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PPAR-alpha: a novel target in pancreatic cancer

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PPAR-ALPHA: A NOVEL TARGET IN PANCREATIC CANCER

by

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ABSTRACT

Background: Current targeted therapies in pancreatic cancer have been ineffective. The tumor stroma, including intra- and peri-tumoral inflammation and fibrosis, is increasingly implicated in pancreatic cancer. Pancreatic cancer is characterized by a highly fibrotic tumor environment resulting in stromal resistance to chemotherapy. Peroxisome proliferator-activated receptor-alpha (PPARα), a ligand-activated nuclear receptor/transcription factor, is a negative regulator of inflammation. In PPARα deficient mice, stromal processes inhibit tumor growth, resulting in dormant tumors. The presence of PPARα in the tumor cells as well as in the host is necessary for unabated tumor growth. **Objective:** We hypothesized that blocking the PPARα pathway with a small molecule PPARα antagonist (NXT) may prevent pancreatic cancer progression by targeting tumor cells as well as non-neoplastic cells in the tumor microenvironment. **Methods:** Growth inhibitory activity of the PPARα antagonist was assessed in murine as well as human pancreatic tumor cell lines (Panc0H7 and BxPC3) and in a murine macrophage cell line (RAW 264.7). Cell viability was determined by trypan blue exclusion assay. AKT, P-AKT, PCNA, BAX, and p27 levels were analyzed by western blot analysis. Cell cycle changes were detected by flow cytometry. Cellular senescence was determined by senescence-associated β-gal (SA-β-gal) staining. **Results:** The PPARα antagonist inhibited cell growth in macrophages and in pancreatic tumor cells as confirmed by reduced protein level expression of PCNA and activated AKT. Treatment

of the PPARα antagonist was non-cytotoxic to tumor cells. Inhibition of PPARα induced cell cycle arrest at G_0/G_1 in tumor cells and macrophages. The induction of cellular senescence was observed in pancreatic cancer cells. Interestingly, we observed a reduction in protein level expression of BAX, a marker for apoptosis, and p27, an inhibitor of the cell cycle. **Conclusion:** We now demonstrate that a PPARα antagonist exerts its anti-growth activity by inducing G_0/G_1 cell cycle arrest, thereby inducing cellular senescence without cell death. These findings provide a mechanism for the anti-tumorigenic activity of PPARα inhibition, and the rationale to use PPARα antagonists as a novel therapeutic approach to pancreatic cancer.

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LIST OF ABBREVIATIONS

AKT/PKB ..... Protein kinase B

BAX .....Bcl-2-associated X protein

CDKN2B..... Cyclin-dependent kinase inhibitor 2B

CoR/SMRT .... Co-repressor/silencing mediator for retinoid and thyroid hormone receptor

DMSO .....Dimethyl sulfoxide

FOLFIIRINOX. .....Folinic acid, 5-fluorouacil, irinotecan, and oxaliplatin

Hsp ..... Heat shock protein

K-ras..... Kristen rat sarcoma

LTB_4..... Leukotriene B_4

mTOR .....Mammalian target of rapamycin

PaIN .....Pancreatic intraepithelial neoplasia

PaINs.....Pancreatic intraepithelial neoplasias

P-AKT/PKB..... Phosphorylated Protein kinase B

PDAC.....Pancreatic ductal adenocarcinoma

PI.....Propidium iodide

PPAR.....Peroxisome proliferator-activated receptor

PPARα .....Peroxisome proliferator-activated receptor-alpha

PPARs.....Peroxisome proliferator-activated receptors

PPRE.....Peroxisome proliferator response element

RXR .....Retinoic X receptor

SA-β-gal.....Senescence associated β-galactosidase

X-gal .....5-bromo-4-chloro-3-indolyl-beta galactopyranoside

INTRODUCTION

Biology and clinical aspects of pancreatic ductal adenocarcinoma

Pancreatic cancer is a highly aggressive disease and is the 4th leading cause of cancer-related deaths in the United States. The one year survival rate is 20 %, and the five year survival rate is 6 % (Howlader *et al.*, 2014). In 2014, in the United States alone, an estimated 45,440 patients were newly diagnosed with pancreatic cancer and approximately 39,590 patients died of the disease. Most patients are between 60 to 70 years of age, and the disease is more common in men than in women. African Americans and Caucasians report the highest prevalence rate of pancreatic cancer compared to all other races (Howlader *et al.*, 2014). The two most reported risk factors for pancreatic cancer are age and cigarette smoking (Li *et al.*, 2004). Other associated risk factors include diabetes, chronic pancreatitis, family history, genetic syndromes, carcinogen exposure, and lifestyle factors such as a high fat diet (Yeo *et al.*, 2002; Li *et al.*, 2004). To date, an effective treatment for pancreatic cancer has yet to be established in the clinic.

The pancreas is located in the abdominal cavity between the stomach and the spine, is approximately 6 inches long, and functions to aid in digestion and to regulate blood glucose levels (Bockman, 1993). The organ is made of an exocrine gland (releasing its products either inside or outside the body) and an endocrine gland (releasing its products directly into the bloodstream). The exocrine gland is comprised of acinar cells and ductal cells. Acinar cells synthesize, store, and secrete digestive enzymes into the pancreatic

duct to aid in breaking down proteins, nucleic acids, carbohydrates, and lipids in food. Ductal cells secrete mucous and bicarbonate to neutralize the acidic gastric chyme. The endocrine gland of the pancreas releases hormones into the blood stream that are important in maintaining glucose homeostasis. Endocrine cells are arranged in clusters called islet of Langerhans. Within each islet are alpha and beta cells that regulate blood glucose levels by releasing insulin and glucagon respectively, and delta cells that produce somatostatin to inhibit the release of pancreatic hormones (Barrett *et al.*, 2010; Costanzo, 2011).

Exocrine tumors account for 95 % of all pancreatic cancer types (Öberg & Eriksson, 2005; Tempero *et al.*, 2010). Pancreatic ductal adenocarcinomas are considered to be aggressive, and tumors are rarely palpable due to the location of the pancreas. Symptoms will often present in later stages when the tumor has spread to neighboring tissues. Approximately 40 % of patients will demonstrate symptoms of distant metastases at the time of diagnosis (Li *et al.*, 2015). Endocrine pancreatic tumors are uncommon, representing less than 5 % of all pancreatic cancer types (Öberg & Eriksson , 2005).

The progression of the pathogenesis of pancreatic ductal adenocarcinoma (PDAC) arises from abnormal cells lining the pancreatic duct (precursor intraductal papillary lesions), which ultimately may progress into invasive PDAC if left untreated (Cubilla & Fitzgerald, 1976; Hruban, Wilentz, & Kern, 2000; Li *et al.*, 2004). Intraductal papillary lesions, also referred to as pancreatic intraepithelial neoplasias (PaINs), can be

subdivided into four groups beginning from low grade PaIN-IA, PaIN-IB, PaIN-2, to high grade PaIN-3. PaIN staging is classified according to the increasing degrees of cellular changes, morphological alterations, and genetic mutation accumulation, as shown in Figure 1 (Bardeesy & DePinho, 2002; Vincent *et al.*, 2011). Normal ductal cells are characterized by a low cuboidal shape and a single cell layer. PaIN-1A is characterized by elongated cells and mucin production. PaIN-B is characterized by papillary architecture. PaIN-2 is characterized by nuclear abnormalities e.g. enlargement, loss of polarity, and crowding. PaIN-3 is characterized by budding into lumen, severe atypia, abnormal mitosis, and is most associated with the development of pancreatic ductal adenocarcinoma (Oliveira-Cunha, Siriwardena, & Byers, 2008).

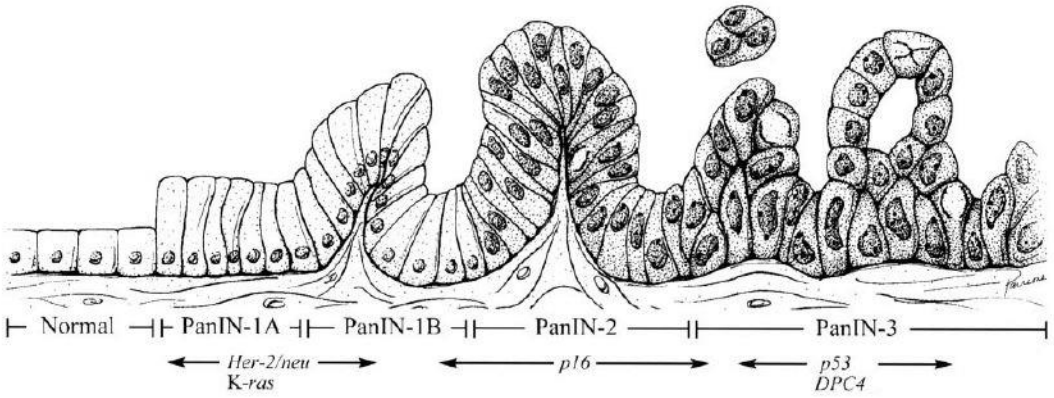


Figure 1 | PaIN progression model.
Histological representation from normal (left) to high grade PaIN-3 (right) is associated with the accumulation of genetic mutations. Figure adapted from Hruban *et al.*, 2000.

The development of PaINs from normal tissue to pancreatic cancer is the result of the overexpression of oncogenes and / or the loss of function of tumor suppressor genes (Li *et al.*, 2004; Sakar, Banerjee, & Li, 2007). Oncogenes are "bad" genes that convert

normal cells into cancerous cells, and are derived from normal genes called proto-oncogenes that have been mutated or altered. The most common oncogene implicated in pancreatic cancer is the K-ras (Kristen rat sarcoma) proto-oncogene, and is considered the "signature" of pancreatic cancer. K-ras mutation is found in 85 % of all pancreatic cancers (Li *et al.*, 2004; Oliveira-Cunha, Siriwardena, & Byers, 2008). Another protooncogene is Her/neu and its genetic mutation is present in 65 to 70 % of pancreatic cancers. Tumor suppressor genes function to slow down cell division and to protect against unabated cell proliferation. Mutation of tumor suppressor genes leads to unregulated cell growth (Yeo *et al.*, 2002). The tumor suppressor gene, p16, is a critical regulator of the cell cycle at G_1/S phase. Approximately 27 to 98 % of pancreatic cancer patients have an inactivated p16 (Sakar, Banerjee, & Li, 2007). Other tumor suppressor genes that have been identified as genes commonly mutated in pancreatic cancer include p53 (40 - 75 %), p19 (27 - 82 %), and CDKN2B (27 - 48 %) (Li *et al.*, 2004).

In early stages of the disease, patients with pancreatic cancer are often asymptomatic (DiMagno, 1999; Li *et al.*, 2015). As the tumor spreads to affect surrounding tissues, common symptoms include muscle weakness (86 %), anorexia (83 %), weight loss (85 %), and abdominal pain (79 %) (Modelell, Guarner, & Malagelada, 1999; Vincent *et al.*, 2011). Jaundice (yellowing of the skin) is the most common sign observed as a result of tumor blockage of the common bile duct (Yeo *et al.*, 2002; Porta *et al.*, 2005). Early clues to identify at-risk patients include chronic pancreatitis, recent onset of diabetes mellitus in patients beyond the 6^{th} decade, intraductal papillary mucinous tumors, and familial

pancreatic cancer (DiMagno, 1999). The diagnosis of pancreatic adenocarcinoma can be confirmed by abdominal CT scans, tissue biopsy, and blood tests (Yeo *et al.*, 2002; Li *et al*., 2015).

Prognosis and long term survival of patients presenting with pancreatic ductal adenocarcinoma varies depending on tumor staging upon initial diagnosis (Snady *et al*., 2000). To date, surgical resection of the tumors remains to be the closest curative treatment (Snady *et al.*, 2000; Fernandez-del Castillo & Michael, 2015). However, only 15 -20 % of patients have resectable tumors while the majority of patients (80 - 85 %) have unresectable or metastatic tumors at the time of diagnosis. The current standard of care for patients with advance pancreatic adenocarcinoma is the chemotherapeutic agent gemcitabine (Li *et al*., 2004; Gresham *et al*., 2014; Vincent *et al.*, 2014). Gemcitabine, an anti-metabolite, prevents normal and tumorigenic cells from growing by specifically inhibiting DNA synthesis (Li *et al*., 2004). Gemcitabine is often used in combination with other chemotherapeutic agents in order to improve patient outcomes (Vincent *et al*., 2011; Gresham *et al*., 2014). For example, in a clinical trial comparing gemcitabine plus nab-paclitaxel (Abraxane) versus gemcitabine alone, the combination resulted in an increased overall survival (8.5 months) compared to gemcitabine alone (6.7 months) (Von Hoff *et al.*, 2013). Other agents that have been studied in combination with gemcitabine include oxaliplatin, capectiabine, cisplatin, and a four-drug regimen including FOLFIIRINOX (folinic acid, 5-fluorouacil, irinotecan, and oxaliplatin). Treatment of FOLFIRINOX and gemcitabine increased overall survival (11.1 months)

compared to gemcitabine alone (6.8 months), however, was associated with increased toxicity (Conroy *et al*., 2011). To date, there are no optimal therapeutic regimens established and despite extensive efforts, targeted therapies for pancreatic cancer have failed. Pancreatic cancer is characterized by a highly fibrotic environment that impedes the delivery of chemotherapy to the tumor cells resulting in chemoresistance (Feig *et al.*, 2012). Therefore, a better understanding of the molecular biology of pancreatic cancer is critical if we are to improve patient survival.

PPAR (Peroxisome Proliferator-Activated Receptor)

In the 1960s, a group in Switzerland observed that ethyl-α-(p-chlorophenoxy) isobutyrate (CPIB or Clofibrate), a known compound with lipid lowering (hypolipidaemic) properties in humans, resulted in the enlargement of the rat liver (hepatomegaly) when administered in the diet. More interestingly, the hepatomegaly was accompanied with the accumulation of what was then described as "microbodies" or peroxisomes (Figure 2). This observation of peroxisome proliferation was further confirmed by using potent hypolipadaemic analogs of clofibrate (Moody & Reddy, 1978). The relationship between the effect of hypolipidaemic drugs and the increase in production of peroxisomes was later confirmed with the discovery of two structurally unrelated compounds to clofibrate possessing hypolipidaemic properties (Reddy & Krishnakantha, 1975). This group of chemicals, capable of inducing massive formation of peroxisomes, became referred to as peroxisome proliferators (Lock, Mitchell, & Elcombe, 1989; Desvergne & Wahli, 1999).

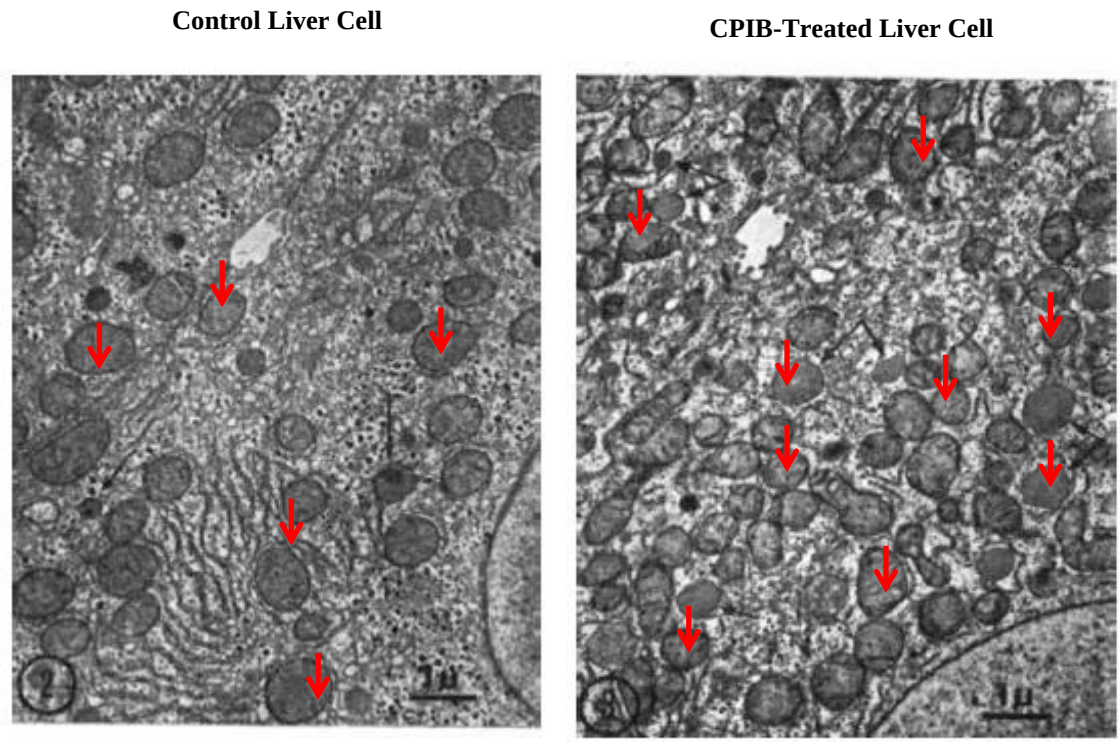


Figure 2 | Peroxisome proliferation in CPIB-treated rat liver cell.
Electron microscopy of a normal liver cell untreated (left) and treated with 500 mg/kg/day of CPIB (right) for 10 days. The liver cell treated with CPIB reveals an increase in the number of peroxisomes (peroxisomes are indicated by the red arrows). Adapted from Hess, Staubli, & Riess, 1965**.**

Peroxisome Proliferation-Activator Receptors (PPARs) were first isolated from mouse cDNA in 1990, and they represent a separate group in the nuclear receptor superfamily (Issemann & Green, 1990). Other groups in the nuclear receptor superfamily include the retinoic acid receptors, thyroid hormone receptors, steroid receptors, and the orphan receptors (Desvergne & Wahli, 1999). Since its discovery in mouse, PPARs have been isolated in multiple species including rats, hamsters, and humans (Gottlicher *et al.*, 1992; Sher *et al.*, 1993; Aperlo *et al.*, 1995). PPARs are a group of ligand-activated transcription factors (Kota, Huang, & Roufogalis, 2005), and play an important role in

lipid metabolism (Whali, Braissant, & Desvergne, 1995). PPARs are most commonly found in tissues with high activity of fatty acid oxidation, but are not just limited to the liver, heart, kidney, brown adipose tissue, muscle and small intestines (Pyper *et al.*, 2010).

The PPAR family in humans consists of three isoforms: PPARα, PPARβ, PPARϒ (Kota, Huang, & Roufogalis, 2005). The PPAR gene consists of five distinguishable domains: A/B, C, D, E, and F, as shown in Figure 3 (Daynes & Jones, 2002). The A/B-domain at the N-terminus contains the activation function-1 (AF-1) region and serves as the independent ligand-binding domain. Phosphorylation at this site regulates the activation of PPAR (Burns & Vanden Heuvel, 2007). Following the A/B-domain is the DNA-binding domain (C-domain) containing two zinc finger motifs capable of inserting itself into DNA at the peroxisome proliferator response element (PPRE) site (Desvergne & Wahli, 1999). The flexible hinge domain (D-domain) connects the DNA-binding domain to the ligand-binding domain (E-domain). The D-domain serves as a docking site for co-repressor proteins in PPAR's unliganded bound state (Kota, Huang, & Roufogalis, 2005; Pyper *et al.*, 2010). The ligand-binding domain (E-domain) is responsible for ligand specificity and receptor dimerization with retinoic X receptor (RXR) (Daynes & Jones, 2002). The ligand dependent activation domain, containing the activation function-2 (AF-2 domain) region, serves to recruit co-activators to assist in gene transcription (Kota, Huang, & Roufogalis, 2005; Pyper *et al.*, 2010). The most conserved regions found in

PPARs are the DNA-binding domain and the ligand-binding domain (Daynes *et al.*, 2002).

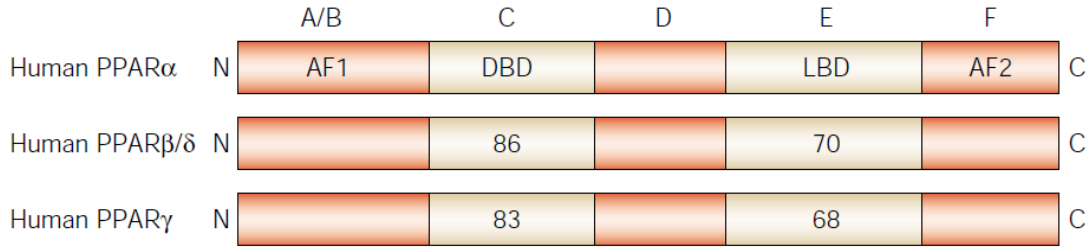


Figure 3: Schematic representation of the functional domains of PPAR.
PPAR isoforms in humans include PPARα, PPARβ, PPARϒ. The A/B-domain at the N-terminus contains the independent activation function-1 region while the F-domain at the C-terminus contains the dependent activation function-2 region. The C-domain contains the DNA-binding domain and is connected to the E-domain containing the ligand-binding domain by the hinge region (D-domain). The percentage represents the percent homology with respect to PPARα. Adapted from Daynes *et al*., 2002.

Receptor activation occurs after the ligand binds to PPAR at the ligand-binding domain (Figure 4). In its unliganded state, PPAR is bound to its co-repressor complex N-CoR/SMRT (co-repressor/silencing mediator for retinoid and thyroid hormone receptor) in the nucleus. Upon ligand-binding, PPAR undergoes a conformational change resulting in the dissociation of its co-repressor. Activation of PPAR allows PPAR to heterodimerize with retinoic X receptor (RXR) forming a PPAR:RXR heterodimer complex. Heterodimerization induces a second conformational change leading to the recruitment of the co-activator-acetyl transferase complex. PPAR:RXR attached to its co-activator complex binds to the peroxisome proliferator response element (PPRE) in DNA. Co-activators, containing histone acetyltransferase activity, alter the chromatin structure by acetylating histone tails thereby regulating the transcription of PPAR target genes

(Daynes & Jones, 2002). About 10% of all human genes have the potential to be directly regulated by PPARs (Heinaniemi *et al.*, 2007).

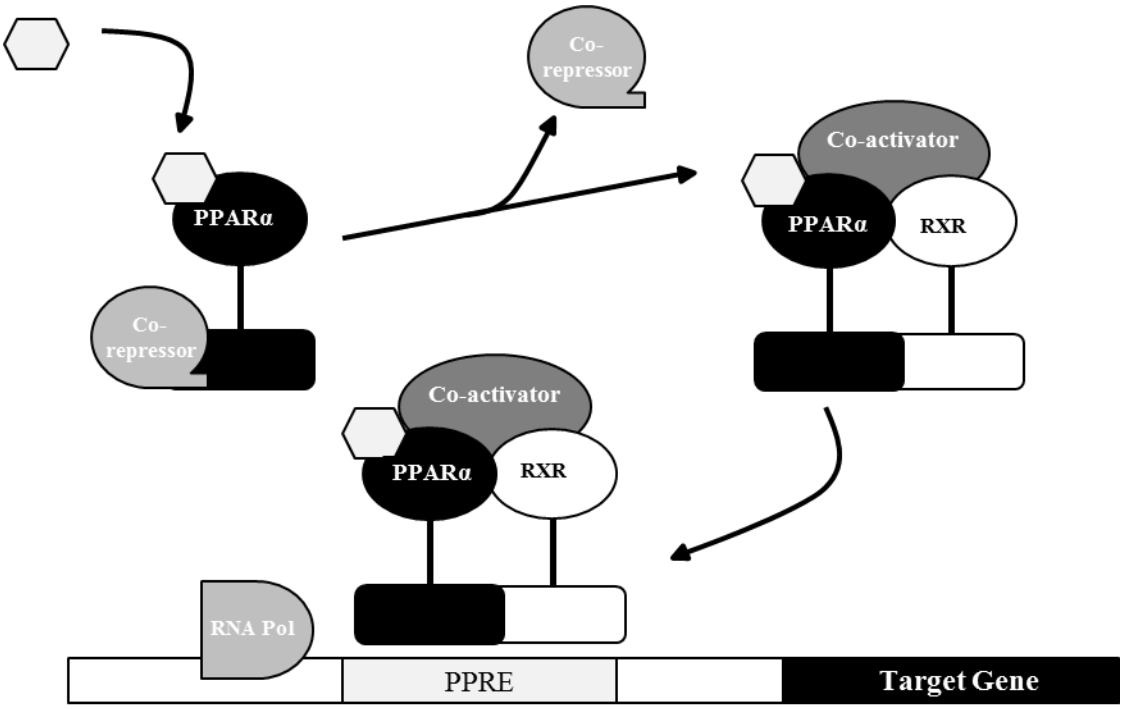


Figure 4: Ligand induced activation of PPAR.
In the unliganded bound state, PPARα is attached to its co-repressor to inhibit gene transcription. PPARα heterodimerizes with RXR after endogenous (fatty acid derivatives) or exogenous (drugs) ligand activation, and recruits co-activators to assist in regulating gene transcription.

Endogenous ligands of PPARα include fatty acids and fatty acid derivatives (Desvergne & Wahli, 1999; Kota, Huang, & Roufogalis, 2005). In ligand binding studies *in vitro*, unsaturated long fatty acyl-CoAs as wells as unsaturated long chain fatty acids both exhibited high PPARα affinity (Hostetler *et al*., 2005). PPARα showed preferential binding to saturated long chain fatty acyl-CoAs, but not to saturated long chain fatty acids (Hostetler *et al*., 2005). PPARα functions as a lipid sensor to maintain lipid

homeostasis. During high fatty acid states, PPARα promotes the metabolism of fatty acids (Reddy, 2004).

Leukotriene B_4 (LTB_4) is an inflammatory mediator produced by inflammatory cells in response to infection or injury (Crooks & Stockley, 1998). Increased levels of LTB_4 promote the recruitment of innate immune cells to the site of infection, and ultimately prolong the inflammatory response duration. LTB_4 activates PPARα through a negative feedback mechanism. At high concentrations, binding of LTB_4 to PPARα stimulates fatty oxidation and LTB_4 breakdown (Devchand *et al.*, 1996).

The fibrate class including fenofibrate, gemfibrozil, clofibrate are exogenous ligands of PPARα (Kota, Huang, & Roufogalis, 2005). By increasing fatty acid metabolism, these compounds have the ability to lower plasma triglyceride levels (Van Raalte *et al.*, 2004). Fibrates are used in the clinic to treat hypercholesterolemia and to prevent metabolic and cardiovascular diseases (Van Raalte *et al*., 2004; Kota, Huang, & Roufogalis, 2005; Pyper *et al.*, 2010).

Inflammation: The link between PPAR and Cancer

A connection between PPARs and cancer was first observed in 1975 after chronic administration of nafenopin, a potent PPARα agonist, caused hepatocellular carcinoma in mice (Reddy, Rao, & Moody, 1976). In 2002, it was demonstrated that PPARα knockout

mice resulted in resistance to ligand-activated tumor growth suggesting that PPARα plays an important role in cancer progression (Gonzalez, 2002).

Interactions between tumor cells and non-malignant cells in the tissue stroma can feed into cell autonomous as well as non-cell autonomous pathways that can contribute to tumor growth (De Visser, Eichten, & Coussens, 2006; Chu *et al.*, 2007). Inflammation is associated with pancreatic cancer, as pancreatitis is a known contributing factor for the development of pancreatic tumors (Guerra *et al.*, 2007; Hagemann, Balkwill, & Lawrence, 2007). Pancreatic cancer can be characterized by a highly fibrotic tumor stroma formation consisting of non-neoplastic fibroblastic, vascular, and inflammatory cells, as well as other cell types that surround and interact with tumor cells (Chu *et al*., 2007). The cells in the microenvironment can promote inflammation by releasing pro-inflammatory cytokines, pro-angiogenic, and pro-lymphangiogenic growth factors (De Visser, Eichten, & Coussens, 2006; Hagemann, Balkwill, & Lawrence, 2007). Although inflammation has been demonstrated to exhibit pro-tumorigenic activity, it has recently been shown that inflammation can initiate the development of pancreatic tumors (Guerra *et al*., 2007). Embryonic mice with K-Ras oncogene mutation in acinar cells develop PanINs that progress into PDAC. However, adult mice with the same K-Ras oncogene mutation will not develop pancreatic tumors unless mild pancreatitis is induced (Guerra *et al*., 2007). Therefore, targeting inflammation in the tumor microenvironment may be a novel approach to prevent pancreatic cancer.

PPARα is a known negative regulator of inflammation and is necessary for unabated tumor growth (Devchand *et al.*, 1996; Kaipainen *et al.*, 2007). When PPARα negative tumors are injected into wildtype mice, a 41 % tumor growth inhibition is observed, and more dramatically, if PPARα is absent in both the tumor and the host, a 97 % tumor growth inhibition is observed, as shown in Figure 5 (Kaipainen *et al.*, 2007). These results suggest that PPARα plays a key role in providing a favorable microenvironment for tumors to grow. Thus, PPARα antagonists could be novel therapy for pancreatic cancer.

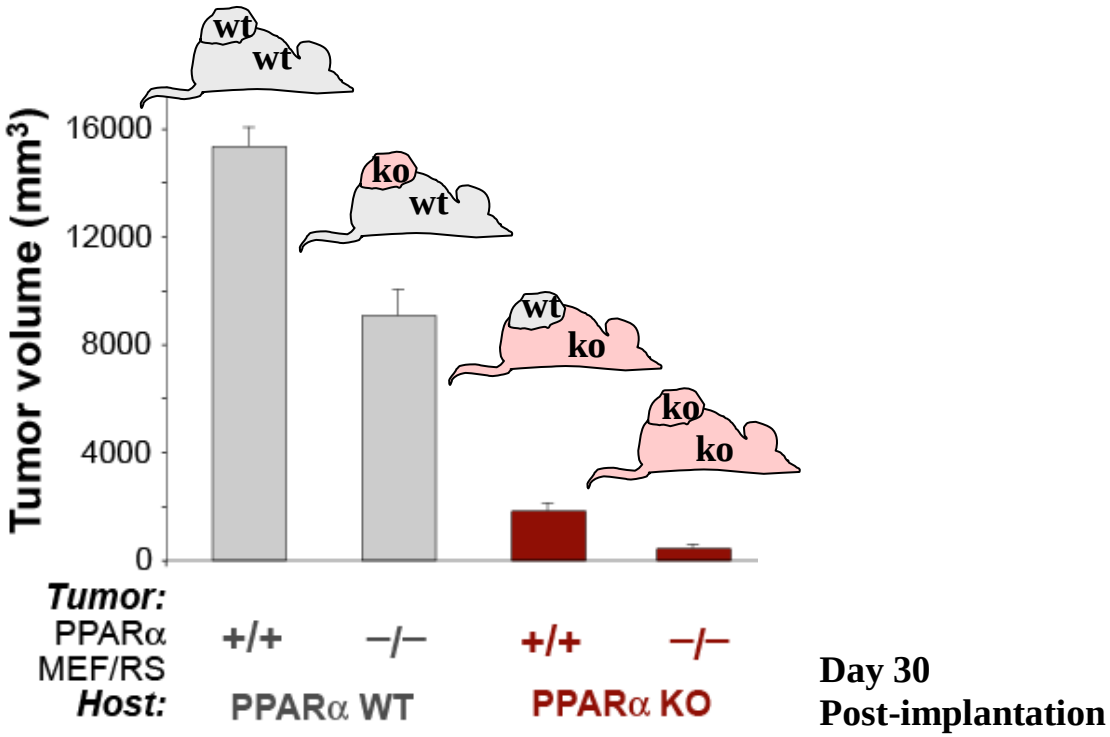


Figure 5 | Tumor growth is inhibited in absence of PPARα.
Wildtype or PPARα negative tumors were subcutaneously injected into PPARα wildtype or deficient mice. PPARα negative tumors were created from isolating mouse embryonic fibroblast (MEF) from a PPARα knockout mouse and transformed with SV40 large T antigen and H-ras to obtain an isogenic tumorigenic cell line.

Specific Aim and Objectives

Pancreatic ductal adenocarcinoma is a highly aggressive tumor, and is resistant to current chemotherapy and radiation treatments (Farrow, Albo, & Berger, 2008; Feig *et al.*, 2012). Molecular targeted therapy provides an opportunity to customize cancer treatments in order to effectively treat patients and improve clinical outcomes. Peroxisome proliferator-activated receptor (PPAR), a ligand-activated transcription factor, is a negative regulator of inflammation (Devchand *et al.,* 1996). Genetic knockout of PPARα in mice resulted in suppression of tumor growth (Kaipainen *et al.,* 2007). We hypothesized that blocking the PPARα pathway with a small molecule PPARα antagonist, NXT, may inhibit pancreatic cancer by targeting tumor cells as well as non-neoplastic cells in the tumor microenvironment.

The present study addresses three main goals:

1. To investigate the anti-growth activity of a PPARα antagonist
2. To characterize the activity of PPARα inhibition on the cell cycle
3. To elucidate the molecular mechanism by which a PPARα antagonist inhibits cell growth

Our study aims to provide a potential mechanism for cell growth inhibition mediated by PPARα. This study will present evidence for the potential use of PPARα antagonists as a novel, therapeutic approach to complement current therapeutic regimens in the treatment of pancreatic cancer.

METHODS

Cell Lines

Human pancreatic adenocarcinoma cell line, BxPC3, was obtained from American Type Culture Collection (ATCC Manassas, VA), and maintained in Roswell Park Memorial Institute-1640 medium supplemented with 10 % fetal bovine serum (Life Technologies Grand Island, NY) and 200 mM L-glutamine, 10,000 units penicillin, and 10 mg/mL streptomycin (Sigma-Aldrich St Louis, MI) at 37 ºC and 10 % CO_2.

Murine pancreatic adenocarcinoma cell line, Panc0H7, was obtained from Diane Bielenberg Lab (Children's Hosptial, Boston, MA) and maintained in Dulbecco's Modified Eagle Medium (Life Technologies Grand Island, NY) supplemented with 10 % fetal bovine serum and 200 mM L-glutamine, 10,000 units penicillin, and 10 mg/mL streptomycin at 37 ºC and 10 % CO_2.

Murine leukaemic monocyte macrophage cell line, RAW 264.7, was obtained from American Type Culture Collection and maintained in Dulbecco's Modified Eagle's Medium (DMEM, ATCC 30-2002) (ATCC, Manassas, VA) supplemented with 10 % fetal bovine serum and 200 mM L-glutamine, 10,000 units penicillin, and 10 mg/mL streptomycin at 37 ºC and 5 % CO_2

Observation of cell growth

Cells (RAW 264.7, Panc0H7, and BxPC3) were plated in 10 cm cell culture plates (1 x 10^6 cells/plate) at room temperature. A day later, cells were treated with the PPARα antagonist (30 μM) or equal volume of DMSO (control). Differences in cell density in PPARα-treated cells were compared to control at 24 and 48 h. Images were captured under a bright-field compound microscope at a 10X magnification.

Preparation of cytosolic and nuclear protein extracts for Western blot analysis

Cells (BxPC3, Panc0H7, RAW 264.7) were plated in 6 wells cell culture plates (300,000 – 500,000 cells/well) at room temperature. A day later cells were treated with the PPARα antagonist (15, 30 μM) or equal volume of DMSO for 48 h. Cell lysates were obtained by washing cells with cold Dulbecco's phosphate buffered saline (Sigma-Aldrich St Louis, MI), and then lysed with cell lytic reagent (Sigma-Aldrich St Louis, MI) containing protease inhibitor (Roche South San Francisco, CA) and phosphatase inhibitor (Roche South San Francisco, CA) over ice. Cells were placed on Rocker I platform (Boekel Feasterville-Trevose, PA) for five minutes at 4 ºC. Cells were scraped off cell culture plate over ice using a cell scrapper. Cells plus lysis buffer was collected in 1.5ml Eppendorf microcentrifuge tube. Cells plus lysis buffer was centrifuged at 13.2 RPM for 5 minutes in 4 ºC. Supernatant (lysate) was collected over ice and placed in new 1.5 ml Eppendorf microcentrifuge tube. Protein concentration in the supernatant was determined by the Bradford method using Bio-Rad Protein Assay reagent (BIO-RAD Herculues, CA)

and bovine serum albumin (Cell Signaling Danvers, MA) (Zor et al., 1996). Samples were diluted with 6X reducing SDS sample buffer (Boston BioProducts Inc, Boston, MA). Samples were boiled for 5 minutes at 95 ºC, cooled over ice for 5 minutes, centrifuged at 13.2 RPM for 30 seconds, vortexed gently, and loaded into 10 - 12 % NuPage tris-acetate gels (Life Technologies Grand Island, NY., USA). Samples were allowed to migrate and separate for 1 h at 150 V, unlimited mAMPs until dye front reaches the bottom of the gel. Page ruler plus pre-stained protein ladder (BIO-RAD Herculues, CA) was used as reference molecular weight. Proteins were transferred onto a supported nitrocellulose membrane (BIO-RAD Herculues, CA) using semi-dry transfer unit (Hoefer Holliston, MA) at 15 V, unlimited mAmps for 1 - 2 h. After transfer was completed, membrane was stained with ponceau red (Sigma-Aldrich St Louis, MI) and transfer issues (bubbles, uneven loading) were noted. Ponceau red was washed away with distilled deionized water for 5 minutes on rocker platform (VWR Radnor, PA). Membrane was blocked with 5 % BSA in tris-buffered saline and 0.2 % tween 20 and incubated with primary antibodies overnight on red rotor (Hoefer Holliston, MA) at 4°C. Gels were washed with in tris-buffered saline and 0.2 % tween 20 on rocker I platform for 5 minutes, repeating the process four more times for a total of five washes, and incubated with the horseradish peroxidase-conjugated secondary antibodies for 1 hour at room temperature on rocker I platform. Membranes were washed with in tris-buffered saline and 0.2 % tween 20 for 5 minutes, repeating the process four more times for a total of five washes. Signal was detected by enhanced chemiluminescence using supersignal west pico chemoluminescence substrate (LifeTechnologies Grand Island, NY) and

supersignal west femto chemiluminescent substrate (LifeTechnologies Grand Island, NY). The signal was detected on an autoradiography film 8 x 10 inches (MIDSCI St. Louis, MI). For re-probing, membrane was stripped by restore western blot stripping buffer (Thermo Scientific Waltham, MA) according to manufacturer's instructions.

Cell Cycle Analysis

Cells were plated in 10 cm cell culture plate (1 x 10^6 cells/well) at room temperature. A day later, cells were treated with the PPARα antagonist (30 μM) or equal volume of DMSO for 24 and 48 h. Cell cycle analysis was performed utilizing BD CycleTest plus DNA reagent kit (BD Biosciences San Jose, CA) according to manufacturer's instruction using propidium iodide and processed for flow cytometry using BD LSR II flow cytometry machine (BD Biosciences San Jose, CA). Cell cycle profiles were obtained in ModFit LT.

Cell Viability Assay

Cells were divided into two groups: Washout group and No Washout group. The Washout group was divided into 3 subgroups (Control, PPARα antagonist, and Release) with two treatment periods and a washout procedure in between. The No Washout group was divided into 2 subgroups (Control and PPARα antagonist) with one treatment period and no washout. Cells were plated in 6 wells cell culture plates (50,000 cells/well) at

room temperature. A day later, cells were administered the first treatment for 24 h. In the Washout group, cells in the PPARα group received 30 μM of the PPARα antagonist and control group received equal volume of DMSO. Cells in the Release group received 30 μM of the PPARα antagonist. In the No Washout group, cells received 30 μM of the PPARα antagonist or equal volume DMSO. After 24 hour incubation, all cells in the Washout group were washed three times with phosphate buffered solution and administered the second treatment for 48 h. Control cells and PPARα antagonist-treated cells received equal volume of DMSO or 30 μM of the PPARα antagonist respectively. The Release group was administered equal volume of DMSO. Control and PPARα antagonist cells in the No Washout group remained in their respective treatments for the remaining 48 h of the experiment for a total of 72 h. Shortly at the end of the second treatment, adherent and floating cells in each subgroup in the Washout and No Washout groups were collected separately into one single cell suspension.

The viability of cells was assessed by Trypan blue exclusion assay (Life Technologies, Grand Island, NY) (Strober, 2001). Cells were diluted 1:1 using Trypan Blue and 10 μl of the sample was loaded onto a counting slide. The number of viable cells and the percent viability was determined using TC 20 automated cell counter (BIO-RAD Herculues, CA).

Senescence Assay

Cells were plated on lab-trek chamber slides (15,000 cells/well) (Thermo Scientific Waltham, MA). A day later, cells were treated with the PPARα antagonist (30 μM) for 48 h. Cells were stained for senescence-associated β-galactosidase activity utilizing senescence β-galactosidase staining kit according to manufacturer's instructions (Cell Signaling Technologies Danvers, MA).

Statistical Analysis

Statistical Analyses were performed by Student's *t*-test. The results were considered statistically significant at $p < 0.05$.

Antibodies for Western Blot

Specificity	Species/isotype	Concentration	Company
AKT	Rabbit	1:1000	Cell Signaling
Anti-Mouse	Sheep	1:1000	GE Healthcare
Anti-Mouse	Horse	1:1000	Cell Signaling
Anti-Rabbit	Donkey	1:1000	GE Healthcare
Anti-Rabbit	Goat	1:1000	Cell Signaling
BAX	Rabbit	1:1000	Cell Signaling
P27 Kip	Rabbit	1:1000	Cell Signaling
P-AKT (Ser473)	Rabbit	1:1000	Cell Signaling
PCNA	Mouse	1:1000	Cell Signaling

Software Programs

Image J	National Institutes of Health
ModFit LT	Verity Software House
Prism	GraphPad Software

RESULTS

PPARα inhibition is associated with the reduction of cell density in macrophages and tumor cells.

To determine whether the PPARα antagonist reduced cell growth, differences in cell density were observed in macrophages and tumor cells treated with the PPARα antagonist or control for 24 or 48 h. The PPARα antagonist reduced cell density in macrophages (RAW 264.7), and pancreatic tumor cells (Panc0H7 and BxPC3) as early as 24 h after treatment (Figure 6, 7, & 8). PPARα antagonist-treated cells were less dense at 48 h after treatment, as observed by eye using a light microscope (Figure 6, 7, & 8). These results suggest that inhibition of PPARα leads to a reduction of cells after 48 h treatment.

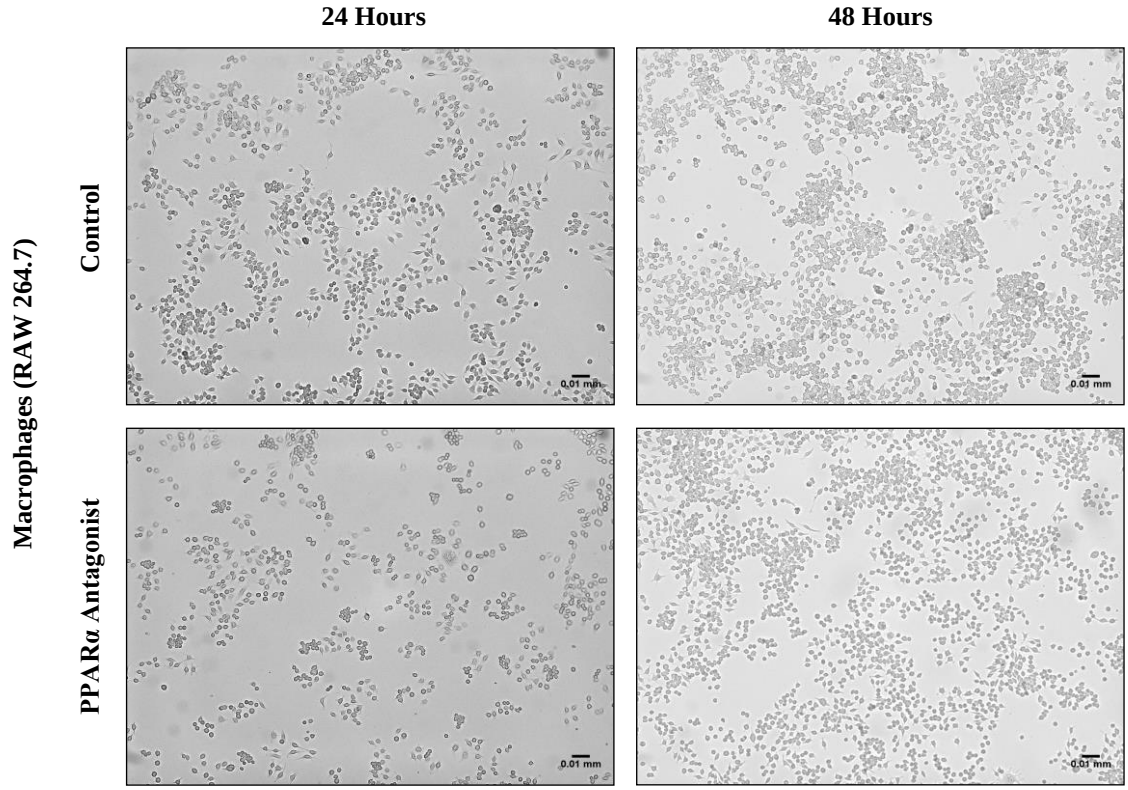


Figure 6 | PPARα antagonist reduces cell growth in macrophages at 24 and 48 h.
Macrophages (RAW 264.7) were plated in a 10 cm cell culture plate (1 x 10^6 cells/well) and treated with the PPARα antagonist (30 μM) or control for 24 or 48 h. Images were captured under a bright field compound microscope (10X).

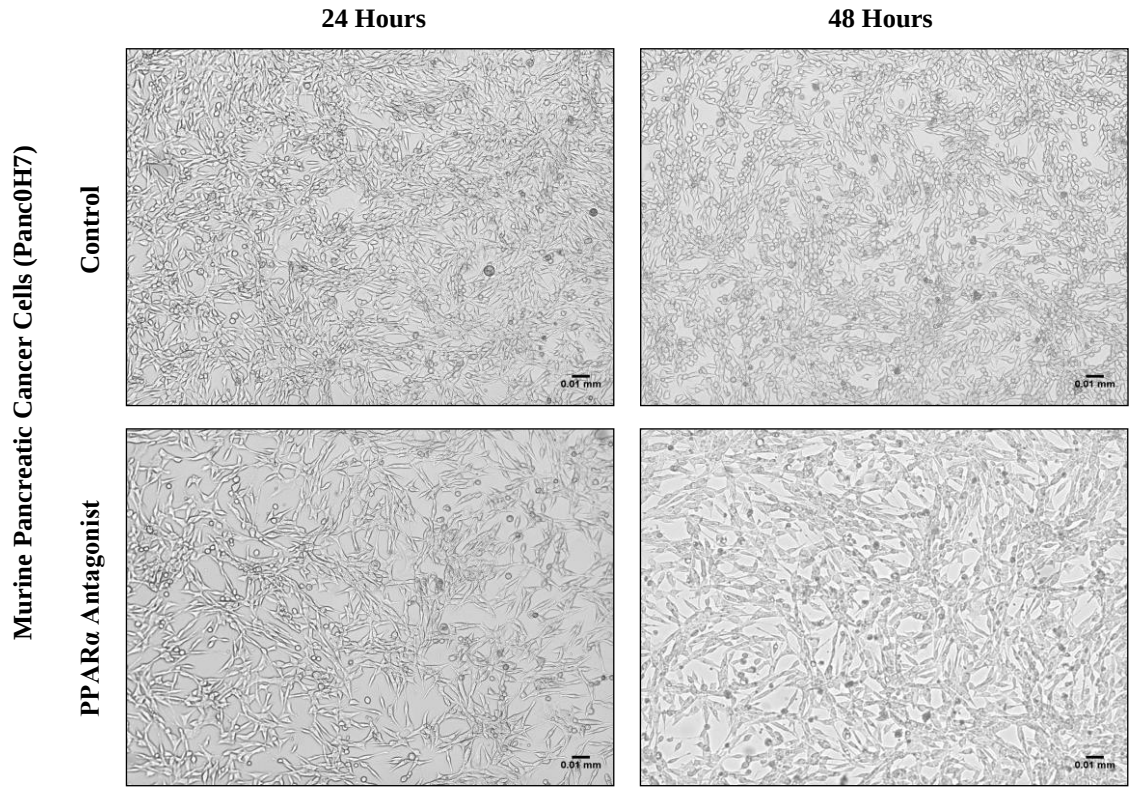


Figure 7 | PPARα antagonist reduces cell growth in murine pancreatic tumor cells at 24 & 48 h.
Murine pancreatic cancer cells (Panc0H7) were plated in 10 cm plate (1 x 10^6 cells/well) and treated with the PPARα antagonist (30 μM) or control for 48 h. Images were captured under a bright-field compound microscope (10X).

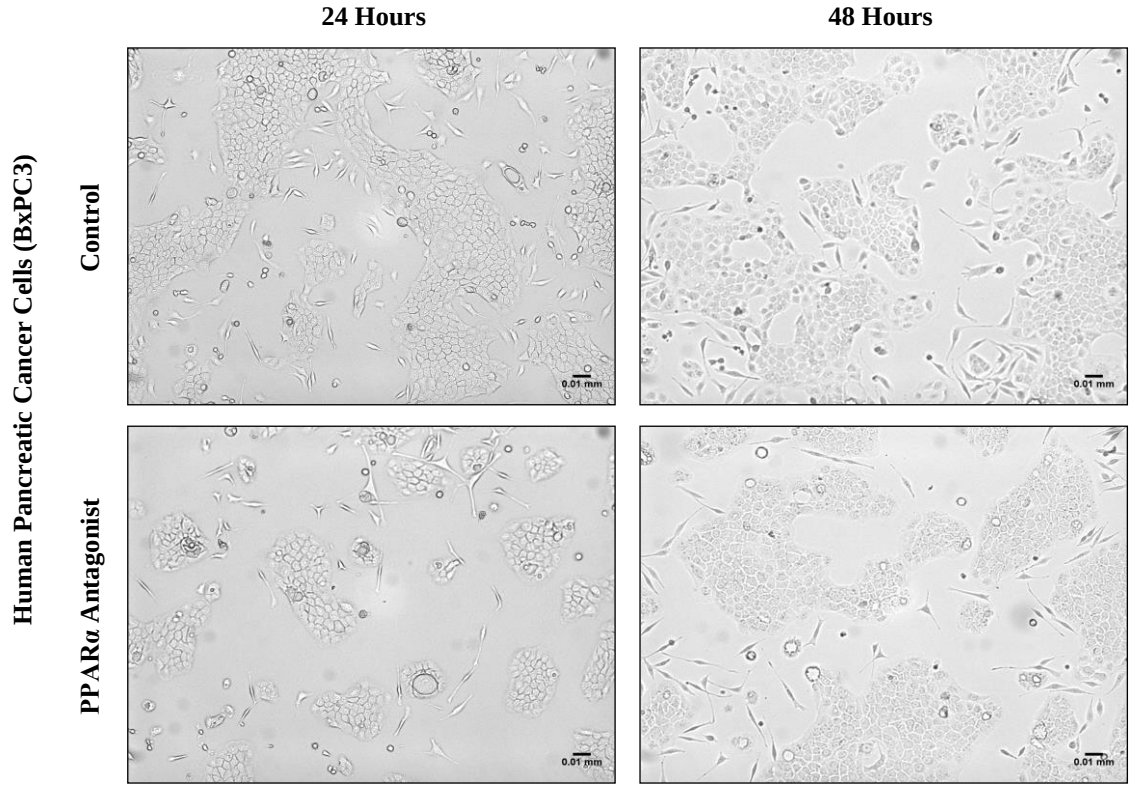


Figure 8 | PPARα antagonist reduces cell growth in human pancreatic tumor cells at 24 & 48 h.
Human pancreatic cancer cells (BxPC3) were plated in 10 cm plate (1 x 10^6 cells/well) and treated with the PPARα antagonist or control for 48 h. Images were captured under a bright-field compound microscope (10X).

Treatment with the PPARα antagonist is non-cytotoxic to tumor cells.

To examine whether the PPARα antagonist exhibited toxicity, cell viability was determined by Trypan blue exclusion assay. More specifically tumor cells were treated with the PPAR antagonist for 24 or 72 h (Figure 9).

The Trypan blue exclusion assay is used to determine the number of live cells and dead cells in a cell suspension. Live cells have intact membranes that will pump out cationic dyes such as trypan blue, while dead cells will uptake the dye and become permanently stained. Live cells or viable cells will have a clear cytoplasm and will appear white, whereas non-viable cells or dead cells will have a blue cytoplasm and will appear blue (Srober, 2001). The percent viability of the cell suspension is calculated by dividing the number of viable cells (white) by the total number of cells (white + blue). The fraction is multiplied by 100 to obtain the percent viability. The percent cell death can then be calculated by subtracting 100 from the percent viability.

Figure 10 shows the number of viable cells and percent cell death in each group. In the "washout group", the number of viable cells was highest in the control ($380 \times 10^4 \pm 15 \times 10^4$ cells/ml), lowest in the PPARα antagonist group ($94 \times 10^4 \pm 6 \times 10^4$ cells/ml), and in the release group, the number viable cells was between either groups ($276 \times 10^4 \pm 11.7 \times 10^4$ cells/ml). The percent death in the control, PPARα antagonist group, and release group were 1.5 ± 0.2 %, 1.16 ± 0.2 %, and 1.5 ± 0.3 % respectively. These results suggest

that when the PPARα antagonist is removed from the growth media, tumor cells are able to resume growth suggesting that the PPARα antagonist is not killing the cells.

In order to confirm the non-cytotoxic effect of the PPARα antagonist is not due to the washout itself, in which cells were washed with phosphate buffered saline three times to clear any residual PPARα antagonist found in the cell media, and thus removing floating dead cells, the number of viable cells and the percent death was obtained in the No washout group. In the No washout group, no washes are carried out throughout a 72 h treatment with the PPARα antagonist. The PPARα antagonist reduced the number of viable cells in the treated group ($135 \times 10^4 \pm 11 \times 10^4$) compared to vehicle ($343 \times 10^4 \pm 12 \times 10^4$ cells/ml). The percentage of cell death in the control and the treated group were 2 ± 0.4 %, 1.7 ± 0.6 % respectively. We confirmed that the growth inhibitory activity of the PPARα antagonist is not due to the result of the "washout."

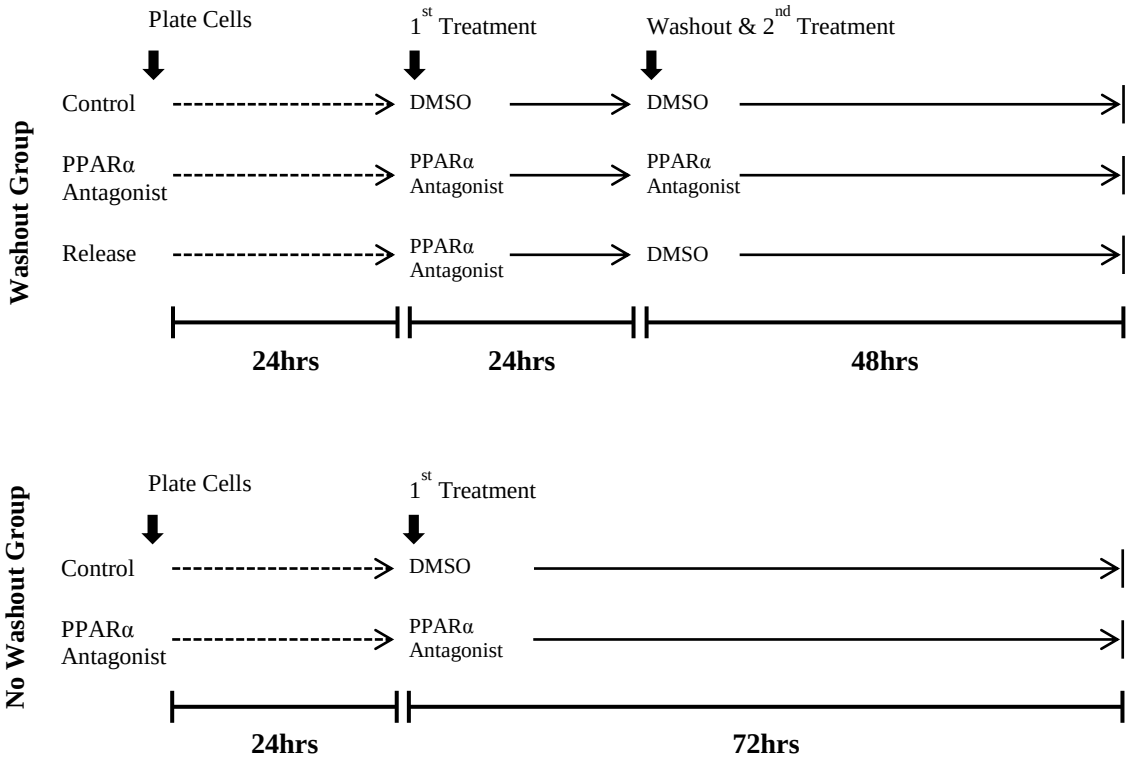


Figure 9 | Schematic representation to determine cytotoxicity of a PPARα antagonist.
Timeline for the experimental procedure of the Washout and the No Washout groups.

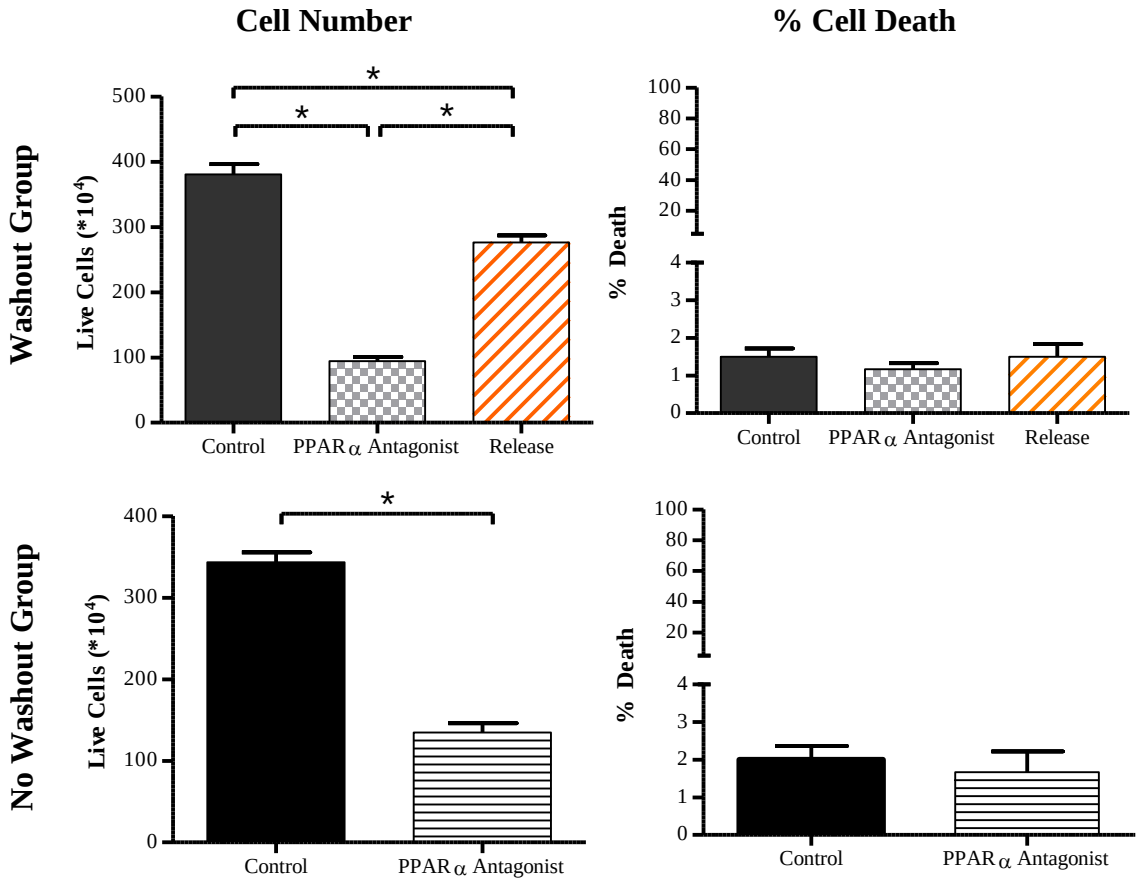


Figure 10 | PPARα antagonist is not cytotoxic to tumor cells.
Tumor cells were plated at in 6 wells cell culture plate (50,000 cells/well) and treated with the PPARα antagonist or control according to experimental design. Cytotoxicity was assessed by trypan blue exclusion assay. The data shown are representative of two independent experiments. The results are means +/- SEM (n=6); *p< 0.05

Antagonizing PPARα is associated with the reduction of P-AKT, AKT, BAX, PCNA in macrophages and tumor cells.

To determine whether the PPARα antagonist regulates cell survival (P-AKT & AKT), apoptosis (BAX), and cell proliferation (PCNA), western blot analysis was performed on macrophages and pancreatic cancer cells treated with the PPARα antagonist or control for 48 h.

AKT, also called PKB, is an important signaling protein and is a marker for cell survival (Manning & Cantley, 2007; Pickhard *et al.*, 2014). Activation of AKT requires the phosphorylation within the catalytic domain (Thr308) and within the hydrophobic motif (Ser473) (Tolker & Marmiroli, 2014). AKT exerts its activity by phosphorylating other downstream molecules, including mTOR (mammalian target of rapamycin) which is found to promote cell survival (Guertin *et al.*, 2007). Treatment with the PPARα antagonist was associated with the reduction in phosphorylation of AKT as wells as the total levels of AKT in macrophages as well as in tumor cells, as shown in Figure 11. These results demonstrate that the PPARα antagonist may have a role in mediating the activation of the AKT signaling pathway, a marker for cell survival.

Bax is a pro-apoptotic protein activated in the mitochondria extrinsic apoptotic pathway in response to cellular stress (Dewson & Kluck, 2009). Apoptosis is a process of programmed cell death, and naturally occurs in a number of cellular physiological

conditions such as in tissue remodeling and early embryogenesis (Ker, Wyllie, & Currie, 1972; Penaloza *et al*., 2006). At the onset of apoptosis, BAX translocates from the cytosol to the mitochondrial outer membrane to increase the membrane's permeability. The leakage of a pro-apoptotic molecule, cytochrome c, into the cytosol initiates caspase activation, which mediates apoptosis (Jeong & Seol, 2008). Cancer cells downregulate BAX to evade apoptosis. Surprisingly, treatment with the PPARα antagonist was associated with a reduction in BAX levels in macrophages and tumor cells (Figure. 11). The strongest reduction was observed at 30 μM of the PPARα antagonist in all three macrophage and tumor cell lines. These results suggest PPARα antagonism may play a role in the modulation of BAX expression, a marker for apoptosis.

PCNA (Proliferating cell nuclear antigen) is a DNA replication accessory protein and is widely used as a marker of cell proliferation (Stoimevnov & Helleday, 2009; Wang, 2014). PCNA acts as a sliding clamp forming a ring around DNA, and serves as a scaffold for specialized proteins involved in DNA replication (Stomevnov & Helleday, 2009). Treatment with the PPARα antagonist reduced PCNA levels in RAW 264.7, Panc0H7 and BxPC3 cell lines (Figure. 11). The most potent reduction was observed at the 30 μM of the PPARα antagonist in both macrophages and tumor cells. These results suggest that the PPARα antagonist mediates the expression of PCNA, a marker for cell proliferation.

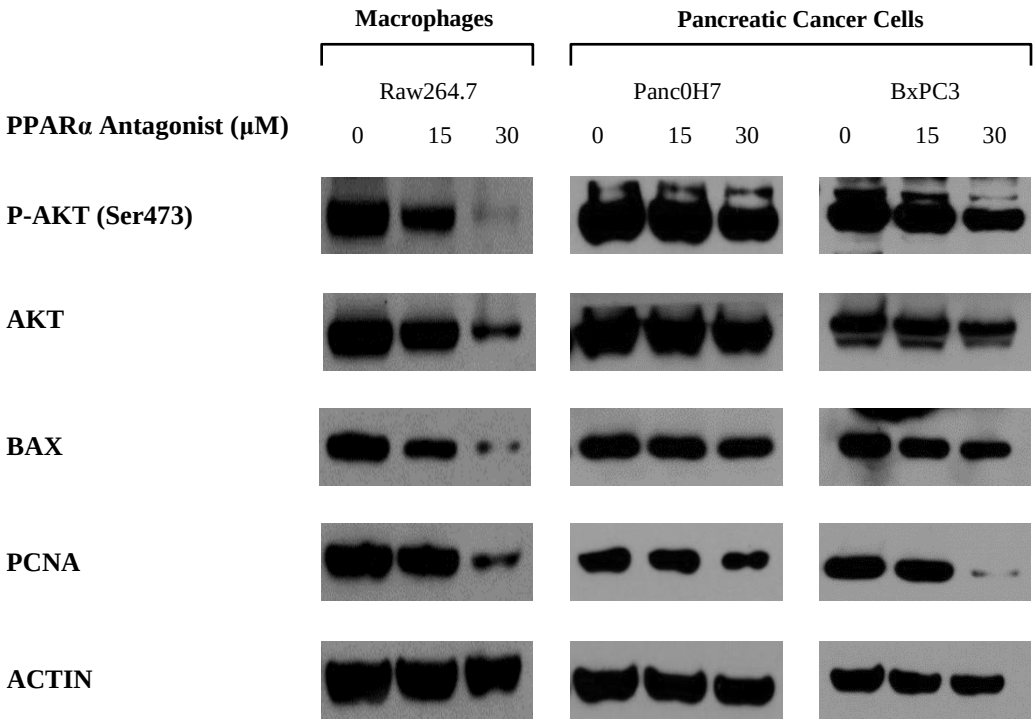


Figure 11 | PPARα inhibition reduces P-AKT, AKT, BAX, & PCNA expression in macrophages and tumor cells. Western blot analysis of protein lysate obtained from macrophages and tumor cells treated with the PPPARα antagonist or control for 48 h at the indicated concentration (15, 30 μM). Results are representative of at least three independent experiments with similar results. Quantifications are provided in Supplemental Figure 3, 4, & 5.

PPARα antagonist induces G_0/G_1cell cycle growth arrest in macrophages and tumor cells.

To determine the role of PPARα on the cell cycle, flow cytometry was performed on macrophages (RAW264.7) and tumor cells (BxPC3) treated with the PPARα antagonist for 24 and 48 h, and 24 h, respectively. Cells were stained with propidium iodide (PI) and the cell cycle profiles were obtained by using fluorescence-activated cell sorting (Figure 12 & 13).

PI is a fluorochrome DNA binding dye. The amount of DNA can be determined by using a flow cytometer, in which a laser detects the stained cells, excites the dye, which then emits light. The emitted light is proportional to the amount of DNA in cells. The cell cycle consists of several stages: G_0/G_1, S, G_2, and M. G_0/G_1 is an intermediate stage between the end of the last cell division and the beginning of DNA replication. S-phase is the phase where DNA is replicated in order to allow cells to undergo division. G_2 and M is characterized as a growth and mitotic phase. Cells in G_2 have twice as much DNA and will proceed to divide during mitosis. At the end of G_2/M, two daughter cells emerge (Luttmann, 2006).

The cell cycle profile is obtained to determine the fraction of cells in the various stages of the cell cycle. The X-axis, "Channels (PI-A)" corresponds to the strength of the fluorescence signal, which is the intensity of the dye. In our analysis, we set the G_0/G_1 to

be at 50 fluorescence intensity (relative to a non-fluorescence cell). Because amount of PI binding is proportional to DNA content, we set G_2/M to be at 100 fluorescence intensity. The S-phase corresponds to the intensity between G_0/G_1 and G_2/M. An algorithm is employed by the analysis software to unbiasedly account for the overlap that occurs between the phases. The Y-axis corresponds to the number of cells in G_0/G_1, S, or G_2/M. We set the flow cytometer to record 10,000 cells or events in each sample. Following the acquisition of the cell cycle profile, the data can be analyzed in a histogram where the X-axis represents the phases of the cell cycle, and the Y-axis represents the percentage of events recorded in G_0/G_1, S, or G_2/M.

The fraction of cells in G_0/G_1 is increased in the PPARα antagonist-treated tumor cells and macrophages at 24 h and was accompanied with a decrease in the fraction of cells in S and G_2/M, as shown in Figure 12 and 13. The fraction of cells in G_0/G_1 is increased at 48 h compared to 24 h (63.8 ± 0.1 % to 75.1 % ± 0. 6%) in macrophages treated with the PPARα antagonist, (Figure 13 & 14). Therefore, antagonizing PPARα in macrophages (RAW 264.7) and tumor cells (BxPC3) resulted in cell cycle arrest at G_0/G_1.

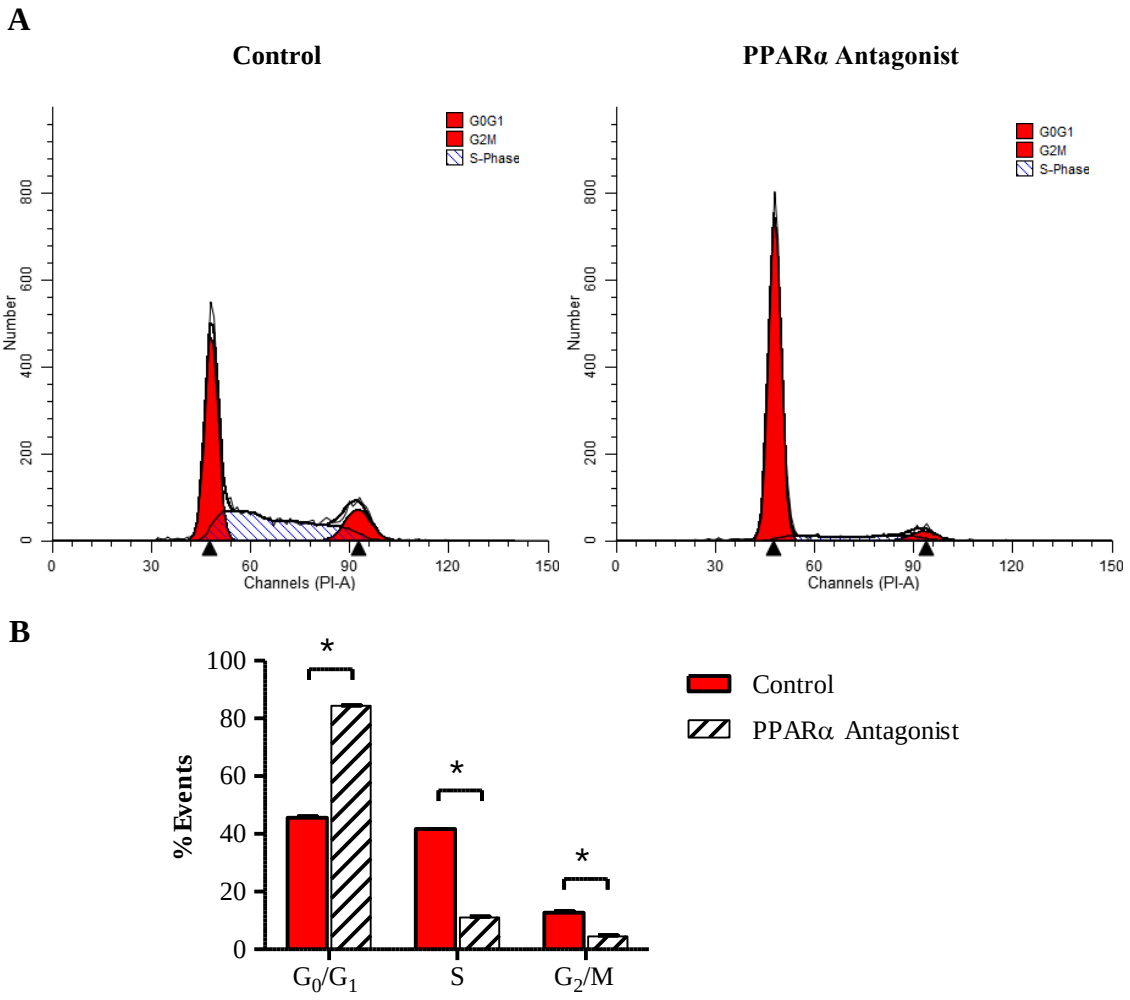


Figure 12 | PPARα antagonist induces cell cycle arrest at G_0/G_1 in pancreatic tumor cells at 24 h.
(A) Cell Cycle analysis by flow cytometry in BxPC3 cells treated with the PPARα antagonist (30 μM) or control for 24 h. The X-axis corresponds to the fluorescence intensity at each channel, and the Y-axis corresponds to number of events at each channel. (B) Histogram showing the percentage of cells in each phase of the cell cycle. The results are means +/- SEM (n=2); *p< 0.05

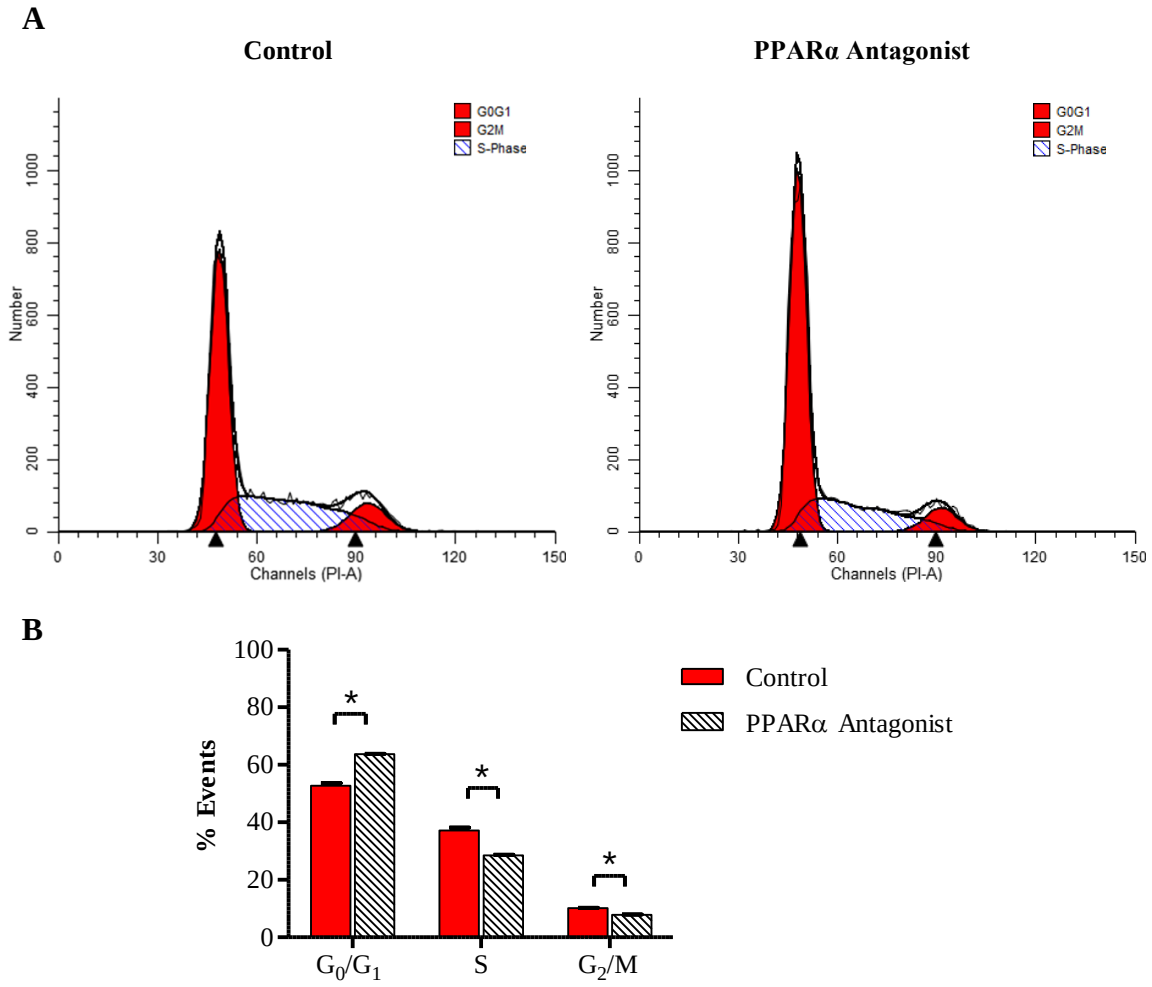


Figure 13 | PPARα antagonist induces cell cycle arrest at G_0/G_1 in macrophages cells at 24 h.
(A) Cell Cycle analysis by flow cytometry in RAW 264.7 cells treated with the PPARα antagonist (30 μM) or control for 24 h. The X-axis corresponds to the fluorescence intensity at each channel, and the Y-axis corresponds to number of events at each channel. (B) Histogram showing the percentage of cells in each phase of the cell cycle. The results are means +/- SEM (n=2); *p< 0.05

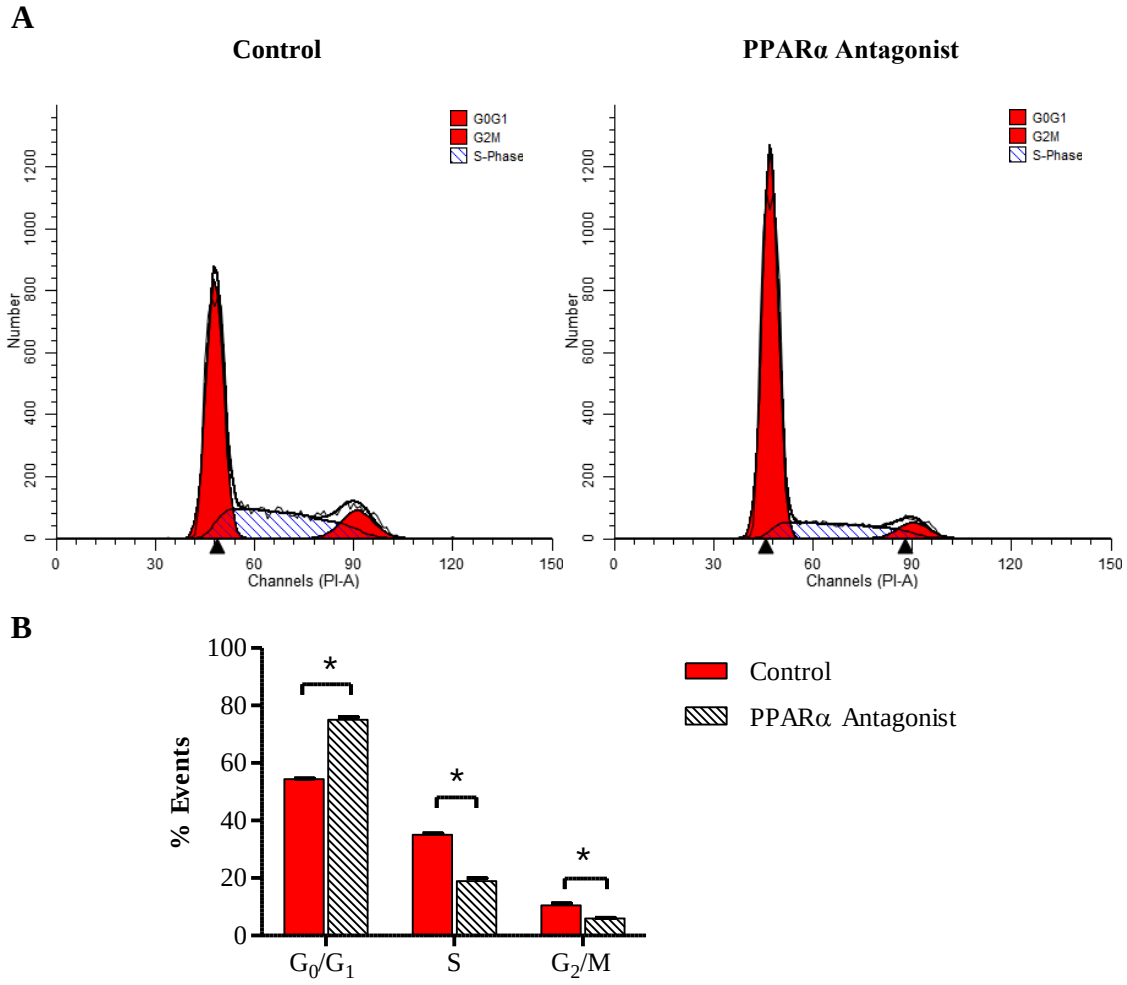


Figure 14 | PPARα antagonist induces cell cycle arrest at G_0/G_1 in macrophages at 48 h.
(A) Cell Cycle analysis by flow cytometry in RAW 264.7 cells treated with the PPARα antagonist (30 μM) or control for 48 h. The X-axis corresponds to the fluorescence intensity at each channel, and the Y-axis corresponds to number of events at each channel. (B) Histogram showing the percentage of cells in each phase of the cell cycle. The results are means +/- SEM (n=2); *p< 0.05

Antagonizing PPARα associated with reduction p27 in tumor cells.

To determine whether the PPARα antagonist modulates p27 protein expression, the expression of p27 was determined by Western blot analysis in tumor cells treated with the PPARα antagonist (15, 30 μM) or equal volume of DMSO for 48 h.

p27 (KIP1) is a cyclin-dependent kinase inhibitor of the CDK2/cyclin E complex. Inhibition of CDK2/cyclin E prevents cell cycle progression, thus p27 acts as a negative regulator of the cell cycle at the G_1/S phase (Bretones, Delgado, & Leon, 2014). In cancer, p27 is downregulated or impaired (Sgambato *et al.* 2000). Surprisingly, treatment of pancreatic cancer cells (Panc0H7) with the PPARα antagonist was associated with the reduction of p27 levels (Figure 15). The most potent reduction in p27 expression was observed at 30 μM of the PPARα antagonist. Therefore, inhibition of PPARα may play a role in regulating p27 activity.

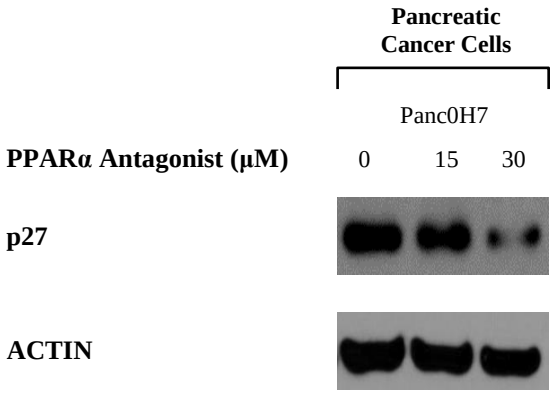


Figure 15 | PPARα antagonist inhibits expression of p27 in tumor cells.
Western blot analysis of protein lysate obtained from human pancreatic cancer cells (Panc0H7) treated with the PPPARα antagonist or control for 48 h at the indicated concentrations (15, 30 μM). Results are representative of at least two independent experiments with similar results. Quantifications are provided in Supplemental Figure 4.

PPARα antagonist induces cellular senescence in pancreatic tumor cells.

To determine whether the PPARα antagonist's anti-growth activity is mediated by therapy-induced senescence, β-galactosidase activity was measured in pancreatic cancer cells (Panc0H7) treated with the PPARα antagonist for 48 h.

Cellular senescence is defined as a state of stable exit from the cell cycle and an irreversible cell growth arrest in G_0/G_1 (Itahana, Campisi, & Dimri, 2007; Perez-Mancera, Young, Narita, 2014). One biomarker used for detecting senescence in cells is β-galactosidase activity *(*Gary & Kindell, 2005). Enzyme activity is measured by staining cells with an artificial substrate X-gal (5-bromo-4-chloro-3-indolyl-beta galactopyranoside). β-galactosidase will catalyze the hydrolysis of X-gal, which produces a blue color in senescent cells (Gary & Kindell, 2005). Cells positive for senescence are stained blue while non-senescent cells are unstained.

Treatment with the PPARα antagonist increased the number of Panc0H7 cells with SA-β-gal activity (senescent associated β-galactosidase) compared to control (Figure 16). No or little SA-β-gal activity was observed in non-treated cells, whereas treated cells were stained blue. These results suggest a mechanism whereby the PPARα antagonist reduces cell growth by therapy-induced senescence.

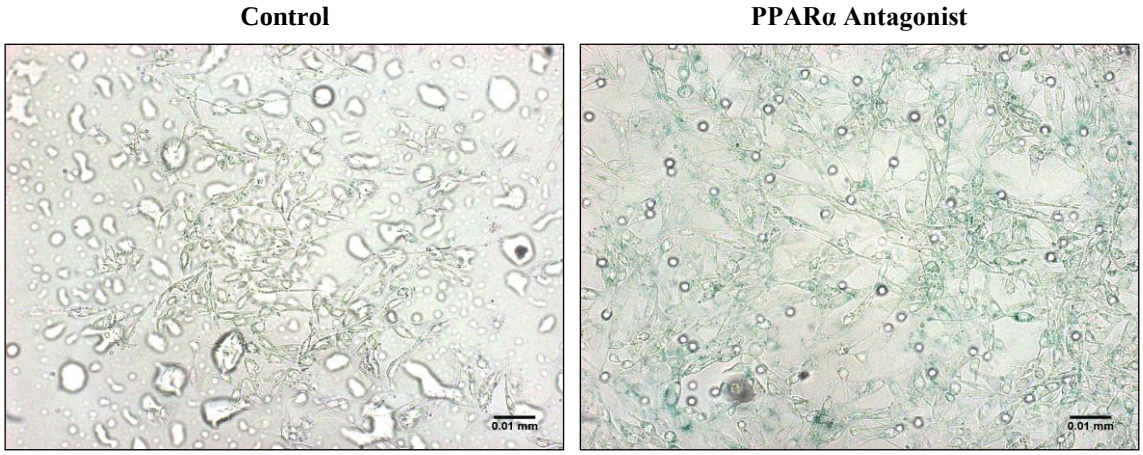


Figure 16 | PPARα antagonist induces cellular senescence in pancreatic cancer cells.
Tumor cells were plated in chamber slides (15,000 cells/well). SA-β-gal activity was measured in Panc0H7 cells treated with PPARα antagonist (30μM) for 48 h. Images were captured under a bright-field compound microscope (20X).

DISCUSSION

Pancreatic ductal adenocarcinoma (PDAC) is a highly aggressive tumor and is characterized by a highly fibrotic tumor environment resulting in stromal resistance to chemotherapy (Farrow, Albo, & Berger, 2008; Feig *et al.*, 2012). Only 6% of patients diagnosed will survive more than five years (Howlander *et al.*, 2014). Current approaches, such as surgery, radiation, chemotherapy, or combination therapies, have had little effect in long term survival outcome (Li *et al.*, 2004; Lockhart, Rothenberg, & Berlin, 2005; Stathis & Moore, 2010). Thus, a better understanding of the molecular basis and progression of pancreatic cancer is needed in order to diagnose, treat, and prevent this disease.

PPARα is a ligand-activated transcription factor that belongs to the nuclear receptor superfamily (Kota, Huang, & Roufogalis, 2005). In addition to its role in β-oxidation and lipid metabolism, PPARα activation is involved in regulating inflammation and ligand-induced tumor growth (Reddy & Azarnoff, 1980; Devchand *et al.*, 1996). Accordingly, PPARα knockout mice exhibited a prolonged inflammatory response and are resistant to the hepatocarcinogenic effect of PPARα agonists (Devchand *et al.*, 1996; Peters, Cattley, & Gonzalez, 1997). More recently, PPARα activation has been observed with increased proliferation in breast cancer cells (Suchanek *et al.*, 2002). Conversely, PPARα agonists also play an anti-tumorigenic role by inhibiting cancer progression of melanoma, endometrial, and fibroblast cancer cells (Grabacka *et al.*, 2006; Saidi *et al.*, 2006; Pozzi

& Capdevilla, 2008). Thus, despite our current knowledge of PPARα, whether PPARα promotes or inhibits cancer remains unclear (Panigrahy *et al*., 2008). This suggests the complexity of the receptor and its resulting biological, pleiotropic effects. Future studies will be necessary in order to clarify the role of PPARα in human cancer development.

The tumor microenvironment plays a crucial role in promoting cancer progression (Farrow, Albo, & Berger, 2008). The active role of non-neoplastic cells including immune cells, fibroblasts, and other cell types can induce inflammation in the microenvironment and exacerbate tumor growth (Farrow, Albo, & Berger 2008). As a basis for this study, we have found the presence of PPARα in both the tumor and the host is necessary for tumor growth (Kaipainen *et al*., 2007). Although studies have demonstrated the anti-proliferative effects of PPARϒ activation in pancreatic cancer, the role of PPARα has been less characterized (Eibl, 2008). Our study is the first to evaluate the role of PPARα inhibition in pancreatic cancer. The aims of the present study were (1) to investigate the anti-growth activity of a PPARα antagonist, (2) to characterize the activity of PPARα inhibition on the cell cycle, and (3) to elucidate the molecular mechanism by which a PPARα antagonist inhibits cell growth.

To elucidate the mechanism by which the PPARα antagonist inhibits cell growth, treatment with the PPARα antagonist in macrophages and pancreatic cancer cells reduced protein level expression of P-AKT, AKT, PNCA, and BAX (Figure 11). AKT is a marker for cell survival, and the protein exists in a complex with Hsp27. Heat shock proteins

(Hsp) are a group of molecular chaperons, and the interaction between Hsp27 and AKT is necessary for AKT to promote cell survival (Guo *et al.*, 2009). PPARα antagonists may act by directly reducing the expression levels of Hsp27 or indirectly by disrupting the Hsp27/AKT interaction. Future studies will be needed to characterize expression levels of Hsp27 in PPARα antagonist-treated tumor cells and macrophages. Interestingly, we observed a decrease in BAX, a marker for apoptosis, thus suggesting that cells have prolonged survival. Recently it has been shown BAX may play a dual role by regulating cell proliferation as well as apoptosis depending on the specific genetic context. In p53 deficient mice, BAX can accelerate tumor growth (Knudson et al., 2001). Our trypan blue exclusion assay results, which demonstrated a decrease in cell growth with low cytotoxicity (Figure 10), are consistent with BAX as a regulator cell proliferation. Future studies may look at the effect of the PPARα antagonist on the ratio of p53: BAX.

We observed cell cycle arrest at G_0/G_1 in both macrophages and tumor cells. To provide evidence for a molecular mechanism, we next examined the protein level of p27, a known checkpoint inhibitor between G_1 and S. Surprisingly, our western blot analysis demonstrated that inhibition of PPARα reduced p27 expression. p27 function may depend on its cellular localization (Coqueret, 2003). Recently, it was observed cytoplasmic p27 is oncogenic *in vitro* and *in vivo* (Serres *et al*., 2011). Future studies will be required to determine the change in the ratio of p27 in the nucleus and cytoplasm in response to PPARα inhibition, as well as examining other G_0/G_1 cell cycle markers.

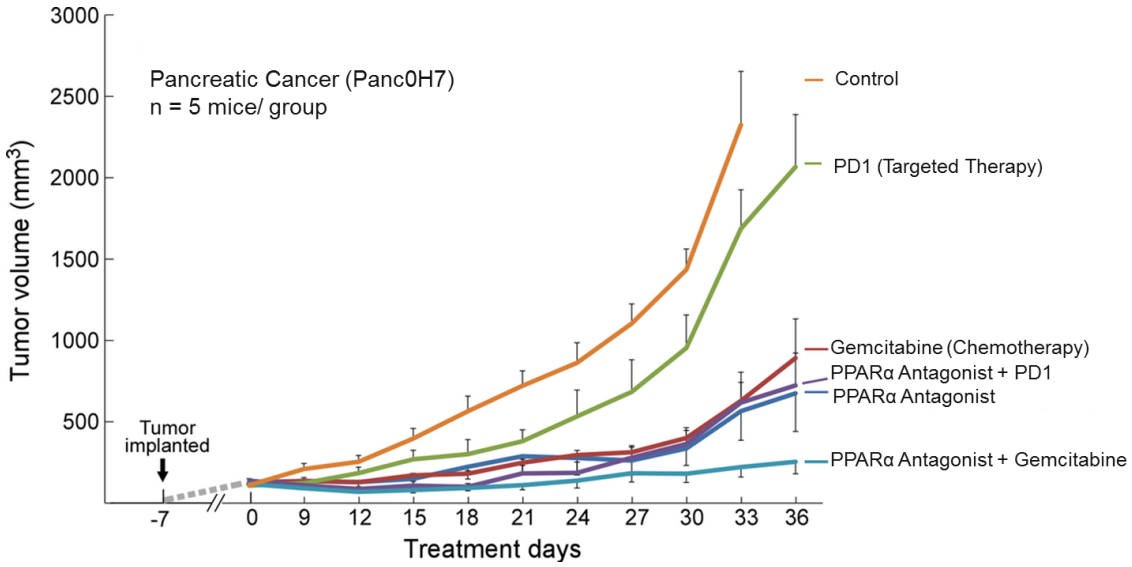
The senescence phenotype is characterized as a stable state of cell arrest. PPARα inhibition induced cellular senescence in tumor cells, as shown in Figure 16. The clinical relevance of therapy-induced senescence remains poorly characterized. Senescent cells can secrete cytokines that can either induce a pro-inflammatory and pro-tumorigenic state, or possess anti-tumorigenic activity by initiating the clearance of senescent cells through an immune response (Perez-Mancera, Young, & Narita, 2014). Future experiments are needed to fully characterize the effect of the PPARα antagonist on tumor cells in the context of senescence.

To our knowledge, PPARα inhibition is a novel approach in cancer progression, and only recently has PPARα antagonists been studied for its anti-tumor activity. GW6471, a different PPARα, has been shown to inhibit renal cell carcinoma, also by inducing cell cycle arrest and apoptosis, thus partially confirming the results of our study, but moreover the role of PPARα as novel therapeutic target (Aboud, Wettersten, & Weiss, 2013). To further extend the potential of PPARα inhibition in pancreatic cancer, we sought to examine the activity of the PPARα antagonist with gemcitabine, the standard of care chemotherapy for pancreatic cancer. In mice bearing pancreatic tumors, the combination treatment of the PPARα antagonist and gemcitabine inhibited tumor growth more than either treatment alone (Supplemental Figure 1). In a murine orthotopic pancreatic cancer model, in which pancreatic tumor cells were injected directly in the pancreas, the combination treatment prolonged survival over 120 days compared to the

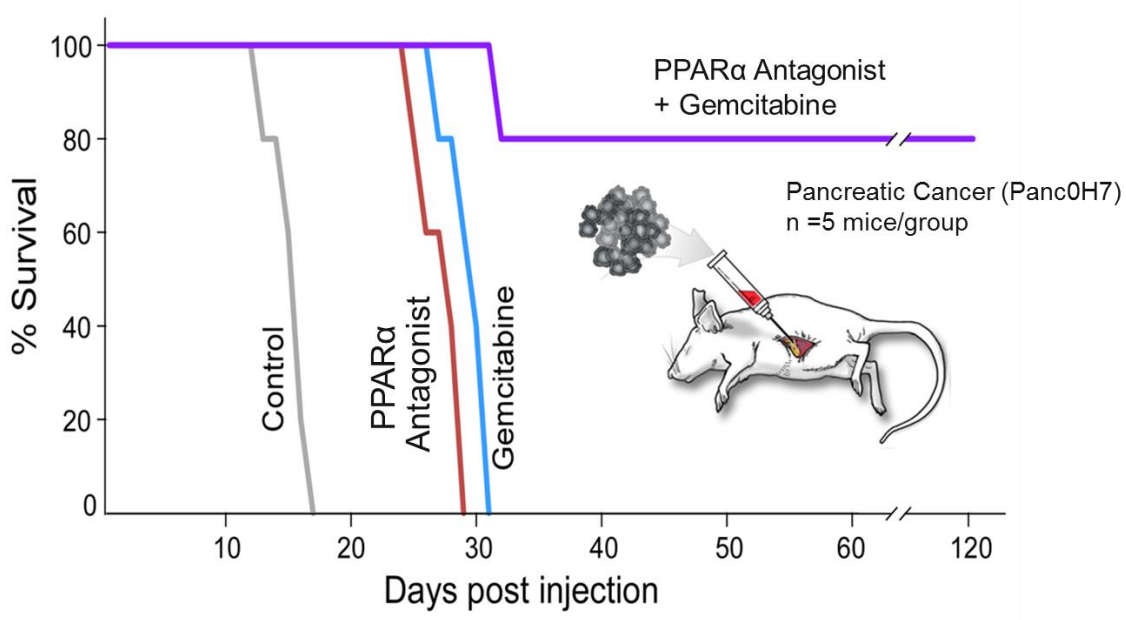
control (10 - 20 days) and the PPARα antagonist and gemcitabine treated groups alone (25 - 35 days) (Supplemental Figure 2).

Our study provides evidence for the synergistic anti-tumorigenic action of the PPARα antagonist and gemcitabine, and the potent suppression of macrophage and pancreatic cancer cell growth through PPARα antagonism. The PPARα antagonist exerts its anti-growth activity by inducing cell cycle arrest at G_0/G_1 thereby inducing cellular senescence without cell death. Our findings provide a mechanism for the anti-tumor activity of PPARα inhibition, and the rationale to use PPARα antagonists to complement current treatment regimens as novel therapeutic approach to pancreatic cancer.

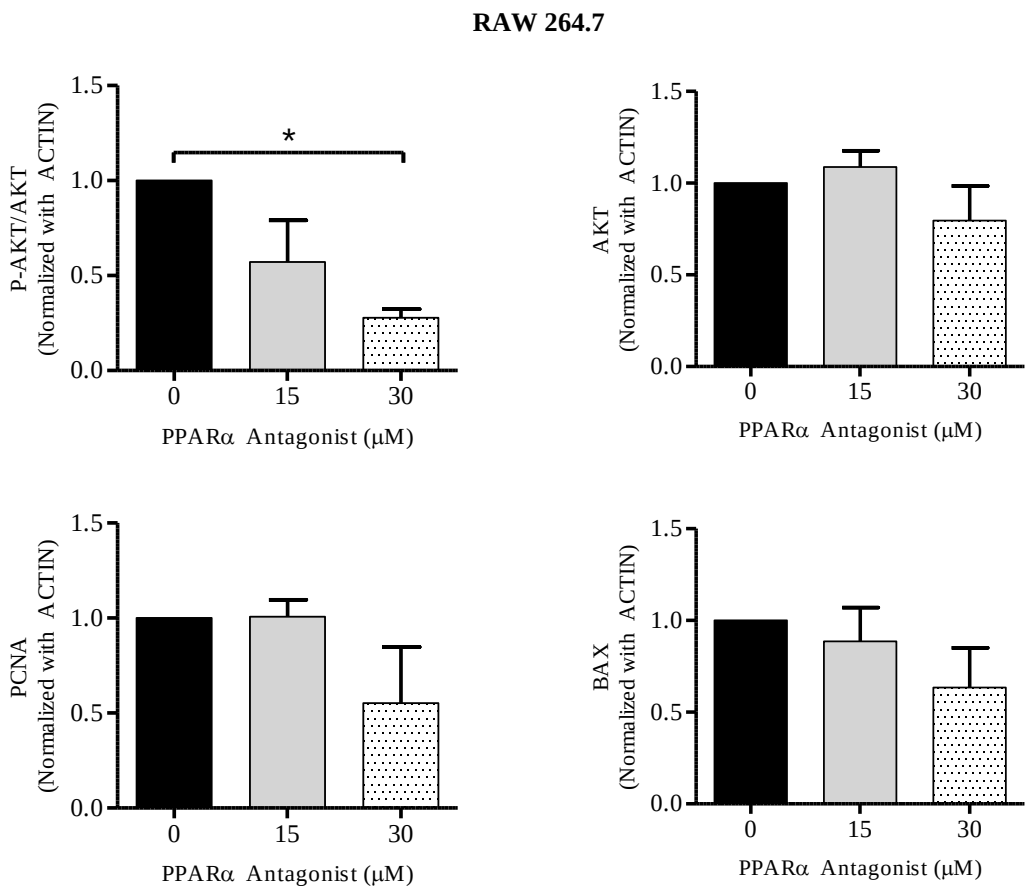
APPENDIX



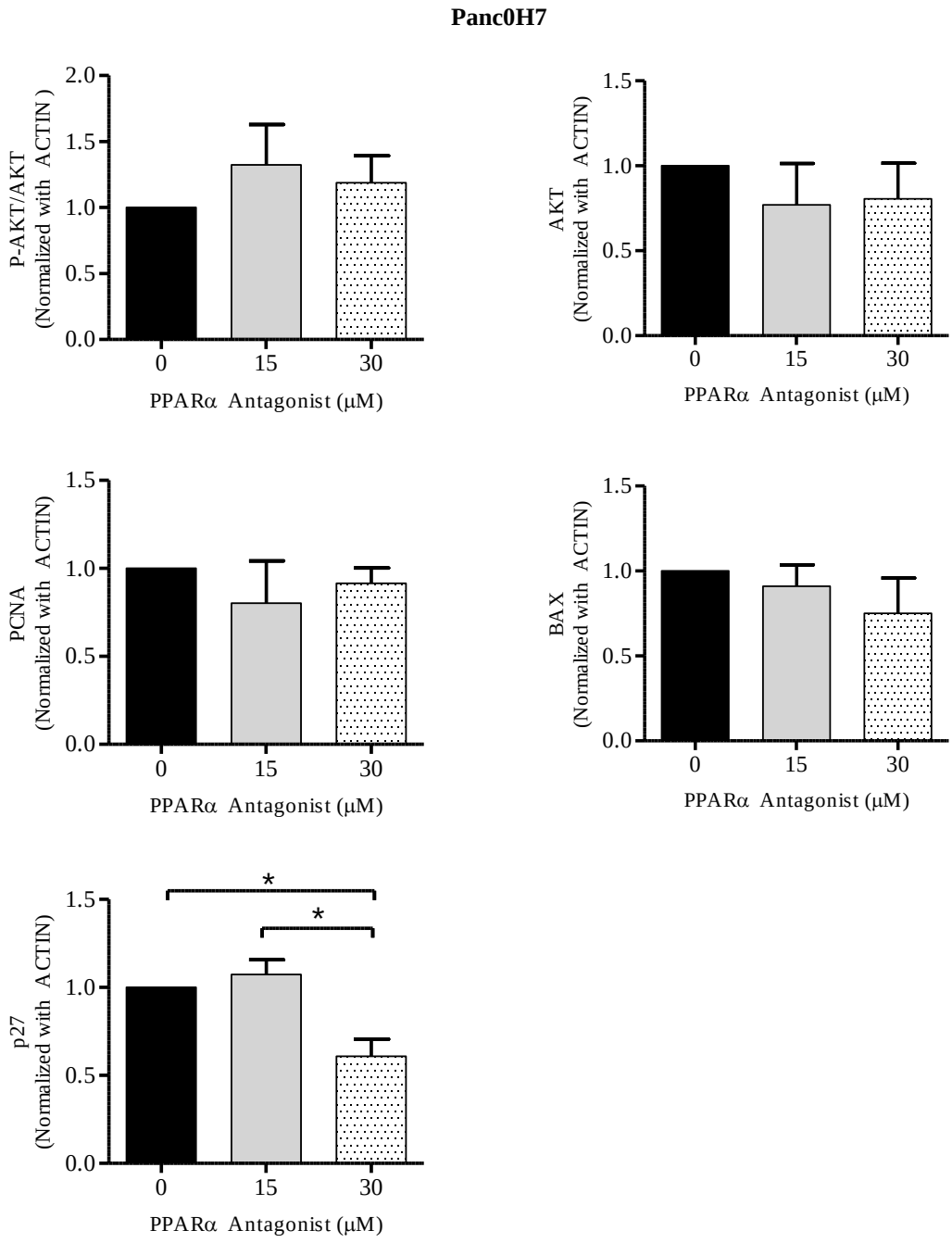
Supplemental Figure 1 | PPARα antagonist and gemcitabine potently suppress pancreatic tumor growth (Panc0H7).
Treatment was initiated when subcutaneous tumors were 100 to 200 mm^3 in size in immunocompetent mice. The PPARα antagonist was compared to gemcitabine (current standard of care chemotherapy), PD1 (checkpoint inhibitor), and various combinations.



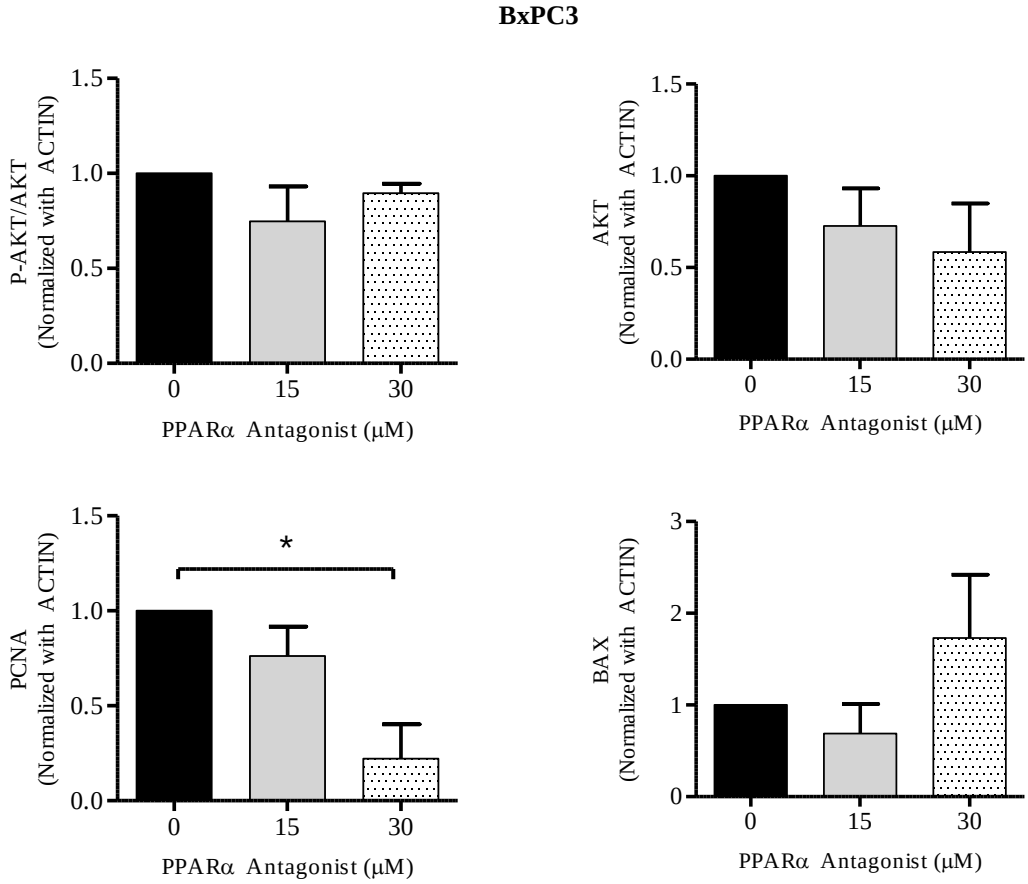
Supplemental Figure 2 | The PPARα antagonist and gemcitabine prolong survival in an orthotopic murine pancreatic cancer model.
Pancreatic tumor cells were injected directly into the pancreas of the mouse and treatment was started after injection. Experiment was performed in collaboration with Dr. Diane Bielenberg (Children's Hospital, Boston, MA).



Supplemental Figure 3 | RAW 264.7 Western blot quantification.
P-AKT/AKT normalized with control, and AKT, PCNA, BAX normalized to ACTIN. The results are means +/- SEM (n=3); *p< 0.05



Supplemental Figure 4 | Panc0H7 Western blot quantification.
P-AKT/AKT normalized with control, and AKT, PCNA, BAX, and p27 normalized to ACTIN. The results are means +/- SEM (n=3); *p< 0.05



Supplemental Figure 5 | BxPC3 Western blot quantification.
P-AKT/AKT normalized with control, and AKT, PCNA, and BAX normalized to ACTIN. The results are means +/- SEM (n=3); *p< 0.05

REFERENCES

Aboud, O., Wettersten, H., & Weiss, R. (2013). Inhibition of PPARα induces cell cycle arrest and apoptosis, and synergizes with glycolysis inhibition in kidney cancer cells. *PLoS One*. *8*(8), e71115-e71123.

Aperlo, C., Pognonec, P., Saladin, R., Auwerx, J., & Boulukos, K. (1995). cDNA cloning and characterization of the transcriptional activities of the hamster peroxisome proliferator-activated receptor PPAR gamma. *Gene, 162*(2), 297-302.

Bardeesy, N., & DePinho, R. (2002). Pancreatic cancer biology and genetics. *Nature Reviews: Cancer, 2*(12), 897-909.

Barrett, K., Boitano, S., Barman, S., & Brooks, H. (2012). Chapter 24. Endocrine Functions of the Pancreas & Regulation of Carbohydrate Metabolism. In Barrett, K., Boitano, S., Barman, S., Brooks, H. (Eds), Ganong's Review of Medical Physiology(pp.24e) NewYork. NY: Mc Graw Hill

Bockman, D. (1993). Anatomy of the pancreas. In V. Go, E. DiMagno, J. Gardner, E. Lebenthal, H. Reber, and V. Scheele (Eds), The Pancreas Biology, Pathology, and Disease (pp.1-8) New York, NY: Raven Press

Bretones, G., Delgado, M., & Leon, J. (2014). Myc and cell cycle control. *Biochimica et Biophysica Acta*. *1849*(2015), 506-516

Burns, K., & Vanden Heuvel, J. (2007). Modulation of PPAR activity via phosphorylation. *Biochimica et Biophysica Acta, 1771*(8), 952-960.

Chu, G., Kimmelman, A., Hezel, A., & DePinho, R. (2007). Stromal biology of pancreatic cancer. *Journal of Cellular Biochemistry*, *101*(4), 887-907.

Conroy, T., Desseigne, F., Ychou, M., Bouche, O., Guimbaud, R., & Becouarn, Y. (2011). FOLFIRINOX versus gemcitabine for metastatic pancreatic cancer. *New England Journal of Medicine, 364*(19), 1817-1825.

Coqueret, O. (2003). New roles for p21 and p27 cell-cycle inhibitors: a function for each cell compartment? *Trends in Cell Biology*, *13*(2), 65-70.

Costanzo, L. (2011). Chapter 6 Gastrointestinal Physiology. In Costanzo, L. (Eds), Physiology (pp.190-221) Philadelphia, PA: Lippincott Williams & Wilkins.

Crooks, S., & Stockley, R. (1998). Leukotriene B4. *International Journal of Biochemistry and Cell Biology*, *30*(2), 173-178.

Cubilla, A., & Fitzgerald, P. (1976). Morphological lesions associated with human primary invasive nonendocrine pancreas cancer. *Cancer Research,* 36(7 PT 2), 2690-2698.

Daynes, R., & Jones, D. (2002). Emerging roles of PPARs in inflammation and immunity. *Nature Reviews: Immunology, 2*(10), 748-759.

De Visser, K., Eichten, A., & Coussens, L. (2006). Paradoxical roles of the immune system during cancer development. *Nature Reviews: Cancer, 6*(1), 24-37.

Desvergne, B., & Wahli, W. (1999). Peroxisome proliferator-activated receptors: nuclear control of metabolism. *Endocrine Reviews, 20*(5), 649-688.

Devchand, P., Keller, H., Peters, J., Vazquez, M., Gonzalez, F., & Wahli, W. (1996). The PPARalpha-leukotriene B4 pathway to inflammation control. *Nature, 384*(6604), 39-43.

Dewson, G., & Kluck, R. (2009). Mechanisms by which Bak and Bax permeabilise mitochondria during apoptosis. *Journal of Cell Science, 122*(16), 2801-2808.

DiMagno, E. (1999). Pancreatic cancer: clinical presentation, pitfalls and early clues. *Annals of Oncology, 10 Suppl* 4, 140-142.

Eibl, G. (2008). The role of PPAR-ϒ and interactions with COX-2 in Pancreatic Cancer. *PPAR Research,* 2008(326915), 1-6.

Farrow, B., Albo, D., & Berger, D. (2008). The role of the tumor microenvironment in the progression of pancreatic cancer. *Journal of Surgical Research, 149*(2), 319-328.

Feig, C., Gopinathan, A., Neesse, A., Chan, D., Cook., & Tuveson, D. (2012). The Pancreas Cancer Microenvironment. *Clinical Cancer Research 18*(16), 4266-4276.

Fernandez-del Castillo, C., & Michael, L . (2015). Overview of surgery in the treatment of exocrine pancreatic cancer and prognosis. Retrieved from http://www.uptodate.com/contents/clinical-manifestations-diagnosis-and-staging-of-exocrine-pancreatic cancer?source=search_result&search=exocrine+pancreatic+cancer&selectedTitle=1~150

Gary, R., & Kindell, S. (2005). Quantitative assay of senescence-associated beta-galactosidase activity in mammalian cell extracts. *Analytical Biochemistry, 343*(2), 329-334.

Gonzalez, F. (2002). The peroxisome proliferator-activated receptor alpha (PPARalpha): role in hepatocarcinogenesis. *Molecular and Cellular Endocrinology, 193*(1-2), 71-79.

Gottlicher, M., Widmark, E., Li, Q., & Gustafsson, J. (1992). Fatty acids activate a chimera of the clofibric acid-activated receptor and the glucocorticoid receptor. *Proceedings of the National Academy of Sciences of the United States of America, 89*(10), 4653-4657.

Grabacka, M., Plonka, P., Urbanska K., Reiss, K. (2006). Peroxisome proliferator-activated receptor alpha activation decreases metastatic potential of melanoma cells in vitro via down-regulation of Akt. *Clinical Cancer Research, 12*(10), 3028-3036.

Gresham, G., Wells, G., Gill, S., Cameron, C., & Jonker, D. (2014). Chemotherapy regimens for advanced pancreatic cancer: a systematic review and network meta-analysis. *BMC Cancer, 14*, 471.

Guerra, C., Schumacher, A., Canamero, M., Grippo, P., Verdaguer, L., Perez-Gallego, L., Dubus, P., Sandgren, E., &Barbacid, M. (2007). Chronic pancreatitis is essential for induction of pancreatic ductal adenocarcinoma by K-Ras oncogene in adult mice. *Cancer Cell, 11*(3), 291-302.

Guertin, D., & Sabatini, D. (2007). Defining the role of mTOR in cancer. *Cancer Cell, 12*(1), 9-22.

Guo, K., Kang, N., Li, Y., Sun, L., Gan, L., Cui, F., Liu, K. (2009). Regulation of HSP27 on NF-kappaB pathway activation may be involved in metastatic hepatocellular carcinoma cells apoptosis. *BMC Cancer, 9(*100), 1-10.

Hagemann, T., Balkwill, F., & Lawrence., T. (2007). Inflammation and cancer: a double-edged sword. *Cancer Cell, 12*(4), 300-301.

Heinaniemi, M., Uski, J., Degenhardt, T., Carlberg, C. (2007). Meta-analysis of primary target genes of peroxisome proliferator-activated receptors. *Genome Biology, 8*(7), R147.29.

Hess, R., Staubl, W., & Riess, W. (1965). Nature of the hepatomegalic effect produced by ethyl-chlorophenoxy-isobutryate in the rat. *Nature, 208*(5013), 856-858.

Hostetler, H., Petrescu, A., Kier, A., & Schroeder, F. (2005). Peroxisome proliferator-activated receptor alpha interacts with high affinity and is conformationally responsive to endogenous ligands. *Journal of Biological Chemistry, 280*(19), 18667-18682.

Howlader, N., Krapcho, M., Garshell, J., Miller, D., Altekruse. S., Kosary, C., Yu., Ruhl, J., Tatalovich, Z.,Mariotto, A., Lewis, D., Chen, H., Feuer, E., & Cronin, K. (2014). SEER Cancer Statistics Review, 1975-2011.

Hruban, R., Wilentz, R., & Kern, S. (2000). Genetic progression in the pancreatic ducts. *American Journal of Pathology*, 156(6), 1821-1825.

Issemann, I., & Green, S. (1990). Activation of a member of the steroid hormone receptor superfamily by peroxisome proliferators. *Nature, 347*(6294), 645-650.

Itahana, K., Campisi, J., & Dimri, G. (2007). Methods to detect biomarkers of cellular senescence: the senescence-associated beta-galactosidase assay. *Methods in Molecular Biology, 371*(2007), 21-31.

Jeong, S., & Seol, D. (2008). The role of mitochondria in apoptosis. *BMB Rep, 41*(1), 11-22.

Kaipainen, A., Kieran, M., Huang, S., Butterfield, C., Bielenberg, D., Mostoslavsky, G., & Panigrahy, D. (2007). PPARalpha deficiency in inflammatory cells suppresses tumor growth. *PloS One, 2*(2), e260.

Kerr, J., Wyllie, A., & Currie, A. (1972). Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *British Journal of Cancer, 26*(4), 239-257.

Knudson, C., Johnson, G., Lin, Y., & Korsmeyer, S. (2001). Bax accelerates tumorigenesis in p53-deficient mice. *Cancer Research, 61*(2), 659-665

Kota, B., Huang, T., & Roufogalis, B. (2005). An overview on biological mechanisms of PPARs. *Pharmacological Research, 51*(2), 85-94.

Li, D., Xie, K., Wolff, R., & Abbruzzese, J. (2004). Pancreatic cancer. *The Lancet, 363*(9414), 1049-1057.

Li, H., Cui, Z., Chen, J., Guo, X., & Li, Y. (2015). Pancreatic cancer: diagnosis and treatments. *Tumour Biology*, *36*(3), 1375-84

Lock, E., Mitchell, A., & Elcombe, C. (1989). Biochemical mechanisms of induction of hepatic peroxisome proliferation. *Annual Review of Pharmacology and Toxicology,1989*(29), 145-163.

Lockhart, A., Rothenberg, M., & Berlin, J. (2005). Treatment for Pancreatic Cancer: current Therapy and Continued Progress. *Gastroenterology, 128*(6), 1642-1654.

Luttmann, W. (2006). Immunology. London, England : Academic Press

Manning, B., & Cantley, L. (2007). AKT/PKB signaling: navigating downstream. *Cell, 129*(7), 1261-1274.

Modolell, I., Guarner, L., & Malagelada, J. (1999). Vagaries of clinical presentation of pancreatic and biliary tract cancer. *Annals of Oncology, 10 Suppl 4*, 82-84.

Moody, D., & Reddy, J. (1978). The hepatic effects of hypolipidemic drugs (clofibrate, nafenopin, tibric acid, and Wy-14,643) on hepatic peroxisomes and peroxisome-associated enzymes. *American Journal of Pathology, 90*(2), 435-446.

Öberg, K., & Eriksson, B. (2005). Endocrine tumours of the pancreas. *Best Practice & Research Clinical Gastroenterology, 19*(5), 753-781.

Oliveira-Cunha, M., Siriwardena, A., & Byers, R. (2008). Molecular diagnosis in pancreatic cancer. *Diagnostic Histopathology, 14*(5), 214-222.

Panigrahy, D., Kaipaninen, A., Kieran, M., Huang, S. (2008). PPARs: A double-edged sword in cancer therapy?. *PPAR Research, 2008*(350351), 1-2.

Penaloza, C., Lin, L., Lockshin, R., & Zakeri, Z. (2006). Cell death in development: shaping the embryo. *Histochemistry and Cell Biology, 126*(2), 149-158.

Perez-Mancera, P., Young, A., & Narita, M. (2014). Inside and out: the activities of senescence in cancer. *Nature Reviews: Cancer, 14*(8), 547-558.

Peters, J., Cattley, R., & Gonzalex. (1997). Role of PPAR alpha in the mechanism of action of the nongenotoxic carcinogen and peroxisome proliferator Wy-14,643. *Carcinogenesis, 18*(11), 2029-2033.

Pickhart, A., Grober, S., Haug, A., Piontek, G., Wirth, M., Straben, U., Rudelius, M., & Reiter, R. (2014). Survivin and pAKT as potential prognostic markers in squamous cell carcinoma of the head and neck. *Oral and Maxillofacial Pathology, 117*(6), 733-742.

Porta, M., Fabregat, X., Malats, N., Guarner, L., Carrato, A., de Miguel, A., & Real, F. (2005). Exocrine pancreatic cancer: symptoms at presentation and their relation to tumour site and stage. *Clinical & Translational Oncology, 7*(5), 189-197.

Pozzi, A., & Capdevila, J. (2008). PPARα ligands as antitumorgenic and antiangiogenic agents. *PPAR Research, 2008*(906542). 1-8.

Pyper, S., Viswakarma, N., Yu, S., & Reddy, J. (2010). PPARalpha: energy combustion, hypolipidemia, inflammation and cancer. *Nucl Recept Signal, 8,* e002.

Reddy, J. (2004). Peroxisome proliferators and peroxisome proliferator-activated receptor alpha: biotic and xenobiotic sensing. *American Journal of Pathology, 164*(6), 2305-2321.

Reddy, J., & Azarnoff, D. (1980). Hypolipidaemic hepatic peroxisome proliferators form a novel class of chemical carcinogens. *Nature, 283*(24), 397-398.

Reddy, J., & Krishnakantha, T. (1975). Hepatic peroxisome proliferation: induction by two novel compounds structurally unrelated to clofibrate. *Science, 190*(4216), 787-789.

Reddy, J., Rao, S., & Moody, D. (1976). Hepatocellular carcinomas in acatalasemic mice treated with nafenopin, a hypolipidemic peroxisome proliferator. *Cancer Research, 36*(4), 1211-1217.

Saidi, S., Holland, C., Charnock-Jones, D., & Smith, S. (2006). In vitro and in vivo effects of the PPAR-alpha agonists fenofibrate and retinoic acid in endometrial cancer. *Molecular Cancer, 5*(13), 1-14.

Sarkar, F., Banerjee, S., & Li, Y. (2007). Pancreatic cancer: pathogenesis, prevention and treatment. *Toxicology and Applied Pharmacology, 224*(3), 326-336.

Serres, M., Zlotek-Zlotkiewicz, E., Concha, C., Gurian-West, M., Daburon, V., Roberts, J., & Besson, A. (2011). Cytoplasmic p27 is oncogenic and cooperates with Ras both in vivo and in vitro. *Oncogene, 30*(25), 2846-2858.

Sgambato, A., Cittadini, A., Faraglia, B., & Weinstein, I. (2000). Multiple functions of p27(Kip1) and its alterations in tumor cells: a review. *Journal of Cellular Physiology, 183*(1), 18-27.

Sher, T., Yi, H., McBride, O., & Gonzalez, F. (1993). cDNA cloning, chromosomal mapping, and functional characterization of the human peroxisome proliferator activated receptor. *Biochemistry, 32*(21), 5598-5604.

Snady, H., Bruckner, H., Cooperman, A., Paradiso, J., & Kiefer, L. (2000). Survival advantage of combined chemoradiotherapy compared with resection as the initial treatment of patients with regional pancreatic carcinoma. An outcomes trial. *Cancer, 89*(2), 314-327.

Stathis, A., & Moore M. (2010). Advanced pancreatic carcinoma: current treatment and future challenges. *Nature Review Clinical Oncology, 7*(13), 163-172.

Stoimenov, I., & Helleday, T. (2009). PCNA on the crossroad of cancer. *Biochemical Society Transactions, 37*(Pt 3), 605-613.

Strober, W. (2001). Trypan blue exclusion test of cell viability. *Current Protocols in Immunology, Appendix 3,* Appendix 3B.

Suchanek, K., May, F., Robinson, J., Lee, W., Holman, N., Monteith, G., & Roberts-Thomson, S. (2002). Peroxisome proliferator-activated receptor alpha in the human breast cancer cell lines MCF-7 and MDA-MB 231. *Molecular Carcinogenesis, 34*(4), 165-171.

Tempero, M., Arnoletti, J., Behrman, S., Ben-Josef, E., Benson, A., & Berlin, J. (2010). Pancreatic adenocarcinoma. *Journal of the National Comprehensive Cancer Network, 8*(9), 972-1017.

Toker, A., & Marmiroli, S. (2014). Signaling specificity in the Akt pathway in biology and disease. *Advances in Biological Regulation, 55,* 28-38.

Van Raalte, D., Li, M., Pritchard, P., & Wasan, K. (2004). Peroxisome proliferator-activated receptor (PPAR)-alpha: a pharmacological target with a promising future. *Pharmaceutical Research, 21*(9), 1531-1538.

Vincent, A., Herman, J., Schulick, R., Hruban, R. & Goggins, M. (2011). Pancreatic cancer. *Lancet, 378*(9791), 607-620.

Von Hoff, D. D., Ervin, T., Arena, F. P., Chiorean, E. G., Infante, J., Moore, M., Renschler, M. F. (2013). Increased survival in pancreatic cancer with nab-paclitaxel plus gemcitabine. *New England Journal of Medicine, 369*(18), 1691-1703.

Wahli, W., Braissant, O., Desvergne, B. (1995). Peroxisome proliferator activated receptor: transcriptional regulators of adipogenesis, lipid metabolism and more. *Chemistry & Biology*, *2*(5), 261-266.

Wang, S. (2014). PCNA: a silent housekeeper or a potential therapeutic target? *Trends in Pharmacological Sciences, 35*(4), 178-186.

Yeo, T., Hruban, R., Leach, S., Wilentz, R., Sohn, T., Kern, S., &Yeo, C. (2002). Pancreatic cancer. *Current Problems in Cancer*, *26*(4), 176-275.

Zor, T., & Selinger, Z. (1996). Linearization of the Bradford Protein Assay Increases Its Sensitivity: Theoretical and Experimental Studies. *Analytical Biochemistry*, *236*(2), 302-308.

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