MECHANISMS OF PIPERLONGUMINE-INDUCED CANCER CELL DEATH

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

DOCTOR OF PHILOSOPHY

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ABSTRACT

Piperlongumine (PPLGM), a bioactive agent obtained from long pepper plants, possesses potent antitumor activity by inducing reactive oxygen species (ROS). However, the mechanisms for PPLGM's antitumor actions are not well defined. We investigated PPLGM's antitumor effects and molecular mechanisms against pancreatic and colon cancer, two of the leading causes of cancer death for both men and women in the U.S. We found that PPLGM activated a ROS-mediated DNA damage pathway that lead to pancreatic cancer cell death *in vitro*. Further, mice treated with PPLGM showed reduced pancreatic tumor volume, which was associated with a decrease in tumor cell proliferation and enhanced oxidative stress levels. To elucidate the target pathways responsible for PPLGM-mediated cell death, RNA sequencing was performed. 684 genes were differentially expressed in pancreatic cancer cells treated with PPLGM compared to the control. Genes related to ER-stress and UPR pathways were activated in PPLGM-treated pancreatic cancer cells. To determine the therapeutic efficacy of PPLGM in combination with currently used chemotherapy *in vivo*, an orthotopic mouse model of pancreatic cancer was used. The combination of PPLGM with gemcitabine resulted in greater reduction of tumor weight and volume than either agent alone, and PPLGM-treated mouse tumors showed decreased expression of Ki-67, a proliferation marker. *In vitro* studies supported the *in vivo* results where the combination of PPLGM with gemcitabine and erlotinib significantly decreased pancreatic cancer cell viability and survival, and induced apoptosis compared to control cells or cells treated with the chemotherapeutic agents alone. PPLGM inhibited the growth of colorectal cancer cells to a greater degree than normal colon cells and activated p-ERK protein expression. The use of a MEK inhibitor attenuated the activation of p-ERK and partially blocked PPLGM-mediated cell death, indicating the involvement of the MEK/ERK pathway in colon cancer cell death. These

results suggest PPLGM holds potential as a therapeutic agent to treat pancreatic and colon cancer in the clinics.

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I am indebted to my thesis advisor, Dr. Katie Reindl, for her assistance, guidance, timely feedback, and constructive criticisms. She not only helped me grow as a scientist but as a writer as well. I am grateful for her immense support, understanding, and encouragement all the way. Thank you for everything!

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Finally, thanks to my true father 'Waheguru' for always guiding me and being a pillar of strength for me. "Mera mujh meh kichh nahi jo kichh hai so tera". Translation: "Nothing within myself is mine. Whatever there is, is Yours" (Page 1375 of Aad Sri Guru Guru Granth Sahib Ji).

DEDICATION

This thesis is dedicated to my late father, S. Lakhbir Singh Randhawa. His words of inspiration, encouragement, and unconditional moral and financial support are largely the reason that this PhD is completed in the United States.

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LIST OF ABBREVIATIONS

PPLGM…….Piperlongumine

μM…….Micromolar

mM……...Millimolar

NAC……..N-acetyl cysteine

DMSO……...Dimethyl sulfoxide

ERK……..Extracellular signal-regulated kinase

MEK…….Mitogen-activated protein kinase kinase

ROS……..Reactive oxygen species

Chk1…….Checkpoint kinase 1

FBS……...Fetal bovine serum

EDTA…….....Ethylenediaminetetracetic acid

nm…….Nanometer

μl……..Microliter

ng……..Nanogram

mg……Milligram

μg/ml……Microgram/milliliters

SDS……..Sodium dodecyl sulfate

PAGE……....Polyacrylamide gel electrophoresis

TBS……..Tris-buffered saline

PBS……...Phosphate buffered saline

GAPDH……Glyceraldehyde 3-phosphate dehydrogenase

KRAS……...Kirsten rat sarcoma viral oncogene homolog

DNA…….Deoxyribonucleic acid

RNA……..Ribonucleic acid

EGFR……....Epidermal growth factor receptor

ATP……...Adenosine triphosphate

FDA……...Food and drug administration

Ca^{2+}……...Calcium ion

ER…….Endoplasmic reticulum

VEGF……Vascular endothelial growth factor

MMP…….Matrix metalloproteinase

GSH……..Reduced glutathione

GSSG……Oxidized glutathione

IgG……Immunoglobulin G

UPR……..Unfolded protein response

mRNA……...Messenger ribonucleic acid

rRNA…….Ribosomal ribonucleic acid

DAPI…….4',6-diamidino-2-phenylindole

q-PCR……Quantitative-polymerase chain reaction

FDR……...False discovery rate

cDNA……Complementary-deoxyribonucleic acid

i.p……..Intraperitoneal

O.D……Optical density

ELISA……...Enzyme-linked immunosorbent assay

mg/kg……milligram/kilogram

CHAPTER 1. GENERAL INTRODUCTION; OBJECTIVES AND SPECIFIC AIMS

Introduction

Plants are a natural source of bioactive agents which have been used to improve human health for thousands of years. According to the World Health Organization, approximately 80% of the population in Asian and African countries relies on herbal medicine for their primary health care needs (WHO 2008). Interestingly, natural products and their derivatives represent more than 50% of the clinically used drugs in the world (Sofowora 1996; Cowan 1999). From 1981 to 2010, 79.8% of the anticancer drugs were either natural products or mimics of natural products in one form or another, suggesting the importance of bioactive agents in treatment of human cancer (Newman and Cragg 2012).

Treatment options for cancer patients depend on several factors including the type and stage of cancer, location of cancer, possible side effects, and the patient's preferences and overall health. Current anticancer therapy relies on several treatment approaches including surgery, chemotherapy, and radiation therapy to block the growth and spread of cancer cells. Chemotherapeutic drugs circulate in the bloodstream and directly damage cells that are actively dividing. A major limitation to chemotherapy is that any cell undergoing DNA replication and mitosis is susceptible to drug-induced toxicity, as these agents cannot differentiate between dividing cells of normal tissues versus cancer cells (Swift and Golsteyn 2014). Therefore, cellular damage to healthy tissues is unavoidable and accounts for the negative side effects linked to chemotherapeutics. For example, gemcitabine, a chemotherapeutic agent used to treat pancreatic cancer patients can cause serious side effects including neutropenia, thrombocytopenia, diarrhea, and neuropathy (Conroy, Desseigne et al. 2011). Rapid drug metabolism and both intrinsic and acquired drug resistance are major factors responsible for

ineffectiveness of chemotherapy. Also, multidrug resistance (MDR) has been the most unpredictable factor associated with chemotherapy. MDR is mainly due to increased efflux pumps such as P-glycoprotein in the cell membrane which are responsible for transport of various anticancer drugs out of cells (Stavrovskaya 2000).

While chemotherapy is a systemic treatment approach that targets any dividing cell, radiation therapy is usually a local treatment aimed primarily at tumor cells. Radiation therapy induces large quantities of reactive oxygen species (ROS) which cause single or double-stranded DNA breaks inside cells, often resulting in cell death (Yu 2012). The major limitations to radiation therapy are development of resistance to the therapy and non-specific toxicity to healthy tissues. Higher doses of radiation can cause varying side effects during treatment (acute side effects), in the months or years following treatment (long-term side effects) or after re-treatment (cumulative side effects) (Ottoman, Langdon et al. 1963). Furthermore, there are certain clinical situations where the success of radiotherapy is limited either by an extremely radioresistant neoplasm or by a tumor invading a radiosensitive normal tissue (Kennedy, Teicher et al. 1980). Residual malignant cells inside hypoxic tumor environment are an obstacle to effective cancer treatment and are capable of proliferating causing the tumor to recur (Harrison, Chadha et al. 2002).

Surgery is the best option for patients whose cancer has not invaded into surrounding tissues or metastasized. Many types of surgeries are done to diagnose, stage, and treat cancer depending on condition of the patient. For instance, diagnostic surgery is used to diagnose cancer in a patient by doing biopsy. Staging surgery is used to determine clinical as well as pathological stage of cancer. When the cancer is localized to particular part/organ of body, curative surgery is done to remove benign cancer. However, in advanced stages of cancer when cancer has

metastasized to other organs of body, debulking surgery is used to remove some, but not all of the cancer.

Advances in molecular biology have tremendously improved our understanding of cancer pathogenesis and have provided the foundation for targeted therapy. This type of therapy targets a cancer's specific genes, proteins, or the tissue environment that contributes to cancer growth and survival. Targeted therapeutics may be aimed at specific genes or proteins involved in a signaling pathway present in cancer cells but not normal cells. For example, EGFR (epidermal growth factor receptor) inhibitors are used for treatment of many types of cancers including lung cancer (Paez, Janne et al. 2004) and metastatic colorectal cancer (Van Cutsem, Kohne et al. 2009) in which a mutation in the EGFR gene leads to activation/ overexpression of EGFR protein allowing increased growth and metastasis of cancer cells. Even though targeted therapies are a promising way to personalize cancer treatment, cancer cells often become resistant to the drug or the drug becomes less effective over time. This is because cancer is not driven by a single gene or pathway; rather, a network of pathways is involved (Vogelstein and Kinzler 2004). While targeting a single pathway could be effective for some time, eventually the protein may signal through a different pathway. Therefore, treatments involving a combination of agents targeting multiple pathways might be a more effective approach as compared to a drug targeting a single pathway (Grant 2008; Vanneman and Dranoff 2012).

In recent years, there has been a growing interest in the therapeutic use of bioactive agents derived from natural plants. The revival of interest in plant-derived compounds is due to several reasons such as ineffectiveness and negative side effects of conventional medicine, abusive use of synthetic drugs, and inaccessibility of conventional therapy by a large percentage of population due to the increasing cost of synthetic drugs and healthcare. Moreover, folk

medicine suggests that bioactive agents derived from plants are effective and safe to use. For example, curcumin, a bioactive agent present in *Curcuma longa*, has anticancer potential against a variety of cancers including colorectal, pancreatic, breast, and prostate cancer (Hanif, Qiao et al. 1997; Mehta, Pantazis et al. 1997; Mukhopadhyay, Bueso-Ramos et al. 2001; Li, Braiteh et al. 2005). Promising effects have been observed in clinical trials using curcumin alone as well as in combination with other chemotherapeutic agents (Duarte, Han et al. 2010; Moorthi and Kathiresan 2013). In one study, administration of curcumin to colorectal cancer patients after diagnosis and before surgery increased body weight, decreased serum TNF-α level, increased the number of apoptotic cells, and enhanced p53 expression (He, Shi et al. 2011). The authors concluded that curcumin treatment could improve the general health of patients by inducing p53 expression.

Cancer cells differ from normal cells in that they exhibit sustained proliferative signaling, evasion of growth suppressor proteins, and resistance to apoptosis and cell death due to telomerase expression (Hanahan and Weinberg 2011). In regards to metabolism, cancer cells display enhanced anaerobic glycolysis even in the presence of oxygen, and consume more than 20 times as much glucose for ATP production as do normal cells (Annibaldi and Widmann 2010). Additionally, cancer cells have heightened ROS and antioxidant systems compared to normal cells due to their high demand for ATP that contributes to aberrant cellular proliferation (Cairns, Harris et al. 2011). Recent studies have shown the potential of bioactive agents to harness this biochemical difference in cancer cells for chemoprevention and/ chemotreatment of cancer (Ding, Han et al. 2009; Al Dhaheri, Attoub et al. 2014; Gundala and Aneja 2014).

The use of bioactive agents with pro-versus antioxidant properties for cancer therapy has been an issue of debate. Various studies have reported that antioxidants prevent the free radical

damage associated with cancer development (Khan, Afaq et al. 2008). For example, berries, due to their high phenolic and flavonoid content, display high antioxidant potential and have been shown to inhibit cancer initiation (Stoner, Wang et al. 2008). On the contrary, nine randomized controlled clinical trials do not provide evidence towards the beneficial role of antioxidant supplements in cancer prevention. For example, the Selenium and Vitamin E cancer prevention trial (SELECT) investigated the role of daily supplementation of selenium and vitamin E for 5 years on the incidence of prostate cancer in men aged 50 and older. The results show that the use of these supplements did not reduce the incidence of prostate cancer (Lippman, Klein et al. 2009). A study by Sayin and colleagues (Sayin, Ibrahim et al. 2014) reported that the antioxidants, NAC and vitamin E accelerate tumor growth by reducing ROS, DNA damage, and p53 expression in mouse and human lung tumor cells. The authors concluded that antioxidants increase tumor growth by disrupting the ROS-p53 pathway. One plausible explanation for the failure of antioxidants to show beneficial effects is that these antioxidants do not function in mitochondria where ROS are produced, rather, the antioxidants accumulate at scattered sites inside the cells (Sheu, Nauduri et al. 2006; Chandel and Tuveson 2014).

Cancer cells have elevated ROS levels and an enhanced antioxidant system. The higher levels of antioxidants in cancer cells act as a natural defense mechanism to keep ROS in check to allow cell growth (Szatrowski and Nathan 1991; Schumacker 2006; Trachootham, Zhou et al. 2006). According to the authors, ROS-inducing therapy or antioxidant inhibition might be a useful therapeutic approach for cancer treatment, whereas, antioxidant supplementation might stimulate the growth of cancer cells (Schumacker 2006; Chandel and Tuveson 2014). However, the challenge is to find the antioxidant proteins and pathways exclusively used by cancer cells and not healthy cells.

Reactive oxygen species as a cancer therapy

Reactive oxygen species (ROS) are chemically reactive molecules derived from molecular oxygen that include superoxide anions, peroxides, and hydroxyl radicals. Inside a cell, ROS are produced as byproducts during cellular respiration or metabolic reactions primarily in the mitochondria, peroxisomes, and endoplasmic reticulum. Mitochondria are considered a major source of ROS production. As much as 2% of oxygen consumed by the mitochondria during the electron transport chain is converted to superoxide which gets dismutated to hydrogen peroxide (Boveris and Chance 1973). Superoxide and hydrogen peroxide are collectively referred to as mitochondrial ROS. In addition to the electron transport chain, ROS are generated by various enzymes such as NADPH oxidase (NOX), xanthine oxidase, uncoupling of endothelial nitric oxide synthase (eNOS), arachidonic acid, and metabolic enzymes such as cytochrome P450, lipoxygenase, and cyclooxygenase (Curtin, Donovan et al. 2002).

Modulation of ROS

Cells maintain a balance of ROS with the help of antioxidant enzyme system. Antioxidants inhibit the oxidation of other molecules. Oxidation reactions can produce free radicals that are capable of damaging macromolecules such as DNA, proteins, and lipids. Antioxidants prevent or repair the damage caused by ROS. Some cellular antioxidant enzymes include superoxide dismutase, glutathione peroxidase, catalase, peroxiredoxin, glutaredoxin, and thioredoxin. Vitamin E, vitamin C, β-carotene, and coenzyme Q are known as the non-enzymatic antioxidants.

Glutathione (GSH) is a tripeptide consisting of glutamate, cysteine, and glycine which acts a major antioxidant inside the cells by serving as a reducing agent. The glutathione system includes glutathione, glutathione reductase, glutathione peroxidases (GPX), and glutathione S-

transferases (GST). For example, the ROS, hydrogen peroxide is reduced to water by glutathione peroxidase using reduced glutathione (GSH) as a cofactor (Brigelius-Flohe 1999). During the process, GSH is oxidized to oxidized glutathione (GSSG) which in turn gets reduced to GSH by glutathione reductase (Carlberg and Mannervik 1975) (Fig.1). Glutathione S-transferases are detoxification enzymes that catalyze the conjunction of GSH to a variety of exogenous and endogenous electrophilic compounds (Townsend and Tew 2003; Sharma, Yang et al. 2004; Hayes, Flanagan et al. 2005). Glutathione S-transferases are overexpressed in a wide variety of tumors and are also involved in the development of resistance to chemotherapeutics via direct detoxification and regulation of MAPK pathways (Townsend and Tew 2003).

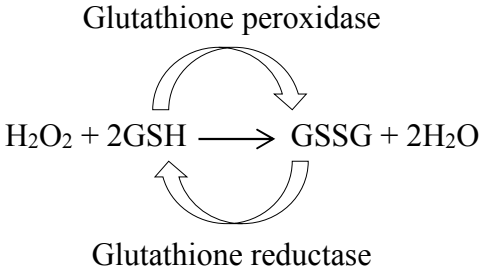


Figure 1. Conversion of reduced glutathione (GSH) to oxidized glutathione (GSSG).

ROS in cancer versus normal cells

Redox homeostasis is maintained in normal cells through a balance between ROS production and elimination. However, cancer cells have elevated ROS levels compared to normal cells due to increased ROS production or impaired ROS detoxification (Toyokuni, Okamoto et al. 1995). Oxidative stress results when ROS are produced at a faster rate than their removal by antioxidant system. Some of the intrinsic factors responsible for ROS generation in cancer cells are metabolic alterations, mitochondrial dysfunction, activation of oncogenes, and loss of functional p53 gene (Irani, Xia et al. 1997; Brandon, Baldi et al. 2006; Horn and Vousden 2007;

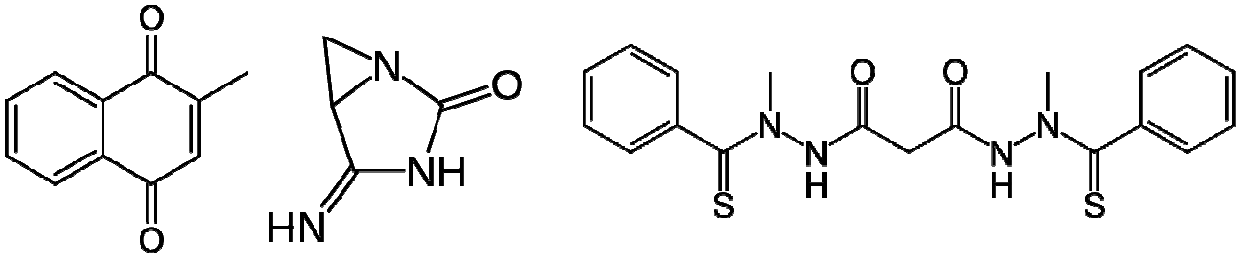
Rodrigues, Reddy et al. 2008). The p53 protein plays an important role in maintaining redox balance by sensing and removing oxidative damage to nuclear and mitochondrial DNA. More than 50% of cancers have a loss of or mutation in the p53 gene resulting in redox imbalance and tumor growth. The extrinsic factors which generate ROS include inflammatory cytokines, hypoxic conditions, and an imbalance of nutrients (Cook, Gius et al. 2004; Azad, Rojanasakul et al. 2008). Cancer cells also have defective antioxidant capacity as compared to their normal counterparts. For instance, the expression level and enzymatic activity of SOD and catalase are reduced in various types of cancer resulting in elevated ROS levels especially H_20_2 (Oberley and Buettner 1979; Skrzydlewska, Sulkowski et al. 2005). In contrast to cancers with defective antioxidant capacity, some cancers can adapt to increased ROS levels, generated by exogenous or endogenous sources, by increasing the level of ROS-scavenging enzymes or GSH (Ray, Batra et al. 2000; Skrzydlewska, Kozuszko et al. 2003).

Strategies to induce ROS-mediated cell death

Trachootham et al. proposed a 'redox adaptation' theory which suggests that cancer cells adapt to increased ROS levels by enhancing their antioxidant capacity (Trachootham, Alexandre et al. 2009). On the contrary, normal cells can tolerate further increase in ROS due to their 'reserve' antioxidant capacity. However, cancer cells being more dependent upon their antioxidant system are more vulnerable to further increases in ROS or antioxidant inhibitors. Therefore, the intrinsic oxidative stress of cancer cells is a feature that can be exploited therapeutically. The strategies to induce ROS-mediated cell death include: 1) exposing cancer cells to ROS-inducing agents, 2) inhibiting antioxidant enzymes, 3) modulating cellular buffering capacity, and 4) a combination of the above.

ROS-inducing compounds in clinical use

Modulation of ROS signaling alone is not an ideal chemotherapeutic approach due to adaptation of cancer cells to ROS stress, activation of growth redundant pathways by ROS molecules, and toxicity associated with ROS-inducing anticancer drugs. The combination of ROS-inducing drugs with pharmaceuticals that can block the redox adaptation might be a better strategy for enhancing cancer cell death. Several antineoplastic drugs currently in clinical trials or approved by FDA for cancer chemotherapy induce high levels of oxidative stress (Fig. 2). These agents have been used alone and in combination with different pharmaceuticals to enhance cancer cell cytotoxicity. This section describes compounds currently in clinical trials as anti-cancer chemotherapeutics in which ROS has a primary cell-death effect. The following section discusses ROS-producing bioactive agents obtained from natural sources and their mechanisms of action.



Menadione Imexon Elesclomol

Figure 2. ROS-inducing clinical drugs for cancer treatment.

Menadione: Menadione is a polycyclic aromatic ketone that generates intracellular ROS through futile redox cycling. At low concentrations (2 μM), menadione-mediated oxidants trigger redox-dependent gene expression (Chuang, Chen et al. 2002). However, high concentrations (30-300 μM) of this compound have been shown to induce mitochondrial DNA damage and programmed cell death in rat pulmonary arterial endothelial cells and pancreatic

acinar cells (Grishko, Solomon et al. 2001; Criddle, Gillies et al. 2006). Gerasimenko et al. reported that menadione causes apoptosis in pancreatic acinar cells by inducing mitochondrial depolarization and intracellular release of Ca^{2+} (Gerasimenko, Gerasimenko et al. 2002). Further, Loor et al. explored the subcellular distribution of oxidative stress and the specific cell-death related genes in genetically modified cells lacking specific cell-death associated proteins. They reported an induction of oxidative stress in both the cytosol and mitochondrial matrix, decrease in mitochondrial potential, and release of cytochrome c in cardiomyocytes. Also, they concluded the involvement of poly (ADP-ribose) polymerase-1 (PARP-1) in all the cell-death pathways. Thus, menadione induces cell death through generation of oxidative stress in multiple subcellular compartments and activation of multiple cell death pathways in which PARP is the essential mediator (Loor, Kondapalli et al. 2010).

The combination of menadione and ascorbate induced ROS levels and exhibited anticancer activity against a wide variety of cell lines. This combination of a reducing agent (ascorbate) and a redox active quinone (menadione) generates redox cycling leading to formation of reactive oxygen species. Ascorbate reduces menadione to semiquinone, which in turn reduces molecular oxygen to produce superoxide anions with reoxidation of semiquinone back to the quinone menadione (Verrax, Delvaux et al. 2005). The reported mechanisms by which menadione and ascorbate together induce cell death involve glycolysis inhibition (Verrax, Vanbever et al. 2007), calcium homeostasis deregulation (Dejeans, Tajeddine et al. 2010), and an impairment of the chaperone function of Hsp90 (Beck, Verrax et al. 2009). Injection or oral administration of the agents has been shown to retard cancer growth and metastasis in tumor-bearing mice (Verrax, Stockis et al. 2006). The authors suggest that this therapy is well tolerated without evident damage to healthy tissues and should be assessed in clinical trials.

Imexon: Imexon is a thiol-reactive small molecule that rapidly forms thiol-adducts by binding to reduced sulfhydryls such as cysteine and glutathione. This binding causes a decrease in glutathione levels, accumulation of reactive oxygen species, loss of mitochondrial membrane potential, release of cytochrome c, and activation of caspases, thereby mediating apoptosis in myeloma cells (Dvorakova, Waltmire et al. 2001; Dvorakova, Payne et al. 2002; Evens, Prachand et al. 2004). Imexon is used for the treatment of Stage III and IV metastatic melanoma in combination with dacarbazine (Weber, Samlowski et al. 2010). Recently, a phase II clinical study has been conducted for the treatment of metastatic pancreatic cancer in combination with the chemotherapeutic agent gemcitabine (NCT00637247).

Elesclomol: A thio-hydrazide amide elesclomol is an oxidative stress inducer that triggers apoptosis in cancer cells. Transcriptome profiling of Hs294T melanoma cells showed an upregulation of heat shock proteins, metallothioneins, and Nrf2 which is a key transcription factor regulating oxidative stress-responsive genes (Kirshner, He et al. 2008). Elesclomol has been tested in several clinical trials with mixed results. In a phase II clinical trial in patients with stage IV metastatic melanoma, treatment with elesclomol in combination with paclitaxel doubled the survival time of patients (112 days) as compared to paclitaxel treatment alone (56 days) (O'Day, Gonzalez et al. 2009). However, phase III studies of elesclomol in combination with paclitaxel did not significantly improve the survival rate of metastatic melanoma patients (O'Day, Eggermont et al. 2013). It was observed that patients with normal serum lactate dehydrogenase levels responded better to the combination as compared to patients with high lactate dehydrogenase levels, suggesting LDH is the predictive factor for the response of melanoma patients to elesclomol in combination with paclitaxel. Table 1 summarizes a brief list of ROS-inducing antineoplastic agents in clinical trials that have been investigated.

Table 1. List of clinically used ROS-inducing agents

Name	**Mechanism of action**	**Cancer types**	**Stage of development**	**References**
Artesunate	Artemisinin derivative, induces ROS, and damages DNA	Hepatocellular carcinoma, uveal, melanoma, and breast cancer	Phase I	(Berger, Dieckmann et al. 2005), ClinicalTrials.gov Identifiers: NCT00764036, NCT02304289, NCT02354534
Motexafin gadolinium	Synthetic metallotexaphyrin, disrupts redox balance in cells by futile redox cycling	Lymphoma, leukemia, breast cancer, lung cancer, and renal cancer	Approved	ClinicalTrials.gov Identifiers: NCT00100711, NCT00076401, NCT00365183, NCT00134186, NCT00305864
Darinaparsin	Synthetic organic arsenical, generates cytotoxic arsenic compounds which induce ROS, cause cell-cycle arrest, and induce apoptosis	Hepatocellular carcinoma, myeloma, lymphoma	Phase I/II	ClinicalTrials.gov Identifiers: NCT00592046, NCT00421213, NCT00423306.
NOV-002	Disodium glutathione disulfide mimetic, alters intracellular GSH/GSSG ratio	Lung, breast, ovarian cancer	Approved	(P. Fidias 2010), ClinicalTrials.gov Identifiers: NCT00347412, NCT00345540, NCT00499122.
Buthionine sulfoximine	Gamma-glutamylcysteine synthetase inhibitor, inhibits glutathione synthesis	Melanoma, breast, neuroblastoma, leukemia, and ovarian cancer	Approved	(O'Dwyer, Hamilton et al. 1996), ClinicalTrials.gov Identifiers: NCT00005835, NCI-T93-0176O, NCI-T92-0206D.

ROS-inducing bioactive agents derived from natural products

Although numerous bioactive agents derived from natural plants are being exploited for anticancer therapy, several alkaloids isolated from plant sources have been reported to have pro-oxidant properties. Table 2 summarizes ROS-inducing naturally-isolated alkaloids and their mechanisms of action. Alkaloids are secondary metabolites containing basic nitrogen atoms isolated from various sources including plants. They are known to be an important component of current cancer chemotherapy as anti-microtubule agents which block cell division by preventing microtubule function (Dumontet and Jordan 2010).

Alkaloids isolated from different natural sources are inducers of mitochondrial-dependent apoptosis, where mitochondrial membrane dissipation accompanied by increased ROS leads to release of pro-apoptotic factors such as cytochrome c resulting in caspase activation and degradation of PARP (Kaminskyy, Kulachkovskyy et al. 2008). Also, an increase in ROS can activate signaling proteins such as extracellular-signal-regulated kinases and c-Jun N-terminal kinase as is the mechanism reported for ellipticine. This is a natural alkaloid isolated from the leaves of the tree *Ochrosia elliptica* with anticancer activity in multiple human malignant tissues including breast, ovarian, thyroid, and human endometrial adenocarcinoma (Kim, Lee et al. 2011).

Table 2. List of ROS-inducing alkaloids, isolated from plant sources.

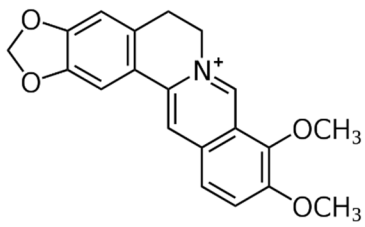
Compound	**Plant source**	**Mechanism of cell death**	**Type of alkaloid**	**Cell type**	**References**
 Berberine	*Coptis chinensis*	Increase of ROS levels, mitochondrial membrane potential dissipation, release of intracellular Ca^{2+}, activation of caspase-3, release of cytochrome c, decrease in Bcl-2 protein	Isoquinoline alkaloid	Human leukemia cells	(Lin, Kao et al. 2006; Hsu, Hsieh et al. 2007; Lin, Yang et al. 2007; Lin, Yang et al. 2007; Lin, Yang et al. 2008; Meeran, Katiyar et al. 2008; Chen, Lai et al. 2009; Ho, Lu et al. 2009; Eom, Kim et al. 2010; Burgeiro, Gajate et al. 2011)

Table 2. List of ROS-inducing alkaloids, isolated from plant sources (continued).

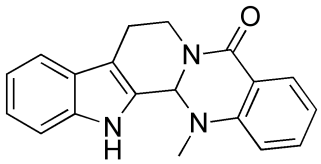
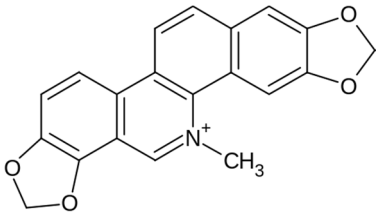
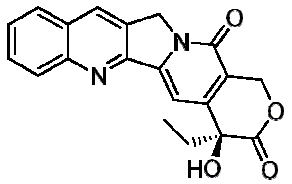
Compound	Plant source	Mechanism of cell death	Type of alkaloid	Cell type	References
 Evodiamine	*Evodia rutaecarpa Bentham*	Increase in ROS and NO levels, induction of mitochondrial membrane permeability, increase in Bax/Bcl-2 ratio, activation of autophagy-related proteins	Plant alkaloid	HeLa cells	(Lee, Kim et al. 2006; Yang, Wu et al. 2007; Yang, Wu et al. 2008)
 Sanguinarine	*Sanguinaria canadensis*	Generation of ROS, activation of caspase, PARP, disruption of mitochondrial membrane potential, release of cytochrome c and Smac/DIABLO, release of intracellular Ca^{2+}	Benzyisoquinoline alkaloid	Human melanoma cells	(Burgeiro, Bento et al. 2013)
 Camptothecin	*Camptotheca acuminata*	Loss of mitochondrial membrane potential, decrease in Bcl-2 protein, cytochrome c release, ROS generation, depletion of GSH	Quinoline alkaloid	Human cervical and uterine tumor cells	(Ha, Kim et al. 2009)

Table 2. List of ROS-inducing alkaloids, isolated from plant sources (continued).

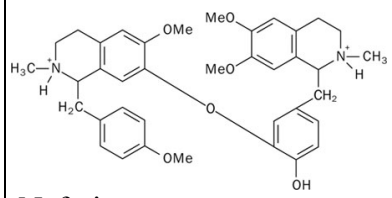
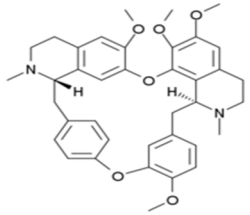
Compound	Plant source	Mechanism of cell death	Type of alkaloid	Cell type	References
 Neferine	*Nelumbo nucifera*	Increase of ROS levels, decrease in GSH, reduction in mitochondrial membrane potential, increase of apoptotic proteins, down-regulation of anti-apoptotic proteins, up-regulation of tumor suppressor proteins (p53 and PTEN)	Bisbenzylisoquinoline alkaloid	Liver cancer cells	(Poornima, Quency et al. 2013)
 Tetrandrine	*Stephaniae tetrandrae*	Increase ROS production, suppression of Akt activation, caspase-8, 9, and PARP activation	Bisbenzylisoquinoline alkaloid	Human hepato--cellular carcinoma	(Liu, Gong et al. 2011)

Table 2. List of ROS-inducing alkaloids, isolated from plant sources (continued).

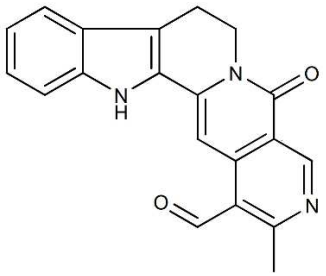
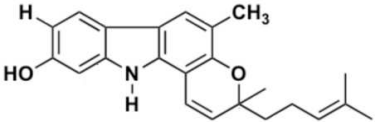
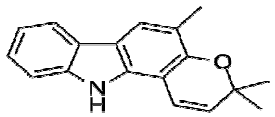
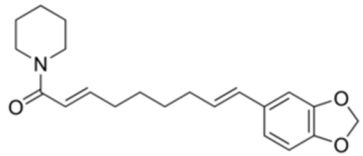
Compound	Plant source	Mechanism of cell death	Type of alkaloid	Cell type	References
 Subtidine	*Nauclea subtida*	Increase of ROS levels, increase expression of glutathione reductase, induction of DNA fragmentation, reduction mitochondrial membrane potential, release of cytochrome c, and down-regulation of Bcl-2 and Bcl-xl	Monoterpenoid indole alkaloid	Human prostate cancer cells	(Liew, Looi et al. 2014)
 Mahanine	*Murraya koenigii* and *Micromelum minutum*	Accumulation of ROS leading to dysfunction of Hsp-90 and disruption of Hsp90-Cdc37 complex, activation of caspase-3, 7 and cleaved PARP proteins	Carbazole alkaloid	Human pancreatic cancer cells	(Sarkar, Dutta et al. 2013)

Table 2. List of ROS-inducing alkaloids, isolated from plant sources (continued).

Compound	**Plant source**	**Mechanism of cell death**	**Type of alkaloid**	**Cell type**	**References**
 Girinimbine	*Murraya koenigii*	ROS generation, up-regulation of apoptotic proteins, down-regulation of anti-apoptotic proteins, increase in lysosomal membrane permeability, activation of caspase 3/7, 8, 9	Carbazole alkaloid	Human lung cancer cells	(Mohan, Abdelwahab et al. 2013)
 Pipernonaline	*Piper longum Linn.*	G_0/G_1 phase arrest, ROS generation, intracellular Ca^{2+} release, mitochondrial membrane depolarization, activation of caspase-3 and cleaved PARP	Piperidine alkaloid	Human prostate cancer cells	(Lee, Kim et al. 2013)

Apoptosis mediated by mitochondria

Mitochondria are considered central to cell death mechanisms. In cancer cells, mitochondria have an essential role in the neoplastic phenotype, especially with respect to apoptosis resistance, unlimited proliferation, and metabolic reprogramming (Galluzzi, Morselli et al. 2010; Tennant, Duran et al. 2010). A pro-death stimulus increases endogenous levels of ROS, especially in the mitochondria, stimulating the apoptotic machinery by promoting membrane permeabilization through mitochondrial permeability transition (Trachootham, Lu et al. 2008). This leads to a series of catastrophic events sealing the cell's fate (Fig. 3). These events include dissipation of mitochondrial transmembrane potential resulting in arrest of ATP synthesis, and translocation of various proteins from mitochondria to the cytosol where they execute cell death. These proteins can be direct caspase activators (eg. cytochrome c), indirect caspase activators (eg. Smac/Diablo), and caspase-independent cell death effectors (eg. apoptosis inducing factor) (Kroemer, Galluzzi et al. 2007). Experiments using antioxidants show that ROS act upstream of mitochondria membrane depolarization (Fleury, Mignotte et al. 2002), Bax relocalization, cytochrome c release, caspase activation, and nuclear fragmentation. Exogenous sources of ROS such as hydrogen peroxide can induce programmed cell death or necrosis depending upon the dose added (Guenal, Sidoti-de Fraisse et al. 1997). Therefore, the level of intracellular ROS at any moment can determine the fate of cell; low levels of ROS can induce programmed cell death whereas the accumulation of high levels of ROS promote necrosis or can lead programmed cell death-committed cells toward necrosis (Fleury, Mignotte et al. 2002).

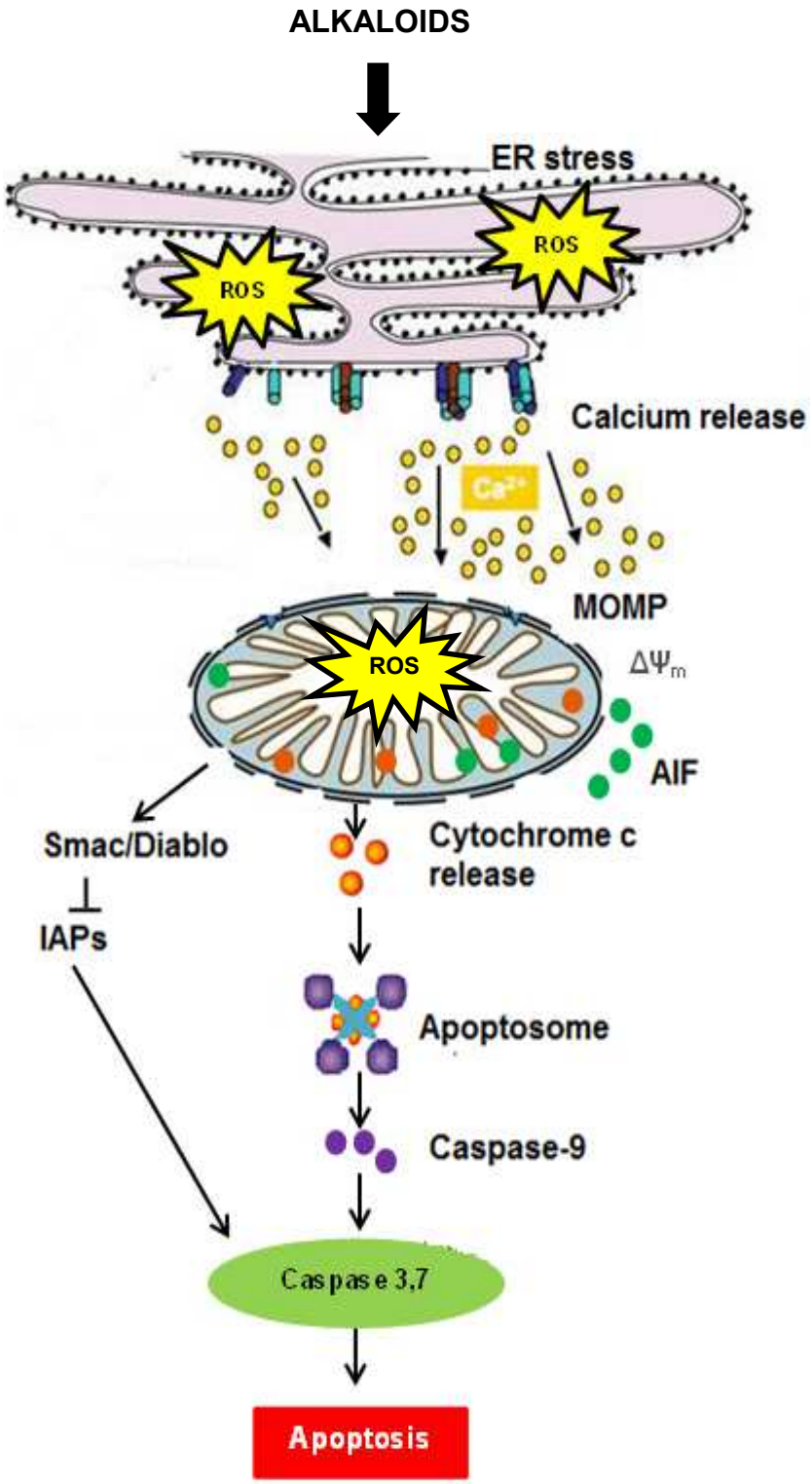


Figure 3. Mitochondria-mediated apoptosis induced by alkaloids. Adapted from (Chaudhari, Talwar et al. 2014; Ralph, Pritchard et al. 2015).

As an example, berberine, an isoquinone alkaloid and ROS inducer, selectively caused cell death in prostate cancer cells (PC-3) without affecting non-neoplastic human prostate epithelial cells (PWR-1E). Berberine disrupted the mitochondrial membrane potential and resulted in release of apoptotic molecules from mitochondria to the cytosol. The effects on mitochondrial membrane integrity and protein translocation was blocked by an antioxidant, N-acetyl cysteine (Meeran, Katiyar et al. 2008). Interestingly, berberine induced apoptosis in human glioblastoma T98G cells which was mediated by ER-stress, mitochondrial dysfunction, and involved production of ROS (Eom, Kim et al. 2010). Therefore, berberine triggers pro-oxidative events by disrupting the ER-mitochondria crosstalk, which eventually leads to cell death.

Piperlongumine

Piperlongumine (PPLGM) (also known as piplartine) is an amide alkaloid (Fig. 4) isolated from the fruits and roots of a variety of pepper plants including *Piper longum L.* that is found in abundance in the tropical regions of India, known as "Pippalli"(Chatterjee and Dutta 1967).

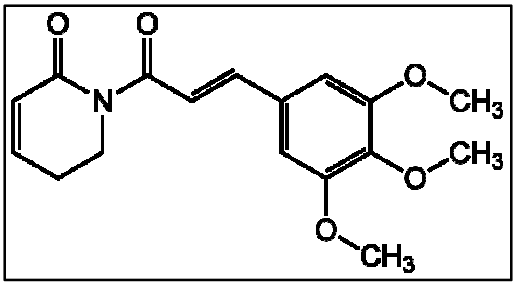


Figure 4. Structure of piperlongumine (1-[(2*E*)-3-(3,4,5-Trimethoxyphenyl)prop-2-enoyl]-5,6-dihydropyridin-2(1*H*)-one).

Long pepper is a flowering vine native of the Indo-Malaya region. It is wildly found in the tropical rainforests of India, Nepal, Indonesia, Malaysia, Sri Lanka, Timor, and Philippines

(Chatterjee and Dutta 1967). Long pepper has traditionally been used in ayurvedic medicine to treat various ailments such as bronchitis, asthma, cough, respiratory infections, cholera, malaria, gonorrhea, viral hepatitis, and tumors (Chopra RN 1956; Chaudhury, Chandrasekaran et al. 2001; Kumar, Kamboj et al. 2011). Alkaloids are the main constituents of the fruits and roots of the long pepper plant and include the following compounds; piperine, piperlongumine, piperlonguminine, and methyl-3,4,5- trimethoxycinnamate (Chatterjee and Dutta 1963).

PPLGM and cancer

PPLGM displays anticancer activity against numerous cancer cell lines as well as patient-derived breast, colon, and osteosarcoma tumor samples (Lee and Mandinova 2009) as shown in Table 3. PPLGM kills several types of cancer cells including colon, breast, lung (Duh, Wu et al. 1990), prostate, hematological, pancreatic, nasopharyngeal (Duh, Wu et al. 1990), glioblastoma (Bezerra, de Castro et al. 2008), bladder (Raj, Ide et al. 2011), and renal (Bokesch, Gardella et al. 2011). Interestingly, PPLGM is cytotoxic in tumor cells but shows only a weak activity in normal cells indicating its selective cytotoxic nature.

Table 3. Selective cytotoxicity of PPLGM in cancer cells with minimal effects on normal cells.

Cell lines	**Cell type**	**IC_{50}(μM)**	**References**
HL-60, K562, Jurkat, Molt-4	Human leukemia cells	5.28, 5.53, 2.49, 4.11	(Bezerra, Militao et al. 2007)
PC-3, DU-145	Human prostate cancer cells	4.9, 3.4	(Ginzburg, Golovine et al. 2014)
HT-29, HCT 116	Human colorectal cancer	10.1, 6.4	(Randhawa, Kibble et al. 2013)
AMC-HN3, AMC-HN7, AMC-HN8	Human head and neck cancer	~2.5, 6	(Roh, Kim et al. 2014)
MCF7, MDAMB231, MDAMB435	Human breast cancer	~12, ~7	(Raj, Ide et al. 2011; Jin, Lee et al. 2014)
MCF10A	Immortalized breast epithelial	>16	(Jin, Lee et al. 2014)
HEK293T	Human embryonic kidney cell line	60.23	(Gong, Chen et al. 2014)
A2780, OVCAR3	Human ovarian carcinoma	6.18, 6.20	(Gong, Chen et al. 2014)
HOK-1, HOK-2, HOF-1, HOF-2, HEK	Normal human cells [oral keratinocytes (HOK), oral fibroblasts (HOF), and human skin keratinocytes (HEK)]	>15	(Roh, Kim et al. 2014)
IMR32	Human neuroblastoma cells	>25	(Jyothi, Vanathi et al. 2009)
PAE, 76N, HKC, HDF, 184B5, MCF10A	Human normal cells [aortic endothelial (PAE), breast epithelial (76N), keratinocytes (HKC), skin fibroblasts (HDF), and breast epithelial cells]	>15	(Raj, Ide et al. 2011)
PANC-1, MIA PaCa-2, BxPC-3	Human pancreatic carcinoma	4.2, 4.6, 4.2	(Dhillon, Chikara et al. 2014)
U2OS, Saos-2	Human osteosarcoma	~7	(Raj, Ide et al. 2011)

Some of the reported anticancer mechanisms of PPLGM (Fig. 5) include activation of autophagy (Wang, Wang et al. 2013), necrosis (Bezerra, Militao et al. 2007), apoptosis, cell cycle arrest in G_1 or G_2/M phase (Kong, Kim et al. 2008), suppression of adhesion and invasion (Ginzburg, Golovine et al. 2014), and activation of cell death-related proteins. Intriguingly, PPLGM causes DNA damage responses including DNA double strand breaks, mutagenesis, and recombination (Bezerra, Moura et al. 2008).

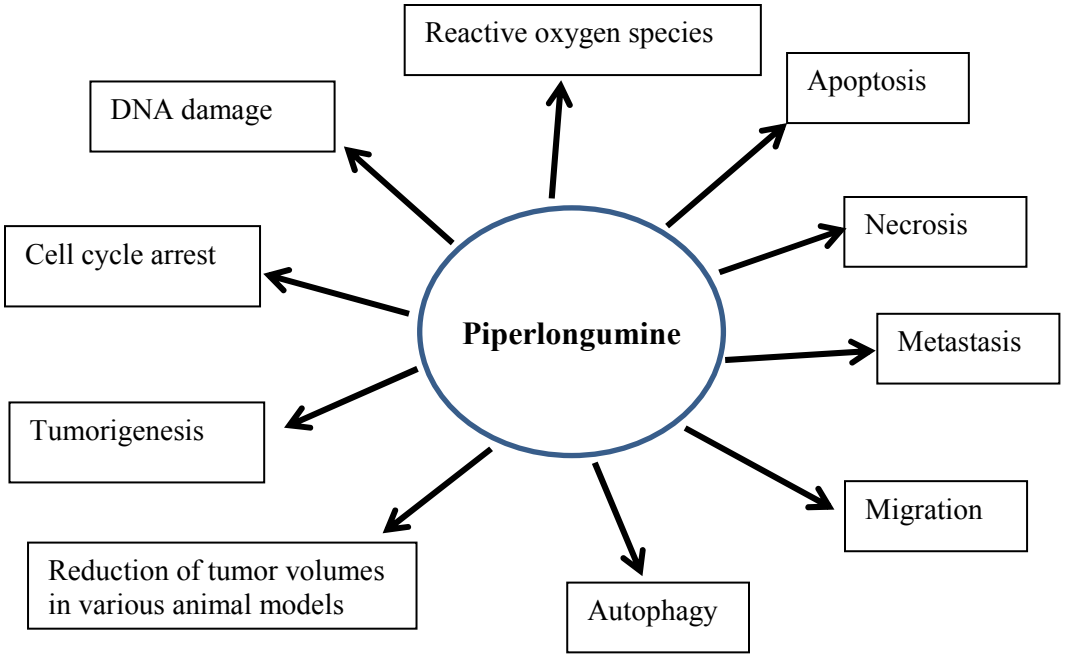


Figure 5. Anticancer properties of piperlongumine.

The cancer-selective cell death induced by PPLGM has been attributed to selective induction of ROS in cancer cells with no effect on normal cells. PPLGM has been shown to elevate nitric oxide and hydrogen peroxide levels with no effect on superoxide radicals, which can be reversed with an antioxidant, NAC. PPLGM increases ROS levels and cancer-selective cell death by directly binding and inhibiting the antioxidant enzyme, glutathione S-transferase pi 1 (GSTP1). Also, PPLGM decreases reduced glutathione levels, and increases oxidized

glutathione levels in cancer cells (Raj, Ide et al. 2011). These results indicate that PPLGM modulates redox and ROS homeostasis by binding to oxidative stress regulating proteins.

In addition to *in vitro* studies, the antitumor effects of PPLGM have been studied in various xenograft mouse models of human bladder carcinoma (EJ), human breast carcinoma (MDA-MB-436), human lung carcinoma (A549), and in a transgenic mouse model of spontaneous breast cancer (Raj, Ide et al. 2011). PPLGM-treated mice showed significantly reduced (50-80% reduction) tumor sizes. The mechanisms associated with the anti-tumor effects of PPLGM in a xenograft mouse model included enhanced expression of CDKN1A, PUMA, caspase-3, and reduced expression of VEGF. Together, the results indicate that PPLGM has pro-apoptotic and anti-angiogenic effects in vivo (Raj, Ide et al. 2011).

While numerous studies have investigated the anticancer potential of PPLGM, the anticancer mechanisms and signaling pathways associated with PPLGM-mediated cell death have not been studied in colorectal and pancreatic cancer; and was the focus of this study.

Colorectal cancer

Colorectal cancer is the third leading cause of cancer death in men and women in the United States (ACS). According to National Cancer Institute, 49,700 people will die due to colorectal cancer in 2015. Treatment for colorectal cancer patients varies with the disease stage. Surgical resection of the primary tumor and regional lymph nodes is the primary treatment for early colorectal cancer. However, adjuvant therapy such as chemotherapy and radiotherapy is recommended for patients with advanced stages of disease. Targeted therapy based on the genetic background of colorectal cancer patients has also been studied in clinical trials. EGFR is overexpressed in up to 85% of colorectal tumors; expression of which has been correlated with metastatic disease and poor prognosis (Aggarwal and Chu 2005). Furthermore, mutations in the

oncogene *KRAS*, which transduces signals from a cell surface receptor (ex. EGFR) to intracellular proteins, are detected in approximately 50% of colorectal tumors (Burmer and Loeb 1989). Typical chemotherapy for colon cancer consists of a combination of drugs that have been found to be the most effective, such as FOLFOX 4 (oxaliplatin, 5-fluorouracil (5-FU), and leucovorin) or FOLFIRI (folinic acid, 5-FU, and irinotecan) (Tournigand, Andre et al. 2004). However, chemoresistance and adverse side effects are major hurdles in the treatment of colorectal cancer.

Recent studies show that natural agents are effective in combating chemoresistance. For instance, curcumin can inhibit the development of chemoresistance to FOLFOX through effects on insulin-like growth factor 1 receptor (IGF-1R) and/or endothelial growth factor receptor (EGFR) (Patel, Gupta et al. 2010). Use of curcumin in combination with the targeted drug dasatinib eliminated colon cancer cells' resistance to FOLFOX (Nautiyal, Kanwar et al. 2011). Curcumin has also been shown to sensitize colorectal cancer cells to the lethal effects of radiation therapy (Sandur, Deorukhkar et al. 2009).

A 25-fold difference in the incidence of colorectal cancers has been observed in geographical areas within North America, Australia, New Zealand, Western Europe, and selected areas of Eastern Europe having the highest rates (Parkin 2004). A diet containing whole foods with nutrients and fibers provides protection against colorectal cancer. A study using the Dietary Approaches to Stop Hypertension (DASH) diet, which is high in whole grains, fruit, and vegetables; moderate amounts of low-fat dairy; and lower amounts of red or processed meats, desserts, and sweetened beverages, found that DASH diet reduced the risk of colon cancer by nearly 20% and rectal cancers by 27% (Fung, Hu et al. 2010). Another study of patients with stage III colon cancer divided their dietary habits into two dietary patterns. The "Prudent" pattern

was characterized by high intakes of fruits and vegetables, poultry, and fish; and the "Western" pattern was characterized by high intakes of meat, fat, refined grains, and dessert. Results showed that patients with "Prudent" diet had less recurrence of colon cancer and were more likely to still be alive after five years (Meyerhardt, Niedzwiecki et al. 2007).

Pancreatic cancer

Pancreatic cancer is the fourth leading cause of cancer-related death in the United States. It is an aggressive malignant disease characterized by invasiveness, rapid proliferation, and resistance to treatment (Bardeesy and DePinho 2002). Although it only accounts for 3% of all diagnosed cancers, the 5-year survival rate of pancreatic cancer patients is less than 7% (Howlader N 2015). This extremely low survival rate is attributable to poor prognosis and late stage diagnosis, such that surgical removal is ineffective. Pancreatic cancers can be formed either by exocrine or endocrine cells of pancreas.

Exocrine tumors: The most common type of pancreatic cancer originates in the exocrine regions of the pancreas. Of the exocrine pancreas tumors, 95% are classified as adenocarcinomas (a cancer originating in the glandular cells). These tumor cells usually originate in the pancreatic ducts, but also develop from the exocrine gland cells that produce pancreatic enzymes (acinar cell carcinomas). Other cancers of the exocrine pancreas include adenosquamous carcinomas, squamous cell carcinomas, signet ring cell carcinomas, undifferentiated carcinomas, and undifferentiated carcinomas with giant cells.

Endocrine tumors: Pancreatic tumors can also arise in the endocrine regions of the pancreas; however, these tumors make up less than 4% of all pancreatic cancers. They are also known as pancreatic neuroendocrine tumors (NETs) or islet cell tumors. About half of the

endocrine tumors make hormones that are released into the blood, hence, they are called functioning tumors.

The first genetic changes detected in the progression of pancreatic cancer are mutations in *KRAS*, a member of the *RAS* family of small GTPases that is involved in intracellular signaling (Bardeesy and DePinho 2002; Wong and Lemoine 2009). Recent findings in mice suggest that pancreatic cancer initiation, progression, and maintenance depend on KRAS-PI3K-PDK1 signaling (Eser, Reiff et al. 2013). Oncogenic *KRAS* engages the PI3K-PDK1-AKT or RAF-MEK1/2-ERK1/2 pathway to drive the initiation, progression, and maintenance of pancreatic cancer and more than 85% of pancreatic cancer patients have *KRAS* mutation at the early stage of cancer development (Li, Xie et al. 2004).

KRAS mutation

RAS, a GTPase protein, is a molecular switch which controls the transduction of signals from the cell-surface receptors (such as EGFR) to intracellular targets. It is an integral part of the RAS-RAF-MAPK pathway downstream of EGFR, and is associated with promotion of cell growth and survival (Adjei 2001).

All mammalian cells express three closely related RAS proteins, namely, HRAS, KRAS, and NRAS, and mutations in all three of these RAS proteins promotes oncogenesis (Rodenhuis 1992). It is estimated that about 15% to 20% of all human tumors contain a point mutation in one of the three *RAS* genes. Intriguingly, mutations in *KRAS* predominate in carcinomas. Studies have reported *KRAS* mutations in about 30% of lung adenocarcinomas, 50% of colon carcinomas and more than 90% of pancreatic carcinomas (Minamoto, Mai et al. 2000). The majority of *RAS* mutations involve codon 12 of the gene, with a smaller number involving other regions such as codons 13 or 61 (Oudejans, Slebos et al. 1991). As a consequence of *RAS* mutations, the signal

transduction function of the RAS protein is consistently active, promoting cell proliferation and constant cell division. Different RAS inhibitors under investigation include tipifarnib (a farnesyltransferase inhibitor), romidepsin (a histone deacetylase inhibitor), and fanesylthiosalicylic acid (salirasib).

Numerous FDA-approved drugs are being used for treatment of pancreatic cancer patients irrespective of *KRAS* gene status. One of the drugs currently being used for advanced stage or metastasized pancreatic cancer is gemcitabine hydrochloride. Gemcitabine, sold under the brand name, Gemzar, belongs to a class of drugs known as antimetabolites that stop or slow the growth of cancer cells by inhibiting DNA replication. Inside the cells, gemcitabine is converted into active metabolites, difluorodeoxycytidine di- and triphosphate (dFdCDP, dFdCTP). dFdCDP decreases the deoxynucleotide pool available for DNA synthesis by inhibiting ribonucleotide reductase, whereas dFdCTP is incorporated into the DNA, thereby causing DNA strand termination and apoptosis. Although gemcitabine by itself is marginally effective in treating pancreatic cancer, several combination strategies are being explored in order to increase the survival of pancreatic cancer patients (Haller 2003). Many clinical trials combining gemcitabine with other agents have demonstrated the effectiveness of combinatorial treatment of advanced stage pancreatic cancer. For example, in a phase III trial of gemcitabine versus gemcitabine and nab-paclitaxel, the survival of metastatic pancreatic adenocarcinoma patients was increased by 1.8 months in the nab-paclitaxel group as compared to gemcitabine group (Von Hoff, Ervin et al. 2013).

In addition to *KRAS* mutations, over-expression of EGFR (a transmembrane receptor tyrosine kinase) and its ligands EGF and TGF-α (43, 46 and 54%, respectively) is frequently observed in pancreatic cancer (Yamanaka, Friess et al. 1993; Wong and Lemoine 2009).

Erlotinib hydrochloride, a small molecule tyrosine kinase inhibitor (TKI), sold under the brand name, Tarceva, is used to treat several types of cancer including pancreatic cancer. Erlotinib is an orally active TKI that binds to ATP binding site of EGFR. In a phase III Canadian clinical trial, erlotinib in combination with gemcitabine improved the median overall survival (6.24 versus 5.91 months) and 1-year survival rate (23% versus 17%) in advanced pancreatic cancer patients as compared to patients with gemcitabine plus placebo, which led to its approval as the first targeted therapy for patients with advanced pancreatic cancer (Moore, Goldstein et al. 2007). A phase II/III clinical trial is undergoing with gemcitabine with or without erlotinib and radiotherapy in pancreatic cancer patients whose tumor has been surgically removed (NCI-2011-01987).

Although erlotinib in combination with gemcitabine is being explored in pancreatic cancer patients, the high percentage of *KRAS* mutations in pancreatic cancer patients limits the efficacy of chemotherapy. For example, *KRAS* mutant pancreatic cancer patients treated with erlotinib had a median survival of 5.2 months compared to wild-type *KRAS* pancreatic cancer patients who had a median survival of 9.7 months (Kim, Lim do et al. 2011). Similarly, a German study correlated patients with advanced pancreatic cancer harboring *KRAS* mutation with a poor survival rate as compared to wild type *KRAS* patients (Boeck, Vehling-Kaiser et al. 2010). This points to a critical need for alternative therapeutic agents for pancreatic cancer patients with *KRAS* mutations.

Objective and specific aims

The objectives for this study were to determine the *in vitro* anticancer mechanisms of PPLGM in *KRAS* mutant colon and pancreatic cancer cells, and to investigate the *in vivo* anticancer effects of PPLGM and its associated mechanisms using *KRAS* mutant pancreatic

cancer mouse models. We hypothesized that PPLGM induces *KRAS* mutant pancreatic and colon cancer cell death *in vitro* and *in vivo* by inducing oxidative stress and activating apoptotic pathways. Therefore, the following specific aims were addressed in this thesis:

Specific aim 1: To investigate the mechanisms by which PPLGM induces *KRAS* mutant pancreatic cancer cell death *in vitro* and *in vivo*. This aim is addressed in Chapter 2, which is a published paper.

Specific aim 2: To identify the signaling pathways by which PPLGM induces *KRAS* mutant pancreatic cancer cell death by using an RNA sequencing approach. This aim is addressed in Chapter 3.

Specific aim 3: To determine the efficacy of PPLGM in combination with chemotherapeutic agents, gemcitabine and erlotinib, in *KRAS* mutant pancreatic cancer cells *in vitro* and *in vivo*. This work is the focus of Chapter 4.

Specific aim 4: To determine the anticancer potential of PPLGM in *KRAS* mutant and wild-type colon cancer cells**,** and to investigate the mechanism**s** of cell death. This aim is addressed in Chapter 5, which is a published paper.

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CHAPTER 2. PIPERLONGUMINE INDUCES PANCREATIC CANCER CELL DEATH BY ENHANCING REACTIVE OXYGEN SPECIES AND DNA DAMAGE[1]

Abstract

Pancreatic cancer is one of the most deadly cancers with a nearly 95% mortality rate. The poor response of pancreatic cancer to currently available therapies and the extremely low survival rate of pancreatic cancer patients point to a critical need for alternative therapeutic strategies. The use of reactive oxygen species (ROS)-inducing agents has emerged as an innovative and effective strategy to treat various cancers. In this study, we investigated the potential of a known ROS inducer, piperlongumine (PPLGM), a bioactive agent found in long peppers, to induce pancreatic cancer cell death in cell culture and animal models. We found that PPLGM inhibited the growth of pancreatic cancer cell cultures by elevating ROS levels and causing DNA damage. PPLGM-induced DNA damage and pancreatic cancer cell death was reversed by treating the cells with an exogenous antioxidant. Similar to the *in vitro* studies, PPLGM caused a reduction in tumor growth in a xenograft mouse model of human pancreatic cancer. Tumors from the PPLGM-treated animals showed decreased Ki-67 and increased 8-OHdG expression, suggesting PPLGM inhibited tumor cell proliferation and enhanced oxidative stress. Taken together, our results show that PPLGM is an effective inhibitor for *in vitro* and *in vivo* growth of pancreatic cancer cells, and that it works through a ROS-mediated DNA damage pathway. These findings suggest that PPLGM has the potential to be used for treatment of pancreatic cancer.

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Introduction

Pancreatic cancer is one of the most deadly cancers in the United States with a 5-year survival rate of less than 6% (Siegel, Naishadham et al. 2013). The vast majority of pancreatic cancer patients present with advanced disease, at which point surgery is no longer an option (Heinemann and Boeck 2008). The best chemotherapy currently available has a minimal impact on advanced pancreatic cancers, and extends patients' lives by only a couple of months (Stathis and Moore 2010). There is a critical need to develop new therapeutic strategies to enhance the survival of pancreatic cancer patients.

A new approach for pancreatic cancer treatment is the use of reactive oxygen species (ROS)-inducing small molecules that take advantage of the altered redox state in cancer cells (Qanungo, Das et al. 2005; Zhang, Humphreys et al. 2008; Jutooru, Chadalapaka et al. 2010). Cancer cells exhibit elevated levels of ROS as well as antioxidant enzymes (Fruehauf and Meyskens 2007). As a result, cancer cells are more vulnerable than normal cells to agents that induce further oxidative stress or impair the antioxidant response (Pelicano, Carney et al. 2004).

Several ROS-inducing small molecules have been tested in clinical trials for the treatment of pancreatic cancer. The ROS inducer β-lapachone causes cytotoxicity in NAD(P)H:quinone oxidoreductase (NQO1)-overexpresssing pancreatic tumors by modulating PARP, NAD^+/ATP levels, leading to single-stranded DNA breaks, and necrosis (Ough, Lewis et al. 2005; Li, Bey et al. 2011). Clinical trials of β-lapachone in combination with gemcitabine have been used for the treatment of metastatic pancreatic adenocarcinoma. Further, phase I and II clinical studies for imexon, a small molecule pro-oxidant have been conducted in pancreatic cancer patients (Cohen, Zalupski et al. 2010). Imexon induces apoptosis in pancreatic cancer cells by elevating ROS levels and causing cell cycle arrest (Dorr, Raymond et al. 2005).

Given the promise of ROS-inducing agents for cancer treatment, we investigated the effects of the ROS-inducer piperlongumine (PPLGM) on pancreatic cancer cell death *in vitro* and *in vivo*. PPLGM is an alkaloid found in the fruits of long pepper plants that displays potent growth-inhibitory properties in a variety of cancer cell lines and various animal models. Interestingly, PPLGM has been shown to be non-toxic to several normal cell types and tissues (Raj, Ide et al. 2011; Randhawa, Kibble et al. 2013). In this study, PPLGM's effect on ROS levels, DNA damage, and cell death were evaluated in cell culture and animal models to evaluate the potential of PPLGM as an alternative therapeutic approach to treating pancreatic cancer.

Materials and Methods

Materials

Piperlongumine (PPLGM) was purchased from Indofine Chemical Company (Catalog#: P-004, 97%, Hillsborough, NJ). PPLGM was dissolved in 100% DMSO at a stock concentration of 10 mM and then diluted in water to a working concentration. The final concentration of PPLGM was in the range of 0.1-20 μM. pChk1 (S296) and total Chk1 antibodies were purchased from Cell Signaling Technologies (Danvers, MA). Mouse anti Ki-67 primary antibody (clone MM1) was purchased from Vector Labs (Burlingame, CA). Anti-8-hydroxy-2′ deoxyguanosine (8-OHdG) monoclonal (N45.1) primary antibody was purchased from Japan Institute for the Control of Aging (JaICa, Shizuoka, Japan). CF633-conguated goat anti-mouse IgG secondary antibody was purchased from VWR (Atlanta, GA).

Cell culture

The PANC-1 and MIA PaCa-2 cell lines were obtained from ATCC in 2013 (Manassas, VA) and cultured at 37 °C with 5% carbon dioxide in Dulbecco's Modified Eagle's (Thermo

Scientific; Waltham, MA) medium supplemented with 10% fetal bovine serum (Atlanta Biologicals; Lawrenceville, GA). The BxPC-3 cell line was also obtained from ATCC and cultured in RPMI-1640 (Thermo Scientific; Waltham, MA) medium supplemented with 10% fetal bovine serum. The cell lines were subcultured by enzymatic digestion with 0.25% trypsin/1 mM EDTA solution (Thermo Fisher) when they reached approximately 70% confluency.

AlamarBlue® cell toxicity assay

PANC-1, MIA PaCa-2, and BxPC-3 cells (5.0×10^3) were seeded into individuals wells of a 96-well plate, and 24 hours later were treated with PPLGM (0-20 μM) after which alamarBlue® (AbD Serotech; Raleigh, North Carolina) was added at a final concentration of 10% and incubated at 37 °C for 4 hours. The oxidized form of the dye is converted into the reduced form by a mitochondrial enzyme present in the viable cells. Absorbance was measured at 570 and 600 nm on a plate reader. The cells were monitored daily over a 3-day period to gauge potential shifts in absorbance. The percent reduction in alamarBlue® over time for each treatment was calculated by using the following formula:

$$\% \text{ reduction in alamarBlue®} = \frac{((117216)(A_1) - (80586)(A_2))}{((155677)(B_1) - (14652)(B_2))} \times 100$$

In the formula, 117216 and 80586 are constants representing the molar extinction coefficients of alamarBlue® at 570 and 600 nm, respectively, in the oxidized form; whereas 115677 and 14652 are constants representing the molar extinction coefficients of alamarBlue® at 570 and 600 nm, respectively, in the reduced form. A_1 and A_2 represent absorbance of wells treated with PPLGM at 570 and 600 nm, respectively. B_1 and B_2 represent absorbance of untreated wells at 570 and 600 nm, respectively. A reduction in alamarBlue® absorbance

correlates to a decrease in cell viability. The data represent the % cell viability relative to control ± standard deviation in eight replication wells per treatment for three independent experiments. The half maximal inhibitory concentrations (IC_{50}) were calculated by fitting the dose-response curves derived after plotting the percent cell viability against the log concentration. Eight replicate wells were used per treatment and the experiments were performed in triplicate for each cell line.

Clonogenic-survival assay

The clonogenic-survival assay tests the long term survival ability of cells in the presence of an anticancer agent. PANC-1, MIA PaCa-2, and BxPC-3 cells (5×10^2) were seeded into individual wells of a 24-well plate. The next day, the cells were treated with 0-20 μM PPLGM for 24 hours. The cells were allowed to grow and form colonies for 14 days. After 14 days, the colonies were fixed in a solution of methanol and acetic acid (3:1), stained with 0.5% crystal violet, and counted manually. Four replicate experiments were performed for each cell line.

Measurement of ROS by the 2,7-dicholorodihydrofluorescein diacetate (DCF-DA) assay

Approximately 5.0×10^5 cells/ml of all three pancreatic cancer cell lines were suspended in culture medium and treated with 10 μM PPLGM for 6 hours. After treatment, cells were harvested by centrifugation and re-suspended in 10 μM DCF-DA (Life Technologies, Carlsbad, CA) in PBS. The cells were incubated at 37 °C for 30 min before flow cytometric analysis using an Accuri C6 Flow Cytometer. The experiments were performed in triplicate for each cell line.

Q-PCR

Total RNA was isolated from PANC-1 cells (1.0×10^6cells) using the Fisher SurePrep Kit (Waltham, MA) as per the manufacturer's instructions. 100 ng of total RNA were reverse transcribed into cDNA using the qScript cDNA synthesis kit (Quanta Biosciences; Gaithersburg,

MD). Primers against human SOD1, GSTP1, and HO1 were designed using Primer Express software (version 2.0, Applied Biosystems), and synthesized by Integrated DNA Technologies (Coralville, IA). Primer sequences were as follows, HO-1 forward: AATTCTCTTGGCTGGCTTCCT; HO-1 reverse: CATAGGCTCCTTCCTCCTTTCC; GSTP1 forward: CAGGAGGGCTCACTCAAAGC; GSTP1 reverse: AGGTGACGCAGGATGGTATTG; SOD1 forward: GCCTGCATGGATTCCATGTT, SOD1 reverse: TGGCCCACCGTGTTTTCT. Steady-state mRNA levels of antioxidant genes were determined for the cDNAs by real-time PCR using PerfeCTa SYBR Green FastMix (Quanta Biosciences). The cycling parameters were 95 °C for 10 min followed by 40 cycles of 95 °C for 30 sec and 60 °C for 1 min and a dissociation program that included 95 °C for 1 min, 55 °C for 30 sec, and 95 °C for 30 sec ramping up at 0.2 °C/sec. One distinct peak was observed for the primer sets. The fold change in mRNA expression was calculated by comparing the 18S rRNA-normalized threshold cycle numbers (C_T) in the PPLGM-treated cancer cells compared to the DMSO-treated cancer cells. Duplicate wells were run for each experiment and the experiments were performed in triplicate.

Cell survival assay

For determining cell survival, human pancreatic cancer cell lines (4.0×10^5cells) were treated with the vehicle control (0.1% DMSO), 10 μM PPLGM, 3 mM N-acetyl cysteine (NAC), and 10 μM PPLGM in combination with 3 mM NAC for 24 hours. The next day, images were taken using a Leica DMIL inverted microscope with a DFC290 digital color camera. The effect of treatments on the number of surviving pancreatic cancer cells can be viewed in the images.

DNA fragmentation assay

MIA PaCa-2 cells (5.0×10^5cells) were seeded into individual wells of a 6-well plate, and 24 hours later were treated with vehicle control (0.1% DMSO), 10 μM PPLGM, 3 mM N-acetyl cysteine (NAC), and 10 μM PPLGM in combination with 3 mM NAC for 24 hours. After 24 hours, the cells were washed twice with PBS, and the DNA was extracted using DNAzol reagent (Molecular Research; Cincinnati, OH, USA). Isolated DNA was resolved on 1.5% agarose gels containing ethidium bromide (EtBr) and images were captured using the MultiImage™ Light Cabinet (Alpha Innotech; San Leandro, CA). The experiments were performed in triplicate.

Western blot

MIA PaCa-2 cells (1×10^6cells) were treated with or without 10 μM PPLGM and 5 μM GEM for 24 hours. Cell pellets were lysed using an SDS lysis buffer (Cell Signaling Technologies) containing protease and phosphatase inhibitors (Roche; Indianapolis, IN). Samples were briefly sonicated to dissociate cell membranes. Sixty micrograms of total protein isolated from PPLGM or GEM-treated MIA PaCa-2 cells were separated on 10% SDS-polyacrylamide gels at 100 V for 1 h. Proteins were transferred to nitrocellulose membranes at 100 V for 70 min at 4 °C. Blots were then probed overnight at 4 °C with primary antibodies. The next day, blots were rinsed with 1X TBS-tween (0.1%) and probed with secondary antibody for 1 h at room temperature. The western blots were analyzed using SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific; Rockford, IL) and images were captured using the MultiImage™ Light Cabinet (Alpha Innotech; San Leandro, CA). pChk1 (S296) levels were normalized to total Chk1 expression. Immunoblots were performed in triplicate and the image in the figure represents one typical replicate.

Nude mouse studies

Six- to 8-week old female athymic nude mice (Nu/Nu) were purchased from Charles River Laboratories (Wilmington, MA). The mice were maintained in sterile conditions using the Innovive IVC System (Inovive; San Diego, CA), following the protocol approved by North Dakota State University's Institutional Animal Care and Use Committee (IACUC). The mice were acclimated for 1 week before experimental manipulation. Tumor xenografts were established by subcutaneous injection of 2.0×10^6 PANC-1 cancer cells in 0.1 ml PBS in the rear flank of the animals. Once tumor masses became established, the mice were randomly divided into two groups (n = 8). Group 1 served as the control group and received dimethyl sulfoxide (DMSO, 1%) by intraperitoneal injection. Group 2 received 2.4 mg PPLGM/kg body weight daily for 30 days by intraperitoneal injection. PPLGM was dissolved initially in DMSO and further diluted in PBS before administering to the mice, and the final concentration of DMSO was 1%. Two axes of the tumor (L, longitudinal axis; W, shortest axis) were measured with a caliper three times a week and each mouse was weighed weekly. The tumor volume was calculated as: $V = L \times W^2)/2$. Following four weeks of experimental treatment, the animals were euthanized in an isoflurane chamber followed by cervical dislocation.

Immunohistochemistry

Tumor tissues from control and PPLGM-treated mice were collected and fixed for 24 hours in formaldehyde. Paraffin-embedded 5 μm thick sections of tumor tissues were prepared. Sections were deparaffinized with histoclear and ethanol, followed by antigen retrieval in 10 mM sodium citrate buffer (0.05% Tween 20, pH 6.0) using an autoclave method. The sections were blocked for 20 minutes in blocking buffer (10% normal goat serum in TBST) and incubated with primary antibodies (Ki-67 [1:100] and 8-OHdG [1:20]) overnight at 4 °C. The

next day, sections were incubated with CF633-conjugated goat anti-mouse secondary antibody (1:250) for an hour at room temperature, and were visualized using a Zeiss inverted Axio Observer Z1 microscope after mounting a coverslip using Hardset Mounting media with DAPI (Vector Labs; Burlingame, CA).

Statistical analyses

Data are presented as means ± standard deviation for at least 3 independent experiments. The significance of differences between groups was determined using a student's t-test with statistical significance defined as $p < 0.05$. Statistical analyses were performed using SigmaPlot v12.

Results

PPLGM causes concentration- and time-dependent growth inhibition of pancreatic cancer cells

The short-term, growth inhibitory effects of PPLGM on three pancreatic cancer cell lines were investigated using the alamarBlue® assay. The cell lines were treated with either the vehicle control (DMSO, <0.1%) or increasing concentrations of PPLGM (0.1-20 μM) for 0-3 days, and cell growth was assessed each day. PPLGM induced a concentration and time-dependent decrease in the viability of PANC-1, MIA PaCa-2, and BxPC-3, with IC_{50} values of 4.2, 4.6, and 4.2 μM, respectively, at 72 hours (Fig. 6 A, B, and C). Twenty micromolar PPLGM significantly inhibited the growth of all pancreatic cancer cell lines by Day 1, while 5 μM PPLGM significantly inhibited growth by Day 2. BxPC-3 cells were slightly more sensitive to lower concentrations of PPLGM than PANC-1 and MIA PaCa-2 cells, where the 2.5 μM and 1 μM treatments significantly reduced BxPC-3 growth at Days 2 and 3, respectively.

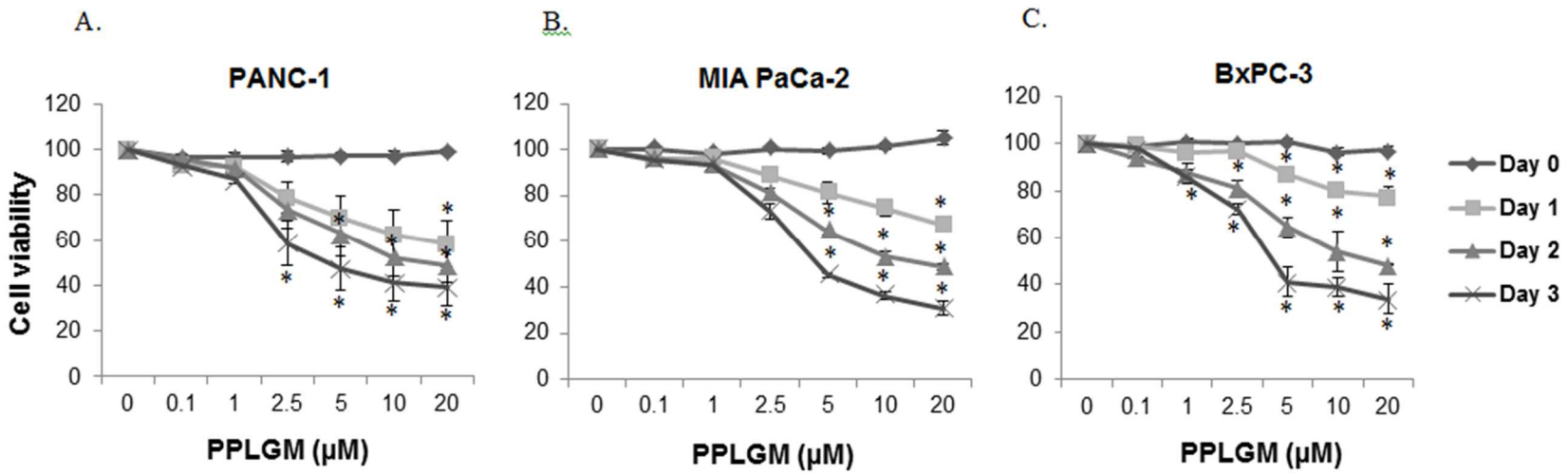

Figure 6. Effect of PPLGM on the short-term growth of three pancreatic cancer cell lines. The three pancreatic cancer cell lines (A) PANC-1, (B) MIA PaCa-2, and (C) BxPC-3 were treated with various concentrations (0-20 μM) of PPLGM for up to 3 days. The short-term growth of pancreatic cancer cells was measured spectrophotometrically using an alamarBlue® assay at Day 0, 1, 2, and 3. The metabolic activity of living cells is detected by a change in color of alamarBlue® from an oxidized (blue) form to a reduced (purple) form. The data represent the average percent cell viability relative to control ± standard deviation in eight replicate wells per treatment for three independent experiments. Statistical significance was determined by Student's t-test ($p < 0.05$ for PPLGM-treated vs. vehicle control).

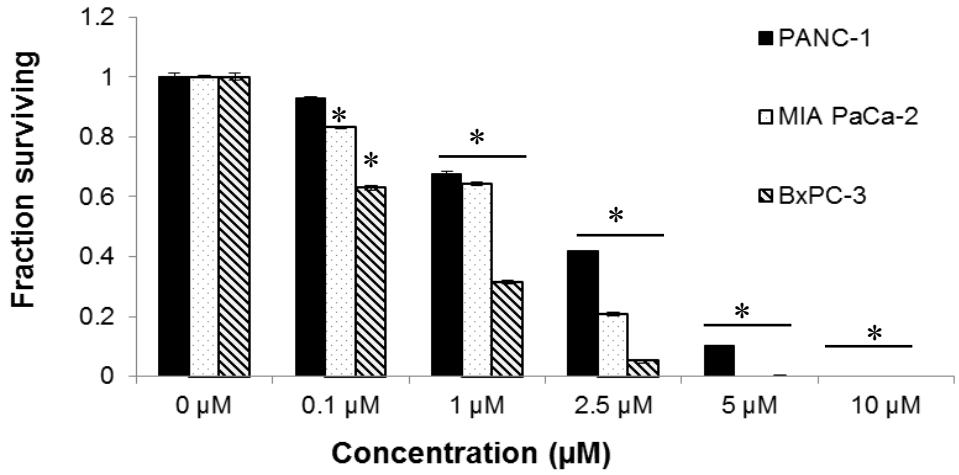

Figure 7. Effect of PPLGM on the long-term growth of three pancreatic cancer cell lines. The long-term growth of all three cell lines was determined by calculating the fraction of cells surviving in the presence of PPLGM (0-10 µM) over the course of 14 days by clonogenic survival assay. The data represent the average ± standard deviation for three independent experiments for PANC-1, MIA PaCa-2, and BxPC-3 cells. Statistical significance was determined by Student's t-test ($p < 0.05$ for PPLGM-treated vs. vehicle control).

PPLGM causes a concentration-dependent decrease in long-term survival of pancreatic cancer cells

To evaluate the effects of PPLGM on long-term survival of pancreatic cancer cell lines, a clonogenic survival assay was performed. PANC-1, MIA PaCa-2, and BxPC-3 cells were seeded at a low density in culture dishes, allowed to adhere overnight, treated with either the vehicle control (DMSO) or increasing concentrations of PPLGM (0.1-10 µM), and then incubated, undisturbed, for 14 days. The total number of colonies formed was counted for each culture dish. The data represent the average number of colonies formed for three replicates of the respective treatment groups. The results show that PPLGM caused a concentration-dependent decrease in the number of colonies formed for all three cell lines as compared to the controls (Fig. 7). MIA PaCa-2 and BxPC-3 cells were particularly sensitive to PPLGM, and no cells survived for the 5 and 10 µM treatments.

PPLGM elevates ROS levels in pancreatic cancer cell lines without significantly altering the expression of antioxidant response enzymes

PPLGM is known to cause elevated levels of ROS in cancer cell lines (Raj, Ide et al. 2011), and enhanced ROS levels are associated with cancer cell death for various agents (Renschler 2004). Therefore, we examined the role of ROS in PPLGM-induced pancreatic cancer cell death. PANC-1, MIA PaCa-2, and BxPC-3 cells were treated with 10 μM PPLGM for 6 hours followed by staining for 30 minutes with the redox-sensitive fluorescent probe DCFDA. Intracellular ROS oxidize DCFDA to the highly fluorescent DCF compound which can be detected by flow cytometry. Fig. 8A shows PPLGM increases the production of ROS in all three pancreatic cancer cell lines compared to the vehicle control treatment. The PPLGM-induced ROS levels were quantified and the results show that the MIA PaCa-2 cell line experienced a 24-fold increase in ROS relative to the control-treated cells, followed by PANC-1 cells with a 9-fold increase, and Bx-PC3 cells with an 8-fold increase (Fig. 8B).

ROS levels can increase within a cell by two primary mechanisms: 1) increased ROS production or 2) decreased antioxidant scavenging. To determine if PPLGM elevates ROS by inhibiting the antioxidant capacity of pancreatic cancer cells, we performed qPCR to measure the mRNA transcript levels of SOD1, GSTP1, and HO1. The mRNA levels for these antioxidant enzymes were slightly, but not significantly ($p > 0.05$) elevated in PPLGM-treated pancreatic cancer cells (Fig. 9).

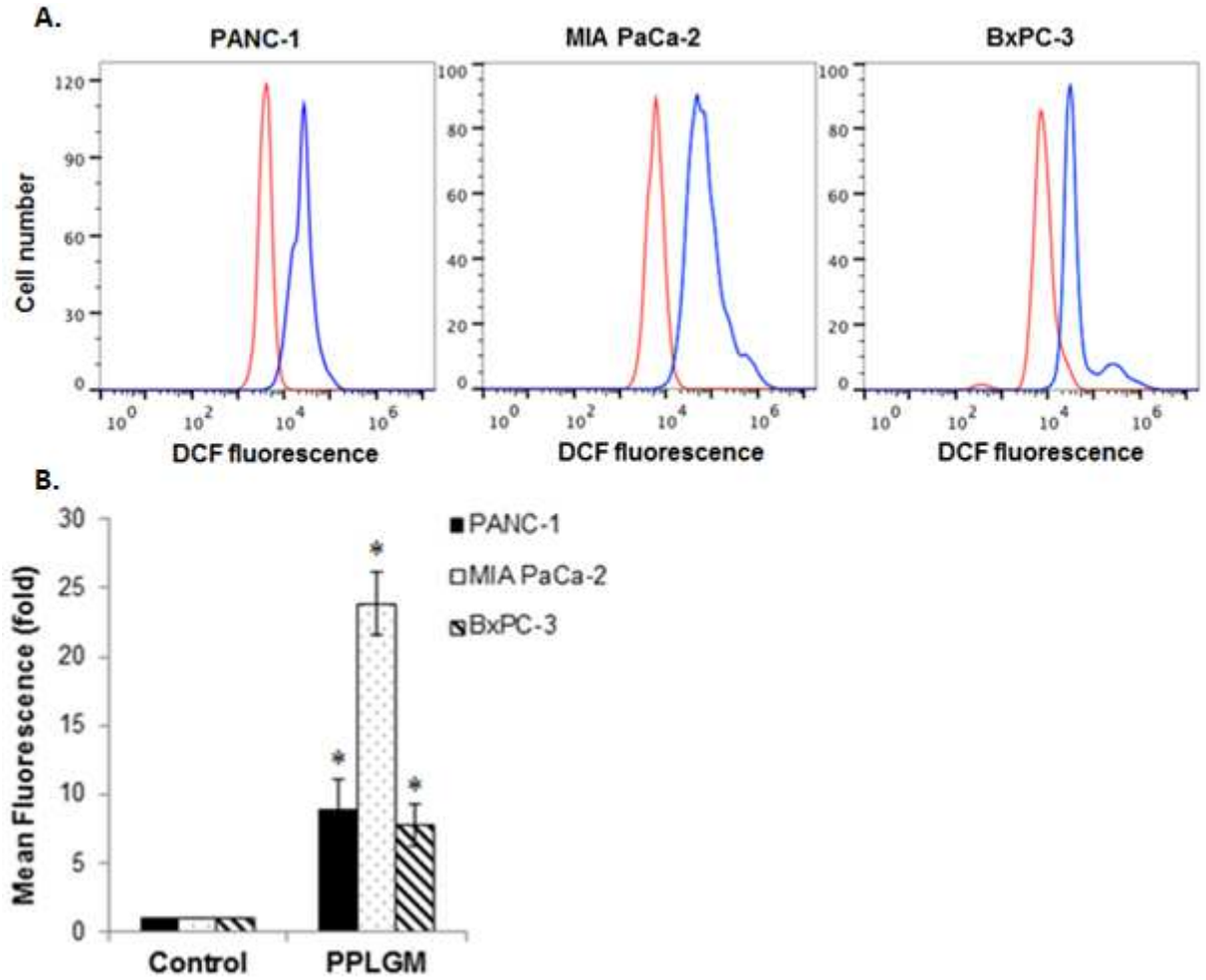

Figure 8. ROS-dependent effects of PPLGM on pancreatic cancer cells. (A) PANC-1, MIA PaCa-2, and BxPC-3 cells were treated with or without 10 μM PPLGM for 6 hours and analyzed for DCF (an indicator of ROS levels) fluorescence. The red trace shows the fluorescence intensity for DCF in the control-treated cells while the blue trace shows values for the PPLGM-treated cells. (B) The DCF fluorescence intensity obtained in part A was quantified for each PPLGM-treated cell line and related to its respective control and represented as mean fluorescence.

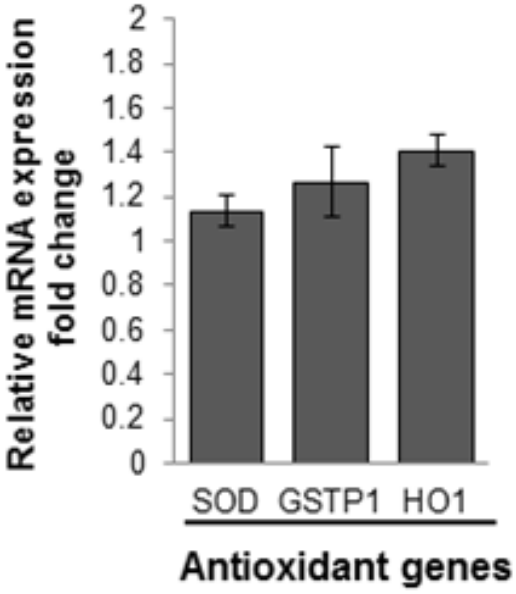

Figure 9. Effect of PPLGM on antioxidant genes. PANC-1 cells were treated with 0 or 10 μM PPLGM for 24 hours and mRNA expression of the antioxidant genes SOD, GSTP-1, and HO-1 was assessed by Q-PCR. 18S rRNA expression in the control and PPLGM-treated cells was used to normalize the data. The mRNA expression fold change for SOD, GSTP-1, and HO-1 are shown for PPLGM-treated cells compared to the control cells.

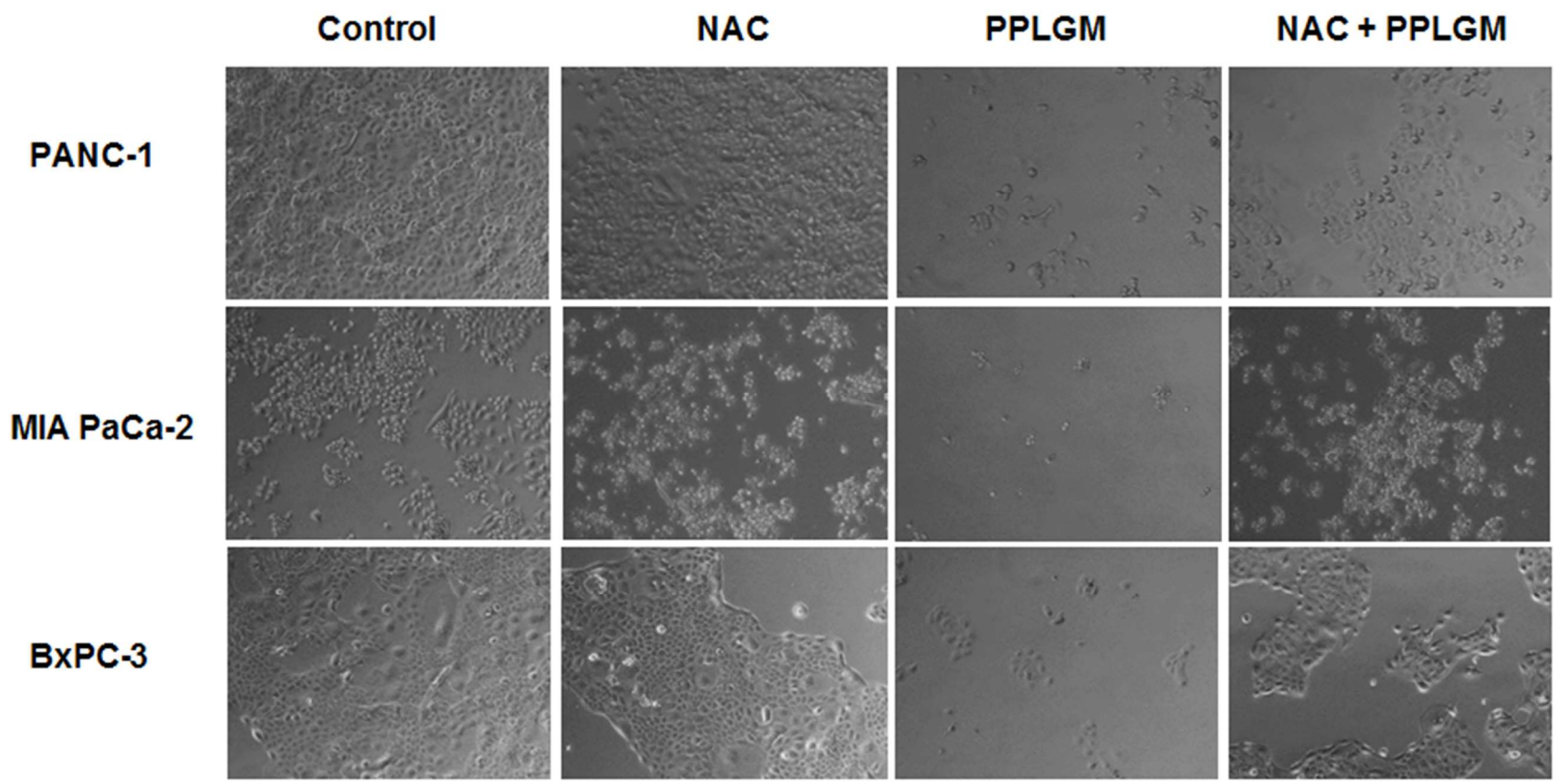

Figure 10. Effect of PPLGM and NAC on pancreatic cancer cell death. PANC-1, MIA PaCa-2, and BxPC-3 cells were treated with the vehicle (Control), the antioxidant NAC (3 mM), 10 μM PPLGM, or 3 mM NAC and 10 μM PPLGM for 24 hours. The images representing cell survival were taken the next day. The data represent a typical experiment or the average ± standard deviation for three independent experiments.

An exogenous antioxidant partially blocks PPLGM-induced pancreatic cancer cell death

We next evaluated the ability of an exogenous antioxidant to reverse the effects of PPLGM for pancreatic cancer cell death. The three pancreatic cancer cell lines were treated for 24 hours with 3 mM N-acetyl cysteine (NAC) alone and in combination with PPLGM. Images of the culture dishes reveal that PPLGM reduced the number of surviving pancreatic cancer cells and that NAC could partially block this effect (Fig. 10).

PPLGM induces DNA damage which is blocked by the antioxidant NAC

Increased ROS levels are known to cause DNA damage. Therefore, a DNA laddering assay was done to investigate the potential involvement of PPLGM-induced ROS in causing DNA damage resulting in pancreatic cancer cell death. MIA PaCa-2 cells were treated with, DMSO (0.1%), 10 μM PPLGM, or gemcitabine (GEM) as a positive control for 24 hours. DNA was isolated from the cells and loaded onto an agarose gel. Increased DNA laddering was observed in PPLGM-treated cells compared to the control cells (Fig. 11A and 11B) indicating that PPLGM causes DNA damage for pancreatic cancer cells. Further evidence of DNA damage was shown by enhanced pChk1 (S296) protein expression in PPLGM-treated cells as compared to the control cells (Fig. 11C and 11D). Cells that were treated with the antioxidant NAC + PPLGM showed reduced DNA damage compared to those treated with PPLGM alone (Fig. 12A, 12B).

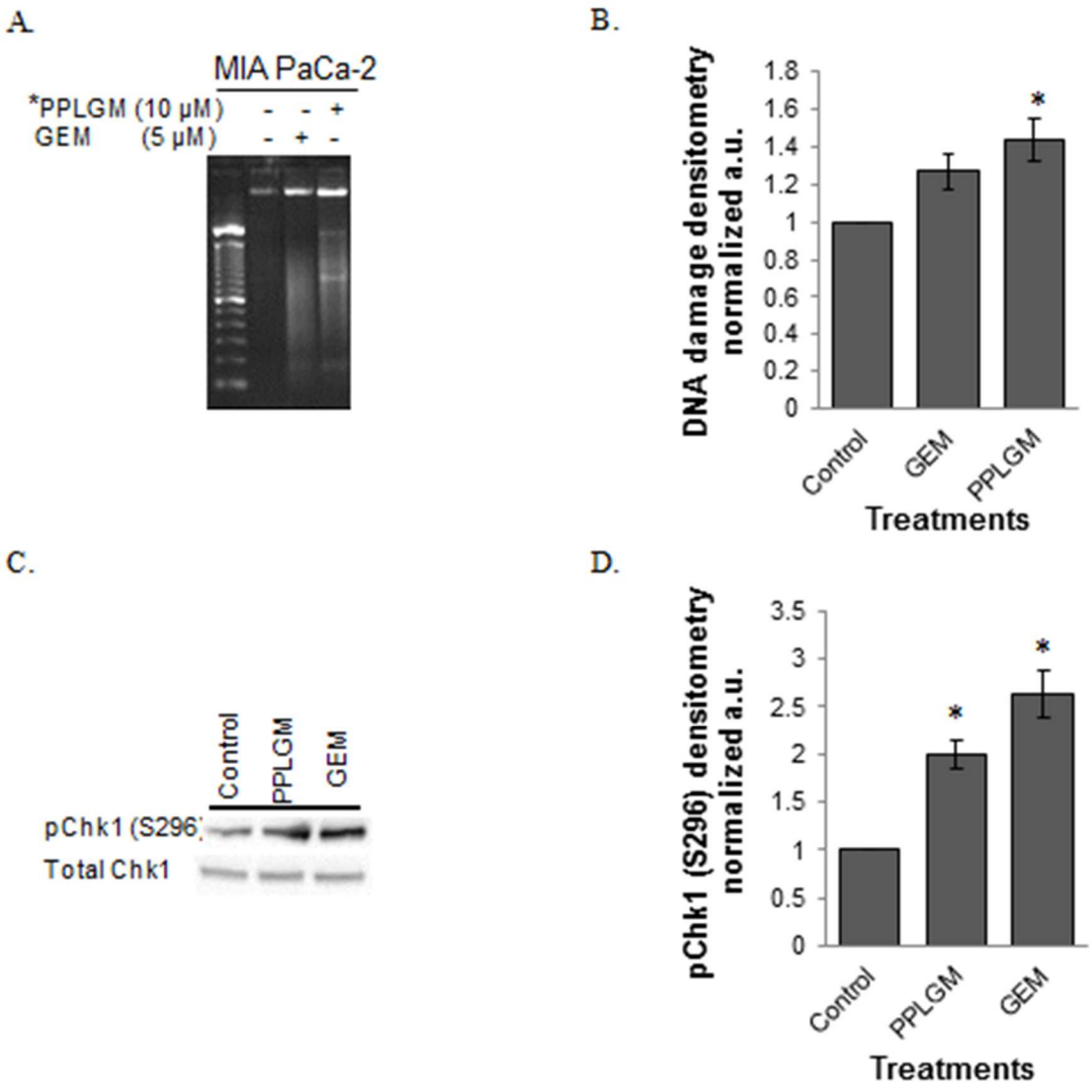

Figure 11. Effect of PPLGM on DNA damage. (A) MIA PaCa-2 cells were treated for 24 hours with 10 μM PPLGM or 5 μM gemcitabine (GEM) as positive control. The DNA damage was assessed by DNA fragmentation assay on a 1.5% agarose gel and images were captured. (B) Images were quantified using ImageJ software. The intensity of DNA damage in the control lane was compared to the PPLGM and GEM-treated lanes. Densitometry shows the results of PPLGM and GEM treatment on DNA fragmentation normalized to the amount of DNA fragmentation in the control in three replicate experiments ± standard deviation. (C) MIA PaCa-2 cells were treated for 24 hours with 10 μM PPLGM or 5 μM GEM as positive control. The cells were lysed to collect protein for western blotting. 60 μg protein was loaded into each lane and probed with pChk1 (S296) and total Chk1 antibodies. (D) Quantification of pChk1 (S296) relative to total Chk1 protein was performed by ImageJ software. Densitometry shows the results of PPLGM and GEM treatment on pChk1 (S296) expression normalized to total Chk1 in three replicates ± standard deviation.

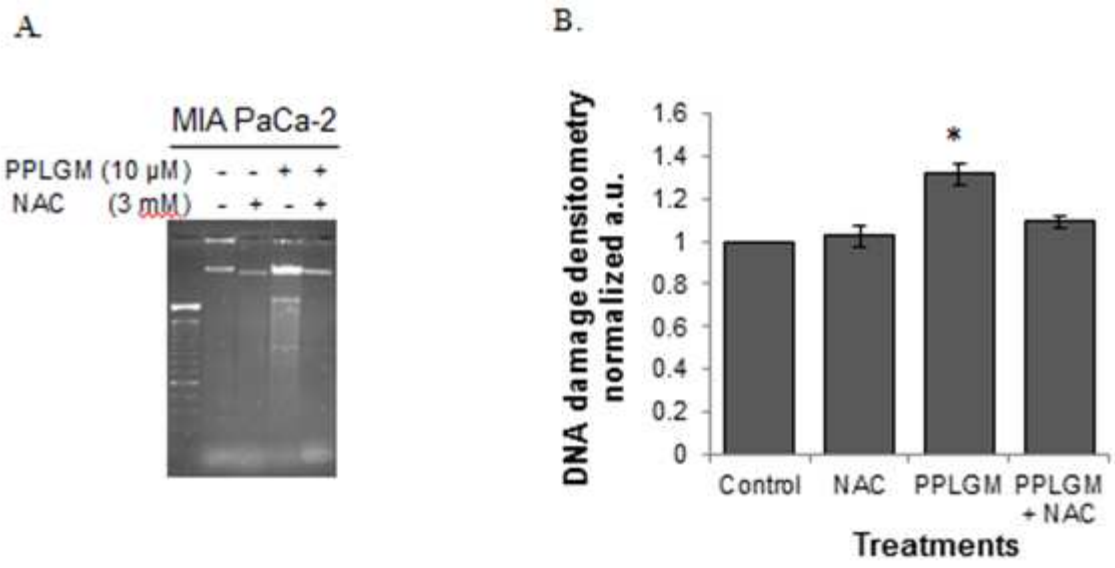

Figure 12. Effect of PPLGM and NAC on DNA damage. (A) MIA PaCa-2 cells were treated with the vehicle, 10 µM PPLGM alone, 3 mM NAC alone, or PPLGM in combination with NAC for 24 hours and DNA damage was assessed by DNA fragmentation assay. (B) Densitometry shows the results of PPLGM and NAC treatment on DNA fragmentation normalized to the amount of DNA fragmentation in the control in three replicate experiments ± standard deviation where * denotes statistical significance ($p < 0.05$).

PPLGM suppresses the growth of tumor in a mouse xenograft model

Given the promising response of pancreatic cancer cell lines to PPLGM, we chose to further investigate the anti-cancer effects of PPLGM in an animal model of pancreatic cancer. PANC-1 human pancreatic cancer cells were injected subcutaneously into athymic nude mice. Tumor growth was significantly suppressed in mice treated with 2.4 mg PPLGM/kg (daily for 30 days) as compared to those treated with DMSO (1%) vehicle (Fig. 13). PPLGM-treated animals had, on average, tumors that were 50% the mass of tumors from control-treated animals (Table 4). The PPLGM treatment was well tolerated and no significant changes were detected in overall animal mass or organ mass, as shown in Table 4.

Table 4. Effect of PPLGM on tumor, organ, and final weight of mice.

	Control	PPLGM
Tumor (mg)	55.9 ± 19.1	27.7 ± 4.7
Kidney (mg)	354.3 ± 13.9	330.8 ± 11.6
Liver (mg)	1105.9 ± 24.1	1088.4 ± 58.0
Lung (mg)	172.6 ± 8.8	169.5 ± 5.3
Spleen (mg)	102.4 ± 12.8	103.7 ± 7.2
Final Weight (g)	21.8 ± 1.1	21.7 ± 0.6

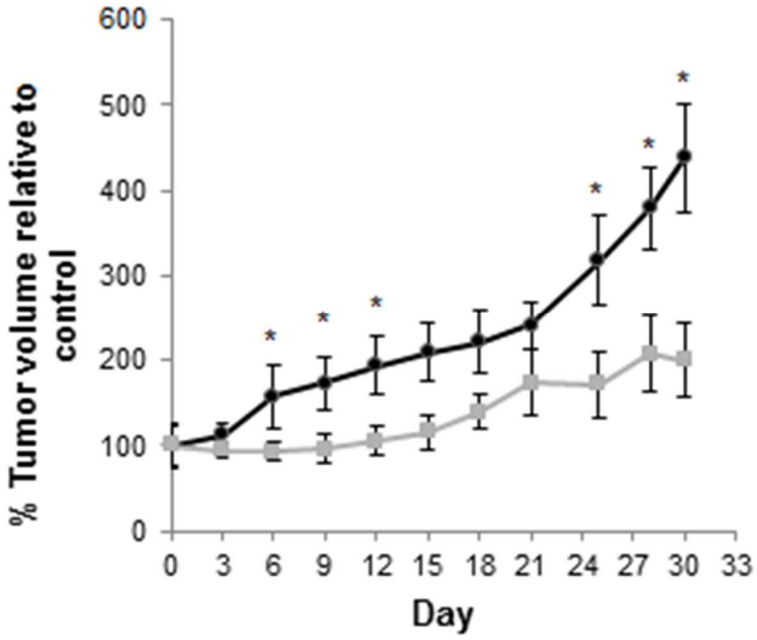

Figure 13. In vivo effects of PPLGM in a PANC-1 xenograft mouse model. Intraperitoneal injections of DMSO (1%) and PPLGM (2.4 mg/kg) were administered daily in untreated (●) and PPLGM-treated mice (□), respectively, for 30 days. Subcutaneous PANC-1 tumors were measured every three days. The data represent mean % tumor volume relative to control ± standard deviation for 8 animals per group, where * denotes statistically significant differences ($p < 0.05$).

PPLGM decreases proliferation and increases DNA damage in pancreatic tumors

Ki-67 cell proliferation and 8-hydroxy-2′-deoxyguanosine (8-OHdG) oxidative DNA damage markers were examined by immunohistochemical staining for PPLGM-treated and vehicle-treated tumors harvested from the mice. The tumors obtained from the PPLGM-treated mice exhibited notably less Ki-67-positive cells compared with tumors from the control mice. Furthermore, tumors from PPLGM-treated mice showed markedly higher 8-OHdG levels compared with tumors from control mice (Fig. 14).

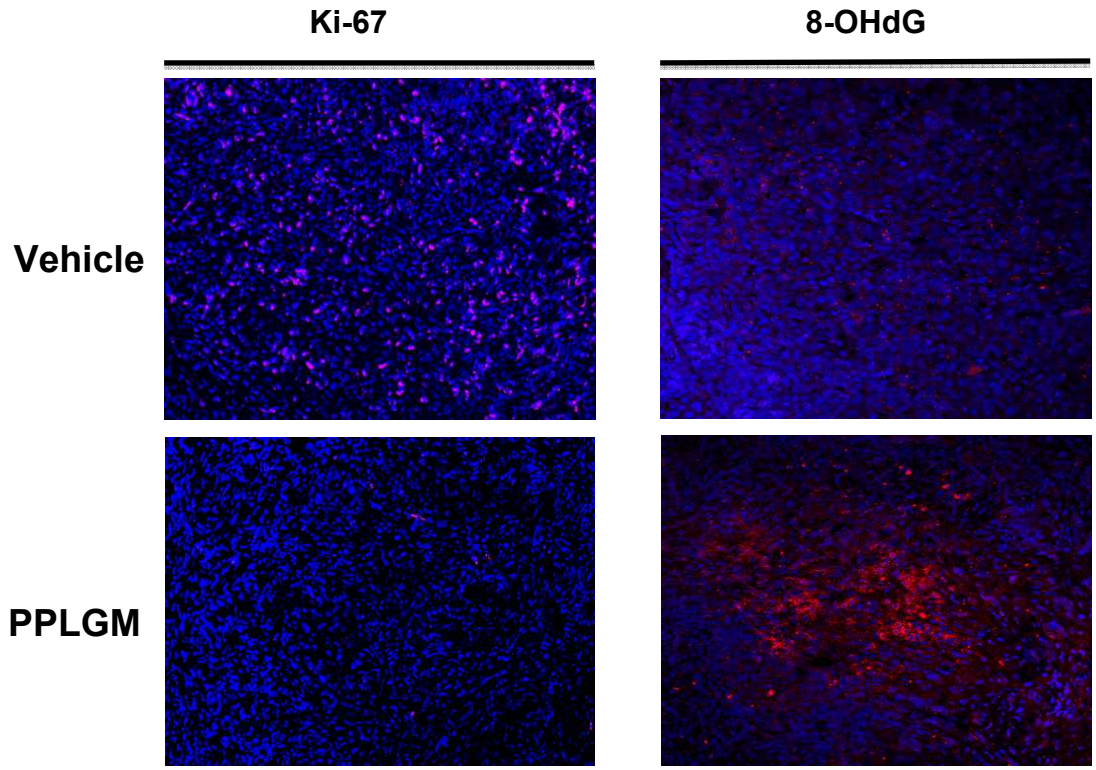

Figure 14. Effects of PPLGM on cell proliferation and the oxidative stress response in PANC-1 xenograft tumors. The tumors were harvested from control and PPLGM-treated animals and were sectioned and stained for Ki-67 (pink) and 8-OHdG (red) expression. DAPI (blue) was used to stain the nuclei. The images show one representative slide for control and PPLGM-treated animals for both Ki-67 and 8-OHdG.

Discussion

ROS-inducing agents are an attractive alternative approach for treating pancreatic cancer. The ROS-inducing agents β-lapachone and imexon induce apoptosis and necrosis in pancreatic cancer cells, and have been investigated in clinical trials for the treatment of pancreatic cancers (Ough, Lewis et al. 2005; Raj, Ide et al. 2011; Siegel, Naishadham et al. 2013). Therefore, we investigated the potential of the small-molecule ROS inducer, PPLGM, for pancreatic cancer cell death. PPLGM is known to cause cancer-selective cell death through a ROS-dependent mechanism (Raj, Ide et al. 2011). Here we show that PPLGM is toxic to PANC-1, MIA PaCa-2, and BxPC-3 pancreatic cancer cells. The cytotoxic effects of PPLGM have previously been shown in PANC-1 and MIA PaCa-2 cells (Raj, Ide et al. 2011). Both of these cells lines are *KRAS* mutant whereas BxPC-3 cells contain wild-type *KRAS*. Our data supports previous results and extends the research to pancreatic cancer cell lines harboring wild type *KRAS*. Further, PPLGM interferes with redox homeostasis by elevating ROS levels in all three cell lines without affecting antioxidant enzymes. Additionally, PPLGM causes DNA damage in the MIA PaCa-2 cells. PPLGM- induced DNA damage was reduced in cells treated with an antioxidant NAC, suggesting PPLGM-induced ROS is responsible for DNA damage in these cells. PPLGM also showed anti-cancer effects *in vivo*. For PANC-1 xenografts in nude mice, PPLGM significantly reduced tumor volume compared to the vehicle control. PPLGM-treated tumors displayed elevated levels of an oxidative DNA damage marker (8-OHdG) and reduced expression of a proliferation marker (Ki-67). We suggest that ROS-mediated DNA damage plays a role in PPLGM-induced cell death *in vitro* and *in vivo* for pancreatic cancer.

Mutations in the *KRAS* oncogene are detected in the vast majority (>90%) of pancreatic cancers and are thought to play an important role in tumor development, progression, and

resistance to chemotherapy and radiotherapy (Kim, Lim do et al. 2011). Cancer patients harboring *KRAS* mutations often have poor prognoses and limited treatment options (Pao, Wang et al. 2005; Massarelli, Varella-Garcia et al. 2007). In this study, we compared the effects of PPLGM on the growth of three different pancreatic cancer cell lines, PANC-1, MIA PaCa-2 and BxPC-3. PANC-1 and MIA PaCa-2 cell lines harbour *KRAS\s* mutation whereas BxPC-3 cell line contains wild-type *KRAS*. We found that PPLGM was sensitive to all three cell lines irrespective of the *KRAS* mutation (Fig. 6). This is a promising result since it suggests that PPLGM can be an effective treatment option even for pancreatic cancer patients with oncogenic *KRAS* mutation that are resistant to the currently available therapies.

ROS are constantly generated and eliminated in biological systems, and play an important role in cell signaling pathways (Dickinson and Chang 2011). PPLGM has previously been shown to cause enhanced production of hydrogen peroxide and nitric oxide levels in cancer cells but not healthy cells. The anticancer effects of PPLGM in cancer cells were attributed to modulation of glutathione S-transferase (GSTP1) activity and glutathione (GSH) levels (Raj, Ide et al. 2011). However, our study shows that PPLGM elevated ROS levels in all three pancreatic cancer cells without significantly affecting the mRNA expression of three key antioxidant enzymes. Excessive production of ROS can result in many types of DNA damage including single- or double-strand breaks, base modifications, deoxyribose modification, and DNA cross linking. Mild DNA damage can be repaired with or without cell cycle arrest. However, severe DNA damage leads to mutations or induction of cell death or senescence (Surova and Zhivotovsky 2013). We found that PPLGM increases DNA laddering and pChk1 (S296) expression in pancreatic cancer cells, and the antioxidant NAC could not only block PPLGM-induced pancreatic cancer death but also offer significant protection against DNA damage.

Therefore, ROS-mediated DNA damage is the mechanism for pancreatic cancer cell death but the type of cell death via DNA damage needs further investigation.

This study is the first to investigate the anti-tumor efficacy of PPLGM for an animal model of pancreatic cancer. We found that PPLGM (2.4 mg/kg/day for 30 days) significantly inhibited pancreatic tumor growth in the nude mice relative to the vehicle control treatment. The final tumor masses from PPLGM-treated animals were 50% lower than tumors obtained from control-treated animals. Our results are in agreement with previous studies that show intraperitoneal administration of 1.5 mg/kg PPLGM suppresses the growth of bladder, breast, lung and melanoma xenografts by 50-80% nude in mice (Raj, Ide et al. 2011). However, several mechanisms including oxidative stress can be involved in tumor growth inhibition. Interestingly, the most abundant oxidative DNA lesion produced in response to oxidative stress is 8-hydroxydeoxy guanosine (8-OHdG). Many studies have reported an elevation of 8-OHdG levels in various human cancers and animal models of tumors (Hsieh, Lien et al. 2005; Valavanidis, Vlachogianni et al. 2009). Our results show that PPLGM markedly increased the levels of 8-OHdG in tumors which may contribute to decreased cell proliferation and tumor growth inhibition.

Taken together, our results demonstrate that PPLGM-induced cell death in human pancreatic cancer cells is mediated by ROS-induced DNA damage. Moreover, PPLGM significantly suppressed the growth of pancreatic tumor xenografts by causing oxidative DNA damage and reduced cell proliferation. Our studies provide a rationale for the development of PPLGM as chemotherapeutic agent against pancreatic cancer in the clinical setting. Further studies evaluating the anti-tumor effects of PPLGM for normal and *KRAS* mutant pancreatic

tumors in combination with chemotherapy are needed to advance this treatment approach to a clinical setting.

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CHAPTER 3. TRANSCRIPTOME ANALYSIS OF HUMAN PANCREATIC CANCER CELLS EXPOSED TO PIPERLONGUMINE REVEALS INVOLVEMENT OF THE OXIDATIVE STRESS AND ER-STRESS PATHWAYS

Abstract

Piperlongumine (PPLGM), a phytochemical obtained from the long pepper plant, has shown anticarcinogenic activity including ROS-induction, apoptosis, and cell cycle arrest for various cancer cell types. However, the anticancer mechanisms of PPLGM mediated cell death have not been fully elucidated. In this study, we report changes in the transcriptome for MIA PaCa-2 human pancreatic cancer cells exposed to 10 μM PPLGM, through the use of an RNA-sequencing approach. After short-term exposure (6 hours) to PPLGM, 684 genes were differentially expressed, 351 of which were up-regulated and 333 were down-regulated. Genes related to oxidative stress, ER-stress, and UPR pathways were significantly activated with PPLGM treatment. The changes in expression of these genes were further confirmed in independent studies with RT-qPCR. IRE1α protein was activated after *in vitro* exposure to PPLGM in a time-dependent manner. This study suggests that PPLGM significantly alters gene expression in human pancreatic cancer cells, and highlights several potential mechanisms by which PPLGM can induce cancer cell death.

Introduction

Pancreatic cancer is a highly aggressive disease that results in very poor clinical outcomes even when treated with the best available chemotherapy and radiation therapy. Although pancreatic cancer accounts for only 3% of all cancer cases, it is the fourth leading cause of cancer-related deaths in both men and women in the United States. Despite therapeutic advances, pancreatic cancer remains the most deadly cancer with a median overall 5-year

survival rate of less than 7% (Siegel, Miller et al. 2015). In recent years, bioactive food compounds have gained attention as alternative approaches for treating pancreatic cancer due to their ability to sensitize tumor cells to chemotherapeutic agents (Bharti and Aggarwal 2002). Particularly, chemopreventive agents obtained from pepper plants have demonstrated potent anticancer activity in cell cultures (Zhang, Zhu et al. 2015) and animal models (Bai and Xu 2000; Sunila and Kuttan 2004).

Piperlongumine (PPLGM), an alkaloid present in the fruits and roots of the long pepper (*Piper longum*) plant, displays anti-proliferative and anti-tumorigenic properties in a variety of cancer cell types *in vitro* and *in vivo* xenograft mouse models (Raj, Ide et al. 2011). Interestingly, PPLGM selectively kills cancer cells with minimal death in normal cells owing to its reactive oxygen species (ROS)-induction potential. PPLGM has been reported to activate various pro-apoptotic genes (PUMA, NOXA, BIM, caspase-3) and suppress anti-apoptotic genes (survivin, Bcl2, XIAP) in different cancer cell lines (Raj, Ide et al. 2011). In addition to apoptosis, PPLGM has been shown to cause cell cycle arrest (Kong, Kim et al. 2008), induce autophagy (Wang, Wang et al. 2013), and inhibit migration (Liu, Liu et al. 2014), adhesion, and invasion (Ginzburg, Golovine et al. 2014). Various proteins such as activation of p38 signaling (osteosarcoma cells), inhibition of NF-kB (prostate cells) (Ginzburg, Golovine et al. 2014), and Akt/mTOR signaling (prostate, breast, and renal carcinoma cells) (Makhov, Golovine et al. 2014) play a role in the PPLGM-mediated phenotypic effects. With respect to pancreatic cancer, we have previously shown that PPLGM inhibits the growth of both *KRAS* mutant and wildtype human pancreatic cancer cell lines both *in vitro* and *in vivo* through an ROS-dependent mechanism that leads to DNA damage and cell death (Dhillon, Chikara et al. 2014).

While multiple studies have reported the cancer-specific cytotoxicity of PPLGM, the gene expression profile and pathways associated with PPLGM-mediated cancer cell death have not been fully elucidated. RNA-sequencing (RNA-seq) is an unbiased sequencing tool that allows transcriptome profiling to detect gene expression changes in a cell or tissue sample. In this study, we used RNA-seq as an approach to identify the mechanisms by which PPLGM induces anti-proliferative and apoptotic effects in a human pancreatic cancer cell line, MIA PaCa-2.

Materials and Methods

Cell culture and treatment of cells

MIA PaCa-2 cells were obtained from ATCC (Manassas, VA) and cultured at 37 °C with 5% carbon dioxide in Dulbecco's Modified Eagle's (Thermo Fisher Scientific Inc; Waltham, MA) medium supplemented with 10% fetal bovine serum (Atlanta Biologicals; Lawrenceville, GA). Cells were subcultured by enzymatic digestion with 0.25% trypsin/ 1 mM EDTA solution (Thermo Fisher Scientific Inc.) when they reached approximately 70% confluency. PPLGM (Indofine Chemical Company; Hillsborough, NJ) was dissolved in 100% DMSO at a stock concentration of 10 mM and then diluted in water to a working concentration. The cells were seeded in 100x20 mm dishes (Greiner Bio-One; Monroe, NC) at 5×10^5 cells/dish at 37°C with 5% carbon dioxide. Twenty- four hours later, they were treated with 10 μM PPLGM or with 0.1% DMSO (vehicle control) for 6 hours. The time point and concentration was chosen based on our previous study reporting the induction of ROS (Dhillon, Chikara et al. 2014). The experiment was conducted in triplicate.

RNA sequencing

Following PPLGM treatment, total RNA was extracted using a Fisher SurePrep Kit (Waltham, MA) as per the manufacturer's instructions. RNA was quantified using a Nanodrop (Thermo Scientific Fisher Inc.). 3 μg of total RNA/sample was sent off for sequencing to the University of Minnesota Genomics Center. All of the samples passed QC with a RNA integrity number >8. Six barcoded libraries were created which were pooled together and sequenced in one full lane of the flow cell on an Illumina HiSeq 2500 (single read, 50 bp).

Tophat (Kim, Pertea et al. 2013) was used to map reads to the reference genome (build 38, ensemble 78) for each sample (max mismatches 15, maximum insertion and deletion lengths 18). Cufflinks (Trapnell, Williams et al. 2010) was used to assemble the transcripts from each library with multiread count (-u) and upper quartile normalization (-N). The transcripts from each of these libraries were combined together using cuffmerge. Cuffdiff was used to identify differentially expressed transcripts. The following cutoff criteria was used to identify the differentially expressed transcripts:

1) Log2 fold change ≥ 1 or < -1
2) Q-value (FDR) < 0.01
3) Fragments per kilobase of exon per million fragments mapped (FPKM) value ≥ 1

RT-qPCR

cDNA was synthesized using 100 ng of total RNA and the qScript cDNA synthesis kit (Quanta Biosciences; Gaithersburg, MD). Primers were designed using Primer Express software (version 2.0, Applied Biosystems), and synthesized by Integrated DNA Technologies (Coralville, IA). Primer sequences are listed in Table 5. Steady-state mRNA levels of genes were determined for the cDNAs by real-time PCR using the PerfeCTa SYBR Green FastMix kit (Quanta

Biosciences). The cycling parameters were 95°C for 10 min followed by 40 cycles of 95°C for 30 sec and 60°C for 1 min and a dissociation program that included 95°C for 1 min, 55°C for 30 sec, and 95°C for 30 sec ramping up at 0.2°C/sec. One distinct peak was observed for the primer sets. The fold change in mRNA expression was calculated by comparing the 18S rRNA-normalized threshold cycle numbers (C_T) in the PPLGM-treated cancer cells compared to the DMSO-treated cancer cells. Duplicate wells were run for each experiment, and the experiments were performed in triplicate.

Table 5. List of primers for qPCR validation.

Name	Forward Sequence	Reverse Sequence
IRE1α	GAA GAC GTC ATT GCA CGT GAA TT	AGG TCC TGA ATT TAC GCA GGT
sXBP1	CTG AGT CCG AAT CAG GTG CAG	ATC CAT GGG GAG ATG TTC TGG
usXBP1	CAG CAC TCA GAC TAC GTG CA	ATC CAT GGG GAG ATG TTC TGG
ASNS	CAC TCC GCG ACT CCC TTT T	ACC ATT TCC ACG GAT GCA A
HERPUD1	AAA CCA GCC TGC CAA TCA GA	CTT CCA CAA TAG GGC CAC CTT
ABCB10	CCA GTG TGG CTG AGA TCC AA	GGA AGG GTC ATC AGC ACC AT
Sugt1	TGG AGG GAG ATG CAG CTT TAA	CCA CCC GAC TCC ATA AAG GAT
PARP9	CTG CCA GGG ACA TCC GTT AA	AGG TTT CAG GGC TGG AGA CA
BCL2L2	TCA GCC CAA CAA CGC TTC A	CAC AGT GCA GCC CCA AAG A
NLRC5	CCA GCA GAC AGG CTA TGC TTT	CTG TAC CCA GCG GGA ATG G
ATF4	CGTTGCCATGATCCCTCAGT	GCTGAGGAGACCCCAGATAGG
TRIB3	AAGAAGCGGTTGGAGTTGGA	GCACGATCTGGAGCAGTAGGT
OSGIN1	GATGCTGTACCCCGAGTACCA	GCCTGGCAGTCTTCCTTGAAG
TNFR	AACAAAACCGGGCGAGATG	GAGCTCAACAAGTGGTCCTCAA

SDS–PAGE and western blotting

MIA PaCa-2 cells were treated for 6, 12, and 24 hours with 10 μM PPLGM or 0.1% DMSO. Cell pellets were lysed using an SDS lysis buffer (Cell Signaling Technologies) containing protease and phosphatase inhibitors (Roche; Indianapolis, IN). Samples were briefly sonicated to dissociate cell membranes. Mitochondrial and cytosolic fractions were isolated using mitochondria isolation kit (Thermo Fisher Scientific Inc.). Twenty five micrograms of total protein and cytosolic fractions isolated from PPLGM or DMSO-treated MIA PaCa-2 cells were separated on 10% SDS-polyacrylamide gels at 100 V for 1 hour. Proteins were transferred to PVDF membranes at 100 V for 70 min at 4 °C. Blots were then probed overnight at 4 °C with IRE1α, HO1 (Cell Signaling Technologies; Danvers, MA), cytochrome c (BD Biosciences; Franklin Lakes, NJ), and ASNS (Proteintech Group Inc; Chicago, IL) primary antibodies. The next day, blots were rinsed with 1X TBS-tween (0.1%) and probed with their respective anti-rabbit/ anti-mouse secondary antibodies (Jackson Immuno Research; West Grove, PA) for 1 hour at room temperature. The western blots were analyzed using SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific; Waltham, MA) and images were captured using the MultiImage™ Light Cabinet (Alpha Innotech; San Leandro, CA). Protein levels were normalized to GAPDH expression. Immunoblots were performed in triplicate and the images in the figures represent one typical replicate.

Software

Gene-annotation enrichment analysis was completed using the functional annotation clustering tool of DAVID Bioinformatics Resources 6.7 using differentially expressed genes (Sherman, Huang da et al. 2007). Pathway analysis was completed with the Reactome pathway analysis tool (Reactome V51) (Matthews, Gopinath et al. 2009).

Results

PPLGM treatment changes gene expression profile in MIA PaCa-2 cells

RNA seq was used to determine the effects of PPLGM on the transcriptome of MIA PaCa-2 cells following 6 hours of 10 μM PPLGM treatment or vehicle control. A total of 143,100,347 reads of 50 base pairs in length were obtained for 6 samples (3 replicates each of control and PPLGM-treated cells). An average of 23,850,058 reads was obtained per sample. Approximately 88% of the reads explicitly mapped to a unique region of the human genome; whereas ~30% of the reads mapped to multiple locations of the genome. A total of 33,572 transcripts were differentially expressed in PPLGM-treated cells as compared to control (Fig.15). A cutoff criterion as mentioned in materials and methods was used to generate significantly different transcripts (q-value <0.01) in PPLGM-treated cells. Seven protein coding genes with known function were exclusively expressed whereas 4 protein coding genes were suppressed in PPLGM-treated cells. Importantly, 684 protein-coding genes were differentially expressed with PPLGM treatment, 351 of which were up-regulated and 333 were down-regulated (Fig. 16).

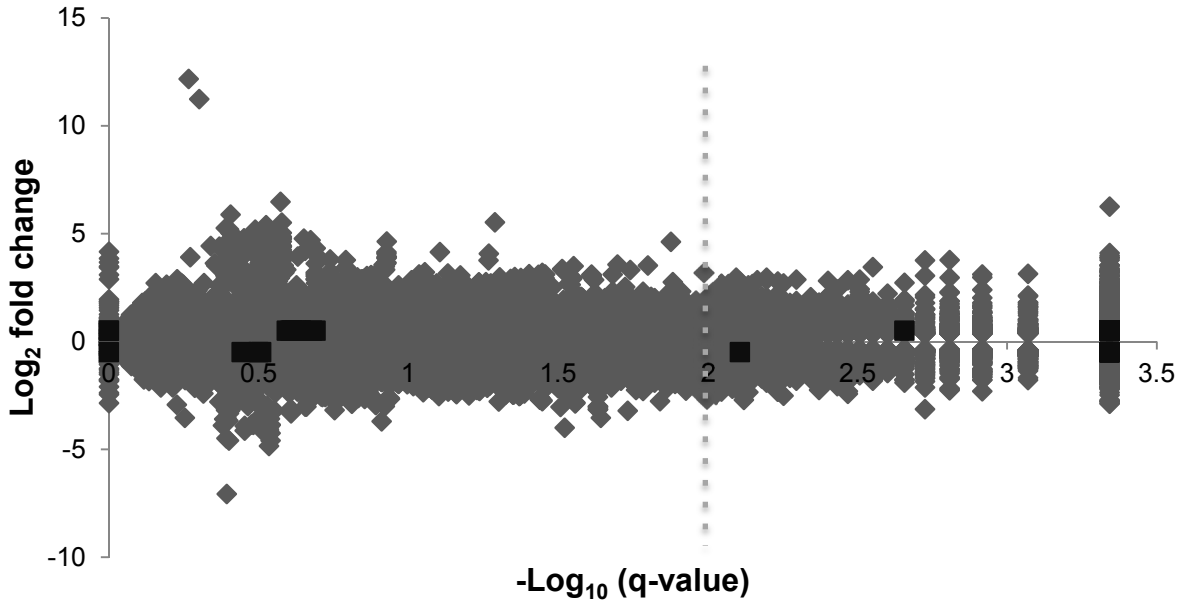


Figure 15. Volcano plot of differentially expressed genes in PPLGM-treated MIA PaCa-2 cells. The vertical axis is the $\log_2$ fold change between control and PPLGM-treated cells. The $-\log_{10}$ (q-value) is plotted on the horizontal axis. Each transcript is represented by one point on the graph. Points to the right of the dotted red line indicate sequences that pass the $q < 0.01$ significance threshold. The two horizontal rows represented by black points in chart are the genes for which expression was only detected either in control or PPLGM treatment, so the $\log_2$ fold change values were artificially set to (±) 0.5 (instead of +/- infinity) as appropriate.

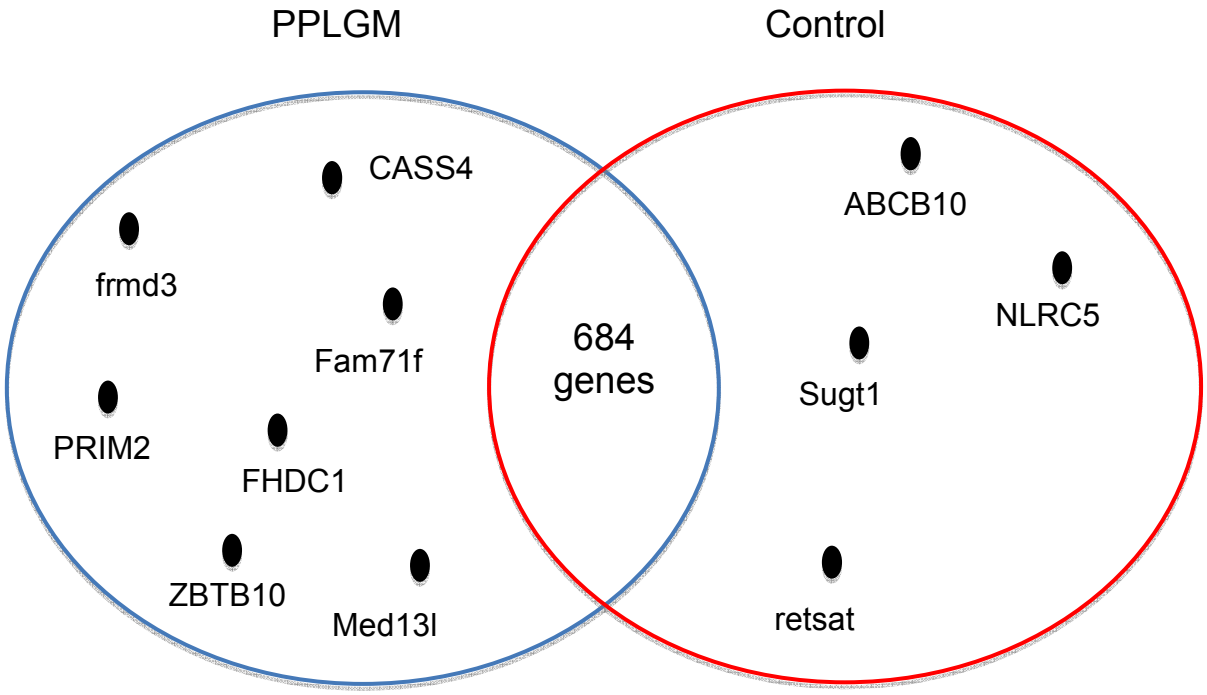


Figure 16. Venn diagram showing the number of genes commonly and exclusively expressed in control and PPLGM-treated MIA PaCa-2 cells. 684 protein-coding genes were differentially expressed with PPLGM treatment. Seven protein-coding genes were exclusively expressed and 4 genes were suppressed in PPLGM-treated cells. These genes were significantly expressed with a q-value <0.01.

PPLGM-treated MIA PaCa-2 cells were highly enriched with transcription and cell death-associated categories

In order to illustrate the phenotypic behavior associated with PPLGM treatment in MIA PaCa-2 cells, gene ontology enrichment analysis was performed using DAVID software. Interestingly, genes associated with the transcription category were significantly enriched (p-value = 6.30E-10) suggesting a gene regulatory role for PPLGM. In addition, genes related to cell death, apoptosis, and negative regulation of growth were also significantly enriched with PPLGM treatment. The RAS-association category was enhanced in *KRAS* mutant MIA PaCa-2 cells. Other categories with greatest significance (p-value < 0.01) included membrane-enclosed lumen, negative regulation of biosynthetic process, zinc finger (C2H2-like), RNA biosynthetic process, BTB/POZ fold, zinc ion binding, heat shock protein binding, and collagen (Fig. 17).

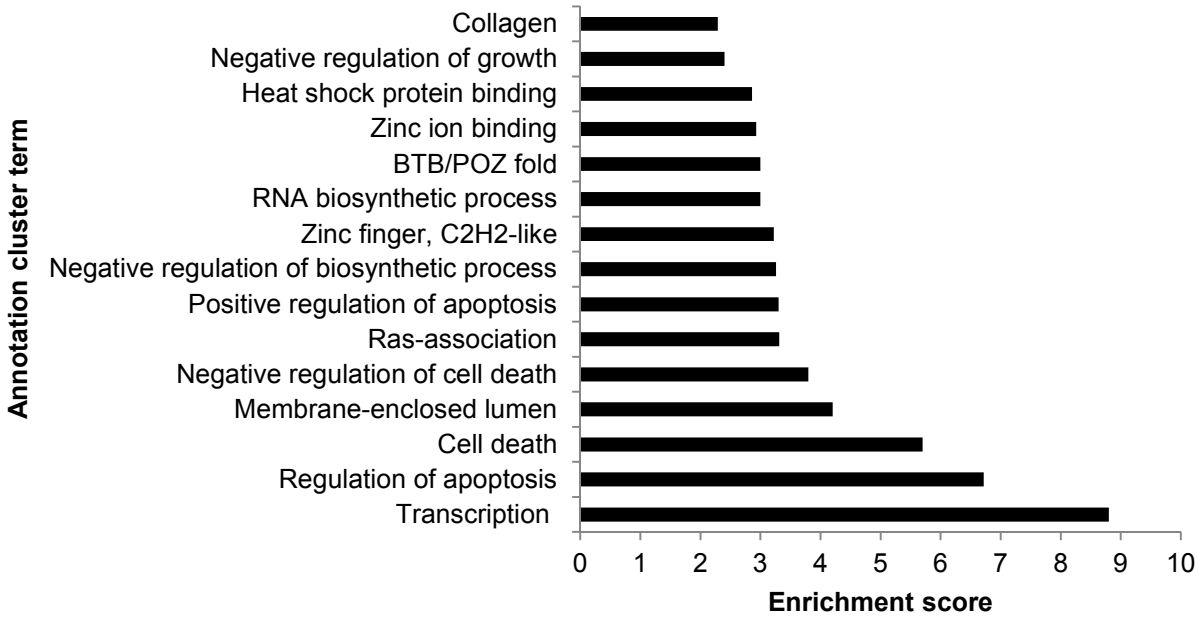


Figure 17. Gene ontology enrichment analysis for differentially expressed transcripts in PPLGM-treated MIA PaCa-2 cells. The enrichment score represents the negative logarithm of the p-value associated with the gene ontology terms for the differentially expressed transcripts. The top 15 annotation clusters are listed as derived from the DAVID functional annotation tool.

PPLGM activates the endoplasmic reticulum stress-associated pathway

To further investigate the relationships among genes regulated by PPLGM in MIA PaCa-2 cells, pathway analysis was completed using Reactome software. This software contains a large database of human pathways spanning signaling cascades and metabolic and cellular processes. Pathways related to initiation of DNA replication and telomere C-strand synthesis were significantly expressed in PPLGM-treated cells only (p-value <0.01) (Table 6). However, pathways involving mitochondrial ABC transporters and inflammasomes were significantly suppressed in PPLGM-treated cells. Cell signaling pathways related to ATF-4, PERK, unfolded protein response, and HSF1 were significantly altered with PPLGM treatment. This suggests that PPLGM activates a cellular stress response pathway, mainly associated with the endoplasmic reticulum.

Table 6. Pathway analysis for genes regulated by PPLGM treatment in MIA PaCa-2 cells. In this table, entities found represents the number of submitted genes found in the Reactome database, entities total represents the total number of genes within the associated pathway in Reactome the database, and entities p-value represents a statistical test for over-representation of the submitted data set.

Pathways exclusively expressed in PPLGM-treated cells	**# Entities found**	**# Entities total**	**Entities p-value**
DNA replication initiation	1	6	0.006009
Telomere C-strand synthesis initiation	1	6	0.006009
Pathways suppressed in PPLGM-treated cells	**# Entities found**	**# Entities total**	**Entities p-value**
Mitochondrial ABC transporters	1	4	0.002007
The NLRP3 inflammasome	1	13	0.006511
Inflammasomes	1	21	0.010502
Pathways altered in PPLGM-treated cells	**# Entities found**	**# Entities total**	**Entities p-value**
Unfolded Protein Response (UPR)	21	140	0.005273
HSF1-dependent transactivation	11	56	0.006255
PERK regulates gene expression	9	38	0.004027
ATF4 activates genes	9	32	0.001299

RT-qPCR results validated the RNA-seq results in MIA PaCa-2 cells

To validate the gene expression data obtained from RNA sequencing, RT-qPCR was performed. Eight up-regulated genes were selected based on high expression (OSGIN1), and involvement in the ER-stress pathway (ATF-4, TRIB3, ASNS, HERPUD1, IRE1α, sXBP1, and TNFR). In each case, RT-qPCR confirmed the significant increase in mRNA expression observed by RNA-seq after 6 hours of PPLGM treatment. Additionally, five genes which were

down-regulated by PPLGM treatment were also validated by RT-qPCR. Additional genes were evaluated that did not support the RNA-seq results. Overall, 80% of the tested genes confirmed the results obtained by RNA-seq as shown in Table 7. A time course was performed to evaluate the selected genes' expression values at 3, 6, and 12 hours in PPLGM-treated versus control-treated MIA PaCa-2 cells. A significant elevation in mRNA expression at 3 and 12 hours was observed in up-regulated genes consistent with the result obtained after 6 hours of PPLGM treatment (Fig. 18A). However, maximum expression with an average fold change of 22 was observed in OSGIN1 mRNA expression after 12 hours of PPLGM treatment (Fig. 18B). Similarly, a significant decrease in mRNA expression at 3 and 12 hours was observed in down-regulated genes consistent with the result obtained after 6 hours of PPLGM treatment (Fig. 18A).

Table 7. List of genes validated by q-PCR.

		RNA seq	RT-qPCR
Gene name	Gene symbol	Log2 fold change	Average fold change
oxidative stress induced growth inhibitor 1	OSGIN1	4.11	9.7887
tribbles homolog 3 (Drosophila)	TRIB3	2.3	3.1986
activating transcription factor 4	ATF4	1.6	2.0361
tumor necrosis factor receptor superfamily, member 10b	TNFR	1.28	1.3730
asparagine synthetase	ASNS	1.32594	1.8645
endoplasmic reticulum to nucleus signaling 1	IRE1α	1.44325	2.0582
homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1	HERPUD1	2.25073	3.1935
X-box binding protein 1	XBP1	1.55823	6.3497
ATP-binding cassette, sub-family B (MDR/TAP), member 10	ABCB10	0	0.7737
NLR family, CARD domain containing 5	NLRC5	0	0.8503
poly (ADP-ribose) polymerase family, member 9	PARP9	-1.08126	0.7565
BCL2-like 2	BCL2L2	-1.29782	0.6800

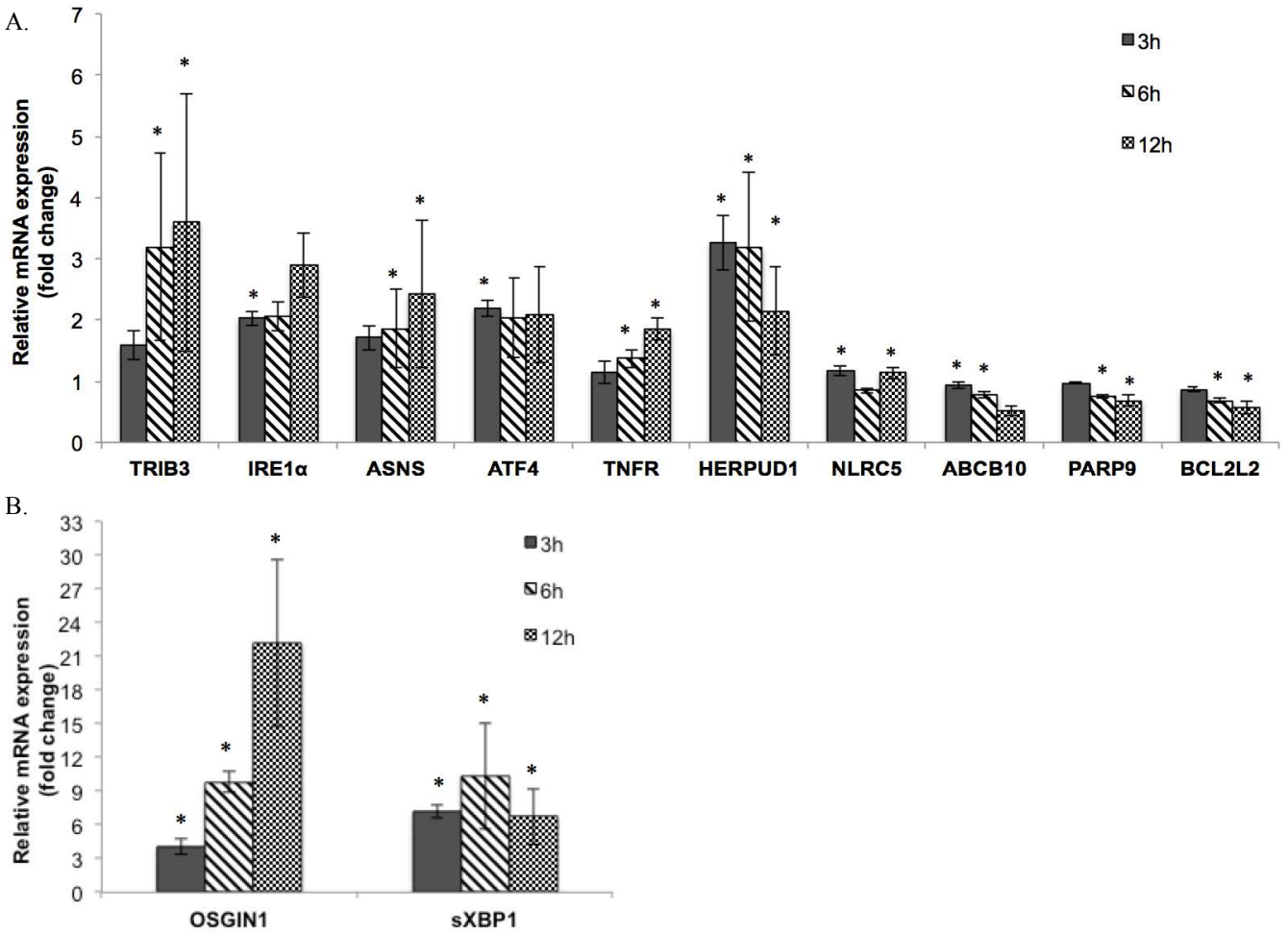
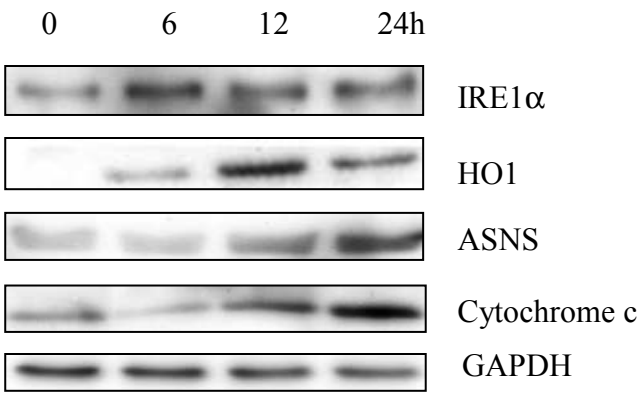


Figure 18. qRT-PCR results to validate RNA-seq results. The mRNA expression fold change for (A) up-regulated (TRIB3, IRE1α, ASNS, ATF4, TNFR, HERPUD1) and down-regulated (NLRC5, ABCB10, PARP9, BCL2L2) genes and (B) highly up-regulated OSGIN1 and sXBP1 genes are shown for PPLGM-treated cells compared to the vehicle-treated control cells. MIA PaCa-2 cells were treated with 0 or 10 μM PPLGM for 3, 6 or 12 hours and mRNA expression of genes identified with RNA-seq was assessed by RT-qPCR. 18S rRNA expression in the control and PPLGM-treated cells was used to normalize the data. Statistically significant differences ($p < 0.05$) from DMSO treated cells are denoted by *.

PPLGM enhances Ire1α, HO1, ASNS, and cytochrome c protein expression in MIA PaCa-2 cells

To determine the correlation between mRNA and protein expression, western blotting was performed. MIA PaCa-2 cells were treated with 0 or 10 μM PPLGM for 6, 12, and 24 hours and assessed for Ire1α, HO1, ASNS, and cytochrome c protein expression. We found that 10 μM PPLGM significantly enhanced IRE1α, ASNS, and cytochrome c protein expression after 24 hours. A significant increase in HO1 protein expression was observed after 12 hours of PPLGM treatment (Fig. 19).

A.



B.

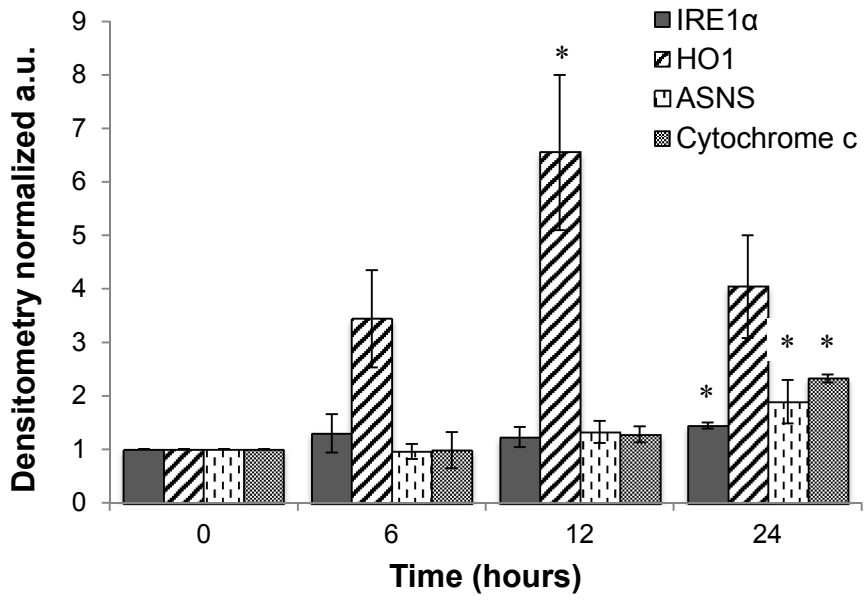


Figure 19. PPLGM increases IRE1α, HO1, ASNS, and cytochrome c protein expression. Western blot analysis (A) and quantitative densitometry (B) of protein expression in MIA PaCa-2 cells treated with 10 μM PPLGM for 6, 12, and 24 hours. Densitometry shows the results of PPLGM treatment on IRE1α, HO1, ASNS, and cytochrome c expression normalized to GAPDH in three replicate experiments ± standard deviation.

Discussion

We have previously shown that PPLGM mediates pancreatic cancer cell death through a ROS-mediated DNA damage pathway (Dhillon, Chikara et al. 2014). In this study, our goal was to more fully understand the underlying mechanisms mediating PPLGM-induced pancreatic cancer cell death. We used an RNA sequencing approach to investigate the gene expression

changes in MIA PaCa-2 human pancreatic cancer cells exposed to PPLGM, and then confirmed the expression changes for a subset of genes by RT-qPCR and western blotting.

RNA sequencing revealed 684 differentially expressed genes with known functions in PPLGM-treated tumor cells. Genes related to cell death and apoptosis were highly enriched, which is consistent with numerous reports on PPLGM's anticancer effects (Roh, Kim et al. 2014; Shrivastava, Kulkarni et al. 2014; Xiong, Liu et al. 2015). In addition, the GADD 45 family of genes was up-regulated in response to the treatment with PPLGM. This gene family is involved in the regulation of growth, DNA repair, and induction of apoptosis (Yin, Bruemmer et al. 2004).

Seven protein-coding genes were exclusively expressed and four protein-coding genes were exclusively suppressed in PPGLM-treated cells. Among the exclusively expressed genes, FERM domain containing 3 (FRMD3) was significantly up-regulated in PPLGM-treated cells. FRMD3 is identified as a putative tumor suppressor gene in lung cancer. FRMD3 expression was reduced in 54 out of 58 primary non-small cell lung tumors, and overexpression of FRMD3 resulted in reduced clonogenicity, suggesting that loss of FRMD3 may play an important role in the progression of lung cancer (Haase, Meister et al. 2007). On the other hand, ATP-binding cassette sub-family B, member 10 (ABCB10) was silenced in PPLGM-treated cells. Recent evidence suggests that ABCB10, a regulator of heme synthesis, helps protect against oxidative stress *in vivo* by peptide export and/or other molecules specifically regulating antioxidant function and/or avoiding mitochondrial oxidative damage (Liesa, Luptak et al. 2011; Hyde, Liesa et al. 2012). Therefore, PPLGM may elevate oxidative stress in pancreatic cancer cells by down regulating ABCB10.

The generation of ROS results in the oxidation of phospholipids. Oxidized phospholipids, such as 1-palmitoyl-2-arachidonyl-sn-glycero-3-phosphorylcholine (OxPAPC), have been shown

to stimulate the expression of several genes including unfolded protein response genes (such as ATF3), redox genes (such as heme oxygenase 1 {HO1}), and OKL38 (Li, Chen et al. 2007). HO1 is the gene most strongly stimulated by oxidized phospholipids (Gargalovic, Gharavi et al. 2006).

We observed a 6-fold up-regulation in HO1 expression in PPLGM-treated cells. Moreover, OKL38, also known as oxidative stress induced growth inhibitor 1 (OSGIN1), was up-regulated 4-fold in PPLGM-treated cells. OSGIN1 is a tumor suppressor gene that inhibits tumor cell growth (Huynh, Ng et al. 2001; Wang, Xia et al. 2005). Its expression is reduced or altered in cancer cell lines and tumor tissues (Huynh, Ng et al. 2001; Ong, Ng et al. 2004). OKL38 has been shown to interact with the p53 protein in osteosarcoma cells. OKL38 induced formation of megamitochondria, increased cellular ROS levels, and resulted in release of cytochrome c from mitochondria in U2OS cells (Hu, Yao et al. 2012). Based on these previous studies and our RNA-sequencing results, we propose that PPLGM is involved in activating an OKL38-mediated apoptotic pathway by increasing p53 and cytoplasmic cytochrome c protein expression (Fig. 20).

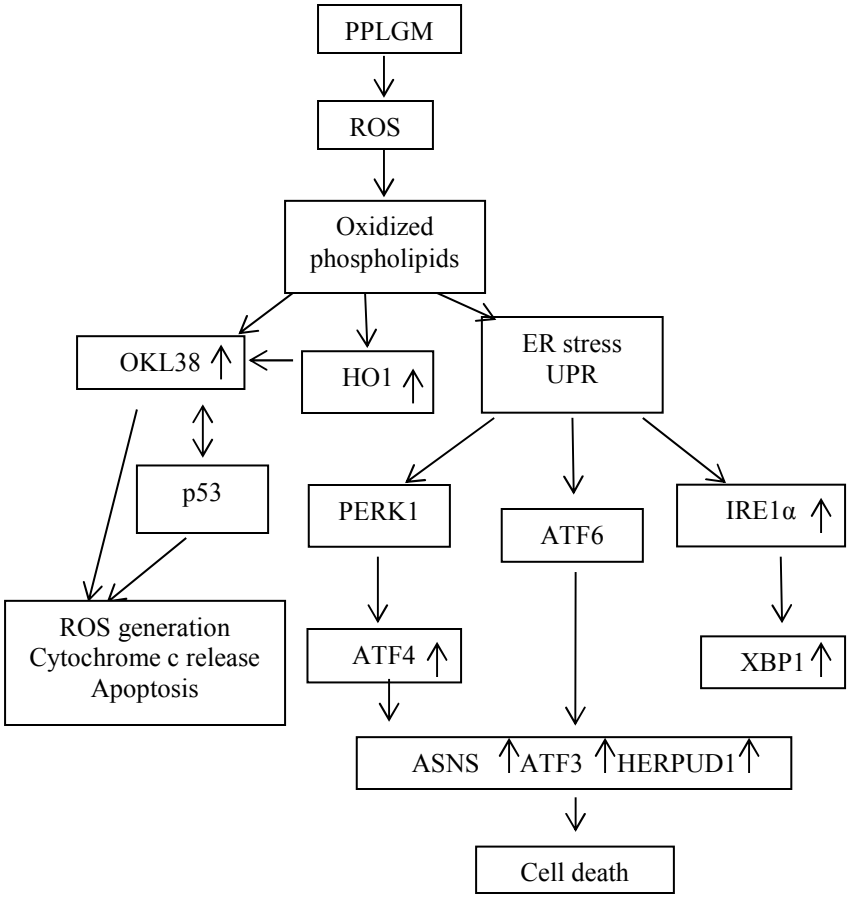


Figure 20. A hypothesized model for PPLGM-mediated MIA PaCa2 pancreatic cancer cell death. Genes with arrow represent upregulation at mRNA or protein level as assessed by qRT-PCR or western blot respectively.

Disturbances in cellular redox regulation and generation of ROS (Fedoroff 2006), faulty calcium regulation (Gorlach, Klappa et al. 2006), and hypoxia (Feldman, Chauhan et al. 2005) act as stress signals to alter ER homeostasis. This elicits a protective response in the ER known as the unfolded-protein response (UPR) that aims to restore ER homeostasis. However, under conditions of prolonged/severe stress, the ER triggers cell death pathways (Szegezdi, Logue et al. 2006; Kim, Xu et al. 2008; Cheng and Yang 2011). We observed an activation of the ER-stress response gene IRE1α, a transmembrane ER sensor which cleaves and activates XBP1 in PPLGM-treated MIA PaCa-2 cells. Also, increased expression of ATF4, ASNS, HERPUD1,

TRIB3, and TNFR UPR mRNA was seen in MIA PaCa-2 cells treated with PPLGM for 6 hours. The transcription factor ATF4 is known to activate the C/EBP homologous protein (CHOP) transcription, which is required for ER-stress-mediated apoptosis both *in vitro* and *in vivo* (Marciniak, Yun et al. 2004; Song, Scheuner et al. 2008). However, we did not detect elevated levels of CHOP in our RNA-sequencing experiments. A longer duration of PPLGM treatment (more than 6 hours) might be needed to activate CHOP expression and induce apoptosis in MIA PaCa-2 cells.

While this is the first genomics-scale study to identify an association between PPLGM and ER-stress pathways in pancreatic cancer cells, recent studies by others have shown an increase in the ER-stress response with PPLGM in other tumor models. Kim *et al.* reported that PPLGM caused an ROS-mediated increase in ER-stress and unfolded protein response markers (CHOP and p-eIF2α) and apoptosis in spheroid-cultured high-grade glioma cells (Kim, Song et al. 2014). The authors suggested that inactivation of peroxiredoxin 4, a PRDX gene family member present in ER, which detoxifies hydrogen peroxide generated during protein folding, is responsible for inducing ROS and ER-stress. Similarly, Jin *et al*. demonstrated the role of ROS in PPLGM-mediated CHOP activation leading to apoptosis in breast cancer cells (Jin, Lee et al. 2014). Taken together, these data confirm ROS-mediated ER-stress induction with PPLGM treatment. Further studies to overexpress or knockdown ER-stress and UPR genes are needed to confirm the results shown here.

Overall, this study shows that PPLGM alters the expression of numerous genes in the human pancreatic cancer cell line, MIA PaCa-2. Particularly, PPLGM treatment significantly activated genes involved in the ER-stress and UPR pathways. Further studies evaluating the

mechanistic response of non-cancerous pancreatic cells to PPLGM are required to consider it as a potential anticancer agent for clinical trials.

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CHAPTER 4. PIPERLONGUMINE POTENTIATES CHEMOTHERAPY-INDUCED PANCREATIC CANCER CELL DEATH *IN VITRO* AND *IN VIVO*

Abstract

Pancreatic cancer is one of the most deadly cancers with a nearly 95% mortality rate. The poor response of pancreatic tumors to currently available chemotherapy and the extremely low survival rate of pancreatic cancer patients point to a critical need for alternative therapeutic strategies. The use of bioactive agents derived from natural products has emerged as an innovative and effective strategy to treat various cancers. In this study, we investigated the anticancer effects of piperlongumine (PPLGM), a bioactive agent found in long peppers, alone and in combination with the chemotherapeutic agents gemcitabine and erlotinib in pancreatic cancer cell culture and animal models. We found that the combination of PPLGM, gemcitabine, and erlotinib resulted in a greater reduction in cell viability, survival, and induction of apoptosis in MIA PaCa-2 and BxPC-3 pancreatic cancer cells when compared to any agent alone. Further, PPLGM in combination with gemcitabine caused a reduction in tumor weight and volume in an orthotopic mouse model of pancreatic cancer. These results suggest that the use of PPLGM in combination with chemotherapeutic agents could be a successful strategy for achieving better treatment outcomes in *KRAS* mutant pancreatic cancer patients.

Introduction

Pancreatic cancer is one of the deadliest forms of cancer for both men and women in the United States with a survival rate of <7% (Siegel, Miller et al. 2015). The poor prognosis for patients with pancreatic cancer is attributed to late stage disease diagnosis when the cancer has already metastasized. Gemcitabine, a deoxycytidine analog, is the first-line conventional chemotherapy for treatment of advanced pancreatic cancer patients. However, gemcitabine

treatment results in only a marginal survival advantage of patients with an objective tumor response rate of <10% (Burris, Moore et al. 1997). Additionally, gemcitabine produces only a modest survival benefit (6-8 months) for pancreatic cancer patients (Burris, Moore et al. 1997; Mancuso, Calabro et al. 2006), particularly those with *KRAS* mutations (Kim, Lim do et al. 2011). Mutations in the *KRAS* oncogene occur in the vast majority (>90%) of pancreatic cancers where oncogenic *RAS* contributes to tumor development, progression, and resistance to therapies (Kim, Lim do et al. 2011).

Although gemcitabine monotherapy or its combination with other agents has become the standard chemotherapeutic approach for treatment of pancreatic cancer, the outcomes are very disappointing. Combination of gemcitabine with erlotinib, a tyrosine kinase inhibitor that inactivates the epidermal growth factor signaling pathway, has resulted in a modest survival benefit in large phase III clinical trial in pancreatic cancer (Moore, Goldstein et al. 2007). Another study involving the combination of gemcitabine with cetuximab (a monocloncal antibody against epidermal growth factor) revealed marginal effects on survival and objective tumor response against cancer in a multicenter phase II trial (Xiong, Rosenberg et al. 2004). In addition to dismal outcomes of chemotherapeutics, chemoresistance and unwanted side effects in healthy tissues remain a problem. Therefore, there is a need for novel therapeutic agents that are less toxic to sensitize cancer cells to chemotherapy.

Numerous lines of evidence suggest the potential for bioactive agents derived from natural products to enhance the anticancer activity of chemotherapeutic drugs without contributing additional toxicity in healthy tissues. For example, curcumin (a derivative of the spice, turmeric) potentiated the antitumor activity of gemcitabine by suppressing the transcription factor, NF-kB and its regulated gene-products such as cyclin D1, c-myc, MMP, and

VEGF (Kunnumakkara, Guha et al. 2007). Another study reported enhanced apoptosis in pancreatic cancer cells in response to the combination of gemcitabine with genistein, an isoflavone derived from soy. The authors suggested an upregulation of Akt and NF-kB proteins as potential mechanisms for chemoresistance to gemcitabine which is suppressed by genistein treatment (Banerjee, Zhang et al. 2005). Together, this evidence suggests natural agents in combination with chemotherapeutics may provide effective therapeutic approaches for treating pancreatic cancer.

Piperlongumine (PPLGM) is an alkaloid derived from long pepper plants. PPLGM has been reported to exert potent antitumor activity in a variety of human cancer cell lines in vitro and in several xenograft mouse models with minimal or no toxicity to normal human cells (Raj, Ide et al. 2011). Intracellularly, PPLGM has been shown to inhibit phosphorylation and degradation of IkBα (Han, Gupta et al. 2014), inactivate peroxiredoxin 4 (Roh, Kim et al. 2014), and directly inhibit STAT3 (Bharadwaj, Eckols et al. 2015). Additionally, previous reports have shown that PPLGM regulates genes related to control of cell proliferation, cell cycle, apoptosis, oncogenesis, and regulation of transcription (Raj, Ide et al. 2011; Golovine, Makhov et al. 2013; Han, Son et al. 2013). Low doses of PPLGM (0.1-1 μM) have been shown to synergize with cisplatin (0.1-1 μM) and paclitaxel with a combination index < 1 to inhibit the growth of human ovarian cancer cells (Gong, Chen et al. 2014).

Preclinical studies *in vitro* from our laboratory have shown that PPLGM induces pancreatic cancer cell death by activating reactive oxygen species (ROS) mediated DNA damage pathway (Dhillon, Chikara et al. 2014). Therefore, in the present study, we sought to examine whether PPLGM can potentiate the antitumor effects of gemcitabine and erlotinib against pancreatic cancer *in vitro*. Importantly, we also tested the chemotherapeutic potential of PPLGM

in combination with gemcitabine *in vivo* using an orthotopic model of pancreatic cancer in nude mice. We found that PPLGM potentiated the cell death and apoptotic effects of the chemotherapeutic agents gemcitabine and erlotinib in pancreatic cancer cells *in vitro* and significantly enhanced the antitumor effects of gemcitabine in pancreatic tumors in nude mice.

Materials and Methods

Cell culture and reagents

The PANC-1 and MIA PaCa-2 cell lines were obtained from ATCC (Manassas, VA) and cultured at 37 °C with 5% carbon dioxide in Dulbecco's Modified Eagle's (Thermo Fisher Scientific Inc; Waltham, MA) medium supplemented with 10% fetal bovine serum (Atlanta Biologicals; Lawrenceville, GA). The BxPC-3 cell line was also obtained from ATCC and cultured in RPMI-1640 (Thermo Fisher Scientific Inc.) medium supplemented with 10% fetal bovine serum. The cell lines were subcultured by enzymatic digestion with 0.25% trypsin/1 mM EDTA solution (Thermo Fisher Scientific Inc.) when they reached approximately 70% confluency. PPLGM (Indofine Chemical Company; Hillsborough, NJ) was dissolved in 100% DMSO at a stock concentration of 78.78 mM and then diluted in water to a working concentration. Gemcitabine (Sigma-Aldrich; St Louis, MO) was dissolved in water at a stock concentration of 10 mg/ml. Erlotinib Hydrochloride (ChemieTek; Indianapolis, IN) was dissolved at a stock concentration of 20 mg/ml in 100% DMSO.

MTT assay

PANC-1, BxPC-3 (3.0×10^3), and MIA PaCa-2 cells (2.5×10^3) were seeded into individual wells of a 96-well plate, 24 h (PANC-1 and BxPC-3) and 48 h (MIA PaCa-2) later the cells were pre-treated with PPLGM (8 μM) for 6 h after which erlotinib (2 μM) and gemcitabine (10 nM) were added for 72 h. After 72 h, 10 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-

diphenyltetrazolium bromide (MTT) reagent was added to each well and incubated for 2 h. Upon termination, the supernatant was aspirated from each well and the MTT formazan formed by metabolically active cells was dissolved in 100 µl of dimethyl sulfoxide (DMSO). The absorbance was measured at 570 nm using a plate reader (Bio-rad; Hercules, CA).

Cell death ELISA assay

The cell death detection ELISA plus kit (Roche; Palo Alto, CA) was used to detect apoptosis in PANC-1, MIA PaCa-2, and BxPC-3 cells with different treatments according to the manufacturer's protocol. Briefly, PANC-1, BxPC-3 (3.0×10^3), and MIA PaCa-2 (2.5×10^3) were treated with 5 µM PPLGM, 2 µM erlotinib, and 10 nM gemcitabine alone or in combination for 72 h. After treatment, the cytoplasmic histone DNA fragments from PANC-1, MIA PaCa-2, and BxPC-3 cells were extracted and bound to an immobilized anti-histone antibody. Subsequently, the peroxidase-conjugated anti-DNA antibody was used for the detection of immobilized histone DNA fragments. After addition of the peroxidase substrate, the spectrophotometric absorbance of the samples was determined using a plate reader (Bio-rad; Hercules, CA) at 405 nm. The level of cytoplasmic histone-associated DNA fragments (mono- and oilgonucleosomes) in pancreatic cancer cells were expressed as an enrichment factor, calculated using the following formula: Enrichment factor = Absorbance for treated sample/ Absorbance for untreated control.

Clonogenic survival assay

The clonogenic-survival assay tests the long-term survival ability of cells and their capacity to proliferate and form a colony in the presence of an anticancer agent. PANC-1, MIA PaCa-2, and BxPC-3 cells (2×10^2) were seeded into individual wells of a 12-well plate. The next day, the cells were treated with 3 µM PPLGM, 0.3 µM gemcitabine, and 8 µM erlotinib

alone or in combination for 72 h. After 72 h, the medium was changed and the cells were allowed to grow and form colonies for 14 days. After 14 days, the colonies were fixed in a solution of methanol and acetic acid (3:1), stained with 0.5% crystal violet, and counted manually. Three replicate experiments were performed for each cell line.

Orthotopic mouse model

Six- to 8-week old female athymic nude mice (Nu/Nu) were purchased from Charles River Laboratories (Wilmington, MA). The mice were maintained in sterile conditions using the Innovive IVC System (Innovive; San Diego, CA), following a protocol approved by North Dakota State University's Institutional Animal Care and Use Committee (IACUC). The mice were acclimated for 1 week before experimental manipulation. MIA PaCa-2 cells were harvested and resuspended in PBS. Mice were anesthetized with a ketamine-xylazine solution, a small left abdominal flank incision was made, and MIA PaCa-2 cells (~8 x 10^5 in 30 µl) were injected into the pancreas using a 27-gauge needle. The abdomen was closed using a 2-layer suture technique involving chromic catgut and ethilon sutures. After 2 weeks of cancer cell implantation, the mice were randomized into 4 groups: (i) untreated control (DMSO 5% i.p. 3x a week every 3 days), (ii) PPLGM treated (5 mg/kg body weight, i.p. 3x a week), (iii) gemcitabine treated (25 mg/kg body weight, i.p. 3x a week), and (iv) PPLGM + gemcitabine (5 mg/kg and 25 mg/kg body weight, respectively), i.p. 3x a week. PPLGM was dissolved in DMSO at a stock concentration of 9.9 mg/ml and further diluted in PBS before administering to the mice. The final concentration of DMSO in the PPLGM working solution was 5%. Gemcitabine was dissolved in sterile saline at a stock concentration of 50 g/L. Each treatment group contained 13-14 animals. After 4 weeks of treatment, the animals were euthanized by an overdose of ketamine-xylazine solution followed by cervical dislocation. The primary tumors in the pancreas were excised and

measured for tumor weight and volume [V= $(width)^2$ x length/2]. The tumor weight and volume were compared between the groups using an unpaired student t-test. Tumors from half of the mice for each treatment were paraformaldehyde-fixed and paraffin-embedded for immunohistochemistry. The other of the mouse tumors were snap-frozen in liquid nitrogen and stored at -80°C.

Immunohistochemistry

Tumor tissues from control and treated mice were collected and fixed for 24 h in paraformaldehyde. Paraffin-embedded 5 μM thick sections of tumor tissues were prepared. Sections were deparaffinized with Histo-Clear and ethanol, followed by antigen retrieval in a 10 mM sodium citrate buffer (0.05% Tween 20, pH 6.0) using an autoclave method. The tissue sections were blocked for 20 min in blocking buffer (10% normal goat serum in TBST) and incubated with Ki-67 (1:100) primary antibody overnight at 4°C. The next day, the sections were incubated with CF633-conjugated goat anti-mouse secondary antibody (1:250) for an hour at room temperature, and were visualized using a Zeiss inverted Axio Observer Z1 microscope after mounting a coverslip using Hardset Mounting media with DAPI (Vector Labs; Burlingame, CA).

Statistical analyses

Data are presented as means ± standard deviation for at least 3 independent experiments. The significance for differences between groups was determined using a Student's *t*-test and two-way ANOVA with statistical significance defined as $p < 0.05$. Statistical analyses were performed using SAS version 9.3.

Results

PPLGM enhances pancreatic cancer cell death in combination with gemcitabine and erlotinib

Cell viability was determined using an MTT assay for pancreatic cancer cell lines pre-treated with PPLGM (8 μM for 6 h) followed by a 72 h-incubation with a suboptimal dose of either gemcitabine (10nM) or erlotinib (2 μM). PPLGM alone resulted in a 70% decrease in cell viability whereas erlotinib and gemcitabine had no effect on cell viability in MIA PaCa-2 cells at the concentrations tested. The combination of PPLGM with erlotinib and gemcitabine caused a significant potentiation in the inhibition of MIA PaCa-2 cell viability (90%) as compared to either agent alone (Fig. 21). Interestingly, the combination of PPLGM with gemcitabine and erlotinib did not significantly reduce PANC-1 cell viability as compared to the PPLGM treatment alone (Fig. 21). In BxPC-3 cells, however, the combination of PPLGM with erlotinib and gemcitabine resulted in a significant decrease in cell survival (70%) as compared to PPLGM alone (40%) (Fig. 21).

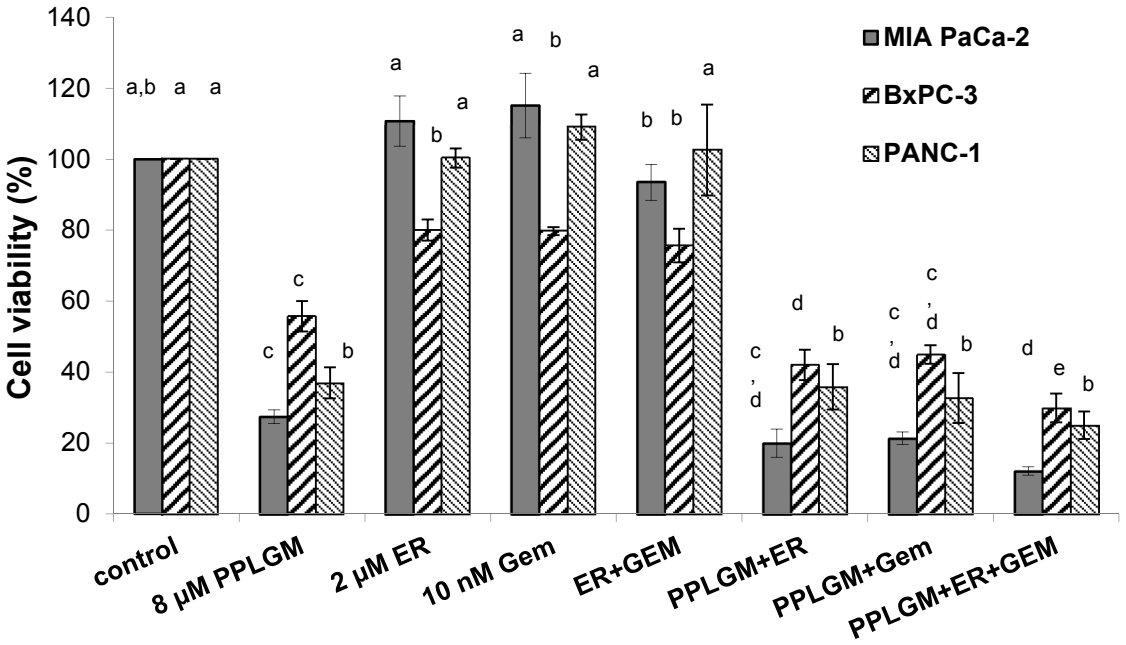


Figure 21. Effect of PPLGM and chemotherapy on the viability of pancreatic cancer cell lines using an MTT assay. MIA PaCa-2, BxPC-3, and PANC-1 cells were either untreated or pre-treated with 8 µM PPLGM for 6 hours followed by 10 nM gemcitabine (Gem) and 2 µM erlotinib (ER) treatment for 72 hours. Upon termination of incubation, absorbance was measured at 570 nm. The cell viability percentage was calculated using the following formula: % viable cells = (O.D. of drug-treated sample/ O.D. of untreated sample)×100. The data represent the average cell viability ± standard deviation for three independent experiments for MIA PaCa-2, BxPC-3, and PANC-1 cells. Statistical significance was determined by two-way ANOVA.

PPLGM in combination with erlotinib and gemcitabine decreases long-term survival of pancreatic cancer cells

To determine the effect of PPLGM, gemcitabine, and erlotinib on long-term survival, cells were treated with each of the single agents alone or in combination and assessed for survival by a clonogenic assay. The combination of PPLGM with erlotinib and gemcitabine resulted in a significant inhibition of colony formation in MIA PaCa2, PANC-1, and BxPC-3 cells when compared to either agent alone (Fig. 22).

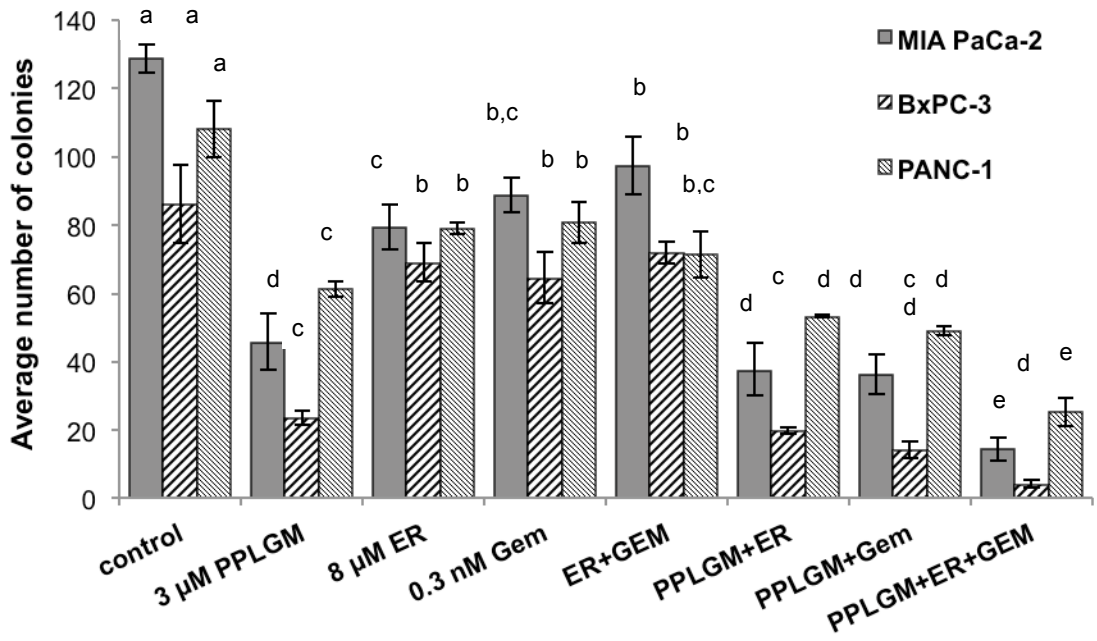


Figure 22. Effect of PPLGM and chemotherapy on survival of pancreatic cancer cell lines using a clonogenic survival assay. The long-term growth of MIA PaCa2, PANC-1, and BxPC-3 cells was determined by calculating the average number of colonies in the presence of 3 μM PPLGM, 8 μM erlotinib (ER), and 0.3 nM gemcitabine (Gem) alone or in combination over the course of 14 days. The data represent average ± standard deviation for four independent experiments for MIA PaCa-2, BxPC-3, and PANC-1 cells. Statistical significance was determined by two-way ANOVA.

PPLGM in combination with gemcitabine and erlotinib enhances apoptosis in pancreatic cancer cells

To assess if enhanced cytotoxicity by PPLGM in combination with chemotherapeutic agents was mediated by apoptosis, a cell death ELISA assay was performed. Relative to single agents, PPLGM in combination with gemcitabine and erlotinib elicited significantly higher apoptosis in MIA PaCa-2 and BxPC-3 cells, suggesting that the loss of viable cells by PPLGM/gemcitabine/erlotinib combination is due to the induction of an apoptotic pathway (Fig. 23).

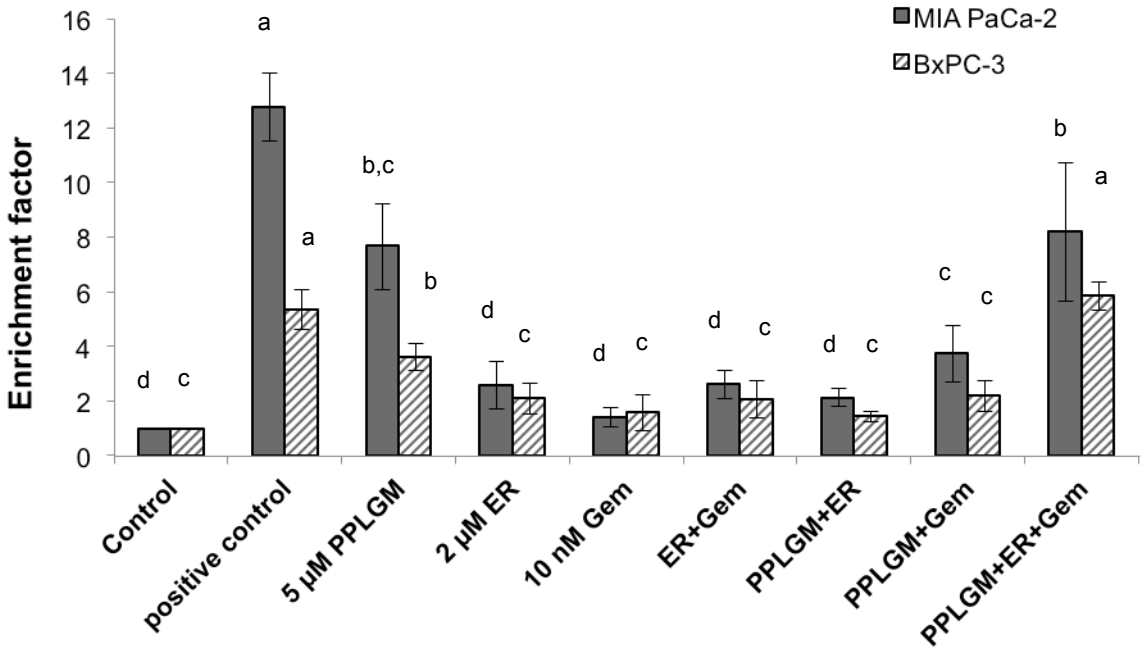


Figure 23. Effect of PPLGM and chemotherapy on apoptosis of pancreatic cancer cell lines using cell death ELISA assay. The apoptotic response was assessed in MIA PaCa2 and BxPC-3 cells by cell death detection ELISA after treatment with 5 µM PPLGM, 2 µM erlotinib (ER), and 10 nM gemcitabine (Gem) alone or in combination for 24 hours represented as relative fold increase in apoptosis as compared with control. The bars represent average ± standard deviation (n=3). Statistical significance was determined by two-way ANOVA.

PPLGM enhances the *in vivo* therapeutic effect of gemcitabine in an orthotopic mouse model

Building on aforementioned results which support better killing of pancreatic cancer cells when PPLGM is combined with chemotherapeutic agents, we evaluated the therapeutic advantage of combining PPLGM and gemcitabine in nude mice bearing orthotopically implanted MIA PaCa-2 cells. Doses of 5 mg/kg of PPLGM and 25 mg/kg gemcitabine were selected for intraperitoneal administration three times a week (Fig. 24) based on previously published reports (Kunnumakkara, Guha et al. 2007; Harikumar, Kunnumakkara et al. 2010; Park, Prasad et al. 2011). The treatment efficacy was determined by considering the mean pancreatic tumor weight and volume immediately following euthanization. Administration of PPLGM caused a 36% and 67% reduction in tumor weight and volume, respectively. Gemcitabine alone caused a 50% reduction in tumor weight and a 64% reduction in tumor volume (Fig. 26). However, the combination of PPLGM and gemcitabine showed a significant decrease ($p<0.01$) in tumor weight and volume relative to DMSO alone, PPLGM alone, and gemcitabine-alone treated mice. No macroscopic evidence of spreading to other visceral organs was evident for any experimental groups (Fig. 25). These results, for the first time, confirm the chemosensitization effect of PPLGM in an *in vivo* orthotopic pancreatic cancer model. The PPLGM and gemcitabine treatments alone were well-tolerated, and no significant changes were detected in overall organ mass (Table 8). A significant reduction in liver mass was observed when PPLGM is used in combination with gemcitabine, which may indicate potential liver toxicity associated with the combined treatment.

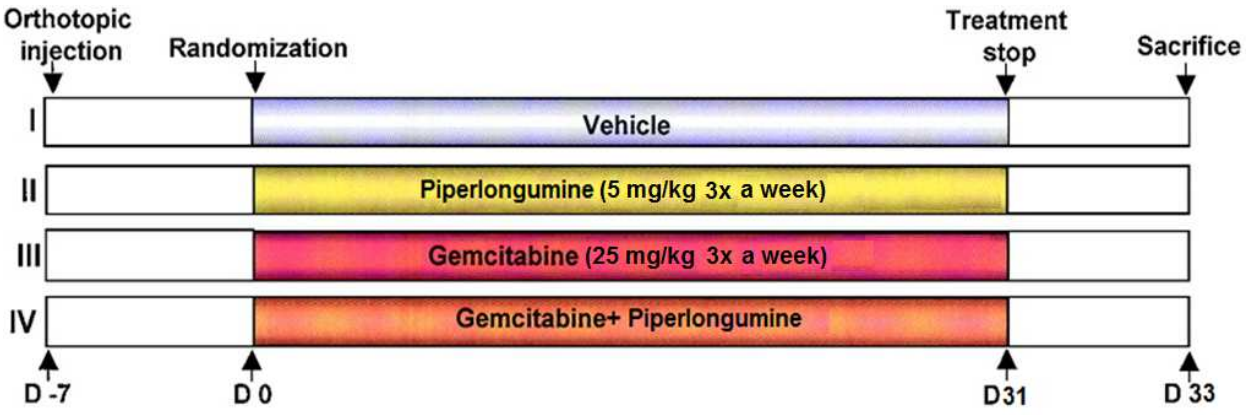


Figure 24. Schematic representation of the experimental protocol for the orthotopic pancreatic cancer mouse model. Group I was given DMSO (5%, i.p., 3x a week), group II was given PPLGM (5 mg/kg, i.p., 3x a week), group III was given gemcitabine (25 mg/kg, i.p., 3x a week), and group IV was given gemcitabine (25 mg/kg, i.p., 3x a week) and PPLGM (5 mg/kg, i.p., 3x a week); n=14/group.

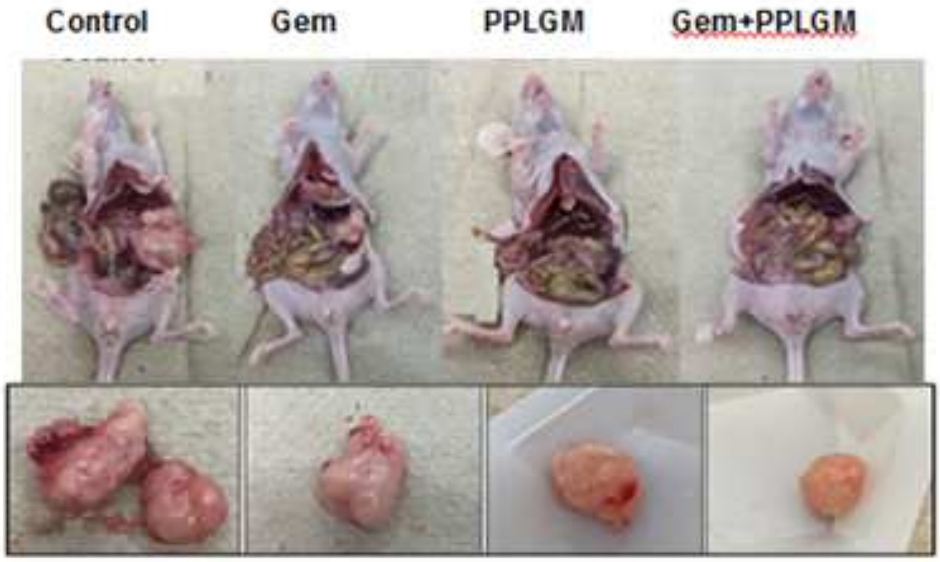


Figure 25. *In vivo* effects of PPLGM and gemcitabine in an orthotopic model of MIA PaCa-2 pancreatic cancer. Necropsy images of mice bearing orthotopically implanted pancreatic tumors from all four groups of mice treated with i.p. injections of DMSO (control), gemcitabine (Gem), PPLGM, and Gem + PPLGM.

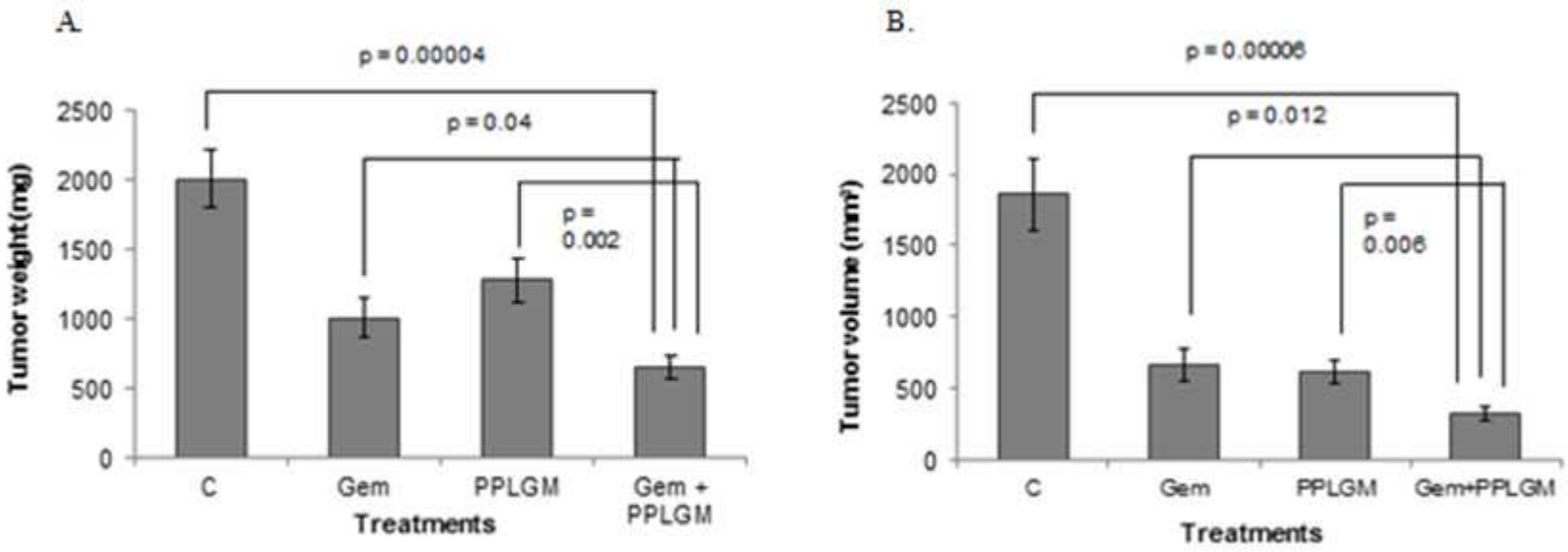


Figure 26. Effect of PPLGM and gemcitabine on tumor weight and volume in an orthotopic model of MIA PaCa-2 pancreatic cancer. (A) Tumor weights in mice were measured on the last day of the experiment at autopsy. (B) End point tumor volumes in mice were measured using vernier calipers and calculated using the formula mentioned in the Materials and Methods section. The final tumor weights and volumes were compared among groups using an unpaired Student's t-test. Statistically significant groups are represented by $p<0.05$.

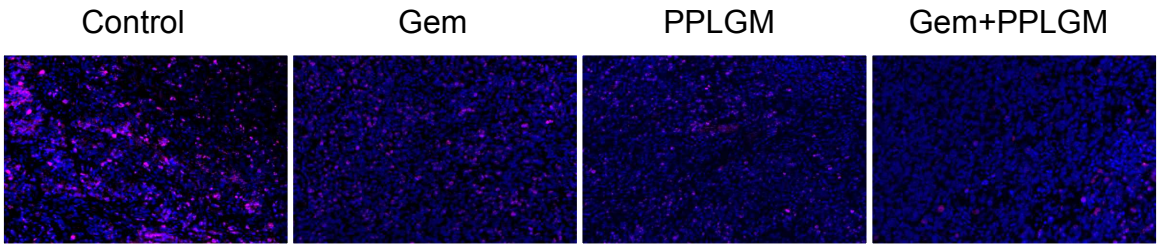
Table 8. Effect of PPLGM and gemcitabine alone and in combination on organ weights of mice

	Control	**Gem**	**PPLGM**	**Gem+PPLGM**
Lung (mg)	180 ± 13.2	187.7 ± 11.5	185.7 ± 10.0	185.4 ± 6.3
Liver (mg)	1283.8 ± 70.3[a]	1240 ± 68.5[a]	1210 ± 58.1[a]	1007.7 ± 45.7[b]
Spleen (mg)	289.1 ± 37.5	212.5 ± 24.7	257.8 ± 15.2	243.3 ± 31.9
Kidney (mg)	314.6 ± 13.8	292.3 ± 14.6	319.3 ± 14.8	296.1 ± 13.9

Combination of PPLGM with chemotherapeutic agents decreases proliferation in pancreatic tumors

Ki-67 protein expression, a cell proliferation marker, was investigated in pancreatic tumors harvested from all four groups of mice treated either with DMSO, PPLGM, and gemcitabine alone or in combination. Tumors obtained from PPLGM and gemcitabine alone-treated mice exhibited significantly less Ki-67-positive cells as compared to DMSO-treated mice. Interestingly, mice treated with a combination of PPLGM and gemcitabine displayed a significant reduction in proliferative cells relative to DMSO alone, PPLGM alone, and gemcitabine-alone treated mice (Fig. 27).

A.



B.

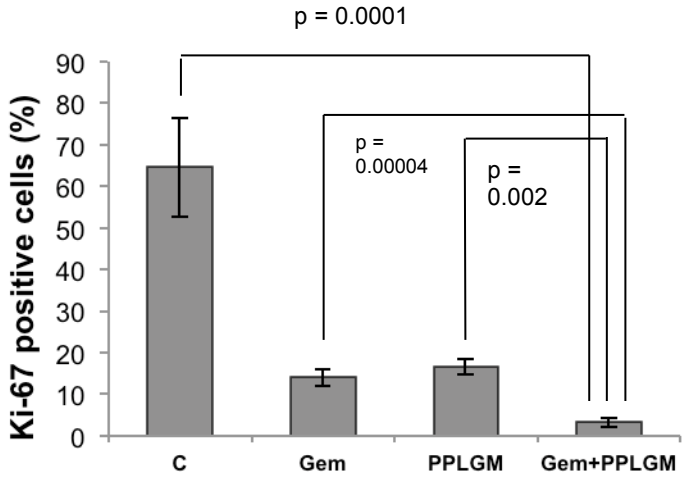


Figure 27. Effects of PPLGM and gemcitabine alone and in combination on a cell proliferation marker in pancreatic tumors. (A) Immunohistochemical analysis of the proliferation marker Ki-67 in DMSO, PPLGM, gemcitabine (Gem) alone or Gem+PPLGM-treated animals. (B) Quantification of Ki-67 positive cells, represented as the percentage of Ki-67 positive cells ± standard deviation per 20X magnification. A total of four 20X fields were examined and counted from three tumors for each of the treatment groups. The values were compared among groups using an unpaired Student's t-test.

Discussion

Pancreatic cancer remains one of the most difficult malignancies to treat due to *de novo* chemoresistance and limited effectiveness of chemotherapeutics. Numerous approaches involving a combination of different chemotherapeutic agents have been used that result in only marginal survival benefits mainly due to toxicity of drug. Therefore, new strategies using a combination of toxic chemotherapeutics in low doses with non-toxic natural agents are recently

being investigated. This preclinical study demonstrates that potentiation of anticancer activity could be achieved by a natural agent, PPLGM with gemcitabine and erlotinib, as shown by more pronounced cell death in the combination approach compared to single-agent treatment. This effect was independent of *KRAS* gene status of pancreatic cancer cells.

PPLGM has been shown to synergize with cisplatin in head and neck cancer cells as documented by enhanced apoptosis and antitumor activity *in vitro* and *in vivo* (Roh, Kim et al. 2014). Similarly, another study reported synergistic effects of PPLGM with cisplatin and paclitaxel, as observed by an antiproliferative effect in human ovarian cancer cells (Gong, Chen et al. 2014). We have recently demonstrated the anticancer role of PPLGM in pancreatic cancer cells is mediated by ROS-induced DNA damage. Further, we have shown PPLGM is effective at inhibiting subcutaneous pancreatic tumor xenograft progression in nude mice (Dhillon, Chikara et al. 2014). Here we show that PPLGM pretreatment followed by chemotherapeutics, gemcitabine and erlotinib, significantly enhanced pancreatic cancer cell death compared with either agent alone. This observation is highly significant because a significantly greater (70-88%) growth inhibition was achieved using the same concentration of chemotherapeutics which resulted in 0-20% growth inhibition when used alone. Inhibition of cell growth was correlated with the induction of apoptosis in MIA PaCa-2 and BxPC-3 pancreatic cancer cells.

This study is the first to investigate the anti-tumor efficacy of PPLGM in an orthotopic animal model. We found that the combination of PPLGM (2.5mg/kg/day, 3x a week) and gemcitabine (2.5mg/kg, 3x a week) significantly inhibited pancreatic tumor growth in nude mice relative to either agent alone. The final tumor volume of PPLGM and gemcitabine treated animals was 80% lower than tumors obtained from control-treated animals. On the other hand, tumor weight of animals treated with PPLGM and gemcitabine combination was 67% lower than

control-treated animals. Our results are in agreement with numerous reports showing potent antitumor effects of dietary agents in combination with gemcitabine in orthotopic models of pancreatic cancer (Banerjee, Zhang et al. 2005; Banerjee, Kaseb et al. 2009; Kunnumakkara, Sung et al. 2010). Although PPLGM and gemcitabine treatment alone was well-tolerated in mice, a significant reduction in liver weight was observed in mice treated with the combination of PPLGM and gemcitabine. This result might indicate enhanced liver toxicity is associated with combined PPLGM and gemcitabine treatment, and will need to be further investigated before moving forward with human clinical trials.

In conclusion, our results demonstrate that the combination of PPLGM with the chemotherapeutic agents, gemcitabine and erlotinib, enhances pancreatic cancer cell death *in vitro*. Moreover, the combination of PPLGM with gemcitabine significantly suppressed the growth of pancreatic tumor xenografts by reducing cell proliferation. Our results suggest that the combination of PPLGM with gemcitabine has significant potential as an effective therapy for pancreatic cancer patients to enhance the effect of gemcitabine. Further studies evaluating the underlying molecular mechanisms are needed to fully support this strategy for treating patients with pancreatic tumors.

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CHAPTER 5. ACTIVATION OF ERK SIGNALING AND INDUCTION OF COLON CANCER CELL DEATH BY PIPERLONGUMINE[2]

Abstract

Piperlongumine (PPLGM) is a bioactive compound isolated from long peppers that shows selective toxicity towards a variety of cancer cell types including colon cancer. The signaling pathways that lead to cancer cell death in response to PPLGM exposure have not been previously identified. Our objective was to identify the intracellular signaling mechanisms by which PPLGM leads to enhanced colon cancer cell death. We found that PPLGM inhibited the growth of colon cancer cells in time- and concentration-dependent manners, but was not toxic toward normal colon mucosal cells at concentrations below 10 μM. Acute (0–60 min) and prolonged (24 h) exposure of HT-29 cells to PPLGM resulted in phosphorylation of ERK. To investigate whether ERK signaling was involved in PPLGM-mediated cell death, we treated HT-29 cells with the MEK inhibitor U0126, prior to treating with PPLGM. We found that U0126 attenuated PPLGM-induced activation of ERK and partially protected against PPLGM-induced cell death. These results suggest that PPLGM works, at least in part, through the MEK/ERK pathway to result in colon cancer cell death. A more thorough understanding of the molecular mechanisms by which PPLGM induces colon cancer cell death will be useful in developing therapeutic strategies to treat colon cancer.

Introduction

Bioactive food components extracted from botanicals are effective chemopreventive and therapeutic agents for various human cancers (Greenwald, Milner et al. 2002). Specifically,

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* This author performed and troubleshot all the experiments (Figures 28 – 36, Table 9). She also wrote all the sections of this chapter which were revised by the last author.

extracts from *Piper* (pepper) plants have been used for medicinal purposes for centuries and show numerous pharmacological activities such as anti-cancer (Bezerra, Pessoa et al. 2005), anti-inflammatory (Bang, Oh da et al. 2009), anti-platelet (Fontenele, Leal et al. 2009), anti-depressant (Lee, Hong et al. 2005), analgesic (Vedhanayaki, Shastri et al. 2003), and cardioprotective effects (Wakade, Shah et al. 2008). A primary constituent of *Piper longum* (long pepper) is piperlongumine (PPLGM; aka piplartine), an alkaloid which is found not only in the fruits but also the roots of the plant (Park, Son et al. 2007).

PPLGM displays potent growth-inhibitory properties in a cancer cell-selective manner in a variety of cancer cell lines including colon cancer (Raj, Ide et al. 2011). A proposed mechanism by which PPLGM induces cancer-selective cell death is through the elevation of reactive oxygen species (ROS). Relative to healthy cells, cancer cells have heightened levels of ROS and a highly active antioxidant defense system (Szatrowski and Nathan 1991). As a result, cancer cells, but not healthy cells, are unable to recover from additional oxidative stress and die (Schumacker 2006; Trachootham, Alexandre et al. 2009). PPLGM directly binds to and inhibits the antioxidant enzyme glutathione S-transferase pi 1 (GSTP1) resulting in elevated levels of ROS and subsequent cancer-selective cell death (Raj, Ide et al. 2011). However, little is known about the signaling mechanisms by which PPLGM causes cell death in cancer cells, particularly colon cancer.

The extracellular signal-regulated kinase pathway mediates signal transduction involved in cell proliferation, differentiation, and migration (Dhillon, Hagan et al. 2007). Paradoxically, a growing number of studies have shown that activation of ERK by chemopreventive compounds (i.e. resveratrol and quercetin) results in anti-proliferative effects such as apoptosis, senescence, or autophagy in cancer cells (She, Bode et al. 2001; Shih, Davis et al. 2002; Aggarwal and

Shishodia 2006; Kim, Lee et al. 2008). Once activated, ERK can activate apoptotic enzymes or phosphorylate transcription factors that regulate the expression of pro-apoptotic genes (Cagnol and Chambard 2010). Previous studies have shown that cell death in PPLGM-treated cells was accompanied by increased ROS levels, increased p53 and PUMA expression, decreased Bcl-2 expression, depolarization of the mitochondrial membrane, cleaved caspase-3, and DNA fragmentation (Kong, Kim et al. 2008; Raj, Ide et al. 2011). The association of chemopreventive compounds with enhanced ERK signaling and the fact that elevated levels of ROS can activate ERK signaling led us to hypothesize that PPLGM induces apoptosis in colon cancer cells via activation of the MEK/ERK pathway.

In the present study, we investigated the effect of PPLGM on MEK/ERK signal transduction activity in HT-29 colon cancer cells. Further, we identified the contribution of the MEK/ERK pathway in PPLGM-mediated colon cancer cell death. Colorectal cancer is a leading cause of cancer-related deaths in the US. A better understanding of the signal transduction pathways used by PPLGM to induce colon cancer cell death will provide a stronger rationale for the use of PPLGM as an adjuvant therapy for colon cancer treatment.

Materials and methods

Materials

Piperlongumine (PPLGM) was purchased from Indofine Chemical Company (Catalog #: P-004, 97%, Hillsborough, NJ). PPLGM was dissolved in 100% DMSO at a stock concentration of 10 mM and then diluted in water for experiments. The final concentration of PPLGM was in the range of 1–40 μM with a maximum DMSO concentration of 0.2%. In all experiments, DMSO was added to control cells at a final concentration of 0.2% in culture medium. Antibodies (total and phospho-ERK, total and cleaved caspase-3, and GAPDH) and the MEK inhibitor

U0126 were purchased from Cell Signaling Technologies (Danvers, MA). The anti-rabbit secondary antibody was purchased from Jackson Immuno Research (West Grove, PA).

Cell culture

HT-29 and HCT 116 cell lines were obtained from ATCC (Manassas, VA) and cultured at 37 °C with 5% carbon dioxide in McCoy's 5a (Thermo Fisher Scientific Inc; Waltham, MA) medium supplemented with 10% fetal bovine serum (Atlanta Biologicals; Lawrenceville, GA). NCM460, a normal human colon mucosal epithelial cell line was obtained from INCELL Corporation (San Antonio, TX) and grown in M3 medium (INCELL) supplemented with 10% FBS and 1% penicillin streptomycin (Thermo Fisher Scientific). Cell lines were subcultured by enzymatic digestion with 0.25% trypsin/1 mM EDTA solution (Thermo Fisher Scientific) when they reached approximately 70% confluency. Both floating and attached cells were harvested in the case of NCM460 for subsequent analysis.

AlamarBlue® cell toxicity assay

HT-29, HCT 116, and NCM460 cells (5×10^3) were seeded into individuals wells of a 96-well plate, and 24 h later were treated with PPLGM (0–40 μM) after which alamarBlue® (AbD Serotech; Raleigh, North Carolina) was added at a final concentration of 10% and incubated at 37 °C for 4 h. The oxidized form of the dye is converted into the reduced form by a mitochondrial enzyme in the viable cells. Absorbance was measured at 570 and 600 nm on a plate reader and the cell viability was calculated by plotting percent reduction in alamarBlue® over time for each treatment. The cells were monitored daily over a 3-day period to gauge a shift in absorbance. The data represent the average percent reduction of alamarBlue® +/− standard deviation in eight replicate wells per treatment for three independent experiments. A reduction in alamarBlue® absorbance correlates to a decrease in cell viability. The half maximal inhibitory

concentrations (IC_{50}s) were calculated by fitting the dose–response curves derived after plotting the percentage reduction of alamarBlue® against the log concentration.

Trypan blue exclusion assay

HT-29 cells in logarithmic growth phase were seeded at 8×10^3/ml into each well of a 48-well plate, and were treated with different concentrations of PPLGM (0–40 μM). Cells were collected each day after 24, 48, and 72 h, and counted under a light microscope after trypan blue exclusion. There were six replicates for each concentration and the experiment was performed three times.

Apoptosis assay by acridine orange/ethidium bromide staining

HT-29 cells were seeded in a 6-well plate and treated with either DMSO alone or increasing concentrations of PPLGM (2.5–40 μM) for 24 h. Alternatively, HT-29 cells were pretreated with 10 μM U0126 for 2 h followed by 15 μM PPLGM for 24 h. The next day, the cells were washed with PBS and stained with 100 μg/ml acridine orange and 100 μg/ml ethidium bromide in PBS just prior to microscopy. The cells were viewed using a Zeiss inverted Axio Observer Z1 microscope with excitation at 480 nm and emission at 535 nm. For quantification of cell viability, the total number of viable cells was determined for eight replicate microscopic views for each of three independent experiments. A minimum of 200 cells were counted for each replicate well in each independent experiment. The average numbers of viable cells in the treatment groups were then expressed as percentages of the control.

SDS–PAGE and western blotting

Cell pellets were lysed using an SDS lysis buffer (Cell Signaling Technologies) containing protease and phosphatase inhibitors (Roche; Indianapolis, IN). Samples were briefly sonicated to dissociate cell membranes. Fifty micrograms of total protein isolated from PPLGM

or U0126-treated HT-29 cells were separated on 10% SDS-polyacrylamide gels at 100 V for 1 h. Proteins were transferred to nitrocellulose membranes at 100 V for 75 min at 4 °C. Blots were then probed overnight at 4 °C with primary antibodies. The next day, blots were rinsed with 1X TBS-tween (0.1%) and probed with anti-rabbit secondary antibody for 1 h at room temperature. The western blots were analyzed using SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific; Rockford, IL) and images were captured using the MultiImage™ Light Cabinet (Alpha Innotech; San Leandro, CA). Phospho-ERK levels were normalized to total ERK or GAPDH expression. Immunoblots were performed in triplicate and the images in the figures represent one typical replicate.

Statistical analyses

Data are presented as means ± standard deviation for at least three independent experiments. The significance of differences between groups was determined using a Student's *t*-test with statistical significance defined as $p < 0.05$. Statistical analyses were performed using SigmaPlot v12.

Results

PPLGM causes concentration and time-dependent growth inhibition of HT-29 and HCT 116 cells and is less toxic toward NCM460 normal colon mucosal cells

We examined the growth inhibitory effect of PPLGM on HT-29 and HCT 116 colon cancer cells and NCM460 normal colon mucosal cells using the alamarBlue® assay. Exponentially growing HT-29 and HCT 116 cells were treated with either the vehicle control (DMSO) or increasing concentrations of PPLGM (1–40 μM) over a course of 3 days and cell growth was assessed each day. PPLGM induced a concentration and time-dependent decrease in the viability of both HT-29 and HCT 116 cells, with an IC_{50}value of 10.1 μM in HT-29 and 6.4 μM in HCT

116 cells at 48 h (Fig. 28A and B), but only inhibited the growth of NCM460 normal colon mucosal cells at concentrations of 10 μM after 48 h (Fig. 28C). Moreover, total cell counts determined by trypan blue exclusion assay in HT-29 cells treated with different concentration of PPLGM (5–40 μM) for 24, 48 and 72 h demonstrated that PPLGM is inhibiting the proliferation of and inducing cell death in HT-29 cells (Fig. 29).

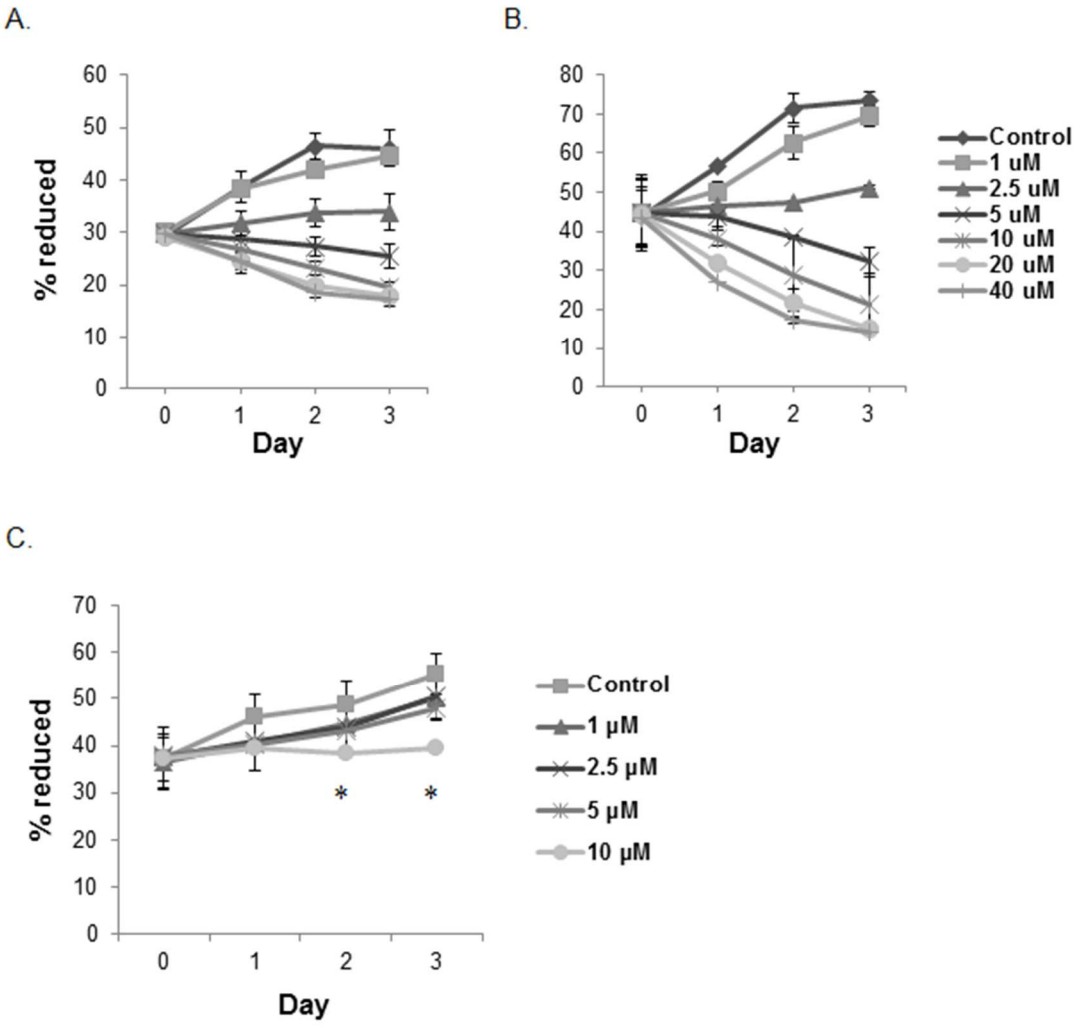

Figure 28. Growth curve showing the concentration and time-dependent effects of PPLGM on the viability of colon cancer and normal colon mucosal cells. The growth of (A) HT-29 and (B) HCT-116 colon cancer cells and (C) NCM460 normal colon mucosal cells was assessed by the percentage of alamarBlue® reduction in the presence of PPLGM (0–40 µM) over the course of 3 days. Data represent the average ± standard deviation of eight replicate wells for three independent experiments for HT-29, HCT 116, and NCM460 cells. PPLGM (2.5–40 µM) significantly inhibited the growth of the colon cancer cells at all time points; whereas, PPLGM (10 µM) inhibited the growth of NCM460 cells after 48 h of treatment, where ∗ denotes statistical significance ($p < 0.05$).

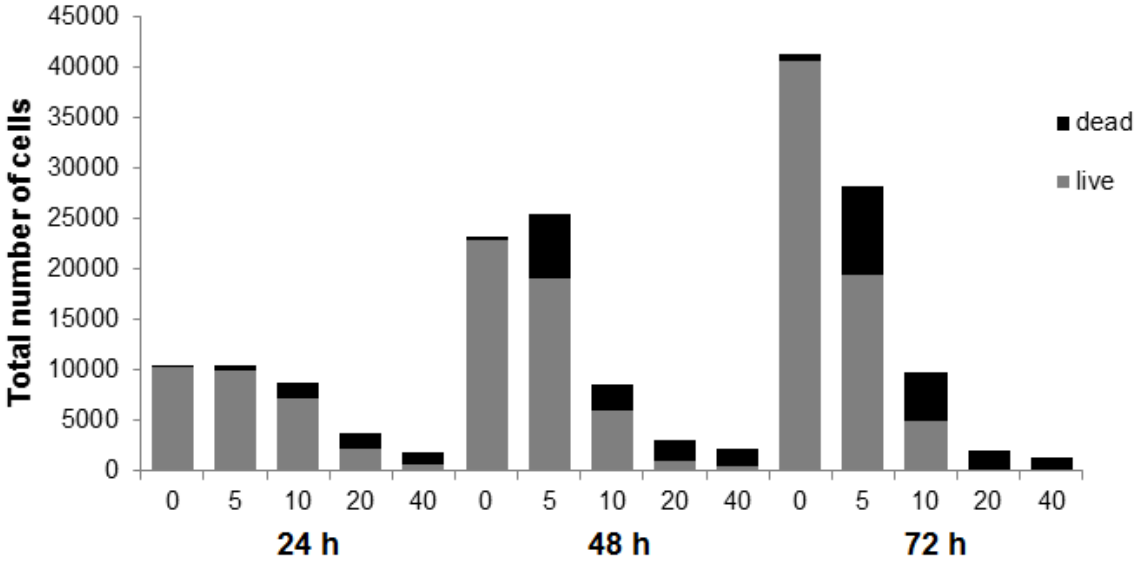

Figure 29. Total cell count of HT-29 cells showing the number of live and dead cells assessed by the trypan blue exclusion assay upon treatment of PPLGM (0–40 μM) for 24, 36, and 72 h. The bar graph shows average cell counts of six replicate wells for three independent experiments.

PPLGM induces apoptosis in HT-29 colon cancer cells

To determine whether the inhibition of colon cancer cell proliferation by PPLGM was due to the induction of apoptosis or necrosis, we assessed cell death using the acridine orange/ethidium bromide method. HT-29 cells were treated with DMSO alone or different concentrations of PPLGM (5–40 μM) for 24 h and stained using acridine orange and ethidium bromide. Fig. 30 shows a decrease in the number of cells upon treatment with increasing concentrations of PPLGM. Live cells were uniformly green whereas apoptotic cells were characterized by chromatin condensation, loss of membrane integrity, and apoptotic blebbing (Fig. 31). In addition, lower concentrations of PPLGM (1–10 μM) enhanced the expression of cleaved caspase 3 (Fig. 32).

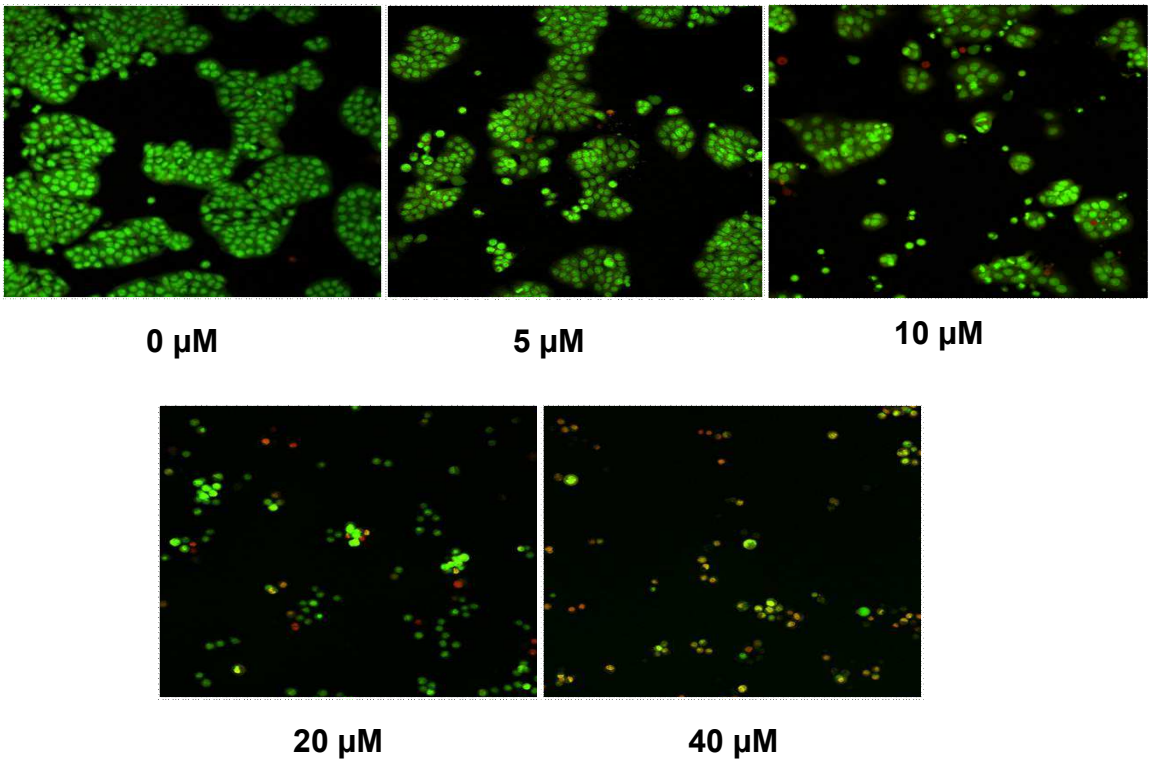

Figure 30. Acridine orange/ethidium bromide staining of HT-29 cells treated with increasing concentrations of PPLGM. PPLGM treatment (0–40 µM) of HT-29 cells for 24 h resulted in concentration-dependent cell death as assayed by acridine orange/ethidium bromide staining. Three independent experiments were performed, and the image shown here is representative of one of these experiments.

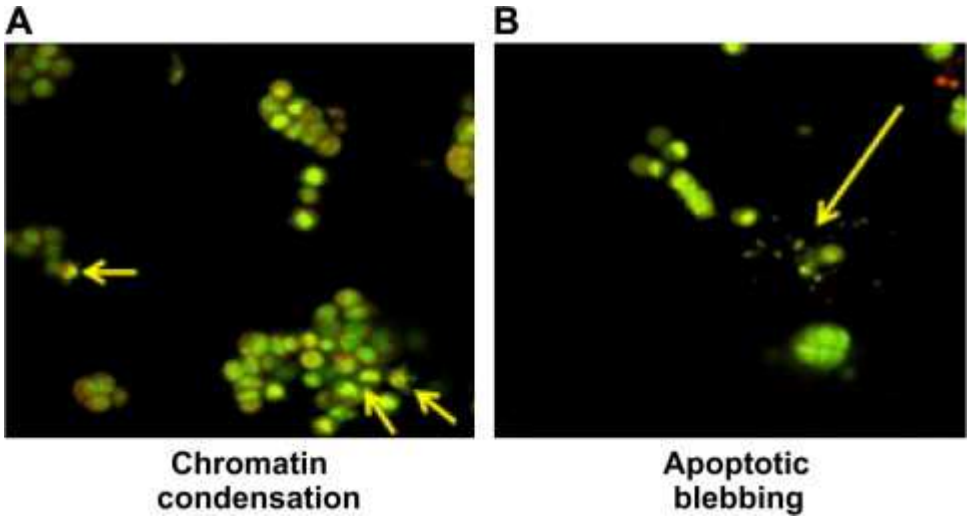

Figure 31. Acridine orange/ethidium bromide staining of HT-29 cells treated with PPLGM reveals characteristics of apoptotic cell death. PPLGM treatment (10 μM for 24 h) induced apoptosis as observed by (A) chromatin condensation and (B) apoptotic blebbing in HT-29 cells assayed using acridine orange/ethidium bromide. Three independent experiments were performed, and the image shown here is representative of one of these experiments.

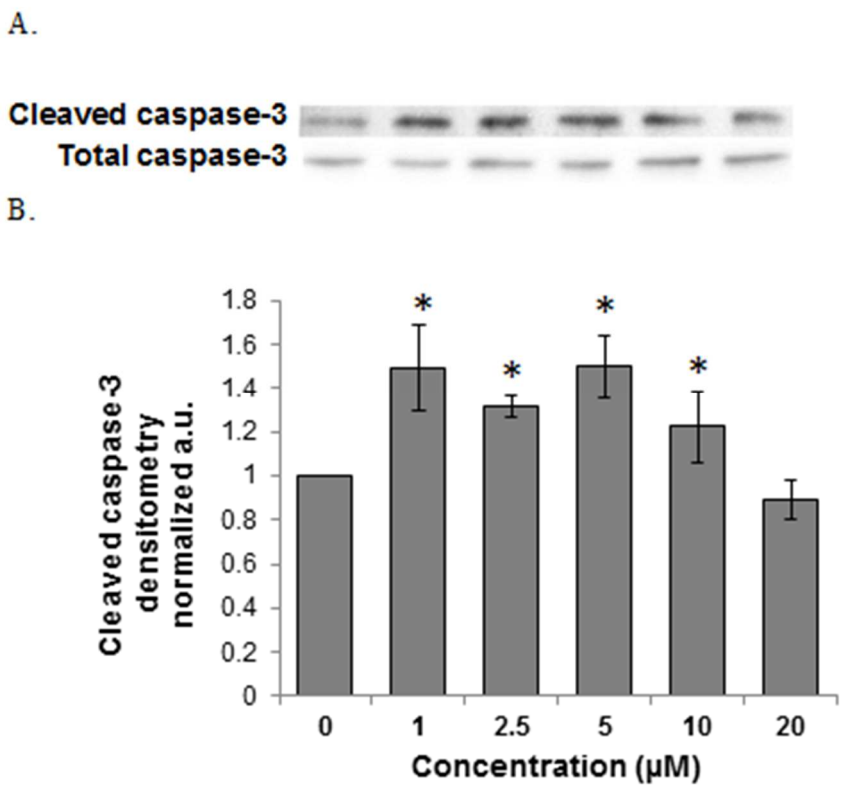

Figure 32. Western blot analysis and quantitative densitometry of cleaved caspase-3 expression in HT-29 cells treated with various concentrations of PPLGM. (A) Treatment (24 h) of HT-29 cells with PPLGM (0–20 μM) resulted in enhanced cleaved caspase-3 expression. (B) Densitometry shows the results of PPLGM treatment on cleaved caspase-3 expression normalized to total caspase-3 in three replicate experiments ± standard deviation, where *denotes statistical significance ($p < 0.05$).

PPLGM induces phosphorylation of ERK in time- and concentration-dependent manners

We investigated the signaling mechanisms by which PPLGM induced colon cancer cell death. We found that 10 μM PPLGM results in a rapid (5–60 min) activation of phosphorylated ERK (p-ERK) in HT-29 cells (Fig. 33) that was sustained for at least 24 h (Fig. 34). Maximal increases in p-ERK expression were observed with the lower concentrations (2.5–10 μM) of PPLGM; whereas, higher concentrations of PPLGM (20–40 μM) produced only slightly elevated levels of p-ERK (Fig. 35). To further investigate the signaling mechanisms mediated by PPLGM, we pre-treated HT-29 cells with 10 μM of the MEK inhibitor U0126 for 1 h followed by 10 μM PPLGM for 15 min. MEK acts upstream of ERK to lead to its activation. An increase in p-ERK

protein expression was observed upon treatment of HT-29 cells with PPLGM alone, when compared to the vehicle-treated control cells. Pre-treatment with U0126 followed by PPLGM treatment abrogated this effect (Fig. 36).

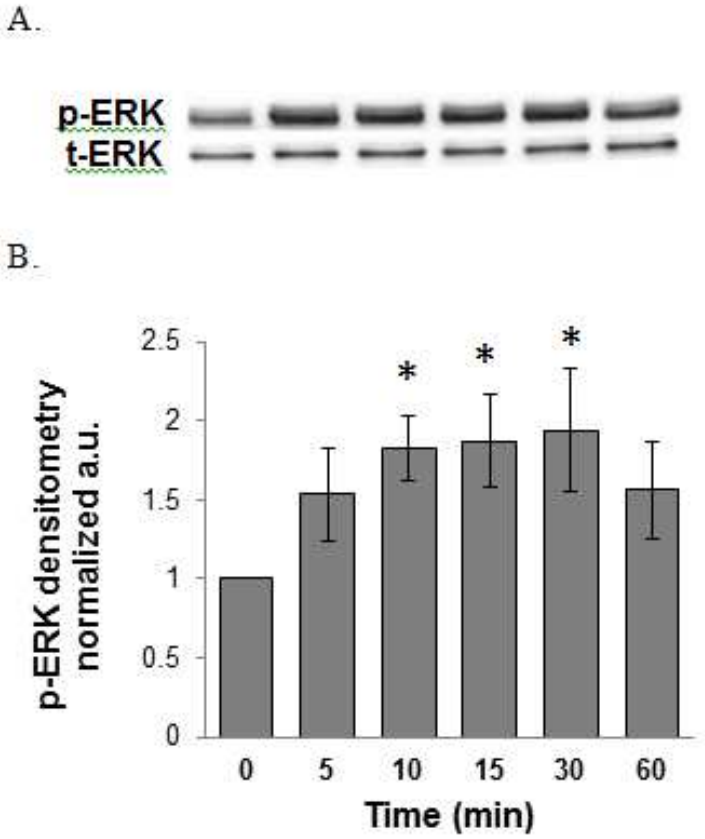

Figure 33. Western blot analysis and quantitative densitometry of phosphorylated ERK expression in HT-29 cells treated with PPLGM for various durations. (A) Short-term treatment (5–60 min) of HT-29 cells with PPLGM (10 μM) resulted in enhanced phosphorylated ERK (p-ERK) expression, but no effect on total ERK (t-ERK) expression. (B) Densitometry shows the results of PPLGM treatment on p-ERK expression normalized to t-ERK in three replicate experiments ± standard deviation, where * denotes statistical significance ($p < 0.05$).

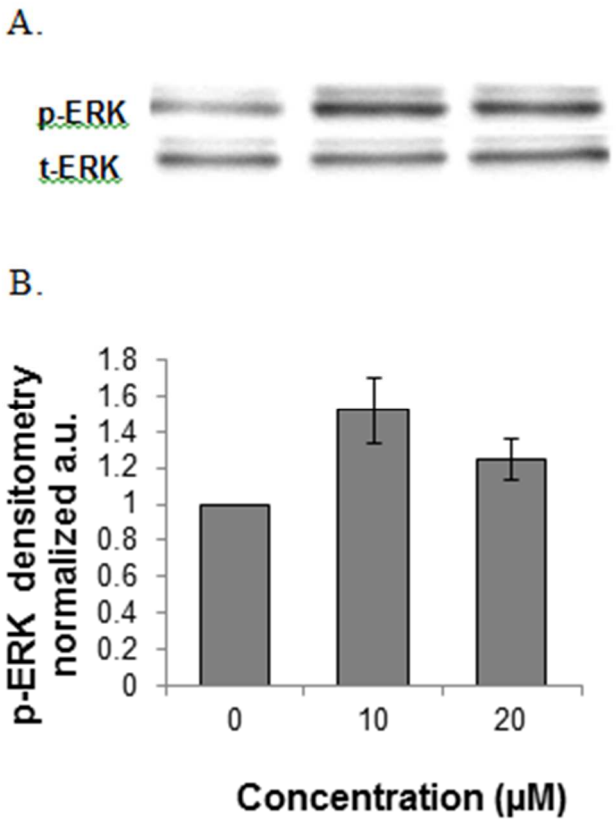

Figure 34. Western blot analysis and quantitative densitometry of phosphorylated ERK expression in HT-29 cells treated with varying concentrations of PPLGM for 24 h. (A) Treatment (24 h) of HT-29 cells with PPLGM (10 and 20 μM) resulted in enhanced phosphorylated ERK (p-ERK) expression. (B) Densitometry shows the results of PPLGM treatment on p-ERK expression normalized to t-ERK in three replicate experiments ± standard deviation.

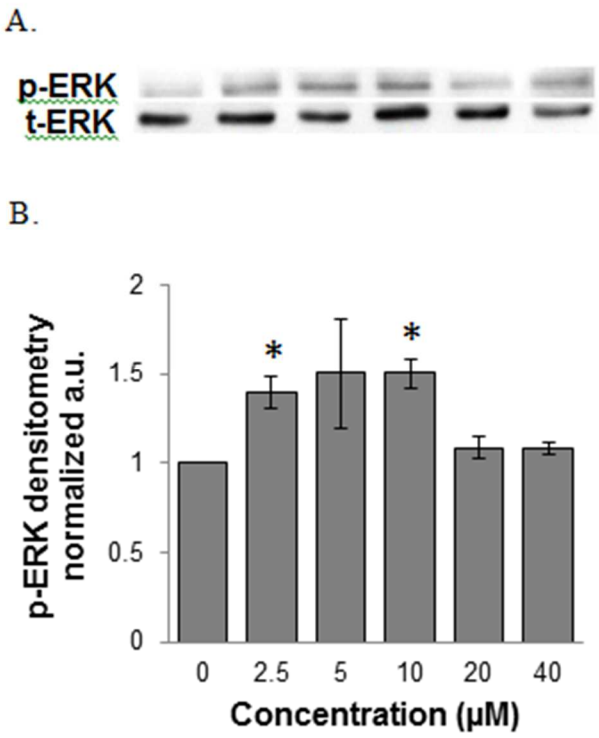

Figure 35. Western blot analysis and quantitative densitometry of phosphorylated ERK expression in HT-29 cells treated with varying concentrations of PPLGM for 10 min. (A) Treatment (10 min) of HT-29 cells with PPLGM (0–40 μM) resulted in enhanced phosphorylated ERK (p-ERK) expression. (B) Densitometry shows the results of PPLGM treatment on p-ERK expression normalized to t-ERK in three replicate experiments ± standard deviation, where * denotes statistical significance ($p < 0.05$).

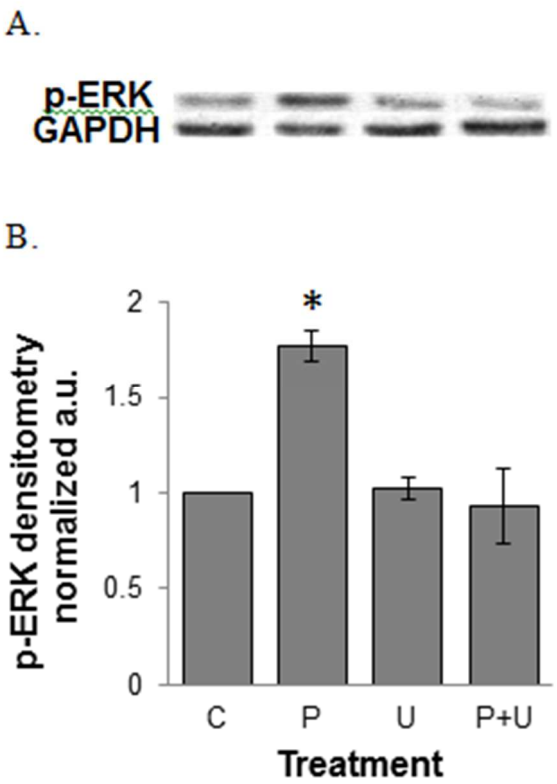

Figure 36. Western blot analysis and quantitative densitometry of phosphorylated ERK expression in HT-29 cells treated with PPLGM ± a MEK inhibitor. HT-29 cells were treated with the vehicle (C), 10 μM PPLGM alone for 15 min (P), 10 μM U0126 alone for 1 h (U), or pre-treated with 10 μM U0216 for 1 h followed by treatment with 10 μM PPLGM for 15 min (P + U). (A) The western blot shows that PPLGM alone enhanced p-ERK expression while U0126 abrogated this effect. (B) Densitometry shows the results of PPLGM and/or U0126 treatment on p-ERK expression normalized to GAPDH in three replicate experiments ± standard deviation, where * denotes statistical significance ($p < 0.05$).

Inhibition of ERK signaling by U0126 attenuates cell death induced by PPLGM

Based upon our independent observations that PPLGM induces apoptosis and PPLGM activates ERK signaling in colon cancer cells, we next investigated the involvement of ERK signaling in PPLGM-induced apoptosis in HT-29 cells. Two hour treatment of HT-29 cells with the MEK inhibitor U0126 (10 μM) alone did not influence the average percentage of cell viability. Conversely, as shown in previous figures, PPLGM was toxic to HT-29 cells and reduced their viability by over 60%. The toxic effects of PPLGM were partially blocked by pre-

treating cells with U0126. U0126 + PPLGM treatment resulted in a 25% increase in cell viability compared to PPLGM treatment alone (Table 9).

Table 9. Average percent of viable HT-29 cells relative to the vehicle-treated control following treatment with 15 μM PPLGM for 24 hours and/or pre-treatment with 10 μM U0126 for 2 hours. A minimum of 200 cells were counted for each well and the average number of viable cells were expressed as the percentages of the control. S.D. denotes standard deviation for eight replicate wells from three independent experiments.

	Average % viability
Control	100^{a}
U0126	99.6 ± 4.4^{a}
PPLGM	$48.3 \pm 25.8^{b,**}$
U0126 + PPLGM	$73.4 \pm 25.5^{c,*}$

Superscript letters denote statistically distinct groups. * $p<0.05$, ** $p<0.01$.

Discussion

The natural compound piperlongumine (PPLGM) displays potent growth-inhibitory effects in many different types of cancers (Bezerra, Castro et al. 2006; Bezerra, Militao et al. 2007; Raj, Ide et al. 2011). However, the intracellular signaling mechanism involved in PPLGM-mediated cancer cell death has never been evaluated and, thus, was the focus of this study. Here we show that PPLGM is toxic to both HT-29 and HCT 116 colon cancer cells, and less toxic to NCM460 normal colon cells. Further, we showed that PPLGM induced apoptosis in HT-29 cells as evidenced by apoptotic blebbing, chromatin condensation, and caspase-3 cleavage. PPLGM enhanced ERK phosphorylation in time- and concentration-dependent manners. PPLGM-induced ERK signaling and cell death were reduced in cells pre-treated with a MEK inhibitor. We suggest that activation of ERK signaling plays a role in PPLGM-induced apoptosis in HT-29

cells. Further studies using RNAi to knock-down MEK or ERK are needed to confirm the results shown here.

The selective anti-cancer effects of PPLGM have been recently reported (Raj, Ide et al. 2011). PPLGM, as high as 15 μM, did not cause toxic effects in six different noncancerous cell types; whereas, in thirteen cancer cell lines, PPLGM showed an average IC_{50} of 7 μM (Raj, Ide et al. 2011). Similarly, our data show that PPLGM is toxic toward HT-29 and HCT 116 colon cancer cells (IC_{50}s of 6.4 and 10.1 μM, respectively) and only slightly inhibits the growth of NCM460 normal colon mucosal cells at concentrations 10 μM and below (Fig. 28). The mechanisms by which PPLGM causes cancer-selective cell death have been explored and found to be dependent upon elevation of reactive oxygen species (ROS).

ROS are essential for normal cell function where they play important roles in regulating signal transduction events, enzyme activity, and cytokine production (Valko, Leibfritz et al. 2007). However, deregulation of redox homeostasis can cause irreparable cell damage and is associated with cancer initiation, promotion, and therapy resistance (Behrend, Henderson et al. 2003; Wu 2006; Valko, Leibfritz et al. 2007). Cancer cells become more dependent on elevated ROS levels and a highly functional anti-oxidant system than healthy cells, and as a consequence, are more vulnerable to agents that impair antioxidant capacity or induce further oxidative stress levels (Pelicano, Carney et al. 2004). Recent studies have revealed that increasing reactive species above a certain threshold can induce cancer cell death (Fruehauf and Meyskens 2007). Many natural products, including sulforaphane (SFN), curcumin (CUR), phenethyl isothiocyanate (PEITC), and PPLGM increase ROS in cancer cells leading to cell cycle arrest or cell death (Pham, Jacobberger et al. 2004; Qanungo, Das et al. 2005; Jutooru, Chadalapaka et al. 2010; Naumann, Fortunato et al. 2011; Raj, Ide et al. 2011). The presence of elevated ROS can

lead to sustained ERK activity resulting in cell death (Kong, Kim et al. 2008; Son, Cheong et al. 2011). Indeed, we found that PPLGM treatment of colon cancer cells induced ERK signaling (Fig. 33 and Fig. 35) that was sustained for at least 24 h (Fig. 34), at which point cell death was noted (Fig. 29 and Fig. 30).

A cascade of signaling events initiated by RAS small GTPases can lead to ERK activation and its associated cellular responses such as enhanced proliferation. RAS proteins phosphorylate and activate downstream effectors such as BRAF which, in turn, phosphorylate MEK1/2 leading to activation of ERK1/2 (Roberts and Der 2007). Mutations in *KRAS*, one form of *RAS*, are detected in approximately 50% of colorectal cancers (Vogelstein, Fearon et al. 1988) and are thought to play an important role in disease progression and resistance to chemotherapies (Haigis, Kendall et al. 2008; Lievre and Laurent-Puig 2009). In this study, we compared the effects of PPLGM on the growth of two different colon cancer cell lines, HCT 116 and HT-29. The HCT 116 cell line contains a *KRAS* mutation, but has wildtype BRAF and p53, while the HT-29 colon cancer cell line contains wild-type *KRAS*, but has BRAF and p53 mutations (Yeh, Routh et al. 2009). We found that the HCT 116 was slightly more sensitive to PPLGM than the HT-29 cell line (Fig. 28A and B). Interestingly, recent studies have revealed that small molecule inducers of oxidative stress can effectively kill *KRAS* mutant cancer cells (Lebedeva, Su et al. 2005; Shaw, Winslow et al. 2011). This is a promising result since it suggests that PPLGM may be an effective treatment strategy even for colon cancer patients with oncogenic *RAS* mutations that may not respond to current treatments. Further evaluation of the effect of PPLGM on normal and mutant *KRAS* colon tumors and a potential role for elevated ROS levels in resulting cancer cell death is needed.

While the ERK pathway is normally associated with enhanced cell proliferation, many studies on bioactive food components have shown that ERK activation up-regulates the expression of apoptotic genes which contribute to enhanced cell death (Blagosklonny, Schulte et al. 1996; Ellington, Berhow et al. 2006; Cagnol and Chambard 2010). The ERK pathway has been shown to modulate the activity and expression of numerous protein targets including pro- and anti-apoptotic proteins (Lee, Kang et al. 2005). Activation of ERK signaling leads to transcriptional events such as enhanced gene expression of p53 and p53 up-regulated modulator of apoptosis (PUMA) and decreased expression of the anti-apoptotic protein Bcl-2 (She, Bode et al. 2001; Liu, Mao et al. 2008). Interestingly, PPLGM enhances p53 and PUMA expression and reduces Bcl-2 expression in various cancer cells (Raj, Ide et al. 2011). It is likely that ERK signaling plays a role in the apoptotic response of cancer cells to PPLGM via these targets.

A previous study showed that lower concentrations (2.5 μg/ml) of PPLGM induced apoptosis in HL-60 human leukemia cells as assessed by caspase-3 activation (Bezerra, Militao et al. 2007). However, higher concentrations of PPLGM (10 μg/ml) resulted in an increased number of necrotic cells as determined by acridine orange and ethidium bromide staining. Our data show that lower concentrations of PPLGM increase pERK and cleaved caspase-3 levels; whereas higher concentrations do not (Fig. 32 and Fig. 35). We suggest that lower concentrations of PPLGM lead to ERK activation resulting in apoptosis; whereas higher concentrations of PPLGM trigger necrosis without activating ERK.

In our study, PPLGM lead to rapid elevation of p-ERK levels in a concentration dependent manner (Fig. 34 and Fig. 35) which was determined for total cell lysates. It is possible that we would have observed greater pERK expression in PPLGM-treated cells from nuclear extracts. Further, we showed that the MEK inhibitor U0126 could partially block PPLGM-

induced colon cancer cell death (Table 9; U0126 resulted in a 25% increase in cell viability compared to cells treated with PPLGM alone). It is likely that ERK is not solely responsible for PPLGM-mediated cell death given that inhibition of its upstream activator MEK was unable to completely block PPLGM-induced cell death.

ERK activity is regulated by dual-specificity phosphatases (DUSPs) that dephosphorylate and inactivate MAPKs. Interestingly, DUSP9 was 12× repressed by PPLGM (Raj, Ide et al. 2011), which would enhance ERK signaling in the presence of PPLGM. Additionally, DUSPs are sensitive to ROS; therefore, elevated levels of ROS triggered by PPLGM treatment would inhibit DUSPs which would result in activation of ERK and promote cancer cell death. These findings support our results showing that the ERK pathway plays a role in PPLGM-induced cell death.

Collectively, our data support the conclusion that PPLGM induces ERK phosphorylation leading to colon cancer cell death. This study suggests a mechanism by which PPLGM can trigger colon cancer cell death and supports the need for further investigation of PPLGM as a potential cancer therapy.

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CHAPTER 6: CONCLUSIONS AND FUTURE DIRECTIONS

Conclusions

Numerous studies have substantiated the anticancer activity of PPLGM, a bioactive agent derived from long pepper plants. In the studies presented here, we have investigated the anticancer mechanisms of PPLGM in pancreatic cancer cell lines *in vitro* and pancreatic cancer mouse models *in vivo*, identified the target pathways affected by PPLGM, revealed the anticancer activity of PPLGM in combination with a known chemotherapeutic agent, gemcitabine, and investigated the effects of PPLGM on human colorectal cancer and non-cancerous cell lines.

We found a significant decrease in cell viability and an induction of cell death with PPLGM treatment in *KRAS* mutant (MIA PaCa-2, PANC-1) and wild-type (BxPC-3) pancreatic cancer cells. ROS levels were elevated and DNA damage was observed in *KRAS* mutant MIA PaCa-2 pancreatic cancer cells treated with PPLGM. Additionally, PPLGM-induced DNA damage and pancreatic cancer cell death was reversed by .treatment of cells with an exogenous antioxidant, NAC. Furthermore, PPLGM treatment reduced pancreatic tumor volume in a subcutaneous xenograft mouse model of human pancreatic cancer. Tumors from PPLGM-treated mice showed a reduction in Ki-67, a proliferation marker, and an increase in 8-OHdG, an oxidative stress marker, compared to tumors from DMSO-treated mice. These results suggest that PPLGM causes pancreatic cancer cell death *in vitro* and *in vivo* through a ROS-mediated DNA damage pathway.

To investigate the target pathways responsible for PPLGM-mediated cell death in a *KRAS* mutant pancreatic cancer cell line, MIA PaCa-2, RNA-sequencing was performed. We identified over 20 million reads per sample in control and PPLGM-treated cells. Seven protein coding

genes with known function were exclusively expressed; whereas 4 protein coding genes were suppressed in PPLGM treated cells. 684 protein-coding genes were differentially expressed with PPLGM treatment. Pathway analysis revealed activation of ER-stress pathway genes such as IRE1α, ATF-4, ASNS, HERPUD1, XBP1, and TNFR. These results were further validated by q-PCR in independent experiments confirming the results of RNA-sequencing. Overall, the results suggests that PPLGM alters gene expression in a pancreatic cancer cell line, particularly the genes involved in ER-stress and the UPR pathway.

Gemcitabine, a conventional chemotherapy for pancreatic cancer patients, has been evaluated in a great deal of preclinical studies and clinical trials alone and in combination with other agents. However, chemoresistance, adverse effects, and poor outcomes still remain a problem for pancreatic cancer patients treated with gemcitabine. Therefore, we evaluated the therapeutic effects of combined PPLGM and gemcitabine for *in vitro* and *in vivo* pancreatic cancer models. PPLGM in combination with gemcitabine and erlotinib enhanced the reduction in cell viability, survival, and induction of apoptosis in MIA PaCa-2 and BxPC-3 cells. Significantly, PPLGM in combination with gemcitabine reduced the tumor volume and weight in an orthotopic mouse model of pancreatic cancer compared to either agent alone. Tumors from PPLGM+gemcitabine treated mice showed a reduction in the Ki-67 marker indicating a reduction in proliferative ability in response to the treatment. These findings indicate that the therapeutic efficacy of gemcitabine was greatly potentiated by PPLGM both *in vitro* and in a pancreatic cancer orthotopic mouse model.

In addition to pancreatic cancer cells, the effect of PPLGM was studied in colorectal cancer cell lines. We observed an inhibition in growth and induction of apoptosis in HT-29 and HCT-116 colon cancer cells treated with PPLGM. Interestingly, PPLGM was not toxic towards

NCM460, normal colon mucosal cells, at lower concentrations. PPLGM treatment resulted in a rapid (5-60 min) activation of phosphorylated ERK with a maximal increase observed at lower concentrations (2.5-10 μM) of PPLGM. Treatment of colon cancer cells with a MEK inhibitor, U0126, attenuated PPLGM-induced ERK activation and partially protected against PPLGM-mediated cell death. These results indicate that PPLGM induces the MEK/ERK pathway resulting in colon cancer cell death.

From the results above, it is clear that PPLGM effectively kills *KRAS* mutant and wild-type colon and pancreatic cancer cells *in vitro* and in pancreatic cancer mouse models. The mechanisms of cell death involve induction of a reactive oxygen species mediated DNA damage pathway, the ER, and oxidative-stress pathways in pancreatic cancer cells. PPLGM activates the MEK/ERK pathway to cause colon cancer cell death. These findings provide a rationale for investigating the therapeutic efficacy of PPLGM in clinical studies for the treatment of pancreatic cancer patients.

Future directions

The future directions of this project include:

1) Investigating the anticancer mechanisms of PPLGM in combination with gemcitabine: Although we have reported an enhanced anticancer activity of PPLGM in combination with gemcitabine for pancreatic cancer cell lines and an orthotopic mouse model using, more work needs to be done to specifically identify the mechanisms of action. We have shown an activation of a ROS-mediated DNA damage pathway as well as ER and oxidative-stress pathways in response to PPLGM treatment. However, further studies need to be done to examine additional pathways that are altered in response to the combination of PPLGM with gemcitabine to better elucidate their mechanisms of action.

2) Determine the effect of PPLGM in a non-cancerous pancreatic cell line: We focused on the effects of PPLGM in three different pancreatic cancer cell lines in this study. Future studies investigating the role of PPLGM in a non-cancerous pancreatic cell line are required to better understand the cancer-selective cell death action of PPLGM. A comparison of PPLGM's effects in a cancer cell line relative to a non-cancerous cell line will better illustrate the differential responses of healthy versus diseased tissues that are needed to evaluate this agent in future clinical trials.