Oncology

KEYWORDS: Pancreatic cancer, Pao Pereira, Cancer stem cells, xenograft models

EXTRACT OF THE MEDICINAL PLANT PAO PEREIRA INHIBITS PANCREATIC CANCER STEM CELL IN VITRO AND IN VIVO

Volume-3, Issue-1, January - 2018

Ruochen Dong Department of Pharmacology, Toxicology and Therapeutics, KU Integrative Medicine, University of Kansas Medical Center, Kansas City, KS, USA

Jun Yu Department of Pharmacology, Toxicology and Therapeutics, KU Integrative Medicine, University of Kansas Medical Center, Kansas City, KS, USA

Ping Chen* Department of Pharmacology, Toxicology and Therapeutics, KU Integrative Medicine, University of Kansas Medical Center, Kansas City, KS, USA*Corresponding Author qchen@kumc.edu.

Qi Chen Department of Pharmacology, Toxicology and Therapeutics, KU Integrative Medicine, University of Kansas Medical Center, Kansas City, KS, USA

Article History
Received: 29.07.2017
Accepted: 12.10.2017
Published: 10.01.2018

ABSTRACT:

Pancreatic cancers are enriched with cancer stem cells (CSCs), which are resistant to chemotherapies, and are responsible for tumor metastasis and recurrence. Here we investigated an extract from the medicinal plant Pao Pereira (Pao) for its activity against pancreatic CSCs. Pao inhibited overall proliferation of human pancreatic cancer cell lines with IC50 ranging from 125 to 325 µg/mL, and had limited cytotoxicity to normal epithelial cells. Pancreatic CSC population, identified using surface markers CD24+CD44+EpCam+ or tumor spheroid formation assay, was significantly reduced, with IC50s of ~100 µg/mL for 48 hours treatment, and ~27 µg/mL for long-term tumor spheroid formation. Nuclear β-catenin levels were decreased, suggesting suppression of wnt/β-catenin signaling pathway. In vivo, Pao at 20 mg/kg, 5 times/week gavage, significantly reduced tumorigenicity of PANC-1 cells in immunocompromised mice, indicating inhibition of CSCs in vivo. Further investigation is warrant in using Pao as a novel treatment targeting pancreatic CSCs.

Introduction

Pancreatic cancer is the 4th leading cause of cancer-related death in the United States with a 5-year overall survival rate of only 6% (Siegel, Miller, & Jemal, 2017). The American Cancer Society estimated that 53,670 (men=27,970, women=25,700) people will be diagnosed with pancreatic cancer in 2017, and 40,560 (men=22,300, women=20,790) will die from it (Siegel et al., 2017). Treatment outcomes are far from satisfaction (Fingerhut, Vassiliu, Dervenis, Alexakis, & Leandros, 2007; Y. Yu, Ramena, & Elble, 2012). Because of the lack of efficient early detection methods, only about 9% of patients are diagnosed with local disease. For them the 5-year survival rate is only 26%. For the majority of patients (about 53%) who are diagnosed at an advanced stage, the 5-year survival rate is less than 5%, which is among the lowest of all types and stages of malignancies (Hidalgo, 2010). Gemcitabine was the first line standard chemotherapy for nearly 2 decades, but it only provides limited benefit on the overall survival of patients with locally advanced and metastatic pancreatic cancer (Oettle & Neuhaus, 2007; Renouf & Moore, 2010). New treatment regimens either by adding chemo-drugs, such as nab-paclitaxel (Von Hoff et al., 2013) to gemcitabine, or using gemcitabine-free combination, such as FOLFIRINOX (Conroy, Gavoille, Samalin, Ychou, & Ducreux, 2013; Faris et al., 2013), show some improvement in survival and response rates, however, they significantly increase toxic side effects (Conroy et al., 2011; Vishnu & Roy, 2011). New treatments are urgently needed for pancreatic cancer.

The poor treatment outcomes may partially attribute to an enriched cancer stem cell (CSC) population in pancreatic cancer. Accumulating evidence have shown that pancreatic CSCs are resistant to current treatments, and therefore survive and eventually generate new tumors, either at the primary or metastatic sites (Du et al., 2011; Lonardo, Cioffi, Sancho, Crusz, & Heeschen, 2015; Schmied, Ulrich, Matsuzaki, Li, & Pour, 1999). CSCs share characteristics with normal stem cells. An important characteristic is the ability of self-renew. Depending on the microenvironment, a stem cell can divide and generate daughter cells which do not differentiate but keep the full potential of differentiation as the parent stem cell (self-renew), and/or raise daughter cells which will differentiate (Reya, Morrison, Clarke, & Weissman, 2001). CSCs possess self-renewal ability and are able to give rise to all cell types found in a particular bulk of tumor (Ajani, Song, Hochster, & Steinberg, 2015). CSCs are resistant to current chemo and radiation therapy (Vinogradov & Wei, 2012), are responsible for tumor metastasis (Shiozawa, Nie, Pienta, Morgan, & Taichman, 2013) and recurrence (Y. Yu et al., 2012), which are the main reasons of cancer related death. Therefore, therapies that inhibit cancer stem cells would hold great promises in eliminating the whole cancer cell population.

Natural products have been a rich resource for bioactive anticancer agents, are used in folk medicines all over the world, and are used by oncologic patients and integrative medicine practitioners for many years. Pao Pereira is an Amazonian tree species Geissospermum vellosii. The extract of the bark of Pao Pereira (Pao) has long been used in complementary and alternative medicine on cancer patients, and has been reported recently to have tumor inhibitory effect toward prostate, ovarian and pancreatic cancers (Bemis, Capodice, Desai, Katz, & Buttyan, 2009; Chang et al., 2014; J. Yu & Chen, 2014; J. Yu, Drisko, & Chen, 2013). We previously reported that Pao induced pancreatic cancer cells apoptosis, and inhibited pancreatic tumor growth in mice (J. Yu et al., 2013). The combination of Pao and gemcitabine showed synergistic anti-tumor effects (J. Yu et al., 2013). Here we investigated the activities of Pao in inhibiting pancreatic cancer stem cells both in vitro and in vivo.

Materials and methods

Cell lines and reagents

Human pancreatic cancer cell lines PANC-1, AsPC-1, HPAF-II, BxPC-3 and MiaPaCa-2 were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained in the lab. An

immortalized human lung epithelial cell line MRC-5 were provided by Dr. Sitta Sittampalam at the National Center for Advancing Translational Sciences, NIH, and were used as a comparison to the cancer cells. All cells were cultured at 37°C in 5% CO2/95% air in recommended growth media containing 10% fetal bovine serum and 1% antibiotics. The extract of Pao Pereira (Pao) was provided by the Natural Source International Ltd. (New York, NY, USA) and were prepared in sterile PBS in 10 mg/mL stock solutions and stored at −20°C.

Cell viability assay

Cells were assessed for viability by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay at 72 h of treatment. Cells in exponential growth phase were exposed to serial dilutions of Pao for 72 h. Cells were then changed into fresh media containing MTT and were incubated for 4 h. The colorimetric MTT assay assesses relative proliferation, based on the ability of living, but not dead cells, to reduce MTT to formazan. Cells did not reach plateau phase during the incubation period. Fifty percent inhibitory concentration (IC50) was defined as the concentration of drug that inhibited cell growth by 50% relative to the untreated control. Pilot experiments for each cell line were performed to optimize cell density and assay duration and to center drug dilution series approximately on the Ic50.

Tumor spheroid formation assay

Single-cell suspension was plated into 24 well ultra-low attachment plates (Corning Inc., Corning, NY) at a density of 5000 cells/well in stem cell media and incubated at 37°C in a humidified atmosphere of 95% air and 5% CO2. The stem cell media consist of DMEM (Corning Inc., Corning, NY) supplemented with 1X B27 Supplement, 20 ng/ml human basic fibroblast growth factor, 20 ng/ml epidermal growth factor, 100 units/ml penicillin/streptomycin (Invitrogen, Grand Island, NY), and 4µg/ml heparin calcium salt (Fisher Scientific, Pittsburg, PA). Spheroids were counted after 4 weeks under the microscope.

Flow Cytometry for detection of cancer stem cells surface markers
Cells were exposed to various concentrations of Pao for 24 hours or 48 hours. Cells were then washed with PBS trice, and re-suspended in binding buffer (PBS supplemented with 0.1% Bovine serum albumin) for 15 minutes. PE conjugated anti-CD24 antibody, PE-Cy7 conjugated anti-CD44 antibody and APC conjugated anti-EpCam antibody (Biolegend, San Diego, CA) were added into cell suspension and incubated for 15 mins according to the manufacturer's protocol. Cells were washed in PBS trice after staining and then analyzed by BD LSR II Flow Cytometer. The data was normalized to cell death (Normalized CSC population = original CSC population detected with flow cytometry x % cell viability detected with MTT assay).

SDS PAGE and western blot

Cells were lysed with RIPA buffer containing protease inhibitors and phosphatase inhibitors (Sigma Al), followed by sonication for 10 seconds. Either whole cell lysate or supernatant were used for further experiment, depending on the proteins of interest. BCA method was used for protein quantification (Pierce BCA protein assay kit, Waltham, MA). SDS-PAGE and Western blot was performed as routine. A goat anti-rabbit and anti-mouse polyclonal horseradish peroxidase conjugated secondary antibody (Cell Signaling Technology, Beverly, MA) was used. Blots were established using a chemiluminescence detection kit (Pierce ECL or ECL+ western blotting substrate, Thermo Scientific, Rockford, IL).

RNA isolation, cDNA synthesis, and Real-time PCR

Total RNA was extracted from cells or tissue samples by using TRIZOL reagent according to the protocol of the manufacturer (Invitrogen, Grand Island, NY). cDNA synthesis was performed with 1µg of total RNA using Omniscript RT kit according to manufacturer's protocol (Qiagen, Valencia, CA). cDNA was diluted 1:5 in DEPC treated nanopure water and used for further analysis. Real-time PCR was performed using Bio-Rad iQ iCycler detection system with iQ SYBR green supermix (Bio-Rad Laboratories Ltd, Hercules, CA). Reactions were performed in a total volume of 10 µl, including 5 µl of 2X iQ SYBR green supermix, 0.4 µl of primers at 20 pmol/µl and 0.4 µl of cDNA template. All reactions were carried out in at 4 repeats for every sample and 3 independent experiments for each group. GAPDH was used as housekeeping gene for normalization. Primers used in Real-time PCR were according to previous study (Amini, Fathi, Mobalegi, Sofimajidpour, & Ghadimi, 2014).

Pancreatic cancer mouse model

All animal studies followed a protocol approved by the Institutional Animal Care and Use Committee of the University of Kansas Medical Center. One-time treatment and repeated treatment were each used for measurement of tumorigenecity. In the one-time treatment model, pancreatic cancer cells PANC-1 at different numbers were used for tumor inoculation: 2x104 cells per injection, 2x105 cells per injection, or 1 x106 cells per injection. PANC-1 cells were suspended in PBS as single cell suspension and then mixed with either 200 mg/mL Pao or PBS. At each cell injection number, cells mixed with Pao were injected subcutaneously into the left flank of the mouse, and cells mixed with PBS into the right flank of the same mouse. Ten mice were used for each cell number. Formation of tumors were monitored daily, and longitudinal tumor growth was measured by a caliper.

In the repeated treatment model, single cell suspension of PANC-1 cells were mixed with 200 mg /ml Pao, and then inoculated into 10 mice at 2 x 105 cells per injection, at both left and right flanks. Treatment started the next day with oral gavage of 20 mg/kg Pao, 5x per week for 3 weeks. Control group (10 mice) was inoculated with the same number of cells in PBS, and then was gavaged with equivalent volume of saline solution. Tumor formation were monitored daily, and longitudinal tumor growth was measured by a caliper.

Data analysis

Statistical analysis was performed using SPSS software for student T-test and log-rank test. A difference was considered significant at the $p < 0.05$ level.

Results

Pao inhibits pancreatic cancer tumor spheroids formation in vitro

Five different human pancreatic cancer cell lines (PANC-1, MiaPaCa-2, AsPC-1, HPAF-II, and BxPC-3) and an immortalized epithelial cell line (MRC-5) were treated with Pao, and cell viability was detected after 72 h. Pao inhibited proliferation of all five cancer cells (Fig. 1A), with IC50 values ranging from 125 to 325 µg/mL. The non-cancerous epithelial cell MRC-5 was less affected, with a higher IC50 value of 547 µg/mL (Fig. 1B). These results are consistent with our previous studies that Pao inhibited the overall proliferation of pancreatic cancer cells (J. Yu et al., 2013).

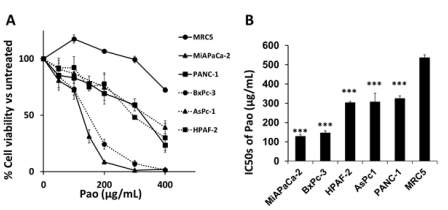


Figure 1. Inhibition of the proliferation of pancreatic cancer cells by Pao. (A) Dose-response curves. Human pancreatic cancer cells PANC-1, AsPC-1, HPAF-II, BxPC-3 and MiaPaCa-2 were exposed to serial concentrations of Pao for 72 h. Cell viability was detected by MTT assay. An immortalized non-cancerous epithelial cell MCR-5 was subjected to the same treatment. (B) IC50 values of Pao in pancreatic cancer cells and MRC-5 cells. *** $p<0.001$ compared to the IC50 of MRC5 cells. All values are expressed as means ± SD of 3 independent experiments each done in triplicates.

To investigate inhibition in CSCs, tumor spheroid formation was detected. The ability of forming tumor spheroids is an in vitro indication for CSCs' self-renew and tumorigenic capacity. When cancer cells are cultured in serum-free, non-adherent conditions, non-CSC population die by anoikis, whereas CSCs overcome anoikis and go through division leading to formation of tumor spheroids (Kim et al., 2016; Vermeulen et al., 2008). At the concentration of 50 µg/mL, Pao significantly reduced the number of the PANC-1 tumor spheroids (Fig. 2A, B). At the concentration of 100 µg/mL and above, Pao completely eliminated the PANC-1 tumor spheroids (Fig. 2A, B). The estimated IC50 value for tumor spheroids inhibition is 27 µg/mL. In comparison, the IC50 value of Pao to the bulk of PANC-1 cells is about 300 µg /mL (Fig. 1A). In the bulk PANC-1 cell population, 100 µg /mL of Pao inhibited the overall proliferation by 20%, whereas 100% tumor spheroids were inhibited at this concentration (Fig. 2A). These results indicate that Pao possesses preferential inhibitory effects against pancreatic CSCs.

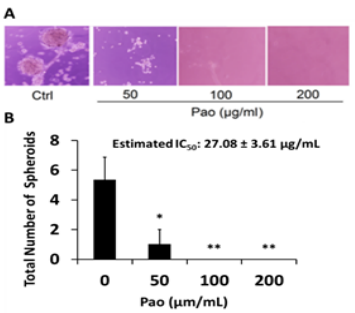
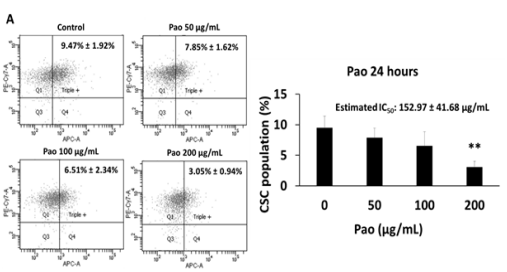


Figure 2. Inhibition of pancreatic tumor spheroids by Pao. PANC-1 single-cell suspension was plated into 24 well ultra-low attachment plates at a density of 5000 cells/well in stem cell media. Tumor spheroids were counted after 4 weeks. (A) Representative images of the spheroids with and without Pao treatment. (B) Number of Panc-1 spheroids (means ± SD of 3 independent experiments). * p<0.05, ** p<0.001 compared to untreated control.

Pao reduces number of pancreatic cancer stem cells in vitro

CSC population can be identified by specific cell surface markers. In pancreatic cancer, a sub-population of cells with high expression of surface markers CD44, CD24 and EpCAM (CD44+ CD24+ EpCAM+ cells) were reported to possess strong self-renewal ability and the ability to produce differentiated progeny and to generate new tumors in mice that were histologically identical to parent tumors (Li, Lee, & Simeone, 2009). Here, we use these markers as indicative markers for pancreatic CSCs, and detected changes in these markers with Pao treatment. PANC-1 cells were treated with Pao for 24 hours or 48 hours at 50, 100 or 200 µg/mL. CD44, CD24, and EpCAM were examined by immune staining and flow cytometry analysis. Pao reduced the CD44+CD24+EpCam+ population at both 24 h and 48 h treatment (Fig. 3A, B). In control group, CD44+CD24+EpCam+ cells consist 7.5% - 9% of the whole population. At the concentration of 200 µg/mL, Pao significantly reduced CD44+CD24+EpCam+ cells to 3.05% at 24 h treatment (Fig. 3A), and to 0.37% at 48 h (Fig. 3B). At a lower concentration of 100 µg/mL, Pao reduced the triple positive cells to 2.31% at 48 h treatment (Fig. 3B), which was still a significant change compared to control (Fig. 3B). We estimated that the IC50 value at 24 hours treatment is 152.97 ± 41.68 µg/mL, and at 48 hours treatment it is 99.53 ± 6.95 µg/mL (Fig. 3A, B).



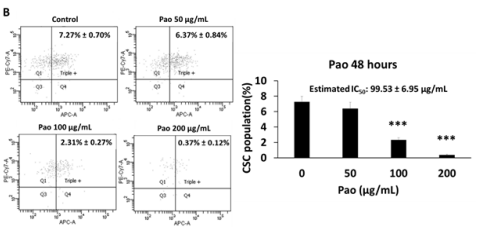
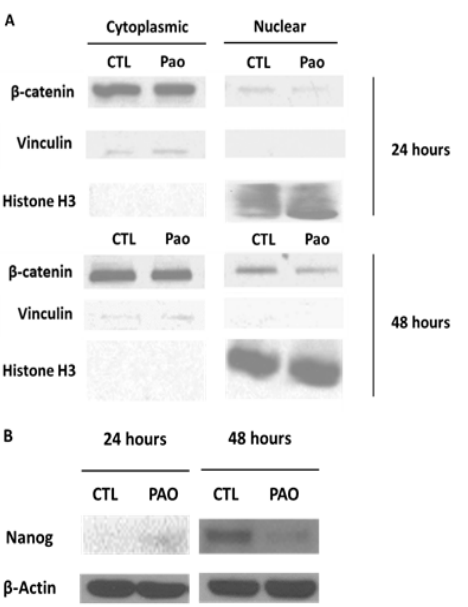


Figure 3. Inhibition of CSCs populations by Pao. PANC-1 cells were treated with Pao for 24 hours (A), and 48 hours (B) at indicated concentrations. Cells were then stained with fluorescent conjugated antibodies for CD24, CD44 and EpCam, followed by flow cytometry analysis. Left panels show the EpCam (APC) and CD44 (PE-Cy7) positive cells under the CD24 (PE) positive gate. The percentages of CD24+CD44+EpCam+ cells were quantified and shown in the bar graph (Mean ± SD of 3 experiments). The data was normalized to cell death. ** p<0.01, *** p<0.001 compared to untreated group.

Canonical wnt/β-catenin signaling pathway plays an important role in maintaining the self-renewal and spheroid formation capacities of CSCs (Ajani et al., 2015; Takebe, Harris, Warren, & Ivy, 2011). Accumulation of β-catenin in the nuclear as a transcriptional factor is a hallmark of wnt/ β-catenin pathway activation (MacDonald, Tamai, & He, 2009). Here, the cytoplasmic and nuclear fractions of the PANC-1 cells were each examined for β-catenin levels with or without Pao treatment. Pao 100 µg/mL at 24 h and 48 h reduced the level of β-catenin in the nuclear (Fig. 4A), while the cytoplasmic β-catenin levels were not changed (Fig. 4A). Studies have shown that a stem cell related gene Nanog can induce β-catenin phosphorylation and therefore enhance its degradation, and consequently inhibit wnt signaling pathway (Cheng et al., 2015). We therefore examined the expression of Nanog by western blot. Nanog was increased at 24 h of Pao treatment, however was decreased at 48 h of Pao treatment (Fig. 4B). We postulate that increase in Nanog at earlier time point suppressed nuclear β-catenin levels, and then the decreasing β-catenin levels feedback and caused inhibition in Nanog expression at a later time point (Takao, Yokota, & Koide, 2007; Yong et al., 2016). As a result, both Nanog and wnt signaling pathway were inhibited by Pao. A panel of other CSCs related genes were also examined by RT-PCR which are reported to be important for CSC initiation and maintenance (Amini et al., 2014). Data showed that the expressions of these genes were not changed with 24 h of Pao treatment (Fig. 4D).



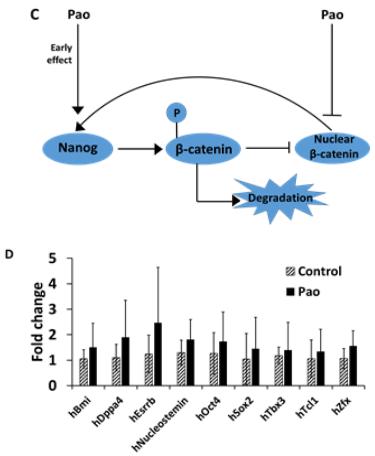


Figure 4. Decrease of nuclear β-catenin by Pao. PANC-1 cells were treated with Pao at 100 μg/mL for 24 hours and 48 hours. (A) The expression of β-catenin was detected by western blots in cytoplasmic and nuclear fractions. Vinculin was a loading control for cytoplasmic proteins, and histone H3 was a loading control indicative for the nuclear fraction. (B) The expression of Nanog was detected by western blots. (C) Postulated mechanism of Pao inhibiting Nanog and nuclear β-catenin. Pao treatment has an early effect in increasing Nanog expression, which leads to β-catenin phosphorylation and degradation, therefore represses nuclear β-catenin level. The decreasing nuclear β-catenin level negatively influences Nanog expression. Pao treatment may also directly inhibits β-catenin nuclear accumulation. Both can result in an overall suppression of both Nanog and nuclear β-catenin levels. (D) The expression of CSCs related genes were examined by RT-qPCR. Error bar shown standard deviation with 12 repeats.

Pao inhibits pancreatic cancer stem cells in vivo

Tumorigenicity was examined in immunocompromised mice to evaluate the inhibitory activity of Pao against pancreatic CSCs in vivo. A one-time treatment was performed first using inoculation of different numbers of PANC-1 cells at limited dilutions. Respectively, 2x104 cells, 2x105 cells, and 1x106 cells were mixed with 200 mg/mL Pao and injected subcutaneously into the left flanks of nude mice (N=10). As control, the same number of cells were mixed with PBS and inoculated into the right flanks of the same mouse. At all three numbers of cell injections, neither a delay nor a reduction of rate in tumor formation was found (Fig. 5A, C, E). The one-time Pao treatment tended to reduce the size of tumors at the 2x104 cells and 2x105 cells groups, but there was no significant difference compared to control groups (Fig. 5B, 5D, 5F).

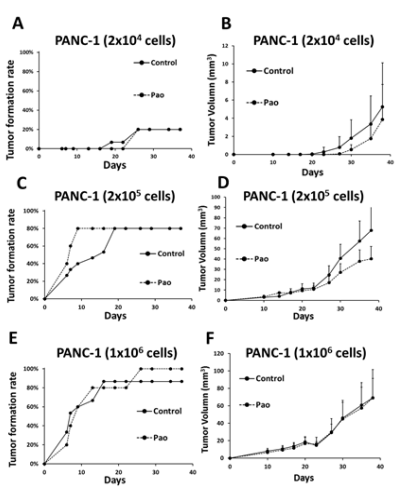


Figure 5. Effects of one-time Pao treatment on PANC-1 tumor formation in nude mice. (A, B) 2x104 PANC-1 cells (C, D) 2x105 PANC-1 cells (E, F) 1x106 PANC-1 cells were mixed with 200 mg/ml Pao Pereira, and then inoculated at the left flank of each mouse. The same density of PANC-1 cells were mixed with PBS, and inoculated at the right flank of each mouse. Totally 10 mice were used for each cell number. The tumor formation rate (A, C, E) was described as the number of tumors observed at specific day / 10 x100%. Tumor size (B, D, E) was monitored weekly by caliper, and the tumor volume was calculated using following formulations: Tumor volume = width x width x length/2.

As the one-time Pao treatment failed to reduce the rate of tumor formation, we conducted repeated treatment with oral administration of Pao. The cell number was selected to be 2x105 per injection. Mice (N=10) were injected subcutaneously at both left and right flanks with PANC-1 cells mixed with 200 mg/mL of Pao. Treatment started the next day and lasted for 3 weeks with oral gavage of 20 mg/kg Pao, 5x per week. Control mice (N=10) were inoculated with the same number of cells mixed with PBS, and were gavaged with equivalent volumes of saline.

Both the rate of tumor formation and time of tumor formation were significantly different between the control and treated groups (Fig. 6A). At day 6, tumor formation rate in control group reached 80%, while in Pao-treated group it was only 10%. At Day 20 when the treatment stopped, all mice in control group were bearing tumors on both flanks (100% tumor formation), while the Pao treated group only had 30% tumor formation. All mice were kept for 2 more months after treatment had stopped. At the end of the experiment, the Pao treatment group had a maximum of 65% tumor formation, compared to the 100% tumor formation in the control group. These data indicate that Pao administration at 20 mg/kg orally eliminated CSCs in 35% of the injection sites.

Growth of the formed tumors was also inhibited by Pao treatment compare to the control group (Fig. 6B). A long-term inhibitory effect in tumor growth was observed after treatment had stopped (Fig. 6B). No adverse effects were observed in both groups during the treatment.

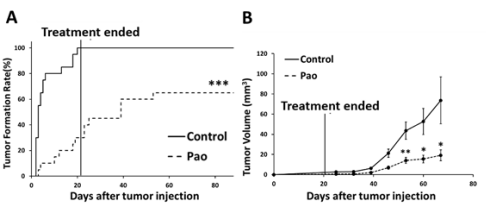


Figure 6. Effects of repeated Pao treatment on PANC-1 tumor formation and tumor growth in nude mice. (A) Tumor formation rate. 2x105 PANC-1 cells were mixed with 200 mg/ml Pao (Pao) or PBS (Control), and inoculated at both flanks of nude mice (N=10 for each group). Treatment started the next day and lasted for 3 weeks with oral gavage of 20 mg/kg Pao (Pao) or saline (Control) 5x per week. ***, P<0.001 by log-rank test. (B) Longitudinal tumor growth. Tumor size was measured weekly by caliper. Tumor volume = width x width x length/2. *, p<0.05; **, p<0.01.

Discussion

Cancer stem cells (CSCs) are a small population in the bulk of cancer cells which are responsible for generation of new tumors. They possess self-renewal ability and are able to give rise to all cell types found in a particular bulk of tumor . Traditional anti-tumor chemo drugs lack the ability to eliminate CSCs, who then survive and later raise recurred tumors often at metastatic sites . CSCs are also responsible for drug resistance . The mechanism by which CSCs become drug resistant is not very clear, probably attribute to the upregulated expression of ABCG2 transporter, which facilitates efflux of chemotherapeutic drugs from the cytosol . Other properties contribute to CSC's drug resistance include the overexpressed detoxifying enzymes, enhanced DNA repair ability

and overexpression of anti-apoptotic proteins . Developing CSC inhibitors has been challenging, yet, new drugs inhibiting CSCs holds the hope to comprehensively inhibit tumor growth, metastasis, recurrence, and conquer drug resistance. In this study, we demonstrated both in vitro and in vivo that the extract of the plant Pao Pereira (Pao) inhibited pancreatic CSCs. Previously, we have reported that Pao induces apoptosis in pancreatic cancer cells and sensitizes pancreatic cancer cells to gemcitabine treatment . Independent of its apoptosis-inducing activity, the CSCs inhibition could be another reason contributing to Pao-induced gemcitabine sensitivity. Taken together, the benefits of Pao in pancreatic cancer treatment are worth investigation clinically, especially in combination with current chemotherapies.

To date there has not been an efficient method to pin-point a pancreatic CSC and maintain/amplify it for drug development purposes. Functional assays such as tumor spheroid assay and tumorigenicity in mice are commonly used . The use of several cell surface markers are powerful to identify and isolate a sub-population enriched with stem-like features . In our studies here, CSCs were not isolated and separately treated, first because it's difficult to obtain and maintain a pure CSC population . Secondly, because isolated CSCs might lose their natural environment in the bulk population . Instead, we treated the bulk of pancreatic cancer cells, and examined the CSC specific outcomes. The inhibitory results from our studies are not likely due to the general cytotoxicity of Pao to the bulk of cancer cells, because Pao has an IC50 value of 300 µg/mL in 72 hours of treatment towards the bulk of PANC-1 cells, and has a much less IC50 value of 153 µg/mL for the reduction of CD44+CD24+EpCam+ cells at a shorter treatment time of 24 hours, and 99.53 µg/mL at 48 hours. Furthermore, in the tumor spheroid formation assay, Pao has an IC50 of 27 µg/mL in inhibiting the number of spheroid. These data suggest that Pao has a preferential inhibitory activity towards pancreatic CSCs.

The mechanism(s) by which Pao induces CSC inhibition needs to be further investigated. Our study showed that Pao reduced both Nanog and nuclear β-catenin level of PANC-1 cells, which are important in stem cell initiation and maintenance. In-depth mechanism on how Pao interacts with Nanog and/or β-catenin singling pathway needs to be further investigated. Moreover, as this plant preparation contains a complex mixture of natural compounds, it is possible that Pao also impacts other molecular targets and pathways that lead to CSC inhibition.

Previous studies on the extract of Pao showed the inhibitory effect on proliferation on pancreatic, ovarian and prostate cancers . Our animal data here showed promising effects of Pao in inhibiting tumorigenicity and tumor growth, at a dose and administration route that can be easily translated into clinical use. No toxic side effects were observed in mice at this dosage. The inhibition in tumorigenicity implies a possible role of Pao in the prevention of cancer, in addition to data indicating a treatment role. Given that the extracts of Pao Pereior are consumed by the American public as a health supplement, the safety, toxicity, and effects of Pao as an anti-cancer agent should be further investigated clinically.

Acknowledgement

This work is supported by a research grant from the Beljanski Foundation. The Beljanski Foundation has no influence on the study design, the experiments performance, data collection, analysis and manuscript preparation. We thank Dr. Sitta Sittampalam at the National Center for Advancing Translational Sciences, NIH, for providing MRC-5 cells, and our previous technician Iman Joker for exploratory work on this project.

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