1-1-2010

Synergistic Effects Of Garcinol And Gemcitabine In Enhancing Therapeutic Efficacy In Pancreatic Adenocarcinoma Cells And Its Effect On Microrna Profile

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SYNERGISTIC EFFECTS OF GARCINOL AND GEMCITABINE IN ENHANCING THERAPEUTIC EFFICACY IN PANCREATIC ADENOCARCINOMA CELLS AND ITS EFFECT ON MICRORNA PROFILE

by

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DISSERTATION

Submitted to the Graduate School

of Wayne State University,

Detroit, Michigan

in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

2010

MAJOR: NUTRITION AND FOOD SCIENCE

Approved by:

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Advisor Date

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Dedication

I dedicate this dissertation to my family, especially…

to my wonderful parents, Kanta and Anand Parasramka who have raised me to be the person I am today;

to my siblings Pramod, Priti, Vikas and Parul for their constant motivation and encouragement;

to Tanushree, Rohan and Aanya for their love and affection;

to my grandparents for all their pampering.

Acknowledgement

Though only my name appears on the cover of this dissertation, a great many people have contributed to its production. I owe my gratitude to all those people who have made this dissertation possible and because of whom my graduate experience has been one that I will cherish forever.

My deepest gratitude is to late Dr. S. D. Telang, my thesis mentor during my Master's program at the Biochemistry Dept., MSU, Baroda, India. I have been amazingly fortunate to have a mentor like her who gave me freedom to explore on my own and at the same time the guidance to recover when my steps faltered. Ms. Telang taught me how to question thoughts and express ideas in research. I will always be thankful to her.

I am especially indebted to my talented dissertation committee. First and foremost I offer my sincerest gratitude to my supervisor, Dr. Smiti Gupta, who encouraged me to not only grow as an experimentalist but also as an instructor and an independent thinker. I am not sure how many graduate students are given the opportunity to develop their own individuality and self-sufficiency by being allowed to work with such independence. You provided me the warmth and care that was needed, when I was going through the toughest phase of my life and for that, I and my entire family will always be indebted to you. For everything you've done for me, Dr. Gupta, I thank you.

I was delighted to interact with Dr. Ahmad Heydari by attending his classes and having him as my co-advisor on my committee. I am thankful for all your valuable suggestions and inputs to my research project. I would also like to thank Dr. Pramod Khosla for his assistance and guidance in getting my graduate career started on the right foot at Wayne State University and constantly encouraging me in my teaching assignments.

I would like to thank Dr. Fazlul Sarkar, for providing me a well-rounded experience in research. I learnt the maximum techniques in his lab and I am thankful to everyone in the team, to make feel one among them. Despite his hectic schedule as a Professor in the School of Medicine, Dr. Sarkar was always available to chat with me and cheer me up. I apologize for always interrupting during your lunch breaks but at the same time will always cherish the long discussions that I had with you during that time which helped me develop a deeper appreciation for the mysteries of life and what it means to “get inside” a text. His insights to cancer research and chemoprevention are second to none.

Dr. Sanjeev Banerjee, whom I can say played all roles from a co-advisor, a colleague, a friend or family, has always been there to listen and advise me. His infectious enthusiasm and unlimited zeal have been a major driving force through my graduate studies at Wayne State University. I am deeply grateful to him for the long discussions that helped me sort out the technical details of my work. I am also thankful to him for encouraging me in my writings and for carefully reading and commenting on countless revisions of this manuscript. I fall short on words to express my gratitude towards you. Thank you!

I would like to extend my gratitude to Ms. Shadan Ali for accommodating me in her work space and taking time off her busy schedule to help me solve the technical difficulties I experienced during the study. I will always cherish and miss those coffee breaks with you.

I am thankful to Dr. Kong and Dr. Kim for helping me with the experimental details in my study. My dissertation would have been incomplete without the efforts of Ms. Tara Deryavoush, Ms. Shaistha Zaheeruddin, Ms. Jennifer Wrubel and Ms. Amrah Ali. They started to assist me as a part of their undergraduate research credits, but eventually took full responsibility of their duties and worked with great enthusiasm on this project. It’s been an honor to work with each one of you. Thank you!!

I express my gratitude to the Dept. Chairperson Dr. Jen, who always kept a watch on each one of us like a guardian and made sure that things were all moving in the right direction. I am also thankful to you for accompanying me to the ASN award ceremony reception; it was a moment I will cherish for long.

I owe sincere gratitude to Ms. Debbie Zebari and Ms. Pat Hanserd. They have helped me manage all my paper work and university documentation over the past five years. I will miss you'll deeply.

A warm hug to Nadia and Shaistha for all the special moments I have shared with you'll. You both made me feel like a family, while I was away from home. I will always cherish the time I have spent with you'll. I will miss you both…..but I am just a phone call away!!!

Special thanks to Ms. Han Pham, for all her wonderful gestures and adding humor to my otherwise monotonous work routine. I am thankful to my lab colleagues Mr. Xiangming Ji, Ms. Andreea Geamanu and Ms. Monika Wadhera for their support and encouragement over the years. I am also thankful to Ms. Archana Unnikrishnan and Ms. Deepa Kushwaha for all their helpful advices over the years.

.

Many friends have helped me stay sane through these difficult years. Their support and care helped me overcome setbacks and stay focused on my graduate study. I greatly value their friendship and I deeply appreciate their belief in me. Indraneel Page, Vaibhav Lidbide, Parag Zatye, Rohan Neelgund, Khyati and Kashyap, Siddharth Mayekar, Palak Patel, Nisha Narang and Fazila Qazi for their constant support, love and care.

Animesh helped me develop a vocabulary for my emotions at the most difficult stages in this entire process. I am grateful to my best friend as he gave me the strength to keep working till completion. He helped me grow more comfortable in my own skin.

From his proofreading to his constant encouragement, he has been with me through and through. Thank you!!

None of this would have been possible without the love and patience of my family. Papa, thank you for always encouraging me and spending all the sleepless nights to accompany me throughout my education. I am glad I was able to fulfill your dream. I couldn't have asked for anything better.

I feel proud of my brothers, Pramod Parasramka and Vikas Parasramka for their talents. They have been a role model for me to follow unconsciously since I was a teenager and they have always been one of my best counselors.

I deeply appreciate the faith and support from my sister-in-law, Priti Parasramka and Parul Parasramka for always encouraging me to go on. I am grateful to God for the wonderful additions to my family, Tanzy, Rohan and Aanya. I am thankful to all my extended family members for their love and support throughout the years.

The last person to be mentioned in the acknowledgments tends, more often than not, to be the one you wanted to mention first. My very special thanks to the person whom I owe everything I am today, my Mom, Kanta Parasramka. She allowed me to be as ambitious as I wanted. I cannot ask for more from my ma as she is simply perfect. I have no suitable word that can fully describe her everlasting love to me. Mom, you mean the world to me and I will try to the best of my abilities to fulfill all your dreams. Momzie, I love you.

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List of Abbreviations

PaCa – Pancreatic Cancer

PanIN - Pancreatic Intraepithelial Neoplasia

EGFR – Epidermal Growth Factor Receptor

MAPK – Mitogen Activated Protein Kinase

MMP – Matrix MetalloProteinases

NFκB – Nuclear Factor Kappa B

TNF-α – Tumor Necrosis Factor alpha

TGF-β – Transforming Growth Factor beta

COX-2 – Cyclooxygenase - 2

VEGF – Vascular Endothelial Growth Factor

IL – Interleukins

PTCH HHR – Patched HedgeHog Receptor

IGF-IR – Insulin like Growth Factor Receptor

PDGFR – Platelet Derived Growth Factor Receptor

PI3K/Akt – Phospatidylinositol 3 Kinase

NRP-1 - Neuropilin-1

SHH – Sonic HedgeHog

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CHAPTER ONE

BACKGROUND

1.1 CANCER

In a healthy body, normal tissues exhibit well maintained homeostasis between cell death and cell proliferation. During the early years of life, normal cells divide faster allowing a person to grow, but on reaching adulthood most cells divide only to replace worn out, damaged or dying cells. When this equilibrium is disturbed, cells lose their ability to undergo apoptosis, proliferate in defiance to normal restraints on cell division and begin to grow, invade and colonize territories normally reserved for other cells. This type of cell behavior is characterized as "*cancer*".

Cancer is the second leading cause of death and is responsible for one in four deaths in US [1]. It is caused by either internal factors (hormones, inherited mutations or immune conditions) or by external factors (diet, tobacco, chemical exposure, radiations, infectious organisms to name a few) acting together or in sequence in the process of carcinogenesis. This process occurs in three phases: initiation, promotion and progression. During initiation, the DNA of the cell is mutated by chemical or physical carcinogens, leading to activation of oncogenes and/or inactivation of tumor suppressor genes. The second phase, tumor promotion involves clonal expansion of initiated cells, owing to increased cell proliferation and reduced cell death. In the third phase, tumor progression, tumor size increases along with invasion and metastasis. In this stage, additional mutations acquired confer a further growth advantage and more malignant phenotype [2].

Cancers can develop anywhere in the body. No matter where the cancer cells spread, they are characterized by tissue and cell type from which they arise. From histological viewpoint carcinoma arise from epithelial cells, sarcomas from connective tissue or muscle cells, leukemia from blood cells, and lymphomas originate in lymph nodes and immune system tissues. This study is focused on novel therapeutic strategies for pancreatic cancer (PaCa) originating from acinar ductal cells of the pancreas. Hence an overview of the clinicopathologic characteristics of PaCa has been discussed with an emphasis on the commonly occurring types of pancreatic carcinomas.

1.2 Pancreatic Cancer

Pancreas is a digestive organ present deep in the abdominal area between the stomach and spine surrounded by liver, intestine and other organs. Pancreas can be divided into six cellular compartments: 1) *Acinar cells* produce the digestive enzymes 2) *Ductal cells* transport these enzymes to the duodenum. Both these cells together regulate the exocrine functions of the organ; 3) *Islets of Langerhans* form the endocrine compartment and are involved in glucose regulation by production of hormones which regulate the metabolic activities; 4) *Supportive elements* like nerves, fibrous tissue, vessels and other connective tissue elements play a very crucial role; 5) *Ambiguous cells* and 6) *Stem cells* are less well characterized and have not been clearly identified in histologic sections [3]. Recent reports have shown that most of the cell types that exist in the pancreas are associated with one or more neoplastic counterparts.

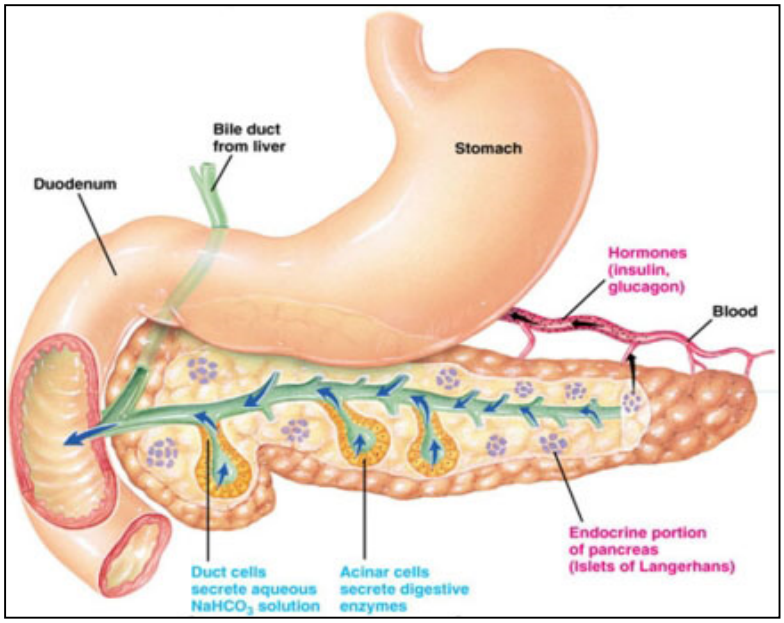


Figure 1: Anatomy of human Pancreas
(Ref: Anatomy, Histology, & Embryology of the Pancreas, 2008)

Pancreatic cancer (PaCa) is the fourth major cause of cancer related death worldwide with an extremely low 5 year survival rate of < 5%. According to NIH-NCI and American Cancer Society statistics, 43,140 new cases will be detected in 2010 and 36,800 deaths will be reported due to this disease [1]. It is also responsible for a substantial number of cases with carcinomas of unknown primacy [4], because it often escapes early diagnosis due to lack of explicit symptoms and early metastasis. Thus at the time of diagnosis, regional lymph node and liver metastasis of pancreatic cancer has already occurred, limiting the effectiveness of current therapeutic strategies.

Risk factors for PaCa are as follows [5]:

Age – Risk of PaCa increases with age. 90% of the patients diagnosed with PaCa are older than 55.

Gender – Men are at a higher risk of getting PaCa than women.

Race – African Americans are more prone to this disease than are Caucasians.

Smoking – Smokers and tobacco chewers are two to three times more susceptible.

Obesity and exercise – Overweight people based on BMI and those who don't get enough exercise are at approximately 1.7 – 2 fold higher risk of this disease.

Diabetes – People with type 2 diabetes are at approximately two fold higher risks. In some patients, cancer is known to be the cause of diabetes.

Chronic pancreatitis – People suffering from long term inflammation might also be at risk of PaCa. Reason is not clear but greatest in patients with inherited chronic pancreatitis.

Cirrhosis of liver – Patients suffering from liver damage due to hepatitis and alcohol abuse are at increased risk of this disease.

Family history – Cancer of the pancreas seems to run in some families.

Work exposure – Heavy exposure to pesticides, certain chemicals and dyes may increase the risk of PaCa.

GI disorders – Excessive stomach acids or presence of H. pylori bacteria in the stomach may increase the risk of PaCa.

Diet – Increased consumption of meats, cholesterol rich foods and nitrosamines, increases the risk of PaCa.

Similar to cancers of other major epithelial organs, pancreatic adenocarcinoma is most common in the seventh decade (mean age 63), and relatively uncommon in patients younger than 40 years old [6]. Invasive ductal carcinoma is the most common neoplasm of the pancreas, constituting >85% of pancreatic tumors that come to clinical attention and due to severity of this disease only 20% of the cases are deemed

resectable at the time of early diagnosis [6]. There are other types of invasive carcinomas of pancreatic ductal lineage which are studied separately from the conventional ductal adenocarcinoma. These include: 1) undifferentiated carcinoma, 2) undifferentiated carcinoma with osteoclast – like giant cells, 3) colloid carcinoma and 4) medullary carcinoma.

Noninvasive carcinomas are another group of pancreatic lesion which may be associated or later progress into invasive carcinoma [3]. Pancreatic Intraepithelial Neoplasia (PanIN) is precancerous lesion affecting the cells lining the smaller side branches in the pancreatic duct. There are different grades of PanIN lesions and as the disease progresses these lesions become severe and are a more direct indicator of PaCa (Figure 2). PanIN-1A microscopic lesion is the most benign type with gradual increments in severity leading to PanIN-3 lesions, which often evolve into PaCa.

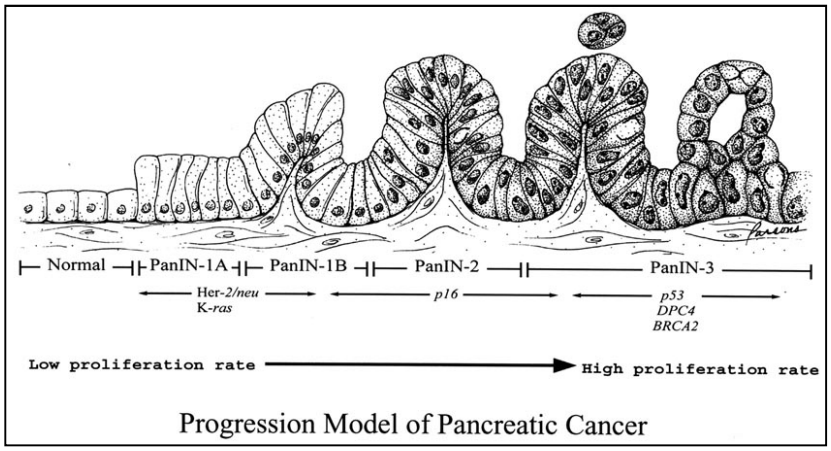


Figure 2: Histologic – genetic model for pancreatic cancer
(Ref: Wilentz et al, 2010)

By the time a patient is diagnosed with this disease, PaCa has often spread and is already in advanced stage, thus a large majority of patients are not suitable for

surgical interventions. Efforts are consistently being made in diagnosing this disease in its early stages, which will help us to detect PaCa while they are clinically manageable by surgery.

1.3 Genetic alterations in pancreatic cancer

Carcinogenesis is a multistep process where several pathways are deregulated and genome stability is compromised. Considerable progress in the identification and characterization of somatic and/or germline genetic alterations have provided a limited understanding as regards to the mechanistic basis of this multigenic mutational disease. Distinct chromosomal aberrations such as amplification, deletion or hypermethylation are often unified with smaller, more subtle alterations affecting the open reading frames of proto-oncogenes, tumor suppressors, and genome caretaker genes dictating the overall development of this disease [7-10] (Figure 3). Although the frequent expression of specific genes might be associated with distinct events at each stage of pancreatic carcinogenesis, pancreatic tumors harbor heterogeneous mutational backgrounds that give each tumor a unique molecular signature leading to very little variation in clinical outcome.

Oncogenic mutations

Oncogenes are derivatives of normal cellular genes called proto-oncogenes. These oncogenes are either constitutively active or become active under specific conditions and play critical role in cell growth and differentiation by maintaining cellular transformation. One of the main oncogenes involved in pancreatic cancer is *K-ras*

(12p12. 1) which functions as a GTP- binding second messenger in growth factor receptor signaling pathways enhancing the transition through the G1 phase of the cell cycle. Somatic mutations can alter the GTP-binding pocket, thus deregulating the ability of *K-ras* to dephosphorylate GTP, rendering the protein constitutively active. 95% of pancreatic cancers have *K-ras* mutations, representing the highest fractions of any tumor type [11]. Gain-of-function owing to mutation of *K-ras* has been reported to occur in a great majority of pancreatic adenocarcinomas leading to the activation of transcription factor *NF-κB* [12]. Normal pancreatic tissue rarely harbors mutations in the *K-ras* oncogene [13], whereas activation of cellular pathways involving *ras* is essential for pancreatic carcinogenesis and occurs early during the development of pancreatic malignancy [14].

Growth factor signaling cascades

The deregulation of certain genes suggests a key role for them in the development of pancreatic cancers. Several reports have confirmed that the stimulation of *EGFR*, *PTCH* HedgeHog receptor, *IGF-IR*, and *PDGFRs* signaling by their corresponding ligands through autocrine and paracrine loops contributes to the sustained proliferation, survival, migration, and metastasis of pancreatic cancer cells [15]. The activation of these growth factor receptors results in the stimulation of distinctive mitotic and antiapoptotic signaling cascades, which get co localized at close proximity in the microdomains of the plasma membrane, thus playing an important role by allowing a more rapid transduction of the mitogenic and survival signals.

Epidermal Growth Factor Receptor signaling cascades

EGF is a polypeptide and one of the maintaining factors in continuous renewal of epithelial cells by binding to its receptor *EGFR*. The latter is characterized by its ligand dependent tyrosine kinase activity, and the subfamily consists of four closely related receptors: *ErbB/ HER 1- 4*. Coexpression of *EGFR* and its ligands *EGF* and *TGF-α*, in pancreatic cancer has been notably recognized as a molecular event linked with tumor growth, metastasis and a decrease in survival span and is reported to occur in 38% of human pancreatic carcinomas [16-18].

Reports have shown that the enhanced activation of *EGFR* signaling in pancreatic cancer could be in part by the down regulated expression of *EGFR*-related protein (*ERRP*), a negative regulator of ligand induced *EGFR* activity [19]. Sustained activation of *EGF-EGFR* systems assumes a crucial role in pancreatic cancer progression. This activation leads to stimulation of numerous mitotic, antiapoptotic and angiogenic signaling cascades including *MAPK*, *PI3K/Akt*, *Src*, *NF-κB*, and the coreceptor for *VEGF*, neuropilin-1(*NRP-1*) which contributes in the sustained proliferation, survival and metastasis of cancer cells.

Sonic Hedgehog, Notch, and Wnt signaling cascades

The aberrant reactivation of distinct developmental signaling pathways like *Sonic Hedgehog, Notch* and *Wnt* in cancer epithelial cells and the molecular crosstalk between these mitogenic cascades plays a major role in the initiation and progression of pancreatic cancer [20-22]. Briefly, the activation of *GL1* transcription factor causes the upregulated expression of Indian and *Sonic Hedgehog* ligands in pancreatic cancer.

Similarly, the reactivation of *Notch* pathway in epithelial cells contributes to the initiation of pancreatic tumorigenesis at the earliest phases. In addition, a defect in *Wnt/β-catenin* signaling through *β-catenin* stabilization also occurs in pancreatic ductal neoplasms. However, the establishment of specific functions associated with enhanced activation of *Wnt/β-catenin* requires further in depth studies.

Vascular endothelial growth factor

Vascular endothelial growth factor (*VEGF*), the most well-characterized angiogenic factor, is known to play a vital role in tumor associated microvascular invasion [23-25]. In human pancreatic cancer, *VEGF* has been found to be over-expressed [25, 26]. Many studies have documented that *VEGF* is a critical mediator of angiogenesis and regulates most of the steps in the angiogenic cascade including proliferation, migration, and tube formation of endothelial cells [27, 28]. *VEGF* exists, at least, in six isoforms with variable amino acid number produced through alternative splicing.

VEGF is subjected to multi-level regulation to ensure proper expression under physiologic and pathologic conditions. Numerous studies have been devoted for understanding the mechanism by which *VEGF* expression can be regulated at the transcriptional level. A wide range of cytokines, oncogenic proteins or transcription factors such as insulin-like growth factor1 (*IGF-1*), transforming growth factor β (*TGF-β*), *c-Src, Ras, NF-κB* and *SP1*, regulate *VEGF* gene transcription [29-31]. Hypoxic condition can also affect *VEGF* mRNA stability [32] and translation initiation [33]. Extracellular cleavage of *VEGF* by uPA or plasmin and processing by *MMPs* can

regulate its mitogenic effect, bioavailability and vascular patterning in tumors [34, 35]. These multi-level regulated patterns of *VEGF* provide various targets for the inhibition of angiogenesis induced by tumors.

Mitotic and survival signaling cascades

Mitogen Activated Protein Kinase (*MAPK*)

EGFR and other growth receptors stimulate the *MAPK* cascade, leading to the expression of numerous mitotic genes involved in growth, survival and invasion of pancreatic cancer cells. Unlike *K-ras* which is required in the initial stages of cancer development, *MAPK* pathway contributes not only in triggering the proliferative signals in pancreatic cancer cells, but also seems to be absolutely essential for the survival of metastatic cancer cells.

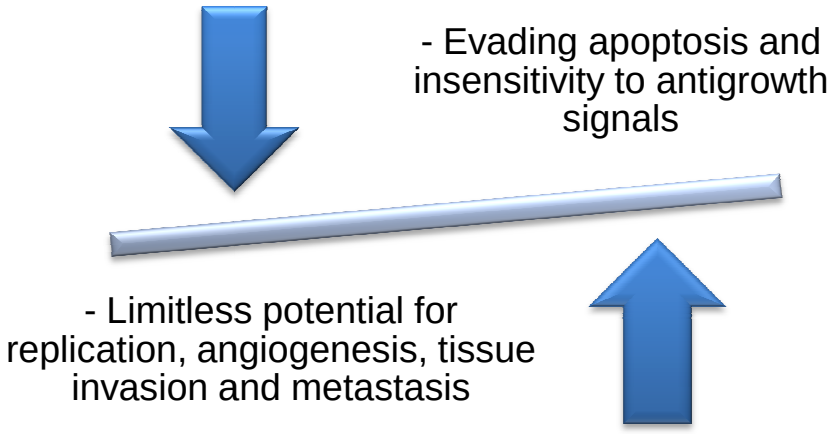


Figure 3: Disruption of signaling mechanisms in cancer

PI3K/Akt signaling

As mentioned previously, gain-of-function mutation of *K-ras* is crucial for pancreatic carcinogenesis [14] which causes the downstream activation of *p21* protein resulting in the activation of various proliferation linked signaling, one of them being *PI3K/Akt* pathway. *PI3K* phosphorylates serine/threonine kinase *PKB/Akt*, which thereby controls the transcription of genes involved in cell survival [36]. *PI3K* cascade regulates a variety of cellular functions in carcinogenesis, such as growth, cell cycle progression, apoptosis and cell migration [37].

NF-κB signaling cascade

Transcription factor *NF-κB* is a family of pleiotropic transcription factors that regulates several downstream growth factors and seems to be constitutively active in about 67% of pancreatic adenocarcinomas but not in immortalized, non-tumorigenic pancreatic epithelial cells [38] (Figure 4).

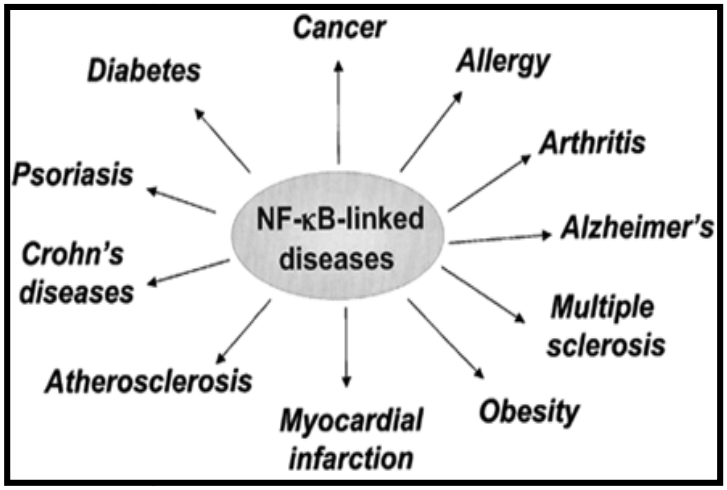


Figure 4: NFκB linked Diseases

NF-κB regulates the expression of a wide array of genes involved in growth, proliferation, metastasis, differentiation, apoptosis and inflammation. Activation of *EGF-EGFR* cascade causes the stimulation of *NF-κB1* (*p50*)/*Rel A* (*p65*) in some pancreatic cancer cells. Binding with inhibitor protein *IκBα*, controls and regulates the dissociation and translocation of active *NF-κB* to the nucleus. On translocation of *NF-κB* to the nucleus, several genes get activated leading to enhanced activity of pro-inflammatory and pro-oncogenic messenger molecules such as *TNF-α, IL-1, IL-6, COX-2*, and *cyclin D1* [39].

Several reports suggest that the main mechanism for *NF-κB* to promote pancreatic cancer growth is via inhibition of apoptosis [40-42]. Additionally, recent works have also revealed that radiation or chemotherapy can evoke cellular stress that causes the activation of *NF-κB*, promoting tumor cell survival. This has been identified as one major cause limiting therapy in PaCa patients [39].

Cyclooxygenase activity

Cyclooxygenase (*COX*) is the enzyme catalyzing the first two steps of synthesis of the prostaglandins from arachidonic acid. There are two isoforms of this enzyme which are similar in structure and activity, but *COX-1* is constitutively expressed in most tissues whereas *COX-2* is absent under normal physiological conditions and is rapidly expressed as a result of stimulation by growth factors, cytokines, inflammatory mediators and tumor promoters [43].

COX-2 mRNA increases by more than 60 fold in pancreatic cancer as compared to adjacent non tumorous tissue, and reportedly present in 90% of pancreatic cancers

[44]. The exact mechanism by which *COX-2* promotes pancreatic carcinogenesis is not clear, but is speculated to increase *VEGF* production and cell proliferation through an autocrine mechanism, inducing angiogenesis and invoking anti-apoptotic effect [43].

Matrix metalloproteinase

Matrix metalloproteinases (*MMPs*), a family of zinc-dependent endopeptidases are critically involved in the processes of tumor cell invasion and metastasis [45-47]. Tumor metastasis occurs by a series of steps including cell invasion, degradation of basement membranes and the stromal extracellular matrix ultimately leading to tumor cell invasion and metastasis [48, 49]. *MMPs* are proteinases that hydrolyze a variety of extracellular matrix protein, which are considered to be important factors in the spread of tumor [49-51]. Among these *MMPs*, matrix metalloproteinase-9 (*MMP-9*, gelatinase B, *M*r 92,000 type IV collagenase) has been shown to be highly expressed in pancreatic cancer [46, 52, 53]

Pancreatic cancer patients with over expression of *MMP-9* revealed a trend towards poor prognosis than those who did not express it [54]. *MMP-9* is also thought to play a role in the degradation of basement membrane collagen IV and, hence, may contribute to the invasive ability of pancreatic cancer cells. Previous studies demonstrated that *MMP-9* expression can be induced by a variety of stimuli, including transforming growth factor-β, and epidermal growth factor through *NF-κB* pathway in pancreatic cancer cells [55]. Inhibition of *MMP-9* expression and/or activity results in reduction of tumor invasion and metastasis in animal studies [56]. Therefore, *MMP-9* is

considered to be an important factor in facilitating invasion and metastases in pancreatic cancer.

Mutations in tumor suppressor genes

Tumor suppressor genes normally function to prevent uncontrolled cellular growth. A minimum of 11 tumor suppressor genes have been identified in human cancers, with inactivation of genes *p16, SMAD4* and *p53* as common events in the development and progression of pancreatic cancer [14, 57, 58]. *p16* (9p21.3) is a cyclin-dependent kinase inhibitor that binds to cyclin-dependent kinase 4(*CDK4*). This is one of the most classical tumor suppressor genes which are most frequently altered in cancers. It normally regulates cell cycle progression by inhibiting the *Cyclin D1-CDK4/6* responsible for G1/S transition by regulating the ability of retinoblastoma tumor suppressor protein (*Rb*) to prevent cell proliferation. As discussed previously, mutation of *K-ras* occurs early on during pancreatic carcinogenesis, whereas alteration of *p16* is associated with the progression of malignancy [59]. This mutation increases the selective advantage of subsequent mutation in *SMAD4* [60].

Some genetic mutations are not always required, but provide a supportive role in development or progression of cancer. *SMAD4* is one such gene which gets inactivated in almost 45% of pancreatic cancers, but less common in other cancer types. *SMAD4/ DPC4* is a tumor suppressor protein which translocates to the nucleus to activate expression of genes involved in growth inhibition [61] and mediates the downstream effects of *TGF-β*-signaling pathway [62] which is otherwise involved in regulation of

several cellular processes such as cell division, differentiation, motility, adhesion and apoptosis [63].

Another frequently altered gene in almost all cancers is *TP53* (p53, 17p13.1). *p53* is a tumor suppressor gene which plays a very crucial role in regulating cell cycle during DNA damage by upregulating the expression of genes in response to cellular stresses. *p53* also directly influences cell cycle machinery by inducing *p21(WAF1/CIP1)* which in turn negatively regulates *CyclinD/Cdk*, thereby arresting cells in the G1 phase of cell cycle and inhibiting cell growth. Mutations in p53 gene occur in almost 70% of pancreatic tumors. Deletion of *p53* by itself does not develop pancreatic neoplasias, but combining it with *K-ras* activation leads to a rapidly progressing and lethal pancreatic adenocarcinoma phenotype [60].

A network is established by the crosstalk between oncogenes, tumor suppressor genes and many other signaling molecules contributing to the molecular pathogenesis of PaCa, suggesting that targeted inactivation of these important signaling pathways could be a novel and newer approach for the prevention and/or treatment of PaCa. Some other genes which are not capable of inducing cancer by themselves but crosstalk with certain signaling pathways or stages of cancer progression and confer an important role in synergism with the other major pathways as listed below.

Cell cycle checkpoints

Various proteins are involved in the regulation of cell cycle. Regulating cell cycle commitment is a very important step which helps in maintaining the balance at different check points. The retinoblastoma protein (*Rb*) plays a crucial role in controlling this

event by its association with *E2F*. When the cells need to undergo a cell division, the growth factors are stimulated which cause the release of *E2F* from *pRb*, allowing the expression of S-phase critical genes. However when cells are not stimulated to divide, *pRb* inhibits *E2F* transcriptional activity thereby restricting the progression in cell cycle. Though it plays a very important role, it is rarely mutated in pancreatic cancers [64]. *Cyclin D1* works in association with either cyclin dependent kinase 4 or 6 to phosphorylate and inactivate *pRb*.

Several reports have been published with different mutation frequencies for this gene, but the discrepancies in frequencies between these studies are not readily explained. Another cyclin, *Cyclin E1* combines with *Cdk2* causing phosphorylation of several substrates important for G1/S transition and DNA replication activities. *Cyclin E1* is found to be overexpressed in ~6% of pancreatic cancers.

Double stranded DNA break repair

Sequence integrity of the genome is very important for normal cells to thrive, which would otherwise be predisposed to cancer. A special class of genes known as genome- maintenance genes plays a primary role in DNA repair and maintenance. *BRCA2* participates with *Rad51* and *BRCA1* to prevent and/or repair DNA double stranded breaks through homologous recombination. Studies show that if this inactive allele is inherited, it confers a 3.5 to 20 fold increased risk of developing pancreatic cancer [65]. The mutational frequency for the homozygous inactivation of this gene has been found to be ~7% in pancreatic cancers [66].

Apoptosis

Apoptosis is a hallmark of cancer progression. Cell survival is regulated by the balance between pro-apoptotic and anti-apoptotic stimuli. Dysregulations in the apoptotic pathway can alter this equilibrium, which is the key step in carcinogenesis. The entire apoptotic mechanism is interplay of several molecules. The tumor suppressor protein *p53* is one of many proteins that contributes to the activation of the intrinsic signaling pathway [67]. Inactivation of this protein or the *p53* pathway (upstream activators and/or downstream effectors), due to mutation, is seen in as many as half of all human cancers [68]. Promoting the alternative extrinsic pathway, operating independently of *p53* or activation of downstream molecules in the intrinsic signaling pathway may be the mechanism by which anti-cancer agents can trigger apoptosis.

MicroRNA in Pancreatic Cancer

Pancreatic cancer is a fatal disease and is usually diagnosed at a later stage, mostly when the tumor has already advanced to an unresectable stage. Recent research has shown that expression patterns of miRNAs are a richer source of pathognomonic tumor profiling compared to messenger mRNA expressions.

miRNA are a family of highly conserved, non-coding, 17-25 nucleotide long RNA products, that play an important role in controlling gene expression at the post transcriptional level [69]. Biosynthesis of miRNAs is a multistep process occurring in both the nuclear and cytoplasmic compartments of the cell (Figure 5). Initially, miRNAs are transcribed into a longer several kilobase pair primary transcript (pri-miRNA) by RNA polymerase II. The pri-miRNA has a characteristic 5' 7- methyl guanylate (m7G)

cap and a 3' poly (A) tail [70, 71]. This pri-miRNA is processed in two stages resulting in the formation of mature miRNA [72]. In the first step *Drosha/ DGCR8*, a ribonuclease III endonuclease cleaves the pri-miRNA into a 65-70 bp stem loop precursor miRNA (pre-miRNA) in the nucleus [72-74]. The pre-miRNA is then transported to the cytoplasm by *Exportin-5-RanGTP* dependent mechanism [75] where it undergoes a series of cuts to generate a mature RNA duplex by RNase III endonuclease *Dicer/ TRBP* and *AGO2* [76, 77]. A pictorial representation of the sequence of events leading to biogenesis of miRNA is shown below.

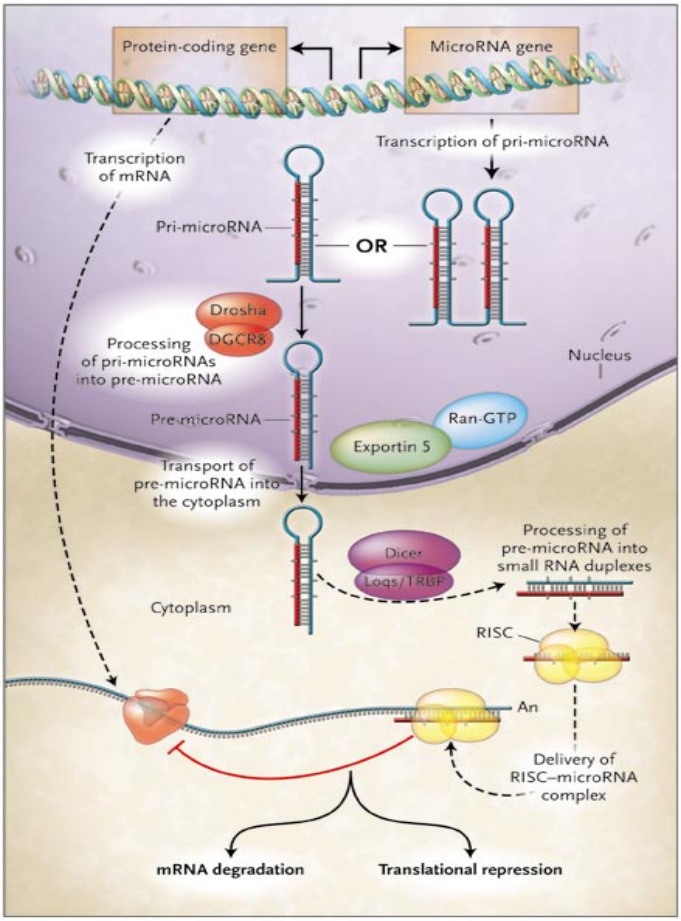


Figure 5: Biogenesis of microRNA
(Ref: Chen, 2005)

The helicase activity causes the RNA duplex to unwind and mature resulting in the formation of a single stranded miRNA which enters into the RNA-induced silencing complex (*RISC*) [78]. This combination then targets mRNA through a poorly defined mechanism. Depending on the complementarity between the two regions the fate is decided. Perfect base pairing is extremely rare but if strong base pairing is present between the 5' half of the miRNA i.e., the "seed" region nucleotides 2-8, and its target may be sufficient to mediate mRNA regulation in animals [79].

Partial complementarity between the seed region of miRNA and 3'UTR region of the target gene leads to translational inhibition [80], whereas perfect complementarity results in target mRNA degradation [81]. Since there is no specific complement sequence to target, each miRNA is predicted to target an average of 200 genes. The genomic distribution of miRNAs is discrete and represents 1-3% of the human genome, controlling 20-30% of the protein-coding genes in the human genome. Approximately 70% of the miRNAs are located in the introns and/or exons while the remaining 30% are situated in intergenic regions or gene deserts [82].

Transcriptional regulation of miRNA expression is controlled by the host tissue genes, which make their expression tissue specific, or regulated independently via their own regulatory elements. Several studies have documented the presence of miRNA genes in genomic regions associated with cancer, chromosomal break points, as well as in the minimal regions of the loss of heterozygosity/ amplifications, suggesting their role in human tumorigenesis [83]. Depending on the tissue and progression of cancer, miRNA can function as oncogenes or tumor suppressor. The exact mechanism of action of miRNAs is not very well documented, but CpG methylation is one of the epigenetic

mechanisms for miRNA deregulation in cancer tissue. Therefore, a thorough analysis of miRNAs in tumor cells could help us in restoring the expression of healthy genes provoking malignant cells to revert into either a benign or normal state.

miRNA profiles can be studied and quantified using a number of approaches, including cDNA arrays [84], Invader assay [85] and Real time PCR [84]. Using these advanced technologies, several studies have recently shown the deregulation of miRNA expression in pancreatic cancer tumor tissues and this information might be useful in differential diagnosis of pancreatic cancer from other tumors. For example, miR - 375 and - 376 expressions were significantly high in the mouse pancreas and pancreatic islet cells as compared to brain, heart and liver tissue [86]. Studies have documented the significant up regulation in miR-21 levels in pancreatic cancer [87-89].

Another advanced study using the QuantiMir system analyzed the differential expression of 95 miRNAs in 10 pancreatic cancer cell lines and 17 pairs of pancreatic cancer/normal tissues. A significant up regulation of miR-196a, miR-190, miR-186, miR-221, miR-222, miR-200b, miR-15b, and miR-95 in most pancreatic cancer tissues and cell lines was reported. The up regulation of these eight miRNAs ranged from 70 – 100% between normal control and tumor cells or tissues [90].

Gusev et al., analyzed and predicted miRNA targets and recommended that co-expressed miRNA collectively provide abnormal phenotypic changes in cancer cells by either targeting the function and/ or the signaling pathways known to be affected in a particular cancer [91]. MiRNA expression profiling may generate a unique molecular signature for any given cancer type. However, pancreatic cancer therapy still remains a

major challenge to all clinicians. This is a uniformly fatal disease and creates a major hurdle due to its late diagnosis and limited therapeutic options.

Pancreatic cancer treatment

There are some standard treatment options for patients with pancreatic cancer.

Surgical treatment

Potentially curative surgery is done when diagnostic tests suggest that it is possible to remove the entire tumor. This attempt might be successful in some patients, since only about 10% of pancreatic cancers are present entirely within the pancreas at the time of diagnosis. In situation, when there appears to be no further spread beyond the pancreas, cancer cells might have already disseminated to other parts of the body. Approximately 30 to 50% patients may suffer from complications due to surgery, such as leakage from various surgical connections, infections and bleeding.

Radiation treatment

Patents with resectable pancreatic cancer are often subjected to radiation before or after the surgery. Radiation therapy is often given along with other treatment modalities to increase the effectiveness of the treatment.

Chemotherapeutic treatment

Less than 20 percent of patients with pancreatic cancer are candidates for surgery, because the disease is often detected in the late stages. In such cases,

chemotherapy is often used since pancreatic cancers have already metastasized. The scheduling of chemotherapy is based on the type of cells, rate at which they divide, and the time at which a given drug is likely to be effective. This is why chemotherapy is typically given in cycles. Unfortunately, chemotherapy does not know the difference between the cancerous cells and the normal cells. Chemotherapy will kill all cells that are rapidly dividing. The "normal" cells will grow back and be healthy but accompanying side effects occur. The "normal" cells most commonly affected by chemotherapy are the blood cells, the cells in the mouth, stomach and bowel, and the hair follicles; resulting in low blood counts, mouth sores nausea, diarrhea, and/or hair loss. Different drugs may affect different parts of the body [92].

PaCa cells do not harbor normal checks and balances in place that control and limit cell division. The process of cell division in all kinds of cells, whether normal or cancerous, is through the cell cycle; hence the ability of chemotherapy to kill cancer cells depends on its ability to halt cell division. Usually, the drugs work by damaging the RNA or DNA that dictates the cell how to copy itself through cell division. If the cells are unable to divide, they die. The faster the cells are dividing, the more likely it is that chemotherapy will kill the cells, causing the tumor to shrink and inducing cell suicide or apoptosis [93].

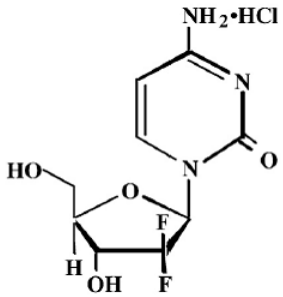


Figure 6: Chemical Structure of Gemcitabine

Gemcitabine has been a standard treatment for patients with advanced (and inoperable) pancreatic cancer for a decade. Mechanistically, gemcitabine belongs to the family of drugs called antimetabolites, which are cell-cycle specific and very similar to normal substances within the cell attacking them at very specific phases in the cycle. When the cells incorporate these substances into the cellular metabolism, they are unable to divide. Chemically gemcitabine is a nucleoside analog in which the hydrogen atoms on the 2' carbons of deoxycytidine are replaced by fluorine atoms thus intracellularly converting to the active metabolites difluorodeoxycytidine di- and triphosphate (dFdCDP, dFdCTP) (Figure 6). dFdCDP inhibits ribonucleotide reductase, thereby decreasing the deoxynucleotide pool available for DNA synthesis; dFdCTP is incorporated into DNA, resulting in DNA strand termination and apoptosis [94] (Figure 7).

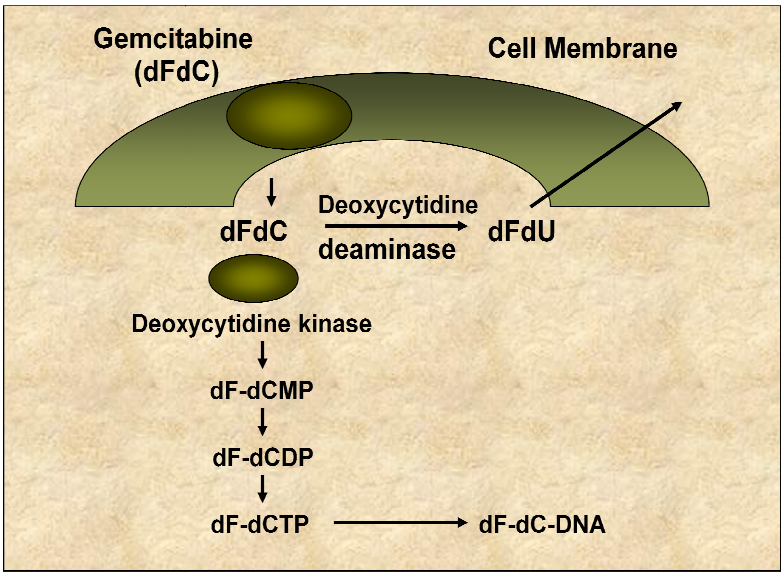


Figure 7: Gemcitabine metabolism

Although gemcitabine remains the backbone of chemotherapy in pancreatic cancer, it has only a 5.4% partial response rate and imparts a progression-free survival interval ranging from 0.9 to 4.2 months [95]. Many patients diagnosed with pancreatic cancer show initial sensitivity to gemcitabine therapy followed by the rapid development of resistance—a feature that essentially characterizes this fatal disease. Several mechanisms have been proposed for acquired chemoresistance after gemcitabine treatment. *HMGA-1* is an architectural transcription factor often upregulated in pancreatic cancer and is known to promote chemoresistance through *Akt* dependent mechanism [96]. Development of resistance is also associated with alteration in activities of enzymes such as deoxycytidine kinase, which is involved in drug metabolism [97, 98].

Although the rapid acquisition of resistance to gemcitabine treatment inevitably translates into poor patient outcomes, the tumor's initial vulnerability and subsequent resistance strongly suggest either the preexistence of resistant cell subpopulation(s) or the rapid development of resistant cells from the tumor itself or from tumor/stromal alterations. Thus, a better understanding of the origins of gemcitabine resistance is critical to the development of superior combination therapies or replacement of gemcitabine as the gold standard in pancreatic cancer. Several genetic and/or epigenetic alterations have been associated with gemcitabine resistance. Not surprisingly, these include gene products associated with gemcitabine transport and metabolism.

However, a more optimistic possibility is that targeted therapy aimed at critical common elements in resistance will restore sensitivity. Gemcitabine can be used as a

neoadjuvant and adjuvant therapy along with other agents which can work synergistically to reduce the effect of chemo resistance providing long term benefits.

Nutrition and pancreatic cancer

It is important for the human body to maintain a balance between the oxidant-antioxidant levels in the body for ideal cell regulation and reduced inflammation. Several bioactive food components have been documented over centuries for their crucial role in maintaining a homeostasis in cell proliferation and integrity. The contribution of diet and nutrition to cancer risk, prevention and treatment has been a major focus of research in recent years because certain nutrients and dietary agents appear to protect the body against diseases such as cancer.

A healthy diet can act as multimodal cancer prevention strategy. Buiatti et al [99] determined that individuals who consumed more meats, salted fish, cold cuts and seasoned cheeses had the highest risk of gastric cancers, while those consuming more fresh fruit, raw vegetables, onion, garlic and spices were associated with a lower risk. Some of the spices commonly used in the diet are obtained from different parts of plants such as fruits, bark, roots or leaves.

Curcumin obtained from the spice turmeric is known to possess anti-inflammatory, anti-proliferative, and anti-angiogenic properties. Anti-carcinogenic properties of curcumin have been very well documented and have shown to be a potent agent against pancreatic cancer. In this study, curcumin was used as a positive control to evaluate the anti-cancer properties of a novel bioactive compound, garcinol.

Garcinol

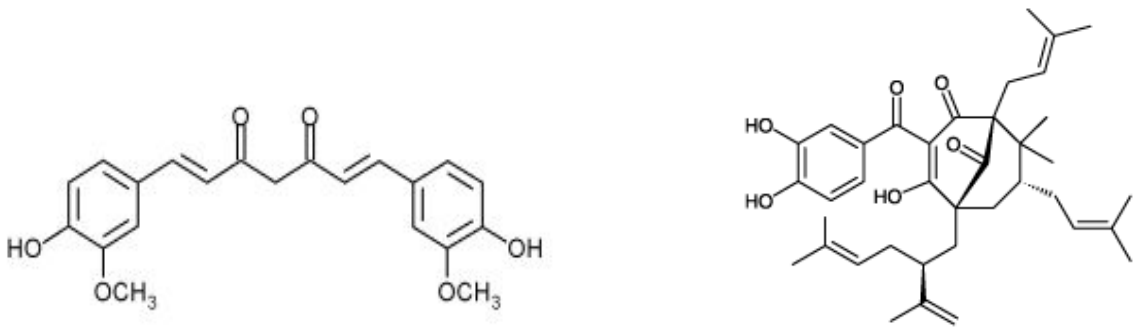
Garcinol, harvested from *Garcinia indica* (Family: Clusiaceae; Genus: Garcinia), has traditionally been used in ayurvedic medicine. The genus Garcinia includes some 200 species found in the tropics, especially Asia and Africa [100]. It has many culinary, pharmaceutical and industrial uses; however its biological properties are only beginning to be elucidated [101]. The plant grows extensively on the western coast of India and is known by various names across the world, such as Mangosteen, wild Mangosteen, or Red Mango (Figure 8).

Extracts from *Garcinia indica* are rich in polyisoprenylated benzophenone derivatives such as yellow colored Garcinol and its colorless isomer, Isogarcinol. These derivatives are primarily found in the rind of the fruit along with other components such as hydroxycitric acid (HCA), hydroxycitric acid lactone, citric acid and oxalic acid. The fruit also contains other compounds including malic acid, polyphenols, carbohydrates, anthocyanin, pigments and ascorbic acid [102]. Garcinol with a molecular weight of 602, is the active principle of *Garcinia indica*, which is crystallized out as yellow needles (1.5%) from the hexane extract of the fruit rind.

Figure 8: Mangosteen fruit – source of garcinol

Putative role of garcinol as a free radical scavenger and anti-ulcer agent was tested by Nakazawa et al. Garcinol suppressed hydroxyl radical, superoxide anion and methyl radical suggesting its ability to scavenge both hydrophilic and hydrophobic ones including reactive oxygen species. In this study, garcinol was shown to be three times more effective in free radical scavenging than DL-R-tocopherol [103, 104].

Garcinol (Figure 9 B) shows strong antioxidant activity since it contains both phenolic hydroxyl groups as well as a β-diketone moiety, and in this respect it resembles with the well-known antioxidant of plant origin, viz. Curcumin [104] (Figure 9 A).



A **B**

Figure 9: Chemical structure of (A) Curcumin and (B) Garcinol

Anti-oxidant properties of garcinol were confirmed in a recent study analyzing the effects of polyisoprenylated benzophenones on lipid and protein oxidation in vitro. Oxidative/ nitrative protein damage in human blood platelets and plasma was induced on treatment with peroxynitrite and the beneficial antioxidant properties of garcinol on lipid peroxidation was analyzed by levels of thiobarbituric acid reactive species

(TBARS). Role of garcinol as a protecting factor against diseases associated with oxidative stress is possible [105].

EGR-1, a transcription factor for Early Growth Response, autoregulates its activation and is a key vascular phenotypic switch modulated by phosphorylation and acetylation of Histone H3. In this study they used garcinol as a histone H3 acetyltransferase inhibitor which inhibited IL-1beta-inducible EGR-1 transcription [106].

Garcinol turned out to be more potent than resveratrol as a bactericidal agent against H. pylori. Also, this study proved equivalent or better bactericidal activity of garcinol compared to clarithromycin, a standard drug against H. pylori at 6 and 12 h incubation, indicating a potential role for this antioxidant in treatment for H. pylori infection[107].

Garcinol showed antitumor activity against human leukemia HL-60 cells more effectively than curcumin, which was used as a reference compound in these studies [104]. Garcinol-induced apoptosis was triggered by the release of cytochrome c into the cytosol, procaspase-9 processing, activation of caspase-3 and caspase-2, degradation of PARP, and DNA fragmentation caused by the caspase-activated deoxyribonuclease through the digestion of DFF-45. The induction of apoptosis by garcinol was proven to be a pivotal mechanism for its cancer chemopreventive action in human leukemia cells.

Anti-inflammatory and anti-cancer properties of garcinol in intestinal cancer cells was evaluated and compared to its oxidative derivatives. Garcinol was found to be more effective in inhibiting growth of intestinal cancer cell growth than that of normal immortalized cells. However, it should be pointed out that at low concentrations garcinol can stimulate cell growth [108]. In yet another study evaluating the anti-cancer

properties of garcinol in colon cancer cells confirmed that garcinol reduced cell invasion and survival through the inhibition of focal adhesion kinase, MMP-7 and downstream signaling [109, 110].

A recent study explaining the anti-cancer properties of garcinol in colon cancer suggested the involvement of TRAIL mediated apoptosis through upregulation of death receptors along with downregulation of antiapoptotic proteins[111]. Recently the anticancer properties of garcinol in breast cancer cells have also been evaluated. The induction of apoptosis via activation of caspase -3/ -7 and cleavage of PARP was modulated by downregulation of NFκB in breast cancer cells. [112]. Traditional uses of garcinol for the treatment of gastrointestinal ailments are known.

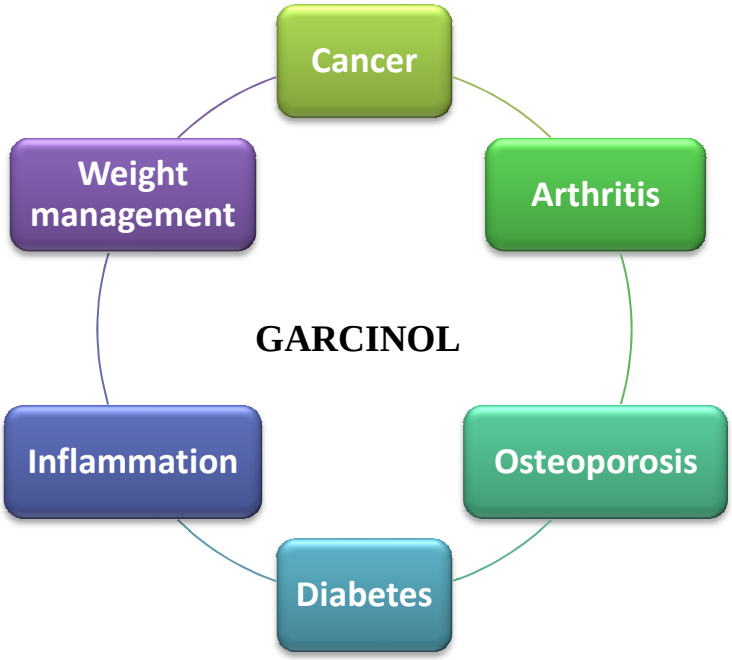


Figure 10: Known therapeutic targets of Garcinol

In another study, the mechanistic role of garcinol in preventing aberrant crypt foci was evaluated [113]. Garcinol could suppress O (2) (-) and NO generation and

expression of iNOS and COX-2 proteins. These findings support possible chemopreventive ability of garcinol, through induction of liver GST and QR, inhibition of O (2) (-) and NO generation and/or suppression of iNOS and COX-2 expression, on colon tumorigenesis.

The effect of dietary feeding of garcinol on oral cancers was evaluated in a study by [114]. Dietary garcinol significantly reduced the incidence and multiplicity of tongue neoplasms and/or preneoplasms as compared to the control diet. Also, the COX-2 expression in the tongue lesions was suppressed by feeding with garcinol. The potent anticancer properties of garcinol in oral cancers were suggested.

Thus, current literature provides evidence for the potential of garcinol as a chemopreventive agent in carcinogenesis. Additionally, feeding garcinol-containing diets does not cause retardation of body weight gain and pathological alterations in liver and other organs including kidney, lung, heart, and esophagus, which is indicative of the low toxicity of the compound, which is a very attractive feature of any anti-cancer agent. However, the precise role of garcinol against pancreatic cancer remains unknown.

Overall hypothesis

Pancreatic cancer is the fourth major cause of cancer related deaths in the United States in both men and women. It often escapes early diagnosis due to lack of explicit symptoms and early metastasis. Current treatment modalities include surgery, radiotherapy and chemotherapy. Gemcitabine remains the standard chemotherapeutic agent for treatment of PaCa. However, there is only a marginal survival advantage with multiple side effects and drug resistance. Thus, it is important to elucidate the molecular mechanisms involved in cancer and design alternative therapeutic strategies with better outcomes. Bioactive components from food have been traditionally used for medicinal purposes and it would be interesting to test these components in combination with standard chemotherapeutic agents for PaCa therapy.

Garcinol, the bioactive agent from the rind of fruiting bodies of Mangosteen, is structurally similar to the known anti-cancer agent curcumin. Anti-inflammatory and anti-carcinogenic properties of garcinol have been recently recognized in oral, tongue, breast, skin and colon cancers. It would be interesting to analyze the anti- proliferative properties of garcinol individually and in combination with standard chemotherapeutic agent gemcitabine in PaCa cells.

Also there is an obvious need to discover unique therapeutic markers and molecular signatures to combat pancreatic cancer. Evidence suggests that

expression patterns of miRNAs are a richer source of diagnostic tumor profiling as compared to messenger mRNA expressions. MiRNA are tissue specific and can target multiple genes and thus have important regulatory functions in carcinogenesis.

The hypothesis for this study is that garcinol as a single agent or in combination with gemcitabine will prove to be an effectual therapeutic agent in human pancreatic cancer cells.

In order to test the hypothesis, the following specific aims were proposed:

Specific Aim 1: To evaluate the anti-proliferative properties of garcinol in PaCa cells

Specific Aim 2: To evaluate the combined effect of Garcinol with (A) Curcumin or (B) Gemcitabine against PaCa cells.

Specific Aim 3: To identify the alterations in microRNA expression profile in PaCa cells on treatment with garcinol and/or gemcitabine.

CHAPTER TWO

METHOD

Media and Chemicals

The established human pancreatic cancer cell lines BxPC-3 and Panc-1, and non-cancerous NIH3T3 were obtained from American Type Culture Collection (Manassas, VA, USA). NIH3T3 cells are mouse fibroblast cells developed by NIH and 3 day transfer of 3 x 10^5 cells is the normal passage time for this cell line. The cell lines were maintained in continuous exponential growth by twice a week passage in RPMI-1640 medium (Cellgro Manassas, VA; BxPC-3 cells) and Dulbecco modified Eagle's medium (DMEM, Cellgro Manassas, VA; Panc-1 cells) respectively and supplemented with 10% fetal bovine serum, 4mM glutamine, sodium pyruvate, 100 units/ml penicillin and 10 mg/ml streptomycin in a humidified incubator containing 5% CO_2 in air at 37°C for a maximum of 72 hours before each experiment. Each cell line was split regularly before attaining 70-80% confluence.

Antibodies were obtained from the following commercial sources: caspase-3 was purchased from Cell Signaling (Beverly, MA, USA); *XIAP, cIAP* from R&D Systems (Minneapolis, MN, USA), anti-*Bcl-xL* antibody from Trevigen Inc(Gaithersburg, MD, USA); anti-*PARP* antibody was from Biomol Research (Plymouth, PA, USA) and anti-β-actin antibody was from Sigma Chemical Co. (St. Louis, MO, USA). Garcinol (Biomol International, USA) and Curcumin (Sigma Aldrich, USA) were reconstituted in DMSO to make 20 mM stock solution. This stock was stored at -20°C wrapped in aluminum foil to prevent dimer formation. The stock was diluted in DMEM to produce to concentrations to be tested.

Cell proliferation

Both PaCa cells were harvested using 0.025% trypsin at specific time points (24, 48, 72 hr) after different concentrations of garcinol treatment (0-40 µM) and proliferation was inferred by counting the cells with a haemocytometer under microscope using Trypan Blue (Sigma Chemical Co.,St. Louis, MO,USA). Trypan Blue staining facilitates the visualization of cell morphology. Viable cells cannot take up the dye due to intact membrane whereas the non-viable cells get stained since the dye is permeable through damaged membrane, hence the proportion of viable cells in a population can be estimated. Proliferation was expressed as number of cells counted for each treatment group relative to the cell number in absence of garcinol (untreated, control cells).

Cell Viability

For cell viability assay, cells were seeded at a density of $3x10^3$ cells/well in a 96 well micro-titer culture plate. After overnight incubation the medium was removed and replaced with fresh medium containing different concentrations of garcinol (0-40 µM) alone or in combination with curcumin (0-50 µM) or gemcitabine (0-1 µM). After specific time points (24, 48 and 72 hrs), 20µl of MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt) solution was added to each well and incubated further for one hour at room temperature. Color development due to reduction of tetrazolium in MTS to formazan is directly proportional to the number of living cells. This was measured spectrophotometrically at 595nm on a plate reader (BIO-TEK Instruments) and KC4 software was used for quantification. Cell proliferation was quantified as per the manufacturer's protocol (Promega, USA).

Cell viability has been expressed as a percentage, for each treatment group relative to control in absence of garcinol. The MTS assay calculates the % of live cells using the following equation: (sample - blank)/ (control - blank). The blank well contains only the MTS solution and is subtracted out in order to remove background absorbance. The control wells contain untreated cells, while the sample wells represent the treat cells. To monitor the effects of concurrent or sequential drug exposure the data obtained from the KC4 analysis was further subjected to analysis by Calcusyn (Biosoft ®) software to predict additive, synergistic, or antagonistic effects.

Quantification of Apoptosis

Apoptosis is characterized by membrane blebbing, condensation of cytoplasm and the activation of endogenous endonucleases. This leads to internucleosomal cleavage of DNA and the generation of mono- and oligonucleosomes, which are tightly complexed with histones. The DNA contained in these nucleosomes is protected from cleavage since it is tightly bound to the histones. The quantitative detection of these histone-associated DNA fragments in mono- and oligonucleosomes are a marker for apoptotic cells.

The Cell Apoptosis ELISA Detection Kit (Roche, Palo Alto, CA) was used to detect apoptosis according to manufacturer's protocol. Briefly, after 48 hrs treatment of BxPC-3 and Panc-1 cells with different concentrations of garcinol (0-40 µM) alone or in combination with curcumin (0-50 µM) or gemcitabine (0-1 µM), the cytoplasmic histone/DNA fragments from cells were extracted and bound to immobilized anti-histone antibody as per the manufacturer's protocol. Subsequently, a peroxidase-conjugated

anti-DNA antibody was used for the detection of immobilized histone/DNA fragments. After addition of the peroxidase substrate, the absorbance by the samples was determined at 405nm with an ULTRA Multifunctional Microplate Reader (BIO-TEK Instruments).

Morphological changes

DAPI (diamidino-2-phenylindole) is a blue fluorescent probe that fluoresces brightly when it is selectively bound to the minor groove of double stranded DNA where its fluorescence is approximately 20-fold greater than in the unbound state [115]. Morphological changes characteristic of apoptosis were determined by DAPI staining as per manufacture's protocol (Invitrogen, USA).

Briefly $5x10^3$ cells were seeded into 6 well culture plates containing 1-2 ml medium. After 24-36 hrs, garcinol (0-40 µM) alone or in combination with curcumin (0-50 µM) or gemcitabine (0-1 µM), was added and incubated for another 48hrs. Cells were harvested by trypsinization, washed with PBS and subsequently incubated for 30 minutes with DAPI at room temperature in dark for 30 minutes. Prior to microscopic analysis the cells were stained with Prolong Gold Antifade reagent and visualized under Fluorescence Microscope (Nikon Eclipsia, 80i) with an Excitation maximum at 358 nm and an Emission maximum at 461 nm).

Cell Cycle Analysis

After seeding cells in 6 well microplates, the cells were synchronized in the G1 phase of cell cycle by serum starvation for 24 hours [116, 117]. Thereafter, the cells

resumed cell cycle in media containing serum and garcinol (0-40 µM). After treatment with garcinol the cells were collected by trypsinization, washed in cold PBS and fixed in 450 µl ice-cold methanol by incubation for 1 h at 4℃. The cells were then centrifuged at 1100 rpm, the pellet resuspended in 500 µl of PBS, incubated with RNase (20 µg/ml, final concentration) for 30 min at 37℃, stained with propidium iodide (50 µg/ml, final concentration) for 1 h, and analyzed using FACScan flow cytometer (Becton Dickinson, USA).

Caspase Activity

Caspase are a family of enzymes essential in cells for apoptosis. *Caspase-3* and *9* activities play a very important role in executing the process of apoptosis. Cell death was measured in whole-cell lysates prepared from samples treated with garcinol (0-40 µM) alone or in combination with curcumin (0-50 µM) or gemcitabine (0-1 µM), using commercially available assay kit (R&D Assay System, Minneapolis, MN) according to manufacturer's instruction. Briefly, for both the assays cells were treated with garcinol and cell pellets were collected by scarping the attached cells using extraction buffer and diluted using the calibrator diluents provided with the kit. Sample or standard were added to the plate, covered and incubated at room temperature for two hours. After a series of washing with wash buffers provided, samples were incubated with Caspase 3/9 conjugates followed by 30 minute incubation in dark with the substrate solution. The reaction was stopped using stop solution and plates were mixed by gentle tapping. Optical density was determined using a microplate reader at 450 nm with wavelength correction set at 540nm.

Wound healing Assay

Cells were cultured in 35 mM culture dishes and maintained till 90% confluence was achieved. Using a sterile pipette tip, a scratch was made on the dish creating a wound through the cells. Microphotographic images were captured at the beginning and at regular intervals during cell migration till the wound closed in the control cells. The images were compared to determine the migration rate of the cells.

Angiogenic factor Analysis

VEGF, MMP-9, IL-8 and PGE_2 activity were measured in conditioned media collected from 1 x 10^6 cells treated with garcinol (0-40 µM) alone or in combination with gemcitabine (0-1 µM), using a commercially available assay kit (R & D Assay System, Minneapolis, MN) according to manufacturer's instruction. Briefly, cell culture supernates were collected and particulates were removed by centrifugation. Samples were stored at < -20°C until further use.

VEGF, IL-8 and PGE2: Each well was soaked with the assay diluent followed by addition of standard, control or sample solution and incubated for 2 hours at room temperature. After a series of washing steps, the conjugate solution was added to each well, covered and incubated at room temperature for two hours. This was followed by addition of substrate solution and stop solution and incubation. The plate was read at 450nm using a correction wavelength of 570 nm with the help of a microplate reader.

MMP-9: Each well was soaked with the assay diluent followed by addition of standard, control or sample solution and incubated for 2 hours at room temperature on a horizontal orbital microplate shaker set at 500 ± 50 rpm. Following a series of washing

steps, p-aminophenylmercuric acetate (APMA) solution was added and plates were incubated for 2 hours at 37° C in a humidified envir onment. This step causes the activation of any potentially active form of *MMP-9* in the sample. Substrate solution was added and the plate was incubated for 17 - 20 hours at 37° C in a dark, humidified environment. The relative fluorescence units (RFU) of each well was determined using a fluorescence plate reader set with the following parameters: excitation wavelength set to 320 nm or 340 nm and emission wavelength set to 405 nm; endpoint mode; 1 x 20 mS integration time; plate speed = 6.

Protein extraction and Western blot analysis

The pancreatic cancer cells- BxPC-3 and Panc-1 were plated in 100mm dish and allowed to attach for 36 hours. Garcinol (0-40 µM) alone or in combination with curcumin (0-50 µM) or gemcitabine (0-1 µM), was directly added to cell cultures at the indicated concentrations and incubated for 48 hrs. Control cells were incubated in the medium with equivalent concentration of DMSO. After the incubation period, the cells were harvested in PBS. Cellular lysates were prepared by suspending the cells in 200µl of lysis buffer (150mM NaCl, 1mM EGTA, 0.1% Triton X-100; 0.1mM sodium orthovanadate, 1mM phenylmethylsulfonyl fluoride and 2µg/ml leupeptin, 2µg/ml aprotinin). The cells were disrupted by sonication and extracted at 4°C for 30 minutes at maximal microfuge speed to remove debris. Cytosolic and Nuclear extracts were prepared using the Cytosolic and Nuclear extraction kit as per the manufacturer's protocol (Pierce Biotechnology, USA).

For Western blot analysis, each extract prepared was used for preparation of loading sample equivalent to 35-50µg total protein and was separated on SDS-PAGE,

electro-transferred onto nitrocellulose membranes. The membrane was soaked in 5% milk solution for blocking at 4C on a slow speed overnight. After several washes, the membrane was soaked in primary antibody solution prepared in 5% BSA for 2-3 hrs. Depending on the protein in question, the antibody dilutions were done ranging from 1:500 to 1: 2000. The membrane was washed and soaked in secondary antibody solution prepared in 5% milk solution with dilution of 1:5000. The membrane was washed several times to avoid background intensities. Detection of specific proteins (*PARP, X-IAP, cIAP Pan, Bcl-xL*) was carried out with an enhanced chemiluminescence Western blotting kit according to manufacturer's instructions (Pierce Biotechnology, USA).

Electrophoretic Mobility Shift Assay

The pancreatic cancer cells- BxPC-3 and Panc-1 were plated in 100mm dish and allowed to attach for 36 hours. Garcinol (0-40 µM) alone or in combination with curcumin (0-50 µM) or gemcitabine (0-1 µM), was directly added to cell cultures at the indicated concentrations and incubated for 48 or 72 hrs. Nuclear extracts were prepared according to the method described by Chaturvedi *et al* [118]. Briefly, the cells were washed with cold PBS and suspended in 0.15 ml of lysis buffer [10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 1 mM PMSF, 2 µg /ml leupeptin, 2µg /ml aprotinin, and 0.5 mg/ml benzamidine]. The cells were allowed to swell on ice for 20 min and then 4.8 µl of 10 % Nonidet P-40 was added. The tubes were vortexed for a few seconds and centrifuged. The nuclear pellet was resuspended in nuclear extraction buffer [20mM HEPES (pH 7.9), 0.4M NaCl, 1mM EDTA, 1mM EGTA, 1mM DTT, 0.5mM PMSF, 2 µg/ml leupeptin, 2 µg/ml aprotinin, and 0.5 mg/ml benzamidine] and incubated on ice. The

tubes were then centrifuged for 5 min in a microfuge at 4°C, and the supernatant (nuclear extract) was collected in a cold eppendorf tube and stored at –70°C for later use. The protein content was measured by BCA method. EMSA was performed by incubating 10µg of nuclear extract with IRDye^{TM}–700 labeled NF-κB oligonucleotide. The incubation mixture included 2 µg of poly (dI-dC) in a binding buffer. The DNA-protein complex formed was separated from free oligonucleotide on 8.0% native polyacralyamide gel using buffer containing 50mM Tris, 200mM glycine, pH 8.5, and 1mM EDTA, and then visualized by Odyssey Infrared Imaging System using Odyssey Software Release 1.1.

Clonogenic Assay

To test the proliferation and differentiation of cells treated with garcinol, gemcitabine, or the combination, Panc-1 cells were plated (50,000 – 100,000 per well) in a six well plate and incubated overnight at 37°C. After 72-h exposure to 10 µM /L garcinol and 100nM gemcitabine, and the combination of the two, the cells were trypsinized, and the viable cells were counted (trypan blue exclusion) and plated in 100 mm Petri dishes in a range of 100 to 1,000 cells to determine the plating efficiency as well as assess the effects of treatment on clonogenic survival. Based on the plating efficiency, 500 cells were then incubated for 10 to 12 days at 37°C in a 5% CO2/ 5% O2/ 90% N2 incubator. The colonies were stained with 2% crystal violet solution and counted. The surviving fraction was normalized to untreated control cells with respect to clonogenic efficiency [119].

Cell Adhesion Assay

96 well plate was coated with *Laminin-1* (10 µg/ml) at 4 degrees C overnight. After two washes with washing buffer (0.1% BSA in DMEM medium), the plate was blocked with 0.5% BSA in DMEM medium and kept in an incubator at 37 degrees C for 1 hr. Wash with washing buffer and chill on ice. Add 50µl of garcinol (0-40 µM) and/or gemcitabine (0-1 µM) treated cells ($4x10^5$ cells/ ml) to each well and incubate in an incubator at 37 degrees C for 30 minutes. Shake the plate at 2000 rpm for 15 sec and wash with washing buffer thrice before fixing it with 4% paraformaldehyde. Incubate at room temperature for 15 minutes. Wash with washing buffer and stain for 10 minutes with crystal violet stain. Wash with water and drain off all the water to get dry plates. Incubate the plate with 2% SDS for 30 minutes and read at 550 nm [120].

Analysis of Cytotoxic Synergy

Cells were plated as described above and allowed to attach overnight. The culture medium was replaced with fresh medium containing garcinol and gemcitabine or curcumin, individually and in combination in different ratios (Table 1) and the effect on cell growth was examined by the MTS assay method as described above.

Table 1: Treatment concentration for Synergy Analysis

Treatment Ratio	Garcinol (μM)	Gemcitabine (nM)
100:1	10	100
	5	50
	2.5	25
50:1	5	100
	2.5	50
	1.25	25
200:1	20	100
	10	50
	5	25

Treatment Ratio	Garcinol / Curcumin (μM)	Curcumin / Garcinol (μM)
1:2.5	4	10
	2	5
	1	2.5
1:5	4	20
	2	10
	1	5
1:10	2	20
	1	10
	0.5	5

Data obtained was analyzed using the Calcusyn (Biosoft ®) software program, which utilizes the T.C. Chou method of determining synergy and antagonism. The combination index was determined at various toxicity levels for each cell line and at each drug ratio. For each ratio, three different concentration combinations were tested. The median-effect equation and combination index analysis was used to calculate the

interaction between treatment modalities. The combination index values (CI) for effective dose (ED50) at which 50% cells were viable was evaluated. This analysis determines if the effect is antagonistic, additive or synergistic. The analysis is termed median effect analysis and used extensively in biological systems [121, 122].

The mathematical equation of the median-effect equation is: $f_a/f_u + (D/D_m)^m$, where f_a is the fraction affected by dose, f_u is the fraction unaffected by the dose, D is the drug dosage, D_m is the dosage of the drug required to inhibit the growth of 50% of the cells and is determined from the x-intercept, and m is the coefficient signifying the sigmoidicity of the dose-effect curve. The logarithmic form of the median-effect equation was used to linearize the dose-response curves, and can be plotted to generate the median-effect plot. Log (D) plotted vs. log (f_a/f_u), the x-intercept obtained is log (D_m), from which the IC_{50} can be evaluated and the slope is the m value. The m and the D_m values obtained thereafter can be used to calculate the effective dose for any effect level [123].

The combination index (CI) is used to determine synergism or antagonism. The equation is based on the multiple-drug effect equation. A CI value of one indicates that the effect of one drug is additive to the second, a CI value of greater than one indicates antagonism between the two drugs, and a CI value of less than one indicates synergism between the drugs (Table 2). The equation used to calculate CI is as follows: $(D)_1/(D_x)_1 + (D)_2/(D_x)_2 + \alpha\ (D)_1(D)_2/(D_x)_1(D_x)_2$, where $(D_x)_1$ and $(D_x)_2$ are the doses for x% inhibition by drug 1 and drug 2 alone [121]. These values are obtained from the median-effect equation. $(D)_1$ and $(D)_2$ are the doses in combination that inhibit cell growth by x%. A

more detailed description of degrees of synergism and antagonism is listed in the table 6.1 adapted from Chou and Hayball (1996).

Table 2: Combination Index Values (CI)

Range of Combination Index Values	Description of Effect
< 0.1	Very strong synergism
0.10 – 0.30	Strong synergism
0.30 – 0.70	Synergism
0.70 – 0.85	Moderate synergism
0.85 – 0.90	Slight synergism
0.90 – 1.10	Nearly additive
1.10 – 1.45	Moderate antagonism
1.45 – 3.30	Antagonism
3.30 – 10	Strong antagonism
> 10	Very strong antagonism

RNA Isolation

Total RNA isolation was done using the miRNA mini easy kit from Qiagen (Valencia, CA, USA). Briefly, PaCa cells were treated with different 10µM garcinol and 100nM gemcitabine individually and in combination for 36 hour. Cells were directly detached from the plate using lysis reagent, disrupted and homogenized by vortexing on full speed for one minute. Homogenate was incubated at room temperature for five minutes after which, chloroform was added and shaken vigorously to induce phase

separation. After leaving the samples at room temperature for two minutes, the samples are cold centrifuged for 15 minutes at 12,000 x g. Upper aqueous phase with the RNA is collected and 100% ethanol is added before passing it through the RNeasy mini columns and centrifuged. A series of buffers are added to isolate pure RNA from the column. Lastly, RNA is dissolved in RNase free water and stored frozen till use. Purity of RNA was checked by determining the 260/280 ratio.

MicroRNA Microarray and Profiling

Isolated total RNA samples were shipped on dry ice to LC Sciences (Houston, TX, USA) for microRNA enrichment and profiling using the microarray analysis. Dual chip analysis was done using Cy3 and Cy5 dye. For miRNA Human chip, five or more redundant regions are included. Each region further comprises a miRNA probe region, which detects miRNA transcripts listed in Sanger miRBase Release 7.1. Multiple control probes are included in each chip. The control probes are used for quality controls of chip production, sample labeling and assay conditions. From Cy3 and Cy5 images one may directly read miRNA profiles and from Ratio images one may get a quick sense of differential expressions between the corresponding samples [124]. Mature miRNAs and miRNA*s are sorted separately according to differential ratios. The ratio values are presented in log2 scale for quick and easy assessing differential direction as well as magnitude. A positive log2 value indicates an up regulation and a negative log2 value indicates a down regulation. One can easily convert a log2 value into a arithmetic ratio on a calculator by typing in 2^ (value). The signal values are derived by background

subtraction and normalization. Blank spaces represent signal values below detection level.

A transcript to be listed as detectable must meet at least two conditions: signal intensity higher than 3× (background standard deviation) and spot CV < 0.5. CV is calculated by (standard deviation)/ (signal intensity). When repeating probes are present on an array, a transcript is listed as detectable only if the signals from at least 50% of the repeating probes are above detection level. Signals are listed in median signal values of repeating probes of p-value < 0.01. During data process, “bad spots” that have signal values deviated more than 50% of average values of repeating spots and/or spot CV larger than 0.5 are discarded [125].

The images are displayed in pseudo colors so as to expand visual dynamic range. In the Cy3 and Cy5 intensity images, as signal intensity increases from 1 to 65,535 the corresponding color changes from blue to green, to yellow, and to red. In the Cy3/Cy5 ratio image, when Cy3 level is higher than Cy5 level the color is green; when Cy3 level is equal to Cy5 level the color is yellow; and when Cy5 level is higher than Cy3 level the color is red. Multiple control probes are included on the microarrays for assessing various chip and assay qualities such as uniformity and specificity. Chips were scanned and the data was normalized. The t tests were done and the predicting target genes for various miRNAs were analyzed.

MiRNA Real Time Reverse Transcription – Polymerase Chain Reaction (qRT-PCR)

To verify the alterations in the expression of specific miRNAs that were found to be altered in miRNA microarray analysis, we chose representative miRNA (miR - 21,

miR -495, miR - 638, miR - 605 and miR – 196a) with varying expression profiles for real-time miRNA reverse transcription PCR (RT-PCR) analysis using TaqMan MicroRNA Assay kit (Applied Biosystems) following the manufacturer's protocol. Briefly, Reverse Transcription master mix was prepared by adding 100mM dNTP mix, Reverse Transcriptase, 10x RT buffer, RNase inhibitor and nuclease free water. Each 15µl reaction consists of 7µl of Mastermix prepared above, 3µl respective primer and 5µl total RNA sample (5ng/15µl reaction). The thermocycler was programmed as follows:

Step Type	Time(Minutes)	Temperature (C)
HOLD	30	16
HOLD	30	42
HOLD	5	85
HOLD	∞	4

RT-PCRs were then carried out in a total of 25µl reaction mixture. Each tube composition was 1.66 µl RT product, 1.25 µl 20x TaqMan miRNA Assay mix, 12.5 µl of 2x TaqMan Universal PCR Master Mix and 9.59 µl distilled water. The Smart Cycler II was programed as follows:

Step Type	Time	Temperature (C)
Initiation	10 Minutes	95
40 Cycles	15 Sec	95
(each cycle)	1 Minute	60

Statistical analysis

All the experiments were done in triplicate for each of the observations. Each of the data represents the mean ± SD from three separate experiments. To analyze the statistical significance of the differences found in the data, Analysis of Variance (ANOVA) was done. Statistical significance was assumed at $p<0.05$.

CHAPTER THREE

RESULTS

Specific Aim 1: To evaluate the anti-proliferative properties of garcinol in PaCa cells

Human pancreatic cancer cell lines- Panc-1 and BxPC-3 were evaluated for putative therapeutic effect of garcinol from multiple viewpoints including its efficacy to potentiate loss of viable tumor cells, apoptosis stimulation, cell cycle arrest and further evaluate known cellular and molecular targets involved in the execution of these effects. Results obtained are described below.

Effect of garcinol on PaCa cell proliferative status and viability - To evaluate the effect of garcinol on viability of PaCa cells *in vitro*, cells were cultured in 10% FBS containing medium for 24, 48 and 72 hrs with increasing concentrations of garcinol (0-40µM). As noted in Figure 11A in PaCa cell lines, garcinol suppressed cell proliferation (as inferred from trypan blue exclusion assay), and significantly reduced the percentage of metabolically viable cells (as inferred by MTS relative to vehicle control) in a dose and time responsive manner (Figure 12). Inhibition of proliferation and < 50% loss of cell viability was evident with 20µM garcinol concentration in BxPC-3 cells. In contrast, in Panc-1 cells 10µM of garcinol at 72 hrs, caused <50% loss of viable cells ($p < 0.01$). The % cell viability observed at 72hrs with 10, 20, 30 and 40µM of garcinol in BxPc3 cells were 55, 45, 30 and 20 % relative to control while in Panc-1 the figure tended to 40, 35, 20 and 10 % respectively (relative to untreated control). Further, the phenotypic alterations following treatment showing loss of viable cells are depicted in Figure 11B. Taken together these results suggest that garcinol is an effective inhibitor of PaCa cell

viability and interferes with cell proliferation irrespective of their molecular genetic make-up. However, BxPC-3 cells harboring wild type *K-ras* oncogene and mutated *p53* was relatively more resistant to the effect of garcinol as compared to Panc-1 cells with mutant *K-Ras* and mutated *p53*. Further, to exclude the possibility of adverse effect of garcinol on normal epithelial cells, similar experiment was performed using MTS assay in immortalized mouse fibroblast NIH3T3 cells. The effect of garcinol was negligible at equivalent concentrations of this compound in these cells relative to PaCa cells (Figure 12C)

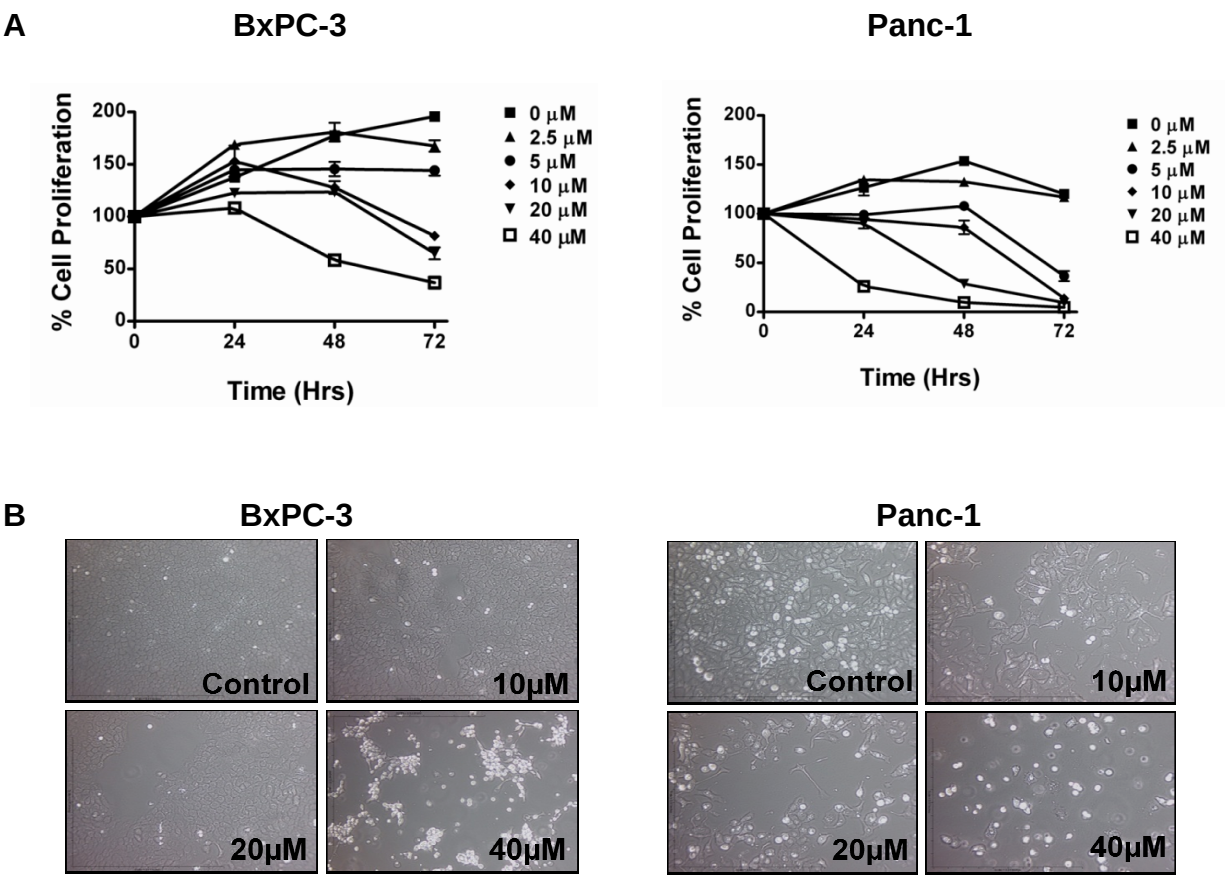
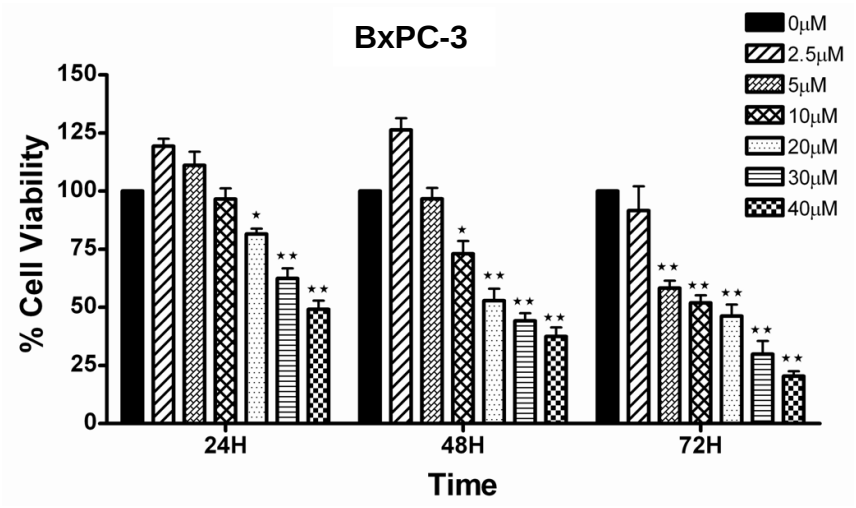


Figure 11: Effect of garcinol on PaCa cell proliferation.

A: Garcinol inhibited cell proliferation in a dose and time responsive manner as demonstrated by trypan blue exclusion assay in both PaCa cell lines, BxPC-3 and Panc-1. **B:** Micrographic pictures depicting dose dependent phenotypic changes showing loss of viable cells upon 48 hour treatment with garcinol in both BxPC-3 and Panc-1 cells.

A



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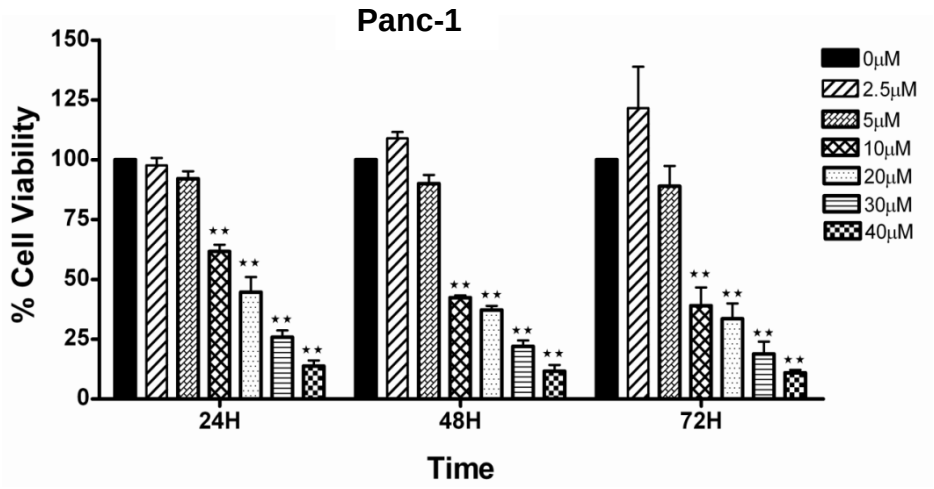

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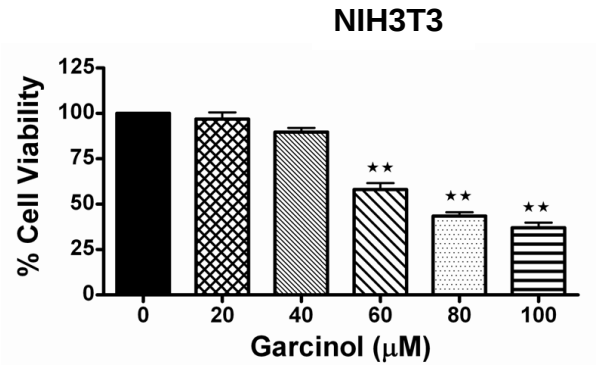


Figure 12: Dose and time response of garcinol on PaCa cell viability.

Garcinol significantly inhibited metabolically viable cells in a dose and time responsive manner as inferred by MTS assay in human PaCa cells, **A:** BxPC-3 **B:** Panc-1. IC50 values were attained at ~15μM garcinol treatment in BxPC-3 cells as compared to ~10μM in Panc-1 cells on 72 hr treatment. Garcinol exhibited no adverse effect on mouse fibroblast cell line **C:** NIH3T3 at relative concentrations on 72hr treatment. * $p < 0.05$, ** $p < 0.01$ relative to untreated control.

Garcinol induces apoptotic effects in PaCa cells - To investigate whether the loss of cell viability by garcinol treatment could in part be due to induction of apoptosis, we evaluated apoptosis based on criterion such as assessing nuclear morphological changes by DAPI staining, ELISA- Histone DNA analysis and additionally examining the extent of sub-G1 DNA by flow cytometry of fixed cells. As shown in Figure 13A, a significant increase in apoptotic cells was observed with increase in concentration of garcinol. This closely parallels to the loss of cell viability by garcinol treatment (48h) in both BxPC-3 and Panc-1 cells. Complimenting these findings, cells harboring abnormal nuclear morphology, typical of apoptosis such as apoptotic body formation, membrane blebbing, cell shrinkage, reduction in cell number, and nuclear condensation was scored under fluorescence microscopy in at least 500 garcinol treated PaCa cells after DAPI staining (Figure 13B). The observed morphological changes correlated well with Histone DNA ELISA findings following garcinol treatment in dose responsive manner in both cell lines investigated.

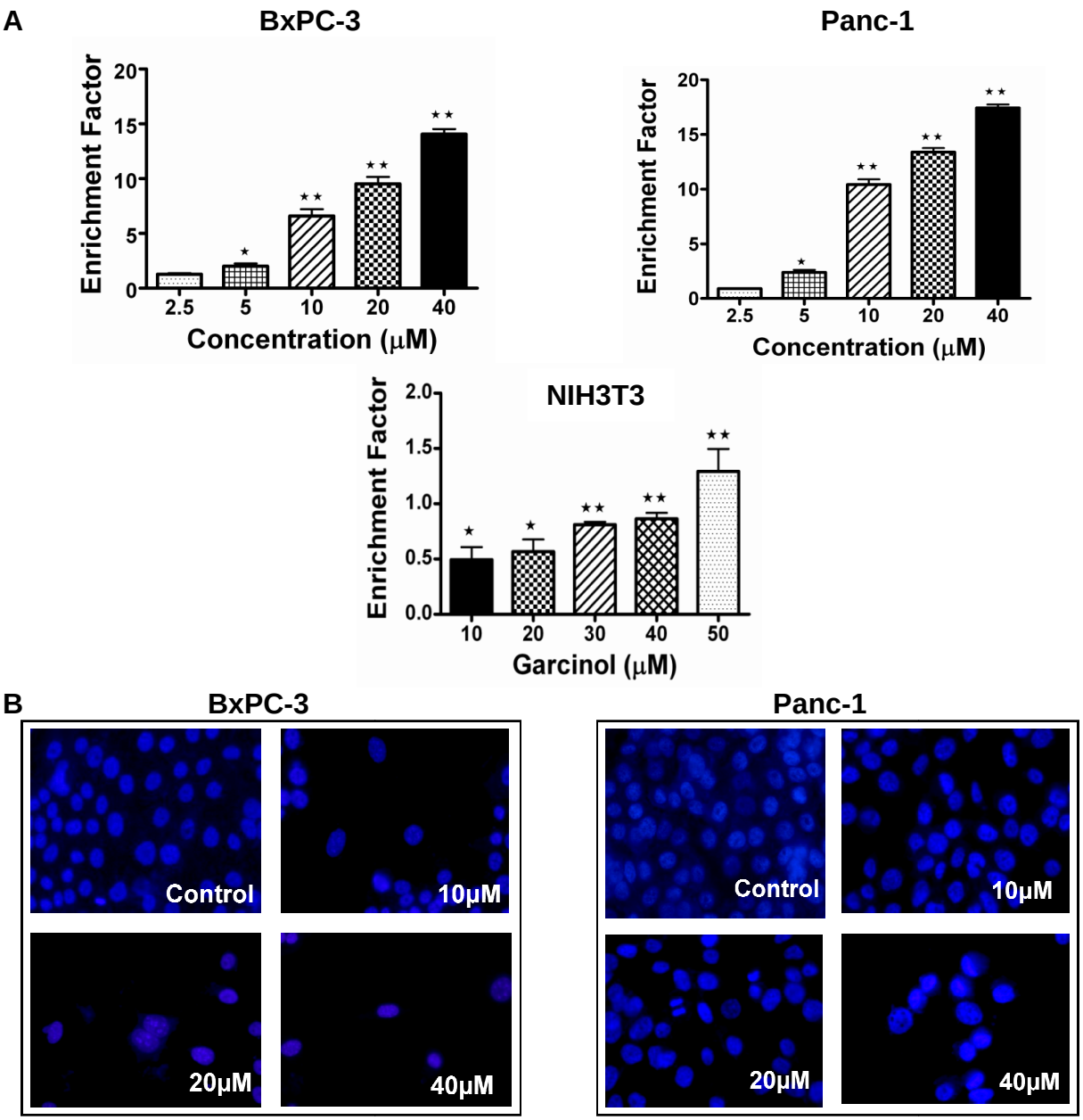


Figure 13: Apoptosis inducing effect of garcinol in PaCa cells

A: Significant induction in apoptotic cells as demonstrated by enrichment of oligosomes and nucleosomes in the cytosol on undergoing apoptosis was estimated using ELISA- Histone DNA analysis on BxPC-3 and Panc-1 cells. Statistical significance was calculated based on optical density values and enrichment factor was calculated by subtracting background signal (* $p < 0.05$, ** $p < 0.01$ relative to control) **B:** Apoptotic morphological changes such as abnormal nuclear morphology, apoptotic body formation, reduction in cell number, all typical of apoptosis was scored in both PaCa cell lines, BxPC-3 and Panc-1 under fluorescence microscopy in 48 hr garcinol treated cells using DAPI staining.

Effect of garcinol on disruption of cell cycle in PaCa cells- The effect of garcinol treatment on cell cycle progression in PaCa cultured for 24 hrs was investigated by flow cytometry. Herein, again we noticed a relatively higher percentage of Panc-1 cells (0.3% *vs*. 36% cells at 0 and 40µM concentrations garcinol, respectively) being killed by garcinol compared to BxPC-3 cells (2% *vs*. 10% cells at 0 and 40µM concentrations of garcinol, respectively) as seen in Figure 14, 15 and Table 3. The effect was dose dependent. Cumulatively, these results indicate that PaCa cells undergo apoptosis following garcinol treatment, and a good correlation exists between apoptosis, loss of cell viability and reduced proliferation of cells.

Garcinol treatment also resulted in a significant dose-dependent increase of cell population in the G0-G1 phase of the cell cycle in BxPC-3 cells (18% vs 65% cells at 0 vs 40µM garcinol concentration, Figure 14A and B, Figure 15A and Table-3) and in Panc-1 cells (31% vs 58% cells at 0 vs 40µM garcinol concentrations respectively, Figure 14A and B, Figure 15B and Table-3). This increase in cell population in the G0-G1 phase was found to be associated with concomitant decrease in cell population in the S and G2-M phase compared to corresponding controls. Overall, these results support the notion that the observed decline in cell population by garcinol is due to cell cycle arrest in G0-G1 phase of cell cycle and induction of apoptosis.

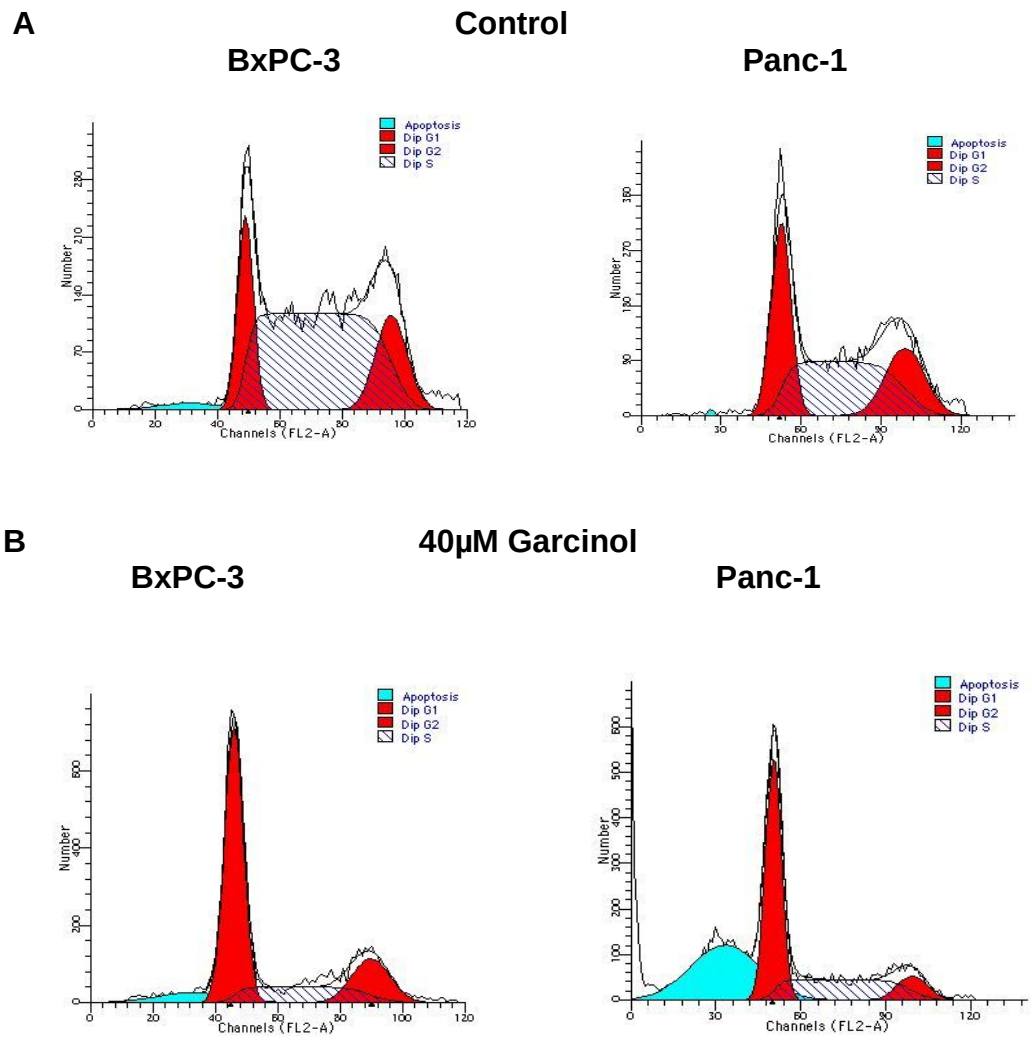
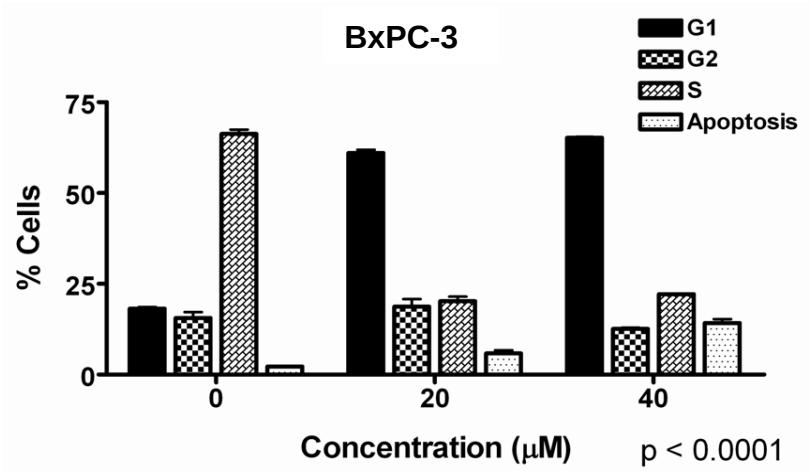


Figure 14: Histograms on FACS analysis of PaCa cells

Cell cycle analysis was performed on cells synchronized in G1 phase by serum starvation in **A:** untreated control cells **B:** 40µM garcinol treated PaCa cells, BxPC-3 and Panc-1. Cells were fixed using propidium iodide and analyzed using flow cytometry analysis. Percentage of cells in each phase of cell cycle was depicted using histograms. Garcinol treatment resulted in G1 phase arrest with increased apoptosis in PaCa cells relative to untreated control, with more significant effect on Panc-1 cells compared to BxPC-3 cells.

A



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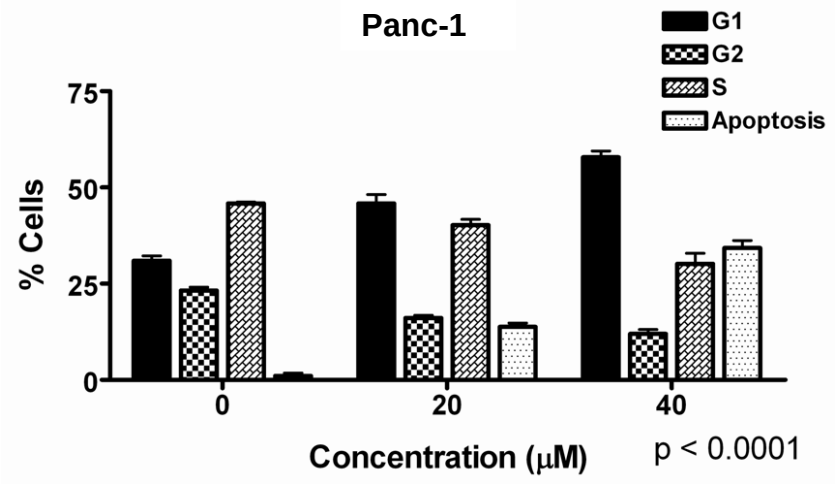


Figure 15: Flow cytometric analysis of PaCa cells

Bar diagram depicting percentage of cells in different phases of cell cycle on treatment with (0, 20 and 40 µM) garcinol as estimated by FACS analysis in both PaCa cell lines, BxPC-3 and Panc-1. Significant dose dependent arrest in G1 phase along with reduction in percentage of cells in S and G2-M phase and increase in apoptosis was observed upon garcinol treatment.

Table 3: Flow cytometry analysis of PaCa cells on treatment with garcinol

	% G0-G1	% S	% G2-M	% Apoptosis
BxPC-3: Control	18.1 ± 0.4	66.3 ± 1.1	15.6 ± 1.6	2.2 ± 0.09
20μM Garcinol	60.3 ± 1.4	24.2 ± 5.1	15.4 ± 2.0	5.0 ± 1.3
40μM Garcinol	65.2 ± 0.2	22.1 ± 0.04	12.6 ± 0.3	14.1 ± 1.1
Panc-1: Control	31.0 ± 1.3	45.8 ± 0.4	23.2 ± 0.9	1.1 ± 0.7
20μM Garcinol	69.0 ± 2.0	14.5 ± 1.7	16.6 ± 3.2	3.7± 0.2
40μM Garcinol	57.8 ± 1.6	30.2 ± 1.9	12.0 ± 1.0	34.3 ± 1.9

The mean values (± SE) represent the percentage of cells in the indicated phase(s) of the cell cycle from atleast two independent experiments.

Garcinol inhibits apoptotic molecules in PaCa cells- The molecular mechanism by which garcinol induces apoptosis in PaCa cells were investigated. Caspases are important mediators of apoptotic stimuli and therefore, we determined the enzyme activity levels of caspase-3 and caspase-9 in BxPC-3 and Panc-1 cells treated with garcinol. As seen in Figures 16A and 16B, garcinol significantly ($p < 0.05$) increased caspase-3 and caspase-9 activity by ~ 2 - 3 folds.

Caspases are sequentially activated by proteolytic processing of their inactive procaspase forms into two smaller units, which in turn leads to cleavage of key cellular proteins including DNA repair enzyme poly ADP-ribose polymerase (PARP). As shown under Figure 17A, the Western immunobloting indicate that garcinol treatment resulted in the cleavage of PARP and appearance of the 85kDa cleaved intermediate in a dose dependent manner in Panc-1 cells. These results suggest that garcinol induces apoptosis in PaCa cells that may be mediated, at least in part, by the mitochondrial pathway.

Inhibitors of apoptosis proteins (IAP) are over-expressed in human PaCa and they are thought to antagonize cell death by interacting with and repressing caspases. We therefore assessed protein expression levels by Western immunobloting using total cellular protein extract from garcinol treated cells. Relative to control, XIAP and cIAP protein expression was down-regulated in cells in a dose dependent manner when exposed to garcinol for 48hrs (Figure 17A). This provides mechanistic evidence of promotion of apoptosis induction by garcinol treatment in PaCa cells.

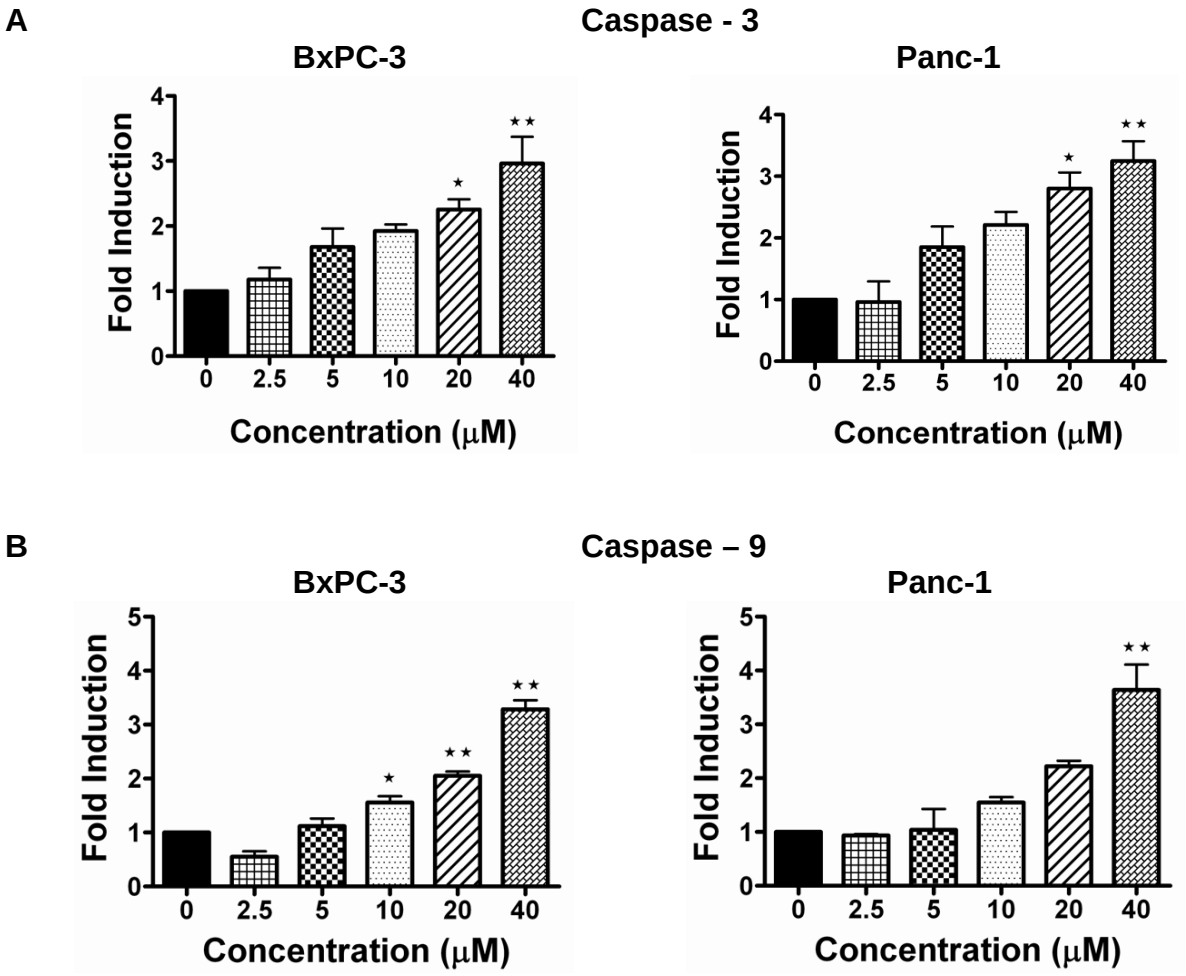


Figure 16: Induction of caspases on treatment with garcinol.

Garcinol significantly increased **A:** *Caspase 3* and **B:** *Caspase 9* activity by ~ 2-4 folds in both PaCa cell lines, BxPC-3 and Panc-1 relative to untreated control on 48 hour treatment. Caspase activity was measured in garcinol treated whole cell extracts using colorimetric assay (* $p < 0.05$, ** $p < 0.01$ relative to control).

Garcinol inhibits activation of NF-κB- As discussed in the introduction, constitutively active NF-κB in pancreatic tumors contributes to the survival of cells by inhibiting apoptosis. To investigate whether garcinol could abrogate constitutively expressed NF-κB, PaCa cells were treated with different concentrations of garcinol for 48 hrs. As shown in Figure 17B, treatment with garcinol resulted in a concentration dependent downregulation of NF-κB DNA binding ability in both BxPC-3 and Panc-1 cells. These results are consistent with down-regulation of XIAP proteins because the latter are transcriptionally regulated by NF-κB.

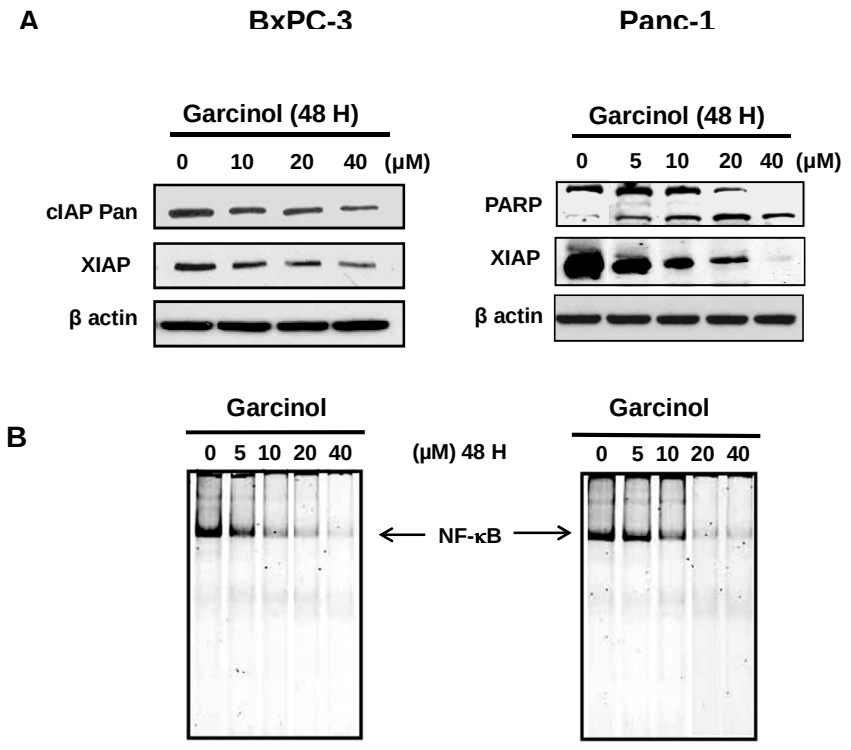


Figure 17: Garcinol downregulates apoptotic pathways.

A: Western immunoblot analysis to detect expression of apoptotic proteins on garcinol treatment. Relative to control, garcinol treatment downregulated protein expression of IAPs, XIAP and cIAP Pan in PaCa cells. Cleaved band of PARP was observed in Panc-1 cells upon treatment with garcinol in a dose responsive manner **B:** Downregulation of *NF-κB* DNA binding ability on garcinol treatment as demonstrated by EMSA in both PaCa cell lines, BxPC-3 and Panc-1 in a dose responsive manner.

Effects of garcinol on MMP-9, VEGF, IL-8 and cell invasion abilities- MMP-9, VEGF and IL-8 are pivotal molecules in angiogenesis, invasion and metastasis of cancer cells and hence, we investigated whether garcinol could inhibit these angiogenic molecules. After 48hrs treatment relative to control, 20μM garcinol reduced the basal MMP-9 activity (Figure 18A) by 72% and 88% in BxPC-3 and Panc-1 cells respectively. IL-8 production (Figure 18B) was suppressed by approximately 78% in BxPC-3 cells and by 84% in Panc-1 cells. VEGF levels (Figure 18C) were inhibited by approximately 90% of basal level in BxPC-3 and Panc-1 cells. Inhibition of these molecules suggests that garcinol may be a potent suppressor of tumor neovascularization. PGE_2 plays a role in augmenting VEGF in human PaCa cells. BxPC-3 which has over expressed COX-2 enzyme, revealed significant reduction (~40% and 65%) in PGE_2 levels following garcinol treatment at 10 and 20 μM concentration respectively ($p < 0.01$; Figure 19A).

We therefore, examined, using *in vitro* wound healing assay, cell migration by creating scratch wounds in confluent monolayers of PaCa cells. After 24-hour incubation in 10% serum-containing media supplemented with 20μM garcinol concentration, the number of cells migrating from the wound edge decreased over time in both BxPC-3 and Panc-1 cells as illustrated in representative microphotographs (Figure 19B). Taken together, the above mentioned results indicate that garcinol is potentially effective in reducing angiogenesis and along with other invasive mediators- such as MMP-9, IL-8 and VEGF may lead to inhibition of tumor progression.

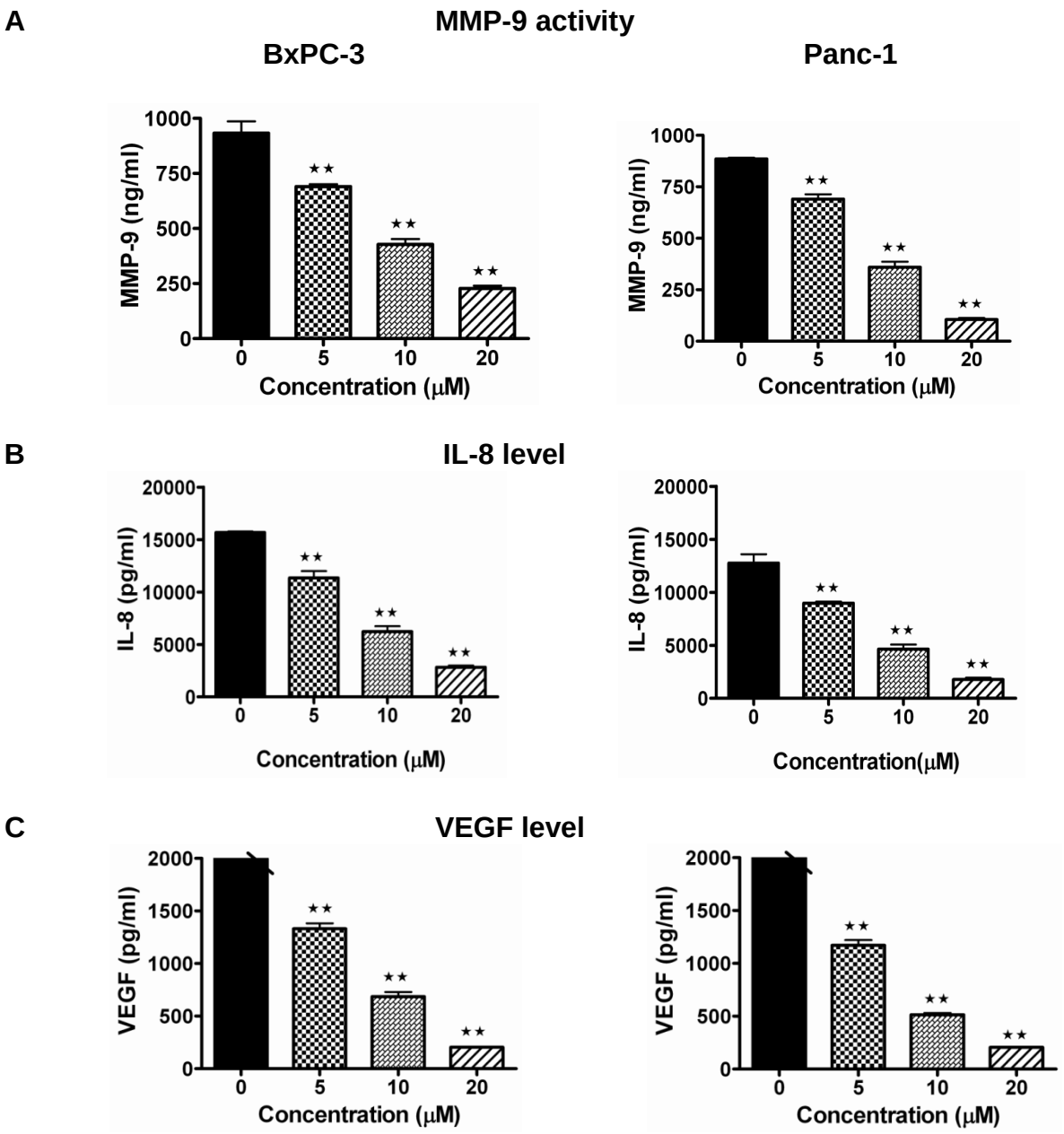


Figure 18: Effects of garcinol on angiogenic markers

Effect of garcinol on angiogenic markers **A:** MMP-9 **B**: IL-8 and **C**: VEGF in PaCa cells, BxPC-3 and Panc-1. Garcinol treatment suppressed MMP-9 activity, IL-8 levels and VEGF concentration in a dose responsive manner in $1x10^6$ cells using quantikine and fluorokine assays (** $p < 0.01$ relative to control).

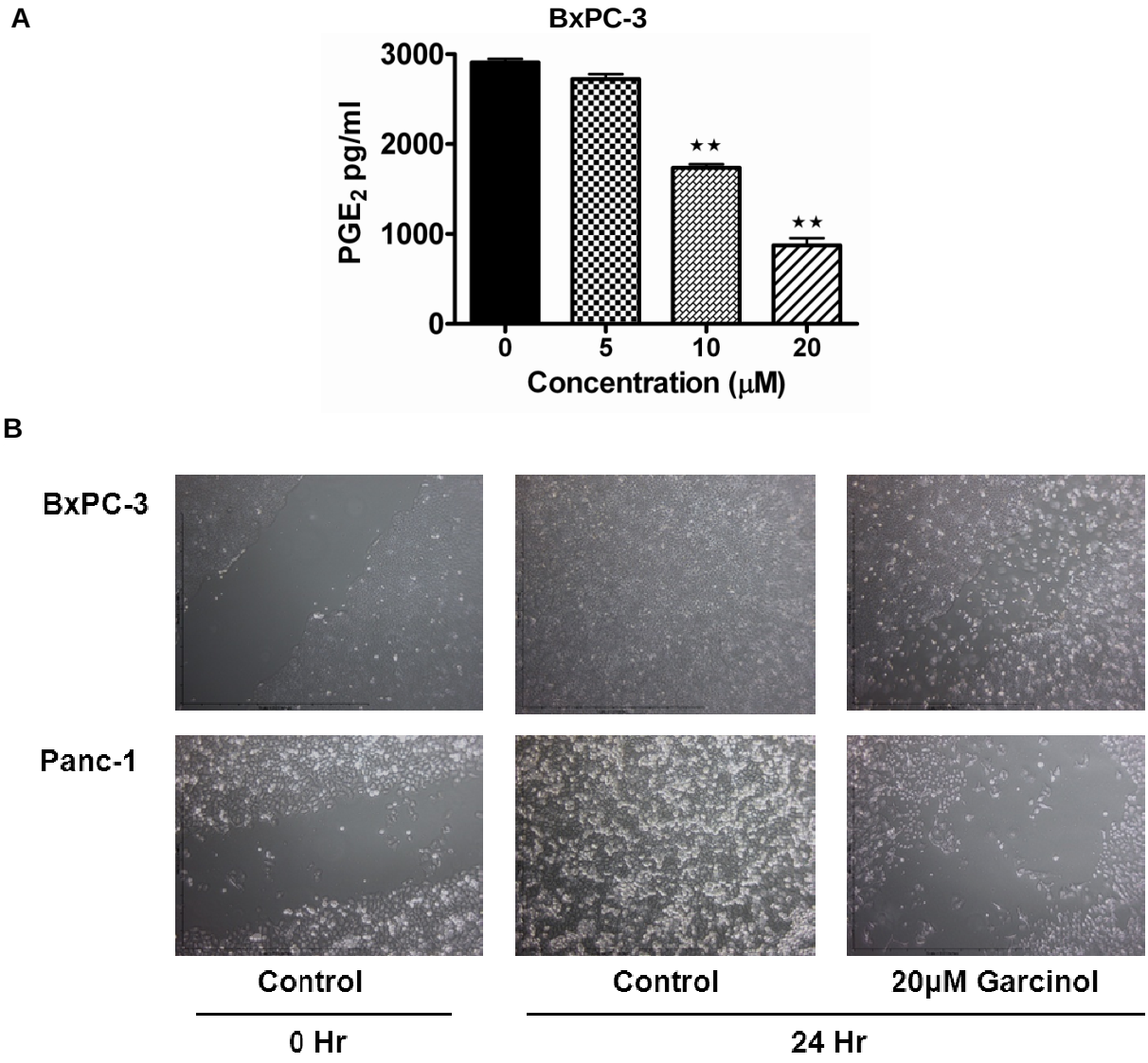


Figure 19: Effects of garcinol on PaCa cell invasion markers

A: Effect of garcinol on PGE_2 levels in BxPC-3 cells. Garcinol significantly reduced COX-2 enzymatic product, PGE_2 in dose responsive manner on 48hr treatment as observed by Quantikine assay. (** $p < 0.01$ relative to control) **B:** cell migration and proliferation was estimated by in vitro wound healing assay. Upper panel: BxPC-3 and Lower panel: Panc-1. After 24hr treatment with 20μM garcinol, number of cells migrating from wound edge reduced relative to control cells more apparently in Panc-1 cells compared to BxPC-3 cells.

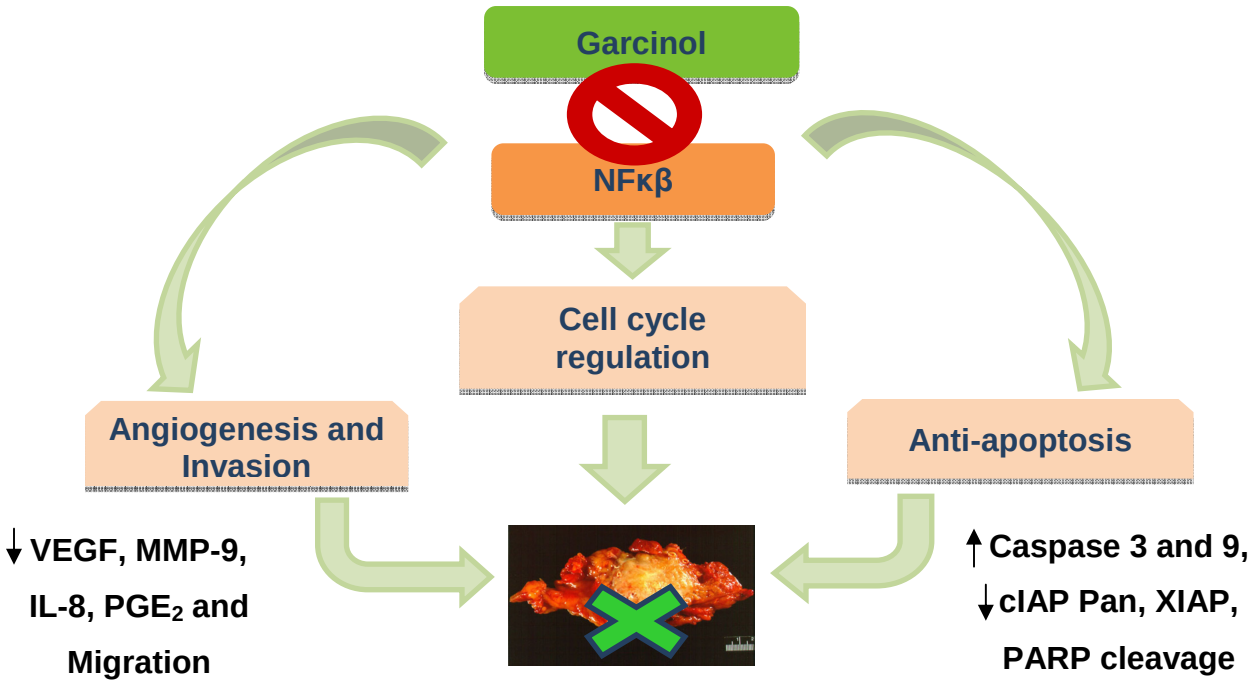


Figure 20: Summary of specific aim 1

Garcinol downregulates *NFκB* in PaCa cells thereby influencing other downstream pathways involving angiogenesis, invasion, cell cycle and apoptotic modulators, thus inhibiting PaCa cell growth by inducing apoptosis and cell cycle arrest.

Specific Aim 2A: To evaluate the combined effect of Garcinol with Curcumin against PaCa cells.

Dietary polyphenol curcumin has been extensively investigated as an anti-cancer agent and is currently undergoing several Phase-I, II and III clinical trials. This part of the study was designed to test the hypothesis that curcumin may synergistically interact with garcinol leading to therapeutic or chemoprotective advantage in PaCa. Results presented include evaluation of garcinol and curcumin as individual agents in human pancreatic cancer cell lines- Panc-1 and BxPC-3, and their combined effect compared for synergistic outcome.

Effect of garcinol and curcumin on cell viability- PaCa cells, BxPC-3 and Panc-1 were tested for their effects on cell viability under the influence of curcumin and garcinol as single agent regimen. It was noted both agents under identical experimental conditions, significantly ($p < 0.01$) reduced cell viability in both cell lines in a dose dependent manner on 48 hrs treatment, but to different extents. Garcinol exhibited more potent effect than curcumin (IC 50 = ~7 µM) in Panc-1 cells, which was comparable to efficacy of curcumin in BxPC-3 cells (IC 50 = ~10 µM) as shown in Figure 21. Garcinol and curcumin hold some structural resemblance but our results suggest that their therapeutic mechanistic targets might be different. In BxPC-3 cells (Figure 21 A) IC50 for garcinol treatment (upper panel) was approximately 12µM as compared to 10µM of curcumin (Figure 21 B). In contrast, in Panc-1 cells (Figure 21) IC50 values for garcinol (upper panel) and curcumin (lower panel) treatment were 7µM and 20µM respectively. We hypothesized these two agents in combination could be a better strategy for the

treatment of PaCa. We earlier mentioned no effect of garcinol on surrogate untransformed cells (NIH3T3).

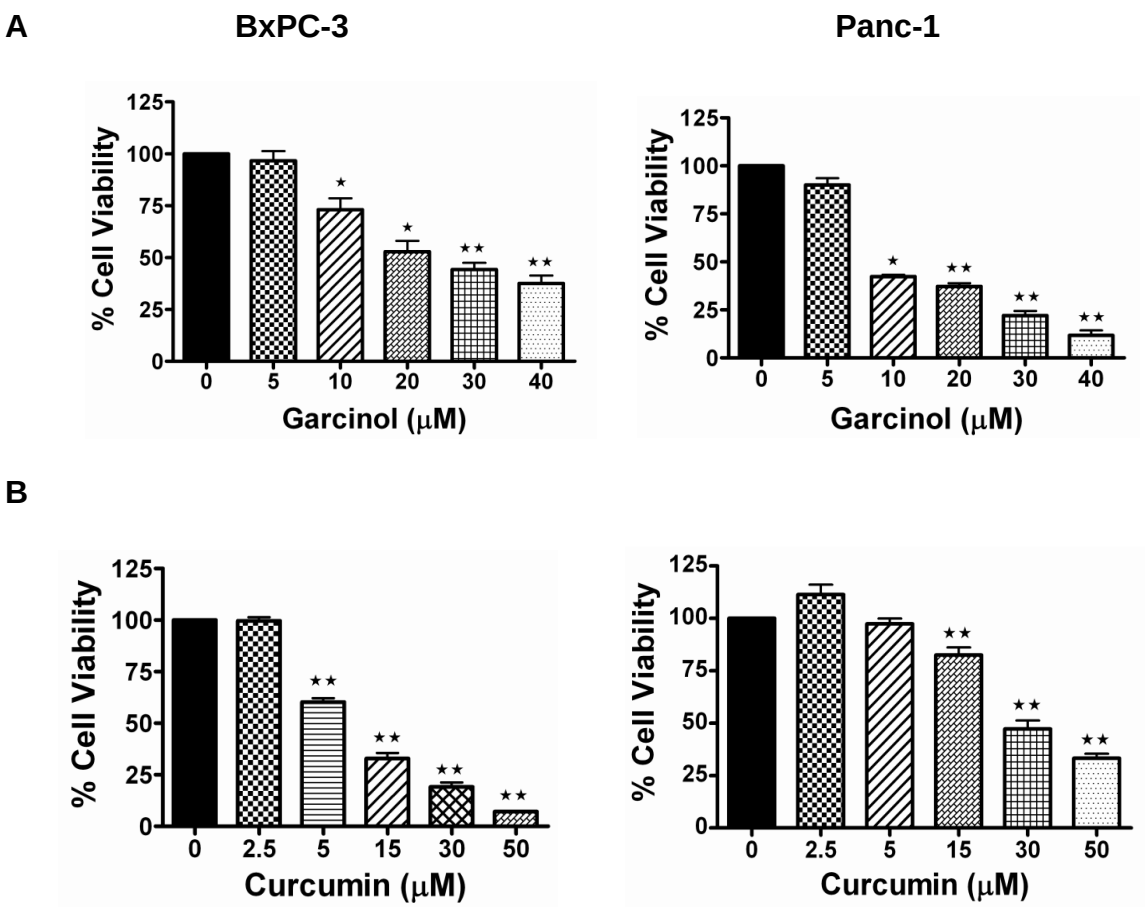


Figure 21: Effect of curcumin and garcinol on PaCa cell viability

Percentage of metabolically viable cells was reduced in a dose responsive manner on 48hr treatment with **A:** garcinol or **B:** curcumin on both PaCa cell lines, BxPC-3 and Panc-1 as analyzed using MTS assay. * $p < 0.05$, ** $p < 0.01$ relative to control.

Garcinol and curcumin induce apoptosis in PaCa cells- To confirm apoptosis, we performed Histone DNA ELISA assay and observed that both curcumin and garcinol were effective in inducing apoptosis in a dose dependent manner (Figure 22). Relative to control the enrichment factor in BxPC-3 cells was found enhanced by ~ 7, 10 and14 folds with garcinol at 10, 20 and 40µM concentrations respectively. The fold increase in histone enrichment factor on treatment with 15, 30 and 50µM curcumin concentrations was 10, 12 and 17 folds respectively. A similar trend was seen for Panc-1 cells. The results obtained corroborate well with morphological changes associated with apoptosis such as formation of apoptotic bodies and reduction in cell numbers evaluated using DAPI stain in both Panc-1 and BxPC-3 cells (Figure 23).

Based on the objective outlined under Aim-2, we sought to investigate the effect of curcumin and garcinol in combination in induction of apoptosis. Figure 24 depicts the changes seen when curcumin and garcinol were combined in different ratios of 1:4 and 1:10 (2.5µM curcumin/garcinol: 10µM garcinol/curcumin; and 2µM curcumin/garcinol: 20µM garcinol/ curcumin) of respective concentrations. As noted under Figure 24 in both cell lines the combination regimen had pronounced effect on induction of apoptosis compared to respective individual agent treatment supporting our hypothesis that curcumin and garcinol have synergistic effect in reducing cell viability and augmenting apoptosis.

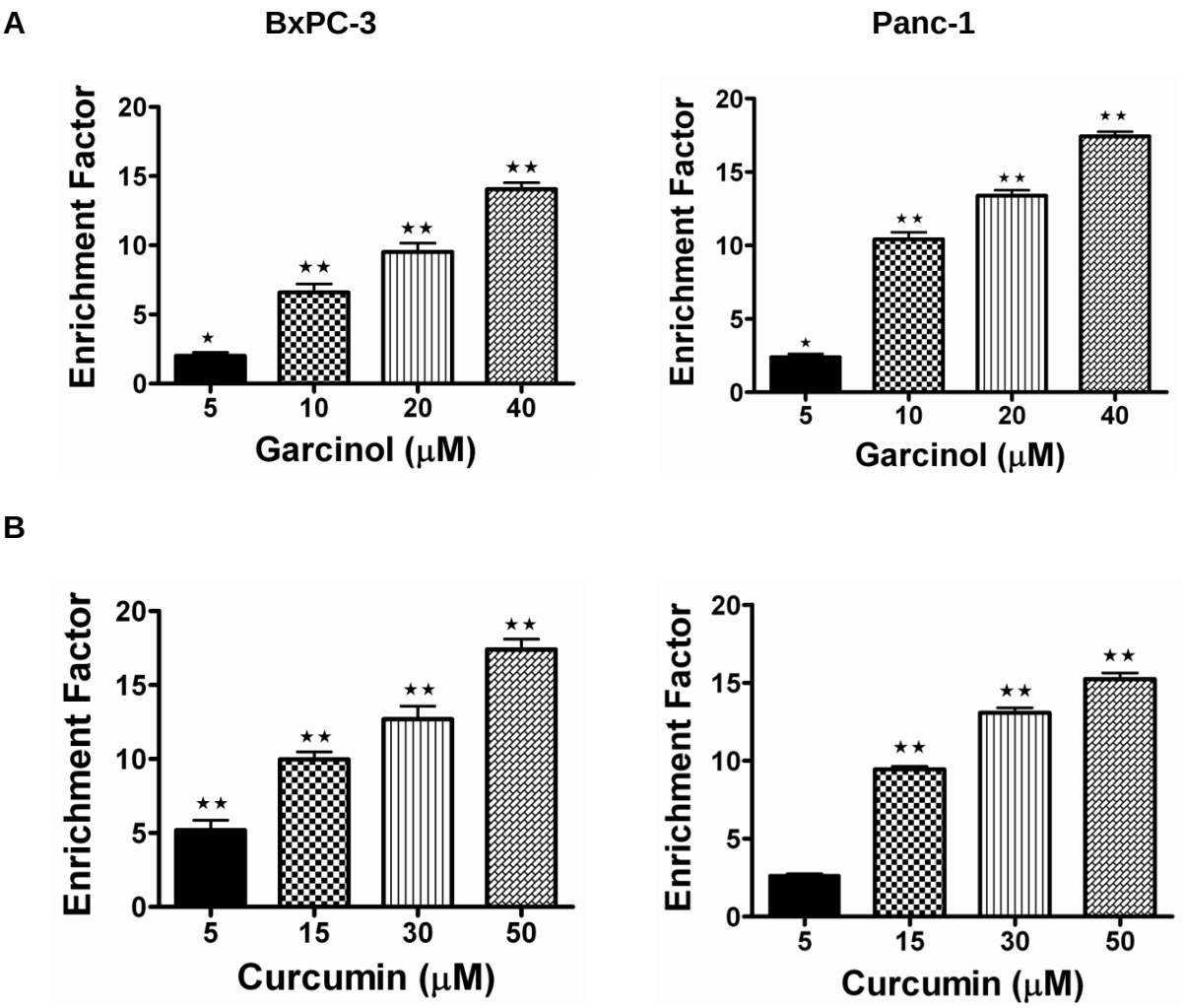


Figure 22: Induction of apoptosis by curcumin and garcinol

A: garcinol or **B:** curcumin treated cytosolic extracts were used to evaluate induction of apoptosis in PaCa cells, BxPC-3 and Panc-1 using ELISA - Histone DNA enrichment assay. Results demonstrate a significant dose dependent increase in apoptotic cells on individual treatment with either agent. Enrichment factor was measured using subtraction of background signal. * $p < 0.05$, ** $p < 0.01$ relative to control.

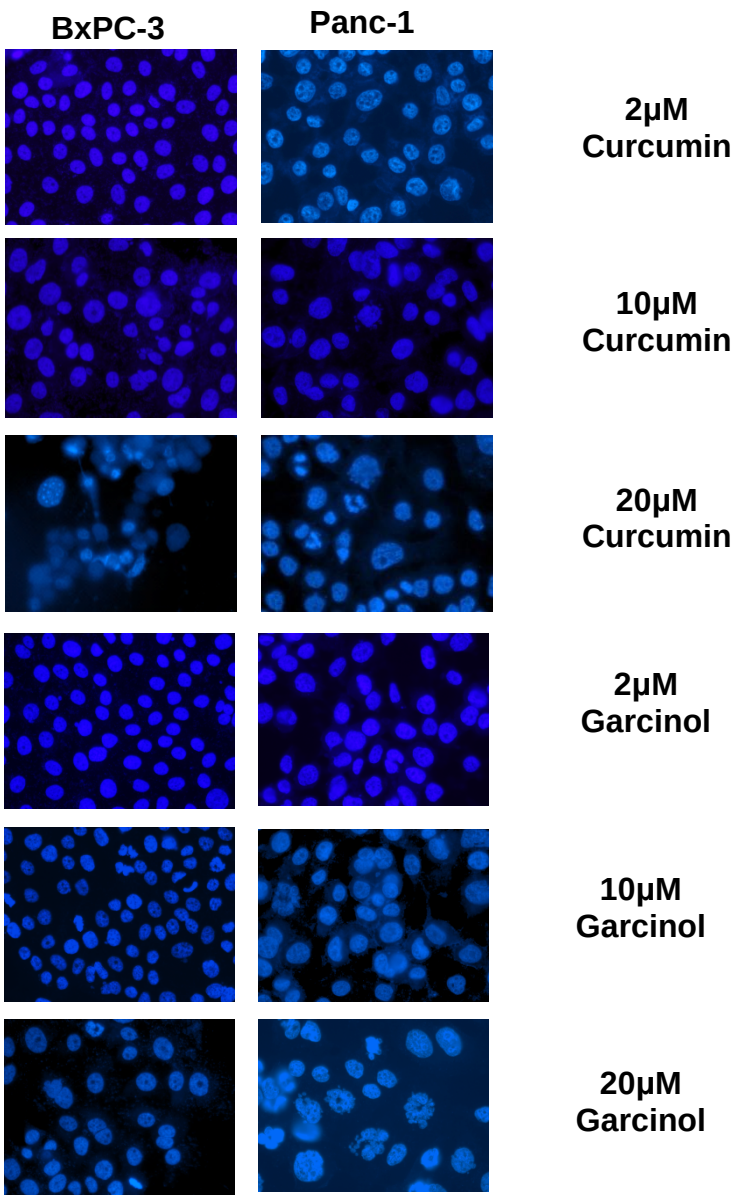


Figure 23: Apoptotic morphological changes on treatment with curcumin and garcinol

Apoptotic morphological changes such as abnormal nuclear morphology, reduction in cell number with apoptotic body formation and cell shrinkage was observed in a dose responsive manner on 48 hour treatment with curcumin or garcinol. BxPC-3 and Panc-1 cells were fixed with DAPI stain and visualized using fluorescence microscopy with an excitation maximum at 358nm and emission maximum at 461nm.

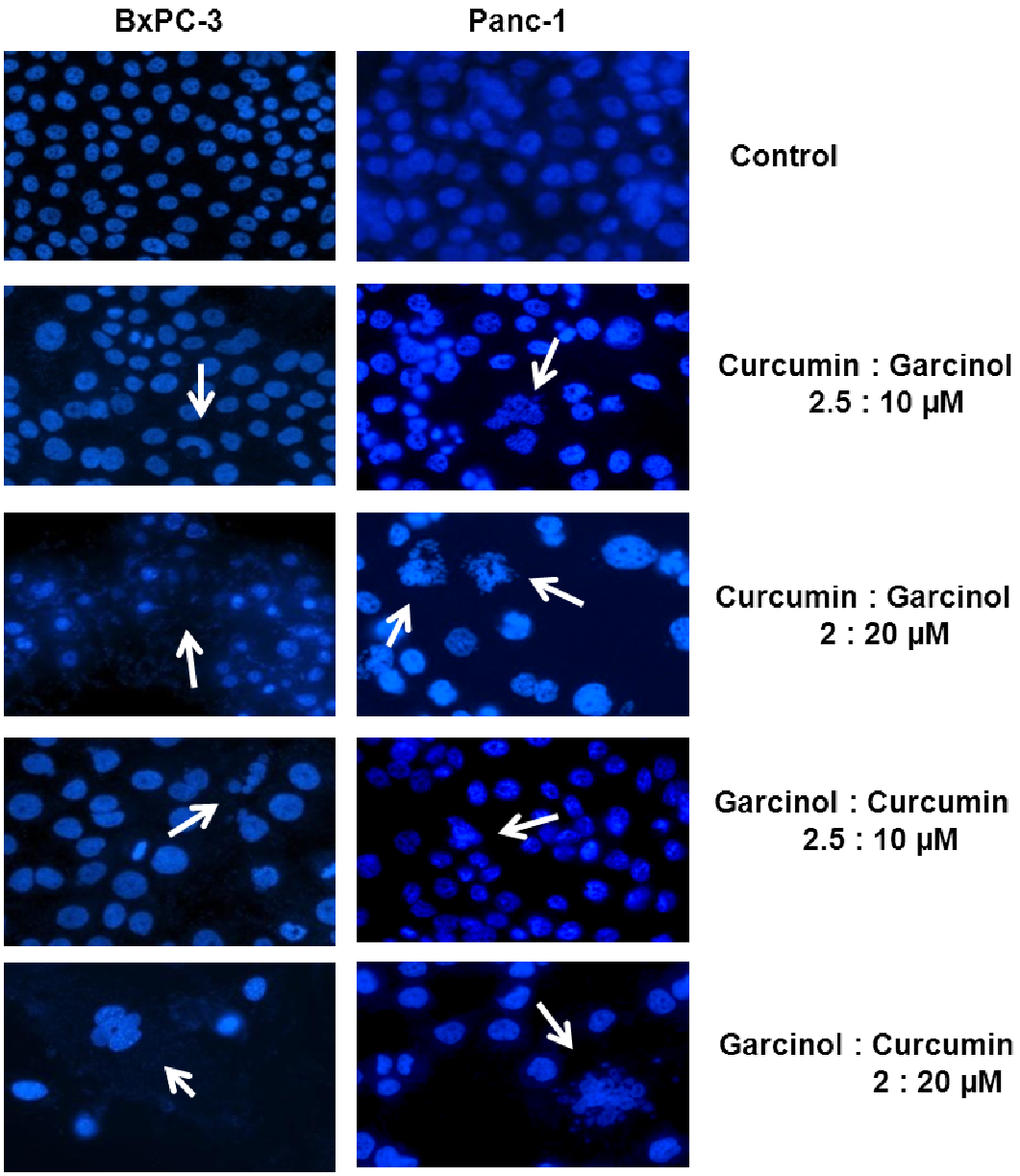


Figure 24: Apoptotic morphological changes on combination treatment

Apoptotic morphological changes such as abnormal nuclear morphology, reduction in cell number with apoptotic body formation and cell shrinkage was observed in a dose responsive manner on 48 hour treatment with curcumin and garcinol in different combination ratio. BxPC-3 and Panc-1 cells were fixed with DAPI stain and visualized using fluorescence microscopy with an excitation maximum at 358nm and emission maximum at 461nm.

Garcinol and curcumin exhibit synergistic relation in PaCa therapy- To evaluate the extent of synergism, the combination effect of garcinol and curcumin (in variable ratios) was tested for cell viability. Cell viability was reduced significantly ($p < 0.01$) in Panc-1 cells upon treatment and the loss of viable cells were more drastic in combinatorial regimen group compared to individual doses (Figure 25A). Using CalcuSyn software for Isobologram analysis, the combination index values (CI) for different ratios of treatment in Panc-1 cells was determined.

Table 4 and 5 depict the different CI values obtained upon treatment. CI = 1 indicates additive effect, < 1 indicates synergistic effect and > 1 suggests antagonistic effect. The CI values for ED50 (Effective dose for 50 % inhibition) when curcumin and garcinol were administered in the ratios of 1:10, 1:5 and 1:2.5 were 0.201, 0.422 and 0.659 respectively (Table 4). Three combinations were tested for each ratio.

Furthermore, Dm defined as the median-effect dose or concentrations signifying the potency of the treatment were 26µM and 17.65µM for curcumin and garcinol, respectively. Dm is an important parameter which helps in establishing dose reduction which leads to lowered toxicity in the host, thus retaining overall therapeutic efficacy. It is usually represented in correlation with ED50 values. We observed that when Panc-1 cells were treated with curcumin and garcinol, in the ratios of 1:10, 1:5 and 1:2.5 the Dm values were 4.57, 8.57 and 10.79 µM respectively. Based on the CI ED50 values and the Dm values one may conclude 2- to 5- fold lower concentration requirement in combination treatment than individual treatments in order to achieve similar effect.

Similarly when the ratio of garcinol and curcumin were reversed (Table 5) and the cells were treated with 1:10, 1: 5 and 1: 2.5 of garcinol to curcumin, the CI values at

ED50 were 0.756, 0.747 and 0.921 respectively. The Dm values recorded for the same ratios were 1.25, 2.32 and 5.11 µM respectively.

In related context, ‘m value’ is another parameter describing the sigmoidicity of the dose effect curve; m = 1, > 1, < 1 indicates hyperbolic, sigmoidal and negative sigmoidal shape respectively. It was noticed that the m value was > 1 in all our samples except when curcumin: garcinol were in the ratio of 1:10 suggesting sigmoidicity of the curve. Further, the linear correlation coefficient of the median effect plot is represented as’ r value’. An r value equal or close to 1 indicates perfect conformity of the data as was seen in our experimental results showing an r value close to 1 (0.97 – 1.00) in all our samples. The foregoing results clearly narrate that garcinol and curcumin have potent synergistic effect in reducing PaCa cell viability.

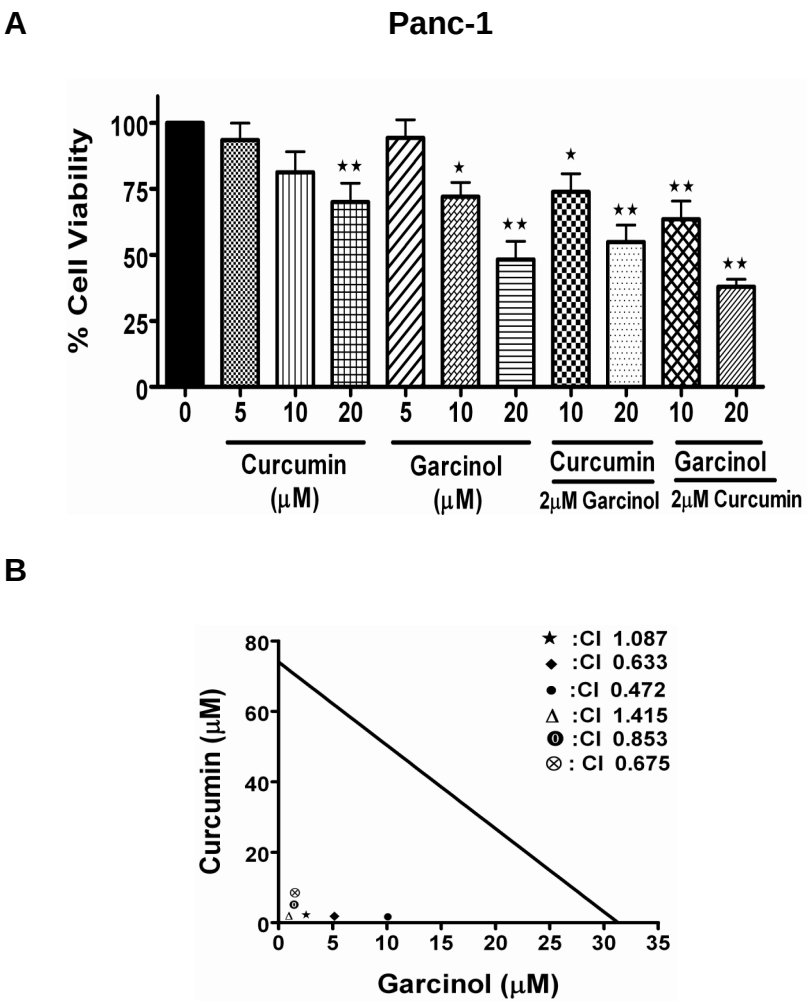


Figure 25: Cytotoxic synergy: curcumin and garcinol

A: Combination effect of curcumin and garcinol on Panc-1 cell viability was determined using MTS assay. Combinatorial treatment significantly reduced cell viability more effectively than monotherapy on 48hr treatment. * $p < 0.05$, ** $p < 0.01$ relative to control. **B:** Isobologram analysis to evaluate the extent of synergism on combining curcumin and garcinol for therapeutic effect.

Table 4: Combination index values for curcumin- garcinol synergy

Drug	ED 50	ED 75	ED 90	Dm	m	r
Curcumin	N/A	N/A	N/A	26.00	1.60	0.99
Garcinol	N/A	N/A	N/A	17.65	2.27	0.99
Curcumin: Garcinol 1:10	0.201	0.472	1.111	4.57	0.72	0.99
Curcumin: Garcinol 1:5	0.422	0.633	0.957	8.47	1.05	1.00
Curcumin: Garcinol 1: 2.5	0.659	1.087	1.812	10.79	0.98	0.99

When ratio of garcinol concentration was higher in the combination than curcumin, the CI value at ED75 (Effective dose at which 75 % cells are non-viable) suggested a moderate to high synergism between the two agents under investigation.

Table 5: Combination index values for curcumin- garcinol synergy

Drug	ED 50	ED 75	ED 90	Dm	m	r
Curcumin	N/A	N/A	N/A	26.00	1.60	0.99
Garcinol	N/A	N/A	N/A	17.65	2.27	0.99
Garcinol : Curcumin 1: 10	0.756	0.675	0.603	1.25	2.88	0.99
Garcinol: Curcumin 1:5	0.747	0.853	0.978	2.32	1.72	1.00
Garcinol : Curcumin 1: 2.5	0.921	1.415	2.188	5.11	1.15	0.97

When ratio of curcumin concentration was higher in the combination than garcinol, the CI value at ED75 (Effective dose at which 75 % cells are non-viable) suggested a low to moderate synergism between the two agents under investigation.

Specific Aim 2B: To evaluate the combined effect of Garcinol with Gemcitabine against PaCa cells.

As reported earlier the pyrimidine nucleoside analog-gemcitabine is the standard drug for treatment of PaCa, but conventional dosing with this drug at maximal tolerated dose cause adverse side effects and patients fail to respond adequately after multiple cycles of therapy due to the emergence of drug-resistance. Thus, we sought to examine the chemo-sensitization potential of garcinol in combination with gemcitabine to reduce viable tumor cells and induce apoptosis using low concentrations of gemcitabine.

Garcinol and gemcitabine inhibit cell viability and apoptosis- As shown in Figure 26A, gemcitabine inhibited cell viability in a dose dependent manner in the two investigated PaCa cell lines with more than 50% loss of cell viability with ~50nM gemcitabine in BxPC-3 cells and ~250nM in Panc-1 cells upon 72 hour treatment. This finding is in concordance with other studies reported in literature [126] and accordingly Panc-1 cells being classified as 'gemcitabine resistant' cells. To address the objective as outlined under Aim- 2B we used sub-optimal concentrations of gemcitabine for chemosensitization of PaCa cells for therapy. Thus, the efficacy was explored using combination comprising 3 different concentrations of garcinol (1, 2.5, and 5μM in BxPC3 cells and 2.5, 5 and 10μM in Panc-1 cells) co-incubated with 2 different concentrations of gemcitabine (25 and 50nM in BxPC3 cells, whereas 50 and 100 nM concentration in Panc-1 cells) and the results are shown in Figure 26C. From the results presented, it is inferred that a significant loss of cell viability occurs when relatively low dose of gemcitabine is combined with garcinol relative to monotherapy. In BxPC-3 cells, the loss

of viable cells was ~75 % (10µM garcinol and 50nM gemcitabine) compared to ~50 % with 10µM of only garcinol and ~55 % with 50nM gemcitabine. A similar trend was also noticed in Panc-1 cells with only ~30 % of viable cells following combination treatment comprising 20µM garcinol and 100nM gemcitabine as compared to their respective individual treatments. These figures compare significantly less than single agent treatment for similar duration of time ($p < 0.01$).

Further, to confirm that the loss in viability owes to cell death by apoptosis, quantification of Histone-DNA ELISA and alterations in cellular morphological features were evaluated as described previously. As depicted in Figure 27 A and B, following combinatorial regimen of garcinol and gemcitabine in both investigated cell lines, a significant increase in apoptotic cells along with morphological alterations closely mimicking the loss of viable cells is evident. This data attests chemosensitization potential of garcinol in PaCa therapy.

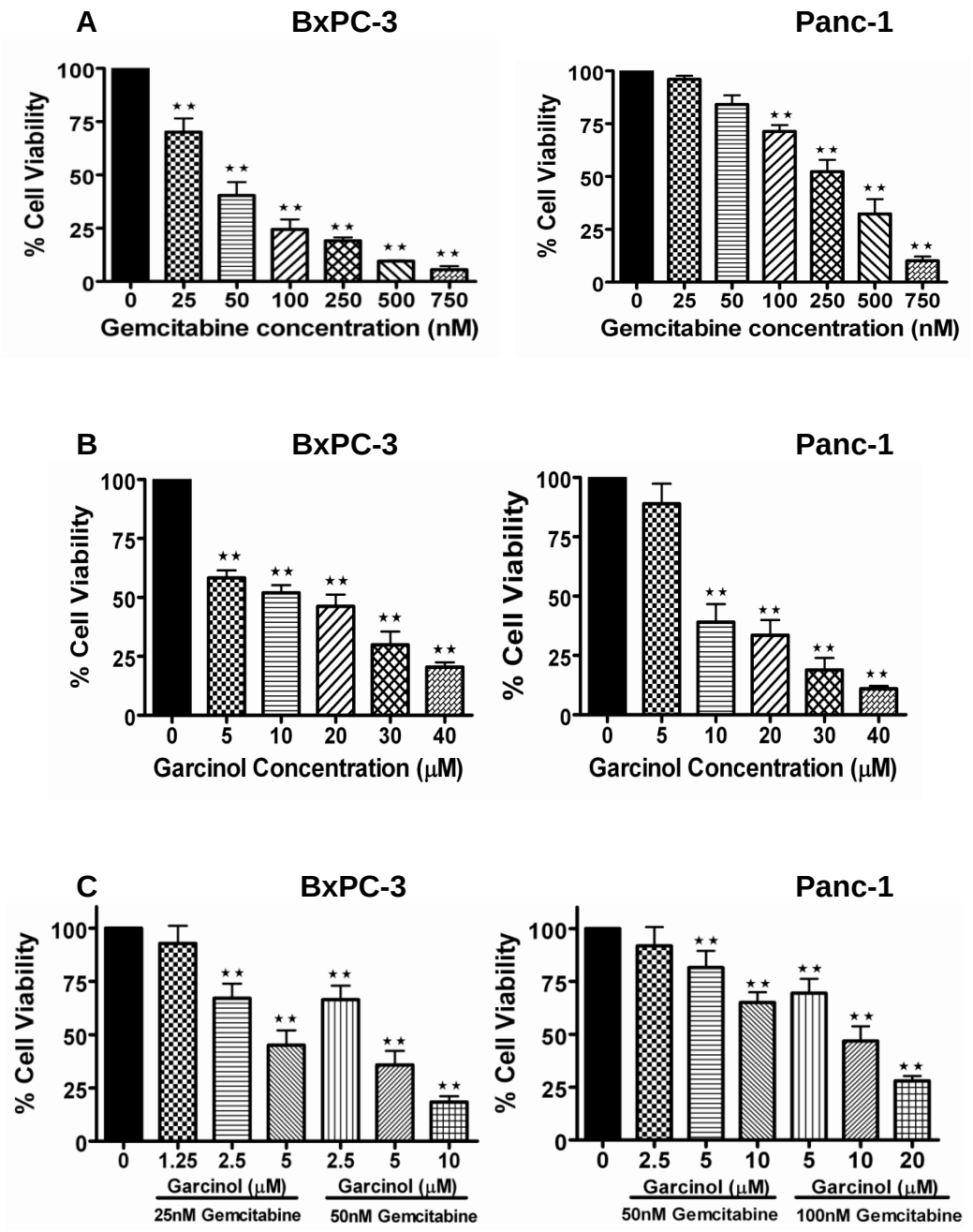
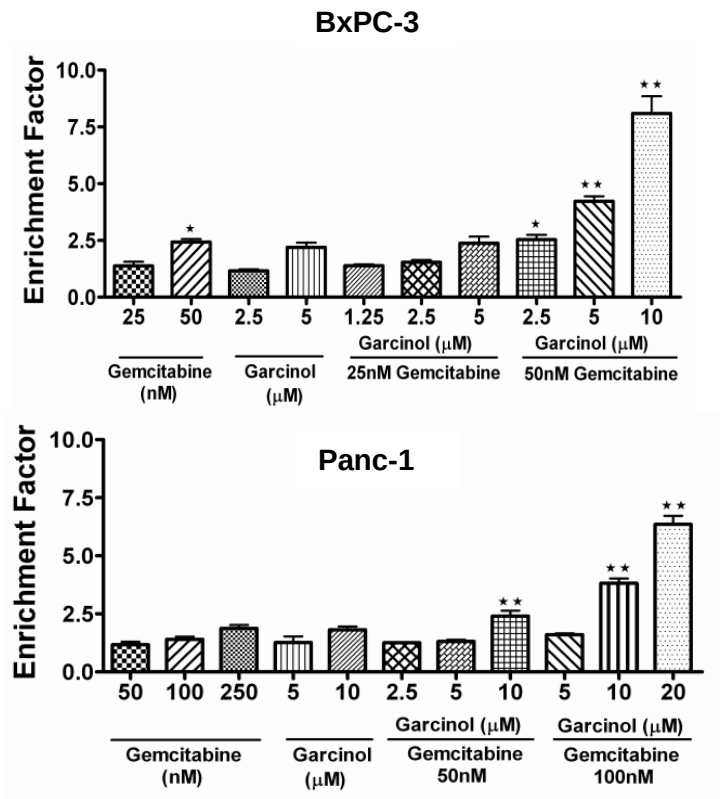


Figure 26: Effect of garcinol and gemcitabine on PaCa cell viability

Significant dose dependent reduction in cell viability upon 72 hour treatment with **A:** gemcitabine. IC50 value in BxPC-3 cells was attained at 50nM concentration as compared to 250nM in Panc-1 cells. **B:** garcinol, IC50 value was attained at ~15μM treatment in BxPC-3 cells compared to 10μM in Panc-1 cells and **C:** sub-optimal concentrations of gemcitabine were combined with three different concentrations of garcinol to achieve enhanced loss in cell viability. ** $p < 0.01$ relative to control.

A



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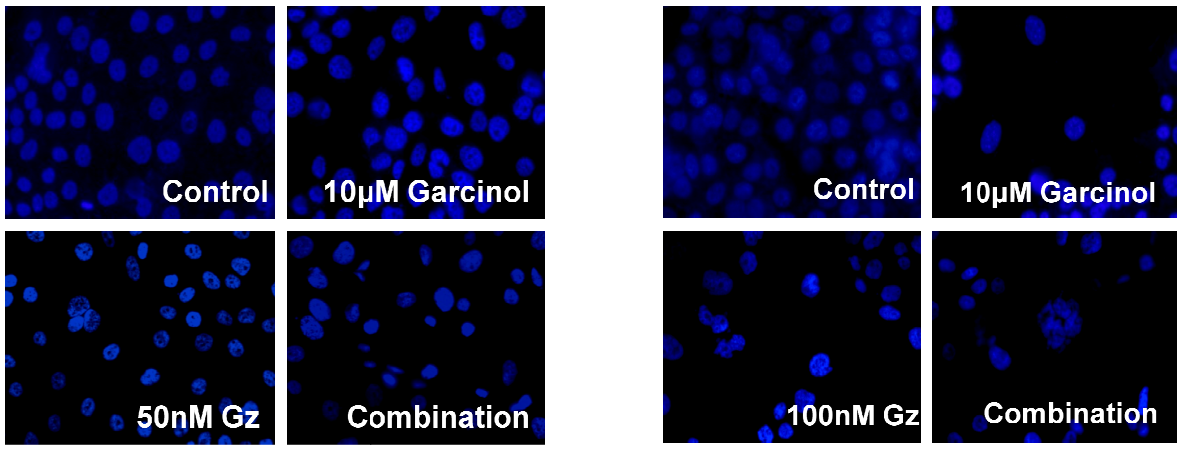


Figure 27: Apoptotic induction of PaCa cells on treatment

Induction of apoptotic cells on treatment with garcinol and gemcitabine individually and in combination was observed using **A:** ELISA histone DNA analysis. Sub-optimal dose of gemcitabine was combined with garcinol which induced significant increase in apoptosis as measured by estimation of enrichment of cytosolic nucleosomes. * $p < 0.05$, ** $p < 0.01$ relative to control. **B:** Apoptotic morphological changes due to single agent or combination treatment were observed using DAPI stain. Combination treatment: 10μM garcinol + 50 or 100 nM gemcitabine.

Garcinol treatment enhances apoptosis by gemcitabine inducing caspase activity- To confirm sensitization and apoptosis by garcinol and gemcitabine treatment, we assessed caspase-3 and caspase-9 activity in the investigated cell lines- BxPC-3 and Panc-1 cells (Figure 28A and B). Cells were exposed to garcinol and gemcitabine as depicted in figure 28, either as single agent or in combination for 48hrs. At end of incubation period cells were collected (including floating cells) and caspase activity was determined as per manufacture's instruction. Data shows that while garcinol treatment at investigated dose do not cause a significant increase in caspase-3 and 9 relative to control, the combination regimen revealed significant increase in their activity being elevated more than one and half - two fold relative to control ($p < 0.05$; $p < 0.01$, Figure 28).

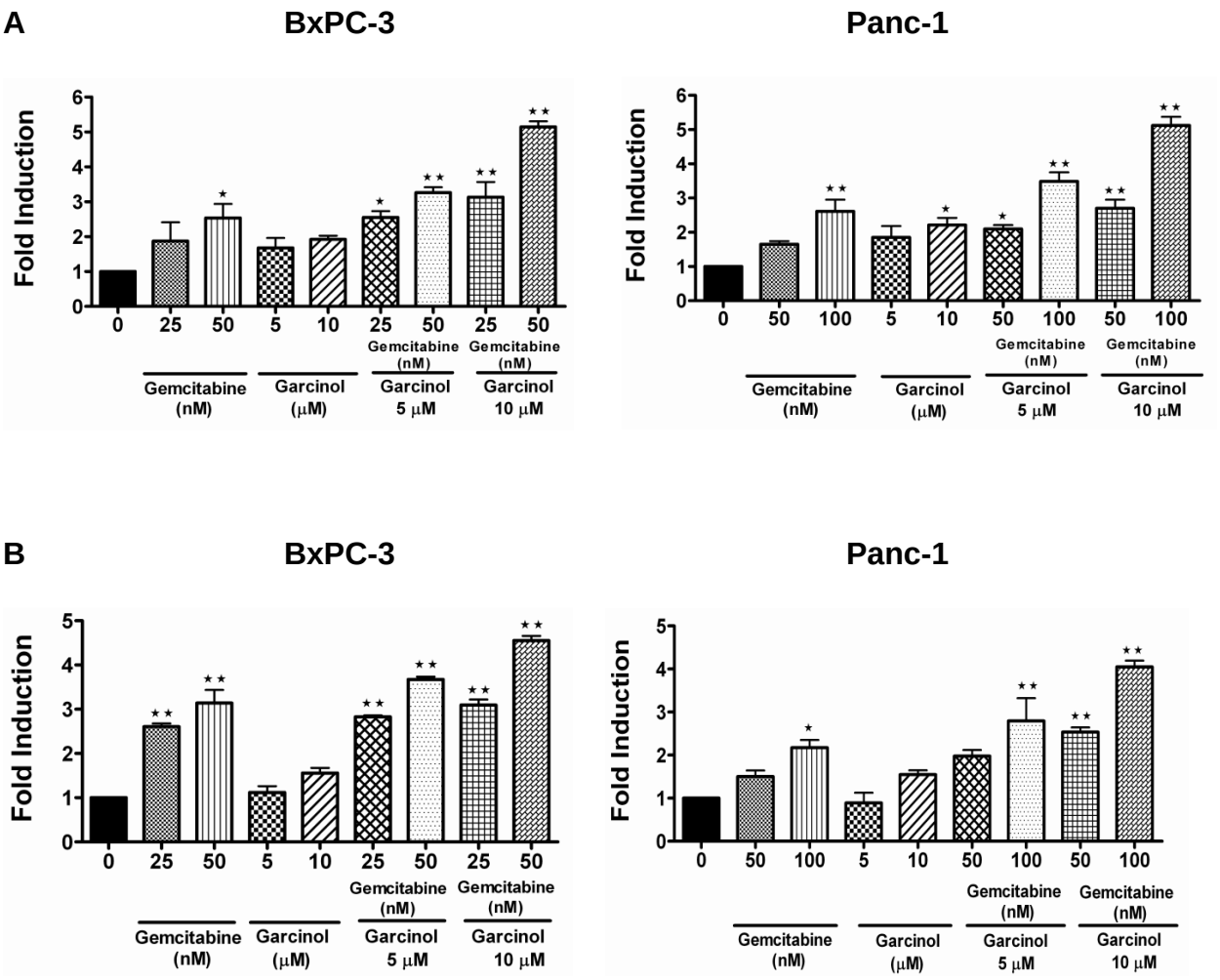


Figure 28: Induction of caspase activity on treatment with garcinol and gemcitabine

Colorimetric analysis for **A:** Caspase 3 and **B:** Caspase 9 activity in PaCa cells, BxPC-3 and Panc-1 cell line. Gemcitabine individually or in combination with garcinol depicted a significant increase in enzyme activity . * $p < 0.05$, ** $p < 0.01$ relative to control.

Garcinol inhibits activation of NF-κB and down-regulates NF-κB activation stimulated by gemcitabine and augments apoptosis by gemcitabine - We previously mentioned that garcinol could downregulate constitutively expressed NF-κB in PaCa, cells (Figure 17B).and regulated many anti-apoptotic proteins. We therefore assessed NF-κB on treatment with garcinol. Experiments were performed exposing BxPC-3 cells and Panc-1 to 10μM garcinol and gemcitabine (25 or 100nM concentration respectively; Figure 29A) treatment for 72 hrs and thereafter prepared the nuclear extract as described under Materials and Methods section and subjected it to DNA binding assay by EMSA. It was concluded that 10μM garcinol treatment of cells could significantly reduce the NF-κB DNA binding activity in combination treatment (Figure 29A). These results demonstrate that garcinol is effective in down-regulating NF-κB DNA binding activity in PaCa cells, but together in combination with gemcitabine could further downregulate NF-κB in PaCa cells, which is consistent with our hypothesis.

Mechanistically, to confirm chemosensitization effects of garcinol via induction of apoptosis, we assessed the alteration of key apoptotic (PARP cleavage) and anti-apoptotic (Bcl-xL) protein molecules expression in Panc-1 cells by Western immunobloting (Figure 29B). Our data show that while treatment with garcinol or gemcitabine alone show no appearance of cleaved PARP; their combination showed strong cleaved PARP band indicative of apoptosis induction. Consistent with the results obtained with PARP cleavage, significant down-regulation of the anti-apoptotic Bcl-xL in the combination treatment group (Figure 29B) was also noted indicating that garcinol indeed sensitizes PaCa cells to the cytotoxic effect of gemcitabine.

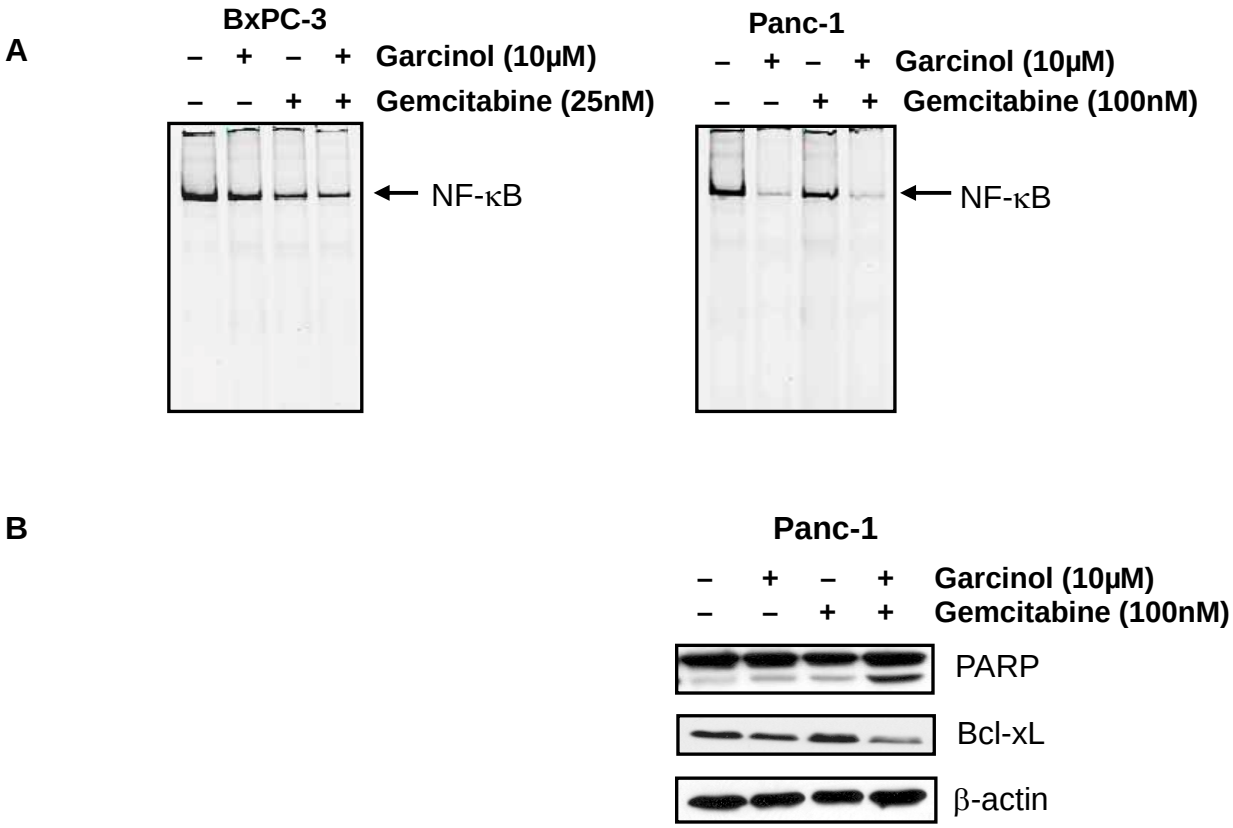


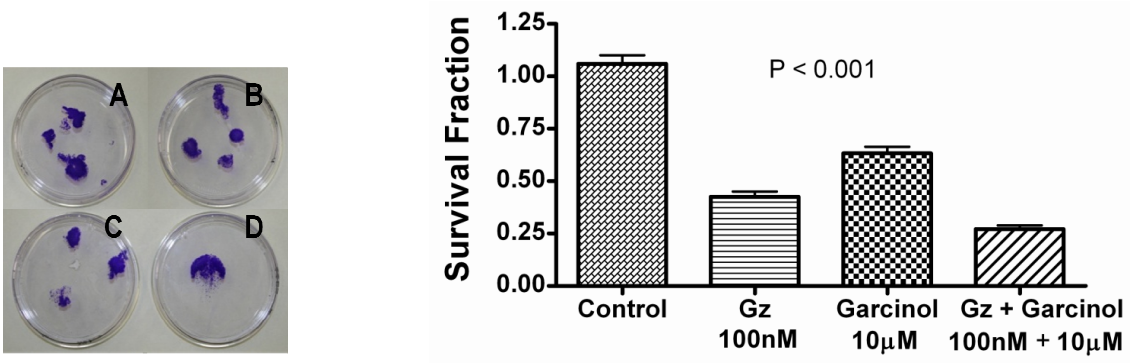
Figure 29: Effect of garcinol and gemcitabine on NFκB levels and apoptotic markers

A: Transcription factor NF-κB levels were analyzed in garcinol and gemcitabine treated nuclear extracts using EMSA analysis in PaCa cells, BxPC-3 and Panc-1. A down-regulation in NF-κB DNA binding ability was observed in combination treated cells relative to control. **B:** Western immunobloting to assess influence of garcinol and gemcitabine treatment in Panc-1 cells was done. Strong cleaved band of PARP was observed in combination treatment relative to individual treatments along with downregulation in expression of anti-apoptotic protein Bcl-xL.

Garcinol inhibits clonogenic survival of cells with gemcitabine - Based on our results showing apoptosis, the clonogenic survival of cells treated with garcinol was moderately reduced relative to control, while gemcitabine treatment lead to a significant decrease in clonogenic survival of Panc-1 cells. Most interestingly, the combination group showing more cytotoxicity resulted in fewer clones compared to control and single agent-treatment alone ($p < 0.001$; Figure 30A).

Cell adhesion is a crucial step in cancer progression playing a pivotal role in recurrent, invasive and distant metastasis. Several cell adhesion molecules (CAM) regulate the entire process. We performed a quantitative analysis as described under methods to check for effect of garcinol and gemcitabine on cell adhesion ability. In BxPC-3 cells, cell adhesion reduced by half in combination (50nM gemcitabine and 5µM garcinol) treatment as compared to individual effect of 50nM gemcitabine ($p < 0.01$) (Figure 30B). Similar trend was observed in Panc-1 cells on treatment with 100nM gemcitabine and 10µM garcinol than respective individual treatments.

A **Clonogenic Assay: Panc-1 cells**



B **Cell Adhesion Assay**

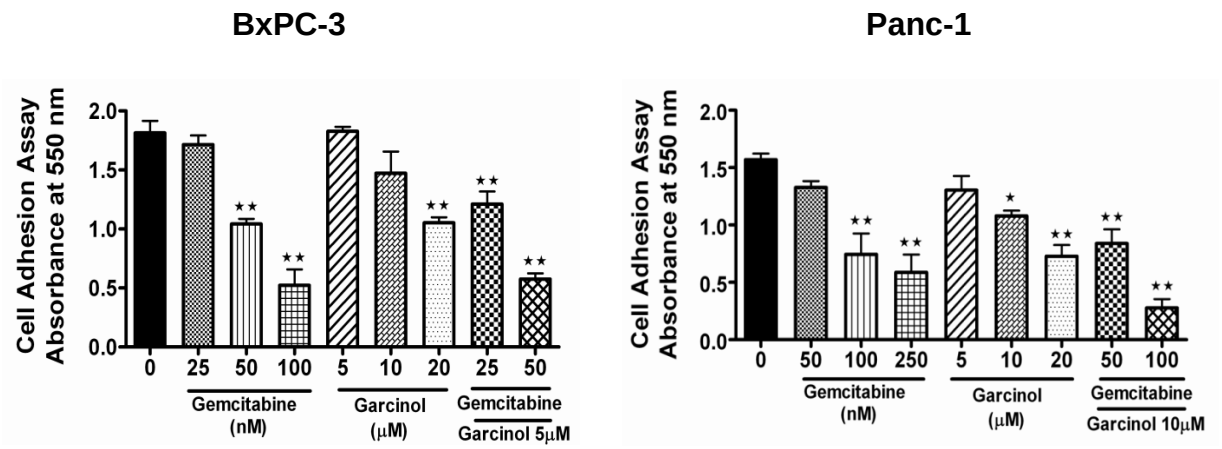


Figure 30: Action of garcinol and gemcitabine on colony formation and cell adhesion

A: Clonogenic assay in Panc-1 cells to test for cell proliferation and migration abilities. Panc-1 cells were treated with garcinol alone or in combination with gemcitabine (Gz) and fewer clones were formed in combination treatment relative to monotherapy. Overall significance was determined by one way ANOVA ($p < 0.001$). **B:** Effect of garcinol and/or gemcitabine on cell adhesion using laminin coated plates. Gemcitabine individually and in combination restricted cell adhesion more potently than garcinol treatment. * $p < 0.05$, ** $p < 0.01$ relative to control.

Effects of garcinol and gemcitabine on MMP-9, VEGF, IL-8 and PGE_2 - As mentioned under Aim-1 of the dissertation, these are important molecules implicated in angiogenesis, invasion and metastasis of cancer cells and garcinol exhibited dose dependent effect in inhibition of these molecules (Figure 18 and 19) . We further investigated the potential of garcinol in chemosensitization reference to these molecules under the conditions of combination treatment using sub-optimal dose of gemcitabine. The experimental details have been described under Methods section. Results of this experiment indicate downregulation of these molecules in both the investigated cell lines by single agents; however the effect was significantly pronounced ($p < 0.01$) in each of the combination group which may have a profound impact in progression of PaCa tumor (Figure 31A-C).

Additionally, PGE_2 assay also done under the same experimental conditions and a similar trend was noted, being significantly reduced in the combination group (Figure 32A). A qualitative wound healing assay (Figure 32B) was performed to test for individual effects of garcinol and gemcitabine on cell migration. We observed an expansion in the wound on treatment with either agent after 24 hour as compared to a closed wound in untreated control cells.

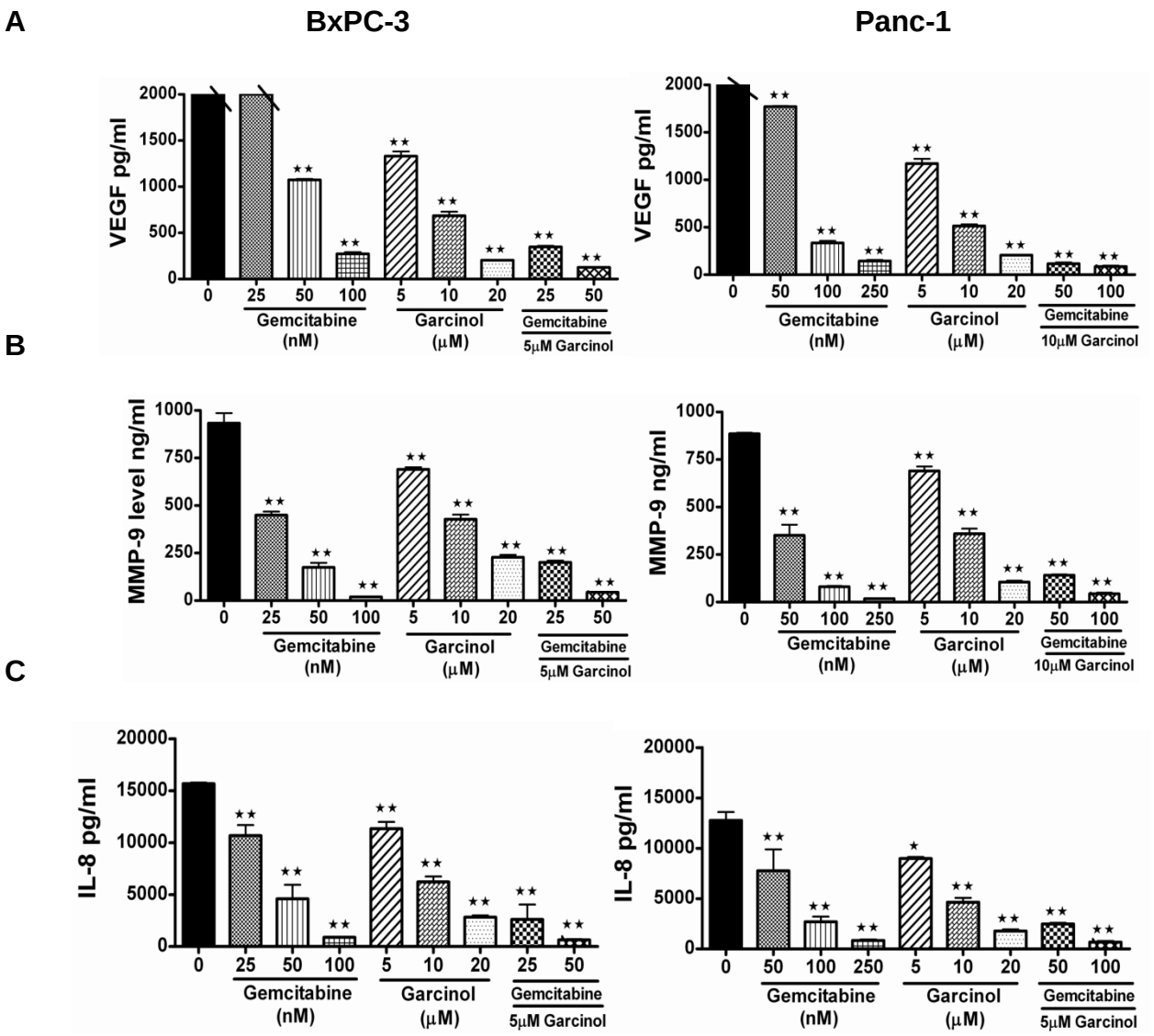


Figure 31: Downregulation of angiogenic markers

Effect of garcinol and gemcitabine treatment on levels of angiogenic markers **A:** *VEGF*, **B:** *MMP-9 activity* and **C:** *IL-8* in PaCa cells, BxPC-3 and Panc-1. $1x10^6$ cells were treated with gemcitabine and/or garcinol for 48hr and supernate was collected and evaluated for various angiogenic markers using quantikine and fluorokine assay. Significant reduction in levels of angiogenic markers was observed in combination treatment relative to individual therapies. * $p < 0.05$, ** $p < 0.01$ relative to control.

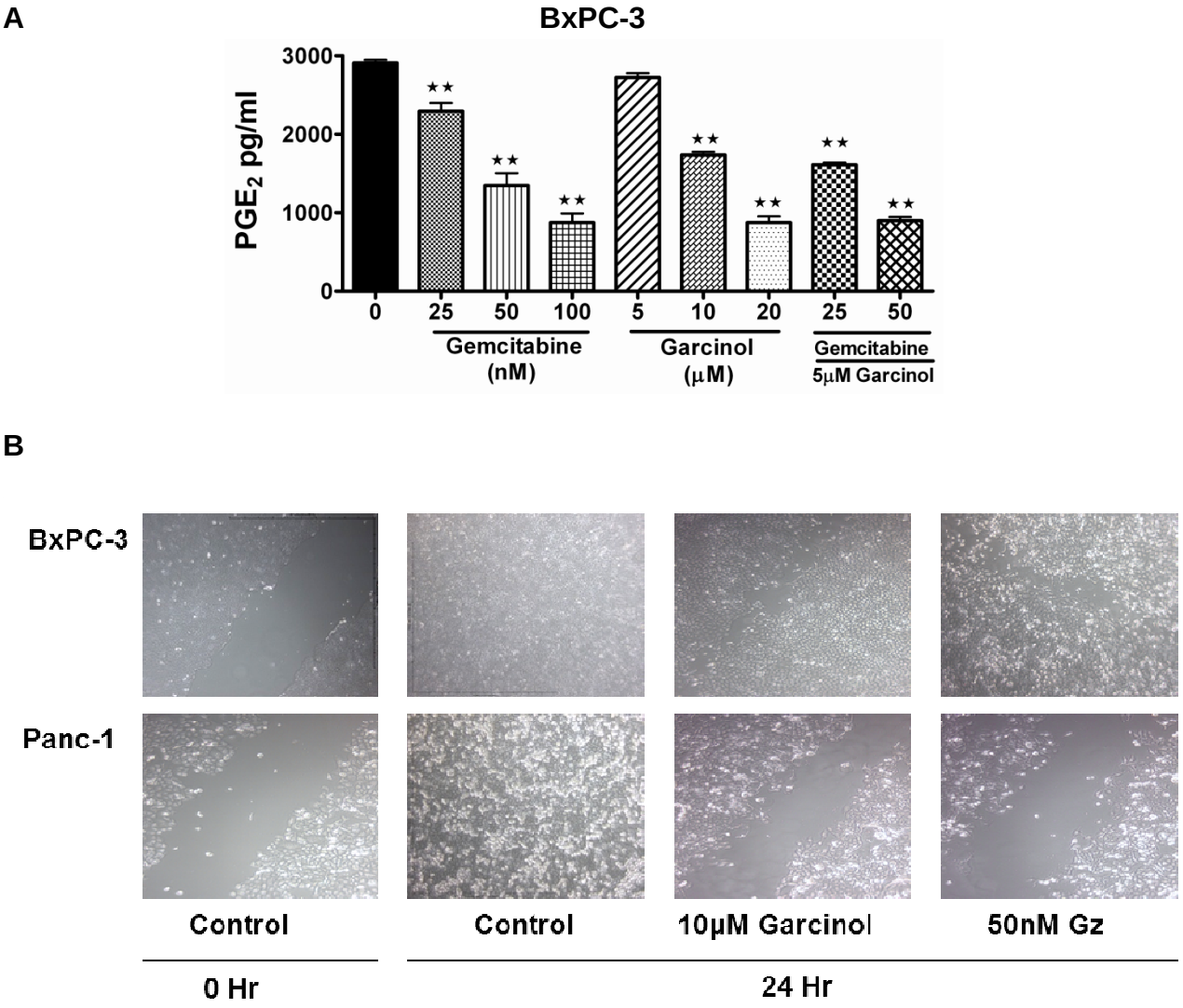


Figure 32: Effect of garcinol and gemcitabine on invasion and migration

A: *PGE_2* concentration as determined by quantikine assay in COX-2 over-expressing BxPC-3 cells was significantly down-regulated on single agent treatment but more effectively in combination dose. * $p < 0.05$, ** $p < 0.01$ relative to control. **B:** Microphotograph of wound healing assay on treatment with garcinol and gemcitabine depicting the ability of these agents in restricting cell migration and proliferation upon treatment.

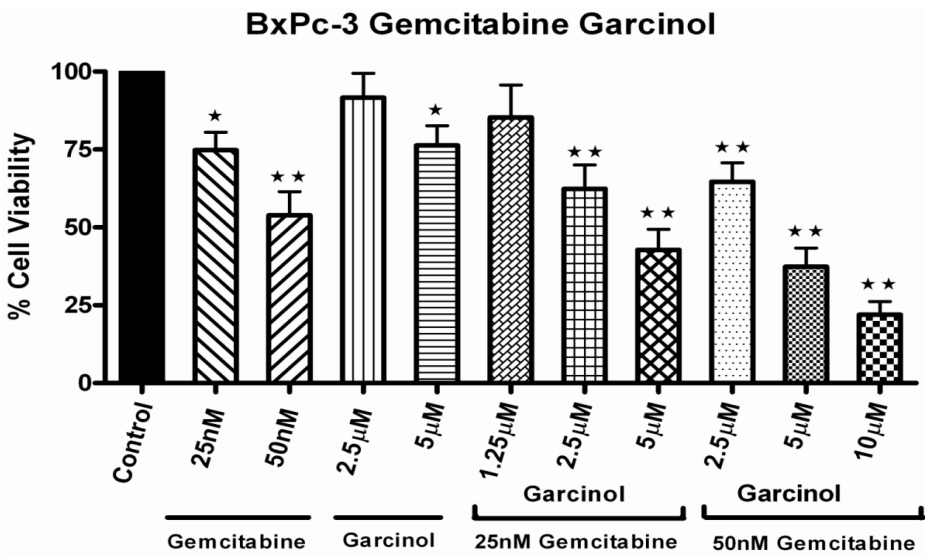
Synergistic effect of garcinol and gemcitabine combination – The concept related to 'synergistic interaction' between garcinol and curcumin has been mentioned earlier. On similar platform, we evaluated the combination of garcinol and gemcitabine for chemosensitization of PaCa as described in the experimental setup under Methods. Based on cell viability data, the combination index values (CI), Dm, m and R values were obtained using different ratios of treatment with garcinol and gemcitabine in BxPC3 and Panc-1 cells (Figure 33A and Figure 34A) using CalcuSyn software and presented in Table-6 and 7. As depicted in Figures 33B and 34B, the CI values tend to be either 1 or < 1 indicating a synergistic or additive effect of the combination.

Table 6: Cytotoxic synergy of garcinol and gemcitabine in BxPC-3 cells

Drug	ED 50	ED 75	ED 90	Dm	m	r
Gemcitabine (Gz)	N/A	N/A	N/A	9706.2	1.77	1.00
Garcinol	N/A	N/A	N/A	5624.1	1.35	1.00
Garcinol: Gz 50:1	1.47	1.02	0.717	3209.3	2.72	1.00
Garcinol: Gz 100:1	1.02	0.88	0.768	3659.2	1.87	1.00
Garcinol: Gz 200:1	0.86	0.76	0.69	4499.7	1.86	1.00

Combination index values on treatment with garcinol and gemcitabine in combination resulted in synergism (CI < 1) in higher ratios of 100:1 and 200:1 in BxPC-3 cell.

A



B

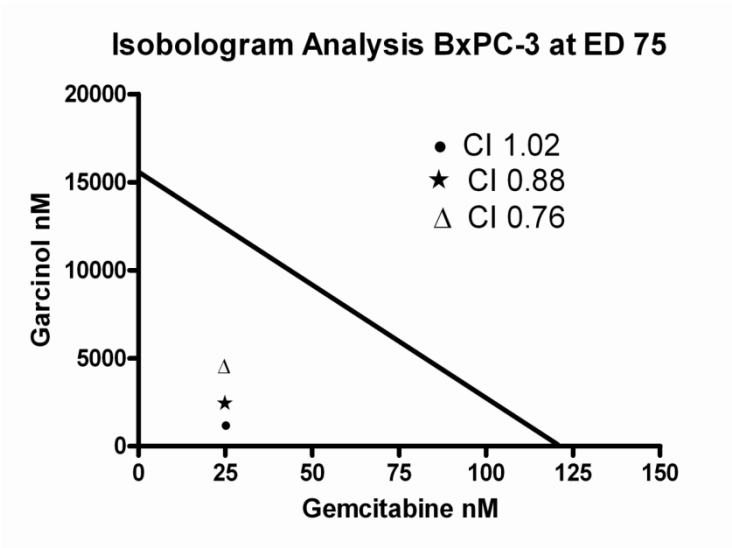


Figure 33: Cytotoxic synergy of garcinol and gemcitabine in BxPC-3 cells

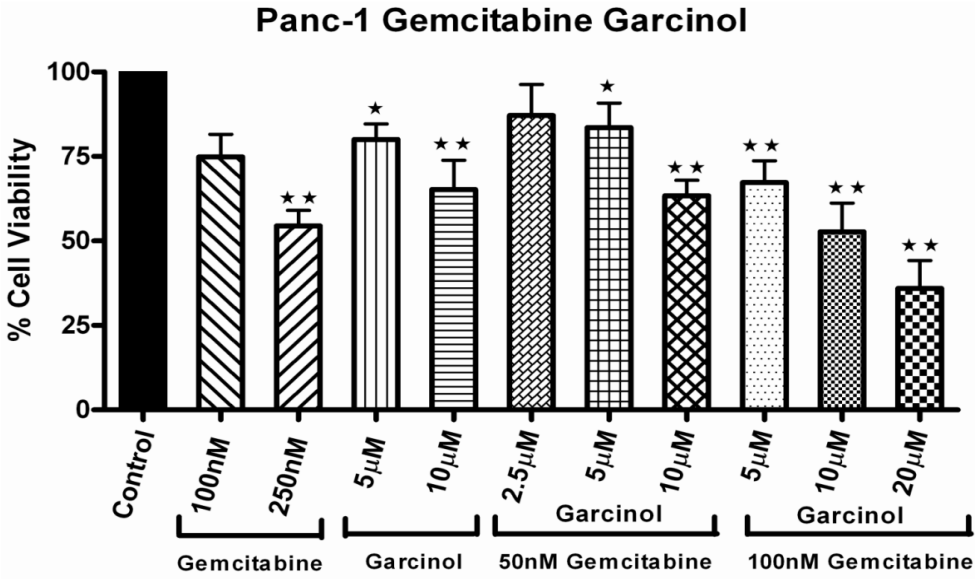
A: Combination effect of gemcitabine and garcinol on BxPC-3 cell viability was determined using MTS assay. Combinatorial treatment significantly reduced cell viability more effectively than monotherapy on 48hr treatment. * $p < 0.05$, ** $p < 0.01$ relative to control. **B:** Isobologram analysis to evaluate the extent of synergism on combining gemcitabine and garcinol for therapeutic effect.

Table 7: Cytotoxic synergy of garcinol and gemcitabine in Panc-1 cells

Drug	ED 50	ED 75	ED 90	Dm	m	r
Gemcitabine (Gz)	N/A	N/A	N/A	299.97	0.99	1.00
Garcinol	N/A	N/A	N/A	17585	1.11	1.00
Garcinol: Gz 50:1	1.504	1.068	0.760	12175	1.55	0.99
Garcinol: Gz 100:1	0.821	0.654	0.523	9104.5	1.37	1.00
Garcinol: Gz 200:1	0.967	0.571	0.338	13158	2.26	1.00

Isobologram analysis used to evaluate combination index values on treatment with garcinol and gemcitabine in combination resulted in synergism (CI < 1) in higher ratios of 100:1 and 200:1 in Panc-1 cells which are categorized as gemcitabine resistant phenotype. CI value suggests the potential of garcinol in sensitizing PaCa cells to the effect of gemcitabine.

A



B

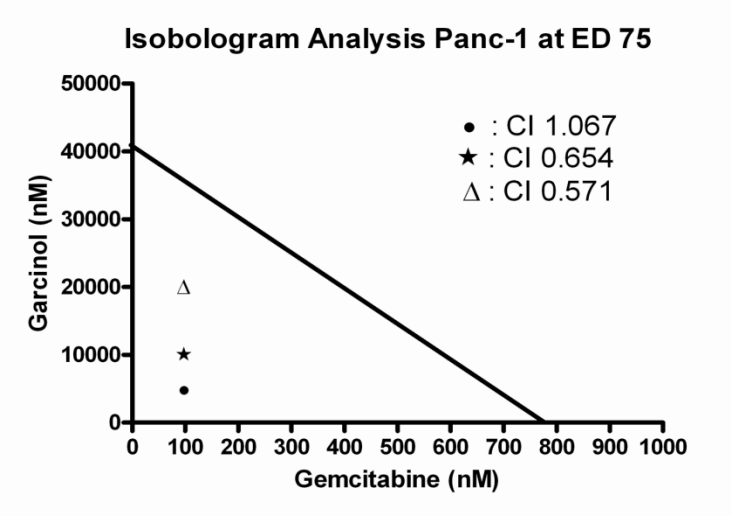


Figure 34: Cytotoxic synergy of garcinol and gemcitabine in Panc-1 cells

A: Combination effect of curcumin and garcinol on Panc-1 cell viability was determined using MTS assay. Combinatorial treatment significantly reduced cell viability more effectively than monotherapy on 48hr treatment. ** $p < 0.05$, ** $p < 0.01$* relative to control. **B:** Isobologram analysis to evaluate the extent of synergism on combining curcumin and garcinol for therapeutic effect.

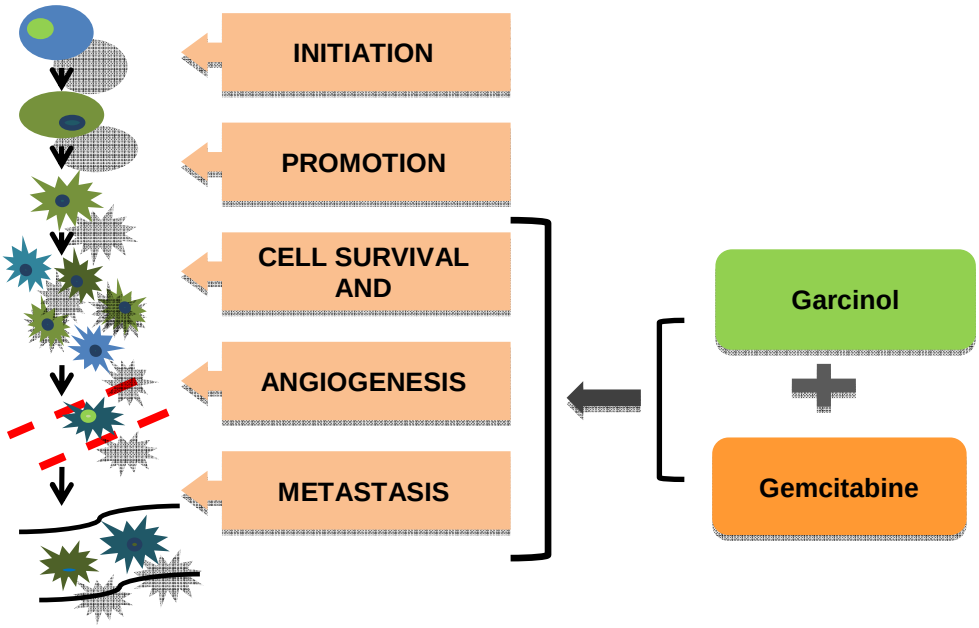


Figure 35: Summary of specific aim 2

Carcinogenesis is a multi-step process. Normal cells exhibit a balance between cell division and cell death, when this equilibrium is disrupted, cells lose their ability to undergo apoptosis and continue to proliferate and grow. They start to invade and colonize in areas normally reserved for other cells. We observed the efficacy of garcinol in combination with gemcitabine to target molecules regulating multiple pathways in carcinogenesis. Dietary interactions of garcinol and curcumin proved to be synergistic in inhibiting cell viability in PaCa cells. The effect of this combination on other mechanistic pathways needs to be explored further.

Specific Aim 3: To identify the alterations in microRNA expression profile in PaCa cells on treatment with garcinol and/or gemcitabine.

MicroRNAs regulate gene expression at transriptional levels targeting multiple genes involved in carcinogenesis. The current aim was conducted to test the hypothesis that garcinol individually or in combination with gemcitabine will alter the miRNA expression in PaCa cells, downregulating oncogenes and upregulating apoptotic and tumor suppressor genes. The resuts obtained are as discussed below.

Garcinol induces changes in miRNA expression profile on treatment- To evaluate the alterations in miRNA expression induced by garcinol, microRNA microarray analysis was done on 10µM garcinol treated Panc-1 cells and miRNA enrichment was done from total RNA. Using one way ANOVA, signal intensities between the two groups were analyzed for statistical significance. Comparison of signal intensity between Group 1 (untreated control cells) vs. Group 2 (10µM garcinol treated cells) was made. Log2 (G2/G1) represents the fold change in specific miRNA upon treatment (Table 8). On treatment with garcinol a downregulation in expression of miRNA – 21 (0.56 fold), - 495 (1.14 fold), -494 (0.97 fold), -1977 (0.80 fold) was observed as compared to untreated control implying their oncogenic role in PaCa. An upregulation in expression of miR – 453 (4.69 fold), - 128 (1.82 fold), - 1280 (0.46 fold) – 720 (0.43 fold) compared to control suggest tumor suppressor activity of these miRNAs. During analysis, a systematic dye bias was found on probes for miR - 377 and was suggested to be excluded from further investigation.

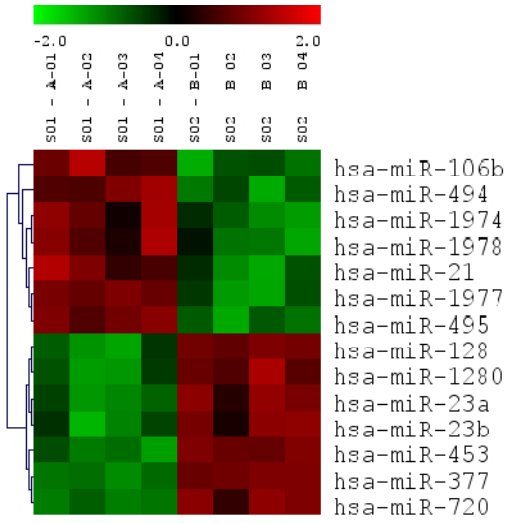


Figure 36: Heat map of alterations in miRNA due to Control vs. Garcinol treatment

Panc-1 cells were treated with 10µM garcinol and the miRNA profile was compared to untreated control cells ($p < 0.01$). Those miRNAs that were different between the two groups have been listed in table 8.

Table 8: Alterations in miRNA profile in Control vs. Garcinol treatment

			Group 1	Group 2	
			A	B	Log2 (G2/G1)
No.	Reporter Name	p-value	Mean	Mean	
499	hsa-miR-377	1.77E-06	20	1,986	6.64
371	hsa-miR-23a	8.20E-04	3,183	4,233	0.41
126	hsa-miR-1280	9.01E-04	5,772	7,922	0.46
824	hsa-miR-720	2.09E-03	1,824	2,463	0.43
329	hsa-miR-21	2.93E-03	3,857	2,609	-0.56
292	hsa-miR-1974	3.48E-03	3,919	3,113	-0.33
373	hsa-miR-23b	3.58E-03	1,266	1,490	0.24
295	hsa-miR-1977	4.00E-03	906	519	-0.80
Following transcripts are statistically significant but have low signals (signal < 500)					
568	hsa-miR-495	1.45E-04	218	99	-1.14
543	hsa-miR-453	2.30E-04	8	199	4.69
567	hsa-miR-494	6.14E-04	94	48	-0.97
31	hsa-miR-106b	1.16E-03	89	59	-0.59
125	hsa-miR-128	3.27E-03	115	405	1.82
296	hsa-miR-1978	5.17E-03	187	122	-0.61

Signal intensity comparisons between Group 1 (untreated control cells) vs. Group 2 (100nM gemcitabine treated cells) was made. On treatment with gemcitabine a downregulation in expression of miRNA – 605 (1.46 fold) was observed as compared to untreated control implying their oncogenic role in PaCa.

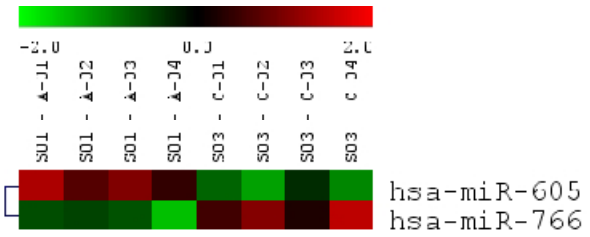


Figure 37: Heat map of alterations in miRNA due to Control vs. Gemcitabine treatment

Panc-1 cells were treated with 100nM gemcitabine and the miRNA profile was compared to that of untreated control cells ($p < 0.01$). Signifiant changes between the two groups have been listed in Table 9.

Table 9: Alterations in miRNA profile in Control vs. Gemcitabine treatment

			Group 1	Group 2	
			A	C	Log2 (G2/G1)
No.	Reporter Name	p-value	Mean	Mean	
Following transcripts are statistically significant but have low signals (signal < 500)					
741	hsa-miR-605	1.90E-03	69	25	-1.46
834	hsa-miR-766	6.11E-03	36	64	0.84

To evaluate the alterations in miRNA expression induced by combination treatment of 10µM garcinol and 100nM gemcitabine, microRNA microarray analysis was done on combination treated Panc-1 cells and miRNA enrichment was done from total RNA. A comparative analysis (Table 10) of signal intensities between Group 1 (untreated control cells) vs. Group 2 (combination treated cells) revealed a downregulation in expression of miRNA – 196a (0.82 fold), - 495 (1.26 fold), -1914 (1.85 fold), -605 (2.42 fold) and -483-3p (1.06) as compared to untreated control implying their oncogenic role in PaCa. An upregulation in expression of miR – 638 (0.65 fold), - 720 (0.41 fold), - 453(4.78 fold) and – 663 (0.92 fold) compared to control suggest tumor suppressor activity of these miRNAs. During analysis, a systematic dye bias was found on probes for miR - 377 and was suggested to be excluded from further investigation.

Based on the fold change in miRNA expression between the two groups (Figure 39), we extended our analysis on six miRNAs miR - 21, -196a, - 495, - 453, - 638 and – 605.

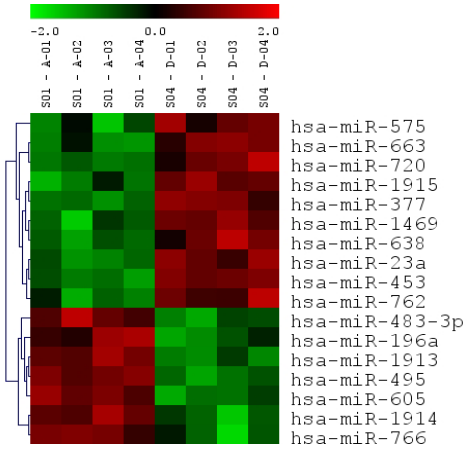


Figure 38: Heat map of miRNA due to Control vs. Combination treatment

Panc-1 cells were treated with combination of 100nM gemcitabine and 10µM garcinol. The miRNA profile was compared to that of untreated control cells. ($p < 0.01$). Signifiant changes between the two groups have been listed below.

Table 10: Alterations in miRNA profile in Control vs. Combination treatment

			Group 1	Group 2	
			A	D	Log2 (G2/G1)
No.	Reporter Name	p-value	Mean	Mean	
371	hsa-miR-23a	3.99E-04	3,183	4,476	0.49
499	hsa-miR-377	5.87E-04	20	633	4.99
780	hsa-miR-638	5.14E-03	758	1,189	0.65
285	hsa-miR-196a	5.75E-03	500	284	-0.82
824	hsa-miR-720	7.88E-03	1,824	2,427	0.41
Following transcripts are statistically significant but have low signals (signal < 500)					
568	hsa-miR-495	1.00E-04	218	91	-1.26
543	hsa-miR-453	2.27E-04	8	210	4.78
270	hsa-miR-1913	3.26E-04	29	16	-0.80
741	hsa-miR-605	4.18E-04	69	13	-2.42
548	hsa-miR-483-3p	1.85E-03	27	13	-1.06
271	hsa-miR-1914	1.88E-03	42	12	-1.85
806	hsa-miR-663	2.36E-03	70	131	0.92
273	hsa-miR-1915	3.07E-03	166	305	0.87
195	hsa-miR-1469	3.19E-03	52	121	1.21
831	hsa-miR-762	4.06E-03	70	131	0.90
834	hsa-miR-766	8.52E-03	36	14	-1.34
707	hsa-miR-575	8.99E-03	41	68	0.73

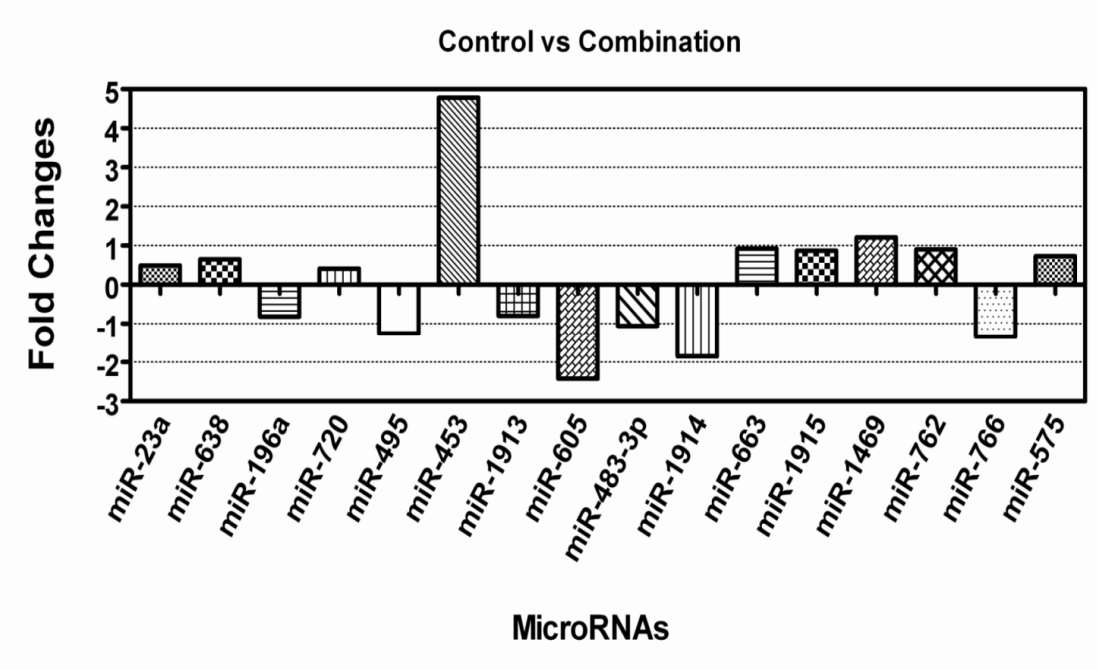


Figure 39: Alterations in miRNAs between Control vs. Combination treatment.

A bar diagram depicting the fold change in miRNA expression profile of untreated control cells and combination treated cells.

Data obtained by microRNA microarray analysis was validated using quantitative Real Time PCR analysis (RT-PCR). 5ng/ reaction tube of total RNA was used for preparation of assay reaction and the experiment was performed as per the manufacturer's instructions. RNU6B was used as a standard and a 2^ (-delta delta Ct) value was evaluated for comparative analysis of specific miRNA under investigation (Figure 40). We further performed a statistical analysis to check for correlation in expression between the two methods (Table 11).

An R^2 value of 0.73, 0.90, 0.81, 0.78, 0.74 and 0.92 was obtained for expression of miR – 196a, - 495, - 21, - 453, - 638 and – 605 respectively suggesting average to good correlation between the two analytical methods. TargetScan 5 software analysis revealed the potential targets for each of these miRNAs from the current database. This database was built using the Wilcoxon test, to identify those miRNAs which are present significantly more often in set of changed genes compared to background of unchanged genes. A *p-value* is calculated signifying that the changed and unchanged sets were produced by the same distribution and the differences between them were due to chance alone. An equivalent negative natural logarithm of the p-value was used as a more intuitive measure for analysis. Table 12- 17 list the potential target pathways, their corresponding genes, statistical significance and KEGG pathway ID for the respective miRNAs.

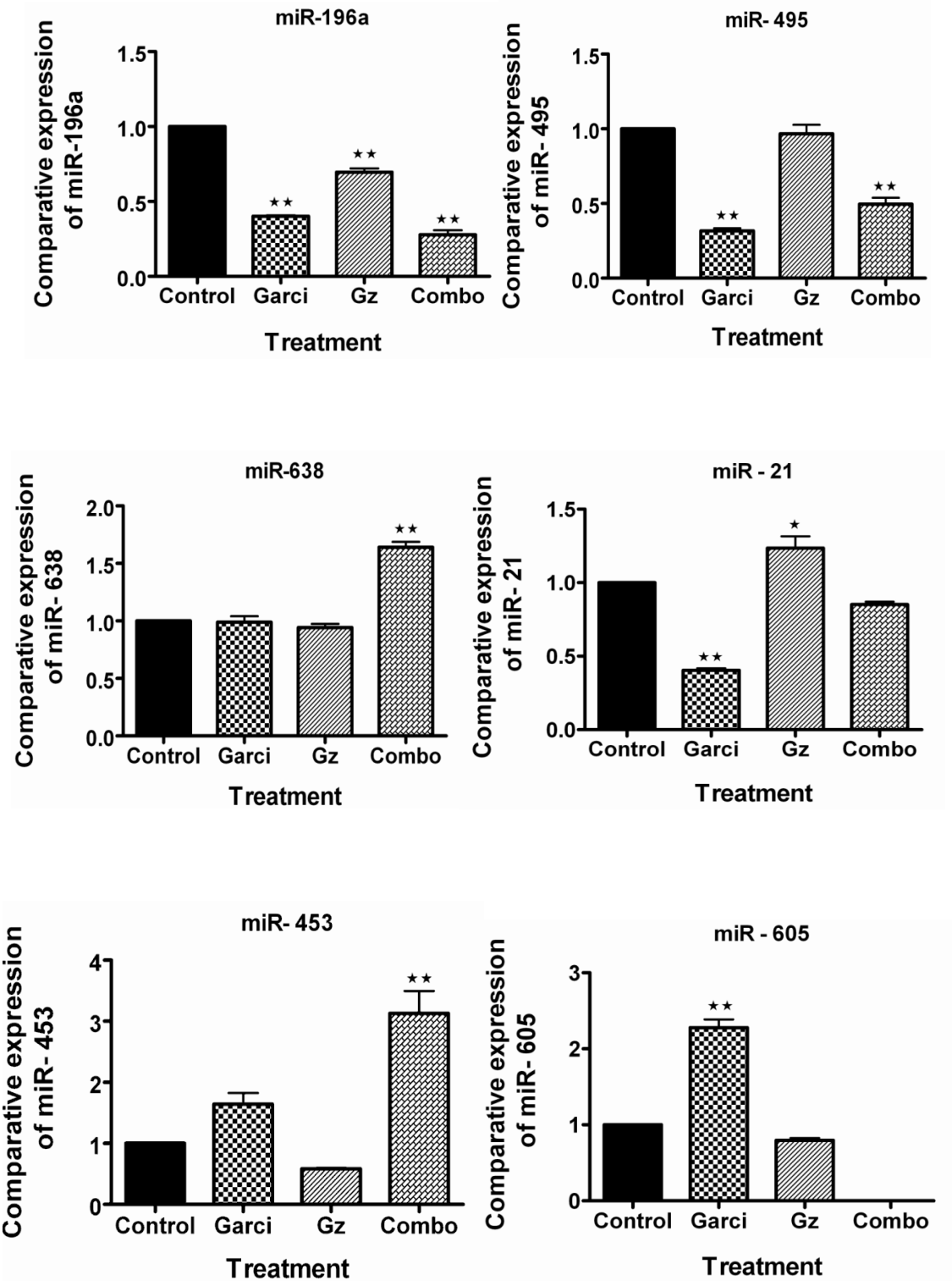


Figure 40: Validation of miRNA profile by Real Time RT-PCR analysis

Comparative expression of miRNA levels in Panc-1 cells was detected using microarray profiling and validated using Taqman RT-PCR method. * $p < 0.05$, ** $p<0.01$ relative to control. R^2 values are listed in Table 11.

Table 11: Correlation coefficient for qRT-PCR and microarray analysis

miRNA	R^2 value
hsa-miR-196a	0.7308
hsa-miR-495	0.9008
hsa-miR-21	0.8182
hsa-miR-453	0.7760
hsa-miR-638	0.7361
hsa-miR-605	0.9165

Table 12: Signaling pathways targeted by hsa-miR-21

Targeted Pathway	Gene Name	-ln (p-value)	KEGG Pathway ID
Cytokine-cytokine receptor interaction	*CCL1, CNTFR, LIFR, CCL22, CCL20, FASLG, CCR7, ACVR2A, IL12A, TGFBR2, BMPR2*	9.77	hsa04060
TGF-beta signaling pathway	*SMAD7, ACVR2A, TGFBR2, ACVR1C, BMPR2, PITX2*	9.5	hsa04350
MAPK signaling pathway	*MAP2K3, NTF3, MAP3K1, FASLG, FGF1, DUSP8, PPP3CA, TGFBR2, ACVR1C, RPS6KA3*	7.73	hsa04010
Pancreatic cancer	*CDK6, PIK3R1, TGFBR2, ACVR1C*	4.54	hsa05212
Jak-STAT signaling pathway	*CNTFR, LIFR, PIK3R1, IL12A, SPRY1, SPRY2*	4.39	hsa04630
Apoptosis	*BCL2, FASLG, PIK3R1, PPP3CA*	3.82	hsa04210
VEGF signaling pathway	*PIK3R1, PPP3CA, NFAT5*	2.23	hsa04370
Notch signaling pathway	*JAG1, RBPJ*	1.62	hsa04330
Focal adhesion	*BCL2, VCL, ITGB8, PIK3R1, COL4A1*	1.56	hsa04510
mTOR signaling pathway	*PIK3R1, RPS6KA3*	1.25	hsa04150

Ref: DNA Intelligent Analysis Database.

Table 13: Signaling pathways targeted by hsa-miR-196a

Targeted Pathway	Gene Name	-ln (p-value)	KEGG Pathway ID
ECM-receptor interaction	*COL1A1, COL3A1, COL5A2, COL1A2*	10.59	hsa04512
Focal adhesion	*PDGFRA, COL1A1, COL3A1, COL5A2, COL1A2, CCND2*	9.73	hsa04510
Cell Communication	*COL1A1, COL3A1, COL5A2, COL1A2*	5.59	hsa01430
Jak-STAT signaling pathway	*SOCS4, OSMR, CCND2, RNF31*	4.83	hsa04630
Gap junction	*CSDE1, PDGFRA, ADCY9*	4.35	hsa04540
MAPK signaling pathway	*MAP4K3, CSDE1, PDGFRA, MAX*	2.19	hsa04010
mTOR signaling pathway	*TSC1, IGF1*	2.11	hsa04150
ErbB signaling pathway	*CDKN1B, CSDE1*	1.59	hsa04012
TGF-beta signaling pathway	*CHRD, SMAD6*	1.53	hsa04350
Regulation of actin cytoskeleton	*CSDE1, PDGFRA, PPP1R12B*	1.28	hsa04810
Cell cycle	*CDKN1B, CCND2*	1.05	hsa04110

Ref: DNA Intelligent Analysis Database.

Table 14: Signaling pathways targeted by hsa-miR- 495

Targeted Pathway	Gene Name	-ln (p-value)	KEGG Pathway ID
Insulin signaling pathway	*PPARGC1A, IRS2, PRKY, KRAS, RHEB, CBL, PIK3R1, SOS1, PRKX, IRS1, PRKCI, RHOQ, PPP1CC, ELK1, PRKAB2, MAPK10, TRIP10, SHC4, PRKACB, PIK3R3, AKT3, RAF1, EIF4E*	14.81	hsa04910
Wnt signaling pathway	*PRKY, FZD5, TBL1X, DKK1, CAMK2G, PRKX, APC, CREBBP, SIAH1, FRAT2, PPP3CA, NLK, CCND2, NFAT5, TCF7L1, MAPK10, PRKACB, JUN, FZD6, CTBP2, FZD10, PPP3CB*	11.7	hsa04310
MAPK signaling pathway	*MAP4K4, HSPA2, PDGFRA, TGFBR1, MAP3K1, KRAS, RRAS2, STK4, SOS1, FGF2, CDC42, RAP1B, NLK, NF1, FGF5, PTPRR, ELK1, EGF, TGFB2, MAP3K5, MAPK10, JUN, AKT3, RAF1,*	11.08	hsa04010
Pancreatic cancer	*TGFBR1, KRAS, CDK6, PIK3R1, CDC42, ACVR1B, JAK1, EGF, TGFB2, MAPK10, PIK3R3, AKT3, RAF1*	8.19	hsa05212
VEGF signaling pathway	*SH2D2A, KRAS, PIK3R1, KDR, CDC42, PPP3CA, PTK2, NFAT5, PIK3R3, AKT3, RAF1, PPP3CB*	7.03	hsa04370
mTOR signaling pathway	*RHEB, PIK3R1, IGF1, PIK3R3, AKT3, EIF4E*	7	hsa04150
Cell cycle	*DBF4, CDKN2C, YWHAB, SMC1A, ANAPC1, CDK6, HDAC2, CDC25C, CREBBP, TGFB2*	5.1	hsa04110

Ref: DNA Intelligent Analysis Database.

Table 15: Signaling pathways targeted by hsa-miR- 453

Targeted Pathway	Gene Name	-ln (p-value)	KEGG Pathway ID
ErbB signaling pathway	*PAK2, PIK3R3, CAMK2B*	9.14	hsa04012
T cell receptor signaling pathway	*NFKBIB, PAK2, PIK3R3*	8.91	hsa04660
B cell receptor signaling pathway	*NFKBIB, PIK3R3*	5.08	hsa04662
Phenylalanine, tyrosine and tryptophan biosynthesis	*GOT2*	4.5	hsa00400
Regulation of actin cytoskeleton	*PAK2, SLC9A1, PIK3R3*	3.28	hsa04810
One carbon pool by folate	*SHMT1*	2.79	hsa00670
Wnt signaling pathway	*PRICKLE2, CAMK2B*	1.74	hsa04310
Focal adhesion	*PAK2, PIK3R3*	1.13	hsa04510

Ref: DNA Intelligent Analysis Database.

Table 16: Signaling pathways targeted by hsa-miR- 638

Targeted Pathway	Gene Name	-ln(p-value)	KEGG Pathway ID
Adherens junction	*IGF1R, NLK, ACTN4, ACVR1C, SMAD4*	10.21	hsa04520
Wnt signaling pathway	*CSNK1A1L, NLK, CACYBP, FZD4, NFAT5, SMAD4*	6.27	hsa04310
Pancreatic cancer	*NFKB1, KRAS, CDK6, SMAD4*	6.05	hsa05212
Hedgehog signaling pathway	*GLI3, CSNK1A1L, PTCH1*	4.13	hsa04340
MAPK signaling pathway	*RPS6KA2, NFKB1, KRAS, BDNF, NR4A1, NLK, ACVR1C*	3.87	hsa04010
ErbB signaling pathway	*KRAS, CBL, ABL2*	2.08	hsa04012
Cell cycle	*CDC27, SMC1A, SMAD4*	1.32	hsa04110

Ref: DNA Intelligent Analysis Database.

Table 17: Signaling pathways targeted by hsa-miR- 605

Targeted Pathway	Gene Name	-ln(p-value)	KEGG Pathway ID
ABC transporters - General	*ABCC9*	7	hsa02010
Glycolysis / Gluconeogenesis	*PGK1*	2.6	hsa00010
Purine metabolism	*AK2*	2.36	hsa00230
ErbB signaling pathway	*PAK2*	1.86	hsa04012
Ubiquitin mediated proteolysis	*BRCA1*	1.25	hsa04120

Ref: DNA Intelligent Analysis Database.

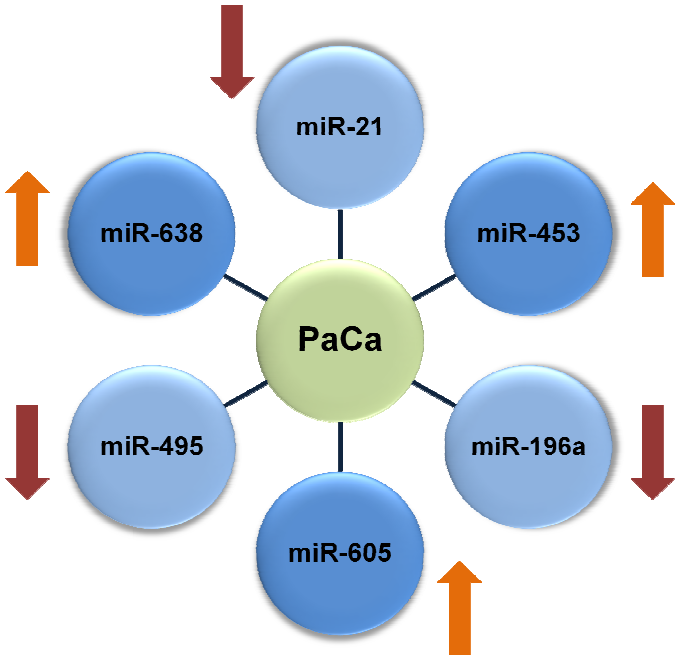


Figure 41: Summary of specific aim 3

MicroRNA is tissue specific in carcinogenesis. The alterations in expression profile of six miRNAs on exposure to garcinol and/or gemcitabine was evaluated. MiRNA-21, -495, - 196a are oncogenic miRNA. The signal intensity reduced significantly on treatment with garcinol or gemcitabine as a single agent or in combination. MiR-453 expressions increased only on treatment with garcinol alone or in combination with gemcitabine, suggesting garcinol specific effect. MiR-638, a tumor suppressor, is often deregulated in gastric cancers. A significant up regulation in miR-638 expression was noted on treatment with garcinol and/or gemcitabine. The role of miR-605 is not very clear. Garcinol treatment increased the signal intensity to almost double whereas the signal reduced to less than half on treatment with gemcitabine alone or in combination. Further analysis is needed to confirm the expression analysis of this miRNA.

CHAPTER FOUR

DISCUSSION

Despite advancements in modern medicine, pancreatic cancer continues to be a major public health problem in US and many parts of the world with dismal prognosis. According to American Cancer Society and NCI/NIH statistics, 43,140 new cases will be detected in 2010 and 36,800 deaths will be reported due to this disease due to futility of present treatment protocols and strategies in successfully treating this disease. This is in part, due to inability of clinicians to address the highly chemoresistant nature of these neoplasms because of which majority of the patients relapse despite continued therapy. Numerous clinical trials have been tried using various drugs or combinations involving chemo- and radiotherapy/ immunotherapy/ targeted therapy in PaCa, but unfortunately none of these therapeutic alternatives have reasonably increased medium survival of patients diagnosed with PaCa.

Several epidemiological studies, overwhelming data demonstrate an inverse relationship between intake of dietary chemopreventive agents as a promising strategy for reducing human cancer risk. Of late, there has been a great deal of interest in developing chemotherapeutic trials that are based on natural dietary agents. A few previous studies have reported garcinol- the bioactive component derived from Garcinia indica to inhibit tumor growth in animal models [111, 114, 127]. In the present study we investigated putative therapeutic potential of garcinol in PaCa cells. This is the first study reporting the therapeutic properties of garcinol on PaCa encompassing multidirectional aspects - as single agent, combination with other known dietary

chemopreventive agent (curcumin), chemosensitization efficacy with gemcitabine, and its influence on microRNA expression profile. Unfortunately, bioavailability of this compound in either healthy human subjects or rodent models has not yet been explored. However, based on subjective indirect evidence, garcinol is speculated to reach systemic circulation but detail pharmacokinetics and pharmaco-dynamics of this compound needs to be analyzed.

Over the years we have realized that no single agent by itself can eliminate or treat cancer, instead combination of different naturally occurring phytochemicals can afford protection. However, therapeutic efficacy of interactions amongst various dietary agents needs further in-depth analysis. Data thus far, suggests that increased consumption of curcumin isolated from *Curcuma longa* (a.k.a. Turmeric), contributes to lowering risk of cancer in Asian (Indian) populations compared to industrialized western nations. Additionally, based on our limited preliminary findings, garcinol appears to exhibit promising therapeutic efficacy in PaCa cells. Epidemiological studies report that Asian (Indian) populations consume turmeric and kokum as a component of their diet regularly. This lead to our hypothesis that interactions between two bio-active components- turmeric (Curcumin) and kokum (Garcinol) can influence PaCa cell growth and proliferation. Based on this rationale, we evaluated whether the combination of these two bio-active components have any additive, synergistic or antagonistic effect on human pancreatic cancer cells in overcoming intrinsic tumor cell resistance to apoptosis and inhibition of angiogenesis in PaCa cells.

PaCa results from the successive accumulation of gene mutations and is a multi-faceted process. Thus, targeting multiple metabolic pathways involved at different

stages by using combinatorial approaches could either delay or halt progression of disease. Gemcitabine is an FDA approved standard cytotoxic chemotherapeutic agent used for the treatment of PaCa. One reason for the limited success of gemcitabine in treatment of PaCa is due to development of resistance due to over expression of proliferation linked signaling molecules like *EGFR, NF-κB, Akt, STAT*, etc. or anti-apoptotic molecules such as *survivin, Bcl-2, Bcl-X_L XIAP's* etc. and mutations in several oncogenes like *K-ras, p53, p16 and DPC4.* Thus, finding an effective therapeutic strategy for PaCa is the primary need of the situation. An ideal therapy would be the one that would target multiple signaling pathways at low doses and be able to discriminate between normal cells and cancer cells, specifically targeting only the latter. We report feasibility of an alternate approach which was tested and found to be synergistically effective in improving overall therapeutic efficacy.

As reported in literature more than 80% of pancreatic cancer patients present with unresectable, locally advanced or metastatic disease, with dismal prognosis of having not more than 6 months of median survival. Thus, in addition to novel therapeutic modalities and approaches there is also need to investigate therapeutic targets specific for this disease. This would be useful in designing efficient strategies for better survival and monitor clinical outcome in patients. In this context the role of microRNAs in carcinogenesis is emerging and it is now known that microRNAs control gene expression at the post transcriptional levels and are more informative than mRNA expression profiles for pathological evidence. Each miRNA can target approximately 200 genes depending on their complementarity and thus can act as tumor suppressors or oncogenes. Therefore, in this study the microRNA profile of human PaCa cells

following treatment with gemcitabine and garcinol was pursued to gain better molecular insight for future implementation.

In the current study, garcinol significantly inhibited PaCa cell viability and cell proliferation irrespective of their molecular genetic makeup as demonstrated by MTS assay and trypan blue exclusion method, respectively (Figure 11-12). The two PaCa cell lines used were *Panc-1,* which as mentioned earlier is a poorly differentiated PaCa cell line that possesses mutated *K-ras* as well as *p53* mutation, and *BxPC-3* cells which is a moderately differentiated cell line with wild type *K-ras* and point mutated *p53.* BxPC-3 cells were found relatively more resistant to the effect of garcinol as compared to Panc-1 cells (Figure 12). Since Panc-1 cells have been classed under category of 'Gemcitabine resistant' the potential of garcinol in inhibiting cell viability and proliferation in these cells should not be under estimated. Moreover, based on our results garcinol treatment complied to induction of apoptosis and cell cycle arrest in G0-G1 phase (Figure 13-15); thus the ability of garcinol to induce apoptosis and halt cell division by cell arrest makes it a promising candidate for further investigations including phase-I clinical trial.

To elucidate the mechanism involved in apoptosis, levels of various pro- and anti-apoptotic molecules which regulate cell death were analyzed. Among these, caspases are important mediators of apoptosis and are sequentially activated by proteolytic processing of their inactive procaspase forms into two smaller units, which in turn leads to cleavage of key cellular proteins including DNA repair enzyme poly ADP-ribose polymerase (PARP). Caspase 9 belongs to the initiator Caspase tier and plays a crucial role in activating the effector Caspase 3 which ultimately induces apoptosis.

Caspases are known to be induced by various agents including phytochemicals and apoptotic stimuli; therefore we determined the *invitro* activities of caspase-3 and caspase-9 in both cell lines upon treatment. Colorimetric assay data revealed increasing activity of caspase-3 and caspase-9 in PaCa cells following garcinol treatment (Figure 16). This was complimented by the presence of cleaved PARP band confirming apoptosis (Figure 17). In addition, several Inhibitor of Apoptotic Proteins (IAPs) such as XIAP, cIAP (Pan) that negatively regulate apoptosis were also investigated and found to be downregulated in dose responsive manner by garcinol treatment. Both these mechanisms need further analysis**.** The nuclear transcription factor- NF-κB is constitutively active in pancreatic tumors, and contributes to the survival of cells by inhibiting apoptosis, thus playing a central role in pathogenesis, progression, invasion and metastasis in PaCa. Analysis of this transcription factor in PaCa cells revealed its downregulation by garcinol in a dose responsive manner (Figure 17).

NF- κB is believed to be a critical determinant of drug resistance in pancreatic and other cancers and inhibition of this nuclear factor sensitizes PaCa cells to chemotherapy induced apoptosis. NF- κB also targets proteasome inhibitors, as they normally prevent the degradation of IκB, thus blocking nuclear translocation of NFκB. Thus, inhibition of NF- κB by garcinol carries significant consequence in enhancing the sensitivity to cytotoxic drugs. This has been confirmed under another aim of this study.

Angiogenesis, invasion and metastasis are the mechanisms by which cancer cells spread and strengthen their foundation. Pancreatic tumors are highly vascular and produce multiple pro-angiogenic factors and cytokines such as VEGF, MMP-9, and IL-8 which help in invasion and progression of cancer cells. Epidemiological evidence shows

that over expression of VEGF and IL-8 in pancreatic cancer patients correlates with poor survival. Interleukins (IL-8) belongs to family of pro-inflammatory cytokines that assist in the process of invasion and angiogenesis. Mechanisms to block cytokines, exhibit a tumorostatic effect rather than a tumoricidal effect, hence their performance can be enhanced by using them for adjunct therapy with suitable drugs. Hence, we investigated the fate of these angiogenic and metastatic markers on treatment with garcinol. We observed a significant dose dependent reduction in levels of VEGF, MMP-9 and IL-8 (Figure 18). These findings supports the contention that garcinol may be a potent suppressor of tumor neovascularization and may predictively be effective in reducing angiogenesis and other invasive mediators leading to inhibition of tumor progression.

Also, Prostaglandin E_2 (PGE_2),which is the enzymatic product of COX-2 activity is commonly over expressed in pancreatic tumors and thought to be an important mediator of chemoresistance and therefore considered as promising chemotherapeutic target for the treatment of pancreatic cancer. Panc-1 cells have very low levels of COX-2 expression as compared to BxPC-3 cells and thus we determined the effect of garcinol on PGE_2levels in COX-2 overexpressing cell line BxPC-3 only. There was a significant reduction in PGE_2 levels upon treatment with garcinol (Figure 19) It is well known that promoter sequence of COX-2 contains binding sites for NF-κB, which overall forms a very crucial network promoting the survival of tumor cells. Since we noticed a dose dependent decrease in levels of PGE_2 and NF-κB, one may speculate that garcinol treatment would be effective in down regulating COX-2 enzyme by inhibiting NF-κB

activity. This mechanism needs further investigation along with analysis of upstream molecular events leading to downregulation of NF-κB by garcinol.

Based on foregoing discussion and our initial conviction that garcinol may have therapeutic advantage, its effect in combination with another potent chemo dietary agent- curcumin was investigated (Figure 25). Appraisal of combination index values along with apoptosis induction by this combination proved to be synergistic / additive with greater inhibition of cell viability and induction of apoptosis through the upregulation of caspase activity. Other related parameters such as Dm, m and r values, the significance of which have been mentioned earlier, favors advantage of this combination in prospective future studies concerning therapy of PaCa relative to single agent and requires to be tested more rigorously in future.

Another aspect under this study paradigm was to explore whether garcinol could be therapeutically combined with gemcitabine for the treatment of PaCa in addition to conventional therapeutics that have thus far shown limited benefit. Results presented conclude that garcinol is effective as a general inducer of apoptosis in PaCa cells by down-regulating anti-apoptotic proteins and stimulating caspase activity without any adverse effect on normal epithelial cells (NIH 3T3). The combination regimen also severely restricted the growth of clonogenic colonies compared to single therapy regimens. Moreover, the transcription factor NF-κB as described earlier, is intimately involved with *de novo* and *acquired* chemoresistant phenotype of PaCa cells [38, 126, 128]. Numerous studies also support that inactivation of NF-κB leads to sensitization of cancer cells to conventional therapeutics [129-133]. Zhang *et al* recently reported that IKK inhibitor could confer sensitivity to PaCa cells and xenograft tumors by blocking

events upstream of NF-κB pathway and thus in consequence also affect NF-κB downstream genes, that lead to enhanced effect on TNF-α in abrogating growth of tumor cells, activating apoptosis [134]. Our findings clearly demonstrate that garcinol in combination with gemcitabine interferes in synergistic loss of cell viability through the upregulation of apoptosis and downregulating NF-κB activation in both PaCa cell lines. Moreover, it is known that antiapoptotic Bcl-xL, regulated by NF-κB at the transcriptional level also contributes to PaCa chemo-resistance which can be suppressed by NF-κB inhibition. Moreover, VEGF, MMP-9 and PGE_2 levels exhibited greater inhibition in the combination treatment group relative to single agent therapy (Figure 31). PGE_2 being enzymatic product of COX-2 expression is elevated in pancreatic cancer tissues over normal controls, thereby increasing VEGF synthesis via EP_2 receptor. We also report maximum inhibition of IL-8 in combinatorial treatments as compared to individual therapies (Figure 32). We thus conclude that adding garcinol to standard therapy for PaCa might be a viable and promising therapy to control invasion and spread of cancer cells. Although search for the development of alternative gemcitabine schedules and chemotherapy combinations continues, the present findings support that garcinol is a novel agent that profoundly impacts chemosensitization of PaCa.

As discussed earlier, pancreatic ductal adenocarcinoma has the poorest overall prognosis amongst gastrointestinal cancers due to its asymptomatic nature, thus limiting therapeutic options. Gemcitabine remains the standard care for PaCa patients today, however multiple side effects and development of chemoresistance is a major hurdle in developing successful strategy to handle this disease. Thus there is much room for

improvement in all aspects of treatment for PaCa. In this study we aimed to evaluate the potential microRNA targets of garcinol and gemcitabine for PaCa therapy.

The role of microRNAs in carcinogenesis has been recognized not too long ago. In the current study, total RNA isolation was performed on garcinol and/or gemcitabine treated Panc-1 cells. A quality check using the 260/280 ratio was done to determine the quality of isolation. The total RNA samples were enriched for microRNA, labeled and hybridized to a chip and cluster analysis was performed at a separate facility by a technical expert. Only miRNAs with significant signal intensity over background and those that showed a differential intensity between groups were used for further analysis. Untreated control group was compared with either 10μM garcinol or 100nM gemcitabine or the combination treatment. Based on the fold changes in the respective groups, six microRNAs were used for further analysis to identify the target pathways of respective treatments.

As part of the constant efforts that are being made to identify microRNAs specific for PaCa we evaluated the targeted pathways of six microRNAs under the influence of treatment. MiR-21 is involved in inducing gemcitabine resistance in PaCa cells [135]. A downregulation in signal intensity of miR-21 by 32%, 1% and 22% was noted on treatment with garcinol, gemcitabine and combination respectively compared to untreated control. A significant (*p-value: 2.93E-03*) reduction miRNA-21 signal intensity on treatment with garcinol alone as compared to untreated control cells was observed (Figure 36, Table 8). Role of miRNA-21 in chemoresistance was clearly demonstrated on treatment with gemcitabine alone and in combination with garcinol. Using TargetScan5 software analysis, we listed a few common targets for miRNA-21 (Table

12). Among these are, cytokine receptor, TGF-β signaling, MAPK signaling, Jak-STAT signaling, apoptosis, VEGF, Notch, mTOR signaling and Focal adhesion pathways. Recent study provided evidence of positive correlation between miRNA-21 and mRNA expression of angiogenic factors such as VEGF, MMP-9 and others [88]. This information is parallel to our findings. Thus treatment with garcinol can be a potent strategy to inhibit miR-21 and overcome the effect of cytotoxic chemoresistance in PaCa cells.

Another oncogenic miRNA, miR-196a was evaluated and downregulated by garcinol (25%), gemcitabine (33%), and combinatorial (43%) treatment compared to control. A gradual reduction in signal was observed on treatment with single agent; however the combination treatment (*p-value: 5.75E-03*) reduced miR-196a signal intensity by almost half suggesting a potent therapeutic effect on PaCa cells (Figure 38, Table 10).. Molecular targets of miRNA-196a as determined by TargetScan5 software are known to be associated with ECM receptor interactions, focal adhesion pathway, cell communication, Jak-STAT signaling, mTOR, actin cytoskeleton regulation and cell cycle regulation (Table 13). Yekta and colleagues first described miR-196a-directed cleavage of specific homebox genes (*HoxB8, HoxC8, HoxD8 and HoxA7*) in mouse embryos and mammalian cells thus promoting tumor progression by cell detachment and tumor cell dissemination in vitro via activation of *Akt* pathway [136]. A recent study describing the role of miR-196a in colorectal cancer suggested that miRNA was mainly involved in enhanced cell detachment, migration, invasion and chemosensitivity towards platin drugs [137]. Also, studies using miRNA chip analyses in pancreatic cancer found that 75% of tumors expressed high levels of miR-196a [91, 138]. Overall downregulation

of this miRNA can be a promising strategy for therapy, since it influences major pathways of carcinogenesis.

90% of PaCa cases have K-ras mutation [139]**.** The role of miR-495 on treatment with both agents was evaluated in PaCa cells since it is known to be overexpressed (>10 folds) in *K-ras* positive tumors [140]. Signal intensity of miR-495 was downregulated by 55%, 15% and 59% on treatment with garcinol, gemcitabine and combination respectively. Garcinol alone or in combination with gemcitabine showed significant (*p-value: 1.45E-04*) reduction (Figure 36, Table 8).. Target analysis using TargetScan5 software revealed its association with pathways involving Insulin signaling, mTOR, VEGF, cell cycle, Wnt and MAPK molecules (Table 14). Since K-ras mutation is commonly associated with PaCa, targeting miR-495 by garcinol alone or in combination would be a key step in designing efficient therapeutics.

Certain microRNAs are known to regulate carcinogenesis as a tumor suppressor. MiRNA-638 was downregulated in gastric cancers suggesting a tumor suppressor activity [141]. We observed an upregulation in signal intensity of miR-638 on treatment as compared to untreated PaCa cells. Garcinol upregulated miR-638 signal intensity by 31%, gemcitabine by 7% and combination by 57% relative to control cells. Our results clearly suggest that garcinol in combination with gemcitabine could induce the tumor suppressor activity of miR-638 more potently (*p-value: 5.14E-03*) than either agent alone (Figure 38, Table 10). Also, gemcitabine by itself was not very efficient in upregulating miR-638 levels, but a synergistic effect on combining with garcinol is possible as suggested by the signal intensity values. Known targets of miR-638 are molecules of glucose metabolism, EGFR pathway, purine metabolism and ubiquitin

mediated proteolytic activities (Table 16). It would be interesting to explore the exact mechanism by which garcinol mediates the induction of this miRNA.

The exact function of miRNA – 605 and – 453 in gastrointestinal cancers is not well documented. However, on treatment with garcinol ~1.81 fold increase in miR-605 signal intensity was observed as compared to ~1.46 fold (*p-value: 1.90E-03*) and ~2.42 fold (*p-value: 4.18E-04*) downregulation on treatment with gemcitabine and combination respectively (Figure 37, Table 9). This result suggests the possibility of miR-605 being a tumor suppressor due to its upregulation on treatment with a natural agent as compared to downregulation by a chemotherapeutic drug. This miRNA is associated with Wnt signaling, MAPK signaling, EGFR pathway, Hedgehog signaling and cell cycle regulation (Table 17). So far in literature, miR-605 is thought to be a non-conserved miRNA, influencing the mRNA expression through its 5'UTR region which is specific to the 3' end of miRNA [142]. However its exact role in cancer is not very clear.

In the current study expression of miR-453 was observed to increase (*p-value: 2.27E-04*) by several folds (~25-26 fold) on treatment with garcinol alone and in combination with gemcitabine but there was no change observed on treatment with gemcitabine alone (Figure 36, Table 8)... Target analysis revealed that miRNA-453 targeted several pathways such as EGFR signaling, amino acid biosynthesis, regulation of actin cytoskeleton and Wnt signaling (Table 15). The role of miR-453 is not known in PaCa but a recent report has documented the role of miR-453 in breast carcinogenesis [143].

In order to validate the data obtained by microarray profiling, real time-reverse transcription PCR (q-RT-PCR) was done for these six miRNAs. Total RNAs from the

same preparation used for microarray analysis were reverse transcribed and the results obtained were comparable (R^2 value 0.73 - 0.92, Table 11) with and followed the microarray analysis closely. Together, these results provide sufficient evidence supporting the superior antitumor activity of the combination treatment garcinol with gemcitabine in pancreatic cancer cells. The functional roles of all miRNAs are not completely understood, but may act as oncogenes and/or tumor suppressors regulating cellular differentiation, proliferation and apoptosis. Computational algorithms indicate that one miRNA may regulate hundreds of genes at different levels. More importantly, from the point of personalized medicine and cancer therapy miRNAs may prove to be useful diagnostic and prognostic markers for cancer treatment.

Overall, with the use of preclinical model system (the PaCa cell lines – BxPC-3 and Panc-1) that recapitulates the complexity of this disease, we successfully established new priorities and strategies for development of new treatments. The genetic complexity of PaCa indicates that it is a heterogeneous cancer and that methods to individualize therapy will be required. These are findings reported in *in vitro* models and still need to be evaluated at preclinical and clinical levels.

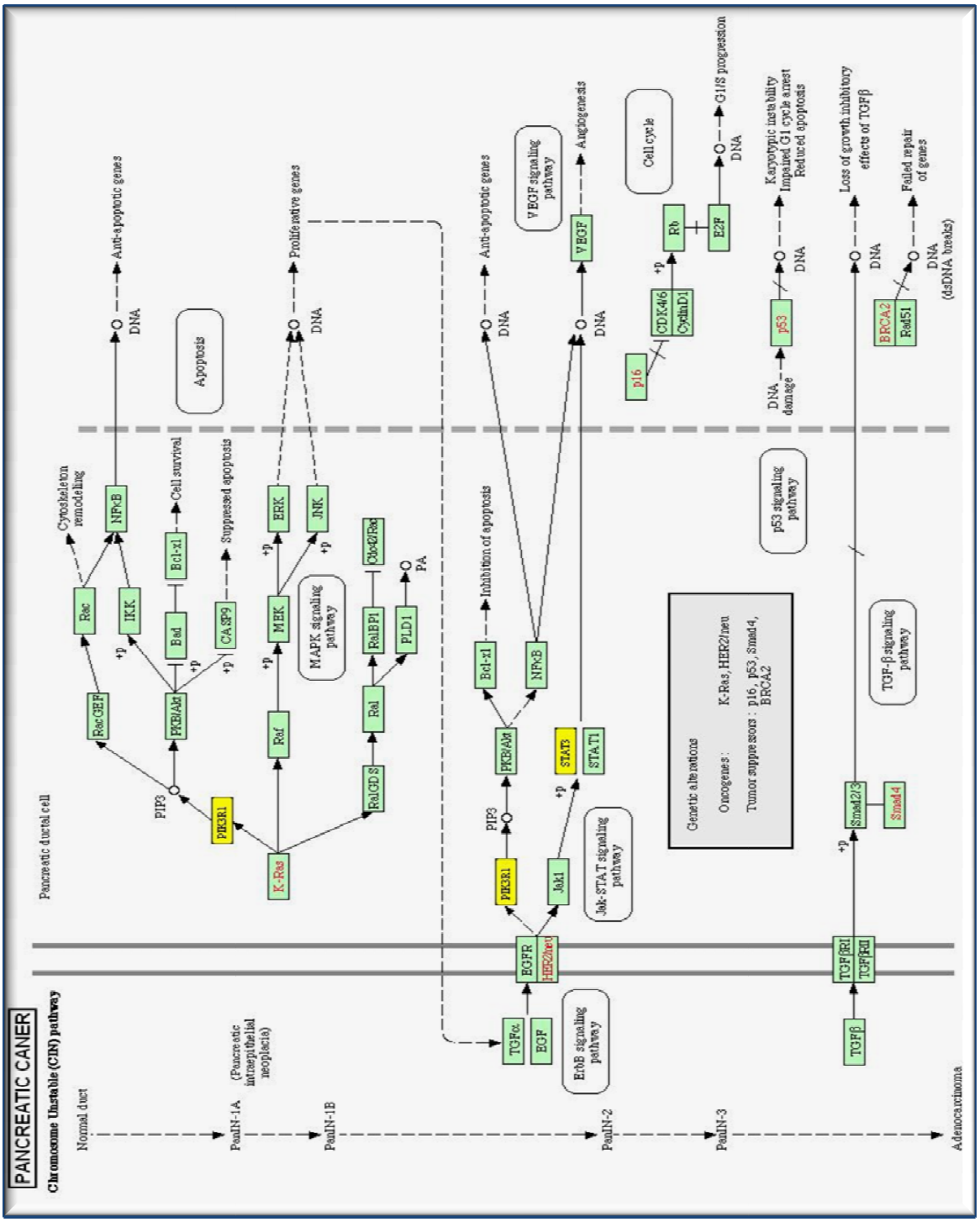


Figure 42: Pancreatic cancer signaling mechanism

Ref: KEGG Pathway

Appendix: Alterations in microRNAs measured by microarray analysis

		Control	Garcinol	Gemcitabine	Combination
No.	**Reporter Name**	**Avg Signal**	**Avg Signal**	**Avg Signal**	**Avg Signal**
1	hsa-let-7a	2,338	2,038	2,030	1,995
2	hsa-let-7a*	8	5	8	12
3	hsa-let-7a-2*	10	18	11	18
4	hsa-let-7b	335	339	338	347
5	hsa-let-7b*	20	16	18	14
6	hsa-let-7c	467	495	485	422
7	hsa-let-7c*	11	13	13	13
8	hsa-let-7d	653	550	435	388
9	hsa-let-7d*	13	19	17	16
10	hsa-let-7e	415	320	345	269
11	hsa-let-7e*	11	14	15	5
12	hsa-let-7f	955	864	622	572
13	hsa-let-7f-1*	21	18	29	12
14	hsa-let-7f-2*	9	11	9	13
15	hsa-let-7g	192	186	165	151
16	hsa-let-7g*	7	20	5	14
17	hsa-let-7i	164	180	155	170
18	hsa-let-7i*	6	9	9	9
19	hsa-miR-1	7	13	7	12
20	hsa-miR-100	248	202	260	218
21	hsa-miR-100*	9	13	5	13
22	hsa-miR-101	9	14	6	12
23	hsa-miR-101*	7	8	7	8
24	hsa-miR-103	129	92	124	102
25	hsa-miR-103-2*	13	9	12	13
26	hsa-miR-103-as	10	9	12	18
27	hsa-miR-105	14	13	14	12
28	hsa-miR-105*	6	9	9	16
29	hsa-miR-106a	68	55	74	66
30	hsa-miR-106a*	3	12	7	18
31	hsa-miR-106b	89	59	108	80
32	hsa-miR-106b*	7	22	10	19
33	hsa-miR-107	94	92	104	90
34	hsa-miR-10a	389	335	320	262
35	hsa-miR-10a*	5	17	14	14
36	hsa-miR-10b	117	112	99	98
37	hsa-miR-10b*	7	10	16	11
38	hsa-miR-1178	4	10	11	12
39	hsa-miR-1179	7	7	6	10

40	hsa-miR-1180	27	27	21	26
41	hsa-miR-1181	30	34	37	20
42	hsa-miR-1182	8	7	8	6
43	hsa-miR-1183	12	10	8	11
44	hsa-miR-1184	4	23	2	18
45	hsa-miR-1185	10	10	10	10
46	hsa-miR-1197	9	11	5	5
47	hsa-miR-1200	5	11	6	24
48	hsa-miR-1201	21	24	21	27
49	hsa-miR-1202	4	10	4	22
50	hsa-miR-1203	6	2	6	14
51	hsa-miR-1204	9	13	5	11
52	hsa-miR-1205	9	11	8	11
53	hsa-miR-1206	7	9	7	14
54	hsa-miR-1207-3p	7	19	3	14
55	hsa-miR-1207-5p	14	16	14	22
56	hsa-miR-1208	11	13	11	24
57	hsa-miR-122	12	13	17	19
58	hsa-miR-122*	5	12	9	11
59	hsa-miR-1224-3p	18	26	31	3
60	hsa-miR-1224-5p	30	15	34	25
61	hsa-miR-1225-3p	13	14	19	12
62	hsa-miR-1225-5p	6	12	9	14
63	hsa-miR-1226	10	20	23	21
64	hsa-miR-1226*	7	11	9	13
65	hsa-miR-1227	15	20	26	13
66	hsa-miR-1228	20	34	18	14
67	hsa-miR-1228*	14	19	9	20
68	hsa-miR-1229	31	38	56	14
69	hsa-miR-1231	18	17	12	22
70	hsa-miR-1233	19	20	18	12
71	hsa-miR-1234	36	33	36	16
72	hsa-miR-1236	17	17	13	7
73	hsa-miR-1237	36	26	35	15
74	hsa-miR-1238	32	19	24	15
75	hsa-miR-124	16	55	10	38
76	hsa-miR-124*	10	6	13	12
77	hsa-miR-1243	7	13	9	3
78	hsa-miR-1244	17	8	18	17
79	hsa-miR-1245	10	7	12	5
80	hsa-miR-1246	32	15	19	27
81	hsa-miR-1247	21	36	33	12
82	hsa-miR-1248	4	11	8	13
83	hsa-miR-1249	40	34	53	17
84	hsa-miR-1250	10	12	11	10
85	hsa-miR-1251	7	6	4	7

86	hsa-miR-1252	10	7	8	12
87	hsa-miR-1253	13	5	9	5
88	hsa-miR-1254	9	7	11	14
89	hsa-miR-1255a	11	8	11	9
90	hsa-miR-1255b	4	14	6	4
91	hsa-miR-1256	12	11	15	9
92	hsa-miR-1257	14	12	16	13
93	hsa-miR-1258	10	8	16	7
94	hsa-miR-1259	10	8	27	14
95	hsa-miR-125a-3p	13	10	14	16
96	hsa-miR-125a-5p	263	206	220	189
97	hsa-miR-125b	776	772	877	858
98	hsa-miR-125b-1*	11	10	13	11
99	hsa-miR-125b-2*	9	9	15	8
100	hsa-miR-126	34	35	45	31
101	hsa-miR-126*	7	8	7	7
102	hsa-miR-1260	74	66	60	69
103	hsa-miR-1261	7	3	9	10
104	hsa-miR-1262	7	5	11	8
105	hsa-miR-1263	7	17	10	9
106	hsa-miR-1264	4	12	10	10
107	hsa-miR-1265	7	4	7	17
108	hsa-miR-1266	6	8	4	15
109	hsa-miR-1267	10	19	7	8
110	hsa-miR-1268	56	88	90	139
111	hsa-miR-1269	7	11	3	9
112	hsa-miR-1270	8	4	8	8
113	hsa-miR-1271	9	10	4	9
114	hsa-miR-1272	10	8	11	6
115	hsa-miR-1273	9	10	10	7
116	hsa-miR-127-3p	112	104	79	131
117	hsa-miR-1274a	64	68	59	65
118	hsa-miR-1274b	39	51	37	60
119	hsa-miR-1275	149	182	172	210
120	hsa-miR-127-5p	11	8	10	15
121	hsa-miR-1276	11	17	10	14
122	hsa-miR-1277	21	33	20	25
123	hsa-miR-1278	10	5	11	11
124	hsa-miR-1279	8	10	9	15
125	hsa-miR-128	115	405	128	170
126	hsa-miR-1280	5,772	7,922	5,321	6,597
127	hsa-miR-1281	39	59	61	46
128	hsa-miR-1282	11	7	14	6
129	hsa-miR-1283	6	7	8	15
130	hsa-miR-1284	1	11	8	21
131	hsa-miR-1285	9	7	9	13

132	hsa-miR-1286	3	9	4	18
133	hsa-miR-1287	9	13	9	14
134	hsa-miR-1288	7	13	13	16
135	hsa-miR-1289	12	17	60	75
136	hsa-miR-129*	14	16	28	10
137	hsa-miR-1290	6	10	12	19
138	hsa-miR-1291	5	12	4	17
139	hsa-miR-1292	9	6	9	18
140	hsa-miR-1293	9	11	10	16
141	hsa-miR-129-3p	16	29	23	8
142	hsa-miR-1294	12	8	200	338
143	hsa-miR-1295	4	13	8	23
144	hsa-miR-129-5p	14	18	9	21
145	hsa-miR-1296	6	13	4	13
146	hsa-miR-1297	6	17	7	14
147	hsa-miR-1298	8	12	6	14
148	hsa-miR-1299	12	10	8	13
149	hsa-miR-1301	8	8	6	16
150	hsa-miR-1302	6	4	9	7
151	hsa-miR-1303	12	9	18	14
152	hsa-miR-1304	10	8	8	14
153	hsa-miR-1305	17	25	12	26
154	hsa-miR-1306	8	12	8	11
155	hsa-miR-1307	20	26	31	35
156	hsa-miR-1308	304	394	273	328
157	hsa-miR-130a	12	12	17	21
158	hsa-miR-130a*	8	12	12	13
159	hsa-miR-130b	13	8	13	18
160	hsa-miR-130b*	7	9	11	13
161	hsa-miR-132	24	64	49	59
162	hsa-miR-132*	6	6	6	18
163	hsa-miR-1321	3	10	5	23
164	hsa-miR-1322	8	7	10	8
165	hsa-miR-1323	12	8	15	13
166	hsa-miR-1324	7	7	4	13
167	hsa-miR-133a	16	19	21	14
168	hsa-miR-133b	17	14	22	11
169	hsa-miR-134	68	45	74	61
170	hsa-miR-135a	12	6	9	8
171	hsa-miR-135a*	11	15	7	13
172	hsa-miR-135b	10	5	9	4
173	hsa-miR-135b*	13	5	8	11
174	hsa-miR-136	9	9	11	12
175	hsa-miR-136*	12	12	6	14
176	hsa-miR-137	13	14	11	13
177	hsa-miR-138	15	16	14	15

178	hsa-miR-138-1*	9	18	6	14
179	hsa-miR-138-2*	6	10	9	12
180	hsa-miR-139-3p	8	9	3	15
181	hsa-miR-139-5p	11	11	7	13
182	hsa-miR-140-3p	7	21	2	29
183	hsa-miR-140-5p	6	13	5	8
184	hsa-miR-141	10	6	6	7
185	hsa-miR-141*	8	9	5	10
186	hsa-miR-142-3p	9	4	12	8
187	hsa-miR-142-5p	9	6	8	12
188	hsa-miR-143	14	13	10	13
189	hsa-miR-143*	9	7	9	5
190	hsa-miR-144	7	9	9	9
191	hsa-miR-144*	7	8	10	5
192	hsa-miR-145	11	16	13	10
193	hsa-miR-145*	10	10	13	8
194	hsa-miR-1468	7	10	8	9
195	hsa-miR-1469	52	61	66	123
196	hsa-miR-146a	415	402	410	394
197	hsa-miR-146a*	8	14	11	11
198	hsa-miR-146b-3p	6	10	10	11
199	hsa-miR-146b-5p	12	11	16	14
200	hsa-miR-147	12	8	28	13
201	hsa-miR-1470	26	31	29	12
202	hsa-miR-1471	7	13	8	16
203	hsa-miR-147b	10	12	11	15
204	hsa-miR-148a	9	10	11	7
205	hsa-miR-148a*	10	9	16	10
206	hsa-miR-148b	26	21	21	22
207	hsa-miR-148b*	12	6	18	5
208	hsa-miR-149	13	16	27	14
209	hsa-miR-149*	103	160	68	143
210	hsa-miR-150	14	12	31	11
211	hsa-miR-150*	11	14	14	19
212	hsa-miR-151-3p	95	111	76	98
213	hsa-miR-151-5p	502	511	563	596
214	hsa-miR-152	18	15	12	20
215	hsa-miR-153	10	4	8	14
216	hsa-miR-1537	10	10	9	9
217	hsa-miR-1538	7	11	6	12
218	hsa-miR-1539	7	13	3	10
219	hsa-miR-154	18	11	14	13
220	hsa-miR-154*	16	13	17	11
221	hsa-miR-155	39	44	46	54
222	hsa-miR-155*	7	17	7	12
223	hsa-miR-15a	14	12	13	12

224	hsa-miR-15a*	6	13	5	12
225	hsa-miR-15b	1,107	932	1,168	930
226	hsa-miR-15b*	10	6	12	10
227	hsa-miR-16	678	567	761	689
228	hsa-miR-16-1*	7	10	8	8
229	hsa-miR-16-2*	14	14	14	17
230	hsa-miR-17	66	65	53	69
231	hsa-miR-17*	3	14	8	5
232	hsa-miR-181a	56	41	58	61
233	hsa-miR-181a*	7	13	12	13
234	hsa-miR-181a-2*	8	10	7	15
235	hsa-miR-181b	20	19	25	22
236	hsa-miR-181c	16	14	19	22
237	hsa-miR-181c*	7	9	6	13
238	hsa-miR-181d	12	11	11	14
239	hsa-miR-182	324	290	327	272
240	hsa-miR-182*	10	9	6	11
241	hsa-miR-1825	27	30	25	19
242	hsa-miR-1826	19,512	17,879	16,472	17,219
243	hsa-miR-1827	5	16	9	17
244	hsa-miR-183	123	109	103	98
245	hsa-miR-183*	9	7	8	11
246	hsa-miR-184	10	9	7	6
247	hsa-miR-185	41	35	28	33
248	hsa-miR-185*	9	8	6	12
249	hsa-miR-186	7	10	8	14
250	hsa-miR-186*	5	14	6	16
251	hsa-miR-187	9	9	7	14
252	hsa-miR-187*	11	15	10	11
253	hsa-miR-188-3p	2	18	5	22
254	hsa-miR-188-5p	15	17	17	26
255	hsa-miR-18a	14	15	18	18
256	hsa-miR-18a*	8	12	8	10
257	hsa-miR-18b	7	9	16	11
258	hsa-miR-18b*	12	21	28	13
259	hsa-miR-190	6	2	11	10
260	hsa-miR-1908	15	19	12	27
261	hsa-miR-1909	4	10	5	8
262	hsa-miR-1909*	10	7	12	10
263	hsa-miR-190b	8	6	11	15
264	hsa-miR-191	1,180	926	1,140	973
265	hsa-miR-191*	16	14	30	13
266	hsa-miR-1910	8	14	2	22
267	hsa-miR-1911	13	14	15	14
268	hsa-miR-1911*	6	8	10	9
269	hsa-miR-1912	9	16	9	6

270	hsa-miR-1913	29	27	47	16
271	hsa-miR-1914	37	23	37	12
272	hsa-miR-1914*	16	12	11	9
273	hsa-miR-1915	166	239	197	305
274	hsa-miR-1915*	7	13	10	13
275	hsa-miR-192	17	17	12	17
276	hsa-miR-192*	8	8	8	7
277	hsa-miR-193a-3p	13	13	13	7
278	hsa-miR-193a-5p	106	91	89	112
279	hsa-miR-193b	14	18	13	7
280	hsa-miR-193b*	23	17	17	19
281	hsa-miR-194	15	16	14	13
282	hsa-miR-194*	11	10	10	10
283	hsa-miR-195	36	23	25	33
284	hsa-miR-195*	23	15	11	9
285	hsa-miR-196a	500	373	336	284
286	hsa-miR-196a*	16	5	6	9
287	hsa-miR-196b	18	12	16	10
288	hsa-miR-196b*	10	10	7	12
289	hsa-miR-197	42	37	64	24
290	hsa-miR-1972	5	14	4	23
291	hsa-miR-1973	8	10	5	16
292	hsa-miR-1974	3,919	3,113	4,272	3,421
293	hsa-miR-1975	2,997	2,808	3,489	2,903
294	hsa-miR-1976	15	27	22	15
295	hsa-miR-1977	906	519	848	612
296	hsa-miR-1978	187	122	169	232
297	hsa-miR-1979	6,640	5,762	6,615	6,208
298	hsa-miR-198	8	10	11	10
299	hsa-miR-199a-3p	8	10	13	9
300	hsa-miR-199a-5p	9	11	13	10
301	hsa-miR-199b-5p	7	14	12	12
302	hsa-miR-19a	11	13	12	12
303	hsa-miR-19a*	11	6	29	13
304	hsa-miR-19b	124	113	102	99
305	hsa-miR-19b-1*	8	20	10	11
306	hsa-miR-19b-2*	11	5	14	11
307	hsa-miR-200a	9	11	10	7
308	hsa-miR-200a*	9	11	10	14
309	hsa-miR-200b	8	14	15	14
310	hsa-miR-200b*	7	11	4	14
311	hsa-miR-200c	11	11	7	7
312	hsa-miR-200c*	13	17	10	12
313	hsa-miR-202	11	12	8	6
314	hsa-miR-202*	6	9	9	6
315	hsa-miR-203	7	12	10	16

316	hsa-miR-204	12	14	10	18
317	hsa-miR-205	13	15	7	12
318	hsa-miR-205*	11	8	12	12
319	hsa-miR-2052	9	11	8	12
320	hsa-miR-2053	9	7	12	10
321	hsa-miR-2054	9	11	6	14
322	hsa-miR-206	10	13	7	13
323	hsa-miR-208a	9	2	11	10
324	hsa-miR-208b	15	10	9	8
325	hsa-miR-20a	89	67	84	90
326	hsa-miR-20a*	12	10	8	14
327	hsa-miR-20b	17	19	20	22
328	hsa-miR-20b*	12	9	10	8
329	hsa-miR-21	3,857	2,609	3,792	3,007
330	hsa-miR-21*	4	17	5	12
331	hsa-miR-210	15	14	15	25
332	hsa-miR-211	10	16	9	10
333	hsa-miR-2110	8	20	14	20
334	hsa-miR-2113	7	7	9	13
335	hsa-miR-2114	12	18	16	8
336	hsa-miR-2114*	3	12	8	19
337	hsa-miR-2115	9	13	5	6
338	hsa-miR-2115*	7	10	5	20
339	hsa-miR-2116	7	7	14	23
340	hsa-miR-2116*	11	15	16	13
341	hsa-miR-2117	9	10	7	13
342	hsa-miR-212	15	18	12	11
343	hsa-miR-214	7	18	14	15
344	hsa-miR-214*	5	22	3	27
345	hsa-miR-215	10	10	11	8
346	hsa-miR-216a	6	11	5	10
347	hsa-miR-216b	8	7	9	9
348	hsa-miR-217	9	10	6	15
349	hsa-miR-218	8	11	7	15
350	hsa-miR-218-1*	11	15	6	14
351	hsa-miR-218-2*	10	19	6	20
352	hsa-miR-219-1-3p	7	9	9	10
353	hsa-miR-219-2-3p	12	11	11	13
354	hsa-miR-219-5p	6	13	8	16
355	hsa-miR-22	27	26	24	28
356	hsa-miR-22*	15	15	13	11
357	hsa-miR-220a	5	19	14	14
358	hsa-miR-220b	7	15	13	20
359	hsa-miR-220c	5	10	13	13
360	hsa-miR-221	121	97	132	139
361	hsa-miR-221*	10	8	20	13

362	hsa-miR-222	155	117	198	148
363	hsa-miR-222*	5	8	11	10
364	hsa-miR-223	22	30	35	11
365	hsa-miR-223*	7	8	8	10
366	hsa-miR-224	9	13	6	10
367	hsa-miR-224*	11	12	11	4
368	hsa-miR-2276	12	15	11	15
369	hsa-miR-2277	12	13	9	16
370	hsa-miR-2278	13	9	11	11
371	hsa-miR-23a	3,183	4,233	3,376	4,476
372	hsa-miR-23a*	36	39	29	42
373	hsa-miR-23b	1,266	1,490	1,171	1,472
374	hsa-miR-23b*	10	6	11	16
375	hsa-miR-24	931	752	1,111	860
376	hsa-miR-24-1*	9	9	8	11
377	hsa-miR-24-2*	15	19	8	17
378	hsa-miR-25	298	217	268	218
379	hsa-miR-25*	12	12	9	20
380	hsa-miR-26a	507	644	537	585
381	hsa-miR-26a-1*	7	14	2	12
382	hsa-miR-26a-2*	5	15	8	8
383	hsa-miR-26b	33	22	20	21
384	hsa-miR-26b*	7	14	1	16
385	hsa-miR-27a	176	114	148	109
386	hsa-miR-27a*	5	12	6	21
387	hsa-miR-27b	98	67	90	80
388	hsa-miR-27b*	9	17	11	20
389	hsa-miR-28-3p	8	10	9	15
390	hsa-miR-28-5p	12	20	13	19
391	hsa-miR-296-3p	6	8	7	14
392	hsa-miR-296-5p	30	25	49	17
393	hsa-miR-297	8	12	14	12
394	hsa-miR-298	3	16	8	14
395	hsa-miR-299-3p	8	13	18	15
396	hsa-miR-299-5p	36	41	59	28
397	hsa-miR-29a	646	732	738	775
398	hsa-miR-29a*	4	12	20	11
399	hsa-miR-29b	8	17	27	23
400	hsa-miR-29b-1*	34	61	48	64
401	hsa-miR-29b-2*	5	4	8	9
402	hsa-miR-29c	12	14	9	9
403	hsa-miR-29c*	8	8	9	9
404	hsa-miR-300	6	8	19	7
405	hsa-miR-301a	7	8	11	8
406	hsa-miR-301b	6	8	9	9

407	hsa-miR-302a	7	3	10	10
408	hsa-miR-302a*	7	8	12	11
409	hsa-miR-302b	11	14	14	8
410	hsa-miR-302b*	8	7	12	9
411	hsa-miR-302c	11	8	12	12
412	hsa-miR-302c*	12	14	9	9
413	hsa-miR-302d	19	12	6	8
414	hsa-miR-302d*	10	14	8	12
415	hsa-miR-302e	12	12	9	13
416	hsa-miR-302f	14	14	10	24
417	hsa-miR-30a	56	52	47	55
418	hsa-miR-30a*	29	41	24	42
419	hsa-miR-30b	89	83	79	77
420	hsa-miR-30b*	6	7	8	14
421	hsa-miR-30c	393	375	388	351
422	hsa-miR-30c-1*	11	17	13	19
423	hsa-miR-30c-2*	12	13	44	87
424	hsa-miR-30d	52	59	45	36
425	hsa-miR-30d*	9	8	7	7
426	hsa-miR-30e	30	23	23	23
427	hsa-miR-30e*	14	16	13	24
428	hsa-miR-31	379	350	464	448
429	hsa-miR-31*	11	14	17	19
430	hsa-miR-32	7	9	12	10
431	hsa-miR-32*	2	18	9	13
432	hsa-miR-320a	189	163	173	175
433	hsa-miR-320b	103	99	97	118
434	hsa-miR-320c	128	132	113	112
435	hsa-miR-320d	25	25	11	18
436	hsa-miR-323-3p	9	14	23	15
437	hsa-miR-323-5p	7	16	7	24
438	hsa-miR-324-3p	11	21	13	21
439	hsa-miR-324-5p	22	21	16	18
440	hsa-miR-325	7	8	5	12
441	hsa-miR-326	13	21	7	13
442	hsa-miR-328	15	26	15	13
443	hsa-miR-329	31	28	26	32
444	hsa-miR-330-3p	6	11	4	11
445	hsa-miR-330-5p	10	22	4	17
446	hsa-miR-331-3p	14	20	11	20
447	hsa-miR-331-5p	9	9	9	13
448	hsa-miR-335	5	13	11	12
449	hsa-miR-335*	6	13	12	14
450	hsa-miR-337-3p	7	15	12	13
451	hsa-miR-337-5p	10	10	12	13
452	hsa-miR-338-3p	6	10	7	10

453	hsa-miR-338-5p	9	9	9	14
454	hsa-miR-339-3p	6	24	5	24
455	hsa-miR-339-5p	13	20	12	14
456	hsa-miR-33a	8	7	11	10
457	hsa-miR-33a*	3	5	9	14
458	hsa-miR-33b	9	7	12	13
459	hsa-miR-33b*	10	22	11	15
460	hsa-miR-340	5	9	12	7
461	hsa-miR-340*	8	12	12	16
462	hsa-miR-342-3p	47	65	54	51
463	hsa-miR-342-5p	12	13	19	11
464	hsa-miR-345	20	43	15	18
465	hsa-miR-346	9	18	15	9
466	hsa-miR-34a	12	7	9	10
467	hsa-miR-34a*	8	16	6	4
468	hsa-miR-34b	13	14	8	13
469	hsa-miR-34b*	11	13	10	4
470	hsa-miR-34c-3p	15	21	16	16
471	hsa-miR-34c-5p	10	7	11	14
472	hsa-miR-361-3p	14	22	19	10
473	hsa-miR-361-5p	278	248	280	257
474	hsa-miR-362-3p	11	11	14	9
475	hsa-miR-362-5p	23	16	13	15
476	hsa-miR-363	20	7	11	15
477	hsa-miR-363*	11	10	4	10
478	hsa-miR-365	18	20	24	8
479	hsa-miR-365*	16	10	9	15
480	hsa-miR-367	13	11	8	9
481	hsa-miR-367*	10	9	10	10
482	hsa-miR-369-3p	14	9	9	10
483	hsa-miR-369-5p	10	6	6	9
484	hsa-miR-370	9	14	8	14
485	hsa-miR-371-3p	6	18	9	17
486	hsa-miR-371-5p	11	16	9	19
487	hsa-miR-372	10	8	14	14
488	hsa-miR-373	7	10	13	9
489	hsa-miR-373*	6	13	9	19
490	hsa-miR-374a	4	10	9	9
491	hsa-miR-374a*	4	11	6	11
492	hsa-miR-374b	5	12	13	13
493	hsa-miR-374b*	6	3	7	22
494	hsa-miR-375	8	15	11	17
495	hsa-miR-376a	51	33	27	33
496	hsa-miR-376a*	9	10	17	14
497	hsa-miR-376b	7	7	12	15
498	hsa-miR-376c	82	68	70	67

499	hsa-miR-377	20	1,986	25	768
500	hsa-miR-377*	11	9	12	11
501	hsa-miR-378	28	23	36	29
502	hsa-miR-378*	17	15	10	13
503	hsa-miR-379	68	55	81	52
504	hsa-miR-379*	24	23	20	21
505	hsa-miR-380	9	10	16	13
506	hsa-miR-380*	7	14	10	14
507	hsa-miR-381	9	12	16	11
508	hsa-miR-382	86	70	90	96
509	hsa-miR-383	12	6	9	16
510	hsa-miR-384	8	13	6	12
511	hsa-miR-409-3p	41	40	27	23
512	hsa-miR-409-5p	16	12	9	15
513	hsa-miR-410	14	16	11	17
514	hsa-miR-411	16	14	11	19
515	hsa-miR-411*	48	27	32	32
516	hsa-miR-412	10	11	11	9
517	hsa-miR-421	16	19	16	16
518	hsa-miR-422a	13	15	6	15
519	hsa-miR-423-3p	22	18	16	10
520	hsa-miR-423-5p	504	355	696	544
521	hsa-miR-424	10	11	8	16
522	hsa-miR-424*	22	28	22	44
523	hsa-miR-425	121	115	132	115
524	hsa-miR-425*	33	21	30	14
525	hsa-miR-429	12	8	10	7
526	hsa-miR-431	26	26	33	35
527	hsa-miR-431*	10	14	15	25
528	hsa-miR-432	323	282	424	377
529	hsa-miR-432*	8	11	7	7
530	hsa-miR-433	34	27	35	36
531	hsa-miR-448	6	9	11	16
532	hsa-miR-449a	10	7	12	9
533	hsa-miR-449b	18	9	13	10
534	hsa-miR-449b*	23	28	12	13
535	hsa-miR-449c	10	10	11	13
536	hsa-miR-449c*	9	17	22	14
537	hsa-miR-450a	6	6	9	6
538	hsa-miR-450b-3p	8	12	15	9
539	hsa-miR-450b-5p	7	11	9	12
540	hsa-miR-451	10	11	6	14
541	hsa-miR-452	9	10	8	10
542	hsa-miR-452*	7	9	8	12
543	hsa-miR-453	8	199	4	210
544	hsa-miR-454	38	22	21	23

545	hsa-miR-454*	11	12	7	7
546	hsa-miR-455-3p	8	13	5	18
547	hsa-miR-455-5p	10	13	9	8
548	hsa-miR-483-3p	30	31	21	13
549	hsa-miR-483-5p	135	140	89	139
550	hsa-miR-484	30	25	30	17
551	hsa-miR-485-3p	35	45	32	17
552	hsa-miR-485-5p	25	19	15	19
553	hsa-miR-486-3p	17	21	9	11
554	hsa-miR-486-5p	39	30	31	29
555	hsa-miR-487a	12	15	15	13
556	hsa-miR-487b	88	59	88	85
557	hsa-miR-488	8	7	11	4
558	hsa-miR-488*	10	14	11	14
559	hsa-miR-489	11	7	14	13
560	hsa-miR-490-3p	6	11	12	10
561	hsa-miR-490-5p	10	12	10	12
562	hsa-miR-491-3p	6	14	13	6
563	hsa-miR-491-5p	15	13	17	11
564	hsa-miR-492	12	8	10	8
565	hsa-miR-493	13	19	17	16
566	hsa-miR-493*	31	19	28	19
567	hsa-miR-494	94	48	72	64
568	hsa-miR-495	218	99	186	90
569	hsa-miR-496	16	12	13	12
570	hsa-miR-497	9	11	6	15
571	hsa-miR-497*	10	13	7	11
572	hsa-miR-498	32	56	41	52
573	hsa-miR-499-3p	13	12	7	10
574	hsa-miR-499-5p	14	11	11	10
575	hsa-miR-500	15	14	7	17
576	hsa-miR-500*	15	13	9	16
577	hsa-miR-501-3p	14	16	7	16
578	hsa-miR-501-5p	24	15	14	16
579	hsa-miR-502-3p	14	19	6	13
580	hsa-miR-502-5p	14	12	9	12
581	hsa-miR-503	38	20	20	22
582	hsa-miR-504	7	10	8	10
583	hsa-miR-505	18	11	6	12
584	hsa-miR-505*	28	19	14	22
585	hsa-miR-506	10	17	14	10
586	hsa-miR-507	7	8	7	11
587	hsa-miR-508-3p	7	10	7	15
588	hsa-miR-508-5p	3	10	2	17
589	hsa-miR-509-3-5p	5	11	6	14
590	hsa-miR-509-3p	10	6	8	12

591	hsa-miR-509-5p	6	10	2	15
592	hsa-miR-510	7	14	7	23
593	hsa-miR-511	8	8	9	10
594	hsa-miR-512-3p	5	7	9	9
595	hsa-miR-512-5p	6	11	7	18
596	hsa-miR-513a-3p	7	7	12	15
597	hsa-miR-513a-5p	6	10	5	14
598	hsa-miR-513b	8	14	9	9
599	hsa-miR-513c	7	12	14	16
600	hsa-miR-514	9	11	15	14
601	hsa-miR-515-3p	10	9	22	13
602	hsa-miR-515-5p	11	7	14	9
603	hsa-miR-516a-3p	10	3	15	5
604	hsa-miR-516a-5p	9	7	16	11
605	hsa-miR-516b	12	7	14	14
606	hsa-miR-517*	10	6	6	5
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608	hsa-miR-517b	14	16	14	15
609	hsa-miR-517c	12	5	16	13
610	hsa-miR-518a-3p	8	19	7	12
611	hsa-miR-518a-5p	10	8	11	6
612	hsa-miR-518b	21	17	12	16
613	hsa-miR-518c	13	9	6	8
614	hsa-miR-518c*	21	12	10	16
615	hsa-miR-518d-3p	11	5	7	13
616	hsa-miR-518d-5p	11	10	4	10
617	hsa-miR-518e	11	14	4	9
618	hsa-miR-518e*	13	8	67	169
619	hsa-miR-518f	9	8	8	5
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621	hsa-miR-519a	9	9	10	5
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623	hsa-miR-519c-3p	17	9	7	12
624	hsa-miR-519d	23	13	13	8
625	hsa-miR-519e	20	12	9	13
626	hsa-miR-519e*	13	6	6	8
627	hsa-miR-520a-3p	15	12	13	11
628	hsa-miR-520a-5p	24	13	6	6
629	hsa-miR-520b	15	9	14	12
630	hsa-miR-520c-3p	10	6	10	10
631	hsa-miR-520d-3p	12	6	12	13
632	hsa-miR-520d-5p	7	11	11	17
633	hsa-miR-520e	11	7	12	6
634	hsa-miR-520f	11	17	18	11
635	hsa-miR-520g	12	8	14	8
636	hsa-miR-520h	11	9	13	6

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638	hsa-miR-522	21	13	15	11
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642	hsa-miR-525-3p	10	8	6	7
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668	hsa-miR-548h	41	42	32	23
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671	hsa-miR-548k	18	11	11	9
672	hsa-miR-548l	16	10	11	10
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781	hsa-miR-639	11	13	11	8
782	hsa-miR-640	132	324	14	7
783	hsa-miR-641	19	14	21	11
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794	hsa-miR-652	8	11	11	17
795	hsa-miR-653	16	9	9	8
796	hsa-miR-654-3p	36	39	45	37
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806	hsa-miR-663	72	115	79	131
807	hsa-miR-663b	5	19	7	14
808	hsa-miR-664	11	16	21	6
809	hsa-miR-664*	6	13	15	12
810	hsa-miR-665	15	24	13	12
811	hsa-miR-668	26	21	17	11
812	hsa-miR-670	8	12	5	7
813	hsa-miR-671-3p	13	25	11	15
814	hsa-miR-671-5p	27	32	16	33
815	hsa-miR-675	21	18	9	12
816	hsa-miR-675*	26	29	29	8
817	hsa-miR-7	15	14	13	15
818	hsa-miR-708	9	7	8	9
819	hsa-miR-708*	4	8	8	9
820	hsa-miR-7-1*	7	13	8	24

821	hsa-miR-711	6	21	3	14
822	hsa-miR-718	30	38	34	16
823	hsa-miR-7-2*	6	16	6	12
824	hsa-miR-720	1,824	2,463	1,989	2,427
825	hsa-miR-744	9	15	7	12
826	hsa-miR-744*	11	9	12	14
827	hsa-miR-758	14	16	16	17
828	hsa-miR-759	6	10	8	1
829	hsa-miR-760	7	8	6	17
830	hsa-miR-761	6	7	11	11
831	hsa-miR-762	70	120	89	131
832	hsa-miR-764	26	36	14	13
833	hsa-miR-765	40	39	39	57
834	hsa-miR-766	36	31	64	14
835	hsa-miR-767-3p	19	27	17	12
836	hsa-miR-767-5p	8	6	11	6
837	hsa-miR-769-3p	9	14	14	20
838	hsa-miR-769-5p	12	13	13	12
839	hsa-miR-770-5p	11	13	6	12
840	hsa-miR-802	8	3	3	6
841	hsa-miR-873	10	12	9	9
842	hsa-miR-874	6	12	5	23
843	hsa-miR-875-3p	8	9	5	10
844	hsa-miR-875-5p	14	18	12	9
845	hsa-miR-876-3p	7	8	8	8
846	hsa-miR-876-5p	9	9	10	13
847	hsa-miR-877	65	47	78	71
848	hsa-miR-877*	21	23	24	10
849	hsa-miR-885-3p	10	13	6	20
850	hsa-miR-885-5p	18	15	8	9
851	hsa-miR-886-3p	8	8	5	8
852	hsa-miR-886-5p	7	3	8	14
853	hsa-miR-887	9	22	4	19
854	hsa-miR-888	8	15	7	10
855	hsa-miR-888*	7	8	8	16
856	hsa-miR-889	7	6	8	5
857	hsa-miR-890	9	5	5	13
858	hsa-miR-891a	6	8	11	4
859	hsa-miR-891b	5	8	8	6
860	hsa-miR-892a	9	8	3	10
861	hsa-miR-892b	6	9	4	6
862	hsa-miR-9	9	8	9	11
863	hsa-miR-9*	16	15	9	9
864	hsa-miR-920	8	8	6	9
865	hsa-miR-921	9	5	7	11
866	hsa-miR-922	9	10	5	25

867	hsa-miR-924	11	11	5	7
868	hsa-miR-92a	1,369	1,405	1,492	1,526
869	hsa-miR-92a-1*	11	11	7	22
870	hsa-miR-92a-2*	9	6	8	7
871	hsa-miR-92b	496	465	520	517
872	hsa-miR-92b*	9	15	4	10
873	hsa-miR-93	54	35	47	47
874	hsa-miR-93*	16	23	6	11
875	hsa-miR-933	29	24	26	11
876	hsa-miR-934	20	5	11	12
877	hsa-miR-935	8	23	4	22
878	hsa-miR-936	31	12	12	23
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882	hsa-miR-940	63	63	64	42
883	hsa-miR-941	7	4	4	8
884	hsa-miR-942	7	7	6	7
885	hsa-miR-943	7	15	11	9
886	hsa-miR-944	8	8	11	8
887	hsa-miR-95	5	7	5	12
888	hsa-miR-96	7	14	7	4
889	hsa-miR-96*	6	4	7	9
890	hsa-miR-98	18	26	12	19
891	hsa-miR-99a	19	24	33	51
892	hsa-miR-99a*	5	11	9	15
893	hsa-miR-99b	57	61	68	65
894	hsa-miR-99b*	3	7	5	18

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Abstract

SYNERGISTIC EFFECTS OF GARCINOL AND GEMCITABINE IN ENHANCING THERAPEUTIC EFFICACY IN PANCREATIC ADENOCARCINOMA CELLS AND ITS EFFECT ON MICRORNA PROFILE

by

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Human Pancreatic Cancer (PaCa) is one of the most hostile and fourth leading cause of cancer deaths in the United States. Current standard chemotherapeutic agent for advanced PaCa is gemcitabine, a cytotoxic nucleoside analogue which results in modest response due to high degree of inherent and acquired chemo resistance. Forthcoming evidence strongly supports that non-nutritive food components have therapeutic benefits attributable to pleiotropic effects including down-regulation of survival signaling and simultaneously activating multiple death pathways in tumors. Garcinol from the plant *Garcinia indica* have exhibited anti-inflammatory, antioxidant, and anticarcinogenic activity in several cancer types including colon, tongue, breast, skin and liver. This study aims to test 1) therapeutic efficacy of garcinol against PaCa (BxPC-3 and Panc-1) cells 2A) its role in dietary interactions with curcumin and 2B) drug sensitization with gemcitabine and 3) identify microRNA targets in PaCa cells on

treatment with garcinol and gemcitabine. The activity of transcription factor nuclear factor – κB (*NF-κB*) has long been associated with pancreatic tumor growth and angiogenesis, playing an important role in cell survival, chemotaxis and inflammation. Angiogenesis, a very crucial process in tumorigenesis requires the interplay between *NF-κB* with other proangiogenic and antiangiogenic factors such as vascular endothelial growth factor (*VEGF*), Interleukin-8 (*IL-8*) and matrix metalloproteinase (*MMP-9*). We hypothesize that molecular targeting of critical signaling pathways (*NF- κB, VEGF, IL-8 and MMP-9*) with gemcitabine in combination with garcinol may improve efficacy by enhancing the signaling response to treatment. Emerging evidence suggests that microRNAs regulate carcinogenesis by controlling gene expression either by their tumor suppressor or oncogenic abilities. We observed that garcinol individually or in combination with curcumin or gemcitabine exhibited potent anticancer abilities in PaCa cells by down-regulating cancer promoting pathways and up-regulating the tumor suppressor mechanisms. Decreased *NF- κB* activity was observed in both cell lines in combination treatment along with significantly reduced levels of angiogenic factors *MMP-9, VEGF, IL-8* and PGE_2. We also performed target analysis after treatment to identify those microRNAs that regulate several cancer signaling pathways. We observed a downregulation in expression of oncogenic miRNA – 21, - 196a and – 495 along with upregulation of tumor suppressor miRNA – 638 and – 453 on treatment with garcinol alone or in combination with gemcitabine. Overall, our results demonstrate that garcinol and gemcitabine in combination have higher efficacy in modulating levels of various factors involved in tumorigenesis and have potential in chemotherapy against PaCa.

Autobiographical Statement

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B.S. in Biochemistry, Gujarat University, Gujarat, India 2002
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M.S. in Biochemistry, Maharaja Sayajirao University, Gujarat, India 2004
Title: Improvement in surface chemistry for enhancing biomolecular interaction – use of Ferrofluids

PhD in Nutrition and Food Science, Wayne State University, Detroit, MI, USA 2010
Title: Synergistic effects of garcinol and gemcitabine in enhancing therapeutic efficacy in pancreatic adenocarcinoma cells and its effect on microRNA profile

Fellowship and Awards

- American Institute of Cancer Research Scholarship 2009
- Wayne State University Travel Award 2009
- Linus Pauling Institute, Young Investigator Award 2009
- Dissertation Research Support Award, Wayne State University 2009
- Wayne State University Travel Award 2009
- American Society of Nutrition, Clinical Emerging Leader Award 2009
- Annual Graduate Professional Scholarship, Wayne State University 2007
- Wayne State University Travel Award 2007
- Gold Medal for Academic Achievements, Gujarat Education Board 1999

Publications:

- **Parasramka M,** Deryavoush T and Gupta SV: Proceedings of Food, Nutrition, Physical Activity and Cancer conference by American Institute of Cancer Research.
- **Parasramka M,** Ali A and Gupta SV: Proceedings of the fifth International conference on Diet and Optimum Health (2009) by Linus Pauling Institute at Oregon State University.
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