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Co-treatment with a C1B5 peptide of protein kinase C γ and a low dose of gemcitabine strongly attenuated pancreatic cancer growth in mice through T cell activation

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Abstract

Although gemcitabine is an effective chemotherapeutic for pancreatic cancer, severe side effects often accompany its use. Since we have discovered that locally administered C1B domain peptides effectively control tumor growth without any side effects, the efficacy of co-treatment with this peptide and a low dose of gemcitabine on the growth of pancreatic cancer was examined. Two- and three-dimensional cell culture studies clarified that a co-treatment with C1B5 peptide and gemcitabine significantly attenuated growth of PAN02 mouse and PANC-1 human pancreatic cancer cells in 2D and 3D cultures. Although treatment with the low dose of gemcitabine alone (76%) or the C1B5 peptide alone (39%) inhibited tumor growth moderately, a co-treatment with C1B5 peptide and a low dose of gemcitabine markedly inhibited the growth of PAN02 autografts in the mouse peritoneal cavity (94% inhibition) without any noticeable adverse effect. The number of peritoneal cavity-infiltrating neutrophils and granzyme B⁺ lymphocytes was significantly higher in the co-treatment group than in the control group. A significant increase of granzyme B mRNA expression was also detected in human T cells by the co-treatment. Taken together, the current study suggests that C1B5 peptide offers a remarkably effective combination treatment strategy to reduce side effects associated with gemcitabine, without losing its tumoricidal effect.

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Keywords

C1B domain peptides; C1B5 peptide; Protein kinase C γ (PKC γ); pancreatic cancer; T cell activation; apoptosis

Introduction

Pancreatic cancer is the fourth leading cause of cancer related deaths in the US with 53,670 estimated new cases and 43,090 estimated deaths in 2017 [1]. This cancer is difficult to diagnose early, to remove surgically, and is poorly responsive to conventional chemotherapies [2, 3]. Since Gemzar® (Eli Lilly and Company, Indianapolis, IN) was approved by the FDA (FDA Application No. NDA 020509) in 1996, gemcitabine has been used as a first-line treatment for patients with locally advanced (non-resectable Stage II/III) or metastatic (Stage IV) pancreatic adenocarcinoma [4, 5]. However, the efficacy of gemcitabine as a single agent remains modest with a median survival of approximately 6 months in randomized trials [6, 7]. In addition, unacceptable side effects often associate with gemcitabine treatment, which include dose-limiting myelosuppression, flu-like symptoms, *etc.* [8]. For these reasons, there has been an interest in developing strategies that reduce systemic toxicity while improving the therapeutic potential of gemcitabine, which includes the development of novel combination chemotherapies.

Recently, synthetic C1B subdomain peptides of protein kinase C (PKC) γ isozymes, C1B1 and C1B5, have been demonstrated to be effective in the attenuation of the growth of human colon carcinoma cells in both cell culture and mouse study without any recognizable side effects [9]. The primary mechanism by which these peptides attenuate cancer growth is associated with an induction of apoptosis by stimulation of p53 phosphorylation and an attenuation of cell proliferation [9]. These peptides were also found to interact with PKC β I/ β II isozymes and stimulate apoptosis in carcinoma cells [9]. These results suggest that the combination treatment (co-treatment) with C1B peptide, which does not possess any side effects and reduced doses of typical chemotherapeutics, such as gemcitabine, may theoretically be a good combination to treat pancreatic cancer since both agents induce cell death through completely different mechanisms, and since reduced doses of chemotherapeutics should show much less off-target side effects.

In the present study, we sought to determine the effects of C1B5 peptide and reduced doses of gemcitabine, either alone or in combination, on murine and human pancreatic carcinoma cell growth in two-dimensional (2D) and three-dimensional (3D) spheroid culture, and *in vivo* tumor growth in a peritoneal dissemination model using the PAN02 murine pancreatic tumor cell line.

Materials and Methods

Materials

The C1B domain peptide, C1B5 peptide (PKC residues 141–151, RCVRSVPSLCG) was synthesized by the University of Ioannina Chemistry department (University of Ioannina, Greece) using standard Fmoc-/tBu solid phase peptide synthesis (SPPS) as described in the

supplementary materials and methods. Gemcitabine hydrochloride was purchased from Sigma-Aldrich.

Cell culture

Murine pancreatic cancer cell line, PAN02, were obtained from the National Cancer Institute. Human pancreatic ductal carcinoma cell line, PANC-1 (CRL-1469) and human T lymphocyte cell line, Jurkat (TIB-152) were purchased from American Type Culture Collection. PAN02 and Jurkat cells were cultured with RPMI1640 medium (Mediatech, Inc.) with 10% v/v fetal bovine serum (FBS, Atlanta Biologicals, Inc.), 1% v/v penicillin-streptomycin (Gibco). PANC-1 cells were maintained in DMEM (Mediatech, Inc.) with 10% FBS, 1% penicillin-streptomycin. These cells were cultured at 37°C with 5% CO₂.

Effect of gemcitabine and/or C1B5 treatment in 2D cell culture and 3D spheroid culture

MTT cell proliferation assay and 3D spheroid assay were performed to evaluate the effect of treatment as described in the supplementary materials and methods. Cell proliferation in 2D culture was evaluated at 48 hrs after treatment. The spheroid growth was evaluated at 9 (PAN02) and 12 (PANC-1) days after seeding cells by determining spheroid volumes, and the resulting data represented as fold changes to the untreated spheroid volume were compared.

Animals

Five to six week-old female C57BL/6 mice were obtained from Charles River Laboratories International, Inc. All mice were housed in a clean facility. All experiments were approved by Kansas State University Institutional Animal Care and Use Committee (IACUC) and Institutional Biosafety Committee, and carried out under strict adherence to the IACUC protocols set by Kansas State University.

Effect of reduced dose of gemcitabine and/or C1B5 treatment on PAN02 tumor growth in peritoneal dissemination mouse model

PAN02 cells (2.5×10^5 cells in 200 μ l PBS) were intraperitoneally injected into C57BL/6 mice. On day 5 after the inoculation, all the mice were divided into four groups (n = 5); (1) PBS control, (2) C1B5 peptide alone (20 mg/kg), (3) gemcitabine alone (15 mg/kg), and (4) the co-treatment. C1B5 was injected intraperitoneally on day 5, 7, 11 and 13. Gemcitabine was injected intraperitoneally on day 5, 8, 11 and 14. PBS was injected with same schedules. On day 17, all the mice were euthanized, and the tumors containing mesentery were collected and weighed. All the tumor tissues were dissected and fixed in 10% formalin for histological analysis.

Histological analysis

Tissue sections were stained with hematoxylin and eosin (H & E) and the number of tumor nodules were counted. Relative tumor area (40 $\times$ magnification) in 5 randomly selected areas were evaluated (n = 4-5) using NIH digital-image analyzing software, Image J 1.50b (NIH).

Immunohistochemical analysis of apoptosis in PAN02 tumor

TUNEL assay and cleaved caspase-3 detection was carried out as previously described [10]. The average number of TUNEL⁺ and anti-cleaved caspase-3⁺ cells (400× magnification) in 5-10 random fields (n = 5) were calculated.

Effect of gemcitabine and/or C1B5 treatment on intraperitoneal infiltration of leukocytes

Leukocytes infiltrated in the peritoneal cavity were collected from mice treated as described above on day 17 after the cancer cell inoculation as described in the supplementary materials and methods. Total cell number was estimated by the hemocytometer. These cells were smeared on slide glasses, air-dried and stained by Giemsa solution (Sigma-Aldrich). Cell population in ten fields (400× magnification) in each sample were evaluated based on their morphological features, and percentages of leukocyte types were estimated.

Granzyme B⁺ cells in intraperitoneally infiltrated leukocytes were analyzed by immunocytochemical staining as described in the supplementary materials and methods. The number of granzyme B⁺ cells in ten fields (400× magnification) was counted under a microscope, and its percentage in total leukocytes was estimated based on the leukocytes counted after Giemsa staining.

Effect of gemcitabine and/or C1B5 treatment on granzyme B, IL-2 and IFN- γ expressions in Jurkat cells

Jurkat cells (1×10^5 cells) were seeded into 6-well plate. After 48 hrs, the cells were treated with 1 μ M C1B5, 5 nM gemcitabine, or the combination. At 6, 12, 24 and 36 hrs after treatment, total RNA was extracted using TRIzol reagent (Invitrogen). Real-time PCR was carried out as described in the supplementary materials and methods. *The gene expression was determined by the comparative Ct method [11] and is represented as relative quantity.*

Statistical analysis

All data are reported as mean $\pm$ standard deviation (SD). Statistical significance was assessed by unpaired t-test and P<0.05 was considered to be statistically significant.

Results

Co-treatment with C1B5 peptide and gemcitabine significantly attenuated the spheroid growth of both PAN02 and PANC-1 cells in 3D spheroid culture

In 2D cell culture, although both C1B5 peptide alone and gemcitabine alone treatment showed only negligible attenuation of the growth of two cell lines, *a co-treatment with the two drugs clearly inhibited the growth of both cell lines* (Fig. 1A, PAN02; P<0.05, PANC-1; n.s.). These results suggest that PAN02 cells are more sensitive to this treatment than PANC-1 cells.

In the 3D spheroid culture, the growth of PAN02 spheroid was inhibited by gemcitabine alone and C1B5 peptide alone (P<0.05) treatment as compared to PBS control. The co-treatment further inhibited spheroid growth of PAN02 more than the other two treatment groups (Fig. 1B and C). Although the growth inhibitory effect by the co-treatment in

PANC-1 spheroid was weaker than that in PAN02 spheroid *despite an increase in concentration of gemcitabine for PANC-1 (50 nM) compared to that of PAN02 (20 nM)*, spheroid growth was significantly inhibited as compared to the PBS control (Fig. 1C).

The co-treatment with C1B5 peptide and a low dose of gemcitabine markedly attenuated tumor growth in mice

While the PBS-injected group exhibited a significant tumor growth at the end of the study period of 17 days after the tumor cell inoculation, the low dose of gemcitabine alone and co-treatment groups showed almost invisible tumor growth in macroscopic observations. The average weight of tumors in gemcitabine alone and co-treatment groups were significantly smaller than those of PBS and C1B5 peptide alone groups (Fig. 2A). Interestingly, although the tumor inhibitory effect of C1B5 peptide alone was not strong, co-treatment with gemcitabine and C1B5 peptide significantly decreased tumor weight compared to gemcitabine alone (Fig. 2A). Histological examination of tumors developed in the mesentery showed that tumor nodule numbers and tumor areas in gemcitabine alone and co-treatment groups were also markedly smaller than those in the other two groups (Fig. 2B–D). Immunohistochemical analysis of apoptotic cells in tumor nodules clearly indicated that the C1B5 peptide alone and the co-treatment significantly increased apoptotic cells in tumor nodules in both TUNEL assay and cleaved caspase 3⁺ cell numbers as compared to PBS control (Fig. 2E and F). These results clearly indicate that co-treatment with C1B5 and a low dose of gemcitabine is capable of attenuating the tumor growth of the PAN02 cells better than the low dose of gemcitabine alone *in vivo* by increasing apoptosis.

Treatment with C1B5 with or without gemcitabine significantly decreased lymphocyte ratio but increased neutrophil ratio in the peritoneal cavity-infiltrating leukocytes

Leukocytosis is one of the typical side effects in the patients treated with chemotherapeutics [12]. However, the number of the total leukocytes was higher in both gemcitabine alone and C1B5 peptide alone groups than in the control group, but the total leukocyte numbers in the co-treatment group was similar to the control group (Fig. 3A). On the other hand, cell population distribution in infiltrating leukocytes was drastically changed in the presence of the C1B5 peptide. As shown in Figure 3B, the ratio of the lymphocytes were significantly lower in C1B5 peptide alone and the co-treatment groups (Fig. 3B). Instead, the ratio of the neutrophils were significantly higher in these groups.

The co-treatment with C1B5 peptide and a low dose of gemcitabine significantly increased granzyme B⁺ cells in the peritoneal cavity-infiltrating leukocytes

T cell activation can be detected by monitoring the granzyme B expression in the lymphocytes. Neutrophils often play an important role in priming and sustaining CD8⁺ cytotoxic T lymphocytes (CTLs) [13, 14]. Since the present study suggested that C1B5 treatment with or without gemcitabine increases neutrophil proportion in intraperitoneally-infiltrating leukocytes (Fig. 3B), the proportion of the granzyme B⁺ cells was determined by immunocytochemistry. The number of granzyme B⁺ cells was significantly higher in the C1B5 peptide alone and co-treatment group compared to the PBS control (Fig. 3C and D). Although gemcitabine alone treatment also increased granzyme B⁺ cells as compared to PBS control, that was not statistically significant due to its high variation. These results

suggest that the treatment with C1B5 plays an important role in activation of CTLs/natural killer (NK) cells in the peritoneal cavity in mice bearing intraperitoneal tumors.

The co-treatment with C1B5 peptide and gemcitabine significantly increased granzyme B, IL-2 and IFN- γ expression in Jurkat cells

Co-treatment of C1B5 peptide and gemcitabine significantly increased granzyme B⁺ cell population in intraperitoneally-infiltrating leukocytes in peritoneal tumor-bearing mouse ascites (Fig. 3C and D). To further analyze whether C1B5 peptide-dependent activation is consistent in naïve T cells, human T lymphocytes (Jurkat cells) were treated with C1B5 peptide and gemcitabine, and the expressions of granzyme B, IL-2 and IFN- γ were determined by real-time PCR. As shown in Figure 4A, significant increases of granzyme B, IL-2 and IFN- γ expressions were observed in Jurkat cells co-treatment group (Fig. 4). *Since IL-2 is released from activated T cells and play a central role in sustained T cell activation and increasing production of cytotoxic molecules from CTLs, such as granzyme B, IFN- γ , etc.* [15, 16], *it may be reasonable to observe early IL-2 responses at 24 hrs time point.* Interestingly, IL-2 (Fig. 4B) and IFN- γ (Fig. 4C) expressions were sensitively increased by the treatment with a low concentration of gemcitabine, while granzyme B expression was only significantly increased in co-treatment group. These results may suggest that co-treatment with C1B5 peptide and gemcitabine effectively stimulate T cell activation, thereby producing an activated T cell-produced apoptosis inducer molecule, granzyme B.

Discussion

In our previous study, peptides derived from regulatory domain of PKC γ protein (C1B1, C1B5) inhibited the growth of human colon adenocarcinoma cells, but not in normal intestinal epithelium, by inducing G2 arrest of the cell cycle and apoptosis both *in vitro* and *in vivo* without showing noticeable adverse effects [9]. These results clearly indicate that C1B5 peptide is potentially useful for human colon cancer treatment. Co-treatment with therapeutic peptide and a low dose of chemotherapeutics for cancer is expected to reduce unexpected side effects and to overcome chemoresistance by cancer cells. In the present study, therefore, the combination effect of C1B5 peptide and a low dose of gemcitabine on the growth of pancreatic carcinoma was explored.

Although co-treatment with C1B5 peptide and gemcitabine showed weak inhibition on the growth of PAN02 (Fig. 1A, 6.3% decrease) and PANC-1 (Fig. 1A, 5.3% decrease) in 2D cell culture, it showed strong inhibition in 3D spheroid culture, particularly, in the growth of PAN02 spheroid (Fig. 1B and C, 69.5% decrease). Three-dimensional culture mimics actual tumor structure, therefore, it can be used to screen drug effect and its efficiency [17]. In the solid tumor, surface structure becomes a physical barrier to the drug penetration [18, 19]. It is known that PKC mediates cell to cell and cell to ECM interactions [20-23]. The difference in our current results in 2D and 3D spheroid culture suggests that C1B5 peptide inhibits 3D growth of cancer cells rather than inhibiting 2D growth presumably *via* a modulation of PKC activity. In support of this speculation, it is shown that an activation of PKC inhibits E-cadherin trafficking, thereby inhibiting cell to cell interaction [23].

To assess the effect of co-treatment with C1B5 peptide and a low dose of gemcitabine in *in vivo* tumor growth, C1B5 peptide, a low dose of gemcitabine, or the combination, was intraperitoneally injected into mice bearing PAN02 murine pancreatic carcinoma cells in the peritoneal cavity. *Peritoneal dissemination is one of the pathological conditions in late stages of pancreatic adenocarcinoma* [24]. *Therefore, the peritoneal dissemination model with PAN02 murine pancreatic carcinoma cells is appropriate and useful to explore the effect and impact of therapeutics* [25]. Co-treatment with C1B5 peptide and gemcitabine attenuated tumor growth compared to PBS control and monotherapy groups macroscopically and microscopically (Fig. 2). As shown in Figure 2E and F, one of the mechanisms of this tumor attenuating effect by co-treatment with C1B5 peptide and gemcitabine is suggested to be the induction of apoptosis in cancer cells. Notably, the number of apoptotic cells was also significantly increased in tumors treated only with C1B5 peptide (Fig. 2E and F). *These results corroborate well with our previous observation in which C1B5 peptide treatment induced caspase-3-mediated apoptosis in colon carcinoma cells* [9]. However, the tumor attenuation effect of C1B5 peptide alone was significantly weaker than that of the co-treatment. These results may suggest that strong tumor growth inhibition by the co-treatment may involve another mechanism in addition to the induction of apoptosis. In order to clarify the other potential mechanisms of this tumor attenuating effect by co-treatment with C1B5 peptide and gemcitabine, an analysis of intraperitoneally-infiltrating immune cells in tumor-bearing mice was conducted (Fig. 3). The treatment with C1B5 peptide alone and the co-treatment increased neutrophil population compared to lymphocyte and macrophage populations in the total infiltrating cells (Fig. 3B). This suggests that the C1B5 peptide might be causing a pro-inflammatory response in these mice. On the other hand, activated neutrophils express high levels of the *Arginase 1 (ARG1)* gene, encode the protein arginase, and result in suppression of T cell proliferation by depleting extracellular L-arginine [26]. This neutrophil-dependent T cell proliferation inhibition may be involved in the decrease of lymphocyte population in ascites collected from tumor-bearing mice in the present study.

In our present study, granzyme B⁺ cells were also increased in the infiltrating lymphocytes in mouse ascites of C1B5 peptide alone and co-treatment groups (Fig. 3C and D). Similar results were also observed in an *in vitro* cytokine profiling study in human T lymphocytes (Jurkat cells, Fig. 4A). *Jurkat cells have been widely used as model cells for human T cells* [27, 28]. *Thus, it is suggested that the T cell activation observed in the present study is not a rodent-specific incidence, but is also applicable in human.* Furthermore, it is reported that tumor-associated neutrophils (TANs) stimulate T cell responses in the earliest stages of lung cancer [29]. Concomitantly, these results may pinpoint that co-treatment of C1B5 peptide and gemcitabine, but not either single treatment, increase anti-tumorigenic TANs, which increase granzyme B⁺ CTLs/NK cells, thereby attenuating tumor growth. However, further cytological and functional analysis on the tumor-infiltrating cells is required to clarify this speculation.

In conclusion, we evaluated the effects of a co-treatment with C1B5 peptide and a low dose of gemcitabine on the growth of pancreatic carcinoma in peritoneal dissemination mouse models. Co-treatment with C1B5 peptide and a low dose of gemcitabine significantly attenuated pancreatic tumor growth compared to the single treatment by either C1B5 peptide or gemcitabine. The tumor growth attenuation in the co-treatment group was caused by an

induction of apoptosis of tumor cells due to the T cells/NK cells' activation, which was apparently stimulated by neutrophils infiltrated into tumor microenvironment by C1B5 peptide treatment. Further analysis may lead the C1B5 peptide to clinical use as an attractive therapeutic in animal and human cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

PKC	protein kinase C
2D	two-dimensional
3D	three-dimensional
IL-2	interleukin-2
IFN-γ	interferon-gamma
CTLs	CD8 ⁺ cytotoxic T lymphocytes
TANs	tumor-associated neutrophils

References

1. Siegel RL, Miller KD, Jemal A. Cancer Statistics, 2017. CA: a cancer journal for clinicians. 2017; 67:7–30. [PubMed: 28055103]
2. Whatcott, CJ., Posner, RG., Von Hoff, DD., et al. Desmoplasia and chemoresistance in pancreatic cancer. In: Grippo, PJ., Munshi, HG., editors. Pancreatic Cancer and Tumor Microenvironment. Transworld Research Network; Trivandrum (India): 2012.
3. Long J, Zhang Y, Yu X, et al. Overcoming drug resistance in pancreatic cancer. Expert opinion on therapeutic targets. 2011; 15:817–828. [PubMed: 21391891]
4. Sultana A, Tudur Smith C, Cunningham D, et al. Systematic review, including meta-analyses, on the management of locally advanced pancreatic cancer using radiation/combined modality therapy. Br J Cancer. 2007; 96:1183–1190. [PubMed: 17406358]
5. Tempero MA, Malafa MP, Behrman SW, et al. Pancreatic adenocarcinoma, version 2.2014: featured updates to the NCCN guidelines. Journal of the National Comprehensive Cancer Network : JNCCN. 2014; 12:1083–1093. [PubMed: 25099441]
6. Burris HA 3rd, Moore MJ, Andersen J, et al. Improvements in survival and clinical benefit with gemcitabine as first-line therapy for patients with advanced pancreas cancer: a randomized trial. J Clin Oncol. 1997; 15:2403–2413. [PubMed: 9196156]

7. Saif MW. Pancreatic cancer: highlights from the 42nd annual meeting of the American Society of Clinical Oncology, 2006. JOP. 2006; 7:337–348. [PubMed: 16832131]
8. Aapro MS, Martin C, Hatty S. Gemcitabine - a safety review. Anti-cancer drugs. 1998; 9:191–201. [PubMed: 9625429]
9. Kawabata A, Matsuzuka T, Doi C, et al. C1B domain peptide of protein kinase Cgamma significantly suppresses growth of human colon cancer cells in vitro and in an in vivo mouse xenograft model through induction of cell cycle arrest and apoptosis. Cancer Biol Ther. 2012; 13:880–889. [PubMed: 22785210]
10. Ishiguro S, Cai S, Uppalapati D, et al. Intratracheal Administration of Hyaluronan-Cisplatin Conjugate Nanoparticles Significantly Attenuates Lung Cancer Growth in Mice. Pharmaceutical research. 2016; 33:2517–2529. [PubMed: 27335023]
11. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C(T) method. Nature protocols. 2008; 3:1101–1108. [PubMed: 18546601]
12. Connolly GC, Khorana AA, Kuderer NM, et al. Leukocytosis, thrombosis and early mortality in cancer patients initiating chemotherapy. Thrombosis research. 2010; 126:113–118. [PubMed: 20684071]
13. Lukens MV, van de Pol AC, Coenjaerts FE, et al. A systemic neutrophil response precedes robust CD8(+) T-cell activation during natural respiratory syncytial virus infection in infants. Journal of virology. 2010; 84:2374–2383. [PubMed: 20015982]
14. Beauvillain C, Delneste Y, Scotet M, et al. Neutrophils efficiently cross-prime naive T cells in vivo. Blood. 2007; 110:2965–2973. [PubMed: 17562875]
15. Liao W, Lin JX, Leonard WJ. Interleukin-2 at the crossroads of effector responses, tolerance, and immunotherapy. Immunity. 2013; 38:13–25. [PubMed: 23352221]
16. Gillis S, Baker PE, Ruscetti FW, et al. Long-term culture of human antigen-specific cytotoxic T-cell lines. The Journal of experimental medicine. 1978; 148:1093–1098. [PubMed: 308989]
17. Friedrich J, Seidel C, Ebner R, et al. Spheroid-based drug screen: considerations and practical approach. Nature protocols. 2009; 4:309–324. [PubMed: 19214182]
18. Sagar JK, Yu M, Tan Q, et al. The tumor microenvironment and strategies to improve drug distribution. Frontiers in oncology. 2013; 3:154. [PubMed: 23772420]
19. Tannock IF, Lee CM, Tunggal JK, et al. Limited penetration of anticancer drugs through tumor tissue: a potential cause of resistance of solid tumors to chemotherapy. Clinical cancer research : an official journal of the American Association for Cancer Research. 2002; 8:878–884. [PubMed: 11895922]
20. McHarg S, Hopkins G, Lim L, et al. Down-regulation of desmosomes in cultured cells: the roles of PKC, microtubules and lysosomal/proteasomal degradation. PloS one. 2014; 9:e108570. [PubMed: 25291180]
21. Nguyen TA, Takemoto LJ, Takemoto DJ. Inhibition of gap junction activity through the release of the C1B domain of protein kinase Cgamma (PKCgamma) from 14-3-3: identification of PKCgamma-binding sites. The Journal of biological chemistry. 2004; 279:52714–52725. [PubMed: 15459208]
22. Nguyen TA, Boyle DL, Wagner LM, et al. LEDGF activation of PKC gamma and gap junction disassembly in lens epithelial cells. Experimental eye research. 2003; 76:565–572. [PubMed: 12697420]
23. Le TL, Joseph SR, Yap AS, et al. Protein kinase C regulates endocytosis and recycling of E-cadherin. American journal of physiology Cell physiology. 2002; 283:C489–499. [PubMed: 12107059]
24. Hidalgo M. Pancreatic cancer. The New England journal of medicine. 2010; 362:1605–1617. [PubMed: 20427809]
25. Doi C, Maurya DK, Pyle MM, et al. Cytotherapy with naive rat umbilical cord matrix stem cells significantly attenuates growth of murine pancreatic cancer cells and increases survival in syngeneic mice. Cytotherapy. 2010; 12:408–417. [PubMed: 20345214]
26. Rotondo R, Bertolotto M, Barisione G, et al. Exocytosis of azurophil and arginase 1-containing granules by activated polymorphonuclear neutrophils is required to inhibit T lymphocyte proliferation. Journal of leukocyte biology. 2011; 89:721–727. [PubMed: 21330347]

27. Huang C, Bi E, Hu Y, et al. A novel NF-kappaB binding site controls human granzyme B gene transcription. *Journal of immunology*. 2006; 176:4173–4181.
28. Abraham RT, Weiss A. Jurkat T cells and development of the T-cell receptor signalling paradigm. *Nature reviews Immunology*. 2004; 4:301–308.
29. Eruslanov EB, Bhojnagarwala PS, Quatromoni JG, et al. Tumor-associated neutrophils stimulate T cell responses in early-stage human lung cancer. *The Journal of clinical investigation*. 2014; 124:5466–5480. [PubMed: 25384214]

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Highlights

- Co-treatment of C1B5 peptide and gemcitabine inhibits pancreatic cancer cell growth *in vitro*.
- Co-treatment of C1B5 peptide and a low dose of gemcitabine attenuates the growth of pancreatic cancer autografts in mice.
- Co-treatment of C1B5 peptide and gemcitabine increases neutrophils and granzyme B⁺ lymphocytes in tumor microenvironment.

Figure 1.

Co-treatment with C1B5 peptide and gemcitabine decreased the growth of PAN02 murine and PANC-1 human pancreatic carcinoma cells in both 2D cell and 3D spheroid cultures. (A), The attenuating effects of C1B5 peptide, gemcitabine (Gem), and the co-treatment *in 2D cultures* were evaluated at 48 hrs after treatment by MTT assay (n = 3). (B), Typical image of PAN02 spheroid in each group. (C), The attenuating effects of C1B5 peptide, gemcitabine, and the co-treatment *in PAN02 and PANC-1 spheroid cultures* were evaluated at Day 9 (PAN02) and Day 12 (PANC-1) after the initiation of spheroid culture (n = 5). *, $P < 0.05$.

Figure 2.

Co-treatment with C1B5 peptide and gemcitabine significantly decreased the growth of pancreatic tumors in mouse peritoneal cavity. (A), Average tumor weight in mesentery in PBS control, 15 mg/kg gemcitabine (Gem) alone, 20 mg/kg C1B5 peptide alone, and the combination group (n = 4-5). (B), Typical image of H & E stained tumor tissues in PBS control (a), gemcitabine alone (b), C1B5 peptide alone (c), and co-treatment with C1B5 and gemcitabine (d). 40× magnification. (C-D), The number of tumor nodules (C) and its relative tumor area (D) in 5 randomly selected areas in H & E stained samples (n = 4-5). (E-F), The

evaluation of apoptosis-induced cells in tumors (n = 4-5) by TUNEL (E) and the cleaved caspase-3 (F). a-c, $P < 0.05$ between different characters.

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Figure 3.

Treatment with C1B5 and/or gemcitabine did not alter total leukocyte numbers, but significantly increased neutrophil and granzyme B⁺ cell populations in the peritoneal cavity. (A), The number of leukocytes in ascites analyzed by Giemsa staining. (B), The ratio of subpopulations of the intraperitoneally-infiltrated leukocytes. (C-D), Immunocytochemical analysis on granzyme B expression in intraperitoneally-infiltrating lymphocyte. (C) Granzyme B⁺ cells (arrow head) in control and co-treatment groups. (D), The percentage of granzyme B⁺ cells in each group. *, P<0.05 vs PBS control. † in panel B, P<0.05 vs gemcitabine alone.

Figure 4.

Co-treatment with C1B5 peptide and gemcitabine significantly increased expression of granzyme B (GZMB) and activated T cell-associated cytokines (IL-2, and IFN- γ) in Jurkat cells. *, $P < 0.05$ vs PBS control at 6 hrs (6H).