Lipoxygenase Inhibitors Attenuate Growth of Human Pancreatic Cancer Xenografts and Induce Apoptosis through the Mitochondrial Pathway

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Abstract

Several studies have suggested that high dietary fat intake, particularly essential fatty acids, is associated with pancreatic cancer development and growth. Our previous studies have demonstrated that blockade of either the 5-lipoxygenase (LOX) or 12-LOX pathway of arachidonic acid metabolism inhibited pancreatic cancer cell proliferation and induced apoptosis. This study investigated the underlying mechanisms for LOX inhibitor-induced apoptosis and the potential of LOX inhibitors as antipancreatic cancer agents using the athymic mice xenograft model. Apoptosis of pancreatic cancer cells induced by LOX inhibitors (including the nonselective LOX inhibitor nordihydroguaiaretic acid, the 5-LOX inhibitor Rev-5901, and the 12-LOX inhibitor baicalein) was confirmed by growth inhibition, annexin V binding, and terminal deoxynucleotidyl transferase-mediated nick end labeling assay in MiaPaCa-2 and AsPC-1 human pancreatic cancer cells. Expression of the antiapoptotic proteins Bcl-2 and Mcl-1 was significantly decreased after LOX inhibitor treatment while that of the proapoptotic protein bax was increased. LOX inhibitors also markedly induced the release of cytochrome c from mitochondria into the cytosol. Caspase-9, caspase-7, and caspase-3 but not caspase-8 were activated after treatment, concomitant with cleavage of the capase-3 substrate poly(ADP-ribose) polymerase. In vivo studies in the athymic mice xenograft model also confirmed the growth inhibitory effect and induction of apoptosis by these LOX inhibitors in pancreatic cancer. In conclusion, LOX inhibitors block pancreatic cancer cell proliferation and induce apoptosis through the mitochondrial pathway both in vivo and in vitro. LOX inhibitors are likely to be valuable for the treatment of human pancreatic cancer.

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Introduction

Pancreatic adenocarcinoma is the fourth leading cause of cancer death for both men and women in the United States (1, 2). The 5-year survival rate is <2%, and the median survival after diagnosis is <6 months (3). Lack of early diagnosis, the aggressiveness of this cancer, and poor response to therapy contributes to this low survival. Because surgical resection cures only a very small minority of patients, we need to find new drugs to fight this devastating disease (4). Multiple lines of evidence have linked pancreatic cancer growth with dietary fat intake, especially diets containing omega-6 polyunsaturated fatty acids (5, 6). The pathways involved in the conversion of unsaturated fatty acid arachidonic acid and linoleic acid to their bioactive lipid metabolites such as prostaglandins and leukotrienes appear to be involved in development and growth of multiple human cancers (7, 8). Several pathways exist in the metabolism of arachidonic acid, which is derived from membrane phospholipids by the action of phospholipase A2. Of these, the COX pathway produces prostaglandins as their final products, whereas the LOX pathway produces HETEs and leukotrienes (9, 10). The COX pathway has two different isoenzymes, COX-1 and COX-2, whereas the LOX pathway can be additionally divided into 5-LOX, 12-LOX, and 15-LOX pathways, named after the position of insertion of oxygen into the fatty acid (9, 10).

Early investigations into the role of arachidonic acid metabolism in cancer mainly focused on the COX pathway because of the epidemiological observation that the incidence of colonic cancer was significantly reduced in regular users of aspirin and other nonsteroid anti-inflammatory drugs (11, 12). In the past few years, several studies have suggested the importance of the LOX pathways in the development of human cancers, including pancreatic, breast, prostate, and colon cancers (13–15). Previous studies in our laboratory have shown that both 5-LOX and 12-LOX mRNA and proteins are expressed in all human pancreatic cancer cell lines studied but not in normal human pancreatic ductal cells (16). Furthermore, LOX inhibitors block proliferation of human pancreatic cancer cells (16, 17), whereas the LOX metabolites 5-HETE and 12-HETE stimulate cancer growth through activation of p44/42 mitogen-activated protein kinase and PI3/Akt kinase pathways (18).

The abbreviations used are: COX, cyclooxygenase; LOX, lipoxygenase; HETE, hydroxyeicosatetraenoic acid; NDGA, nordihydroguaiaretic acid; Rev-5901, (6-pentyl)-4-[2-(quinolinylmethyl) benzene methanol; baicalein, 5, 6, 7-trihydroxyflavone; PARP, poly(ADP-ribose) polymerase; TdT, terminal deoxynucleotidyl transferase; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling.
Apoptosis or programmed cell death is a physiological cell suicide program that is critical for the development and maintenance of healthy tissues. Deregulation of this pathway occurs in cancer, autoimmune diseases, and neurodegenerative disorders. Two major apoptotic pathways have been identified thus far, the death receptor-mediated and mitochondrial-mediated pathways, respectively (19, 20). The former is initiated by triggering of cell surface death receptors of the tumor necrosis factor receptor superfamily by their ligands and the activation of caspase-8. The latter is initiated by some chemicals, growth factor deprivation, or irradiation. It involves cytochrome c release from mitochondria and activation of caspase-9. The Bcl-2 protein family plays an important role in regulating apoptosis. This family includes bad and bax proteins, which are presumed to form pores in the outer mitochondrial membrane through which cytochrome c can be released into the cytosol (21). Antiapoptotic Bcl-2 family members such as Bcl-2 and Mcl-1 appear to provide negative regulation of apoptosis by impeding bad/bax-induced pore formation and cytochrome c release in response to death-inducing stimuli, thereby preventing apoptosis. In contrast, the proapoptotic Bcl-2 family members such as bax, bad, bik, and bak promote cytochrome c release and enhance apoptosis (22, 23). Once caspase-8 and caspase-9 are activated, they can further activate the downstream effector caspases, including caspase-3, caspase-6, and caspase-7, which can then cleave their respective substrates and induce characteristic apoptotic changes. Previous studies in our laboratory and those of others have shown that blockade of either the 5-LOX or the 12-LOX pathway can induce apoptosis in multiple human cancer cell lines, including pancreatic cancer (16–18). However, the mechanism of LOX inhibitor-induced apoptosis and the effect of LOX inhibitors on pancreatic cancer growth in vivo have not been well studied.

On the basis of the above evidence, this study was designed to investigate the mechanism of LOX inhibitor-induced apoptosis of human pancreatic cancer cells and the effect of LOX inhibitors on pancreatic cancer growth in athymic mice xenograft model. The results show that LOX inhibitors induce cytochrome c release and activate the caspase cascade. The Bcl-2 protein family is also involved in this process. In vivo studies also confirmed the inhibitory effect of these LOX inhibitors on pancreatic cancer cell growth in athymic mice.

Materials and Methods

Chemicals and Antibodies. MEM media, penicillin-streptomycin solution, trypsin-EDTA solution, proteinase K, propidium iodide, and RNase A were purchased from Sigma Chemicals (St. Louis, MO). Fetal bovine serum was from Atlanta Biologicals (Norcross, GA). [3H]Methyl-thymidine was from Amersham (Arlington Heights, IL). NDGA, a nonspecific LOX inhibitor, baicalein, a selective 12-LOX inhibitor, and Rev-5901, a selective 5-LOX inhibitor, were purchased from Calbiochem (La Jolla, CA). The DeadEnd Colorimetric Apoptosis Detection System for tissue sections was from Promega (Madison, WI). The APO-BRDU kits for TUNEL assay, polyclonal rabbit antihuman caspase-3 antibody (CPP32), and monoclonal mouse antihuman caspase-9 and caspase-7 antibodies were from PharMingen (San Diego, CA). The monoclonal mouse antihuman PARP antibody was from Biomol (Plymouth Meeting, PA). The Annexin V Apoptosis Detection Kit and the monoclonal mouse antihuman Bcl-2, Mcl-1, bax, and cytochrome c antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase-conjugated goat-antirabbit and rabbit antimouse secondary antibodies were purchased from New England BioLabs (Beverly, MA). All other chemicals were purchased from Sigma Chemicals.

Human Pancreatic Cancer Cells and Cell Culture. The MiaPaCa-2, HPAC (both poorly differentiated), and AsPC-1 (pleomorphic) human pancreatic cancer cells used in this study were purchased from the American Type Culture Collection (Manassas, VA). Cells were grown as monolayers in MEM media supplemented with 10% fetal bovine serum in a humidified atmosphere of 95% O2 and 5% CO2 at 37°C. The cells were regularly seeded into a 75-cm2 flask with media changed every other day.

DNA Synthesis by [3H]Methyl-thymidine Incorporation. Cells were plated in either 12- or 24-well plates at a concentration of 50,000/well. After reaching 50% confluence, they were incubated in serum-free media for 24 h, which was then replaced with fresh serum-free media with or without the LOX inhibitors. After the appropriate periods of culture, cellular DNA synthesis was assayed by adding 0.5 μCi [3H]methyl-thymidine/well and incubated for another 2 h. Then the cells were washed twice with PBS, fixed with 10% trichloroacetic acid for 30 min and solubilized by adding 250 μl of 0.4 N NaOH to each well. Radioactivity, indicating incorporation of [3H]methyl-thymidine into DNA, was measured by adding scintillation mixture and counted on a Wallac LKB RackBeta scintillation counter (Turku, Finland).

Annexin V Staining. Cells grown on coverslips were treated with or without LOX inhibitors for 6 h. The cells were then rinsed with PBS and 500 μl of assay buffer once. Then 200 μl of assay buffer, 4 μl of annexin-V FITC, and 20 μl of propidium iodide were added to each coverslip. The coverslips were then incubated at room temperature for 15 min in the dark and washed once with PBS. Cells were finally viewed under fluorescence microscope using a dual filter set for FITC and rhodamine, and pictures were taken using a Kodak DC120 digital zoom camera (Eastman Kodak Company, Rochester, NY).

TUNEL Assay. Cells treated with various LOX inhibitors for 24 h were digested with trypsin-EDTA, washed with ice-cold PBS twice, and fixed in 10% paraformaldehyde on ice for 20 min. Cells were then washed with PBS, permeabilized with 70% ethanol for at least 4 h, washed again with PBS, and incubated with 2.5 units of TdT enzyme and 100 pmol Br-dUTP in DNA-labeling solution for 1 h at 37°C. Cells were then rinsed twice in PBS, resuspended in 0.1 ml of fluorescein-labeled anti-BrdUrd antibody solution in the dark for 30 min. Then 0.5 ml of propidium iodide/RNase A solution was added, and the cells were analyzed by flow cytometry at 488 nm excitation.
Preparation of Cytosolic and Mitochondrial Extracts. Preparation of cytosolic extracts was performed as described by Bossy-Wetzel and Green (24). Briefly, cell pellets from control or LOX-inhibitor-treated MiaPaCa-2 and AsPC-1 cells were suspended in 600 μl of extraction buffer [220 mM mannitol, 68 mM sucrose, 50 mM HEPES-KOH (pH 7.4), 50 mM KCl, 5 mM EGTA, 2 mM MgCl2, 1 mM DTT, and protease inhibitors]. After 30 min of incubation on ice, cells were homogenized with a glass dounce and a B pestle (40 strokes). Cell homogenates were spun at 14,000 × g for 15 min. The supernatants (cytosolic fraction) and the pellets (mitochondrial fraction) were stored at −80°C until ready for Western blotting.

Western Blotting. After treatment of cells with or without LOX inhibitors, cells were scraped into lysing buffer [20 mM Tris-HCl (pH 7.4), 2 mM sodium vanadate, 1.0 mM sodium fluoride, 100 mM NaCl, 2.0 mM phosphate substrate, 1% NP40, 0.5% sodium deoxycholate, 25 μg/ml each aprotinin and leupeptin, 25.0 μg/ml pepstatin, and 2.0 μM each EDTA and EGTA] at 4°C. Cell lysates were clarified by microcentrifugation at 12,000 × g after incubation on ice for 25 min. The supernatants were recovered and their protein concentrations measured using Bio-Rad Protein Assay reagent. Equivalent amounts of cell lysate protein (30 μg) were resolved by SDS-PAGE (15%). Proteins were transferred to nitrocellulose membranes by electroblotting using a Bio-Rad semidy transfer blotting apparatus. Membranes were subsequently blocked in Tris-buffered saline and 0.1% Tween 20 (TBST), then incubated with appropriate antibodies (Bcl-2 and Mcl-1 1:2,000 dilution; bax and caspase-3 1:10,000 dilution; caspase-9, caspase-7, cytochrome c, and PARP 1:1,000 dilution) in 5% nonfat milk overnight at 4°C. The membrane bound protein antibody complexes were then incubated with horseshadish peroxidase-conjugated goat anti-rabbit (for polyclonal antibodies) or rabbit antimouse (for monoclonal antibodies) secondary antibodies at 1:2000 dilution for 2 h at room temperature. The membranes were then detected by chemiluminescence, and light emission was captured on Kodak X-ray films (Eastman Kodak Company, Rochester, NY).

Animal Study. Athymic mice (BALB/c nu/nu, 5-week-old females) were purchased from NCI-Frederick Cancer Research and Development Center Charles River Laboratories (Wilmington, MA). Mice were acclimatized to the animal facility for 1 week before receiving xenografts. Xenografts of MiaPaCa-2 and AsPC-1 cells were originally attempted in MiaPaCa-2 and AsPC-1 human pancreatic cancer cells after LOX inhibitors treatment for 6 h but no staining or only very weak staining can be seen in control cells.

In Situ Tissue TUNEL Assay. Paraffin-embedded tissue sections were deparaffinized in xylene for 5 min, then rehydrated in gradient ethanol, and fixed with 4% paraformaldehyde. After permeabilizing tissue with proteinase K, refixed with 4% paraformaldehyde, the sections were then incubated with TdT enzyme and biotinylated nucleotide mix and blocked with 0.3% hydrogen peroxide. The sections were then incubated with 3,3′-diaminobenzidine and chromagen for 10 min and viewed using light microscope, and pictures were taken.

Statistical Analysis. Data were analyzed by ANOVA with Dunnett’s or Bonferroni’s corrections for multiple comparisons as appropriate. This analysis was performed with the Prism software package (GraphPad, San Diego, CA). Data were expressed as mean ± SE.

Results

LOX Inhibitors Inhibit Proliferation of Human Pancreatic Cancer Cells in Vitro. The nonselective LOX inhibitor NDGA, the 5-LOX inhibitor Rev-5901, and the 12-LOX inhibitor baicalein all caused concentration-dependent and time-dependent inhibition of thymidine incorporation in both MiaPaCa-2 (Fig. 1, A and C) and AsPC-1 (Fig. 1, B and D) human pancreatic cancer cells.

LOX Inhibitors Induce Apoptosis in Human Pancreatic Cancer Cells in Vitro. During early apoptosis, phosphatidylserine, a phospholipid usually located on the inner surface of plasma membrane, translocates to the outer plasma membrane because of the loss of membrane phospholipid asymmetry. Annexin V preferentially binds to negatively charged phosphatidylserine. By conjugating fluorescein to annexin V, it can be used to identify early apoptosis by flow cytometry or fluorescence microscopy. Early apoptotic cells bind annexin V but do not exhibit intracellular staining with propidium iodide. As cells progress through apoptosis, the integrity of the plasma membrane is lost, allowing propidium iodide to penetrate and label the cells with a strong yellow-red fluorescence. The results in Fig. 2 showed strong annexin V staining in both MiaPaCa-2 and AsPC-1 pancreatic cancer cells after LOX inhibitors treatment for 6 h but no staining or only very weak staining can be seen in control cells.

LOX inhibitor-induced apoptosis was also confirmed by the TUNEL assay. The 3′-end break of double-stranded DNA during apoptosis can be detected by incorporation of fluorescence-labeled dUTP in the presence of the TdT enzyme and analyzed by flow cytometry. Treatment of MiaPaCa-2 and AsPC-1 cells with 10 μM NDGA, 15 μM Rev-5901, or 20 μM baicalein for 24 h greatly increased the percentage of apoptotic cells from 0.5 to 53.1%, 49.5 and 43.2% in MiaPaCa-2 cells, and from 1.2 to 59.2%, 40.1 and 39.2% in AsPC-1 cells, respectively (Fig. 3).

LOX Inhibitors Decrease Bcl-2 and Mcl-1 and Increase bax Expression in Human Pancreatic Cancer Cells. The Bcl-2 protein family plays a very important role in the regulation of apoptosis. Their major site of action is on the regulation of cytochrome c release from the mitochondria into
Our results showed that the levels of the anti-apoptotic proteins Bcl-2 (Fig. 4A) and Mcl-1 (Fig. 4B) were greatly decreased after LOX inhibitor treatment for 24 h. In contrast, the levels of proapoptotic protein bax were increased markedly by LOX inhibitor treatment (Fig. 4C).

LOX Inhibitors Induce Cytochrome c Release and Caspase-9 Activation. Cytochrome c is a mitochondrial protein that is released from the mitochondria to cytosol during apoptosis when mitochondrial membrane permeability is disrupted. This process is regulated by the Bcl-2 protein family. An increase in the amount of cytochrome c in the cytosolic fraction was seen in both MiaPaCa-2 and AsPC-1 cells after LOX inhibitor treatment (Fig. 5A). Once cytochrome c is released from the mitochondria, it complexes with apoptotic protease-activating factor 1 to activate caspase-9 in the presence of dATP or ATP. Once caspase-9 is activated, it can additionally activate downstream caspases such as caspase-3, caspase-6, and caspase-7. An increase in the activation of caspase-9 was seen after LOX inhibitor treatment for 24 h, as indicated by the increased active caspase-9 level that was produced by cleavage of procaspase-9 (Fig. 5B).

LOX Inhibitors Induce Effector Caspase Activation and Substrate Cleavage. Caspase-3 is the most important executioner in the apoptotic process. During apoptosis, the M₇ 32,000 procaspase-3 is cleaved into M₇ 17,000 and M₇ 11,000 forms of active caspase-3 (26). Activated caspase-3 in turn cleaves its substrate PARP from M₇ 116,000 protein to produce an M₇ 85,000 fragment (27). Our results showed LOX inhibitors induced marked caspase-3 activation (Fig. 6A) and PARP cleavage (Fig. 6B) in both MiaPaCa-2 and AsPC-1 cells after 24 h of treatment. Activation of caspase-7 but not caspase-6 was also seen after LOX inhibitor treatment (data not shown). On the other hand, caspase-8, which
is usually activated in death receptor-mediated apoptosis, was not affected by LOX inhibitor treatment.

**LOX Inhibitors Block Pancreatic Cancer Growth and Induce Apoptosis in Vivo.** The effect of LOX inhibitors was confirmed in an *in vivo* study. Both NDGA and baicalein at 250 mg/kg/day greatly inhibited the growth of s.c. transplanted HPAC (Fig. 7, A and C) and AsPC-1 (Fig. 7, B and D) cells in athymic mice after 4 weeks of treatment, as measured by both tumor volume and tumor weight. In AsPC-1 xenografts, the nonselective LOX inhibitor NDGA seemed more potent than the selective 12-LOX inhibitor baicalein in attenuating tumor growth. Whereas in HPAC xenografts, both NDGA and baicalein were equally potent in attenuating tumor growth. No toxicity of these drugs were seen during the whole treatment period, and there was no significant difference between the body weight of control and treated animals.

The *in situ* tissue TUNEL assay was performed on the tumor tissues from the animal experiments. A marked increase in apoptosis in tumor tissues from both NDGA- and baicalein-treated HPAC (Fig. 8, *top panels*) and AsPC-1 (Fig. 8, *bottom panels*) tumor xenografts was seen, as indicated by the dark brown nuclear staining of apoptotic cells. On the other hand, very few apoptotic cells were seen in control tumor tissues.

**Discussion**

Epidemiological studies and animal experiments support the hypothesis that dietary fats, especially essential fatty acids
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such as arachidonic acid, play an important role in pancreatic cancer growth and development (5, 6). Of the two major metabolic pathways of arachidonic acid, early investigations have mainly focused on the COX pathway. This was based on the epidemiological observation that the incidence of colonic cancer was significantly reduced in regular users of aspirin. Although a correlation of COX pathway and cancer development has been widely studied, investigation of the role of the LOX pathway in regulating cancer growth and development has been limited. In previous studies, we have shown that both 5-LOX and 12-LOX mRNA and proteins are expressed in several human pancreatic cancer cell lines but not in normal human pancreatic ductal cells (17). Blockade of either the 5-LOX or 12-LOX pathway inhibits proliferation and induces apoptosis in human pancreatic cancer cells (16, 17). In this study, we investigated the mechanisms of LOX inhibitor-induced apoptosis and the effect of LOX treatment in vivo. Our results indicate that the mitochondria-mediated apoptosis pathway was involved in LOX inhibitor-induced apoptosis, with cytochrome c release and caspase activation as well as changes in the Bcl-2 protein family. LOX inhibitor treatment also blocked tumor growth and induced apoptosis in vivo.

The results with LOX inhibitors demonstrate that blocking either the 5-LOX or 12-LOX pathway using their specific inhibitors greatly inhibits pancreatic cancer cell proliferation in vitro, suggesting that both 5-LOX and 12-LOX are required for pancreatic cancer cell proliferation. The apoptosis induced by LOX inhibitors was also confirmed by annexin V staining and TUNEL assay, which indicates the disruption of cellular membrane and nuclear fragmentation, respectively.

Induction of apoptosis is the characteristic of chemotherapy and irradiation therapy of human cancers. The ability to induce apoptosis in tumor cells is a very attractive feature of antitumor agents (28, 29). Two apoptosis pathways are well established, the death receptor-mediated and mitochondria-mediated pathways, which involve the activation of the initiator proteases caspase-8 and caspase-9, respectively. Activated caspase-8 and caspase-9, in turn, activate a common cascade, which involves the activation of effector caspases and substrate cleavage (30, 31). Our results show that LOX inhibitor treatment increases cytosolic cytochrome c and induces activation of caspase-9, whereas caspase-8 remains inactive. This confirms our hypothesis that the mitochondria-mediated pathway is the mechanism for LOX inhibitor-induced apoptosis. The final stage of apoptosis involves the activation of effector caspases (including caspase-3, caspase-6, and caspase-7) that can be activated by either caspase-8 or caspase-9. This is followed by substrate cleavage. Caspases exist in cells in an inactive zymogen form called procaspases that can be activated by proteolytic cleavage to produce active caspases. Activated caspases can cleave their substrates after specific aspartic acid residues. Caspase-3 is the most important among these proteases. The substrates for caspase-3 include PARP, retinoblastoma protein, actin, and laminin (32). Our results showed that after LOX inhibitor treatment, there is a marked activation of caspase-3 and caspase-7 as well as the cleavage of PARP.

The Bcl-2 protein family plays an important role in regulating cytochrome c release during apoptosis (33, 34). The Bcl-2 protein family has more than 10 members. These are broadly classified into two categories, according to their role in apoptosis, as antiapoptotic members such as Bcl-2, Mcl-1, and Bcl-xl and proapoptotic members such as bax, bad, bak, and bag. The Bcl-2 protein family controls the release of cytochrome c from the mitochondria into the cytosol, thereby indirectly regulating the activation of the caspase cascade (33, 34). It is believed that the ratio between antiapoptotic proteins and proapoptotic proteins de-
terms whether cells will enter apoptosis or not. Proapoptotic and antiapoptotic Bcl-2 family proteins can form homodimers or physically interact with each other to form heterodimers to regulate apoptosis (33, 34). Our studies show a marked decrease in Bcl-2 and Mcl-1 levels and a concomitant increase in bax levels after LOX inhibitor treatment, which is accompanied by cytochrome c release from mitochondria.

The in vivo studies confirm the inhibitory effect of LOX inhibitors on human pancreatic cancer xenografts in athymic mice. Throughout the 4 weeks of LOX inhibitor treatment, there was a dramatic inhibition on tumor growth, and tumor weight was decreased at the end of the experiment. All animals tolerated LOX inhibitor treatment very well, and no toxicity was apparent. All animals survived the treatment period, and there was no significant difference in body weight between treated and control animals, although control animals seemed less active because of large tumor burden. These experiments suggested that LOX inhibitors may be very safe and effective when given p.o. to inhibit tumor growth.

In summary, this study showed that blockade of the LOX pathways induces apoptosis of human pancreatic cancer cells through the mitochondria-mediated pathway, with cytochrome c release and caspase activation. LOX inhibitor treatment also blocked growth of human pancreatic cancer cell xenografts in athymic mice and induced apoptosis in vivo. Blockade of LOX pathway may provide new treatment for pancreatic cancer.

References
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