A small molecule inhibits pancreatic cancer stem cells

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Abstract
Pancreatic cancer is the fourth highest cause of cancer-related deaths in the United States, with a projected 60,430 new cases diagnosed and 48,220 patients dying in 2021. We employed a small chemical, N-(6-Chloro-2-enzothiazolyl)-3, 4-dimethoxy-benzene propanamide (KY-02111), to target suppression of tumori-sphero-genesis of PANC1ORGRCD¹⁹+³¹+⁴⁵+¹³³+, to propose a novel therapeutic strategy against drug registrant pancreatic cancer stem cells (PANC1ORGRCD¹⁹+³¹+⁴⁵+¹³³+). According to our findings, the pancreatic stem cell indicators (CD¹⁹+³¹+⁴⁵+¹³³+) are found to be more strongly expressed in pancreatic cancer tissues than in normal pancreatic tissues. The flow cytometry, immunoblot and immunofluorescence analysis showed that the expression of these markers (CD¹⁹+³¹+⁴⁵+¹³³+) in PANC1ORGR spheroid cells was lowered by treatment of our new therapeutic approach. Therefore, this study identified the significant relationship of inhibition of tumori-sphero-genesis of PANC1ORGR with associated novel biomarkers (CD¹⁹+³¹+⁴⁵+¹³³+) which could be target candidates in designing drugs against pancreatic cancer. Further investigation and funding are needed to find the molecular mechanism of inhibition of tumori-sphero-genesis by this small molecule. This work was partly used the financial support from award money of 2017 Translational Research Award, Society of Toxicology and 2018 AACR Minority and Minority-Serving Institution Faculty Scholar award of Dr. Jayanta K. Das.

Keywords: Pancreatic cancer, drug registrant, cancer stem cells, tumori-sphero-genesis, new molecule.
Introduction
Pancreatic cancer is the fourth highest cause of cancer-related deaths in the United States, with a projected 60,430 new cases diagnosed and 48,220 patients dying in 2021 (American Cancer Society, 2021). Until 2040, around 355,317 additional cases are expected worldwide. On the other hand, PC patients have a poor prognosis, with a 5-year overall survival rate of just 9% (Rawla et al., 2019). Pancreatic cancer is classified histologically into two types: exocrine and endocrine. Exocrine cancers account for more than 95% of all pancreatic cancers, whereas endocrine tumours are hormone-producing endocrine cells. About 90% of all exocrine tumours were found to be pancreatic ductal adenocarcinoma (PDAC) (Sun et al., 2020). Surgical excision remains the only possibly curative therapy option for pancreatic patients. Unfortunately, due to late diagnosis, tumour resistance to conventional medicines, or a lack of effective early identification of pancreatic cancer biomarkers, fewer than 20% of patients are in a suitable disease state for surgery. For most pancreatic cancer patients, metastases and drug-resistant cancer stem cells are the leading causes of mortality. Within 12 months of surgical resection, around 30% of patients developed metastatic pancreatic cancer, and the accompanying chemo- and radiation had little effect on patient survival during the previous two decades. For the improvement of patient outcomes, this illness offers a significant challenge to extremely aggressive, metastatic, and diverse pancreatic stem cells (Farhana et al., 2013; Ortega et al., 2020; Das et al., 2020; Das et al., 2021).

Gemcitabine (2′-2′-difluoro-deoxycytidine; GEMZAR®) has been used to treat pancreatic cancer as first-line therapy. Unfortunately, disease recurrence occurs in the majority of instances as a result of gemcitabine resistance, and this is the primary reason for pancreatic cancer patients' poor survival rates. As a result, overcoming gemcitabine resistance remains a significant problem in properly treating this lethal cancer. When used in conjunction with other treatment drugs such as platinum analogues, anti-metabolites, or topoisomerase inhibitors, Gemcitabine has failed to show any increase in therapeutic result or survival rate. Because of the enhanced median overall survival of 11.1 months compared to 6.8 months with gemcitabine-based treatment, FLOFIRINOX has lately been advocated as an alternative to a gemcitabine-based therapy. However, there is currently no general agreement on the best therapy regimen for pancreatic cancer, with most chemotherapeutic clinical studies ending in phase II or III owing to negative or inconsequential results. According to a recent evaluation of clinical studies on second-line therapy in patients with locally advanced or metastatic pancreatic cancer, neither FLOFIRINOX nor a gemcitabine-based regimen has been able to establish a standard of care. The FDA has authorized the use of Abraxane, an albumin-bound paclitaxel nano complex in conjunction with gemcitabine, to prolong patient life for a few months (Rocha Lima et al., 2004; Frese et al., 2012; Sun et al., 2020). As a result, there is a pressing need to reassess the treatment strategy and develop new techniques to combat pancreatic cancer.
Our present research aims to develop a novel pancreatic cancer therapeutic approach using a small chemical (KY-02111) for the prevention of tumori-sphero-genesis in heterogenic pancreatic cancer stem cells.

Materials and Methods

Cell culture and treatment conditions

The PANC-1ORGR cell lines (established Gemcitabine resistant PANC-1 cells, a gift from Dr. Jonathan Celli of the University of Massachusetts Boston) were maintained in DMEM/F12 (1:1) with 5% FBS. Cells were grown at 37°C in a humidified environment containing 5% CO₂. KY-02111 was purchased from LKT Laboratories (St Paul, MN) and dissolved in dimethyl sulfoxide (DMSO). Equal volumes of KY-02111 as in KY-02111 treatment group were added to vehicle control with the final percentage of DMSO in each group to be less than 0.1%.

Tumori-sphero-genesis assay

Cells were suspended in serum-free DMEM/F12 (1:1) culture medium supplemented with B27. For tumori-sphero-genesis, approximately 100 cells per well were seeded in an ultra-low-attachment 96-well plate (Corning Inc, Lowell, MA). The LC₅₀ doses of KY-02111 was determined by treating the spheroid cells with 0µM, 5µM, 10µM, 20µM, 40µM, 80µM exposure with KY-02111 on the day of seeding cells and continued for 10 days. The tumori-sphero-genesis was studied, and LC₅₀ (lethal concentration for 50% survival) of KY-02111 were determined at 40µM for spheroid sizes, diameter and number of viable cells by Trypan blue exclusion method. This method is based on the principle that live (viable) cells do not take up trypan blue dyes, whereas dead (non-viable) cells do. Staining facilitates the visualization of spheroids' morphology. The effect of KY-02111 was studied by treating the spheroids for 10 days in liquid culture in the absence or presence of 20µM KY-02111 below LC₅₀ dose for the same time periods. Each experimental group had a total of 15 spheroids with a minimum diameter of 50 µm. The ANOVA and Tukey HSD tests were used to evaluate the data for multiple comparisons.

3-D PANC-1ORGR CD31+tumori-sphero-genesis cell culture

Magnetic-activated cell sorting (MACS) CD31+ cells were initially isolated from 5-day old spheroids. According to the manufacturer's instructions, these cells were extracted using the Dynal MPC®-L Magnetic Particle Concentrator (Invitrogen Dynal AS, Oslo, Norway) and anti-CD31 conjugated magnetic Dynabeads® (Invitrogen Dynal AS). DMEM/F12 (1:1) supplemented with B27* was used to suspend CD31-positive PANC-1ORGR cells in serum-free stem cell media. For 3-D CD31+tumori-spherogenesis, 100–150 cells per well were plated in DMEM/F12 (1:1), including B27* serum-free supplement with or without 20µM KY-02111 treatment in an ultra-low-attachment 96-well plate (Corning Inc, Lowell, MA). On the day of cell seeding, 20µM KY-02111 was introduced. Spheroids were transplanted onto a 24-well plate after 5 days in a cell culture insert constructed with collagen matrix and decreased growth factor Matrigel (Corning Inc, Corning, NY). The cell culture insert was carefully filled with DMEM/F12 serum-free B27* media, inserted in the 24 well plates, and cultured.
at 37°C for 30 days with or without treatment with 20µM KY-02111. Every 15 days, the culture medium was replaced.

**MTT, SRB, and BrdU Assays**

After 5 days of spheroid development, PANC-1ORGR spheroid cells were collected, trypsinized, and seeded in 96-well plates at a density of 1104 cells/mL, then treated with or without KY-02111(20µM) for 24 hours. Each well was filled with a methyl thiazole tetrazolium (MTT) solution and incubated at 37°C for 30 minutes. After that, the MTT solution was withdrawn, and the formazan crystals were dissolved using DMSO. Absorbance was measured at 560 nm (reference at 700 nm) in a Tecan Genios plate reader. For the SRB experiment, spheroid-derived cells were fixed for 1 hour at 4°C (50 L/well) with 50% trichloroacetic acid. The plate was rinsed five times with tap water, dried, then stained with SRB dye (0.057% in 1% acetic acid) for 30 minutes before being washed with 1% acetic acid to remove unbound dye. After the plate was air-dried, the bound protein dye was solubilized using 100 L of a 10 mM Tris base. A Tecan Genios microplate reader was used to measure the absorbance at 540 nm. In cells treated with KY-02111 (20µM), bromodeoxyuridine (BrdU) incorporation was measured as a biological indication of DNA synthesis.

**Cell Invasion Assay**

In the top chamber of a transwell insert, cells from 5 days old spheroids (5 × 10³ cells/mL) were sown in serum-free media, 10% FBS medium was introduced to the bottom chamber as a chemoattractant. Invaded cells were stained with 0.2% crystal violet after 24 hours of incubation, and photos were taken using a microscope. Invasive cells were counted and graded in triplicate from the bottom chamber.

**Colony Formation Assay**

In six-well plates, cells from five-day-old spheroids were sown on top of soft agar (0.3%) with a bottom layer of 0.7% agar in DMEM/F12 and kept in full media for five days. KY-02111(20µM) or DMSO as a vehicle was used to treat the cells. The cells were rinsed in PBS after 5 days, fixed in methanol for 15 minutes, then stained with 0.005% crystal violet for 15 minutes. The colonies were counted after the plates were photographed. For each test, at least three separate experiments were conducted.

**Immunofluorescence Detection**

Methanol was used to fix cells from 5-day-old spheroids, and immunofluorescence staining was done according to conventional techniques. In a nutshell, cells were blocked with 3 percent normal goat serum before being treated overnight at 4°C with antibodies such as anti-CD19-FITC, anti-CD31-FITC, anti-CD45, or anti-CD133. After washing, cells were treated with anti-mouse-IgG Alexa Fluor® 633 for anti-CD133 and anti-rabbit-IgG Alexa Fluor® 546 for CD45 (Life Technologies Corporation, Carlsbad, CA, USA).
Then, using a Nikon C1 laser scanning confocal microscope, cells were mounted using Fluoromount-GTM reagent and examined. PANC-1ORGR 5 days old spheroids cells were sown in the eight-chambered slides wells for 24 hours with or without KY-02111 (20µM) treatment.

**FITC Annexin V Apoptosis detection**

FITC Annexin V Apoptosis Detection Kit I (BD Pharmingen) was used to analyze the apoptosis of PANC-1ORGR cells from 5-day old spheroids, according to the manufacturer’s procedure. Cells were rinsed in cold PBS, resuspended in 1X binding buffer, then stained with 5µlAnnexin V-FITC and 5µlpropidium iodide in 100µl of the solution containing cells (PI). Following a 15-minute incubation period in the dark, 100 µl of 1X binding buffer was added, and cells were examined using Guava easy Cyte’s CytoSoft software application, as directed by the manufacturer.

**CD31, CD45, and CD133 expression in tissue microarrays**

In order to uncover new biomarker levels linked with pancreatic cancer, CD31, CD45, and CD133 expressions were measured in a pancreatic cancer tissue microarray (Cat. #PA242c, US Biomax, Inc). In order to prepare paraffin-embedded tissue slices for immunohistochemistry, they were put in xylene and progressively re-hydrated with ethanol (100%, 95%, 70%) and PBS, pH 7.4. The tissues were then blocked with 3% normal goat serum and treated overnight at 4°C with anti-CD31, CD45, and CD133 antibodies. Next, tissues were treated with anti-rabbit-IgG. The tissues were then soaked in xylene and mounted with histology mounting media on a coverslip.

**Immunoblotting**

Whole-cell lysates from 5-day spheroids were produced in lysis buffer comprising [25 mM Tris-Cl buffer (pH 8.0), 150 mM NaCl, 0.2 percent NP-40, 10% glycerol, 8 mM-glycerophosphate, 2.5 mM sodium pyrophosphate, 10 mM NaF, 0.2 mM Na$_3$VO$_4$, 1 mM DTT, and 10 µl/ml (Sigma Aldrich). According to the manufacturer's instructions, the Bradford Assay Reagent (Bio-Rad) was used to quantify proteins. Proteins (35–75 µg) were separated on a 10% SDS-PAGE gel and transferred to PVDF membranes (Millipore). Membranes were blocked with 5% nonfat milk and incubated with the antibodies CD31, CD133, CD45, and CD19 (Santa Cruz BioTechnology), and actin (Santa Cruz BioTechnology). Antibody dilutions were employed according to the manufacturer's instructions for immunoblot detection. Membranes were then treated with secondary IgG antibodies coupled to horseradish peroxidase and imaged using ECL Plus Western blot reagents (GE Healthcare, Amersham). As a loading control, the membranes were re-probed for -actin. A Bio-Rad Versa Doc equipment was used to visualize and quantify the electrochemiluminescence (ECL) intensity of discovered target proteins. For each experiment, all immunoblots were done at least three times.

**Results**

**Inhibition of PANC-1ORGR tumori-sphero-genesis**

The tumori-sphero-genesis or sphere-forming assays are widely reported in stem...
cell biology to culture cells under non-adherent conditions in order to characterize stemness. The PANC-1ORGR cells enriched with biomarkers (CD19+31+45+133+) cells were inhibited by treating 20 µM KY-02111 in 10 days (Fig. 1).

The 20 µM KY-02111 dose was used for these tumori-sphero-genesis assay after observing the effect of different concentrations of KY-02111 for the LC50 value (found 40 µM, Fig. 2) of KY-02111.

Fig. 1. Treatment with 20 µM KY-02111 of PANC-1ORGR spheroids at 5 days (D), 10 days, showed inhibition of tumori-sphero-genesis.

Fig. 2. Dose response curve of KY-02111 showed LC50 dose as 40 µM studied by trypan blue dye exclusion assay of spheroid cells of PANC-1ORGR. We used 20 µM KY-02111 for our experimental studies which is below LC50.
Inhibition of tube formation or epithelial mesenchymal morphological transition of 3D-PANC-1ORGR CD31+ enriched tumori-spheroids was compared up to 30 days with or without treatment of 20µM KY-02111 (Fig. 3).

Fig. 3. Inhibition of tube formation or epithelial mesenchymal morphological transition as cancer cell migration of 3D-PANC-1ORGR CD31+ enriched tumori-spheroids with or without treatment of 20 µM KY-02111 in different days interval.

**Observed characteristics of spheroid cells of PANC-1ORGR by MTT, SRB, and BrdU Assays**

Inhibition of growth characteristic due to metabolic capacity of spheroid cells (5 days old) were observed by MTT assay, cellular protein content by sulforhodamine B (SRB) and DNA synthesis by BrdU incorporation assay at 24hrs with or without 20µM KY-02111 (Fig. 4, 5 & 6).
Fig. 4. MTT assay from 5 days old spheroid cells with or without treatment of 20 µM KY-02111 at 24 hours.

Fig. 5. SRB assay from 5 days old spheroid cells with or without treatment of 20 µM KY-02111 at 24 hours.

Fig. 6. BrdU assay from 5 days old spheroid cells with or without treatment of 20 µM KY-02111 at 24 hours.
Cell Invasion assay, colony formation assay and immunofluorescence detection of novel biomarkers (CD$^{19+31+45+133+}$) of spheroids cells of PANC-1ORGR

Treatment with DMSO and 20µM KY-02111 were assessed for anchorage-independent growth, migration and invasion. The quantitative changes in colonies, invaded cells and immunofluorescence detection of novel biomarkers (CD$^{19+31+45+133+}$) of PANC-1ORGR spheroids were observed when were treated with DMSO (control) and 20µM KY-02111 for 5 days, respectively (Fig. 7, 8 & 9).

Fig. 7. The quantitative changes of colonies of PANC-1ORGR spheroid cells with or without treatment of 20 µM KY-02111 for 5 days.

Fig. 8. The number of invaded cells associated with cell migration from PANC-1ORGR spheroids with or without treatment of 20 µM KY-02111 for 5 days.
Fig. 9. The immunofluorescence detection of novel biomarkers (CD$^{19+31+45+133+}$) from PANC-1ORGR spheroids with or without treatment of 20 µM KY-02111 for 5 days. The expression of these novel biomarkers were reduced with sizes of spheroids by 20 µM KY-02111 at 5th day.

Apoptosis of spheroids cells treated with 20 µM KY-02111 found by FITC Annexin V Apoptosis detection
The increased number of apoptotic cells were observed by FITC Annexin V Apoptosis detection among PANC-1ORGR cells from 5 days old spheroids (Fig. 10).

Identifying novel bio marker levels associated with pancreatic cancer
The highest expression of novel bio markers (CD$^{31+45+133+}$) levels were observed in pancreatic cancer tissues compared to normal pancreatic tissues (Fig. 11). These novel biomarkers were also observed highly expressed in PANC-1ORGR spheroids.

Immunoblotting analysis revealed the reduced pancreatic cancer novel bio markers (CD$^{19+31+45+133+}$) by 20 µM KY-02111
We observed the decreased level of pancreatic cancer novel bio markers (CD$^{19+31+45+133+}$) expression in the PANC-1ORGR cells from 5 days old spheroids by 20 µM KY-02111 as observed with immunoblotting analysis (Fig. 12).
Fig. 10. The FITC Annexin V Apoptosis detection showed the increased number of apoptotic cells by 20 µM KY-02111 treatment in PANC-1ORGR cells from 5 days old spheroids. The upper panel showed the representative pictures of the assay. The lower panel showed the histogram of apoptosis analysis.
Fig. 11. The immunofluorescence detection of novel biomarkers (CD$^{31+45+133^+}$) from adjacent normal pancreas tissue compared to adenocarcinoma pancreas tissue.

Fig. 12. The immunoblotting analysis of novel biomarkers (CD$^{19+31+45+133^+}$) from PANC-1ORGR cells from 5 days old spheroids with or without treatment of 20 µM KY-02111.
Discussion

Our findings support the stochastic acquisition of many heterogenic cancer stem cell new biomarkers (CD$^{19+31+45+133+}$) linked with PANC-1ORGR spheroids, which were decreased by KY-02111(20µM). According to the Cancer Stem Cells Therapeutic Target Database Version 2.0 (CSCTTv2.0), high CD133 CSCs are PAN CSCs that are resistant to anticancer treatments such docetaxel, 5-FU, irinotecan, and oxaliplatin (Das et al., 2020). As a result of our current studies, KY-02111 seems to be a promising small chemical for inhibiting the proliferation and tumorigenic progression of pancreatic cancer stem cells, with the potential for future therapeutic development. Although the MTT test is only a modestly reliable measure of viability, its convenience of use and potential for high throughput analysis in multiwell plates has made it quite popular. SRB (sulforhodamine B), a red fluorescent amino-xanthene dye, detects the presence of live cells by producing a colourimetric shift when its sulfonic group attaches to basic amino acid residues in proteins, yielding an estimate of total protein mass, which is directly proportional to cell number. The trypan blue exclusion experiment is a simple and commonly used method to count the proportion of live cells. Menyhárt et al., (2016) employed the BrdU (bromoeoxyuridin) test to examine de novo DNA synthesis as a direct indicator of cell proliferation. KY-02111(20µM) treatment decreased the metabolic capacity, live cell counts, and proliferation of PANC-1ORGR spheroids, according to our investigations using MTT, SRB, and BrdU. Furthermore, multiple scientific research has shown that heterogenic stem cell populations are responsible for cancer development, metastatic transformation, epithelial mesenchymal morphological transition as cancer cell migration and reprogramming in a variety of drug registrant malignancies (Das and Roy, 2015; Das and Roy, 2017; Das et al., 2018; Ortega et al., 2020; Das et al., 2021). Our recent study saw the inhibition of progression, invasion, metastatic transformation, epithelial mesenchymal morphological transitionas cancer cell migration and reprogramming of heterogenic drug registrant pancreatic cancer stem cells (PANC1ORGRCD$^{19+31+45+133+}$) investigations. Apoptosis is a kind of programmed cell death that includes nuclear fragmentation, chromatin condensation, chromosomal DNA fragmentation, and cell shrinkage in response to medication effectiveness against cancer stem cells (Das and Roy, 2015; Das and Roy, 2017). FITC Annexin V Apoptosis detection among PANC-1ORGR cells from 5 days old spheroids revealed an increased number of apoptotic cells in our investigations. When comparing pancreatic cancer tissues to normal pancreatic tissues, the pancreatic tissue array revealed significantly expressed new biomarkers (CD$^{19+31+45+133+}$) (12 in 24 tissues). According to Western blot analysis, the KY-02111 reduced the expression of pancreatic cancer new bio markers (CD$^{19+31+45+133+}$) in PANC-1ORGR cells from 5 days old spheroids.

In conclusion, the major new findings of our studies show that a new small molecule (KY-02111) has a promising effect in inhibiting drug-registrant pancreatic cancer stem cells (PANC1ORGRCD$^{19+31+45+133+}$), thus pointing
to a new therapeutic approach in pancreatic cancer research. Inhibition of heterogeneous PANC1ORGRCD\textsuperscript{19+31+45+133+} by KY-02111 should better understand the molecular process, opening up new paths for pancreatic cancer therapy.

Acknowledgements

This work was partly used the financial support from award money of 2017 Translational Research Award, Society of Toxicology and 2018 AACR Minority and Minority-Serving Institution Faculty Scholar award of Dr. Jayanta K. Das.

References


