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Piperlongumine induces pancreatic cancer cell death by enhancing reactive oxygen species and DNA damage

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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.

1. Introduction

Pancreatic cancer is one of the most deadly cancers in the United States with a 5-year survival rate of less than 6% [1]. The vast majority of pancreatic cancer patients present with advanced disease, at which point surgery is no longer an option [2]. The best chemotherapy currently available has a minimal impact on advanced pancreatic cancers, and extends patients' lives by only a couple of months [3]. 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 [4–6]. Cancer cells exhibit elevated levels of ROS as well as antioxidant enzymes [7]. As a result, cancer cells are more vulnerable than normal cells to agents that induce further oxidative stress or impair the antioxidant response [8].

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

Abbreviations: 8-OHdG, 8-hydroxy-2′-deoxyguanosine; DCFDA, 2,7-dichlorodihydrofluorescein diacetate; DMSO, dimethyl sulfoxide; GSTP1, glutathione S-transferase P1; HO1, heme oxygenase 1; NAC, N-acetyl cysteine; PPLGM, piperlongumine; pChk1, phospho checkpoint kinase 1; ROS, reactive oxygen species; SOD1, superoxide dismutase 1.

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NAD(P)H:quinone oxidoreductase (NQO1)-overexpressing pancreatic tumors by modulating PARP, NAD^+/ATP levels, leading to single-stranded DNA breaks, and necrosis [9,10]. 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 [11]. Imexon induces apoptosis in pancreatic cancer cells by elevating ROS levels and causing cell cycle arrest [12].

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 [13,14]. 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.

2. Materials and methods

2.1. 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).

2.2. 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.

2.3. AlamarBlue® cell toxicity assay

PANC-1, MIA PaCa-2, and BxPC-3 cells (5.0×10^3) were seeded into individual wells of a 96-well plate, and 24 h later were treated with PPLGM (0–20 μM) after which alamarBlue® (AbD Serotech, Raleigh, NC) 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 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:

$$\%\,\text{reduction in alamarBlue}^{\circledR} = \frac{117,216(A_1) - 80,586(A_2)}{155,677(B_1) - 14,652(B_2)} \times 100$$

In the formula, 117,216 and 80,586 are constants representing the molar extinction coefficients of alamarBlue® at 570 and 600 nm, respectively, in the oxidized form; whereas 115,677 and 14,652 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.

2.4. 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 h. 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.

2.5. Measurement of ROS by the 2,7-dichlorodihydrofluorescein 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 h. 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.