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Original Article

A novel gemcitabine analog as a potential anticancer agent: synthesis and *in-vitro* evaluation against pancreatic cancer

Joy Okoro[1], Raviteja Bulusu[1], Esther Frimpong[1], Xue Zhu[1], Sherise Rogers[2], Edward Agyare[1]

[1]College of Pharmacy and Pharmaceutical Sciences, Florida A&M University, Tallahassee, Florida, USA; [2]Department of Medicine, Division of Hematology and Oncology, University of Florida College of Medicine, Gainesville, Florida, USA

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Abstract: Gemcitabine (Gem) is approved for use in pancreatic cancer chemotherapy. However, Gem undergoes rapid metabolism in the blood, producing an inactive metabolite. Due to this rapid metabolism, the effective dose of Gem is high, thereby predisposing patients to severe adverse effects. This study aimed to improve Gem's metabolic and therapeutic stability by modifying the amine group (4-NH_2) with hydroxylamine to form 4-N-hydroxylGem hydrochloride (GemAGY). Micro-elemental analysis and Nuclear Magnetic Resonance (NMR) were used to characterize GemAGY, and its anticancer activity was investigated against MiaPaCa-2, BxPC-3, and PANC-1 pancreatic cancer cell lines. The GemAGY metabolic stability was evaluated in human liver microsomal solution. In the 2D cytotoxicity assay, the IC_{50} values of GemAGY-treated MiaPaCa-2, PANC-1, and BxPC-3 cells were significantly lower when compared to GemHCl-treated cultures. More so, in 3D spheroid assay results, GemAGY IC_{50} values were found to be 9.5 ± 1.1 μM and 12.6 ± 1.0 μM when compared to GemHCl IC_{50} values of 24.1 ± 1.6 μM and 30.2 ± 1.8 μM in MiaPaCa-2 and PANC-1 cells, respectively. GemAGY was stable, with 60% remaining intact after 2 hours of digestion in microsomal enzymes, compared to GemHCl, which had less than 45% remaining intact after 30 minutes. GemAGY-treated MiaPaCa-2 and PANC-1 cells at 3.12 and 6.25 μM concentrations demonstrated a significantly reduced cell migration towards the wound area compared to the GemHCl-treated cultures at the same concentrations. Further, GemAGY-treated MiaPaCa-2 cells significantly increased the expression of p53 and BAX compared to GemHCl-treated cells. GemAGY demonstrated significant anticancer activity and improved metabolic stability compared to GemHCl and is most likely to have potential anticancer activity against pancreatic cancer.

Keywords: Gemcitabine analog, 4-N-hydroxylGem hydrochloride, pancreatic cancer, synthesis, cytotoxicity, metabolic stability

Introduction

Pancreatic cancer is a disease of national importance in the United States because pancreatic cancer is the fourth leading cause of cancer death in men and women of all ages. In both sexes, the rate of new cases of pancreatic cancer has increased steadily by around 1% each year since the 1990s. In 2024, it is estimated that over 66,000 adults in the United States will be diagnosed with pancreatic cancer, and over 51,750 people (24,480 women and 27,270 men) will die of pancreatic cancer. The disease accounts for approximately 3% of all cancers [1]. In recent years, progress has been made in the early detection and treatment of pancreatic cancer [2]. Despite the progress made in the last decade, the overall median survival of this deadly malignancy is less than one year [2, 3], and when compared with many other cancers, pancreatic cancer has an accumulated five-year survival rate of about 5 to 10 percent, which is very low [4]. This is primarily attributed to the late detection and less effective therapies for pancreatic cancer [5].

Among the modalities of pancreatic cancer treatment, surgical resection combined with adjuvant systemic chemotherapy currently provides a higher chance of long-term survival [6,

7]. Hence, chemotherapy remains an important treatment option for pancreatic cancer patients [2, 8]. In considering chemotherapy, the first-line treatment option used clinically for people with locally advanced or metastatic pancreatic cancer includes the use of FOLFIRINOX (5-FU, leucovorin, irinotecan, and oxaliplatin) and Gem plus nab-paclitaxel. However, severe adverse effects have limited their use [8, 9].

Gem, a pyrimidine nucleotide antimetabolite, was approved by the Food and Drug Administration (FDA) for use as a monotherapy for the treatment of inoperable pancreatic cancer for decades following a promising randomized controlled trial of Gem versus 5-fluorouracil (5-FU) [10, 11]. Burris and colleagues reported that Gem had a higher efficacy than 5-FU in ameliorating some disease-attributable symptoms in patients with metastatic, symptomatic pancreatic cancer [12]. In evaluating its effectiveness, Gem was found to reduce cancer-related pain, contribute to an increased Karnofsky's performance score and quality of life, and subsequently result in a moderate improvement in survival time [13, 14]. After administration, Gem enters the cancer cell, and this step is followed by an initial phosphorylation mediated by the enzymes deoxycytidine kinase (dCK) and the extra-mitochondrial thymidine kinase 2 [15]. Subsequently, a series of phosphorylation steps are taken for Gem to be incorporated into both Deoxyribonucleic acid (DNA) and Ribonucleic acid (RNA) as its active phosphorylated form, which is Gem triphosphate (dFdCTP) [15]. Moreover, Ribonucleotide Reductase (RR), an enzyme in the nucleotide pathway that is responsible for enabling cancer cells to manage their pools of deoxynucleotides, is also inhibited by Gem diphosphate (dFdCDP) [15].

Despite Gem's modest improvement in cancer survival rate, following its systemic administration, Gem is rapidly metabolized to the inactive metabolite (2',2'-difluorouridine) by the enzyme cytidine deaminase. This process is known to shorten Gem's half-life and decrease its systemic stability, which eventually limits Gem's anticancer effect and application in clinical settings [16]. Owing to its poor systemic stability (plasma circulation half-life < 12 min) and rapid metabolism [16], Gem is usually administered at high doses (usually 1,000 mg/m^2) for 30 minutes via an intravenous infusion to improve its therapeutic levels [16], thereby causing severe adverse effects which present as renal, hematological, hepatic, and pulmonary toxicities [16].

Numerous pieces of evidence from the literature have demonstrated that the limited efficacy of Gem is primarily due to the deamination of its 4-NH_2 group by cytidine deaminase in the blood [17, 18]. Another limitation of this drug is that, after initial cancer remission, some cancerous cells may develop various types of drug resistance, for instance, resistance related to nucleoside transporter deficiency [17].

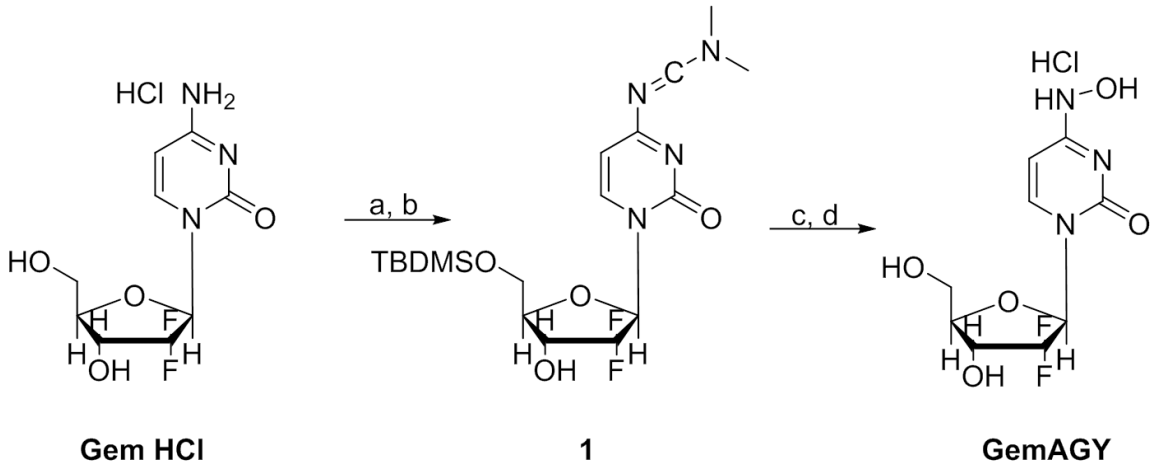
Despite the challenges associated with Gem, this drug remains the backbone of numerous regimens for chemotherapy, and diverse strategies or approaches have been developed to improve its clinical efficacy and stability [19]. One such approach is the chemical modification of drugs employed to address some metabolic stability issues associated with a drug. For example, some anticancer agents such as paclitaxel and Gem have been chemically modified (Paclitaxel was chemically modified to form paclitaxel-2'-*O*-3-pentadecylhemiglutarate, 10-Deacetylpaclitaxel, and Gem to PEG-gemcitabine, 4-(N)-stearoyl-gemcitabine, and 4-(N)-Tris-nor-squalenoylgemcitabine) to enhance their metabolic stability [20-22], increase half-life [22, 23], improve cellular uptake [22], and prolong cell retention [22].

Overall, we designed, synthesized, and evaluated GemAGY's metabolic stability and anticancer activity in this study.

Materials and methods

Materials

All analytical-grade reagents and chemicals were purchased from Sigma-Aldrich (St. Louis, Missouri, USA), and we purchased GemHCl (Gemcitabine Hydrochloride) from AK Scientific (Union City, CA). Pancreatic cancer cell lines MiaPaCa-2 (ATCC® CRL1420™), BxPC-3 (ATCC® CRL-1687™), and PANC-1 (ATCC® CRL1469™) were bought from American Type Culture Collection (ATCC) (Manassas, VA).



Scheme 1. Synthesis of GemAGY. Reagents and conditions: a) Pyridine, TBDMSCl, rt, 12 hrs; b) DMF-DMA, Toluene, reflux, 2 hrs; c) NH_2OH·HCl, Butanol/H_2O (1:1), reflux, 12 hrs; d) con. HCl, MeOH, rt.

Synthesis and characterization of GemAGY

Chemistry

The synthesis of GemAGY started from GemHCl with multiple steps of transformations, as depicted in **Scheme 1.** Protection of the primary hydroxy group by conversion of GemHCl into its 5'-TBDMS silyl ether was finished quantitatively. Transformation of the 4-NH_2 amine into its 4-dimethylamino imine Gem 1 by condensation of Gem 5'-TBDMS silyl ether with DMF-DMA (N, N-Dimethylformamide dimethyl acetal). The reaction was conducted by refluxing the toluene solution, and the reaction was monitored by TLC till the complete disappearance of the starting material Gem 5'-TBDMS silyl ether in two hours. Hydroxylamine was installed by refluxing 4-dimethylamino imine Gem 1 with hydroxylamine hydrochloride in butanol/H_2O (1:1). At the same time, the 5'-TBDMS silyl ether was removed to provide 4-N-hydroxyl Gem free base which then was converted into its HCl salt GemAGY.

Experimental

Unless otherwise specified, the reagents were purchased from commercial suppliers and used without further purification. NMR (Nuclear Magnetic Resonance) spectra were recorded on a Mercury 300 spectrometer operating at 300 (^{1}H NMR) and Bruker 600 MHz (^{13}C NMR) in DMSO-d^6 or $CDCl_3$. All ^{1}H and ^{13}C NMR chemical shifts were reported in parts per million and coupling constants J are given in Hz. The following abbreviations are used to describe peak patterns where appropriate: singlet (s), doublet (d), triplet (t), quartet (q), multiplet (m), and broad resonances (BR). All reactions were monitored by TLC (silica gel GF254, Merck, Kenilworth, New Jersey), and spots were visualized with UV light. Flash column chromatography on silica gel (200-300 mesh) was used for the routine purification of reaction products. Elemental analysis was done by Atlantic Microlab (Norcross GA).

4-amino-1-((2R,4R,5R)-5-(((tert-butyldimethylsilyl)oxy)methyl)-3,3-difluoro-4-hydroxytetrahydrofuran-2-yl)pyrimidin-2(1H)-one: To a solution of GemHCl (3.0 g, 10 mmol) in pyridine (20 mL) was added TBDMSCl (1.7 g, 11.3 mmol) at room temperature. After stirring for 12 hrs at room temperature, the reaction solution was diluted with EtOAc (300 mL) and washed with 10% HCl solution (200 mL), water (2 × 200 mL), sat. $NaHCO_3$ (50 mL). The organic layer was dried over Na_2SO_4 and filtered. The filtrate was concentrated in vacuo and followed by column chromatography on silica gel afforded Gem 5'-TBDMS silyl ether, 3.76 g, in a yield of 100%.

^{1}H NMR (DMSO-d^6): δ 7.60 (1H, d, *J* = 6.9 Hz), 7.35 (2H, s), 6.30 (1H, d, *J* = 6.0 Hz), 6.11 (1H, t, *J* = 8.1 Hz), 5.73 (1H, d, *J* = 7.5 Hz), 4.02-4.14 (1H, m), 3.92 (1H, d, *J* = 11.2 Hz), 3.76-3.85 (2H, m), 0.88 (9H, s), 0.07 (6H, s).

1-((2R,4R,5R)-5-(((tert-butyldimethylsilyl)oxy) methyl)-3,3-difluoro-4-hydroxytetrahydrofuran-2-yl)-4-(((dimethylamino)-l3-methylene) amino)pyrimidin-2(1H)-one: A solution of Gem 5'-TBDMS silyl ether (1.9 g, 5 mmol) and DMF-DMA (1.2 g, 10 mmol) in toluene (20 mL) was refluxed for 2 hrs. After cooling to room temperature, the reaction was directly purified through column chromatography on silica gel afforded 4-dimethylamino imine Gem 1, 1.92 g, in a yield of 90%.

^{1}H NMR ($CDCl_3$): δ 8.80 (1H, s), 7.84 (1H, d, *J* = 7.8 Hz), 7.26 (1H, s), 6.40 (1H, dd, *J* = 6.6, 8.7 Hz), 6.03 (1H, d, *J* = 7.2 Hz), 4.37 (1H, td, *J* = 7.5, 11.7 Hz), 3.95-4.04 (2H, m), 3.90 (1H, dd, *J* = 1.5, 11.4 Hz), 3.16 (3H, s), 3.12 (3H, s), 0.92 (9H, s), 0.10 (6H, s).

1-((2R,4R,5R)-3,3-difluoro-4-hydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl)-4-(hydroxyamino)pyrimidin-2(1H)-one hydrochloride: A mixture of 4-dimethylamino imine Gem 1 (2.15 g, 5 mmol), hydroxylamine hydrochloride (2 g, 30 mmol) in butanol-H_2O (1/1) (15 mL) was refluxed with stirring. After refluxing for 12 hours, the solid was filtered off, the solvent was removed in vacuo. The residue was purified through column chromatography on silica gel, producing a free base of 4-N-hydoxylGem, converted into hydrochloride salt, and further recrystallized with MeOH/EtOAc to produce GemAGY.

^{1}H NMR (DMSO-d^6): δ 10.04 (1H, s), 9.76 (1H, brs), 6.88 (1H, d, *J* = 8.1 Hz), 6.18 (1H, brs), 5.90 (1H, t, *J* = 9.0 Hz), 5.54 (1H, d, *J* = 8.1 Hz), 5.12 (1H, s), 4.00-4.10 (1H, m), 3.62-3,69 (2H, m), 3.48-3.56 (1H, m).

4-N-hydoxyl Gem, hydrochloride: ^{1}H NMR (DMSO-d^6): δ 10.49 (1H, brs), 7.31 (1H, d, *J* = 8.1 Hz), 6.09 (3H, brs), 5.98 (1H, t, *J* = 8.7 Hz), 7.31 (1H, d, *J* = 8.1 Hz), 5.98 (1H, t, *J* = 8.7 Hz), 4.13 (1H, dt, *J* = 8.1, 12.9 Hz), 3.76-3.80 (1H, m), 3.73 (1H, d, *J* = 12.9 Hz), 3.59 (1H, dd, *J* = 3.6, 12.3 Hz).

^{13}C NMR (DMSO-d^6): δ 148.84, 148.10, 135.35, 123.46 (t, *J* = 256.5 Hz), 95.37, 83.64 (t, *J* = 33.5 Hz), 81.40, 68.93 (t, *J* = 21.9 Hz), 59.30.

Micro elemental analysis of GemAGY

GemAGY ($C_9H_{11}F_2N_3O_5$·HCl) Calculated: (C) 34.25, (H) 3.83, (N) 13.31; Found: (C) 34.06, (H) 3.93, (N) 13.09.

Human liver microsome stability of GemAGY

The metabolic stability of GemAGY was evaluated using PBS (Phosphate-buffered Saline) and human liver microsomes purchased from Thermo Fischer Scientific. Usually, microsomes are used as the enzyme source for measuring metabolic stability [24]. Liver microsomes or PBS were used to spike GemAGY in concentrations of 10 μM. The samples were incubated in a water bath set at a temperature of 37°C for 7 hours. At designated time points of 0, 15, 30, 60, 120, 180, 220, and 240 minutes of incubation, 100 μL aliquots were taken, reconstituted with cool methanolic solution which contained an internal standard, vortexed for 4 minutes, and centrifuged (11,000×g, 12 minutes). The supernatants were immediately collected. The test and blank samples were prepared instantly and analyzed using HPLC. Triplicates of all samples were prepared [25].

Cell viability studies

The cell viability assays used pancreatic cancer cell lines (MiaPaCa-2, PANC-1, and BxPC-3). PANC-1 and MiaPaCa-2 cells were cultured with Dulbecco's modified Eagle medium (DMEM) with high glucose and L-glutamine and augmented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin (PenStrep) and 2.5% 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES). BxPC-3 cells were cultured with Roswell Park Memorial Institute (RPMI)-1640 medium with high glucose, L-glutamine, and HEPES. T75 flasks were used to plate the cells for the cell culture process. Thereafter, the cells were seeded in 96-well flat bottom plates after attaining 75-80% confluency.

2D-cell-viability studies

MiaPaca-2, PANC-1, and BxPC-3 cells were seeded in 96-well plates at 8,000 cells/well density and incubated at 5% CO_2 and 37°C. Stock solutions of GemHCl and GemAGY were prepared in 200 μM concentrations, and using the supplemented DMEM, the stock solutions

were serially diluted to obtain the following concentrations: 100, 50, 25, 12.5, 6.25, and 3.125 μM. 200 μL of each drug concentration in triplicates was used to treat all cells, followed by a 48-hour incubation at 37°C. At the termination of the treatment, 20 μL of 0.15% resazurin sodium salt (Alamar blue®) was added to each well, and the plates were incubated for 4 hours under optimal conditions (5% CO_2, 37°C). Fluorometric analysis was determined at an excitation wavelength of 560/580 nm and an emission wavelength of 590/610 nm, and the percent of viable cells per concentration was calculated [26].

3D-cell viability studies

Nunclon Sphera® 96-well plates were used to seed PANC-1 and MiaPaCa-2 cells at a seeding density of 10,000 cells/well using 100 μL of fresh complete media per well. The plates were incubated at conditions of 5% CO_2 and 37°C for 48 hours to form the 3D spheroids. A hundred microliter (100 μL) of each drug in the growth medium prepared following the procedure described for the 2D viability studies was used during treatment [26]. At the termination of treatment, 50 μL of 0.15% resazurin sodium salt (Alamar blue®) in growth medium was added to each well and carefully dispersed by pipetting before being incubated for 4 hours [26]. After that, fluorometric analysis was measured as described above.

Cell cycle studies

Cell cycle assay was carried out to investigate underlying basic mechanisms and evaluate the therapeutic efficacies of GemAGY [27]. MiaPaCa-2 and PANC-1 cells (1×10^6 cells per flask) were seeded in T-25 flasks and cultured using DMEM medium supplemented with 10% FBS, 10 mM HEPES, and 1% penicillin/streptomycin followed by incubation at 37°C [25]. At 75% confluency, the cells were treated using 3 concentrations (0.35 μM, 0.7 μM, and 1.5 μM) of GemHCl and GemAGY for MiaPaCa-2 and 0.5, 1.0 and 2.0 μM of GemAGY and GemHCl for PANC-1 cells. After 24 hours of drug treatment, the cells were harvested, washed with PBS, centrifuged at 350×g for 5 minutes, then resuspended the pellets in 100 μL of PBS, and passed through a 29-gauge needle to disperse the cells singly. Thereafter cells were fixed using ice-cold 70% ethanol whilst steadily vortexing. The cells were stored at a temperature of -20°C overnight [25]. After that, the cells were resuspended in 0.1 mg/mL RNase, stained with 200 μL of 50 μg/mL Propidium Iodide, and the phase distribution was examined using flow cytometry (FACScalibur-Becton Dickinson) [25].

Cell migration assay

A cell migration assay was conducted to determine the effect of GemAGY on the motility of MiaPaCa-2 and PANC-1 cells. Cell culture inserts obtained from Ibidi were used for this assay. The cells were grown in such a way as to have two confluent monolayers separated by a "wound" that was created by the inserts [25]. The cells were seeded into 12-well plates at a seeding density of 4×10^4, per well in 140 μL of serum-free media for 24 hours at 37°C. After 24 hours, adherent monolayers were formed on the two sides of the tissue culture insert. Hence, the inserts were removed gently to create a gap or "wound" between the two confluent layers of cells, and the monolayers were gently washed with experimental media [25]. Subsequently, the cells were treated with different concentrations of GemHCl and GemAGY treatments. The cells invading the wound area were imaged after 24 hours with a Nikon Ti Eclipse microscope [28].

Clonogenic assay

To carry out the colony formation assay, MiaPaCa-2 cells were seeded into T-25 cm^2 culture flasks at a density of 1×10^6 cells and cultured in DMEM medium supplemented with 2 mM L-glutamine, 10 mM HEPES, 10% FBS, and 1% penicillin/streptomycin [25]. On attaining 75% confluency, the cells were exposed to two concentrations of (1 μM and 3 μM) of GemHCl and GemAGY and incubated for 48 hours. Controls were also used, which had none of the treatments. After 48-hour exposure, treatment was terminated, cells harvested, then re-plated onto 6-well plates at a density of 1,000 cells per well, and incubated with a growth medium. The controls were used to monitor the confluency of the cell. At 75% confluency of the cells, the experiment was terminated, and the cells were fixed for 30 minutes using a methanol and acetic acid mixture in a 7:1 ratio. After that, the plates were stained with 0.5% crystal violet solution for 2 hours [25]. The stained colonies

(fifty per colony) were counted using a Jenco™ Stereomicroscope [25]. The plating efficiency (PE) and survival fraction (SF) were calculated, and a graph of the percentage of the survival fraction versus the concentration was obtained [29].

3D spheroid assays

To determine the effects of GemAGY on a 3D spheroid culture, MiaPaCa-2 and PANC-1 cells (10,000 cells/well) were seeded into 96 U round bottom Nunclon Sphera plates and incubated for 48 hours for the spheroids to form [28]. Thereafter, two treatments with GemHCl and GemAGY at 24 and 48 hours, respectively, were introduced into the cells. Three replicates were prepared for each treatment concentration. After 72 hours of the spheroids retaining the treatment, the spheroids were stained with 5 µg/mL of an acridine orange/ethidium bromide (AO/EB) solution. The spheroid cultures were observed for 72 hours for signs of degeneration, and images of the spheroids were captured using a Nikon Ti Eclipse microscope. Using the NIS Element software version 4.30.02 (Nikon Instruments Inc., Melville, NY, USA), the ratio of the fluorescent intensities of AO over EB for each GemHCl and GemAGY treatment concentration was computed, analyzed using Microsoft Excel, and plotted using GraphPad Prism version 10.0 for Windows [28].

Western blot assay

Western blot assay was performed as previously described [30]. Briefly, MiaPaCa-2 cells were seeded in T-75 culture flasks containing DMEM supplemented with 10% FBS at a cell density of 1.5×10^6 cells per flask [31]. Cells were exposed to drug treatment at $IC_{50}/2$ and IC_{50} concentrations of GemHCl and GemAGY for 12 hours upon attaining optimum confluency. Further, cell culture flasks were washed in PBS, and whole cell lysate was prepared using RIPA buffer containing protease and phosphatase inhibitor (1:100) (Sigma Aldrich, Louis, MO, USA) while the cell culture flasks were placed on ice. Incubation on ice was done for 30 minutes, and subsequently, the supernatant was collected in 2 ml Eppendorf tubes after centrifuging at 12,000 rpm for 15 minutes. Thereafter, the protein content of the supernatant was estimated using the BCA protein assay protocol [32]. To proceed with gel electrophoresis, a loading sample was prepared by heating at 98°C for 10 minutes using a heating block [31]. Samples containing 40 µg equivalent of the extracted proteins were allowed to run on a gel electrophoresis machine and then transferred onto a Polyvinylidene fluoride membrane (PVDF). Following the transfer, blocking was performed for 1 hour using 5% BSA (Bovine Serum Albumin) in Tris-Buffered Saline Tween 20 (TBST). Then the blots were cut according to their appropriate molecular sizes and incubated in a fridge overnight on a shaker in the primary antibodies prepared in TBST. Primary antibodies used were p53 (1:1,000), PARP (Poly (ADP-ribose) polymerase) (1:1,000), β-actin (1:1,000), HER2 (1:1,000), BAX (1:1,000) and EGFR (1:1,000).

The next day, the blots were washed 3 times (5 minutes for each wash) and subsequently incubated with Horseradish peroxidase (HP) - conjugated secondary antibodies (Cell signaling technology, USA) (1:20,000) for 1 hour [31]. After that, the blots were washed with the wash buffer, prepared for detection using a chemiluminescence solution (Thermo Fisher), and visualized using the ChemiDoc™ XRS+ imaging system (Bio-Rad).

Statistical analysis

Statistical analysis of the data obtained from experiments was performed using GraphPad Prism 10 Software. ANOVA followed by Tukey's Multiple Comparison Test or ANOVA with post hoc Dunnett's tests was used to analyze the GemHCl and GemAGY treatment groups. Statistical significance was considered for p-value < 0.05. We ensured that we repeated all experiments at least three times.

Results

Considering the pyrimidine analogs, Gem (2',2'-difluorodeoxycytidine, dFdC; Gemzar®) is one of the most widely used drugs in clinical oncology and ranked third as the most prescribed anticancer agent worldwide [15]. Gem enters the tumor cell to exert its effect and becomes activated by phosphorylation [33]. However, Gem is rapidly cleared from the body through renal excretion and undergoes enzymatic conversion to the inactive and more soluble metabolite 2',2'-difluorodeoxyuridine (dFdU) by the enzyme Cytidine deaminase

Table 1. Micro-elemental analysis of GemAGY

Element	Theory (%)	Found (%)
C	34.25	34.06
H	3.83	3.93
N	13.31	13.09

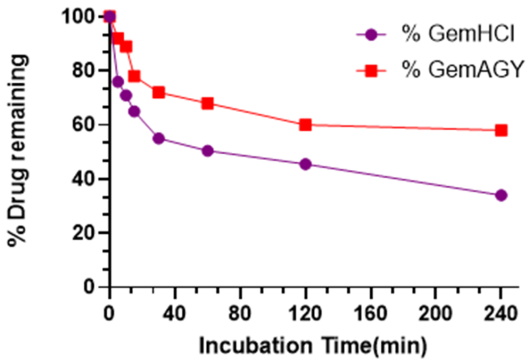


Figure 1. *In-vitro* metabolic stability of GemAGY and GemHCl in human liver microsomes after exposure for 4 hours.

(CDA) expressed in blood, liver, kidney, and various tumor tissues [34, 35]. CDA deaminates and deactivates Gem, thereby transforming Gem into inactive metabolites [36, 37]. More so, high CDA activity in patients who are on Gem activity correlates with disease progression [37]. Gem (< 10%) and its main metabolite, dFdU, account for 99% of the excreted dose after administration. As a result of this rapid metabolism and inactivation by CDA, Gem has a very short plasma half-life (8-17 min) [38, 39], and to attain therapeutic drug levels, Gem is currently administered at a high dose of 1,000 mg/m^2 as a 30-minute intravenous infusion [34].

To counter these limitations of rapid metabolism and short half-life associated with Gem, we designed and synthesized GemAGY. Our rationale for modifying GemHCl to obtain GemAGY was to improve GemHCl's metabolic stability and cytotoxic activity. We hypothesized that the hydroxyl group at the 4NH$_2$ position would shield the amine group and delay Gem's rapid deamination by the enzyme CDA. Our aim is that if the amine is shielded by the hydroxyl group, deamination by CDA is delayed, and circulation time will be increased, leading to improved metabolic stability and therapeutic efficacy. Our aim in this modification is supported by evidence in the literature that points out that inhibiting or delaying the deamination of Gem by cytidine deaminase increases the sensitivity of Gem and its cytotoxicity in human cancer cell lines [40, 41].

Synthesis and characterization of GemAGY

GemAGY was successfully synthesized and had a good yield of 90%. GemAGY was characterized using NMR and micro elemental analysis. GemAGY's NMR spectra were observed using ^{1}H and ^{13}C NMR (See Figures S1 and S2).

Micro-elemental analysis

The elemental analysis of GemAGY is presented in **Table 1** and Figure S3. It shows that 34.06% of carbon, 3.93% of hydrogen, and 13.09% of Nitrogen were found in GemAGY, which was comparable with the theoretical values of 34.25% carbon, 3.83% hydrogen, and 13.31% nitrogen. The data presented in **Table 1** showed that the purity of GemAGY was 99.6% based on the elemental analysis.

Human liver microsome stability of GemAGY

We investigated the in-vitro metabolic stability of GemAGY in human liver microsomes using HPLC. The graph (**Figure 1**) shows our findings at the end of the experiment. The percentage of drug remaining or intact GemAGY against the different time points was plotted. The HPLC analysis of the percentage of GemAGY remaining was 60% after 2 hours. However, on performing the same study to determine the stability of GemHCl in vitro using the liver microsomes, less than 45% of intact GemHCl remained after 2 hours. Our findings suggest that GemAGY may have better metabolic stability than GemHCl.

Cytotoxic effect of GemAGY on MiaPaCa-2, PANC-1 and BxPC-3 cells

The cytotoxic activity of GemAGY was evaluated in comparison to the standard drug GemHCl in MiaPaCa-2, PANC-1, and BxPC-3 via 2D culture and 3D spheroids using the Resazurin assay. As shown in **Table 2**, GemAGY exhibited higher cytotoxic activity in MiaPaCa-2 culture with an IC_{50} value of 1.6 ± 0.2 μM, compared to GemHCl after 48-hour treatment with a greater than two-fold increase for GemHCl (4.0 ± 1.7 μM)

Table 2. IC_{50} values of GemHCl and GemAGY treated 2D MiaPaCa-2, PANC-1 and BxPC-3 cells

Drug/Analog	MiaPaCa-2	PANC-1	BxPC-3
GemHCl	4.0 ± 1.7	5.6 ± 1.3	5.5 ± 1.0
GemAGY	1.6 ± 0.2	1.7 ± 0.2	3.2 ± 1.1

treated groups, respectively. A similar trend was observed in PANC-1 and BxPC-3 treated cells. In PANC-1 cells, GemAGY exhibited a higher cytotoxic effect with an IC_{50} value of 1.7 ± 0.2 μM compared to GemHCl (5.6 ± 1.3 μM) after 48-hour treatment. Similarly, in 3D spheroids, GemAGY exhibited a higher cytotoxic effect when compared to GemHCl. While IC_{50} values were expectedly higher in the 3D treated MiaPaCa-2 cultures compared to that of 2D MiaPaCa-2 cultures (**Figure 2A**, **2B** and **Table 3**), GemAGY treated 3D MiaPaCa-2 culture (IC_{50} = 9.46 ± 1.1 μM) showed lower IC_{50} values compared to GemHCl treated 3D MiaPaCa-2 culture (IC_{50} = 24.12 ± 1.6 μM). A similar trend was observed for studies performed with PANC-1 cells, in which even though the IC_{50} values were higher in 3D models than in 2D models, the IC_{50} values for the 3D cultures treated with GemAGY were still lower than those of GemHCl-treated cultures. Comparing the IC_{50} values, as shown in **Table 3**, indicates that GemAGY demonstrated a significantly higher cytotoxic activity compared to GemHCl in 2D and 3D cultures.

Cell cycle assay

To evaluate the stages in the cell cycle promoting a decreased cell proliferation rate, the cell cycle assay was conducted using MiaPaCa-2 and PANC-1 cells. The cells were cultured in T-25 flasks and thereafter divided into four groups, corresponding to the treatment concentrations, which were 0, 0.35, 0.70, and 1.50 μM concentrations for MiaPaCa-2 cells and 0, 0.5, 1.0, and 2.0 μM for PANC-1 cells. Treatment lasted for 24 hours, as previously described. Our findings indicated that GemAGY significantly impeded the cell cycle progression by increasing the percentage of cells arrested at the G_1 phase (**Figure 3A**). As shown in **Figure 3A**, at 0.70 μM, GemAGY-treated cells revealed a higher G_1 population (60.24%) in MiaPaCa-2 cells compared to the corresponding GemHCl treatment (53.24%). At 1.50 μM, GemAGY significantly increased the percentage of cells arrested in the G_1 phase (72.80%) compared to GemHCl treatment (55%). Similarly, in PANC-1 cells, GemAGY increased the percentage of cells arrested in the G_1 phase (58.20% at 2.0 μM) compared to GemHCl treatment (52.70%), as shown in **Figure 3C** and **3D**. The GemAGY-treated groups exhibited a significant concomitant reduction in MiaPaCa-2 cells entering the S and G_2 phases (**Figure 3B**). GemAGY stimulated a decrease in cell population entering the G_2 phase at 1.50 μM concentration in MiaPaCa-2 cells (0.26%) compared to GemHCl treatments, which showed a higher cell population of 7.20% and 9.54%. Our findings in this assay indicated that GemAGY induced G_1-phase cell cycle arrest at low concentrations (0.35, 0.7, 1 μM), impeding cell transition to the S and G_2 phases.

GemAGY cell migration assay

The mobility of cells or migration towards a gap/wound uniformly made in confluent monolayer cultures of MiaPaCa-2 and PANC-1 cells was evaluated. The mobility of the cells toward the wound was evaluated at 0, 1.56, 3.125, and 6.25 μM after 24 hours. As seen in **Figure 4A** and **4C**, the controls (untreated MiaPaCa-2 and PANC-1 cells) migrated towards the wound and covered the entire surface area of the Ibidi cell culture inserts, which was expected. GemAGY-treated MiaPaCa-2 cells at 3.12 and 6.25 μM concentration significantly reduced cell mobility towards the wound area with (102.3 ± 4.1) and (65.7 ± 6.0) number of cells migrating respectively compared to GemHCl treatments which showed (192 ± 3.0) and (174.3 ± 4.0) MiaPaCa-2 cells at 3.12 and 6.25 μM concentrations (**Figure 4B**). Analogous to our findings in MiaPaCa-2 cells, GemAGY-treated PANC-1 cells at 3.12 and 6.25 μM concentrations significantly reduced the migration of the cells towards the wound area with (232.0 ± 15.5) and (97.7 ± 5.6) number of cells migrating respectively compared to GemHCl treatments which showed (392 ± 4.0) and (253.7 ± 6.7) at 3.12 and 6.25 μM concentrations (**Figure 4C**, **4D** and **Table 4**).

Clonogenic assay of GemAGY

The colony formation assay assessed the proliferative property of MiaPaCa-2 cells after exposure to two concentrations of GemHCl and GemAGY. This assay was conducted to assess

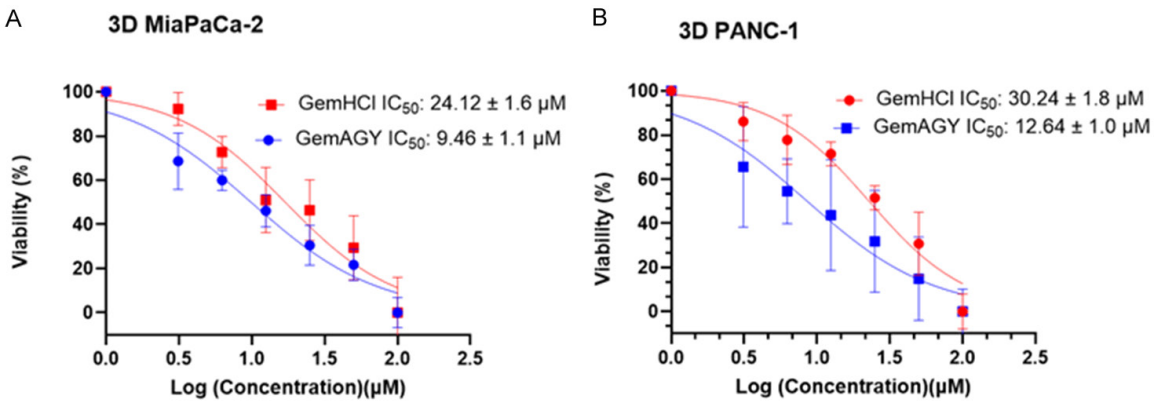


Figure 2. Cytotoxic activity of GemHCl and GemAGY on MiaPaCa-2 and PANC-1 spheroid cultures using Resazurin assay. A and B. 3-D spheroid cultures showing GemHCl and GemAGY-treated MiaPaCa-2 and PANC-1 cells, respectively.

Table 3. Comparison of IC_{50} values of GemHCl and GemAGY treated 2D and 3D MiaPaCa-2, PANC-1 cells

Cell line	GemHCl ± SD (μM)	GemAGY ± SD (μM)	*p*-value
MiaPaCa-2 (2D)	4.0 ± 1.7	1.6 ± 0.2	< 0.01
PANC-1 (2D)	5.6 ± 1.3	1.7 ± 0.2	< 0.01
BxPC-3 (2D)	5.5 ± 1.0	3.2 ± 1.1	< 0.05
MiaPaCa-2 (3D)	24.1 ± 1.6	9.5 ± 1.1	< 0.001
PANC-1 (3D)	30.2 ± 1.8	12.6 ± 1.0	< 0.001

Data represented as ± SEM, *n* = 3.

GemAGY's ability to prevent tumor relapse after treatment. As shown in **Figure 5A**, GemAGY induced concentration-dependent inhibition of clonogenic cell survival on MiaPaCa-2 cells. The difference in percent survival of GemHCl and GemAGY-treated MiaPaCa-2 cells revealed a reduction in colony formation for GemAGY-treated cells at different concentrations. This assay establishes GemAGY's ability to disrupt the proliferation of cancer cells compared to GemHCl (**Figure 5A**). As shown in **Figure 5B**, the survival curve depicts the differences in both treatment groups. The clonogenic assay result specifies the effectiveness of GemAGY and its potential to significantly inhibit the proliferation and survival of cancer cells when compared to GemHCl.

GemAGY induces degeneration of 3D spheroid of MiaPaCa-2 and PANC-1 cells

To elucidate drug response characteristics in a system that proffers a simulation of the tumors in an in vivo environment, the effect of GemAGY was studied using 3D spheroids. MiaPaCa-2 and PANC-1 cells were seeded in Nunclon Sphera 96-well, U-shaped-bottom microplates and were allowed to grow and form compact spheroids. The spheroid cultures were then treated with varying concentrations of GemHCl and GemAGY. The effects of GemHCl and GemAGY were captured using the Nikon Ti Eclipse microscope (4× magnification) with the Nikon DS Qi2 camera. As seen in **Figure 6**, the spheroids disintegrate at higher concentrations of GemHCl and GemAGY. The effects of the two drugs on the spheroid cultures of MiaPaCa-2 and PANC-1 were analyzed by measuring the areas of the spheroid bodies using the NIS-Element software. GemAGY induced higher disintegration in PANC-1 and MiaPaCa-2 spheroids when compared to GemHCl. The results are the mean ± SEM, n = 3. Statistical significance (*P < 0.05, **P < 0.01 and ***P < 0.001) was determined using one-way ANOVA with post hoc Dunnett's tests.

GemAGY induces concentration-dependent cell death in MiaPaCa-2 and PANC-1 spheroids

Following our results from the degeneration of MiaPaCa-2 and PANC-1 spheroids, we demonstrated the induction of cell death by GemAGY in MiaPaCa-2 and PANC-1 spheroids. The spheroids were stained with Acridine Orange/Ethidium Bromide (AO/EB) (5 μg/mL), and the fluorescent images were taken at 4× magnification using the Nikon Ti Eclipse microscope. Following the capture of images, the mean fluorescence intensity ratios were calculated (AO

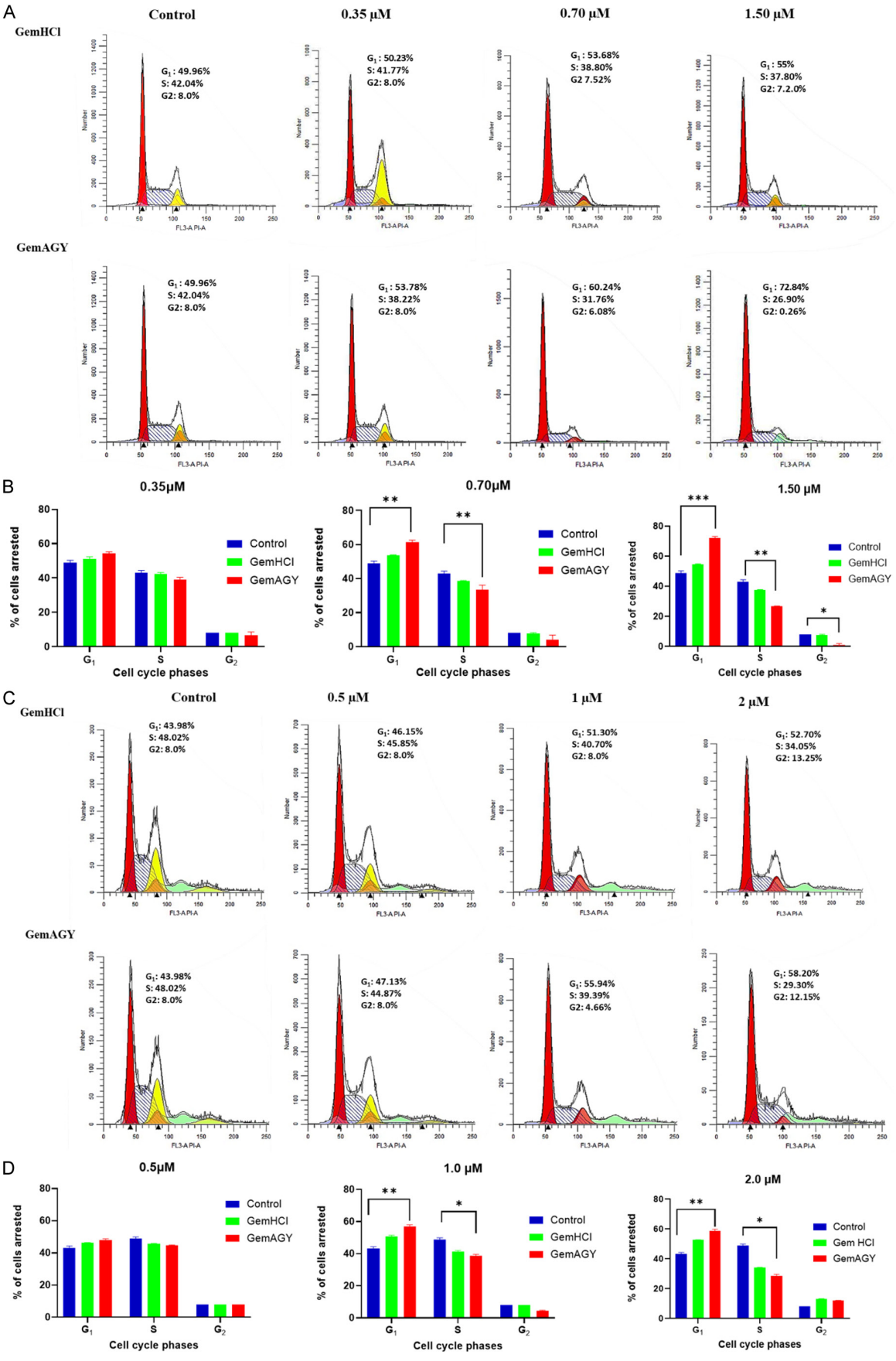

A
Control
0.35 µM
0.70 µM
1.50 µM
GemHCl
G1 : 49.96%
S: 42.04%
G2: 8.0%
G1 : 50.23%
S: 41.77%
G2: 8.0%
G1 : 53.68%
S: 38.80%
G2 7.52%
G1 : 55%
S: 37.80%
G2: 7.2.0%
GemAGY
G1 : 49.96%
S: 42.04%
G2: 8.0%
G1 : 53.78%
S: 38.22%
G2: 8.0%
G1 : 60.24%
S: 31.76%
G2: 6.08%
G1 : 72.84%
S: 26.90%
G2: 0.26%
B
0.35µM
0.70µM
1.50 µM
% of cells arrested
Control
GemHCl
GemAGY
G1
S
G2
Cell cycle phases
C
Control
0.5 µM
1 µM
2 µM
GemHCl
G1: 43.98%
S: 48.02%
G2: 8.0%
G1: 46.15%
S: 45.85%
G2: 8.0%
G1: 51.30%
S: 40.70%
G2: 8.0%
G1: 52.70%
S: 34.05%
G2: 13.25%
GemAGY
G1: 43.98%
S: 48.02%
G2: 8.0%
G1: 47.13%
S: 44.87%
G2: 8.0%
G1: 55.94%
S: 39.39%
G2: 4.66%
G1: 58.20%
S: 29.30%
G2: 12.15%
D
0.5µM
1.0 µM
2.0 µM
% of cells arrested
Control
GemHCl
GemAGY
Gem HCl
G1
S
G2
Cell cycle phases

Figure 3. Cell cycle assay on MiaPaCa-2 and PANC-1 cells following treatment with GemHCl and GemAGY. A and C. Flow cytometry images of MiaPaCa-2 and PANC-1 cells arrested at different cell cycle phases. B and D. MiaPaCa-2 and PANC-1 cells show the percentage of cells at different cell cycle phases, respectively.

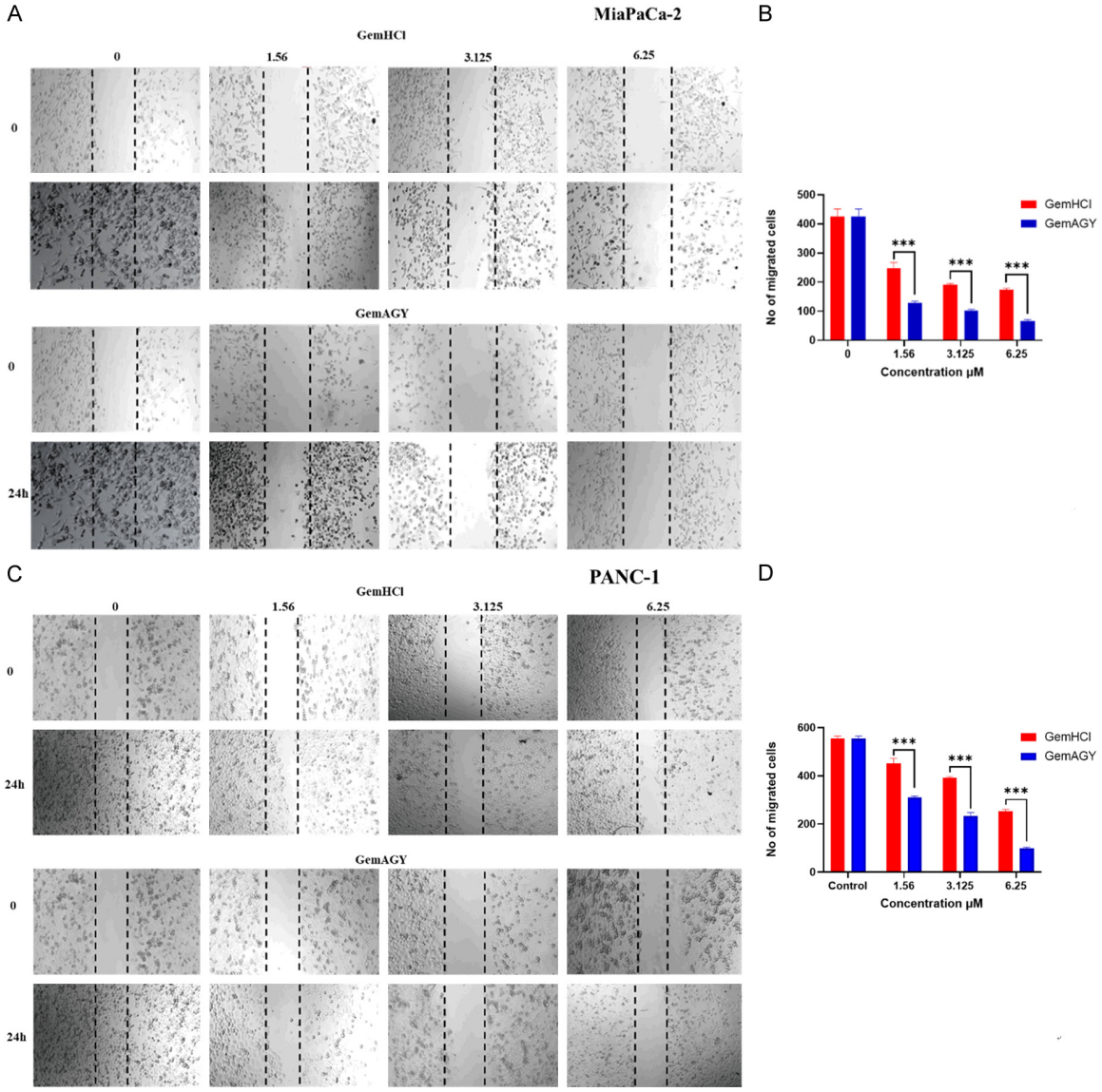


Figure 4. Cell migration study of GemAGY compared to GemHCl using MiaPaCa-2 and PANC-1 cells. (A and C) show the MiaPaCa-2 and PANC-1 cells that migrated when treated with 1.56, 3.125 and 6.25 μM of GemHCl and GemAGY for 24 hours. GemAGY exhibited higher inhibition of the migration of cells toward the wound area compared to GemHCl. closures of wounds were captured at 40× magnification using the Nikon Eclipse Ti inverted fluorescent microscope. (B and D) are the plots of the means of the number of cells that migrated ±SD, n = 3. Significance (GemHCl and GemAGY $***P < 0.01$) was determined by t-test.

(FITC)/EB (TRITC)) and analyzed using the Two-Way ANOVA with Tukey's Multiple Comparison Test. Statistical significance (*P < 0.05 and ***P < 0.001) was established following the analysis. As shown in **Figure 7**, a significant disruption of already compact spheroids generated with MiaPaCa-2 and PANC-1 cells was induced by GemAGY when compared to MiaPaCa-2 or PANC-1 cells treated with GemHCl. More so, acridine orange/ethidium bromide (AO/EB) staining at 48 h post-GemHCl and GemAGY-treatments revealed significant cell

Table 4. Estimation of GemHCl and GemAGY treated cells that migrated toward the wound area

The quantification of migrated cells				
Concentration (μM)	MiaPaCa-2		PANC-1	
	GemHCl	GemAGY	GemHCl	GemAGY
0 (control)	420 ± 9	430 ± 5	525 ± 10	555 ± 2
1.56	248 ± 10	128 ± 7	452 ± 16	311 ± 4
3.12	192 ± 3	102 ± 4	392 ± 4	232 ± 15
6.25	174 ± 4	66 ± 3	254 ± 7	98 ± 6

death in the spheroids as the concentration increased (**Figure 7A** and **7B**). On evaluating the spheroids' mean AO/EB intensity, concentration-dependent decreases could be observed in MiaPaCa-2 and PANC-1, respectively (**Figure 7C**, **7D**). GemAGY significantly induced higher cell death in both cell lines as the concentration increased compared to GemHCl.

Western blot analysis

The level of tumor suppressor protein p53 expression significantly increased with an increase in the concentration of GemAGY-treated MiaPaCa-2 cells compared to GemHCl-treated cells and the controls, which had no treatment (**Figure 8**). Similarly, the expression of the pro-apoptotic protein BAX was significantly more upregulated with an increase in the concentration of MiaPaCa-2 cells treated with GemAGY compared to cells treated with GemHCl. The PARP protein was significantly downregulated in GemAGY-treated MiaPaCa-2 cells compared to GemHCl-treated cells. Similarly, a significant concentration-dependent downregulation was observed for the EGFR and HER2 proteins in GemAGY-treated cells compared to GemHCl-treated cells. Inhibition of the EGFR signaling pathway is an attractive therapeutic target. Hence, GemAGY may be useful in targeting the inhibition of EGFR.

Discussion

In recent decades, Gem has become a first-line treatment for pancreatic cancer chemotherapy and a critical target drug for research [42-44]. The ability of Gem to suppress tumor growth in pancreatic cancer cell lines has been well documented. However, the use of Gem in clinical practice is limited by its short half-life, systemic toxicity, and high doses needed to attain therapeutic levels [34, 38, 39]. Despite the issues associated with Gem, this drug remains an important drug in cancer therapy. This study highlights the importance and benefits of a synthesized Gem analog (GemAGY) in in vitro studies using MiaPaCa-2, PANC-1, and BxPC-3 pancreatic cancer cell lines.

Milovica and colleagues provided evidence that non-toxic hydroxylamine-containing inhibitors of ODC (1-aminooxy-3-aminopropane, APA) and SAMDC (*S*-(5'-deoxy-5'-adenosyl)-methylthioethyl-hydroxylamine, AMA) induced growth arrest and enhanced the antiproliferative actions of standard cytostatic drugs such as 5-FU [45]. Also, hydroxylamine-containing ODC and SAMDC blockers APA and AMA are potent cytostatic agents in colon cancer cells [45]. Additionally, Lianshuang Zhang and colleagues, in their study on human carcinoma cell lines, reported that in evaluating the biological activity of a series of hydroxyl-substituted double Schiff-base 4-piperidone/cyclohexanone derivatives, they showed better anticancer activity than cyclohexanone derivatives [46].

In this study, we began by confirming the structure and purity of GemAGY using NMR and micro-elemental analysis. NMR was used to determine molecular identity and structure, confirm the hydroxylamine linkage, and characterize GemAGY. The micro-elemental analysis matched GemAGY's calculated carbon, hydrogen, and nitrogen presence, confirming the compounds' purity to be 99.6% as shown in **Table 1**. The purity of GemAGY was within the acceptable range. Usually, the measured values have to fit the gold standard of ± 0.40% of the calculated values to guarantee that sufficient purity has been attained [47].

Following characterization, we determined GemAGY's in vitro metabolic stability. Our findings indicated that GemAGY was very stable both in vitro and in human liver microsomes, as we reported that over 60% of the drug remained intact after the 2-hour exposure, suggesting that GemAGY had higher metabolic stability in comparison to GemHCl as reported in previous studies [48, 49].

The 2D cell viability data obtained after treating MiaPaCa-2, PANC-1, and BxPC-3 cells with

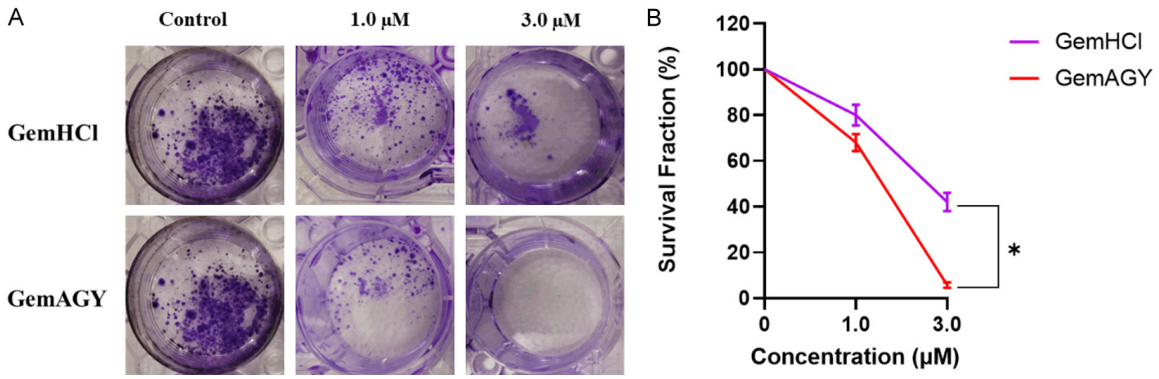


Figure 5. Colony formation studies of GemHCl and GemAGY against MiaPaCa-2 cells. A. Colony formation assay post-treatment images of GemHCl and GemAGY treated MiaPaCa-2 cells. B. The Survival curve of GemHCl and GemAGY treated MiaPaCa-2 cells. Data represents mean ± SD, *n* = 3 with 20× magnification.

GemAGY suggested that this compound could be a potential therapeutic agent in treating pancreatic cancer (**Table 2**). The IC_{50} values for GemAGY were significantly lower than those of GemHCl for all three cell lines. This suggests that GemAGY has a higher cytotoxic effect than GemHCl (**Table 3**). In MiaPaCa-2 and PANC-1 cells, GemAGY exhibited a higher anticancer effect, with over 2-fold and 3-fold increased effects over GemHCl. Since MiaPaCa-2 and PANC-1 cells were more sensitive to GemAGY when compared to the BxPC-3 cell line based on our 2D cell viability data, we decided to exclude the BxPC-3 cell line from future studies. More so, some studies have shown that BxPC-3, Capan-1, and PancTu-1 cells resist Gemcitabine-induced apoptosis. Therefore, for the 3D assays on spheroids and most of the in vitro experiments, we focused on MiaPaCa-2 and PANC-1 cell lines, and in the western blot and clonogenic assays, we focused only on MiaPaCa-2 due to its high sensitivity to Gem when compared to the other two cell lines [50].

In 3D spheroids of MiaPaCa-2 and PANC-1, GemAGY exhibited higher cytotoxic activity and spheroid structure degeneration than GemHCl. 3D spheroids were used to simulate the tumor environment in vivo closely, and our results provide evidence of GemAGY's superior cytotoxic activity over GemHCl.

In comparing the IC_{50} values of GemAGY in MiaPaCa-2 and PANC-1 cells after 48-hour treatment to other studies on modified Gemcitabine, we observed that GemAGY had a lower IC_{50} value than reported in the literature for other modified Gemcitabine compounds [51-55]. A higher cytotoxicity was expected. This is because we theorized that improved metabolic stability of GemAGY will inherently lead to improved cytotoxic activity. Based on our in vitro metabolic assay in liver microsomes, it was evident that GemAGY was more stable than GemHCl. This means that GemAGY is not rapidly metabolized and will potentially have more contact time in vivo, leading to a longer half-life and improved therapeutic efficacy compared to GemHCl. However, the exact mechanism GemAGY uses to inhibit cell proliferation is unknown.

The cell cycle is a highly regulated process that enables cell growth, duplication of genetic material, and cell division [56, 57]. This regulation of the cell cycle progression in cancer metastasis is a promising strategy to control tumor growth [25]. Gem, as reported in the literature, is a cell cycle-specific and antimetabolic chemotherapeutic drug [58]. Gem can arrest the cell cycle in the G_1/S phase and inhibit DNA synthesis [58, 59]. Our research findings on the cell cycle are in line with the literature. Our results show that GemAGY-treated MiaPaCa-2 and PANC-1 cells at 1.5 μM and 2 μM concentrations, respectively, exhibited a significant arrest of cell cycle progression at the G_1-phase. This cell cycle arrest at the G_1-phase observed at 1.5 μM and 2 μM concentrations suggests a cell-cycle-specific nature observed in Gem treatments. This cell cycle arrest at the G_1-phase shown in this experiment could explain the increased cell count at the G_1-phase, which blocked the cells from advancing to the S-phase for DNA replication. Hence, this

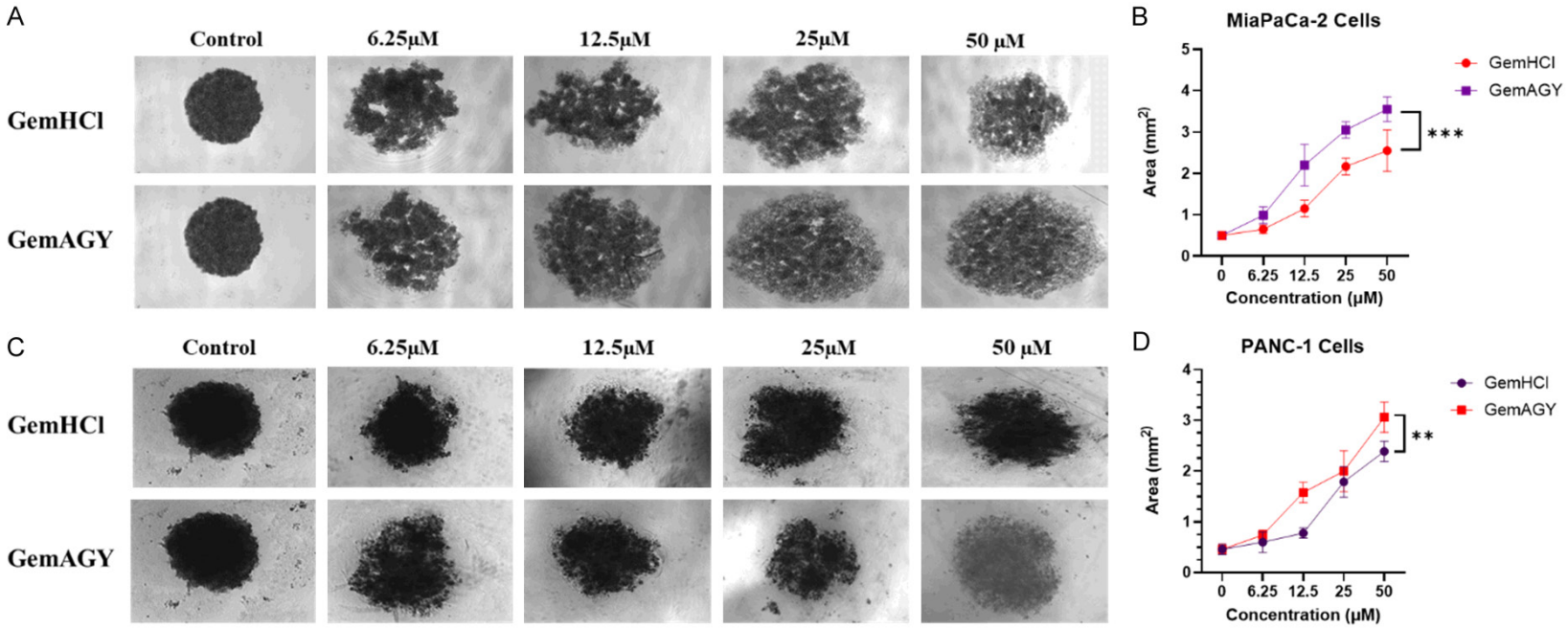


Figure 6. Degeneration of MiaPaCa-2 and PANC-1 spheroid cultures. GemHCl and GemAGY induce degeneration of MiaPaCa-2 and PANC-1 (A, C) spheroid cultures. The effects of GemHCl and GemAGY were captured using the Nikon Ti Eclipse microscope (4× magnification) with the Nikon DS Qi2 camera. (B) shows graph of mean areas of formed MiaPaCa-2 spheroids as concentration was increased and (D) shows graph of mean areas of formed PANC-1 spheroids as concentration was increased.

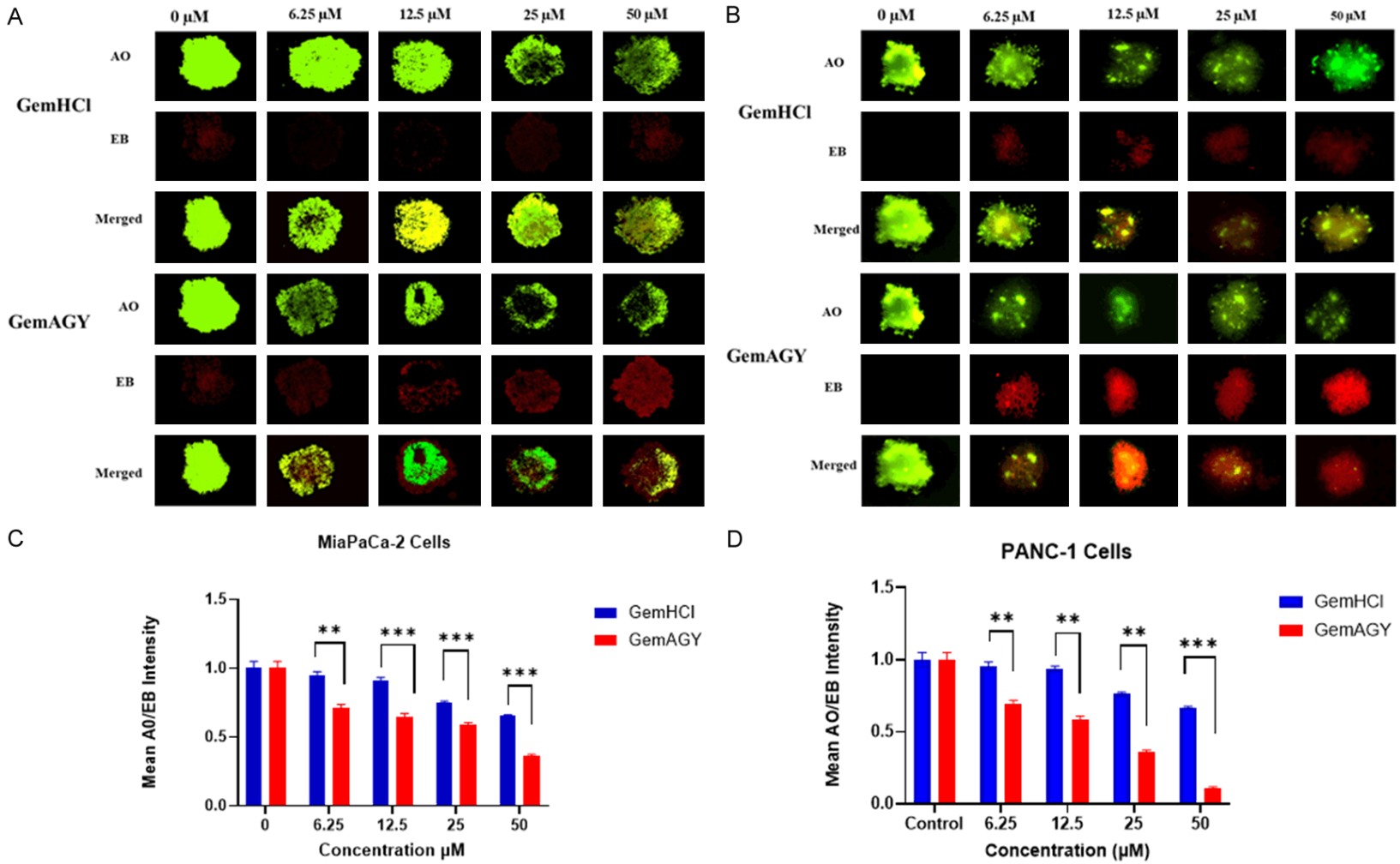


Figure 7. GemAGY treatment significantly induced higher cell death in MiaPaCa-2 and PANC-1 spheroids. A, B. Images were captured at 4× magnification using a Nikon Ti Eclipse microscope. The mean intensity ratios AO/EB equivalent to the viable-to-dead cells in the spheroids were determined. C, D. Images are obtained from three replicates. Statistical significance (*, $P < 0.05$; ***, $P < 0.001$) was determined by Two-way ANOVA with Tukey's Multiple Comparison Test.

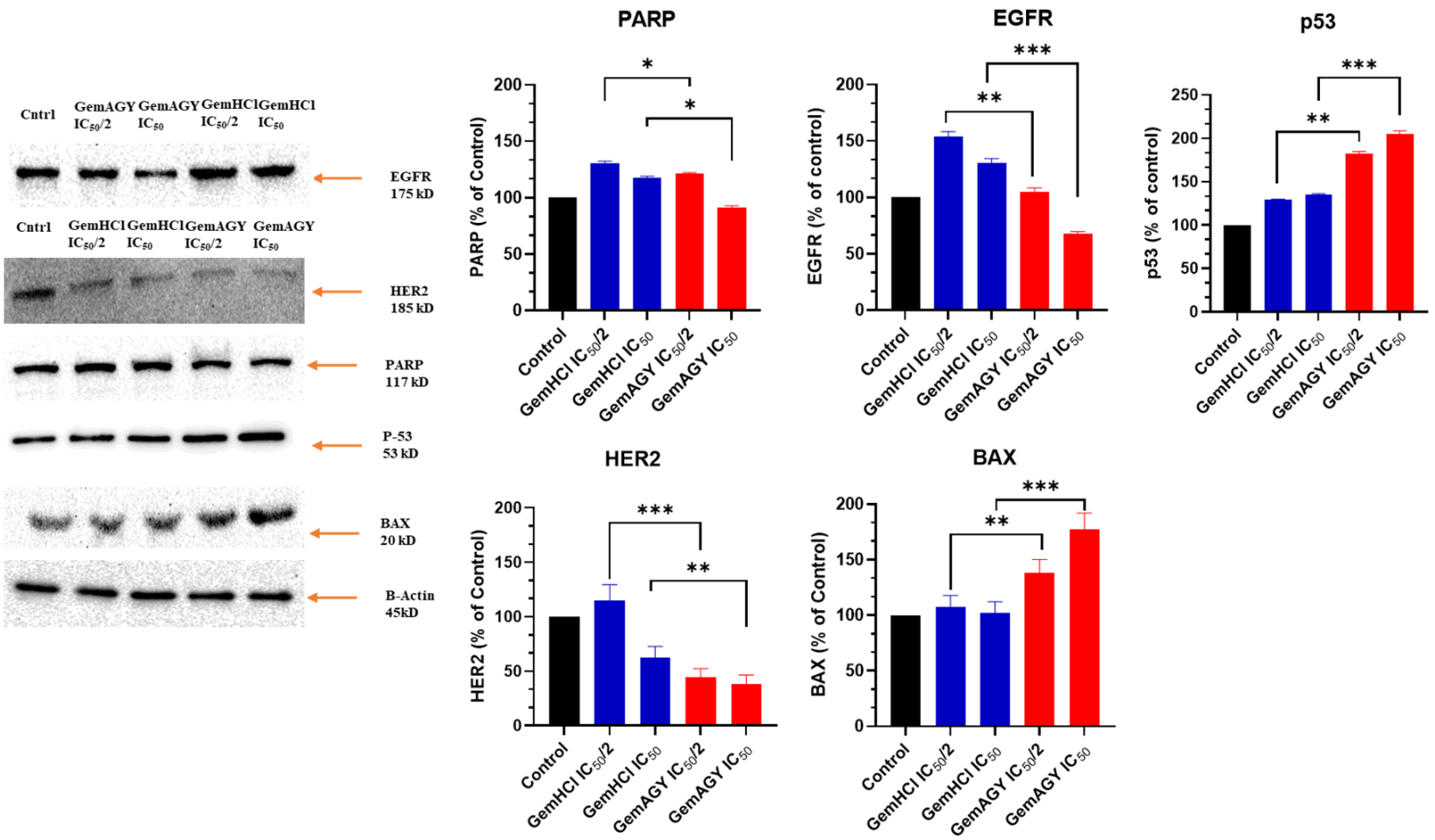


Figure 8. Protein bands after treatment of MiaPaCa-2 cells with GemHCl and GemAGY. GemAGY treatment significantly increased the expression of p53 and BAX and decreased/downregulated the expression of PARP, HER2, and EGFR.

assay provides evidence to support our claim that GemAGY exhibited a higher anticancer effect than GemHCl.

Cell migration is crucial in many biological functions [60] and is involved in pathophysiological processes such as cardiovascular diseases and cancer metastasis [60, 61]. The cell migration assay explored the ability of GemAGY to inhibit cell mobility and proliferation. Our research findings indicated that GemAGY significantly inhibited cell mobility towards the 'wound area' in MiaPaCa-2 and PANC-1 cells. GemAGY-treated MiaPaCa-2 and PANC-1 cells at 3.125 and 6.25 μM concentrations had a small number of cells present in the wound area, and this suggested that GemAGY's potential to inhibit motility of cells was higher in comparison to GemHCl at the same concentration.

The clonogenic assay is an in vitro cell survival assay based on the ability of a single cell to grow into a colony. The colony is defined as consisting of at least 50 cells [62]. This assay is useful for quantifying reproductive cell survival in vitro [63]. Our findings suggested that GemAGY was more effective in inhibiting the cells' ability to proliferate when compared to GemHCl (**Figure 5A** and **5B**).

Generally, cells are exposed to various cellular stresses, such as DNA damage. These cellular stresses finally introduce genomic aberrations, including mutation, deletion, and/or translocation into the cellular genome, thereby inducing genomic instability. Accumulation of genomic aberrations often results in the development of cancers [64]. p53 is a nuclear transcription factor with a pro-apoptotic function, i.e., it is an inducer of cell cycle arrest and programmed cell death. Since over 50% of human cancers carry loss of function mutations in the p53 gene, p53 has been considered a classical type of tumor suppressor [64, 65].

Similarly, BAX is a pro-apoptotic protein, and overexpression of the BAX gene has been found to induce apoptotic death in pancreatic cancer cells [66]. In our western blot studies, GemAGY exhibited a significantly higher expression of p53 and BAX proteins in MiaPaCa-2 cells than GemHCl. We postulate that the improved metabolic stability observed in GemAGY may account for the higher expression of p53 and BAX proteins compared to GemHCl in this assay. Moreover, PARP activity has been reported to be upregulated in certain types of cancer [67]. Studies have revealed that patients suffering from hepatocellular carcinoma tumors presented with significantly increased levels of ADP ribosylated PARP in their tumor tissues than their adjacent non-cancerous tissues [67]. The PARP pathway and its inhibition provide several avenues for therapeutic intervention in metastasis and other diseases [67, 68]. Our results indicated that in MiaPaCa-2 cells, GemAGY significantly inhibited PARP as evidenced by a lower expression of the PARP protein compared to GemHCl.

Also, in pancreatic cancer and various solid tumors, HER2 and EGFR, which is a subclass of tyrosine kinase receptors, are highly expressed [69]. Several studies have demonstrated that EGFR is over-expressed in pancreatic cancer [70], and over-expression correlates with more advanced disease [71] or poor clinical outcomes in the case of overexpression of HER2 protein [72]. In this study, EGFR and HER2 proteins had lower expressions in GemAGY-treated MiaPaCa-2 cells when compared to GemHCl-treated MiaPaCa-2 cells (Figure S4). In the literature, EGFR signaling and expression in pancreatic cancer is a promising field for developing new and improved treatment strategies [73], as inhibiting this protein may be beneficial. Based on our results from the western blot assay, we could suggest that treatment with GemAGY may have resulted in significant inhibition of EGFR and HER2 proteins.

Conclusion

In this study, we successfully designed and synthesized GemAGY, a novel analog of GemHCl. GemAGY demonstrated significantly higher anticancer activity against pancreatic cancer cells than GemHCl. GemAGY significantly reduced cell viability, cell migration, and the formation of colonies compared to GemHCl. Further studies will be focused on determining the pharmacokinetics and biodistribution profile as well as the in vivo antitumor activity of GemAGY using PDX (Patient Derived Xenograft) mouse models of pancreatic cancer.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Edward Agyare, Division of Basic Pharmaceutical Sciences, College of Pharmacy and Pharmaceutical Sciences, Florida A&M University, 1415 South Martin Luther King Blvd, Tallahassee, Florida 32307, USA. Tel: 850-599-3581; Fax: 850-599-3934; E-mail: edward.agyare@famu.edu

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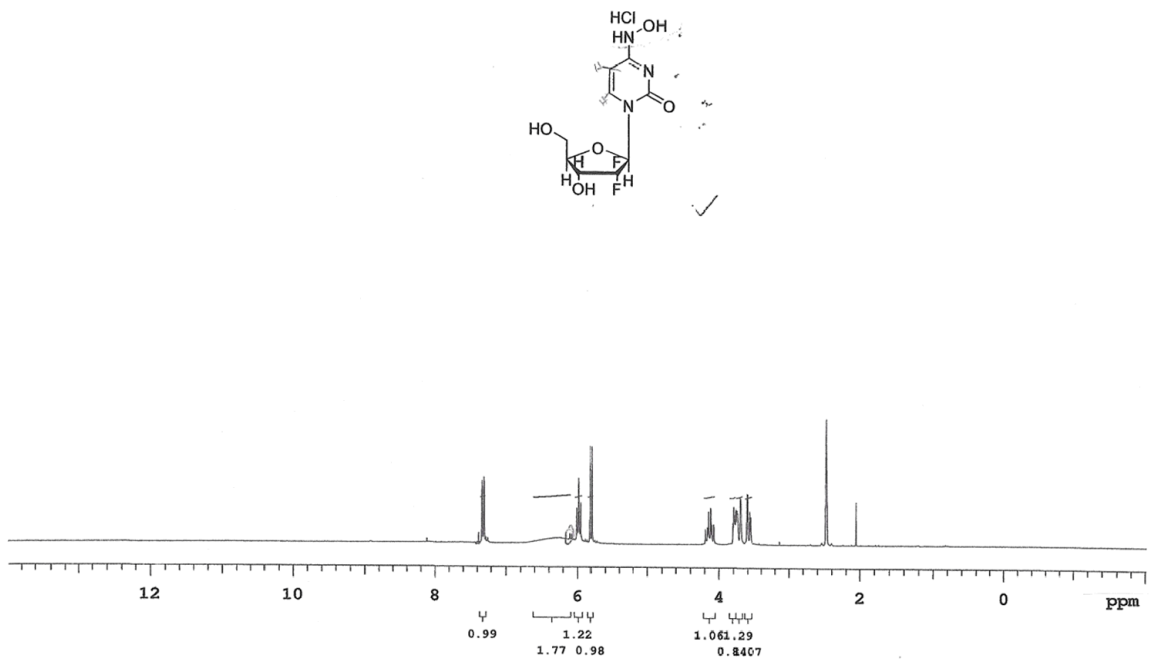

Figure S1. ^{1}H NMR spectra for GemAGY.

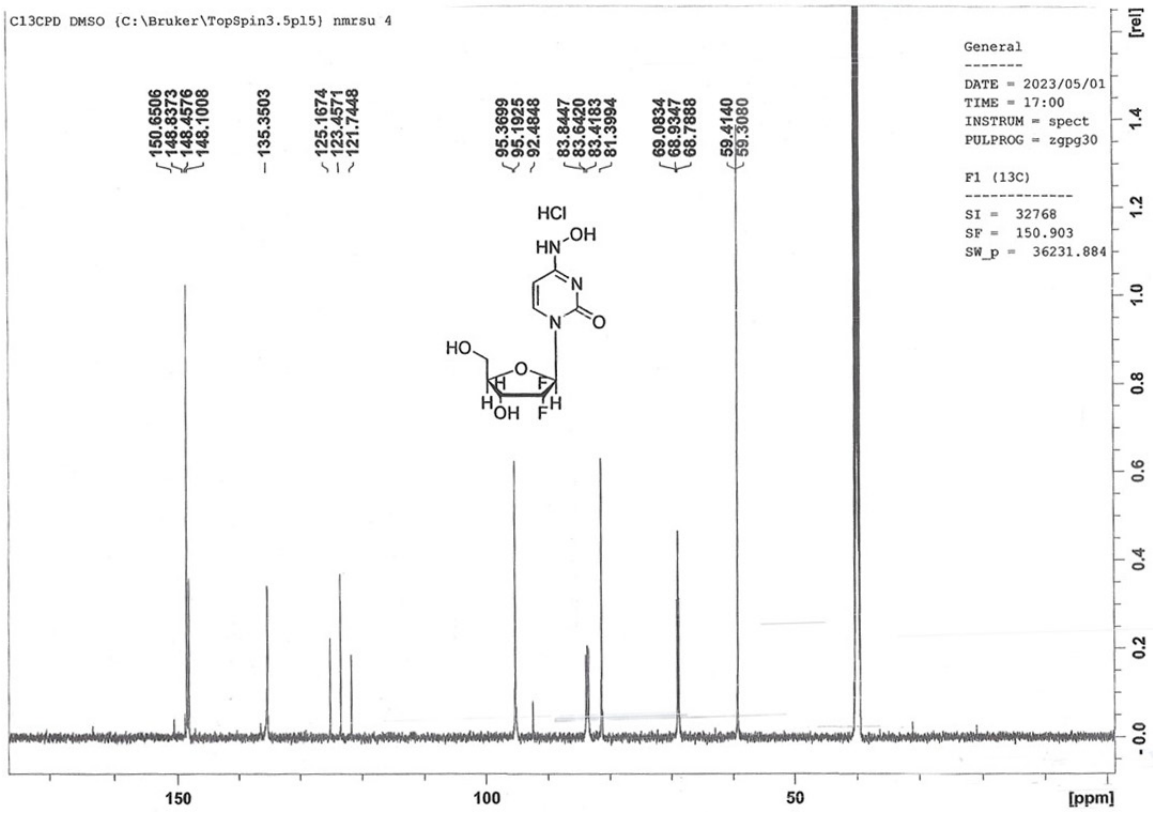

Figure S2. C-13 NMR spectra for GemAGY.

Atlantic Microlab, Inc.

Sample No. GemAGY

6180 Atlantic Blvd. Suite M
Norcross, GA 30071
www.atlanticmicrolab.com

Company/School Florida A&M Univ
Dept. College of Pharmacy
Address
City, State, Zip

Professor/Supervisor: Dr Agyare
PO# / CC# FAM01-0000222274

Name Xue You Zhu Date 04/06/2023
Phone

Element	Theory	Found	
C	34.25	34.06	
H	3.83	3.93	
N	13.31	13.09	

Single ☐ Duplicate ☐
Elements Present: C9H12ClF2N3O5
Analyze for: C H N
Hygroscopic ☐ Explosive ☐
M.P. B.P.
To be dried: Yes ☒ No ☐
Temp. rt Vac. yes Time 24 h
Rush Service ☐ Rush service guarantees analyses will be completed and results available by 5 PM EST on the day the sample is received by 11 AM.
Include Email Address or FAX # Below
xue.zhu@famu.edu

Date Received APR 10 2023 Date Completed APR 11 2023
Remarks:

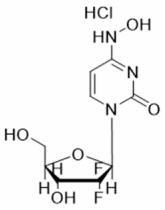

Figure S3. Micro-elemental analysis of GemAGY showing the percent by mass of the elements carbon, hydrogen, and nitrogen in the structure of GemAGY compared to theoretical estimates.

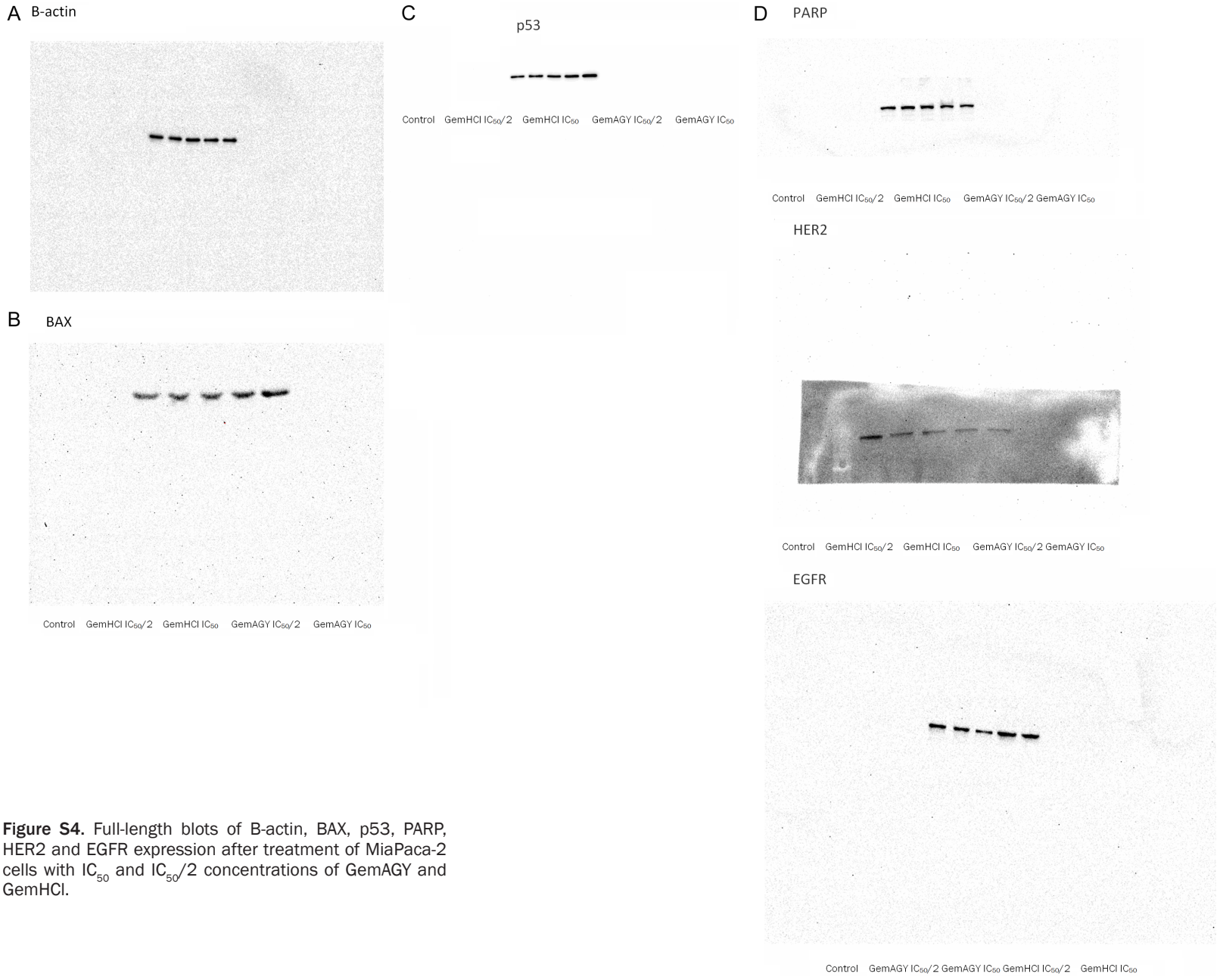

Figure S4. Full-length blots of B-actin, BAX, p53, PARP, HER2 and EGFR expression after treatment of MiaPaca-2 cells with IC_{50} and $IC_{50}/2$ concentrations of GemAGY and GemHCl.