Small-Molecule Inhibition of GCNT3 Disrupts Mucin Biosynthesis and Malignant Cellular Behaviors in Pancreatic Cancer

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Abstract

Pancreatic cancer is an aggressive neoplastic disease with almost uniform lethality and a 5-year survival rate of 7%. Several overexpressed mucins that impede drug delivery to pancreatic tumors have been therapeutically targeted, but enzymes involved in mucin biosynthesis have yet to be preclinically evaluated as potential targets. We used survival data from human patients with pancreatic cancer, next-generation sequencing of genetically engineered Kras-driven mouse pancreatic tumors and human pancreatic cancer cells to identify the novel core mucin-synthesizing enzyme GCNT3 (core 2 β-1,6 N-acetylglucosaminyltransferase). In mouse pancreatic cancer tumors, GCNT3 upregulation (103-fold; \( P < 0.0001 \)) was correlated with increased expression of mucins (5 to 87-fold; \( P < 0.04\) to \( P < 0.0003 \)). Aberrant GCNT3 expression was also associated with increased mucin production, aggressive tumorigenesis, and reduced patient survival, and CRISPR-mediated knockout of GCNT3 in pancreatic cancer cells reduced proliferation and spheroid formation. Using in silico small molecular docking simulation approaches, we identified talinumlate as a novel inhibitor that selectively binds to GCNT3. In particular, docking predictions suggested that three notable hydrogen bonds between talinumlate and GCNT3 contribute to a docking affinity of \(-8.3\) kcal/mol. Furthermore, talinumlate alone and in combination with low-dose gefitinib reduced GCNT3 expression, leading to the disrupted production of mucins in vivo and in vitro. Collectively, our findings suggest that targeting mucin biosynthesis through GCNT3 may improve drug responsiveness, warranting further development and investigation in preclinical models of pancreatic tumorigenesis. Cancer Res; 76(7): 1965–74. ©2016 AACR.

Introduction

Pancreatic cancer is a lethal disease, and its management is an ongoing challenge. Pancreatic cancer is the fourth leading cause of deaths due to cancer in the United States (1). It is a highly aggressive cancer that is usually diagnosed at an advanced stage and has the worst prognosis of any malignancy, with a 7% 5-year survival rate due to high chemoresistance. This chemoresistance is due in part to altered expressions of mucins, which form a mesh that makes target sites inaccessible to drugs (2–9). Clinical and preclinical studies have shown aberrant expression of mucins during pancreatic cancer development. The mucins may prevent drugs from accessing their sites of action. The expression of several mucins, including MUC1, MUC4, MUC5AC, and MUC16, is highly upregulated in pancreatic ductal adenocarcinoma (PDAC), pancreatic intraepithelial neoplasia (PanIN), intrapapillary mucinous neoplasms, and mucinous cystic neoplasms from patients with pancreatic cancer (2–18). The extent to which the dense mucin mesh influences the antiproliferative activity of 5-fluorouracil (5-FU) was investigated using human pancreatic cancer cells (2, 3). These studies have led to increased recognition of mucins as potential diagnostic markers and therapeutic targets in pancreatic cancer. However, there are no reports evaluating the mucin-synthesizing genes as potential therapeutic targets.

Over 95% of human pancreatic cancers are associated with Kras mutations. Activation of the Kras and EGFR signaling pathways is involved in increased cell proliferation, angiogenesis, metastasis, and decreased apoptosis. Clinical studies also suggest a correlation between overexpression of mucins and pancreatic cancers (10–13). One important issue is whether synthesis of mucins by core enzymes is regulated coordinately. We have shown that p48\(^{12/12} \)–1.5L-Kras\(^{12/12} \) genetically engineered mice (GEM) with Kras mutations display significant dysregulation of EGFR and upregulation of mucin biosynthesis in PanIN lesions (19). We have observed that inhibition of PDAC with the EGFR inhibitor

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gefitinib is associated with suppression of mucin synthesis in p48\(^{Cre/+}\)–LSL-Kras\(^{G12D/+}\) GEM (19). Although EGFR inhibitors (gefitinib) are effective, higher doses lead to significant skin toxicity. Hence, new approaches to target mucin synthesis are urgently needed to improve the efficacy of existing drugs or to design new drugs. Although several mucins, like Muc1 and Muc5, have been targeted, we have little knowledge about potential intervention approaches involving mucin-synthesizing genes.

The mucin-synthesizing core 2 β-1,6 N-acetylglucosaminyltransferase (GCNT3/C2GNT) plays a significant role in mucin biosynthesis. Aberrant GCNT3 expression leads to overexpression of mucins (17, 18, 20). GCNT3 activity plays important roles in physiologic processes, including inflammatory and immune responses. Thus, GCNT3 is an attractive novel target for pancreatic cancer treatment (17–18, 20–21). To date, nearly all reports on pancreatic cancer mucins have focused on mucin peptide gene expression or on aspects of the glycosylated mucins, with relatively few reports addressing the enzymes that catalyze mucin biosynthesis.

In this article, using human pancreatic cancer tissues, GEM tissues, transcriptome analysis, in silico experiments, Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR), spheroid assay, and in vitro and in vivo studies, we identified GCNT3 as a novel target for pancreatic cancer intervention. Then, we determined that talinumate effectively binds to GCNT3 with low binding energy. Finally, we found that talinumate decreased GCNT3 expression alone or in combination with gefitinib in vitro and in vivo.

Materials and Methods

Cell lines and reagents

Human pancreatic cancer cell lines (MIA PaCa, BxPC-3, and PANC-1) were obtained from the ATCC. The cell lines were authenticated by short-tandem repeat analysis by the ATCC. Cell lines and reagents

PANC-1) were obtained from the ATCC. The cell lines were

previously published method (19). Mucin staining was performed per our earlier publication (19).

Mouse model, diet, and handling

All animal research was performed under the auspices of animal protocols approved by the University of Oklahoma Health Sciences Center (OUHSC, Oklahoma City, OK) Institutional Animal Care and Use Committee. Animals were housed in ventilated cages under standardized conditions (21°C, 60% humidity, 12-hour light/12-dark cycle, 20 air changes/hour) in the university rodent barrier facility. Semipurified modified AIN-76A diet ingredients were purchased from Bioserv, Inc. Generation of p48\(^{Cre/+}\)–LSL-Kras\(^{G12D/+}\) mice expressing the activated KrasG12D oncogene has been described previously (22–27). The EGFR inhibitor gefitinib was procured from the center for cancer prevention and drug development repository. Mice were allowed ad libitum access to the respective diets and to automated tap water purified by reverse osmosis.

Breeding and genotyping analysis

LSL-Kras\(^{G12D/+}\) and p48\(^{Cre/+}\) mice were maintained on a C57BL/6 heterozygous genetic background. LSL-Kras\(^{G12D/+}\) and p48\(^{Cre/+}\) mice were bred, and offspring of activated p48\(^{Cre/+}\)–LSL-Kras\(^{G12D/+}\) and C5BL/6 wild-type mice were generated at the required quantities as previously described (22–27).

Tissue processing and histologic analysis of PanIN lesions and PDAC

After euthanizing the mice, pancreata and other key organs, including liver, spleen, kidney, and lung, were collected and weighed. Tissues were fixed in 10% formalin for 24 hours and were routinely processed and embedded in paraffin. Formalin-fixed 4-μm tissue sections from each pancreas were stained with hematoxylin & eosin and were histologically evaluated by a pathologist blinded to the experimental groups. PanIN lesions and carcinoma were classified according to histopathologic criteria, as recommended elsewhere (19, 22–27). To quantify the progression of PanIN lesions, the total number of ductal lesions and their grades were determined (19, 22–27). Similarly, pancreatic carcinoma and normal-appearing pancreatic tissue were evaluated in all animals.

Transcriptome analysis/next-generation sequencing/RNA-seq and data analyses

Pancreatic tumor tissues and normal pancreas were collected from at least 6 10-month-old Kras mice and 6 10-month-old wild-type mice. Pancreatic tumor tissues were also collected from at least 3 untreated control mice and 3 gefitinib-treated mice, after 25 weeks of treatment. Total RNA was extracted with Trizol reagent (Life Technologies) and subjected to cDNA library construction, followed by next-generation sequencing (NGS), per the NGS protocol, with a Solid or Illumina sequencer in the OUHSC core facility (Microgen). The sequence data were deposited to the Geospiza/Perkin Elmer company server and analyzed with GeneSifter bioinformatics software.

Synthesis of talinumate

Talinumate was synthesized with the synthetic method and characterized by comparison of spectral data; it was fully in...
agreement with those reported in the literature (28, 29). 1H-
NMR spectroscopy was performed on a Varian Mercury VX-300
NMR Spectrometer at the NMR Facility (University of Okla-
ahoma). High-resolution electrospray mass (ES-HRMS) spectral
analysis was performed at the OUHSC Molecular Biology-
Proteomics Facility using a Qstar Elite Q-TOF (Applied Biosys-
tems) mass spectrometer. High-performance liquid chromatog-
raphy (HPLC) solvents consisted of water containing 0.1%
trifluoroacetic acid (solvent A) and acetonitrile containing
0.1% trifluoroacetic acid (solvent B). A Sonoma C18 (ES
Industries, 10 μm, 100 Å, 4.6 × 250 mm) column was used
with a flow rate of 1.5 mL/min. The HPLC gradient system
began with an initial solvent composition of 95% A and 5% B
for 2 minutes, followed by a linear gradient to 5% A and 95% B
in 30 minutes, after which the column was reequilibrated.
The absorption detector was set at 254 nm.

In silico molecular docking studies
Talniflumate was obtained from NCBI's PubChem database
(PubChem CID 48229). The crystal structure for GCNT3 was
downloaded from the RCSB Protein Data Bank (PDBID 2GAM).
Chain A and the reference ligand, GALB1,3GALNAc, were sepa-
rated from the pdb file into two separate files for docking, and all
water molecules were removed. Using AutoDockTools, nonpolar
hydrogens and partial charges were added to the receptor GCNT3,
and to the ligands GALB1,3GALNAc and talniflumate. For flexi-
ble ligand docking, rotatable bonds were defined for both
GALB1,3GALNAc and talniflumate, with a total of 12 and 6
rotatable bonds, respectively. To accomplish a blind docking
simulation, six search grids were created with AutoGrid4 to
surround the entire protein with the following grid center coor-
dinates: (84.029, −5.002, 65.752), (51.029, −5.002, 65.752),
(67.529, 11.498, 65.752), (67.529, −21.502, 65.752), (67.529,
−5.002, 68.752), (67.529, −5.002, 62.752). Each grid was set to
126 × 126 × 126 points with a spacing of 0.35 Å. The Lamarckian
search algorithm was used to dock the ligands in all six energy
grids. The GA population size was increased to 500, energy
evaluations to 25,000,000, number of generations to
10,000,000, and the number of runs to 500. Other default search
parameters were unchanged. All docking results were concatenat-
ed and sorted by binding free energies to identify the conforma-
tion with the lowest binding free energy. Figures were created
using Pymol.

MTT assay, Western immunoblotting, and real-time PCR
3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
(MTT) assay for viability, Western immunoblotting, and real-time
PCR techniques were used to analyze the expression of GCNT3,
Muc1, Muc13, Muc4, Muc5ac, PCNA, and cyclin D1.

CRISPR knockout for GCNT3 and spheroid assay
CRISPR/Cas9 KO plasmid transfection. In a 6-well tissue culture
plate, 1 × 10^5 to 2.5 × 10^5 cells were seeded in 3 mL of
antibiotic-free standard growth medium per well, 24 hours prior
to transfection. Cells were grown to 40% to 80% confluency.
Healthy and subconfluent cells are used for successful human
GCNT3 CRISPR/Cas9 KO Plasmid transfection (Santa Cruz Bio-
technology) as per the manufacturer's instructions. After incuba-
tion, successful transfection of CRISPR/Cas9 KO Plasmid was
visually confirmed by detection of the GFP via fluorescent micro-
scopy. Cell viability was determined using the MTT method.

Spheroid assay. The Algimatrix 3D Culture System (Life Tech-
ologies) was used for multicellular tumor spheroid assays,
following the vendor's instructions. After GCNT3 transfection,
untransfected and transfected cells were trypsinized and resus-
pended (1 × 10^6 cells/mL) in standard cell culture medium.
Then, 10% (v/v) Algimatrix Firming Buffer was added to this
suspension. An Algimatrix 3D Culture System plate was inco-
minated with the suspension, which was added to the top of each
dry sponge with a pipette. Approximately 5 minutes after
rehydration, additional volumes of culture medium were added to the top of each sponge, without forming buffer, and were incubated at 37°C in a humidified atmosphere of 5% CO2 in air. Culture medium was changed based on cell proliferation or when the medium began to change color. Spheroid formation was monitored for 3 weeks, and the number and size of spheroids were recorded.

Statistical analysis
Survival time was compared between men and women and between high and low expressions of GCNT3 using the log-rank test. Survival rates across time by gender and by GCNT3 expression were estimated and plotted using the Kaplan–Meier method. The other data are presented as mean ± SE. Differences in body weights were analyzed using ANOVA. Statistical differences between control and treated groups were evaluated using the Fisher exact test for PDAC incidence. Unpaired t test with the Welch correction was used for PanIN and PDAC lesions. Differences between groups were considered significant at P < 0.05.

Results
Correlation between GCNT3 expression and patient survival in human pancreatic cancer
To determine the association between GCNT3 expression and patient survival, we performed an IHC analysis for GCNT3 expression on a tissue microarray containing 180 specimens from individuals with pancreatic cancer (90) or matched controls (90). The median age was 62 years, and 36.1% of patients were female. Based on the seventh edition of the American Joint Committee on Cancer criteria, there were 4 stage IA, 35 stage IB, 13 stage IIA, 33 stage IIB, and 2 stage IV tumors. Pathologically, the tumors were adenocarcinoma, ductal adenocarcinoma, mucinous adenocarcinoma, and adenosquamous carcinoma. There were 51.3% poorly differentiated tumors and 48.6% well-to-moderately differentiated tumors. All tumors that were tested for GCNT3 expression were evaluated by an independent pathologist.

There were 88 patients (32 females, 56 males) with pancreatic cancer in the study. The median follow-up time was 12 months. Fifty-nine patients died during the study. Women had a
marginally nonsignificant longer survival time than men (median survival: 30 vs. 12 months, \( P = 0.053 \); Supplementary Fig. S1A). A 5.5% staining score as a cutoff point was used to determine low GCNT3 (<5.5%) versus high GCNT3 (>5.5%) expression. Patients with low expression of GCNT3 had a longer survival time than patients with high expression of GCNT3 (median survival: 17.5 vs. 10.5 months, \( P = 0.036 \); Fig. 1A). In patients with high expression of GCNT3, there was no significant difference in survival time between women and men (median survival: 17 vs. 10, \( P = 0.2894 \); Supplementary Fig. S1B). Importantly, those patients with history of smoking, drinking, or diabetes comprised 62.9% of the sample with high GCNT3. These data indicate that GCNT3 may be an important prognostic factor for patients with pancreatic cancer. Further, after analyzing additional samples (total 103 pancreatic cancer samples) for the percent positive cells stained for GCNT3, 24.2% (25/103) of male patients with PDAC showed >50% GCNT3 expression, 30.23% (13/43) showed 5% to 50% GCNT3 expression, and 48.83% (21/43) showed <5% GCNT3 expression. In female patients with PDAC, 20.9% (9/43) showed >50% GCNT3 expression, 30.23% (13/43) showed 5% to 50% GCNT3 expression, and 48.83% (21/43) showed <5% GCNT3 expression (Fig. 1B). Similarly, high expression of GCNT3 was observed in human PanINs (Fig. 1C). Figure 1D shows the expression of GCNT3 and mucin in PanINs and PDAC.

Pancreatic cancer progression in GEM

Over the past few years, we have demonstrated the utility of the p48\(^{(+/-)}\)-LSL-Kras\(^{G12D}\) (Kras) mouse model for testing the efficacy of pancreatic cancer chemopreventive agents (19, 22–27). In this study, we used pancreata from 10-month-old Kras and wild-type mice (\( N = 6 \)/group). A significant increase in pancreas weight was observed in Kras mice compared with wild-type mice (Fig. 2A). As summarized in Fig. 2B, the Kras mice developed PanIN lesions (mean ± SEM; PanIN1: 190 ± 28, PanIN2: 172 ± 23, PanIN3: 160 ± 18), whereas no evidence of PanIN lesions was observed in wild-type mice. Out of 6 Kras mice, 4 mice (66%) showed carcinoma incidence, with 32.5% carcinoma spread
Gene expression profiling in pancreatic tumor tissues by next-generation transcriptome analysis

We next collected the pancreata and determined GCNT3 expression using PCR. We observed that tumors showed significantly increased GCNT3 compared with normal pancreas. GCNT3 mRNA levels are significantly increased GCNT3 compared with normal pancreas. We observed that tumors showed significantly increased GCNT3 compared with normal pancreas. We then determined GCNT3, EGFR, and top hits related to mucin expression using transcriptome analysis (Fig. 2D and E). We observed a significant increase in the GCNT3 (103.16-folds; \( P < 0.001 \)) in correlation with increased mucins Muc4 (50-fold; \( P < 0.04 \)), Muc5ac (87-fold; \( P < 0.01 \)), Muc6 (67-fold; \( P < 0.008 \)), Muc 1 (5-fold; \( P < 0.009 \)), Muc 16 (5-fold; \( P < 0.0003 \)), and Muc 20 (17-fold; \( P < 0.007 \)), along with EGFR (\( P < 0.001 \)), in the pancreatic tumors from 10-month-old mice compared with pancreata from wild-type mice, as determined by NGS/RNAseq analysis (Fig. 2E). Further, IHC and mucin staining revealed high expression of GCNT3 and mucins in PanIN lesions and PDAC (Fig. 2F). Importantly, the GCNT3 expression in the pancreatic tissue was observed to increase progressively as the GEM age (Supplementary Fig. S2A and S2B).

Talniflumate is a novel drug that targets GCNT3

We synthesized talniflumate for our studies using the following method (Fig. 3A; refs. 28, 29). Quality and purity were determined using NMR, HRMS, and HPLC (Fig. 3B–D). Using bioinformatics tools, the binding confirmation of the noncompetitive inhibitor talniflumate (Fig. 4A) to GCNT3 was explored using in silico small molecular docking simulations. Our blind docking simulations revealed that talniflumate binds to GCNT3 with a better affinity than does the reference ligand GALB1,3GALNAC. Although talniflumate is positioned in the same pocket of GCNT3 that GALB1,3GALNAC occupies, they do not occupy the same exact space (data not shown). Talniflumate’s best docking affinity of –8.3 kcal/mol is achieved deeper in the pocket of GCNT3, whereas GALB1,3GALNAC’s best docking affinity of –7.51 kcal/mol is achieved closer to the surface of GCNT3. The docking predictions suggest three notable hydrogen bonds between talniflumate and GCNT3: Arg192 (3.0 Å), Tyr288 (3.5 Å), and Ala287 (2.9 Å). Although side chain hydrogen bond contacts are suggested between both the hydrophilic residues, Arg192 and Tyr288, the docking suggests a main chain hydrogen bond contact between talniflumate and the hydrophobic Ala287 (Fig. 4B–D). One drawback with performing rigid receptor docking simulations is that the side chains do not adjust to the docked ligand, which could explain the longer hydrogen bonds between talniflumate and both Arg192 and Tyr288. In vivo, these side chains could move closer to the ligand, shortening the hydrogen bond and, therefore, making this a stronger interaction.

Talniflumate inhibits GCNT3 and mucins

We observed a consistent expression of GCNT3 and mucins in the human pancreatic cancer specimens, GEM tissues, and human pancreatic cancer cell lines that were analyzed (Figs. 1 and 2; Supplementary Figs. S2–S4). We then sought to determine whether talniflumate inhibits GCNT3 and mucins in GEM in vivo. Pancreata from 6-week-old Kras mice treated with talniflumate for 1 week showed a significant decrease in GCNT3 and mucin expression in PanIN lesions (Fig. 5A). Further, GCNT3 protein expression was significantly decreased in the pancreas of talniflumate-treated Kras mice compared with untreated control mouse pancreas (Fig. 5B). mRNA expression of GCNT3 was also observed to be lower in pancreatic tissues from talniflumate-treated mice (Fig. 5C). These results support that talniflumate within the pancreas. Only 6% of the pancreata from Kras mice were free from PanIN lesions and PDAC, whereas 100% of pancreata were normal in wild-type mice.
(400 ppm) can inhibit GCNT3, and, thereby, mucins, in GEM in vivo. Further, we also used next-generation Illumina sequencing to evaluate pancreatic cancer in gefitinib-treated mice and controls (Fig. 5D–G). The results clearly demonstrate that gefitinib treatment (100 ppm) significantly decreases EGFR, GCNT3, and mucins in the pancreas. Gefitinib significantly reduced PanIN lesions (PanIN1, 2, and 3) and carcinoma (Fig. 5F). Mice treated with gefitinib showed more normal pancreatic tissues (i.e., free from PanIN lesions and carcinoma) than did untreated mice (Supplementary Fig. S2C and S2D).

Combination of gefitinib and talniflumate inhibits GCNT3 and mucins
We further determined the expression of mucins and GCNT3 in the human pancreatic cancer Mia PaCa cells treated with talniflumate, gefitinib, and the combination of talniflumate and gefitinib (Fig. 6). A significant decrease in cell viability, GCNT3 expression, and number of mucin-expressing cells was seen after treatment compared with untreated cells (Fig. 6). We also observed a decrease in cell viability upon treatment with talniflumate and gefitinib in PANC-1 and BxPc-3 cells (Supplementary Figs. S3–S5). Along with GCNT3, individual and combination treatments also reduced mucins, PCNA, and cyclin D1 (Supplementary Fig. S4). These results justify the use of talniflumate for mucin synthesis disruption, thereby enhancing the efficacy of EGFR inhibitors for pancreatic cancer treatment.

CRISPR GCNT3-KO leads to reduced cell viability and spheroid formation
We next sought to determine whether knocking out GCNT3 inhibits cell growth and spheroid formation. Pancreatic cancer cells were knocked out for GCNT3 using CRISPR technology following the vendor’s instruction. Transfection efficiency was determined by GFP fluorescence in GCNT3-KO cells (Fig. 7A). After 48 hours, cells were harvested and evaluated for cell viability via MTT assay (Fig. 7B) and spheroid formation in algimatrix 3D.
culture system (Fig. 7C). We observed a decrease in cell proliferation by 32% on day 1 and by 55% on day 2. Further, talniflumate significantly reduced spheroid formation, and GCNT3-KO cells did not form significant numbers of spheroids (Fig. 7C). Further, implanting GCNT3-KO cells into nude mice for in vivo studies will help us to evaluate the tumor-forming ability of these cells.

Discussion

Pancreatic cancer has an aggressive nature and ranks among the worst in survival rates of all epithelial cancers. The difficulties in early diagnosis and the lack of effective therapies contribute to its poor prognosis. One of the challenges in pancreatic cancer therapy is the failure of existing drugs to access their target sites due to excess mucin production. Clinical and preclinical studies have shown that various mucins (Muc1, Muc4, Muc13, and Muc5ac) are significantly overexpressed in pancreatic tumors. A large body of evidence supports the role of these mucins in tumor progression. Some have been studied as biomarkers or targeted for pancreatic cancer treatment. Clinical studies suggest a correlation between the overexpression of mucins and pancreatic cancer (10–13), and have demonstrated a link between the aberrant expression and differential overexpression of mucin glycoproteins and the initiation, progression, and poor prognosis of the disease (14–16, 19, 30). However, little advancement has been made in this area. We sought to evaluate whether a mucin-synthesizing gene could be an ideal target for pancreatic cancer intervention, rather than simply targeting individual mucins. In this direction, we identified GCNT3 as a potential target that is significantly overexpressed in human pancreatic cancer and is known to decrease patient survival by 7 months (Fig. 1A). Further, 62.9% of the patients with high GCNT3 expression had a history of alcohol use, tobacco use, and/or diabetes. Our results suggest that excessive mucin synthesis may be associated with these factors.
The GCNT3-KO cells did not yield spheroids after 3 weeks of treatment. A decrease in cell viability was seen with GCNT3-KO. C, talnifluate significantly reduced the number of spheroids after 3 weeks of treatment. The GCNT3-KO cells did not yield spheroids.

We have shown that p48Cre/LSL-KrasG12D+ GEM with Kras mutations display significant dysregulation of EGFR and upregulation of mucin biosynthesis in PanIN lesions and PDAC (Fig. 2; ref. 19). Further, next-generation transcriptome analysis revealed that GCNT3 is aberrantly expressed in pancreatic tumors from GEM compared with normal pancreas, and has increased mucin subtypes. GCNT3 is a mucin core-synthesizing gene, and its overexpression is associated with pancreatic cancer tumorigenesis and enhanced proliferation (Fig. 3). Jones and colleagues (in Supplementary Table S9; 20) demonstrated that normal pancreas do not express GCNT3, whereas GCNT3 is overexpressed significantly in tumors from patients with pancreatic cancer. GCNT3 is also expressed highly in human pancreatic cancer cell lines. Our studies, preliminary reports, and the available literature support the notion that GCNT3 is involved in mucin synthesis in pancreatic cancer, and is an attractive novel target for pancreatic cancer treatment. However, no mucin synthesis inhibitors that affect GCNT3 have been tested, and there are no published data that support a direct role of GCNT3 in altering mucin synthesis during pancreatic cancer growth.

Talnifluate is an orally available, small-molecule inhibitor, and a potential muco-regulator that inhibits mucin synthesis, blocks mucus overproduction in patients with chronic mucous respiratory problems, and reduces cystic fibrosis (31, 32). Talnifluate is also reported as calcium-activated chloride channel (hCLCA1/mCLCA3) blocker; it reduces mucin synthesis in cell culture and animal models. Its anti-inflammatory actions are via inhibition of cyclooxygenases and inhibit Cl-/HCO3- exchanger activity. Further, it increased survival in a cystic fibrosis mouse model of distal intestinal obstructive syndrome (31, 32). The combination of these properties in an orally tolerated drug may be useful in the treatment of pancreatic cancer. Although talnifluate was shown to exert its effects by an anti-inflammatory mechanism, studies have not supported its role as a mucous inhibitor in pancreatic cancer. Hence, after synthesizing talnifluate and fully characterizing it using NMR, HMR, and HPLC purification, we used in silico molecular modeling approaches to investigate the binding capability of talnifluate to GCNT3.

Compared with known ligand GALB1,3GALNAC, talnifluate showed a best docking affinity of −8.3 kcal/mol toward GCNT3, with three notable hydrogen bonds between GCNT3 and talnifluate (Fig. 4; Supplementary Fig. S6). After confirming that talnifluate effectively binds to GCNT3, we evaluated the effects of talnifluate on GCNT3 expression in PanIN lesions from GEM. Talnifluate significantly reduced GCNT3 protein expression, mucins, and GCNT3 mRNA compared with untreated controls. These data further confirm that talnifluate has an effect on GCNT3.

Use of the EGFR inhibitor gefitinib (Iressa) significantly reduced tumor weight, PanINs, and PDAC, with an accompanying significant decrease in mucin synthesis and GCNT3, suggesting a potential role of EGFR in the regulation of mucin biosynthesis through GCNT3 (Fig. 3). Given that clinical doses of most FDA-approved EGFR inhibitors (gefitinib) are associated with skin toxicity, it would be beneficial to use lower doses of gefitinib along with talnifluate to produce synergistic effects on inhibition of tumor cell proliferation. The combination of talnifluate and gefitinib showed a significant decrease in GCNT3 and mucins with reduced cell proliferation. Further, talnifluate inhibited spheroid formation, and CRISPR transfection of GCNT3 decreased cell proliferation and spheroid formation.

However, further studies are warranted to evaluate the mechanism of GCNT3 regulation and talnifluate’s effects on pancreatic cancer development in detail. Understanding the function of GCNT3 and mucin synthesis in the setting of pancreatic cancer is critically important to understanding the mechanisms of pancreatic cancer tumorigenesis and to developing new targeted therapies that reduce chemoresistance.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors’ Contributions**

Conception and design: C.V. Rao, N.B. Janakiram, V. Madka, A. Mohammed Development of methodology: N.B. Janakiram, V. Madka, G. Kumar, A. Mohammed
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