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Micelle Mixtures for Co-administration of Gemcitabine and GDC-0449 to treat Pancreatic Cancer

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Abstract

Hedgehog (Hh) signaling plays an important role in the development and metastasis of pancreatic ductal adenocarcinoma (PDAC). Although gemcitabine (GEM) has been used as a first-line therapy for PDAC, its rapid metabolism and short plasma half-life restrict its use as a single chemotherapy. Combination therapy with more than one drugs is a promising approach for treating cancer. Herein, we report the use of methoxy poly(ethyleneglycol)-block-poly(2-methyl-2-carboxyl-propylenecarbonate)-graft-dodecanol (mPEG-b-PCC-g-DC) copolymer for conjugating GEM and encapsulating an Hh inhibitor, Vismodegib (GDC-0449) into its hydrophobic core for treating PDAC. Our objective was to determine whether the mixed micelle formulation of these two drugs could show better response in inhibiting Hh signaling pathway and restraining the proliferation and metastasis of pancreatic cancer. The *in vivo* stability of GEM significantly increased after conjugation, which resulted in its increased antitumor efficacy. Almost 80 % of encapsulated GDC-0449 and 19 % conjugated GEM were released *in vitro* at pH 5.5 in 48 h in a sustained manner. The invasion, migration and colony forming features of MIA PaCa-2 cells were significantly inhibited by micelle mixture carrying GEM and GDC-0449. Remarkable increase in PARP cleavage and Bax proved increased apoptosis by this combination formulation compared to individual micelles. This combination therapy efficiently inhibited tumor growth, increased apoptosis and reduced Hh ligands PTCH-1, Gli1, and lowered EMT-activator ZEB-1 when injected to athymic nude mice bearing subcutaneous tumor generated using MIA PaCa-2 cells compared to monotherapy as observed from immunohistochemical analysis. In conclusion, micelle mixtures carrying GEM and GDC-0449 have the potential to treat pancreatic cancer.

Graphical Abstract

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ASSOCIATED CONTENT

Supporting Information

¹H NMR and plot of fluorescence intensity and critical micelle concentrations of polymers mPEG-b-PCC-g-GEM-g-DC and mPEG-b-PCC-g-DC, HPLC chromatogram of mPEG-b-PCC-g-GEM-g-DC after incubation with 1 N NaOH.

Keywords

pancreatic cancer; hedgehog signaling; gemcitabine; GDC-0449; polymeric micelles; combination therapy

1. INTRODUCTION

Amongst the malignant cancers leading to fatal mortality, pancreatic ductal adenocarcinoma (PDAC) deserves special mention and expected to become the second among cancer-related deaths in the United States before 2030.^{1,2} Surgical resection is the only beneficial option for early and advanced stage PDAC that further requires adjuvant therapeutic approaches for patients developing metastasis and post-surgery local recurrence.^{2,3} Despite various therapeutic approaches, 5-year survival rate remains < 5 % due to the development of chemoresistance and toxicity.⁴

Gemcitabine (GEM), (2',2'-difluorodeoxycytidine; dFdC) has been used as a chemotherapeutic agent for more than 15 years.^{5,6} Its anti-tumor action is manifested due to blocking of the chromosomal replication and inducing apoptosis through caspase signaling.^{7,8} However, overall clinical potential of GEM is compromised by rapid metabolism into its inactive metabolite 2',2'-difluorodeoxyuridine (dFdU) by cytidine deaminase and fast kidney excretion thus requiring higher doses to meet therapeutic level at the tumor site that leads to undesirable side effects and toxicity.⁹ The modest results of the drug regimen also arise from the hydrophilic nature of this drug that prevents it from prolonged release and subsequent extravasation to cancerous tissues.¹⁰

Delivery approaches to increase the overall intracellular GEM concentration via improved pharmacokinetics and chemoresistance have been widely applied.¹¹⁻¹⁴ Nano- formulations have been extensively applied to address the plasma instability, metabolic inactivation and subsequent deamination of GEM into its inactive uracil derivatives. Both conjugation and

encapsulation strategies have been explored to overcome the barriers for attenuated delivery of GEM. Particularly, polymer-drug conjugates resulted better efficacy with the use of modified polyethylene glycol (PEG) to increase its circulation half-life, enhanced permeability and retention (EPR) effect and thus augmenting extravasation to tumor tissues.¹⁵ Squalene (SQ) conjugated GEM encapsulated in liposomes has shown promising anticancer efficacy in leukemia cancer model.¹⁶ For elevated intracellular drug fate, Bildstein et al demonstrated that SQdFdC application lead to achieve better pharmacokinetic profiles and increased tumor accumulation in resistant cancer cells. However, these formulations were taken up by the cells of reticuloendothelial system (RES), leading to high accumulation in the liver and spleen.¹⁷ Consequent increased toxicity demands new delivery approaches. In our previous study, we conjugated GEM to methoxypoly(ethylene)-block-poly(2-methyl-2-carboxyl-propylene carbonate) (mPEG-b-PCC) copolymer, which self-assembled into micelles and resulted in its improved plasma stability, enhanced internalization and apoptosis.¹⁸

Cancer stem cells (CSCs), stromal depletion and excess production of the extracellular matrix (ECM) proteins lead to the pathology of PDAC and its metastasis.¹⁹ Correlation between PDAC and hedgehog (Hh) signaling has been well established in various studies, where dysregulation of this pathway has resulted in significant disturbances in the cancer microenvironment.²⁰ Hh signaling is responsible for cellular differentiation during embryonic development in non-pathological conditions and when activated leads to various cancers including PDAC.²¹ Hh antagonists, such as cyclopamine, vismodegib (GDC-0449) and erismodegib block Hh signaling pathway by binding to the Smoothened (SMO) thus disturbing the downstream intracellular cascade, leading to the inactivation of the transmembrane protein PTCH-1 and Gli transcription factors.²² When Hh is repressed alone, tumor progression continued thus inferring that only Hh inhibition is unable to ameliorate the metastasis.^{23,24} GDC-0449 at a very high dose (100 mg/kg) suppressed Hh signaling but increased the angiogenesis subsequently.²⁵ In fact, combination with GEM/GDC-0449/nab-paclitaxel was superior than GEM/nab-paclitaxel.²⁶ Bahra et al. have demonstrated the *in vivo* efficacy of the combination therapy of cyclopamine and GEM administrated intraperitoneally causing synergistic effect in PDAC tumor growth reduction.²¹ Feldmann et al. efficiently used the combination of these two drugs in orthotopic xenograft model to illustrate the reduction in primary tumor size and thus disseminating metastasis.²⁰ Co-administration of GEM and IPI-926 (a semisynthetic derivative of cyclopamine) resulted in enhanced GEM efficacy extending the median survival of mice from 11 days to 25 days and this combination treatment significantly decreased liver metastasis.²⁷ This study proved tumor perfusion was augmented in the presence of the stromal depletion agent, the Hh inhibitor that indirectly increased the intracellular concentration of GEM metabolite. However, the SMO inhibitor monotherapy did not affect the overall cellular proliferation significantly and particularly in metastatic PDAC, GDC-0449/GEM combination in pilot clinical trial did not improve the median overall survival or progression free survival compared to GEM alone.²⁸ Thus, to have an effective treatment regimen, a balanced Hh inhibition with a chemotherapy can be a pivotal approach. We hypothesize a rationale design of formulation strategy for sustained release of combination drugs could serve as an inevitable alternative for effective treatment.

Additionally, relationship between epithelial-to-mesenchymal transition (EMT) and Hh signaling has been established in various reports.²⁹⁻³² EMT is a developmental process where the polar, non-motile phenotype of epithelial cells changes to non-polar, motile mesenchymal cells thus accelerating tumor progression and metastasis.³³ Tumor sensitivity to cytotoxic agents can be increased via reversal of EMT by inhibiting Hh signaling which has been demonstrated in a study where blocking of Hh signaling abrogated resistance of non-small lung cancer cells to erlotinib and cisplatin.²² Suppression of EMT was effective in increasing the sensitivity to GEM treatment.³⁴ Several clinical studies showed the association between increased epithelial features and improved survival in different tumor types.^{35,36} Islam et al. demonstrated the benefits of targeting Hh signaling pathway that resulted in reversed EMT phenotype and decreased tumorigenicity of bladder cancer.³⁷

In our previous studies, we have demonstrated the application of micelle formulation to increase the bioavailability of the hydrophobic drug, GDC-0449.³⁸ Herein, we report, the use of micelle mixtures carrying GEM (mPEG-b-PCC-g-GEM-g-DC) micelles and GDC-0449 (mPEG-b-PCC-g-DC). When these micelle mixtures were injected subcutaneously pancreatic tumor bearing mice the synergistic effect of these two drugs showed comparable inhibition of tumor growth. Combination therapy with micelle mixtures can potentially elude multiple resistance mechanisms that limit the activity of individual anticancer drugs.

2. MATERIALS AND METHODS

2.1. Materials and Reagents

Gemcitabine and Vismodegib (GDC-0449) were purchased from Ark Pharma (Libertyville, IL) and LC Laboratories (Woburn, MA), respectively. 2,2-bis(hydroxymethyl) propionic acid, methoxy polyethylene glycol (mPEG; Mn= 5000, polydispersity index (PDI)=1.03), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide HCL (EDC), 1-hydroxybenzotriazole (HOBt), 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), benzyl bromide and all other reagents were purchased from Sigma-Aldrich (St. Louis, MO) and used without further purification.

2.2. Synthesis and Characterization of Copolymers

Monomer 2-methyl-2-benzoyloxycarbonyl-propylene carbonate (MBC) was synthesized in two steps as white crystals according to our previous report.¹⁸ Ring opening polymerization was performed with mPEG and MBC for the synthesis of mPEG-MBC in the presence of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) catalyst at the room temperature for 3 h under nitrogen atmosphere. mPEG-MBC was in tetrahydrofuran (THF): methanol (1:1, v/v)) hydrogenated in presence of 10 wt % palladium on charcoal (Pd/C) to obtain the copolymer containing carboxyl pendant groups (mPEG-b-PCC). Finally, GEM and/or dodecanol (DC) were conjugated by carbodiimide coupling reaction at the room temperature for 48 h to obtain mPEG-b-PCC-g-DC and mPEG-b-PCC-g-GEM-g-DC using EDC/HOBt as reported previously.¹⁸ These final products were precipitated in isopropyl alcohol followed by diethyl ether. mPEG-b-PCC-g-GEM-g-DC was dissolved in acetone and dialyzed against water (M.W. Cut off 2000 Da) followed by lyophilization overnight (Fig. 1). Copolymers were

characterized by ^1H NMR, recorded with a Bruker (500 MHz, $T=25^\circ\text{C}$) using deuterated dimethyl sulfoxide ($\text{DMSO}-d_6$, Cambridge Isotope Laboratories, Inc., MA)

Critical micelle concentration (CMC) of both polymers (mPEG-b-PCC-g-GEM-g-DC and mPEG-b-PCC-g-DC) was measured according to pyrene probe method ($\lambda_{\text{excitation}} = 338$ and 333 nm and $\lambda_{\text{emission}} = 390$ nm) by spectrofluorometer. Plots of intensity ratio of two excitation wavelengths (I_{338}/I_{333}) versus logarithm of polymer concentration were used to calculate the critical micelle concentration (CMC) values of the micelles (Fig. S3).

2.3. Preparation of Micelles

GEM conjugated micelles and GDC-0449 loaded micelles were prepared by thin film hydration as described previously.^{18,38} Briefly, 20 mg of copolymers were dissolved in 0.4 mL of chloroform. Organic solvent was evaporated under pressure followed by storing overnight at vacuum. Rehydration of the film with phosphate buffered saline (PBS; pH 7.4), followed by vortexing, sonication, centrifugation at 5,000 g (5 min) and filtration (0.22 μm) were performed for preparing micelles. Micelle mixtures were prepared by mixing the individual micelle formulations (containing required equivalent amount of drugs; GEM micelles and GDC-0449 micelles) at the ratio of 50:50 (Fig. 1).

2.4. Characterization of Formulations

Mean particle size and size distribution of the micelles were determined by dynamic light scattering using a Zeta SizerTM (Malvern 3800-ZLS, Boston, MA).

2.4.1. Quantification of Gemcitabine Loading in Polymer-Drug Conjugate—The degree of GEM conjugation to the copolymer was determined by alkaline hydrolysis in the presence of 1 N NaOH. The amount of GEM in the conjugated polymer was determined by HPLC-UV analytical method using Inertsil ODS 3 column (250×4.60 mm, 5 μm) and methanol : sodium acetate buffer (20 mM, pH 5.5) mobile phase = 07:93 v/v (20 μL injection volume; 268 nm). Drug payload calculated using the following equation.

$$\text{Drug Payload (w/w \%)} = (\text{Weight of Drug Loaded} / \text{Total weight of formulation}) \times 100$$

2.4.2. Quantification of GDC-0449 Encapsulation Efficiency and Drug Loading

—Micelles loaded with GDC-0449 with a 5% theoretical loading were dissolved in dichloromethane (DCM) and bath sonicated for 30 min at 37°C followed by withdrawal of the lower layer of the dual phase that includes dissolved GDC-0449 in DCM. After evaporating DCM, acetonitrile was added and vortexed for 5 min. Encapsulated drug amount was determined by HPLC using Phenomenex Aqua C18 column (250×4.60 mm, 5 μm) with acetonitrile : water mobile phase (60:40, v/v; 1.0 mL / min) at 230 nm (UV). Drug loading and encapsulation efficiency were calculated using the following equations:

$$\begin{aligned} \text{Encapsulation efficiency (\%)} &= (\text{Weight of drug encapsulated} / \text{Initial weight of drug taken}) \times 100 \\ \text{Drug loading (\%)} &= (\text{Weight of drug encapsulated} / \text{total weight of formulation}) \times 100 \end{aligned}$$

2.4.3. In vitro Release of Gemcitabine and GDC-0449 from Combination

Micelles—*In vitro* release of GEM from the micelles and micelle mixtures were determined in PBS at pH 5.5 and pH 7.4 separately in dialysis bags (M.W. cutoff 2000 Da). Samples (1mL) were withdrawn at a regular interval and replaced with equivalent volume of fresh media. GEM amount in the samples was determined by HPLC-UV method. All parameters kept the same for GDC-0449 release from the micelles except the addition of 2% Tween 80 to maintain sink conditions. GDC-0449 concentration was determined by HPLC-UV as described in the earlier section.

2.5. In Vitro Cell Culture Studies

2.5.1. Cytotoxicity Assay—DMEM (containing 10% FBS and 1% antibiotic-antimycotic (streptomycin, penicillin and amphotericin B)) were used to culture MIA PaCa-2 cells in 5% CO₂ at 37° C in a 95% humidified atmosphere. MIA PaCa-2 cells were seeded on a 96-well plate in 100 PL media (5×10^3 cells / well) and incubated for 24 h. The cells treated with different concentrations of GEM conjugated micelles, GDC-0449 encapsulated micelles and micelle mixture were incubated for 72 h at 37° C. MTT assay (absorbance at 570 nm and cell debris corrected by subtracting at 630 nm) was performed to assess cell viability. Cell viability was calculated using the formulation below:

$$\text{Cell viability (\%)} = (\text{Absorbance of test sample}) / (\text{Absorbance of control}) \times 100$$

2.5.2. Western Blotting—MIA PaCa-2 cells (2×10^5 cells/well) were seeded in 6-well plates containing 2 ml media. After 24 h, GEM conjugated micelles, GDC-0449 loaded micelles and micelle mixture carrying equivalent amount of GEM and GDC-0449 were added and incubated for 72 h. Untreated cells were taken as the control. After 72 h, cells were incubated with RIPA buffer and protease inhibitor cocktail (Sigma, St. Louis, MO) followed by the determination of total protein concentration by BCA protein assay kit (Thermo Scientific, Rockford, IL). 4–15% mini PROTEAN polyacrylamide gel was used for separation of 40 Pg total proteins from cells treated with three different formulations and the control group followed by transferring to Polyvinylidene fluoride (PVDF) (Life Technologies Carlsbad, CA) membranes by iBlot gel transfer system. Odyssey Blocking Buffer was used to block membranes for 1 h at room temperature. Membranes were incubated overnight with rabbit polyclonal to Gli-1 (SC-20687), Bax (SC-6236), total PARP (CS-9532S) (Cell Signaling Tech., Danvers, MA), goat polyclonal to β -actin (SC-1616) (1:1000), Patched-1 (SC-6147) (1:1000) (Santa Cruz Biotech., Dallas, TX). Following washing with TBST buffer, the membranes were incubated with their corresponding anti-rabbit and anti-goat IR dye conjugated secondary antibodies (1:10000) (LI-COR Biosciences, Lincoln, NE) (60 min) and visualized using LI-COR imaging system. β -actin (SC-1616) protein expression level were used for normalizing protein expression levels.

2.5.3. Invasion, Migration and Colony Forming Assays—Matrigel invasion assay was performed using Transwell membrane filter inserts (8 Pm pore size). Invaded cells were visualized under a microscope by staining with 0.2 % crystal violet.³⁹

Migration assay was carried out to evaluate the contribution of GDC-0449 on the migratory ability of MIA PaCa-2 cells. Cell monolayer was scraped using a micropipette tip and 48 to 72 hours after treatment with GEM, GDC-0449 and mixed micelle formulations, the residual gap length was calculated from photo micrographs.⁴⁰ Clonogenic assay was carried out by treating 250 cells with micellar formulations for 10 days. Visible colonies (50) were counted following Crystal Violet (0.2% in 2% ethanol) staining and the % colonies was calculated compared with the control as described earlier.⁴¹

2.6. In vivo Study

Animal experiments were carried out in accordance to the protocol approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Nebraska Medical Center (UNMC, Omaha, NE). Flank tumors were established in 8–10 week old male athymic nude mice by subcutaneous injection of 3×10^6 MIA PaCa-2 cells suspended in a total 200 μL of 1:1 serum-free media and Matrigel (BD Biosciences, CA). When the tumor volume reached 200–300 mm^3 animals were randomly divided into four groups (n= 6): blank micelles, GEM conjugated micelles, GDC-0449 loaded micelles and combination micelles (GEM micelles: GDC-0449 micelles, 50: 50). Formulations were administered intratumorally thrice a week for 2 weeks at an equivalent dose of 40 mg/kg GEM and 10 mg/kg GDC-0449. Tumor size was measured at regular intervals using a vernier caliper. Body weight of the animals was recorded thrice a week. At the end of the study, tumor tissues were excised, weighed and snapped frozen for further analysis.

2.7. Histochemical and Immunofluorescence Assays

Excised tumors embedded in OCT compound (Sakura Finetek, Torrance, CA), frozen and stored in -80°C for further experiments. To stain Gli-1 and active caspase-3, sections (4 Pm) were fixed in pre-cold acetone for 30 min and washed with PBS for 3 times. Permeabilization was done by washing the slides 2 times for 5 min in TBS + 0.025% Triton X-100. After blocking in 10% goat serum with 1% BSA in TBS for 2 h at the room temperature, sections were incubated with Gli-1 antibody (SC-20687) (1:50) (Santa Cruz Biotech., Dallas, TX) and active caspase-3 (SC-1225) (1:50) overnight at 4°C . Next day, slides were washed with TBS + 0.025% Triton X-100 and further incubated with anti-rabbit IR dye conjugated secondary antibody.

To evaluate tissue morphology, sections (4 Pm) were stained with hematoxylin and eosin (H&E) and analyzed blindly. For cell proliferation, marker Ki-67 and EMT-activator ZEB-1, sections were probed with rabbit polyclonal Ki-67 antibody (1:50) (ab-15580) and rabbit polyclonal ZEB-1 antibody (1:150) (sc-25388), respectively. Sections were incubated for 45 min at the room temperature with anti-rabbit horse raddish peroxidase (HRP) conjugated secondary antibody diluted to 1:500 in 2% BSA/1X PBS solution. All stained slides were visualized under microscope (Leica, Germany).

2.8. Statistics

Data values were expressed as the mean $\pm$ standard deviation (S.D.) Student's t test was performed for statistical evaluation. Value of $p < 0.05$ was considered as statistically significant.

3. RESULTS

3.1. Synthesis and Characterization of Copolymers

GEM and/or DC were conjugated to free carboxylic acid groups of mPEG-b-PCC using EDC/HOBt. ¹H NMR was used to characterize the final purified compound (Supporting Information, Fig. S1, S2). PEG-PCC showed copolymer backbone peaks corresponding to PEG (-CH₂-CH₂-O) at δ 3.5, PCC (-CH₂-) at δ 4.2. After the removal of pendant benzyl group by hydrogenation process characteristic peak of phenyl ring at δ 7.3 disappeared and carboxyl group peak was observed at δ 13 as reported earlier by our group. Average molecular weight of mPEG-b-PCC was 10196 Da with 24 PCC units that was determined by integration of corresponding protons of mPEG and PCC units. CMC values were 0.2×10^{-3} mg/ml and 0.15×10^{-3} mg/ml for mPEG-b-PCC-g-GEM-g-DC and mPEG-b-PCC-g-DC, respectively (Fig. S3).

3.2. Formulation Characterization

GEM conjugated micelles and GDC-0449 loaded micelles were prepared by film hydration showed a mean size of 24 nm (PDI=0.121) and 36 nm (PDI=0.131), respectively (Fig. 2B). GDC-0449 loading was 5% as it was confirmed with the calculation of corresponding HPLC peak at 5.5 min. GEM payload was approximately 14% proven by HPLC analysis after alkaline hydrolysis of GEM conjugated micelles that showed a peak at 10.8 min corresponding to GEM (Fig. S4). *In vitro* release profile of GEM conjugated micelles, GDC-0449 encapsulated micelles were determined in PBS at 37 °C. We chose two different pH values to stimulate the drug release in blood (pH 7.4) and the tumor cells (pH 5.5).⁴² GDC-0449 release from micelles at pH 5.5 was more rapid (80% at 48 h) than GEM release (19% at 48 h) since GEM is conjugated to the polymer (Fig. 2A).

3.2.1. *IN VITRO* Cytotoxicity Assay—MIA PaCa-2 cells were used to test different concentrations of GEM micelles (8, 16, 32, 62, 125, 250, 500 nM), GDC-0449 micelles (1.5, 3, 6, 12, 25, 50, 100 PM) and micelle mixture (combination of the above concentrations) for 72 h. The formulations were cytotoxic against MIA PaCa-2 cells (Fig. 3A).

3.2.2. Mechanism of micelle mixture-induced cell death—PARP cleavage in MIA PaCa-2 cell line was performed following 72 h of treatment with with GEM micelles, GDC-0449 micelles, and micelle mixtures carrying these two drugs. Untreated cells were considered as the control group. The PARP cleavage was greater in micelle mixtures than micelles carrying either GEM or GDC-0449, indicating that the combination therapy causes more apoptosis. Similarly, micelle mixtures carrying GDC-0449 and GEM significantly increased the expression level of Bax protein in MIA PaCa-2 cells which confirms that apoptosis was effectively induced by the combination therapy (Fig. 3B).

To study the effect of GDC-0449 micelles and micelle mixtures carrying GDC-9448 and GEM on the inhibition of Hh ligands, we performed Western Blot of Gli-1 and PTCH-1 in MIA PaCa-2 cells after 72 h treatment. Cells treated with micelle mixtures showed significantly decreased Gli-1 expression than the cells treated with micelles carrying GDC-0449 alone (Fig. 3B).

3.2.3. Invasion, Migration and Colony Forming Assays—Treatment by micelle mixtures significantly inhibited the clonogenic potential, invasion and migration ability of MIA PaCa-2 as observed by the decreased number of colonies and migrated cells after treatment with micelle mixtures carrying GEM and GDC-0449 (Fig. 4).

3.3. *IN VIVO* evaluation

The *in vivo* efficacy of micelles containing GEM and/or GDC-0449 was determined in subcutaneous tumor bearing athymic nude mice generated using MIA PaCa-2 cells. Only two animals showed loss of body weight during the treatment period. Mice treated with micelles carrying either GEM or GDC-0449 showed low tumor growth compared to the control group (804 mm³ vs. 68.4 mm³). However, significant tumor growth inhibition was observed in mice that received the combination treatment (Fig. 5).

Immunofluorescence and immunohistochemistry analyses were performed to elucidate the superior anti-tumorigenic effect of the combination therapy. Gli-1 expression was high in the control group and the mice treated with micelles carrying only GEM. Mice treated with micelles carrying GDC-0449 displayed lesser level of Gli-1 expression while mice treated with micelle mixtures carrying both GEM and GDC-0449 showed modest decrease in fluorescence signal (Fig. 6A). No caspase-3 signal was observed in the sections from the control mice. Modest increase in active caspase-3 signal was seen in the combination treatment group compared to single drug micellar treatment groups (Fig. 6B). Mice treated with micelle mixtures carrying GEM and GDC-0449 displayed suppressed ZEB-1 compared to the control and monotherapy groups (Fig. 6C). Cell proliferation marker Ki-67 staining was lower in the treated group compared to untreated group (Fig. 7A).

4. DISCUSSION

Treatment of pancreatic cancer faces multiple challenges owing to the complexities of the tumor microenvironment and its multi-faced signaling networks that lead to the failure of the therapeutic of most drugs. Understanding the roles played by the individual biochemical pathways and their influences could be effective in controlling the pathology of PDAC otherwise refractory to blockbuster drug candidates. Attempts are being made to increase the quality and span of pancreatic cancer patients' lives. Investigation of the cross talks among the key players of invasion, progression and metastasis of pancreatic cancer is expected to address current ambiguities in the PDAC treatment. In this regard, the activation of Hh signaling pathway deserves special mention along with the EMT among the tumor cells. Hh pathway when dysregulated from its physiological functions lead to overexpression of transcription factors such as Gli 1 and Gli 2. The inhibition of Hh pathway by antagonists such as cyclopamine, GDC-0449, erismodegib, saridegib (IPI-926) has shown promising results in multiple studies.^{43,44} Thus, combination therapy to address multiple factors has been important avenue to ameliorate the failures of monotherapy. Synergistic effect of GDC-0449 with docetaxel in inhibiting human pancreatic cancer cells has been reported where the inhibition of Hh signaling has been proved to improve the anti-carcinogenic effect of docetaxel in prostate cancer.⁴⁵ FOLFIRINOX (combination of 5-fluorouracil, irinotecan and oxaliplatin) has been proved to be significantly effective compared to GEM alone.⁴⁶

Similarly, combining GEM and an Hh inhibitor has been tested to mitigate the disease progression and to achieve optimal benefits.²⁰ Olive et al. demonstrated the inhibition of Hh pathway using IPI-926 lead to enhanced delivery of GEM to pancreatic cancer KPC mouse model.²⁷ The rationale for conjugating GEM to our amphiphilic polymer mPEG-b-PCC-g-DC to form mPEG-b-PCC-g-GEM-g-DC is well justified by our earlier reports which showed these self-assembled micelles compromised the drug hydrophilicity thus decreasing its rapid plasma metabolism and increased antitumor efficacy through sustained GEM release. We have already published our results with GEM as a free drug where we have found the free GEM did not cause significant cytotoxicity and tumor growth inhibition compared to GEM conjugated polymer. Treatment with GEM conjugated micelles was significantly more effective compared to the GEM with respect to higher drug payload, tumor growth inhibition and apoptotic cell death.¹⁸ For GDC-0449 which is a hydrophobic drug, study with free drug is not possible. However, we also explored the same polymeric system in a separate study where we encapsulated GEM into amphiphilic copolymeric micelles with or without miRNA let7b.³⁸ Encapsulation of GDC-0449 within the amphiphilic copolymer resulted in increased solubility from 0.1 $\mu\text{g}/\text{mL}$ to $2450 \pm 50 \mu\text{g}/\text{mL}$.

In the present study, we investigated the synergistic effect between GEM and GDC-0449 by preparing micelle mixtures using GEM conjugated and GDC-0449 encapsulated micelles. Our goal was to compare the efficacy of micelle mixtures carrying GEM and GDC-0449 with micelles carrying either GEM or GDC-0449. Combination of these two drugs has also been tested in a clinical pilot study where GDC-0449 was administered orally at the dose of 150 mg and GEM was intravenously infused at the dose of $1000 \text{ mg}/\text{m}^2$ to a group of 25 patients with metastatic pancreatic adenocarcinoma.²⁸ There was no significant improvement in the overall survival of the patients by the combination therapy compared to monotherapy. However, there is still a need to investigate about the failure of the combination therapy in metastatic environment. In a separate clinical by Infinity Pharmaceuticals showed difference in survival favoring GEM monotherapy due to a higher rate of progressive disease in the combination therapy of saridegib and GEM.⁴⁷ Elevated drug toxicity reflected in the relatively shorter progression free survival due to the high doses of the combination therapy, suggesting the need of drug delivery modifications. Among other possible mechanisms, Atwood et al have reported SMO mutations for development of resistance in basal cell carcinoma.⁴⁸

Herein, we focused on the formulation strategy to deliver both GEM and GDC-0449 effectively to the tumor site in a sustained release manner to decreased toxicity with increased effectiveness even at lower doses. We previously reported that the stealth effect of our PEGylated micelles also has an advantage of increased mean residence time at the tumor site.⁴⁹ Since the micelles were nanosized they could bypass the RES. Almost 80 % of encapsulated GDC-0449 and 19 % GEM were released from the micelles in a sustained manner *in vitro* when the formulations were mixed together (Fig. 2A). The release profile of GDC-0449 between two pH values depends on the solubility difference of GDC-0449 in acidic solutions. The solubility of GDC-0449 is pH dependent with 0.1 $\mu\text{g}/\text{mL}$ at pH 7.0 and 0.99 mg/mL at pH 1.0.⁶² Moreover, since GDC-0449 was not conjugated to polymer

backbone, faster release compared to neutral conditions was expected as the drug was encapsulated.

MIA PaCa-2 cells are known to express high levels of Hh ligands as well as mesenchymal markers.⁵⁰ Based on our earlier work where the cytotoxicity effects of GEM conjugated micelles and GDC-0449 encapsulated micelles were pronounced on MIA PaCa-2 cells compared to other cell lines. Therefore, we selected this cell line for in vitro and in vivo evaluation of the micelle mixtures carrying GEM and GDC-0449.^{18,38} We found that when GEM conjugated and GDC-0449 encapsulated micelles were added together, cytotoxic effect was much higher than single micelle formulation of GEM and GDC-0449 (Fig. 3A). GEM is a potent drug with its IC₅₀ value of 7.6 nM compared to GDC-0449 whose IC₅₀ is 6.3 μ M for MIA PaCa-2 cell-lines.¹⁸ The IC₅₀ of GEM conjugated micelles was 500 nM, but 10.7 μ M for GDC-0449 encapsulated micelles. For the micelle mixtures, we found 5 nM of GEM and 1 μ M of GDC-0449 had showed 50 % cell viability (Fig. 3A).

Apoptosis plays a key role in maintaining the homeostasis of normal cells. Cellular shrinkage, DNA fragmentation, membrane blebbing and variance at the molecular level are characteristics acquired during apoptosis.⁵¹ Among various apoptotic events, the cleavage of poly(ADP-ribose) polymerase-1 (PARP-1; chromatin-associated nuclear enzyme) deserves mention due to its role in repairing and stabilizing damaged DNA as well as transcriptional regulation. During DNA damage in cells, PARP-1 gets activated and its level increases in cells thus acting as a DNA repair machinery. However, the induction of apoptosis leads to cleaving of PARP-1 by caspase-3 into two fragments: 89 and 24 kDa as active site and DNA binding domain.⁵² In our study, after 72 h of incubation of MIA PaCa-2 cells with the micelle mixtures, increased PARP-1 cleavage was observed compared to single drug formulations (Fig. 3B). Mazumdar et al. have reported the role of sonic hedgehog (SHH) in colon cancer cell lines by inhibiting PARP cleavage and caspase-3 deactivation as one of the mechanisms for controlling cellular proliferation.⁵³ Their study was supported by the work of Singh et al where the authors investigated the role of GDC-0449 as an Hh inhibitor in pancreatic cancer stem cells.⁵⁴ Treatment with 10 μ M GDC-0449 showed significant enhancement of PARP cleavage. Interactions of Hh inhibitor, PARP and apoptosis are in good agreement with our results. Another important intermediate in the apoptosis signaling pathway, Bax (a proapoptotic protein) accelerates cell death in response to apoptotic stimuli.⁵⁵ We observed an increased Bax expression after the incubation of MIA PaCa-2 cells with the micelle mixtures carrying GEM and GDC-0449 compared to micelles containing either GEM or GDC-0449 alone (Fig. 3B). Under physiological conditions, the transmembrane protein PTCH-1 inhibits the seven span transmembrane protein Smo. However, upon binding of Hh ligand to its transmembrane PTCH-1 receptor, Smo inactivation is repressed which leads to further activation of Gli-1 transcription factors. GDC-0449 as a potent Smo inhibitor perturbs Gli-1 activation indirectly by binding to the G-protein coupled receptor (Smo).⁵⁶ As a result, occurrence of PTCH-1 loss of function resulted in downregulation of this transmembrane protein. We measured PTCH-1 and Gli-1 expression levels of MIA PaCa-2 cells after 72 h treatment with these formulations. PTCH-1 and Gli-1 expressions were inhibited more efficiently with the micelle mixtures carrying GEM and GDC-0449 compared to single drug treatment (Fig. 3B).

EMT enables epithelial cells to lose their polarity and cell-cell adhesion resulting in mesenchymal characteristics such as migration and invasion, which contributes to the aggressiveness of PDAC.⁵⁷ Thus, we evaluated the effects of Hh pathway inhibition on invasion, migration and colony forming potential of MIA PaCa-2 cells. After 48 h, while the control group, GEM-conjugated and GDC-0449 encapsulated micelle treated groups contained migrated cells at the scratched area, the cells treated with micelle mixtures lost their migration ability as observed from almost absence of cells at the scratched area. Clonogenic assay was carried out to evaluate the long-term cell survival as well as to compare the colony forming potential of cells after treatment with the formulations (Fig. 4B). Ten days post-incubation of MIA PaCa-2 cells with the micelle mixtures significantly decreased the colony forming potential of the cells.

In vivo efficacy of combination drug formulations was evaluated in athymic nude mice bearing ectopic tumor generated by subcutaneous implantation of MIA PaCa-2 cells. Treatment of these mice with combination formulation resulted in a significant reduction in the tumor growth rate and tumor weight compared to the control group (Fig. 5). High expression of Ki-67 protein was observed in the control group, whereas for GEM conjugated micelles, GDC-0449 encapsulated micelles and their micelle mixtures reduced proliferation to a significantly low level.⁵⁸ Morphological observation of tumor specimens by H&E staining implied the compact mass of epithelial cells in the control group and loose epithelial cell aggregates with a larger amount of interspersed mesenchymal cells treated with the micelle mixtures. Immunofluorescence assays demonstrated that Gli-1 expression was upregulated in tumor-associated stromal cells in pancreatic cancer tissue specimens. Treatment with only GEM conjugated micelles did not affect Gli-1 expression, which is in agreement with *in vitro* studies. Down-regulation of Gli-1 expression was observed in the tissue specimen of mice, which received combination drug formulation (Fig. 6A). ZEB-1 protein correlates with EMT activation and promotion of metastasis.⁵⁹ To analyze whether combination therapy can decrease ZEB-1 expression, we performed another immunohistochemistry assay where the micelle mixtures strongly suppressed ZEB-1 expression compared to GEM conjugated micelles alone (Fig. 6C). Only GEM conjugated micelles treated group displayed ZEB-1 expression almost in the same pattern with the control group, which is in good agreement with the data published previously.⁶⁰ Tumor specimen of the mice treated with the micelle mixture showed increased level of active caspase-3 signal and decreased level of Ki-67, which supports the anti-proliferative and apoptotic effects induced by the synergistic effect of GDC-0449 and GEM (Fig. 7A). Targeting Hh signaling single handedly was unable to repress tumor growth where aggressive tumor growth continues even after Hh deletion.^{24,28,61} It has been shown that tumor promotion is specifically linked to increased angiogenesis mediated by tumor stroma and it is mediated by a dose-dependent manner.²⁵ Furthermore, we analyzed the morphology of tumor tissues and found loosened groups of epithelial cells in combination treated mice tumor tissues. However, dense cells within tumor mass were visualized in the control group (Fig 7B).

Our results have shown that the delivery of micelle mixtures carrying GEM and GDC-0449 significantly inhibited MIA PaCa-2 xenograft tumor growth, induced apoptosis and suppressed EMT by the connection between Hh signaling and EMT. Our observations leads

to the fact that tumor regression phenomenon could be achieved by the synergistic effect of both potent drugs, GEM and GDC-0449 when delivered in a dose dependent manner. Although the actual mechanistic study behind this regression requires further investigations, yet it can be inferred that a sustained release formulation of these two drugs can overcome the drawbacks of known combination therapy otherwise leading to increased toxicity.

5. Conclusions

GEM conjugated and GDC-0449 encapsulated micelles were used in combination as a platform to provide a synergistic effect of both these two drugs. Downregulation of PTCH-1 and Gli-1 expression, suppression of ZEB-1, upregulation of Bax protein and PARP cleavage by the micelle mixtures carrying GEM and GDC-0449 overcome the drawbacks of simply mixing these drug solutions. In conclusion, our study showed that the micelle mixtures carrying GEM and GDC-0449 rather micelles carrying a single drug present a promising approach to inhibit pancreatic cancer addressing the delivery challenges.

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Figure 1.
 (A) Synthesis scheme of mPEG-b-PCC-g-GEM-g-DC or mPEG-b-PCC-g-DC. (B)
 Schematic illustration of GDC-0449 loaded micelles. (C) GEM conjugated micelles.

Figure 2.

(A) Drug release profiles of GEM conjugated and GDC-0449 loaded micelles. (B) Particle size distribution of GEM conjugated micelles. (C) GDC-0449 encapsulated micelles.

Figure 3.

(A) Cell viability of GEM micelles, GDC-0449 micelles and micelle mixtures carrying these two drugs in MIA PaCa-2 cell line. MTT assay was carried out at the end of 72 h treatment. Results are presented as the mean $\pm$ SD (n=3). (B) Western blot analysis for PTCH-1, Gli-1, Bax, Intact PARP, Cleaved PARP and β -actin.

Figure 4.

(A) Effect of GEM conjugated micelles, GDC-0449 encapsulated micelles and micelle mixtures carrying these two drugs on MIA PaCa-2 cells motility. Bars represent the means $\pm$ S.D. from three independent experiments. (B) Colony formation potential. Statistical analysis was performed for clonogenic potential, * $p < 0.01$; ** $p < 0.001$.

Figure 5.

In vivo efficacy of GEM micelles, GDC-0449 micelles and micelle mixture carrying these two drugs in tumor bearing mice. Tumors were developed by subcutaneous injection of 3×10^6 of MIA PaCa-2 cells in the right flank of athymic nude mice. When the tumor size reached 200 mm^3 , mice were injected intratumorally with micellar formulation of GEM, GDC-0449 and GEM + GDC-0049. Dose was 40 mg/kg GEM and 10 mg/kg GDC-0449. Doses were kept the same for the combination treatment. (A) Animal tumor volume during the treatment, (B) representative tumor and (C) tumor weight, and (D) change in animal body weight. Data expressed as the mean $\pm$ SE (n=6).

Figure 6.

(A) Immunostaining of cryosections for Gli-1 as Hh signaling marker, (B) Caspase-3 as an apoptosis marker. Tumor samples were cryosectioned, fixed and stained for Gli-1 (green), Caspase-3 (red) and DAPI (blue). Scale bar, 100 μ m. (C) Immunohistochemical analysis for ZEB-1 as EMT marker, scale bar, 20 μ m.

Figure 7.

(A) Immunohistochemical analysis of tumor samples for Ki-67 staining, scale bar, 50 μm .

(B) H&E staining, scale bar, 100 μm .