation of Junction Plakoglobin and induced methylation loss and N-acetylation gain of Keratins 14 and 16 (Fig 8, A). The reduced association of MUC1 with E-cadherin, junction plaque antigen 2, 14-3-3, and Cadherin 14-3-3 further confirmed disruption of the co-
Fig 6. MUC1-CT tyrosine mutants show differential sensitivity to TAB004. (A): A schematic of the MUC1-CT with the 7 tyrosine residues in red and the kinases that are known to phosphorylate the tyrosines. (Aii): Western blot showing increased phosphorylation of MUC1-CT at Tyr 1229 at 10, 20, and 30 minutes after treatment with 10 μg/ml of TAB004. CFPAC cells were serum-starved for 72 hours and then treated with 10 μg/ml of IgG and TAB0004. Total MUC1 levels are shown, and β-actin was used as the endogenous loading control. Densitometric analysis showing increased phosphorylation of MUC1-CT at Tyr 1229 at 10 minutes that goes up at 20 minutes and then comes down at 30 minutes. (Aiii) A schematic of the WT and the point mutations of tyrosine to phenyl alanine substitution. Authenticity of the various mutated fragments carrying individual Y-F mutations was verified by sequencing. (B) Graph showing the percentage of colonies formed by the ten MiaPaca2 MUC1-CT mutant cell lines after treatment with increasing concentrations of TAB004 (5, 10, 15, and 20 μg/ml). IC50 value of TAB004 in μg/ml for each of the mutants listed in a chart. (C) Images of colonies formed by the MiaPaca2 MUC1-CT mutant cells after treatment with 10 μg/ml IgG and TAB004 after 7 days. (D) Graph showing the percentage of colonies formed by the MiaPaca2 MUC1-CT mutant cells after treatment with 10 μg/ml IgG and TAB004 after 7 days. (E) Representative fluorescence images of MiaPaca2 Neo, MiaPaca2 Y0, and MiaPaca2 MUC1 cells after treatment with 80 μg/ml of IgG and TAB004 for 48 hours followed by JC-1 staining for 30 minutes. Percentage of area positive for JC1 staining in IgG and TAB004 treated cells was plotted as a graph and a P value of < 0.05 was considered as significant. All experiments were performed in 3 independent replicates and data are shown as + -SEM. * P < 0.05 *, ** P < 0.01 ***, *** P < 0.001 ****
transcriptional activity of MUC1 on treatment with the antibody (Fig 8, A). Other novel MUC1 associations disrupted were EF2 (Elongation factor 2) and Arginase 1 (Fig 8, A). Microscopic images of single cells treated with TAB004 showed that it significantly reduces the capacity of cancer cells to remain attached to a matrix thus destroying their colony forming potential (Fig 8, B).

Albeit, not statistically significant, it appears that there was an increased association of MUC1 with the ER stress response and the proteasomal machineries, for instance, BIP, GRP-75, ER Resident Protein, Proteasomal subunit and Proteasomal Ubiquitin receptor on TAB004 treatment (Fig 8, C). This indicates that TAB004 induced ubiquitination of tMUC1. To confirm this, we pulled down MUC1 after treatment with TAB004.
**Fig 8.** TAB004 compromises the desmosomal assembly and disrupts colony forming factors by degrading their association with MUC1. (A) Mass spectrometry data plotted as fold change of relative abundance or PSM value (Peptide Spectrum Matches) of cytoskeletal proteins and known tumorigenic factors found in Co-IP samples from CFPAC lysates treated with 10 μg/ml IgG or TAB004 for 20 minutes. The mass spectrometry was performed on 2 independent biological replicates of CFPAC treated cells. (B) Microscopic images of CFPAC cells treated with 20 μg/ml IgG or TAB004 after 5 hours showing detachment from the surface. Quantification was performed by counting the number of detached cells in IgG and TAB004 treated wells and plotted as a percentage of total number of cells. (C) Mass spectrometry data plotted as fold change of relative abundance or PSM value of proteins associated with ER stress and proteasomal degradation found in Co-IP samples from CFPAC lysates treated with 10 μg/ml IgG or TAB004 for 20 minutes. Data are shown as ± S.E.M. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 (D) Co-IP on CFPAC cell lysates after 20 minutes of treatment with 10 μg/ml of IgG or TAB004. Lysates were pulled down with isotype IgG control or MUC1-CT2 antibody and WB was performed for Ubiquitin and MUC1. Total MUC1 was used to show input. Similar results were obtained in at least 2 independent experiments and one representative image has been shown. (E) A schematic diagram showing the mechanism of action of TAB004 in blocking tMUC1-oncogenic signaling.
IgG or TAB004 for 20 minutes and checked the level of ubiquitination. Indeed, there was increased ubiquitination of MUC1 after TAB004 treatment (Fig 8, D).

The mechanism of action of TAB004 in blocking tMUC1 oncogenic signaling and anoikis-resistance is shown as a schematic diagram (Fig 8, E). On binding of TAB004 to tMUC1, there is phosphorylation and ubiquitination of MUC1-CT which tags it for autophagy-lysosomal degradation, thus decreasing its binding with desmosomal proteins as well as transcriptional binding partners, in turn, blocking the oncogenic signaling. This blockage prevents activation of PI3K-PTRH2-BCL2 mediated antiapoptotic gene functions, and reduces overall expression of STAT3, c-Src and c-Myc (Fig 5). Furthermore, TAB004 binding to tMUC1 reduces the binding of EGF to EGFR, enhancing the inhibition of downstream signaling (Fig 3).

**Discussion**

Our study demonstrates the antitumor effects of a tMUC1 antibody, TAB004, in vitro and in vivo and identifies the underlying mechanisms associated with the antitumor effects of the antibody. The DEGs in normal vs PDA from the TCGA database included 9 anoikis-resistance genes (Fig 1, C), with 7 of these genes correlating with MUC1 expression (Fig 1, D) including EGF, EGFR, PI3KR1, PI3KCB, PI3KCD, c-Myc, and BCL2. The genes that positively correlated with MUC1 also correlated with poor overall survival (Fig 1, E). These data are in line with other studies that have shown MUC1 to confer anoikis-resistance to other epithelial cancer cells.14,15 Further bioinformatics analysis illustrated that these genes were significantly associated with various pathways including stemness, drug resistance, autophagy, apoptosis, rearrangement of the cytoskeleton, and focal adhesion (Fig 1, F). MUC1 is known to enhance the expression and function of the ABC transporters to confer chemoresistance via upregulation of EGFR.16,37 Thus, it was interesting to observe that TAB004 treatment significantly reduced phosphorylation of EGFR (Fig 3) and increased the sensitivity of PDA cells to chemotherapeutic drugs like Gemcitabine and 5-FU (Supplementary Fig 1, B).

Because tMUC1 and EGFR-PI3K associated signaling collaborate extensively to enhance many of the oncogenic signaling pathways including anoikis-resistance and drug resistance (Fig 1),34,38 we hypothesized that TAB004 may block activation of EGFR-PI3K pathway via blocking signaling through its CT. Indeed, pretreatment of CFPAC with TAB004 failed to significantly phosphorylate EGFR and PI3K even after 10 minutes of exposure to EGF (Fig 3, A and B). Although not proven in this paper, we believe that TAB004 binding to surface tMUC1-N prevents binding of EGF to EGFR (due to steric hindrance), thus inhibiting phosphorylation of EGFR and PI3K.

To confirm that the effects of TAB004 binding to tMUC1-N triggers signaling through its CT, we show that inhibition of colony formation induced by TAB004 treatment was completely rescued in cells (Mia-Paca-2-Y0, Y6, and Y7) that lacked the critical tyrosines necessary for TAB004-mediated blockage of tMUC1-associated downstream oncogenic signals (Fig 6, A–E). The differential sensitivity of the tyrosine mutants to TAB004 may be due to differential tMUC1 turnover rate. tMUC1 turnover is maintained via ubiquitination at lysine 1231 by the Ubiquitin E3 ligase WWPE1.39 Thus, we hypothesize that both tyrosines 1229 (Y6) and 1243 (Y7) aid in binding of WWPE1 to K1231, and therefore, when these 2 sites are mutated to phenylalanine, there is less binding of WWPE1 to MUC1-CT, leading to reduced degradation by the proteasomal machinery. Ubiquitination of membrane proteins triggers their internalization and targets them for degradation by the lysosomal pathway.40,44 Therefore, mutation of the critical tyrosines block phosphorylation and hamper the binding of the Ubiquitin proteins, thus decreasing MUC1 degradation and resisting TAB004 induced apoptosis. The Y7 of MUC1-CT is critical for binding of MUC1 to GRB2 and getting targeted for internalization.45 Tyrosine Y2 has been shown to bind to adaptor protein AP2 and its mutation led to reduced internalization of MUC1.46 Our data shows that phosphorylation of Y1229 by c-Src is crucial for TAB004-induced blockage of tMUC1 signaling (Fig 5) and that Y6 and Y7 are the most critical residues for blocking TAB004-induced antitumor effects (in the colony formation assay).

TAB004 was most potent in reducing the colony forming potential of the PDA cells and induced detachment and rounding of the cells by disruption of the desmosomal assembly, a phenomenon consistent with anoikis (Fig 8, B). In normal cells, loss of attachment to the ECM induces anoikis. Resistance to anoikis in cancer cells promotes their ability to survive in circulation, with subsequent colonization to distant anatomic sites, leading to tumor metastasis.47,48 TAB004 treatment reversed anoikis-resistance in these cells in both a ligand dependent and independent manner. In a ligand-dependent manner, TAB004 blocked the binding of EGF to EGFR and reduced activation of PI3K, thus inhibiting growth signal and inducing cell detachment and apoptosis (Fig 2 and 3).

Mass spectrometry data also showed significantly decreased binding of tMUC1 to Desmoplakin, Myosin 9 (MYH9), Junction Plakoglobin (γ-catenin), Desmoglein, Keratin, Galectin-7 post TAB004 treatment indicative of disruption of the desmosomal assembly in the cells (Fig 8, A). Additional modifications post-treatment with TAB004 were the loss of phosphorylation at Ser 2209 of Desmoplakin and loss of acetylation of Plakin (Fig 8, A). The role of these post-translational modifications of the desmosomal proteins have been well documented to keep cell membrane integrity and potential for adhesion.49 MUC1 is known to bind to desmosomal proteins and aid in transendothelial migration.50,52 MUC1 has been shown to bind to Junction Plakoglobin or γ-catenin (that can substitute for the function of β-catenin in some cells), translocate into the nucleus and drive expression of oncogenes.52 There was decreased association of tMUC1 with other pro-tumorigenic binding partners including 14-3-3 Stratifin and β-catenin,52-54 thus blocking majority of the co-transcriptional activity of tMUC1 (Fig 8, A). In addition, we found decreased binding of tMUC1 with EF2 (elongation factor 2) and Arginase1 (Fig 8, A) which are novel interactions and need to be further explored. Therefore, in a ligand-independent manner, TAB004 binding to tMUC1 compromised the capability of the cells to form colonies and/or attach to the ECM or the neighboring cells. The role of desmosomal proteins in regulation of tumorogenesis has been described extensively.52

Data suggest that degradation of tMUC1 destroyed the docking site of crucial pro-survival factors like EGFR, PI3K, c-Src, STAT3, and c-Myc (Fig 5), thus blocking the entire oncogenic cascade. Transcription factors like γ-catenin, β-catenin, NF-κB, STAT3, and c-Myc, out of many, use MUC1-CT as an adaptor protein or co-transcription factor to regulate expression of oncogenes. Therefore, TAB004 destroys the hub of this oncogenic nexus and renders tumor cells unable to revive and colonize to different locations after dissemination. Fig 8, E is an attempt to summarize the mechanism of action of TAB004 antibody and its antitumor effects.

Multiple studies on the mechanism of anti-MUC1 antibodies as monotherapy or in combination with drugs have been published before, showing activation of the apoptotic pathways and blocking of EGFR and PI3K pathways.55,56 However, this is the first time that a tMUC1 antibody (TAB004) has been shown to induce phosphorylation of MUC1-CT by activating c-Src and reverse anoikis-resistance by degrading tMUC1 with an in-depth transcriptomic and proteomic analysis leading to the functional effect. In addition, our study has explored the domain of desmosomal assembly and its association with MUC1 that plays a huge role in migration and colony formation. We have shown the effect of TAB004 with respect to both MUC1-N terminal and C-terminal domains. Recently, there has been an increasing interest in targeted protein degradation owing to its potential to therapeutically modulate proteins that have been difficult to target with conventional small molecules. Proteolysis-targeting chimera protein degraders are being developed that are heterobifunctional small molecules consisting of 2 ligands joined by a linker: one ligand recruits and binds a protein of interest (POI) while the other simultaneously recruits and binds an E3 ubiquitin ligase. This induces ubiquitylation of the POI followed by degradation by the
ubiquitin-proteasome system, and then proteolysis-targeting chimera is recycled to target another copy of the POL.57 TAB004 naturally targets tMUC1 for degradation and further research with it could aid in development of advanced molecular therapies to enhance drug efficacy.

In keeping with the in vitro data, we confirm our findings in vivo in a xenograft model. Mice treated with TAB004 alone or in combination with 5FU showed the slowest tumor growth rate compared to the control group and significantly enhanced the survival (Fig 7, F–H), without any mal-effects on their body weight (Fig 7, I). The most significant observation with TAB004 treatment of PDA cells was its ability to reduce colony formation of PDA cells. Therefore, our objective was to deliver TAB004 along with a small number of cancer cells to assess its efficacy as a prophylactic treatment. One limitation of this experiment was that the number of initial cells was already too large (0.5 to 1 x 10⁶ cells) compared to the dose of antibody administered. Nevertheless, we observed significant reduction in tumor growth (Fig 7, A). In future, we aim to optimize the ratio of starting number of cells to the dose of antibody to better assess the prophylactic potential of TAB004.

In summary, TAB004 treatment triggered tyrosine-phosphorylation and ubiquitination of tMUC1, thus inducing its degradation by the autophagy-lysosomal pathway. This reversed the tMUC1-mediated anoikis resistance in vitro and in vivo. We provide an in-depth transcriptomics and proteomics analysis of how TAB004 binding to its receptor tMUC1 significantly alters several intracellular oncogenic signaling cues and induces its effects. We suggest that TAB004 may be further explored as a prophylactic agent post-surgery and/or in combination therapies to reverse drug and small molecule inhibitor resistance and its impact on minimal residual disease should be assessed to diminish recurrence and metastasis in epithelial cancers. Although the data presented thus far are in PDA cells, we found that other epithelial tumor cell lines responded to TAB004 as effectively as PDA. TAB004 treatment significantly reduced the colony forming potential of several other human epithelial cancer cell types, including, hepatocellular (SNU449 and SNU475), ovarian (SKOV3 and CAOV3) and triple-negative breast cancer cell lines (HCC1937 and HCC70) (Supplementary Fig 1, C).

We recognize that other MUC1 antibodies have not been clinically very successful mostly because of 2 major reasons, one being their binding to circulating MUC1 and not reaching the tumor site, and second being their binding to normal MUC1 expressed in normal epithelial cells. There are a couple of reasons why we believe that TAB004 is a better candidate antibody that has the potential to be successful in the clinic. TAB004 does not bind to MUC1 in solution and does not bind normal MUC1. However, TAB004 has not been tried clinically and we do not know the challenges that may come about. In the future, we will also use TAB004 as a cargo in liposomes or nanoparticle that will help fusion with cell membrane thus reducing binding to serum tMUC1. The other way to circumvent binding of TAB004 to serum tMUC1 is to administer it directly to the tumor site likely after surgery. Intratumoral injections boost the antitumor efficacy and therapeutic index of drugs and overcome barriers to systemic immunotherapy efficacy. The feasibility of intratumoral delivery of antitumor therapy has been discussed previously.58,59

Supplementary Fig 1. TAB004 increases the efficacy of chemotherapeutic drugs and reduces colony forming potential of multiple epithelial cancer cells. (A) Western blot showing MUC1 expression in PDA cell lines with MUC1-CT antibody. (B) MTT cell survival assay on CFPAC cells with 10 μM of Gemcitabine, Paclitaxel and 5FU alone or in combination with 20 μg/ml TAB004 for 48 hours. Percentage of survival was calculated as a fold change of combination over drug alone. (C) Colony forming assay on 2 hepatocellular carcinoma cell lines (SNU449 and SNU475), 2 ovarian cancer cell lines (SKOV3 and CAOV3) and 2 TNBC cell lines (HCC1937 and HCC70) with increasing concentration of TAB004 (5, 10, 15, and 20 μg/ml). All experiments were performed in 3 independent replicates and data are shown as + S.E.M. The P values for comparison between TAB004 and control for each cell line are shown in the table. * P < 0.05, ** P < 0.01, *** P < 0.001, and **** P < 0.0001.

D. Western blot showing MUC1 expression in MiaPac2 cells expressing the empty vector Neo and MUC1-CT tyrosine mutants (Y0, Y1F through Y7F and MUC1). β-actin was used as endogenous control.

Supplementary Fig 2. TAB004 induces phosphorylation of MUC1-CT through c-Src. (A) Western blot showing increased phosphorylation of c-Src at 10, 20, and 30 minutes after treatment with 10 μg/ml of TAB004. Total c-Src was used as the endogenous control. (Right) Densitometric analysis showing increased phosphorylation of c-Src after TAB004 treatment. CFPAC cells were serum-starved for 24 hours and then treated with 10μg/ml of IgG and TAB0004. (B) Western blot showing phosphorylation of MUC1-CT at Tyr 1229 (left) and c-Src (right) after CFPAC cells were serum-starved for 24 hours and treated with Src inhibitor PP2 for 30, 45, and 60 minutes followed by treatment with 10μg/ml of TAB004 for 20 minutes. Total MUC1 and c-Src were used as respective endogenous controls. (Right) Densitometric analysis showing that pretreatment with PP2 led to decreased phosphorylation of MUC1 at Tyr1229 (left) and p-c-Src (right) after TAB004 treatment. Similar results were obtained in at least 2 independent experiments and one representative image has been shown.

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Data availability statement: The RNA-seq data used for analysis are openly available at the Genomics Data Commons (GDC) portal (https://portal.gdc.cancer.gov).

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Supplementary materials

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