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Zyflamend Suppresses Growth and Sensitizes Human Pancreatic Tumors to Gemcitabine in an Orthotopic Mouse Model Through Modulation of Multiple Targets

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Abstract

Agents that can potentiate the efficacy of standard chemotherapy against pancreatic cancer are of great interest. Because of their low cost and safety, patients commonly use a variety of dietary supplements, although evidence of their efficacy is often lacking. One such commonly used food supplement, Zyflamend, is a polyherbal preparation with potent anti-inflammatory activities, and preclinical efficacy against prostate and oral cancer. Whether Zyflamend has any efficacy against human pancreatic cancer alone or in combination with gemcitibine, a commonly used agent, was examined in cell cultures and in an orthotopic mouse model. *In vitro*, Zyflamend inhibited the proliferation of pancreatic cancer cell lines regardless of p53 status and also enhanced gemcitabine-induced apoptosis. This finding correlated with inhibition of NF-κB activation by Zyflamend and suppression of cyclin D1, c-myc, COX-2, Bcl-2, IAP, survivin, VEGF, ICAM-1, and CXCR4. In nude mice, oral administration of Zyflamend alone significantly inhibited the growth of orthotopically transplanted human pancreatic tumors, and when combined with gemcitabine, further enhanced the antitumor effects. Immunohistochemical and Western blot analyses of tumor tissue showed that the suppression of pancreatic cancer growth correlated with inhibition of proliferation index marker (Ki-67), COX-2, MMP-9, NF-κB, and VEGF. Overall, these results suggest that the concentrated multiherb product Zyflamend alone can inhibit the growth of human pancreatic tumors and, in addition, can sensitize pancreatic cancers to gemcitabine through the suppression of multiple targets linked to tumorigenesis.

Keywords

Zyflamend; pancreatic cancer; inflammation

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Introduction

Pancreatic cancer (PaCa), one of the most lethal malignant diseases, has a five-year survival, even with the best treatment available today, of about 5%. More than 35,000 men and women in the United States died of pancreatic cancer in 2009 [1]. Gemcitabine and erlotinib are the only agents that have been approved by the FDA for treatment of this cancer. However, they produce responses in <10% of patients and are associated with multiple adverse events and the development of drug resistance. Therefore, the need to think "out of the box" for treatment options against this lethal disease is needed. There are known to be around 25,431 human genes, out of which 2995 have been linked with 153 different biochemical pathways; about 350 genes have been specifically linked with cancer development and metastases. Thus, mono-targeted or "smart" drugs and single chemical entities are unlikely to treat diseases as complex as cancer. Rather, therapies that are multi-targeted, are needed. In fact, most of the drugs recently approved by the FDA are multi-targeted and these include sunitinib, sorafenib, and vandetanib.

Activation of the transcription factor nuclear factor-kappaB (NF-κB) has been linked with tumorigenesis and chemoresistance in most tumors [2, 3] including pancreatic cancer. Evidence indicates that NF-κB is constitutively active in pancreatic cancer cells [4] but not in immortalized, nontumorigenic pancreatic ductal epithelial cells [5]. NF-κB activation has been reported in animal models of pancreatic cancer [6] and in human pancreatic cancer tissue [4]. NF-κB promotes pancreatic cancer growth in part by opposing apoptosis [4, 7] and mediates the induction of mitogenic gene products [8]. These specific genes are over-expressed in human pancreatic cancer tissue and are inversely correlated with patient survival [9]. Additionally, activation of NF-κB enhances the angiogenic potential of pancreatic cancer cells via increased expression of proangiogenic factors [10], while other NF-κB-regulated gene products promote the migration and invasion of the tumor [11]. NF-κB has also been linked with gemcitabine resistance in pancreatic cancer [3]. Together, these findings implicate a role of activated NF-κB in development of pancreatic cancer and suggest that agents that block this pathway might inhibit tumor growth, and may also sensitize cells to gemcitabine.

The inability of standard chemotherapy regimens to improve the prognosis of pancreatic cancer has led to reconsideration of the potential of traditional medicines. Over 63% of anticancer drugs introduced over the last 25 years are natural products or can be traced back to a natural products source [12]. Butler showed that 79 drugs between 2005–2007 that were entered into clinical trials as anticancer agents were natural products or natural product analogues [13]. Numerous agents have been identified from isolated nutrients, herbal products, and dietary supplements with potential to modulate physiological functions and critical biological activities. Although safe and relatively inexpensive, their effectiveness against cancer is uncertain and thus requires investigation. Zyflamend is a polyherbal formulation comprised of 10 standardized, concentrated herbal extracts (rosemary, turmeric, ginger, holy basil, green tea, hu zhang, Chinese goldthread, barberry, oregano, and baikal skullcap). It is a successful food supplement sold for support of a wide variety of ailments that commonly involve inflammation and/or pain. Each of these herbs contains unique constituents that have been reported to possess anti-inflammatory and anticancer activities through modulation of different targets [14–24]. But the exact mechanisms by which Zyflamend mediates its anti-cancer effect are poorly understood. Recent published reports suggest that Zyflamend can suppress cyclooxygenase (COX)-1 and COX-2 activities in human prostate cancer cells [25–28], inhibit 5-lipooxygenase (5-LOX) and prevent 7,12-dimethylbenz[α]anthracenene (DMBA)-induced oral carcinogenesis in a hamster cheek pouch model of oral carcinoma [29]. Studies from our laboratory have reported Zyflamend's ability to suppress NF-κB cell signaling pathway and NF-κB regulated gene products [30], mechanisms that underlie the effectiveness of many traditional anticancer medicines [31, 32].

The possibility that Zyflamend may potentiate the effect of chemotherapeutic agents such as gemcitabine, makes this product all the more intriguing as a part of pancreatic cancer therapy. In the present study, we investigated whether Zyflamend alone can inhibit the growth of human pancreatic cancer tumors in cell culture and in orthotopic mouse models; and whether Zyflamend can sensitize the tumors to gemcitabine. We demonstrated that Zyflamend inhibits the *in vitro* proliferation of various pancreatic cancer cells, enhances gemcitabine-induced apoptosis, and potentiates the antitumor activity of gemcitabine against orthotopically implanted human pancreatic tumors through downregulation of various biomarkers of this disease.

Materials and Methods

Materials

Zyflamend, that contains holy basil (12.8%), turmeric (14.1%), ginger (12.8%), green tea (12.8%), rosemary (19.2%), Hu zhang (10.2%), barberry (5.1%), oregano (5.1%), baikal skullcap (2.5%) and Chinese goldthread (5.1%); was kindly supplied by New Chapter, Inc. (Brattleboro, VT). It was dissolved in dimethyl sulfoxide (DMSO) as a 100 mg/ml stock solution and stored at −20 °C. The following polyclonal antibodies against p65 (recognizing the epitope within the NH_2-terminal domain of human NF-κB p65) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA): intercellular adhesion molecule-1 (ICAM-1), cyclin D1, matrix metalloproteinase 9 (MMP-9), survivin, cellular inhibitor of apoptosis protein 1 (cIAP-1), procaspase-3, and procaspase-9. Also obtained from Santa Cruz Biotechnology were monoclonal antibodies against COX-2, c-myc, Bcl-2, and Bcl-xL. Antibodies against VEGF and Ki-67 were purchased from Thermo Fisher Scientific (Fremont, CA). The liquid DAB+ Substrate Chromogen System-HRP used for immunocytochemistry was obtained from Dako (Carpinteria, CA). Penicillin, streptomycin, RPMI 1640, and fetal bovine serum (FBS) were obtained from Invitrogen (Grand Island, NY). Tris, glycine, NaCl, sodium dodecyl sulfate (SDS), and bovine serum albumin (BSA) were obtained from Sigma Chemical (St. Louis, MO). Gemcitabine (Gemzar; kindly supplied by Eli Lilly, Indianapolis, IN) was stored at 4°C and dissolved in sterile phosphate-buffered saline (PBS) on the day of use.

Cell lines

The pancreatic cancer cell lines AsPC-1, BxPC-3, MIA PaCa-2, and PANC-1 were obtained from the American Type Culture Collection (Manassas, VA). All cell lines were cultured in RPMI 1640 supplemented with 10% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin. The above-mentioned cell lines were procured more than 6 months ago and have not been tested recently for authentication in our laboratory. The human pancreatic duct epithelial (HPDE) cells were a generous gift from Dr. Ming-Sound Tsao (University of Toronto, Ontario, Canada). These cells were cultured in keratinocyte growth medium (KGM) supplied with 5 ng/mL epidermal growth factor (EGF) and 50 μg/mL bovine pituitary extract (Lonza, Walkersville, MD). The mouse embryonic fibroblast (MEF) derived from *p65* $^{-/-}$ C57Bl/6J mice and its wild type were kindly provided by Dr. David Baltimore (California Institute of Technology, Pasadena, CA). Cells were cultured in DMEM supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin.

Proliferation assay

The effect of Zyflamend on cell proliferation was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) uptake method as described previously [33]. Cells (2,000 cells/well) were incubated with Zyflamend in triplicate in a 96-well plate and then incubated for 2, 4, or 6 days at 37°C. An MTT solution was added to each well and incubated for 2 h at 37°C. An extraction buffer (20% SDS and 50%

dimethylformamide) was added, and the cells were incubated overnight at 37°C. The absorbance of the dissolved cell suspension was measured at 570 nm with an MRX Revelation 96-well multiscanner (Dynex Technologies, Chantilly, VA).

Apoptosis assay

To determine whether Zyflamend could potentiate the apoptotic effects of gemcitabine in pancreatic cancer cells, we used a LIVE/DEAD cell viability assay kit (Invitrogen), which is used to determine intracellular esterase activity and plasma membrane integrity [34]. Briefly, cells (5,000/well) were incubated in chamber slides, pretreated with Zyflamend solution for 4 h, and treated with gemcitabine for 24 h. Cells were then stained with the assay reagents for 30 min at room temperature. Cell viability was determined under a fluorescence microscope by counting live (green) and dead (red) cells.

Annexin V assay

One of the early indicators of apoptosis is the rapid translocationand accumulation of the membrane phospholipid phosphatidylserinefrom the cell's cytoplasmic interface to the extracellular surface. This loss of membrane asymmetry can be detected using the bindingproperties of annexin V. To detect apoptosis, we used annexinV antibody conjugated with the fluorescent dye fluorescein isothiocyanate(FITC). Briefly, cells (1×10^6) were treated with Zyflamend for 24 h, and then subjected to annexin V and propidium iodide (PI) staining. Cells were washed, stained with FITC-conjugated anti-annexin V antibody, and then analyzed with a flow cytometer (FACSCalibur, BD Biosciences).

Cell-cycle distribution

To determine the effect of Zyflamend on the cell cycle, cells were treated with Zyflamend for 24 h, and then were stained with PI as mentioned earlier [35].

Animals

Male athymic *nu/nu* mice (6–8 weeks old) were obtained from the breeding colony of the Department of Experimental Radiation Oncology at M. D. Anderson Cancer Center. The animals were housed in standard plexiglass cages (five per cage) in a room maintained at constant temperature and humidity and in a 12 h:12 h light-dark cycle. Their diet consisted of regular autoclave chow and water *ad libitum*. None of the mice exhibited any lesions, and all were tested pathogen-free. Before initiating the experiment, all of the mice were acclimatized to a standard rodent chow pulverized diet for 7 days. Our experimental protocol was reviewed and approved by the Institutional Animal Care and Use Committee at M.D. Anderson Cancer Center.

Orthotopic implantation of MIA PaCa-2 cells

MIA PaCa-2 cells were stably transfected with luciferase as previously described for PANC-1 cells [36]. The MIA PaCa-2 cells were orthotopically implanted as described previously [34]. Briefly, luciferase-transfected MIA PaCa-2 cells were harvested from subconfluent cultures after a brief exposure to 0.25% trypsin and 0.2% ethylenediaminetetraacetic acid (EDTA). Trypsinization was stopped with medium containing 10% FBS. The cells were washed once in serum-free medium and resuspended in PBS. Only suspensions consisting of single cells, with >95% viability, were used for the injections. After mice were anesthetized with ketamine-xylazine solution, a small incision was made in the left abdominal flank, and MIA PaCa-2 cells (1×10^6) in 50 μL PBS were injected into the subcapsular region of the pancreas with a 27-gauge needle and a calibrated push button-controlled dispensing device (Hamilton Syringe Co., Reno, NV). A cotton swab

was held for 1 min over the site of injection to prevent leakage. The abdominal wound was closed in one layer with wound clips (Braintree Scientific, Inc., Braintree, MA).

Experimental protocol

One week after tumor implantation, mice were randomized into the following treatment groups (n = 6/group) based on the bioluminescence first measured with an *in vivo* imaging system (IVIS 200, Xenogen Corp., Alameda, CA): (a) untreated control (olive oil, 100 μL by gavage, daily); (b) Zyflamend (1g/kg once daily orally [p.o.]); (c) gemcitabine alone (25 mg/kg twice weekly by intraperitoneal [i.p.] injection); and (d) combination (Zyflamend, 1g/kg once daily p.o., and gemcitabine, 25 mg/kg twice weekly by i.p. injection). Tumor volumes were monitored weekly with the bioluminescence IVIS, which includes a cryogenic cooling unit, and a data acquisition computer running Living Image software (Xenogen Corp.). Before imaging, the animals were anesthetized in an acrylic chamber with 2.5% isoflurane/air mixture and injected i.p. with 40 mg/mL D-luciferin potassium salt in PBS at a dose of 150 mg/kg body weight. After 10 min of incubation with luciferin, each mouse was placed in a right lateral decubitus position and a digital grayscale animal image was acquired, followed by the acquisition and overlay of a pseudo-color image representing the spatial distribution of detected photons emerging from active luciferase within the animal. Signal intensity was quantified as the sum of all detected photons within the region of interest per second. The mice were subjected to imaging on days 0, 7, 16, 22, and 29 of treatment. Therapy was continued for 4 weeks, and the animals were euthanized 1 week later. Primary tumors in the pancreas were excised, and the final tumor volume was measured as $V = 2/3\pi r^3$, where r is the mean of the three dimensions (length, width, and depth). Half of the tumor tissue was fixed in formalin and embedded in paraffin for immunohistochemistry and routine hematoxylin and eosin (H&E) staining. The other half was snap frozen in liquid nitrogen and stored at −80°C. H&E staining confirmed the presence of tumor(s) in each pancreas.

NF-κB activation in pancreatic cancer cells

To assess NF-κB activation, we isolated nuclei from pancreatic cancer cell lines and carried out electrophoretic mobility shift assays (EMSA) essentially as described previously [34]. Briefly, nuclear extracts prepared from pancreatic cancer cells (1×10^6/mL) and tumor samples were incubated with ^{32}P-end-labeled 45-mer double-stranded NF-κB oligonucleotide (4 μg of protein with 16 fmol of DNA) from the HIV long terminal repeat (5′-TTGTTACAAGGGACTTTCCGCTGGGGACTTTCCAGG GGGAGGCGTGG-3′; underline indicates NF-κB–binding sites) for 15 min at 37°C. The resulting DNA-protein complex was separated from free oligonucleotide on 6.6% native polyacrylamide gels. The dried gels were visualized, and radioactive bands were quantitated with a PhosphorImager and ImageQuant software (GE Healthcare, Piscataway, NJ).

Immunolocalization of NF-κB p65, COX-2, VEGF and MMP-9 in tumor samples

The nuclear localization of p65 and the expression of COX-2 and VEGF were examined via an immunohistochemical method described previously [37]. Briefly, pancreatic cancer tumor samples were embedded in paraffin and fixed with paraformaldehyde. After being washed in PBS, the slides were blocked with protein block solution (Dako) for 20 min and then incubated overnight with rabbit polyclonal anti-human p65, mouse monoclonal anti-human VEGF, and anti-COX-2 antibodies (1:400, 1:50, and 1:75, respectively). After incubation with the antibodies, the slides were washed and then incubated with biotinylated link universal antiserum followed by horseradish peroxidase-streptavidin detection with the $LSAB^+$ kit (Dako). The slides were rinsed, and color was developed with 3,3′-diaminobenzidine hydrochloride used as a chromogen. Finally, sections were rinsed in distilled water, counterstained with Mayer's hematoxylin, and mounted with DPX mounting

medium (Sigma) for evaluation. Pictures were captured with a Photometrics CoolSNAP CF color camera (Nikon, Lewisville, TX) and MetaMorph software version 4.6.5 (Universal Imaging, Downingtown, PA).

Ki-67 immunohistochemistry

Formalin-fixed, paraffin-embedded sections (5 μm) were stained with anti-Ki-67 (rabbit monoclonal clone SP6) antibody as described previously [34]. Results were expressed as the percentage of Ki-67$^+$ cells ±standard error per ×40 magnification. A total of ten 40×fields were examined and counted from three tumors of each of the treatment groups.

Western blot analysis

Pancreatic cancer cells were harvested and incubated on ice for 30 min in 0.5 mL of ice-cold whole-cell lysate buffer (10% NP40, 5 mol/L NaCl, 1 mol/L HEPES, 0.1 mol/L EGTA, 0.5 mol/L EDTA, 0.1 mol/L PMSF, 0.2 mol/L sodium orthovanadate, 1 mol/L NaF, 2 μg/mL aprotinin, 2 μg/mL leupeptin). The proteins were then separated by SDS-PAGE, electrotransferred to nitrocellulose membranes, blotted with each antibody, and detected by enhanced chemiluminescence reagents (GE Healthcare).

Statistics

In vitro experiments were repeated twice. The values were initially subjected to one-way analysis of variance (ANOVA), which revealed significant differences between groups, and then they were compared between groups with an unpaired Student's t-test, which revealed significant differences between two sample means. Values from *in vivo* experiments were initially subjected to one-way ANOVA and then later compared among groups with an unpaired Student's t-test.

Results

This study was designed to determine whether Zyflamend a) could inhibit the growth of human pancreatic cancer cells; b) could enhance the antitumor effects of gemcitabine *in vitro*; and c) could potentiate the effects of a chemotherapeutic agent *in vivo* in models of human pancreatic cancer. In addition, we sought to explore potential mechanism(s) by which Zyflamend might enhance the effects of the chemotherapeutic agent. We used four different human pancreatic cancer cell lines-AsPC-1, BxPC-3, MIA PaCa-2, and PANC-1-of different origins for this investigation. To monitor tumor growth *in vivo*, we used noninvasive imaging of the luciferase-transfected MIA PaCa-2 cell line.

Zyflamend inhibits the proliferation of pancreatic cancer cells *in vitro*

We examined whether Zyflamend could inhibit the proliferation of human pancreatic cancer cell lines AsPC-1, PANC-1, BxPC-3, and MIA PaCa-2. Cell lines were treated with different doses of Zyflamend for various time periods following which the inhibition of proliferation was determined by examining mitochondrial activity via the MTT uptake method. As shown in Fig. 1A, Zyflamend suppressed the proliferation of all four pancreatic cancer cell lines in a concentration-dependent manner, irrespective of the cell genetic background (Fig. 1A). Proliferation of pancreatic cancer cells was also significantly suppressed by 25 μg/mL dose, while 10 μg/mL had minimal effect. MIA PaCa-2 and BxPC-3 cell lines were more sensitive to Zyflamend than AsPC-1 or PANC-1 (Fig. 1A).

The anti-proliferative effect of Zyflamend on non-tumorigenic pancreatic ductal cell was also examined. As shown in Fig. 1B, in pancreatic cancer cell lines, MIA PaCa-2 and AsPC-1, Zyflamend suppressed viability in a dose-dependent manner but had minimal effect

on human pancreatic ductal epithelial (HPDE) cells, suggesting the effect of Zyflamend was more specific to tumor cells.

Zyflamend induces apoptosis and cell cycle arrest in pancreatic cancer cells

How Zyflamend induces growth inhibition was examined. MIA PaCa-2 were treated with different doses of Zyflamend for 24 h following which the apoptosis and cell cycle arrest were determined by flow cytometry analysis. As shown in Fig. 1C, Zyflamend increased annexin V-positive cells in a dose-dependent manner from 7% to 35%.

We next examined whether Zyflamend could affect cell cycle distribution. We found that after 24 h treatment with Zyflamend, a significant accumulation of cells in G2-M phase was observed (Supplement Table 1).

Zyflamend sensitizes human pancreatic cancer cells to gemcitabine

Gemcitabine is a standard treatment for patients with pancreatic cancer. To determine whether Zyflamend enhances the apoptotic effects of gemcitabine in pancreatic cell lines, we performed the Live/Dead assay. Zyflamend (10μg/mL) and gemcitabine (200 nM) alone had minimally toxic effects, but the two together induced a high level of apoptosis in pancreatic cancer cell lines *in vitro* (Fig. 2). No significant difference was observed between the cell lines with respect to their sensitivity to the combination.

Zyflamend inhibits constitutive NF-κB activation and proteins associated with inflammation, proliferation, invasion, and angiogenesis in pancreatic cancer cells

Because NF-κB has been linked with both proliferation and chemoresistance, we next examined whether Zyflamend could inhibit constitutive NF-κB activation in MIA PaCa-2 cell lines. Our results showed that this multi-herb product inhibited constitutive NF-κB activation (Fig. 3A, left panel).

The dose of Zyflamend employed to examine its effect with gemcitabine, was 10 μg/mL. Whether this dose can suppress constitutive NF-κB activation, was examined. For this, MIA PaCa-2 cells were exposed to 10 μg/mL Zyflamend for different times and then examined for NF-κB. We found that Zyflamend suppressed constitutive NF-κB activation in a time-dependent manner in these cells (Fig. 3A, right panel).

Next, we investigated the effects of Zyflamend on the constitutive expression of antiapoptotic proteins Bcl-2, IAP-1, and survivin. The expression of all these proteins was downregulated by Zyflamend in a concentration-dependent manner (Fig. 3B). The same was true for the constitutive expression of proliferation-associated proteins cyclin D1, c-myc, and COX-2 (Fig. 3C). Likewise, Zyflamend downregulated the expression of proteins linked to invasion, adhesion, and angiogenesis--MMP-9, ICAM-1, VEGF, and CXCR4--in a concentration-dependent manner (Fig. 3D).

Zyflamend downmodulates NF-κB-regulated gene products involve in inflammation, proliferation, invasion, and angiogenesis in all pancreatic cancer cell lines

Up to this point, our all studies were carried out with MIA PaCa-2 cells. Whether Zyflamend downregulates expression of inflammation, proliferation, invasion, and angiogenesis-associated gene products in other pancreatic cancer cells, was examined. Cells were treated with 100μg/mL Zyflamend for 24 h and then examined for gene expression by Western blot analysis. Zyflamend downregulated the expression of antiapoptotic proteins (Bcl-2, IAP-1 and survivin) in all four different pancreatic cancer cell lines (Fig. 4A). The expression proteins associated with cell proliferation (cyclin D1, c-myc, and COX-2), invasion, and

angiogenesis (MMP-9, ICAM-1, VEGF, and CXCR4), was also suppressed by Zyflamend in all four different pancreatic cancer cell lines (Fig. 4B and C).

Deletion of NF-κB enhances the effect of Zyflamend on cell proliferation

Whether the effect of Zyflamend is mediated through inhibition of NF-κB was examined by using $p65^{-/-}$ cells. Results showed that $p65^{-/-}$ cells that lack NF-κB were more sensitive to Zyflamend as compared to wild type cells (Fig. 4D). Overall, these results indicated that NF-κB activation plays an essential role in tumor cell proliferation.

Zyflamend potentiates the antitumor activity of gemcitabine in an orthotopic pancreatic tumor model in nude mice

Based on our *in vitro* results, we designed studies to determine the effects of Zyflamend on gemcitabine in orthotopically implanted human pancreatic tumors in nude mice (Fig. 5A).

Luciferase-transfected MIA PaCa-2 cells were implanted in the tails of the pancreas in nude mice. One week later, based on the IVIS imaging, the mice were randomized into four groups, at which point treatment with Zyflamend or gemcitabine alone or Zyflamend plus gemcitabine was started; treatment was continued for 4 weeks. The animals were euthanized 38 days after tumor cell injection and 31 days from the date of treatment (Fig. 5A). To determine tumor development and the effects of Zyflamend and gemcitabine, we assessed the tumors with the bioluminescence IVIS on days 7, 16, 22, and 29 after the start of treatment. The bioluminescence imaging results (Fig. 5B and C) indicated a gradual increase in tumor volume in the control group compared to the three treatment groups. The imaging results showed that the tumor volume in the combination treatment group was significantly lower than in the group treated with Zyflamend or gemcitabine alone as well as in the vehicle-treated control group ($P < 0.001$ versus gemcitabine; $P < 0.001$ versus vehicle). On the 42nd day, we euthanized the mice and measured the tumor volume with vernier calipers. Additionally, there was no significant change in average animal weight between the vehicle group and Zyflamend-treated groups (Fig. 5D), suggesting lack of toxicity. The results were in concordance with those from bioluminescence imaging and showed that the combination treatment reduced the tumor volume more than in the other three groups (Fig. 5E). The reduction of tumor volumes in combination treatment group was statistically significant when compared with Zyflamend-treated group ($P < 0.05$). The imaging result also revealed that Zyflamend alone was at least as active as gemcitabine alone in inducing tumor regression.

Zyflamend inhibits proliferative index in orthotopic pancreatic tumors

We next examined the expression of the cell proliferation marker Ki-67 in tumor tissues from the four groups. The results in Fig. 6A show that Zyflamend plus gemcitabine significantly downregulated the expression of Ki-67 in tumor tissues compared with the control group ($P < 0.001$ versus vehicle). The results also showed that Zyflamend alone significantly suppressed the expression of Ki-67 ($P < 0.005$ versus vehicle; Fig. 6B). The decrease of proliferation index Ki-67 in combination group was statistically significant when compared with gemcitabine alone group ($P = 0.001$)

Zyflamend inhibits NF-κB, COX2, VEGF, and MMP-9 in orthotopic pancreatic tumors

Immunohistochemical analysis showed that Zyflamend alone suppressed various biomarkers in orthotopically grown human pancreatic tumor samples (Fig. 6C). Zyflamend also further suppressed NF-κB activation in gemcitabine-treated tissues. Immunohistochemical analysis further demonstrated that Zyflamend suppressed the expression of COX-2 (Fig. 6C, second

panel), VEGF (Fig. 6C, second panel), and MMP-9 (Fig. 6C, bottom panel) in human pancreatic tumor tissues, and this effect was enhanced in tissues treated with gemcitabine.

Discussion

Zyflamend is a polyherbal preparation taken by many people to prevent as well as treat various chronic diseases that typically involve inflammation. Although pharmacologically safe and affordable, the ability of Zyflamend to inhibit pancreatic cancer has not previously been established. Whether this agent can affect the activity of standard chemotherapy used by cancer patients is also not clear. In the present study we used pancreatic cancer models to examine the activity of Zyflamend alone and in combination with chemotherapeutic agent. We selected this cancer because pancreatic cancer as it is one of most lethal types of malignant disease with an estimated 5-year survival rate of around 4% despite the best treatment available today.

We found that Zyflamend alone suppressed the proliferation of various pancreatic cancer cell lines including PANC-1, MIA PaCa-2, AsPC-1, and BxPC-3 cells. This is the first report to describe the antiproliferative effects of this agent against PaCa cells. How Zyflamend mediates an antiproliferative effect also was investigated in detail. We found that Zyflamend suppressed the expression of antiapoptic (Bcl-2, IAP-1, and survivin) and cell proliferative proteins (cyclin D1, c-myc, and COX-2). Consistent with these effects, Zyflamend also suppressed the constitutive activation of NF-κB in pancreatic tumor cell lines. These results on suppression of NF-κB by Zyflamend are in agreement with a previous report on other tumor cell lines [30] and with reports that berberine, resveratrol, epigallocatechins, gingerol, curcumin and ursolic acid, all present within Zyflamend, are linked with NF-κB suppression [14, 17, 19, 24, 38].

We also found that Zyflamend can enhance the apoptotic effect of gemcitabine in various pancreatic cancer cell lines *in vitro*. We found that this effect may be mediated through the down-regulation of cell survival proteins such as Bcl-2, IAP-1, and survivin. Moreover, constitutive activation of NF-κB expressed in human pancreatic cancer cell lines has been linked with chemoresistance [3], and the suppression of NF-κB activation can also sensitize cells to chemotherapeutic agents. These results are consistent with those previously reported for curcumin [34] and resveratrol [39].

Interestingly, oral administration of Zyflamend alone inhibited the growth of human pancreatic tumors in an orthotopic nude mouse model. Tumor growth was inhibited by almost 50% by 1g/kg Zyflamend alone, and this level of inhibition was comparable to that produced by gemcitabine alone. Zyflamend was very well tolerated by the animals. When the two agents were used in combination, they were even more effective.

How Zyflamend exhibited its effects *in vivo* was also investigated in several ways. First, Ki-67 expression in tumor tissues, a measure of proliferation, was inhibited by Zyflamend. Likewise, Zyflamend alone inhibited constitutive activation of NF-κB. Zyflamend significantly down-regulated the expression of proinflammatory enzyme COX-2, suppressed the expression of invasion biomarker MMP-9, and inhibited the angiogenic biomarker VEGF in tissues. All of these effects were further enhanced by gemcitabine as indicated by immunohistochemical analysis. These data indicate for the first time the mechanisms by which Zyflamend may exert its effects against human pancreatic cancer.

Our results are in agreement with a previous report describing Zyflamend's anticancer activities against oral cancer in a hamster cheek pouch model [29]. Yang's studies showed that reduction of leukotriene (LT) B4 generated by 5-LOX played a major role in the action of Zyflamend. Recently the effect of oral administration of Zyflamend (780 mg capsule 3

×daily) was examined in men (23 subjects) with high-grade prostatic intraepithelial neoplasia [26–28, 40]. Zyflamend was found to be safe, and after 18 months, 48% of the subjects showed a 2–50% decrease in prostate-specific antigen, and a reduction in serum C-reactive protein as well as a reduction in expression of NF-κB. We used 1g/kg dose in mice, which corresponds to 7 gram/day in man, thus, this dose is achievable in humans.

Overall, our results show for the first time that Zyflamend alone can inhibit the growth of human pancreatic tumors in mice and it can further potentiate the antitumor activity of gemcitabine by inhibiting various biomarkers of the disease, leading to the inhibition of proliferation. However, further studies are necessary to confirm our findings in patients with pancreatic cancer. Considering that Zyflamend can modulate multiple targets, it merits further exploration.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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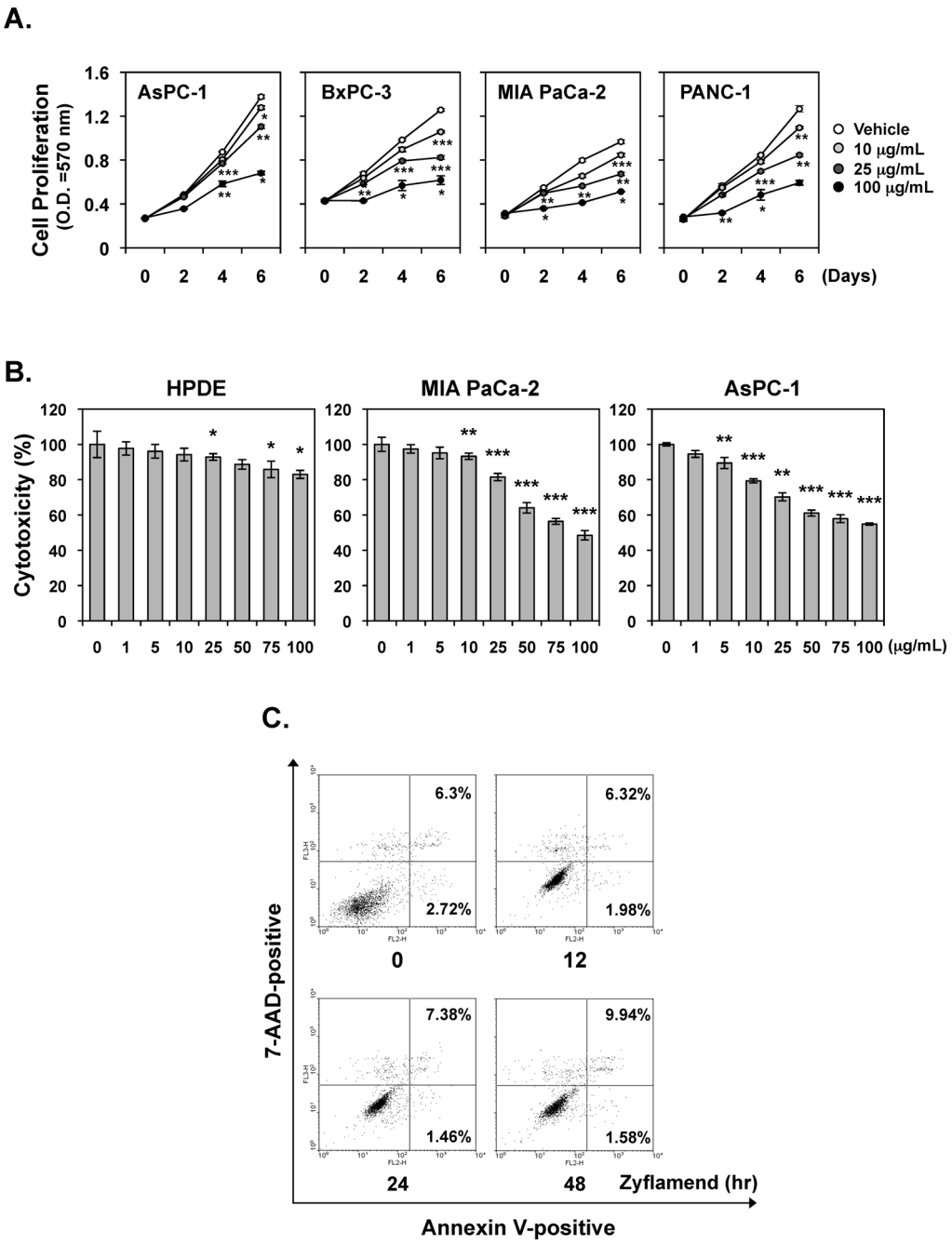

Figure 1. Zyflamend inhibits proliferation, induces apoptosis and cell cycle arrest in pancreatic cancer cells in vitro

(A) Zyflamend inhibits the proliferation of pancreatic cancer cells. MTT assay results showed dose-dependent suppression of cell proliferation in all four pancreatic cancer cell lines tested. Points, mean of triplicate; bars, SE. (B) Cells (2000 cells/well) were seeded in triplicate onto 96-well plates, treated with the indicated concentration of Zyflamend for 72 h, and then measured cell viability by the MTT method and presented as percent cell viability. Points, mean of triplicate; bars, SE (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. untreated cells). (C) MIA PaCa-2 cells were incubated with the indicated concentration of Zyflamend for 24

h and then incubated with anti-annexin V antibody conjugated with fluorescein isothiocyanate and then analyzed with a flow cytometer for early apoptotic effects.

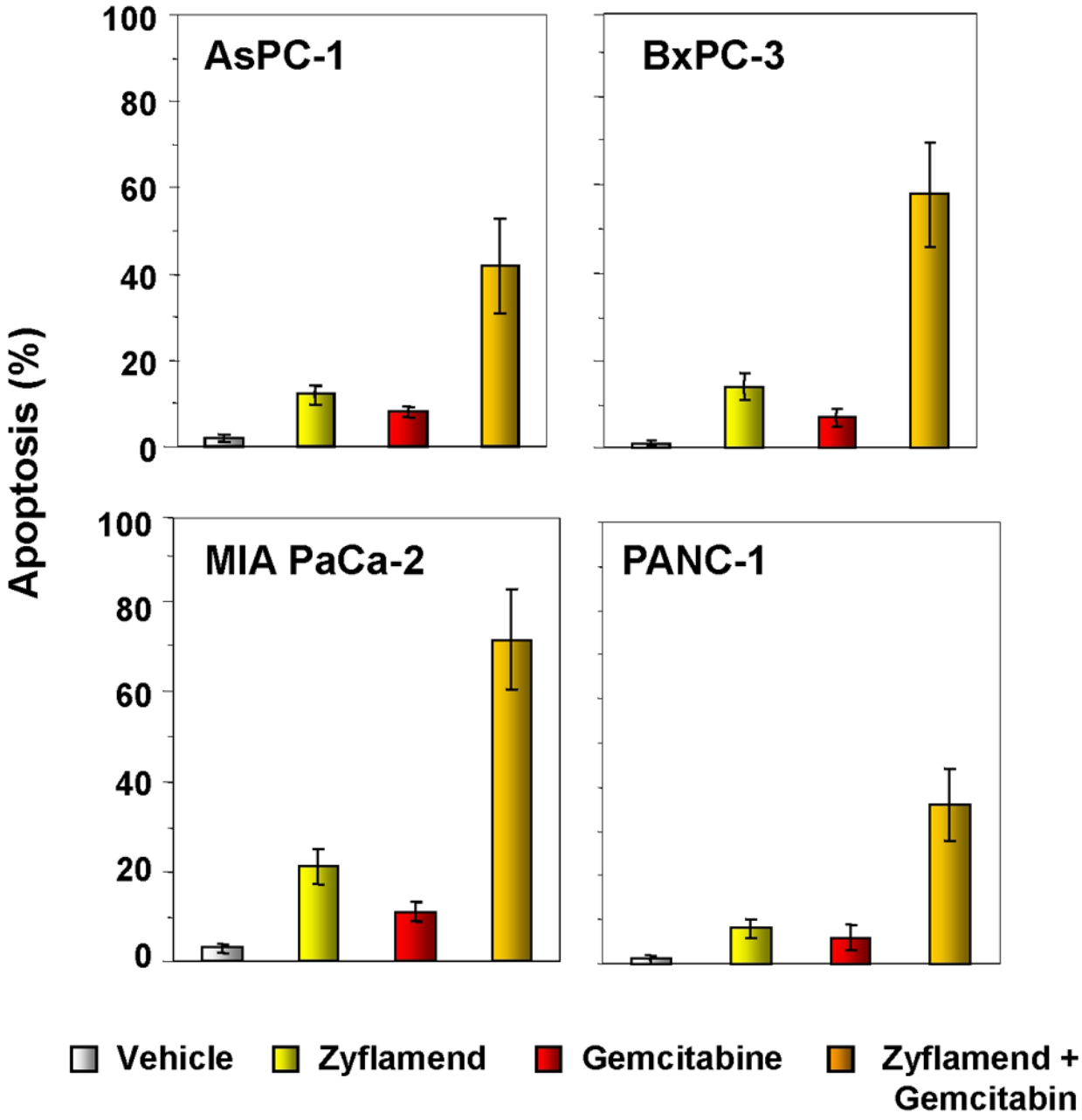

Figure 2. Zyflamend potentiates the apoptotic effects of gemcitabine in pancreatic cancer cells in vitro

Live/Dead assay results indicated that Zyflamend potentiates gemcitabine-induced cytotoxicity. Percentages, proportions of apoptotic pancreatic cancer cells. Data are representative of two independent experiments.

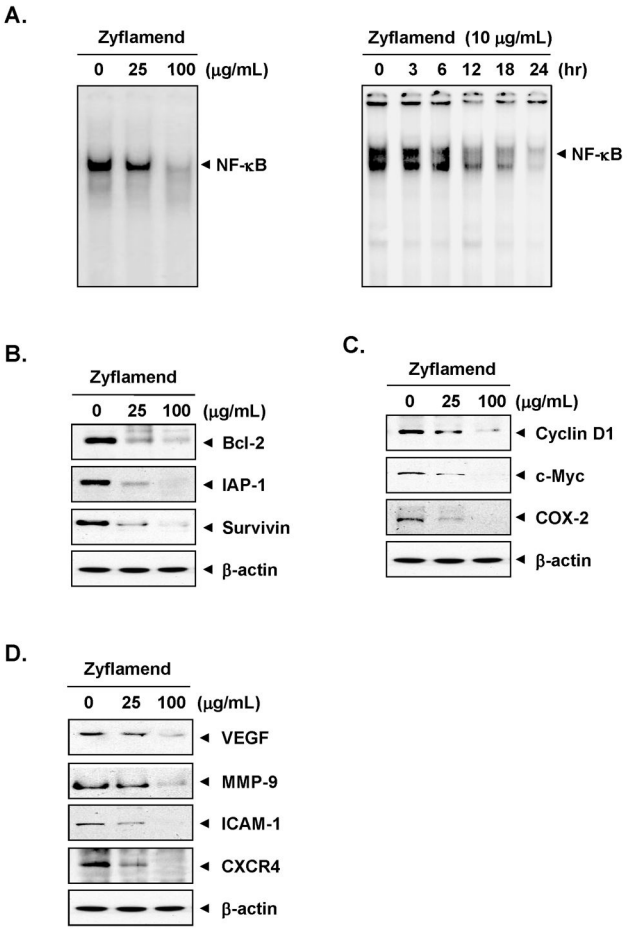

Figure 3. Zyflamend inhibits constitutive activation of NF-κB and various biomarkers in vitro

(A, *left*), MIA PaCa-2 (1×10^6) cells were treated with Zyflamend (25 and 100 μg/mL) for 4 h, nuclear extracts were prepared and then assayed for NF-κB activation by EMSA; (*Left*) MIA PaCa-2 cells were incubated with Zyflamend (10 μg/mL) for indicated time, nuclear extracts were prepared and then assayed for NF-κB activation by EMSA. (B–D), Western blot analysis showed that Zyflamend inhibited constitutive expression of NF-κB-regulated gene products that regulate apoptosis (B), proliferation (C), and metastasis and angiogenesis (D) in pancreatic cancer cells. The MIA PaCa-2 (1×10^6) cells were treated with Zyflamend (25 and 100 μg/mL) for 24 h. Whole-cell lysates were prepared and assayed for NF-κB-regulated gene products by Western blotting. Data represent two independent experiments.

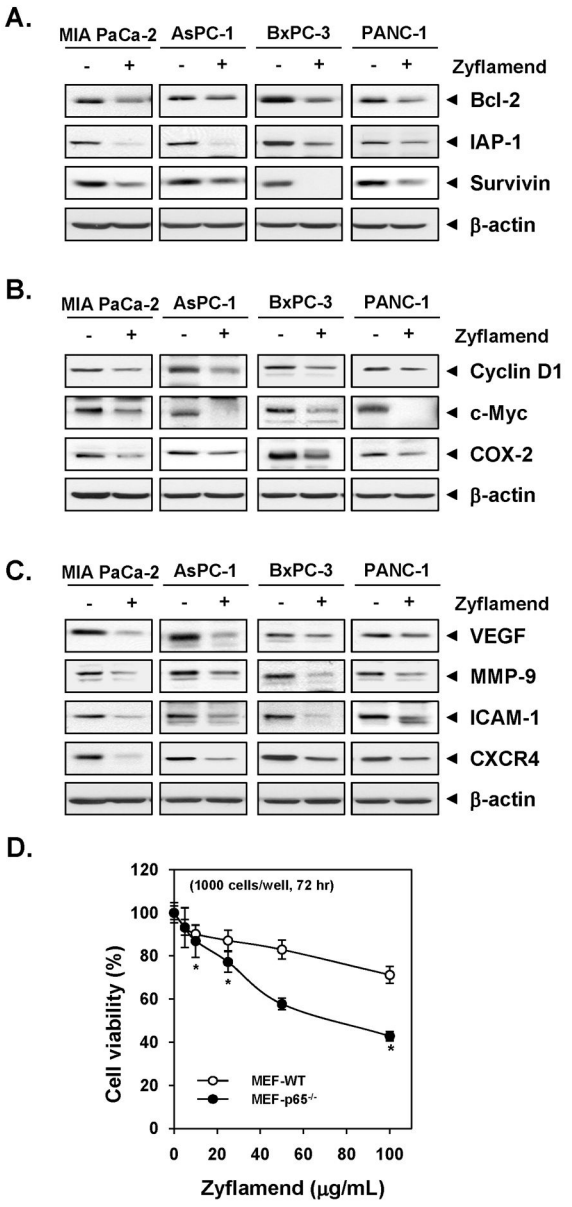

Figure 4. Zyflamend downmodulates expression of proteins associated with inflammation, proliferation, invasion, and angiogenesis in pancreatic cancer cells

(A–C) Cells (1×10^6) cells were treated with Zyflamend (100 μg/mL) for 24 h, whole-cell lysates were prepared and subjected to Western blotting, using antibodies as indicated. Data represent two independent experiments. (D) The wild-type and $p65^{-/-}$ deficient (1×10^3) cells were treated with indicated concentrations of Zyflamend for 72 h, and then measured cell viability by the MTT method. Results are presented as percent cell viability. Points, mean of triplicate; bars, SE (*$P < 0.05$ vs. wild-type cells).

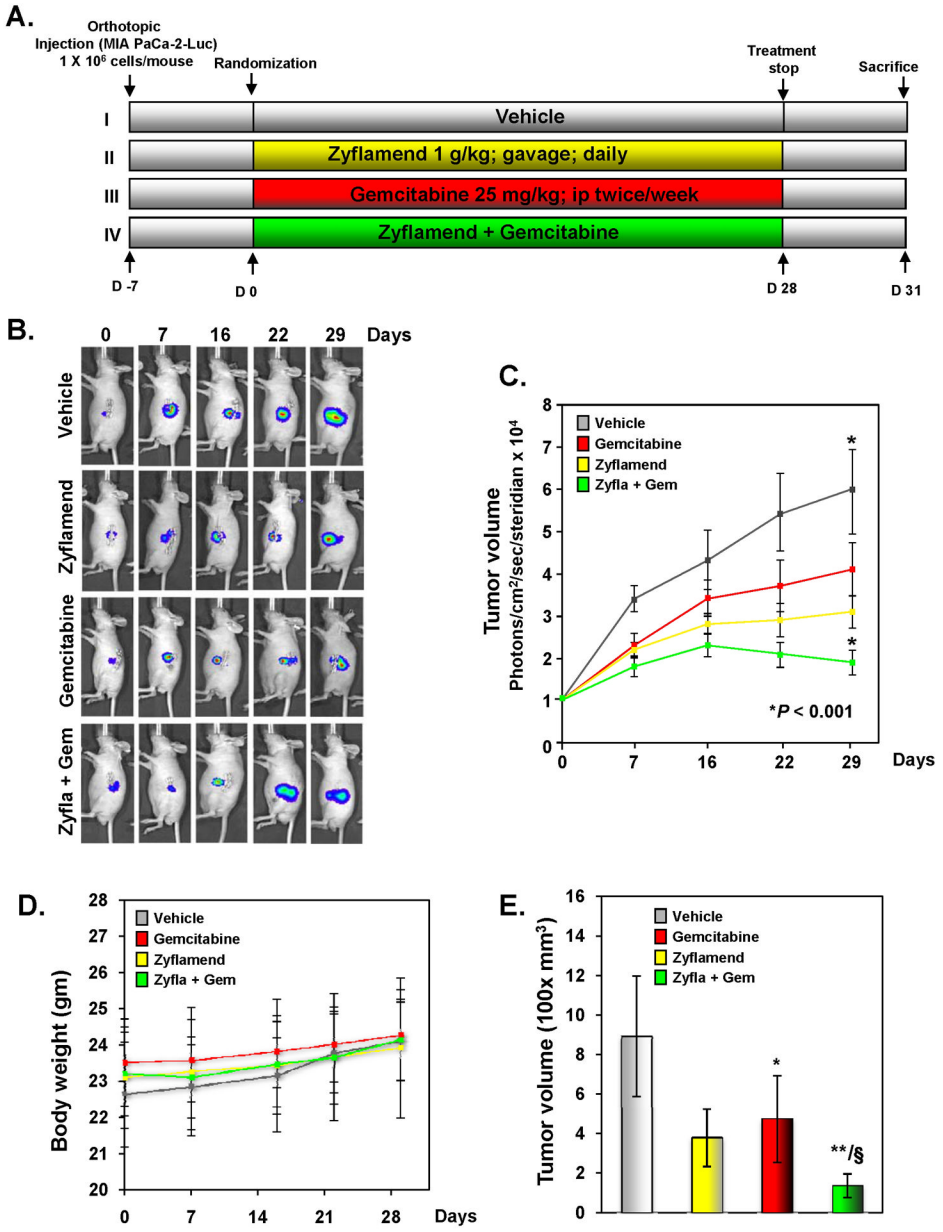

Figure 5. Zyflamend potentiates the effect of gemcitabine in blocking the growth of pancreatic cancer in nude mice

(A), Schematic of experimental protocol described in Materials and Methods. Group I was given olive oil (100 μL, p.o., daily) and PBS (100 μL, i.p., twice weekly), group II was given Zyflamend (1g/kg in 100 μL olive oil, p.o., daily), group III was given gemcitabine (25 mg/kg in 100 μL PBS, i.p., twice weekly), and group IV was given Zyflamend (1g/kg in 100 μL olive oil, p.o., daily) and gemcitabine (25 mg/kg in 100 μL PBS, i.p., twice weekly). (B), Bioluminescence IVIS images of orthotopically implanted pancreatic tumors in live, anesthetized mice. (C), Measurements of photons per second depicting tumor volume at various time points using live IVIS imaging at the indicated times (n = 6). Points, mean; bars, SE (*$P < 0.05$ vs. vehicle). (D), Body weight changes of mice was measured at indicated times (n = 6). Points, mean; bars, SE. (E), Tumor volumes in mice measured on the last day of the experiment at autopsy with vernier calipers and calculated using the

formula $V = 2/3\pi r^3$ (n = 6). Columns, mean; bars, SE (*$P < 0.05$, **$P < 0.01$ vs. vehicle; §$P < 0.05$ vs. Zyflamend alone).

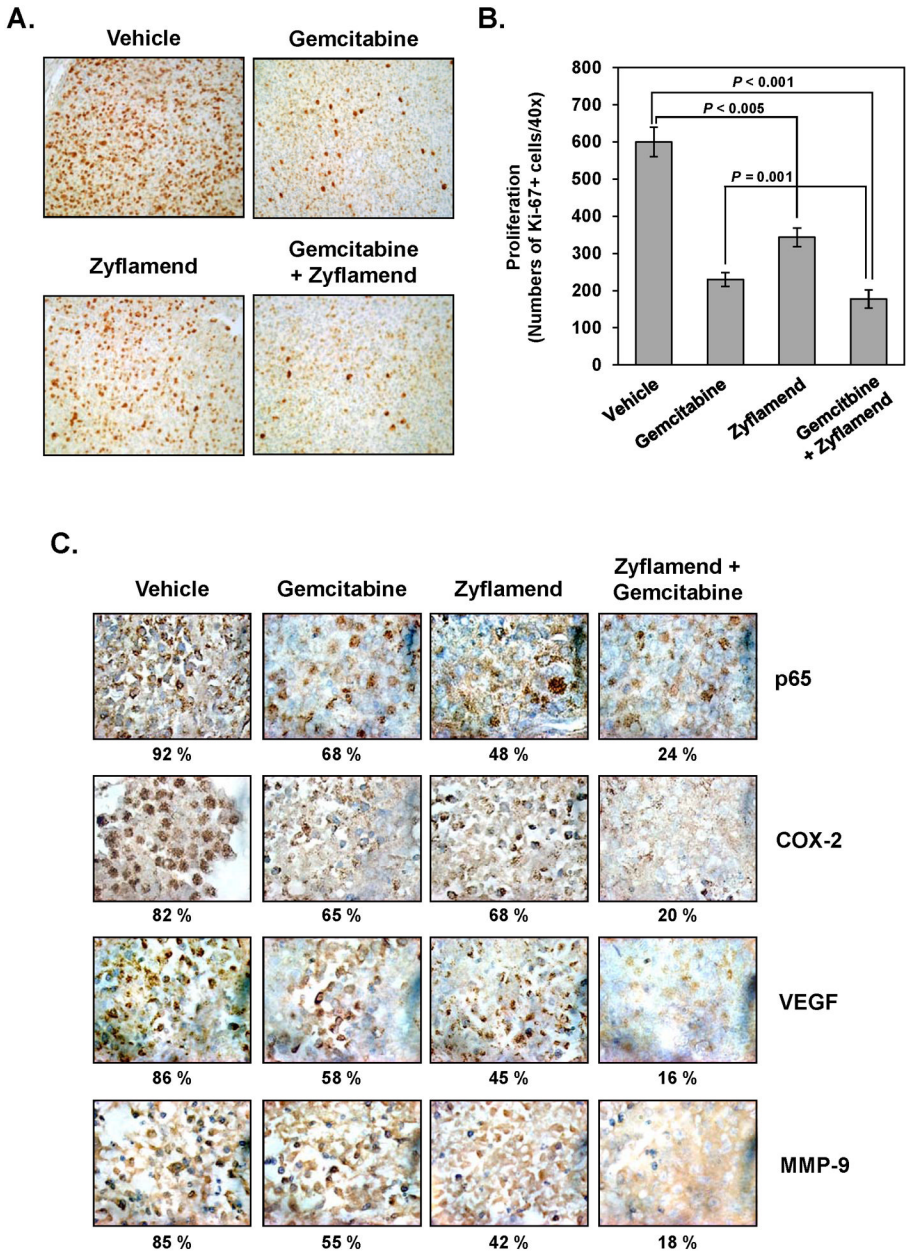

Figure 6. Zyflamend inhibits the expression of various biomarkers and further enhances the effect of gemcitabine in pancreatic cancer

(A) Immunohistochemical analysis of NF-κB-regulated genes COX-2, VEGF, and MMP-9 in pancreatic tumor tissues from mice. Percentages indicate positive staining for the given biomarker. Samples from three animals in each group were analyzed, and representative data are shown. (B), Mechanism of action of Zyflamend against pancreatic tumors.