

Targeting Pancreatic Cancer with Synergy

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Master of Science
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Abstract

Pancreatic Cancer is one of the deadliest forms of cancer that one can acquire. Following diagnoses, only 8.5% with invasive pancreatic cancer will live 5 years beyond diagnosis. While cancer survival rates have improved overall, the rates for pancreatic cancer have remained unchanged. Numerous risk factors including aging, smoking, obesity, chronic pancreatitis, familial history, and diabetes can all effect pancreatic cancer outcomes. The biology and cellular signaling that drives pancreatic cancer progression and survival is intricate and multifactorial. In addition, pancreatic cancer often develops resistance to chemotherapeutics such as gemcitabine and cisplatin. This lack of effectiveness has driven the demand for new and innovative approaches, aimed at targeting pancreatic cancer and enhancing the known therapies. This need for novel approaches, has led to new discoveries regarding the effectiveness of herbal and natural compounds against pancreatic cancer. Phytochemicals such as ginger, turmeric, cannabidiol (CBD) and resveratrol have all shown potential against pancreatic cancer. Zyflamend is a polyherbal combination of ten extracts that was originally created to target inflammation. Interestingly, cancer research began to identify Zyflamend as a promising treatment option. Zylamend has proven effective against cancers of the skin, mouth, bone, kidney, colon and pancreas. However, the molecular mechanisms of Zyflamend are yet to be well-characterized. Therefore, we sought to investigate the effects of Zyflamend treatment on pancreatic cancer. Utilizing an *in vitro* pancreatic cancer model, we were able to identify the mechanism through which Zyflamend induced cell death and apoptosis.

Table of Content

CHAPTER 1 INTRODUCTION	1
Cancer	2
America Cancer Statistics.....	2
Overview	2
Pancreatic Cancer	3
Epidemiology and Current Statistics	3
Etiology and Pathology.....	3
Risk Factors	4
Aging.....	4
Familial History.....	5
Smoking	5
Smoking and Pancreatic Cancer.....	5
Obesity	6
Chronic Pancreatitis	6
Glucose Homeostasis, Diabetes and the Stromal Environment	7
Therapies, Limitations and Alternatives	8
Chemotherapy and Radiotherapy	8
Limitations of Current Therapies	9
Dietary/ Herbal/ Alternative Medicine.....	9
Resveratrol.....	10
BITC	10
CBD	11
DRE/EGCG	11
Zyflamend	12

Introduction	12
Recipe	12
Zyflamend and Cancer.....	12
Zyflamend and Pancreatic Cancer	13
Individual Components of Zyflamend in Pancreatic Cancer Research	13
Zyflamend Signaling Pathways: Ampk, Apoptosis, Etc.	15
Conclusion	15
Research Objectives.....	16
Specific Aims	16
Specific Aim 1: We will explore the in vitro effects of Zyflamend, both alone and in combination, with known chemotherapeutic compounds.....	17
Specific Aim 2: Illustrate the in vivo effects of Zyflamend, both alone and in combination, with known chemotherapeutic compounds on the initiation and progression of pancreatic cancer.	17
Specific Aim 3: Characterize the molecular mechanisms in vitro.....	17
CHAPTER 2 RESEARCH PROJECT	19
Abstract	21
Significance.....	22
Introduction.....	23
Material and Methods	24
Chemicals and reagents.	24
Cell culture.	25
Zyflamend treatment.....	25
Western blotting analysis.....	25
Morphological analysis of apoptosis.	25
Annexin V staining.....	25

Statistical analysis.....	27
Results.....	27
Discussion	50
CHAPTER 3 CONCLUSION and FUTURE PERSPECTIVES	57
Conclusion	58
Future Perspectives	59
LIST OF REFERENCES	61
VITA.....	81

List of Figures

Figure 1. Zyflamend reduces cell survival while inducing cell death in a dose dependent manner.	29
Figure 2. Zyflamend induces apoptotic cell death in Beta-TC-6 cells.....	33
Figure 3. Zyflamend induces inflammatory, ER stress, and autophagy responses in Beta-TC-6 cells.	36
Figure 4. Zyflamend-induced cell death is mediated through the ER stress/Jnk/autophagy pathways.	41
Figure 5. Zyflamend exacerbates the effects of cisplatin and gemcitabine on Zyflamend-induced inflammatory, ER stress, and autophagy in Beta-TC-6 cells.....	47

List of Tables

Table 1. List of primary antibodies and conditions of use.	29
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CHAPTER 1

INTRODUCTION

Cancer

America Cancer Statistics

You have cancer! According to the NIH approximately, 1,735,350 Americans will receive this dreaded diagnosis in 2018 [1]. Furthermore, approximately 38.4% of Americans will be diagnosed with cancer at some point in their life span [1]. It was also estimated in 2016 that there were over 15 million cancer survivors in America [1]. In addition, the extensive therapy and follow up care cancer patients must receive places a substantial burden on the medical system. The result is staggering and led to around \$147.3 billion in healthcare cost in 2017 [1]. The encumbrance is not only placed upon the individual and economy but is often shared by friends and loved ones of the cancer survivor. As a result, cancer has become the unified enemy of mankind and continues to stump even the brightest of minds. Fortunately, innovative ideas and groundbreaking research show promise in developing better treatment strategies and contribute valuable insight to uncovering the enigmatic pathologies of cancer.

Overview

The human organism experiences a high rate of cellular turnover on a daily-basis. This process is intended to be a highly controlled and orderly series of events that allow us to thrive and regenerate at the cellular level. In many cases, toxic levels of environmental or chemical exposure, genetic factors, viruses and increasing age can modulate the effectiveness of proper cellular turnover. Furthermore, this interference within the cell-cycle occurs frequently and can result in DNA lesions [2]. If the body does not handle these DNA lesions appropriately then genome integrity can be compromised [3]. Dysregulation of the cell cycle DNA replication machinery can lead to premature cell death and if left uncorrected tumors can develop [3]. Genetic mutations occur frequently and under normal circumstances, the body is capable of sensing the mutation and handles it accordingly. However, if one somatic mutation remains uncorrected then a series of complex events can lead to the downstream development of cancer cells exhibiting a unique epigenetic profile, capable of metastasizing and invading neighboring tissue [4]. Three categories of genes have been isolated in the pathological development of cancer: tumor suppressors, oncogenes and DNA repair genes. Mutations in any of these genes can disrupt the cell replication machinery and if unregulated will lead to tumor formation [5]. The individual “fingerprint” and sheer number of cancer mutations leads to difficulties in isolating, treating, and understanding the pathological nature of this disease [4].

Various types of environmental exposure can increase the risk for cancer. For example, ultraviolet radiation from prolonged sun exposure may lead to the development of melanomas [6], smoking may lead to lung cancer [7], and recently, exposure to compounds such as bisphenol A (BPA) has been identified as a potential carcinogen [8]. Furthermore, cancer often originates in tissue specific locations such as the pancreas, lungs, kidney, brain, reproductive organs, colon, and beyond. Cancer can also target and hijack immune system functioning, as seen in lymphomas and leukemias [9]. The multivariable nature and complex genetic profile that accompanies cancer has created a difficult quest towards curing and treating the disease. In order to understand the complexities, it is imperative that efforts towards dissecting the precise cellular mechanisms and pathology of the various forms of cancer be made. Through this process, valuable insight into the mechanistic profile allow advancement towards better therapeutic options and potentially even the coveted cure.

Pancreatic Cancer

Epidemiology and Current Statistics

Pancreatic cancer is one of the deadliest forms of cancer that one can acquire with GLOBOCAN 2012 estimating around 331,000 deaths per year globally [10]. The outcome for a pancreatic cancer diagnosis, in the majority of cases is death. The United States National Cancer Institute data showed that as of 2014 only 8.5% exhibiting invasive pancreatic cancer would live 5 years beyond diagnosis [11]. Much to the demise of the patient, the diagnosis of pancreatic cancer often occurs beyond the early stages of development. In many cases, this late diagnosis leads to difficulty treating the cancer, for it has progressed into the metastatic phase [12, 13]. Advancements in science and health care have led to decreased mortality from numerous forms of cancer. However, pancreatic cancer survival rates have not improved significantly over the past several decades and the need for solutions to this problem remain.

Etiology and Pathology

The risk for developing pancreatic cancer has been associated with numerous biological, environmental, pathological, and genetic factors. These factors include variables such as, familial history [14], chronic pancreatitis [15], smoking [16], obesity [17], diabetes[18] and stage of diagnosis. In addition, familial hereditary factors and

germline mutations could contribute to increased risk. The survival outcome is highly dependent upon the time of diagnosis. Problematically, symptoms such as pain, nausea and weight loss often occur after the cancer has metastasized. This leads to a large majority of pancreatic cancer diagnoses occurring beyond the early stages of development. In a majority of the cases, pancreatic cancer is discovered with the use of computed tomography (CT) scan and in many cases is performed for an unrelated condition.

There are two main types of pancreatic cancer, exocrine cancers, such as pancreatic adenocarcinomas (the majority), and the rarer endocrine cancers known as neuroendocrine tumors. The development of pancreatic cancer often revolves around a complex array of unique inter-related events. The initial growth and progression of lesions recognized as pancreatic intraepithelial neoplasia (PanIN) can further transform into pancreatic ductal adenocarcinoma (PDAC) [19]. The formation of PanIN and subsequent development of PDAC can result from events such as: the formation of oncogenic mutations in genes, such as *KRAS* [19], pancreatic acinar cell reconfiguration through acinar-to-ductal metaplasia (ADM) [20, 21], and inflammatory conditions leading to the potential activation of NF- κ B [22]. Events such as these, can initiate the signaling cascades required for pancreatic cancer to develop invasive and proliferative potential. Further understanding of how pancreatic cancer develops could lead to better preventative therapeutic options in the future.

Risk Factors

Aging

Of all the risk factors associated with pancreatic cancer aging shows the strongest association, with 72 being the median age of diagnosis in the United States [23, 24]. Through a natural process known as antagonistic pleiotropy, genes important for survival in the early stages of development can become debilitating later in life [25]. This senescent response can lead to metabolic alterations such as: disease-promoting phenotypes, disrupted tissue function, increased inflammatory response and eventually cancer [26]. When properly functioning tumor suppressor pathways such as: p16^{INK4a}/pRB and p53/p21 are capable of halting the progression of tumorigenesis [26]. However, in aging organisms these tumor-suppressing pathways can become overloaded and dysregulated in response to excessive genomic damage, shortened telomeres, accumulating mutations and stress [27]. For instance, it has been demonstrated that p16^{INK4a} levels can increase with age and inhibit the ability of beta cells to proliferate, regenerate and respond to

injury [28-30]. In the end, one mutation left uncorrected can initiate the required cascade for the development of cancer. These senescent factors likely play a key role in why aging is the major risk factor for pancreatic cancer.

Familial History

Epidemiological studies have identified that familial history may contribute to approximately 10% of pancreatic cancer occurrences [31-33]. Moreover, germline mutations in genes such as *BRCA2* [34], *p16/CDKN2A* [35], and *STK11* [36] can all lead to increased risk of pancreatic cancer. In search of gene-specific therapy, research targeting various pancreatic cancer cell lines harboring *BRCA2* mutations, found that they were highly sensitive to PARP (Poly[ADP-ribose] polymerase) and mitomycin C inhibitors [32, 37, 38]. Furthermore, the identification of high-risk genes and familial history profile can warrant frequent screening. When promptly diagnosed, curable neoplasms can be surgically resected thus preventing tumorigenic progression of pancreatic cancer [39]. It is important to understand the relationship between familial factors and pancreatic cancer based on the potential for improved therapy and advanced screening that could save lives.

Smoking

The carcinogenic effects of tobacco smoking have been well documented in numerous cancer types [40]. The idea that smoking correlated with the development of lung cancer has been known for over 50 years and over 80% of United States lung cancer cases correlate to smoking [41, 42]. Smoking leads to significant increases in environmental and avoidable risk factors for developing pancreatic cancer [43-45]. In addition, research has continually showed that smoking directly correlates with major increases in pancreatic cancer incidence [46, 47].

Smoking and Pancreatic Cancer

Tobacco smoke contains high levels of carcinogenic compounds including nicotine which metabolizes into numerous carcinogens [48]. Over time continued or chronic exposure to carcinogens can lead to extreme consequences to numerous aspects of human biology and metabolic function. The earliest *in vivo* study revealing the relationship between smoking and pancreatic cancer dates back to 1979 [49]. However, animal model studies have generally been inconsistent, with large variations in the experimental design,

along with their reported findings [45, 50, 51]. In one study, the hypothesis that smoking could act in conjunction with Kras to promote PDAC development, was investigated. Through injecting transgenic mice exhibiting pancreatic acinar cell specific Kras expression (EL-Kras) with the nitrosamine and nicotine metabolite 4-(methylnitrosamine)-1-(3-pyridyl)-1-butanone (NNK), the researchers revealed a potential synergistic mechanism [45]. The authors proposed that NNK and Kras could synergistically promote the formation of PanIN lesions and pancreatic carcinogenesis [45]. These diverse findings across nearly half a century, promote a strong argument towards abstaining from smoking tobacco in order to reduce the risk for developing pancreatic cancer.

Obesity

The obesity problem stems in many different directions, including increased risk for developing various chronic illnesses along with cancer [52, 53]. In addition, it has been approximated that excess weight and obesity causes one fifth of all cancers [54]. Understanding how obesity influences the development and risk for pancreatic cancer has been no easy challenge. One way that obesity could contribute is through the increased chronic inflammatory response [55]. Supportively, studies have shown that adipocyte derived peroxisome proliferator-activated receptor- γ (PPAR- γ) could play a role in the development of cancer in obesity [56]. Furthermore, adipocytes could engage in crosstalk with tumor cells, resulting in increased tumorigenesis as seen in a breast cancer model [57]. However, the precise mechanisms through which obesity is a risk factor for pancreatic cancer remains murky. More research targeting the specific relationship between obesity and pancreatic cancer could provide valuable insight.

Chronic Pancreatitis

Chronic pancreatitis is a major risk factor for pancreatic cancer. Lowenfels et al, were the first researchers to isolate the causality between the two diseases, revealing a mild association where 56 of 2,015 chronic pancreatitis patients developed cancer [15]. In a more recent systematic review, meta-analysis revealed an eightfold increase in risk of pancreatic cancer, in chronic pancreatitis patients having been diagnosed five years prior [58]. Another matched cohort included 41,669 Denmark hospital patients that had been diagnosed with acute pancreatitis between the years 1980-2012 [59]. They showed that having been diagnosed once with acute pancreatitis led to an approximated twofold increased risk of pancreatic cancer [59]. In addition, heavy alcohol consumption can lead to increased risk for both chronic pancreatitis and pancreatic cancer [60].

Problematically, due to symptomatic and pathological similarities, it can be difficult to diagnose and differentiate between pancreatic cancer and chronic pancreatitis [61].

Chronic pancreatitis is driven by an inflammatory response that can progressively intensify, if left untreated. Inflammation has been linked with the development of most cancers. Therapy targeting Cox-2 has shown promise against chronic pancreatitis [62]. Nevertheless, controversy surrounds the use of the Cox inhibitor aspirin and the risk for pancreatic cancer [63, 64]. It has also been shown that increased NF- κ B activity was directly associated with pancreatitis severity [65]. In addition, the inflammation associated with chronic pancreatitis could lead to the diverse activation and recruitment of cytokines and stromal cells, promoting the microenvironment changes necessary for pancreatic cancer progression. Recently, Liao et al., investigated how modifying arachidonic acid metabolism and outcomes could decrease chronic pancreatitis associated inflammatory biomarkers. They demonstrated that treatment with a dual inhibitor of RAF1 proto-oncogene serine/threonine kinase (c-RAF) and soluble epoxide hydrolase (sEH) resulted in decreased levels of macrophages, neutrophils, and inflammatory cytokines in the pancreas of *LSL-Kras^{G12D}/Pdx1-Cre* mice [66]. Furthermore, this was accompanied by a significant reduction in pancreatic injury and fibrosis. These findings support an emerging concept that through sEH inhibition, stabilization of epoxyeicosatrienoic acids (EETs) and subsequent modifications of the eicosanoid profile may result in desirable anti-inflammatory results [66, 67].

Glucose Homeostasis, Diabetes and the Stromal Environment

Type 2 diabetes (T2D) has become one of the leading health epidemics worldwide. The development of T2D involves β cell dysfunction leading to decreased insulin secretion, and decreased glucose uptake as related to insulin resistance [68-70]. Pancreatic β cells are responsible for the release of insulin and essential in maintaining glucose homeostasis. β cell function or dysfunction is often measured by the levels of insulin secreted and/or urinary excretion of C-peptide [68, 71]. Insulin secreted from β cells responds to a complex array of interconnected biological factors [72, 73]. Glucose-stimulated insulin secretion (GSIS) occurs through the uptake of glucose into β cells, which leads to an eventual rise in intracellular Ca^{2+} , resulting in the subsequent insulin secretion [74]. This process is dependent upon the depolarization of the K_{ATP} channel in response to an increased

ATP/ADP ratio [74, 75]. When exposed to prolonged hyperglycemic conditions, the β cells can begin to exhibit decreased mass and impaired function, leading to inflammation and impaired insulin secretion [76]. Analysis and research targeting the relationship between diabetes and pancreatic cancer, continues to progress. The relationship appears bi-directional in relation with the development of diabetes and pancreatic cancer [77, 78]. These findings suggest that the recent diagnosis or onset of diabetes could be an early detection marker for the diagnosis of pancreatic cancer [79]. Moreover, late onset diabetic patients over the age of 50 have a significantly higher risk and warrant screening for pancreatic cancer [80]. Further understanding of the bi-directional relationship between diabetes and pancreatic cancer could be essential in improving preventative and therapeutic application.

Additionally, PDAC has been identified as exhibiting a high level of stromal content such as growth factors and fibroblasts [81]. Studies have shown that the stroma could provide the tumors with advantages through the expression of certain surface markers such as CD10 seen in pancreatic cancer associated fibroblasts [82]. One study has shown that diabetes can increase the level of inflammation, fibroblasts and overall atrophy of the pancreas [83]. Stromal environmental effectors, such as increased hedgehog signaling [84] and PDAC immunosuppression resulting in a high level of cytokines [81], have been seen. Interestingly, the hedgehog pathway has been identified in conflicting roles in pancreatic function, diabetes, and pancreatic cancer [85]. Understanding how this highly active stromal environment, diabetes progression, and pancreatic cancer are connected could be essential in uncovering the secrets to the pathology of the disease. The following section will explore the limitations of modern therapies and possible alternative solutions.

Therapies, Limitations and Alternatives

Chemotherapy and Radiotherapy

In most cases, the chemotherapeutic approach towards treating pancreatic cancer remains the primary recommendation within the medical community. In addition, adjunct therapy through a combination of various treatment approaches such as, surgery and radiotherapy often compliment this choice. If the cancer appears to be resectable, then either the adjuvant or neoadjuvant approach will be adopted. Chemotherapeutic compounds such as gemcitabine, cisplatin, FOLFIRINOX and 5-fluorouracil (5-FU) often play a central role in these approaches [86]. In addition, these therapies remain largely ineffective and this is highly apparent in the rate of survival statistics [23].

Limitations of Current Therapies

Current pharmacological therapies and treatments targeting pancreatic cancer have too often proven in-effective. These treatments typically include chemotherapeutic compounds such as gemcitabine, 5-fluorouracil, and cisplatin. These compounds show increased effectiveness at higher doses, but this directly exacerbates the risk for cytotoxicity and collateral side effects [87]. In addition, adjunct therapy through a combination of various treatment approaches, such as surgery and radiotherapy, often compliment this choice. Surgery can lead to beneficial outcomes if performed in a prompt manner [88]. However, the stage of diagnosis and degree of difficulty required to perform a successful surgery on pancreatic cancer make this option unrealistic for many [89]. In addition, the low levels of effectiveness and burden of side effects, leave patients weighing their options. In pursuit of survival and improved quality of life, patients often seek to enhance the effectiveness of conventional therapies through dietary and supplemental means. The search for more effective therapies has illuminated new possibilities and continues to explore beyond the known pathology and treatments. One such promising avenue for research is uncovering the potential for combined and synergistic enhancement of known therapies [90, 91].

Dietary/ Herbal/ Alternative Medicine

A growing body of literature endorses the potential for numerous phytochemicals based on their anti-tumorigenic and pro-apoptotic effects, as demonstrated in murine and cellular experimental cancer models [92-94]. Having shown potential to treat a myriad of diseases, scientists, medical professionals, and the public continue to seek clarification. Fortunately, progress has been made and scientists have begun isolating the precise function and mechanisms through which natural compounds could prove beneficial. For instance, phytonutrients such as: resveratrol [94], benzyl isothiocyanate [93], cannabidiol [92], and dandelion root extract have all shown potential in experimental models of pancreatic cancer. In pancreatic cancer, natural compounds such as pachymic acid, cannabinoids, triptolide, and their synthetic analogues such as diarylmethanes, have been shown to activate ER stress and autophagy to induce apoptotic cell death [95]. Triptolide, is a Chinese herbal extract that shows promise through its ability to constrain pancreatic cancer cell growth [96-98]. Researchers identified that triptolide treatment resulted in

pancreatic cancer cell death accompanied by ER stress response including increased eIF2 α phosphorylation, Ire1 α expression, and modified expression patterns of XBP1, CHOP, and GPR78 [98]. Similarly, pachymic acid a purified triterpene from a fungal extract can trigger apoptosis in both MIA PaCA-2 and PANC-1 cells through ER stress induction as indicated by increased expression of phospho-eIF2 α , XBP-1s, CHOP, ATF-4, and Hsp70 [99]. ER stress signaling was confirmed as the initiator of apoptosis when ER stress inhibitor tauroursodeoxycholic acid (TUDCA) blocked the effect [99]. Although many of these natural compounds were shown to exhibit anti-proliferative and pro-apoptotic activities in pancreatic cancer cells at relatively high concentrations, at low concentrations (similar to doses achieved with 200 ug/ml of Zyflamend), many of these compounds fail to exhibit cell death-inducing activity [100].

Resveratrol

Resveratrol a compound found primarily in grapes has drawn vast interest in age and metabolic related studies [101]. In addition, resveratrol has drawn an accumulating level of interest in cancer related research [102]. The effect of resveratrol on pancreatic cancer has been explored. In one study, the authors identified that resveratrol was able to inhibit genes involved in tumorigenesis through suppressed NF- κ B activation [103]. The combination of resveratrol and gemcitabine enhanced the effectiveness both *in vitro* and *in vivo* [103]. Furthermore, resveratrol has shown potential to regulate cellular functions integral to the survival of pancreatic cancer; including the Pi3k/Akt pathway [94] and apoptosis induction [104],

BITC

Cruciferous vegetable compound, benzyl isothiocyanate (BITC) is another phytonutrient that has drawn interest in pancreatic cancer research. BITC treatment reduced STAT3 activation and protein expression in a variety of pancreatic cancer cell lines. Correspondingly, BITC treatment induced apoptosis and reduced cell survival in these same cell lines, while immortalized human pancreatic cells (HPDE-6) remained unaffected [105]. The growth of BxPC-3 tumors *in vivo* was suppressed in response to BITC feeding and this effect was accompanied by a downregulation in STAT3 expression, concomitant with elevated levels of apoptosis [105]. Supportively, it was recently discovered that BITC treatment increased ROS levels and subsequently inhibited the survival, growth, and invasive potential of pancreatic cancer [93].

Interestingly, this effect was attributed to pSTAT3/STAT3 inhibition and glutathione co-treatment reversed the response [93].

CBD

In recent times and for numerous reasons, the science behind marijuana and the cannabidiol (CBD) receptor system, has been spotlighted. CBD is an extract of the marijuana plant that lacks tetrahydrocannabinol (THC) and the psychoactive response associated with being high. Pioneering this research, Ferro *et al* showed that through the inhibition of G protein-coupled receptor GPR55, CBD was effective against PDAC [92]. CBD effectively inhibited cell cycle progression and growth in both HPAFII and PANC1 cells. Moreover, it was revealed that CBD inhibited the phosphorylation of ERK1/2 in HPAFII cells, showing that CBD may have multiple avenues through which it could prove beneficial in targeting pancreatic cancer. Interestingly, CBD treatment significantly enhanced the effectiveness of gemcitabine (GEM) to prolong survival in an *in vivo* PDAC model involving KPC mice [92]. The KPC mice treated with combined GEM and CBD exhibited an extraordinary increase in lifespan. The mean length of survival went from 27.8 (GEM alone) to 52.7 days (combination GEM/CBD) compared to only 18.6 days in the control [92]. This study warrants future research into the potential of both CBD and GPR55 inhibition in targeting and treating pancreatic cancer.

DRE/EGCG

Numerous other herbal extracts and dietary compounds have been researched for their potential to treat and kill pancreatic cancer. The following will be a brief insight into recent research regarding a variety of these. Genistein, a flavonoid found in soy, exhibits *in vitro* anti-pancreatic cancer activity through cell cycle arrest and increased ROS induced mitochondrial apoptosis [106]. Green tea polyphenol, epigallocatechin-3-O-gallate (EGCG), through combined non-invasive treatment with low strength pulsed electric field (PEF), resulted in a synergistic anticancer effect on PANC-1 cells [107]. In addition, dandelion root extract (DRE) induces autophagy and apoptosis in PANC-1 and BxPC-1 cells [108].

Zyflamend

Introduction

New Chapter, Incorporated, first launched Zyflamend based on the theory that it could act as a natural anti-inflammatory supplement. Individually, the extracted components of Zyflamend have proven to exhibit anti-cancer activity. However, the extremely high doses required to optimize effectiveness against cancer could prove infeasible for the majority of patients [109-111]. In theory, the combined effects generated through integrating these unique and powerful herbs could grant superior benefit over their singular form [112]. A large volume of research, has emerged over the last two decades supporting Zyflamend's anti-inflammatory role. Initially, the research centralized around the effectiveness of Zyflamend as a Cox inhibitor. Since then, this theory has proven true as research shows that Zyflamend acts as a Cox inhibitor in models such as: prostate [113], melanoma [114], and oral cancer models [115]. Additionally, Zyflamend has shown to modify a variety of integral cellular signaling pathways beyond Cox. These signaling pathways and mechanistic interactions of Zyflamend include: Ampk [116, 117], NF- κ B [118-120], mtorC1 [117], apoptosis, cell growth [113, 121], ER stress [119, 122, 123] and autophagy [114]. Taken together, these findings show that Zyflamend could exhibit profound potential in therapeutic applications.

Recipe

Zyflamend (New Chapter, Inc., Brattleboro, VT) is a combined supplement that makes use of 10 herbal extracts including the following in a two capsule once-daily dose; turmeric (110 mg), rosemary (150 mg), ginger (100 mg), holy basil (100 mg), organic green tea (100 mg), hu zhang (80 mg), chinese goldthread (40 mg), barberry (40 mg), organic oregano (40 mg), and Chinese skullcap (20 mg). Zyflamend has been highly regarded as an anti-inflammatory agent. Individually, the extracted components of Zyflamend have proven to exhibit anti-cancer activity. The encapsulated composition of Zyflamend revolves around the idea of either an additive or a synergistic response. In theory, the combined effects generated through integrating these unique and powerful herbs could grant superior benefit over their singular form [112].

Zyflamend and Cancer

The combination supplement of 10 herbal extracts, has been implicated for its therapeutic potential in a wide variety of cancer research platforms [113-115, 118-120, 124]. A growing body of literature indicate that Zyflamend exhibits anti-survival and pro-apoptotic effects on several

cancer models [119, 121, 125-127]. Multiple pathways have been proposed through which Zyflamed could exert its effect including, down-regulation of NF- κ B signaling in bone cancer [119], decreased expression of cyclooxygenase Cox-2 in melanoma [114] and inhibition of the arachidonic pathway through targeting 12-Lox and 5-Lox in prostate cancer [124]. Human equivalent dosing of Zyflamend in a xenograft prostate cancer cell model, resulted in decreased *in vivo* tumor growth [128]. In addition, Zyflamend treatment resulted in the decreased expression of histone deacetylases, protein kinase B (Pkb) phospho-Akt, prostate specific antigen and androgen receptor, all biomarkers associated with the progression of prostate cancer [128]. Furthermore, Zyflamend has proven to reduce the overall viability of various prostate cancer cell lines [113, 124, 129]. Beyond prostate cancer, Zyflamend has shown anti-tumor potential in other areas of cancer research targeting the skin, mouth, bone, kidney, colon and pancreas [119, 121, 125, 126]. Research targeting the specific effects of Zyflamend on pancreatic cancer has been limited [120].

Zyflamend and Pancreatic Cancer

To date, only one study has targeted the effects of Zyflamend on pancreatic cancer [130]. Zyflamend treatment induced an antiproliferative response in numerous pancreatic cancer cell lines. This effect was attributed to its ability to suppress proliferation and induce apoptosis through a variety of gene and protein modifications. They reported that antiapoptotic proteins Bcl-2, survivin and Iap-1 were all down regulated. Additionally, cellular proliferation proteins Cox-2, cyclin D1 and c-Myc, were also suppressed in response to treatment. The *in vitro* findings also showed that Zyflamend suppressed NF- κ B expression. Moreover, the effects of orally administered Zyflamend (1g/kg) on nude mice with orthotopically implanted human tumors, was revealed. The murine model showed Zyflamend treatment reduced the growth of pancreatic tumors by nearly 50%. The *in vivo* findings correlated with the *in vitro* results. They showed that through either an additive or synergistic effect Zyflamend could increase the effectiveness of gemcitabine against tumors [130].

Individual Components of Zyflamend in Pancreatic Cancer Research

Individually, the extracts contained have shown promise in a wide variety of experimental models. For instance, curcumin, a polyphenol found in turmeric, has shown potential in a wide variety of cancer research [131-133]. Curcumin could inhibit

carcinogenesis through a variety of proposed pathways and processes including: angiogenesis and tumorigenesis inhibition [134] and disruption of the mtor-raptor complex and subsequently decreased phosphorylation and activation of Akt/mtor signaling [135, 136]. In addition, curcumin can lead to the inactivation of the Pi3k/Akt pathway and apoptosis induction through increased mitochondrial p53 and Bax translocation [137], and can regulate autophagy turnover in an Ampk/Ulk1 dependent manner [133]. Moreover, modifying Ampk levels could have profound consequential effects to mitochondrial homeostasis and the overall energetic profile within the tumor microenvironment [138]. Recently, the effects of curcumin treatment on a strain of gemcitabine-resistant PDAC cells (BxPC3-GemR) revealed a novel mechanism of interaction that involves the modulation of the PRC2-PVT1-c-Myc axis. Similarly, ginger (another component of Zyflamend) and its derivatives, have been evaluated for its anticancer properties [139, 140] and their ability to modulate major signaling pathways important for cancer survival and proliferation of numerous cancer models including the NF- κ B, Pi3k/Akt/mtor, inflammation, cell cycle, angiogenesis, and apoptotic signaling pathways [141-145]. In pancreatic cancer research, the effects of ginger show vast potential in both murine and *in vitro* models. Ginger and its major pungent components were shown reportedly to downregulate and/or inhibit NF- κ B signaling across a variety of pancreatic cancer cell lines [146-148]. 6-gingerol, in particular, was demonstrated to induce cell cycle arrest at the G1 phase along with blocking entry into the S phase potentially through decreased cyclin-dependent kinase (Cdk) expression and reduced phosphorylation of retinoblastoma (Rb). Likewise, 6-shogaol, an active constituent of ginger, was reported to sensitize pancreatic cancer to gemcitabine through inhibiting NF- κ B, cyclin D1, Bcl-2, Cox-2, resulting in increased apoptosis [147]. Moreover, zerumbone, another isolated component of ginger, demonstrated pro-apoptotic effects on PANC-1 cells through the upregulation of P21, P53 and increased ROS production [149]. In corroboration, another study demonstrated that zerumbone could inhibit NF- κ B and block the translation of proangiogenic genes [140, 146].

Furthermore, rosemary (another main component of Zyflamend) and its constituents have proven effective in a wide variety of cancer research models. For instance, *in vitro* findings reveal the effectiveness of rosemary extracts (RE) against numerous cancers [150-154] through a variety of proposed mechanisms. Petiwala and colleagues demonstrated that RE treatment activated an ER stress response accompanied by the induction of apoptosis in prostate cancer cells [155]. RE

led to the increased expression of Bax, cleaved caspase 3, CHOP and IRE1 α , a mechanism similar to our findings [155]. Moreover, cancer research has targeted isolated polyphenols of RE such as carnosic acid (CA) and rosmarinic acid (RA) [154]. In pancreatic cancer research, the antitumor activity of RE against rat insulinoma (RINm5F) cells has been explored. In this *in vitro* model, RE treatment led to increased Tnf α and nitrate accumulation accompanied by the induction of apoptosis [152].

Additionally, another component of Zyflamend, holy basil, has shown promise in pancreatic cancer research. Holy basil treatment inhibited tumorigenesis in both murine and *in vitro* models and promoted apoptosis in pancreatic cancer cells [156]. Murine intraperitoneal injections of holy basil resulted in an influx in the expression of metastatic inhibitory (*BAD*) and apoptotic genes (*E-cadherin*). In addition, the injections simultaneously downregulating pro-survival genes (*Bcl-2* and *Bcl-xL*) and genes promoting treatment resistance *Chk1*, *AURKA*, and *survivin* [156]. Taken together these findings demonstrate the potential for combining these herbal extracts to target pancreatic cancer.

Zyflamend Signaling Pathways: Ampk, Apoptosis, Etc.

While the individual components of Zyflamend show potential in targeting cancer, the doses often required to elicit response exceed physiological relevance. For instance, the curcumin dose required to elicit the inhibition of Akt phosphorylation occurred at high doses (>40 μ M) in a previously mentioned study [135]. In addition, the dose required to generate antitumor response to polyphenolic extract from ginger, 6-shogaol, was relatively high both *in vitro* (15-20 μ M) and *in vivo* (50 mg/Kg) in a pancreatic cancer model [147]. Studies on RE shows huge variation in the doses used in cancer experimental models with *in vivo* doses reported from a low of 100 mg/kg/day to a high of 3333.3 mg/kg/day [154]. Furthermore, a dose of 6 grams of green tea per day given to prostate cancer patients led to varying degrees of collateral cytotoxicity [157]. Understanding the translatable potential of phytochemical research cannot be achieved without proper allometric scaling.

Conclusion

Zyflamend has been identified as a modifier of numerous cancer related pathways including the NF- κ B, ER stress, autophagy, and cell death pathways [114, 117, 121, 158] that play a major role in tumorigenesis. Through simultaneously and safely targeting

numerous aspects of tumorigenesis, Zyflamend could continue to prove effective in adjunct and preventative therapy. While the individual components of Zyflamend show potential in targeting cancer, the doses often required to elicit response exceed physiological relevance. For instance, curcumin dose required to elicit the inhibition of Akt phosphorylation occurred at high doses ($>40\ \mu\text{M}$) in a previously mentioned study [135]. In addition, the dose required to generate antitumor response to polyphenolic extract from ginger, 6-shogaol, was relatively high both *in vitro* (15-20 μM) and *in vivo* (50 mg/Kg) in a pancreatic cancer model [147]. Future research targeting the full scope of Zyflamend and its interactions with pathways such as ER stress, autophagy, and apoptosis could reveal critical insight into its full potential against pancreatic cancer. Finally, the pursuit to develop solutions to the enigmatic killer that is pancreatic cancer will not end. Through exploring the dynamic potential possessed by Zyflamend, science could begin to uncover novel therapies that alleviate side effects.

Research Objectives

Specific Aims

Pancreatic cancer is one of the most lethal malignancies. The overall prognosis and survival rate of a pancreatic cancer diagnosis are grim, and in the vast majority of cases, terminal. Current pharmacological therapies and treatments targeting pancreatic cancer have too often proven ineffective. These treatments typically include chemotherapeutic compounds such as gemcitabine, 5-fluorouracil, and cisplatin. These compounds could prove effective at higher doses, but this increases the risk for cytotoxicity and undesirable side effects [87]. Research on more effective therapies has illuminated new possibilities by exploring beyond the known pathology and treatments. One such promising avenue for research is uncovering the potential for combined and synergistic enhancement of known therapies [90, 91]. A growing body of literature endorses the potential value of several phytochemicals based on their anti-proliferative, anti-metastatic, anti-angiogenic and pro-apoptotic effects, which have been demonstrated both *in vitro* and *in vivo* using a plethora of experimental cancer models.

The objective and aim of the current proposal is to target pancreatic cancer with Zyflamend, an anti-inflammatory herbal compound that has proven effective in various *in vitro* and *in vivo* studies against cancers of the skin, mouth, bone, kidney, colon and pancreas and isolate the precise mechanisms of action [119, 122, 123, 125, 126]. The effectiveness of Zyflamend relates to the modification of a variety of cell signaling pathways, including, apoptosis, inflammation,

autophagy, and ER stress [119, 122, 123]. Furthermore, our preliminary data revealed that Zyflamend could initiate a cell death response through these same pathways in pancreatic cancer cell lines. However, can this be reproduced in an *in vivo* experimental model of pancreatic cancer and can the precise mechanism mediating Zyflamend's action on cancer be determined? We hypothesize that Zyflamend, in conjunction with chemotherapeutic compounds, could exhibit an additive or synergistic effect against pancreatic cancer, a concept that is backed by previous findings [122, 123]. It is our objective to assess the ability of Zyflamend to both target pancreatic cancer and enhance known therapies by using multiple, complementary approaches. This will include isolating the precise mechanisms of Zyflamend both with and without chemotherapeutic compounds cisplatin and gemcitabine. Based on our preliminary findings, we expect to demonstrate a synergistic mechanism through which Zyflamend can enhance the effectiveness of these compounds in pancreatic cancer.

Specific Aim 1: We will explore the *in vitro* effects of Zyflamend, both alone and in combination, with known chemotherapeutic compounds. We will utilize a well-established *in vitro* model that uses Min-6 pancreatic cancer cells [159]. Utilizing state-of-the-art pharmacological, imaging, and proteomic approaches, we expect to uncover how the treatments, both alone and in combination, induce Min-6 cell death. In addition, we will strive to use translatable human dose equivalences, based on the known cytotoxicity of gemcitabine and cisplatin, along with normal Zyflamend dosing.

Specific Aim 2: Illustrate the *in vivo* effects of Zyflamend, both alone and in combination, with known chemotherapeutic compounds on the initiation and progression of pancreatic cancer. We will examine the effects of treatments on the initiation of pancreatic cancer using experimental model such as the genetic: PDX-1-Cre; LSL-KrasG12D or the Pdx1-Cre; KrasG12D Smad4flox/flox models or chemically induced models (repeated induction of pancreatitis with choline-deficient diet combined with DL-ethionine and L-methionine after initiation with N-nitrosobis(2-oxopropyl) amine.

Specific Aim 3: Characterize the molecular mechanisms *in vitro*. We expect to dissect the precise mechanisms through which cell death was induced. Preliminary findings show that Zyflamend acts through modification of the JNK signaling pathway. Utilizing a

comprehensive approach, we will characterize the molecular mechanisms mediating the cellular actions of Zyflamend.

CHAPTER 2

RESEARCH PROJECT

Zyflamend Induces Apoptosis in Pancreatic Cancer cells via Modulation of the Jnk Pathway

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Abstract

Pancreatic cancer is the fourth leading cause of death in the United States with over 53,670 new cases and 80% mortality in 2017. The overall prognosis and survival rate of this diagnosis are grim, and in the vast majority of cases, terminal. Current pharmacological therapies and treatments targeting pancreatic cancer have proven ineffective far too often. Therefore, there is an urgent need for alternative therapeutic approaches. Zyflamend, an anti-inflammatory herbal compound, that has proven effective in various in-vitro and in-vivo cancer platforms, shows promise. However, its effects on pancreatic cancer in particular, remain largely unexplored. The objective of this study is to investigate the effects of Zyflamend on pancreatic cancer cell survival and decipher the underlying molecular mechanisms. Following treatment of pancreatic cancer cells with Zyflamend, key signaling pathways involved in the survival and proliferation were investigated. Our studies revealed that Zyflamend treatment resulted in a dose-dependent decrease in cell proliferation, consistent with an increase in apoptotic cell death and cell cycle arrest in the G2/M phase. Additionally, Zyflamend in conjunction with chemotherapeutic compounds exhibited an additive or synergistic effect against pancreatic cancer. At the molecular level, treatment with Zyflamend led to the activation of the Jnk pathway. Notably, pharmacological inhibition of Jnk abrogated the pro-apoptotic effects of Zyflamend. These studies identify Zyflamend as a potential novel approach to treat pancreatic cancer via modulation of the Jnk pathway.

Significance

Through investigating the actions of Zyflamend as an agent against pancreatic cancer, we discovered novel therapeutic potential of this polyherbal blend. These findings could help pioneer future advancements in our understanding of how phytochemicals and natural compounds could prove effective against pancreatic cancer through synergistic alterations of cancer cell's survival and proliferation. Furthermore, the evidence presented within, promotes Zyflamend as an adjuvant prospect, where it could enhance the effectiveness of standard practice therapies. In addition, we firmly believe that these novel findings have significant translational implications and will be of major interest to a wide spectrum of scientists.

Introduction

Pancreatic cancer remains one of the deadliest types of cancer in the United States with over 53,670 new cases and 80% mortality rate in 2017, ranking it as the fourth leading cause of death. Recent epidemiological studies predict that in 2019 pancreatic cancer will be the third leading cause of cancer-related death [11]. Although, advancements in science and health care have led to decreased mortality from numerous forms of cancer, pancreatic cancer survival rates have not improved significantly over the past several decades, leaving a desperate need for more effective treatment options [160]. The risk for developing pancreatic cancer has been associated with numerous biological, environmental, pathological, and genetic factors. These factors include variables such as, familial history [14], chronic pancreatitis [15], smoking [16], obesity [17], and diabetes [18]. In addition, familial hereditary factors and germline mutations could contribute to increased risk [161, 162]. The survival outcome is highly dependent upon the time of diagnosis.

Current pharmacological therapies and treatments targeting pancreatic cancer have too often proven in-effective [163]. These treatments typically include chemotherapeutic compounds such as gemcitabine, 5-fluorouracil, and cisplatin. These compounds show increased effectiveness at higher doses, but this directly exacerbates the risk for cytotoxicity and collateral side effects [87]. In addition, adjunct therapy through a combination of various treatment approaches such as, surgery and radiotherapy often compliment this choice. In pursuit of survival and improved quality of life, patients often seek to enhance the effectiveness of conventional therapies through dietary and supplemental means [164, 165].

One such promising avenue for research is uncovering the potential for combined and synergistic enhancement of known therapies [90, 91]. A growing body of literature endorses numerous phytochemicals based on their anti-tumorigenic and pro-apoptotic effects, as demonstrated in cell culture experiments as well as murine experimental cancer models [92-94]. Having shown potential to treat a myriad of diseases, scientists, medical professionals, and the public continue to seek clarification on the world of natural healing and prevention. Fortunately, progress has been made and scientists have begun investigating natural compounds such as; resveratrol [94], benzyl isothiocyanate [93], cannabidiol [92], and dandelion root extract [108], and they all show some level of promise against pancreatic cancer.

New Chapter (Brattleboro, VT) first launched Zyflamend based on the idea of combining ten different herbs to effectively reduce inflammation through Cox inhibition [166]. A large-

volume of research has emerged over the last two decades that supports Zyflamend's anti-inflammatory role. Research has revealed that Zyflamend acts as a Cox inhibitor in models such as; prostate [113], melanoma [114], and oral cancer models [115]. Individually, many of the extracted components of Zyflamend, have proven to exhibit anti-cancer activity [131, 140, 156, 167-169]. However, the high doses required to optimize effectiveness against cancer could prove infeasible for the majority [109-111]. In theory, the combined effects generated through integrating these unique and powerful herbs could grant superior benefit over their isolated form [112]. Additionally, Zyflamend has shown the capability to interact with a variety of integral cellular signaling pathways beyond Cox. These signaling pathways and mechanisms of interaction include; Ampk [116, 117], NF- κ B [118-120], mtorc1 [117], apoptosis, cell growth [113, 121], ER stress [119, 122, 123] and finally autophagy [114].

While these findings show that Zyflamend could exhibit profound potential in therapeutic application, more research is required to isolate the precise molecular mechanisms. In the current study, we investigated the effects of Zyflamend on the survival of Beta-TC-6 pancreatic insulinoma cells and deciphered the underlying molecular mechanisms.

Material and Methods

Chemicals and reagents. Media, sera and trypsin for cell culture were purchased from Gibco (Thermo Fisher Scientific, Waltham, MA). Primary antibodies and secondary antibodies were acquired from varying sources (Table 1). General caspases inhibitor (Z-VAD.fmk) was obtained from Calbiochem (La Jolla, CA). Zyflamend™ whole body was purchased from New Chapter (New Chapter, Inc. Brattleboro, VT). Zyflamend composition is indicated in **Fig. 1A**. Quality assurance is in full compliance with Good Manufacturing Practicing Standards (GMP) as mandated by 21 CFR Part 111. Additionally, full description and characterization of Zyflamend and its preparation has been described previously in detail [128]. Chemical reagents such as dithiothreitol (DTT), percoll, digitonin, phenylmethylsulfonyl fluoride (PMSF), protease inhibitors cocktail, sodium deoxycholate, Triton X-100, ethylene glycol-bis-(2-aminoethyl)-N,N,N',N'-tetraacetic acid (EGTA), sodium fluoride (NaF), Sodium phenylbutyrate (4-PBA), Hoechst 33258, propidium iodide, autophagy inhibitor 3-Methyladenine (3-MA), and Jnk inhibitor (SP600125) were acquired from Millipore-Sigma (Burlington, MA). Finally, Ampk inhibitor compound C, were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and Millipore-Sigma, respectively

Cell culture. Beta-TC-6 pancreatic insulinoma cells (ATCC® CRL-11506™) were cultured as monolayers in Eagle's modified Dulbecco medium plus L-glutamine (2mM), sodium pyruvate (1mM) and 15% foetal bovine serum (Gibco-Thermo Fisher Scientific). Cells were maintained in tissue culture plates (Thermo Fisher Scientific) at 37°C in a humidified atmosphere of 10% CO₂. Medium was replaced with fresh medium 24h before experiments.

Zyflamend treatment. Zyflamend was dissolved in DMSO at a concentration of 800 mg/ml. Cells were treated with Zyflamend at the indicated concentrations and for the indicated durations. Treatments were stopped by two washes with ice-cold phosphate buffer saline (PBS). Plates were then flash-frozen in liquid nitrogen and stored at -80°C until further analyses.

Western blotting analysis. Cells were lysed in radio-immunoprecipitation assay (RIPA) buffer as previously described [170]. Lysates were clarified by centrifugation at 15,000 g for 10 min, and protein concentrations determined using bicinchoninic acid assay kit (Pierce Chemical, Dallas, TX). Proteins (5-30 µg) were resolved by SDS-PAGE and transferred to PVDF membranes. Immunoblotting of lysates was performed with primary antibodies (Table 1), and after incubation with secondary antibodies, proteins were visualized using Luminata™ Western Chemiluminescent HRP Substrate (Millipore). Pixel intensities of immunoreactive bands were quantified using FluorChem Q Imaging software (Alpha Innotech Corp, San Leandro, CA). Data for phosphorylated proteins are presented as phosphorylation normalized to protein expression while total protein expression was normalized to β-actin as a loading control.

Morphological analysis of apoptosis. Beta-TC-6 cells were exposed to Zyflamend for the indicated duration then washed with PBS and labelled with Hoechst 33258 (50 µg/ml in PBS) (blue-green fluorescence) (Millipore-Sigma). Hoechst binds to condensed nuclear chromatin [170] and was used to visualize apoptotic cells (green fluorescence) by fluorescence microscopy (Leica DMI8, Leica Microsystems Inc. Buffalo Grove, IL). For each condition, at least 500 cells were counted. Percentages of apoptotic cells were calculated relative to total cells.

Annexin V staining. Annexin V staining was performed as previously described [171] with modification. Briefly, 50-60% confluent Beta-TC-6 cells (10⁶/ml) were exposed to Zyflamend for 24 hrs then washed with PBS and resuspended in 0.2ml of PBS containing 10% FBS. Equal volumes of the Guava Nexin reagents were added and cells were incubated for 10 min at room temperature in the dark. Cells (5,000) were then analyzed using the Guava® easyCyte Flow Cytometer (Millipore-Sigma). Intensities of fluorescence emitted by Annexin V- Fluorescein

Table 1: List of primary antibodies and conditions of use.

Antibodies	Source	Host	Dilution
Acc	Cell Signaling Technology	Rabbit	1:2,000
Ampk	Cell Signaling Technology	Rabbit	1:5,000
Atg5	Cell Signaling Technology	Rabbit	1:1,000
Atg7	Cell Signaling Technology	Rabbit	1:1,000
Beclin	Santa Cruz Biotechnology	Mouse	1:1,000
Chop	Santa Cruz Biotechnology	Mouse	1:5,000
c-Jun	Santa Cruz Biotechnology	Mouse	1:1,000
Cleaved caspase 3	Cell Signaling Technology	Rabbit	1:5,000
Cleaved Parp	Santa Cruz Biotechnology	Rabbit	1:1,000
eIF2 α	Santa Cruz Biotechnology	Mouse	1:1,000
Erk1/2	Cell Signaling Technology	Rabbit	1:5,000
Ikk	Cell Signaling Technology	Rabbit	1:2,500
Ire1	Santa Cruz Biotechnology	Rabbit	1:1,000
IkB α	Cell Signaling Technology	Rabbit	1:1,000
Jnk1/2	Santa Cruz Biotechnology	Mouse	1:1,000
Lc3-I/II	Bio-Rad	Rabbit	1:2,500
NF- κ Bp65	Cell Signaling Technology	Rabbit	1:5,000
P38	Santa Cruz Biotechnology	Mouse	1:1,000
Perk	Santa Cruz Biotechnology	Rabbit	1:1,000
Phospho- NF- κ Bp65 ^{S536}	Cell Signaling Technology	Rabbit	1:5,000
Phospho-Akt ^{S473}	Cell Signaling Technology	Rabbit	1:2,500
Phospho-Ampk ^{T172}	Cell Signaling Technology	Rabbit	1:2,500
Phospho-c-Jun ^{S63}	Santa Cruz Biotechnology	Mouse	1:1,000
Phospho-eIF2 α ^{S51}	Santa Cruz Biotechnology	Rabbit	1:1,000
Phospho-Erk1/2 ^{T202/Y204}	Cell Signaling Technology	Rabbit	1:5,000
Phospho-Ikk α ^{S178/180}	Cell Signaling Technology	Rabbit	1:2,500
Phospho-Ire1 ^{S724}	Abcam	Rabbit	1:10,000
Phospho-IkB α ^{S32}	Cell Signaling Technology	Rabbit	1:2,500
Phospho-Jnk1/2 ^{T183/Y185}	Santa Cruz Biotechnology	Mouse	1:1,000
Phospho-P38T ^{180/Y182}	Cell Signaling Technology	Rabbit	1:10,000
Phospho-Perk ^{T980}	Santa Cruz Biotechnology	Rabbit	1:1,000
sXbp1	Santa Cruz Biotechnology	Goat	1/500
β -Actin	Santa Cruz Biotechnology	Mouse	1:20,000

isothiocyanate (FITC) and 7-amino-actinomycin D (7-AAD) were collected on PM1 and PM2 channels, respectively. Both viable (negative for Annexin V and 7-AAD staining) and apoptotic cells (early apoptosis: positive for Annexin V, but negative for 7AAD staining, and late apoptosis positive for Annexin V and 7AAD) were quantified using the InCyte™ and GuavaSuite Software package (Luminex Corp.; Austin, TX). **Cell cycle analysis.** Cell cycle analysis was determined by examining the DNA content of cells stained with propidium iodide as previously described [172] with modification. Briefly, Beta-TC-6 cells were exposed to Zyflamend for the indicated duration then washed with PBS and fixed in 70% ethanol for 12 hrs at 4°C. Next, cells were washed twice with cold PBS and then incubated in RNase solution in PBS (100 U/ml) for 30 min at 37°C. Cells were then incubated in solution of propidium iodide solution (10 µg/ml in PBS) overnight at 4°C. Fluorescence was measured using the Guava® easyCyte flow cytometer and analyzed using the InCyte™ and GuavaSuite Software package.

Statistical analysis. Data were analyzed using JMP Pro 13.2 program (SAS Institute, NC) and presented as means $\pm$ standard error of the mean (SEM). Unpaired heteroscedastic two-tail Student's t test was used for all statistical analyses and differences were considered significant at $p < 0.05$ and highly significant at $p < 0.01$. Single symbol (such as *) corresponds to $p < 0.05$, while double (such as **) corresponds to $p < 0.01$.

Results

Zyflamend Decreases Cell Proliferation, Causes G2/M Cell-Cycle Arrest, and Induces Apoptotic Cell Death in Pancreatic Cancer Cells. We first examined the effects of varying doses of Zyflamend on the proliferation of pancreatic insulinoma Beta-TC-6 cells. Zyflamend caused a significant dose- and time-dependent decrease in cell growth (**Fig. 1-B**). Our study shows that a Zyflamend dose of 25 µg/ml is sufficient to inhibit cell proliferation by 58% after 36 hrs of treatment. A dose of 800 µg/ml was sufficient to completely abolish cell proliferation (**Fig. 1B**). In line with these findings, cell cycle analysis demonstrated that Zyflamend alters cell cycle distribution in a dose-dependent manner. Indeed, Zyflamend treatment resulted in the enrichment of the G2/M fraction with 2N DNA content, which was accompanied by a reduction in cell cycle progression through the G0/G1 and S phases (**Fig. 1C-D**). These results suggest that Zyflamend-induced inhibition of cell proliferation is mediated, at least in part, by cell cycle arrest in the G2/M phase.

In order to determine whether Zyflamend-induced inhibition of cell proliferation was associated with apoptotic cell death, we determined changes in apoptosis in the Beta-TC-6 cells treated with increasing doses of Zyflamend (0, 25, 50, 100, 200, 400, and 500 µg/ml) for 24 hrs using two approaches: the Guava Nexin Annexin V assay and Hoechst 33258 stain. Hoechst 33258 binds to condensed chromatin in the nucleus of apoptotic cells thus giving an assessment of overall

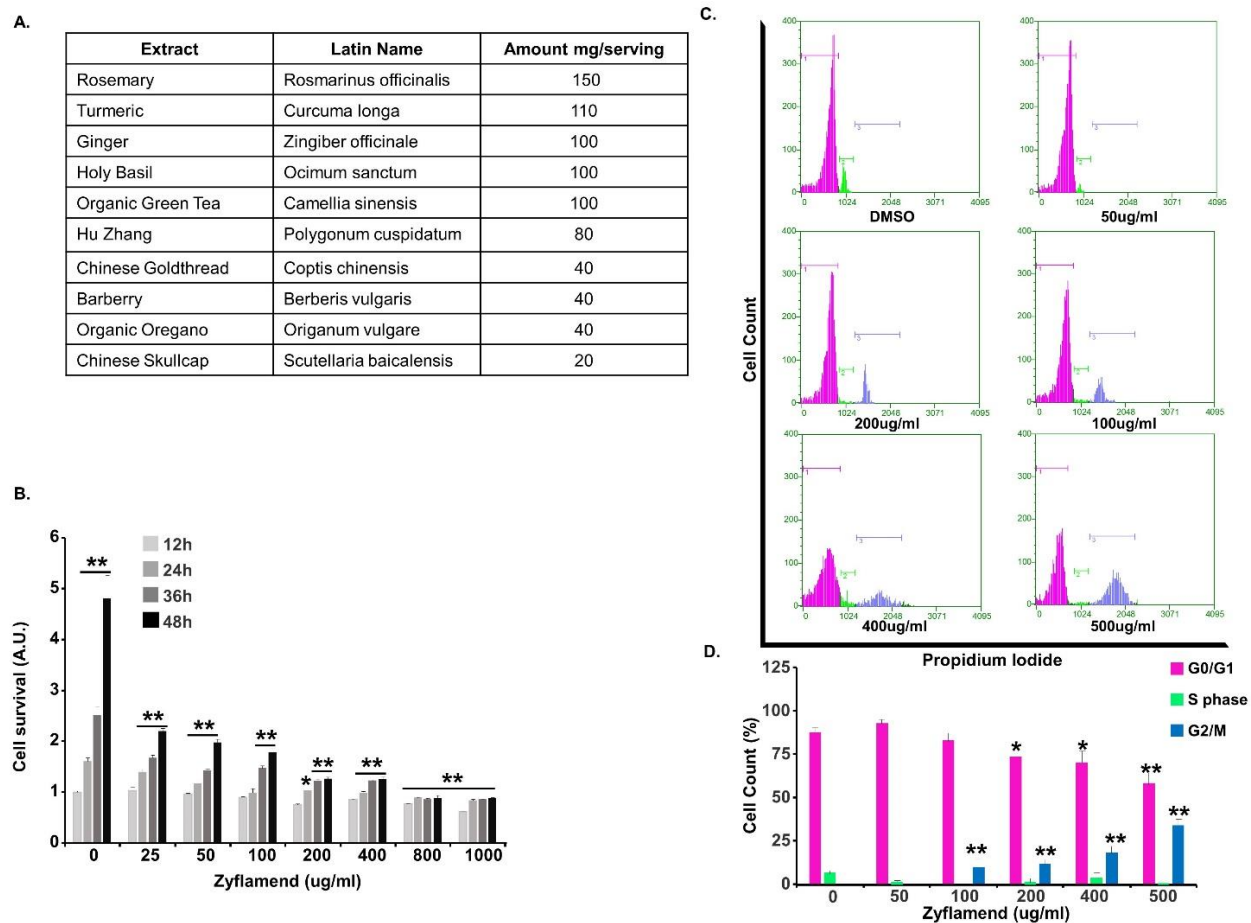
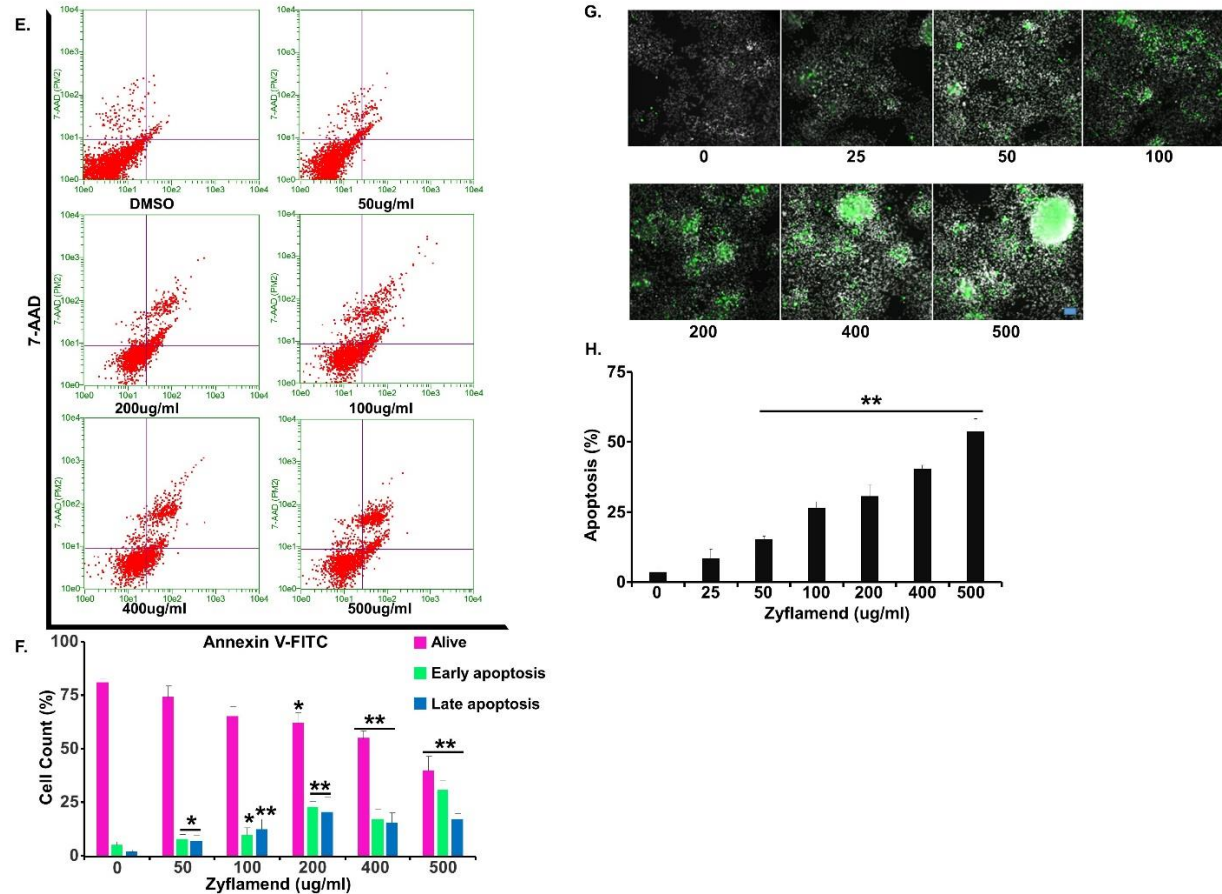


Figure 1. Zyflamend reduces cell survival while inducing cell death in a dose dependent manner. A) Phytochemical composition of Zyflamend. **B)** Effects of Zyflamend on cell survival and proliferation: cells were treated with increasing doses of Zyflamend for 24 hrs. Bar graphs represent the intensity of SRB staining reflective of the cell number and presented as means + SEM. **C-D)** Cell cycle analysis and assessment of DNA content in Beta-TC-6 treated with DMSO or the indicated concentration of Zyflamend. Representative histogram distributions for each treatment are shown. **D)** Bar graphs represent the percentages of cells in each phase of the cell cycle which were estimated using the GuavaSuite Software package and are presented as means + SEM from three independent experiments. * $p < 0.05$, ** $p < 0.01$ indicate significant difference the indicated concentration and control cells treated with the vehicle DMSO.



E-F) Zyflamend treatment induces apoptosis in Beta-TC-6: 50% confluent cells were treated with increasing concentrations of Zyflamend, and then labelled with Annexin V-FITC and 7-AAD. Representative dot plots are shown. Annexin V positive and 7-AAD negative cells (lower right quadrants) represent early stages of apoptosis, whereas cells that are positive for both Annexin V and 7-AAD (upper right quadrants) are in late stages of apoptosis. Bar graphs represent live, early, and late apoptotic cells are presented as means + SEM of at least three independent experiments. * $p < 0.05$, ** $p < 0.01$ indicate significant difference the indicated concentration and control cells treated with the vehicle DMSO. **G-H)** Chromatin condensation in cells treated with increasing doses of Zyflamend for 24 hrs. Representative images are shown. Scale bar: 50μm. **D)** Bar graphs represent the number of apoptotic cells (Hoechst positive) as means + SEM. of at least three independent experiments. * $p < 0.05$, ** $p < 0.01$ indicate a significant difference between untreated and Zyflamend treated cells.

apoptotic cell death [173]. Using the Annexin V assay, the percentages of both Annexin V positive /7-AAD negative cells, reflective of early apoptotic cells, and Annexin V positive /7-AAD positive cells, reflective of late apoptosis, exhibited a dose-dependent and significant increase in response to Zyflamend treatment (**Fig. 1E-F**). Consistent, with this observation, the number of Hoechst-positive cells were also higher in Zyflamend-treated cells compared to control cells (**Fig. 1G-H**). At a dose of 200, 400 and 500 $\mu\text{g/ml}$, the percentages of apoptotic cells were $30.44 \pm 4.07\%$, $40.30 \pm 1.30\%$, and $53.75 \pm 4.45\%$ respectively, further emphasizing the pro-apoptotic effects of Zyflamend on these cells.

A Human Equivalent Dose of Zyflamend Induces Apoptotic Cell Death in Beta-TC-6 Cells. In order to address changes in apoptotic cell levels we conducted a time course analysis utilizing a physiological relevant fixed dose of Zyflamend (200 $\mu\text{g/ml}$) [174]. This dose is representative of the maximum plasma concentration of curcumin (one of Zyflamend's constituents) that was reported in humans upon supplementation [175]. At this dose, a marked increase in apoptotic cell number was observed after 24 hrs of treatment (**Fig. 2A-B**). Subsequently, markers of apoptosis and cell survival were investigated using Western blotting. Zyflamend induced cleavage of caspase 3 and its downstream target Parp (**Fig. 2C-D**). Next, we examined changes in the mitogen-activated protein kinases (Map kinases) pathways in response to Zyflamend. This revealed that Beta-TC-6 cells treated with Zyflamend exhibited a marked decrease in Akt and Erk phosphorylation, particularly after 12 hrs of treatment.

To determine whether Zyflamend-induced inhibition of cell proliferation and cell death are associated with the caspase dependent pathways of apoptosis, we tested whether blocking caspases' activity using carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]- fluoromethylketone (Z-VAD-FMK) could inhibit Zyflamend-induced chromatin condensation and apoptosis (**Fig. 2E**). Z-VAD-FMK is a potent cell permeable pan-caspase inhibitor, which acts by irreversibly binding to the catalytic site of the caspase proteases, and thus inhibiting their activities. Our study shows that Z-VAD-FMK decreased levels of chromatin condensation in Zyflamend treated cells (**Fig. 2E-F**). Taken together, our findings indicate that Zyflamend treatment reduces cell viability and induces cell death through the activation of the apoptosis machinery.

Zyflamend Induces NF- κ B Pathway, ER Stress, Apoptosis and Autophagy Responses in Beta-TC-6 Cells. A plethora of intrinsic and extrinsic pathways can lead to apoptosis in response to stressors, including ER stress, autophagy, and inflammation among many more.

Accordingly, we examined the activation of key signaling molecules by Western blotting. Here we report that Zyflamend (200 $\mu\text{g/ml}$) significantly increased the activation of the NF- κB pathway over time (**Fig. 3A**). Additionally, we determined the effects of Zyflamend on ER stress through evaluation of the activation of ER transmembrane sensors Perk and Ire1 α , along with downstream targets such as eIF2 α and Chop by Western blotting. Zyflamend induced ER stress as evidenced by increased Perk (Thr⁹⁸⁰), eIF2 α (Ser⁵¹) and Ire1 α (Ser⁷²⁴) phosphorylation (**Fig. 3C**). Furthermore, the level of Chop expression was elevated, a direct downstream target of both the Perk and Ire1 pathways. The activation of Chop, a potent inducer of apoptotic cell death [176, 177] in response to ER stress (**Fig. 3C-D**) strengthen our findings on Zyflamend-induced apoptosis in these cells. Moreover, Zyflamend has been shown to activate Ampk, and our findings recapitulate these findings [117]. The Ampk signalling pathway has been shown to regulate autophagy and cell death [178]. Therefore, in order to assess whether Zyflamend induces autophagy in Beta-TC-6 cells we immunoblotted for autophagy related proteins. We observed a time dependent increase in Beclin, Lc3-I & II, Atg5, and Atg7 (**Fig.3E**) indicative of increased autophagy in these cells. Because ER stress, inflammation and autophagy can all lead to apoptosis, we used the pan-caspases inhibitor Z-VAD-FMK to determine which pathway might be responsible for the pro-apoptotic effects of Zyflamend. Our data shows that, while Z-VAD-FMK prevented the cleavage of caspase3 and its downstream target Parp, we observed no effect on Ampk, autophagy, or ER stress signaling (**Fig. 3G-H**). These findings confirm that these signaling pathways are upstream of the apoptotic signaling cascade and may be mediating the pro-apoptotic effects of Zyflamend in Beta-TC-6 cells.

Zyflamend-induced Cell Death is Mediated through the ER Stress/Jnk/Autophagy Pathway. The exact molecular mechanism(s) leading to apoptosis by Zyflamend in cancer cells has/have not been revealed yet, although recent studies have supported the role of Ampk in the regulation of cancer cell growth, bioenergetics, autophagy, and cell death. To investigate the potential role of Ampk in Zyflamend-induced apoptosis, we pre-treated cells with the Ampk inhibitor (compound C, 5 μM) 12 hrs prior to adding Zyflamend or DMSO (as a vehicle control) for an additional 24 hrs. The dose and duration of exposure were determined based on the ability of compound C to reverse the Ampk-dependent inhibitory phosphorylation of ACC (data not

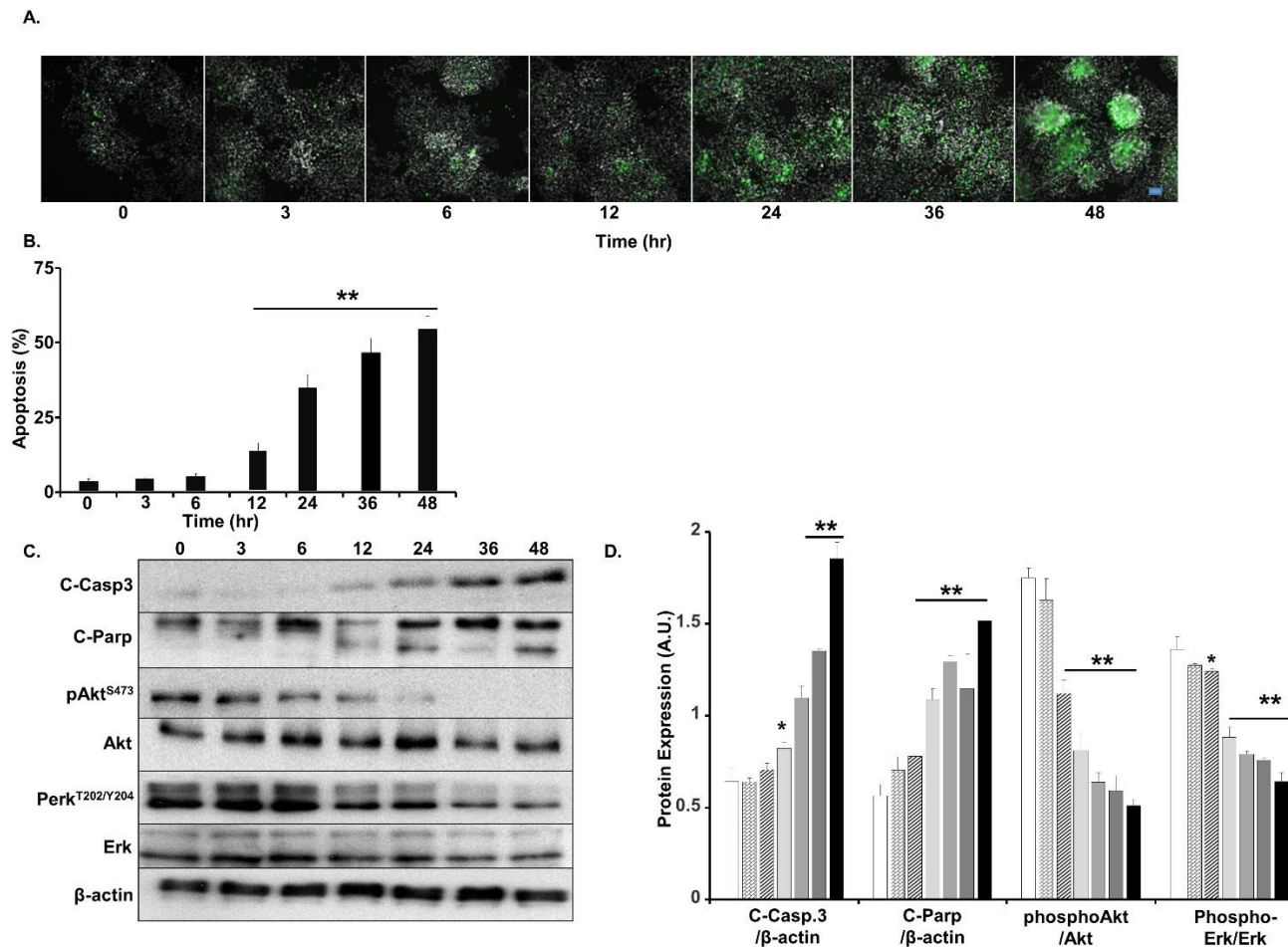
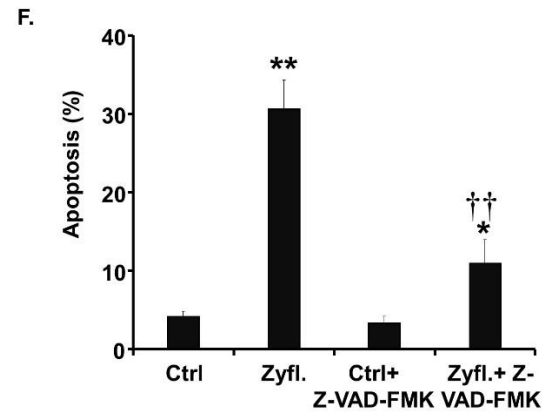
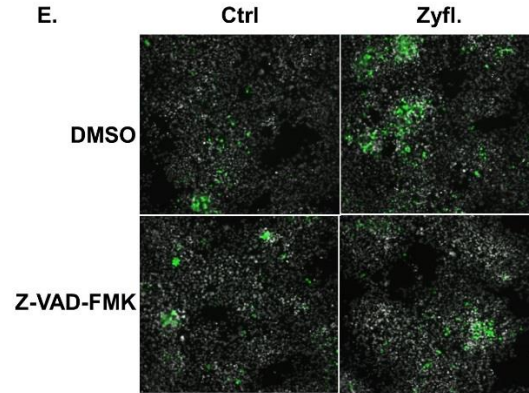


Figure 2. Zyflamend induces apoptotic cell death in Beta-TC-6 cells. **A)** Chromatin condensation in cells treated with 200 µg/ml Zyflamend for the indicated time. Representative images are shown. Scale bar: 50 µm. **B)** Bar graphs represent the number of apoptotic cells (Hoechst positive) as means + SEM. ** $p < 0.01$ indicates a significant difference between untreated and Zyflamend treated cells. **C)** Immunoblots of key proteins in cell survival and apoptosis markers in cells treated with 200 µg/ml of Zyflamend for the indicated time. **D)** Bar graphs represent cleaved caspase 3 (C-Casp.3)/β-Actin, cleaved Parp (C-Parp)/β-Actin, pAkt/Akt, and Perk/Erk, as means + SEM. * $p < 0.05$, ** $p < 0.01$ indicates a significant difference between untreated and Zyflamend treated cells.



E) Chromatin condensation in Beta-TC-6 cells treated with 200 $\mu\text{g/ml}$ Zyflamend with and without the pan-caspase inhibitor Z-VAD-FMK. Representative images are shown. Scale bar: 50 μm . **F)** Bar graphs represent the number of apoptotic cells (Hoechst positive) as means + SEM. * $p < 0.05$, ** $p < 0.01$ indicate a significant difference between untreated and Zyflamend treated cells. † $p < 0.05$, †† $p < 0.01$ indicate a significant difference between untreated and Z-VAD-FMK treated cells.

Cells were then examined for Ampk activation as well as activation of inflammation, ER stress, autophagy, and cell death (**Fig. 4A**). While the level of phosphorylated Ampk was reduced in the co-treated cells, compound C treatment had no effects on Zyflamend-induced Jnk phosphorylation, ER stress, autophagy, nor cell death (**Fig. 4A-B**) suggesting that Zyflamend-induced apoptosis in β -TC6 cells is independent of Ampk activation. Next, we sought to examine whether blocking autophagy using 3-MA could protect cells against Zyflamend-induced apoptosis. 3-MA inhibits autophagy by blocking autophagosome formation via the inhibition of class I and class III phosphatidylinositol 3-kinases (Pi3k) [179]. Cells were pre-incubated with 3-MA (5 nM) for 12 hrs prior to Zyflamend treatment. Co-treatment with Zyflamend and 3-MA significantly decreased the expression of Beclin, Lc3, and cleaved caspase-3 but had no effects on ER stress markers nor on Ampk phosphorylation (**Fig. 4C-D**). These data suggest that autophagy mediates Zyflamend-induced apoptosis and that both Ampk and ER stress activation by Zyflamend occur upstream of autophagy and apoptosis.

The relationship between these two fundamental processes; ER stress and autophagy, is complex and poorly understood. Recent literature demonstrates that both pathways display dual roles in cell survival in multiple cancer cell lines. Similar to ER stress, autophagy has been shown to promote cell survival by clearing unwanted components from the cells. Nonetheless, a considerable body of evidence also shows that both autophagy and ER stress can lead to apoptosis in tumor cells. In addition to this, a growing body of literature support a crosstalk between the two pathways [180-182]. It is yet unclear which pathway is upstream of which, although our data suggests that Zyflamend-induced autophagy is likely to be downstream of ER stress. To test this hypothesis, we pre-treated β -TC6 cells with the ER stress inhibitor 4-phenylbutyrate (4-PBA; 5 mM for 12 hrs) prior to Zyflamend treatment and examined changes in inflammation, ER stress, autophagy, and cell death (**Fig. 4E-F**). 4-PBA is a cell permeant chemical chaperone that has been shown to inhibit ER stress and ER stress-induced apoptosis in many cancer cell types including pancreatic cancer cells [183, 184]. Our findings show a profound decrease in ER stress, autophagy, and cell death markers in response to Zyflamend (**Fig. 4E-F**) when cells were pre-treated with 4-PBA. Additionally, 4-PBA treatment alleviated the decrease in cell proliferation (**Fig. 4G**) caused

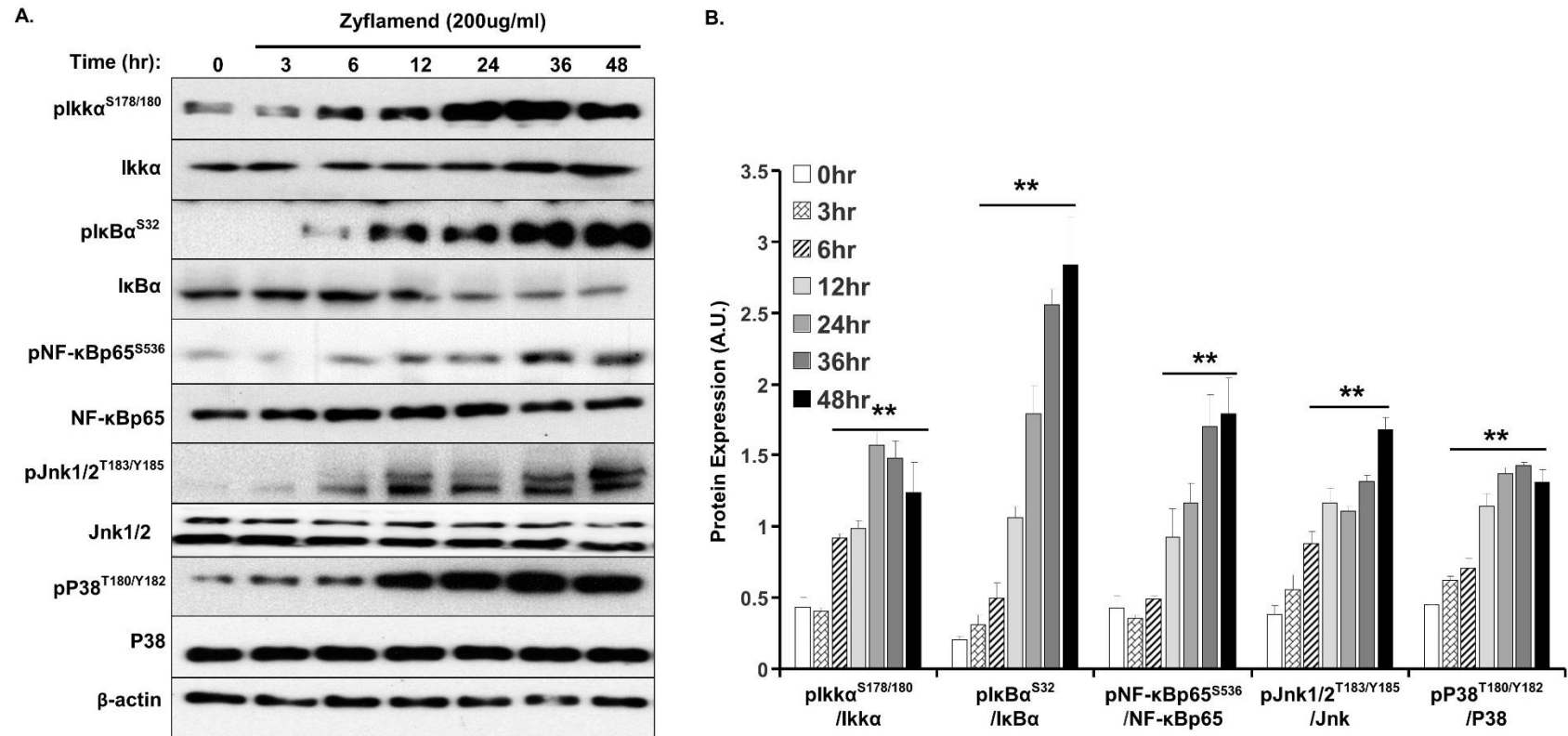
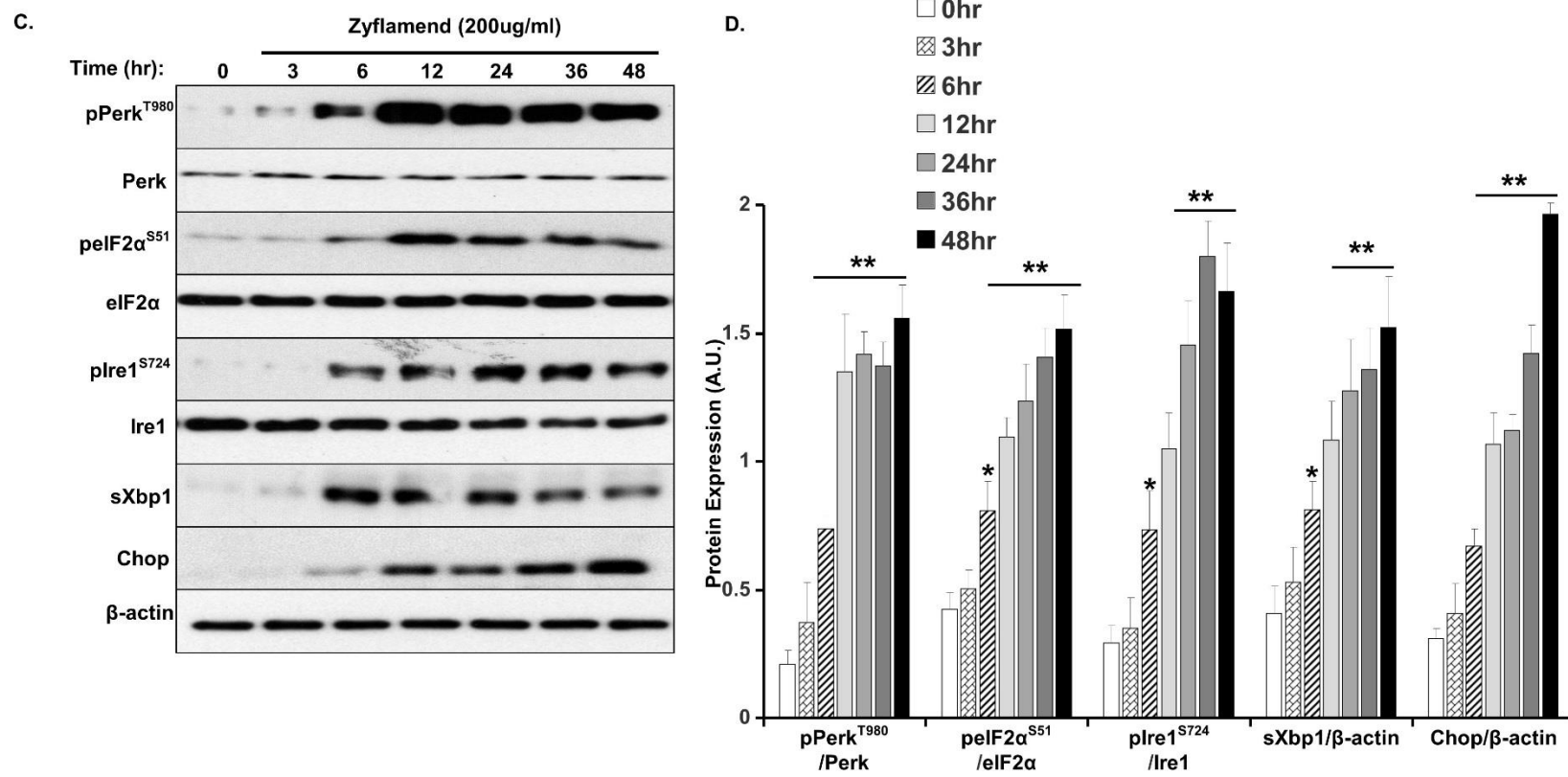
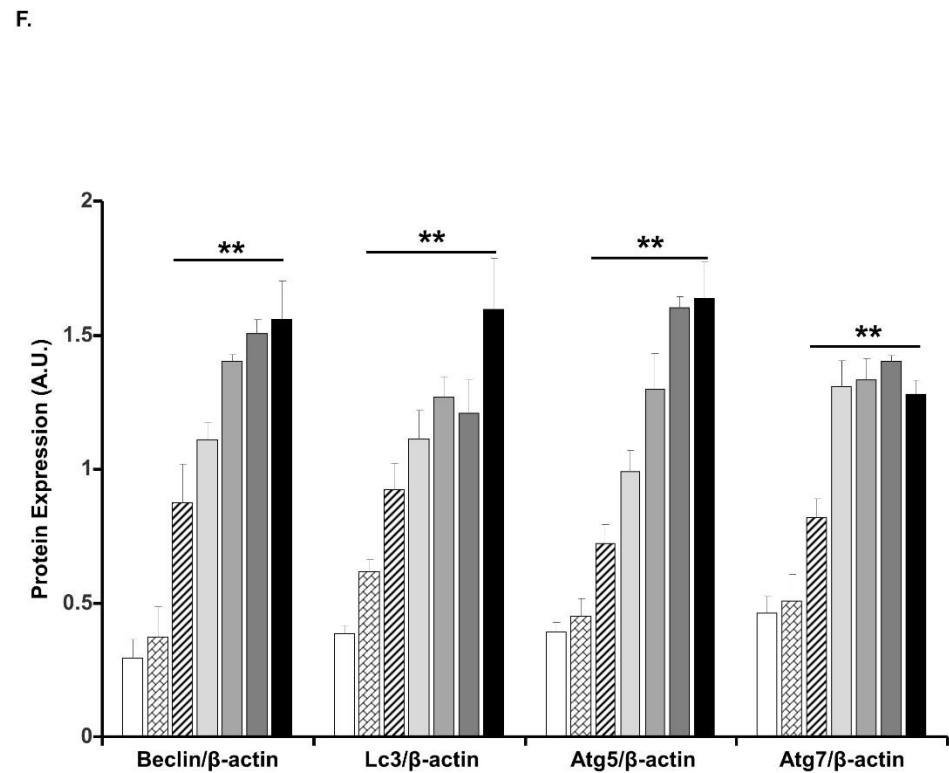
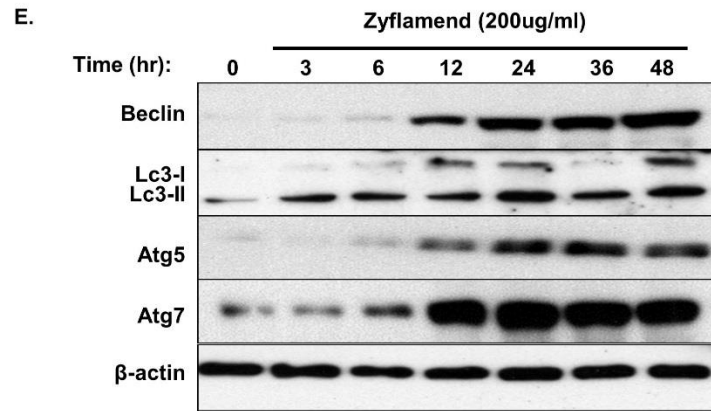


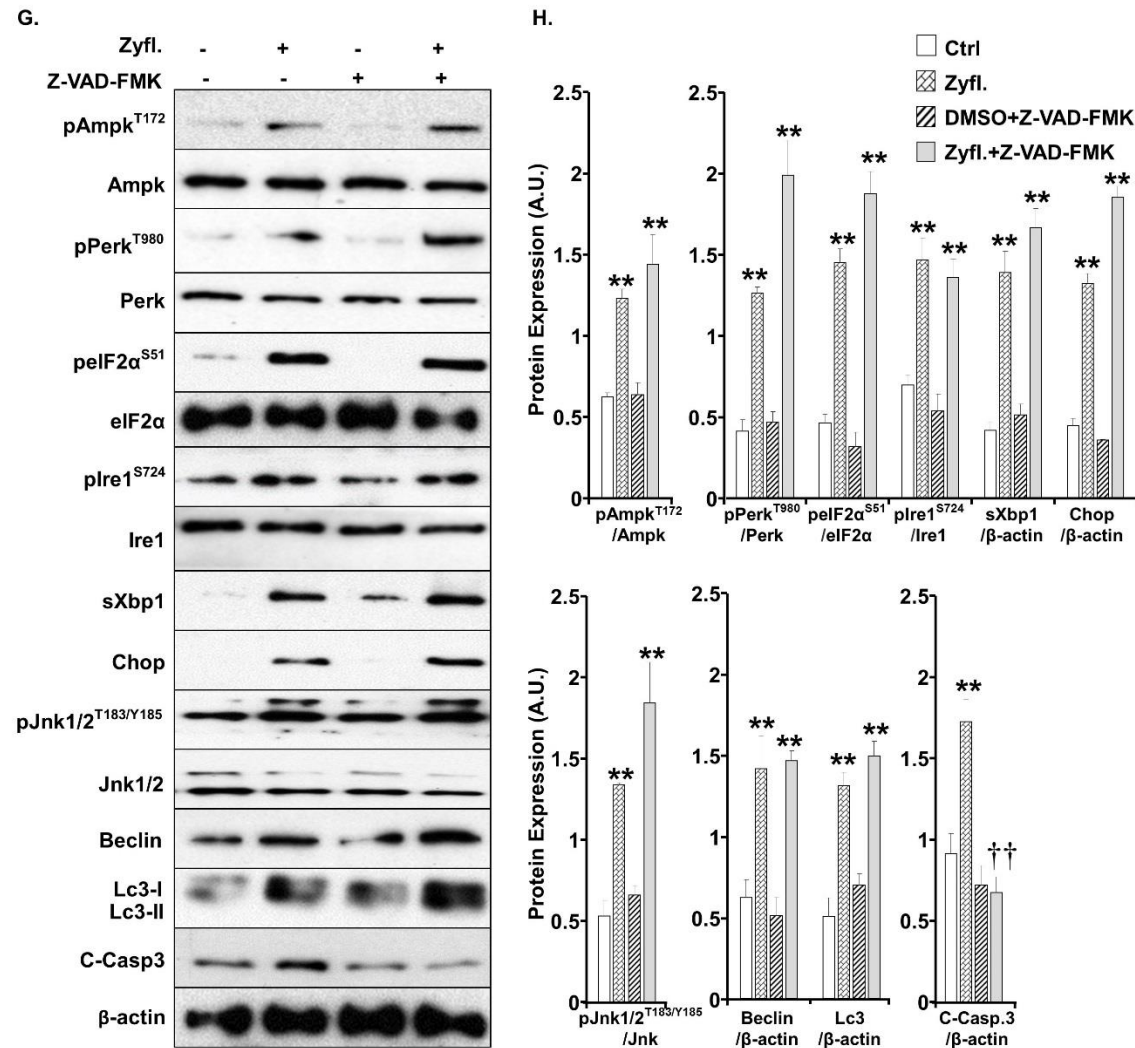
Figure 3. Zyflamend induces inflammatory, ER stress, and autophagy responses in Beta-TC-6 cells. **A)** Total cell lysates from control and Zyflamend treated cells for 3, 6, 12, 24, 36 and 48 hrs were immunoblotted for pIkk α , pI κ B, pNF- κ Bp65, pJnk, pp38, their respective unphosphorylated proteins, and β -actin as a loading control. Representative immunoblots are shown. **B)** Bar graphs represent pIkk/Ikk α , pI κ B/I κ B α , pNF- κ Bp65/NF- κ Bp65, pJnk/Jnk, and pp38/p38 as means + SEM. * $p < 0.05$, ** $p < 0.01$ indicate a significant difference between untreated and Zyflamend treated cells.



C) The same lysates were immunoblotted for pPerk, pEIF2α, pIre1α, their respective unphosphorylated proteins, sXbp1, Chop, and β-Actin as a loading control. Representative immunoblots are shown. **D)** Bar graphs represent pPerk/Perk, pEIF2α/eIF2α, pIre1/Ire1, sXbp1/β-Actin, and Chop/β-Actin as means + SEM. *p<0.05, **p<0.01 indicate a significant difference between untreated and Zyflamend treated cells.



E) Markers of autophagy were examined in the same lysates using antibodies against Beclin, Lc3, I&II, Atg5, Atg7, and β -Actin as a loading control. **F)** Bar graphs represent Beclin/ β -Actin, Lc3/ β -Actin, Atg5/ β -Actin, and Atg7/ β -Actin as means + SEM. * $p < 0.05$, ** $p < 0.01$ indicate a significant difference between untreated and Zyflamend treated cells.



G) Immunoblots of key proteins in autophagy, Ampk, ER stress, and apoptosis signaling in Beta-TC-6 cells treated with 200μg/ml Zyflamend with and without the pan-caspase inhibitor Z-VAD-FMK. Representative immunoblots are shown. **H)** Bar graphs represent pAmpk/Ampk, pPerk/Perk, pEIF2α/EIF2α, pIre1α /Ire1α, sXbp1/β-Actin, Chop/β-Actin, pJnk/Jnk, Beclin/β-Actin, Lc3I&II/β-Actin, and cleaved caspase3/β-Actin as means + SEM. *p<0.05, **p<0.01 indicate a significant difference between untreated and Zyflamend treated cells. †p<0.05, ††p<0.01 indicate a significant difference between untreated and Z-VAD-FMK treated cells.

by Zyflamend and reduced Zyflamend-induced chromatin condensation (**Fig. 4H-I**), but had no effects on Ampk activation by Zyflamend treatment. Taken together, these data suggest that the ER stress pathway occurs upstream of autophagy and apoptotic cell death.

Previous studies have shown that the ER stress sensor Ire1 may promote autophagy through the Traf2/Ask1/Jnk pathway [185, 186]. To test this hypothesis, we treated β -TC6 cells with SP600125, a selective Jnk inhibitor and changes in inflammation, ER stress and proliferation in response to Zyflamend treatment were investigated (**Fig. 4J-N**). As expected, cells pre-treated with SP600125 exhibited a significant reduction in the phosphorylation of Jnk and a reduced expression of autophagy and cell death markers in response to Zyflamend (**Fig. 4J-K**). Likewise, Jnk inhibition protected β -TC6 cells from Zyflamend-induced reduction in cell survival (**Fig. 4L**) and chromatin condensation (**Fig. 4M-N**). However, pre-treatment with SP600125 did not alter Zyflamend-induced activation of Ampk or ER stress. Altogether, the above findings suggest that Zyflamend is capable of reducing survival and inducing apoptosis in the β -TC6 murine insulinoma cells through a complex mechanism involving ER stress-mediated activation of Jnk, which in turn induces autophagy followed by the activation of apoptosis.

Zyflamend Enhances the Effects of Cisplatin and Gemcitabine. In order to test the ability of Zyflamend to enhance the effectiveness of chemotherapeutics used to treat pancreatic cancer, we tested and compared the combined effects of Zyflamend treatment with cisplatin or gemcitabine for 12 hrs. Total cell lysates were then immunoblotted for various markers of inflammation, ER stress, autophagy, and cell death. The combined treatment led to significantly enhanced activation and phosphorylation of $\text{I}\kappa\kappa$, $\text{I}\kappa\text{B}\alpha$, and NF- κB . In addition, both Jnk and p38 showed significantly increased phosphorylation in response to the combined treatment, over cisplatin, gemcitabine, or Zyflamend alone (**Fig. 5A-B**). Similarly, there was a significant increase in ER stress signalling pathway activation including; increased phosphorylation of both Perk and its downstream target EIF2 α , along with increased phosphorylation of Ire1 and the splicing of its downstream target Xbp1. Furthermore, the expression Chop was significantly higher in cells co-treated with Zyflamend and cisplatin or gemcitabine (**Fig. 5C-D**). Likewise, Zyflamend enhanced cisplatin and gemcitabine-induced expression of autophagy (beclin, Lc3-I & II, Atg5, and Atg7) and apoptosis (cleaved caspase 3) markers (**Fig. 5E-F**). Overall, our study revealed that Zyflamend treatment leads to either synergistic or additive effects when combined with cisplatin or gemcitabine.

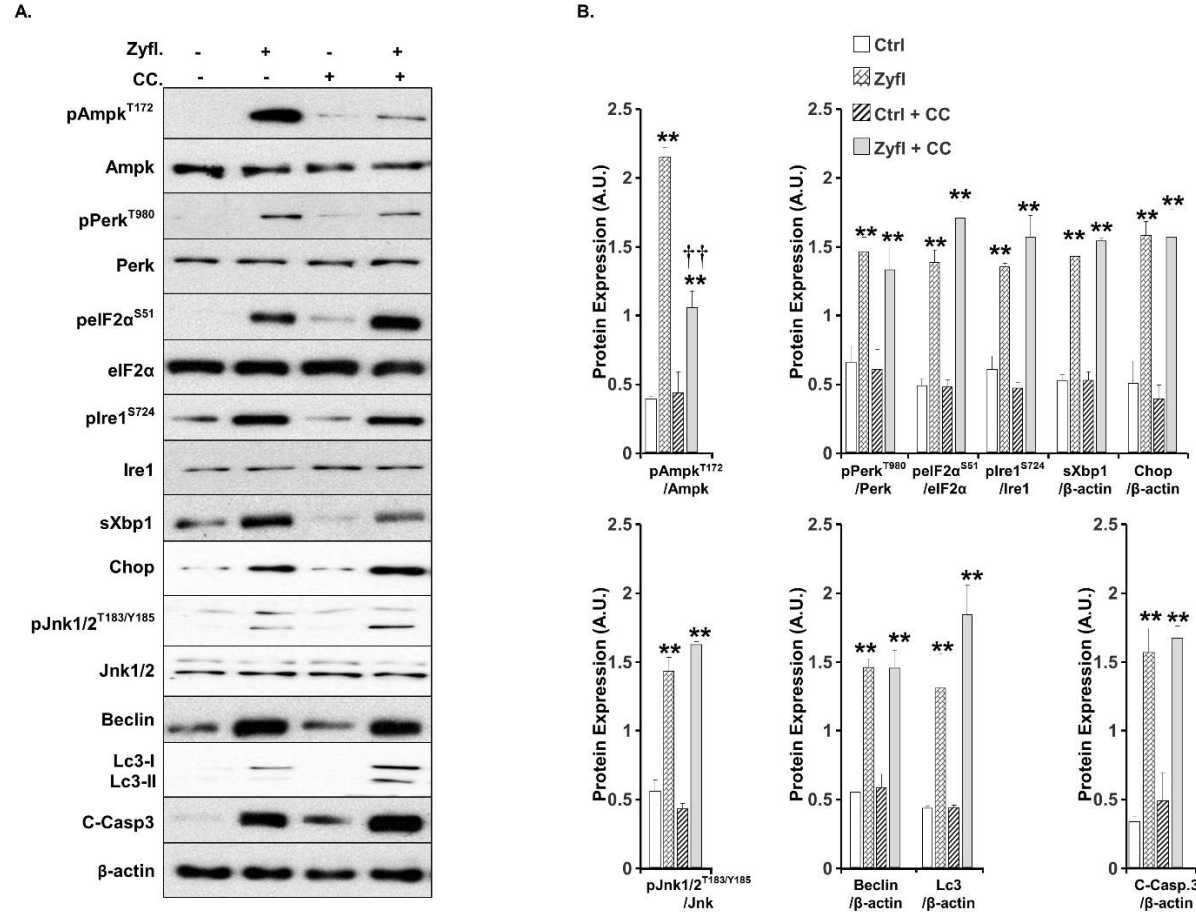
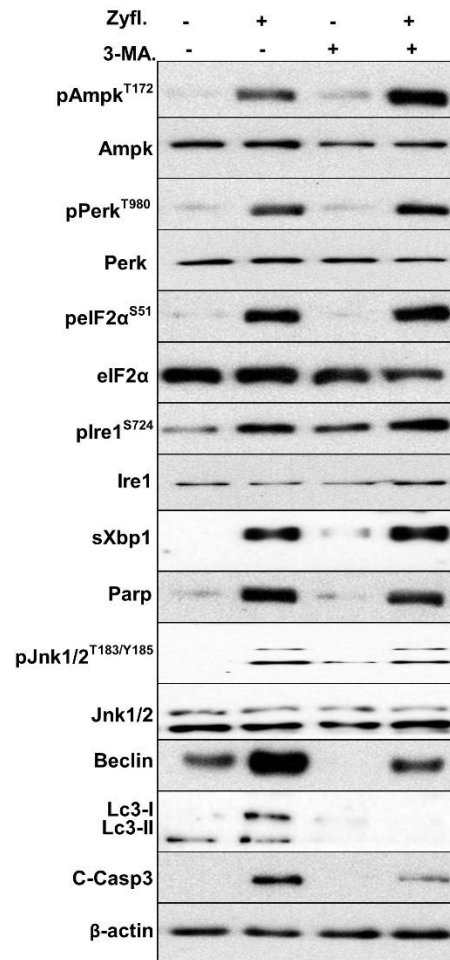
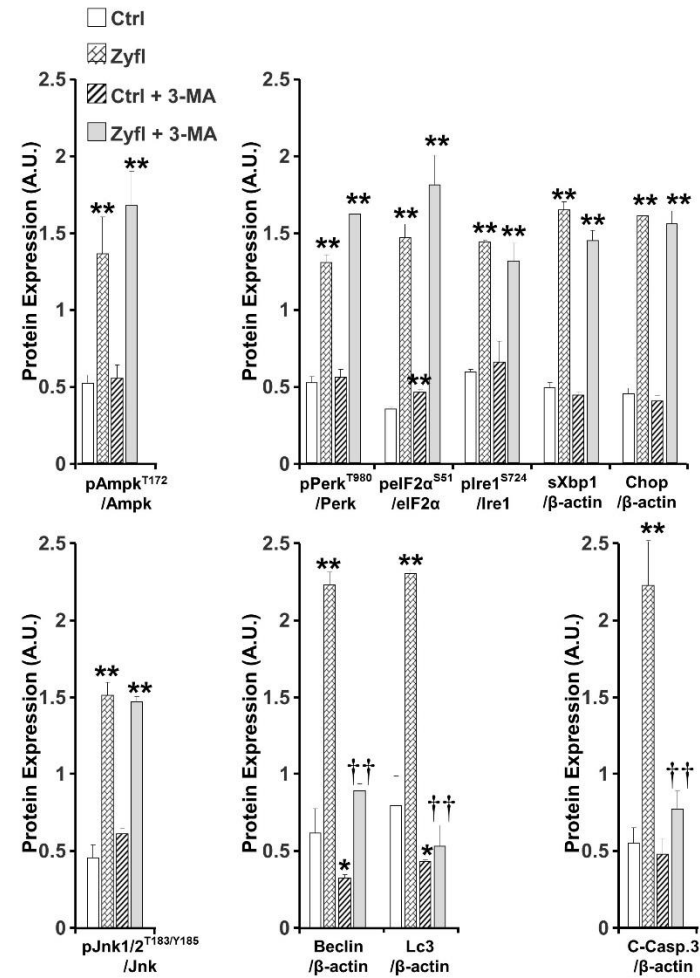


Figure 4. Zyflamend-induced cell death is mediated through the ER stress/Jnk/autophagy pathways. Total cell lysates from control untreated and Zyflamend treated cells with or without (A) Ampk inhibitor (Compound C: CC), (A-B), autophagy inhibitor (3-MA) (C-F) ER stress inhibitor (4-PBA) or (G-J) Jnk inhibitor (SP600125) were immunoblotted for markers of inflammation, ER stress, autophagy, and cell death. Cells were pre-treated with the indicated inhibitors 12 hrs prior adding Zyflamend or DMSO (as a vehicle control) for an additional 24 hrs. A-C, G) Bar graphs represent pAmpk/Amk, pPerk/Perk, pEIF2α/eIF2α, pIre1α/Ire1α, sXbp1/β-Actin, Chop/β-Actin, pJnk/Jnk, Beclin/β-Actin, Lc3II&II/β-Actin, and cleaved caspase3/β-Actin as means + SEM. ** $p < 0.01$ indicates

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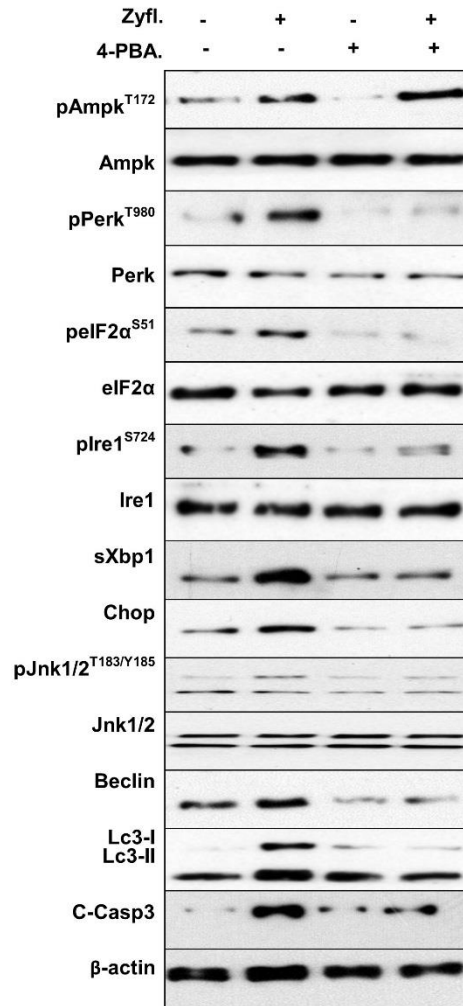


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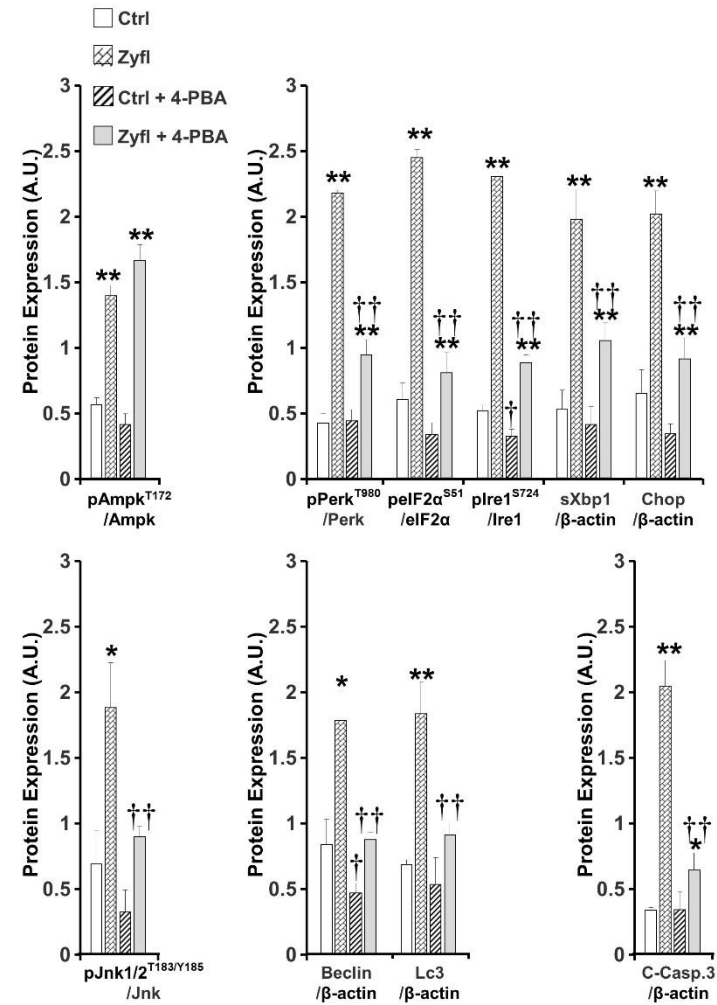


a significant difference between untreated and Zylflamend treated cells. * $p<0.05$, ** $p<0.01$ indicate a significant difference between untreated and Zylflamend treated cells. † $p<0.05$, †† $p<0.01$ indicate a significant difference between untreated and cells treated with the indicated inhibitor (BML275, DBeQ, SP600125, or 4-PBA). C) Total cell lysates from control untreated and Zylflamend treated cells with or without the ER stress inhibitor (4-PBA; Sodium 4-phenylbutyrate) were immunoblotted for markers of inflammation, ER stress,

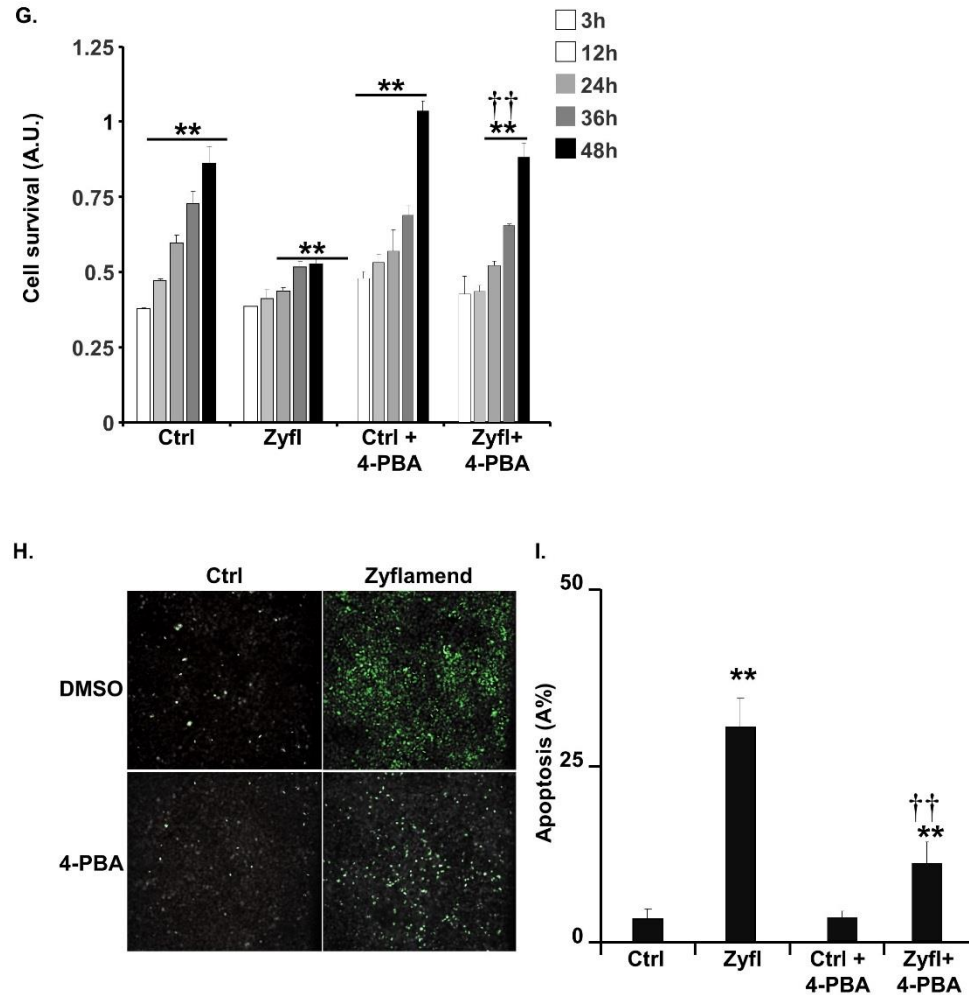
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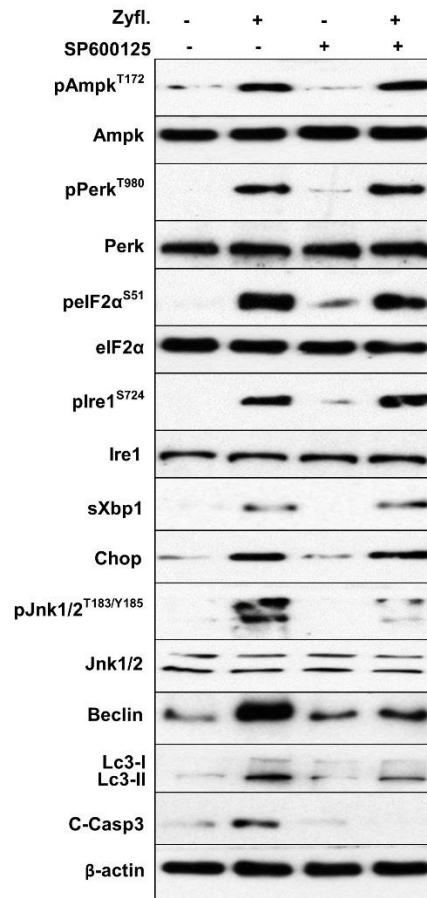


autophagy, and cell death. Bar graphs represent pAmpk/Ampk, pPerk/Perk, pEIF2α/EIF2α, pIre1α/Ire1α, sXbp1/β-Actin, Chop/β-Actin, pJnk/Jnk, Beclin/β-Actin, Lc3II&II/β-Actin, and cleaved caspase3/β-Actin as means + SEM. * $p < 0.05$, ** $p < 0.01$ indicate a significant difference between untreated and Zyflamend treated cells. † $p < 0.05$, †† $p < 0.01$ indicate a significant difference between untreated and

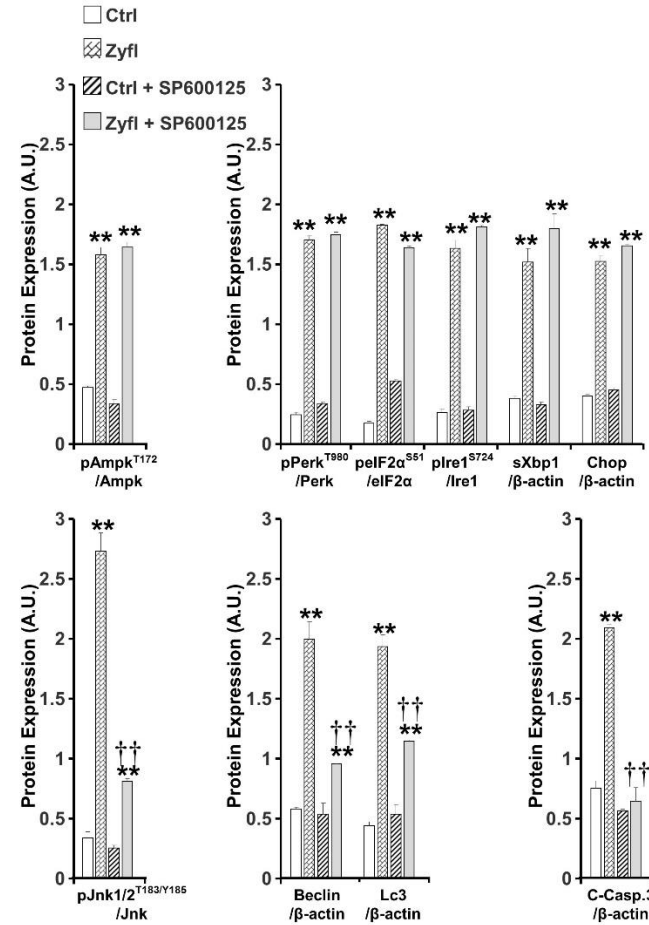


cells treated with 4-PBA. **D)** Cells were treated with 200 $\mu\text{g/ml}$ of Zyflamend for 24 hrs with or without 4-PBA. Bar graphs represent the intensity of SRB staining reflective of the cell number and presented as means + SEM from at least three independent experiments. * $p < 0.05$, ** $p < 0.01$ indicate a significant difference between untreated and Zyflamend treated cells. † $p < 0.05$, †† $p < 0.01$ indicate a significant difference between untreated and cells treated with 4-PBA. **E)** Chromatin Condensation in cells treated with 200 $\mu\text{g/ml}$ of

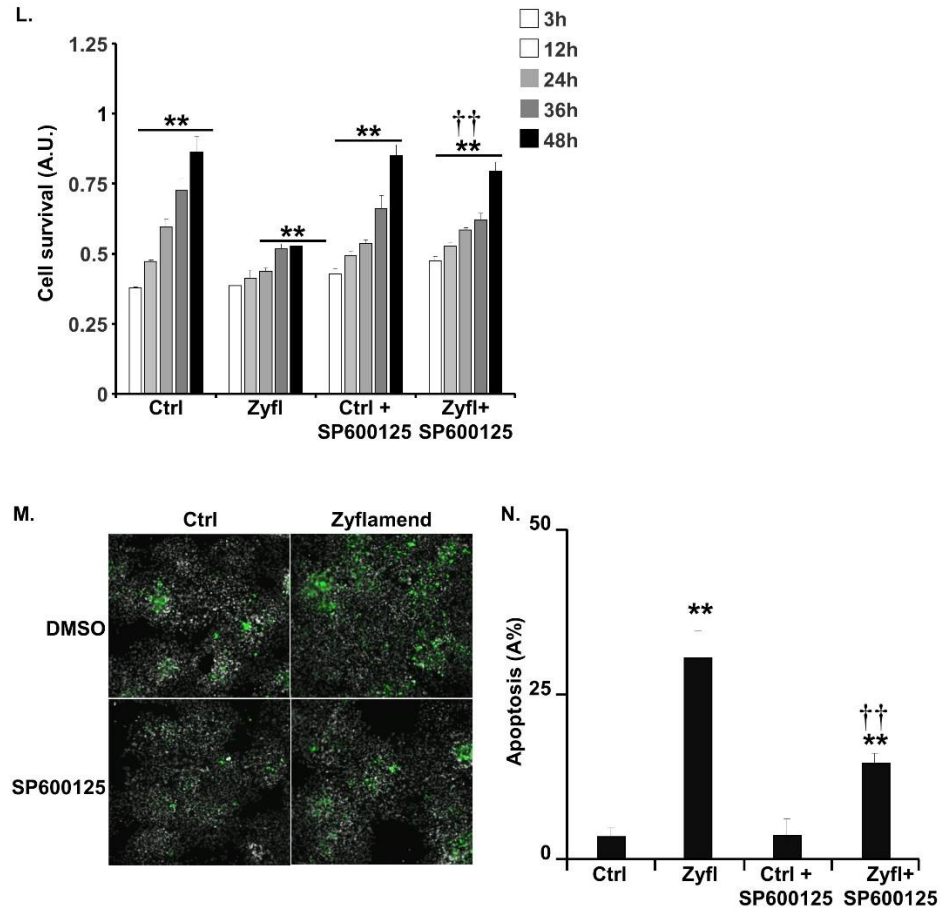
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K.



Zyflamend for 24 hrs with or without 4-PBA. Representative images are shown. Scale bar: 50μm. **F)** Bar graphs represent the number of apoptotic cells (Hoechst positive) as means + SEM from at least three independent experiments. ** $p < 0.01$ indicates a significant difference between untreated and Zyflamend treated cells. †† $p < 0.01$ indicates a significant difference between untreated and cells treated with 4-PBA. **G)** Cells were treated with 200μg/ml of Zyflamend for 24 hrs with or without SP600125. Bar graphs represent pAmpk/Ampk, pPerk/Perk, pEIF2α/eIF2α, pIre1α/Ire1α, sXbp1/β-Actin, Chop/β-Actin, pJnk/Jnk, Beclin/β-Actin, Lc3II&II/β-Actin, and cleaved caspase3/β-Actin as means + SEM. * $p < 0.05$, ** $p < 0.01$ indicate a significant difference between untreated and Zyflamend



treated cells. $\dagger p < 0.05$, $\dagger\dagger p < 0.01$ indicate a significant difference between untreated and cells treated with SP600125. **H)** Cells were treated with 200 $\mu\text{g/ml}$ of Zyflamend for 24 hrs with or without SP600125. Bar graphs represent the intensity of SRB staining reflective of the cell number and presented as means + SEM from at least three independent experiments. $*p < 0.05$, $**p < 0.01$ indicate a significant difference between untreated and Zyflamend treated cells. $\dagger p < 0.05$, $\dagger\dagger p < 0.01$ indicate a significant difference between untreated and cells treated with SP600125. **I)** Chromatin Condensation in cells treated with 200 $\mu\text{g/ml}$ of Zyflamend for 24 hrs. Representative images are shown. Scale bar: 50 μm . **F)** Bar graphs represent the number of apoptotic cells (Hoechst positive) as means + SEM from at least three independent experiments. $**p < 0.01$ indicates a significant difference between untreated and Zyflamend treated cells. $\dagger\dagger p < 0.01$ indicates a significant difference between untreated and cells treated with SP600125.

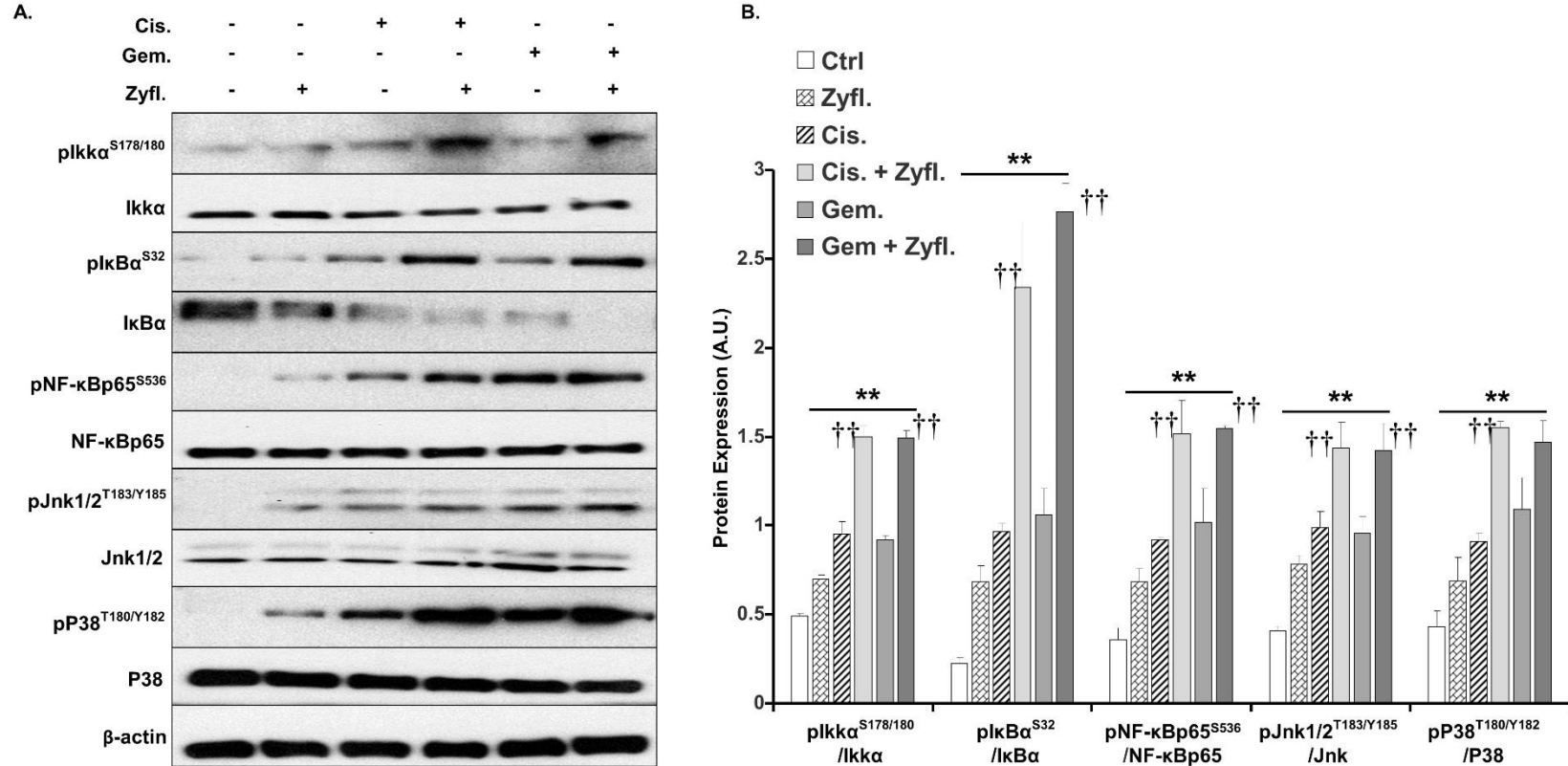
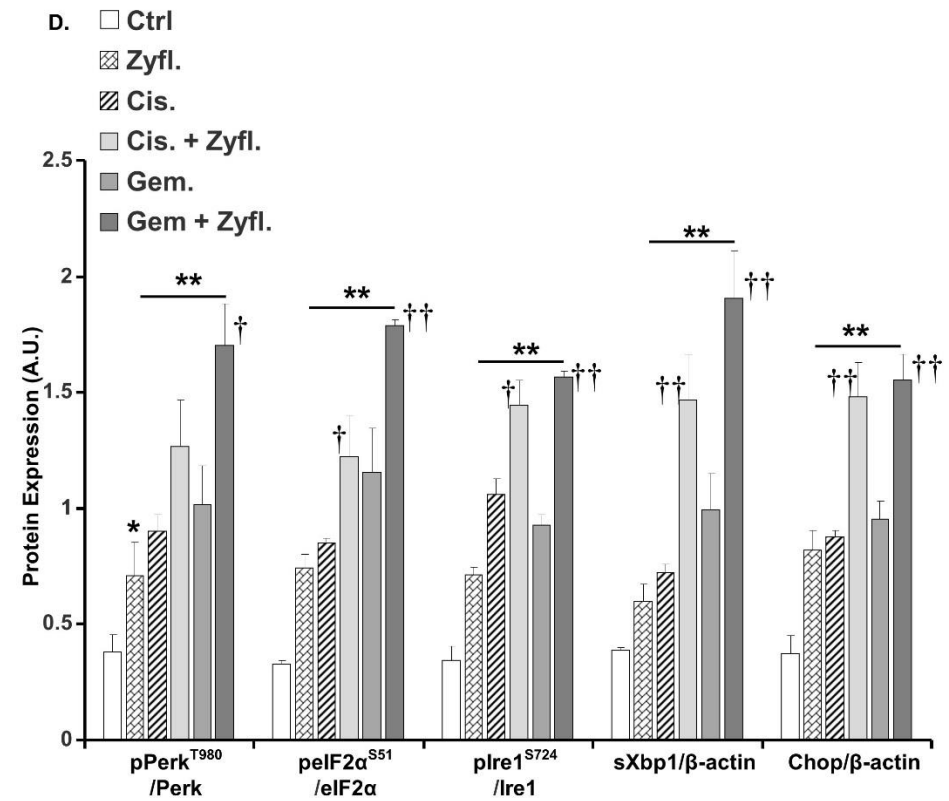
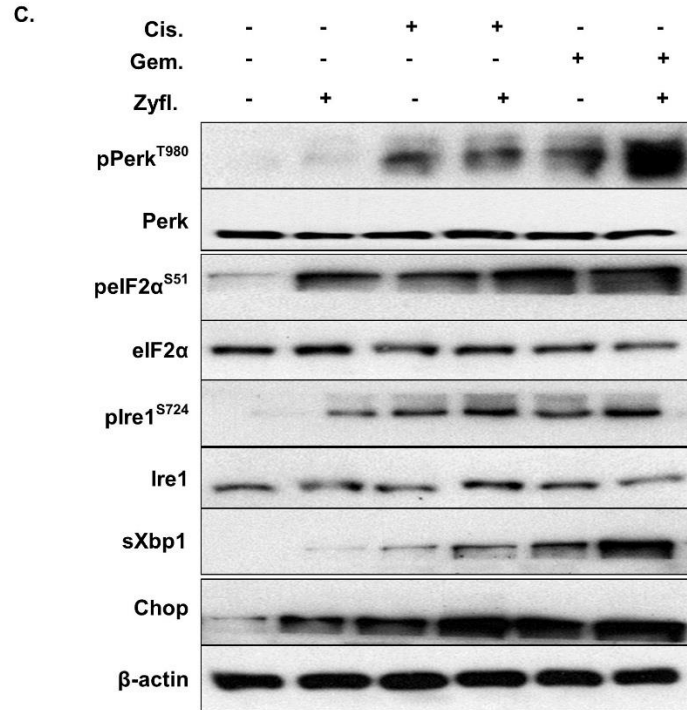
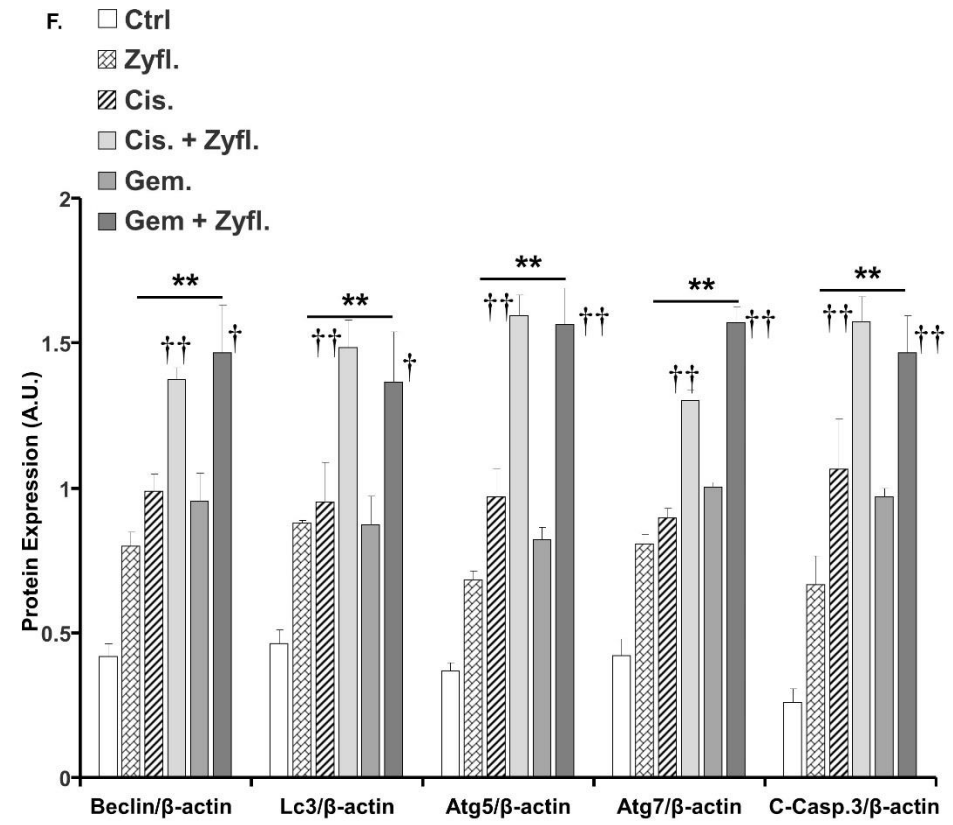
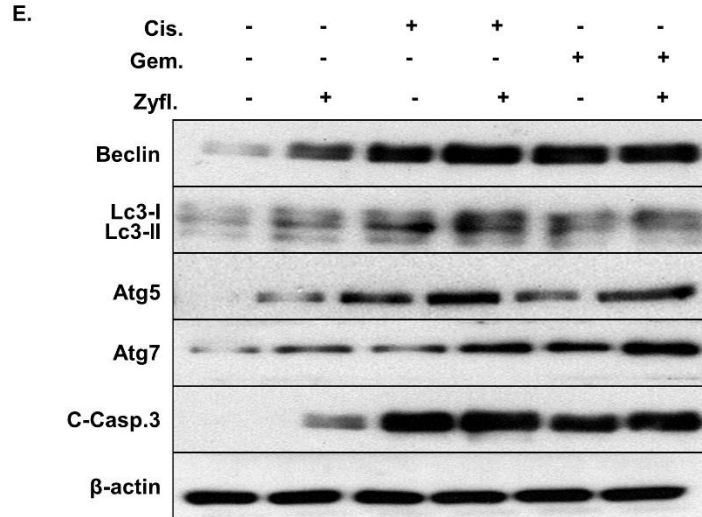


Figure 5. Zyflamend exacerbates the effects of cisplatin and gemcitabine on Zyflamend-induced inflammatory, ER stress, and autophagy in Beta-TC-6 cells. Total cell lysates from control untreated and Zyflamend treated cells with or without cisplatin or gemcitabine for 12 hrs were immunoblotted for markers of (A-B) inflammation, (C-D) ER stress, (E-F) autophagy, and cell death. Representative immunoblots are shown. **B)** Bar graphs represent pIkk α /Ikk α , pIkkB α /IkkB α , pNF-κBp65/NF-κBp65, pJnk/Jnk, and pp38/p38 as means + SEM. *p<0.05, **p<0.01 indicate a significant difference between untreated and Zyflamend treated cells. **D)** Bar graphs represent pPerk/Perk, pEIF2 α /EIF2 α , pIre1/Ire1, sXbp1/β-Actin, and Chop/β-Actin as means + SEM. *p<0.05, **p<0.01 indicate



a significant difference between untreated and Zyflamend treated cells. F) Bar graphs represent Beclin/β-Actin, Lc3/β-Actin, Atg5/β-Actin, Atg7/β-Actin, and cleaved caspase 3/ β-Actin as means + SEM. *p<0.05, **p<0.01 indicate a significant difference between



untreated and Zyflamend treated cells. † $p < 0.05$, †† $p < 0.01$ indicate a significant difference between cells treated with Zyflamend combined with the chemotherapeutic agents (cisplatin or gemcitabine) and cells treated only with cisplatin or gemcitabine only.

Discussion

Pancreatic cancer is one of the most untreatable forms of malignancy. The survival rates for pancreatic cancer diagnosis are abysmal, leaving patients hopeless. The success of current therapies has been extremely limited, and in many cases, completely ineffective. In addition, these therapies often rely on invasive procedures and high doses that can lead to cytotoxicity and collateral side effects. The need for innovative solutions to these problems has never been higher and the search for more effective therapies has illuminated new possibilities and continues to explore beyond the known pathology and treatments. One such promising avenue for research is uncovering the potential for combined and synergistic enhancement of known therapies [90, 91] through the use of phytochemicals such as Zyflamend. Emerging evidence supports the anti-tumor and pro-apoptotic effects of Zyflamend on various types of cancers such as prostate [113], melanoma [114], and oral cancer models [115]. In this study, we uncover novel insight into the molecular mechanisms through which Zyflamend could prove beneficial both alone and as an adjunct therapy in targeting pancreatic cancer. We demonstrate that Zyflamend is capable of inducing cell death and reducing cell survival in a time and dose dependent manner. Furthermore, we demonstrate that Zyflamend induces ER stress, that in turn activates the Jnk pathway leading to autophagy and caspase mediated cell death. Additionally, Zyflamend exacerbates the effects cisplatin and gemcitabine on cell death and survival, two chemotherapeutic compounds commonly used to treat pancreatic cancer. Taken together, these findings supports the anti-cancer potential of Zyflamend.

Our findings are in line with other studies supporting the anti-cancer properties of Zyflamend in a wide variety of cancer research platforms [113-115, 118-120, 124]. Research has revealed that the individual extracts and constituents contained within Zyflamend could possess therapeutic potential and value as backed by a large of number of investigations. For instance, curcumin, a polyphenol found in turmeric, has shown promise in a wide variety of cancer research [131-133]. Curcumin could inhibit carcinogenesis through a variety of proposed pathways and processes including; angiogenesis and tumorigenesis inhibition [134] and disruption of the mtor-raptor complex and subsequently decreased phosphorylation and activation of Akt/mtor signaling [135, 136]. In addition, curcumin can lead to the inactivation of the Pi3k/Akt pathway and apoptosis induction through increased mitochondrial p53 and Bax translocation [137], and can regulate autophagy turnover in an Ampk/Ulk1 dependent manner [133]. Furthermore, curcumin

can induce cell cycle arrest and apoptosis supporting its potential as an adjuvant therapy through increasing the sensitivity to gemcitabine treatment in pancreatic cancer tumors resistant to chemotherapy [132].

Similarly, ginger (another component of Zyflamend), has been evaluated for its anticancer properties [139, 140]. Ginger and its derivatives have been shown to modulate major signaling pathways important for cancer survival and proliferation of numerous cancer models including the NF- κ B, Pi3k/Akt/mTOR, inflammation, cell cycle, angiogenesis, and apoptotic signaling pathways [141-145]. In pancreatic cancer research, the effects of ginger showed vast potential in both murine and *in-vitro* models. For example; ginger and its major pungent components (gingerol, shogaol, and zerumbone) were shown reportedly to downregulate and/or inhibit NF- κ B signaling across a variety of pancreatic cancer cell lines [146-148]. 6-gingerol, in particular, was demonstrated to induce cell cycle arrest at the G1 phase along with blocking entry into the S phase potentially through decreased cyclin-dependent kinase (Cdk) expression and reduced phosphorylation of retinoblastoma protein (pRb). Likewise, 6-shogaol, an active constituent of ginger, was reported to sensitize pancreatic cancer to gemcitabine through inhibiting NF- κ B, cyclin D1, Bcl-2, Cox-2, resulting in increased apoptosis [147]. Moreover, zerumbone, another isolated component of ginger, demonstrated pro-apoptotic effects on PANC-1 cells through the upregulation of p21, p53 and increased ROS production [149].

Furthermore, rosemary and its constituents, another component of Zyflamend has proven effective in a wide variety of cancer research models. For instance, *in vitro* findings reveal the effectiveness of rosemary extracts (RE) against numerous cancers [150-154] through a variety of proposed mechanisms. Petiwala and colleagues demonstrated that RE activate an ER stress response accompanied by the induction of apoptosis in prostate cancer cells. RE were also shown to increase the expression of Bax, cleaved caspase 3, Chop and Ire1 α , a mechanism similar to our findings [155]. Moreover, isolated polyphenols of RE, such as carnosic acid and rosmarinic acid, were to increased TNF α and nitrate accumulation, accompanied by the induction of apoptosis in rat insulinoma (RINm5F) cells [152, 154]. Finally, the Zyflamend component holy basil has also shown promise in pancreatic cancer research as it inhibited tumorigenesis in both murine and *in vitro* models and promoted apoptosis [156]. Taken together, these findings demonstrate the potential for combining these herbal extracts to target pancreatic cancer.

The development and progression of pancreatic cancer has been linked to the activation and inhibition of a variety of cell signaling pathways. In this study, we explored the role of Zyflamend on cell survival, cell cycle, and cell death. We demonstrate that Zyflamend attenuates cell survival, causes G2/M cell cycle arrest and promote apoptotic cell death in a dose and time dependent manner. While 800 µg/ml completely inhibited cell growth, 200 µg/ml was sufficient to significantly reduce cell survival. Based on previously described findings, we chose 200 µg/ml for further exploration because this dose represents the maximum plasma concentration of one of Zyflamend's constituents, curcumin, in humans following supplementation [175]. Importantly, previous studies have revealed that bioactive compounds such as those found in Zyflamend, can be more effective when combined. Supportively, compounds exhibiting relatively low bioactivity in isolation often show increased activation when combined with another bioactive originating from the same source [187]. Moreover, Zyflamend doses at or below 200 µg/ml have to varying degrees, proven effective at either inducing apoptosis and or inhibiting proliferation of several tumor-derived cell lines, including pancreatic [130] and prostate [188] cancer cells.

In agreement with these studies, here we report that this dose was sufficient to significantly induce apoptotic cell death in pancreatic cancer cells. On the other hand, while the individual components of Zyflamend show potential in targeting cancer, the doses often required to elicit response exceed physiological relevance. For instance, curcumin dose required to elicit the inhibition of Akt phosphorylation occurred at high doses (>40 µM) in a previously mentioned study [135]. In addition, the dose required to generate anti-tumor response to polyphenolic extract from ginger, 6-shogaol, was relatively high both *in vitro* (15-20 uM) and *in vivo* (50 mg/Kg) in a pancreatic cancer model [147]. Research on RE shows huge variation in the doses used in cancer research with *in vivo* doses reported from a low of 100 mg/kg/day to as high as 3333.3 mg/kg/day [154]. Furthermore, a dose of 6 g of green tea per day given to prostate cancer patients, lead to varying degrees of collateral cytotoxicity [157]. In order to develop translationally valid phytochemical research, it is imperative that dosing and human equivalence be carefully considered.

In order to determine the molecular mechanisms mediating Zyflamend's actions, we explored pathways that are critical for pancreatic cancer cell survival and proliferation. We found that Zyflamend induced the cleavage of caspase 3 along with its downstream target Parp. This is in alignment with other studies showing similar activation of the apoptotic cascade in response to

Zyflamend treatment [121, 189]. Next, we probed for changes in the Mapk pathways in response to Zyflamend. As aforementioned, the Mapk pathways include: Mek1/Erk 1/2/p90 RSK, p38 Mapk, and Jnk/c-Jun pathways. In conjunction with the Pi3k/Akt/mtor pathway, these proteins and pathways are responsible for key physiological responses such as cell survival, protein synthesis, cell proliferation, growth, migration, and apoptosis in both normal and cancerous cells [190-193]. In addition, elevated activation of the phosphoinositide 3-kinase (Pi3k) signaling cascade is observed in a majority of cancers. Therefore, targeting the Pi3k pathway with natural compounds could provide invaluable therapeutic potential to cancer patients. At much higher doses, individual components found in Zyflamend such as curcumin [194], ginger (6-shogaol) [195], and rosemary [169] have all been shown to inhibit the phosphorylation of Akt and cell survival of various cancer types. In addition, curcumin has been shown to inhibit the phosphorylation of Akt in a pancreatic cancer cell line (PANC-1) [196]. In line with these findings, our data demonstrates that Zyflamend treatment caused a significant reduction in both Erk1/2 and Akt phosphorylation.

It has been previously demonstrated, that specific inhibition of Akt results in both autophagy and apoptotic cell death in pancreatic cancer cells [197, 198]. However, depending on factors such as; cell line, duration, and the inhibitor being used, Akt inhibition can in some cases lead to decreased autophagy and increased apoptosis [199, 200]. Through its effects on autophagy signaling and outcomes, targeting Akt through inhibition possesses substantial potential as an emerging therapeutic approach to pancreatic cancer. Our findings revealed that while Zyflamend inhibited the phosphorylation of Akt and Erk, it also induced autophagy as judged by the increased levels of Lc3 I&II, Atg5, and Atg7. Inhibition of autophagy abrogated Zyflamend-induced cell death. Together, these results indicate that Zyflamend-induced autophagy in our experimental model is independent of Akt. While this may seem contradicting, previous cancer studies on the role of Akt and autophagy in cancer cell survival and proliferation, have indicated that phytochemicals in general, present diverse roles in the regulation of autophagy [201]. Numerous phytochemicals including those found in Zyflamend such as; gingerol [167], curcumin [202], [6]-shogaol [203], and epigallocatechin gallate (EGCG) [204], have all been shown to induce autophagy-mediated cell death and are in agreement with our findings. Further research targeting the diverse relationship between Akt and autophagy will be required to better isolate how these biological responses interrelate.

A key finding from this research is that, in addition to inducing autophagy, Zyflamend also upregulates ER stress and through this could reduce the survival of pancreatic cancer cells. The ER stress response is a set of highly regulated mechanisms sensitive to a wide variety of physiological and pathological conditions that effect protein synthesis and folding. One of the mechanisms through which misfolded and/or unfolded proteins are handled by the ER is through ER-associated degradation (ERAD). As improperly folded aggregated proteins reach excessive thresholds, the primary functions of the ER stress response can be overwhelmed. To cope with this rising stress level, the autophagy-lysosome dependent ERAD (type II) machinery can be activated [205, 206]. If the level of unfolded proteins continues to rise, the stress can overwhelm both autophagy and the ER's capacity to function leading to the induction of programmed cell death [206]. In tumorigenesis, the environment leads to elevated levels of metabolic stress and can often lead to increased activation of autophagy and ER stress responses [207, 208]. However, the activation of autophagy and proper functioning ER stress responses acts in duality where properly functioning can protect cancer from the harsh microenvironment and when critical thresholds are reached, can initiate cell death [208]. In this study, we demonstrated that Zyflamend treatment led to the activation of two branches of the ER stress mediated UPR through increased phosphorylation of Perk and Ire1 α and activation of their downstream targets eIF2 α and sXbp1. In addition, this was accompanied by elevated levels of Chop, an ER stress mediated apoptosis inducer. Taken together, these findings show that Zyflamend can act as an inducer of ER stress, mediating autophagy and leading to cell death. Our findings support targeting ER stress and autophagy to induce cell death in cancer. In recent times, both natural and synthetic compounds have been explored regarding ER stress and autophagy mediated apoptosis in various cancer models [95]. However, this study is the first to delineate how natural compounds can act synergistically in the coordination of ER stress, MAP Kinases, and autophagy signaling to induce cell death in pancreatic cancer cells.

In pancreatic cancer, natural compounds such as pachymic acid, cannabinoids, triptolide, and their synthetic analogues such as diarylmethanes, have been shown to activate ER stress and autophagy to induce apoptotic cell death [95]. Triptolide, is a Chinese herbal extract that shows promise through its ability to constrain pancreatic cancer cell growth [96-98]. Previously, triptolide treatment resulted in pancreatic cancer cell death accompanied by ER stress response, including increased eIF2 α phosphorylation, Ire1 α expression, and modified expression patterns of Xbp1,

Chop, and GPR78 [98]. Similarly, pachymic acid, a purified triterpene from a fungal extract, can trigger apoptosis in both MIA PaCA-2 and PANC-1 cells through ER stress induction as indicated by increased expression of phospho-eIF2 α , Xbp1s, Chop, Atf-4, and Hsp70 [99]. ER stress signaling was confirmed as the initiator of apoptosis when ER stress inhibitor tauroursodeoxycholic acid (TUDCA) blocked the effect [99]. Although many of these natural compounds were shown to exhibit anti-proliferative and pro-apoptotic activities in pancreatic cancer cells at relatively high concentrations, at low concentrations (similar to doses achieved with 200 μ g/ml of Zyflamend), many of these compounds fail to induce cell death. It has been demonstrated that the apoptotic effect of curcumin in human hepatoma-derived (Huh-7) cells likely occurs through its ability to enter the cell. After cellular entry, curcumin can cause lysosomal destabilization, ROS accumulation, Bax translocation, increased calcium and cytochrome c release [209]. Importantly, the findings of Moustapha, A., et al, promote the idea that apoptosis and autophagy crosstalk mechanisms play an integral role to cellular outcomes [209].

The activation of ER stress related Ire1 α can stimulate the downstream activation of Jnk and p38 Mapk signaling to promote Bax-dependent apoptosis [210]. Moreover, the activation of p38 can lead to the phosphorylation of Chop leading to alterations in gene expression that promote apoptosis [211, 212]. Although it has been shown that Zyflamend activates TRAIL [121], which is well established that it can activate Jnk [213], our study is the first to show direct activation of Jnk by ER stress in response to Zyflamend. Activation of the Jnk signaling can act in a dual natured role, where it either promotes or suppresses tumor expansion in a manner highly dependent upon the experimental design and type of cancer [214]. Recently, the role of Jnk signaling in pancreatic cancer was explored. In response to high glucose conditions, proportional accumulations of ROS occurred, resulting in reduced Jnk signaling and enhanced pancreatic cancer cell proliferation [215]. In addition, ROS production and accumulation has been shown to play a critical role in mediating the activation of the ER stress response [216]. Here, we report that Zyflamend treatment induced the phosphorylation of both Jnk and p38. We also demonstrate that activation of Jnk through phosphorylation resulted in decreased proliferation of pancreatic cancer cells. This downstream signaling activation supports the idea that the mechanism through which cell death occurred was mediated by ER stress regulation of the Jnk signaling pathway. Previously, epigallocatechin-3-gallate (EGCG), an extract from green tea and one constituent of Zyflamend,

was shown to cause elevated intracellular levels of ROS, activation of Jnk/p38 signaling, along with increased caspase cleavage and apoptosis in human leukaemic cells [217]. These findings suggest that these events were interrelated. Furthermore, in a study by Yang and colleagues, a similar mechanism of EGCG on p38/Jnk signaling was described. EGCG treatment led to increased levels of ROS and Ask1-p38/Jnk pathway activation in order to induce cell death [218]. In general, these findings are in line with our results, yet, further research investigating whether Zyflamend causes alteration in redox homeostasis and oxidative ROS contribute to the pro-apoptotic effects of Zyflamend is warranted.

In summary, while previous studies on Zyflamend has shown that through combining these 10 unique herbs the treatment potential for targeting various cancers, could be enhanced in an additive or synergistic manner [175], it is our hope that findings from this study may pave the way towards pre-clinical validation of the beneficial effects of Zyflamend as a potential adjuvant for the treatment for pancreatic cancer. It is also our hope that finding from this study could be soon translated into clinical trials aiming at developing safer and more potent therapeutic approaches capable of improving pancreatic cancer patient outcomes and quality of life.

CHAPTER 3

CONCLUSION and FUTURE PERSPECTIVES

Conclusion

While still largely enigmatic, potential solutions and discoveries centralized around novel approaches, could prove invaluable to uncovering the secrets of pancreatic cancer. Unfortunately, those diagnosed and afflicted with pancreatic cancer have little reason to hope in the hands of current standard medical therapies. Promisingly, innovative research focused on delineating the unknown aspects of how this malignancy forms and progresses continue to push the known boundaries. However, many questions remain and the need for improved therapies is critical. Investigations targeting alternative therapies have revealed the vast potential for approaching this disease from a holistic point of view. Holism is a concept where all parts are considered for their interconnectedness and shared relationships. Traditional pharmacological approaches such as gemcitabine and cisplatin often target pancreatic cancer specifically without regard for the full impact on the patient. This approach often leads to collateral side effects and can result in patients having a low quality of life, throughout what is often their final days on earth. Novel research into alternative therapies including heat or cold shock, hyperbaric chambers, CBD, nutraceuticals, and many unique phytonutrients, have been underway. Phytonutrients are compounds that exist in a natural state and are consumed regularly in the foods that we eat, to varying degrees dependent upon the diet. In addition, phytochemicals and nutrients have been isolated and extracted to potentiate and determine how they can be effective against pancreatic cancer. Zyflamend, is a polyherbal extract that contains a high level of phytonutrients, that has shown potential in a wide variety of cancer research models. Furthermore, while often at doses that exceed human physiological relevance, some of the individual extracts that comprise Zyflamend, have shown promise in a variety of pancreatic cancer research models. However, these extracts when combined, can act synergistically to enhance their effectiveness.

In pursuit of understanding Zyflamend and its potential as a nutraceutical, we investigated its effects on pancreatic cancer cell survival and proliferation. We discovered that Zyflamend treatment resulted in a dose-dependent decrease in cell proliferation, consistent with an increase in apoptotic cell death and cell cycle arrest in the G2/M phase. Additionally, Zyflamend in conjunction with chemotherapeutic compounds exhibited an additive or synergistic effect against pancreatic cancer. At the molecular level, treatment with Zyflamend led to the activation of the JNK pathway. Notably, pharmacological inhibition of JNK abrogated the pro-apoptotic effects of Zyflamend. These studies identify Zyflamend as a potential novel approach to treat pancreatic

cancer via modulation of the JNK pathway. Although it has been shown that Zyflamend activates TRAIL, which is well established that it can activate JNK, our study is the first to show direct activation of JNK by ER stress in response to Zyflamend. Activation of the JNK signaling can act in a dual natured role, where it either promotes or suppresses tumor expansion in a manner highly dependent upon the experimental design and type of cancer. It has been revealed that in response to high glucose conditions proportional accumulations of ROS occurred, resulting in reduced JNK signaling and enhanced pancreatic cancer cell proliferation. In addition, ROS production and accumulation has been shown to play a critical role in mediating the activation of the ER stress response. Here, we report that Zyflamend treatment induced the phosphorylation of both JNK and p38. We also demonstrate that activation of JNK through phosphorylation resulted in the decreased proliferation of pancreatic cancer cells. This downstream signaling activation supports the idea that the mechanism through which cell death occurred was mediated by ER stress regulation of the JNK signaling pathway. In addition, EGCG a constituent of Zyflamend has been shown to induce cell death through elevating intracellular levels of ROS and activating JNK/p38 in several cancer models. In general, these findings are in line with our results, yet, further research investigating whether Zyflamend causes alteration in redox homeostasis and oxidative ROS contribute to the pro-apoptotic effects of Zyflamend is warranted.

Future Perspectives

Targeting the effects of Zyflamend on various murine models of pancreatic cancer could significantly aid in the translation of our findings. Murine experimental models can be designed to effectively mimic human pancreatic cancer through inducing chronic inflammation and or through the insertion or overexpression of oncogenic mutations such as *KRAS* that promote the development and progression of the disease as seen in humans. These methods allow for investigations into pancreatic cancer that are biologically relevant across species. These methods will help to provide preclinical validation of using Zyflamend as an adjuvant with medically-approved standard therapies, or in a preventative manner. Beyond strengthening our discoveries, further research into the unknown aspects and mechanisms of how natural compounds can act synergistically against pancreatic cancer possesses profound potential. Therefore, research illustrating the *in vivo* effects of Zyflamend both alone and in combination with known chemotherapeutic compounds on the onset and progression of pancreatic cancer is warranted. Through these examinations, the potential capability of Zyflamend to act as a nutraceutical in both

the prevention and treatment of pancreatic cancer will be explored. In addition, through analyzing the combined effects with standard therapies we will generate a more complete understanding of Zyflamend's potential as an adjuvant. Finally, with perseverance and dedication, solutions to the mysteries of pancreatic cancer could be discovered through the pursuit of novel research into the less understood aspects of this malignancy.

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VITA

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