MOLECULAR MECHANISMS OF BIOACTIVE COMPOUNDS IN FRUITS AND VEGETABLES THAT INHIBIT PANCREATIC CANCER AND THEIR RELATIONSHIP WITH INFLAMMATION

BY

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DISSERTATION

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ABSTRACT

For all stages of pancreatic cancer, the 5-year survival rate from the time of diagnosis is 6%, with the median survival rate being only 6 months. This poor prognosis for pancreatic cancer is due to its aggressive nature and, too frequently, to its late diagnosis due to the absence of clinical symptoms at early stages in this disease. A potential therapeutic target in pancreatic cancer is the serine/threonine kinase glycogen synthase kinase-3β (GSK-3β) that is known to translocate into the nuclei of pancreatic cancer cells and act as a key regulator of NF-κB transcriptional activity. The constitutively active NF-κB resulting from misregulation of this signaling cascade is an essential component in the progression and tumorigenic properties of this disease. Fruit and vegetable bioactive compounds have the potential to be used as therapeutic agents for pancreatic cancer due to their anti-oxidant, -carcinogenic, -inflammatory, -proliferation and -progression properties. The objective of this research was to examine the effect of bioactive compounds present in fruits and vegetables on pancreatic cancer proliferation, apoptosis, and the role of the GSK-3β/NF-κB signaling pathway using in vitro and in vivo models.

In aim 1, we evaluated the ability of fruit and vegetable bioactive compounds (flavonoids, limonoids, phenolic acids and ascorbic acid) to bind within GSK-3β's catalytic site and inhibit its activity using an in vitro enzymatic assay and computational modeling. Of the 22 bioactive compounds tested, the flavonoids luteolin, apigenin, and quercetin had the highest inhibitory effects on GSK-3β activity, with 50% inhibitory values of 1.5, 1.9, and 2.0 µM, respectively. Molecular dockings were then performed to determine the potential interactions of each flavonoid with GSK-3β. Luteolin, apigenin and quercetin were predicted to fit within the binding pocket of GSK-3β with low interaction energies (-76.4, -76.1, and -84.6 kcal·mol⁻¹).
respectively). Our results indicated that several flavonoids inhibit GSK-3β activity and suggest that these have potential to suppress the growth of pancreatic tumors.

In aim 2, we tested the inhibitory potential of fruit and vegetable bioactive compounds on the proliferation of BxPC-3 and PANC-1 pancreatic cancer cells, in vitro, and gene and protein expressions of molecular markers in the GSK-3β/NF-κB signaling pathway. Of the 22 bioactive compounds tested, apigenin and luteolin were the most effective at inhibiting pancreatic cancer cell proliferation (IC$_{50}$ = 23 and 31 µM for BXPC-3 cells, respectively, and IC$_{50}$ = 71 and 90 µM for PANC-1 cells, respectively, for 24 h). Further investigation with apigenin into the molecular mechanism by which these flavonoids induced pancreatic cell death demonstrated that it was through the GSK-3β/NF-κB signaling pathway. Apigenin arrested cell cycle at G$_2$/M phase (36% and 32% at 50 µM for BxPC-3 and PANC-1, respectively) with concomitant decrease in the expression of cyclin B1. Apigenin activated the mitochondrial pathway of apoptosis (44% and 14% at 50 µM for BxPC-3 and PANC-1, respectively) and modified the expression of apoptotic proteins. Apigenin highly upregulated the expression of cytokine genes IL17F (114.2-fold), LTA (33.1-fold), IL17C (23.2-fold), IL17A (11.3-fold), and IFNB1 (8.9-fold) in BxPC-3 cells, which potentially contributed to the anti-cancer properties. These results suggest that flavonoids have a protective role in pancreatic tumorigenesis.

In aim 3, we assessed the potential interactions of either apigenin or luteolin with the anti-cancer activity of chemotherapeutic drugs in BxPC-3 cells in vitro, and evaluated their mechanisms of action related to inhibition of the GSK-3β/NF-κB signaling pathway. The effect of simultaneous treatment of flavonoids and chemotherapeutic drugs as well as a range of flavonoid pretreatment times (0, 6, 24 and 42 h) and concentrations (0-50 µM) on pancreatic cancer cell proliferation were assessed using the MTS cell proliferation assay. Simultaneous
treatment with either flavonoid (13, 25 or 50 µM) and the chemotherapeutic drug 5-fluorouracil (5-FU, 50 µM) or gemcitabine (Gem, 10 µM) for 60 h resulted in less-than-additive effects on inhibition of cell proliferation. Pretreatment for 24 h with 13 µM of either apigenin or luteolin, followed by the addition of either 5-FU or Gem for 36 h was determined to optimally inhibit cell proliferation. These combinations led to 67 or 60% inhibition when apigenin or luteolin was used as a pretreatment for 5-FU and 66 or 69% inhibition when they were each used as a pretreatment for Gem. Pretreatment of cells with 11-19 µM of either flavonoid for 24 h resulted in 43-72% growth inhibition when followed by 5-FU (50 µM, 36 h) and 59-73% growth inhibition when followed by Gem (10 µM, 36 h). Pretreatment of cells with 11-15 µM of either flavonoid for 24 h resulted in 15-35% inhibition when followed by cisplatin (Cis, 10 µM, 36 h) and 36-63% growth inhibition when followed by oxaliplatin (Oxa, 0.1 µM, 36 h). Isobolographic analyses using the IC_{20}s or IC_{30}s for the flavonoids and chemotherapeutic drugs demonstrated an additive effect for either flavonoid in combination with 5-FU or Gem and an antagonistic effect when combined with Cis. When apigenin was used in combination with Oxa this caused an additive effect, while luteolin in combination with Oxa caused an antagonistic effect. Luteolin (15 µM, 24 h) pretreatment followed by Gem (10 µM, 36 h), significantly decreased the protein expressions of nuclear GSK-3β and NF-κB p65 and increased pro-apoptotic cytosolic cytochrome c. The results suggest that pretreatment of pancreatic cancer cells for 24 h with low concentrations of flavonoids effectively aid in the anti-cancer activity of chemotherapeutic drugs through inhibition of the GSK-3β/NF-κB signaling pathway leading to increased apoptosis.

In aim 4, we evaluated the potential of luteolin to enhance the anti-tumor effects of Gem on pancreatic cancer using an orthotopic mouse model. Male athymic nude mice (6 wk old) were injected with BxPC-3 human pancreatic cancer cells directly into the pancreas and randomized
into four treatment groups: (1) control (n = 14); (2) luteolin [84 mg/kg body weight (bw)] 7 times/wk for wk 1 and 5 times/wk for wks 2-6 (n = 12) by intraperitoneal (i.p.) injection; (3) Gem (125 mg/kg bw) twice/wk by i.p. injection for wks 2-6 (n = 14); and (4) luteolin (84 mg/kg bw) 7 times/wk for wk 1, followed by luteolin 5 times/wk and Gem (125 mg/kg bw) 2 times/wk for wks 2-6 by i.p. injection (n = 12). The combination treatment of luteolin and Gem significantly lowered (p = 0.048) the pancreatic tumor mass compared to the control group.

Luteolin, Gem and their combination significantly reduced proliferating cell nuclear antigen expression by 25%, 37% and 37%, respectively. Immunohistochemical and western blot analyses showed that combination treatment led to a significant reduction in the expressions of K-Ras (46%, p=0.0006), GSK-3β (34%, p=0.014), p(Tyr216)GSK-3β (16%, p=0.033), p(Ser311)NF-κB p65 (27%, p=0.036) and the Bcl-2/Bax ratio (68%, p=0.006) while significantly increasing the expressions of cytochrome c (44%, p=0.035) and caspase 3 (417%, p=0.003). The combination treatment of luteolin and Gem promoted apoptotic cell death in pancreatic tumor cells through inhibition of the GSK-3β/NF-κB signaling pathway leading to a reduction in the Bcl-2/Bax ratio, release of cytochrome c and activation of caspase 3. These results suggest the potential for luteolin to enhance the anti-tumor activity of Gem in a pancreatic cancer in vivo model.

In summary, we first demonstrated that fruit and vegetable flavonoids, specifically luteolin, apigenin and quercetin, were able to optimally bind within the catalytic site of GSK-3β and inhibit its activity. Next, the inhibitory effects of these bioactive compounds against the pancreatic cancer cell lines BxPC-3 and PANC-1 were determined, showing that apigenin and luteolin were the most potent. The inhibitory effects of the flavonoids on GSK-3β enzymatic activity and on BxPC-3 cell proliferation significantly correlated (r = 0.87 for 24 h exposure and
r = 0.86 for 48 h exposure) suggesting that the flavonoids may be decreasing pancreatic cancer in vitro through inhibition of GSK-3β. However, the correlations were not statistically significant with the inhibitory results of the PANC-1 cells, potentially due to the higher tumor grade of this cell line. Investigation into the specific molecular mechanism of action demonstrated that apigenin is inhibiting proliferation of pancreatic cancer cells due to suppression of the GSK-3β/NF-κB signaling cascade and the induction of apoptosis. From there, we showed the ability of apigenin and luteolin to enhance the anti-cancer activity of chemotherapeutic drugs 5-FU, Gem and Oxa in BxPC-3 cells, and showed that this enhancement was due to inhibition of the GSK-3β/NF-κB signaling pathway. Lastly, these in vitro findings were translated to an in vivo model of pancreatic cancer demonstrating the ability of luteolin to enhance the anti-tumor effects of Gem through inhibition of the GSK-3β/NF-κB signaling pathway leading to increased apoptosis.

In conclusion, the results from this research demonstrate the potential of fruit and vegetable flavonoids, specifically apigenin and luteolin, to protect against pancreatic carcinogenesis, and provide the foundation for future studies in this area.
Dedicated to my family, especially my husband Blake, without their unconditional love and support this would not have been possible
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# ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-FU</td>
<td>5-fluorouracil</td>
</tr>
<tr>
<td>Api</td>
<td>Apigenin</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>Cis</td>
<td>Cisplatin</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's minimum essential medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>Gem</td>
<td>Gemcitabine</td>
</tr>
<tr>
<td>GSK-3β</td>
<td>Glycogen synthase kinase-3β</td>
</tr>
<tr>
<td>H and E</td>
<td>Hematoxylin and eosin</td>
</tr>
<tr>
<td>HPDE</td>
<td>Human pancreatic ductal epithelium</td>
</tr>
<tr>
<td>IC₅₀</td>
<td>Concentration needed to inhibit 50% of cell growth</td>
</tr>
<tr>
<td>IFNB1</td>
<td>Interferon beta 1</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IL17</td>
<td>Interleukin 17</td>
</tr>
<tr>
<td>KEGG</td>
<td>Kyoto Encyclopedia of Genes and Genomes</td>
</tr>
<tr>
<td>K-Ras</td>
<td>Kirsten rat sarcoma virus</td>
</tr>
<tr>
<td>LTA</td>
<td>Lymphotoxin alpha</td>
</tr>
<tr>
<td>Lut</td>
<td>Luteolin</td>
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<tr>
<td>Abbreviation</td>
<td>Full Name</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>MOE</td>
<td>Molecular Operating Environment</td>
</tr>
<tr>
<td>MTS</td>
<td>3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl-2-(4-sulfophenyl)-2H-tetrazolium</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa B</td>
</tr>
<tr>
<td>Oxa</td>
<td>Oxaliplatin</td>
</tr>
<tr>
<td>PanIN</td>
<td>Pancreatic intraepithelial neoplasia</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCNA</td>
<td>Proliferating cell nuclear antigen</td>
</tr>
<tr>
<td>PES</td>
<td>Phenazine ethosulfate</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodine</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene iodide</td>
</tr>
<tr>
<td>RIPA</td>
<td>Radio immunoprecipitation assay</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>SAS</td>
<td>Statistical analysis software</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris-buffered saline Tween 20</td>
</tr>
<tr>
<td>XIAP</td>
<td>X-linked inhibitor of apoptosis protein</td>
</tr>
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</table>
CHAPTER 1

OVERVIEW

1.1 Synopsis

Pancreatic cancer is the fourth leading cause of cancer-related death among both men and women in the United States, with the 5-year survival rate for all stages of this disease being only 6%. Moreover, an estimated 45,000 new cases and 38,000 deaths are expected from this disease in 2013 (Figure 1.1) [1]. The poor prognosis for pancreatic cancer is due to its aggressive nature and, too frequently, to its late diagnosis due to the absence of clinical symptoms at early stages in this disease [2]. Two common risk factors associated with pancreatic cancer are chronic pancreatitis as well as glucose intolerance due to obesity and type 2 diabetes mellitus, and in both instances glycogen synthase kinase-3β (GSK-3β) is overexpressed. The overexpression of GSK-3β promotes pancreatic cancer progression (Figure 1.2) [3] by translocating into the nuclei of these cells and acting as a key regulator of NF-κB transcriptional activity [4, 5]. The constitutively active NF-κB resulting from misregulation of this signaling cascade is an essential component in the progression and tumorigenic properties of this disease [6]. With much known about GSK-3β [3-5, 7, 8], it is now apparent that the identification of bioactive compounds that can inhibit it in pancreatic cancer is critical for developing novel targeted therapies aimed at slowing the progression of this disease. Fruit and vegetable bioactive compounds (Figure 1.3) have the potential to be used as therapeutic agents for pancreatic cancer due to their anti-oxidant, -carcinogenic, -inflammatory, -proliferation and -progression properties [9], however due to their low bioavailability (Figure 1.4) higher concentrations or alternative routes of administration are needed to overcome this obstacle. Our long term goal was to understand the molecular mechanisms by which bioactive compounds present in fruits and vegetables can inhibit pancreatic cancer using in vitro and in vivo models. We hypothesized that certain fruit and
vegetable bioactive compounds would inhibit GSK-3β, thereby decreasing NF-κB transcriptional activity and decrease the expression of genes involved in proliferation and anti-apoptosis. The overall objective was to examine the effect of bioactive compounds present in fruits and vegetables on pancreatic cancer proliferation, apoptosis and the role of GSK-3β/NF-κB signaling pathway using in vitro and in vivo models. The first aim of this research was to evaluate the ability of 22 fruit and vegetable bioactive compounds (flavonoids, limonoids, phenolic acids and ascorbic acid) to bind directly within GSK-3β's catalytic site and inhibit its activity using an enzymatic assay and computational modeling. The flavonoids, specifically apigenin, luteolin and quercetin, were found to optimally bind within GSK-3β's catalytic site and most effectively inhibit its activity. These specific flavonoids were shown to be more effective at inhibiting GSK-3β activity than the other bioactives that were examined due to the presence of an unsaturated C-ring that allows them to remain planar within the binding cavity of the enzyme and thereby avoid unfavorable steric interactions. This study demonstrated the capability of fruit and vegetable flavonoids to inhibit the activity of GSK-3β, a key therapeutic target of interest in pancreatic cancer. Aim 2 of this research was to examine the potential of the 22 fruit and vegetable bioactive compounds to inhibit the proliferation of BxPC-3 and PANC-1 pancreatic cancer cells, in vitro. Pearson correlations of flavonoids (apigenin, luteolin, quercetin, kaempferol, hesperetin, naringenin and nobiletin) between their inhibitory effects on GSK-3β activity from aim 1 and on proliferation of BxPC-3 cells were statistically significant, but not with the inhibitory results for PANC-1 cells. Apigenin and luteolin were the most effective flavonoids at inhibiting the proliferation of pancreatic cancer cells, however to a lesser degree in the PANC-1 cells. Further analysis of apigenin demonstrated that it was able to exert its anti-proliferative effects in both cells lines through inhibition of GSK-3β leading to the suppression
of NF-κB activity and the induction of apoptosis. In addition for the BxPC-3 cell line, analysis of various inflammatory and cancer genes revealed that apigenin treatment highly upregulated the expression of cytokines IL17 (A, C, F), LTA and IFNB1. These findings demonstrated the capability of various flavonoids to inhibit pancreatic cancer cell proliferation and suggest that the potential molecular mechanism of action is through inhibition of the GSK-3β/NF-κB signaling cascade. The third aim of this research was to investigate the interactions between apigenin or luteolin and the chemotherapeutic drugs 5-fluorouracil (5-FU), gemcitabine (Gem), cisplatin (Cis) or oxaliplatin (Oxa) in BxPC-3 cells. Low pretreatment (24 h) concentrations of apigenin and luteolin were able to similarly enhance the anti-proliferative effects of 5-FU, Gem and Oxa. This enhanced anti-proliferative effect was due, in part, to inhibition of the GSK-3β/NF-κB signaling pathway leading to increased apoptosis, as demonstrated by the combination treatment of luteolin and Gem. However, simultaneous treatment (60 h) with either flavonoid and a chemotherapeutic drug caused less-than-additive effects. These results suggest the potential of apigenin and luteolin to enhance the anti-proliferative effects of chemotherapeutic drugs in pancreatic cancer, however the timing of flavonoid treatment and the concentration is of great importance. Aim 4 of this research was to evaluate the potential of luteolin to enhance the anti-tumor effect of Gem in an orthotopic mouse model of pancreatic cancer. Combination treatment of luteolin and Gem led to a significant reduction in pancreatic tumor mass compared to the control group. Apoptosis was increased in tumor tissue of mice in the combination treated group due to, in part, inhibition of the GSK-3β/NF-κB signaling pathway. These results suggest the potential role of flavonoids to enhance the anti-pancreatic tumor activity of chemotherapeutic drugs, in vivo. In summary, we expect that the results of this research would provide a better
understanding of the molecular mechanisms by which fruit and vegetable flavonoids, specifically apigenin and luteolin, can be used to aid in the protection against pancreatic carcinogenesis.

1.2 References

1.3 Figures

**Figure 1.1.** Ten leading cancer types for the estimated new cancer cases and deaths by sex, United States, 2013 [1].

<table>
<thead>
<tr>
<th>Estimated New Cases</th>
<th>Males</th>
<th>Females</th>
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<tbody>
<tr>
<td>Prostate</td>
<td>238,560</td>
<td>232,340</td>
</tr>
<tr>
<td>Lung &amp; bronchus</td>
<td>118,060</td>
<td>110,110</td>
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<tr>
<td>Colorectum</td>
<td>73,680</td>
<td>69,140</td>
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<tr>
<td>Urinary bladder</td>
<td>54,610</td>
<td>49,560</td>
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<td>Melanoma of the skin</td>
<td>45,060</td>
<td>46,310</td>
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<td>Kidney &amp; renal pelvis</td>
<td>40,430</td>
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<tr>
<td>Non-Hodgkin lymphoma</td>
<td>37,660</td>
<td>31,630</td>
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<tr>
<td>Oral cavity &amp; pharynx</td>
<td>29,620</td>
<td>24,720</td>
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<tr>
<td>Leukemia</td>
<td>27,880</td>
<td>31,630</td>
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<td>Pancreas</td>
<td>22,740</td>
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<td>854,700</td>
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<tr>
<th>Estimated Deaths</th>
<th>Males</th>
<th>Females</th>
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</thead>
<tbody>
<tr>
<td>Lung &amp; bronchus</td>
<td>87,260</td>
<td>72,220</td>
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<tr>
<td>Prostate</td>
<td>29,720</td>
<td>39,620</td>
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<tr>
<td>Colorectum</td>
<td>26,300</td>
<td>24,530</td>
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<tr>
<td>Leukemia</td>
<td>13,660</td>
<td>10,060</td>
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<tr>
<td>Esophagus</td>
<td>12,220</td>
<td>8,430</td>
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<tr>
<td>Liver &amp; intrahepatic bile duct</td>
<td>14,890</td>
<td>14,030</td>
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<tr>
<td>Kidney &amp; renal pelvis</td>
<td>10,820</td>
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<tr>
<td>Non-Hodgkin lymphoma</td>
<td>10,590</td>
<td>6,780</td>
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<tr>
<td>Pancreas</td>
<td>19,480</td>
<td>18,980</td>
</tr>
<tr>
<td>All Sites</td>
<td>366,920</td>
<td>273,430</td>
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</tbody>
</table>

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**Note:** Figures and data are illustrative and should be referenced to the source material for accurate and detailed information. The data reflects estimated cases and deaths for the year 2013.
Figure 1.2. Systemic and total effects of aberrant GSK-3β on risk factors (glucose intolerance and chronic inflammation) and progression of pancreatic cancer [3].
**Figure 1.3.** Some bioactive compounds found in fruits and vegetables that have been shown to inhibit pancreatic cancer.
Figure 1.4. Scheme for digestion, absorption and metabolism of flavonoids using luteolin-7-O-glucoside as an example [10].
CHAPTER 2
LITERATURE REVIEW

DIETARY FACTORS AND PANCREATIC CANCER: THE ROLE OF FOOD BIOACTIVE COMPOUNDS

2.1 Abstract

Pancreatic cancer is the fourth leading cause of cancer mortality among both men and women in the United States with a five year survival rate of only 6%. Several dietary factors may influence the risk of developing pancreatic cancer and its recurrence. Some of these factors may offer innovative therapies for prevention of this disease. The goal of this review is to provide an overview of pancreatic cancer, as well as current knowledge on the epidemiological, in vitro, in vivo and clinical studies conducted about this disease using various dietary agents. The main focus is on food-based approaches for preventing this disease particularly, citrus fruits, and foods containing flavonoids and curcumin.

2.2 Introduction

Pancreatic cancer is the fourth leading cause of cancer death among both men and women in the United States [1]. This disease is so aggressive that it kills more than half of all its victims within 6 months of diagnosis, with the remaining patients having only a five year survival rate of 6%. Although no one knows the exact causes of pancreatic cancer, research shows that people with certain risk factors are more likely to develop this disease. The main risk factors of pancreatic cancer include age, smoking, type 2 diabetes, family history, race, obesity, poor physical activity, and chronic pancreatitis [2] (www.cancer.gov). Age is the most significant un-modifiable risk factor, while smoking is the most predominant modifiable risk factor [3] and

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1This chapter is part of the paper: Johnson, J., Gonzalez de Mejia, E., Dietary factors and pancreatic cancer: the role of food bioactive compounds. Mol. Nutr. Food Res. 2011, 55, 58-73. Permission granted by John Wiley & Sons, Inc.
changes in the diet may also play a role. Pancreatic cancer is often known as the silent killer because symptoms, when they occur at all, are typically vague and non-localizing. Some of these symptoms include jaundice, pain in the upper abdomen, weakness, loss of appetite, nausea, vomiting, and weight loss [2]. By the time most symptoms are noticeable, the cancer has already spread to other organs and surgical intervention is no longer a viable option [4]. It is because of such a poor outlook for patients diagnosed with pancreatic cancer that there is a need to expand our knowledge on different approaches that can be used to prevent and/or treat this disease. The goal of the present review was to provide an overview of pancreatic cancer, as well as an analysis of current knowledge on food-based approaches for preventing pancreatic cancer, particularly the role of bioactive compounds in citrus fruits, flavonoids and curcumin.

2.3 Physiology of the Pancreas

The pancreas is a glandular organ located deep within the abdomen. This 6 inch organ consists of three main parts: the head, the body and the tail, with the head being the widest part. The pancreas is composed of two glands that have two completely different functions. The endocrine gland, which comprises only 1-2% of the pancreas’ mass, is made up of millions of cell clusters called islets of Langerhans. Its function is to produce and secrete into the bloodstream hormones such as insulin, glucagon, somatostatin, pancreatic polypeptide, and ghrelin. The exocrine gland, which comprises 90-95% of the pancreas’ mass, is made up of cell clusters called acini that surround the islets of Langerhans. Its function is to synthesize and secrete digestive enzymes including trypsin, chymotrypsin, lipase and amylase into the duodenum that help breakdown proteins, lipids and carbohydrates [5].
2.4 Pancreatic Cancer

2.4.1 The Prevalence of Pancreatic Cancer

There are two types of pancreatic tumors: exocrine ductal epithelial adenocarcinoma and endocrine cancer, also called islet cell cancer. The more common of the two tumors is exocrine pancreatic cancer and about 95% of these tumors are pancreatic adenocarcinomas [6]. Of the people diagnosed with this disease, more than 90% are over the age of 55, with the average age of diagnosis being 72 years [6].

2.4.2 Pancreatic Intraepithelial Neoplasias and the Development of Pancreatic Cancer

Pancreatic adenocarcinomas are believed to arise from noninvasive precursor lesions in the pancreatic ducts that undergo histological and genetic mutations towards invasive cancer. These histologically well-defined precursor lesions are termed pancreatic intraepithelial neoplasias (PanINs) [7, 8]. Figure 2.1 shows the three PanIN grades that normal ductal epithelium undergo before they become invasive adenocarcinoma. In PanIN-1A, the epithelial lesions are flat and composed of tall columnar cells with basally located nuclei and abundant mucin [8]. In PanIN-1B, the epithelial lesions are papillary, but otherwise identical to PanIN-1A. In these two early sub-categories, mutations lead to the overexpression/amplification/activation of EGFR, K-ras, Shh, Notch and Wnt, and to the deletion or loss of function of p21, PTEN, Telomere and attrition [7]. In PanIN-2, the epithelial lesions are papillary and have some nuclear abnormalities (loss of polarity, nuclear crowding, enlarged nuclei, pseudo-stratification and hyperchromatism) [8]. In this intermediate grade, mutations lead to the overexpression/amplification/activation of Hes-1, cyclin D1 and ErbB2, and the loss of function of p16 [7]. In PanIN-3 (or carcinoma in situ), the epithelial lesions show the budding off of clusters of cells (cribriforming) into the lumen as well as luminal necrosis [8]. Mutations in PanIN-3 lead to overexpression/amplification/activation of
c-Met, Ki-67, Topo IIα, mesothelin and telomerase; and the deletion or loss of function of p53, BRCA2, Smad4 and TGF-βR [8].

2.4.3 K-ras Signaling Pathway in Pancreatic Cancer

One of the most frequently activated oncogenes in all human cancers is Kirsten Rat Sarcoma virus (K-ras), with over 25% of cancers having a mutation in this gene. In pancreatic cancer, constitutively active K-ras is found in over 95% of tumors, making it a molecular signature of this disease [9]. K-ras, a member of the small GTPase superfamily, is 21 kDa membrane-bound protein. This protein is used to transmit signals from the extracellular to the intracellular environment and when it is in its active form, it mediates several cellular activities including proliferation, survival, migration and metabolism [7, 10]. Normally the K-ras activation pathway is regulated by GTPase activating proteins (GAP), but single point mutations on K-ras codons 12, 13 or 61 render this protein resistant to GAP inactivation and therefore constitutively active [7]. K-ras is thought to be an initiator of pancreatic cancer due to the research conducted using multiple mouse models that show that mutations to this protein cause the formation of lesions that closely resemble human PanINs. Continuous production of K-ras has also been shown to be necessary in order to maintain tumor progression. However, the exact mechanism of how K-ras contributes to these factors of cancer is still unknown. What is known is that K-ras propagates at least part of its signal through three Ras pathways. The first pathway is the cell survival promoting pathway of phosphoinositide-3-kinase (PI3K). The second is the RAF-MEK-ERK pathway and it plays an important role in cell proliferation. The third pathway is the Ral guanine nucleotide exchange factor pathway (RalGEFs) and it is responsible for activating the G proteins, RalA and RalB, which are critical for K-ras induced transformation and tumorigenesis of human cells [7]. Because K-ras plays such a key role in
pancreatic cancer there has been considerable interest in discovering compounds that can inhibit it and the pathways they affect. Figure 2.2 shows how some of the dietary factors discussed in this review affect K-ras and these three pathways.

### 2.4.4 GSK-3β/NFκB Signaling Pathway in Pancreatic Cancer

An important therapeutic target in pancreatic cancer downstream of K-ras is the serine/threonine protein kinase glycogen synthase kinase-3β (GSK-3β). This enzyme was first identified to regulate glucose/glycogen metabolism under the control of insulin signaling [11]. GSK-3β has been implicated as therapeutic target for inflammation, diabetes, cancer and central nervous system diseases [12]. Disregulated GSK-3β expression and activity are fundamental characteristics of gastrointestinal cancers, and this enzyme helps to maintain survival and proliferation of these tumor cells [13]. More specifically, GSK-3β has been shown to be overexpressed in pancreatic cancer cells [14-16] where it stimulates NFκB transcription activity and as a consequence activates genes that control cell proliferation and survival [14, 17-19]. Several reports have demonstrated that inhibition or genetic depletion of GSK-3β leads to decreased NFκB transcriptional activity in pancreatic cancer [14, 17-19]. The potential mechanisms proposed for how this occurs are (i) inhibition of GSK-3β prevents it from phosphorylating NFκB p65, (ii) inhibition of GSK-3β may result in chromatin remodeling and therefore prevent NFκB accessibility to the promoter regions of target genes or (iii) both occur [20]. Recently, Zhang et al. [21] demonstrated that upstream regulation of GSK-3β gene expression in pancreatic cancer is controlled by the K-ras/ MAPK (aka ERK)/ ETS cascade which increases binding of ETS2 to GSK-3β promoter elements and recruitment of the histone acetylase p300 to form a transcriptionally active complex. Research on the identification of novel compounds that can inhibit GSK-3β is of great interest because this could reduce pancreatic
cancer survival and proliferation, and also lead to an indirect decrease in K-ras activity, a key oncogene of this disease.

2.4.5 Common Pancreatic Cancer Risk Factors

There are several common risk factors that can contribute to the development of pancreatic cancer. They can be divided into two categories, un-modifiable and modifiable. Un-modifiable risk factors include heredity, age, sex and race. Modifiable risk factors include smoking, obesity and physical inactivity, type 2 diabetes, high fat diet, and excessive alcohol consumption [6].

2.4.6 Food-based Approaches for Treating Pancreatic Cancer

Every year about $881 million is spent on pancreatic cancer treatment in the United States [22]. The main treatment options for patients with pancreatic cancer are surgery, chemotherapy, radiation and targeted therapy. The treatment option that is best for the patient depends on the location of the tumor, whether or not the disease has spread and the patient’s age and general health [2].

There are two major diet-related prevention strategies involved in cancer research; cancer chemoprevention and dietary cancer prevention [23]. Cancer chemoprevention is defined as the use of natural, synthetic or biologic chemical agents for pharmacologic intervention to prevent, inhibit or reverse carcinogenesis [24]. Dietary cancer prevention, on the other hand, involves the modification of food consumption patterns that are often accompanied by lifestyle changes in order to decrease the risk [23, 25]. The use of food-based approaches for the prevention of pancreatic cancer have been explored in recent years based on epidemiological evidence that shows an inverse association between consumption of fruits and vegetables and the
risk of developing pancreatic cancer [26-28]. Figure 2.3 depicts the structures of some of these compounds of interest.

2.5 Pancreatic Cancer and Whole Foods

2.5.1 Citrus Fruits

Citrus fruits are one of the most important and largest fruit crops produced worldwide, with approximately 100 million metric tons produced annually [29]. This type of fruit is most commonly consumed as fresh produce and juice. It is known for its diverse and high phytonutrient content that contributes to its health benefits [30]. Flavonoids, one of the major classes of compounds in citrus, have been shown to have antioxidant, antiproliferative, antitumor, antiinflammatory and pro-apoptotic activities [31]. The most abundant flavonoids found in citrus are hesperidin, narirutin, neohesperidin, eriocitrin, neoeriocitrin, rutin, diosmin, neoponcirin and nobiletin [30]. Carotenoids are also found in abundance in citrus fruits, with over 115 of them that have been identified [32]. Numerous studies have demonstrated that high carotenoid intake may decrease the risk of cardiovascular disease, age-related macular degeneration and cancer [33]. Among the carotenoids, β-carotene, lutein, zeaxanthin, β-cryptoxanthin and lycopene are found the most in citrus fruits [30]. Citrus limonoids, a class of compounds that are responsible for the delayed bitterness in citrus juices, have been found to have several beneficial properties as well including, antioxidant, hypocholesterolemic, antiviral, insecticidal and anticancer activities [34]. Limonin, nomilin and obacunone are the major limonoids present in citrus [35].

Several case-control and cohort epidemiological studies have examined the effects of citrus fruits on the risk of developing pancreatic cancer [26-28, 36-43, 102]. The majority of these studies show an inverse association between the consumption of citrus fruit and the risk of
developing this disease. However, the existing data has inconsistencies and diversity in the study designs limit the ability to fully interpret the published observations. Relevant details about the studies are shown in Tables 2.1 and 2.2. Six of the studies took place in the United States [28, 36-48, 102], while the rest were conducted in Italy [26], Sweden [27], the Netherlands [40], Finland [41], China [42], and Japan [43]. All of the studies adjusted for age and cigarette smoking, except for that in reference [28] which did not. In all twelve of the studies the results showed that the higher the consumption of citrus fruit the lower the risk of developing pancreatic cancer.

More recent studies have shown that citrus bioactive compounds have the ability to inhibit multiple stages of breast [44], colon [45], prostate [46], lung [47], and pancreatic cancers [48, 49]. In an in vitro investigation conducted by Patil et al. [48] the effects of freeze-dried lime (Citrus aurantifolia Swingle) juice extracted with chloroform, acetone, methanol or methanol/water (8:2) on pancreatic cancer cells were tested. The results showed that all four lime juice extracts inhibited the growth of cancer cells in a dose-dependent manner, with the methanol extract having the highest activity. The protein levels of p53, Bax, Bcl-2 and caspase-3 demonstrated that the lime juice extracts favored the induction of apoptosis in the cancer cells. It was also determined by high-performance liquid chromatography that the bioactive components in the lime juice were the flavonoids: rutin, neohesperidin, hesperidin and hesperetin; and the limonoids: limonexic acid, isolimonexic acid and limonin. In another study conducted by the same research group [49] they found that limonoids (limonin glucoside, limonixic acid, isolimonixic acid, limonin and obacunone) extracted from the seeds of the same type of lime also inhibited pancreatic cancer cells through apoptosis.
2.6 Pancreatic Cancer and Bioactive Foods Components

2.6.1 Flavonoids

Flavonoids are the largest class of polyphenols with over 5,000 of them described. They are water-soluble secondary metabolites that possess a common C6-C3-C6 structure and provide much of the flavor and color to fruits and vegetables. There are six major subclasses of flavonoids: flavones, flavonols, flavonones, flavanols, anthocyanidins and isoflavones [50]. In humans, flavonoids have a wide range of bioactivities including anticancer, anti-inflammatory and antihypertensive properties [51]. Tables 2.3 and 2.4 show the in vitro and in vivo studies that have been conducted on compounds in each of the subclasses of flavonoids and pancreatic cancer.

One class of flavonoids is the flavones and these include apigenin, luteolin, tangeretin and nobiletin, among others. Apigenin, an isoconformer of the isoflavone genistein, has been shown to possess antioxidant properties as well as antiinflammatory and anticarcinogenic effects [52]. In pancreatic cancer, apigenin has been shown to have an antiproliferative effect on cell growth by downregulating the expression of geminin protein and glucose uptake through inhibition of the GLUT-1 transporter [53, 54]. It has also been reported to induce cell cycle arrest at G2/M phase through the downregulation of cyclin B-cdc2 activity [55]. In addition, apigenin has been shown to enhance the inhibitory and apoptotic effects of the chemotherapeutic drug gemcitabine both in vitro and in vivo by downregulating NFκB activity with the suppression of Akt [56, 57].

The second class of flavonoids is the flavonols, which are commonly present in many fruits and vegetables, and some examples are quercetin, myricetin and kaempferol [58]. One flavonol of great interest due to its anticancer properties is quercetin. Quercetin has been shown to be a free-radical scavenging antioxidant, as well as having the ability to induce apoptosis and block
different phases of the cell cycle in a variety of cancer cell lines [59]. *In vitro* and *in vivo* quercetin has been shown to reduce apoptosis resistance in pancreatic cancer by downregulating Hsp70 protein [60]. In addition, it has been shown to inhibit the expression and function of P-glycoprotein in resistant to daunorubicin pancreatic cancer cell line EPP85-181RDB [61]. Another flavonol, kaempferol, has also been found to possess anticancer properties, such as the ability to inhibit DNA synthesis and growth in cancer cells. In pancreatic cancer cells kaempferol has been shown to provide an additive inhibitory effect when combined with 5-fluorouracil [62].

The third class of flavonoids is flavonones, commonly represented in the diet by hesperidin, hesperetin, naringenin and rutin. Hesperidin and rutin have recently been discovered to have antiproliferative effects on pancreatic cancer cells [48]; however, more research needs to be conducted to determine their exact mechanisms of action.

Flavanols, the fourth class of flavonoids, have a 2-phenylchromanol skeleton. Examples of this class of compounds are catechin, epicatechin, epicatechin 3-gallate, epigallocatechin and epigallocatechin 3-gallate. Epigallocatechin 3-gallate (EGCG), the major green tea catechin, has been found to act as an antioxidant, antiproliferative, antitumor and antiangiogenic agent in several types of cancer. Specifically in pancreatic cancer, it has been reported to induce apoptosis, both *in vitro* and *in vivo*, through the activation of caspase-3, the upregulation of proapoptotic Bax, Bak, Bcl-X<sub>S</sub> and PUMA, and the downregulation of antiapoptotic Bcl-2 and Bcl-X<sub>L</sub> [63-65]. Catechin, another green tea flavanol, has also been shown to have anticarcinogenic effects on pancreatic cancer by inducing early apoptosis [66].

The last class of flavonoids reported to have antipancreatic cancer effects is isoflavones. Isoflavones differ from the other classes of flavonoids due to the fact that they have the B-ring
attached at the C3 position rather than at C2. Some naturally occurring isoflavones include genistein, daidzein and genistin. Genistein, found in soybeans, has been shown to enhance the inhibitory and apoptotic effects of the chemotherapeutic drugs gemcitabine (in vitro and in vivo), erlotinib (in vitro) and cisplatin (in vitro and in vivo) by downregulating NFκB activity [67-70]. Most importantly, genistein has been shown to abrogate NFκB activity caused by gemcitabine and cisplatin [67, 69]. In vitro studies on the use of genistein alone to treat pancreatic cancer have shown that it has the ability to inhibit cancer cell growth and induce apoptosis through the downregulation of FoxM1, NFκB and Notch-1 activities [71-74]. In March 2010, a phase II clinical trial was completed on the use of genistein in combination with gemcitabine and erlotinib to treat patients with locally advanced or metastatic pancreatic cancer. The results of this study have not yet been published. As of July 2010 there is one phase II clinical trial recruiting that will look at the effect of genistein treatment on patients with resectable pancreatic cancer [75].

2.6.2 Curcumin

Curcumin is a fat-soluble polyphenolic compound that is the main and most active curcuminoid found in turmeric (Curcuma longa L.) [76]. This compound is commonly used as a spice in Asian countries as well as a natural food-coloring agent [77, 78]. Curcumin has been a bioactive compound of interest for many types of cancer including breast [79], prostate [80], oral [81], ovarian, endometrial [82] and others. The reason curcumin is so widely studied in a variety of cancers, as well as other diseases, is because of its many pharmacological properties including antioxidant, antiinflammatory, antimicrobial, antitumor, antidepressant and antiatherogenic activities [83, 84]. In pancreatic cancer studies, curcumin has been used as a bioactive agent in in vitro, in vivo, and phase I, II and III clinical trials.
The anticancer effects of curcumin on pancreatic cancer *in vitro* have been widely researched and documented. Early studies on the use of curcumin on pancreatic cancer showed that it has the ability to suppress NFκB activity through the inhibition of IkB kinase (IKK) and therefore decrease the expression of the NFκB regulated gene products COX-2, prostaglandin E$_2$ and interleukin-8 [85]. Curcumin has been shown to inhibit ERK activity, as well as downregulate EGFR and Notch-1 signaling leading to increased apoptosis in pancreatic cancer [86, 87]. Curcumin has also been shown to augment the cytotoxic effect on pancreatic adenocarcinoma cell lines when used in combination with the chemotherapeutic drugs gemcitabine or celecoxib [88, 89]. A more recent study conducted by Glienke *et al.* [90] was aimed to determine the effect of curcumin on Wilms’ tumor gene 1 (WT1), a gene frequently expressed in pancreatic cancer. The results showed that WT1 expression was able to be downregulated in a dose-dependent manner by curcumin. Another *in vitro* study conducted by the same research group [91] found that curcumin has the ability to inhibit signal transducer and activator of transcription 3 (STAT3) protein and induce apoptosis by the downregulation of the expression of the antiapoptotic gene Survivin/BIRC4 in pancreatic cancer cells. Sahu *et al.* [92] used the single dose of 2.5 µM of curcumin to determine the mechanism by which it causes G2/M phase cell cycle arrest. The results showed that G2/M phase cell cycle arrest was achieved by increased phosphorylation of H2A.X, Chk1 and ATM, as well as decreased DNA polymerase-β levels and expression of cyclin B1 and Cdk1. Lastly, a recently published study showed that curcumin-encapsulated MePEG/PCL (40:60) diblock copolymeric micelles could have a promising future for the controlled delivery of curcumin as cancer therapy [83]. In this investigation, the authors found that the curcumin-encapsulated micelles had up to 2.95 times more uptake into the pancreatic cancer cells than the un-modified curcumin. The results from
this study are of great importance because research has shown that curcumin, a naturally
hydrophobic compound, has very poor bioavailability when administered alone either orally or
intravenously [93].

Bar-Sela et al. [94] published a detailed review about the clinical trials that have been
conducted or are currently on-going that use curcumin as an anticancer agent. To summarize the
completed trials, two research groups published their results from their Phase II studies that used
8 g per day of curcumin as a treatment for patients with advanced pancreatic cancer. In the study
conducted by Epelbaum et al. [95], they used a combination of curcumin and gemcitabine to
treat seventeen patients and the results showed that this combined treatment option is tolerable in
patients but suggested that the oral dose of 8 g per day of curcumin should be reduced. In the
study conducted by Dhillon et al. [96], they only used curcumin as the 1st line treatment for the
twenty-five patients and the results showed that curcumin was able to downregulate the
expression of NFκB, COX-2 and phosphorylated STAT3 in peripheral blood. As of July 2010
there are currently three on-going clinical trials using curcumin as a treatment option for
pancreatic cancer, two phase II and one phase III [75]. The phase III clinical trial, being carried
out at the Tev-Aviv Sourasky Medical Center in Israel, is the first one for the use of curcumin to
treat pancreatic cancer. In this study the researchers are using a combination of curcumin and
the drugs gemcitabine and celecoxib to treat patients with advance or inoperable pancreatic
cancer.

2.7 Summary and Conclusion

Pancreatic cancer is a devastating disease with a dismal outlook for diagnosed patients.
Although there have not been a sufficient number of clinical trials, promising dietary factors to
prevent pancreatic cancer include citrus fruits, flavonoids and curcumin. Curcumin is one of the
most important bioactive compounds that have been studied for its chemoprotective effects on pancreatic cancer. *In vitro* studies have shown that curcumin has the ability to inhibit a diverse range of molecular targets in pancreatic cancer cells including NFκB, EGFR, WT1 and STAT3. Phase II clinical trials of curcumin have shown encouraging chemoprotective effects in patients with pancreatic cancer and have determined that curcumin can be safely administered to patients at oral doses up to 8 g/d. However, in order to validate curcumin as a pharmaceutical for pancreatic cancer treatment more large-scale trials are needed. Another important limitation of curcumin research is its poor bioavailability and more studies are needed to develop ways to effectively deliver curcumin to its target sites.

Several flavonoids found in a variety of fruits and vegetables have been shown to also inhibit pancreatic cancer at various molecular targets including cell cycle, Akt, NFκB, ERK and many others. The isoflavone genistein is one of the more studied flavonoids in pancreatic cancer. It has been shown to effectively inhibit NFκB and its regulated genes *in vitro*, both alone and in combination with chemotherapeutic drugs. Another important finding about genistein is that it has the ability to abolish the activation of NFκB induced by the chemotherapeutic drugs gemcitabine and cisplatin when used as a pretreatment for either of them. Currently there is one on-going phase II clinical trial on the use of genistein in treating resectable pancreatic cancer patients. However, more clinical trials are needed to explore the efficacy and application of genistein in treating pancreatic cancer.

The use of citrus fruit extracts to treat pancreatic cancer has become of interest only in the past few years. Using citrus fruit extracts instead of individual compounds to treat pancreatic cancer is of great interest because it allows the use of low doses of multiple bioactive compounds and nutrients instead of large doses of single compounds, therefore reducing the possibility of
reaching toxic effects. When comparing the inhibitory effects of different extraction methods of lime juice on pancreatic cancer, it was found that the methanol extract exhibited the highest inhibitory effect. While the results from this study provide insight into the best options for extracting citrus fruits, more research needs to be conducted on various types of citrus fruits extracts and their mechanisms of action by which they affect pancreatic cancer.

The role of food bioactive compounds in the prevention and/or treatment of pancreatic cancer is very promising. With a better understanding of how specific bioactive substances affect pancreatic cancer cell growth, scientists will be able to develop therapeutic treatment options that have less harmful side effects than current chemotherapeutic drugs.

2.8 References


Stewart, I., Wheaton, T., Conversion of β-citraurin to reticulataxanthin and β-apo-8'-carotenal to citranaxanthin during the isolation of carotenoids from citrus. *Phytochemistry* 1973, 12, 2947-2951.


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### Tables and Figures

**Table 2.1: Summary of Case-control Citrus Fruit and Pancreatic Cancer Studies.**

<table>
<thead>
<tr>
<th>Reference</th>
<th>Enrollment Period</th>
<th># of cases/ # of controls</th>
<th>Age range</th>
<th>Gender</th>
<th>Factors controlled for in analysis of citrus fruit intake</th>
<th>Type of citrus fruit</th>
<th>Comparison of exposure level</th>
<th>OR (95% CI)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>[26]</td>
<td>1991-2008</td>
<td>326/652</td>
<td>34-80</td>
<td>Italian male/female</td>
<td>Gender, age, cigarette smoking, alcohol consumption, body mass index, year of interview, education, self-reported history of diabetes and total energy</td>
<td>Citrus fruits</td>
<td>0 servings/wk vs 10.5 servings/wk</td>
<td>OR: 0.92 (0.54-1.57)</td>
</tr>
<tr>
<td>[28]</td>
<td>1995-1999</td>
<td>526/1,701</td>
<td>21-85</td>
<td>Male/female</td>
<td>Age, gender and energy intake</td>
<td>Orange, grapefruit, orange juice, grapefruit juice</td>
<td>&lt;2 servings/day vs &gt;4 servings/day</td>
<td>OR: 0.78 (0.58-1.0)</td>
</tr>
<tr>
<td>[36]</td>
<td>1980-1983</td>
<td>212/220</td>
<td>40-84</td>
<td>Caucasian male</td>
<td>Age, cigarette smoking, alcohol consumption, reported history of diabetes mellitus, educational level, and meat and vegetable consumption</td>
<td>Oranges, grapefruit, orange juice</td>
<td>&lt;5 times/mo vs &gt;20 times/mo</td>
<td>OR: 0.6 (0.3-1.1)</td>
</tr>
<tr>
<td>[37]</td>
<td>1986-1989</td>
<td>436/2003</td>
<td>30-79</td>
<td>African American and Caucasian male/female</td>
<td>Age at diagnosis/interview, race, study area, calories from food, diabetes mellitus, cholecystectomy, body mass index, cigarette smoking, alcohol consumption, income (men) and marital status (women)</td>
<td>Grapefruit; oranges or tangerines</td>
<td>&lt;1.5 servings/wk vs &gt;4 servings/wk</td>
<td>OR: 0.9 in men, OR: 1.2 in women</td>
</tr>
<tr>
<td>[40]</td>
<td>1984-1988</td>
<td>164/480</td>
<td>35-79</td>
<td>Dutch male/female</td>
<td>Age, gender, response status, total smoking and total energy</td>
<td>Citrus fruits</td>
<td>Q1 vs Q5 (by level of daily intake in grams)</td>
<td>OR: 0.95</td>
</tr>
<tr>
<td>[42]</td>
<td>1990-1993</td>
<td>451/1552</td>
<td>30-74</td>
<td>Chinese male/female</td>
<td>Age, income, smoking, green tea drinking (females only) and response status</td>
<td>Oranges/tangerines</td>
<td>&lt;2 servings/mo vs &gt;4 servings/mo</td>
<td>OR: 0.65 (0.42-0.99) in men, OR: 0.58 (0.34-0.99) in women</td>
</tr>
<tr>
<td>[102]</td>
<td>2004-2009</td>
<td>384/983</td>
<td>24-94</td>
<td>Male/female</td>
<td>Age, sex, smoking (current, former, never), body mass index, energy intake (per 1,000 kcal), number of drinks of alcohol per week</td>
<td>Orange/grapefruit juice</td>
<td>Q1 vs Q5 (grams/1,000 kcal)</td>
<td>OR: 0.52 (0.35-0.79)</td>
</tr>
</tbody>
</table>

* OR, odds ratio; CI, confidence interval.
<table>
<thead>
<tr>
<th>Reference</th>
<th>Enrollment Period</th>
<th># of cases/ # of noncases</th>
<th>Age range</th>
<th>Gender</th>
<th>Factors controlled for in analysis of citrus fruit intake</th>
<th>Type of citrus fruit</th>
<th>Comparison of exposure level</th>
<th>OR/HR/RR (95% CI)</th>
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</thead>
<tbody>
<tr>
<td>[27]</td>
<td>1998-2004</td>
<td>135/81,787</td>
<td>40-75</td>
<td>Swedish male/female</td>
<td>Age, gender, education, body mass index, physical activity, cigarette smoking status and pack-years of smoking, history of diabetes, multivitamin supplement use, intakes of total energy and alcohol</td>
<td>Oranges, grapefruits, orange juice, grapefruit juice and other citrus fruits</td>
<td>&lt;1 servings/wk vs ≥7 servings/wk</td>
<td>HR: 1.12 (0.68-1.83)</td>
</tr>
<tr>
<td>[38]</td>
<td>1993-1996</td>
<td>434/161,716</td>
<td>45-75</td>
<td>African American, Japanese American, Latino, Native Hawaiian and Caucasian male/female</td>
<td>Gender, time on study, race-ethnicity, age at cohort entry, smoking status, pack-years of smoking, family history of pancreatic cancer, energy intake, intakes of red meat and processed meat, and body mass index</td>
<td>Citrus fruits</td>
<td>&lt;13.4 g·1000 kcal⁻¹·day⁻¹ vs ≥93.9 g·1000 kcal⁻¹·day⁻¹</td>
<td>RR: 1.08 (0.82-1.43)</td>
</tr>
<tr>
<td>[39]</td>
<td>1982</td>
<td>3751/1,098,557</td>
<td>45-71 (male), 43-71 (female)</td>
<td>Male/female</td>
<td>Age, race, years of education, family history of pancreatic cancer in a first degree relative, history of gallstones, body mass index, cigarette smoking history, alcohol consumption, total red meat consumption, vegetable consumption and history of diabetes</td>
<td>Citrus fruits and juices</td>
<td>Q1 vs Q4</td>
<td>RR: 1.0 (0.8-1.2) in men, RR: 0.9 (0.7-1.1) in women</td>
</tr>
<tr>
<td>[41]</td>
<td>1985-1988</td>
<td>163/26,948</td>
<td>50-69</td>
<td>Male smokers</td>
<td>Age, years of smoking and energy intake by residue method (except coffee and tea)</td>
<td>Citrus fruits</td>
<td>≤5.3 g/day vs &gt;90.9 g/day</td>
<td>HR: 0.79 (0.47-1.31)</td>
</tr>
<tr>
<td>[43]</td>
<td>1988-1990</td>
<td>300/105,138</td>
<td>40-79</td>
<td>Japanese male/female</td>
<td>Age, area and pack-years of smoking</td>
<td>Citrus fruits</td>
<td>0-2/mo vs almost every day</td>
<td>HR: 0.85 (0.47-1.51) in men, HR: 1.07 (0.57-1.98) in women</td>
</tr>
</tbody>
</table>

*OR, odds ratio; HR, hazard ratio; RR, relative risk; CI, confidence interval.*
Table 2.3: Summary of *in vitro* and *in vivo* Anticarcinogenic Activities of Flavonoids against Pancreatic Cancer.

<table>
<thead>
<tr>
<th>Flavones / Flavanols</th>
<th>Mechanism of anti-cancer action (dose, exposure time, route of administration)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Apigenin</strong></td>
<td>Antiproliferative (100 µM for 24-72 h), inhibits DNA synthesis (6.5-100 µM for 24 h) and induces cell cycle arrest at G2/M phase through downregulation of cyclin A and B, phospho-activatedcdc2, cdc25A and cdc25C in AsPC-1, MIA PaCa-2, CD18 and S2-013 cells (100 µM for 24 h)</td>
<td>[55]</td>
</tr>
<tr>
<td></td>
<td>Inhibits proliferation (5-100 µM for 72 h) and induces apoptosis (10-80 µM for 48 h), as well as enhances the inhibitory and apoptotic effects of gemcitabine through the downregulation of NFκB activity with suppression of Akt activation and the reduction of Bcl-2 expression in MIA PaCa-2 and AsPC-1 cells (25 or 50 µM apigenin with 0-10 µM gemcitabine for 72 h)</td>
<td>[56]</td>
</tr>
<tr>
<td></td>
<td>Decreases glucose uptake through downregulation of GLUT-1 in CD18 and S2-013 cells and interferes with the PI3K/Akt pathway (0-100 µM for 24 h to assess glucose uptake, 6.5-50 µM for 24 h to assess GLUT-1 gene expression)</td>
<td>[54]</td>
</tr>
<tr>
<td></td>
<td>Increases the anticancer effects of gemcitabine on AsPC-1 and CD18 cells through induction of S and G2/M phase cell cycle arrest and increased apoptosis; downregulates pAkt expression induced by gemcitabine (25 µM apigenin for 6 h followed by the addition of 10 µM gemcitabine for 24 h); inhibits proliferation of gemcitabine-resistant cell line AsPC-1 (25-100 µM apigenin 24 h)</td>
<td>[57]</td>
</tr>
<tr>
<td></td>
<td>Downregulates geminin and Cdc6 at both mRNA and protein levels in the cell lines CD18 and S2-013 (25-50 µM for 24 h and 50 µM for 6-24 h); inhibits geminin promoter activity (50 µM for 6 h)</td>
<td>[53]</td>
</tr>
<tr>
<td><strong>Kaempferol</strong></td>
<td>Inhibits cell growth (17.5-70 µM for 4 days) and induces apoptosis (17.5-70 µM for 3 days) in MIA PaCa-2 and PANC-1 cells; provides an additive effect on the inhibition of MIA PaCa-2 cell proliferation when combined with 5-fluorouracil (35 µM kaempferol with 7.7 µM 5-fluorouracil for 5 days)</td>
<td>[62]</td>
</tr>
<tr>
<td></td>
<td>Antiproliferative effects on BsPC-3, MIA PaCa-2 and PANC-1 cells, induces apoptosis by upregulating caspase-3 and caspase-9 and downregulating Hsp70 protein (IC50 ~ 50 µM/L at 24 h for MIA PaCa-2 and PANC-1 cell lines)</td>
<td>[60]</td>
</tr>
<tr>
<td></td>
<td>Inhibits expression and function of P-glycoprotein in resistant to daunorubicin pancreatic cancer cell line EPP85-181RDB, as well as decreases the expression of ABCB1 (IC50 ~ 12 µM at 72 h)</td>
<td>[61]</td>
</tr>
<tr>
<td><strong>Quercetin</strong></td>
<td>Inhibits tumor growth and Hsp70 (50 mg/kg for 18 days, injection, 6 wk old male nude mice)</td>
<td>[60]</td>
</tr>
<tr>
<td></td>
<td>Antiproliferative effects on PANC-28 cells (IC50 = 147.28, 26.29 and 16.68 µg/mL at 24, 48 and 72 h, respectively)</td>
<td>[48]</td>
</tr>
<tr>
<td></td>
<td>Antiproliferative effects on PANC-28 cells (IC50 = 187.20, 49.47 and 41.73 µg/mL at 24, 48 and 72 h, respectively)</td>
<td>[48]</td>
</tr>
<tr>
<td><strong>Hesperidin</strong></td>
<td>Reduces cellular proliferation (100 µM, 24-72 h); induces early apoptosis in MIA/PACA and PANC-1 cells (100 µM for 18 h)</td>
<td>[66]</td>
</tr>
<tr>
<td></td>
<td>Antiproliferative effects on MIA PaCa-2 cells through apoptosis by caspase-3 mediated PARP cleavage (0.2 mM for 24 h); arrests cancer cells at an early phase of the cell cycle; causes mitochondrial membrane depolarization (0.2 mM for 14 h) and BAX oligomerization (0.1 mM) which facilitate the release of cytochrome c into the cytosol; facilitates the activation of ROS-mediated JNK (0.1 mM for 24 h)</td>
<td>[97]</td>
</tr>
<tr>
<td></td>
<td>Inhibits growth in PANC-1, MIA PaCa-2, AsPC-1 and Hs 766T cell lines (0-80 µM for 24 h); induces apoptosis through ROS-mediated caspase-3 and -9 activation (10-80 µM for 0-360 min for ROS activity; 0-80 µM for 12 h for caspase 3 activity), increased expression of Bax, Bak, Bcl-X(+) and PUMA, and inhibition of Bcl-2 and Bcl-X(–) (0-40 µM for 48 h); regulates MAP kinase pathways through the inhibition of Raf, Rafl, ERK 1/2 activities and p90 RSK, and the induction of MEKK1, JNK 1/2, p38 and aJUN activities (40 µM for 0-48 h); induces cell cycle arrest at G1 phase of the cell cycle through the upregulation of p21WAF1/CIP1 and p27KIP1 and the downregulation of cyclin D1, cdks4 and cdks6 (0-40 µM for 24 h for cell cycle; 40 µM for 0-48 h molecular markers)</td>
<td>[63]</td>
</tr>
<tr>
<td></td>
<td>Mediates apoptosis in MIA PaCa-2 cells through the activation of caspase-8 and -9, the disappearance of Bid protein, the upregulation of death receptor related genes and the downregulation of survivin (50 µg/mL for 8-24 h); provides synergistic effect when combined with TRAIL on apoptosis due to caspase-3 mediated PARP cleavage (50 µg/mL of EGCGR and 5 mg/mL for 16 h)</td>
<td>[98]</td>
</tr>
<tr>
<td></td>
<td>Inhibits cell growth (IC50&lt;50 µM at 48 h) in MIA PaCa-2 cells by binding directly to the C-terminal region of Hsp90 and preventing its association with the cochaperones p23 and Hsc 70 (60-200 µM); decreases cellular levels of Hsp90 client proteins Her-2, Akt, Cdk4, Raf-1 and pERK (80 µM for 0-24 h or 60 µM/24 h for up to 72 h); induces apoptosis through the activation of caspase-3 (60 µM/24 h)</td>
<td>[65]</td>
</tr>
</tbody>
</table>
Table 2.3 (cont.)

<table>
<thead>
<tr>
<th>Flavonoids (Cont.)</th>
<th>Mechanism of anti-pancreatic cancer action (dose, exposure time, route of administration)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>EGCG</strong></td>
<td><strong>In vivo Studies</strong></td>
<td>Decreases tumor growth in AsPC-1 xenografts through inhibition of cell proliferation caused by reduced Ki-67 and PCNA staining and induced growth arrest caused by induced p21&lt;sub&gt;WAF1/CIP1&lt;/sub&gt; expression; induces apoptosis through caspase-3 activation; regulates MAP kinase pathways through the inhibition of ERK activity and the induction of JNK and p38 activities; inhibits angiogenesis by reducing the expression of VWF, VEGF and CD31; blocks metastasis by inhibiting the expression of matrix metalloproteinases MMP-2, -7, -9 and -12 (60-100 mg/kg, 5 days a wk for entire experiment, 4-6 wks old athymic nude mice, oral)</td>
</tr>
<tr>
<td><strong>Isoflavones</strong></td>
<td><strong>In vitro Studies</strong></td>
<td>Inhibits cell growth (30 μM for 72-96 h), induces apoptosis (30 μM for 72-96 h or 50 μM for 72 h), and inhibits NFκB activity (30 or 50 μM for 2 h) in BxPC-3 cells; potentiates effects of cisplatin and docetaxel by inhibiting cell growth and inducing apoptosis (30 μM genistein for 24 h followed by 100 nM cisplatin or 1 nM docetaxel for 48 h); inhibits NFκB activity (30 μM of genistein and 100 or 150 nM of cisplatin for 24 h)</td>
</tr>
<tr>
<td>Genistein</td>
<td></td>
<td>Enhances cell growth inhibition of gemcitabine on COLO 357 and L3.6pl cell lines (25 μM genistein for 24 h followed by 25 nM of gemcitabine for 72 h); abrogates gemcitabine-induced activation of NFκB DNA-binding activity (30 μM for 48 h followed by 25 nM of gemcitabine for 6 or 24 h); sensitizes gemcitabine-treated cells to apoptosis by induction of caspase-3 mediated PARP cleavage and the downregulation of Bcl-2, Bcl-X&lt;sub&gt;L&lt;/sub&gt; and p-Akt (30 μM genistein for 48 h followed by 25 μM [COLO 357] or 100 nM [L3.6 pl] gemcitabine)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reduces NFκB activity in BxPC-3 cells thus downregulating Bcl-2 and Bcl-X&lt;sub&gt;L&lt;/sub&gt; (30 μM for 24 h); induces apoptosis (30 μM for 72 h); inhibits cell growth (30 μM for 72 h); decreases the penetration of pancreatic cancer cells through the matrigel-coated membrane (50 µM)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Inhibits cell growth of BxPC-3 cells (10-50 μM for 24-72 h); induces apoptosis through the downregulation of Notch-1 which leads to inhibition of IKK protein and therefore reduced NFκB activity (25 μM for 24-72 h)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Downregulates Notch-1 activity thus leading to a reduction in Hes-1, Bcl-X&lt;sub&gt;L&lt;/sub&gt; and cyclin D1 (25 μM for 24-72 h); inhibits cell growth in BxPC-3 cells (15-50 μM for 72 h); induces apoptosis (25 μM for 24-72 h) and inhibits NFκB DNA-binding activity (10-50 μM for 48 h)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Inhibits cell growth (0-50 μM for 24-72 h) and the activation of NFκB (25 and 50 μM for 72 h) in COLO 357 and L3.6pl cells; induces apoptosis through the downregulation of Bcl-2, Bcl-X&lt;sub&gt;L&lt;/sub&gt; and p-Akt, and the upregulation of caspase-3 (5-100μM for 72 h); sensitizes COLO-357 and L3.6pl cells to cisplatin induced growth inhibition (30 μM genistein for 24 h followed by coincubation with 1 and 2 μM of cisplatin for 72 h); augments apoptosis by cisplatin (30 μM genistein for 24 h followed by coincubation with 1 and 2 μM of cisplatin for 72 h) through the upregulation of cleaved caspase-3 and cleaved PARP and the downregulation of Bcl-2 and Bcl-X&lt;sub&gt;L&lt;/sub&gt;; downregulates NFκB activation caused by cisplatin (30 μM genistein for 72 h followed by 2.5 μM cisplatin for 2.5 h)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Potentiates the growth inhibition of erlotinib-treated cells (25 μM genistein and 2 μM cisplatin for 72 h); increases the apoptotic effect of erlotinib in BxPC-3 cells (25 μM genistein and 2μM erlotinib for 72 h) through the downregulation of EGFR, pAkt, NFκB activation, and survivin; potentiates the growth inhibition and apoptotic effects of combined gemcitabine and erlotinib treatment in COLO 357 cells by downregulating EGFR, survivin and Bcl-X&lt;sub&gt;L&lt;/sub&gt; (20 μM genistein, 1 μM erlotinib and 10 nM genistein)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Enhances growth inhibition and sensitizes the apoptotic effects of cisplatin on BxPC-3 cells (25 μM genistein for 24 h followed by 0.5 μM for 72 h); abrogates cisplatin-induced activation of NFκB activity thus downregulating Bcl-2 and Bcl-1-2 (10-50 μM genistein for 24 h followed by 0.5 μM of cisplatin for 72 h)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Increases tumor weight when combined with gemcitabine treatment and inhibits gemcitabine-induced activation of NFκB activity (1 mg/day of genistein, orally and 80 mg/kg body weight every other day of gemcitabine, injection, 13 days total, 4-6 wks old ICR-SCID female nude mice)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Inhibits cell proliferation in BxPC-3, HPAC, Mia PaCa-2 and PANC-28 cell lines (10-100 μM for 72 h); induces apoptosis (25-100 μM for 72 h); downregulates expression of FoxM1 (25-100 μM for 72 h) thus leading to the inhibition of cdk25α, survivin, MMP-9 and VEGF (0-100 μM for 72 h); decreases the penetration of pancreatic cancer cells through the matrigel-coated membrane (50 μM)</td>
</tr>
<tr>
<td><strong>In vivo Studies</strong></td>
<td></td>
<td>Reduces tumor weight when combined with gemcitabine treatment and inhibits gemcitabine-mediated activation of NFκB (9 mg/kg body weight of cisplatin as single intraperitoneal bolus injection and 1 mg/day of gemcitabine for 10 days, orally, 4-6 wks old ICR-SCID female mice)</td>
</tr>
<tr>
<td>Genistein</td>
<td></td>
<td>Reduces tumor weight when combined with cisplatin; abrogates cisplatin-mediated NFκB activation; increases apoptosis in cisplatin-treated tumors through the upregulation of PARP cleavage and the downregulation of Bcl-X&lt;sub&gt;L&lt;/sub&gt; (9 mg/kg body weight of cisplatin as single intraperitoneal bolus injection and 1 mg/day of gemcitabine for 10 days, orally, 4-6 wks old ICR-SCID female mice)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reduces tumor weight when combined with cisplatin; abrogates NFκB activity induced by cisplatin (800 μg/kg genistein for 5 days orally and 9 mg/kg cisplatin given as an intraperitoneal bolus injection, 4-wk old ICR-SCID female mice)</td>
</tr>
</tbody>
</table>

EGCG, Epigallocatechin gallate
Table 2.4: Summary of *in vitro* and *in vivo* Anticarcinogenic Activities of Flavonoids against Pancreatic Cancer since Manuscript Publication in 2011.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Mechanism of anti-pancreatic cancer action (dose, exposure time, route of administration)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Flavones</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>In vitro Studies</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apigenin</td>
<td>Blocks hypoxia-induced upregulation of HIF-1α, GLUT-1 and VEGF in CD18 and S2-013 cells (0-50 μM, 0-24 h)</td>
<td>[103]</td>
</tr>
<tr>
<td></td>
<td>Reduces cell proliferation (0-100 μM, 24-48 h) and induces apoptosis (0-25 μM, 24 h) through transcription-independent p53 function via interactions with BclXL and PUMA (0-25 μM, 48 h) in MIA PaCa-2 and BxPC-3 cells</td>
<td>[104]</td>
</tr>
<tr>
<td></td>
<td>Decreases tobacco-derived carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)-induced proliferation in BxPC-3 and MIA PaCa-2 cells (0-100 μM, 48 h) through inhibition of Fak and ERK activation (50 μM, 0-30 min)</td>
<td>[105]</td>
</tr>
<tr>
<td>Luteolin</td>
<td>Antiproliferative effects on MIA PaCa-2 cells (IC50 = 75 μM, 48 h), inhibits fatty acid palmitate de novo synthesis (1.56 and 0.46% at 50 and 100 μM, respectively) as well as nucleotide RNA ribose turnover via oxidative branch of the pentose cycle (16.15 and 6.86% at 50 and 100 μM, respectively), controls energy production (50 μM, 48 h)</td>
<td>[106]</td>
</tr>
<tr>
<td></td>
<td>Inhibits proliferation (0-80 μM, 12-72 h) of PANC-1, CoLo-357 and BxPC-3 cells, induces apoptosis (0-80 μM, 24 h) by decreasing Bcl-2, and increasing Bax, cleaved caspase 3 and cleaved PARP, and suppresses angiogenesis by inhibiting VEGF production (0-80 μM, 24 h, PANC-1 only) and secretion (0-100 μM, 24 h)</td>
<td>[107]</td>
</tr>
<tr>
<td><strong>In vivo Studies</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apigenin</td>
<td>Non-significantly reduces tumor weight and significantly induces apoptosis (0.2% apigenin-supplemented diet, orally, 6 wks, 4 wk old male nude mice)</td>
<td>[104]</td>
</tr>
<tr>
<td>Luteolin</td>
<td>Inhibits angiogenesis by reducing vessel growth (5-10 nmol/egg, 72 h) in the chicken chorioallantoic membrane of chicken embryos</td>
<td></td>
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<tr>
<td><strong>Flavonols</strong></td>
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<tr>
<td><strong>In vitro Studies</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Quercetin</td>
<td>Antiproliferative effects on MIA PaCa-2 cells (IC50 = 178 μM, 48 h) through inhibition of glycogen synthesis and turnover (23.8%, 100 μM, 48 h)</td>
<td>[106]</td>
</tr>
<tr>
<td></td>
<td>Inhibits proliferation and induces apoptosis alone (0-75 μM, 48 h), as well as enhances the inhibitory effects of gemcitabine in MIA PaCa-2 and BxPC-3 cells (10 μM quercetin with 0.001 μg/mL gemcitabine for 48 h)</td>
<td>[108]</td>
</tr>
<tr>
<td></td>
<td>Reduces cell proliferation (0-200 μM, 24 h), induces apoptosis in MIA PaCa-2, PANC-1 and S2-013 cells through increased caspase-3 and -9 activity (0-200 μM, 6 h) and inhibition of pAkt (0-100 μM, 24 h, MIA PaCa-2 and S2-013 cells only)</td>
<td>[109]</td>
</tr>
<tr>
<td>Myricetin</td>
<td>Reduces tumor volume and tumor weight alone or combined with gemcitabine treatment by decreasing proliferation and increasing apoptosis (1% quercetin-supplemented diet, daily, orally and 120 mg/kg gemcitabine every 7 days starting at day 10, intraperitoneal injection, 38 days, nude mice)</td>
<td>[108]</td>
</tr>
<tr>
<td></td>
<td>Decreases tumor growth (50 mg/kg in the MIA PaCa-2 model for 35 days, 50 mg/kg in the S2-013 model for 18 days, intraperitoneal injection, 4-6 wk old female nude mice) and pAkt (S2-013 model only)</td>
<td>[109]</td>
</tr>
<tr>
<td><strong>Flavanones</strong></td>
<td></td>
<td></td>
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<tr>
<td><strong>In vitro Studies</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Naringenin</td>
<td>Inhibits proliferation of AsPC-1 (IC50 = 347 μM, 72 h) and PANC-1 (IC50 = 541 μM, 72 h) cells, enhances the antiproliferative effects of gemcitabine in AsPC-3 cells (IC50 = 2.6 μM, 96 h), reverses TGF-β1-induced resistance to gemcitabine in AsPC-3 cells (0-10 μM gemcitabine and 50 μM naringenin, 72 h), suppresses TGF-β1-induced migration and invasion in both cell lines (0-100 μM, 36-72 h), and downregulates EMT markers by inhibiting the TGF-β1/Smad3 pathway (0-100 μM, 24 h) in both cell lines</td>
<td>[110]</td>
</tr>
</tbody>
</table>

35
<table>
<thead>
<tr>
<th>Compound</th>
<th>Mechanism of anti-pancreatic cancer action (dose, exposure time, route of administration)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Flavanols</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>In vitro Studies</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EGCG&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Antiproliferative effects on PANC-1 cells under hypoxic conditions (0-160 µg/mL, 12-48 h) through inhibition of HIF-1α and P-gp (0-80 µg/mL, 24 h)</td>
<td>[111]</td>
</tr>
<tr>
<td></td>
<td>Decreases migration and invasion (0-60 µM, 24 or 48 h, respectively), and induces apoptosis (0-40 µM, 48 h) through inhibition of the JAK3/STAT3 pathway (0-60 µM, 48 h) in AsPC-3 and PANC-1 cells; enhances the antiproliferative effects of gemcitabine (0.5 µM gemcitabine, 0-60 µM EGCG, 72 h) and the induction of apoptosis (0.5 µM gemcitabine, 0-60 µM EGCG, 72 h); increases the antiproliferative activity of CP690550 (0.5 µM CP690550, 0-60 µM EGCG, 72 h) and the induction of apoptosis (0.5 µM CP690550, 0-60 µM EGCG, 72 h)</td>
<td>[112]</td>
</tr>
<tr>
<td></td>
<td>Synergistically inhibits IL-1-induced apoptosis in CoLo-357 cells when combined with celecoxib by increasing caspase activity (50 µM EGCG, 0.01-10 µM celecoxib, 24 h), reduces angiogenesis through downregulation of IL-8 (50 µM EGCG, 0.01-10 µM celecoxib, 24-72 h) and VEGF (50 µM EGCG, 0.01-10 µM celecoxib, 24 h), and decreases invasion by inhibiting MMP-2 secretion (50 µM EGCG, 0.01-10 µM celecoxib, 24 h)</td>
<td>[113]</td>
</tr>
<tr>
<td><strong>In vivo Studies</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EGCG</td>
<td>Reduces tumor growth (0-100 mg/kg five times/wk for 28 days, gavage, 4-6 wk old Balb/c nude mice) through inhibition of the PI3K/AKT and ERK pathways and activation of FKHRL1/FOXO3α, induces apoptosis by activating caspase 3, decreases angiogenesis through inhibition of VEGF, and suppresses metastasis through inhibition of MMP-2 and -7</td>
<td>[114]</td>
</tr>
<tr>
<td><strong>Isoflavones</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>In vitro Studies</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Genistein</td>
<td>Suppresses cell survival of Notch-1 overexpressing AsPC-3 cells (0-60 µM, 72 h), inhibits Notch-1-mediated EMT phenotype (0-60 µM, 72 h), migration and invasion (30-60 µM, 24 h), formation of pancreatospheres (0-60 µM, 1-3 wks) and expression of CD44 and EpCAM (0-60 µM, 1 wk)</td>
<td>[115]</td>
</tr>
<tr>
<td></td>
<td>Inhibits TGF-β1-induced invasion and migration (0-50 µM, 48 h) in PANC-1 cells through downregulation of uPA and MMP-2 (0-50 µM, 24 h), decreases TGF-β1-induced EMT by downregulating E-cadherin, upregulating vimentin (0-50 µM, 48 h) and through the inhibition of Smad4-dependent and -independent (p38 MAPK) pathways (0-50 µM, 48 h)</td>
<td>[116]</td>
</tr>
</tbody>
</table>

<sup>a</sup>EGCG, Epigallocatechin gallate
Figure 2.1 A schematic diagram and histopathological images that show the progression of normal pancreatic ducts into invasive pancreatic adenocarcinomas (adapted from [100], histopathological images from [8, 100, 101].
Figure 2.2 Modulation of K-ras and the signaling pathways it affects in pancreatic cancer cells by citrus fruit extracts, apigenin, epigallocatechin 3-gallate (EGCG), genistein and curcumin.
Figure 2.3 Structures of some compounds reviewed in this paper that were found to inhibit pancreatic cancer. A) Citrus flavonoids and limonoids, B) Flavonoids, C) Curcumin.
CHAPTER 3

HYPOTHESIS AND OBJECTIVE

3.1 Long term goal

To understand the molecular mechanisms by which bioactive compounds present in fruits and vegetables can inhibit pancreatic cancer using \textit{in vitro} and \textit{in vivo} models.

3.2 Central hypothesis

Bioactive compounds present in fruits and vegetables will inhibit GSK-3β, thereby decreasing NF-κB transcriptional activity and decrease the expression of genes involved in proliferation and anti-apoptosis.

3.3 Overall objective

To examine the effect of bioactive compounds present in fruits and vegetables on pancreatic cancer proliferation, apoptosis, and the role of GSK-3β/NF-κB signaling pathway using \textit{in vitro} and \textit{in vivo} models.

3.4 Specific aims

\textbf{Aim 1 (Chapter 4): Evaluate the ability of fruit and vegetable bioactive compounds (flavonoids, limonoids and phenolic acids) to bind within GSK-3β’s catalytic site and inhibit its activity using an \textit{in vitro} enzymatic assay and computational modeling.}

\textit{Hypothesis:} Fruit and vegetable bioactive compounds will bind within GSK-3β’s catalytic site and inhibit its activity to different degrees depending on their structural properties.

\textit{Objectives:}

1.1 To quantify the inhibitory effects of bioactive compounds on the enzyme activity of GSK-3β using a luminescence assay.
1.2 To investigate the interactions between GSK-3β and bioactive compounds using molecular docking.

**Aim 2 (Chapter 5):** Test the inhibitory potential of fruit and vegetable bioactive compounds on the proliferation of BxPC-3 and PANC-1 human pancreatic cancer cells, *in vitro*, and the expressions of molecular markers in the GSK-3β/NF-κB signaling pathway.

**Hypothesis:** Flavonoids will inhibit the proliferation of pancreatic cancer cells through the inhibition of GSK-3β activity and modulation of markers of inflammation.

**Objectives:**

2.1 To examine the effect of bioactive compounds on the proliferation of BxPC-3 and PANC-1 pancreatic cancer cell lines, *in vitro*.

2.2 To determine the effect of apigenin treatment on the GSK-3β/NF-κB signaling pathway, cell cycle distribution and apoptosis in BxPC-3 and PANC-1 cells.

2.3 To evaluate the expression of genes involved in inflammation and cancer in BxPC-3 cells after apigenin treatment.

**Aim 3 (Chapter 6):** To assess the potential interactions of either apigenin or luteolin with chemotherapeutic drugs in BxPC-3 cells *in vitro*, and evaluate their mechanisms of action related to inhibition of the GSK-3β/NF-κB signaling pathway.

**Hypothesis:** Apigenin and luteolin will enhance the anti-cancer activity of chemotherapeutic drugs in BxPC-3 cells through inhibition of GSK-3β leading to the suppression of NF-κB activity.

**Objectives:**

3.1 To determine the interactions of apigenin and luteolin with chemotherapeutic drugs (cisplatin, 5-fluorouracil, gemcitabine and oxaliplatin) on BxPC-3 cell proliferation.
3.2 To evaluate the mechanisms of action on cell proliferation and apoptosis related to the GSK-3β/NF-κB signaling pathway.

Aim 4 (Chapter 7): To evaluate the potential of luteolin to enhance the anti-tumor effect of gemcitabine on pancreatic cancer using an orthotopic mouse model, and investigate their mechanism of action.

Hypothesis: Mice exposed to luteolin prior to gemcitabine chemotherapy will have less tumor growth compared to either treatment of luteolin or gemcitabine alone. The chemosensitizing effect of luteolin will be related to inhibition of the GSK-3β/NF-κB signaling pathway.

Specific Objectives:

4.1 To determine the effect of luteolin exposure followed by chemotherapy on the growth of pancreatic tumors in nude mice.

4.2 To evaluate the mechanism of action on cell proliferation and apoptosis related to the GSK-3β/NF-κB signaling pathway.
CHAPTER 4

CITRUS FLAVONOIDS LUTEOLIN, APIGENIN AND QUERCETIN INHIBIT GLYCOGEN SYNTHASE KINASE-3β ENZYMATIC ACTIVITY BY LOWERING THE INTERACTION ENERGY WITHIN THE BINDING CAVITY²

4.1 Abstract

Pancreatic cancer studies have shown that inhibition of glycogen synthase kinase-3β (GSK-3β) leads to decreased cancer cell proliferation and survival by abrogating nuclear factor kappa B (NFκB) activity. In this investigation, various citrus compounds including flavonoids, phenolic acids and limonoids were individually investigated for their inhibitory effects on GSK-3β using a luminescence assay. Out of the 22 citrus compounds tested, the flavonoids luteolin, apigenin and quercetin were found to have the highest inhibitory effects on GSK-3β, with IC₅₀ values of 1.5, 1.9 and 2.0 μM, respectively. Molecular dockings were then performed to understand the potential interactions of each citrus flavonoid with GSK-3β. Luteolin, apigenin and quercetin were predicted to fit within the binding pocket of GSK-3β with low interaction energies (-76.4, -76.1 and -84.6 kcal·mol⁻¹, respectively) and low complex energies (-718.1, -688.1 and -719.7 kcal·mol⁻¹, respectively). Our results indicate that several citrus flavonoids inhibit GSK-3β activity, and suggest that these have potential to suppress pancreatic cancer tumor growth.

4.2 Introduction

Pancreatic cancer is the fourth most common cause of cancer-related death among both men and women in the United States with a five year survival rate of only 4% [1]. It is estimated

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²This chapter is part of the paper: Johnson, J., Rupasinghe, S., Stefani, F., Schuler, M., Gonzalez de Mejia, E., Citrus flavonoids luteolin, apigenin and quercetin inhibit glycogen synthase kinase-3β enzymatic activity by lowering the interaction energy within the binding cavity. J. Med. Food 2011, 14, 325-333. Permission granted by Mary Ann Liebert, Inc.
that 43,140 new patients will be diagnosed with pancreatic cancer in 2010, with about half of these likely to die within 6 months of diagnosis [2]. Because of this poor prognosis for patients diagnosed with pancreatic cancer, there is a great need to expand our knowledge on the mechanisms causing this disease and to develop new preventive and treatment strategies.

One potential therapeutic target is glycogen synthase kinase 3 (GSK-3), a serine/threonine kinase that has become of interest due to its implications in many diseases including diabetes [3-5], Alzheimer’s disease [6-8] and cancer [9-13]. This kinase has two homologous mammalian isoforms, GSK-3α and GSK-3β, that are closely related (85% identical) but not functionally identical [14]. This is especially apparent in studies linking GSK-3β, but not GSK-3α, to pancreatic cancer [15-17]. It is now known that GSK-3β is overexpressed in the nucleus of pancreatic cancer cells where it stimulates nuclear factor kappa B (NFκB) activity and, as a consequence, activates the inflammatory response cascade [11]. Although the mechanism by which GSK-3β regulates NFκB is unknown, several studies have shown that inhibition of GSK-3β activity decreases NFκB activity and leads to decreased cancer cell proliferation and survival [11, 12].

Citrus fruits are of interest in cancer research due to the substantial amounts of bioactive compounds that they contain and the health benefits that these compounds confer. The bioactive compounds found in citrus fruits are fiber, folate, potassium, ascorbic acid (vitamin C) and phytochemicals (monoterpenes, limonoids, flavonoids, carotenoids and hydroxycinnamic acid) [18]. In vitro studies conducted with flavonoids and limonoids have shown that they inhibit the proliferation of human pancreatic cancer cells [19, 20] and have been accompanied by epidemiological studies showing an inverse association between the consumption of citrus fruits and the risk of pancreatic cancer [21-31]. Further investigation of the effects of citrus
compounds on this type of cancer is certainly warranted. The objective of our present study was to identify specific citrus compounds that inhibit GSK-3β activity. Inhibitor data collected using biochemical luminescence assays and computational molecular dockings provide direct evidence that several flavonoids in citrus fruit inhibit GSK-3β activity and predict binding modes for these compounds.

4.3 Materials and methods

Reagents

Human recombinant GSK-3β and phospho-glycogen synthase peptide-2 substrate were purchased from Millipore (Billerica, MA). Kinase-Glo Luminescent Kinase Assay™ was provided by Promega (Madison, WI). Citrus compounds purchased from Sigma-Aldrich (St. Louis, MO) included luteolin (≥ 98%), apigenin (≥ 95%), quercetin (≥ 98%), kaempferol (≥ 97%), rutin hydrate (≥ 94%), naringenin (≥ 95%), neohesperidin (≥ 90%), flavone (97%), naringin (≥ 90%), hesperidin (≥ 80%), caffeic acid (≥ 98%), chlorogenic acid (≥ 95%) and L-ascorbic acid (≥ 99%). Hesperetin (≥ 95%) was purchased from SAFC (Wicklow, Ireland) and limonin (≥ 90%) from MP BioMedicals (Solon, OH). Nobiletin (94.9%), tangeretin (96.4%), narirutin (93.9%), nomilin (87.7%), eriocitrin (97.4%), obacunone (85.8%) and azadirachtin (90.7%) were purchased from Chromadex (Irvine, CA). UltraPure water was purchased from Cayman Chemical (Ann Arbor, MI). ATP and all other reagents were purchased from Sigma-Aldrich (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA). Assay buffer contained 50 mM 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES) (pH 7.5), 15 mM magnesium acetate, 1 mM ethylenediaminetetraacetic acid (EDTA) and 1 mM ethylene glycol-bis(2-aminoethylether)-N,N′,N″,N‴-tetraacetic acid (EGTA). Enzyme buffer contained 50 mM Tris/HCl (pH 7.5), 150 mM NaCl, 0.1 mM EGTA, 0.03% Brij-35, 270 mM sucrose, 0.2 mM
phenylmethylsulfonyl fluoride (PMSF), 1 mM benzamidine and 0.1% 2-mercaptoethanol.

Below illustrates the scheme for the experimental procedures.

Glycogen synthase kinase-3β (GSK-3β) biochemical assay

GSK-3β activity was determined using the Kinase-Glo Luminescent Kinase Assay™ from Promega, as optimized by Baki et al. [32] In a typical assay, the test inhibitor was dissolved in dimethyl sulfoxide at a 10 mM concentration and then diluted to the desired concentrations (0.1, 1, 10, 25, 50, 100, 200 and 300 μM) using the assay buffer. The test inhibitor was then mixed in a black 96-well plate with 10 μL (20 ng) of GSK-3β and 20 μL assay buffer containing 25 μM substrate and 1 μM ATP. The mixture was incubated at 30°C for 30 min and the reaction was stopped by adding 40 μL of Kinase-Glo reagent. After an additional 10 min of incubation at 30°C, the luminescence was recorded using the luminescence option on the
Synergy 2 multi-mode microplate reader (BioTek Instruments, Inc., Winooski, VT). All plates carried negative controls (100% inhibition), which were achieved by adding 5 μM of the known GSK-3β inhibitor SB 415286 (Tocris Bioscience, Ellisville, MI), and positive controls (0% inhibition), which contained no test inhibitors and only assay buffer. The percent inhibition was calculated for each test inhibitor as follows:

\[
\% \text{ inhibition} = 100 \times \frac{\text{luminescence of test inhibitor} - \text{average positive control}}{\text{average negative control} - \text{average positive control}}
\]

Each citrus compound was assayed in at least two independent trials with three replicates of each concentration per trial and their inhibitory activities were expressed as the concentration of that particular citrus compound inhibiting GSK-3β activity by 20% and 50% (IC\textsubscript{20} and IC\textsubscript{50}).

**Molecular modeling**

**Preparation of GSK-3β structure**

Of the 35 GSK-3β crystal structures available in the Protein Data Bank (http://www.rcsb.org), only one structure (PDB 1I09) [33] had no ligand bound in the active site and our initial docking experiments were initiated with this structure. Another structure (PDB 1H8F) [34] had the smallest ligand (HEPES) in the active site and was used to fill in two large internal gaps in the ligand-free structure. In the 1I09 file, these missing loop regions include residues 120-126 and 286-300 and missing terminal regions include 24 residues at the N-terminus and 36 residues at C-terminus (Figure 4.1). The complete 420 amino acid GSK-3β sequence was obtained from the Protein Knowledgebase (http://www.uniprot.org) and used to build 10 different models of the combined GSK-3β structure in the Molecular Operating Environment (MOE) Program [35] using its HOMOLOGY function. The model with the best
residue packing score calculated by MOE’s residue packing quality function was selected and hydrogen atoms were added to the structure before it was energy-minimized using the CHARMM22 force field [36] and docked with inhibitors.

Preparation of structures of citrus compounds and synthetic inhibitor SB 415286

The chemical structures of most citrus compounds were downloaded from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (http://www.genome.jp/kegg/). Some of the rare citrus compounds and the GSK-3β inhibitor SB 415286 [37] were built in MOE using the MOLECULE BUILDER function and then energy-minimized using the MMFF94 force field [38].

Docking experiment

Ligands were docked within the active site of the energy-minimized GSK-3β protein model using the DOCK function within MOE, which docks each ligand by initially placing it in the identified binding cavity and allowing it to vary through Monte Carlo simulations that remove biases due to manual placement. For each ligand, fifty possible conformations were generated while maintaining rigid protein side chains. The interaction energies between the ligand conformations and the protein were then calculated using the potential energy function in MOE and the five with the lowest energies were selected as the optimal conformations for each ligand and subjected to further energy minimization using the MMFF94 force field while allowing full protein side chain relaxation. The interaction energies between the minimized protein and the ligands were calculated as the difference between the total potential energy of the minimized complex and the sum of the individual protein and ligand components of the complex. The potential energy function contains the sum of the ligand/protein internal energy,
van der Waals and electrostatic energy, and terms the conformation with the lowest sum [39, 40]. The conformation with the lowest calculated interaction energy was selected as the most possible binding conformation.

**Statistical analysis**

The sigmoidal dose-response analysis of the concentrations needed to inhibit 20% and 50% (IC\textsubscript{20} and IC\textsubscript{50}) of GSK-3β activity were determined by nonlinear regression (curve fit) using the GraphPad Prism software [41]. Pearson correlation analyses were performed using SAS 9.2 statistical software [42].

4.4 **Results and discussion**

*Citrus compounds inhibit GSK-3β activity*

Among the citrus compounds tested (flavonoids, limonoids, phenolic acids and ascorbic acid), the flavonoids (Figure 4.2A presents the basic chemical structure of flavonoids) were found to have the overall highest inhibitory activity on GSK-3β and their results are displayed in Tables 4.1 and 4.2. In particular, the flavonoids luteolin (IC\textsubscript{50} = 1.5 µM), apigenin (IC\textsubscript{50} = 1.9 µM), quercetin (IC\textsubscript{50} = 2.0 µM), kaempferol (IC\textsubscript{50} = 3.5 µM), rutin (IC\textsubscript{50} = 10.3 µM), hesperetin (IC\textsubscript{50} = 26.9 µM) and naringenin (IC\textsubscript{50} = 45.7 µM) all inhibited GSK-3β activity by at least 50% at concentrations of 50 µM or lower. Based on the structures of the flavonoids (Figure 4.2B and C) tested, it is apparent that side group substitutions affect their capability to inhibit GSK-3β activity and that flavonoids with larger side groups have lower inhibitory activity. For instance, hesperidin, narirutin, eriocitrin, naringin and neohesperidin, all of which have sugar substitutions, have low inhibitory activities ranging from 3-34% at concentrations of 100 or 300 µM. Other classes of citrus compounds, such as the limonoids (nomilin, obacunone, limonin
and azadirachtin), phenolic acids (caffeic acid and chlorogenic acid) and ascorbic acid, also have less of an effect on GSK-3β activity with inhibition ranging from 1-40% at concentrations of 100, 200 or 300 µM (data not shown).

*Molecular interactions of GSK-3β and citrus compounds*

The citrus flavonoids were selected for further molecular docking analysis because of their high inhibitory activity on GSK-3β. Because no crystal structure of GSK-3β bound with SB 415286 or a flavonoid exists, the first step in our docking process was to identify the potential binding site for citrus flavonoids. For this, the crystal structure of GSK-3β bound to another anilinomaleamide of comparable size to SB 415286, 2-chloro-5-[(4-(3-chloro-phenyl)-2,5-dioxo-2,5-dihydro-1H-pyrrol-3-yl]-amino}benzoic acid (I-5) (PDB 1Q4L) [43], was used to locate the probable binding site for flavonoids within the active site of the GSK-3β structure. Figure 4.3 shows the orientation of I-5 bound in the active site of the GSK-3β crystal structure, located between the N-terminal β-strand domain and the C-terminal α-helical domain and bordered by a glycine-rich loop and a hinge region. For each citrus flavonoid, the conformation closest to the binding position of I-5 and with the least predicted interaction energy was selected as the most possible binding conformation.

Analysis of the predicted binding site for flavonoids (Figure 4.4A) showed a planar cleft with a narrow opening of about 11 Å in length and about 4.5 Å in width. The top and bottom surfaces of the cleft were formed by hydrophobic residues and the edges were formed by polar residues from the hinge and the glycine-rich loop of the GSK-3β structure. The edge directly opposite to the cavity opening had fewer polar residues. This architecture appears to make the site uniquely suitable for planar aromatic ligands with polar substituted groups. All the flavonoids were predicted to bind in almost the same orientation with the B-ring hydroxyls
stabilized by hydrogen bonding with Arg141 and Tyr134 in the hinge, A-ring hydroxyls
stabilized by hydrogen bonding with Asn64, Gly65, Lys85 and Asp200 residues in the glycine-
rich loop, and the carbonyl oxygen on the C-ring facing towards the back of the cavity.

Tables 4.1 and 4.2 compare the IC$_{20}$, IC$_{50}$, interaction energy (IE) and complex energy
(CE) values of the flavonoids tested and the two known GSK-3β inhibitors, as well as their
physicochemical properties. Luteolin, apigenin, quercetin, kaempferol and flavone all have an
unsaturated C-ring (Figure 4.2B) that allows them to remain planar in the binding cavity of the
enzyme, thereby avoiding unfavorable steric interactions and allowing them to minimize their
CEs (-626.7 to -719.7 kcal·mol$^{-1}$). The fact that, among these five flavonoids, flavone has no
hydroxyl residues capable of hydrogen bonding with residues in the active site causes its IE to be
quite high (-24.1 kcal·mol$^{-1}$) compared to the others (IE = -67.1 to -84.6 kcal·mol$^{-1}$). Further
analysis showed that there is a negative correlation ($r = -0.83$) between the number of hydroxyl
substituted groups on the flavonoids (includes all 15 flavonoids tested) and the predicted IE
values of the flavonoids. The lower IE values calculated for the various hydroxylated flavonoids
indicate that their interactions are comparatively more favorable and likely to more effectively
block GSK-3β activity, as observed in our biochemical assays.

A number of other studies have reported the importance of the presence of hydroxyl
groups on the flavonoid structure for some of its biochemical activities. Particularly relevant to
our discussion of cancer prevention, a study conducted by Ueda et al. [44] determined that
flavonoids with hydroxyl groups on the A-5, A-7 and B-4′ positions, such as luteolin, apigenin,
quercetin and kaempferol, had the highest inhibitory effect on tumor necrosis factor α (TNF-α)
production in vitro. Several studies have also demonstrated that the total number and location of
hydroxyl groups on flavonoids greatly influence their impact on several mechanisms of antioxidant activity [45-49].

Both hesperetin and naringenin contain hydroxyl groups on their A- and B-rings that form hydrogen bonds with residues in the active site and have lower inhibitory capacities (IC$_{50}$ = 26.9 µM for hesperetin and 45.7 µM for naringenin) and slightly higher IE values (IE = -56.1 kcal·mol$^{-1}$ for hesperetin and -60.7 kcal·mol$^{-1}$ for naringenin) than luteolin (IC$_{50}$ = 1.5 µM and IE = -76.4 kcal·mol$^{-1}$). Comparison of their structures (Figure 4.2B) shows that the C-ring of luteolin is unsaturated, while the C-rings in both hesperetin and naringenin are saturated, causing bulging in the middle of these molecules and unfavorable steric interactions in the narrow binding site. A study conducted by Chen et al. [50] assaying for proteasome inhibition also found that flavonoids having saturated C-rings are much less potent than flavonoids having unsaturated C-rings. In particular, unsaturated luteolin and apigenin were approximately 11- and 21-fold more effective at inhibiting 20S and 26S proteasome activities than their saturated counterparts eriodictyol and naringenin, respectively.

Both nobiletin and tangeretin contain multiple methoxy side groups (Figure 4.2B) that decrease their ability to form hydrogen bonds in the active site, as well as decrease their inhibitory capacities. In addition to the lower number of potential hydrogen bonds, the large methyl groups on these molecules lead to steric interactions that increase their IEs and CEs. Analyses of their docking conformations did not provide an explanation for why the inhibitory activity of nobiletin (IC$_{50}$ = 52.5 µM) with six methoxy groups was so much higher than that of tangeretin (IC$_{50}$ > 100 µM) with five methoxy groups. The IC$_{50}$ values of flavonoids that were found to inhibit GSK-3β activity, including luteolin, apigenin, quercetin, kaempferol, rutin, hesperetin, naringenin and nobiletin correlated with their IE values (r = 0.71).
In comparing the non-glycosylated citrus flavonoids (Table 4.1) with the glycosylated citrus flavonoids (Table 4.2), it was determined that conjugation of bulky sugar groups to the flavonoid core structure drastically decreased the inhibitory capacity of the tested flavonoids. Docking results indicate that the complex energies calculated for GSK-3β bound to these glycosylated flavonoids is generally higher than for flavonoid aglycones, causing the interactions to be less stable and have shorter lifetimes. These results are similar to a study conducted by Lin et al. [51] that found, through molecular modeling and docking, flavonoids conjugated with sugars had weaker interactions with xanthine oxidase, an enzyme that causes gout and is responsible for oxidative damage to tissues. In both our work and that of Lin et al. [51], it is likely that glycosylation decreases the hydrophobicity of the flavonoids thereby reducing hydrophobic interactions between these ligands and their target proteins. Rutin is the exception to this conclusion in that it has a much greater inhibitory capacity (IC$_{50}$ = 10.3 µM) compared to the other glycosylated flavonoids. Examination of the rutin structure (Figure 4.2C) indicates that its sugar group is conjugated to the C-ring of the flavonoid skeleton rather than to the A-ring, as in all other tested glycosylated flavonoids. This orientation is much more favorable for binding to the GSK-3β active site since it causes the sugar group to orient to the middle of the opening in the binding site (Figure 4.5), rather than very close to the edge of the opening as is the case for narirutin (also shown in Figure 4.5) and other glycosylated flavonoids (not shown).

To the best of our knowledge, this is the first report of the inhibition of GSK-3β activity by citrus bioactive compounds evaluated biochemically and computationally. In 2009, Bustanji et al. [52] studied the effects of curcumin, another polyphenol, on GSK-3β activity. In their study they used a different software program, FRED software, and a different GSK-3β crystal structure, 1Q5K [53]. However, both studies found that either ligand, curcumin [52] or
flavonoids (present study), helped stabilize themselves within the GSK-3β active site by hydrogen bonding with the amino acid residues Lys85 and Arg141. The researchers of the curcumin study also validated their docking results by in vitro studies, which showed curcumin potently and more effectively inhibited GSK-3β (IC$_{50} = 66.3$ nM) than the GSK-3β known inhibitor TDZD-8 (IC$_{50} = 1.5$ μM). Additional in vivo analyses by these researchers showed that curcumin significantly increased liver glycogen reserves in fasting Balb/c mice in a dose-dependent manner, possibly as the result of GSK-3β inhibition. These results, along with our findings, provide critical evidence documenting the need for further investigation into the mechanisms of inhibition of GSK-3β and the downstream effects this may cause. A limitation of our study is that it is not of physiological relevance at this time. However, in our laboratory we are currently studying the effects of citrus compounds in pancreatic cancer cells to determine if inhibition of GSK-3β activity is indeed part of their mechanism of action. Future studies will consider bioavailability and metabolism of these flavonoids.

In conclusion, our study demonstrated that a variety of citrus flavonoids can inhibit GSK-3β activity directly by binding in the active site of the enzyme. Flavonoids with hydroxyl side groups that are available for hydrogen bonding with the amino acid residues in the enzyme were the most favorable. Flavonoids with large side groups (i.e. methoxy groups or sugar conjugations) were much more unfavorable due to the drastic alterations the enzyme had to make in order to accommodate them into its binding site.

4.5 References


[41] GraphPad Prism Software, Version 4.0. 2003. GraphPad Software Inc., La Jolla, CA, USA.


### 4.6 Tables and figures

#### Table 4.1. Physicochemical properties, GSK-3β inhibition and predicted interaction and complex energies of citrus flavonoids

<table>
<thead>
<tr>
<th>Compound</th>
<th>Molecular Mass (mg/mmol)</th>
<th>Free OH Groups</th>
<th>IC$_{20}$ Value (μM)</th>
<th>IC$_{50}$ Value (μM)</th>
<th>Interaction Energy (kcal•mol$^{-1}$)</th>
<th>Complex Energy (kcal•mol$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Luteolin</td>
<td>286.24</td>
<td>4</td>
<td>0.48 ± 0.03</td>
<td>1.51 ± 0.05</td>
<td>-76.4</td>
<td>-718.1</td>
</tr>
<tr>
<td>Apigenin</td>
<td>270.24</td>
<td>3</td>
<td>0.48 ± 0.03</td>
<td>1.91 ± 0.06</td>
<td>-76.1</td>
<td>-688.1</td>
</tr>
<tr>
<td>Quercetin</td>
<td>302.24</td>
<td>5</td>
<td>0.68 ± 0.00</td>
<td>2.04 ± 0.00</td>
<td>-84.6</td>
<td>-719.7</td>
</tr>
<tr>
<td>Kaempferol</td>
<td>286.24</td>
<td>4</td>
<td>0.83 ± 0.03</td>
<td>3.47 ± 0.11</td>
<td>-67.1</td>
<td>-626.7</td>
</tr>
<tr>
<td>Hesperetin</td>
<td>302.28</td>
<td>3</td>
<td>5.89 ± 0.38</td>
<td>26.92 ± 0.88</td>
<td>-56.1</td>
<td>-710.2</td>
</tr>
<tr>
<td>Naringenin</td>
<td>272.26</td>
<td>3</td>
<td>10.97 ± 0.36</td>
<td>45.71 ± 0.00</td>
<td>-60.7</td>
<td>-688.1</td>
</tr>
<tr>
<td>Flavone</td>
<td>222.24</td>
<td>0</td>
<td>44.68 ± 1.45</td>
<td>&gt;100</td>
<td>-24.1</td>
<td>-675.0</td>
</tr>
<tr>
<td>Nobiletin</td>
<td>402.39</td>
<td>0</td>
<td>12.30 ± 0.00</td>
<td>52.48 ± 0.00</td>
<td>-51.8</td>
<td>-586.8</td>
</tr>
<tr>
<td>Tangeretin</td>
<td>372.37</td>
<td>0</td>
<td>&gt;100 (13.2%, 100 μM)</td>
<td>&gt;100</td>
<td>-48.6</td>
<td>-592.1</td>
</tr>
</tbody>
</table>

**Flavonoids**

**Methoxyflavones**

[a] Data represent the mean ± standard deviation. The lower the IC value, the higher the potency.

[b] The interaction energies and complex energies were calculated using the potential energy function in MOE. The lower the energy, the stronger the binding.
Table 4.2. Physicochemical properties, GSK-3β inhibition and predicted interaction and complex energies of citrus flavonoids and synthetic inhibitors SB 415286 and I-5.  

<table>
<thead>
<tr>
<th>Compound</th>
<th>Molecular Mass (mg/mmol)</th>
<th>Free OH Groups</th>
<th>IC_{50} Value (μM)</th>
<th>IC_{50} Value (μM)</th>
<th>Interaction Energy (kcal·mol(^{-1}))</th>
<th>Complex Energy (kcal·mol(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Glycosylate Flavonoids</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Rutin</td>
<td>610.52</td>
<td>10</td>
<td>2.58 ± 0.34</td>
<td>10.28 ± 1.33</td>
<td>-98.0</td>
<td>-576.8</td>
</tr>
<tr>
<td>Hesperidin</td>
<td>610.57</td>
<td>8</td>
<td>133.79 ± 15.22</td>
<td>&gt;300</td>
<td>-136.2</td>
<td>-560.5</td>
</tr>
<tr>
<td>Narirutin</td>
<td>580.53</td>
<td>8</td>
<td>&gt;100 (11.3%, 100 μM)</td>
<td>&gt;100</td>
<td>-142.9</td>
<td>-556.5</td>
</tr>
<tr>
<td>Eriocitrin</td>
<td>596.53</td>
<td>9</td>
<td>&gt;100 (10.3%, 100 μM)</td>
<td>&gt;100</td>
<td>-88.9</td>
<td>-606.0</td>
</tr>
<tr>
<td>Naringin</td>
<td>580.53</td>
<td>8</td>
<td>&gt;300 (15.0%, 300 μM)</td>
<td>&gt;300</td>
<td>-134.6</td>
<td>-566.5</td>
</tr>
<tr>
<td>Neohesperidin</td>
<td>610.56</td>
<td>8</td>
<td>&gt;100 (2.8%, 100 μM)</td>
<td>&gt;100</td>
<td>-122.4</td>
<td>-529.8</td>
</tr>
<tr>
<td><strong>Synthetic Inhibitors</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SB 415286</td>
<td>359.73</td>
<td>1</td>
<td>0.02 ± 0.00</td>
<td>0.07 ± 0.00</td>
<td>-94.0</td>
<td>-641.4</td>
</tr>
<tr>
<td>I-5</td>
<td>377.18</td>
<td>1</td>
<td>N/A</td>
<td>0.160</td>
<td>-99.0</td>
<td>-682.5</td>
</tr>
</tbody>
</table>

\(^{a}\) Data represent the mean ± standard deviation. The lower the IC value, the higher the potency.  
\(^{b}\) The interaction energies and complex energies were calculated using the potential energy function in MOE. The lower the energy, the stronger the binding.  
\(^{c}\) Value reported in literature [43].
**Figure 4.1. Sequence alignments.** Amino acid sequences of the GSK-3β structures reported in PDB 1H8F [34] and 1I09 [33] and the complete 420 amino acid coding sequence of GSK-3β are aligned. Both 1H8F and 1I09 are missing residues from their N- and C-termini; 1I09 is missing two loop regions between residues 120-126 and 286-300.

<table>
<thead>
<tr>
<th></th>
<th>1H8F</th>
<th>1I09</th>
<th>GSK3B_2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amino acid sequences of the GSK-3β structures reported in PDB 1H8F [34] and 1I09 [33] and the complete 420 amino acid coding sequence of GSK-3β are aligned. Both 1H8F and 1I09 are missing residues from their N- and C-termini; 1I09 is missing two loop regions between residues 120-126 and 286-300.</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>
Figure 4.2. Flavonoids and known GSK-3β inhibitor structures. (A) Basic flavonoid structure. The A-ring is commonly hydroxylated at positions 5 and 7, while the B-ring is hydroxylated at positions 4’, 3’4’ or 3’4’5’. (B) Structures of flavonoids presented in Table 1. (C) Some of the flavonoid structures and the two known inhibitor structures presented in Table 4.2.

A

B

luteolin
apigenin
quercetin
kaempferol
flavone

hesperetin
naringenin
nobiletin
tangeretin

C

rutin
narirutin

SB 415286

I-5
Figure 4.3. Predicted structure of GSK-3β complexed with I-5. The predicted protein backbone of GSK-3β is shown in ribbon format with α-helices shown in red, β-sheets in yellow and loops in blue. The active site is located between the β-rich N-terminus and the α-rich C-terminus and the predicted binding mode of I-5 is shown in space-filling format.
Figure 4.4. Predicted docking mode for luteolin in the binding cavity of GSK-3β. (A) Surface representation of the binding cavity of GSK-3β is shown with the predicted mode for luteolin binding and the interacting amino acid residues. Binding cavity residues are shown with acidic residues in red, basic residues in dark blue, hydrophobic residues in green and hydrophilic residues in light blue. (B) Two-dimensional representation of the luteolin interacting residues following the method of Clark and Labute [54]. Binding cavity residues that are shown with green circles indicate residues with no polar or charged side chains. Residues with light mauve circles indicate polar side chains that can be acidic, indicated by a red ring, or basic, indicated by a blue ring. The arrows indicate hydrogen bonds to side chain residues in green and backbone residues in blue.
Figure 4.5. Predicted docking modes for rutin and narirutin in the binding cavity of GSK-3β. Surface representation of the binding cavity of GSK-3β is shown with the predicted modes for rutin in blue and for narirutin in orange. The binding cavity residues are shown with acidic residues in red, basic residues in dark blue, hydrophobic residues in green and hydrophilic residues in light blue.
CHAPTER 5

FLAVONOID APIGENIN MODIFIED GENE EXPRESSION ASSOCIATED WITH INFLAMMATION AND CANCER AND INDUCED APOPTOSIS IN HUMAN PANCREATIC CANCER CELLS THROUGH INHIBITION OF GSK-3β/NF-κB SIGNALING CASCADE

5.1 Abstract

Scope: The objective was to examine the inhibitory effects of citrus fruit bioactive compounds on BxPC-3 and PANC-1 human pancreatic cancer cells, focusing on the anti-proliferative mechanism of action of the flavonoid apigenin related to the glycogen synthase kinase-3β (GSK-3β)/NF-κB signaling pathway.

Methods and results: Flavonoids, limonoids, phenolic acids and ascorbic acid were tested for cytotoxic effects on BxPC-3 and PANC-1 cells; apigenin was the most potent (IC₅₀ = 23 and 12 µM for 24 and 48 h for BxPC-3 and IC₅₀ = 71 and 41 µM for 24 and 48 h for PANC-1).

Apigenin induced pancreatic cell death through inhibition of the GSK-3β/NF-κB signaling pathway. Apigenin arrested cell cycle at G₂/M phase (36% and 32% at 50 µM for BxPC-3 and PANC-1, respectively) with concomitant decrease in the expression of cyclin B1. Apigenin activated the mitochondrial pathway of apoptosis (44% and 14% at 50 µM for BxPC-3 and PANC-1, respectively) and modified the expression of apoptotic proteins. Apigenin highly upregulated the expression of cytokine genes IL17F (114.2-fold), LTA (33.1-fold), IL17C (23.2-fold), IL17A (11.3-fold), and IFNB1 (8.9-fold) in BxPC-3 cells, which potentially contributed to the anti-cancer properties.

Conclusion: Flavonoids have a role in prevention of pancreatic cancer tumorigenesis.

5.2 Introduction

Pancreatic cancer is a significant health problem with an estimated 45,000 new diagnoses and 38,000 deaths anticipated in 2013 [1]. The poor prognosis for pancreatic cancer is due to its aggressive nature and to its late diagnosis as a result of the absence of clinical symptoms at early stages in this disease [2]. The most common therapy options for cancer patients are surgery, chemotherapy and radiation. More recently, targeted therapies have gained interest due to their ability to block the growth of cancer cells by interfering with specific molecules required for tumor development and proliferation [3].

One potential molecular target in pancreatic cancer is glycogen synthase kinase-3β (GSK-3β), a multifunctional serine/threonine kinase. GSK-3β is of significant interest because alterations in its regulation have been implicated in several types of cancers [4] including pancreatic [5], colon [6] and ovarian [7]. Overexpression of GSK-3β due to the upstream K-Ras/MAPK/ETS cascade has been implicated in pancreatic cancer [8], leading to the stimulation of NF-κB transcriptional activity [9-12] and as a consequence, activating genes that control cell proliferation and apoptosis [13]. Although the exact mechanism by which GSK-3β alters NF-κB-mediated gene transcription is unclear, it might involve regulation of transcriptional binding partners through phosphorylation [14]. It is critical to develop novel targeted therapies aimed at slowing the progression of pancreatic cancer through the identification of natural compounds that can inhibit GSK-3β.

Citrus fruit compounds including flavonoids, limonoids, phenolic acids and ascorbic acid, have the potential to be used as therapeutic agents for pancreatic cancer. This is supported by epidemiological evidence demonstrating that consumption of citrus fruits leads to a decrease in the risk for developing pancreatic cancer [15-17]. It is also supported by in vitro pancreatic
cancer studies conducted with citrus flavonoids (rutin, neohesperidin, hesperidin and hesperetin) and limonoids (limonexic acid, isolimonexic acid, limonin, limonin glucoside and obacunone) showing that these compounds inhibit cell proliferation and induce apoptosis [18-20]. The flavonoid apigenin is not only found in citrus fruits, but also in herbs (chamomile, parsley), vegetables (celery, onions) and beverages (tea, red wine and beer) [21]. Apigenin possesses anti-inflammatory [22], anti-proliferative [23] and anti-carcinogenic [24] properties. However, there is a need to study the molecular mechanisms of action by which apigenin exerts its anti-proliferative effects in pancreatic cancer.

The objective of this study was to examine the effect of 22 pure citrus compounds on proliferation of BxPC-3 and PANC-1 human pancreatic cancer cells, including the specific effect of apigenin on cell cycle, apoptosis and the GSK-3β/NF-κB signaling pathway. In addition, we investigated the effect of apigenin treatment in BxPC-3 cells on the expression of genes involved in inflammation and cancer. The results showed that apigenin inhibited the GSK-3β/NF-κB signaling pathway leading to an induction of the mitochondrial pathway of apoptosis in both cell lines. Gene expression analysis revealed apigenin treatment (50 µM, 24 h) upregulated 59 genes and downregulated 63 genes related to inflammation and cancer in BxPC-3 cells. It was demonstrated that apigenin highly upregulated the cytokine genes interleukins (IL) 17A, 17C, and 17F, lymphotoxin alpha (LTA) and interferon beta 1 (IFNB1).

5.3 Materials and methods

5.3.1 Materials

Roswell Park Memorial Institute (RPMI)-1640 and DMEM growth mediums, and 0.25% (w/v) trypsin-0.53 mM EDTA were purchased from American Type Culture Collection (ATCC) (Manassas, VA). Keratinocyte-SFM (1X) growth medium supplied with human recombinant
Epidermal Growth Factor (EGF) and Bovine Pituitary Extract (BPE), 1X antibiotic-antimycotic, 0.5% trypsin-EDTA and soybean trypsin inhibitor were purchased from Life Technologies (Carlsbad, CA). Penicillin-streptomycin and sodium pyruvate were purchased from Corning Inc. (Corning, NY), fetal bovine serum (FBS) from Hyclone (Thermo Scientific Hyclone, Logan, UT), and dimethyl sulfoxide (DMSO) from Sigma-Aldrich (St. Louis, MO). Citrus compounds (% purity) purchased from Sigma-Aldrich were apigenin (>95%), caffeic acid (>98%), chlorogenic acid (>95%), flavone (>99%), hesperidin (>80%), hesperetin (>95%), L-ascorbic acid (>99%), limonin (>90%), naringenin (>95%), naringin (>90%), neohesperidin (>90%), quercetin (>98%) and rutin hydrate (>94%). Luteolin (>98%) was purchased from Cayman Chemical (Ann Arbor, MI) and kaempferol (>97%) from MP BioMedicals (Solon, OH). Azadirachtin A (90.7%), eriocitrin (97.4%), narirutin (93.9%), nobiletin (94.9%), nomilin (87.7%), obacunone (85.8%) and tangeretin (96.4%) were purchased from ChromaDex, Inc. (Irvine, CA). Natural controls genistein (99%) and genistin (91%) were kindly provided by Dr. Mark Berhow (USDA, Peoria, IL), and epigallocatechin gallate (>95%) was purchased from Sigma-Aldrich. The GSK-3β known synthetic inhibitor SB 415286 (>99%) was obtained from Tocris Cookson, Inc. (Ellisville, MO) and the chemotherapeutic drug cisplatin (>99.9%) from Sigma-Aldrich. Primary antibodies Bcl-2 (sc-56018), procaspase 3/caspase 3 (sc-56053), cyclin B1 (sc-166757), cytochrome c (sc-13560), GADPH (sc-47724), GSK-3β (sc-9166), K-Ras (sc-30), NF-κB p65 (sc-71675), nucleolin (C23, sc-17826), p(Tyr216)GSK-3β (sc-135653), and XIAP (sc-55552) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-mouse and anti-rabbit IgG horseradish conjugate secondary antibodies were purchased from GE Healthcare Biosciences (Pittsburg, PA). All other chemicals were purchased from Sigma-Aldrich or Thermo Fisher Scientific Inc. (Pittsburgh, PA) unless otherwise stated.
5.3.2 Cell culture

Two human pancreatic cancer cell lines, BxPC-3 and PANC-1, were selected based on their characteristics. The BxPC-3 cell line is a grade 2 primary pancreatic ductal adenocarcinoma, while PANC-1 is a grade 3 primary pancreatic ductal adenocarcinoma. Additionally, BxPC-3 has wildtype K-Ras [25], underexpresses p53 [25] and p16 [26], and has homozygous deletion of DPC4/smad4 [27]. PANC-1 overexpresses K-Ras, underexpresses p53, has a homozygous deletion of p16 and has wildtype DPC4/smad4 [28]. These cells were obtained from ATCC. BxPC-3 cells were cultured in RPMI-1640 growth medium containing 1% penicillin-streptomycin and 10% FBS. PANC-1 cells were cultured in DMEM growth medium containing 1% penicillin-streptomycin, 1% sodium pyruvate and 10% FBS. Human pancreatic ductal epithelium (HPDE) cells (normal cells) were provided as a generous gift from Dr. Ming-Sound Tsao, University of Toronto, Canada. HPDE cells were cultured in keratinocyte-SFM growth medium supplemented with EGF, BPE and 1% antibiotic-antimycotic [29, 30]. All cells were maintained at 37°C in a humidified atmosphere with 5% CO₂.

5.3.3 Cell proliferation assay

Cell proliferation was determined using the CellTiter 96® AQ ueous One Solution Cell Proliferation Assay from Promega (Madison, WI). Briefly, BxPC-3 and PANC-1 cells were seeded in 96-well plates at a density of 5 x 10⁴ cells/well, allowed to adhere for 24 h at 37°C and then treated with different compounds (0-300 µM) for an additional 24 and 48 h. HPDE cells were seeded in 96-well plates at a density of 5 x 10⁴ cells/well, allowed to adhere for 24 h at 37°C and then treated with apigenin (0-100 µM) for an additional 24 h. After treatment the spent medium was replaced with 100 µL of fresh growth medium and 20 µL of MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) / PES (phenazine ethosulfate) was
added to each well. Plates were incubated for 2 h at 37°C before absorbance was read at 515 nm on an ELx 808 Ultra Microplate Reader (BioTek Instruments, Inc., Winooski, VT). The percent inhibition was calculated with respect to cells treated with 0.5% DMSO as the control. Six replicates were used for analysis. Below illustrates the scheme for the experimental procedures.

5.3.4 Cell cycle analysis

Analysis of cell cycle was performed by flow cytometry following a previously reported protocol [31]. Briefly, BxPC-3 and PANC-1 cells were seeded at a density of 2 x 10^6 cells/T75 flask and allowed to grow for 24 h at 37°C. The cells were then treated with apigenin (0-100 µM) for additional 24 h at 37°C. After treatment, supernatants and cells were collected, centrifuged (300 x g for 10 min at 4°C) and pellets were washed twice with phosphate buffered saline (PBS). The cell pellets were resuspended in 300 µL of PBS, then fixed with ice-cold 70% ethanol and
incubated on ice overnight at 4°C. The fixed cells were then centrifuged and pellets were washed again with PBS. After resuspension in 1 ml PBS, the cells were incubated with RNase (10 µL) (Sigma-Aldrich) and propidium iodide (50 µL) (Sigma-Aldrich) and shaken for 1 h at room temperature in the dark. Cell solutions were then filtered through cell strainer caps (BD Falcon, Franklin Lakes, NJ) before analysis. Cell cycle distribution analysis was performed using a LSR II flow cytometer (BD Biosciences, San Jose, CA) and FCS Express 4 Flow Research Edition Software (De Novo Software, Los Angeles, CA). A total of 10,000 events were collected for each sample. The analysis was performed in duplicate.

5.3.5 Apoptosis assay

Apoptosis was measured using the Annexin V-FITC apoptosis detection kit (Sigma-Aldrich, Catalog number APOAF) and flow cytometry following our previously reported protocol [31]. Briefly, BxPC-3 and PANC-1 cells were seeded in 6-well plates at a density of 2 x 10^5 cells/well and allowed to adhere for 24 h at 37°C. The cells were then treated with apigenin (0-100 µM) for additional 24 h at 37°C. After treatment, supernatants and cells were collected, centrifuged (500 x g for 10 min at 4°C) and pellets were washed with PBS. After resuspension in 500 µL of 1X Binding Buffer, the cell solution was stained with 5 µL Annexin V-FITC and 10 µL propidium iodide at room temperature for 10 min in the dark. The cells were analyzed by a LSR II flow cytometer (BD Biosciences, San Jose, CA). The analysis was performed in triplicate.

5.3.6 Computational modeling

The computational modeling of apigenin within the active site of GSK-3β was obtained using the methodology previously described for luteolin by Johnson et al. [32].
5.3.7 Western blot analysis of Bcl-2, cyclin B1, K-Ras, procaspase 3/caspase 3, p(Tyr 216)GSK-3β, GSK-3β and XIAP on whole cell lysates

BxPC-3 and PANC-1 cells were seeded at a density of 2 x 10^5 cells/well in 6-well plates and allowed to adhere for 24 h at 37°C. The cells were then treated with apigenin (0-100 µM) for an additional 24 h. Cells were lysed with 150 µL of RIPA buffer (Sigma-Aldrich) for 5 min, sonicated for 30 s, centrifuged at 8,000 x g for 10 min (4°C) and the supernatants were collected. Protein concentrations of the whole cell lysates were determined using the DC Protein Assay (Bio-Rad Laboratories, Inc., Hercules, CA). Laemmli buffer containing 5% β-mercaptoethanol was added to the cell extractions (1:1), samples were boiled for 5 min and then stored at -80°C until use. For western blot analysis, 15 µg of protein/well was loaded in 4–20% gradient SDS-polyacrylamide gels (Bio-Rad Laboratories, Inc.). The separated proteins were transferred onto PVDF membranes (GE Healthcare Biosciences) and blocked with 5% nonfat dry milk in 0.1% Tris-buffered saline Tween 20 (TBST) for 1 h at room temperature. After blocking, the membranes were washed with 0.1% TBST (5 times, 5 min each) and incubated with either Bcl-2, cyclin B1, K-Ras, procaspase 3/caspase 3, GSK-3β, p(Tyr216)GSK-3β, or XIAP primary antibodies (1:500) for 1 h at room temperature. The membranes were washed again and incubated with anti-mouse or anti-rabbit IgG horseradish peroxidase conjugate secondary antibodies (1:2500) for 1 h at room temperature. After incubation and repeated washing, the membranes were prepared for detection using a 1:1 mixture of chemiluminescent reagents A (luminol solution) and B (peroxide solution) (GE Healthcare Biosciences). The membrane pictures were taken on a GelLogic 4000 Pro Imaging System (Carestream Health, Inc., Rochester, NY). The relative amount of each target protein was normalized to GAPDH. The analysis was performed at least in triplicate.
5.3.8 Western blot analysis of nuclear NF-κB p65 and cytoplasmic cytochrome c

Separation of nuclear and cytoplasmic fractions was conducted using NE-PER nuclear and cytoplasmic extraction kit (Thermo Scientific, Waltham, MA). Briefly, BxPC-3 and PANC-1 cells were plated at a density of 2 x 10^5 cells/well and allowed to grow for 24 h at 37°C. The cells were then treated with apigenin (0-50 µM) for an additional 24 h. After treatment, the cells were harvested by washing with PBS (1 mL, twice), followed by washing with trypsin-EDTA solution (1 mL, twice), then they were collected with 0.5 mL PBS and pelleted by centrifugation at 500 x g for 14 min. After centrifugation, cell pellets were treated with 100 µL of ice-cold cytoplasmic extraction reagent I, vortexed for 30 s and incubated on ice for 10 min. Ice-cold cytoplasmic extraction reagent II (5.5 µL) was then added, tubes were vortexed for 10 s and then incubated on ice for 1 min. After incubation, the tube was vortexed for 10 s and centrifuged at 16,000 x g for 10 min at 4°C. The supernatant (cytoplasmic extract) was immediately transferred to a clean pre-chilled 1.5-mL microcentrifuge tube. The pellet was then resuspended in 25 µL nuclear extraction reagent, vortexed for 15 sec and incubated on ice for 40 min with vortexing for 15 s every 10 min. The mixture was centrifuged at 16,000 x g for 10 min at 4°C and the supernatant (nuclear extract) was collected. Protein concentrations of the nuclear and cytoplasmic extracts were determined using the DC Protein Assay. Laemmli buffer containing 5% β-mercaptoethanol was added to the cell extractions (1:1), samples were boiled for 5 min and then stored at -80°C until use. For western blot analysis, 8 (nuclear extract) or 15 (cytoplasmic extract) µg of protein/well was loaded in 4–20% gradient SDS-polyacrylamide gels (Bio-Rad Laboratories, Inc.). The separated proteins were transferred in PVDF membrane and blocked with 5% nonfat dry milk in 0.1% TBST for 1 h at room temperature. After blocking, the membranes were washed with 0.1% TBST (5 times, 5 min each) and incubated with cytochrome
c or NF-κB p65 primary antibodies (1:500) for 1 h at room temperature. The membranes were washed again and incubated with anti-mouse IgG horseradish peroxidase conjugate secondary antibody (1:2500) for 1 h at room temperature. After incubation and repeated washings, the membrane pictures were taken as described above in section 2.7. The relative amount of each target protein was normalized to nucleolin for the nuclear extracts and GAPDH for the cytoplasmic extracts. The analysis was performed at least in triplicate.

5.3.9 RNA extraction, real-time RT-PCR gene expression assays and analysis of gene data
BxPC-3 cells were seeded at a density of 2 x 10⁶ cells in T75 flasks and allowed to grow for 24 h before treatment with apigenin (0-50 µM, 24 h). Cells were harvested by trypsinization and total RNA was isolated using an RNeasy Mini Kit with an on-column DNase treatment step (Qiagen, Valencia, CA). RNA was quantified using a NanoDrop-1000 Spectrophotometer (Thermo Fisher Scientific Inc.). Two thousand nanograms of RNA from each sample was reverse transcribed into cDNA using the High Capacity cDNA Reverse Transcription Kit (Life Technologies Carlsbad, CA) according to the manufacturer's instructions. The PCR components mixes were then prepared by combining each cDNA sample with 20X Taqman Gene Expression Assay, 2X Taqman Gene Expression Master Mix and RNase-free water. Mixes were then plated in a 384-well plate and gene expression was determined using the 7900HT Real-Time PCR System (Life Technologies). Cycling conditions were 2 min at 50°C for 1 cycle, then 10 min at 95°C for 1 cycle followed by 15 s at 95°C and 1 min at 60°C for 40 cycles. Gene expression was quantified using the ΔΔCt method and fold-change values were reported as 2^(ΔΔCt). The Taqman Gene Expression Assays (Life Technologies) used for this study were K-Ras (Hs00364284_g1; also for PANC-1 cells), GSK-3β (Hs01047719_m1; also for PANC-1 cells), IL17F (Hs00369400_m1), IL17C (Hs00171163_m1), IL17A (Hs00174383_m1), LTA
(Hs04188773_g1), IFNB1 (Hs01077958_s1) and GAPDH (endogenous control, Hs02758991_g1). The relative amount of each target gene was normalized to GAPDH. At least six replicates were used for analysis.

Additionally, two RT	extsuperscript{2} Profiler PCR Arrays purchased from Qiagen were used in this study, the Inflammatory Cytokines and Receptors Array (PAHS-011ZA) and the Cancer PathwayFinder Array (PAHS-033). RNA extracted from BxPC-3 cells treated with either 50 µM apigenin or control (0.5% DMSO) was used for these experiments. Briefly, four hundred nanograms of total RNA from each sample was used to carry out reverse transcription using a RT	extsuperscript{2} First Strand Kit (Qiagen). The PCR components mixes were then prepared by combining each cDNA sample with RT	extsuperscript{2} SYBR Green Mastermix and RNase-free water. The mixes were then plated in the RT	extsuperscript{2} Profiler PCR Arrays and the gene expressions were determined using the 7900HT Real-Time PCR System (Life Technologies). Cycling conditions were 10 min at 95°C for 1 cycle, 15 s at 95°C and 1 min at 60°C for 40 cycles, followed by a dissociation stage. Gene expressions were quantified using the $\Delta\Delta Ct$ method. The five endogenous housekeeping genes used for normalization in the $\Delta\Delta Ct$ method were B2M, HPRT1, RPL13A, GADPH and ACTIN. Fold-change values were reported as $2^{-\Delta\Delta Ct}$ and when the value was <1 it was converted to its negative inverse to report downregulated genes. The analysis was performed in duplicate.

5.3.10 Statistical analysis

The sigmoidal dose-response analysis of the IC\textsubscript{50} values of each compound on the proliferation of BxPC-3, PANC-1 and HPDE cells was performed by nonlinear regression (curve fit) using GraphPad Prism Software, version 4 (La Jolla, CA). Pearson correlation analyses between the computational modeling and proliferation results were performed by using Statistical Analysis System (SAS) Software, version 9.2 (Cary, NC). Cell cycle, apoptosis, western blot and real-
time RT-PCR (K-Ras and GSK-3β) data were analyzed by analysis of variance. Means were
generated and adjusted with Tukey’s post hoc comparisons using SAS Software. Significant
differences were reported at \( p \)-values <0.05.

5.4 Results

5.4.1 Citrus flavonoids inhibited proliferation of BxPC-3 and PANC-1 human pancreatic
cancer cells

Among the various citrus bioactive compounds tested (15 flavonoids, 2 phenolic acids, 4
limonoids and ascorbic acid), the most effective at inhibiting proliferation (as seen by lower \( IC_{50} \)
values) of BxPC-3 and PANC-1 cells were the aglycone flavonoids in particular, apigenin and
luteolin (Table 5.1). The \( IC_{50} \) values for BxPC-3 cells treated with either apigenin or luteolin for
24 h was 23 and 31 µM, respectively; and the \( IC_{50} \) values after 48 h of treatment was 12 and 14
µM, respectively. The \( IC_{50} \) values for PANC-1 cells exposed to either apigenin or luteolin
treatment for 24 h was 71 and 90 µM, respectively; and the \( IC_{50} \) values after 48 h of exposure
was 41 and 27 µM, respectively. Treatment of the cancer cells with glycosylated flavonoids such
as hesperidin did not produce strong inhibitory effects (\( IC_{50} = 258 \) and 269 µM for BxPC-3 and
PANC-1 cells, respectively, after 48 h). Treatment of the cancer cells with phenolic acids,
limonoids, or ascorbic acid had little effect on the proliferation of either cell line; the exception
was the limonoid obacunone which appeared to improve PANC-1 cell growth (-59% at 48 h).

Pearson correlations of flavonoids (apigenin, luteolin, quercetin, kaempferol, hesperetin,
naringenin and nobiletin) between their inhibitory effect on GSK-3β activity [29] and BxPC-3
cell proliferation was 0.87 (\( p=0.0002 \)) for 24 h exposure and 0.86 (\( p<0.0001 \)) for 48 h exposure.
These correlations were not statistically significant for PANC-1 cells. The \( IC_{50} \) value for HPDE
normal cells treated with apigenin for 24 h was greater than 100 µM (data not shown).
5.4.2 Apigenin arrested G₂/M phase of cell cycle in BxPC-3 and PANC-1 human pancreatic cancer cells

Apigenin treatment in BxPC-3 and PANC-1 cells after 24 h exposure resulted in G₂ phase cell cycle arrest, with a concomitant decrease in G₁ phase (Fig. 5.1A and C). The number of cells in the G₂ phase increased from 15.0% in BxPC-3 cells treated with 0 µM apigenin (0.5% DMSO) to 35.7% when treated with 50 µM apigenin, while the number of cells in the G₁ phase decreased from 42.5% to 34.8%. The number of PANC-1 cells in the G₂ phase increased from 17.7% in the 0 µM apigenin (0.5% DMSO) treated cells to 31.7% in the 50 µM apigenin treated cells, while the G₁ phase decreased from 54.7% to 29.4%. To understand the mechanism by which apigenin caused G₂ cell cycle arrest, we measured the protein expression of cyclin B1. Apigenin treatment at 10, 25 and 50 µM inhibited cyclin B1 in BxPC-3 cells (Fig. 5.1B) by 28.6, 39.7 and 54.2%, respectively. Apigenin treatment at 25, 50 and 100 µM inhibited cyclin B1 in PANC-1 cells (Fig. 5.1D) by 1.1, 22.8 and 53.2%, respectively. These results indicate that apigenin treatment caused G₂ phase cell cycle arrest in BxPC-3 and PANC-1 cells by decreasing cyclin B1 protein expression.

5.4.3 Apigenin promoted apoptosis in BxPC-3 and PANC-1 human pancreatic cancer cells

Treatment with apigenin for 24 h resulted in a dose-dependent increase in the number of cells undergoing apoptosis in both BxPC-3 and PANC-1 cell lines. In BxPC-3 cells (Fig. 5.2A), the percentage of cells undergoing apoptosis increased from 8.4% in cells treated with 0 µM apigenin (0.5% DMSO) to 43.8% in cells treated with 50 µM apigenin. In PANC-1 cells (Fig. 5.3A), the percent of apoptotic cells increased from 3.2% in cells treated with 0 µM apigenin (0.5% DMSO) to 13.8% in cells treated with 50 µM apigenin. To further elucidate the
mechanism involved in apigenin-mediated apoptosis, we measured the expression of Bcl-2, XIAP, caspase 3, procaspase 3 (inactive form) and cytosolic cytochrome c proteins which are associated with the mitochondrial pathway of apoptosis. In BxPC-3 cells (Fig. 5.2B and C), the protein expressions of the anti-apoptotic Bcl-2 and XIAP were significantly decreased by 25.4% and 39.9%, respectively due to apigenin (50 μM) treatment. Apigenin increased protein expressions of the pro-apoptotic cytosolic cytochrome c (91.2% at 25 μM) and caspase 3 (215% at 50 μM), while decreasing procaspase 3 (inactive form of caspase 3) by 31.0% at 50 μM. In PANC-1 cells (Fig. 5.3B and C), apigenin significantly decreased XIAP (41.4% at 100 μM) and procaspase 3 (47.5% at 100 μM) protein expressions, while increasing cytochrome c (71.4% at 50 μM) protein expression. In PANC-1 cells, Bcl-2 and caspase 3 were not quantifiable due to the low intensity of the bands. Apigenin caused apoptosis in BxPC-3 and PANC-1 cells by decreasing anti-apoptotic protein expressions and increasing the release of cytochrome c, a caspase activator.

5.4.4 Apigenin binds within the active site of GSK-3β

Fig. 5.4A shows the orientation of apigenin bound in the active site of the GSK-3β crystal structure. Fig. 5.4B and C demonstrated how apigenin is predicted to bind within and stabilize itself in the active site of GSK-3β. We found that apigenin was able to stabilize itself within GSK-3β’s active site through hydrogen bonding with Asp200, Asn64, Arg141 and Pro136 amino acid residues.
5.4.5 Apigenin modifies the expression of genes and proteins involved in the GSK-3β/NF-κB signaling pathway in BxPC-3 and PANC-1 human pancreatic cancer cells

It can be seen in Fig.5.5A that treatment of BxPC-3 cells with 50 µM of apigenin led to a significant reduction of both K-Ras (upstream GSK-3β effector) and GSK-3β at the mRNA level compared to cells treated with 0 µM apigenin (0.5% DMSO). In PANC-1 cells treated with 50 µM apigenin, compared to 0 µM apigenin (0.5% DMSO) treated cells, there was a significant increase in K-Ras mRNA expression but no significant effect on GSK-3β mRNA (Fig. 5.5B). Apigenin affected K-Ras and GSK-3β gene expressions differently in BxPC-3 and PANC-1 cells, likely due to differences in their cellular characteristics.

In BxPC-3 cells (Fig. 5.5C and D), apigenin treatment also led to a significant decrease in the protein expressions of K-Ras (38.7% at 50 µM), total GSK-3β (36.0% at 50 µM), p(Tyr216)GSK-3β (active form, 48.7% at 50 µM) and nuclear NF-κB p65 (43.7% at 25 µM). In PANC-1 (Fig. 5.5E and F), apigenin treatment significantly decreased K-Ras (33.5% at 100 µM), total GSK-3β (47.0% at 100 µM), p(Tyr216)GSK-3β (69.0% at 100 µM) and nuclear NF-κB p65 (77.1% at 50 µM) protein expressions.

5.4.6 Apigenin upregulated the gene expression of inflammatory IL17 family and LTA, and the expression of the cancer gene IFNB1 in BxPC-3 human pancreatic cancer cells

Table 5.2 summarizes the effect of apigenin on the expression of human inflammatory cytokine and receptor genes in BxPC-3 cells. From the 84 genes that were included in the array, 39 genes were undetermined, while apigenin treatment caused the upregulation of 27 genes and the downregulation of 18 genes. The most upregulated genes were IL17F (114.2-fold), LTA (33.1-fold), IL17C (23.3-fold) and IL17A (11.3-fold). The most downregulated gene was chemokine ligand 10 (CXCL10) (4.2-fold).
Tables 5.3 and 5.4 summarize the effect of apigenin on the expression of genes involved in cell cycle, apoptosis, signal transduction, adhesion, angiogenesis, and invasion and metastasis in BxPC-3 cells. Out of the 84 genes measured, 7 were undetermined, while 32 genes were upregulated and 45 genes were downregulated due to apigenin treatment. Interestingly, the most upregulated genes were IFNB1 (8.9-fold) and TIMP metallopeptidase inhibitor 1 (TWIST1) (5.6-fold), and the most downregulated was tumor protein factor (TP53) (5.6-fold). IL17F, LTA, IL17C and IFNB1 gene expressions validations, using Taqman Gene Expression Assays, are shown as footnotes of Tables 2 and 4.

5.5 Discussion

Citrus fruit bioactive compounds exhibited different cytotoxic effects against human pancreatic cancer cells. The flavonoids, in particular apigenin and luteolin, were the most effective inhibitors of proliferation on BxPC-3 and PANC-1 cells, at comparable concentrations to the chemotherapeutic drug cisplatin and the GSK-3β inhibitor SB414286.

Apigenin most potently affected the proliferation of BxPC-3 cells. Apigenin arrested G_2 phase of BxPC-3 and PANC-1 cell cycles due to the decrease in cyclin B1 protein expression. Cyclin B1 is part of a protein kinase complex needed for cells to enter mitosis, therefore when cyclin B1 levels are reduced this leads to cell cycle arrest at the G_2 phase [33]. Ujiki et al. [34], demonstrated that apigenin caused G_2 phase cell cycle arrest, in different pancreatic cancer cells AsPC-1, CD18, MiaPaCa2 and S2-013, through decreased cyclin B-cdc2 activity.

Apigenin treatment resulted in a significant increase in the percentage of BxPC-3 and PANC-1 cells undergoing programmed cell death through activation of the mitochondrial pathway of apoptosis. Activation of this pathway causes the release of cytochrome c from the mitochondria allowing for the formation of the apoptosome multiprotein complex, which activates caspase 9
and in turn leads to the cleavage of caspase 3 (executioner caspase) [35]. Our results indicated that apigenin treatment led to the release cytochrome c from the mitochondria (a late apoptotic event) and therefore the activation of caspase 3 through inhibition of Bcl-2 (a regulator of mitochondrial membrane permeability, demonstrated in BxPC-3 cells only) and the caspase inhibitor XIAP.

We propose that apigenin inhibits GSK-3β activity in pancreatic cancer, both directly and indirectly, causing induction of apoptosis. We have previously demonstrated that luteolin was able to inhibit GSK-3β enzymatic activity directly [32] by binding within its active site and preventing it from catalyzing a luminescent reaction. This is the first time that the docking figures for apigenin within the active site of GSK-3β are presented. Apigenin was able to optimally bind within GSK-3β due to the presence of an unsaturated C-ring that allows it to remain planar in the active site of the enzyme and thereby avoid unfavorable steric interactions.

In BxPC-3 and PANC-1 cells, the results demonstrated that apigenin was able to decrease protein expression of both total GSK-3β and p(Tyr216)GSK-3β (active form) leading to a decrease in NF-κB and its transcriptional targets Bcl-2 and XIAP. Apigenin treatment decreased GSK-3β protein expression due to direct binding within its active site and by the upstream inhibition of K-Ras. Zhang et al. [8] suggested that upstream regulation of GSK-3β gene expression in pancreatic cancer cells is controlled by a K-Ras/MAPK/ETS cascade that increases binding of ETS2 to GSK-3β promoter elements and recruitment of the histone acetylase p300 to form a transcriptionally active complex. This supports the results of our study showing downregulation of GSK-3β gene expression when K-Ras protein expression was reduced in BxPC-3 cells. Interestingly, in PANC-1 cells K-Ras gene expression significantly increased with apigenin treatment (50 µM), while K-Ras gene expression decreased in BxPC-3 cells. K-Ras is
overexpressed in the PANC-1 cells [28], but not in BxPC-3 cells [25], and this overexpression was potentially trying to compensate the inhibitory action of apigenin.

We discovered that the inflammatory genes IL17 (A, C and F) and LTA were highly upregulated due to apigenin treatment in BxPC-3 cells. Interleukins 17A, C and F are pro-inflammatory cytokines that together with IL17B, D and E make up the IL17 cytokine family [36]. IL17 cytokines are found in various tumors and their role in anti-tumor immunity is controversial because they have been reported to have both pro- and anti-tumor roles depending on the type of tumor. For instance, IL17A has been shown to promote T cell-mediated tumor rejection in lung melanoma [37], in contrast it has been shown to support tumor growth in lung adenocarcinoma by facilitating angiogenesis [38]. Recently, IL17F was shown to play a protective role in colon tumorigenesis through the inhibition of angiogenesis [39], while Chae and Bothwell [40] determined that IL17F was critical for small intestinal tumorigenesis. This is the first study to establish the relevance of increased IL17 (specifically A, C and F) mRNA in inhibition of pancreatic cancer cells. To date, only two studies have reported the potential of IL17s to reduce tumor growth in pancreatic cancer models [41,42]. Another pro-inflammatory cytokine that apigenin was found to highly upregulate was LTA. Fahrig et al. [43] demonstrated that upregulation of LTA by RP101 [(E)-5-(2-bromovinyl)-2’-deoxyuridine (BVDU)] contributed to enhanced anti-tumor immunity in CAPAN-2 and AsPC-1 pancreatic cancer cells.

Apigenin treatment of BxPC-3 cells led to the upregulation of the anti-cancer gene IFNB1, which encodes for the protein interferon beta (IFN-β), a member of the type 1 IFN family of cytokines. Administration of recombinant IFN-β or the transfer of IFN-β genes to pancreatic cancer cells has demonstrated anti-proliferative and pro-apoptotic effects using both in vitro and in vivo models [44,45]. Furthermore, IFN-β is currently being used as a therapeutic drug for
cancer treatment in clinical trials (clinicaltrials.gov). Wang et al. [46] showed in dendrite cells that IFN-β activates the JAK/PI3K/Akt signaling pathway leading to a decrease in GSK-3β activity through phosphorylation at its Ser 9 residue. Interestingly, studies conducted by Huang et al. [47] and Shen et al. [48] have also demonstrated the ability of IL17 to lead to inhibition of GSK-3β. We have discovered, for the first time, that apigenin has the ability to increase IL17s (A, C, F) and IFNB1 in BxPC-3 pancreatic cancer cells and their upregulation may have potentiated the inhibition of GSK-3β leading to the downregulation of NF-κB and therefore inhibition of anti-apoptotic proteins Bcl-2 and XIAP.

As discussed by Rao et al. [49], several laboratories have found that flavonoids are effective as anti-proliferative agents at relatively high concentrations. We found that apigenin concentrations ranging from 10 to 100 μM were effective at inhibiting proliferation, as well as modulating protein and gene expressions in pancreatic cancer cells, in vitro. In a clinical study, Meyer et al. [50] determined that the average maximum apigenin plasma concentration after consumption of one oral bolus of parsley (65.8 μM) was 127 nM, meaning that only about 0.2% was found in plasma. Based on these findings, current dietary consumption may not be able to achieve physiological plasma levels of 10-100 μM apigenin due to its low bioavailability. If the preferred via of administration of this anti-proliferative flavonoid is oral, the alternative approach to achieve the needed concentrations in plasma could be through appropriately designed drug formulations. For example, the use of nanoparticles for the encapsulation of the bioactive compound curcumin has been useful in overcoming its low bioavailability and allowing it to effectively exert its anti-cancer effects [51]. In humans, studies are needed to determine the effect of constant dietary consumption of fruits and vegetables on plasma concentrations of apigenin, as well as the effect of the microbiota on the bioactivity of this flavonoid.
In summary, we demonstrated that apigenin was able to induce cell death in BxPC-3 and PANC-1 human pancreatic cancer cells through the inhibition of the GSK-3β/NF-κB signaling cascade causing induction of apoptosis. Our results also showed, for the first time, that apigenin treatment increased gene expression of the cytokines IL17s, LTA and IFNB1 in BxPC-3 cells and that the upregulation of these cytokines may have facilitated cancer cell growth inhibition. Flavonoids are important in cancer therapy research and we have proposed the underlying mechanism of their anti-cancer properties targeting key cellular cascades in pancreatic carcinogenesis.

5.6 References


### 5.7 Tables and figures

**Table 5.1.** Cell proliferation (IC$_{50}$) values of citrus compounds and other natural and synthetic compounds against BxPC-3 and PANC-1 cells $^{a,b}$

<table>
<thead>
<tr>
<th>Compound</th>
<th>BxPC-3</th>
<th>PANC-1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC$_{50}$ Value (μM) at 24 h</td>
<td>IC$_{50}$ Value (μM) at 48 h</td>
</tr>
<tr>
<td><strong>Flavonoids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apigenin</td>
<td>23.1 ± 0.4</td>
<td>12.4 ± 0.4</td>
</tr>
<tr>
<td>Luteolin</td>
<td>31.3 ± 3.1</td>
<td>14.3 ± 0.8</td>
</tr>
<tr>
<td>Quercetin</td>
<td>81.9 ± 3.4</td>
<td>43.4 ± 0.9</td>
</tr>
<tr>
<td>Kaempferol</td>
<td>66.9 ± 1.4</td>
<td>76.4 ± 3.2</td>
</tr>
<tr>
<td>Hesperetin</td>
<td>127.7 ± 1.6</td>
<td>130.1 ± 0.8</td>
</tr>
<tr>
<td>Naringenin</td>
<td>&gt;300</td>
<td>292.9 ± 0.0</td>
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<tr>
<td>Flavone</td>
<td>&gt;300</td>
<td>&gt;300 (47.2%)</td>
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<tr>
<td><strong>Methoxyflavones</strong></td>
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<tr>
<td>Nobiletin</td>
<td>147.4 ± 8.2</td>
<td>155.6 ± 0.0</td>
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<tr>
<td>Tangeretin</td>
<td>86.3 ± 0.6</td>
<td>96.2 ± 0.8</td>
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<tr>
<td><strong>Glycosylated flavonoids</strong></td>
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<tr>
<td>Rutin</td>
<td>&gt;200</td>
<td>&gt;200 (-2.6%)</td>
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<tr>
<td>Hesperidin</td>
<td>&gt;300</td>
<td>258.2 ± 4.7</td>
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<tr>
<td>Narirutin</td>
<td>&gt;100</td>
<td>&gt;100 (-1.0%)</td>
</tr>
<tr>
<td>Eriocitrin</td>
<td>&gt;100</td>
<td>&gt;100 (2.2%)</td>
</tr>
<tr>
<td>Naringin</td>
<td>&gt;100</td>
<td>&gt;100 (-1.0%)</td>
</tr>
<tr>
<td>Neohesperidin</td>
<td>&gt;200</td>
<td>&gt;200 (-0.5%)</td>
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<tr>
<td><strong>Phenolic Acids</strong></td>
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<tr>
<td>Caffeic Acid</td>
<td>&gt;300</td>
<td>&gt;300 (-15.2%)</td>
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<tr>
<td>Chlorogenic Acid</td>
<td>&gt;300</td>
<td>&gt;300 (3.5%)</td>
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<tr>
<td><strong>Limonoids</strong></td>
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<tr>
<td>Nomilin</td>
<td>&gt;100</td>
<td>&gt;100 (-13.1%)</td>
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<tr>
<td>Obacunone</td>
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<td>Azadirachitin</td>
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<td><strong>Other natural compounds</strong></td>
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<td>Ascorbic Acid</td>
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<tr>
<td>Epigallocatechin gallate</td>
<td>241.8 ± 8.7</td>
<td>153.0 ± 1.8</td>
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<td>Genistein</td>
<td>224.9 ± 5.4</td>
<td>187.7 ± 2.3</td>
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<tr>
<td>Genistin</td>
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<td>291.1 ± 1.8</td>
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<tr>
<td><strong>Synthetic inhibitor</strong></td>
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<tr>
<td>SB 415286</td>
<td>25.0 ± 2.3</td>
<td>24.3 ± 0.6</td>
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<tr>
<td><strong>Chemotherapeutic drug</strong></td>
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<td></td>
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<tr>
<td>Cisplatin</td>
<td>25.7 ± 1.6</td>
<td>13.6 ± 0.2</td>
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$^{a}$Data are expressed as the mean ± standard error. The lower the IC$_{50}$ value (the concentration needed to inhibit cell proliferation by 50%), the higher the potency, n = 6.

$^{b}$Values in parentheses represent the percentage of cells that were inhibited at the highest treatment concentration.
Table 5.2. Effect of apigenin (50 µM) on gene expression of inflammatory cytokines and receptors in BxPC-3 cells after 24 h of exposure.

<table>
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<td>Aminoacyl tRNA synthetase complex-interacting multifunctional protein 1</td>
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<td>BMP2</td>
<td>Bone morphogenetic protein 2</td>
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<td>C5</td>
<td>Complement component 5</td>
<td>NM_001735</td>
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<td>CCL17</td>
<td>Chemokine (C-C motif) ligand 17</td>
<td>NM_002987</td>
<td>-1.9</td>
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<td>CCL2</td>
<td>Chemokine (C-C motif) ligand 2</td>
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<td>IL1RN</td>
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<td>IL7</td>
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<td>IL8</td>
<td>Interleukin 8</td>
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<td>Lymphotoxin alpha (TNF superfamily, member 1)</td>
<td>NM_000595</td>
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<td>Nicotinamide phosphoribosyltransferase</td>
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<td>SPP1</td>
<td>Secreted phosphoprotein 1</td>
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<td>VEGFA</td>
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*a* Fold-change was calculated as \(2^{\Delta\Delta Ct}\) and when the value was <1 it was converted to its negative inverse to report downregulated genes, n=2.

*b* Bold values represent fold-changes \(>1.5\).

*Gene expressions validated using Taqman Gene Expression Assays (IL17F = 12.3 ± 0.4-fold, LTA = 6.7 ± 0.3-fold and IL17C = 108.0 ± 2.9).*
Table 5.3. Effect of apigenin (50 µM) on the expression of genes involved in cancer cell cycle, apoptosis and signal transduction pathways in BxPC-3 cells after 24 h of exposure \(^{a,b}\)

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Gene Description</th>
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</tr>
</thead>
<tbody>
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<td>ATM</td>
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<td>CCNE1</td>
<td>Cyclin E1</td>
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<td>CDC25A</td>
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### Table 5.3 (cont.)

<table>
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<th>Gene Symbol</th>
<th>Gene Description</th>
<th>GenBank ID</th>
<th>Fold Change</th>
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<tbody>
<tr>
<td><strong>Apoptosis &amp; Cell Senescence (cont.)</strong></td>
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<td></td>
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<tr>
<td>CFLAR</td>
<td>CASP8 and FADD-like apoptosis regulator</td>
<td>NM_003879</td>
<td>-1.5</td>
</tr>
<tr>
<td>FAS</td>
<td>Fas (TNF receptor superfamily, member 6)</td>
<td>NM_000043</td>
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</tr>
<tr>
<td>GZMA</td>
<td>Granzyme A (granzyme 1, cytotoxic T-lymphocyte-associated serine esterase 3)</td>
<td>NM_006144</td>
<td>1.6</td>
</tr>
<tr>
<td>HTATIP2</td>
<td>HIV-1 Tat interactive protein 2, 30kDa</td>
<td>NM_006410</td>
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<td>TERT</td>
<td>Telomerase reverse transcriptase</td>
<td>NM_198253</td>
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<td>TNFRSF10B</td>
<td>Tumor necrosis factor receptor superfamily, member 10b</td>
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<td>TNFRSF1A</td>
<td>Tumor necrosis factor receptor superfamily, member 1A</td>
<td>NM_001065</td>
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<td>TNFRSF25</td>
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<td>AKT1</td>
<td>V-akt murine thymoma viral oncogene homolog 1</td>
<td>NM_005163</td>
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<tr>
<td>ETS2</td>
<td>V-Ets erythroblastosis virus E26 oncogene homolog 2 (avian)</td>
<td>NM_005239</td>
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<tr>
<td>FOS</td>
<td>FBJ murine osteosarcoma viral oncogene homolog</td>
<td>NM_005252</td>
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<tr>
<td>JUN</td>
<td>Jun proto-oncogene</td>
<td>NM_002228</td>
<td>1.1</td>
</tr>
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<td>MAP2K1</td>
<td>Mitogen-activated protein kinase kinase 1</td>
<td>NM_002755</td>
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</tr>
<tr>
<td>MYC</td>
<td>V-myc myelocytomatosis viral oncogene homolog (avian)</td>
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<td>NFKB1</td>
<td>Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1</td>
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<tr>
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<td>PIK3R1</td>
<td>Phosphoinositide-3-kinase, regulatory subunit 1 (alpha)</td>
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<td>RAF1</td>
<td>V-raf-1 murine leukemia viral oncogene homolog 1</td>
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<tr>
<td>SNCG</td>
<td>Synuclein, gamma (breast cancer-specific protein 1)</td>
<td>NM_003087</td>
<td>-1.4</td>
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</table>

*a* Fold change was calculated as $2^{(ΔΔCT)}$ and when the value was <1 it was converted to its negative inverse to report downregulated genes, n=2.

*b* Bold values represent fold-changes >1.5.
Table 5.4. Effect of apigenin (50 µM) on the expression of genes related to cancer adhesion, angiogenesis, and invasion and metastasis pathways in BxPC-3 cells after 24 h of exposure.

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Gene Description</th>
<th>GenBank ID</th>
<th>Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Adhesion</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>ITGA1</td>
<td>Integrin, alpha 1</td>
<td>NM_181501</td>
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<tr>
<td>ITGA2</td>
<td>Integrin, alpha 2 (CD49B, alpha 2 subunit of VLA-2 receptor)</td>
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<td>ITGA3</td>
<td>Integrin, alpha 3 (antigen CD49C, alpha 3 subunit of VLA-3 receptor)</td>
<td>NM_002204</td>
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<tr>
<td>ITGA4</td>
<td>Integrin, alpha V (vitronectin receptor, alpha polypeptide, antigen CD51)</td>
<td>NM_002210</td>
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</tr>
<tr>
<td>ITGB1</td>
<td>Integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12)</td>
<td>NM_002211</td>
<td>1.1</td>
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<tr>
<td>ITGB3</td>
<td>Integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61)</td>
<td>NM_000212</td>
<td><strong>1.5</strong></td>
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<tr>
<td>ITGB5</td>
<td>Integrin, beta 5</td>
<td>NM_002213</td>
<td>-1.2</td>
</tr>
<tr>
<td>MCAM</td>
<td>Melanoma cell adhesion molecule</td>
<td>NM_006500</td>
<td>-1.3</td>
</tr>
<tr>
<td>MTSS1</td>
<td>Metastasis suppressor 1</td>
<td>NM_014751</td>
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<tr>
<td>PNN</td>
<td>Pinin, desmosome associated protein</td>
<td>NM_002687</td>
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<td>SYK</td>
<td>Spleen tyrosine kinase</td>
<td>NM_003177</td>
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<td>EPDR1</td>
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<td><strong>Angiogenesis</strong></td>
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<td>ANGPT2</td>
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<td>COL18A1</td>
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<td>IFNB1</td>
<td>Interferon, beta 1, fibroblast</td>
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<td>IL8</td>
<td>Interleukin 8</td>
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<td>--------------------------------------------------------</td>
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<td>Angiogenesis (cont.)</td>
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<td>Invasion &amp; Metastasis</td>
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</tr>
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<td>-1.2</td>
</tr>
<tr>
<td>NME4</td>
<td>Non-metastatic cells 4, protein expressed in</td>
<td>NM_005009</td>
<td>1.1</td>
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<td>-1.3</td>
</tr>
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<td>TIMP metalloproteinase inhibitor 1</td>
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<td>TWIST1</td>
<td>Twist homolog 1 (Drosophila)</td>
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<sup>a</sup>Fold-change was calculated as \(2^{(-\Delta\Delta C_t)}\) and when the value was <1 it was converted to its negative inverse to report downregulated genes, n=2.

<sup>b</sup>Bold values represent fold-changes ≥1.5.

<sup>*</sup>Gene expression validated using Taqman Gene Expression Assay (IFNB1 = 2.3 ± 0.1).
Figure 5.1. Apigenin arrested cell cycle progression at G$_2$/M phase and decreased the expression of cyclin B1 in BxPC-3 and PANC-1 pancreatic cancer cells. Cell cycle progression analysis of (A) BxPC-3 cells as affected by apigenin treatment. Means with different lower-case letters are significantly different among apigenin concentrations for G$_1$ phase, capital letters are different among apigenin concentrations for S phase and superscript lower-case letters are different among apigenin concentrations for G$_2$ phase as determined by Tukey's post hoc comparisons (p<0.05). A total of 10,000 events were collected for each sample. The analysis was performed in duplicate. Data are expressed as the mean ± standard error. Cyclin B1 expression in (B) BxPC-3 cells as affected by apigenin treatment. Cyclin B1 protein expression means with different lower-case letters are significantly different from each other as determined by Tukey's post hoc comparisons (p<0.05). Data are expressed as the mean ± standard error, n = 3. Cell cycle progression analysis of (C) PANC-1 cells as affected by apigenin treatment. Means with different lower-case letters are significantly different among apigenin concentrations for G$_1$ phase, capital letters are different among apigenin concentrations for S phase and superscript lower-case letters are different among apigenin concentrations for G$_2$ phase as determined by Tukey's post hoc comparisons (p<0.05). A total of 10,000 events were collected for each sample. The analysis was performed in duplicate. Cyclin B1 expression in (D) PANC-1 cells as affected by apigenin treatment. Cyclin B1 protein expression means with different lower-case letters are significantly different from each other as determined by Tukey's post hoc comparisons (p<0.05). Data are expressed as the mean ± standard error, n = 3.
Figure 5.1 (cont.)

B)  
![Graph showing Cyclin B1/GAPDH levels with Apigenin (µM) as x-axis.](image)

C)  
![Histograms showing cell cycle phase distribution in PANC-1 cells with Apigenin (µM) as x-axis.](image)

D)  
![Graph showing Cyclin B1/GAPDH levels with Apigenin (µM) as x-axis.](image)
Figure 5.2. Apigenin induced the mitochondrial pathway of apoptosis through modification of Bcl-2, XIAP, caspase 3, procaspase 3 and cytochrome c protein expressions in BxPC-3 pancreatic cancer cells. (A) Apoptosis analysis (quadrant 4) was measured by treatment of confluent cells for 24 h with different concentrations of apigenin. Percent apoptotic cell means with different lower-case letters are significantly different from each other as determined by Tukey’s post hoc comparisons (p<0.05). Data are expressed as the mean ± standard error, n = 3. (B) Apigenin decreased the expression of the anti-apoptotic proteins Bcl-2 and XIAP, and increased pro-apoptotic caspase 3, while decreasing its inactive form procaspase 3. Different lower-case letters are significantly different among Bcl-2 means, capital letters are different among XIAP means, superscript lower-case letters are different among caspase 3 means and italicized capital letters are different among procaspase 3 means as determined by Tukey’s post hoc comparisons (p<0.05). Data are expressed as the mean ± standard error, n = 3. (C) Apigenin treatment increased the protein expression of cytosolic cytochrome c. Different lower-case letters are significantly different among cytochrome c means as determined by Tukey’s post hoc comparisons (p<0.05). Data are expressed as the mean ± standard error, n = 3.
Figure 5.2 (cont.)

B) 

Relative Target Protein Expression/GAPDH

0 µM
10 µM
25 µM
50 µM

0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0

Bcl-2
XIAP
Caspase 3
Pro caspase 3

C) 

Relative cytochrome c/GAPDH

0 µM
10 µM
25 µM
**Figure 5.3:** Apigenin induced the mitochondrial pathway of apoptosis through modification of XIAP, procaspase 3 and cytochrome c protein expressions in PANC-1 pancreatic cancer cells. (A) Apoptosis analysis (quadrant 4) was measured by treatment of confluent cells for 24 h with different concentrations of apigenin. Percent apoptotic cell means with different lower-case letters are significantly different from each other as determined by Tukey’s post hoc comparisons (p<0.05). Data are expressed as the mean ± standard error, n = 3. (B) Apigenin decreased the expression of the anti-apoptotic protein XIAP, and decreased procaspase 3 (inactive form of caspase 3). Different capital letters are different among XIAP means and italicized capital letters are different among procaspsase 3 means as determined by Tukey’s post hoc comparisons (p<0.05). Data are expressed as the mean ± standard error, n = 3. (C) Apigenin treatment increased the protein expression of cytosolic cytochrome c. Different lower-case letters are significantly different among cytochrome c means as determined by Tukey’s post hoc comparisons (p<0.05). Data are expressed as the mean ± standard error, n = 3.
Figure 5.4. Apigenin binds and stabilizes itself within GSK-3β’s active site through hydrogen bonds with Asp200, Asn64, Arg141 and Pro136 amino acid residues. (A) Predicted structure of GSK-3β complexed with apigenin. (B) Surface representation of the binding cavity of GSK-3β is shown with the predicted mode for apigenin binding and the interacting amino acid residues. Binding cavity residues are shown with acidic residues in red, basic residues in dark blue, hydrophobic residues in green and hydrophilic residues in light blue. (C) Two-dimension representation of the apigenin interacting residues following the method of Clark and Labute [52]. Binding cavity residues that are shown with green circles indicate residues with no polar or charged side chains. Residues with light mauve circles indicate polar side chains that can be acidic, indicated by a red ring, or basic, indicated by a blue ring. The arrows indicate hydrogen bonds to side chain residues in green and backbone residues in blue.
Figure 5.4 (cont.)

B)

Cavity Opening

C)
Figure 5.5. Apigenin decreased the expression of genes and proteins involved in the GSK-3β/NF-κB signaling pathway in BxPC-3 and PANC-1 pancreatic cancer cells. Apigenin treatment modified the gene expression of K-Ras and GSK-3β in (A) BxPC-3 and (B) PANC-1 cells. Different lower-case letters are significantly different among K-Ras means and capital letters are different among GSK-3β means as determined by Tukey's post hoc comparisons (p<0.05). Data are expressed as the mean ± standard error, n = 6. Apigenin decreased the protein expression of K-Ras, GSK-3β and p(Tyr216)GSK-3β (active form) in whole cell lysates in (C) BxPC-3 cells. Different lower-case letters are significantly different among K-Ras means, capital letters are different among GSK-3β means and superscript lower-case letters are different among p(tyr216)GSK-3β means as determined by Tukey's post hoc comparisons (p<0.05). Data are expressed as the mean ± standard error, n = 3. Apigenin treatment decreased the nuclear protein expression of NF-κB p65 in (D) BxPC-3 cells. Different lower-case letters are significantly different among NF-κB p65 means as determined by Tukey's post hoc comparisons (p<0.05). Data are expressed as the mean ± standard error, n = 3. Apigenin treatment decreased the nuclear protein expression of NF-κB p65 in (E) PANC-1 cells. Different lower-case letters are significantly different among K-Ras means, capital letters are different among GSK-3β means and superscript lower-case letters are different among p(tyr216)GSK-3β means as determined by Tukey's post hoc comparisons (p<0.05). Data are expressed as the mean ± standard error, n = 3.
Figure 5.5 (cont.)

D) 

\[
\begin{array}{c}
\text{kDa} \\
65 \\
110
\end{array}
\]

```
0.0 0.2 0.4 0.6 0.8 1.0 1.2 1.4
NF-\kappa B p65
```

E) 

```
kDa 47 37
21 37
```

```
0.0 0.5 1.0
K-Ras \\
GSK-3\beta \\
p(Tyr216)GSK-3\beta
```

```
0 µM \\
25 µM \\
50 µM \\
100 µM
```

F) 

```
kDa 65 110
```

```
0.0 0.5 1.0
NF-\kappa B p65
```

```
0 µM \\
25 µM \\
50 µM
```
CHAPTER 6

INTERACTIONS BETWEEN DIETARY FLAVONOIDS APIGENIN OR LUTEOLIN AND CHEMOTHERAPEUTIC DRUGS TO POTENTIATE ANTI-PROLIFERATIVE EFFECT ON HUMAN PANCREATIC CANCER CELLS, IN VITRO

6.1 Abstract

The objectives were to assess the potential of dietary flavonoids apigenin (Api) and luteolin (Lut) to enhance the anti-proliferative effects of chemotherapeutic drugs on BxPC-3 human pancreatic cancer cells using the MTS cell proliferation assay and to investigate the potential molecular mechanism of action using western blot analysis. Simultaneous treatment with either flavonoid (13, 25 or 50 µM) and chemotherapeutic drugs 5-fluorouracil (5-FU, 50 µM) or gemcitabine (Gem, 10 µM) for 60 h resulted in a less-than-additive effect (p<0.05). Flavonoid pretreatment trials (13 µM for 0, 6, 24, or 42 h) determined that 24 h pretreatment with 13 µM of either Api or Lut, followed by 5-FU or Gem for 36 h was optimal to inhibit cell proliferation. Pretreatment of cells with 11-19 µM of either flavonoid for 24 h resulted in 59-73% growth inhibition when followed by Gem (10 µM, 36 h). Lut (15 µM, 24 h) pretreatment followed by Gem (10 µM, 36 h), significantly decreased the protein expression of nuclear GSK-3β and NF-κB p65 and increased pro-apoptotic cytosolic cytochrome c. Pretreatment of BxPC-3 human pancreatic cancer cells with low concentrations of Api or Lut effectively aid in the anti-proliferative activity of chemotherapeutic drugs.

6.2 Introduction

Pancreatic cancer is an aggressive disease that kills more than half of its victims within 6 months of diagnosis, and the remaining patients have a five-year survival rate of only 6% [1].

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*This chapter is part of the paper: Johnson, J., Gonzalez de Mejia, E., Interactions between dietary flavonoids apigenin or luteolin and chemotherapeutic drugs to potentiate anti-proliferative effect on human pancreatic cancer cells, in vitro. (Submitted for publication to Food Chem. Toxicol., FCT-D-13-01063).*
Poor prognosis of this disease is due to its rapid progression and the lack of disease-specific signs and symptoms, which prevents early diagnosis and curative treatment [2]. The current standard treatment for patients with pancreatic cancer is gemcitabine (Gem) alone or in combination with other chemotherapeutic drugs such as cisplatin (Cis), 5-fluorouracil (5-FU), irinotecan, capecitabine or oxaliplatin (Oxa) [3]. However, resistance towards chemotherapy due to the activation of anti-apoptotic proteins is believed to be a major cause of treatment failure in pancreatic cancer [4].

Novel strategies for sensitizing pancreatic cancer cells with naturally occurring dietary compounds have gained considerable attention because of their beneficial effects in overcoming tumor cell resistance to apoptosis [5]. In particular, the dietary flavonoids apigenin (Api) and luteolin (Lut), which are found in a variety of fruits, vegetables and herbs, have demonstrated the ability to induce apoptosis in pancreatic cancer cells [6, 7] and therefore have the potential to overcome chemoresistance. A possible target for these flavonoids is the serine/threonine kinase glycogen synthase kinase-3β (GSK-3β), an enzyme that is known to translocate into the nuclei of pancreatic cancer cells and act as a key upstream regulator of NF-κB transcriptional activity [8, 9]. It has been shown that many conventional chemotherapeutic agents activate NF-κB, thereby resisting apoptosis [10]. Our laboratory has previously shown that Api and Lut can bind within GSK-3β’s catalytic site and inhibit its activity directly [11]. In addition, we have demonstrated the inhibitory effects of these flavonoids against pancreatic cancer cell proliferation in vitro, as well as the ability of Api to induce apoptosis through inhibition of the GSK-3β/NF-κB signaling pathway [7]. These findings support the feasibility that inactivation of the GSK-3β/NF-κB signaling pathway by Api or Lut could also sensitize pancreatic cancer cells to chemotherapeutic drugs, becoming likely an important and novel strategy for the treatment of this disease.
The main objectives of this study were to evaluate the interactions of either Api or Lut and chemotherapeutic drugs 5-FU, Gem, Cis or Oxa, on the anti-proliferative activity of BxPC-3 human pancreatic cancer cells, and to investigate the potential mechanism of action related to the GSK-3β/NF-κB signaling pathway.

6.3 Materials and methods

6.3.1. Materials and reagents

Growth medium Roswell Park Memorial Institute (RPMI)-1640 and 0.25% (w/v) trypsin-0.53 mM EDTA were purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA). Keratinocyte-SFM (1X) growth medium supplied with human recombinant Epidermal Growth Factor (EGF) and Bovine Pituitary Extract (BPE), 1X antibiotic-antimycotic, 0.5% trypsin-EDTA and soybean trypsin inhibitor were purchased from Life Technologies (Carlsbad, CA). Penicillin-streptomycin was purchased from Corning Inc. (Corning, NY, USA), fetal bovine serum (FBS) from HyClone (Thermo Scientific HyClone, Logan, UT, USA), and dimethyl sulfoxide (DMSO) from Sigma-Aldrich (St. Louis, MO, USA). Api (≥95% pure), Cis (≥99.9%), 5-FU (≥99%), Gem (gemcitabine hydrochloride, ≥98%) and Oxa (≥98%) were purchased from Sigma-Aldrich. Lut (≥98%) was purchased from Cayman Chemical (Ann Arbor, MI, USA) (Fig. 6.1A and B present the chemical structures of flavonoids and chemotherapeutic drugs studied). Western blot control lysates for GSK-3β (293T lysate, sc-120654) and NF-κB p65 (293T lysate, sc-122027) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Primary antibodies cytochrome c (sc-13560), GAPDH (sc-47724), GSK-3β (sc-9166), NF-κB p65 (sc-71675) and nucleolin (C23, sc-17826) were purchased from Santa Cruz Biotechnology, Inc. Anti-mouse and anti-rabbit IgG horseradish conjugate secondary antibodies were purchased from GE Healthcare Biosciences (Pittsburg, PA). All other chemicals were...
purchased from Sigma-Aldrich or Thermo Fisher Scientific Inc. (Pittsburgh, PA) unless otherwise stated.

6.3.2. Cell culture

The human pancreatic cancer cell line BxPC-3 was purchased from ATCC. BxPC-3 cells were cultured in RPMI-1640 medium supplemented with 1% penicillin-streptomycin and 10% FBS. Human pancreatic ductal epithelium (HPDE) cells (normal cells) were provided as a generous gift from Dr. Ming-Sound Tsao, University of Toronto, Canada. HPDE cells were cultured in keratinocyte-SFM growth medium supplemented with EGF, BPE and 1% antibiotic-antimycotic [12, 13]. All cells were maintained at 37°C in a humidified atmosphere with 5% CO₂.

6.3.3. Cell proliferation assay

Cell proliferation was determined using the CellTiter 96® AQ™ueous One Solution Cell Proliferation Assay from Promega (Madison, WI) and the experimental design is outlined in Fig. 6.1C. Briefly, BxPC-3 and HPDE cells were seeded in 96-well plates at a density of 3 x 10³ to 5 x 10³ cells/well and allowed to adhere for 24 h at 37°C. BxPC-3 cells were treated with 0-50 µM of either Api or Lut and/or a chemotherapeutic drug (50 µM 5-FU, 10 µM Gem, 10 µM Cis or 0.1 µM Oxa) for up to 78 h. HPDE cells were treated with 0-100 µM Api or Lut for 24 h. Then the spent medium was replaced with 100 µL of fresh growth medium and 20 µL of MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) / PES (phenazine ethosulfate) was added to each well. Plates were incubated for 2 h at 37°C before absorbance was read at 515 nm on an ELx 808IU Ultra Microplate Reader (BioTek Instruments,
Inc., Winooski, VT). The percent inhibition was calculated with respect to cells exposed to DMSO (0-0.25%) as the vehicle.

6.3.4. Western blot analysis

Separation of nuclear and cytoplasmic fractions was conducted using NE-PER nuclear and cytoplasmic extraction kit (Thermo Scientific, Waltham, MA). Briefly, BxPC-3 cells were plated at a density of 1 x 10^5 cells/well and allowed to grow for 24 h at 37°C. The cells were then treated with 10 µM of Gem for 36 h, 15 µM of Lut for 60 h, or Lut pretreatment for 24 h followed by the addition of Gem for 36 h (total time = 60 h). After treatment the cells were harvested by washing with PBS (1 mL, twice), followed by washing with trypsin-EDTA solution (1 mL, twice), then they were collected with 0.5 mL PBS and pelleted by centrifugation at 500 x g for 14 min. After centrifugation, cell pellets were treated with 100 µL of ice-cold cytoplasmic extraction reagent I, vortexed for 30 s and incubated on ice for 10 min. Ice-cold cytoplasmic extraction reagent II (5.5 µL) was then added, tubes were vortexed for 10 s and then incubated on ice for 1 min. After incubation, the tube was vortexed for 10 s and centrifuged at 16,000 x g for 10 min at 4°C. The supernatant (cytoplasmic extract) was immediately transferred to a clean pre-chilled 1.5-mL microcentrifuge tube. The pellet was then resuspended in 25 µL nuclear extraction reagent, vortexed for 15 s and incubated on ice for 40 min with vortexing for 15 s every 10 min. The mixture was centrifuged at 16,000 x g for 10 min at 4°C and the supernatant (nuclear extract) was collected. Protein concentrations of the nuclear and cytoplasmic extracts were determined using the DC Protein Assay. Laemmli buffer containing 5% β-mercaptoethanol was added to the cell extractions (1:1), samples were boiled for 5 min and then stored at -80°C until use. For western blot analysis, 7 µg (nuclear extract) or 14 µg (cytoplasmic extract) of protein/well was loaded in 4–20% gradient SDS-polyacrylamide gels (Bio-Rad Laboratories,
Inc.). The separated proteins were transferred to a PVDF membrane and blocked with 5% nonfat dry milk in 0.1% TBST for 1 h at room temperature. After blocking, the membranes were washed with 0.1% TBST (5 times, 5 min each) and incubated with GSK-3β, NF-κB p65 or cytochrome c primary antibody (1:500) for 1 h at room temperature. The membranes were washed again (5 times, 5 min each) and incubated with anti-mouse or anti-rabbit IgG horseradish peroxidase conjugate secondary antibody (1:2500) for 1 h at room temperature. After incubation and repeated washing, the membranes were prepared for detection using a 1:1 mixture of chemiluminescent reagents A (luminol solution) and B (peroxide solution) (GE Healthcare Biosciences). The membrane pictures were taken on a GelLogic 4000 Pro Imaging System (Carestream Health, Inc., Rochester, NY). The relative amount of each target protein was normalized to nucleolin for the nuclear extracts and to GAPDH for the cytoplasmic extracts.

6.3.5. Statistical analysis

The Statistical Analysis System (SAS) Software, version 9.2 (Cary, NC, USA) was used. The results were expressed as mean ± standard error and analyzed through one-way analysis of variance (ANOVA) followed by Tukey's test. Significant differences were reported at $p < 0.05$. The sigmoidal dose-response analysis of the IC values of flavonoid pretreatment for 24 h followed by the addition of a chemotherapeutic drug for 36 h on the proliferation of BxPC-3 cells was performed by nonlinear regression (curve fit) using GraphPad Prism Software, version 4 (La Jolla, CA, USA). To better understand the interactions between compounds tested, isobolographic analyses were performed [14]. Briefly, IC$_{30}$ or IC$_{20}$ values for flavonoids and chemotherapeutic drugs were used in constructing the isobolograms. A straight line connecting the IC values of these compounds predicts the IC value of an additive effect. If results of the isobolographic analysis deviated significantly to the left of the additivity line with 95%
confidence interval, the interaction was confirmed as synergistic; if it deviated to the right, the interaction was antagonistic.

6.4 Results and discussion

6.4.1. Effect of simultaneous treatment of flavonoid and chemotherapeutic drug on BxPC-3 human pancreatic cancer cell proliferation

The effects of the flavonoids Api and Lut were very similar throughout the cell proliferation studies. These findings are likely due to the fact that both are flavones, a subclass of flavonoids that differ from each other by the addition of a second hydroxyl group on the B ring of Lut (Fig. 6.1A). Fig. 6.2 shows the dose-response percent cell growth inhibition for Api (Fig. 6.2A) or Lut (Fig. 6.2B) treatment alone for 60 h on BxPC-3 pancreatic cancer cells. Cells were treated with concentrations ranging from 3.125-50 µM, with Api treatment causing 10-96% cell growth inhibition and Lut treatment causing 8-97% cell growth inhibition. Fig. 6.3 shows the effects of treating BxPC-3 cells simultaneously for 60 h with either Api or Lut (13, 25 or 50 µM) and 5-FU (50 µM, Fig. 6.3A and C) or Gem (10 µM, Fig. 6.3B and D) on the percent inhibition of cell proliferation. Treatment of pancreatic cancer cells with either 5-FU (50 µM) or Gem (10 µM) alone for 60 h resulted in 59 or 69% cell growth inhibition, respectively. Simultaneous treatments of 5-FU (50 µM) with 13 µM of either Api or Lut caused 71% and 70% cell growth inhibition, respectively. Statistical analysis indicated that the combinations were more effective than Api, Lut, or 5-FU treatments alone (p<0.05). More studies are needed to understand the mechanism of action of these simultaneous treatment effects. However, 13 µM Api or Lut treatment combined with Gem (10 µM) was not significantly more effective than Gem treatment alone (p<0.05); a 72% inhibition was obtained for simultaneous treatments compared to 69% inhibition for Gem treatment alone. Although simultaneous treatments with higher flavonoid
concentrations (25 or 50 µM) were significantly better at inhibiting cell growth than either chemotherapeutic drug alone, the combinations were only as effective as or less effective than either flavonoid treatment alone. Therefore, at higher concentrations, simultaneous treatments of flavonoid (either Api or Lut) and chemotherapeutic drugs (either 5-FU or Gem) produced less-than-additive effects on the inhibition of cell growth.

A possible explanation for this observation is the potential competition between antioxidant and pro-oxidant activities. Api and Lut, as with most flavonoids, are regarded as antioxidant substances [15, 16]. These substances directly scavenge reactive oxygen species (ROS) or indirectly upregulate antioxidant defenses and inhibit ROS production [17]. ROS are a diverse group of reactive short-lived, oxygen-containing species, such as superoxide (O₂•−), hydrogen peroxide (H₂O₂) and hydroxyl radical (•OH) that when produced in excess result in oxidative stress and damage to cellular proteins, lipids and DNA [18]. Because most chemotherapeutic agents, including 5-FU, Gem, Cis and Oxa mediate their cytotoxic effects through the generation of ROS [18], and thus pro-oxidant action, some evidence suggests that antioxidant use might reduce the effectiveness of these chemotherapies [18-20]. However, the research in this area remains controversial as to whether the use of antioxidants, in the form of flavonoids, is harmful or negates the effect of chemotherapy [21]. Therefore the specific timing of when these flavonoids are administered, in relation to chemotherapy treatment, may be important in avoiding potential antioxidant/pro-oxidant interactions that may prevent enhancement of anti-proliferative activity.
6.4.2. Effect of time of flavonoid pretreatment on ability to potentiate the anti-proliferative activity of chemotherapeutic drugs in BxPC-3 human pancreatic cancer cells

Fig. 6.4 shows the effects of Api (13 µM, Fig. 6.4A) and Lut (13 µM, Fig. 6.4B) alone or as pretreatments for 0, 6, 24 and 42 h before the administration of 5-FU (50 µM, 36 h) or Gem (10 µM, 36 h). The concentrations needed for each individual drug to cause between 20-40% cell growth inhibition, at the optimal drug exposure time of 36 h, were based on our preliminary experiments (data not shown). In Fig. 6.4A and B, a flavonoid pretreatment time of 0 h represents the effect of 5-FU (33% inhibition) or Gem (30% inhibition) alone on cell proliferation after 36 h exposure. Pretreatment of cells with a flavonoid for 6, 24 and 42 h followed by the addition of 5-FU resulted in 56, 67 and 57% cell growth inhibition for Api pretreatment and 52, 60 and 56% cell growth inhibition for Lut pretreatment, respectively. The percent of cell growth inhibition from flavonoid pretreatment for 6, 24 and 42 h followed by the addition of Gem was 30, 66 and 63% for Api pretreatment and 35, 69 and 60% for Lut pretreatment, respectively. Statistical analysis demonstrated that 24 h pretreatment with either flavonoid followed by 5-FU or Gem was the most effective at inhibiting pancreatic cancer cell proliferation. After 6 h flavonoid pretreatment, the combination of Api and Gem was less effective than Api alone, while the Lut and Gem combination was only as effective as Lut alone.

6.4.3. Effect of flavonoid concentration on ability to potentiate the anti-proliferative activity of chemotherapeutic drugs in BxPC-3 human pancreatic cancer cells

We determined the flavonoid dose-response relationship with respect to pancreatic cancer cell growth inhibition. Fig. 6.5 shows the effects of different pretreatment concentrations (0-50 µM) of Api (Fig. 6.5A) or Lut (Fig. 6.5B) for 24 h on the cell growth inhibitory potential of different chemotherapeutic drugs (50 µM 5-FU, 10 µM Gem, 10 µM Cis or 0.1 µM Oxa). A
flavonoid pretreatment concentration of 0 µM represents the effect of 5-FU (27% inhibition), Gem (36% inhibition), Cis (20% inhibition) or Oxa (24% inhibition) alone on cell proliferation after 36 h exposure. Pretreatment of cells with 11-19 µM of either Api or Lut for 24 h followed by the addition of 5-FU resulted in 45-72% or 43-71% cell growth inhibition, respectively; a 59-73% or 60-70% cell growth inhibition when Gem was combined with either Api or Lut, respectively. Pretreatment of cells with 11-15 µM of either Api or Lut for 24 h followed by the addition of Oxa resulted in 49-63% or 36-54% cell growth inhibition, respectively. Only Api or Lut (15 µM) pretreatment for 24 h was significantly effective at enhancing the anti-proliferative activity of Cis in cells, causing 34% and 35% inhibition, respectively. Although high flavonoid concentrations of 25 or 50 µM were effective at enhancing the anti-proliferative effects of 5-FU and Gem, the combinations were not statistically different from the flavonoid treatments alone (p<0.05). Table 6.1 shows the IC_{20} and IC_{50} values due to either Api or Lut pretreatment for 24 h followed by the addition of a chemotherapeutic drug for 36 h. The combinations of Gem plus either Api or Lut were the most potent at inhibiting cell proliferation, with both combinations having IC_{50} values less than the lowest flavonoid concentration (11 µM) tested. These results demonstrated that pretreatment of low concentrations of Api or Lut for 24 h followed by the addition of a chemotherapeutic drug for 36 h were optimal for inhibiting pancreatic cancer cell proliferation. The IC_{50} values for HPDE normal cells treated with Api or Lut for 24 h were >100 µM and >50 µM, respectively.

When investigating the effect of flavonoids in combination with standard chemotherapeutic agents, it is essential to consider not only the total time of treatment, but also the time scheduled of addition of flavonoids in relation to the addition of chemotherapeutic drugs. In this study, we examined the effect of simultaneous treatment (60 h) of flavonoids and
chemotherapeutic drugs as well as a range of flavonoid pretreatment times (0, 6, 24 and 42 h) and concentrations (0-50 µM) on pancreatic cancer cell proliferation. Simultaneous treatment of cells with flavonoids and chemotherapeutic drugs caused less-than-additive effects ($p<0.05$) on inhibition of cell proliferation, potentially due to competing antioxidant/pro-oxidant interactions. However, the flavonoid pretreatment time (24 h) at low concentrations (11-19 µM) were determined to optimally enhance the anti-proliferative activity of some chemotherapeutic drugs, such as 5-FU and Gem, in BxPC-3 pancreatic cancer cells.

Fig. 6.6 presents the isobolographic analyses for the flavonoids and chemotherapeutic drugs tested. Combination of the IC$_{30}$ concentrations of Api (Fig. 6.6A and B) or Lut (Fig. 6.6E and F) and Gem or 5-FU resulted in an additive effect in inhibiting pancreatic cancer cell proliferation. Combination of the IC$_{20}$ concentrations of Api (Fig. 6.6C) or Lut (Fig. 6.6G) and Cis resulted in a clear antagonistic effect in inhibiting pancreatic cancer cell proliferation. Interestingly, combination of the IC$_{20}$ concentrations of Api and Oxa (Fig. 6.6D) was additive, while Lut and Oxa (Fig. 6.6H) was antagonistic. ČipáK et al. [22] demonstrated a difference in the effect of Api and Lut when used in simultaneous combination with Cis to inhibit the proliferation of leukemia cells. They demonstrated that when Lut was used in combination with Cis it caused a synergistic effect; however when Api was used in combination with Cis, on leukemia cells, it caused an additive to antagonist effect depending on the concentrations used. They suggested that the difference in chemical structure of these flavonoids was the potential reason for why they interacted differently with Cis.

Lee et al. [23] and Strouch et al. [24] have demonstrated that combination treatment with Api and Gem enhanced anti-proliferation in MiaPaCa-2, AsPC-3 and CD18 pancreatic cancer cells in vitro, and decreased tumor growth in a MiaPaCa-2 xenograft model. However, our
present study is the first to compare the ability of Api and Lut to potentiate, either simultaneously or as pretreatment, the BxPC-3 human pancreatic cancer cell growth inhibitory effects of the chemotherapeutic drugs 5-FU, Gem, Cis and Oxa. It is important to focus on the ability of bioactive compounds to enhance the anti-proliferative effects of Gem, because it is the standard treatment for pancreatic cancer patients. However, it is also of relevance to investigate the effect of such bioactive compounds combined with other chemotherapeutic drugs in pancreatic cancer treatment. In recent years, there have been attempts to augment treatment efficacy by using a combinatorial approach. A randomized clinical trial conducted by Conroy et al. [25] demonstrated that the multidrug regimen FOLFIRINOX (5-FU, leucovorin, Oxa and irinotecan) was superior to Gem treatment alone with respect to the median overall survival and median progression-free survival. Thus, combination of chemotherapeutic drug treatments for pancreatic cancer could be further enhanced through pretreatment with bioactive compounds, such as Api or Lut. While our results demonstrated the abilities of these flavonoids to enhance the anti-proliferative effects of single chemotherapeutic drugs by 24 h pretreatment, future directions would involve looking at the ability of flavonoids to enhance the anti-proliferative potential of combined chemotherapeutic drugs. The translation of these in vitro results to in vivo models of pancreatic cancer is underway in our laboratory.

6.4.4. Effect of luteolin, gemcitabine and their combination treatment on the GSK-3β/NF-κB signaling pathway and cell apoptosis in BxPC-3 human pancreatic cancer cells

Lastly, we investigated the potential molecular mechanism by which Lut enhanced the anti-proliferative effects of Gem in BxPC-3 cells. Fig. 6.7 displays the effects of Lut (15 µM, 60 h), Gem (10 µM, 36 h) and Lut pretreatment (15 µM, 24 h) followed by Gem (10 µM, 36 h) treatment on protein expression of nuclear GSK-3β (Fig. 6.7A), nuclear NF-κB p65 (Fig. 6.7B)
and cytosolic cytochrome c (Fig. 6.7C) in pancreatic cancer cells. Our results showed that whereas treatment of cells with Gem alone had no significant change in nuclear GSK-3β and NF-κB p65 levels, the combination with Lut pretreatment substantially reduced these protein expression levels by 32 and 67%, respectively compared to control cells. Combination treatment of pancreatic cancer cells with Lut and Gem also led to a significant increase in the cytosolic protein expression of the pro-apoptotic cytochrome c compared to either treatment alone or the control treated cells. Cytochrome c is an important marker of apoptosis because when released from the mitochondria it allows for the formation of the apoptosome multiprotein complex (cytochrome c, caspase 9 and Apaf). This complex is then able to activate caspase 3, which is the executioner caspase that initiates cell degradation [26].

It is widely accepted that several chemotherapeutic agents act primarily by inducing apoptosis and that alterations in the apoptotic pathway can make cancer cells resistant to therapy [27]. GSK-3β is a serine/threonine kinase that plays a key role in the regulation of numerous cell survival signaling pathways and has been shown to be activated in several types of cancer [28, 29]. Studies have shown that synthetic inhibitors of GSK-3β, such as AR-A014418 or ZM336372, induce apoptosis in pancreatic cancer cells in vitro [29-31] and inhibit tumor growth in vivo [29, 31]. One potential mechanism by which inhibition of GSK-3β leads to apoptosis is through suppression of NF-κB activity. NF-κB is a transcription factor that is necessary for the production of many anti-apoptotic proteins, including Bcl-2 and XIAP [32]. In the current study we demonstrated that pretreatment of cells with Lut followed by the addition of Gem caused a significant decrease in nuclear GSK-3β accumulation leading to decreased NF-κB transcriptional activity. This decrease in NF-κB transcriptional activity allowed for apoptosis to occur, as shown
through increased cytochrome c expression, through reduced production of anti-apoptotic proteins.

Api is abundantly present in oranges, grapefruit, parsley, onions, wheat sprouts and chamomile tea [15]. Rich sources of Lut include apple skins, parsley, celery, broccoli, onion leaves, carrots, peppers, cabbages and chrysanthemum flowers [16]. More studies are needed to demonstrate the effectiveness of these flavonoids in in vivo models.

6.5 Conclusion

The results suggest that in vitro pretreatment of BxPC-3 human pancreatic cancer cells for 24 h with 11-19 µM of either Api or Lut were able to effectively influence the efficacy of the anti-proliferative effects of 5-FU and Gem. The timing of Api and Lut treatments and their concentrations were of great importance; we demonstrated that a simultaneous application of both compounds, flavonoid and chemotherapeutic drug, will produce less-than-additive effects. We also demonstrated that flavonoid Lut (15 µM, 24 h) enhanced the anti-proliferative effects of Gem (10 µM, 36 h) through inhibition of the GSK-3β/NF-κB signaling pathway, leading to the induction of apoptosis. More studies are needed to understand other potential mechanisms of action.

6.6 References


### 6.7 Tables and figures

**Table 6.1.** Effect of either Api or Lut pretreatment (24 h) followed by the addition of a chemotherapeutic drug (36 h) on BxPC-3 human pancreatic cancer cell proliferation.

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC$_{20}$ (µM)$^1$</th>
<th>IC$_{50}$ (µM)$^{1,2}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apigenin</td>
<td>11.7 ± 0.1</td>
<td>16.4 ± 0.1a</td>
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<tr>
<td>Apigenin + 5-Fluorouracil$^3,4$</td>
<td>&lt;11</td>
<td>12.6 ± 0.1b</td>
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<tr>
<td>Apigenin + Gemcitabine$^3,5$</td>
<td>&lt;11</td>
<td>&lt;11</td>
</tr>
<tr>
<td>Apigenin + Cisplatin$^3,6$</td>
<td>12.5 ± 0.3</td>
<td>Not calculable</td>
</tr>
<tr>
<td>Apigenin + Oxaliplatin$^3,7$</td>
<td>&lt;11</td>
<td>11.5 ± 0.2c</td>
</tr>
<tr>
<td>Luteolin</td>
<td>11.6 ± 0.1</td>
<td>16.3 ± 0.3A</td>
</tr>
<tr>
<td>Luteolin + 5-Fluorouracil$^3,4$</td>
<td>&lt;11</td>
<td>12.8 ± 0.4B</td>
</tr>
<tr>
<td>Luteolin + Gemcitabine$^3,5$</td>
<td>&lt;11</td>
<td>&lt;11</td>
</tr>
<tr>
<td>Luteolin + Cisplatin$^3,6$</td>
<td>11.5 ± 0.5</td>
<td>Not calculable</td>
</tr>
<tr>
<td>Luteolin + Oxaliplatin$^3,7$</td>
<td>&lt;11</td>
<td>14.4 ± 0.3B</td>
</tr>
<tr>
<td>5-Fluorouracil</td>
<td>21.2 ± 9.1</td>
<td>&gt;200</td>
</tr>
<tr>
<td>Gemcitabine</td>
<td>0.11 ± 0.06</td>
<td>&gt;200</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>6.6 ± 0.9</td>
<td>21.7 ± 1.7</td>
</tr>
<tr>
<td>Oxaliplatin</td>
<td>&lt;0.1</td>
<td>0.3 ± 0.0</td>
</tr>
</tbody>
</table>

$^1$Data are expressed as the mean ± standard error. The lower the IC value (the concentration needed to inhibit cell proliferation by 20 or 50%), the higher the potency.

$^2$Different lower-case letters are significantly different among Api means, capital letters are different among Lut means.

$^3$A concentration that inhibited cell growth between 20-40% at 36 h was selected for each chemotherapeutic drug.

$^4$5-FU treatment concentration for 36 h was 50 µM.

$^5$Gem treatment concentration for 36 h was 10 µM.

$^6$Cis treatment concentration for 36 h was 10 µM.

$^7$Oxa treatment concentration for 36 h was 0.1 µM.
**Fig. 6.1.** Chemical structures of (A) flavonoids and (B) chemotherapeutic drugs used in this study, and (C) the experimental design for the interaction studies between flavonoids and chemotherapeutic drugs on BxPC-3 human pancreatic cancer cell proliferation.

**A)**

- Flavonoids = Apigenin (0-50 µM) and Luteolin (0-50 µM)
- Chemotherapeutic drugs = Cisplatin (10 µM), 5-Fluorouracil (50 µM), Gemcitabine (10 µM) and Oxaliplatin (0.1 µM)
- BxPC-3 pancreatic cancer cells
- MTS (Proliferation) assay
- Western blot analysis

**B)**

- 5-Fluorouracil
- Gemcitabine Hydrochloride
- Cisplatin
- Oxaliplatin

**C)**

- Materials and Treatments:
  - 60 h flavonoid treatment (3.125-50 µM)
  - 6 h flavonoid pretreatment (13 µM)
  - 24 h flavonoid pretreatment (0-50 µM) + 36 h chemotherapeutic drug
  - 42 h flavonoid pretreatment (13 µM)
  - 60 h simultaneous treatment of flavonoid (13, 25 or 50 µM) + chemotherapeutic drug
Fig. 6.2. Dose-response inhibitory effect of either (A) Api or (B) Lut treatment on BxPC-3 human pancreatic cancer cell proliferation. Cells were incubated for 60 h at 37°C with flavonoid concentrations ranging from 3.125 to 50 µM. Two independent experiments performed in triplicates were used for the analysis.
**Fig. 6.3.** Effect of concentration of flavonoid on simultaneous treatment with chemotherapeutic drugs on BxPC-3 human pancreatic cancer cell proliferation. Cells were incubated for 60 h at 37°C with (A) Api (13, 25 or 50 µM) and 5-FU (50 µM), (B) Api (13, 25 or 50 µM) and Gem (10 µM), (C) Lut (13, 25 or 50 µM) and 5-FU (50 µM) or (D) Lut (13, 25 or 50 µM) and Gem (10 µM). Different lower-case letters are significantly different among means of 13 µM samples, capital letters are different among means of 25 µM samples and italicized lower-case letters are different means of 50 µM samples as determined by Tukey's post hoc comparisons ($p<0.05$). Data are expressed as the mean ± standard error from at least two independent experiments performed in triplicates.
**Fig. 6.4.** Effect of time of flavonoid pretreatment on the ability to potentiate the anti-proliferative activity of chemotherapeutic drugs in BxPC-3 human pancreatic cancer cells. Cells were pretreated for 0, 6, 24 and 42 h with 13 µM of either (A) Api or (B) Lut followed by the addition of either 5-FU (50 µM) or Gem (10 µM) for 36 h at 37°C. The value indicated at 0 h flavonoid pretreatment time, represents 5-FU or Gem treatment alone for 36 h without Api or Lut pretreatment. Different lower-case letters are significantly different from each other as determined by Tukey’s post hoc comparisons (p<0.05). Data are expressed as the mean ± standard error from at least two independent experiments performed in triplicates.
**Fig. 6.5.** Effect of flavonoid concentration on potentiation of the anti-proliferative activity of chemotherapeutic drugs in BxPC-3 human pancreatic cancer cells. Cells were pretreated for 24 h with 0-50 µM of either (A) Api or (B) Lut followed by the addition of 5-FU (50 µM), Gem (10 µM), Cis (10 µM) or Oxa (0.1 µM) for 36 h at 37°C. The value indicated at 0 µM flavonoid pretreatment concentration, represents 5-FU, Gem, Cis or Oxa treatment alone for 36 h without Api or Lut pretreatment. Data are expressed as the mean ± standard error from at least two independent experiments performed in triplicates. Combinations that were statistically more effective than either compound alone were indicated by an *.

![Graph A](image1)

![Graph B](image2)
Fig. 6.6. Isobolograms for the interaction of either Api or Lut (24 h pretreatment) with 5-FU, Gem, Cis or Oxa (36 h treatment) on inhibition of BxPC-3 human pancreatic cancer cell proliferation. (A) Api and 5-FU, (B) Api and Gem, (C) Api and Cis, (D) Api and Oxa, (E) Lut and 5-FU, (F) Lut and Gem, (G) Lut and Cis, (H) Lut and Oxa. The line of additivity (solid line) is shown with its 95% confidence interval (dashed lines). When results deviate significantly to the left of the additivity line with 95% confidence interval, the interaction is synergistic; when it deviates to the right, the interaction is antagonistic.
Fig. 6.6 (cont.)

E) 

\[ IC_{50}^{5-FU} (\mu M) \]

\[ IC_{30} \text{ Lut (\mu M)} \]

F) 

\[ IC_{50} \text{ Gem (\mu M)} \]

\[ IC_{30} \text{ Lut (\mu M)} \]

G) 

\[ IC_{20} \text{ Cis (\mu M)} \]

\[ IC_{20} \text{ Lut (\mu M)} \]

H) 

\[ IC_{20} \text{ Oxa (nM)} \]

\[ IC_{20} \text{ Lut (\mu M)} \]
Fig. 6.7. Effect of Lut, Gem or combination treatment on the protein expression of (A) nuclear GSK-3β, (B) nuclear NF-κB p65 and (C) cytosolic cytochrome c. BxPC-3 cells were pretreated with Lut (15 µM) for 24 h followed by the addition of Gem (10 µM) for 36 h at 37°C. Nuclear and cytoplasmic cell lysates were extracted and protein expression of GSK-3β, NF-κB p65 and cytochrome c were analyzed by Western blot. Means with different lower-case letters are significantly different (p<0.05) as determined by Tukey’s post hoc comparisons. Data are expressed as the mean ± standard error. A representative western blot image is shown in A, B and C containing an individual control (represented as C) for each sample due to differences in % DMSO and exposure times. The membrane pictures were taken on a GelLogic 4000 Pro Imaging System (Carestream Health, Inc., Rochester, NY). The relative amount of each target protein was normalized to either nucleolin for the nuclear extracts, or GAPDH for the cytoplasmic extracts. The analysis was performed in triplicate.
Fig. 6.7 (cont.)

C)

- Cytochrome c/GAPDH

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Gem</th>
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<td>0.0</td>
<td>0.5</td>
<td>1.0</td>
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</tr>
</tbody>
</table>

Legend:
- Control
- Gem
- Lut
- Lut + Gem
CHAPTER 7
LUTEOLIN POTENTIATES THE ANTI-TUMOR EFFECT OF GEMCITABINE IN AN ORTHOTOPIC MOUSE MODEL OF PANCREATIC CANCER THROUGH INHIBITION OF THE GSK-3β/NF-κB PATHWAY LEADING TO INCREASED APOPTOSIS\textsuperscript{5}

7.1. Abstract

Natural compounds that can sensitize cancer cells to chemotherapeutic drugs are urgently needed. The objective was to evaluate the ability of luteolin (Lut) to enhance the anti-tumor effects of gemcitabine (Gem) on pancreatic cancer using an orthotopic mouse model. Male athymic nude mice (6 wk old) were injected with human BxPC-3 pancreatic cancer cells directly into the pancreas and randomized into four treatment groups: (1) control (n = 14); (2) Lut [84 mg/kg body weight (bw)] 7 times/wk for wk 1 and 5 times/wk for wks 2-6 (n = 12) by intraperitoneal (i.p.) injection; (3) Gem (125 mg/kg bw) twice/wk by i.p. injection for wks 2-6 (n = 14); and (4) Lut (84 mg/kg bw) 7 times/wk for wk 1, followed by Lut 5 times/wk and Gem (125 mg/kg bw) 2 times/wk for wks 2-6 by i.p. injection (n = 12). The combination treatment of Lut and Gem significantly lowered (p = 0.048) the pancreatic tumor mass compared to the control group. Lut, Gem and their combination significantly reduced proliferating cell nuclear antigen expression by 25\%, 37\% and 37\%, respectively. Immunohistochemical and western blot analyses showed that combination treatment led to a significant reduction in the expressions of K-Ras (46\%, p=0.0006), GSK-3β (34\%, p=0.014), p(Tyr216)GSK-3β (16\%, p=0.033), p(Ser311)NF-κB p65 (27\%, p=0.036) and the Bcl-2/Bax ratio (68\%, p=0.006) while significantly increasing the expressions of cytochrome c (44\%, p=0.035) and caspase 3 (417\%, p=0.003). The combination treatment of Lut and Gem promoted apoptotic cell death in

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pancreatic tumor cells through inhibition of the K-Ras/GSK-3β/NF-κB signaling pathway leading to a reduction in the Bcl-2/Bax ratio, release of cytochrome c and activation of caspase 3.

7.2. Introduction

Pancreatic cancer is the fourth leading cause of cancer-related death for both men and women in the United States, with a 5-year relative survival rate for all stages of only 6%. Of the approximately 45,000 Americans that will be diagnosed with pancreatic cancer in 2013, 38,000 are expected to succumb to this disease [1]. Currently, the chemotherapeutic drug gemcitabine (Gem) is regarded as the standard of care for patients with pancreatic cancer [2,3]. However, the development of resistance is a major problem in the use of chemotherapeutic drugs and limits their clinical utility [4]. A key mechanism by which this resistance occurs in pancreatic cancer is through activation of the NF-κB signaling pathway leading to the blockage of apoptosis [4,5]. Naturally occurring phytochemicals have gained considerable attention as part of a synergistic approach to suppress pancreatic cancer due to their ability to inhibit NF-κB and therefore overcome chemoresistance [6,7].

Luteolin (Lut), 3',4',5,7-tetrahydroxyflavone, is a member of the flavone subclass of flavonoids and it exists abundantly in fruits, vegetables and herbs such as apples, beets, broccoli, celery, peppers, parsley and thyme [8-10]. Previous studies have reported the ability of Lut to inhibit pancreatic cancer cell proliferation, in vitro [11-14]. In addition, we have demonstrated the ability of Lut to enhance the anti-proliferative effects of Gem in BxPC-3 pancreatic cancer cells, in vitro, through inhibition of glycogen synthase kinase-3β (GSK-3β) leading to a decrease in NF-κB transcriptional activity and an increase in apoptosis [14]. Shimasaki et al. [15] and Kitano et al. [16] showed that inhibition of GSK-3β by its known synthetic inhibitor AR-A014418 in in vitro and in vivo models led to the sensitization of pancreatic cancer cells to Gem.
GSK-3β, a serine/threonine protein kinase, has been demonstrated to translocate into the nuclei of pancreatic cancer cells where it stimulates NF-κB transcriptional activity and as a consequence promotes the production of anti-apoptotic genes [17,18]. These previous studies suggest that Lut could be useful as an adjuvant to conventional chemotherapies such as Gem through inhibition of the GSK-3β/NF-κB signaling pathway; however, in vivo studies are lacking in support of this statement and our current findings fill that gap.

The objectives were to evaluate the ability of Lut to enhance the anti-tumor effect of Gem using an orthotopic mouse model injected with human pancreatic cancer cells, as well as to investigate the potential mechanism of action related to the GSK-3β/NF-κB signaling pathway. The results showed that only the combination treatment of Lut and Gem led to a significant reduction in pancreatic tumor mass when compared to the control group. The combination treatment significantly reduced the expressions of K-Ras, GSK-3β, p(Tyr216)GSK-3β and p(Ser311)NF-κB p65, while significantly increasing the expressions of pro-apoptotic cytochrome c and caspase 3. These findings suggest that Lut has the potential to enhance the anti-tumor activity of Gem through inhibition of the GSK-3β/NF-κB signaling pathway leading to increased apoptosis.

7.3. Materials and Methods

7.3.1 Materials

The human pancreatic cancer cell line BxPC-3, growth medium Roswell Park Memorial Institute (RPMI)-1640 and 0.25% (w/v) trypsin-0.53 mM EDTA were purchased from American Type Culture Collection (ATCC, Manassas, VA). Penicillin-streptomycin was purchased from Corning Inc. (Corning, NY) and fetal bovine serum (FBS) from Hyclone (Thermo Scientific Hyclone, Logan, UT). Lut (>98%) was purchased from Cayman Chemical (Ann Arbor, MI) and
dissolved in 25% polyethylene glycol 200 (PEG, Sigma-Aldrich, St. Louis, MO). Gem
(gemcitabine hydrochloride, >99%) was purchased from Selleck Chemicals (Houston , TX) and
dissolved in PBS (Corning, Inc.). Primary antibodies for proliferating cell nuclear antigen
(PCNA, sc-7907), GSK-3β (sc-9166), p(Tyr216)GSK-3β (sc-135653), p(Ser311)NF-κB p65 (sc-
135769), cytochrome c (sc-13560), Bax (sc-493), Bcl-2 (sc-56018), caspase 3 (sc-56053, sc-
7272) and GAPDH (sc-47724) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The
primary antibody for K-Ras (ab137739) was purchased from Abcam (Cambridge, MA).
ImmunoCruz mouse (sc-2017) and rabbit (sc-2018) ABC staining systems were purchased from
Santa Cruz Biotechnology, Inc. Anti-mouse and anti-rabbit IgG horseradish conjugate secondary
antibodies were purchased from GE Healthcare Biosciences (Pittsburg, PA). All other chemicals
were purchased from Sigma-Aldrich or Thermo Fisher Scientific Inc. (Pittsburgh, PA) unless
otherwise stated.

7.3.2 Animals, Care, and Diets
The protocol was approved by the Institute of Animal Care and Use Committee at the University
of Illinois at Urbana-Champaign (IACUC at UIUC). Fifty-two 6-week-old male athymic nude
mice were purchased from Harlan Laboratories (Madison, WI). The mice were housed singly in
ventilated wire cages under standardized conditions (21 °C, 60% relative humidity, 12 h light/12
h dark cycle, 20 air changes/h), at the University of Illinois, Institute for Genomic Biology
Animal Facility. All mice received an irradiated AIN-93G pelleted diet (TD.97184, Harlan
Laboratories) and autoclaved drinking water ad libitum throughout the study.
7.3.3 Experimental Design

All mice were acclimated for one week before starting the experiments. BxPC-3 human pancreatic cancer cell viability was assessed prior to injections and was always $\geq 90\%$. Cells were suspended in PBS at a concentration of 1 million cells per 50 µL PBS and kept on ice until injections. Mice were anesthetized (2.5-5% isoflurane: air mixture) and the surgical procedure was carried out according to the IACUC at UIUC. Briefly, a small incision on the left side immediately inferior to the ribcage line was made, and the spleen and pancreas were exteriorized. Cells were injected into the pancreas with a 30-gauge needle attached to a 1-mL Hamilton syringe. Both layers of the wound were closed with a wound stapler. Three to seven days after tumor transplantation mice were weighed and randomized into 4 groups as follows: (**Group 1**) control group received 100 µL of 25% PEG (vehicle for Lut) by intraperitoneal (i.p.) injection 7 times/wk for wk 1, and 5 times/wk for wks 2-6 (n = 14); (**Group 2**) Lut [84 mg/kg body weight (bw)] suspended in 100 µL 25% PEG by i.p. injection 7 times/wk for wk 1, and 5 times/wk for wks 2-6 (n = 12); (**Group 3**) 100 µL of 25% PEG by i.p. injection 7 times/wk for wk 1, followed by 100 µL of 25% PEG 5 times/wk and Gem (125 mg/kg bw) suspended in 100 µL of PBS 2 times/wk (treatments were not on same days) for wks 2-6 (n = 14); and (**Group 4**) Lut (84 mg/kg bw) suspended in 100 µL 25% PEG by i.p. injection 7 times/wk for wk 1, followed by Lut (84 mg/kg bw) suspended in 100 µL 25% PEG 5 times/wk and Gem (125 mg/kg bw) suspended in 100 µL of PBS 2 times/wk (treatment were not on same days) for weeks 2-6 (n = 12). The dose of Lut (84 mg/kg bw) was extrapolated from our previous *in vitro* study [14] which demonstrated that 15 µM Lut was effective at enhancing the anti-cancer effects of Gem in BxPC-3 cells. The Gem treatment concentration of 125 mg/kg bw was based on what previous studies using pancreatic cancer xenograft models have shown to be effective [19]. Body weight
was recorded twice weekly. Mice were euthanized by carbon dioxide asphyxia after 41 days of treatment. Total body weight, pancreas, liver, lung and spleen weights were determined during necropsy. Each pancreas tumor was divided into two pieces: one fixed in 10% formalin buffered solution and the other snap frozen in liquid nitrogen and stored at -80°C. Below illustrates the scheme for the experimental procedures, grouping and treatment schedule.

**7.3.4 Hematoxylin and eosin staining of pancreatic tumor tissue**

Paraffin-embedded pancreatic tumor tissue was cut at 5 µm thickness, transferred to a slide and deparaffinized prior to hematoxylin and eosin (H and E) staining. H and E staining was conducted using the standard method. Hematoxylin was used to stain the nuclei blue and eosin was used to stain the cytoplasm pink. Stained slides were visualized using Nanozoomer Digital Pathology (Olympus Hamamatsu, Bridgewater, NJ).

**7.3.5 Immunohistochemical (IHC) analyses of pancreatic tumor tissue**

Paraffin-embedded pancreatic tumor tissue was cut at 5 µm thickness, transferred to a slide, deparaffinized and antigen retrieval (K-Ras, GSK-3β, p(Tyr216)GSK-3β and p(Ser311)NF-κB
p65 only) was performed using Tris-EDTA buffer prior to immunohistochemical (IHC) analyses. IHC for PCNA, K-Ras, GSK-3β, p(Tyr216)GSK-3β, p(Ser311)NF-κB p65 and cytochrome c was performed following the manufacturer's protocol (Santa Cruz Biotechnology, Inc.). Stained slides were visualized using Nanozoomer Digital Pathology (Olympus Hamamatsu). Intensity of staining was quantified using Teton machine (MediaCybernetics, Inc., Bethesda, MD) equipped with Axio Vision analysis software (Carl Zeiss, Jena, Germany).

7.3.6 Western blot analysis of pancreatic tissue

One hundred mg of snap frozen pancreatic tissue was mixed with 300 µL RIPA buffer containing protease cocktail inhibitor, sodium orthovanadate and phenylmethylsulfonylfluoride (Santa Cruz Biotechnology, Inc.). The mixture was homogenized for 1 min, incubated on ice for 30 min, centrifuged at 10,000 x g for 10 min (4 °C) and the supernatants were collected. Protein concentrations were determined using the DC Protein Assay (Bio-Rad Laboratories, Inc., Hercules, CA). Laemmli buffer containing 5% β-mercaptoethanol was added to the supernatants (1:1), samples were boiled for 5 min and then stored at -20°C until use. For western blot analysis, 100 µg of protein/well was loaded in 4–20% gradient SDS-polyacrylamide gels (Bio-Rad Laboratories, Inc.). After which, proteins were transferred onto PVDF membranes (GE Healthcare Biosciences) for 1 h at 110 V, 4 °C. Blocking was performed using 5% nonfat dry milk in 0.1% Tris-buffered saline Tween 20 (TBST) for 1 h at 4 °C. After blocking, the membranes were washed with 0.1% TBST (5 times, 5 min each) and incubated with Bax, Bcl-2 or caspase 3 primary antibodies (1:300) overnight at 4 °C. The membranes were washed again and incubated with anti-mouse or anti-rabbit IgG horseradish peroxidase conjugate secondary antibodies (1:1500) for 1 h at room temperature. After incubation and repeated washing, the membranes were prepared for detection using a 1:1 mixture of chemiluminescent reagents A
(luminol solution) and B (peroxide solution) (GE Healthcare Biosciences). The membrane pictures were taken on a GelLogic 4000 Pro Imaging System (Carestream Health, Inc., Rochester, NY). The relative amount of each target protein was normalized to GAPDH.

7.3.7 Statistical analysis

GraphPad Prism Software, version 4 (La Jolla, CA) was used. The results were expressed as the mean ± standard error and a non-parametric Mann-Whitney test was used to test for statistical differences between groups. Significant differences were reported at $p < 0.05$.

7.4. Results

7.4.1 Luteolin, gemcitabine and their combination modified mouse body weight, organ weights and pancreatic tumor mass

Table 7.1 shows the average body weight change, liver, lung and spleen weights, in relation to final body weight, among the groups after 41 days of treatment. Gem and the combination group of Lut and Gem had significant changes in body weight compared to the control ($p=0.0259$ and $p=0.0004$, respectively), while Lut treatment alone did not ($p=0.1108$). There were no significant differences in mean lung weights as compared to the control. However, the Gem treated group had a significantly lower ($p = 0.045$) mean liver weight than the control group; and groups treated with either Lut alone or in combination with Gem had significantly higher ($p = 0.025$ or $0.010$, respectively) mean spleen weights compared to the control group.

Treatment with Lut, Gem or their combination led to a reduction in tumor mass, shown as pancreas weight (g) over body weight (g) (Fig. 7.1A). Mean tumor mass per body weight was reduced to 0.021 in the Lut group and to 0.014 in the Gem group, however, only the combination group with a mass of 0.013 was significantly lower ($p = 0.048$) than the control group tumor.
mass of 0.027. Three representatives of pancreas pictures from each group are presented in Fig. 7.1B.

### 7.4.2 Luteolin, gemcitabine and their combination reduced the expression of proliferating cell nuclear antigen

Fig. 7.2A shows representative slides of H and E staining of pancreas tumor sections from each treatment group. Fig. 7.2B shows the effect of Lut, Gem and their combination on the expression of PCNA in pancreatic tumor tissue. All three treatments caused a significant reduction in PCNA expression with Lut causing a 25% reduction (p=0.005), Gem causing a 37% reduction (p=0.0002) and their combination causing a 37% reduction (p=0.0008).

### 7.4.3 Combination treatment of luteolin and gemcitabine decreased the expressions of K-Ras, GSK-3β, p(Tyr216)GSK-3β and p(Ser311)NF-κB p65 in pancreatic tumor tissue

Fig. 7.3 displays the effect of Lut, Gem and their combination on the expression of K-Ras, GSK-3β, p(Tyr216)GSK-3β (active form) and p(Ser311)NF-κB p65 (active form) in pancreatic tumor tissue. In Fig. 7.3A, single-agent Lut and Gem treatments presented non-significant reductions on the expression of K-Ras (upstream GSK-3β effector) of 20% (p=0.122) and 16% (p=0.293), respectively, while the combination treatment resulted in a significant reduction of 46% (p=0.0006). In Fig. 7.3B, Lut and Gem treatments presented non-significant reductions on the expression of GSK-3β of 8% (p=0.762) and 17% (p=0.593), respectively, while the combination treatment resulted in a significant reduction of 34% (p=0.0014). In Fig. 7.3C, Lut and Gem treatments presented non-significant reductions on the expression of p(Tyr216)GSK-3β of 10% (p=0.261) and 8% (p=0.174), respectively, while the combination treatment resulted in a significant reduction of 16% (p=0.033). In Fig. 7.3D, Lut and Gem treatments presented non-
significant reductions on the expression of p(Ser311)NF-κB p65 of 12% (p=0.418) and 11% (p=0.503), respectively, while the combination treatment resulted in a significant reduction of 27% (p=0.036).

7.4.4 Luteolin, gemcitabine and their combination modified the expression of some proteins involved in apoptosis in pancreatic tumor tissue

Fig. 7.4 shows the effect of Lut, Gem and their combination on the expression of the apoptotic markers cytochrome c and caspase 3, as well as the effect on the Bcl-2/Bax ratio in pancreatic tumor tissue. Taking into account the expressions of Bax and Bcl-2, Gem treatment resulted in a significant reduction of Bcl-2/Bax ratio from 1.00 (control group) to 0.34 (p=0.022), while the combination treatment of Lut and Gem reduced this ratio to 0.32 (p=0.006) (Fig. 7.4A). On the other hand, Lut treatment alone did not affect the Bcl-2/Bax ratio (p=0.960). This reduction on Bcl-2/Bax ratio allowed for the release and activation of other pro-apoptotic markers cytochrome c and caspase 3. As shown in Fig. 7.4B the combination treatment resulted in a significant increase (p=0.035) in cytochrome c expression of 44%, and Fig. 7.4C showed a statistically significant increase in the expression of the executioner of apoptosis, caspase-3 in the combination by 417% (p = 0.003) when compared to the control group.

7.5. Discussion

Lut has been reported to exhibit anti-cancer effects in various cancer cells as well as to potentiate the cytotoxic effects of celecoxib in breast cancer cells [20], of oxaliplatin, bleomycin and doxorubicin in lung cancer cells [21], and cisplatin in leukemia cells [22]. However, the effect of Lut against pancreatic cancer cells in vitro has only recently began to be investigated [11-14] and an in vivo study on its effects on pancreatic cancer has yet to be published. The present study
examined the ability of Lut to enhance the anti-tumor activity of Gem in an orthotopic mouse model. We did not administer Lut and Gem on the same day as our previous *in vitro* study showed that simultaneous treatment of Lut with chemotherapeutic drugs produced less-than-additive effects [14]. The results showed that only the combination treatment of Lut and Gem was able to significantly lower pancreatic tumor mass compared to the control group; however, this effect was not significantly different than Gem alone.

PCNA expression is associated with the late G1 and S phases of the cell cycle [23]. Its significance as a measure of tumor growth and metastasis has been investigated in pancreatic cancer. For instance, strong expression of PCNA was reported in cell-line induced pancreatic tumors in rats [24] while suppression of PCNA oncogenic activity is associated with development of novel drug therapy for pancreatic cancer [25]. Our results showed that expression of PCNA was significantly reduced by all the treatments indicating that Lut, Gem and their combination have the capability to reduce proliferation of pancreatic cancer *in vivo*. This was further supported by our observation that these groups had lower pancreatic tumor burden as compared to the control group. However, there was no statistical difference between PCNA expression in Gem and combination treatment groups, suggesting that Lut was not enhancing Gem anti-tumor effects through direct inhibition of proliferation.

Inhibition of cell proliferation and/or induction of apoptosis are two major mechanisms by which cancer cells are killed by chemotherapeutic agents. Unfortunately, resistance to apoptosis induced by chemotherapeutic agents, including Gem, constitutes a major obstacle in the use of these drugs for pancreatic cancer treatment [26]. Several studies have shown that inhibition of a key target, NF-κB, in cancer pathogenesis by novel bioactive compounds leads to the sensitization of pancreatic cancer cells to chemotherapeutic drugs [27-29]. In the present
study, the combination treatment of Lut and Gem caused a significant decrease in GSK-3β, p(Tyr216)GSK-3β (active form) and NF-κB [as measured by a decrease in the active form of p65 subunit, p(Ser311)NF-κB p65], as well as an increase in apoptosis, specifically the mitochondrial pathway of apoptosis. Activation of this pathway of apoptosis causes the release of cytochrome c from the mitochondria allowing for the formation of the apoptosome multiprotein complex, which activates caspase 9 and in turn leads to the cleavage of caspase 3 (executioner caspase) [30]. The results indicated that combination treatment of Lut and Gem led to the release of cytochrome c from the mitochondria and therefore the activation of caspase 3 through a reduction in the Bcl-2/Bax ratio, regulators of mitochondrial membrane permeability. This suggests that Lut is enhancing the anti-pancreatic tumor effects of Gem through increased apoptosis.

K-Ras is an oncogene that is found to be mutated in up to 90% of diagnosed pancreatic tumors [31]. Zhang et al. [32] suggested that K-Ras increases GSK-3β gene expression via the MAPK signaling pathway in pancreatic cancer cells. Although K-Ras is not found to be mutated in the BxPC-3 cell line [33] used in the present in vivo study, combination treatment of Lut and Gem did lead to a significant reduction in K-Ras expression and this could potentially explain the reduction in GSK-3β expression. We have previously demonstrated the ability of apigenin, a flavonoid that differs from Lut by one hydroxyl group, to inhibit K-Ras expression in both BxPC-3 (wildtype K-Ras) and PANC-1 (mutated K-Ras [34]) pancreatic cancer cells in vitro [13]. Taking this into account, Lut also has the potential to inhibit mutated K-Ras as well, however more studies are needed to determine the mechanism of action.

Chemotherapeutic drugs often cause various adverse effects in patients, including anorexia, constipation or diarrhea, which can cause under nourishment and loss of body weight.
We found that the final body weight of mice treated with Gem alone or in combination with Lut was significantly less than the control or Lut groups. Wei et al. [35] also reported a significant body weight loss at the end of their study compared to the control group when 125 mg/kg bw Gem was used in a pancreatic cancer xenograft model. However, they reported that combination treatment of evodiamine (a bioactive compound in *Evodiae fructus*) and a lower dose of Gem (80 mg/kg bw) was able to effectively inhibit pancreatic tumor growth without leading to a significant body weight loss.

Collectively, the present study demonstrated the potential of Lut to enhance the anti-tumor activity of Gem in a pancreatic cancer orthotopic mouse model. Combination treatment of Lut and Gem promoted apoptotic cell death in pancreatic tumor cells through inhibition of the K-Ras/GSK-3β/NF-κB signaling pathway and subsequently a reduction in the Bcl-2/Bax ratio allowing for the release of cytochrome c from the mitochondria and activation of caspase 3. Since the bioavailability of Lut in plasma is low (0.44%) in humans [36], future studies may use encapsulation to overcome this obstacle if oral administration is the preferred route.

### 7.6. References


7.7. Tables and figures

Table 7.1: Effect of luteolin, gemcitabine and combination treatment on change in body weight, and liver, lung and spleen weights in an orthotopic pancreatic cancer mouse model\textsuperscript{a,b}

<table>
<thead>
<tr>
<th>Group</th>
<th>Change in Body wt (g)\textsuperscript{c}</th>
<th>Liver wt (g) / Body wt (g)</th>
<th>Lung wt (g) / Body wt (g)</th>
<th>Spleen wt (g) / Body wt (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.3 ± 0.4</td>
<td>0.0550 ± 0.0009</td>
<td>0.0063 ± 0.0004</td>
<td>0.0055 ± 0.0004</td>
</tr>
<tr>
<td></td>
<td>n = 14</td>
<td>n = 10</td>
<td>n = 10</td>
<td>n = 10</td>
</tr>
<tr>
<td>Lut</td>
<td>2.1 ± 0.6</td>
<td>0.0589 ± 0.0027</td>
<td>0.0073 ± 0.0003</td>
<td>0.0086 ± 0.0009*</td>
</tr>
<tr>
<td></td>
<td>p = 0.1108</td>
<td>p = 0.364</td>
<td>p = 0.088</td>
<td>p = 0.025</td>
</tr>
<tr>
<td></td>
<td>n = 12</td>
<td>n = 7</td>
<td>n = 10</td>
<td>n = 10</td>
</tr>
<tr>
<td>Gem</td>
<td>2.2 ± 0.2*</td>
<td>0.0505 ± 0.0017*</td>
<td>0.0070 ± 0.0005</td>
<td>0.0068 ± 0.0006</td>
</tr>
<tr>
<td></td>
<td>p = 0.0259</td>
<td>p = 0.045</td>
<td>p = 0.504</td>
<td>p = 0.085</td>
</tr>
<tr>
<td></td>
<td>n = 14</td>
<td>n = 11</td>
<td>n = 11</td>
<td>n = 11</td>
</tr>
<tr>
<td>Lut + Gem</td>
<td>-0.2 ± 0.9*</td>
<td>0.0596 ± 0.0029</td>
<td>0.0076 ± 0.0004</td>
<td>0.0098 ± 0.0013*</td>
</tr>
<tr>
<td></td>
<td>p = 0.0004</td>
<td>p = 0.230</td>
<td>p = 0.088</td>
<td>p = 0.010</td>
</tr>
<tr>
<td></td>
<td>n = 12</td>
<td>n = 7</td>
<td>n = 7</td>
<td>n = 7</td>
</tr>
</tbody>
</table>

All values are shown as mean ± standard error.

\(a\)To account for differences in body weight of the mice liver, lung and spleen weights were divided by individual body weights to get a ratio.

\(b\)Treatment groups were individually compared to the control group for each type of weight and groups that were statistically different as determined by Mann-Whitney analysis were indicated by an *.

\(c\)Change in body wt = Final body wt - Initial body wt
Fig. 7.1. Effect of luteolin, gemcitabine and their combination on pancreas weight (adjusted by final total body weight) in an orthotopic mouse model. (A) * indicates statistically significant differences for combination treatment of luteolin and gemcitabine vs. the control group as determined by Mann-Whitney analysis. Values are the mean ± standard error. P-values < 0.05 indicate significant differences. (B) Representative pancreas pictures from each group.
Fig. 7.2. Effect of luteolin, gemcitabine and their combination on the expression of proliferating cell nuclear antigen (PCNA). (A) Representative pancreatic tissues of hematoxylin and eosin stained sections from each treatment group. Arrows indicate normal cells. (B) Immunohistochemical staining for PCNA in pancreatic tumor tissue showed that treatment with luteolin, gemcitabine and their combination resulted in the reduction of its expression when compared to the control group. * indicates statistically significant differences in PCNA expression vs. the control group as determined by Mann-Whitney analysis (n = 6). Values are the mean ± standard error. P-values < 0.05 indicate significant differences. On the basis of the immunohistochemical peroxidase staining assay the color produced was brown.
Fig. 7.3. Inhibitory effect of luteolin, gemcitabine and their combination on the expression of molecules in the GSK-3β/NF-κB signaling pathway. (A) Immunohistochemical staining for K-Ras in pancreatic tumor tissue showed that combination treatment of luteolin and gemcitabine resulted in a significant (indicated by *) reduction in its expression when compared to the control group as determined by Mann-Whitney analysis (n = 6). (B) Combination treatment of luteolin and gemcitabine significantly (indicated by *) reduced the expression of GSK-3β in pancreatic tumor tissue compared to the control group as determined by Mann-Whitney analysis (n = 5). (C) Combination treatment of luteolin and gemcitabine significantly (indicated by *) reduced the expression of p(Tyr216)GSK-3β in pancreatic tumor tissue compared to the control group as determined by Mann-Whitney analysis (n = 6). (D) Combination treatment of luteolin and gemcitabine significantly (indicated by *) reduced the expression of p(Ser311)NF-κB p65 in pancreatic tumor tissue compared to the control group as determined by Mann-Whitney analysis (n = 5). Values are the mean ± standard error. P-values < 0.05 indicate significant differences. On the basis of the immunohistochemical peroxidase staining assay the color produced was brown.
Fig. 7.3 (cont.)

C) 

![Graph showing area stained (μm²) for different treatments: Control, Lut, Gem, Lut + Gem.](image)

- **p(Tyr216)GSK-3β**
  - Control
  - Lut
  - Gem
  - Lut + Gem

D) 

![Graph showing area stained (μm²) for different treatments: Control, Lut, Gem, Lut + Gem.](image)

- **p(Ser311)NF-κB p65**
  - Control
  - Lut
  - Gem
  - Lut + Gem

300 μm
**Fig. 7.4.** Effect of luteolin, gemcitabine and their combination on the expression of apoptotic markers. Protein lysates from pancreatic tissue were extracted and subjected to western blot analysis for Bax, Bcl-2 and caspase 3 protein expressions. (A) Treatment with gemcitabine or the combination of luteolin and gemcitabine significantly (indicated by *) decreased the Bcl-2/Bax ratio compared to the control group as determined by Mann-Whitney analysis (n ≥ 4). (B) Immunohistochemical analysis of pancreatic tumor tissue showed that combination treatment of luteolin and gemcitabine significantly (as indicated by *) increased the expression of pro-apoptotic cytochrome c compared to the control group as determined by Mann-Whitney analysis (n=6). (C) Combination treatment of luteolin and gemcitabine significantly (indicated by *) increased the expression of pro-apoptotic caspase 3 compared to the control group as determined by Mann-Whitney analysis (n ≥ 6). Values are the mean ± standard error. P-values < 0.05 indicate significant differences. On the basis of the immunohistochemical peroxidase staining assay the color produced was brown.
Fig. 7.4 (cont.)

C)
CHAPTER 8

CONCLUSIONS

- Of the 22 bioactive compounds tested, the flavonoids luteolin, apigenin and quercetin had the overall highest inhibitory effect on GSK-3β activity and they optimally fit within the enzyme’s binding cavity.

- The inhibitory effects of flavonoids (luteolin, apigenin, quercetin, kaempferol, rutin, hesperetin, naringenin and nobiletin) on GSK-3β activity correlated with their interaction energy values (r = 0.71).

- The inhibitory effects of flavonoids (apigenin, luteolin, quercetin, kaempferol, hesperetin, naringenin and nobiletin) on GSK-3β activity and on BxPC-3 cell proliferation correlated, r = 0.87 for 24 h exposure and r = 0.86 for 48 h exposure.

- Apigenin and luteolin were the most effective bioactive compounds to inhibit the growth of both BxPC-3 and PANC-1 human pancreatic cancer cells.

- Apigenin inhibited BxPC-3 and PANC-1 pancreatic cancer cell proliferations through inhibition of the K-Ras/GSK-3β/NF-κB signaling cascade.

- Apigenin arrested G2/M cell cycle phase through inhibition of cyclin B1 and induced the mitochondrial pathway of apoptosis in BxPC-3 and PANC-1 cells by allowing the release of cytochrome c from the mitochondria and therefore the activation of caspase 3 through inhibition of Bcl-2 (a regulator of mitochondrial membrane permeability, demonstrated in BxPC-3 cells only) and the caspase inhibitor XIAP.

- Apigenin treatment led to an upregulation in the gene expression of potential anti-tumor cytokines IL17 (A, C, F), LTA and IFNB1 in BxPC-3 cells.
• Pretreatment of BxPC-3 cells for 24 h with low concentrations of apigenin or luteolin, followed by the addition of 5-fluorouracil, gemcitabine or oxaliplatin for 36 h (total time 60 h) was effective at inhibiting cancer cell proliferation.

• Simultaneous treatment for 60 h with either apigenin or luteolin and 5-fluorouracil or gemcitabine caused a less-than-additive effect in BxPC-3 cells.

• Luteolin enhanced the anti-proliferative effects of gemcitabine in BxPC-3 cells through inhibition of GSK-3β leading to a decrease in NF-κB transcription activity and an increase in apoptosis (as measured by an increase in pro-apoptotic cytochrome c).

• Combination treatment of luteolin and gemcitabine significantly lowered the pancreatic tumor mass compared to the control group, in vivo.

• Luteolin, gemcitabine and their combination all significantly reduced proliferating cell nuclear antigen expression compared to the control group showing that they have the capability to reduce proliferation of pancreatic cancer in vivo.

• Combination treatment of luteolin and gemcitabine promoted apoptotic cell death in pancreatic tumor cells in vivo through inhibition of K-Ras/GSK-3β/NF-κB signaling pathway leading to a reduction in the Bcl-2/Bax ratio, release of cytochrome c and activation of caspase 3. This suggests that luteolin is able to enhance the anti-tumor activity of gemcitabine in vivo by increasing apoptosis.

• Bioactive compounds in fruits and vegetables, specifically apigenin and luteolin, have the potential to reduce pancreatic cancer growth through inhibition of the GSK-3β/NF-κB signaling pathway leading to the induction of apoptosis.
CHAPTER 9  
INTEGRATION AND FUTURE WORK

Since the five-year survival rate for patients with pancreatic cancer is only 6% there is a great need to identify novel treatments that can effectively inhibit cancer cell proliferation and prolong the lives of patients with this disease. The long term goal of this research was to understand the molecular mechanisms by which bioactive compounds present in fruits and vegetables can inhibit pancreatic cancer using \textit{in vitro} and \textit{in vivo} models. Figure 9.1 depicts the overall strategy of this study.

GSK-3\(\beta\), a serine/threonine protein kinase, is an important therapeutic target of interest because it’s disregulated expression has been shown to maintain survival and proliferation in pancreatic cancer cells. This study showed that fruit and vegetable flavonoids were able to optimally bind within the catalytic site of GSK-3\(\beta\) and effectively inhibit its activity. The presence of an unsaturated C-ring and the lack of bulky side groups were among the reasons that apigenin, luteolin and quercetin were superior to the other flavonoids at exerting these effects. These findings suggest the importance of chemical structure in defining inhibitory potential of a bioactive compound on GSK-3\(\beta\) enzymatic activity.

Fruit and vegetable bioactive compounds may represent potential agents that can aid in the treatment of pancreatic cancer. This study showed that flavonoids were the most effective at inhibiting pancreatic cancer cell proliferation in two pancreatic cancer cell lines, BxPC-3 (wildtype K-Ras) and PANC-1 (mutated K-Ras), \textit{in vitro}. There was a significant correlation between the inhibitory effect of flavonoids on GSK-3\(\beta\) activity and on BxPC-3 cell proliferation. This suggests that flavonoids, most notably apigenin, luteolin and quercetin, may be decreasing pancreatic cancer cell proliferation through inhibition of GSK-3\(\beta\). The results have demonstrated
that apigenin was able to exert its anti-proliferative effects through inhibition of the K-Ras/GSK-3β/NF-κB signaling pathway leading to increased apoptosis (Figure 9.2). We propose that apigenin was able to suppress GSK-3β activity both directly by binding within its catalytic site and indirectly by inhibiting K-Ras (an upstream GSK-3β effector) leading to a reduction in GSK-3β gene expression. Decrease in total/active GSK-3β reduced its ability to translocate into the nucleus and activate NF-κB transcription activity therefore leading to a decrease in the expression of anti-apoptotic proteins Bcl-2 and XIAP. Decrease in these two proteins allowed for pro-apoptotic cytochrome c to be released from the mitochondria and the formation of the apoptosome multiprotein complex, which activated caspase 9. Caspase 9 was then able to cleave caspase 3 causing the induction of the mitochondrial pathway of cell death. Examination into the effect that apigenin treatment has on expression of genes related to inflammation and cancer identified that it led to a high increase in the expression of cytokines IL17 (A,C and F), LTA and IFNB1. Further study to evaluate the specific mechanisms by which apigenin upregulates these genes and the downstream effect this causes is needed. Also the determination of whether or not this same effect occurs in pancreatic cancer cell lines that have mutated K-Ras is warranted.

Chemotherapy is one of the three main treatment options for patients with pancreatic cancer, however chemoresistance due to the inhibition of apoptosis is a major challenge when using this type of treatment. Data showed that either apigenin or luteolin were able to enhance the anti-proliferative effects of chemotherapeutic drugs 5-fluorouracil, gemcitabine and oxaliplatin, but not cisplatin in BxPC-3 pancreatic cancer cells. The timing of apigenin and luteolin treatments and their concentrations were of great importance. Luteolin was able to enhance the anti-proliferative effects of gemcitabine through inhibition of the GSK-3β/NF-κB signaling pathway leading to increased apoptosis. Future studies to evaluate the mechanism of
action by which these flavonoids enhanced the anti-proliferative activity of 5-fluorouracil and oxaliplatin, as well as examination into why they were not able to enhance the effects of cisplatin are needed.

An orthotopic mouse model of pancreatic cancer showed that combination treatment of luteolin (84 mg/kg bw) and gemcitabine (125 mg/kg bw) led to a significant reduction in tumor mass compared to the control group, while either treatment alone did not cause a significant reduction. Although combination treatment with luteolin did not directly enhance the anti-proliferative effects of gemcitabine, it did increase apoptosis through inhibition of the K-Ras/GSK-3β/NF-κB signaling pathway. Future studies should focus on increasing the sample size per treatment group and dose optimization for the combination of luteolin and gemcitabine to prevent adverse side effects due to treatment such as weight loss. Since the bioavailability of flavonoids in plasma is low in humans, future studies should also investigate the use of encapsulation to overcome this obstacle if oral administration is the preferred route.

Additionally, studies should investigate the combination effect of luteolin or apigenin with other chemotherapeutic drugs, including 5-fluorouracil and oxaliplatin, in an in vivo pancreatic cancer model.

In conclusion, the results provide support for the beneficial effects of the flavonoids apigenin and luteolin against pancreatic carcinogenesis and provide the foundation for future studies on their benefits as adjuvants for chemotherapy in the treatment of this disease.
Figures

Figure 9.1. Illustration of the overall strategy for each aim in this study.
Figure 9.2. Proposed mechanisms of action by which apigenin and luteolin inhibit pancreatic carcinogenesis.
APPENDIX

Appendix A.1. Dose-response inhibitory effect of (A) 5-Fluorouracil, (B) Gemcitabine, (C) Cisplatin and (D) Oxaliplatin treatment on BxPC-3 human pancreatic cancer cell proliferation. Cells were incubated for 36 h at 37°C with chemotherapeutic drug concentrations ranging from 0.01 to 200 µM. Two independent experiments performed in triplicates were used for the analysis.

\[ IC_{30} = 2.0 \pm 1.8 \mu M \]

\[ IC_{30} = 62.8 \pm 25.7 \mu M \]

\[ IC_{30} = 10.4 \pm 1.1 \mu M \]

\[ IC_{30} = 0.14 \pm 0.01 \mu M \]
The research was performed under the direction and supervision of Dr. Elvira Gonzalez de Mejia, Professor in the Department of Food Science and Human Nutrition at the University of Illinois at Urbana-Champaign.