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In Vitro Investigation on Therapeutic Potential of Juglone, a Naphthoquinone from Walnuts against Pancreatic Cancer

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IN VITRO INVESTIGATION ON THERAPEUTIC POTENTIAL OF JUGLONE, A NAPHTHOQUINONE FROM WALNUTS AGAINST PANCREATIC CANCER

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To my husband, Subarna Raj Kandel

&

My family

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

$^{\cdot}OH$	hydroxyl radical
Acetyl-CoA	acetyl coenzyme A
AIF	apoptosis-inducing factor
Akt	protein kinase b
ANOVA	analysis of variance
AP	alkaline phosphatase
ATCC	the American type culture collection
ATP	adenosine tri-phosphate
Bcl-2	b cell lymphoma-2
Bcl-xl	b cell lymphoma-extra large
BSA	bovine serum albumin
CAD	caspase-activated DNAse
Caspases	cysteine-dependent aspartate-specific proteases
CAT	collective to amoeboid transition
c-Flip	flice-inhibitory protein
CI	combination index
CM	conditioned media
CO_2	carbon dioxide
COX-2	cyclo oxygenase 2
CTL	cytotoxic T lymphocytes
CV	crystal violet
DCFH-DA	dichloro-dihydro-fluorescein diacetate
DIABLO	direct IAP binding protein with low pi
DIC	differential interference contrast
DISC	death inducing signal complex
DMEM	dulbecco's modified eagle medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
EGCG	epigallocatechingallate
EGF	epidermal growth factor
ELISA	enzyme linked immunosorbent assay
EMT	epithelial to mesenchymal transition
ErbB2	erythroblastic leukemia viral oncogene homolog 2
ERK	extracellular signal-related kinase
Fa	fraction affected
FADD	fas-associated death domain
FAMMM	familial atypical multiple mole melanoma
FDA	Federal Drug Administration

FITC	fluorescein isothiocyanate
FLICE	FADD-like IL-1β-converting enzyme
FOXO3 A	forkhead box o
GLUT1	glucose transporter 1
H_2O_2	hydrogen peroxide
Her2	human epidermal growth factor 2
HIF-1α	hypoxia inducible factor-1α
HNPCC	hereditary nonpolyposis colorectal cancer
HPLC	high performance liquid chromatography
HRP	horse radish peroxidase
HtrA2	high temperature requirement protein a
HUVEC	human umbilicial vein endothilial cells
IAP	inhibitors of apoptosis
IC50	the half maximal inhibitory concentration
IPMN	intraductal papillary mucinous neoplasm
LL	lower left
LR	lower right
LSGS	low serum growth supplement
MAPK	mitogen-activated protein kinase
MAT	mesenchymal to amoeboid transition
Mcl1	myeloid cell leukemia 1
MCN	mucinous cystic neoplasm
MEK 1/2	MAPK/ERK kinase
MET	mesenchymal to epithelial transition
mTOR	mammalian target of rapamycin
MTT	thiazolyl blue tetrazolium bromide
MVA	mevalonic acid
NADPH	nicotinamide adenine dinucleotide phosphate
NF-KB	nuclear factor kappa-light-chain-enhancer of activated b cells
NK	natural killer
NQO1	NADPH:quinone oxidoreductase 1
$O2^{\cdot-}$	superoxide radical
OD	optical density
$ONOO^-$	peroxynitrite
OSB	*ortho*-succinylbenzoic acid
P95HER-2	the p95 truncated portion of her-2
PanIN	pancreatic intraepithelial neoplasia
PARP	poly ADP ribose polymerase
PBS	phosphate buffer saline
PC	pancreatic cancer

PI	propidium iodide
PI3K	phosphatidylinositol 3-kinase
Pin1	peptidyl prolyl isomerase
PIP2	phosphatidylinositol 4,5-bisphosphate
PIP3	phosphatidylinositol (3,4,5)-trisphosphate
PKM1/M2	pyruvate kinase isozymes m1/m2
Ppm	parts per million
PS	phosphatidyl serine
PVDF	polyvinylidene difluoride
RAF	the rapidly accelerated fibrosarcoma
Ras	rat sarcoma
KRAS	kristen rat sarcoma virus proto-oncogene
RB	retinoblastoma-associated
ROS	reactive oxygen species
RT	room temperature
SAS	statistical analysis system
SDS	sodium dodecyl sulfate
SEER	surveillance, epidemiology, and end results
SM	secondary metabolite
SMAD4	mothers against decapentaplegic homolog 4
STAT3	signal transducer and activator of transcription
TBS	tris-buffered saline
TCA	tricarboxylic acid
TE	tris-edta
TNF	tumor necrosis factor
TP53	tumor protein p53
TPA	12-o-tetradecanoylphorbol-13-acetate
TQ	thymoquinone
TRADD	TNF receptor-associated death domain
TSP-1	thrombospondin-1
UL	upper left
UR	upper right
UV	ultraviolet
VEGF	vascular endothelial growth factor
Zeb ½	zinc finger e-box-binding homeobox 1

ABSTRACT

Juglone, a naphthoquinone found in *Juglandaceae* family, which includes black walnut, European walnut, and butter nut possess various biological activities. The anti-cancer properties of juglone has been reported; however, the effect of juglone in pancreatic cancer (PC) has not been elucidated yet. PC is an aggressive lethal, highly metastatic disease associated with poor prognosis and high mortality rate. PC is usually diagnosed in advanced stage and chemotherapy is provided as a first line of treatment. The *de novo* chemoresistance that develops with chemotherapeutic treatment creates a critical need for identification of novel therapeutic agents for effectively targeting the disease.

The effects of juglone on PC cell proliferation, level of reactive oxygen species (ROS) production, and expression of various oncogenic signal transduction molecules in MIA Paca-2, pancreatic carcinoma cells were investigated. The major findings indicate that treatment with juglone dose dependently suppressed the *in vitro* proliferation and induced cell death of rapidly dividing human PC cells with an IC_{50} value of 5 μM at 24 h. Long-term colonies forming ability of PC cells was also significantly inhibited. The molecular mechanisms behind juglone-induced apoptosis of PC cells indicated activation of caspase-3, cleavage of PARP, upregulation of Bax, down regulation of Akt, ERK, HER-2, Cox-2, and Bcl-2 and very high production of ROS leading to chromatin condensation, DNA damage and cell death. Changes in morphological features of cell treated with juglone were obtained by confocal microscopy using Hoechst staining, which specified apoptotic features in treated cells. The results also revealed the anti-angiogenic and anti-metastatic potential of juglone. PC cell migration and invasion was significantly reduced with juglone treatment and the potential of endothelial cells to form tubes was also limited when treated with juglone. Key angiogenic regulators such as HIF-1α and VEGF were also downregulated with

juglone treatment. Taken together, our data suggest that of ROS-inducing agent juglone could provide a novel therapeutic approach for PC treatment.

CHAPTER 1. REVIEW OF LITERATURE

1.1. Natural Phenolic Compounds

Medicinal properties of natural herbs and botanicals provide a major contribution as a source for new pharmaceutical discoveries [1, 2]. Plant derived components are gaining attention for promoting good health and defined as functional foods and nutraceuticals. The phenolic content of plants has been categorized into more than 20 different groups.

Natural products comprise a significant percentage of anti-cancer drugs that are currently used for treatment. Globally, more than fifty percent of drugs that have been used in cancer treatment from 1940 to 2006 were reported to be either natural products or their derivatives [2, 3]. The National Cancer Institute of the United States began screening plant extract for anti-tumor activities in the 1960s [5] and since significant progress has been made in the pharmacology of natural products. Drugs made from natural products include paclitaxel, docetaxel and others [6].

1.2. Secondary Metabolites from Plants

Secondary metabolites (SM) are the low molecular weight non-essential components of the plant and are produced as a defense mechanism by plants against their competitors, insects, herbivores, microbial pathogens or to attract insects for pollination. Other abiotic factors that induce production of SM by plants include UV exposure [7]. SM play an integral role in a plant life cycle for its survival and reproduction. The pharmacological values of many SM have been recognized. SM represent a huge variety and diversity; a single plant can produce a complex mixture of SM. Thousands of structures of SM have been identified and can be divided into two different groups; nitrogen containing compounds and compounds with no nitrogen, which are listed in Table 1.1.

Table 1.1: Number of Structurally known Secondary Metabolites [8, 9]

Secondary Metabolite (SM)		Number of Structures
Nitrogen Containing SM	Amines	100
	Alkaloids	21000
	Non protein amino acids	700
	Cyanogenic glucosides	60
	Glucosinolates	100
	Alkamides	150
	Lectins, peptides	2000
SM containing no nitrogen	Monoterpenes	2500
	Sesquiterpenes	5000
	Diterpenes	2500
	Triterpenes	5000
	Tetraterpenes	500
	Polyketides, quinones	750
	Polyacetylenes, fatty acids, waxes	1500
	Flavonoids	4000
	Phenylpropanoids, lignin , coumarins, lignans	2000

1.2.1. Quinones

Quinones are secondary metabolites derived from plants or microorganisms. Quinones are a class of organic compound usually derived from aromatic compounds such as benzene or naphthalene. They are found especially in the plant kingdom in higher plants, fungi and bacteria and in the animal kingdom in arthropods and echinoderms. They range from pale yellow to black in color and are used in the food industry as food colorants. They are also used as dyeing agents and are valued for their therapeutic benefits as well. Diet, drugs and environment are some of the

medium for exposure of quinones to human. There are different types of quinones with remarkable therapeutic applications. Based on the number of fused benzene rings in the structural skeleton, they have been divided mainly into four different groups; benzoquinone, naphthoquinone, phenanthrenequinone, and anthraquinone. Examples of these quinones and their basic structure are listed in Table 1.2.

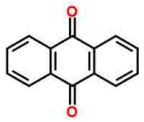
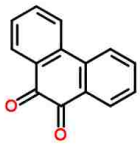
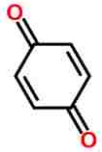
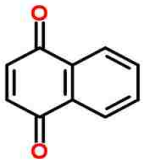
Out of different groups of quinones, anthraquinones are the largest group of naturally occurring quinones. The name of quinone-based compounds are usually derived from their parent aromatic compound [10]. For instance, naphthoquinones are derived from naphthalene, benzoquinones are derived from benzene, and anthraquinones from anthracene. Quinones are abundantly found in nature.

More than 1200 types of quinones have been reported in literature [11]. They primarily function as electron carriers in plants and are very well known for their cytotoxicity by radical formation though electron transport where the quinone ring is of major importance [12]. Other mechanisms that have been associated with quinone-induced cytotoxicity are its ability to form cell adducts, intercalate DNA, cause alkylation, and inhibit proteins and enzymes such as topoisomerases [12].

1.2.2. Quinones for Anti-Cancer Therapy

Quinones make one of the largest group of compounds used against cancer [14]. Some of the various drugs against cancer that have been approved and those going through clinical trials are quinone-based compounds such as actinomycin D, anthracylines, daunorubicin, menadione, mitomycin C, mitoxantrones, saintopin, and trenimon [15, 16]. This validates the importance of naturally occurring quinones for drug development in cancer research.

Table 1.2: Examples and basic structures of some quinone [9, 13]

Types of Quinones	Examples	Basic Structure
Anthraquinones	Rhein, emodin, aloe-emodin	
Phenanthraquinones	Denbinonbin, bulbophyllanthrone	
Benzoquinones	Embelin, embelinol, thymoquinone	
Naphthoquinones	Juglone, Plumbagin, Shikonin, Lawsone, Menaquinones	

1.2.3. General Mechanisms behind Quinone-based Toxicity

Bio-reductive activation of quinones and generation of reactive oxygen species are the major events behind the biological activity of quinones [10]. Quinones can undergo reduction via 2 different pathways; a. one electron reduction b. two electrons reduction as shown in Figure 1.1. Semiquinones are formed via one electron reduction of quinones, which is catalyzed by enzymes like NADPH-cytochrome P_{450} reductase, resulting in generation of O_2^- and H_2O_2. Hydroquinones are usually formed by 2 electron pathway, and this reaction is catalyzed by NADPH:quinone oxidoreductase 1 (NQO1) or DT-diaphorase. Quinones are electrophilic in nature and this

promotes the reaction with cellular components especially with macromolecules containing thiol groups. This electrophilic reaction provides quinone with a cytotoxic property [11].

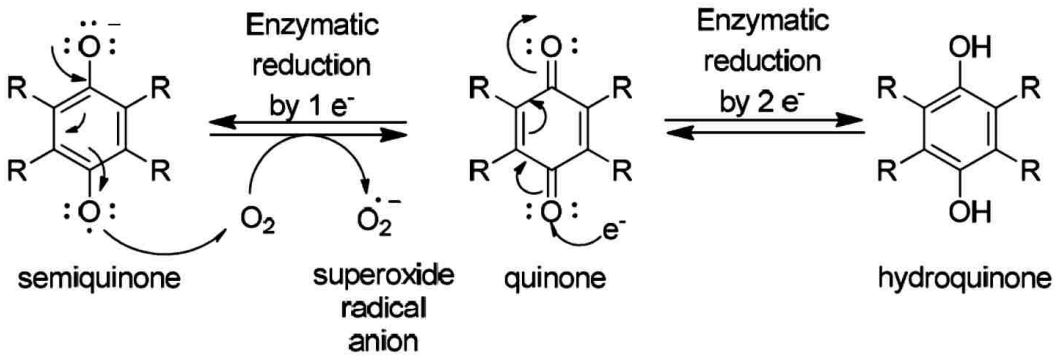


Figure 1.1. Reduction of quinone

1.3. Juglone (5-hydroxy- 1, 4- naphthoquinone)

Naphthoquinones are found in almost 20 families of higher plants like *Ebenaceae, Droseraceae, Balsaminaceae, Juglandaceae, Plumbagnaceae* and others. Juglone, a naphthoquinone is a major component found in the nuts, hulls, leaves, roots, and bark of plants in *Juglandaceae* family, which includes black walnut (*Juglans nigra* L.), European walnut (*Juglans regia* L.), butter nut (*Juglans cinerea*) and others [17]. Plants in the *Juglandaceae* family contain about 50 species in 8 genera and usually grow in the northern temperate and subtropical climate zone [18]. These plants are widely distributed in southern Europe, northern Africa, eastern Asia, the USA, and western South America.

The amount of juglone in plants varies depending on the source and varieties. The molecular weight of juglone is 174.14 and its melting point is 155^{o}C. It is soluble in alcohol, acetone, acetic acid, chloroform, benzene, DMSO and is slightly soluble in hot water [18]. The structure of juglone is shown in Figure 1.2. In a sample of 1121 different plants, the amount of juglone ranged from 13 to 1500 mg/100g dry weight [19]. Normal-phase liquid chromatography is a simple and rapid method used for the determination of juglone with a reported retention time

of 4.27 min [18]. Juglone was used in ancient medicine for ringworm, fungal, bacterial and viral infections, and for its allelopathic activity in plants [20].

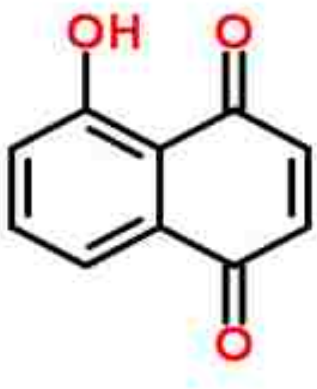


Figure 1.2. Structure of juglone

1.3.1. Biosynthesis of Juglone

Juglone is biosynthesized in plants from the *ortho*-succinylbenzoic acid (OSB) of the shikimic acid pathway [21]. The shikimic acid pathway is the major route for the formation of secondary metabolites in higher plants [22]. This pathway does not occur in animals, but only in higher plants and microbes. Aromatic compounds and some of the essential amino acids are formed via this pathway. An intermediate that is reported to be involved in the biosynthesis of juglone is 1, 4 naphthoquinone and its decarboxylation and hydroxylation results in the formation of juglone as illustrated in Figure 1.3. Juglone is present in both free as well as glucosidic form in plants. The glucosidic form of juglone (1, 5-dihydroxy-4-naftalenyl-β-D-glucopyranoside) is usually decomposed by the enzyme hydrojuglone-β-D-glucopyranosid-β-glucosidase to juglone [23].

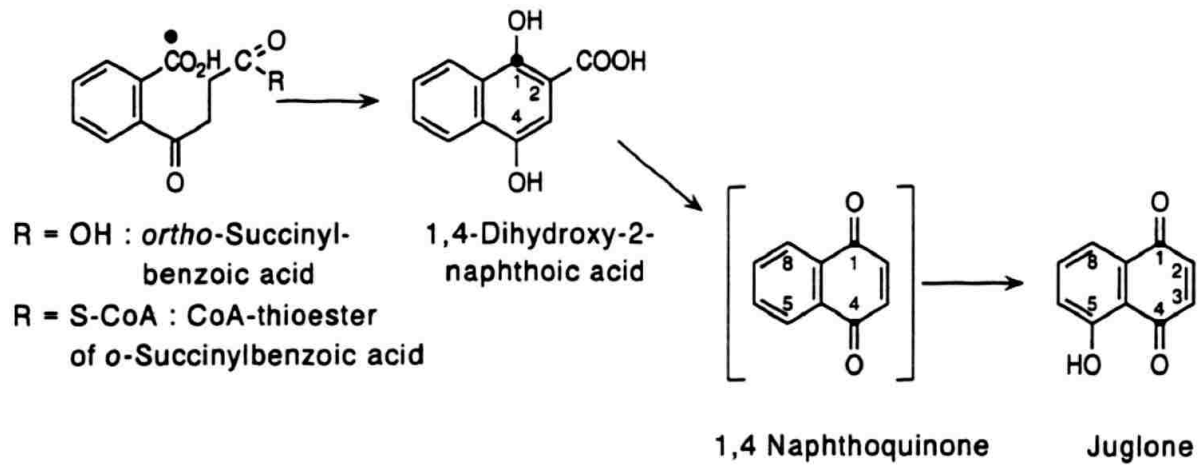


Figure 1.3. Biosynthesis of juglone [24]

1.3.2. Chemo-preventive Activity of Juglone

There are very limited studies on the anti-cancer activities of juglone. However, a very closely related compound plumbagin (2-methoxy -5-hydroxy,1,4 naphthoquinone), an analogue of juglone has been extensively investigated for its anti-cancer properties. Plumabgin and juglone at 200 ppm reduced the occurrence of multiple intestinal tumors in azoxymethane (carcinogen)-induced colon cancer in rats [25]. Both plumbagin and juglone had significant effect against tumor initiation and multiplication, but the underlined mechanism was not detailed.

Juglone dose-dependently inhibits melanoma cells viability. The IC_{50} for juglone in B16F1 melanoma cells at 1, 24 and 48 h was found to be 9.9 µM, 7.46 µM and 6.92 µM respectively [17]. Very high intracellular production of ROS was linked to the mode of action of juglone mediated apoptosis in melanoma cells. Peptidyl prolyl isomerase Pin1 activity is very high in various cancers, which is induced by oncongenes such as Ras and Neu. Pin 1 is also associated with regulation of cyclin D1. Juglone has been reported to inhibit Pin1 activity [26]. Expression of oncogenic Ras is highly elevated in pancreatic cancer and juglone being an inhibitor of Pin1, which

is a downstream molecule of oncogenic Ras signaling pathway [27], juglone seems to be a promising compound against pancreatic cancer.

Juglone has been reported to be the substrate for NADPH-cytochrome P450 reductase and DT-diaphorase [11], which means it can either be reduced by one electron or 2 two electron pathways.

1.4. Thymoquinone (TQ)

TQ (2-isopropyl-5-methyl-benzoquinone) is the principal bioactive component of the volatile oil of *Nigella sativa* (black seed). The structure of TQ is shown in Figure 1.4. The content of TQ in black seed oil as analyzed by HPLC has been reported to be 27.8% [28]. Black seed oil has been extensively used in traditional medicine in the Mediterranean region and Asian continent [29] for its anti-inflammatory [30], anti-diabetic [31], and anti-microbial [32] properties.

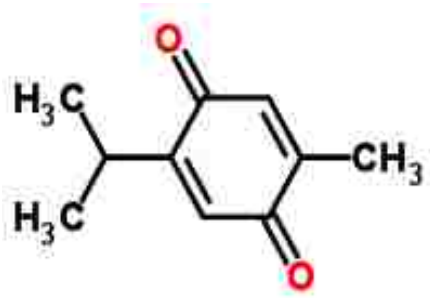


Figure 1.4. Structure of thymoquinone

The compound was isolated from black seed 50 years ago and has been studied ever since [1]. Various studies have been carried out to understand the effect and mechanism of TQ on different types of cancer cells. TQ inhibits various cancer cells *in-vitro* and *in vivo* and has been suggested to undergo clinical trials [33].The toxicity of TQ has proven to be very low in experimental animal models. The LD_{50} in rats and mice models after intraperitoneal injection was found to be 57.5 mg/kg and 104.7mg/kg whereas in oral administration the LD_{50} was 794.3 mg/kg and 870.9 mg/kg, respectively [34].

All different hallmarks of cancer has been found to be affected by TQ except “evasion of immune system” [1]. As illustrated in Figure 1.5., generation of reactive oxygen species [35], translocation of phosphatidylserine [33], chromatin condensation [36], inhibition of angiogenesis [37], and control of invasion and metastasis [38] are some of the approaches by which TQ acts on cancer cells. As TQ possesses so many desirable effects against cancer and has already been used in many combinatorial therapies with chemotherapeutic agents, hormones or ionizing radiation [1, 39], combination of juglone and TQ was therefore designed in order to target the multi-pathophysiological processes in pancreatic cancer as discussed in Chapter 2.

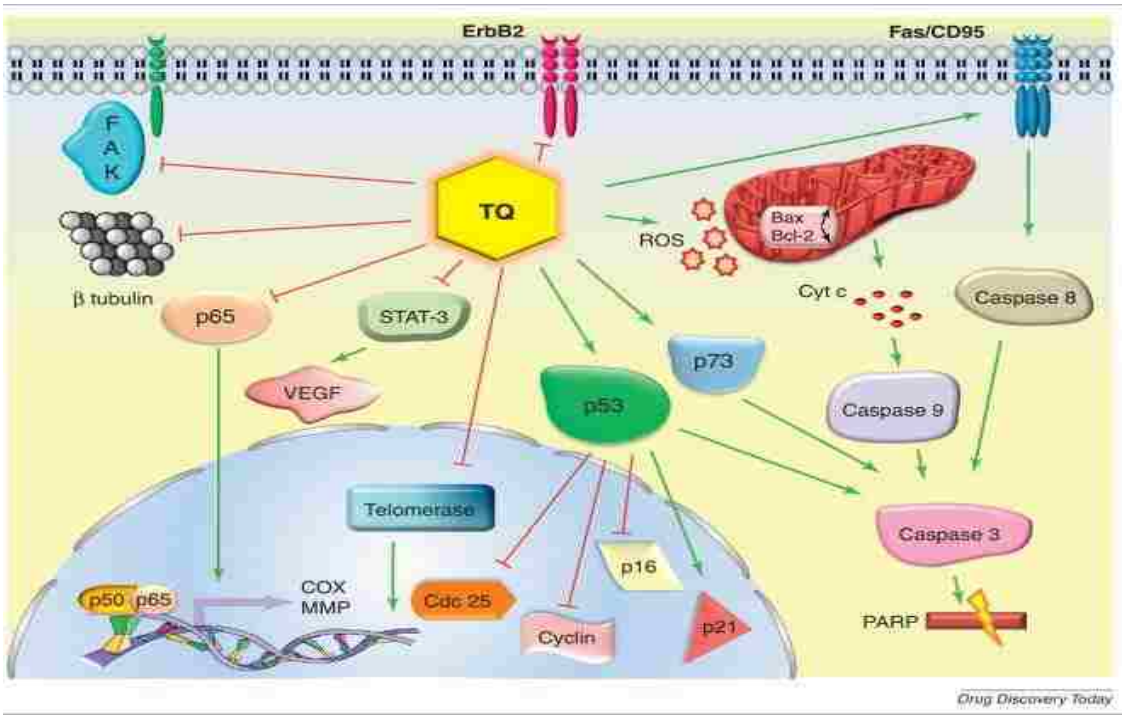


Figure 1.5. Mode of action of TQ [1]

1.5. Hallmarks of Cancer

Hanahan and Weinberg in 2000 had summarized the key features of cancer cells in six hallmarks [40], which are listed and discussed below. These six hallmarks of cancer are shared by all types of cancer. The concept of the hallmarks has remained the same since the last decade, but

in 2011 a few new features were added to the list namely including cancer cell metabolism and evasion of immune-surveillance [41].

1. Self-sufficiency in growth signals
2. Abrogation of Growth Suppressors
3. Evasion of Apoptosis
4. Unlimited Replicative Potential
5. Induction of Angiogenesis
6. Activation of invasion and metastasis
7. Metabolism
8. Evasion of immune surveillance

1.5.1. Self-sufficiency in Growth Signals

Normal cells require reception of growth signals in order to proceed through cell division and growth cycle, which helps them to maintain homeostasis in a cellular environment and ensures formation of a normal tissue. In contrast to normal cells, tumor cells act independently to growth signals and have deregulated mechanisms that disrupt the balance in cell numbers and results in chronic proliferation of cancer cells. Various factors that contribute to reduced dependency of cancer cells to growth factor signals have been identified. Cancer cells have the ability to generate growth factors on their own and respond to it via expression of numerous growth factor receptors. Alternatively, they can also signal surrounding normal cells to release growth factors required for their growth [42]. Other differences that are seen in cancer cells include overexpression of growth factor receptors that enables amplification of cancer cell signaling in response to a limited amount of growth factor, and structural modification in growth factor receptors which causes cancer cell signaling even in absence of growth factor ligand. Cancer cells containing these overexpressed

growth receptors are found to contain somatic mutation that triggers hyper-activation of downstream signaling cascades inside the cells such as activated Ras-Raf-MAPK (mitogen-activated protein kinase) pathway and phosphoinositide 3-kinase (PI3K)/Akt signal transduction pathway which can be related to increased proliferation.

1.5.2. Abrogation of Growth Suppressor Signals

In order to maintain tissue homeostasis and cells senescence, normal cells exhibit anti-proliferative mechanism by responding to anti-growth factors or growth inhibitors. These inhibitors are either in solubilized or immobilized forms embedded in the extracellular matrix. These inhibitors are recognized by the cell surface receptors. These receptors further induce intracellular anti-growth signaling in cells resulting in their quiescent stage or differentiated stage losing their ability to proliferate. In contrast, cancer cells resist anti-growth signals by disrupting action of many tumor suppressor genes. These genes encode tumor suppressor proteins like RB (retinoblastoma-associated) and TP53 (tumor protein p53) that play a major role in controlling proliferation and cell cycle progression by activating senescence and apoptosis. However both of these proteins are found to be defective in most of the cancers. Besides inhibiting tumor suppressor proteins, tumor cells can also evade differentiation by overexpression of the oncoprotein c-Myc [43].

1.5.3. Evasion of Apoptosis

Apoptosis is also called programmed cell death. It is derived from the Greek words meaning “falling off” like leaves in autumn and was first coined by Kerr, Willie, and Currie in 1972 [44]. Physiological significance of apoptosis is to maintain a balance in cell population and the process is very high during embryonic development, when the tissues are going through sculpting and during aging [45]. Dysregulation of apoptotic process leads to various pathological

conditions such as cancer, Alzheimer's disease and Parkinson's disease. Cancer cells acquire the ability to resist apoptosis.

The apoptotic process includes upstream regulators and downstream effector components. The regulators of apoptosis can either be extrinsic or intrinsic. The extrinsic regulation includes different cell surface death ligand receptors including the Fas ligand and TNF receptor. The intrinsic regulation monitors condition of the cells and activates apoptosis in detection of abnormalities within the cells such as DNA damage, hypoxia or survival factor insufficiency. These apoptotic signals converge in the induction of release of mitochondrial pro-apoptotic protein cytochrome C whose release is regulated by the Bcl-2 family of proteins. Both kinds of regulation eventually initiate the caspase cascade and converge to the activation of common executioner protease, caspase-3 responsible for the execution of apoptosis.

In case of cancer, the process of apoptosis is unregulated. Tumor cells evade apoptosis through a variety of mechanisms. Some of the mechanisms responsible for evasion of apoptosis include mutation of tumor suppressor gene p53, overexpression of anti-apoptotic regulators like Bcl-2 and c-myc, and downregulation of pro-apoptotic factors like Bax and cytochrome-C. The PI3K/Akt pathway, which is responsible for transmission of anti-apoptotic cell survival signals is also activated in human tumors and this alteration also enables the evasion of apoptosis [46]. Apoptosis is triggered in cancer cells as a vital anti-cancer therapeutic approach. The signaling circuitry regulating apoptosis is further discussed in details in the following sections.

1.5.3.1. Cellular Changes during Apoptosis

Various morphological and biochemical changes occur in cells undergoing apoptosis. Rounding up of the cells, chromatin condensation or pyknosis followed by nuclear fragmentation or karyorrhexis, membrane blebbing, modification in structures of cyto-organelles, and loss of

membrane integrity are some of the physical changes that are observed during apoptosis [4]. Apoptotic cells also go through numerous biochemical changes; activation of caspases, degradation of DNA, and changes in plasma membrane like, externalization of phosphatidylserine (PS) molecules. Expression of PS on the outer membrane of dead cells allows it to be recognized by the macrophages and hence phagocytosis occurs. The process of dead cells undergoing phagocytosis is just as important because if not the apoptotic bodies can induce neighboring cells to induce production of pro-inflammatory cytokines that could have deleterious effect.

1.5.3.2. Mechanism of Apoptosis

Understanding the mechanism of apoptosis is very important with regard to drug development. Schematic diagram of extrinsic and intrinsic pathway of apoptosis is shown in Figure 1.6. Caspases (Cysteine-dependent aspartate-specific proteases) play a major role in initiating and executing apoptosis. They are usually present as inactive proenzyme and when activated initiates activation of other caspases leading to a cascade of caspase activation. Activated caspases lead to cleavage or change in many structural proteins, nuclear proteins or enzymes eventually causing cell death. There are 12 different types of caspases found in human cells and only seven types of caspases are involved with apoptosis. Caspases involved in apoptosis are divided as initiators (-2, -8, -9, -10) and executioners (caspase -3, -6, and -7) [45]. Caspases are proteolytic in nature and function through two major pathways as discussed below. Both of these pathways (extrinsic and intrinsic) eventually lead to the execution of apoptosis.

1.5.3.2.1. Extrinsic Pathway: It is also referred to as the death receptor pathway which starts with binding of death ligands to the transmembrane death receptors that belong to the tumor necrosis factor (TNF) receptor gene superfamily [47]. These receptors have a cysteine rich

extracellular domain and a cytoplasmic death domain. The death domain transmits intracellular signals from the cell surface and initiates a series of events [48].

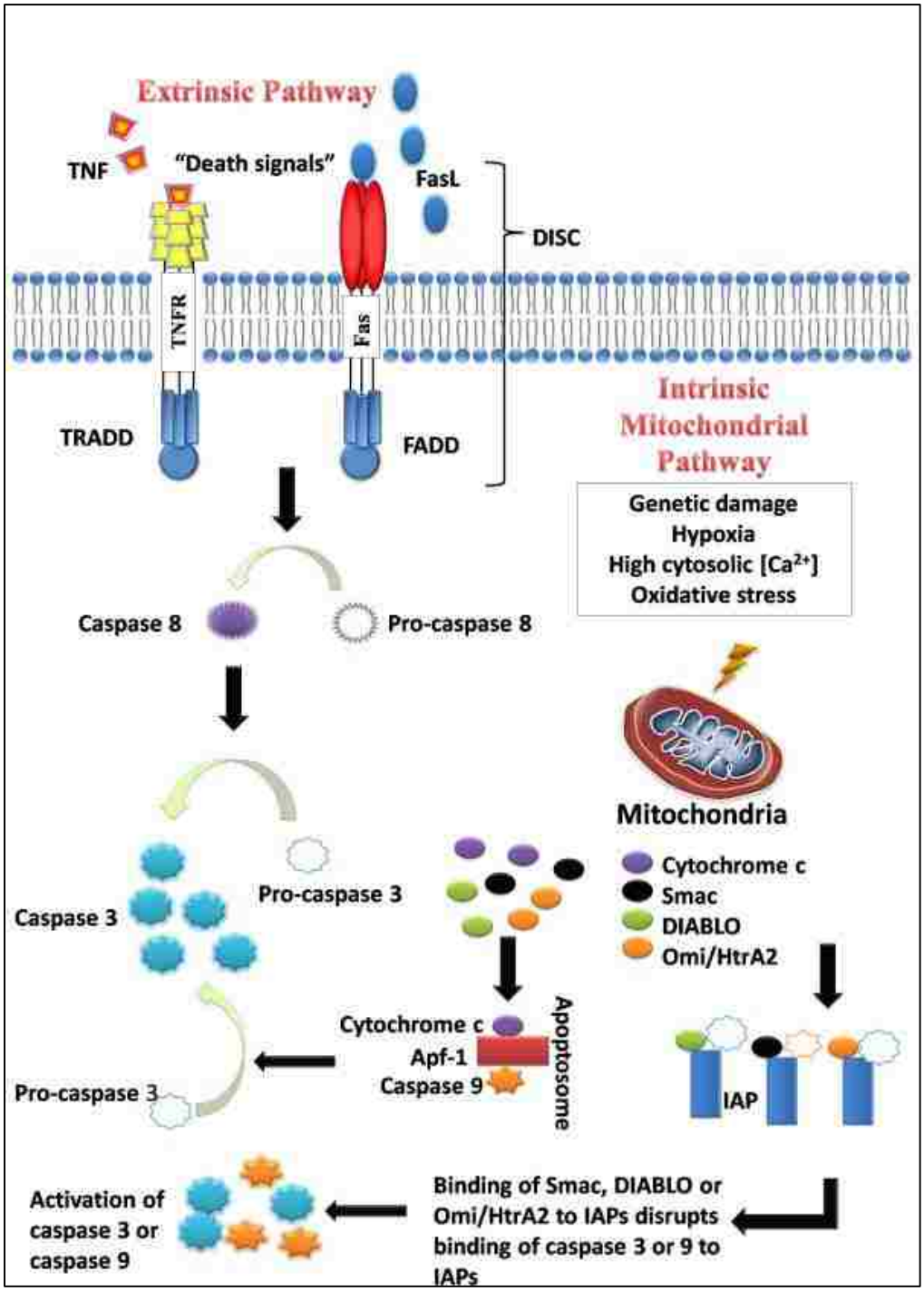


Figure 1.6. Extrinsic and intrinsic pathways of apoptosis [4]

TNF receptors and FasL receptors are some of the receptors that receive the death signals from their respective ligands, TNF and Fas [49]. Binding of the ligands to the receptor causes recruitment of adapter proteins in the binding site of intracellular death domain of the receptor forming the Death Inducing Signal Complex (DISC) [50]. TNF receptor-associated death domain (TRADD) and Fas-associated death domain (FADD) are the adapter proteins for TNF receptor and Fas receptor. DISC then activates pro-caspase 8. Activated pro-caspase 8 is the initiator caspase and triggers the execution of apoptosis. FLICE (FADD-like IL-1β-converting enzyme)-inhibitory protein (c-FLIP) and Toso are some of the inhibitor proteins of death receptor mediated apoptosis [45].

1.5.3.2.2. Intrinsic Pathway: As the name suggests, this pathway begins from the mitochondria within the cell. Stimuli like oxidative stress, hypoxia, toxins, radiation, and free radicals trigger high mitochondrial permeability, loss of mitochondrial potential, which results in opening of pores in mitochondria with release of pro-apoptotic proteins from the inner membrane of mitochondria to the cytosol of cells. There are typically 2 groups of pro-apoptotic proteins released in this manner. The first group consists of cytochrome C, second mitochondria derived activator of caspase (Smac), direct inhibitors of apoptosis (IAP) binding protein with low pI (DIABLO), and high temperature requirement protein A (HtrA2)/Omi and these proteins activate the caspases. Cytochrome C acts by forming an apoptosome complex by binding with Apaf-1 and pro-caspase 9 and the apoptosome complex activates the initiator caspase, caspase-9. Smac/DIABLO and HtrA2/omi proteins bind to IAP, and promotes apoptosis by preventing it from binding with caspase-9 or -3. The other group of proteins released from the mitochondria are endonuclease G, Caspase-activated DNAse (CAD) and Apoptosis-inducing Factor (AIF). AIF and endonuclease G function get translocated to the nucleus and cause DNA fragmentation and work

in a caspase-independent mechanism whereas CAD after being translocated to nucleus has to be first cleaved by caspase-3 in order to create distinct DNA fragmentation and chromatin condensation.

The Bcl-2 family of proteins are accountable for the regulation of mitochondrial permeability in this specific pathway. The major groups of proteins within Bcl-2 family can be classified into pro-apoptotic and anti-apoptotic proteins. Anti-apoptotic proteins such as Bcl-2, Bcl-x, Bcl-XL, Bcl-w, BAG play their role by blocking the mitochondrial release of cytochrome C whereas the pro-apoptotic proteins such as Bcl-10, Bax, Bak, Bid, Bad, Bim, Bik and Blk promote the release of cytochrome C.

1.5.3.2.3. The common Execution Pathway: Both the extrinsic and the intrinsic apoptotic pathways converge to the common execution pathway, which is considered to be the final step of apoptosis. Executioner caspases (caspase-3, -6, -7) are activated in this phase and stimulate the activity of various endonucleases and proteases to degrade the nuclear material and cytoskeletal proteins. Some of the substrates that are cleaved by executioner caspases are DNA repair proteins, PARP (poly ADP ribose polymerase), cytokeratins and nuclear mitotic apparatus protein 1 (NuMA). These cumulative action of caspases and endonucleases causes the morphological and biochemical changes seen in cells undergoing apoptosis. Once the cells undergo apoptosis the next immediate step is the phagocytosis of the cellular components.

1.5.4. Unlimited Replicative Potential

Cancer cells acquire the property of unlimited replicative potential that results in macroscopic tumors. Normal cells can only go through limited cell divisions. Senescence and crisis restrict uncontrolled proliferation of normal cell. Senescence is an irreversible state where the cells enter into a viable but non-proliferative stage and cells escaping senescence enter crisis stage which

refers to cell death. There are rare occasions where cells from population of crisis escape and achieve unlimited replicative potential termed as immortalization.

Telomeres are the TTAGGG sequences of DNA that are present at the ends of the chromosomes and function to protect the chromosomal ends. In normal cells, with each cell division, the length of telomeres shortens and eventually a limit is reached where the cells are directed to arrest or senescence. The natural process of telomere shortening in normal cells with each cell division leads consequently to a point where the cells lose their protective function and senescence or apoptosis is triggered. This process is evaded by cancer cells with activation and up regulation of telomerase, a DNA polymerase enzyme that adds telomere segments in the end of the chromosome. The activity of telomerase is almost lacking in non-immortalized cells and found significantly higher in malignant cancer cells, which helps cancer cells resist the induction of senescence or crisis/apoptosis. Therefore, suppression of the enzyme telomerase in cancer cells leads to telomere shortening and to activation of barriers to proliferation namely, senescence or apoptosis.

1.5.5. Induction of Angiogenesis

Tumor cells need a supply of nutrients and oxygen and also require a system to evacuate metabolic waste and carbon dioxide like normal cells do. These needs of tumor cells are met by the process of neo-vascularization or angiogenesis. Normally, angiogenesis is very active only in few cases during embryogenesis such as wound healing and female reproductive cycling, and remains quiescent most of the time. A tumor cannot grow in size beyond 1-2 mm until it is supplied with nutrients and oxygen. In case of tumor progression, the “angiogenic switch” is always turned on which refers to a state where the balance of pro-angiogenic and anti-angiogenic factors is disrupted and more inclined towards the pro-angiogenic factors such as vascular endothelial

growth factors [51]. This process of angiogenic switch causes activation of normal quiescent blood vasculature to form new blood vessels for growth of tumors which eventually leads to formation of malignant tumors [51].

The angiogeneic switch in tumors are regulated by pro-angiogenic growth factors like vascular endothelial growth factor (VEGF) or inhibitors like thrombospondin-1 (TSP-1). In some tumors, oncogenes such as Ras and Myc are known to upregulate expression of pro-angiogenic growth factors. Tumor-associated blood vessels formed by chronic activation of angiogenesis are usually irregular with enormous sprouting of capillaries, distorted with enlarged vessels, and contains inconsistent blood flow and leakiness [52]. Traditionally, tumor angiogenesis used to be considered important only after the formation of macroscopic tumors, but recent data of histological analyses of premalignant and non-invasive lesions have shown induction of angiogenesis in early steps of tumor growth [53] both in animal and human models, making angiogenesis a basic hallmark of cancer.

1.5.6. Activation of Invasion and Metastasis

Metastasis is a process where neoplastic cells disseminate, invade adjacent tissues and grow at a site distant from the site of origin as depicted in Figure 1.7. [54]. It is the cause for more than 90% of cancer related deaths. The process of invasion and metastasis is a multi-step process and referred as the invasion-metastasis cascade. It consists of a series of the following steps [55]:

i. Infiltration of cancer cells to adjacent tissue (local invasion)
ii. Intravastation (migration of cancer cells into blood vessels)
iii. Transit of cancer cells through lymphatic and hematogenous systems
iv. Extravastation (movement of cancer cells from vessels to distant tissues)

v. Formation of micro-metastases

vi. Growth of micro-metastatic tumor to macroscopic tumors also called (colonization)

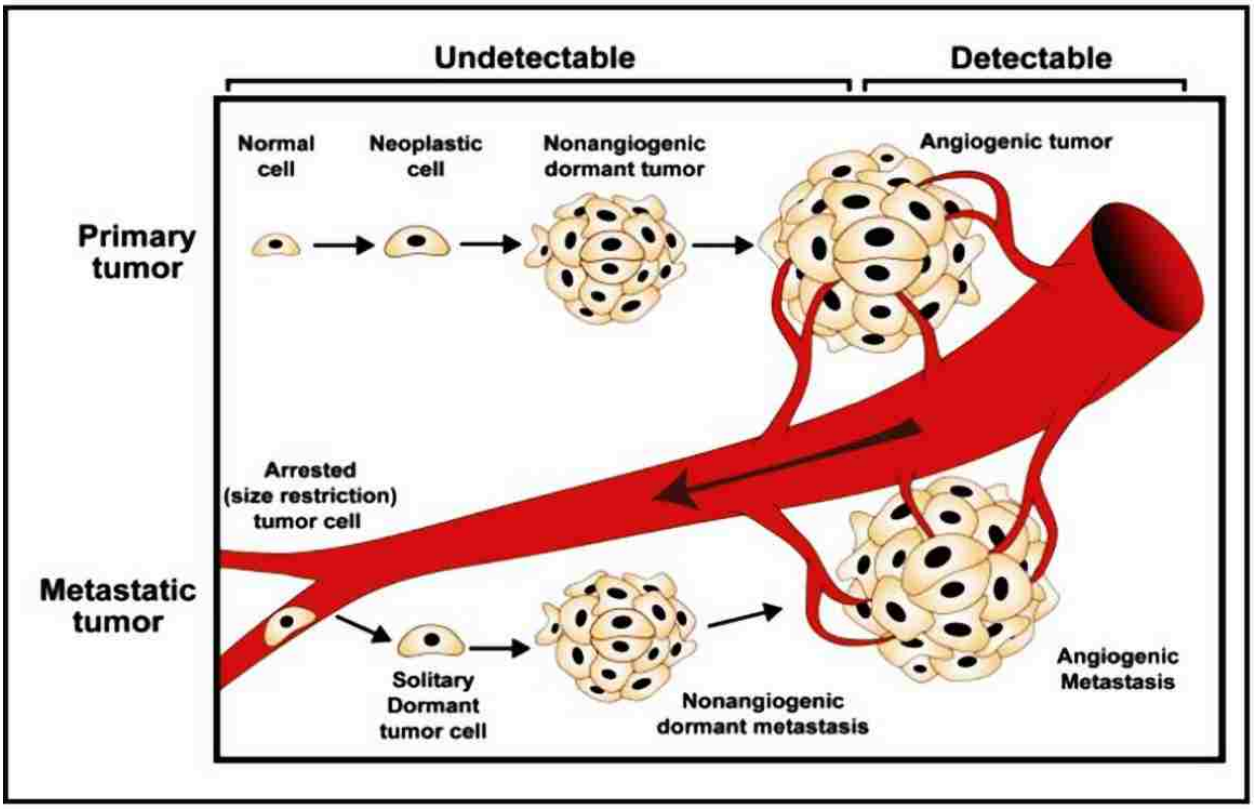


Figure 1.7. Progression of cancer cells at primary and metastatic sites [56]

Tumor cells can invade the adjacent tissue in form of epithelial sheets, detached clusters, single cells or amoeboid cell types [57]. Morphological and phenotypical changes that are seen in metastasizing tumor cells are epithelial to mesenchymal transition (EMT), collective to amoeboid transition (CAT) and mesenchymal to amoeboid transition (MAT) as illustrated in Figure 1.8.

EMT represents a crucial event in metastasis of cancer cells. This transformation of epithelial cells provides the cells with capabilities to invade, to resist apoptosis and to disseminate. EMT is regulated by a set of transcriptional factors like Snail, Slug, Twist, and Zeb1/2 [58]. Changes that are induced in cellular traits by these transcriptional factors are transformation from a polygonal/epithelial to a spindly/fibroblastic morphology, expression of matrix degrading enzymes, resistance to apoptosis, and loss of adherence and high motility which all contributes to

invasion and metastasis of cells [59]. Reversal of EMT or mesenchymal to epithelial transition (MET) also occurs in cancer cells after invading and metastasizing in order to re-colonize at a secondary location and this feature helps cancer cells retain their histopathologic properties [60].

Besides EMT or mesenchymal invasion, the other two modes of invasion are collective and amoeboid form of invasion. Cancer cells that undergo collective invasion maintain their intact epithelial cell to cell contact and are infrequently metastatic. Unlike collective and mesenchymal transition, the amoeboid kind of transition is protease-independent and instead of making a passage in the extracellular matrix, involves sliding of cells through spaces in the extracellular matrix, and is the fastest migratory phenotype [57].

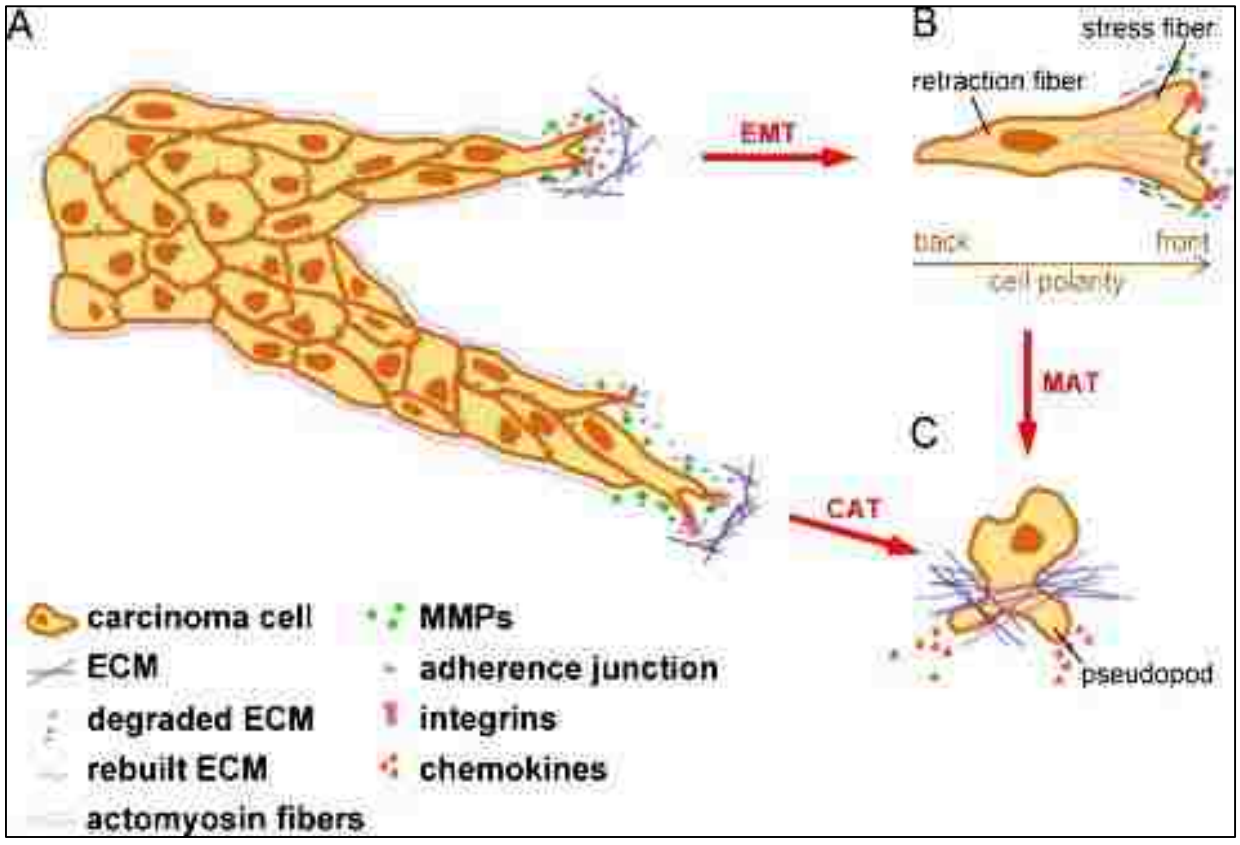


Figure 1.8. Ongoing changes in invading cancer cells [54]. A. Sheets of cancer cells. B. Single mesenchymal cell. C. Amoeboid cell movement. CAT, collective to amoeboid transition; EMT, epithelial to mesenchymal transition; MAT, mesenchymal to amoeboid transition; MMP, matrix metalloproteinase.

1.5.7. Reprogrammed Energy Metabolism

Cancer cells require many nutrients and energy to fuel the ongoing metabolism in order to maintain uncontrolled growth and proliferation. Changes in energy metabolism in cancer cells are considered as a key regulator of tumorigenesis and has been added to the list of general hallmarks of cancer [61].

For energy metabolism in normal cells in an aerobic condition pyruvate generated from glycolysis is further converted to acetyl-CoA and CO_2 via tricarboxylic acid (TCA) cycle and oxidative phosphorylation with generation of 36 molecules of ATP per glucose molecule and only in anaerobic condition pyruvate is converted to lactate. But in tumor cells, even in presence of oxygen, most of the pyruvate generated through glycolysis is converted to lactate and only yields 2 ATPs. This phenomenon was first observed by Otto Warburg in the 1930s and is termed "aerobic glycolysis" [61] and is illustrated in Figure 1.9. To compensate the lower yield of ATPs, glycolysis is highly upregulated in cancer. Upregulation in glucose uptake and utilization via elevated expression of glucose transporters like GLUT 1 and activity of glycolytic enzymes in cancer cells are associated with higher expression of oncogenes like Ras and myc [62].

Glycolytic switch benefits cancer cells by utilization of intermediates formed during glycolysis in the biosynthesis of macromolecules, lipids, nucleotides, amino acids and NADPH [63]. For instance, metabolic intermediates like glucose-6-phosphate is used in the synthesis of nucleic acids [64]. This formation and accumulation of biomass is eventually used in the promotion of growth and proliferation of cancer cells. Besides that, lactate formed via aerobic glycolysis aids invasion and metastasis as it lowers the pH of the surrounding environment, which activates metalloproteases and helps in tumor progression by degradation of the extracellular matrix [65].

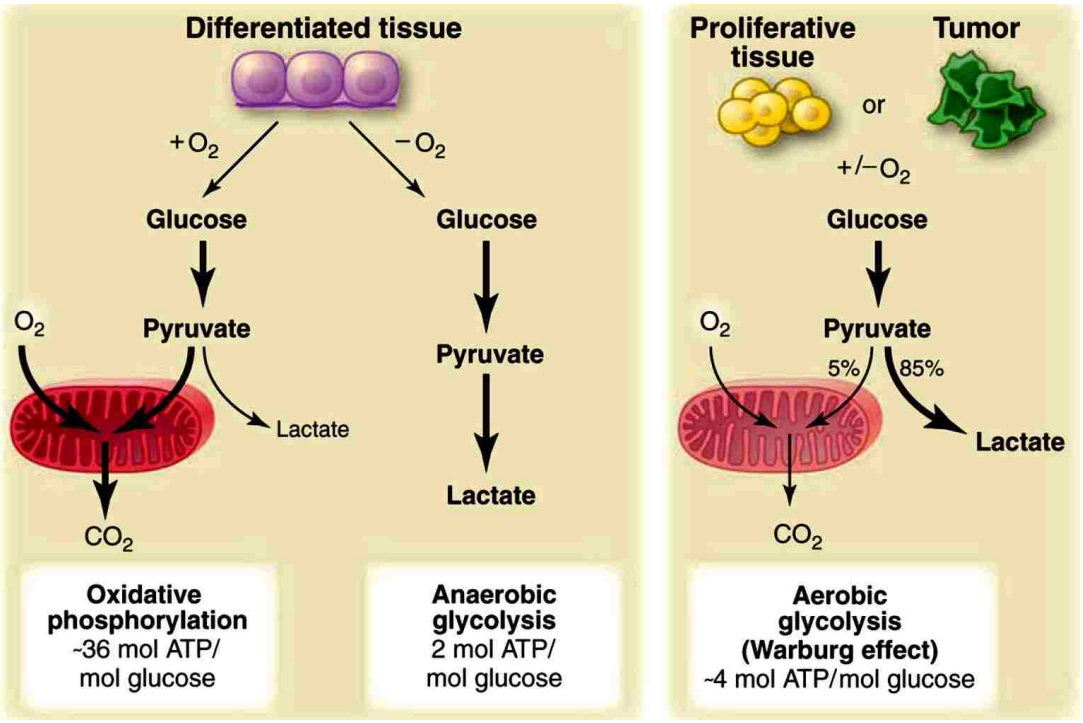


Figure 1.9. Schematic representation of oxidative phosphorylation, anaerobic glycolysis and aerobic glycolysis [66]

1.5.8. Evasion of Immune Surveillance

Immuno-evasion by cancer cells is another feature of cancer cells that has been added to the existing list of hallmarks. The immune system of the body is responsible for the recognition and elimination of cancer cells before they are established as a tumor mass. However, cancer cells go through immune-editing and are found to escape detection and decrease the immunological deleterious effect. Cancer cells disable the immune recognition system and evade immune-surveillance. For example, the action of cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells are restricted by release of immunosuppressive factors such as TGF-β [67].

The increased rate of cancer prognosis in colon and ovarian cancer patients with high CTLs and NK cells depicts the importance of the immune system in anti-tumorigenesis. [68-70]. It has also been shown that an immune-compromised organ recipient has a higher chance of donor

derived cancer development. This suggests that the tumor free donor had a dormant state of cancer cells well monitored by the active immune system [71].

1.6. Pancreatic Cancer

Pancreatic cancer (PC) is the fourth leading cause of cancer related death in the United States. According to the American Cancer Society, in 2015 in the US, the estimated number of new cases of PC was 48,960 and number of deaths was 40,560 [72]. PC is highly chemo-resistant and the median survival rate due to PC is six months while the five years survival rate is less than 3-5% [73]. According to the Surveillance, Epidemiology and End Results registries, data on treatment outcomes of patients from last three decades (1981-2010) have been analyzed and it has been reported that there was a mere improvement in 1 year survival from 17% to 28.2% whereas improvement in long term survival (≥ 5 years) remained low from 3.1 to 6.9% [74]. Several risk factors have been identified for PC, which includes family history, smoking, alcohol, diet, diabetes mellitus, obesity, and some genetic disorders [75].

The pancreas plays a major role in the digestion process and in maintenance of glucose homeostasis. Exocrine and endocrine compartments are present in the pancreas for its functionality. Exocrine compartment is primarily responsible for the production of digestive enzymes and endocrine for hormones insulin and glucagon. Because of the variation of cell composition in the pancreas, the types of tumors found in the pancreas are different. This study is focused on pancreatic ductal adenocarcinoma or pancreatic cancer, which usually refers to exocrine centered pancreatic tumor, for which there is a great need for therapies [76].

1.6.1. Risk Factors Associated with Pancreatic Cancer

Several risk factors associated with pancreatic cancer can be classified into exogenous and endogenous types. Smoking, alcohol, diet, and workplace exposure are some of the examples of

exogenous risk factors. Inherited predisposition of family history, Peutz-Jeghers syndrome, familial atypical multiple mole melanoma (FAMMM) syndrome, hereditary nonpolyposis colorectal cancer (HNPCC), hereditary pancreatitis, chronic pancreatitis, and diabetes mellitus are categorized as the endogenous risk factors [75].

The risk of pancreatic cancer associated with cigarette smoking is 1.74-fold higher than the non-smokers [77]. Incidence of type II diabetes increases the risk 1.8-fold, and it is even worse in cases of newly diagnosed diabetes with an increased risk of 2.9-fold [78, 79]. Individuals with body mass index (BMI) of >35 have a 1.55 fold higher risk than the ones with BMI of 18.9-24.9 [80]. Rate of heavy alcohol consumption (more than six drinks a day) is also linked to higher incidence of pancreatic cancer with 1.46-fold increased risk than compared to cases with one drink per day [81].

The rate of incidence of pancreatic cancer in the African-Americans population is relatively higher [82]. The adjusted rates of pancreatic cancer in African American males are 17.5 per 100,000 whereas in white males the rates are 13.5 per 100,000. Similarly in females the rates are 14.8 per 100,000 in African American and 10.5 per 100,000 in white females [82].

1.6.2. Pancreatic Cancer Progression: From a Histological Perspective

Pancreatic ductal adenocarcinoma is the predominant form of pancreatic cancer which comprises more than 95% of pancreatic cancer. Similar to other types of epithelial cancers, pancreatic cancers do not arise *de novo* but undergo a series of stepwise development from premalignant lesions to invasive cancer. The three non-invasive forms of pancreatic cancer are: 1. pancreatic intraepthelial neoplasia (PanIN), 2. mucinous cystic neoplasm (MCN), and 3. intraductal papillary mucinous neoplasm (IPMN) [83]. PanINs are the most common type of precursor lesions in pancreatic cancer and are detailed below.

The standard nomenclature and classification of the precursor lesions are based on the grades of "pancreatic intraepithelial neoplasia" (PanIN). PanINs are small microscopic lesions in the smaller pancreatic ducts, which are less than 5 mm and are morphologically classified into three types: PanIn-1 (low-grade PanINs), PanIN-2 (intermediate grade PanINs), and PanIN-3 (high-grade PanINs) which further advances to the invasive carcinoma [83].

The histopathological features of each stage is shown in Figure 1.10. PanIN 1 can be either flat (1A) or papillary (1B). The nuclear polarity and round morphology of nucleus in the cells are conserved in PanIN-1 stage. The lesions in PanIN-2 bear more nuclear abnormalities, loss of nuclear polarity, pleomorphism (differences in size of nucleus), nuclear hyperchromasia (increased DNA content), and nuclear pseudostratification. Mitoses are generally not seen. PanIN-3 is also called as "carcinoma-*in situ*". Features of this lesion include enlarged nuclei, budding of clusters of cells in the lumen, recurrent mitoses, dystrophic goblet cells with nucleus towards the lumen and mucinous cytoplasm towards the basement.

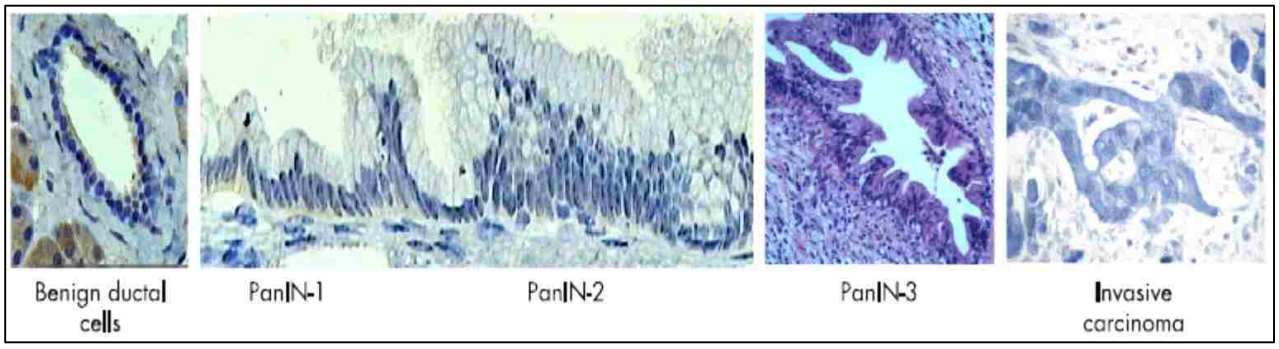


Figure 1.10. Histological images of benign pancreatic ductal cells, PanIN lesion and invasive carcinoma [84].

1.6.3. Abnormal Signaling Pathways in Pancreatic Cancer

Besides the histo-pathological changes in features, distinct genetic mutations occur in each grade of PanINs. Activation of Kristen Rat sarcoma virus oncogene homolog (KRAS), inactivation of INK4A, and depletion of tumor suppressor TP53 and Mothers against decapentaplegic homolog

4 (SMAD4) are some of the successive genetic mutations occurring in the process of progression of pancreatic tumors [85]. Pancreatic cancer harbors deregulation of 12 core signaling pathways with 63 types of abnormal genetic changes [86]. Some of the molecular pathways that are involved in survival, proliferation, progression, and the metastasis of pancreatic cancer are discussed below:

1.6.3.1. The Ras Activated Pathway

Mutations and deregulation in the KRAS signaling pathway are very common phenomena identified in 90% of pancreatic cancer [86-88]. KRAS mutation occurs in a very high frequency in PC [89, 90]. Mutation in the KRAS oncogene is one of the earliest events that take place in the progression towards the development of PC [91].

The Ras protein is localized at the inner wall of the cell membrane and acts as a principal signal regulator. It is encoded by the human *RAS* genes. In a normal physiological condition, the Ras protein usually cycles between "GDP-bound inactive state" and "GTP-bound active state". This cycle is regulated by the Guanine-nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs). The schematic diagram of this process is illustrated in Figure 1.11. The GEFs promote Ras activation by assisting in the exchange of GDP for GTP whereas GAPs hydrolyze the GTP and thus cause inactivation of Ras protein. On reception of signals from the growth factors, the bound GDP is removed by GEFs and activation of Ras protein occurs with binding to GTPs. The GTP bound Ras protein can further trigger kinase activity of down-streaming effector molecules like the Raf family of proteins and eventually initiate the MAPK cascade. This cycle of activation is terminated by GAPs, which converts GTP bound Ras to a GDP bound inactive form. But in cancer cells, Ras proteins are frequently mutated (single point mutations at residues G12, G13 or Q61) and because of these mutations, the Ras proteins are often insensitive to GAP activity which results in a constant GTP bound active state.

Activation of Ras protein is associated with, but not limited to the mitogen-activated protein kinase (MAPK), the Rapidly Accelerated Fibrosarcoma (RAF)/MAPK/ERK kinase (MEK)/ Extracellular signal-Related Kinase (ERK), and the Phosphatidylinositol 3-kinase (PI3K)/AKT signaling pathways for cellular proliferation, transformation and apoptosis. Aforementioned pathways are the key pathways activated in pancreatic cancer because of the frequent KRAS mutation and are briefly discussed below.

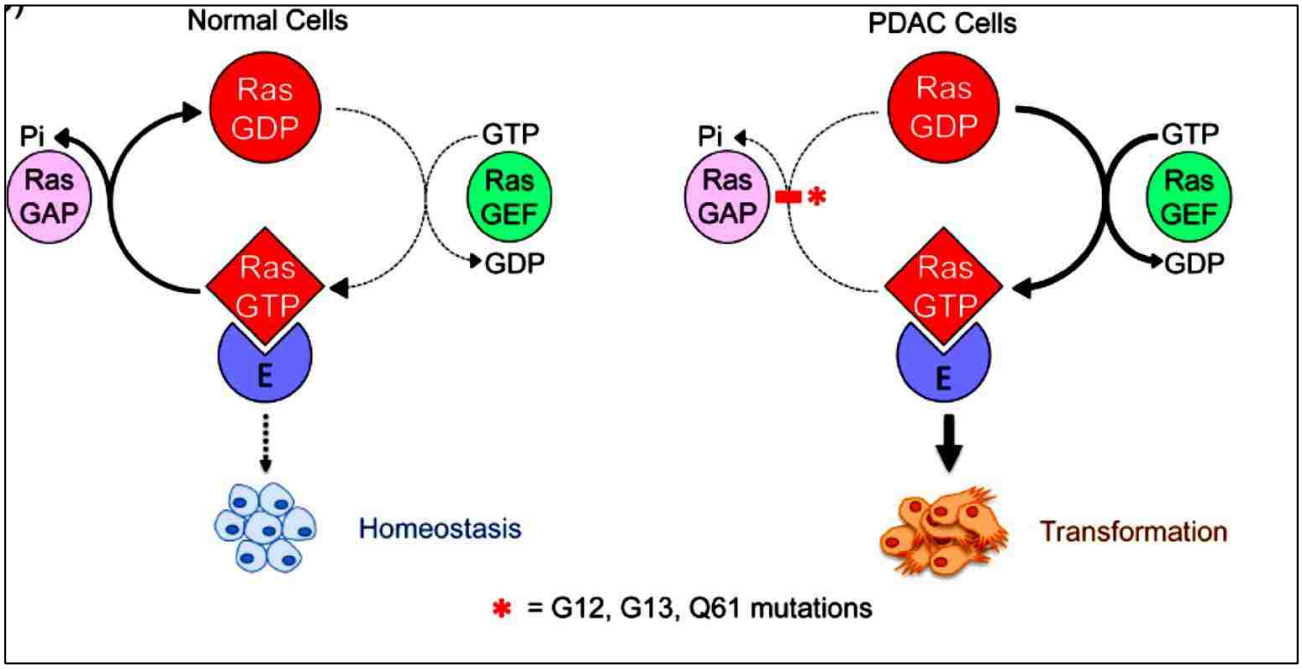


Figure 1.11. The cycle of Ras protein in normal cells and in pancreatic cancer cells. The activated state is GTP bound and the inactive state is GDP bound. Proteins like GEF and GAP aid in regulating the activation cycle of Ras protein.

1.6.3.2. The Ras/Raf/MAPK Signaling Pathway

The oncogenic Ras/Raf/MAPK pathway plays an important role in cancer cell growth and division, differentiation and migration, tissue healing and angiogenesis [92]. The Raf family proteins consists of serine/threonine kinases which bind to activated Ras. This further stimulates a series of phosphorylation and activates kinases belonging to the MAPK family including MEK 1/2, ERK1/2. Finally, the activated ERK1/2 translocate to the nucleus and activates transcription

factors and stimulates expression of growth factors, cytokines or growth regulators such as cyclins resulting in continuous proliferation of cancer cells. Cyclin D1, signal transducer and activator of transcription (STAT3), nuclear factor kappa B (NF-κB), and cyclooxygenase-2 (COX-2) are some of the downstream molecules linked to the ERK pathway [93]. It has been demonstrated that inhibition of these kinases result in cell cycle arrest and decreased proliferation of PC cells [94]. Currently there are thirteen ongoing clinical trials with MEK inhibitors in pancreatic cancer [79].

1.6.3.3. The PI3K/Akt Pathway

Even though KRAS is such a major element in PC, therapy directed at its expression has not been successful [95]. The other pathway that is activated with activated Ras is the PI3K/Akt pathway. The activated Ras complex can activate PI3K [96] which phosphorylates membrane bound phosphatidylinositol (3, 4)-biphosphate (PIP2) and forms phosphatidylinositol (3, 4, 5)-trisphosphate (PIP3). The phosphorylated PIP3 then attracts Akt or Protein Kinase B to the cell membrane [97]. Akt are a family of serine/threonine protein kinases that once phosphorylated further phosphorylates multi-targets. Once activated, Akt affects various downstream processes such as inhibition of apoptosis [98], activation of protein synthesis [99], inhibition of tumor suppressor proteins like Forkhead box O (FOXO3A) [100], stimulation of glucose uptake [101], and promotion of angiogenesis. In most of the cancers, the expression of PI3K and Akt is very high and is of major importance. Therefore targeting PI3K/Akt and MAPK signaling pathways is of great importance in anti-cancer therapy [102, 103]. There are at least 27 ongoing clinical trials with drugs aimed at targeting the PI3K/Akt pathway in pancreatic cancer [79].

1.6.3.4. The Epidermal Growth Factor Receptor (EGFR) Signaling Pathway

The epidermal growth factor family of receptors also plays a major role in pancreatic cancer cell growth and progression. There are four types of receptors within this family; EGFR1, HER2,

EGFR3 and EGFR4. The expression of these receptors is highly upregulated by KRAS and has shown to be crucial in KRAS associated carcinogenesis of the pancreas [104]. Its overexpression is associated with advanced disease, poor survival and metastatic pancreatic cancer [105]. The EGFR family possess a strong ability to activate both the MAPK and PI3K pathways. Pancreatic cancer MIA Paca-2 cells have been found to extensively express these receptors [106]. Figure 1.12. represents the schematic overview of pathways and cross talk involved with expression of EGFR.

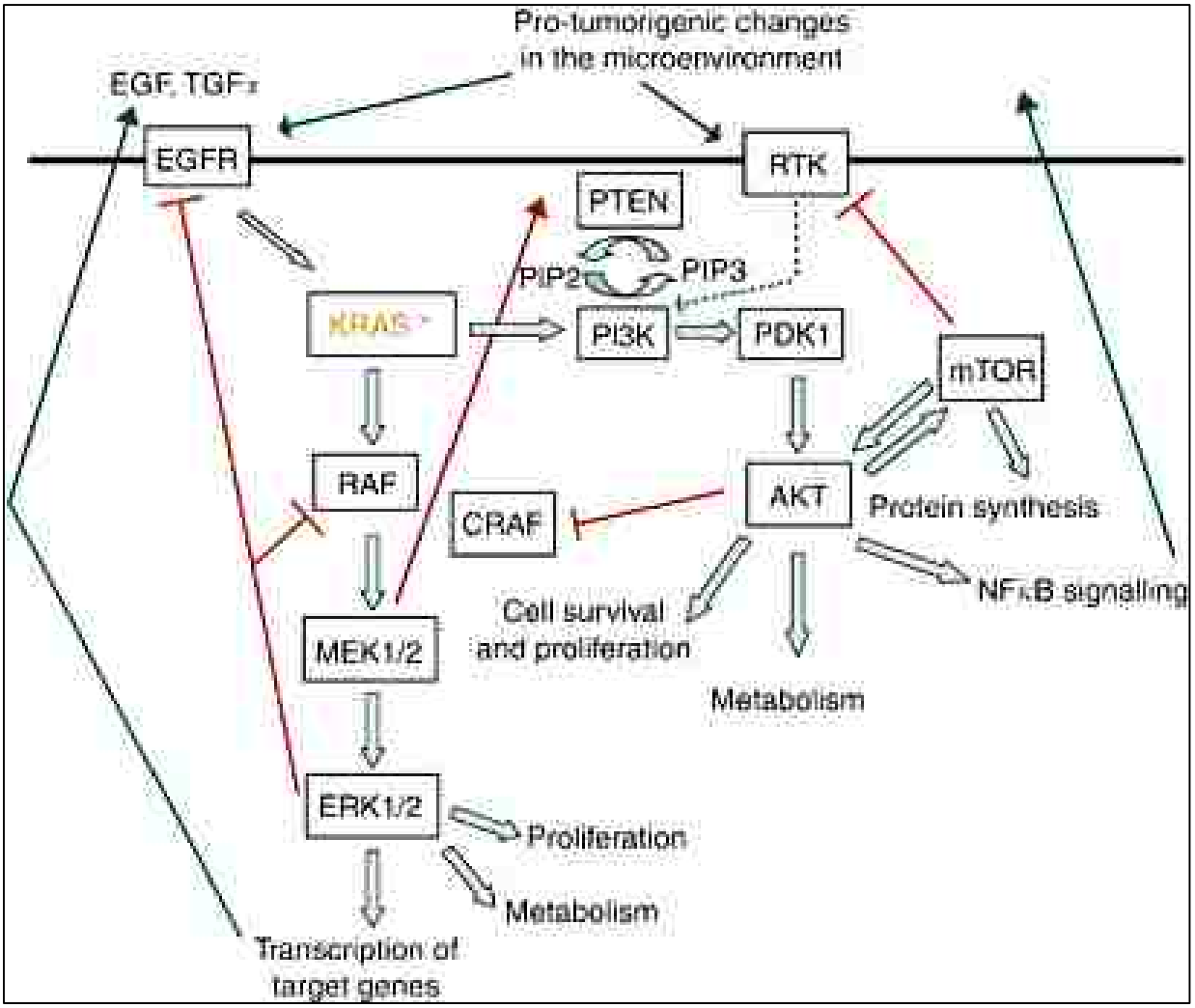


Figure 1.12. Schematic diagram of dysregulated expression of EGFR and oncogenic KRAS. Activation of the Ras/Raf/MEK/ERK and PI3K/Akt/mTOR signaling pathways is linked to pancreatic cancer. Adapted from Eser et al. [107].

1.6.3.5. Irregular Metabolism

KRAS oncogenes are also reported to be responsible for stimulation of genes responsible for glycolysis and glutaminolysis [108]. Normal differentiated cells depend upon mitochondrial oxidative phosphorylation for energy production but rapidly proliferating cancer cells undergo aerobic glycolysis which is also called the Warburg effect. Activation of NF-κB is also linked to increased Warburg effect in cancer cells. Increased Warburg effect is considered one of the hallmarks of cancer [109]. High NF-κB activity in cancer cells is also associated with down regulation of apoptotic proteins and development of chemo-resistance [110]. Therefore targeting NF-κB is one of the major steps towards drug development against cancer [111].

In normal cells, in the presence of oxygen, pyruvate generated by glycolysis is metabolized via the tricarboxylic acid (TCA) cycle generating CO_2 and adenosine triphosphate (ATP), but in absence of oxygen, pyruvate is converted to lactate, whereas in cancer cells, regardless of the presence or absence of oxygen, about 85% of pyruvate is metabolized to form lactate with very low generation of ATPs [66]. Previously, the process was misinterpreted that cancer cells have defective mitochondria, which was the reason for dependence of cancer cells in glycolytic process [112]. High rate of glycolysis is not efficient for cancer cells in terms of ATP production, however the glycolytic intermediates formed are used by the cancer cells for the synthesis of nucleotides, amino acids, and lipids, which are molecules required for the foundation of cellular structure and function [66].

Cancer cells harboring the Ras oncogene have high rates of glycolysis and the expression of glucose transporter GLUT1 facilitates a higher uptake of glucose [112]. Hexokinase, pyruvate kinase and lactate dehydrogenase are some of the many enzymes involved in the glycolytic pathway and account for the conversion of phosphoenolpyruvate to pyruvate and lactate. It has

been suggested that targeting these enzymes can inhibit the Warburg effect, one of the hallmarks of cancer [113].

Despite knowledge about molecular mechanism of PC, there is a limited progress in effective drug development because PC is highly resistant to therapeutic agents. Therefore there is a dire need in the improvement of pancreatic cancer research by finding for better chemotherapeutic approaches. Since oncogenic regulation of KRAS is one of the main common changes found in PC, studies are more focused towards targeting KRAS.

1.6.4. Therapeutic Approaches to Pancreatic Cancer

Treatment options for pancreatic cancer vary depending on the stages of tumor. The sign and symptoms for pancreatic cancer include indigestion, nausea, weight loss, back pain, jaundice and abdominal pain. These symptoms could be misinterpreted as signs of other diseases and dysfunctions. That is why, by the time a patient is diagnosed with pancreatic cancer, the median size of the tumor is already about 3.1 cm [114]. For a surgical resection, pancreatic tumors usually have to be less than 2 cm in diameter and without advanced metastasis [115]. As a result only 15-20% of the cases can be resected at the time of diagnosis [116]. Even after undergoing surgical resection, the 5-year survival rate of the patients is 20% [117]. Adjuvant therapies, chemo-radiotherapy followed by chemotherapy is provided to patients for prolonged survival after resection [118].

For locally advanced or metastatic pancreatic cancer, surgical resection is not suitable; rather chemotherapy is considered. Among various chemotherapeutic agents, gemcitabine is considered as the first line standard chemotherapeutic treatment approved by the FDA for advanced and metastatic pancreatic cancer [119]. Some of the other drugs that are administered for treatment of pancreatic cancer are FOLFOX, and FOLFIRINOX [120]. Gemcitabine only

increases the median survival from 4.5 months to 6 months [121]. Therefore to attain better clinical benefits of gemcitabine for a prolonged survival, several clinical trials have been carried out combining gemcitabine with other chemotherapeutic agents like cisplatin [122] and paclitaxel [123]. The combination therapy has proven to be better than gemcitabine alone, but still the rate of overall improvement is still low with deleterious side effects [124]. As a result there have been serious ongoing efforts in discovery of novel compounds for treatment of pancreatic cancer with better advantages. In this regard, bioactive components from plants and herbs are studied as the source of pharmaceutical agents.

1.6.5. Dietary Chemo-preventive Agents against Pancreatic Cancer

Anti-cancer properties of various dietary bio-actives from fruits, vegetables, nuts, or spices have been reported in literature. A recent review by Azimi et al. have summarized the data on the use of phytomedicine for the treatment of pancreatic cancer up to the year 2013 [120]. Phytochemicals have been shown to target the disease and are known for the ability to modulate various abnormal cell signaling pathways associated with cancer [125]. For that reason modification of dietary pattern can help prevent various forms of cancer including pancreatic cancer [126]. The common targeted mechanisms of phytomedicines in pancreatic cancer include antiangiogenic, autophagy, cell cycle arrest, cytotoxic, anti-metastatic, cytotoxic, and apoptotic activities [120].

There are 12 different frequently studied flavonoids namely; apigenin, genistein, quercetin, resveratrol [127], epigallocatechin gallate [128, 129], benzyl isothiocyanate, sulforaphane [130], curcumin [131], thymoquinone, dihydroartemisinin, cucurbitacin B and perillyl alcohol that target pancreatic cancer via 4 or more mechanisms [120]. These findings confirms the beneficial properties of phytochemicals. Many of the phytochemicals are already in human clinical trials with

positive outcome. For instance extract of ukrain (extract of *Chelimodium majus*) was shown to improve the quality of life of pancreatic cancer patients and also when given in combination with gemcitabine doubled the survival time [132] and extract of garlic was found to improve the activity of NK cells [133]. Cancer treatment and prevention using dietary phytochemicals has gained attention for their availability and essential biological activity.

1.7. Research Objective and Specific Aims

The main objective of this doctoral dissertation research is to evaluate the cytotoxic effect of bioactive natural quinoid compound juglone and to provide a better understanding of its effect on molecular mechanism associated with human pancreatic cancer. The molecular biology involved in the pathogenesis of pancreatic cancer has been considered in order to investigate the properties of juglone as an anti-cancer agent. Based on the evidence provided in the literature [134], targeting KRAS related signaling molecules can be a promising therapeutic strategy against pancreatic cancer and chemo-resistance [135]. We hypothesized that juglone acts as a cytotoxic agent against pancreatic cancer cells through down-regulation of critical oncogenic markers. The goal of this project was achieved with the following three specific aims:

- **Aim 1:** Screen the cytotoxic effect of juglone at various concentrations and determine the IC_{50} value in pancreatic adenocarcinoma cells
- **Aim 2:** Evaluate the mode of action and analyze the therapeutic efficacy of juglone in various signal transduction markers associated with pancreatic cancer
- **Aim 3:** Investigate the anti-angiogenic and anti-metastatic potential of juglone and examine its effect on modulation of key angiogenic factors active in PC cells

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CHAPTER 2. CYTOTOXICITY OF JUGLONE AND THYMOQUINONE AGAINST PANCREATIC CANCER CELLS

2.1. Introduction

The mortality rate due to pancreatic cancer in both men and women is very high [1]. For instance, in 2015 the estimated new cases of pancreatic cancer in the United States for male and female were 24,840 and 24,120 and the number of deaths for male and female were estimated to be 20,710 and 19,850 respectively [1]. The median five-year survival rate for pancreatic cancer patients is less than 5% [2]. Pancreatic cancer is usually diagnosed at an advanced stage and gemcitabine is the first-line chemotherapeutic agent that is used for the treatment [3]. The tumor response rate to gemcitabine is reported to be <10% [3] with a survival benefit of 5.8 months only. Moreover due to the development of chemo-resistance associated with the use of this drug, there is a very limited clinical benefit [4]. Therefore, there is an unmet need in the field of drug discovery processes for treatment of pancreatic cancer and novel strategies are required.

Juglone (5-hdroxy-1, 4-napthoquinone) is a naphthoquinone found in leaves, roots, husks and barks of the plants in the *Juglandaceae* family. Juglone is formed through the shikimic acid pathway [5]. It is basically dark orange-brown in color and is also used as a coloring agent. The concentration of juglone in the husk is 1.54-1.66 mg/g [6]. Juglone has anti-tumor effects on ovarian cancer cells [7], breast cancer cells [8], prostate cancer cells [9], colon cancer cells and cervical cancer cells [10]. However, the anti-proliferative effect of juglone in human pancreatic MIA Paca-2 cells has never been demonstrated.

Combinatorial effect using juglone in conjunction with thymoquinone (TQ) in PC cells survival is studied. TQ is a principle active component of *Nigella sativa* seeds' oil which belongs to *Ranunculacea*e family, and is considered as a compound of interest for its potential medicinal properties. It has been reported to be used in traditional medicine for the treatment of dysentery,

asthma, gastrointestinal diseases, hypertension and obesity [11]. TQ has also been proven to have antitumor activities in various kinds of cancers such as breast, colon, ovarian, lungs, and larynx [12] including pancreatic cancer [13].

The primary objectives of this research were to examine the effect of juglone on cell growth inhibition, colony formation, and apoptosis induction in pancreatic cancer MIA Paca-2 cells. The effect of juglone in combination with thymoquinone (TQ) was also investigated.

2.2. Materials and Methods

2.2.1. Reagents

Juglone, thiazolyl blue tetrazolium bromide (MTT), crystal violet, dimethyl sulfoxide (DMSO), and thymoquinone were purchased from Sigma Aldrich (St. Louis, MO); Trypsin EDTA, Dulbecco's Modified Eagle Medium (DMEM), Hoechst 3342, Phosphate Buffer Saline (PBS), penicillin streptomycin was obtained from Invitrogen (Carlsbad, CA) and fetal bovine serum was from Atlanta Biologicals (Flowery Branch, GA).

2.2.2. Cell lines and Culture Conditions

MIA Paca-2, PANC-1 and BxPC-3 are adherent epithelial type pancreatic carcinoma cell lines from ATCC (the American Type Culture Collection) and were generously provided by The William Hansel Cancer Prevention Laboratory, PBRC. As recommended by ATCC, the cell lines were maintained in culture in DMEM supplemented with 10 % fetal bovine serum, penicillin and streptomycin and grown at 37^{o}C in a humidified incubator with 5% CO_2 until 70-80% confluency. After reaching confluency, it was split at the ratio of 1:3 to1:8. The growth medium was changed 2-3 times a week and 0.25% trypsin EDTA was used to detach the cells for subculture.

2.2.3. Screening of Cytotoxicity

Stock solution of juglone was prepared in DMSO, the final concentration of DMSO when in culture was < 0.1%. In order to determine the dose- and time- dependent cytotoxicity of juglone, MIA Paca-2, BxPC-3, and PANC-1cells were plated at a cell density of 5 $x10^3$ cells/well in a 96-well plate. After seeding overnight, cells were treated with juglone and incubated for 4 h or 24 h. Cytotoxicity was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) assay. Cytotoxicity of juglone at 24 h was also tested against BxPC-3 and PANC-1.

After required incubation, 20 µl of MTT (5mg/ml) was added and incubated for 4 h. Formazan product formed from MTT was dissolved with addition of DMSO and absorbance was recorded at 590 nm. The number of viable cells is directly proportional to the absorbance at 590 nm. Cell viability results are shown as percentage of control. All experiments were performed in triplicates. Data collected from cytotoxicity assay were used to calculate the IC_{50} value for juglone.

2.2.4. Morphological Changes

Juglone-treated MIA Paca-2 cells were observed under inverted microscope and images were recorded at 10X magnification. To observe the change in nuclear morphology, cells treated with or without juglone were first fixed with 4% (v/v) formaldehyde for 10 min at RT, washed with PBS and stained with Hoechst 3342 (1 µg/ml) for at least 15 min. The nuclear morphology was observed with a fluorescent microscope at 40X magnification.

2.2.5. Long Term Survival Colony Formation Assay

The method of Woolston et al. was used [14]. Logarithmically growing MIA Paca-2 cells were plated at a density of 3.5 x 10^5 cells per well in a 6- well plate. After allowing the cells to attach properly, cells were treated with juglone for 6 h. After treatment, juglone-treated and untreated viable MIA Paca-2 cells ($2x10^3$) were immediately re-plated in a 6- well plate with fresh

medium containing no treatments. Cells were incubated at 37^{o}C in a humidified incubator with 5% CO_2. The plates were undisturbed during the entire incubation period and cells were allowed to grow until colonies consisting of more than 50 cells were seen, which was equivalent to about 12 days for MIA Paca-2 cells.

After colonies were formed, growth media was aspirated and cells were washed with PBS. Colonies were fixed with sufficient volume of methanol: saline (1:1) to cover the colonies and the methanol: saline solution was removed after 15 min. Methanol (100%) was added to the colonies and after 15 min colonies were stained with 0.5% crystal violet (CV) solution for at least 30 min at RT. CV solution was aspirated and colonies were washed carefully with an indirect flow of water. Plates were inverted and allowed to dry at RT. Experiments were conducted in triplicates. Cell colonies containing at least more than 50 cells were counted manually. Retained CV by colonies was dissolved with 33% acetic acid, optical density was read and results from juglone-treated colonies were compared to colonies formed with untreated MIA Paca-2 cells.

2.2.6. Detection of Apoptosis by Annexin V Staining and Propidium Iodide Labeling

To confirm apoptosis, the translocation and externalization of phospholipid phosphatidylserine from cytoplasm to extracellular surface was detected by Annexin V staining and PI staining [15]. Annexin V- FITC Apoptosis detection kit was purchased from Sigma. Cells were plated in 6-well plates at a cell density of 350 K cells/well. After treatment with juglone for 24 h, cells were collected with 0.25% trypsin, centrifuged at 1000 rpm for 5 min and re- suspended in the supplied binding buffer. Cells were stained with Annexin V-FITC following the manufacturer's protocol (Sigma). FITC (fluorescein isothiocyanate)-conjugated Annexin V was added to the cell suspension at a ratio of 1:100 and propidium iodide (PI) at 1:50. Cells were incubated for at least 10 min in the dark. Stained cells were analyzed by flow cytometry.

2.2.7. Drug Combinatorial Effect of Juglone with Thymoquinone

Combination therapy is mostly used in the drug development process to target multi oncogenic markers and avoid drug resistance. In this experimental design, the effect of the combination of juglone and TQ on MIA Paca-2 cells was also evaluated. When two or more drugs are combined, there are three possible outcomes with treatment; synergy, additive effect or antagonist effect. There multi-drug effect was evaluated using the combination index method of Chou and Talalay [16, 17].

In order to determine whether Jug had an additive, synergistic or antagonistic effect with TQ first the IC_{50} for each drug had to be established. MTT Assay as described in section 2.2.2 was performed using TQ to determine its IC_{50}. The dose range that would be used for drug combination was designed as explained by Chou and Talalay [17]. A total of six different concentration combinations (at least two data points above and below the IC_{50} and a zero concentration) with three replicates per condition were used. As recommended by Chou [16], 'Constant combination ratio' was used where two drugs were mixed simultaneously at their respective IC_{50} value and dilutions were made accordingly. For that, serial dilution of both drugs was performed yielding 4, 2, 1, 0.5, 0.25, 0.125 times the IC_{50} of each compound and MIA Paca-2 cells were treated at the respective concentrations. After 24 h of incubation, MTT assay was performed as described earlier.

The affected fraction of cells after treatment was then calculated and the dose-effect curves for juglone or TQ alone and for two-drug combinations juglone-TQ were created. Isobolograms were made using the values for affected fraction of cells and combination index (CI) was also determined using CompuSyn software. The CI value correlates with the effect of combination treatment. A CI of < 0.9 is considered synergistic, a CI of $\geqslant 0.9$ or $\leqslant 1.1$ is considered additive, and a CI of > 1.1 is considered antagonistic [17].

The formula for CI for two drug combinations [16, 18] is:

$$CI = \frac{C_{Jug,50}}{IC_{50,Jug}} + \frac{C_{TQ,50}}{IC_{50,TQ}}$$

Where $C_{Jug,\ 50}$ = Corresponding concentration of juglone at the IC_{50} level of drug combination
$C_{TQ,\ 50}$ = Corresponding concentration of TQ at the IC_{50} level of drug combination
$IC_{50,\ Jug}$ or $IC_{50,\ TQ}$ = IC_{50} for juglone or TQ alone

2.2.8. Statistical Analysis

All the experiments were conducted in triplicates. Results are expressed as means ± SD of experiments. Analysis of variance (ANOVA) was conducted to examine the differences between treatments followed by Tukey analysis using SAS software (Cary, NC). A P-value of <0.05 was considered to be statistically significant and indicated with different letters.

2.3. Results

2.3.1. Effect of Juglone on Pancreatic Cancer Cell Viability

To determine the effect of juglone in various types of human pancreatic cancer cells, BXPC-3, Panc-1 and MIA Paca-2 cells were used. Cancer cells were treated with equimolar concentration of juglone for 24 h. Results in Figure 2.1. show that juglone significantly inhibited the growth and proliferation of all of the pancreatic cancer cells investigated. However, the inhibitory effect of juglone was higher in MIA Paca-2 cells than BXPC-3 or Panc-1 and among these pancreatic cancer cell lines MIA Paca-2 cells contains high level of mutant KRAS whereas BxPC-3 has the wild type KRAS [19, 20]. Therefore, remaining experiments to achieve the outlined aims of this study were done using MIA Paca-2 cells.

Figure 2.2. shows that juglone exhibited cytotoxic effect in MIA Paca-2 cells in a concentration- and a time-dependent manner. In order to calculate the IC_{50} value of juglone in MIA Paca-2 cells, MTT assay was performed. Cells were treated with at least seven different

concentrations of juglone in a range of 0.5 µM-20µM for 24 h. Juglone significantly inhibited the proliferation of MIA Paca-2 cells in a dose-dependent manner.

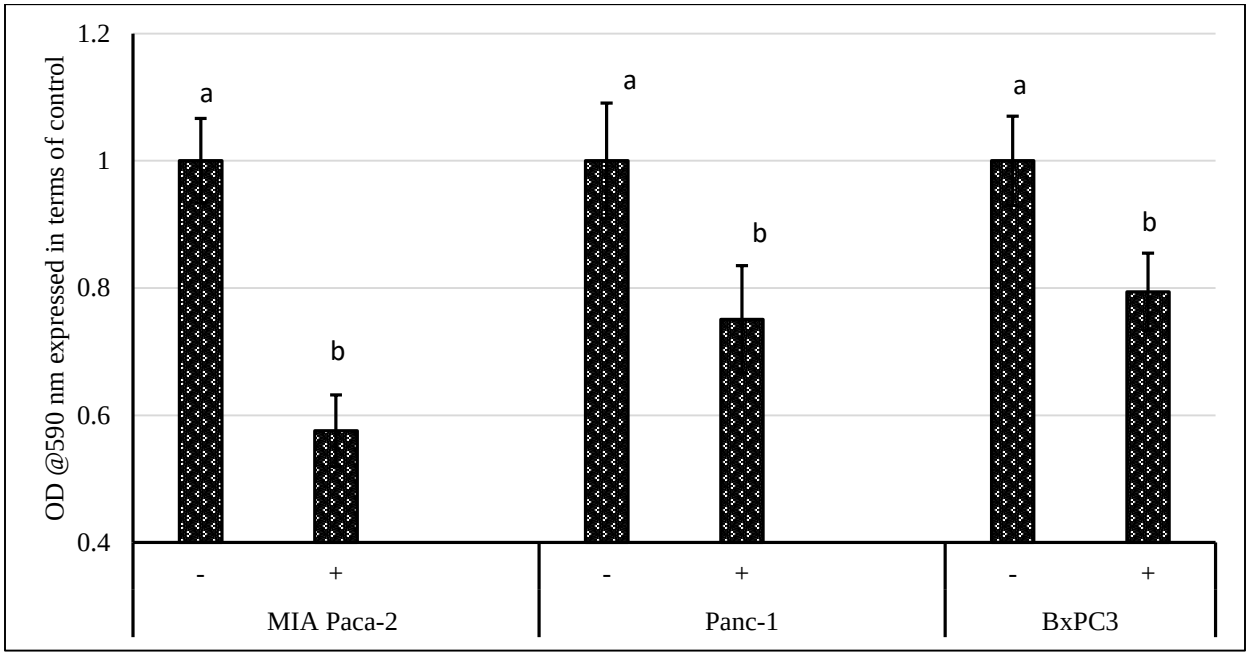

Figure 2.1. Comparative inhibitory effect of juglone (+) at equimolar concentration on the proliferation of various types of pancreatic cancer cells.

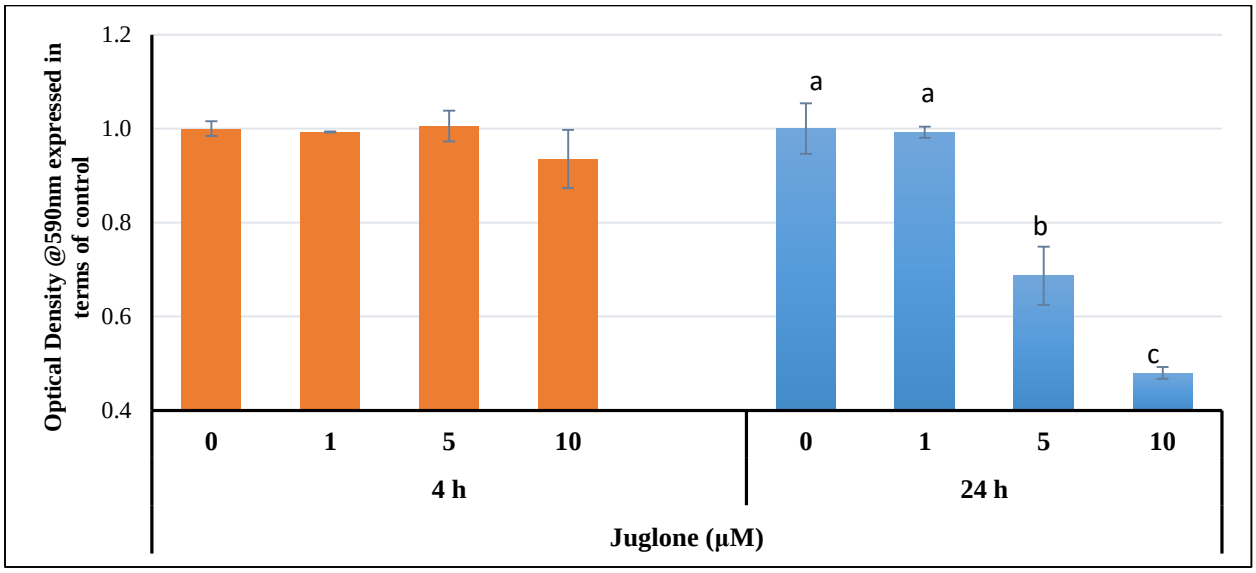

Figure 2.2. Concentration and time dependent inhibition of juglone on the growth of MIA Paca-2 cells.

Fractions of affected cells at various concentrations were calculated and a dose effect curve was created as shown in Figure 2.3. The IC_{50} value of juglone in MIA Paca-2 cells was calculated

using the concentration effect relationship and reported to be 5.05 µM. These data indicate that juglone exerts concentration- and time- dependent growth inhibitory effect in pancreatic cancer cells.

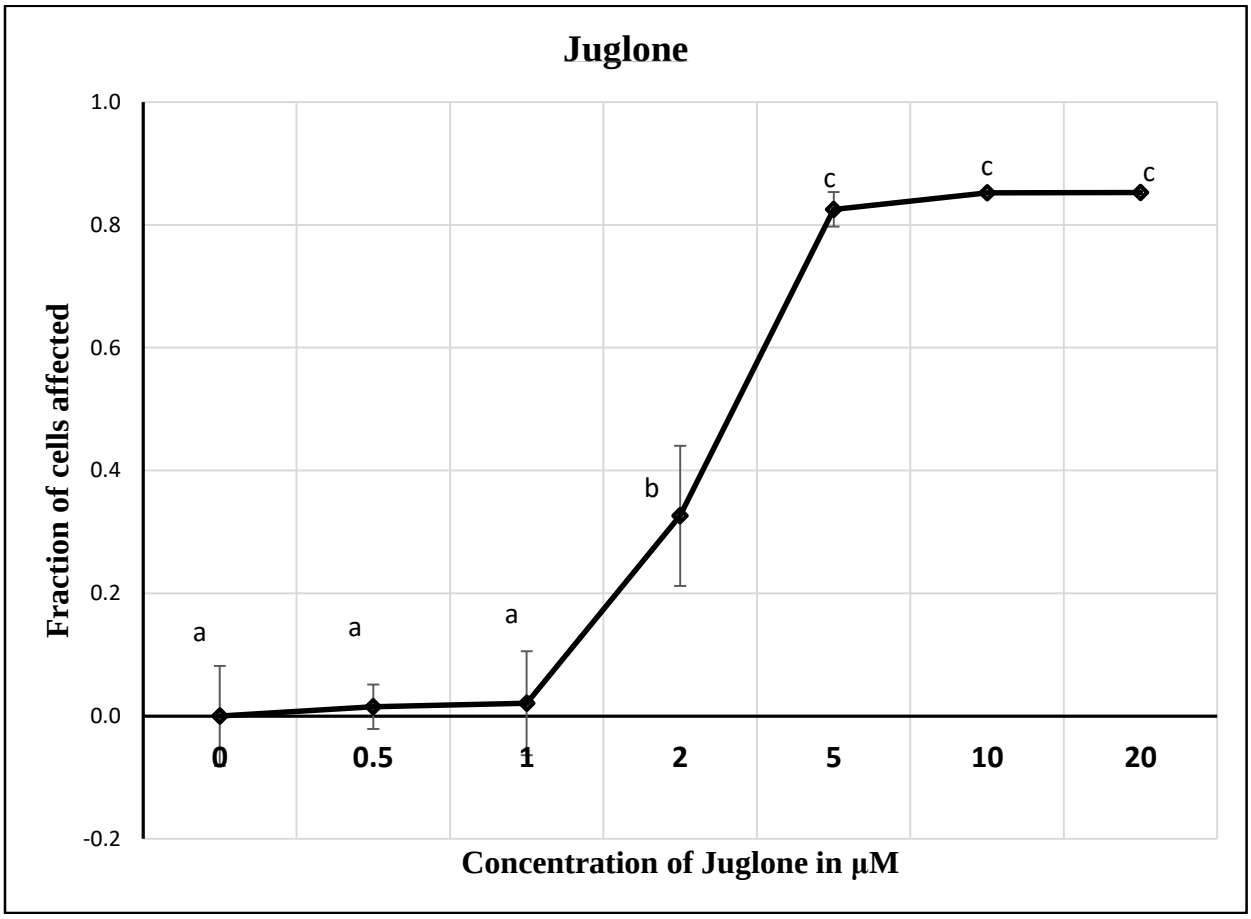

Figure 2.3. Dose-effect curve of juglone on MIA Paca-2 cells on the basis of the results from the MTT assay.

2.3.2. Juglon**e** Induced Morphological Changes in Pancreatic Cancer MIA Paca-2 Cells

Results indicate that treatment with juglone controlled the proliferation of pancreatic cancer cells. Morphological changes such as cell shrinkage, nuclear condensation and formation of apoptotic bodies are observed in apoptotic cells. Chromatin condensation is one of the hallmarks of apoptosis. As shown in Figure 2.4., untreated cancer cells had normal morphology, and possessed cell to cell contacts, but when treated with juglone, the cells shrunk and rounded up, an indication that cells underwent apoptosis.

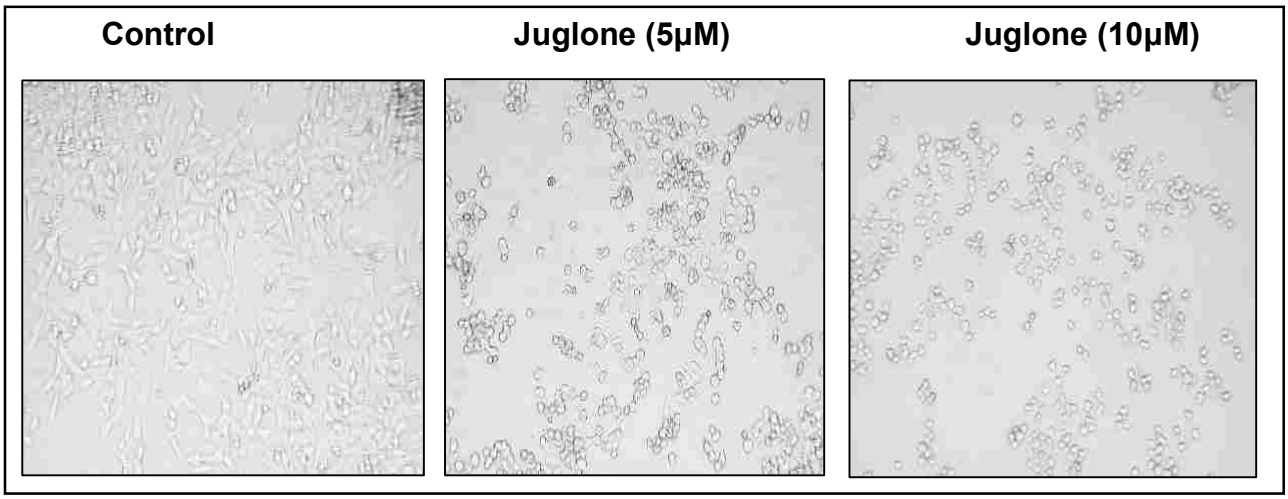

Figure 2.4. Morphological changes in juglone-treated MIA Paca-2 cells. Images were recorded at 10X magnification.

The nucleus of cells treated with or without juglone for 24h was also observed by Hoechst staining with a fluorescent microscope. Results of nuclear morphology as depicted in Figure 2.5. indicate that the untreated cancer cells had intact nuclear architecture whereas presence of small, condensed and bright nuclei and apoptotic bodies were seen in juglone-treated MIA Paca-2 cells due to the cytotoxic effect of juglone.

2.3.3. Effect of Juglone on Colony Forming Ability of Pancreatic Cancer MIA Paca-2 Cells

Clonogenic assay has been widely used to assess the sensitivity of cancer cells to anti-cancer drugs. To determine long term effect of juglone on survival and proliferation, clonogenic assay was performed. Results of colony formation assay are presented in Figure 2.6. and show that there was a dose-dependent inhibition of juglone on clonogenicity of pancreatic cancer cells.

Formed colonies were stained with CV (Figure 2.6., A). Quantification of the colonies formed were either done manually or using a spectrophotometer. Retained CV in the colonies was dissolved using 33% acetic acid, optical density was measured, and results were expressed in percentage of control (Figure 2.6., B). Colonies were also counted manually and the number of

colonies formed are presented in Figure 2.6., C. MIA Paca-2 cells treated with juglone at 5 and 10 μM for 6 h significantly lost the ability to form colonies.

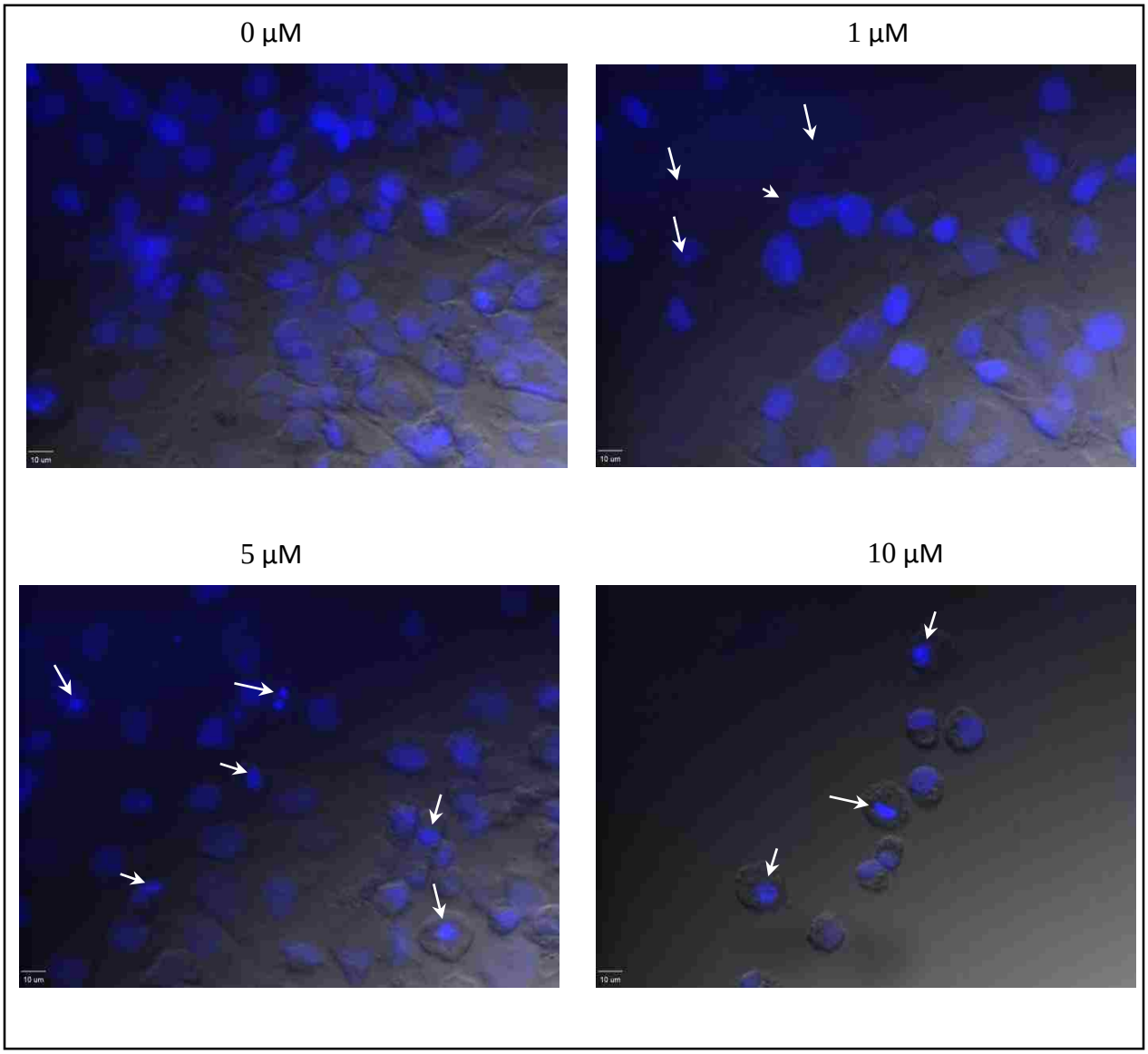

Figure 2.5. Changes in cellular and nuclear morphology of MIA Paca-2 cells upon treatment with juglone. MIA Paca-2 cells were treated for 24 h and then fixed and stained with DNA binding dye Hoechst 3342 (1μg/ml). Images were captured with a white field confocal microscope. Differential interference contrast (DIC) gray scale images of cells with blue nuclei in blue color are shown. Small, condensed and fragmented nuclei was observed in juglone-treated cells. Juglone-treated cells lose their cellular integrity and appear smaller and less dense than the untreated MIA Paca-2 cells. Arrows indicate chromatin condensation and apoptotic bodies. Scale bar represents 10 μM.

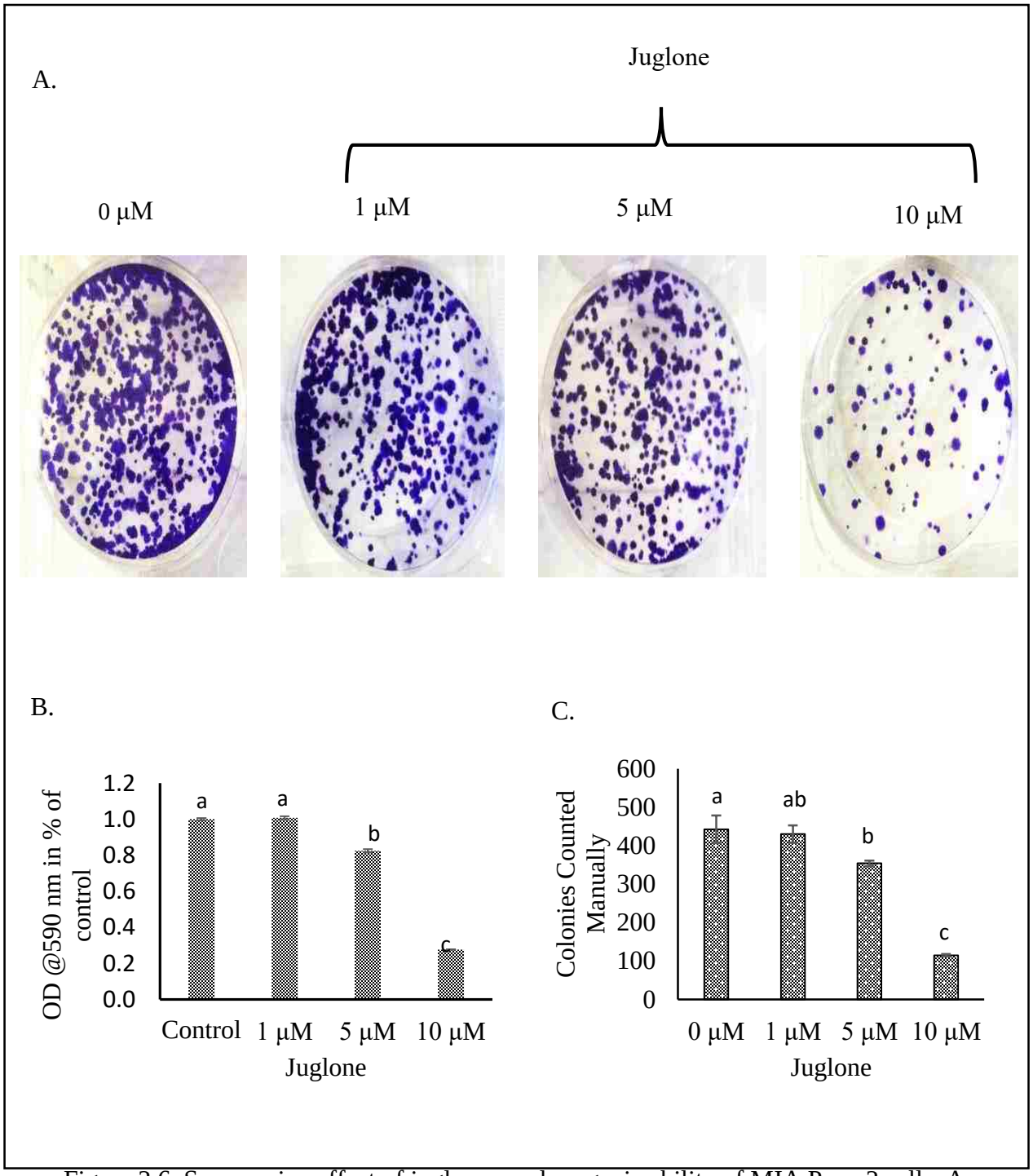

Figure 2.6. Suppressive effect of juglone on clonogenic ability of MIA Paca-2 cells. A. Representative images of colony formation assay. MIA Paca-2 cells were treated with juglone for at least 6 h and the treated cells were plated and incubated for 12 days. Formed colonies were stained with crystal violet. B. Retained CV by the colonies were dissolved in 33% acetic acid and optical density was measured at 590 nm and reported in terms of control. C. Colonies were also manually counted and reported.

2.3.4. Juglone Induced Apoptosis of MIA Paca-2 Cells

Apoptotic cell death was identified by double staining with FITC-conjugated Annexin V and PI using flow cytometry. In a live cell, the phosphatidylserine molecules are located in the inner surface of the cell membrane and are not accessible to the annexin molecules for binding as illustrated in Figure 2.7. Externalization of the phosphatidylserine (PS) molecules from inner to outer membrane in cells is a very common feature of cells in the early stage of apoptosis. Annexin V is a special protein that specifically binds to the PS molecules, and when conjugated to fluorescent dye like FITC, makes it possible for detecting apoptosis with a flow cytometer. When cells die, they also lose membrane integrity, permeability in the plasma membrane mostly occurs in the late stage of apoptosis. Dyes such as propidium iodide that only binds to the nucleic acids of cells get access into the cell only during the late stage of apoptosis when the membrane of the cells are permeable. Therefore, labeling cell with Annexin V and PI distinguishes and confirms if cells are viable or are in an early or a late stage of apoptosis.

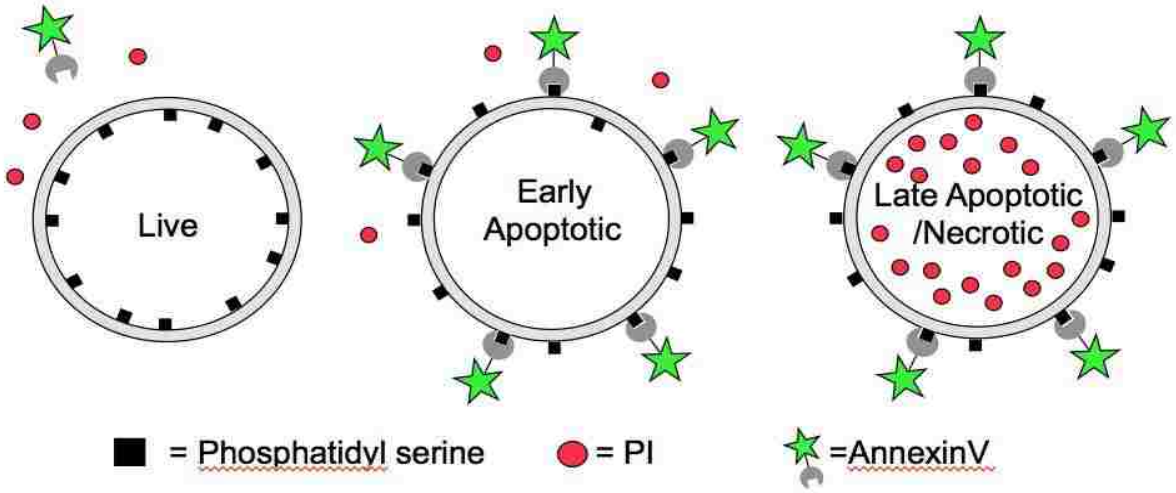

Figure 2.7. Schematic diagram demonstrating the principle of Annexin V and PI (propidium iodide) staining.

MIA Paca-2 cells were treated with juglone at 1, 5 or 10 µM concentrations and co-stained with Annexin V and PI. Cells in the early phase of apoptosis are located in the LR quadrant

(Annexin V^{+ve}/PI^{-ve}), those in the late stage of apoptosis in the UR quadrant (Annexin V^{+ve}/PI^{+ve}), and viable cells (Annexin V^{-ve}/PI^{-ve}) in the LL quadrant. The results as depicted in Figure 2.8. show that apoptosis was observed in juglone-treated MIA Paca-2 cells within 24 h. Results from flow cytometry are presented in dot-plot graph (Figure 2.8., A). The graph is divided into four quadrants; Lower Left (LL), Lower Right (LR), Upper left (UL) and Upper Right (UR). The LL quadrant represents Annexin V negative and PI negative cell population, the LR quadrant represents Annexin V positive and PI negative cell population and UR quadrant represents both Annexin V and PI positive cell population.

Our results show that when MIA Paca-2 cells were treated with increasing concentration of juglone, the percentage of cells in the early and late stage of apoptosis increased simultaneously. At 1 µM of juglone , ~42.31 % of cells were in the early stage of apoptosis, ~ 35.92% cells in the late stage of apoptosis and ~20.13% cells were viable. When cells were treated with 5 µM of juglone, ~52.09% of cells were in the early stage of apoptosis, ~68.06% cells in the late stage of apoptosis and ~2.44% cells were viable. At 10 µM juglone, most of the cells were in the late stage of apoptosis with ~14.63% in the early stage of apoptosis, ~85.05% in the late stage of apoptosis and only ~1.16% cells were viable. These data strongly show that upon treatment with juglone MIA Paca-2 cells undergo apoptosis.

2.3.5. Combinatorial Effect of Juglone and Thymoquinone

To determine the drug combination effect, first IC_{50} value of each compound was determined by MTT assay. As described in section 2.3.1, IC_{50} value for juglone was found to be 5 µM. From the dose effect curve (Figure 2.9.), IC_{50} value for thymoquinone was found to be 24.15 µM. After determining the IC_{50} values, juglone and thymoquinone were mixed at a constant ratio and combination index was determined at each concentration using Compusyn software.

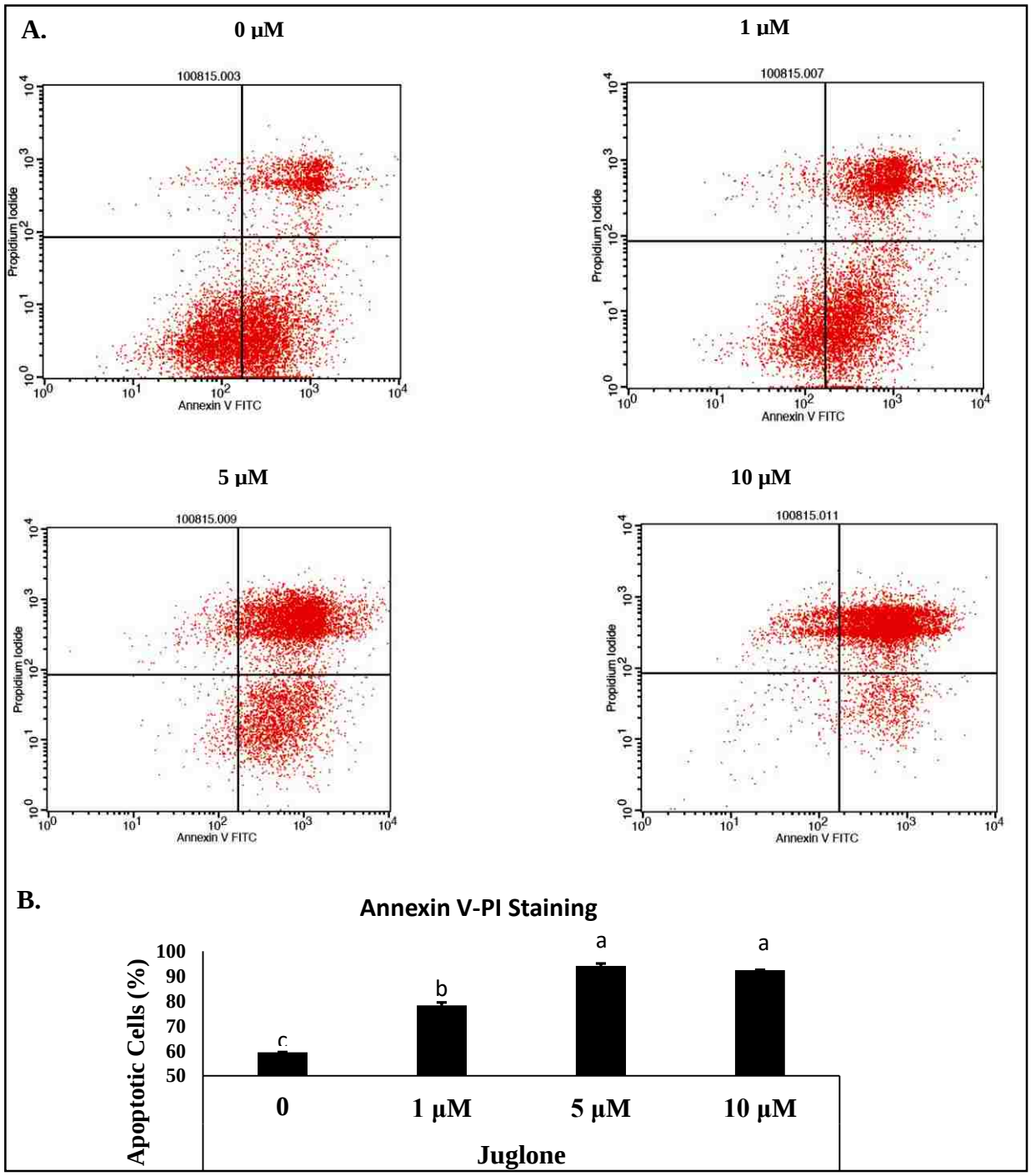

Figure 2.8. Juglone induces apoptosis in MIA Paca-2 cells. Cells were treated with juglone as indicated and then stained with annexin V-FITC and PI. A. Results from flow cytometry analysis are presented in a dot-plot graph. B. Percentage of apoptotic cells (in the early and late stage of apoptosis).

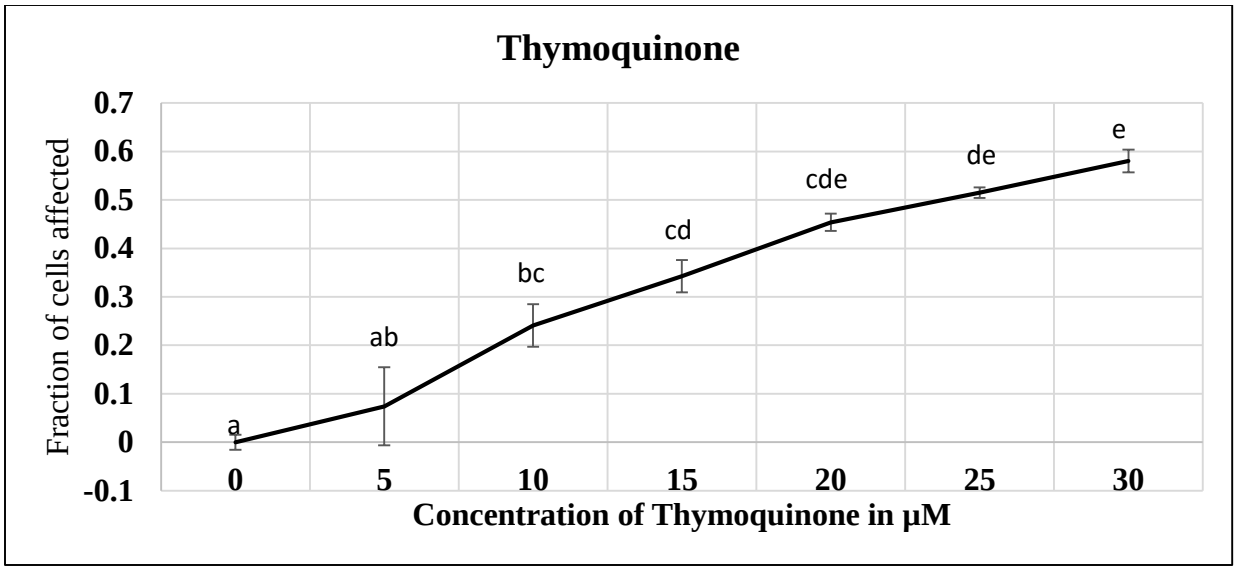

Figure 2.9. Dose-effect curve of thymoquinone against MIA Paca-2 cells on the basis of the results from MTT assay.

The effect of juglone in combination with TQ is summarized in Tables 2.1., 2.2., and 2.3. The IC_{50} value for juglone and TQ in combination was found to be 24.75 µM, which was higher than juglone or TQ alone. At concentrations where 10, 20 or 50% of cells were affected, there existed a moderate antagonistic relationship between juglone and TQ (Table 2.2.) as indicated by the combination index (CI) value determined by the Compusyn software. At concentrations that affected 75% and 90% of cells, there were nearly an additive effect with CI value of 1.09249 and 0.92391, respectively. Moderate synergism was only seen at concentration where 95% of cells were affected, and the corresponding concentration of juglone and TQ at that combination was 40.8950 µM and 511.1870 µM respectively.

The graphical representation of combinatorial effect of juglone and TQ are also presented via isobolograms (Figures 2.10. and 2.11.) that were created using Compusyn software. The nature of interaction between two drugs can be evaluated by isobologram analysis as well in a two co-ordinate plot. The x and y coordinates in an isobologram represent the concentrations of individual drugs required to produce a defined effect. Respective line connecting two data points in two

different axes is the line of additivity. Points lying below the line of equivalency or line of additivity in the isobologram indicate synergistic effect between two drugs and points above the line of equivalency represent antagonistic effect [21]. The resulting isobolograms in our study show that juglone and TQ act antagonistically at most of the concentrations and only exert some synergy at very high concentration.

Table 2.1. IC_{50} values for juglone, thymoquinone and their combination in human pancreatic cancer MIA Paca-2 cells

Drug	IC_{50} value (μM)	m	r
Juglone	5.27353	0.89120	0.99055
Thymoquinone	24.1519	0.80316	0.99713
Juglone + Thymoquinone	24.7545	0.94838	0.92373

Where m refers to the shape of the dose **effect curve (m=1, >1, <1 indicate hyperbolic, sigmoidal and negative sigmoidal) and r to the linear co-relation coefficient**

Table 2.2. Assessment of combinatorial effect of juglone and thymoquinone at Fa: 0.1, 0.2 and 0.5. Combination index was determined by Compusyn software.

Fa	CI Value	Total Dose	Juglone (μM)	Thymoquinone (μM)
0.1	1.84625	2.44049	0.18078	2.25971
0.2	1.61803	5.73887	0.42510	5.31377
0.5	1.29674	24.75447	1.83367	22.9208

Table 2.3. Assessment of combinatorial effect of juglone and thymoquinone at Fa: 0.75, 0.90 and 0.95. Combination index was determined by Compusyn software.

Fa	CI Value	Total Dose	Juglone (μM)	Thymoquinone (μM)
0.75	1.09249	78.8392	5.8399	72.9992
0.90	0.92391	251.091	18.5993	232.4917
0.95	0.82626	552.082	40.8950	511.1870

2.4. Discussion

Recent advances in the use of phytochemicals for promoting health and reducing risk of cancer have been documented in literature. Dietary bioactive compounds such as resveratrol [22], curcumin [23], sulforaphane [24], epigallocatechingallate [25], piperlongumine [26] and others

have proven efficacious in inhibiting the growth and proliferation of pancreatic cancer cells in various *in vitro* and *in vivo* experiments.

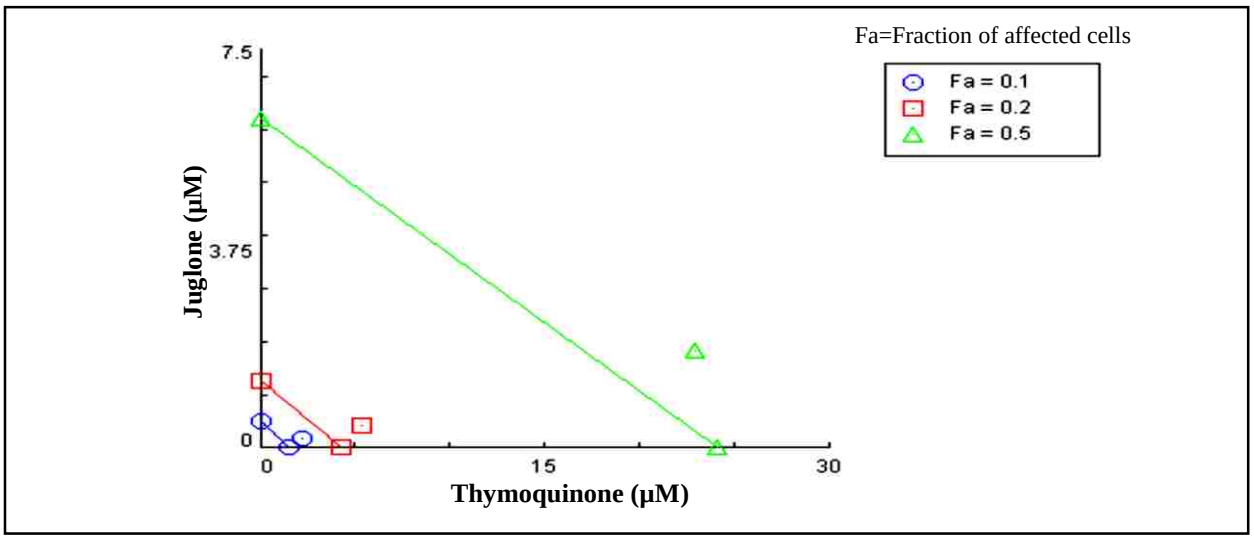

Figure 2.10. Juglone exerts an antagonistic relationship with thymoquinone at lower concentrations. Isobologram was created with Compusyn software. The blue, red and green line represents line of additivity at which there is 10%, 20% and 50% effect on the cells respectively. Points lying above the line of additivity indicate antagonistic effect.

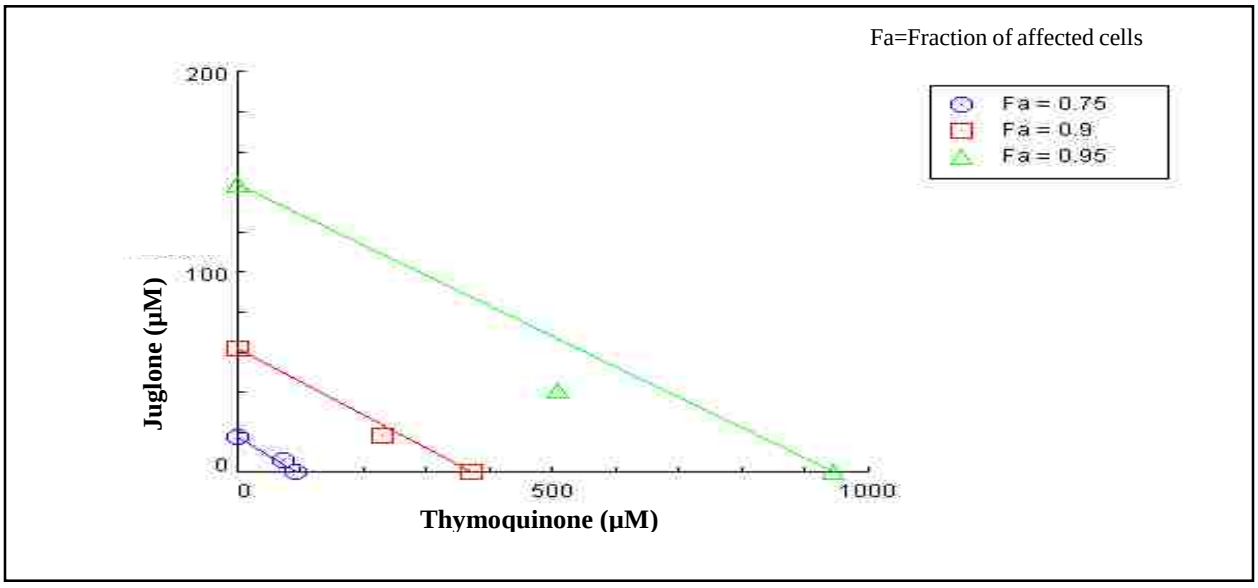

Figure 2.11. Juglone exerts an additive and synergistic relationship with thymoquinone at higher concentrations. Isobologram was created with Compusyn software. The blue, red and green line represents line of additivity at which there is 75%, 90% and 95% effect on the cells respectively. Points lying below the line of additivity indicate synergistic effect and points lying on the equivalence line indicate additive effect.

Many quinones-based drugs have been proven to be effective against cancer and are already in clinical trials, which makes juglone an attractive compound to be explored against PC. Quinones are abundantly found in nature; more than 1200 types of quinones have been reported in literature [27]. They function as electron carriers in plants and are very well known for their redox potential. Some of the various drugs against cancer that have been approved and those going through clinical trials are quinones-based compounds such as actinomycin D, anthracyclines, daunorubicin, menadione, mitomycin C, mitoxantrones, saintopin, and trenimon [28, 29]. This shows the importance of naturally occurring quinones such as juglone in cancer research.

Abnormal growth and proliferation is an important characteristic of malignant tumors. In this study, the effect of juglone on the proliferation and apoptosis of human pancreatic cancer MIA Paca-2 cells was analyzed. We determined the IC_{50} value of juglone in pancreatic cancer MIA Paca-2 cells to be 5.27 µM at 24 h. The IC_{50} value for juglone on ovarian cancer SKOV3 cells was 30.13 µM at 24 h [7]. Juglone has also shown to be effective against melanoma cells in a dose and time dependent manner. The IC_{50} for juglone on B16F1 melanoma cells at 1, 24, and 48 h was 9.9 µM, 7.46 µM, and 6.92 µM, respectively [30]. Very high intracellular production of ROS was linked to the mode of action of juglone mediated apoptosis in melanoma cells. These studies with juglone on various cancer models show that the same compound reacts in different ways depending upon the type of cancer and therefore it is very critical to establish the IC_{50} values of juglone in specific cell lines.

There are studies that have also utilized animal models to understand the anti-cancer effect of juglone. In one study model, the dietary effect of juglone was investigated. When juglone was administered at a concentration of 200 ppm, occurrence of multiple intestinal tumors was significantly reduced in azoxymethane (carcinogen) –induced colon cancer rats [31]. Results

indicated that juglone had significant effect against tumor initiation and multiplication, but the underlined mechanism was not been detailed. Another recent study conducted by Asano et al. demonstrated that when LnCap tumor cells bearing mice were intraperitoneally injected with 40 µg of juglone once a week for 4 weeks, there was a significant reduction of tumor size and volume with no significant reduction in weight of the mice [9]. These studies suggest that juglone as an anti-cancer agent when administered in animal models proved to be safe and effective.

In this study we also investigated whether juglone induced apoptosis in pancreatic cancer cells. Cancer cells evade apoptosis or "programmed cell death" by amplifying various anti-apoptotic mechanisms, which leads to its uncontrollable growth, tumor progression and metastasis [32]. Anti-cancer agents are known to induce apoptosis as well as inhibit cell growth and proliferation of cancer cells [33]. The morphological changes that occur during apoptosis are cell shrinkage, membrane blebbing, chromatin condensation and nuclear fragmentation [34]. Our results clearly demonstrated that cells treated with juglone was positively stained with annexin V and PI. As cells undergoing apoptosis have condensed chromatin, fluorescent dye such as Hoechst is known to stain the nucleus of apoptotic cells more brightly than that of the normal cells [35], and this phenomena of bright fluorescence in nucleus of apoptotic cells was also recorded in MIA Paca-2 cells treated with juglone (Figure 2.5.). Morphological changes, cell shrinkage, translocation of phosphatidylserine molecules were observed in MIA Paca-2 cells exposed to juglone at concentration range of 1-10 µM; similar concentration of juglone has been reported to cause increase in annexin V binding in juglone-treated erythrocytes [36] and cancer cells [37].

Colony formation assay was used to determine juglone associated long term inhibition of the proliferation of MIA Paca-2 cells. As demonstrated in Figure 2.6., there was a significant reduction in the number of colonies formed in juglone-treated pancreatic cancer cells in

comparison to the untreated MIA Paca-2 cells. These findings are in line with other reports of juglone treatment in other types of cancer. Studies performed by Pedrosa et al. showed a reduction in the number of colonies in MCF-7 cells when treated with 10 µM of juglone [38].

The above findings indicate the cytotoxic effect of juglone on pancreatic cancer cells and determine the effectiveness of juglone as an anti-proliferative agent against MIA Paca-2 cells. Pancreatic cancer cell proliferation is regulated by juglone in a dose-dependent manner.

Drug combination studies was also carried out using juglone with TQ, the principal bioactive component of the volatile oil of *Nigella sativa* (black seed). Each of these compounds was demonstrated to be cytotoxic to MIA Paca-2 cells individually. Based on their cytotoxicity, we hypothesized that combination treatment of MIA Paca-2 cells with juglone and TQ would result in synergistic cytotoxicity. There are many *in-vitro* and *in-vivo* studies that have reported synergistic action of TQ in conjunction with other phytochemicals such as EGCG against prostate cancer [39], lycopene against cervical cancer [40], gemcitabine against pancreatic cancer [12], doxorubicin against breast cancer cells [41], 5-fluorouracil against colon cancer [42], and many more [40]. However, our results demonstrated that TQ in combination with juglone, did not exert favorable synergistic relationship against pancreatic cancer cells. TQ acts as an anti-oxidant (free radical scavenger) at lower concentrations and at higher concentrations acts as a pro-oxidant [43], which might be one of the possible mechanisms that might be responsible for the antagonistic effect between juglone and TQ as juglone induced cytotoxicity in cancer cells majorly involves production of reactive oxygen species through redox activation [44]. Synergy between juglone and TQ was only seen at very high dose combination where physiological concentrations of each dietary bioactive compound could be very difficult to achieve.

2.5. Conclusion

Inducing apoptosis in cancer cells is an important defense mechanism against cancer. Our findings indicate that juglone exerts cytotoxic effect on pancreatic cancer MIA Paca-2 cells and also controls its proliferation. The treated cells die through apoptosis and this has been confirmed by Annexin V/PI staining. These data suggest that juglone may play a pivotal role in obstructing pancreatic cancer development and progression. To further delineate the molecular mechanisms of juglone-induced apoptosis of pancreatic cancer MIA Paca-2 cells, additional *in vitro* studies were designed, performed and discussed in chapter 3.

2.6. References

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CHAPTER 3. JUGLONE MODULATES INTRACELLULAR LEVEL OF REACTIVE OXYGEN SPECIES AND SUPPRESSES PANCREATIC CANCER PROLIFERATION BY INHIBITION OF THE KRAS-DRIVEN SIGNALING PATHWAYS

3.1 Introduction

Despite the high incidence and mortality rate due to pancreatic cancer, progress in drug discovery for an operative chemotherapeutic option has still not been very effective. One of the challenges chemotherapy/radiotherapy faces in pancreatic cancer is the de novo chemo resistance that pancreatic cancer cells develops [1]. Resistance to chemotherapeutic agents makes drug development effort very crucial and therefore the identification of novel cytotoxic agents against pancreatic cancer is much needed. Naturally occurring bioactives have played a significant role in the drug discovery process and are recommended to be studied extensively for discovery of novel source of efficient chemotherapeutic agents against cancer [2]. Among many bioactive compounds present in nature, quinones comprise one of the largest group of compounds studied as anticancer agents [3].

Juglone (5-hydroxy-1,4-naphthoquinone)is a naturally occurring naphthoquinone found in the roots, leaves, nut-hulls, bark and wood of *Juglans regia* (English, Persian and California walnut), *J. cinerea*,(butternut and white walnut) and *J. nigra* (black walnut). Juglone is known to possess anti-tumor, anti-bacterial, anti-fungal anti-inflammatory and anti-parasitic activities [4-6]. Use of juglone-containing plants for therapeutic benefits has been documented in traditional medicine practices for many years in countries like China and India [7, 8]. The anti-tumor activities of naphthoquinones are primarily related to their pro-oxidant property [9].

Juglone inhibits a wide range of cancer cells and juglone inhibitory effect differs depending upon cell type. Xu et al. has demonstrated that the cytotoxic effect of juglone on human leukemia HL-60 cells was through reactive oxygen species (ROS)-dependent and mitochondria-dependent pathways. Similarly, juglone was reported to induce ROS and cause apoptosis via mitochondrial

mediated apoptosis in gastric cancer SGC-7901 cells [10]. There are no studies on the interaction of juglone and pancreatic cancer cells. The aim of the present study was to investigate the molecular mechanism of juglone induced cytotoxicity in human pancreatic cancer cells.

3.2. Material and Methods

3.2.1. Chemicals and Antibodies

Juglone, dichloro-dihydro-fluorescein diacetate (DCFH-DA), and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO). Primary antibodies against COX-2 (#4842s), PARP (#9532s), Bax (#2772) and BCl-2 (#2876) were purchased from Cell Signaling (Danvers, MA). Primary antibody against HER-2 (#21070-2) was purchased from SAB® Signalway Antibody (College Park, MD). HRP conjugated secondary anti-rabbit antibodies and bovine serum albumin were purchased from Santa Cruz (Dallas, TX). Trypsin EDTA, Dulbecco's Modified Eagle Medium (DMEM), phosphate buffer saline (PBS), penicillin streptomycin was purchased from Invitrogen (Carlsbad, CA) and fetal bovine serum from Atlanta Biologicals (Flowery Branch, GA).

3.2.2. Cell Culture

MIA Paca-2 cells were purchased from American Type Cell Culture. This cell line contains mutation in *KRAS* genes with high levels of activated KRAS protein [11] and is appropriate for the study, which is to target the down-stream molecules involved in the oncogenic *KRAS* pathway. As recommended by ATCC, the cell line was maintained in culture in DMEM supplemented with 10 % fetal bovine serum, penicillin and streptomycin and grown at 37^{o}C in a humidified incubator with 5% CO_2 until 70-80% confluency. After reaching confluency, it was split at the ratio of 1:3 to1:8. The growth medium was changed 2-3 times a week and 0.25% trypsin EDTA was used to detach the cells for subculture.

3.2.3. Measurement of ROS Production

Level of intracellular ROS with treatment was measured as previously described [12]. MIA Paca-2 cells were plated in a black 96-well plate at a density of 10, 000 cells/well. After 24 h of seeding, cells were treated with various concentrations of juglone. A stock of dichloro-dihydro-fluorescein diacetate (DCFH-DA) (20mM) was prepared in DMSO. To determine the level of intracellular ROS, supernatant was removed and replaced with 100 µM of DCFH-DA in PBS. After 1h of incubation with DCFH-DA, cells were washed with freshly prepared Hank's balanced salt solution and fluorescence intensity was measured by a fluorescence reader using the excitation wavelength of 485 nm and emission wavelength of 530 nm. Experiments were performed in triplicate.

3.2.4. Western Blot Analysis

MIA Paca-2 cells were seeded at a density of 5 x10^5cells/well in a 6- well plate. After seeding overnight, cells were treated with Juglone. Whole cell lysates were prepared by using cell lysis buffer (Cell Signaling Technology (Danvers, MA) and supernatant were saved. Protein concentrations of the samples were determined using the Bradford assay. Protein separation was resolved in 4-12 % gradient Bis-Tris SDS polyacrylamide gels (Invitrogen, Carlsbad, CA) and transferred to polyvinylidene difluoride (PVDF) membranes (Invitrogen, Carlsbad, CA). Membranes were blocked with 5% BSA in PBS containing 0.05% Tween 20 (TBS-Tween) for 1h. The PVDF membranes were then probed with primary antibody (Akt, Bax, Bcl-2, Cox-2, or HER-2) in PBS-Tween containing 5% BSA overnight at 4ºC with gentle agitation. Detection of protein expression was done by probing the blots with horseradish peroxidase–conjugated secondary antibodies (Santa Cruz) and exposure to chemiluminescent substrate (Thermo Fisher Scientific).

Chemiluminescence was recorded by BioRad Imaging system and the densities of the bands were quantified by Quantity One Software 1-D analysis software.

3.2.5. DNA Fragmentation Assay

Nuclear fragmentation and formation of apoptotic bodies occurs in cells undergoing apoptosis. To demonstrate this process, the DNA fragmentation assay was performed. MIA Paca-2 cells were plated at a density of 2×10^5 cells/well in 6-well plates and were treated with juglone for 24 h. After required incubation, cells were collected by scraping and centrifuged. DNA of the cells was then collected and purified using a genomic DNA extraction protocol [13]. Briefly, cell pellets were suspended in Tris-EDTA (TE) buffer and 10% SDS buffer was added along with proteinase K. The mix was incubated at 37ºC for 1 h. After incubation, equal volume of phenol/chloroform was added, mixed by inverting the tubes and incubated at 65ºC for 10 min. Phase separation was seen, the upper aqueous phase was collected and one tenth volume of sodium acetate was added to the mix. Ethanol precipitation was done by adding 2 volumes of 100 % ethanol and the samples were stored at -80ºC overnight. The samples were centrifuged for 30 min. The supernatants were discarded and 100% of ethanol was added to the pellet again and the mixture was centrifuged for 15 min. The pellet was allowed to dry at 37ºC and further dissolved in TE buffer. Purified DNA was further separated using 1% agarose gel containing 0.5 µg/ml ethidium bromide at 120 V, 50 A for 40 min. Gels were visualized under UV trans-illuminator and pictures were captured. DNA from viable cells remained on the top of the gel whereas DNA from apoptotic and necrotic cells formed ladder or smear.

3.2.6. Colorimetric Assay for Activated Caspase-3 Cell-Based ELISA

Caspase-3 activation was analyzed by using the LSBio^{TM} Caspase 3-Cell Based ELISA kit (Seattle, WA). Approximately, 2 x 10^4 cells were plated in a 96-well plate and allowed to attach

overnight. Cells were then treated with juglone for 6h and after required incubation the media was replaced, washed with PBS and fixed with 4% formaldehyde. Fixed cells were quenched and blocked for at least 1h at room temperature. Cells were incubated overnight at 4^{o}C with anti-caspase-3 antibody at a dilution of 1:100. After overnight incubation cells were incubated with HRP conjugated secondary antibodies for 1.5 h at room temperature, the substrate was added and the mixture was incubated for 30 min at room temperature. Optical density was then read at 450 nm with a plate reader after addition of the stop solution. Once the readings were taken, cells were then washed and further stained with crystal violet (CV) for 30 min. Stained cells in the plates were washed with indirect flow of running water and TBS. Retained CV was then dissolved with 33% acetic acid and OD was measured at 595 nm. The readings at 450 nm were then normalized with CV readings at 595 nm of the respective wells. Results are expressed in percentage of control.

3.2.7. Determination of phospho-ERK by Cell- Based ELISA

In order to determine whether juglone inhibited the phosphorylation of ERK, the level of p-ERK/ERK was measured by a cell-based ELISA using a kit from R&D Systems (Minneapolis, MN). MIA Paca-2 cells were plated in a black 96-well plate at a cell density of $1x10^4$ cells per well. After allowing to attach overnight, cells were treated with juglone for 6h. After required incubation, the media was removed and the cells were washed with PBS. Immediately the cells were fixed with 4% formaldehyde for 15 min. After 15 min fixation, the cells were washed with PBS and endogenous peroxidase activity of the cells was blocked by incubation with 30% hydrogen peroxide for 15 min. The cells were incubated with blocking buffer for 1 h, washed with PBS at least 3 times and incubated overnight at 4^{o}Cwith phospho-ERK and total ERK antibodies. After incubation with primary antibodies, secondary antibodies either conjugated to HRP or AP (alkaline phosphatase) were added and incubated at room temperature for at least 2 h. Substrates

provided in the kit were added to each well and fluorogenic detection was done using a fluorescence plate reader (Perkin Elmer, MN). Readings with excitation at 540 nm and emission at 600 nm represented the amount of phosphorylated ERK and readings with excitation at 360 nm and emission at 450 nm represented the amount of total ERK in the cells. The readings were expressed as the normalized value of the amount of phosphor-ERK fluorescence to that of total ERK fluorescence.

3.2.8. Kinase Activities

The effect of juglone on enzymes that are actively involved in glycolysis including hexokinase and pyruvate kinase was studied by determining hexokinase or pyruvate kinase activity. Hexokinase activity was determined using a colorimetric kit from Sigma-Aldrich (St. Louis, MO). Pyruvate kinase colorimetric was determined using a kit from Abcam® (Cambridge, MA). Protein concentration of cell lysates was measured and glycolytic enzymes activities were measured. Enzyme activity was measured accordingly at the respective wavelength. Hexokinase activity was measured at 450 nm and pyruvate kinase at 570 nm.

$\Delta OD_{450 \text{ or } 570}$ (Optical Density at 450 nm or 570 nm) = A_2-A_1, where A_2 and A_1 are final and initial absorbance at 450 nm or 570 nm respectively. Then amount of NADH or Pyruvate generated (B) at the respective $\Delta OD_{450 \text{ or }} \Delta OD_{570}$ was calculated and enzyme activity was then finally calculated. Enzyme activity (nmol/min/ml or milliunit/ml) = B/ ($\Delta T \times V$) × dilution factor, where B is the amount of NADH generated (nmol) in case of hexokinase activity or amount of pyruvate generated in case of pyruvate kinase activity, ΔT is the reaction time (min), and V is the sample volume added (ml).

3.2.9. Statistical Analysis

All the experiments were conducted in triplicates. Results are expressed as means ± SD of experiments. Analysis of variance (ANOVA) was conducted to examine the differences between treatments followed by Tukey analysis using SAS. A P-value of < 0.05 was considered to be statistically significant. Means with different letters indicate significantly different

3.3. Results

3.3.1. Juglone Induced ROS Production in Pancreatic Cancer MIA Paca-2 cells

The level of intracellular production of ROS in MIA Paca-2 cells after treatment with 1, 5 or 10 µM juglone was detected by the DCFH-DA method. There was a 1.48-, 1.81- and 1.72- fold increase in fluorescence, respectively (Figure 3.1.). There was a significant increase in the generation of ROS in juglone-treated cells, the level of ROS at 5 and 10 µM of juglone treated cells were significantly higher than in the untreated control group.

3.3.2. Juglone Activated Caspase-3 and Caused PARP Cleavage in MIA Paca-2 cells

Results of caspase-3 colorimetric assay indicate that juglone significantly induced caspase-3 activation in a dose dependent manner (Figure 3.2., A). As the activation of caspase-3 was observed in juglone-treated cells, we next determined whether PARP, a substrate for caspase-3 was cleaved. PARP cleavage is indicative of caspase-mediated cell apoptosis [14]. PARP assists the DNA repair mechanism in cancer cells, and when cleaved by caspases they lose their functionality [15]. Western blot analysis of PARP in juglone-treated cells clearly showed the presence of cleaved fragments of PARP and the intensity of PARP increased as juglone concentration increased starting at 1µM concentration (Figure 3.2., B). These results suggest that juglone-induced apoptosis in MIA Paca-2 cells is the result of activation of effector caspase,

caspase-3 and cleavage of PARP, which ultimately results in biochemical and morphological changes seen in apoptotic cells.

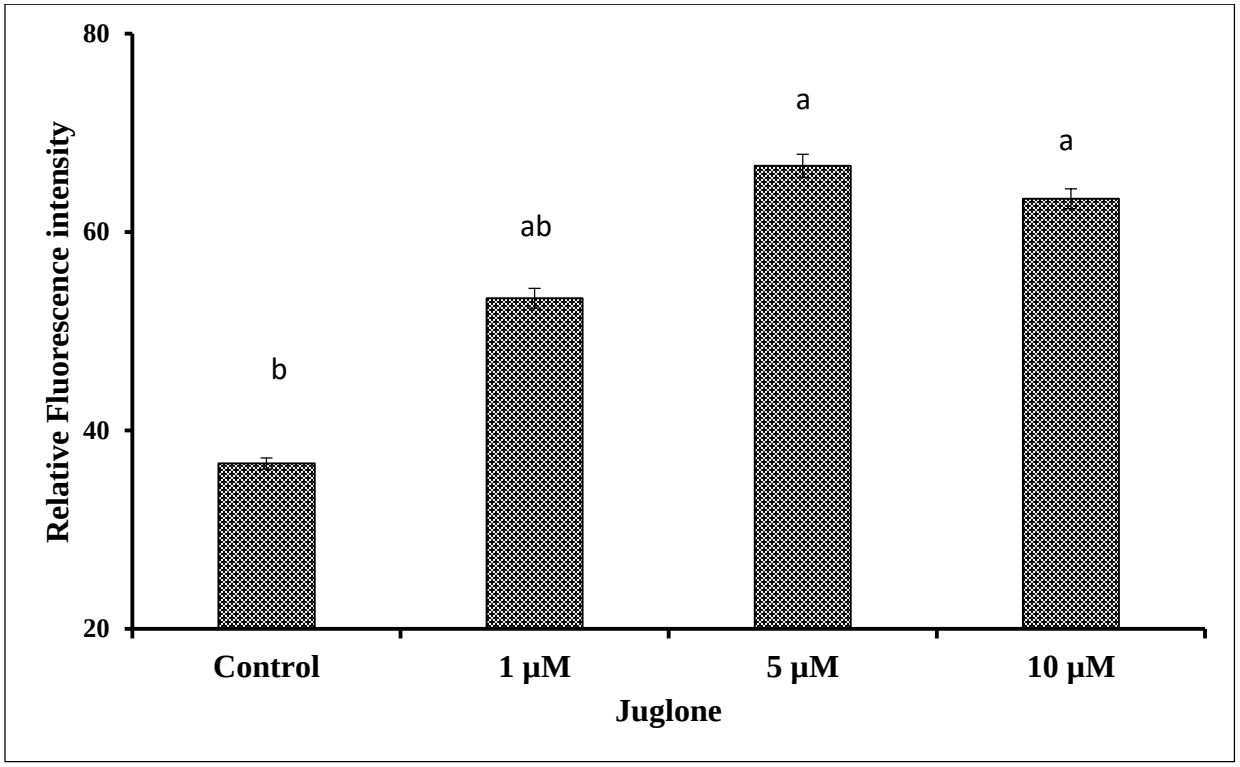

Figure 3.1. Intracellular measurement of reactive oxygen species (ROS). The levels of intracellular reactive oxygen species in pancreatic cancer cells treated with juglone were determined by dichloro-dihydro-fluorescein diacetate (DCFHDA) assay. Data represent average of triplicates ± SD.

3.3.3. Juglone Induced DNA Fragmentation in MIA Paca-2 cells

Apoptosis in juglone treated MIA Paca-2 cells was also characterized by the DNA fragmentation assay. Genomic DNA from juglone-treated and untreated MIA Paca-2 cells were isolated and separated by agarose gel electrophoresis. Visualization of ethidium bromide bound DNA fragments were done under UV illumination. Fragmentation of DNA was observed in juglone-treated cells (Figure 3.2, C). These results also confirmed that dose-dependent apoptosis occurred in MIA Paca-2 cells when exposed to juglone.

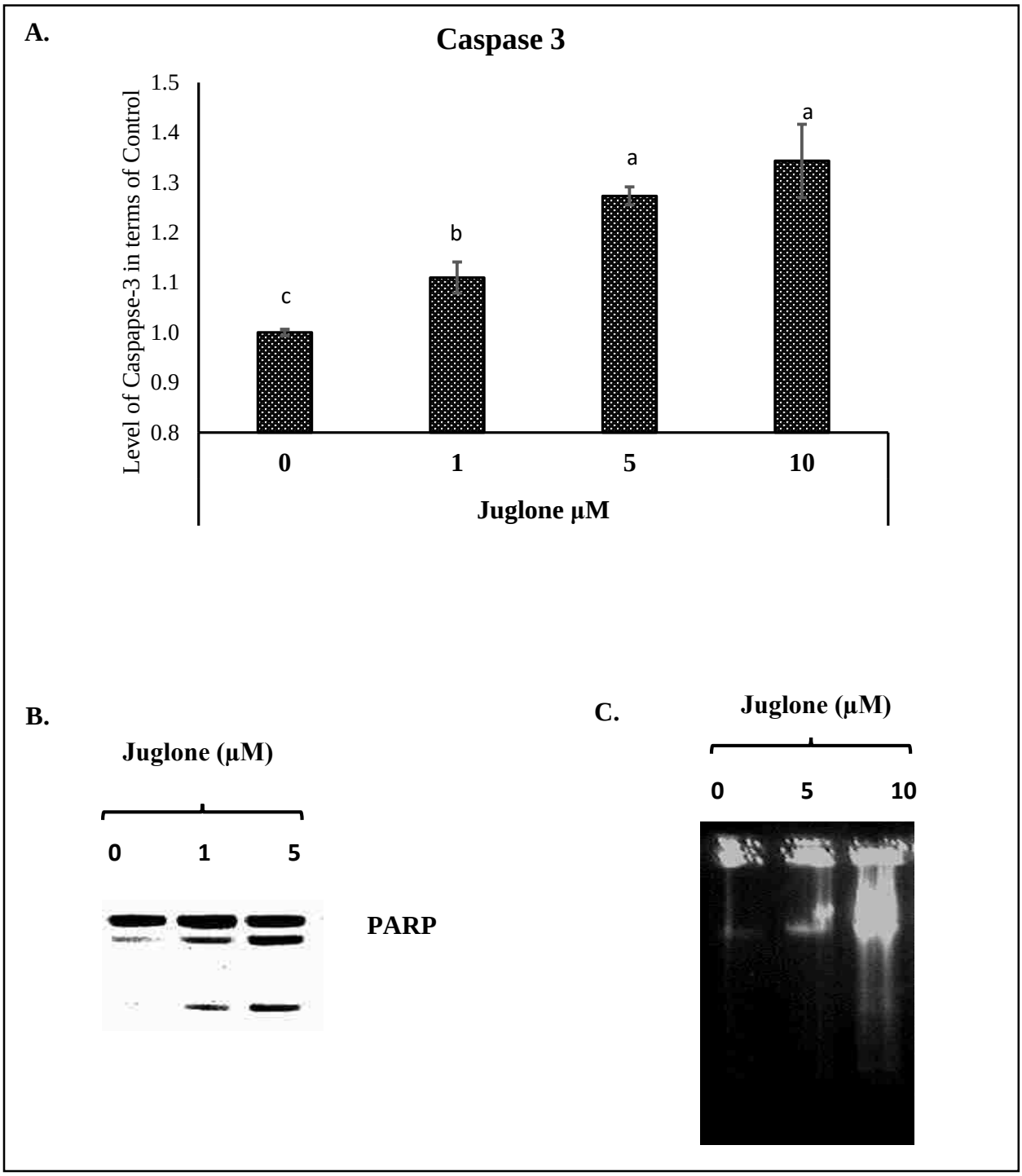

Figure 3.2. A. Juglone-mediated activation of caspase-3 in MIA Paca-2 cells as measured by cell- based ELISA. B. PARP cleavage in cells treated with juglone as demonstrated by Western Blot. C. Chromosomal DNA ladder formation in cells treated with juglone following agarose gel electrophoresis under UV. Lane 1: DNA from MIA Paca-2 cells without juglone, Lane 2 and 3: DNA from juglone-treated (5 and 10 µM) MIA Paca-2 cells.

3.3.4. Juglone Modulated the Expression of Pro-apoptotic Protein Bax and Anti-apoptotic Protein Bcl-2

The BCl-2 family of proteins includes pro-apoptotic and anti-apoptotic proteins, which are primarily involved in the regulation of apoptosis. To further explore the mechanism of apoptosis, the characterization of Bax and Bcl-2 protein levels in juglone-treated MIA Paca-2 cells at 1 µM and 5µM was done by western blot analysis. When compared with the untreated group, juglone-treated group of MIA Paca-2 cells showed significant downregulation of anti-apoptotic Bcl-2 and upregulation of pro-apoptotic Bax (Figure 3.3., A). The ratio of Bcl-2 to Bax decreased by 37.5% and 61.40% in 1 and 5 µM of juglone-treated MIA Paca-2 cells, respectively. Densitometric analysis of expression of Bax and Bcl-2 is presented in Figures 3.3., B and 3.3., C, respectively.

3.3.5. Effect of Juglone on EGFR Signaling and Downstream pAKt and pERK Pathways in MIA Paca-2 cells

Results in Figure 3.4 show that treatment of MIA Paca-2 cells with juglone resulted in a dose-dependent decrease in HER-2 expression. Both full length HER2 and p95HER-2 were downregulated with increasing concentrations of juglone. HER-2 down streaming pathways include Akt and MAPK pathways, which are highly upregulated in pancreatic cancer cells. The phosphorylation levels of Akt or Erk were determined. Juglone inhibited the phosphorylation of Akt (Figure 3.5.) and Erk (Figure 3.6.) in MIAPaca-2 cells

3.3.6. Juglone Downregulated COX-2 in MIA Paca-2 Cells

Western blot analysis of cell lysates from juglone-treated cells showed that juglone at concentrations 1, 5, or 10 µM significantly downregulated the expression of COX-2 in MIAPaca-2 pancreatic cancer cells (Figure 3.7., A). Densitometric analysis of the bands further elucidated the dose-dependent inhibition of COX-2 expression (Figure 3.7., B).

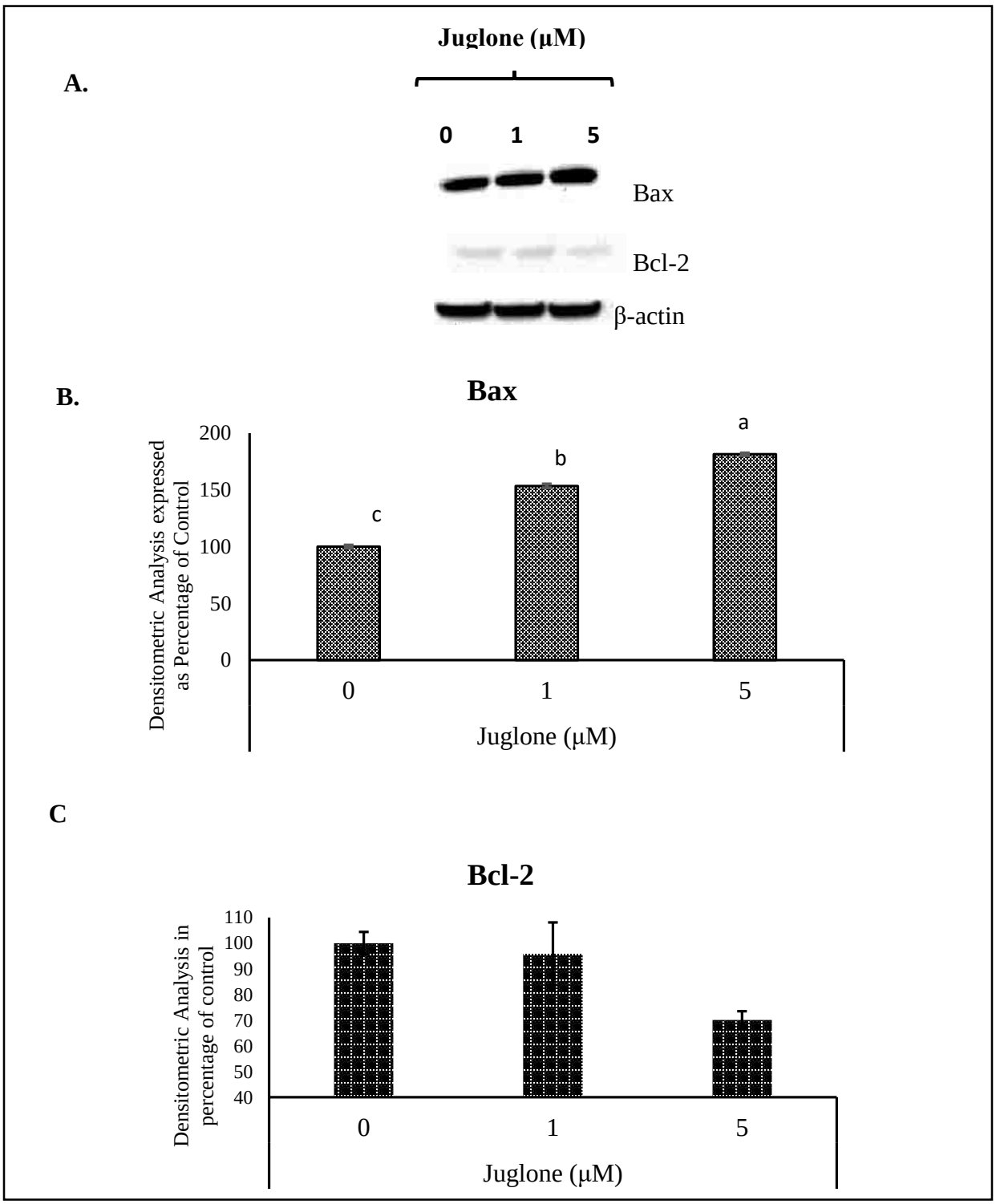

Figure 3.3. Effect of juglone in expression of Bcl-2 family proteins in MIA Paca-2 cells. A. MIA Paca-2 cells were treated with juglone at 1 and 5 µM for 6 h. After incubation cells were lysed and subjected to western blot analysis of Bcl-2 and Bax. For loading control, β-actin was used.

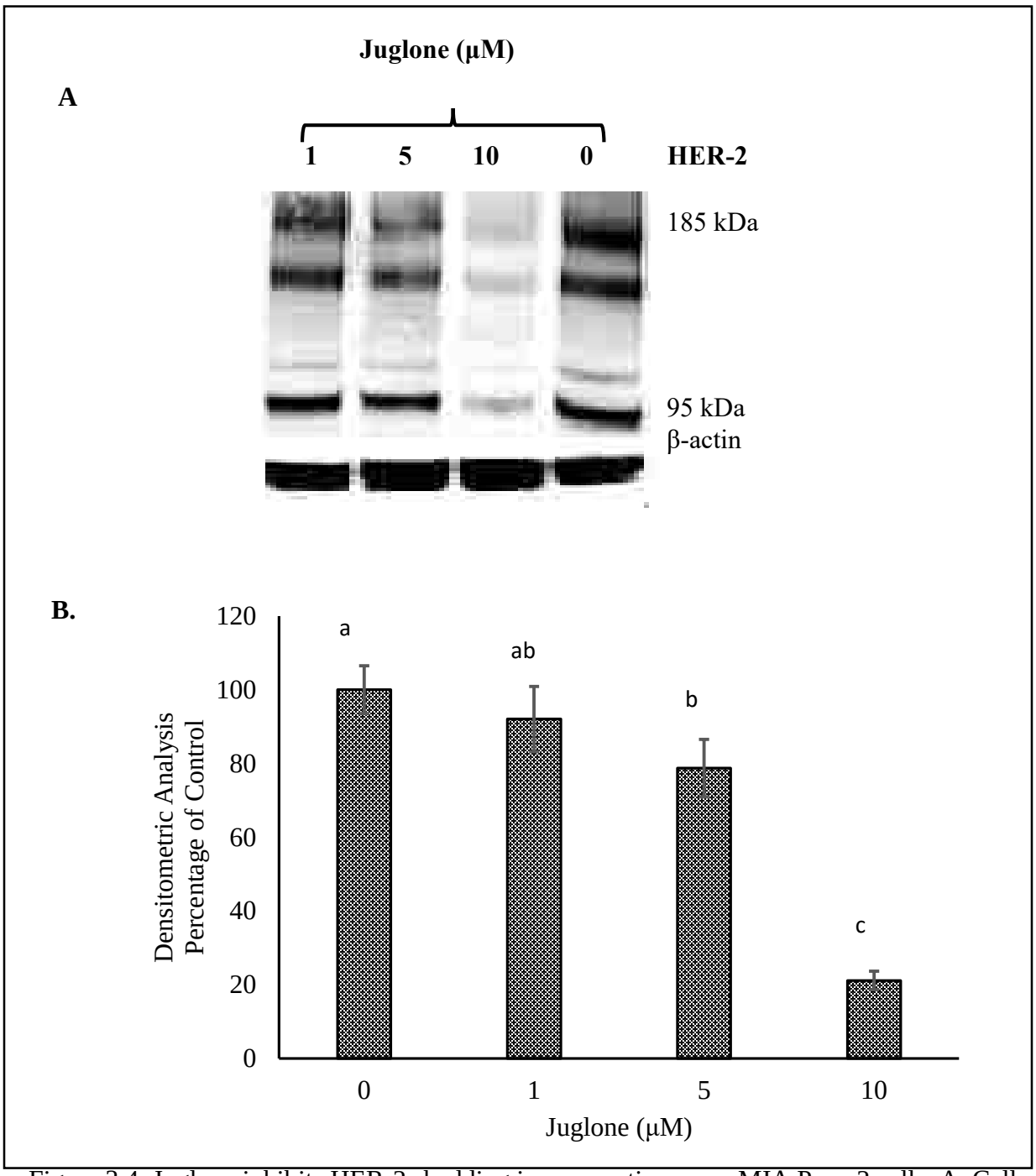

Figure 3.4. Juglone inhibits HER-2 shedding in pancreatic cancer MIA Paca-2 cells. A. Cells were lysed and the 30 µg of whole cell lysate was subjected to western blot analysis. Basal level of both full length (p185 HER-2) and truncated (p95 HER2) forms of HER-2 were inhibited with treatment of juglone. B. Densitometry analysis of the bands are expressed in terms of untreated control.

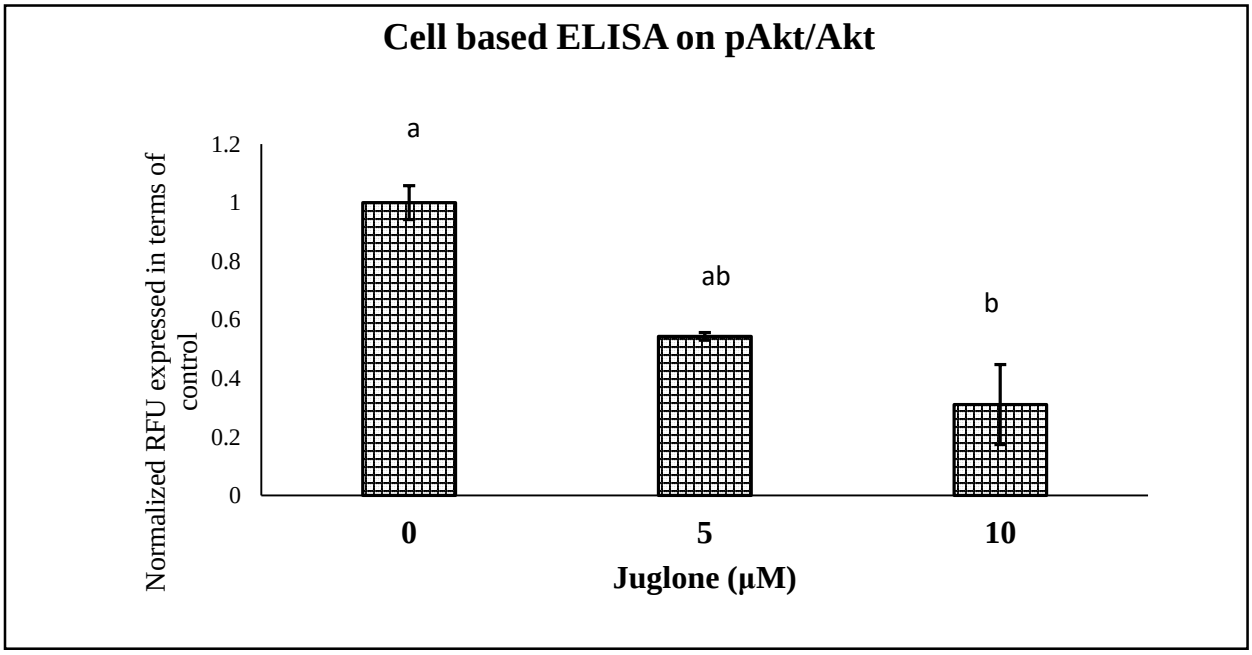

Figure 3.5. Effect of juglone on the phosphorylation of Akt. MIA Paca-2 cells were plated in black 96-well plate and treated with juglone (5 µM and 10 µM) for 6 h. After required incubation cells were fixed and p- Akt in cells was measured using Phospho-Akt Cell based ELISA.

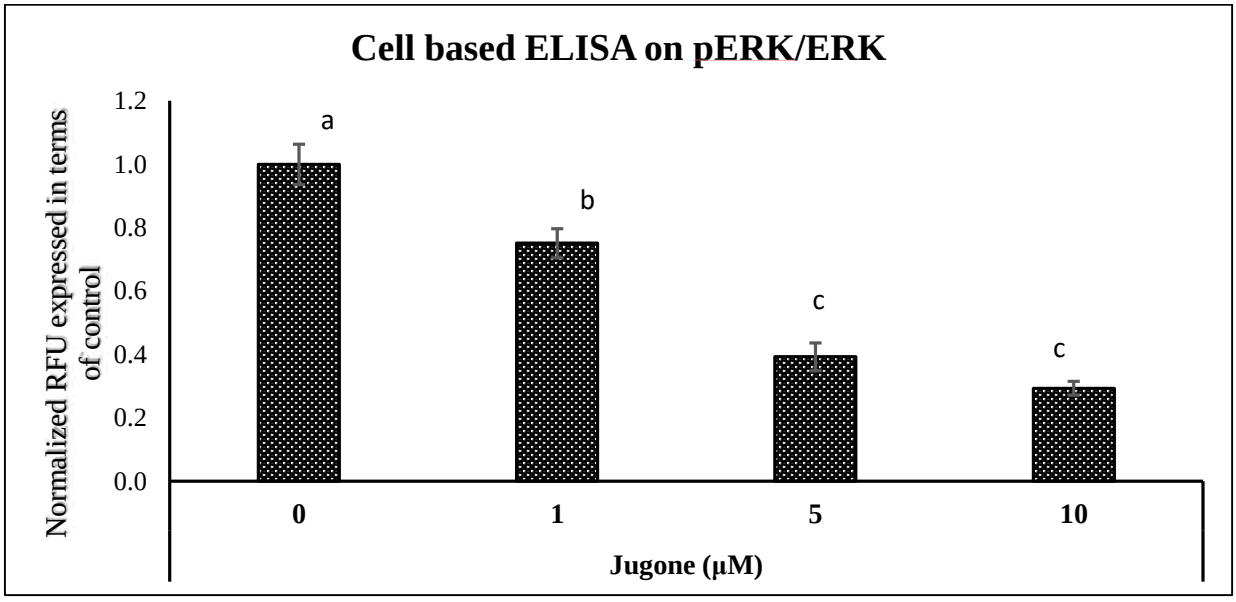

Figure 3.6. Effect of juglone on the phosphorylation of ERK. MIA Paca-2 cells were plated in black 96 well plate and treated with juglone (1 µM, 5 µM, or 10 µM) for 6 h. After required incubation, cells were fixed and p- Akt in cells was measured using Phospho-ERK Cell based ELISA.

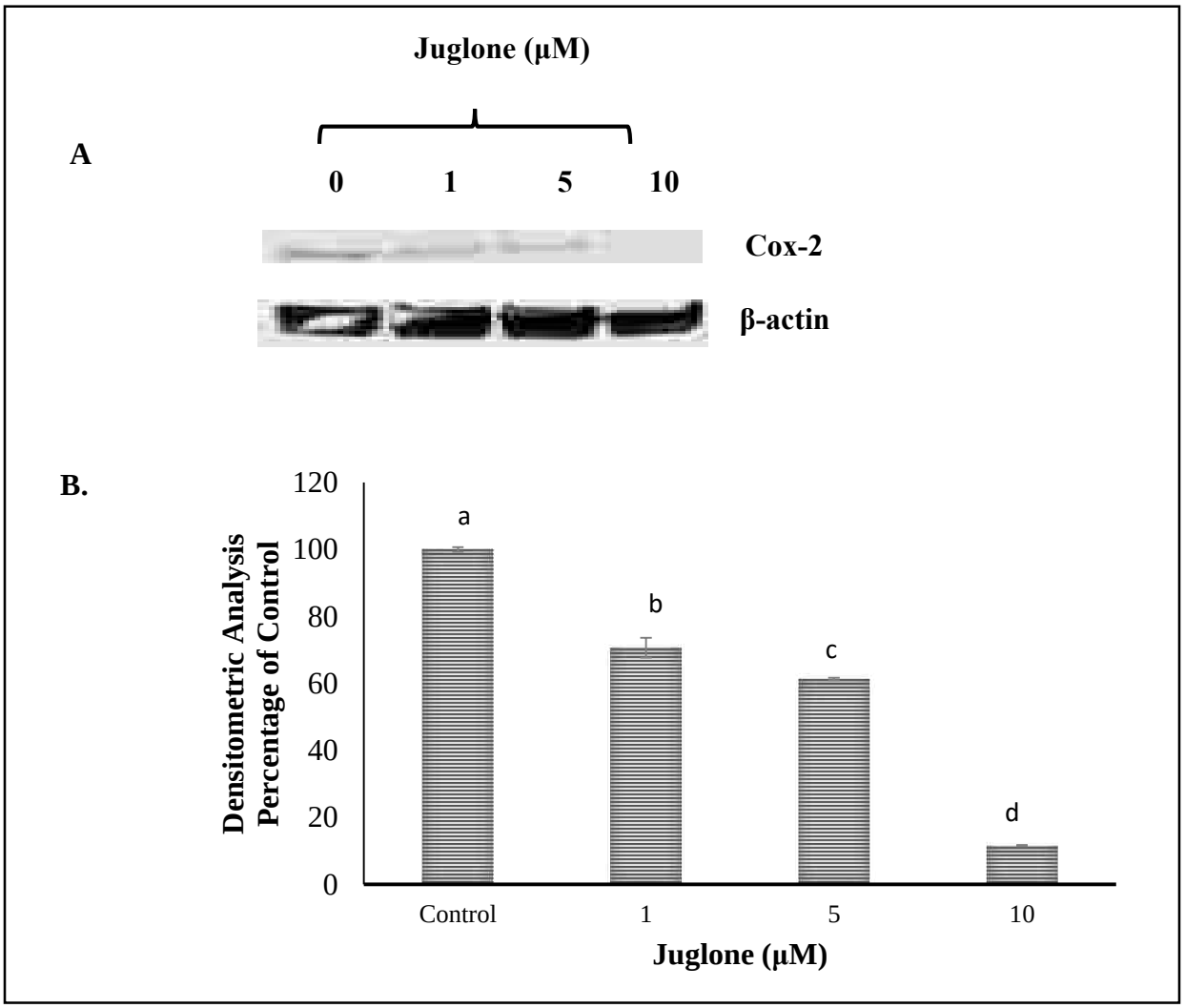

Figure 3.7. Effect of juglone on the expression of COX-2 in pancreatic cancer MIA Paca-2 cells. MIA PAca-2 cells were treated with juglone at 1, 5, or 10 µM. A. Whole cell lysates were prepared and 30 µg of cell lysate was subjected to western blot analysis. B. Densitometric analysis of the bands using Quantity One software was done and results are expressed in terms of untreated control.

3.3.7. Juglone Inhibits Aerobic Glycolysis via Downregulation of Hexokinase

The influence of juglone on enzymes involved in glycolysis was also investigated in this study. Results in Figure 3.8 indicate that juglone dose-dependently inhibited glycolysis by downregulating the expression of hexokinase in MIA Paca-2 cells. However, at similar concentration juglone had no effect on pyruvate kinase (Figure 3.9.).

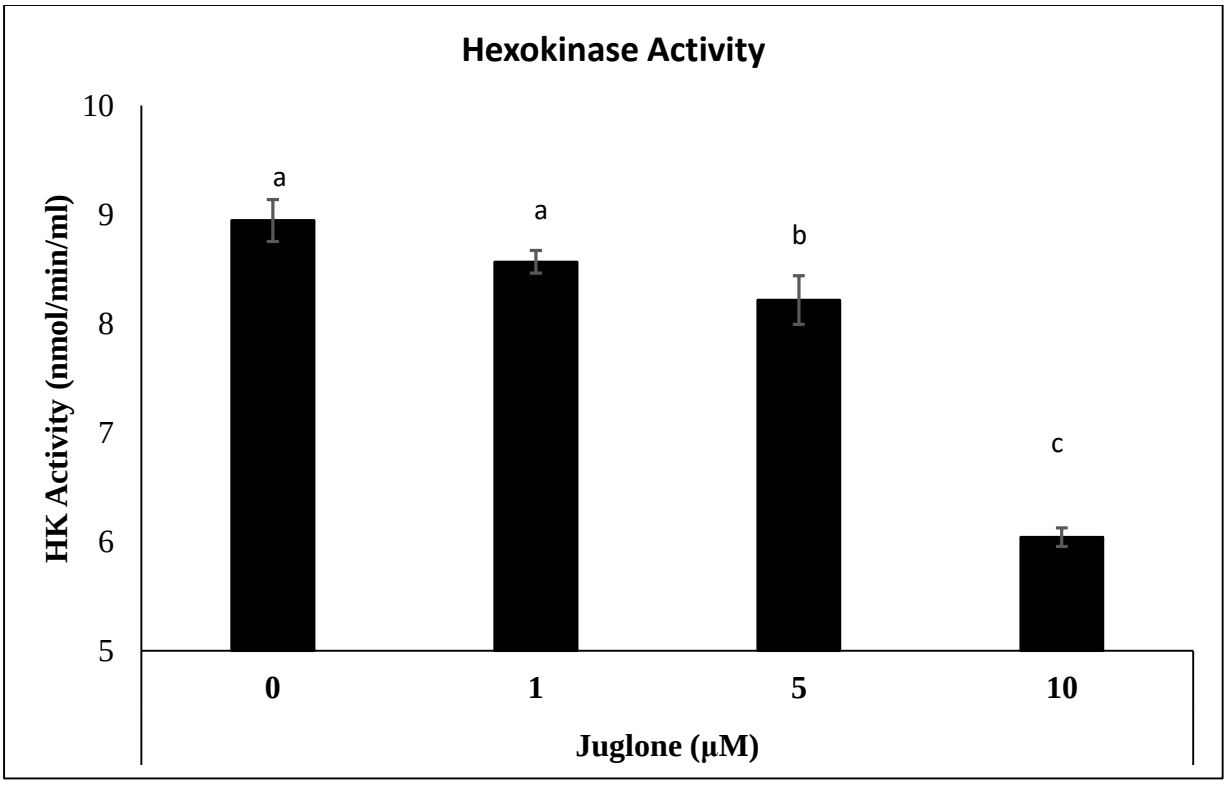

Figure 3.8. Effect of juglone on the activity of hexokinase MIA Paca-2 cells were treated with juglone for 6 h. Whole cell lysates were collected and hexokinase activity was measured.

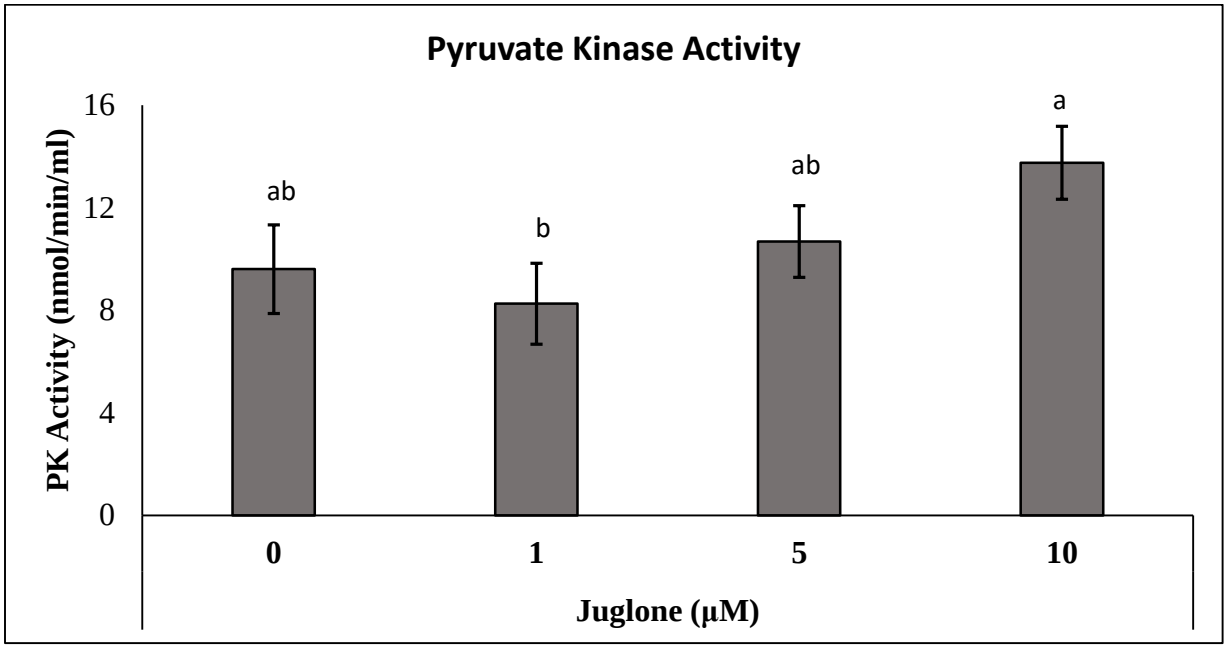

Figure 3.9. Effect of juglone on pyruvate kinase activity. MIA Paca-2 cells were treated with juglone for 6 h. Whole cell lysates were collected and pyruvate kinase activity was measured.

3.4. Discussion

In the present study the molecular mechanism behind juglone-induced cytotoxicity in pancreatic cancer MIA Paca-2 cells was investigated. In a previous study it was shown that juglone inhibited the proliferation and exerted cytotoxic effect in pancreatic cancer MIA Paca-2 cells with an IC_{50} of 5.05 µM. To confirm that the cytotoxic effect of juglone was induced by apoptosis, juglone treated cells were analyzed for DNA fragmentation assay, caspase activation and expression of pro-apoptotic and anti-apoptotic proteins. Results indicate that juglone increased caspase-3 activation, which in turn cleaved PARP, induced DNA fragmentation, increased the expression of Bax and inhibited Bcl-2.

Results also indicate that juglone induced almost a 2-fold increase in ROS generated by MIA Paca-2 cells (Figure 3.1.). A 2-fold increase in ROS production is very high [16]. ROS that are produced by mitochondria usually include superoxide anions (O_2^-), hydroxyl radicals (·OH), hydrogen peroxides (H_2O_2), and peroxynitrite ($ONOO^-$) [17]. The role of ROS against cancer cells have been studied extensively [18, 19]. Cancer cells are found to have a higher concentration of endogenous ROS than normal cells [20] and the endogenous level of ROS assists cancer cell in proliferation and survival through MAPK pathway and DNA damage [21]. When ROS production is further induced in cancer cells, the disproportional increase of ROS leads to cell cycle arrest, senescence and apoptosis, and is a direct way of specific-targeting of cancer cells [22, 23]. Juglone caused a disproportionate increase in ROS leading to MIA Paca-2 cells apoptosis. Single electron reduction causes juglone to form semiquinone radicals which are catalyzed by enzymes like NADPH-cytochrome P45 reductase. The semiquinone radicals are capable of reducing molecular oxygen to form the anion radical ($O_2^{\cdot-}$). Further enzymatic reaction with $O_2^{\cdot-}$ forms other reactive species like H_2O_2 and $OH^{\cdot}$ [24]. This excessive generation of ROS leads to oxidative stress in cells

and eventually causes apoptosis. Other studies have reported that juglone-induced ROS production in cancer cells could cause apoptosis of gastric cancer cells [10], leukemia cells [7], or breast cancer cells [25]. Juglone is a naphthoquinone. Plumbagin [12] and shikonin [26, 27] are among the best investigated naphthoquinones that have cancer cell inhibitory activities. . Plumbagin and shikonin inhibit cancer viability and proliferation via generation of ROS suggesting that naphthoquinones in general cause cancer cell apoptosis by generating more ROS in the cells. .

Mitochondria-mediated apoptosis involves regulation by pro-apoptotic and anti-apoptotic BCl-2 family of proteins [28]. Bax and Bad are among the best characterized pro-apoptotic proteins and Bcl-2, Bcl-xl and Mc11 are among the best characterized anti-apoptotic proteins. When apoptotic signals like excess generation of ROS are triggered in cells, the Bax proteins translocate to mitochondrial outer-membrane and creates permeability transition pore, which allows the release of pro-apoptotic proteins such as cytochrome C that are responsible for the activation of caspases. Bcl-2 acts as an anti-apoptotic protein by heterodimerization with Bax and not permitting Bax to create pores in the membranes of mitochondria [28]. Therefore, the ratio of Bcl-2 to Bax is very important in determining whether proliferative or apoptotic events occur in a cell [29]. The Bcl-2/Bax ratio was lower in juglone-treated MIA Paca-2 cells than the control cells suggesting an increase in cell apoptosis (Figure 3.3.). Xu et al. showed that Bcl-2/Bax ratio was lower in juglone-treated leukemia cells than the control [7].

Caspases play an essential role in the induction of apoptosis. Caspases remain as inactive zymogens in cells and only undergo proteolytic activation when undergoing apoptosis. Caspase-3 are the effector caspases and involved in initiation of apoptosis [30]. Our data suggest that when MIA Paca-2 cells were treated with juglone, caspase-3 was activated in a dose-dependent manner (Figure 3.2., C). Confirmation of activated caspase-3 is also provided with the cleavage of PARP

which are substrates of caspase-3 and undergo cleavage upon caspase activation (Figure 3.2, A) [14]. Taken together, these results confirm that juglone-induced apoptosis is mediated by activation of caspase as well as reduction of Bcl-2/Bax ratio, the latter is associated with mitochondrial apoptotic pathway.

Many types of cancers; breast, ovarian, gastric, colorectal including pancreatic cancer are known to express HER-2 receptors, a transmembrane receptor tyrosine kinase of the epidermal growth factor family [31]. One of the other abnormalities that is seen in cases of pancreatic cancer is the overexpression of epidermal growth-factor receptors. In particular pancreatic cancer MIA Paca-2 cells overexpress HER-2 [32]. Expression of HER-2 occurs in 7-61% of pancreatic cancer cases [33, 34]. Overexpression of HER-2 is responsible for various oncogenic processes like cancer cell survival, proliferation, invasion and angiogenesis via activation of various signal transduction pathways such as Ras/Raf/MAPK and PI3k/Akt/mTOR [35]. Peptidyl prolyl isomerase (Pin1) enzyme activity is very high in various cancers, is induced by oncogenes such as Ras and Neu, and is associated with regulation of HER-2 expression and cellular transformation [36]. Pin 1 catalyzes phosphorylation and amplification of EGF pathways and interacts with MEK to induce HER-2 expression which suggests that Pin1 plays an important role in overexpression of HER-2 receptors in tumors [36]. Importantly, juglone has been reported to be a known inhibitor of Pin1 activity [37] and therefore is considered as an important molecule to investigate for its activity against cancer.

Targeting HER-2/*neu* receptors in pancreatic cancer cells has been described as one of the treatment approaches [33] as HER-2 overexpression is found to be responsible for chemo-resistance and low survival of cancer patients [31, 38]. Tyrosine kinase inhibitors like Trastuzumab (Herceptin) were developed to treat cancers that overexpress HER-2 [39]. Anti-tumor effect of

Trastuzumab has also been reported in HER-2 overexpressing pancreatic cancer xenograft [40]. However, the full length HER-2 (p185) is known to undergo proteolytic cleavage, which generates a 95 kDa HER-2 fragment [41]. The p95 truncated portion of HER-2 (p95HER-2) is shown to possess higher kinase activity causing increase in intracellular signaling and its presence has been linked to lymph node metastasis in breast cancer [42]. Trastuzumab acts against HER-2 by binding to the extracellular domain and one of the major events that is accountable for the resistance to Trastuzumab is the shedding or cleavage of HER-2 receptors [43]. Presence of truncated p95HER-2 fragments in tumors is associated with chemo resistance to HER-2 targeted therapies. It is suggested that treatment approaches have to be modified in such cases [43]. The importance of p95HER-2 fragments in HER2-targeted therapies have been studied in tumor samples from breast cancer patients; 51.4% of patients positively responded to Trastuzumab and the response was lowered to only 11.1% in cases where tumor samples were p95HER-2 positive [44]. In our study, occurrence of basal level of full length and truncated forms of HER-2 was seen in MIA Paca-2 cells and treatment of juglone was significantly able to block the expression of both in a dose dependent manner (Figure 3.4.). Therapies that have been developed by targeting this specific receptor have been documented as an efficient anti-cancer approach. Ability of juglone to inhibit the expression of truncated and full length HER-2 makes it a very attractive therapeutic agent for treating p95HER-2-positive tumors, and this study is first of a kind to report inhibition of truncated p95HER-2 fragments in pancreatic cancer cells.

Yun et al. demonstrated using *in vitro* and *in vivo* experiments that juglone at 50 µM in combination with 50 µM of Trastuzumab inhibited the expression of HER-2 receptors and enhanced the effectiveness of Trastuzumab in both breast BT474 cancer cells and tumor xenograft model in *BALB*/C mice [45]. Similarly, plumbagin (5-hydroxy-2-methyl-1, 4-naphthoquinone), a

quinoid compound isolated from root of *Plumabago zeylanica* [46] which is very closely related molecule to juglone has been recognized to downregulate the expression of HER-2 and p-Akt in lung cancer H460 cells [47]. However, plumbagin was not able to downregulate ERK signaling. In our study, juglone was shown to inhibit the expression of HER-2 as well as its 95kDa protein fragment as shown in Figure 3.4. Juglone (5μM) in 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced mouse skin epidermal JB6 CI41 cells downregulated the activities of PI3K, an important event required for cancer cell metabolism and growth [48]. In the same study, it was also demonstrated that juglone could inhibit TPA-induced phosphorylation of Akt, which remains in agreement with our findings. Herein our study has reported successful findings on juglone-induced inhibition of phosphorylation of ERK, which was not observed in other studies carried out using juglone in TPA-induced mouse epidermal cells [48]. The results of this investigation suggest that juglone inhibits the expression of HER-2 receptors by inhibiting phosphorylation of ERK in pancreatic cancer MIA Paca-2 cells.

Cyclooxygenases are a group of enzymes responsible for conversion of essential fatty acids, arachidonic acid to prostaglandins and are found in two different isoforms; COX-1 and -2. The level of COX-2 is very low in normal cells and is found to be prominent in pancreatic cancer tumors. The level of COX-2 in pancreatic cancer was reported to be sixty times higher than in their normal counterpart and was detected in 90% of pancreatic cancer [49]. Inhibition of cell intrinsic COX-2 expression has been linked to delay in pancreatic cancer development and progression [50]. COX-2 is shown to upregulate tumor aggressiveness by inducing expression of anti-apoptotic protein Bcl-2 and activating Akt. Phosphorylation of Akt and activation of COX-2 are interrelated [50]. Drugs that target both COX-2 and Akt are required to develop an effective chemotherapy in order to limit tumor growth and overcome chemo-resistance. Therefore, to investigate whether

downregulation of p-Akt also results in inhibition of COX-2, western blot analysis of MIA Paca-2 cancer cells treated with juglone was done. Celecoxib, a COX-2 inhibitor has shown promising results in decreasing tumor weight and inhibiting metastasis in mice bearing pancreatic cancer xenograft [51]. COX-2 inhibitors execute their role by releasing cytochrome C, activation of caspases, and cleavage of PARP [52]. The upregulation of COX-2 in pancreatic cancer and its role in tumor growth provides an important rationale to target COX-2 pathway. Our results indicate that the level of COX-2 molecules was significantly suppressed in juglone-treated cells in a dose dependent manner at concentration as low as 1 μM. Like other COX-2 inhibitors [53], in this study juglone was found to inhibit COX-2 (Figure 3.7) along with Bcl-2 (Figure 3.3) and Akt (Figure 3.5.). Similar inhibition of COX-2 activity using juglone was previously been determined in TPA-induced COX-2 activation in mouse epidermal cells [48].

Increased aerobic glycolysis is another abnormal phenomena observed in cancer cells. This process was first described by Otto Warburg in 1926 and therefore is also known as the "Warburg effect". The Warburg effect has been recognized as the seventh hallmark of cancer [54, 55]. Normal cells usually depend upon mitochondrial oxidative phosphorylation for generation of energy, and only undergo aerobic glycolysis in absence of oxygen whereas cancer cells mostly rely on aerobic glycolysis even in presence or absence of oxygen [56]. Generation of ATP via aerobic glycolysis (2 ATP/glucose) is more significantly energy inefficient than oxidative phosphorylation (36 ATP/glucose), therefore utilization of glucose by cancer cells is a lot higher in order to produce enough ATPs required for their vigorous metabolism. Dependency of cancer cells on glycolytic pathways for energy can be exploited to design therapies that can target the key biomolecules involved in the pathway [57].

The first step in glycolysis is the formation of glucose-6-phosphate from glucose, which is irreversibly catalyzed by hexokinase enzyme [58]. Hexokinases are predominantly expressed in many tumor cells and function in catabolism of glucose in rapidly proliferating tumor cells. In this study, we report for the first time that juglone inhibited glycolysis via downregulation of hexokinase in human pancreatic cancer cell. Hexokinase has also been linked to the mitochondrial apoptotic related BCl-2 family proteins [59]. Downregulation of hexokinase also leads to upregulation of mitochondrial pro-apoptotic protein Bax [60] which was observed in this study using juglone on MIA Paca-2 cells. Other known glycolytic inhibitors such as iodoacetate and 3-bromopyruvate are reported to decrease pancreatic cancer cell survival and proliferation [61]. Shikonin, a quinone based compound similar to juglone was reported to inhibit hexokinase in glioblastoma cells [62]. In the same study, shikonin in combination with a commercial glycolytic inhibitor showed promising results in inhibiting proliferation and angiogenesis in *in vitro* and *in vivo* experiments [62]. These studies support the importance of inhibiting glycolytic pathways in cancer cells by naphthoquinone derivatives as a promising therapeutic strategy.

Our results of juglone-induced inhibition of hexokinase (Figure 3.8) are comparable to the study performed using curcumin in HT29 colon cancer cells [63]. Curcumin was shown to significantly inhibit hexokinase activity but had no effect on other glycolytic enzymes. Other natural flavonoids that are documented with anti-glycolytic effect include oroxylin A, a molecule derived from root of *Scutellaria baicalensis* used in traditional Chinese medicine [64]. Oroxylin A indirectly inhibited peptidyl-prolyl cis/trans isomerase (Pin1) activity in breast cancer cells which eventually caused inhibition of hexokinase and glycolytic activity. Oroxylin A, like juglone is a known Pin1 inhibitor [65]. Taken together, our results suggest that juglone could modulate the glycolytic activity in pancreatic MIA Paca-2 cancer cells by inhibiting hexokinase activity.

3.5. Conclusion

Juglone possess various biological activities against pancreatic cancer MIA Paca-2 cells. Findings that are listed in this chapter indicate that juglone-induced cytotoxicity is based on generation of reactive oxygen species, upregulation of pro-apoptotic molecules, downregulation of anti-apoptotic molecules, and activation of caspase-3, followed by cleavage of PARP which eventually causes DNA damage and subsequent cell death. Other depicted molecular events are altered expression of oncogenic markers, downregulation of activation of Akt, COX-2, Erk, tyrosine receptors like HER-2, and inhibition of glycolytic enzyme. These findings provide a rationale for further *in vivo* and pre-clinical evaluation of juglone on pancreatic cancer.

3.6. References

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CHAPTER 4. ANTI-ANGIOGENIC AND ANTI-METASTATIC ACTIVITIES OF JUGLONE AGAINST PANCREATIC CANCER CELLS

4.1. Introduction

Pancreatic cancer accounts for 3% of all cancers in the United States and still is the fourth leading cause of cancer related deaths in the US. The estimated number of new cases in the year 2015 was 48,960 and number of death was 40,560 [1]. The five- year survival rate for patients with pancreatic cancer is 6% with median survival time of 3-6 months [2]. Surgical resection, chemotherapy or radiotherapy are the current treatments for early stage pancreatic cancer; however there is no curative option for advanced pancreatic cancer, which is usually metastatic in nature. Better treatment approaches are needed to target pancreatic cancer metastasis.

The metastatic ability of malignant tumors plays an important role in tumor progression. Cancer metastasis is accountable for more than 90% of cancer related deaths ([3-5]. The principal sites of metastasis for pancreatic cancer cells are liver and lungs [6]. Metastasis involves a series of four sequential steps: detachment, migration, invasion and adhesion described as the "invasion-metastasis cascade" [7, 8]. The adhesion and detachment ability of cells, interaction with other cells and with the extracellular matrix defines the extent of invasiveness of tumor cells. Angiogenesis, or the formation of new blood vessels, is an essential process of tumor metastasis [9].

Tumor cells produce growth factors, like vascular endothelial growth factor (VEGF), which regulates angiogenesis and stimulates blood vessel formation. The newly formed blood vessels are a primary route for tumor cells to escape the primary site and enter into circulation [10]. Several anti-angiogenic therapeutic approaches have been developed. Many natural products, phytochemicals and dietary agents such as gallic acid, rhein, luteolin, and quercetin have been extensively studied for their anti-angiogenic potential as a therapy against cancer [11].

Juglone (5-hydroxy-1,4-naphthoquinone) is a natural quinone based compound and is a derivative of 1, 4-naphthoquinone. Juglone is isolated from plants of the *Juglandaceae family* and commonly found in black walnut, English walnut, butternut and others [12]. Juglone has been reported to be a component of ancient medicine for ringworm, fungal, bacterial and viral infections [13], and only limited studies have been conducted to understand the anti-tumor properties of juglone [12, 14, 15].

No study has yet demonstrated the anti-metastatic potential of juglone in pancreatic cancer cells. Therefore, the present experiments were designed to study the inhibitory activities of juglone against the migration and invasion of human pancreatic cancer MIA Paca-2 cells *in vitro*.

4.2. Materials and Methods

4.2.1. Reagents

Juglone was purchased from Sigma-Aldrich (St. Louis, MO). Primary and secondary antibodies for vascular endothelial growth factor (VEGF, #sc-7269) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). β-actin ((#4967), Akt (#2966) and p-Akt (#2531s) were purchased from Cell Signaling Technology (Danvers, MA).

4.2.2. Cell Culture

Human Umbilical Vein Endothelial Cells (HUVEC) and pancreatic cancer MIA Paca-2 cells were purchased from American Type Cell Culture. Cells were cultured in a humidified incubator containing 5% CO_2 at 37ºC. HUVEC were maintained in Medium 200 (Invitrogen) supplemented with Low Serum Growth Supplement (LSGS kit, Invitrogen). MIA Paca-2 cells were cultured in Dulbecco's Modified Eagle Medium supplemented with 10 % fetal bovine serum, penicillin and streptomycin and both cell lines were sub-cultured after reaching 70-80% confluency.

4.2.3. In-vitro Endothelial Cell Tube Formation Assay

This assay was performed as suggested by the manufacturer using the *in vitro* angiogenesis assay kit from Millipore™. Briefly, 50 µl of matrigel was added to the wells of a 96-well plate at 4°C and allowed to polymerize at 37°C for at least 1 h. Human umbilical vein endothelial cells (HUVEC) were then seeded at a density of $1x10^3$ cells per well on the surface of the matrix. Cells were then treated with juglone at concentrations ranging from 100 nm to 10 µM.

In a different set, HUVEC were also treated with conditioned media collected from MIA Paca-2 cells treated with juglone. Cells were then allowed to incubate at 37°C for at least 6 h and tube formation was analyzed and photographed using a Leitz phase-contrast inverted microscope at 10X magnification. Length of the capillaries were measured by using Image-Pro software (Media Cybernetics, Inc, Warrendale, PA).

4.2.4. Wound Healing Migration Assay

MIA Paca-2 cells were plated in 96-well plates overnight to achieve a confluent cell layer. A linear wound was created with micropipette tips and cell layers were washed twice with serum free media to remove the floating cells. Cells were then treated with juglone (0, 1, 5 or 10 µM). The effect of juglone on tumor cell migration was observed after 24 h incubation. Each well of the plate was photographed at the same site at 0 h and 24 h and compared. The width of the wound at 0h and 24 h was measured using Image-Pro Software. Results are expressed as the ratio of wound width at 0 and 24 h after treatment [16].

4.2.5. Transwell Migration Assay

Transwell Migration is also referred to as Boyden chamber assay. MIA Paca-2 cells at 70% confluence were treated with juglone (0, 1, 5 or 10 µM)) for 6 h and after treatment viable cells were harvested with 0.25% trypsin EDTA (Invitrogen, CA) and plated at a density of $5x10^4$ MIA

Paca-2 cells in 200 µl serum-free growth media per insert in the apical chamber of Transwell with 8 µM pore size (Corning). The basal chamber was filled with 500 µl of medium with 20% FBS as a chemoattractant. After 72 h of incubation, media from the apical and basal chambers of transwell were removed and non-migrated cells in the apical chamber were scraped off by moist cotton swab.

Cells in the undersurface of the transwell inserts were washed with phosphate buffer saline (PBS) and then fixed with 70% ethanol for 15 min at room temperature. Fixed cells were then stained with 0.25% crystal violet (CV). After 30 min of incubation with CV, excess dye was removed and the inserts were washed in running water. Images of stained cells were captured with Leitz phase-contrast inverted microscope. Retained CV in cells were dissolved with 33 % acetic acid and optical density was read at 595 nm (Benchmark Plus, BioRad, Hercules, CA).

4.2.6. Matrigel Invasion Assay

Matrigel invasion assay is used to evaluate chemotaxis and the ability of tumor cells to invade the extracellular matix [16, 17]. The matrigel invasion assay is similar to the transwell migration assay however, the difference in the set-up is that the inserts in the transwell insert are first coated with ECM matrix (matrigel). Matrigel (BD Biosciences) was thawed overnight at $4^{o}C$, diluted with serum-free media in the ratio of 1:1 and 50 µl of matrigel was added to each transwell insert. Transwell inserts with matrigel were placed in the incubator at $37^{o}C$ for at least 1h to form a thin layer gel. The rest of the protocol was similar to the one described in the migration assay.

4.2.7. Expression of HIF-1α and VEGF

MIA Paca-2 cells were plated in a black 96 well plate (R&D Systems, Minneapolis, MN) at a cell density of $1x10^4$ cells per well. After allowing cells to attach overnight, cells were treated with juglone (1, 5 or 10 µM) for 6h. After required incubation, media were removed and cells were washed with PBS. Immediately, cells were fixed with 4% formaldehyde for 15 min. After 15 min,

cells were washed with PBS and endogenous peroxidase activity of cells was blocked by incubation with 30% hydrogen peroxide for 15 min. Cells were then incubated with blocking buffer for 1 h, washed with PBS at least 3 times and incubated overnight with HIF-1α antibody.

VEGF was analyzed in cell lysates by Western Blot. In brief, cells were plated at a cell density 2.5 $x10^5$cells/well in a six well plate. After seeding overnight, cells were treated with juglone for 6 h. After 6 h, media was removed and cells washed with phosphate buffer saline. Whole cell lysate was prepared by using cell lysis buffer from Cell Signaling Technology. Protein concentrations of the samples were determined using the BCA assay.

Fifty µg of protein was loaded in SDS polyacrylamide gel; 4-12 % Bis-Tris (Invitrogen, Carlsbad, CA). Prepared samples were separated by gel electrophoresis and transferred to polyvinylidene difluoride (PVDF) membranes (Invitrogen, Carlsbad, CA). Membranes were blocked with 5% BSA in TBS containing 0.05% Tween 20 (TBS-Tween) for 1h. The PVDF membranes were then incubated with primary antibody in TBS-Tween containing 5% BSA overnight at 4^{o}C with gentle agitation.

Visualization of the bound primary antibody was done by probing the membrane with horseradish peroxidase–conjugated secondary antibodies and exposure to chemiluminescent substrate (Thermo Fisher Scientific). Chemiluminescence was recorded by BioRad Imaging system. For quantification, densitometric analysis of the bands were done by Quantity One 1-D analysis software (BioRad, CA).

4.2.8. Statistical Analysis

All the experiments were conducted in triplicates. Results are expressed as means ± SD of experiments. Analysis of variance (ANOVA) will be conducted to examine the differences

between treatments followed by Tukey's analysis using SAS. A P-value of <0.05 was considered to be statistically significant. Means with different letters indicate significantly different.

4.3. Results

4.3.1. Juglone Inhibits *in-vitro* Tube Forming Ability of Endothelial Cells

The results of this experiment showed dose-dependent inhibition of endothelial cell alignment (Figures 4.1. and 4.2.). When endothelial cells were treated with juglone there was a dose dependent inhibition. Juglone was effective in reducing the length of the tubes *in vitro* at a concentration as low as 250 nM (Figure 4.1.) and at concentration of 5 and 10 µM, the network was completely disrupted and was not readable (NR).

Highly organized, extensively aligned capillary-like structures were formed in untreated HUVEC. Similarly, highly organized and extensively aligned capillary-like structures were formed when HUVEC were treated with conditioned media from MIA Paca-2 cells (Figure 4.2.).

4.3.2. Juglone Suppresses Pancreatic MIA Paca-2 Cells Migration

The wound scratch assay was performed for analyzing the effect of juglone in inhibiting migration. The width of the wound was measured after 24 h of treatment and the ratio of wound size at 0 h and 24 h was calculated (Figure 4.3., A). All the treatments with juglone resulted in inhibition of closure of wounds.

In untreated cells, the ratio was 1.3. In cells treated with 1, 5 and 10 µM juglone, the ratio dropped to 1.24, 1.11, and 0.91, respectively. These ratios are presented in terms of percentage of untreated cells in Figure 4.3, B. A similar pattern was observed in the transwell chamber migration assay where juglone treatment significantly decreased the number of migrated cells at 5 µM and 10 µM (Figure 4.4.).

4.3.3. Juglone Inhibits Pancreatic Cancer MIA Paca-2 Cell Invasion

The Boyden Chamber assay was used to evaluate the effect of juglone on MIA Paca-2 cell invasion. To rule out the cytotoxic effect of juglone on migration and invasion, cells were treated with juglone (1, 5 or 10 μM) for 6h and only viable cells were collected and utilized for both migration and invasion assay.

Results in Figure 4.5. show that juglone dose-dependently inhibited the invading potential of MIA Paca-2 cells. When MIA Paca-2 cells were treated with 1 μM juglone, 34.8% of the pancreatic cancer cells maintained their ability to invade the matrigel. When MIA Paca-2 cells were treated with 5 μM juglone, 28.9% of the pancreatic cancer cells were able to invade the matrigel. And when MIA Paca-2 pancreatic cancer cells were treated with 10 μM, only 0.3% of MIA Paca-2 pancreatic cancer cells maintained their ability to invade the matrigel.

4.3.4. Juglone Inhibits Expression of Proteins Associated with Cell Migration and Invasion *in vitro*

The level of HIF-1α in MIA Paca-2 cells after treatment of juglone was measured by cell based ELISA and is reported in Figure 4.6. The results of western blot on analysis of p-Akt and VEGF are also shown in Figures 4.7 and 4.8. Results indicate that juglone dose-dependently inhibited the levels of VEGF, p-Akt and HIF-1α (Figures 4.6-4.8).

4.4. Discussion

Several studies suggest that juglone possesses numerous anti-cancer properties. The effect of juglone as an anti-cancer agent against ovarian cancer cells was reported [18]. Zhang et al. [19] demonstrated the inhibitory activities of juglone against cervical cancer cells. However, there are no reports on the effect of juglone in pancreatic cancer cells. This study investigated the inhibitory activity of juglone against the migration and invasion of human pancreatic cancer MIA Paca-2 cells *in vitro*.

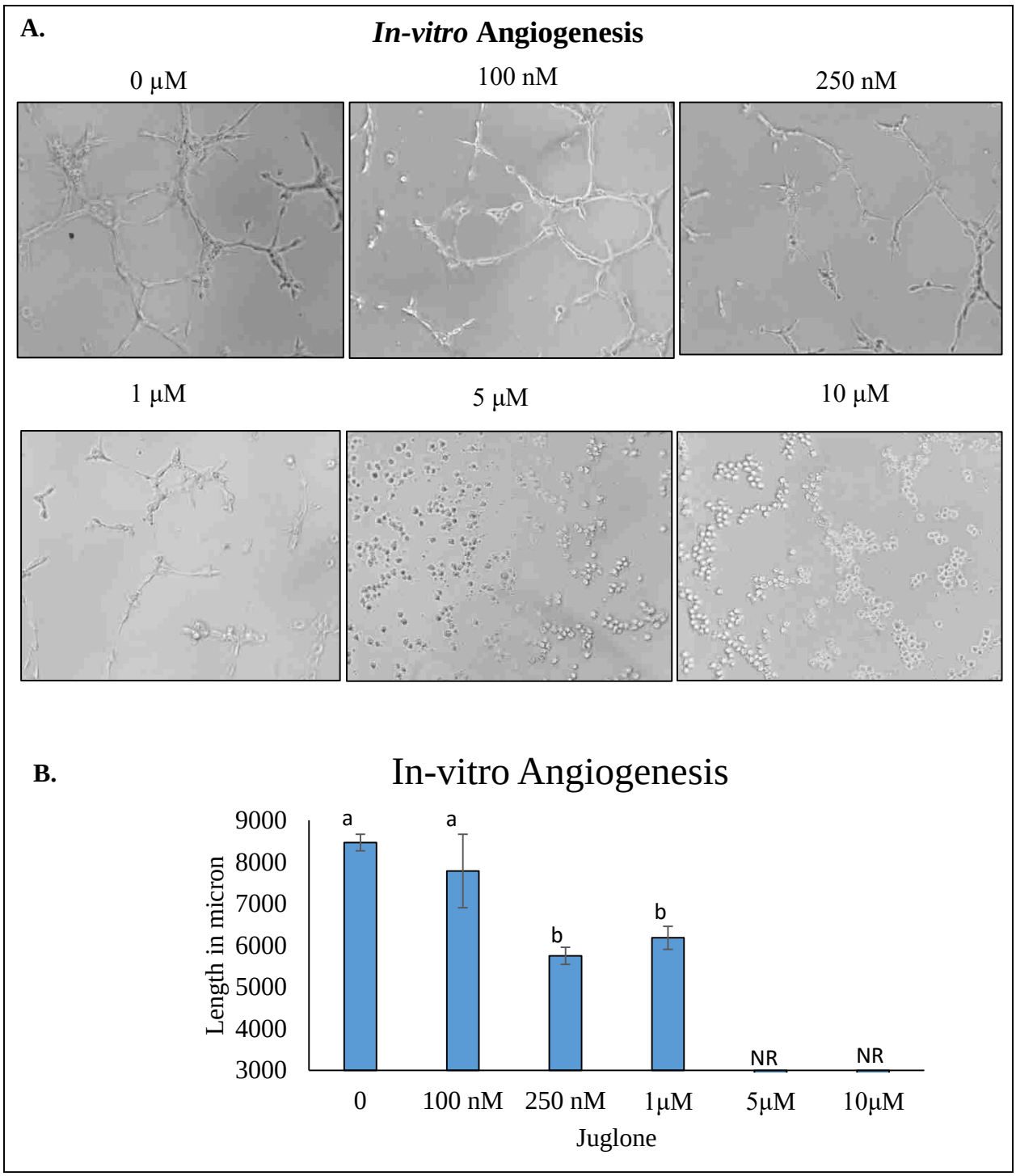

Figure 4.1. Effect of juglone on HUVEC tube formation. HUVEC were plated on top of polymerized matrigel, incubated with various concentrations of juglone and tube formation was observed after 6h of incubation (A). The length of capillaries formed was measured using ImagePro software and represented (B). At concentrations 5 and 10 µM the lengths were not readable (NR).

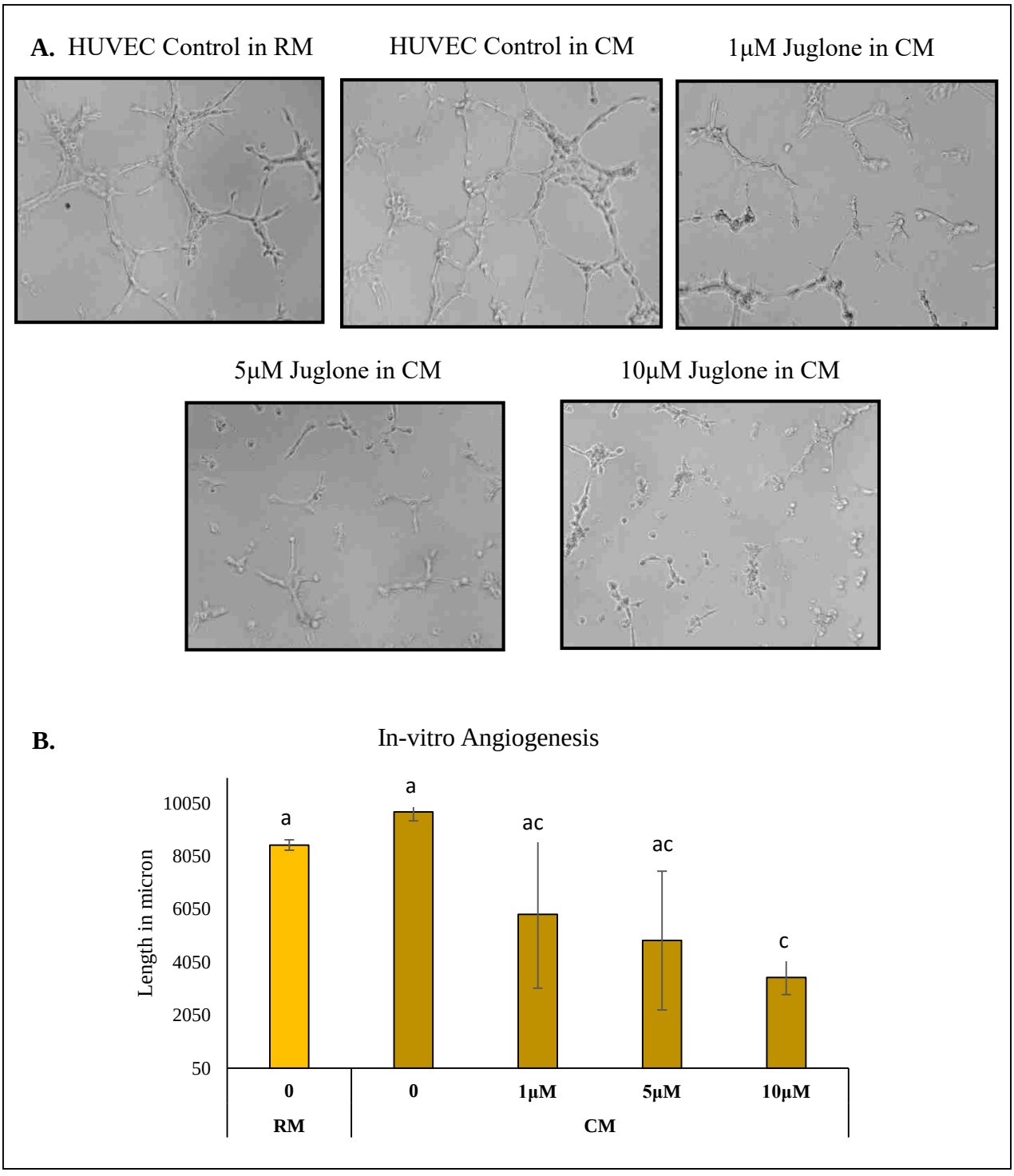

Figure 4.2. Effect of juglone on HUVEC tube formation using conditioned media (CM) from MIA Paca-2 cells. Results were compared to HUVEC grown in regular media (RM). HUVEC were plated on top of polymerized matrigel, incubated with various concentrations of juglone containing conditioned media (CM) from MIA Paca-2 cells and tube formation was observed after 6h of incubation (A). The length of capillaries formed was measured using ImagePro software and represented (B).

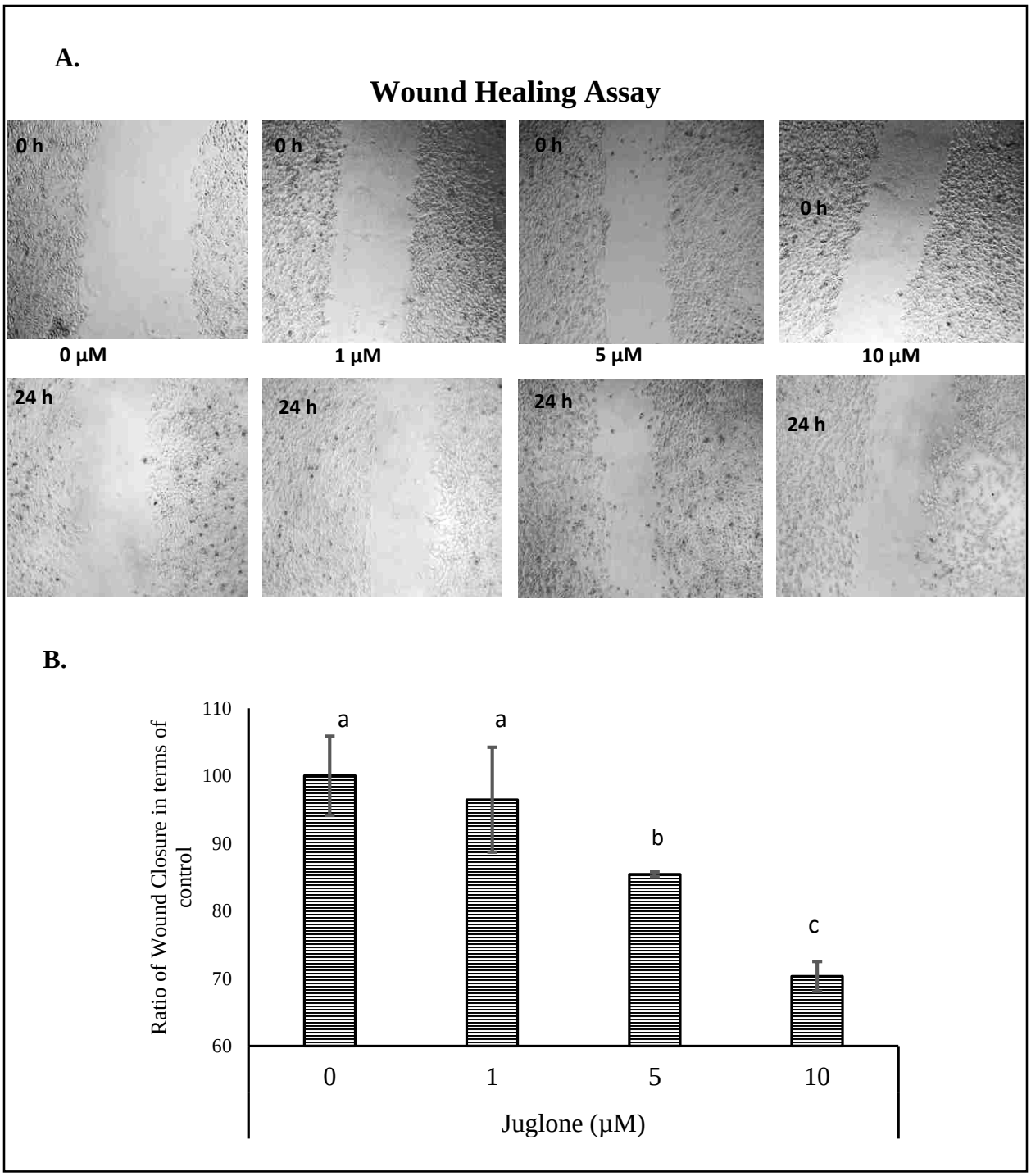

Figure 4.3. Effect of juglone on wound closure. A. Confluent layer of MIA Paca-2 cells were scratched with micropipette tips (upper panel A) and cells with wounds were incubated with varying concentrations (1-10 µM) of juglone for 24 h. Photographs of the wound were taken at 0 h (upper panel A) and at 24 h (lower panel B) of incubation. Quantitative representation of wound closure was measured using Image Pro software and ratio of width of the wound before and after treatment is presented (B).

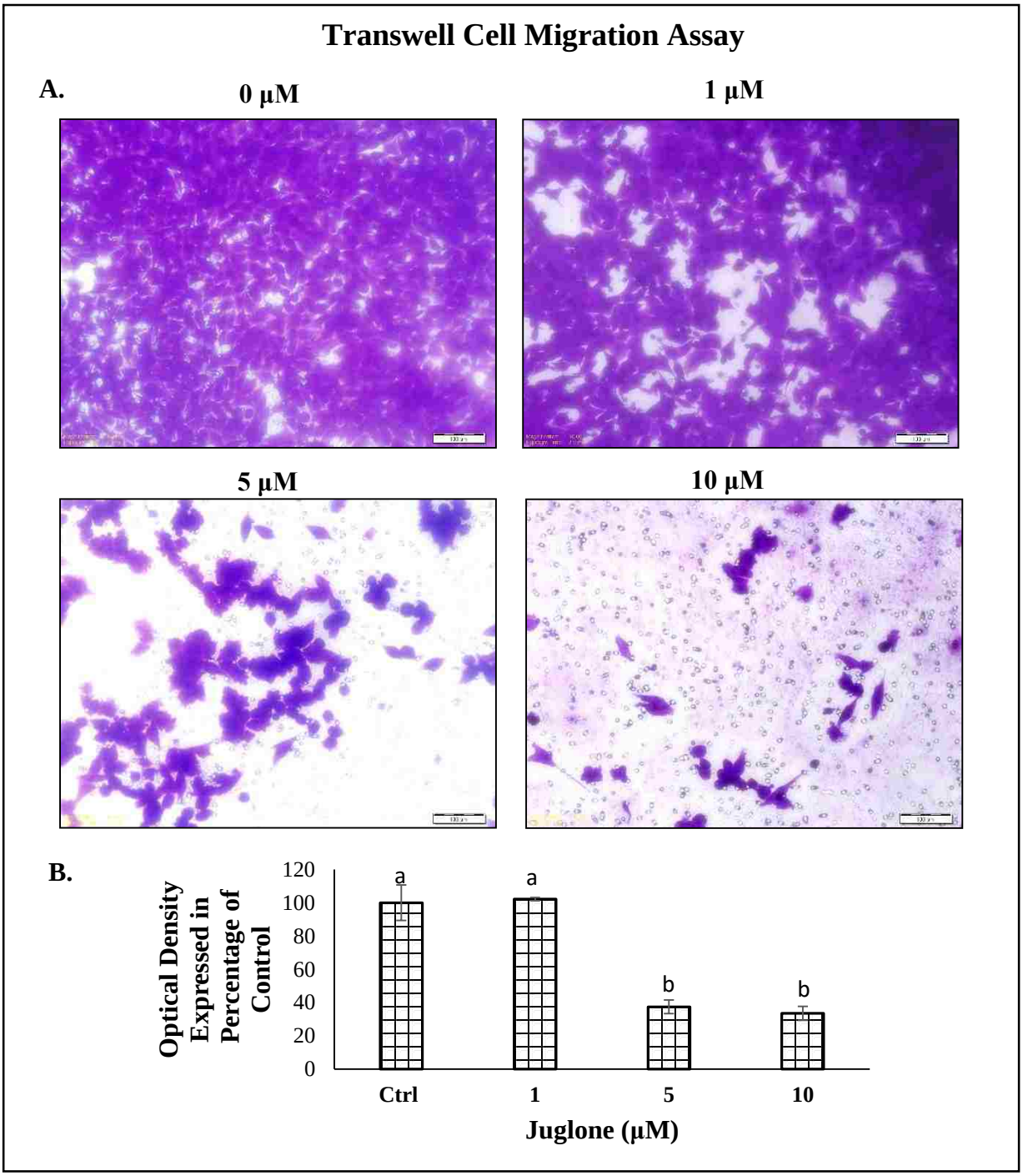

Figure 4.4. Effect of juglone on the migration of MIA Paca-2 cells. Cells were treated with various concentrations of juglone for 6h. After treatment, cells were harvested and live cells were plated ($5x10^4$) on the upper chamber of transwell inserts and allowed to migrate for 48h. Invaded cells on the undersurface of the inserts were stained with crystal violet and photographs were captured with a phase contrast microscope (A). Retained crystal violet by migrated cells were dissolved in acetic acid and optical density was measured at 595 nm and expressed as percentage of control (B).

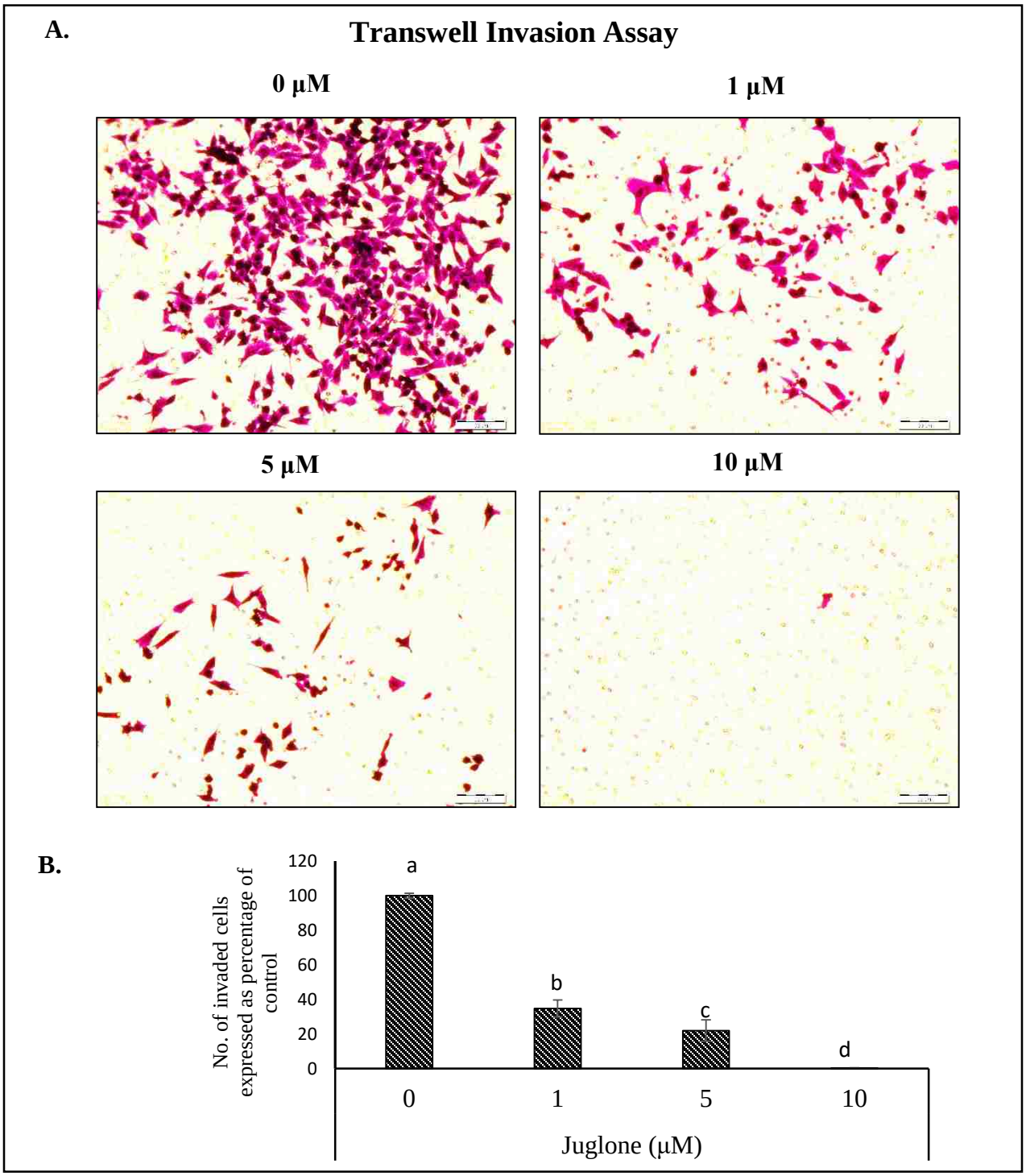

Figure 4.5. Effect of juglone on the invasion ability of MIA Paca-2 cells. Cells were treated with various concentrations of juglone for 6h and after treatment, cells were harvested and plated ($1x10^5$) on the upper chamber of transwell inserts with matrigel layer and allowed to invade for 48h. Invaded cells on the undersurface of the inserts were stained with crystal violet and photographs were captured with a phase contrast microscope.

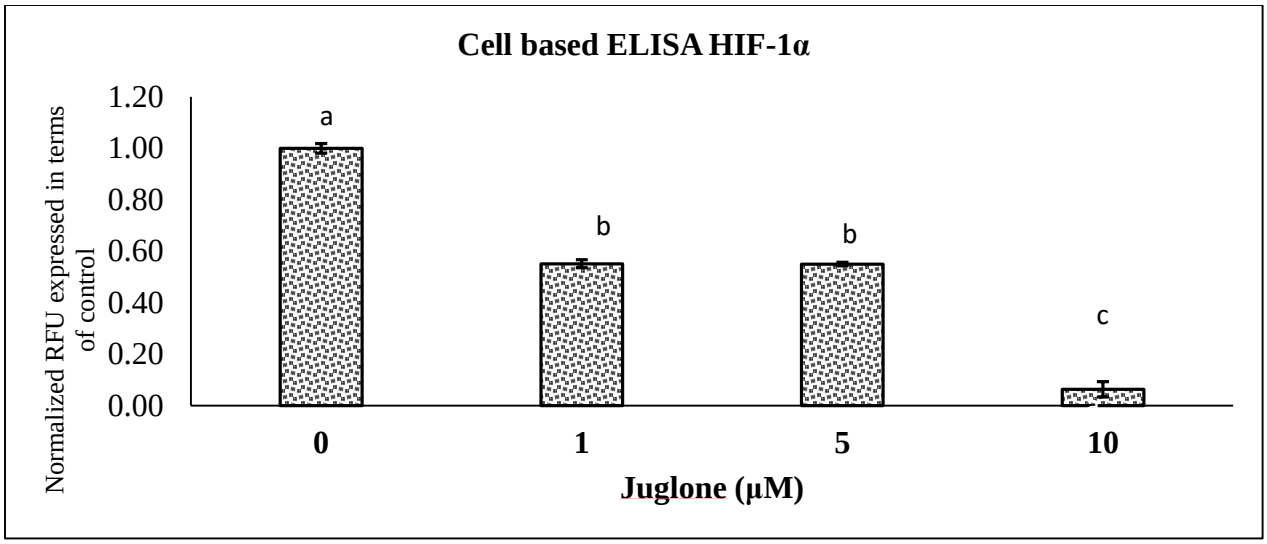

Figure 4.6. Expression of HIF-1α in MIA Paca-2 cells treated with juglone as measured by a cell based ELISA assay.

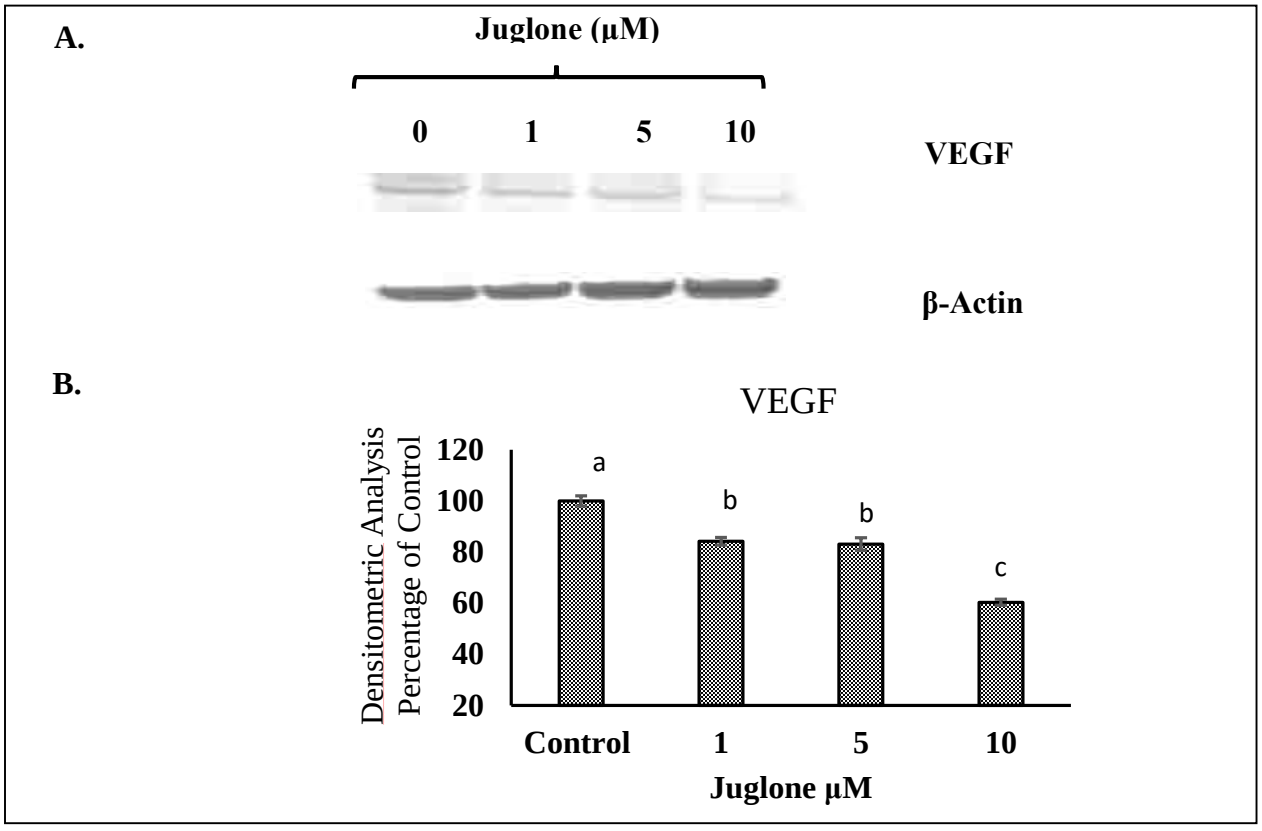

Figure 4.7. Western Blot analysis of VEGF expression in MIA Paca-2 cells. Cells were treated with various concentrations of juglone (1-10 µM) for 6h and cell lysates were collected. Western blot was performed with 30 µg of protein/lane (A). Expression of VEGF was quantified using Quantity One software and reported as percentage of control (B).

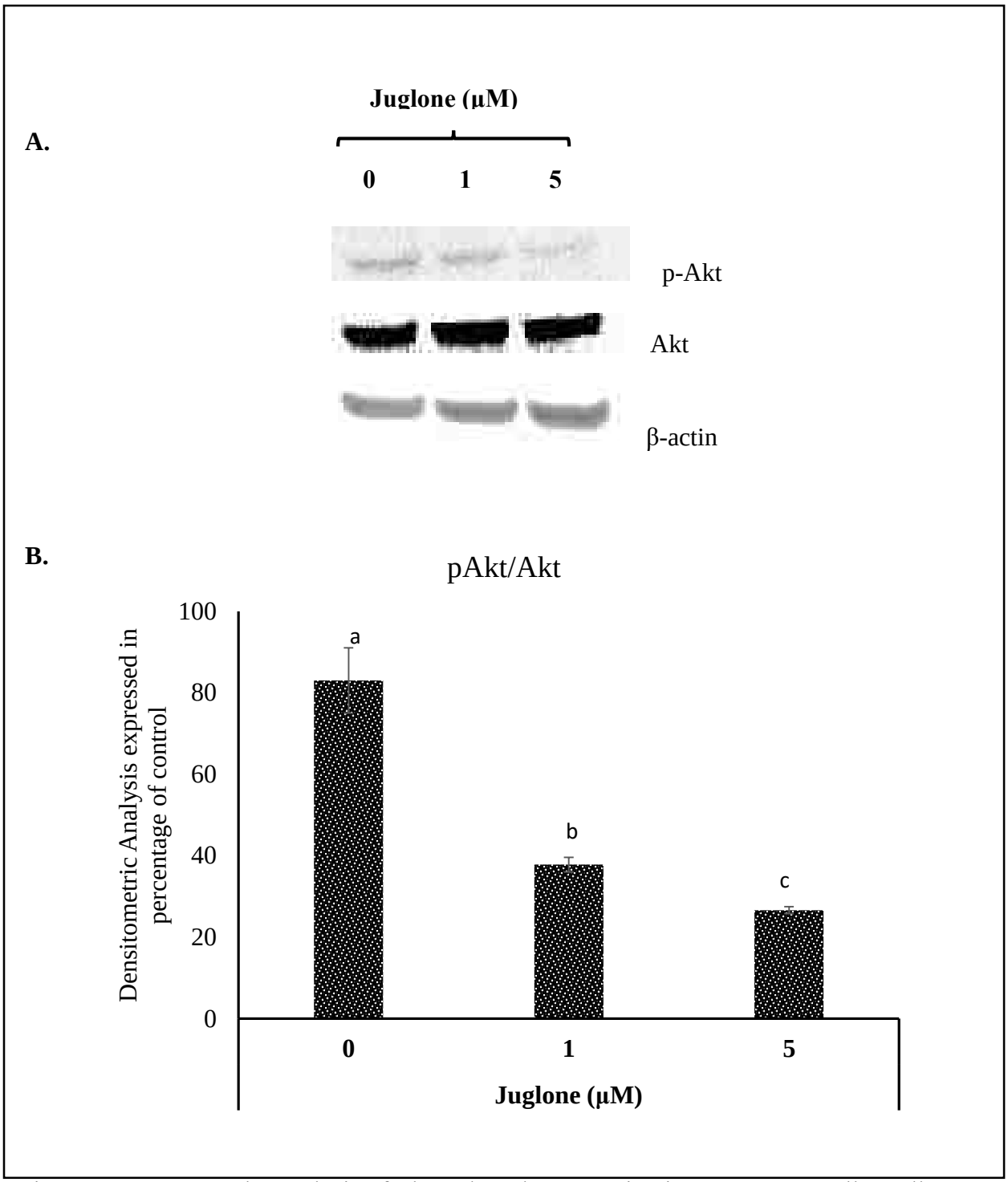

Figure 4.8. Western Blot analysis of Akt and p-Akt expression in MIA Paca-2 cells. Cells were treated with juglone (1-5 µM) for 6h and cell lysate were collected. Western blot was performed with 70 µg of protein/lane (A). Quantification of p-Akt/Akt ratio was done using Quantity One software and reported as percentage of control (B).

Juglone at 1μM significantly inhibited the migration and invasion capabilities of pancreatic cancer MIA Paca-2 cells. Cancer cells grow rapidly and are efficient in migrating and closing the artificial scratch wound. Decrease in the ratio of the size of the wound with time and dose of juglone suggests that cancer cell migration can be significantly reduced with juglone treatment.

To understand the molecular mechanism behind angiogenesis, studying the expression of angiogenic markers such as Vascular Endothelial Growth Factor (VEGF) is important. VEGF overexpression is regulated by Hypoxia inducible factor (HIF-1α) [20, 21] and is associated with significant metastasis, angiogenesis and poor prognosis of PC [22]. Angiogenesis inhibitors are beneficial in reducing tumor growth and metastasis. Many naphthoquinone derivatives like Vitamin K2 [23] have been reported to act as inhibitors of angiogenesis. In this study, the anti-angiogenesis mechanism of juglone, a naphthoquinone derivative was studied in functions of HUVEC and results demonstrate that juglone strongly inhibits the formation of capillary-like network as shown in Figure 4.1. Our results agree with the findings from a study where 1, 4-naphthoquinone was used as a drug of interest and inhibited HUVEC tube formation at a similar concentration as juglone [24]. The tube formation assay was also performed using conditioned media from MIA Paca-2 cells and results are presented in Figure 4.2. The tube formation in HUVEC was higher when media from cancer cells was used when compared to regular media used in culturing HUVEC (Figure 4.2.), which might be due to the increased level of growth factors released by cancer cells in culture. Even in this set of experiments, juglone containing conditioned media inhibited tube formation. These results of tube formation assays taken together suggest juglone as a strong anti-angiogenesis agent, which can be beneficial in treating cancer.

Akt plays a central role in regulating angiogenesis as it transmits signals received from cytokines and growth factors to upregulate HIF-1α and VEGF expression in cancer cells. There

exists an autocrine loop between Akt and VEGF expression and Akt activation is a requirement for HIF-1α and VEGF expression [25]. Various natural compounds e.g. resveratrol from grapes, capsaicin from pepper have shown inhibitory effect on HIF-1α and VEGF by inhibiting the Akt pathway [26]. Juglone at 20 μM in combination with ascorbate (1mM) was recently shown to inhibit p-Akt in breast cancer MCF7 cells [27]. Our results indicate that juglone by itself is very effective in inhibiting p-Akt at 5μM in pancreatic cancer MIA Paca-2 cells (Figure 4.8.).

The transcription factor hypoxia inducible factor - 1α is very closely related with the regulation of numerous genes associated with angiogenesis [28], erythropoiesis, glycolysis, iron metabolism, and cell survival [29]. Besides, the level of oxygen tension there are other oxygen independent factors like nitric oxide [30], cytokines, serum, insulin, and insulin-like growth factors [31] that can enhance the activity of HIF-1α. Overexpression of HIF-1α in human tumors is connected to metabolic pathways that might lead to the activation of tumor angiogenesis, invasion, and metastasis [32, 33]. In this present study, our results demonstrated that juglone significantly decreased the level of HIF-1α compared to the untreated control (Figure 4.6.). Previous studies with anthraquinones such as emodin or rhein [34] have shown its efficacy in inhibiting migration and invasion of cancer cells with down regulation of associated proteins like HIF-1α and VEGF [35], which is in agreement with our data (Figures 4.6. and 4.7.).

One of the downstream signaling molecule of HIF-1α is VEGF, which is considered as an important factor for regulating angiogenesis and is related to growth and metastasis of pancreatic adenocarcinoma [21, 36]. VEGF is highly expressed in physiological cases like wound healing and embryonic development and in pathological cases like cancer. A recent study has demonstrated that juglone inhibited tumor proliferation, angiogenesis and migration of breast cancer MCF7 cells *via* downregulation of VEGF and cyclin E proteins [37]. In our study, juglone

significantly inhibited VEGF expression in MIA Paca-2 cells (Figure 4.7.). Juglone inhibited pancreatic cancer cells wound closure, migration and invasion (Figures 4.3.-4.5.) and one of the underlying molecular mechanisms may involve reduced expression of VEGF.

4.5. Conclusion

In summary, juglone appears to have the ability with cell-based models to inhibit angiogenesis and metastasis behavior of pancreatic cancer MIA Paca-2 cells. While we have demonstrated that juglone appears to be a promising anti-cancer agent and inhibits metastasis and angiogenesis of pancreatic cancer cells *in vitro*, these results further warrant future preclinical animal studies and, if successful, clinical trials need to be followed to confirm the efficacy of juglone in *in-vivo* models for the treatment of pancreatic cancer.

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CHAPTER 5. SUMMARY AND CONCLUSIONS

Many studies have been carried out to understand the biological function of juglone in various types of cancer. The purpose of this dissertation was to evaluate the antitumor effect of naturally occurring quinone juglone in human pancreatic cancer cell model and have revealed its molecular targets. Evaluation on effect of juglone as an anti-angiogenic/anti-metastatic agent was also performed.

We have found out that juglone exerted cytotoxic effect and inhibited proliferation of pancreatic cancer cells *in vitro.* Morphological changes associated with apoptosis upon treatment of MIA Paca-2 cells with juglone was recorded. Significant inhibition in the clonogenic potential of cancer cells treated with juglone was also observed. Induction of apoptosis was further confirmed by occurrence of externalization of phosphatidylserine molecule, which was recorded by Annexin V staining and analysis of various molecular markers of apoptosis. The activity of caspases were found to be upregulated followed by cleavage of PARP in cells with exposure to juglone. Anti-apoptotic molecules like Bcl-2 were downregulated and there was an upregulation of pro-apoptotic molecules like Bax.

Most quinones exert its cytotoxic effect with their general redox cycling mechanism and production of reactive oxygen species. Similarly, our data showed that juglone also induced production of ROS. Furthermore, it was found that like other quinones, juglone targeted DNA of cells and pattern of DNA fragmentation was observed in cells treated with juglone. Other cellular events that were affected by juglone included inhibition of epidermal growth factor receptor (HER-2), phosphorylation of ERK, inhibition of COX-2, and reduced activity of the glycolytic enzyme hexokinase.

Anti-angiogenic and anti-metastatic actions of juglone were also investigated. Numerous quinone moiety containing drugs have been proven effective in inhibiting angiogenesis making

quinones an attractive agent for cancer therapy. Our findings suggest that juglone possess strong potential to inhibit angiogenesis and metastasis. The ability of endothelial cells to form capillary like network was disrupted with influence of juglone. The anti-angiogenic effect of juglone has been supported by the findings that juglone inhibited the central pro-angiogenic growth factor VEGF along with its regulators HIF-1α and Akt. Further, the effect of juglone on the ability of cancer cells to migrate and invade was conducted by using cell migration and invasion assays. It was found that pancreatic cancer cells treated with juglone significantly lost its ability to migrate and invade. These results indicate that juglone can inhibit the metastatic spreading of pancreatic tumor cells.

Based on our data, it can be extrapolated that juglone possess anti-cancer properties against pancreatic cancer and this compound can be further utilized in the process of drug discovery. The limitation of this research project is that all the studies were performed in cell culture models. It is unknown how the results will be translated in mouse models and human samples. Therefore future studies using juglone needs to be carried out in animal models in order to validate our findings and also to understand the absorption, distribution, metabolism and excretion (ADME) profile of juglone.

VITA

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