CEP1430 and CEP1507 Novel Drug Candidates for Targeting Pancreatic Cancer Stem Cells and for Circulating Tumor Cells as Potential new Combinational therapy for Advanced Pancreatic Cancer Patients

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Introduction

Pancreatic cancer is the fourth leading cause of cancer mortality in the US, despite significant improvements in diagnostic imaging and surgical resections. The 5-year survival rate remains less than 6%. The low survival can be attributed to positive resection margins, poor tumor differentiation, a large tumor size, lymph node involvement, high levels of preoperative carbohydrate antigen 19-9 (CA19-9), and persistently elevated levels of postoperative CA 19-9. Other contributing factors to low survival include microscopic or gross metastatic disease at time of diagnosis. The treatment of non-surgical candidate pancreatic cancer patients remains a huge challenge. However with the development of new strategies and trial designs, the treatment of non surgical pancreatic cancer patients is entering a new era. Novel therapeutic agents, combinations and hypothesis driven trial designs with the objective to identify robust prognostic and predictive markers will unravel new targets and relevant pathways for anti-tumor benefit. Over the past decade, increasing evidence suggests that stem cells play a crucial role in the development and progression of malignant diseases. Most cancers from a variety of origins contain a subset of distinct cancer phenotype responsible for tumor initiation and propagation. These cells have been termed cancer stem cells or tumor-initiating cells and are highly resistant to chemotherapeutic agents. Three-dimensional (3D) cancer stem cell culture mimics several important growth characteristics of tumorigenicity *in-vivo*. These *in vitro 3*D models serve as simple high throughput systems to rapidly screen/select active drugs against solid tumors and minimize the use of experimental animals. Chemotherapy or chemo-radiotherapy have been used increasingly to improve survival but the approach leads to significant morbidity and mortality. The availability of reliable chemosensitivity assays will be crucial to predict response to chemotherapy and guide the selection and treatment of cancer patients. The purpose of this study was to determine optimum drug candidates *on the* chemosensitivity of patient derived tumor tissues or the corresponding cancer stem cell cultures. CEP1430 shows promise as an effective therapeutic agent against Pancreatic CSC without signs of toxicity when tested in SCID mice PDX model administered twice daily via IP injections for 30 days. CEP1430 reduced the tumor volume by 80-90 % in the treated group when compared with the control group. CEP1507 inhibited circulating tumor cells from proliferating at the metastatic site in comparison to control groups. The combination treatment group had localized tumor reduction of 85%.

Methods:

In-vitro study: Pancreatic tumor cells (parental) and CSCs were isolated from 10 terminal donor patients that had under gone chemotherapy and radiation treatments. The ages ranged from 35-65 years old, and included both genders. The tissues were consented and obtained under IRB and HIPPA guidelines. The tissues were transported from the surgical suites to Celprogen in Human Pancreatic CSC complete growth media [M36115-42S] within 24 hours following surgical resection. Upon receipt, the tissue was sectioned into three equal pieces and processed. One section was maintained as the heterogeneous tumor population and cultured as parental cell culture. The other section was processed further and isolated with CSC biomarkers, in Celprogen Media [M36115-42S] and ECM [E36115-42-T25] combination. The third section was utilized for generating PDX model in SCID mice. For the CSC generation, once the cell cultures were established within 7-14 days the cells were characterized by Flow, IHC, Western Blot and Real Time PCR for Pancreatic Cancer stem cell markers. Both the parental and the Human Pancreatic CSCs were evaluated for tumorgenicity by injecting 1000 cells subcutaneously in SCID mice. For in vitro studies, following characterization the cells were seeded at 10,000 cells per well in a 96 well format, pre-coated with Celprogen ECM [E36115-42-96Well] and cultured in complete growth media [M36115-42S]. The drugs were tested on the cultures at various concentrations for 72 hours at 5% carbon dioxide, 5% oxygen, humidified 37 ^{0}C incubator. Real time cell proliferation and viability were determined with Incucyte Zoom (Essen Bioscience). IC_{50} curves were generated for the test compounds CEP1430 and CEP1507. In addition to the two test compounds we also tested Gemcitabine, Taxol, Fluorouracil, Leucovorin, Irinotecan, and Oxaliptin and found that they were not effective against Pancreatic Cancer Stem cell (CSC) but were effective on tumor cells (differentiated CSCs). We found that CEP1430,was effective against Pancreatic CSC targeting selected pathways whereas, CEP1507 was very effective against Pancreatic CTCs.

In-vivo study: One thousand viable human pancreatic CSCs and parental cells were subcutaneously injected on the hind limb of SCID mice. After 10 days post injection when visible tumors were observed the mice were separated into control or experimental group of 10 mice/group. Mice received IP injections three times per week for a period of two weeks of the test drugs or diluent control. Each week the tumor growth measurements were performed with calipers and tabulated. At the end of the two weeks the mice were sacrificed and the tumor tissues were fixed, H&E and IHC stained, cultured, Real-time PCR performed for specific genes from total RNA, and cells evaluated by staining and flow cytometry with various Stem cell. Table 2 indicates the study design.

Group	Treatments	Cell Types			PDX
		CSC / #* animals	CTC/#* animals	Parental/# *animals	# * animals
1	Control	10	10	10	10
2	CEP1430	10	10	10	10
3	CEP1507	10	10	10	10
4	CEP1430+ 1507	10	10	10	10
5	Vehicle	10	10	10	10

Table 2. In-vivo study design in SCID mice. * 5 females and 5 Males.

Results:

Figure 2. Generation of donor specific cells from solid tumor. Utilizing Celprogen Pancreatic cancer complete growth media and ECM. Also used in generation of PDX models in SCID mice.

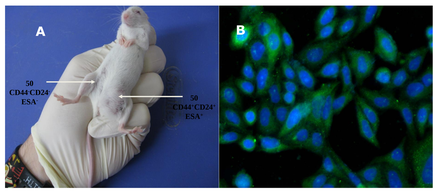

Figure 3. A. SCID mice injected with 50 $CD44^+CD24^+ESA^+$ cells and 50 $CD44^-CD24^-ESA^-$ cells. Tumor formation within 20 days after subcutaneous injections. **B.** Human Pancreatic CSC stained positive for Ephrin type-B receptor 4 (Eph B4) marker.

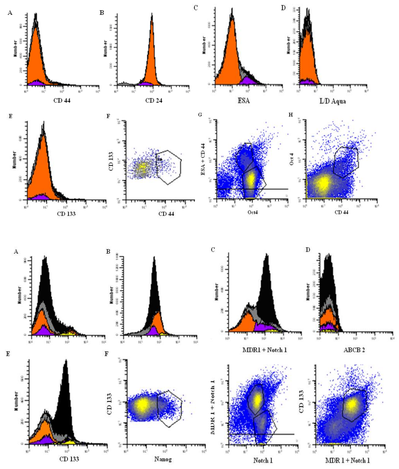

Figure 4. Flow Cytometry characterization of Human Pancreatic Cancer Stem Cells. The dissociated cells were counted and transferred to 5ml tube, washed with IXPBS and resuspended in million cells per 100 µM. Antibodies were added incubated for 20 minutes washed and secondary antibodies added when required. The antibodies utilized were anti-CD44, CD24, ESA, Nanog, Notch 1 and MDR1 (Shankar S *et al*. 2011 6(1):e16530. doi:10.1371/journal.pone.0016530)

1.

Cancer Stem Cell Markers	Commonly Expressed Markers in PDX
CD133	☑
Ability to for tumors <1000 cells in SCID mice	☑
Telomerase	☑
SSEA ¾	☑
OCT-4	☑

2.

Parental Cancer	Cancer Stem Cells	CTC
CA19-9	CD133, CD44,	CD133,CD44
CEA	SSEA3/4, OCT4	CEA
GAD	Tumorigenicity (<1000 cells)	Tumor growth potential
alpha-1-antitrypsin	Alkaline Phosphatase	Telomerase
Mucin	Aldehyde dehydrogenase	Nestin
Keratin	Telomerase	GAD
CK7	Nestin	Mucin

Table 1. Cancer Stem Cell general characterization Markers , and PDX model. **2.** Positive Cells Markers for Human Pancreatic Parental Cancer cells , CTC and Cancer Stem Cells.

Figure 1 Model for Human Pancreatic CSC screening. Human Pancreatic Cancer stem cells were inoculated subcutaneously (1000 cells/mouse). 6-10 days post injection blood samples were obtained from animals 200-300 mm^3 sized tumors for PK/PD and ex-vivo Biochemical/IHC analysis. PDX model was generated from patient's tumor.

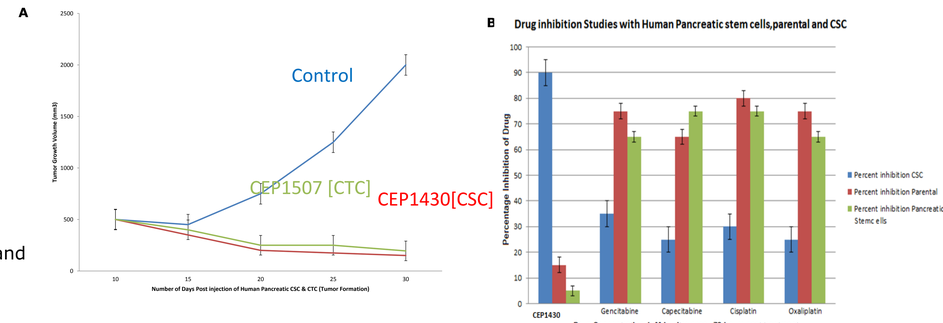

Figure 5. A. Tumor growth Inhibition curve for mice injected with 1000 cells of Human Pancreatic CSC and CTC. **B.** Comparison of % drug inhibition between Human Pancreatic CSC, Human Pancreatic Parental and Human Pancreatic Stem Cell. **C.** IC50 curve for Human Pancreatic CSC treated with drug CEP1430. D. IC50 curve for Human Pancreatic CTC. E. Pancreatic Parental, F. Pancreatic CSC, G. Pancreatic CTC cultured in 1% XFS2 media M36115-42S

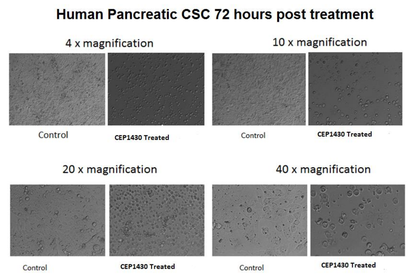

Figure 6. Human Pancreatic CSC treated with CEP 1430 1 µM concentration for 72 hours in the 96 well format and an initial seeding of 10000 cells/well.

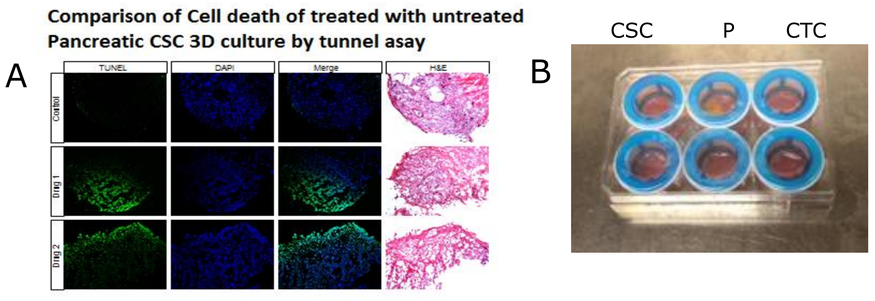

Figure 7. A. Human Pancreatic CSC treated with CEP 1430 [Drug1] 0.9nM & CEP1430 [Drug 2] 1 uM concentration for 72 hours in 3D histo-culture system of patients Tumor approximately 10000 CSCs /well seeding density. **B.** Human Pancreatic 3D Cell Culture System E36115-42-3D, with CSC, P (parental) and CTC culture.

Conclusions:

The efficacy of various therapeutic agents targeting major pathways (wnt,Notch,PI3K,MAPK,STAT) and chemotherapy agents were tested using DNA uptake and TUNNEL assay anti-cancer agents was calculated according to the inhibition index. The same compounds were tested utilizing the patient's Pancreatic Cancer Stem Cell Cultures established with Celprogen's Media and ECM. Expression of PDX-1, SHH, CD24, CD44, CD133, EpCAM, CBX7, OCT4, SNAIL, SLUG, TWIST, Ki-67, E-cadherin, β-catenin and vimentin were quantified by qPCR or immunocytochemistry. We cultured the cells in low oxygen since Tumor hypoxia induces epithelial-mesenchymal transition (EMT), which induces invasion and metastasis, and is linked to cancer stem cells (CSCs). Among the compounds screen tested Gemcitabine, Taxol, Fluorouracil, Leucovorin, Irinotecan, and Oxaliptin were not effective against Pancreatic Cancer Stem cell (CSC) but were effective on tumor cells (differentiated CSCs). We were able to show CEP1430 was effective against Pancreatic CSC targeting selected pathways. CEP1430 shows promise as a better therapeutic agent against Pancreatic CSC and when tested in SCID mice model with once daily IP injections for 30 days showed no apparent adverse effects. CEP1430 reduced the tumor volume in the treated group by 80-90 %, when compared with the control group. CEP1507 was effective in stopping the pancreatic CTC for metastasizing and the combination therapy of CEP1430 and CEP1507 reduced the tumor to 85% and stopped the circulating tumor Cells form metastasizing at the primary sites when compared to the control group.

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