Studies on the Regulation of Hypoxia: Molecular Signaling and Their Intervention

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"I believe in evidence. I believe in observation, measurement, and reasoning, confirmed by independent observers. I'll believe anything, no matter how wild and ridiculous, if there is evidence for it. The wilder and more ridiculous something is, however, the firmer and more solid the evidence will have to be."

Isaac Asimov (1997) The Roving Mind. Prometheus Books. p. 349.

Abstract

Oxygen is the most abundant element on earth; essential for survival, and physiology of living organisms so called oxygen homeostasis. Organisms and cells have developed number of adaptive mechanisms to survive in oxygen-deprived (hypoxia) conditions. Hypoxia-inducible factor-1 (HIF-1α) serves as a master regulator of these adaptive processes. It binds directly to the hypoxia responsive element in the promoter region of hypoxia-inducible genes. Upregulation of HIF-1α has been tightly related to carcinogenesis, metastasis and angiogenesis. With a growing understanding of the HIF-1 pathway, inhibition and stimulation of its transcriptional activity via small molecules has become an attractive goal in disease therapeutics.

In light of above information and my interest in hypoxia signaling, I have established a cell-based screening system to search for phytochemicals with hypoxia-modulating effects by recruiting hypoxia responsive element (HRE) driven luciferase reporter, and identified few bioactive natural compounds as pro-hypoxia and anti-hypoxia factor. In the first study, I have chosen a pro-hypoxia molecule (C-021) Caffeic acid phenethyl ester (CAPE), identified as pro-hypoxia factor, which is known as major component of New Zealand propolis, known for a variety of health promoting and therapeutic potentials. Several studies shown selective toxicity to cancer cells while treated with CAPE. I have investigated the anticancer and anti-metastasis activities of CAPE and its direct binding partner. Initial molecular docking studies suggested that CAPE is showing capability of mortalin-p53 complexes disruption. Invitro evidences clearly demonstrated that CAPE disrupts mortalin-p53 complexes which lead to nuclear translocation and activation of p53 resulting in growth arrest in cancer cells. In addition, CAPE-treated cells shown reduction in mortalin and several other key regulators of cell migration which is accountable for its anti-metastasis activity. Here, I have identified CAPE as a new anti-mortalin molecule.

Most drugs used as anticancer agents to treat various cancers like lung, breast, pancreatic etc. also promote drug resistance and finally unlimited tumor growth. In

extension of this study, I tried to identify the effect of CAPE on drug resistance in cancer cells. Drug resistance is closely related to cancer cell stemness, that is acquired along with resistance to various anticancer agents. Mortalin/mthsp70, a member of Hsp70 family, has been shown to promote proliferation, metastasis and angiogenesis, and downregulate apoptotic signaling. Mortalin/mtHsp70 is a member of Hsp70 family of proteins. Enriched in a large variety of cancers, it has been shown to contribute to the process of carcinogenesis by multiple ways including, inactivation of tumor suppressor p53 protein, deregulation of apoptosis and activation of EMT signaling. As I already established CAPE as an anti-mortalin molecule, here I found that upregulation of mortalin contributes to cancer cell stemness by using a mortalin overexpression model. Several cancer cell stemness markers, such as ABCG2, OCT4, CD133, ALDH1, CD9, MRP1 and connexin were upregulated in mortalin overexpressing cells that showed higher ability to form spheroids. These cells also showed higher migration and were less responsive to a variety of cancer chemotherapeutic drugs. Of note, knockdown of mortalin by specific shRNA or CAPE sensitized these cells to all the drugs used in this study. I used low doses of anti-mortalin molecules, MKT-077 (already known) and CAPE and found that these caused similar sensitization of cancer cells to chemotherapeutic drugs and hence are suggested as potential candidates for effective cancer chemotherapy.

I provide experimental evidence that the low doses of CAPE, that did not cause cytotoxicity or anti-migratory effect, rather activated HIF-1α and inhibited stress-induced protein aggregation which is a cause in diseases like Alzheimer etc.

In second study, I have chosen (C-024) Withaferin-A (Wi-A) identified as anti-hypoxia molecule, a natural compound from *Withania somnifera* (Ashwagandha) plant, an important medicinal plant, is commonly known as “Indian ginseng” or “winter cherry”. In previous studies, Nuclear Factor-κB (NF-κB) a redox-sensitive transcriptional factor identified as major transcription factor responding to cellular stress. So perhaps it will not surprising that it is activated by hypoxia, or decreased oxygen availability.

Withaferin-A was shown to inhibits NF-kB (nuclear factor kappaB) in previous studies. More recently, NF-kB binding to this site in the HIF-1α promoter has been shown also under hypoxic conditions. Thus, these two major pathways regulating the responses to inflammation and oxidative stress on the one hand, and hypoxia on the other hand, appear to be intimately linked. Constitutional activation of NFκB in cancer cells has been related to their anti-apoptosis and continued proliferation characteristics. Other than these facts, NF-kB also showed to have a major role in cellular senescence. Hypoxia modulates senescence in various ways. Excess oxygen expose to mitochondria produces reactive oxygen species (ROS) that can promote senescence. Hypoxia leads to the stabilization of HIF, which impacts many pathways that can affect senescence. So, I recruited low dose of Wi-A that caused slow growth arrest in cancer cells, and were relatively safe for normal cells in short term (2-3 days) viability assays. Consistently, I detected nuclear translocation of NF-κB and activation of p38MAPK in cancer, but not in normal, cells. Experimental data revealed an increased level of phosphorylated IκBα in Wi-A treated cells, suggesting an activation of IKK complex that was supported by nuclear translocation of NF-κB. It was supported by downregulation of NF-κβ effectors, Cyclin D and Cyclin E. I further investigated the DNA damage signaling pathway and found that Wi-A caused upregulation of CARF (Collaborator of ARF) demonstrating an activation of DNA damage and oxidative stress response in both cancer and normal cells. In line with this, and in contrast to selective activation of NF-κB and p38MAPK in cancer cells, growth arrest and senescence-determining proteins $p21^{WAF1}$, $p16^{INK4A}$, hypophoshorylated-pRB showed upregulation both in cancer and normal cells. Indeed, long term (4-8 days) viability assays revealed induction of senescence both in cancer and normal cells demonstrating that Wi-A-induced senescence is mediated by multiple pathways in which CARF-mediated DNA damage and oxidative stress play a major role.

Taken together, these results provide a unique way to identify new small molecules to modulate hypoxia. For future perspective, these candidates could be used as potential therapeutic target for oxidative stress related diseases.

Keywords: cancer, HIF 1 α, stemness, drug resistance, senescence.

Contents

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Abbreviations

HIF	Hypoxia Inducible Factor
HRE	Hypoxia Responsive Elements
CAPE	Caffeic Acid Phenethyl Ester
BSA	Bovine serum alibumin
CARF	Collaborator of ARF
ABCG2	ATP Binding Cassette Transporter G2
OCT4	OCtamer-binding Transcription factor 4
ALDH1	ALdehyde DeHydrogenase 1
MRP1	Multidrug-associated Protein 1
shRNA	Short Hairpin RiboNucleic Acid
CAME	Caffeic Acid Methyl Ester
OGA	2-OxoGlutaric Acid
FIH	Factor Inhibiting Hypoxia
Wi-A	Withaferin-A
NF-κB	Nuclear Factor-κB
p38MAPK	p38 Mitogen-Activated Protein Kinase
CDKs	Cyclin Dependent Kinases
DDR	DNA Damage Response
DMEM	Dulbecc's Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide
DNA	DeoxyriboNucleic Acid
DOX	Doxorubicin
ECL	Enhanced Chemiluminescence
EDTA	EthyleneDiamineTetraAcetic acid
FBS	Fetal Bovine Serum
GFP	Green Fluorescent Protein

γH2AX	Gamma Histone Variant H2AX
HRP	HorseRadish Peroxidase
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PDs	Population Doublings
PVDF	Poly VinyliDene Fluoride
Rb	Retinnoblastoma
RNA	RiboNucleic Acid
ROS	Reactive Oxygen Species
SDS	Sodium Dodecyl Sulphate
WS	*Withania somnifera*

Chapter-I

1. Introduction

1.1 Hypoxia and Its molecular mechanism

Organism and cells have developed number of adaptive mechanism to survive in oxygen-deprived (hypoxia) conditions. Oxygen homeostasis is really required for normal cellular function. (Fig. 1-1) At the organism level, hyperventilation, neovascularization and increased erythropoiesis in combination deliver oxygen from the atmosphere to the tissues. Whereas at the cellular level, glycolysis activation, rapid increases in glucose uptake, cell survival proteins induction and transcriptional induction at gene levels are main survival or adaptation mechanism (1). It mainly regulates through a transcriptional factor, hypoxia-inducible factor-1 (HIF-1α) which binds with the hypoxia responsive element and regulates about 40 hypoxia-inducible genes, directly (2). HIF is a heterodimer consisting α and β subunits, in which α subunit is oxygen regulated, expressed in all human cell types whereas β subunit binds with α subunit in the nucleus and activates hypoxia signaling through recruitment of p300 and CBP. (3).

In normoxia condition, HIF-1 α is degraded by pVHL (Von Hippel-Lindau tumor suppressor protein, a part of E3 ubiquitin ligase protein complex by ubiquitination and proteosomal degradation or by Factor Inhibiting HIF (FIH) by inhibition of its interaction with co-activator p300/CBP. (i) The mechanism underlying in normoxia, prolyl hydroxylase domain enzymes (PHDs) hydroxylate Proline564 or Proline402 on HIF-1 α with the help of iron, 2-oxoglutarate (2-OG) and molecular oxygen as a substrate and recognized by pVHL, resulting inhibition of enzymatic reaction of prolyl hydroxylation precluding the binding of pVHL and stabilize HIF-1 α (4). Another mechanism in which FIH hydroxylates, asparginyl 803 on HIF-1 α with the help of 2-OG and iron and inhibits its HIF-1 alpha interaction with its co-activator p300/CBP(5;6). Prolyl hydroxylation (P564 or P402) is more sensitive than

asparaginyl hydroxylation (A803) (another hydroxylation site on HIF) to inhibition by iron chelators (7).

1.2 Hypoxia in cancer

Cancer is generally characterized as a disease of cell proliferation and spreading of abnormally growing cells to other parts of the body. It continues to be a killer throughout the world although past decade has seen rapid developments in cancer diagnostics and treatment. The primary reason for treatment failure and high mortality has been attributed to the highly invasive nature of cancer cells resulting in rapid cancer progression and metastasis. Hypoxia-inducible transcription factor (HIF) has long been linked to malignant tumor phenotypes in different types of cancers and known as transcriptional factor (8-14). Hypoxia appeared in tumors through the uncontrolled oncogene-driven proliferation of cancer cells in the absence of sufficient blood and oxygen supply, which increase proliferation of cancer cells, resulting tumor quickly exhausts the nutrient and oxygen supply from the normal vasculature, and becomes hypoxic (Fig. 1-2). The major regulator acts as a heterodimer (HIF-1α and HIF-1β). Whereas HIF-1α is regulated by oxygen, HIF-1β is constitutively expressed (15). A Per-ARNT-Sim (PAS) domain is present in both the subunits. It is centrally involved in oxygen homeostasis and activated in a large majority of tumors (16;17). Under normoxia conditions, α-subunit undergoes hydroxylation by Proline-hydroxylase-2. The hydroxylated HIF-1α undergoes degradation by proteasome mediated degradation pathway involving tumor suppressor VHL (von Hippel–Lindau protein) (18). Another oxygen-dependent modification (Asparaginyl hydroxylation) catalysed by dioxygenase called Factor Inhibiting HIF-1α (FIH-1) occurs in the C-terminal (Asn803) transactivation domain of HIF-1α (13;19). Under hypoxia conditions that prevent HIF-1α hydroxylation and degradation, HIF-1α accumulates, translocates to the nucleus, dimerizes with HIF-1β, and transactivates several effector proteins (20;21). It is also regulated by CREB binding protein (CBP) and p300 that interact with the carboxy-

terminal transactivation domain of HIF-1α and act as its transcriptional co-activators (22) (Fig. 1-3). It has been shown that hydroxylation at Asn803 under normoxic conditions inhibits interactions of HIF-1α to CBP and p300 (6). Since HIF-1 is a key driver of hypoxia signalling, involved in cancer progression in one hand and several brain-disorders on the other, the identification of HIF-modulating drugs/factors and molecular mechanism of their action has been warranted for disease therapeutics (11).

1.3 Hypoxia in oxidative stress and stress protection

Under normal cellular conditions there is a constant balancing act between the oxygen concentration and reactive oxygen species (ROS) and their elimination is done by antioxidant system including enzymes such as superoxide dismutase, glutathione peroxidase, and catalase. In general, low levels of ROS can delay cellular signaling and disrupt intracellular reactions, while high levels of ROS create oxidative stress which can lead to the improper oxidation of lipids, proteins and DNA damage resulting eventually resulting in apoptosis, necrosis, and other cellular damage. Oxidative stress during hypoxia may sound self-contradictory (23). Oxidative stress can be accommodated in cells in lower or higher oxygen tension. (Fig. 1-4) The hypoxic cell relies on anaerobic respiration through glycolysis to generate ATP, whereas low oxygen supply supports some level of oxidative production of ATP through the tricarboxylic acid cycle and electron transport chain (ETC). Electrons leakage from the mitochondrial ETC generate an excess of reactive oxygen species (ROS), i.e., oxidative stress. Reoxygenation or high oxygen levels following severe hypoxia further exaggerate ROS generation (23). So, a balance is must in oxygen tension to support normal cellular functions.

In previous studies, PHD which is a hydroxylation partner of HIF, inhibition consistently provide neuroprotection in various neurological disease models e.g. stroke (24;25) Parkinson's disease (PD) (26), Oxidative stress (27), Alzheimer's disease (AD) (26;28;29) and mitochondrial dysfunction (30). PHD's have been recognized as direct

targets for iron chelators such as desferoxamine which shown beneficial in many neurological disorders with the neuroprotective effects (24).

Wei et al (31) reported that CAPE prevents neonatal hypoxic-ischaemic (HI) insult caused brain injury in rat pups. The main mechanism underlying of neuroprotection by CAPE was direct inhibition of HI induced inflammation and neuronal death. Other than these reports, CAPE has been reported as a potent inhibitor of HIF prolyl hydroxylase (HPH), a key enzyme for von Hippel-Lindau-dependent HIF-1 α degradation. Inhibition of HPH leads to induction of HIF-1 α protein and transactivating HIF-1 targeting genes VEGF (Vascular Endothelial Growth Factor) and heme oxygenase-1 (HO-1), which play a protective role in I/R injury (8). Since HIF-1 α regulation is really important and its link with oxidative stress relates pathology of brain disorders like Alzheimer's and Parkinson's etc., so there is a need of identification of small molecule regulators of hypoxia.

1.4 Cancer metastasis and drug resistance

Recent investigations in cancer diagnostic and therapeutic have increased the success rate and cancer patient's survival. However, metastasis cancer treatment is still a challenge. Cancer metastasis involves a process including translocation of cancer cells from their primary site and travel at either the surrounding or distant tissues by invasion through the extracellular matrix (ECM) or the blood/lymph streams, respectively. It is a complex multi-step process which involves several factors that enable cancer cells to acquire aggressiveness, invasiveness and intravasate into circulatory system, survive in the blood or lymph circulation, form microvasculature and extravagate to form tumor mass at distant organs (32-34). (Fig. 1-4) These also include several processes at cellular level including epithelial-to-mesenchymal transition (EMT), motility, adhesion and invasiveness that are regulated by oncogene activation, proteases activation and other tumor-microenvironment components activation including hypoxia and inactivation of tumor suppressor proteins and apoptosis-mediating factors (35-38).

When cells sense a decrease in oxygen availability (hypoxia), they develop adaptive responses in order to sustain this condition and survive. There is a define levels of hypoxia called acute and chronic hypoxia. In general tumor metastasis, acute hypoxia stabilizes within the microenvironment and develop vascularization leads to metastasis. But (39) If hypoxia lasts too long or is too severe, the cells eventually die. Hypoxia is also known to modulate the p53 pathway, in a manner dependent or not of HIF-1 (hypoxia-inducible factor-1), the main transcription factor activated by hypoxia. The p53 protein is a transcription factor, which is rapidly stabilized by cellular stresses and which has a major role in the cell responses to these stresses. In the tumor microenvironment, oxidative stress potentiates not only necrosis but also limiting O_2 diffusion (Hypoxia) within the tumors, which decreased sensitivity to cell death signals and increased signaling to promote angiogenesis and metastasis (40). This stress conditions particularly responsible in cancer stem cells to escape from the radiotherapy and chemotherapy. Many Heat shock proteins (hsp's) are up regulated in cells while exposed to different environmental stress. Mortalin a mitochondrial protein also named as Glucose-regulated protein (Grp) 75 is a member of HSP70 family, known for p53 interaction partner in cancer cells and a selective for cancer therapy (41). Mortalin plays an important role in human carcinogenesis by enhancing proliferation, apoptosis protection and angiogenesis (42). Mortalin's role in hemolysis and the ninth component of complement (C9) polymerization suggests that it promotes resistance in cancer cells (43). Elevated levels of mortalin expression increases cancer cell resistance to cisplatin-induced cytotoxicity and identify mortalin as a potential target in drug-resistant ovarian cancer (44). (Fig. 1-5) Chemotherapeutic drugs and pro-cancers signaling pathways are correlated with each other's. (Fig. 1-4) However, the major concern is most of the available drugs also target the non-cancer normal dividing cells in the body resulting in undesired adverse side-effects including low-immune response, hair loss, fatigue, nausea and hormone-imbalance. In addition, most of the anticancer drugs show low efficacy for metastatic cancer cells resulting either treatment failure or tumor relapse.

Taken together, Chemoresistance and radioresistance in cancer cells and nonspecific toxicity of chemical drugs towards normal cells are the major hurdles the cancer treatment, so there is a need of identification of new small molecules, which can revert chemoresistance with no cytotoxic effect on normal cells.

1.5 Thesis structure and overall impact

This whole study provides a novel method to identify new molecular targets (natural compounds) for hypoxia regulation and link to oxidative stress related diseases like stress, aging, cancer and inflammation. This work provides a new strategy for the development of new screening method and identification of new hypoxia target molecules which could be further modified chemically in the future for better therapeutic purpose.

Chapter 1 Introduction

In this chapter, the major signaling which is hypoxia is described with informative background and tried to connect hypoxia with different oxidative stress related diseases. The purpose and motivation are also addressed in this chapter for better understanding of hypoxia and related diseases.

Chapter 2 Screening of drug library by HRE driven luciferase reporter system-Identification of CAPE (C-021) as prohypoxia factor and its anti-stress effect by stress models.

In this chapter, CAPE is defined as pro-hypoxia factor by biochemical approach. Together, pro-hypoxia effect of CAPE is described on cell stress models.

Chapter 3 Identification of CAPE (C-021) as anti-mortalin molecule and studied its effect on drug resistance.

In this chapter, CAPE is identified as anti-mortalin molecule and described its potential anti-metastasis activity with help of other supportive metastasis markers. In addition, cancer stem cell model has been established by mortalin overexpression cell lines. For same purpose drug resistance properties and stemness markers have been studied.

Chapter 4 Screening of drug library by HRE driven luciferase reporter System-Identification of Withaferin-A (C-024) as anti-hypoxia factor and its major role in NF-kB signaling.

In this chapter, lower dose effect of Wi-A has been shown on NF-kB signaling and senescence which is related to oxidative stress.

Chapter 5 Conclusions and future researches

In this chapter, the main results are summarized. In particular, the future research points were also directed.

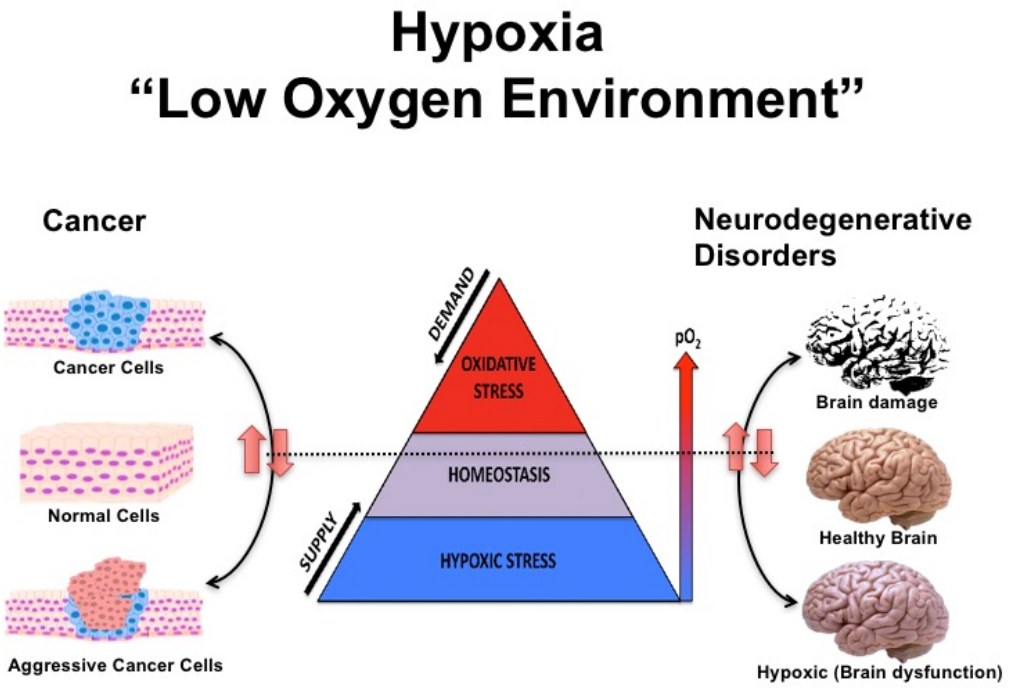


Fig. 1- 1 A general description of oxygen homeostasis and its effect on diseases

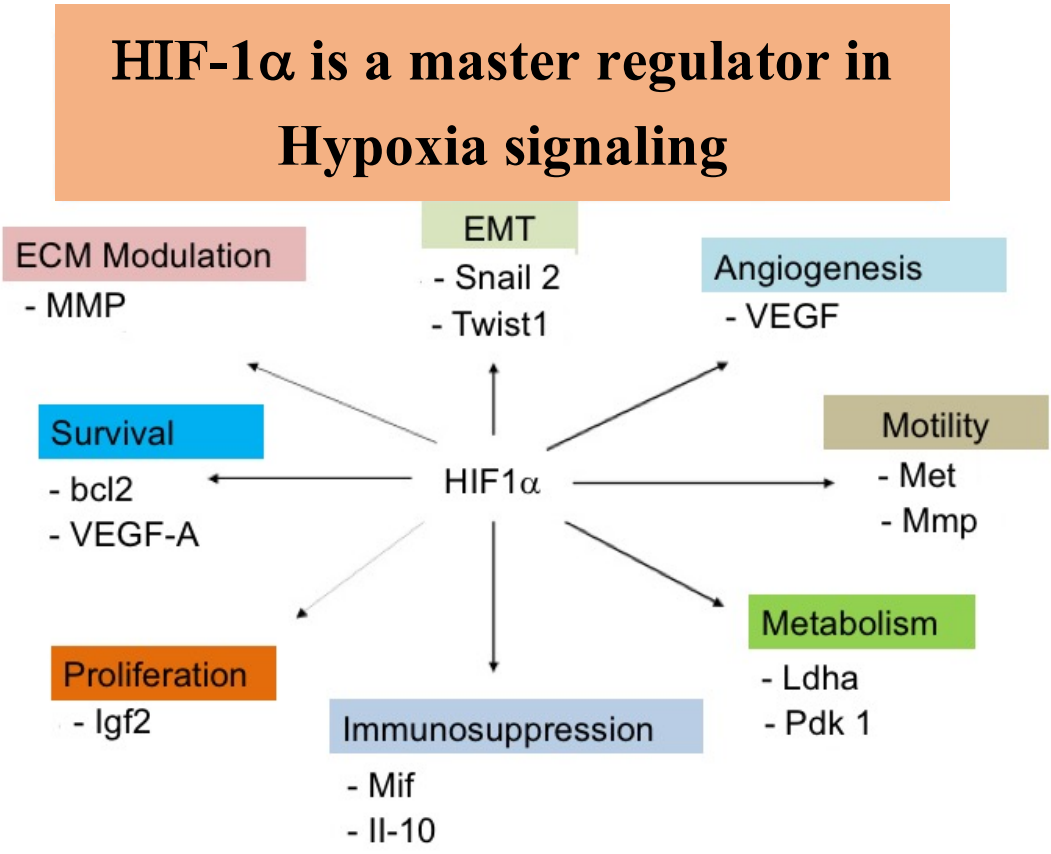


Fig. 1- 2 Hypoxia and related cellular mechanism

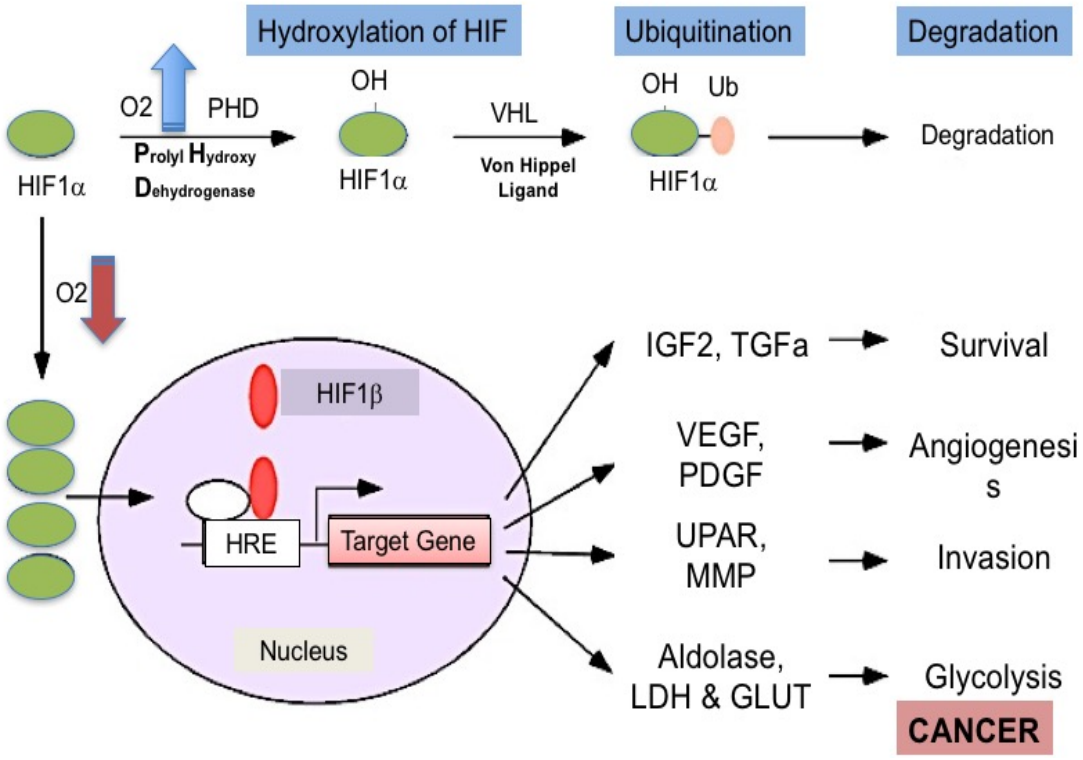


Fig. 1- 3 Oxygen driven regulation of hypoxia

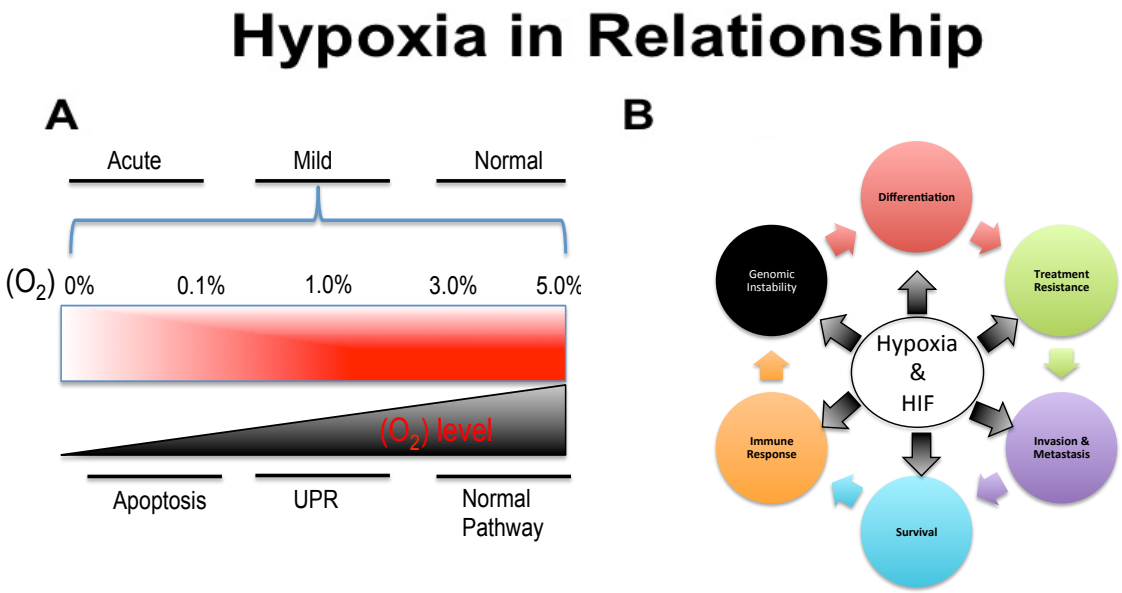
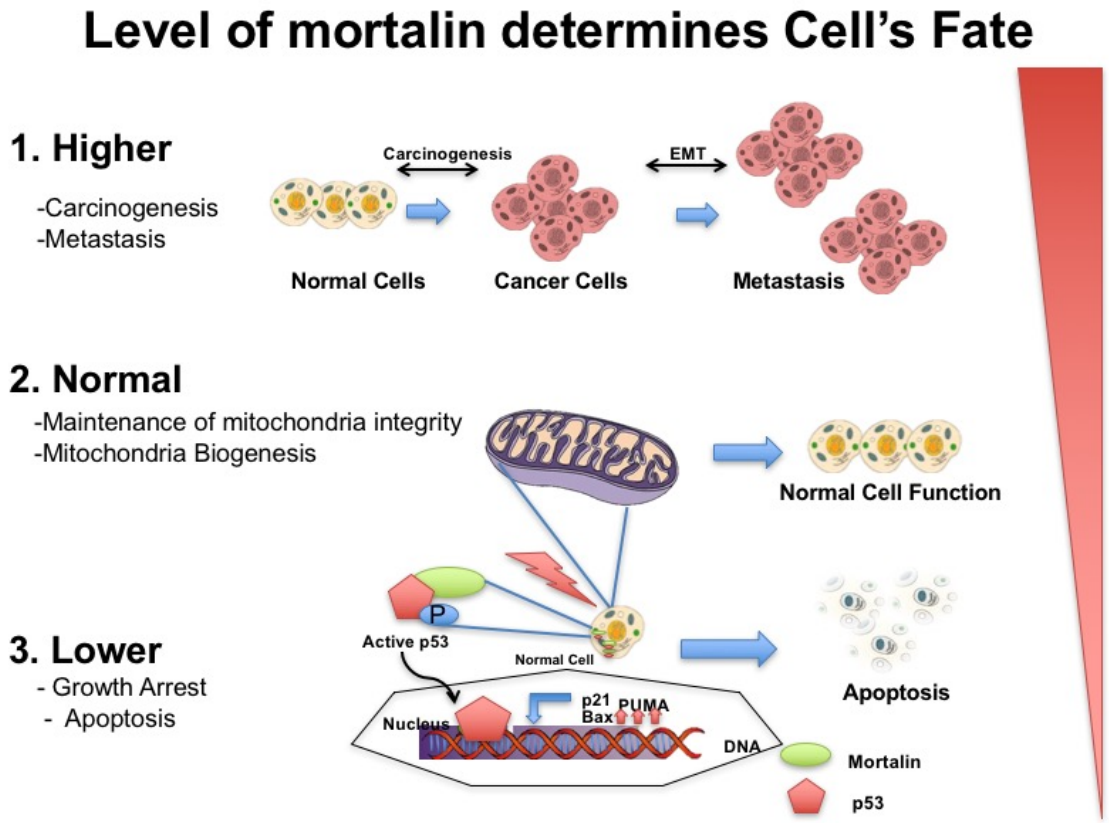


Fig. 1- 4 Oxygen tension and its impression on various pathologies



Mitochondria picture taken from-https://sites.google.com/site/mrstewartjfr/matter-energy-in-cells-noteson to metastasis

Fig. 1- 5 Description of mortalin role from normal cell function

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Chapter 2

Screening of drug library by HRE driven luciferase reporter system- Identification of CAPE (C-021) as prohypoxia factor and its anti-stress effect by stress models.

2.1 Introduction

Cancer is generally characterized as a disease of cell proliferation and spreading of abnormally growing cells to other parts of the body. It continues to be a killer throughout the world although past decade has seen rapid developments in cancer diagnostics and treatment. The primary reason for treatment failure and high mortality has been attributed to the highly invasive nature of cancer cells resulting in rapid cancer progression and metastasis. Hypoxia-inducible transcription factor (HIF) has long been linked to malignant tumor phenotypes in different types of cancers (1-7). It is a transcription factor that acts as a heterodimer (HIF-1α and HIF-1β). Whereas HIF-1α is regulated by oxygen, HIF-1β is constitutively expressed (8). A Per-ARNT-Sim (PAS) domain is present in both the subunits. It is centrally involved in oxygen homeostasis and activated in a large majority of tumors (9;10). Under normoxia conditions, α-subunit undergoes hydroxylation by Proline-hydroxylase-2. The hydroxylated HIF-1α undergoes degradation by proteasome mediated degradation pathway involving tumor suppressor VHL (von Hippel–Lindau protein) (11). Another oxygen-dependent modification (Asparaginyl hydroxylation) catalysed by dioxygenase called Factor Inhibiting HIF-1α (FIH-1) occurs in the C-terminal (Asn803) transactivation domain of HIF-1α (6;12). Under hypoxia conditions that prevent HIF-1α hydroxylation and degradation, HIF-1α accumulates, translocates to the nucleus,

dimerizes with HIF-1β, and transactivates several effector proteins (13;14). It is also regulated by CREB binding protein (CBP) and p300 that interact with the carboxy-terminal transactivation domain of HIF-1α and act as its transcriptional co-activators (15). It has been shown that hydroxylation at Asn803 under normoxic conditions inhibits interactions of HIF-1α to CBP and p300 (16;17). Since HIF-1 is a key driver of hypoxia signalling, involved in cancer progression in one hand and several brain-disorders on the other, the identification of HIF-modulating drugs/factors and molecular mechanism of their action has been warranted for disease therapeutics (4). In view of this, I performed a natural drug screening to search for hypoxia-modulating effects using cell-based HIF-1α reporter assay, and identified caffeic acid phenethyl ester (CAPE) as one of the prohypoxia factors.

CAPE is a bioactive compound found in many plants (18). It is a key component of propolis obtained from honeybee hives (19-22). The presence of hydroxyl groups in the catechol ring of this polyphenol has been ascribed to be responsible for many of its biological activities, such as anticancer, anti-microbial, anti-viral, neuroprotective and anti-inflammatory properties (20-24). Choi et al. reported that CAPE is a potent inhibitor of HIF prolyl hydroxylase (HPH-2) raising the question on its relevance to modulate hypoxia signaling in cancer cells (1). Roos et al. also reported the stabilization of HIF-1α and subsequent induction of heme oxygenase-1 (HO-1) during CAPE induced growth arrest in PDGF-activated vascular smooth muscle cells (25). In view of these information and identification of CAPE as a candidate pro-hypoxia factor in our screenings, I explored the docking potential of CAPE with HIF-1α and FIH-1 (inhibits HIF-1α by hydroxylation at Asn803). I found that whereas CAPE was incapable of interacting with HIF-1α, it docked efficiently into the active site of FIH-1. I provide experimental evidence for the prohypoxia activity of CAPE that may be the result of attenuation of HIF-1α inhibition by FIH, and its impact on stress-induced protein aggregation and cell-migration phenotypes. Furthermore, structural analogues of CAPE with stronger activity are reported.

2.2 Material and Methods

2.2.1 Transfection and reagents

Human osteosarcoma (U2OS) was purchased from JCRB Japan, and cultured in DMEM (Life technologies). Transfection was performed using lipofectamine 2000 (invitrogen) in opti-mem (Gibco, Life Technologies) media. The pGL4-p53-3'UTR were gifted from Dr. Chae-Ok Yun (Hanyang University, Seoul, South Korea). GFP-tagged mortalin MOT/GFP was expressed from pEGFP-C1/mot-2. $NaAsO_2$ (Sodium (meta) Arsenite) was purchased from Sigma Aldrich.

2.2.2 Generation of hypoxia responsive cells

U2OS cells were transfected with the plasmid expressing luciferase driven by promoter containing Hypoxia Response Element (HRE). (Fig. 2-1A) Twenty-four hours post-transfections, the media was replaced with DMEM supplemented with hygromycin B (Roche) (700μg/mL) for 10-15 days. The clones showing 100- to 1000-folds increase in luciferase in fixed and live cell assays were selected. (Fig. 2-1B) The selected clones were amplified and established into cells stably expressing hypoxia responsive luciferase reporter.

2.2.3 Cell viability assay

Vital dye, MTT (molecular probes, Invitrogen) (0.5 mg/ml), was added to the U2OS cell culture medium for 4 h in a humidified incubator (37°C and 5% CO_2). MTT-containing medium was replaced with DMSO (100 μl) to dissolve purple formazan crystals. Absorbance of the blue chromogen was observed at 550 nm using spectrophotometer (TECAN, Switzerland). The statistical significance of the data was calculated from triplicates and 3-4 independent experiments, respectively.

2.2.4 Screening for hypoxia modulating natural drugs

U2OS-HRE cells were plated in 96-well plates at about 70% confluency. After the cells had attached well to the surface, they were treated with phytochemical library (for 48 h) at sub-toxic doses, determined by independent MTT-based cell viability assays as described above. HIF-1 activity in the treated cells was determined by HRE-dependent luciferase by Dual Luciferase reporter assay system (Promega, Madison, WI). Luciferase activity was calculated per microgram protein following the manufacturer's protocol, and reported as the relative activity normalized against untreated cells. Three independent experiments were performed for statistical significance.

2.2.5 Western blotting

Cells were cultured in DMEM in 6 well plates and lysed using 1% Nonidet P-40 buffer containing a protease inhibitor cocktail (WAKO). The protein concentrations of whole cell lysate were measured by bicinchonic acid assay (BCA) (Thermo Fisher Scientific, Rockford, IL). The cell lysates (15-20 μg) were separated in 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA). Blocked membranes were probed with target protein-specific primary antibodies, Luciferase (luci17), HIF-1 α (Santa Cruz Biotechnology INC. CA, United States), overnight at 4°C. The blots were incubated with the following secondary antibodies conjugated to horseradish peroxidase: anti-mouse IgG (Santa Cruz Biotechnology INC. CA, United States) and developed by enhanced chemiluminescence reaction (ECL).

2.2.6 Immunofluorescence staining

Cells were fixed on a glass coverslip placed in a 12 well culture dish (TPP, Switzerland) and fixed with 4% paraformaldehyde in PBS. Later, cells were permeabilized with 0.1%

Triton X-100 for 10mins, blocked with 0.2% BSA/PBS for 1 h and then incubated with specific antibody Vimentin, MMP2, MMP7, MMP9, hnRNAP-k (Santa Cruz Biotechnology INC. CA, United States) for overnight at 4^0C. Mortalin antibody was raised in our lab. Cells were washed and probed with fluorophore conjugated Goat-anti mouse secondary antibody (molecular probes, Invitrogen). Counter staining was performed with Hoechst 33342 (Sigma) for 10 mins in dark, then coverslips visualized by Carl Zeiss microscope (Axiovert 200 M, Tokyo, Japan).

2.2.7 RNA isolation and cDNA synthesis

Total RNA was extracted from U2OS and U2OS-HRE cell lines by using TRIzol Reagent (Life Technologies) following supplier-described protocol. Quality and quantity were determined by spectrophotometry method (Nano Drop, 1000 spectrophotometer). For cDNA synthesis, total RNA (1 μg) was reverse-transcribed into cDNA using Quantitect Reverse Transcriptase Kit (QIAGEN) following the manufacture's protocol. cDNA was stored at -20^{o}C for PCR.

2.2.8 Quantitative real-time PCR

Gene expression was quantified by quantitative real time PCR using Syber Select Master mix (Applied Biosystem, Life Technologies), luciferase specific primers (F-5'-GGACTTGGACACCGGTAAGA-3' and R-5'-CTTGTCGATGAGAGCGTTTG-3'), HIF-1α specific primers (F-5'-GTTTACTAAAGGACAAGTCACC-3' and R-5'-TTCTGTTTGTTGAAGGGAG-3' and EcoTM Real-Time PCR System (Illumina, San Diego, CA). Relative level of expression of target gene was normalized against the internal control 18S by ΔC_T method. An amplification plot between fluorescence signals and cycle number was plotted and the mean values in the triplicated samples of targeted genes and internal control 18S were calculated. The relative quantitative

value was expressed as $2^{-\Delta C}{}_{T}$. Statistical significance of the results was calculated from three independent experiments.

2.2.9 Effect of CAPE on cell migration and growth of cancer cells: wound-scratch assays

The *in vitro* migration capability of U2OS cells with or without CAPE treatment was observed with a wound-scratch assay. The cells were seeded in a 6-well plate and wounded by uniformly scratching the cells with a pipette tip. Cells were washed several times with PBS to remove damaged cells, and continue culture on either the control or CAPE-supplemented medium, respectively. The time of wound scratching was taken as time 0. Movement of cells into the scratched area was captured during next 24 h, up to 72-96 h under a phase contrast microscope with a 10 X phase objective.

2.2.10 Effect of CAPE in stress-induced protein aggregation

Sodium arsenite-based aggregation-deaggregation model was used. U2OS cells ($4X10^3$/well) stably expressing mortalin-GFP (U2OS-mot-GFP) were seeded in 96-well plates for overnight followed by treatment with sodium arsenite (20 μM) for 24 h. Cells were recovered either in normal culture media or the one supplemented with CAPE for 24-48 h. Cell viability was determined by MTT assay as described above. Statistical significance of results was determined from 3-4 independent experiments including triplet or quadruplet sets in each experiment. For immunofluorescence, same method was adopted in 12 well plates with coverslips.

For heat-shock experiment, cells were seeded in 6 well plate and transfected with pGL4-p53-3'UTR for 24 Hrs. After 24 Hrs. cells were then incubated at 42^0 C temperature and 5% CO_2 for 2 Hrs. for heat shock. Post 2 Hrs. stress, media was changed with or without CAPE for next 48 Hrs. At the end, Cells were harvested and

lysed to measure luciferase activity as described earlier. For immunofluorescence, same method was adopted in 12 well plates with coverslips.

2.2.11 Statistical analysis

All the experiments were performed in triplicate. Data are expressed as mean ±SEM of triplicate experiments. Unpaired t-test (GraphPad Prism GraphPad Software, San Diego, CA) has been performed to determine the degree of significance between the control and experimental samples. Statistical significance was defined as p-values (*), where *<0.05, **<0.01, ***<0.001 represented significant, very significant and very very significant, respectively.

2.3 Results and Discussion

2.3.1 Identification of CAPE as a prohypoxia factor

I investigated hypoxia-modulating potential of 60 purified phytochemicals by Hypoxia Responsive Element (HRE) driven luciferase in stably transfected cells (as described in Material and Methods and Fig. 2-1A, B). Cells were treated with sub-toxic doses, determined by independent experiments (MTT assay and morphological observations), of phytochemicals (data not shown). Through three rounds of screenings, I identified 5 prohypoxia and 4 anti-hypoxia compounds. (Fig. 2-1C) CAPE was identified as one of the prohypoxia factors. I investigated dose-dependent effect of CAPE on HRE-luciferase activity in comparison to a standard pro-hypoxia drug, cobalt chloride ($CoCl_2$). As shown in Fig. 2-2A, HRE-Luciferase activity showed dose dependent increase when cells were treated with sub-toxic doses of CAPE (5-15 μM). Of note, the effect of CAPE was stronger than $CoCl_2$, effective at 50-150 μM. Consistent with luciferase reporter assays, qPCR for luciferase showed increase in CAPE- and $CoCl_2$-treated cells (Fig. 2-2B). Detection of luciferase protein by immunofluorescence using

anti-luciferase antibody also showed increase in CAPE-treated cells (Fig. 2-2C), supported by increase detected by Western blotting (Fig.2-2D). I also examined the expression of endogenous HIF-1α protein in control and CAPE-treated cells, and found that it was increased in CAPE-treated cells (Fig.2-2E). $CoCl_2$-treated cells were used as positive control (Fig. 2-2E).

2.3.2 Prohypoxic doses of CAPE and its anticancer potential

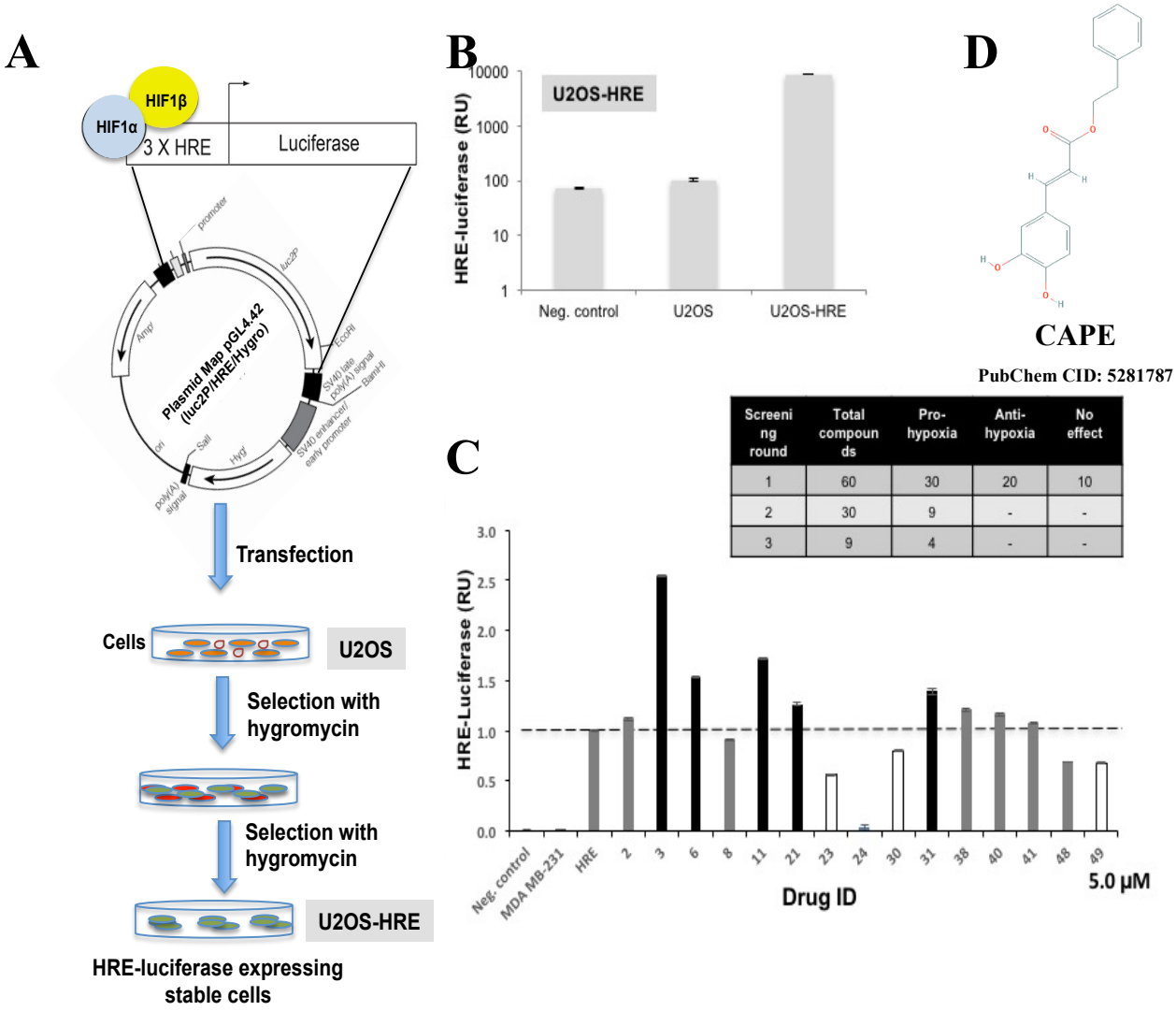
Anticancer potential of CAPE has been well documented in several studies (23;25-28). In view of the above findings on prohypoxia activity of CAPE, I next investigated the effect of low dose of CAPE on cell survival and migration. Cells treated with low doses (5-10 μM) showed only a minor toxicity in 48 h treatment as detected by MTT assay (Fig. 2-3A). I also examined the effect of CAPE on cell migration by Wound-scratch assays and found that low doses of CAPE failed to affect cell migration (Fig. 2-3B). The observation thus made was consistent with other results where no change was observed in protein markers of cell migration studied in control and CAPE (low non-toxic dose)-treated cells (Fig. 2-3C).

2.3.3 Pro-hypoxia doses of CAPE and protein de-aggregation

Accurate protein folding determines their subcellular niche and functional characteristics (29). Environmental conditions such as adverse temperature, pH, metals and oxidative stress have been established as the prominent factors leading to protein mis-folding and aggregation that are toxic for cells and have been associated with diseases including diabetes, neurodegenerative disorders, and cancer (30-33). Endoplasmic Reticulum (ER) plays an essential role in protein quality control by activating Unfolded Protein Response (UPR) in response to altered conditions of nutrients and oxygen that trigger protein misfolding (34). Activated UPR regulates expression of several pro-survival proteins (35). Interestingly, lower doses of CAPE as

well as mild hypoxia have been shown to activate UPR (35;36). Several studies have shown that HIF-1α stabilization provides stress protection in various neurological disease models e.g. stroke (37;38), Parkinson's disease (39), oxidative stress (40), Alzheimer's disease (39;41;42) and mitochondrial dysfunction (43). The beneficial effects of HIF-1α stabilization arise mainly through the activation of HIF-1 target genes that combat oxidative stress, improve glucose metabolism and block cell death pathways (44).

In view of this, I recruited metal (sodium arsenite) and high temperature-induced protein aggregation in cells exogenously transfected with either GFP or luciferase proteins that allowed direct readout of protein aggregation. As shown in Fig. 2-4A, GFP-labelled cells were treated with sodium arsenite (24 h) followed by recovery (48 h) either in the control or CAPE-supplemented medium. As expected, I found that the cells possessed pancytoplasmic fluorescence of mortalin-GFP protein (Fig. 2-4B). Of note, whereas sodium arsenate-treated cells recovered in normal medium still showed strong aggregation (Fig. 2-4B), protein de-aggregation was seen in the cells recovered in CAPE supplemented medium (Fig. 2-4B). Consistently, I found that the sodium arsenite stressed cells treated with CAPE led to 20-30% increase in viability (Fig. 2-4C). We, next, confirmed protein de-aggregation and anti-stress effect of CAPE by using heat shock-induced protein misfolding and aggregation of luciferase reporter (Fig. 2-5A) (45). As shown in Figure 8B, heat-induced aggregation of luciferase led to decrease in its activity. Cells recovered in normal medium for 48 h showed reversal of misfolding (46), and was associated with increase in luciferase activity. Of note, recovery in CAPE-supplemented medium caused 2-fold increase in luciferase activity. The results were confirmed by immunostaining of luciferase by specific antibodies (Fig. 2-5B). These data demonstrated that the prohypoxic doses of CAPE protected cells against stress-induced aggregation of proteins.



Screening round	Total compounds	Pro-hypoxia	Anti-hypoxia	No effect
1	60	30	20	10
2	30	9	-	-
3	9	4	-	-

Fig. 2-1 Screening for hypoxia modulating drugs. (A) Schematic diagram showing the screening assay protocol for hypoxia modulating drugs. (B) HIF-1α driven Luciferase assay for U2OS cells stably transfected with HRE-luciferase plasmid. (C) HIF-reporter assay in cells treated with natural drugs (5.0 μM). Out of the 60 drugs tested, 5 compounds (shown by dark bars) showed strong prohypoxia activity; 4 compounds (shown by white bars) showed anti-hypoxia activity. (D) Structure of CAPE (Obtained from PubChem)

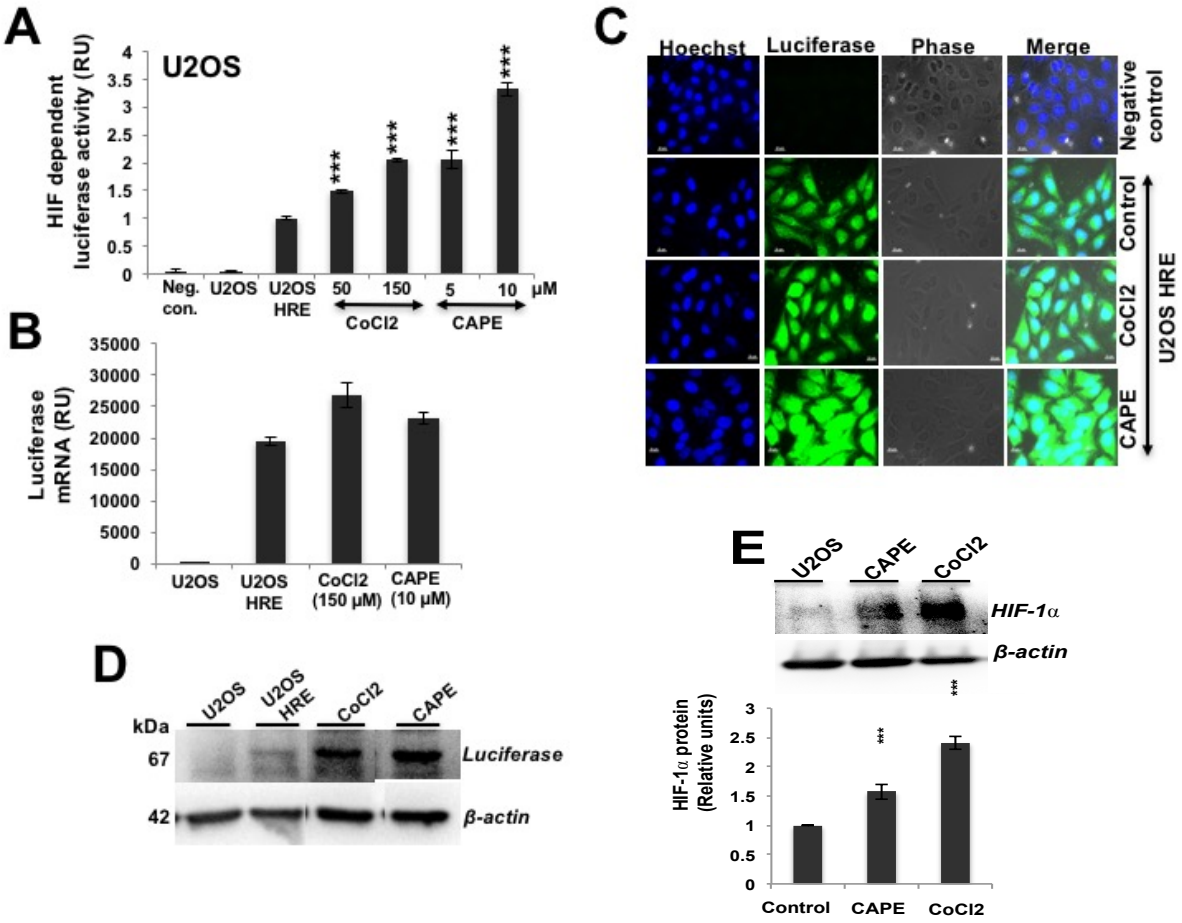


Fig. 2-2 HIF-1α driven prohypoxia effect of CAPE on U2OS. (A) Effect of CAPE on HIF-1α driven luciferase reporter assays in comparison to a standard hypoxia inducing drug, CoCl2. (B, C and D) Effect of CAPE on the luciferase transcript (B) and protein as detected by immunostaining (C) and Western blotting with anti-luciferase specific antibody (D). Effect of CAPE on the Luciferase protein and protein (C). Effect of CAPE on endogenous HIF-1α protein; CoCl2 was used as positive control.

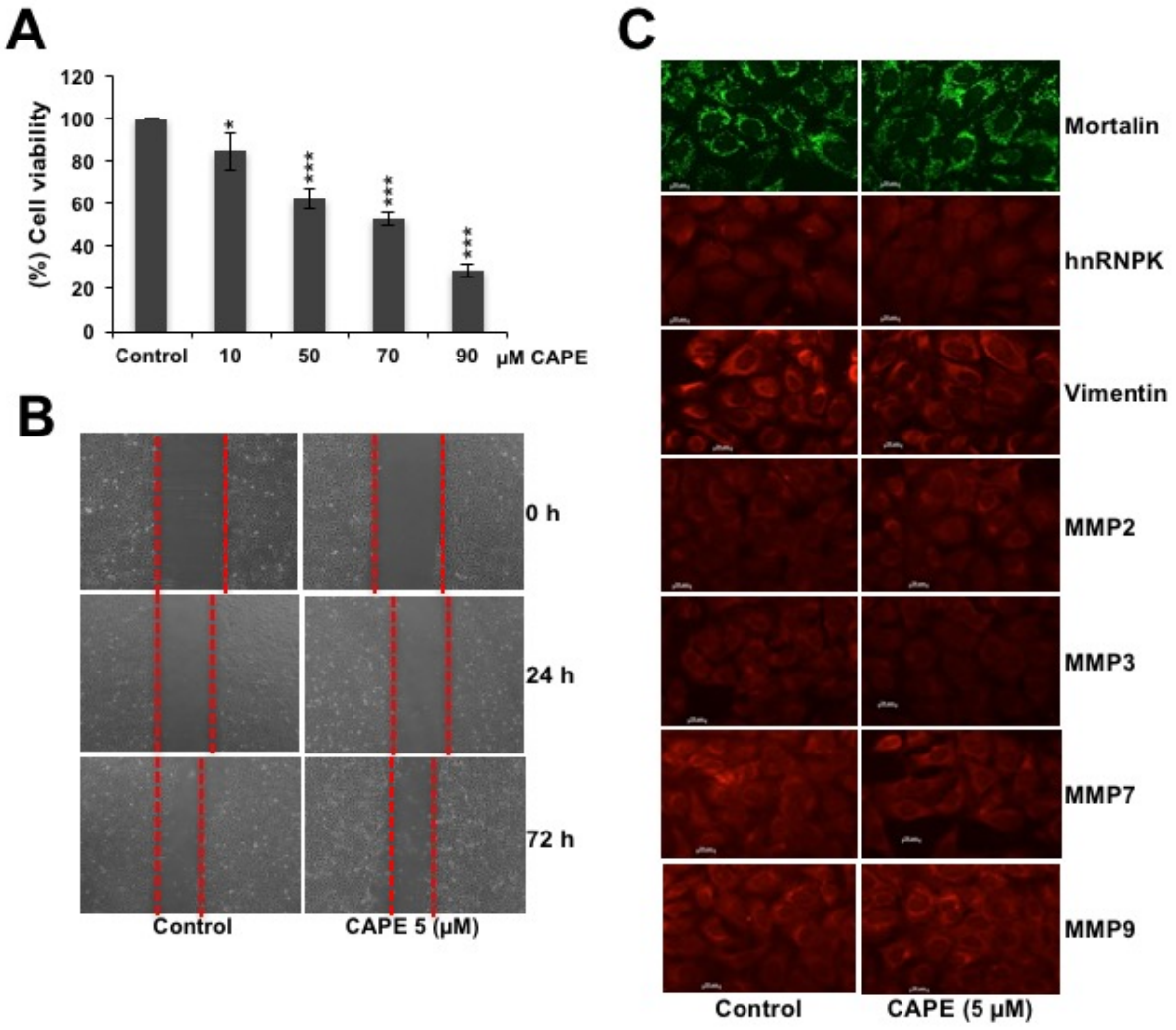
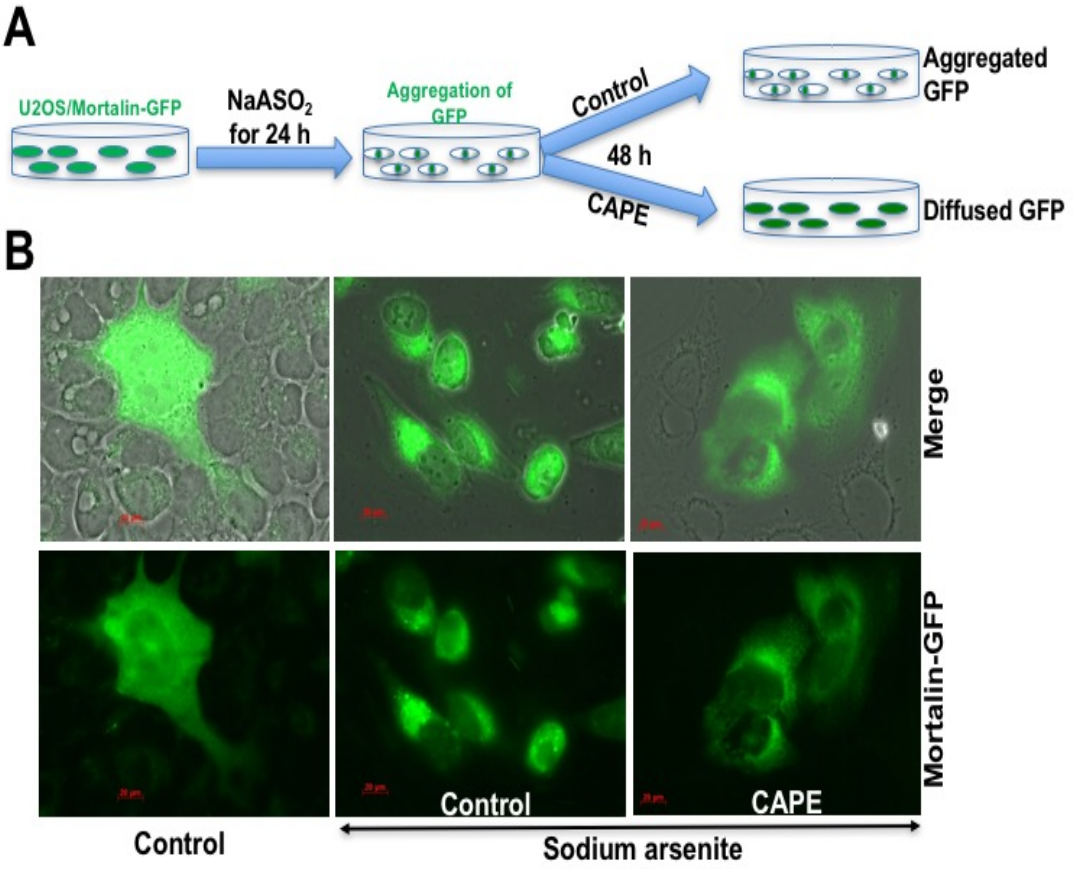


Fig. 2-3 Effect of CAPE at low doses on U2OS cell viability (A), migration (B) and proteins (C) involved in cell migration.



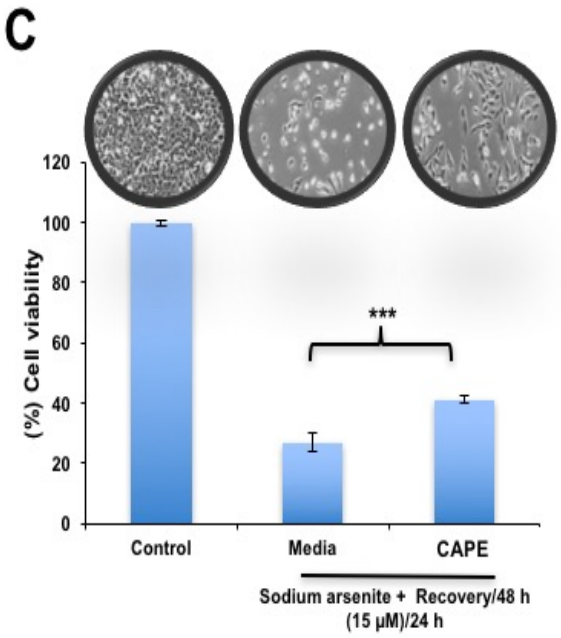


Fig. 2-4 Effect of CAPE at low doses (15 µM)on sodium arsenate-induced aggregation in U2OS-mot-GFP and cell survival in U2OS cell lines. (A) Schematic diagram showing the protein aggregation and recovery assay, (B) aggregation of GFP in sodium arsenate-treated and recovered (either in control or CAPE-supplemented medium) and (C) viability of cells treated with sodium arsenate followed by recovery either in control or CAPE-supplemented medium.

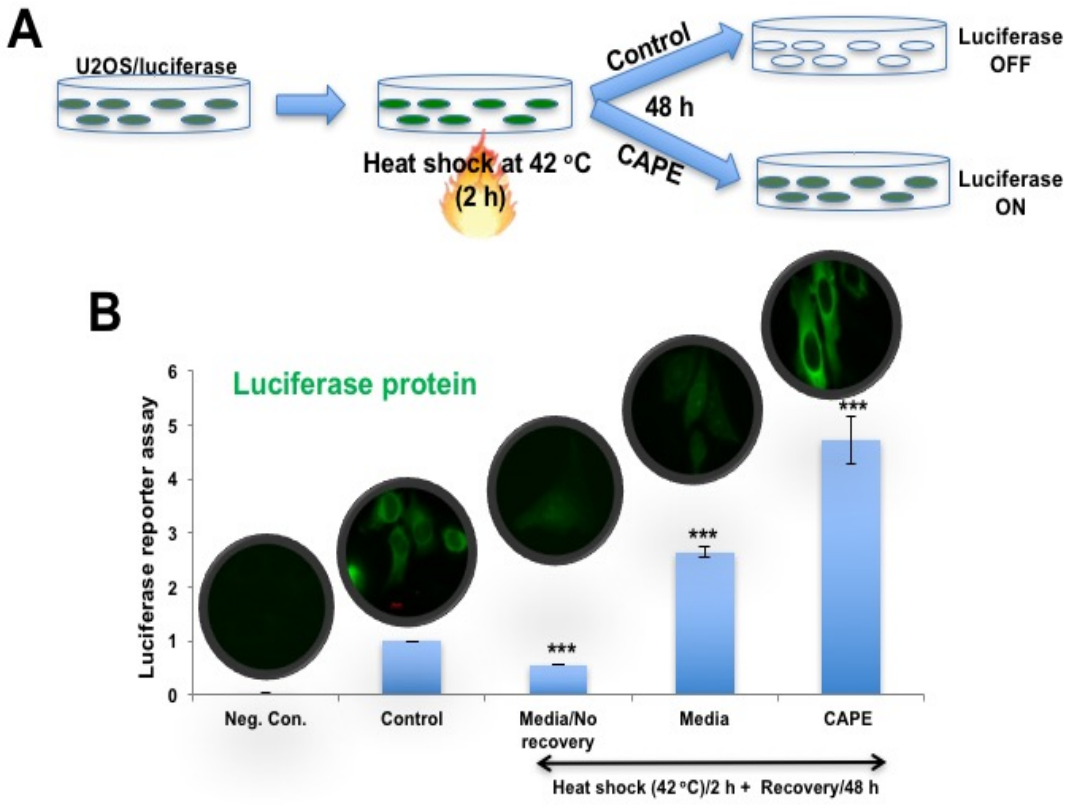


Fig. 2-5 Effect of CAPE at low doses on heat shock-induced aggregation of luciferase reporter and activity in U2OS. (A) Schematic diagram showing the protein aggregation and recovery assay, (B) luciferase activity and expression in sodium arsenate-treated and recovered either in control or CAPE-supplemented medium is shown.

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Chapter 3

Identification of CAPE (C-021) as anti-mortalin molecule and studied its effect on drug resistance.

3.1 Introduction

Chemoresistance and radioresistance in cancer cells and non-selectivity of chemical drugs towards normal cells are the major reasons of treatment failure. Several epidemiological studies have observed that the daily meal of fruits and vegetables lower cancer risk. Several natural plant-derived polyphenols including curcumin, genistein, silymarin, caffeic acid phenethyl ester, resveratrol, green tea polyphenols, flavopiridol, emodin and piperine have been investigated for their chemo-therapeutic and chemo-preventive properties and for sensitization of tumor cells to chemo- and radio-therapeutic agents (1-7). Looking at, availability and economic aspects, natural chemicals are gaining increasing focus in disease research because of their cancer-preventive and -curative activities, and their mechanism(s) of action. A minority of cancer cell population, called cancer stem cells (CSC), with CD44(+/high)CD24(-/low) signature, has been identified in a large variety of cancers. These cells have been ascribed as the key determinants of malignant transformation, metastasis and multi-drug resistance characteristics that form a prime cause of failure in cancer chemotherapy leading to fatality (8-10). CSC are also distinguished by enriched expression of several other markers referred to as stemness factors. These include aldehyde dehydrogenase, ATP-binding cassette transporter protein-ABCG2/BCRP1, 5-transmembrane glycoprotein-CD133, and transcriptional factor Oct-4 (11-16). Tumor progression, especially in case of solid tumors, is often accompanied by generation of hypoxia microenvironment that in turns promotes proliferation, EMT, invasion and metastasis (17;18). It has been shown that cancer cells survive during

hypoxia by up-regulation of stemness factors (18). Furthermore, CSC-enriched tumors have been shown to display chemoresistance and poor prognosis, indicating that these cells are an important target for therapeutic success (19;20). In view of these reports, research on CSC biology is deemed important for understanding the process of tumorigenesis, tumor progression, treatment, prognosis and recurrence.

Cancer cells depend heavily on mitochondria, a key organelle for regulation of metabolism, survival and death signalings (21). Mortalin/mthsp70, a member of Hsp70 family, has been shown to promote proliferation, metastasis and angiogenesis, and downregulate apoptotic signaling. It has been shown to interact with p53, telomerase and hnRNP-K in cancer cells (22-28). Whereas p53 is inactivated by mortalin in cancer cells, telomerase and hnRNP-K are activated and were shown to contribute to malignant transformation (29). Mortalin was shown to inhibit p53-BAX interactions and activate AKT that are required for apoptotic signaling (25;30;31). It was also shown to interact with complement C9, a major component of membrane attack complexes that are released in membrane vesicles from complement attacked cells accounting for resistance of cancer cells to complement-dependent cytotoxicity (32). Increased mortalin expression was shown to mediate resistance of ovarian cancer cells to cisplatin (33). Based on these data and our recent findings on role of mortalin in EMT, I hypothesized that it may also be involved in cancer cell stemness. I therefore investigated several cell stemness markers and drug resistance in mortalin overexpressing breast cancer cells. I demonstrate that mortalin overexpressing cells were enriched with stemness markers and exhibit resistance to cytotoxicity induced by several chemotherapeutic drugs. Furthermore, treatment of the cells with mortalin shRNA or inhibitors like CAPE (C-021) reverted the drug resistance of cells and dampened their migration and invasion potentials.

Propolis is a natural mixture produced by honeybees by mixing plant materials with wax and resin to build their hives. In ancient medicines, it has been used for anti-bacterial, anti-inflammatory, wound healing, skin infections, gastro-intestinal disorders,

immunomodulation and cardiovascular benefits. CAPE (Caffeic Acid Phenethyl Ester) is an active phenolic component derived from propolis. It was known for several activities, previously detected in propolis, including anti-mitogenic and anticancer natural drug (34;35). Several studies suggested that CAPE and its derivative compounds contain selective toxicity to transformed/cancer cells (36-42). CAPE- and Artepillin C (ARC)-rich propolis extracts were demonstrated as natural anti-PAK1 inhibitors and NF1/NF2 remedies (43;44). CAPE was shown to inhibit TPA (12-O-tetradecanoylphorbol-13-acetate)-induced tumors (45;46), and suggested as an anticancer agent for treatment of colorectal carcinoma (47), hepatoma (48) and gastric carcinoma (49). CAPE-induced cell cycle arrest and apoptosis are mediated by down-regulation of beta-catenin (50;51) and p38MAPK (52) signaling pathways. It was demonstrated to inhibit NF-kappaB, COX-2 (53;54) levels and stimulate human anti-oxidative response element-mediated gene expression of the NAD(P) H:quinone oxidoreductase (NQO1) gene (55). CAPE was reported for (i) restoring intercellular communication through gap junctions, regulation of phosphorylation of connexin 43 (54), (ii) helps in rearrangement of actin cytoskeleton and loss of focal adhesion plaques causing reduced cell invasion (56). It inhibited growth of cancer cells on soft agar through downregulation of $p21^{ras}$ protein (57). Together with the *in vitro* reports, anticancer activity of CAPE was supported by *in vivo* studies in mice (58-60). CAPE and its derivative, were shown to inhibit PI3-K/Akt, AMPK and m-TOR signaling cascades both *in vitro* and *in vivo* (60). Although such reports present CAPE as a natural drug for cancer treatment but molecular studies are warranted to clarify and clarity to its mechanism of action and rational designing of its derivatives.

3.2 Material and methods

3.2.1 Cell culture and reagents

Human normal cells (TIG-3 and MRC5) and Cancer cell lines, breast cancer (MDA-MB 231, MCF-7), osteosarcoma (U2OS, Saos-2), cervical carcinoma (HeLa), hepatocellular carcinoma (HUH-6, HUH-7), ovarian carcinoma (SKOV3), adenocarcinoma (A549) and colorectal adenocarcinoma (DLD-1, COLO 320 and HCT116), procured from JCRB or DS Pharma, Japan, were cultured in DMEM (Life technologies). Human melanoma (G361)(JCRB, Japan) was cultured in McCoy's 5A (Life technologies). Mortalin overexpressing cells were generated by retroviral expression vector as described previously(61;62). Mortalin-targeting adeno-oncolytic virus expressing mortalin shRNA (#009-GAATGA GGCTAGACCTTTA) was generated and used as described earlier (63). Plasmid based mot-shRNA 2166 (5′-ACCATCTCGCACACAGCAATTCAAGAGATTGCTGTGTGCGAGATGGTT-3') was constructed and used as described earlier (24). Chemotherapeutic drugs were purchased from Sigma (Nocodazole, Paclitaxel, Doxorubicin hydrochloride, Cyclophamide monohydrate) or Wako (Methotrexate, Epirubicin hydrochloride and Docetaxel). Anti-mortalin antibodies (polyclonal and monoclonal) were raised in our laboratory. Mortalin targeting shRNA and adeno-oncolytic viruses were generated as described earlier (63).

3.2.2 Cell proliferation assay

Cytotoxicity and cell survival were assessed using MTT {3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (Life Technologies) assays in which the cell viability was estimated by the conversion of yellow MTT by mitochondrial dehydrogenases of living cells to purple formazon (MTT assay). Statistical significance of results was determined from 3-4 independent experiments including triplet or quadruplet sets in each experiment.

3.2.3 Cell migration and invasion assay

Cells migration and invasion assays were performed using Transwell chamber (Corning, NY) and BD BioCoat Matrigel Invasion Chamber (BD Bioscience, MA), respectively, as described earlier (62).

3.2.4 Western blotting

Cells were cultured in DMEM (5% fetal bovine serum) in 100-mm plates and lysed using 1% Nonidet P-40 buffer containing a protease inhibitor cocktail (Sigma Aldrich). The protein concentrations of whole cell lysate were measured by bicinchonic acid assay (BCA) (Thermo Fisher Scientific, Rockford, IL). The cell lysates (10-20 ng) were separated in 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA). Blocked membranes were probed with target protein-specific primary antibodies, ABCG2 (Novus Biologicals, Littleton, CO) and OCT4 (Cell signaling, Beverly, MA), CD24 (Santa Cruz Biotechnology INC. CA, United States), Anti-Calreticulin (Abcam, Cambridge UK) overnight at 4°C. overnight at 4°C. The blots were incubated with the following secondary antibodies conjugated to horseradish peroxidase: anti-rabbit IgG and anti-mouse IgG (Cell signaling technology) and developed by enhanced chemiluminescence reaction (ECL) (Elpis Biotech, Daejeon, Korea).

3.2.5 Immunofluorescence staining

Cells were fixed on a glass coverslip placed in a 12 well culture dish with 4% paraformaldehyde in PBS, permeabilized with 0.1% Triton X-100 for 10mins, blocked with 0.2% BSA/PBS for 1 h and were then incubated with specific antibody (as described above) for overnight at 4^0C. For mito-tracker staining MitoTracker®Red CMXRos (Invitrogen, M7512) were added into the cell culture media at final concentration of 50nM. The cells were incubated under normal culture condition for 20

mins then visualized by Carl Zeiss microscope (Axiovert 200 M, Tokyo, Japan). Counter staining was performed with Hoechst 33342 (Sigma) for 10 mins in dark. To examine the localization and sections of cells z-stack has been acquired with laser scanning confocal microscopy (Zeiss LSM 700). The files were transferred o a graphic workstation and analyzed with IMARIS software (Bitplane, Zurich, Switzerland).

3.2.6 Flow cytometric analysis

For the assessment of cancer stem cell maker expression, cells were collected and suspended at a density of $1x10^6$ cells/ml. The cells were stained with fluorescein isothiocyanate (FITC)-conjugated anti-CD24, allophycocyanin (APC)-conjugated anti-CD44, or phycoerythrin (PE)-conjugated anti-CD19 following the manufacturer's instructions. Cells were incubated at 4^oC for 1h followed by washings (twice) with PBS and analysis using a flow-cytometer (FACS Caliber, Becton Dickinson).

3.2.7 Mammosphere formation assay

For mammosphere formation, cells were plated onto 6-well plates at a density of $1x10^3$ cells/ml in DMEM/F-12 (Hyclone, Logan, UT) supplemented with 2% B27 (Gibco, Carlsbad, CA), 10 ng/ml EGF, and 10 ng/ml FGF (ProSpec,East Brunswick, NJ). Growth factors were added to the mammosphere cultures every 3 days, and mammospheres (> 40 μm in size) were counted on Day 7.

3.2.8 RNA isolation and cDNA synthesis

Total RNA was extracted from MDA-MB231, U2OS and G361 cells and their mortalin-overexpressing variants using TRIzol Reagent (Life Technologies) following supplier-described protocol. Quality and quantity were determined by spectrophotometery (Nano Drop, 1000 spectrophotometer). For cDNA synthesis, total RNA (1 μg) was reverse-transcribed into cDNA using Quantitect Reverse

Transcriptase Kit (QIAGEN) following the manufacture's protocol. cDNA was stored at -20^{o}C for PCR.

3.2.9 Quantitative real-time RT-PCR

Gene expression was quantified by quantitative real time PCR using Syber Select Master mix (Applied Biosystem, Life Technologies), gene specific primers (Table 1) and EcoTM Real-Time PCR System (Illumina, San Diego, CA) wherein the relative level of expression of target gene was normalized against the internal control 18S by ΔC_T method. An amplification plot between fluorescence signals vs. cycle number was plotted. The difference between the mean values in the triplicated samples of targeted genes and internal control 18S ere calculated by Microsoft excel and the relative quantitative value was expressed as $2^{-\Delta C}{}_T$. All the experiments were performed, at least thrice, for statistical significance.

3.2.10 Metabolic rate assay

MCF-7 and their mortalin overexpressing derivatives were suspended in serum-free, phenol red-free and glucose-free RPMI-1640 medium containing antibiotics and glutamine (4 mM). Cells were dispensed into PM-M1 microplates (2,500 cells/50 μL per well) and incubated for 24 -72h followed by addition of Redox Dye Mix MA (10 μL). Plates were sealed with tape (LMT-SEAL-EX, Phenix Research Products, Hayward, CA) to prevent CO2 loss, and incubated at 37°C in an OmniLog instrument (Biolog, Hayward, CA) for 18-36 hours to obtain kinetic record (X-axis- time and the Y-axis-OmniLog color density units) of formazan as per manufacturer's instructions.

3.2.11 Statistical analysis

All the experiments were performed in triplicate. Data are expressed as mean ±SEM of triplicate experiments. Unpaired t-test (GraphPad Prism GraphPad Software, San Diego, CA) has been performed to determine the degree of significance between the

control and experimental samples. Statistical significance was defined as p-value proliferation (green boxes), cell stasi*<0.05, **<0.01, ***<0.001 while no mark denotes insignificant correlation.

3.3 Results and Discussion

3.3.1 Anticancer and anti-metastasis potential of CAPE

I examined the toxicity effect of CAPE in a variety of human cancer cell lines including HT1080, U2OS, MCF-7, MDA-MB-231 and G361 and found that it induces apoptosis in all cell types at doses higher than 10-20 μM. (Fig. 3-1A). Low doses (5-10 μM) were observed to cause growth arrest. The data was consistent with other reports on anticancer activity of CAPE. In order to determine if CAPE could also be used to treat aggressive and metastatic cancers, I prepared genetically isogenic breast cancer cells that are differed in their migration and metastasis potentials by retrovirus-mediated overexpression of stress chaperone mortalin (61;62). In short- and long-term survival assays, I found that the CAPE caused growth arrest of parent and metastatic-derivatives of isogenic cells to a significant comparable level (Fig. 3-1 B&C). Similar results were observed in other breast cancer cell lines suggesting that CAPE (10μM) may also be used for aggressive and malignant cells.

3.3.2 CAPE causes induction of p53 function by targeting mortalin-p53 interactions

Based on the observed fact that GADD45α is a downstream effector of p53 tumor suppressor and it is also involved in growth arrest of cells in response to DNA damage, I hypothesized that CAPE-induced GADD45α could be mediated by activation of p53. Examination of p53 in control and CAPE-treated cells indeed revealed that p53 upregulates and translocates into the nucleus and increase in its downstream effector $p21^{WAF1}$ in MCF-7, U2OS as well as A549 cells (Fig. 3-2 A). Upregulation of p53

and $p21^{WAF1}$ in treated cells was confirmed by Western blotting (Fig. 3-2 B). In addition, p53-dependent reporter assays also shown increase in transcriptional activation function of p53 in CAPE-treated cells (Fig. 3-2 C). The involvement of mortalin and p53 in complex formation was already known so, I predicted that the increase in p53 might be due to targeting of mortalin (stress chaperone known to inhibit p53 activities) by CAPE. Mortalin is well known to promote carcinogenesis and metastasis signaling. I next checked the expression level of mortalin in control and CAPE-treated cells. As shown in Fig. 3-3 A-C, there was a decrease in mortalin at both protein and transcript level in CAPE-treated cells. I examined the expression level of other cell migration regulators in these cells and found decrease in vimentin, MMP-2, MMP-3, β-catenin, TGF-β and WNT-3a transcripts (Figs. 3-3 C&D). Of note, CAPE-γCD performed better and showed higher decrease (Fig. 3-3 A).

3.3.3 Mortalin overexpressing cells possess higher level of expression of cancer cell stemness markers

Mortalin is enriched in a large variety of cancer cells (61;64-68). In this study, I first investigated the expression level of mortalin and CD24 in parallel in some normal, immortalized and tumor derived cells (Fig. 3-9 A). As expected, mortalin was upregulated in all cancer cell lines examined as compared to the normal cells. Interestingly, CD24 expression showed variability. Whereas SV40- immortalized (JFCF-6B and 4D) and several tumor-derived cells (MCF-7, G361, SKOV3, HUH-6, A549, DLD1, COLO 320, HCT 116) showed increase in CD24 expression as compared to the control cells, others (MDA-MB 231, Saos-2, HeLa, HUH-7, H1299) (Fig. 3-9 A) showed decrease. Based on these data, I selected breast adenocarcinoma MDA-MB 231 (low level of CD24) and MCF-7 (high level of CD24) for the current study and determined the role of mortalin by generating their overexpressing derivatives. In order to examine the role of mortalin in cancer cell stemness characteristics, I first investigated the expression of two major stem cell markers, ABCG2 and OCT4 in

control and their mortalin-overexpressing derivatives (Mot-OE) by Western blotting with specific antibodies. As shown in Fig. 3-4 A, Mot-OE MCF-7 cells possessed higher expression of both ABCG2 and OCT4 as compared to the control and, showed high efficacy of spheroid formation (Fig. 3-4 B). Consistent with these, Mot-OE cells exhibited $CD44^{high/+}$ (97.3%) and $CD24^{low/-}$ (17.0%) level of expression as compared to the parent MCF-7 cells (Fig. 3-4 C). The results were also validated by qPCR that confirmed higher level of expression of CD9, MRP1, CD133 and ALDH1 (Fig. 3-4 D) in Mot-OE as compared to the control cells. Furthermore, mortalin overexpression caused decrease in CD61 and CD24 expression at the transcription level (Fig. 1D). Mortalin-overexpressing MDA-MB 231 cells also showed higher level of expression of CD9 and lower level expression of CD24 and CD61 as compared to the control parental cells (Fig. 3-5 A). These cells also showed high spheroid-forming capability, about 10-fold higher expression of cancer stem cell marker, CK19 (Fig. 3-5 B) and significant upregulation of ABCG2 and OCT4. Similar results were obtained in U2OS (Fig. 3-5 B) and G361 cells (Fig. 3-5 C) showing that mortalin upregulation enhances the cancer stem cell characteristics as determined by upregulation of ABCG2, MRP1, CD133, and downregulation of CD61 and CD24.

Examination of cell migration characteristics of control and Mot-OE cells revealed that MCF-7/mot-OE cells possess higher migration and invasion ability and was consistent with the earlier findings[22]. Furthermore, both migration and invasion were compromised in cells treated with mortalin-shRNA cells as compared to the respective controls. Similar results were also obtained for MDA-MB 231 cells (68). Taken together, these data suggested that upregulation of mortalin overexpression in cancer cells contributes to cancer cell stemness in a cell line-unspecific manner.

3.3.4 Mortalin contributes to drug-resistance in cancer cells and offers a target for better cancer therapy

Since resistance of cancer stem cells to chemotherapeutic drugs is one of their major characteristics that possess a foremost hurdle to cancer treatment, I next investigated if mortalin overexpression contributes to drug resistance of cancer stem cells. As shown in Fig. 3-6 A&B, mortalin-overexpressing derivatives of both MDA-MB 231 and MCF-7 cells showed resistance to several anti-cancer drugs. Of note, mortalin-knockdown using shRNA plasmid sensitized the cells to the drugs (Fig. 3-6 C). In order to further validate these findings, I recruited mortalin-targeting adeno-oncolytic virus (harboring independent mortalin targeting site as described in material and methods section) that was shown to cause mortalin knock-down and tumor suppression *in vitro* and *in vivo* (63). Cells treated with subtoxic doses of mortalin-targeting adeno-oncolytic virus, as described earlier (63;68), showed better drug response. In line with this data, (33) have also reported that elevated levels of mortalin expression were responsible for resistance of ovarian cancer cells to cisplatin (33).

In light of above data, I next used mortalin inhibitors MKT-077 (a cationic rhodacyanine dye) (69;70) (24;71) and CAPE (Caffeic Acid Phenethyl Ester, a bioactive compound in honey bee propolis) (72) to sensitize cancer cells to various drugs. Since MKT-077, CAPE and other drugs used in this study could cause cancer cell apoptosis by themselves, their subtoxic doses (as determined by independent experiments) were used in order to avoid their cytotoxic effect per se, and to achieve knockdown of mortalin as reported earlier (24;69-71). As shown in Fig. 3-10 A, I found that pretreatment of MCF-7 cells with sub-toxic dose of MKT-077 (0.2~0.5 μM), sensitized them to various chemotherapeutic drugs) (Table 2). CAPE (0.8 μM) that was seen to cause reduction in mortalin expression both at the transcriptional and translational level (Fig. 3-10 A&B) also caused similar sensitization of cancer cells to a variety of drugs (Fig. 3-10 B). Similar results were obtained in MDA-MB 231 and

U2OS cells (Fig. 3-10 c-f) suggesting that targeting of mortalin by shRNA or small molecules including natural drugs potentiated the effect of anticancer drugs.

Mortalin has been shown to play key role in mitochondria biogenesis (28). It has been identified as the only ATPase component of the mitochondrial import complex and is essential for the translocation of most mitochondrial inner membrane and matrix proteins. It binds to Tim44 (inner mitochondrial membrane translocase) and serves as the ATP-driven force generating motor during protein import (73-75). Mortalin depletion altered mitochondrial bioenergetics, depolarized mitochondrial membrane, decreases oxygen consumption and extracellular acidification and increased oxidative stress in medullary thyroid carcinoma cells (76). I have earlier detected expression of retrovirally-expressed mortalin in mitochondria (62), and hence the mitochondrial functions of upregulated mortalin may contribute to increasing metabolic demand that accompanies increased proliferation capacity of cancer stem cells. Glucose uptake, ATP content, OCR and membrane potential are higher in CSC as compared to non-CSC. Analysis of metabolic curves of MCF-7 and their mortalin overexpressing derivatives by real time automated kinetic cell assays (OMNILOG) confirmed the higher metabolic rate of mortalin overexpressing derivatives (Fig. 3-9 B). Mitochondria has been implicated in cell stemness and differentiation properties (77) (78) (79) also due to its key role in cellular oxygen consumption and extracellular acidification characteristics. Mortalin, Hsp60 and several other mitochondrial proteins have been shown to be essential regulators of oxidative stress (28;29;80-83). Role of mortalin in protection against oxidative stress was also confirmed in Alzheimer disease model where suppression in mortalin expression significantly increased mitochondrial membrane depolarization and reduced the cellular ATP levels in neuroblastoma cells (84). It was shown to regulate hematopoietic stem cell function by controlling oxidative stress (80;85). In light with these reports, increased mortalin expression in cancer cells was predicted to increase (i) mitochondrial potential resulting in lower ROS

level, (ii) oxygen consumption in hypoxia condition, cellular ATP levels and (iii) maintain cancer stemness and drug resistance properties.

A variety of stressful environmental conditions induce heat shock proteins. Cancer, a physiologically stressed conditions that challenge cell survival in limited nutirents and oxygen supply, has been associated with upregulation of heat shock proteins (Hsp) including mortalin, Hsp70, Hsp90 and Hsp27. These have been predicted to hold diagnostic and prognostic clinical applications (86-88) and hence warranted studies. Multifunctional stress chaperone mortalin, enriched in cancers and found in various subcellular sites (the mitochondrion, plasma membrane, endoplasmic reticulum, cytosol and nucleus) has been shown to interact with tumor suppressor protein-p53, inactivates its transcriptional activation and apoptotic functions in cancer cells resulting in activation of proproliferation signaling (24-26;77;89-91). Nuclear mortalin has been shown to promote cancer cell metastasis by mechanism involving functional activation of telomerase and heterogeneous ribonucleoprotein K (hnRNP-K)(62). Role of mortalin in cancer cell stemness has not been elucidated and hence warrated investigations. I found that core markers of cancer cell stemness (i) ABCG2, the ATP-binding cassette transporter protein that reduces the intracellular concentration of drugs and (ii) Oct-4 transcriptional factor that is critically involved in the self-renewal and drug resistance (11-16) were upregulated in mortalin-overexpressing cells. Furthermore, stem cell surface markers proteins, CD133, MRP1, ALDH1, CD9 also showed increase in mortalin overexpressing cells. In light of these information and data, I next considered if the anti-mortalin small molecules MKT-077 and CAPE affected a specific pool of subcellular mortalin. MKT-077 has been reported to cause significant decrease in mortalin levels leading to short/compressed/fragmented mitochondria (32;69;76;92-94). It was also shown to abrogate mortalin-p53 interactions, causing nuclear translocation and reactivation of p53 in cancer cells (24;25;69;71). (32) have also shown Mortalin-MAC (Membrane Attack Complex) complexes are released in membrane vesicles from complement attacked cells causing resistance of cancer cells

to complement-dependent cytotoxicity (32). siRNA-mediated knock down of mortalin or MKT-077 reduced MAC elimination and resulted in enhanced cell sensitivity to MAC-induced cell death.

I examined effect of CAPE on mortalin by expression and dual-immunocytostaining assays. As shown in Figs. 3-7 A-D, CAPE treated cells showed decrease in level of expression at mRNA as well as protein level, decrease in intensity and shift of mortalin staining from perinuclear to pancytoplasmic type. Furthermore, decrease in mortalin was clearly observed in the cell nucleus by analysis of the images by IMARIS software (Fig. 3-7 D). Dual co-immunostaining of mortalin and ER resident protein calreticulin in control and CAPE treated cells revealed that although high doses of CAPE caused decrease in both proteins, the low doses were more effective to mortalin (Fig. 3-8 A). Similar analysis with hnRNP-K revealed significant decrease in mortalin in the nucleus of CAPE treated cells (Figs. 3-8 B&C). Taken together, it was concluded that targeting of mortalin by subtoxic doses of CAPE caused drug sensitization by multiple ways including its functions in mitochondria, ER and nucleus (28); nuclear clearing seemed most remarkable of all. Of note, nuclear mortalin has been assigned roles in EMT and malignant transformation (62;68). Other drugs that have been shown to target mortalin or its functions include Withanone, Withaferin-A and Embelin that caused activation of Tumor Suppressor protein p53 and deactivation of metastatic signaling in cancer cells (95-97), and veratridine that sensitized cancer cells to chemotherapeutic drugs by UBXN2A-dependent inhibition of mortalin (98).

The most effective chemotherapeutic agents in breast cancer are doxorubicin, taxol, cyclophosphamide, methotrexate, 5-fluorouracil and their combination chemotherapy program. Although each of these drugs possesses appreciable tumor regression and anti-metastatic properties, they are often complicated drug-resistant cancer cell sub-population (CSCs) responsible for tumor relapse. Vargas-Roig et al. reported increased level of nuclear Hsp70 in the clinical samples of breast cancer that showed with drug resistance to doxorubicin, cyclophosphamide, methotrexate and epirubicin

(85). I have earlier demonstrated that mortalin promotes malignant transformation of cancer cells by increasing their proliferative and migration capacity and hence mortalin is a promising target for cancer therapy (24-26;61-63;68;89). In the present study, I report that the CSC cells have high expression of mortalin that contributes to their stemness and drug resistance characteristics. In recent studies, I have also shown that like CAPE, Artepillin C a brazillian propolis major component also interfere in p53-mortalin complex (99). I demonstrate that anti-mortalin drugs, CAPE and MKT-077, reverted the drug resistance and hence could be useful new adjuvants for increasing the efficacy and outcome of chemotherapy.

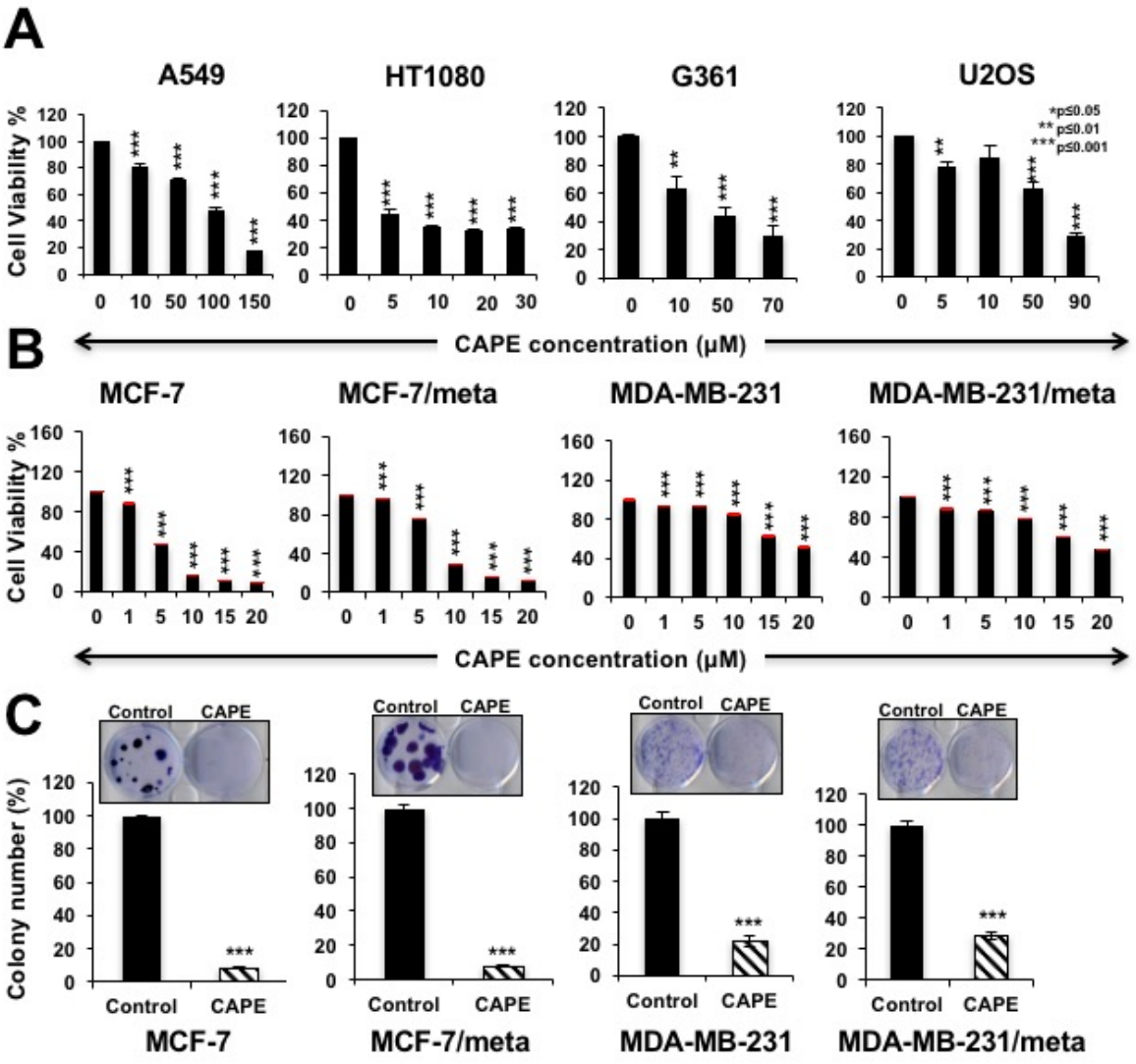


Fig. 3-1 Cytotoxicity of CAPE to human cancer cells. Dose dependent loss in cell viability in cells treated with CAPE is shown. IC50 for A549, HT1080, G361 and U20S were ~100, 5, 20, 60 μM respectively (A). Effect of CAPE on cell viability (B) and colony forming efficiency (C) of MCF-7, MDA-MB-231 cells and their mortalin-overexpressing metastatic deriatives showing that CAPE (10μM) inhibited cell proliferation in both short term (A) and long term (B) viability assays.

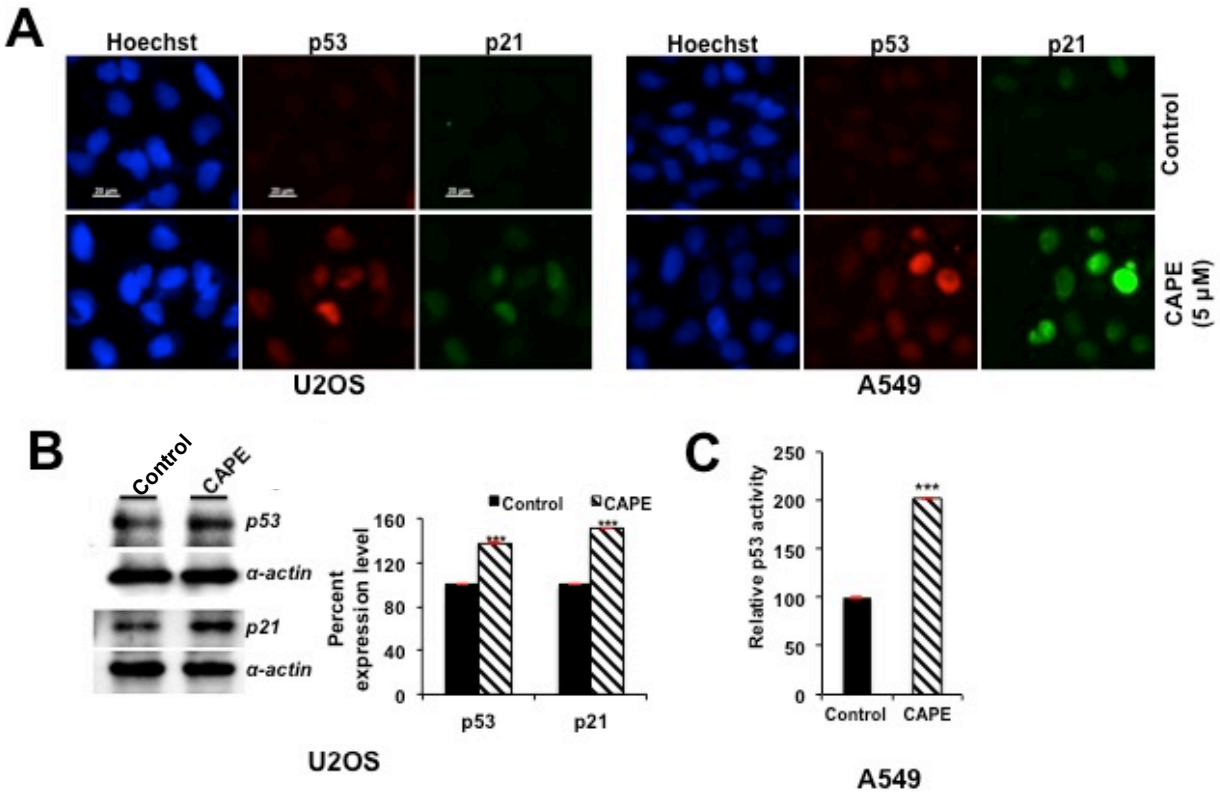


Fig. 3-2 Activation of p53-p21 pathway by CAPE. (A) Immunostaining of p53 and p21 in control and CAPE (5μM) treated cells showing their increase. (B) Increase in p53 and p21 as determined by Western blotting with specific antibodies. (C) p53-dependent reporter assay showing increase in transcriptional activation function of p53 in CAPE-treated (5μM) than in control cells.

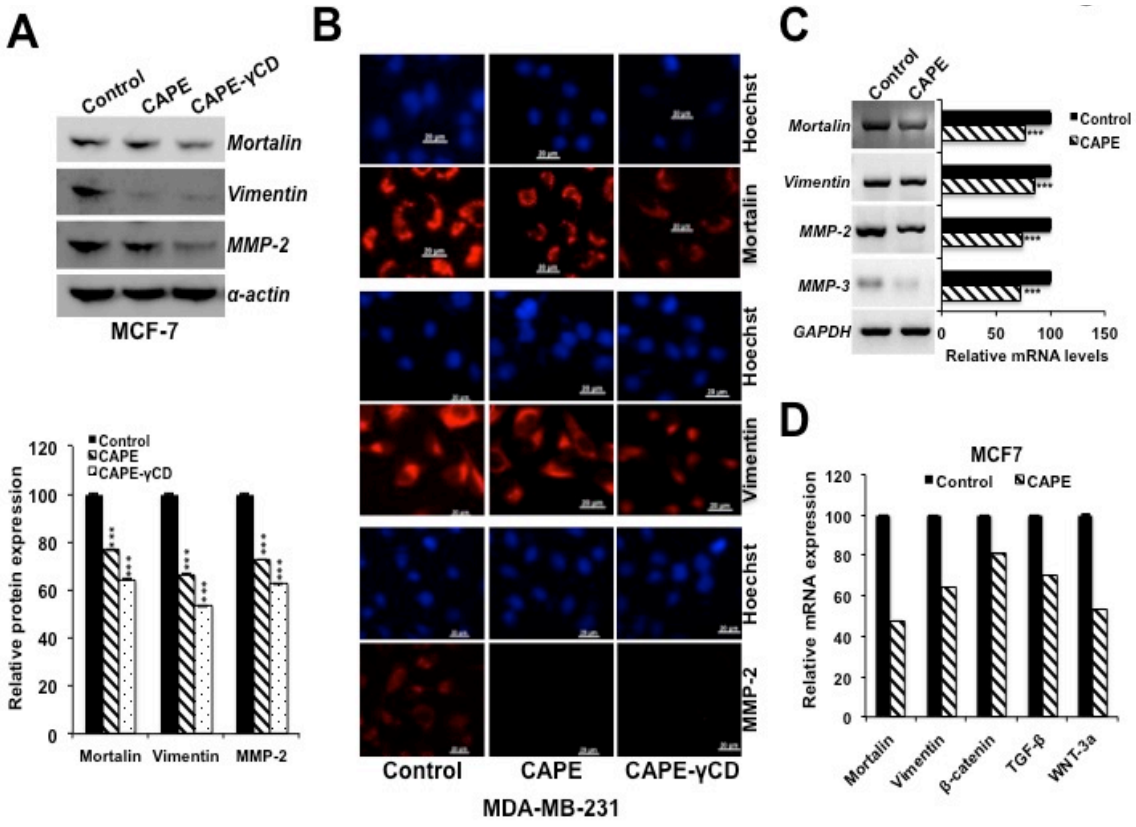


Fig-3-3 Downregulation of mortalin and other metastasis-regulatory proteins by CAPE. Effect of CAPE on mortalin and cell migration regulatory proteins. Decrease in mortalin, vimentin, MMP-2 was obserevd by Western blotting (A), immunostaining (B) and RT-PCR (C). Decrease in mortalin, vimentin, β-catenin, TGF-β and Wnt-3a as determined by qPCR is shown (D)

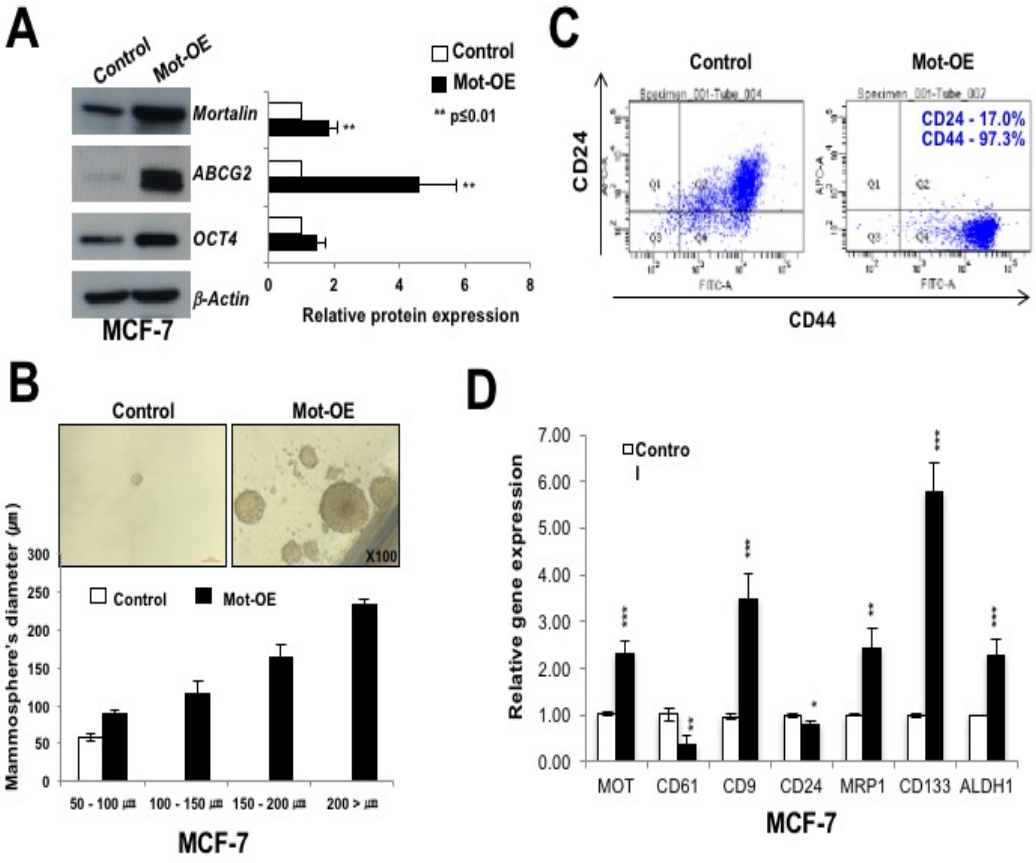


Fig. 3-4 Mortalin overexpressing MCF-7 cells showed higher expression of cancer stem cell marker proteins ABCG2, OCT4 as determined by Western blotting (**A**) and showed high degree of spheroid formation (**B**). FACS and RT-qPCR analysis revealed higher level of expression of CD44 (**C**), CD9, MRP1, CD133 and ALDH1 (**D**) and low level of expression of CD24 (**C** and **D**), CD61 (**D**) in mortalin-overexpressing as compared to the control cells.

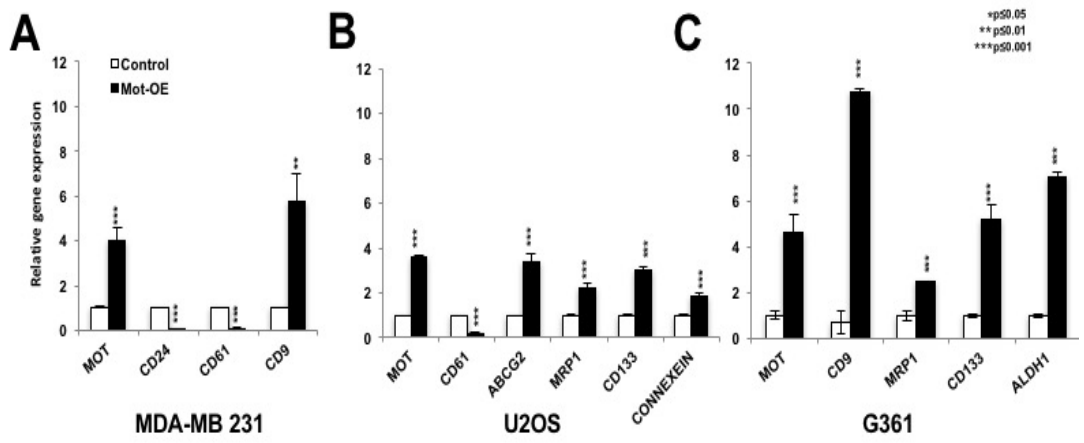


Fig. 3-5 Mortalin overexpressing MDA-MB 231 cells showed low level of expression of CD24, CD61 and high level of expression of CD9 as compared to the control cells (**A**). Mortalin overexpressing U2OS cells showed low level of expression of CD61 and high level of expression of ABCG2, MRP1, CD133, & Connexein as compared to the control cells (**B**) Mortalin overexpressing G361 cells showed high level of expression of CD9, MRP1, CD133 and ALDH1 (**C**).

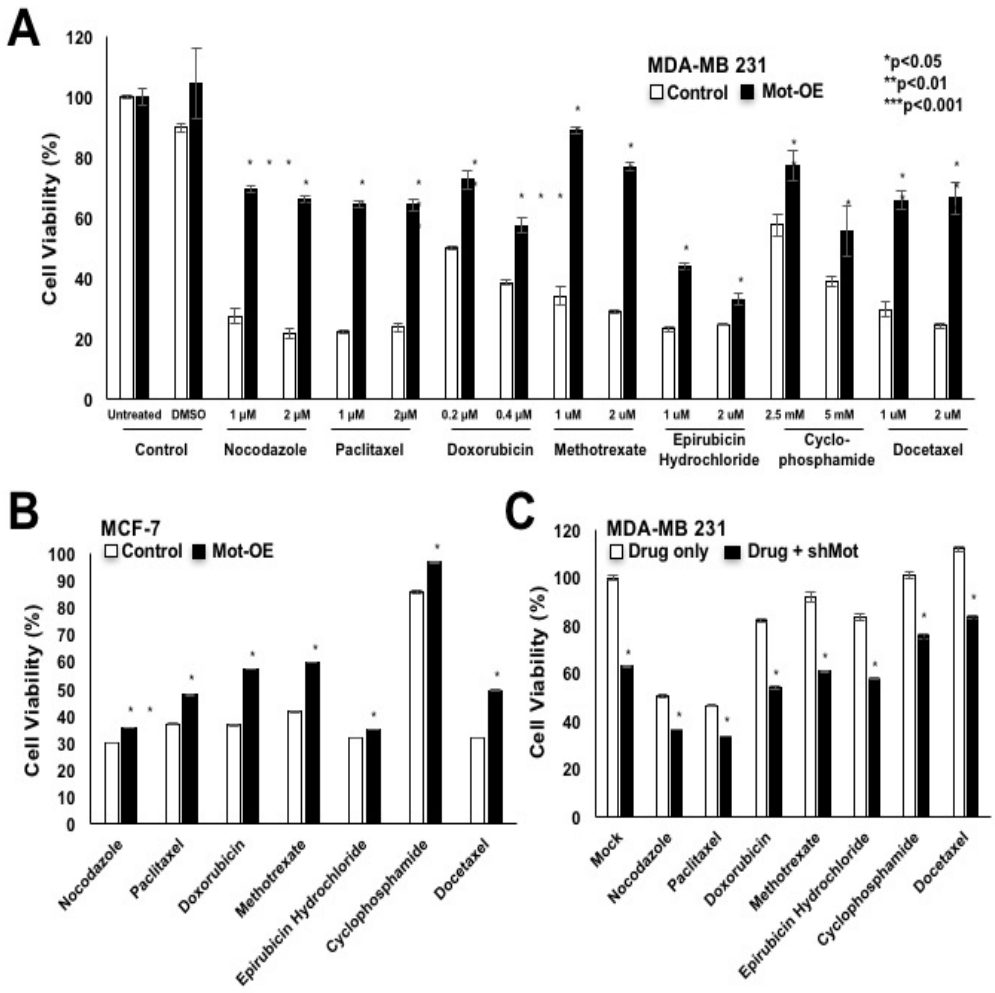


Fig. 3-6 Mortalin overexpressing MDA-MB 231 (**A**) and MCF-7 (**B**) cells showed higher viability when treated with a variety of drugs indicating drug resistance characteristics. Knockdown of mortalin with shRNA caused sensitization to the drugs (**C**).

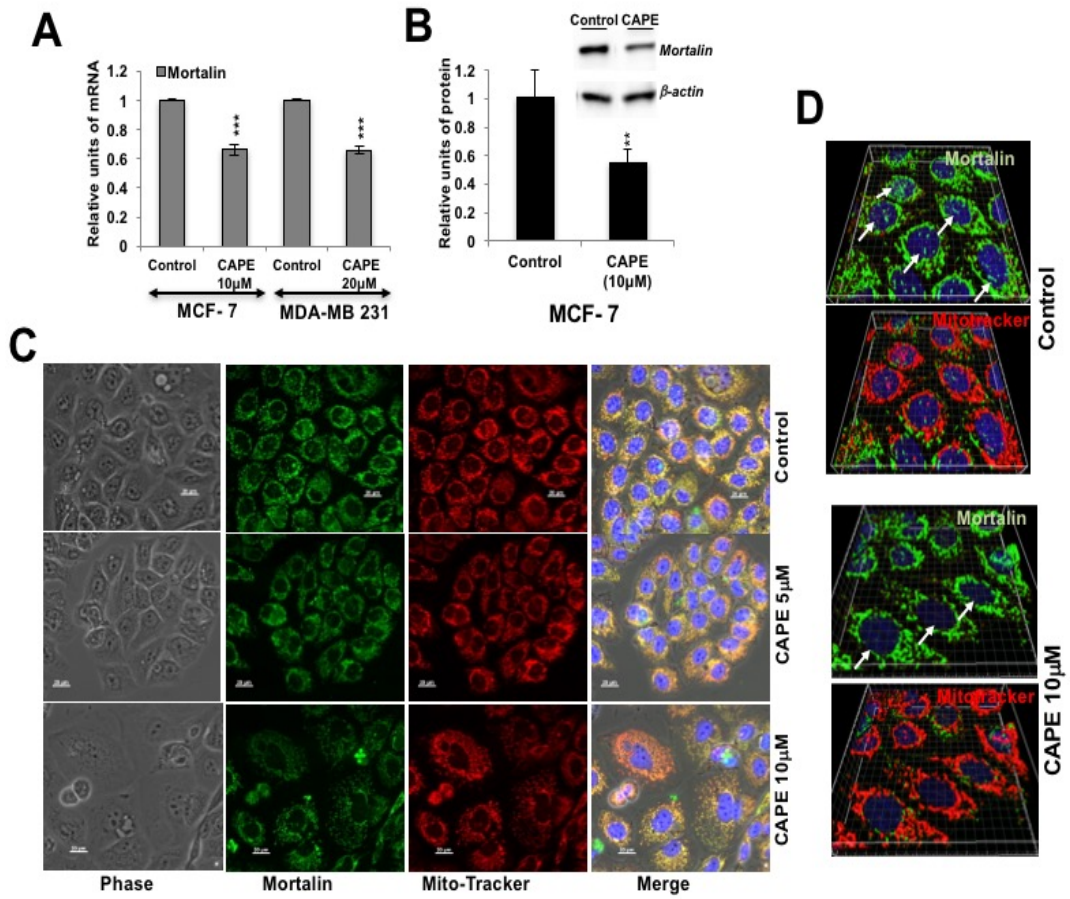


Fig. 3-7 MCF-7 & MDA-MB 231 cells treated with CAPE showed downregulation of mortalin mRNA (**A**) and protein (**B**) as compared to the control cells. Dual immunostaining of control and CAPE treated cells with mortalin antibody and mito-tracker showed decrease in mortalin intensity (**C**). IMARIS analysis of images showed remarkable reduction in nuclear mortalin, shown by white arrows (**D**).

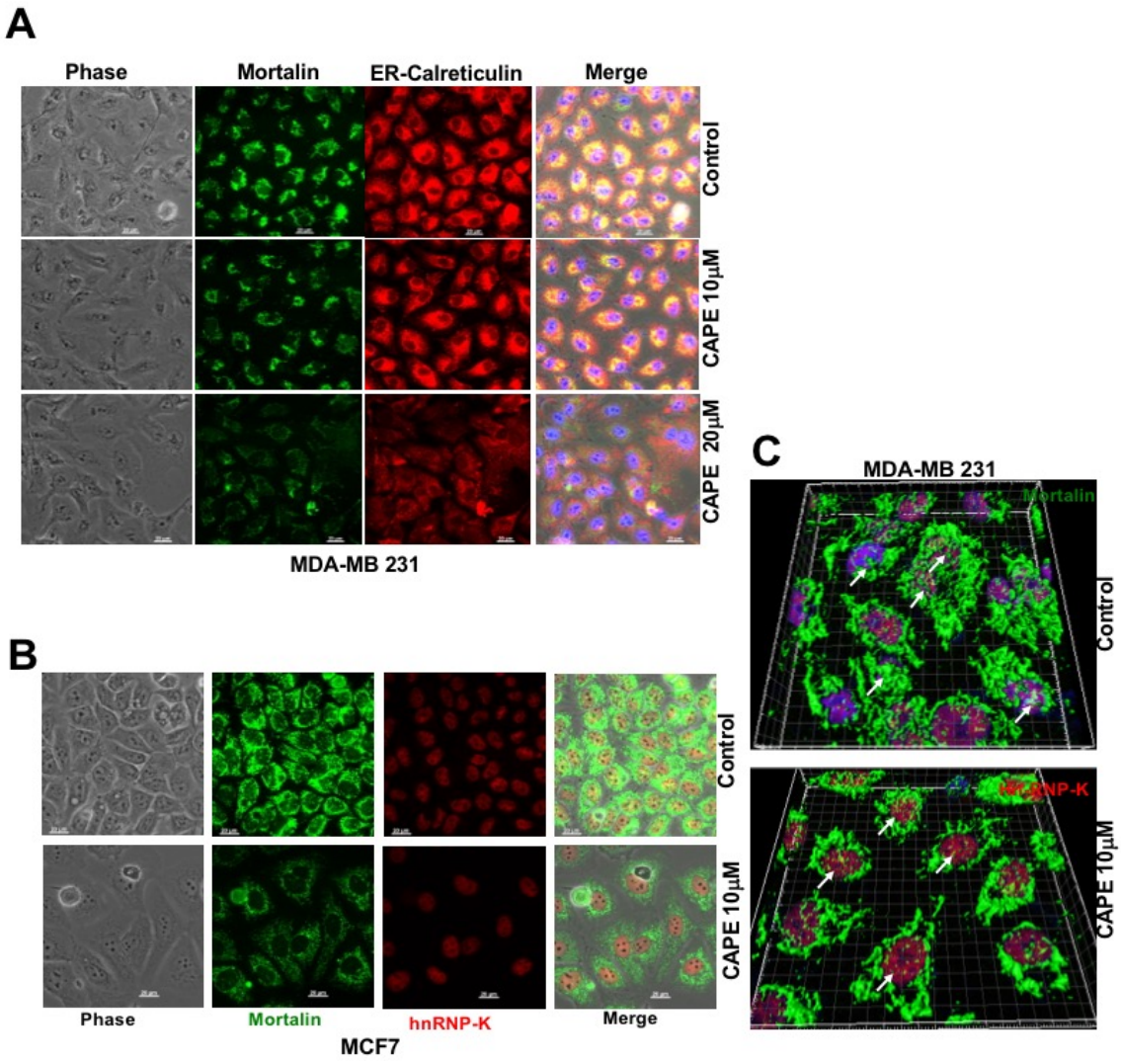
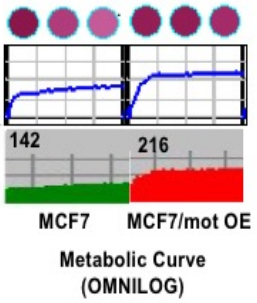


Fig. 3-8 CAPE treated MDA-MB 231 cells showed downregulation of mortalin & calreticulin. Dose response analyses showed stronger effect on mortalin than calreticulin (**A**). Coimmunostaining of mortain and nuclear protein hnRNP-K showed reduction in mortalin and its remarkable clearance from the nucleus (**B**) IMARIS images of control and CAPE treated cells showing reduction in nuclear mortalin, shown by white arrows (**D**)

A

Relative units of mortalin and CD24 expression as determined by Western blotting with specific antibodies. Endogenous β-actin was used as internal control.				
	Cell Lines Name	Description	Mortalin Expression (Relative units)	CD24 Expression (Relative units)
Normal cells				
1	TIG	Normal Skin Fibroblast	1.0	1.0
2	MRC5	Normal Lung Fibroblast	1.0	1.0
Immortalized cell lines				
3	JFCF-6B	SV 40 Immortalized Fibroblast	2.2	2.1
4	JFCF-4D	SV 40 Immortalized Fibroblast	1.3	1.6
Tumorigenic cell lines				
5	MDA-MB 231	Breast Adenocarcinoma	1.8	0.3
6	Saos-2	Osteogenic Sarcoma	4.5	0.8
7	HeLa	Epithelioid Cervical Carcinoma	4.1	0.5
8	HUH-7	Hepatocellular Carcinoma	1.4	0.6
9	H1299	Non-Small Lung Cell Carcinoma	2.2	0.6
10	MCF-7	Breast Adenocarcinoma	1.8	1.9
11	G361	Skin Malignant Melanoma	2.1	1.4
12	SKOV3	Ovarian Carcinoma	2.2	1.5
13	HUH-6	Human Hepatoblastoma	2.0	1.8
14	A549	Adenocarcinoma	3.4	2.8
15	DLD-1	Colorectal Adenocarcinoma	1.4	1.2
16	COLO320	Colorectal Adenocarcinoma	4.0	2.1
17	HCT116	Colorectal Carcinoma	6.7	2.0

B



Metabolic Curve (OMNILOG)

Fig. 3-9 Quantitation of the protein signals obtained by Western blotting with specific anti-mortalin and anti-CD24 antibodies is shown. The expression was normalized against endogenous protein, ㉾-actin as a loading control **(A).** Real time metabolic rate of control and mortalin overexpressing MCF-7 cells as determined by Omnilog incubator (**B**).

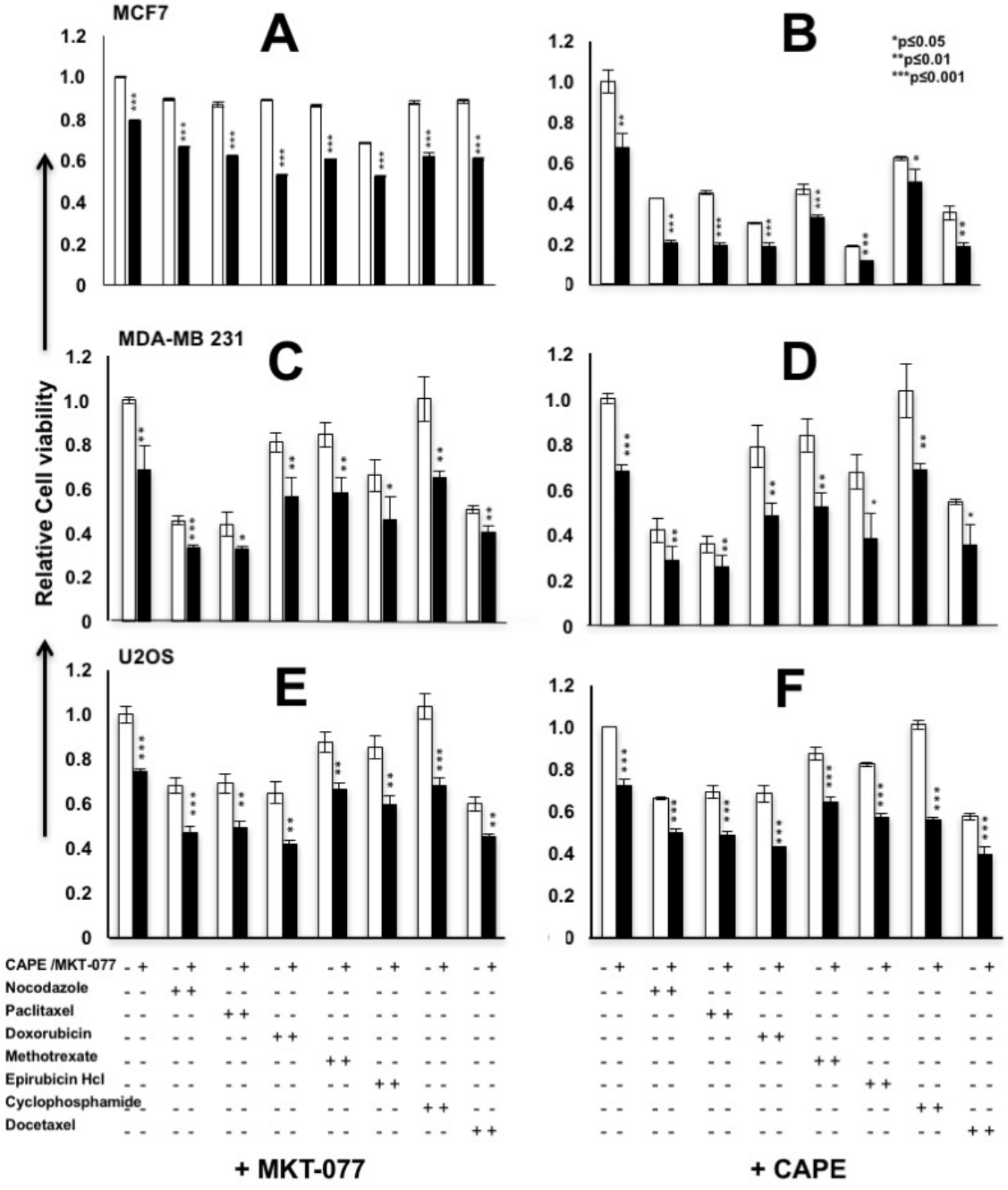


Fig. 3-10 MCF-7 (**A**) and MDA-MB231 (**B**) and U2OS (**C**) cells when treated with mortalin inhibitors (MKT-077 or CAPE) showed better response to a variety of anticancer drugs.

Table 1. Sequence of primers used for real time PCR analysis.

ABCG2	Forward	5′-TTCTCCATTCATCAGCCTCG-3′
	Reverse	5′-TGGTTGGTCGTCAGGAAGA-3′
CD61	Forward	5′-ATGGGACACAGCCAACAACC-3′
	Reverse	5′-GTGGCACAGGCTGATAATGA-3′
CD-133	Forward	5′-GCATTGGCATCTTCTATGGTT-3′
	Reverse	5′-CGCCTTGTCCTTGGTAGTGT-3′
MRP1	Forward	5′-TCTGGGACTGGAATGTCACG-3′
	Reverse	5′-CCAGGAATATGCCCCGACTTC-3′
Connexin 43	Forward	5′-CTACTCAACTGCTGGAGGGAAG-3′
	Reverse	5′-GGCCACCTCAAAGATAGACTTG-3′
ALDH 1	Forward	5′-CGCAAGACAGGCTTTTCAG-3′
	Reverse	5′-TGTATAATAGTCGCCCCCTCTC-3′
CD 24	Forward	5′-CCCACGCAGATTTATTCCAG-3′
	Reverse	5′-GACTTCCAGACGCCATTTG-3′
CD 9	Forward	5′-ATGATGCTGGTGGGCTTC-3′
	Reverse	5′-GCTCATCCTTGGTTTTCAGC-3′
Mortalin	Forward	5′-AGC TGG AAT GGC CTT AGT CAT-3′
	Reverse	5′-CAG GAG TTG GTA GTA CCC AAA TC-3′
CD61	Forward	5′-ATGGGACACAGCCAACAACC-3′
	Reverse	5′-GTGGCACAGGCTGATAATGA-3′
HIF-1α	Forward	5′-CCAGCAGACTCAAATACAAGAACC-3′
	Reverse	5′-TGTATGTGGGTAGGAGATGGAGAT-3′

Table 2. Drug doses used for different cell lines.

	MCF-7 (μM)	MDA-MB231 (μM)	U2OS (μM)
MKT-077 / CAPE	0.5 / 10	0.4 / 20	0.6 / 15
Nocodazole	2.0	1.0	1.0
Paclitaxel	2.0	1.0	1.0
Doxorubicin	0.4	0.2	0.2
Methotrexate	2.0	1.0	1.0
Epirubicin Hydrochloride	2.0	1.0	1.0
Cyclophosphamide	5000	5000	5000
Docetaxel	2.0	2.0	2.0

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Chapter 4

Screening of drug library by HRE driven luciferase reporter System-Identification of Withaferin-A (C-024) as anti-hypoxia factor and its major role in NF-kB signaling.

4.1 Introduction

NFκB (Nuclear Factor kappa-light-chain-enhancer of activated B cells) family of proteins is a class of transcription factors that possess a Rel homology domain in their N-terminus and control expression of genes involved in regulation of growth, apoptosis, immuno-regulatory and inflammatory processes (1;2). It regulates cellular response to oxidative stress, cytokines, bacterial and viral antigens and plays a key role in regulating the immune response to infection (3). NFκB1 and NFκB2 are transcription factors synthesized as large precursors, p105, and p100 that are cleaved to mature NFκBp65 and p50 proteins by selective degradation of their C-terminal region containing ankyrin repeats (4). These are present in cells in an inactive (cytoplasmically sequestered) state by binding to their inhibitors, called IκBs (Inhibitor of NFκB). Intracellular or extracellular stresses, cytokine signals that activate its upstream regulator, IKK (Inhibitor of IκB Kinase), phosphorylate IκBα at Ser32 and Ser36 residues. The phosphorylated IκBα undergoes proteasomal degradation resulting in active NFκB that translocates to the nucleus and performs transcriptional activation function (5;6). The NFκB proteins lack intrinsic transcriptional activation ability and function by binding as homodimers (7). Its crucial role in processes like proliferation, apoptosis and invasiveness requires controlled activation (8). Dysregulated activation has been shown to be associated with various conditions like arthritis, asthma and inflammatory disorders (1;9;10). Aberrant or constitutive activation of NFκB has been observed in

various types of cancer cells marking it as a potential therapeutic target (1;2;11;12). In line with this, identification and functional characterization on natural and synthetic inhibitors of NFκB have been initiated. Inhibition of NFκB by small molecules, β-Mangostin (βM) isolated from *Cratoxylum arborescens* (13) and Wi-A (Withaferin-A) isolated from medicinal herb *Withania somnifera* (Ashwagandha) (10;11;14;15) has been reported. It was shown that Wi-A inhibits DNA binding of NFκB resulting in ROS generation, mitochondrial dysfunction, oxidative stress and apoptosis in cancer cells (14-17).

DNA damage response is differentially regulated in cancer and normal cells. Whereas normal cells respond by executing growth arrest, cancer cells are refractory and keep proliferating (18). Several studies have established that DNA damage response (DDR) signaling is essential for execution of senescence, an established powerful tumor suppressor mechanism (19;20). The p38MAPK is a kinase that is activated through environmental stress and DNA damage stress including ionizing radiation, UV, chemotherapeutic drugs, and lead to the induction of a G2/M cell cycle checkpoint through p53-dependent and independent mechanisms (21;22). p38MAPK has been shown to transcriptionally activate NFκB leading to senescence (23-25), mediated by upregulation of p53-$p21^{WAF1}$ pathway. CARF (Collaborator of ARF), an ARF (Alternative Reading Frame, p14ARF)-interacting protein, has been shown to regulate activities of p53-tumor suppressor protein in an ARF-dependent or -independent manner (26-28). It has been shown to regulate DNA damage response in a dose dependent manner and regulates cell proliferative fates in normal and cancer cells (28;29).

Wi-A has been shown to cause inhibition of IKK activity in some earlier studies. (10;15;30) Through bioinformatics analysis, I had previously shown that Wi-A disrupts important hydrophobic interaction between IKKβ and Nemo chain residues, L93:F734, T735:F92, F734:M94, W739:F97, W741:A100, W741:R101, thereby inhibiting their complex (31). However, an experimental study failed to reveal the inhibition of IKKβ-

Nemo interaction by Wi-A, instead it inhibited IκB kinase activity by interacting with C179 residue located in catalytic domain of IKKβ (10). It was also demonstrated that Wi-A hyper-phosphorylates IKKβ at S181 causing inhibition of TNF-induced IKK activity and, thereby, causing inhibition of IκB degradation and p65 translocation (15). These reports described three different binding sites of Wi-A on IKK complex suggesting its interaction at multiple sites. Inhibition of IKK activity by Wi-A and/or NFκB translocation to the nucleus has been reported in some studies (10;15;30). However, the molecular insights and overall impact of interactions of Wi-A with IKKβ or NFκB structure and activity remain undefined. I provide experimental evidence to the activation of kinase activity of IKK complex resulting in phosphorylation of IκBα and nuclear translocation of NFκB. Wi-A was further seen as not disrupting NFκB-DNA interactions. Of note, I found that the cancer cells that showed activated NFκB and p38MAPK possessed decreased level of expression of cyclin D1, cyclin E and CDK-2/4. I provide evidence that Wi-A trigger DNA-damage response, as supported by upregulation of γH2AX and CARF that yielded senescence in both cancer and normal cells.

4.2 Material and Methods

4.2.1 Cell culture and antibodies

Human lung carcinoma (H1299 and A549) and normal fibroblasts (MRC5) were cultured in RPMI-1640 and DMEM mediums, respectively, supplemented with 10% (V/V) FBS (fetal bovine serum) in 5% CO2 and 95% air humidified incubator. The antibodies against NFκB (p65), p-IκBα p53, p38MAPK, $p16^{INK4A}$, CDK2, Cyclin E, Cyclin D1 and Cdk-4 (Santa Cruz Biotech Inc. CA, USA) and HP1γ, p-p38MAPK, pRb (S780), γH2AX, $p21^{WAF1}$ (Cell signaling, Beverly, MA, USA) were purchased. Anti-β-actin antibody was purchased from Abcam, Cambridge, UK. Anti-CARF antibodies were generated in our laboratory.

4.2.2 Cell viability assay

The cells were seeded in 96-well tissue culture plates and treated with different concentrations of Wi-A for indicated time intervals followed by incubation with 0.5 mg/ml MTT (Invitrogen, Carlsbad, CA) in media for 4 h at 37°C in CO_2 incubator. 100 µl of dimethyl sulfoxide were added to each well for cell lysis. Absorbance (570 nm) was measured using a microplate reader (Infinite 200 PRO; Tecan Group Ltd., Mannedorf, Switzerland). All assays were performed independently, at least, three times.

4.2.3 Colony forming assay

H1299 cells were treated with Wi-A (0.2 µg/ml and 1 µg/ml). After 24 h, 5 x 10^2 cells were re-plated on 6-well culture plate and observed for colonies every alternate day up to 10 days with regular change of medium (without drugs). Colonies were washed with PBS, and fixed with a pre-chilled methanol for 10 min at 4^oC followed by staining with 0.1% Crystal violet (Sigma-Aldrich, MO). The stained plates were dried, photographed and colonies were counted.

4.2.4 Western blot analysis

The cells were treated with Wi-A (0.2 µg/ml and 1 µg/ml) for 24 h following which cell lysates were prepared in RIPA buffer (Thermo Scientific, Rockford, IL) containing complete protease inhibitor cocktail (Roche Applied Science, Mannheim, Germany). Cell lysates (containing 20 µg protein) were separated on a SDS-polyacrylamide gel using Mini-PROTEAN® Tetra cell equipment (Bio-Rad, Hercules, CA), and transferred onto PVDF membrane (Millipore, Bedford, MA) by Mini Trans-Blot® Electrophoretic Transfer Cell (Bio-Rad). Membranes were blocked in 0.2% Tween 20 in TBS containing 3% BSA followed by incubation with specific antibodies (1-2 h), washing buffer (0.2% Tween 20 in TBS; 3x10 min) and then horseradish peroxidase-conjugated secondary antibody (45 min), and washing buffer (3x10 min). The blots

were then developed using chemiluminescence (GE Healthcare, UK) and visualized using Lumino Image Analyzer equipped with CCD camera (LAS3000-mini; Fuji Film, Tokyo, Japan). Nuclear and cytoplasmic fractions were prepared using the Qproteome cell compartment kit (Qiagen, Hilden, Germany).

4.2.5 Immunostaining

Cells (1 x 10^4), cultured on a glass coverslips placed in a 12-well plate, were treated with Wi-A (0.2 and 1.0 μg/ml). Briefly, cells were washed with cold phosphate-buffered saline (PBS) and fixed with pre-chilled methanol: acetone (1:1) for 5 min at 4°C. Fixed cells were washed with PBS, permeabilized with 0.2% Triton X-100 in PBS and incubated in blocking buffer (2% BSA in PBS) containing anti-NFκB (p65), p-IκBα, p53, $p21^{WAF1}$, Cyclin E, CDK2, CDK6, p-p38MAPK, pRb (S780), γH2AX, HP1γ or anti-CARF primary antibodies at 4°C overnight. Cells were washed extensively in washing buffer (0.2% Triton X-100 in PBS) before incubation with the fluorochrome-conjugated secondary antibodies (Alexa-488-conjugated goat anti-rabbit, anti-mouse, Alexa-594-conjugated goat anti-rabbit or anti-mouse (Molecular Probes, OR)) for another 30 min. After six washes in washing buffer, cells were overlaid with a coverslip with FA Mounting Fluid (VMRD Inc. Pullman, WA). The cells were examined on a Zeiss Axiovert 200M microscope (Carl Zeiss Microimaging, Thornwood, NY) and analyzed by AxioVision 4.6 software (Carl Zeiss Microimaging). To examine and confirm the localization of p65 in cells, z-stack had been acquired with laser scanning confocal microscopy (Zeiss LSM 700). The files were transferred to a graphic workstation and analyzed with IMARIS software (Bitplane, Zurich, Switzerland).

4.2.6 Cell cycle analysis

H1299 and MRC5 cells were treated with indicated doses of Wi-A for 24 h. Cells were collected in 1.5 ml tube, washed with cold PBS and fixed with 70% ethanol at 4°C

for 12 h. The fixed cells were centrifuged (2000 rpm for 10 min), washed with cold PBS twice and then re-suspended in 0.25 mL PBS. The cells were stained with Guava Cell Cycle Reagent (Millipore) for 30 min in dark. To avoid false DNA-PI staining, RNA was removed by adding 5 µl of 1 mg/ml RNAse A at 37^{o}C for 1 h in cells suspension before PI staining. The stained cells were applied for cycle analysis using Guava® PCA-96 System (Millipore) and cell cycle status was analyzed by CytoSoft TM Software, version 2.5.6 (Millipore).

4.2.7 Apoptosis assay

H1299 cells were seeded at a density of 2 X 10^5 cells/well in 6-well plates for 24 h followed by treatment with Wi-A. Apoptosis assay was executed using Guava Nexin Reagent (EMD Millipore Corporation) following the protocol recommended by the manufacturer. Apoptotic cells were quantitated with the help of Flow Jo Software.

4.2.8 Senescence-associated β-galactosidase (SA-β-gal) assay

SA-β-gal detection kit (Cell Signaling Technology, Danvers, MA, USA) was used following manufacturer's instructions. Cells showing blue staining were considered positive staining and considered as senescence cells. Three independent experiments were performed for data reproducibility.

4.2.9 Statistical analysis

All the experiments were performed in triplicate, independently. Variables were expressed as mean ±SEM of triplicate experiments. Unpaired t-test (GraphPad Prism GraphPad Software, San Diego, CA) has been performed to determine the degree of significance between the control and experimental samples. Statistical significance was defined as p-value and represented by *P<0.05, **P<0.01, ***P<0.001 while no mark denotes insignificant correlation.

4.3 Results and Discussion

4.3.1 Wi-A, at low dose, triggers growth arrest in human lung carcinoma

Human lung cancer (H1299) cells were treated with two doses Wi-A (low, 0.2 and high, 2.0 μg/ml). As shown in Fig. 4-1 A, whereas high dose caused 50% reduction in viability within 48 h, low dose caused only ~10% reduction followed by slow growth arrest. Annexin IV cytometric analysis revealed that the high, not low, dose instigated apoptosis in about 48 h (Fig. 4-1 B). In contrast to 5.12% in control, 8.71% and 34.59% cells were detected in apoptosis in low and high dose treated cultures, respectively (Fig. 4-1 C). Low dose-induced slow growth arrest lead to reduction in colonigenicity in long-term colony forming assays (Fig. 4-1 C) and most interestingly, I found that whereas low dose (24-48 h treatment) induced Go/G1 cell cycle arrest in H1299 cells, the normal (MRC5) cells remained unaffected (Fig. 4-1 D).

4.3.2 Low dose of Wi-A caused nuclear translocation of NFκB in cancer cells

I next treated H1299 (cancer) and MRC5 (normal) cells with low dose of Wi-A (0.2 to 1.0 μg/ml) for 24-48 h and examined the localization of NFκB in control and treated cells. As shown in Fig. 4-2 A NFκB showed nuclear translocation only in cancer cells. The results were confirmed by confocal laser scanning microscopy (Fig. 4-2 B) as well as biochemical analyses including cell fraction and Western blotting with anti-NFκB antibody (Fig. 4-2 C&D). I found that the expression of NFκB (p65) was enhanced in Wi-A treated H1299 and A549 cells. (Fig. 4-2 C) The nuclear translocation of NFκB in response to treatment was also confirmed by cell fractionation and Western blotting analyses using with specific antibodies against cytoplasmic (lamina/C) and nuclear (α-tubulin) proteins (Fig. 4-2 D). The nuclear enrichment of NFκB (p65) suggested its activation in cancer cells, and was in contrast to the earlier reports that have predicted inactivation of NFκB by Wi-A (10;15).

4.3.3 Wi-A caused nuclear translocation of NFκB and p38MAPK

In order to confirm the above computational findings, I performed experiments using human lung cancer (H1299 and A549) and normal (MRC5) cells. As shown in Fig. 4-3A, immunostaining of control and Wi-A treated H1299 cells with anti-phosphorylated IκBα antibody revealed its increased level in cancer, but not in normal, cells. Furthermore, Wi-A induced increase in phosphorylated IκBα supporting the nuclear translocation of NFkB in cancer cells (Fig. 4-3A). Several studies have shown that NFκB is regulated by p38MAPK, activated in response to various environmental stresses including DNA damage signaling, UV, ionization radiation, oxidative stress and cytokines (32). Activation causes its phosphorylation, essential for its nuclear translocation, (21) that in turn stimulates NFκB signaling (33). In view of these reports, I investigated whether Wi-A-induced activation of NFκB was mediated by p38MAPK. As shown in Fig. 4-4 A&B I found nuclear p38MAPK in cancer cells. It was confirmed to be in activated phosphorylated form by Western blotting with phosphorylation specific antibodies (Fig. 4-4 C). Normal cells did not show increase in p38MAPK (Fig. 4-4 A). The data suggested that Wi-A preferentially instigated DNA damage response and activation of NFκB in cancer cells only.

4.3.4 Wi-A caused upregulation of DNA damage response in cancer and normal cells In order to further clarify, I examined the level of CARF expression, an established important regulator of DNA damage response and proliferation fate of cells (26;28). As shown in Fig. 4-6 A&C, I found an upregulation of CARF in Wi-A treated cancer cells. In contrast to the activation of NFκB and p38MAPK, occurred selectively in cancer cells (Fig. 4-4 A and 4-6 A), the level of CARF was upregulated in normal cells in response to Wi-A treatment. In line with this data, both cancer and normal cells also showed increase in γH2AX foci (Fig. 4-6 A) and ROS (Fig. 4-6 A) consequent to Wi-A treatment, suggesting that it may cause growth arrest/senescence both in cancer and

normal cells. Analyses of $p21^{WAF1}$ and $p16^{INK4A}$ proteins that regulate growth arrest and senescence in cells revealed heir increase in Wi-A treated cancer and normal cells (Fig. 4-6 C&D). As expected, the downstream effector of these proteins CDK4 and CDK2 showed decrease that in turn decrease the phosphorylated pRB leading to cell cycle arrest (Fig. 4-8 A-D). Although normal cells did not show decrease in expression of Cyclin D1 (Fig. 4-5 A) or CDK4 (Fig. 4-7 A&D) expression levels, phosphorylated pRB showed distinct decrease in Wi-A treated cells (Fig. 4-7 D) suggesting decrease in kinase activity of CyclinD1-CDK4 complex. Indeed, induction of senescence was confirmed by β-gal staining and HP1γ foci in Wi-A treated both cancer and normal cells (Fig. 4-8 A-B). Of note, since H1299 cells lacked p53 protein (Fig. 4-8 C), an increase in $p21^{WAF1}$ was therefore attributed to p53-independent mechanism, such as increase in CARF, resulting in decreased levels of HDM2 and stabilization of $p21^{WAF1}$ (26;29;34;35).

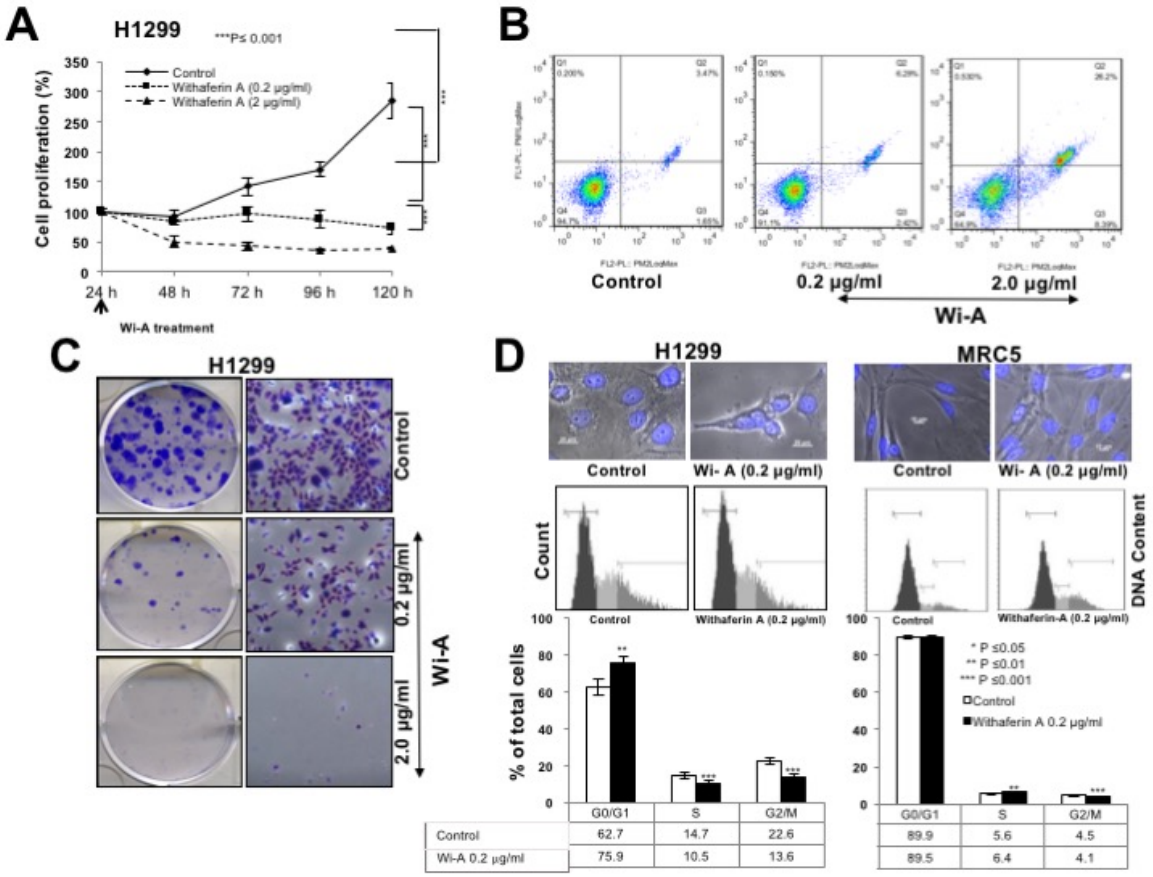

Fig. 4-1 Dose-dependent cytotoxicity of Wi-A to human lung carcinoma and normal cells. Growth curves of control and Wi-A (low- 0.2 µg/ml and high-2.0 µg/ml dose) treated lung carcinoma (H1299) (A). Induction of apoptosis by high dose of Wi-A was confirmed by Annexin-V cytometric analysis. As shown, increase in apoptotic cells was observed in cultures treated with high doses of Wi-A (B). Crystal violet staining of the plates showing decrease in colony forming efficiency of Wi-A (both low and high dose) treated cells (C). Cell cycle analysis showed arrest of H1299, but not the MRC5, cells in G_0/G_1 (D)

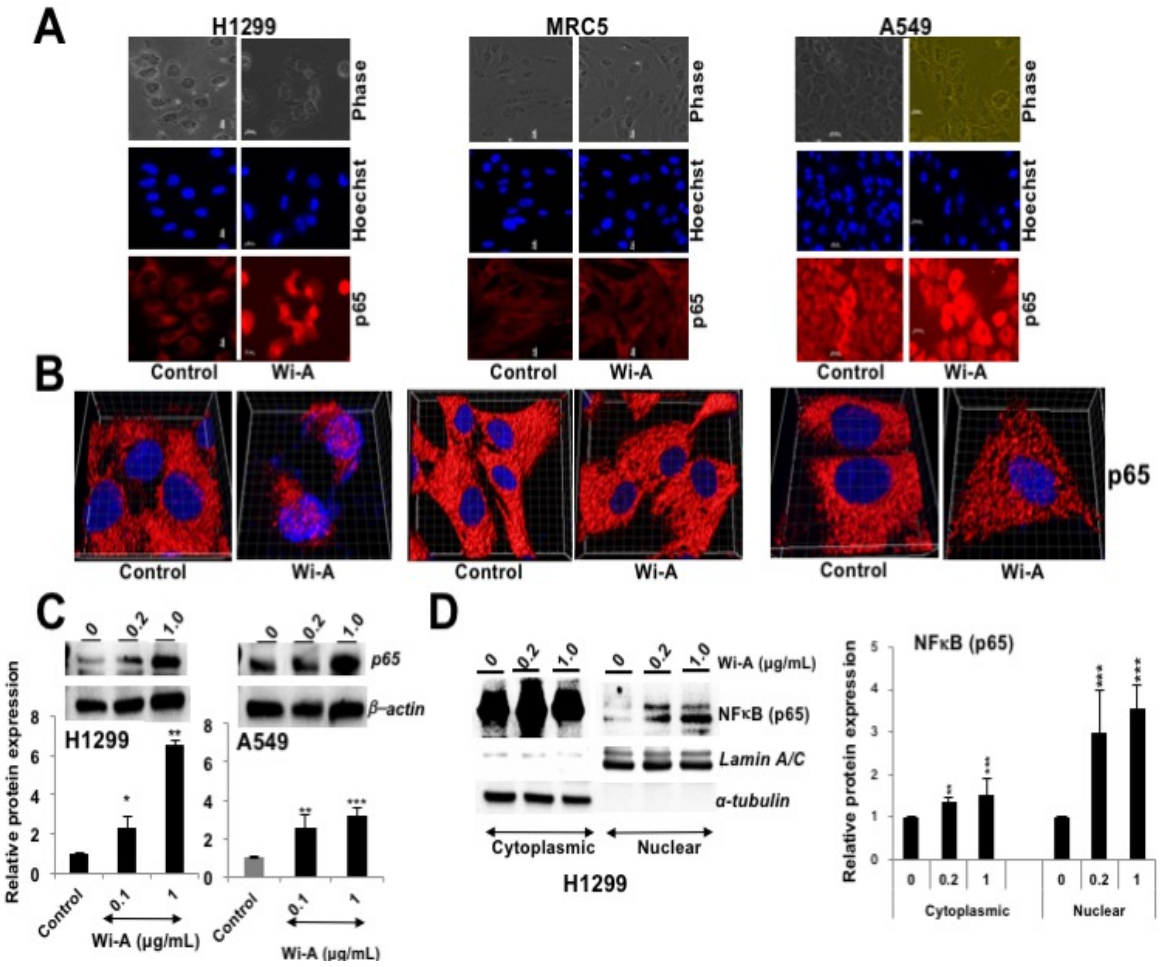

Fig. 4-2 Low dose of Wi-A caused nuclear translocation of NFκB in lung carcinoma. Immunostaining of NFκB, in control and Wi-A treated cells, showed its nuclear translocation in cancer (H1299 and A549) cells (A & B). Normal cells (MRC5) did not show nuclear translocation of NFκB. (B) Confocal images showing nuclear NFκB staining in cancer cells. Western blotting exhibited increase in NFκB expression in Wi-A treated cells (C). Cell fractionation and Western blotting analyses confirmed the nuclear translocation of NFκB in Wi-A treated cells (D)

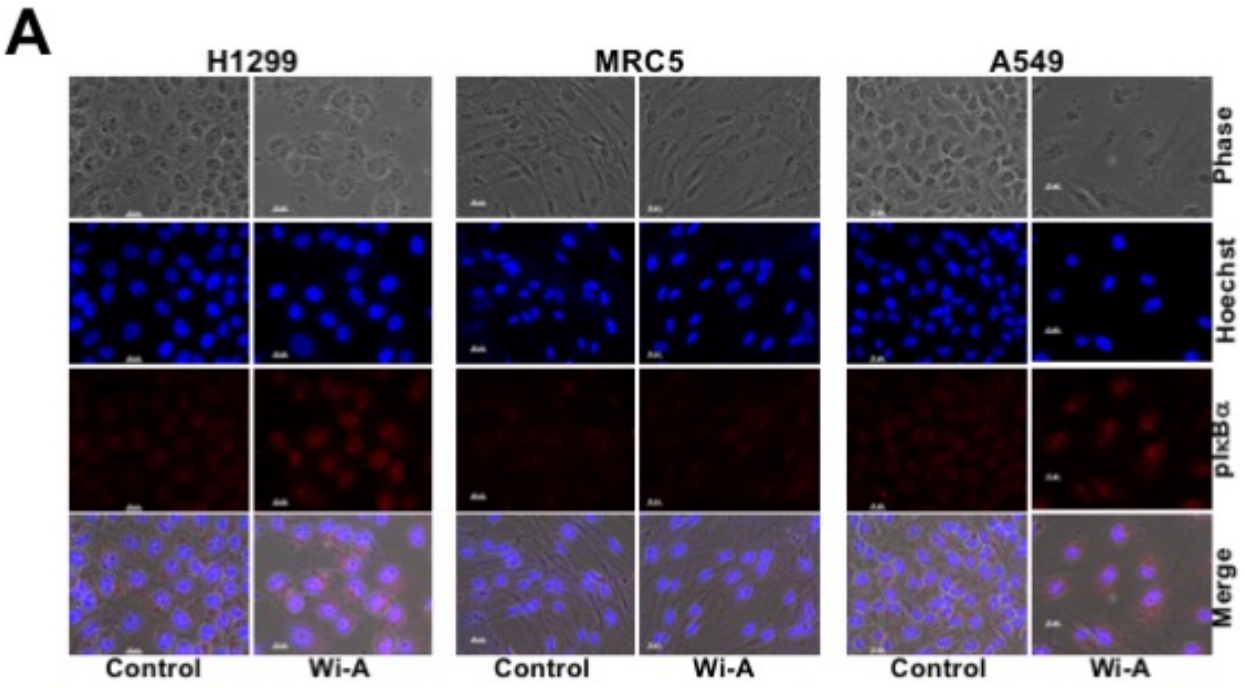

Fig. 4-3 Upregulation of IκBα in Wi-A treated cells. Immunostaining of phosphorylated IκBα (A)

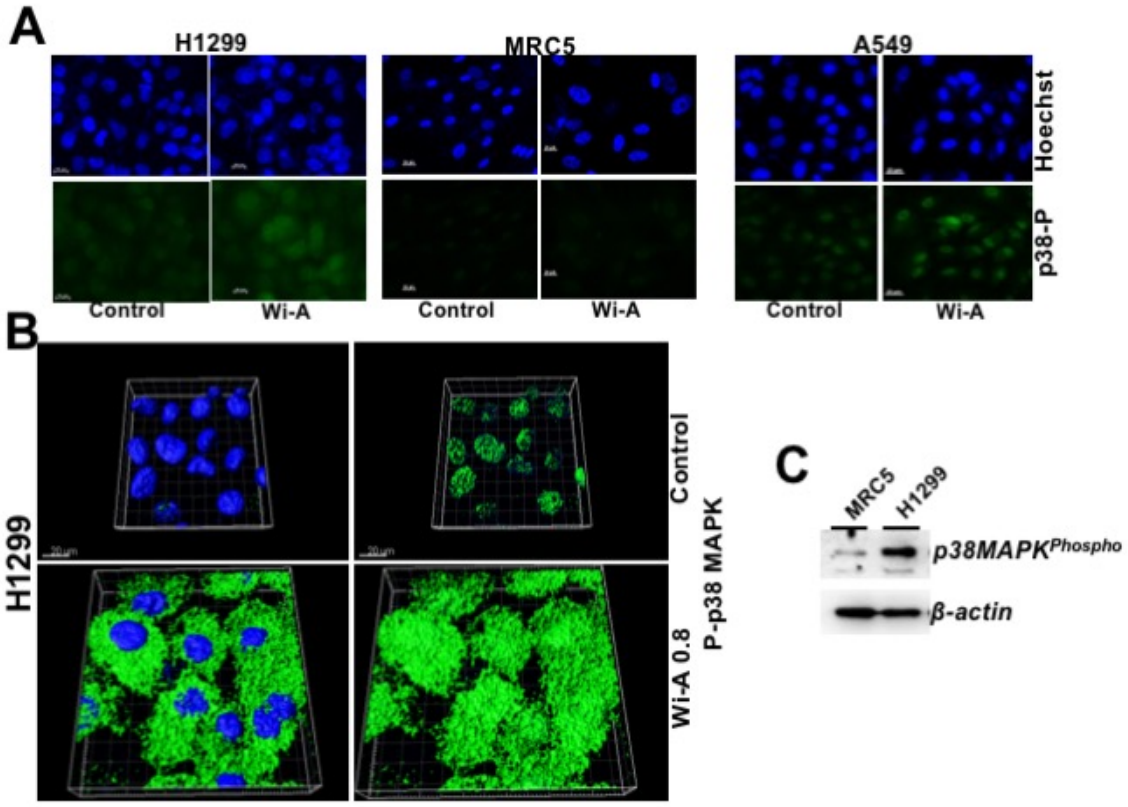

Fig. 4-4 Upregulation of p-p38MAPK in Wi-A treated cells. Immunostaining of phosphorylated p38MAPK (A) in control and Wi-A treated lung carcinoma and normal cells revealed their upregulation in cancer cells only. Nuclear translocation of p38MAPK and its phosphorylation was confirmed by confocal laser scanning microscopy (B) and Western blotting (C) with phosphorylation specific antibody.

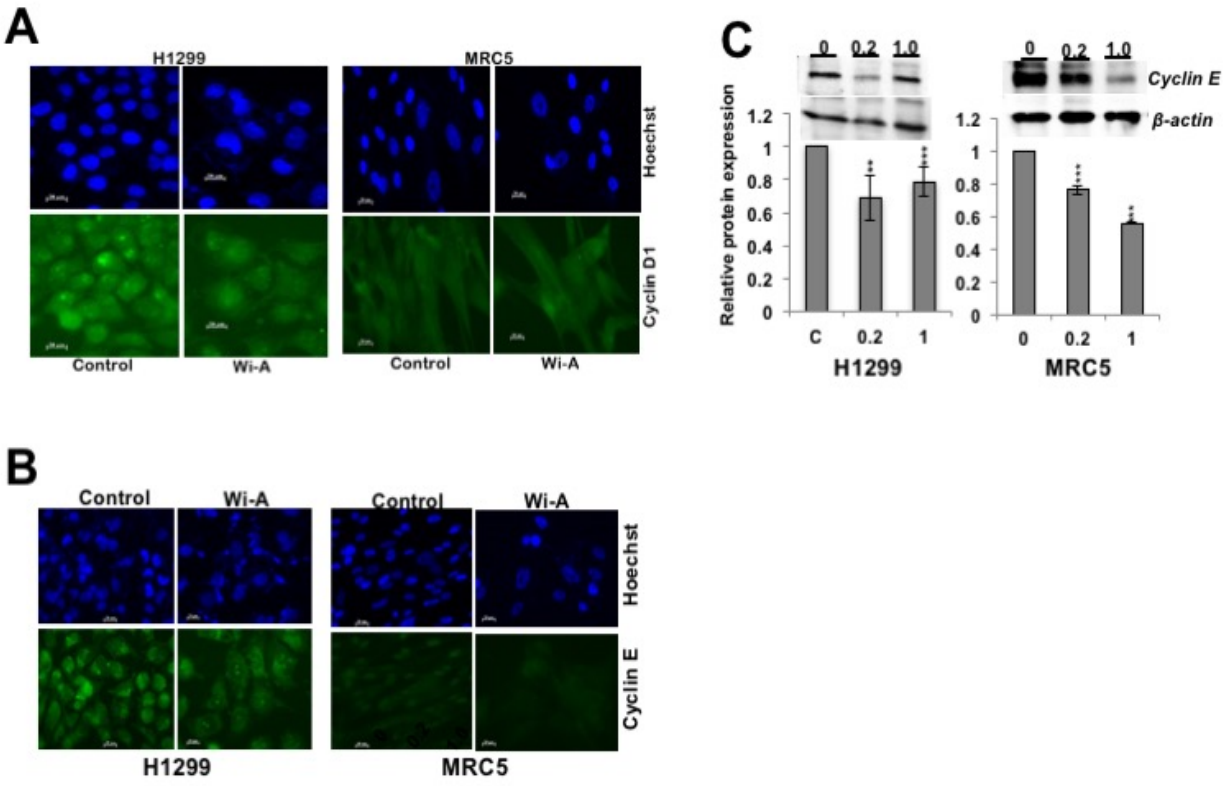

Fig. 4-5 Immunostaining and Western blotting (A, B, C) for Cyclin D1 and Cyclin E in control and Wi-A treated cancer and normal cells showed downregulation of Cyclin D1 and Cyclin E cancer cells only. Normal cells showed downregulation of Cyclin E, but not of Cyclin D1.

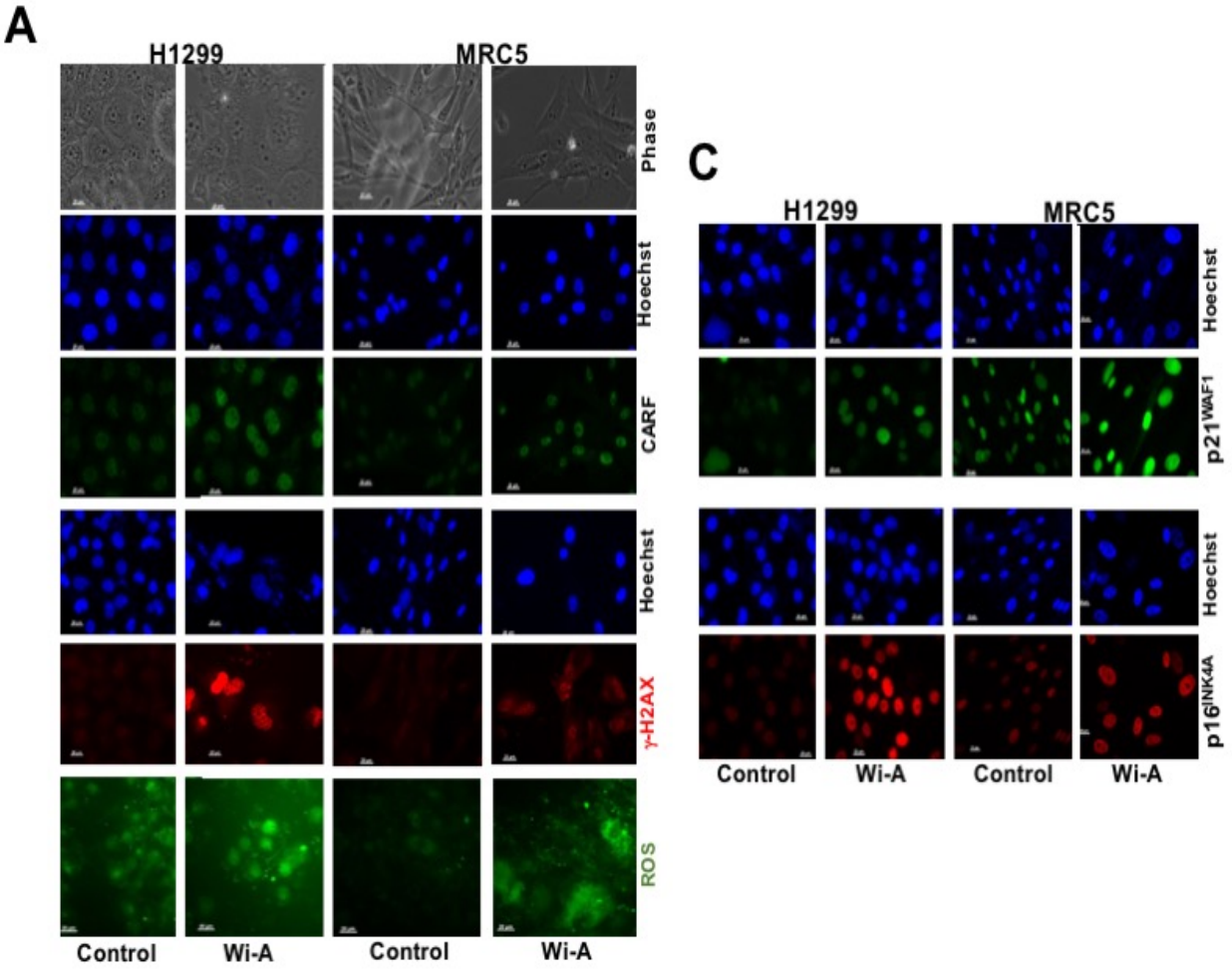

Fig. 4-6 Upregulation of DNA damage response in Wi-A treated cancer and normal cells. Immunostaining of CARF, γH2AX and ROS revealed their upregulation in Wi-A treated both cancer and normal cells (A and B). Upregulation of $p21^{WAF1}$ and $p16^{INK4A}$ was also observed both in cancer and normal cells (C and D).

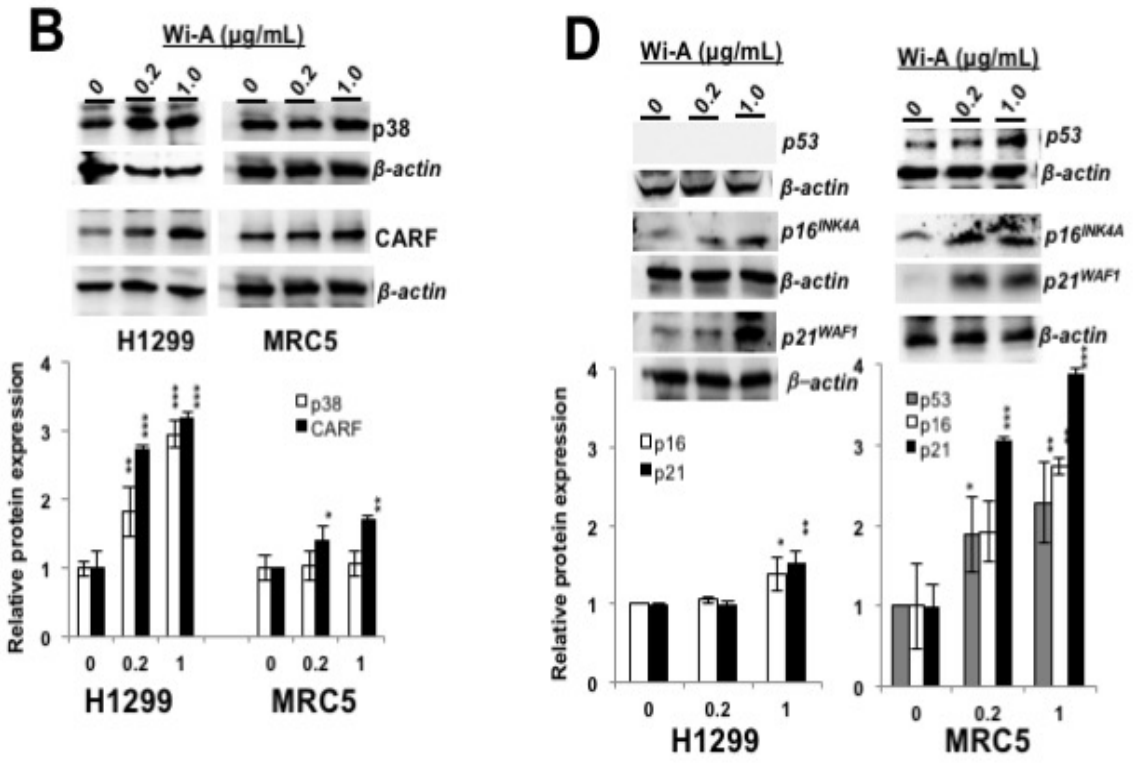

Fig. 4-6 Upregulation of DNA damage response in Wi-A treated cancer and normal cells. Immunostaining of CARF, γH2AX and ROS revealed their upregulation in Wi-A treated both cancer and normal cells (A and B). Upregulation of $p21^{WAF1}$ and $p16^{INK4A}$ was also observed both in cancer and normal cells (C and D).

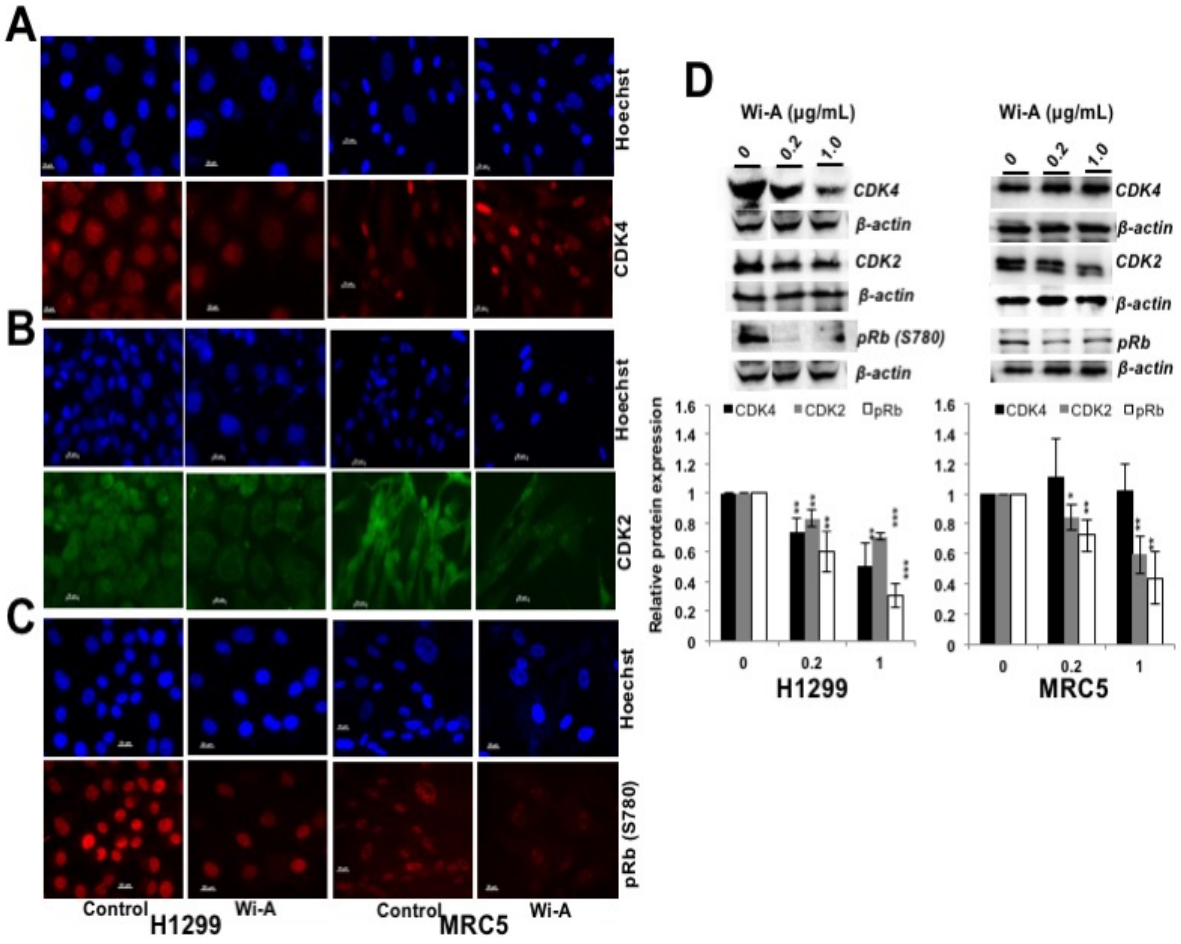

Fig. 4-7 Downregulation of CDK4, CDK2 and pRB phosphorylation in Wi-A treated cancer and normal cells. Immunostaining of CDK4, CDK2 and phosphorylated pRB showed their downregulation in Wi-A treated both cancer cells (A and C). Normal cells showed decrease in CDK2 and phosphorylated pRB (A-C). (D) Western blotting showing decrease in CDK2 and phosphorylated Rb both in cancer and normal cells; CDK4 showed decrease in cancer cells.

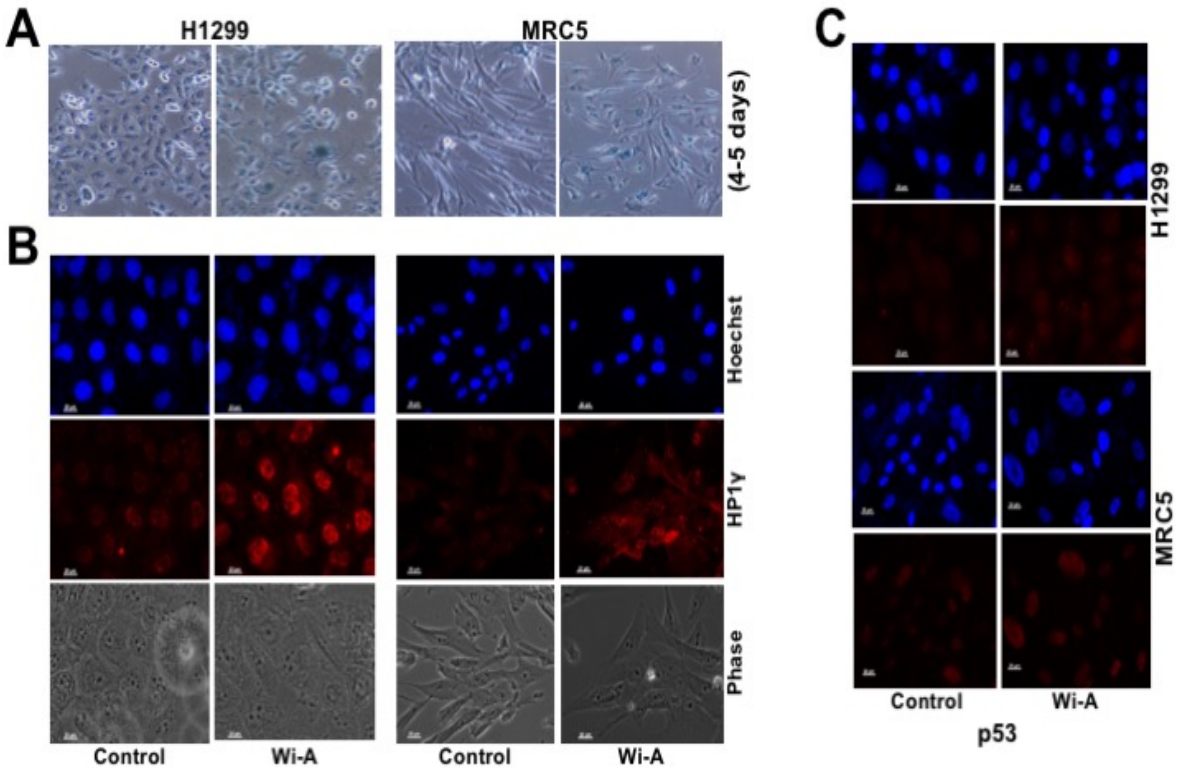

Fig. 4-8 Induction of senescence in Wi-A treated cancer and normal cells. Senescence associated β-gal staining (A) and HP1γ (B) foci appeared both in H1299 (p53 -/-) and MRC5 (p53+/+) cells subsequent to Wi-A treatment. Increase in p53 was observed in MRC5 cells (C).

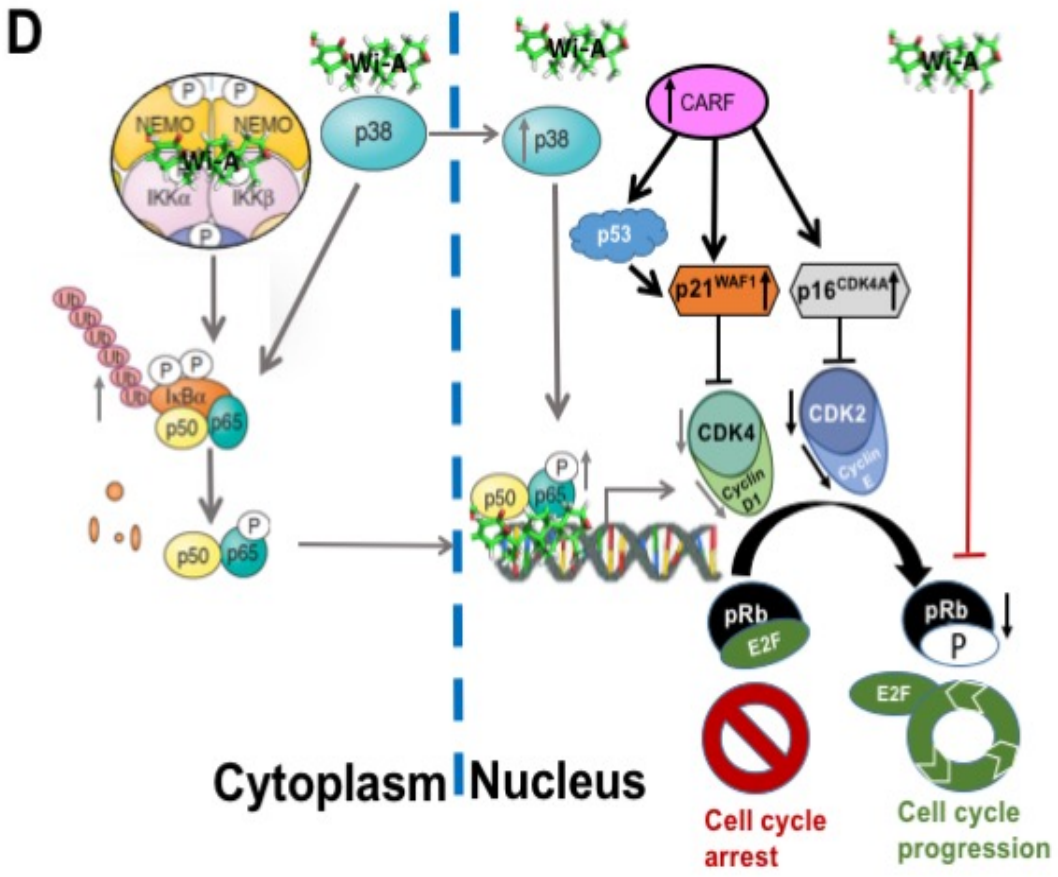

Fig. 4-9 (A) Schematic diagram showing the effect of Wi-A on DNA damage signaling in cancer and normal cells. Whereas p38 and NFkB axis were selectively activated in cancer cells (shown by grey arrows), CARF and its downstream activators p53, $p21^{WAF1}$ and $p16^{INK4A}$ were activated both in cancer and normal cells (shown by black arrow) resulting in induction of senescence.

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Chapter 5 Conclusions and future research

The anticancer activity of CAPE and cytotoxic doses for different cancer cell lines was similar with other reports (1-6). Computational and molecular analyses together support and revealed that CAPE targets mortalin-p53 complex interactions that are specific in cancer cells and hence a worth observation, at least in part, for selective toxicity of CAPE to cancer cells (1-6). Furthermore, CAPE treated cells showed reduction in mortalin, that is enriched in cancer cells and known to promote carcinogenesis and metastasis, suggesting that CAPE is an anti-mortalin molecule suitable for cancer and metastatic therapy.

The mechanisms of the anti-tumor activity in CAPE have not been well known. It was shown to suppress 12-O-tetradecanoylphorbol-13-acetate-mediated cell transformation and induce apoptosis in mouse epidermal cells through p53-dependent and independent pathways (7). CAPE was shown to inhibit production of nitric oxide and iNOS protein expression which are involved in inflammatory and autoimmune-mediated tissue destruction induced by lipopolysaccharide (LPS) plus interferon-gamma (IFN-gamma), and show anti-inflammatory, anti-viral and anticancer effects (8). CAPE caused cell cycle arrest and growth inhibition were shown to be mediated via activation of Skp2, p53, p21Cip1 and p27Kip1 proteins (9). It was shown to inhibit NFkB, activate survival MAPK family proteins p38 and JNK resulting in apoptosis (10). Anti-metastasis activity of CAPE (decreased expression of matrix metalloproteinases) was supported by many studies (11-14). Taken together and addition to these reports, I demonstrate that CAPE targets mortalin a stress chaperon leading to (i) growth arrest activation through p53-GADD45α-p21 signaling and (ii) reduction in cell migration through matrix metalloproteinases. I therefore investigated several cell stemness markers and drug resistance in mortalin overexpressing breast cancer cells. I demonstrate that mortalin overexpressing cells were enriched with stemness markers and exhibit resistance to cytotoxicity induced by several chemotherapeutic drugs. (iii) Furthermore, treatment of the cells with mortalin shRNA or inhibitors LIKE CAPE

reverted the drug resistance of cells and dampened their migration and invasion potentials.

Metals interfere with the biological activity of native, folded proteins by diverse modes of action (i) binds with other functional groups in protein or with free thiols (ii) catalyze oxidation of amino acid side chains (iii) displace essential metals ions (15;16). All things considered, it is abundantly cleared that protein aggregation or mis-folding is also a cause of many neurodegenerative diseases and it also regulates neuronal health and length. Using small molecules CAPE could be a better therapeutic approach to understand selective neuronal loss in the common neurodegenerative disease.

Senescence associated β-gal and HP1γ assays, indeed, endorsed Wi-A induced senescence both in cancer and normal cells. The study demonstrated that Wi-A caused activation of DNA damage response by pleiotropic mechanisms. Although one axis of activated DNA damage signaling, p38MAPK-NFκB, was activated selectively in cancer cells, the other one, CARF-$p21^{WAF1}$/$p16^{INK4A}$-Cyclin/CDK-pRB was activated both in cancer and normal cells suggesting that CARF plays an important role in Wi-A induced senescence.

Future research

1. I have established a new screening system to identify new hypoxia modulating small molecules. On basis of that, other identified pro-hypoxia and anti-hypoxia molecules will be further studied in different disease models like oxidative stress, cancer and aging.

2. The current study will be extended to identify other molecular pathways and targets by these molecules to make a bridge in between these diseases, connecting with aging, which will be helpful to improve these small molecule therapeutic effects.

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